KLF4 gene expression is inhibited by the notch signaling pathway that controls goblet cell differentiation in mouse gastrointestinal tract

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1Department of Pathology, Microbiology and Immunology, University of South Carolina, Columbia, South Carolina; 2Division of Gastroenterology, School of Clinical Sciences, University of Liverpool, Liverpool, United Kingdom; 3Division of Digestive and Liver Diseases, Department of Medicine, Columbia University Medical Center, New York, New York; and 4Sealy Center for Cancer Cell Biology and Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, Texas

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Zheng H, Pritchard DM, Yang X, Bennett E, Liu G, Liu C, Ai W. KLF4 gene expression is inhibited by the notch signaling pathway that controls goblet cell differentiation in mouse gastrointestinal tract. Am J Physiol Gastrointest Liver Physiol 296: G490–G498, 2009. First published December 24, 2008; doi:10.1152/ajpgi.90393.2008.—In Kruppel-like factor (KLF)-4-deficient mice, colonic goblet cell numbers are significantly reduced. Goblet cell development is regulated by the Notch signaling pathway. The aim of this study was to examine whether Notch represses KLF4 expression to regulate goblet cell differentiation. We first detected that KLF4 gene expression was upregulated in a human progastrin-overexpressing mouse model where goblet cell hyperplasia has been observed. We then found that mice treated with a γ-secretase inhibitor (compound E, 10 μmol/kg) for 24 h, which inhibits the Notch signaling pathway, had significantly increased KLF4 mRNA levels in small intestine and colon, accompanied by an increased number of KLF4-expressing cells at the bottom of crypts in small intestine and colon. In a colon cancer cell line (HCT116 cells), KLF4 promoter activity was inhibited by a constitutively active form of Notch1 (ICN1) by transient cotransfection assays. This inhibition was significantly compromised by a dominant-negative RBPjk, a repressive mediator of the Notch signaling pathway. An ICN1-responsive element was then mapped in the human KLF4 promoter between −151 and −122 nucleotides upstream of the transcriptional start site. It was also found that an intact ICN1-responsive element is required for ICN1 to inhibit KLF4 promoter activity by transient cotransfection assays. Our findings thus reveal a possible mechanism by which KLF4 is inhibited by Notch, which controls goblet cell differentiation in mouse gastrointestinal tract.

Kruppel-like factor-4; notch signaling; gene expression; goblet cells
following recruitment of coactivators, including Mastermind-like proteins and CBP/p300, then activates gene expression of downstream target genes such as Hairy and Enhancer of split homologue-1 (Hes1) (16). Hes1 is a bHLH transcriptional factor that plays a critical role in the differentiation processes of the gastrointestinal tract. It has an antagonistic effect on Math1, another transcriptional factor required for the development of secretory cell lineages in the mouse intestine (38).

In this study, we provide in vivo evidence that KLF4 is inhibited by the Notch signaling pathway that controls goblet cell differentiation in mouse small intestine and colon. Additionally, in vitro data indicate that KLF4 promoter activity is inhibited by Notch, and this inhibition is significantly compromised by a dominant-negative RBPJk, a repressing isoform of the Notch signaling pathway.

MATERIALS AND METHODS

RNA extraction and quantitative RT-PCR analysis. Total RNA was extracted from mouse tissues and culture cells using Trizol Reagent (Invitrogen) according to the manufacturer’s instructions. About 3–5 μg of total RNA was used to generate reverse transcriptase mix (cDNA) that was used as template in quantitative RT-PCR as described previously (1). New primers that were used in the PCR reactions are as follows: mouse (m) glyceraldehyde-3-phosphate dehydrogenase, top strand: 5'-GCATCAAGAAGGGTGAAGCAG-3', bottom strand: 5'-ATACAGAGAATGAGACCTGAACAAA-3'; mouse KLF4, top strand: 5'-AGAGGAGCCCAAGCCAAAGAGG-3', bottom strand: 5'-CCACAGCGGCCGTCCAGTCAAGT-3'.

Protein extraction and Western blotting analysis. Total proteins from mouse tissues were prepared using RIPA buffer, and 50 μg of proteins were separated by SDS-PAGE gel followed by Western blotting analysis as previously described (1). The antibodies used were as follows: anti-KLF4 antibody (1:1,000, catalog no. IMG-3231 from IMGENEX) and anti-β-actin (1:3,000, catalog no. ab8227 from Abcam).

