which we could induce NICD1 expression in a tetracycline- or doxycycline (DOX)-dependent manner (TET-on NICD1 cells), we examined whether the expression of Dll1 and Dll4 was regulated by intracellular Notch activity. Immunoblot analysis of TET-on NICD1 cells confirmed that expression of NICD1 was induced upon the addition of DOX (Fig. 2A). We also found that the mRNA expression of Hes1 was significantly upregulated upon the addition of DOX to TET-on NICD1 cells (Fig. 2B). Further analysis of gene expression showed that Dll1, Dll4, MUC2, and Atoh1 expression were all significantly downregulated in these cells. In sharp

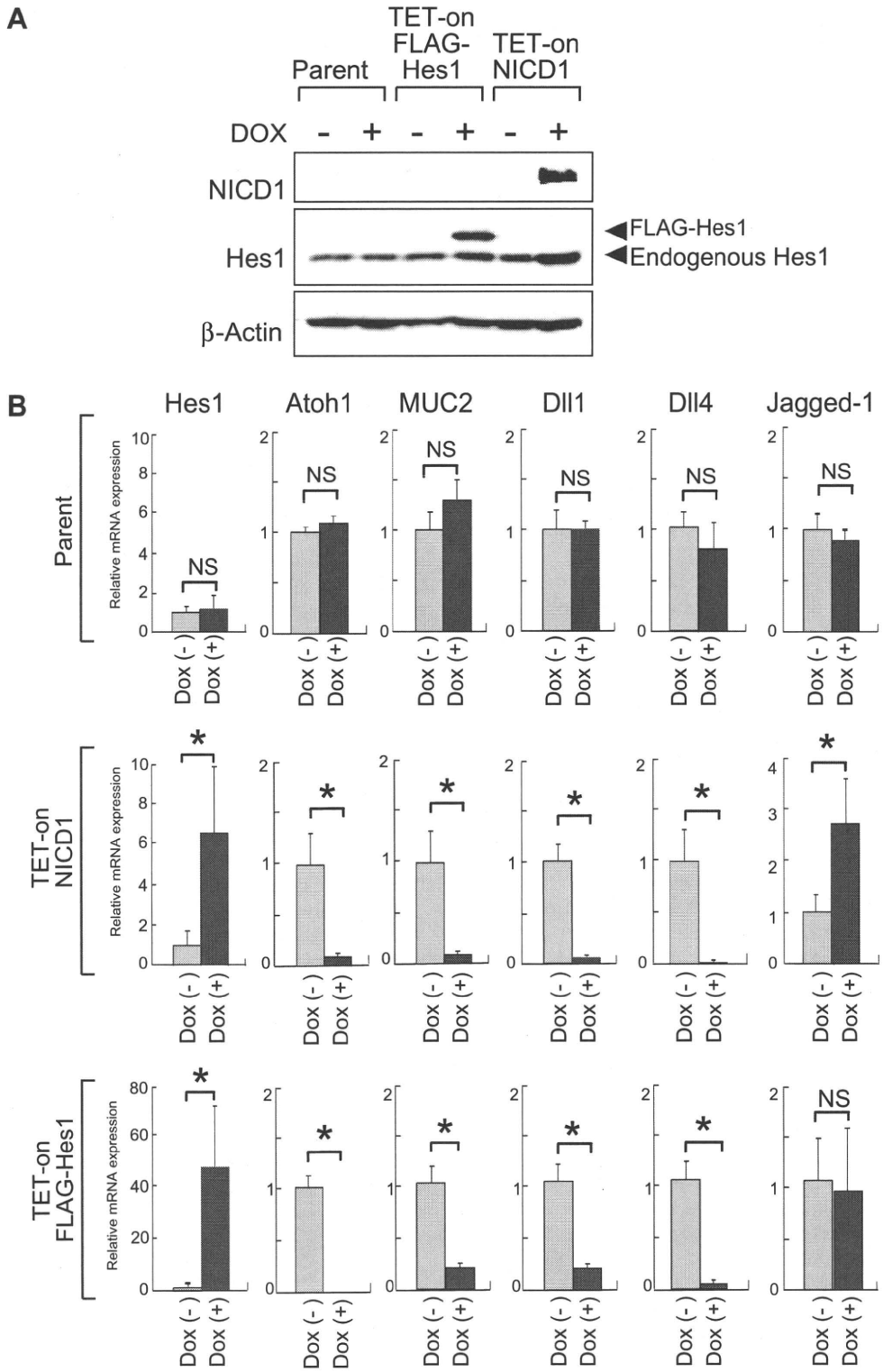

Fig. 2. Both the goblet cell phenotype and expression of Delta-like ligands are downregulated upon activation of the Notch-Hes1 pathway in human colonic epithelial cells. (A) Immunoblot analysis showing forced expression of Notch1 intracellular domain (NICD1) or FLAG-tagged Hes1 upon doxycycline (DOX) addition in TET-on NICD1 cells and TET-on Hes1 cells, respectively. Parent LS174T cells (Parent) served as the control. Cell lysates were prepared 72 h after DOX addition. (B) Expression of the indicated genes was analysed by quantitative RT-PCR. Data are means ± SD, normalized to the expression level of β-actin. $^*P < 0.05$. NS indicates that the comparison was not significant.

contrast, Jagged-1 demonstrated slight upregulation (up to 2.6-fold) when NICD1 expression was induced. These results suggested that expression of Dll1 and Dll4, but not Jagged-1 was regulated by intracellular Notch activity and that this occurred in-parallel with the expression of genes required for goblet cell differentiation.

