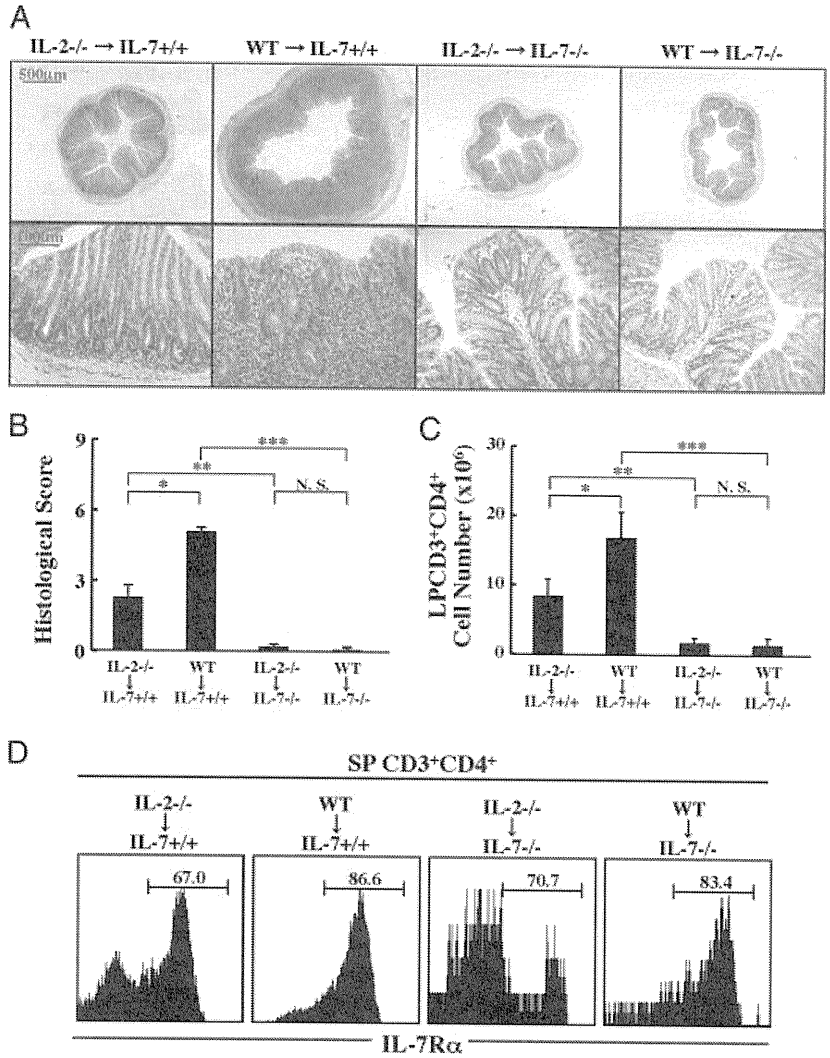

Figure 6. IL-7 is absolutely essential for the development of colitis in mice transferred with IL-2$^{-/-}$ LP CD4$^+$ T cells despite down-modulated IL-7Rα expression. We performed one independent experiment. (A) Histopathology of the distal colon 8 wk after retransfer. Original magnification, × 40 (upper) and × 200 (lower). (B) Histological score 8 wk after retransfer. Data are expressed as the mean ± SEM of five mice *per* group. $^*p = 0.009$, $^{**}p = 0.016$, $^{***}p = 0.009$ (Mann-Whitney *U*). (C) Number of LP CD3$^+$CD4$^+$ T cells recovered 8 wk after retransfer. Data are expressed as the mean ± SEM of five mice *per* group. $^*p = 0.049$ (Student's *t*), $^{**}p = 0.033$ (Welch's *t*), and $^{***}p = 0.014$ (Mann-Whitney *U*). (D) Expression of IL-7Rα on SP CD3$^+$CD4$^+$ T cells was determined using FACS. Representative FACS data of five mice are shown.

IL-2$^{-/-}$ CD4$^+$CD45 RB^{high} T cells do not induce colitis, even if they were developed in IL – 2$^{-/-}$ and WT BM chimaeric mice

To precisely prepare similar naïve WT and IL-2$^{-/-}$ CD4$^+$ CD45RB^{high} T cells in the same *in vivo* setting, we generated mixed BM chimeras. To this end, irradiated GFP mice were first transplanted with a mixture of the same number of BM cells obtained from young WT and IL-2$^{-/-}$ mice (Fig. 7A). Six weeks after the BM transplantation, various cell markers were compared between the transplanted WT and IL-2$^{-/-}$ BM-derived GFP$^-$ CD4$^+$ cells. As shown in Fig. 6B, almost all the cell markers, including IL-7Rα, were identical in the transplanted WT and IL-2$^{-/-}$ BM-derived CD4$^+$ cells. Thus, GFP$^-$ Ly5.1$^+$ WT and Ly5.2$^+$ IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells were isolated, and then the separated cells or a mixture of the same number of the two cell types was transferred into RAG-2$^{-/-}$ mice. Again, we found that RAG-2$^{-/-}$ mice transferred with GFP$^-$ Ly5.1$^+$ WT CD4$^+$ CD45RB^{high} T cells or a mixture of GFP$^-$ Ly5.1$^+$ WT and Ly5.2$^+$ IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells showed similar signs of wasting disease (Fig. 7B), clinical symptoms (Fig. 7D), and clinical and histological scores (Fig. 7E and F) 8 wk after transfer, which differs from the findings for non-colitic RAG-2$^{-/-}$ mice transferred with GFP$^-$ Ly5.2$^+$ IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells (Fig. 7C–F). The number of LP CD4$^+$ T cells recovered from RAG-2$^{-/-}$ mice transferred with GFP$^-$ Ly5.1$^+$ WT CD4$^+$CD45RB^{high} T cells or a mixture of GFP$^-$ Ly5.1$^+$ WT and Ly5.2$^+$ IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells was significantly higher than that recovered from mice transferred with Ly5.2$^+$ IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells (Fig. 8A). The production of IFN-γ,

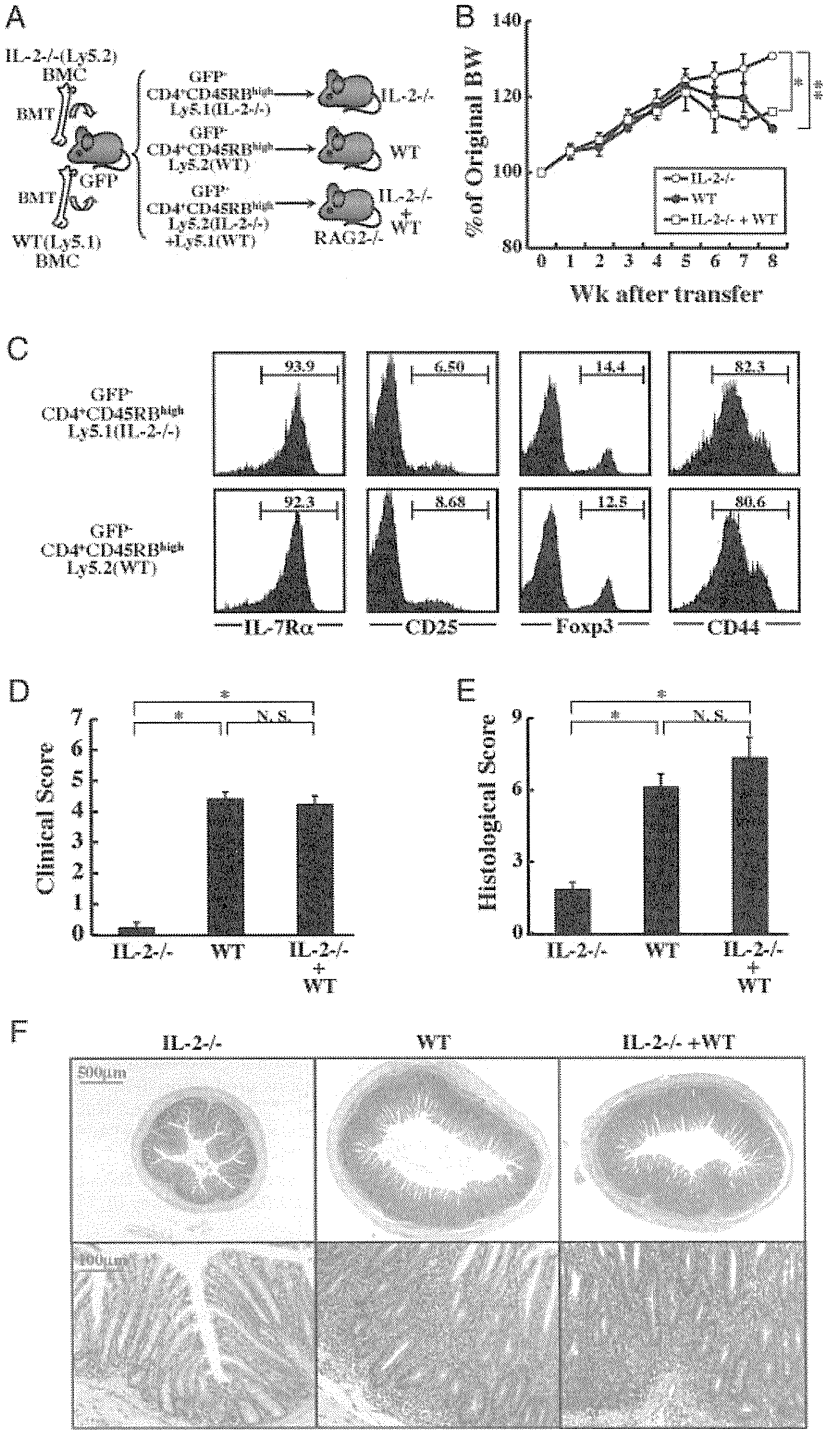

Figure 7. RAG-2$^{-/-}$ mice transferred with SP IL-2$^{-/-}$ CD4^{+}CD45RB^{high} T cells from GFP mice previously transplanted with WT and IL-2$^{-/-}$ BM cells do not develop colitis. We performed one independent experiment. (A) Experimental design. Irradiated (7.5 Gy) GFP mice were transplanted with a mixture of the same number (5×10^6 cells) of CD3-depleted BM cells from WT (Ly5.1^{+}) and IL-2$^{-/-}$ (Ly5.2^{+}) mice. Eight weeks after the BM transplantation, WT (Ly5.1^{+}) and IL-2$^{-/-}$ (Ly5.2^{+}) GFP^{-} CD4^{+}CD45RB^{high} T cells were isolated using FACSAria. The cells (3×10^5 cells *per* mouse) or cell mixtures (3×10^5 cells of each) were transferred into new RAG-2$^{-/-}$ mice ($n = 5$). Mice were sacrificed 8 wk after transfer. (B) The change in body weight over time is expressed as a percentage of original weight. Data are expressed as the mean ± SEM of five mice *per* group. $^{*}p = 0.008$, and $^{**}p = 0.041$ (Mann-Whitney *U*). (C) Phenotypic characterization of SP GFP^{-} WT and IL-2$^{-/-}$ CD4^{+}CD45RB^{high} donor T cells after BM transplantation. (D) Clinical score 8 wk after transfer. Data are expressed as the mean ± SEM of five mice *per* group. $^{*}p = 0.0143$ (Mann-Whitney *U*). (E) Histological score 8 wk after transfer. Data are expressed as the mean ± SEM of five mice *per* group. $^{*}p = 0.0143$ (Mann-Whitney *U*). (F) Histopathology of the distal colon 8 wk after transfer. Original magnification, × 40 (upper) and × 200 (lower).

