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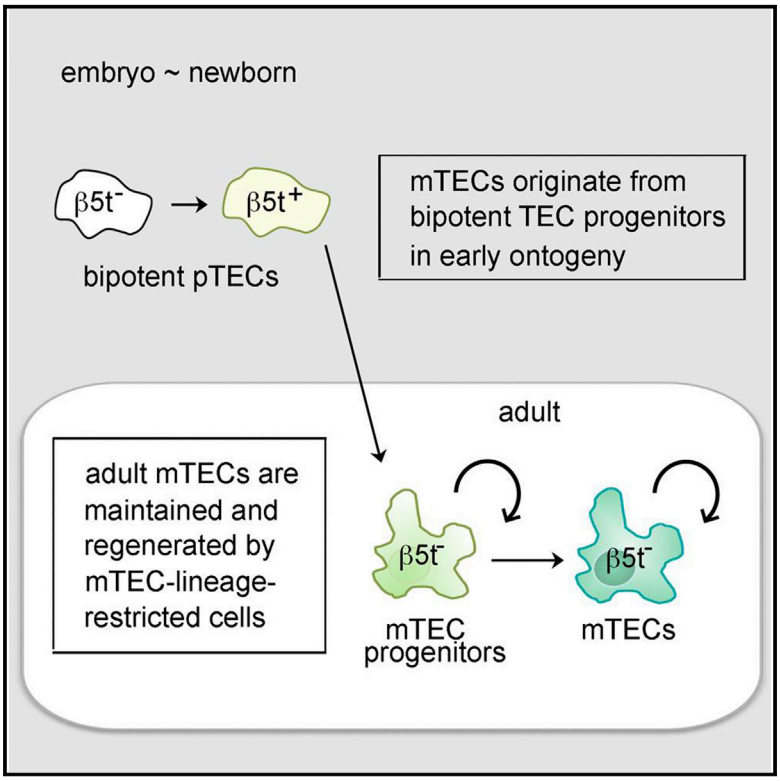
Adult Thymic Medullary Epithelium Is Maintained and Regenerated by Lineage-Restricted Cells Rather Than Bipotent Progenitors

Graphical Abstract



Authors

Izumi Ohigashi, Saulius Zuklys, Mie Sakata, ..., Nagahiro Minato, Georg A. Hollander, Yousuke Takahama

Correspondence

takahama@genome.tokushima-u.ac.jp

In Brief

Medullary thymic epithelial cells (mTECs) are essential for establishing self-tolerance in T cells. Findings by Ohigashi et al. indicate that adult mTECs are maintained and regenerated by mTEC-lineage-restricted cells rather than bipotent TEC progenitors.

Highlights

- Adult thymic medulla is maintained and regenerated by mTEC-lineage-restricted cells
- mTEC-restricted progenitors originate from bipotent TEC progenitors

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Adult Thymic Medullary Epithelium Is Maintained and Regenerated by Lineage-Restricted Cells Rather Than Bipotent Progenitors

Izumi Ohigashi,[1] Saulius Zuklys,[2,3] Mie Sakata,[1] Carlos E. Mayer,[2,3] Yoko Hamazaki,[4] Nagahiro Minato,[4] Georg A. Hollander,[2,3,5] and Yousuke Takahama[1,*]

[1]Division of Experimental Immunology, Institute for Genome Research, University of Tokushima, Tokushima 770-8503, Japan
[2]Paediatric Immunology, Department of Biomedicine, University of Basel, Basel 4058, Switzerland
[3]University Children's Hospital Basel, Basel 4058, Switzerland
[4]Department of Immunology and Cell Biology, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan
[5]Developmental Immunology, Department of Paediatrics and Weatherall Institute of Molecular Medicine, University of Oxford, Oxford OX3 8LL, UK
*Correspondence: takahama@genome.tokushima-u.ac.jp
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SUMMARY

Medullary thymic epithelial cells (mTECs) play an essential role in establishing self-tolerance in T cells. mTECs originate from bipotent TEC progenitors that generate both mTECs and cortical TECs (cTECs), although mTEC-restricted progenitors also have been reported. Here, we report in vivo fate-mapping analysis of cells that transcribe β5t, a cTEC trait expressed in bipotent progenitors, during a given period in mice. We show that, in adult mice, most mTECs are derived from progenitors that transcribe β5t during embryogenesis and the neonatal period up to 1 week of age. The contribution of adult β5t$^+$ progenitors was minor even during injury-triggered regeneration. Our results further demonstrate that adult mTEC-restricted progenitors are derived from perinatal β5t$^+$ progenitors. These results indicate that the adult thymic medullary epithelium is maintained and regenerated by mTEC-lineage cells that pass beyond the bipotent stage during early ontogeny.

