Milk Fat Globule–Epidermal Growth Factor 8 Is Decreased in Intestinal Epithelium of Ulcerative Colitis Patients and Thereby Causes Increased Apoptosis and Impaired Wound Healing

Qiu-jie Zhao,[1,2] Yan-bo Yu,[1,2] Xiu-li Zuo,[1] Yan-yan Dong,[1] and Yan-qing Li[1]

[1]Department of Gastroenterology, Qilu Hospital, Shandong University, Jinan, Shandong, China; and [2]The Key Laboratory of Cardiovascular Remodeling and Function Research, Chinese Ministry of Education and Chinese Ministry of Health, Qilu Hospital, Shandong University, Jinan, Shandong, China

Milk fat globule–epidermal growth factor 8 (MFG-E8) plays an important role in maintaining intestinal barrier homeostasis and accelerating intestinal restitution. However, studies of MFG-E8 expression in humans with ulcerative colitis are lacking. We examined MFG-E8 expression in colonic mucosal biopsies from ulcerative colitis patients and healthy controls (n = 26 each) by real-time quantitative polymerase chain reaction (PCR), Western blot analysis and immunohistochemistry. MFG-E8 mRNA and protein expression was lower in ulcerative colitis patients than in controls. MFG-E8 expression was inversely correlated with mucosal inflammatory activity and clinical disease activity in patients. MFG-E8 was present in human intestinal epithelial cells both *in vivo* and *in vitro*. Apoptosis induction was also detected in the intestinal epithelium of ulcerative colitis patients by terminal-deoxynucleotidyl transferase mediated nick-end labeling assay. We used lentiviral vectors encoding human MFG-E8 targeting short hairpin RNA to obtain MFG-E8 knockdown intestinal epithelia cell clones. MFG-E8 knockdown could promote apoptosis in intestinal epithelial cell lines, accompanied by a decrease in level of the antiapoptotic protein B-cell lymphoma 2 (BCL-2) and induction of the proapoptotic protein BCL2-associated protein X (BAX). The addition of recombinant human MFG-E8 led to decreased BAX and cleaved caspase-3 levels and induction of BCL-2 level in intestinal epithelia cells. MFG-E8 knockdown also attenuated wound healing on scratch assay of intestinal epithelial cells. The mRNA level of intestinal trefoil factor 3, a pivotal factor in intestinal epithelial cell migration and restitution, was downregulated with MFG-E8 knockdown. In conclusion, we demonstrated that decreased colonic MFG-E8 expression in patients with ulcerative colitis may be associated with mucosal inflammatory activity and clinical disease activity through basal cell apoptosis and preventing tissue healing in the pathogenesis of ulcerative colitis.

INTRODUCTION

Ulcerative colitis (UC) is a chronic and relapsing inflammatory disorder involving the mucosal layer of the colon and rectum (1). Studies suggest that abnormal innate and adaptive immunities, genetic susceptibility and environmental factors collaboratively lead to the disease (1–4). UC is characterized by elevated production of proinflammatory mediators, repeated intestinal injury and cell restitution and increased apoptosis of intestinal epithelial cells (IECs), which leads to impaired mucosal barrier function (5–8). However, the underlying molecular basis has not been completely elucidated.

Milk fat globule–epidermal growth factor 8 (MFG-E8), also named lactadherin, BA46 or SED1, is a secreted glycoprotein present in several cell types, including macrophages, mammary epithelial cells and epidermal keratinocytes (9–11). It participates in engulfing apoptotic cells. *MFG-E8*–null mice show severe autoimmune disease–like human systemic lupus erythematosis because of accumulation of uncleared apoptotic cells (9,12–14). MFG-E8 also participates in cell events such as the promotion of mammary gland branching and facilitation of sperm–egg binding (10,15).

Several studies of animal models demonstrated that MFG-E8 has an important role in intestinal homeostasis. In mice with sepsis, MFG-E8 promoted enterocyte migration along the crypt–villus axis and accelerated intestinal mucosal healing (16). Also, MFG-E8 expression was impaired in inflamed mouse colons during the acute phase of dextran sodium sulfate (DSS)-induced colitis, but pretreatment with recombi-

***Address correspondence to** Yan-qing Li, Department of Gastroenterology, Qilu Hospital, Shandong University, Jinan, Shandong, China. Phone: +86-531-82169508; Fax: +86-531-82169236; E-mail: mx8902@163.com.*

nant MFG-E8 had a protective role against inflammation (17). Moreover, a recent study reported that recombinant MFG-E8 administered during the recovery phase of DSS-induced colitis in mouse could attenuate inflammation and enhance epithelial repair, which suggests a therapeutic role for MFG-E8 in DSS-induced colitis (18). However, we lack reports of MFG-E8 activity in humans with UC. MFG-E8 may be decreased in such patients and involved in the disease process. We aimed to delineate the expression of intestinal MFG-E8 in UC patients and evaluated the consequences of MFG-E8 knockdown by lentiviral vector *in vitro* in the context of UC.

MATERIALS AND METHODS

Patients and Controls

We enrolled 26 patients with UC who underwent colonoscopy for disease surveillance. The diagnosis was based on well-established clinical, endoscopy and histopathological criteria. Control subjects were 26 healthy volunteers matched by age and sex who underwent colonoscopy for physical examination, surveillance of polyp recurrence or functional abdominal pain. Our study was approved by the Clinical Ethical Committee of Qilu Hospital of Shandong University. All subjects gave written informed consent before biopsy sampling. Table 1 includes demographic features, colitis extent and drug therapy.

During colonoscopies, intestinal mucosal biopsies were obtained from the macroscopically inflamed area for UC patients or from normal areas for healthy controls in the sigmoid colon or rectum. For each subject, we obtained two adjacent biopsies: one for histology and the other for RNA or protein analysis. For histology, biopsies were immediately fixed in 10% neutral buffered formaldehyde, and those for RNA or protein analysis were snap frozen in liquid nitrogen and stored at –80°C. Biopsies from subjects underwent routine histology, and all

Table 1. Demographic features of participants.

	UC patients	Healthy controls
n	26	26
Sex ratio (M/F)	15/11	14/12
Mean age (years (range))	47.9 (22–73)	46.5 (25–74)
Extent of colitis[a]		
No colitis	0	26
Ulcerative proctitis	3	0
Left-sided colitis	14	0
Pancolitis	7	0
Treatment		
SASP or 5-ASA only	10	0
SASP/5-ASA + corticosteroids	14	0
Immunosuppressive agents	0	0
None	2	26

ASA, acetylsalicylic acid; SASP, sulfasalazine.
[a]Colitis was classified according to the Montreal classification (40): ulcerative proctitis, limited to the rectum; left-sided colitis, distal to the splenic flexure; pancolitis (total colitis), proximal to the splenic flexure.

biopsies from healthy controls were confirmed as histologically normal.

Mucosal inflammation severity was classified as mild, or moderate or severe, by neutrophil infiltration according to Matts grade (19). The Mayo disease activity index (0–12 scale) calculated for each UC patient at the time of colonoscopy was on the basis of frequency of bowel movements, rectal bleeding, endoscopy findings and the physician's overall assessment (20).

