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Features of Medullary Thymic Epithelium Implicate Postnatal Development in Maintaining Epithelial Heterogeneity and Tissue-Restricted Antigen Expression

Geoffrey O. Gillard and Andrew G. Farr

Although putative thymic epithelial progenitor cells have been identified, the developmental potential of these cells, the extent of medullary thymic epithelium (mTEC) heterogeneity, and the mechanisms that mediate the expression of a wide range of peripheral tissue-restricted Ags (TRAs) by mTECs remain poorly defined. Here we have defined several basic properties of the mTEC population that refine our understanding of these cells and impose important constraints for any model of mTEC differentiation and function. We report here that mTECs from adult mice are mitotically active, implying continual turnover, differentiation, and replacement of mTEC populations in the adult thymus. The mTEC population in adult thymus expresses transcription factors implicated in the maintenance of multipotential progenitor cell populations, suggesting that epithelial progenitors in the adult thymus may not be restricted to a thymic fate. mTECs also express multiple transcription factors required for the specification of multiple epithelial lineages in peripheral tissues. Thus, expression of some TRAs by mTECs may represent coordinated gene expression that reflects alternate programs of epithelial differentiation among mTECs. Analysis of TRA expression in individual and small pools of sorted mTECs show that mTECs are highly heterogeneous; each individual mTEC expresses a limited spectrum of TRAs, and the frequency of mTECs that express any individual TRA is quite low (>0.4–2%). Collectively, these findings suggest that the differentiation of mTECs can involve some of the developmental programs used by other epithelial lineages and that expression of some TRAs by mTECs may reflect this activity.

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Abbreviations used in this paper: TE, thymic epithelium; arie, autoimmune regulator; CK, cytokeratin; Csnb, casein; Csb, casein β; Cng, casein γ; Csnk, casein κ; Dppa3, developmental pluripotency associated 3; Epcam, epithelial cell adhesion molecule; Foxn1, forkhead N1; Gip, gastric inhibitory protein; Ins2, insulin II; mTEC, medullary thymic epithelial cell; Pdx1, pancreatic and duodenal homeobox 1; Ppy, pancreatic polypeptide; Pp34, Purkinje cell protein 4; Sst, somatostatin; Spt1, salivary protein 1; Spy2, salivary protein 2; TRA, tissue-restricted Ag; CMF, Ca2+ and Mg2+-free; TBE, Tris-buffered EDTA; mup, major urinary protein; RT, reverse transcriptase.

that were previously thought to be expressed only by specialized cells in peripheral tissues (3–7).

Understanding the basis for this epithelial heterogeneity has been hampered by a lack of knowledge regarding TE differentiation. Although a putative progenitor population has been identified in the fetal thymus (8–10), their persistence in the postnatal thymus has not been established. Furthermore, the developmental potential of these putative progenitor cells is not known. At one extreme, they could be specified to a thymic fate and have a very restricted developmental potential. Alternatively, they could possess multilineage potential that becomes restricted in response to proximal environmental cues. Understanding how TE differentiation is controlled will be of great assistance in determining the basis for mTEC heterogeneity and TRA expression by mTECs.

The purpose of this study was to define the properties of TE that would help refine testable models of mTEC differentiation. One property that has not been closely examined concerns the mitotic activity of TE. Compared with hematogenous cells in the thymus, TE is relatively radiation resistant; this property allows for the generation of radiation bone marrow chimeras. That TE is relatively radiation resistant has led to the notion that TE is composed predominantly of differentiated postmitotic cells, although the deleterious effect of conditioning regimens used in bone marrow transplantation suggests that epithelial turnover and maintenance may also be impaired by these treatments (11, 12). The extent of turnover among TECs, and mTEC in particular, may indicate the extent to which TEC maturation is a postmitotic event.

To account for mTEC heterogeneity, and the expression of some TRAs, we have previously suggested that some epithelial progenitors in the postnatal thymus are multipotential and retain the ability to follow differentiation programs of other epithelial lineages (13, 14). According to this view, subsets of mTECs would express
transcription factors typically associated with multipotential progenitor cells and/or those known to be involved in the specification of other epithelial lineages. The absence of these transcription factors in mTECs would require a reassessment of this model.

Finally, as a potential indicator of mTEC heterogeneity, the expression of TRAs by these cells must be assessed with greater precision. Morphological and immunohistochemical data have shown that expression of individual TRAs is restricted to scattered single cells (4, 5, 15) and small multicellular foci (16) of mTECs. These data support the notion that mTECs are a heterogeneous mixture of cells. In contrast, analyses of TRA expression by mTECs, using large pools of cells, have assumed that TRA-expressing mTECs represent a homogeneous population. Use of large populations imposes an important limitation on interpreting these results, because they could reflect either a transcriptional profile common to a homogeneous population of cells or the summed expression of a heterogeneous mTEC population. Although the former interpretation is currently favored, our recent demonstration that TRAs associated with respiratory epithelium are preferentially expressed by a small subset of mTECs favors the latter interpretation (16). A more detailed analysis of the pattern of TRA expression by mTECs would facilitate efforts to determine the mechanisms controlling this process.

In this study, we report that mTECs in adult thymus are a mitotically active population, raising the possibility that morphological heterogeneity and TRA expression by mTECs could be associated with ongoing epithelial differentiation and turnover. The mTEC population expresses transcription factors that are associated with multipotency, consistent with the possibility that epithelial progenitor cells in thymus have a degree of developmental plasticity. The observation that mTECs also express transcription factors involved in the differentiation of other epithelial lineages, lineages that are represented by the spectrum of TRAs expressed within the mTEC population, raises the possibility that the mechanisms controlling this process.