INTRODUCTION

The medullary region of the thymus provides a specialized microenvironment for the establishment of self-tolerance in T cells (Kyewski and Klein, 2006; Anderson and Takahama, 2012). Following positive selection, newly generated TCRαβ$^+$ thymocytes migrate from the thymic cortex to the thymic medulla via CCR7-mediated attraction by medullary thymic epithelial cells (mTECs) (Ueno et al., 2004; Kwan and Killeen, 2004). In the thymic medulla, mTECs promiscuously express numerous tissue-restricted self-antigens and, in cooperation with dendritic cells, eliminate self-reactive T cells and promote the generation of regulatory T cells (Liston et al., 2003; Gallegos and Bevan, 2004; Koble and Kyewski, 2009; Lei et al., 2011; Cowan et al., 2013; Perry et al., 2014). Thus, mTECs play an essential role in the formation and maintenance of the immune system by establishing self-tolerance in T cells.

The mTECs originate from Foxn1-expressing endodermal epithelial progenitors that are generated in the thymic primordium of the third pharyngeal pouch (Blackburn and Manley, 2004; Rodewald, 2008). Thymic epithelial progenitors contain the potential to give rise to both mTECs and cortical TECs (cTECs) (Rossi et al., 2006; Bleul et al., 2006). It has been shown recently that mTECs are derived from bipotent TEC progenitors that express molecular traits specific for cTECs, including β5t and CD205 (Ohigashi et al., 2013; Baik et al., 2013; Ribeiro et al., 2013; Alves et al., 2014), indicating that bipotent TEC progenitors pass through a stage during which they express cTEC-specific molecules (Figure 1A). On the other hand, it also has been shown that mTECs are derived from mTEC-lineage-restricted progenitors (Rodewald et al., 2001; Hamazaki et al., 2007). Recent studies have found that mTEC-lineage-restricted progenitors/stem cells express the tight junction molecules claudin-3 and claudin-4 as well as the stem cell-associated molecule SSEA-1 (Hamazaki et al., 2007; Sekai et al., 2014). However, it remains unknown whether these bipotent and lineage-restricted progenitors are developmentally linked and, more importantly, how these different progenitors contribute to the maintenance and regeneration of mTECs in the adult thymus. In vitro culture and transplantation experiments have indicated that both bipotent and mTEC-lineage-restricted progenitors can be isolated from the adult mouse thymus (Wong et al., 2014; Ucar et al., 2014; Sekai et al., 2014). However, whether either of those progenitors contributes to maintenance and regeneration of mTECs in the adult thymus has not been addressed.

In this study, we conducted a fate-mapping analysis of thymic epithelial progenitors that transcribe β5t in vivo at a given period in mice. Our results show that the majority of mTECs in adult mice are derived from bipotent progenitors that transcribe β5t early in ontogeny (<1 week of age), whereas adult β5t$^+$ progenitors are very minor contributors to the formation and maintenance of the adult thymic medulla. Interestingly, the contribution

