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Alterations of the Medullary Epithelial Compartment in the Aire-Deficient Thymus: Implications for Programs of Thymic Epithelial Differentiation1,2

James Dooley,* Matthew Erickson,* and Andrew G. Farr3*†‡

A widely held model of thymic epithelial differentiation is based on patterns of keratin expression, where a K8*K5+ progenitor gives rise to K8*K5/K14− cortical thymic epithelium (CTEC), and medullary thymic epithelium (MTEC) are K8−K5*K14+. The thymic phenotype of p63-deficient mice indicates that p63 is an important regulator of proximal stages of thymic epithelial differentiation. In this study, we have examined several features of the thymic medullary compartment in wild-type and Aire-deficient thymi in an effort to integrate the proapoptotic activity of Aire with these different perspectives of TE differentiation. Patterns of keratin and p63 expression by MTEC described here are difficult to reconcile with postmitotic MTEC that express a K8−K14− phenotype and suggest that the patterns of p63 and keratin expression reflecting differentiation programs of other epithelial tissues provide a useful framework for revising models of TE differentiation. Alterations of the Aire+/− MTEC compartment included reduced expression of p63, increased frequency of MTEC expressing truncated Aire protein, and shifts in the pattern of keratin expression and epithelial morphology. These data suggest a scenario where cellular targets of Aire-mediated apoptosis are postmitotic MTEC that have not yet completed their terminal differentiation program. According to this view, the minor population of globular K8−K14−/low MTEC observed in the Aire+/− thymus and significantly expanded in the Aire−/− thymic medulla represent end-stage, terminally differentiated MTEC. These Aire-dependent alterations of the MTEC compartment suggest that the activity of Aire is not neutral with respect to the program of MTEC differentiation. The Journal of Immunology, 2008, 181: 5225–5232.

Thymic epithelium contributes to an environment that recruits lymphoid progenitor cells from the circulation, directs a number of important fate choices during thymocyte development, and shapes the repertoire of T cell Ag diversity by imposing MHC restriction during positive selection. Thymic epithelial cells (TEC)4 also project a spectrum of self-Ags that contribute to negative selection of thymocytes with autoreactive phenotypes of these cells are not known (5). It is widely held that TEC coexpressing keratin (K) 8 and K5 represent precursors to TEC destined to be K8+K5/K14− cortical TEC (CTEC) (6, 7) and that TEC bearing a K5/K14−K8+ phenotype define the majority of medullary TEC (MTEC). The differentiation program of medullary thymic epithelia (TE) remains obscure.

Recently, p63 has been implicated in TE differentiation by promoting the survival of progenitor/transit amplifying cells (8, 9). Some of this effect may reflect alteration of FGFR2IIib signaling, since FGFR2IIib is a downstream target of p63 (8), FGFR2IIib signaling can affect levels of p63 expression (10), and the thymic phenotypes of FGFR2-deficient and p63-deficient thymi are quite similar (8, 9, 11, 12).

p63 has long been considered as a marker of basal progenitor/transit amplifying cells in stratified or pseudostratified epithelia of epidermis, prostate, trachea, and esophagus (9, 13–17). In the epidermis, down-regulation of p63 expression in suprabasal cells is associated with cessation of cell division and initiation of terminal differentiation, with additional differentiation occurring within this postmitotic population (9, 13).

A third view of MTEC differentiation has emerged from the patterns of Aire expression among MTEC populations defined by cell surface phenotype, where Aire is preferentially expressed by MTEC bearing a CD80+MHCIIhigh phenotype. This phenotype is considered to reflect terminally differentiated MTEC based on a similar CD80/MHCII phenotype displayed by differentiated dendritic cells (18) and the observation that Aire+MTEC are postmitotic and display a high degree of turnover (19). Transfection studies with TE cell lines suggested that Aire expression compromises cell survival and enhances apoptosis (19). Pleomorphic alterations of the medullary thymic compartment as a consequence of Aire deficiency has also led to the suggestion that Aire may play a broader role in MTEC differentiation (20).

