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*J Immunol* 2015; 195:5678-5687; Prepublished online 4 November 2015; doi: 10.4049/jimmunol.1502010
http://www.jimmunol.org/content/195/12/5678

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2015/11/04/jimmunol.1502010.DCSupplemental

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Foxn1 Protein Expression in the Developing, Aging, and Regenerating Thymus

Immanuel Rode,* Vera C. Martins,*-1 Günter Küblbeck,∗ Nicole Maltry,* Claudia Tessmer,‡ and Hans-Reimer Rodewald*†

The forkhead box N1 (Foxn1) protein is the key regulator of thymic epithelial cell (TEC) development, yet how Foxn1 functions remains largely unknown. All mature TECs arise from Foxn1-expressing progenitors/immature TECs and it is widely assumed that TECs as a whole are defined by Foxn1 expression. However, data on the Foxn1 protein are virtually lacking. In this study, we developed novel tools to visualize Foxn1 protein expression at single-cell resolution. We generated Foxn1 knock-in mice expressing a C-terminal hemagglutinin-tagged Foxn1 protein, and a cytometry-grade monoclonal anti-Foxn1 Ab. We evaluated Foxn1 expression patterns in TEC subsets and its dynamics during normal thymus development, aging, injury, and regeneration. Upon challenges, upregulation of Foxn1 was a common feature of thymus regeneration, but the timing of Foxn1 expression changed and the responding TEC subsets depended on the type of treatment. Whereas dexamethasone and recombinant human fibroblast growth factor 7 promoted expansion of Foxn1+Ly51+CD80+ TECs, castration led to expansion of Foxn1Ly51CD80+ TECs. Collectively, Foxn1 expression is highly heterogeneous in the normal thymus, with large fractions of Foxn1low or Foxn1− TECs accumulating with age. Furthermore, Foxn1 expression is responsive to perturbations.

The Journal of Immunology, 2015, 195: 5678–5687.

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Received for publication September 9, 2015. Accepted for publication October 13, 2015.

This work was supported in part by Deutsche Forschungsgemeinschaft-Sonderforschungsbereich Grant 873-Project B11, as well as by a grant from the Helmholtz Alliance Preclinical Comprehensive Cancer Center (to H.-R.R.).

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The online version of this article contains supplemental material.

Materials and Methods

Mice and targeting

Foxn1Z/foxn1Z (8), Foxn1-Cre (16), Rosa26-YFP (17), and Rag2−/− (18) mice were described earlier. C57BL/6J mice were either bred at the animal facility of the German Cancer Research Center or purchased from Charles River Laboratories. Foxn1foxn1 knock-in mice were generated by conventional gene targeting using the construct shown in Supplemental Fig. 1A. All animal experiments were performed in accordance with institutional and government regulations and were approved by the Regierungsräsidium (Karlsruhe, Germany).

Epithelial cell isolation

Thymic stromal cell isolation was done with slight modifications as described earlier (19). In contrast to the published method, minced thymi...
were directly subjected to digestion without discarding any fractions, and lymphoid cells (anti-CD45 beads, Miltenyi Biotec) and erythrocytes (anti-Ter119 beads, Miltenyi Biotec) were manually depleted using MACS technology. Embryonic thymi were digested in 96-well V-bottom plates, and the resulting single-cell suspensions were directly used for flow cytometry without depletion.

Alveolar epithelial cells were isolated from the lungs as described (20). Small intestinal epithelial cells were isolated by incubating cleaned and finely minced small intestine fragments three times for 20 min each at 37°C in RPMI 1640 (5% FCS, 1.25 mM EDTA) while shaking. Supernatants were filtered (70 and 30 μm) to yield single-cell suspensions.

**Flow cytometry and counting**

For intracellular stainings (Foxp3 staining kit, eBioscience), cells were first incubated with a Live/Dead fixable violet dead cell stain kit (Life Technologies) and then fixed and blocked, and surface stainings were directly blocked. Simultaneous staining of YFP and Foxn1 required replacing the eBioscience fixative with 3.7% paraformaldehyde in PBS. Fluorescein-di-tetra-galactopyranoside (FDG) staining was done with the FluoroReporter lacZ flow cytometry kit (Life Technologies). Abs used were CD45 (30F11) Brilliant Violet 421/PE-Cy7, Aire (5H12) Alexa Fluor 488, K67 (SolA15) PE-Cy7 (eBioscience), CD326 (G8.6) PerCy-Cys5.5/PE-Cy7/Alexa Fluor 647, CD80 (16-10A1) Brilliant Violet 605, rat IgG1, κ (RTK2071) PE-Cy7 (BioLegend), Ly51 (6C3) PE, CD45 (30F1) FITC (BD Biosciences), aprotinin, hemagglutinin (HA; GG8-1F3.3.1) PE (Miltenyi Biotec), and Foxn1 (2/41) (RTK2071) PE-Cy7/Alexa Fluor 647 (available on request).

