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Altered T Cell Differentiation and Notch Signaling Induced by the Ectopic Expression of Keratin K10 in the Epithelial Cells of the Thymus

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Abstract Transgenic mice expressing hK10 under the keratin K5 promoter display several alterations in the epidermis including decreased cell proliferation, and reduced susceptibility to tumor development. Given that K5 promoter is also active in the epithelial cells of the thymus, we explored the possible alterations of the thymus because of K10 transgene expression. We found severe thymic alterations, which affect not only the thymic epithelial cells (TEC), but also thymocytes. We observed altered architecture and premature thymus involution in the transgenic mice associated with increased apoptosis and reduced proliferation of the thymocytes. Interestingly, prior to the development of this detrimental phenotype, thymocytes of the transgenic mice also displayed altered differentiation, which is aggravated later on. Molecular characterization of this phenotype indicated that Akt activity is reduced in TEC, but not in thymocytes. In addition, we also observed altered expression of Notch family members and some of their ligands both in TEC and T cells. This produces reduced Notch activity in TEC but increased Notch activity in thymocytes, which is detectable prior to the disruption of the thymic architecture. In addition, we also detect altered Notch expression in the epidermis of bK5hK10 transgenic mice. Collectively the present data indicate that keratin K10 may induce severe alterations not only in a cell autonomous manner, but also in neighboring cells by the modulation of signals involved in cell–cell interactions. J. Cell. Biochem. 95: 543–558, 2005. © 2005 Wiley-Liss, Inc.

Key words: keratin; cell cycle; signal transduction; epidermis; transgenic mice; Akt; thymus; T lymphocytes; Notch

The issue of keratin protein function in epithelial cells and tissues is a matter of controversy. Keratin genes display a differential regulation in the various epithelia of the body and inherited mutations affecting keratin genes are responsible for a variety of epithelial fragility syndromes [Fuchs, 1995; Takahashi et al., 1999]. In addition to this shared family-wide functions, keratin filaments appear to carry out cell type- and context-dependent functions that include protection against metabolic stress, modulating apoptotic signals, cell cycle progression, and promotion of specific epithelial cytoarchitecture [Coulombe and Omary, 2002; Paramio and Jorcano, 2002; Herrmann et al., 2003; Kirfel et al., 2003]. Despite the recent progress in this field, our understanding of the relationship between keratin proteins and the differentiated epithelial cells remains poor. In particular, regarding functional explanation that may clearly justify the diversity and the tissue- and differentiation-specific expression patterns of these proteins.

Recently, we have investigated the possible existence of such specific keratin functions focusing on keratin K10. This protein is char-

Abbreviations used: SP, single positive T lymphocytes; DP, double positive T lymphocytes; FACS, fluorescence-activated cell sorter; TEC, thymic epithelial cells; NICD, Notch intracellular domain; PKC, protein kinase C.

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acteristic of epidermal keratinocytes that have entered the differentiation program and become postmitotic [Fuchs and Green, 1980]. Interestingly, K10 expression is severely reduced and even absent in conditions of hyperproliferation, including skin tumors [Roop et al., 1988]. We have shown that the expression of this keratin inhibits cell proliferation in cultured cells in a pRb-dependent manner through the inhibition of Akt kinase [Paramio et al., 1999, 2001a]. More recently, these results have been extended to include in vivo situations through the generation of transgenic mice in which human keratin K10 gene expression was targeted to the basal layer of the epidermis (bK5hK10 transgenic mice) [Santos et al., 2002b]. These animals displayed epidermal hypoplasia and reduced susceptibility to tumor formation upon chemical skin carcinogenesis protocols [Santos et al., 2002b]. These data suggested that keratin K10 might control proliferation in cells committed in the differentiation program. This hypothesis seemed to be confirmed by the epidermal hyperplasia displayed by adult keratin K10-null mice [Reichelt and Magin, 2002]. However, in these animals, hyperproliferation was confined to basal cells, indicating that K10 can mediate effects in the neighboring cells [Reichelt and Magin, 2002]. The mechanism involving the signaling between suprabasal and basal keratinocytes remains unknown.

The bK5hK10 mice display early lethality [Santos et al., 2002b]. Necropsy analyses showed that these mice are frequently immunocompromised. Indeed peripheral blood analyses showed a dramatic reduction in granulocytes, macrophages, T and B lymphocytes (Fig. 1). However, we did not observe ectopic expression of the transgene in spleen or bone marrow (not shown). Nevertheless, the keratin K5 promoter used in the generation of the bK5hK10 mice is active in the medullar epithelial cells of the thymus [Ramirez et al., 1994]. Given that thymus and T cells are involved in the maturation of other immune cells, this might suggest that the observed defect is due to the ectopic expression of K10 in these cells.

Among the different organs of the body, the thymus is unique as it has an epithelial compartment that cannot be strictly classified as simple or stratified epithelium. Thymic epithelial cells (TEC) are not organized as the characteristic epithelial sheets in other organs, but rather form a three-dimensional network. This

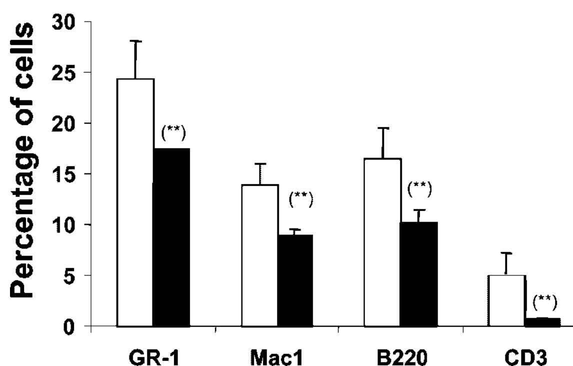

Fig. 1. Peripheral blood parameters are affected in bK5hK10 transgenic mice. Blood from control (open bars) and bK5hK10 transgenic mice (closed bars) was extracted at 8 weeks post-birth and stained with monoclonal antibodies to identify the different leukocyte subpopulations. A clear reduction in the percentage of lymphoid lineages, and lymphoid and myeloid lineages was observed at 2 and 8 weeks, respectively. Statistical significance (*t* test) is 0.995 (**).

distribution of TEC facilitates the thymocyte migration and the interaction with thymocyte subsets located in the cortex, and medullary regions. The different TEC populations can be distinguished by the expression of specific keratin subsets [Klug et al., 1998]. TEC in the thymic cortex predominantly express K8 and K18, whereas in the medulla they express K5 and K14. Finally, there are some TEC concentrated at the corticomedullary junction that coexpress K8 and K5 [Klug et al., 1998]. The changes in keratin expression that take place during the differentiation of these cells and the development of the thymus [Klug et al., 1998, 2002], makes this organ a very attractive system where the issue of different keratin functions can be studied. Importantly, these specific TEC subsets affect the development and differentiation of thymocytes in early embryos [Klug et al., 1998, 2002], whereas thymocyte-derived signals are required during late fetal stages for continued development and maintenance of TEC subsets [Klug et al., 2002]. The developmental pathways leading to the generation of mature CD4 and CD8 single positive (SP) T cells from immature $CD4^{-}CD8^{-}$ double-negative (DN) thymocyte precursors in the thymus have been extensively studied (reviewed in [Ellmeier et al., 1999; Manley, 2000; Chidgey and Boyd, 2001]. During the T-lineage commitment process, $CD44^{+}CD25^{-}$ progenitors in the DN compartment up-regulate CD25, down-regulate CD44, and initiate T cell antigen receptor (TCR) γ-, δ-, and β-gene rearrangements. $CD44^{-}CD25^{+}$

pre-T cells that productively rearrange the TCRβ locus and express pre-TCR/CD3 complexes proliferate and differentiate to the $CD4^{+}CD8^{+}$ double-positive (DP) stage. The DN to DP transition is accompanied by loss of CD25 expression, prohibition of TCRβ locus rearrangements, and induction of TCRα locus rearrangements. Signaling through the α βTCR/CD3 complexes on DP thymocytes mediates the positive and negative selection processes that shape the T cell repertoire. DP thymocytes that are positively selected by self-peptide/major histocompatibility complex molecules presented on cortical epithelial cells terminate CD8 or CD4 expression and migrate to the thymic medulla. TEC are not only involved in the selection of DP cells, but also promote differentiation of early DN thymocyte precursors. Nevertheless, the factors which govern TEC development, the possible influence of keratin cytoskeleton in such process, and how the differentiation of TEC might affect T cell developmental processes are incompletely understood.