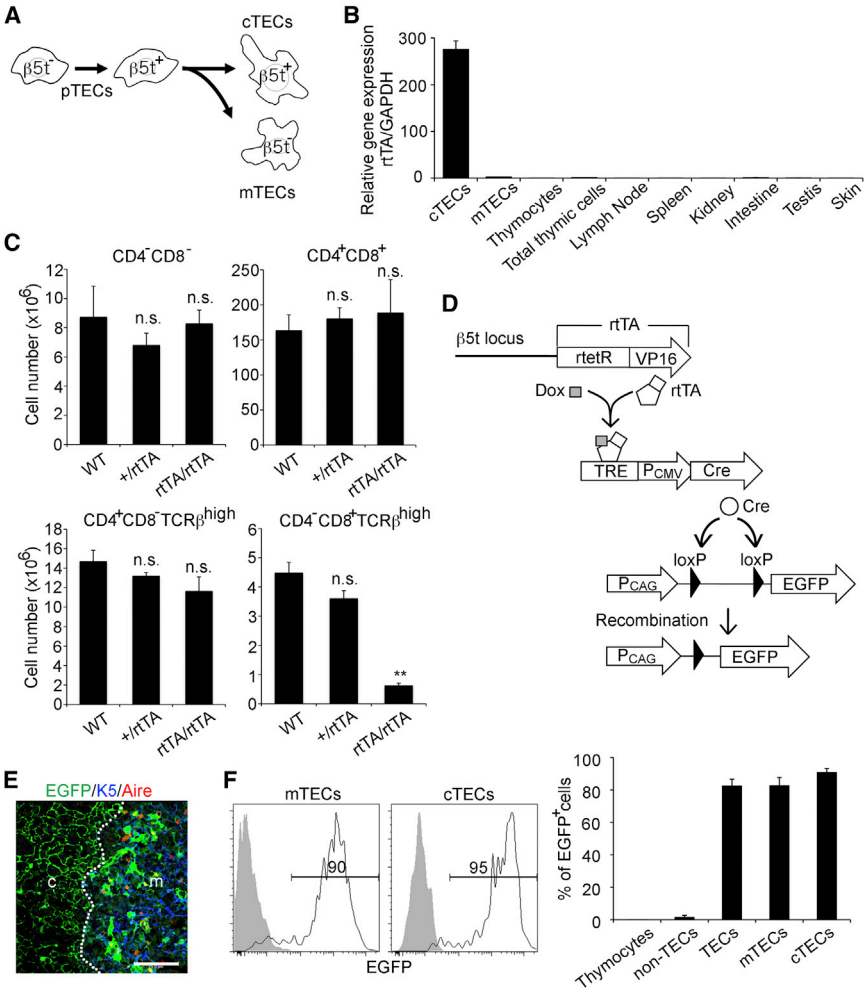

Figure 1. β5t-rtTA Knockin Allows Dox-Induced Labeling of β5t$^+$ TECs

(A) Schematic illustration shows thymic epithelial cell (TEC) development. pTECs, TEC progenitors; mTECs, medullary TECs; cTECs, cortical TECs.

(B) mRNA expression of rtTA in indicated cells and organs isolated from 2- to 3-week-old heterozygous β5t-rtTA knockin mice. Expression levels (means and SEs, n = 3) of rtTA measured by qRT-PCR were normalized to those of GAPDH and compared with the levels measured in total thymic cells.

(C) Numbers (means and SEs, n = 3) of indicated thymocyte subpopulations in wild-type (WT), heterozygous β5t-rtTA knockin (+/rtTA), and homozygous (rtTA/rtTA) mice at 4–6 weeks old are shown. **p < 0.01; n.s., not significant.

(D) Schematic illustration shows doxycycline (Dox)-dependent β5t-specific EGFP expression in β5t-rtTA knockin × tetO-Cre-transgenic × CAG-loxP-stop-loxP-EGFP (β5t-rtTA × Cre × EGFP) mice.

(E and F) β5t-rtTA × Cre × EGFP mice were treated with Dox from embryonic day (E)0 to 4 weeks old. (E) Immunofluorescence analysis of thymus tissues for EGFP (green), keratin 5 (K5) (blue), and Aire (red) is shown. c, cortex; m, medulla. Scale bar, 75 μm. (F) Flow cytometric analysis of EGFP expression in CD45$^-$CD326$^+$UEA1$^+$CD205$^-$ mTECs and CD45$^-$CD326$^+$UEA1$^-$CD205$^+$ cTECs from β5t-rtTA × Cre × EGFP mice (solid lines) or littermate control mice (shaded lines) is shown. Representative results of three independent experiments are shown. Numbers in histograms indicate frequencies of EGFP$^+$ cells within the indicated area. Graph shows frequencies (means and SEs, n = 6) of EGFP$^+$ cells within indicated cells.