For TEC counting, Accudrop beads (BD Biosciences) of a fixed concentration were spiked into all samples just before they were acquired on the LSRFortessa.

**Ab generation and labeling**

Full-length, native, recombinant mouse Foxn1 was used to immunize mice in collaboration with the mAb Facility at the German Cancer Research Center. One purified clone (2/41, mouse Ig2b) was labeled for all further experiments using the Alexa Fluor 647 Ab labeling kit (Life Technologies).

**Dexamethasone treatment**

Seven- to eight-week-old Foxn1HA/HA mice received a single i.p. injection of either 20 mg/kg dexamethasone (DEX; Sigma-Aldrich) dissolved in DMSO (Sigma-Aldrich) or an equal volume of DMSO.

**Recombinant human fibroblast growth factor 7 injection**

Six-day-old C57BL/6J mice were i.p. injected with either 5 mg/kg recombinant human fibroblast growth factor 7 (rhFGF7; Kepivance [palifermin], Sobi) or an equal amount of solvent (PBS) for 3 consecutive days. Three (i.e., postnatal day 9) and six days later (i.e., postnatal day 12), mice were analyzed by flow cytometry.

**Surgical castration**

For surgical castration, 9- to 11-wk-old C57BL/6J mice were anesthetized and a small ventral abdominal midline incision was made to reveal the testes. These were removed along with surrounding fatty tissue. Sham surgery involved the removal of the testes. These were removed along with surrounding fatty tissue. Sham surgery involved the removal of the testes.

**Histology**

Embryonic day 14.5 embryos, used for thymus staining in Fig. 1A, were fixed overnight (4% paraformaldehyde, 4˚C), and 1-wk-old thymus lobes (Supplemental Fig. 2B) from C57BL/6J and Foxn1HA/HA mice were fixed for 1.5 h. Fixation was followed by cryoprotection in 20% sucrose/PBS (w/v). After OCT embedding in −80°C isopentane, embryonic sections were stained with anti-ΔNp63 (Poly6190, BioLegend) and anti-α-HA (3F10, Roche). Anti-ΔNp63 was detected with anti-rabbit Alexa Fluor 488 (A-11008, Thermo Fisher Scientific), and anti-HA was detected with anti-rabbit Alexa Fluor 546 (A-11081, Thermo Fisher Scientific). Endogenous biotin in thymus sections was blocked (E-21390, Thermo Fisher Scientific) before incubation with anti-Foxn1Bio (2/41) and anti-HA (3F10, Roche). The biotinylated (EZ-Link-kit 21327, Thermo Fisher Scientific) Foxn1 Ab was detected with anti-biotin-Cy3 (Jackson ImmunoResearch Laboratories), and anti-HA was detected with anti-rabbit Alexa Fluor 488 (A-11006, Thermo Fisher Scientific).

**Southern blotting**

Nonradioactive Southern blotting was performed on SspI/SphI-digested tail DNA of Foxn1HA/HA knock-in mice using a biotinylated Foxn1 probe (Supplemental Fig. 1A).

**Expression systems**

Foxn1 and fragments of various lengths were cloned into pEGFP-C1 and electroporated (Gene Pulser Xcell, Bio-Rad) into Cos-1 cells for transient expression.

For immunization, Foxn1 cDNA was cloned into pET-22b(+) (Novagen). Expression of the protein in BL21-CodonPlus (DE3)–RIL bacteria (Agilent Technologies) was induced with isopropyl β-D-thiogalactopyranoside (Sigma-Aldrich).

**X-gal staining**

Newborn thymi were fixed for 15 min with 2% paraformaldehyde on ice, transferred into X-gal staining solution, and incubated at 37°C with gentle agitation. The staining reaction was stopped by repeated washes with unsupplemented PBS. Staining solution (in unsupplemented PBS) included 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl2, 0.01% sodium deoxycholate, 0.02% Nonidet P-40, and 1 mg/ml X-gal.