The epidermis and the thymus share common molecular circuits that govern cell fate decisions and differentiation processes. Among the different developmental pathways involved in both tissues, Notch signaling has emerged as key player [Kopan and Weintraub, 1993; Deftos et al., 1998; Robey, 1999; Deftos and Bevan, 2000; Anderson et al., 2001; Rangarajan et al., 2001; Nicolas et al., 2003]. Notch family of proteins are transmembrane receptors implicated in cell differentiation in many tissues (for review, see [Mumm and Kopan, 2000]. The mammalian Notch pathway includes at least four Notch receptor genes (*Notch1–Notch4*) and multiple ligands (*Jagged1–Jagged2* and the *Delta* family). Upon ligand binding, the Notch protein is proteolytically cleaved to release its intracellular domain (NICD), which represents an activated form of the Notch receptor. NICD then translocates into the nucleus and binds the transcription factor RBP-Jκ. In absence of NICD, RBP-Jκ is a DNA-binding protein that normally represses transcription by virtue of its interaction with several corepressor complexes. The binding of NICD converts RBP-Jκ into a transcriptional activator and thus induces the expression of target genes, most notably the Hes family of transcriptional repressors. Here, we have analyzed the functional consequences of ectopic expression of keratin K10 in the epithelial cells of the thymus in bK5hK10 transgenic mice. This allowed us to study the mechanism by which the changes in the keratin expression pattern in this cell population may affect the structure and function of this organ in the transgenic mice. Moreover, since our previous data suggested that K10 expression modulates epidermal cell proliferation and differentiation, this transgenic mouse model permit us to understand how alterations in these processes in TEC affect the whole organ including the possible mechanisms that connect TEC and T cell differentiation in vivo. We report that the expression of keratin K10 leads to a severe thymic phenotype. This affects proliferation and apoptosis of TEC and leads to severely perturbed T cell differentiation associated with alterations in Notch signaling mediated by the ectopic expression of keratin K10.

MATERIALS AND METHODS

Transgene Construction and Generation of Transgenic Mice

The generation of the transgenic mice and identification have been described previously [Santos et al., 2002b].

Histological Analysis

Thymi were dissected and fixed either in formalin or ethanol and embedded in paraffin prior to sectioning. Sections (5 μm) were cut and stained with haematoxylin–eosin. For specific labeling purposes, thymi were also frozen immediately after sacrifice and embedded in OCT compound. Skin and thymi cryosections (7 μm) were fixed in cold acetone (5 min) and used in immunofluorescence labeling as described [Paramio et al., 2001a,b; Santos et al., 2002b]. At least five different mice were analyzed at each time point.

5-Bromo-2′-Deoxyuridine (BrdU) and TUNEL Labeling

Mice were injected i.p. with 0.1 mg per 1 g of animal weight BrdU in 0.9% NaCl, and thymi were obtained 1 h later. To detect BrdU incorporation, deparaffinized thymic sections were incubated in 2N HCl for 1 h at room temperature. After washing in PBS, the sections were incubated with rat anti-BrdU (a generous gift from Dr. S. Mittnacht) for 1 h at room temperature. To detect TUNEL-positive cells, 5 μm frozen sections were processed using an In Situ cell death detection kit (Roche, Basel,

Switzerland) per manufacturer's instructions. Negative control slides were incubated in reaction mixture without TdT.

Antibodies

The antibodies used included polyclonal antibodies to Notch 1 (Santa Cruz sc-114, Santa Cruz, CA), Notch 2 (Santa Cruz sc-372), Notch 3 (Santa Cruz sc-5593), Delta (Santa Cruz sc-371), Delta like (Santa Cruz sc-7182), Jagged 1 (Santa Cruz sc-8032) Jagged 2 (Santa Cruz sc-8032), Stat3 (R&D, Minneapolis, MN), keratin K10 (K8.60, Sigma), keratin K5 (Covance, Richmond, CA), keratin K8 (TROMA1 rat mAb, kindly provided by Dr. R. Kemler), Akt1/2 (Santa Cruz), and P473 Akt (Cell Signaling, Beverly, MA). Antibodies used in FACS were purchased from Pharmingen (San Jose, CA) and included anti Mac-1, GR-1, B220, CD45, CD44, CD3, CD4, or CD8 antigens and were directly conjugated with either fluorescein isothiocianate (FITC) or phycoerythrin (PE).

Western Blots

Whole thymus were collected in lysis buffer (Tris pH 7.5, NaCl 150 mM, EDTA 1 mM, EGTA 1 mM, β-glycerophosphate 40 mM, sodium orthovanadate 1 mM, PMSF 0.1 mM, aprotinin 2 μg/ml, leupeptin 2 μg/ml, NP-40 1%). To obtain TEC and thymocyte extracts, thymi from 2-month-old mice were minced gently, and the fragments were pipetted in a layer on fetal calf serum (FCS) to collect thymocytes as floating cells. FCS was removed from thymocyte preparations by exhaustive washing with ice-cold PBS and centrifugation. Afterwards, protein extracts were obtained in lysis buffer. Protein mixtures (10 μg) were separated on 4%–20% sodium dodecyl sulfate–polyacrylamide gradient gels (SDS–PAGE) and transferred to nitrocellulose filters (Hybond ECL, Amersham, Little Chalfont, UK). Filters were blocked with 5% non-fat dry milk in PBS-0.1% Tween 20 at 4°C overnight, washed three times in PBS-0.1% Tween 20, and incubated with the indicated antibodies. After washing, membranes were incubated with a peroxidase-conjugated secondary antibody, washed again, and analyzed using the enhanced chemiluminiscence method (West Picosignal, Pierce, Rockford, IL), according to the manufacturer's instructions. Membranes were stripped by incubation with 62.5 mM Tris-HCl (pH 6.7), 2% sodium dodecyl sulfate (SDS), 100 mM β-mercaptoethanol at 55°C for 30 min, and reprobed with different antibodies. Anti actin antibody (Santa Cruz) was used to monitor the equal loading.

Flow Cytometry Studies

Cells obtained from peripheral blood and thymus were incubated for 10 min with ammonium chloride lysis solution (0.155 mM NH_4Cl, 0.01 mM $KHCO_3$, 10^{-4} mM EDTA) to eliminate erythrocytes and washed with PBA (PBS plus 0.1% bovine serum albumin plus 0.02 NaN_3). Then, cells from peripheral blood were stained for 30 min with antibodies that recognize Mac-1, GR-1, B220, CD4, or CD8 antigens. For CD3 evaluation, cells were incubated with anti CD3 biotinylated antibody (Pharmingen), washed and incubated again with streptavidin-Tricolor (Caltag, Burlingame, CA). Cells were washed, resuspended in PBA with 2 μg/ml propidium iodide (PI) and analyzed in an EPICS XL flow cytometer (Coulter Electronics, Hialeah, FL). A minimum number of 10^4 cell were acquired. Off-line analysis was done with WinMDI free software package (a kind gift from Dr. J. Trotter, The Scripps Research Institute, La Jolla, CA).

Band Shift Analysis

Electrophoretic mobility shift assays (EMSA) were performed by incubating whole cell extracts with a labeled oligonucleotide corresponding to a palindromic κB site. The sequence of the κB oligonucleotide coding strand was 5′-GATCCAACGGCAGGGGAATTCCCCTCTCC-TTA-3′.

Complexes were separated on 5.5% native polyacrylamide gels in 0.25× Tris-borate-EDTA buffer, dried and exposed to Hyperfilm-MP (Amersham) at −70°C. The analysis and composition of the κB complexes were determined as previously described [Perez et al., 2000; Santos et al., 2003].

Northern Blots

Total RNA from freshly harvested TEC or thymocyte preparations was isolated by Trizol (Sigma) extraction. Northern blots containing total RNA (15 μg/lane) were probed for expression of HES-1 employing DNA probes prepared by random primed reactions using the complete mouse cDNA [Kageyama et al., 2000]. The membranes were also hybridized with a ribosomal 7S RNA probe to verify that equal

amounts of RNA were loaded and transferred. Quantification was performed using Phosphorimager (Bio-Rad) plus Quantity1 software (Bio-Rad).

