HB-EGF promotes epithelial cell migration in eyelid development

Naoki Mine, Ryo Iwamoto* and Eisuke Mekada

Department of Cell Biology, Research Institute for Microbial Diseases, Osaka University, 3-1, Yamadaoka, Suita, Osaka 565-0871, Japan

*Author for correspondence (e-mail: riwamoto@biken.osaka-u.ac.jp)

Accepted 2 August 2005

Development 132, 4317-4326
Published by The Company of Biologists 2005
doi:10.1242/dev.02030

Summary

Heparin-binding EGF-like growth factor (HB-EGF) is a member of the EGF family of growth factors that binds to and activates the EGF receptor (EGFR) and ERBB4. Here, we show that HB-EGF-EGFR signaling is involved in eyelid development. HB-EGF expression is restricted to the tip of the leading edge of the migrating epithelium during eyelid closure in late gestation mouse embryos. Both HB-EGF null ($HB^{del/del}$) and secretion-deficient ($HB^{uc/uc}$) mutant embryos exhibited delayed eyelid closure, owing to slower leading edge extension and reduced actin bundle formation in migrating epithelial cells. No changes in cell proliferation were observed in these embryos. In addition, activation of EGFR and ERK was decreased in $HB^{del/del}$ eyelids. Crosses between $HB^{del/del}$ mice and waved 2 mice, a hypomorphic EGFR mutant strain, indicate that HB-EGF and EGFR interact genetically in eyelid closure. Together with our data showing that embryos treated with an EGFR-specific kinase inhibitor phenocopy $HB^{del/del}$ embryos, these data indicate that EGFR mediates HB-EGF-dependent eyelid closure. Finally, analysis of eyelid closure in TGFα-null mice and in HB-EGF and TGFα double null mice revealed that HB-EGF and TGFα contribute equally to and function synergistically in this process. These results indicate that soluble HB-EGF secreted from the tip of the leading edge activates the EGFR and ERK pathway, and that synergy with TGFα is required for leading edge extension in epithelial sheet migration during eyelid closure.

Key word: HB-EGF, TGFα, EGFR, ERK, Eyelid, Leading edge, Epithelial cell migration, Mouse

Introduction

The epidermal growth factor (EGF)-ERBB signaling network includes multiple ligands of the EGF/neuregulin superfamily and four related tyrosine kinase receptors, which include EGF receptor (EGFR)/ERBB1 and the ERBB receptors (ERBB2-4) (Holbro and Hynes, 2004). The EGF family ligands that bind EGFR include EGF, transforming growth factor α (TGFα), amphiregulin (AR), heparin-binding EGF-like growth factor (HB-EGF), epiregulin (EPR), betacellulin (BTC) and epigen (Harris et al., 2003).

The importance of ERBB signaling during early mouse development has been demonstrated by gene targeting studies (Holbro and Hynes, 2004). Disruption of the EGFR locus results in embryonic or perinatal lethality, depending on the genetic background (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). The phenotypes observed in these mice suggest several physiological roles for EGFR, including epithelial development. Newborn homozygous null EGFR mice exhibit immature development of epithelial cells in the skin, lung, gastrointestinal tract, tooth and eyelid (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995).

Eyelid closure represents a typical model for epidermal development. Although TGFβ/activin signaling is known to be essential for this process, EGFR signaling also is required (Xia and Karin, 2004). EGFR-deficient mice have an open eye at birth (EOB) phenotype (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995), and mice lacking the EGFR ligand TGFα occasionally exhibit an EOB phenotype (Luetteke et al., 1993).

HB-EGF is a member of the EGF family of growth factors that binds to and activates EGFR and ERBB4 (Elenius et al., 1997; Higashiyama et al., 1991). HB-EGF is synthesized as a type I transmembrane protein (proHB-EGF) and, like other EGF family members (Massague and Pandiella, 1993), is cleaved at the juxtamembrane domain, resulting in the shedding of soluble HB-EGF (sHB-EGF) (Goishi et al., 1995). sHB-EGF is a potent mitogen and chemoattractant for a number of cell types (Raab and Klagsbrun, 1997), while proHB-EGF acts as a juxtacrine growth factor that signals to neighboring cells in a non-diffusible manner (Iwamoto and Mekada, 2000). HB-EGF has been implicated in a number of physiological and pathological processes (Raab and Klagsbrun, 1997). Importantly, analysis of HB-EGF-null mice has shown that HB-EGF is a crucial factor for proper heart development and function (Jackson et al., 2003; Iwamoto et al., 2003), and for skin wound healing (Shirakata et al., 2005).

Here, we show a novel role for HB-EGF in the process of eyelid closure. Our data indicate that HB-EGF functions synergistically with TGFα in leading edge formation during eyelid closure, by promoting epithelial sheet migration through activation of the EGFR-ERK signaling cascade.

Development

Materials and methods

Mice

Generation of HB-EGF-null mice (HB^{del}) and the knock-in mice expressing the uncleavable mutant form of proHB-EGF (HB^{uc}) was previously described (Iwamoto et al., 2003; Yamazaki et al., 2003). These mice were maintained on a mixed background of C57BL/6J, ICR and CBA. TGFα-null mice and waved 2 mice were purchased from Jackson Laboratory. TGFα-null mice were maintained on a background of C57BL/6J, while waved 2 mice were maintained on a mixed background of C57BL/6JEi and C3H/HeSnJ.

Hematoxylin/eosin staining and *lacZ* detection

Hematoxylin/eosin staining and *lacZ* detection were performed as previously described (Iwamoto et al., 2003). The rates of eyelid closure and the eyelid formation were measured by microscopy as indicated in Fig. 2C and in Fig. 4A, respectively.

BrdU incorporation

E15.5 pregnant mice were injected with BrdU (Nakalai) (100 μg/g of body weight). Two hours after injection, embryos were harvested, fixed with 4% PFA, dehydrated and embedded in paraffin. Sections (4 μm) were stained with anti-BrdU monoclonal antibody (ABCAM). The BrdU-positive cells in serial sections were measured by microscopy.

Phalloidin staining

Embryos were fixed with 4% PFA, washed with PBS and incubated for overnight with FITC-phalloidin (Molecular Probes), as previously described (Zhang et al., 2003).

Immunohistochemistry

Mouse anti-mouse HB-EGF ectodomain monoclonal antibody (clone 4D9) was prepared by immunizing HB$^{del/del}$ mice with an abdominal injection of the recombinant ectodomain of mouse HB-EGF, prepared by the baculovirus expression system. Lymphoid node cells from the immunized mice were fused with P3U1 myeloma cells, as previously described (Iwamoto et al., 1991), and the hybridoma producing an antibody reacting to mouse HB-EGF was selected. Purified 4D9 mAb was biotinylated. Rabbit anti-phosphorylated EGFR antibody, anti-EGFR antibody, anti-phosphorylated ERK antibody and anti-phosphorylated JNK antibody were purchased from Cell Signaling Technology. For immunohistochemistry, embryos were fixed by 4% PFA, dehydrated and embedded in paraffin. Sections (5 μm) were incubated with each antibody in the blocking solution Block Ace (Dainihon Seiyaku), permeabilized with 0.1% Triton X-100, and then incubated with Alexa fluorophore-labeled streptavidin or Alexa fluorophore-labeled secondary antibodies (Molecular Probes). After incubation, sections were washed with PBS and observed by fluorescence microscopy.

Data analysis

Statistical significance was assessed with the Student's *t*-test. A value of $P<0.05$ was considered statistically significant.

Results

HB-EGF expression during eyelid development

A mutant allele of HB-EGF was previously generated by replacing exons 1-3 of the HB-EGF locus with loxP-flanked HB-EGF cDNA linked to the *lacZ* gene (Iwamoto et al., 2003). Removal of the HB-EGF cDNA by Cre-mediated recombination generates a null allele (HB^{del}) and leaves the *lacZ* gene under the control of the HB-EGF promoter, allowing us to use this allele to examine the expression pattern of HB-EGF during eyelid formation.