RNA Isolation, Reverse Transcription–Polymerase Chain Reaction (PCR) and Real-Time PCR

A total of 25 biopsy specimens (12 from UC patients and 13 from controls) were used for RNA analysis. Total RNA was isolated by the Trizol reagent method (Invitrogen, San Diego, CA, USA). In total, 500 ng RNA from each specimen was reverse-transcribed into cDNA in a final volume of 20 μL containing 1 μL Moloney murine leukemia virus (MMLV)-derived reverse transcriptase (Invitrogen, San Diego, CA, USA) and 1 μL oligo dT primer. For real-time polymerase chain reaction (PCR), 2 μL cDNA in total was amplified with SYBY Green reagent (Takara, Japan) in a fluorescence thermocycler

(LightCycler; Roche Diagnostics, Mannheim, Germany). Reverse transcription (RT)-PCR was performed with ExTaq DNA polymerase (Takara, Japan) in a Mastercycler thermal cycler (Eppendorf, German). Human β-actin was used as an internal control. Sequences of primers used in this study are listed in Table 2. Targeted gene mRNA levels were normalized to those of β-actin by the $2^{-\Delta\Delta CT}$ method (21).

Western Blot Analysis

Total protein was extracted from 27 biopsy samples (14 from UC patients and 13 from controls) in radioimmunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology, Shanghai). Protein was quantified by using a BCA protein quantification kit (Beyotime). An amount of 20 μg total protein from each sample was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (0.22 μm pore; Millipore, Bedford, MA, USA). After being blocked with 5% skim milk powder diluted in TBS containing 0.1% Tween-20 for 1 h, the membrane was incubated with primary antibodies at 4°C overnight. Horseradish peroxidase–conjugated secondary

Table 2. Sequences of primers used in RT-PCR and real-time PCR analysis.

Gene name	Primers	Product length
Human *MFG-E8*	sense 5′-CCTGCCACAACGGTGGTTTAT-3′; antisense 5′-GCGATCTGTGAGTTGGCAATGT-3′	169 bp
Human *IL-8*	sense 5′-TGAGAGTGATTGAGAGTGGACCA-3′; antisense 5′-TCAGCCCTCTTCAAAAACTTCTCC-3	113 bp
Human *BCL-2*	sense 5′-TTCTTTGAGTTCGGTGGGGTC-3′; antisense 5′-GCCAGGAGAAATCAAACAGAGG-3′	196 bp
Human *BAX*	sense 5′-GACGAACTGGACAGTAACATG-3′; antisense 5′-AGGAAGTCCAATGTCCAGCC-3′	230 bp
Human *TGF-β1*	sense 5′- CGCGTGCTAATGGTGGAAA-3′; antisense 5′-CGCTTCTCGGAGCTCTGATG-3′	102 bp
Human *TFF3*	sense 5′-AACCGGGGCTGCTGCTTTG-3′; antisense 3′-GAGGTGCCTCAGAAGGTGC-3′	92 bp
Human *β-actin*	sense 5′- TGGACATCCGCAAAGAC-3′; antisense 5′-GAAAGGGTGTAACGCAACTA-3′	323 bp

antibodies (Zhongshan Gold Bridge, Beijing, China) were probed the next day, and an enhanced chemiluminescent substrate (Millipore) was used to detect the protein bands. Primary antibodies for human MFG-E8 and β-actin were from Abcam (Cambridge, MA, USA). BCL-2 and BAX primary antibodies were from Cell Signaling Technology (Beverly, MA, USA). Cleaved caspase-3 primary antibody was from Bioworld (Atlanta, GA, USA). The polyvinylidene fluoride membrane probed with MFG-E8 primary antibody initially was rinsed in stripping buffer and reprobed with anti-human β-actin antibody. Densitometry of protein bands was quantified by use of Quantity One 4.6.2 (Bio-Rad Laboratories, Hercules, CA, USA).

Immunohistochemistry

Paraffin-embedded tissues were cut into 3-μm-thick sections and underwent routine deparaffinizing and rehydrating. For antigen retrieval, sections were immersed in a Tris–ethylenediaminetetraacetic acid (EDTA) buffer, pH 9.0 (Dako, Carpentaria, CA, USA) and boiled in a microwave oven for 15 min. After sections were cooled at room temperature for 20 min, 3% hydrogen peroxide was applied to quench endogenous peroxidase. Mouse anti-human MFG-E8 monoclonal antibody (Abcam) was incubated at 4°C overnight. The secondary detection was

performed with the Envision system (Dako) with 3′3-diaminobenzidine (DAB) as a chromogen for visualization. Nuclei were counterstained with hematoxylin. For a negative control, primary antibodies were substituted with normal goat IgG.

For *in situ* apoptosis detection, fragmented DNA was stained by terminal-deoxynucleoitidyl transferase-mediated nick-end labeling (TUNEL) assay with an *in situ* cell death detection kit (Roche Applied Science, Mannheim, Germany). After deparaffinizing and rehydrating, sections were permeabilized with 20 μg/mL proteinase K (Merck, Germany) for 20 min at ambient temperature. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide. Sections were subsequently incubated with TUNEL reaction mixture containing terminal-deoxynucleoitidyl transferase and label solution at 37°C for 1 h.
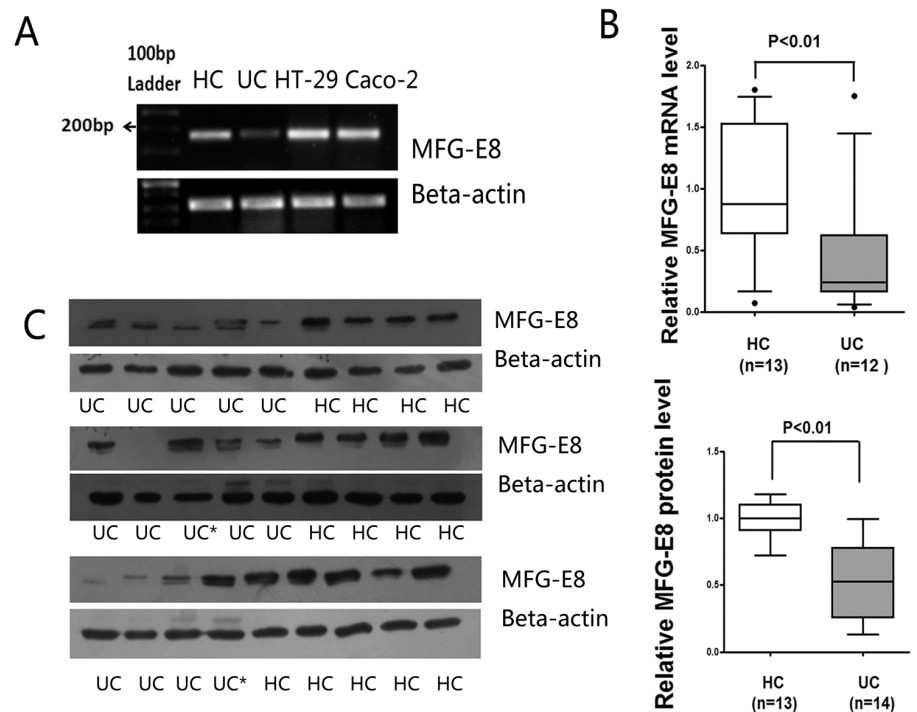

