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Expression pattern of Notch1, 2 and 3 and Jagged1 and 2 in lymphoid and stromal thymus components: distinct ligand–receptor interactions in intrathymic T cell development

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Abstract

The suggested role of Notch1 or its mutants in thymocyte differentiation and T cell tumorigenesis raises the question of how the different members of the Notch family influence distinct steps in T cell development and the role played by Notch ligands in the thymus. We report here that different Notch receptor–ligand partnerships may occur inside the thymus, as we observed differential expression of Notch1, 2 and 3 receptors, their ligands Jagged1 and 2, and downstream intracellular effectors *hairy* and *Enhancer of Split* homolog 1 (HES-1) and *hairy* and *Enhancer of Split* homolog 5 (HES-5), depending on ontogenetic stage and thymic cell populations. Indeed, while Jagged2 is expressed in both stromal cells and thymocytes, Jagged1 expression is restricted to stromal cells. Moreover, a differential distribution of Notch3, with respect to Notch1, was observed in distinct age-related thymocyte subsets. Finally, Notch3 was preferentially up-regulated in thymocytes, following the induction of their differentiation by interaction with thymic epithelial cells expressing the cognate Jagged1 and 2 ligands, suggesting that, besides Notch1, Notch3 may also be involved in distinct steps of thymocyte development. Our results suggest that the Notch signaling pathway is involved in a complex interplay of T cell developmental stages, as a consequence of the heterogeneity and specific expression of members of the Notch receptor family and their cognate ligands, in distinct thymic cell compartments.

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Introduction

Most of the events that lead T cells to phenotypic and functional differentiation occur in the thymus. T cell precursors enter the thymus and, through a coordinate series of events, including a cascade of proliferation and differentiation phases, acquire the ability to recognize and react to antigenic stimuli (1).

The complex lympho-stromal structure of the thymus and the close association of thymocytes with stromal cells highlight a role for specific intrathymic cell–cell interactions in T cell development. Several reports demonstrate that complex and direct interactions between developing thymocytes and thymic stromal cells promote the survival and proliferation of thymocytes, and also direct their progression from immature $CD4^-CD8^-$ double-negative (DN) towards $CD4^+CD8^+$ double-positive (DP) phenotype and their $CD4^+CD8^-$ versus $CD4^-CD8^+$ single-positive (SP) lineage choice (reviewed in 2,3).

Notch is a transmembrane receptor containing an extracellular domain carrying EGF-like repeats, and is known to mediate cell interactions between adjacent cells and to regulate diverse cell-fate decisions during development in both invertebrate and vertebrate tissues (4–6). In a number of developmental models, the Notch1 signaling pathway controls the ability of equivalent non-terminally differentiated cells to respond to differentiation signals, by choosing between primary and secondary cell fates or keeping as uncommitted precursors, according to the lateral specification model (for review see 7). In other systems, Notch1 is involved in signaling among non-equivalent cells (in terms of cell properties and differentiation status, including the repertoires of receptors and ligands) to promote differentiation (8). In mammals four distinct Notch genes have been cloned (Notch1–4) (9–13). Notch receptor-specific ligands (Jagged1 and 2 as well as *Delta1* and *3*) have also been identified in vertebrates (14–19).

It has been recently reported that the Notch1 receptor may play a role in T cell lineage decision, since Notch1 has been detected in DN thymocytes (20), and its deregulation has been suggested to control the choice between either the $\alpha\beta$ versus $\gamma\delta$ and the $CD8^+$ SP versus $CD4^+$ SP thymocyte lineages (21,22).

The suggested role of Notch1 receptor in lymphocyte differentiation raises the question of how the different members of the Notch family may influence distinct stages in T cell development. Moreover, the expression and tissue partitioning of the different Notch ligands within the thymus has yet to be elucidated. This has prompted us to investigate the spatio-temporal expression of these genes in the different lymphoid and non-lymphoid cellular components of murine thymus, in order to study a putative involvement of the different members of the Notch receptor family and their specific ligands in lympho-stromal interactions that support T cell development. Furthermore, we have examined modulation of Notch receptors and Notch ligand expression following lympho-stromal cell–cell interaction, in an experimental model of thymic epithelial cell-driven thymocyte maturation *in vitro* (23). We report that differential Notch receptor–ligand partnerships may occur in the lymphoid and stromal compartments of the thymus, and suggest that, in addition to Notch1, Notch3 may also be involved in distinct stages of thymocyte development.

Methods

Animals

C57BL/6 (B6) mice (Charles River, Calco, Italy) were used at different stages of embryonic and post-natal life. The age of embryos was determined according to the vaginal plug (E0.5). Animals were killed by cervical dislocation and thymuses were surgically removed and processed for further analysis as described below.

Cell preparation and cultures

Thymocytes were prepared from thymuses obtained from 4- to 5-week-old male B6 mice. The non-lymphoid thymic stromal cell line TC-1S was established from B6 mice, as previously described (24), and cultured in RPMI 1640 medium supplemented with 10% FCS and 0.1 mg/ml sodium pyruvate (Hyclone Europe, Cramlington, UK).

For thymocyte/thymic stromal cell co-culture studies, $CD4^-CD8^-$ thymocyte precursors were obtained from freshly isolated thymocytes by a two-step killing technique to remove $CD4^+$ and $CD8^+$ T cells, as previously reported (25). The efficiency of the isolation procedures and the purity of the DN cell population ($> 96\%$) were assessed by two-color (CD4 versus CD8) and one-color (anti-Ig) flow cytometric analysis (FCA). DN thymocyte were layered onto TC-1S stromal cells in a Petri dish for 4–24 h, then removed, washed, and yield and viability determined prior to FCA or mRNA analysis.

$CD4^-CD8^-$ DN, $CD4^+CD8^+$ DP and $CD4^+$ or $CD8^+$ SP thymocytes used for RNA extractions were isolated by a sorting procedure (FACStar Plus; Becton Dickinson, Palo Alto, CA), as previously described (23). Electronic gates were set to collect highly purified thymocyte subsets.

mAb used for thymocyte phenotypic characterization

Phycoerythrin (PE)-conjugated anti-CD8 (clone 53-6.7) and FITC-conjugated anti-CD4 (clone H 129.19) used for two-color analysis were obtained from Boehringer Mannheim (Mannheim, Germany).

