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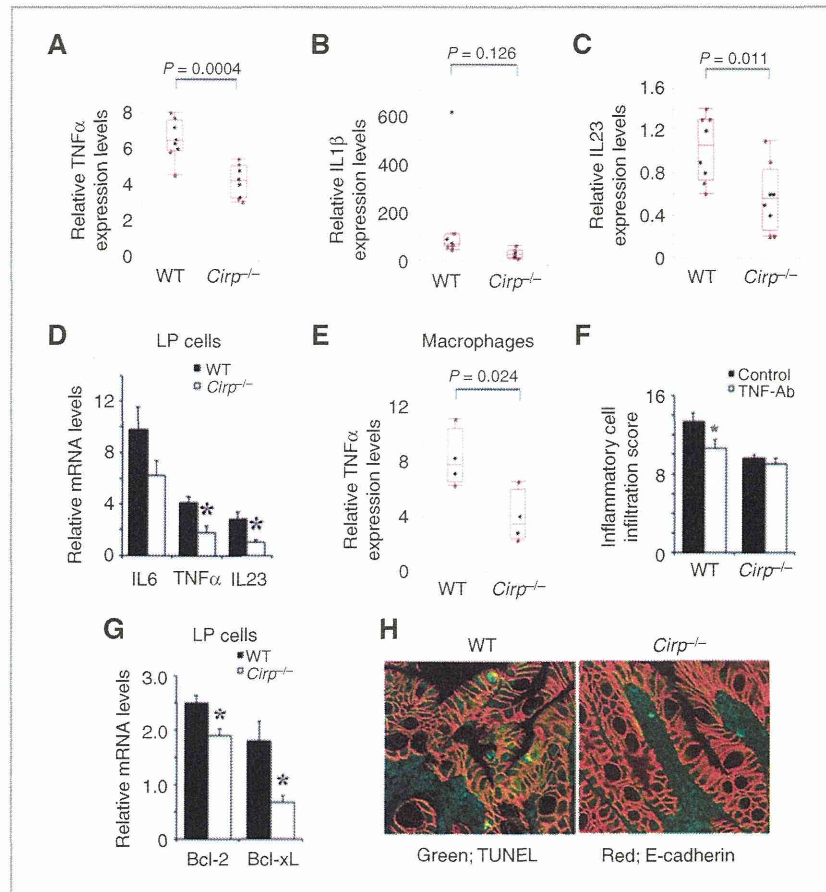

Figure 4. Cirp contributes to TNFα and IL23 production and Bcl-2 and Bcl-xL expression in inflammatory cells. A–C, relative levels of TNFα (A), IL1β (B), and IL23 (C) in colonic tissues from mice treated with DSS, as determined by real-time qPCR ($n = 8$ per group). Expression level in colonic tissues from nontreated WT mice was set as 1. D and E, effect of Cirp on gene expression in colonic lamina propria (LP) cells (D) and macrophages (E). Lamina propria cells and macrophages were isolated, and cytokine mRNA expression was analyzed by real-time qPCR. The mRNA expression levels in lamina propria cells or macrophages from nontreated WT mice were set as 1, respectively. Results are expressed as means ± SEM ($n = 4$ per group). *, $P < 0.05$ compared with WT mice. F, inflammatory cell infiltration into colonic tissues of WT and *Cirp*$^{-/-}$ mice 7 days after the initiation of DSS administration with or without anti-TNFα antibody treatment. Scoring was performed as described in Materials and Methods. $n = 3$ per group. *, $P < 0.05$ compared with control. G, effect of Cirp on gene expression in colonic lamina propria cells. Lamina propria cells were isolated, and cytokine mRNA expression was analyzed by real-time qPCR. The mRNA expression levels in lamina propria cells from nontreated WT mice were set as 1. *, $P < 0.05$ compared with WT LP cells. H, representative images of immunohistochemical detection of E-cadherin, a marker for epithelial cells, and TUNEL staining of colonic tissues from DSS-treated mice.

derived from WT bone marrow exhibited marked upregulation of TNFα mRNA relative to those derived from *Cirp*$^{-/-}$ bone marrow (Supplementary Fig. S4C). Cirp has been reported to activate NF-κB (25, 27). Consistently, the presence of Cirp increased IκBα phosphorylation in bone marrow–derived macrophages (Supplementary Fig. S4D). Treatment with anti-TNFα antibody reduced inflammatory cell infiltration in WT mice but not in *Cirp*$^{-/-}$ mice (Fig. 4F). These data indicate that Cirp in inflammatory cells augments the inflammatory response by producing cytokines such as TNFα and IL23. In addition, *Bcl-2* and *Bcl-xL* mRNA expression was significantly reduced in Cirp-deficient inflammatory cells (Fig. 4G), and more apoptosis was found in *Cirp*$^{-/-}$ immune cells than WT immune cells (Fig. 4H), which might at least partially contribute to the attenuated inflammatory responses by Cirp deficiency.

