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Notch signaling is necessary for GATA3 function in the initiation of T cell development

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GATA3 and Notch1 are essential for T cell development at the earliest stage, but their mutual roles in this process remain to be clarified. In this study, we demonstrated that impairment of T lymphopoiesis in hematopoietic progenitor cells (HPC) of GATA3-deficient fetal liver (FL) on day 11.5 of gestation (E11.5) was rescued only by introduction of both GATA3 and the intracellular region of Notch1 but not by either alone. However, the introduction of GATA3 only was sufficient for T cell induction in GATA3-deficient FL cells at the advanced stage, where Notch signaling is well detectable. This indicates that Notch signaling is necessary for GATA3 to function for T cell fate specification but is not sufficient without GATA3. On the other hand, Notch signaling is sufficient for blockage of B cell development without GATA3, suggesting that T cell fate specification at the branching point does not result simply from the developmental arrest of B cell lineage by Notch signaling.

Key words: GATA3 · Notch signaling · Thymopoiesis

Introduction

Within the complex process of lymphopoiesis, the differentiation of hematopoietic progenitor cells (HPC) requires a thymic environment for T cell development, while B cells differentiate in a bone marrow (BM) environment. Studies on B cell fate specification have revealed an appropriate *in vitro* induction system in which B cell lineage can be induced from HPC on BM-derived stromal cells. This system is useful for determining the environment-specific molecules in the B cell development process [1]. In contrast, because the three-dimensional structure of the thymic lobe is always required to induce T cell fate *in vitro*, it has been difficult to investigate the signal molecules involved in T cell development. However, several recent studies have demonstrated that lineage specification of T cells is determined by Notch signaling instead of the thymic environment [2].

Notch genes encode transmembrane receptors that are highly conserved from invertebrates to mammals, and Notch-mediated signaling regulates cell fate specification in a large number of developmental systems [3, 4]. Such signals are transmitted through direct contact between cells expressing Notch receptors and their ligands. The mutual interactions of Notch receptors and their ligands result in proteolysis of Notch and consequent movement of the intracellular region of Notch (ICN) into the nucleus with a DNA-binding protein, RBP-J. This translocation is an essential part of the Notch signal transduction process.

Using Cre-*loxP*-mediated gene targeting, it was clearly shown that the specific gene deletion of *Notch1* in hematopoietic cells leads to complete failure of T cell development and a substantial amount of B cells in the thymus, whereas B cell development in BM remains intact [5]. In contrast to the study with loss-of-function by gene targeting, the enforced expression of the active form of

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Notch1, ICN1 in HPC, showed the ectopic appearance of CD4 and CD8 double-positive cells and the inhibition of B cell development in BM [6]. Moreover, there are *in vitro* studies showing that if HPC receiving Notch signaling by ICN or Notch ligand-expressing BM-derived stromal cells are cultured, they are forced to T cell lineage despite the fact that the culture system is specific for B cell induction [7–9]. These findings clearly indicated that Notch1-mediated signals are indispensable for T cell specification and exclude B cell lineage at the branching point of T versus B cells, and that Notch signaling may serve as a substitute for the environmental function of the thymus.

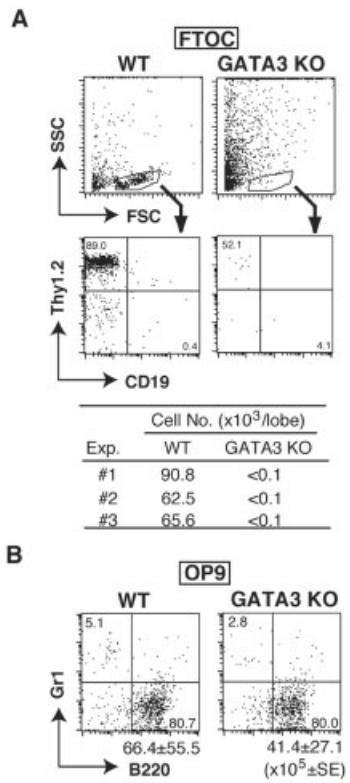
A phenotype similar to that in Notch1-deficient mice has been reported in the GATA3-defective condition; no T cells, but intact B cells were observed in chimeric mice with RAG2-deficient embryo and GATA3-null ES cells [10]. This means that GATA3, as well as Notch1, specifically contributes to the initiation of T cell development at the branching point. GATA3 is the zinc finger transcription factor and is expressed in hematopoietic cells and the nervous system [11]. Among hematopoietic cells, GATA3 expression is limited to T cells including thymocytes, with same way as Pax5 expression is limited to B cells. GATA3 has DNA-binding sites in a number of T cell-specific genes [12, 13], including regulatory regions of TCR α, β, γ and δ, and CD8 α. However, it is still unclear whether GATA3 and Notch signaling are involved in the determination of T cell fate in a cooperative or in a parallel manner. Loss-of-function studies did not answer these questions, although both are actually required for T cell development.

For addressing this issue, the *in vitro* induction system for T cells is ideal, because it allows manipulation of Notch signaling and GATA3 expression in the developmental stages. Past studies showed that, while GATA3 is certainly necessary for T cell development, its overexpression seems to impair T cell development [14, 15]. Thus, in this study, we used GATA3-deficient mice [16] and introduced the *GATA3* gene into their HPC to determine how GATA3 functions in T cell development. The results demonstrated that introduced GATA3 rescues T lymphopoiesis from GATA3-deficient HPC receiving Notch signaling, which is detectable during organogenesis of fetal liver (FL). In contrast, B lymphopoiesis is inducible in GATA3-deficient HPC but is blocked by Notch signaling independently of GATA3. These results suggest that Notch signaling functions in a different manner at the branching point for regulating T and B cell inductions.