In an attempt to integrate these different views of TE differentiation and the possible role of Aire in this process, we have related Aire and

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1 Department of Biological Structure and 2 Department of Immunology and 3 Institute for Stem Cell and Regenerative Medicine, University of Washington, Seattle, WA 98195.

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3 Address correspondence and reprint requests to Dr. Andrew Farr, Department of Biological Structure; Box 357420, School of Medicine, University of Washington, Seattle, WA 98195-7420. E-mail address: farr@u.washington.edu

4 Abbreviations used in this paper: TEC, thymic epithelial cell; CTEC, cortical TEC; MTEC, medullary TEC; TE, thymic epithelial; PE, prostate epithelium.

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p63 expression with the expression of keratins considered to define stages of TE differentiation in Aire+/+ and Aire−/− thymi. The data presented here raise a number of questions regarding some of the current views regarding the process of TE differentiation in general and MTEC differentiation in particular. First, extensive coexpression of K8 and K14 by MTEC suggest that the current binary model of K8/K5/K14+ CTEC and K8/K5/K14− MTEC differentiation should be revised. We also demonstrate that MTEC in the Aire−/− thymus display altered morphology, altered patterns of p63 and keratin expression, and increased frequencies of MTEC that expressed truncated non-functional Aire protein and that may define MTEC that would be eliminated in the Aire+/+ thymus. Although some of these alterations are consistent with the interpretation that Aire is involved in the elimination of end-stage, terminally differentiated MTEC, other alterations of the Aire−/− medullary compartment can be interpreted to indicate that Aire exerts proapoptotic effects at a more proximal stage of MTEC differentiation and eliminates postmitotic MTEC that have not completed their terminal differentiation program.

Materials and Methods

Mice

C57BL/6 mice were obtained from Charles River Laboratories. One strain of Aire-deficient mice (21) was obtained from the Jackson Laboratory and the other (22) was obtained from Dr. L. Peltonen (Department of Molecular Medicine, National Public Health Institute, Helsinki, Finland). 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.

Antibodies

Primary Abs for immunohistochemistry included anti-EpCAM (G8.8; Ref. 23), anti-podoplanin (8.1.1; Ref. 24), an Ab that detects a 110-kDa protein expressed by medullary TC (10.1.1; Ref. 25), and a rat IgM Ab that reacted with K14 (3G10; Ref. 26). G8.8 and 8.1.1 are available from the Developmental Studies Hybridoma Bank (http://dshb.biology.uiowa.edu/). Two mAbs preferentially reacting with cortical thymic epithelium were used; anti-DEC205 (NLDC145; Ref. 27) and CDR1 (Ref. 28). Polyclonal anti-Aire Abs (D-17; SC-17986 and M-300; SC-33189) and a polyclonal anti-p63 (H-137, SC-8343) were from Santa Cruz Biotechnology. Polyclonal anti-Aire Abs (D-17; SC-17986 and M-300; SC-33189) and a polyclonal anti-p63 (H-137, SC-8343) were from Santa Cruz Biotechnology. Polyclonal rabbit anti-K8 and K14 Abs were purchased from Covance; monoclonal anti-K8 (Troma-1) (29) was obtained from the Developmental Studies Hybridoma Bank (http://dshb.biology.uiowa.edu/), and anti-claudin Abs were purchased from Zymed Laboratories. 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 reagent Abs 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 cacodylate buffer (pH7.4) containing 4% paraformaldehyde 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 before PBS to cryoprotection (30% sucrose in PBS) and embedded in OCT (Sakura Finetech) for cryosectioning. Ag retrieval was accomplished by incubating paraformaldehyde-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. To detect Ags sensitive to aldehyde fixation, freshly isolated thymic tissue was embedded in OCT, and sections mounted on glass slides were fixed in cold (~20°C) acetone for 20 min. mAbs were used as hybridoma supernatants, while polyclonal Abs were used at dilutions of 1/500–2000. Best results with the anti-Aire and anti-p63 Abs were obtained with aldehyde fixation before tissue sectioning and the Ag retrieval protocol described above.

