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Hes1 promotes the IL-22-mediated antimicrobial response by enhancing STAT3-dependent transcription in human intestinal epithelial cells

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ABSTRACT

Notch signaling plays an essential role in the proliferation and differentiation of intestinal epithelial cells (IECs). We have previously shown that Notch signaling is up-regulated in the inflamed mucosa of ulcerative colitis (UC) and thereby plays an indispensable role in tissue regeneration. Here we show that in addition to Notch signaling, STAT3 signaling is highly activated in the inflamed mucosa of UC. Forced expression of the Notch target gene Hes1 dramatically enhanced the IL-22-mediated STAT3-dependent transcription in human IECs. This enhancement of STAT3-dependent transcription was achieved by the extended phosphorylation of STAT3 by Hes1. Microarray analysis revealed that Hes1-mediated enhancement of IL-22-STAT3 signaling significantly increased the induction of genes encoding antimicrobial peptides, such as REG1A, REG3A and REG3G, in human IECs. Conversely, the reduction of Hes1 protein levels with a γ-secretase inhibitor significantly down-regulated the induction of those genes in IECs, resulting in a markedly poor response to IL-22. Our present findings identify a new role for the molecular function of Hes1 in which the protein can interact with cytokine signals and regulate the immune response of IECs.

1. Introduction

The proliferation and differentiation of intestinal epithelial cells (IECs) are finely regulated by distinct molecular pathways, such as Notch, Wnt and BMP signaling [1]. Among these signaling pathways, Notch signaling has been shown to play a major, indispensable role in several stages of the differentiation process [2].

Notch signaling is a molecular pathway that acts between adjacent cells [2]. Studies have shown that Hes1 is expressed by intestinal stem cells and progenitor cells and is therefore one of the most important genes downstream of Notch in IECs [3]. Mouse intestines lacking Hes1 exhibit a dramatic increase in goblet cells and endocrine cells [4]. Accordingly, we have shown that Hes1 can directly suppress the expression of Atoh1, a key transcription factor for secretory lineage cell differentiation [5]. Thus, the Notch–Hes1 pathway functions as a major molecular pathway that determines the cell fate of IECs.

Our previous study also showed that the Notch–Hes1 pathway has an indispensable role in the tissue regeneration of ulcerative colitis (UC) patients [6]. In the colitic mucosa of UC patients, Notch–Hes1 signaling is highly activated in an increased number of IECs. Blocking Notch–Hes1 activation in DSS-colitis mice severely impaired epithelial regeneration. A recent study has also shown that silencing Notch signaling in IECs leads to the development of spontaneous colitis [7]. Thus, Notch–Hes1 signaling in IECs plays a critical role in both the initiation and the recovery from colitis. However, little is known about the potential interactions of Notch–Hes1 with inflammatory signals in IECs.

IL-22 is a cytokine that is produced by various inflammatory cells, and it is up-regulated in active UC patients [8,9]. IL-22 activates receptor-associated kinases and thereby phosphorylates STAT3 [8]. The activation of STAT3 induces the expression of its targets genes, including REG family genes. Studies have shown that the activation of the IL-22-STAT3 pathway in IECs plays a crucial role in the regeneration of the inflamed colonic mucosa [10,11]. A recent study suggested that its receptor, IL-22R1, is expressed exclusively by intestinal stem and progenitor cells [12]. Therefore, it is possible that the Notch–Hes1 and IL-22-STAT3 pathways may be activated within the same population of IECs.

Abbreviations: UC, ulcerative colitis; IECs, intestinal epithelial cells; p-STAT3, phosphorylated STAT3; DOX, doxycycline; GSI, γ-secretase inhibitor.

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In the present study, we show that both Hes1 and phosphorylated STAT3 are co-expressed in IECs residing within the inflamed mucosa of UC patients. Intracellular protein level of Hes1 can dramatically enhance or restrict the response to IL-22 in IECs. This Hes1-dependent regulation of the IL-22-STAT3 pathway appears to regulate the induction of REG family genes in IECs. Our present findings reveal a new aspect of the molecular function of Hes1 and demonstrate that it can regulate the response of IECs to inflammatory stimuli such as IL-22.

2. Materials and methods

2.1. Immunohistochemistry of human intestinal tissues

Tissue specimens were obtained from patients who underwent surgery for the treatment of UC at Yokohama Municipal General Hospital. Written informed consent was obtained, and the study was approved by the ethics committee of the Yokohama Municipal General Hospital and Tokyo Medical and Dental University. Paired tissue sections were prepared from macroscopically inflamed and non-inflamed regions of a surgical specimen. Immunohistochemical analysis using human intestinal tissues was performed as previously described [13]. We used anti-Hes1 (1:20,000, a kind gift from Dr. T. Sudo, Toray Industry) [14] and anti-phosho-STAT3 (Tyr^{705}) (1:100, clone D3A7, Cell Signaling Technology, Danvers, Massachusetts, USA) primary antibodies.