Results

GATA3-deficient HPC exhibit potential to differentiate into B cells but not T cells *in vitro*

A previous study using chimeric mice with GATA3$^{-/-}$ ES cells showed that GATA3 is indispensable for T cell development but not for B cells [10], but it remained unclear as to how GATA3 is involved in determining T cell fate. To address this issue, we used the *in vitro* induction system for T and B cell lineages from GATA3-deficient HPC. Since GATA3-deficient mice are known to be embryonically lethal on day 12.5 of gestation (E12.5) [16], we prepared FL cells from E11.5 embryos for fetal thymus organ culture (FTOC) or OP9 stromal cell monolayer culture [8, 17] for the induction analysis of T or B cell lineages (Fig. 1). In FTOC, most wild type (WT) FL cells developed into Thy-1$^+$ T cells, which could be distinguished from a few NK cells with low density of Thy-1. These were associated with relatively high cell proliferation (Fig. 1A, left panel), and several of these Thy-1$^+$ cells expressed CD4/CD8 or CD25 but not NK1.1 (data not shown). In contrast to WT cells, most FL cells from GATA3-deficient mouse appeared to have died during FTOC because the FSC/SSC FACS profile showed abundant small cell debris but few cells in the lymphoid gate (encircled area in Fig. 1A, right panel). In OP9 stromal cell monolayer culture, which is known as a B cell induction system, GATA3-deficient FL cells developed into B cells as well as WT FL cells (Fig. 1B), indicating that GATA3-deficient FL cells retain the potential for becoming B cells *in vitro*. Thus, FTOC of GATA3-deficient FL cells is a useful tool for analyzing the ability of GATA3 to induce T cell development by manipulation of its expression.



Exp.	Cell No. (x10^3/lobe) WT	GATA3 KO
#1	90.8	<0.1
#2	62.5	<0.1
#3	65.6	<0.1

Figure 1. GATA3-deficient FL cells differentiate into B cells but not T cells *in vitro*. FL cells as HPC were prepared from E11.5 wild type (WT) or GATA3-deficient (GATA3 KO) embryos, and cultured in FTOC for 13 days (A) or on a monolayer of OP9 stromal cells with IL-7 (10 ng/ml) for 7 days (B). After FTOC, collected cells were analyzed for FSC and SSC to determine the gate for live lymphocytes, and their expression of Thy1.2 and CD19 are shown with the gate (A). Alternatively, live cells from the cultures on OP9 stromal cells were analyzed for expression of B220 and Gr1 (B). Numbers in the table or under the dot-plots represent the cell number (× 10^3/lobe in A, × 10^5 ± SE in B) obtained from the cultures of three independent experiments. Numbers in the dot-plots represent the relative percentages for each corresponding quadrant.

Introduction of GATA3 into GATA3-deficient FL cells does not rescue impairment of T cell development

Next, we examined whether exogenous GATA3 could rescue impairment of T cell development of GATA3-deficient FL cells if they were cultured in FTOC. To introduce certain genes into FL cells, we chose the retrovirus-mediated gene-transfection system and monitored the transfected cells by the expression of GFP as described in *Materials and methods*. Surprisingly, the introduction of GATA3 into E11.5 GATA3-deficient FL cells did not induce T cell development; Thy-1$^+$ cells were almost undetectable in FTOC (Fig. 2A, "GATA3 KO", upper right panel). However, when an active form of Notch1, the intracellular region of Notch1 (ICN1), was simultaneously introduced with GATA3 by double infection of retroviruses, a high proportion of Thy-1$^+$ cells became detectable from GATA3-deficient FL cells (Fig. 2A, "GATA3 KO", lower right panel). These cells were in the T cell lineage because they expressed CD4/CD8 or CD25 but not NK1.1 (Fig. 2B). To confirm whether both genes were actually transfected into a cell, GATA3-introduced cells were distinguished by using the retrovirus vector with a distinct marker, human nerve growth factor receptor (hNGFR), in addition to GFP (Fig. 2C). Thy-1$^+$ cells derived from GATA3-deficient FL cells gated out by FACS expressed both GFP (ICN1) and hNGFR (GATA3) (Fig. 2C, left panel). At the same time, we confirmed that the appearance of Thy-1$^+$ cells was not detected when two populations of GATA3- and ICN1-transfected cells were simply mixed (data not shown). We also showed that introduction of ICN1 alone was not able to induce T cell development without GATA3 (Fig. 2A, "GATA3 KO", lower left panel), although ICN1 can induce T lymphopoiesis in WT FL cells on a monolayer culture of OP9 stromal cells [8]. These results indicated that only progenitors expressing both GATA3 and ICN1 could develop into T cells in GATA3-deficient FL cells.