See also Figure S1.

of adult β5t$^+$ progenitors remained poor even during injury-triggered thymic regeneration. We further show that claudin-3/4$^+$ SSEA-1$^+$ mTEC-restricted progenitors in adult mice are derived from β5t$^+$ progenitors. These results indicate that the adult thymic medullary epithelium is maintained and regenerated by the cells that pass through the β5t$^+$ progenitor stage early in ontogeny.

RESULTS

Tracing the Fate of Thymic Epithelial Progenitors that Transcribe β5t at a Specific Period

We previously established β5t-Cre knockin/knockout mice that express the recombinase Cre instead of β5t, a proteasome subunit that is expressed abundantly in cTECs and not detected in other cell types including mTECs, and we demonstrated that virtually all mTECs are derived from β5t-expressing progenitor cells (Ohigashi et al., 2013; Figure 1A). In this study, we prepared a new line of β5t-rtTA knockin/knockout mice, in which a reverse tetracycline-controlled transactivator (rtTA)-encoding gene sequence was inserted in the β5t genome sequence and the original β5t-encoding sequence was removed. Those knockin mice indeed expressed rtTA in a cTEC-specific manner (Figure 1B), similar to β5t (Ripen et al., 2011; Ohigashi et al., 2013). Homozygous knockin mice exhibited defective generation of CD4$^-$CD8$^+$TCR^{high} thymocytes (Figure 1C) as a result of β5t deficiency, similar to previously generated β5t-deficient mouse lines (Murata et al., 2007; Ohigashi et al., 2013). Therefore, the β5t-rtTA knockin mice faithfully expressed rtTA instead of β5t. To trace the fates of β5t-expressing cells in functionally undisturbed mice, we used mice heterozygous rather than homozygous for the β5t-rtTA knockin allele in the following experiments described in this paper.

We crossed β5t-rtTA knockin mice with tetO-Cre-transgenic mice and CAG-loxP-stop-loxP-EGFP-transgenic mice to produce β5t-rtTA-knockin × tetO-Cre-transgenic × CAG-loxP-stop-loxP-EGFP-transgenic mice, herein abbreviated as β5t-rtTA × Cre × EGFP mice. In β5t-rtTA × Cre × EGFP mice, β5t gene transcription leads to the expression of rtTA protein, which binds to and activates the tetO operator of the tetO-Cre transgene only in the presence of doxycycline (Dox). Dox-induced tetO-mediated Cre expression would then induce excision of the loxP-flanked stop sequence from the CAG-loxP-stop-loxP-EGFP transgene, resulting in ubiquitous CAG promoter-driven EGFP expression in cells

