

Krüppel-like factor 5 activates MEK/ERK signaling via EGFR in primary squamous epithelial cells

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ABSTRACT Rapid cell proliferation is a hallmark of transit amplifying cells, but the mechanisms of this localized proliferation are not well understood. The *Krüppel*-like factor family member Klf5 (IKLF; BTEB2) promotes cell proliferation and is highly expressed in squamous epithelia, in regions of active proliferation. Here, using mouse primary esophageal keratinocytes as a model, we identify a critical role for Klf5 in regulating squamous epithelial proliferation via the epidermal growth factor receptor (EGFR), which, like Klf5, is localized to basal cells in squamous epithelia. We show that Klf5 increases proliferation, transcriptionally up-regulates *EGFR*, and activates MEK/ERK signaling, as indicated by increased phosphorylation of MEK and ERK. By chromatin immunoprecipitation, we demonstrate that Klf5 binds directly to the 5' regulatory region of *EGFR*. In addition, we show that regulation of proliferation by Klf5 is dependent on EGFR and MEK/ERK signaling, as the proliferative response to Klf5 is blocked by pharmacologic inhibition of EGFR or MEK. Inhibition of EGFR or MEK also decreases *Klf5* expression. Thus, Klf5 regulates MEK/ERK signaling via EGFR and is also downstream of MAPK signaling, providing a novel mechanism for signal amplification or suppression and control of proliferation in basal cells.—Yang, Y., Goldstein, B. G., Nakagawa, H., Katz, J. P. *Krüppel*-like factor 5 activates MEK/ERK signaling via EGFR in primary squamous epithelial cells *FASEB J.* 21, 543–550 (2007)

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WITHIN THE EPITHELIA of the luminal gastrointestinal tract is a spatial separation of cell proliferation and differentiation (1). Stem cells give rise to transit amplifying cells, which migrate and undergo further differentiation to form the functional epithelial cells of the esophagus, stomach, small intestine, and colon. The esophagus in particular is lined by a stratified squamous epithelium, the most common epithelial type in the human body, which also lines the skin, oral cavity, and several other organs. These stratified squamous epithelia contain proliferative basal cells and differentiated cells in the suprabasal (spinous) and superficial layers (2). Within the basal layer of these epithelia are the slow-cycling stem cells, which rarely divide within the

stem cell niche, and the transit amplifying cells, which undergo rapid cell division before migrating out of the basal layer (3, 4). These processes are tightly regulated, and abnormalities of epithelial homeostasis form the basis of numerous benign and malignant diseases, including esophagitis and cancer (5). While a great deal has been learned about squamous epithelial stem cell biology (6), little is known about the mechanisms regulating the localized rapid proliferation of the transit amplifying cells.

The *Krüppel*-like factors (KLFs) are DNA-binding transcriptional regulators with diverse roles in proliferation and differentiation in a number of tissues and cell types (7, 8). Members of this family bind similar 'CACCC' elements on DNA. *Klf5* (IKLF; BTEB2) is a tissue-restricted KLF highly expressed in epithelial cells in the proliferative compartments of the gastrointestinal tract and epidermis, including in basal cells of the esophagus and skin (9, 10). In NIH3T3 cells, Klf5 promotes proliferation, mediates the transforming properties of oncogenic H-ras, and activates the cyclin B1/Cdc2 complex during mitosis (11–13). KLF5 also enhances proliferation and tumorigenesis of a human bladder cancer cell line (14). However, the function of Klf5 appears to be context-dependent, as Klf5 enhances cell growth and activates cyclin D1 in nontransformed intestinal cell lines, such as IEC-18 and IMCE cells, but is negatively correlated with cell growth in colon cancer cell lines (15). *KLF5* has also been suggested to be a tumor suppressor in esophageal, breast, prostate, and intestinal cancers (15–18). In esophageal cancer cells, for example, KLF5 inhibits proliferation and invasion and promotes apoptosis, including that in response to anchorage-independent growth. Homozygous null mice for *Klf5* die early in embryogenesis, and studies of heterozygous mice demonstrate a key role for *Klf5* in cardiovascular remodeling and adipocyte differentiation (19, 20).

Given its expression in basal cells, we hypothesized that Klf5 may play a key role in regulating squamous

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epithelial proliferation. In other tissues, Klf5 has been reported to be either upstream (14) or downstream (12, 21) of the mitogen-activated protein kinase (MAPK) pathway, which may be regulated by the epidermal growth factor receptor (EGFR). Activation of EGFR occurs frequently in both benign and malignant hyperproliferative diseases and triggers a cascade of downstream intracellular signaling pathways, including the highly conserved Ras-Raf-MEK-ERK, which results in cell survival, proliferation, migration, angiogenesis, and inhibition of apoptosis (22, 23). In esophageal keratinocytes, EGFR mediates increased proliferation, migration, and aggregation (24) and, EGFR, like Klf5, is localized to cells in the basal layer of esophageal epithelia (25). Nonetheless, while the biochemical properties of EGFR have been extensively studied, the transcriptional regulation of *EGFR* is not well understood.

