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*J Immunol* 2009; 182:2997-3007; doi: 10.4049/jimmunol.0713723
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Stabilized β-Catenin in Thymic Epithelial Cells Blocks Thymus Development and Function

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Thymic T cell development is dependent on a specialized epithelial microenvironment mainly composed of cortical and medullary thymic epithelial cells (TECs). The molecular programs governing the differentiation and maintenance of TECs remain largely unknown. Wnt signaling is central to the development and maintenance of several organ systems but a specific role of this pathway for thymus organogenesis has not yet been ascertained. In this report, we demonstrate that activation of the canonical Wnt signaling pathway by a stabilizing mutation of β-catenin targeted exclusively to TECs changes the initial commitment of endodermal epithelia to a thymic cell fate. Consequently, the formation of a correctly composed and organized thymic microenvironment is prevented, thymic immigration of hematopoietic precursors is restricted, and intrathymic T cell differentiation is arrested at a very early developmental stage causing severe immunodeficiency. These results suggest that a precise regulation of canonical Wnt signaling in thymic epithelia is essential for normal thymus development and function. The Journal of Immunology, 2009, 182: 2997–3007.

The T cells generated mainly in the thymus are from blood-borne lymphoid precursors and are critical for immune system adaptive response to Ag. Immature intrathymic T cell precursors are characterized by an absence of surface CD3, CD4, and CD8 expression, designated double negative (DN) thymocytes. These cells subsequently differentiate into CD4CD8 double positive thymocytes (1). Following the successful expression of a complete αβ T cell Ag receptor and the selection of appropriate receptor specificities, double positive cells mature to CD4 or CD8 single positive T cells that are now largely self-tolerant but responsive to foreign Ags (1). Correct intrathymic T cell maturation relies upon signals provided by a functionally competent three-dimensional network of stromal cells (2). This compartment consists of epithelial and mesenchymal cells that collectively enable the attraction, survival, expansion, migration, and differentiation of T cell precursors. Thymic epithelial cells (TECs) constitute the most abundant cell type within the thymic microenvironment and can be subdivided into distinct subpopulations according to function, structure, and specific antigenic features (3, 4).

Thymus organogenesis is initiated at around embryonic day 10.5 (E10.5) of development when epithelial cells of the third pharyngeal pouch commit to a thymic cell fate (reviewed in Ref. 5). Expression of the transcription factor Foxn1, which is detected as early as E10.5 (6, 7), has identified the ventral aspect of the third pharyngeal pouch endoderm as the anatomical site from where the thymus primordium emerges. The first lymphoid precursors colonize the thymus anlage at E11.5, a time when TECs are still organized as a two-dimensional cell layer (8). Independent of signals provided by immigrating hematopoietic cells, TECs adopt by E13.5 their typical three-dimensional organization and differentiate into specific cytokeratin (CK) expressing cortical (CK8+/CK5–) and medullary (CK8–CK5+) subsets (9). Mesenchymal cells have been demonstrated to be required for the creation and maintenance of a functional thymic microenvironment (10, 11). The preservation of the cortical compartment and its correct TEC architecture relies in addition on the presence of DN cells (9, 12). Similarly, the expansion of the initially small medullary islands at E18.5, the maturation and the homeostatic maintenance of its epithelial subpopulation correlate with the emergence of mature single positive thymocytes (13). Hence, a complex combination of inductive molecular cues provided by cells other than TECs are responsible for the formation of an architecturally correct and functionally competent epithelial microenvironment. However, the precise nature of the signals and the specific signaling pathways by which epithelial cells committed to a TEC fate respond to these signals are presently not well defined.

Wnt belongs to a family of highly conserved, secreted signaling molecules implicated in the development and maintenance of several organ systems (14, 15). Wnt proteins that activate the canonical pathway bind at the cell surface to the serpentine receptor...
Frizzled (Fz) and the single-span transmembrane low-density lipoprotein receptor-related protein Lrp5/6 (reviewed in Ref. 16). This interaction generates signals that engage the adenomatous polyposis coli protein complex and eventually inhibit the catalytic activity of glycogen synthase kinase 3β, which results in the stabilization and accumulation of β-catenin. Translocated to the nucleus, β-catenin interacts with the transcription T cell factor Tcf and lymphoid enhancer factor Lef and allows for transcription of Wnt target genes to occur (17, 18). The activity of Wnt proteins is controlled at the cell surface by several soluble antagonists including Wnt inhibitory factor (Wif)-1 and Dickkopf (Dkk) receptors (19). Wif-1 acts as a competitive inhibitor of serpine receptor Frizzled by sequestering Wnt factors that activate both the canonical and noncanonical Wnt pathways (20). In contrast, Dkk-1 acts via binding to the receptor Kremen, which induces the endocytotic removal of Lrp5/6 complex from the cell surface thus blocking canonical Wnt signaling (21, 22).