In three-color analysis, PE-conjugated anti-CD4 and biotinylated anti-CD8a were obtained from Boehringer Mannheim and PharMingen (San Diego, CA) respectively. Streptavidin–Red 613, used as the third chromogen in three-color FCA, was obtained from Gibco/BRL (Grand Island, NY). Rabbit polyclonal antisera to the Notch1, 2 and 3 proteins, raised against the extracellular domain of the three Notch receptors, are described elsewhere (26). FITC-conjugated affinity purified goat anti-rabbit IgG was used as second antibody (Sigma Biosciences, St Louis, MO).

FCA

CD8 and CD4 immunofluorescence staining was performed as previously described (23). Notch protein immunofluorescence staining was performed on 2% paraformaldehyde-fixed cells. After washing in ice-cold PBS, cells were incubated for 15 min at 4°C with anti-Notch antibodies followed by FITC-conjugated anti-rabbit IgG. After staining, cells were resuspended in 0.3 ml of PBS and analyzed on FACScan (Becton Dickinson, Mountain View, CA) with at least 1×10^4 events scored. In two- and three-color analysis, $1–5\times10^4$ events were scored. Positive cells were defined as those cells with

greater immunofluorescence, on a logarithmic scale, than control cells incubated with anti-Ig reagents only. Dead cells were detected by adding 1 μg/ml of propidium iodide and gated out by setting an electronic gate to exclude propidium iodide-positive cells. Fluorescence data were analyzed by FACScan or Consort 30 software (Becton Dickinson).

In situ hybridization and immunofluorescence

For cryostat sections, thymuses were surgically removed and embedded in OCT compound (Miles, Elkhart, IN), rapidly frozen in liquid nitrogen and stored at –80°C until analysis. For *in situ* hybridization studies, digoxigenin-labeled sense (as negative control) and antisense riboprobes for Notch1 and 3 (26) were synthesized following the manufacturer's instructions (Boehringer Mannheim, Germany). The riboprobes were diluted to 1 μg/ml in the hybridization mixture. *In situ* hybridization on cryostat sections was performed as previously described (17). The hybridization signal was visualized with NBT/BCIP (Boehringer Mannheim).

For TC-1S cell Jagged1 immunostaining, cells were allowed to grow on chamber culture slides, washed with PBS and fixed with absolute ethanol for 5 min at –20°C, stained with the rat anti-human Jagged1 mAb overnight at 4°C and monitored with FITC-conjugated goat anti-rat IgG (Kirkegaard & Perry, Gaithersburg, MD). The rat mAb against the intracellular domain of human Jagged1(TS1 15H2) was prepared by cloning the PCR amplified product from nucleotides 3646–4025 (amplification was performed using the 5′-CTACGGATCCCTGCGGAAGCG GCGGAAGCCGGGCAGC TRANSMEM and 5′- CGGTGAATTCTACGATGTACTCCATTC GGTTTAAG STOP) into the pGex2T vector (Amrad, Sydney, Australia), which resulted in a construct coding for a fusion protein with glutathione-S-transferase. The fusion protein was produced in bacteria and used to immunize rats (27).

RNA isolation and Northern blot analysis

Total RNA was isolated in guanidine isothiocyanate and poly(A)$^+$ RNA by using the FastTrack kit (Invitrogen, San Diego, CA), and were further processed for Northern blot analysis as previously described (23,28,29).

RT-PCR

RT-PCR was performed by a first step of reverse transcription from total RNA isolated as described above followed by PCR, essentially as previously reported (23,30), using a Perkin-Elmer Gene-Amp RNA PCR kit (Perkin-Elmer Cetus, Norwalk, CT). Samples of the PCR reactions were taken at different cycles throughout the amplification allowing accurate quantitation of the product during the exponential phase of DNA amplification. Negative controls included RT-PCR without reverse transcriptase or without RNA. Amplification of β-actin using 5′-GTGGGCCGCTCTAGGCACCAA-3′ and 5′-CTCTTTGATGTCACGCACGA TTTC-3′ as 5′ and 3′ primers was used as an internal control for both reverse transcription, PCR and as a measure of the relative amount of RNA. Amplifications of Notch 3 cDNAs were performed at an annealing temperature of 55°C using the following primers: 5′ (5′-ACACTGGGAGTTCTCTGT-3′) and 3′ (5′-GTCTGCTGGCATGGGATA-3′) (mouse Notch3 sequence X74760). Amplification of Jagged-1 cDNA was performed using the 5′ (5′-CTTGAGCCTTCTGCTCGCC-3′) and 3′ (5′-TGCAGGAGCCATGCTTGG-3′) primers (rat Jagged1 sequence L38483), with an annealing temperature of 58°C. Amplification of Jagged2 cDNA was performed using the 5′ (5′-GTCCTTCCCACATGGGAGTT-3′) and 3′ (5′-GTTTCCACCTTGACCTCGGT-3′) primers (rat Jagged2 sequence U70049 and U70050) with an annealing temperature of 59.4°C. PCR products were analyzed by agarose gel electrophoresis followed by either ethidium bromide staining or Southern blotting. Hybridizations were performed using specific oligomers internal to the amplified sequences. Autoradiographic bands were quantitated by scanning densitometry using a BioRad GS-670 scanning densitometer (BioRad, Richmond, CA) and employing Molecular Analist PC^{TM} software.

Results

Expression of Notch receptors in lymphoid and stromal components of murine thymus

We studied the distribution pattern of Notch 1, 2 and 3 mRNAs in the lymphoid and non-lymphoid compartments of murine thymus by Northern blot analysis. Transcripts of Notch1 and 3 genes were readily detected in freshly isolated thymocytes, whereas a lower expression for Notch 2 was observed (Fig. 1A).

In thymic tissue, thymocytes are closely associated with thymic stromal cells which play a critical role in specifying T cell development. Since Notch receptors are known to