In WT FL cells, GATA3-introduction generated almost no Thy-1$^+$ cells (Fig. 2A, "WT", upper right panel). Although GATA3-transfected cells reached 27.4% immediately after the gene was introduced, these GFP$^+$ cells disappeared over 13 days after FTOC, resulting in a majority (>99.5%) of exogenous GFP$^-$ cells that expressed Thy-1 (data not shown). Monitoring dual markers for transgenes in the transfected cells showed that when Thy-1$^+$ cells were gated, the majority of cells derived from WT FL cells were composed of ICN1-single transfected (GFP$^+$, hNGFR$^-$; 52%) and non-transfected cells (GFP$^-$, hNGFR$^-$; 40%) (Fig. 2C, right panel). Moreover, the density of hNGFR on double-expressing cells derived from WT FL cells was lower than that on cells derived from GATA3-deficient FL cells (Fig. 2C). Thus, it was suggested that WT E11.5 FL cells with endogenous GATA3 require very limited exogenous GATA3 to become Thy-1$^+$ cells, but when the total amount of endogenous and exogenous GATA3 is excessive, T cell development from HPC is inhibited if exogenous ICN1 is present.

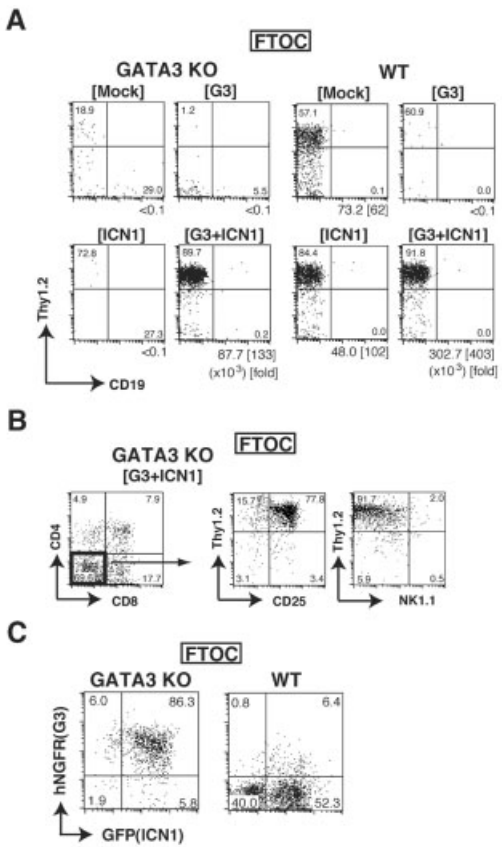


Figure 2. Simultaneous introduction of *ICN1* and *GATA3* genes can rescue T cell development of GATA3-deficient FL cells. (A) WT or GATA3-deficient E11.5 FL cells, infected with the retroviruses shown at the top of the profiles (G3, GATA3), were cultured in FTOC and analyzed for the expression of Thy1.2 and CD19. The FACS profiles shown have been gated to detect only GFP$^+$ CD45.2$^+$ cells, *i.e.* retrovirus-infected, FL-derived cells (See *Materials and methods*). Numbers under the dot-plots represent the cell number of GFP$^+$ cells ($\times$ 10^3/lobe) obtained from the cultures and the relative increase of cellularity during the culture in square brackets. (B) After the culture of GATA3-deficient FL cells with GATA3 and ICN1, GFP$^+$ cells were further analyzed for the expression of CD4, CD8, CD25 or NK1.1. FACS profile of CD4 and CD8 within GFP$^+$ cells (left panel), and that of CD25 or NK1.1 with Thy1.2 within GFP$^+$CD4$^-$CD8$^-$ cells (right panels) are shown. (C) Alternatively, the GATA3 gene was transfected into WT or GATA3-deficient FL cells by another vector containing hNGFR to identify the transfected cells as described in the *Materials and methods*. The mixture of retroviruses (one encoded ICN1 with GFP and the other GATA3 with hNGFR) was used as shown in (A). FACS profiles are shown with the gate (Thy1.2$^+$CD45.2$^+$) as FL-derived T cells. Numbers in the dot-plots represent the relative percentages for each corresponding quadrant.

GATA3 function for T cell development is effective after organ culture of E11.5 FL

As described above, impairment of T cell development in GATA3-deficient FL cells of E11.5 was rescued only by introduction of both GATA3 and ICN1 but not by either GATA3 or ICN1 alone. This led to the assumption that E11.5 FL cells do not become T cell lineage presumably because they are too immature to generate efficient Notch signaling for GATA3 to induce T cell development. To test

this assumption, FL organ culture (FLOC) of GATA3-deficient E11.5 embryos was performed to advance the organ developmental stage (Fig. 3A). During 2-day FLOC, FL cells seemed to maintain the differentiation potential of HPC because WT FL cells obtained after organ culture differentiated into T cells in FTOC (Fig. 3B, left panel). After organ culture, GATA3-deficient FL cells were transfected with GATA3 alone or with both GATA3 and ICN1, and then submitted to FTOC. As shown in Fig. 3B, 2-day-cultured GATA3-deficient FL cells developed into Thy-1$^+$ cells in FTOC by GATA3 introduction alone, although the efficiency seemed to be lower than that of FL cells transfected with both GATA3 and ICN1. This suggests that 2-day FL organ culture may induce immature E11.5 FL cells to a more advanced stage where GATA3 is able to function efficiently for T cell induction under newly generated Notch signaling.

To further confirm whether 2-day FLOC actually contributes to generating Notch signaling required for T cell development, we added a γ-secretase inhibitor (GSI), which blocks the cleavage of Notch receptors for generating ICN1, into the organ culture of E11.5 FL. After FLOC with GSI, the isolated FL cells were submitted into FTOC. As seen in Fig. 3C, the effect of organ culture on T cell development of GATA3-deficient FL cells by GATA3 introduction was completely abrogated; this phenotype was similar to that of GATA3-deficient FL cells without FLOC (Fig. 2A, "GATA3 KO").