2.2. Cell culture

LS174T and DLD-1 cells were maintained as described elsewhere [6]. LY411575 was purchased from SIGMA (Buchs, Switzerland). Human recombinant IL-6, IL-22, IL-24, EGF, and TNF-α were all purchased from Pepro-Tech Inc. (Rocky Hill, New Jersey, USA). Human IL-31 was purchased from R&D Systems (Minneapolis, Minnesota, USA). The establishment of a cell line expressing FLAG-tagged Hes1 under the control of doxycycline (DOX, Clontech Laboratories, Mountain View, California, USA) was previously described [15].

2.3. Plasmid construction

Hes1p-Luc was a kind gift from Dr. R. Kageyama (Kyoto University, Japan) [16]. The response element specific for STAT3-dependent transcription was amplified by PCR from 4xM67 pTATA TK-Luc (Addgene plasmid 8688) [17] and cloned into pTL-Luc (Panomics, Fremont, California, USA) to construct STAT3p-Luc.

2.4. siRNA-mediated gene-knockdown

An siRNA specific for STAT3 or a non-targeting control (100 nM, Dharmacon, Lafayette, Colorado, USA) was transfected into cells using DharmaFECT Transfection Reagents (Dharmacon, Lafayette, Colorado, USA), according to the manufacturer's instructions.

2.5. Luciferase reporter assays and DNA transfections

Luciferase reporter assays were performed as previously described [6]. Each assay was performed in triplicate, and the results were normalized using the *Renilla* luciferase activity. The data were statistically analyzed using paired Student's *t*-tests.

2.6. Immunoblot analysis

Immunoblot assays were performed as described previously [13]. We used anti-phospho-STAT3 (Tyr^{705}) (1:1000, Cell Signaling Technology, Danvers, Massachusetts, USA), anti-STAT3 (1:1000, Cell Signaling Technology, Danvers, Massachusetts, USA), anti-FLAG (1:1000, Sigma–Aldrich, Buchs, Switzerland), anti-Hes1 (1:4000, a kind gift from Dr. T. Sudo, Toray Industry, Japan), and anti-β-actin (1:1000, Sigma–Aldrich, Buchs, Switzerland) primary antibodies. Densitometry analysis was performed using Image J software [18].

2.7. Microarray analysis

Total RNA was isolated as described previously [6] and subjected to microarray analysis using Human Oligo Chip 25 k V2.1 (TORAY, Kamakura, Japan). The complete dataset was submitted to the NCBI Gene Expression Omnibus (accession number GSE43012).

2.8. Quantitative RT-PCR analysis

Total RNA was prepared, and RT-PCR was performed as described previously [6]. The following primer sets were used for the analysis: REG1A, 5′-TGGAAGGATGTGCCTTGTGAAGACA-3′ (sense) and 5′-AGGCAAACTCAGCAGAGAAGAGAGT-3′ (antisense); REG3A, 5′- GAGAGTGACTCCTGATTGCCTCCT-3′ (sense) and 5′-GG GACAGCGGATCCGTGCAG-3′ (antisense); REG3G, 5′-GTGACCCGATT GCCTCCTCAAGTC-3′ (sense) and 5′-GGCAGGAAAGCAGCATCCAGG AC -3′ (antisense); and β-actin, 5′-CCTAAGGCCAACCGT GAAAAG -3′ (sense) and 5′-TCTTCATGGTGCTAGGAGCCA -3′ (antisense). Each assay was performed in triplicate, and the results are presented as the means of the data collected from three rounds of assays. We used paired two-sample Student's *t*-tests to analyze these data.

2.9. ELISA

ELISA assays for REG1A (Uscn Life Science Inc., Wuhan, China) and REG3A (Abnova, Taipei, Taiwan) were performed as per the manufacturers' instructions. The assays were performed in triplicate, and the results are shown as the means of the data. The data were statistically analyzed using paired Student's *t*-tests.

3. Results

3.1. Both Hes1 expression and STAT3 phosphorylation are up-regulated in IECs residing in the inflamed mucosa of UC patients

We first performed immunostaining of Hes1 and phosphorylated STAT3 (p-STAT3) using tissues derived from UC patients and compared their expression levels between the inflamed and non-inflamed mucosa (Fig. 1A). Consistent with our former reports [5,6], Hes1-positive IECs were restricted to the lower part of the crypt in non-inflamed mucosa, whereas a marked increase of Hes1-positive IECs was clearly observed in the inflamed mucosa (Fig. 1A). In the adjacent section, immunostaining for p-STAT3 also clearly showed an increased number of p-STAT3-positive cells in the inflamed mucosa. Furthermore, double immunostaining showed frequent co-expression of both Hes1 and p-STAT3 in the IECs of the inflamed colonic mucosa (Fig. 1B). These results indicated that these two transcription factors may possibly co-operate within IECs residing at the inflamed mucosa of UC patients.