**Results**

New tools to measure Foxn1 expression at the single-cell level

Most information on Foxn1 expression is based on RNA data or knock-in/transgenic reporter alleles (8, 14, 21). However, Foxn1 protein and reporter levels may differ due to transcriptional variations, posttranslational modifications, or differences in protein stability comparing the reporter and the native Foxn1 protein. To overcome these disadvantages, we followed two different experimental approaches. First, we engineered a mouse carrying a tagged version of Foxn1, and second, we generated a monoclonal, flow cytometry grade Ab. Because modifications of the Foxn1 locus had previously resulted in hypomorphic alleles (22), we decided to attach the small HA tag of 9 aa to the C terminus of Foxn1 by gene knock-in in embryonic stem cells (Supplemental Fig. 1A, 1B). Homozygous Foxn1HA/HA mice were born with fur and thymus, indicating that the mutant allele encodes a functional Foxn1 protein. We compared total thymus cell numbers and phenotypic stages of T cell development in Foxn1HA/HA to Foxn1−/− C57BL/6 mice (Supplemental Fig. 1C–E). All parameters were comparable, except for an increase in cell numbers at 6 wk of age in early backcrosses of Foxn1HA/HA mice compared with C57BL/6 controls (Supplemental Fig. 1C). Reanalysis of 10th generation backcrosses of Foxn1HA/HA mice revealed comparable cell numbers also in 6- to 8-wk-old mice (Supplemental Fig. 1C, 1E, red symbols). We next analyzed whether the HA-tagged Foxn1 protein showed a nuclear localization in TECs. Double staining against Foxn1-HA and the epithelial cell–specific transcription factor ΔNp63 revealed colocalization within the nuclei of TECs (Fig. 1A). Collectively, these data show that HA-tagged Foxn1 correctly localizes to the nucleus and functions well in lieu of the normal protein.

In the second approach, we immunized mice with full-length recombinant native Foxn1 to produce mouse mAbs. One mAb (clone 2/41) was selected for detailed characterization. This Ab stained Cos-1 cells transiently transfected with Foxn1 cDNA (Supplemental Fig. 2A). As further evidence for the specificity of both reagents, double staining with anti-Foxn1 and anti-HA Abs identified the same cells in Foxn1HA/HA thymus sections (Supplemental Fig. 2B). Epitope mapping with deletion constructs determined that clone 2/41 binds the C terminus of Foxn1 between amino acids 475 and 542 (Supplemental Fig. 2C).

Next, we tested the capacity of Abs against HA or Foxn1 (clone 2/41) to recognize the Foxn1-HA fusion protein or normal Foxn1 protein, respectively, by flow cytometry. Newborn TECs from Foxn1−/− or Foxn1HA/HA mice were intracellularly stained with Abs against HA, the new Foxn1 Ab (clone 2/41), or a combination of both, and analyzed by flow cytometry. Whereas the anti-HA Ab stained the Foxn1HA/HA TECs exclusively (Fig. 1B, left column),
the tag-independent anti-Foxn1 Ab stained TECs of both genotypes (Fig. 1B, middle column). When both Abs were used in combination, Foxn1HA/HA but not Foxn1+/+ TECs were double positive, that is, both Abs stained the same cells (Fig. 1B, right column). To further exclude nonspecific labeling, we used Foxn1LacZ knock-in mice, in which Foxn1 is nonfunctional whereas β-galactosidase is produced under the control of the endogenous Foxn1 promoter (8). According to their allelic composition, TECs of this strain expressed homozygously Foxn1 (Foxn1+/+) or homozygously β-galactosidase (Foxn1LacZ/LacZ) or both heterozygously (Foxn1LacZ/+) or the presence of the C-terminal HA tag (middle). One representative out of three experiments is shown. (C) Newborn thymic stroma (live, CD45+) of Foxn1+/+ (left), Foxn1LacZ+/+ (middle), or homozygous nude Foxn1LacZ/LacZ mice (right) was stained with either FDG (upper panel) or anti-Foxn1 (lower panel). Note that the live, CD45+ EpCAM+ population shows no signs of Foxn1 staining. One representative out of three independent experiments is shown.

FIGURE 1. New Foxn1 tools are TEC specific. (A) E 14.5 thymus sections of either Foxn1+/+ (upper panel) or Foxn1HA/HA (lower panel) mice were stained with Abs against HA (red, left) and ΔNp63 (green, middle) and counterstained with DAPI (blue, merge on the right; scale bars, 20 μm). One representative out of two independent experiments is shown. (B) Newborn thymic stroma (live, CD45−) of either Foxn1+/+ (upper panel) or Foxn1HA/HA mice (lower panel) was intracellularly stained with Abs against HA (left), Foxn1 (middle), or a combination of both (right). Note that the epitope of the Foxn1 Ab is not affected by the presence of the C-terminal HA tag (middle). One representative out of three experiments is shown. (C) Newborn thymic stroma (live, CD45−) of Foxn1+/− (left), Foxn1LacZ+/− (middle), or homozygous nude Foxn1LacZ/LacZ mice (right) was stained with either FDG (upper panel) or anti-Foxn1 (lower panel).
Simultaneous Foxn1 and FDG staining of cells was technically incompatible, and hence we stained cells either by FDG (indicating at least one Foxn1LacZ allele) (Fig. 1C, top row) or with anti-Foxn1 Ab (indicating at least one Foxn1+ allele) (Fig. 1C, bottom row) versus the pan-epithelial marker EpCAM. Wild-type Foxn1+/+ TECs stained for Foxn1 only (Fig. 1C, left column), heterozygous Foxn1+/LacZ TECs were positive for FDG and anti-Foxn1 (Fig. 1C, center column), and homozygous nude Foxn1LacZ/LacZ TECs were exclusively positive for FDG (Fig. 1C, right column). Therefore, cells with “TEC identity” could be identified in the Foxn1-deficient thymic rudiment by the active Foxn1 promoter (evident by staining with FDG), but these “wannabe” TECs cannot express Foxn1, and lack of their staining proves the specificity of the monoclonal Foxn1 Ab (clone 2/41). In line with this specificity, the CD45EpCAM+ non-TEC stroma remained unstained (Fig. 1C, bottom row). Moreover, we also stained nonthymic epithelium and found that the tested alveolar (Supplemental Fig. 2E) and intestinal (Supplemental Fig. 2F) epithelial cells were not recognized by the Foxn1 Ab based on flow cytometry.