In the present study, we have used mouse primary esophageal epithelial cells as a model to demonstrate that Klf5 increases cell proliferation, binds to the 5' regulatory region of *EGFR* and up-regulates *EGFR* expression, and specifically enhances MEK/ERK signaling mediated by EGFR. We also show that Klf5 is itself regulated by the MEK/ERK pathway, offering a mechanism for the rapid amplification or suppression of signaling and, consequently, proliferation in epithelial basal cells.

MATERIALS AND METHODS

Cell culture and treatment

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania. The isolation and culture of mouse primary esophageal keratinocytes has been described elsewhere (24). Briefly, the esophagus was removed and incubated in Hank's solution containing 1 U/ml dispase (BD Biosciences, San Jose, CA, USA) for 10 min at 37°C. Esophageal epithelium was then dissected away from the other tissues, minced, and incubated in 0.05% trypsin (Invitrogen, Carlsbad, CA, USA) for 10 min at 37°C to generate single cells. The reaction was stopped with soybean trypsin inhibitor (Sigma, St. Louis, MO, USA), and cells were washed with Hank's solution and centrifuged. Esophageal epithelial cells were grown in keratinocyte serum-free medium (K-SFM, Invitrogen) supplemented with 40 µg/ml bovine pituitary extract (Invitrogen), 1.0 ng/ml epidermal growth factor (EGF) (Invitrogen), 100 U/ml penicillin, and 100 µg streptomycin (Invitrogen).

Except as noted, all experiments were begun when cells reached 70% confluence. For studies of effects of EGFR and MEK inhibition, cells were starved for 48 h without EGF and then treated for 4 h with 0 or 10 ng/ml of EGF, along with DMSO alone, 100 nM of the EGFR inhibitor AG1478 (Sigma) in DMSO, or 50 µM of the MEK inhibitor PD98059 (Cell Signaling, Danvers, MA, USA) in DMSO.

Retroviral expression vector and infection

The full-length mouse *Klf5* cDNA (GenBank Accession Number NM_009769) was excised from pMT3-Klf5 (gift from Dr. Vincent Yang) and subcloned into the pFB-neo retroviral

vector (Stratagene, La Jolla, CA, USA). A *Klf5* siRNA expressing retrovirus was constructed using the pSR-neo vector (OligoEngine, Seattle, WA, USA) by subcloning at the *Bgl*II and *Hind*III sites complementary double-stranded oligonucleotides (GATCCCCACATGAACGTCTTCCCTCCCTTCAAGAGAGGGAGG AAGACGTTTCATGTTTTTA) directed against nucleotides 676–694 (italics) of mouse *Klf5*. The resulting constructs were packaged in Phoenix-Ampho cells (Stanford University, Stanford, CA, USA) by transfection with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Culture supernatants from individual Phoenix-Ampho cells were used to infect mouse primary keratinocytes at a 1:6 dilution in K-SFM. Cells were passaged after 24 h and selected with 300 µg/ml G418 for 14 d.

MTT assay

Cell growth rates were evaluated by MTT assay as described previously (18). In brief, 1×10^4 cells per well were seeded in triplicate onto 48-well plates in K-SFM without EGF. After 24 h, the cells were treated according to the experimental design. After each treatment, media were removed, and cells were washed with PBS. MTT reagent (USB, Cleveland, Ohio, USA) was added at 2 mg/ml in Hank's buffer (Invitrogen) and incubated for 1 h until dark-blue crystals were seen in the cytoplasm under light microscopy. The crystals were dissolved in DMSO, and the absorbance measured at 570 nm with background subtraction at 650 nm in a Beckman DU 600 spectrometer. Results were expressed as the mean of absorbance relative to time zero or control \pm SEM.

Cell transfection and reporter assays

Primary esophageal epithelial cells containing retroviral vectors as described above, were transfected at 50% confluence in triplicate on 24 well plates using FuGENE 6 Transfection Reagent (Roche, Indianapolis, IN, USA). To analyze the effects of *EGFR* expression on cell growth, we cloned full-length human *EGFR* cDNA into pCI-neo (Promega, Madison, WI, USA) and performed MTT assays as described above. For *EGFR* reporter assays, we amplified by polymerase chain reaction (PCR) a 709 bp region of *EGFR*, immediately upstream of the translation start site, and cloned this fragment into pGL3-Basic (Promega). After 48 h, cells were lysed with BD Pharmingen Cell Lysis Buffer (BD Biosciences). We analyzed luciferase reporter activity using Luciferase Assay Reagent (Promega) with a Microtiter Plate Luminometer (Dynex Technologies, St. Paul, MN, USA). Luciferase activity was normalized to β -galactosidase and expressed relative to control.