Cirp deficiency attenuated tumorigenesis in the murine CAC model

Chronic inflammation increases intestinal cancer risk in IBD (10). To investigate the precise pathogenic mechanisms underlying IBD-associated colorectal carcinogenesis, we used the AOM plus DSS mouse model to study the role of Cirp in CAC. In the AOM/DSS protocol, a significant decrease was noted in the number and maximum size of tumors in the *Cirp*$^{-/-}$ mice compared with WT mice (Fig. 5A and B). Histologic

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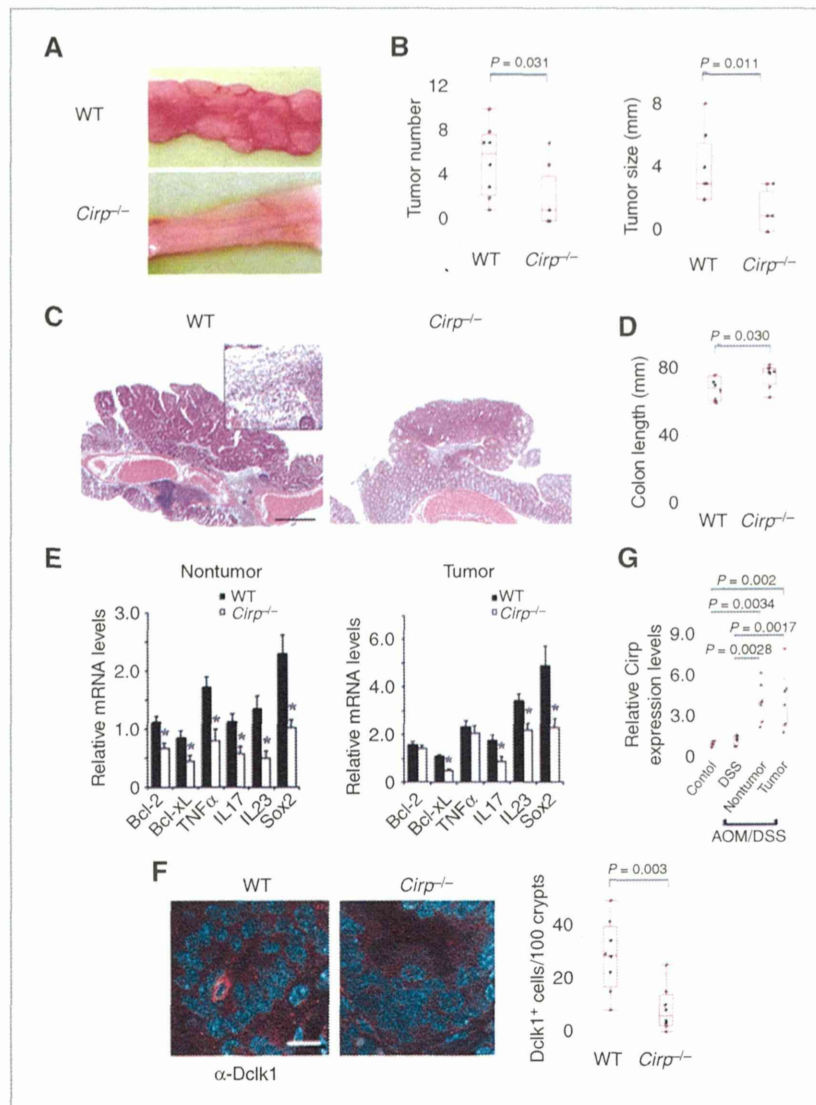

Figure 5. Cirp deficiency affects colonic tumorigenesis in a murine model of CAC. A, typical examples of macroscopic tumorigenesis in the CAC model. Colons were cut longitudinally. B, tumor number and maximum size (WT mice, $n = 8$; *Cirp*$^{-/-}$ mice, $n = 8$). C, typical examples of microscopic tumorigenesis in the CAC model with magnified insets. Scale bar, 500 μm. D, colon length after treatment with AOM and DSS. E, RNA was extracted from nontumorous colon and tumor tissues. Relative amounts of mRNA, as determined by real-time qPCR normalized to the amount of actin mRNA. The amount of each mRNA in the untreated colon was given an arbitrary value of 1.0. *, $P < 0.05$ compared with WT mice. F, immunohistochemical findings of colon sections of AOM/DSS-treated WT and *Cirp*$^{-/-}$ mice. The tumor bases stained with anti-Dclk1 antibody were identified by confocal microscopy. Scale bar, 15 μm. G, RNA was extracted from the colonic tissues of WT mice 7 days after the initiation of DSS administration (DSS, $n = 8$) and nontumorous colon and tumor tissues of WT mice after administration of AOM and DSS (DSS + AOM, $n = 6$). Relative amounts of mRNA as determined by real-time qPCR and normalized to the amount of actin mRNA. The mean value of mRNA in untreated colon (control, $n = 5$) was given an arbitrary value of 1.0. *P* values were calculated by *post hoc* Tukey–Kramer HSD multiple comparison.