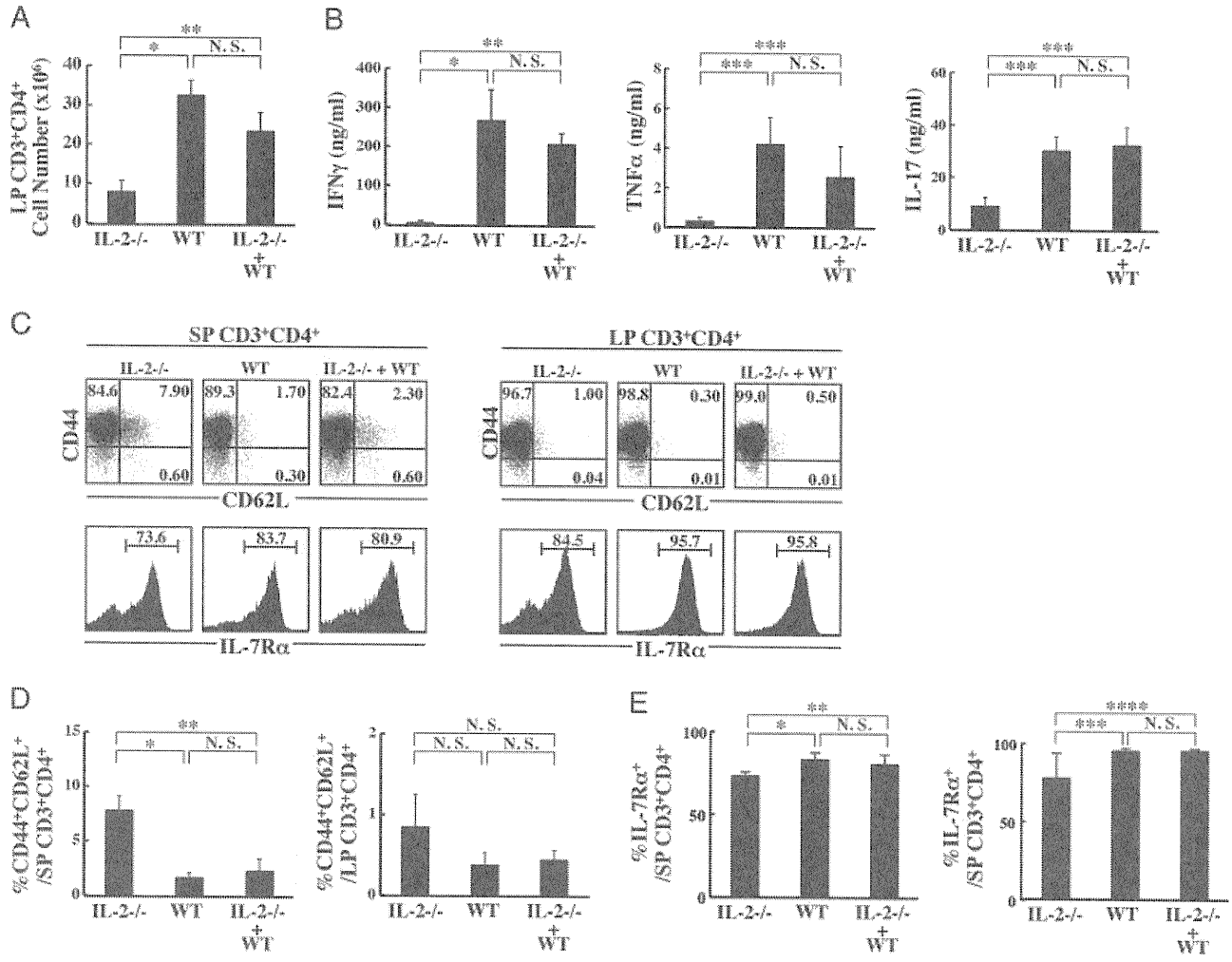

Figure 8. Marked down-modulation of IL-7Rα expression in non-colitic RAG-2$^{-/-}$ mice transferred with SP IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells. (A) Numbers of LP CD3$^+$CD4$^+$ T cells recovered from each mouse 8 wk after transfer. Data are expressed as the mean ± SD of five mice *per* group. $^*p = 0.037$ (Student's *t*), $^{**}p = 0.05$ (Mann-Whitney *U*), and N.S., not significant (B) Cytokine production (IFN-γ, TNF-α, and IL-17) by LP CD4$^+$ T cells. Data are expressed as the mean ± SEM of five mice *per* group. $^*p = 0.003$, $^{**}p = 0.007$, $^{***}p = 0.0143$, and N.S., not significant. (C) Expression of various cell surface markers on SP (left) and LP (right) CD3$^+$CD4$^+$ T cells of each group was assessed using FACS. (D) The percentage of SP (left) and LP (right) T_{CM} cells was determined using FACS. Data are expressed as the mean ± SEM of five mice. $^*p = 0.0015$, $^{**}p = 0.0017$ (Mann-Whitney *U*), and N.S., not significant. (E) Percentage of SP (left) and LP (right) CD3$^+$CD4$^+$ T cells positive for IL-7Rα$^+$. Data are expressed as the mean ± SEM of five mice *per* group. $^*p = 0.0002$ (Welch's *t*), $^{**}p = 0.0065$ (Mann-Whitney *U*), $^{***}p = 0.0027$ (Mann-Whitney *U*), $^{****}p = 0.011$ (Mann-Whitney *U*), and N.S., not significant.

TNF-α, and IL-17 by anti-CD3/CD28 mAb-stimulated LP CD4$^+$ T cells obtained from RAG-2$^{-/-}$ mice transferred with WT CD4$^+$ CD45RB^{high} T cells or a mixture of WT and IL-2$^{-/-}$ CD4$^+$ CD45RB^{high} T cells was significantly higher than that produced in RAG-2$^{-/-}$ mice transferred with IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells (Fig. 8B).

We next examined cell surface markers in terms of T_{CM}/T_{EM} and IL-7Rα after transfer. Again, the proportion of T_{CM} in the SP of RAG-2$^{-/-}$ mice transferred with IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells was significantly higher than that in RAG-2$^{-/-}$ mice transferred with WT CD4$^+$CD45RB^{high} T cells or a mixture of WT and IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells (Fig. 8C and D). In contrast, LP CD4$^+$ T cells were exclusively T_{EM} cells irrespective of the cells transferred (Fig. 8C and D). It is noteworthy that the positive frequency of IL-7Rα expression on the SP and LP of RAG-2$^{-/-}$ mice transferred with IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells significantly reduced, as compared with that in the paired cells of other groups, irrespective of the presence or absence of colitis (Fig. 8C and E).

Discussion

In the present work, we demonstrated that IL-2 is essential for the development and perpetuation of chronic colitis. First, IL-2 is needed for the normal expression of IL-7Rα on naïve CD4$^+$ T cells during the development of mature naïve CD4$^+$ T cells. Second, IL-2 is required for retaining IL-7Rα expression on colitogenic

$CD4^+$ T cells during conversion from effector to memory $CD4^+$ T cells, resulting in the acquisition of IL-7 dependency for the perpetuation of chronic colitis.

$IL\text{-}2^{-/-}$ mice, as well as $IL\text{-}2R\alpha$ $(CD25)^{-/-}$, $IL\text{-}2R\beta$ $(CD122)^{-/-}$, and $IL\text{-}2R\gamma$ $(CD132)^{-/-}$ mice [12], all of which are severely defective in the IL-2/IL-2R signalling pathway, are known to spontaneously develop chronic colitis. Thus, it was considered possible that the abnormal naïve $CD4^+$ T cells in $IL\text{-}2^{-/-}$ mice themselves might be critically involved in the development of colitis in such colitis models. It was also considered possible that IL-2 itself may not be needed for the development of pathogenic effector and memory $CD4^+$ T cells, but is solely essential for the development and maintenance of Treg, as was recently emphasized [16–18]. To solve these issues, we used an adoptive transfer model of colitis using WT or $IL\text{-}2^{-/-}$ $CD4^+$ $CD45RB^{high}$ T cells, which are almost all naïve T cells, excluding Treg. Surprisingly, we found that $RAG\text{-}2^{-/-}$ mice transferred with $IL\text{-}2^{-/-}$ $CD4^+CD45RB^{high}$ T cells did not develop colitis until 8 wk after transfer. Furthermore, $IL\text{-}2^{-/-}$ $CD4^+CD45RB^{high}$ T-cell-derived cells differentiated into $CD44^{high}$ memory T cells in the LP and SP, similar to WT T-cell-derived cells (Figs. 2 and 3). It should be noted that inducible $CD4^+CD25^+Foxp3^+$ Treg were present in $RAG\text{-}2^{-/-}$ mice transferred with WT, but not $IL\text{-}2^{-/-}$, $CD4^+CD45RB^{high}$ T cells. These results clearly demonstrate that IL-2 is positively involved in the development of colitis without the effect of naturally occurring Treg, although IL-2 supports the development of inducible Treg, which are generally thought to suppress the development of colitis. However, an unresolved discrepancy remains between the present finding that IL-2 is positively important for the development and perpetuation of colitis and the previous finding that $IL\text{-}2^{-/-}$ mice spontaneously develop colitis together with various autoimmune diseases. Indeed, we showed that adoptive retransfer of $IL\text{-}2^{-/-}$ LP $CD4^+$ T cells obtained from colitic $RAG\text{-}2^{-/-}$ mice previously transferred with a mixture of WT and $IL\text{-}2^{-/-}$ $CD4^+CD45RB^{high}$ T cells induced colitis, albeit less severely than adoptive retransfer of WT LP $CD4^+$ T cells, suggesting that IL-2 is not absolutely essential for the development of colitis, but is a fine tuner for the full development of colitis. Indeed, we confirmed that $RAG\text{-}2^{-/-}$ mice transferred with $IL\text{-}2^{-/-}$ $CD4^+CD45RB^{high}$ T cells develop mild colitis 20 wk after transfer (data not shown). It is also possible that other cells, such as B cells and NK T cells may affect the development of colitis in $IL\text{-}2^{-/-}$ mice. Further investigation of this issue is needed.

