GPR48 Regulates Epithelial Cell Proliferation and Migration by Activating EGFR during Eyelid Development

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PURPOSE. Eyelid development is a dynamic process involving cell proliferation, differentiation, and migration regulated by a number of growth factors and cytokines. Mice deficient in the orphan G protein-coupled receptor 48 (GPR48) showed an eye open at birth (EOB) phenotype. In this study, the authors attempted to clarify the role of GPR48 in eyelid development and the molecular mechanisms leading to the EOB phenotype.

METHODS. Phenotypic analysis of the eyelids of Gpr48+/− mice was carried out using histology and scanning electron microscopy. GPR48 expression pattern was determined using X-gal staining. In vitro scratch assay was used to determine cell motility defects in Gpr48−/− keratinocytes. The molecular mechanism underlying GPR48-mediated eyelid closure was explored using Western blot and immunostaining analyses. Expression levels of EGFR and its phosphorylated counterpart were examined in Gpr48−/− and wild-type keratinocytes and in eyelids.

RESULTS. GPR48 is highly expressed in the epithelium and apical mesenchymal cells of eyelids during embryonic development. Detailed analysis revealed that Gpr48−/− mice exhibited delayed leading-edge extension, reduced filopodia formation, and decreased rounded periderm cell formation around eyelid margins. Keratinocytes lacking GPR48 are defective in cell proliferation and migration with reduced F-actin staining. In addition, the phosphorylation of EGFR was dramatically decreased in cultured keratinocytes and developing eyelids in the absence of GPR48.

CONCLUSIONS. Inactivation of GPR48 induces the EOB phenotype by reducing epithelial cell proliferation and migration, indicating that GPR48 plays an essential role in eyelid development. Furthermore, GPR48 contributes to eyelid development through the regulation of the EGFR signaling pathway. (Invest Ophthalmol Vis Sci. 2008;49:4245–4253) DOI:10.1167/iovs.08-1860

Mammalian eyelid development requires growth of the eyelid across the eye, fusion of its tips, and subsequent reopening. Mouse eyelid development starts at embryonic day (E) 11.5, characterized by a profusion of rounded periderm cells at the leading edge of the growing eyelid. The eyelids then grow, extend over the cornea, and move progressively toward the center of the eye. Fusion of the peridermal and epidermal layers of the epithelial sheets leads to eyelid closure at E15.5 to E16.5. The closed eyelid forms a protective covering over the ocular surface and permits normal eye development. At 12 to 14 days postpartum, the eyelids reopen. Failure in eyelid closure results in the eye open at birth (EOB) phenotype, leading to severe inflammation of the cornea and to partial blindness in adult mice.1,2

Embryonic eyelid formation is a temporally specific, morphogenetically organized developmental process that is easily observed in murine models. Studies of mice with the EOB phenotype led to the identification of various genes encoding growth factors and their receptors involved in embryonic eyelid closure, such as activin of the transforming growth factor (TGF)-β family, transforming growth factor (TGF)-α, and epidermal growth factor receptor (EGFR).3-9 Ablation of MEK kinase 1 (MEK1) and transcription factor c-Jun also results in the EOB phenotype.1,10-12 With the use of keratinocyte migration to examine the underlying mechanism, two signaling pathways have emerged as responsible for epithelial cell migration.13 One is the TGFβ/activin-induced MEKK1-JNK pathway, and the other is the TGFα/EGFR-induced ERK signaling pathway. Although TGFβ/activin signaling is essential in eyelid development, EGFR signaling is also indispensable for eyelid morphogenesis because interruption in EGFR signaling elicits the EOB phenotype.1,3,4,7,12,14-16

GPR48, also known as LGR4, belongs to the leucine-rich, G protein-coupled receptor (LGR) family. Members of the LGR family form a subfamily of G protein-coupled receptors.17 These transmembrane receptors are characterized by a large extracellular domain with multiple leucine-rich repeats at the N-terminus, which is proposed to be important for ligand binding.18 The LGRs appear very early in evolution, signifying their functional importance. The mouse Gpr48 gene contains 18 exons. Exon 1 encodes the signal peptide and the N-terminal leucine-rich repeat region, whereas exons 2 to 17 encode 17 leucine-rich repeats, and exon 18 encodes the seven-transmembrane domain and the intracellular region.19 However, the ligand(s) and functions for GPR48 are still unclear. To elucidate the physiological roles of the orphan receptor GPR48, Gpr48−/− deficient mice were generated using a se-
cretory trap approach to delete most of the Gpr48 gene or by targeted deletion of part of exon 18.2,20–22 Studies have implicated GPR48 as essential in development because Gpr48 null mice show in utero growth retardation and early neonatal lethality.21 The cause of early neonatal lethality remains unknown but is presumed to be related to renal dysfunction because null mice showed hypoplastic kidneys with an increased plasma concentration of creatinine.22 In addition, phenotypic interpretation of Gpr48−/− mice identified defective development in the renal, mammary, opthalmologic, and male reproductive tracts.22–24 Weng et al.2 also demonstrated that GPR48 regulates ocular anterior segment development and dysgenesis through the regulation of Pitx2, a key transcription factor.