RESULTS

Thymus Involution in bK5hK10 Transgenic Mice

Transgenic mice in which the expression of K10 is driven by the keratin K5 promoter were generated to study the keratin K10 functions in vivo. This leads to the ectopic expression of the transgene in the basal layer of stratified epithelia such the epidermis and the epithelial cells of the thymus. Five different transgenic lines were generated. All the animals appeared healthy at birth, but by day 21 those animals bearing high copy number of the transgene displayed a clear detrimental phenotype leading to death between days 34 and 79 [Santos et al., 2002b]. This effect is in agreement with previous findings indicating that the functional specific effects of keratin K10 are related to the expression level [Paramio et al., 1999, 2001a]. We generated two transgenic mice lines in which only homozygous transgenic animals displayed epidermal phenotypic alterations and this early lethality.

The cause of death in these mice is still unknown. However, peripheral blood analyses indicated that bK5hK10 mice are immunodeficient (Fig. 1). However, we failed to detect transgene expression in the spleen or bone marrow (not shown). On the other hand, given the involvement of thymus and T cells in the maintenance of other immune cells and that the K5 promoter is active in TEC of the thymic medulla [Ramirez et al., 1994], the observed immunodeficiency can be due to alterations in this organ. We first analyzed the possible expression of the K10 transgene in parallel with other keratins expressed in TEC.

By 2 weeks of age, prior to the development of any detrimental phenotype, immunohistochemical analyses demonstrated that K10 (Fig. 2A″) is expressed in most of the K5-expressing cells of the thymus (Fig. 2A), whereas most of the K8 positive cells located in the cortex (Fig. 2A′) are K10-negative (Fig. 2A″). It is worth mentioning that similar distribution of K5+ and K8+ cells was observed in non transgenic thymi, although K10 expression was not detected (not shown). In this regard, the expression of K10 has never been reported in TEC [Klug et al., 1998, 2002; Kuo, 2000].

We next examined the possible histological alterations because of the K10 ectopic expression. The thymus of the bK5hK10 transgenic mice (Fig. 2B′) is indistinguishable from the control, non-transgenic littermates (Fig. 2B) by 2 weeks of age. However, by 8 weeks of age while the control wt thymus (Fig. 2C) preserves most of the characteristics observed at 2 weeks of age, several alterations were observed in the transgenic mice. These include the reduction of the cortex (Fig. 2C′), paralleled with a reduction of the thymic cellularity (Fig. 2E). In the extreme situation, this leads to the presence of only small thymic rudiments in which the separation between cortex and medulla was no longer obvious (Fig. 2D).

Ectopic Expression of K10 Leads to Altered T Cell Differentiation

The histological defects in the thymus lead us to investigate the differentiation of cells in this organ. We detected an acute decrease in the number of DP and CD3+ cells in the bK5hK10 thymus (Fig. 3A,A′), becoming more evident at 8 weeks of age (Fig. 3A′), where all the cell subtypes are affected. This general reduction might reflect the overall decrease in the thymus cellularity observed (Fig. 2E). Because other cell types were not affected by 2 weeks of age (Fig. 3A), we suggest that differentiation processes can be also affected. Consequently, we also monitored the percentage of the different cell types. By 2 weeks of age (Fig. 3B), prior to the altered architectural defects, a moderate increase in the percentage of DN, CD4 SP, and CD8 SP cells was detected, whereas the DP percentage decreases. In 8 weeks transgenic mice, we found increased CD3+, CD4 SP, and CD8 SP percentages whereas the percentage of the DN population decreases (Fig. 3B′). Collectively, the above commented results demonstrated that the ectopic expression of K10 does not inhibit thymocyte differentiation at a certain stage, but rather the overall thymocyte differentiation is dramatically perturbed, besides inducing a dramatic decrease in the different cell types in the thymus.

The development of thymocytes is associated with the development and differentiation of different TEC subpopulations. In particular, the minor K8+;K5+ subset, which is highly represented at the corticomedullary junction,

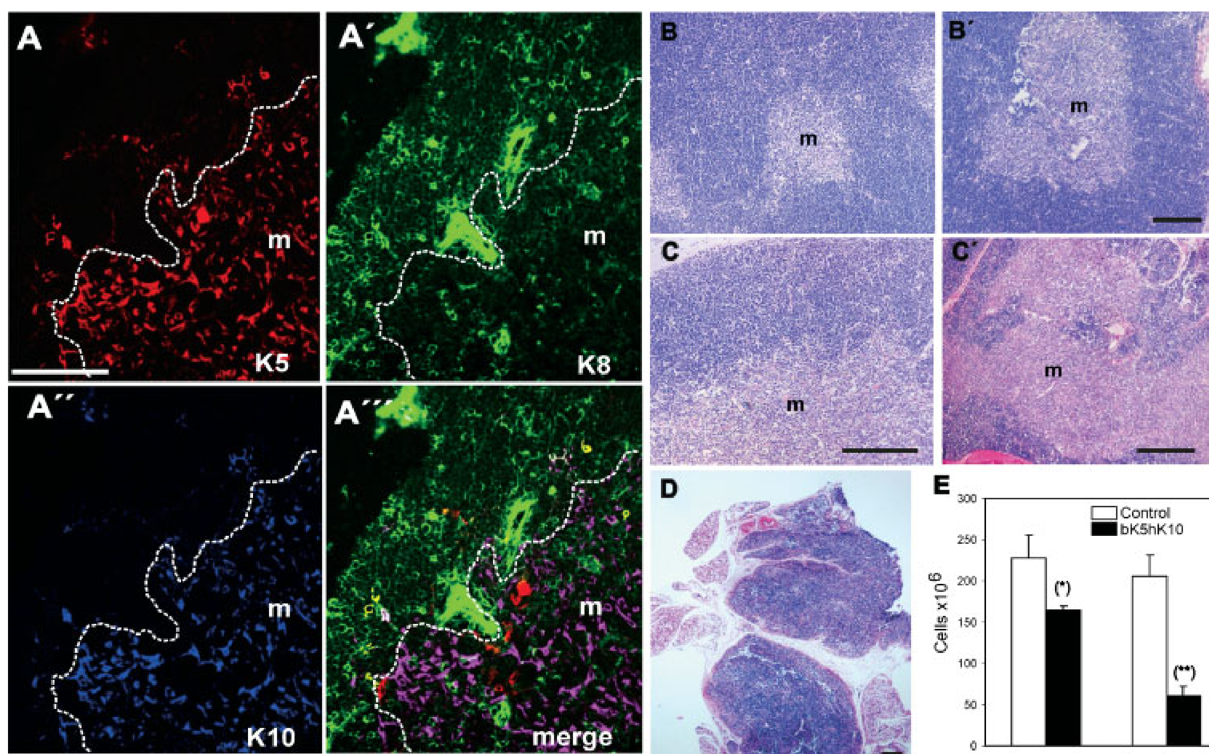

Fig. 2. Keratin K10 is expressed in the thymus of bK5hK10 transgenic mice and induces premature thymus involution. The expression of keratin K5 (**A**), K8 (**A′**), and K10 (**A″**) was monitored by triple immunofluorescence. Merged image (A‴) demonstrated that K10 is co-expressed with K5. **B–C′**: Histology of thymus from control (B, C) and bK5hK10 transgenic mice (B′, C′) at 2 weeks (B, B′) and 8 weeks (C, C′) of age: Note no major alterations in the architecture of this organ at 2 weeks of age, whereas at 8 weeks transgenic mice thymi showed increased medulla and decreased cellularity in the cortex. **D**: Example of a severe phenotype in bK5hK10 transgenic mice at 8 weeks of age. Note that medulla and cortex are not distinguishable and only small thymus rudiments are present. **E**: Cellularity of the thymus from control (open bars) and transgenic mice (closed bars) at 2 and 8 weeks of age. Note the decreased cell content in the thymus of the transgenic mice, which is more evident at 8 weeks of age. Data in E come from six to eight different mice and are shown as mean ± SD. Statistical significance (*t* test) is 0.95 (*) and 0.995 (**). m denotes the medulla. Bars = 150 μm.

has been involved in a differentiation process concomitant with T cell lineage commitment [Klug et al., 1998]. Consequently, we monitored whether the expression of K10 affected this cell subpopulation. Thus, the expression of K5 and K8 were monitored in control and transgenic mice thymi. By 2 weeks of age, no major alterations in the distribution of the different TEC subpopulations were observed among control and transgenic mice thymi (not shown, see also Fig. 2A–A‴). However, we observed by 8 weeks an increased proportion of K8+ cells in the medulla of the transgenic thymus (Fig. 4B) compared to the controls. Moreover, a significant proportion of these cells also expressed K5 (Fig. 4A), leading to altered distribution of K8 + K5+ TEC subsets (Fig. 4C), which are also located in the medullary region rather than in the corticomedullary junction (arrows in Fig. 4C,E). These data demonstrate that altered distribution of the TEC population in the transgenic mice only takes place by 8 weeks of age. They also suggested that the altered differentiation of thymocytes is not an indirect consequence of the overall disturbances attributable to a possible toxic effect of the transgene, as these alterations were already observed by 2 weeks of age, when no major alterations in the in thymus architecture were found.

