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*J Immunol* 2009; 183:5042-5049; Prepublished online 28 September 2009;
doi: 10.4049/jimmunol.0901371

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Lessons from Thymic Epithelial Heterogeneity: FoxN1 and Tissue-Restricted Gene Expression by Extrathymic, Endodermally Derived Epithelium

James Dooley,* Matthew Erickson,* and Andrew G. Farr2*†‡

Modeling of thymic epithelial differentiation has been guided by several important underlying assumptions. One is that within epithelial tissues derived from pharyngeal endoderm, FoxN1 expression is signature for the thymic epithelial lineage. Another is that expression of tissue-restricted Ag (TRA) is a unique feature of thymic epithelium. In this murine study, we evaluate the thymic expression of a subset of TRA, parathyroid hormone, calcitonin, and thyroglobulin, as part of an effort to better define the heterogeneity of medullary thymic epithelial cells. In this study, we demonstrate that both conventional and cystic epithelial cells display a history of FoxN1 expression using a cre-lox approach. We also document that extrathymic epithelial tissues that originate from pharyngeal endoderm also have a history of FoxN1 expression, indicating that FoxN1 expression per se is not a signature for the thymic lineage and suggesting that FoxN1 expression, whereas necessary for thymic epithelium, development, is not sufficient for this process to occur. Both cystic and conventional medullary thymic epithelial cells express these TRAs, as do extrathymic epithelial tissues that are not usually considered to be sources of these molecules. This finding supports the proposition that promiscuous gene expression is not unique to the thymus. Furthermore, the pattern of promiscuous gene expression in these extrathymic epithelia is consistent with developmental regulation processes and suggests that it is premature to discard the possibility that some promiscuous gene expression in the thymus reflects normal differentiation programs of epithelia. The Journal of Immunology, 2009, 183: 5042–5049.

The spectrum of self-Ags expressed by medullary thymic epithelial cells (MTEC) plays an important role in maintaining self-tolerance and is remarkably diverse, with a marked skewing toward molecules that have been associated with ectodermal, endodermal, and neuroectodermal derivatives (1, 2). The finding that mutations of the AIRE gene are associated with an autoimmune syndrome in humans (3) led to the finding that MTEC from mice lacking functional Aire have a reduced spectrum of tissue-restricted Ag (TRA) expression and also display features of autoimmunity (4, 5). Although it is clear that MTEC expression of some TRAs is strictly Aire-dependent, there are a large number of TRAs that are expressed via an Aire-independent mechanism (2). By one estimate, 90% of the TRAs expressed by MTEC are Aire-independent (H. Petrie, unpublished observation). Although it is not clear how the expression of Aire-independent TRAs is regulated, it is widely held that the expression of many Aire-independent TRAs by MTECs also reflects derepressed transcription that occurs in terminally differentiated MTEC, similar to Aire-dependent TRAs (2).

Gaps in our understanding of the differentiation of thymic epithelium have hampered better understanding of TRA expression by thymic epithelium and the mechanisms that regulate this activity. The thymus is derived from endoderm of the third pharyngeal pouch, where domains of endoderm that give rise to thymus and parathyroid are demarcated by the expression of FoxN1 and Gcm2, respectively (6, 7), and do not receive a contribution of pharyngeal ectoderm (8). It is clear that the activity of FoxN1 is required for productive thymic epithelial differentiation, as evidenced by the nonfunctional thymic rudiment of nude mice (9), but the nature of contribution of FoxN1 to this process is not clear. Based on the thymus-appropriate mediastinal location of an epithelial structure in the nude mouse, it is thought that the initial specification of pharyngeal endoderm to a thymic fate is FoxN1-independent, and that FoxN1 is required for the subsequent differentiation of thymus-restricted cells to proceed (10, 11). However, the striking respiratory character of the nude thymic rudiment and the lack of any molecular features of this rudiment indicative of thymic lineage specification (12) would be consistent with a role for FoxN1 in the specification of endoderm to the thymic lineage.

Cortical and medullary epithelial compartments have been commonly defined on the basis of reciprocal patterns of keratin expression, where cortical thymic epithelium express the K8/K18 simple keratin pair and medullary thymic epithelium have been defined by their expression of K5/K14, a keratin pair usually associated with basal progenitor or transit amplifying epithelial cells within stratified and pseudostatified epithelia (13, 14). A subset of thymic epithelium at the corticomedullary junction that coexpress K5 and K8 have been proposed to represent the immediate precursors to the K8+K5+/K14– cortical thymic epithelium and the K8–K5/K14– MTEC (15–17). Although it is clear that single

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1 Abbreviations used in this paper: MTEC, medullary thymic epithelial cell; TRA, tissue-restricted Ag; Pth, parathyroid hormone; YFP, yellow fluorescent protein.

