mice (Iwamoto et al., 2003; Shirakata et al., 2005) and infected with adenoviruses encoding either Cre or GFP. Deletion of the *Hb-egf* gene and the absence of its mRNA transcript were confirmed in Cre recombinase-expressing MEFs ($HB^{-/-}$ cells); in contrast, the gene and transcript were retained in GFP-expressing MEFs ($HB^{+/+}$ cells) (Fig. 4A–C). HB-EGF protein production in $HB^{+/+}$ and $HB^{-/-}$ cells was detected by Western blotting (Fig. 4B, lower part). Stimulation of EGF induced *c-Myc* expression in quiescent $HB^{+/+}$ cells, and up-regulation of *c-Myc* by EGF was also attenuated in $HB^{-/-}$ cells (Fig. 4D). Strikingly, phosphorylation of EGFR and Erk1/2 was stimulated equally in response to EGF in $HB^{+/+}$ and $HB^{-/-}$ cells (Fig. 4E).

Negative effects of uncleavable mutant of proHB-EGF on c-Myc gene expression and cell cycle progression

We next performed a recovery assay with adenovirus infection of MEFs to introduce exogenous expression of wild-type proHB-EGF. Protein production by wild-type or mutant proHB-EGF in $HB^{-/-}$ cells is shown in Fig. 5A. Infection with viruses carrying the wild-type protein restored *c-Myc* induction in $HB^{-/-}$ cells by EGF (Fig. 5B). Although the quiescent MEFs proceeded into S phase following EGF stimulation, entry into this phase was somewhat delayed in $HB^{-/-}$ cells compared to $HB^{+/+}$ cells with statistical significance (Fig. 5C). On the other hand, expression of an uncleavable mutant of proHB-EGF (Nanba et al., 2003) by adenovirus infection severely depressed *c-Myc* expression in both $HB^{-/-}$ and $HB^{+/+}$ cells (Fig. 5B). Moreover, adenovirus-driven overexpression of the uncleavable mutant completely inhibited cell cycle progression into S phase, although overproduction of wild-type proHB-EGF in $HB^{-/-}$ cells accelerated entry into S phase (Fig. 5C). It is presumably due to a potent dominant negative effect of the uncleavable form.

Elevated transcription of *c-Myc* was induced in quiescent MEFs stimulated with bFGF (Fig. 6A). The increased transcription of *c-Myc* mRNA in response to bFGF was significantly depressed in $HB^{-/-}$ cells (Fig. 6A). Expression of wild-type proHB-EGF recovered the induction of the *c-Myc* gene by bFGF in $HB^{-/-}$ cells, but expression of the uncleavable mutant of proHB-EGF did not do so (Fig. 6B). These data raise the possibility that the release of overexpressed HB-EGF restored *c-Myc* induction by bFGF. However, induction by bFGF was not inhibited by treatment with HB-EGF-neutralizing antibodies under the expression of proHB-EGF (Fig. 6B). bFGF induced quiescent MEFs into S phase, but S phase entry was delayed in $HB^{-/-}$ cells compared to $HB^{+/+}$ cells (Fig. 6C). Furthermore, expression of the uncleavable mutant markedly suppressed S phase entry, similarly to the result with EGF.

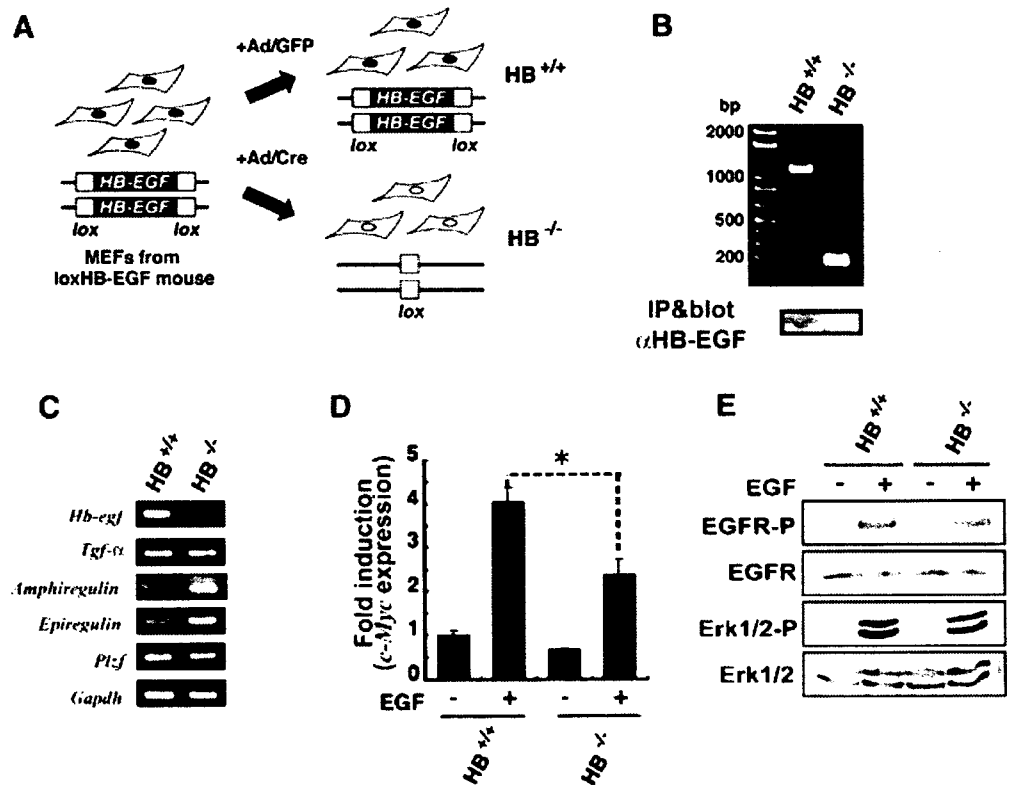

Fig. 4. Involvement of HB-EGF in *c-Myc* transcription induced by EGFR signaling. A: Schematic representation of generation of proHB-EGF-deficient MEFs from loxHB-EGF mice. B: The upper part shows PCR detection of the *Hb-egf* gene in loxHB-EGF MEFs after infection with adenoviruses expressing GFP ($HB^{+/+}$) or Cre ($HB^{-/-}$). The upper band represents the intact *Hb-egf* gene flanked by *loxP* sites, and the lower band reflects the fragment size decrease after *Hb-egf* gene deletion. The bottom part shows the protein level of proHB-EGF in each type of cell. Data were collected for 10^6 cells for each condition. C: RT-PCR analysis of mRNAs for EGFR ligands (*Hb-egf*, *Tgf-α*, *Amphiregulin*, and *Epiregulin*) and *Plzf* mRNA expression. D: Analysis of *c-Myc* mRNA expression in $HB^{+/+}$ and $HB^{-/-}$ cells by quantitative PCR analysis. Serum-starved MEFs were stimulated with 10 ng/ml EGF for 1 h. Expression of *Gapdh* mRNA was examined as a normalization control, and densitometric analysis was performed. *$P < 0.05$ versus $HB^{+/+}$ cells treated with EGF. E: Phosphorylation of EGFR and Erk1/2 induced by addition of EGF for 15 min in $HB^{+/+}$ and $HB^{-/-}$ cells. All experiments were performed independently in triplicate.

