Metalloproteinase-Mediated, Context-Dependent Function of Amphiregulin and HB-EGF in Human Keratinocytes and Skin

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Human keratinocytes (KCs) express multiple EGF receptor (EGFR) ligands; however, their functions in specific cellular contexts remain largely undefined. To address this issue, first we measured mRNA and protein levels for multiple EGFR ligands in KCs and skin. Amphiregulin (AREG) was by far the most abundant EGFR ligand in cultured KCs, with >19 times more mRNA and >7.5 times more shed protein than any other family member. EGFR ligand expression in normal skin was low (<8‰ of RPLP0/36B4); however, HB-EGF and AREG mRNAs were strongly induced in human skin organ culture. KC migration in scratch wound assays was highly metalloproteinase (MP)- and EGFR dependent, and was markedly inhibited by EGFR ligand antibodies. However, lentivirus-mediated expression of soluble HB-EGF, but not soluble AREG, strongly enhanced KC migration, even in the presence of MP inhibitors. Lysophosphatidic acid (LPA)-induced ERK phosphorylation was also strongly EGFR and MP dependent and markedly inhibited by neutralization of HB-EGF. In contrast, autocrine KC proliferation and ERK phosphorylation were selectively blocked by neutralization of AREG. These data show that distinct EGFR ligands stimulate KC behavior in different cellular contexts, and in an MP-dependent fashion.

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INTRODUCTION

Substantial evidence implicates EGF-like growth factor activity in the regulation of cell migration, proliferation, survival, and differentiation of normal and malignant epithelial cells (Hashimoto *et al.*, 1994; Piepkorn *et al.*, 1998; Yarden and Sliwkowski, 2001). Human keratinocytes (KCs) express multiple EGF-like growth factors including amphiregulin (AREG), betacellulin (BTC), epiregulin (EREG), heparin-binding EGF-like growth factor (HB-EGF), and TGF-α in an autocrine fashion (Coffey *et al.*, 1987; Barnard *et al.*, 1994; Hashimoto *et al.*, 1994; Piepkorn *et al.*, 1998; Shirakata *et al.*, 2000). All EGF receptor (EGFR) ligands are synthesized as membrane-bound precursors that require MP-mediated proteolytic cleavage to produce the soluble, mature forms (for review see (Sanderson *et al.*, 2006)). Although paracrine/juxtacrine signaling by transmembrane precursors has been shown to mediate biological effects in some experimental systems (Miyoshi *et al.*, 1997; Iwamoto and Mekada, 2000; Singh *et al.*, 2004; Willmarth and Ethier, 2006), findings from numerous studies strongly suggest that major EGF-like growth factor functions including cell proliferation depend on proteolytic release of soluble EGFR ligands from their membrane-bound precursors (Peschon *et al.*, 1998; Yamazaki *et al.*, 2003; Sanderson *et al.*, 2006).

EGF-like growth factors bind to one or more members of the ErbB family of receptor tyrosine kinases, which include the EGFR, also known as ErbB1 or HER1, ErbB2 (HER2 or neu), ErbB3 (HER3), and ErbB4 (HER4) (Sanderson *et al.*, 2006). Ligand binding results in conformational changes of the extracellular receptor domains (Burgess *et al.*, 2003) initiating signaling mechanisms that regulate multiple cellular responses such as migration, proliferation, differentiation, and survival (Yarden and Sliwkowski, 2001; Citri and Yarden, 2006).

Human KCs express substantial levels of EGFR, ErbB2, and ErbB3 but no detectable ErbB4 protein (Press *et al.*, 1990; Prigent *et al.*, 1992; De Potter *et al.*, 2001; Stoll *et al.*, 2001) suggesting that EGF-like growth factor signaling in KCs proceeds through the formation of EGFR homo- or EGFR/ErbB2 and/or EGFR/ErbB3 heterodimers.

Although the function of EGFR ligands in human KCs appears to be highly redundant (Coffey *et al.*, 1987; Cook *et al.*, 1991; Barnard *et al.*, 1994; Hashimoto *et al.*, 1994;

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Abbreviations: Ab, antibody; AREG, amphiregulin; BTC, betacellulin; EGF, epidermal-growth factor; EGFR, EGF receptor; ELISA, enzyme-linked immunosorbent assay; EPGN, epigen; EREG, epiregulin; ERK, extracellular signal-regulated kinase; GF, growth factor; GPCR, G protein-coupled receptor; HB-EGF, heparin-binding EGF-like growth factor; KC, keratinocyte; LPA, lysophosphatidic acid; MP, metalloproteinase; QRT-PCR, quantitative real time polymerase chain reaction; TET, tetracycline; TGF-α, transforming growth factor-α

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Shirakata *et al.*, 2000; Strachan *et al.*, 2001), the importance of individual growth factors in specific cellular contexts has not been elucidated. From animal models and other experimental systems it is known that EGF-like growth factors have distinct roles in various tissues. For example, HB-EGF has been shown to be important for wound healing (Marikovsky *et al.*, 1993; Stoll *et al.*, 1997; Tokumaru *et al.*, 2000), arteriosclerosis (Nakata *et al.*, 1996), blastocyst implantation (Das *et al.*, 1994), and heart function (Iwamoto *et al.*, 2003; Jackson *et al.*, 2003; Yamazaki *et al.*, 2003), whereas AREG has been implicated in mammary gland development (Sternlicht *et al.*, 2005) and targeted expression of AREG in the epidermis results in a dermatosis with many similarities to psoriasis (Cook *et al.*, 1997, 2004). Both AREG and HB-EGF have been shown to be important for retinoic acid-induced epidermal hyperproliferation (Varani *et al.*, 2001; Rittié *et al.*, 2006). TGF-α is implicated in hair follicle development and eye formation (Luetteke *et al.*, 1993), whereas EREG appears to be a mediator of dermatitis and lung metastasis (Shirasawa *et al.*, 2004; Sternlicht *et al.*, 2005; Gupta *et al.*, 2007). BTC null mice have no detectable defects (Luetteke *et al.*, 1999) however, in transgenic animals it was recently shown that BTC regulates hair follicle development and angiogenesis during wound healing (Schneider *et al.*, 2008).

In this study, we asked whether the autocrine expression of different EGFR ligands by KCs reflects the existence of nonredundant signaling mechanisms that become activated in different cellular contexts, and whether MPs are necessary for their activation. In pursuit of these objectives, we measured the effects of inhibitors of MP, EGFR, and EGF-like growth factor function in the contexts of wound-induced KC migration and proliferation. We also assessed the potency of various EGF-like growth factors on EGFR activation, as well as their effects on autocrine and LPA-induced ERK phosphorylation.