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3 This work was supported by Grant AI09575 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0901371
epithelial progenitor cells can contribute to both cortical and medullary epithelial compartments in fetal (18) and postnatal (19) mice, the binary aspect of this thymic epithelium differentiation model based on keratin expression has been recently called into question by demonstrations that the bulk of the MTEC population express both K8 and K5/K14 (20–22).

The differentiation program of MTEC is of particular interest because it is related to the ability of these cells to expression of TRAs and to mediate self-tolerance. In addition to the patterns of keratin expression described above, MTEC differentiation has also been modeled on the phenotype of differentiating dendritic cells for which the expression of CD80 and high levels of MHC class II has been considered to represent terminal differentiated MTEC (2). The preferential expression of Aire and Aire-dependent TRAs by sorted MTEC bearing a CD80^+ MHCII^{high} phenotype (2, 23) and the demonstration that MTEC with this phenotype display low levels of DNA synthesis and are rapidly eliminated (21) have contributed to a model in which Aire and Aire-dependent TRA expression are features of end-stage terminally differentiated MTEC. This view of MTEC differentiation, in which the spectrum of MTEC heterogeneity would be largely defined by phenotypic changes and TRA expression, does not account for organizational and morphological heterogeneity of MTEC.

There are a number of important unresolved issues regarding the phenomenon of TRA expression as it relates to thymic epithelium differentiation. One concerns the relationship between TRA expression and MTEC populations that are not accommodated by prevailing views of MTEC differentiation. Another issue is whether TRA expression is unique to thymic epithelium or whether it represents a more general property of epithelia that has acquired functional significance in the thymus because it occurs in a context in which TRA expression would have important immunological consequences. We have previously suggested that expression of some TRAs could represent transient transcription of “irrelevant” genes during epithelial differentiation (20, 22, 24). According to this view, TRA expression would not be restricted to thymic epithelium.

In this study we have begun to address some of these questions by evaluating the expression of subset of TRAs in the thymus. The TRAs examined, parathyroid hormone (Pth), calcitonin, and thyroglobulin are hormones that are considered to be signature for parathyroid gland, parafollicular cells of the thyroid gland, and follicular epithelial cells of the thyroid gland, respectively (25–27). Thyroid expression of Pth and calcitonin is considered to be Aire-independent (GDS2274, Gene Expression Omnibus; www.ncbi.nlm.nih.gov/projects/geo/), whereas MTEC expression of thyroglobulin appears to be partially Aire-dependent (28, 29). These TRAs were of particular interest because the tissues that express them are derived from pharyngeal endoderm, and thus have a developmental origin similar to the thymus. To gain a better understanding of the basis for observed heterogeneity of MTEC, we evaluated their history of FoxN1 expression using a cre-lox approach. We also evaluated extrathymic tissues for evidence of “promiscuous” gene expression to test the hypothesis that this property is unique to thymic epithelium. We report that there is extensive heterogeneity in terms of the MTEC populations that express these TRAs. In addition to conventional MTEC, epithelial cells lining thymic cysts also expressed Pth, calcitonin, and thyroglobulin. Despite obvious morphological and organizational differences, conventional MTEC, and the cyst epithelium displayed considerable phenotypic similarities and both displayed a history of FoxN1 expression. However, parathyroid and follicular thyroid epithelial cells also displayed a history of FoxN1 expression, indicating that FoxN1 expression per se is not a definitive marker for thymic lineage specification among epithelial tissues derived from pharyngeal endoderm. We also demonstrate instances in which extrathymic tissue express Pth and calcitonin, which suggests that mechanisms that lead to TRA expression are not unique to thymic epithelium. Finally, in the course of these studies, we document multiple instances in which parathyroid tissue is ectopically associated with the thymus. Features of the ectopic parathyroid tissue are consistent with aberrant segregation of pharyngeal endoderm during thymic organogenesis and suggest one possible mechanism for the formation of some thymic cysts.

Materials and Methods

Mice

C57BL/6 mice were obtained from Charles River Breeding Laboratories. FoxN1-cre mice (30) were provided by Dr. N. Manley (University of Georgia, Athens, GA), and the ROSA-stop^{flox}—yellow fluorescent protein (YFP) mice (31) were obtained from Dr. A. Y. Rudensky (Memorial Sloan-Kettering Cancer Center, New York, NY), now available from The Jackson Laboratory (http://jaxmice.jax.org/index.html). All mice were maintained in the University of Washington Specific Pathogen Free facility and used in accordance with protocols approved by the University of Washington Institutional Animal Care and Use Committee.

