Title: 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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Running head: Klf5 increases proliferation in esophageal basal cells

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Abstract

Krüppel-like factor 5 (Klf5; IKLF; BTEB2), a zinc-finger transcription factor with pro-proliferative 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 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 one year of age, and showed no evidence of esophageal dysplasia or cancer. Staining for 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 which 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 sum, while Klf5 positively regulates proliferation in basal cells, it is not sufficient to maintain proliferation in the esophageal epithelium.

Key Words  keratinocytes, transgenic mice, homeostasis
**Introduction**

Diseases of the esophagus are among the most common ailments in the United States and throughout the world. Gastroesophageal reflux disease (GERD) affects up to 44% of the U.S. population at least monthly and approximately 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, more than 300,000 people worldwide contract esophageal cancer and nearly that many die of the disease (24). In the United States, esophageal cancer affects more than 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). The KLFs are DNA-binding transcriptional regulators that play a diverse role in proliferation, differentiation, and development. Members of the KLF family bind similar “CACCC” DNA elements. Among the KLFs, two tissue-restricted factors, Klf4 (GKLF) and Klf5 (IKLF; 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.
This pattern of expression for \textit{Klf5} is opposite that of \textit{Klf4}, which has been shown to negatively regulate proliferation and promote differentiation, \textit{in vitro} and \textit{in vivo} (8, 15, 28-30). Notably, Klf4 opposes the effect of Klf5 on a number of promoters, including \textit{Klf4} and \textit{LAMA1} (6, 26).

Substantial data point to a role for \textit{Klf5} as a positive regulator of cell proliferation with transforming properties \textit{in vitro}. In NIH 3T3 cells, for example, transfection of \textit{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), activation of which plays a key role in the development of colorectal cancer, and activates \textit{LAMA1}, which has been linked to malignant progression (26). In esophageal keratinocytes, Klf5 increases proliferation via EGFR, MEK, and ERK (37). However, other data suggest that \textit{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, \textit{Klf5} inhibits proliferation and invasion and promotes apoptosis, including in response to anchorage-independent growth.

To date, little information about the role of \textit{Klf5} in epithelial homeostasis and tumorigenesis has been gained from animal models. Homozygous null mice for \textit{Klf5} have been generated but die by E8.5 for unexplained reasons (31). Heterozygous \textit{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 \textit{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 \textit{in vivo} have not been established. In this study, we have utilized \textit{ED-L2/Klf5} transgenic mice, which express \textit{Klf5} throughout the esophageal epithelium, to analyze the function of Klf5 \textit{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 \textit{ED-L2/Klf5}**

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania. To express Klf5 in esophageal epithelia, we excised full length \textit{Klf5} cDNA from a cDNA library in pSPORT2 (gift of Dr. Klaus Kaestner, University of Pennsylvania) and cloned this into the pL2 plasmid (gift of Dr. Anil Rustgi, University of Pennsylvania), containing 782 bp of the EBV \textit{ED-L2} promoter (17). The pL2-\textit{Klf5} plasmid was sequenced, and the \textit{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 nine \textit{ED-L2/Klf5} founder lines by PCR. Offspring were screened for transgene expression by RNase protection assay and immunohistochemistry, as described below. Mice utilized for these experiments were on a mixed genetic background.

**Histology**

Esophagi were removed from one month-old and one year-old \textit{ED-L2/Klf5} mice, examined grossly, and then processed for histology as previously described (15). Briefly,
esophageal tissue was fixed in 4% paraformaldehyde, embedded in paraffin, and 5 μm sections were applied to Probe-on Plus™ slides (Fisher). Slides were stained with hematoxylin and eosin (H&E) and images were captured on a Nikon Eclipse E600 microscope with a Photometrics CoolSNAP CCD camera (Roper Scientific, Tucson, Arizona). The following numbers of matched littermate control and ED-L2/Klf5 transgenic mice were examined histologically: age one month, 3 controls and 3 mutants; age one year, 2 controls and 4 mutants.