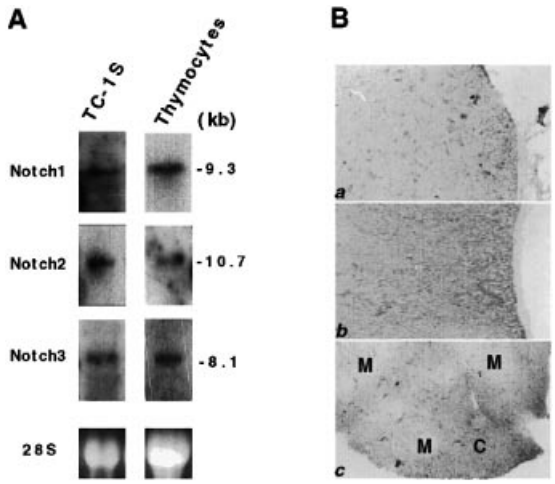

Fig. 1. Expression pattern of Notch1, 2 and 3 receptors in thymus. (A) The expression of the three Notch receptors in unfractionated thymocytes and in TC-1S thymic epithelial cells was analyzed by Northern blot. Total RNA (20 μg) was probed with RT-PCR amplified cDNA fragments of Notch1, 2 and 3 as described in Methods. The lowest panel shows the 28S ethidium bromide-stained RNA of the corresponding agarose gel lanes. (B) Notch1 and 3 expression in thymus was analyzed by *in situ* hybridization with digoxigenin-labeled antisense or sense (as negative control, not shown) riboprobes. Notch1 is expressed in the subcapsular region of the thymic cortex (*a*), whereas Notch3 transcripts are detected more extensively in the cortex (*b*). A general view of thymus displaying an intense and broad Notch3-expression in cortex is shown in (*c*). Note that Notch3 mRNA is absent in medulla (M) (*c*).

determine cell fate in both equivalent and non-equivalent cells in a number of models, we further investigated the pattern of Notch receptor expression in the non-lymphoid compartment of murine thymus. For this purpose the presence of Notch receptors was analyzed in a previously established thymic epithelial cell line, TC-1S, derived from mouse thymic stroma (24). This cell line is comprised of cortical epithelial cells and is functionally competent in promoting T cell differentiation (23). Northern blot analysis indicated that Notch1, 2 and 3 mRNAs were also expressed in TC-1S thymic epithelial cells (Fig. 1A).

In order to better clarify the localization of the Notch receptors within the thymus we performed *in situ* hybridization on tissue sections. The expression pattern of Notch1 and 3 receptors was analyzed in adult mouse thymus cryostat sections by RNA *in situ* hybridization using sense, as negative control, or antisense-specific probes (Fig. 1B). Figure 1(B, panel *a*) shows that the thymic cortex is positive for Notch1 expression, highest in the subcapsular region. Conversely, Notch3 expression was observed more extensively throughout the cortex (Fig. 1B, panel *b*). Both Notch1 (not shown) and Notch3 expression were not readily detected in medulla (Fig. 1B, panel *c*). These data indicate that Notch 1 and 3 are highly expressed in the subcapsular cortical region and throughout in the thymic cortex, the site of accumulation of immature $CD4^-CD8^-$ DN and intermediate-maturity $CD4^+CD8^+$ DP thymocytes respectively, but barely detectable in the medulla, the site were more mature SP thymocytes are located.

Notch receptor downstream effectors: HES-1 and HES-5 expression in murine thymus

The presence of Notch receptors in thymocytes and stromal cells of the thymus (Fig. 1A) and the previously described role played by Notch1 in thymocyte development (21,22) suggest that a number of the molecular events related to the Notch signaling pathway may be active in the thymus. A number of genes, including the basic helix-loop-helix protein complex HES-1 and HES-5, are activated following Notch–ligand interaction (6,31,32). Therefore, we analyzed the expression of HES-1 and HES-5 in stromal cell component (TC-1S cells), in thymocytes and in whole thymus, by Northern blotting. Figure 2 shows the presence of significant levels of HES-1 and HES-5 mRNAs in thymocytes and TC-1S epithelial cells.

Whereas the pattern of HES-1 expression in lymphoid and stromal thymic tissues confirms a previous report of the wide distribution of this gene product (33), HES-5 expression has so far only been described in the nervous system (28). Therefore, the HES-5 expression by thymocytes and thymic stromal cells broadens the spectrum of tissues in which HES-5 may play a role.

Notch1, 2 and 3 receptor expression in thymocyte subpopulations during development

The thymocyte differentiation process proceeds from immature subcapsular $CD4^-CD8^-$ DN cells through an intermediate stage of cortical $CD4^+CD8^+$ DP thymocytes, and terminates in mature CD3-expressing $CD4^+CD8^-$ and $CD4^-CD8^+$ SP thymocytes, located within the medulla. In order to analyze Notch protein expression on the cell surface of freshly isolated thymocyte subpopulations, that differentially express either CD4 or

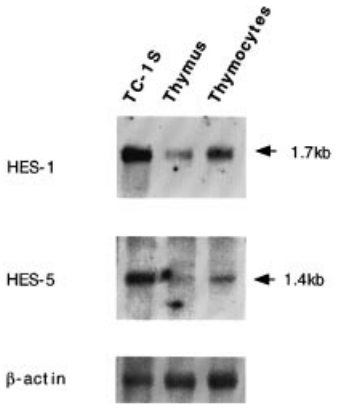

Fig. 2. Expression of HES-1 and HES-5 in thymic tissues. The expression of HES-1 and HES-5 was analyzed by Northern blot of 8.5 μg of poly(A)$^+$ RNAs extracted from TC-1S cells, whole thymus and thymocytes using cDNA probes. β-Actin hybridization to the same blot is shown as a loading control.

CD8 markers, during development, antibodies raised against the extracellular domain of the three transmembrane Notch receptors were used for three-color FACS analysis (Notch1, 2 or 3 versus CD4 versus CD8). Figure 3 shows that Notch1 protein in $CD4^+CD8^+$ DP thymocytes was observed at steady-state level in a low percentage of cells [range from 5 to 1% in embryo (E16.5) and adult (P1m) respectively] throughout thymus development, was not detected in $CD4^-CD8^-$ DN embryonic thymocytes but expression had significantly increased by birth (P0d) and continued into adulthood (range from 9 to 5%). $CD8^+$ and $CD4^+$ SP cells show a peak of Notch1 expression during the neonatal period (P3d), decreasing thereafter to reach the expression levels observed in DN cells. Notch2 protein was undetectable in E16.5 embryonal thymocytes, was slightly increased in P3d and, to a lesser extent, in adult thymocytes, which exhibited a few positive cells, displaying the $CD8^+$ SP and DN phenotype. Notch3 expression was readily detectable in a significant percentage of $CD4^+CD8^+$ DP (~15%) and in $CD4^-CD8^-$ DN (~4%) embryonal thymocytes, and displayed a significant decrease in expression at birth (P0d). After birth, the percentage of Notch3-expressing thymocytes progressively increased throughout neonatal and adult periods with the highest percentage observed in adult DN and $CD8^+$ SP cells (~30% for both). Lower expression was observed in DP and $CD4^+$ SP cells (~13%). These data indicate that Notch3 exhibits significantly elevated expression compared to Notch1 and 2 in both embryonal and adult thymocytes, despite variable expression at different ages. Figure 4 shows a FACS analysis of Notch3 expression by different thymocyte subsets obtained from embryonal and adult thymuses. It is noteworthy that embryonal DN thymocytes displayed a significant lower percentage of positive cells (3.42%) than adult DN cells (27.44%).