RESULTS

Expression of EGFR ligands in human KCs and normal and organ-cultured human skin

To characterize the context-dependent function of EGFR ligands in KC physiology, we first measured EGF-like growth factor mRNA expression and protein shedding in subconfluent cultures of proliferating KCs using QRT-PCR and a multiplex EGFR ligand assay. As shown in Figure 1a, normal human KCs express high levels of AREG (transcript levels were 596‰ of 36B4 control gene expression) whereas EPGN, EREG, HB-EGF and TGF-α were expressed at significantly lower levels (transcript levels 5.8, 17.3, 31.3, and 26.9‰ of 36B4, respectively). BTC message levels in proliferating KCs were nearly 40,000 times less abundant than AREG transcripts. AREG was also the most abundant EGFR ligand protein shed into the culture medium (Figure 1b), whereas EREG, HB-EGF, and TGF-α were present at much lower levels. BTC and EPGN were not included in the study due to the lack of reliable detection reagents.

We next measured EGFR ligand mRNA levels in normal and organ-cultured human skin. As depicted in Figure 1c, expression of all EGF-like growth factors in normal human skin was very low (<8‰ of 36B4 control gene transcript levels). However, HB-EGF was strongly induced after 4 hours of human skin organ culture (>32-fold, increasing to 140‰ of 36B4). AREG expression was also markedly induced after

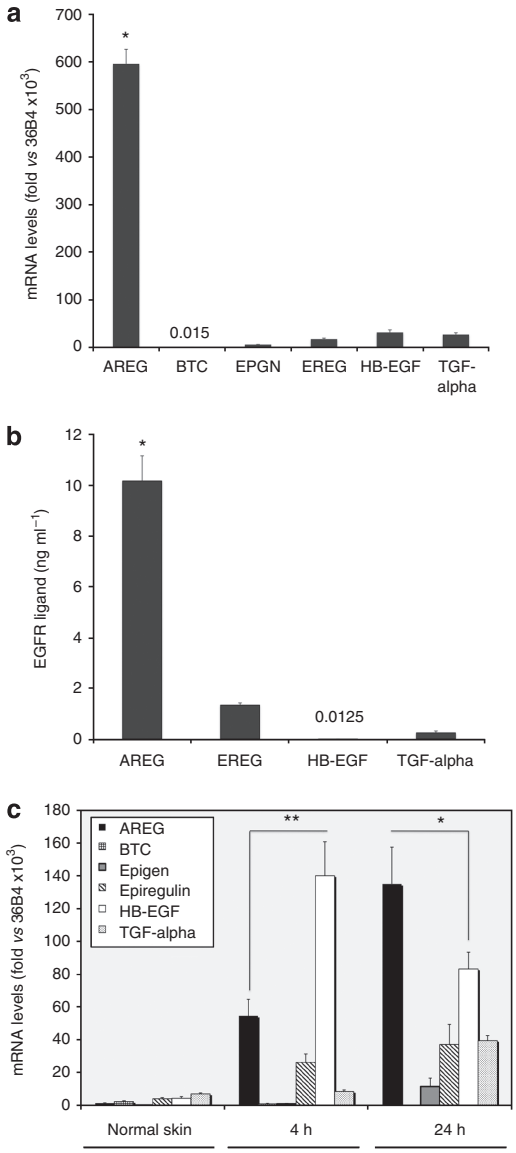

Figure 1. EGFR ligand expression and shedding in human KCs and normal and organ-cultured human skin. (**a**) Normal human KCs were plated at 5% confluence, grown as described in Materials and Methods and harvested ~70–95% confluence. Relative EGFR ligand mRNA expression was analyzed by QRT-PCR. Values represent fold-change *versus* the control gene 36B4 times 10^3 (mean ± SEM, $n=8$). Asterisk denotes a significant difference in AREG expression relative to other ligands with $P<10^{-6}$. (**b**) KCs were plated in 60 mm dishes and grown as described above and 24 hours accumulation of shed EGFR ligands in the culture medium of near-confluent KC cultures were assayed using a Multiplex Ligand Assay as described in Materials and Methods. Data are expressed as ng of shed growth factor protein per ml of KC culture medium, mean ± SEM, $n=8$, $^*P<0.0001$ *versus* all other growth factors shown. Soluble HB-EGF could only be detected in four out of eight samples. (**c**) Total RNA was isolated from full-thickness 3 mm punch biopsies of normal human skin either immediately (control) or from biopsies subjected to human skin organ culture for 4 or 24 hours and EGFR ligand mRNA was analyzed by QRT-PCR. Data represent fold-change *versus* the control gene 36B4 times 10^3, $n=5$–8, $^*P<0.05$, $^{**}P<0.007$.

4 hours of organ culture, but to a significantly lesser extent than HB-EGF. After 24 hours of organ culture HB-EGF expression declined by nearly half whereas AREG expression doubled, making it the most strongly expressed EGFR ligand at this time point (135‰ of 36B4).

HB-EGF is a potent mediator of KC migration *in vitro*

To determine the relative importance of various components of the EGFR system for KC migration, we performed scratch wound assays on near-confluent KC monolayers in the presence or absence of inhibitors of EGFR signaling, EGF-like growth factor function, and MP activity. As shown in Figure 2a, KCs showed vigorous migration in scratch wound assays that could only be slightly improved through addition of 10 ng ml^{-1} EGF. Interfering with EGFR signaling using the EGFR blocking antibody (Ab) 225 IgG or the pan-ErbB RTKI PD158780 or MP activity using either GM6001 or MMPI-3, markedly reduced scratch wound-induced migration.

