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GPR48-Induced keratinocyte proliferation occurs through HB-EGF mediated EGFR transactivation

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ABSTRACT

GPR48 can mediate keratinocyte proliferation and migration. Our investigations showed that AG1478, an inhibitor of EGFR tyrosine kinase, could block GPR48-mediated cellular processes. AG1478 treatment of *Gpr48*$^{+/+}$ cells also decreased phosphorylation of EGFR, ERK and STAT3. Subsequent screening using conditioned media immunodepleted of EGFR ligands identified HB-EGF as the ligand responsible for phosphorylation of EGFR, ERK and STAT3. HB-EGF was reduced in *Gpr48*$^{-/-}$ cell culture medium, but its addition restored the phosphorylation of EGFR, ERK, STAT3, as well as cell proliferation. Confirmation that GPR48 mediates EGFR signaling pathway through HB-EGF was subsequently performed using an inhibitor of HB-EGF.
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1. Introduction

The signaling pathways controlling the proliferation and migration of epidermal keratinocytes serve as a model for the understanding of epithelial cell migration and growth responsible for wound healing and tumorigenesis. Studies had demonstrated that activation of the epidermal growth factor (EGF) receptor by several ligands, such as EGF, heparin binding EGF (HB-EGF), and transforming growth factor-α (TGF-α), is a central event in the regulation of epithelial cell proliferation and migration [1,2]. EGFR activation can trigger several signaling pathways including extracellular signal-regulated kinase 1/2 (ERK1/2), signal transducer and activator of transcription 3 (STAT3), and c-Jun, which are all important for eyelid closure and keratinocyte migration [2–4]. Similarly, deficiencies in members of the EGFR pathway including TGF-α, HB-EGF and EGFR using knockout mice have been shown to be associated with the eye open at birth (EOB) phenotype [2,3].

We have demonstrated previously that inactivation of G protein-coupled receptor 48 (GPR48) also induced the EOB phenotype by reducing epithelial cell proliferation and migration through EGFR signaling [5]. *Gpr48* deficient mice, generated using a secretory trap approach to delete the majority of the *Gpr48* gene or by targeted deletion of part of exon 18, are responsible for a wide range of developmental defects ranging from the renal system, the ocular system, eyelids and the male reproductive tracts [5–11]. Although GPR48 is an orphan receptor, the physiologic functions that are being attributed to this receptor continue to intrigue us by its diversity [12–14]. In this study, we explored the signaling pathways that GPR48 utilizes in keratinocyte proliferation and migration using *Gpr48*$^{-/-}$ keratinocytes. With the help of various chemical inhibitors, each step of the EGFR pathway was sampled and compared with a *Gpr48*$^{-/-}$ background to confirm its necessity in EGFR transactivation.

2. Materials and methods

2.1. Mice

Gpr48$^{+/-}$ mice were generated as previously described [11]. Heterozygous mice were intercrossed to generate homozygous

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Gpr48$^{-/-}$ *mice. All studies were approved by the Wenzhou Medical College Animal Care and Use Committee.*

2.2. BrdU assay in vitro

Isolation of primary murine keratinocytes was performed according to the protocols described previously [5]. To measure cell proliferation, cultured primary keratinocytes were pre-treated with AG1478 (1 μM; Calbiochem) or HB-EGF (20 ng/ml; R&D Systems) for 48 h, then incubated with BrdU (50 μg/ml; Sigma) for 5 h, fixed in methanol/acetone (1:1), and subsequently incubated with 95% formamide at 70 °C for 45 minutes. Immunochemical staining for BrdU was performed with the use of a monoclonal antibody (DAKO, 1:100; DakoCytomation) and a mouse ABC kit (UniTect; Calbiochem). The number of BrdU-positive cells was counted for ten random high power microscopic fields (400X objective).

2.3. In vitro scratch assay

To determine cell motility, primary keratinocytes grown to ~80% confluence were scratched using a 200-μl pipette tip, and continued culture with AG1478 (1 μM) or HB-EGF (20 ng/ml) for 48 h. The number of migrating cells was quantified as described previously [5].