In this study, we attempted to clarify the role of GPR48 in eyelid development and to elucidate the molecular mechanism of GPR48 in the regulation of the EOB phenotype. First, we showed the expression pattern of GPR48 during eyelid formation and closure, and then we provided a detailed phenotypic analysis of eyelid development in Gpr48 mutant embryos. Subsequently, we demonstrated that GPR48 is essential in epithelial cell proliferation and migration during mouse eyelid development. Finally, we provided evidence that GPR48 promotes cell proliferation and migration through the activation of EGFR by phosphorylation during eyelid development.

Materials and Methods

Mice

Gpr48−/− mice were generated as previously described.2 These mice were maintained on a mixed 129 × C57BL/6 background. Heterozygous mice were intercrossed to generate homozygous Gpr48−/− mice and wild-type littermate controls. For the timing of embryonic development after overnight mating, E0.5 was defined as noon of the day on which the vaginal plug was detected. Genotypes were determined by PCR analysis of tail DNA using three primers, as follows: primer A (5′ GCA GTC ACC ACT CTG ACA TCA CTA CA 3′), primer B (5′ ATT CCC GTA GGA GAT AGC GTC CTA G 3′), and primer C (5′ GGT CTT TGA GCA CAG GAC 3′). The size of the PCR product was approximately 1 kb from the wild-type allele and approximately 700 bp from the targeted allele. All studies and procedures were approved by the Wenzhou Medical College Animal Care and Use Committee. Experimental animals used in this study were handled with strict adherence to the guidelines set forth in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

X-Gal Staining

To perform X-gal staining, embryo mouse heads were embedded in frozen section medium (Neg50; Richard-Allen Scientific, Kalamazoo, MI) in preparation for making 10-μm thick frozen sections. The frozen sections were then placed on the slides. The slides were immediately incubated with the β-galactosidase fixative buffer (0.2% glutaraldehyde, 1.5% formaldehyde, 2 mM MgCl2, 5 mM EDTA, 0.1 M sodium phosphate buffer [pH 8]) for 30 minutes and washed three times for 15 minutes each in the washing buffer (0.1 M sodium phosphate buffer [pH 8], 2 mM MgCl2, 5 mM EDTA, 0.01% [wt/vol] sodium deoxycholate, 0.02% [vol/vol] Nonidet P40). Subsequently, the slides were incubated in the staining solution (washing buffer containing 1 mg/mL X-gal [Amersco, Solon, OH], 5 mM K4Fe(CN)6, 5 mM K3[Fe(CN)6] × 3 H2O) until results were optimized. The slides were then counterstained with eosin (Shanghai SSS Reagent Co., Shanghai, China) before they were evaluated and photographed under a microscope (Imager Z1; Carl Zeiss, Oberkochen, Germany).

Histology, Electron Microscopy, and Immunostaining

For histologic analysis, the heads of Gpr48−/− and Gpr48+/+ littermates at various stages of development (E12.5-E16.5) were collected, fixed in 4% paraformaldehyde, dehydrated with a graded ethanol series, and embedded in paraffin. Five-micrometer-thick sections were deparaffinized by immersion in xylene and rehydrated. Hematoxylin and eosin (H&E) staining was performed according to standard protocol.

For immunostaining, deparaffinized sections of specimens from E15.5 were first blocked with nonimmune goat serum in 0.05 M Tris buffer for 30 minutes. Primary antibodies for total EGFR and phosphorylated-EGFR (Cell Signal, Danvers, MA) were applied and incubated at 4°C overnight. After incubation, slides were washed with 1× PBS, incubated for 30 minutes with a biotinylated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature, and washed and treated with peroxidase-conjugated streptavidin for 30 minutes. After washing, 0.1% 3,3′-diaminobenzidine-4 HCl (DAB) in Tris buffer containing 0.01% H2O2 was used as the coloring reagent, and hematoxylin was used as the counterstain.

For ultramicrostructural analysis, the heads of Gpr48−/− and Gpr48+/+ embryos at E14.5, E15, and E16 were fixed in 2.5% glutaraldehyde and processed for scanning electron microscope observation according to standard procedures. The samples were evaluated and photographed under a scanning electron microscope (S-3000N; Hitachi, Yokohama, Japan).

Brdu Incorporation and TUNEL Assay

For Brdu uptake in embryos, pregnant mice were injected intraperitoneally with Brdu (Sigma, St. Louis, MO) at a dose of 100 μg/g body weight and then euthanatized 1 hour later. Immunohistochemical staining for Brdu was carried out with the use of a monoclonal antibody (DAKO, 1:100; DakoCytomation, Copenhagen, Denmark) and a mouse ABC kit (UniTect; Calbiochem, Darmstadt, Germany) according to the manufacturer’s instructions. Eighty cells in the epithelium of the eyelid (along the line outlining the contour of the epithelium, as shown in Figs. 4A, 4D) were counted starting from the bottom of the groove. The percentage of Brdu-positive cells out of the total 80 epithelial cells was determined. Every paraffin block was serially sectioned at 5-μm thickness, with three sections (1 in every 10 sections) counted for each embryo. To quantify the number of proliferating mesenchymal cells, the area close to the leading edge of the eyelid primordia was outlined as depicted in Figure 4A. Each area measured approximately 40,000 μm2. The number of Brdu-positive cells was then counted. TUNEL staining of apoptotic cells was performed using the in situ cell death detection kit (Roche, Mannheim, Germany).