Expression of Notch receptor ligands in murine thymus during development: Jagged 1 expression is restricted to the thymic epithelium

Given that Notch receptors are expressed in both thymic stromal cells and thymocytes of developing mouse, we exam-

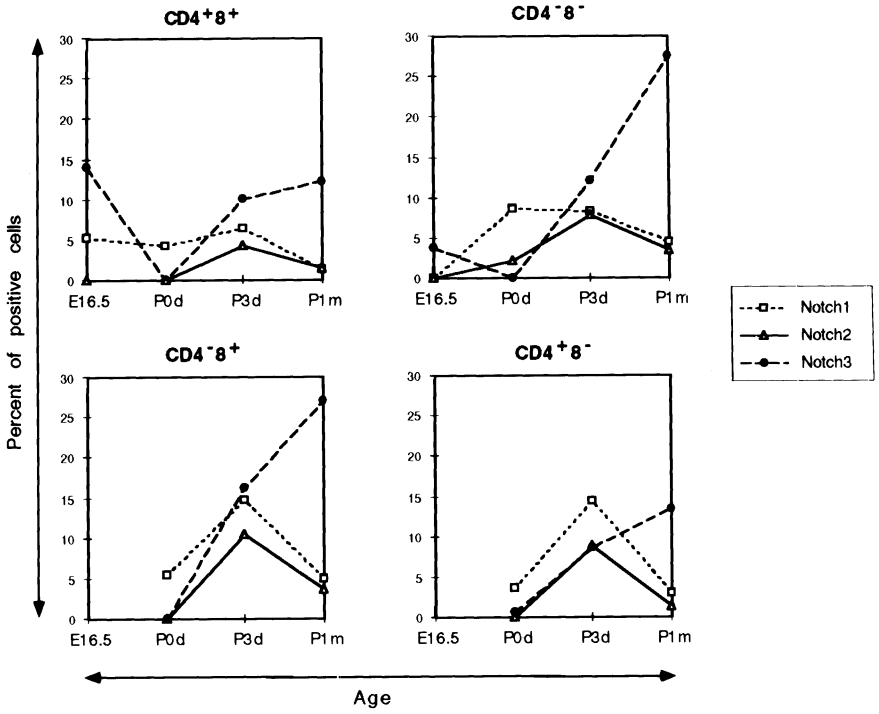

Fig. 3. Ontogenetic pattern of Notch receptor expression by different thymocyte subsets in developing thymus. Unfractionated thymocytes at different developmental ages were subjected to three-color flow cytometric analysis to study the expression of Notch1, 2, and 3 in electronically gated $CD4^-CD8^+$, $CD4^+CD8^+$, $CD4^+CD8^-$ and $CD4^-CD8^-$ subsets. The percentage of Notch1 (□)-, Notch2 (△)- and Notch3 (●)-expressing cells (*y*-axis) in different thymocyte subsets observed at the different ages (*x*-axis) are represented. The results shown are representative of three similar experiments.

ined the pattern of Notch receptor-specific ligand, Jagged1 and 2, expression.

The expression of both ligands was analyzed in whole thymus total RNA by Northern blotting and RT-PCR. Jagged1 and 2 mRNAs were detected in embryonic thymus (E16.5) and thymuses from mice at different stages of post-natal life (P0d, P3d and P1m). The identity of the RT-PCR amplified bands was confirmed by nucleotide sequence analysis. As depicted in Fig. 5(A), the expression of Jagged1 in developing thymus progressively decreased from embryonic to post-natal life. The higher level of expression of Jagged1 in embryonal thymus was also observed by Northern blot analysis of Fig. 5(B). In contrast, a steady-state of Jagged2 mRNA expression was observed throughout thymus development (Fig. 5A).

Given the main role played by intrathymic lympho-stromal interactions in determining the survival and the differentiation of thymocytes, and the role of Notch–ligand interactions in determining cell fate in different cell systems, the expression of Jagged1 and 2 was analyzed in the stromal and lymphoid components of thymus. As shown in Fig. 5(C), Jagged1 and 2 were expressed in the thymic stromal cell line TC-1S and in freshly excised whole thymus. However, RT-PCR performed on RNA from isolated cell populations indicated that, whereas Jagged2 was expressed at similar levels in both stromal and lymphoid components, Jagged1 exhibited preferential expression in TC-1S cells when compared to the various thymocyte subsets (Fig. 5D).

Similarly, Jagged1 protein was not detected in thymocyte subpopulations, assessed by FCA (not shown), whereas ~30% of thymic epithelial TC-1S cells exhibited immunoreactivity for Jagged1 (Fig. 5E).

Modulation of Notch receptor expression by signals triggering thymocyte maturation

The changes in Notch protein levels observed in thymocyte subsets at different stages along the T cell lineage differentiation/maturation pathway suggest that Notch expression may be regulated by signals that influence T cell development. TC-1S thymic epithelial cells have been previously reported to promote thymocyte maturation *in vitro* via generation of DP and SP thymocytes from DN precursors (23). In order to study the expression pattern of Notch receptors and ligands in TC-1S-driven T cell maturation *in vitro*, isolated DN thymocytes were cultured over a TC-1S cell monolayer or in medium alone. Thymocytes were harvested at different times during the co-culture (4, 12 and 24 h) and processed for FACS analysis, by staining with antibodies against the extracellular domain of either Notch1, 2 or 3.