Sections were incubated in PBS containing 10% v/v of normal 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 serum). After incubation with primary Abs overnight, sections were washed repeatedly with PBS and then incubated with appropriate secondary Abs. After 1–5 h of incubation, sections were again washed and then coverslips were applied with Fluoromount G (Southern Biotech Associates). Processing of aceton-fixed samples was similar, except that primary Ab incubation times were reduced to 1 h.

Images were captured with a fluorescence microscope equipped with a monochrome digital charge-coupled device camera (Orca-ER; Hamamatsu) and assembled into RGB images with Photoshop (Adobe). Determination of tissue areas examined and quantitation of labeled cells was accomplished with Image J (http://rsb.info.nih.gov/ij/). The Wilcoxon two-sample test was used to determine statistical significance of the resulting data http://www.fon.hum.uva.nl/Service/Statistics/Wilcoxon_Test.html.

Detection of apoptotic cells

TUNEL assay was performed as previously described (30) using streptavidin–Alexa Fluor 555 for detection of incorporated biotinylated nucleotides. Labeling procedures in the absence of TdT served as a negative control. A fluorescent pan-caspase inhibitor (catalog no. G7461; Promega) was mixed with primary Abs at a final concentration of 2.5 μM using tissue sections of thymus fixed by perfusion with 1% paraformaldehyde.

PCR analyses

Procedures for enzymatic dissociation of thymic tissue, flow cytometric purification of MTEC, retention of RNA from these cells, and generation of cDNA have been described previously (12, 20, 31). Primer sequences are available upon request. Determination of relative quantitation values using three independently generated pools of sorted MTEC cDNA has been described previously (12, 20, 31) and SDs of real-time data were calculated according to the manufacturer’s suggestions (www.appliedbiosystems.com).

Results

Detection of Aire protein in wild-type and Aire-deficient thymi

Recent data indicating that the MTEC expressing Aire are postmitotic and display a high degree of turnover led to the suggestion that Aire expression contributes to the relatively short half-life of Aire−/− MTEC (19). The MTEC eliminated by Aire activity may be an end-stage, terminally differentiated population or may correspond to MTEC that are postmitotic, but have not completed their terminal differentiation program. In the latter case, the absence of functional Aire protein would reveal the full program of MTEC terminal differentiation. We first evaluated the ability of commercially available anti-Aire Abs to detect the expression of truncated Aire protein expressed by two independently generated lines of Aire−/− mice as a means to identify MTEC that would normally be eliminated by Aire activity. One line has a Cre-mediated deletion in exons 1 and 2 (21), while the other carries a disruption of exons 5 and 6 of the Aire gene (22).

As shown in Fig. 1a, two polyclonal anti-Aire Abs reacted with a subset of medullary epithelial cells of Aire−/+ thymi with the speckled and punctate staining patterned described previously with other anti-Aire Abs (32–34). The M300 Ab was raised in rabbits against a peptide fragment (253–552) mapping at the C terminus of mouse Aire, while the D17 Ab was raised in goats against an internal peptide of the human AIRE protein. The staining pattern of D17 and M300 were totally overlapping in the normal thymus, indicating that they were detected epitopes associated with a common structure. As expected, thymic epithelial cells from mutant mice expressing an exon 1 and 2 truncation of Aire failed to react with either the M300 or D17 Abs (Fig. 1b, middle column). The M300 Ab also did not react with thymic tissue from Aire−/− mice bearing a deletion of exons 5 and 6 of the Aire gene and also lacking the carboxyl-terminal Aire epitope. However, the D17 Ab did detect the truncated Aire protein produced by the Peltonen strain of Aire-deficient mice (Fig. 1b, right column). The pattern of D17 staining of MTEC in this strain of Aire−/− mice was sensitive to tissue preparation, whereas the pattern of Aire detection in the Aire−/− thymus was not. When sections of fresh frozen tissue were fixed with cold acetone or paraformaldehyde, staining with D17 usually presented as one or two punctate dots over the nucleus, with some diffuse nuclear and
Aire staining in the Aire reacts with MTEC from mice with an exon 5–6 disruption of the gene. This demonstrates the specificity of these Abs; neither reacts with MTEC from mice with an exon 1–2 deletion of Aire (Aire−/−; Mab), D17 but not M300 reacts with MTEC from mice with an exon 5–6 disruption of the Aire gene (Aire−/−; Peltonen). This lower set of panels also demonstrates that pattern of Aire staining in the Aire−/−; Peltonen mice is sensitive to tissue processing. Sections counterstained with Abs to K8. The diffuse nuclear localization of the truncated Aire protein observed in the Peltonen Aire−/− mice presumably reflects the retention of amino-terminal nuclear localization signals and the absence of Aire domains that interact with DNA.