examination of H&E-stained sections from the rolled-up colons revealed larger adenomas with a complex tumor growth pattern in WT tumors compared with *Cirp*$^{-/-}$ tumors (Fig. 5C). Extensive infiltration of inflammatory cells into the lamina propria and submucosal layer surrounding the tumors suggest the involvement of inflammatory responses in the tumorigenesis seen in the AOM/DSS-treated mice (Fig. 5C). Colon length was measured as one parameter to assess the severity of inflammation and was found to be significantly longer in *Cirp*$^{-/-}$ mice than in WT mice (Fig. 5D). The expression of TNFα was decreased in nontumorous tissue, but not in tumors, of *Cirp*$^{-/-}$ mice challenged with AOM and DSS (Fig. 5E). Tumor and nontumor cells would use different mechanisms to regulate gene expression. In tumor cells, expression of TNFα might be upregulated in a Cirp-independent manner. IL23 and IL17 inhibit antitumor immunity and promote tumorigenesis (6–8). Expression of IL23 and IL17 was decreased in *Cirp*$^{-/-}$ tumors and nontumor colons compared with WT counterparts (Fig. 5E). *Bcl-2* and *Bcl-xL* mRNA expression that is upregulated by Cirp in inflammatory cells (Fig. 4G) was significantly reduced in Cirp-deficient colons (Fig. 5E). Cirp deficiency decreased proliferating cell nuclear antigen (PCNA) expression in DSS-treated colons, whereas in established tumors, neither apoptosis nor PCNA expression was significantly affected by Cirp deletion (Supplementary Fig. S6). The expression of stemness factor Sox2 was decreased in Cirp-deficient colons

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and tumors compared with WT tissues (Fig. 5E and Supplementary Fig. S5A). Dclk1 is a candidate tumor stem cell marker in the gut (37). Deletion of Cirp decreased the number of Dclk1^{+} cells at the tumor base (Fig. 5F).

Cirp expression was increased in the colonic mucosa of tumor-harboring mice given DSS and AOM, whereas short-term inflammation induced by DSS administration for 7 days did not upregulate Cirp expression (Fig. 5G and Supplementary Fig. S5B). Coupled with the findings in humans (Fig. 2B and E), these results suggest that Cirp is induced by long-term intestinal inflammation.

Cirp promotes tumorigenesis through hematopoietic cell populations

To functionally characterize the contribution of different cell populations to colorectal tumorigenesis, we created Cirp chimeric mice using a combination of γ-irradiation and BMT. Nontransplanted controls survived less than 2 weeks after irradiation, indicating there was ablation of the endogenous marrow. Transplanted animals were allowed to recover for 2 months before placing them on the AOM/DSS protocol. WT mice rescued with *Cirp*$^{-/-}$ bone marrow had a significantly smaller tumor burden than those rescued with WT bone marrow (Fig. 6A and B). Both WT and *Cirp*$^{-/-}$ mice rescued with WT bone marrow had equivalent tumor sizes (Fig. 6B). Chimeras harboring *Cirp*$^{-/-}$ bone marrow diminished expression of TNFα (Fig. 6C), which indicates that Cirp in hematopoietic cells is involved in upregulation of TNFα. Cirp-chimeric mice with Cirp-deficient bone marrow showed a smaller number of Dclk1^{+} and Sox2^{+} cells in tumor than in the WT mice (Fig. 6D and E). Taken together, at least in this model, the absence of Cirp in hematopoietic cellular compartments protects against AOM/DSS-induced tumorigenesis.

Discussion

The association between IBD and colorectal cancer is well established; the cumulative risk of developing colorectal cancer after 20 years is 7% in ulcerative colitis and 8% in Crohn disease (10). Optimal IBD management would reduce the risk of CAC (35). While it is clear that chronic mucosal inflammation plays a causative role in the transition to adenocarcinoma, the molecular link between inflammation and cancer remains to be elucidated. AOM is a procarcinogen that is metabolically activated to a potent alkylating agent that forms O^6-methylguanine (38). Its oncogenic potential is markedly augmented in the setting of chronic inflammation, such as that induced by repeated cycles of DSS treatment (39). TNFα, a key mediator of inflammation in IBD (3), contributes to tumorigenesis by creating a tumor-supportive inflammatory microenvironment

