# Differential Expression of Notch Signaling-related Transcripts Accompanies Pro-thymocyte Proliferation and Phenotype Transition Induced by Epidermal Growth Factor plus Insulin in Fetal Thymus Organ Cultures

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*Thymus regression upon stressing stimuli, such as infectious diseases, is followed by organ reconstitution, paralleling its development in ontogeny. A narrow window of thymus development was here studied, encompassing the pro-T lymphoid precursor expansion during specification stages, by the use of epidermal growth factor plus insulin (INS) in murine fetal thymus organ cultures. Aiming to disclose signaling pathways related to these stages, cultured thymus lobes had their RNA extracted, for the search of transcripts differentially expressed using RNAse protection assays and reverse transcriptase-polymerase chain reactions. We found no difference that could explain INS-driven thymocyte growth, in the pattern of transcripts for death/proliferation mediators, or for a series of growth factor receptors and transcriptional regulators known as essential for thymus development. Thymocyte suspensions from cultured lobes, stained for phenotype analysis by fluorescence activated cell sorting, showed a decreased staining for Notch1 protein at cell surfaces upon INS addition. We analyzed the expression of Notch-related elements, and observed the recruitment of a specific set of transcripts simultaneous and compatible with INS-driven thymocyte growth, namely, transcripts for Notch3, for its ligand Jagged2, and for Deltex1, a mediator of a poorly characterized alternative pathway downstream of the Notch receptor.*

Key words: development - thymus - T-lymphocytes - Notch - growth factors

Thymus plays a central role in the development of immunity. As a target organ in infectious diseases, it displays a plastic behavior, its regression in response to infectious or stressing agents being followed, as soon as these stimuli are discontinued, by a prompt reconstitution that in many aspects mimics ontogeny (Savino et al. 1992). The organ development involves an ordered sequence of bi-directional signaling events between two cellular compartments, a complex microenvironment that eventually turns permissive to seeding by pluripotent hematopoietic precursors, and the progeny of the last (Anderson 2000). These lead, finally, to the exportation of mature αβTCR-bearing T-lymphocytes to periphery, besides minority subsets as products of divergent pathways (Rodewald 1995). Therefore, under microenvironmental influence, pluripotent precursors undergo a progressive specification, narrowing their potential to generate non-T descendants, and acquiring essential T-lymphoid characteristics (Rothenberg 2002). While intrathymic double-negative ($CD4^-CD8^-$) pro-T cells (DN1 to 3) can still diverge to other lymphoid (B, NK) and, under defined conditions, even to myeloid fates, the pre-T subset (DN4) represents cells committed to the mainstream αβTCR fate. They show differentiated requirement for growth factors, down-regulating c-kit (stem cell receptor), expressing interleukin-7 receptor (IL7R) only at low levels (Hattori et al. 1996a, DiSanto et al. 2000), and expanding upon pre-TCR signaling. Once thymocytes acquire a DP (double-positive, $CD4^+CD8^+$) phenotype, they must endure a series of TCR-mediated checkpoints, encompassing the survival to selective events that result in massive death, and the expansion of positively selected subsets, finally leaving thymus as mature $CD4^+$ or $CD8^+$ T- lymphocytes (Von Boehmer et al. 1997).

Precursor growth in the mouse thymus occurs at many points of the journey. While DN1 cells ($CD44^+25^-$) show limited expansion, CD25 acquisition is followed by 2 to 3 cell cycles, at the DN2 ($CD44^+25^+$) and DN3 ($CD44^-25^+$) stages (Pénit et al. 1995). Two more cell cycles, corresponding to the transition from a DN3 pro-T to a DN4 ($CD44^-25^-$) pre-T phenotype, result from the productive TCRβ-chain gene rearrangement and CD3-driven, $p56^{lck}$-mediated signaling (Pénit et al. 1995). While most DN3 cells express intracellular (IC) CD3ε, only 10-30% of them can productively rearrange the gene for TCRβ- chain and express IC TCRβ as well, otherwise they undergo apoptosis at DN4 (Falk & Eichman 2002). At this point, cell death signal involves the adaptor protein Fas-associated-with-a-death-domain (FADD) that would, conversely, promote proliferation in cells able to express a pre-TCR, in

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the so-called beta-selection (Newton et al. 2000). Thus, in normal physiology, cells cycling in DN3 stage are likely to be a mixture of cells finishing the cell cycles induced at the DN2 step and those starting the β-chain-, lck- mediated proliferation at DN4 (Pénit et al. 1995).