**Patterns of Aire expression by MTEC in Aire+/+ and Aire−/− thy mi**

We used the D17 Ab in conjunction with other anti-stromal cell Abs to better characterize the phenotype of MTEC that express Aire protein in Aire+/+ and Aire−/− thymi. As shown in Fig. 2, a–c, Aire expression was preferentially associated with K14+ K8+ MTEC in the Aire+/+ thymus, with rare K8−K14− MTEC also expressing Aire. As described previously (12, 20) and shown here, K14+ K8+ cells comprises a prominent MTEC subset in the Aire+/+ thymus and display a range of K14/K8 expression ratios, making it difficult to determine the stage of MTEC differentiation, the mixture of Aire+ and Aire− MTEC in the Aire−/− medulla may represent progeny of Aire+ and Aire− precursors or may indicate that surviving progeny of Aire+ precursors acquire an Aire− phenotype as they undergo additional differentiation.

A similar situation was seen in neonatal thymic tissue, where Aire expression was preferentially associated with K8+ K14+ MTEC and a few K8− K14−/−/− cells (cf Fig. 2, g–i with j–l). The accumulation of Aire+ cells in the Aire−/− neonatal thymus was less pronounced compared with thymi of older mice, suggesting that this phenotype is acquired over time, consistent with the notion that the accumulation of these cells is due to altered developmental kinetics rather than direct Aire activity.

The relative density of D17+ cells in Aire+/+ and Aire−/− thymus tissue shown in Fig. 2, indicated a higher density of Aire+ cells in the Aire−/− thymus. Enumeration of D17+ cells in three sets of Aire−/− and Aire+/+ thymi confirmed this and demonstrated that this difference was statistically significant (Fig. 3).

**Aire deficiency alters p63 expression in the medullary epithelial compartment**

It has recently been reported that p63 plays an important role in maintaining progenitor epithelial cells in the thymus (9), perhaps in part through the regulation of FGFR2IIIb and Jagged expression by thymic epithelium (8). Because of the general alterations of the Aire-deficient medullary compartment described here and previously (20), we evaluated the impact of Aire deficiency on p63 expression by TE, reasoning that Aire could affect other pathways...
Expression of selected p63 targets that are positively regulated by the coincidence of Aire and K14 expression by MTEC was evident in the Aire-deficient thymus. Multiple thymus sections from three individual mice were used to quantitate the number of Aire+ cells within the medullary compartment defined by K8 and K14 expression.

Reducing MTEC differentiation. As shown in Fig. 4, and in agreement with previous reports (35–38), p63 expression was widespread among epithelial cells in cortical and medullary compartments, with a higher density of p63+ cells in the medulla (Fig. 4a). Within the medullary compartment, there was little overlap between p63 expression and either Aire or high levels of K8 (Fig. 4, b and c). In contrast, there was good concordance between K14 and p63 expression and between K14 and Aire expression (Fig. 4, g and h), indicating that p63 and Aire are expressed by different subsets of MTEC or by a common population of MTEC at different stages of their differentiation. In the Aire−/− thymus, the density of medullary p63+ cells was noticeably reduced, again with few cells expressing both Aire and p63, similar to the Aire+/? thymus (Fig. 4b). The enhanced expression of truncated Aire protein by K8+ MTEC described above is also evident in Fig. 4, e and i. A lower coincidence of Aire and K14 expression by MTEC was evident in the Aire−/− thymus, while the coincidence of K14 and p63 expression was maintained (Fig. 4, g and j).

