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Altered intestinal epithelial homeostasis in mice with intestine-specific deletion of the Krüppel-like factor 4 gene

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ABSTRACT

The zinc finger transcription factor, Krüppel-like factor 4 (KLF4), is expressed in the post-mitotic, differentiated epithelial cells lining the intestinal tract and exhibits a tumor suppressive effect on intestinal tumorigenesis. Here we report a role for KLF4 in maintaining homeostasis of intestinal epithelial cells. Mice with conditional ablation of the *Klf4* gene from the intestinal epithelium were viable. However, both the rates of proliferation and migration of epithelial cells were increased in the small intestine of mutant mice. In addition, the brush-border alkaline phosphatase was reduced as was expression of ephrine-B1 in the small intestine, resulting in mispositioning of Paneth cells to the upper crypt region. In the colon of mutant mice, there was a reduction of the differentiation marker, carbonic anhydrase-1, and failure of differentiation of goblet cells. Mechanistically, deletion of *Klf4* from the intestine resulted in activation of genes in the Wnt pathway and reduction in expression of genes encoding regulators of differentiation. Taken together, these data provide new insights into the function of KLF4 in regulating postnatal proliferation, migration, differentiation, and positioning of intestinal epithelial cells and demonstrate an essential role for KLF4 in maintaining normal intestinal epithelial homeostasis *in vivo*.

Introduction

The mammalian intestinal epithelium is a dynamic system in which cell proliferation, differentiation, migration, and apoptosis are stringently coordinated to achieve homeostasis. The epithelium of the small and large intestine consists of a crypt/villus and crypt/surface epithelium unit, respectively. The bulk of the villus and surface epithelium is composed of differentiated columnar epithelial cells that are divided into absorptive cells (enterocytes) and secretory cells (including goblet, enteroendocrine, and Paneth cells; the last unique to the small intestine). The differentiated epithelial cells are descendants of the crypt progenitor cells, which are themselves derived from the multi-potent stem cells, also located in the crypt compartment (Barker et al., 2008; Scoville et al., 2008).

The zinc finger transcription factor, Krüppel-like factor 4 (KLF4) (Garrett-Sinha et al., 1996; Shields et al., 1996), is normally expressed in the differentiated epithelial cells of the intestine, suggesting that KLF4 may function in the switch from proliferation to differentiation. *In vitro*, KLF4 inhibits cell proliferation by functioning as a cell cycle checkpoint protein (Chen et al., 2001; Shields et al., 1996). *In vivo*, KLF4 exhibits a tumor suppressive effect on intestinal tumorigenesis (Ghaleb et al., 2007). Consistent with this finding, *KLF4* is down-regulated in a variety of human cancers including esophageal, gastric, colorectal, and urinary bladder cancers (Kanai et al., 2006; Ohnishi et al., 2003; Wang et al., 2002; Wei et al., 2005; Zhao et al., 2004). However, KLF4 can promote tumorigenesis in a different context, for example, in the absence of $p21^{CIP1}$ (Rowland et al., 2005; Rowland and Peeper, 2006).

Delineation of the physiologic function of KLF4 in the intestinal epithelium is hampered by the early lethality of mice lacking *Klf4* (Katz et al., 2002; Segre et al., 1999). *Klf4*-null mice die within 1 day after birth and suffer from a loss of barrier function of the epidermis (Segre et al., 1999). Additionally, the colons of the *Klf4*-null mice have a 90% reduction in the number of goblet cells, suggesting that KLF4 plays a crucial role in colonic epithelial cell differentiation *in vivo* (Katz et al., 2002). Mice with conditional deletion of *Klf4* from specific tissues have been described. Targeted deletion of *Klf4* from the stomach and esophagus causes altered differentiation and precancerous changes (Katz et al., 2005; Tetreault et al., 2010). Here, we use the Cre recombinase system under control of the *villin* promoter to drive specific deletion of *Klf4* from the intestinal epithelium. The resultant mutant mice had significantly altered homeostasis that involved proliferation, migration, differentiation, and positioning of intestinal epithelial cells. This study provides the first definitive evidence that KLF4 exerts a crucial function in maintaining intestinal epithelial cell homeostasis *in vivo*.

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Materials and methods

Generation of mice with intestine-specific deletion of the Klf4 gene

C57BL/6 mice carrying floxed *Klf4* gene (*Klf4*$^{fl/fl}$) were previously described (Katz et al., 2002). C57BL/6 mice carrying *Cre* recombinase gene under the regulation of *villin* promoter (*Vil*/*Cre*) were purchased from The Jackson Laboratory in Bar Harbor, ME (Madison et al., 2002). Mice lacking *Klf4* in their intestinal epithelium were generated by mating *Klf4*$^{fl/fl}$ mice with *Vil*/*Cre* mice followed by backcrossing to obtain *Vil*/*Cre*;*Klf4*$^{fl/fl}$ mice (designated *Klf4*$^{\Delta IS}$ for intestine-specific deletion). All protocols involving mouse work have been approved by the Institutional Animal Care and Use Committee of Emory University (protocols #098-2007 and 099-2007).

Histology

The small and large intestines were removed from age-matched littermates of *Klf4* mutant mice (*Klf4*$^{\Delta IS}$) and control (*Klf4*$^{fl/fl}$) mice for histological and immunohistochemical characterization of the intestinal tract. Isolated small and large intestines were flushed with modified Bouin's fixative (50% ethanol, 5% acetic acid, and 10% formaldehyde), and cut open longitudinally for gross examination. The intestines were then rolled into a Swiss-roll, fixed, and embedded in paraffin. Five-μm sections were cut and stained with hematoxylin and eosin (H&E). Age-matched littermate control and mutant mice were examined histologically at ages 3 weeks, 4, 7, and 10 months.