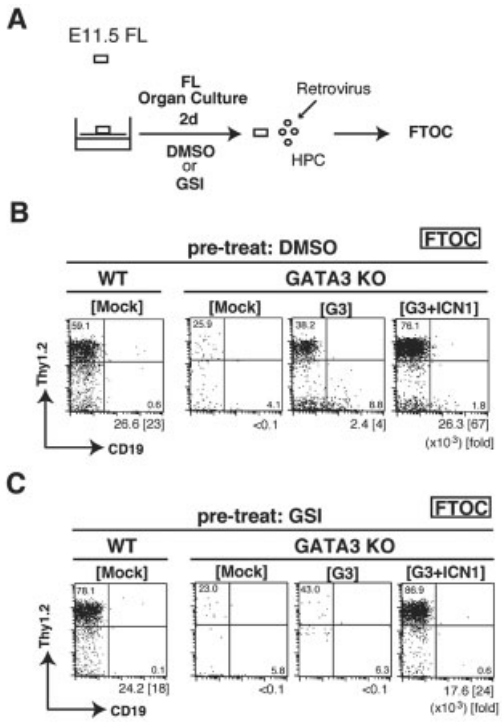


Figure 3. GATA3-deficient FL cells advance the competent stage for GATA3 during FLOC by Notch signaling. (A) Experimental procedure for FLOC and gene transfection. FL obtained from E11.5 WT or GATA3-deficient embryos were cultured on the filter for 2 days in the presence or absence of GSI. FL cells were harvested, infected with the retrovirus and cultured in FTOC for 13 days. (B, C) After FLOC with (pre-treat: GSI, (C) or without (pre-treat: DMSO, (B) GSI for 2 days, the cells were infected with the retroviruses encoding the genes shown above the profiles and cultured in FTOC. Live cells were harvested and analyzed for the expression of CD19 and Thy1.2 with GFP$^+$CD45.2$^+$ gate as described in Fig. 2. Numbers under the profiles represent the cell number of GFP$^+$ cells obtained from FTOC ($\times$ 10^3/lobe) and the relative increase of cellularity during the culture in square brackets.

Notch signaling is detectable in FL after day 12.5 of gestation

In order to confirm that Notch signaling is substantially generated in FL after E11.5, we investigated the cleaved Notch1 fragment in WT FL cells. Lineage marker-negative, c-kit-positive FL (LK-FL) cells were fixed and stained with anti-cleaved Notch1 Ab for detecting the intracellular fragment. Cleaved Notch1 fragments were found in LK-FL cells after E12.5 but rarely at E11.5 (Fig. 4A and B). This means that Notch1 protein is enzymatically cleaved by interaction with its ligand in the FL environment at about E12.5. To ascertain the appearance of Notch signaling during organ culture of E11.5 FL, we also examined the presence of cleaved Notch1 in LK-FL cells obtained from organ cultured FL. The frequency of cells expressing cleaved Notch1 significantly increased after FLOC for 1 or 2 days (Fig. 4C), although it was lower than that found *in vivo*. Moreover, the addition of GSI to FLOC suppressed the appearance of cleaved Notch1 after culture (Fig. 4C). These findings suggested that Notch signaling, which is critical for GATA3 function to induce T cell development, is generated during organogenesis of FL and organ culture of E11.5 FL.

In addition to the cleaved Notch1 fragments, LK-FL cells were submitted to quantitative RT-PCR to check the Notch targeting genes [18]. *Hes1* gene is known to be a target of Notch signaling and a useful marker for tracing Notch signaling. Significant transcription of the *Hes1* gene was basically absent at E11.5 but was found after E12.5 (Fig. 4D). This also indicated that Notch signaling becomes effective at a particular stage of the organogenesis of FL. In contrast to cleaved Notch1, post organ culture of E11.5 FL did not show any significant increase in Hes1 transcript (data not shown). Hes1 transcription may be delayed coincident with the lower level of cleaved Notch1 in the cultured FL cells in comparison with the *in vivo* level as described in Fig. 4C. Although at the moment, the reason for the delayed expression of Hes1 transcript in FL culture is not clear, it is possible to assume that transcription of the *Hes1* gene may not always be critical for T cell induction. In fact, we found that enforced expression of *Hes1* gene, instead of ICN1, could not induce T lymphopoiesis on the monolayer culture (data not shown). Weak transcripts of other Hes family members such as Hes5 and Hey1 were also detected but were not substantially altered during organogenesis, and Hey2 transcript was not observed (data not shown).

Consistent with the appearance of cleaved Notch1 or Hes1 transcript, one of the Notch ligands, Jagged1, was detected in E12.5 FL, especially on the cells not expressing cytokeratin, while little Jagged1 expression was detected at the E11.5 stage (Fig. 5A). Moreover, cells expressing Jagged1 were stained with anti-PDGFR mAb (Fig. 5B). The cytokeratin-positive cells seemed to be

