

Original Article

Hsa-miRNA-26b regulates SPAK expression during intestinal epithelial cell differentiation

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Abstract: The Ste20-like proline/alanine rich kinase (SPAK) is involved in variety of physiological cellular processes such as cellular volume regulation and epithelial barrier integrity, as well as some abnormal conditions such as intestinal inflammation, but the underlying mechanisms remain largely unknown. MicroRNAs also plays important roles in numerous cellular functions like apoptosis and proliferation by post-transcriptionally regulating the production of target gene expression. Here, we studied if microRNAs are involved in the regulation of SPAK-mediated cellular differentiation and inflammation. We demonstrated that miRNA-26b can directly bind to the 3'-untranslated region (UTR) of SPAK mRNA, by which negatively regulates its expression in Caco2-BBE cells. The expression of SPAK is always inversely related to the production of miRNA-26b, in both well-differentiated and poor-differentiated Caco2-BBE cells and mouse jejunum villus/crypt cells. Further, miRNA-26b can reverse SPAK overexpression-mediated epithelial cell barrier dysfunction in Caco2-BBE cell, suggesting that miRNA-26b modulates the integrity of epithelial cell and expression of junction proteins (such as occludin) by regulating SPAK expression, which in turn could affect differentiation. In a pathological context, the pro-inflammatory cytokine interferon-gamma (IFN- γ) increases SPAK expression in Caco2-BBE cells by decreasing miRNA-26b levels. Consistent with these *in vitro* data, miRNA-26b levels were decreased in actively inflamed colonic tissues in ulcerative colitis, where SPAK expression was up-regulated, compared with normal tissues. Taken together, these results reveal a novel mechanism underlying the regulation of SPAK expression by miRNA-26b during the differentiation of epithelial cells, and in inflammatory conditions, which raises miRNA-26b as promising therapeutic targets for intestinal inflammation.

Keywords: SPAK, miRNA-26b, epithelial differentiation, barrier function, inflammatory bowel disease

Introduction

Intestinal epithelial cells undergo turnover every 3-5 days [1], during which cell differentiation, together with cell proliferation and migration, plays important roles in maintaining the integrity of intestinal epithelial monolayer and homeostasis of human innate and adaptive immune system [2-4], evidenced by the fact that dysregulated differentiation of epithelial cells is always accompanied with tumorigenesis and inflammation [5-13]. For example, loss of epithelial differentiation contributes to pathogenesis of carcinomas and specially the progression of most carcinomas toward malignancy characterized by increased cell motility and invasion [14], resulting in tumor metastasis [15], even cancer-drug resistance [16]. Kruppel-

like factor 4 (Klf4) is critical for normal epithelial homeostasis and regulates goblet cell differentiation in the colon [17], loss of Klf4 in mice can lead to abnormal proliferation and differentiation and precancerous changes in the adult stomach [18]. In intestine, activation of Oct-4 causes dysplasia in epithelial tissues by inhibiting cellular differentiation in a manner similar to that in embryonic cells [19]. Inflammatory bowel diseases (IBD), mainly Crohn's diseases (CD) and ulcerative colitis (UC), are relapsing, idiopathic intestinal inflammatory conditions, caused by inappropriate and continuing immunologic responses to aberrant intestinal microorganisms in genetically susceptible individuals under certain environmental conditions [20]. CD displays increased differentiation of naive T cells into Th1/Th17 cells in intestinal lamina

propria cells (LPCs) [21-23]. However, in ulcerative colitis (UC), naïve T cells predominately differentiate into Th2-like cells [24]. Further, in a murine T cell transfer model of colitis, methyltransferase G9A alters the development of intestinal inflammation by regulating T cell differentiation [25]. Opposing effects of smoking in ulcerative colitis and Crohn's disease may attribute to its distinct differentiation effect on dendritic cells (DC) [26]. However, the underlying mechanisms involved in the dysregulated differentiation of intestinal epithelium, especially the upper portion of the crypts-villus axis, during tumorigenesis and inflammation are still largely unknown.

MicroRNAs (miRNAs), 20-23 nucleotides long non-coding RNA, modulate gene expression by inhibiting the translation or promoting the degradation of target mRNAs [27]. To date, currently almost 2000 microRNA sequences have been identified through experimentation or by *in silico* analyses [28]. Studies have shown that miRNAs plays important regulatory roles in differentiation in various cell types, such as embryonic stem cells [29], dendritic cells [30], mesenchymal stem cells [31] and intestinal epithelial cells [32]. Our lab found miR-7 down-regulates hCD98 expression by directly targeting the 3'-UTR of hCD98 mRNA, further, to modulate integrin-laminin interactions, which in turn affects proliferation and differentiation of enterocytes across the crypt-villus axis [32]. miR-200 was also implied in the regulation of differentiation processes during epithelium development and epithelial-to-mesenchymal transition (EMT) [33]. But the differentiation process of epithelium development and the EMT, especially, during intestinal inflammation and intestinal tumorigenesis is complicated, in which other miRNAs may get involved are to be identified.

Ste20-like proline-/alanine rich kinase (SPAK), also called serine threonine kinase 39 (STK39), belongs to the germinal center kinase (GCK) IV subfamily, containing a series of proline and alanine repeats (PAPA box) upstream of N-terminal catalytic domain followed by a C-terminal regulatory region with a nuclear localization signal (NLS) and a consensus caspase cleavage motif in the middle [34]. SPAK functions as one MAP4K and regulates various cellular processes, including development, cell-cycle control,

cell growth, apoptosis, cell stress responses [35, 36], and regulation of chloride transport [37-39] as a sensitive cell volume sensor. We previously showed that SPAK expression was increased in intestinal mucosa from IBD patients including CD [40] and UC [41] and other inflammatory conditions [40-42]. Further studies demonstrated that SPAK regulates the integrity of intestinal epithelial cells like barrier function through modulating the expression and function of some junction proteins.

Using an online program (<http://www.microrna.org/microrna/getGeneForm.do>), we found that SPAK is one of the potential targets of hsa-miRNA-26b. In this study, we employed mouse model and intestinal epithelial cell line- the enterocyte-like Caco2-BBE to investigate how miRNA-26b regulates the differentiation of epithelium. We found that as the miRNA-26b expression decreases significantly along the crypt-villus axis, the expression of SPAK increases inversely. Further studies found that miRNA-26b can physically bind to SPAK mRNA and compromise its stability at the posttranscriptional level. Inhibiting expression of miRNA-26b or increasing expression of SPAK can increase the differentiation of the intestinal epithelial cells and the overall transepithelial resistance (TER). Consequently, we establish the concept that miRNA-26b regulates the differentiation and barrier function of Caco2-BBE cells by altering the expression of SPAK.