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table

**Materials and Methods**

**Mice**

Adult B6 mice (5–10 wk) were obtained either directly from the National Cancer Institute, from Charles River Laboratories, or from our colony maintained at the University of Washington SPF facility. Age-matched 4- to 6-wk-old BALB/c mice for BrdU labeling experiments were obtained from the National Cancer Institute. All mice were handled and used in accordance with protocols approved by the University of Washington’s Institutional Animal Care and Use Committee.

**Sorting and flow cytometry**

Enzymatic dissociation of thymi was performed as described (16). Briefly, thymi were removed and diced in HBSS (Ca²⁺- and Mg²⁺-free; CMF) + 2% FBS + HEPES using forceps and scalpels, washed, and sequentially digested using collagenase D (Roche) in CMF HBSS followed by a mixture of collagenase and neutral dispase (Roche) in CMF HBSS to obtain a single-cell suspension. Single-cell suspensions were treated with rat anti-FcγR II/III mAb (clone 24G2) before staining. After staining, cells were washed twice in HBSS CMF + 2% FBS and resuspended in the same containing 7-aminoactinomycin D (Molecular Probes) at a final concentration of 0.5 μg/ml. Cells were then stained with either a BD Vantage or Anni cell sorter directly into Trizol (Invitrogen; for analyzing large populations) or into lysis/reverse transcriptase (RT) buffer in 96-well plates for small pools and single-cell analyses. Analysis of mTEC cell cycle status was performed

on an Annius Imagestream (Annnis) using the same dissociation method and primary Abs. DraG5 (Biostatus) was used to stain DNA.

Primary Abs were: monoclonal anti-murine epithelial cell adhesion molecule (Epcam; clone G8.8) either directly conjugated to Alex 647 (Molecular Probes) or conjugated to digoxigenin; anti-CD45 PE (clone 30-F11; eBiosciences); anti-MHC class II (clone Y3P, conjugated to digoxigenin; a generous gift of A.Y. Rudensky); a mixture of purified anti-BP-1 Abs (clones 6C3 and CD1R, directly conjugated to Alexa 647), CD4 FITC (BD Pharmingen); and CD24 APC (eBiosciences). Secondary Ab was anti-digoxigenin-FITC (Boehringer Mannheim).

**Isolation of RNA**

RNA samples in Trizol were vortexed and passed five times each through three (18-, 22-, and 25-gauge) needles. RNA was then isolated using standard Trizol isolation protocol and resuspended in 10 μl of 1× DNase Turbo buffer (Ambion). We added 100 U of SUPERasein (Ambion) to each sample, followed by 5 μl of DNase I (Ambion). Each sample was incubated at 37°C for 30 min. The DNase-treated RNA was then reisolated using RNeasy columns (Qiagen), concentrated, and linearly amplified following a modified Eberwine procedure as described in Ref. 17. From 3 to 5 μg of each amplified cRNA was used to generate dsDNA for PCR analysis with random primers and Arrayscript RT (Ambion) to generate the first strand. PCR analysis was conducted using primers for multiple molecules. All primers were designed using Primer Express (Applied Biosystems) to have a Taₘ of 60°C and to work well under the following conditions: 25- or 50-μl reactions with a final concentration of 10 mM Tris, 2.5 mM MgCl₂, 0.2 mM dNTPs, 0.4 μM primers, and 0.625 U Taq DNA polymerase in buffer B (Promega). All primers, where possible, were designed to span introns. No polymerase controls were used to ensure cDNA specificity for amplification. Assay control primers do not span introns. All primer sequences are available upon request. Cycling conditions were 94°C for 5 min; cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 45 s, 72°C for 10 min; 4°C hold. For cDNA from large mTEC populations, HPRT, Epcam, air, and CD45 reactions were amplified through 28 cycles; all other PCR were amplified through 38–42 cycles. PCR products were examined on 1.8% Tris-buffered EDTA (TBE) gels stained with Syber safe DNA gel stain (Invitrogen and Molecular Probes).

**BrdU labeling studies**

Age-matched cohorts of BALB/c mice (NCl) were maintained either on BrdU drinking water (0.8 μg/ml; prepared and changed daily) or normal drinking water in the University of Washington SPF facility for up to 5 wk. After 5 wk, experimental groups were returned to normal drinking water for an additional 5 wk. At indicated intervals, 10 mice from each group were sacrificed, and thymi were dissociated to form a single-cell suspension and enriched for epithelial cells by anti-CD45 bead depletion as described in Ref. 18. After bead depletion, the remaining cells were washed, suspended in PBS, and spun onto aminosilane-coated slides with a cytocentrifuge. Slides were air dried overnight, fixed in a 75% acetone-25% ethanol for 3 min at room temperature, rehydrated in PBS (pH 7.4), and then stained with primary Abs in PBS containing 0.1% BSA + 10% FBS for 1 h at room temperature. Slides were washed PBS three times, incubated in PBS + 0.1% BSA for 5 min, before addition of Alexa 488-conjugated or biotin-conjugated antibiot-BrdU mAb (Invitrogen and Molecular Probes) and all fluorochrome-conjugated secondary Abs (if biotin or digoxigenin conjugated; all fluorochrome-conjugated reagents and anti-BrdU mAbs were applied after HCl treatment), washed, and fixed at room temperature for 20 min in PBS containing 4% paraformaldehyde. Slides were washed three times in PBS before immersion in 4 N HCl for 30 min at room temperature. Slides were rinsed in PBS and transferred to 0.2 M sodium borate (pH 8.5) for 3 min to neutralize any residual acid. Slides were then incubated in PBS + 0.1% BSA for 5 min, before addition of Alexa 488-conjugated or biotin-conjugated antibiot-BrdU mAb (Invitrogen and Molecular Probes) and all fluorochrome-conjugated secondary/tertiary Abs and/or staining reagents.