Ab production

Primary Abs for immunohistochemistry included polyclonal goat or rabbit Abs to Aire (M-300; SC-31389, D-17; SC-17986), Pth (N18, SC-9676), thyroglobulin (M-20, SC-7837), and calcitonin (G-18, SC-7784) from Santa Cruz Biotechnology. The rabbit anti-K5 and anti-K14 Abs were purchased from Covance; monoclonal anti-K8 (Troma-1) (32) was obtained from the Developmental Studies Hybridoma Bank (http://dshb.biology.uiowa.edu/). The Troma-1 hybridoma was exhaustively grown in medium supplemented with 10 mM glucose to maximize Ab concentration in the supernatant. Anti-claudin Abs were purchased from Zymed Laboratories and anti-surfactant C Ab was purchased from Chemicon International. Abs raised against GFP, which are highly cross-reactive with YFP, were purchased from Rockland Immunochemicals. Secondary reagents for immunofluorescence microscopy (donkey anti-goat IgG, donkey anti-rabbit IgG, goat anti-rabbit IgG, goat anti-rat IgG, chicken anti-rat IgG and streptavidin-conjugated with Alexa Fluor 488, Alexa Fluor 546, or Alexa Fluor 647) were purchased from Molecular Probes.

Immunohistochemistry

Different tissue processing protocols were used to accommodate the requirements of the reagents used. Wherever possible, aldehyde fixation was used to minimize the diffusion artifacts introduced by acetone fixation. Frozen sections collected on microscope slides were fixed in 0.1 M cacoodylate buffer, pH (7.4), containing 4% parafomaldehyde and 5 mM CaCl2. In some instances, thymus tissue was perfused via the heart with 10 ml of 1% paraformaldehyde fixative or immersed in this fixative for 4–16 h at 4°C, then washed repeatedly in PBS before cryoprotection (30% sucrose in 1% paraformaldehyde fixative) overnight at 4°C, then washed repeatedly in PBS before cryoprotection (30% sucrose in PBS) and embedment in OCT (Sakura Finetech) for cryosectioning. This latter approach without Ag retrieval was used for detection of YFP expression. Samples were mounted to harvest thymic sections in the coronal plane to maximize thymic area. Ag retrieval was accomplished by incubating parafomaldehyde-fixed sections in 20 mM Tris buffer (pH 9.0) for 25 min in a steam device (HS2776; Black&Decker) and then allowing the sections to cool for ~45 min before washing in PBS and subsequent processing. Monoclonal Abs were used undiluted, and hybridoma supernatants, whereas polyclonal Abs were used at dilutions of 1/500-2000.

The anti-hormone and anti-Aire Abs worked best with aldehyde fixation and Ag retrieval as described or with paraffin sections. For paraffin sections, tissue was fixed in modified Carnoy’s fixative (60% ethanol, 30% formalin solution (37%), and 10% galacical acetic acid) overnight at 4°C, then dehydrated through ascending concentrations of ethanol, followed by standard paraffin embedding. Following removal of paraffin and rehydration, the sections were subjected to Ag retrieval as described.

After Ag retrieval, aldehyde-fixed sections were incubated in PBS containing 10% v/v of normal mouse serum for 1 h before application of primary Abs (polyclonal Abs diluted in hybridoma supernatant or PBS containing 1% (w/v) BSA and 10% normal mouse serum. After incubation with primary Abs overnight, sections were washed repeatedly with PBS and then incubated with appropriate secondary Abs diluted in PBS containing 1% BSA (w/v) and 10% (v/v) of normal sera matched to the secondary Abs used. After 1–1.5 h of incubation, sections were again washed...
and then coverslips were applied with Fluoromount G (Southern Biotechnology Associates). For nuclear counterstaining with DAPI (4’ 6-diamidino-2-phenylindole), it was included in the secondary Ab solution at 0.1 μg/ml. Images were captured with a fluorescence microscope equipped with a monochrome digital CCD camera (Orca-ER) and assembled into RGB images with Photoshop (Adobe).

