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Hoxa3 and Pax1 Transcription Factors Regulate the Ability of Fetal Thymic Epithelial Cells to Promote Thymocyte Development

Dong-ming Su and Nancy R. Manley

Thymocyte maturation into T cells depends on interactions between thymocytes and thymic epithelial cells. In this study, we show that mutations in two transcription factors, Hoxa3 and Pax1, act synergistically to cause defective thymic epithelial cell development, resulting in thymic ectopia and hypoplasia. Hoxa3+/−Pax1+/− compound mutant mice exhibited more severe thymus defects than Pax1−/− single mutants. Fetal liver adoptive transfer experiments revealed that the defect resided in radio-resistant stromal cells and not in hematopoietic cells. Compound mutants have fewer MHC class II+ epithelial cells, and the level of MHC expression detected was lower. Thymic epithelial cells in these mutants have reduced ability to promote thymocyte development, causing a specific block in thymocyte maturation at an early stage that resulted in a dramatic reduction in the number of CD4+8+ thymocytes. This phenotype was accompanied by increased apoptosis of CD4+8+ thymocytes and their immediate precursors, CD44+25− (CD3−4−8) cells. Our results identify a transcriptional regulatory pathway required for thymic epithelial cell development and define multiple roles for epithelial cell regulation of thymocyte maturation at the CD4−8− to CD4+8+ transition.

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The vertebrate thymus is responsible for the production of self-restricted, self-tolerant T cells. Within the mature thymus, T cell precursors (thymocytes) undergo a series of developmental stages via interactions with thymic stromal cells, resulting in the generation of mature T lymphocytes that are then exported to the periphery. Epithelial cells constitute a major component of the thymic stroma and are required both for thymus organogenesis and for thymocyte maturation (1–7). Thymocyte development is characterized by the sequential expression of cell surface markers at particular stages of maturation. The main stages are CD4−8− (double negative (DN)) to CD4+8− (double positive (DP)) to mature CD4+ or CD8+ (single positive (SP)) cells. Interactions with thymic epithelial cells are required for both the DN to DP and DP to SP transitions (1, 2, 4, 8, 9). Thymic epithelial cells play a variety of roles in promoting thymocyte development, from providing maturation signals such as cytokines, chemokines, adhesion molecules, and matrix components to promoting MHC-mediated positive selection. However, the molecular mechanisms regulating thymic epithelial cell differentiation and function are poorly understood.

Although the role of epithelial cells in positive selection at the DP to SP transition has been studied extensively, their role in the DN to DP transition is less clear. Thymocyte development stages within the DN population are defined by their expression of the CD44 and CD25 cell surface markers, in stages progressing from CD44+25− to CD44+25+, to CD44+25+, and to CD44+25− (10). Many if not all of these stages require epithelial cell interactions to proliferate and/or differentiate to the next stage. MHC class II-positive epithelial cells are required for thymocytes to develop from DN to DP stages in reaggregate cultures (2) and for induction of β and δ TCR rearrangements (11). Epithelial cells are known to provide a number of cytokines that are necessary both for early expansion of DN cells and at later thymocyte development stages (12, 13). However, for the most part the signals provided by epithelial cells to promote specific stages of early thymocyte differentiation and proliferation have not been defined.

Although the central role of epithelial cells in thymic development and function is widely acknowledged, little is known about the molecular pathways by which these cells become competent to perform these functions. Mutations in several transcription factors, including relB, wnh, Pax9, and Hoxa3, have been shown to cause severe defects in thymus organogenesis and thymic epithelial cell development, leading to the absence of a functional thymus or deletion of a specific epithelial cell type (14–17). Although all of these transcription factors have profound effects on thymic epithelial cell development, the severity of their phenotypes has made it difficult to use these mutants to investigate epithelial cell function. In contrast to these more severe phenotypes, mutation of the Pax1 gene has more subtle effects on thymus development and function.