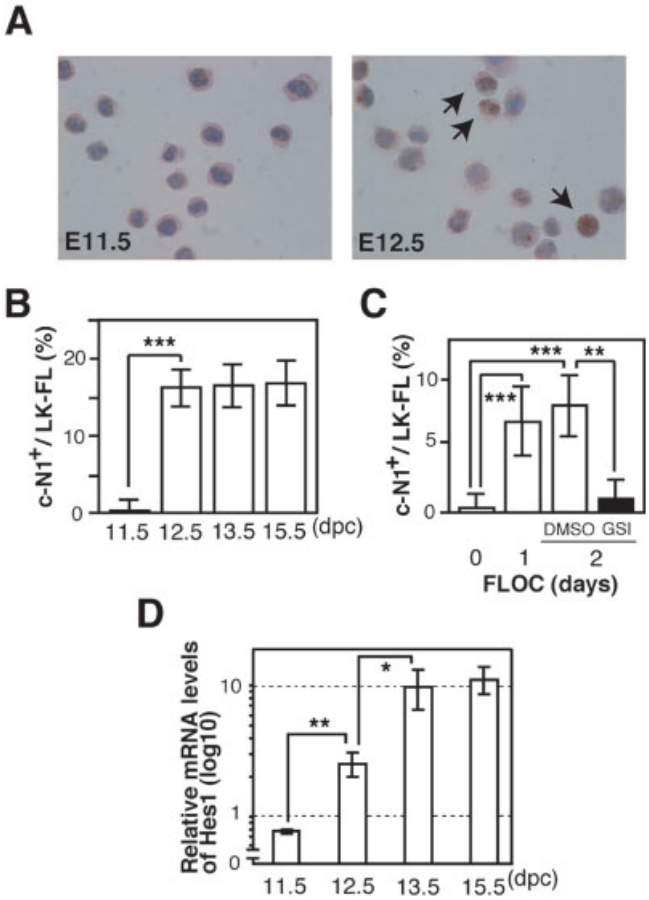


Figure 4. Notch signaling occurs during organogenesis of FL. (A, B) The cleaved Notch1 fragment was found in LK-FL cells. Cells prepared from embryos at various stages of gestation were fixed and stained with anti-cleaved Notch1 Ab. The cells with cleaved Notch1 in the nucleus (brown) are depicted by arrows (A). Frequencies of cells with cleaved Notch1 (c-N1$^+$) (percentage ± SE from five fields in a slide with more than 100 cells) were counted (B). (C) FL of E11.5 embryos were cultured for 1 or 2 days as FLOC in the presence (filled) or absence (open) of GSI. LK-FL cells were prepared after the organ cultures, fixed, and stained with anti-cleaved Notch1 Ab. Frequencies of cells with cleaved Notch1 were counted. (D) Transcripts of *Hes1* gene in LK-FL cells from embryos at various stages of gestation were detected by real-time RT-PCR as described in *Materials and methods*. Representative relative mRNA levels of *Hes1* gene (relative gene expression ± SE averaged from three independent experiments) were calculated from the ratio of Hes1 transcripts compared with 18S ribosomal RNA. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

hepatoblasts, precursors of both hepatocytes and cholangiocytes, because they also expressed E-cadherin (data not shown). These findings indicated that Jagged1 found after E12.5 in FL is expressed on mesenchymal cells.

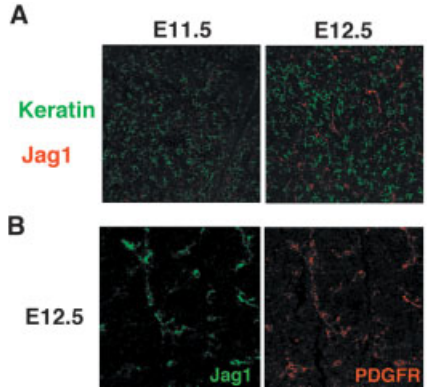


Figure 5. Expression of Jagged1 is increased on E12.SFL compared to E11.5 FL. (A) Tissue sections prepared from E11.5 or E12.5 FL were stained with anti-Jagged1 (red) and anti-cytokeratin (green) Abs, and analyzed by confocal laser microscopy. (B) The section from E12.5 FL was stained with anti-Jagged1 (green) and anti-PDGFR (red) Ab, and analyzed as in (A).

GATA3 is not required for inhibition of B cell, but is necessary for the induction of T cell, development by Notch signaling

As shown in Fig. 2, Notch signaling alone could not lead to T lymphopoiesis in FTOC without GATA3. To confirm the involvement of GATA3 in the determination of B cell fate at the branching point of T/B cells, whether Notch signaling abrogates the appearance of B lineage cells on the monolayer culture of OP9 stromal cells in the absence of GATA3 was examined. GATA3-deficient FL cells differentiated into B cells on OP9 stromal cells (Fig. 6A, "GATA3 KO", upper left panel and left bar graph), but the introduction of ICN1 completely blocked B cell development ("GATA3 KO", lower left panel and left bar graph), which was the same as that seen with WT FL cells ("WT", lower left panel and right bar graph). This indicated that GATA3 is not required for inhibition of B cell development by Notch signaling. This is in clear contrast to the appearance of T lineage cells, which expressed Thy-1 with CD25 but not NK1.1 (Fig. 6B), requiring the presence of both GATA3 and Notch signaling (Fig. 6A, "GATA3 KO", lower right panel and left bar graph).