Immunohistochemistry and Quantitation of Cells

We injected control and ED-L2/Klf5 transgenic mice with BrdU Labeling Reagent (Zymed, South San Francisco, California) 90 minutes prior to sacrifice, removed the esophagi, and prepared them as above. Rabbit polyclonal anti-Klf5 and anti-Klf4 were generated (Biosource International/QCB, Hopkinton, MA) as previously described (36). We performed microwave antigen retrieval and processed the tissues as described (15), followed by incubation with one of the following primary antibodies: 1:500 sheep anti-BrdU (US Biological, Swampscott, Massachusetts), 1:150 mouse anti-cytokeratin 4 (Lab Vision, Fremont, California), 1:750 rabbit anti-caspase 3 (R&D Systems, Minneapolis, Minnesota), 1:5000 rabbit anti-Klf4 (13), and 1:5000 rabbit anti-Klf5. For co-labeling immunofluorescence, 1:100 goat anti-Klf4 (Santa Cruz Biotechnology, Santa Cruz, California) and 1:5000 rabbit anti-Klf5 were used. Species-specific secondary antibodies were added and antibody binding detected as described (15). For fluorescent labeling, 1:200 anti-rabbit Alexa Fluor 488 (Invitrogen) and 1:600 Cy3 (Jackson ImmunoResearch. West Grove, Pennsylvania) were used. For studies of Klf4 and Klf5 co-expression, we counted cells staining for both Klf4 and Klf5 in five fields at 400x magnification from one year-old mice. The proliferative index was determined by counting the
number of BrdU-labeled cells per 100 basal cells in at least 5 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 number of labeled cells ± SEM.

RNA Analyses

RNA was extracted from esophagi or primary esophageal keratinocytes using the RNaseasy Mini Kit (Qiagen, Valencia, California) following manufacturer’s instructions. Ribonuclease (RNase) protection assays were performed as described previously using 1 µg of total RNA per sample (14). 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-nt cyclophilin probe was employed as an internal standard. The RNA fragments obtained were separated on a Novex 6% TBE-urea acrylamide gel (Invitrogen, Carlsbad, California) and the radioactive bands visualized on a Storm 840 phosphorimager (GE Healthcare Bio-Sciences, Piscataway, New Jersey). 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, California) protocol. Reverse transcription was performed with random hexamers and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, California). TBP was used as the internal control. Primer sequences are available upon request.

Microarray Analyses

All protocols were conducted as described in the Affymetrix GeneChip Expression Analysis Technical Manual. Briefly, whole esophageal RNA from 3 control and 3 ED-L2/Klf5
transgenic mice at 3 months 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. The cRNA products were fragmented to 200 nucleotides or less, heated at 99ºC for 5 min and hybridized for 16 h at 45ºC to GeneChip Mouse Expression Arrays MOE430A v2 (Affymetrix, Santa Clara, California), containing over 22,600 probe sets representing transcripts and variants from over 14,000 well-characterized mouse genes. The microarrays were then washed at low and high stringency and stained with streptavidin-phycoerythrin. Fluorescence was amplified by adding biotinylated anti-streptavidin and an additional aliquot of streptavidin-phycoerythrin stain. A confocal scanner was used to collect fluorescence signal 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 (EMBL-EBI) ArrayExpress repository ([http://www.ebi.ac.uk/arrayexpress/](http://www.ebi.ac.uk/arrayexpress/)) under accession number 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. The data were imported into GeneSpring GX version 7.3.1 (Agilent, Santa Clara, California), and filtered based on “present” in at least 2 out of 6 samples. Finally, SAM version 2.2.1 (Stanford University, Stanford, California) was applied using a two-class unpaired analysis, and differentially expressed genes were found using a fold change cutoff of greater than or equal to 2.0 and a false discovery rate of 5%.
**Western Blots**

Cells were lysed with 150 mM NaCl, 50 mM Tris pH 7.5, 1% NP 40, 0.5% sodium deoxycholic acid, and Complete Protease Inhibitor Cocktail Tablets (Roche, Indianapolis, Indiana), 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 PVDF membrane (Millipore Corp, Bedford, Massachusetts) in 1X NuPage transfer buffer (Invitrogen) for 75 min at 4°C. Membranes were blocked with 5% nonfat dry milk in TBST for two hours at room temperature and incubated overnight at 4°C with 1:5000 rabbit anti-Klf5 or 1:200 mouse anti-cytokeratin 13 (Lab Vision). 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, New Jersey), washed twice for 10 min, and developed with the Enhanced Chemiluminescence 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 pFB-\textit{Klf5} retrovirus, containing the full-length mouse \textit{Klf5} cDNA subcloned into the pFB-\textit{neo} retroviral vector (Stratagene), as described previously (37), to produce stable overexpression of \textit{Klf5}, or with the pFB-\textit{Klf4} retrovirus, containing the full-length mouse \textit{Klf4} cDNA subcloned into pFB-\textit{neo}, to stably overexpress \textit{Klf4}. To overexpress \textit{Klf4} and \textit{Klf5} simultaneously, primary esophageal keratinocytes stably infected with pFB-\textit{Klf5} were transfected at 50% confluence with pMT3-\textit{Klf4} (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, 1x10^4 cells per 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 epidermal growth factor (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, Ohio) was added at 2 mg/ml in Hank’s buffer (Invitrogen) and incubated for 1 hour 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 control ± SEM.