Decreased Proliferation and Increased Apoptosis in T Cells of bK5hK10 Transgenic Mice

The ectopic expression of keratin K10 is associated with a decreased proliferation and increased apoptosis in epidermal cells in vivo and in vitro [Paramio et al., 2001a; Santos et al., 2002a,b, 2003]. Thus, a possible explanation for the above-described alterations in the bK5hK10 thymi might be altered proliferation or apoptosis in the epithelial medullary cells of the

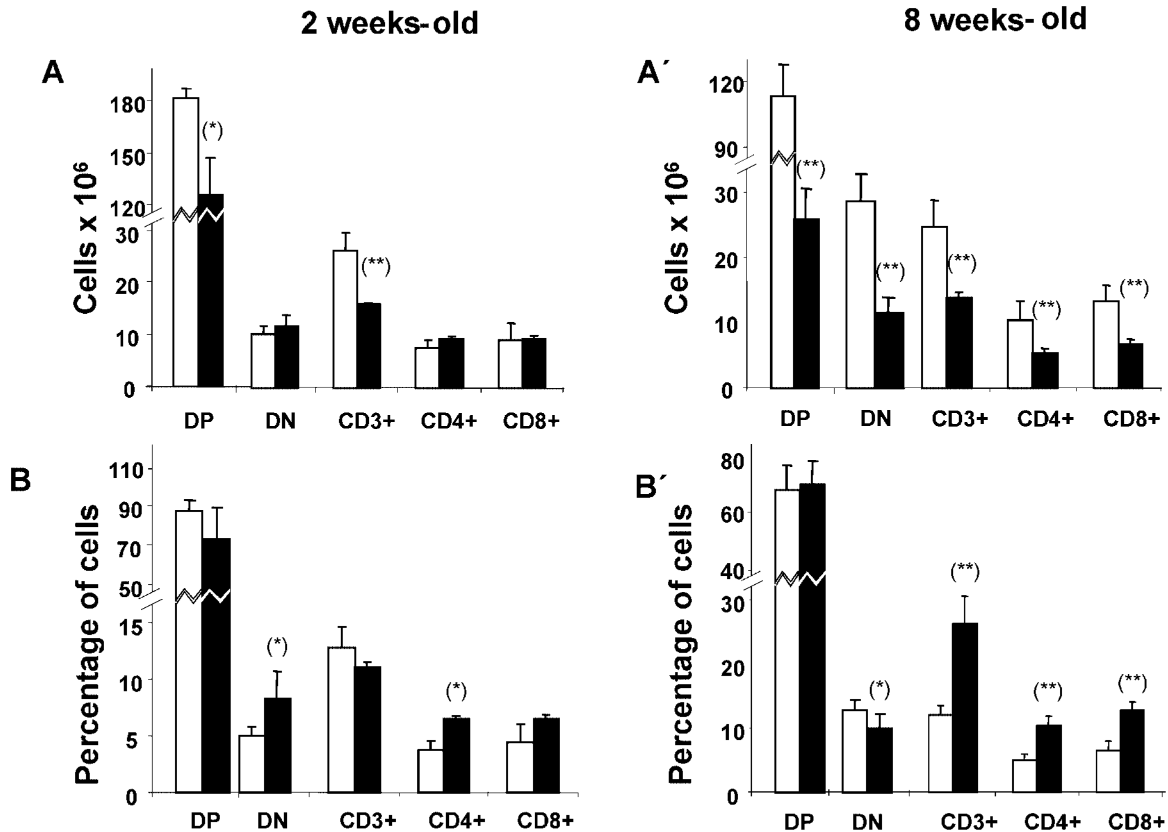

Fig. 3. Impaired T cell differentiation in bK5hK10 transgenic mice. Mononuclear cells from thymi of control (open bars) and bK5hK10 transgenic mice (closed bars) were extracted and analysed by flow cytometry for the expression of different T cell markers at 2 (**A**, **A′**) and 8 weeks (**B**, **B′**) after birth. A: Total number of cells of each phenotype present in the thymus. B: Percentage of the different thymocyte phenotypes is represented. A clear disbalance in the normal rates of cell differentiation and an overall reduction in the number of cells in 8-week-old transgenic mice is observed. Data come from five different mice and are shown as mean ± SD. Statistical significance (*t* test) is 0.95 (*) and 0.995 (**).

transgenic mice. To monitor this possibility, we studied the BrdU incorporation and TUNEL positive cells. In 2-weeks-old mice, BrdU incorporation was similar in control (Fig. 5A) and in transgenic mice (Fig. 5A′), but, interestingly, in both cases the proliferation appeared to take place almost exclusively in K5 negative cells, probably lymphocytes. By 8 weeks of age, the proliferative rate in control mice was maintained in cells of the cortex and corticomedullary region, again mostly in K5 negative cells (Fig. 5B). On the contrary, a clear inhibition of BrdU incorporation was observed in the transgenic mice at this age (Fig. 5B′). A possible explanation for this effect can be that such decrease in BrdU+ cells is due to the net loss of DPs and DNs cells which are the majority of thymocytes in cycle. The apoptosis studies render equivalent results to those of BrdU incorporation. TUNEL labeling was essentially similar between control and transgenic mice by 2 weeks of age (Fig. 5C,C′, respectively). However, an increase in the TUNEL positive cells was observed in 8-weeks-old transgenic mice thymi (Fig. 5D,D′). Interestingly, the increase in apoptosis seems to affect to both the K5-negative cells and the K5-positive cells in the thymic medulla of these mice.

Collectively, our data indicated that the expression of K10 in the TEC population initially induces alterations not only in these cells but also in the neighboring thymocytes, which display altered differentiation. Later on, this phenotype is aggravated and reduced proliferation and increased apoptosis were found in the thymocytes of the transgenic mice. The differentiation, proliferation, and apoptosis are interconnected processes that might account to the gross phenotype of thymi of the bK5hK10 transgenic mice by 8 weeks of age, in which not only the medulla, but also the cortex is severely affected. Altogether these defects might account

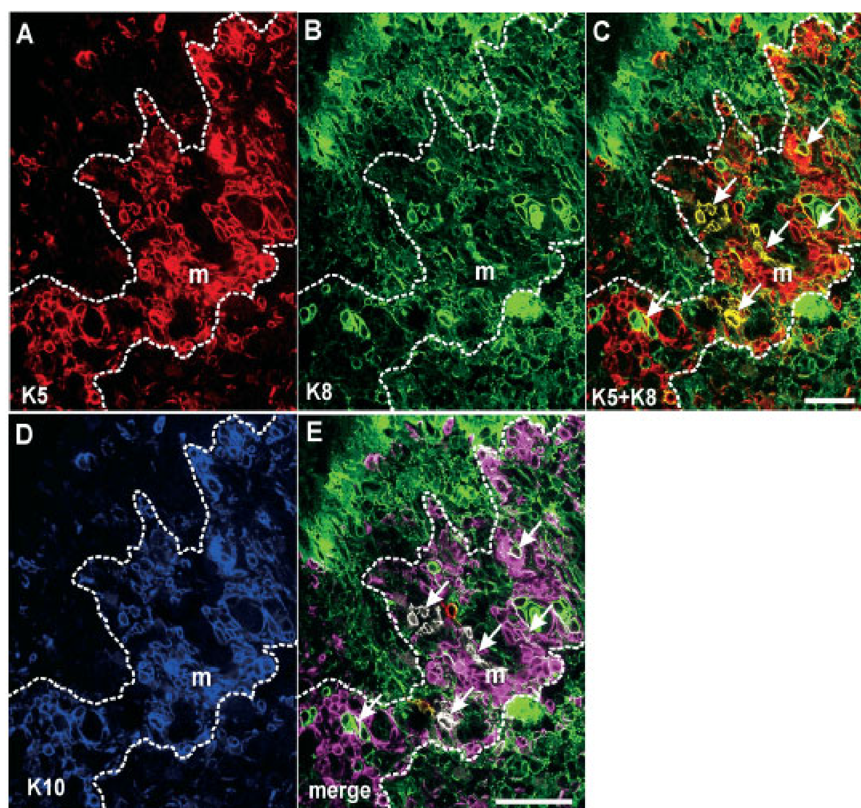

Fig. 4. Distribution of epithelial cells in the bK5hK10 thymus is altered at 8 weeks of age. Immunofluorescence of bK5hK10 transgenic mice thymus at 8 weeks of age showing the expression of K5 (**A**), K8 (**B**), and K10 (**D**). Note the co-expression of K5 and K8 in epithelial cells of the medulla (arrows in **C**), which also express K10 (arrows in **E**). C: Merged image of A and B. E: Merged image of A, B, and D. m denotes the medulla. Bars = 100 μm.

for the overall decrease in immune cells observed in peripheral blood in adult mice (Fig. 1). In addition, these observations also indicate that keratin K10 is acting in a non cell autonomous manner to influence thymocyte physiology already at 2 weeks of age, prior to the development of the detrimental phenotype of the thymus. Consequently, we monitored different signaling pathways that may account for these alterations.