Pax1 mutants have a hypoplastic thymus that is deficient in thymocyte development (18, 19). Pax1 is expressed in the third pharyngeal pouch endoderm where thymus organogenesis originates, in a subset of thymic epithelial cells throughout fetal development, and in the adult thymus (19). Our previous studies have suggested that there is a functional link between Hoxa3 and Pax1 in thymus development (20). Hoxa3 is a member of the Hox family of transcription factors, which specify positional identity in the developing embryo (21). Mutation of the Hox3 gene in mice results in athymia, as part of a spectrum of pharyngeal region defects (17, 20, 22–24). Hoxa3 is expressed in both the third pharyngeal pouch endoderm and the neural crest mesenchyme, which contribute to
early thymus organogenesis and may act in both of these cell types (6, 20, 24–26). Pax1 is specifically down-regulated in the third pharyngeal pouch endoderm in Hoxa3+/− embryos at embryonic day 10.5 (E10.5) (20). This result indicates that Hoxa3 and Pax1 may be in a common pathway regulating thymus development.

To investigate this possible link between Hoxa3 and Pax1, we generated mice carrying mutations in both genes. To detect an interaction between these genes, we looked for the ability of the Hoxa3 mutation to enhance the Pax1+/− thymus phenotype in double heterozygotes (Hoxa3+/−/Pax1+/−) and in Hoxa3+/−/Pax1+/+ congenic compound mutants. The thymus phenotype of Hoxa3+/−/Pax1+/−/+ double heterozygotes was indistinguishable from that of Pax1+/−/Pax1+/−/+ single mutants, indicating that mutations in these genes show nonallelic non-complementation. In addition, the Hoxa3+/−/Pax1+/−/+ compound mutant mice had a strikingly more severe thymus phenotype than Pax1+/−/Pax1+/−/+ single mutants did, displaying ectopic thymus lobes, increased thymic hypoplasia, and more severe effects on thymocyte maturation. Hoxa3+/−/Pax1+/−/+ compound mutants have fewer MHC class II+ thymic epithelial cells and reduced levels of epithelial I-Aβ expression. Thymocyte maturation defects in these compound mutants included a 10-fold reduction in CD4+8+ thymocytes. Cell death analysis indicated that this decrease is associated with increased apoptosis in both CD44+/− and DP cells. This analysis also identified an apparent effect of the Pax1 mutation on specific subsets of triple negative (TN) cells. Our results may define multiple roles for epithelial cell regulation of thymocyte maturation at the DN to DP transition.

Materials and Methods

Strains and genotyping

The Hoxa3 mutant strain and genotyping by PCR have been described (17, 20). Pax1m−/− mice and embryos were genotyped by Southern blot with the pSacII-3 probe, as described (18, or by PCR. PCR primers were designed from a size-selected genomic library probed with the pSacII-3 probe (D.-m.S., unpublished data). The sequences of the oligos were as follows: common forward primer, 5′-GCCACCCAAAGGTCTCTGTGC-3′; wild-type reverse primer, 5′-CCACACAAAAAGGATCTGATCTGC-3′; un-ex reverse primer, 5′-GGTGTAGTTTATAGGATGAA-3′; and un-ex specific reverse primer, 5′-GGGTAGTTCATAGAGATCA-3′. The wild-type allele yields a 430-bp product, and the un-ex allele gives a 300-bp product. Both the Hoxa3 and Pax1m−/− strains are congenic on the C57BL/6J genetic background. Compound heterozygotes were viable and fertile. Noon of the day of vaginal plug was considered E0.5; embryos were also staged using somite number and morphological criteria. All animal use procedures were approved by the Georgia Committee on Animal Use for Research and Education.

Histology

Embryos were collected at E17.5, fixed in 4% formaldehyde, and processed for paraffin embedding using standard techniques. Sections were cut at 6 μm and stained with hematoxylin and eosin.

Flow cytometry and Abs

Thymocytes were collected by filtering through a cell strainer. Three-color flow cytometry was performed using the following mAbs (PharMingen, San Diego, CA): biotin or PE anti-mouse CD3ε (clone 145-2C11), R-PE anti-mouse CD4 (clone RM4-5), FITC or PE anti-mouse CD8a (clone 53-6.7), biotin anti-mouse CD69 (clone H1.2F3), biotin anti-mouse αβTCR (clone H57-597), biotin anti-mouse γδ TCR (clone GL-3), biotin anti-mouse CD44 (clone Pgg-1), and FITC anti-mouse CD25 (clone 7D4). Biotinylated Abs were visualized using streptavidin-conjugated Cy-chrom (PharMingen). Data generation and analyses were performed using a FACStar™ flow cytometer. All data was analyzed using CelQuest software. Significant differences were determined by the unpaired Student’s t test or ANOVA analysis.