**Results**

To investigate the role of Klf5 in esophageal epithelial proliferation, differentiation, and tumorigenesis in vivo, we utilized the Epstein Barr Virus 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 RNAase protection assay is shown in Figure 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 (Figure 1B), levels of Klf5 protein were markedly increased in esophagi from ED-L2/Klf5 transgenic mice, compared to controls, while Klf5 expression in whole stomach was essentially unchanged. Immunohistochemistry of control mice at one month (Figure 1C) and one year of age (Figure 1E) 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 one month (Figure 1D) and 1 year of age (Figure 1F). 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 one year of age. The esophagi and other organs of the ED-L2/Klf5 mice appeared grossly normal. Compared to littermate controls at one month (Figure 2A) and one year of age (Figure 2C), esophageal epithelia of one month-old (Figure 2B) and one year-old (Figure 2D) ED-L2/Klf5 transgenic mice appeared normal. All three epithelial cell layers from ED-L2/Klf5 mice were indistinguishable from control, 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 S-phase with 5-bromo-2-deoxyuridine (BrdU). Surprisingly, in control mice aged one month (Figure 3A) and one year (Figure 3C) and ED-L2/Klf5 mice at one month (Figure 3B) and one year of age (Figure 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 (Figure 1B, D). Thus, Klf5 expression was
not sufficient to promote cell proliferation in differentiating squamous epithelial cells \emph{in vivo}. To quantitate the effect of Klf5 on cell proliferation \emph{in vivo}, we determined the proliferative index by counting the number of BrdU-labeled cells per 100 basal cells. \textit{ED-L2/Klf5} mice had a statistically significant two-fold increase in the number of proliferating cells at both one month and one year of age (Figure 3E).

Thus, while the \textit{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 (Figure 4A) and \textit{ED-L2/Klf5} mice (Figure 4B) showed no changes in staining for the differentiation marker keratin 4, and even at one year of age, keratin 4 expression in control (Figure 4C) and \textit{ED-L2/Klf5} mice (Figure 4D) was unchanged. In addition apoptosis was not significantly altered in \textit{ED-L2/Klf5} mice at any age by staining for activated caspase 3, with fewer than one apoptotic cell seen per esophageal cross-section in both control and mutant mice (data not shown).

The KLF family member Klf4 inhibits proliferation and promotes differentiation both \emph{in vitro} and \emph{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 \textit{Klf4} transcriptional activity \emph{in vitro}. To examine whether ectopic expression of Klf5 altered the expression of Klf4 in esophageal epithelia \emph{in vivo}, we performed co-labeling immunofluorescence for Klf4 and Klf5. In control mice (Figure 5A), Klf5 was expressed in basal cells, while expression of Klf4 was seen in cells of the suprabasal and superficial layers. Note that some transient co-expression of Klf4 and Klf5 was normally seen in cells migrating from the basal to the suprabasal layer. Surprisingly, in \textit{ED-L2/Klf5} mice (Figure 5B), Klf4 was
present in numerous suprabasal cells which also expressed Klf5, suggesting that \textit{Klf4} is not repressed by Klf5 in esophageal epithelial cells \textit{in vivo}. Overall, \textit{ED-L2/Klf5} mice had a 50\% increase in the number of esophageal epithelial cells co-expressing Klf4 and Klf5 (14 ± 0.9 cells/field in controls versus 21 ± 2 cells/field in mutants; \textit{p}=0.03). Quantitative real-time PCR (qPCR) provided additional evidence that \textit{Klf4} was not altered in esophageal epithelia of \textit{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 containing a \textit{Klf5} expressing retrovirus, pFB-\textit{Klf5}, with the \textit{Klf4} expression vector pMT3-\textit{Klf4}. pFB-\textit{Klf5} infection leads to stable overexpression and increases proliferation in mouse primary esophageal keratinocytes (37). When pMT3-\textit{Klf4} was introduced into mouse primary esophageal keratinocytes infected with pFB-\textit{Klf5}, the rate of cell proliferation was significantly decreased by MTT assay, compared to control (Figure 6A). In addition, levels of the differentiation marker keratin 13 were increased by Western blot in pFB-\textit{Klf5} infected cells with overexpression of \textit{Klf4}, compared to controls (Figure 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 \textit{ED-L2/Klf5} mice. We identified a total of 18 probe sets covering 15 different genes which were differentially regulated by at least two-
fold in mutant versus control 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 Tcf23, which inhibits terminal differentiation of myoblasts (20), was increased 3.6-fold in ED-L2/Klf5 mice. Crisp1 (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 there has not yet been determined (25). To confirm the findings on microarray, we performed qPCR on all known genes (excluding Klf5) identified by microarray analyses. Of these 12 genes, we identified 8 genes which had statistically significant changes by qPCR 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, Gpsm1, which is downregulated in ED-L2/Klf5 mice compared to controls, and Sorcs2, which is upregulated in ED-L2/Klf5 mice. In primary esophageal keratinocytes, Klf5 reduces Gpsm1 expression while Klf4 upregulates Gpsm1 (Figure 7a). In contrast, Sorcs2 expression is 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 to cells with overexpression of Klf5 alone, overexpression of both Klf4 and Klf5 resulted in increased Gpsm1 expression (Figure 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* had 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 one year 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 (Figure 1). Alternatively, the consequences of increased Klf5 expression may be tissue dependent and may vary even between the stratified squamous epithelia of the skin and esophagus. To this end, it would be interesting to examine the esophageal epithelia of the K5-Klf5 mice. Finally, K5 but not ED-L2 may target Klf5 to stem
cells, and ectopic Klf5 expression in stem cells may lead to the stem cell depletion, while 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 two-fold increase in proliferation could be compensated for by subtle changes in apoptosis and/or cell migration, in order to maintain homeostasis. For example, apoptosis in esophageal basal cells is extremely rare, with fewer than one 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 the ED-L2/Klf5 mice, we have 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 non-transformed cells (2, 36), and Klf5 has been implicated as a tumor suppressor in a number of cancers, including breast and prostate (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 the 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 the ED-L2/Klf5 mice. One hypothesis consistent with our findings, the results obtained from the K5-Klf5 mice (34), and the 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, these studies offer valuable new insights into the regulation of esophageal epithelial proliferation and differentiation in vivo.