We and others have previously demonstrated that TECs and thymocytes at all stages of development produce Wnt glycoproteins, and that established TEC lines respond in vitro to Wnt proteins (6, 23, 24). Using a Cre-mediated ablation of APC expression in various epithelia, severe defects in the architecture and composition of the thymic microenvironment were observed in postnatal mice (25). However, the loss of APC expression in epithelia other than TECs resulted in a premature death of these mice precluding a detailed analysis of TEC phenotype and function. Moreover, neither the specific cell type nor the time during development when Cre is expressed in these mice has been documented. An in vivo role for Wnt signaling has also been suggested in experiments with mice ubiquitously lacking the expression of the membrane-bound Wnt inhibitory receptor Kremen (26). Although these animals display an aberrant thymic microenvironment featuring epithelial free zones and an increase in TECs concomitantly expressing cortical and medullary markers, the general loss of Kremen expression also affects lymphoid and mesenchymal cells in the thymus. Consequently, the thymic phenotype of these mice cannot be exclusively attributed to increased Wnt signaling in TECs.

The complex cellular nature of the thymic microenvironment has rendered the identification of molecular pathways required in vivo for TEC differentiation and function difficult. Explicitly, the lack of tools to direct genetic manipulations specifically to TECs has hindered the analysis of both direct and indirect consequences of gain- or loss-of function mutations within defined signaling pathways. Although several keratin promoters drive expression of transgenes in distinct subsets of epithelia, their nonuniform expression is stained using directly conjugated Abs specific for CD4, CD8, CD24, CD25, CD44, CD45, and CD117 (BD Biosciences). Anti-EpCAM mAb (G8.8) was purified and conjugated in our laboratory. The labeled cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences) using the CellQuest Pro software (BD Biosciences) as previously reported (30). TECs (CD45 EpCAM+) were sorted using a FACSaria (BD Biosciences) and the routinely assessed cell purity was constantly >95%.

Immunohistochemistry

Frozen thymic sections (8 µm) were obtained either from whole embryos (Figs. 3, A–D and 6A) or isolated thymic lobes (see Figs. 2, C–F and 5, A–C). Tissue sections containing the largest cross-section through the thymus were fixed in acetone and stained using Abs against CK5 (BabCO), CK8 (ProGene), β-catenin (BD Biosciences), Foxn1 (rabbit polyclonal) (31), phospho-Ser 10 histone H3 (Upstate Biotechnology), ETRR7 provided by W. van Ewijk (Utrecht, Netherlands), CD45 (BD Biosciences), CD4, CD24, CD25, CD44, CD45, and CD117 (BD Biosciences). Anti-EpCAM was stained using directly conjugated Abs specific for CD4, CD8, CD24, CD25, CD44, CD45, and CD117 (BD Biosciences). Anti-EpCAM mAb (G8.8) was purified and conjugated in our laboratory. The labeled cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences) using the CellQuest Pro software (BD Biosciences) as previously reported (30). TECs (CD45 EpCAM+) were sorted using a FACSaria (BD Biosciences) and the routinely assessed cell purity was constantly >95%.

Materials and Methods

Mice

Mouse strains Rosa26loxLox(LacZ) (B6;129S-Gtrosa26tm1Sor) and Z/EGLobe21(B6. Cg-Tg(ACB-BgeoGFP)21IbeJf) were obtained from The Jackson Laboratory and maintained as a homozygous and heterozygous colony, respectively. The CITAlox/lox/Cre(15) mice of low expression (CITAlox1tmWith), generated and provided by Dr. W. Reith (University of Geneva, Geneva, Switzerland) and the Ctnnb1lox/loxCre23/3 mice have previously been reported (27, 28) and were kept as homozygotes. Because a robust activation of the canonical Wnt signaling pathway in these mice is already achieved by a single transgene (27), all our experiments were conducted using mice compound heterozygous for the exon 3 deletion of the β-catenin gene. For developmental staging, the day of the vaginal plug was designated as E0.5. Mice were 5- to 10-wk-old for experiments unless stated otherwise and housed in our animal facility in accordance with Institutional and Can-
zeins) was used to isolate cells from the ventral aspect of the third pharyngeal pouch. RNA was extracted using the Micro RNAeasy kit (Qiagen). cDNA was synthesized as described and PCR was initially performed with the following primers: Foxn1 5'-aagcgcgaagttcacaacact-3', 5'-tcaacgggagaggcaagcaatg-3'; and Cre 5'-cagcataacaatgctgcaacag-3', 5'-tactcagcacacacagac-3'. Subsequently, PCR was followed by using the following primers: Foxn1 5'-tcaacatctggtcagcaacag-3', 5'-tcaacctgtaaagcaagttg-3'; and Cre 5'-tcgcttggtcagacagcaacag-3', 5'-tgttgtctgctgcacgttgc-3'. To detect the deletion of β-catenin exon 3 in sections of E16.5 thymic tissue, microdissected tissue was lysed (1 h; 37°C) in 1X PCR buffer (Sigma-Aldrich) supplemented with 1.7 μM SDS (Sigma-Aldrich) and 50 μg/ml proteinase K (Merck Chemicals). Lysates were inactivated (1 min; 85°C) and subjected to PCR as detailed (32).

