Overexpression of Krüppel-like factor 5 in esophageal epithelia in vivo leads to increased proliferation in basal but not suprabasal cells

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Goldstein BG, Chao H-H, Yang Y, Yermolina YA, Tobias JW, Katz JP. Overexpression of Krüppel-like factor 5 in esophageal epithelia in vivo leads to increased proliferation in basal but not suprabasal cells. Am J Physiol Gastrointest Liver Physiol 292: G1784–G1792, 2007. First published March 29, 2007; doi:10.1152/ajpgi.00541.2006.—Krüppel-like factor 5 (Klf5; also called IKLF or BTEB2), a zinc-finger transcription factor with proproliferative and transforming properties in vitro, is expressed in proliferating cells of gastrointestinal tract epithelia, including in basal cells of the esophagus. Thus, Klf5 is an excellent candidate to regulate esophageal epithelial proliferation in vivo. Nonetheless, the function of Klf5 in esophageal epithelial homeostasis and tumorigenesis in vivo has not previously been determined. Here, we used the ED-L2 promoter of the Epstein-Barr virus to express Klf5 throughout esophageal epithelia. ED-L2/Klf5 transgenic mice were born at the appropriate Mendelian ratio, survived to at least 1 yr of age, and showed no evidence of esophageal dysplasia or cancer. Staining for bromodeoxyuridine (BrdU) demonstrated increased proliferation in the basal layer of ED-L2/Klf5 mice, but no proliferation was seen in suprabasal cells, despite ectopic expression of Klf5 in these cells. Notably, expression of the KLF family member Klf4, which binds the same DNA sequences as Klf5 and which inhibits proliferation and promotes differentiation, was not altered in ED-L2/Klf5 transgenic mice. In primary esophageal keratinocytes that overexpressed Klf5, expression of Klf4 still inhibited proliferation and promoted differentiation, providing a possible mechanism for the persistence of keratinocyte differentiation in ED-L2/Klf5 mice. To identify additional targets for Klf5 in esophageal epithelia, we performed functional genomic analyses and identified a total of 15 differentially expressed genes. In summary, while Klf5 positively regulates proliferation in basal cells, it is not sufficient to maintain proliferation in the esophageal epithelium.

keratinocytes; transgenic mice; homeostasis

DISEASES OF THE ESOPHAGUS are among the most common ailments in the United States and throughout the world. Gastro-esophageal reflux disease (GERD) affects up to 44% of the United States population at least monthly, and ~20% have weekly symptoms (16). Some of these patients will develop altered proliferation and differentiation in the esophageal mucosa, with a disease spectrum ranging from symptomatic GERD to erosive esophagitis to Barrett’s esophagus to adenocarcinoma of the esophagus (35). Chronic irritation of the mucosa by factors such as alcohol or tobacco use also alters the normal mechanisms of esophageal homeostasis, predisposing to squamous cell cancers of the esophagus (7). Each year, >300,000 people worldwide contract esophageal cancer, and nearly that many die of the disease (24). In the United States, esophageal cancer affects >14,000 individuals each year and is almost universally fatal (11). More than 90% of esophageal cancers arise in the epithelium (32). The common feature of GERD, esophageal cancer, and numerous other esophageal and gastrointestinal diseases is the dysregulation of normal epithelial homeostasis (12).

A number of proteins have been implicated in the control of epithelial homeostasis and carcinogenesis, including members of the Krüppel-like factor (KLF) family (9). KLFs are DNA-binding transcriptional regulators that play a diverse role in proliferation, differentiation, and development. Members of the KLF family bind similar “CACC” DNA elements. Among the KLFs, two tissue-restricted factors, Klf4 (GKLF) and Klf5 (IKLF or BTEB2), are highly expressed in epithelial cells of the gastrointestinal tract including the esophagus (5, 30, 37). In the adult, Klf5 is localized to regions of active cell proliferation, including basal cells of the esophagus (5, 21). During development, Klf5 is first expressed throughout the primitive gut at embryonic day 10.5 (E10.5) and, by E17.5, becomes restricted to the base of the intestinal crypts (21). This pattern of expression for Klf5 is opposite that of Klf4, which has been shown to negatively regulate proliferation and promote differentiation, in vitro and in vivo (8, 15, 28–30). Notably, Klf4 opposes the effect of Klf5 on a number of promoters, including Klf4 and the laminin1 gene (Lama1) (6, 26).