While studying the effect of growth factors upon thymus development in vitro, using fetal thymus organ cultures (FTOC), we observed that exogenously added epidermal growth factor (EGF) could, complete but reversibly, block both growth and differentiation of the αβTCR, but not of γδTCR lymphocytes (Freitas et al. 1998a), while provoking extensive microenvironment disarrangement. Cells remnant from EGF-FTOC showed a DN1/2 surface phenotype. The simultaneous addition of insulin (INS) prompted pro-T cells to acquire a majority DN3 phenotype (Freitas et al. 1998b), besides inducing microenvironment changes (unpublished results). These observations provided us with a narrow window of analysis of growth and differentiation in the thymus during T-cell specification stages.

Aiming to disclose signaling pathways related to this particular window of thymus development, we searched for transcripts differentially expressed by EGF- and EGF+INS-FTOC, starting with those coding for factors implied in early thymus development and/or T-lymphoproliferative diseases. These included death-related mediators, growth factor receptors and transcription regulators essential during T-cell specification stages, as well as members of Notch family and their ligands, known to intermediate lympho-stromal relationships.

Notch family, in vertebrates, comprises a group of cell surface proteins homologous to *Drosophila* Notch that, upon binding to ligands such as Delta-like and Jagged (respective homologous of *Drosophila* Delta and Serrate), mediate interactions between contiguous cells (Osborne & Miele 1999). Being essential for embryonic development, Notch action resumes further in many organs and systems, included the immunological system, mediating the microenvironmental inductive events that lead to growth, death and differentiation decisions (Osborne & Miele 1999). For example, Notch and its ligands are essential for the Common Lymphoid Precursors decision between a B- or T- lymphoid fate (Radtke et al. 1999, Schmitt & Zúñiga-Pflücker 2002, Hozumi et al. 2003).

We here report the sudden expression of a specific set of Notch-related transcripts, simultaneous and compatible with the lymphoid precursor growth and the one-step-limited thymocyte differentiation here analyzed.

## MATERIALS AND METHODS

*Animals* - C57BL6/J mice, originated from the animal facilities of the National Cancer Institute of Rio de Janeiro, were bred in the Laboratory of Transgenic Animals, Federal University of Rio de Janeiro. Females bred overnight were separated from males in the morning (day 0). At day 14, pregnant females were killed by cervical dislodgement, and fetuses were harvested under sterile conditions.

*FTOC* - The cultures were performed as described in the literature, with some modifications. Fourteen-day-fetal thymuses, containing hematopoieitc precursors up to the pro-T stage, were used. They were excised by the use of watchmaker forceps, cleaned from contaminant tissues with the aid of surgical needles, and assembled (5-10 per dish) on a 0.22 µm Millipore membrane sustained by a stainless steel grid inside a delta Nunc plate (Nunclon, Roskilde, Denmark), containing 1.5 ml of culture medium (DMEM 310 mOsm) plus 10% fetal calf serum (FCS) (defined serum, Hyclone, Logan, UT, US), glutamine, non-essential amino acids (Life Technologies, Gaithersburg, MD, US), 60 mg/l penicillin and 100 mg/l streptomycin. Medium was changed after 3 days, and lobes were harvested after 7 days of culture, when were pooled and stored at –70ºC before processing. A series of cultures were performed, and two different pools of lobes cultured in each condition were used for RNA extraction.

*Cytokines* - Natural EGF from mouse submaxillary glands (Sigma Chem. Co., St. Louis, MO, US) was added to a 100 ng/ml final concentration. Purified porcine and bovine insulin was obtained from CEME (Brazil), and added to a 20 nM final concentration.

*Reverse transcriptase-polymerase chain reactions (RT-PCR)* - Cultured lobes from FTOC-C, FTOC-EGF, and FTOC-EGF+INS were harvested, pooled, and their total RNA extracted by the use of TRIZOL (Life Technologies). The cDNA was synthesized from 2 µg each RNA by the use of Superscript TMII RT and oligo-dT (Life Technologies). PCR amplification used Taq DNA polymerase (Promega, Madison, WI, US), and specific primers (Invitrogen, SP, Brazil) as described in Table I. PCR assays started with 3 min at 94ºC, followed by 35 cycles of 94ºC for 45 s, variable annealing temperatures for 30 s, and 72ºC for 1.5 min, with a final extension for 10 min at 72ºC. Reaction products were analyzed in 1.5% agarose gels in TAE containing ethidium bromide.

*RNAse protection assays (RPA)* - Five µg of total RNA extracted from pools of intact cultured lobes were subjected to RPA, using [α-$^{32}$P] UTP radiolabelled mAPO-2 and mAPO-3 probe sets as specified by the manufacturer (BD Pharmingen, CA, US). The resulting protected RNAs were resolved on 5% denaturing polyacrylamide gels and exposed to x-ray films.