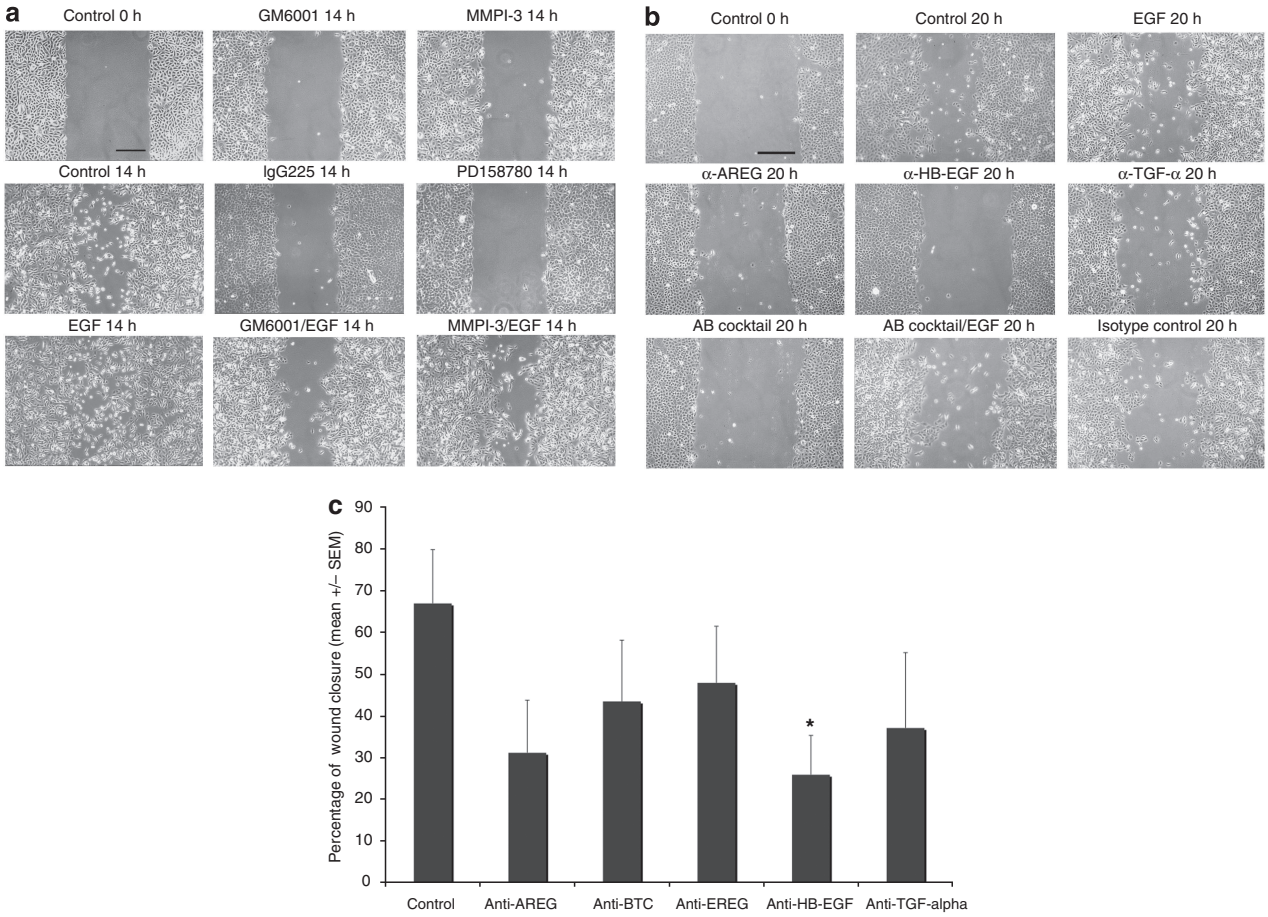

Figure 2. HB-EGF is a potent inducer of KC migration *in vitro*. (**a**) Scratch-wounded, confluent KC monolayers were incubated in basal M154 medium in the presence or absence of EGF (10 ng ml^{-1}), GM6001 (40 μM), MMPI-3 (25 μM), PD158780 (1 μM) and/or IgG225 (5 μg ml^{-1}). Scratch wounds were photographed by phase contrast microscopy after the indicated times. The results for individual panels are representative of at least two independent experiments. Scale bar = 400 μm. (**b**) Scratch-wounded KC monolayers were incubated in basal M154 in the presence or absence of neutralizing Abs against EGFR ligands alone or in combination (Ab cocktail) or isotype control Abs (each at 5 μg ml^{-1}) with and without EGF (10 ng ml^{-1}) for 20 hours. Scale bar = 400 μm. (**c**) Confluent KC cultures were scratch wounded and incubated for 18–24 hours as described above (b). Digital images of representative areas were quantitated by measuring the scratch surface area using AxioVision-LE software. Data are expressed as percent wound closure relative to controls at $t = 0$ hour (% wound closure = (100−((scratch surface area at $t = 18$–24 hours/surface area at $t = 0$ hour) × 100)), $n = 4$ independent experiments, $^*P \leqslant 0.05$ *vs* control. (**d**) N-TERT, N-TERT-TR, and N-TERT, or N-TERT-TR stably infected with lentivirus constructs encoding transmembrane (tm) and soluble (s) AREG (N-TERT-tmAREG, N-TERT-sAREG) and transmembrane and soluble HB-EGF (N-TERT-TR-tmHB-EGF, N-TERT-TR-sHB-EGF) were grown to confluence, scratch wounded and incubated in basal KSFM medium for 36 hours in the presence or absence of 40 μM GM6001 (MPI). HB-EGF expression in N-TERT-TR-HB-EGF cells was induced with 1 μg ml^{-1} TET at least 3 hours before scratch wounding. Scale bar = 400 μm. (**e**) Confluent cultures of N-TERT and N-TERT-TR with and without lentivirus-mediated expression of sAREG or sHB-EGF were scratch wounded and incubated for 18–36 hours in basal KSFM in the presence or absence of 40 μM GM6001 or 25 μM MMPI-3 as described above (d). Tetracycline-induced expression of HB-EGF in N-TERT-TRsHB-EGF cells is indicated by "+ TET". Digital images of representative areas were quantitated by measuring the scratch surface area as described in c, $n = 3$, except N-TERT and N-TERT-TR $n = 2$, $^*P < 0.05$. (**f**) Equal amounts of RIPA cell lysates of control N-TERT or N-TERT stably infected with lentiviruses encoding AREG and HB-EGF were analyzed by ELISA for AREG or HB-EGF expression, respectively, as described in Materials and Methods. Data are expressed as ng of AREG or HB-EGF protein per ml of RIPA lysates, $n = 3$ for AREG and controls and $n = 7$–12 for HB-EGF and controls, $^*P < 0.001$ relative to uninfected controls. (**g**) N-TERT-TR-sHB-EGF KC were grown and scratch wounded as described above and followed by incubation in basal KSFM medium for 24 hours in the presence or absence of TET with and without 1 μg ml^{-1} mitomycin C. Results are representative of two separate experiments. Bar = 400 μm.