**Analysis of gene expression**

Pieces of esophagus and trachea were carefully dissected to exclude cervical thymic tissue (33, 34) and also included a 1-mm interval of esophagus and trachea immediately caudal to the parathyroid/thyroid tissue to avoid contamination of these samples with thyroid/parathyroid tissue. Parathyroid and thyroid tissue was examined with a dissection microscope to remove any tissue that might correspond to ectopic thymic tissue. The fetal mix sample was whole E14 embryos. Total RNA from these tissues was isolated with Absolute RNA RT-PCR kits (Stratagene) and used to generate cDNA. Polymerase chain reactions were performed with the following primer sets: Pth (forward) ctagcagctggtttctca (reverse) caaataaatccttacactc; thyroglobulin (forward) tgggaagaagaggtttt; cact; and (reverse) ggctgaaagtggattctgggt; HPRT (forward) gttgatacaggcagactttgttg (reverse) gagggtattctggcctatggct; K8 (forward) gcatt (reverse) gcatcttagagaacggtttc; and FoxN1 (forward) ggactgacctgg (reverse) gagggtattctggcctatggct; E-cadherin (forward) aggaaatgcacccctc and (reverse) geatcagagaaggtttt; and FoxN1 (forward) ggactgacctgg (reverse) gagggtattctggcctatggct.

The following conditions were used for all PCR: at 3 min start at 94°C, with denaturation (94°C) and annealing (60°C) periods of 30 s each, followed by an extension period (72°C) of 45 s. Magnesium concentration was 2.5 mM. Real-time PCR was performed with an ABI instrument, and cycle threshold determinations were determined according to the manufacturer’s recommendations.

**Results**

**Heterogeneity of MTEC expressing Pth, calcitonin, and thyroglobulin**

Immunohistochemistry was used to characterize the cellular sources of these hormones within the thymus. The specificity of the anti-Pth Ab is demonstrated in Fig. 1a, in which parathyroid tissue was labeled and adjacent thyroid tissue was not. MTEC that expressed Pth also expressed K8 and K14 (Fig. 1b) and/or K8 and K5 (Fig. 1c), Cystic epithelial structures that were universally K8+ and variably expressed K14 or K5 also expressed Pth (Figs. 1, d and e). Within individual cysts, the organization of epithelium ranged from columnar/pseudostratified columnar to squamous. Pth immunoreactivity was detected in many of these cysts and with more uniformity among epithelial domains that were columnar/pseudostratified, although scattered, more squamous epithelial cells were also noted (Fig. 1, d and e). The conventional Pth-positive MTEC were variably claudin3-positive (Fig. 1f), and variably expressed claudin4 (Fig. 1g).

The epithelium of the cysts, including Pth-positive cells, were also uniformly claudin3-positive (Fig. 1b) and showed some heterogeneity in claudin4 expression in some instances, Pth-positive lining cells expressed little, if any, claudin4 (Fig. 1i). MTECs that expressed Pth were very rarely Aire-positive (Fig. 1j). The overlapping claudin and Pth coexpression and lack of Aire and Pth codistribution suggested that the pattern of Aire and claudin expression in postnatal MTEC was different from that of fetal MTEC, in which a high degree of claudin and Aire coexpression was reported (35). The extent of Aire coexpression with either claudin3 (Fig. 1k) or claudin4 (Fig. 1l) was not particularly high in the postnatal thymus. Although double positive MTEC were evident, MTEC expressing either Aire or claudins alone were also present. In contrast to conventional MTEC, epithelial cells lining the cysts did not express Aire (Fig. 1m).

Evaluation of calcitonin expression used an Ab that selectively labeled the parafollicular cells of the thyroid gland (Fig. 2a). Calcitonin was expressed by MTEC that were K8- K14+ (Fig. 2b) or K8+ K5+ (data not shown). There was also regional calcitonin labeling in the epithelium lining the cysts (Fig. 2c), although the staining was less punctate than the labeling observed in conventional MTECs. The Ab used to detect thyroglobulin strongly labeled the contents of thyroid follicles, labeled the surrounding follicular epithelial cells less strongly, and failed to label the adjacent parathyroid tissue (Fig. 2d). Simultaneous localization of Pth in adjacent parathyroid tissue also demonstrated that the secondary Abs used in these studies were species specific. MTECs expressing thyroglobulin were often organized in multicellular clusters as previously described (36). There was considerable overlap among MTEC expressing thyroglobulin and calcitonin (Fig. 2e) or thyroglobulin and Pth (Fig. 2f). For technical reasons involving two primary Abs derived from the same species, we were not able to simultaneously assess the expression of calcitonin and Pth. Many of the epithelial lining cells of the cyst that were thyroglobulin-positive were also Pth-positive (Fig. 2g). The relationship between thyroglobulin and Aire expression by individual MTEC was variable. In some instances, no coexpression was evident (Fig. 2h), whereas there was coexpression of Aire and thyroglobulin in other cells (Fig. 2i).
and in close proximity to the ectopic parathyroid tissue. The cysts were located at the base of the clefts as previously described. Three different thymic samples are depicted in Fig. 3. Quite often, capsule that penetrated toward the thymic medulla. Examples from observed ectopic parathyroid tissue located in clefts of the thymic tissue were often found to express Pth and in one case, thyroglobulin as well (Fig. 2c). Although subcapsular PTH-producing cells in the thymus have been previously described (25), the association of these cells with cystic structures was not appreciated.