Figure 1. MFG-E8 mRNA and protein level in colonic biopsies from control subjects and UC patients. (A) Semiquantitative PCR of the mRNA level of MFG-E8 in colons of controls (HC) and UC patients and in human colon cancer cell lines HT-29 and Caco-2. β-Actin was an internal control. Real-time quantitative PCR analysis of mRNA level (B) and Western blot analysis of protein level of MFG-E8 (C) in colons from UC patients and healthy controls. *Not representative outliers. Human β-actin was a loading control. Quantification is median (line within the box) and interquartile range (top and bottom of the box). Upper and lower whiskers indicate the 95th and 5th percentile, respectively.
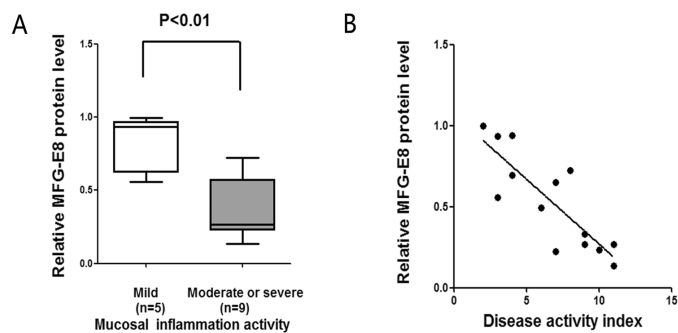
Figure 2. Association of colonic MFG-E8 protein expression with inflammation severity and clinical disease activity. (A) MFG-E8 protein expression in UC biopsies with colitis classified as mild (n = 5) and moderate or severe (n = 9) according to Matts grade (19). (B) Spearman rank correlation of MFG-E8 protein level with Mayo clinical disease activity in UC patients (r = –0.7713; P < 0.01).
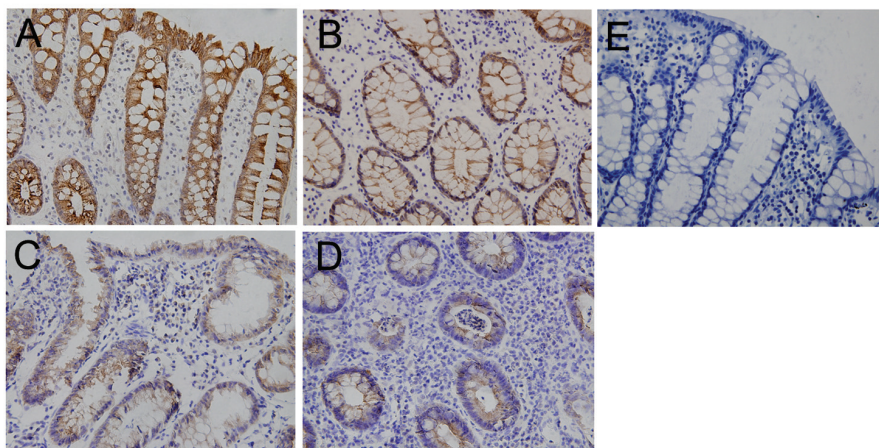

Figure 3. Immunohistochemical staining for MFG-E8 expression in human colonic biopsies. (A, B) Normal colons from control subjects. (C, D) Inflamed colons from UC patients. (E) Negative control. Original magnification: 400×.
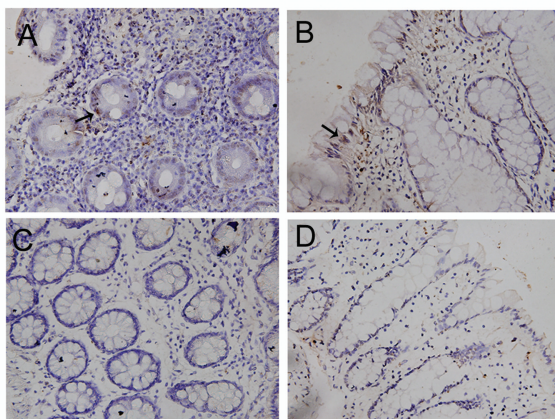

Figure 4. TUNEL staining of inflamed colons of patient with UC (A, B) and normal colons from healthy controls (C, D). Arrows indicate TUNEL-positive IECs. Original magnification: 400×.

Converted peroxidase (POD) was added later. DAB was used as a chromogen for final visualization, as described above.

Cell Culture and Lentiviral Vector–Mediated MFG-E8 Knockdown

Human colorectal cancer-derived IEC lines Caco-2 and HT-29 were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco by Invitrogen, CA, USA) supplemented with 10% (v/v) fetal calf serum (FCS; Gibco) at 37°C in a humidified incubator containing 5% CO_2. Lentiviral particles encoding short hairpin RNA (shRNA) sequences targeting human MFG-E8 mRNA (5′-CAACC ACTGT GAGAC GAAA-3′, LV-M) and control lentiviral particles encoding a scrambled shRNA sequence (5′-TTCTC CGAAC GTGTC ACGT-3′, LV-C) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lentiviruses all carried a puromycin-resistant gene. copGFP control lentiviral particles encoding green fluorescence protein (GFP) (Santa Cruz Biotechnology) was used to monitor transduction efficiency and optimize the multiplicity of infection. Cells were seeded in 12-well plates at 1×10^5 cells per well in 1 mL complete medium. When cells were at 50% to 60% confluence, lentiviral vectors were added with 10 μg/mL polybrene (Santa Cruz Biotechnology). Transduction efficiency was monitored under an inverted fluorescence microscope 72 h after transduction. When cells reached 80% to 90% confluence, they were trypsinized and seeded into 100-mm dishes. Puromycin (Santa Cruz Biotechnology) was added to adherent cells at a final concentration of 100 μg/mL for selection. Medium was changed to fresh puromycin-containing medium every 2 d. After 7 d, transduced clones encoding the puromycin-resistant gene survived and nontransduced cells died. Stable clones were selected and expanded. MFG-E8 knockdown levels were validated by real-time quantitative PCR and Western blot analysis.

Flow Cytometry

Apoptosis was detected by flow cytometry with an annexin-V/fluorescein isothiocyanate (FITC) kit (Biotech, Nanjing, China). Briefly, floating and adherent cells were washed with phosphate-buffered saline (PBS). After cells were resuspended in binding buffer, FITC-conjugated annexin-V and propidium iodide were added for 10 min in the dark. Annexin-V binding was determined in a BD FACSCalibur flow cytometer (BD, Franklin Lakes, NJ, USA).

Apoptotic Cell Morphology

To determine apoptotic nucleus changes, cells were incubated with Hoechst 33342 staining solution (Sigma, St. Louis, MO, USA) in the dark for 10 min. Apoptotic cells with nuclear chromatin condensation and fragmentation were viewed under a fluorescence microscope (Olympus IX-70; Olympus, Prague, Czech Republic).

Wound Healing Assay

HT-29 cells transduced with LV-C or LV-M were seeded in six-well plates and allowed to reach confluence overnight; then wounds were made by scratching cell monolayers with a sterile pipette tip. Detached cells were rinsed off three times with PBS. Serum-deprived culture medium (DMED with 0.1% FCS) was added for further culture. Cells were photographed 0 and 24 h after wounding. Distances covered by migrated cells were quantified.