Eyelid formation occurs between embryonic day 11.5 (E11.5) and E17, and can be broken down into five steps. First, from E11.5 to E14.5, in the primitive eyelid region, the ectoderm layer surrounding the mesoderm undergoes increased cell proliferation as well as morphological changes to form the immature eyelid. Here, we refer to this immature eyelid structure as the 'eyelid root' (Fig. 1K). During root formation, no HB-EGF expression is detected (Fig. 1A,F,K). Next, the leading edge begins to extend from the tip of root epithelium at E15.0. HB-EGF expression is detected exclusively in the tip of the leading edge at this stage (Fig. 1B,G,L). Between E15.0 and E16.0, the leading edge extends, followed by root extension over the cornea. During this step, HB-EGF continues

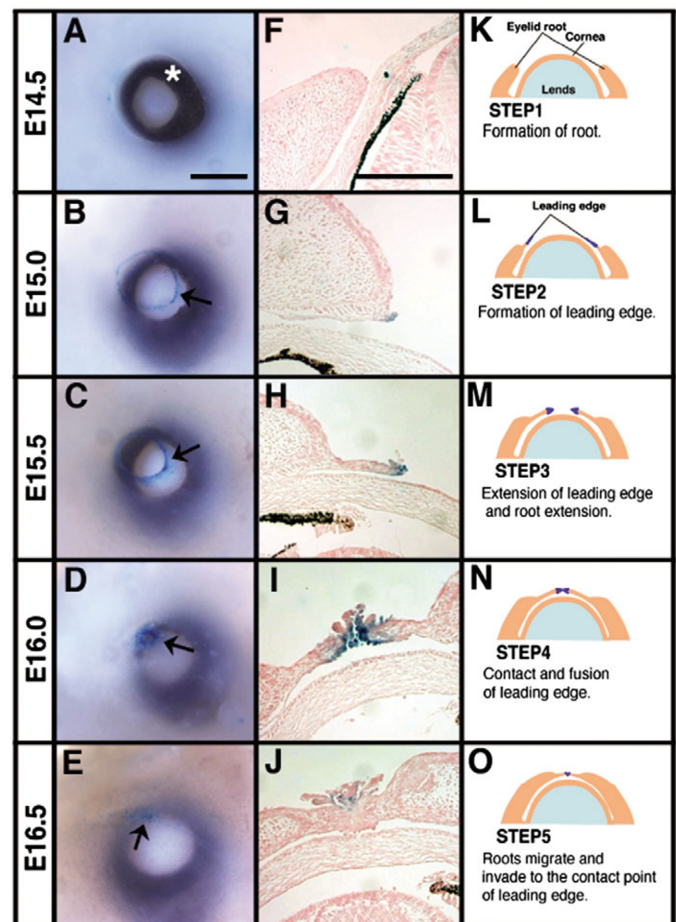

Fig. 1. HB-EGF expression during eyelid development. (A-E) Whole-mount *lacZ* staining of HB$^{del/+}$ eyelids (black arrow) at (A) E14.5, (B) E15.0, (C) E15.5, (D) E16.0 and (E) E16.5. The densely blue zone (asterisk) is the iris. (F-J) *lacZ* and Hematoxylin/Eosin stained sections of HB$^{del/+}$ eyelids at (F) E14.5, (G) E15.0, (H) E15.5, (I) E16.0 and (J) E16.5. (K-O) Schematic illustration of eyelid development. (K) At E14.5, leading edge formation has still not occurred. (L) At E15.0, the leading edge starts to extend from the eyelid root on both sides. (M) At E15.5, extension of both the leading edge and the root region continues (arrowheads). (N) At E16.0, the leading edges from both sides contact each other. (O) At E16.5, the root regions migrate and contact each other. (G-J) The expression of HB-EGF is restricted to the tip of the leading edge during this process. Scale bars: 500 μm for A-E; 100 μm for F-J.

Development

Development

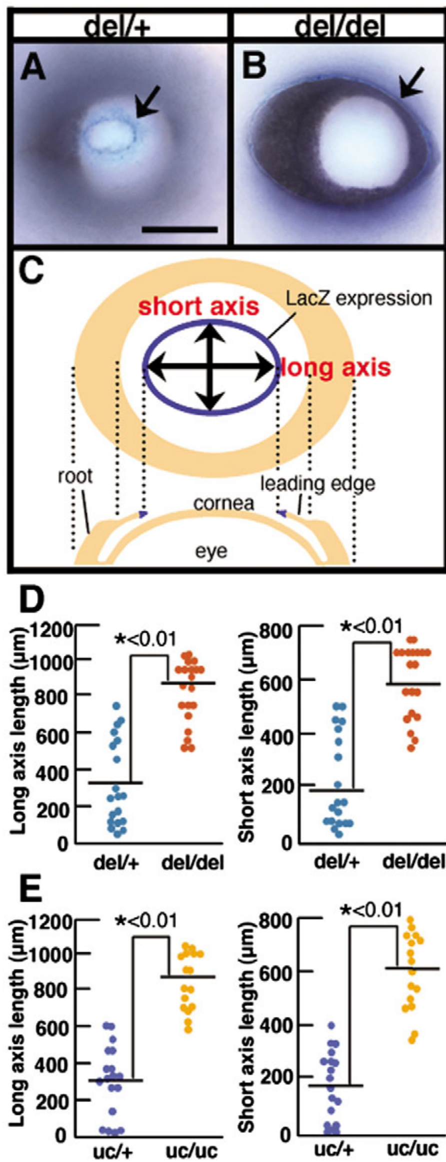

Fig. 2. Both HB-EGF null embryos and embryos with an uncleavable mutant form of HB-EGF show delays in eyelid closure. (A,B) Representative whole-mount *lacZ* stained (black arrow) $HB^{del/+}$ eyelids (A) and $HB^{del/del}$ eyelids (B) at E15.5. Scale bar: 500 µm for A and B. (C) Schematic diagram showing how the progression of eyelid closure was measured. (D) Comparison of the progression of eyelid closure along the long axis (left panel) and the short axis (right panel) between $HB^{del/+}$ (blue dots, n=19) and $HB^{del/del}$ (orange dots, n=20) embryos. (E) Comparison of the progression of eyelid closure along the long axis (left panel) and the short axis (right panel) between $HB^{uc/+}$ (blue dots, n=18) and $HB^{uc/uc}$ (yellow dots, n=16) embryos. Horizontal bars in the graphs indicate the mean value (D,E).

to be expressed at the tip of the leading edge (Fig. 1C,H,M). At E16.0, the two leading edges from both sides of the eyelid root meet and fuse at the center of eye. At this stage, HB-EGF is still expressed at the contact point of the leading edges (Fig. 1D,I,N). Finally, after E16.5, the root epithelium migrates towards the contact point of the leading edges. At this stage, HB-EGF expression is decreased but remains at the contact point of the leading edges (Fig. 1E,J,O). After eyelid closure is complete, HB-EGF expression is greatly diminished (data not shown). The observed pattern of HB-EGF expression during eyelid closure suggests that HB-EGF might play a fundamental role in eyelid development.

HB-EGF is involved in the progress of eyelid closure

To investigate the role of HB-EGF in eyelid closure, we examined eyelid closure in $HB^{del/del}$ embryos and their heterozygous littermates. At E15.5, eyelid closure was obviously delayed in $HB^{del/del}$ embryos compared with heterozygous littermates (Fig. 2A,B). To analyze this phenotype more quantitatively, the progression of eyelid closure was compared between $HB^{del/del}$ and $HB^{del/+}$ embryos during this stage. As *lacZ* expression was detected at the margin of extending leading edge of the eyelid in both genotypes, the progression of eyelid closure was determined by measuring the long and short axis of the *lacZ*-stained ellipse (Fig. 2C). Along both axes, the average progression of eyelid closure in $HB^{del/del}$ embryos was approximately three times lower than in $HB^{del/+}$ embryos (Fig. 2D). However, by birth, eyelid closure was completed and showed no abnormalities in mice of either genotype (Fig. 6C, Fig. 7G). These results indicate that HB-EGF contributes to the progress of eyelid closure.

Soluble form of HB-EGF is required for the progress of eyelid closure

HB-EGF is first synthesized as a membrane-anchored form (proHB-EGF), and the soluble form (sHB-EGF) is subsequently released from the cell surface by ectodomain shedding (Goishi et al., 1995). ProHB-EGF acts as a juxtacrine growth factor that signals to neighboring cells in a non-diffusible manner (Iwamoto and Mekada, 2000). To investigate which form of HB-EGF is involved in eyelid closure, we analyzed knock-in mice expressing an uncleavable form of proHB-EGF (HB^{uc}) (Yamazaki et al., 2003). As was the case with HB-EGF-null embryos, the average progression of eyelid closure in $HB^{uc/uc}$ embryos was approximately one-third that in $HB^{uc/+}$ embryos (Fig. 2E), indicating that the soluble form of HB-EGF is required for the progress of eyelid closure.

