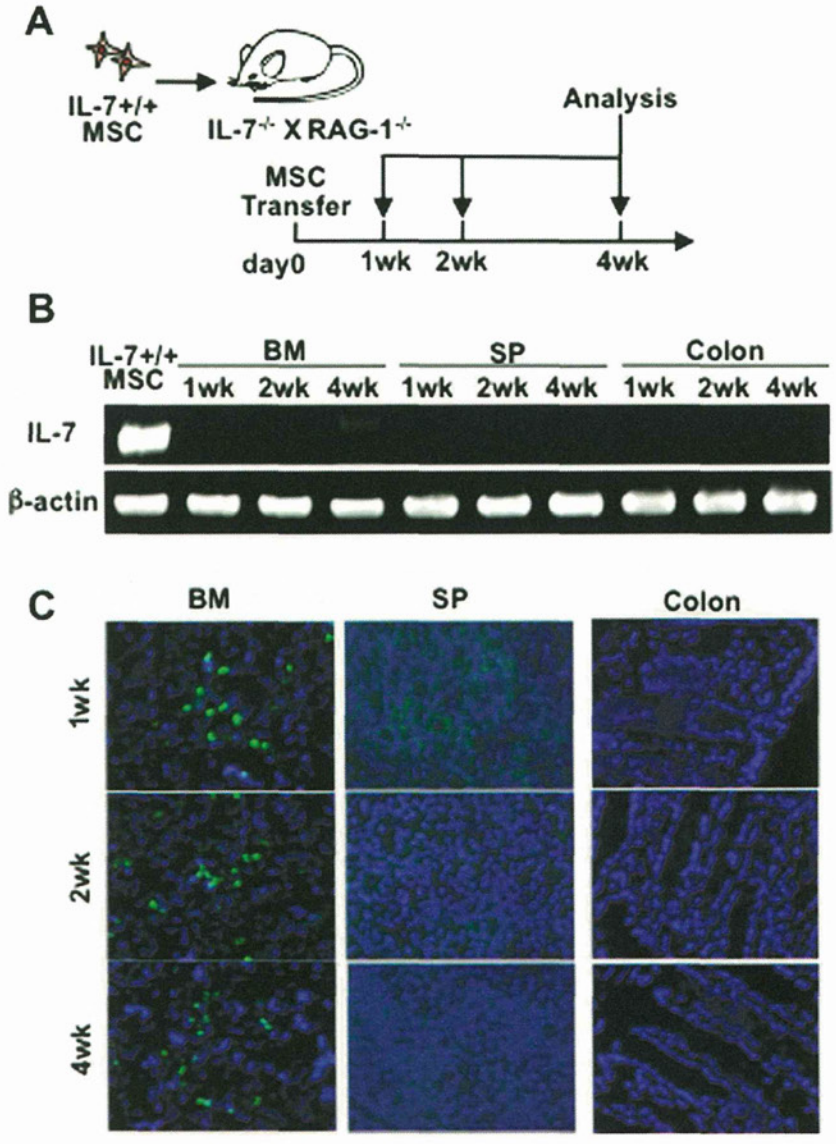

Figure 4 Time course analysis of IL-7 expression in the bone marrow (BM), spleen and colon of IL-7$^{-/-}$×RAG-1$^{-/-}$ mice pre-injected with IL-7$^{+/+}$ mesenchymal stem cells (MSC). (A) Experimental design. IL-7$^{-/-}$×RAG-1$^{-/-}$ mice were injected intravenously with IL-7$^{+/+}$ MSC. At 1, 2 and 4 weeks after the transfer, the BM, spleen (SP) and colon of the mice were collected and checked for the expression of IL-7. (B) IL-7 mRNA expression in the BM, spleen and colon at each time point as assessed by quantitative reverse transcription PCR. (C) IL-7 protein expression in the BM, spleen and colon at each time point as assessed by immunohistochemistry. IL-7 (green) and DAPI (blue).

into four groups as follows: RAG-1$^{-/-}$ mice injected with CD4^{+}CD45RB^{high} T cells and IL-7$^{+/+}$ MSC (IL-7$^{+/+}$ MSC); RAG-1$^{-/-}$ mice injected with CD4^{+}CD45RB^{high} T cells and IL-7$^{-/-}$ MSC (IL-7$^{-/-}$ MSC); RAG-1$^{-/-}$ mice injected with CD4^{+}CD45RB^{high} T cells (RB^{high}) as a positive control; and RAG-1$^{-/-}$ mice injected with CD4^{+}CD45RB^{high} T cells and CD4^{+}CD25^{+} T cells (RB^{high}+T$_{reg}$) as a negative control (figure 6A). Both IL-7$^{+/+}$ MSC and IL-7$^{-/-}$ MSC groups developed a wasting disease and colitis with a thickened colon and splenomegaly to the same extent as that in the RB^{high} group, while the RB^{high}+T$_{reg}$-negative control group gained weight and did not develop colitis (figure 6B–F). A large number of LP CD4 T cells was recovered from IL-7$^{+/+}$ MSC, IL-7$^{-/-}$ MSC, and RB^{high} groups, while only a small number of LP CD4 T cells was recovered from the RB^{high}+T$_{reg}$ group (figure 6G). As shown in figure 6H, on in-vitro stimulation, LP CD4 T cells from IL-7$^{+/+}$ MSC, IL-7$^{-/-}$ MSC and RB^{high} groups produced equal and significantly higher amounts of IFN-γ, TNFα and IL-17 than those by the RB^{high}+T$_{reg}$ group. These data indicated that, at least in our present in-vivo model, neither IL-7$^{+/+}$ MSC nor IL-7$^{-/-}$ MSC could suppress the development of colitis, even when they were transferred in combination with CD4^{+}CD45RB^{high} T cells. Although we performed multiple injections of IL-7$^{+/+}$ and IL-7$^{-/-}$ MSC to suppress colitis and evaluate a therapeutic effect, colitis could not be suppressed in terms of clinical and histological scores, the number of infiltrated LP CD4 T cells and cytokine production (see supplementary figure S6A–G, available online only).

DISCUSSION

BM MSC have previously been identified as progenitors of mesenchymal tissues by migrating to injured tissues to repair them,[19–21] and transplantation of BM MSC for tissue repair has been proposed based on their stem cell qualities. Moreover, recent studies suggest that cultured MSC play a second role in the induction of peripheral tolerance by inhibiting the release of proinflammatory cytokines and interacting with various kinds of immune cells. However, the present study clearly shows that: (1) BM MSC produce IL-7; (2) MSC have the potential to support the proliferation and survival of colitogenic CD4 T$_{EM}$ cells; and notably (3) transplantation of BM MSC into IL-7$^{-/-}$×RAG-1$^{-/-}$ mice induces colitis when the mice are later injected with CD4^{+}CD45RB^{high} T cells; and (4) IL-7 expression is maintained in the BM of IL-7$^{-/-}$×RAG-1$^{-/-}$

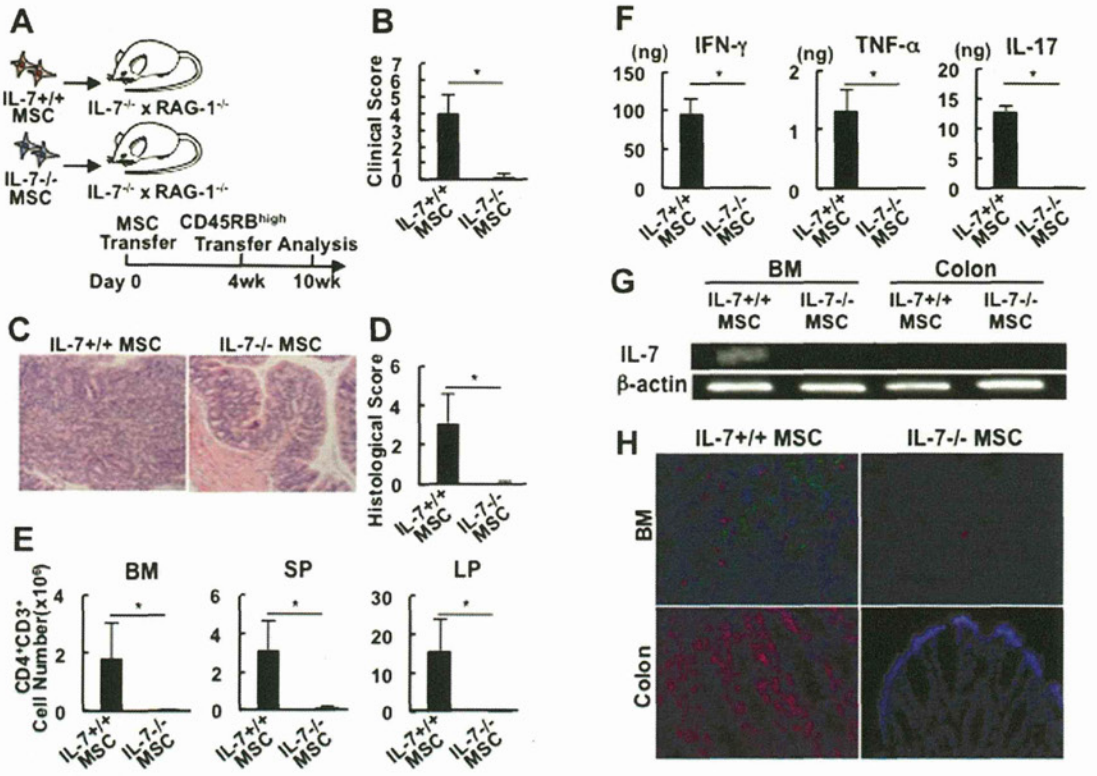

Figure 5 $CD4^+CD45RB^{high}$ T-cell-injected IL-$7^{-/-}$×RAG-$1^{-/-}$ mice pretransplanted with IL-$7^{+/+}$, but not IL-$7^{-/-}$, mesenchymal stem cells (MSC) develop colitis. (A) Experimental design. Mice were divided into two groups (n=5). Each group was injected intraperitoneally with $CD4^+CD45RB^{high}$ T cells at 4 weeks after MSC transfer. (B) Clinical scores. Data are shown as the mean±SEM for five mice in each group, *p<0.05. (C) Histological results for the colons of each group. Original magnification ×200. (D) Histological scores. Data are shown as the mean±SEM for five mice in each group, *p<0.05. (E) Absolute number of lamina propria (LP) $CD3^+CD4^+$ T cells from the colon at 10 weeks after transfer. Data are shown as the mean±SEM. N.S. not significant, *p<0.01. (F) Cytokine production by LP CD4 T cells stimulated in vitro. IFN-γ, TNFα and IL-17 concentrations in culture supernatants were measured by ELISA. Data are shown as the mean±SEM for five mice in each group, *p<0.05. (G) Expression of IL-7 mRNA in the bone marrow (BM) and LP of IL-$7^{+/+}$ MSC- or IL-$7^{-/-}$ MSC-transplanted mice as measured by reverse transcription PCR. (H) Expression of IL-7 protein in the BM and LP of each group of mice as detected by immunohistochemistry.

mice transplanted with BM MSC. The present study thus suggests the possible participation of IL-7-producing BM MSC as niche cells to maintain colitogenic CD4 memory T cells. Although it is possible that IL-7 produced in BM leads to levels of circulating IL-7 that support local (intracolonic) expansion of T-cell populations, rather than facilitating the formation of a niche in BM for these cells, we previously demonstrated that intrarectally administered colitogenic CD4 T cells surprisingly egress to the colon, migrate to BM.[18] In addition, we have reported that IL-$7^{-/-}$×RAG-$1^{-/-}$ host mice combined with colitic RAG-$2^{-/-}$ donor mice as a parabiosis develop colitis without IL-7 expression in any organ.[12] Therefore, we concluded that colitogenic memory CD4 T cells as 'memory stem cells' may be supported in some specific niche, such as BM, in which IL-7 is abundant even when in the acute phase of colitis.