Adoptive transfer

Adoptive transfers of fetal hematopoietic stem cells were performed as previously described (27). Briefly, 5- to 8-wk-old Rag1−/− mice were lethally irradiated and injected with 2.5 × 107 E15.5 fetal liver cells from control, Pax1+/−/+ and Hoxa3+/−/Pax1+/−/+ embryos. After 2 mo, the thymus, spleen, and peripheral lymph nodes were analyzed by flow cytometry for CD4, CD8, and CD3 expression as described above.

TUNEL analysis

TUNEL analysis was modified from the previously described method (28, 29). Briefly, for three-color analysis of CD4 and CD8 populations, E17.5 thymocytes were collected and stained with PE-CD4 and FITC-CD8. Cells were then fixed in 2% PFA for 1 h, permeabilized with 0.1% Triton X-100, and equilibrated in 1× TdT buffer. Terminal transferase reactions were conducted in 50-μl reactions containing 1× TdT buffer, 5 μM biotin-dUTP, and 100 U/ml TdT (Boehringer Mannheim, Indianapolis, IN). TUNEL cells were identified with streptavidin-peridinin chlorophyll-protein (PharMingen). Four-color analysis of TUNEL− cells in TN subsets was performed using cell surface staining with PE-CD4, PE-CD8, PE-CD3, APC-CD44, and FITC-CD25. Cells were then fixed, permeabilized, and the TdT reaction and streptavidin staining conducted as described above. Results were analyzed by gating on cells negative for staining with PE-CD4, PE-CD8, and PE-CD3 and measuring the percentage of TUNEL− cells within each quadrant defined by labeling with APC-CD44 and FITC-CD25 as described above.

Fetal thymic organ culture and epithelial cell staining

Thymic lobes from E15.5 embryos were isolated and cultured at 37°C in high oxygen subculture medium as described previously (30, 31). Lobes were cultured in 1 ml RPMI 1640 medium with 10% FBS and 5 × 10−5 M 2-ME containing 1.35 μM deoxyguanosine (dGuo) for 5 days to deplete thymocytes and dendritic cells (32, 33). After depletion, thymic lobes were washed twice with PBS and then were incubated with 0.25% Trypsin/0.02% EDTA for 30 min at 37°C. Cells were dissociated by pipetting, washed, and counted, stained using the anti-I-Aε mAb M5/114 and a FITC-conjugated anti-rat IgG secondary Ab (PharMingen), and analyzed by flow cytometry. The M5/114 hybridoma cell line was obtained from the American Type Culture Collection (Manassas, VA); culture supernatant was purified using a protein G column. Ab staining was analyzed by flow cytometry as described above.

Results

Hoxa3 enhances the Pax1 mutant phenotype

As a genetic test of whether Hoxa3 and Pax1 are in the same pathway, we investigated whether mutation of one copy of Hoxa3 could enhance the Pax1 mutant phenotype. At E17.5, control mice had two large thymic lobes located directly cranial to the heart (Fig. 1a). Pax1m−/−/− mice were as previously described (18, 19), with somewhat smaller thymic lobes that often contained cysts (Fig. 1b). Thymic lobes from Hoxa3+/−/Pax1+/−/+ and Pax1+/−/+/− mice were indistinguishable in size and morphology (Fig. 1, b and c), indicating that these two mutations do show a genetic interaction. Even more strikingly, Hoxa3+/−/Pax1m−/−/+ mice at E17.5 had a dramatically more severe thymus phenotype than Pax1+/−/+/+ single mutants. Histological analysis showed that all five Hoxa3+/−/Pax1m−/−/+ mice examined had very small thymic lobes that were often ectopically located anterior to their normal position (Fig. 1, d–f). Ectopic lobes were never seen in wild-type, single heterozygote Hoxa3+/−/Pax1+/−/+ or Pax1+/−/+/+ animals. Both ectopic and normally placed Hoxa3+/−/Pax1m−/−/+ thymic lobes at E17.5 had numerous cysts that were often very large relative to the size of the thymic lobe (Fig. 1, d and e). These cysts were morphologically similar to those seen in Pax1m−/−/+/+ thymic lobes. All mutant and control thymus had identifiable cortical and medullary development. However, in the Hoxa3+/−/Pax1m−/−/+ animals the density of cortical thymocytes was noticeably reduced (Fig. 1, g and h). These results indicated a surprisingly strong Hoxa3+/−/Pax1+ genetic interaction.