A significant increase of Notch3-expressing thymocytes was observed after a 12 h co-culture period, which was further increasing following 24 h of co-culture (Fig. 6A). A lesser increase in Notch1 expression was also observed after 12 h, which persisted up to 24 h of co-culture. No modification in Notch2 protein expression was observed in thymocytes during the entire co-culture period. The increase in Notch3 and 1 expression preceded the acquisition of CD4 and CD8 phenotypic maturation markers, since no significant

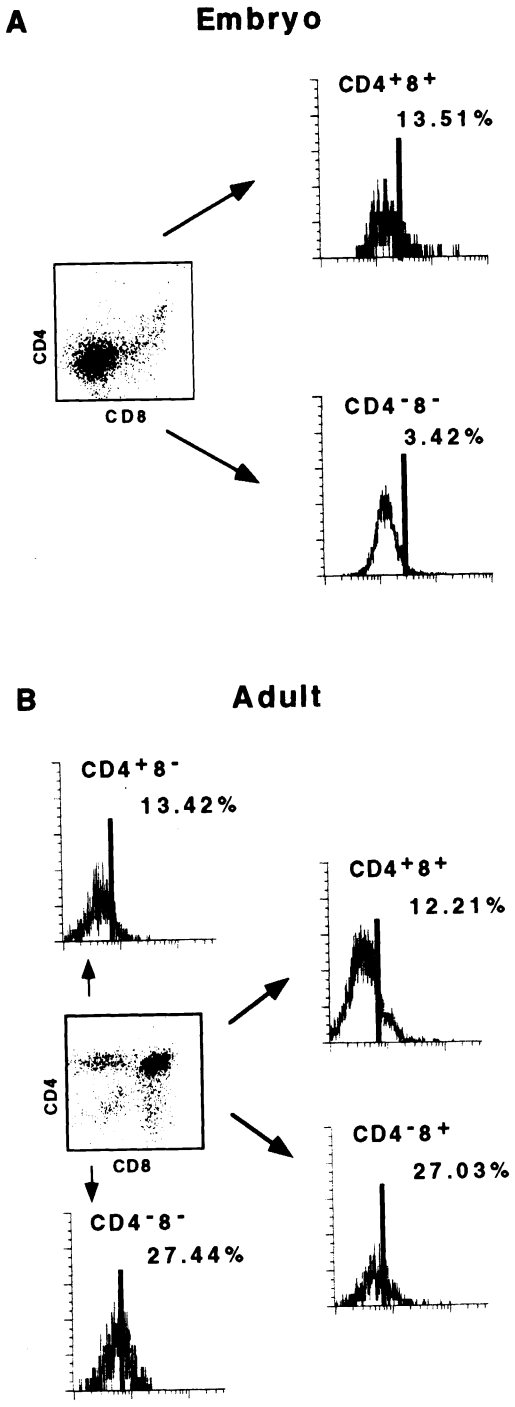

Fig. 4. Notch3 expression in embryonal and adult mouse thymocyte subsets. A three-color analysis of Notch3 versus CD4 versus CD8 expression was performed in embryonal (A) and adult (B) mouse unfractionated thymocytes. The expression of Notch3 (versus relative cell number) in electronically gated $CD4^{-}CD8^{+}$, $CD4^{+}CD8^{+}$, $CD4^{+}CD8^{-}$ and $CD4^{-}CD8^{-}$ thymocyte subsets is shown. In all histogram panels the bars indicate fluorescence levels of cells stained with FITC-conjugated affinity-purified goat anti-rabbit IgG without previous staining with anti-Notch3 antibody. Numbers indicate the percentage of Notch3-positive cells. Results shown are representative of three similar experiments.

generation of $CD8^{+}$ and/or $CD4^{+}$ thymocytes was observed during the 24 h co-culture period (these cells accumulated after 48 h) (data not shown and 23,34). No modification in the percentage of Notch3-expressing cells was observed in thymocytes cultured in the absence of TC-1S cell monolayers (Fig. 6B). It is intriguing that significantly lower levels of Notch1 and 3 receptor expression were observed in DN thymocytes obtained following isolation procedures when compared to fresh preparations. The possibility that this was dependent upon the thymocyte isolation technique employed is under examination. A significant increase in Notch3 mRNA levels was also observed in thymocytes as early as 12 h following co-culture with TC-1S cells (Fig. 6C).

In order to determine whether *in vitro* lympho-stromal cell interactions modulate stromal Notch ligand expression, Jagged1 and 2 mRNA levels were examined in TC-1S cells co-cultured with thymocyte precursors. No significant modification of stromal (TC-1S) expression of either Jagged1 or 2 mRNA or protein expression was detected (not shown).

Discussion

T cell development is mainly regulated by a close interaction between thymocytes and thymic stromal cells, and occurs throughout the complex lympho-stromal structure of the thymus. Thymic stroma provides a microenvironment suitable for homing and sustaining differentiation programs of thymocyte progenitors, by producing soluble factors (including stroma-derived cytokines and growth factors) and extracellular matrix components (which contribute to survival and development-related processes by mediating direct cell to cell interactions), and by expressing MHC class I and II structures required to shape the T cell repertoire (reviewed in 3).

Notch1 transmembrane receptor is activated during development and controls the ability of non-terminally differentiated cells to acquire or maintain a particular cell fate in a variety of tissues ranging from *Drosophila* to mammals (7,8). The heterogeneity of Notch-mediated differentiation models may involve the complexity of possible Notch receptor/ligand partnerships and the subsequent intracellular signaling that would be expected to occur in the multifaceted scenario of equivalent and non-equivalent interacting cell populations. In this respect, the thymocyte differentiation process is a suitable model to address the mechanisms of Notch receptor signaling, based on the heterogeneity of cell populations that participate to intrathymic T cell development and on the role played by at least one member of the Notch family (Notch1) in T cell differentiation (21,22).

Selective putative role of different Notch receptors in thymocytes

The expression pattern of Notch1 and 3 during thymus development reported here indicates that receptor expression begins at the embryonal stage and is maintained in the adult thymus where Notch1 and 3 are distributed in both lymphoid and stromal components. This suggests potential involvement in multiple differentiation pathways (lymphoid and epithelial lineages). The overlapping expression pattern of both receptors in thymocytes suggests a combined role for Notch1 and