**OP9-DL1 cultures**

E13.5 thymocytes (4 × 10^5) in fully supplemented medium containing IL-7 were seeded onto irradiated (30 Gy) OP9-DL1 stroma cells and harvested after 7 days for flow cytometric analysis (33).

**Statistical analysis**

Values are mean ± SD. For two-group comparisons, the two-sided t test was used.

**Results**

**Gene targeting in TECs**

To investigate a role for canonical Wnt signaling in early thymus development, the expression of a β-catenin gain-of-function mutation was exclusively targeted to TECs using the Cre-loxP recombination system. Because Foxn1 is expressed within the thymus only by TECs (34), we first generated mice transgenic for a PAC combination system. Because restriction to TECs but could not be detected in thymocytes (Fig. 1, top and middle panels). Analyzing mice at E16.5 and later, β-catenasidaase activity could also be detected in the skin (but no other additional tissues) where Foxn1 is physiologically expressed (Fig. 1B and data not shown). Monitoring GFP expression in the reporter strain Z/EGLobe21, comparable results were obtained when analyzing thymic tissue by immunohistochemistry and flow cytometry: thymic GFP expression in Foxn1cre/Z/EGLobe21 mice was exclusively restricted to TECs but could not be detected in thymocytes (Fig. 1, C and D). The efficiency of Cre recombination was further assessed in Foxn1-Cre:CITTAloxP/loxP crosses in which the recombination of loxP sites results in a deficiency of the transactivator CITTA required for MHC class II expression by TECs (28). These mice lacked positively selected CD4^+CD8^+ thymocytes and hence revealed a phenotype identical with that of animals with a germline deletion of CITTA (Fig. 1E). Thus, Cre expression in Foxn1-Cre mice faithfully mirrors that of Foxn1 and hence allows for efficient Cre-mediated recombination exclusively in TECs but not other thymic cells.

**Conditional stabilization of β-catenin alters thymic organogenesis**

The loss of β-catenin exon 3 results in a constitutively active form of β-catenin due to an absence of the phosphorylation site necessary for protein degradation (36). To target such a stabilized form of β-catenin to TECs, Foxn1-Cre mice were crossed with mice in which exon 3 of β-catenin is flanked by loxP sites (Cumb/Hoxbox35) (27). In contrast to controls (termed βcat wt), the thymic lobes of E16.5 double mutant embryos (designated βcatΔex3) were greatly reduced in size, displayed a rounded shape, and had not descended to their normal precordia position (Fig. 2, A and B). The failure to descend was first apparent at E13.5 and thymic lobes remained positioned high in the neck at later stages of fetal development and in postnatal mice (data not shown). Staining thymic tissue sections from βcat wt control mice with H+E, different CK and the mesenchymal marker ERTR7 (4) revealed abundant cortical (CK5^− CK8^+) epithelium and the emergence of medullary (CK5^− CK8^-) islands, a slender organ capsule, developing trabeculae, and perivascular tissue (Fig. 2, C and E). In contrast, thymus sections of E16.5 βcatΔex3 embryos revealed an unusual organ architecture, with a central epithelial-like core structure largely negative for CK5 and CK8, surrounded by layers of mesenchymal ERTR7^+ cells (Fig. 2, D and F). Specifically, a few CK5^- CK8^- and CK5^− CK8^- cells were localized to the rim of an epithelial-like core. None of these cells within this core coexpressed CK5 and CK8 or the cell surface marker MTS24, both phenotypic hallmarks of thymic epithelial precursors (Fig. 2F and data not shown) (37). The outer aspect of the E16.5 βcatΔex3 thymus was marked by CK5^− CK8^- cells (Fig. 2F). These changes in organ architecture and CK expression were already apparent at E14.5 (data not shown). Most of the cells positioned within the central core stained positively for CKs using a mixture of specific Abs, whereas all of these cells stained positively for the epithelial marker E-cadherin (Fig. 2H and see Fig. 5A). Moreover, a significant fraction of cells in the central core of the βcatΔex3 thymus expressed involucrin, a protein typically present in differentiated skin epithelium (38) (Fig. 2J). The mRNA of both involucrin and loricrin, a major protein component of the cornified cell envelope found in terminally differentiated epithelial cells, was highly up-regulated in the βcatΔex3 thymus at E14.5 and E16.5 when compared with βcat wt control mRNA (data not shown), suggesting a fate-shift of TECs toward terminally differentiated skin epithelium.