*Fluorescence activated cell sorting (FACS) analyses* - Thymus lobes from FTOCs were smashed under a glass coverslip, and the lymphocytes recovered were surface-stained with anti-mouse NK1.1 monoclonal antibodies (moAbs) (clone PK136, PE-conjugated, Southern Biotechnology Associates, AL, US), or with anti-Notch1 moAbs (clone 18G, kindly sent by Dr David Flowers, Seattle, WA, US) plus PE-conjugated goat anti-rat IgG (Gibco/BRL, Life Technologies). Unspecific labeling was prevented by pre-incubation of the cells with Fcγ-blocker (BD Pharmingen). Dead cells were excluded from analyses by the addition of propidium iodide (2 µg/ml final). Intracellular TCRβ staining followed fixation of the cells with 1% paraformaldehyde and permeabilization with 0.01% Tween-20; anti-mouse TCRβ antibodies (rat Ig, clone H57-597, biotin-conjugated, Gibco) and Streptavidin-Tricolor (Caltag, CA, US) were used. Cytofluorometric acquisition was performed in a FACScan apparatus (Becton Dickinson, San Jose, CA, US) equipped with a 15-mW air-cooled 488-nm argon-ion laser. Data acquisition was carried out using

TABLE

Primer pairs utilized in reverse transcriptase-polymerase chain reaction assays

| Oligos | Sequences 5'-3' | Band lenght | Reference | NCBI access | Bases | Ann. Temp. |
|---|---|---|---|---|---|---|
| pT α S<br>pT α AS | CTGCAACTGGGTCATGCTTC<br>TCAGAGGGGTGGGTAAGATC | 640/<br>320 bp | Hattori et al. 1996a | gi 6755215 | 86-105<br>758-739 | 55 |
| c-kit S<br>c-kit AS | GGATCATTGTGATGGTGCTC<br>TATGCAGTGGCCTCAACGAC | 271 bp | Taubenbergeret al. 1996 | gi 20832867 | 1651-1670<br>1867-1848 | 57 |
| RAG 1 S<br>RAG 1 AS | TGCAGACATTCTAGCACTCTGG<br>ACATCTGCCTTCACGTCGAT | 556 bp | Taubenbergeret al. 1996 | gi 20857179 | 38-59<br>591-572 | 55 |
| RAG 2 S<br>RAG 2 AS | CACATCCACAAGCAGGAAGTACAC<br>GGTTCAGGGACATCTCCTACTAA | 471 bp | Hozumi et al. 1996 | gi 6677660 | 91-114<br>562-540 | 55 |
| IL7Rα S<br>IL7Rα AS | AGCAACTGGACGCATGTATC<br>TCACCATCTCTGTAGTCAGG | 656 bp | Taubenbergeret al. 1996 | gi 20897210 | 573-592<br>1228-1209 | 55 |
| TCF1a S<br>TCF1a AS | CATGAAGGAGATGAGAGCCA<br>GCCTGTCTCTGAGATTCTTG | 529 bp | Taubenbergeret al. 1996 | gi 20880453 | 777-796<br>1306-1287 | 55 |
| GATA 3 S<br>GATA 3 AS | TCGGCCATTCGTACATGGAA<br>GAGAGCCGTGGTGGATGGAC | 299 bp | Hattori et al.1996b | gi 6679950 | 299-319<br>583-564 | 55 |
| PU.1 S<br>PU.1 AS | CCCGGATGTGCTTCCCTTAT<br>TCCAAGCCATCAGCTTCTCC | 120 bp | Anderson et al. 2002 | gi 200971 | 499-518<br>619-600 | 59 |
| SCL S<br>SCL AS | TCCCCATATGAGATGGAGATTT<br>ATTGATGTACTTCATGGCAAGG | 197bp | Herblot et al. 2000 | gi 6755715 | 655-676<br>852-831 | 42 |
| C-MYB S<br>C-MYB AS | CCTCTAGGAGCTCATTTGTG<br>TTCAAGGCCAGCATTCTTGC | 682 bp | Dai et al. 2000 | gi 199928 | 3214-3233<br>3896-3877 | 55 |
| C-MYC S<br>C-MYC AS | ACCAACAGGAACTATGACCTC<br>AAGGACGTAGCGACCGCAAC | 219 bp | Douglas et al. 2001 | gi 20902322 | 603-623<br>822-803 | 55 |
| E47 S<br>E47 AS | TTGACCCTAGCCGGACATACA<br>GCATAGGCATTCCGCTCACT | 120 bp | Anderson et al. 2002 | gi 13310808 | 295-315<br>415-396 | 50 |
| Id2 S<br>Id2 AS | CCGCTGACCACCCTGACC<br>ATAAGCTCAGAAGGGAATTCAGATG | 70 bp | Anderson et al. 2002 | gi 13905247 | 363- 378<br>433- 409 | 50 |