The positive frequency of IL-7Rα expression on LP $IL\text{-}2^{-/-}$ $CD4^+CD44^{high}$ T cells was significantly reduced, as compared with the paired WT cells, indicating that IL-2 was needed for the expression of IL-7Rα on some subpopulation of $CD4^+$ T cells during the differentiation of memory $CD4^+CD44^{high}$ T cells in all the adoptive transfer experiments. Importantly, since we demonstrated that mice transferred with isolated $IL\text{-}7R\alpha^{high}$ or $IL\text{-}7R\alpha^{low}$ $CD4^+CD45RB^{high}$ T cells obtained from young $IL\text{-}2^{-/-}$ mice did not develop colitis (Fig. 3), it is not likely that $IL\text{-}7R\alpha^{low}$ cells suppress $IL\text{-}7R\alpha^{high}$ cells when they are co-transferred into $RAG\text{-}2^{-/-}$ mice. Rather, IL-2 may be critically involved in the retainment of IL-7Rα expression on colitogenic $CD4^+$ T cells. In other words, the lack of IL-2 may induce the exhaustion of colitogenic $CD4^+$ T cells, resulting in the apoptosis of those cells. Furthermore, it is a matter of controversy whether IL-2 positively or negatively controls IL-7Rα expression [13, 30]. One group demonstrated that IL-2 is a negative regulator of IL-7Rα expression during *in vitro* stimulation with anti-CD3/anti-CD28 mAb [30], whereas another group argued that IL-2 promotes the generation of $CD4^+IL\text{-}7R\alpha^+$ memory T cells based on findings from a physiologically relevant *in vitro* and *in vivo* priming system with antigen and antigen-presenting cells [15]. Our result from adoptive transfer in the *in vivo* colitis model supports the latter result. In our previous study with the same model of colitis using IL-2-sufficient $CD4^+CD45RB^{high}$ T cells [27], we observed that the expression of IL-7Rα on $CD4^+$ T cells was down-modulated approximately 1 wk after transfer and was then up-regulated until 4 wk after transfer. Thus, our current result suggests that IL-2 is needed for re-expression of IL-7Rα during the differentiation of functionally colitogenic memory $CD4^+$ T cells. In accordance with this, we showed that $IL\text{-}7^{-/-} \times RAG\text{-}2^{-/-}$ mice retransferred with $IL\text{-}2^{-/-}$ LP $CD4^+$ T cells obtained from colitic $RAG\text{-}2^{-/-}$ mice previously transferred with a mixture of WT and $IL\text{-}2^{-/-}$ $CD4^+CD45RB^{high}$ T cells did not develop colitis, whereas $IL\text{-}7^{+/+} \times RAG\text{-}2^{-/-}$ mice retransferred with those cells did develop colitis, albeit to a lesser extent than $IL\text{-}7^{+/+} \times RAG\text{-}2^{-/-}$ mice retransferred with WT $CD4^+CD45RB^{high}$ T-cell-derived colitic LP $CD4^+$ T cells. IL-2 may play a crucial role in the re-expression of IL-7Rα on colitogenic memory $CD4^+$ T cells, which may be critical for the acquisition of their IL-7 dependency in chronic colitis. Furthermore, since we showed that mice transferred with $IL\text{-}2^{-/-}$ $CD4^+CD45RB^{high}$ T cells retained $IL\text{-}7R\alpha^{high}$ $CD4^+$ T-cell population partly as well as $IL\text{-}7R\alpha^{low}$ population, but did not develop colitis, it is possible that non-colitogenic $CD4^+$ memory T cells could re-express IL-7Rα highly in IL-2-independent manner. Further investigation of this issue is needed.

Nevertheless, our model is open to criticism concerning the role of IL-2 in the development of functional memory $CD4^+$ T cells when impaired $IL\text{-}2^{-/-}$ $CD4^+CD45RB^{high}$ T cells are used because their IL-7Rα expression is down-modulated before transfer. To this end, we next used donor WT and $IL\text{-}2^{-/-}$ $CD4^+$ $CD45RB^{high}$ T cells after preparing a mixed BM chimera that received a mixture of WT and $IL\text{-}2^{-/-}$ BM cells. As mentioned previously, the phenotypic characteristics of the $IL\text{-}2^{-/-}$ $CD4^+$ T cells in the BM chimera mice, including IL-7Rα expression, were similar to those of WT $CD4^+$ T cells, indicating that paracrine IL-2 secretion from WT $CD4^+$ T cells supports the normal development of naïve $IL\text{-}2^{-/-}$ $CD4^+CD45RB^{high}$ T cells in the BM and/or thymus and prevents preclinical autoimmunity *via* Treg that develop from WT precursor cells. However, we found that $RAG\text{-}2^{-/-}$ mice transferred with the $IL\text{-}2^{-/-}$ $CD4^+CD45RB^{high}$ T cells sufficiently expressing IL-7Rα- did not develop colitis along with the down-modulated expression of IL-7Rα on both LP and SP $CD4^+$ T cells after the transfer, which is in contrast to mice transferred with the WT $CD4^+CD45RB^{high}$ T cells, which

developed severe Th1/Th17-mediated colitis. These results reinforce our evidence that IL-2 is needed for the development of colitogenic memory $CD4^+$ T cells.

Finally, it should be noted that our study might not reflect the pathogenesis of human IBD directly because our colitis model is in the extreme "lymphopenic" environment for the induction of colitis, in which IL-7 and IL-2 might be important for rapid proliferation of T cells. Further studies will be needed to address this point using other colitis models in non-lymphopenic conditions.

Collectively, our findings indicate that, at least in the absence of Treg, IL-2 is critically involved in the development and perpetuation of colitis in three ways: (i) in the normal development of naïve $CD4^+$ T cells, (ii) in the re-expression of IL-7Rα on colitogenic memory $CD4^+$ T cells, and (iii) in the conversion from T_{CM} to T_{EM}/effector T cells. Although it will be necessary to consider the double-edged role of IL-2 in colitogenic $CD4^+$ T cells and Treg in other models of colitis, which may affect the balance between these two cell types, the current study appears to shed light on the positive involvement of IL-2 in chronic colitis. Thus, it will be necessary to assess when strategies for blocking and promoting IL-2 for IBD treatment should begin.

Materials and methods

Mice

C57BL/6-Ly5.1 and C57BL/6-Ly5.2-RAG-$2^{-/-}$ mice were obtained from Taconic Laboratory (Hudson, NY) and the Central Experimental Animal Institute (Kawasaki, Japan). IL-$2^{-/-}$ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). C57BL/6-Ly5.2- IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice were kindly provided by Dr. Rosa Zamoyska (National Institute for Medical Research, London, UK) [31]. GFP transgenic mice were originally generated by Dr. Masaru Okabe (Osaka University, Japan) [32]. All mice were maintained under specific pathogen-free conditions at the Animal Care Facility of Tokyo Medical and Dental University. All experiments were approved by the regional animal study committees.

Antibodies

Biotin-conjugated anti-IL-7Rα (A7R34) was obtained from Immuno-Biological Laboratories (Takasaki, Japan). The following mAb were obtained from BD PharMingen (San Diego, CA): 145–2C11, FITC- or PerCP- anti-CD3; RM4–5, FITC-, PE-, PerCP- or APC- anti-CD4; 16A, FITC- or APC- anti-CD45RB; IM7, PE-anti-CD44; PC61, PE-anti-CD25; MEL-14, FITC-anti-CD62L; H1.2F3, FITC-anti-CD69; A20, FITC- or PE-anti-Ly5.1 (CD45.1); and 104, FITC- or PE-anti-Ly5.2 (CD45.2). Biotinylated antibodies were detected with PE–streptavidin (BD Phar Mingen).

In vivo adoptive transfer experiments

Adoptive transfers of WT or IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells into RAG-$2^{-/-}$ mice were performed to avoid the effect of $CD4^+CD25^+$ Treg. Exp. 1: Ly5.1-derived WT (IL-$2^{+/+}$) or Ly5.2-derived IL-$2^{-/-}$ ($n = 8$) $CD4^+CD45RB^{high}$ T cells (3×10^5 cells) were injected intraperitoneally into RAG-$2^{-/-}$ mice. As a negative control, RAG-$2^{-/-}$ mice were transferred with $CD4^+CD45RB^{high}$ T cells (3×10^5 cells) and Treg (1×10^5 cells). Exp. 2: $CD4^+CD45RB^{high}$ T cells from SP of 4- to 5-wk-old IL-$2^{-/-}$ mice were divided into two populations, IL-$7R\alpha^{high}$ or IL-$7R\alpha^{low}$, by cell sorting and then each population was transferred into RAG-$2^{-/-}$ hosts. As a positive control, we transferred the same number of WT $CD4^+CD45RB^{high}$ T cells into RAG-$2^{-/-}$ mice. ($n = 5$). Exp. 3: First, SP $Ly5.1^+$ and $Ly5.2^+$ $CD4^+$ T cells were separately isolated from mice transferred with $Ly5.1^+$ WT and $Ly5.2^+$ IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells using FACSAria. Second, the isolated $Ly5.1^+$ or $Ly5.2^+$ T cells were separately transferred into new IL-$7^{+/+}$ × RAG-$2^{-/-}$ or IL-$7^{-/-}$ × RAG-$2^{-/-}$ mice. Exp. 4: First, preparations from the femurs and tibias of WT or IL-$2^{-/-}$ mice were incubated with biotin-conjugated anti-CD3 mAb and anti-biotin magnetic beads, followed by bead depletion using a MACS separation system (Miltenyi Biotec, Auburn, CA). Then 5×10^6 T-cell-depleted BM cells from each mouse were injected intravenously into lethally irradiated (7.5 Gy) GFP transgenic mice. Second, mice were sacrificed to isolate SP naïve GFP^- $Ly5.1^+$ WT and GFP^- $Ly5.2^+$ IL-$2^{-/-}$ $CD4^+$ $CD45RB^{high}$ T cells 8–12 wk post-transplantation. Third, the isolated $Ly5.1^+$ WT or $Ly5.2^+$ IL-$2^{-/-}$ $CD4^+CD45RB^{high}$ T cells were transferred into RAG-$2^{-/-}$ mice.

Disease monitoring and histological examination

The recipient mice were weighed immediately after T-cell transfer and three times *per* week thereafter. They were observed for clinical signs of illness as previously described [9]. Histological examination was performed as described before [9].

Flow cytometry

To detect the surface expression of a variety of molecules, isolated splenocytes or LP mononuclear cells were pre-incubated with an FcγR-blocking mAb (CD16/32; 2.4G2, BD PharMingen) for 20 min, followed by incubation with specific antibodies for 30 min on ice. Biotinylated antibodies were detected with PE–streptavidin. Intracellular Foxp3 staining was performed using a PE-anti-mouse Foxp3 staining set (eBioscience) according to the manufacturer's instructions. Background fluorescence was assessed by staining with control-irrelevant isotype-matched mAb.

Cytokine ELISA

To measure cytokine production, 1×10^5 LP $CD4^+$ T cells were cultured in 200 μL of culture medium at 37°C in a humidified

atmosphere containing 5% CO_2 in 96-well plates (Costar, Cambridge, MA) precoated with 5 μg/mL of hamster anti-mouse CD3ε mAb (145–2C11, BD PharMingen) and 2 μg/mL of hamster anti-mouse CD28 mAb (37. 51, BD PharMingen) in PBS overnight at 4°C. Culture supernatants were removed after 48 h and assayed for cytokine production. Cytokine concentrations were determined by specific ELISA according to the manufacturer's recommendations (R&D Systems, Minneapolis, MN).

Statistical analysis

We examined the normality of each group. If either group was not normally distributed, we assessed the difference between the two groups using the Mann–Whitney *U*-test. If both groups were normally distributed, we assessed the variance of the population to which each group belonged using the *f*-test. When homoscedasticity of both populations occurred, we assessed the difference between two groups using Student's *t*-test. In the absence of homoscedasticity, we assessed the difference using Welch's *t*-test. We used Statcell software for all statistical analyses. Results are expressed as the mean+SEM. Differences were considered statistically significant when $p<0.05$.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **IBD:** inflammatory bowel disease · **LP:** lamina propria · **SP:** spleen · **T_{CM}:** central memory T cell · **T_{EM}:** effector memory T cell

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Suppression of *Hath1* Gene Expression Directly Regulated by Hes1 Via Notch Signaling Is Associated with Goblet Cell Depletion in Ulcerative Colitis

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Background: The transcription factor *Atoh1/Hath1* plays crucial roles in the differentiation program of human intestinal epithelium cells (IECs). Although previous studies have indicated that the Notch signal suppresses the differentiation program of IEC, the mechanism by which it does so remains unknown. This study shows that the undifferentiated state is maintained by the suppression of the *Hath1* gene in human intestine.