HB-EGF does not contribute to cell proliferation during eyelid closure

The process of eyelid closure coordinates both cell proliferation and migration. We therefore examined the cell proliferation during eyelid closure in $HB^{del/del}$ embryos and their heterozygous littermates at E15.5 by measuring BrdU incorporation. BrdU-positive cells were mainly detected in the eyelid dermis of the root region, but not in epidermal cells at the leading edge (Fig. 3A-D). There was no significant difference between the number of BrdU-positive cells in $HB^{del/+}$ and $HB^{del/del}$ eyelids (Fig. 3E). These results indicate that HB-EGF is not involved in cell proliferation during eyelid closure, and may instead play a role in cell migration.

HB-EGF contributes to the leading edge extension during eyelid closure

Two primary morphological changes occur during eyelid formation: extension of the root region (root formation) and extension of the leading edge (leading edge formation). We

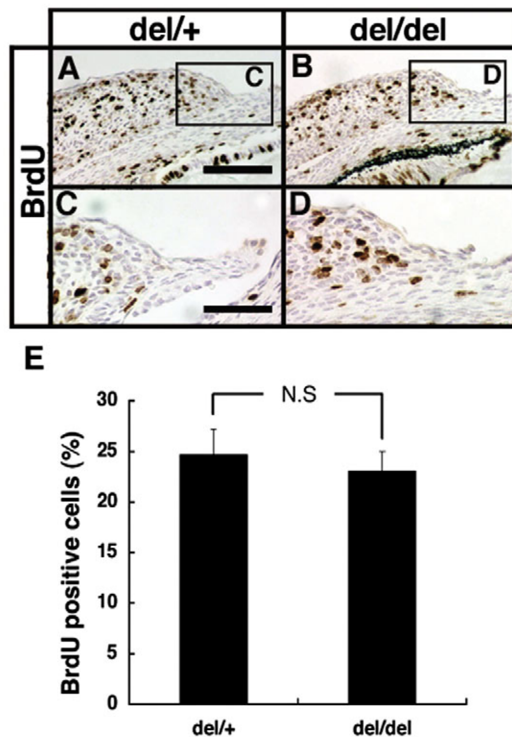

Fig. 3. HB-EGF is not required for cell proliferation in eyelid closure. (A,B) BrdU incorporation in eyelids of $HB^{del/+}$ (A) and $HB^{del/del}$ (B) embryos at E15.5. (C,D) High magnification of A and B, focusing on the leading edge. BrdU-positive cells (brown nuclei) are mostly in the dermis but not in the epidermis. Scale bars: 100 μm for A and B; 50 μm for C and D. (E) Scoring of BrdU-positive cells based on the data shown in A and B. Data represent the mean value±s.e.m. of the results obtained in three individual embryos. N.S, not significant.

therefore compared the progression of total eyelid formation, root formation and leading edge formation in $HB^{del/+}$ and $HB^{del/del}$ embryos (Fig. 4A).

The average progression of all three processes was significantly slower in $HB^{del/del}$ embryos than in $HB^{del/+}$ embryos (Fig. 4B), consistent with the previously observed delay in eyelid closure in $HB^{del/del}$ embryos (Fig. 2D). The progression of total eyelid formation, leading edge formation and root formation were ~2.0-, 4.2- and 1.6-fold slower, respectively, in $HB^{del/del}$ embryos compared to $HB^{del/+}$ embryos. Thus, the reduction in the progression of leading edge formation contributed more strongly than the reduction in the progression of root formation to the overall delay in eyelid formation in $HB^{del/del}$ embryos. These results support the

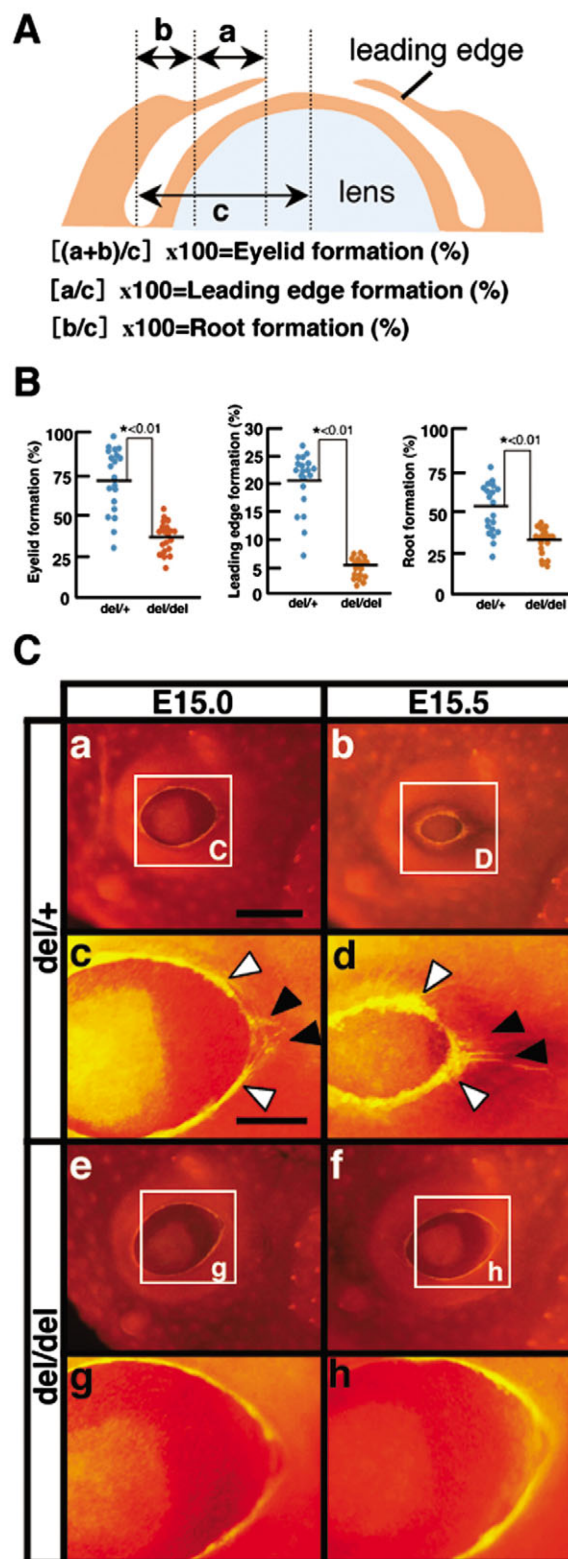

Fig. 4. HB-EGF contributes to the epithelial sheet migration in leading edge formation. (A) Schematic diagram showing how the progression of eyelid formation was measured. Contribution of leading edge formation and root formation to total eyelid formation was estimated as shown. (B) Progression of total eyelid formation (left panel), leading edge formation (middle panel) and root formation (right panel) in $HB^{del/+}$ (blue dots, n=19) and $HB^{del/del}$ (orange dots, n=20) animals. Horizontal bar in the graph indicates the mean value. (C) HB-EGF promotes F-actin reorganization in the developing eyelid epithelium. Whole-mount staining of $HB^{del/+}$ (a-d) and $HB^{del/del}$ (e-h) embryos at E15.0 (a,c,e,g) and E15.5 (b,d,f,h) was performed using FITC-phalloidin and propidium iodide to visualize F-actin and DNA, respectively. High-magnification views of boxed areas in a,b,e,f are shown in c,d,g,h, respectively. Both actin cable formation (white arrowheads) and radial F-actin fiber (black arrowheads) can be detected more clearly in $HB^{del/+}$ eyelids than in $HB^{del/del}$ eyelids. Scale bars: 500 μm for a,b,e,f; 200 μm for c,d,g,h.

Development

Development

possibility that HB-EGF functions predominantly in the formation of the leading edge during eyelid closure.

HB-EGF is involved in F-actin polymerization in the developing eyelid epithelium

The role of HB-EGF in leading edge extension during eyelid closure strongly suggests that HB-EGF functions in epithelial cell sheet migration. Previous studies have shown that migrating epithelial cells exhibit actin reorganization (Zhang et al., 2003). During eyelid closure, F-actin in the form of actin cables are detected at the margin of the migrating epithelial sheet, and radial F-actin fibers align with the axis of extension of the leading edge (Xia and Karin, 2004). To examine whether epithelial cell migration during eyelid closure is normal in HB$^{del/del}$ embryos, we used FITC-phalloidin staining as a marker for actin polymerization.