Substantial data point to a role for Klf5 as a positive regulator of cell proliferation with transforming properties in vitro. In NIH 3T3 cells, for example, transfection of Klf5 promotes proliferation, results in loss of contact inhibition, and mediates transformation by oncogenic H-Ras via cyclin D1, cyclin B1, and Cdc2 (18, 19, 33). Klf5 is a downstream target of the Wnt pathway (38), the activation of which plays a key role in the development of colorectal cancer, and activates Lama1, which has been linked to malignant progression (26). In esophageal keratinocytes, Klf5 increases proliferation via EGF receptor (EGFR), MEK, and ERK (37). However, other data suggest that Klf5 may function as a tumor suppressor in esophageal, breast, prostate, and intestinal cancers (2–4, 36), leading to some controversy about its role in carcinogenesis. In esophageal cancer cells, for example, Klf5 inhibits proliferation and invasion and promotes apoptosis, including in response to anchorage-independent growth.

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To date, little information about the role of Klf5 in epithelial homeostasis and tumorigenesis has been gained from animal models. Homozygous null mice for Klf5 have been generated but die by E8.5 for unexplained reasons (31). Heterozygous Klf5 mice show abnormalities in cardiovascular remodeling and adipogenesis (22, 31) and reportedly have “misshapen” small intestinal villi, but the description and implication of this finding are unclear. Recently, ectopic expression of Klf5 in epidermis by the keratin 5 promoter produced embryonic defects in epidermal and craniofacial development (34). However, the function of Klf5 may be tissue and/or context dependent, and thus the definitive role for Klf5 in epithelial homeostasis and carcinogenesis remains to be elucidated. Moreover, the specific epithelial targets for Klf5 in vivo have not been established. In this study, we used ED-L2/Klf5 transgenic mice, which express Klf5 throughout the esophageal epithelium, to analyze the function of Klf5 in vivo. Using these mice, we determine that Klf5 is an important regulator of esophageal epithelial cell proliferation but is not sufficient to maintain proliferation in these cells.

**MATERIALS AND METHODS**

**Generation of ED-L2/Klf5.** All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. To express Klf5 in esophageal epithelium, we excised full-length Klf5 cDNA from a cDNA library in pSPORT2 (a gift of Dr. Klaus Kaestner, University of Pennsylvania) and cloned this into the pL2 plasmid (a gift of Dr. Anil Rustgi, University of Pennsylvania), which contained 782 bp of the Epstein-Barr virus (EBV) ED-L2 promoter (17). The pL2-Klf5 plasmid was sequenced, and the ED-L2/Klf5 fragment was excised for injection. Derivation of transgenic mice was accomplished by the University of Pennsylvania Transgenic and Chimeric Mouse Facility. We documented transgene integration in the pL2 plasmid was sequenced, and the ED-L2/Klf5 fragment was excised for injection. Derivation of transgenic mice was accomplished by the University of Pennsylvania Transgenic and Chimeric Mouse Facility. We documented transgene integration in

**Histology.** Esophagi were removed from 1-mo-old and 1-yr-old ED-L2/Klf5 mice, examined grossly, and then processed for histology. Esophagi were removed from 1-mo-old and 1-yr-old ED-L2/Klf5 mice, examined grossly, and then processed for histology. The following number of matched littermate control and ED-L2/Klf5 transgenic mice were examined histologically: age 1 mo, three controls and three mutants; and age 1 yr, two controls and four mutants.