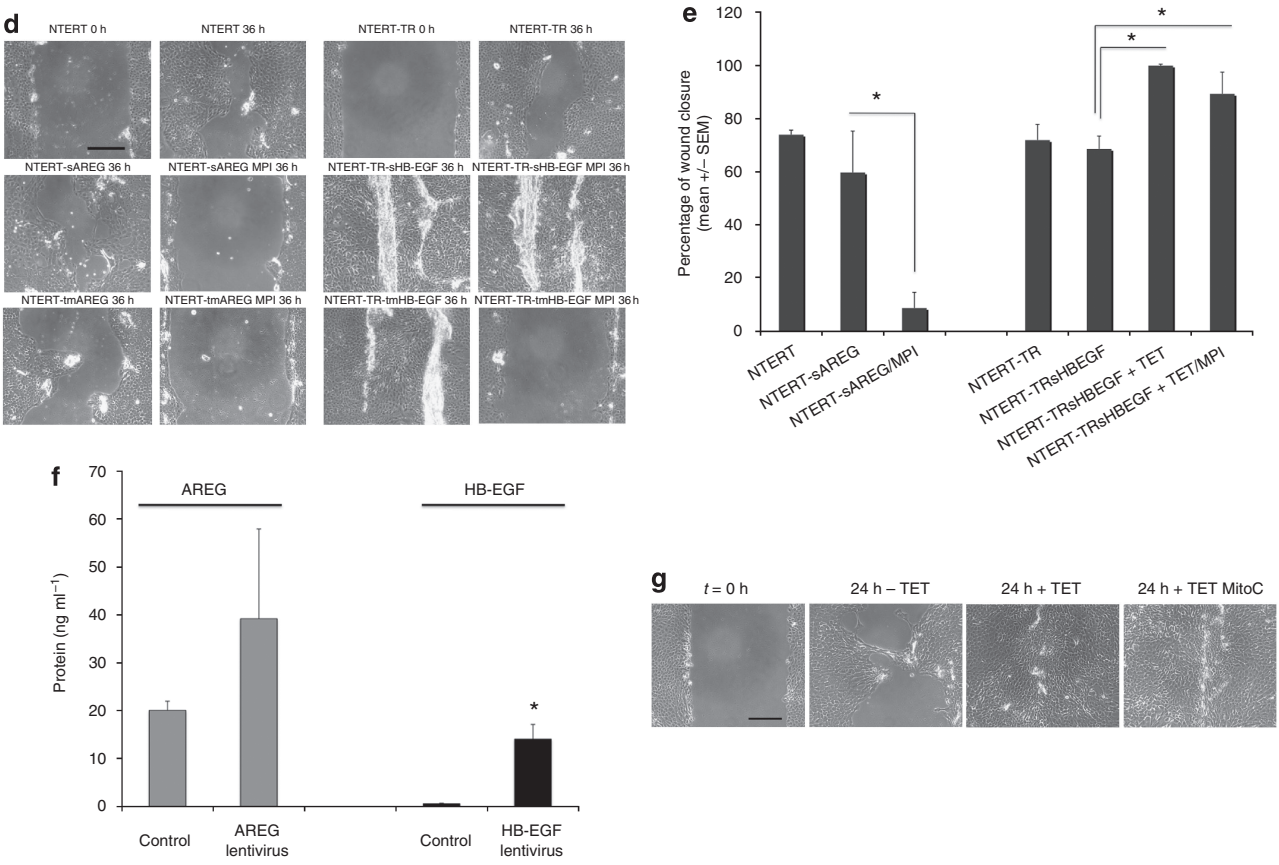

Figure 2. Continued.

However, addition of EGF to MP inhibitors could only partially overcome the block, suggestive of MP-mediated aspects of KC migration other than EGFR activation. Similarly, scratch wound-induced migration was markedly reduced in the presence of EGFR-neutralizing Abs (Figure 2b and c) with HB-EGF and AREG Abs displaying the strongest inhibition of KC migration. Addition of EGF to Ab-treated cultures restored their migratory phenotype, demonstrating a lack of cellular toxicity from Ab treatment.

Because KCs express and shed much less HB-EGF than AREG (Figure 1), yet neutralizing Abs against HB-EGF were as effective as AREG Abs to block KC migration, we wondered whether exogenous expression of HB-EGF might improve KC migration to a greater extent than exogenous expression of AREG. To address this, N-TERT KCs stably infected with lentivirus constructs encoding transmembrane and soluble forms of HB-EGF and AREG were tested in scratch wound assays as above. As shown in Figure 2d and quantitated in Figure 2e, TET-induced expression of the full-length transmembrane (tm) form of HB-EGF (tmHB-EGF) or a secreted form lacking the transmembrane and cytoplasmic domains (sHB-EGF) markedly improved KC migration compared with N-TERT-TR control cells or N-TERT-TR-HB-EGF without TET-induced expression of sHB-EGF. Interestingly, TET-induced expression of HB-EGF resulted in a marked piling up of cells upon closure of scratch wounds. In contrast, lentivirus-mediated, constitutive expression of soluble AREG (sAREG) or its transmembrane form (tmAREG) did not improve KC migration relative to control cells (N-TERT). As expected, MP inhibitor (MPI) treatment of KCs reduced migration in cells expressing tmHB-EGF, but not in cells expressing sHB-EGF. Unexpectedly, however, MPI treatment significantly reduced cell migration in KCs expressing soluble AREG. Our data in Figure 2f confirmed increased expression of AREG and HB-EGF in lentivirus infected N-TERT KCs and show that scratch wound migration is not blocked by the proliferation inhibitor mitomycin C (Figure 2g).

Autocrine KC proliferation and ERK phosphorylation are selectively regulated by MP-dependent release of AREG

To address the importance of individual growth factors on KC growth, we performed growth assays in the presence or absence of EGFR ligand-neutralizing Abs. As depicted in Figure 3, incubation of KCs with anti-AREG Abs significantly reduced KC growth by more than 66 % ($P<0.005$), whereas Abs directed against other ligands had only modest effects on KC growth. Similar to their effects on KC migration, PD158780 and GM6001 markedly reduced KC growth. Addition of exogenous EGF (1 ng ml^{-1}) to KCs incubated with GM6001 could only partially restore growth but had no effect on KCs incubated in the presence of PD158780. In contrast, EGF treatment markedly improved KC growth in cell cultures incubated with a cocktail of all three neutralizing Abs (AREG, HB-EGF, and TGF-α), demonstrating a lack of Ab toxicity.