Isolation and Culture of Primary Keratinocytes and Fibroblasts and In Vitro Brdu Assay

Isolation and culture of primary keratinocytes were performed according to the protocols described by Li et al.,7 with minor modifications. Briefly, 1- to 3-day-old mice were euthanatized, and their skins were removed, washed, and incubated in Disperse medium (defined keratinoocyte-serum free medium [defined K-SFM; Gibco, Grand Island, NY], 5 U/mL Disperse II [Sigma], 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL gentamicin) at 4°C overnight. The dermis was separated from the epidermis with forceps. The epidermis was then minced and digested in 0.25% trypsin for 15 minutes. Mouse keratinocyte culture medium, which consisted of defined K-SFM supplemented with 10 ng/mL EGF (PeproTech, Rocky Hill, NJ) and 10–15 M chola toxin, was used to culture the isolated primary keratinocytes.

To isolate fibroblasts, the dermis was separated from the epidermis, as described, and was minced and digested in 0.25% trypsin for 30 minutes. Cells were cultured in Dulbecco modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco).

To measure cell proliferation in vitro, primary keratinocytes or second-passaged fibroblasts were grown in a 12-well culture dish (Becton Dickinson Labware, Franklin Lakes, NJ) in 1 mL medium. The cells were incubated with Brdu (final concentration, 50 μg/mL) at 37°C in 5% CO2 for 5 hours, then fixed in methanol/acetone (1:1) for
5 minutes, washed in 1× PBS three times, and subsequently incubated with 95% formamide at 70°C for 45 minutes. Cell immunocytochemistry staining for BrdU was performed as described. The number of BrdU-positive cells was counted for 10 random high-power microscopic fields (400× objective) on each dish. The percentage of BrdU-positive cells was then calculated.

To determine cell motility, primary keratinocytes or second-passaged fibroblasts isolated from Gpr48lox/lox and Gpr48−/− mice were grown in a 12-well culture dish in 1 mL medium. At approximately 80% confluence, the cell monolayers were scratched using a 200-μL pipette tip, then washed twice with Hanks medium to remove the floating cells. Fresh medium (1 mL) was added so that culturing could be continued. Defined K-SFM containing 10 ng/mL EGF was added to culture keratinocytes, and DMEM without FBS was added to culture fibroblasts. Photographs were taken immediately after scratching and 48 hours after culturing. The number of cells that migrated to the gap (the line was approximately 1 mm long on both sides) was counted. For immunostaining, the cells were washed twice with 1× PBS and fixed in 4% paraformaldehyde solution in 1× PBS at room temperature for 20 minutes. Phalloidin (Alexa Fluor 568; Molecular Probes, Eugene, OR) was used to stain for F-actin. Photographs were taken under a microscope (Imager Z1; Carl Zeiss).

Western Blot Analysis
Gpr48lox/lox and Gpr48−/− primary keratinocytes were collected, washed three times with ice-cold 1× PBS, resuspended in 100 μL lysis buffer (50 mM Tris-HCl [pH 8], 1 mM EDTA, 2% SDS, 5 mM dithiothreitol, 10 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM Na3VO4, and protease inhibitor cocktail), denatured in boiling water for 5 minutes, and sonicated for 30 seconds. Lysates were centrifuged at 12,000 rpm for 20 minutes. Supernatant fractions were collected for Western blot analysis. The protein concentration was determined by the BCA assay kit (Pierce, Rockford, IL). Protein lysates (40 μg each) were separated by 6% (for phosphorylated-EGFR and total EGFR), 10% (for phosphorylated-Akt and total Akt), or 12% (for phosphorylated-c-Jun, total c-Jun, and α-tubulin) SDS-PAGE and were transferred to nitrocellulose membranes. After blocking nonspecific binding with blocking buffer (TBS containing 1% milk, 5% BSA, and 0.1% Tween 20) for 2 hours, the blots were probed with antibodies for phosphorylated EGFR (Tyr1173), phosphorylated Akt (Ser473), phosphorylated c-Jun (Ser63), and total EGFR, Akt, c-Jun, and α-tubulin. Blots were developed by a chemiluminescence (ECL) detection system (Pierce). Bands were quantified using ImageJ (for phosphorylated-Akt and total Akt), or 12% (for phosphorylated-c-Jun, total c-Jun, and α-tubulin) SDS-PAGE and were transferred to nitrocellulose membranes. After blocking nonspecific binding with blocking buffer (TBS containing 1% milk, 5% BSA, and 0.1% Tween 20) for 2 hours, the blots were probed with antibodies for phosphorylated EGFR (Tyr1173), phosphorylated Akt (Ser473), phosphorylated c-Jun (Ser63), and total EGFR, Akt, c-Jun, and α-tubulin. Blots were developed by a chemiluminescence detection system (ECL) and visualization system (Imager Z1; Carl Zeiss). The Student’s t-test was used to determine the significance of differences between population means. P < 0.05 was considered statistically significant.