**Immunohistochemistry and quantitation of cells.** We injected control and ED-L2/Klf5 transgenic mice with bromodeoxyuridine (BrdU) Labeling Reagent (Zymed, San Francisco, CA) 90 min prior to euthanization, removed the esophagi, and prepared them as above. Rabbit polyclonal anti-Klf5 and anti-Klf4 antibodies were generated (Biosource/QCB, Hopkinton, MA) as previously described (36). We performed microwave antigen retrieval and processed the tissues as previously described (15), followed by an incubation with one of the following primary antibodies: 1:500 sheep anti-BrdU (US Biological, Swampscott, MA), 1:150 mouse anti-cytokeratin 4 (Lab Vision, Fremont, CA), 1:750 rabbit anti-caspase 3 (R&D Systems, Minneapolis, MN), 1:5,000 rabbit anti-Klf4 (13), and 1:5,000 rabbit anti-Klf5. For colabeling immunofluorescence, a 1:100 goat anti-Klf4 (Santa Cruz Biotechnology, Santa Cruz, CA) and 1:5,000 rabbit anti-Klf5 antibodies were used. Species-specific secondary antibodies were added, and antibody binding was detected as previously described (15). For fluorescent labeling, 1:200 anti-rabbit Alexa Fluor 488 (Invitrogen) and 1:600 Cy3 (Jackson Immunoresearch, West Grove, PA) antibodies were used. For experiments studying Klf4 and Klf5 coexpression, we counted cells staining for both Klf4 and Klf5 in five fields at ×400 magnification from 1-yr-old mice. The proliferative index was determined by counting the numbers of BrdU-labeled cells per 100 basal cells in at least five distinct regions of esophagi from at least two ED-L2/Klf5 mice and two littermate controls at each time point. Results were expressed as mean numbers of labeled cells ± SE.

**RNA analyses.** RNA was extracted from esophagi or primary esophageal keratinocytes using the RNeasy Mini Kit (Qiagen, Valencia, CA) following manufacturer’s instructions. RNA protection assays were performed as described previously (14) using 1 μg total RNA/sample. Probes were designed to span the 3' end of the ED-L2/Klf5 transgene, from the Klf5 cDNA to just upstream of the polyadenylation signal (17), to protect fragments of 344 bp for endogenous Klf5 and 499 bp for the ED-L2/Klf5 transgene. A 103-bp cyclophilin probe was employed as an internal standard. Transgenic Klf5 fragments were obtained on a Novex 6% Tris-borate-EDTA (TBE)-urea acrylamide gel (Invitrogen), and the radioactive bands visualized on a Storm 840 phosphorimagery (GE Healthcare Bio-Sciences, Piscataway, NJ). Quantitative real-time PCR analysis was performed on a Stratagene Mx4000 Multiplex QPCR System using conditions and primer concentrations suggested by the Brilliant SYBR Green (Stratagene, La Jolla, CA) protocol. Reverse transcription was performed with random hexamers and SuperScript II Reverse Transcriptase (Invitrogen). The TATA box binding protein (TBP) was used as the internal control. Primer sequences are available upon request.

**Microarray analysis.** All protocols were conducted as described in the Affymetrix GeneChip Expression Analysis Technical Manual. Briefly, whole esophageal RNA from three control and three ED-L2/Klf5 transgenic mice at 3 mo of age was extracted using the RNeasy Mini Kit (Qiagen) and converted to first-strand cDNA using SuperScript II Reverse Transcriptase (Invitrogen) primed by a poly(T) oligomer that incorporated the T7 promoter. Second-strand cDNA synthesis was followed by in vitro transcription for linear amplification of each transcript and incorporation of biotinylated CTP and UTP. cRNA products were fragmented to 200 nt or less, heated at 99°C for 5 min, and hybridized for 16 h at 45°C to GeneChip Mouse Expression Arrays MOE430A v2 (Affymetrix, Santa Clara, CA), which contained >22,600 probe sets representing transcripts and variants from >14,000 well-characterized mouse genes. Microarrays were then washed at low and high stringency and stained with streptavidin-phycocerythrin. Fluorescence was amplified by adding biotinylated anti-streptavidin and an additional aliquot of streptavidin-alkaline phosphatase (Roche Molecular Biochemicals, Branchburg, NJ) was applied using a two-class unpaired analysis, and differential expression values for the probe set were calculated using GCRMA. Affymetrix “present” or “absent” marginal flags were also calculated. Data were input into GeneSpring GX version 7.3.1 under Accession No. E-MEXP-923. For analyses, probe intensity signals at 3-μm resolution after excitation at 570 nm. The average signal from two sequential scans was calculated for each microarray feature. A complete set of data from these microarray experiments was submitted to the European Bioinformatics Institute ArrayExpress repository (http://www.ebi.ac.uk/arrayexpress/) under Accession No. E-MEXP-923. For analyses, probe intensity data were input into ArrayAssist Lite version 3.4 (Stratagene), and expression values for the probe set were calculated using GCRMA. Affymetrix “present” or “absent” marginal flags were also calculated. Data were imported into GeneSpring GX version 7.3.1 (Agilent) and filtered based on “present” in at least two of six samples. Finally, SAM version 2.2.1 (Stanford University, Stanford, CA) was applied using a two-class unpaired analysis, and differentially expressed genes were found using a fold change cutoff of ≥2.0 and a false discovery rate of 5%.