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References


**Figure Legends**

**Figure 1.** Transgenic expression in *ED-L2/Klf5* mice. (A) RPA 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 esophagus, stomach, and duodenum, but not liver. *Cyclophilin* was used as a loading control. (B) Western blot revealed a marked increase in Klf5 protein levels in esophagus but not stomach of *ED-L2/Klf5* mice, compared to controls. (C-F) While staining for Klf5 (arrows) was restricted to basal cells in one month-old control mice (C), *ED-L2/Klf5* mice at one month 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 one year-old control (E) and *ED-L2/Klf5* mice (F). Magnification x200 (C-F).

**Figure 2.** *ED-L2/Klf5* transgenic mice had normal appearing esophageal mucosa. Compared to one month-old littermate controls (A), H&E stained esophageal epithelia, along with the underlying lamina propria and muscular layers, appeared normal in one month-old *ED-L2/Klf5* mice (B). Note the preservation of surface keratinization. Similar findings were seen in H&E stained esophagus from one year-old control (C) and *ED-L2/Klf5* mice (D). Magnification x400.

**Figure 3.** *ED-L2/Klf5* transgenic mice had increased proliferation in esophageal basal cells. At one month of age, BrdU labeled cells were confined to the basal layer in both control (A) and *ED-L2/Klf5* mice (B). No evidence of cell proliferation was seen within the suprabasal or superficial layers. In one year-old mice, proliferation was still restricted to basal cells of control (C) and *ED-L2/Klf5* mice (D). However, by quantitation, the number of proliferating cells was increased two-fold in *ED-L2/Klf5* mice at both one month and one year of age (E). Not
surprisingly, a slight increase in cell proliferation was seen in the younger mice, which had not yet reached full adult size. Magnification x400 (A-D).

**Figure 4.** Esophageal epithelial cells underwent normal differentiation in *ED-L2/Klf5* transgenic mice. Staining of one month-old control (A) and *ED-L2/Klf5* mice (B), as well as one year-old control (C) and *ED-L2/Klf5* mice (D), revealed no changes in the expression of the keratinocyte differentiation marker keratin 4. Magnification x400.

**Figure 5.** Klf5 did not inhibit Klf4 expression in esophageal epithelial cells *in vivo*. In control mice (A), Klf5 (green) was expressed in cells of the basal layer while 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 co-expression of Klf5 and Klf4 was observed in many suprabasal cells as well (arrows). Magnification x400.

**Figure 6.** Klf4 expression inhibited proliferation and promoted differentiation of esophageal keratinocytes with overexpression of Klf5. Primary esophageal keratinocytes were stably infected with the retrovirus pFB-*Klf5* to overexpress Klf5 and then transiently transfected with the *Klf4* expression vector pMT3-*Klf4*. Expression of Klf4 in these cells inhibited growth in MTT assays (A) and promoted differentiation, as indicated by Western blot for the differentiation marker keratin 13 (B).
Figure 7. Klf4 opposed the effects of Klf5 on Gpsm1 in esophageal keratinocytes. (A) In primary esophageal keratinocytes stably infected with pFB-Klf5 to overexpress Klf5, expression of Gpsm1 was decreased while 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.
Table 1. Genes differentially regulated in ED-L2/Klf5 mice compared to littermate controls

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*p positive fold change indicates higher expression in mutant than control mice

Table 2. Differentially regulated genes on microarray confirmed by qPCR

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</table>

*p positive fold change indicates higher expression in mutant than control mice
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7