Methods: To assess the effect of Notch signaling, doxycycline-induced expression of Notch intracellular domain (NICD) and Hes1 cells were generated in LS174T. *Hath1* gene expression was analyzed by quantitative reverse-transcription polymerase chain reaction (RT-PCR). Hath1 promoter region targeted by HES1 was determined by both reporter analysis and ChIP assay. Expression of Hath1 protein in ulcerative colitis (UC) was examined by immunohistochemistry.

Results: Hath1 mRNA expression was increased by Notch signal inhibition. However, Hath1 expression was suppressed by ectopic HES1 expression alone even under Notch signal inhibition. Suppression of the *Hath1* gene by Hes1, which binds to the 5′ promoter region of Hath1, resulted in suppression of the phenotypic gene expression for goblet cells. In UC, the cooperation of aberrant expression of HES1 and the disappearance of caudal type homeobox 2 (CDX2) caused Hath1 suppression, resulting in goblet cell depletion.

Conclusions: The present study suggests that Hes1 is essential for *Hath1* gene suppression via Notch signaling. Moreover, the suppression of Hath1 is associated with goblet cell depletion in UC. Understanding the regulation of goblet cell depletion may lead to the development of new therapy for UC.

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Key Words: ulcerative colitis, Hath1, Hes1, Notch signaling

The gut epithelium undergoes continual renewal throughout adult life, maintaining the proper architecture and function of the intestinal crypts. This process involves highly coordinated regulation of the induction of cellular differentiation and the cessation of proliferation, and vice versa.[1–3] Many studies of the regulation of intestinal differentiation have shown that cellular formation of the villi in small and large intestine is affected by various intracellular signaling pathways such as Notch, Wnt, and BMP.[4–7] Moreover, recent studies have also shown that dysregulation of the differentiation system for prompt intestinal epithelial cell formation induces the pathology of such intestinal diseases as colon cancer, Crohn's disease and ulcerative colitis (UC).[8] Then it was suggested that crucial genes for the differentiation of intestinal epithelium cells (IECs) become corrupt by aberrant cell signaling on the pathogenesis of intestinal diseases.

One of the most important genes for cell formation is a basic helix-loop-helix (bHLH) transcription factor, *Atoh1*, and its human homolog, *Hath1*, which is essential for the differentiation toward secretory lineages in small and large intestine.[9] Using a ubiquitin proteasomal system, we demonstrated that regulation of Hath1 protein in colon carcinogenesis is regulated by glycogen synthase kinase 3β (GSK3β) via Wnt signaling. Moreover, Hath1 and β-catenin protein are reciprocally regulated by GSK3β in Wnt signaling for the coordination between cell differentiation and

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proliferation. These findings together indicate that the deletion of adenomatous polyposis coli (APC) in colon carcinogenesis causes Hath1 protein degradation by switching the target of GSK3β from β-catenin to Hath1, resulting in maintenance of the undifferentiated state.[10] The dysregulation of prompt differentiation of IEC thus causes major intestinal diseases, and elucidation of the roles of various cell-signaling pathways in intestine is therefore important in understanding the pathogenesis of intestinal diseases.

We have also recently reported aberrant expression of Notch intracellular domain (NICD) in lesions showing goblet cell depletion in UC patients.[8] Moreover, forced expression of NICD caused the suppression of phenotypic genes for goblet cells in human intestinal epithelial cells. It has also been reported that forced expression of NICD in murine intestinal epithelial cells caused the depletion of goblet cells with the decrease of Atoh1 expression.[5] Thus, it is likely that *Atoh1* gene expression is regulated by Notch signaling, leading to subsequent control of intestinal epithelial cell lineage decision of the crypt cells.

The regulation of Hath1, however, is less well understood in human intestine. In previous reports, regulation of *Atoh1* gene expression was assessed using the mouse or chicken promoter region,[11,12] but the critical domains of the mouse and chicken sequences are not completely conserved in the Hath1 promoter region and enhancer region. To date, the regulation of *Hath1* gene expression has not been assessed using the human sequence. In particular, it remains unknown how *Hath1* gene expression is suppressed by Notch signaling in the intestine. It also remains unknown whether goblet cell depletion in UC is affected by Hath1 expression in intestinal epithelial cells.

In this study we demonstrated that Hes1 expression via Notch signaling is enough to suppress the *Hath1* gene by directly binding to the 5′ promoter region of Hath1. In UC, the cooperation of Hes1 and caudal type homeobox 2 (CDX2) caused the suppression of Hath1, resulting in the goblet cell depletion.

MATERIALS AND METHODS

Cell Culture

Human colon carcinoma-derived LS174T cells were maintained in minimum essential medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, 4 mM L-glutamine. Except where indicated otherwise, cells were seeded at a density of 5×10^5 cells/mL in each experiment. Cell cultures and transfections of plasmid DNA were performed as previously described.[6] A cell line expressing Notch1 intracellular domain (NICD), Hes1, HeyL (Tet-On NICD, Tet-On Hes1, Tet-On HeyL cells) under the control of doxycycline (DOX, 100 ng/mL, ClonTech, Palo Alto, CA) was generated as previously described.[8] The cell lines were supplemented with Blastcidin (7.5 μg/mL, Invitrogen, La Jolla, CA) and Zeocin (750 μg/mL, Invitrogen) for maintenance. The inhibition of Notch signaling was achieved by the addition of LY411,575 (1 μM).

Quantitative Real-time Polymerase Chain Reaction (PCR)

Total RNA was isolated with Trizol reagent (Invitrogen) according to the manufacturer's instructions. Aliquots of 1 μg of total RNA were used for cDNA synthesis in 20 μL of reaction volume. One microliter of cDNA was amplified with Cyber Green in a 20-μL reaction as previously described.[6] The primer sequences in this study are summarized in Supporting Information Table S1.

Plasmids

5′ Hath1 reporter plasmid was generated by cloning a 1031-bp sequence 5′ of the human *Hath1* gene (corresponding to −1,029 to +2 of the promoter region) into a pGL4 basic vector (Promega, Madison, WI). Hath1 reporter plasmid containing the 3′ region was generated by cloning a 4811-bp sequence 3′ of the human *Hath1* gene (corresponding to +1401 to +6211 of the Hath1 genome) into the 5′ Hath1 reporter plasmid. Internal deletion mutants of the 5′ Hath1 reporter plasmid in which three Hes1 binding sites CACGCG (−305 to −300, −269 to −264, −159 to −154) were replaced with GTCGAC were constructed by PCR-mediated mutagenesis.[13] Doxycycline-dependent expression of NICD was achieved by cloning the gene encoding the intracellular portion of the mouse Notch1 into the pcDNA4/TO/myc-his vector (Invitrogen).[8] Doxycycline-dependent expression of Hes1 was achieved by cloning the gene encoding rat Hes1 into the pcDNA4/TO/myc-his vector (Invitrogen). Doxycycline-dependent expression of HeyL was achieved by cloning the gene encoding human HeyL into the pcDNA4/TO/myc-his vector (Invitrogen). All constructs were confirmed by DNA sequencing.

Luciferase Assays

LS174T cell seeded in a 6-well plate culture dish were transfected with 4 μg of reporter plasmid along with 10 ng of pRL-tk plasmid (Promega). Cells were harvested 36 hours after transfection, lysed by three cycles of freezing and thawing, and the luciferase activities in each sample as indicated by arbitrary unit were normalized against Renilla luciferase activities as previously described.[10]

Chromatin Immunoprecipitation Assay

A chromatin immunoprecipitation (ChIP) assay was performed essentially as previously described with some modifications.[6] LS174T/Hes1 cells were seeded onto a 150-mm dish, then stimulated with DOX or left untreated for 12 hours. Immunoprecipitation was performed overnight at 4°C with 10 μg of an anti-Hes1 (a kind gift from Dr. T. Sudo), normal mouse immunoglobulin G (sc-2025, Santa Cruz Biotechnology, Santa Cruz, CA), or an anti-histone H3 antibody (Abcam,

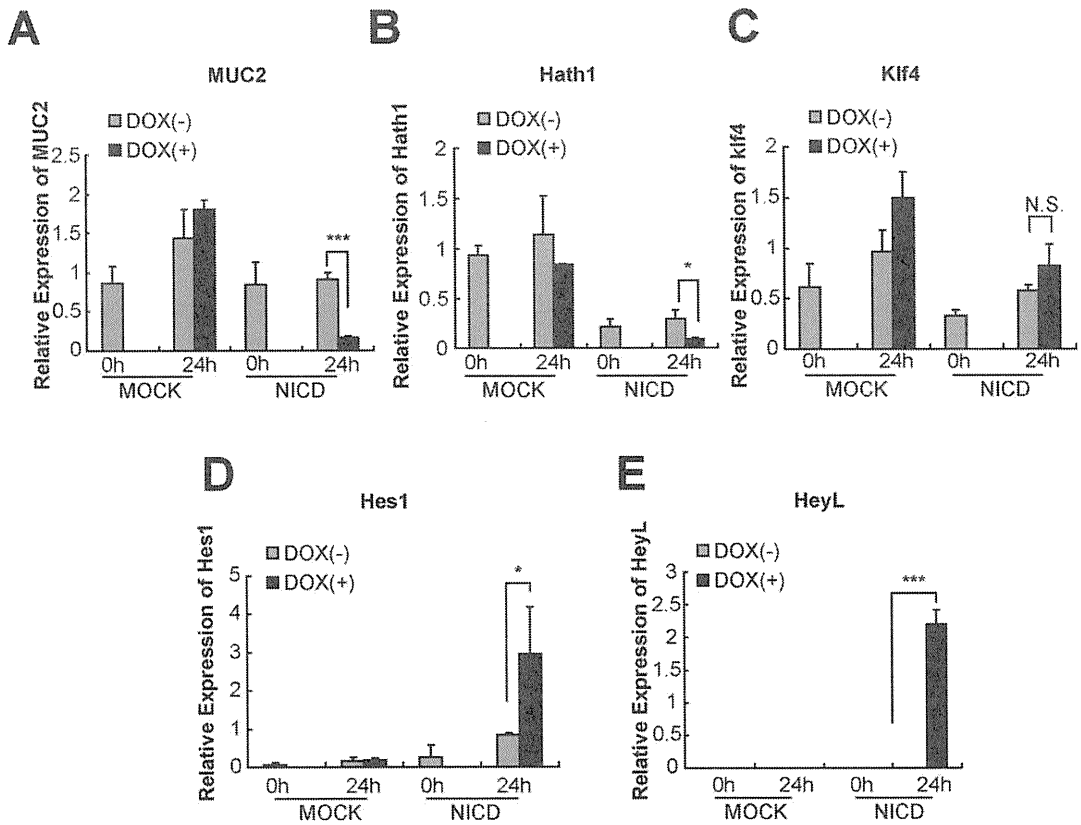

FIGURE 1. Gene alternation in LS174T cells by the expression of NICD. NICD is induced by DOX using the Tet-on system to mimic the acceleration of the Notch signal in LS174T cells. NICD expression by DOX decreased the expression of MUC2 (A) and Hath1 (B) genes. Klf4 gene expression was not affected (C). NICD also induced expression of Hes family genes such as Hes1 (D) and HeyL (E). ($^{*}P < 0.05$, $^{***}P < 0.001$, $n = 3$).

Cambridge, MA). The genomic DNA fragments in the immunoprecipitated samples were analyzed by PCR using primers indicating the positions on the genomic DNA relative to the translation start site (Supporting Information Table 1). The same amounts of DNA samples were analyzed by conventional PCR in parallel with the following parameters: denaturation at 94°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 68°C for 60 seconds for 45 cycles. The products were resolved by agarose gel electrophoresis, stained with ethidium bromide, and visualized using an ImageQuant TL system (GE Healthcare, Milwaukee, WI).[6] The primer sequences in this study are summarized in Supporting Information Table S1.