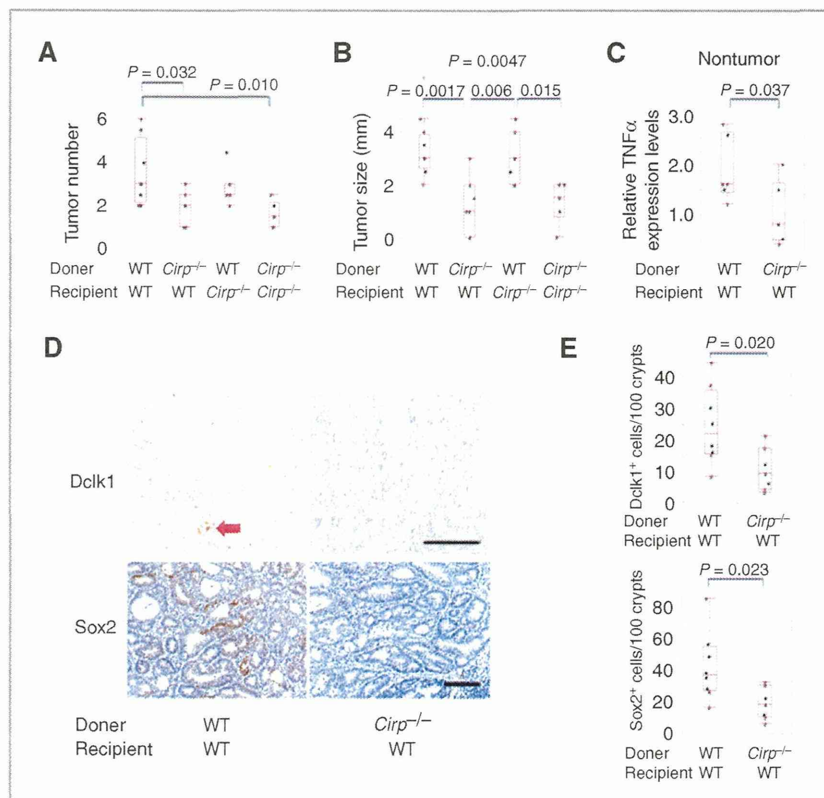

Figure 6. Cirp promotes tumorigenesis through hematopoietic cell populations. WT mice with WT or *Cirp*$^{-/-}$ bone marrow (BM) and *Cirp*$^{-/-}$ mice with WT or *Cirp*$^{-/-}$ bone marrow were generated by BMT. Number (A) and maximum size (B) of the tumors (WT-BM/WT mice, $n = 8$; *Cirp*$^{-/-}$-BM/WT mice, $n = 7$; WT-BM/*Cirp*$^{-/-}$ mice, $n = 7$; *Cirp*$^{-/-}$-BM/*Cirp*$^{-/-}$ mice, $n = 6$). *P* values were calculated by *post hoc* Tukey–Kramer HSD multiple comparison. C, RNA was extracted from nontumorous colon. Relative amounts of mRNA as determined by real-time qPCR normalized to the amount of actin mRNA. The amount of TNFα mRNA in the untreated colon was given an arbitrary value of 1.0. $n = 6$ per group. D, representative images of immunohistochemical detection of Dclk1 at the tumor base and Sox2 in tumors. Scale bar, 100 μm. E, the number of Dclk1^{+} cells at the tumor base and Sox2^{+} in tumors.

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and through its direct effect on malignant cells (40). Interactions between tumor and immune cells regulate tumorigenesis. IL23/IL17 signaling has been correlated with promotion of tumor growth, as well as IBD pathogenesis. In the tumor microenvironment, IL23/IL17 signaling suppresses antitumorigenic immune response during tumor initiation, growth, and metastases (6–8). In the present study, Cirp deficiency decreased the production of TNFα and IL23 in inflammatory cells and attenuated DSS-induced colitis. In the murine CAC model, we found that TNFα, IL23, and IL17 expression were increased in Cirp-deficient mice and that Cirp was required for inflammation-associated colonic carcinogenesis. Cirp expression was positively associated with the levels of TNFα and IL23 in the colonic mucosa of patients with IBD. Increased Cirp expression, seen in refractory IBD, would promote tumorigenesis by enhancing TNFα and IL23 production. Given the contribution of Cirp in hematopoietic cells to tumor formation (Fig. 6), Cirp likely promotes tumorigenesis through its action in inflammatory cells.

Adult somatic stem cells of the colon sustain self-renewal and are targets for cancer initiation (41), and perturbation in stem cell dynamics is generally considered the first step toward colon tumorigenesis. High levels of stemness factor Sox2 expression are associated with poor prognosis and recurrence in patients with colorectal cancer (42). In patients with IBD, mucosal Cirp expression correlated with the expression of Sox2. We also showed that Cirp is important for sustained expression of Sox2 in the colonic mucosa during colorectal carcinogenesis. Cirp deficiency decreased the number of cells positive for an intestinal cancer stem cell marker Dclk1 at the tumor base. These data suggest a possible function of Cirp in influencing stem cell behavior.