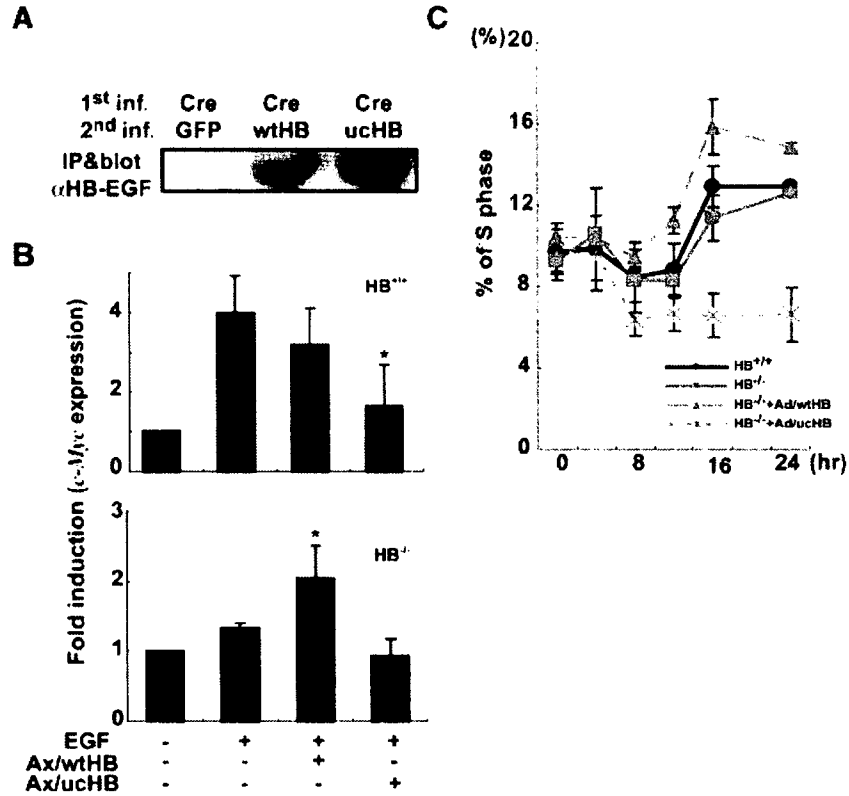

Fig. 5. EGFR signaling-induced *c-Myc* transcription and cell cycle progression by expression of proHB-EGF and an uncleavable mutant. A: The protein level of each proHB-EGF in adenovirus-infected cells. Immunoprecipitation and the Western blotting assay were each performed using 10^6 cells. 1st inf. and 2nd inf. indicate first and second infection respectively. B: RPA of *c-Myc* transcription induced by EGF in $HB^{+/+}$ and $HB^{-/-}$ cells infected with adenoviruses encoding either proHB-EGF (Ax/wtHB) or its uncleavable mutant (Ax/ucHB). The expression level of *c-Myc* was normalized using the corresponding expression level of *Gapdh*, and fold induction is shown based on a control value of 1. C: Flow cytometry analysis of cell cycle progression in cells stimulated with EGF. All experiments were performed independently in triplicate.

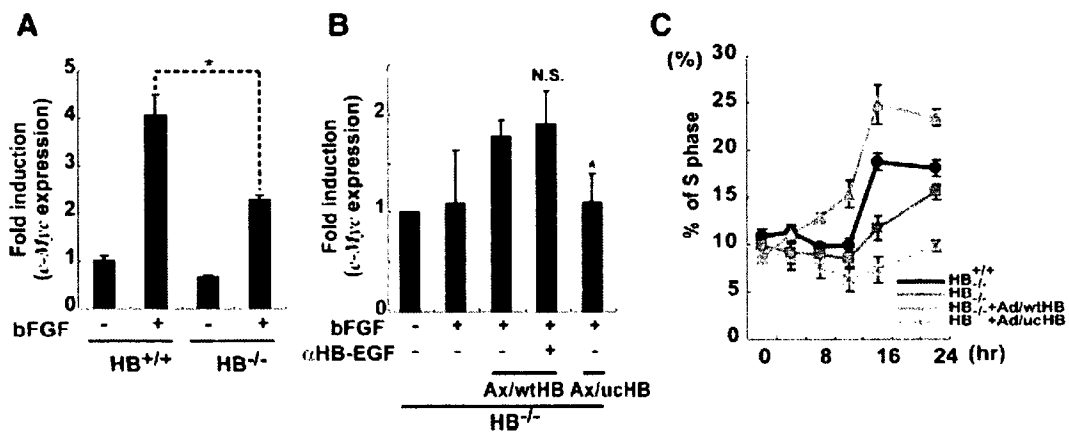

Fig. 6. Involvement of proHB-EGF shedding in *c-Myc* transcription induced by bFGF. A: Quantitative PCR analysis of *c-Myc* transcription induced by bFGF in $HB^{+/+}$ and $HB^{-/-}$ cells. Serum-starved MEFs were stimulated with 1 ng/ml bFGF for 1 h. Expression of *Gapdh* mRNA was examined as a control. B: RPA of *c-Myc* transcription induced by bFGF in $HB^{-/-}$ cells infected with Ax/wtHB and Ax/ucHB. A HB-EGF-neutralizing antibody (αHB-EGF) was used to inhibit the function of mature HB-EGF. The expression level of *c-Myc* was normalized using the corresponding expression level of *Gapdh*, and fold induction is shown based on a control value of 1. C: Flow cytometry analysis of cell cycle progression in cells stimulated with bFGF. $^{*}P < 0.05$ versus $HB^{+/+}$ cells. All experiments were performed independently in triplicate.

These results suggest that proHB-EGF shedding and subsequent HB-EGF-CTF signaling can modulate bFGF signaling-induced *c-Myc* expression and cell cycle progression.

Epigenetic effect of uncleavable proHB-EGF on Mouse c-Myc gene promoter

We have previously reported that HB-EGF-CTF inactivates PLZF, a transcriptional repressor, by induction of translocation to the cytoplasm. On the other hand, a recent study has shown that PLZF represses expression of human *c-Myc* (McConnell et al., 2003). When we checked the PLZF-binding motifs in the 5 kb region from the transcriptional start site of the mouse *c-Myc* gene, six putative sites were found (Fig. 7A). To examine chromatin modification in MEFs infected by adenoviruses carrying GFP or the uncleavable mutant of proHB-EGF, a chromatin immunoprecipitation (ChIP) assay was performed. After stimulation with EGF, the level of acetylated histone H3 increased at the region III (Fig. 7B), where the acetylation, however, was not induced with expression of the uncleavable form of proHB-EGF. These data suggest that production of HB-EGF-CTF followed by shedding is required for histone modification. We also examined the participation of PLZF in this region in the ChIP assay. However, the fragment of the region III was not detected with anti-PLZF antibodies (data not shown). We also tried detection of this region with anti-FLAG antibodies in FLAG-tagged PLZF-overexpressed cells, but the region III was not detected again (Fig. 7C).

Discussion

Induction of *c-Myc* transcription by receptor tyrosine kinases (RTKs) is regulated by at least two distinct intracellular signaling pathways: the Ras/Raf/MEK/ERK (MAPK) pathway (Kerkhoff et al., 1998) and the Src pathway (Barone and Courtneidge, 1995; Chiariello et al., 2001). The MAPK pathway induces proHB-EGF shedding by the activation of metalloproteases (Gechtman et al., 1999; Umata et al., 2001). The activated metalloproteases cleave proHB-EGF at the plasma membrane, generating both the EGFR ligand HB-EGF and the transcription-modulating protein HB-EGF-CTF (Nanba et al., 2003). The present study demonstrates that signal transduction mediated by HB-EGF-CTF modulates induction of *c-Myc* transcription and cell cycle progression by EGF and bFGF.

Reiss et al. provided evidence that proteolysis of transmembrane proteins on the cell surface is involved in *c-Myc* expression, by showing decreased expression of *c-Myc* in ADAM10-deficient MEFs (Reiss et al., 2005). ADAM10 cleaves N-cadherin, an event that causes redistribution of β-catenin from the plasma membrane to the cytoplasmic pool, thereby accelerating β-catenin/Tcf signaling and the resulting *c-Myc* expression (He et al., 1998). ADAM10 is also a sheddase for proHB-EGF and other EGFR ligand precursors (Sahin et al., 2004). Therefore, it is possible that KB-R7785, a metalloproteinase inhibitor, could inhibit the increase in *c-Myc* expression by blocking redistribution of β-catenin (Fig. 3A, C). However, expression of the *c-Myc* gene by growth factors diminished in MEFs in which the *Hb-egf* gene was removed by Cre recombinase (Figs. 4D, 5B, and 6A). Moreover, expression of an uncleavable mutant of proHB-EGF did not recover expression of *c-Myc*, rather inhibited it (Figs. 5B and 6B), suggesting that full induction of the *c-Myc* gene by growth factors requires shedding of proHB-EGF. CTF signaling derived from proHB-EGF may also contribute to *c-Myc* transcription induced by growth factors.

c-Myc participates in a variety of biological processes, including cell proliferation, differentiation, apoptosis, metabolism, and tumorigenesis (Dang, 1999; Murphy et al., 2005). We performed cell cycle analysis to determine the effects caused by a change in *c-Myc* expression. Delayed S phase entry was observed in *Hb-egf*-depleted MEFs following stimulation with either EGF or bFGF (Figs. 5C and 6C). Expression of the uncleavable mutant of proHB-EGF in the presence of the wild-type protein completely stopped the cell