3.2. Induction of IL-22-STAT3 signaling does not change the level of intracellular Notch–Hes1 activity in IECs

We first sought to identify stimuli that could induce STAT3 phosphorylation in human IECs. Various cytokines that can

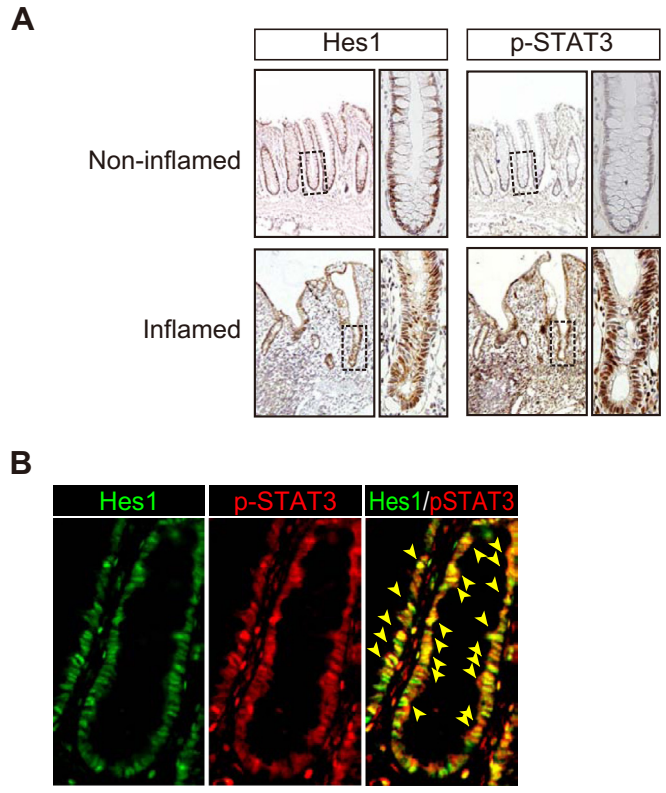


Fig. 1. The expression of both Hes1 and phosphorylated STAT3 are up-regulated in IECs residing in the inflamed mucosa of ulcerative colitis (UC) patients. (A) Serial sections analysis of inflamed and non-inflamed colonic tissues taken from a UC patient (original magnification: 120x). A magnified view of the indicated square is shown on the right side (original magnification: 600x). Positive signals are shown as the brown staining of 3,3′-diaminobenzidine (DAB). Representative data from three patients are shown. (B) Double immunostaining of Hes1 (green) and phosphorylated STAT3 (p-STAT3, red) of the inflamed colonic tissues of a UC patient (original magnification: 600×). Data representative of three independent analyses are shown. The yellow arrowhead indicates cells that express both Hes1 and p-STAT3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

activate STAT3, such as IL-6 or IL-22, are up-regulated in the mucosa of UC patients [9,19]. Using LS174T and DLD-1 cells, we tested the response to various cytokines that may induce the phosphorylation of STAT3. Of the tested cytokines, IL-22 alone was able to clearly induce the phosphorylation of STAT3 in both DLD-1 and LS174T cells (Supplementary Fig. S1A). Therefore, we focused on the IL-22-mediated phosphorylation of STAT3 throughout the rest of the study.

Next, we tested whether the IL-22-mediated phosphorylation of STAT3 altered the level of Notch–Hes1 activity in human IECs. Immunoblot analysis showed that p-STAT3 could be clearly detected at 10 min post-IL-22 stimulation and maintained for up to 3.5 h in DLD-1 cells (Supplementary Fig. S1B). However, no significant change was observed in the expression level of Hes1. Furthermore, a reporter assay using Hes1p-luc, a reporter plasmid that contains tandem repeats of the RBP-Jk-specific response element, showed no significant change upon IL-22 stimulation in DLD-1 cells (Supplementary Fig. S1C). Thus, the activation of the IL-22-STAT3 pathway has no detectable effect upon the Notch–Hes1 activity in human IECs.

3.3. Overexpression of Hes1 can enhance IL-22-induced STAT3-dependent transcription in human IECs

To complement the experiments described above, we also tested whether the expression level of Hes1 might change the IL-22-induced response in IECs. We previously generated a cell line in which we can induce the overexpression of Hes1 in a doxycycline (DOX)-dependent manner in LS174T cells (LS174T-TetON-Hes1 cells) [5,15]. We generated a new similar cell line using DLD-1 cells (DLD-1-TetON-Hes1 cells), and we subjected both cell lines to the assays described below (Fig. 2A).