Cancer stem cells, the microenvironment, and the immune system interact with each other through cytokines. In the context of chronic inflammation, cytokines, secreted by immune cells, activate the necessary pathways required by cancer stem cells (43). The number of $Sox2^+$ and $Dclk1^+$ cells in tumor was decreased upon Cirp deletion in the hematopoietic compartment (Fig. 6), suggesting that the absence of Cirp in inflammatory cells decreased production/secretion of these cytokines. There were statistically significant relationships between TNFα and Dclk1 expression and between IL23/IL17 and Sox2 expression in colonic mucosa of patients with ulcerative colitis (data not shown). Thus, Cirp-driven immune responses such as activation of TNFα and IL23/IL17 signaling would affect proliferation of stem cells and increase the expression of stem cell markers. It should be noted, however, that the direct causal link between Cirp and the stem cell markers has not been established in this study. In this regard, the reduced expression of the stem cell markers, such as Dclk1 and Sox2, seen in the absence of Cirp might be due to the secondary effects associated with reduced inflammation.

Apoptotic cell death has been implicated as a major homeostatic and pathogenic mechanism of the intestinal epithelium (2). The lower susceptibility to apoptosis observed in the $Cirp^{-/-}$ intestinal epithelial mucosa in our *in vivo* experiment was unexpected because a previous report showed that Cirp attenuates TNFα-mediated apoptosis by activating ERK and NF-κB in murine embryonic fibroblasts (27). However, expression of Cirp did not affect the sensitivity of murine embryonic fibroblasts to busulfan, and the numbers of apoptotic testicular cells was not different between $Cirp^{-/-}$ and WT mice after busulfan treatment (28). Thus, the role of Cirp may vary depending on cell type and kind of stimuli. In fact, in the DSS-treated colon, Cirp deficiency did not attenuate ERK activity (Supplementary Fig. S4A). In $Cirp^{-/-}$ mice, more inflammatory cells died because of decreased Bcl-2 and Bcl-xL expression than in WT mice (Fig. 4G and H), which would attenuate inflammatory response in $Cirp^{-/-}$ mice. Cell death and inflammation are intimately linked through a self-amplifying loop, making it difficult to distinguish between causes and effects. The attenuated mucosal immune activity due to augmented apoptosis of inflammatory cells likely contributed to the decreased apoptosis of epithelial cells in Cirp-deficient colon.

Bcl-2–mediated apoptosis resistance in inflammatory cells has been shown to attenuate therapeutic efficacy and exacerbate inflammation in IBD (33, 34). In chronically inflamed mucosa seen in refractory ulcerative colitis, Cirp expression is induced in inflammatory cells, which likely inhibits the apoptosis of inflammatory cells, augments proinflammatory cytokine production and treatment resistance via the upregulation of Bcl-2 and Bcl-xL expression. Thus, persistent inflammation resulting from insufficient treatment might further drive resistance to therapy through increased expression of Cirp and subsequent attenuated apoptosis in inflammatory cells. Hypoxia that is enhanced in chronic inflammatory diseases, including IBD, upregulates Cirp expression by a mechanism that involves neither hypoxia-inducible factor (HIF)1 nor mitochondria (20). This may be one explanation for Cirp induction by chronic inflammation. However, the exact mechanisms by which long-term inflammation upregulates Cirp expression remain to be elucidated.

It has been reported that Cirp released into the circulation stimulates the release of TNFα from macrophages via TLR4 and NF-κB activation and triggers an inflammatory response to hemorrhagic shock and sepsis (25). Here, we have shown that in bone marrow–derived macrophages, the presence of Cirp increased IκBα phosphorylation. NF-κB activation would be one of the mechanisms by which Cirp produces proinflammatory cytokines such as TNFα, IL17, and IL23 and upregulates expression of antiapoptotic genes such as Bcl-2 and Bcl-xL (Figs. 4 and 5). A recent study reported the involvement of Cirp in regulating expression of IL1β, another NF-κB target gene, in cultured fibroblasts (44). In bone marrow–derived macrophages, *IL1β* mRNA level was decreased in the absence of Cirp (data not shown). Although in DSS-induced colitis, Cirp protein was not detected in the blood (data not shown), it is conceivable that Cirp released from injured epithelial cells could function as damage-associated molecular pattern molecules *in situ* to activate NF-κB in immune cells of the colon. Furthermore, Cirp can bind the 3′untranslated region of specific transcripts to stabilize them and thus facilitate their transport to ribosomes for translation (22–24). Cirp might regulate the expression of cytokines and antiapoptotic genes posttranscriptionally as well.