Because we used IL-7 as a marker of MSC in this setting, it is possible that transferred MSC can spread to many tissues including inflamed colonic mucosa to become differentiated cells but lose expression of IL-7. However, it remains unknown why IL-7 was not detected in the inflamed colon of IL-$7^{-/-}$× RAG-$1^{-/-}$ mice after transfer of IL-$7^{+/+}$ MSC with $CD4^+CD45RB^{high}$ T cells, although MSC-derived adipocytes that expressed IL-7 could not be detected (figure 2C,D). Nevertheless, it is noteworthy that IL-7 production by the transferred MSC was maintained only in the BM regardless of their differentiation status. Therefore, we propose that, in addition to the two major roles previously reported, namely tissue repair[19–21] and immune suppression,[19 20] BM MSC-derived IL-7 is positively involved in the perpetuation of chronic inflammatory diseases by forming the niche for pathogenic CD4 memory T cells in BM (figure 7). Although many differences exist between our colitis model induced by a lymphopenic driver, other murine models, such as dextran sodium sulfate-induced acute colitis model, and human IBD, we propose a pathological role of IL-7-producing MSC at least in our model. Furthermore, the present study supports a conceptual change of IBD from an intestinal to a systemic disease, and suggests therapeutic approaches that target BM MSC-derived IL-7 for the treatment of IBD.

From a clinical viewpoint, we have previously demonstrated that IL-7 protein in the serum of patients with ulcerative colitis (UC) is higher than that in healthy controls.[24] Furthermore, *IL7R* has previously been identified as one of the disease susceptibility genes of UC.[25] Therefore, it may be interesting to compare the IL-7 levels in BM, especially in BM MSC, between IBD patients and healthy controls to determine whether BM MSC are responsible for IL-7 production in the pathogenesis of human UC, and a strategy targeting IL-7 might be a feasible clinical approach for the treatment of UC. Furthermore, the current approach for the induction of remission (the acute stage of the disease) using autologous or allogeneic MSC in patients with intractable UC would be considered based on the present finding that MSC may play a pathological role in the maintenance of colitogenic memory T cells (remission stage). However, we would like to emphasise

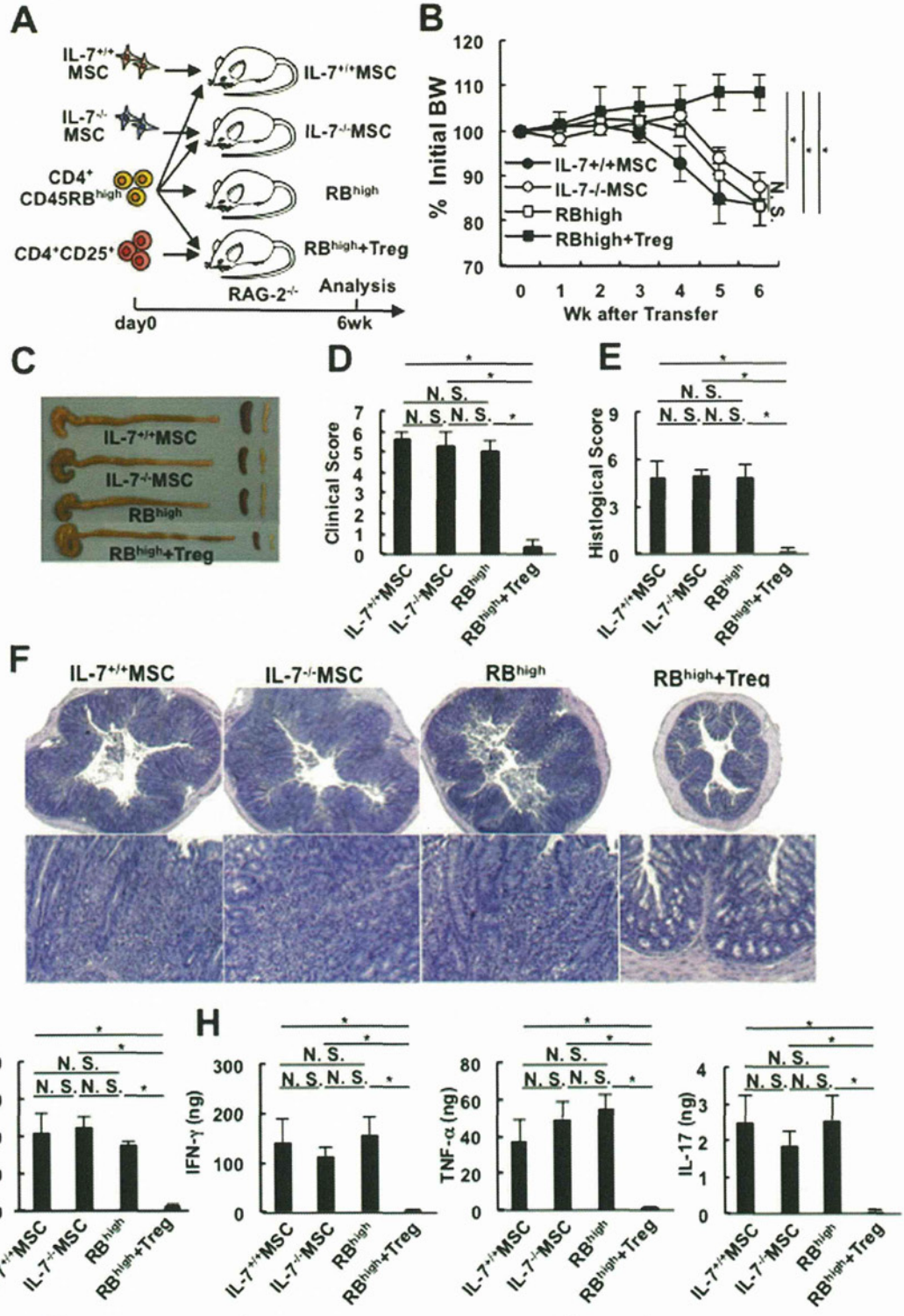

Figure 6 $CD4^+CD45RB^{high}$ T-cell-injected RAG-$1^{-/-}$ recipients pre-injected with either IL-$7^{+/+}$ or IL-$7^{-/-}$ mesenchymal stem cells (MSC) develop colitis. (A) Experimental design. Mice were divided into four groups as follows: RAG-$1^{-/-}$ mice that were pre-injected with $CD4^+CD45RB^{high}$ T cells and IL-$7^{+/+}$ MSC (n=3, IL-$7^{+/+}$ MSC); RAG-$1^{-/-}$ mice that were pre-injected with $CD4^+CD45RB^{high}$ T cells and IL-$7^{-/-}$ MSC (n=3, IL-$7^{-/-}$ MSC); RAG-$1^{-/-}$ mice that were pre-injected with $CD4^+CD45RB^{high}$ T cells (n=3, RB^{high} cells); and RAG-$1^{-/-}$ mice that were pre-injected with $CD4^+CD45RB^{high}$ T cells and $CD4^+CD25^+$ T cells (n=3, RB^{high} + T_{reg} cells). (B) Percentage of the initial body weight (BW) of each group. Data are shown as the mean±SEM for three mice in each group, *p<0.05. (C) Representative gross appearance of the spleen, mesenteric lymph nodes and colon of each group. (D) Clinical scores. Data are shown as the mean±SEM for three mice in each group, *p<0.05. (E) Histological scores. Data are shown as the mean±SEM for three mice in each group, *p<0.05. (F) Histopathology of the distal colon of the indicated mice at 6 weeks after transfer. Original magnification, ×40 upper panel and ×200 lower panel. (G) Absolute number of lamina propria (LP) $CD3^+CD4^+$ T cells from the colon at 6 weeks after transfer. Data are shown as the mean±SEM. N.S. not significant, *p<0.01. (H) Cytokine production by LP CD4 T cells. LP CD4 T cells were isolated at 6 weeks after transfer and stimulated with anti-CD3 and anti-CD28 antibodies for 48 h. IFN-γ, TNFα and IL-17 concentrations in culture supernatants were measured by ELISA. Data are shown as the mean±SEM for three mice in each group, *p<0.05.

