Krüppel-like Factor 4 Regulates Intestinal Epithelial Cell Morphology and Polarity

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Krüppel-like Factor 4 Regulates Intestinal Epithelial Cell Morphology and Polarity

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Abstract

Krüppel-like factor 4 (KLF4) is a zinc finger transcription factor that plays a vital role in regulating cell lineage differentiation during development and maintaining epithelial homeostasis in the intestine. In normal intestine, KLF4 is predominantly expressed in the differentiated epithelial cells. It has been identified as a tumor suppressor in colorectal cancer. KLF4 knockout mice demonstrated a decrease in number of goblet cells in the colon, and conditional ablation of KLF4 from the intestinal epithelium led to altered epithelial homeostasis. However, the role of KLF4 in differentiated intestinal cells and colon cancer cells, as well as the mechanism by which it regulates homeostasis and represses tumorigenesis in the intestine is not well understood. In our study, KLF4 was partially depleted in the differentiated intestinal epithelial cells by a tamoxifen-inducible Cre recombinase. We found a significant increase in the number of goblet cells in the KLF4-depleted small intestine, suggesting that KLF4 is not only required for goblet cell differentiation, but also required for maintaining goblet cell numbers through its function in inhibiting cell proliferation. The number and position of Paneth cells also changed. This is consistent with the KLF4 knockout study using villin-Cre [1]. Through immunohistochemistry (IHC) staining and statistical analysis, we found that a stem cell and/or tuft cell marker, DCAMKL1, and a proliferation marker, Ki67, are affected by KLF4 depletion, while an enteroendocrine cell marker, neurotensin (NT), was not affected. In addition, we found KLF4 depletion altered the morphology and polarity of the intestinal epithelial cells. Using a three-dimensional (3D) intestinal epithelial cyst formation assay, we found that KLF4 is essential for cell polarity and crypt-cyst formation in human colon cancer cells. These findings suggest that, as a tumor suppressor in colorectal cancer, KLF4 affects intestinal epithelial cell morphology by regulating proliferation, differentiation and polarity of the cells.

Introduction

Colorectal cancer is the second most commonly diagnosed cancer among men and women and the second leading cause of cancer deaths in the United States [2,3]. Different genetic variations could lead to abnormal epithelial development and polyp formation, which could be further induced to progression of colorectal carcinomas [4]. Wnt signaling plays an important role in early stages of colorectal carcinogenesis; abnormality in the gene APC or β-catenin leads to aberrant crypt formation [5,6]. Mutations in other oncogenes and tumor suppressor genes, such as K-ras and p53, also contribute to colorectal carcinogenesis [4].

KLF4 is a zinc finger transcription factor initially found to be enriched in the epithelium of intestine and skin [7,8]. Later, it was found in a variety of other tissues, such as thymus, cornea, cardiac myocytes and lymphocytes [9,10,11,12]. KLF4 plays an important role in development and cell differentiation [8,13,14]. In normal intestine, KLF4 is predominantly expressed in differentiated epithelial cells near the luminal surface and goblet cells in the crypts [15,16]. KLF4 is down-regulated in colorectal cancers and has been identified as a tumor suppressor [17,18,19]. As one of the four factors that induce pluripotent stem cells, KLF4 plays a role in cell fate reprogramming and self-renewal of embryonic stem (ES) cells [20,21]. The roles of KLF4 in differentiated intestinal cells are not well understood.

Mice homozygous for a null mutation in KLF4 had defects in terminal differentiation of goblet cells, while further study of KLF4 in mouse intestine was hampered due to early lethality of mutant mice [14]. Using Villin-Cre recombinase system, another study found that conditional ablation of KLF4 from the intestinal epithelium led to failure of goblet cell differentiation [15], which also highlights the role of KLF4 in maintaining intestinal epithelial morphology and homeostasis. Interestingly, depletion of KLF4 from two-week-old mice using vil-CreER, an inducible Cre recombinase, had no effect on goblet cell differentiation [22]. The discrepancy may be due to differential expression of the villin gene in early and later stages of gut development [23]. In this study, we analyzed the role of KLF4 in the adult intestine using an
inducible Cre recombinase, which is driven by native promoter of KLF4.