Discussion

To understand the role of Notch signaling in T cell fate, past studies regarding gain-of-function have examined the effect of enforced expression of ICN1 [6, 8] or cultures of stromal cells expressing Notch ligands [2, 7, 9]. All of them showed that Notch signaling might be sufficient for T cell fate specification in HPC even under conditions of induction of B cell development. In these studies, however, it was not determined whether Notch signaling actually induces T cell lineage from HPC in cooperation with GATA3. Here, we used E11.5 FL cells from GATA3-deficient mice and introduced ICN1 into these FL cells. The results showed that impairment of

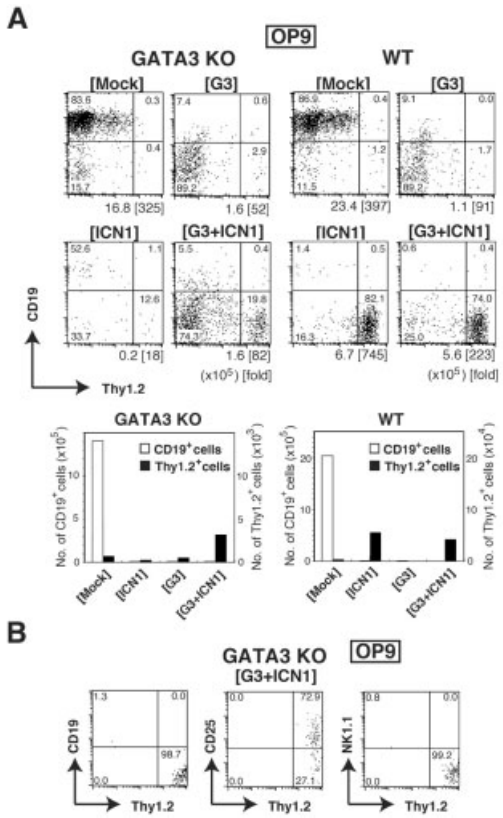


Figure 6. Active Notch signaling inhibited B cell development without GATA3. (A) WT or GATA3-deficient E11.5 FL cells were infected with the retrovirus encoding the genes shown above the profiles and cultured on OP9 stromal cells with IL-7 for 7 days. Live cells were harvested and analyzed for the expression of CD19 and Thy1.2 with GFP$^+$ CD45.2$^+$ gate as described in Fig. 2. Numbers under the FACS profile indicate the number of GFP$^+$ cells obtained from the culture ($\times$ 10^5) and the relative increase of cellularity during the culture in square brackets. The absolute cell numbers of GFP$^+$CD19$^+$Thy1.2$^-$ (CD19$^+$ cells, plain bar) and GFP$^+$CD19$^-$Thy1.2$^+$ (Thy1.2$^+$ cells, filled bar) fractions obtained from the cultures are represented in the bar graphs. (B) GATA3 gene was transfected into GATA3-deficient FL cells by another vector containing hNGFR as shown in Fig. 2C. After culture on OP9 stromal cells, double-transfected cells with GATA3 and ICN1 were identified as GFP$^+$NGFR$^+$ cells, and the expression of CD19, Thy1.2, CD25 or NK1.1 on the double-transfected cells was analyzed.

T cell development in GATA3-deficient FL cells was not rescued by ICN1 introduction but was clearly rescued when both GATA3 and ICN1 were simultaneously introduced into the same FL cells. These *in vitro* studies first indicated that Notch signaling is not sufficient for determining T cell lineage in HPC in the absence of GATA3 even if it is provided exogenously in ICN1 form.

At the same time, it is noteworthy that introduction of the GATA3 gene alone was also not effective in T cell development from GATA3-deficient E11.5 FL cells but was sufficient with the addition of ICN1. From this finding, it can be assumed that introduction of GATA3 does not contribute to T cell development from GATA3-deficient E11.5 FL presumably because the cells are still immature, and this may be improved if Notch signaling appears at a more advanced stage. This assumption was supported by evidence. The levels of cleaved Notch1 fragment and gene expression of Hes1, a downstream target of Notch signaling, were extremely low in LK-FL cells of E11.5 WT embryos, but increased after E12.5. Moreover, if E11.5 FL underwent FLOC, E11.5 derived LK-FL cells obtained from post 2-day organ culture clearly showed cleaved Notch1 fragment, which disappeared in the presence of a GSI. In GATA3-deficient mice, FL cells obtained from a similar organ culture acquired the potential to develop into T cells in the presence of exogenous GATA3 alone, but this was also completely abrogated by adding GSI during the organ culture. Thus, it is suggested that Notch signaling generated during 2-day organ culture or post E12.5 contributes to the GATA3 function of T cell fate specification in HPC. In *in vivo* development, GATA3 expression in FL cells is first detectable at E12.5 [19, 20], which is about the same as the developmental day on which Notch signaling was detected (Fig. 4), although the amount of GATA3 transcripts was much less than that in thymocytes (data not shown). Accordingly, when E11.5 FL cells are cocultured with the thymic lobe (FTOC), WT FL cells may receive Notch signaling via Notch ligands expressed in the thymic lobe prior to relative GATA3 expression. Conversely, if E11.5 FL cells with exogenous GATA3 introduced are applied to FTOC, both WT and GATA3-deficient FL cells thereafter receive Notch signaling. Taken together, it is likely that Notch signaling and/or the consequently induced intracellular events are involved in T cell fate specification before or simultaneously, but not after GATA3 appears *in vivo*.