Human Intestinal Tissue Specimens

Human tissue specimens were obtained from patients who underwent endoscopic examination or surgery at Yokohama Municipal General Hospital or Tokyo Medical and Dental University Hospital. Normal colonic mucosa was obtained from patients with colorectal cancer who underwent colectomy. Each of three patients with UC and colon cancer were examined. Written informed consent was obtained from each patient and the study was approved by the Ethics Committee of both Yokohama Municipal General Hospital and Tokyo Medical and Dental University.

Immunohistochemistry

Hath1 antibody (1:5000) was originally generated as previously described. Hes1 antibody (1:10,000) was the same as in the ChIP assay. Fresh frozen tissue was used after microwave treatment (500W, 10 minutes) in 10 mM citrate buffer for Hath1 and Hes1. The standard ABC method (Vectastain; Vector Laboratories, Burlingame, CA) was used, and staining was developed by addition of diaminobenzidine (Vector Laboratories).

Statistical Analyses

Quantitative real-time PCR analyses were statistically analyzed with Student's *t*-test. *P* less than 0.05 was considered statistically significant.

RESULTS

Notch Signaling Suppresses *Hath1* Gene Expression But Not Kuppel-like Factor 4 (Klf4) Gene in Human IECs

Expression of Atoh1 seems to be regulated at its transcriptional level, as forced expression of NICD in murine IECs causes the decrease of Atoh1 mRNA expression and subsequent depletion of goblet cells in vivo.[5] We therefore assessed the effect of the Notch signal on the expression of

Hath1 in a human intestinal epithelial cell line, LS174T cells. NICD is induced by DOX using a Tet-on system to mimic the acceleration of the Notch signal. NICD expression showed not only the decrease of Mucin2 (MUC2) expression but also a significant decrease of *Hath1* gene expression (Fig. 1A,B). We also assessed Klf4 gene expression by NICD expression because Klf4 is also essential to goblet cell differentiation.[14] Klf4 gene expression, however, was not affected by forced NICD expression (Fig. 1C), since it is suggested that the suppression of goblet cell phenotypic gene expression by Notch signaling is independent of Klf4 expression.

To assess how Notch signaling suppresses the gene expression of Hath1, we selected the Hes1 and HeyL genes as possible suppressors, based on previous identification of the Hes family genes induced by NICD in LS174T cells using a microarray system.[8] We confirmed that the gene expression of Hes1 and HeyL was markedly induced by NICD expression (Fig. 1D,E).

Hes1 But Not HeyL Suppresses *Hath1* Gene Expression in Human IECs, Resulting in the Decrease of MUC2 Gene Expression

To assess which genes suppress the *Hath1* gene expression, we generated cells (LS174T Tet-on Hes1 cells and LS174T Tet-on HeyL cells) in which either Hes1 or HeyL is induced by DOX using the Tet-on system, respectively. Forced expression of Hes1 alone showed a significant decrease of MUC2 gene expression following the decrease of *Hath1* gene expression (Fig. 2A,B). In contrast, HeyL induction alone did not change the expression of either MUC2 (Fig. 2A) or Hath1 genes (Fig. 2B). Moreover, neither Hes1 nor HeyL induction affected Klf4 gene expression (Fig. 2C). These results are compatible with previous reports that the depletion of Hes1 in a mouse model upregulated Atoh1 mRNA expression in intestinal epithelial cells, resulting in the hyperplasia of the goblet cells.[15] Conversely, the finding that Klf4 was not affected by the Notch signaling differs from previous reports.[16]

Hes1 Expression Alone Is a Sufficient Condition for the Repression of the Phenotypic Gene Expression of Goblet Cells by Notch Signaling

To further analyze the functional role of Notch signaling in the differentiation of IECs, we next asked whether Hes1 expression alone is enough to compensate for the suppression of *Hath1* gene expression in Notch signaling. To inhibit the Notch signaling, LS174T Tet-on Hes1 cells were treated with gamma-secretase inhibitor (GSI), which prevents the separation of NICD from the Notch receptor. Notch signal inhibition by GSI treatment alone showed a significant decrease of Hes1 gene expression (Fig. 3A), in contrast to marked induction of MUC2

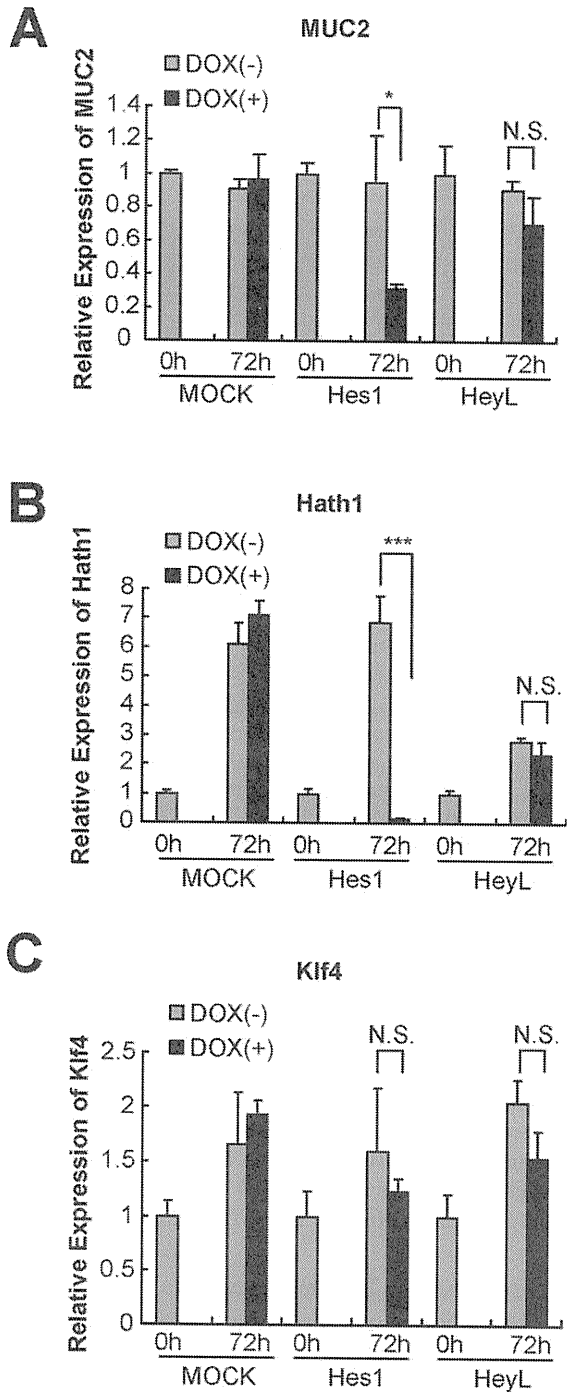

FIGURE 2. Gene alternation in LS174T cells by the expression of either Hes1 or HeyL. (A) Hes1 or HeyL was induced by DOX in LS174T Tet-on Hes1 cells or LS174T Tet-on HeyL cells, respectively. Hes1 induction significantly decreased MUC2 gene expression. (B) Hes1 induction resulted in a significant decrease of Hath1. (C) Neither Hes1 nor HeyL induction affected Klf4 gene expression. (*$P < 0.05$, ***$P < 0.001$, $n = 3$).

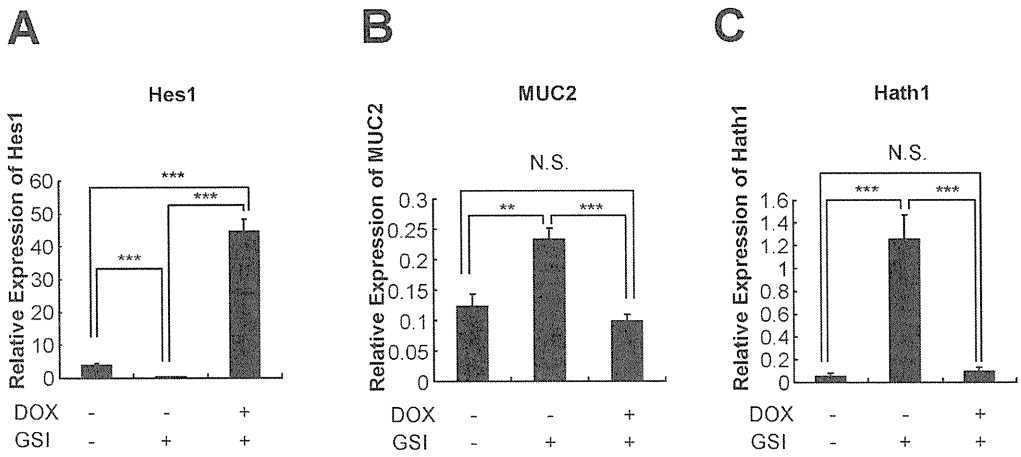

FIGURE 3. Hes1 expression is enough to suppress intestinal cell differentiation by Notch signaling. (A) LS174T Tet-on Hes1 cells were treated with GSI, which prevents the separation of NICD from the Notch receptor. GSI treatment alone significantly decreased Hes1 gene expression. Hes1 was induced by DOX in addition to GSI. (B) GSI markedly induced MUC2 gene expression. Hes1 induction by DOX in GSI-treated cells restored MUC2 gene expression to the level in untreated cells. (C) GSI markedly induced *Hath1* gene expression. Hes1 induction by DOX in GSI-treated cells restored *Hath1* gene expression to the level in untreated cells. (**$P < 0.01$, ***$P < 0.001$, $n = 3$).

gene expression (Fig. 3B) following the induction of the *Hath1* gene (Fig. 3C). Interestingly, the Hes1 gene was expressed by DOX when Notch signaling was inhibited by GSI (Fig. 3A), while Hath1 expression was restored to the level in untreated cells (Fig. 3C). Moreover, MUC2 gene expression was also decreased by Hes1 expression alone (Fig. 3B).

These results indicate that Hes1 might be a mainstream of Notch signaling to suppress the phenotypic gene expression of goblet cells in human intestine.

Previous results raised the question of whether Hath1 is essential for expression of the MUC2 gene by Notch signaling inhibition. To assess the importance of the *Hath1* gene for MUC2 expression, the effect of silencing the *Hath1* gene using siRNA system was examined in LS174T cells in the Notch signaling-inhibited state. *Hath1* gene silencing resulted in cancellation of the *Hath1* gene expression induced by GSI treatment and restoration of MUC2 expression to the level in untreated cells (Supporting Information Fig. 1).

These results together suggest that Notch signaling affects the gene expression of Hath1 but not Klf4 to decide the fate of IECs.

HES1 Suppresses the Transcriptional Activity of Hath1 Via the 5′ Promoter Region

It has been reported that expression of Math1, the mouse homolog of Atoh1, was suppressed by ZIC1 or HIC1 via its 3′ region.[12,17] However, it has never been shown how Hes1 suppresses the transcriptional activity of Hath1 via Notch signaling. To assess the regulation of Hath1 transcriptional activity, we constructed a reporter plasmid containing the 1000-bp upstream 5′ region of Hath1. Hath1 reporter plasmid was transfected into LS174T Tet-on Hes1 cells or LS174T cells transfected with a mock plasmid. Hes1 induction by DOX showed a significant decrease of the transcriptional activity on Hath1, whereas the mock plasmid did not change its transcriptional activity (Fig. 4A). We then found three regions that matched the consensus sequence for binding Hes1, the Class C site,[18] in the 1000-bp upstream region of Hath1. We therefore constructed a reporter plasmid in which all regions of the Hes1 binding site in the 1000-bp upstream region of Hath1 were deleted. As expected, reporter activity of the deletion mutant construct was not suppressed by Hes1 expression. We next constructed mutants in which one of the binding sites of Hes1 in the 1000-bp upstream region of Hath1 was deleted. Interestingly, only the mutant construct lacking the second region of the Hes1 binding site was not affected by Hes1, indicating that Hes1 might directly suppress the Hath1 transcriptional activity to bind to the second region of the Hes1 binding site (Fig. 4A).