RESULTS
GPR48 Is Required for Embryonic Eyelid Closure
Mice heterozygous for Gpr48 were created as previously reported and had no apparent abnormalities. Homozygous Gpr48−/− mice were generated by crossing Gpr48lox/lox mice. Genotypic analysis identified 58 Gpr48lox/lox mice out of a total of 350 mice. Interestingly, 49 of these 58 mice (84.5%) exhibited the EOB phenotype (Fig. 1A). In contrast, all 97 Gpr48lox/lox wild-type and 195 Gpr48−/− littermates showed the normal eyelid closed at birth phenotype. Eyelid development is critical for normal eye development. Without the protection of the eyelids, Gpr48−/− mutant adult mice exhibited exposure keratitis (data not shown). Therefore, to determine the specific role of GPR48 in eyelid development, we first sought to examine eyelid closure in Gpr48lox/− embryos and their wild-type littermates at different embryonic stages using H&E staining. In the wild-type mice, eyelids generally started to develop at E11.5. The eyelids folded up, extended over the cornea at E14.5, moved toward the center of the eye at E15.5, and closed by E16.5 (Fig. 1B). At E12.5, the eyelid morphology of Gpr48lox/− embryos looked similar to that of the wild-type controls except that the eyelid grooves between the eyelid and eyeball in the mutant embryos appeared to be shallower than those in the wild-type littermates (Fig. 1B). By E14.5, the eyelid grooves were growing even shallower in the mutant embryos than in the wild-type controls. By E15.5, the eyelids of Gpr48lox/− embryos moved toward the center more slowly than Gpr48lox/lox eyelids. The leading edges of the upper and lower eyelids started to extend from the eyelid roots in Gpr48lox/− embryos but not in Gpr48−/− embryos. No leading edge of the eyelids was formed in the mutant embryos at this stage. By E16.5, the eyelids of Gpr48lox/− embryos remained wide apart and failed to close, leaving the ocular surface exposed, whereas the Gpr48−/− embryos formed completely closed eyelids (Fig. 1B). These results indicate that GPR48 is crucial during the development of eyelids and eyelid closure.

GPR48 Is Expressed Mainly in the Basal Layer of Epithelium and Mesenchymal Cells of Eyelid Tips in the Developing Eyelid
To identify the specific role that GPR48 plays in eyelid development, we studied its expression pattern in mouse eyelid primordia during embryonic development. Because the gene trap vector used to generate Gpr48-deficient mice contains the β-galactosidase gene, the expression of β-galactosidase correlates with GPR48 expression. Thus, the expression of GPR48 can be determined by staining tissues with X-gal in Gpr48 heterozygous mice and homozygous mice.

X-gal staining of heterozygous embryos at E12.5 demonstrated that GPR48 was highly expressed in the epithelium and the mesenchymal cells of the eyelid, notably in the epithelium of the groove between the eyelid and the eyeball (blue staining; Figs. 2A–2A′). GPR48 was also intensely expressed in the eyelid (Figs. 2A–2A′). At E14.5, GPR48 was predominantly expressed in the basal layer of the epithelium and the mesenchymal cells of the eyelid tip (Figs. 2B–2B′), whereas at E16.5, GPR48 was expressed mainly in the basal layer of the epithelium and the mesenchymal cells next to the junctional area of the fused eyelids (Figs. 2C–2C′). Taken together, these results show that GPR48 is predominantly expressed in the cells with high proliferative potential, such as cells in the basal layer of epithelium, mesenchymal cells in the developing eyelid tips, and rudimentary cells of the eyelid. Conversely, cells that are already differentiated, such as cells in the superficial layer of epithelium and cornea stroma late in the embryonic period, expressed little or no detectable level of GPR48. Thus, the expression pattern of GPR48 in the eyelid during embryonic development suggested that GPR48 is involved in cell proliferation and plays an essential role in eyelid development.

Morphology of Eyelid Epithelium Is Defective in Gpr48−/− Embryos
To search for detailed morphologic changes in the eyelid epithelium of mutant embryos, we performed scanning electron microscopy. Gpr48lox/− and Gpr48−/− embryos displayed a similar eye opening morphology at E14.5 except that the
Eyelid grooves appeared to be deeper in wild-type embryos (Fig. 3A). However, higher magnification showed that filopodia (or microvilli) were markedly fewer and shorter in the Gpr48/H11002/H11002 epithelium at E14.5 than in the wild-type epithelium (Fig. 3B), indicating that GPR48 is important for the elongation and growth of filopodia. Filopodia have been shown to be essential for fusion between epithelial sheets.\textsuperscript{11,12} Thus, the deletion of Gpr48 may affect the closure of upper and lower eyelids by reducing filopodia formation, ultimately leading to the EOB phenotype in Gpr48/H11002/H11002 mice. At E15, the upper and lower eyelids started to move toward each other in wild-type embryos (oval shaped). In mutant embryos, however, the eyelids stayed wide apart (round shaped). Higher magnification views showed that rounded periderm cells were infrequently seen at the inner canthus and not seen at the outer canthus or the upper or lower eyelid margins (Fig. 3C). The rounded periderm cells had high proliferation and migration potential.\textsuperscript{27} Therefore, deficiency in GPR48 may affect cell proliferation and migration during eyelid development and eyelid closure. At E16, the eyelids were closed in the Gpr48\textsuperscript{+/+} fetuses but still open in the Gpr48\textsuperscript{--/} fetuses (Fig. 3A).