Western blotting

Cells were lysed with 150 mM NaCl; 50 mM, Tris pH 7.5; 1% Nonidet P-40; 0.5% sodium deoxycholic acid; and Complete Protease Inhibitor Cocktail Tablets (Roche) and protein was isolated. Total protein (30 µg) from each sample were separated on a NuPAGE 4–12% bis-tris acrylamide gel (Invitrogen) and transferred onto PVDF membrane (Millipore Corp., Bedford, MA, USA) in 1× NuPage transfer buffer (Invitrogen) for 75 min at 4°C. Membranes were blocked with 5% nonfat dry milk in TBST for 2 h at room temperature and incubated overnight at 4°C with the following primary antibodies: 1:5000 rabbit anti-Klf5 (generated against amino acids 95–111 of the mouse Klf5 protein by Biosource International/QCB); 1:1000 mouse anti-EGFR (NeoMarker); 1:500 mouse anti-ERK1/2 (Santa Cruz Biotech, Santa Cruz, CA, USA); 1:500 mouse antiphospho-ERK1/2 (Santa Cruz Bio-

tech); 1:1000 rabbit anti-MEK1/2 (Cell Signaling); 1:1000 rabbit antiphospho-MEK1/2 (Cell Signaling); 1:1000 rabbit anti-p38 (Cell Signaling); 1:1000 rabbit antiphospho-p38 (Cell Signaling); 1:300 rabbit anti-JNK2 (Santa Cruz Biotech); 1:1000 rabbit antiphospho-JNK2 (Cell Signaling); 1:500 mouse anti- β -actin (Sigma); and 1:2000 mouse anti- α -tubulin (Sigma). Membranes were then incubated for 45 min at room temperature with a 1:3000 dilution of anti-rabbit/horseradish peroxidase or anti-mouse/horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ, USA), washed twice for 10 min, and developed with the Enhanced Chemiluminescence Plus Western blot Analysis Kit (Amersham Pharmacia Biotech).

Quantitative PCR

Total RNA was isolated with the RNeasy Micro Kit (Qiagen, Valencia, CA, USA), and cDNA was synthesized with Superscript II Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was performed in triplicate on three samples for each experimental condition using a Stratagene Mx4000 Multiplex Quantitative PCR System and Brilliant SYBR Green QPCR Reagents (Stratagene). *TATA box binding protein (TBP)* was used as the internal control. Primer sequences are available on request.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed with the ChIP Assay Kit (Upstate Biotechnology, Lake Placid, NY, USA), according to the manufacturer's protocol. Mouse primary esophageal keratinocytes at 80% confluence were treated with 1% formaldehyde for 10 min to cross-link associated protein to DNA. Cells were lysed as above and sonicated with an Ultrasonic Processor (Sonics & Materials, Newtown, CT, USA) for 3 sets of 10 s pulses at 30% power. After a 10-fold dilution, the samples were precleared with protein A agarose/salmon sperm DNA for 30 min at 4°C. Samples were incubated overnight at 4°C with 1:500 anti-Klf5 or 1:500 anti-mouse IgG (Sigma), as a negative control, and then precipitated with protein A agarose for 1 h. Samples were heated at 65°C for 4 h, treated with proteinase K, and DNA extracted with phenol/chloroform. Primers for amplification of the *EGFR* gene were designed as indicated in Fig. 2C. PCR was performed with puReTaq Ready-To-Go PCR beads (Amersham Biosciences) for 25 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. PCR products were separated on a 2% agarose gel and visualized by a Gel Doc XR system (Bio-Rad Laboratories, Hercules, CA, USA)

RESULTS

Klf5 is important for cell proliferation *in vitro*

Primary esophageal epithelial cells provide a valuable platform for the study of normal epithelial homeostasis (24). Primary esophageal cell lines can be readily grown in culture, show morphological, cytogenetic, and biochemical properties of normal cells, reaching senescence after ~45–50 population doublings, and can be retrovirally transduced, permitting the investigation of specific factors and pathways (26).

Klf5 has previously been shown to increase cell growth and cell cycle progression in NIH3T3 cells by accelerating both the G1/S transition and entry into mitosis (12, 13). To investigate the role of Klf5 in esophageal epithelial proliferation, we stably infected mouse primary esophageal keratinocytes with retrovirus to express *Klf5* (pFB-Klf5) or siRNA directed against nucleotides 676–694 of mouse *Klf5* (pSR-siKlf5). By Western blot, we confirmed successful overexpression and knockdown of *Klf5* by pFB-Klf5 and pSR-siKlf5, respectively, in mouse primary esophageal epithelial cells (Fig. 1A). We next performed MTT assays to examine the effects of Klf5 on cell proliferation. *Klf5* overexpressing cells showed a slight increase in prolif-