To further confirm the role of the canonical Notch-Hes1 pathway, we generated another sub-line of LS174T cells in which we could induce FLAG-tagged Hes1 expression upon DOX addition (TET-on FLAG-Hes1 cells). Immunoblot (Fig. 2A) and quantitative RT-PCR analysis (Fig. 2B, which shows the total amount of both endogenous Hes1 mRNA and FLAG-Hes1 mRNA in these cells) confirmed that Hes1 expression is upregulated upon DOX addition to TET-on FLAG-Hes1 cells. We also found that the expression of Dll1 and Dll4, and the expression of Atoh1 and MUC2, was significantly downregulated upon the addition of DOX to TET-on FLAG-Hes1 cells (Fig. 2B). In sharp contrast, Jagged-1 expression remained unchanged. These results further confirmed that the in-parallel downregulation of Dlls and the genes required for goblet cell differentiation were mediated by the Notch-Hes1 axis in human colonic epithelial cells.

Both the goblet cell phenotype and Dll ligand expression are upregulated upon inactivation of the Notch-Hes1 signaling pathway in human colonic epithelial cells

The results of our previous experiments suggested that both Dll ligand expression and the acquisition of goblet cell phenotype are under control of the intracellular Notch-Hes1 signaling pathway. To further confirm that this regulatory system exists in human colonic epithelial cells, we next sought to examine the expression of Dll1 and Dll4 upon the induction of goblet cell differentiation. In our previous report, we showed that treatment with a gamma-secretase inhibitor, LY-411,575, completely blocked intracellular Notch activity and thereby induced significant upregulation of MUC2 expression in LS174T and HT29 cells [9]. Therefore, we examined the expression of Dll1 and Dll4 in this model of goblet cell differentiation.

Following the protocol used in our previous study, we analysed LS174T and HT29 cells by quantitative RT-PCR after they were treated with either DMSO or LY-411,575 (Fig. 3A). Treatment with LY-411,575 led to a significant downregulation of Hes1 mRNA