Next we determined the time point at which these alterations in βcatΔex3 thymus organogenesis became first apparent. As early as E12.5, β-catenin encoding mRNA lacking exon 3 and increased amounts of β-catenin protein could be detected in thymic tissue and in TECs, respectively (Fig. 3A, left panels, and data not shown). The total thymic amount of β-catenin mRNA was not yet altered in E12.5 βcatΔex3 embryos and only slightly (1.5 times) increased in E13.5 thymic tissue, which suggests a lack of a feedback inhibition effect by an increased concentration of stabilized β-catenin protein. The known direct Wnt target genes Axin2 (39) and Lgr5 (40) were up-regulated 14-fold in TECs at E13.5 revealing activation of the canonical Wnt signaling pathway as a consequence of β-catenin stabilization (Fig. 3A, right). Thymus sections of E12.5 βcatΔex3 embryos contained areas with fewer CK8^- and CK5^- TECs, despite still normal size of the epithelial anlage and a regular appearance of a condensed mesenchymal capsule (Fig. 3B, top left). At E13.5, the thymus of βcatΔex3 embryos was already decreased in cellularity and revealed multiple areas in
FIGURE 1. A PAC based transgenic expression of the Cre recombinase targeted to thymic epithelial cells using the Foxn1 promotor. A, Targeting strategy and expression of Cre. The codon-improved sequence for the Cre recombinase was placed into exon 2 of the Foxn1 gene following its endogenous translation initiation site to preserve all regulatory elements of the Foxn1 locus (top). kan, kanamycin resistance gene; ex, exon; FRT, flipase recombination target. RT-PCR-based Cre expression analysis is shown in the ventral aspects of the third pharyngeal pouch (3 pp) at embryonic day (E) 10.5 in transgene-negative (−) and transgene-positive (+) mice (bottom). The tissue for analysis was isolated by laser capture microscopy. The expression of Foxn1 was used as a tissue-specific control. B, Timing and specificity of Cre recombination in Foxn1-Cre:RosaloxLacZ mice. Immunofluorescence analysis of thymic sections using Abs specific for the epithelial markers CK8 (green) and Foxn1 (red) at E11.5 and E12.5 (upper panels). Detection of β-galactosidase activity by LacZ staining on thymic sections from E11.5 (middle panel left) (the inset represents a magnification of the ventral part of the thymus anlage) from E12.5 (middle panel right), and from E14.5 embryos (lower panels) is shown. The thymus anlage is outlined with a dotted line (middle panels). Successful recombination (arrows) was first detected in the ventral aspects of the third pharyngeal pouch at E11.5. Sections were counterstained with Nuclear Fast Red. C, Detection of Cre-mediated recombination in thymic tissue of E12.5 Z/EG (left panel) and E12.5 Foxn1-Cre:Z/EG mice (three right panels) as revealed by enhanced GFP expression and immunofluorescence for the epithelial marker CK8 (red). D, Flow cytometric analysis of Cre-controlled enhanced GFP expression in thymic epithelial cells (CD45<sup>+</sup>EpCAM<sup>+</sup>) from E14.5 Z/EG mice (black histogram), in thymic epithelial cells (CD45<sup>+</sup>EpCAM<sup>+</sup>) from E14.5 Foxn1/Z/EG mice (light gray histogram) and in thymocytes (CD45<sup>+</sup>EpCAM<sup>+</sup>) from Foxn1/Z/EG mice (dark gray histogram). E, Flow cytometric analysis of thymocyte maturation in adult Foxn1-Cre:CIITA<sup>lox/lox</sup> mice using the developmental markers CD4 and CD8. The relative frequency of the distinct subpopulations is provided as the percentage in each quadrant. Note that the extent of the CD4 deficiency is identical with that reported for mice with a germline deletion of CIITA (28). Scale bar represents 50 μm.
which the staining for CK8 and CK5 was either reduced or absent (Fig. 3B, top right, and Fig. 3C). Moreover, the initiation of the typical lobular partition by in-growing mesenchymal cells was missing in the βcatΔex3 thymus at E13.5 (Fig. 3B, top right).
We next investigated whether the decreased thymus size of cat ex3 embryos could be related to a down-regulation of FgfR2IIb, the receptor specific for the TEC-mitogenic ligands Fgf7 and Fgf10 (43). Real-time RT-PCR analysis for FgfR2IIb transcripts revealed a significant decrease in βcatΔex3 thymic tissue and in isolated TECs from E13.5 embryos in comparison to the wt controls (Fig. 4F). Hence, a limited capacity of TECs to proliferate was consistent with a smaller thymus size in βcatΔex3 embryos, whereas the survival of TECs seemed unaffected by the expression of stabilized β-catenin.