GPR48 Regulates Cell Proliferation in the Early Phase of Eyelid Development

The process of eyelid closure requires the coordination of cell proliferation and migration. To investigate the mechanisms underlying the observed defects in the mutant eyelids, we first examined cell proliferation during eyelid development using a BrdU incorporation assay. At E12.5, the epithelium of the upper eyelid of Gpr48\textsuperscript{--/} embryos showed a significant decrease in cell proliferation compared to wild-type embryos. At E14.5 and E15.5, this decrease was more pronounced, indicating that GPR48 is involved in regulating cell proliferation during the early phase of eyelid development.
crease in proliferation because only 30.8% ± 1.6% of these cells were BrdU positive, whereas 42.8% ± 4.1% of Gpr48−/− cells were BrdU positive (n = 4; Figs. 4A, 4B). Although the epithelia of the lower eyelids in mutant embryos showed decreased proliferation compared with the epithelia of the wild-type littermates, the changes were less prominent (Figs. 4A, 4B). The total number of proliferating mesenchymal cells in the eyelid tip was also assessed. As shown in Figs. 4A and 4C, significantly reduced numbers of proliferating mesenchymal cells were observed in the upper eyelids of the mutant embryos in comparison with their wild-type littermates (82.3 ± 4.2 vs. 115.8 ± 2.2; n = 3; P < 0.05). The difference in proliferation detected in the lower eyelid, however, did not reach statistical significance (76.7 ± 1.3 vs. 90.4 ± 6.5; n = 3). By E14.5, there was no significant difference in the number of proliferating epithelial cells or mesenchymal cells between

Figure 3. Eyelid epithelium defects in Gpr48−/− mice. Scanning electron micrographs of the eyelids from Gpr48+/+ and Gpr48−/− embryos are shown. (A) The eyelid is closed at E16 in the Gpr48−/− embryo but not in the Gpr48+/+ embryo. (B) Filopodia (microvilli) are rare and short in the Gpr48−/− eyelid epithelium compared with those in the Gpr48+/+ embryo at E14.5. (C) Higher magnification of the eyelid at E15 is shown. In, out, up, and low indicate different regions of the eyelid, as shown in (A). Clumps of rounded peridermal cells are present along the eyelid margin of Gpr48−/− embryo (arrows) but are rarely seen in Gpr48+/+ eyelid. in, inner canthus region; out, outer canthus region; up, upper eyelid; low, lower eyelid. Scale bars: (A) 500 μm; (B) 5 μm; (C) 50 μm.

Figure 4. Impaired cell proliferation in the developing eyelid epithelium in Gpr48−/− mice. BrdU analysis of the E12.5 eyelid is shown in (A–C), whereas the E14.5 eyelid is shown in (D, E). (A) Histologic sections for BrdU staining of the eyelid at E12.5. (B) Mean percentage of BrdU-positive cells in upper eyelid or lower eyelid based on the data from (A). Eighty cells along the contour of the epithelium (starting from the bottom of the groove) were counted, and the percentage of BrdU-positive cells was determined. Results are expressed as the mean ± SEM for the data obtained from four embryos of each genotype. (C) The total number of proliferating mesenchymal cells in the eyelid primordia, per 40,000 μm² as depicted in (A), was determined. Results are expressed as the mean ± SEM for the data obtained from three embryos of each genotype. (D) Histologic sections for BrdU staining of the eyelid at E14.5. (E) The percentage of BrdU-positive cells from the experiment depicted in (D) was determined. Results are expressed as the mean ± SEM for the data obtained from three embryos of each genotype. *Differences in cell proliferation in the eyelid from Gpr48+/+ and Gpr48−/− mouse embryos were significant (P < 0.05). up, upper eyelid; low, lower eyelid. Scale bars, (A, D) 100 μm.
Gpr48<sup>−/−</sup> and Gpr48<sup>+/−</sup> eyelids (Figs. 4D, 4E, and data not shown). These results indicate that GPR48 is only partially required for cell proliferation during the early phase of eyelid development.

To determine whether the reduced extension of Gpr48<sup>−/−</sup> eyelid was exacerbated by an increase in apoptosis, we also analyzed sections using an in situ TUNEL assay. No noticeable changes in the apoptotic cell numbers were detected in Gpr48<sup>−/−</sup> eyelids compared with their wild-type counterparts at E15.5 (data not shown). Taken together, these results demonstrate that GPR48 is important for cell proliferation at the onset of eyelid tip development but is not required for cell death.

**GPR48 Is Involved in Keratinocyte Proliferation and Migration In Vitro**

To further determine whether the absence of GPR48 affected cell proliferation in vitro, we isolated and cultured primary keratinocytes, which are the major constituents of eyelid epidermis, from newborn wild-type and mutant mice. Cells were cultured in the presence of low levels (10 ng/mL) of EGF. A dramatic decrease in proliferation was observed in Gpr48<sup>−/−</sup> keratinocytes compared with wild-type cells (26.8% ± 2.8% vs. 41.2% ± 1.2%; n = 3; P < 0.05; Figs. 5A, 5B). To determine the cell-type specificity of GPR48 function, we also isolated and cultured dermal fibroblasts, another major component of the developing eyelids. In contrast to what was observed in primary keratinocytes, Gpr48<sup>−/−</sup> fibroblasts exhibited no detectable difference in proliferation when compared with wild-type cells (31.5% ± 1.2% vs. 31.7% ± 1.0%; n = 3; Figs. 5A, 5B).

Cell migration plays an integral role in epithelial fusion, leading to eyelid closure. Therefore, it is possible that GPR48 also regulates the epithelial cell migration required for eyelid closure. To determine the importance of GPR48 in eyelid closure, we carried out an in vitro scratch assay. Far fewer Gpr48<sup>−/−</sup> keratinocytes than wild-type keratinocytes migrated to the gap within 48 hours (112.7 ± 11.5 vs. 196.2 ± 19.1; n = 6; P < 0.05; Fig. 6A). In addition, no significant difference in cell migration was observed between Gpr48<sup>+/−</sup> and Gpr48<sup>−/−</sup> fibroblasts (69.0 ± 6.6 vs. 69.7 ± 4.8; n = 3; Fig. 6F).