Specific decrease in CD4+8+ cells in Hoxa3/Pax1 mutants

The reduced thymocyte density and smaller thymus size suggested that there could be defects in thymocyte maturation in
Hoxa3+/−Pax1un-ex mice had even more severe reductions in total thymocyte cell number and the CD4+CD8− compartment than did controls or Pax1 mutants. Cell number was reduced to 25–30% of controls, caused by a 10-fold reduction in CD4+ cells (Fig. 2b). Although the number of CD4−CD8− cells in Hoxa3+/−Pax1un-ex mutants was consistently lower than in Pax1un-ex mutants, this decrease was not significant (p > 0.05). The percentage of CD4+CD8+ cells was significantly decreased, with a compensatory increase in the percentage of CD4−CD8− cells (Fig. 2b).

**TCR and CD69 expression is not changed**

We investigated whether the expression of TCR had been affected in these mutants (Table I). Developing thymocytes rearrange and express either γδTCR or αβTCR, with the αβTCR lineage representing the majority of thymocytes during late fetal and adult stages. Expression of αβTCR is coincident with development from DN to DP cells. Although the total number of cells expressing αβTCR was reduced significantly in Hoxa3+/−Pax1un-ex mutants, this decrease was not significant (p > 0.001). This reduction was due to a specific decrease in the number of CD4+CD8− cells, as the number of more immature CD4−CD8− cells was the same as it was in controls.
selection. A decrease in CD4\(^{+}\)8\(^{-}\)69\(^{+}\) cells was previously reported for the Pax1 undulated shorttail allele (19). Again, although the total number of CD4\(^{+}\)8\(^{-}\)69\(^{+}\) cells was significantly decreased in Hoxa3\(^{+/−}\)Pax1\(^{+/-}\) mice, the percentage of DP and SP cells expressing CD69 was the same in controls and mutants (Table I; \(p = 0.87\)). Taken together, these results suggest that there is a defect in the generation and/or proliferation of CD4\(^{+}\)8\(^{-}\) cells; however, those CD4\(^{+}\)8\(^{-}\) cells that are produced can undergo positive selection and generate mature SP cells. In support of this conclusion, although the numbers of mature CD4\(^{+}\) and CD8\(^{+}\) SP thymocytes were significantly reduced in these mutants, the percentages of these SP cells were not significantly decreased (data not shown).

**Phenotypes are not due to thymocyte defects**

It is well-documented that hematopoietic cells in the thymus play an important role in early thymus organogenesis (5). Although neither Hoxa3 nor Pax1 has been shown to be expressed in hematopoietic cells, other Hox genes, including the closely related gene Hoxb3, are expressed in hematopoietic stem cells and can affect hematopoiesis when overexpressed (34). To exclude any contribution to the observed phenotype by hematopoietic cell defects, we irradiated 5- to 8-wk-old Rag1\(^{-/-}\) mice and reconstituted their immune systems with fetal liver cells from E15.5 control and Hoxa3\(^{+/−}\)Pax1\(^{+/-}\) embryos. After 2 mo, the thymus, spleen, and peripheral lymph nodes were analyzed by flow cytometry for CD3, CD4, and CD8 expression. Our analysis showed no difference in the ability of control and Hoxa3\(^{+/−}\)Pax1\(^{+/-}\) fetal liver cells to reconstitute the thymus and peripheral lymphoid organs (Fig. 3). Total thymocyte cell numbers in the reconstituted thymic lobes were similar to normal adult cell numbers (data not shown). These results showed that the thymocyte maturation defects seen were not due to intrinsic defects in hematopoietic cells.