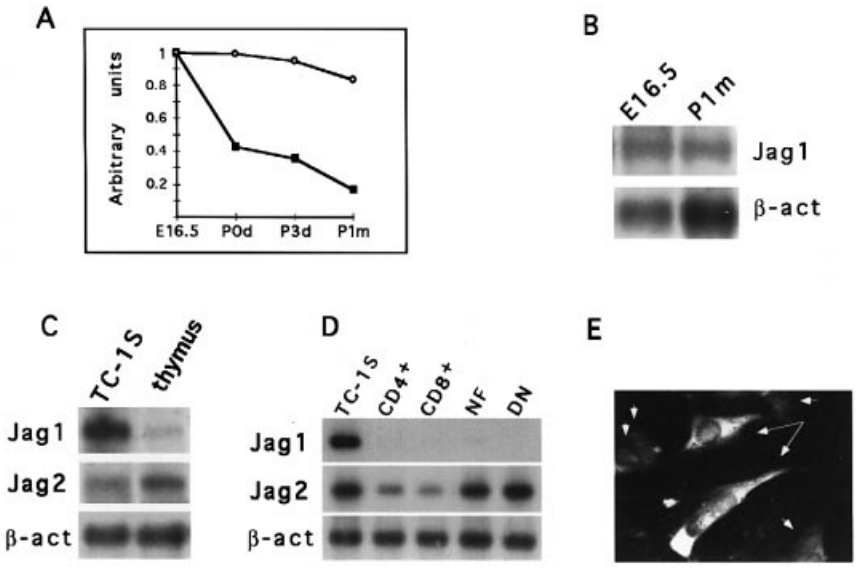

Fig. 5. Expression of Jagged1 and 2 in murine developing thymus, thymic epithelial cells and thymocytes. (A) Time course of Jagged1 (■) and Jagged2 (○) gene expression during thymus development. The expression of Jagged1 and 2 was evaluated by RT-PCR of total RNA extracted from E16.5, P0d, P3d and P1m murine thymus. Jagged1 and 2 amplified products (relative to the levels observed at E16.5 for the two genes, assigned the value of 1) are represented as the densitometric analysis of the autoradiographic bands normalized to β-actin amplified products evaluated at the same times. Results shown represent one point of the multiple samples taken during the exponential phase of DNA amplification for appropriate quantitation, as described in Methods. (B) Expression of Jagged1 (Jag1) mRNA in E16.5 embryonic (E16.5) and 1 month post-natal (P1m) murine thymus. Then 2.5 and 15 μg of total RNA, obtained from E16.5 and P1m murine thymus respectively was analyzed by Northern blot using murine Jagged1 cDNA as a probe. β-Actin (β-act) hybridization to the same blot is shown as a loading control. (C) The expression of Jagged1 (Jag1) and Jagged2 (Jag2) mRNAs in TC-1S cells and in whole thymus was evaluated by Northern blot analysis of 3 μg of poly(A)$^+$ RNA using either mouse Jagged1 or 2 cDNA probes respectively. β-Actin (β-act) hybridization to the same blot is shown as a loading control. (D) The expression of Jagged1 (Jag1) and 2 (Jag2) was evaluated by RT-PCR of total RNAs extracted from unfractionated (NF), CD4$^-$CD8$^-$, CD4$^-$CD8$^+$ and CD4$^+$CD8$^-$ thymocytes and thymic epithelial TC-1S cells (TC-1S). Southern hybridization of Jagged1, 2 and β-actin amplified products is shown. Results shown represent one point of the multiple samples taken during the exponential phase of DNA amplification for appropriate quantitation, as described in Methods. (E) Immunofluorescence staining of Jagged1 expression in TC-1S cells. Arrows show Jagged1 immunoreactive cells, which amount to 30%. Arrowheads indicate cells devoid of any immunoreactivity against Jagged1.

3 as already observed in other cell systems. Indeed, recently it has been reported that Notch1 and 2 receptors, co-expressed in myeloid progenitors, perform distinct differentiation functions within the same cell in response to different cytokines (35). Furthermore, distinct patterns of Notch1 and 3 expression have also been described in different regions of the developing central nervous and peripheral nervous system arising from common neural progenitors, suggesting that different receptor combinations may determine different neural cell types (36). Similar events may occur in the thymus, since we have observed that Notch1 and 3 co-expressed by thymocytes exhibited a differential expression pattern (higher Notch3 versus 1) in DN cells (27.44 versus 5% respectively). This suggests that Notch1 and 3 may have different functions in DN thymocytes. This observation also suggests that Notch3-dependent signal transduction may dominate in DN thymocytes. This supports a potential role for Notch3 in thymocyte development and is the subject of current investigation in our laboratory.

A different role for Notch3 with respect to Notch1 is also suggested by the significant increase in the percentage of Notch3-expressing cells in the precursor thymocyte population that was induced following interaction with thymic epithelial cells. In this model, cell–cell interactions between T cell precursors and TC-1S epithelial cells trigger the commitment of DN cells to differentiate into DP cells and up-regulates Notch3 expression prior to DP cell generation. This suggests that the transition from the DN to DP phenotype may involve Notch3.

Expression of Notch pathway elements in non-equivalent thymic stromal and lymphoid cell populations

The Notch signaling pathway is triggered by a number of ligands that exhibit tissue-specific distribution, suggesting specific functions. The distribution of Notch ligand, Jagged1 and 2, expression in lymphoid and stromal components of the thymus may be critical for Notch-dependent T cell development.

It has been previously reported that the Notch ligand Jagged2 is expressed with Notch 1 and 3 in rat embryonic thymus (stage E16.5) (15). Furthermore, Jagged2 expression has been reported in embryonic and post-natal murine thymus (16). However, there is no information concerning the distribution of Notch ligands and ligand-induced HES effectors in lymphoid and stromal cell thymic compartments.

We report that Jagged 2 and HES-1 and HES-5 effectors are expressed in both lymphoid and stromal cell compartments of the thymus. Jagged1 expression, however, is restricted to the thymic epithelium. We also show that both Jagged transcripts are abundant in the embryonic thymus and that Jagged1