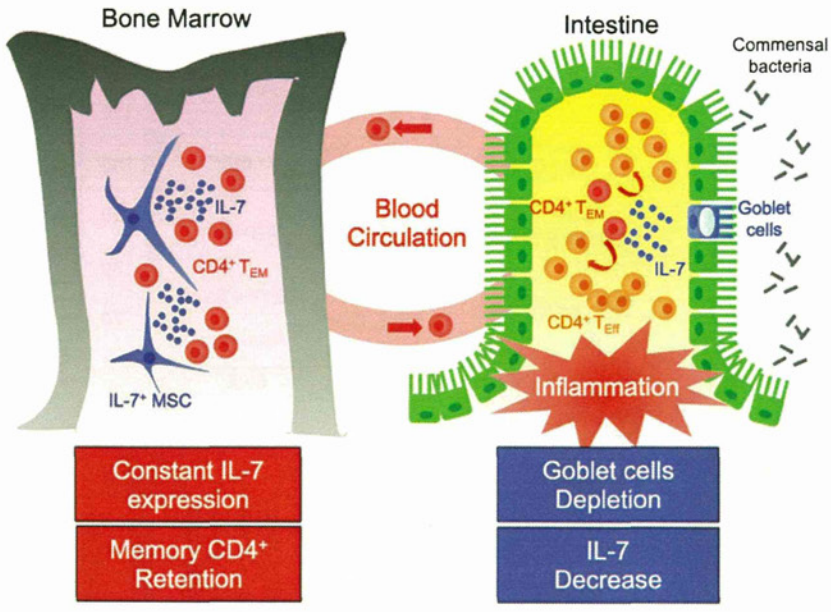

Figure 7 Model for the maintenance of colitogenic CD4 T_{EM} cells by bone marrow (BM) mesenchymal stem cell (MSC)-derived IL-7. BM MSC-derived IL-7 plays a role in the maintenance of colitogenic CD4 T_{EM} cells, and may be a clinical target for the treatment of inflammatory bowel diseases.

that the present results are consistent with the current concept of using MSC to treat human IBD in ongoing clinical trials, because we also confirmed that our cultured MSC suppressed the proliferation of CD4 T cells in the short-term culture system. Finally, it should be emphasised that the strategy of IL-7 blockade is at an immature stage at this time, because IL-7 is essential not only for colitogenic CD4 T cells but also protective memory CD4 T cells such as regulatory T cells. Therefore, further investigation in this field is warranted.

Overall, in support of previous evidence that BM is a reservoir organ for CD4 memory T cells, we demonstrated for the first time that BM MSC express IL-7 and comprise the key population that forms the niche for colitogenic memory CD4 T cells and causes the persistence of chronic colitis.

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Contributors YN helped to design the study, performed experiments, analysed the data, and wrote the paper; TK conceived and designed the study, analysed the data, and wrote the paper; MT performed experiments; SO, TN, RO and KT helped to design the study and MW supervised the study.

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Bone marrow-mesenchymal stem cells are a major source of interleukin-7 and sustain colitis by forming the niche for colitogenic CD4 memory T cells

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The acquisition of malignant potential in colon cancer is regulated by the stabilization of Atonal homolog 1 protein

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ABSTRACT

The transcription factor Atonal homolog 1 (Atoh1) plays crucial roles in the differentiation of intestinal epithelium cells. Although we have reported that the Atoh1 protein was degraded in colon cancer by aberrant Wnt signaling, a recent study has indicated that the Atoh1 protein is expressed in mucinous colon cancer (MC) and signet ring cell carcinoma (SRCC). However, the roles of the Atoh1 protein in MC are unknown. To mimic MC, a mutated Atoh1 protein was stably expressed in undifferentiated colon cancer cells. Microarray analysis revealed the acquisition of not only the differentiated cell form, but also malignant potential by Atoh1 protein stabilization. In particular, Atoh1 enhanced Wnt signaling, resulting in the induction of Lgr5 as a representative stem cell marker with the enrichment of cancer stem cells. Moreover, the fluorescent ubiquitination-based cell cycle indicator system with time-lapse live imaging demonstrated cell cycle arrest in the G0/G1 phase by Atoh1 protein stabilization. In conclusion, the Atoh1 protein regulates malignant potential rather than the differentiation phenotype of MC, suggesting the mechanism by which MC and SRCC are more malignant than non-mucinous adenocarcinoma.

1. Introduction

Pathological differentiation has been one of the most reliable histological criterion to predict the effectiveness of chemotherapy and the prognosis of colon cancer. Many reports support the idea that pathological differentiation is closely related to the progression and chemoresistance of a cancer [1]. However, "pathological differentiation" has often been confused with cell differentiation because the classification of pathological differentiation is based on the ductal formation of cancer cells. Interestingly, cancer cells that maintain the differentiated form such as mucinous carcinoma (MC) and signet ring cell carcinoma (SRCC) have often been classified as undifferentiated tumors on the basis of pathological findings. Nevertheless, the cell differentiation mechanism of colon cancer has not been investigated. One of the most important genes for cell formation is the basic helix–loop–helix (bHLH) transcription factor, Atonal homolog 1 (Atoh1), which is essential for differentiation toward secretory lineages in the small and large intestine [2]. Previous reports have suggested that the Atoh1 gene was suppressed by Wnt signaling in some colon cancers [3]. Moreover, we have demonstrated that the Atoh1 protein was actively degraded in colon cancer by the ubiquitin proteasomal system resulting in the disappearance of the Atoh1 protein in colon cancer despite Atoh1 gene expression [4]. Collectively, the deletion of adenomatous polyposis coli (APC) in colon carcinogenesis causes Atoh1 protein degradation by switching it to become the target of glycogen synthase kinase 3β GSK3β rather than β-catenin, resulting in maintenance of the undifferentiated cellular state [4]. On the other hand, it has been reported that the Atoh1 protein was expressed in MC and SRCC, both of which have secretory capacity [5]. It is notable that MC and SRCC are often classified as poorly differentiated tumors from pathological findings, resulting in a poorer

Abbreviations: Atoh1, Atonal homolog 1; MC, mucinous colon cancer; SRCC, signet ring cell carcinoma; GSK3, glycogen synthase kinase 3; Lgr5, Leucine-rich repeat-containing G-protein coupled receptor 5; IEC, intestinal epithelial cells; MUC2, mucin 2; TFF3, trefoil factor 3; HD6, human defensin 6; TCF4, T-cell factor 4; RT-PCR, reverse-transcription polymerase chain reaction; RLU, relative light units.

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prognosis than sporadic colon cancer. The relationship between pathological differentiation and cellular differentiation in colon cancer has not been clarified. Moreover, the significance of Atoh1 expression in cancer cells for malignant potential is controversial. It has been reported that the deletion of Atoh1 prevents cerebellar neoplasia in a mouse model of medulloblastoma in brain [6], suggesting that Atoh1 acts as a tumor accelerator. In contrast, Atoh1 is reported to be a tumor suppressor gene in Merkel cell carcinoma and colon cancer because Atoh1 suppressed cell proliferation [7]. However, there is no evidence except for the suppression of cell proliferation to confirm that in colon cancer Atoh1 acts as a tumor suppressor. Moreover, previous studies of Atoh1 function in colon cancer have analyzed the Atoh1 gene expression; however, because the expressed Atoh1 protein is degraded, its function has not been elucidated in detail.

In this study, we investigated the functions of Atoh1 in colon cancer to clarify the definition of cancer cell differentiation. We demonstrate that stable expression of the Atoh1 protein in colon cancer induces not only differentiation, but also the promotion of the malignant potential of colon cancer.

2. Materials and methods

2.1. Cell culture and chemicals

Sporadic human colon cancer-derived SW480, DLD1 cells and human embryonic kidney-derived 293T cells were cultured as described previously [8] Plasmid DNA was transfected as described previously [8]. Lentivirus infection was performed according to the manufacturer's protocols. The infected cell lines were supplemented with Blasticidin (7.5 μg/ml, Invitrogen Carlsbad, CA, USA) during maintenance. Oxaliplatin (L-OHP) was used (Tocris Cookson, Ellisville, MI, USA) for evaluating chemoresistance.

2.2. Plasmids

The mCherry-Atoh1 vector was generated by inserting Atoh1 gene into the mCherry DNA template PG27188 (DNA 2.0, Menlo Park, CA, USA). The Atoh1 mutant (5SA-Atoh1) was constructed by PCR-mediated mutagenesis in which five serine residues, TCC (160–162) and AGC (172–174, 328–330, 340–342, 352–354), were replaced with alanine residue GCC. The Atoh1-lentivirus vector was generated by inserting the PCR-amplified mCherry-Atoh1 gene or mCherry-5SA-Atoh1 into pLenti 6.4 (Invitrogen). The S/G2/M-green-lentivirus vector was generated by inserting the PCR-amplified pFucci-S/G2/M-green DNA sequence into pLenti 6.4 (Invitrogen). The lentivirus was generated according to the procedure manual. 5′ Lgr5 reporter plasmid was generated by cloning a 1000 bp sequence 5′of the human Lgr5 gene into a pGL4 basic vector (Promega, Madison, WI). The promoter region of Lgr5 was gradually shortened by 200–1000 bp was generated into a pGL4 basic vector. Polymerase chain reaction-mediated mutagenesis was used to construct internal deletion mutants of the 5′ Lgr5 reporter plasmid in which the following base pair sets −300 to −310, −310 to −320, −320 to −330, −330 to −340, and −340 to −350 were deleted separately.

2.3. Quantitative real-time PCR

Total RNA was isolated with TRIzol® reagent (Invitrogen) according to the manufacturer's instructions as described previously [8]. The primer sequences in this study are summarized in Supplementary Table S1. In all examinations, the expression in LS174T cells (mucinous phenotype colon cancer cell line) was used as standard.

2.4. Western blot analysis

Cells were extracted with 1% sodium dodecyl sulfate (SDS)-containing radioimmunoprecipitation assay (RIPA) buffer as described previously [8]. The membranes were immunoblotted with anti-mCherry (Clontech, Mountain View, CA, USA) and anti-USF2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies before incubation with secondary antibodies.

2.5. Immunofluorescence analysis

The cells were fixed as described previously [8]. The antibodies used were anti-human MUC2 (Ccp58; Santa Cruz Biotechnology), anti-human TFF3 (ab57752; Abcam, Cambridge, UK) and anti-human Lgr5 (TA301323; OriGene, Rockville, MD, USA). Anti-mouse IgG Alexa Fluor® 594 or Alexa Fluor® 488 (Invitrogen) were used as the secondary antibody. Cells were mounted with VectaShield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and visualized by confocal laser fluorescent microscopy (BZ-8000 [Keyence, Tokyo] and FLUOVIEW FV10i [Olympus, Tokyo]).

2.6. MTS assay

1×10^4 DLD1 cells were cultured in a 96-well tissue culture plate at 37 °C, and 5% CO_2. After a 48-h incubation period in the presence or absence of oxaliplatin, CellTiter96® Aqueous One Solution was added (20 μl/well) and incubated for 1 h at 37 °C and 5% CO_2. The absorbances at 490 nm were measured with an ARVO™MX plate reader (Perkin Elmer, Boston, MA, USA). Background absorbances from medium-containing wells were subtracted from those of the sample wells.

2.7. Migration assay

The Oris™ Pro Cell Migration Assay kit (Platypus Technologies, LLC USA) was used. This assay is formatted for 96 well-plates and uses a non-toxic biocompatible gel to form a cell-free zone on cell culture surfaces. DLD1 cells (1×10^4) were seeded into 96-well plates and incubated for 1 h. Phase contrast images were taken for pre-migration reference. After 6 h of incubation, images were captured using phase contrast microscopy. The ratio of the vacant area between pre- and post-migration was analyzed.