**Increased cell death in specific thymocyte populations**

To better define the thymocyte differentiation block at the DN to DP transition, we investigated DN cell development by assaying CD44 and CD25 expression in CD3\(^{-}\)4\(^{-}\)8\(^{-}\) TN cells (Refs. 10 and 35 and Fig. 4). No dramatic changes in the percentage of cells in each subset were seen. However, small (<2\%) but significant changes in the distribution of thymocytes were seen in both Pax1\(^{+/-}\) and Hoxa3\(^{+/-}\) mice. The percentage of CD44\(^{+}\)25\(^{+}\) cells was reduced by ~40% relative to single heterozygote controls (\(p < 0.01\)), whereas the percentage of CD44\(^{+}\)25\(^{-}\) cells was increased by ~25% (\(p < 0.001\)). The percentage of CD44\(^{-}\)25\(^{-}\) cells was not significantly different. Because these differences were seen in both Pax1\(^{+/-}\) and Hoxa3\(^{+/-}\) mutants, these changes are likely due to the Pax1 mutation alone and cannot account for the exacerbated decrease in DP cell numbers seen in the compound mutants.

To investigate the mechanism underlying these phenotypes, we performed TUNEL analysis of cell death both in DN and DP populations and in the subpopulations of TN cells defined by CD44 and CD25. TUNEL\(^{+}\) cells in each subpopulation were identified by flow cytometry. Only Hoxa3\(^{+/-}\)Pax1\(^{+/-}\) mutants, but not Pax1\(^{+/-}\) single mutants, had significant increases in TUNEL\(^{+}\) populations. Both DN and DP compartments had a significant increase in TUNEL\(^{+}\) cells of over 2-fold compared with those of single heterozygote and wild-type controls (Fig. 5a). An even more striking difference was seen in the analysis of TN subpopulations. A significant change in apoptosis was seen only in the CD44\(^{-}\)25\(^{-}\) TN cells, with a more than 5-fold increase in TUNEL\(^{+}\) cells in Hoxa3\(^{+/-}\)Pax1\(^{+/-}\) compound mutants (Fig. 5b). No significant differences in the frequency of TUNEL\(^{+}\)

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**Table I. TCR and CD69 expression within thymocyte subpopulations are not changed**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% γ8TCR(^{+}) Among DN Cells (n)</th>
<th>% αβTCR(^{+}) Among DP/SP Cells (n)</th>
<th>% CD69(^{+}) Among DP Cells (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pax1(^{+/-})</td>
<td>48.40 ± 12.47 (31)</td>
<td>77.56 ± 8.15 (31)</td>
<td>16.86 ± 5.00 (29)</td>
</tr>
<tr>
<td>Hoxa3(^{+/-})</td>
<td>49.24 ± 10.98 (14)</td>
<td>73.14 ± 16.35 (14)</td>
<td>17.39 ± 3.32 (13)</td>
</tr>
<tr>
<td>Hoxa3(^{+/-})Pax1(^{+/-})</td>
<td>56.06 ± 15.45 (27)</td>
<td>79.27 ± 10.82 (27)</td>
<td>16.46 ± 4.51 (21)</td>
</tr>
<tr>
<td>Pax1(^{+/-})</td>
<td>51.23 ± 6.23 (11)</td>
<td>78.44 ± 2.37 (11)</td>
<td>15.56 ± 4.70 (11)</td>
</tr>
<tr>
<td>Hoxa3(^{+/-})Pax1(^{+/-})</td>
<td>37.63 ± 7.47 (4)</td>
<td>75.97 ± 2.22 (5)</td>
<td>15.79 ± 3.26 (5)</td>
</tr>
</tbody>
</table>

* Results shown are mean ± SD. Values within each column are not significantly different by ANOVA analysis.