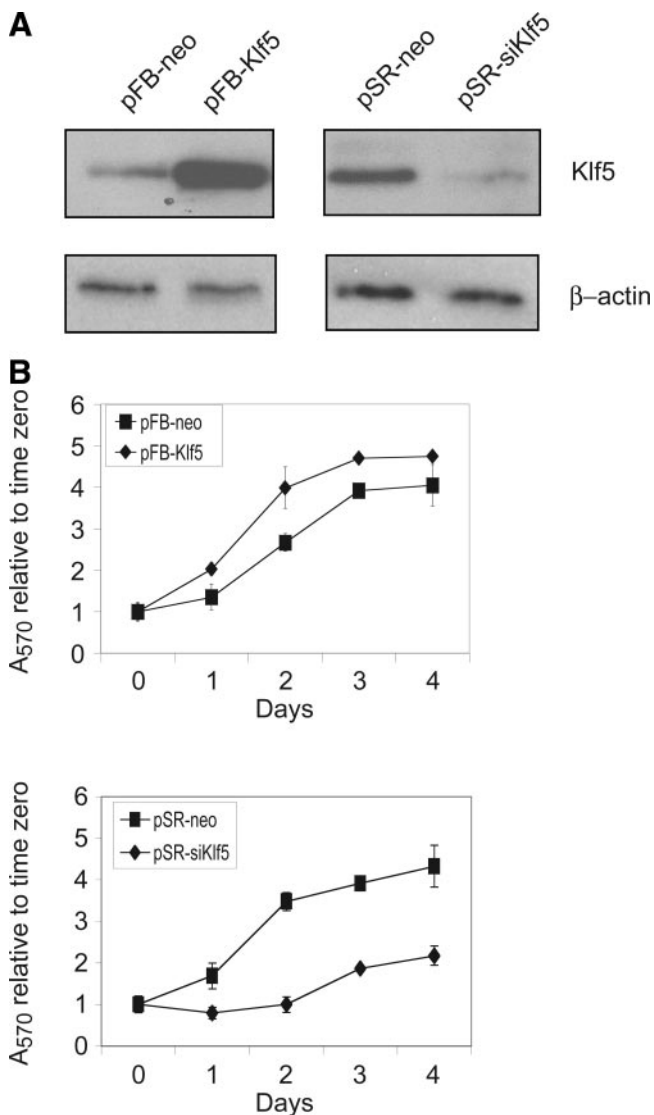


Figure 1. Klf5 promotes proliferation in mouse primary esophageal cells. A) Western blot demonstrated successful overexpression and suppression of Klf5 in esophageal keratinocytes by pFB-Klf5 and pSR-siKlf5, respectively. pFB-neo and pSR-neo infected cells served as controls. B) By MTT assay, overexpression of Klf5 increased proliferation while inhibition of Klf5 with siRNA reduced cell growth. The data represented the average value of triplicate samples from three individual experiments.