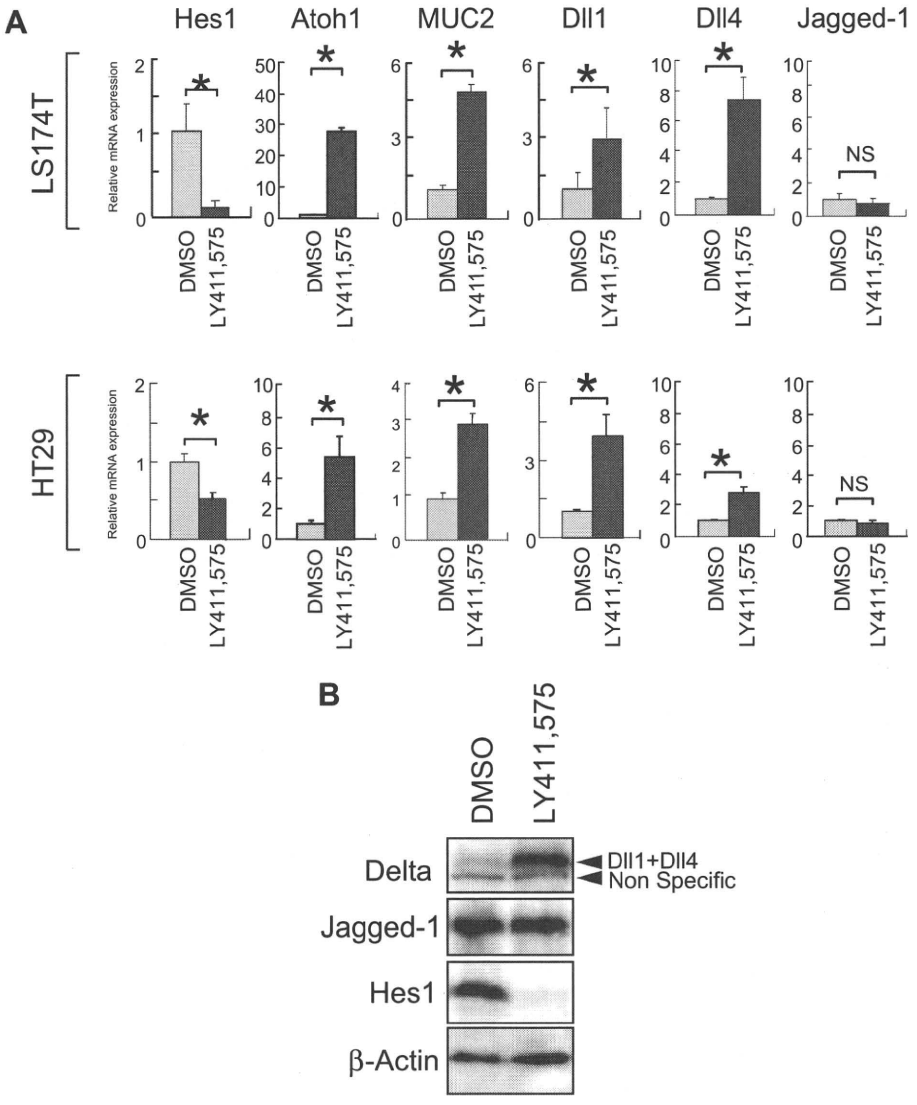

Fig. 3. Both the goblet cell phenotype and expression of Delta-like ligands are upregulated upon inactivation of the Notch pathway in human colonic epithelial cells. (A) Expression of the indicated genes in LS174T cells and in HT29 cells was analysed by quantitative RT-PCR. Total RNA was prepared after 72 h of culture with either LY-411,575 (1 μM) or DMSO. Data are means ± SD, normalized to the expression level of β-actin. *$P < 0.05$. NS indicates that the comparison was not significant. (B) Immunoblot analysis showing upregulation of Delta-like ligand proteins (total Dll1 and Dll4 content) upon Notch inactivation in LS174T cells. Cell lysates were prepared after 72 h of culture with either LY-411,575 (1 μM) or DMSO.