Materials and methods

Human material

The diagnosis of ulcerative colitis (UC) was based on the protocol described by our lab previously [43]. Clinical records for patients suffering from UC were obtained by medical record review approved by the Human Study Ethic Committees at the Affiliated Hospital of Hangzhou Normal University. The biopsy samples were collected with the approval from the Institutional Review Board of Hangzhou Normal University and the Human Study Ethic Committees at the Affiliated Hospital of Hangzhou Normal University. Mucosal biopsy specimens were collected from 6 UC active patients and 6 volunteers undergoing colonoscopy for colorectal cancer screening with written informed consent.

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Cell culture

The human intestinal cancer cell line Caco2-BBE, when grown as a confluent monolayer [44] or exposed to treatment of Calcium-activated chloride channel family member 1 (CLCA1) [45] and sodium butyrate (NaBT) [46, 47], differentiate to mimic phenotypically and functionally mature colonic epithelium, exhibiting featured enterocytes, for example, Caco2-BBE spontaneously differentiate into absorptive epithelial cells, therefore represent a good *in vitro* model to study human enterocytes [48, 49]. In this study, Caco2-BBE cells were grown in DMEM supplemented with 14 mM NaHCO₃, 10% fetal bovine serum, and 1.5 µg/ml plasmocin (Life technology, Carlsbad, CA, USA), at 37°C in a 5% CO₂ atmosphere and 90% humidity.

miRNAs, plasmid construction, transfection, and luciferase assay

Mature hsa-miRNA-26b and mature mmu-miRNA-26b are highly conserved in evolution with completely the same RNA sequence (UUCAAGUAAUUCAGGAUAGGUU). Here, miRNA-26b represents both hsa-miRNA-26b and mmu-miRNA-26b, miRNA-26b (Pre-miR Precursor AM17100), anti-miRNA-26b (anti-miR inhibitor AM17000), pre-miR negative vector (scrambled, AM17110), and anti-miRTM negative control (scrambled, AM17010) were obtained from Ambion (Life technology, Carlsbad, CA, USA). Caco2-BBE cells cultured on 24-well plastic plates, coverslips, or filter supports were transfected with 40 nM miRNA precursors or 40 nM miRNA anti-sense oligoes using Lipofectamine 2000 (Life technology, Carlsbad, CA, USA).

The SPAK/pcDNA3.1/V5 was constructed as we described previously [34]. Caco2-BBE cells were transfected with the SPAK/pcDNA3.1/V5 or the pcDNA3.1/V5 vector, SPAK siRNA and scramble siRNA (Life technology, Carlsbad, CA, USA) using Lipofectamine 2000 (Life technology, Carlsbad, CA, USA) and stably selected in culture medium supplemented with 1.2 mg/ml geneticin (Life technology, Carlsbad, CA, USA). SPAK mRNA 3'-UTR was cloned into the Spel/HindIII sites of the pMIR-REPORTTM Luciferase vector (Life technology, Carlsbad, CA, USA) or the XhoI/HindIII sites of the pEGFP-C1 vector (BD Biosciences Clontech). For luciferase assay, Caco2-BBE cells on 24-well plastic plates

were transfected with 1 µg of the SPAK3'-UTR-luciferase construct in the presence or absence of 20 nM the indicated miRNA precursors using Lipofectamine 2000 (Life technology, Carlsbad, CA, USA). Firefly luciferase activity was measured at 48-h post-transfection using the Dual-Luciferase Reporter Assay System (Promega, San Luis Obispo, CA, USA) and a Luminoskan Ascent luminometer (Thermo Electron Corp., Waltham, MA, USA). Values were normalized to lysate protein concentration. For GFP repression experiment, Caco2-BBE cells seeded on coverslips were transfected with 1 µg of the SPAK3'-UTR-GFP construct in the presence or absence of 40 nM miRNAs using Lipofectamine 2000. After 48 h of transfection, GFP expression was assessed by Western blot analysis and fluorescent microscopy using a Zeiss Axioskop2 plus microscope.

Isolation of epithelia from mouse intestinal crypts and villi

Isolation of epithelial cells from the intestinal crypt-villus axis was performed as previously described by Merlin's lab [32].

RNA extraction and real time PCR

Total RNAs were extracted using TRIzol method (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using the first-strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA). Real time RT-PCR was performed using an iCycler sequence detection system (Bio-Rad, Hercules, CA), with specific SPAK primers and internal control GAPDH primers as described previously [43]. Real time PCR data were presented using the $\delta\text{-}\delta$ cycle threshold ($\Delta\Delta C_t$) method [50].

Protein extraction and western blot analysis

Cells or tissue were re-suspended in radioimmune precipitation assay (RIPA) buffer (150 mM NaCl, 0.5% sodium deoxycholate, 50 mM Tris-HCl, pH 8, 0.1% SDS, 0.1% Nonidet P-40) supplemented with protease inhibitors (Roche Diagnostics, Indianapolis, IN), centrifuged, quantified, and subjected to western blot with relevant primary antibodies: anti-mouse SPAK (Cell Signaling Technology), anti-ALP (Santa Cruz Biotechnology), Lgr5 (Cell Signaling Technology), and monoclonal anti-GAPDH (Life technology, Carlsbad, CA, USA), followed by

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incubation appropriate horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology), and blots were detected using the Enhanced Chemiluminescence Detection kit (Amersham Biosciences).

Quantification of mature miRNAs

Total RNAs were extracted with mirVana miRNA Isolation Kit (Life technology, Carlsbad, CA, USA), and polyadenylated and reverse transcribed using the NCode™ miRNA first-strand cDNA synthesis kit (Life technology, Carlsbad, CA, USA). Levels of mature miRNAs were quantified by real time PCR using the universal reverse primer provided in the kit and the following forward primers: miRNA-26b: 5'-CCGG-GACCCAGTTCAAGTA-3' and 5'-CCGGTCCCCGT-GCCTTGTA-3'. 18 S was used as housekeeping genes.

Immunohistochemistry and immunocytochemistry

Immunostaining was performed according to the protocol with rabbit SPAK antibody (Cell signaling technology Inc, Danvers, MA) developed by our lab previously [43].

Caco2-BBE cells grown on slide were washed and fixed with 4% paraformaldehyde in PBS with calcium for 20 min. The cells were then permeabilized with 0.1% Triton/PBS for 30 min at room temperature. Cells were incubated with rabbit SPAK antibody; monolayers were then stained with Alexa Fluor 488 goat-anti rabbit IgG (Molecular Probes, Carlsbad, CA, USA). Samples were mounted in p-phenylenediamine/glycerol (1:1) and analyzed by confocal microscopy (Zeiss dual-laser confocal microscope).