Statistical Analysis

The nonparametric Mann-Whitney test was used to compare colonic MFG-E8 mRNA and protein expression between UC patients and healthy controls. Spearman correlation analysis was used to correlate MFG-E8 expression and clinical disease activity index in UC patients. For *in vitro* experiments, the Student *t* test was used. Data are shown as means ± standard deviation. All analyses involved use GraphPad Prism 5.01 (Graphpad Software, San Diego, CA, USA). Differences were considered statistically significant at $P < 0.05$.
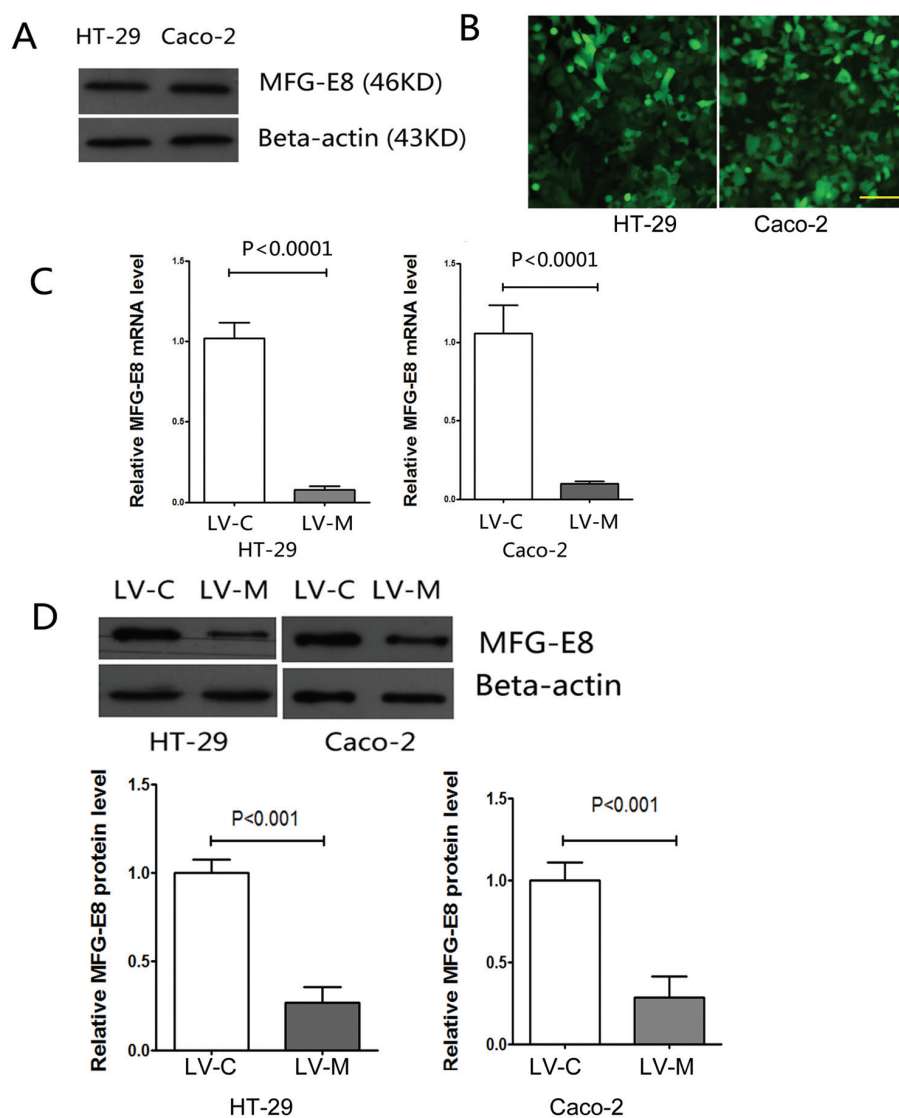

Figure 5. Validation of MFG-E8 expression in IEC lines and MFG-E8 knockdown. (A) Western blot analysis of MFG-E8 protein level in HT-29 and Caco-2 cells. (B) Fluorescence microscopy of HT-29 and Caco-2 cells 72 h after transduction with copGFP. Scale bar, 100 μm. (C) Real-time quantitative PCR analysis of MFG-E8 mRNA expression in LV-M– and LV-C–transduced HT-29 and Caco-2 cells. LV-M, lentiviral particles encoding short hairpin RNA sequences targeted to human MFG-E8 mRNA. LV-C, control lentiviral particles encoding a scrambled shRNA sequence. (D) Western blot analysis of MFG-E8 protein level in LV-M– and LV-C–transduced HT-29 and Caco-2 cells.

RESULTS

Decreased MFG-E8 Expression in Biopsies of Inflamed Mucosa from Patients with UC

RT-PCR analysis revealed that MFG-E8 mRNA was abundant in normal intestinal biopsies and lower in inflamed UC biopsies (Figure 1A). Similarly, real-time PCR analysis showed a 58% decrease of relative MFG-E8 mRNA level in inflamed intestinal tissue of UC patients compared with healthy tissue ($P < 0.01$; Figure 1B). MFG-E8 protein level was significantly downregulated in inflamed UC biopsies. Relative protein level of MFG-E8 was decreased 70% in inflamed intestinal tissue of UC patients
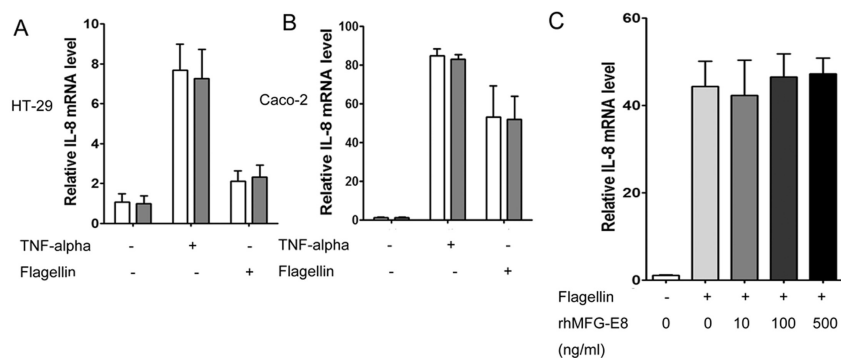
Figure 6. Basal and TNF-α– and flagellin-induced IL-8 mRNA expression in LV-C– and LV-M–transduced HT-29 (A) and Caco-2 cells (B). To detect TNF-α– and flagellin-induced IL-8 mRNA expression, total RNA was isolated after stimulation with 100 ng/mL TNF-α or 100 ng/mL flagellin for 12 h. (C) IL-8 mRNA levels in flagellin-stimulated Caco-2 cells pretreated with rhMFG-E8 at different concentrations for 12 h. A, B: □, LV-C; ▓, LV-M.

Figure 7. Apoptosis assay in MFG-E8 knockdown IEC lines. (A) Flow cytometry of annexin V staining with LV-M or LV-C in HT-29 and Caco-2 cells. (B) Hoechst 33342 staining results. Arrows indicate typical apoptotic nuclei with chromatin condensation or fragmentation. Scale bar, 50 μm. □, LV-C; ▓, LV-M.

compared with healthy tissue ($P < 0.01$; Figure 1C).