Epithelium of thymic cysts and extrathyroidal tissues derived from pharyngeal endoderm have a history of FoxN1 expression

These thymic cysts may represent remnants of pharyngeal endoderm that have not been incorporated during thymic organogenesis and hence have not undergone thymic lineage specification. Alternatively, they may contain epithelial cells that are progeny of pharyngeal endoderm that has previously undergone thymic specification and hence would represent an alternative differentiation outcome of committed thymic epithelial progenitors. Based on the prevalent view that FoxN1 expression is a hallmark of the thymic lineage among tissues derived by pharyngeal endoderm, evidence that the epithelium lining the cysts has a history of FoxN1 expression would indicate that the cysts arose from epithelium previously committed to the thymic lineage. To address this question, we examined thymic tissue from mice expressing FoxN1-cre (30) and ROSA-stop^{lox}-YFP (31). Consistent with previous flow cytometric analysis of enzymatically dissociated thymic stroma from these mice, (38), immunohistochemical detection of YFP with anti-GFP Abs showed widespread expression by thymic epithelium and included the epithelium lining cysts (Fig. 4, a and b). Association of claudin4 (Fig. 4a) and K14 (Fig. 4b) with the labeled cysts indicated they had a medullary location. Although this finding was consistent with the possibility that the cyst-lining cells were derived from thymic lineage-specified epithelium, evaluation of other tissues from the same mice revealed other tissues derived from pharyngeal endoderm also displayed anti-GFP reactivity. Labeling was seen in parathyroid (Fig. 4, c-f), thyroid (Fig. 4, g and h), and by scattered epithelial cells lining the trachea (data not shown) in tissue samples from four individual mice. The area of parathyroid tissue that shows a history of FoxN1 expression displayed the follicular epithelial organization characteristic of that tissue (Fig. 4, g and h). Labeling was not observed when the anti-GFP primary Ab was replaced with normal rabbit Ig (Fig. 4i) or when anti-GFP primary Ab and appropriate secondary Abs were applied to tissue of Rosaflox^{stop}-YFP mice (Fig. 4j). The patchy epithelial cells lining the cyst in the vicinity of the parathyroid tissue were often found to express Pth and in one case, thyroglobulin (green) (arrowhead); K8 (blue). Scale bar represents 50 microns.

FIGURE 2. Thymic expression of calcitonin and thyroglobulin. a. Specificity of anti-calcitonin Ab. Ab labels parafollicular cells, but not follicular epithelial cells of thyroid tissue with calcitonin (red) and K8 (blue). b, K14\(^{+}\)K8\(^{-}\) (green/blue) MTEC express calcitonin (red) with a punctate distribution pattern. c. Cystic epithelium expresses calcitonin (red) with K14 (green) K8 (blue). d, Specificity of anti-thyroglobulin Ab. Ab labels the lumen and associated cells of thyroid follicles in thyroid tissue (T), but not parafollicular thyroid cells or parathyroid tissue (PT). There is no cross-reactivity of secondary Abs with Pth (red), thyroglobulin (green), and K8 (blue). e, Simultaneous detection of calcitonin (green) and Pth (red) reveals coexpression by MTEC K8 (blue). f, Expression of thyroglobulin (green) and Pth (red) by MTEC. The clustered distribution of thyroglobulin-positive MTEC is evident with K8 (blue). g, An example of thyroglobulin (green) and Pth (red) expression by cystic epithelium with K8 (blue). h and i, Variable coexpression of Aire (red) and thyroglobulin (green) with K8 (blue). j, An MTEC coexpressing surfactant C (green) and Pth (red) with K8 (blue). k, Expression of Surfactant C by cyst lining cells. Most of the lining cells in this section coexpress surfactant C (green) and Pth (red) with K8 (blue).

As we had previously described the expression of surfactant C by MTEC and epithelium lining thymic cysts (37), we wanted to determine whether the thymic epithelium expressing Pth and surfactant C represented separate, nonoverlapping populations. As shown in Fig. 2, j and k, both conventional and cyst-associated thymic epithelium can coexpress surfactant C and Pth.