Altered Akt and NFκB Signaling in bK5hK10 Thymus

K10 sequesters Akt kinase precluding its activation at the cell membrane [Paramio et al., 2001a]. Similar inhibition is also observed in the epidermis of bK5hK10 transgenic mice preventing the tumor formation upon chemical carcinogenesis protocols [Santos et al., 2002b]. Consequently, we monitored the activity of Akt in the thymus using an immunohistochemical approach employing an antibody that specifically reacts with the phosphorylated active Akt. We found strong decrease in phosphorylated Akt in transgenic thymus compared to controls at 2 weeks of age, being such decrease more evident by 8 weeks of age (Fig. 6A,A′,B,B′). To further confirm this observation a biochemical analysis was performed. Whole thymus extracts or purified TEC or thymocyte extracts from 2-week-old transgenic or control mice were obtained. The purity of these fractions was monitored by Western blotting against keratins K5 and K10 (Fig. 6D, upper panels). Regarding the Akt expression and activity, whereas similar levels of Akt protein were observed among transgenic and control samples, the levels of phosphorylated Akt were decreased in transgenic TEC extracts (Fig. 6D). This indicates that Akt activity was severely impaired in the TEC population of the transgenic mice but not in control TEC or thymocytes or in transgenic thymocytes.

It has been recently reported that Akt kinase may activate Stat3 [Kortylewski et al., 2002; Sun and Steinberg, 2002]. In addition, mice

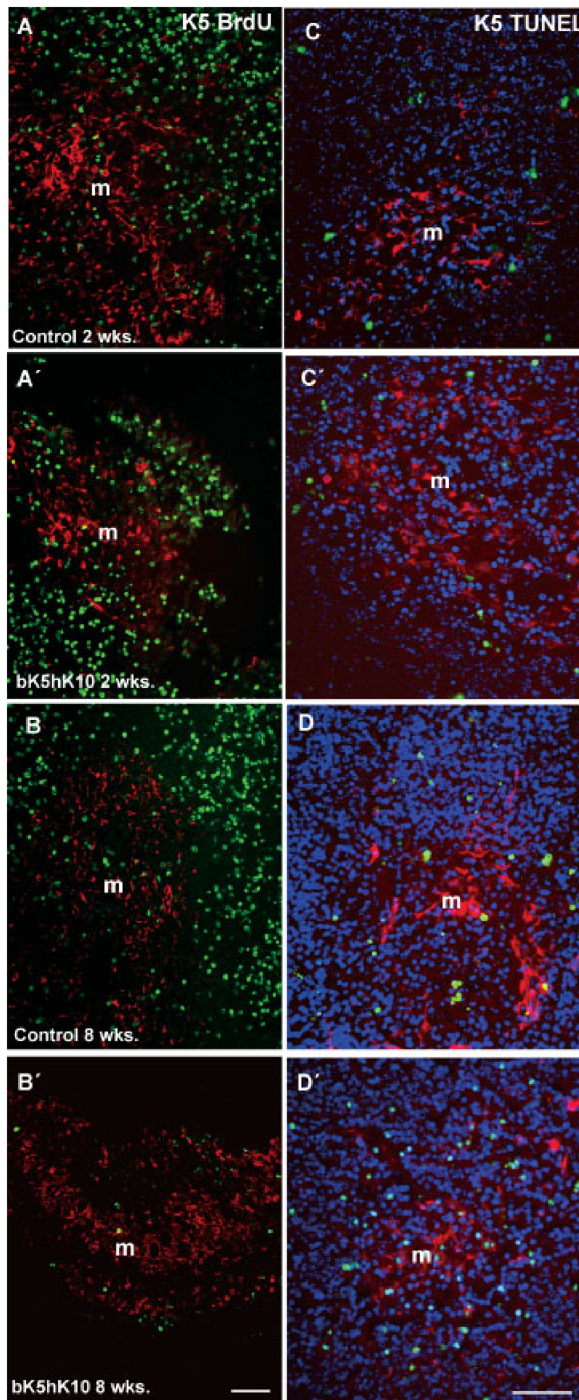

Fig. 5. Apoptosis and proliferation in bK5hK10 thymus. Immunofluorescence of control (**A**, **B**, **C**, and **D**) and bK5hK10 transgenic mice (**A′**, **B′**, **C′**, **D′**) thymus at 2 (A–C′) and 8 weeks of age (B–D′) showing the proliferation, as BrdU incorporation (A–B′), and apoptosis, detected by TUNEL labeling (C–D′) together with K5 (red). DAPI (blue labeling) was used to counterstain nuclei in TUNEL labeling experiments. Note the decrease in proliferation and the increase in thymocyte apoptosis observed in the transgenic mice thymus that takes place only at 8 weeks of age. m denotes the medulla. Bars = 100 μm.

lacking Stat3 gene in the TEC population display several defects, including premature involution, similar to some of those reported here [Sano et al., 2001]. Therefore, we next investigated the localization and expression of Stat3 in the thymus of bK5hK10 transgenic mice. We did not observe any change in the expression of Stat3 when compared with controls at 2 (Fig. 6D) or at 8 weeks of age (not shown). Moreover, we found that Stat3 expression and nuclear localization at 8 weeks of age was similar in both in TEC and thymocytes of the transgenic mice and the controls (Fig. 6C,C′). These data indicate that the defects found were primarily not attributable to altered Stat3 signaling in the transgenic mice.

We have recently reported that the K10-mediated Akt inhibition in epidermis leads to impaired NFκB signaling [Santos et al., 2003]. Similarly, we observed decreased basal NFκB activity in the transgenic TEC population (Fig. 6E), in agreement with the observed decreased Akt activity. Surprisingly, we also found decreased basal NFκB in transgenic thymocytes (Fig. 6E′). Given the well reported roles of NFκB signaling in T cell differentiation and function, the observed inhibition of NFκB activity might explain some of the observed defects in these parameters in thymocytes. Indeed, this is in agreement with the decreased proliferation and increased apoptosis observed in this population. The possible mechanism responsible for such decreased NFκB basal activity in thymocytes does not seem to be due to altered Akt or Stat3 activation in these cells. The partial NFκB activity observed in T cells can be triggered by the activation of specific protein kinase isotypes or through the activation of non-canonical signaling pathway (reviewed in [Hayden and Ghosh, 2004]. In this regard, it is worth mentioning that K10 also interferes with certain PKC isotypes, such as λ and ζ [Paramio et al., 2001a; Santos et al., 2002b]. Nevertheless, as K10 is not expressed in T cells (Fig. 6D see also [Kuo, 2000]) it is possible that the remaining basal NFκB activity in these cells is mediated through this pathway.