2.4. Immunoblot and co-immunoprecipitation analysis

Gpr48$^{+/+}$ and *Gpr48*$^{-/-}$ cells were harvested and analyzed by Western blotting as described previously [5]. Pretreatment of wildtype keratinocytes with CRM197 (Sigma) was performed at different concentrations prior to cell harvesting [15]. Protein lysates (50 μg each) were separated by 6% (for p-Tyr, p-EGFR and total EGFR), 10% (for p-STAT3 and total STAT3), 12% (for p-ERK1/2, JNK, and total ERK1/2, JNK), 15% (for HB-EGF) SDS–PAGE, and transferred to Schleicher and Schuell nitrocellulose membranes (Whatman). After incubating with blocking buffer, the blots were probed with antibodies for p-Tyr (PY99), p-EGFR (Tyr 1173), p-STAT3 (Tyr 705), p-ERK(Ser 42/44), p-JNK (Thr 183/Tyr 185), total EGFR, STAT3, ERK, JNK, HB-EGF. The blots were developed by the supersignal chemiluminescence system (Pierce). Antibodies for total EGFR, STAT3, ERK, JNK, and p-STAT3, ERK, JNK were from Cell Signal; antibodies for p-Tyr (PY99), p-EGFR, total HB-EGF were from Santa Cruz Biotechnology. For co-immunoprecipitation, cell lysates were pre-cleared by incubation with nProtein A Sepharose 4 Fast (GE healthcare) for overnight at 4 °C with gentle rotation. Beads were washed extensively with washing buffer and immune complexes were eluted in 2X loading buffer, boiled, microcentrifuged and subjected to immunoblot analysis.

2.5. Immunodepletion of EGFR ligands from conditioned medium

Conditioned medium (CM) was prepared as described previously [16]. One milliliter of CM was immunoprecipitated overnight at 4 °C with 10 μg of EGFR ligand antibodies (TGF-α, HB-EGF, and EGF) or control antibodies pre-coupled to nProtein A Sepharose 4 Fast. The immunocomplexes were separated from the CM by centrifugation through a 0.45 mM Ultrafree-MC filter unit (Millipore), and the CM was tested for its ability to activate EGFR, ERK and STAT3 of *Gpr48*$^{-/-}$ primary keratinocytes. TGF-α, HB-EGF, and EGF antibodies were from Santa Cruz Biotechnology.

2.6. Analysis of HB-EGF in conditioned medium

The CM, containing 1000 μg of protein, was immunoprecipitated overnight at 4 °C with 10 μg of HB-EGF antibody. The immunocomplexes were separated by 15% SDS–PAGE and analyzed by Western blotting.

2.7. Statistical analysis

All applicable data are shown as mean values ± S.E.M. The Student's *t*-test was used to determine the significance of differences between population means.

3. Results

3.1. GPR48 is required for EGFR phosphorylation

Western blot analysis showed that although the total EGFR expression was unaffected in mutant keratinocytes, the activation of EGFR was reduced in *Gpr48*$^{-/-}$ keratinocytes (Fig. 1A). Introduction of AG1478 [17] also reduced EGFR phosphorylation in *Gpr48*$^{+/+}$ keratinocytes, to a level comparable to that observed in *Gpr48*$^{-/-}$ keratinocytes (Fig. 1A). The densities of the phosphorylated-EGFR bands were 219.7 ± 36.1 and 114.3 ± 54.2 for wildtype and mutant keratinocytes without AG1478 (Fig. 1B). The density measured 130.7 ± 40.9 and 78.0 ± 15.6 for wildtype and mutant keratinocytes with AG1478, respectively.

3.2. EGFR phosphorylation is required for keratinocyte proliferation and migration

A decrease in proliferation was observed in *Gpr48*$^{-/-}$ keratinocytes compared with *Gpr48*$^{+/+}$ cells, as shown previously [5]. After the addition of AG1478 to cultured *Gpr48*$^{+/+}$ keratinocytes, cell proliferation decreased to levels similar to *Gpr48*$^{-/-}$ keratinocytes (Fig. 2A and B). In BrdU assays, wildtype cells without treatment with AG1478 showed a 49.6 ± 1.2% ($n = 3$) rate of proliferation,

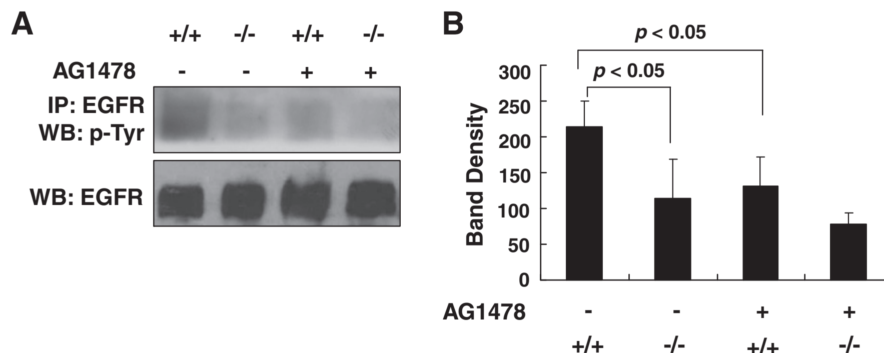

Fig. 1. GPR48 is required for the phosphorylation of EGFR. (A) Western blot analysis of EGFR and p-EGFR in wildtype and *Gpr48*$^{-/-}$ keratinocyte is shown. Presence of AG1478 or absence of GPR48 led to lower density of p-EGFR. (B) Quantification of band density in (A) is shown.