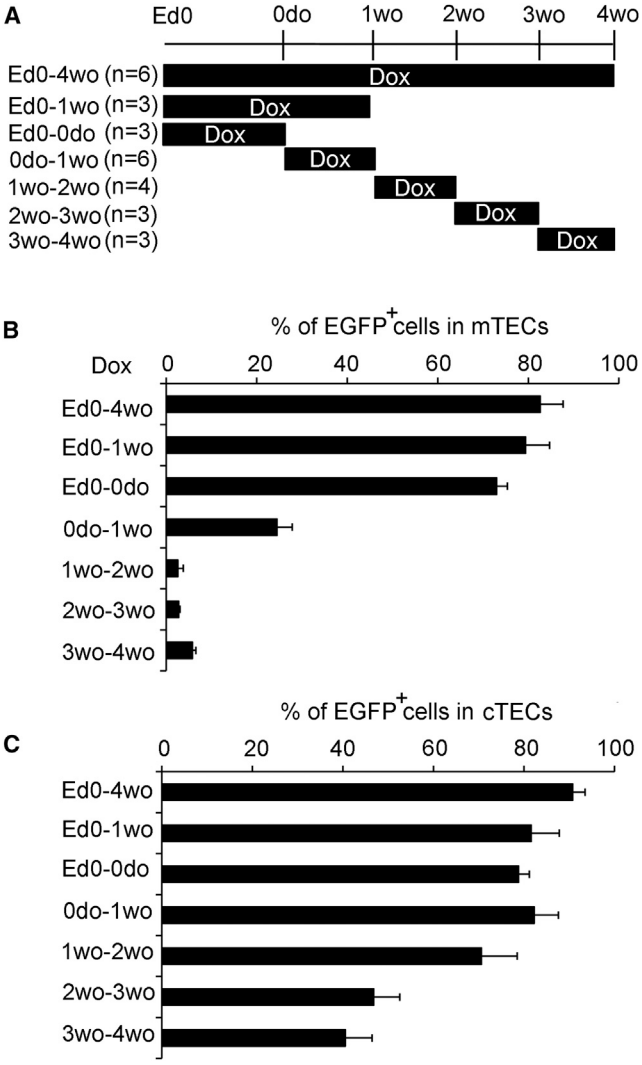

Figure 2. Tracing the Fates of β5t-Expressing TECs at Specific Time Periods

(A) Scheme shows Dox treatment in β5t-rtTA × Cre × EGFP mice (number of mice examined).

(B and C) Frequencies (means and SEs) of EGFP$^+$ cells in CD45$^-$CD326$^+$UEA1$^+$CD205$^-$ mTECs (B) and CD45$^-$CD326$^+$UEA1$^-$CD205$^+$ cTECs (C) were analyzed at 4 weeks old.

See also Figure S2.

(Figure 1D). In other words, in β5t-rtTA × Cre × EGFP mice, EGFP expression should label cells that transcribe β5t during the Dox administration period and the progeny of those cells.

To verify the fidelity of β5t- and Dox-dependent EGFP expression in β5t-rtTA × Cre × EGFP mice, we examined the specificity and efficiency of EGFP expression with or without Dox administration. Upon Dox administration throughout the period from embryonic day (E)0 to 4 weeks old, EGFP expression was detectable throughout thymus sections, in both the keratin 5 (K5)-negative cortical region and K5-positive medullary region, which contained Aire-expressing mTECs (Figure 1E). EGFP signals were neither detected in tissues other than the thymus in Dox-treated mice nor in any tissues, including the thymus, in the absence of Dox administration (data not shown). Flow cytometric analysis of collagenase-digested thymic cells showed that EGFP$^+$ cells were confined to the TEC compartment (CD326/EpCAM$^+$CD45$^-$) and were not detectable among other thymic cells, including thymocytes (Figure 1F; Figure S1). In addition to the majority of cTECs (UEA1$^-$CD205$^+$ TECs) that expressed β5t, the majority of mTECs (UEA1$^+$CD205$^-$ TECs) that did not express β5t were also labeled (Figure 1F), in agreement with the notion that mTECs are derived from β5t$^+$ progenitors (Ohigashi et al., 2013; Figure 1A). EGFP expression was detectable in 83% ± 5% (n = 6) of mTECs and 91% ± 3% (n = 6) of cTECs (Figure 1F). These results indicate that β5t-rtTA × Cre × EGFP mice specifically and efficiently allow in vivo EGFP labeling of cells that express β5t during the Dox administration period.

Nearly all cTECs continuously express β5t (Ripen et al., 2011) and nearly all mTECs are derived from β5t-expressing progenitors (Ohigashi et al., 2013). Thus, the EGFP-labeling rates of ~80%–90% in both mTECs and cTECs likely reflected the maximal labeling efficiency in Dox-treated β5t-rtTA × Cre × EGFP reporter mice rather than the presence of β5t-negative cTECs or mTECs that were not derived from β5t-expressing progenitors.

Most mTECs Are Derived from Embryonic and Neonatal β5t$^+$ Progenitors

The mTECs, most of which do not express β5t, are derived from β5t$^+$ progenitors (Ohigashi et al., 2013), so that the EGFP expression detected in the majority of mTECs in β5t-rtTA × Cre × EGFP mice (Figure 1F) reflected previous β5t expression in progenitors that later differentiated into β5t$^-$ mTECs (Figure 1A). Consequently, via periodic Dox administration to β5t-rtTA × Cre × EGFP mice, we examined the dynamics of mTEC generation from β5t$^+$ progenitors during postnatal ontogeny (Figure 2A). As shown in Figure 2B, 83% ± 5% (n = 6) of mTECs were EGFP$^+$ after Dox treatment from E0 to 4 weeks old. The frequency of EGFP$^+$ cells among mTECs was 79% ± 5% (n = 3) by Dox treatment from E0 to 1 week old, 73% ± 2% (n = 3) with Dox treatment only during the embryonic period, and 24% ± 3% (n = 6) by Dox treatment from 0 days old to 1 week old (Figure 2B). On the other hand, the frequency of EGFP$^+$ mTECs was 2% ± 1% (n = 4) by Dox treatment from 1 week old to 2 weeks old, 3% ± 0.3% (n = 3) by Dox treatment from 2 weeks old to 3 weeks old, and 6% ± 1% (n = 3) by Dox treatment from 3 weeks old to 4 weeks old (Figure 2B). Therefore, the majority (~95%; i.e., ~79% among ~83%) of mTECs in 4-week-old mice were derived from cells that transcribed β5t by the first week of age, whereas the contribution of β5t-expressing cells after 1 week old was minor (~5%). These results indicate that most mTECs are derived from embryonic and neonatal β5t$^+$ progenitors. EGFP-labeling rates during embryogenesis and during the first week after birth were 70%–80% and 20%–30%, respectively, whereas labeling after 1 week old was minor (~5%), indicating that the contribution of β5t$^+$ progenitors to mTECs decreases rapidly during postnatal ontogeny.