Morphometric analyses of the frequency of p63+ MTEC in thymus tissue confirmed visual impressions, where the medullary density of these cells was reduced by ~50% in the Aire−/− thymus, with no discernable differences in cortical expression of p63 (Fig. 5a). The reduced expression of p63 by Aire−/− MTEC was also evident in sorted MTEC populations as measured by quantitative PCR. This reduction primarily involved the expression of the truncated ΔNp63 isoforms; reduced expression of the full-length trans-activating isoforms of p63 did not appear to be significantly affected. Furthermore, there were comparable reductions in expression of the α, β, and γ ΔN isoforms (Table I).

Reduced expression of p63 target genes in the Aire-deficient thymus
The reduction of p63 expression in the Aire-deficient thymus demonstrated by Immunohistochemistry and PCR analysis was indirectly confirmed by the demonstration of corresponding reduced expression of several p63 targets as judged by quantitative PCR. These included Perp (39), K14 (15, 40, 41), Edar, FGFR2IIb, Jagged 1, and Jagged 2 (8, 10, 16) (Fig. 5b and data not shown). Expression of selected p63 targets that are positively regulated by trans-activating p63 isoforms, such as αα kinase α (42) or p21 (43) was minimally altered in the Aire−/− MTEC (data not shown).

Patterns of MTEC apoptosis in Aire−/+ and Aire−/− thymus
Due to the high levels of apoptosis exhibited by TE recovered by enzymatic digestion (Ref. 19 and Gillard and Farr, unpublished observations), a comparison of TE apoptosis in Aire−/+ and Aire−/− thymi required an in situ approach. We used either a TUNEL assay or a fluorescent caspase inhibitor in combination with immunohistochemistry to characterize patterns of MTEC apoptosis in the Aire−/+ and Aire−/− thymus. As shown in Fig. 6, both approaches yielded similar results, demonstrating that apoptotic cells in the Aire−/+ thymus were predominantly K8+ K14−, while in the Aire−/− thymus, apoptotic MTEC were predominantly K8− K14−. Because the frequency of apoptotic cells in the medulla was fairly low and only a subset of apoptotic cells could be clearly identified as keratin + MTEC, the number of MTEC analyzed was too low to provide quantitative data. However, there was a clear shift in the phenotype of MTEC that displayed evidence of DNA fragmentation or caspase activation.

Discussion
This study raises a number of important issues regarding models of TE differentiation. First, data presented here indicate that Aire deficiency provides new insight into the terminal differentiation program of MTEC that is normally obscured by the apoptotic activity of Aire.
in the wild-type thymus. Although it has been convincingly demonstrated that MTEC expressing Aire represent a postmitotic population that turn over rapidly (19), the differentiation status of these cells is not known. This is an important issue because cessation of proliferation, widely considered to represent the initiation of the terminal differentiation program, can be followed by extensive differentiation by postmitotic cells. For instance, progeny of the mitotically active epithelial cells in the basal layer of epidermis become postmitotic as they enter the suprabasal spinous layer and then undergo substantial subsequent differentiation as they progress through the granular layer to form the cornified layer, which represents completion of their terminal differentiation program (44) (shown diagrammatically in Fig. 7a). Thus, the MTEC eliminated by Aire may be postmitotic end-stage cells that have completed their program of terminal differentiation or may be postmitotic cells that are eliminated before this program is completed. These two scenarios make very different predictions regarding the impact of Aire deficiency on the composition of the medullary compartment. If the MTEC eliminated by Aire represent an end-stage, terminally differentiated population, loss of Aire activity in Aire<sup>−/−</sup> mice should lead to accumulation of MTEC that are indistinguishable from the end-stage Aire<sup>+</sup> MTEC in Aire<sup>−/−</sup> mice. This scenario also predicts that apoptotic MTEC in Aire<sup>−/−</sup> and Aire<sup>−/−</sup> thymi would be indistinguishable because they would represent cells eliminated at the same end stage of terminal differentiation (shown diagrammatically in Fig. 7b). Alternatively, if Aire eliminates MTEC that are postmitotic but have not completed their program of terminal differentiation, Aire<sup>−/−</sup> MTEC could complete this program, leading to the accumulation of terminally differentiated MTEC. In this case, subsets of MTEC that may normally represent minor populations of terminally differentiated MTEC in the Aire<sup>+/+</sup> thymus would be expanded in the Aire<sup>−/−</sup> thymus (shown diagrammatically in Fig. 7c).