At E15.0, although no overt morphological differences were detected in the eyelids of HB$^{del/+}$ and HB$^{del/del}$ embryos, actin cable formation at the margin of the eyelid epithelium was lower in HB$^{del/del}$ embryos than in HB$^{del/+}$ embryos (Fig. 4C, parts a,c,e,g). Moreover, at E15.5, when the leading edge is extending, progressive eyelid closure was accompanied by both actin cable formation and radial F-actin fiber formation in HB$^{del/+}$ eyelids (Fig. 4C, parts b,d), while in HB$^{del/del}$ embryos, eyelid closure was retarded and both actin cable and radial F-actin fiber formation were quite low (Fig. 4C, parts f,h). These results suggest that HB-EGF plays a role in epithelial cell sheet migration during leading edge formation by promoting F-actin polymerization.

HB-EGF activates the EGFR-ERK signaling pathway during leading edge formation

To investigate the molecular mechanism underlying HB-EGF-mediated eyelid closure, we performed immunohistochemistry for HB-EGF protein in the developing eyelid using an anti-HB-EGF ectodomain monoclonal antibody. As shown in Fig. 5C,E, HB-EGF protein was detected broadly in the leading edge of HB$^{del/+}$ eyelids, although HB-EGF mRNA was at the tip of the leading edge (Fig. 1, Fig. 5A). As expected, no HB-EGF protein was detected in HB$^{del/del}$ eyelids (Fig. 5D,F), although the *lacZ* expression pattern resembled that of HB$^{del/+}$ embryos (Fig. 5B). These results indicate that sHB-EGF is secreted at the tip of the leading edge, and diffuses only in this region.

EGFR is a receptor for HB-EGF (Higashiyama et al., 1991) and is essential for eyelid closure (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). To investigate whether HB-EGF activates EGFR during eyelid closure, we examined HB$^{del/+}$ and HB$^{del/del}$ eyelids for phosphorylated EGFR. In HB$^{del/+}$ embryos, phosphorylated EGFR protein was detected predominantly in the extending leading edge (Fig. 5G), while total EGFR protein was detected more broadly in the epithelial cells of the leading edge as well as the root region (Fig. 5I). Phosphorylated EGFR was detected in the same region as HB-EGF (Fig. 5E,G). By contrast, the level of phosphorylated EGFR was drastically reduced in HB$^{del/del}$ embryos, especially in the leading edge (Fig. 5H), while total EGFR protein appeared normal (Fig. 5I,J). These results suggest that HB-EGF activates EGFR in the leading edge and is restricted to this region.

Next, we used antibody staining to examine the phosphorylation state of ERK, a major downstream effector of EGFR signaling (Jorissen et al., 2003). In HB$^{del/+}$ embryos, phosphorylated ERK protein was detected predominantly in the extending leading edge (Fig. 5K), as was phosphorylated EGFR (Fig. 5G). By contrast, the level of phosphorylated ERK was decreased in HB$^{del/del}$ eyelids, especially in the leading edge (Fig. 5L). Thus, activation of ERK in the leading edge is correlated with HB-EGF-mediated EGFR activation during eyelid closure.

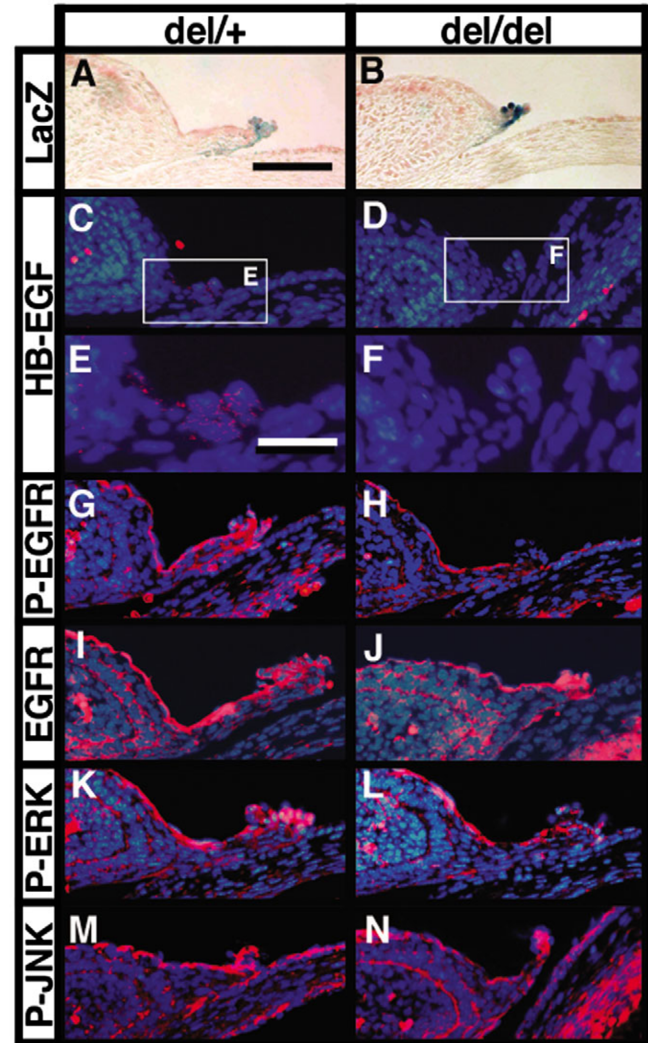

Fig. 5. sHB-EGF activates EGFR-ERK signaling but not JNK signaling in the extending leading edge. (A,B) *lacZ* expression at the tip of the leading edge of HB$^{del/+}$ (A) and HB$^{del/del}$ (B) eyelids. (C,D) Detection of sHB-EGF protein in the leading edge of HB$^{del/+}$ (C) and HB$^{del/del}$ (D) eyelids. (E,F) High-magnification views of boxed areas in C and D. (G-N) State of activation of EGFR and downstream signaling molecules in the leading edge of HB$^{del/+}$ (G,I,K,M) and HB$^{del/del}$ (H,J,L,N) eyelids. (G,H) Phosphorylated EGFR. (I,J) Total EGFR protein. (K,L) Phosphorylated ERK. (M,N) Phosphorylated JNK. Scale bars: 100 μm for A-D,G-N; 20 μm for E,F.

JNK signaling has also been implicated in eyelid closure (Grose et al., 2003; Li et al., 2003; Weston et al., 2004; Zenz et al., 2003; Zhang et al., 2003). Moreover, the transcription factor Jun, a target of JNK signaling, has been shown to be

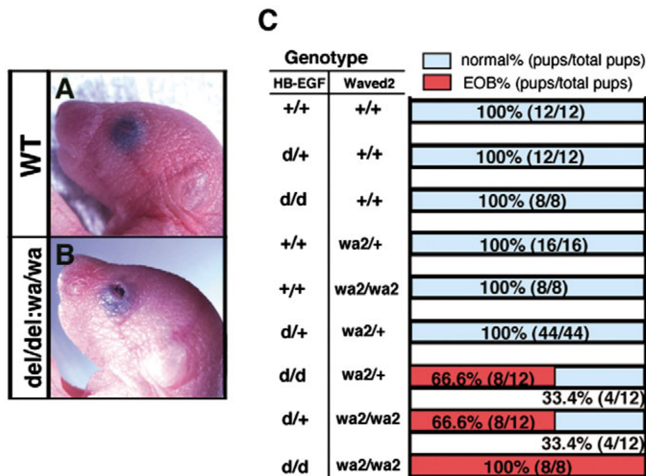

Fig. 6. EGFR contributes to HB-EGF-dependent eyelid closure process. (A,B) Neonatal eyelid of a wild-type (A) and a double mutant mouse carrying the HB-EGF null allele and the waved 2 EGFR allele, showing an EOB phenotype (B). (C) Penetrance of the EOB phenotype in compound mutants carrying the HB-EGF null ('d') and waved 2 ('wa2') alleles. Blue bars indicate pups with normal closed eyes; red bars indicate pups with EOB.

necessary for HB-EGF expression in keratinocytes (Zenz et al., 2003). No difference in the level of phosphorylated JNK was detected between $HB^{del/+}$ and $HB^{del/del}$ eyelids (Fig. 5M,N), suggesting that JNK signaling is not downstream of HB-EGF in leading edge formation.

These data strongly suggest that secreted sHB-EGF at the tip of the leading edge of the eyelid diffuses and locally activates EGFR and a downstream ERK signaling cascade during eyelid closure.