**Results**

KLF4 loss leads to change in number of goblet cells and morphology of the small intestinal epithelium

In order to test the function of KLF4 in adult intestinal epithelium cells, we generated inducible KLF4 knockout (Klf4/−−/−) mice, which are KLF4/CreER (+/−) and KLF4/lox/lox double transgenic. The Cre recombinase δSNA fused with tamoxifen-inducible estrogen receptor gene was inserted into BAC clone at mice. Induction of KLF4/CreER (+/−) and KLF4/lox/lox double transgene with tamoxifen led to activation of Cre recombinase. The KLF4 function in the skin was studied using this model. KLF4 depletion resulted in a significant increase of hair follicle density, as well as changes of suprabasal cytes [24]. In the small intestine, the Cre recombinase was recapitulating expression pattern of endogenous KLF4 (Fig. 1A). In order to further examine the role of KLF4 in intestinal epithelial cells, we generated inducible KLF4 knockout (Klf4/−−/−) mice, which are KLF4/CreER (+/−) and KLF4/lox/lox double transgenic mice. Induction of KLF4/CreER (+/−) and KLF4/lox/lox double transgene with tamoxifen-mediated Cre recombinase activation resulted in partial depletion of KLF4 when compared with non-induced transgenic mice (Fig. 1A).

Haematoxylin and cosin (H&E) staining results indicated an increase in the number of secretory cells in Klf4/−−/− intestine; the position of these cells appeared to be dislocated compared with control intestine (Fig. 1B). To analyze the effects of KLF4 depletion on goblet cells, which are one of the secretory cell lineages in the small intestine, tissue sections were stained with both Periodic acid-Schiff (PAS) and Alcian Blue (AB), respectively (Fig. 1C left panel). An enlargement in size and an increase in the numbers of PAS and AB positive cells indicated an increase in goblet cell proliferation in small intestine of Klf4/−−/− mice (Fig. 1C right panel), which highlights the role of KLF4 in maintaining numbers of goblet cells in mature small intestine. Time point-specific changes in number of PAS positive cells due to tamoxifen treatment further indicated that KLF4 is critical for goblet cell number maintenance (Fig. 2A). It is worth noticing that our result is distinct from the finding that KLF4 knockout leads to loss of Goblet cells in the colon [14], and that conditional ablation of KLF4 also leads to loss of goblet cells in the intestinal epithelium [15]. The difference is due to the stage of KLF4 knockout before or after goblet cell differentiation. KLF4 depletion had no effect on neuroendocrine cells, as indicated by immunofluorescent staining for neurotensin (NT) (Fig. 1D), suggesting that function of KLF4 in neuroendocrine cells, as indicated by immunofluorescent staining for neurotensin (NT) (Fig. 2A). An enlargement in size and an increase in the number of PAS positive cells due to tamoxifen treatment further indicated that KLF4 is critical for goblet cell number maintenance (Fig. 2A). It is worth noticing that our result is distinct from the finding that KLF4 knockout leads to loss of Goblet cells in the colon [14], and that conditional ablation of KLF4 also leads to loss of goblet cells in the intestinal epithelium [15]. The difference in size and number of goblet cells in small intestine of Klf4/−−/− mice were stained positive for lysozyme in small intestine from control intestine; the morphology change was analyzed in Klf4/−−/− mouse intestine. PCNA is another proliferation marker; and its change in response to KLF4 loss is consistent with the results from Ki67 staining (Fig. 2A). This further highlights the role of KLF4 in inhibiting intestinal cell proliferation.

In addition to goblet cell staining, the role of KLF4 in intestinal cell proliferation was confirmed by staining for other cell types including Paneth cells and tuft cells. Tissue slides from both normal and Klf4/−−/− intestine were stained for lysozyme, which is a marker for Paneth cells (Fig. 2A and C). A larger proportion of cells stained positive for lysozyme in small intestine from Klf4/−−/− mice compared with control mice, and these cells were dislocated through the crypt-villus axis, indicating that KLF4 loss also led to an increase in Paneth cell population and has an effect on position of these cells. This result re-emphasizes the role of KLF4 in controlling Paneth cells and strongly supports the finding from the KLF4 knockout study using villin Cre [15].

Based on the current model, small intestine is composed of the Paneth cell region (bottom of crypt), the stem cell zone (through +4 location), an amplification compartment (up to top of crypt) and a differentiation compartment (including crypt-villus junction) [25, 26]. To further analyze the effect of KLF4 on intestinal homeostasis, tissue sections from Klf4/−−/− mouse intestine were stained for stem cell and/or tuft cell marker DCAMKL-1 [27] (Fig. 2A C). Surprisingly, DCAMKL-1 positive cells were increased in the Klf4/−−/− mouse small intestine along the villus, but were not restricted to the crypt base, indicating an increase in number of tuft cells due to loss of KLF4.