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**FIGURE 3.** Flow cytometry analysis of thymocytes and splenocytes from irradiated Rag1\(^{-/-}\) host mice reconstituted with E15.5 fetal liver cells. The genotypes of the donor embryos are listed to the left. No differences between control and mutant donor cells were seen. Values shown are percentage of total cell number in each quadrant.
cells were seen in any other TN cell population. Because these increases in cell death are specific to the compound mutants, this result suggests that increased apoptosis of CD44^−/− cells and DP cells could be responsible for the dramatic decrease in DP cells in Hoxa3^+/− Pax1^+/− compound mutants.

**Hoxa3^+/− PAX1^+/− thymic epithelial cells have reduced MHC class II expression**

As an initial assay for thymic epithelial cell differentiation, we cultured E15.5 thymic lobes in the presence of dGuo for 5 days to deplete thymocytes and dendritic cells (32, 33) and then measured expression of MHC class II (anti-I-Ab). Epithelial cells normally deplete thymocytes and dendritic cells (32, 33) and then measured cultured E15.5 thymic lobes in the presence of dGuo for 5 days to decrease MHC class II expression. E15.5 thymic lobes were cultured in the presence of dGuo for 5 days, dissociated, and assayed for the expression of MHC class II (I-Ab).

Thymic lobes from double heterozygote and in independent of interactions with thymocytes (36, 37). Interestingly, thymic lobes from double heterozygote and Pax1^+/−/−/− compound mutants had significantly higher levels of TUNEL^− cells in the CD44^/−/− subpopulation.

**Discussion**

Our results show that mutations in Hoxa3 and Pax1 result in defective thymic epithelial cell development, characterized by reduced MHC class II expression and a decreased ability to promote thymocyte development. These results identify specific stages in thymocyte development that are dependent on Hoxa3 and Pax1 function in thymic epithelial cells. The most dramatic defect is a partial block at the DN to DP transition in Hoxa3^+/− PAX1^+/−/− compound mutants resulting in a 10-fold reduction in the number of DP cells relative to controls. Our TUNEL analysis localizes this defect to the CD44^/−/− and DP stages, both of which showed increases in cell death only in the compound mutants. Although they usually copurify with DN cells and are CD3^−, almost all CD44^−/− cells have begun to express very low levels of CD4^+ CD25^+ cells in the CD44^/−/− subpopulation.

![FIGURE 5. Increased thymocyte apoptosis in Hoxa3^+/− PAX1^+/−/−/− compound mutants. Each data point represents data from an individual mouse; bars are average values. a, Percentage of TUNEL-positive thymocytes in CD44^−/− and CD44^+/− populations. In both populations, Hoxa3^+/− PAX1^+/−/−/− average values were significantly higher than in control mice (p < 0.05). Values for Pax1^+/−/−/−/− mutants were not significantly different from controls. b, Percentage of TUNEL-positive cells in CD3^+ 4^/−/− subpopulations defined by expression of CD44 and CD25. Average values in CD44^−/− and CD44^+ subpopulations were not significantly different between genotypes. Hoxa3^+/− PAX1^+/−/−/− compound mutants had significantly higher levels of TUNEL^− cells in the CD44^−/− subpopulation.](http://www.jimmunol.org/)

![FIGURE 6. Hoxa3^+/− PAX1^+/−/− epithelial cells at E15.5 have reduced MHC class II expression. E15.5 thymic lobes were cultured in the presence of dGuo for 5 days, dissociated, and assayed for the expression of MHC class II (I-Ab). a, The total number of epithelial cells was significantly decreased in the depleted lobes from Hoxa3^+/− PAX1^+/−/− mutants (+). Hoxa3^+/− PAX1^+/−/− epithelial cells also had a significantly lower number (b) and percentage (c) of MHC class II^+ cells than any other genotype class (+). d, Histograms showing levels of MHC class II expression in representative cultures of selected genotypes. Dashed vertical line shows the peak of fluorescence intensity in control and Pax1^+/−/−/− cells. Filled bar (+) shows shift in fluorescence intensity of MHC class II staining in Hoxa3^+/− PAX1^+/−/− cells.](http://www.jimmunol.org/)
for the development of large numbers of DP cells, and this pro-
liferation is likely to be controlled by epithelial cells (41, 42). Our
results are consistent with Hoxa3-Pax1 compound mutants having
a specific effect on the survival of pre-DP and DP cells. Because
the frequency of pre-DP cells does not appear to be affected, we
suggest that these cells may be undergoing apoptosis as they dif-
erentiate into DP cells. Therefore, these mutants may provide an
experimental system for identifying the factors expressed by ep-
thelial cells that control the proliferation and survival of pre-DP
cells.