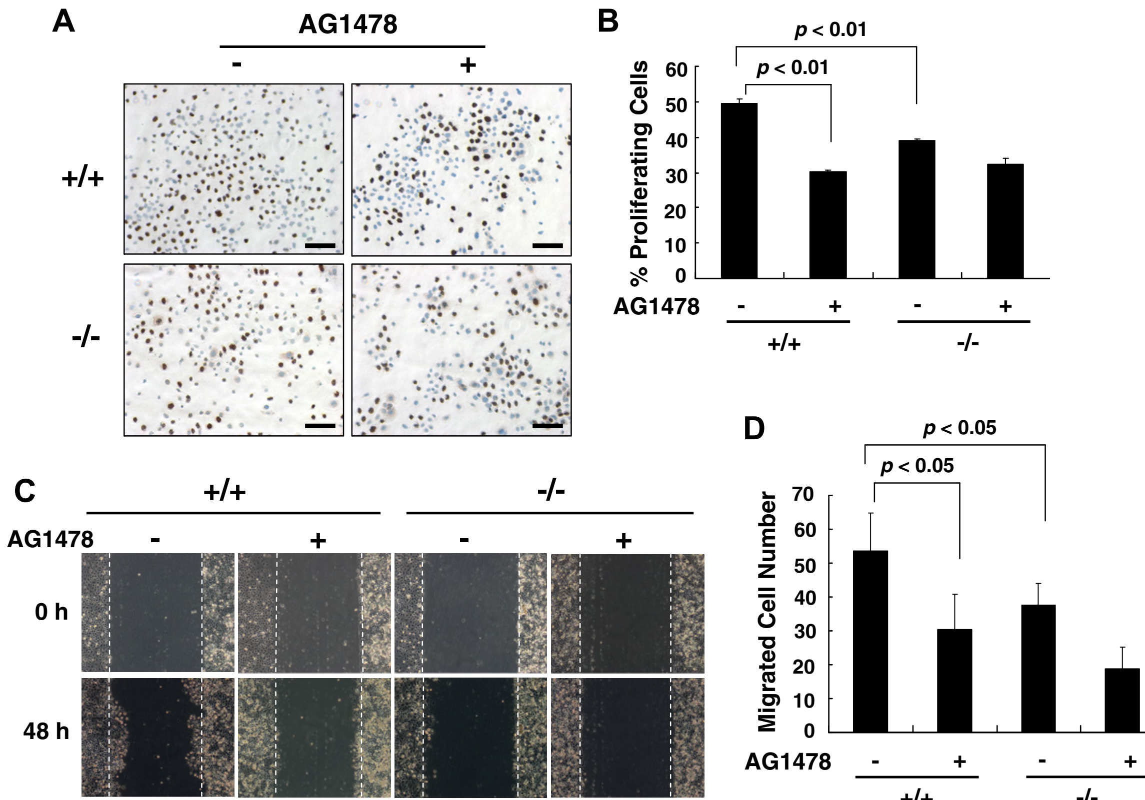

Fig. 2. GPR48-induced EGFR activation is important for keratinocyte proliferation and migration. (A) Treatment with AG1478 or the absence of GPR48 inhibited the proliferation of keratinocytes in BrdU assays. (B) Quantification of BrdU-positive cells from (A) is shown. (C) Monolayers of keratinocytes were subjected to *in vitro* scratch assays. Both $Gpr48^{-/-}$ and treatment with AG1478 inhibited the migration of keratinocytes. (D) The quantity of migrating cells from (C) was determined by counting the number of the cells that migrated across the gap.

which was significantly higher when compared to wildtype with treatment (30.2 ± 0.2%), mutants without (38.9 ± 0.7%) and with (32.1 ± 1.8%) treatment.