Primary Abs were: G8.8 (purified or digoxigenin conjugate); 6C3 (anti-BP-1; purified or digoxigenin conjugate); rabbit anti-pancytokeratin (CK; Sigma-Aldrich); and biotin- or Alexa 488-conjugated antibiot-BrdU mAb (Invitrogen Life Technologies and Molecular Probes). Secondary/tertiary Abs and antigens were: anti-rat Ig Alexa546 conjugate; anti-rat Ig Alexa 488 conjugate; streptavadin-Alexa 546 conjugate (Molecular Probes); anti-rabbit biotin conjugate (Sigma-Aldrich), anti-digoxigenin FITC conjugate (Roche).

**Generation of pooled cDNAs from sorted mTECs**

Generation of cDNA from pools of mTECs sorted from adult (6–12-wk-old) mice thymi were removed and diced in HBSS (Ca²⁺- and Mg²⁺-free; CMF) + 2% FBS and resuspended in the same containing 7-aminoactinomycin D (Molecular Probes) at a final concentration of 0.5 μg/ml. Cells were sorted on either a BD Vantage or Aria cell sorter directly into Trizol (Invitrogen; for large-scale pooling) or into lysis/reverse transcriptase (RT) buffer in 96-well plates for small pools and single-cell analyses. Analysis of mTEC cell cycle status was performed...
0.5 U reaction SUPERasein RNase inhibitor (Ambion), 0.5 U reaction Ribolock RNase inhibitor (Fermentas), 0.5% Nonidet P-40) heated to 70°C for 3 min 45 s, and cooled on ice for 5 min before addition of 0.5 μl of a 1:1 mixture of lysis/RT buffer-SuperscriptII RT (200 U/μl; Invitrogen Life Technologies). Plates were vortexed briefly, spun down, and incubated at 37°C. After 30 min, samples were heated to 70°C for 10 min to inactivate the RT enzyme and chilled on ice. Next, 5 μl of 2× TdT mix (6 mM dATP, 3 mM CoCl2, 2× tailing buffer + 25 Urease TdT (Roche) was mixed into each reaction and incubated at 37°C. After 15 min, samples were heated to 65°C for 15 min to inactivate TdT. Three parallel PCRs were run consisting of 3.3-μl aliquots each of the TdT reactions added to 21.7 μl of primary PCR mix (0.9 mM dNTP mix, 10 μM first primer, 1× HotStarTag buffer, 0.5× Q solution containing 2.5 U HotStarTag (Qiagen), and 0.125 U Pfu Turbo (Stratagene) per reaction), mixed, and run on a thermocycler using the following program: 94°C for 15 min; 1 cycle of 94°C for 45 s, 50°C 2 min, 72°C for 3 min and 15 s; 36 cycles of 94°C for 45 s, 58°C for 45 s; 72°C for 3 min; 72°C for 10 min; 4°C hold. Primary PCRs were combined and purified using Qiaquick PCR purification columns (Qiagen) and eluted in a total volume of 100 μl. Secondary PCR were prepared using 40 μl of purified primary PCR products mixed with 60 μl of secondary PCR mix (0.2 mM dNTP mix, 5 μM second primer (ACCTACTATAG GGAAGCGCGTGT), 1% HotStarTag buffer, 0.5× Q solution, 2 U Taq (Promega) and 0.25 U Pfu Turbo per reaction) and cycled with the following program: 94°C for 5 min; 32 cycles of 94°C for 30 s; 55°C for 45 s; 72°C for 3 min; 72°C for 10 min; 4°C hold. Secondary reactions were column purified and eluted in 100-μl volumes. PCR analysis for multiple genes were performed using 2 μl of purified secondary PCR products in standard 25-μl PCR mixtures with 60°C annealing temperatures (38 cycles for CD45Epcam, Pax1, and lymphotoxin) and cycled with the following program: 94°C for 5 min; 36 cycles of 94°C for 45 s, 50°C for 30 s; 60°C for 30 s; 72°C for 45 s; 72°C extension for 10 min; 4°C hold. Primary PCRs were prepared using 1 μl of the outer nest reaction added to 49 μl of individual inner nest PCR mixture (1× PCR buffer, 2.5 mM MgCl2, 0.4 μM gene-specific outer nesting primers, + 2.5 U Taq per reaction). Thermocycler conditions for the outer nesting PCR: 94°C for 5 min; 36 cycles of 94°C for 30 s; 60°C for 30 s; 72°C 1 min; 72°C extension for 10 min; 4°C hold. Outer nesting PCR were performed using 1 μl of the outer nest reaction added to 49 μl of individual innernesting PCR mix (1× PCR buffer, 2.5 mM MgCl2, 0.4 μM Mgene-specific inner nesting primers, 1.25 U Taq). Thermocycler conditions for the inner nest PCR: 94°C for 5 min; 36 cycles of 94°C for 30 s; 60°C for 30 s; 72°C for 45 s; 72°C 1 min; 4°C extension for 10 min: 4°C hold. Inner nesting PCR products were visualized on 1.8% TBE gels stained with Sybrsafe DNA stain (Invitrogen Life Technologies and Molecular Probes).