expression in both LS174T cells and HT29 cells, confirming that the intracellular Notch-Hes1 pathway had been inactivated. Consistent with our previous results, treatment with LY-411,575 upregulated Atoh1 and MUC2 expression in both cell lines. Under these conditions, expression of Dll1 and Dll4 was significantly upregulated in both cell lines, whereas Jagged-1 expression remained unchanged. This upregulation of Dlls was also confirmed at the protein level in LS174T cells, as immunoblot analysis of cells treated with LY-411,575 showed a significant increase in Dll protein levels (Fig. 3B, total Dll1 and Dll4 protein content). Thus, these results further confirmed that both expression of Dlls and the acquisition of the goblet cell phenotype are under the control of the intracellular Notch-Hes1 signaling pathway in human colonic epithelial cells. Also, these results are consistent with the results of our immunohistochemical analyses (Fig. 1B) in which we found that Dll1 and Dll4 were expressed in a goblet cell-specific manner, whereas Jagged-1 was expressed in a lineage-non-specific manner.

Upregulation of Dll1 is required for the upregulation of the goblet cell phenotype upon inactivation of the Notch-Hes1 signaling pathway in human colonic epithelial cells

The results from the experiments described above demonstrated that the expression of Dlls, Atoh1, and MUC2 are upregulated in-parallel upon inactivation of intracellular Notch signaling in human colonic epithelial cells. MUC2 has been reported to be one of the direct targets of Atoh1 [5]. Thus, it remains unknown whether the upregulation of Dll1 and Dll4 expression has any role in promoting cells to differentiate into goblet cells. If Dlls do play a role in this process, it also remains unknown whether they do so through the Atoh1-MUC2 axis.

To further study this process, we performed a knockdown experiment targeting Dlls to examine the functional role that Dll expression played in goblet cell differentiation. Despite our finding that treatment with gene-specific siRNAs significantly downregulated Dll1 and Dll4 expression at the mRNA level, our primary experiment showed a marked decrease of the total Dll1 and Dll4 protein content by the siRNA targeted for Dll1, but not for Dll4 (Supplemental Fig. 1). Thus, the dominant Dll protein expressed by LS174T cells appeared to be Dll1, not Dll4. Therefore, further analysis was performed using the Dll1-specific siRNA.

Combined treatment with LY-411,575 and a Dll1-targeted siRNA was performed using LS174T cells. Immunoblot analysis showed knockdown of Dll proteins in cells treated with Dll1-targeted siRNA (Fig. 4A). Also, Hes1 protein was completely undetectable in LY-411,575 treated cells. Quantitative RT-PCR analysis revealed that Dll1 mRNA expression was also clearly knocked down by treatment with the Dll1-specific siRNA, completely abrogating any increase in Dll1 expression upon treatment with LY-411,575 (Fig. 4B). In contrast, Dll4 expression was not affected by treatment with the siRNA targeted to Dll1. Knockdown of Dll1 significantly downregulated the expression of both Atoh1 and MUC2 in cells treated with DMSO alone (52% and 42% reduction for Atoh1 and MUC2, respectively). However, upon knockdown of Dll1 expression, a significant downregulation of Atoh1 and MUC2 expression was also observed in cells treated with LY-411,575 (64% and 70% reduction for Atoh1 and MUC2, respectively). Knockdown of Dll1 completely abrogated the upregulation of MUC2 expression by LY-411,575, suggesting that induction of the goblet cell phenotype in Notch-inactivated colonic epithelial cells is dependent upon the upregulation of Dll1 expression.

Based on these collective findings, we suggest a modified model describing the cell fate decision pathway that determines if a human intestinal epithelial cell will develop into an absorptive or goblet cell within the human intestinal epithelium (Fig. 4C). In this proposed model, the development of the absorptive cell phenotype