Unlike mTECs, most cTECs express β5t (Ripen et al., 2011). Accordingly, the labeling efficiency with 1-week Dox

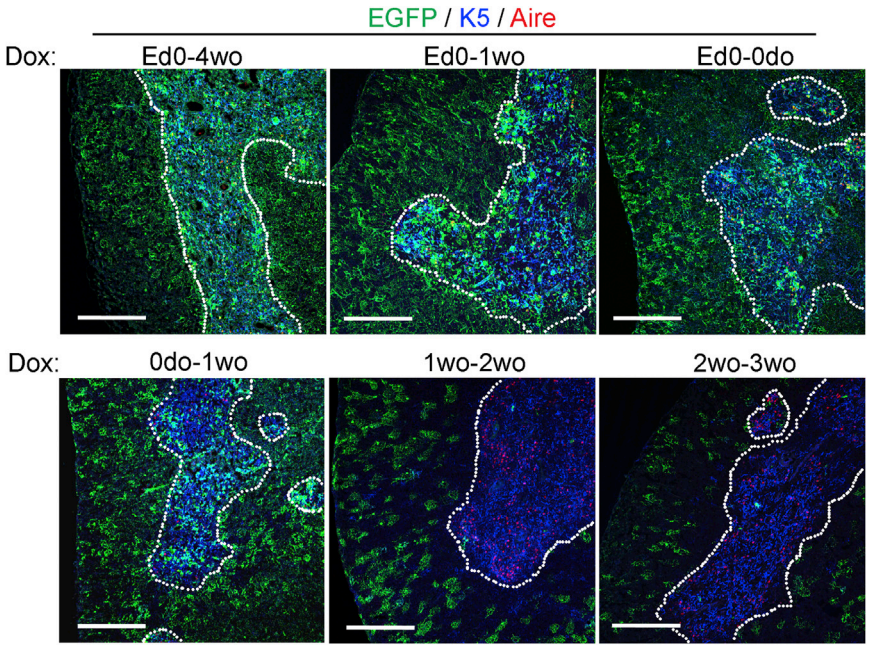

Figure 3. Immunofluorescence Analysis of Thymus Tissues

β5t-rtTA × Cre × EGFP mice were treated with Dox during the indicated periods, and thymus sections were examined at 4 weeks old for the expression of EGFP (green), K5 (blue), and Aire (red). Data are representative of at least three separate experiments using three mice each. Scale bar, 300 μm.

administration remained high (>80%) until 1 week old (Figure 2C). Labeling with 1-week Dox administration decreased to ~40%–50% after 2 weeks old (Figure 2C), which may have been due to reduced labeling efficiency resulting from reduced β5t transcription during postnatal ontogeny (Figure S2).