Ectopic parathyroid tissue frequently associated with thymic cysts

During the course of evaluating Pth expression in the thymus, we observed ectopic parathyroid tissue located in clefts of the thymic capsule that penetrated toward the thymic medulla. Examples from three different thymic samples are depicted in Fig. 3. Quite often, cysts were located at the base of the clefts as previously described (37) and in close proximity to the ectopic parathyroid tissue. The
Thyroid tissue from the thyroid gland display a history of FoxN1 expression. Shown are parathyroid tissue and are not ectopic thymus tissue. of YFP expression (green) have the same tissue organization as adjacent e, except the anti-GFP labeling has been removed to demonstrate that areas anti-GFP Abs. There is widespread YFP expression in both cortical and subcortical areas of the thyroid gland. Scale bar represents 50 microns.

These real-time PCR data provide qualitative corroborating support for the immunohistochemical demonstration of GFP. Scale bar represents 50 microns.

FIGURE 4. History of FoxN1 expression demonstrated with FoxN1-cre ROSA26fox<sup>cre</sup> YFP mice. a and b, Thymic tissue processed to demonstrate YFP expression immunohistochemically with highly cross-reactive anti-GFP Abs. There is widespread YFP expression in both cortical and medullary compartments, including epithelium lining cysts. a, Claudin4 expression (red) is associated with MTEC and with some cystic epithelial cells; GFP (green). b, MTEC and some cystic epithelial cells express K14 (red) and GFP (green). c–f, Some cells of the parathyroid gland display a history of FoxN1 expression. Parathyroid tissue from two individual mice is representative of tissue from four mice. Samples have been counterstained with DAPI (red) to provide tissue detail. d and f, Duplicate of c and e, except the anti-GFP labeling has been removed to demonstrate that areas of YFP expression (green) have the same tissue organization as adjacent parathyroid tissue and are not ectopic thymus tissue. g and h, Some cells of the thyroid gland display a history of FoxN1 expression. Shown are samples from two mice that are representative of tissue from four mice. i, Thyroid tissue from foxn1cre ROSA26fox<sup>cre</sup> YFP mice in which the primary anti-GFP Ab has been omitted. j, Representative thyroid tissue form ROSA26fox<sup>cre</sup> YFP mice that has been processed for the immunohistochemical demonstration of GFP. Scale bar represents 50 microns.

pattern of FoxN1 expression randomly dispersed in these tissues would be consistent with a clonal, transient, and perhaps stochastic expression of FoxN1 by thyroid or parathyroid epithelial cells at intermediate stages of differentiation. Comparison of cycle threshold (C<sub>T</sub>) values from real-time PCR analysis of cDNA from thymus, a mixture of thyroid and parathyroid tissue, and salivary gland indicated that there was modest transcription of FoxN1 in thyroid/parathyroid tissue. Normalizing FoxN1 expression to E-cadherin within each tissue sample, the threshold value for parathyroid/thyroid tissue was ~12% of the value obtained for thymus, whereas a FoxN1 signal was not detected in salivary gland at 40 cycles. Although we cannot exclude the possibility that ectopic thymic tissue might contribute to this signal, microscopic examination of the thyroid/parathyroid complex did not reveal the small whitish structures indicative of ectopic thymic tissue. As thyroid and parathyroid tissue was pooled in these analyses, the relative expression of FoxN1 in these two tissues was not determined. These real-time PCR data provide qualitative corroborating support for the immunohistochemical approach described and indicates that some of the GFP signal observed corresponds to epithelial cells actively transcribing FoxN1.

Thymus is not unique in expressing TRA ectopically

It has been widely assumed that TRA expression is a unique feature of thymic epithelium. Although this assumption seems reasonable for the subset of TRAs that are Aire-dependent because Aire expression is restricted to thymic epithelium (22, 39–41), there has been little assessment of the expression of TRAs by extrathympic tissues. If, as we have suggested, expression of some TRAs reflect a general property of transient transcriptional activity of epithelial cells discrete stages of differentiation, other epithelial tissues would be predicted to also express gene products that are not associated with the primary function of that tissue. To begin exploration of this question, we examined the expression of Pth, calcitonin, and thyroglobulin in other epithelia, focusing attention on epithelial tissues that have a pharyngeal/ventral foregut endodermal derivation. As shown in Fig. 5 a and b, stratified squamous epithelium of the esophagus expressed Pth. Using either patterns of K5 or K14 expression to delineate the basal progenitor and transit amplifying epithelial compartment (Fig. 5a and data not shown), Pth expression was clearly associated with suprabasal and presumably postmitotic epithelial compartment in the stratified squamous epithelium of the esophagus. The stratified nature of esophageal epithelium facilitates staging epithelial cells at different stages of differentiation and it was possible to relate patterns of claudin3 expression to esophageal epithelial differentiation and Pth expression. As shown in Fig. 5b, claudin3 was expressed by the suprabasal epithelial cells that are more mature than the K5<sup>high</sup>-basal cells, but less mature than the adjacent, more luminal layer of epithelial cells that expressed Pth. It is noteworthy that stratified claudin and Pth expression in the esophagus appears to define discrete stages of differentiation of a single epithelial lineage, and by doing so, provides an alternative interpretation to the
model in which claudin expression in the thymus defines different lineages of epithelium (35).