To assess downstream targets of canonical Wnt signaling that may affect a change in TEC phenotype, we analyzed at an early stage of thymus development the expression of the transcriptional repressor Msx1 (44). The homeoprotein Msx1 is not only present in a variety of embryonic tissues that require epithelial-mesenchymal interactions for their morphogenesis but is also associated with inhibition of the differentiation of several cell types (45). Before any apparent structural changes, a 4-fold up-regulation in the expression of Msx1 had already occurred in the thymus of E12.5 βcatΔex3 embryos and dramatically increased within the following 24 h (Fig. 4G, left). The abundance of Msx1 expression was specifically detected in TECs but not in nonepithelial stroma or thymocytes (Fig. 4G and data not shown). In contrast to Msx1, decreased amounts of Foxn1 mRNA were present in the thymus at E13.5 (Fig. 4H).

Stabilizing mutation in β-catenin leads to loss of TEC identity

To further establish the identity of the cells in the inner core of the E16.5 βcatΔex3 thymus, tissue sections were stained for the expression of Foxn1 protein and the epithelial cell marker E-cadherin. Foxn1 was detected by immunohistochemistry only in a few epithelial cells in the central core of the thymus (Fig. 5, A–D). As expected, Foxn1 mRNA expression could still be detected in βcatΔex3 thymic tissue at this stage, but its abundance had already significantly decreased starting at day E13.5 (Fig. 4H). We next determined whether the abundant Foxn1 negative epithelia had originated from cells committed to a thymic fate. For this purpose, βcatΔex3 mice were crossed to Rosa26loxLacZ reporter mice and their thymic tissue was stained for β-galactosidase activity. In these mice, any cell that had once committed to a TEC fate, and hence expressed Foxn1, was genetically marked independently of a later adopted change in cell identity secondary to the expression of a stabilized form of β-catenin. As demonstrated in Fig. 5, E and F, epithelia in the inner core of the thymus stained positively for β-galactosidase and had deleted β-catenin gene exon 3. These cells had therefore adopted a thymic fate but had subsequently lost the expression of Foxn1, a typical marker of TEC identity. In contrast, the N-cadherin positive cells in the outer part of the βcatΔex3 thymus were negative for β-galactosidase and retained normal genomic sequence. Therefore, these cells did not originate from TECs by way of epithelial-mesenchymal transition.

Thus, considerable changes in the thymic epithelial compartment were already present as early as E12.5 following the constitutive expression of a stable form of β-catenin.

Constitutively active β-catenin changes proliferation of TECs

We next investigated whether the decreased thymus size of βcatΔex3 mice was due to either an increase in apoptotic cell death of developing TECs or, alternatively, a decrease in cell proliferation. Using TUNEL, no difference in programmed cell death could be detected at E12.5 (Fig. 4, A and B) and at E13.5 (data not shown) when comparing thymic tissue from βcat wt and βcatΔex3 E12.5 embryos. This observation argues against a general toxic effect of the stabilized β-catenin on TEC viability. In contrast, a substantial decrease in the frequency of cycling TECs (defined as cells double positive for Foxn1 and the mitosis marker phospho-Ser10 histone H3) was noted in βcatΔex3 embryos (Fig. 4, C–E). As the proliferation of epithelial cells has been linked to canonical Wnt signaling and fibroblast growth factors (41, 42), we investigated whether the smaller thymic size of βcatΔex3 embryos could be related to a down-regulation of FgfR2IIb, the receptor specific for the TEC-mitogenic ligands Fgf7 and Fgf10 (43).
similar or possibly slightly reduced in the βcatΔex3 thymus when compared with the control (Fig. 6A, left row). At E13.5, the number of intrathymic CD45<sup>+</sup> cells was lower in mutant mice and correlated with an accumulation of these cells adjacent to the thymus anlage (Fig. 6A, right row). As the attraction and subsequent colonization of lymphoid precursors to the prevascular thymus anlage are dependent on the chemokines CCL21 and CCL25 (8), we investigated whether a change in thymic expression of these molecules could account for this early impairment in homing. Analyzed by real-time RT-PCR, the amount of CCL25-specific transcripts (but not that of CCL21) was decreased 2- to 3-fold at E12.5, 10-fold at E13.5 (in both whole thymic tissue and isolated TECs), and remained low at E14.5 and E16.5, when compared with age-matched controls (Fig. 6B).

