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Expression of *REG* family genes in human inflammatory bowel diseases and its regulation

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ABSTRACT

The pathophysiology of inflammatory bowel disease (IBD) reflects a balance between mucosal injury and reparative mechanisms. Some regenerating gene (*Reg*) family members have been reported to be expressed in Crohn's disease (CD) and ulcerative colitis (UC) and to be involved as proliferative mucosal factors in IBD. However, expression of all *REG* family genes in IBD is still unclear. Here, we analyzed expression of all *REG* family genes (*REG Iα*, *REG Iβ*, *REG III*, *HIP/PAP*, and *REG IV*) in biopsy specimens of UC and CD by real-time RT-PCR. *REG Iα*, *REG Iβ*, and *REG IV* genes were overexpressed in CD samples. *REG IV* gene was also overexpressed in UC samples. We further analyzed the expression mechanisms of *REG Iα*, *REG Iβ*, and *REG IV* genes in human colon cells. The expression of *REG Iα* was significantly induced by IL-6 or IL-22, and *REG Iβ* was induced by IL-22. Deletion analyses revealed that three regions (− 220 to − 211, − 179 to − 156, and − 146 to − 130) in *REG Iα* and the region (− 274 to − 260) in *REG Iβ* promoter were responsible for the activation by IL-22/IL-6. The promoters contain consensus transcription factor binding sequences for MZF1, RTEF1/TEAD4, and STAT3 in *REG Iα*, and HLTF/FOXN2F in *REG Iβ*, respectively. The introduction of siRNAs for MZF1, RTEF1/TEAD4, STAT3, and HLTF/FOXN2F abolished the transcription of *REG Iα* and *REG Iβ*. The gene activation mechanisms of *REG Iα/REG Iβ* may play a role in colon mucosal regeneration in IBD.

1. Introduction

Crohn's disease (CD) and ulcerative colitis (UC), the two primary forms of idiopathic human inflammatory bowel disease (IBD), are both characterized by chronic, destructive intestinal inflammation of unknown cause(s). Despite advances over the past decade in our understanding of cellular and molecular mechanisms underlying chronic inflammation, the precise etiopathogenic factors in IBD remain undefined. Current theories suggest that interplay between environmental, genetic, microbial, and immunologic factors results in the chronic gut inflammation that characterizes CD or UC.

Regenerating gene (*REG*) family proteins are structurally similar proteins belonging to the calcium-dependent (C-type) lectin superfamily. In humans, five *REG* family genes (i.e., *REG Iα*, *REG Iβ*, *REG*-related sequence (pseudogene), *HIP/PAP* (*INGAP*), and *REG III*) are tandemly ordered in the 95 kbp region of chromosome 2p12, whereas *REG IV* is located on chromosome 1q12-q21 [1,2]. The first *Reg* gene was discovered in rat regenerating pancreatic islets and involved in β-cell regeneration [3], and Reg proteins have since been found in other physiological and pathophysiological processes [1,2]. Their basic biological effects seem to be induction of cellular proliferation [4–7]. Reg family proteins have been suggested to be involved in cellular

Abbreviations: CD, Crohn's disease; CDX2, caudal-type homeobox transcription factor 2; FOXN2, forkhead box protein N2; GATA6, GATA DNA-binding protein 6; HLTF, helicase-like transcription factor; IBD, inflammatory bowel disease; IL, interleukin; MZF1, myeloid zinc finger 1; *REG*, regenerating gene; RTEF1, related transcriptional enhancer factor-1; siRNA, small interfering RNA; SOCS3, suppressors of the cytokine signaling 3; STAT3, signal transducer and activator of transcription 3; TEAD4, TEA Domain transcription Factor 4; UC, ulcerative colitis

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proliferation in gastrointestinal cells [1,2,8]. Elsewhere in the gastrointestinal system, these proteins are found during tissue injury [1,2]. They are also overexpressed in gastric and colorectal cancers [9–12] and in colorectal cancer cell lines [13].

Concerning IBD, overexpression of *REG Iα* and *REG Iβ* mRNA in resected colonic tissue from CD and UC was first reported by Lawrance et al. [14]. We also showed the overexpression of *HIP/PAP* and *REG III* in IBD [15]. Subsequently, overexpression of *REG Iα*, *REG Iβ*, and *REG III* in IBD colon was reported [16,17]. Overexpression of *REG Iα* mRNA and protein in UC, particularly in dysplasia or cancer, and a possible role for REG Iα as a marker for UC-associated neoplasia were also reported [18]. Recently, Granlund et al. analyzed four of *REG* family genes in five functional human *REG* family members, and found that the analyzed four genes (*REG Iα*, *REG Iβ*, *HIP/PAP*, and *REG IV*) were overexpressed in IBD samples [19].

Although we and others have previously suggested that REG family proteins have a trophic effect on mammalian epithelial cells and are involved in IBD, expression of all functional members of *REG* family genes (*REG Iα*, *REG Iβ*, *REG III*, *HIP/PAP*, and *REG IV*) in IBD is still unclear. In the present study, we examined all five *REG* family genes in IBD and found overexpression of *REG Iα*, *REG Iβ*, and *REG IV* mRNAs in CD and that of *REG IV* mRNA in UC. Reporter gene assays and siRNA-mediated knockdown experiments indicated that the overexpression of *REG Iα*, *REG Iβ*, and *REG IV* mRNA was mediated through several transcription factors, including myeloid zinc finger 1 (MZF1), related transcriptional enhancer factor-1 (RTEF1)/TEA Domain transcription Factor 4 (TEAD4), and signal transducer and activator of transcription 3 (STAT3) in *REG Iα*, helicase-like transcription factor (HLTF)/forkhead box protein N2 (FOXN2) in *REG Iβ*, and GATA DNA-binding protein 6 (GATA6) in *REG IV* gene expression.