As previously shown, $Gpr48^{-/-}$ keratinocytes had decreased migration in *in vitro* scratch assays [5]. The addition of AG1478 resulted in significantly fewer $Gpr48^{+/+}$ cells migrating into the gap when compared to non-treated $Gpr48^{+/+}$ keratinocytes, but comparable to $Gpr48^{-/-}$ keratinocytes (Fig. 2C and D). Total number of cells that migrated into the gap in wildtype without AG1478 treatment was 53.6 ± 11.1 ($n = 3$), which was significantly different from wildtype with treatment (30.3 ± 10.5) or mutants without (37.5 ± 6.4) or with (18.9 ± 6.4) treatment.

3.3. GPR48 is required for the activation of ERK and STAT3 in the EGFR signaling pathway

Western blot analysis showed that although total ERK and STAT3 expression was unaffected in the mutant keratinocytes, the activation of ERK and STAT3 by phosphorylation was reduced in $Gpr48^{-/-}$ keratinocytes compared with $Gpr48^{+/+}$ keratinocytes (Fig. 3A). Unexpectedly, $Gpr48^{-/-}$ keratinocytes expressed similar levels of both total and phosphorylated JNK compared with wildtype keratinocytes (Fig. 3A). As shown in Fig. 3B, the treatment of $Gpr48^{+/+}$ keratinocytes with AG1478 for 20 minutes resulted in diminished levels of phosphorylated ERK and STAT3, down to levels observed with $Gpr48^{-/-}$ cells.

3.4. GPR48-mediated EGFR transactivation occurs through HB-EGF

In EGFR transactivation, external stimuli activate metalloproteinases of the zinc-dependent disintegrin and metalloproteinase family, resulting in the release of precursor forms of the EGFR ligands such as EGF, HB-EGF and TGF-α [18–21]. $Gpr48^{+/+}$ conditioned medium immunodepleted of EGF or TGF-α did not affect EGFR, ERK and STAT3 phosphorylation of $Gpr48^{-/-}$ keratinocytes (Fig. 4A). In contrast, $Gpr48^{+/+}$ CM immunodepleted with HB-EGF antibody harbored a reduced ability to stimulate EGFR, ERK and STAT3 phosphorylation. Conditioned medium obtained from confluent cultures of $Gpr48^{+/+}$ and $Gpr48^{-/-}$ primary keratinocytes showed that $Gpr48^{-/-}$ cells expressed much lower levels of soluble HB-EGF when compared to $Gpr48^{+/+}$ cells (Fig. 4B). Phosphorylation

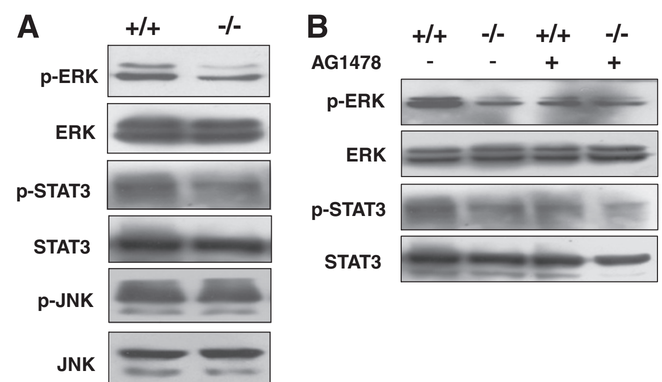

Fig. 3. GPR48 increases ERK and STAT3 phosphorylation. (A) Western blot analysis of ERK, STAT3, JNK and phorphorylated ERK, STAT3, and JNK in wildtype and $Gpr48^{-/-}$ keratinocytes. (B) Following treatment with AG1478, Western blot analysis of wildtype cell lysates showed decreased phosphorylation of ERK and STAT3 in comparison to control, but similar to the amount produced by $Gpr48^{-/-}$ keratinocytes.