2.8. Cell cycle assay and live cell imaging

Live imaging was performed on the DeltaVision system (Applied Precision, Washington, USA) incorporating a fluorescent microscope IX-71 (Olympus, Tokyo, Japan) using a 20× 0.75NA Olympus UPlanSApo objective. Differential interference contrast (DIC) and fluorescent images were acquired at 15-min intervals for 72 h. The data were processed using softWoRx® (Applied Precision). Maximum intensity projections of the time series were exported into QuickTime format for presentation as Supplementary movies. The ratio of cells in the S/G2/M phase was analyzed by FACS caliber to detect cells expressing S/G2/M green fluorescence.

2.9. Chromatin immunoprecipitation assay

A chromatin immunoprecipitation (ChIP) assay was performed as described previously with some modifications [9]. The primer sequences in this study are summarized in Supplementary Table S1.

2.10. Statistical analysis

Quantitative real-time PCR analyses were statistically analyzed with the Student's *t*-test. *P* values of <0.05 were considered statistically significant.

3. Results

3.1. Mutant Atoh1 protein stabilized in colon cancer acquires mucinous secretion and Lgr5 expression

To assess the function of the Atoh1 protein in colon cancer, we attempted to construct a mutant Atoh1 protein that was stably expressed in undifferentiated colon cancer cells. In our previous study, the SA-Atoh1 protein, in which two serine residues were replaced by alanine, was transiently expressed in colon cancer cells, leading to a slight increase in mucin 2 (MUC2) gene expression [8]. However, SA-Atoh1 was not stably expressed in colon cancer cells (data not shown). Therefore, a mutant Atoh1 gene (5SA-Atoh1) in which five serine residues were replaced by alanine was generated in order to avoid phosphorylation by GSK3 and proteasomal degradation in colon cancer cells (Supplementary Fig. S1). Both of these Atoh1 proteins were expressed in 293T cells, in which Wnt signaling is normal. In contrast, only the 5SA-Atoh1 protein was stably expressed in sporadic colon cancer derived DLD1 cells and SW480 cells (Fig. 1A). Fluorescence analysis showed the expression of 5SA-Atoh1 in the nuclei of DLD1 cells (Fig. 1B). Moreover, the transcriptional activity of 5SA-Atoh1 was conserved through the E-box sequence even in DLD1 cells (Fig. 1C). Atoh1 protein stabilization also resulted in the induction of the secretory phenotypic genes and proteins such as MUC2, trefoil factor 3 (TFF3) and human defensin 6 (HD6) (Fig. 1D and E). The expression in LS174T cells that are mucinous colon cancer cell line were used as standard in all PCR examination, since the quantity of gene expression in LS174T cells were defined as 1. Thus, Atoh1 protein-expressing cells were generated for the first time in colon cancer, resulting in the acquisition of a mucinous phenotype, although MUC2 expression in Atoh1 expressing cells was lower than in LS174T.

To assess whether the expression of the Atoh1 protein affects the malignant potential of colon cancer, comprehensive genes induced by the Atoh1 protein were detected by microarray analysis. A gene set enrichment analysis (GSEA) indicated the malignant potential by identifying genes involved in the Wnt pathway, cell adhesion, cell cycle arrest, and metastasis in 5SA-Atoh1 cells (Supplementary Table S2). Moreover, cancer stem markers upregulated by Atoh1 were found in the microarray gene list (Supplementary Table S3). Therefore, we confirmed the expression of cancer stem markers in 5SA-Atoh1 cells. Interestingly, a leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) gene was markedly increased in 5SA-Atoh1 DLD1 cells (Fig. 1F). These findings that stabilized Atoh1 protein induces both mucinous phenotype and Lgr5 gene were also shown in SW480 cells (data not shown).

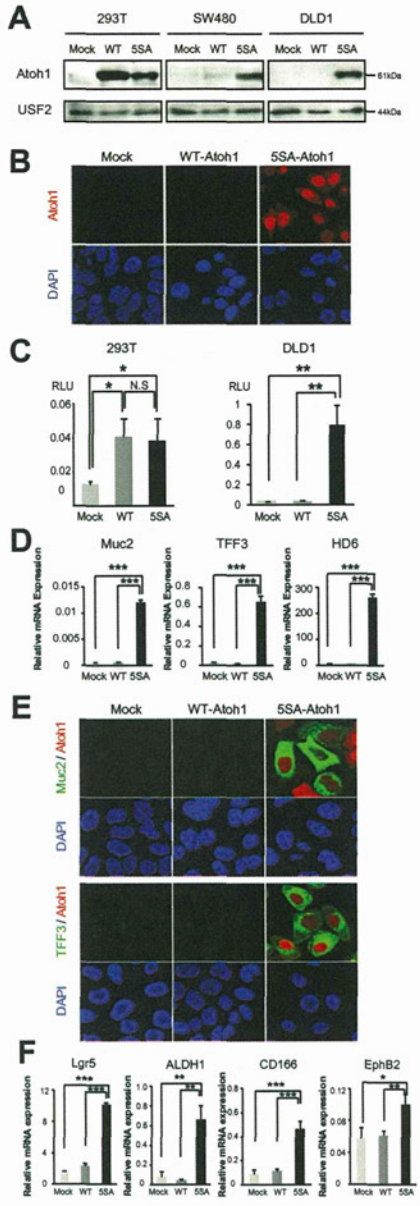

Fig. 1. Mutant Atoh1 protein stabilized in colon cancer acquires mucinous secretion and Lgr5 expression. (A) WT-Atoh1 and 5SA-Atoh1 were transfected into 293T, SW480, and *DLD1 cells*. The protein expression of WT-Atoh1 and 5SA-Atoh1 was analyzed by Western blot. (B) Immunofluorescence staining showed the nuclear localization of the 5SA-Atoh1 protein in DLD1 cells. (C) A reporter activity via the E-box sequence (E-box-Luc) was analyzed. In DLD1 cells, 5SA-Atoh1 induced significantly higher reporter activity than WT-Atoh1, with correspondingly higher protein expression. (D) The expression of the differentiation phenotypic genes was analyzed by RT-PCR. The MUC2 TFF3 and HD6 genes were significantly upregulated in 5SA-Atoh1 DLD1 cells. The expression level in LS174T cells was defined as 1, (E) Immunofluorescence staining of MUC2 and TFF3 showed the expression of both proteins only in 5SA-Atoh1 DLD1 cells and (F) The expression of the cancer stem cell marker genes was analyzed by RT-PCR. Lgr5 was significantly upregulated in 5SA-Atoh1DLD1 cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $n = 3$.

3.2. Atoh1 protein directly upregulates the transcriptional activity of Lgr5

The Lgr5 protein was detected in peripheral nuclei and the cell membrane showed a punctate appearance, consistent with a previous report (Fig. 2A) [10]. As Lgr5 is one of the Wnt target genes, we assessed Wnt signaling activity by examining T cell factor 4 (TCF4)-dependent transcriptional activity in 5SA-Atoh1 cells. TCF4 transcriptional activity was upregulated in 5SA-Atoh1/DLD1 cells but not in WT-Atoh1 cells (Fig. 2B), suggesting that Atoh1 has the potential to promote transcription through the TCF4 binding site. The promoter assay with human Lgr5 also showed an upregulation by the Atoh1 protein. The next critical region on the Lgr5 promoter was limited to within 350 bp upstream (Fig. 2C). Finally, deletion mutants of the Lgr5 promoter revealed that the critical region for transcription by Atoh1 lay between 340 bp and 350 bp upstream (Fig. 2D). Because, The transcriptional activity for Lgr5 was also upregulated by Atoh1 expressed transiently in 293T cells

(Fig. 2E), We confirmed the binding of Atoh1 to the Lgr5 promoter region by ChIP assay (Fig. 2F and G), suggesting that Atoh1 directly upregulates Lgr5 promoter activity in the region located between 340 bp and 350 bp upstream of Lgr5.

3.3. Atoh1 protein stabilization enriches the cancer stem cells in vitro

We next assessed the cancer stemness by Atoh1 protein stabilization, because various stem cell markers including Lgr5 were induced