Using the described cell lines, we tested whether the expression level of Hes1 might alter the IL-22-induced response in IECs. A reporter assay using STAT3p-Luc, a plasmid that contains tandem repeats of a STAT3-specific response element [17], showed that the addition of IL-22 up-regulates the reporter activity by up to 5-fold in both the LS174T-TetON-Hes1 cells (Hes1) and the parental cells (Control) (Fig. 2B, left panel). The addition of DOX alone had no effect in Control cells but induced a slight up-regulation (up to 2-fold) in the LS174T-TetON-Hes1 cells, indicating that Hes1 may activate STAT3-dependent transcription independently from IL-22, but at a much lower level. However, the addition of both IL-22 and DOX gave the most dramatic increase of the reporter activity, reaching up to an 80-fold increase in the LS174T-TetON-Hes1 cells. This additional effect was completely absent in the Control cells. These results were also confirmed in DLD-1-TetON-Hes1 cells (Hes1) and their parent cells (Control) (Fig. 2B, right panel). Collectively, the response to IL-22 was dramatically enhanced and led to extremely high level of STAT3-dependent transcription by the overexpression of Hes1 in IECs.

3.4. Overexpression of Hes1 can prolong the IL-22-induced phosphorylation of STAT3

To further analyze the mechanism by which Hes1 enhances IL-22-induced STAT3-dependent transcription, we examined the phosphorylation status of STAT3. Immunoblot analysis of LS174T-TetON-Hes1 cells showed that p-STAT3 could be detected as early as 10 min post-stimulation with IL-22, but its level started to decrease after 2 h when DOX was not given (DOX-) to those cells (Fig. 2C). However, when DOX was added to the cells (DOX+), p-STAT3 was maintained at a high level up to 4 h post-stimulation by IL-22 (Fig. 2C). We also confirmed that the same result could be clearly observed in DLD-1-TetON-Hes1 cells (Fig. 2D), suggesting that a robust and prolonged phosphorylation of STAT3 might be the underlying mechanism of how Hes1 can enhance IL-22-induced STAT3-dependent transcription in IECs.

3.5. IL-22-mediated induction of genes encoding antimicrobial proteins can be significantly enhanced by over-expression of Hes1 in IECs

To analyze the functional relevance of the co-operation between Hes1 and IL-22-STAT3 signaling, we next tried to identify the group of genes that are targeted and co-regulated by these two pathways. A microarray analysis was performed to compare the differences between LS174T-TetON-Hes1 cells that were stimulated by IL-22 alone, DOX alone, or both (Fig. 3A). Genes associated with anti-apoptosis, cell cycle regulation and angiogenesis did not respond to any condition. However, genes associated with the antimicrobial response, such as REG1A, REG3A and REG3G, clearly showed an enhanced induction by the combined treatment with IL-22 and DOX (Fig. 3A). Therefore, we focused on REG family genes and further confirmed the co-regulation of their expression by IL-22 and Hes1.

REG1A, REG3A and REG3G belong to the secreted C-type lectin protein family that has antimicrobial activity against Gram-positive bacteria [20]. Quantitative RT-PCR analysis confirmed that these genes are clearly up-regulated upon IL-22 stimulation in both LS174T-TetON-Hes1 cells (Hes1) and parent cells (Control) (Fig. 3B). However, co-stimulation with DOX had a significant