Previous studies have shown that migrating epithelial cells exhibited actin reorganization.11 Cellular levels and distribution of F-actin are essential for cell migration. Therefore, we assessed the cellular levels and distribution of F-actin by immunofluorescent staining with phalloidin. As seen in Figure 6B, actin staining was intense, with organization into stress fibers in Gpr48<sup>+/−</sup> keratinocytes. In contrast, Gpr48<sup>−/−</sup> cells stained less avidly for actin and displayed a more flattened morphology (Fig. 6C). Furthermore, filopodia were observed abundantly only in Gpr48<sup>+/−</sup> keratinocytes, with fewer and shorter extensions seen in Gpr48<sup>−/−</sup> keratinocytes (Figs. 6D, 6E). These results corresponded to the in vivo data shown in Figure 3B. However, no noticeable difference in F-actin between Gpr48<sup>+/−</sup> and Gpr48<sup>−/−</sup> fibroblasts was observed (Figs. 6G-6J). Taken together, these results also demonstrate that GPR48 plays an important role only in keratinocyte cell migration during eyelid development.

**GPR48 Activates the EGFR Signaling Pathway**

To explore the molecular mechanism underlying GPR48-mediated eyelid closure, we examined the expression levels of EGFR and phosphorylated EGFR and c-Jun and phosphorylated c-Jun in Gpr48<sup>−/−</sup> and wild-type keratinocytes. Activation of EGFR signaling has been shown to be essential in the regulation of eyelid development, as evidenced by phenotypic abnormalities seen in spontaneous mutation or knockout mice in which EGFR signaling is interrupted.1,3,4,7,12,14–16 Western blot analysis showed that though total EGFR expression was unaffected in the mutant keratinocytes (Fig. 7A), the activation of EGFR by phosphorylation was reduced 1.6- to 2.1-fold in Gpr48<sup>−/−</sup> keratinocytes compared with Gpr48<sup>+/−</sup> keratinocytes (n = 3; Fig. 7A). In the developing eyelid, EGFR phosphorylation was also readily detected in Gpr48<sup>−/−</sup> eyelid sections at E15.5 but was reduced in Gpr48<sup>−/−</sup> eyelids (Fig. 7B). Expression of the EGFR protein in the developing eyelid was not substantially affected in the absence of GPR48 (Fig. 7B). Thus, these results demonstrated that the deletion of Gpr48<sup>−/−</sup> affected the activation of EGFR signaling, which in turn is critical for embryonic eyelid closure.

Given that the EOB phenotype was also identified in mice with a specific ablation of transcription factor c-Jun in keratinocytes,1,12 we reasoned that the GPR48-EGFR cascade may function through c-Jun, whose phosphorylation could be one of the end points of GPR48-EGFR signaling. Unexpectedly, Gpr48<sup>−/−</sup> keratinocytes expressed similar levels of phosphorylated c-Jun compared with wild-type keratinocytes (Fig. 7A). Total c-Jun expression was similar in wild-type and mutant keratinocytes (Fig. 7A).

EGFR signaling has also been linked to the regulation of cell survival through Akt activation.28 Thus, expressions of Akt and phosphorylated Akt were also examined in those cells. As shown in Figure 7A, neither total Akt nor phosphorylated Akt expression was altered in the absence of GPR48, consistent with the observation that the number of apoptotic cells was
 approach, to delete most of the staining was observed in anti–EGFR antibody. Reduced p-EGFR migration was observed between anti–p-EGFR antibody (Fig. 2). First we characterized the EOB phenotype of its high expression levels in epithelial cells of the growing normal eyelid morphogenesis, an observation consistent with regulator in embryonic eyelid closure. GPR48 is essential for metastasis.21–25,29 In this study, we demonstrated that GPR48, a member of the G protein–coupled receptors, is an important metastasis.21–25,29 In this study, we demonstrated that GPR48, a member of the G protein–coupled receptors, is an important regulator in embryonic development, cell motility, and tumor migration. Scanning electron microscopy showed a significant reduction of rounded proliferative periderm cells around the eyelid margins in Gpr481/1/– mice (Fig. 3C). BrdU incorporation analysis of Gpr481/1/– eyelid sections further revealed that the number of proliferating epithelial cells was decreased at E12.5, indicating that GPR48 may affect epithelial cell proliferation during the early stages of eyelid development (Figs. 4A, 4B).

In vitro studies using isolated keratinocytes from eyelid epidermis showed keratinocytes lacking GPR48 expression exhibited much slower proliferation (Fig. 5) and migration (Fig. 6) than did wild-type keratinocytes. The requirement of GPR48 for cell proliferation was found to be keratinocyte specific because fibroblasts isolated from dermis were unaffected by the absence of GPR48 expression (Fig. 5). In addition, keratinocytes lacking GPR48 expression (Fig. 5) and migration (Fig. 6) than did wild-type keratinocytes. The requirement of GPR48 for cell proliferation was found to be keratinocyte specific because fibroblasts isolated from dermis were unaffected by the absence of GPR48 expression (Fig. 5).