In spite of the fact that GATA3 is indispensable for T cell development, enforced expression of GATA3 in HPC suppressed differentiation to a T cell lineage in FTOC as well as to a B cell lineage on the monolayer culture of OP9 stromal cells (Fig. 2 and 6). As Anderson *et al.* [14] indicated, GATA3 might operate within a narrowly defined window in terms of the effective amount and stage for inducing T cell development. In the present study, we showed that WT FL cells (E11.5) expressing only a small amount of exogenous GATA3 develop T cell lineage with ICN1. This suggests that the proper window of GATA3 expression may be restricted even in the presence of Notch signaling. In the context of the effect of GATA3 on T cell fate determination, another interpretation is possible based on a recent report in which GATA3-overexpressed HPC derived from adult bone marrow skews to differentiate into erythroid/megakaryocytic cells, resulting in differentiation blockage of other lineages including T and B cells [15]. Interestingly, a recent report indicated that GATA1-mediated differentiation to erythroid/megakaryocytic lineage is inhibited by Notch signaling [21]. Therefore, if GATA3 is assumed to possess a function similar to GATA1 in lineage choice of erythroid/megakaryocytic cells [22, 23], the presence of Notch signaling may suppress differentiation of erythroid/megakaryocytic cells induced by GATA1 and/or GATA3, maintaining the possibility of T cell development. Very recently, it has been reported that over-expression of GATA3 induces respecification to the mast cell lineage from HPC or thymocytes, which is inhibited by Notch signaling [24]. These results are basically consistent with our results. On the other hand, it has been reported that Notch signaling directly regulates GATA3 expression during Th2 cell differentiation via binding of RBP-J on the distal promoter [25] of the *GATA3* locus [26, 27], suggesting

that Notch signaling is necessary not only for GATA3 function but also for its induction in the early stage of T cell development.

In various developmental pathways, Notch signaling has been suggested to regulate the developmental progression of undifferentiated cells toward a certain advanced stage and to be involved in the cell fate decision at the developmental branching point. However, it is still unclear whether Notch signaling blocks the primary cell fate of development from undifferentiated cells, leading to the secondary fate, or whether secondary cell development is selected directly. In the central nervous system, for instance, it has been demonstrated that Notch signaling is essential for the inhibition of neural differentiation and for promotion of gliogenesis [28], but it has remained unclear whether these phenomena were induced by identical machinery or not. In the present study, we clearly demonstrated that ectopic expression of ICN1 without GATA3 is sufficient for blocking B cell development (Fig. 6), in contrast to T cell development for which both Notch signaling and GATA3 are required. These results demonstrate that T cell fate is not determined simply as the secondary fate by the blocking of B cell development by Notch signaling in a GATA3-independent manner. In addition to B cell and erythroid/megakaryocytic lineages, the differentiation of HPC to $Gr1^+$ myeloid cells on OP9 stromal cells was also blocked by Notch signaling (data not shown). Taken together, it is suggested that Notch signaling preferentially contributes to the induction of T cell lineage and to actively suppressing differentiation to other lineages.

Associated with the appearance of cleaved Notch1 fragment and *Hes1* gene expression in LK-FL cells, Jagged1 protein was detectable on non-hematopoietic FL cells expressing PDGFR, a marker of mesenchymal cells, after E12.5. This stage-consistent expression of Notch-related molecules suggests that Jagged1 in FL may aid in Notch signaling as a Notch1 ligand. In fact, expression of Jagged1 was observed in the late stage (E18.5-neonate) of FL in mesenchymal cells around the portal vein, and it contributes to cell fate decision of cholangiocytes from hepatoblasts expressing Notch2 [29, 30]. On the other hand, Dll family members, Dll1 and Dll4, induced sufficient Notch signaling for T cell lineage *in vitro*. Among these Dll family members, Dll4 protein, but not Dll1, was detectable on $CD31^+$ endothelial cells through the early stage of FL, even at E11.5 (data not shown). Since this is incompatible with the few cleaved Notch1 fragments and transcripts of *Hes1* gene in HPC at E11.5, Dll4 may contribute little to Notch signaling induced in FL at this stage. This is consistent with the fact that FL is not the microenvironment for T cell development. Since there are many regulatory systems for Notch signaling and probably Notch ligands at the post-translational level, further investigation will be necessary to determine the Notch ligand that actually provides Notch signaling in FL. Understanding how the determination of T cell fate is regulated is a highly significant goal.

Materials and methods

Retroviral constructs and transduction of HPC

The retrovirus vectors MIGR1, MIG ICN1 and GCDNSamI were kindly donated by Dr. W. Pear (University of Pennsylvania Medical Center, Philadelphia, PA) and Dr. M. Onodera (University of Tsukuba, Tsukuba, Japan). GATA3 cDNA was inserted into the EcoRI site of MIGR1 or NotI/XhoI site of GCDNSamI. These vectors were transfected transiently into PLAT-E cells [31], and the culture supernatants were harvested as the source for the retrovirus. Whole FL cells obtained from E11.5 WT or GATA3-deficient [16] embryos were infected with the above retrovirus as described previously [8]. Mitomycin C-treated bone marrow cells from C57BL/6 congeneic mice (CD45.1) were used as carrier cells during the infection process, because they might be ignored by staining with anti-CD45.2 mAb in flow cytometric analysis. Total cells (mixture of infected and non-infected cells) were cultured and the gene-transfected cells were monitored by the expression of GFP (MIGR1) or human NGFR (GCDNSamI). The efficiency of the infection was examined by expression of the above markers following 3-day suspension cultures with stem cell factor (SCF) (100 ng/mL) (Peprotech, London, UK). All mice were maintained in specific pathogen-free conditions and all mouse experiments were approved by the Animal Experimentation Committee, Isehara Campus (Tokai University, Kanagawa, Japan).

In vitro cultures with stromal cells

E11.5 FL cells (10^4) after gene-transfection were seeded on monolayer of OP9 stromal cells [8] in 6-well culture plates (Corning, Corning, NY) with IL-7 (10 ng/mL) (Peprotech) for 1 week. After the culture, growing cells were harvested and analyzed by FACS Calibur (BD Biosciences, San Jose, CA) with the gate of GFP^+ and/or $hNGFR^+$ as the gene-transfected cells. The relative increase of the gene-transfected cells was calculated as a ratio of the numbers of input and harvested cells.