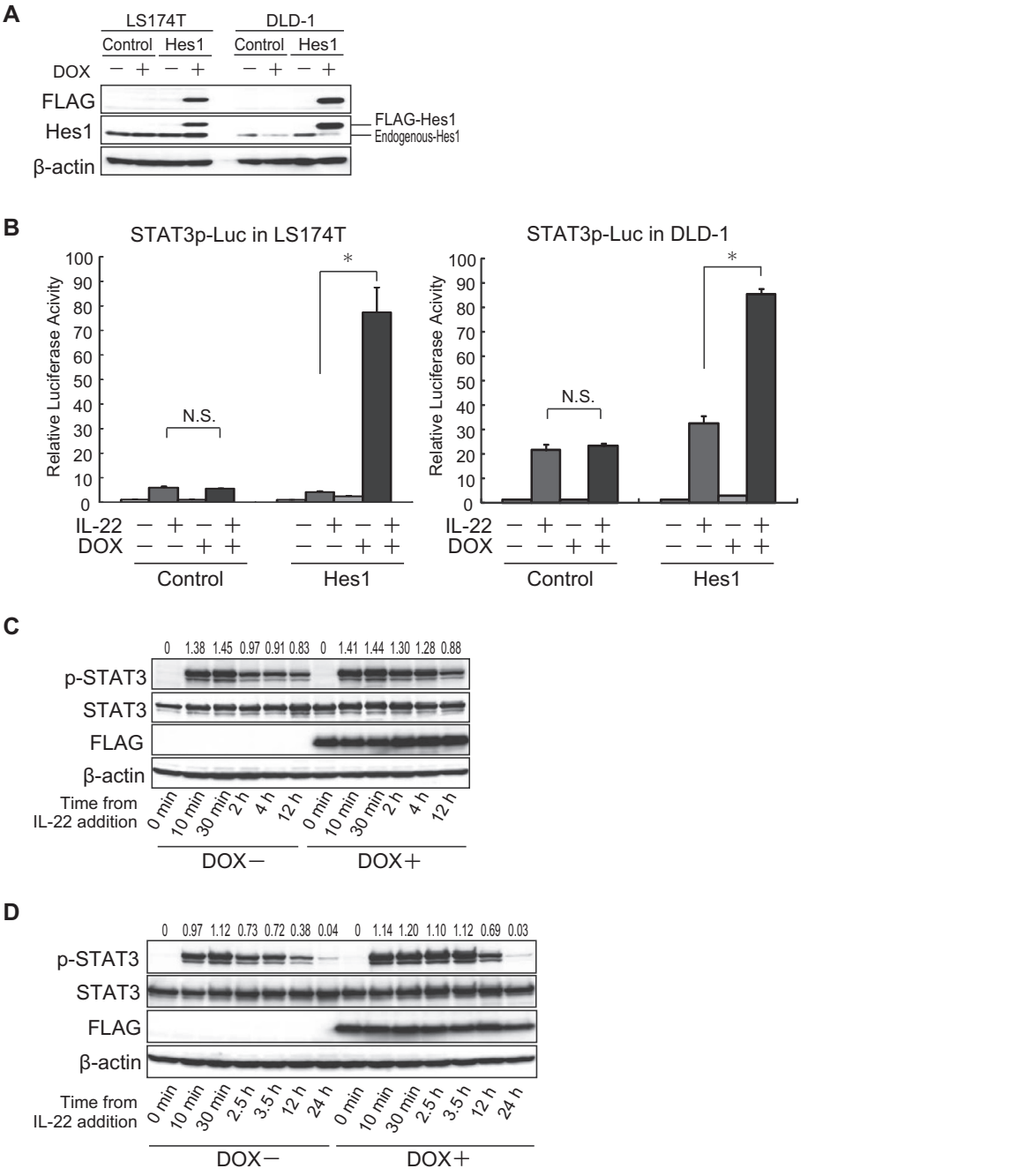
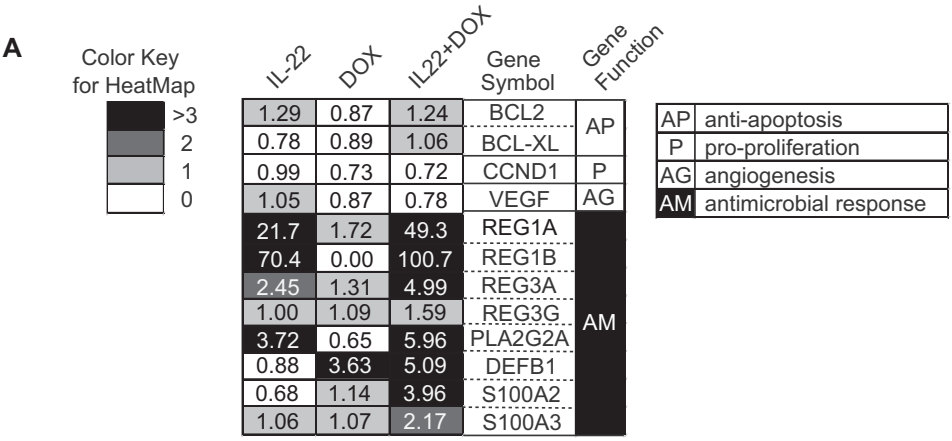