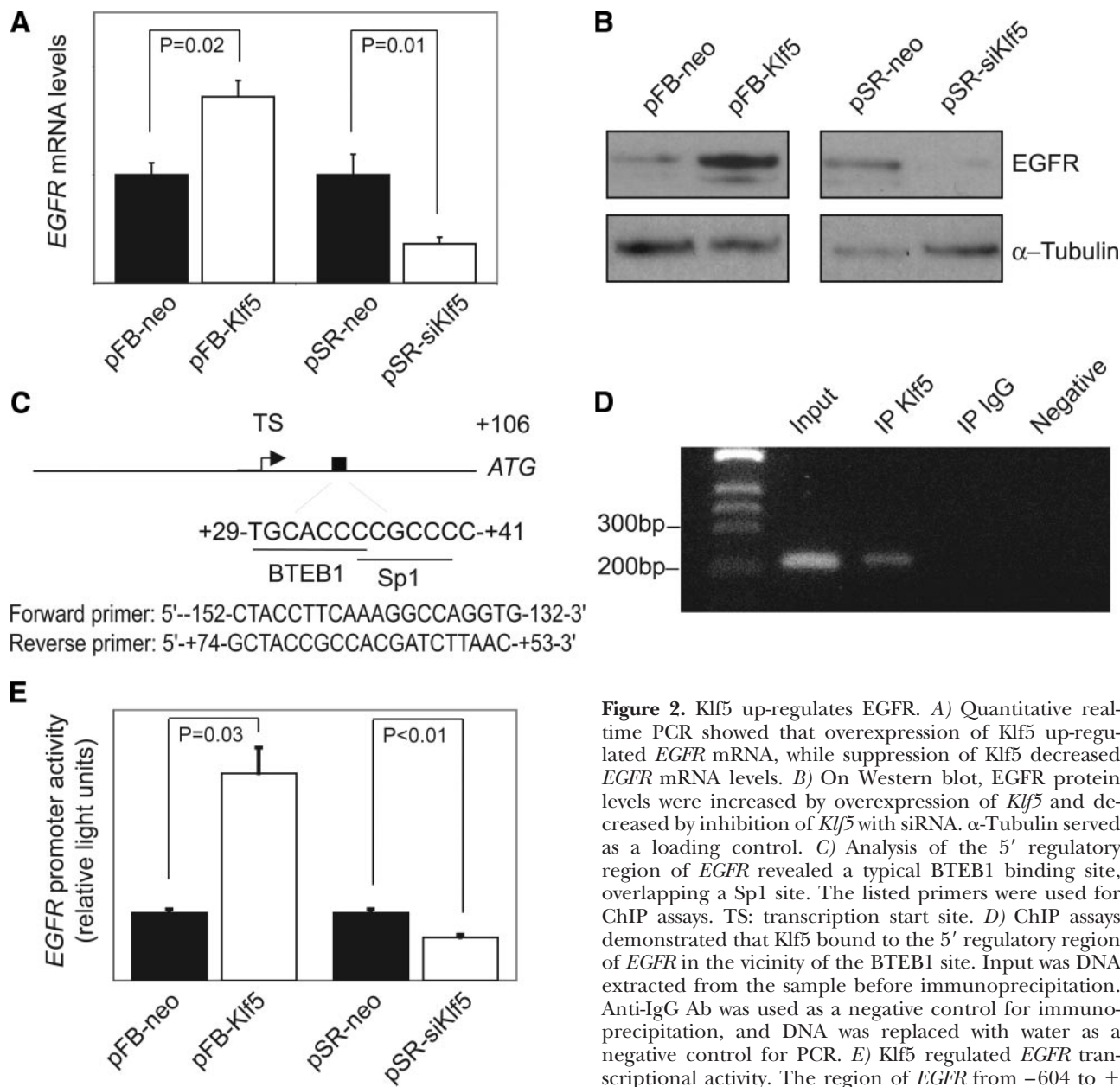


Figure 2. Klf5 up-regulates EGFR. *A*) Quantitative real-time PCR showed that overexpression of Klf5 up-regulated *EGFR* mRNA, while suppression of Klf5 decreased *EGFR* mRNA levels. *B*) On Western blot, EGFR protein levels were increased by overexpression of *Klf5* and decreased by inhibition of *Klf5* with siRNA. α -Tubulin served as a loading control. *C*) Analysis of the 5' regulatory region of *EGFR* revealed a typical BTEB1 binding site, overlapping a Sp1 site. The listed primers were used for ChIP assays. TS: transcription start site. *D*) ChIP assays demonstrated that Klf5 bound to the 5' regulatory region of *EGFR* in the vicinity of the BTEB1 site. Input was DNA extracted from the sample before immunoprecipitation. Anti-IgG Ab was used as a negative control for immunoprecipitation, and DNA was replaced with water as a negative control for PCR. *E*) Klf5 regulated *EGFR* transcriptional activity. The region of *EGFR* from -604 to +105 in the pGL3-Basic luciferase reporter vector was

transfected into primary esophageal cells. While a 3-fold increase in *EGFR* transcriptional activity was seen in pFB-Klf5 cells, compared with control, suppression of *Klf5* in pSR-siKlf5 cells resulted in a 40% decrease in activity.

eration compared with controls, and suppression of Klf5 reduced cell growth dramatically (Fig. 1*B*), indicating that Klf5 is critical for normal proliferation of esophageal keratinocytes. Notably, *Klf5* overexpression in esophageal keratinocytes did not result in signs of cellular transformation, such as loss of contact inhibition or anchorage-independent growth (data not shown), as seen in NIH3T3 cells (11).

Klf5 binds to and regulates EGFR

To elucidate the mechanism by which Klf5 regulates proliferation in esophageal keratinocytes, we investigated the effects of *Klf5* expression and suppression on EGFR. EGFR, like Klf5, is expressed in esophageal basal

cells (25) and plays a key role in the control of cell proliferation (24). By quantitative real-time PCR, *Klf5* overexpression significantly increased *EGFR* mRNA, while suppression of *Klf5* significantly decreased *EGFR* mRNA expression (Fig. 2*A*). EGFR protein levels were also increased by *Klf5* overexpression and decreased by *Klf5* inhibition (Fig. 2*B*).

To determine whether *EGFR* was a direct target for Klf5, we examined the 5' regulatory region of *EGFR* for putative Klf5 binding sites. Using the computational program TESS (<http://www.cbil.upenn.edu/teess>), we identified a typical BTEB1 site, which overlapped a putative Sp1 binding site from +29 to +41 (Fig. 2*C*), upstream of the translation start site (+106). The specificity of DNA binding has been

shown to be similar between BTEB1 and BTEB2/Klf5 (27). To test whether this region binds Klf5 *in vivo*, we performed ChIP assays using an anti-Klf5 antibody (Ab) to precipitate the DNA-protein complex. Using primers specific for this region of *EGFR*, we demonstrated that Klf5 binds directly to the 5' regulatory region of *EGFR* containing the putative BTEB1-BTEB2/Klf5 binding site (Fig. 2D). No Klf5 binding was identified using primers for the 5' regulatory region of *EGFR* between -297 to -147, a region containing putative Klf4, E-box, and HNF3 binding sites by computational analysis (data not shown), demonstrating the specificity of Klf5 binding between -152 and +74. Using a region of *EGFR* from -604 to +105 cloned into the pGL3-Basic luciferase reporter vector, we demonstrated that overexpression of *Klf5* produced a 3-fold induction of *EGFR* transcriptional activity, while suppression of Klf5 resulted in a 40% decrease in activity (Fig. 2E). Taken together, these data show that *EGFR* is a direct transcriptional target for Klf5.

Klf5 activates MEK/ERK signaling

The activation of MAPK signaling is crucial for many fundamental cellular processes, including the spatiotemporal control of cell proliferation (23). MAPKs are part of a phosphorelay system composed of three sequentially activated kinases, and activation of the pathway involves the phosphorylation of MAPKs by MAPK kinases (28). To determine whether activation of MAPK signaling plays a role in the regulation of esophageal epithelial proliferation by Klf5, we examined the phosphorylation of MEK1/2, ERK1/2, and p38 by Western blot (Fig. 3). *Klf5* overexpression increased levels of phospho-MEK1/2 and phospho-ERK1/2 but did not affect levels of total MEK or total ERK (Fig. 3). In contrast, *Klf5* suppression led to inhibition of MEK/ERK signaling. The specificity of Klf5 for MEK/ERK signaling was demonstrated by the failure of *Klf5* expression to alter levels of phospho-p38 (Fig. 3) or phospho-JNK2 (data not shown).