Fluorescence in situ hybridization (FISH) and immunohistochemistry on same slide

FISH was performed with the LNA microRNA FISH optimization kit from Exiqon (miRCURY LNA detection; Exiqon, Vedbaek, Denmark) based on instruction. Briefly, the mucosa was fixed with 4% fresh paraformaldehyde overnight and embedded in paraffin for sections. The slides were deparaffinized and then incubated with proteinase-K. After washes with phosphate-buffered saline (PBS), the slides were dehydrated and hybridized with FITC 5' labeled

locked-nuclei-acid incorporated (LNA) miRNA miRNA-26b probe. This hybridization will be followed by thoroughly washes in saline sodium citrate (SSC) respectively. After the endogenous peroxidase activity was blocked, we incubated slides with diluted mouse anti-FITC antibody (Cell Signaling Technology, Danvers, MA, USA) for overnight at 4°C. Immunofluorescence staining with Rhodamine phalloidin (Invitrogen) to visualize actin was performed on the same slides.

Nuclear run-on assay

Nuclear run-on assay with nuclei isolated from Caco2-BBE cells was performed following the protocol described previously [41]. cDNA was synthesized with the SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA) and amplified with Platinum TaqDNA polymerase (Invitrogen, Carlsbad, CA, USA), GAPDH acts as internal control, here, specific primers (SPAK HQF, SPAK HQR, GAPDH For and GAPDH Rev) are as described previously [41]. The products were visualized with ethidium bromide agarose gel.

SPAK mRNA stability assay

mRNA decay assay in Caco2-BBE cells transfected with 60 nM miRNA-26b were performed as described previously [41, 51]. Here, all the primers used are described previously [41].

Measurement of cell resistance

Resistance of Caco2-BBE cells that reflects cell differentiation and barrier function was monitored as described previously [32].

Statistical analysis

Values in the current study were expressed as means \pm SE. Unpaired two-tailed Student's *t* test by InStat v3.06 (GraphPad) software was employed for the statistical analysis. *P* < 0.05 was considered statistically significant.

Results

SPAK expression is increased during epithelial cell differentiation

Previous study has demonstrated that the expression of SPAK is mainly located at the

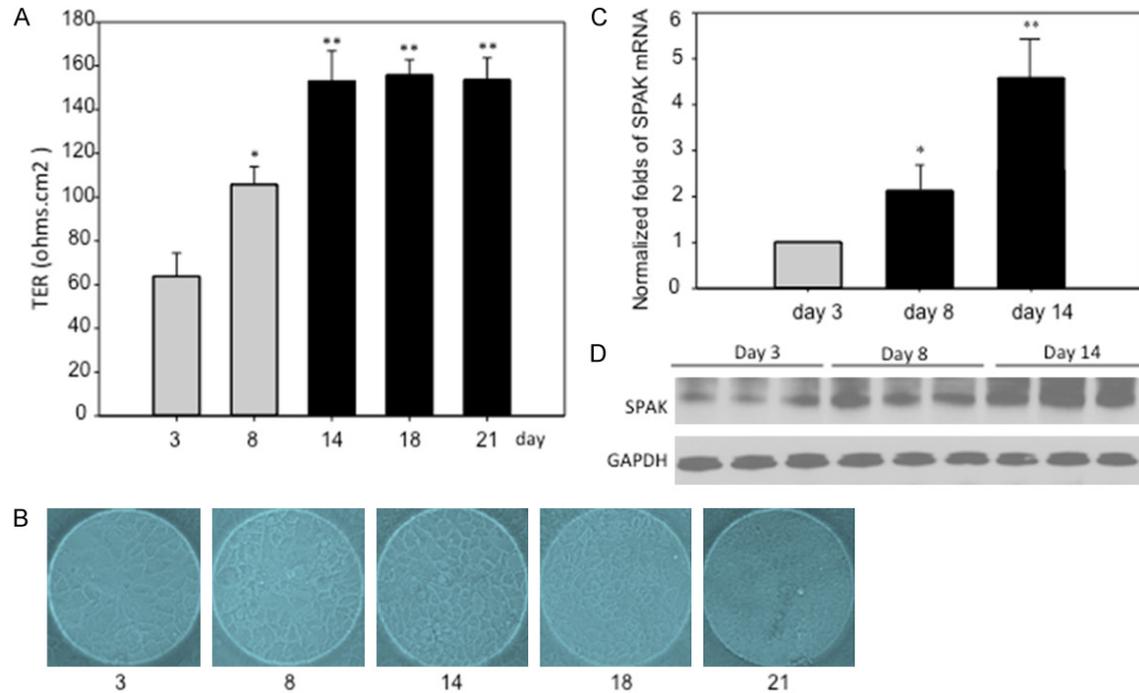


Figure 1. Expression of SPAK is decreased during differentiation of intestinal epithelial cells. (A) Resistance of Caco2-BBE cells (2×10^4 cells/400 μ l/electrode) was measured at 500 Hz, 1 V in real time using ECIS device. (B) Images were taken at different time points post-seeding (3, 8, 14, 18 and 21 days) using a Nikon Eclipse TS100 microscope at $\times 20$ magnification. Each image is representative of quadruplicate electrodes. SPAK expression in Caco2-BBE cells cultured on plastic plates for 3, 8, and 14 days were analyzed by real time PCR (C) and Western blot (D). Values represent means \pm S.E. of three determinations. *, $P < 0.05$; **, $P < 0.01$.

cytosolic pool of IEC [34], however, its expression during the period of IEC differentiation is not yet investigated. We first examined the expression of SPAK in Caco2-BBE cells at different time post-seeding. Caco2-BBE cell line evolved from the parental cell line Caco2, harboring microvilli just like human enterocytes [48] and exhibiting same characteristics as absorptive enterocytes such as differentiation [48, 49]. The differentiation of Caco2-BBE cells was characterized by TER with ECIS technique, as reported previously [32, 52]. Our data showed that Caco2-BBE monolayer underwent gradually and increasingly differentiated over-time, reaching a plateau after 14 days of culture (Figure 1A and 1B). Importantly, SPAK expression at both mRNA and protein levels was enhanced, parallel with increasing levels of cell differentiation; SPAK expression levels were much higher in well differentiated Caco2-BBE cells (day 8 post-seeding) than in non-differentiated cells (day 2 post-seeding) (Figure 1C and 1D).