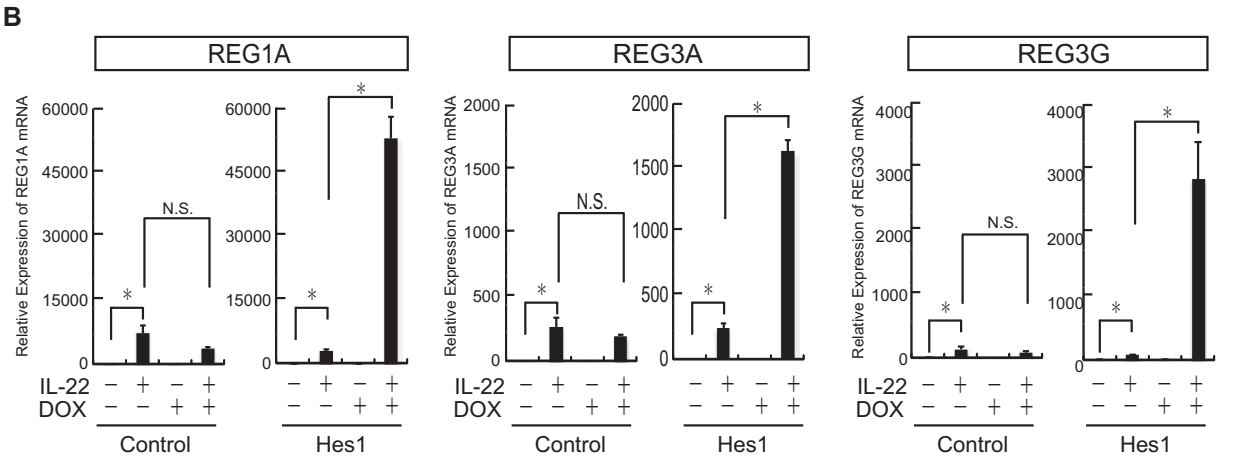
Fig. 2. Hes1 enhances IL-22-induced STAT3-dependent transcription through the prolonged phosphorylation of STAT3. (A) LS174T (LS174T-TetON-Hes1 cells) and DLD-1 cells (DLD-1-TetON-Hes1 cell) in which FLAG-tagged Hes1 is expressed in a doxycycline (DOX)-dependent manner. The cells were cultured with or without DOX (100 ng/ml) for 12 h and subjected to immunoblot analysis. Parental LS174T or DLD-1 cells served as the controls. (B) Luciferase reporter assay using STAT3p-Luc in LS174T-TetON-Hes1 cells or DLD-1-TetON-Hes1 cells (Hes1) and their corresponding parent cells (Control). Cells were transfected with STAT3p-Luc for 12 h and stimulated with IL-22 (20 ng/ml) alone, DOX (100 ng/ml) alone, or both for an additional 24 h. Error bars represent the S.D. of triplicate experiments. $^{*}P < 0.05$. N.S. indicates that the comparison was not significant. (C, D) LS174T-TetON-Hes1 cells (C) or DLD-1-TetON-Hes1 cells (D) were stimulated by IL-22 alone (DOX−) or with DOX (DOX+) and subjected to immunoblot analysis. The signal intensity ratio of p-STAT3 and STAT3 at various time points is shown at the top of each panel.

additive effect in LS174T-TetON-Hes1 cells but not in parent cells. The same result could also be obtained from DLD-1-TetON-Hes1 cells and parent cells (Fig. 3C). This up-regulation in mRNA levels was functionally relevant, as a significant increase in the secretion of both REG1A and REG3A was also clearly confirmed (Supplementary Fig. S2). Thus, the results indicated that Hes1 could enhance the IL-22-mediated induction of STAT3 target genes that encode anti-microbial proteins, such as REG family genes, in IECs.

3.6. Up-regulation of REG family genes by IL-22 is dependent on STAT3 and endogenous Notch–Hes1 activity in IECs

To confirm that the observed effects of IL-22 and Hes1 on the expression of the REG family genes were dependent on STAT3, we performed a siRNA-mediated gene knockdown in DLD-1-TetON-Hes1 cells (Fig. 4A and B). Knockdown of STAT3 almost completely abolished the induction of REG1A, REG3A and REG3G