2. Materials and methods

2.1. Tissue specimens and histological examination

Patients admitted to the Gastrointestinal Endoscopy Unit, Department of Gastroenterology, Saiseikai Nara Hospital (Nara, Japan) or Nara Medical University Hospital (Kashihara, Japan) for colonoscopy were included after informed consent. Colon biopsy samples were obtained from the rectum by endoscopy from 49 patients with CD (36 men and 13 women; mean age 33.21 ± 2.32 years, range 0.4–79) and 39 patients with UC (21 men and 18 women; mean age 43.07 ± 2.58, range 16–75). Normal control specimens were obtained from resected colon for cancer (44 patients, 26 men and 18 women; mean age 66.78 ± 1.65, range 43–91) and were used as normal control. The tissue specimens were fixed in 10% formalin solution, embedded in paraffin, and subjected to histopathological analyses.

This work was done with approval of the Review Board(s) of Saiseikai Nara Hospital and Nara Medical University Hospital. The diagnosis of UC was based on established endoscopic and histologic criteria [20].

2.2. Real-time RT-PCR

Total RNA was isolated from formalin-fixed, paraffin-embedded tissue specimens and from human colonic epithelial cells (HT-29 and LS-174T cells) using RNeasy FFPE Kit (Qiagen, Hilden, Germany) and RNeasy Protect Cell Mini Kit (Qiagen), respectively. The isolated RNA was reverse transcribed to the cDNA using High Capacity cDNA Synthesis Kit (Applied Biosystems, Foster City, CA) for the template of real-time PCR as described [21–27]. The cDNA was subjected to PCR with the following primers: *β-actin* (NM_001101) sense primer, 5'-GCGAGAAGATGACCCAGA-3' and antisense primer, 5'-CAGAGGCGTACAGGGATA-3'; *REG Iα* (NM_002909) sense primer, 5'-AGGAGAGTGGCACTGATGACTT-3' and antisense primer 5'-TAGGAGACCAGGGACCCACTG-3'; *REG Iβ* (NM_006507) sense primer, 5'-GCTGATCTCCTCCCTGATGTTC-3' and antisense primer, 5'-TGTCAGTGATCTTGGTTTGAA-3'; *REG III* (**AB161037**) sense primer, 5'-GAATATTCTCCCCAAACTG-3' and antisense primer, 5'-GAGAAAAG CCTGAAATGAAG-3'; *HIP/PAP* (NM_138937) sense primer, 5'-AGAGAATATTCGCTTAATTCC-3' and antisense primer, 5'-AATGAAGAGACTGAAATGACA-3'; and *REG IV* (**AY007243**) sense primer, 5'-ATCCTGGTCTGGCAAGTC-3' and antisense primer, 5'-CGTTGCTGCTCCAAGTTA-3'. All the PCR primers were synthesized by Nihon Gene Research Laboratories (Sendai, Japan). Real-time PCR was performed using KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems, Boston, MA) and Thermal Cycler Dice Real Time System (Takara Bio Inc, Kusatsu, Japan) as described [21–27]. PCR was performed with an initial step of 3 min at 95 °C followed by 40 cycles of 3 s at 95 °C and 20 s at 60 °C for *β-actin*, *REG III*, and *HIP/PAP*; 40 cycles of 3 s at 95 °C and 20 s at 64 °C for *REG Iα, REG Iβ*, and *REG IV*. Target cDNAs were cloned into pBluescript SK(-) plasmid (Stratagene, La Jolla, CA), and sequential 10-fold dilutions from 10^2 to 10^7 copies/μL were prepared. The serial dilutions were run to verify the specificity and to test the sensitivity of the SYBR Green-based real-time RT-PCR. Target mRNA value was normalized to that of *β-actin* mRNA, which was used to account for differences in the efficiency of reverse transcription between samples.

2.3. Cell culture and treatment

LS-174T and HT-29 human colonic epithelial cells were grown in RPMI 1640 medium (Nakarai Tesque, Inc., Kyoto, Japan) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Wako) [23]. Human interleukin (IL)− 6 and tumor necrosis factor (TNF)α were purchased from Roche Diagnostics (Indianapolis, IN), and human IL-8, IL-17A, IL-22, hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) were from Wako. Cells were treated with 20 ng/mL IL-6, 10 nM IL-8, 1 μg/mL IL-17A, 20 ng/mL IL-22, 20 ng/mL TNFα, 50 ng/mL HGF, 10 nM bFGF, and 10 nM EGF for 24 h.

2.4. Promoter assay

The reporter constructs were prepared by inserting fragments of human *REG Iα* gene promoter (− 1190 to + 26, − 402 to + 26, − 370 to + 26, − 345 to + 26, − 301 to + 26, − 258 to + 26, − 247 to + 26, − 234 to + 26, − 226 to + 26, − 220 to + 26, − 211 to + 26, − 204 to + 26, − 179 to + 26, − 156 to + 26, − 146 to + 26, − 130 to + 26) [25,26], human *REG Iβ* gene promoter (− 978 to + 30. − 806 to + 30, − 622 to + 30, − 562 to + 30, − 525 to + 30, − 478 to + 30, 441 to + 30, − 381 to + 30, − 326 to + 30, − 298 to + 30, − 274 to + 30, − 260 to + 30) [25] and human *REG IV* (− 1053 to + 22) upstream of a firefly luciferase reporter gene in pGL3-Basic vector (Promega, Madison, WI). The cells were grown in 24-well plates to 70–80% confluency and were transfected with reporter plasmids by lipofection. Briefly, 0.5 μg of each reporter plasmid and, as an internal control, 0.05 μg of pCMV-SPORT-β-galactosidase (Invitrogen, Carlsbad, CA) per well were mixed with Lipofectamine®3000 (Invitrogen) in a 24-well plate. After 24 h, the medium of each well was replaced with fresh medium containing stimulants and incubated further for 24 h. Cells were washed twice with PBS and extracts were prepared in extraction buffer (0.1 M potassium phosphate, pH 7.8/0.2% Triton X-100) as described [25–27]. Luciferase activity was measured using a PicaGene® Luciferase assay system (Toyo-ink, Tokyo, Japan) and was normalized by the β-galactosidase activity as described [25–27].