Organ cultures

Fetal thymic lobes were cultured with 1.35 mM 2-deoxyguanosine (dGuo) for 5–6 days and then rinsed with medium without dGuo. FL cells after gene-transfection were aliquotted at 2000/well in Terasaki plates (Sumitomo Bakelite, Tokyo, Japan), and one dGuo-treated lobe per well was added. The cells and lobes were incubated as hanging drop cultures with IL-7 (10 ng/mL). After 48 h, the lobes were removed, rinsed and cultured on insert filters in 24-well plates for 13 days with IL-7 as FTOC. After FTOC, the growing cells were harvested and analyzed with the gate of GFP^+ and/or $hNGFR^+$ as gene-transfected cells. The relative increase of the gene-transfected cells was calculated as the ratio of the

numbers of input and harvested cells. In some experiments, FL obtained from E11.5 WT or GATA3-deficient embryos were cultured on the filters in the presence or absence of GSI (10 μM) (IX, EMB Biosciences, San Diego, CA) for 2 days as FLOC. After the culture, WT or GATA3-deficient FL cells were infected with the retroviruses and then submitted to FTOC.

mAb and flow cytometry

FITC-conjugated and biotinylated TER119, Mac-1 (M1/70), Gr-1 (RB6–8C5), CD19 (MB19–1); PE-conjugated c-kit (2B8), Thy1.2 (53–2.1), B220 (RA3–6B2), CD4 (GK1.5), CD8 (53–6.7); APC-conjugated CD19, CD8, Thy1.2, Cy-5-conjugated Gr-1 and biotinylated CD45.2 (104), CD25 (PC61.5), NK1.1 (PK136) mAb were purchased from eBioscience (Mountain View, CA). PE-conjugated hNGFR (C40–1457), PerCP-Cy5.5-conjugated CD19 mAb and streptoavidin-conjugated PerCP-Cy5.5 were purchased from BD Biosciences. Flow cytometric analysis was carried out as described previously [32]. The gene-transfected FL-derived cells were identified by the expression of GFP or hNGFR and/or CD45.2. Cell sorting was carried out by a JSAN automatic cell sorter (Bay Bioscience, Kobe, Japan).

Real-time PCR

mRNA from sorted LK-FL cells (>98%) was isolated by RNeasy mini kit (Qiagen, Hilden, Germany), and transcribed with random hexamer and Ominiscript reverse transcriptase (Qiagen). In real-time PCR analysis, Hes1 mRNA and 18S rRNA were detected by using primers purchased from Applied Biosystems (Foster City, CA) as TaqMan® Gene Expression Assays. Real-time PCR was carried out by ABI 7700 with diluted cDNA (two times, four points), and the relative mRNA level of the *Hes1* gene was calculated from the Ct value obtained by the comparison of amplified DNA with different primers (Hes1 and 18S) from each sample.

Immunohistochemistry

FL of E11.5 or E12.5 embryos were embedded in OCT compound (Sakura Finechemical, Tokyo, Japan). Cryostat sections (6–10 μm) were cut and fixed in PBS containing 4% PFA at 4°C for 10 min. After sections were blocked with 5% normal goat serum, they were incubated overnight at 4°C with biotinylated goat anti-rat Jagged1 (R&D Systems, Minneapolis, MN) and FITC-conjugated mouse anti-pan cytokeratin Ab (Sigma-Aldrich, St. Louis, MO) or rat anti-mouse CD140α mAb (PDGF Receptor, BD Biosciences). The sections were then incubated in the ABC kit mixture (Vector Laboratories, Burlingame, CA) according to the manufacturer's directions, and visualized using a CSA system (DAKO, Carpinteria, CA). For double staining, sections were also incubated in a mixture containing the following secondary Ab for 1 h: Alexa fluor 488- or 594-conjugated donkey anti-rat IgG or streptoavidin (Molecular Probes, Eugene, OR). The samples were analyzed with a laser-scanning confocal imaging system (LSM 510 Meta, Carl Zeiss Japan, Tokyo, Japan).

For cytospin samples, purified LK-FL cells (5×10^4) were attached to MAS-coated glass slides (Matsunami Glass, Osaka, Japan) by centrifugation. Slides were fixed in PBS containing 4% PFA at 4°C for 10 min. Antigen retrieval was accomplished by autoclave treatment in Target Retrieval Solution (DAKO) at 105°C for 5 min [33]. These were stained with rabbit anti-cleaved murine Notch1 Ab (Cell Signaling Technology, Beverly, MA) or control rabbit IgG (DAKO). The signals were visualized with ABC kit mixture and DAB (Sigma). Five random fields were digitally photographed and printed to count the number of cells with or without cleaved Notch1. The percentage of cleaved Notch1-positive cells was calculated as follows: (No. of stained cells with anti-cleaved Notch1 – No. of stained cells with control rabbit IgG/ total No. of cells counted × 100).

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Abbreviations: **E:** day of gestation · **GSI:** γ-secretase inhibitor · **FL:** fetal liver · **FLOC:** FL organ culture · **FTOC:** fetal thymus organ culture · **hNGFR:** human nerve growth factor receptor · **HPC:** hematopoietic progenitor cells · **ICN:** intracellular region of Notch · **LK-FL:** lineage marker-negative c-kit-positive FL

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