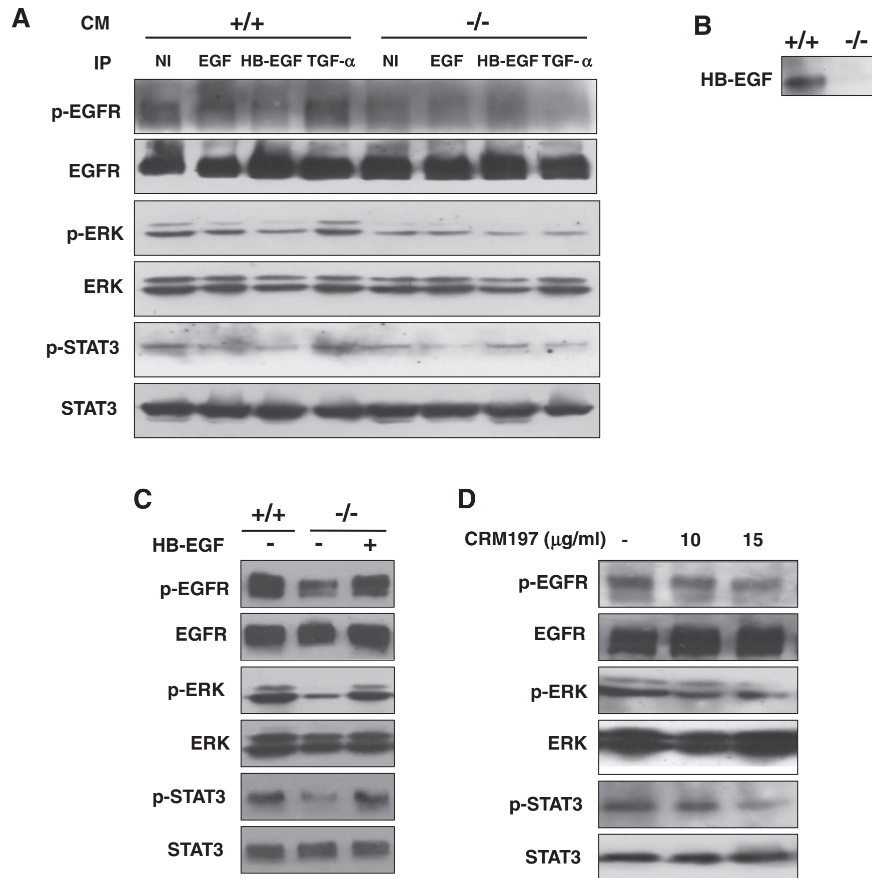

Fig. 4. HB-EGF is required for the GPR48-induced EGFR phosphorylation. (A) Depletion of HB-EGF in CM inhibited the phosphorylation of EGFR and the EGFR downstream target molecules. CM of *Gpr48*$^{+/+}$ keratinocytes immunodepleted of HB-EGF showed a reduced ability to stimulate phosphorylation of EGFR, ERK and STAT3 of *Gpr48*$^{-/-}$ keratinocytes, similar to CM of *Gpr48*$^{-/-}$ keratinocytes. (B) HB-EGF was decreased in the CM of *Gpr48*$^{-/-}$ primary keratinocytes. (C) The treatment of *Gpr48*$^{-/-}$ primary keratinocytes with HB-EGF resulted in a higher level of phosphorylated-EGFR (p-EGFR) in comparison to those without treatment. (D) Wildtype primary keratinocytes were pre-treated with 10 or 15 μg/ml of CRM197, an inhibitor of HB-EGF, for 30 minutes, the cells were harvested and subjected to Western blot analysis against phosphorylated EGFR, ERK and STAT3.

of EGFR, ERK and STAT3 in *Gpr48*$^{-/-}$ keratinocytes could be rescued to a level similar to that of *Gpr48*$^{+/+}$ keratinocytes, after the addition of HB-EGF (Fig. 4C). Pretreatment of *Gpr48*$^{+/+}$ keratinocytes with CRM197, a HB-EGF inhibitor, decreased GPR48-induced EGFR, ERK and STAT3 activation (Fig. 4D).

3.5. HB-EGF is important for GPR48-mediated cell proliferation

As shown in Fig. 5A, the addition of HB-EGF to cultured *Gpr48*$^{-/-}$ keratinocytes allowed cell proliferation to increase to levels similar to those of *Gpr48*$^{+/+}$ keratinocytes. The addition of HB-EGF to cultured *Gpr48*$^{-/-}$ keratinocytes was only partially effective (not statistically significant) in restoring the ability of cells to migrate in the scratch assay (Fig. 5B).