**Western blot analysis.** 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, Indianapolis, IN) were added, and antibody binding was detected as previously described (15). For fluorescent labeling, 1:200 anti-rabbit Alexa Fluor 488 (Invitrogen) and 1:600 Cy3 (Jackson Immunoresearch, West Grove, PA) antibodies were used. For experiments studying Klf4 and Klf5 coexpression, we counted cells staining for both Klf4 and Klf5 in five fields at ×400 magnification from 1-yr-old mice. The proliferative index was determined by counting the numbers of BrdU-labeled cells per 100 basal cells in at least five distinct regions of esophagi from at least two ED-L2/Klf5 mice and two littermate controls at each time point. Results were expressed as mean numbers of labeled cells ± SE.
IN), and protein was isolated. Thirty micrograms of total protein from each sample were separated on a NuPAGE 4–12% bis-Tris acrylamide gel (Invitrogen) and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA) in 1× NuPAGE transfer buffer (Invitrogen) for 75 min at 4°C. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline-Tween for 2 h at room temperature and incubated overnight at 4°C with 1:5,000 rabbit anti-Klf5 antibody or 1:200 mouse anti-cytokeratin 13 antibody (Lab Vision). Membranes were then incubated for 45 min at room temperature with a 1:3,000 dilution of anti-rabbit/horseradish peroxidase or anti-mouse/horseradish peroxidase (Amersham Pharmacia Biotech), washed twice for 10 min, and developed with the Enhanced Chemi-

**Fig. 1.** Transgenic expression in mice containing Krüppel-like factor 5 (Klf5) under the control of the Epstein-Barr virus ED-L2 promoter (ED-L2/Klf5 mice). A: RNase protection assay demonstrated strong ED-L2/Klf5 expression in the esophagus (Es), with weak expression in the stomach (St), and no expression in the duodenum (Du) or liver (Li). This band in the stomach likely represented expression in the squamous-lined forestomach. Endogenous Klf5 expression was seen in the esophagus, stomach, and duodenum, but not the liver. Cyclophilin was used as a loading control.

B: Western blot revealed a marked increase in Klf5 protein levels in the esophagus but not the stomach of ED-L2/Klf5 mice compared with controls.

C–F: whereas staining for Klf5 (arrows) was restricted to basal cells in 1-mo-old control mice (C), ED-L2/Klf5 mice at 1 mo of age (D) demonstrated Klf5 expression throughout the esophageal epithelium, including in cells of the suprabasal and superficial layers. Similar staining patterns were observed in 1-yr-old control (E) and ED-L2/Klf5 mice (F). Magnification: ×200 in C–F.

**Fig. 2.** ED-L2/Klf5 transgenic mice had normal-appearing esophageal mucosa. Compared with 1-mo-old littermate controls (A), hematoxylin and eosin (H&E)-stained esophageal epithelia, along with the underlying lamina propria and muscular layers, appeared normal in 1-mo-old ED-L2/Klf5 mice (B). Note the preservation of surface keratinization. Similar findings were seen in H&E-stained esophagi from 1-yr-old control (C) and ED-L2/Klf5 mice (D). Magnification: ×400.
luminescence Plus Western Blot Analysis Kit (Amersham Pharmacia Biotech).