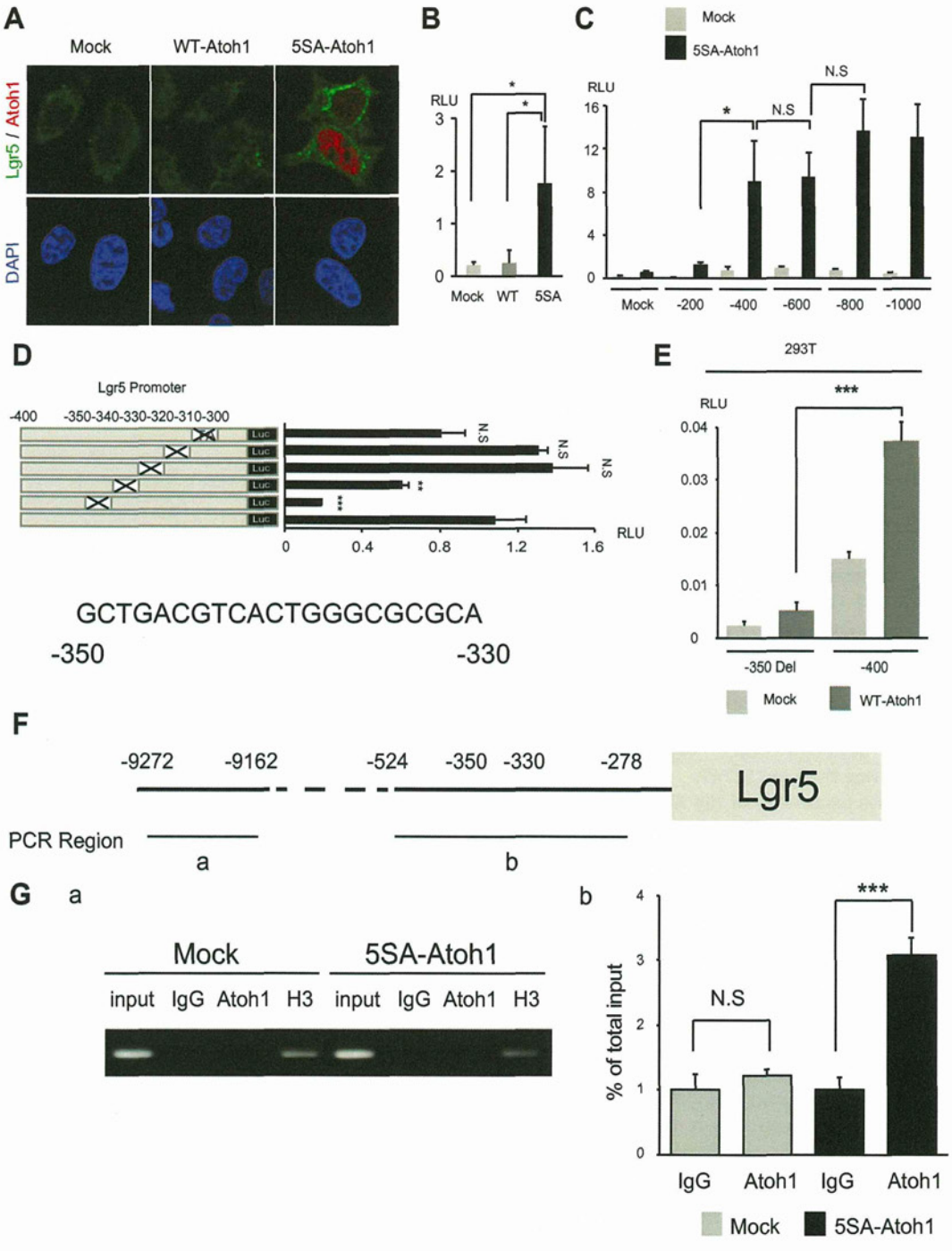

Fig. 2. Atoh1 protein directly upregulates the transcriptional activity of Lgr5. (A) Immunofluorescence staining showed the expression of Lgr5 in 5SA-Atoh1/DLD1 cells. (B) TCF4-dependent reporter activity (TOPflash) was examined. Reporter activity was significantly upregulated in 5SA-Atoh1 DLD1 cells. (C) The reporter activity of Lgr5 promoters that were longer than 400 bp was elevated only in 5SA-Atoh1 DLD1 cells. (D) Mutated Lgr5 reporter vectors each with a 10-bp deleted inform from 300 to 350 bp upstream of the Lgr5 promoter region were generated. The reporter activity by Atoh1 protein expression was significantly decreased between 340 bp and 350 bp of the Lgr5 promoter. The sequence between 330 bp and 350 bp of Lgr5 promoter was shown. (E) WT-Atoh1 upregulated the transcriptional activity through the region between 340 bp and 350 bp of the promoter region in 293T cells. (F) Schematic representation of the human Lgr5 genome and the region amplified by PCR and (G) the ChIP assay was performed using DLD1 cells with or without 5SA-Atoh1. Each region indicated by a schema was amplified from the immunoprecipitant by each antibody. Only the region including the 340–350-bp segment of the Lgr5 promoter (region b) was amplified from the immunoprecipitant by the mCherry antibody. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $n = 3$.

Table 1
Frequency of spheroid-forming cells in DLD1 or 5SA-Atoh1/DLD1 cells by extreme limiting dilution analysis. Various concentrations of DLD1 cells expressing GFP or 5SA-Atoh1 (1000, 500, 100, and 10) were cultured with SCM medium in low attachment dishes. Ten days after, the number of spheroids was counted. The confidence interval (95%) for spheroid forming frequency was calculated using a software application for limiting dilution analysis (LDA), ELDA, as described previously [11].

No. of cells	No. of spheroids	
	Naive	5SA-Atoh1
1000	28.3	50.3
500	10.6	31
100	5.6	13.6
10	0.6	5
Spheroid-forming frequency (95% confidence interval)	1/27559 (1/36913–1/20576)	1/12273 (1/14946–1/10079)
P value	2.75×10^{-6}	

by Atoh1. *In vitro* spheroid formation assay showed the tumorigenicity in 5SA-Atoh1 cells derived spheroids. Analysis by extreme limiting dilution analysis (ELDA) [11] showed that the Atoh1 protein enriched cancer stem cells (Table 1) (Supplementary Fig. S2).

3.4. Atoh1 protein stabilization suppresses cell growth by extension of the G0/G1 phase of the cell cycle

To further analyze the functional role of the Atoh1 protein in colon cancer cells, we examined whether the Atoh1 protein affected cell proliferation. Atoh1 protein stabilization suppressed cell proliferation in all colon cancer cells (Fig. 3A). A previous report has suggested that the Atoh1 protein in Merkel cell carcinoma induces apoptosis, resulting in the suppression of cell proliferation [7]. However, Atoh1 protein stabilization did not induce an apoptotic signal in DLD1 cells (data not shown). As cell cycle may affect cell proliferation in cells expressing the Atoh1 protein, we attempted to visualize the cell cycle using a fluorescent ubiquitination-based cell cycle indicator (Fucci) system in which cells in the S/G2/M phase were marked by Azami-Green 1 fused with geminin [12]. Live-imaging analysis showed an extension of the G0/G1 phase in 5SA-Atoh1 cells, resulting in a longer cell cycle by Atoh1 protein stabilization (Fig. 3B; Supplementary Table S4; Supplementary Movie S1, 2). Moreover, a decreased number of 5SA-Atoh1 cells in the S/G2/M phase were found compared with mock-transfected cells (Fig. 3C).

3.5. Atoh1 protein stabilization promotes cell migration and chemoresistance in colon cancer cells

5SA-Atoh1 cells were transformed cell shape and cell adhesion that were dispersed each cells (Supplementary Movie S2). Cells at rest exhibited accelerated migration (Fig. 3D and E), indicating that the Atoh1 protein may confer not only the secretory phenotype but also a cancer stem cell phenotype. We finally assessed whether the Atoh1 protein conferred chemoresistance. 5SA-Atoh1 DLD1 cells were more resistant to oxaliplatin than both WT-Atoh1 DLD1 cells and mock-transfected DLD1 cells. WT-Atoh1 protein was also expressed by the treatment with oxialiplatin because of the GSK3 inactivation (Supplementary Fig. 3), resulting in the resistance to oxaliplatin in WT-Atoh1 DLD1 cells (Fig. 3F).

4. Discussion

This study revealed that the mutated Atoh1 protein could be stably expressed in colon cancer cells. We also demonstrated that Atoh1 protein stabilization induces both a cancer stem cell phenotype and a mucinous phenotype, resulting in the acquisition of chemoresistance.

One of the most reliable markers for cancer stem cells is Lgr5 because Lgr5 is essential for the maintenance of intestinal epithelial stem cells [13]. Lgr5-positive colon cancer cells have the potential to become cancer stem cells because the Lgr5 gene is selectively expressed in human colon cancer stem cell populations [14,15]. Recently, Lgr5-positive cells were reported to be stem cells in adenoma by a lineage tracing method [16], supporting that Lgr5 may be one of the cancer stem markers in the colon. Although it remains to be elucidated how the Atoh1 protein induces the Lgr5 gene expression, this study showed that the Atoh1 protein directly induces the promoter activity of the Lgr5 gene, resulting in the enrichment of cancer stem cells. Whether Lgr5 induced by Atoh1 is related to the enrichment of cancer stem cells should be clarified more in detail in future.

Another interesting finding was that the Atoh1 protein extended the G0/G1 phase of the cell cycle. Atoh1 gene expression is known to be negatively regulated by the Notch signal [9], which promotes the cell cycle [17]. Therefore, it has been considered that cell cycle arrest during intestinal epithelial cell differentiation could be attributed to Notch signal suppression rather than Atoh1 gene expression. However, a recent study showed that deletion of the Atoh1 gene canceled the suppression of cell proliferation by Notch signal inhibition in colon cancer [18], indicating that Atoh1 may directly regulate cell proliferation. It was only after stabilization of the Atoh1 protein in colon cancer that cell proliferation was markedly suppressed *in vitro*. Because some reports have indicated that slow cycling cancer stem cell populations was included in tumor to survive therapies, cell cycle arrest by Atoh1 might express one of phenotypes for cancer stem cells [19].

Moreover, the Atoh1 protein induced chemoresistance by the avoidance of G2 phase entry, which most alkylating agents target [20]. These results provide useful information on the acquisition of chemoresistance by MC and SRCC that express the Atoh1 protein. Furthermore, WT-Atoh1 DLD1 cells were also resistance to oxaliplatin by the stabilization of Atoh1 protein. Therefore, mRNA expression of Atoh1 in non-mucinous sporadic colon cancer might be important to assess the chemoresistance and how oxaliplatin stabilizes Atoh1 protein should be clarified in future.

The reason why cell cycle arrest does not induce apoptosis has an important bearing on the significance of Atoh1 protein expression in cancer cells. A major difference in Atoh1 protein expression between normal tissue and cancer cells may be the Wnt signaling. β-Catenin and Atoh1 do co-localize in the nuclei of mucinous carcinoma, probably because of GSK3 inactivation, resulting in the increase of TCF4-dependent transcriptional activity in cooperation with β-catenin. Consequently, Lgr5 and MMP9 (data not shown), both of which contribute to cell survival [21], were upregulated by Wnt signaling acceleration with Atoh1 protein stabilization, suggesting that cancer cells may prevent apoptosis in spite of the cell cycle arrest. Although it is unknown how the Atoh1 protein is permanently expressed in mucinous cancer, the investigation of GSK3 kinase inactivation by GSK3 phosphorylation in mucinous cancer may help in clarifying the mechanism by which the Atoh1 protein is stabilized.