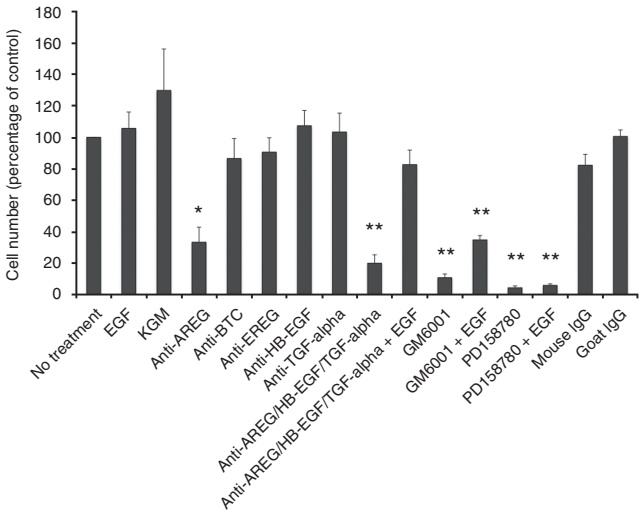

Figure 3. Neutralizing antibodies against AREG selectively block KC growth. KCs were incubated in basal M154 medium in the presence of blocking antibodies against AREG, BTC, EREG, HB-EGF, and TGF-α or isotype controls (each at 5 μg ml^{-1}), 40 μM GM6001 or 1 μM PD158780 with and without 1 ng ml^{-1} EGF. After an additional 7–9 days of incubation, cell growth was assessed using the 3-(4,5dimethyldiazol-2-yl)-2,5-diphenyltetrazolium assay (Roche). KGM denotes fully supplemented KC growth medium. Each data point represents the mean OD570 reading for duplicate culture conditions. Data are expressed as percent of untreated controls, $n = 3$–5, with $^{*}P < 0.005$ and $^{**}P < 0.0005$ relative to "no treatment" controls.

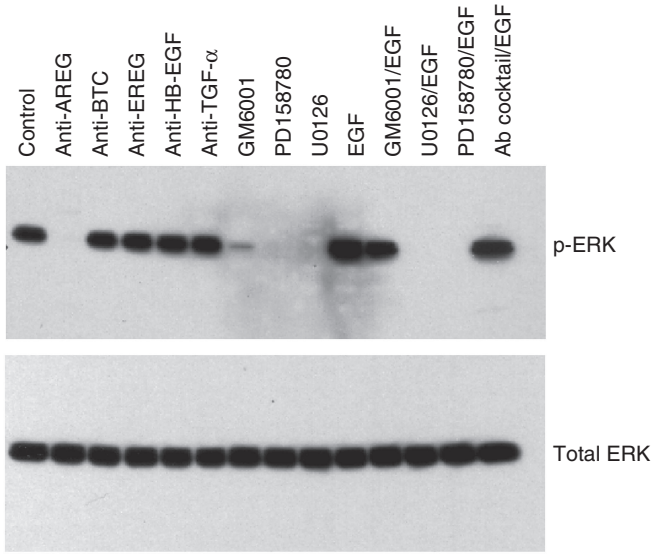

Figure 4. Autocrine ERK phosphorylation is selectively regulated by AREG. KCs were growth factor depleted for 48 hours as described in Materials and Methods and incubated for 2 hours in fresh basal M154 in the presence or absence of neutralizing antibodies against EGF-like growth factors (each at 5 μg ml^{-1}) or GM6001 (40 μM), PD158780 (1 μM), or U0126 (10 μM) in the presence or absence of 100 ng ml^{-1} EGF. Equal amounts of RIPA were analyzed by western blotting with antibodies against ERK and phospho-ERK. Results are representative of three independent experiments.

Because ERK is an important regulator of cell proliferation (Hobbs *et al.*, 2004), we tested whether AREG also selectively regulates the high levels of ERK phosphorylation that we have consistently observed in KCs even after 48 hours of growth factor deprivation (Stoll *et al.*, 2002; Kansra *et al.*, 2004). As shown in Figure 4, neutralizing antibodies against AREG strongly reduced ERK phosphorylation in KCs incubated in growth factor-free medium whereas Abs against four other EGFR ligands had little or no inhibitory activity on this process. As expected, ERK phosphorylation was strongly inhibited by the MEK inhibitor U0126, the pan-ErbB RTKI PD158780, and the MPI GM6001. Addition of EGF restored NHK ERK phosphorylation in the presence of GM6001 and in the presence of an Ab cocktail against all five EGFR ligands but not in cells incubated with PD158780 or U0126.

LPA-induced ERK phosphorylation is mediated by MP-dependent release of HB-EGF

Transactivation of EGFR by GPCR ligands including LPA is a mechanism that requires proteolytic release of EGFR ligands from their transmembrane-bound precursors (Sanderson *et al.*, 2006) and LPA-mediated ERK activation has been previously shown (Kranenburg and Moolenaar, 2001). Therefore, we tested whether LPA could induce ERK phosphorylation in KCs and whether neutralizing Abs against EGFR ligands could block this process. As can be seen in Figure 5, LPA treatment of KCs maintained under autocrine conditions for 48 hours before assay led to a marked increase in ERK phosphorylation that could be blocked after treatment with the MPI GM6001, the MEK inhibitor U0126, and the ErbB RTKI PD158780. Interestingly and in contrast to the data presented in Figure 4, LPA-induced ERK phosphorylation was markedly inhibited by HB-EGF antibodies whereas other EGFR ligand Abs or IgG isotype controls had little or no effect.

ErbB ligands differ in their ability to stimulate EGFR tyrosine phosphorylation

We also measured the potency of the various EGF-like growth factors to induce total tyrosine phosphorylation as well as tyrosine residue-specific EGFR phosphorylation in KCs. As can be seen in Figure 6, EGF, HB-EGF, and BTC were nearly equipotent in their ability to stimulate total tyrosine phosphorylation of proteins in the 170–180 kDa size range (4G10). The same three growth factors also induced a very similar pattern of tyrosine phosphorylation on EGFR residues 1148, 992, and 845. In contrast, TGF-α and particularly AREG were much less potent as inducers of total and residue-specific EGFR tyrosine phosphorylation, with no visible phosphorylation of EGFR pY845 even after treatment with 20 nM AREG.