**Cell culture and analyses.** The isolation and culture of mouse primary esophageal keratinocytes has been described elsewhere (1, 37). Cells were then transduced with the pFB-Klf5 retrovirus, containing the full-length mouse Klf5 cDNA subcloned into the pFB-neo retroviral vector (Stratagene), as described previously (37), to produce stable overexpression of Klf5, or with the pFB-Klf4 retrovirus, containing the full-length mouse Klf4 cDNA subcloned into pFB-neo, to stably overexpress Klf4. To overexpress Klf4 and Klf5 simultaneously, primary esophageal keratinocytes stably infected with pFB-Klf5 were transfected at 50% confluence with pMT3-Klf4 (a gift of Dr. Vincent Yang) or pMT3 control using FuGENE 6 Transfection Reagent (Roche). Cell growth rates were evaluated by MTT assay as described previously (36, 37). In brief, 1 x 10^4 cells/well were seeded in triplicate onto 48-well plates in keratinocyte serum-free medium (K-SFM; Invitrogen) supplemented with 40 g/ml bovine pituitary extract (Invitrogen), 1.0 ng/ml EGF (Invitrogen), 100 U/ml penicillin, and 100 g streptomycin (Invitrogen). At each time point, medium was removed, and cells were washed with PBS. MTT reagent (USB, Cleveland, OH) 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 mean of absorbance values relative to control ± SE.

**RESULTS**

To investigate the role of Klf5 in esophageal epithelial proliferation, differentiation, and tumorigenesis in vivo, we used the EBV ED-L2 promoter to express Klf5 throughout the esophageal epithelium. The ED-L2 promoter has been shown previously to target expression specifically to the tongue, esophagus, and forestomach, all of which are lined by stratified squamous epithelia (1, 17, 23). We documented transgene integration by PCR in nine ED-L2/Klf5 founder lines (data not shown) and confirmed transgene expression in the esophagus of three ED-L2/Klf5 transgenic lines by RNAse protection assays. A representative RNAse protection assay is shown in Fig. 1A. Strong transgenic expression was seen in the esophagus, with faint expression seen in the stomach, likely representing expression in the squamous forestomach. As all transgenic lines appeared similar on initial analyses, subsequent data are shown from one transgenic line with high levels of Klf5 expression.

We performed Western blots and immunohistochemistry to confirm overexpression and demonstrate localization of the Klf5 protein. By Western blot (Fig. 1B), levels of Klf5 protein were markedly increased in esophagi from ED-L2/Klf5 transgenic mice compared with controls, whereas Klf5 expression in the whole stomach was essentially unchanged. Immunohis-
tochemistry of control mice at 1 mo (Fig. 1C) and 1 yr (Fig. 1E) of age demonstrated expression of Klf5 in the basal layer, where cell proliferation occurs. In contrast, Klf5 expression was seen throughout the basal, suprabasal, and superficial layers of ED-L2/Klf5 mice at 1 mo (Fig. 1D) and 1 yr (Fig. 1F) of age. Esophageal epithelial cells normally cease proliferation and undergo differentiation as they migrate through the suprabasal and superficial cell layers (27).

ED-L2/Klf5 transgenic mice were born at the appropriate Mendelian ratio and survived to at least 1 yr of age. The esophagi and other organs of the ED-L2/Klf5 mice appeared grossly normal. Compared with littermate controls at 1 mo (Fig. 2A) and 1 yr (Fig. 2C) of age, esophageal epithelia of 1-mo-old (Fig. 2B) and 1-yr-old (Fig. 2D) ED-L2/Klf5 transgenic mice appeared normal. All three epithelial cell layers from ED-L2/Klf5 mice were indistinguishable from controls, and surface keratinization was preserved. There was no evidence of hypertrophy, hyperplasia, dysplasia, or cancer at any time point in any of the mice.

Given the evidence implicating Klf5 as a positive regulator of cell proliferation, we hypothesized that Klf5 overexpression in esophageal epithelia would lead to increased proliferation. To investigate cell proliferation, we pulse labeled cells in the S phase with BrdU. Surprisingly, in control mice aged 1 mo (Fig. 3A) and 1 yr (Fig. 3C) and in ED-L2/Klf5 mice at 1 mo (Fig. 3B) and 1 yr (Fig. 3D), cell proliferation was confined to the basal layer, despite evidence of Klf5 expression in the suprabasal and superficial layers of ED-L2/Klf5 mice (Fig. 1, D and F). Thus, Klf5 expression was not sufficient to promote cell proliferation in differentiating squamous epithelial cells in vivo. To quantitate the effect of Klf5 on cell proliferation in vivo, we determined the proliferative index by counting the numbers of BrdU-labeled cells per 100 basal cells. ED-L2/Klf5 mice had a statistically significant twofold increase in the numbers of proliferating cells at both 1 mo and 1 yr of age (Fig. 3E).

