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Cutting Edge: Broad Expression of the FoxP3 Locus in Epithelial Cells: A Caution against Early Interpretation of Fatal Inflammatory Diseases following In Vivo Depletion of FoxP3-Expressing Cells

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Dogma that the regulatory T cell (Treg) prevents catastrophic autoimmunity throughout the lifespan relies on the assumption that the FoxP3 locus is transcribed exclusively in Treg. To test the assumption, we used the Rag2−/− and the Rag2−/− mice with the Scurfy (sf) mutation (FoxP3sf/sf or FoxP3 sf/sf) to evaluate FoxP3 expression outside of the lymphoid system. Immunohistochemistry and real-time PCR revealed FoxP3 expression in breast epithelial cells, lung respiratory epithelial cells, and prostate epithelial cells, although not in liver, heart, and intestine. The specificity of the assays was confirmed, as the signals were ablated by the Scurfy mutation of the FoxP3 gene. Using mice with a green fluorescence protein open reading frame knocked into the 3′ untranslated region of the FoxP3 locus, we showed that the locus is transcribed broadly in epithelial cells of multiple organs. These results refute an essential underlying assumption of the dogma and question the specificity of FoxP3-based Treg depletion. The Journal of Immunology, 2008, 180: 5163–5166.

The Forkhead transcription factor FoxP3 has been initially identified as a causative mutation of a fatal autoimmune disease in mouse and human (1–4). More recently, FoxP3 was shown to be a key transcription factor for function of regulatory T cells (Treg)5 (5, 6), where it is expressed at levels comparable to a housekeeping gene in CD4+CD25+ Treg (5). In an attempt to test the physiological function of Treg, two groups have designed strategies to delete Tregs by treating transgenic mice expressing a diphtheria toxin (DT) receptor (DTR) under the control of the FoxP3 locus with DT (7, 8). The acute and fatal autoimmune diseases in adult and/or neonates were used to support a dramatic conclusion that the Treg cells prevent catastrophic autoimmunity throughout the lifespan of mice (7). For this conclusion to be valid, however, one must ascertain that Treg is the only cell type in mice that can express FoxP3 and, by inference, the DTR in the transgenic mice. Unfortunately, no serious effort has been made to confirm this assumption.

We have reported expression of FoxP3 protein in thymic (9) and breast epithelial cells (10). Although these data are inconsistent with the notion of Treg-exclusive expression of FoxP3, activation of the locus in these organs would in theory not affect DT-mediated fatal injury, as these organs are not vital for the survival of mice. In this study, we systematically investigated expression of FoxP3 by immunohistochemistry, real-time PCR, and the knockin mice with a bicistronic FoxP3 locus that coexpresses the enhanced GFP (EGFP) under the control of the endogenous FoxP3 promoter/enhancer elements (11). In this work we report a broad expression of the FoxP3 gene in epithelial cells of multiple lineages. Our data raised a serious concern about interpretation of the fatal inflammation in DT-treated FoxP3-DTR transgenic/knockin mice.

Materials and Methods

Experimental animals

Wild-type (WT) or FoxP3EGFP mice that express both FoxP3 and EGFP under the endogenous regulatory sequence of the FoxP3 locus have been described (11). BALB/c RAG-2−/− and RAG-2−/− mice with the Scurfy (sf) mutation of the FoxP3 gene have also been described (9). All mice were used at 6–8 wk after birth.

Immunohistochemistry and immunofluorescence

Tissues from mice were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned according to the standard procedure. Endogenous peroxidase activity was quenched using 3% H2O2 for 30 min at room temperature; nonspecific binding sites in the sections were blocked using 5% normal BSA (Sigma-Aldrich) in PBS for 1 h at room temperature. Sections of 5 μm were made and reacted with affinity-purified anti-FoxP3 polyclonal Ab (9). The slides were washed in PBS and subsequently incubated with the secondary Ab.

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3 Abbreviations used in this paper: Treg, regulatory T cell; DT, diphtheria toxin; DTR, DT receptor; EGFP, enhanced GFP; Hprt, hypoxanthine phosphoribosyltransferase; sf, Scurfy; WT, wild type.

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analysis to detect expression of FoxP3. Sections from the mammary gland, prostate, lung, and small intestine of either Rag2\(^{-/-}\) BALB/c mice (top row) or FoxP3\(^{WT/Y}\) BALB/c mice (bottom row) were stained with anti-FoxP3 polyclonal Ab and counterstained with hematoxylin. The second Ab used was HRP labeled. Positive staining was found in the nuclei of lung respiratory epithelial (RE) cells, prostate secretory epithelial cells, and mammary epithelial cells. Apart from epithelial cells, no specific staining can be found in other cell types in these organs. Other organs that were analyzed but showed no FoxP3 expression include liver, kidney, and heart (data not shown). M, Male; F, female.