DISCUSSION

Autocrine EGFR receptor signaling controls multiple KC functions including migration, proliferation, differentiation, and survival (Klein *et al.*, 1992; Danilenko *et al.*, 1995; Nanney and King, 1996; Stoll *et al.*, 1998; Pastore *et al.*, 2008). Acute stimulation of KCs with high concentrations of EGF or other EGFR ligands leads to increased expression of multiple EGF family members including AREG, HB-EGF, and TGF-α (Barnard *et al.*, 1994; Stoll and Elder, 1999; Shirakata

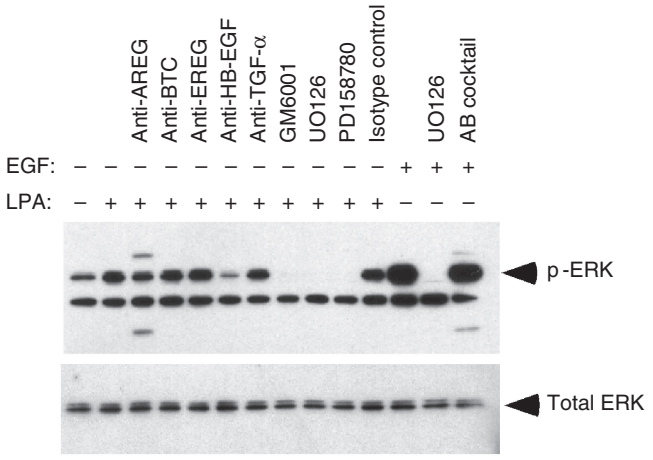

Figure 5. HB-EGF mediates LPA-stimulated ERK phosphorylation. KCs were depleted of growth factors by incubation in basal M154 medium for 48 hours followed by treatment with goat isotype control (for HB-EGF) or neutralizing Abs against EGFR ligands (each at 5 μg ml^{-1}) alone or in combination (AB cocktail), GM6001 (40 μM), PD158780 (1 μM), or U0126 (10 μM) for 1 hour followed by treatment with EGF (10 ng ml^{-1}) or LPA (10 μM) for 10 or 20 minutes, respectively. Protein lysates were assayed for phospho-ERK and total ERK by western blotting as described in Materials and Methods. The slight reduction of ERK phosphorylation in the presence of AREG Abs might be related to the component of the total signal that is due to basal ERK phosphorylation and therefore sensitive to AREG antibodies (see Figure 4). The band underneath the phospho-ERK signal has been consistently observed with the mouse mono- but not with the rabbit polyclonal phospho-ERK Ab (Figure 4) from Cell Signaling Technologies. Its identity is unknown, however, it does not appear to be regulated in response to different treatments. The additional bands in the anti-AREG lane are due to a cross-reaction of the goat anti-mouse-HRP-labeled secondary antibody with the AREG-neutralizing antibody. The results shown are representative for three independent experiments.

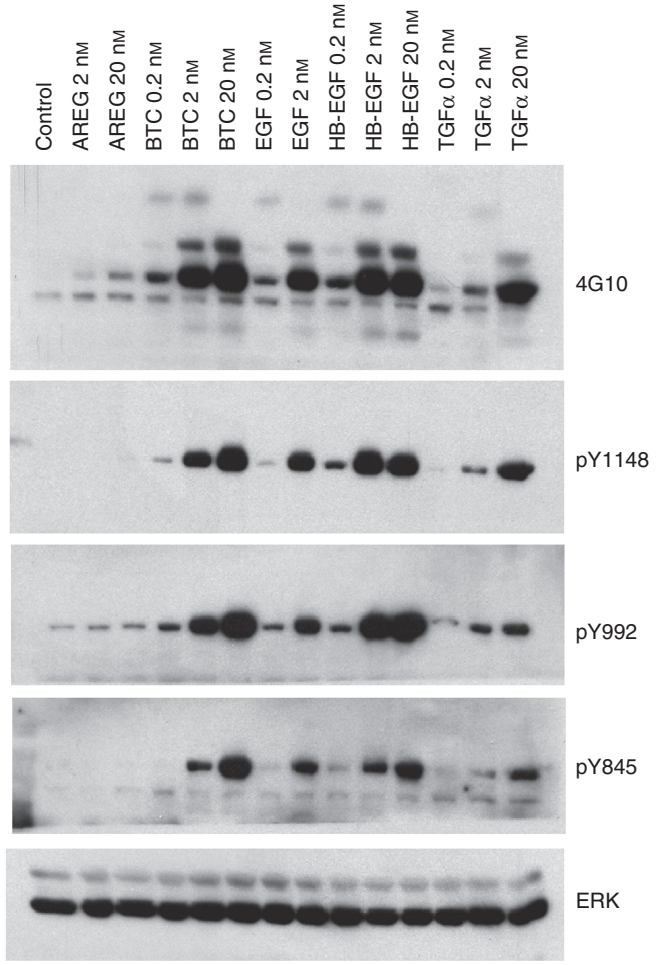

Figure 6. EGFR ligands differ in their ability to stimulate EGFR tyrosine phosphorylation. KCs were grown in M154 medium until ~40–50% confluence, growth factor depleted for 48 hours, followed by growth factor treatment for 10 minutes at 37°C in fresh M154 as indicated. Equal amounts of RIPA lysates were analyzed by western blotting with antibodies indicated to the right of the panels. Total ERK served as a control for equal protein loading. Results are representative of three separate experiments.

et al., 2000). Although KCs express multiple EGF-like growth factors in an autocrine fashion, their importance and specific function in different cellular contexts has been incompletely characterized, and it remains unclear why the ErbB-signaling network relies on multiple ligands. To address these questions, we started our investigation by assessing the relative expression of EGF ligands in cultured KCs and normal and organ-cultured human skin (Figure 1). Using QRT-PCR, we found that proliferating normal human KCs express at least 19 times more AREG mRNA than EPGN, EREG, HB-EGF or TGF-α, and that BTC mRNA was nearly undetectable. Similarly, using a multiplex EGFR ligand assay we found that AREG was also the most abundant EGF-like growth factor shed into the culture medium, whereas EREG, TGF-α, and HB-EGF were very close to our detection limit.

Our finding that AREG is the most abundantly expressed and shed EGF-like growth factor in KCs may largely explain why autocrine KC growth and ERK phosphorylation were selectively blocked by antibodies against AREG but not by antibodies against four other EGF-like growth factors (Figures 3 and 4). However, it may not be the only explanation. AREG has a much lower binding affinity for EGFR than does EGF, due to the lack of a conserved leucine residue necessary for high affinity binding to EGFR (Adam *et al.*, 1995). Interestingly, EPGN is also a low affinity EGFR ligand with a hundred-fold lower binding affinity than EGF, yet its mitogenic potential is far superior to that of EGF or TGF-α (Kochupurakkal *et al.*, 2005). The authors of this study suggested that the high mitogenic potential of EPGN might be due to evasion of desensitization; for example, receptor-mediated endocytosis-targeting receptor-ligand complexes for intracellular degradation. We confirm that HB-EGF, BTC, and EGF are much more potent activators of the KC EGFR than is AREG (Figure 6). Thus, it is possible that the strong dependence of KC proliferation on AREG might be further explained by relatively weak desensitization of ligand-receptor complexes.

Our study also confirms findings from earlier studies showing that AREG antibodies block the growth of cultured KCs under autocrine conditions (Bhagavathula *et al.*, 2005), whereas TGF-α Abs had no effect under these conditions (Pittelkow *et al.*, 1993). However, in those studies the function of EGFR ligands other than AREG and TGF-α for

autocrine KC growth was not assessed. Similarly, we have previously shown that AREG antibodies abrogate autocrine ERK phosphorylation (Kansra *et al.*, 2004). In this study, we show that four other EGFR ligands are not important for this process.