| HEB S<br>HEB AS | AAATCAGATGATGAGTCCTCCC<br>CTCTGGAACTGGCTGATGTTT | 453 bp | Herblot et al. 2000 | gi 6755729 | 1753-1774<br>2206-2186 | 55 |
| Notch1 S<br>Notch1 AS | CGGTGTGAGGGTGATGTCAATG<br>GAATGTCCGGGCCAGCGCCACC | 910 bp | Izon et al. 2002 | gi 208502 | 3865-3886<br>4399-4378 | 55 |
| Notch3 S<br>Notch 3 AS | ACACTGGGAGTTCTCTGT<br>GTCTGCTGGCATGGGATA | 466 bp | Felli et al. 1999 | gi 20899415 | 3532-3549<br>3998-3981 | 42 |
| Jagged1 S<br>Jagged1 AS | CTTGAGCCTTCTGCTCGCC<br>TGCAGGAGCCATGCTTGG | 697 bp | Felli et al. 1999 | rat mRNA<br>gi 9506824 | 425-443<br>1122-1105 | 52 |
| Jagged2 S<br>Jagged 2 AS | GTCCTTCCCACATGGGAGTT<br>GTTTCCACCTTGACCTCGGT | 590 bp | Felli et al. 1999 | rat mRNA<br>gi 1718247 | 2496-2515<br>3086-3067 | 42 |
| Dll 1 S<br>Dll1 AS | GGACTATAACCTCGTTCG<br>GAAAGACTGGCTCATAGG | 586 bp | Bettenhausen et al. 1995 | gi 6681196 | 1731-1755<br>2515-2499 | 50 |
| Dll4 S<br>Dll4 AS | TCTTCCGCATCTGCCTTAAGCACT<br>AGTCTCTGGCCGCAGGTCGTCTCC | 229 bp | Yoneya et al. 2001 | gi 9506546 | 485-508<br>714-691 | 55 |
| Pref 1S<br>Pref 1AS | CGTTCACTCGATTCCACACAT<br>GAGGCTGGTGATGAGGAGATC | 188 bp | Smas & Sul 1993 | gi 6753641 | 1287-1307<br>1475-1455 | 50 |
| Deltex1S<br>Deltex1AS | CACTGGCCCTGTCCACCCAGCCTTGGCAGG<br>GGGAAGGCGGGCAACTCAGGCCTCAGG | 910 bp | Izon et al. 2002 | gi 6679876 | 1028-1057<br>1938-1912 | 57 |
| HES1 S<br>HES1 AS | CAGCCAGTGTCAACACGACAC<br>TCGTTCATGCACTCGCTGAG | 306 bp | Sestan et al. 2001 | gi 17390875 | 287-307<br>593-575 | 55 |
| HES 5 S<br>HES 5 AS | CGCATCAACAGCAGCATAGAG<br>TGGAAGTGGTAAAGCAGCTTC | 268 bp | Sestan et al. 2001 | gi 6754181 | 91-111<br>359-339 | 55 |
| G3PDH S<br>G3PDH AS | ACCACAGTCCATGCCATCAC<br>TCCACCACCCTGTTGCTGTA | 451 bp | Sestan et al. 2001 | gi 25020911 | 572-591<br>1023-1004 | 55 |

the LYSIS II software program (BDIS).

## RESULTS

*RPA assessment of differential expression of death-related transcripts in EGF- and EGF+INS-FTOCs* - Proliferation and rescue from death are hardly unraveled processes in the thymus, sometimes sharing signal transducers, as is the case of FADD (Newton et al. 2000). We thus investigated the expression of death-related transcripts, some of which are developmentally regulated in this organ (Chao & Korsmeyer 1998, Tomayko et al. 1999, Newton et al. 2000). We used RPA with probe sets that cover both pro- and anti-apoptotic factors associated with Bcl-family- and TNFR-family-related signaling. If normalized for loading on the basis of constitutively expressed transcripts (G3PDH and L32), similar patterns of expression were seen in both EGF- and EGF+INS- transcripts, including those for FADD (Fig. 1). Low levels of signal for Fas were present in these RNAs, when compared to Control-FTOC-derived RNAs (Fig. 1A), consistent with Fas expression being restricted, among immature subsets, to DP cells (Newton et al. 2000). The overall ratio of Bcl-family death antagonists to agonists, which determines the susceptibility to death stimuli (Chao & Korsmeyer 1998), was also maintained in EGF- and EGF+INS- derived transcripts (Fig. 1B).