Maintenance of mice and assessment of goblet cell numbers. All mice with C57BL/6 and FVB/N background were bred and housed under clean specific pathogen-free conditions. C57BL/6 mice were maintained and used at the animal facility at Columbia University. Mice at 6–8 wk old were used for the experiments, which were performed under approved protocols from the Institutional Animal Care and Use Committee of Columbia University. Hematoxylin and eosin (HE) and Periodic acid–Schiff (PAS) staining of mouse small intestine and colon were performed by the histology core facility at Columbia. Male hGAS mice (10–12 wk old; see Ref. 36) and their wild-type C57BL/6 counterparts were used. They were housed under conventional animal house conditions, and experiments were conducted under United Kingdom Home Office approval. Following death, colonic tissue was dissected, fixed in 4% formal saline, and paraffin embedded. Sections (3–5 μm) of the distal half of colon were stained with Alcian blue/PAS or alcian blue/PAS-stained goblet cells in the distal colon compared with wild-type FVB/N mice (P < 0.01 by Student’s t-test and significant differences at cell positions 4–38 by modified median test) (Fig. 1, A and B). Because KLF4 is required for colonic goblet cell development, we postulated that the increased goblet cell numbers observed in hGAS mice is accompanied by increased gene expression of KLF4. To test this possibility, mRNA and proteins were extracted from colons of control FVB/N mice and human progastrin-overexpressing FVB/N mice (hGAS/FVB), followed by quantitative RT-PCR analysis. As shown in Fig. 1C, KLF4 mRNA levels in hGAS/FVB mouse colon were increased two- to threefold compared with control mice (P < 0.05 by Student’s t-test). KLF4 protein levels were also increased in the colons of hGAS/FVB mice compared with the control mice (Fig. 1D). By densitometry analysis, they were elevated by about seven- to eightfold (averaged from 3 independent experiments).

Notch inhibition increased KLF4 gene expression. Previously, it has been shown that KLF4 is required for goblet cell development (15). Because the Notch signaling pathway has been shown to regulate the differentiation of a broad spectrum of cell types, including goblet cells in the gastrointestinal tract, we proposed that KLF4 is a downstream target of the Notch signaling pathway that controls differentiation of goblet cells. To test this possibility, 6- to 8-wk-old C57BL/6 mice were used, and Notch signaling was inhibited by using a gamma secretase inhibitor (compound E). Consistent with a previous report (35), 5-day treatment of the mice with compound E at a
dose of 10 μmol·kg⁻¹·day⁻¹ converted amplifying crypt cells within the small intestine into goblet cells that were accompanied by increased KLF4 mRNA levels in small intestine and colon (Figs. 2 and 3, A and B). However, because 5-day treatment with compound E changed the morphology of mouse small intestine, a 1-day treatment with compound E was performed, which resulted in no significant morphological changes of mouse small intestine as observed by HE staining and PAS staining (data not shown). Under this condition, RNA levels of KLF4 were upregulated in mouse small intestine and colon by quantitative RT-PCR (P<0.05 by Student’s t-test) (Fig. 3, C and D). In parallel, KLF4-positive cells at the bottom of the crypt of mouse intestine were significantly increased (compare cells in white boxes in D (6 cells) and G (17 cells) in Fig. 4). Similar results were observed at the bottom of crypts of mouse colon (compare cells in white boxes in A (10 cells) and D (19 cells) in Fig. 5). After counting 50 crypts of small intestine and 50 lower one-third of the colonic crypts before and after 24 h compound E treatment, it was found that the number of KLF4-positive cells was significantly increased in both small intestine (11 cells vs. 20 cells, P<0.01 by Student’s t-test) and colon (14 cells vs. 22 cells, P<0.05 by Student’s t-test) following treatment.

Notch inhibited the promoter activity of KLF4. Because an in vivo approach cannot be used to provide direct evidence of KLF4 inhibition by the Notch signaling pathway, a constitutively active version of Notch (ICN1, a COOH-terminal region of human Notch1) was used to assess the inhibition of KLF4 promoter activity in vitro. In HCT116 cells, we performed cotransfection assays to examine the regulation of KLF4 reporter by ICN1. Consistent with our hypothesis, ICN1 decreased KLF4 promoter activity by ~50%. A dominant-negative version of RBPjk, a repression mediator of the Notch signaling pathway, activated KLF4 promoter activity by ~60%. In addition, dominant-negative RBPjk significantly compromised ICN1-induced KLF4 inhibition (Fig. 6). Similar observation was also true when mKLF4 promoter was used in the same assay (data not shown).