Some of the alteration of the Aire<sup>−/−</sup> medullary compartment described here would be consistent with either interpretation; reduction of p63<sup>+</sup> MTEC and an expansion of MTEC-expressing

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**Table I.** P63 expression by isolated CD45<sup>−</sup>, EpCam<sup>+</sup> MTEC<sup>a</sup>

<table>
<thead>
<tr>
<th>Gene (pan-isoform)</th>
<th>Aire&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Aire&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>p63 (pan-isoform)</td>
<td>1</td>
<td>0.48 ± 0.25</td>
</tr>
<tr>
<td>p63α (TA and AN isoforms)</td>
<td>1</td>
<td>0.24 ± 0.13</td>
</tr>
<tr>
<td>p63β (TA and AN isoforms)</td>
<td>1</td>
<td>0.27 ± 0.12</td>
</tr>
<tr>
<td>ΔN p63 (α, β, and γ isoforms)</td>
<td>1</td>
<td>0.32 ± 0.07</td>
</tr>
<tr>
<td>TA p63 (α, β, and γ isoforms)</td>
<td>1</td>
<td>0.89 ± 0.52</td>
</tr>
</tbody>
</table>

<sup>a</sup>Three independently generated pools of sorted CD45<sup>−</sup>, EpCAM<sup>+</sup> MTEC from Aire<sup>+/+</sup> and Aire<sup>−/−</sup> mice were processed for real-time PCR analysis. Samples were normalized to EpCAM expression. Average relative quantitation values are presented with SD values. TA, Transactivating.
Admittedly, this interpretation of MTEC differentiation runs counter to the prevailing view. However, several lines of evidence indicate that alternative models of TE differentiation based on the differentiation of other epithelial tissues should be explored. One concerns the role of p63. The impact of p63 deficiency on thymic epithelium indicates that p63 contributes to the maintenance and/or behavior of TE at proximal stages of their differentiation (8, 9). Although there are indications that p63 expression identifies progenitor epithelial cells (15), the extensive expression of p63 by thymic epithelial cells (Refs. 35–38 and this report) indicates that p63 expression in the thymus is not restricted to this relatively rare population (4) and includes transit amplifying cells as well. There is debate regarding the role of p63 in epithelial differentiation, where it has been implicated in differentiation, cell fate specification, proliferation, survival, senescence, apoptosis, cell-cell, and cell-matrix interaction programs (reviewed in Refs. 13, 16, and 45). In epidermal epithelium, p63 is expressed prominently by basal, mitotically active K14+ progenitor cells and is down-regulated in more differentiated, postmitotic keratinocytes that express lower levels of K14 and express keratins 1 and 10 instead (14, 46).

FIGURE 7. Schematic representation of stages of epithelial differentiation and how Aire-mediated MTEC apoptosis could affect the pattern of MTEC differentiation. Different stages of epithelial differentiation are indicated by shapes. a. The situation in the epidermis where proliferation is associated with immature progenitor and transit amplifying cells and postmitotic keratinocytes undergo a significant pattern of differentiation. b. The situation where Aire expression is associated with end-stage, terminally differentiated MTEC and c. the scenario where Aire expression is associated with MTEC at an intermediate stage of differentiation at the initiation of the terminal differentiation program. P, Progenitor; TA, transit amplifying.