Klf5 activates the MEK/ERK pathway via EGFR

Stimulation of EGFR activates the MEK/ERK pathway (29). To investigate whether the activation of MEK/ERK by Klf5 in esophageal epithelial cells is dependent on EGFR, cells were starved without EGF for 48 h and then treated with EGF with or without the EGFR inhibitor AG1478 for 4 h. As expected, EGF stimulation in control cells resulted in phosphorylation of EGFR and this EGFR activation was blocked by AG1478 (Fig. 4A). The decline in total EGFR in response to EGF has been reported to result from ligand-induced endocytosis (30). Stimulation with EGF also activated MEK/ERK signaling in esophageal keratinocytes, although in *Klf5* overexpressing cells, this activation was blunted due to higher baseline levels of MEK/ERK phosphorylation. It should

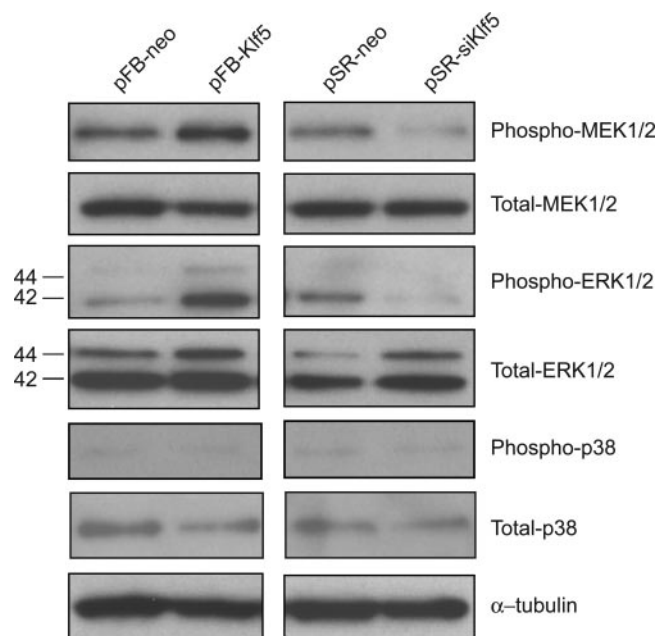


Figure 3. Klf5 activates MEK/ERK signaling. Western blot revealed increased phosphorylation of MEK and ERK in primary esophageal keratinocytes with overexpression of Klf5 and decreased MEK and ERK phosphorylation in cells with suppression of Klf5 by siRNA, compared with controls. Levels of total MEK and total ERK, as well as phosphorylated and total p38 MAPK, were not altered by changes in Klf5 expression, demonstrating that Klf5 activated MAPK signaling specifically through phosphorylation of MEK and ERK.

be noted that the basal level of EGFR tyrosine phosphorylation without exogenous ligand stimulation was higher in *Klf5* transduced cells (Fig. 4A), where EGFR is up-regulated (Fig. 2). In contrast, inhibition of EGFR resulted in reduced MEK/ERK signaling in both control and *Klf5* overexpressing cells. These changes were also reflected in the rate of cell growth (Fig. 4B). In cells with suppression of *Klf5* by siRNA, the MEK/ERK pathway was only minimally activated (Fig. 3), and the effects of further EGFR inhibition were not evident (data not shown). However, overexpression of *EGFR* in pSR-siKlf5 cells by transient transfection restored normal cell growth (Fig. 4C), providing additional evidence that *EGFR* is downstream of Klf5 and mediates the effects of Klf5 on cell proliferation. Of note, Klf5 appeared to increase the expression of the EGFR ligands EGF, TGF- α , and amphiregulin by quantitative real-time PCR, although this result did not reach statistical significance (data not shown). The mechanism by which Klf5 might modulate the expression of EGFR ligands is not yet known.

Klf5 is regulated by the EGFR-MEK/ERK pathway

Klf5 has been reported to be a downstream target of MAPK in other cell types (12, 21). In esophageal keratinocytes, EGF stimulation increased levels of *Klf5*