Overall, Atoh1 protein stabilization appears to act as a tumor suppressor that differentiates colon cancer cells by inducing differentiation phenotypes and suppressing cell proliferation. Nevertheless, the Atoh1 protein conferred cancer stem cell phenotypes, such as cell migration, cancer stem marker expression, and chemoresistance, indicating that in colon cancer, the Atoh1 protein plays a greater role in malignant potential than tumor suppression. The

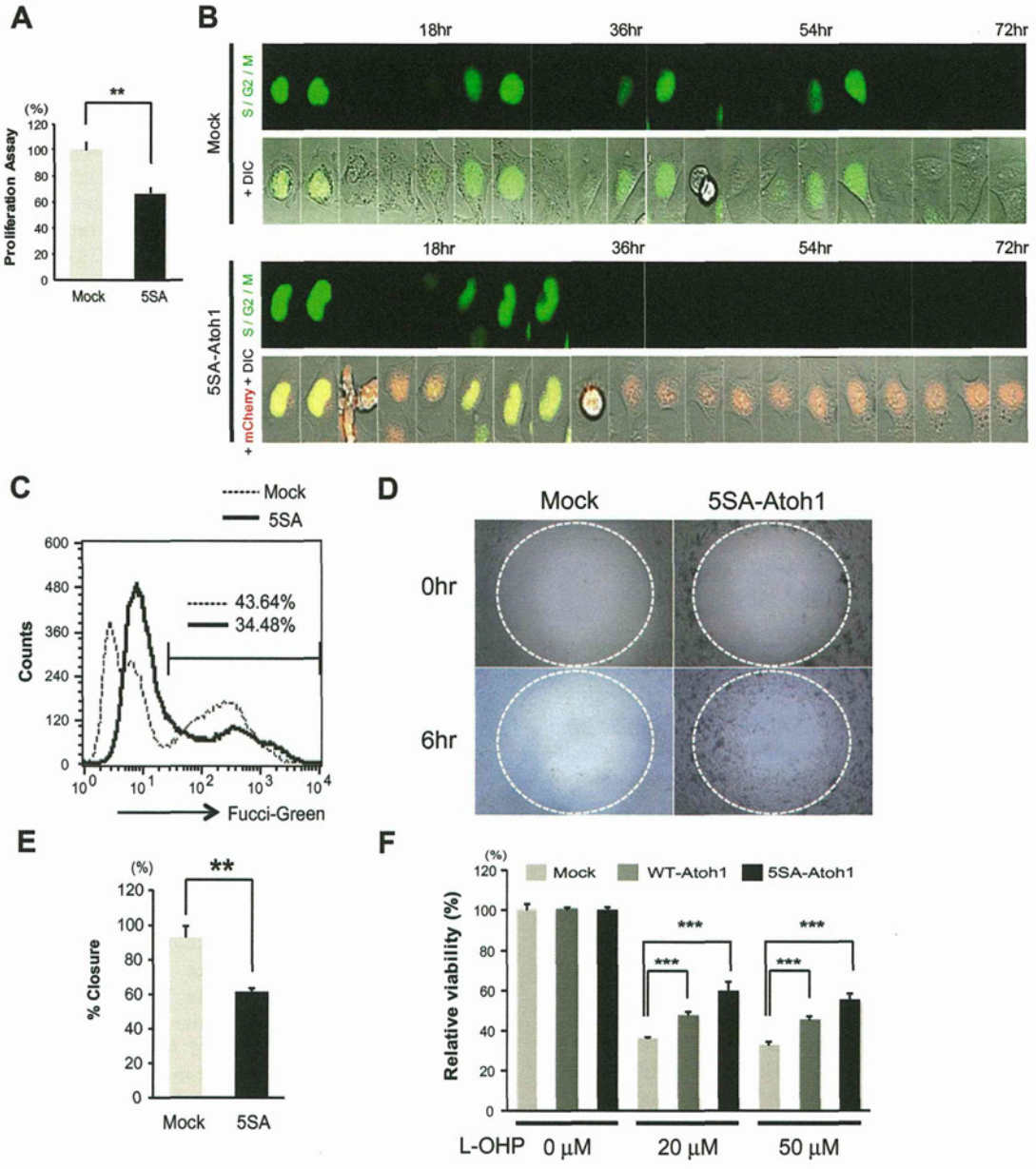

Fig. 3. Atoh1 protein stabilization in colon cancer induces cell cycle arrest, migration and chemoresistance. (A) A proliferation assay revealed that cells expressing 5SA-Atoh1 grew slowly. (B) To visualize the S/G2/M phase of cells, a lentivirus composed of Azami-Green combined with geminin (Green-S/G2/M) was infected into both DLD1 cells and 5SA-Atoh1 DLD1 cells. Time-lapse live imaging of DLD1 cells showed elongation of the G0/G1 phase in 5SA-Atoh1 DLD1 cells. (C) FACS analysis showed a decreased number of 5SA-Atoh1/DLD1 cells in the S/G2/M phase, resulting in the accumulation of 5SA-Atoh1 DLD1 cells in the G0/G1 phase. (D) Migration assay showed that the vacant circular area was occupied by a greater number of 5SA-Atoh1 DLD1 cells than DLD1 cells. (E) The ratio of the remaining vacant area is shown. The vacant area of 5SA-Atoh1 DLD1 cells was smaller than that of DLD1 cells at 6 h after the cells were seeded and (F) An MTS assay showed that the reduction of cells in both 5SA-Atoh1 DLD1 cells and WT-Atoh1 DLD1 cells by treatment with oxaliplatin (L-OHP) was less than that of DLD1 cells. (**$p < 0.01$, ***$p < 0.001$, N.S.: not significant). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

present study also suggests that the cell differentiation phenotype regulated by Atoh1 expression in colon cancer is not linked to the pathological differentiation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.034.

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Meta-analysis of Published Studies Identified Eight Additional Common Susceptibility Loci for Crohn's Disease and Ulcerative Colitis

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Background: Both ulcerative colitis (UC) and Crohn's disease (CD) have a complex etiology involving multiple genetic and environmental factors. Many genome-wide association studies (GWAS) and subsequent replication studies revealed that both diseases share some of the susceptibility loci; however, common genetic factors for both diseases are not fully elucidated. This study is aimed to identify the common genetic factors for CD and UC by a meta-analysis of published studies.

Methods: We first reviewed the 10 GWAS for CD to select candidate single nucleotide polymorphisms (SNPs). Next, we performed a PubMed literature search up to June 30, 2010 and carried out a systemic review of published studies that examined the association of CD susceptibility loci in UC patients. Meta-analysis was carried out using the inverse variance-weighted method or the DerSimonian-Laird method after estimating the heterogeneity among the studies. The data for highly linked SNPs were combined. Finally, we performed a meta-analysis of 43 published studies in 45 SNPs located at 33 loci by using a total of 4852 to 31,125 subjects.

Results: We confirmed the association of 17 reported common susceptibility loci. Moreover, we found associations at eight additional loci: *GCKR, ATG16L1, CDKAL1, ZNF365, LRRK2-MUC19, C13orf31, PTPN2*, and *SBNO2*. The genetic risk of each locus was modest (odds ratios ranged from 1.05–1.22) except *IL23R*.

Conclusions: These results indicate that CD and UC share many susceptibility loci with small genetic effect. Our data provide further understanding of the common pathogenesis between CD and UC.

(Inflamm Bowel Dis 2011;000:000–000)

Key Words: single nucleotide polymorphism, meta-analysis, shared genetic risk, ulcerative colitis, Crohn's disease

Ulcerative colitis (UC) and Crohn's disease (CD), the two most common forms of inflammatory bowel disease (IBD), have a complex etiology involving multiple genetic and environmental factors. Family and twin studies have clearly indicated the involvement of genetic factors in the development of both diseases.[1] Moreover, UC and CD exist in the same family with higher frequency than the co-occurrence by chance alone, suggesting an etiological relationship between the two diseases.[2,3] Since the chronic relapsing intestinal inflammation induced by the dysregulated mucosal immune response to commensal enteric bacteria is one of the common pathogenesis of CD and UC, it is important to understand the shared genetic factors for both diseases.

Recent genome-wide association studies (GWAS) for CD[4–13] have identified more than 30 susceptibility loci and provided new insights into the immunopathogenesis of this disease, implicating an important role of genes of the innate and adaptive immune systems for disease occurrence.[14] Similarly, several GWAS for UC[15–20] have identified more than 10 susceptibility loci. A comparison of the results of these studies and additional association studies has identified 18 common susceptibility loci between CD and UC, including *IL23R, JAK2, STAT3, BSN-MST1, CCNY-CREM, KIF21B, NKX2-3, IL12B, ORMDL3, ICOSLG, LOC441108, IRGM, CCR6, TNFSF15*, 5p13, 6p21, 7p12,

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and 21q21.[16,19–25] However, considering the strong heritability of both diseases, several common genetic factors may not have been found yet and meta-analysis of published studies is one approach by which these factors may be identified. Nevertheless, to our knowledge, only a handful of meta-analysis for common susceptibility loci between UC and CD have been performed, most notably for *NOD2, PTPN22, ATG16L1*, and *IRGM*.[26–31] Therefore, we performed a comprehensive meta-analysis of published studies that examined the association of CD susceptibility loci in UC patients to clarify common genetic factors for both diseases.

MATERIALS AND METHODS

Single Nucleotide Polymorphism (SNP) Selection for a Literature Search

We reviewed the literature of 10 GWAS for CD including meta-analyses[4–13] published before June 30, 2010. Initially, we selected 62 SNPs for the literature search based on the following criteria: 1) SNPs showed a significant level of overall *P*-value less than 5×10^{-7} in an initial GWAS for CD; and 2) located at non-MHC region because of the broad and strong linkage disequilibrium across the MHC region (Supporting Information Table 1).

Literature search strategy and study selection criteria

We performed a PubMed literature search (National Center for Biotechnology Information [NCBI]; http://www.ncbi.nlm.nih.gov/pubmed/) up to June 30, 2010 using the following terms: (ulcerative colitis or inflammatory bowel disease) and (polymorphism* or variant* or loci or locus). References from the selected publications were manually scanned to identify other relevant studies. Studies were included if: 1) they were case–control studies for Caucasian UC; 2) they included at least 100 UC cases; 3) they were published in English; 4) they examined the selected SNPs or the highly linked SNPs with the selected ones ($r^2 \geq 0.95$ in the HapMap Southern Utah residents of European descent [CEU] samples [release #27, build 36]); and 5) they provided enough data to calculate odds ratios (ORs) and 95% confidence intervals (CIs). For publications using overlapping samples, we discarded the smaller dataset (13 studies). The literature search and data extraction were conducted by two authors (K.A. and J.U.). Disagreement over eligibility was resolved by a detailed discussion after review by one additional author (T.M.). Details of this search strategy are shown in Figure 1. Finally, a total of 43 articles[16,19–25,27,29,30,32–63] were included in the meta-analysis (Table 1).

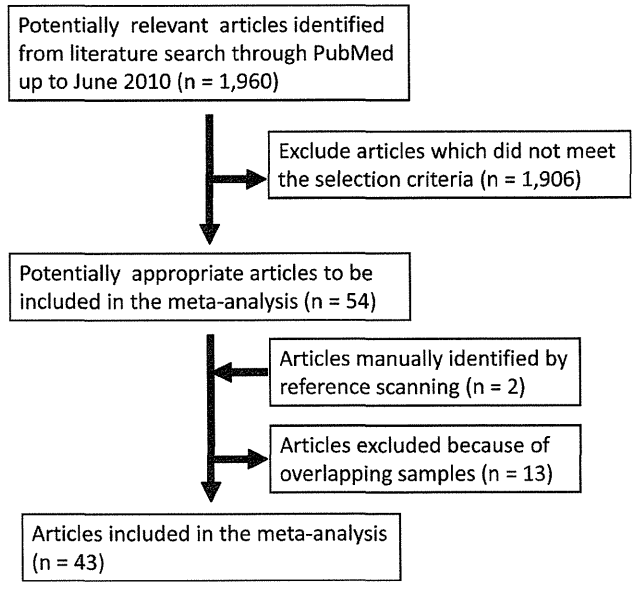

FIGURE 1. Flowchart of search strategy for meta-analysis.