MFG-E8 Expression Is Inversely Correlated with Histological Inflammatory Activity and Clinical Disease Activity but Not Endoscopy Grading

Intriguingly, all of the outlier UC cases exhibiting high MFG-E8 protein expression were graded as mild inflammation on histology and were from patients with mild clinical symptoms. So we presumed that MFG-E8 downregulation in UC patients was correlated with mucosal histological grading and clinical disease activity. Indeed, MFG-E8 protein expression was inversely correlated with mucosal inflammation activity (Figure 2B). The median decrease in MFG-E8 protein level was 33% for biopsies with mild inflammation compared with 77% for biopsies with moderate or severe inflammation ($P < 0.01$; Figure 2A). MFG-E8 protein level was inversely correlated with clinical disease activity in UC patients ($r = –0.7721$, $P < 0.01$) but not endoscopy grading (data not shown).
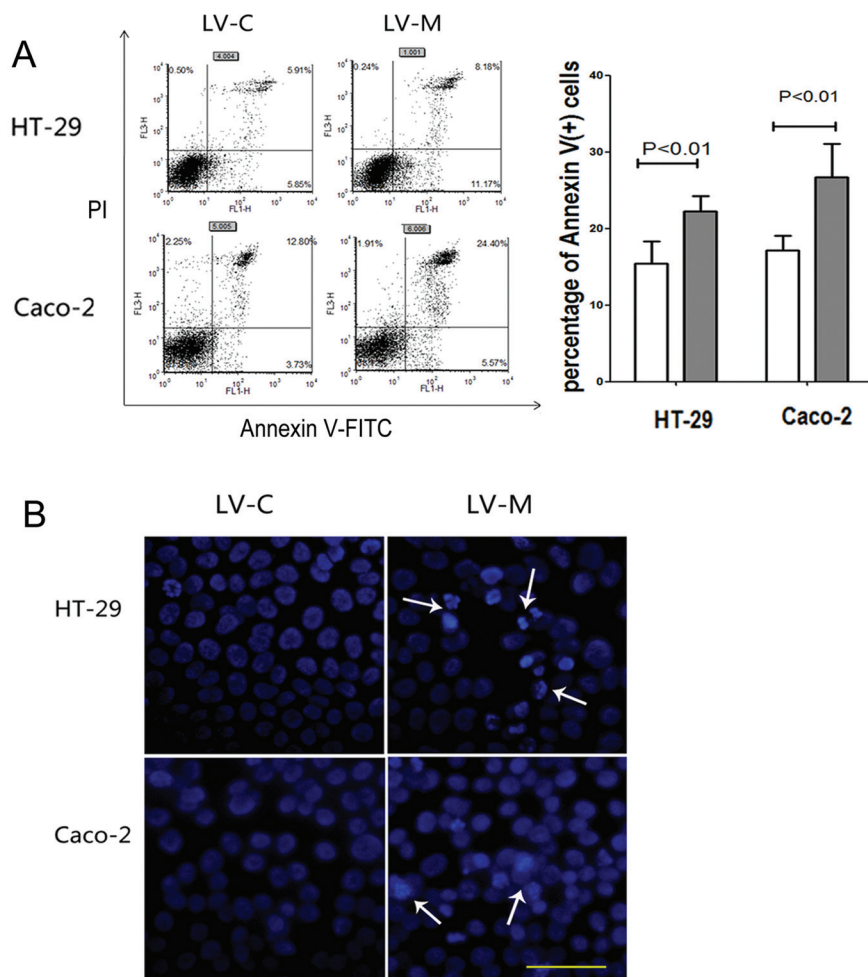
Decrease of MFG-E8 and Induction of Apoptosis in IECs of UC Patients

We used immunohistochemistry to investigate the cellular origin of MFG-E8 in intestinal tissues. The intestinal epithelium of healthy controls showed strong positive staining for MFG-E8 (Figures 3A, B). MFG-E8 was present in both the surface epithelium and crypts of normal intestinal tissues. However, the intestinal epithelium of UC patients showed less staining for MFG-E8 (Figures 3C–E). The specificity of staining was confirmed by the negative control (Figure 3F). Additionally, our TUNEL assay revealed increased apoptotic cells in the epithelium of the inflamed colon from UC patients compared with normal colons from healthy controls (Figure 4).

MFG-E8 in IEC Lines and Validation of MFG-E8 Knockdown

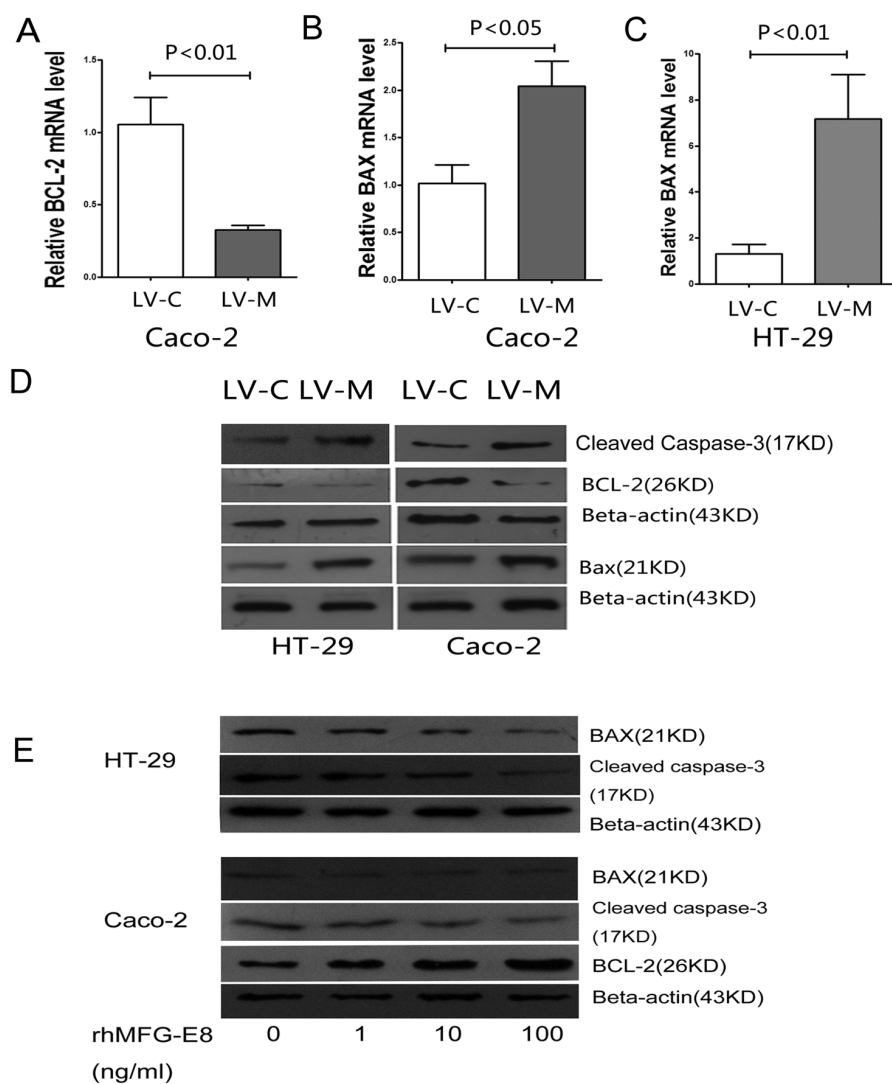MFG-E8 mRNA and protein is expressed in the IEC lines HT-29 and