**Foxn1 expression during ontogeny**

Specification of pharyngeal epithelium toward TECs occurs independent of Foxn1, but further differentiation into cortical and medullary TECs requires Foxn1 (8). During ontogeny, comprehensive information on Foxn1 expression levels and the distribution of expressing TEC subsets is lacking. Analysis of embryonic development (E14.5–18.5) revealed that ~94% of CD45 EpCAM+ epithelial cells in the thymus expressed Foxn1 (Fig. 2A). Non-epithelial (CD45 EpCAM−) stromal cells were Foxn1− (Fig. 2A). These high percentages of Foxn1+ TECs remained constant over time in prenatal development (Fig. 2B, 2C), irrespective of TEC subset and proliferative state. The small fraction of Foxn1− TECs was initially mostly limited to Ly51−CD80+ cells (termed double-negative TECs in this study) and to Ly51+CD80− cells (termed Ly51+cortical TECs [cTECs]), but some Ly51− CD80+ cells (termed CD80+ medullary TECs [mTECs]) were also detected (Fig. 2A).

To determine whether Foxn1− cells (Fig. 2C) represent a pre- or post-Foxn1 stage, we lineage-traced these cells using Foxn1::Cre.
transgenic mice crossed to \textit{Rosa26-YFP} reporter mice (16, 17). In these mice all cells expressing Foxn1 at any time point in their development and their progeny are YFP\textsuperscript{+}. In E14.5 thymi from \textit{Foxn1\textsuperscript{Cre}} transgenic mice, but not \textit{Cre\textsuperscript{−}} control mice, almost all TECs had a history of Foxn1 expression (Fig. 2D). The vast majority of these cells actively expressed Foxn1 (YFP\textsuperscript{Foxn1\textsuperscript{−}}). However, \textsim\textfrac{5}{100} of these lineage-traced cells were Foxn1 protein-negative (YFP\textsuperscript{Foxn1\textsuperscript{−}}) (Fig. 2D). Collectively, during embryonic development, TECs almost homogeneously express Foxn1, and consequently this is true for TECs with cTEC and mTEC phenotypes. A very small fraction of cells (\textsim\textfrac{5}{100}) has gone through a Foxn1 RNA-expressing stage, but fails to express the protein. Hence, these cells are “post-Foxn1 mRNA” cells. Additionally, a minor subset of EpCAM\textsuperscript{+}YFP\textsuperscript{+}Foxn1\textsuperscript{−} cells was also found.

\textbf{Foxn1 expression during adulthood}

We examined Foxn1 expression in \textit{Foxn1\textsuperscript{Rosa26-YFP}} mice using our Foxn1 mAB 2/41 between 11 d after birth and up to \textsim\textfrac{2}{3} y of age. Flow cytometric analysis using anti-Foxn1 revealed that both expression levels and frequencies were highest in the youngest analyzed mice (Fig. 3A–C), which was verified with an independent dataset obtained by anti-HA staining. Frequencies of Foxn1 expressing cells progressively declined with time (Fig. 3A, 3B). Interestingly, this decline began within the first weeks after birth (Figs. 2A, 3A, 3B) and reached a plateau of \textfrac{3}{10} after 50 wk (Fig. 3A, 3B), which remained constant throughout the observation period. On a per-cell basis, however, Foxn1 downregulation continued throughout life (Fig. 3C). Based on expression of the markers Ly51 and CD80, cells marked by Foxn1 expression always included the major TEC populations (Fig. 3D). In contrast, among the Foxn1\textsuperscript{−} cells, double-negative TECs dominated (Fig. 3E). Taken together, we observed a temporal loss of Foxn1-expressing cells together with a long-lasting, yet slow downregulation of Foxn1 levels in the remaining expressers.

To examine whether Foxn1 expression levels are dependent on normally proceeding T cell development, we analyzed Foxn1 expression in \textit{Rag2} deficient mice. In these mutants, T cell development is blocked at the double-negative 3 stage. This state is associated with impaired TEC differentiation, which can be corrected by reconstitution with wild-type thymocytes (23). Intriguingly, in the presence of only immature thymocytes (double-negative 3), a very high fraction of TECs expressed Foxn1 up to 28 wk (Fig. 3F, 3G). Hence, progression beyond the double-negative 3 stage induces loss or downregulation of Foxn1, possibly by inducing TEC maturation.