2.5. RNA interference (RNAi)

Small interfering RNA (siRNA)s directed against human MZF1, RTEF1/TEAD4, HLTF/FOXN2, STAT3, suppressors of the cytokine signaling 3 (SOCS3), caudal-type homeobox transcription factor 2 (CDX2), and GATA DNA-binding protein 6 (GATA6) were synthesized by Nihon

Gene Research Laboratories. The sense sequences of siRNA for human MZF1, RTEF1/TEAD4, HLTF/FOXN2, STAT3, SOCS3, CDX2, and GATA6 were as follows: 5'-GAGGUCCUAUCAGAGAAGAtt-3' for MZF1, 5'-GGGCAGACCUCAACACCAAtt-3' for RTEF1/TESD4, 5'-GGAAUUU UAGCUGAUGAUAtt-3' for HLTF/FOXN2, 5'-GCACCUUCCUG CUAAGAUUtt-3' for STAT3, 5'-CCAAGAACCUGCGCAUCCAtt-3' for SOCS3, 5'-AAGCCUCAGUGUCUGGCUCUGtt-3' for CDX2, and 5'-GACAGAACGUGAUUCUCGUtt-3' for GATA6. siRNA-scramble (Ambion®, Life Technologies) [25–27] was also used as a control. Transfection of siRNA to LS-174T cells was carried out using Lipofectamine® RNAiMAX (Thermo Fischer Scientific, Waltham, MA). Cells were transfected with 5 pmol of siRNA in a 24-well culture dish (4×10^5 cells/mL) as described [25–27].

2.6. Statistical analysis

All values for real-time RT-PCR were expressed as mean ± SE. The data were analyzed by unpaired two-tailed *t*-test using GraphPad Prism6 (GraphPad Software, La Jolla, CA). *P* value of < 0.05 was considered to be statistically significant.

3. Results

3.1. Expression of the REG family genes in IBD colon mucosa

REG family gene activation in the colon was evaluated in 88 patients with IBD (39 UC and 49 CD patients) and 44 controls (normal mucosa of surgical resected colon cancer specimens) from Saiseikai Nara Hospital and Nara Medical University Hospital. Patients with IBD were younger than control patients (mean ± SE ages of UC, CD, and control were 43.07 ± 2.58, 33.21 ± 2.32, and 66.78 ± 1.65 years old, respectively).

We extracted total RNA from the colon specimens and measured all *REG* family (*REG Iα*, *REG Iβ*, *REG III*, *HIP/PAP*, and *REG IV*) mRNA expression by real-time RT-PCR and found that *REG Iα*, *REG Iβ*, and *REG IV* genes were overexpressed in CD colon samples (Fig. 1A, B, and E). The *REG IV* gene was also overexpressed in UC colon samples (Fig. 1E). In contrast, the expression of type III *REG* genes (*REG III* and *HIP/PAP*) was not changed in IBD colon samples (Fig. 1C and D).

3.2. Transcription of REG Iα is activated by IL-22 and IL-6 via MZF1, RTEF1/TEAD4, and STAT3

IL-6 and IL-8 are reported to induce *REG* family mRNA in vitro [8,28,29] and in vivo [22,30], and IL-22 [31], TNFα [32], IL-17A [33], bFGF [34], HGF [35], and EGF [34] are also reported to induce *REG* family mRNA(s). To verify whether *REG Iα* gene expression is up-regulated by cytokine(s)/growth factor(s) in colon epithelial cells, we introduced luciferase reporter plasmid(s) containing *REG Iα* promoter (− 1190 to + 26) into colon epithelial cells (LS-174T and HT-29), stimulated by IL-6, IL-8, IL-17A, IL-22, TNFα, bFGF, HGF, or EGF, and measured transcriptional activities. As shown in Fig. 2A and B, *REG Iα* transcription was significantly increased by the addition of IL-6 and IL-22, and the IL-22-induced promoter activity of *REG Iα* showed greater increase than the IL-6-induced promoter activity in both LS-174T and HT-29 human colon cells.

To map out the *cis*-element of *REG Iα* promoters that are responsible for *REG Iα* transcription in response to IL-22/IL-6 in colon epithelial cells, several lengths of *REG Iα* promoters were fused to the luciferase gene. After transfection of the reporter plasmid, cells were stimulated by IL-22 or IL-6 and transcriptional activity was measured. As shown in Fig. 2C, the deletion down to position − 220 in *REG Iα* promoter did not alter significantly the expression of the IL-22-induced reporter activation, but additional deletions to nucleotide − 211 (− 220 to − 211), − 179 to − 156, and − 146 to − 130 caused remarkable decreases of promoter activities. These results indicate that the regions of − 220 to − 211, − 179 to − 156, and − 146 to − 130 contain essential *cis*-elements for the IL-22 induced *REG Iα* promoter activities. A computer-aided search for sequences similar to known *cis*-acting element(s) revealed that each region has a possible binding site for MZF1, RTEF1/TEAD4, and STAT3, respectively.

We also tested nested deletion constructs of *REG Iα* reporter plasmids in the IL-6-induced *REG Iα* promoter activation. The deletion analyses showed that the essential regions for the IL-22-induced *REG Iα* promoter activation were also essential for the IL-6-induced *REG Iα* promoter (Fig. 2D), indicating that IL-6-induced *REG Iα* activation occurs in the same mechanism as IL-22-induced *REG Iα* activation.

To investigate the significance of MZF1, RTEF1/TEAD4, and STAT3 in IL-22-induced *REG Iα* expression in colon epithelial cells, we used RNA interference of *MZF1*, *RTEF1/TEAD4*, and *STAT3* to identify whether these factors are essential for the IL-22-induced transcription of the *REG Iα* gene. As shown in Fig. 2E, the introduction of either *MZF1*, *RTEF1/TEAD4*, or *STAT3* siRNA into the LS-174T cells significantly reduced the IL-22-induced expression of *REG Iα* mRNA as compared to no siRNA or scrambled siRNA introduction.