Results

The mTEC population in adult mice is mitotically active and undergoing significant turnover

One of the elements necessary for a developmental model of TEC heterogeneity and TRA expression is ongoing differentiation and turnover of mTECs in adult thymus. We used two independent means to assess mitotic activity in mTECs, the first being a set of BrdU labeling experiments. Cohorts of age-matched adult BALB/c mice (4–6 wk old) received BrdU in their drinking water for a 5-wk pulse period and then returned to normal drinking water for a 5-wk chase period. At various intervals during this regimen, enzymatically dissociated thymi from test and control groups were enriched for epithelial cells by anti-CD45 bead depletion (18), and then assessed for CK and BrdU content by immunofluorescence microscopy (Fig. 1A). Triple staining experiments showed that almost all epithelial cells recovered were mTECs (as defined by staining results with Abs to Epcam (a medullary marker), BP-1 (a cortical marker), and CK; data not shown). Due to low recovery of cortical TECs using this method, we were unable to draw any conclusions regarding the mitotic activity of this population. The results of three independent experiments were consistent and showed significant accumulation of BrdU-labeled mTECs through the 5-wk labeling period, followed by a corresponding decrease in the number of cells retaining the BrdU label after cessation of BrdU treatment (Fig. 1B). The kinetics of the loss of the BrdU label following cessation of BrdU administration mirrored the kinetics of the labeling phase. We conclude that the proliferation documented here reflects epithelial turnover, because the adult thymus has already reached maximal size and is not undergoing pubertal expansion (21).

To independently confirm the existence of mitotically active CD45Epcam mTECs, we used the Amnis Imagestream instrument to measure the DNA content of individual mTECs stained for CD45, Epcam, and the DNA stain Draq5. The Amnis Imagestream is a specialized instrument that combines the image-capturing capacity of a high throughput fluorescence microscope to acquire real time images of each individual cell in multiple channels, with the capacity to generate population analyses (similar to a conventional flow cytometer) using the raw imaging data generated from large input populations. This instrument allowed the simultaneous quantitation of signal intensity and visualization of the staining pattern for each individual cell that fell within a gate or population. We assessed the cell cycle status of individual CD45 Epcam mTECs isolated from unmanipulated adult B6 thymus (Fig. 1C) using the DNA stain Draq5. A population profile for mTECs based on the staining intensity of Draq5 demonstrated a main peak of cells that were quiescent and a heterogeneous population in the gated population that were >2N in DNA content or were apoptotic (Fig. 1C, a). Representative images of individual cells representative of these populations include quiescent (2N DNA content) mTECs (Fig. 1C, b), apoptotic mTECs (Fig. 1C, c), and mTECs that have either synthesized DNA (>2N DNA content; Fig. 1C, d). Results of several experiments indicated that ~1–2% of mTECs isolated from unmanipulated adult (6–10 wk) B6 thymus have synthesized DNA or recently divided. These results are consistent with the BrdU labeling data and clearly indicate that there is ongoing mitotic activity within the mTEC population of adult mice. It also appears that the rate of epithelial turnover is rather low, which would be consistent with the relative radiation resistance of this population that allows the generation of radiation bone marrow chimeras.

Sorted mTECs express molecules known to regulate thymic organogenesis and TEC development

Although ongoing epithelial turnover implies ongoing mTEC differentiation, mitosis could be attributed to self-renewal of adult mTECs. We wanted to determine whether the known molecular regulators of thymic organogenesis and mTEC differentiation are expressed within this mitotically active population. We generated cDNA from 1 to 2 × 105 EpcamCD45 BP-1 mTECs isolated from adult B6 thymus. We first evaluated these cDNAs for the expression of aire, Epcam, CD45, and a number of TRAs previously shown to be expressed by mTECs, including casein (Csgn), Purkinje cell protein 4 (Pcp4), and insulin II (Ins2) (3, 22). These PCR results showed that all of these molecules (except CD45) were expressed within the sorted mTEC population (Fig. 2A) and verified that we were examining a cell population comparable with those used in previous studies (3, 22).

We then assessed whether or not the known developmental regulators of thymic epithelial differentiation are expressed within the mTEC population. Although some, such as forkhead N1 (FoxN1), Pax1, and lymphotixin β receptor, have been shown to be expressed by postnatal thymic epithelium, the roles of Eya1, Hoxa3,
and Pax9 in thymic epithelial development have been largely defined by the impact of their targeted inactivation on thymic organogenesis (reviewed in Ref. 23). Here we have determined that all of these known regulators of thymic epithelial development were expressed in bulk, sorted populations of adult mTECs (Fig. 2B). This expression pattern raised the possibility that Eya1, Hoxa3, and Pax9 may continue to play a role in the development and maturation of mTECs in the adult thymus. These results also demonstrate that the molecular regulators of thymic organogenesis and differentiation continue to be expressed within the adult mTEC population and suggest that mTEC differentiation from a resident progenitor population persists in adult thymus.