Our analysis also identified defects within the CD3−4−8− TN
cell compartment that appear to be specific to the Pax1 mutation
alone. In both Pax1un-exon-es and Hoxa3+/−Pax1un-exon-es mu-
tants, the percentage of CD44+25 cells was reduced and the per-
centage of CD44−25 cells was increased relative to controls. These
changes in the TN compartment are identical in Pax1 single
mutants and Hoxa3-Pax1 compound mutants, indicating that these
defects are due only to the loss of Pax1. During the CD44+25 stage,
αβTCR lineage thymocytes are selected for their ability to
express a functionally rearranged TCRβ molecule and to assemble
with the pre-Tα molecule to form the pre-TCR complex (43, 44).
The Pax1 phenotype is similar in some respects to previously de-
scribed mutations in components of the pre-TCR complex, which
cause partial or complete blocks in thymocyte maturation at the
CD44+25 stage (45). Similarities include decreases in total thy-
mocyte number and in the number and percentage of DP cells as
well as increases in the percentages of DN cells and of CD44−25
cells in the TN compartment and specific defects in the αβTCR
lineage but not in γδTCR cells. Despite these similarities, there
are also differences indicating that the Pax1 mutant phenotype may
not be explained entirely by a partial block at the CD44+25 stage.
The magnitude of the differences seen is relatively small
(<2×), and there is no change in the percentage of cells in the next
stage, CD44−25 pre-DP cells. Pre-DP cells are strikingly re-
duced in mutants directly affecting pre-TCR expression (46), al-
though it should be noted that not all mutants that affect pre-TCR
signaling show a decrease in CD44−25 cells (47). The role of
epithelial cells at this step has not been defined, and a specific
ligand for the pre-TCR is unknown (48). However, because thymo-
cytes cannot mature beyond this step in suspension culture,
some as yet undefined interaction or signal is required to proceed
beyond this stage (40), possibly involving cortical epithelial cells.
Our results raise the intriguing possibility that there is an interac-
tion between thymic epithelial cells and developing thymocytes at
the CD44−25 stage that is disrupted in these mutants.

Some candidates for epithelial cell-produced factors that could
mediate the observed phenotypes include cytokines and glucocor-
ticoids. Thymic epithelial cells express a number of cytokines,
beginning as early as E12.5 (49, 50). Thymocytes at different
stages of development have the capacity to respond to different
cytokines, and specific combinations of cytokines can have ad-
tional effects beyond those of individual factors (12, 13, 35, 50,
51). Although the null phenotypes for a number of cytokines have
been described, none match the Hoxa3+/−Pax1un-exon-es pheno-
type (12, 13). It should be emphasized that multiple cytokines
could be reduced or absent in a specific subpopulation of epithelial
cells, so the null phenotypes for the individual candidates could be
different from the Hoxa3-Pax1 compound mutants. Another can-
didate is a decrease in glucocorticoid synthesis. Inhibition of cor-
ticosterone synthesis by thymic epithelial cells or of glucocorticoid
receptor expression in thymocytes results in a phenotype that is
strikingly similar to the Hoxa3+/−Pax1+/− phenotype, with a 95% decrease in DP cell number and no effect on DN cell number or
CD44/CD25 subpopulations (52, 53). These effects were proposed
to be caused both by a defect at the DN to DP transition and
increased DP cell death due to increased negative selection (53).
We are currently investigating possible changes in both cytokines
and glucocorticoids in Hoxa3-Pax1 mutants.