Pth was also detected in tracheal epithelium. Scattered luminal K8−K14− epithelial cells displayed strong apical anti-Pth labeling (Fig. 5c) and there was also diffuse Pth staining associated with the epithelium of the submucosal glands. Both the submucosal glands and the luminal epithelium were uniformly claudin3-positive (Fig. 5d). Calcitonin was not detected in esophageal epithelium, but similar to Pth, was expressed by the epithelium of the tracheal submucosal glands (Fig. 5c). These immunohistochemical demonstrations of ectopic hormone/TRA expression in esophagus and trachea were confirmed by RT-PCR analysis in which trachea and esophagus were found to express message for Pth and calcitonin (Fig. 6).

Discussion

This study raises a number of important issues regarding several key aspects of thymic epithelium differentiation. One concerns the manner in which FoxN1 regulates thymic epithelium differentiation. A two-stage model of thymic epithelium differentiation was proposed in which initial specification of pharyngeal endoderm to a thymic lineage was independent of FoxN1 and that FoxN1 proposed in which initial specification of pharyngeal endoderm to a thymic lineage was independent of FoxN1 and that FoxN1

Because FoxN1 expression cannot be considered as a signature marker for thymic epithelium, the expression of FoxN1 by epithelium comprising the cystic structures within the thymus is not very informative regarding their derivation. Two nonexclusive possibilities are suggested. The cysts may constitute a minor differentiation pathway of thymic epithelium within the medullary compartment that emerges after lineage specification has occurred or they may be remnants of nonspecified parathyroid endoderm that has partitioned with the thymic anlage during development. A circumstantial case can be made for the latter possibility. The evagination of the epithelial cells representing the thymic domain of the third pharyngeal pouch retains continuity with the overlying parathyroid endoderm via a thymopharyngeal duct until this connection is lost upon further differentiation (reviewed in Ref. 43). The presence of cysts in E16 fetal thymus (37) and the regular, but minor contribution of cysts to the postnatal thymus (36, 37, 44) are both consistent with the possibility that some of the thymic cysts are remnants of thymopharyngeal ducts that persist in the postnatal thymus. A lack of precision during the partitioning of thymic and parathyroid primordia from thymopharyngeal duct endoderm would provide an explanation for the frequent occurrence of thymic tissue in the neck or associated with thyroid/parathyroid tissue (33, 34, 45, 46), the association of parathyroid hormone-producing cells with the thymus (Ref. 25 and this report), and the close proximity of ectopic parathyroid tissue and cystic epithelial structures in the thymus (Ref. 47 and this study). In this last situation, parathyroid tissue associated with the thymus may represent progeny of third pharyngeal pouch endoderm that was specified to a parathyroid fate, but failed to partition from the pharyngeal endoderm forming the thymopharyngeal duct, and thus remained associated with the developing thymus.

A second important issue raised in our study concerns the mechanism responsible for TRA expression by thymic epithelium. The preferential association of Aire-dependent TRAs with Aire-positive MTEC and the demonstration that this population displays BrdU labeling properties of cells that are postmitotic with rapid turnover (21) support a model in which Aire-dependent TRA expression is a feature of terminally differentiated MTEC (1). Expression of some Aire-independent TRA expression is also thought to reflect the activity of mature MTEC, whereas others appear to be expressed by less mature MTEC (2). In either case, it has been assumed that TRA expression is a unique feature of thymic epithelium. We have proposed that some TRAs expressed by MTEC may reflect a general feature of epithelial differentiation in which epithelial cells in the course of their differentiation program would transiently transcribe genes that are normally signature features of other epithelia (20, 22, 24). This latter view does not consider the ability to express TRAs to be an intrinsic feature unique to thymic epithelium. The proposal that TRA expression may be a general feature of epithelial differentiation is consistent with the demonstration here that epithelium of the trachea and esophagus can also express Pth and calcitonin. In the case of esophageal epithelium, where the stratified organization reflects progressive differentiation, expression of Pth is clearly associated with a more luminal and distal stage of epithelial differentiation. The expression of Pth and calcitonin by tracheal epithelium and associated submucosal glands represents another instance of extrathyroidal TRA expression. This expression is intriguing because submucosal gland epithelium is thought to contain relatively undifferentiated cells with progenitor activity (48).