Molecules characteristic of multipotential cells are expressed by mTECs

Based on the previous demonstration that epithelial foci in adult thymus ultrastructurally and phenotypically resemble other endodermally derived epithelium (reviewed in Ref. 13), we have proposed the presence of multipotential progenitor epithelial cells in thymus (13, 14). Because the nature of and product-precursor relationships for mTEC subsets remain ill defined, we could not test this hypothesis directly. However, we could determine whether or not some of the transcription factors previously demonstrated to be characteristic of multipotential cell populations were expressed within the mTEC population. The molecules we examined are largely restricted to multipotential cell populations (such as embryonic stem cells and blastocysts) and are either considered to be characteristic markers of these populations or have been directly implicated in the maintenance of pluripotentiality in the cells that express them. These include Stella (developmental pluripotency associated 3; Dppa3) (24), Efox (embryonic homeobox) (25), Foxd3 (also known as Genesis) (26), and undifferentiated transcription factor 1 (27). We determined that each of these molecules is expressed within the mTEC population (Fig. 3). We also evaluated the expression of Sox2, Nanog, and Oct4 (Pouf51) by mTECs because they have been reported to compose

FIGURE 1. mTECs are mitotically active in adult thymus. A and B. Results from studies where dividing cells were labeled in vivo by incorporation of BrdU. A, Representative cytospin of CD45-depleted thymic cells stained for BrdU (green) and CK (red). B, Quantitative analysis of the percentage of total CK− (epithelial) cells that have synthesized DNA during the labeling phase of the experiment (CK−BrdU−). Results are representative of three separate experiments. No BrdU staining was observed in cells from age-matched control animals maintained on normal water throughout the experiment. A minimum of 700 epithelial (keratin+) cells was counted at each time point. C. Results of DNA content analyses of single sorted mTEC isolated from adult mice that was performed with the Amnis Imagestream instrument. a, Draq5 staining intensity histogram of Epcam+CD45− cells. The subset of cells with high DNA staining intensity is indicated by gate R3. b, Representative image of an individual mTEC classified as quiescent (2N DNA content). c, mTEC considered to be apoptotic, based on the dense and blebbed nucleus. d, Individual mTEC with DNA content >2N and lacking nuclear features of apoptosis. A total of 34 of 1906 mTECs (1.8%) were scored as live, >2N DNA content cells. The scoring of Epcam+CD45− Draq5high cells as apoptotic or mitotic was performed by Amnis personnel who lacked direct knowledge of the project, and results are representative of three experiments.
The molecules regulating early thymic development are also expressed by mTECs from adult thymus. PCR on a serial dilution (undiluted, 1/5, 1/25) of cDNA generated from 1 to 2 × 10⁵ sorted mTECs (Epcam⁺ CD45⁺) demonstrated that Epcam, aire, and a range of aire-dependent TRAs characteristic of multiple peripheral tissues (Csng, mammary gland; liver fatty acid binding protein (LFabp), liver; Gip, intestine; Pcp4, neuronal; and Ins2, pancreas) are expressed by this population (A). Additional PCR analysis (B) shows that mTECs in adult thymus express multiple molecular regulators of thymic epithelial development: eyes absent 1 (Eya1); FoxN1 (Whn); homeobox a3 (Hoxa3); lymphotoxin β receptor (LtβR); paired box 1 (Pax1); paired box 9 (Pax9).

FIGURE 2. The molecules regulating early thymic development are also expressed by mTECs from adult thymus. PCR on a serial dilution (undiluted, 1/5, 1/25) of cDNA generated from 1 to 2 × 10⁵ sorted mTECs (Epcam⁺ CD45⁺) demonstrated that Epcam, aire, and a range of aire-dependent TRAs characteristic of multiple peripheral tissues (Csng, mammary gland; liver fatty acid binding protein (LFabp), liver; Gip, intestine; Pcp4, neuronal; and Ins2, pancreas) are expressed by this population (A). Additional PCR analysis (B) shows that mTECs in adult thymus express multiple molecular regulators of thymic epithelial development: eyes absent 1 (Eya1); FoxN1 (Whn); homeobox a3 (Hoxa3); lymphotoxin β receptor (LtβR); paired box 1 (Pax1); paired box 9 (Pax9).

the core transcriptional regulatory circuit active in multipotent cells (28). As shown in Fig. 3, each of these molecules was also expressed within the mTEC population. In the case of Dppa3, FoxD3, and undifferentiated transcription factor 1, these results confirm array data that had identified these molecules as preferentially expressed by mTECs (29). Although it remains to be determined whether or not the expression of this group of transcription factors can be attributed to a restricted subset of mTECs with unique developmental potential, this work identifies new markers that may be useful for identifying mTEC progenitors and establishing product-precursor relationships among mTEC subsets. Given the reported function of these transcription factors in other cell populations referenced above, we hypothesize that their expression will likely be restricted to a discrete subset of immature mTECs.

Multiple regulators of development in peripheral epithelial tissues are expressed by mTECs

Having observed expression of multiple regulators of thymic development and transcription factors characteristic of multipotent cells within the mitotically active mTEC population, we next wanted to determine whether mTECs also expressed transcription factors that regulate the differentiation of peripheral epithelial tissues where TRAs are characteristically expressed. We determined that in addition to FoxA1 and FoxA2, which are centrally involved in the development of a number of endodermal derivatives (30–36), mTECs expressed other transcription factors that are critical for the development of endodermal and neural tissues (Cdx1 (37, 38), Crx (39), Pdx1 (40–43), Hnf-6 (44–46), and Foxg1 (47, 48); Fig. 4A). Multiple TRAs characteristic of the tissues that these transcription factors regulate, including blue cone opsin (retina), fatty-acid binding protein 1 (liver), gastric inhibitory polypeptide (Gip; intestinal epithelium), Ins2 (pancreas), and major urinary protein 1 (liver), were also expressed by mTECs (Fig. 2, Fig. 4B, and data not shown). These results show that the spectrum of TRAs expressed within the mTEC population extends beyond structural or terminal TRAs to include upstream developmental mediators characteristic of peripheral epithelial tissues.