\textbf{Foxn1 during thymic regeneration}

Loss of Foxn1 during adulthood has been linked to thymic involution (14), an assumption that is supported by the premature thymic degeneration caused by genetic reduction of Foxn1 expression (22, 24). Corroborating these findings, elevated Foxn1 expression has been shown to attenuate thymic involution in multiple settings (24–26). Hence, we were prompted to elucidate Foxn1 expression at the single-cell level in response to thymic insults and/or thymic regeneration. Three conditions are commonly induced in mice to study thymus regeneration, that is, glucocorticoid treatment, growth factor treatment, or castration. Glucocorticoids such as DEX are well known for their multiple effects on both thymocytes and TECs. In contrast to thymic aging, the DEX effect is transient, and thymocyte as well as TEC numbers recover (27, 28). Conversely, thymus regeneration can be induced by castration of mice, which results in a dramatic, but transient, increase of thymus cellularity (23, 29–33). Finally, the injection of Kepivance (palifermin), a \DeltaN23KGF form of rhFGF7, has been shown to have regenerating capacity on the thymus (33–35).

DEX injection affected female and male \textit{Foxn1\textsuperscript{Rosa26-YFP}} mice alike by reducing thymocyte and TEC numbers drastically within 2 d. By day 7, there was a clear, albeit not yet complete, recovery of cellularity (Fig. 4A, 4B). After 2 d, Foxn1 expression dropped markedly in response to DEX treatment (Fig. 4C, 4D). In addition to its global TEC diminishing effect, DEX strongly influenced the ratio of Foxn1\textsuperscript{CD80\textsuperscript{+}} mTECs to Foxn1\textsuperscript{Ly51\textsuperscript{+}} cTECs (Supplemental Fig. 3A). The reduction in Foxn1 expression was temporary, that is, 7 d after injection the frequency of Foxn1-expressing cells had already returned to normal (Fig. 4C, 4D). Regrowth of Ly51\textsuperscript{−}CD80\textsuperscript{+}Foxn1\textsuperscript{+} TECs did not occur within the 7-d observation period, and hence the altered ratio of Foxn1\textsuperscript{+} CD80\textsuperscript{−} mTECs to Foxn1\textsuperscript{+}Ly51\textsuperscript{+} cTECs persisted (Supplemental Fig. 3B). In summary, DEX treatment has a profound effect on Foxn1 expression in the thymus, on proportions of Foxn1-expressing TEC subsets and on their cycling properties. Whereas Foxn1 expression recovers within 7 d, TEC homeostasis does not appear to be equilibrated by this time point.

Next, we stimulated mice with rhFGF7, rhFGF7 has been shown to have a direct effect on FgfR2-IIIb\textsuperscript{+} TECs and to cause an initial thymocyte loss followed by a drastic increase in thymic cellularity (33–35). As a result, the stimulated thymus increases in size, in contrast to the recovery to normal size following DEX treatment (28). Injection of rhFGF7 triggered an initial reduction of thymocyte numbers (Fig. 5A) and, already by day 3, an increase in TEC numbers (Fig. 5B), which is consistent with stimulated TEC proliferation on day 3 (Fig. 5C). The frequency of Foxn1-expressing cells declined transiently on day 3 (Fig. 5D). Akin to the DEX recovery, the ratio of Foxn1\textsuperscript{+}CD80\textsuperscript{−} mTECs to Foxn1\textsuperscript{+}Ly51\textsuperscript{+} cTECs was altered (Supplemental Fig. 4A). Among Foxn1\textsuperscript{+} TECs, Ly51\textsuperscript{+} cTECs expanded (Supplemental Fig. 4A). Hence, this early thymic stimulation was accompanied by subset changes remarkably similar to the ones observed late during DEX regeneration. The initially major Ly51\textsuperscript{−}CD80\textsuperscript{−} Foxn1\textsuperscript{−} compartment of control mice was replaced by Ly51\textsuperscript{+} CD80\textsuperscript{−} Foxn1\textsuperscript{+} TECs, which turned into a Ly51\textsuperscript{+}CD80\textsuperscript{−} Foxn1\textsuperscript{+} population by day 6 (Fig. 5E, right column, Supplemental Fig. 4A). Note that the level of Foxn1 expression on these TECs exceeded the level of control TECs (Fig. 5E, left column).