*Availability of transcripts coding for factors critical during T-cell specification stages in the thymus* - Fig. 2 shows results from RT-PCR assays, comparing EGF- and EGF+INS-FTOCs levels of expression of transcripts for factors known to be essential during early thymus development. No difference that could explain the proliferation blockade and its reversal by INS was observed. Transcripts for the growth factor receptors IL7Rα and c-kit were present in EGF-FTOCs, and thus seemingly were not growth-limiting, although their contents in EGF+INS-FTOC seemed to vary in keeping with thymocyte developmental stages (Hattori et al. 1996a, DiSanto et al. 2000).

Transcripts for gene regulators such as GATA 3, that here was not correlated with cellular proliferation as was reported in literature (Hattori et al. 1996b, Hendriks et al. 1999), for TCF1a (Hattori et al. 1996b), PU.1 (Anderson et al. 2002), c-MYB (Rothenberg 2002), c-MYC (Douglas et al. 2001), SCL and HEB (Herblot et al. 2000), E47 (E2A) and its inhibitor Id2 (Anderson et al. 2002), as for the T-cell receptor gene-rearranging enzymes RAG-1 and RAG-2, were seen in both conditions (Fig. 2A). Transcripts for the two pT-α isoforms were also observed in both situa-

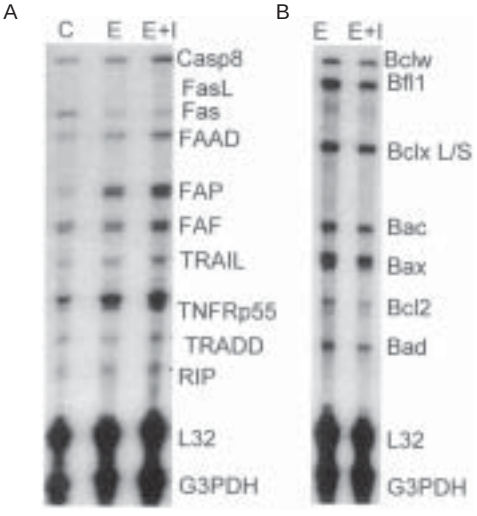


Fig. 1: expression of death-related transcripts assessed by RNAse protection assay. Total RNA was extracted from pools of entire lobes, harvested from fetal thymus organ cultures with epidermal growth factor (E), with epidermal growth factor plus insulin (E+I), or from control lobes, without growth factors (C). Five micrograms from each RNA were used for hybridization with [α-$^{32}$P]UTP radiolabelled m-APO2 (A) and m-APO3 (B) probes. The resulting protected RNAs were resolved on 5% denaturing polyacrylamide gels and exposed to x-ray films.

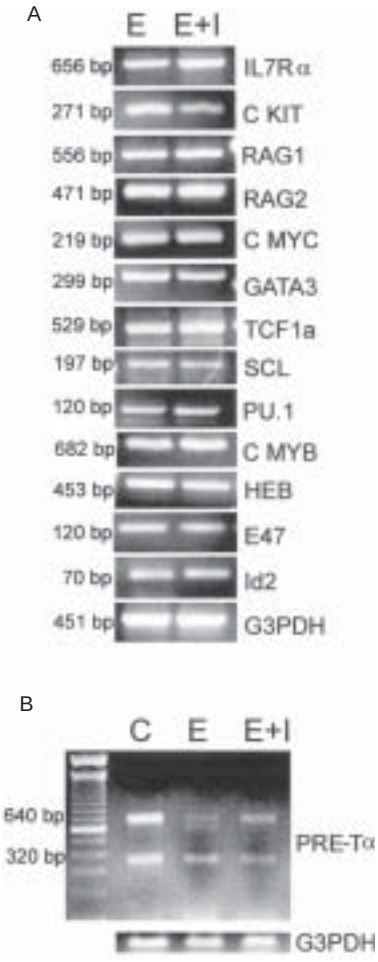


Fig. 2: availability of transcripts essential for early thymus development assessed by reverse transcriptase-polymerase chain reaction (RT-PCR) assays. In A, presence of transcripts for growth factor receptors, T-Cell-receptor-rearranging enzymes, and transcription factors. In B, presence of transcripts for the two isoforms of the pre-Tα chain. Total RNA was extracted from pools of entire lobes, harvested from fetal thymus organ cultures with epidermal growth factor (E), with epidermal growth factor plus insulin (E+I), or control cultures, without growth factors (C). RT-PCR assays were performed as described in Materials and Methods, using primer pairs as in the Table. Experiments were run twice with different sets of pools, with similar results.

tions (Fig. 2B). The smallest ($pT\alpha^a$) was reported as preferentially represented in early thymocyte subsets, besides T-lymphoid malignancies, while the expression of the largest transcript ($pT\alpha^b$) would represent a delayed event in development, preparing beta-selection (Ramiro et al. 2001).