Next, thymic tissues from βcatΔex3 and βcat wt mice were investigated for the presence and relative distribution of the different thymocyte subpopulations. At E13.5, thymocytes from βcat wt mice were either at the DNI (CD44<sup>+</sup>CD25<sup>-</sup>) or at the DNII (CD44<sup>+</sup>CD25<sup>-</sup>) stage with only very few cells having yet progressed to the DNIII phenotype (CD44<sup>+</sup>CD25<sup>+</sup>). The bulk of DNI cells concurrently expressed high levels of heat-stable Ag (CD24) and the receptor c-kit (CD117) (Fig. 7A). In contrast, none of the thymocytes from βcatΔex3 mice had matured beyond the DNI stage and the majority of these cells failed to express high surface levels of either CD24 or CD117 (Fig. 7A). This complete block in thymocyte differentiation persisted at all later embryonic and postnatal stages investigated (Fig. 7A). Hence, the constitutive activation of the canonical Wnt pathway in TECs created a microenvironment unable to support thymocyte development beyond the first stage of maturation.

Wnt signaling promotes the expression of a number of secreted Wnt antagonists (46) that may thus modulate Wnt signaling via a feedback mechanism. Secreted Wnt antagonists have also been found to block intrathymic T cell development in in vitro fetal thymic organ cultures (24, 47). Therefore, we analyzed thymic tissue from βcatΔex3 and βcat wt embryos for the presence of...
transcripts of secreted Wnt antagonists. As demonstrated in Fig. 7B, increased expression of Dkk-1, Dkk-4, and Wif-1 could be detected in the βcatΔex3 thymus at E12.5, E13.5, and in TECs isolated from E12.5 and E13.5 and in TECs isolated by cell sorting from embryos at E13.5 (right side). βcat wt values were arbitrarily set to a value of 1.0 for comparison. Data represent the mean ± SD of triplicate real-time PCR analyses. C, Flow cytometric analysis of thymocytes from βcat wt and βcatΔex3 embryos cultured for 7 days on OP9-DL1 cells. The relative frequency of the distinct subpopulations stained for the cell surface expression of CD4 and CD8 is provided for each quadrant. Data are representative of two independent experiments.

Discussion

Expression of a gain-of-function mutation of β-catenin was efficiently targeted to TECs using a novel Foxn1-Cre mouse strain and this allowed us to investigate the role of the canonical Wnt signaling for TEC development and homeostasis. In this report, we demonstrate that constitutive activation of Wnt signaling changes the earlier commitment of TECs to a thymic fate and as a result causes an atypical composition and architectural organization of the developmental support necessary for DNI thymocytes to progress to more mature thymocyte stages, but do not affect their potential to become T cells.

FIGURE 7. T cell development in βcatΔex3 embryos is arrested at the DNI stage of development. A, Flow cytometric analysis of thymocyte maturation in E13.5 embryos using the developmental markers CD44 and CD25 (left panels), in E16.5 embryos and adult mice using the CD4 and CD8 (right panels). The population of CD44−CD25− thymocytes was further characterized for its expression of CD117 (c-kit) in conjunction with CD24 (arrows) (second row left panels). The CD44+CD25− population represents nonhematopoietic CD45− cells released during enzymatic digestion of the thymic lobes. The relative frequency of the distinct subpopulations is provided as a percentage in each quadrant. Data are representative of two independent experiments. B, Quantitative RT-PCR analysis for the thymic expression of IL-7 and the secreted Wnt inhibitory factors Dkk-1, Dkk-4, and Wif-1 in embryos at E12.5 and E13.5 and in TECs isolated by cell sorting from embryos at E13.5 (right side). βcat wt values were arbitrarily set to a value of 1.0 for comparison. Data represent the mean ± SD of triplicate real-time PCR analyses. C, Flow cytometric analysis of thymocytes from βcat wt and βcatΔex3 embryos cultured for 7 days on OP9-DL1 cells. The relative frequency of the distinct subpopulations stained for the cell surface expression of CD4 and CD8 is provided for each quadrant. Data are representative of two independent experiments.
the stromal compartment. Consequently, only limited numbers of lymphoid precursors immigrated into the thymus anlage, but these cells were arrested at the earliest stage of their development.