truncated, nonfunctional Aire protein would be a sequelae of enhanced survival of postmitotic MTEC regardless of their stage of differentiation. However, other features of the Aire+MTEC are difficult to reconcile with Aire expression and apoptotic elimination targeting end-stage terminally differentiated MTEC and would be consistent with an Aire-dependent interruption of a terminal differentiation process. One is the shift in Aire expression from K14+/low K8+/low cells in the Aire+/+ thymus toward K14−/low K8+ MTEC in the Aire−/− thymus. As will be discussed below, similar shifts in patterns of keratin expression reflect progressive differentiation in other epithelial tissues. This phenotypic change is accompanied by a shift in the morphology of the Aire+ MTEC from a more reticular pattern in the Aire+/+ medulla to more globular cells in the Aire−/− thymus. Furthermore, the predominant K14+ K8+ phenotype of apoptotic MTEC in the Aire+/+ thymus is contrasted by the elevated representation of K14−/low apoptotic MTEC in the Aire−/− thymus, indicating that Aire+/+ and Aire−/− MTEC are eliminated at different stages of differentiation.

A working hypothesis based on these data is that end-stage, terminally differentiated MTEC acquire a K8+ K14−/low phenotype and are represented by the small subset of globular K8−K4/K5−/low MTEC seen in the normal thymus (6, 7, 31). The minor representation of these cells in the normal medulla would reflect Aire-mediated elimination of many MTEC at a more proximal stage of terminal differentiation. Aire deficiency abrogating this apoptotic interruption of the MTEC differentiation program would explain the accumulation of putative terminally differentiated globular K8+K14−/low terminally differentiated MTEC in the Aire−/− thymus (20).
mitotic potential, p63 expression, and down-regulation of K5 and K14 expression, while levels of K8 expression would be maintained or elevated. As a functional K14 enhancer is a direct positively regulated target of p63 (55), coordinate reduction of p63 and K14 expression is not surprising.

Based on the postmitotic status of Aire+ cells shown previously (19) and the p63/keratin phenotype of Aire− MTEC described here, it is suggested that initiation of MTEC terminal differentiation, down-regulation of p63, and cessation of mitotic activity occurs in a subset of K14+/K8+ MTEC. Due to extensive elimination of MTEC at this stage of differentiation, few MTEC complete the terminal differentiation program, accounting for the low frequency of p63+/K14+K8− MTEC in the normal medullary compartment. However, in the absence of Aire-mediated apoptosis, the p63+K14−K8− MTEC can complete down-regulation of K14 expression and acquire a K8−K14− phenotype and globular morphology before they also undergo apoptosis upon completion of their differentiation program. This model proposes a symmetry of CTEC and MTEC differentiation programs in that they would both originate from a K14+K5+ progenitor population and because terminal differentiation would lead to cells expressing high levels of K8 relative to K5/K14 in either compartment. Differences that emerge between CTEC and MTEC, such as the more precipitous down-regulation of K14 expression by CTEC (or persistent K5/K14 expression by MTEC) and other differences in programs of gene expression evidenced by CTEC and MTEC, may reflect the unique set of local signals experienced by TE in these different environments. MTEC likely experience a more complex environment in terms of cellular constituents and the milieu of locally produced microenvironments. MTEC likely experience a more complex environment in terms of cellular constituents and the milieu of locally produced media-

FIGURE 8. Schematic representation of epidermal differentiation occurring in prostatic (a) and thymic (b) epithelium. Patterns of keratin expression during the differentiation of cortical and medullary epithelium bear strong resemblance to the program displayed by PE. The similarities between differentiation programs proposed for cortical and medullary thy-

obseravation that expression of transcription factors associated with progenitor cells, Sox2, Oct4, and Nanog (62) could be detected in pools of sorted MTEC from Aire−/− but not Aire+/− mice (20). Although this may reflect their direct transcriptional control by Aire, these transcription factors may not be detected in sorted Aire−/− MTEC because the representation of immature MTEC that express these transcription factors has been reduced to the point where the levels of these transcription factors are below detection. Similarly, if K14+ MTEC represent a relatively immature population of cells as we suggest here, the reduced expression of K14+ Aire−/− by Aire MTEC (Ref. 20 and this report) would also be consistent with an expansion of mature MTEC at the expense of immature cells.

Finally, the proposal here that Aire expression occurs when MTEC initiate their program of terminal differentiation may be relevant to Aire-dependent regulation of tissue-restricted Ags. If Aire act to de-

References


Disclosures

The authors have no financial conflict of interest.