In chicken and mouse models, Atoh1 expression is regulated only by the 3′ region of Atoh1 that contains both the enhancer region and the repressor region.[12,19] We also found a homologous sequence of the enhancer region in the 3′ region of Hath1, and a Hes1 binding site in this enhancer region of Hath1. We therefore constructed a Hath1 reporter plasmid containing the 3′ region of Hath1 behind the luciferase sequence. As before, Hes1 suppressed Hath1 transcriptional activity. Moreover, deletion mutants of the Hes1 binding site in the 5′ region of Hath1 were also unaffected by Hes1 expression, indicating that the Hes1 binding site of the 3′ region might not affect Hath1 suppression by Hes1 (Fig. 4B).

HES1 Binds Directly to the 5′ Promoter Region of Hath1

To confirm the binding of Hes1 to Hath1 promoter region, we performed a ChIP assay. The region immunoprecipitated by Hes1 antibody was amplified only in the 5′ region

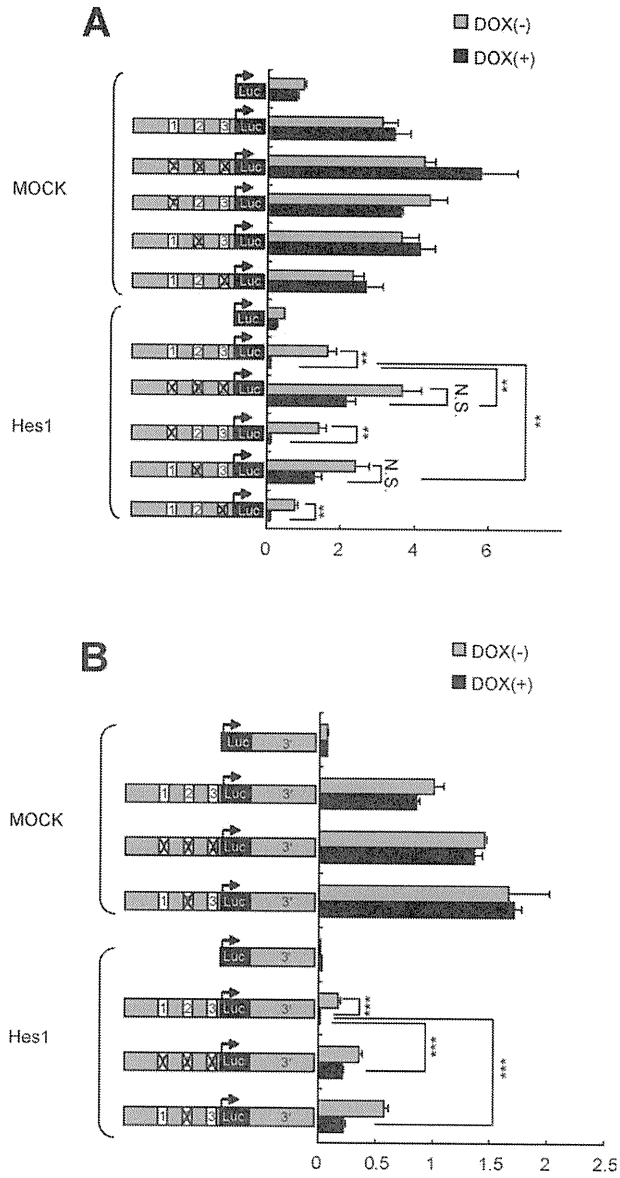

FIGURE 4. Hes1 regulates the transcriptional activity of Hath1 via 5′ promoter region. (A) 5′ Hath1 reporter plasmid containing the 1000-bp upstream region of Hath1 was transfected into LS174T Tet-on Hes1 cells and LS174T cells transfected with a mock plasmid. The induction of Hes1 by DOX significantly decreased the transcriptional activity on Hath1, whereas the transcriptional activity of the mock plasmid did not change. Three regions that matched the consensus sequence for binding Hes1, the Class C site, in the 1000-bp upstream region of Hath1 are indicated as square numbers. Reporter activity of a mutant with all regions of the Hes1 binding site deleted was not suppressed by Hes1 expression. A mutant construct in which only the second region of the Hes1 binding site was deleted was also unaffected by Hes1. (B) Hath1 reporter plasmid containing the 3′ enhancer region of Hath1 behind the luciferase sequence was inserted into 5′ Hath1 reporter plasmid. Hes1 also suppressed Hath1 transcriptional activity enhanced by 3′ enhancer region. The deletion mutants of the Hes1 binding site in the 5′ region of Hath1 were also unaffected by Hes1 expression (B). (**$P < 0.01$, ***$P < 0.001$, $n = 3$).

including the Hes1 binding sites but not 3′ region of the Hes1 binding sites (Fig. 5B), supporting the idea that Hes1 binds directly to the 5′region of Hath1 to suppress the transcriptional activity in IEC.

Hes1 Does Not Completely Block the Transcriptional Activity of Hath1 Promoted by CDX2

To clarify the balance between the enhancer and the repressor in Hath1 transcriptional activity, we next assessed whether CDX2, which promotes *Atoh1* gene transcription in mice, is affected by Notch signaling on Hath1 transcription. Treatment with GSI showed slight induction of CDX2 in LS174T cells (Fig. 6A). Moreover, HES1 expression did not affect the expression of CDX2 (Fig. 6B), suggesting that the expression of CDX2 may be independent of Notch signaling. To assess the effect of CDX2 on Hath1 transcription regulated by HES1, a reporter assay of Hath1 was performed. Although CDX2 did not promote Hath1 transcription via the 5′ promoter region of Hath1 (Fig. 6C), CDX2 cotransfected with the reporter plasmid containing the 3′ enhancer region of Hath1 showed significant increase of transcriptional activity of Hath1 (Fig. 6D). Interestingly, the transcriptional activity of Hath1 promoted by CDX2 was not suppressed by Hes1 induction in LS174T tet-HES1 cells. These results suggest that Hes1 at the 5′ region of Hath1 could not completely abrogate the transcriptional activity of Hath1 promoted via the 3′ enhancer region by CDX2, and *Hath1* gene expression might be regulated by the balance between HES1 and CDX2.

Hath1 Protein Expression Is Decreased in the Goblet Cell Depletion of UC

We finally assessed whether Hath1 is decreased in colon mucosa with goblet cell depletion in line with the former results in vitro. In normal colonic mucosa, Hath1 and CDX2 were expressed in almost all IECs. In contrast, Hes1 was expressed in IECs situated in the lower half of the villi (Fig. 7). In UC patients, both Hath1 and CDX2 disappeared, while Hes1-positive cells were extended at the top of the villi (Fig. 7), indicating that the suppression of Hath1 in goblet cell depletion might be caused by both the disappearance of CDX2 and the extension of Hes1-positive cells.

DISCUSSION

This study reveals for the first time that Hes1 directly suppresses *Hath1* gene expression via the Notch signal, indicating that downregulation of Hath1 is associated with goblet cell depletion in human UC in combination with the disappearance of CDX2. Previous reports have suggested that Notch signaling suppressed the phenotypic gene expression of goblet cells by suppressing *Atoh1* gene expression,[5] although it remains unknown how Notch signaling suppresses

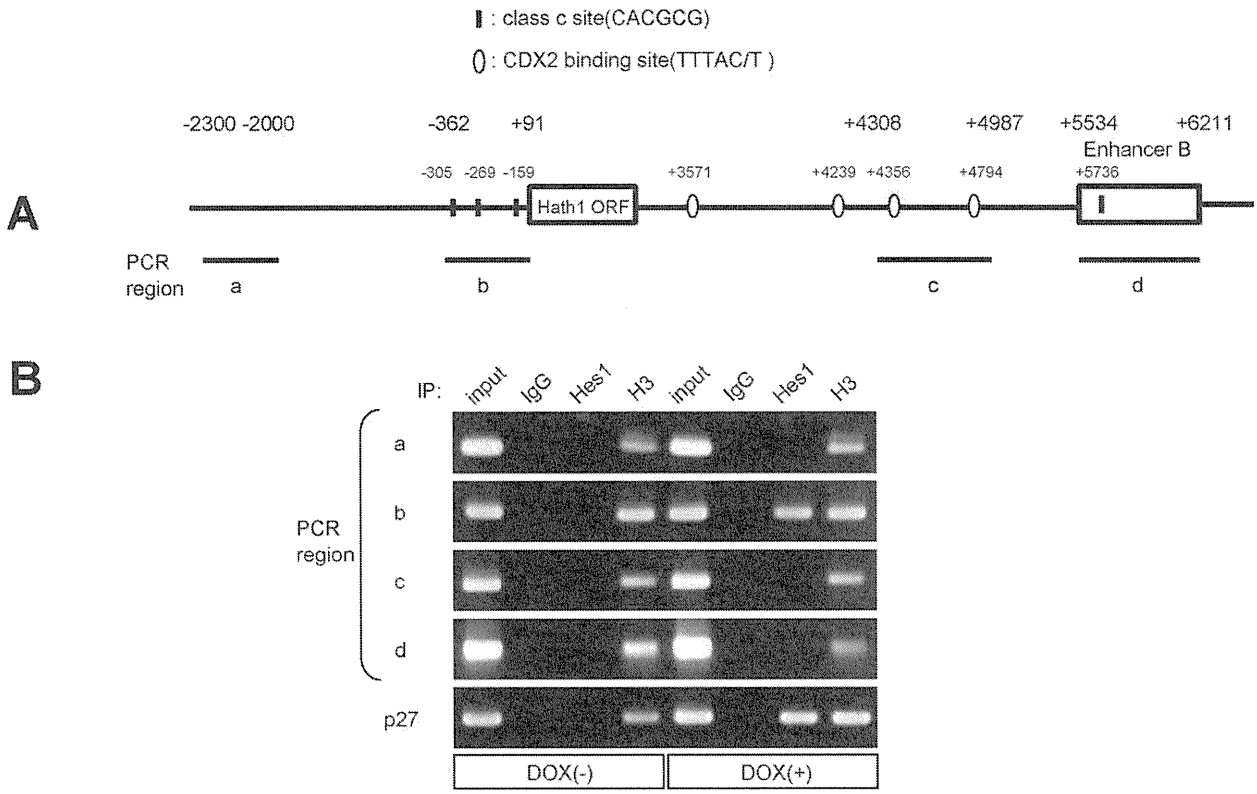

FIGURE 5. Hes1 binds to 5′ Hath1 promoter region. (A) Schematic presentation of Hath1 genome. (B) ChIP assay was performed using LS174T Tet-on Hes1 cells with or without DOX treatment for 24 hours. Each region indicated by a letter in (A) was amplified from the immunoprecipitant by each antibody. The amplification of p27 from the immunoprecipitant by Hes1 antibody was confirmed to be the known region of the Hes1 binding site. Only the 5′ region including the Hes1 binding sites of Hath1 (region b) was amplified from the immunoprecipitant by Hes1 antibody under the induction of Hes1 expression by DOX.