Figure 8. Assessment of apoptotic-related proteins in MFG-E8 knockdown IEC lines. The mRNA level of the antiapoptotic factor BCL-2 in Caco-2 cells (A) and proapoptotic factor BAX in Caco-2 (B) and HT-29 cells (C) with LV-M or LV-C transduction is shown. (D) Western blot analysis of cleaved caspase-3, BCL-2 and BAX protein level with LV-M or LV-C transduction in HT-29 and Caco-2 cells. (E) Western blot analysis of cleaved caspase-3, BCL-2 and BAX protein levels in IECs incubated with rhMFG-E8 at different concentrations for 24 h.

or flagellin (Enzo Life Science, Farmingdale, NY, USA) for 12 h, IL-8 mRNA levels were both upregulated in MFG-E8 knockdown cells and LV-C–transduced cells. And IL-8 mRNA expression induced by TNF-α or flagellin was not altered in MFG-E8 knockdown IECs. To further investigate the role of MFG-E8 in flagellin-induced inflammatory response in IECs, we used recombinant human MFG-E8 (rhMFG-E8; R&D Systems, Minneapolis, MN, USA) to treat Caco-2 cells (since Caco-2 is more responsive to flagellin than HT-29) before flagellin addition. And preincubation with rhMFG-E8 from 10 to 500 ng/mL for 12 h did not alter IL-8 mRNA levels induced by flagellin in Caco-2 cells (Figure 6C).

MFG-E8 Knockdown Promoted Apoptosis in IEC Lines and Altered the Expression of Apoptosis-Related Proteins

Flow cytometry results demonstrated that annexin V–positive cells significantly increased in number in MFG-E8 knockdown IECs (Figure 7A). Hoechst 33342 staining revealed typical apoptotic cell changes, including nuclear chromatin condensation and fragmentation, in MFG-E8 knockdown IECs (Figure 7B). In Caco-2 cells, MFG-E8 knockdown decreased the mRNA and protein levels of the antiapoptotic protein BCL-2 (Figures 8A, D). In HT-29 cell lines, BCL-2 expression was barely detected (Figure 8D). The mRNA and protein levels of the proapoptotic BAX were both upregulated in HT-29 and Caco-2 cells with MFG-E8 knockdown (Figures 8B–D). MFG-E8 knockdown was accompanied by induction of cleaved caspase-3 protein in both two-cell lines (Figure 8D). Addition of rhMFG-E8 led to a decrease of BAX and cleaved caspase-3 protein levels in both two-cell lines and an increase of BCL-2 protein level in Caco-2 cells (Figure 8E). The effect of rhMFG-E8 was dramatic at high concentrations (100 ng/mL). In HT-29 cells, BCL-2 was still barely detected after incubation with rhMFG-E8 (data not shown).

Caco-2 (Figures 1A, 5A). In copGFP lentiviral-transduced HT-29 and Caco-2 cells, transduction efficiencies were both >90% (Figure 5B). After transduction with LV-M, MFG-E8 mRNA levels were decreased by 93.2% and 92.7% in HT-29 and Caco-2 cells, respectively, compared with LV-C transduction (Figure 5C). As well, MFG-E8 protein levels were decreased by 78% and 73% in HT-29 and Caco-2 cells, respectively (Figure 5D).

MFG-E8 Has No Effect on Interleukin-8 Production Induced by Tumor Necrosis Factor-α or Flagellin in IEC Lines

The basal interleukin (IL)-8 mRNA expression level with MFG-E8 knockdown HT-29 and Caco-2 cells was similar to that with LV-C transduction (Figures 6A, B). After stimulation with 100 ng/mL tumor necrosis factor (TNF)-α (PeproTech Inc., Rocky Hill, NJ, USA)
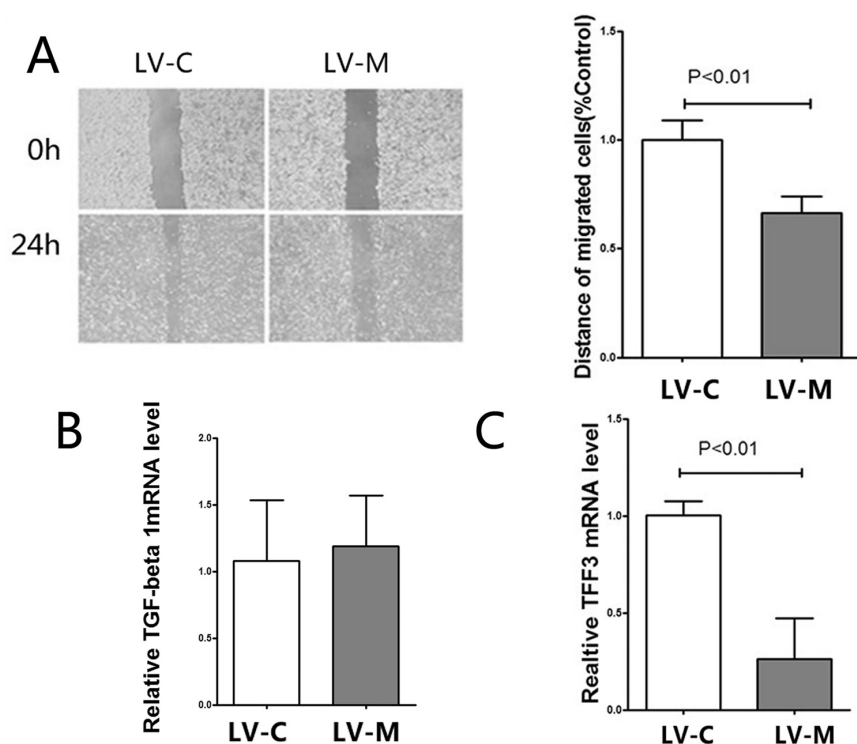
Figure 9. MFG-E8 knockdown attenuated IEC restitution *in vitro*. (A) Wound healing assay of MFG-E8 knockdown HT-29 cells and cells transduced with control lentivirus. The mRNA expression of TGF-β1 (B) and intestinal TFF3 (C) in MFG-E8 knockdown HT-29 cells is shown.

MFG-E8 Knockdown Impaired Wound Healing and Downregulated Intestinal Trefoid Factor 3 mRNA Expression

MFG-E8 knockdown greatly attenuated wound closure of scratched HT-29 cell monolayers 24 h after wounding (Figure 9A). In light of the crucial role of transforming growth factor (TGF)-β1 and intestinal trefoid factor 3 (TFF3) in IEC migration and restitution, we determined their mRNA expression in MFG-E8 knockdown cells. TGF-β1 mRNA levels were not changed (Figure 9B), whereas TFF3 mRNA levels were significantly reduced in MFG-E8 knockdown HT-29 cells (Figure 9C).