Expression of individual TRAs is restricted in mTECs

Several studies have suggested that TRAs are broadly and promisingly expressed by mature mTECs as a unique property of the mTEC lineage (22, 29); however, other studies have suggested that mTECs are highly heterogeneous and that the expression of individual TRAs is highly restricted to relatively rare mTECs (4, 5, 15). To distinguish between these two possibilities, we assessed the TRA expression profiles for small pools of sorted mTECs.

Using the method of Chiang and Melton (19), we generated cDNA from small pools (20 cells/pool) of sorted Epcam high CD45+ BP-1 mTECs from the thymi of adult B6 mice. We analyzed the expression patterns of aire and 15 individual TRAs in pools of cells previously verified by PCR to be Epcam⁺ and MHC II⁺. As shown in Fig. 5, each individual TRA, with the notable exception of casein β (Csnb), was rarely expressed in the pools. The ubiquitous (and atypical) expression of Csnb suggests that this molecule is regulated differently than the other TRAs we assessed and may indicate that Csnb is expressed as part of a global mTEC expression profile. When all TRAs were considered, including Csnb, the average number of TRAs expressed per pool was closest to 2 of 15, with no pool expressing >4 of 15 TRAs. If Csnb is omitted from the analysis, the average dropped to 1 of 14 TRAs expressed per pool; no single pool expressed >3 of 14 TRAs, and 5 pools expressed 0 of 14 TRAs.

The expression frequency for any individual TRA expressed in the pools (excepting Csnb) ranged from 0 of 13 to 3 of 13. If it is assumed (given the relatively low frequency of expression for each individual TRA) that positive expression of a TRA within a pool reflects the presence of an individual mTEC that expresses that TRA, we could estimate the percentage of mTECs that make a given TRA. Based on the data presented here, the percentage of mTECs that expressed a given TRA ranged from <0.4% – 1% (0 of 260 cells to 3 of 260 cells). This estimate is consistent with previously published estimates of the frequency of mTECs that express a given TRA (4, 15).

Expression of individual TRAs by functionally mature mTECs is also restricted to relatively rare cells

It has been proposed that mTECs progressively derepress TRA-containing loci as they mature, resulting in the highest levels of...
The genes within the casein locus would be proposed as a strong example of epigenetic derepression of TRA chromosome 5 (49). Expression of all three by mTECs has been noteworthy, given that these genes reside within the same locus on chromosome 5. The frequency of mTECs that expressed any individual TRA was low. Our analysis of TRA expression within small pools of sorted mTECs clearly showed that Csnb and Csnk appear to be expressed independently from both Csnb and each other; indeed, we did not find Csnb and Csnk coexpressed within any pool (Figs. 5 and 6B). In contrast, Csnb was highly expressed in mTECs and may be expressed by every mTEC or a large, as yet undefined subset of mTECs. Further single-cell analysis will be required to determine whether that is the case.

Although we saw no evidence for coordinated expression of casein genes, we did observe that the pools that expressed multiple TRAs coexpressed Gip, pancreatic polypeptide (Ppy), and somatostatin (Sst). Although chromatin de-repression does not readily account for the coordinated expression of these genes (Gip and Ppy are located over 6 Mb apart on chromosome 11, Sst is located on chromosome 16), developmental mechanisms may explain the possible coordinated expression of these TRAs. The pancreatic transcription factor Pdx1 (pancreatic and duodenal homeobox1, or somatostatin transactivating factor-1) is known to directly regulate the transcription of both Gip (43) and Sst (40); the development of the cells that express Ppy in pancreas is also dependent on Pdx1 (41). It will be important to determine the extent to which other developmentally related genes are coordinately expressed in mTECs and the extent to which expression of developmentally related TRAs is coincident with the transcription factors that regulate them in peripheral tissues.

### FIGURE 4

mTECs express a wide spectrum of transcription factors involved in the development of peripheral tissues. Bulk populations of sorted mTECs express a broad range of transcription factors involved in the development of specialized cell types in peripheral tissues. A, Results of PCR on a serial dilution (undiluted, 1/5, 1/25) of cDNA from sorted mTECs established that a number of transcription factors known to be involved in the development of peripheral epithelial cell lineages, including Cdx1 (embryonic positioning; intestinal epithelium), Cxcr5 (retinal photoreceptor cells), FoxA1 and FoxA2 (Hnf-3α and Hnf-3β, multiple tissues), Hnf-6 (multiple tissues, including pancreas), and Pdx1 (pancreatic organogenesis; transcription of Gip and Ins2). The expression of these developmental transcription factors corresponds to the expression of downstream TRAs that are also found in mTECs (B), including blue cone opsin (Bco; retina), glucagon (Gcg; pancreas), and major urinary protein 1 (Mup1; liver), and Spt1 (salivary gland). For additional examples of TRAs that are representative of the tissues specified by the transcription factors shown in A, see Fig. 2.