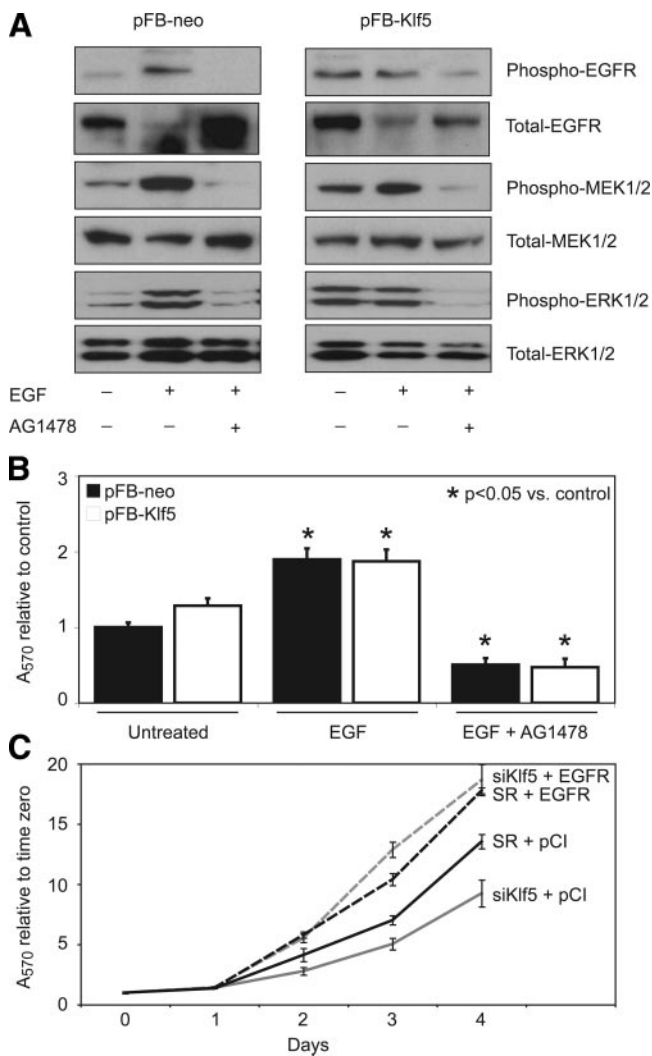


Figure 4. Klf5 activates MEK/ERK signaling and cell proliferation via EGFR. *A*) Western blot was performed on lysates from cells infected with pFB-Klf5 to overexpress *Klf5* or pFB-neo control cells. Cells were treated with DMSO alone or with the EGFR inhibitor AG1478 in DMSO for 4 h. When AG1478 was added to pFB-neo or pFB-Klf5 infected cells stimulated with EGF, activation of EGFR, MEK, and ERK was abrogated. *B*) MTT assay after 4 h of treatment demonstrated that inhibition of EGFR with AG1478 decreased cell growth. The data represented the average value of triplicate samples from three individual experiments. *C*) pSR-neo (SR) and pSR-siKlf5 cells (siKlf5) were transfected in triplicate with pCI-neo (pCI) or with the *EGFR* expression vector pCI-EGFR (EGFR). By MTT assay, pSR-siKlf5 infected cells had decreased cell growth compared with cells infected with pSR-neo control, and transfection of pSR-siKlf5 cells with the *EGFR* expression vector pCI-EGFR (EGFR) restored normal cell growth.

mRNA and protein, while inhibition of EGFR with AG1478 or MEK with PD98059 blocked the induction of *Klf5* by EGF (Fig. 5). This regulation of *Klf5* by EGFR-MEK/ERK signaling allows for the potential of rapid signal amplification to enhance cell proliferation in esophageal keratinocytes. In addition, down-regulation of *Klf5* provides a means to rapidly halt cell proliferation during keratinocyte differentiation.

DISCUSSION

Epithelial stem cells provide the basis for the mature, functional cells that line the luminal gastrointestinal tract, the skin, and many other organs of the body (31). However, the molecular events underlying the transition from these multipotent, slow-cycling stem cells to rapidly proliferating, committed, or partially committed transit-amplifying cells are not well understood. Primary esophageal epithelial cells, which lack the genetic abnormalities of cancer-derived cell lines, are an excellent model to study the mechanisms of normal epithelial homeostasis (24). Using primary esophageal squamous cells as a model, we have identified a novel bidirectional regulatory loop involving *Klf5*, EGFR, MEK, and ERK.

Even in unstimulated mouse primary esophageal cells, MAPK signaling is active, and the pathway is up-regulated by either EGF or *Klf5*. However, the impact on proliferation by increasing *Klf5* is less dramatic than that of *Klf5* suppression (Fig. 1), suggesting