3.3. Transcription of REG Iβ is activated by IL-22 via HLTF/FOXN2

IL-22 showed significant increase in *REG Iβ* promoter activation, but the other cytokines/growth factors did not (Fig. 3A). Progressive deletions of the *REG Iβ* promoter gene were performed, the deleted constructs were transfected into LS-174T cells, and the IL-22-induced promoter activities were measured. The deletion analyses of *REG Iβ* promoter revealed that the − 274 to − 260 region was important for the IL-22-induced *REG Iβ* promoter activation (Fig. 3B), and that the region contains a possible HLTF/FOXN2 binding site.

To investigate the significance of HLTF/FOXN2 for *REG Iβ* expression in colon epithelial cells, we used RNA interference of *HLTF/FOXN2* to verify whether HLTF/FOXN2 is essential for the IL-22-induced transcription of *REG Iβ* gene. As shown in Fig. 3C, the introduction of *HLTF/FOXN2* siRNA significantly reduced the IL-22-induced *REG Iβ* mRNA as compared to no siRNA or scrambled siRNA introduction.

3.4. GATA6 is required for REG IV expression

In *REG IV* promoter, no enhanced promoter activity was detected in all the cytokine/growth factor additions (IL-6, IL-8, IL-17A, IL-22, TNFα, HGF, bFGF, and EGF) in LS-174T cells. On the other hand, TNFα significantly decreased the *REG IV* promoter activity in LS-174T cells (Fig. 4A).

Concerning the TNFα-induced suppression of *REG IV* transcription, we tested siRNAs for *CDX2*, *SOCS3*, and *GATA6* as CDX2 [36] and GATA6 [37] were reported to be involved in *REG IV* gene expression and TNFα was reported to control SOCS3 expression [38], and found that *GATA6* siRNA abolished the TNFα-induced suppression of *REG IV* expression as compared to no siRNA or scrambled siRNA (Fig. 4B). This indicates that GATA6 but not CDX2 nor SOCS3 is an essential transcription factor for *REG IV* expression in colon epithelial cells and is responsible for the TNFα-induced suppression of *REG IV* expression.

4. Discussion

The pathophysiology of IBD such as UC and CD reflects a balance between mucosal injury related to an ongoing inflammatory process and mucosal reparative mechanisms. As proliferative mucosal factors may offer new therapeutic paradigms, Dieckgraefe et al. searched such candidate genes and found that *REG* family genes (*REG Iα*, *REG Iβ*, and *HIP/PAP*) were highly expressed in IBD colonic mucosa [17]. Ogawa et al. found that *Reg IIIβ* and *Reg IIIγ* were overexpressed in sodium dextran sulfate-induced mouse colitis as well as in a murine bacterial reconstitution model, and that *HIP/PAP* was overexpressed in human IBD colonic mucosa [15]. Numerous papers on expression analyses in