Expression of SPAK is reversely correlated to the differentiation of epithelium

To confirm our data *in vitro*; we characterize *in vivo* the expression of SPAK along the intestinal crypt-villus axis in mouse. Villi and crypts were isolated from mouse jejunum as demonstrated in Figure 2A that the collected villi and crypts were intact and pure. It is known that crypt is characterized by proliferation and villus featured by differentiation [53], evidenced here by enhanced expression of intestinal alkaline phosphatase (ALP) [54] in the villus region and leucine-rich-repeat-containing G-protein-coupled receptor 5 (Lgr5) [55] in crypt region (Figure 2B and 2C), which function as common marker for differentiation and proliferation. Real time PCR, immunofluorescence and Western blot analyses showed much higher levels of mouse SPAK expression in well differentiated villus cells compared with undifferentiated crypt cells (Figure 2D-F). Together, these data demonstrate that SPAK expression is increased with increasing levels of IEC differentiation.

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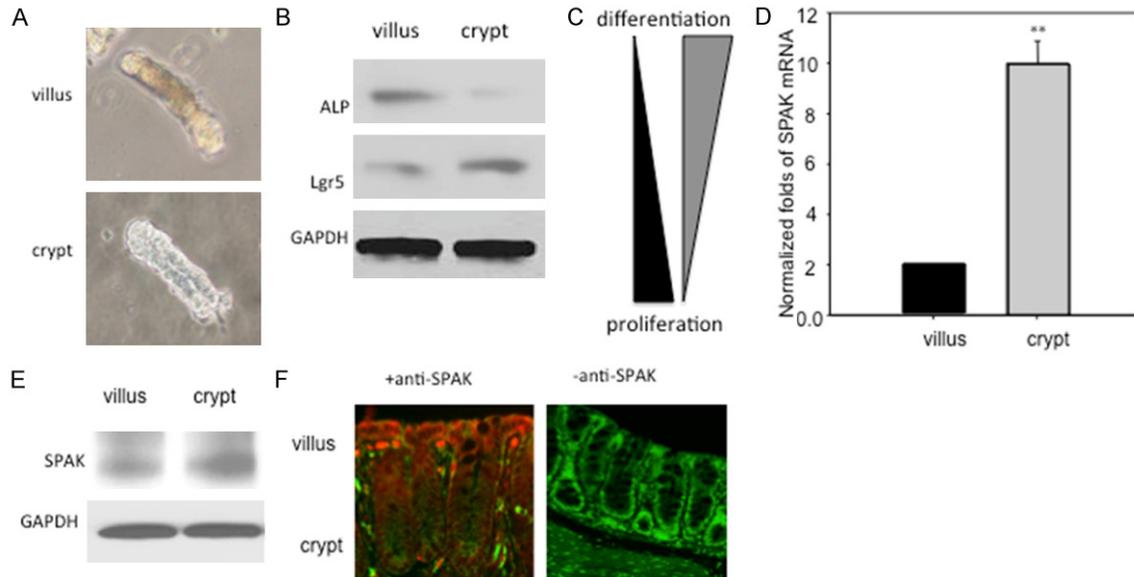


Figure 2. Mouse SPAK expression is increased toward the crypt-villus axis of mouse intestine. (A) Villi and crypts were isolated from jejunum of 6-8-week-old C57BL/6 male mice. Pictures of the extracted villus and crypt fractions were taken using a Nikon Eclipse TS100 microscope at $\times 20$ magnifications, respectively. (B) Western blots with differentiation marker ALP and proliferation marker Lgr5 demonstrate the integrity and characteristics of villi and crypts. (C) Schematic graph shows the pattern of differentiation and proliferation along the villus-crypt axle. SPAK expression in the villus and crypt fractions was assessed by real time PCR (D) Western blot (E) and immunofluorescence (F). Values represent means \pm S.E. of three determinations. *, $P < 0.05$; **, $P < 0.01$.

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Evidence suggested an important role for miRNA in cell differentiation; we hence hypothesized whether miRNAs play important roles in the process of epithelial differentiation and the accompanying alteration of SPAK expression. To test this possibility, we first used the miR-Base website to screen the 3'-UTR sequence of SPAK mRNA against the public database for possible complementation of miRNAs. Both the human and mouse original hsa-miRNA-26b and mmu-miRNA-26b are identified as potential miRNA candidates for SPAK gene, respectively (Figure 3A and 3B). Given the fact that human SPAK has very high similarity in mRNA sequence with mouse SPAK [56], mmu-miRNA-26b have the exact nucleotide sequence with hsa-miRNA-26b. To be simple, we use miRNA-26b to represent both hsa-miRNA-26b and mmu-miRNA-26b. After the synthesis of first-strand cDNA from tailed miRNAs, the levels of mature form of miRNA-26b in Caco2-BBE cells and in mouse intestinal crypt-villus axis were quantified by real time PCR analysis. We found that the levels of mature miRNA-26b were inversely correlated

with SPAK expression levels during Caco2-BBE cell differentiation: miRNA-26b levels were higher in non-differentiated cells than in well-differentiated cells (Figure 3C). In contrast, expression of hsa-mir-626 [32], used as a control miRNA, was not significantly different in undifferentiated and well-differentiated Caco2-BBE cells (Figure 3C). Consistent with this, the levels of mature mouse miRNA-26b were significantly decreased in the villus cells, where SPAK was expressed at a high level, relative to what observed in crypt cells, where SPAK was expressed at a low level (Figure 3D). FISH assay with FITC-labeled LNA confirmed this result that miRNA-26b is predominantly located at the crypt region of intestinal monolayers, in comparison with villus region (Figure 3E). Together, the inverse correlation between miRNA-26b and SPAK expression levels in IECs suggests a role for miRNA-26b in the regulation of SPAK expression during IEC differentiation.

miRNA-26b inhibits the expression of SPAK

To directly examine the regulation of SPAK by potential candidate miRNA-26b, Caco2-BBE cells were transiently transfected with vehicle