In our final model of thymus modulation, we castrated mice and followed Foxn1 expression over time. Castration led to an increase in thymocyte numbers as early as 8 d after surgery (Fig. 6A). Frequencies of Foxn1 expression did not show major alterations (Fig. 6B). On a per-cell basis, however, all Foxn1-expressing cells had shifted toward a higher expression level at day 14 posttreatment, similar to that of young mice (Fig. 6C). This effect was observed as early as day 8, when thymocyte numbers were already increased, in one third of the cases, and in 100% of the animals at day 14 after surgery, when thymocyte numbers were even higher. Therefore, upregulation of Foxn1 does not seem to be an absolute prerequisite for higher thymopoietic capacity at day 8 of recovery.

In marked contrast to the treatments described above, where thymic regeneration led to the appearance of a Ly51\textsuperscript{−}Foxn1\textsuperscript{−} population (Fig. 5E, Supplemental Fig. 3B), CD80\textsuperscript{−}Foxn1\textsuperscript{+} TECs represented the most abundant population in response to castration (Fig. 6D). This shift in TEC distribution could be first observed 8 d after surgery, and became more prominent 14 d after treatment when these cells uniformly turned Foxn1\textsuperscript{hi} (Fig. 6D, Supplemental Fig. 4B). Collectively, compared with DEX and rhFGF7 treatment, castration has a unique effect on the thymus by strongly expanding CD80\textsuperscript{−} Foxn1\textsuperscript{hi} TECs.
FIGURE 3. Loss of Foxn1 expression with age depends on lymphocytes. (A) Flow cytometry for Foxn1 expression by thymic stroma (live, CD45−) isolated at the indicated time points. The indicated frequencies refer to the distribution of Foxn1+ and Foxn1− fractions among EpCAM+ TECs. One representative out of three to four experiments is displayed. (B) Summary of Foxn1 expression by TECs. Frequencies of Foxn1+ (●) and Foxn1− (○) TECs (live, CD45−, EpCAM+, n = 4–5 per age, mean ± SD) are shown. (C) Histogram based on FACS plots shown in (A). Foxn1 stainings of the TEC compartment at various ages are overlaid to visualize differences in expression levels. Phenotype of Foxn1+ (D) and Foxn1− (E) TECs (live, CD45−, EpCAM+) isolated from mice of various ages (as indicated) is shown. One representative out of three to four experiments is displayed. (F) Representative staining depicting Foxn1 expression by thymic stroma (live, CD45−) derived from Rag2-deficient mice of various ages. The indicated frequencies refer to the distribution of Foxn1+ and Foxn1− fractions among EpCAM+ TECs. One representative out of three to four experiments is shown. (G) Summary of the frequencies of Foxn1− (○) and Foxn1+ (●) TECs from Rag2-deficient mice at various ages (n = 6–8 per age, mean ± SD).
Discussion

Reliable detection of the Foxn1 protein has been a notorious hurdle in studies on its roles in thymus organogenesis and thymus epithelium. We generated a C-terminal–tagged Foxn1HA allele and a flow-grade Foxn1 Ab. In contrast to a published hypomorphic Foxn1 allele (22), Foxn1HA/HA mice have a phenotypically and functionally normal thymus.

Although data with anti-Foxn1 serum have been reported (21, 36), commercially available antisera did not work robustly in our hands to detect Foxn1. We therefore, in a second approach, raised a tag-independent mAb against endogenous Foxn1. We have mapped the epitope of this mAb to the activation domain, close to the C terminus of Foxn1. The Ab detects Foxn1 by immunofluorescence, by flow cytometry, and by immunoprecipitation in thymus cell lysates. Foxn1HA/HA mice and the Foxn1-specific mAb enabled us to assess Foxn1 protein expression during thymus development, in thymi with arrested T cell development, during thymus aging, and after thymic insult, as well as regeneration. During prenatal development and up until about 2 wk of age, a very high percentage of all TECs were Foxn1+. By 4 wk of age, still 70% of TECs expressed Foxn1. An earlier report using rabbit anti-Foxn1 serum quantified numbers of Foxn1+ TECs on cytospins and obtained much lower frequencies (36). The reason for this discrepancy is unclear, but it is possible that the representation of Foxn1+ TEC subsets was perturbed on cytospin preparations of TECs, or that the antiserum/assay was less sensitive. The frequencies we report in the present study were comparable in independent experiments using our anti-HA or anti-Foxn1 mAb.

The early “ubiquitous” expression of Foxn1 in the developing thymus is in line with the reported fate-mapping data (37, 38). We addressed the origin of the few Foxn1− TECs in embryonic mice. In line with observations of others, most TECs displayed a history of Foxn1 expression. Costaining of lineage-traced embryonic TECs with anti-Foxn1 Ab identified some EpCAM+ TECs that do not actively express Foxn1 and had not expressed it in the past. The significance of this population is unclear, but it may be of minor importance: due to their EpCAM expression, they cannot represent the recently proposed EpCAM− Foxn1− stem cells, and second, they are unable to rebuild the thymus after prenatal intoxication of Foxn1+ TECs (38, 39). These cells may originate from the incomplete separation of the parathyroid and the thymus (40), as has been proposed previously for the cervical thymus in a reciprocal manner (21).