Identification of an ICN1-responsive element in hKLF4 promoter. To identify an ICN1-responsive element in the hKLF4 promoter, a series of 5'-truncated versions of the KLF4 promoter were generated for the assay (Fig. 7A). Although truncation of 5'-end of the KLF4 promoter to the −151 position retained the inhibitory effect of ICN1, further truncation of KLF4 promoter to the −108 position abrogated the effect of ICN1 (Fig. 7B), suggesting that an ICN1-responsive element of the KLF4 promoter resides in the −151 to −108 region.

Fig. 1. Increased colonic goblet cell numbers correlate with upregulation of Kruppel-like factor (KLF)-4 gene expression in hGAS mice. A: cell positional distribution of goblet cells. B: mean no. of goblet cells in 50 half-crypts of the distal colon of hGAS and FVB/N mice (n = 6 male mice/experimental group). Significant differences were found at cell position 4–38 by the modified median test in A and P<0.01 by Student’s t-test in B. Total RNA and protein were extracted from the colon of adult hGAS mice as described in MATERIALS AND METHODS. Quantitative RT-PCR (C) and Western blotting analysis (D) were performed. Three control mice and three hGAS mice were used for RT-PCR analysis, and a representative Western blotting result from 3 independent experiments was shown.
element was located between the −151 to −108 position at the 5′-region of the hKLF4 promoter. Further mapping using the same approach identified a short region between −151 to −122 of the hKLF4 promoter as an ICN1-responsive element.

This region was conserved in both hKLF4 and mKLF4 promoters in the same area, with the only differences being the three nucleotides shown in lowercase in Fig. 7, suggesting a bona fide regulation of KLF4 gene expression by the Notch signaling pathway. To further narrow down the ICN1-responsive element within this region, several different mutant KLF4 reporters were generated and used to test ICN1-mediated promoter inhibition. Those mutants harbor mutations at different positions between −151 and −122. It was found that, after introduction of the mutations in the underline region of KLF4 promoter between −149 and −138 nucleotide, the promoter activity was decreased, suggesting a positive effect of this element. In addition, although ICN1 showed an inhibitory effect on the wild-type KLF4 promoter, it lost its ability on the mutant KLF4 promoter (Fig. 7D). It should be noted that the mutant promoter was still active, thus ruling out the possibility that it is permanently repressed and could not be further inhibited. This result suggests that an intact ICN1-responsive element is required for ICN1 to inhibit KLF4 promoter activity.

DISCUSSION

KLF4 is required for colonic goblet cell differentiation, a process that is highly regulated by the Notch signaling pathway. Our current in vivo study indicates that KLF4 mRNA levels after normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Fig. 3. Increased KLF4 mRNA levels in mouse small intestine and colon upon inhibition of Notch. Total RNA from mouse colon and small intestine was extracted, and quantitative RT-PCR was performed as described in MATERIALS AND METHODS. A and B: Notch signaling was inhibited by 5-day CompE treatment, and KLF4 expression in mouse small intestine (A) and colon (B) was measured by RT-PCR analysis. C and D: similar to A and B, except that mice were treated with CompE for 24 h. Note that y-axis indicates relative KLF4 mRNA levels after normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).
intestine and colon in mice, since increased KLF4 signals at the cellular levels were observed at the bottom of the crypts upon inhibition of the Notch signaling pathway by the γ-secretase inhibitor compound E (Figs. 3–5). The current observation is consistent with Notch signaling in the intestinal crypts. Both Notch1 and Notch2 are expressed in crypt epithelial cells. Notch ligand Delta-like 1 and 3, and Jagged-1 are also expressed in crypt epithelium (29). The importance of the Notch signaling pathway has been revealed by several different studies. RBP-Jκ conditional knockout mice or Notch inhibition by γ-secretase inhibitors results in secretory cell expansion at the expense of enterocytes and epithelial proliferation (34). Conversely, constitutively active Notch results in depletion of secretory lineage cells and increased proliferation (9). Low levels of KLF4 expression in the proliferation zone of the crypt region are not only consistent with its role in growth arrest and differentiation but also provide a possible mechanism of how a low level of KLF4 is maintained. The Notch signaling at least partly contributes to this KLF4 regulation by inhibiting KLF4 gene expression. In addition, cross talk of KLF4 and β-catenin may provide another possible way of KLF4 regulation in the intestinal crypts (42), at the bottom of the intestinal crypts where KLF4 expression is low and β-catenin signaling is active, which results in proliferation of stem cells and/or progenitor cells. With cells migration upward, cells become more differentiated. This might be because of interaction between KLF4 and β-catenin, resulting in the inhibition of expression of downstream Wnt-responsive genes. Cross talk between KLF4 and Wnt signaling through β-catenin and inhibition of KLF4 gene expression by Notch provide another link between the Wnt and the Notch signaling pathways that play a critical role in homeostasis of the normal intestine and in tumorigenesis of colorectal cancers (19). It should be mentioned that the tests that we are using for Notch signaling are