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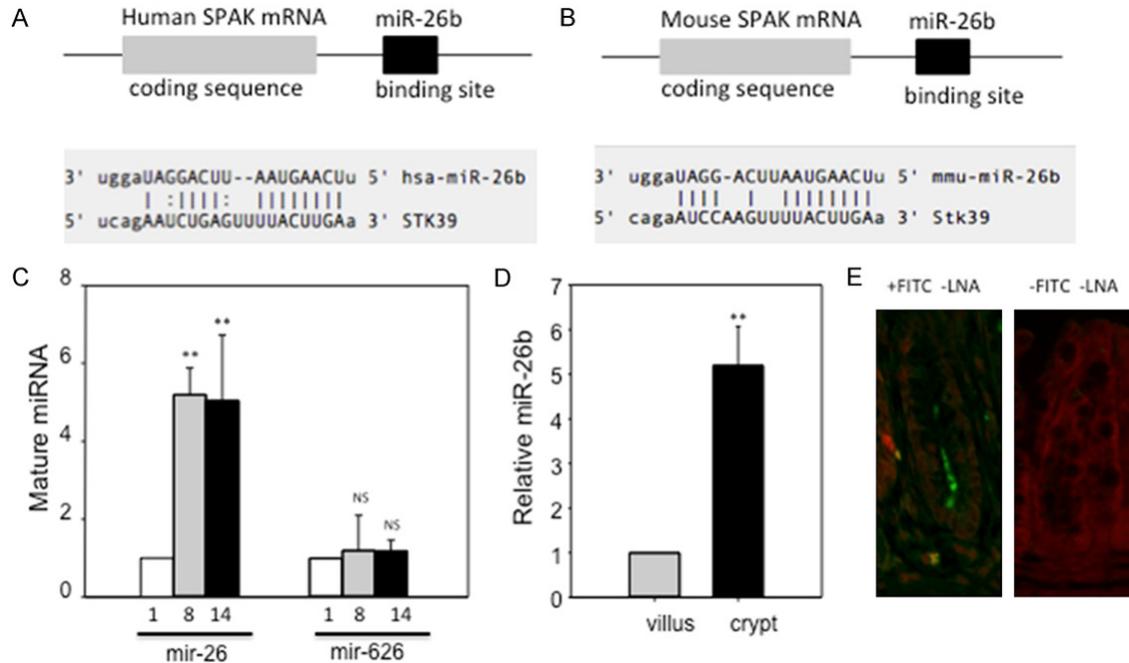


Figure 3. Expression levels of miRNA-26b are decreased during intestinal epithelial cell differentiation. (A) Schematic representation of the human SPAK mRNA with the 3'-UTR hsa-miRNA-26b binding site (upper panel) and sequence alignment of SPAK 3'-UTR miRNA-26b target site (bottom panel) predicted by miRBase. (B) Schematic representation of the mouse SPAK mRNA with the 3'-UTR miRNA-26b binding site (upper panel) and sequence alignment of mouse SPAK 3'-UTR mmu-miRNA-26b target site predicted by miRBase. (C) Levels of mature forms of miRNA-26b or hsa-miR-626, used as an internal control miRNA, in Caco2-BBE cells cultured on plastic plates for 3, 8, and 14 days were analyzed by real time PCR, levels of mature form of miRNA-26b in jejunum of 6-8-week-old c57BL/6 male mice were quantified by real time PCR (D) and FISH assay (E). Values represent means \pm S.E. of three determinations. *, $P < 0.05$; **, $P < 0.01$; NS, not statistically significant.

(Lipofectamine 2000, control), miRNA-26b precursor (miRNA-26b), or a negative control miRNA precursor (miR-control) containing a non-targeting sequence for the indicated time, and SPAK expression levels were assessed by real time PCR and Western blot analyses. We found that miRNA-26b significantly reduced SPAK mRNA and protein levels in Caco2-BBE cells at 48 h post-transfection (**Figure 4A** and **4B**). In contrast, transfection of cells with miR-control or vehicle did not affect SPAK mRNA and protein expression (**Figure 4A** and **4B**).

miRNA-26b antisense increases SPAK expression in Caco2-BBE cells

To confirm the negative regulation of SPAK expression by miRNA-26b, SPAK3'-UTR-luc, miRNA-26b antisense or scrambled miR-control were co-transfected into Caco2-BBE cells. Our data demonstrated that miRNA-26b antisense increased significantly the luciferase activity; however, no significant effect was no-

ticed in scrambled miR-control transfected cells (**Figure 4C**). This data suggested that miRNA-26b antisense competitively inhibits the interactions of miRNA-26b with SPAK3'-UTR-luc. Further, parallel co-transfection with SPAK3'-UTR-luc and miRNA-26b displayed significant lower luciferase activity, implying the functional specificity of miRNA and its antisense. The negative regulation of SPAK expression was further examined by Western blot. Consistent with the luciferase assay results, transfection of cells with miRNA-26b antisense, but not miR-control antisense, resulted in an increase in SPAK expression (**Figure 4D**). Collectively, these results strongly support the finding that miRNA-26b post-transcriptionally represses SPAK expression in Caco2-BBE cells.

Regulation of SPAK by miRNA-26b is primarily at the post transcriptional level

To determine whether the reduction of SPAK gene expression by miRNA-26b is mediated

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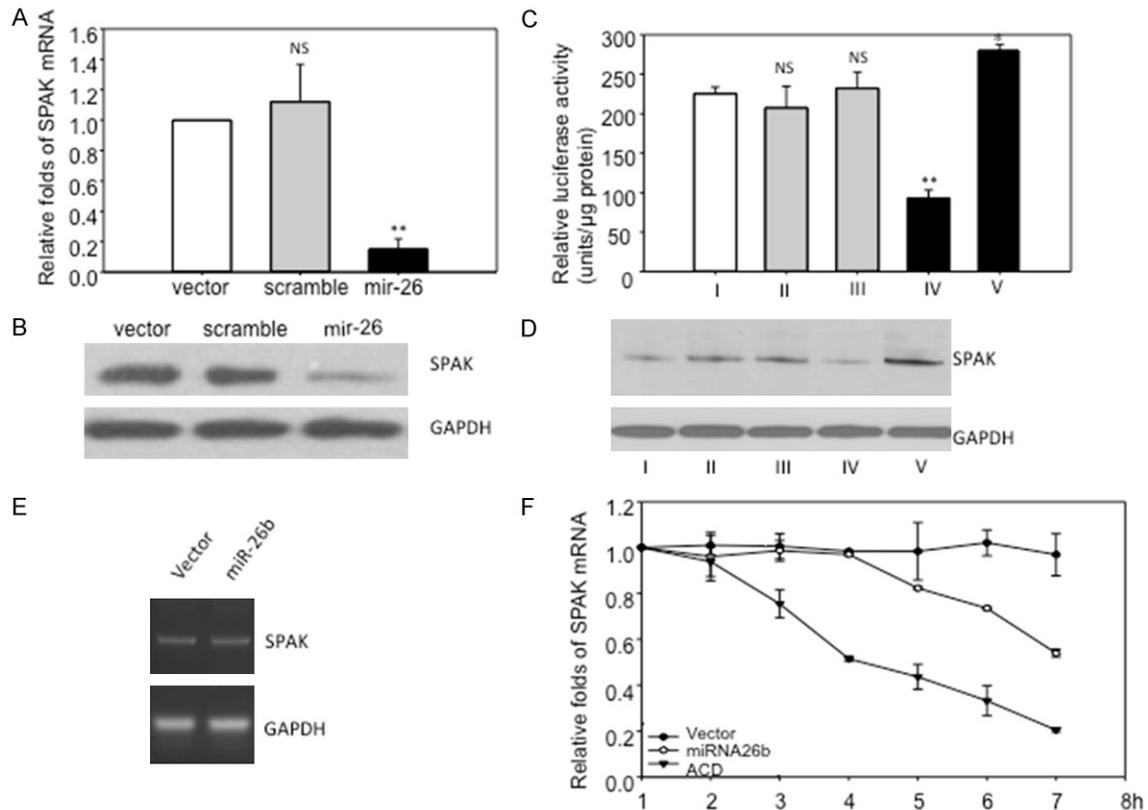