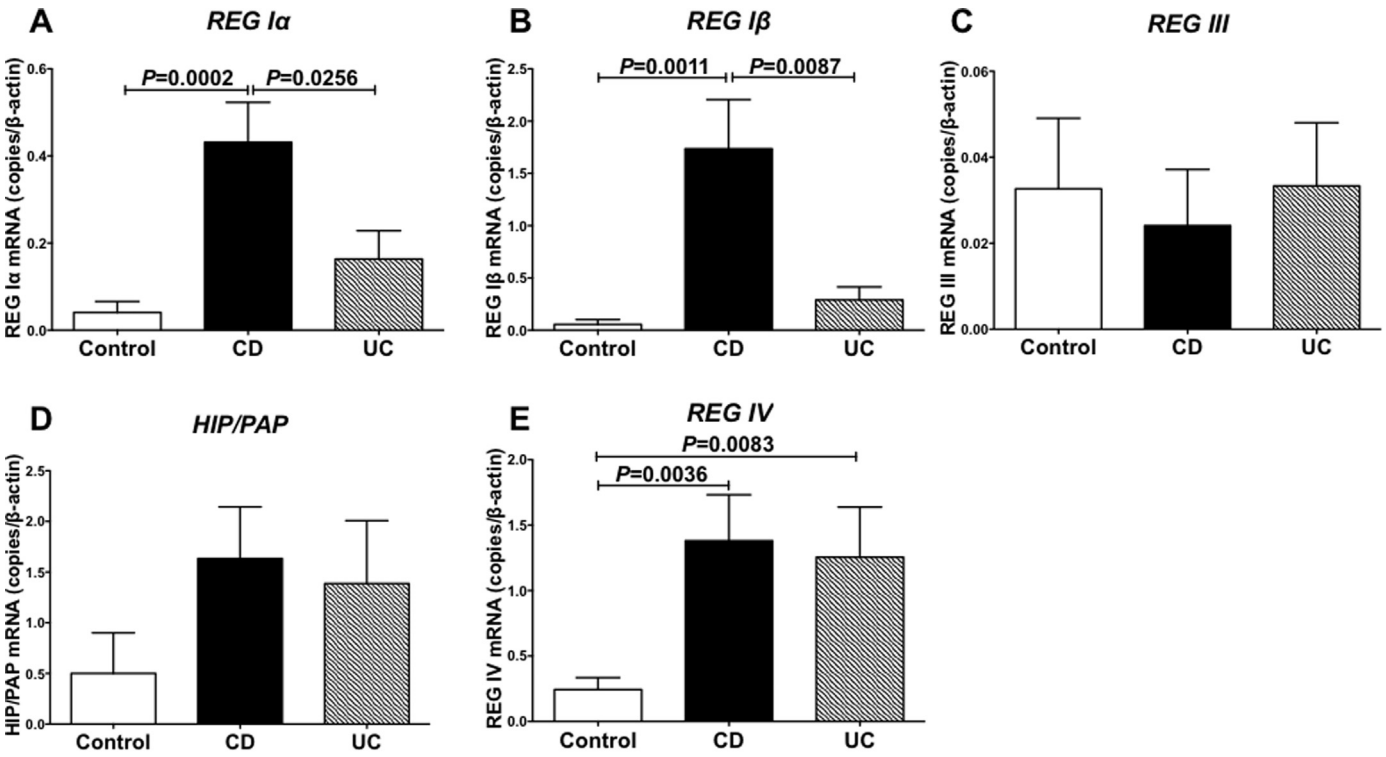

Fig. 1. Expression of the *REG* family mRNAs in CD and UC colons. Expression of *REG Iα* (A), *REG Iβ* (B), *REG III* (C), *HIP/PAP* (D), and *REG IV* (E) was measured by real-time RT-PCR. The mRNA levels of *REG Iα*, *REG Iβ*, and *REG IV* in the CD colon were significantly higher than those of the control ($P = 0.0002$, 0.0011, and 0.0036, respectively). The mRNA level of *REG IV* in the UC colon was higher than that of the control ($P = 0.0083$). The mRNA levels of *REG III* and *HIP/PAP* were not different among control, CD, and UC. Data are expressed as mean ± SE for each group (n = 44 (Control), 49 (CD), and 39 (UC)). The statistical analyses were performed using Student's *t*-test.

one or more members of *REG* family genes in IBD mucosa followed these findings [16,18,19,31,39]. Despite these reports, expression of all members of *REG* family genes in IBD lesions has not been clarified. In the present study, we analyzed expression of all *REG* family mRNAs (*REG Iα*, *REG Iβ*, *REG III*, *HIP/PAP*, and *REG IV*) in CD and UC samples and found that *REG* family genes (*REG Iα*, *REG Iβ*, and *REG IV*) were overexpressed in CD colons and that the *REG IV* gene was also overexpressed in UC samples. In the present study, we used normal parts of resected colon cancer as normal 'control' and therefore the patients (CD and UC) were much younger than 'control'. As Perfetti et al. showed that mouse pancreatic *Reg I* (mouse counterpart of *REG Iα/REG Iβ*) mRNA levels declined with age [40], we additionally analyzed 21 much younger control samples (mean age 15.6 years old) and found that mRNA levels of *REG Iα*, *REG Iβ*, and *REG IV* in the younger controls were not significantly different from those of 'control' in Fig. 1 (data not shown). In addition, we also analyzed *REG* family gene expression in another IBD, Celiac disease [41]. In subjects from Barcelona (12 Celiac disease patients before gluten-free diet and 10 age-related controls), we found that any of the *REG* family genes were not overexpressed in Celiac disease samples (Supplemental Fig. 1), suggesting that the *REG* family gene expression and its role(s) in disease progression are different among CD, UC, and Celiac disease, and the significance of the *REG* family gene expression as biomarkers is different. The autoimmune condition of Celiac disease and the role of some REG proteins as autoantigens [22,42, 43] may contribute to explain these differences.

Originally, *Reg* was isolated as a gene specifically expressed in regenerating rat islets induced by 90% pancreatectomy with poly(ADP-ribose) polymerase inhibitor (such as nicotinamide and 3-aminobezamide) administrations [2,3]. The *Reg* and *Reg*-related genes were isolated and were revealed to constitute a multigene family, the *Reg* gene family [1,2]. Based on the primary structures of the Reg proteins, the members of the family are grouped into four subclasses; types I, II, III, and IV [1]. In humans, five *REG* family genes, i.e., *REG Iα* [3,9], *REG Iβ* [44], *REG*-related sequence (pseudogene) (*RS*) [9], *HIP/PAP* [45, 46], and *REG III* [47] are tandemly ordered in the 95 kbp region of chromosome 2p12 [48], whereas *REG IV* locates on chromosome 1 [49]. In mouse genome, all *Reg* family genes except for *Reg IV* (i.e., *Reg I*, *Reg II*, *Reg IIIα*, *Reg IIIβ*, *Reg IIIγ*, and *Reg IIIδ*) were mapped to a contiguous 75 kbp region of chromosome 6 C [50], whereas *Reg IV* was mapped on chromosome 3. Type I (and type II) Reg proteins are expressed in regenerating islets [1] and are involved in β-cell regeneration [4,5,42, 51]. Reg family proteins have been suggested to be involved in cellular proliferation in gastrointestinal cells [52], hepatic cells [53], cardiovascular cells [30] and neuronal cells. Importantly, mouse Reg III was shown to be a Schwann cell mitogen that accompanies the regeneration of motor neurons [54], and Reg protein functions as a neurotrophic factor for motor neurons [54, 55]. Reg protein was also shown to mediate gastrointestinal epithelial cell proliferation in rats [56, 57]. Yonemura et al. showed that the expression of the *REG Iα* gene is closely related to the infiltrating property of gastric carcinoma, and may be a prognostic indicator of differentiated adenocarcinoma of the stomach [11]. In fact, following publication of this paper, correlations were reported between *REG* family gene expression and cancer prognosis [11,12,24,58]. These observations suggest that the *Reg* gene family is involved in cell proliferation in a variety of cell types, including gastrointestinal cells.