EGFR mediates HB-EGF-dependent eyelid closure

Since EGFR phosphorylation was decreased in leading edge of the eyelid in $HB^{del/del}$ embryos, we used ZD1839, an EGFR-specific kinase inhibitor (Tanno et al., 2004; Von Pawel, 2004), to examine whether loss of EGFR function would phenocopy loss of HB-EGF. We administrated ZD1839 to E14.5 wild-type embryos in utero, and harvested and analyzed them 24 hours later. Embryos with reduced EGFR activity had a similar phenotype to $HB^{del/del}$ and $HB^{uc/uc}$ embryos (data not shown). In ZD1839-treated embryos, eyelid formation was significantly impaired owing primarily to defects in leading edge formation, but BrdU incorporation was normal, indicating that EGFR activity does not promote proliferation of dermal cells in the root region. In addition, formation of actin cables and radial F-actin fibers was drastically decreased in ZD1839-treated embryos. These results indicate that from E15.0 to E16.0, EGFR activity is correlated with HB-EGF activity during leading edge formation. EGFR signaling does not appear to regulate cell proliferation during leading edge formation, but probably instead functions to control epithelial cell sheet migration.

In order to determine whether EGFR acts as a receptor for HB-EGF in eyelid closure, we used a hypomorphic EGFR mutant, waved 2, and tested for a genetic interaction with HB-EGF. In waved 2 mice, the kinase activity of EGFR is decreased to less than 10% that of wild-type EGFR, owing to a point mutation in the kinase domain (Fowler et al., 1995; Luetteke et al., 1994). Although $HB^{del/del}$ and waved 2 (*wa2/wa2*) mice did not exhibit an EOB phenotype, $HB^{del/+}$; *wa2/wa2* (66.6%) and $HB^{del/del}$; *wa2/wa2* (100%) mice did (Fig. 6). These results suggest that eyelid closure relies on dose-dependent HB-EGF-EGFR signaling, and that EGFR mediates HB-EGF-controlled eyelid closure.

TGFα functions synergistically with HB-EGF in eyelid closure

TGFα, another EGFR ligand, also has been reported to be a critical factor in eyelid closure (Luetteke et al., 1993; Wong et al., 2003). As expression of TGFα is regulated by HB-EGF in an autocrine manner in keratinocytes (Hashimoto et al., 1994; Piepkorn et al., 1998), we examined whether the defects in eyelid closure in $HB^{del/del}$ embryos resulted from downregulation of TGFα expression. TGFα mRNA was expressed in the tip of the leading edge in both $HB^{del/+}$ and $HB^{del/del}$ eyelids, and no significant difference in the level of TGFα expression was detected between $HB^{del/+}$ and $HB^{del/del}$ eyelids (data not shown), indicating that the expression of TGFα is not affected by the presence or absence of HB-EGF.

To clarify the functional relationship between HB-EGF and TGFα in eyelid closure, we investigated this process in TGFα-null mice. As was the case with HB-EGF null mice, the BrdU incorporation in TGFα mutant eyelids appeared normal (Fig. 7A-C). We next compared the progression of eyelid closure in $Tgfa^{+/-}$ and $Tgfa^{-/-}$ embryos. The average progression of total eyelid formation, root formation and leading edge formation were significantly lower in $Tgfa^{-/-}$ embryos than in $Tgfa^{+/-}$ embryos (2.0-, 3.0- and 1.8-times, respectively, Fig. 7D), with the progression of total eyelid formation in $Tgfa^{-/-}$ embryos similar to that of $HB^{del/del}$ embryos (Fig. 4B, Fig. 7D). Thus, as with the HB-EGF mutants, the reduction in the progression of leading edge formation contributed more strongly to the reduced progression of total eyelid formation than did root formation in TGFα embryos.

Finally, to test for genetic interactions between HB-EGF and TGFα in eyelid closure, we examined double mutants of TGFα and HB-EGF. As shown in Fig. 7E-G, both $HB^{del/del}$; $Tgfa^{+/-}$ and $HB^{del/+}$; $Tgfa^{-/-}$ embryos exhibited similar frequencies of EOB (10% and 11%, respectively), while $HB^{del/del}$ and $Tgfa^{-/-}$ single homozygotes did not have an EOB phenotype. Moreover, homozygous double mutants ($HB^{del/del}$; $Tgfa^{-/-}$) had an even higher frequency of EOB (50%). These results strongly suggest that TGFα and HB-EGF contribute equally to leading edge formation and function synergistically in this process.

Discussion

Here, we demonstrate a novel role for HB-EGF in eyelid development. Our major findings are as follows: (1) HB-EGF is expressed only at the tip of the leading edge of the migrating epithelial cell sheet during eyelid formation; (2) HB-EGF promotes epithelial cell migration, but not cell proliferation, during leading edge formation; (3) the secreted form of HB-EGF activates EGFR, resulting in the activation of ERK, which becomes localized to the leading edge; and (4) HB-EGF functions synergistically with TGFα in this process.

Development

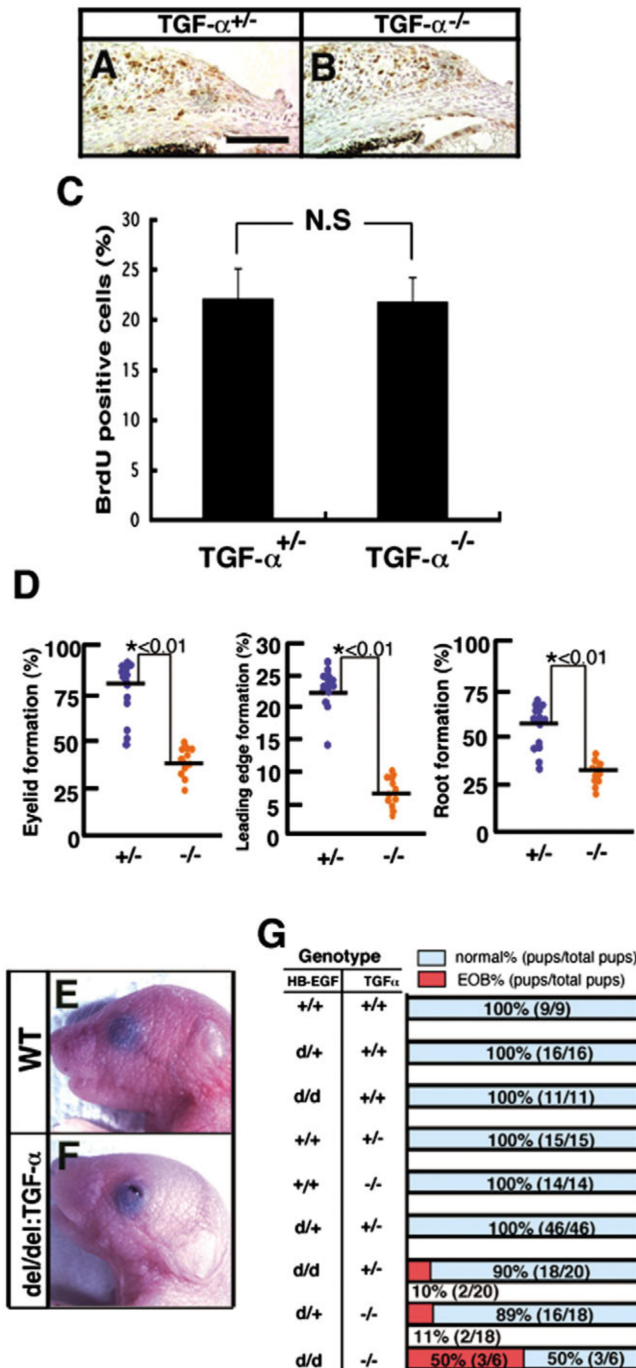

Fig. 7. TGFα functions equally and synergistically with HB-EGF in eyelid closure. (A,B) BrdU incorporation in eyelids of *Tgfa*$^{+/-}$ (A) and *Tgfa*$^{-/-}$ (B) E15.5 embryos. Scale bar: 100 μm for A,B. (C) Scoring of BrdU-positive cells shown in A and B. Data represent the mean value±s.e.m. of the results obtained from three individual embryos. N.S, not significant. (D) Progression of total eyelid formation (left panel), leading edge formation (middle panel) and root formation (right panel) in *Tgfa*$^{+/-}$ (blue dots, *n*=14) and *Tgfa*$^{-/-}$ (orange dots, *n*=12) eyelids. Horizontal bar in the graphs indicates the mean value. (E-G) TGFα functions synergistically with HB-EGF in eyelid closure. Neonatal eyelid of wild-type animal (E) and double homozygote lacking both HB-EGF and TGFα with an EOB phenotype (F). (G) Penetrance of EOB phenotype in compound mutants carrying HB-EGF null and TGFα null alleles. Blue bars indicate pups with normal closed eyes; red bars indicate pups with EOB.