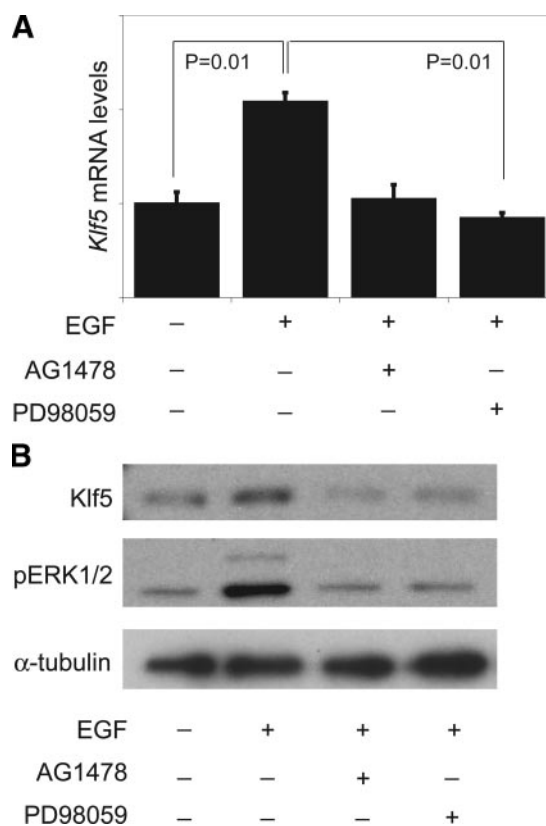


Figure 5. Klf5 is regulated by EGFR-MEK/ERK signaling. pFB-neo infected primary esophageal cells were stimulated with EGF and treated with inhibitors in DMSO or with DMSO alone for 4 h. *A*) Stimulation of cells with EGF up-regulated *Klf5* mRNA expression, as measured by quantitative real-time PCR, and this increase was completely blocked by treatment with the EGFR inhibitor AG1478 or the MEK inhibitor PD98059. *B*) By Western blot, stimulation with EGF resulted in induction of *Klf5* protein and phospho-ERK (pERK), while inhibition of EGFR and MEK resulted in decreased levels of phospho-ERK and *Klf5*. α -Tubulin served as a loading control.

that endogenous Klf5 is sufficient to maintain proliferation in these cells. Nonetheless, the marked reduction in proliferation with *Klf5* suppression demonstrates that Klf5 is critical for proliferation in esophageal keratinocytes. The effect of Klf5 on proliferation may also involve other pathways, as Klf5 has been shown to regulate *cyclin D1*, *cyclin B1/Cdc2*, and p21^{Waf1/Cip1}, for example, in other cell types (12, 13, 15, 18). Studies of the influence of Klf5 on these factors in primary esophageal cells are ongoing.

The function of Klf5 in primary esophageal keratinocytes contrasts with its role in esophageal cancer cells, where *KLF5* overexpression inhibits proliferation (18). Also, while transfection of Klf5 into NIH3T3 cells results in signs of oncogenic transformation (11), we saw no evidence of transformation by Klf5 in esophageal keratinocytes. This could be due to the effects of Ras on Klf5 in transformed *vs.* nontransformed cell lines. In intestinal cell lines, the effect of Klf5 on downstream targets such as cyclin D1 differs in transformed *vs.* nontransformed cells (15). To better understand this, we plan to compare the effects of Klf5 expression on cyclin D1 and other targets in primary esophageal and esophageal cancer cell lines and to investigate the effects of Ras on Klf5 in primary esophageal cells.

The function of EGFR, like Klf5, is also context dependent. The importance of EGFR is highlighted by other studies indicating a key role in hyperproliferation and basal cell hyperplasia in esophageal epithelia *in vitro* and *in vivo* (24, 32). While *EGFR* amplification has been linked to esophageal cancer (33), its specific role in transformation is not clear. In fact, most studies of the biological functions of EGFR have utilized fibroblasts or cancer cells, thus limiting the insight into the impact of *EGFR* overexpression on epithelial cell immortalization and malignant transformation. Recently, EGFR was shown to modulate cell growth but not induce malignant transformation in primary esophageal keratinocytes via the insulin growth factor binding protein IGFBP-3 (34). In oral keratinocytes, EGFR overexpression is one step in neoplastic transformation but is not in itself sufficient for transformation (35). Thus, the interaction with EGFR may play a key role in the context and cell-type specific effects of Klf5.

Klf5 binds to the EGFR promoter region in the vicinity of a typical BTEB1 site and increases transcriptional activity and expression of *EGFR*. Thus, the activation of *EGFR* by Klf5 occurs at the transcriptional level. However, further studies are required to confirm the specific binding site for Klf5 in the 5' regulatory region of *EGFR*. Surprisingly, Klf5 regulates not only *EGFR* expression (Fig. 2) but also EGFR activation (Fig. 4A). This may result from the effects of Klf5 on other factors, including other cell-surface receptors. Integrins, for example, have been shown to trigger ligand-independent phosphorylation and activation of EGFR (36). Like Klf5 and EGFR, integrins are expressed predominantly in basal keratinocytes (37). Investiga-

tions into the role of Klf5 on integrin expression and function are ongoing.

In other cell types, Klf5 is either upstream (14) or downstream (12, 21) of the MAPK pathway. Here we show that in esophageal keratinocytes Klf5 is both upstream, via direct regulation of *EGFR*, and downstream of MAPK signaling. The manner in which Klf5 is regulated by MAPK in esophageal keratinocytes is not clear and may be transcriptional, via phosphorylation of specific effectors (23), or at the level of mRNA or protein stability. Interestingly, MAPK-dependent phosphorylation alters the protein stability and half-life of other transcriptional regulators (38), and KLF5 is an unstable protein that is tightly regulated in breast and prostate epithelial cells (39, 40). Thus, multiple mechanisms may be involved in the regulation of Klf5 by MAPK signaling.

In conclusion, we show that the zinc-finger transcription factor Klf5 is a central player in a regulatory loop, which controls proliferation in squamous epithelial cells. Klf5 both regulates and is regulated by MAPK signaling, which in turn is controlled by EGFR. Klf5/BTEB2 binds to the 5' regulatory region of *EGFR*, which contains a typical BTEB1 binding site, and up-regulates transcription of *EGFR*. These data suggest a mechanism for the rapid modulation of signaling and, consequently, proliferation within basal cells of squamous epithelia. EJ

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