Based on our observation of changes in cell position as well as epithelial apical-basolateral morphology, we proposed that KLF4 is not only responsible for controlling cell differentiation and proliferation, but also cell polarity. As indicated by H&E staining, a great part of the cell nuclei lost polarity in Klf4/−−/− mouse small intestine. Paneth cells change their position due to depletion of KLF4. Meanwhile, most of the tuft cells that were positive for DCAMKL-1 also were out of direction in knockout mice (Fig. 2A, C). In order to investigate the role of KLF4 in regulating cell polarity, we generated a three-dimensional (3D) epithelial cyst formation assay for Caco-2 cells, in which the morphological structure of cyst and apical-basolateral cell polarity can be examined in vitro.

KLF4 ablation leads to abnormal proliferation and differentiation in small intestinal epithelium

In order to further examine the role of KLF4 in intestinal epithelial cells, the morphology change was analyzed in Klf4/−−/− mice compared with non-induced (Day 0) mice by H&E staining (Fig. 2A): The average length of the crypt-villus axis was increased in Klf4/−−/− mouse intestine (Fig. 2A, 2B). The number of secretory-like cells is increased; these cells either have larger volume of vacuoles or contain secreted granules like Paneth cells (Fig. 2A). A large number of cell nuclei lost apical-basolateral polarity, which is typical of the wild-type enterocytes. Instead of a monolayer of well-oriented epithelial cells, Klf4/−−/− intestine had multiple layers of disorganized cells (Fig. 2A, C). Positions of the secretory cells were changed; instead of sitting at the bottom of the crypt, the granule-containing cells dislocated upward in the crypts (Fig. 2A, C). In order to confirm that the morphology change was not due to tamoxifen treatment, small intestine from wild-type (WT) mice treated with tamoxifen was stained as a control; they showed a normal morphology as non-treated transgenic mice.

The cell proliferation marker Ki67 was examined by IHC. The average length of Klf4−− region along the crypt-villus axis, as well as numbers of Klf4−− cells increased (Fig. 2A−C), i.e., proliferation compartment of the intestine was expanded, indicating an increase in proliferation capacity in Klf4−− mouse intestine. PCNA is another proliferation marker; and its change in response to KLF4 loss is consistent with the results from Klf67 staining (Fig. 2A). This further highlights the role of KLF4 in inhibiting intestinal cell proliferation.

KLF4 is essential for cell polarity and crypt-cyst formation in 3D culture of Caco-2 cells

Normally, Caco-2 cells with high polarity form lumen-containing cysts in matrigel-based 3D culture and show apical-basolateral polarity as indicated by ZO-1 as a basolateral marker and α6-integrin as an apical marker, respectively (Fig. 3A, bottom; Fig. 3B, top); low- or no-polarity Caco-2 cells only form cysts without lumen (Fig. 3B, bottom). Staining of α6-integrin and ZO-1 for Caco-2 cells in 2D culture is shown as control, indicating non-polarization of Caco-2 cells in 2D growth conditions (Fig. 3A, top). To determine the role of KLF4 in lumen-cyst formation, KLF4 was depleted in Caco-2 cells by siRNA and shRNA delivery...
KLF4 facilitates cell polarity and crypt-cyst formation in colon cancer cells

In order to confirm the role of KLF4 in facilitating cell polarity formation, 3D culture assay was performed in another colon cancer cell line to test whether KLF4 can enhance cyst formation.
A

KLF4 Regulates Intestinal Cell Morphology

Day 0  Day 3  Day 5  Day 30  WT-Day 5

H&E

Ki67

Lysosome

DCAMKL1

PAS

PCNA

B

Control  Klf4

C

H&E

Ki67

Lysosome

DCAMKL1

ZO-1 (1 month)
in vitro. The LS174T-KLF4 stable cell line expresses KLF4 upon doxycycline induction [18]. LS174T cells seldom form cysts, even under 3D culture conditions. However, induction of KLF4 expression in LS174T cells significantly increased the chances of cyst formation in 3D culture (Fig. 4A, B), indicating that KLF4 indeed enhances cell polarity and thus facilitates cyst formation in 3D formation assay.

In order to address the mechanism by which KLF4 regulates cell polarity both in a knockout mouse model and in a 3D culture system, a panel of cell fate and polarity-related genes were analyzed by semi-quantitative RT-PCR. KLF4 was induced by doxycycline in LS174T-KLF4 colon cancer cells (Fig. 4C). Though we didn’t see significant changes in DCAMKL-1 transcription, several polarity-related genes, LKB1, EPHB2, and EphB3, were down-regulated. Intestinal stem cell markers LGR5 and CD44 were also down-regulated. As controls, the differentiation marker IAP and cell cycle inhibitor P21, which are known KLF4 target genes, were up-regulated by KLF4 (Fig. 4D). These findings suggest that KLF4 regulates epithelial cell polarity by regulating the transcription of multiple genes.