### Results and Discussion

To avoid interference of FoxP3 from lymphocytes, we used RAG-2-deficient mice lacking T and B cells to analyze expression of FoxP3 in nonlymphoid organs. As control for Ab specificity, we generated Rag2\(^{-/-}\) Scurfy mice that have a naturally occurring truncation mutation resulting in an early termination codon. Because of the non-sense-mediated decay of the mRNA, essentially no FoxP3 can be detected in the Scurfy mice (9). We performed immunohistochemical staining to detect expression of FoxP3 in various tissues using affinity-purified polyclonal Ab (9). As shown in Fig. 1, in FoxP3\(^{WT}\) Rag2\(^{-/-}\) mice, FoxP3 was found in mammary epithelial cells, lung bronchial epithelial cells, and prostate epithelial cells. In contrast, no staining can be found in intestine (Fig. 1), kidney, liver, and heart (data not shown). Because none of the tissues from the FoxP3\(^{ sf/Y}\) or \( sf/sf \) Rag2\(^{-/-}\) mice show staining, we conclude that the staining is specific for the FoxP3 protein as it is largely diminished by a FoxP3 mutation that leads to disappearance of mRNA by a non-sense-mediated decay (Fig. 2).

In conjunction with immunohistochemistry, we investigated FoxP3 mRNA levels in various mouse tissues by real-time PCR. A typical positive amplification is shown in Fig. 2A, and the summary data from multiple organs are shown in Fig. 2B. These data demonstrated that FoxP3 was expressed at significant levels in mammary gland, lung, and prostate in FoxP3\(^{WT/Y}\) Rag2\(^{-/-}\) mice, but no expression could be detected in liver. Again, the FoxP3 transcript was reduced by 20- to 500-fold in FoxP3\(^{ sf/Y}\) or \( sf/sf \) Rag2\(^{-/-}\) mice. It is worth noting that the levels of the FoxP3 transcript are 3- to 100-fold lower in other organs in comparison to those in the spleen. The overall lower abundance makes it harder to detect FoxP3 expression in nonlymphoid organs. To determine whether the full-length FoxP3 open reading frame was transcribed, we used primers that encompass the whole open reading frame to amplify FoxP3 mRNA. The PCR products were analyzed by agarose gel electrophoresis. As shown in Fig. 2C, the m.w. of the product was

![FIGURE 1](image1.png)

**FIGURE 1.** Immunohistochemical analysis to detect expression of FoxP3. Sections from the mammary gland, prostate, lung, and small intestine of either Rag2\(^{-/-}\) BALB/c mice (top row) or FoxP3\(^{WT/Y}\) BALB/c mice (bottom row) were stained with anti-FoxP3 polyclonal Ab and counterstained with hematoxylin. The second Ab used was HRP labeled. Positive staining was found in the nuclei of lung respiratory epithelial (RE) cells, prostate secretory epithelial cells, and mammary epithelial cells. Apart from epithelial cells, no specific staining can be found in other cell types in these organs. Other organs that were analyzed but showed no FoxP3 expression include liver, kidney, and heart (data not shown). M, Male; F, female.

![FIGURE 2](image2.png)

**FIGURE 2.** Identification and quantitation of the FoxP3 transcripts in nonlymphoid organs from either Rag2\(^{-/-}\) BALB/c mice or FoxP3\(^{ sf/Y}\) BALB/c mice. A and B, Quantitative analysis of FoxP3 transcripts in mouse tissues by real-time PCR. A. Representative profile of FoxP3 amplification in the prostate tissue. B, The expression level of FoxP3 transcripts was expressed as fraction of Hprt. A sample of WT spleen was used as positive control. Data shown were means ± SD of three mice and triplicate experiments were performed on each mouse. C, RT-PCR analysis of FoxP3 expression in mammary gland and prostate. Lane 1, Prostate from FoxP3\(^{ sf/Y}\)Rag2\(^{-/-}\) mouse; lane 2, prostate from FoxP3\(^{WT/WT}\)Rag2\(^{-/-}\) mouse; lane 3, mammary gland from FoxP3\(^{WT/WT}\)Rag2\(^{-/-}\) mouse. Hprt was used as cDNA control. The m.w. of the product is consistent with it being full length mRNA. This is confirmed by DNA sequencing of the RT-PCR product (data not shown).
cells of vital organs expressed FoxP3 at high levels, such targeting, either alone or in combination with Treg cell depletion, may contribute to lethality caused by DT treatment.

Finally, FoxP3 is an interesting transcription factor that binds to a large number of genes important for a number of fundamental cellular functions (10, 15–17). Our documentation of its broad expression in epithelial cells of multiple organs suggests that it may play a more broad function outside of Treg cells. In this regard, we reported critical roles for FoxP3 as a regulator of thymopoiesis when expressed in thymic epithelial cells. In this regard, we reported critical roles for FoxP3 as a regulator of thymopoiesis when expressed in thymic epithelial cells. However, it is unclear whether the different approaches explain the lack of report on the FoxP3 locus activation by DT treatments (7, 8) the epithelial cells may be among those targeted by DT treatment. Because epithelial cells of vital organs expressed FoxP3 at high levels, such targeting, either alone or in combination with Treg cell depletion, may contribute to lethality caused by DT treatment.

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Disclosures
The authors have no financial conflict of interest.

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