Thus, while the ED-L2 promoter drove Klf5 expression in all three esophageal epithelial layers, the changes in proliferation were confined to basal cells. In addition, suprabasal and superficial cells of the esophageal epithelium underwent normal differentiation. One-month-old control (Fig. 4A) and ED-L2/Klf5 mice (Fig. 4B) showed no changes in staining for the differentiation marker keratin 4, and, even at 1 yr of age, keratin 4 expression in control (Fig. 4C) and ED-L2/Klf5 mice (Fig. 4D) was unchanged. In addition, apoptosis was not significantly altered in ED-L2/Klf5 mice at any age as shown by staining for activated caspase 3, with <1 apoptotic cell seen per esophageal cross-section in both control and mutant mice (data not shown).

Fig. 5. Klf5 did not inhibit Klf4 expression in esophageal epithelial cells in vivo. In control mice (A), Klf4 (green) was expressed in cells of the basal layer, whereas Klf4 (red) was seen in suprabasal and superficial cells. Normally, cells may transiently express both Klf5 and Klf4 (arrows) as they migrate from the basal layer, coincident with the switch from differentiation to proliferation. In ED-L2/Klf5 mice (B), Klf5 was expressed in basal, suprabasal, and superficial cells, and coexpression of Klf5 and Klf4 was observed in many suprabasal cells as well (arrows). Magnification: ×400.
The KLF family member Klf4 inhibits proliferation and promotes differentiation both in vitro and in vivo (8, 15, 28–30). Interestingly, Klf4 and Klf5 have been shown to bind to the same DNA sequence in a number of promoters with opposite effects (6, 9, 26). For example, Klf5 downregulates Klf4 transcriptional activity in vitro. To examine whether ectopic expression of Klf5 altered the expression of Klf4 in esophageal epithelia in vivo, we performed colabeling immunofluorescence for Klf4 and Klf5. In control mice (Fig. 5A), Klf5 was expressed in basal cells, whereas expression of Klf4 was seen in cells of the suprabasal and superficial layers. Note that some transient coexpression of Klf4 and Klf5 was normally seen in cells migrating from the basal layer to the suprabasal layer. Surprisingly, in ED-L2/Klf5 mice (Fig. 5B), Klf4 was present in numerous suprabasal cells, which also expressed Klf5, suggesting that Klf4 is not repressed by Klf5 in esophageal epithelial cells in vivo. Overall, ED-L2/Klf5 mice had a 50% increase in the number of esophageal epithelial cells coexpressing Klf4 and Klf5 (14 ± 0.9 cells/field in controls vs. 21 ± 2 cells/field in mutants, \( P = 0.03 \)). Quantitative real-time PCR provided additional evidence that Klf4 was not altered in esophageal epithelia of ED-L2/Klf5 mice (data not shown). This persistent expression of Klf4 provides a possible explanation for the inability of Klf5 to maintain proliferation in suprabasal and superficial cells and the ability of these cells to undergo further differentiation.

Primary esophageal epithelial cells provide a valuable platform for the study of normal epithelial homeostasis (1, 10). To test whether Klf4 inhibits proliferation and promotes differentiation of esophageal keratinocytes even in the presence of Klf5, we transiently transfected mouse primary esophageal keratinocytes with a Klf4-expressing retrovirus, pFB-Klf4, with the Klf4 expression vector pMT3-Klf4. pFB-Klf5 infection leads to stable overexpression and increases proliferation in mouse primary esophageal keratinocytes (37). When pMT3-Klf4 was introduced into mouse primary esophageal keratinocytes infected with pFB-Klf5, the rate of cell proliferation was significantly decreased by MTT assay compared with controls (Fig. 6A). In addition, levels of the differentiation marker keratin 13 were increased by Western blot in pFB-Klf5-infected cells with overexpression of Klf4 compared with controls (Fig. 6B), confirming that Klf4 promotes differentiation of esophageal keratinocytes, even in the presence of Klf5.