Alcian blue (AB) and periodic acid-Schiff (PAS) staining

Goblet cell staining was carried out as described before (Ghaleb et al., 2008) with slight modifications. For AB staining, sections were deparaffinized in xylene, rehydrated in ethanol and brought to distilled water for 5 min. AB 8GX (Biocare Medical) was applied to the sections for 15 min at RT, followed by a 2-min wash in running tap water, counterstained with Nuclear Fast Red (Biocare Medical), followed by dehydration (twice in 95% EtOH and twice in 100% EtOH) and cover-slipped. For PAS staining, deparaffinized and rehydrated sections were treated with Periodic acid (Biocare Medical) for 5 min at RT. Slides were washed in distilled water then stained with Schiff's reagent (Biocare Medical) for 15 min at RT, followed by a 5-min wash in running tap water. The sections were then counterstained with hematoxylin, washed in running tap water for 2 min, followed by dehydration (twice in 95% EtOH and twice in 100% EtOH) and cover-slipped.

Intestinal alkaline phosphatase staining

Deparaffinized and rehydrated sections were stained for endogenous intestinal alkaline phosphatase activity using Vulcan Fast Red Chromogen kit (Biocare Medical), following manufacturer's recommendations.

Immunohistochemistry (IHC) and immunofluorescence (IF)

Mice were sacrificed by CO_2 asphyxiation prior to IHC and IF examination. The entire small intestine and colon were dissected longitudinally and washed in modified Bouin's fixative (50% EtOH, 5% acetic acid, and 10% formaldehyde). The small intestine was divided into 3 equal segments (proximal, middle, and distal). Both the small and large intestines were cut open along their longitudinal axis, rinsed briefly in phosphate-buffered saline (PBS), and examined under a dissecting microscope. Each segment of the intestine was then rolled in a Swiss-roll to allow for histopathological examination of the entire length of both the small and large intestines. Following the dissecting microscopic examination, intestinal tissues were fixed in 10% formalin in PBS and subsequently embedded in paraffin. Five μm-thick paraffin sections were cut and applied to Superfrost Plus slides (VWR). Some sections were used for standard H&E staining. For IHC, sections were deparaffinized in xylene, incubated in 3% hydrogen peroxide in methanol for 30 min, rehydrated in ethanol gradient, and then treated with 10 mM Na citrate buffer, pH 6.0, at 120 °C for 10 min (except for Muc2 which was for 1 min) in a pressure cooker. For lysozyme staining, antigen retrieval was done by Protienase K (Millipore) digestion (1:10 dilution in PBS, for 15 min at 37 °C). All histological sections were incubated with a blocking buffer (2% non-fat dry milk and 0.01% Tween 20 in PBS) for 1 h at RT. An avidin/biotin blocking kit (Vector Laboratories) was used in conjunction with the blocking buffer according to manufacturer's directions to reduce background and nonspecific secondary antibody binding. Sections were then stained using goat anti-KLF4 (1:300 dilution; R&D), rabbit anti-lysozyme (1:200 dilution; Dako), goat anti-LBP (1:200 dilution; Santa Cruz), rabbit anti-Muc2 (1:500 dilution; Santa Cruz), mouse anti-BrdU (1:50 dilution; BD Pharmingen), rabbit anti-ephrin-B1 (1:500 dilution; Santa Cruz), goat anti-EphB2 and goat ant-EphB3 (1:500 dilution; R&D), rabbit anti-Ki67 (1:800 dilution; Leica Microsystems), rabbit anti-chromogranin A, rabbit anti-cleaved caspase-3 (1:500 dilution; Cell Signal), and rabbit anti-colonic carbonic anhydrase-1 (1:500 dilution; Santa Cruz). Detection of primary antibodies for IHC was carried out using appropriate biotinylated secondary antibodies at 1:500 dilutions for 30 min at 37 °C, and color development was performed using the Vectastain ABC kit (Vector Laboratories). Sections were then counterstained with hematoxylin, dehydrated, and cover-slipped. Detection of primary antibodies for IF was carried out using appropriate AlexaFluor labeled secondary antibodies (Molecular Probes) at 1:500 dilutions in 3% bovine serum albumin (BSA) in PBS for 30 min at 37 °C, counterstained with Hoechst 33258 (2 μg/ml), mounted with Prolong gold (Molecular Probes), and cover-slipped. Images were acquired using an Axioskop 2 plus microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA) equipped with an AxioCam MRc5 CCD camera (Carl Zeiss MicroImaging, Thornwood, NY, USA).

5-Bromo-2-deoxyuridine (BrdU) labeling

Mice were injected intraperitoneally (IP) with BrdU (Sigma) at 50 μg/g body weight, then sacrificed at 4 and 24 h post-injection. Following immunostaining for BrdU, the number and position of BrdU-positive cells were counted from at least 30 crypts per mouse per genotype per time point. Statistical significance for number of BrdU positive cells was performed by *t*-test and for cumulative frequency, Kolmogorov–Smirnov (K–S) test.

Ki67 staining and quantification

Following immunostaining for Ki67, the number of Ki67-positive cells was counted from at least 30 crypts per mouse per genotype. Statistical analysis for number of Ki67-positive cells was performed by *t*-test.

Goblet cells counting and size measuring

The number and diameter of Muc2-positive cells in the colon were counted and measured from at least 30 crypts per mouse per genotype. Diameter measurement was done using AxiovisionLE software (Carl Zeiss MicroImaging). Statistical analysis for number and diameter of Muc2-positive cells was performed by *t*-test.

Western blot analysis

Following euthanasia, intestines were removed, flushed once with cold PBS containing protease and phosphatase inhibitors. The