Immunofluorescence analysis of thymic sections showed that K5-positive mTECs in the thymic medullary region were effectively EGFP labeled following embryonic Dox treatment, and EGFP labeling in both the medulla and cortex was easily detectable with Dox treatment from 0 days to 1 week old (Figure 3). However, EGFP labeling in K5-positive mTECs was barely detectable with Dox treatment after 1 week old, even though EGFP labeling of cTECs remained detectable in the K5-negative cortical region (Figure 3). These results reinforced the flow cytometric results to demonstrate that most mTECs are derived from embryonic and neonatal β5t$^+$ progenitors, whereas the contribution of β5t$^+$ progenitors to mTECs was minor after 1 week old.

mTECs Derived from Embryonic and Neonatal β5t$^+$ Progenitors Maintain Adult Thymic Medullary Epithelium

The results indicating that the majority of mTECs are derived from embryonic and neonatal β5t$^+$ progenitors prompted us to examine how these mTECs contribute to maintenance of the adult thymic medulla. Accordingly, we traced EGFP$^+$ mTECs in mice up to 45 weeks old that were treated with Dox either during embryogenesis or during the first week after birth. In embryonic Dox mice, the frequency of EGFP$^+$ cells among mTECs was ~85% at birth (Figure 4A), a labeling efficiency similar to that achieved with continuous Dox treatment up to 4 weeks old (Figure 2B). During postnatal thymic medullary growth until 4–6 weeks old, mTEC cellularity increased from ~2 × 10^3 per mouse at 0 days old to ~4 × 10^4 per mouse at 4 weeks old (Figure 4B), suggesting that mTECs increased at an average rate of ~10% per day (Figure 4B). During this growth period, the cellularity of embryonically EGFP-labeled mTECs increased from ~1.5 × 10^3 per mouse at 0 days old to ~3.4 × 10^4 per mouse at 6 weeks old (the rate of increase was ~8% per day), whereas the cellularity of neonatally EGFP-labeled mTECs increased from ~1.0 × 10^2 per mouse at 1 week old to ~1.5 × 10^4 per mouse at 7 weeks old (the rate of increase was ~13% per day), suggesting that both embryonically and neonatally EGFP-labeled mTECs vigorously increased in number until 4–6 weeks old (Figure 4B). Consequently, the frequency of neonatally labeled EGFP$^+$ cells increased to ~30% among mTECs by 4–6 weeks old, whereas that of embryonically labeled EGFP$^+$ cells among mTECs decreased to ~60% by 4–6 weeks old (Figure 4A). The sum frequency of embryonically and neonatally labeled cells within mTECs remained unchanged at ~80%–90% (Figure 4A), equivalent to the maximum labeling efficiency of the mice used in this study (Figures 1F and 2B) and in agreement with the notion that, up to 4–6 weeks old, postnatal mTEC growth is primarily mediated by the expansion of cells that had passed through the β5t$^+$ progenitor stage by 1 week old rather than by continuous contributions of β5t$^+$ progenitors even after the first week of life.

After 6 weeks old, mTEC cellularity decreased from ~5 × 10^4 per mouse at 6 weeks old to ~5 × 10^3 per mouse at 45 weeks old (Figure 4B), reflecting age-related involution of the thymus (Hirokawa and Makinodan, 1975; Gray et al., 2006; Dooley and Liston, 2012). Both embryonically and neonatally labeled mTECs decreased with age at similar average rates of ~1% per day (Figure 4B). The frequency of EGFP$^+$ cells among mTECs remained unchanged during age-dependent thymus involution in both embryonic and neonatal Dox mice (Figure 4A). These results indicate that the thymic medullary epithelium is essentially maintained by mTECs derived from embryonic and neonatal β5t$^+$ progenitors throughout post-adolescent adult life up to 45 weeks old, with a minor, if any, contribution of de novo development from adult β5t$^+$ progenitors.

Among cTECs, the frequency of cells labeled either during embryogenesis or in the neonatal first week gradually decreased from ~80% in neonates to ~50% in 45-week-old mice (Figure 4C). The cTEC cellularity reached a maximum at 2 weeks old, showed a significant decrease ($p < 0.05$) of 10-fold to 10–12 weeks old, and remained essentially unchanged (no significant difference) up to 45 weeks old (Figure 4D). These results