To identify additional targets for Klf5 in esophageal epithelia, we performed functional genomic analyses of esophageal tissue from control and ED-L2/Klf5 mice. We identified a total of 18 probe sets covering 15 different genes that were differentially regulated by at least twofold in mutant versus control mice.
mice (Table 1). Among these genes was Klf5 (up 2.2-fold in ED-L2/Klf5 mice) as well as other key regulatory factors. For example, expression of transcription factor 23, which inhibits terminal differentiation of myoblasts (20), was increased 3.6-fold in ED-L2/Klf5 mice. Cysteine-rich secretory protein 1 (down 12.3-fold in ED-L2/Klf5 mice), which was originally identified in the epididymis, where it plays a role in sperm maturation, was recently found to be expressed in murine hair follicles, although its function has not yet been determined (25). To confirm the findings on microarray, we performed quantitative real-time PCR on all known genes (excluding Klf5) identified by microarray analyses. Of these 12 genes, we identified 8 genes that had statistically significant changes by quantitative real-time PCR in the same direction as the microarray results (Table 2).

Using our primary esophageal epithelial cell culture model, we examined the regulation by Klf4 and Klf5 of two of these genes, G protein signaling modifier 1 (Gpsm1), which was downregulated in ED-L2/Klf5 mice compared with controls, and sortilin-related VPS10 domain-containing receptor 2 (Sorcs2), which was upregulated in ED-L2/Klf5 mice. In primary esophageal keratinocytes, Klf5 reduced Gpsm1 expression, whereas Klf4 upregulated Gpsm1 (Fig. 7A). In contrast, Sorcs2 expression was increased by Klf5 and decreased by Klf4. To examine whether Klf4 opposed the effect of Klf5 on Gpsm1 and Sorcs2, we overexpressed Klf4 by transient transfection in pFB-Klf5-infected primary esophageal keratinocytes. Compared with cells with overexpression of Klf5 alone, overexpression of both Klf4 and Klf5 resulted in increased Gpsm1 expression (Fig. 7B). However, Sorcs2 expression was not changed by expression of Klf4 in pFB-Klf5-infected cells. One possible explanation for these divergent findings is that the relative binding affinities for Klf4 and Klf5 may vary with different binding sites, such that simultaneous expression of Klf4 may inhibit Klf5 function on some genes but not others. Nonetheless, the opposing role for Klf4 in the regulation of Gpsm1 by Klf5 was consistent with our hypothesis that persistent expression of Klf4 was responsible for the failure of Klf5 to drive proliferation in suprabasal cells of ED-L2/Klf5 transgenic mice. The functions of Sorcs2 and Gpsm1 in esophageal epithelia are not yet known.

DISCUSSION

While Klf5 has been shown to positively regulate proliferation in vitro, animal models of Klf5 function in regulating epithelial homeostasis in vivo have been lacking, as homozygous deletion of Klf5 in mice results in embryonic lethality (31). Recently, however, transgenic expression of Klf5 in skin under the keratin 5 promoter was shown to result in epidermal hypoplasia and depletion of stem cells (34). Here, using the EBV ED-L2 promoter, we show that Klf5 expression in esophageal epithelial cells leads to increased proliferation in vivo, but expression of Klf5 is not sufficient to restore proliferation in differentiating esophageal epithelial cells. We also identify Klf4 as an inhibitor of Klf5 function in esophageal epithelial cells and demonstrate that, in contrast to prior in vitro models, Klf5 does not repress Klf4 in esophageal epithelial cells in vivo.

How do we reconcile the epidermal hypoplasia seen in mice with Klf5 expression under the control of keratin 5 with our model, in which Klf5 expression resulted in normal appearing epithelia in mice up to 1 yr of age? Several possible explanations exist. First, the effects could be dose dependent. We believe that this explanation is unlikely as Klf5 expression in esophageal epithelia of ED-L2/Klf5 mice was robust (Fig. 1). Alternatively, the consequences of increased Klf5 expression may be tissue dependent and may vary even between the

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<tr>
<th>Gene</th>
<th>Fold Change by qPCR</th>
<th>P Value</th>
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<tr>
<td>Sorcs2</td>
<td>+4.3</td>
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<td>Gpsm1</td>
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<td>Crisp1</td>
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Positive fold changes indicate higher gene expression in mutant mice than in control mice; negative fold changes indicate lower gene expression in mutant mice than in control mice. qPCR, quantitative real-time PCR.