Discussion

As an important regulator in intestinal cell differentiation during early development, KLF4 is also essential in maintaining normal homeostasis and morphology in adult intestine. Previous studies have deleted KLF4 in embryonic stages of mouse intestine; the terminal differentiation of goblet cells was decreased in these mice [14,15]. Here, we reported that in mature mouse intestine, partial depletion of KLF4 resulted in an increase in the number of goblet cells, indicating that KLF4 is required not only for goblet cell differentiation in early stages, but also for maintaining the number of differentiated goblet cells, probably by inhibiting cell proliferation. This is consistent with the observation that KLF4 is strongly expressed in goblet cells [15,16,19]. We found that the average length of crypts was increased in KLF4-depleted small intestine, and the number of Ki67 positive cells was also increased. In agreement with previous findings, the number and position of Paneth cells had also changed [15]. DCAMKL-1 is a marker for tuft cells, and a potential marker for quiescent intestinal stem cells [28,29]. We found that the number and position of DCAMKL-1 positive cells was also altered by KLF4 deletion. Changes in morphology and polarity of intestinal epithelial cells were confirmed by H&E staining. These data suggest that KLF4 plays a key role in maintaining normal intestinal homeostasis and morphology by regulating cell differentiation, proliferation and polarity. The roles of KLF4 in cell polarity were further analyzed in 3D culture, and several novel KLF4 target genes involved in cell differentiation and polarity were identified.

Our results suggest tamoxifen-induced knockout of KLF4 is advantageous in tissue- and stage-specificity. We were able to partially delete KLF4 in the villi of small intestine, where KLF4 normally predominantly expresses. In addition, the inducible knockout strategy allows normal development of small intestine in the early stage of development, which assures that lineage differentiation (i.e., the ability to differentiate Paneth and goblet cells) and intestine function is not affected by KLF4 deletion. The limitation of our model is that KLF4 depletion is not complete.
leads to elevated expression of LKB1 [38]. Since KLF4 expression is dependent on CDX2 in human colon cancer cells [39], our finding is consistent with these reports and suggests that KLF4 regulates cell polarity through multiple genes, including LKB1.

In summary, the results from this study and previous studies suggest that KLF4 has multiple functions. In the early embryonic stage, KLF4 induces goblet cell differentiation in intestinal epithelium; throughout intestinal development, KLF4 maintains homeostasis of normal intestinal growth and keeps epithelial cells from over-proliferation. Meanwhile, KLF4 regulates apical-basolateral polarity of the intestinal epithelial cells. After all, the intestinal homeostasis and morphology are regulated by multiple factors, including KLF4 and its target genes.

Materials and Methods

Transgenic mice and animal work

**Ethics Statement.** Mouse experiments were performed under the approval by the Institutional Biosafety Committee (IBC) and by the Institutional Animal Care and Use Committees (IACUC) of University of South Carolina (Proposal number 1573).

Transgenic mice were generated using a Cre recombinase derived from a bacterial artificial chromosome (BAC, RP23-322L22) containing mouse KLF4 gene [40]. A Cre recombinase cDNA was fused with estrogen receptor gene and was inserted into KLF4 locus at the initiating codon, and the CreER gene transcription is under the control of KLF4 promoter. KLF4

![Image](image-url)
knockout in KLF4/CreER (+/−)/KLF4 (floxed/floxed) double transgenic mice was induced by 100 mg/kg tamoxifen intraperitoneally (i.p.) for 5 consecutive days at 4 weeks old. Expression of KLF4 as well as multiple genes in wild-type (Klf4+/+; Klf4−/−) and knockout (Klf4−/−) mice was analyzed 3, 5 or 30 days after induction by immunofluorescence (IHC) staining of fixed intestine tissues.

Cell culture and 3D formation assay

Caco-2 human colononic epithelial cell line [41] was cultured in high glucose Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. For 3D culture, approximately 1.5×10^5 cells were embedded into 250 µl of 80–90% matrigel. The 3D matrix was allowed to harden in a 24-well plate at 37°C for 30 minutes, then 500 µM of DMEM medium with 2% fetal bovine serum was added and cysts were allowed to form over 5–7 days at 37°C.