In the present study, overexpression of *REG Iα*, *REG Iβ*, and *REG IV* in CD and of *REG IV* in UC colon mucosa was detected. It was reported previously that the *REG* family genes were expressed not only in various human inflammatory diseases such as gastritis [59], pancreatitis [60], salivary glanditis [22,26], and colitis [15], but also in various experimental models of inflammation in animal tissues [30,61]. *REG Iα* and *REG Iβ* were recently reported to be upregulated in human intestine during *Entamoeba histolytica*-infected acute colitis, and intestinal epithelia cells from *Reg I* (mouse counterpart of *REG Iα/REG Iβ*) knockout mice were found to be more susceptible to spontaneous, and parasite-induced, apoptosis [62], suggesting that *REG Iα* and *REG Iβ* could function to protect the intestinal epithelium from parasite-induced

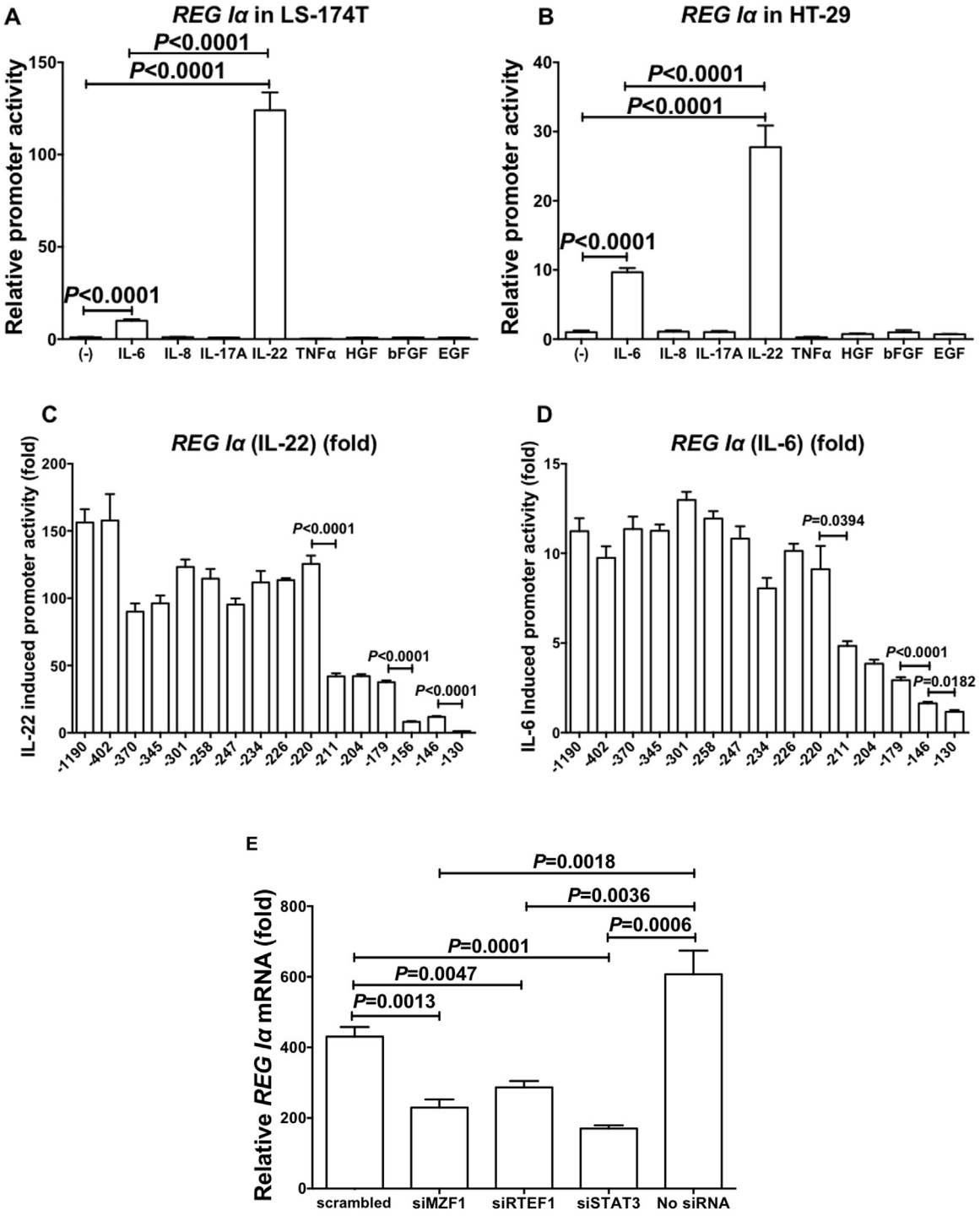

Fig. 2. Transcriptional activation of *REG Iα* by IL-22 and IL-6 via MZF1, RTEF1, and STAT3. Promoter activities of *REG Iα* in LS-174T (A) and HT-29 cells (B) stimulated with IL-6, IL-8, IL-17A, IL-22, TNFα, HGF, bFGF, or EGF were measured. The statistical analyses were performed using Student's *t*-test. Deletion analysis of *REG Iα* stimulated by IL-22 (C) and IL-6 (D). Human LS-174T cells were transfected with constructs containing various deletion mutants of *REG Iα* promoter. Constructs listed on ordinate are numbered according to their 5' terminus in the *REG Iα* promoter. The transfected cells were stimulated with IL-22 (20 ng/mL) (C) or IL-6 (20 ng/mL) (D), after which the luciferase activities were measured. The diagram represents fold increase of luciferase activities to the untreated cells. All data are represented as the mean ± SE of the samples (n = 4). The statistical analyses were performed using Student's *t*-test against no addition. Effects of siRNA transfection on IL-22-induced increase of *REG Iα* mRNA in LS-174T cells (E). After siRNA was introduced, LS-174T human colon epithelial cells were stimulated with IL-22 (20 ng/mL). The expression of *REG Iα* (E) mRNA was measured by real-time RT-PCR using β-actin as an endogenous control. Data are expressed as mean fold (vs no addition) ± SE for each group (n = 4). The statistical analyses were performed using Student's *t*-test.

apoptosis. Thus, it is most likely that inflammation, regardless of whether it is autoimmune associated, is a key event that triggers *REG* family gene expression in many tissues. Therefore, whether *REG* family overexpression is associated directly with the immune disorder in patients with IBD is an interesting question. We performed real-time RT-PCR analyses of all *REG* family genes in IBD samples and found overexpression of *REG Iα*, *REG Iβ*, and *REG IV* in CD and of *REG IV* in UC colon mucosa (Fig. 1). These results support the idea that *REG* family mRNA overexpression is associated with inflammation triggered by autoimmune disorders such as IBD.