HB-EGF-EGFR signaling functions in cell migration but not proliferation during eyelid closure

We demonstrate here using HB-EGF-null embryos that HB-EGF-mediated activation of EGFR promotes epithelial cell sheet migration, but not cell proliferation, during eyelid closure. Among our conclusions are the following: (1) In HB-EGF-deficient embryos, eyelid closure was significantly retarded; (2) the level of cell proliferation was not significantly different between HB$^{del/del}$ and HB$^{del/+}$ eyelids; (3) retardation of eyelid formation in HB$^{del/del}$ embryos was mainly due to defects in leading edge extension rather than root region extension; (4) the level of cell migration, judged by the detection of actin bundle formation in epithelial cells of the leading edge, was decreased in HB$^{del/del}$ eyelids; (5) phosphorylated EGFR was dramatically decreased in the leading edge lacking HB-EGF; (6) inhibition of EGFR activity by the kinase inhibitor ZD1839 phenocopied the loss of HB-EGF; and (7) reduction of EGFR activity in mice lacking both HB-EGF and EGFR function accelerated the defect of eyelid closure.

Eyelid closure occurs between E15.5 and E16.5, with the eyelid epidermis eventually fusing to form a closed eyelid that covers the ocular surface and serves as a protective barrier that is crucial for normal eye development (Findlater et al., 1993; Harris and Juriloff, 1986). Initiation of eyelid formation requires the proliferation of epithelial cells, and impaired cell migration leads to a complete loss of eyelids (Li et al., 2001). Our results indicate that HB-EGF and EGFR signaling is not required for cell proliferation, but is required for cell migration during eyelid closure.

Recently, we demonstrated that in skin wound healing, HB-EGF promotes epithelial cell sheet migration, but not cell proliferation, in the process of re-epithelialization (Shirakata et al., 2005). In adult mice, HB-EGF is not usually expressed in the skin, but wounding induces expression of HB-EGF at the migrating leading edge of the wound. The parallels in both the domain of expression and function of HB-EGF between eyelid formation and skin wound healing suggest that HB-EGF functions to promote cell motility, but not proliferation in epithelial keratinocytes.

Role of EGFR and downstream signaling in eyelid closure

In this study, we present evidence that activation of EGFR by HB-EGF is important for eyelid formation. Although HB-EGF can also bind to and activate ERBB4 receptor, ERBB4 expression is not detectable in the murine epidermis (Kiguchi et al., 2000; Xian et al., 1997) or the human epidermis (Plowman et al., 1993), ruling out a role in eyelid closure. By contrast, both EGFR knockout mice (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995) and the spontaneous EGFR mutant mice *velvet* (Du et al., 2004) showed an EOB phenotype. However, it remained unclear whether EGFR functions in cell proliferation or migration. In the present study, we show that EGFR is essential for epithelial cell sheet migration and promotes leading edge formation during eyelid closure process. These results suggest that the

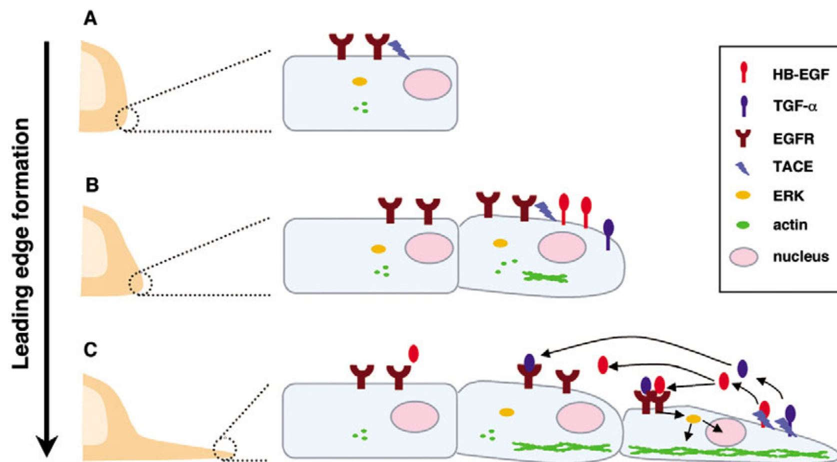

Fig. 8. Proposed model for the function of HB-EGF in leading edge formation during eyelid closure. (A) Before E15.0, the leading edge is still not formed, and HB-EGF expression is not detectable. (B) At E15.0, the leading edge starts to extend, and HB-EGF expression appears at the tip of the leading edge. (C) From E15.0 to E16.0, HB-EGF is constitutively expressed at the tip of the extending leading edge. On the surface of the cells located in this region, ectodomain shedding of proHB-EGF may be mediated by ADAM17 (TACE), allowing sHB-EGF to diffuse throughout the leading edge region. sHB-EGF binds to and activates EGFR and the ERK pathway, resulting in F-actin polymerization and promotion of epithelial sheet migration.

defects in eyelid closure resulting from loss of EGFR activity are mainly caused by retarded epithelial sheet migration.

ERK MAPK (ERK1/2) is a key component in the RAS-MAPK signaling cascade that is activated by a multitude of RTKs. Here, we show that ERK phosphorylation is decreased in HB-EGF mutant eyelids. Consistent with our results, cell migration has been reported to involve activation of ERK in primary cultured keratinocytes (Zhang et al., 2003). Moreover, another recent study has demonstrated that wound-induced activation of ERK1/2 propagates in cell sheets with the cell sheet movement, and that this movement is dependent on the activation of ERK2 (Matsubayashi et al., 2004). Thus, together with our study, these findings suggest that ERK is the key signaling component for promoting epithelial cell migration.

JNK signaling, which can be activated by TGFβ/activin (Xia and Kao, 2004; Xia and Karin, 2004; Zhang et al., 2003), may also play a role in eyelid closure. Mice that lack MEKK1 showed defects in leading edge formation, owing to loss of JNK activation (Zhang et al., 2003). In addition, mice that lack the JNK target JUN showed defects in leading edge formation (Grose, 2003; Li et al., 2003; Zenz et al., 2003). Furthermore, mutant $Jnk1^{-/-}$ $Jnk2^{+/-}$ pups exhibited an EOB phenotype (Weston et al., 2004). Interestingly, it has been reported that expression of HB-EGF depends on JUN activation in keratinocytes (Zenz et al., 2003). However, in our experiments, there was no difference in JNK activation between $HB^{del/+}$ and $HB^{del/del}$ animals. Thus, JNK may function as an upstream factor for HB-EGF-EGFR-ERK signaling pathway in eyelid closure.

Relationship between HB-EGF and TGFα in eyelid closure

TGFα is also known to be required for normal eyelid closure, with TGFα deficient mice occasionally exhibiting an EOB phenotype (Luetteke et al., 1993; Wong, 2003). However, the TGFα-null mice used in the present study did not show an EOB phenotype. This difference could be due to differences in the genetic background of our mice and the previously reported mice. Although it has been reported that TGFα functions upstream of HB-EGF signaling pathway involved in eyelid closure (Hayashi et al., 2005), our data suggest that TGFα and HB-EGF function at equal levels and synergistically in eyelid closure, based on the following findings: (1) expression of TGFα was not affected by HB-EGF expression; (2) TGFα-null mice phenocopy HB-EGF mutants, with defects in epithelial sheet migration but not proliferation; (3) mice that are homozygous null for HB-EGF and heterozygous for a TGFα null mutation showed a variably penetrant EOB phenotype; likewise, mice that are homozygous null for TGFα and heterozygous for a HB-EGF null mutation had a similar phenotype, while both single null mutant mice failed to show any EOB phenotype; and (4) HB-EGF and TGFα doubly homozygous null mice had dramatically increased penetrance of the EOB phenotype. However, in contrast to the complete penetrance of the EOB phenotype in EGFR-null mice (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995), only 50% of these double homozygotes were born with EOB. This suggests that other EGFR ligand(s) might also be involved in eyelid closure. Interestingly, it has been reported that triple null mice lacking EGF, amphiregulin, and TGFα exhibited a more severe EOB phenotype than did TGFα single null mice. However, even in these triple null mice, the EOB phenotype is not fully penetrant (Luetteke et al., 1999). This indicates that the EGFR ligands HB-EGF, TGFα, EGF and amphiregulin may cooperate in eyelid closure.