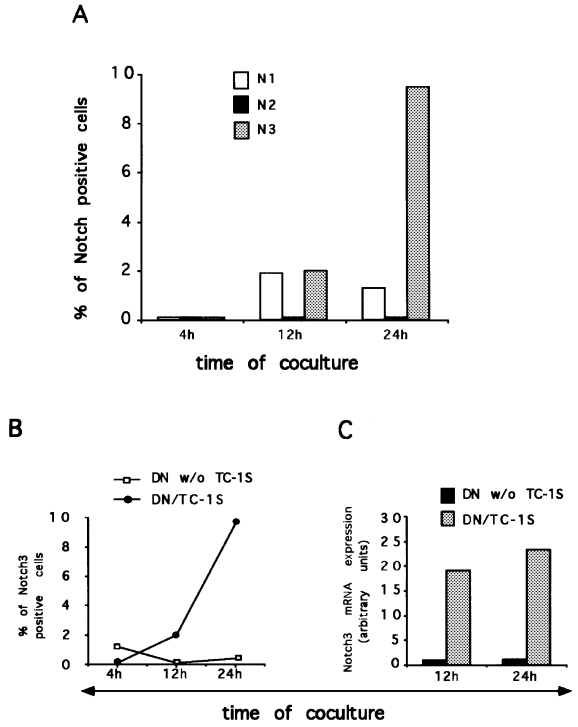

Fig. 6. Modulation of Notch receptor expression in thymocytes following thymic stromal cell/thymocytes co-culture. (A) Following co-culture on TC-1S cell monolayers, thymocytes were harvested at different incubation times (4, 12 and 24 h) and processed for a three-color FACS analysis. The percentages of Notch1 (open bars), Notch2 (solid bars) and Notch3 (shaded bars) receptor-positive cells (*y*-axis) are indicated. Results shown are representative of three similar experiments. No significant modification of Notch1, 2 and 3 expression was observed in DN thymocytes cultured in the absence of TC-1S cells (not shown). (B) Time course of Notch3 receptor expression in DN thymocytes cultured in the presence (DN/TC-1S) or in the absence (DN w/o TC-1S) of TC-1S cells. Viable thymocytes recovered after 4, 12 and 24 h incubation times with TC-1S cells were 60.2, 52.0 and 46.6% respectively. Viability of DN cells cultured in the absence of TC-1S was 59.0, 47.0 and 42.3% respectively. (C) Notch3 mRNA expression in thymocytes cultured for 12 and 24 h with (DN/TC-1S) or without (DN w/o TC-1S) TC-1S cells. The expression of Notch3 was evaluated by RT-PCR of total RNAs. Notch3 amplified products (relative to the levels observed in DN thymocytes cultured for 12 h in the absence of TC-1S cells, assigned the value of 1) are represented as the densitometric analysis of the autoradiographic bands normalized to β-actin amplified products evaluated at the same times. Results shown represent one point of the multiple samples taken during the exponential phase of DNA amplification for appropriate quantitation, as described in Methods.

expression progressively declines from embryonic to post-natal life. Therefore, we speculate that Jagged1 and 2 may play distinct roles in Notch receptor-mediated events in developing thymocytes. Their activity may be exerted through cell–cell contact mediated by receptors and ligands expressed by neighboring thymocytes and/or by promiscuous cell interactions such as those that occur between thymocytes and stromal cells.

Thus, Jagged2 may be involved in events that control reciprocal thymocyte interactions, whereas Jagged1 may regulate the cross-talk between stromal cells and thymocytes. The distinct activities of Jagged1 and 2 may also be a consequence of selective interaction with specific Notch receptors, depending on the receptor repertoire characteristic of a T cell developmental stage. Once the distribution of Notch ligands in thymic tissue has been mapped, functional studies using *in vitro* differentiation cell models or generation of appropriate transgenic animals will dissect the exact role of Jagged1 and 2 in T cell development. To this end, it is interesting to note that Jagged2 mutant mice display a defect in thymic development and impaired differentiation of the γδ T cell lineage (37).

In conclusion, our results suggest a novel function for Notch3 in thymus development and T cell maturation. Furthermore, the asymmetric distribution of Notch ligands and the presence of different Notch receptor downstream effectors suggests a potential multifaceted scenario of equivalent and non-equivalent interacting cell populations that could participate in the T cell developmental process. Indeed, two distinct scenarios are suggested. In the first, Notch receptors (mainly Notch1 and 3) and ligands (Jagged2), expressed by equivalent lymphoid cell precursors, could interact with each other to trigger reciprocal signals that direct diverging T cell fates (αβ versus γδ and $CD8^+$ versus $CD4^+$ subset), according to the previously described lateral specification model (4,7). In the second scenario, Notch receptors and ligands (i.e. Jagged1) are distributed on different cells: thus the cell fate of Notch receptor-carrying thymocytes may be triggered by stromal cells expressing the Notch ligand Jagged1, resulting in the enhancement and recruitment of thymocyte populations undergoing similar differentiation programs. As the ratio between Notch and Jagged1 levels increases throughout thymus development, as a consequence of the decreased ligand expression and increased numbers of Notch receptor-bearing thymocytes, an imbalance would be generated in the lympho-stromal cell interaction that may allow equivalent thymocyte populations to progress towards diverging T cell fates driven by the lateral inhibitory Notch–Jagged2 pathway. All of these events contribute to the highly orchestrated complexity of T cell development as a consequence of the heterogeneity in specific expression patterns of members of the Notch receptor and cognate ligand families, and intracellular transducers expressed by distinct cell compartments.

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Abbreviations

DN	double negative
DP	double positive
FCA	flow cytometric analysis
HES-1	*hairy* and *Enhancer of Split* homolog 1
HES-5	*hairy* and *Enhancer of Split* homolog 5
PE	phycoerythrin
SP	single positive

References

1 Sprent, J., Lo, D., Gao, E. K. and Ron, Y. 1988. T cell selection in the thymus. *Immunol. Rev.* 101:173.

2 Boyd, R. L., Tuceck, C. L., Godfrey, D. I., Izon, D. J., Wilson, T. J., Davidson, N. J., Bean, A. G. D., Ladyman, H. M., Ritter, M. A. and Hugo, P. 1993. The thymic microenvironment. *Immunol. Today* 14:445.

3 Screpanti, I., Modesti, A. and Gulino, A. 1993. Heterogeneity of thymic stromal cells and thymocyte differentiation: a cell culture approach. *J. Cell Sci.* 105:601.

4 Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M. E. 1995. Notch signaling. *Science* 268:225.

5 Greenwald, I. and Rubin, G. M. 1992. Making a difference: the role of cell–cell interactions in establishing separate identities for equivalent cells. *Cell* 68:271.

6 Robey, E. 1997. Notch in vertebrates. *Curr. Opin. Genet. Dev.* 7:551.

7 Kopan, R. and Turner, D. L. 1996. The Notch pathway: democracy and aristocracy in the selection of cell fate. *Curr. Opin. Neurobiol.* 6:594.

8 Fleming, R. J., Purcell, K. and Artavanis-Tsakonas, S. 1997. The NOTCH receptor and its ligands. *Trends Cell Biol.* 7:437.

9 Ellisen, L. W., Bird, J., West, D. C., Soreng, A. L., Reynolds, T. C., Smith, S. D. and Sklar, J. 1991. TAN-1, the human homolog of the *Drosophila* Notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* 66:649.

10 Lardelli, M. and Lendahl, U. 1993. Motch A and motch B—two mouse Notch homologues coexpressed in a wide variety of tissues. *Exp. Cell Res.* 204:364.

11 Weinmaster, G., Roberts, V. J. and Lemke, G. 1992. Notch2: a second mammalian Notch gene. *Development* 116:931.

12 Lardelli, M., Dahlstrand, J. and Lendahl, U. 1994. The novel Notch homologue mouse Notch 3 lacks specific epidermal growth factor-repeats and is expressed in proliferating neuroepithelium. *Mech. Dev.* 46:123.