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Given the long-term impact of the natural history and treatment of IBD, cancer risk is a major lifelong concern for patients and gastroenterologists. Early detection of CAC/dysplasia is typically achieved by colonoscopic surveillance with multiple biopsies and alternatively by chromoendoscopy with targeted biopsies of all suspect areas. It has been reported that in patients with extensive colitis, surveillance should start after colonoscopy screening (8–10 years after disease onset) and be performed every 2 years for 20 years, then once or twice a year for the next 10 years of disease duration (45). However, such surveillance programs have a number of limitations such as low yield, high cost, invasiveness, incomplete patient enrollment, sampling variations, and poor agreement in histopathologic interpretation (46). If we can reliably predict an individual's risk of CAC so that surveillance strategies can be appropriately personalized, surveillance programs would make much progress. A number of molecular markers for predicting CAC have been reported (47–49) but are not feasible yet for the practical management of patients with IBD. Here, we showed significantly increased Cirp expression in mucosal specimens from patients with refractory IBD that is reported to be associated with increased cancer risk (9). Furthermore, in the murine CAC model, longstanding colonic inflammation increased Cirp expression, which led to enhanced AOM/DSS-induced colorectal tumorigenesis. Cirp expression reflects the presence of refractory inflammation and is therefore a potential marker for predicting the risk of CAC development. Analyzing the Cirp level in colonoscopy specimens may increase the identification rate of IBD patients with a high risk for developing CAC. A future large-scale study of Cirp in IBD patients with different duration and anatomical extent of the disease will be crucial for determining whether Cirp status can be used to predict the risk of cancer and prognosis of patients with IBD.

Taken together, Cirp, whose expression is upregulated by chronic inflammation in humans and mice, enhances the inflammatory response and tumorigenesis by increasing Bcl-2 and Bcl-xL expression and TNFα and IL23/IL17 production in inflammatory cells. Suppression and measurement of Cirp expression is a promising approach for advanced treatment and personalized management of patients with IBD.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: T. Sakurai
Development of methodology: T. Sakurai, J. Fujita
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Sakurai, H. Kashida, T. Mizushima, H. Iijima, N. Nishida
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Sakurai, N. Nishida, J. Fujita, M. Kudo
Writing, review, and/or revision of the manuscript: T. Sakurai, H. Kashida, T. Watanabe, J. Fujita, M. Kudo
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Sakurai, S. Hagiwara
Study supervision: T. Sakurai, H. Kashida, H. Higashitsuji, M. Kudo

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Stress Response Protein Cirp Links Inflammation and Tumorigenesis in Colitis-Associated Cancer

Toshiharu Sakurai, Hiroshi Kashida, Tomohiro Watanabe, et al.

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ORIGINAL ARTICLE—ALIMENTARY TRACT

Regulation of anergy-related ubiquitin E3 ligase, GRAIL, in murine models of colitis and patients with Crohn's disease

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Abstract

Background Abrogating tolerance is a critical step in the pathogenesis of Crohn's disease (CD). T cell-anergy is one of the main mechanisms of tolerance and is regulated by the gene related to anergy in lymphocytes (GRAIL). This study investigated the expressions and regulation of GRAIL in CD and murine colitis models.

Methods Expressions of GRAIL mRNA and protein in $CD4^+$ T cells were investigated in the peripheral blood and mucosal tissues of patients with CD, mice with dextran sodium salt (DSS)-induced colitis, and *Il-10*-deficient mice. MicroRNAs responsible for the regulation of GRAIL were examined by miRNA microarray. GRAIL-overexpressing T cells were intravenously injected in mice with DSS-induced colitis.

Results The GRAIL expression was higher in the lamina propria (LP) $CD4^+$ T cells of CD patients than of the control subjects, while it was lower in the peripheral blood $CD4^+$ T cells of the CD patients than of the control subjects. The GRAIL mRNA expression was lower, but the GRAIL protein expression was higher in the LP of colitic mice than that of non-colitic mice. The miRNA microarray identified miR-290-5p as an miRNA that inhibits expression of the GRAIL protein and that is highly expressed in the LP of non-colitic mice. GRAIL-expressing T cells expressed regulatory T cell markers and showed suppressive effects in murine DSS-induced colitis.

Conclusions Our results show that expression of GRAIL is uniquely regulated by the specific miRNA in the intestinal mucosa, and suggest that GRAIL may associate with the pathophysiology of CD.