*Cytofluorometric assays* - TCR beta-chains, however, necessary for the assembly of pre-TCR complexes and for beta-selection to occur, seemed absent, since FACS analyses revealed a negative IC staining with anti-TCRβ chain-Abs in both EGF- and EGF+INS-FTOC (Fig. 3A). Unselected DN thymocytes could still diverge into alternative routes, such as for an NK fate. Surface staining for the NK1.1 marker, however, showed this was not the case (Fig. 3B). Analyses of surface characteristics of the lymphoid remnants from FTOCs revealed a high Notch1 surface staining for EGF-FTOC, with a shift of the fluorescence peak to an intermediate pattern, for EGF+INS-FTOCs (Fig. 3C). Thus, Notch1 down-modulation from thymocyte surfaces suggested an involvement of Notch-mediated signaling in INS-driven effects.

*Differential expression of transcripts involved with Notch signaling* - RT-PCR assays revealed that INS addition to EGF-FTOC resulted in a clear-cut change in the expression of transcripts involved in Notch signaling. Besides transcripts for Notch1, its ligands Jagged1, Preadipocyte factor-1 (Pref-1; delta-like; dlk), Delta-like1 (Dll1), and Delta-like4 (Dll4), and for the Notch target genes "Hairy and Enhancer of Split" 1 (HES1), the EGF+INS-FTOC- derived RNA contained those for Notch3, as well as for Jagged2, and for the downstream modulator of Notch action, Deltex1 (Fig. 4).

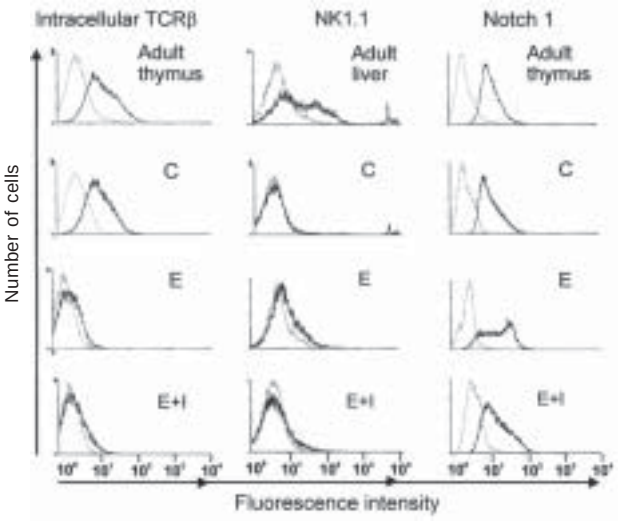


Fig. 3: cytofluorometric analyses of lymphoid populations. Lymphoid cells were recovered from fetal thymus organ cultures with epidermal growth factor (E), with epidermal growth factor plus insulin (E+I), or from control lobes, without growth factors (C). Lobes were pooled, smashed, and cell suspensions stained with specific moAbs plus a fluorochrome-conjugated second layer. Intracellular staining with biotin-conjugated anti-mouse TCRβ antibodies plus Streptavidin-Tricolor followed fixation and permeabilization of the cell suspensions as described in Materials and Methods. Living cells were surface-stained with PE-conjugated anti-mouse NK1.1 moAbs, or with anti-Notch1 moAbs plus PE-conjugated goat anti-rat IgG. Gray lines represent controls of staining with the second layer only. Dead cells were excluded from analyses by the addition of propidium iodide. Controls for positive staining were: adult thymus lymphocytes (for IC TCRβ and Notch1), and adult liver cell suspensions (for NK1.1). Shown are results of one experiment representative of two similar.

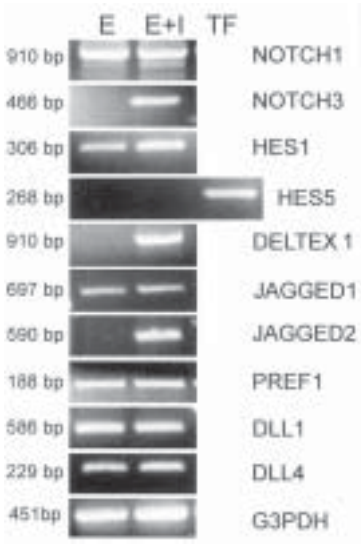


Fig. 4: differential expression of Notch signaling-related transcripts accessed by reverse transcriptase-polymerase chain reaction assays. Total RNA was extracted from pools of entire lobes, harvested from fetal thymus organ cultures with epidermal growth factor (E), or with epidermal growth factor plus insulin (E+I). RT-PCR assays were performed as described in Materials and Methods, using primer pairs as in the Table. Total RNA from total fetuses (TF) was used as a positive control for HES5 expression. Experiments were run twice with different sets of pools, with similar results.