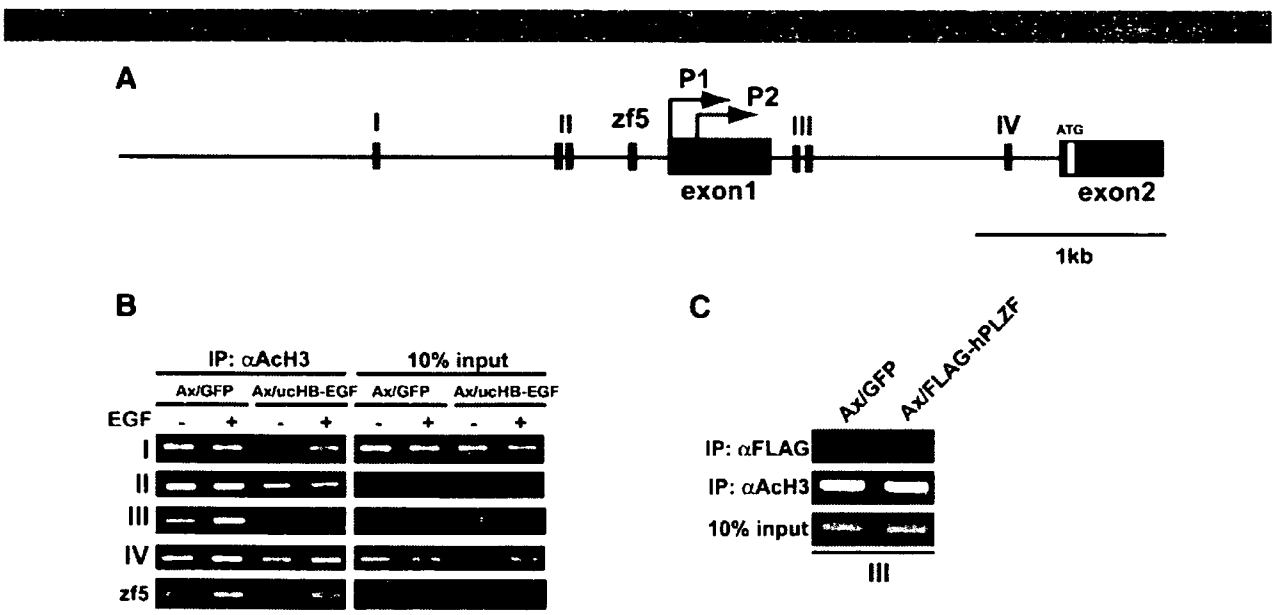

Fig. 7. Inhibition of EGF-induced acetylation of histone H3 at the mouse *c-Myc* intron I by the uncleavable mutant of proHB-EGF. A: Schematic diagram of the *c-Myc* promoter, exon I, intron I, and exon II. Gray boxes show the PLZF-binding motif-like sites. The regions including gray boxes are named as regions I, II, III, and IV; zf5 indicates the binding motif of ZF5, a novel *c-Myc* suppressor; black boxes indicate exons; and P1 and P2 are transcriptional start sites. B: ChIP assay with MEFs infected by adenoviruses including cDNA of GFP or the uncleavable mutant of proHB-EGF, and PLZF (C). All experiments were performed independently in triplicate.

cycle. These results indicate that the shedding event of proHB-EGF is one of the important steps to regulate cell cycle progression, even in the presence of growth factors. Therefore, the present study raises a possibility that cellular behavior involving *c-Myc* expression is controlled by regulating proteolysis of cell surface proteins and signaling by their cell-associated remnant fragments.

Our current studies indicate that the CTFs of other EGF family members such as amphiregulin, TGF-α, and epiregulin bind to PLZF as well as HB-EGF does (unpublished work, Morimoto and Higashiyama), and that these members share shedding enzymes in some extent (Sahin et al., 2004). The former supports the idea that CTF signaling would be redundant in some members of the EGF family for explaining that the lack of HB-EGF-CTF showed partial inhibition of *c-Myc* induction by EGF and bFGF. The latter indicates that overexpression of uncleavable proHB-EGF would competitively block the shedding of other members in the EGF family and works as a dominant negative form, explaining that uncleavable proHB-EGF markedly blocked S phase entry of cell cycle induced by EGF and bFGF.

Stimulation of EGF enhanced acetylation of histone H3 at the keratin 16 and c-fos chromatin promoter (Cheung et al., 2000; Wang et al., 2006). We also observed enhanced levels of acetylated histone H3 with EGF treatment in the mouse *c-Myc* intron I near the boundary of the exon I (Fig. 7B). The activated *c-Myc* allele in Burkitt's lymphoma is associated with a cluster of somatic mutations within a discrete domain of intron I that define protein recognition sequences, designated as *c-Myc* intron factors (MIFs) (Zajac-Kaye and Levens, 1990; Tachibana et al., 1993; Yu et al., 1993). The sequence of the intron I in the mouse *c-Myc* gene is partly homologous to that in the human *c-Myc*, and this region could also be important in the regulation of the expression of *c-Myc* in mouse.

PLZF negatively regulates the human *cyclin A2* and *c-Myc* genes (Yeyati et al., 1999; McConnell et al., 2003) and the consensus binding sequence has been identified. We have recently reported that HB-EGF-CTF targets PLZF to de-repress the human *cyclin A2* gene (Nanba et al., 2003). It was shown here that shedding of proHB-EGF and sequential production of HB-EGF-CTF affected activation of *c-Myc* in MEFs, and PLZF may participate in this event. However, over-expression of PLZF did not result in attenuation of acetylation of histone H3 at the intron I region, which includes two similar PLZF-binding motif sites (Fig. 7C). Moreover, we tested the influence of ZF5, a novel regulatory factor for *c-Myc* expression (Numoto et al., 1993), which has a binding site in the upper region of exon I (Fig. 7A). However, no effects of the uncleavable proHB-EGF mutant were observed at this region in the ChIP assay (Fig. 7B). These results raise the possibility that other C_2H_2 type zinc finger transcriptional repressors with similar features to PLZF might be targeted by HB-EGF-CTF in the expression of mouse *c-Myc*. This speculation might be supported by two pieces of the following important information. (1) HB-EGF-CTF is able to bind Bcl6, a PLZF-like transcriptional repressor (Kinugasa et al., 2007). (2) PLZF-like repressors (Kruppel-type zinc finger repressors) are well-known gene family showing that active rounds of segmental duplication, involving single genes or larger regions and including both tandem and distributed duplication events, have driven the expansion of this mammalian gene family. Comparisons between the human genes and ZNF loci mined from the draft mouse, dog, and chimpanzee genomes highlighted a substantial level of lineage-specific change. Kruppel-type zinc finger genes are widely expressed and clustered genes are typically not coregulated, indicating that paralogs have evolved to fill roles in many different biological processes in each species (Huntley et al., 2006).

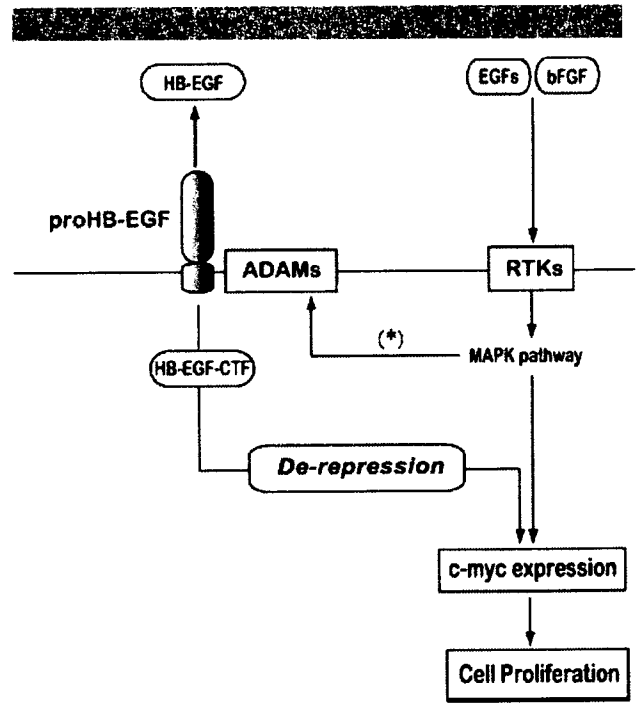

Fig. 8. A schematic diagram for the proposed role of HB-EGF-CTF signaling in *c-Myc* transcription induced by growth factor receptor activation. (*) Activation of ADAMs by MAPK pathway has already been reported (Gechtman et al., 1999; Umata et al., 2001).

In conclusion, we have shown here that shedding of proHB-EGF induces epigenetic changes in the mouse *c-Myc* gene, in support of the induction of expression of *c-Myc* by EGF or bFGF. HB-EGF-CTF may target other transcriptional repressors besides PLZF, and further studies are required to identify the target repressors. We propose a possible model for the mechanism of *c-Myc* regulation by RTKs in Figure 8.

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Human corneal epithelial cell proliferation by epiregulin and its cross-induction by other EGF family members

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Purpose: To investigate the effects of epiregulin, a newly identified member of the epidermal growth factor (EGF) family, on the proliferation of human corneal epithelial cells (HCECs).