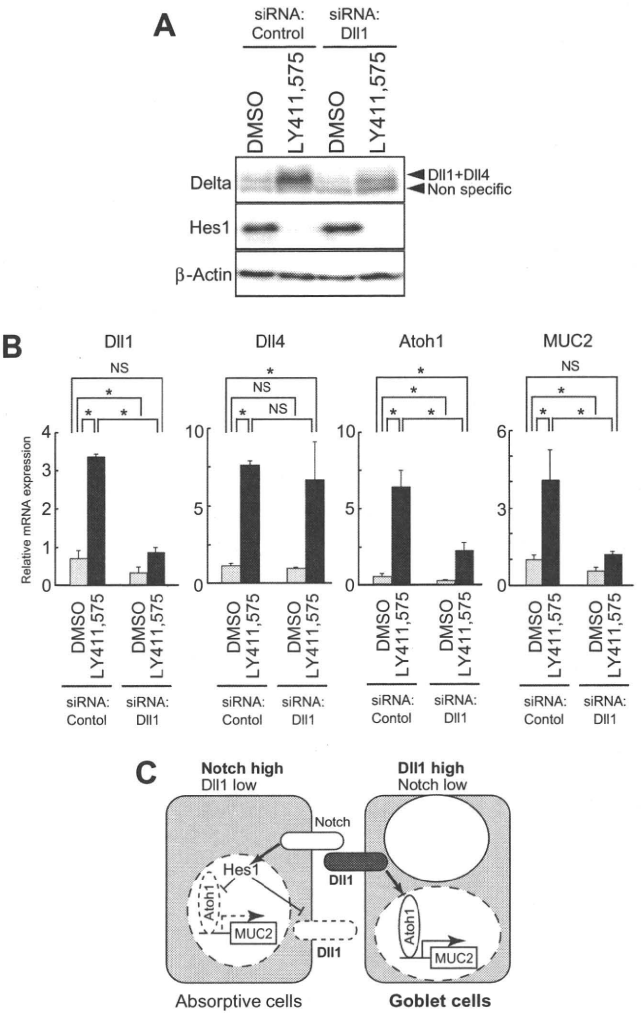

Fig. 4. Upregulation of Delta-like 1 is required for the upregulation of the goblet cell phenotype upon inactivation of the Notch pathway in human colonic epithelial cells. (A) siRNA-mediated knockdown of Delta-like 1 (Dll1) downregulated Delta-like ligand protein expression in LS174T cells. Cells were transfected with either Dll1-specific siRNA or non-targeting siRNA (Control) and cultured for 72 h with either LY-411,575 (1 μM) or DMSO. Immunoblot analysis was performed as described in Fig. 3B. (B) Expression of the indicated genes in LS174T cells was analysed by quantitative RT-PCR. Data are means ± SD, normalized to the expression level of β-actin. $^*P < 0.05$. NS indicates that the comparison was not significant. (C) A modified scheme of absorptive versus goblet cell differentiation that was derived from the present data.

is facilitated by the activation of the Notch-Hes1 pathway, the downregulation of both Atoh1 and Dll1 expression, and the subsequent downregulation of MUC2. However, the development of the goblet cell phenotype is facilitated by both Notch inactivation and Dll1 expression, as both are required for sufficient levels of Atoh1 and MUC2 expression. The present study raises the possibility that Dll1 might not only function in trans as a ligand that activates the Notch-Hes1 pathway in neighboring cells but that it might also function in cis to promote the differentiation of goblet cells in Notch-inactivated colonic epithelial cells.

Discussion

In the present study, we demonstrated for the first time that Dll1 and Dll4 are selectively expressed in goblet cells, whereas Jagged-1 is expressed in a lineage-non-specific manner within the human colonic epithelium. In a previous study, we showed that NICD1 is expressed in cells other than goblet cells, and it is

completely absent in mature goblet cells in the human colon [9]. Thus, the complementary expression of NICD1 and Dlls suggests that Dlls and not Jagged-1, take part in the lateral inhibition of Notch signaling within the colonic epithelium.