![Image](http://ajpgi.physiology.org/content/296/3/E494/F4.large.jpg)
not subtle. First, ICN1 is a constitutively active version of Notch1. This may give an exaggerated effect of Notch that is activated in a ligand-dependent version in a subtle process. Second, compound E, a γ-secretase inhibitor used in our assays, has the potential to have broad and pleiotropic effects other than simply inhibiting Notch activation. Therefore, these are not simple loss-of-functional Notch experiments, although they provide strong evidence for Notch signaling. To better understand the role of Notch signaling in regulation of KLF4 and ultimately how these factors affect goblet cell differentiation by Notch signaling, a floxed Notch1 allele or Notch1 knockdown and the use of FcDelta to activate Notch at a more physiological level should be used in future experiments.

Our in vitro studies suggest that a transcription factor that binds to the ICN1-responsive element in KLF4 promoter may mediate KLF4 inhibition by Notch. To identify the potential transcriptional factor(s), a search of transcriptional factor database was performed using several online programs, including Match (http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi?). FoxD3 was identified as the potential transcriptional factor that binds to the underlined region of the ICN1-responsive element in both hKLF4 and mKLF4 (Fig. 7C, bottom). FoxD3, also named Genesis, is a member of the winged helix/Forkhead family of transcriptional factors that binds to specific DNA sequences having the consensus A(A/T)TRTT(G/T)RYTY (22, 31). Expression of FoxD3 was initially thought to be restricted to embryonic stem cells. It has a crucial role in the maintenance of two disparate embryonic progenitor cells, cells that give rise to the epiblast and embryonic stem cells and those that give rise to the placenta and trophoblast stem cells (13, 33). However, a recent report also indicates that FoxD3 is expressed in adult tissues (24). Our data suggest that FoxD3 is expressed in mouse intestinal epithelium and (data not shown). It is possible that KLF4 is regulated by FoxD3, and this regulation may occur in stem cells or progenitor cells that are located at the bottom areas of crypts, where the regulation of KLF4 by the Notch signaling pathway occurs. This is consistent with the expression of FoxD3 in stem cells and with a critical role of KLF4 in the biology of stem cells (17, 32, 40). It will therefore be very interesting to test the expression of KLF4 in stem cells of intestinal origin. The relationship between Notch and FoxD3 remains largely unclear. FoxD3 may be directly or indirectly regulated by the Notch signaling pathway. Recent studies suggest that Notch1 induces upregu-

Fig. 5. Increased KLF4 cells in mouse colon upon inhibition of Notch. Similar to Fig. 4 except mouse colon was examined instead of mouse small intestine. After CompE treatment, KLF4 positive cells increased from 10 cells (inside the white box in A) to 19 cells (inside the white box in D). Note that all white boxes have the same size in very similar areas.

Fig. 6. ICN1 inhibited the promoter activity of KLF4. Human KLF4 (hKLF4) promoter activity is inhibited by a constitutively active version of human Notch1 (ICN1) by transient transfection and dual luciferase assays using a colon cancer cell line (HCT116). Vector control (PCS2), a constitutively active Notch 1 (ICN1), dominant-negative RBPjk plasmid (dnRBPjk), and combination of ICN1 and dnRBPjk expression constructs were transfected in HCT116 cells with a 2.0-kb hKLF4 promoter reporter construct. After transfection (48 h), dual Luciferase assays were then performed to measure KLF4 promoter activities as described in MATERIALS AND METHODS.
lation of the phosphatidylinositol 3-kinase-protein kinase B pathway (23), which in turn negatively regulates the function of Forkhead box-containing proteins (4). At a molecular level, FoxD3 has been shown to form a complex with another stem cell transcription factor (Oct4) to regulate the transcription of downstream targets (12). The binding of FoxD3 to the ICN1-responsive element in KLF4 promoter, the regulation of FoxD3 by Notch, the regulation of KLF4 by FoxD3, and the involve-
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GRANTS

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