The loss of typical CK and Foxn1 expression by TECs in βcatΔex3 mice revealed a change in their cell identity. The expression of Foxn1 serves as the earliest molecular marker for the commitment of epithelial cells to a thymic cell fate (7, 6) and has been implicated in epithelial cell proliferation and terminal differentiation (49). Nonetheless, the initial patterning of the thymus anlage is independent of Foxn1 (34) because TECs in nude (i.e., Foxn1-deficient) mice display phenotypic features characteristic of the putative epithelial mesenchymal progenitor population (50, 51). The thymi of nude and to a lesser extent also of wt mice contain as well other epithelial cell types (e.g., respiratory and intestinal epithelial cells) that are frequently organized in cysts (52). The thymus of βcatΔex3 mice lacks, however, such features (Fig. 2E and data not shown). Because the epithelia of the inner core of the βcatΔex3 thymus had once adopted a TEC fate (as revealed by the Foxn1-Cre mediated recombination of the Rosa26loxPlacZ locus, Fig. 5E), it is very unlikely that these cells are derived from a non-TEC epithelial origin. As a consequence of activation of the canonical Wnt signaling pathway, it is rather conceivable that the E-cadherin-positive epithelial cell types within the inner core of the βcatΔex3 thymus thymus had shifted their fate to acquire features of other cell types including that of skin epithelia. This notion is supported by up-regulation of involucrin and loricrin, markers associated with the terminal differentiation of skin epithelia (38).

Engagement of the Wnt/β-catenin pathway has been associated with the conversion of E-cadherin-positive epithelia to N-cadherin-positive mesenchyme, a process defined as epithelial-mesenchymal transition (53). Our experiments excluded epithelial-mesenchymal transition as an explanation for the increase in N-cadherin-positive mesenchymal cells in the outer aspects of the βcatΔex3 thymus because these cells had a nonrecombined β-catenin locus and failed to display β-galactosidase activity in the triple mutant βcatΔex3:Rosa26loxLacZ mice. The N-cadherin-positive mesenchymal cells in the βcatΔex3 thymus may have expanded from autochthonous mesenchymal cells in response to brachyury, as increased levels of transcripts for this transcription factor could be detected in the thymic tissue at a stage of development when gross structural changes had not yet occurred (i.e., E12.5 and E13.5, and data not shown). The expression of brachyury is controlled by β-catenin-dependent transactivation (54) and constitutes an early-immediate response to different inducers of mesoderm specification and differentiation (55). Consequently, the stromal cells positioned in the outer areas of the βcatΔex3 thymus must have accumulated in response to molecular cues provided by the altered thymic microenvironment.

The factors that control the initial expression of Foxn1 in thymic epithelia have not yet been identified, as mice with loss of function mutations of the signaling cascade that pattern the thymic pouch (i.e., the Hox-Pax-Eya-Six pathway) fail to reach the developmental stage at which Foxn1 is expressed and a thymus primordium becomes discernible (reviewed in Ref. 5). Wnt signals have been demonstrated in vitro to modulate Foxn1 expression in established TEC lines that characteristically express very low levels of this transcription factor (6). The precise mechanism by which these molecules control the transcription of Foxn1 in vivo is not yet fully established, but may likely engage (in contrast to in vitro data) signaling pathways independent of β-catenin. Following the expression of a stabilized form of β-catenin, potent Wnt inhibitors are secreted within the thymic microenvironment which in turn sequester Wnt molecules precluding their capacity to signal via noncanonical (i.e., β-catenin-independent) Wnt pathways (56).

This might explain why Foxn1 expression is diminished and eventually lost prohibiting the maintenance of early thymic epithelial development. Alternatively, the loss of Foxn1 expression could also have occurred secondary to TECs adopting a new cell fate because enforced activation of canonical Wnt signaling has been demonstrated to effect transdifferentiation (57, 58). In this context, it is possible that Wnts regulate the expression of downstream target genes whose functions are associated with positional fate assignment. Indeed, expression of the homeobox gene Msx1 in the thymus was highly up-regulated in βcatΔex3 thymic epithelia (Fig. 4G). Msx1 is a transcriptional repressor and is present in a variety of embryonic tissues requiring epithelial-mesenchymal interactions for their morphogenesis (59). Msx1 is differentially expressed at E10.5 between the ventral and the dorsal endoderm of the third pharyngeal pouch but is subsequently down-regulated in TECs to low expression levels (M.P.K., K.N. and G.A.H., unpublished data). As the misexpression of Msx1 inhibits the differentiation of multiple epithelial progenitor types (45), it is thus attractive to speculate that a very high and continuous expression of Msx1 in the presence of soluble Wnt inhibitors perturb TEC differentiation (possibly together with a deregulated expression of other ligands to conserved signaling pathways, such as bone morphogenetic proteins). To ascribe a definitive role to Msx1 or other target genes of the canonical Wnt pathway for changes in TEC identity, additional experiments are needed. However, the shift in fate of TECs and the loss in maintaining a regular thymus function following the constitutive activation of the Wnt pathway are consistent with findings in other experimental systems (25–27, 57).