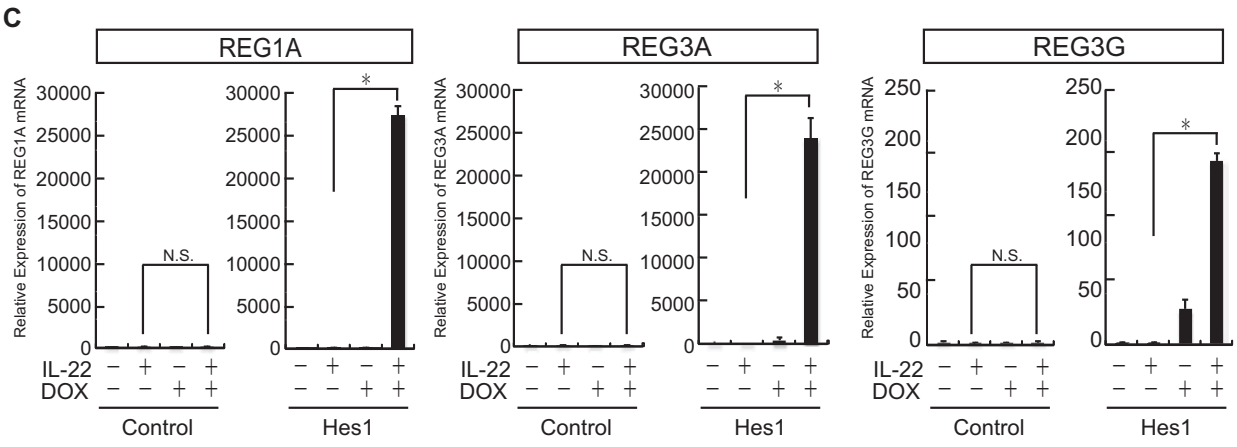


Fig. 3. The IL-22-mediated induction of genes encoding antimicrobial proteins can be significantly enhanced by the overexpression of Hes1 in IECs. (A) LS174T-TetON-Hes1 cells were stimulated for 24 h with IL-22 alone (20 ng/ml), DOX alone (100 ng/ml) or both IL-22 and DOX or left untreated and then subjected to microarray analysis. The numbers represent the fold induction compared to the untreated cells. (B, C) LS174T-TetON-Hes1 cells (B) and DLD-1-TetON-Hes1 cells (C) or their corresponding parent cells (Control) were stimulated for 24 h with IL-22 alone (20 ng/ml), DOX alone (100 ng/ml), or both and subjected to quantitative PCR analysis. The data shown are the means ± SD, normalized to the expression level of β-actin. $^{*}P < 0.05$. N.S. indicates that the comparison was not significant.

expression in response to IL-22 alone and to co-stimulation with IL-22 and DOX (Fig. 4B).

To confirm that the response to IL-22 was also dependent on endogenous Notch–Hes1 activity, we treated cells with a γ-secretase inhibitor (GSI), LY411575, which can decrease the expression of endogenous Hes1 in IECs [6,15] (Fig. 4C). Treatment with LY411575 significantly down-regulated the IL-22-mediated induction of REG1A and REG3A in LS174T cells (Fig. 4D). Thus, the endogenous protein level of Hes1 may restrict the response to IL-22 in IECs.

4. Discussion

In this study, we found that both Notch–Hes1 and STAT3 signaling can be activated in IECs residing within the inflamed region of the intestines of UC patients. We also found that they can