Keywords Inflammatory bowel disease · MicroRNA · GRAIL · Regulatory T cells

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Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) are two major categories of inflammatory bowel diseases (IBD) in the human gastrointestinal tract. Both genetic susceptibility and dysregulation of mucosal immune responses against enteric host flora have pivotal roles in mucosal injury [1–3], and abrogating tolerance against unidentified antigens is a critical step in the pathogenesis

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of IBD [4]. Clonal anergy of T cells is one of the main mechanisms of tolerance [5], and failure to induce clonal anergy is considered to be associated with the pathogenesis of IBD [6, 7]. T-cell anergy is tightly regulated by E3 ubiquitin ligases, including the gene related to anergy in lymphocyte (GRAIL), the casitas B lineage lymphoma (cbl) family, itchy homologue E3 ubiquitin protein ligase (Itch), and the neural precursor cell expressed developmentally downregulated 4 (NEDD4) [8]. Among these, the role of GRAIL in T-cell anergy has been most vigorously investigated. GRAIL is a type I transmembrane protein that localizes to the endocytic pathway and bears homology to the 'really interesting new gene' (RING) zinc-finger proteins [8]. GRAIL has been shown to be expressed in anergic $CD4^{+}$ T cells [5, 8]. The expression of GRAIL in $CD4^{+}$ T cells induces T-cell anergy by limiting interleukin (IL)-2 and IL-4 production. When DO11.10 T cells were infected with an ecotropic retrovirus constitutively expressing wild-type GRAIL, their proliferative capacity in response to antigen and antigen-presenting cells was diminished. Recent studies revealed that GRAIL inhibits the T cell activation cascade by the ubiquitination of CD40 ligand (CD40L), CD83, and T cell receptor (TCR)-CD3 [9–11]. In addition to the importance of GRAIL in converting T cells to an anergic phenotype, a recent study showed that the forced expression of GRAIL in DO11.10 T cells was sufficient for the conversion of these cells to a regulatory phenotype [12]. Additionally, in a Staphylococcal enterotoxin B (SEB)-mediated model of T cell unresponsiveness in vivo, the SEB-exposed forkhead box P3 $(FoxP3)^{+}GRAIL^{+}$ T cells were shown to be highly suppressive and non-proliferative, independent of CD25 expression level and glucocorticoid-induced, tumor necrosis factor receptor-related protein (GITR) [13, 14]. This model system revealed a novel paradigm for chronic, non-canonical TCR engagement leading to the development of highly suppressive $FoxP3^{+}GRAIL^{+}CD4^{+}$ T cells. In fact, GRAIL-deficient ($grail^{-/-}$) mice exhibit a susceptibility to autoimmune disease [11]. The importance of GRAIL in human diseases was further demonstrated by our recent study showing that GRAIL expression was increased in the peripheral blood $CD4^{+}$ T cells of UC patients in remission compared to those of healthy subjects and active UC patients [15]. In addition, GRAIL expression was increased after the effective treatments for active UC patients. To date, no previous reports have investigated the expression of GRAIL in patients with CD and animal models of IBD. In addition, GRAIL expression has not been investigated in the intestine, which comprises the largest pool of immune cells in the human body [16]. We demonstrate here for the first time the expression of GRAIL in patients with CD and in murine colitis models. We also present a novel mechanism in terms of the regulation of GRAIL in the intestine by a specific microRNA (miRNA).

Materials and methods

Human samples

Blood samples were obtained from CD patients who were hospitalized in Osaka University Hospital or from healthy volunteers (HV) recruited in Osaka University Hospital. Intestinal tissues were obtained from the patients with CD or patients with colon cancer who were subjected to surgical resection of the small intestine or the colon in Osaka University Hospital. Patients were diagnosed as having CD according to the endoscopic, radiologic, histological, and clinical criteria provided by the International Organization for the Study of Inflammatory Bowel Disease [17, 18]. The disease activity of CD was evaluated by the Crohn's disease activity index (CDAI) [19]. A CDAI above 150 was defined as active, whereas a CDAI $\leq$150 was defined as remission.

Mice

C57BL/6J mice were purchased from SLC (Shizuoka, Japan). *Il-10*$^{-/-}$ mice (C57BL/6 background) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). To generate dextran sodium sulfate (DSS) colitis, we orally administered 2 % DSS to female C57BL/6J mice (age 8–12 weeks) in their drinking water for 7 days. All mice were kept under specific pathogen-free conditions in an environmentally-controlled, clean room at the Institute of Experimental Animal Sciences, Osaka University Graduate School of Medicine. The spleen (SP) and mesenteric lymph node (MLN) were aseptically extracted, and single-cell suspensions were prepared by a standard mechanical disruption procedure [20, 21]. Mononuclear cells in the intestinal lamina propria (LP) were prepared by an enzymatic dissociation, as described previously [20, 21]. Briefly, after Peyer's patches were removed from the intestine, the epithelial cell layers were removed from intestinal tissue by incubation in RPMI1640 (Sigma Aldrich, St. Louis, MO, USA) containing ethylenediamine tetraacetic acid (EDTA) [20]. The specimens were dissociated in RPMI1640 containing collagenase (Wako, Osaka, Japan) by stirring at 37 °C. Mononuclear cells were isolated using the discontinuous density gradients procedure with Percoll (GE Healthcare, Pewaukee, WI, USA).