LS174T colon cancer cell line [18] was grown in RPMI medium (Mediatech) supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin. Stable cell line LS174T-tet-on-KLF4 has been described previously [Zhang et al., 2006]. LS174T-tet-on-KLF4 cells were plated at approximately 2×10^5 cells per well in a 6-well plate. The following day, doxycycline (1 µg/ml) was added to the culture medium. After 24 h of incubation, cells were trypsinized and counted, then followed by 3D formation assay as indicated with Caco-2 cells.

Western Blotting

Cells were lysed in the appropriate volume of lysis buffer (50 mM HEPES, 100 mM NaCl, 2 mM EDTA, 1% glycerol, 50 mM NaF, 1 mM Na3VO4, 1% Triton X-100, with protease inhibitors). The following antibodies were used: mouse anti-β-Actin (Sigma, A1978), mouse anti-Flag (Sigma, F1804).

RT-PCR

LS174T-tet/on-KLF4 cells were plated at approximately 2×10^5 cells per well in a 6-well plate. The following day, doxycycline (1 µg/ml) was added to the culture medium. After 48 h of incubation, RNA was isolated using the RNeasy kit (Qiagen). Reverse transcriptase PCR (RT-PCR) was performed as described previously [Zhang et al., 2006]. The following primers were used: β-actin, 5′-CAACCGGGAAGATGAC-3′ and 5′-AGGAAGGCTGGAAGAGTG-3′; IAP, 5′-CCATGGCGTTA-CAGGATGAGG-3′ and 5′-CAGGGGCTTCCTACTCTTGTTGTG-3′; p21/WAF1, 5′-CGACTGTGATGCGCTAATGG-3′ and 5′-AGAGATGACCCGGCGTGGTG-3′; LGR5, 5′-CCTGCGTTACTTGAGGAAACG-3′ and 5′-ATGTTCCACTGCTGGATGAC-3′; CD44, 5′-CAGAATGGCGTTACATCTTG-3′ and 5′-CGGAAGATCGGCACAAAAA-3′ and 5′-CTGTGGCAGTATTTCCGTG-3′; MARK2, 5′-GCCGAGATCGCAAAAG-3′ and 5′-ATGATGGTATGTCGGGAGG-3′; BMI1, 5′-AGCAGAAATGCTGACAAAC-3′ and 5′-CCTAACCAGAT- GAAAGGTGCTG-3′; EphB2, 5′-AAAATGACAGGTTGTA-TGGC-3′ and 5′-TCAGAGGTTGTCGTCGTCGTC-3′; EphB3, 5′-AGCAGCTGGTCTGCAAGGT-3′ and 5′-TGCACTGCC- CATGACCTGCC-3′.

Interference RNA, H&E staining, immunohistochemistry, PAS and AB staining

Interference RNA and immunohistochemistry were tested as described previously [Zhang et al., 2006]. Lentiviral stocks were prepared using control shRNA or human KLF4 shRNA on pGIPz vector containing a marker of turbo GFP (Open Biosystems). H&E staining was performed based on standard protocol by Histology Laboratory of the Imaging Facility at University of Kentucky.

For immunohistochemistry staining, the following antibodies were used: KLF4 [Zhang et al., 2006], rabbit anti-human Lysozyme (Diagnostic BioSystems, RP 028-05), rabbit anti-DCAMKL-1 (Abgent, AP7219b), rabbit anti-Ki67 (Novus Biologicals, NB110-89717). PAS staining was performed based on standard protocol using reagents of PAS Staining System from Sigma (395-B). Alcian Blue (AB) staining was performed according to standard protocol using Alcian Blue 8GX and Fast Red from Sigma (kindly provided by Dr. Tianyan Gao).

Immunofluorescent staining

Cells grown on cover glass were fixed in 4% paraformaldehyde in PBS at room temperature for 15 min, washed 3 times with PBS, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and then blocked in 5% goat serum in PBS at room temperature for 1 h. Cells were incubated with primary antibodies at room temperature for 2 hours. Antibodies used include rat anti-human CD49f (86-integrin, BD Pharmingen, 555734) and rabbit anti-ZO-1 (Invitrogen, 61-7300). Then cells were washed 3 times with PBS and further incubated with Alexa-488-labeled anti-rat IgG (1:500) and Alexa-568-labeled anti-Rabbit IgG (1:500) diluted in PBS for 40 min. Nuclei were stained by DAPI (Sigma). The cover glasses were washed, mounted on glass slides, viewed and photographed with an Olympus FW1000 confocal microscope.

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Author Contributions

Conceived and designed the experiments: TY RX CL. Performed the experiments: TY XG JL. Analyzed the data: TY. Contributed reagents/materials/analysis tools: TW WA. Wrote the paper: TY CL.

References