4. Discussion

Transactivation of EGFR by G protein-coupled receptors (GPCR) illustrates the complexities of inter-receptor communication, with the potent EGFR signaling pathway being activated by

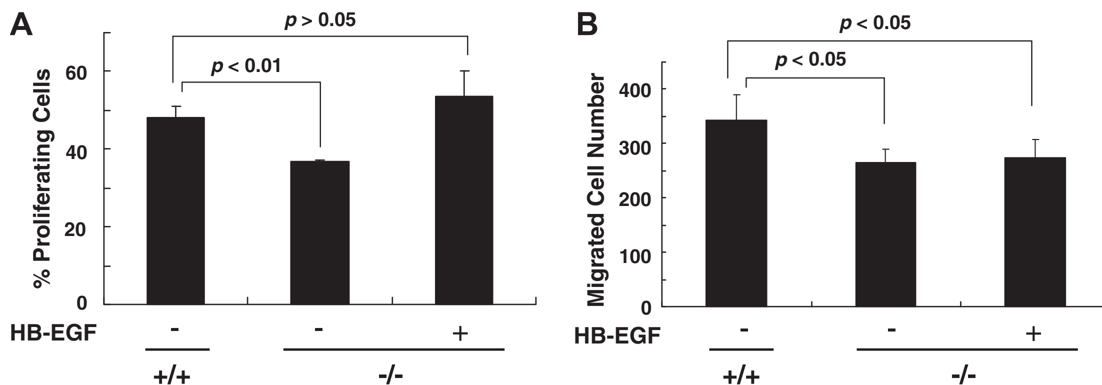

Fig. 5. HB-EGF can rescue the proliferation of *Gpr48*$^{-/-}$ keratinocytes. (A) HB-EGF can rescue the proliferation of *Gpr48*$^{-/-}$ primary keratinocytes based on BrdU assays. (B) Cell migration, though improved, was not significantly increased in *Gpr48*$^{-/-}$ primary keratinocytes after the addition of HB-EGF.

a ubiquitous and incompletely understood receptor, GPCR. GPCR ligands act synergistically leading to autocrine/paracrine loops that contribute to cell proliferation and migration [18]. Aberrant regulation of GPCRs has been implicated in the development of tumors in this microenvironment [21,22].

We have previously demonstrated that GPR48 regulates epithelial cell proliferation and migration by activating EGFR during eyelid development [5]. Initial investigations using inhibitors of EGFR activation by AG1478 demonstrated an essential role of EGFR signaling in epithelial cell proliferation and migration. Addition of AG1478 led to decreased levels of ERK and STAT3 with concurrent anti-proliferative and anti-migratory effects on *Gpr48*$^{+/+}$ keratinocytes (Figs. 2 and 3). Our results suggest that ERK and STAT3 play an essential role in transducing a signal required for proliferation and migration of keratinocytes.

The activation of EGFR is mediated, at least in part, by HB-EGF, which is cleaved from its membrane-anchored form (pro-HB-EGF) by specific metalloproteinases. Release of transmembrane HB-EGF serves as a strong stimulus in the transactivation of EGFR by G-protein-coupled receptors [19], which ultimately leads to increased cell proliferation and migration. In this study, we showed that HB-EGF is the primary ligand responsible for GPR48-mediated EGFR signaling (Fig. 4). Western blot analysis confirmed the role of HB-EGF and discovered that the expression level of phosphorylated EGFR, ERK and STAT3 were all dramatically decreased in *Gpr48*$^{-/-}$ keratinocytes. Functionally, HB-EGF was able to rescue the proliferation of *Gpr48*$^{-/-}$ keratinocytes to a level similar to *Gpr48*$^{+/+}$ keratinocytes (Fig. 5). Blockade of HB-EGF by CRM197 inhibited the GPR48-induced activation of EGFR, ERK and STAT3 in wildtype epidermal keratinocytes.

Although the soluble form of HB-EGF is a potent mitogen and chemoattractant for many cell types, the catalyst in the proteolytic processing of HB-EGF remains elusive. Physiologic roles as a result of proteolytic release of HB-EGF include keratinocyte migration in cutaneous wound healing [4], cell migration in eyelid development [2], cornea epithelial cell migration [23], cardiac hypertrophy [20] and lung epithelial cell movements in response to gram-positive bacterial challenge [24]. Members of the ADAM family of proteinases such as ADAM9, ADAM10, ADAM12, and ADAM17 are implicated in the endoproteolytic release of HB-EGF [20,25–27]. Our own studies have also shown that the expression level of matrix metalloproteinase 7 (MMP-7) is significantly higher in *Gpr48*$^{+/+}$ keratinocytes than in *Gpr48*$^{-/-}$ cells (data not shown).

Overall, our data indicated that GPR48 is one of the constituents responsible for the EGFR signaling pathway with its effects directly linked to its ligand, HB-EGF. The ability of GPR48 to initiate the cascade of events leading to EGFR transactivation is a highly interconnected and coordinated process. Despite the current expanding understanding in this arena, it remains a fertile ground for investigation into the molecular mechanisms behind cellular proliferation and tumorigenesis.

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