It was reported that *Reg* gene expression was regulated by several factors, such as nicotinamide [28,30], glucocorticoids [28], nutrient

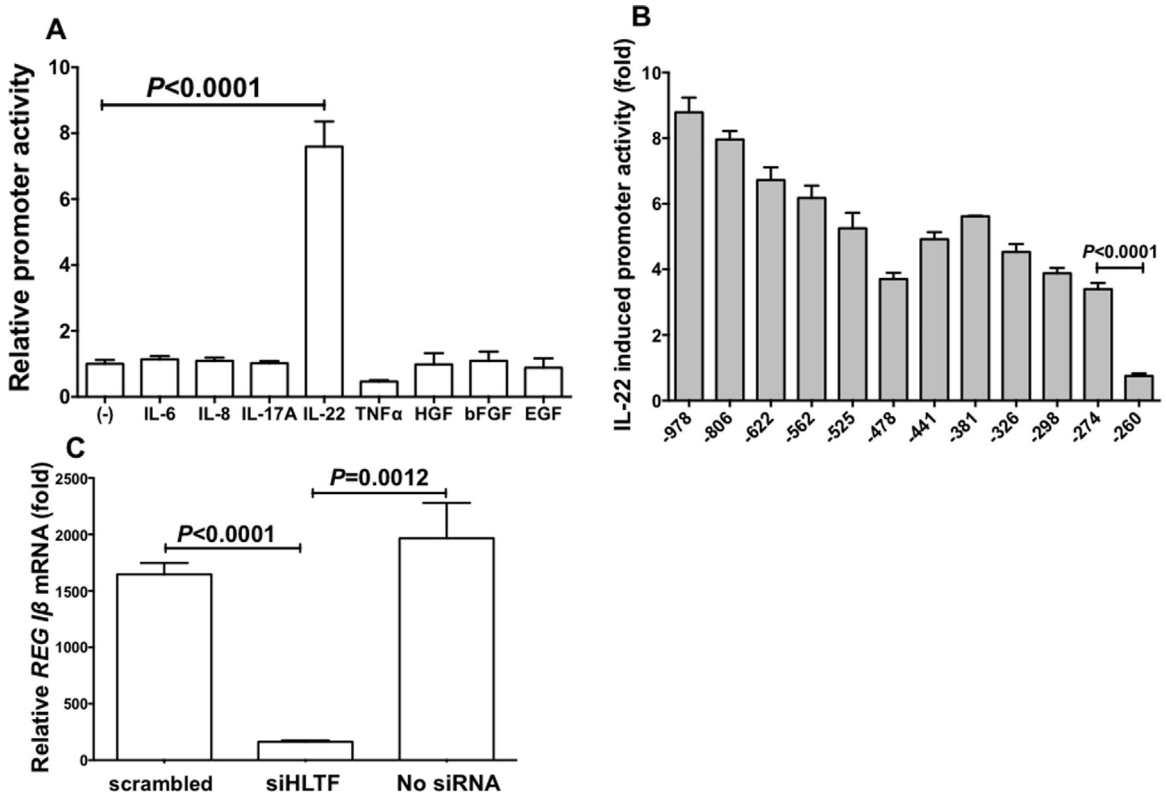

Fig. 3. Transcriptional activation of *REG Iβ* by IL-22 via HLTF. (A) Promoter activities of *REG Iβ* in LS-174T cells stimulated with IL-6, IL-8, IL-17A, IL-22, TNFα, HGF, bFGF, or EGF were measured. The statistical analyses were performed using Student's *t*-test. (B) Deletion analysis of *REG Iβ* promoter. Human LS-174T cells were transfected with constructs containing various deletion mutants of *REG Iβ* promoter. Constructs listed on ordinate are numbered according to their 5' terminus in the *REG Iβ* promoter. The transfected cells were stimulated with IL-22 (20 ng/mL), after which the luciferase activities were measured. The diagram represents fold increase of luciferase activities to the untreated cells. All data are represented as the mean ± SE of the samples (n = 4). The statistical analyses were performed using Student's *t*-test against no addition. (C) Effects of siRNA transfection on IL-22-induced increase of *REG Iβ* mRNA in LS-174T cells. After siRNA was introduced, LS-174T human colon epithelial cells were stimulated with IL-22 (20 ng/mL). The expression of *REG Iβ* mRNA was measured by real-time RT-PCR using β-actin as an endogenous control. Data are expressed as mean fold (vs no addition) ± SE for each group (n = 4). The statistical analyses were performed using Student's *t*-test.

factors [63], IL-6 [25,26,28], IL-8 [29], IL-17A [62], IL-22 [31], TNFα [32], HGF [35], bFGF [34], and EGF [34]. We tested the induction of *REG Iα*, *REG Iβ*, and *REG IV* by IL-6, IL-8, IL-17A, IL-22, TNFα, HGF, bFGF, and EGF and found that *REG Iα* was induced by IL-22/IL-6 via transcription factor(s) MZF1, RTEF1/TEAD4, and STAT3 (Fig. 1) and that *REG Iβ* was induced by IL-22 via HLTF/FOXN2 (Fig. 2). Recent reports have identified a crucial role for IL-22 in the regulation of both gut inflammation and epithelial barrier integrity. *IL-22* gene delivery leads to rapid amelioration of dextran sodium sulfate-induced colitis [65], whereas *IL-22* knockout mice show increased intestinal epithelial damage, along with systemic bacterial burden and significantly increased mortality [66]. Sekikawa et al. reported that IL-22 induced *REG Iα* expression via STAT3 in UC [31]. IL-6 signaling is thought to be of central importance for the maintenance of chronic intestinal