DISCUSSION

MFG-E8 expression was decreased in inflamed mouse colons with colitis, and recombinant MFG-E8 had a protective role against inflammation. However, because of the lack of studies of MFG-E8 expression in humans with UC, we investigated MFG-E8 expression in colonic mucosal biopsies from UC patients and healthy controls. Several studies revealed MFG-E8 expression in mouse macrophages of the intestinal lamina propria but not the intestinal epithelium (16,17). In the present study, we detected positive staining for MFG-E8 in the human colonic epithelium of both control subjects and UC patients, the first evidence of MFG-E8 in human IECs. This disparity may be partially due to the low homology of MFG-E8 between the species (human MFG-E8 shares only 52% sequence identity with mouse MFG-E8) (22) but also due to the conditions of different stages of colitis since UC in humans is a chronic, relapsing form of intestinal disorder. Additionally, we detected a high basal mRNA and protein level of MFG-E8 in human colorectal cancer–derived IEC lines HT-29 and Caco-2, whereas MFG-E8 expression was absent at base-

line in the murine colon cancer cell line MC38 (23).

MFG-E8 has been implicated in intestinal homeostasis and inflammation in animal models (16–18). Here we determined for the first time the expression of MFG-E8 in patients with UC. MFG-E8 mRNA and protein levels were lower in inflamed intestinal biopsies from UC than control biopsies. MFG-E8 staining was decreased in the colonic epithelium of UC patients. In UC patients, intestinal MFG-E8 expression was inversely correlated with Mayo disease activity index, a well-established clinical calibrator for disease severity (20). Also, MFG-E8 expression was lower in UC biopsies with moderate or severe inflammation than mild inflammation, which suggests a potential negative correlation between histology grading and MFG-E8 expression. Our findings agree with previous findings in mice of decreased production of MFG-E8 in inflamed colons during the initiation and acute phase of DSS-induced colitis, with recovered expression during the healing phase (17).

IECs are a pivotal physiological barrier between the environment and the host. They play a key role in gut innate immunity and contribute to inflammatory progression in intestinal bowel disease by secreting various chemokines and cytokines (24,25). Recombinant mouse MFG-E8 administration attenuated inflammatory mediator secretion of macrophages induced by lipopolysaccharide (LPS) in a NF-κB–dependent manner (17). We wondered whether MFG-E8 is involved in the inflammatory response of IECs. Because the IECs are not well responsive to LPS, we used TNF-α and flagellin (another TLR ligand) for IEC stimulation. We did not observe any changes in basal mRNA level of IL-8 or IL-8 mRNA level induced by TNF-α or flagellin in IEC lines with MFG-E8 knockdown. And preincubation with rhMFG-E8 did not change IL-8 levels induced by flagellin in IECs. Maybe this is due to different inflammatory responsive mechanisms in IECs from macrophages and other cell types.

IEC apoptosis has a major role in intestinal epithelial homeostasis and pathogenic mechanisms (26). Increased IEC apoptosis was observed in acute inflammatory sites of patients with inflammatory bowel disease, which led to impaired intestinal barrier function and contributed to disease development (7,27). Here we showed increased IEC apoptosis in patients with UC compared with healthy controls by TUNEL *in situ* apoptosis assay. We also confirmed that MFG-E8 knockdown could promote basal apoptosis in IEC lines, as supported by flow cytometry and the induction of cleaved caspase-3. A previous report demonstrated that knockdown of MFG-E8 with shRNA in human melanoma cells was associated with increased apoptosis (28). Also, in chemotherapeutic agent–resistant MC38 murine colon cancer cells (cells surviving chemotherapeutic agents and showing significant MFG-E8 expression), the addition of anti–MFG-E8 antibodies potentiated the lethality of chemotherapeutic drugs and enhanced caspase-3 activation (23). We found decreased mRNA and protein levels of antiapoptotic BCL-2 and increased levels of proapoptotic BAX in MFG-E8 knockdown IECs. Addition of rhMFG-E8 led to decreased BAX and cleaved caspase-3 levels and induction of BCL-2 level in IECs. These results indicate a potential underlying mechanism involved in MFG-E8 knockdown-induced apoptosis.

The intestinal epithelium is exposed to various stimuli, and IEC injury is constant and inevitable. Especially in patients with inflammatory bowel disease, repeated intestinal epithelial injury is typical (29). During intestinal epithelium injury, IECs rapidly depolarize and migrate to cover the denuded area, independent of proliferation. This process is called restitution, and rapid restitution is required to maintain the integrity of the intestinal barrier (30). Using a classic *in vitro* wound healing model to properly recapitulate the restitution of IECs after injury *in vivo*, we found that MFG-E8 knockdown significantly attenuated closure of scratch-wounded IEC monolayers 24 h after wounding. In a pre-

vious study, recombinant mouse MFG-E8 administration accelerated murine IEC migration *in vitro* and *in vivo* (16).

Intestinal restitution is modulated by a broad spectrum of cytokines and growth factors. Among these, TGF-β1 plays a central role (31–33). The beneficial effect of many cytokines and growth factors in intestinal restitution depends on TGF-β1 because neutralizing TFG-β1 with the anti–TGF-β1 antibody diminished their effect (33,34). However, we did not observe any alterations in TGF-β1 mRNA level after MFG-E8 knockdown in HT-29 IECs.

TFF3 is another crucial factor in intestinal restitution and repair. It is a protease-resistant peptide secreted by goblet cells of the small intestine and colon that can promote IEC migration and intestinal restitution through a mechanism independent of TGF-β1 (35–37). Colonic TFF3 expression was found reduced in trinitro-benzene-sulfonic acid–induced rat colitis (38). In UC patients, reduced TFF3 expression was more pronounced with increasing disease severity (39). We found TFF3 mRNA expression significantly decreased in MFG-E8 knockdown HT-29 IECs, which suggests a potential molecular basis responsible for impaired wound healing.

CONCLUSION

In summary, we confirmed the presence of MFG-E8, important in maintaining intestinal barrier homeostasis and accelerating intestinal cell restitution, in the human intestinal epithelium. And we further demonstrated decrease of MFG-E8 and induction of apoptosis in IECs of patients with UC. We also showed that intestinal MFG-E8 expression in UC was inversely correlated with clinical disease activity and histology grading. Our *in vitro* studies confirmed the expression of MFG-E8 in human colon cancer–derived IEC lines and that MFG-E8 knockdown could promote IEC apoptosis accompanied by decreased level of the antiapoptotic protein BCL-2 and induction of the proapoptotic protein BAX. MFG-E8 knockdown could attenuate wound healing of scratched IEC monolayers. The

level of TFF3, a pivotal factor in intestinal restitution and migration, was decreased with MFG-E8 knockdown in IECs. We suggest a pivotal role for MFG-E8 in UC pathogenesis. Decreased epithelial MFG-E8 expression is a risk factor in inflammatory bowel disease associated with increased apoptosis and impaired wound healing.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES

1. Xavier RJ, Podolsky DK. (2007) Unravelling the pathogenesis of inflammatory bowel disease. *Nature*. 448:427–34.
2. Strober W, Fuss I, Mannon P. (2007) The fundamental basis of inflammatory bowel disease. *J. Clin. Invest.* 117:514–21.
3. Podolsky DK. (2002) Inflammatory bowel disease. *N. Engl. J. Med.* 347:417–29.
4. Yamamoto-Furusho JK, Podolsky DK. (2007) Innate immunity in inflammatory bowel disease. *World J. Gastroenterol.* 13:5577–80.
5. Matsuda R, *et al.* (2009) Quantitive cytokine mRNA expression profiles in the colonic mucosa of patients with steroid naive ulcerative colitis during active and quiescent disease. *Inflamm. Bowel Dis.* 15:328–34.
6. Hagiwara C, Tanaka M, Kudo H. (2002) Increase in colorectal epithelial apoptotic cells in patients with ulcerative colitis ultimately requiring surgery. *J. Gastroenterol. Hepatol.* 17:758–64.
7. Strater J, *et al.* (1997) CD95 (APO-1/Fas)-mediated apoptosis in colon epithelial cells: a possible role in ulcerative colitis. *Gastroenterology*. 113:160–7.
8. Roda G, *et al.* (2010) Intestinal epithelial cells in inflammatory bowel diseases. *World J. Gastroenterol.* 16:4264–71.
9. Hanayama R, *et al.* (2002) Identification of a factor that links apoptotic cells to phagocytes. *Nature*. 417:182–7.
10. Ensslin MA, Shur BD. (2007) The EGF repeat and discoidin domain protein, SED1/MFG-E8, is required for mammary gland branching morphogenesis. *Proc. Natl. Acad. Sci. U S A*. 104:2715–20.
11. Watanabe T, *et al.* (2005) Production of the long

and short forms of MFG-E8 by epidermal keratinocytes. *Cell Tissue Res.* 321:185–93.

12. Hanayama R, *et al.* (2004) Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. *Science.* 304:1147–50.

13. Miksa M, Amin D, Wu R, Ravikumar TS, Wang P. (2007) Fractalkine-induced MFG-E8 leads to enhanced apoptotic cell clearance by macrophages. *Mol. Med.* 13:553–60.

14. Yamaguchi H, *et al.* (2008) Milk fat globule EGF factor 8 in the serum of human patients of systemic lupus erythematosus. *J. Leukoc. Biol.* 83:1300–7.

15. Lyng R, Shur BD. (2007) Sperm-egg binding requires a multiplicity of receptor-ligand interactions: new insights into the nature of gamete receptors derived from reproductive tract secretions. *Soc. Reprod. Fertil. Suppl.* 65:335–51.

16. Bu HF, *et al.* (2007) Milk fat globule-EGF factor 8/lactadherin plays a crucial role in maintenance and repair of murine intestinal epithelium. *J. Clin. Invest.* 117:3673–83.

17. Aziz MM, *et al.* (2009) MFG-E8 attenuates intestinal inflammation in murine experimental colitis by modulating osteopontin-dependent alphavbeta3 integrin signaling. *J. Immunol.* 182:7222–32.

18. Chogle A, *et al.* (2011) Milk fat globule-EGF factor 8 is a critical protein for healing of dextran sodium sulfate-induced acute colitis in mice. *Mol. Med.* 17:502–7.

19. Matts SG. (1961) The value of rectal biopsy in the diagnosis of ulcerative colitis. *Q. J. Med.* 30:393–407.

20. Rutgeerts P, *et al.* (2005) Infliximab for induction and maintenance therapy for ulcerative colitis. *N. Engl. J. Med.* 353:2462–76.

21. Livak KJ, Schmittgen TD. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 25:402–8.

22. Larocca D, *et al.* (1991) A Mr 46,000 human milk fat globule protein that is highly expressed in human breast tumors contains factor VIII-like domains. *Cancer Res.* 51:4994–8.

23. Jinushi M, *et al.* (2009) Milk fat globule epidermal growth factor-8 blockade triggers tumor destruction through coordinated cell-autonomous and immune-mediated mechanisms. *J. Exp. Med.* 206:1317–26.

24. Rescigno M. (2011) The intestinal epithelial barrier in the control of homeostasis and immunity. *Trends. Immunol.* 32:256–64.

25. Catalioto RM, Maggi CA, Giuliani S. (2010) Intestinal epithelial barrier dysfunction in disease and possible therapeutical interventions. *Curr. Med. Chem.* 18:398–426.

26. Edelblum KL, Yan F, Yamaoka T, Polk DB. (2006) Regulation of apoptosis during homeostasis and disease in the intestinal epithelium. *Inflamm. Bowel. Dis.* 12:413–24.

27. Qiu W, *et al.* (2011) PUMA-mediated intestinal epithelial apoptosis contributes to ulcerative colitis in humans and mice. *J. Clin. Invest.* 121:1722–32.

28. Jinushi M, *et al.* (2008) Milk fat globule EGF-8 promotes melanoma progression through coordinated Akt and twist signaling in the tumor microenvironment. *Cancer Res.* 68:8889–98.

29. Iizuka M, Konno S. (2011) Wound healing of intestinal epithelial cells. *World J. Gastroenterol.* 17:2161–71.

30. Sturm A, Dignass AU. (2008) Epithelial restitution and wound healing in inflammatory bowel disease. *World J. Gastroenterol.* 14:348–53.

31. Ciacci C, Lind SE, Podolsky DK. (1993) Transforming growth factor beta regulation of migration in wounded rat intestinal epithelial monolayers. *Gastroenterology.* 105:93–101.

32. Beck PL, *et al.* (2003) Transforming growth factor-beta mediates intestinal healing and susceptibility to injury in vitro and in vivo through epithelial cells. *Am. J. Pathol.* 162:597–608.

33. Dignass AU, Podolsky DK. (1993) Cytokine modulation of intestinal epithelial cell restitution: central role of transforming growth factor beta. *Gastroenterology.* 105:1323–32.

34. Bulut K, *et al.* (2006) Vascular endothelial growth factor (VEGF164) ameliorates intestinal epithelial injury in vitro in IEC-18 and Caco-2 monolayers via induction of TGF-beta release from epithelial cells. *Scand. J. Gastroenterol.* 41:687–92.

35. Dignass A, Lynch-Devaney K, Kindon H, Thim L, Podolsky DK. (1994) Trefoil peptides promote epithelial migration through a transforming growth factor beta-independent pathway. *J. Clin. Invest.* 94:376–83.

36. Mashimo H, Wu DC, Podolsky DK, Fishman MC. (1996) Impaired defense of intestinal mucosa in mice lacking intestinal trefoil factor. *Science.* 274:262–5.

37. Durer U, Hartig R, Bang S, Thim L, Hoffmann W. (2007) TFF3 and EGF induce different migration patterns of intestinal epithelial cells in vitro and trigger increased internalization of E-cadherin. *Cell. Physiol. Biochem.* 20:329–46.

38. Song M, Xia B, Li J. (2006) Effects of topical treatment of sodium butyrate and 5-aminosalicylic acid on expression of trefoil factor 3, interleukin 1beta, and nuclear factor kappaB in trinitrobenzene sulphonic acid induced colitis in rats. *Postgrad. Med. J.* 82:130–5.

39. Longman RJ, *et al.* (2006) Alterations in the composition of the supramucosal defense barrier in relation to disease severity of ulcerative colitis. *J. Histochem. Cytochem.* 54:1335–48.

40. Satsangi J, Silverberg MS, Vermeire S, Colombel JF. (2006) The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. *Gut.* 55:749–53.