## DISCUSSION

The interactions between lymphoid and stromal elements in early thymus still remain partially obscure (Allman et al. 2002, Harman et al. 2003, Hozumi et al. 2003), as is the role of intermediate messengers of transduction pathways in developmental processes of the thymus (Rothenberg 2002). We here intended to depict, among the many parameters reported as taking part of thymus development, those which expression could be associated with the induction of thymocyte growth and one-step-limited phenotype transition in our model. Briefly, after a 7-day culture, typically around 1-4 x $10^4$ thymocytes per lobe could be recovered from EGF-FTOC (DN1/2, surface phenotype: $CD44^+25^{-/int}$, $CD3^-$, $4^-$, $8^-$, $2^-$, $16/32^+$, $\alpha\beta TCR^-$), while 1-2 x $10^5$ thymocytes per lobe from EGF+INS-FTOC (DN3, $CD44^-25^+$, $CD3^-$, $4^-$, $8^-$, $2^-$, $16/32^+$, $\alpha\beta TCR^-$) (Freitas et al. 1998b). Since lymphoid cells recovered from EGF+INS-FTOC in their majority have not missed CD16/32 staining nor acquired CD2 (Freitas et al. 1998b), an impairment to progress to a DN4 stage ($CD44^-25^-$, $CD3^-$, $4^-$, $8^-$, $2^+$, 16/32, $\alpha\beta TCR^-$) was suggested, or alternatively, their putative death at this stage. Being surface-negative for NK1.1 (Fig. 3B), their expansion seemingly did not represent a diver-

sion of precursors into this alternative route. They seemed not yet definitively committed to a T-cell fate, as judged by negative staining for both surface (Freitas et al. 1998b) and IC TCRβ-chain protein (Fig. 3A). The INS-driven thymocyte growth could not be abolished by tyrphostin PP2, a specific lck inhibitor (Freitas et al. 1998b), showing that it indeed represents the "first wave of thymocyte growth" (Pénit et al. 1995), not mediated by pre-TCR-lck. This expansion of intra-thymic precursors occurred even in the absence of EGF, and accounted for an accelerated thymus recovery after EGF subtraction from EGF-FTOCs, when INS was added, compared to lobes in INS absence (unpublished observations). Thus, despite a putative multi-factorial and indirect action, the introduction of the exogenous growth factors to FTOC allowed us to focus specifically at the changes that result in thymocyte proliferation during CD25 acquisition, in the transition from DN1/2 to DN3 T-cell specification stages, taking advantage of an ex-vivo model that does not rely on transformed cells or constitutively active Notch constructs.

Assessment of the expression of death-related mediators in RPA assays, comparing EGF- to EGF+INS-derived RNA, did not provide evidence for any important difference in the availability of transcripts for "death/ proliferation mediators" that could account for INS-induced growth, unless if upon post-translational mechanisms, or if related to the expression of alternative death receptors/ ligands here not tested (Chao & Korsmeyer 1998). The higher levels of transcripts for BclxL/S than for Bcl-2 observed were unexpected, in view of literature. DN thymocytes were reported to express Bcl-2 but not Bcl-xL, in a reciprocal feedback mechanism of regulation (Chao & Korsmeyer 1998). It is possible that here a cell type other than lymphoid contributed to Bcl-xL transcript levels, since intact lobes, containing also stromal components, were used for RNA extraction. Anyway, if an imbalance of the relative levels of transcripts for BclxL/S and Bcl-2 were to be accounted for the inability of DN3 cells to evolve to a DN4 phenotype in our model, what was not tested, it would not account for the INS-driven growth, since the levels were similar in both culture conditions.