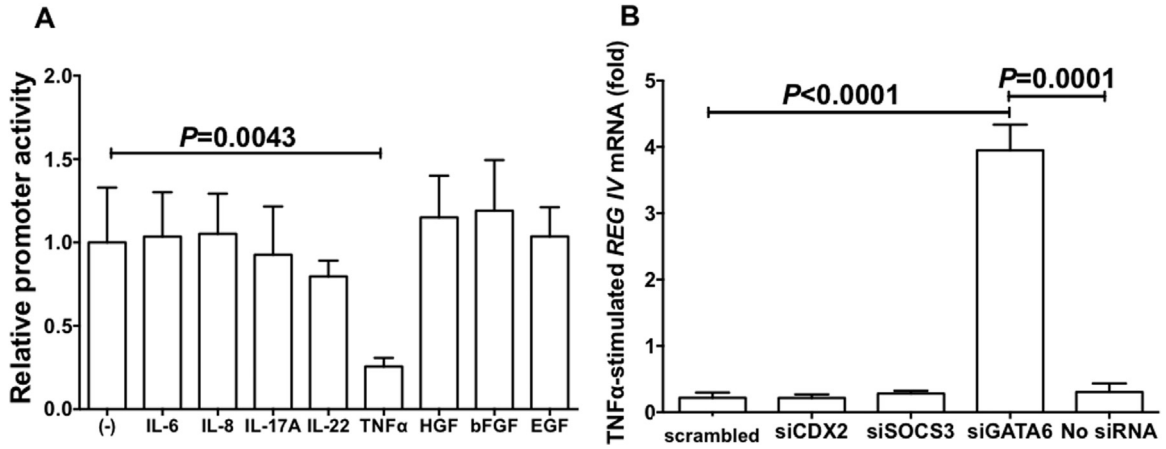

Fig. 4. Requirement of GATA6 in *REG IV* expression. (A) Promoter activities of *REG IV* in LS-174T cells stimulated with IL-6, IL-8, IL-17A, IL-22, TNFα, HGF, bFGF, or EGF were measured. The statistical analyses were performed using Student's *t*-test. (B) Effects of siRNA transfection on TNFα-induced decrease of *REG IV* mRNA. After siRNA was introduced, LS-174T human colon epithelial cells were stimulated with TNFα (20 ng/mL). The expression of *REG IV* mRNA was measured by real-time RT-PCR using β-actin as an endogenous control. Data are expressed as mean fold (vs no addition) ± SE for each group (n = 4). The statistical analyses were performed using Student's *t*-test.

inflammation in IBD such as CD and UC. IL-6 has also been implicated in the pathogenesis of colorectal cancer. In fact, IL-6 directly promotes tumor cell proliferation and survival through STAT3 activation. Due to its role in both types of diseases, IL-6 has been proposed as a missing link between inflammation and tumor development in the colon [67].

In this study, we showed the IL-22/IL-6-induced up-regulation of *REG Iα* and *REG Iβ* in LS-174T and HT-29 human colon cells and their molecular mechanism(s) in which several transcription factors (MZF1, RTEF1/TEAD4, and STAT3 in *REG Iα*, and HLTF/FOXN2 in *REG Iβ*) were involved. Recently, MZF1 was reported to be involved in colorectal cancer [68], and RTEF1/TEAD4 was associated with increased growth of gastric cancer [69]. Accumulating evidence indicates that IL-22/IL-6-STAT3 signaling is important in tumorigenesis and tumor growth of colitis-associated colorectal cancer [70]. HLTF/FOXN2 was reported to be associated with lower prognosis of glioblastoma patients [71]. These associations between transcription factors and cancer tumor growth/poor prognosis of cancer patients may be mediated via up-regulation of *REG Iα/REG Iβ*.

Up-regulation of *REG IV* gene in IBD has been reported previously [34,39,72]. However, the mechanism of *REG IV* expression in the colon is still controversial: Involvements of CDX2 [36] and GATA6 [37] were reported. In the present study, we found that *REG IV* mRNA was significantly up-regulated in UC and CD samples and down-regulated by the addition of TNFα in cultured colon cells. We therefore introduced siRNAs for *CDX2*, *GATA6*, and *SOCS3* into LS-174T colon cells and evaluated the effect of the siRNAs on the TNFα-induced suppression. The introduction of *GATA6* siRNA, but not *CDX2* nor *SOCS3* siRNA, was significantly attenuated the TNFα-induced *REG IV* suppression (Fig. 4), indicating that GATA6 is essential for *REG IV* expression and TNFα-induced *REG IV* suppression in colon epithelial cells.

Up-regulation of *REG Iα*, *REG Iβ*, and *REG IV* may have two conflicting sides like Dr. Jekyll and Mr. Hyde. The expression induces to protect/recover the intestinal epithelium from immune-mediated damages by proliferation of intestinal epithelial cells by REG family proteins in UC and CD. On the other hand, it may sometimes result in an increased risk of colon cancer. Therefore, long lasting IL-22/IL-6 production and the IL-22/IL-6-induced *REG* family gene up-regulation should be monitored and controlled for healthy recovery of intestinal epithelium. After recovery of damaged epithelium, anti-IL-22/anti-IL-6 antibodies or anti-REG family proteins may be a possible new preventive therapy to reduce colon cancer risk in UC and CD patients.

In the present study, we analyzed the expression of all *REG* family genes and found overexpression of *REG Iα*, *REG Iβ*, and *REG IV* in IBD. We tested cytokines that were reported to regulate the expression of the *Reg* family genes, regardless of whether they activate them, and found that IL-22/IL-6 up-regulated *REG Iα* via MZF1, RTEF1/TEAD4, and STAT3 and that IL-22 up-regulated *REG Iβ* via HLTF/FOXN2 in human colon cells. We also found that TNFα down-regulated *REG IV* expression via GATA6 in colon epithelial cells.

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2017.10.003.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2017.10.003.

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