### FIGURE 5

Expression of TRAs is limited to rare cells within small pools of sorted mTECs. Small pools of sorted mTECs (Epcam^CD45^; 20 cells/pool) were assessed for the expression of aire and 15 TRAs. Pools were validated using PCR for Epcam, MHC II, and HPRT expression. The expression profile of multiple TRAs in 13 pools is depicted. Dark squares, positive expression; light squares, lack of expression. Ags are grouped loosely by tissue. The TRAs assessed include those known to be aire dependent (Csnb, mammary; elastase 3b, pancreas; Gip, intestine; Ins2, pancreas; Mup1, liver; Pcp4, neuronal; Spt1 and Spt2, salivary gland) and additional Ags characteristic of other peripheral epithelial cell lineages (cystic fibrosis transmembrane conductance regulator homologue (CFTR), multiple; palate, lung, and nasal epithelium carcinoma associated (PLUNC), multiple; Csnb, Csnk, mammary gland: glucagon (Gcg), pancreatic polypeptide (PPP), somatostatin (Sst), pancreas; and thyroglobulin. The total number of TRAs expressed in a given pool is shown at the bottom of the graph. Note that, despite the widespread expression of aire, a number of aire^+ pools express few if any aire-dependent TRAs.
Nested PCR analysis of cDNA from pools and sorted single cells confirms the observed TRA expression patterns

We wanted to confirm the small pool data described above using a different method (20) and extend our observations to the single-cell level. To accomplish this, nested gene-specific PCR was performed with cDNA from both small pools (25 cells/pool; Fig. 7A) and individual (Fig. 7B) sorted MHC II^highEpcam^H11001CD45^H11002 cells. Although the use of nested PCR with cDNAs generated using gene-specific primers allowed for increased sensitivity, the spectrum of molecules that could be analyzed was significantly smaller. Amplified cDNA from all pools and single cells examined were first validated via secondary PCR for expression of MHC II. We then tested these pools and single cells for the presence of transcripts for aire and the aire-dependent TRA Gip (22). As shown in Fig. 7, Gip was expressed by only 1 of 23 MHC II^+^ pools (Fig. 7A and data not shown), and 0 of 40 MHC II^+^ individual mTECs (Fig. 7B and data not shown). These results were consistent with the pool data in Figs. 5 and 6 and confirm that expression of an individual TRA is limited to rare individual mTECs.

aire expression alone is not sufficient for the coexpression of aire-dependent TRAs in sorted mTECs

The expression of many of the TRAs assessed in Figs. 5 and 6 have been shown to be aire dependent in mTECs, including Csng, elastase 3B, Gip, iFABP, Ins2, major urinary protein (mup), Pcp4, and salivary protein 1 (Spt1; Ref. 22). Although almost all of these pools expressed aire (only pool 13 of the Epcam^high^ mTECs did not; see Fig. 5), few of these pools expressed aire-dependent TRAs, and none of the pools expressed all, or even a majority of them. No individual aire-dependent TRA was expressed in 5 of 21 pools that express aire. These results indicate that the expression of aire in an mTEC was not sufficient for the simultaneous coexpression of aire-dependent TRAs.

The data generated from the pools analyzed by gene-specific nesting (Fig. 7) was consistent with this finding. We found that MHC II^highEpcam^CD45^- mTECs express aire at a relatively low frequency (5 of 40 single cells; Fig. 4B and data not shown). As a population, these MHC II^high^ mTECs have been previously shown to express aire at relatively high levels (29); our finding that only 5 of 40 single cells and 15 of 23 pools tested express aire shows that only a minority of mTECs within the MHC II^high^ population actively express aire, a frequency consistent with immunohistochemical staining patterns for aire observed in adult thymus (Ref. 50 and A. G. Farr and G. O. Gillard, unpublished observations). As in our earlier experiments, expression of aire within an individual cell or pool did not correspond well with Gip expression. Gip, an aire-dependent TRA, was expressed within only 1 of the 15 aire^-^ pools, and by none of the 5 aire^-^ single cells. Whether the expression of Gip requires that aire be coexpressed within the

![FIGURE 6.](http://www.jimmunol.org/) Expression of TRAs is limited to rare cells within small pools of sorted mature (MHC II^high^) sorted mTECs. A. Sort profile used to obtain Epcam^-^CD45^-^MHC II^high^ mTEC. B. Tabular representation of the expression of 17 TRA by 9 pools, each containing 25 of these mTECs using the same format as in Fig. 5. The total number of TRAs expressed in a given pool is indicated at the bottom of each column. C. Representative sample of the raw data used to generate the chart. Only pools expressing Epcam, MHC II, and HPRT were included in the analyses. Tgn, Thyroglobulin.

![FIGURE 7.](http://www.jimmunol.org/) Sorted MHC II^high^ mTECs are heterogeneous. Nested PCR was performed on pools (25 cells/pool, A) or individual (B) sorted MHC II^high^ mTECs to determine expression of MHC II, aire, and the aire-dependent TRA Gip. Data represent 12 of 24 pool samples (reverse transcriptase was not added to the first pool) and 11 of 40 single cells (MHC II^-^ wells were not included).
same cell still remains to be determined, because the frequency of Gip-expressing cells was so low that examination of a significantly large cohort of sorted single cells will be required to address this question.

**Discussion**

In this study, we have defined several important and novel characteristics of thymic epithelium that are central to understanding three elements of thymic epithelial biology that we believe are interrelated: the nature of thymic epithelial progenitor cells; the process of mTEC differentiation; and the generation of mTEC heterogeneity. The properties of mTEC described here lend support to a developmental model for mTEC diversity previously proposed (14), where this population is maintained by the steady state differentiation of an epithelial progenitor population with broad developmental potential within the adult thymus. This model predicts the proliferation and turnover of epithelial cells is ongoing in the adult thymus, the presence of a subset of progenitor cells within the mTEC population that express the molecules characteristic of multipotential progenitor cells, and the expression by mTECs of other transcription factors that are involved in the specification and/or differentiation of other epithelial lineages. The heterogeneity of mTECs is reflected in the molecules they express; these molecules include TRAs. Although we cannot yet test this model directly, we have demonstrated here that the properties of mTECs are compatible with these proposed requirements.