Methods: The proliferation of HCECs was determined by cell counting and BrdU incorporation assays at specific times after exposure to different concentrations of human recombinant epiregulin (0 to 20 ng/ml). Immunohistochemical staining was used to localize epiregulin in cadaveric corneas. RT-PCR and real-time PCR were used to determine the expression levels of epiregulin in cultured and cadaveric HCECs. To examine the interaction between epiregulin and epidermal growth factor receptors (EGFRs), the phosphorylation of ErbB1 and ERK1/ERK2 (ERK1/2) was estimated by western blot analysis in the presence or absence of AG1478, a specific inhibitor of EGFR kinase activity. To search for cross-induction of epiregulin by other EGF family members, the expressions of EGF, heparin-binding epidermal growth factor-like growth factor (HB-EGF), amphiregulin (AR), and transforming growth factor-alpha (TGF-α) mRNA were determined by real-time PCR in the presence of 10 ng/ml of epiregulin. Conversely, the expression of epiregulin was also determined following the incubation of HCECs with 10 nM of either of EGF, HB-EGF, TGF-α, or AR.

Results: The mRNA of epiregulin was expressed in cultured HCECs and HCECs obtained from cadaveric eyes. Epiregulin was strongly detected in the limbal epithelium and basal epithelium of the peripheral cornea, but it was weakly detected in the central corneal epithelium. HCECs proliferated in the presence of epiregulin in a dose-dependent manner as detected by an increase in cell numbers or in BrdU incorporation. When HCECs were incubated with exogenous epiregulin, the expression of the mRNA of epiregulin was up-regulated as detected by real-time PCR, and the phosphorylation of ErbB1 and ERK1/2 was up-regulated in a dose-dependent manner as shown by Western blot analysis. These up-regulations were inhibited by AG1478, a specific inhibitor of EGFR kinase activity. Epiregulin increased the expression of HB-EGF and AR, while TGF-α, HB-EGF, AR, and EGF increased the expression of epiregulin in HCECs.

Conclusions: These findings indicate that epiregulin played an autocrine role in the proliferation of HCECs presumably through cross-induction with other EGF family members.

The corneal epithelium is a multi-layered stratified epithelium that covers the surface of the cornea and acts as a physical barrier to noxious agents. To maintain an effective barrier, a constant renewal of corneal epithelial cells is necessary, and this was accomplished with the aid of different growth factors [1,2]. Of these growth factors, the members of the epidermal growth factor (EGF) family have been most extensively studied. The EGF family consists of EGF, transforming growth factor-alpha (TGF-α), heparin binding EGF-like growth factor (HB-EGF), amphiregulin (AR), epiregulin, betacellulin (BTC), neuregulin 1, neuregulin 2, and neuregulin 3 [3-12]. Among these, TGF-α and HB-EGF are known to stimulate not only the migration and proliferation of corneal epithelial cells, but also the synthesis of the basement membrane and extracellular components [6,11,13-16].

In general the growth factors of the EGF family consist of a transmembrane domain and one or more EGF domains. Soluble mature peptides are released from the extracellular domains by proteolytic cleavage as ligands for EGF receptors [17]. There are four types of EGF receptors: ErbB1 (EGFR/Her1), ErbB2 (Neu/Her2), ErbB3 (Her3), and ErbB4 (Her4) [18-21]. The members of the EGF family can be divided into two groups according to their binding specificity to transmembrane receptors. The first group, which includes EGF, HB-EGF, TGF-α, AR, BTC, and epiregulin, generally binds to ErbB1 [4,5,12,22-24] and plays a crucial role in epithelial development and wound-healing for skin, lungs, and the gastrointestinal tract [25-27]. The members of the second group, the neuregulins, bind to ErbB3 and ErbB4 [3,7,8,28] and are crucial for the development of cardiac muscle and the central nervous system [29-32].

It has been shown that the first group of the EGF family members plays important roles during the healing of corneal injuries [1,16,33-35]. These binding EGFR ligands activate ErbB1, thus activating subsequent intracellular signals such as extracellular signaling-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K), which lead to corneal epithelial cell proliferation and migration [36-39].

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Epiregulin is a relatively new member of the EGF family and was originally purified from conditioned medium of the NIH3T3 cell line T7 [12]. It is made up of 46 amino acids that form a single-chain polypeptide that exhibits 24-25% amino sequence homology with other EGFR ligands [12,40,41]. Epiregulin is unique due to its affinity to bind to all of the ErbB family members [42]. It has been demonstrated that epiregulin plays a role in the proliferation of epithelial cells, e.g., epidermal and urothelial cells [43,44]. In the eye, Zhou et al. [45] reported that the mRNA of epiregulin was preferentially expressed in limbal basal cells of mice; however, the precise role of epiregulin in human corneal epithelial cells has not been determined. Like other members of the first group of the EGF family, epiregulin also binds to ErbB1, and it is thus reasonable to consider that epiregulin may play a role in corneal epithelial cell proliferation.

The purpose of this study was to determine whether the proliferation of HCECs is up-regulated in the presence of epiregulin, and whether epiregulin can act on the HCECs together with other members of the EGF family including EGF, TGF-α, HB-EGF, and AR.

METHODS

Human subjects: All procedures involving human subjects were conducted in accordance with the tenets of the Declaration of Helsinki (JAMA 1997; 277:925-926). The experimental protocol for these experiments was approved by the Institutional Review Board of Ehime University.

Materials: All reagents used for cell cultures were purchased from Invitrogen (Carlsbad, CA) except for the growth factors. Recombinant human epiregulin, EGF, TGF-α, AR, and HB-EGF were purchased from R&D Systems (Minneapolis, MN).

Cell cultures: Primary HCECs were isolated from human corneoscleral buttons dissected from eyes acquired from an American eye bank. The buttons were carefully denuded of the endothelial cells and adherent iris tissues. After digestion with 1.2 U/ml Dispase at 4 °C for 24 h, the loosened epithelial sheets were removed and dissociated into single cells by enzyme digestion with 0.25% trypsin. The isolated HCECs were cultured in serum-free, modified MCDB153 medium containing insulin (5 μg/ml), hydrocortisone (0.5 μM), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), and bovine hypothalamic extract (50 μg/ml). The medium was changed every 2 days, and cells from the third or fourth passage were used for all experiments.

Expression of epiregulin mRNA in cadaveric corneal epithelium: The corneal epithelium was collected from the cadaveric corneoscleral buttons using a surgical knife, and then total RNA was immediately extracted using an RNeasy kit (Qiagen, Valencia, CA) and measured by spectrophotometry (OD at 260 and 280 nm). Total RNA was reverse-transcribed to cDNA using Omniscript Reverse Transcription Reagents (Qiagen) according to the instructions of the manufacturer. The PCR conditions for the initial denaturation step were 95 °C for 15 min and 40 cycles at 95 °C for 10 s, followed by 64 °C for 20 s for annealing and 72 °C for 20 s for extension.

Proliferation of HCECs by epiregulin: HCECs were seeded in 6 well plates at a density of $8x10^4$ cells/well in serum-free modified MCDB153 medium. On the following day, the cells were subcultured in fresh medium containing serial concentrations of recombinant epiregulin (0-20 ng/ml) in the absence of bovine hypothalamic extract because the cells were still 20% confluent. After 6 days, the cells were collected and counted using a hemocytometer.

To determine whether the HCECs had proliferated, the level of BrdU incorporated into the HCECs was determined. HCECs were seeded in 6 well plates at a density of $8x10^4$ cells/well in serum-free modified MCDB153 medium. After reaching about 50% confluency, the cells were subcultured in a medium without bovine hypothalamic extract. On the following day, the cells were subcultured in the same medium containing serial concentrations of recombinant human epiregulin (0-20 ng/ml) and incubated for 48 h. The cells were then incubated with medium containing BrdU for 2 h. BrdU was detected immunohistochemically using the 5-Bromo-2'-deoxy-uridine Labeling and Detection kit 2 (Roche, Indianapolis, IN) according to the instructions of the manufacturer. The number of BrdU-labeled cells/5 mm^2 were counted (n=4).