Figure 4. miRNA-26b inhibits SPAK expression. Caco2-BBE cells were transfected with 50 nM miRNA-26b precursor or the negative control miRNA precursor (miR-control), or vehicle. Expression of SPAK was assessed by quantitative PCR (A) and Western blot (B). (C) MiRNA-26b antisense increases SPAK expression in Caco2-BBE cells. Caco2-BBE cells were transfected with the 3'-UTR-luciferase reporter construct in the absence (control) or presence of 40 nM miRNA-26b antisense or miR-control antisense, or 40 nM miRNA-26b precursors. Luciferase activity was measured at 2 days post-transfection and normalized to lysate protein concentration. Here, I, II, III, IV and V represents separately vector; crumble miRNA; crumble antisense miRNA; miRNA-26b and miRNA-26b antisense. (D) Caco2-BBE cells were transfected with vehicle (Lipofectamine 2000; control), 40 nM miRNA-26b antisense or miR-control antisense, or 40 nM miRNA-26b precursor or miRNA-26b antisense for 2 days. SPAK expression was assessed by Western blot. Here, I, II, III, IV and V represents separately vector; crumble miRNA; crumble antisense miRNA; miRNA-26b and miRNA-26b antisense. (E) Nuclear run-on assay were performed in miR-26 transfected Caco2-BBE cells and visualized by DNA electrophoresis. (F) SPAK mRNA decay assay were performed in miR-26 transfected Caco2-BBE cells, actinomycin (D) functions here as positive control. For real time PCR and mRNA decay assay, values represent means \pm S.E. of three determinations. *, $P < 0.05$; **, $P < 0.01$; NS, not statistically significant versus control.

through transcriptional or posttranscriptional modification, nuclear run-on assays (Figure 4E) were performed on Caco2-BBE cells transfected with miRNA-26b. Data demonstrated that overexpression of miRNA-26b did not change significantly the SPAK mRNA transcribed from nuclei in vitro in comparison with vector-transfect counterpart. This experiment was repeated three times with the similar result. Changes in steady-state mRNA levels may be attributable to changes in the degradation rate of a transcript. Therefore, it was very crucial to further study the relative contribution of miRNA-26b in the regulation of SPAK mRNA at posttranscrip-

tional level. To examine the stability of SPAK mRNA, 5 ng/ml of AcD was employed to treat Caco2-BBE cells to block the synthesis of overall mRNAs; the level of SPAK mRNA was quantified at different time points by real time PCR. 18S rRNA was used as an internal control to normalized SPAK mRNA levels. We found that the half-life of SPAK mRNA is about 240 minutes ($t_{1/2} \leq 240$ minutes) in AcD-treated cells, however, at around 240 minutes, production of SPAK mRNA in miRNA-26b transfected cells was almost the same as that in vector-transfected cells, indicating that miRNA-26b does not change the transcription rate of SPAK

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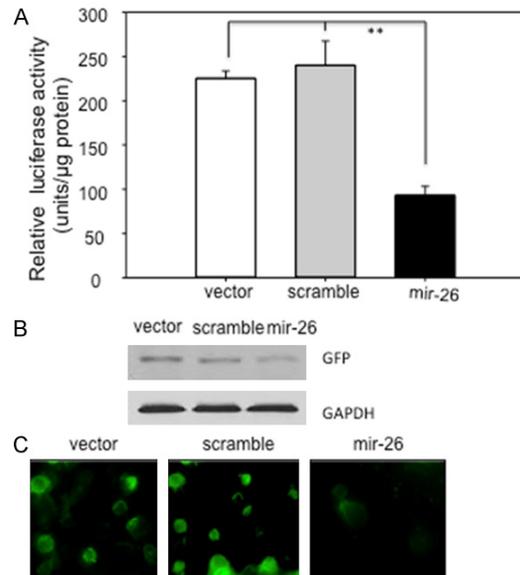


Figure 5. miRNA-26b directly targets the SPAK mRNA 3'-UTR. The full-length SPAK mRNA 3'-UTR was cloned downstream a luciferase reporter gene (SPAK 3'-UTR-luc) or a GFP-coding sequence (SPAK-3'-UTR-GFP). (A) Caco2-BBE cells were transfected with the SPAK 3'-UTR-luc construct in the presence or absence (control) of 40 nM miR-miR-26 precursor or scrambled miR. Luciferase activity was measured at 2 days post-transfection and normalized to lysate protein concentration. (B, C) Caco2-BBE cells were transfected with the SPAK 3'-UTR-GFP construct in the presence or absence of 40 nM miRNA-26b precursor or scrambled miR 48 h. GFP expression was assessed by Western blot. Pictures of cells plated on coverslips were taken using a Zeiss Axioskop2 plus microscope. (C) Values represent means \pm S.E. of three determinations. *, $P < 0.05$; **, $P < 0.01$; NS, not statistically significant versus control.

mRNA. After 4 hours (240 minutes), the amount of SPAK mRNA start reducing, indicating the decay of mRNA, reaching up to 40% (1-60%), in comparison with vector transfected Caco2-BBE cells. Together, these findings indicate that the observed changes in SPAK protein level are attributable to increased decay of SPAK mRNA transcripts, not because of changes in mRNA transcription rate.

3'-UTR of SPAK mRNA is the direct target of miRNA-26b

To determine if the 3'-UTR of SPAK mRNA is the direct target of miRNA-26b, SPAK3'-UTR was cloned into pMIR-REPORT™ Luciferase vector (SPAK3'-UTR-luc), together with miRNA-26b, and subjected to transient transfect into Caco2-

BBE cells, followed by the measurement of luciferase activity. As demonstrated in **Figure 5A**, up to 65% of luciferase activity was inhibited by miRNA-26b at 2-day post-transfection, suggesting that miRNA-26b can bind directly to SPAK3'-UTR. In contrast, miR-control had no effect on luciferase activity in cells transfected with the SPAK3'-UTR-luc (**Figure 5A**).