Hath1 gene expression. We first found that Hes1, but not HeyL, was necessary and sufficient for the suppression of *Hath1* gene expression by Notch signaling in IEC. Canonical Notch signaling leads to transcriptional activation of Hes family and Hey family genes such as Hes1, Hes5, Hes7, Hey1, Hey2, and HeyL by binding NICD to RBP-Jk.[20] Hes and Hey family genes play important roles in the differentiation of various tissues,[21,22] but it has not been clarified how the function of each gene is assigned via Notch signaling. While we found that all Hes and Hey family genes were upregulated by NICD expression in intestinal cells, we also noticed that Hes1 and HeyL were exorbitantly expressed by NICD than other Hes and Hey family genes (data not shown), suggesting that the functional assignment of Notch signaling is regulated by the quantity of each Hes and Hey family gene expressed. HeyL has been identified as one of the target genes of Notch3 receptor, because HeyL is expressed in smooth muscle cells of the digestive tract and the vasculature following Notch3 expression in later stages of development.[23] In this study, we could not identify the function of HeyL in goblet cell differentiation; rather, its function is expected to be assessed in future study of the effect of Notch signaling on IEC.

On the other hand, we found that Hes1 is critical for the differentiation into goblet cells via Notch signaling, since the binding of HES1 to the Hath1 5′ promoter region silences *Hath1* gene expression. Although the 3′ region of Atoh1 has been characterized as the enhancer and repressor region to regulate *Hath1* gene expression by CDX2, Zic1, and Hic1, the function of the 5′ region of Atoh1 has not been clarified. This study revealed that the 5′ region of Hath1 is necessary not only for basic transcription but also for the regulation by HES1 via Notch signaling to presumably suppress the transcriptional activity of the basic transcription factors. It has been reported that Hes1 binds not only to the N-box sequence but also to class C sites to suppress the expression of genes such as $P27^{kip1}$[18] and achaete-scute homolog-1,[24] through which it plays a central role in cell proliferation and differentiation, respectively. In this study we identified a class C site at position −289 of the 5′ region of Hath1, playing a crucial role in the regulation of *Hath1* gene expression by the Notch signal. We therefore suspected that Hes1 might completely shut out the transcriptional activity via the

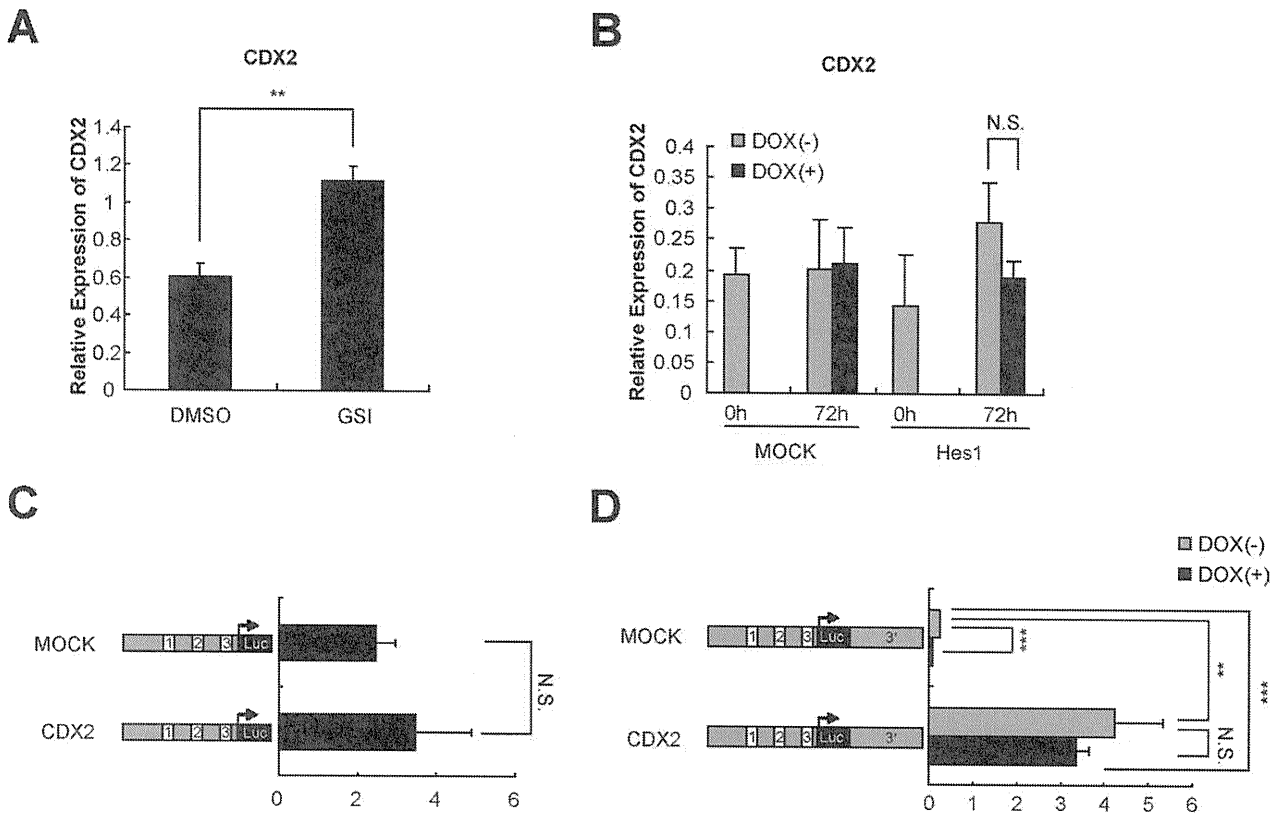

FIGURE 6. CDX2 enhances the transcriptional activity of Hath1 independently of Notch signaling. (A) *CDX2* gene expression was analyzed by treatment of LS174T cells with GSI for 72 hours. CDX2 was slightly upregulated by Notch signal inhibition. (B) *CDX2* gene expression was analyzed by the Hes1 expression induced by DOX in LS174T Tet-on Hes1 cells. *CDX2* gene expression was not affected by Hes1 expression. (C) Transcriptional activity of Hath1 via the 5′ region by CDX2 was assessed in LS174T cells for 72 hours after transfection of both the *CDX2* gene and 5′ Hath1 reporter plasmid. CDX2 did not affect the transcriptional activity via the 5′ region of Hath1. (D) HES1 did not suppress the transcriptional activity via the 3′ region of Hath1 by forced expression of CDX2. The transcriptional activity of Hath1 was assessed for 72 hours after transfection of both the *CDX2* gene and 3′ Hath1 reporter plasmid with or without DOX in LS174T Tet-on HES1 cells. (**$P < 0.01$, ***$P < 0.001$, $n = 3$).

3′ enhancer region, but that forced expression of CDX2 could induce the transcriptional activity of Hath1 even with Hes1 expression. Moreover, the expression of CDX2 was not affected by Notch signaling, suggesting that CDX2 and HES1 independently regulate *Hath1* gene expression. Thus, regulation by Hes1 via Notch signaling is not sufficient to suppress the gene transcription of *Hath1*, indicating that the transcriptional activity of Hath1 is regulated by the balance between CDX2 and HES1 expression.

Importantly, the present study also indicated that Hath1 is essential to regulate goblet cell formation in UC. Although the expression of Hath1 in inflamed mucosa of UC has been reported,[25] the correlation between goblet cell content and Hath1 expression in UC has not been elucidated. We confirmed that Hath1 was expressed in inflamed mucosa with conserved goblet cell formation in UC (data not shown), since goblet cell content might correlate with Hath1 expression in UC. In Atoh1-deficient mice, secretory lineages of IEC including goblet cells are completely lost,[9,26] indicating that Hath1 might have the function of not only mucus production but also differentiation toward goblet cells in human intestine.

Moreover, this study suggested that goblet cell depletion in UC caused by the disappearance of Hath1 required not only HES1 expression but also CDX2 suppression of IEC. CDX2 has been reported to be downregulated in UC mucosa,[27] but it remains unknown how CDX2 expression is suppressed by colonic inflammation even though CDX2 is upregulated by inflammation in the esophagus and stomach.[28,29] One previous report indicated that CDX2 expression is suppressed by hypoxia inducible factor 1 (HIF1).[30] Another report found that HIF1 is overexpressed in UC mucosa,[31] suggesting that HIF1 might suppress CDX2 expression in UC. Whatever the case, the regulation of CDX2 expression of IEC should be assessed to clarify the mechanism of goblet cell depletion in UC.

In conclusion, we have revealed for the first time that Hes1 is sufficient to suppress *Hath1* gene transcription via the Notch signal, but insufficient to suppress *Hath1* gene transcription by CDX2. The cooperation between Hes1 and

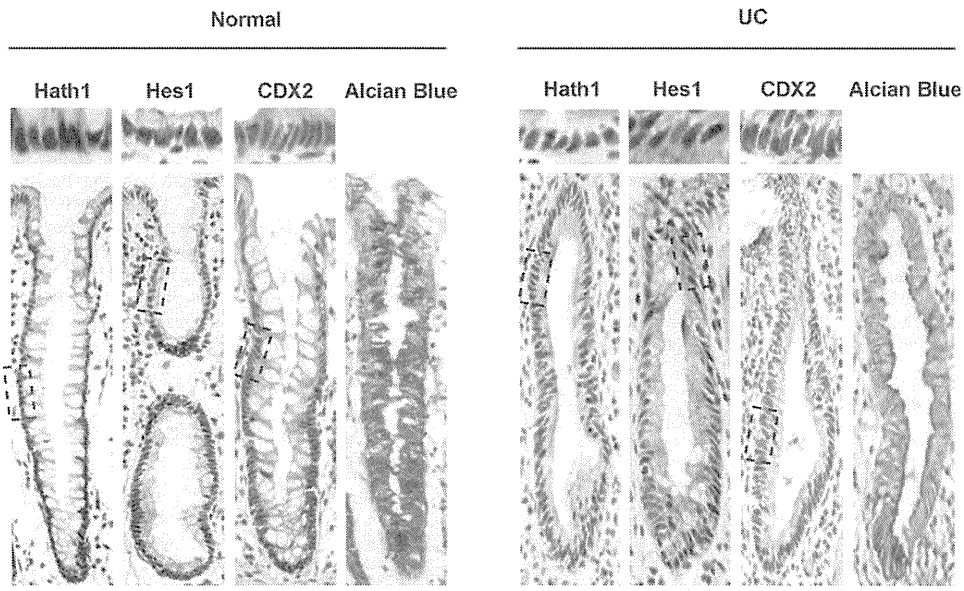

FIGURE 7. Immunohistochemistry of intestinal mucosa in UC. In normal colonic mucosa, Hath1 and CDX2 were expressed in most IEC. Hes1 was expressed in intestinal epithelial cells in the lower half of villi. In UC mucosa with goblet cell depletion, neither Hath1 nor CDX2 was expressed, whereas Hes1 was expressed up to the top of the villi. Upper column shows magnified view of the upper villus areas identified by dashed line in the lower column. Blue staining with Alcian blue represents goblet cells. The examination was performed by using the sections from three different individuals.

CDX2 is important to regulate *Hath1* gene expression, which is involved in goblet cell formation in UC. More detailed analysis of Hath1 expression at various stages of UC or other enteritis diseases associated with goblet cell depletion will lead us understand the regulation of Hath1 reduction under the inflammation state with various cytokines and inflammatory cells infiltration. Finally, elucidation of the mechanism of goblet cell depletion in UC will help us to develop novel therapies for strengthening the barrier function of colonic mucosa.

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Double-Blind, Placebo-Controlled Trial of Oral Tacrolimus (FK506) in the Management of Hospitalized Patients with Steroid-Refractory Ulcerative Colitis

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Background: We report a multicenter study of oral tacrolimus (FK506) therapy in steroid-refractory ulcerative colitis (UC).