FIGURE 6. Deletion of Gpr48 causes impaired keratinocyte migration. Monolayers of primary mouse epidermal keratinocytes (A) or dermal fibroblasts (F) were subjected to in vitro scratch assays. Photographs were taken immediately or 48 hours after scratching, as indicated in (A) and (F). (A) More wild-type keratinocytes than Gpr481/1/– keratinocytes were observed to migrate to the gap within 48 hours. (B–E) Keratinocytes were stained with Alexa Fluor 568 phalloidin for F-actin (red) and DAPI for nuclei (blue). Filopodia were observed abundantly in Gpr481/1/– keratinocytes but not in Gpr481/1/– keratinocytes. (F) No difference in migration was observed between Gpr481/1/– fibroblasts and Gpr481/1/– fibroblasts. (G–J) No morphologic difference in Factin expression was detected between Gpr481/1/– and Gpr481/1/– fibroblasts. 1/1, Gpr481/1/–; 1/–, Gpr481/1/–. Scale bars: (B, C, I, J) 50 μm; (D, E) 20 μm; (G, H) 100 μm.

unchanged in the absence of GPR48 (data not shown). Taken together, these results demonstrate that GPR48 regulates epithelial cell proliferation and migration by regulating EGFR signaling.

DISCUSSION

Recent studies have underscored the importance of GPR48 for its role in embryonic development, cell motility, and tumor metastasis.21–25,29 In this study, we demonstrated that GPR48, a member of the G protein–coupled receptors, is an important regulator in embryonic eyelid closure. GPR48 is essential for normal eyelid morphogenesis, an observation consistent with its high expression levels in epithelial cells of the growing eyelid (Fig. 2). First we characterized the EOB phenotype of the Gpr481/1/– mice, generated through a secretory trap approach, to delete most of the Gpr48 gene with insertion of a β-galactosidase reporter gene immediately after exon 1.β21 Results demonstrated that GPR48 affected epithelial cell proliferation and migration.

FIGURE 7. GPR48 was required for the activation of EGFR. (A) Western blot analysis of EGFR, EGFR phosphorylation, Akt, Akt phosphorylation, and c-Jun, c-Jun phosphorylation in response to EGF (10 ng/mL) treatment in wild-type (+/+) and Gpr481/1/– (1/–) keratinocytes is shown. (B) Histologic sections of Gpr481/1/– and Gpr481/1/– embryos from E15.5 were immunostained with anti–p-EGFR antibody (top) or anti-EGFR antibody. Reduced p-EGFR staining was observed in Gpr481/1/– eyelids compared with Gpr481/1/– eyelids, p-EGFR, phosphorylated EGFR; p-Akt, phosphorylated Akt; p-c-Jun, phosphorylated c-Jun. α-tubulin. Scale bars, (B) 100 μm.
tion, in vitro scratch assay showed that the number of migrating cells was reduced in the absence of GPR48 expression (Fig. 6A). Studies have shown that migrating cells exhibited actin cytoskeleton reorganization. F-actin, in the form of actin cables, has been detected realigning along the leading edges during eyelid development. F-actin staining of keratinocytes in vitro showed that the numbers and lengths of filopodia on the edges of Gpr48−/− cells were substantially reduced (Figs. 6B–6E). Scanning electron microscopy confirmed in vivo the reduction of filopodia in the epithelium of the growing eyelid at E14.5 (Fig. 3B). Specifically, GPR48 is only involved in cell migration and reorganization of the actin cytoskeleton of keratinocytes because cell migration and F-actin staining of fibroblasts were unaffected in the absence of GPR48 expression (Figs. 6F–6I). These studies demonstrated that the inactivation of GPR48 induced an EOB phenotype by reducing cell proliferation and migration in the developing eyelid.

A recent study by Kato et al. generated another Gpr48−/− mutant by deleting part of exon 18, which encodes for the transmembrane and signal-transducing domains of the receptor. Comparison between their null mutant and the one used in this study revealed many phenotypic similarities and several major discrepancies. Phenotypically, our Gpr48−/− mutant mice showed high perinatal mortality, with approximately 60% of newborn mice dying in the first postpartum week (data not shown). Only 84.5% of newborn mice exhibited an EOB phenotype. In contrast, the Gpr48−/− mutant generated by Kato et al. had complete embryonic/neonatal mortality. Mutant mice were born under the 25% expected frequency from cross-breeding of Gpr48 heterozygous mice. Gpr48 null mice that survived in utero died shortly after birth in almost all cases. The typical phenotype of their mutant mice was the EOB phenotype with complete penetrance.

The discrepancy between the Gpr48−/− phenotypes is most likely attributed to species variation and mode of knockout generation. Studies by Mendive et al. have demonstrated that Gpr48−/− mice are viable on the CD1 genetic background, and most of the offspring reached adulthood. In contrast, only 40% of the inbred C57BL/6j knockout strain was viable at birth, and most of the surviving newborns died 1 day after birth. In addition, Hoshii et al. recently reported that approximately 85% of the homozygous mice of the C57BL/6j genetic background died during the postnatal development period. With the CBA genetic background, 60% of the offspring survived. Kato et al. using a 129Ola × C57BL/6 hybrid background, found a higher incidence of embryonic/neonatal lethality than reported in the studies cited here. Different knockout strategies also contribute to the discrepancy in the phenotypes. Compared with the study by Mazzerbourg et al., conducted with the same gene-trapped embryonic stem (ES) cell line technique used in this study, Kato et al. observed a greater incidence of neonatal lethality and a more profound renal histopathologic phenotype through targeted deletion of part of the gene by homologous recombination in ES cells.