13 Uyttendaele, H., Marazzi, G., Wu, G., Yan, Q., Sassoon, D. and Kitajewski, J. 1996. Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian Notch gene. *Development* 122:2251.

14 Lindsell, C. E., Shawber, C. J., Boulter, J. and Weinmaster, G. 1995. Jagged: a mammalian ligand that activates Notch1. *Cell* 80:909.

15 Shawber, C., Boulter, J., Lindsell, C. E. and Weinmaster, G. 1996. Jagged2: a serrate-like gene expressed during rat embryogenesis. *Dev. Biol.* 180:370.

16 Luo, B., Aster, J. C., Hasserjian, R. P., Kuo, F. and Sklar, J. 1997. Isolation and functional analysis of a cDNA for human Jagged2, a gene encoding a ligand for the Notch1 receptor. *Mol. Cell Biol.* 17:6057.

17 Mitsiadis, T. A., Henrique, D., Thesleff, I. and Lendahl, U. 1997. Mouse Serrate-1 (Jagged-1): expression in the developing tooth is regulated by epithelial–mesenchymal interactions and fibroblast growth factor-4. *Development* 124:1473.

18 Bettenhausen, B., de Angelis, M. H., Simon, D., Guènet, J.-L. and Gossler, A. 1995. Transient and restricted expression during mouse embryogenesis of Dll1, a murine gene closely related to *Drosophila* Delta. *Development* 121:2407.

19 Dunwoodie, S. L., Henrique, D., Stephen, M. H. and Beddington, R. S. P. 1997. Mouse *Dll3*: a novel divergent *Delta* gene which may complement the function of other *Delta* homologues during early pattern formation in the mouse embryo. *Development* 124:3065.

20 Hasserjian, R. P., Aster, J. C., Davi, F., Weinberg, D. S. and Sklar, J. 1996. Modulated expression of notch1 during thymocyte development. *Blood* 88:970.

21 Washburn, T., Schweighoffer, E., Gridley, T., Chang, D., Fowlkes, B. J., Cado, D. and Robey, E. 1997. Notch activity influences the alphabeta versus gammadelta T cell lineage decision. *Cell* 88:833.

22 Robey, E., Chang, D., Itano, A., Cado, D., Alexander, H., Lans, D., Weinmaster, G. and Salmon, P. 1996. An activated form of Notch influences the choice between CD4 and CD8 T cell lineages. *Cell* 87:483.

23 Meco, D., Scarpa, S., Napolitano, M., Maroder, M., Bellavia, D., De Maria, R., Ragano-Caracciolo, M., Frati, L., Modesti, A., Gulino, A. and Screpanti, I. 1994. Modulation of fibronectin and thymic stromal cell-dependent thymocyte maturation by retinoic acid. *J. Immunol.* 153:73.

24 Screpanti, I., Meco, D., Scarpa, S., Morrone, S., Frati, L., Gulino, A. and Modesti, A. 1992. Neuromodulatory loop mediated by nerve growth factor and interleukin 6 in thymic stromal cell cultures. *Proc. Natl Acad. Sci. USA* 89:3209.

25 Screpanti, I., Meco, D., Morrone, S., Gulino, A., Mathieson, B. J. and Frati, L. 1991. *In vivo* modulation of the distribution of thymocyte subsets: effects of estrogen on the expression of different T cell receptor V beta gene families in $CD4^-$, $CD8^-$ thymocytes. *Cell. Immunol.* 134:414.

26 Mitsiadis, T. A., Lardelli, M., Lendahl, U. and Thesleff, I. 1995. Expression of Notch 1, 2 and 3 is regulated by epithelial–mesenchymal interactions and retinoic acid in the developing mouse tooth and associated with determination of ameloblast cell fate. *J. Cell Biol.* 130:407.

27 Frorath, B., Scanarini, M., Netter, H. J., Abney, C. C., Liedvogel, B., Lakomek, H. J. and Northemann, W. 1991. Cloning and expression of antigenic epitopes of the human 68-kDa (U1) ribonucleoprotein antigen in *Escherichia coli*. *Biotechniques* 11:364.

28 Akazawa, C., Sasai, Y., Nakanishi, S. and Kageyama, R. 1992. Molecular characterization of a rat negative regulator with a basic helix-loop-helix structure predominantly expressed in the developing nervous system. *J. Biol. Chem.* 267:21879.

29 Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

30 Screpanti, I., Scarpa, S., Meco, D., Bellavia, D., Stuppia, L., Frati, L., Modesti, A. and Gulino, A. 1995. Epidermal growth factor promotes a neural phenotype in thymic epithelial cells and enhances neuropoietic cytokine expression. *J. Cell Biol.* 130:183.

31 de la Pompa, J. L., Wakeham, A., Correia, K. M., Samper, E., Brown, S., Aguilera, R. J., Nakano, T., Honjo, T., Mak, T. W., Rossant, J. and Conlon, R. A. 1997. Conservation of the Notch signaling pathway in mammalian neurogenesis. *Development* 124:1139.

32 Jarriault, S., Briu, C., Logeat, F., Schroeter, E. H., Kopan, R. and Israel, A. 1995. Signaling downstream of activated mammalian Notch. *Nature* 377:355.

33 Sasay, Y., Kageyama, R., Tagawa, Y., Shigemoto, R. and Nakanishi, S. 1992. Two mammalian helix-loop-helix factors structurally related to *Drosophila hairy* and *Enhancer of split*. *Genes Dev.* 6:2620.

34 Maroder, M., Bellavia, D., Meco, D., Napolitano, M., Stigliano, A., Alesse, E., Vacca, A., Giannini, G., Frati, L., Gulino, A. and Screpanti, I. 1996. Expression of trKB neurotrophin receptor during T cell development. Role of brain derived neurotrophic factor in immature thymocyte survival. J Immunol. 157:2864.

35 Bigas, A., Martin, D. I. K. and Milner, L. A. 1998. Notch1 and Notch2 inhibit myeloid differentiation in response to different cytokines. *Mol. Cell Biol.* 18:2324.

36 Lindsell, C. E., Boulter, J., diSibio, G., Gossler, A. and Weinmaster, G. 1996. Expression patterns of Jagged, Delta1, Notch1, Notch2, and Notch3 genes identify ligand–receptor pairs that may function in neural development. *Mol. Cell Neurosci.* 8:14.

37 Jiang, R., Lan, Y., Chapman, H. D., Shawber, C., Norton, C. R., Serreze, D. V., Weinmaster, G. and Gridley, T. 1998. Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. *Genes Dev.* 12:1046.