Fig. 7. Klf4 opposed the effects of Klf5 on G protein signaling modulator (Gpsm1) in esophageal keratinocytes. A: in primary esophageal keratinocytes stably infected with pFB-Klf5 to overexpress Klf5, expression of Gpsm1 was decreased, whereas sortilin-related VPS10 domain-containing receptor 2 (Sorcs2) was increased, relative to control-infected cells. In contrast, overexpression of Klf4 in primary esophageal keratinocytes using the pFB-Klf4 retrovirus resulted in increased Gpsm1 and decreased Sorcs2. B: in pFB-Klf5 infected primary esophageal keratinocytes, overexpression of Klf4 by transient transfection with pMT3-Klf4 increased expression of Gpsm1 relative to control but did not alter Sorcs2 expression.
stratified squamous epithelia of the skin and esophagus. To this end, it would be interesting to examine the esophageal epithelia of keratin 5-Klf5 mice. Finally, keratin 5 but not ED-L2 may target Klf5 to stem cells, and ectopic Klf5 expression in stem cells may lead to the stem cell depletion, whereas Klf5 expression in transit amplifying cells of the basal layer leads to increased proliferation.

As described above, we see changes in proliferation in cells of the basal layer in ED-L2/Klf5 transgenic mice, but no gross changes in tissue morphology. This twofold increase in proliferation could be compensated for by subtle changes in apoptosis and/or cell migration to maintain homeostasis. For example, apoptosis in esophageal basal cells is extremely rare, with <1 apoptotic cell seen per esophageal cross section in both control and mutant mice, making detection of changes extremely difficult. Nonetheless, the changes in proliferation are significant, and while compensation normally occurs, this balance may be perturbed by tissue injury or malignant transformation.

Using ED-L2/Klf5 mice, we identified novel targets for Klf5 in vivo. While some of these have been implicated in proliferation or differentiation in other cell types (20, 25), the functions of many of these target genes have not been established. Interestingly, the role for Klf5 has been suggested to vary in transformed versus nontransformed cells (2, 36), and Klf5 has been implicated as a tumor suppressor in a number of cancers, including breast and prostate cancer (3, 4), suggesting that loss of Klf5 may be critical for tumor progression. Thus, it will be interesting to examine the expression of these target genes during tumorigenesis, and ED-L2/Klf5 mice will likely form a valuable platform for study of the role of Klf5 in esophageal carcinogenesis in vivo.

The failure of Klf5 to regulate Klf4 expression in vivo contrasts with prior in vitro studies. This disparity may be due to tissue- or context-dependent effects and highlights the importance of using in vivo models such as ED-L2/Klf5 mice. One hypothesis consistent with our findings, the results obtained from K5-Klf5 mice (34), and expression patterns of Klf4 and Klf5 in vivo is that Klf5 is critical for the early stages of keratinocyte differentiation, from stem cell to transit amplifying cell, and that Klf4 then takes over to promote further keratinocyte differentiation. In this case, ectopic expression of Klf5 in stem cells would lead to stem cell depletion. Then, in the absence of Klf4 expression, Klf5 would promote and maintain the transit amplifying cell state as described previously (37). However, in the presence of Klf4, keratinocytes would undergo further differentiation, despite persistent Klf5 expression. We would hypothesize that, in the absence of Klf4, the effects of Klf5 overexpression on epithelial homeostasis would be dramatic, and we are currently testing this hypothesis by crossing the ED-L2/Klf5 mice with mice lacking Klf4 in the esophagus, using Klf4 floxed mice described previously (13).

In conclusion, we show that Klf5 is a key regulator of esophageal epithelial proliferation in vivo but is not sufficient to maintain proliferation in these cells. We demonstrate that the KLF family member Klf4 is a key determinant of Klf5 function in esophageal epithelial cells, and we identify other potential mediators of the effects of Klf5 on epithelial homeostasis in vivo by functional genomic analyses. ED-L2/Klf5 mice provide an exciting model for future studies of the role of Klf5 in wound healing and tumorigenesis. Overall, this study offers valuable new insights into the regulation of esophageal epithelial proliferation and differentiation in vivo.

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REFERENCES

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