Phosphorylation of ErbB1 and ERK1/ERK2 by epiregulin: HCECs were seeded in 24 well plates at a density of $5x10^4$ cells/well in serum-free, modified MCDB153 medium. After reaching about 80% confluency, the cells were subcultured in a medium without bovine hypothalamic extract. On the following day, the cells were subcultured in the same medium containing serial concentrations of recombinant human epiregulin (0-20 ng/ml) for 5 min. The cells were rinsed with cold Ca^{++}- free and Mg^{++}-free phosphate buffer saline (PBS^-) and then harvested on ice with a cell scraper. The cells were lysed in 200 μl of lysis buffer containing 62.5 mM Tris-HCl, 2% SDS, 25% glycerol, 0.01% bromophenol blue, and proteinase inhibitors at pH 6.8. After centrifugation, the cell lysates were separated on 7.5% polyacrylamide gel containing sodium dodecyl sulfate (SDS-PAGE) and transferred to a nitrocellulose membrane (Millipore, Billerica, MA). After blocking with 5% non-fat dry milk, the membrane was incubated

Table 1. Specific primer pairs for human epiregulin, HB-EGF, TGF-α, AR, EGF, and GAPDH are listed

Gene (Accession number)	Forward primer	Reverse primer	Product size (bp)
Epiregulin (NM_001432)	CGTGTGGCTCAAGTGTCAAT	TGGAACCGACGACTGTGATA	235
HB-EGF (NM_001945)	GCTCTTTCTGGCTGCAGTTC	AGCTGGTCCGTGGATACAGT	120
TGF-α (NM_003236)	TGATACACTGCTGCCAGGTC	ATCTCTGGCAGTGCTGTCCT	207
AR (NM_001657)	CGGCAGCCGACTATGACTAC	CCATTTTTGCCTCCCTTTTT	172
EGF (NM_001963)	CAGGGAAGATGACCACCACT	CAGTTCCCACCACTTCAGGT	187
GAPDH (AY340484)	CGACCACTTTGTCAAGCTCA	AGGGGTCTACATGGCAACTG	228

In the table, HB-EGF=heparin-binding epidermal growth factor-like growth factor, TGF-α=transforming growth factor-alpha, AR=amphiregulin, EGF=epidermal growth factor, and GAPDH=glyceral-dehyde-3-phosphate dehydrogenase.

with a 1:1,000 dilution of an anti-phospho-EGFR(ErbB1, Tyr1173) antibody (Upstate Biotechnology, Lake Placid, NY) or an anti-phospho-ERK1/ERK2 (ERK1/2) antibody (R&D systems) at 4 °C for 12 h. After washing with PBS, the membrane was incubated with a 1:2,500 dilution of fluorescein-labeled goat anti mouse IgG (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 h at RT. The signal was amplified with an anti-fluorescein alkaline phosphatase conjugate followed by the addition of a fluorescent substrate, Attophos (Amersham Pharmacia Biotech). The membrane was scanned using a FluorImager (Molecular Dynamics, Sunnyvale, CA), and the expression levels of ErbB1 and ERK1/2 were determined relative to that of beta-actin in the same sample using the NIH Image program. The phosphorylation of ErbB1 and ERK1/2 was also determined by Western blot analysis in HCECs exposed to serial concentrations of AG1478 (Calbiochem, La Jolla, CA), a specific inhibitor of EGFR kinase activity, with 100 ng/ml of epiregulin.

Quantitative real time PCR analysis: To determine whether exogenous epiregulin affected the expression of endogenous epiregulin, i.e., an auto-induction mechanism, or other members of the EGF family, i.e., a cross-induction mechanism, HCECs were stimulated by epiregulin, and the expression of the mRNA of the other growth factors was examined by real time PCR. Briefly, HCECs were seeded in 24 well plates at a density of $5x10^4$ cells/well in serum-free, modified MCDB153 medium. After reaching about 80% confluency, the cells were subcultured in a medium without bovine hypothalamic extract. On the following day, the cells were again subcultured in the same medium containing 10 ng/ml of epiregulin and harvested at 0.5, 1, 2, 3, 6, and 12 h. To determine whether other EGF family members affected the expression of epiregulin (a cross-induction mechanism by other EGF family members), HCECs were exposed to 10 nM of HB-EGF, TGF-α, AR, or EGF. In addition, to determine whether AG1478 affected the expression of endogenous epiregulin by exogenous epiregulin stimulation, HCECs were exposed to 10 ng/ml of epiregulin for 2 h with or without 1 h of preincubation in AG1478 (1 μM). Then the total RNA was extracted using an RNeasy kit (Qiagen, Valencia, CA) and

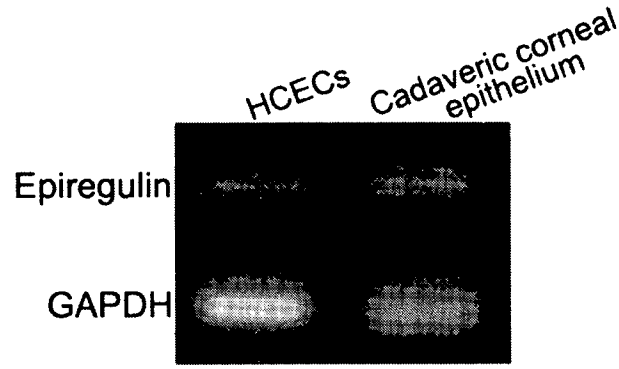


Figure 1. Expression of epiregulin mRNA in HCECs and cadaveric human corneal epithelia. The expression of epiregulin mRNA was detected in HCECs and corneal epithelium collected from cadaveric eyes by RT-PCR.

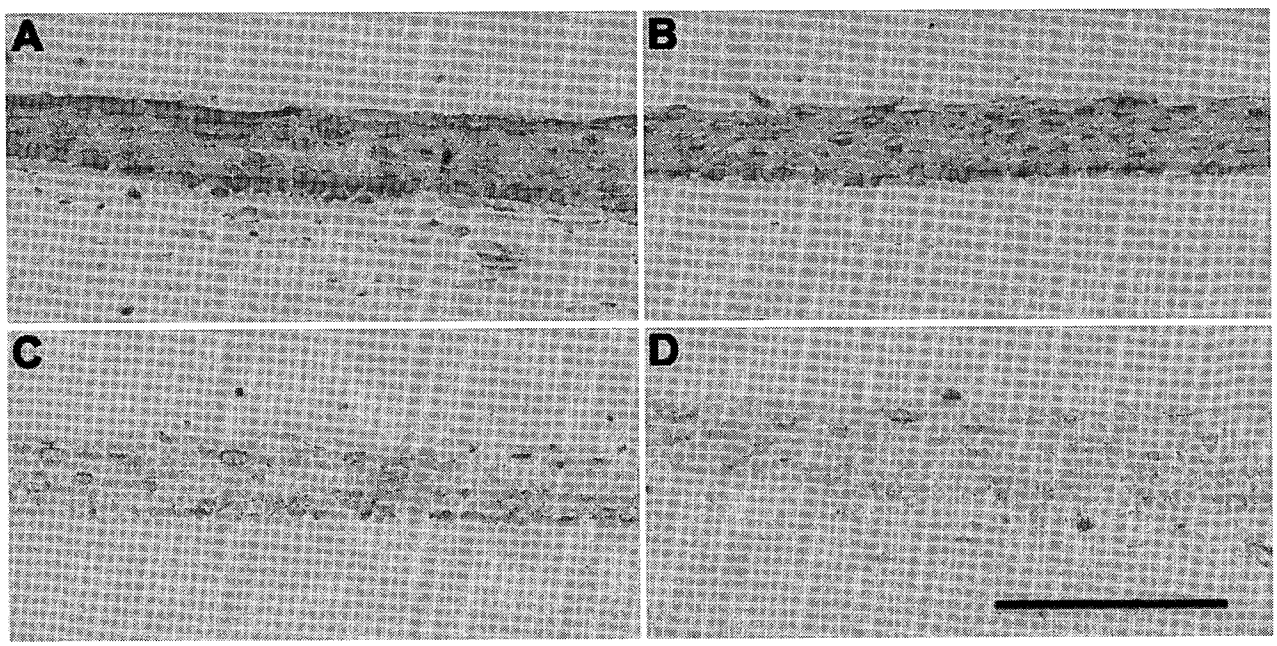
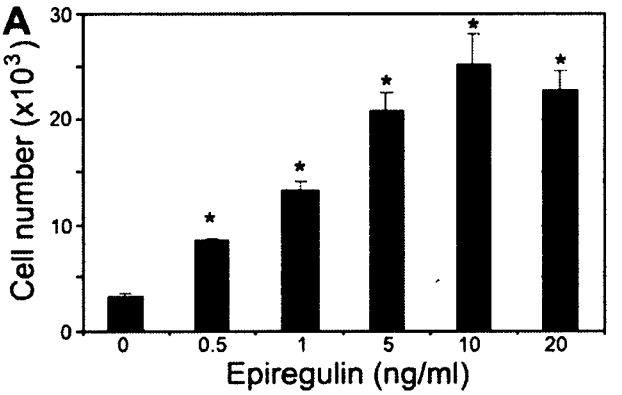


Figure 2. Immunohistochemical localization of epiregulin in human cornea. **A**: Corneal limbus. **B**: Peripheral cornea. **C**: Center of cornea. **D**: Corneal limbus (treated with normal goat Ig G). Immunoreactivity was detected strongly on basal and superficial limbal epithelium, on the basal layer of peripheral corneal epithelium, and some basal cells of central cornea. The scale bar is equal to 100 μm.

measured by spectrophotometry (OD at 260 and 280 nm). Equal amounts (1 μg) of total RNA were reverse-transcribed to cDNA using Omniscript Reverse Transcription Reagents (Qiagen) according to the instructions of the manufacturer. The PCR primers are listed in Table 1. These primers were designed from the full-length cDNA sequence in Genbank, and their specificities were confirmed by BLAST (National Library of Medicine, Bethesda, MD).