Considering that the transition to the DN3 phenotype was not a 100% event, it would not be surprising that transcripts seen in the DN1 and DN2 stages could still be observed among RNAs from EGF+INS-FTOC. Newly expressed messages, however, were considered as representative of the new condition. We found a clear-cut differential expression of Notch signaling- related transcripts, comprising thymocyte/stromal ligands and downstream effectors, simultaneous and compatible with proliferation and DN1/2 to DN3 phenotype transition. This differential expression of Notch transcripts seemed in keeping with that reported for early thymus development. Notch3 expression, for example, was seen at the end of the "HES1-dependent $CD25^+$ proliferation" (Tomita et al. 1999). Notch3-related unregulation of thymocyte development specifically affected late DN cells, while Notch1 impairment would be critical at earlier points (Wolfer et al. 2002). Here, compared to EGF-FTOC-derived cells, lowered levels of Notch1 protein were observed at the surface of cells from EGF+INS-FTOC (Fig. 3C), although transcripts for Notch1 were still present. Inversely related to Notch1 surface levels, newly transcribed messages for Notch3 were observed, simultaneously with those for Deltex1 (Fig. 4). Although Izon et al. (2002) could detect transcripts for Deltex1 from the DN1 stage, contrasting with our EGF-FTOC-derived- DN1/2, they found an up-regulation in the transition from DN2 to DN3. In thymus physiology, Deltex up-regulation would succeed the specifying action of Notch1, its enforced expression in hematopoietic progenitors resulting in B cell development at the expense of T-cells in FTOC (Izon et al. 2002). Besides acting as a modulator of Notch action through the "classical CSL-mediated transduction pathway", Deltex1 was pointed as a mediator of a still poorly characterized pathway, acting as a transcriptional regulator downstream of the Notch receptor (Yamamoto et al. 2001, Allman et al. 2002).

Concerning the "classical downstream effectors" of Notch signaling, HES1 and HES5, we could not, again, observe differences that could explain growth blockade or its reversal upon INS addition (Fig. 4). In our model, HES1 transcripts were seen in EGF-FTOCs, when lymphoid growth was blocked. Unless if dose-dependent (not tested), HES1 transcripts would not be growth-limiting. Neither would be the HES5 transcripts, absent even during INS-driven growth. Active intracellular Notch was reported to augment also a HES-1-independent thymocyte proliferation, giving support to an alternative pathway (Ordentlich et al. 1998, Huang et al. 2003) that could putatively be associated with Deltex1 expression.

Notch signaling is not functional in Common Lymphoid Precursors until they enter the thymic rudiment, where, upon contact with ligands expressed by epithelial stromal cells, its activity turns essential (Harman et al. 2003). T- lineage commitment would require a high Notch expression within a thymic microenvironment, but such specific stromal ligands were not yet completely identified (Allman et al. 2002, Schmitt & Zúñiga-Pflücker 2002, Harman et al. 2003). Transcripts for the Notch ligands Jagged1 and Jagged2 have been described in the thymus, the first expressed by thymic stromal components, and the last by both stromal cells and thymocytes (Felli et al.1999, Kaneta et al. 2000). Here, when assessed by RT-PCR, Jagged2 was co-expressed with Notch3, upon INS action, differently from Jagged1, which expression was seen in both culture conditions. Transcripts for other ligands belonging to the Delta-like- family did not differ among the culture conditions here tested (Fig. 4). Were they: Pref1, reported to regulate the levels of HES1 expression and so influence the cellularity of developing thymus (Kaneta et al. 2000); Dll1, able to direct hematopoietic progenitors to a T/NK fate (Schmitt & Zúñiga-Pflücker 2002); and Dll4, implied in lymphoproliferative disease and T-cell lymphoma in mice (Dorsch et al. 2002).

Thus, while the redundant action of the different members of Notch family, their ligands and downstream targets, is under discussion (Felli et al. 1999, Osborne & Miele 1999, Anderson et al. 2000, Wolfer et al. 2002, Hozumi et al. 2003), our results point to the recruitment of a specific set of Notch signaling-related transcripts, if not causally related, at least simultaneous and compatible with

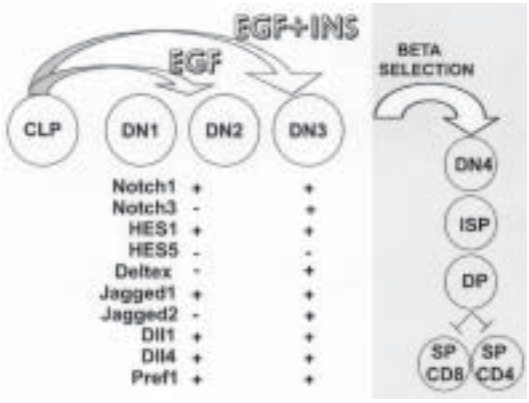


Fig. 5: schematic representation of epidermal growth factor (EGF) and insulin (INS) effects upon thymocyte development and expression of Notch-related transcripts. CLP: common lymphoid precursor; DN: double-negative, $CD4^-CD8^-$; ISP: immature single positive; DP: double-positive, $CD4^+CD8^+$; SP: single positive

thymocyte proliferation and DN1/2 to DN3 transition, as summarized in Fig. 5.

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