The demonstration in this study that esophageal and tracheal Pth expression and Pth or calcitonin provides proof-of-principle that TRA expression is not unique to the thymus. Furthermore, the demonstration in this study that TRA expression may be restricted to specific stages of differentiation in extrathyroidal epithelia raises the possibility that the apparent unique capacity of thymic epithelium to

FIGURE 6. Demonstration of Pth and calcitonin message in trachea and esophagus. cDNA was prepared from the indicated tissues as described in Materials and Methods and then subjected to RT-PCR analyses. Fetal mix represents RNA isolated from whole E14 embryos. In H2O lane, cDNA template was omitted.
express TRAs may reflect a bias in TRA analyses. This is because the same rigor of analyses used to look at fractionated thymic epithelium has never been applied to comparably fractionated extrathythmic epithelia. This issue may be particularly important if the pool sizes of epithelial cells at different stages of differentiation vary among different epithelia. When transcriptional profiles of thymic and extrathymic epithelial cells at comparable stages of differentiation are evaluated, the differences of TRA expression between these populations may be more quantitative than qualitative. As there may be multiple mechanisms effecting TRA expression by epithelium, it will be important to follow up these initial findings with an evaluation of a broader panel of candidate TRAs and their patterns of expression by other epithelia.

This study also fills in some of the gaps in our knowledge regarding MTEC heterogeneity and the relationship of this heterogeneity to the expression of TRAs. First, despite the striking morphological differences between conventional and cyst-associated MTEC, they were phenotypically similar. This may reflect a common ancestry or indicate that these phenotypic criteria are common to multiple epithelia. This phenotypic convergence between conventional MTEC and cyst epithelium extended to the panel of TRAs that we examined. MTEC expressing Pth, calcitonin, or thyroglobulin were readily detected and probably occur with a frequency consistent with previous estimates for abundantly expressed TRAs (1) and is in contrast to the rarity of MTEC that express the Aire-dependent TRA, insulin (49).

Based on the model in which claudin-positive Aire+ cells give rise to claudin-positive Aire-positive cells and other data referenced, indicating that Aire expression is a feature of terminally differentiated MTEC, the rarity of MTEC's coexpressing Aire and Pth (and by virtue of their high degree of coexpression, calcitonin, and thyroglobulin as well) would indicate that these TRAs are not expressed at a terminal stage of differentiation. One could still invoke the terminal differentiation model by proposing that Aire expression does not define all terminally differentiated MTEC. Parenthetically, the finding here that claudin3 expression is restricted to a layer of suprabasal epithelial cells in the esophagus, and the demonstration that claudin3 expression is restricted to the granular layer in human epidermis (50) show that claudin3 expression can define discrete stages of epithelial differentiation within a single lineage of epithelium, and thus provides an alternative interpretation to the consideration of claudin3 as a marker of thymic epithelial lineage heterogeneity (35).

The extent of Pth, calcitonin, and thyroglobulin coexpression by individual MTEC was also noteworthy. The demonstrated specificity of the Abs used to detect these molecules, and the demonstration that these molecules are not always coexpressed by MTEC indicate that the expression patterns reported in this study are accurate. However, immunohistochemistry could not confirm the thyroglobulin expression by cortical thymic epithelium indicated by PCR analyses of enzymatic-dissociated thymic epithelium (1). This may reflect the sensitivity limits of immunohistochemical detection. The previous demonstration that genes promiscuously expressed in the thymus tend to be clustered in the genome and the proposal that the thymic expression of both Aire-dependent and Aire-independent TRAs reflects transcripational “read-through” of neighboring genes (2) could provide an explanation for the coexpression of Pth and calcitonin, which are closely positioned on chromosome 15. That a transgene driven by the Pth promoter showed strong expression in the parathyroid gland but was not expressed in the thymus (51) suggests that Pth expression in the thymus may not conventionally regulated. This would parallel other instances in which a TRA expressed by thymic epithelium is not regulated in the same manner as in the corresponding target tissue (23, 52, 53).

The data presented in this study indicate that Foxn1 expression cannot serve as a definitive marker of thymic epithelial differentiation among derivatives of thymic endoderm, thereby leaving the field in search of molecular signatures unique for the thymic lineage. These data also challenge the view that promiscuous gene expression is a unique feature of thymic epithelium and indicate that it may be premature to exclude a developmental basis for any TRA expression by thymic epithelium.

Disclosures

The authors have no financial conflict of interest.

References