Altered Notch Signaling in bK5hK10 Thymus

The above commented results indicate that the expression of K10 in the TEC population of transgenic mice leads to altered differentiation, increased apoptosis, and decreased proliferation of thymocytes, indicating that a paracrine

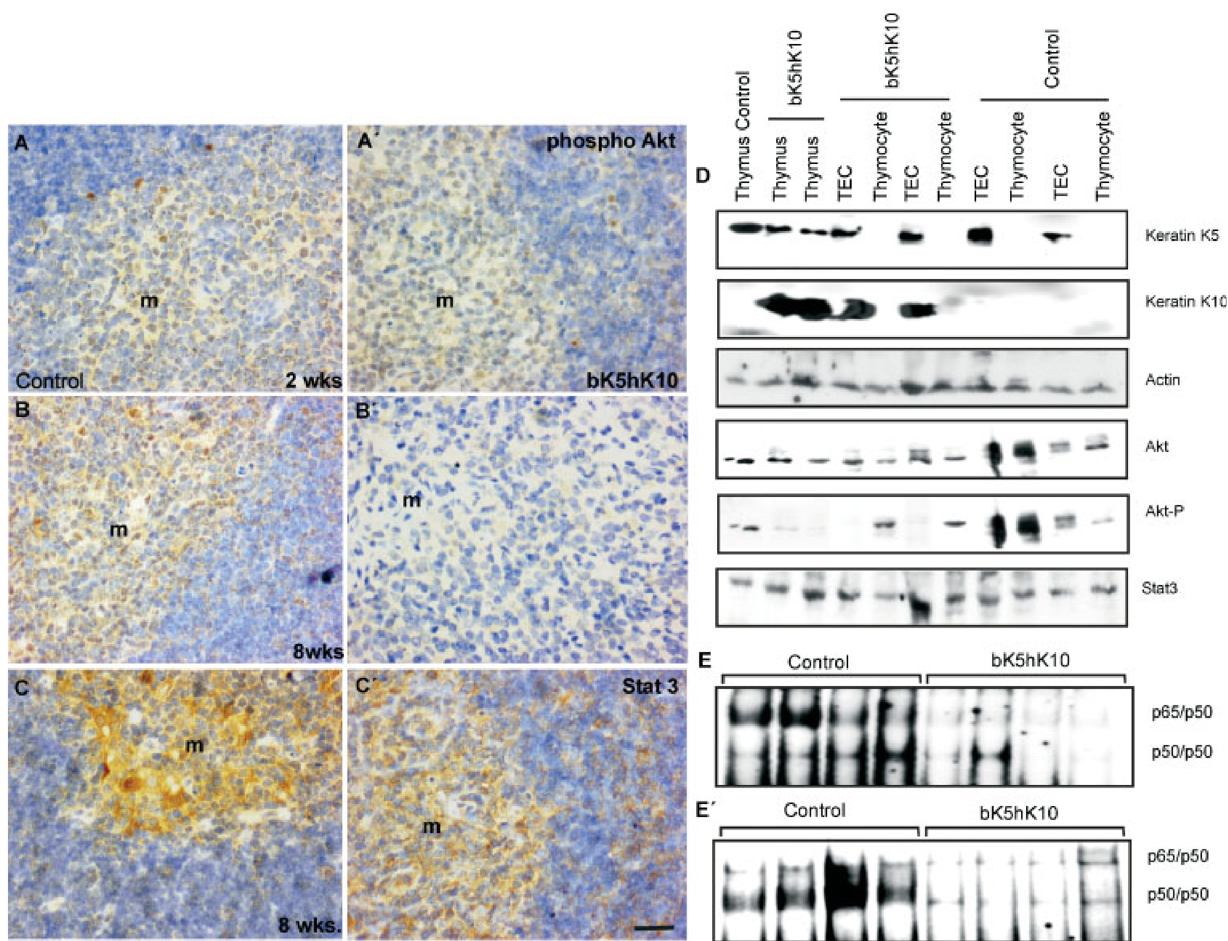

Fig. 6. Altered Akt and NFκB signaling in bK5hK10 thymus. Immunohistochemical detection of phosphorylated Akt in control (**A** and **B**) and bK5hK10 transgenic mice (**A′** and **B′**) thymus at 2 (A, A′) and 8 weeks of age (B–**D′**). **C**, **C′**: Immunohistochemical detection of Stat3 in control (C) and bK5hK10 transgenic mice (C′) thymus at 8 weeks of age. m denotes the medulla. Bars = 100 μm. **D**: Biochemical analysis of protein extracts from whole thymus, TEC or thymocyte extracts from control and bK5hK10 transgenic mice at 2 weeks of age showing the reduction in Akt phosphorylation in TEC population of the transgenic mice. **E**, **E′**: Basal NFκB activity analyzed by EMSA of TEC (E) and thymocytes (E′) from different control and bK5hK10 transgenic mice samples. Note the reduced DNA binding observed in the transgenic samples.

signaling is affected by K10 expression. Among the different possibilities, several observations might indicate that Notch signaling is responsible for the observed alterations. In fact, Notch signaling modulates thymocyte differentiation [Deftos et al., 1998; Robey, 1999; Deftos and Bevan, 2000; Anderson et al., 2001; Guidos, 2002]. Moreover, it has been reported that Notch signaling inhibits basal NFκB activity [Wang et al., 2001; Oakley et al., 2003]. In addition, K10 is normally expressed in the suprabasal layers of epidermis, sites of normal Notch expression [Rangarajan et al., 2001]. Consequently, we monitored the expression of Notch family members and their ligands in TEC and thymocytes from bK5hK10 transgenic mice.

Western blot analyses demonstrate no major changes in Notch1 or Delta among transgenic and control samples (Fig. 7A). On the contrary, we clearly observed a decreased expression of Notch3, Jagged1, and Jagged2 in both TEC and thymocytes from transgenic mice (Fig. 7A). Finally, increased Notch2 expression in both cell populations from bK5hK10 transgenic thymi was observed (Fig. 7A). To further confirm these suggestions, we performed immunostaining for Notch2, Jagged1, and Delta in thymus sections from 2-week-old transgenic and control mice. The results (Fig. 7B–D) confirmed the biochemical data and indicate that the expression of K10 in TEC induces changes in the expression of specific elements of the Notch signaling pathway. The fact that only