Meta-analysis

We assessed heterogeneity across the studies using Cochran's *Q* test and I^2 statistics. *P*-value > 0.10 and I^2 statistics < 25% indicated a lack of heterogeneity.[64] If there was no heterogeneity among the studies, meta-analysis was carried out using the inverse variance-weighted method. This method is a fixed-effect model based on the assumption that the true OR of all studies is the same and no interstudy variance exist. When heterogeneity was present, we used the DerSimonian-Laird method. This method is a random-effect model which considers interstudy variance to estimate the combined OR. Publication bias was investigated by funnel plot and evaluated using Egger's test.[65] Funnel plot is a scatterplot which displays the OR of each study on the X axis against sample size on the Y axis. If there is no publication bias, OR will be distributed symmetrically and its variation may be smaller in larger studies. The degree of symmetry of funnel plot was estimated by Egger's test. We considered the evidence of significant publication bias as an obvious asymmetry of funnel plot and Egger's *P*-value < 0.05. All statistical analyses were undertaken using R (http://www.r-project.org/).

We basically used reported ORs and 95% CIs of the published studies to perform meta-analysis. Since 15 out of 43 articles did not report OR or 95% CI, we calculated OR and 95% CI of each SNP using genotype data in eight studies,[21,27,33,35,38,39,53,61] sample size and minor allele frequency (MAF) in three studies,[32,34,37] *P*-value and OR in three studies,[16,20,59] and *P*-value and MAF in one study.[48] Among the 62 SNPs initially selected, we excluded seven SNPs (rs10801047 [1q31], rs1002922 [5p13], rs10512734 [5p13], rs1373692 [5p13], rs3810936 [*TNFSF15*], rs7848647 [*TNFSF15*], and rs5743289 [*NOD2/CARD15*]) because these SNPs had not been studied in at least two studies. In addition, the data of SNPs in *ATG16L1* (rs2241880, rs10210302, and rs3828309), *BSN-MST1* (rs9858542 and rs3197999), 5p13 (rs4613763 and rs17234657), *IRGM* (rs13361189, rs1000113, and rs11747270), *TNFSF15*

TABLE 1. Studies Included in the Meta-analysis

	Study	Reference	Year	Population	Case	Control
1	Ogura	32	2001	USA	182	287
2	Cuthbert	33	2002	UK	566	290
3	Esters	34	2004	Belgium	173	165
4	Büning	35	2005	Hungary	128	208
5	Martín	36	2005	Spain	544	812
6	Waller	37	2006	UK	512	750
7	Oostenbrug	27	2006	Netherlands	207	276
8	Crawford	38	2007	USA	172	104
9	Cucchiara	39	2007	Italy	186	347
10	Tremelling	40	2007	UK and Scotland	975	1345
11	Büning_1	41	2007	Germany and Hungary	296	707
12	Cummings	42	2007	UK	647	1134
13	Glas	43	2007	Germany	456	1381
14	Economou	44	2007	Greece	180	100
15	Büning_2	45	2007	German and Hungary	294	845
16	Roberts	46	2007	New Zealand	466	591
17	Glas	47	2008	Germany	507	1615
18	Lappalainen	48	2008	Finnland	459	292
19	Márquez	49	2008	Spain	363	546
20	Franke	15	2008	Germany	1103	1817
21	Fisher	21	2008	UK	1740	1492
22	Lakatos	50	2008	Hungary	149	149
23	Okazaki	51	2008	Canada	117	310
24	Roberts	52	2008	New Zealand	475	576
25	Fowler	53	2008	Australia	543	1244
26	Weersma_1	54	2009	Netherlands	1120	1350
27	Anderson	23	2009	UK	2527	3028
28	Silverberg	16	2009	USA and Canada	1052	2571
29	Weersma_2	24	2009	Belgium and Netherlands	1442	1045
30	Einarsdottir	55	2009	Sweden	455	280
31	Glas	56	2009	Germany	476	1503
32	Newman	57	2009	Canada	402	1005
33	Palomino-Morales	29	2009	Spain	425	572
34	Márquez_1	30	2009	Spain	368	745
35	Márquez_2	58	2009	Spain	405	800
36	Törkvist	59	2010	Sweden	935	1460
37	Festen	25	2010	Netherlands	1455	1902
38	Sventoraityte	60	2010	Lithuania	123	186
39	Lacher	61	2010	Germany	132	253
40	Cénit	62	2010	Spain	442	1692
41	Franke	19	2010	Germany	1043	1703
42	McGovern_GWAS1	20	2010	USA	723	2880
	McGovern_GWAS2	20	2010	Sweden	948	1408
	McGovern_GWAS3	20	2010	USA and Canada	1022	2503
	McGovern_Replication1	20	2010	Italy	993	826
	McGovern_Replication2	20	2010	Netherlands	1016	754
43	Perdigones	63	2010	Spain	662	1361

(rs6478108 and rs426389), *NKX2-3* (rs11190140 and rs10883365), and *NOD2/CARD15* (rs17221417, rs2066843, and rs2076756) were combined because these SNPs were in high linkage disequilibrium with each other ($r^2 \geq 0.95$) in the HapMap CEU samples. Finally, we performed a meta-analysis for 45 SNPs located at 33 loci by using a total of 4852 to 31,125 subjects. For an easy understanding of the risk direction, we calculated the OR and 95% CI of each SNP according to the risk allele in the GWAS for CD. A *P*-value less than 0.0015 (0.05/33) was considered statistically significant after applying Bonferroni correction.

RESULTS

We found evidence of heterogeneity among the studies for 19 SNPs: rs2476601 (*PTPN22*), rs2274910 (*ITLN1*), rs2241880-rs10210302-rs3828309 (*ATG16L1*), rs4613763-rs17234657 (5p13), rs2188962 (*LOC441108*), rs10077785 (*LOC441108*), rs4958847 (*IRGM*), rs6908425 (*CDKAL1*), rs1456893 (7p12), rs1551398 (8q24), rs6478108-rs4263839 (*TNFSF15*), rs17582416 (*CCNY-CREM*), rs10995271 (*ZNF365*), rs10761659 (*ZNF365*), rs7927894 (*C11orf30*), rs2872507 (*ORMDL3*), rs2542151 (*PTPN2*), rs1736135 (21q21), and rs762421 (*ICOSLG*). Therefore, the pooled ORs and 95% CIs were calculated using a random-effect model in these variants. We found a significant publication bias at rs9292777 on 5p13 locus (Egger's $P = 0.02$) and excluded this SNP from the analysis.

Among the 45 SNPs included in the meta-analysis, 35 SNPs located at 30 loci were investigated by more than five studies. Among the 33 loci examined in this study, we found significant associations with UC in 14 loci and nominal associations ($P < 0.05$) in 11 loci. We confirmed the associations of 17 susceptibility loci which are commonly associated with both CD and UC in the previous study[20]: *IL23R, KIF21B, BSN-MST1*, 5p13, *LOC441108, IRGM, IL12B, CCR6*, 7p12, *JAK2, TNFSF15, CCNY-CREM, NKX2-3, ORMDL3, STAT3*, 21q21, and *ICOSLG* (Supporting Information Table 2). Moreover, we found associations with UC in eight additional loci (Table 2): *GCKR* (rs780094, $P = 2.47 \times 10^{-2}$, OR 1.05), *ATG16L1* (rs2241880-rs10210302-rs3828309, $P = 4.70 \times 10^{-2}$, OR 1.05), *CDKAL1* (rs6908425, $P = 7.68 \times 10^{-3}$, OR 1.10), *ZNF365* (rs10761659, $P = 4.67 \times 10^{-4}$, OR 1.14), *LRRK2-MUC19* (rs11175593, $P = 1.54 \times 10^{-2}$, OR 1.21), *C13orf31* (rs3764147, $P = 1.80 \times 10^{-2}$, OR 1.07), *PTPN2* (rs2542151, $P = 2.49 \times 10^{-2}$, OR 1.08), and *SBNO2* (rs4807569, $P = 1.72 \times 10^{-2}$, OR 1.06). For all loci showing association, the directions of risk alleles for UC were all the same as those for CD. The OR of *IL23R* locus was relatively high (rs11209026, OR 1.62, 95% CI: 1.48–1.77), whereas ORs of other loci were modest ranged from 1.05–1.22.

DISCUSSION

We comprehensively reviewed the published studies that examined the CD susceptibility loci in UC patients and performed a meta-analysis to clarify the common genetic factors for both diseases. We found associations at 25 out of 33 candidate loci. Among them, we confirmed the associations in 17 loci reported in the previous GWAS,[20] and this study found an additional eight common susceptibility loci for CD and UC, namely, *GCKR, ATG16L1, CDKAL1, ZNF365, LRRK2-MUC19, C13orf31, PTPN2*, and *SBNO2*. Among these additionally identified loci, *GCKR* and *LRRK2-MUC19* have never shown nominal association with UC in any single studies performed to date. Although the genetic risk of each locus was modest, many genes or loci will contribute to the pathogenesis of both CD and UC.

Previous GWAS identified that the autophagy-related genes are associated with the susceptibility of CD.[6,7,9,10,13] In contrast to the strong association with CD, previous association studies for UC showed inconsistent results in these autophagy-related genes.[16,21–24,29–31,56] Our meta-analysis demonstrated nominal association with *ATG16L1* by using 11,466 cases and 19,659 controls ($P = 4.7 \times 10^{-2}$, OR 1.05, 95% CI: 1.00–1.10). Other autophagy-related genes also showed associations with UC in this study ($P = 1.54 \times 10^{-2}$, OR 1.21, 95% CI: 1.03–1.41 for *LRRK2-MUC19*; $P = 1.18 \times 10^{-3}$, OR 1.14, 95% CI: 1.05–1.24 for *IRGM*). These findings suggest a possibility that autophagy might contribute to the development of both UC and CD, but its effect may be weaker for UC.