Overexpression of AREG in transgenic mice leads to a hyperproliferative skin phenotype with many similarities to psoriasis (Cook *et al.*, 1997, 2004). Furthermore, a humanized antibody against AREG also markedly blocked the psoriatic phenotype of human skin grafts on immunodeficient mice (Bhagavathula *et al.*, 2005). Although it is tempting to speculate that the growth-stimulatory properties of AREG in culture are responsible for the profound epidermal hyperplasia characteristic of psoriasis, this remains to be proven. Recently, AREG has been shown to be overexpressed in synovium and synovial fluid as well as synovial fluid-derived mononuclear cells of rheumatoid arthritis (RA) patients, relative to patients with osteoarthritis (Yamane *et al.*, 2008). Notably, transgenic mice engineered to overexpress AREG in basal keratinocytes developed a severe inflammatory arthritis (Cook *et al.*, 1997). Thus, it is possible that AREG may have a role in the inflammatory cascade of psoriasis, instead of or along with a direct effect on KC proliferation.

The low expression of BTC in proliferating KCs was not surprising, as it was previously shown that its expression is restricted to the fully differentiated, upper suprabasal layers of the skin (Piepkorn *et al.*, 2003; Rittié *et al.*, 2006). It is possible that BTC might be more important for KC differentiation rather than proliferation. On the other hand, forced expression of BTC in the basal layer of transgenic mice results in significantly increased KC proliferation without affecting differentiation (Schneider *et al.*, 2008). Basal overexpression of BTC might lead to different physiological effects than its normal expression in suprabasal layers, which also display different ErbB expression profiles (Stoll *et al.*, 2001). In this study we also show that the recently discovered EPGN (Strachan *et al.*, 2001) is another EGFR ligand expressed by KCs (Figure 1).

In contrast to cultured KCs, expression of all EGF-like growth factors in normal skin was very low. However, HB-EGF and AREG were strongly induced in human skin organ culture, an *in vitro* model displaying many similarities to cutaneous wound healing (Sarkany *et al.*, 1965; Reaven and Cox, 1968; Eisen, 1969; Hebda, 1988; Mackie *et al.*, 1988; Bhora *et al.*, 1995; Stoll *et al.*, 1997, 2002) (Figure 1c). Our recent data confirm and extend earlier data from our laboratories about EGFR ligand expression in normal and organ-cultured skin (Stoll *et al.*, 1997, 2002; Rittie *et al.*, 2007). However, using QRT-PCR instead of northern blotting, we were able to quantitate the expression levels of all EGFR ligands and show that EREG and TGF-α are also strongly induced in the organ culture system. Furthermore, our data demonstrate a sequential regulation of HB-EGF and AREG expression, and suggest that HB-EGF may be important in the earliest phases of wound healing, with AREG increasing later during the process. This is interesting because wound healing can be divided into an early phase during which KCs migrate but do not proliferate and a later phase characterized by vigorous KC proliferation (Marks *et al.*, 1972; Stenn, 1978; Hebda, 1988; Bhora *et al.*, 1995; Stoll *et al.*, 1997).

The importance of AREG for autocrine KC proliferation (Figure 3) might explain its increased expression during the later phase of organ culture. Interestingly, increased expression of AREG during wound healing has been reported (Schelfhout *et al.*, 2002). The early expression of HB-EGF in this model and its importance in scratch wound assays (Figure 2), strongly suggest an important function of HB-EGF during the early migration phase of wound healing. Consistent with this, it has been shown that skin wound closure was markedly impaired in KC-specific HB-EGF-deficient mice (Shirakata *et al.*, 2005). Our data also confirm earlier findings that KC migration is sensitive to EGFR, HB-EGF, and MP inhibitors (Tokumaru *et al.*, 2000). However, in those experiments KC migration was assessed on tissue culture plates coated with type-1 collagen. Although KC migration was sensitive to antibodies against several ligands, expression of soluble HB-EGF markedly improved KC migration even in the presence of MP inhibitors (Figure 2). In contrast, our findings demonstrate that soluble AREG by itself is not sufficient to promote KC migration, but instead requires the proteolytic release of one or more additional growth factor(s).

LPA is an important constituent of blood and serum and has been implicated in many cellular processes such as migration, proliferation, cancer, and wound healing (Watterson *et al.*, 2007). The strong activation of EGFR by HB-EGF (Figure 6) and our data showing that LPA-induced ERK phosphorylation (Figure 5) depends on MP-mediated release of HB-EGF further suggest an important role of HB-EGF during the early phases of wound healing. The finding that an anti-HB-EGF mAb blocks LPA-induced ERK phosphorylation is in marked contrast to the specific blockade of autocrine ERK phosphorylation by AREG Abs and the lack thereof in the presence of HB-EGF Abs (Figure 4).

We cannot exclude that differential ligand affinities of neutralizing antibodies affect some of the conclusions of the growth and migration assays or other comparative analyses of this study. Ultimately, these findings will have to be confirmed using RNAi-mediated gene knockdown in human KCs.

In aggregate, our data show that MP-mediated release of membrane-bound EGF-like growth factors is required for EGFR-dependent autocrine ERK phosphorylation, migration, and proliferation of normal human KCs. We find that autocrine KC proliferation and ERK phosphorylation are selectively regulated by MP-dependent release of AREG, whereas proteolytic release of HB-EGF is required for KC migration as well as LPA-induced ERK phosphorylation. These data suggest important but distinct functions of HB-EGF and AREG during the migratory and proliferative phases of cutaneous wound healing, respectively.

MATERIALS AND METHODS

Reagents

The MP inhibitors (MPI) GM6001 and MMP inhibitor III (MMPI-3), the MEK inhibitor U0126, and the pan-ErbB receptor tyrosine kinase inhibitor (RTKI) PD158780 were purchased from Calbiochem

(San Diego, CA). Recombinant human EGF was from Peprotech (Rocky Hill, NJ) and AREG, BTC, EREG, HB-EGF, and TGF-α and their cognate Abs were from R&D Systems (Minneapolis, MN). Anti-ERK and anti-phospho ERK Abs were obtained from Cell Signaling Technology (Beverly, MA). Abs against human EGFR were from Labvision (Freemont, CA), Cell Signaling Technology and from Biosource/Invitrogen (Carlsbad, CA). The anti-phosphotyrosine mAb 4G10 and horseradish peroxidase (HRP) or FITC-conjugated anti-mouse, anti-rabbit, and anti-goat Abs were purchased from Upstate Biotechnology (Lake Placid, NY). All other chemicals were from Sigma (St Louis MO) or Invitrogen (Carlsbad, CA).