Previous reports by Crosnier et al. have shown that the genetic disruption of DeltaD (a zebrafish homologue of Dll) increases the number of secretory-type cells, including goblet cells, in the zebrafish intestine [12]. In their report, however, the increase in the number of secretory-type cells was minimal when the DeltaD protein was completely absent. In contrast, disruption of its ligand function alone resulted in upregulation of the DeltaD protein and a marked increase in the number of secretory-type cells in the intestinal epithelium. These findings are consistent with our present results showing that Dll1 might play a role in promoting goblet cell differentiation in human colonic epithelial cells that is distinct from its function as a ligand for Notch. Previous reports have consistently shown that Notch ligands may have functions other than acting merely as a ligand for Notch. Notch ligands are also cleaved by gamma-secretase upon binding to the Notch receptor, following which, the released intracellular domain localizes to the nucleus [10]. This intracellular domain of Notch ligands might function in cis as a transcriptional co-activator [17] and promote the expression of genes such as Atoh1 and MUC2. Consistently, our immunostaining of Dll1 and Dll4 revealed dominant staining in the nuclei of goblet cells (Fig. 1B). However, because our in vitro studies of goblet cell differentiation were based on the inactivation of Notch by a gamma-secretase inhibitor, it is possible that the observed function of Dll1 might not be dependent upon release of the intracellular domain. Notch ligands bound to the cell membrane may bind with transcriptional co-activators containing a PDZ-domain and could potentially regulate their function by keeping them away from the nucleus [10]. Thus, Dll1 might function as a regulator of intracellular transcription through such a mechanism.

Another finding of the present study was that the expression of the Dlls, Atoh1, and MUC2 were under the control of the Notch-Hes1 axis. A previous report found that Dll1 was a direct target of Hes1 in mice [18]. Our results showed that Dll1 and Dll4 might be one of the downstream targets of Hes1 in human colonic epithelial cells. Such regulation of Dlls, Atoh1 and MUC2 by the Notch-Hes1 axis emphasizes their importance in lateral inhibition, thereby contributing to promote absorptive cell differentiation (Fig. 4C).

Although we found that Dll1 is required for proper goblet cell development in Notch-inactivated cells, Dll4 appeared to be of less importance in this context. Knockdown of Dll1 downregulated total Dll1 and Dll4 protein content in LS174T cells (Fig. 4A). Thus, Dll4 might be expressed in low levels as compared to Dll1 in colonic epithelial cells. Also, previous in vivo studies have shown that the administration of a neutralizing antibody for Dll4 had no effect on murine intestinal epithelium [19]. Thus, although the importance of Dll4 expression remains to be elucidated, as compared to Dll1, its role may be minimal with regard to the induction of goblet cell differentiation.

In conclusion, Dll1 expression is upregulated upon Notch inactivation, which is required for the proper acquisition of the goblet cell phenotype. Further studies focusing on a potential cis-acting function of the Dll1 protein might reveal a yet unknown link between Dll1 expression and the regulation of goblet cell differentiation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.02.048.

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Ⅷ. 研 究 班 構 成

難治性腸管吸収機能障害Microscopic colitisに関する調査研究班

区　　分	氏　　名	所　　属　　等	職　　名
研究代表者	渡辺　守	東京医科歯科大学大学院医歯学総合研究科消化器病態学	教　授
研究分担者	平田　一郎	藤田保健衛生大学医学部消化管内科学	教　授
	松井　敏幸	福岡大学筑紫病院消化器科	教　授
	三浦 総一郎	防衛医科大学校内科学	教　授
	田中　正則	弘前市立病院臨床検査科	医療局長
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	清水　誠治	JR大阪鉄道病院消化器内科	医務部長
	岡本　隆一	東京医科歯科大学大学院医歯学総合研究科消化器病態学	准教授
	土屋 輝一郎	東京医科歯科大学大学院医歯学総合研究科消化器病態学	講　師
事務局	岡本　隆一	東京医科歯科大学大学院医歯学総合研究科消化器病態学 〒113－8519 東京都文京区湯島1-5-45 ＴＥＬ　03－5803－5877 ＦＡＸ　03－5803－0268 e-mail　rokamoto.gast@tmd.ac.jp	
経理事務担当者	増田　晴彦	東京医科歯科大学学術国際部・研究推進課 ＴＥＬ　03－5803－5872 ＦＡＸ　03－5803－0179 e-mail　haruhiko.adm@cmn.tmd.ac.jp	