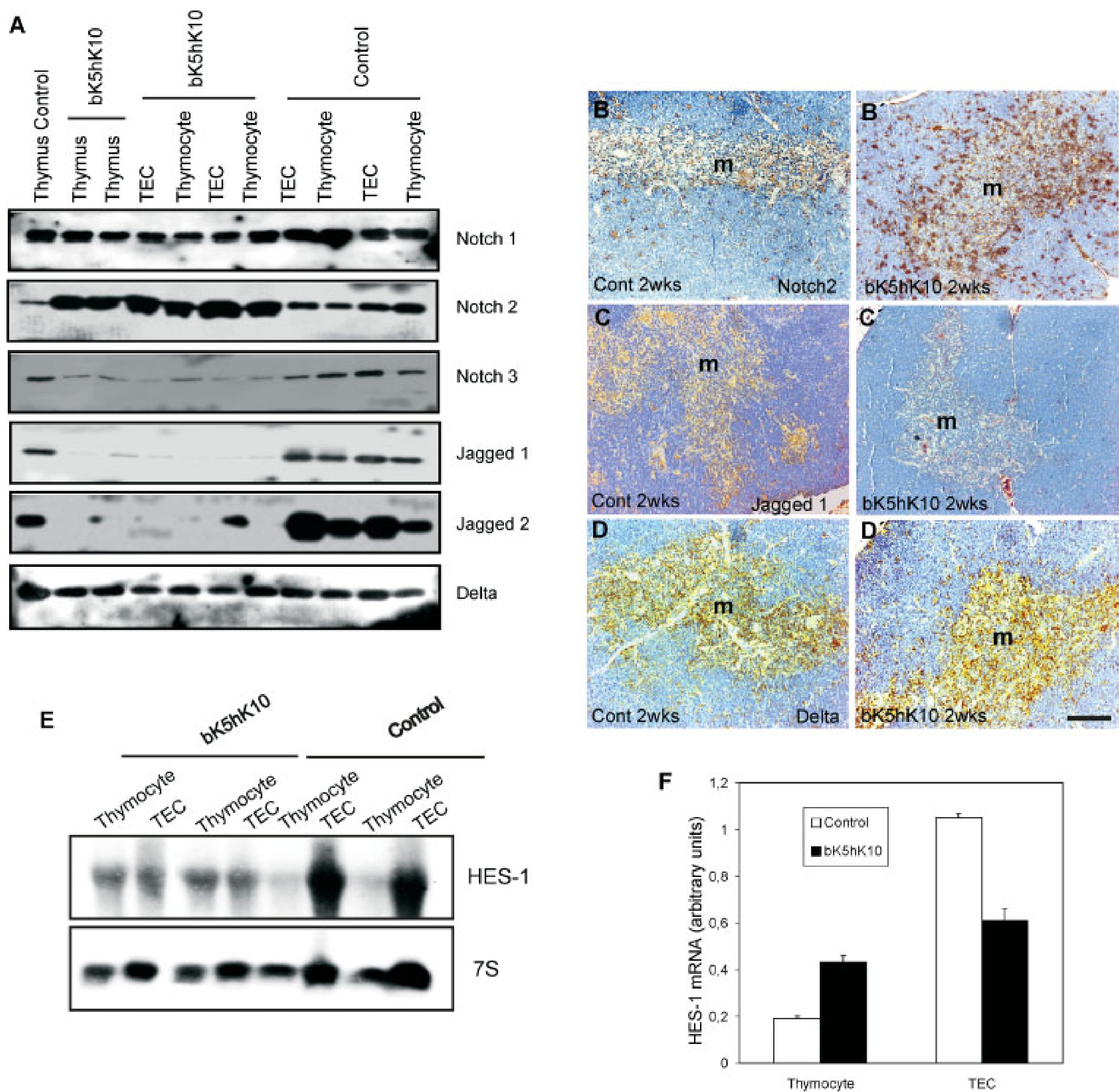

Fig. 7. Altered Notch signaling in bK5hK10 thymus. The expression of the different Notch pathway components was analyzed by Western blot using the same protein extracts as shown in **D** and the quoted antibodies. Note the increase in Notch 2 and reduced Notch 3, Jagged 1 and Jagged 2 in the transgenic mice samples. Immunohistochemical detection of Notch 2 (**B**, **B′**), Jagged 1 (**C**, **C′**), and Delta (D, **D′**) in control (B, C, and D) and bK5hK10 transgenic mice (B′, C′, and D′) thymus at 2 weeks of age. m denotes the medulla. Bars = 100 μm. **E**: Northern blot of TEC and thymocytes from control and bK5hK10 transgenic mice at 2 weeks of age probed for HES-1 cDNA. RNA loading was monitored using a ribosomal 7S probe (lower panel). **F**: Quantification of the Northern results using Quantity 1 software (Bio Rad). Note that HES1 expression decreased in TEC and increased in thymocytes from transgenic mice.

some elements of this pathway showed altered expression suggested once again that the alterations are not attributable to an overall damage to the thymus.

The observed alterations, as they affect both the receptors and the correspondent ligands do not explain the possible status of Notch signaling. Since RBP-Jκ proteins may act as repressors in absence of Notch signaling, one might expect either increased or repressed signaling in the thymus of the transgenic mice. To study this aspect, Northern analyses to monitor the expression of Hes-1 gene, a well known target on Notch signaling, were performed. We found that Hes-1 is expressed in both TEC and thymocytes of transgenic and control mice by 2 weeks of age

(Fig. 7E). However, we found that the expression in transgenic TEC is decreased when compared with the correspondent controls (Fig. 7E,F). On the contrary, Hes1 mRNA levels increased in the thymocytes from bK5hK10 mice compared to non-transgenic samples (Fig. 7E,F). It is worth mentioning that a similar expression of RBP-Jκ and the corepressor CIR proteins were observed in all the samples irrespective of their origin, transgenic or control mice (not shown).

Collectively, these data indicate that the expression of keratin K10 leads to the altered expression of Notch proteins and ligands in the TEC, reducing Notch activity in these cells, but also promoting increased Notch signaling in the thymocytes.

Altered Notch Expression in bK5hK10 Transgenic Mice Epidermis

The above commented data indicated that keratin K10 may influence cell differentiation in paracrine fashion through the modulation of Notch members. It is important to note that cell fate and differentiation is also influenced by Notch signaling in skin, which expresses several members of this pathway (reviewed in [Lefort and Dotto, 2004]. In addition, K10 is normally expressed in the suprabasal layers of epidermis, sites of normal Notch expression [Rangarajan et al., 2001]. However, given that keratin K10 is never expressed in normal thymus, one might argue that this effect is not relevant. Therefore, possible changes in Notch 2 and 3 and their ligand jagged 1 in the epidermis of bK5hK10 were analyzed. We observed (Fig. 8) that the expression of K10 in the basal layer affect also the expression of these molecules. In particular, we found ectopic expression of jagged 1 in the outer root sheath of the follicles in bK5hK10 transgenic skin (hollow arrows in Fig. 8A′), whilst the normal expression of this protein in the basal layer of interfollicular epidermis and in the inner root sheath of the hair follicles persists (arrows in Fig. 8A,A′). We cannot detect Notch 3 expression in control epidermis (Fig. 8B). However, a clear induction of this protein takes place in the basal layer of interfollicular epidermis and in the inner and outer root sheath of the hair follicles (denoted by hollow arrows in Fig. 8B′). A similar induction of Notch 2 was also observed in transgenic epidermis (Fig. 8C′) compared to control, non-transgenic samples (Fig. 8C). Finally, as in the thymus, we did not detect altered expression of Notch 1 or Delta 1 (not shown).

These results indicate that keratin K10 is able to modify Notch family members expression not only in the thymus, but also in its normal expression site, the epidermis, thus reinforcing the suggestion that K10 indeed affect cell signaling events in a paracrine fashion. Importantly, we also found differences in both tissues as Notch3 and jagged expression is reduced in thymus and induced in epidermis. Given the well reported involvement of Notch signaling in epidermis, this aspect would deserve future investigations.

DISCUSSION

The possible existence of specific functions for the different members of keratin families is a matter of controversy. All these proteins contribute to maintain the integrity of epithelial cells in the tissue context. However, the fact that each polypeptide might play unique functions has not been discarded. Our previous work demonstrated that ectopic expression of keratin K10 in cultured cells or in transgenic mice decreased proliferation and increased apoptosis [Paramio et al., 1999, 2001a; Santos et al., 2002a,b]. Altogether, these data indicated the existence of cell-autonomous effect of K10. However, a different situation emerges from the K10-deficient mice [Reichelt et al., 2001; Reichelt and Magin, 2002]. These mice develop with age an epidermal phenotype with increased proliferation of basal keratinocytes and increased cycD1 and c-myc expression [Reichelt and Magin, 2002]. This situation means that the altered composition of suprabasal cytoskeleton in epidermis is able to alter the proliferation state of basal cells, and imply that a mechanism of cell–cell interaction is affected, thus suggesting that K10 might have roles in a non-cell autonomous manner. In the present study, we present evidences for such roles, as the expression of K10 in the TEC of bK5hK10 transgenic mice is able to modulate Notch signaling in TEC and in the neighboring thymocytes. Given that K10 is normally expressed in the suprabasal layers of epidermis, sites of normal Notch expression [Rangarajan et al., 2001], these data might also indicate that K10 is acting to modulate Notch signaling in epidermis. In agreement, we also found altered Notch expression in epidermis of bK5hK10 transgenic mice,