Human subjects and organ culture

Human skin full-thickness punch biopsies (3 mm) were collected from sun-protected skin (buttocks) of healthy volunteers after having obtained informed consent according to a protocol approved by the University of Michigan Institutional Review Board. All experiments involving humans were performed in adherence to the Helsinki Guidelines. Skin samples were snap frozen immediately or after having been subjected to organ culture by incubation in basal M154 medium (Cascade Biologics, Portland, OR) for 4 and 24 hours at 37°C/5% CO_2 as previously described (Stoll *et al.*, 2002) and were processed for RNA isolation as described below.

Cell culture

Normal human KCs (passages 2–4) were cultured in low-calcium, serum-free M154 medium (KGM) as previously described (Stoll *et al.*, 2001). Human embryonic kidney cells (293FT, Invitrogen) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Gibco). The immortalized, non-transformed KC cell line N-TERT-2G was grown in keratinocyte SFM medium (KFSM, Gibco) as previously described (Dickson *et al.*, 2000).

Lentivirus-mediated gene expression

The cDNAs encoding the full-length (transmembrane) or extracellular (soluble) forms of AREG and HB-EGF were cloned into the lentiviral expression vectors pLenti6/CMV/V5-DEST (full-length AREG) or pLenti4/TO/V5-DEST (soluble forms of AREG and HB-EGF and full-length HB-EGF) and used to produce infectious lentivirus particles according to the manufacturer's instructions (Invitrogen). Stably transduced KC cell lines with constitutive expression of soluble or transmembrane AREG were generated by infection of N-TERT-2G KCs with the corresponding lentivirus constructs followed by antibiotic selection with 8 μg ml^{-1} blasticidin or 200 μg ml^{-1} zeocin and the resulting cell lines were termed N-TERT-sAREG (expressing the soluble form of AREG) and N-TERT-tmAREG (expressing full-length, transmembrane AREG). To generate stably transduced cell lines with inducible expression of HB-EGF, N-TERT-2G KCs were first infected with TR lentivirus (pLenti6/TR construct) as above. After selection with blasticidin, the resulting cell line, termed N-TERT-TR, was infected with lentiviruses encoding HB-EGF constructs as described above followed by antibiotic selection with zeocin. Stably transduced cell lines expressing the soluble or transmembrane forms of HB-EGF (N-TERT-TRsHB-EGF and N-TERT-TR-tmHB-EGF, respectively) were used for experiments as described below and gene expression was induced with 1 μg ml^{-1} TET (Invitrogen).

RNA isolation and quantitative reverse transcriptase polymerase chain reaction (QRT-PCR)

Total RNA from KCs or frozen skin was isolated using RNeasy mini kits with on-column DNase digestion (Qiagen, Valencia, CA). Total RNA was reverse transcribed using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit. cDNA equivalent to 5–40 ng of total RNA was used for QRT-PCR using pre-validated TaqMan gene expression assays (Applied Biosystems, Foster City, CA) for AREG (no. Hs00155832), BTC (no. Hs00156140), EREG (no. Hs00914313), EPGN (no. Hs02385425), HB-EGF (no. Hs00181813), TGF-α (no. Hs00608187) and ribosomal protein large P0 (RPLP0 or 36B4, no. Hs99999902) (Laborda, 1991; Minner and Poumay, 2009). Data are expressed as fold-change relative to 36B4 multiplied by 10^3 (fold-change *vs* 36B4 $= 2^{-(\text{CT target}-\text{CT 36B4})}$).

In vitro wound healing assays

KCs were plated, grown and wounded as previously described (Stoll *et al.*, 2003; Kansra *et al.*, 2005). Wounded cultures were incubated with basal M154 medium or KSFM in the presence or absence of EGF (10 ng ml^{-1}) with or without MMPI-3 (25 μM) or GM6001 (40 μM), IgG225 (5 μg ml^{-1}), PD158780 (1 μM), or neutralizing Abs directed against EGFR ligands (each at 5 μg ml^{-1}) or isotype control Abs. KC migration was assessed by phase contrast microscopy and documented by photography. Digital images were quantified using AxioVision-LE software (Carl Zeiss, Germany).

Cell growth assays

KCs were plated at 1,000–2,000 cells per cm^2 in complete M154 and allowed to attach for 20 hours. The cells were then incubated in basal M154 in the presence or absence of EGF (1 ng ml^{-1}) with or without GM6001, PD158780, or blocking Abs and isotype controls as described above. KC growth was assessed using the 3-(4,5dimethyldiazol-2-yl)-2,5-diphenyltetrazolium assay (Roche, Indianapolis, IN).

Western blotting

KCs were grown to 40–50% confluence and deprived of growth factors by incubation in basal M154 medium for 48 hours. The cells were incubated in fresh M154 in the presence or absence of U0126 (10 μM), PD158780 (1 μM), GM6001 (40 μM), or EGFR ligand-blocking antibodies (5 μg ml^{-1} each) with or without stimulation for 10 minutes with EGF (16.5 nM) or 20 minutes with LPA (10 μM). Cells were harvested with RIPA buffer and analyzed by western blotting as previously described (Stoll *et al.*, 2002; Kansra *et al.*, 2004).

Multiplex EGFR ligand assay

A multiplex EGFR ligand assay was developed by cross-linking Abs against AREG, BTC, EREG, HB-EGF, and TGF-α (R&D Systems) with a set of fluorescently dyed Bio-Plex microspheres (Bio-Rad, Hercules, CA) according to the manufacturer's instruction. Briefly, microspheres coupled with EGFR ligand Abs were incubated with KC-conditioned medium on 96-well microplates followed by incubation with EGFR ligand-specific biotinylated Abs. After addition of streptavidin-PE (Bio-Rad), fluorescence was measured using a Bio-Plex 200 system (Bio-Rad). EGFR ligand concentration was determined in duplicates using a 5-parameter logistic curve fit with a cocktail of recombinant EGFR ligands used in threefold dilutions to generate an 8-point standard curve for each ligand.

Enzyme-linked immunosorbent assay

EGFR ligands in RIPA cell lysates were detected by sandwich ELISA (R&D Systems) using anti-AREG and anti-HB-EGF antibodies as previously described (Kansra *et al.*, 2004).

Statistical analysis

Data are expressed as mean ± SEM, with n for independent experiments. Statistical analysis was performed using paired or independent Student's t-test (two-tailed). P-values $\leqslant 0.05$ were considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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