Our results suggest that there are both fewer epithelial cells and
a defect in epithelial cell differentiation in Hoxa3+/−Pax1un-exon-es
embryos. The fetal thymic organ culture experiments showed a sig-
nificant reduction in epithelial cell number, specifically in the
Hoxa3+/−Pax1un-exon-es mutant thymic lobes. Hoxa3-Pax1 com-
 pound mutants also have a smaller thymic rudiment as early as E11.5–
12.5 (data not shown), when thymocytes normally constitute a small
percentage of the thymus. This result supports the conclusion that
there are fewer epithelial cells from the earliest stages of organogen-
esis. A reduction in epithelial cell number alone could account at least
in part for the observed effects on thymocyte differentiation by re-
stricting the availability of epithelial cells capable of supporting thymo-
cyte maturation.

In addition to reduced epithelial cell number, the reduction in
MHC class II expression levels is an indication of defective epith-
elial cell differentiation. Although the role of MHC class II-ex-
pressing epithelial cells in promoting positive selection and the
maturation of DP cells to single positive cells is well-documented
(1, 2), the role of these cells in the development of DN cells into
DP cells is less well-understood. Evidence from reaggregate thy-
mic organ cultures indicates that interactions between thymocytes
and MHC class II+ epithelial cells are required for pro-
gression from late DN to DP stages (54). Induction of TCR rear-
rangements and commitment to the T cell lineage is also controlled
by interactions between IL-7-producing MHC class II+ epithelial
cells and immature DN thymocytes (11). The reduction in MHC
class II expression itself is unlikely to be functionally related to the
observed phenotype in that loss of this expression on thymic ep-
thelial cells is associated with defects in positive selection of
CD4+ cells but not in generation of DP cells (4, 8). We did not see a
reduction in the percentage of CD4+ SP cells or in positive
selection as measured by CD69 expression on DP cells. It should
be noted that relatively little positive selection normally occurs at
these fetal stages. Unfortunately, Hoxa3+/−Pax1+/− mice on the
C57BL6 genetic background die at or soon after birth due to other
effects of the mutations, and we have been unable to recover any
survivors of this genotype for postnatal analysis. Therefore, we
cannot determine as of yet whether the defects in thymocyte mat-
uration we observed reflect a permanent deficit or if they are due to
a developmental delay or whether defects in positive selection
would be apparent postnatally.

Our results show that mutations in Hoxa3 and Pax1 act in a
synergistic and dosage-dependent fashion to affect thymus devel-
opment. Our results represent the first demonstration of genetic
interactions between members of the Hox and Pax transcription
factor families. This result is particularly striking because Hoxa3
and Pax1 show a stronger genetic interaction than Hoxa3 and its
paralogs, Hoxb3 and Hoxd3 (22–24). The nature of this interac-
tion is not yet determined. Although our previous studies suggested that
Pax1 might be downstream of Hoxa3 (20), it is clear from the dif-
fERENCE between the Hoxa3+/−Pax1un-exon-es and Pax1
phenotypes that there is an interaction beyond any transcriptional
regulation of Pax1 by Hoxa3. Hoxa3 and Pax1 may act together to reg-
ulate common downstream target genes. Alternatively, other genes in
the pathway could also be involved, including Pax9 (16, 55).

Another transcription factor important for early thymic epithe-
lial cell development is whn (Hfh11), the gene mutated in the nude
mouse (15). Loss of whn causes a thymic epithelial cell defect that
results in arrest in epithelial cell differentiation and failure of lym-
phocytes to populate the thymus. Nehls et al. (56) proposed that
whn activity functions as a “control point” dividing thymic epithelial cell development into initial formation of the lymphoid thymic rudiment and further differentiation in the presence of hematopoietic cells. The interaction between Hoxa3 and Pax1 may act downstream of this whn checkpoint or in a separate pathway from whn because the epithelial cell defect in Hoxa3-Pax1 mutants appears to occur before E12.5 (data not shown), but hematopoietic precursors do invoke the thymic rudiment and undergo maturation. In support of a separate pathway, our preliminary data suggest that Hoxa3 and whn do not show a genetic interaction, in contrast to the results presented in this paper (C. Raines and Nancy R. Manley, unpublished data). Additional genetic studies and investigation of molecular interactions will be needed to determine the relationships between these transcription factors during early thymus development.

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