Whereas the embryonic and young thymi contain only a small fraction of Foxn1+ TECs, the proportion of Foxn1− cells increases between 2 and 10 wk, reaching a plateau of roughly 50% Foxn1+ TECs. Based on their Foxn1 expression history, these Foxn1+ TECs are post-Foxn1 cells rather than representing a Foxn1− lineage (37, 38). Although the Foxn1+ TECs contain all known mature major TEC subsets as well as few double-negative TECs, the latter fraction dominates the Foxn1− TEC population. Currently, we lack information on the relative location of Foxn1+ and Foxn1− TEC subsets in the thymus. Thus, neither the location nor the function of Foxn1− TECs is known. We speculate that these cells are exhausted or are end-stage cells that nevertheless may have a role as scaffold to support the thymus structure. It is plausible that the detrimental effects of either ablation of Foxn1 expression (by conditional deletion of Foxn1-floxed alleles) or Foxn1-expressing cells (by progressive intoxication of Foxn1-expressing cells) acted on the thymus only via the Foxn1+ TEC population. These data

FIGURE 4. TEC rebound following DEX treatment. Seven- to 8-wk-old Foxn1HA/HA mice were injected once with DMSO or 20 mg/kg DEX in DMSO and thymic regeneration was analyzed at 2 d (●, n = 6 per treatment) and 7 d (○, n = 9 per treatment) after injection. Females (red) and males (blue) were analyzed separately. Data represent three independent experiments with two to three mice per group (mean ± SD). Absolute thymocyte (A) and TEC (B) numbers are shown. The frequency of Foxn1-expressing TECs is depicted in (C). (D) Stroma (live, CD45−)-specific expression of Foxn1 at 2 (left) or 7 d after injection (right). The indicated frequencies refer to the distribution of Foxn1+ and Foxn1− fractions among EpCAM+ TECs. One representative out of three experiments is shown. Note that the alterations in EpCAM staining are a result of differently labeled (dye) EpCAM Abs.
imply that Foxn1 TECs, despite their abundance in the adult thymus, are unable to sustain a functional thymus (24, 38, 41–43). Reduced Foxn1 expression has been linked to thymus aging and involution. Our detailed kinetics of frequencies and levels of Foxn1 expression reveal a surprisingly early decline in Foxn1 expression at a juvenile age. The ubiquitous requirement for Foxn1 expression in all TECs appears to end at around 2 wk of age, and hence in a phase when the thymus is about to enter steady-state function. In this scenario, lower levels of Foxn1 may set the stage for the later occurring involution; however, this process is most likely multifactorial.

The thymus is known for its regenerative capacity in response to thymic insults and thymic stimulation. The observed disappearance of the CD80+Ly51+Foxn1hi TECs in response to DEX (28) and the emergence of a prominent CD80+Ly51− mTEC population after castration (23) are well in line with previous publications. Regeneration after DEX treatment reestablished the frequency of Foxn1-expressing cells without changes in Foxn1 levels, and the regrowth of the thymus in response to DEX was characterized by the appearance of a CD80−Ly51+Foxn1− population.

The long-lasting rhFGF7-induced regeneration (34) relied on the expansion of a different TEC subset than that involved in the transient regeneration observed after castration (32, 44), although each response was accompanied by an upregulation of Foxn1 protein levels. A predominant cTEC-like Ly51+Foxn1+ population could be seen in response to DEX administration and rhFGF7 treatment, as well as in Rag2-deficient mice lacking mature thymocytes. That the transient castration response involved a CD80+Ly51+Foxn1− mTEC-like population clearly pointed toward two different regenerative mechanisms. Concomitantly, Fgf7 deficiency

![FIGURE 5. rhFGF7 treatment generates CD80+Ly51+Foxn1hi TECs. Six-day-old C57BL/6J mice were injected on three consecutive days with either PBS or 5 mg/kg rhFGF7. Thymic cellularity (A), TEC numbers (B), TEC proliferation (C), and Foxn1 expression by TECs (D) were analyzed 3 d (○, three independent experiments with three mice per treatment) or 6 d (●, three independent experiments with three mice per treatment) after the first injection (mean ± SD). (E) Flow cytometric analysis of Foxn1 expression by thymic stroma (live, CD45−; left) and the phenotype of Foxn1− (middle) and Foxn1+ (right) TECs (live, CD45−EpCAM+) 6 d after the first injection. The indicated frequencies refer to the distribution of Foxn1+ and Foxn1− fractions among EpCAM+ TECs. One representative out of three experiments is shown.](http://www.jimmunol.org/)

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does not result in an inability to respond to castration (45). It seems that each thymic injury, and thereby the regenerative outcome, has its own characteristic responding TEC population.