Although some of the earliest T lymphoid precursors could be recovered from the E13.5 βcatΔex3 thymus, a substantial number of these cells nonetheless accumulated in the tissue surrounding the combined thymus/parathyroid primordium. The seeding of T-lymphoid precursor cells to the avascular thymus is dependent on a coordinated action of CCL21/CCR7 and CCL25/CCR9-mediated chemokine signals and a single deficiency for either of these signals is sufficient to cause a decrease in thymocyte cellularity (60). CCL25 transcripts were significantly reduced in the combined thymus/parathyroid primordium of E12.5 βcatΔex3 embryos when the overall composition and organization of the thymus had not yet been greatly affected by the expression of a stable form of β-catenin. This decrease in CCL25 expression correlates with a reduction in early precursor immigration to the βcatΔex3 thymus and may reflect the direct influence of canonical Wnt signaling on CCL25 expression. In contrast, levels of CCL21 transcripts remained unchanged or were even increased, a finding consistent with the preferential expression of CCL21 by parathyroid epithelia within the combined thymus/parathyroid primordium (8).

The earliest thymic population of lymphoid cells, termed early T-lineage progenitors, are found within the DNI subpopulation and express high levels of the cytokine receptor c-kit (CD117) (61). Fetal early T-lineage progenitors are already largely restricted to a T cell fate (62), whereas adult early T-lineage progenitors may or may not have yet made such a decision (63). It is generally accepted that the progeny of early T-lineage progenitors depend on Notch signals for their up-regulation of CD117, subsequent commitment to the T cell lineage and the ability to transit beyond a DNII phenotype (64). At E13.5, thymocytes of βcatΔex3 mice were completely arrested at the DN1 stage and the majority of these cells failed to up-regulate CD117, despite the unchanged expression of Delta-like 1 (DL1) and Delta-like 4 in the thymus of βcatΔex3 embryos (data not shown). A phenotype of low CD117 and CD24 expression on thymocytes may be reflective of a changed developmental potential of the DNI cells of βcatΔex3 mice or, alternatively, the consequence of an altered composition.
and function of the thymic microenvironment. In support of the latter notion, a decreased expression of IL-7 was noted in βcat/Δex3 thymi at E13.5, which may have contributed to the block in T cell development. Coculture of the βcat/Δex3 thymocytes with OP9 stromal cells expressing high levels of the Notch ligand Delta-like 1 (i.e., OP9-DL1) revealed, however, the cell-autonomous potential of these DNI cells to progress to more mature T cell phenotypes. In summary, the extensive changes to the thymic microenvironment of βcat/Δex3 mice preclude, directly or indirectly via the increased production of soluble Wnt inhibitors (24), the regular development of lymphoid precursors to T cells.

Multiple signaling inputs control regular TEC development and homeostasis, which are annulled in the presence of a gain-of-function mutation of β-catenin. As TEC expression of stabilized β-catenin up-regulated the Wnt inhibitor Wnt-1, most likely as part of a physiologically negative feedback loop, the lack of regular noncanonical Wnt signaling may also account for the broad developmental changes observed in βcat/Δex3 mice. Independent of detailed knowledge regarding the contributions by other pathways, our results demonstrate at a cellular and molecular level that a precise regulation of canonical Wnt signaling is essential for the normal development and maintenance of TEC identity and function.

Acknowledgments

We thank N. Copeland (Buffalo, NY), R. Sprengel (Heidelberg, Germany), and E. Casanova (Vienna, Austria) for providing reagents and advice, A. Nebenius and D. Nebenius-Oosthuizen (Transgenic Mouse Core Facility, University of Basel) for generating Foxn1-Cre transgenic mice, A. Offinger and R. Recinos (Center for Biomedicine, University of Basel) for animal husbandry, A. Peter for technical assistance, and U. Schaller for secretarial assistance.

Disclosures

The authors have no financial conflict of interest.

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