Real-time PCR was performed using a DyNAmo SYBR Green qPCR Kit (Finnzymes, Espoo, Finland). Amplifications were performed in a final volume of 20 μl containing 0.5 μM of primer mixture and 2 μl of cDNA. The PCR conditions for the initial denaturation step were 95 °C for 15 min and 40 cycles at 95 °C for 10 s, followed by 60 °C (HB-EGF, AR, EGF and GAPDH) or 64 °C (epiregulin and TGF-α) for 20 s for annealing, and 72 °C for 20 s for extension. All PCR reactions were performed by OPticon2 DNA Engine (BioRad, Hercules, CA), and each run was completed with a melting curve analysis to confirm the specificity of amplification and lack of primer dimers. The comparative cycle threshold (C_t) was calculated for all samples to quantify the relative expression of each mRNA with standardization using that of GAPDH mRNA [46]. All experiments were performed in duplicate for each datum point.



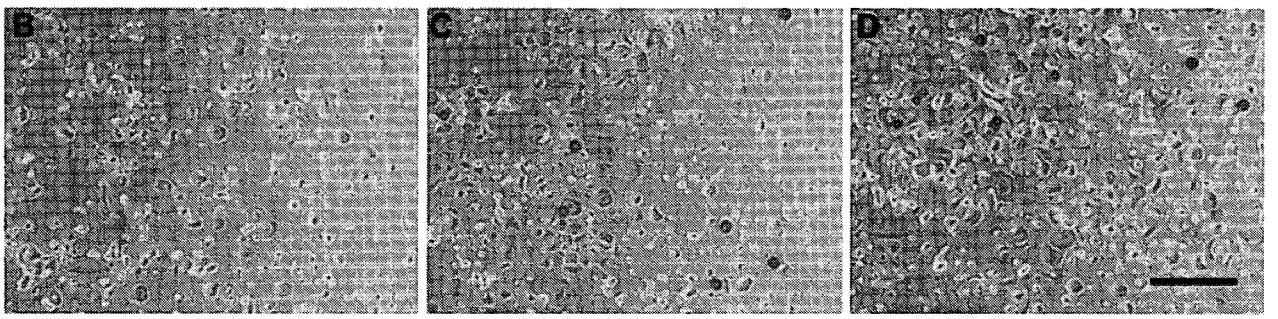
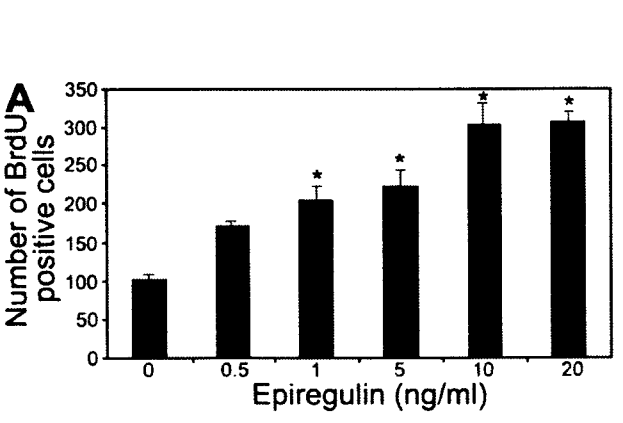


Figure 3. Effects of epiregulin on the proliferation of HCECs. Serial concentrations of recombinant epiregulin were added to the medium, and after 6 days the cell numbers were counted using a hemocytometer. The bottom panel shows representative photographs of HCECs 6 days after the addition of epiregulin. (**B**: 0 ng/ml, **C**: 1 ng/ml, **D**: 10 ng/ml) The scale bar is equal to 400 μm.



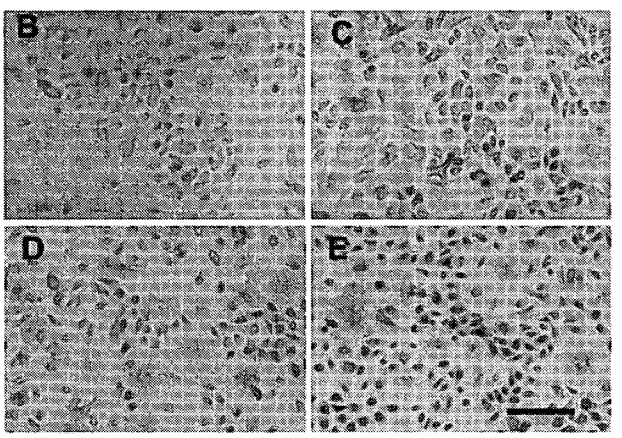


Figure 4. Effect of epiregulin on BrdU incorporation into HCECs. Serial concentrations of epiregulin were added to the medium, and after 48 h the incorporation of BrdU was determined by counting the number of BrdU labeled cells/5 mm^2. The bottom panel shows representative photographs of BrdU labeled cells. (**B**: 0 ng/ml, **C**: 1 ng/ml, **D**: 5 ng/ml, **E**: 10 ng/ml). The scale bar is equal to 200 μm. Asterisks show a significant difference ($p<0.01$) from the corresponding control (no addition of epiregulin). P values were calculated by two-sample t test. Results are representative of four independent experiments.

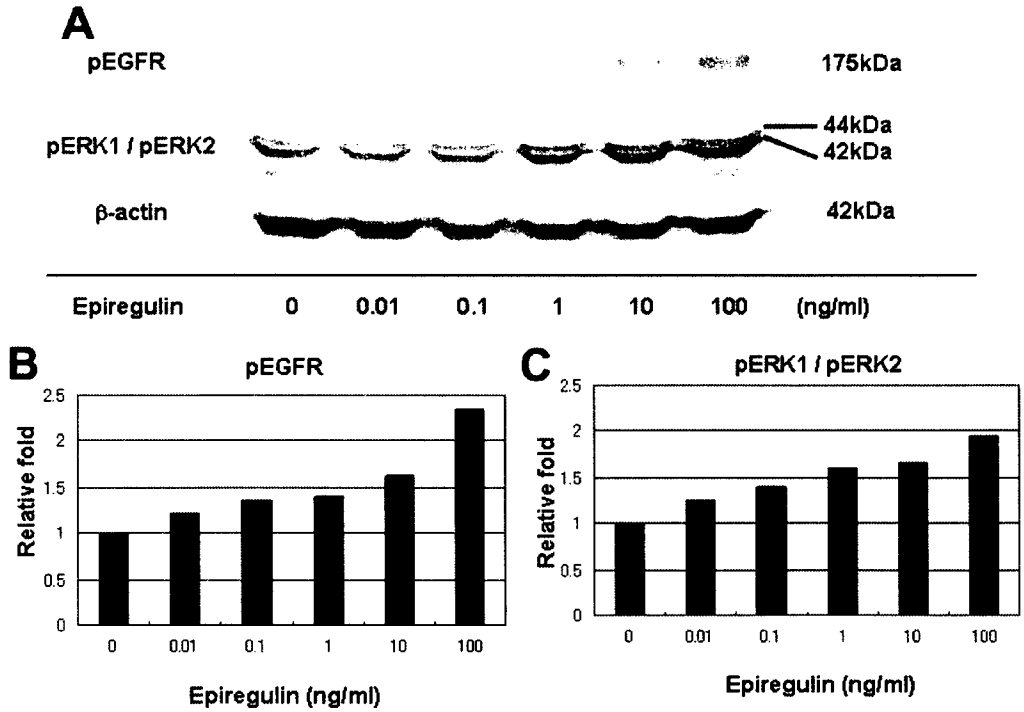


Figure 5. Phosphorylation of ErbB1 (EGFR) and ERK1/2 by epiregulin. HCECs were exposed to serial concentrations of epiregulin for 5 min. The cell lysates were subjected to 7.5% SDS-PAGE and then phosphorylated ErbB1 and ERK1/2 were detected with anti-phosphorylated-ErbB1 or anti-phosphorylated ERK1/2 antibody. The expression levels of ErbB1 and ERK1/2 were measured relative to that of β-actin in the same sample.