Quantitative real-time RT-PCR

$CD4^+$ T cells from human peripheral blood mononuclear cells (PBMCs) or murine lymphoid tissues were isolated by magnetic cell separation using anti-CD4 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). The total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA was obtained by reverse-transcription of mRNA with High-Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA, USA). The miRNA fraction was prepared using an miRNA Isolation kit (Ambion, Austin, TX, USA), and 10 ng of total RNA were reverse-transcribed by a MicroRNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) analyses for GRAIL (human ID, Hs00226053; mouse ID, Mm00506597), FoxP3 (ID, Mm00475162), GITR (ID, Mm00437136), CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4; ID, Mm00486849), β-actin, miR-290-5p (ID, 002590), and snoRNA135 (ID, 001234) were performed using ready-to-use assays in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). β-actin and snoRNA135 were used as the endogenous controls.

Immunoblot analysis

Human $CD4^+$ T cells from PBMC or murine $CD4^+$ T cells were lysed in radio-immunoprecipitation assay (RIPA) buffer. Protein samples were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane, as previously described [15]. The membrane was probed with rabbit anti-human GRAIL antibody (IMGENEX, San Diego, CA), rat anti-mouse GRAIL antibody (BD Biosciences, Franklin Lakes, NJ, USA) or rabbit anti-β-actin antibody (Cell Signaling Technology, Danvers, MA, USA). The blots were developed with horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemiluminescence (GE Healthcare).

Immunohistochemistry

Paraffin-embedded tissue sections were subjected to antigen retrieval by incubation in a pressurized heating chamber (Dako, Carpinteris, CA, USA). Tissue sections were reacted in combination with rat anti-mouse GRAIL antibody (BD Biosciences), rabbit anti-human GRAIL antibody (IMGENEX, San Diego, CA, USA), mouse anti-human CD4 antibody (Abcam, Cambridge, UK), or mouse anti-human FoxP3 antibody (Abcam) as first antibodies, followed by Alexa Flour 488®-labeled anti-rat or anti-rabbit IgG antibody (Molecular Probes, Eugene, OR, USA) or Texas Red-labeled anti-mouse IgG antibody (Abcam) as a secondary antibody. Nuclear staining was performed by DAPI (4′,6-diamidino-2-phenylindole). These stained samples were examined using fluorescence microscopy (Keyence, Osaka, Japan) or three-dimensional, structured illumination microscopy (Carl Zeiss, Oberkochen, Germany). In mice transferred with $GRAIL^{high}$ DO11.10 cells, the tissue sections were stained with anti-mouse GFP antibody (Clontech), and color was developed by 3′-diaminobenzidine using a VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA, USA).

miRNA microarray analysis

For the miRNA microarray analysis, 0.5 μg of total RNA from $CD4^+$ T cells of the SP or LP were labeled using a miRCURY LNA™ microRNA Array Power Labeling kit (Exiqon, Vedbaek, Denmark) according to the manufacturer's instructions. Each sample of RNA labeled with Hy5 was hybridized with a highly sensitive DNA chip, *3D-Gene*™ (Toray Industries, Tokyo, Japan) at 37 °C for 16 h. Hybridization signals derived from Hy5 were scanned using a 3D-Gene Scanner (Toray). The scanned image was analyzed and scaled by global normalization [22]. All microarray experiments were performed in compliance with MIAME (Minimum Information About a Microarray Experiment) guidelines [23].

miRNA target predictions

miRgator (http://mirgator.kobic.re.kr:8080/MEXWebApp/), miRGen (http://www.diana.pcbi.upenn.edu/cgi-bin/miRGen/v3/Targets.cgi), and MicroCosmTargets (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) were used to predict miRNAs that potentially combine with the 3′-untranslated region (3′UTR) of GRAIL mRNA.

Transfection

DO11.10 cells (a murine T cell line, purchased from European Collection of Cell Cultures, Salisbury, UK) were transfected with anti-miR-290-5p inhibitor (50 nM, Ambion) by electroporation (250 V, 950 μF). Anti-miRNA Inhibitors Negative Control #1 (Ambion) was used as endogenous control. Seventy-two hours after the transfection of anti-miR-290-5p inhibitor or negative control #1, cells were collected, and cellular lysates were subjected to western blot using an anti-GRAIL antibody. The GRAIL fragment was inserted into a pAcGFP vector (Clontech, Palo Alto, CA, USA), and the pAcGFP-GRAIL vector or empty vector was transfected into DO11.10 cells by X-tremeGENE HP (Roche, Basel, Switzerland) according to the manufacturer's instructions. $EGFP^+$ DO11.10 cells ($2–5 \times 10^4$ cells/body) sorted by flow cytometry (FACSAria™ II) were intravenously injected into