First, generation of differentiated mTEC from a progenitor population predicts a level of epithelial turnover. We have shown that proliferation of TEC continues in the postnatal thymus, albeit at a relatively low level. A basal level of proliferation of mTECs indicates that this population turns over and likely has the capacity for self-renewal. On the basis of other differentiating systems, one could envision low rate of turnover of the least mature progenitor cells, followed by a high rate of turnover and expansion of a transit amplifying population, which then becomes postmitotic and terminally differentiates. The capacity of TECs to proliferate in response to exogenous keratinocyte growth factor in vivo and in vitro (51) suggest that this signaling pathway may play a critical role in regulating TE turnover in postnatal thymus.

The continuing differentiation of mTECs in the postnatal thymus presents alternative, but not mutually exclusive mechanisms for the generation of mTEC heterogeneity and the regulation of TRA expression. Differentiating epithelial cells could access multiple developmental pathways before specification to a terminal, perhaps thymic fate choice. The ability of immature cell populations to express transcription factors characteristic of alternate lineages before commitment to a specified terminal lineage has been observed in multiple settings (20, 52–55) and may also be occurring in thymic epithelium. A multipotential thymic epithelial progenitor population could respond to developmental cues within the thymic microenvironment to adopt alternate epithelial cell fates. Thymic epithelium shares space with a complex mesenchymal compartment, together forming an environment replete with elements of several signaling pathways (bone morphogenetic protein (56, 57), Wnt (58), fibroblast growth factor (51, 59), Notch (60), and Hedgehog (61, 62)) that play critical roles in the specification and differentiation of a range of other epithelial tissues.

Invoking multipotential epithelial progenitor cells as a basis for mTEC heterogeneity and TRA expression in the adult thymus is partially based on reports that described an epithelial progenitor population in the fetal thymus (8, 9) and the assumption that these cells persist postnatally. mTEC progenitors that are multipotential and not lineage restricted would be predicted to share the molecular signature of other multipotent populations. We have shown that a number of transcription factors normally associated with multipotential cells, including Oct4, nanog, and Sox2, are expressed within the mTEC population. Defining the expression pattern of these transcription factors within the mTEC population may prove useful in parsing mTEC heterogeneity and facilitate the identification of the progenitor epithelial cell population in the adult thymus.

That TECs can adopt alternative epithelial fates is supported by several lines of experimental evidence. In the absence of functional foxn1, the thymic rudiment of nude mice displays the morphology and organization of early fetal lung and a respiratory phenotype (63). One interpretation of this finding is that the progenitor population in the nude thymus is multipotential and that foxn1 is required to specify a thymic epithelial fate. There are also indications that mTEC in normal mice adopt alternative developmental fates. Cystic epithelial structures that are lined, in part, by ciliated epithelial cells and display a wide range of respiratory system-related genes, including plunc, surfactants, and CC10, are commonly found in normal thymus (16). Foci of epithelial cells resembling small thyroid follicles within the normal thymus have also been described (13). Although these latter instances may represent extreme examples of the developmental potential of thymic epithelial progenitor cells where terminal differentiation has occurred, they highlight the potential plasticity of epithelial cells in the thymus.

Our results demonstrating the expression of peripheral developmental transcription factors by mTECs is significant in two respects. First, these findings substantially expand the number of candidate transcription factors that may be involved in mTEC differentiation, thus providing additional tools for analyzing thymic epithelial heterogeneity. Second, accepting the premise that mTEC heterogeneity arises as a consequence of differentiation and that TRA expression is a reflection of this process, it is reasonable to propose that conventional, lineage-restricted developmental transcriptional networks could regulate expression of downstream TRAs in mTECs. We have previously shown that Ttf1 (Nkx2.1), which is centrally involved in lung and thyroid development, is expressed by mTEC (16), as are multiple lung- and thyroid-specific TRAs (3, 16, 22, 29). We have shown here that mTEC also express a number of transcription factors that play key roles in the differentiation of other peripheral tissues; the mTEC population also expresses a number of TRAs characteristic of these peripheral tissues. Because some of these transcription factors are required for efficient expression of TRAs in peripheral tissues, their functional inactivation might result in lacunar, lineage-restricted deficiencies in TRA expression by mTEC. The observation that multiple Pdx1-dependent and lineage-related TRAs are coexpressed within small pools of sorted mTEC supports this notion that tissue-specific transcriptional networks may be functioning in mTEC as in their native peripheral epithelial tissues.

We favor the interpretation that expression of these tissue-specific transcription factors (and their downstream targets) results from conserved developmental mechanisms during mTEC differentiation; however, an alternative interpretation is that the derepression of chromatin in mature mTECs leads to the promiscuous expression of multiple developmental regulators (and their downstream targets) by mature mTECs. Given the gaps in our knowledge regarding the extent of mTEC heterogeneity, and the developmental relationships among mTEC populations, we cannot presently distinguish between these two interpretations.

Our examination of individual and small pools of sorted mTECs revealed that expression of a given TRA is limited to rare mTECs and that individual mTECs do not express a broad range of TRAs. These data strongly support the notion that mTECs represent a diverse population and that TRA expression reflects that diversity.
Previous reports have examined TRA expression in pools of 10^4–10^5 mTECs (3, 22, 29); by using bulk populations, the diversity of the mTEC population is not apparent because the results represent averaged expression from many cells. If the TRA expression results for the pools shown in Fig. 6 are summed, almost all of the TRAs examined were expressed (12 of 17 TRA expressed by 225

References
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