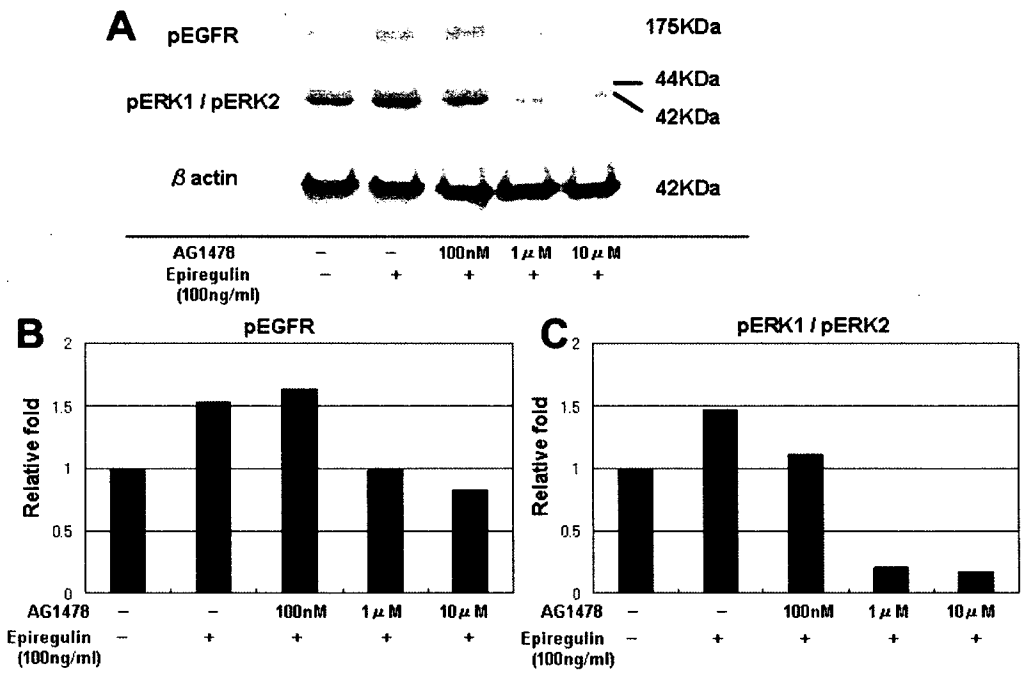


Figure 6. Inhibitory effect of AG1478 on the epiregulin induced phosphorylation of ErbB1 (EGFR) and ERK1/2. HCECs were exposed to serial concentrations of AG1478 for 1 h before incubation with 100 ng/ml of epiregulin for 5 min. The cell lysates were subjected to 7.5% SDS-PAGE, and then phosphorylated ErbB1 and ERK1/2 were detected with anti-phosphorylated-ErbB1 or anti-phosphorylated ERK1/2 antibodies. The expression levels of ErbB1 and ERK1/2 were measured relative to that of β-actin in the same sample.

Immunohistochemical staining: Cadaveric corneas were fixed in methanol, embedded in paraffin, and cut into 5 μm sections. Immunohistochemical staining was carried out with anti-epiregulin antibody (R & D Systems, 1:50 dilution, 16 h at 4 °C) using the VECTASTAIN Elite ABC kit (Vector Lab, Burlingame, CA), according to the manufacturer's protocol for paraffin sections. All sections were developed with AEC and counterstained with hematoxylin. For control, sections were treated with normal goat immunoglobulin G (IgG).

RESULTS

Expression of epiregulin in human cornea and corneal epithelial cells: To determine whether epiregulin is expressed in human corneal epithelial cells, we used RT-PCR to detect epiregulin mRNA in the corneal epithelium collected from the cadaveric eyes and from cultured HCECs. Epiregulin mRNA was detected in both corneal epithelial cells and cultured HCECs (Figure 1). Immunohistochemical staining showed that immunoreactivity was detected in all layers of the corneal epithelium, however, strong staining was detected in the basal and superficial layers of the limbal epithelium and in the basal layer of peripheral corneal epithelium. Immunoreactivity was very weakly detected in the central cornea (Figure 2). Immunoreactivity was also detected in the endothelium and weakly detected in keratocytes. Other than the cornea, epiregulin was detected strongly in the conjunctival epithelium, blood vessel endothelium, and weakly in the subconjunctival fibroblasts.

Recombinant human epiregulin stimulates proliferation of HCECs: To determine whether epiregulin stimulates the proliferation of HCECs, HCECs were cultured in serum-free medium with serially diluted concentrations of epiregulin, and the total number of cells and the number of BrdU-labeled cells were counted. The results showed that epiregulin stimulated the proliferation of HCECs in a dose-dependent manner with an 8.0 fold increase in cell numbers at 10 ng/ml (Figure 3). The number of BrdU labeled cells was also increased by epiregulin in a dose dependent manner with an increase up to 3.0 fold at 10 and 20 ng/ml (Figure 4).

Phosphorylation of ErbB1 and ERK1/2 by epiregulin: Epiregulin is known to bind to ErbB1 and to activate MAP kinase pathways [12]. In the next study, we investigated whether the tyrosine residues of ErbB1 and ERK1/2 in HCECs were phosphorylated in the presence of epiregulin. Western blot analysis revealed that epiregulin enhanced the phosphorylation of ErbB1 and ERK1/2 in a dose-dependent manner (Figure 5). The phosphorylation by 100 ng/ml of epiregulin was suppressed by a 1 μM or higher concentration of AG1478 (Figure 6).

Autoinduction of epiregulin and cross-induction of other EGF family members by epiregulin: We examined whether epiregulin up-regulated the transcription of its own mRNA in HCECs, i.e., an auto-induction mechanism of epiregulin. The results from real-time PCR showed that the expression of epiregulin increased as early as 0.5 h after incubation with 10 ng/ml of epiregulin, reached a peak (4.8 fold increase from 0 h) at 3 h after incubation (Figure 7), and gradually returned to its original level by 12 h. This up-regulation was completely inhibited by AG1478 (Figure 8).

Because EGF-related autocrine growth factors were able to induce other growth factors of the EGF family in a variety of epithelial cells [47], we examined whether epiregulin up-regulated the other members of the EGF family, i.e., a cross-induction mechanism of epiregulin, in HCECs. Real time PCR analysis showed that epiregulin exposure significantly increased the mRNA levels of AR at 2 h and of HB-EGF between 0.5 and 2 h, while the mRNA levels of EGF and TGF-α remained essentially unchanged (Figure 9).

Lastly, we investigated whether the expression of epiregulin was enhanced by other EGF family members, i.e., a cross-induction mechanism by other EGF family members, using real-time PCR analysis. The results showed that the ex-

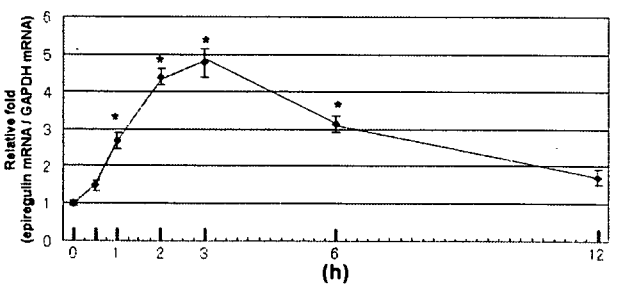


Figure 7. Auto-induction of epiregulin in HCECs. HCECs were incubated with 10 ng/ml of epiregulin for 0.5, 1, 2, 3, 6, and 12 h, and the relative expression of mRNA was determined by real time PCR. The amount of mRNA was calculated relative to the amount of GAPDH mRNA in the same samples. Asterisks indicate a significant difference from the corresponding control (0 h). P values were calculated by the two-sample t test. Results are mean±SEM of five independent experiments.