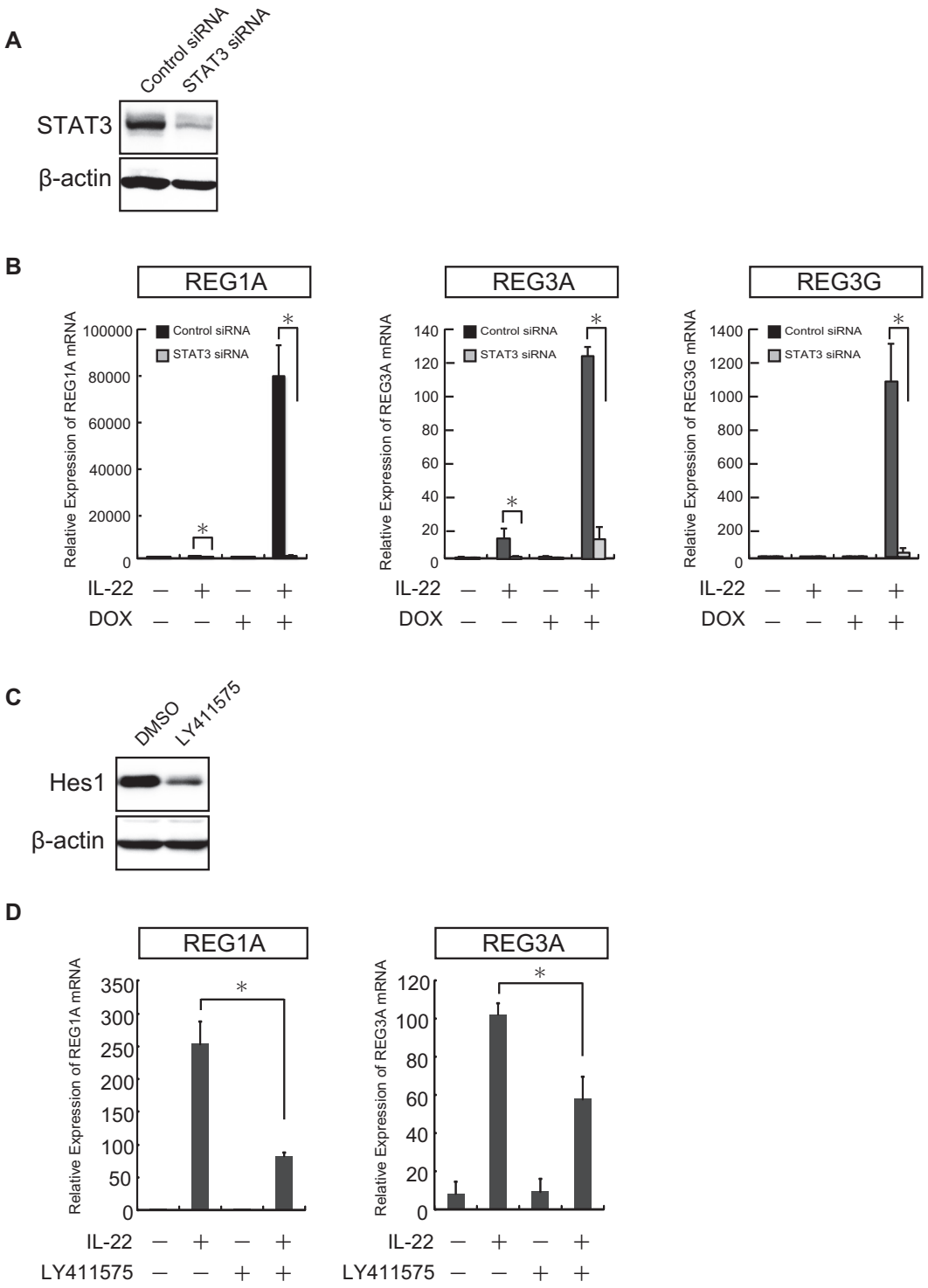


Fig. 4. The up-regulation of REG family genes by IL-22 is dependent on both STAT3 and endogenous Notch–Hes1 activity in IECs. (A) Cells were transfected with 100 μM of siRNA targeting STAT3 (STAT3 siRNA) or siRNA with no target (Control siRNA) for 48 h and were then subjected to immunoblot analysis. The signal intensity showed a 65% reduction in STAT3 in cells transfected with STAT3-specific siRNA (relative intensity of STAT3: Control siRNA, 1.0; STAT3 siRNA, 2.8) (B) DLD-1-TetON-Hes1 cells were transfected with siRNA for 48 h as done in (A) and stimulated for an additional 24 h with IL-22 alone (20 ng/ml), DOX alone (100 ng/ml), or both. The mRNA levels were then analyzed by quantitative PCR. The data shown are the means ± SD normalized to the expression level of β-actin. $^{*}P < 0.05$. (C) LS174T cells were treated with 1 μM of LY411575 for 48 h and then subjected to immunoblot analysis. The signal intensity showed a 70% reduction in the expression of Hes1 by treatment with LY411575 (Relative intensity of Hes1: DMSO, 1.0; LY411575, 3.5). (D) LS174T cells were treated with 1 μM of LY411575 for 48 h and then stimulated with IL-22 for the following 24 h. The quantitative PCR results are shown as the means ± SD normalized to the expression level of β-actin. $^{*}P < 0.05$.

co-operate to promote IL-22-mediated STAT3 signaling in IECs. Our study shows for the first time that Hes1 expression in IECs can interact with cytokine signals and plays major role in determining the response. We suggest that Hes1 is more than just a regulator of proliferation or differentiation in human IECs; it may also have a major role in integrating the signals provided from inflammatory cytokines other than IL-22 and in converting these signals into a beneficial response. Thus, the present study emphasizes the

dominant role of Hes1 in promoting the amelioration of the inflamed human mucosa.

We used microarray experiments to demonstrate that Hes1 overexpression can enhance the expression of antimicrobial genes in response to IL-22. Antimicrobial proteins protect the intestinal epithelia from invasion by intestinal bacteria and have been shown to have an ameliorating effect in several mouse models of colitis [21]. Antibacterial therapy has also been reported to be beneficial for the treatment of UC [22]. Thus, enhancing the antibacterial response in IECs may have an anti-inflammatory effect in the mucosa of UC patients. We focused on the REG family genes and found that the IL-22-mediated induction of REG1A, REG3A and REG3G is regulated by Hes1 protein levels (Fig. 3B, C). Consistently, it has been reported that REG proteins are expressed in the lower part of the colonic crypt in UC [23,24], where we found that Hes1 and p-STAT3 are co-expressed (Fig. 1B). In addition to its antimicrobial function, REGIA has also been shown to have a proliferative effect on intestinal epithelial cells [9,25]. REG3A has also been shown to inhibit the secretion of pro-inflammatory cytokines from IECs [26]. These reports support the idea that Hes1 contributes to the antimicrobial response, epithelial cell proliferation and the anti-inflammatory response via STAT3.

We showed in this study that Hes1 could enhance STAT3 dependent transcription and also induce prolonged phosphorylation of STAT3 (Fig. 2). This is the first report showing that Hes1 can regulate the functions of STAT3 in human IECs. The overall level of p-STAT3 is determined by the balance between its phosphorylation and dephosphorylation. As we found that Hes1 overexpression induces the prolongation in time, but not the increase in level of STAT3 phosphorylation, Hes1 may be able to maintain the phosphorylation/dephosphorylation ratio at a high level, but it may not be able to increase the ratio over a limit. Former study using the neural tissue has shown that Hes1 can activate STAT3 phosphorylation through facilitating the complex between Jak2 and STAT3 [27]. Therefore, further investigations may reveal that Jak family kinases that are bound to the IL-22 receptor might also be responsible for the robust STAT3 phosphorylation induced by Hes1 in human IECs.

In conclusion, we have found that Hes1 acts as a critical factor in determining the response to IL-22 in human IECs. Our findings broaden the functional aspect and importance of Hes1 expression in IECs.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.061.

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