To confirm these results, the SPAK3'-UTR was cloned downstream the GFP-coding sequence, and the effect of miRNA-26b on GFP expression in cells transfected with this construct was examined. We found consistently that miRNA-26b decreased the expression of the GFP by Western blots (**Figure 5B**). Furthermore, immunostaining demonstrated a dramatic reduction of fluorescent intensity in Caco2-BBE cells transfected with miRNA-26b, in comparison with control cells or cells transfected with the miR-control (**Figure 5C**). Together, these data demonstrated that miRNA-26b down-regulates SPAK expression by targeting the 3'-UTR of SPAK mRNA.

miRNA-26b regulates SPAK expression during intestinal inflammation

In an effort to investigate the possible involvement of miRNA-26b in regulation of SPAK expression during intestinal inflammation, we first assessed the expression of miRNA-26b under intestinal inflammatory conditions by real time PCR. Significant decreases of miR-26 in inflamed colon tissues in ulcerative colitis patients ([UC group: 0.69 ± 0.19 -fold, $n = 6$] versus [normal group: 1.00 , $n = 6$]) were demonstrated, indicating miRNA-26b may be involved in the pathogenesis of UC (**Figure 6A**). Pro-inflammatory cytokine IFN- γ is very important in the pathogenesis of intestinal inflammation; IFN- γ itself only is enough to stimulate inflammation in different cell lines. Here we demonstrated by real time PCR that IFN- γ treatment increased SPAK expression at mRNA level in Caco2-BBE cells (**Figure 6B**), however, IFN- γ inhibited the production of miRNA-26b at same time (**Figure 6C**). To test whether miRNA-26b was directly involved in the IFN- γ -mediated SPAK up-regulation, Caco2-BBE cells were transfected with miRNA-26b and subsequently stimulated with IFN- γ . Real time PCR analysis revealed that miRNA-26b inhibits significantly the expression of SPAK mRNA, but IFN- γ

miRNA-26b regulates epithelial differentiation

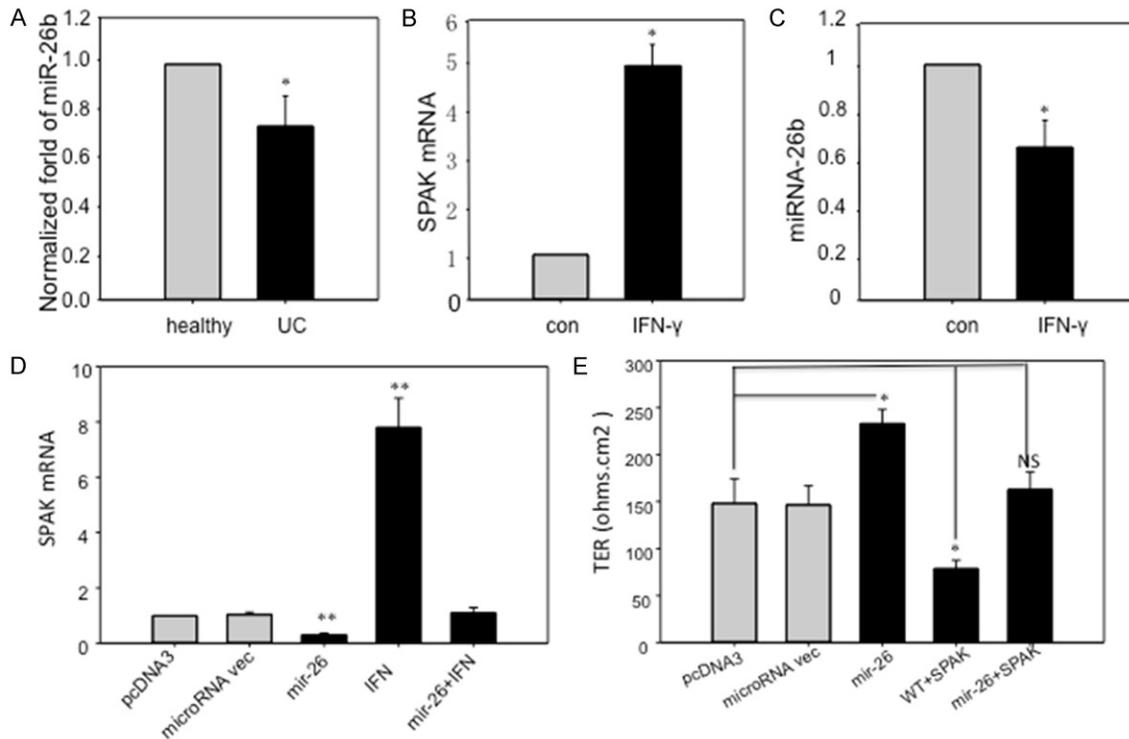


Figure 6. MiRNA-26b is involved in ulcerative colitis (UC). (A) Real time PCR demonstrated that there are lower miRNA-26b in UC patients (n=6) than that in healthy controls (n=6). IFN- γ (10 ng/ml) treatment of Caco2-BBE cells demonstrated significantly increased SPAK expression and decreased miRNA-26b levels (C). Cells were cultured on 12-well plastic plates for 5 days, serum starved for 24 h and stimulated or not with 10 ng/ml of IFN- γ in serum-free Dulbecco's modified Eagle's medium for 24 h. SPAK expression in untreated (control) and IFN- γ -treated cells were analyzed by real time PCR (B). Levels of mature miRNA-26b were quantified by real time PCR (C). (D) miRNA-26b and proinflammatory cytokine IFN- γ work inversely on the production of SPAK. miRNA-26b transfection can block the production of SPAK by real time PCR, however, IFN- γ treatment increased significantly the transcripts level of SPAK; importantly, real time PCR showed that transfections of miRNA-26b together with IFN- γ in Caco-BBE cells can restore the enhanced production of SPAK to normal. (E) miRNA-26b and SPAK work inversely on the transepithelial resistance (TER) in Caco2-BBE cells. Transfection of miRNA-26b increased TER significantly in Caco2-BBE cells, however, overexpression of SPAK compromised TER; co-transfection of miRNA-26b and SPAK restored the TER to its normal level. Values represent means \pm S.E. of three determinations. *, P < 0.05; **, P < 0.01; NS, not statistically significant.

increased SPAK mRNA levels by ~8-fold compared to control, importantly, this effect was reversed when cells were pre-transfected with miRNA-26b (**Figure 6D**). These results indicated that IFN- γ can stimulate the over-expression of SPAK; however, the enhancement is compromised by miRNA-26b. Further, miRNA-26b can increase the TER in Caco2-BBE cells, which can restore the compromised TER caused by over-expression of SPAK (**Figure 6E**).