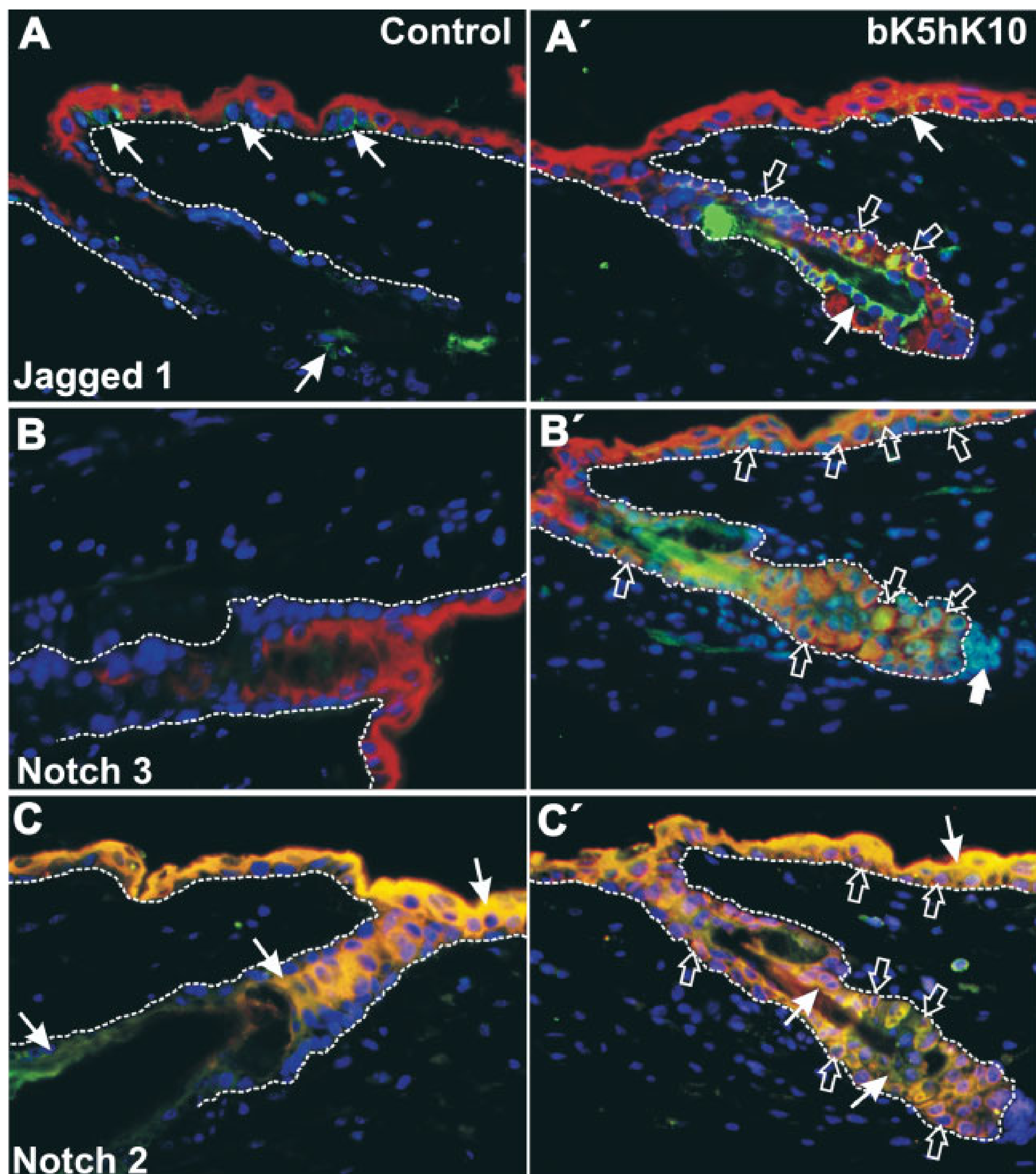

Fig. 8. Altered Notch expression in bK5hK10 epidermis. The expression of the different quoted Notch pathway components was analyzed by immunofluorescence using antibodies against Jagged 1 (green in **A**, **A′**), Notch 3 (green in **B**, **B′**), and Notch 2 (green in **C**, **C′**) in parallel with K10 (in red) in control (A, B, C) and transgenic bK5hK10 (A′, B′, C′) mice epidermis. Note that the normal expression of Jagged 1 and Notch 2 (denoted by arrows) is maintained in transgenic samples. In addition ectopic expression of Notch 3 and Jagged 1 and Notch 2 was observed in transgenic samples (denoted by hollow arrows).

in which ectopic expression of K10 is targeted to the basal layer.

The transgene construction (bK5hK10) used here allows the expression of K10 in the basal keratinocytes [Santos et al., 2002a] and in the epithelial cells of the thymic medulla, site of normal keratin K5 expression [Klug et al., 1998]. Moreover, as keratin K10, at least at the protein level, is not expressed in the thymus [Moll et al., 1982; Kuo, 2000], bK5hK10 transgenic mice are valuable tools to study the phenotypic consequences of ectopic keratin K10 expression. We found that the expression of K10 in these cells leads to a severe thymus phenotype (Fig. 2), affecting the cortex and the medulla, where K10 is expressed (Fig. 2). We also found decreased T cell population in association with impaired proliferation and increased apoptosis (Fig. 5). The alterations in thymus function might account for the observed immunodeficiency displayed by bK5hK10 transgenic mice (Fig. 1). Similar impaired

proliferation and increased apoptosis were observed in the epidermis of bK5hK10 transgenic mice, probably due to reduced Akt activity [Santos et al., 2002b]. A reduced Akt activity was also observed in cells expressing K10 in the thymus (Fig. 6). However, Akt activity was similar in thymocytes from control and transgenic mice, suggesting that the inhibition of this kinase is not directly responsible for the observed T cell phenotype, which also involves the alterations in the differentiation process (Fig. 3).

One may argue that the defect observed can be promoted by a toxic effect of the transgene that may cause an overall disturbance of the thymic environment and, thus the phenotype could be simply an artifact of misexpression. However, the fact that alterations in thymocyte differentiation (Fig. 3) and in the expression patterns of some elements of the Notch pathway (Fig. 7) were observed at 2 weeks of age, when no alterations in the thymic architecture nor in the proliferation or apoptosis of thymocytes or TEC were detected, clearly suggested the existence of an specific effect. Given that Notch signaling is involved in many processes during embryonic development, the possibility that similar or different alterations also take place in embryos is highly suggestive. This possibility is highlighted because thymocyte/TEC interactions in late foetal development seem to be indispensable for maintaining TEC differentiation and organization in the adult thymus. These aspects would be subject of future studies. However, it is worth mentioning that the transgenic mice were born at the expected mendelian ratios, indicating that the possible alterations do not affect overall embryo survival.

Thymic organogenesis is a complex process that depends on mutually inductive thymocyte/TEC interactions to generate a microenvironment that serves as the major site of T cell development. Inductive interactions between thymocytes and TEC are required for the maturation of both cell types [Klug et al., 1998, 2002]. Although numerous investigations have provided insight into the intricacies of T cell developmental progression, the processes governing TEC development, and how alterations in TEC differentiation may affect T cell development generally remain obscure. Here, we show that the alterations of the TEC population in the medulla, due to ectopic keratin K10 expression, is paralleled with altered differentiation of thymocytes. It is interesting to note that K10 is not expressed in these cells suggesting that the ectopic expression of K10 in TEC is responsible of the thymocyte phenotype through the modulation of paracrine signaling events. One obvious aspect is that K10 expression might change the spatial organization of the TEC in the thymus, which appeared to control the differentiation and development of T cells. We found that the expression of keratin K10 in the K5+ TEC leads to a severe alterations in the architecture of this organ (Figs. 2 and 4). This might suggest that the altered architecture due to keratin K10 expression is responsible for the observed alterations, through a not yet known mechanism. However, we found disturbances in thymocyte differentiation and severe decrease in thymus cellularity in the transgenic mice at 2 weeks of age (Fig. 3). Given that the architecture of the thymus in the transgenic mice, including the distribution of K5 + K8+ TEC, is not altered at this time (compare Fig. 2A–A‴ with Fig. 5, compare also Fig. 2B,B′ with Fig. 2C,C′), this does not seem to be the prominent cause of the thymic phenotype, and points to altered cell–cell signaling as a responsible of the observed alterations.

Among the different pathways that may mediate T cell differentiation through cell–cell interactions, Notch signals seem to be prominent [Deftos et al., 1998; Robey, 1999; Deftos and Bevan, 2000; Anderson et al., 2001; Guidos, 2002]. Moreover, our findings of decreased basal NFκB activity in thymocytes (Fig. 6) are consistent with altered Notch signaling in these cells [Oakley et al., 2003]. We characterized the expression and activity of *notch* family both in TEC and thymocytes (Fig. 7) and found that the expression of keratin K10 represses Notch signaling in TEC and, probably as a consequence of such inhibition, T cells display increased Notch activity. This represents the first direct evidence of alterations in a pathway involved in cell–cell communication due to changes in keratin expression.

The possible mechanism by which keratin K10 can mediate the alterations in Notch signaling remain to be elucidated. However, it is conceivable that the inhibition of Akt in TEC might be responsible. Indeed, it has been reported that PTEN transfer alters the expression of several components of the Notch pathway [Matsushima-Nishiu et al., 2001], and vascular

endothelial growth factor induces Notch1 gene expression through a phosphatidylinositol 3-kinase/Akt pathway [Liu et al., 2003]. Therefore, it is possible to envisage a model in which the ectopic expression of K10 inhibits NFκB in TEC through the modulation of Akt. This leads to altered Notch expression, which affect the neighboring T cells leading to secondary alterations in Notch expression, which in turns modulates NFκB in thymocytes. This possibility is also reinforced by the fact that NFκB could also control Notch signaling [Bash et al., 1999; Espinosa et al., 2002, 2003]. On the other hand, it has been recently shown that altered IKK activity may alter Notch signaling through NFκB-independent mechanisms in specific cell types [Ohazama et al., 2004]. Given that K10 also affects IKK activity [Santos et al., 2003], further experiments would be required to determine the actual mechanism. These will be carried out in the near future.

Collectively we present here evidence for two important events. On the one hand, we demonstrate that the ectopic expression of keratin K10 is sufficient to alter cell signaling in a paracrine fashion. On the other hand, our data also demonstrate that the epithelial component of the thymus, and in particular the signaling pathways acting in these cells, are responsible, at least in part, for the developmental events that leads to functional differentiation of thymocytes.

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