Mode of action of HB-EGF in the process of eyelid formation

We show using the $HB^{del/+}$ *lacZ* reporter allele that HB-EGF is specifically expressed at the tip of the leading edge of the migrating epithelial sheet, and only between E15.0 and E16.5. By contrast, HB-EGF protein was detected throughout the leading edge of $HB^{del/+}$ developing eyelids. Moreover, embryos with an uncleavable mutant version of proHB-EGF ($HB^{uc/uc}$) displayed defects in eyelid closure. Together, these findings indicate that sHB-EGF, but not proHB-EGF, functions in eyelid closure, and that ectodomain shedding of proHB-EGF is essential for this process, as is the case in cardiomyocytes and in the valvulogenesis (Yamazaki et al., 2003).

Previous cell culture studies indicate that ectodomain shedding of HB-EGF is regulated by multiple signaling cascades (Izumi et al., 1998; Prentzel et al., 1999; Takenobu et al., 2003; Umata et al., 2001). Interestingly, activation of RTKs stimulates HB-EGF shedding via the RAS-MAPK pathway (Umata et al., 2001). Thus, activation of EGFR by HB-EGF

Development

Development

may induce shedding of HB-EGF, forming an autocrine positive feedback loop. As shown in the present study, HB-EGF, activated EGFR and activated ERK were colocalized in the leading edge during eyelid closure, consistent with the existence of a feedback loop that stimulates HB-EGF shedding.

Among the proteases (convertases) that might induce ectodomain shedding of HB-EGF, ADAM17/TACE, a member of the ADAM family metalloproteases, is a prime candidate for functioning in this process in vivo (Lee et al., 2003). ADAM17-null mice had enlarged cardiac valves resembling those of HB-EGF-null mice (Jackson et al., 2003) and also exhibited an EOB phenotype (Sahin et al., 2004). ADAM17 has also been shown to be the sheddase for TGFα (Lee et al., 2003). These results suggest that ADAM17 may function in eyelid closure as a sheddase for HB-EGF and TGFα.

Although the precise mechanism is still unclear, we propose the following model for the mode of function of HB-EGF in mouse eyelid closure (Fig. 8). Before E15.0, the leading edge is not yet formed, and HB-EGF expression is not yet detected (Fig. 8A). At E15.0, the leading edge starts to extend, and the expression of HB-EGF appears in a few cells at the tip of the leading edge (Fig. 8B). Between E15.0 and E16.0 (Fig. 8C), HB-EGF is constitutively expressed at the tip of the extending leading edge. On the surface of the cells located in this region, ectodomain shedding of proHB-EGF may be mediated by ADAM17 directly or indirectly, and liberated sHB-EGF diffuses throughout the leading edge region. sHB-EGF then binds to and activates EGFR, activating the ERK pathway. This results in actin polymerization and promotion of epithelial cell sheet migration. TGFα also functions synergistically with HB-EGF, possibly in a similar manner to HB-EGF.

Detection of the endogenous proteins is important for the analysis of growth factor function in vivo; however, it is very difficult to obtain suitable antibodies to detect endogenous proteins in mouse tissues. In the present study, we were able to show the pattern of HB-EGF protein localization in the leading edge of eyelids using a monoclonal anti-mouse HB-EGF mAb (4D9), which was generated by immunizing HB-EGF null mice with recombinant mouse HB-EGF protein. Although many previous reports have examined HB-EGF localization using other anti-HB-EGF antibodies, this represents the first clear and specific detection of endogenous HB-EGF protein in mouse tissue. Thus, our new antibody will be a very useful tool for the analysis of HB-EGF function in mice in vivo.

In conclusion, in the present study we have demonstrated for the first time that HB-EGF signaling contributes to eyelid development. HB-EGF functions synergistically with TGFα in leading edge formation during eyelid closure, by promoting epithelial sheet migration through activation of the EGFR-ERK signaling cascade. We have demonstrated also that gene-expression, processing and protein localization of HB-EGF are strictly regulated temporally and spatially during eyelid closure process. Molecular mechanisms regulating these processes are remained unclear. Future study will be necessary to solve these issues.

We thank I. Ishimatsu and T. Yoneda for technical assistance. This work was supported by Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology (14580696 for R.I. and 16207014 for E.M.).

References

Du, X., Tabeta, K., Hoebe, K., Liu, H., Mann, N., Mudd, S., Crozat, K., Sovath, S., Gong, X. and Beutler, B. (2004). Velvet, a dominant Egfr mutation that causes wavy hair and defective eyelid development in mice. *Genetics* **166**, 331-340.

Elenius, K., Paul, S., Allison, G., Sun, J. and Klagsbrun, M. (1997). Activation of HER4 by heparin-binding EGF-like growth factor stimulates chemotaxis but not proliferation. *EMBO J.* **16**, 1268-1278.

Findlater, G. S., McDougall, R. D. and Kaufman, M. H. (1993). Eyelid development, fusion and subsequent reopening in the mouse. *J. Anat.* **183**, 121-129.

Fowler, K. J., Walker, F., Alexander, W., Hibbs, M. L., Nice, E. C., Bohmer, R. M., Mann, G. B., Thumwood, C., Maglitto, R., Danks, J. A. et al. (1995). A mutation in the epidermal growth factor receptor in waved-2 mice has a profound effect on receptor biochemistry that results in impaired lactation. *Proc. Natl. Acad. Sci. USA* **92**, 1465-1469.

Goishi, K., Higashiyama, S., Klagsbrun, M., Nakano, N., Umata, T., Ishikawa, M., Mekada, E. and Taniguchi, N. (1995). Phorbol ester induces the rapid processing of cell surface heparin-binding EGF-like growth factor: conversion from juxtacrine to paracrine growth factor activity. *Mol. Biol. Cell* **6**, 967-980.

Grose, R. (2003). Epithelial migration: open your eyes to c-Jun. *Curr. Biol.* **13**, R678-R680.

Harris, M. J. and Juriloff, D. M. (1986). Eyelid development and fusion induced by cortisone treatment in mutant, lidgap-Miller, foetal mice. A scanning electron microscope study. *J. Embryol. Exp. Morphol.* **91**, 1-18.

Harris, R. C., Chung, E. and Coffey, R. J. (2003). EGF receptor ligands. *Exp. Cell Res.* **284**, 2-13.

Hashimoto, K., Higashiyama, S., Asada, H., Hashimura, E., Kobayashi, T., Sudo, K., Nakagawa, T., Damm, D., Yoshikawa, K. and Taniguchi, N. (1994). Heparin-binding epidermal growth factor-like growth factor is an autocrine growth factor for human keratinocytes. *J. Biol. Chem.* **269**, 20060-20066.

Hayashi, Y., Liu, C.-Y., Jester, J. J., Hayashi, M., Wang, I.-J., Funderburgh, J. L., Saika, S., Roughley, P. J., Kao, C. W.-C. and Kao, W. W.-Y. (2005). Excess biglycan causes eyelid malformation by perturbing muscle development and TGF-α signaling. *Dev. Biol.* **277**, 222-234.

Higashiyama, S., Abraham, J. A., Miller, J., Fiddes, J. C. and Klagsbrun, M. (1991). A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science* **251**, 936-939.

Holbro, T. and Hynes, N. E. (2004). ErbB receptors: directing key signaling networks through life. *Annu. Rev. Pharmacol. Toxicol.* **44**, 195-217.

Iwamoto, R. and Mekada, E. (2000). Heparin-binding EGF-like growth factor: a juxtacrine growth factor. *Cytokine Growth Factor Rev.* **11**, 335-344.

Iwamoto, R., Senoh, H., Okada, Y., Uchida, T. and Mekada, E. (1991). An antibody that inhibits the binding of diphtheria toxin to cells revealed the association of a 27-kDa membrane protein with the diphtheria toxin receptor. *J. Biol. Chem.* **266**, 20463-20469.

Iwamoto, R., Yamazaki, S., Asakura, M., Takashima, S., Hasuwa, H., Miyado, K., Adachi, S., Kitakaze, M., Hashimoto, K., Raab, G. et al. (2003). Heparin-binding EGF-like growth factor and ErbB signaling is essential for heart function. *Proc. Natl. Acad. Sci. USA* **100**, 3221-3226.