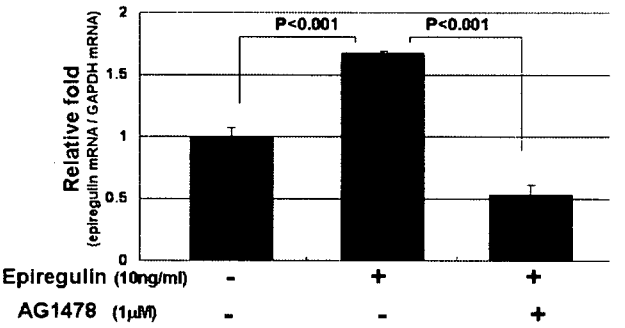


Figure 8. The inhibitory effect of AG1478 on the auto-induction of epiregulin in HCECs. HCECs were exposed to 10 ng/ml of epiregulin for 2 h with or without preincubation of AG1478 (1 μM), and the relative expression of epiregulin mRNA was examined by real-time PCR. The amount of mRNA was calculated relative to the amount of GAPDH mRNA in the same samples. P values were calculated by the two-sample t test. Results are mean±SEM of five independent experiments.

pression of mRNA of epiregulin increased around 2 or 3 h after treatment with all of the EGF family members including TGF-α, HB-EGF, EGF, and AR (Figure 10).

DISCUSSION

The integrity of the EGF receptor-ligand signaling system is of great importance in regulating epithelial cell differentiation and proliferation. Among the members of the EGF family (EGF, TGF-α, AR, and HB-EGF), ligands for ErbB1 are known to be responsible for the migration and proliferation of HCECs and for the synthesis of extracellular matrix during corneal epithelial wound healing [13-16,34,35].

Epiregulin is a relatively new member of the EGF family, and in a previous report on the distribution of epiregulin in human tissues, northern blot analysis showed that epiregulin mRNA was found predominantly in the placenta and peripheral blood leukocytes and to a lesser extent in the heart. A very weak expression was also detected in normal adult bone marrow, ovaries, small intestine, colon, lungs, and liver [41]. Similar to other growth factors belonging to the EGF family, epiregulin has been reported to promote the proliferation of other human epithelial cells such as the urothelium and epidermis [43,44].

We have shown that epiregulin mRNA was expressed in human corneal epithelial cells. Immunohistochemical staining for epiregulin revealed that immunoreactivity was strongly detected in limbal epithelial cells, and in the basal layer of peripheral corneal epithelia in which transient amplifying cells are supposed to exist. Similarly, Zhou et al. [45] reported that the mRNA of epiregulin was preferentially expressed in limbal basal cells of mouse corneal epithelia. The differences in expression patterns may be due to the differences in species and experimental methods. However, in both studies epiregulin expression was strongly detected in the progenitor or proliferating corneal epithelial cells. These results would suggest that epiregulin may contribute to the proliferative capacity of corneal epithelial cells.

We have shown that epiregulin was able to enhance the proliferation of HCECs. It has been suggested that the proliferation of corneal epithelial cell by members of the EGF family was induced through the ErbB1-MAPK pathway [38]. Our results showed that epiregulin was also able to activate ErbB1 following ERK1/2 phosphorylation. Thus, like other EGF family members, epiregulin may induce corneal epithelial cell proliferation as, at least, a ligand for ErbB1.

Most of the EGF-related autocrine growth factors are capable of promoting the expression of other EGF family growth factors in a variety of epithelial cells [47,48], and an increase in the level of EGF family growth factors (EGFR ligand transcripts) is dependent upon signals from their own receptors, "EGFR" [48-51]. Shirakata et al. reported that epiregulin is part of an auto-induction and cross-induction mechanism involving HB-EGF, AR, and TGF-α in keratinocyte proliferation [44]. In this study, we have shown that epiregulin stimulated the induction of its own mRNA as well as the phosphorylation of ErbB1. The significant inhibition of the induction of epiregulin expression by AG1478, a specific inhibitor of EGFR kinase activity, suggests the possible existence of an auto-induction of epiregulin, and phosphorylated ErbB1 may be part of the pathway of the auto-induction mechanism. These results also indicate that epiregulin can be listed as one of the auto- or paracrine growth factors for corneal epithelial cells interaction with ErbB1.

Our results also showed that epiregulin served as an effector of similar auto- and cross-induction mechanisms in corneal epithelial cells. Although an up-regulation by epiregulin was limited to HB-EGF and AR in the case of HCECs, the epiregulin mRNA was up-regulated by EGF, HB-EGF, TGF-α, and AR. These findings suggest that epiregulin most likely

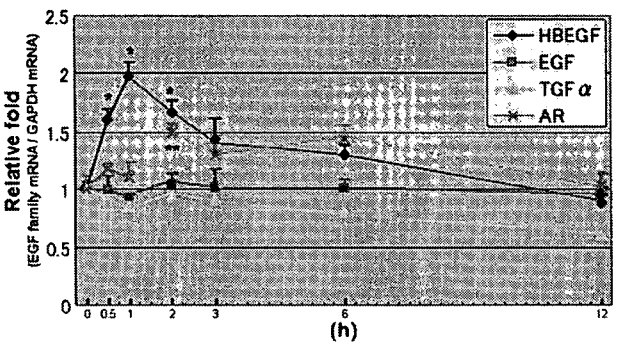


Figure 9. The cross-induction of HB-EGF, TGF-α, EGF and AR mRNA by epiregulin. HCECs were incubated with 10 ng/ml epiregulin for 0.5, 1, 2, 3, 6, and 12 h, and the relative expressions of HB-EGF, TGF-α, EGF, and AR mRNA were examined by real-time PCR. The amount of mRNA was calculated relative to the amount of GAPDH mRNA in the same samples. Asterisks indicate a significant difference from the corresponding control (0 h). P values were calculated by the two-sample t test. Results are mean±SEM of five independent experiments.

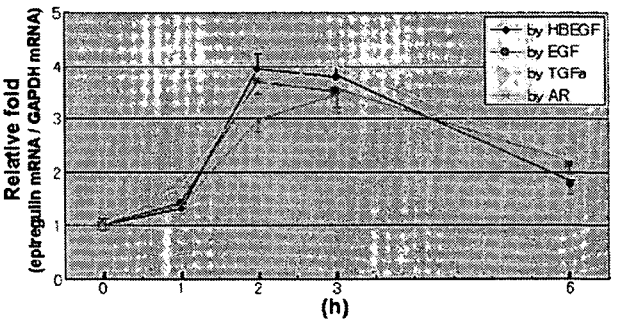


Figure 10. Cross-induction of epiregulin mRNA expression by HB-EGF, EGF, AR, and TGF-α. HCECs were incubated with 10 nM each of EGF, HB-EGF, AR, and TGF-α for 1, 2, 3, and 6 h, and the relative level of expression of epiregulin mRNA was examined by real-time PCR. The amount of mRNA was calculated relative to the amount of GAPDH mRNA in the same samples. Results are the mean±SEM of five independent experiments.

operates in corneal epithelial proliferation in concert with other EGF family members.

The extent of cross-induction differs among the members of the EGF family. It has been reported that each EGF family growth factor has distinct biological functions. The tissue distribution, molecular characteristics, receptor binding, preference to dimerize, and receptor affinity differ among the growth factors [44,47]. Interestingly, Barnard et al. reported results similar to ours that HB-EGF and AR were rapidly induced by EGF-related peptides in the intestinal epithelial cell line, and speculated that differences in the post-receptor processing may contribute to the heterogeneity in the biological responses [47]. TGF-α, HB-EGF, and AR have been reported to be up-regulated on corneal epithelial cells, although the levels of EGF are at trace levels and do not change during the corneal wound healing process [16,52]. Although TGF-α mRNA was not induced by epiregulin under the conditions we employed, it is consistent that HB-EGF and AR were induced in corneal epithelial cells, and these results might be a molecular characteristic of the corneal epithelial cells. Consistent with other reports, EGF was not induced by epiregulin; however, EGF induced the expression of epiregulin in HCECs.

It is known that EGF exists in tear fluid along with HB-EGF and TGF-α, and corneal wounds increase the expression of EGF supplied from lacrimal glands through the sensory nerves [53,54]. Thus, EGF may be able to stimulate corneal epithelial cells, but not be induced in corneal epithelial cells. Together with previous reports, our results suggested that epiregulin may function together with not only endogenously produced EGF family members, but also with those in the tear fluid through cross-induction mechanisms during corneal epithelial wound-healing.

The presence of autocrine or paracrine feedback loops involving EGFR have been documented for the members of the EGF family in different tissues [51]. When it comes to these pathways, an increase in the level of EGFR ligand transcripts is dependent upon the signal from their own receptors, "EGFR" [48-51]. As shown in this study, the phosphorylation of ErbB1 by epiregulin can lead to an increase in transcripts of the other EGF family members. It would be highly efficient for corneal epithelial cells to utilize a common pathway when the integrity of the corneal epithelium is seriously compromised.

In conclusion, epiregulin is present in human corneal epithelial cells and is able to induce HCECs to proliferate as an autocrine growth factor. The intricate cross-induction mechanism involving epiregulin certainly plays a role in a variety of events which occur in the corneal epithelium.

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