Whether the predominant Ly51+Foxn1+ cTEC-like cells of Rag2-deficient mice, as well as the phenotypically identical populations expanding in response to DEX and rhFGF7, represent bona fide cTECs remains to be shown. It seems more likely that some TEC progenitor population may also display the Ly51+Foxn1+ phenotype, as does the vast majority of TECs in unborn mice, and at least some of these cells have been demonstrated to exhibit progenitor function (46). Moreover, cTEC-specific β5t promoter–driven Cre labels mTECs, once more suggesting an overlap between the phenotypes of TEC progenitors and cTECs (47). The appearance of cTEC markers on mTECs might represent a coincidental, transient upregulation of these markers during mTEC development, or it requires a revised order of TEC maturation, as suggested (48).

In summary, our in-depth analysis of Foxn1 expression in TEC subsets in the developing, aging, and regenerating thymus may provide a useful basis to address molecular functions of this transcription factor in the near future.

**Acknowledgments**

We thank Carmen Blum and Susan Schlenner for help with the generation of mouse mutants; the Imaging Core Facility for help with immunofluorescence; the German Cancer Research Center animal facility for expert mouse husbandry; and Andrea Weber, Tabea Arnsperger, and Sven Schäfer for technical assistance.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


Supplementary Figure 1: Foxn1HA/HA mice are phenotypically normal

(A) Schematic display of the targeting construct and the Foxn1 locus before and after neo deletion. (B) Southern blot analysis of Foxn1+/-, Foxn1HA/HA or homozygous Foxn1HA/HA mice. Expected bands and probe as indicated in A. Data represent two independent experiments. (C) Absolute thymic cellularity of female non-littermate Foxn1+/- C57BL/6J (open circle) or female Foxn1HA/HA mice (filled squares) at 2, 6 and 12 weeks of age. Data represent three independent experiments with 2-5 mice per group (n = 9-10 per time point, mean ± SD). Red circles (Foxn1+/- C57BL/6J) and red squares (Foxn1HA/HA) display thymic cellularity of the 10th backcross generation at 6-8 weeks (three independent experiments with 4-5 mice of mixed gender, n = 12-13 per genotype, mean ± SD). (D) Exemplary CD4 vs CD8 thymocyte staining of 2 weeks old Foxn1+/- C57BL/6J (left) or homozygous Foxn1HA/HA (right) mice. (E) Summary of the T-cell subset analysis over time as shown in D (mean ± SD).
Supplementary Figure 2: Characterization of the Foxn1 antibody

(A) Cos-1 cells transiently transfected with either pEGFP-C1 (left), or pEGFP-C1-Foxn1 (right) were stained with α-Foxn1 antibody (red), and counterstained with DAPI (scale bar=20 μm). (B) Thymic sections of Foxn1+/+ (left) and Foxn1fl/fl mice (right) were stained with anti-HA (green) and anti-Foxn1 (red) antibody. The dotted line marks medullary regions (scale bar=20 μm). Note: Co-localization of anti-Foxn1 and anti-HA staining in Foxn1fl/fl mice. (C) Foxn1 fragments fused to EGFP were transiently expressed in Cos-1 cells and tested for their reactivity with the Foxn1 antibody. One out of two representative experiments is shown. (D) X-Gal staining of newborn thymi of Foxn1+/+ (left), Foxn1LacZ/LacZ (middle) and nude Foxn1LacZ/LacZ mice (right). Note: most of the newborn nude-rudiment is actually composed of adipose tissue. (E) Live alveolar epithelial cells and (F) intestinal epithelial cells of Foxn1+/+ (left) and nude Foxn1LacZ/LacZ mice were tested for their reactivity with anti-Foxn1. Note: due to the IRES-lacZ integration Foxn1LacZ/LacZ mice cannot express the epitope containing fragment of Foxn1.
Supplementary Figure 3: Expression of TEC markers during DEX recovery

Stroma (live, CD45) specific expression of Foxn1 (left) or Ly51 vs CD80 on Foxn1+ TEC (live, CD45, EpCAM+) right) at 2 days (A) and 7 days (B) post injection. One representative out of three experiments shown.
Supplementary Figure 4: Early TEC response to rhFGF7 treatment and castration

(A) Flow cytometric analysis of Foxn1 expression by thymic stroma (live, CD45⁻; left) and phenotype of Foxn1⁻ (middle) and Foxn1⁺ (right) TECs (live, CD45 EpCAM⁺) 3 days after the first rhFGF7 injection. One representative out of three experiments is shown.

(B) Foxn1 levels expressed by thymic stroma (live, CD45⁻; left), and expression of CD80 and Ly51 on Foxn1⁻ (middle) and Foxn1⁺ (right) TECs of sham-castrated (top panels) and castrated males (bottom panels) at day 8 after surgery. One representative out of three experiments is shown.