Izumi, Y., Hirata, M., Hasuwa, H., Iwamoto, R., Umata, T., Miyado, K., Tamai, Y., Kurisaki, T., Sehara-Fujisawa, A., Ohno, S. et al. (1998). A metalloprotease-disintegrin, MDC9/meltrin-γ/ADAM9 and PKCδ are involved in TPA-induced ectodomain shedding of membrane-anchored heparin-binding EGF-like growth factor. *EMBO J.* **17**, 7260-7272.

Jackson, L. F., Qiu, T. H., Sunnarborg, S. W., Chang, A., Zhang, C., Patterson, C. and Lee, D. C. (2003). Defective valvulogenesis in HB-EGF and TACE-null mice is associated with aberrant BMP signaling. *EMBO J.* **22**, 2704-2716.

Jorissen, R. N., Walker, F., Pouliot, N., Garrett, T. P., Ward, C. W. and Burgess, A. W. (2003). Epidermal growth factor receptor: mechanisms of activation and signalling. *Exp. Cell Res.* **284**, 31-53.

Kiguchi, K., Bol, D., Carbajal, S., Beltran, L., Moats, S., Chan, K., Jorcano, J. and DiGiovanni, J. (2000). Constitutive expression of erbB2 in epidermis of transgenic mice results in epidermal hyperproliferation and spontaneous skin tumor development. *Oncogene* **19**, 4243-4254.

Lee, D. C., Sunnarborg, S. W., Hinkle, C. L., Myers, T. J., Stevenson, M. Y., Russell, W. E., Castner, B. J., Gerhart, M. J., Paxton, R. J., Black, R. A. et al. (2003). TACE/ADAM17 processing of EGFR ligands indicates a role as a physiological convertase. *Ann. New York Acad. Sci.* **995**, 22-38.

Li, C., Guo, H., Xu, X., Weinberg, W. and Deng, C. X. (2001). Fibroblast

growth factor receptor 2 (Fgfr2) plays an important role in eyelid and skin formation and patterning. *Dev. Dyn.* **222**, 471-483.

Li, G., Gustafson-Brown, C., Hanks, S. K., Nason, K., Arbeit, J. M., Pogliano, K., Wisdom, R. M. and Johnson, R. S. (2003). c-Jun is essential for organization of the epidermal leading edge. *Dev. Cell* **4**, 865-877.

Luetteke, N. C., Qiu, T. H., Peiffer, R. L., Oliver, P., Smithies, O. and Lee, D. C. (1993). TGF alpha deficiency results in hair follicle and eye abnormalities in targeted and waved-1 mice. *Cell* **73**, 263-278.

Luetteke, N. C., Phillips, H. K., Qiu, T. H., Copeland, N. G., Earp, H. S., Jenkins, N. A. and Lee, D. C. (1994). The mouse waved-2 phenotype results from a point mutation in the EGF receptor tyrosine kinase. *Genes Dev.* **8**, 399-413.

Luetteke, N. C., Qiu, T. H., Fenton, S. E., Troyer, K. L., Riedel, R. F., Chang, A. and Lee, D. C. (1999). Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development. *Development* **126**, 2739-2750.

Massague, J. and Pandiella, A. (1993). Membrane-anchored growth factors. *Annu. Rev. Biochem.* **62**, 515-541.

Matsubayashi, Y., Ebisuya, M., Honjoh, S. and Nishida, E. (2004). ERK activation propagates in epithelial cell sheets and regulates their migration during wound healing. *Curr. Biol.* **14**, 731-735.

Miettinen, P. J., Berger, J. E., Meneses, J., Phung, Y., Pedersen, R. A., Werb, Z. and Derynck, R. (1995). Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. *Nature* **376**, 337-341.

Piepkorn, M., Pittelkow, M. R. and Cook, P. W. (1998). Autocrine regulation of keratinocytes: the emerging role of heparin-binding, epidermal growth factor-related growth factors. *J. Invest. Dermatol.* **111**, 715-721.

Plowman, G. D., Culouscou, J. M., Whitney, G. S., Green, J. M., Carlton, G. W., Foy, L., Neubauer, M. G. and Shoyab, M. (1993). Ligand-specific activation of HER4/p180erbB4, a fourth member of the epidermal growth factor receptor family. *Proc. Natl. Acad. Sci. USA* **90**, 1746-1750.

Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C. and Ullrich, A. (1999). EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* **402**, 884-888.

Raab, G. and Klagsbrun, M. (1997). Heparin-binding EGF-like growth factor. *Biochim. Biophys. Acta.* **1333**, F179-F199.

Sahin, U., Weskamp, G., Kelly, K., Zhou, H. M., Higashiyama, S., Peschon, J., Hartmann, D., Saftig, P. and Blobel, C. P. (2004). Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. *J. Cell Biol.* **164**, 769-779.

Shirakata, Y., Kimura, R., Nanba, D., Iwamoto, R., Tokumaru, S., Morimoto, C., Yokota, K., Nakamura, M., Sayama, K., Mekada, E. et al. (2005). Heparin-binding EGF-like growth factor accelerates keratinocyte migration in skin wound healing. *J. Cell Sci.* **118**, 2363-2370.

Sibilia, M. and Wagner, E. F. (1995). Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science* **269**, 234-238.

Takenobu, H., Yamazaki, A., Hirata, M., Umata, T. and Mekada, E. (2003). The stress- and inflammatory cytokine-induced ectodomain shedding of heparin-binding epidermal growth factor-like growth factor is mediated by p38 MAPK, distinct from the 12-O-tetradecanoylphorbol-13-acetate- and lysophosphatidic acid-induced signaling cascades. *J. Biol. Chem.* **278**, 17255-17262.

Tanno, S., Ohsaki, Y., Nakanishi, K., Toyoshima, E. and Kikuchi, K. (2004). Small cell lung cancer cells express EGFR and tyrosine phosphorylation of EGFR is inhibited by gefitinib ('Iressa', ZD1839). *Oncol. Rep.* **12**, 1053-1057.

Threadgill, D. W., Dlugosz, A. A., Hansen, L. A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., Harris, R. C. et al. (1995). Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science* **269**, 230-234.

Umata, T., Hirata, M., Takahashi, T., Ryu, F., Shida, S., Takahashi, Y., Tsuneoka, M., Miura, Y., Masuda, M., Horiguchi, Y. and Mekada, E. (2001). A dual signaling cascade that regulates the ectodomain shedding of heparin-binding epidermal growth factor-like growth factor. *J. Biol. Chem.* **276**, 30475-30482.

Von Pawel, J. (2004). Gefitinib (Iressa, ZD1839): a novel targeted approach for the treatment of solid tumors. *Bull. Cancer* **91**, E70-E76.

Weston, C. R., Wong, A., Hall, J. P., Goad, M. E., Flavell, R. A. and Davis, R. J. (2004). The c-Jun NH2-terminal kinase is essential for epidermal growth factor expression during epidermal morphogenesis. *Proc. Natl. Acad. Sci. USA* **101**, 14114-14119.

Wong, R. W. (2003). Transgenic and knock-out mice for deciphering the roles of EGFR ligands. *Cell. Mol. Life Sci.* **60**, 113-118.

Xia, Y. and Kao, W. W. (2004). The signaling pathways in tissue morphogenesis: a lesson from mice with eye-open at birth phenotype. *Biochem. Pharmacol.* **68**, 997-1001.

Xia, Y. and Karin, M. (2004). The control of cell motility and epithelial morphogenesis by Jun kinases. *Trends. Cell Biol.* **14**, 94-101.

Xian, W., Rosenberg, M. P. and DiGiovanni, J. (1997). Activation of erbB2 and c-src in phorbol ester-treated mouse epidermis: possible role in mouse skin tumor promotion. *Oncogene* **14**, 1435-1444.

Yamazaki, S., Iwamoto, R., Saeki, K., Asakura, M., Takashima, S., Yamazaki, A., Kimura, R., Mizushima, H., Moribe, H., Higashiyama, S. et al. (2003). Mice with defects in HB-EGF ectodomain shedding show severe developmental abnormalities. *J. Cell Biol.* **163**, 469-475.

Zenz, R., Scheuch, H., Martin, P., Frank, C., Eferl, R., Kenner, L., Sibilia, M. and Wagner, E. F. (2003). c-Jun regulates eyelid closure and skin tumor development through EGFR signaling. *Dev. Cell* **4**, 879-889.

Zhang, L., Wang, W., Hayashi, Y., Jester, J. V., Birk, D. E., Gao, M., Liu, C. Y., Kao, W. W., Karin, M. and Xia, Y. (2003). A role for MEK kinase 1 in TGF-beta/activin-induced epithelium movement and embryonic eyelid closure. *EMBO J.* **22**, 4443-4454.