There is another possibility that the association of autophagy-related genes are caused by the contamination of colonic CD cases because rs2241880-rs10210302-rs3828309 (*ATG16L1*) and rs4958847 (*IRGM*) showed heterogeneity among the studies. However, we could not find any consistent set of studies that contributed to this heterogeneity. Moreover, when we assume the possibility of this misclassification for *ATG16L1*, colonic CD cases should be included in more than 20% of UC cases based on the assumption of a case–control study of 11,466 cases and 19,659 controls, an allele test model, a risk allele frequency of 0.571 based on the HapMap-CEU population, an allelic OR of colonic CD for 1.25,[13] a statistical power of 0.80, and a *P*-value of 0.05. Since the diagnosis of UC was made by the established guidelines in each study, we think the association of autophagy-related genes in this study might not be caused by the misclassification of colonic CD cases in the previous studies.

Recent genetic studies have revealed shared genetic components of different immune-related diseases.[66] For the shared susceptibility genes between CD and UC, previous studies have shown the importance of the common pathogenesis of the IL-23/Th17 signaling pathway, which promotes inflammation in the adaptive immune response.[14]

TABLE 2. Results of Meta-analysis for Eight Additionally Identified Common Susceptibility Loci for CD and UC

	Allele* [1/2]	Study	Number		RAF		OR (95%CI)	Combined		Heterogeneity		Publication Bias
			Case	Control	Case	Control		*P*	OR (95% CI)	*P*	I^2 Statistics	*P*
GCKR												
rs780094	T/C	Anderson (2009)	2464	4002	0.40	0.38	1.07(0.99-1.16)	2.47E-02	1.05(1.00-1.09)	0.48	0	0.40
		Franke (2010)	1043	1703	0.42	0.40	1.10(0.99-1.23)					
		McGovern (2010) GWAS#1	723	2880	—	—	1.03(0.88-1.20)					
		McGovern (2010) GWAS#2	948	1408	—	—	1.00(0.94-1.07)					
		McGovern (2010) GWAS#3	1022	2503	—	—	1.08(0.96-1.21)					
		Total	6200	12496								
ATG16L1												
rs2241880	G/A	Büning_1 (2007)	296	707	0.52	0.51	1.10(0.89-1.35)	4.70E-02	1.05(1.00-1.10)	0.11	0.31	0.43
rs10210302	T/C	Roberts (2007)	466	591	0.51	0.50	1.05(0.87-1.25)					
rs3828309	G/A	Glas (2008)	507	1615	0.55	0.52	1.15(0.98-1.36)					
		Lappalainen (2008)	459	190	0.46	0.47	0.96(0.75-1.23)					
		Franke (2008)	1077	1793	0.55	0.53	1.19(1.01-1.41)					
		Fisher (2008)	1739	1491	0.54	0.52	1.08(0.97-1.20)					
		Lakatos (2008)	149	149	0.54	0.50	1.26(0.91-1.74)					
		Okazaki (2008)	117	310	0.50	0.48	1.02(0.61-1.68)					
		Fowler (2008)	543	1244	0.48	0.51	0.87(0.75-1.01)					
		Newman (2009)	402	1005	—	—	1.19(1.00-1.41)					
		Weersma_1 (2009)	1120	1350	0.55	0.56	0.95(0.84-1.08)					
		Palomino—Morales (2009)	414	666	0.54	0.51	1.10(0.92-1.32)					
		Márquez_1 (2009)	368	745	0.51	0.53	0.93(0.78-1.12)					
		Sventoraityte (2010)	123	186	0.53	0.48	1.26(0.91-1.75)					
		McGovern (2010) GWAS#1	723	2880	—	—	1.08(0.95-1.22)					
		McGovern (2010) GWAS#2	948	1408	—	—	0.91(0.79-1.05)					
		McGovern (2010) GWAS#3	1022	2503	—	—	1.08(0.95-1.22)					
		McGovern (2010) Rep#1	993	826	—	—	1.08(0.94-1.23)					
		Total	11466	19659								
CDKAL1												
rs6908425	C/T	Franke (2008)	1102	1794	0.81	0.79	1.18(1.01-1.39)	7.68E-03	1.10(1.02-1.18)	0.13	0.39	0.33
		Anderson (2009)	2453	4034	0.80	0.77	1.18(1.08-1.29)					
		Weersma_2 (2009)	1442	1045	0.81	0.78	1.18(0.99-1.41)					
		McGovern (2010) GWAS#1	723	2880	—	—	1.05(0.90-1.22)					
		McGovern (2010) GWAS#2	948	1408	—	—	1.03(0.91-1.16)					
		McGovern (2010) GWAS#3	1022	2503	—	—	1.11(0.95-1.30)					
		McGovern (2010) Rep#1	993	826	—	—	0.91(0.76-1.09)					
		Total	8683	14490								
ZNF365												
rs10995271	C/G	Törkvist (2010)	935	1460	—	—	1.03(0.89-1.18)	1.37E-01	1.07(0.97-1.17)	0.02	0.68	0.26
		Franke (2010)	1043	1703	0.44	0.40	1.19(1.07-1.33)					

(*Continued*)

TABLE 2. (*Continued*)

	Allele* [1/2]	Study	Number		RAF		OR (95%CI)	Combined		Heterogeneity		Publication Bias
			Case	Control	Case	Control		*P*	OR (95% CI)	*P*	I^2 Statistics	*P*
		McGovern (2010) Rep#1	993	826	—	—	1.09(0.95-1.24)					
		McGovern (2010) Rep#2	1016	754	—	—	1.00(0.96-1.05)					
		Total	3987	4743								
rs10761659	G/A	Franke (2008)	1088	1775	0.58	0.54	1.10(1.02-1.19)	4.67E-04	1.14(1.05-1.23)	0.24	0.28	NA
		Fisher (2008)	1807	1549	0.57	0.54	1.19(1.07-1.31)					
		Total	2895	3324								
LRRK2—MUC19												
rs11175593	T/C	Anderson (2009)	3026	1132	0.02	0.01	1.31(0.99-1.74)	1.54E-02	1.21(1.03-1.41)	0.70	0	0.08
		Törkvist (2010)	935	1460	—	—	1.11(0.68-1.80)					
		Franke (2010)	1043	1703	0.02	0.02	1.18(0.83-1.70)					
		McGovern (2010) Rep#1	993	826	—	—	1.31(0.97-1.76)					
		McGovern (2010) Rep#2	1016	754	—	—	0.94(0.62-1.43)					
		Total	7013	5875								
C13orf31												
rs3764147	G/A	Anderson (2009)	2424	4017	0.22	0.21	1.07(0.98-1.18)	1.80E-02	1.07(1.01-1.13)	0.39	0.03	0.78
		Törkvist (2010)	935	1460	—	—	1.22(1.04-1.42)					
		Franke (2010)	1043	1703	0.25	0.25	1.02(0.89-1.15)					
		McGovern (2010) Rep#1	993	826	—	—	1.04(0.90-1.20)					
		McGovern (2010) Rep#2	1016	754	—	—	1.02(0.89-1.16)					
		Total	6411	8760								
PTPN2												
rs2542151	G/T	Franke (2008)	1005	1779	0.19	0.15	1.33(1.11-1.59)	2.49E-02	1.08(1.00-1.16)	0.14	0.37	0.06
		Fisher (2008)	1735	1488	0.17	0.17	1.07(0.93-1.22)					
		McGovern (2010) GWAS#1[a]	723	2880	—	—	1.14(0.95-1.36)					
		McGovern (2010) GWAS#2[a]	948	1408	—	—	1.04(0.90-1.20)					
		McGovern (2010) GWAS#3[a]	1022	2503	—	—	1.03(0.85-1.24)					
		McGovern (2010) Rep#1	993	826	—	—	1.00(0.92-1.09)					
		McGovern (2010) Rep#2	1016	754	—	—	1.13(0.94-1.35)					
		Total	7442	11638								
SBNO2												
rs4807569	C/A	Anderson (2009)	2425	4047	0.22	0.20	1.10(1.00-1.20)	1.72E-02	1.06(1.01-1.12)	0.57	0	0.85
		Franke (2010)	1043	1703	0.25	0.24	1.03(0.90-1.17)					
		McGovern (2010) GWAS#1[b]	723	2880	—	—	1.00(0.85-1.18)					
		McGovern (2010) GWAS#2[b]	948	1408	—	—	1.03(0.93-1.13)					
		McGovern (2010) GWAS#3[b]	1022	2503	—	—	1.15(0.99-1.33)					
		Total	6161	12541								

*Allele "1" denotes the reported risk allele.
†OR and 95% CI were calculated using the random-effect model because of the heterogeneity among the studies.
[a]rs1893217 is absolutely linked with rs2542151 ($r^2 = 1.0$).
[b]rs2024092 is absolutely linked with rs4807569 ($r^2 = 1.0$).
RAF, risk allele frequency; OR, odds ratio; CI, confidence interval; NA not applicable.

Many genetic variants including in this pathway such as *IL23R, IL12B, JAK2*, and *STAT3* are associated with susceptibility for both diseases. Among the eight additionally identified common susceptibility loci for CD and UC, several genes are reported to be associated with various diseases or traits: *C13orf31* is associated with leprosy.[67] *PTPN2* is associated with type 1 diabetes[68,69] and celiac disease.[70] *CDKAL1* is a susceptibility gene for type 2 diabetes.[71–73] *GCKR* is implicated in metabolic traits such as triglyceride,[74–76] fasting glucose,[77] and serum uric acid.[78] However, there is little information how these genes affect the development of CD and UC. Functional analysis of these genes will provide further understanding of the common pathogenesis of CD and UC.

When we compared our results with those of a recent meta-analysis for UC,[20] we could not find a significant association in the 6q21 locus. In the present study we performed a meta-analysis using the data of rs7746082 that showed the strongest association with CD at the 6q21 locus.[13] However, the GWAS meta-analysis estimated the association using the data of rs6938089, best proxy SNP for rs7746082.[20] Although the r^2 value between rs7746082 and rs6938089 is 0.60 for the HapMap CEU population (release #27, build 36), there is a possibility that the hidden causative variant at the 6q21 locus might be different between CD and UC. Further detailed analysis is necessary to clarify the effect of the 6q21 locus on susceptibility to CD and UC.

Significant publication bias was observed at rs9292777 on 5p13 locus. The funnel plot showed that the largest study[21] had the largest OR, whereas the OR of the smaller studies were all shifted to the smaller ones. Based on this asymmetrical distribution of OR, we excluded this SNP in this study.

In conclusion, in addition to the reported common susceptibility loci, we identified eight common susceptibility loci for CD and UC by a meta-analysis of published studies using more than 30,000 subjects. Our data indicate that UC and CD share many genetic factors with small effect. These findings will help to clarify the common pathway involved in the development of both diseases.

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