Discussion

In the current study, we established for the first time the concept that miRNA-26b regulates the differentiation of intestinal epithelial cells by

modulating the expression of SPAK in *in vivo* murine model and *in vitro* cell line model, further, affects TER during the migration of enterocytes across the crypt-villus axis, which together shed some lights on the mechanistic insight of the rapid turn over of intestinal epithelium.

It is known that proliferative cells are located at the crypts, whereas differentiated cells migrate and reach to the villi [57], here we demonstrated that very low levels of miRNA-26b is expressed at well-differentiated IEC villus cells, which may reflectively increase and/or dis-inhibit SPAK expression. Accordingly, higher level of SPAK expression was observed in well-differentiated intestinal epithelial Caco2-BBE cells,

in comparison with un-differentiated cells, as well as in mouse intestinal villus cells relative to crypt cells, proportional to IEC differentiation, implying its distinct roles in differentiated and proliferative cells, which is consistent with our previous data that SPAK is not involved in IEC proliferation [42]. We showed that miRNA-26b directly binds to 3'UTR region of SPAK gene, increasing the decay process of SPAK mRNA degradation, leading to the decreased expression of SPAK, which is consistent with the concept that posttranscriptional mechanisms play predominant roles in regulating gene expression by miRNA [58]. We also noticed that the increased TER might accredit to the accelerated differentiation driven by the low level of SPAK expression, therefore, the regulation of SPAK expression by miRNA-26b may have an important role in maintaining cellular homeostasis along the crypt-villus axis. Besides the important function in maintaining intracellular and extracellular ion homeostasis by phosphorylating target $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ co-transporter 1 (NKCC1) [59, 60], SPAK can also regulate the intestinal barrier function, decreased expression of SPAK contributes significantly to enhanced intestinal barrier, thus SPAK knockout mice were more tolerant to experimental colitis induced by dextran sodium sulphate (DSS) with decreased intestinal microorganism translocation into the mucosa and inhibition of the production of inflammatory mediators [61]. Overexpression of SPAK aggravates the DSS-induced colitis in mice by compromising the intestinal barrier function [42].

miRNAs can regulate the expression of up to 30 percent of the protein-coding genes in the human genome, highlighting their importance as regulators of gene expression, which further implies their intensive involvement in variety of important biological processes including development, differentiation, autophagocytosis proliferation, and apoptosis [27, 62, 63]. Besides what demonstrated in miRNA-26b in our study, many other miRNAs play important roles in differentiation of different cells [64]. It has been shown that miR-194 is up-regulated during IEC differentiation [65], miR-273, miR-9 and miR-124a are implicated in the neuronal differentiation from ESCs of *Caenorhabditis elegans* [66] and in homo sapiens [67, 68], miR-181 is involved in human hematopoietic cell differentiation [69], miR-1 and miR-133 in skeletal

muscle differentiation [70], and miR-143 is thought to play a role in adipocyte differentiation [71]. Since many inflammations are T lymphocytes-mediated disorder, the role of miRNAs in the differentiation of T cells into distinct effector T cell subsets has been recently intensively investigated; for example, miR-326 regulates differentiation of T_H17 cells both *in vitro* and *in vivo* [72]. MiR-155 is implicated in regulatory T (T_{reg}) cell formation and function, since forkhead box P3 (FOXP3), a transcription factor that is required for the development and function of T_{reg} cells, may directly regulate the expression of this miRNA [73], thus is essential in the regulation of regulatory T cells. Besides, recent studies have explored the role of miR-150, a miRNA specifically expressed by mature lymphocytes, in B cell differentiation [74]. Thus, by the regulation of differentiation, miRNA is critical in the determination of various diseases, such as miRNAs and viral diseases [75], Parkinson disease [76], Alzheimer's disease [77], diabetes [78], liver diseases [79], heart disease [80], kidney diseases [81], neurodegenerative disorders [82], human diseases [83], generally human cancers and inflammation [84-86].

Here, we demonstrated that miRNA-26b is involved in the regulation of SPAK during intestinal inflammation. Exposure of human intestinal epithelial Caco2-BBE cells to pro-inflammatory cytokine IFN- γ can increase SPAK expression and decrease mature miRNA-26b levels. Furthermore, miRNA-26b is directly involved in the IFN- γ -mediated SPAK up-regulation since transfection of cells with miRNA-26b suppresses the IFN- γ -induced SPAK expression. Consistent with the *in vitro* findings, the levels of mature miRNA-26b were markedly decreased in inflamed ulcerative colitis colonic tissues, whereas SPAK expression was up regulated in inflamed ulcerative colitis colonic tissues [41] and in pro-inflammatory cytokines-treated IECs [42], in experimental colitis in mice [42]. To our knowledge, our study is the first experimental supported case showing the involvement of miRNAs in the regulation of SPAK at normal condition and during intestinal inflammation. In a pathological context, such as in inflammatory bowel disease, we propose that an abnormal expression of miRNA-26b could affect SPAK expression and subsequently IEC differentiation along the crypt-villus axis, which can alter

the crypt-villus architecture and epithelial barrier function. In consistent with data in intestinal disorder, it was reported that miRNA-26b was significantly reduced in human hepatocellular carcinoma (HCC) tissues, compared with surrounding non-tumor tissues, and gene network analysis revealed that miRNA-26b expression was inversely correlated with the activation of NF- κ B and IL-6 signaling pathways [87]. Although a causal relationship between miRNA-26b and hepatocarcinogenesis could not be evaluated in this study, Kota et al [88] showed that low miRNA-26b expression played a causal role in hepatocarcinogenesis using a myc-induced mouse HCC model, and induction of miRNA-26b by gene therapy suppressed HCC development. Although the role of miRNAs in immune system development and response has recently become evident, the mechanisms underlying involvement of miRNAs in immunity, such as their role in regulation of cytokine responses, are poorly understood [89]. Therefore, it would be of interest to further study the mechanisms by which the pro-inflammatory cytokines regulate miRNA-26b expression and the role of miRNAs in the regulation of epithelial inflammatory responses in general.

In conclusion, the expressions of miRNA-26b and SPAK are in a converse manner at intestinal crypt-villus axle, along the transition from proliferation to differentiation; miRNA-26b down-regulates SPAK expression by directly targeting the 3'-UTR of SPAK mRNA; miRNA-26b acts via regulating SPAK expression to modulate intestinal epithelium barrier function; and miRNA-26b regulates SPAK expression during intestinal inflammation. Together, our study reveals a novel mechanism underlying the regulation of SPAK during patho-physiological states, and raises miRNAs as a promising target for therapeutic modulations of the multiple functions of SPAK in intestinal inflammatory disorders.

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Disclosure of conflict of interest

None.

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