Differences in cellular phenotype of the two Gpr48 mutant keratinocytes allowed us to study the mechanism of GPR48 signaling transduction. In both studies, GPR48 was important for cell migration, as evidenced by in vitro cell scratch assay, but was unrelated to cell death based on apoptosis assay. However, in contrast to the study by Kato et al., proliferation in cultured keratinocytes in this study was dramatically reduced in the absence of GPR48. More important, we were able to reveal decreased phosphorylation of EGFR in the absence of GPR48 but no change in total EGFR, c-Jun, or phosphorylated c-Jun on Western blot analysis (Fig. 7A). We further substantiated, by immunostaining techniques, our assertion that EGFR phosphorylation was reduced in Gpr48−/− eyelids (Fig. 7B).

To our knowledge, this is the first report linking GPR48 receptor with EGFR activation in epithelial cell proliferation and migration.

Previous studies have shown that activation of EGFR by several ligands, such as EGF, TGFα, and heparin-binding EGF-like growth factor (HB-EGF), is critical for the regulation of epidermal development, including eyelid morphogenesis. Deficiency in EGFR signaling in waved-1, TGFα null, and EGFR-knockout mice is associated with the EOB phenotype. Impaired EGFR signaling has also been implicated in the defective eyelid morphogenesis in mice lacking transcription factor c-Jun. Furthermore, the overexpression of biglycan leads to defects in eyelid development resulting from interruption of the EGFR signaling pathway. Interestingly, in this study, though the expression of EGFR was unaltered, EGFR phosphorylation was dramatically reduced in Gpr48−/− keratinocytes (Fig. 7), indicating that the EGFR signaling pathway was attenuated in the absence of GPR48.

Studies of mice with the EOB phenotype have demonstrated that embryonic eyelid development requires at least two signaling pathways. One is the c-Jun–controlled pathway through the activation of EGFR by its ligands, leading to eyelid closure. The other appears to be the TGFβ/activin-induced MEKK1-JNK pathway, which induces act polymerization and c-Jun phosphorylation. Both pathways are essential for eyelid development because in vivo deletion of either pathway results in the EOB phenotype. MEKK1-deficient mice exhibit several phenotypes, similar to Gpr48−/− mice, including decreased rounded cells along the eyelid margin, reduced keratinocyte migration, and decreased F-actin staining. Given that MEKK1 is also expressed in the eyelid epithelium, it would be interesting to determine whether GPR48 interacts with the TGFβ family, perhaps through activin, which can induce the MEKK1-JNK pathway.

Furthermore, the two pathways have been suggested to be connected through the transcription factor c-Jun. c-Jun appears to be essential for eyelid development because keratinocyte-specific ablation of c-Jun in mice leads to the EOB phenotype. Therefore, on examination of the expression of c-Jun and the phosphorylation of c-Jun in Gpr48−/− keratinocytes, we were surprised to find that both total c-Jun and phosphorylation of c-Jun were unaffected by the absence of GPR48 (Fig. 7A). The possibility that GPR48 is involved in c-Jun regulation still cannot be excluded because Western blot analysis of keratinocytes included proliferating and differentiated cells. Detection of c-Jun expression in proliferating cells may be suppressed by the abundance of quiescent, nonproliferating keratinocytes. Thus, the process by which GPR48 regulates the activation of EGFR, which in turn activates downstream target genes, ultimately leading to cell proliferation and migration, awaits further investigation.

The possibility that alternative pathways leading to the EOB phenotype, such as the bone morphogenetic protein (BMP [members of the TGFβ superfamily]) signaling pathway, should also be considered. BMP signaling is involved in eyelid opening through the regulation of apoptosis and cell differentiation. Overexpression of the BMP antagonist noggin leads to the inhibition of eyelid opening. The effects of the BMP signaling pathway on eyelid opening appear to be distinct from the effects of activin because overexpression of the activin antagonist follistatin results in precocious eyelid opening (EOB phenotype at birth). Mouse embryonic eyelid closure, which involves cell proliferation, migration, and morphogenesis, is a developmental process with significant implications in many physiological and pathologic processes. Epithelial cells are essential in serving as a protective barrier; hence, dysregulation may lead to impaired wound healing and even tumorigenesis. Studies by Wankell et
al.32 substantiated these findings by showing that impairment in activin signaling resulted in delayed healing of skin wounds. Moreover, c-Jun null mice showed not only delayed skin wound healing but also reduced tumor formation, as did mice deficient in TGFα or EGFR signaling.1,12.20 A recent study found that GPR48 can enhance colon cancer cell invasiveness and metastasis, suggesting that GPR48 is involved in carcinoma cell motility and may play an important role in tumorigenesis.39 Understanding the molecular mechanism underlying the GPR48/EGFR-mediated signaling pathway and identifying molecular factors important for eyelid closure might serve as potential targets for pharmaceutical intervention for diseases that result from the dysregulation of epithelial cell migration and morphogenesis. In conclusion, in the present study, we have demonstrated that GPR48 contributes to eyelid development by promoting cell proliferation and migration through regulation of the EGFR signaling pathway.

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References