Methods: In a placebo-controlled, double-blind study, 62 patients with steroid-refractory, moderate-to-severe UC were randomized into either a tacrolimus group or a placebo for 2 weeks. Patients were evaluated using the Disease Activity Index (DAI). As an entry criterion, patients had to have a total DAI score of 6 or more as well as a mucosal appearance subscore of 2 or 3. Clinical response was defined as improvement in all DAI subscores. Mucosal healing was defined as mucosal appearance subscore of 0 or 1. Clinical remission was defined as a total DAI score ≤ 2 with an individual subscore of 0 or 1.

Results: The mean total DAI score at study entry was 9.8 ± 1.61 in the tacrolimus group and 9.1 ± 1.05 in the placebo group. At week 2 the clinical response rate was 50.0% (16/32) in the tacrolimus group and 13.3% (4/30) in the placebo group ($P = 0.003$). The rate of mucosal healing observed was 43.8% (14/32) in the tacrolimus group and 13.3% (4/30) in the placebo group ($P = 0.012$) and the rate of clinical remission observed was 9.4% (3/32) in the tacrolimus group and 0.0% (0/30) in the placebo group ($P = 0.238$). The therapies in this study were well tolerated, with only minor side effects.

Conclusions: Oral tacrolimus therapy in patients with steroid-refractory UC shortened the acute phase and induced rapid mucosal healing. These results suggest that tacrolimus therapy is useful as an alternative therapy for steroid-refractory UC.

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Key Words: ulcerative colitis, immunosuppressive therapy, tacrolimus

Tacrolimus, a macrolide immunosuppressant produced by *Streptomyces tsukubaensis*, a species of *Actinomyces*, was discovered in 1984 on Mt. Tsukuba in Japan. Fellermann et al[1] reported the results of a study of tacrolimus in patients with steroid-refractory, severe ulcerative colitis (UC). With patients initially treated by continuous intravenous infusion and subsequently transferred to oral administration, the study showed improved symptoms in five of six patients, with successful induction of remission and steroid tapering achieved in four patients. A report on oral and injectable formulations of tacrolimus stated, "most importantly, oral tacrolimus therapy appears to be effective and obviates the need for intravenous dosing."[2]

Baumgart et al[3] demonstrated the usefulness of low doses of oral tacrolimus (4–6 ng/mL) and Högenauer et al[4] reported, "Oral tacrolimus might be an effective alternative treatment to intravenous cyclosporine for treatment of steroid-refractory UC."

As no evaluation had yet been made of tacrolimus using a placebo as comparator, we conducted a dose-ranging study to evaluate oral administration over 2 weeks.[5] The study established a placebo group, a group with a target tacrolimus trough concentration of 10–15 ng/mL, and a group with a target tacrolimus trough concentration of 5–10 ng/mL. The results indicated a significant difference in efficacy between the 10–15 ng/mL group and the placebo group over the short 2-week period.

Here we report on a multicenter study which was a double-blind study of oral administration for 2 weeks,

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comparing a placebo group with a group having a target tacrolimus trough concentration of 10–15 ng/mL.

MATERIALS AND METHODS

Patient Selection

Patients with moderate-to-severe, active UC were eligible for inclusion in this study. UC was defined according to standard criteria for symptoms and standard radiographic and endoscopic criteria.[6] Before starting treatment, infectious diarrhea was ruled out by stool cultures and *Clostridium difficile* toxin testing. Endoscopies were performed during the week prior to the first dose of the study drug. The extent of colonic involvement was determined by total colonoscopy. All patients in the study had left-sided colitis and pancolitis and all were hospitalized.

Patients with known renal or severe hepatic dysfunction and pregnant women were excluded from the study. Pretreatment assessment included taking a history of the patient, physical examination, complete blood count, chemistry screening panel, and urinalysis.

Patients were classified as steroid-resistant or steroid-dependent. Patients with active UC were defined as steroid-resistant when the disease failed to respond to a systemic daily dose of 1 mg per kg of body weight, or 40 mg or more of prednisolone given over at least 7 days, or the equivalent of a daily dose of prednisolone of 30 mg or more over at least 2 weeks. Steroid-dependent patients were defined as patients with active UC in whom attempts to taper steroids had been unsuccessful. The steroid dosage remained the same from study initiation for 2 weeks, while only those patients in whom a dose of prednisolone of 60 mg/day or more was effective were permitted to decrease the dosage during this period. Efficacy was based on improvement in the frequency of stools and a decreased amount of blood in the stool.

Patients were evaluated using the Disease Activity Index (DAI).[7] The DAI score is a sum of subscores for the following four factors: stool frequency, rectal bleeding, mucosal appearance, and physician's overall assessment, each of which is graded on a scale from 0 to 3. The DAI score ranges from 0 to 12; the higher the score, the more severe the disease activity. As an entry criterion, the patient was required to have a total DAI score of 6 or more, as well as a mucosal appearance subscore of 2 or 3.

Patients who started taking azathioprine within 3 months prior to entering the study were excluded from the study, and patients were permitted to continue taking azathioprine at an unchanged dose over the period beginning 3 months prior to the start of the study, until completion of the study. Patients were permitted to continue taking 5-aminosalicylic acid during the study, as long as the drug dosage was not changed over the period beginning 2 weeks prior to the start of the study, until completion of the study. Receiving cytapheresis within 14 days prior to entry in the study was a reason for exclusion from the study. Patients receiving concomitant nutritional therapy continued to receive the same therapy during the study.

As UC therapy with cyclosporin, biological therapies, 6-mercaptopurine, or other immunosuppressants was not covered by health insurance in Japan, the concomitant use of these drugs was prohibited.

Protocol Review

The study protocol was reviewed and approved by each Institutional Review Board. Each patient read and signed a consent form before enrollment in the study.

Study Design

We conducted a multicenter study of oral tacrolimus treatment, consisting of a 2-week placebo-controlled, double-blind, randomized study in which patients with active UC were given either placebo or tacrolimus at an oral dose sufficient to achieve and maintain target blood concentrations of 10–15 ng/mL.

Open-label Extension

After week 2, patients received conventional treatment or tacrolimus open-label treatment. Data were collected during an open-label extension phase of the study. The effect of continuous treatment in the tacrolimus group was evaluated by comparing the condition of patients in the tacrolimus group at weeks 2 and 12.

Administration and Monitoring of Study Drug

The tacrolimus capsules used (Tacrolimus, Astellas Pharma, Japan) contained 0.5 mg or 1 mg of FK506. In consideration of safety, tacrolimus therapy was initiated at a small dose of 1–2.5 mg per time, twice daily. Dose adjustments were determined using proportional calculations of "blood trough concentration at steady state" and "target trough concentration" as shown in Table 1. To reach the target trough concentration quickly, the first dose adjustment occurred at an early stage. This increase required blood collection at 12 hours (C12h) and 24 hours (C24h) after the initial dose for determination of the trough concentration of tacrolimus in whole blood. Steady-state values were estimated to be 4 times the value at C12h, 2.5 times the value at C24h, or 3 times the mean value of C12h and C24h. The dose was adjusted by proportional calculation using a target concentration of 12.5 ng/mL. These equations were created based on the known pharmacokinetic profile of tacrolimus in healthy volunteers (data not shown).

For the next adjustment, measured values were checked against the target trough concentration. When the measured value was outside the range of 10–15 ng/mL, the dose was readjusted using blood trough concentration at steady state.

The randomization was performed by the Control Center (Bellsystem24, a third-party organization independent of study physicians and sponsor). To preserve blinding, blood trough

TABLE 1. Dose Adjustment of Tacrolimus

Dosage calculation method using trough concentration
Blood trough concentration under the same food intake condition as at administration should be used (fed/fasted condition).
For 2 weeks:
The dose is increased to a target trough concentration of 10-15 ng/mL (target of 12.5 ng/mL).
Initial adjustment (a, b, or c)
Initial dose

Weight (kg)	30≤<50	50≤<70	70≤<90	90≤ ≤100
Dose per time (mg), twice daily	1	1.5	2	2.5

The blood trough concentration at 12 hours (C12h) and/or 24 hours (C24h) after the initial dose.
a: Initial dose (mg) × target trough concentration (12.5 ng/mL) / (average of C12h & C24h × 3).
b: Initial dose (mg) × target trough concentration (12.5 ng/mL) / (C12h × 4).
c: Initial dose (mg) × target trough concentration (12.5 ng/mL) / (C24h × 2.5).
Next adjustment:
The blood trough concentration (C) was measured at steady-state, after 2 days or more following the prevopus adjustment, to check whether the value was within the range of 10-15 ng/mL.
When the measured value was outside the range of 10-15 ng/mL, the dose was readjusted.
Previous dose × target trough concentration (12.5 ng/mL) / C.

levels were measured by SRL (a third-party organization independent of study physicians and sponsor) and relayed to the Control Center (Bellsystem24). Dosages were calculated at the Control Center based on the trough levels. The clinical sites were informed of the adjusted dosage by 3 days after the blood sample was drawn. Patient doses in the placebo group were pseudo-adjusted to preserve study blinding. The Control Center used the equations shown in Table 1 to carry out dose adjustments.

Symptom Assessment and Study Endpoints

The primary endpoint was clinical response based on the DAI score.[7] Clinical response was defined as a reduction in DAI by at least 4 points and improvements in all categories (stool frequency, rectal bleeding, mucosal appearance, and physician's overall assessment). A worse or unchanged score in any category was considered a treatment failure, even if all other scores improved. Secondary endpoints were mucosal healing and clinical remission.[8] Mucosal healing was defined as mucosal appearance subscore of 0 or 1. Clinical remission was defined as a total DAI score ≤2 with individual subscore (stool frequency, rectal bleeding, mucosal appearance, and physician's overall assessment) of 0 or 1. When a patient's symptoms worsened at any time and the investigator decided the study drug could not be continued, the treatment was considered a failure.

Statistical Analysis

Fisher's exact test was used to compare the tacrolimus group with the placebo group for demography, efficacy, and safety. The Wilcoxon signed rank test was used to compare each timepoint with baseline for demography. All statistical tests were two-sided with a significance level of 0.05 unless otherwise specified.

Sample Size

Based on previous results,[5] the clinical response was assumed to be 50% in the tacrolimus group and 10% in the placebo group. We estimated that randomizing 31 patients to each group would be sufficient to show a difference in efficacy between placebo and tacrolimus based on the above assumptions and a two-sided alpha of 0.025 and power of 0.9 using a normal approximation.

RESULTS

Patient Population

This study was performed between August 2006 and February 2008. Sixty-two patients in total were recruited. The mean total DAI score of patients enrolled was 9.8 ± 1.61 in the tacrolimus group and 9.1 ± 1.05 in the placebo group.

Drug Exposure

The mean trough concentrations in the tacrolimus group were 1.4 ± 0.9 ng/mL at 12 hours, 2.2 ± 1.5 ng/mL at 24 hours, 9.6 ± 3.1 ng/mL at day 7, 10.3 ± 3.1 ng/mL at day 8, 11.6 ± 3.4 ng/mL at day 10, and 13.0 ± 4.4 ng/mL at day 14.

Efficacy

Figure 1 shows that a clinical response was observed in 50.0% (16/32) of patients in the tacrolimus group and 13.3% (4/30) of patients in the placebo group. Significantly more patients in the tacrolimus group showed improvements compared with the placebo group ($P = 0.003$).

The observed rate of mucosal healing was 43.8% (14/32) in the tacrolimus group and 13.3% (4/30) in the placebo group ($P = 0.012$) at week 2, and clinical