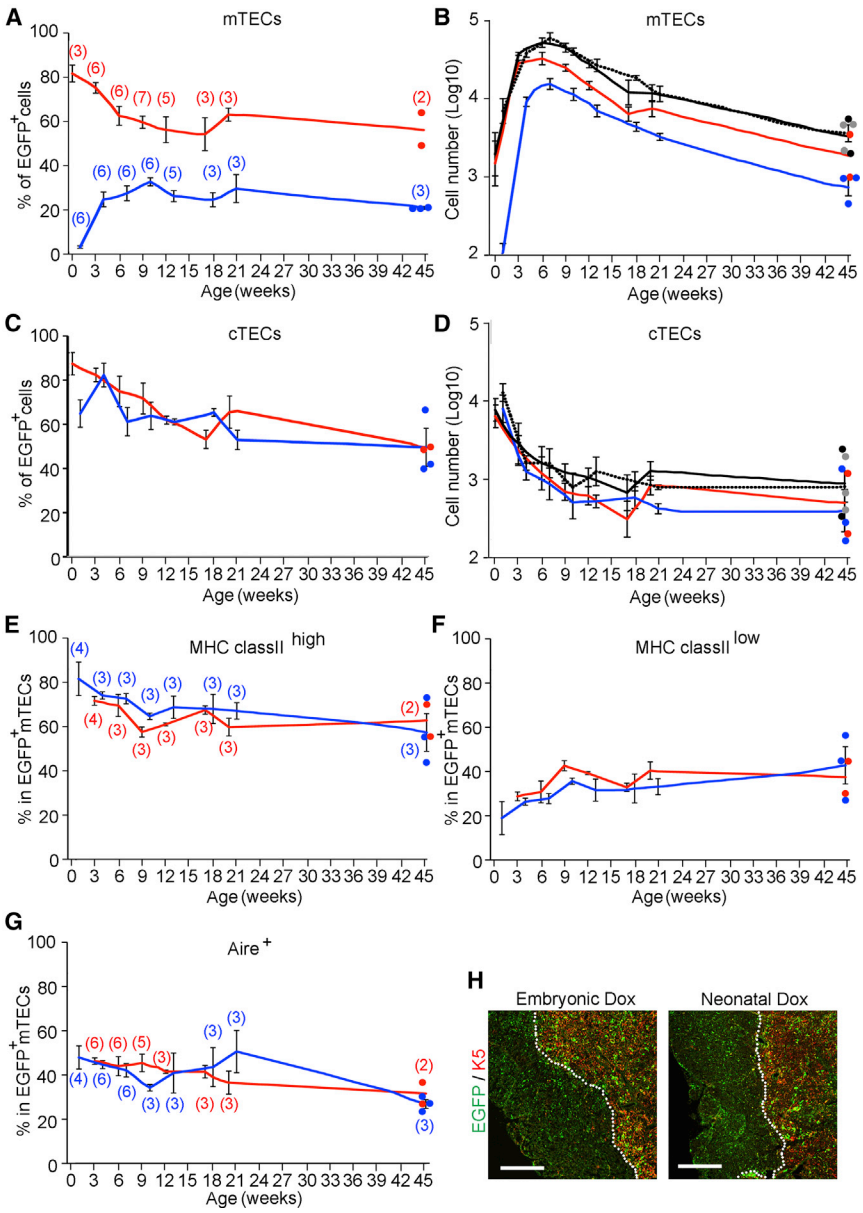

Figure 4. Tracing the Fates of Embryonically and Neonatally Labeled TECs

(A–D) Frequencies (means and SEs, A and C) and absolute numbers (means and SEs, B and D) of EGFP$^+$ cells among mTECs (A and B) and cTECs (C and D) from embryonic Dox mice (red line) and neonatal Dox mice (blue line). Absolute numbers of total mTECs and total cTECs from embryonic Dox mice (black line) and neonatal Dox mice (dashed line) also were plotted. The numbers of measurements for data in (A)–(D) are shown in parentheses in (A).

(E–G) Frequencies (means and SEs) of MHC classII^{high} cells (E), MHC classII^{low} cells (F), and Aire$^+$ cells (G) among EGFP$^+$ mTECs from embryonic Dox mice (red line) and neonatal Dox mice (blue line). The numbers of measurements for data in (E) and (F) are shown in parentheses in (E), and those for data in (G) are shown in parentheses in (G). As two measurements were conducted in embryonic Dox mice at 45 weeks old, the symbols shown for 45-week-old data indicate individual measurements.

(H) Immunofluorescence analysis of thymus sections for EGFP (green) and K5 (red) at 45 weeks old. Representative data from two independent experiments are shown. Scale bar, 300 μm.

See also Figure S3.

suggest that cTECs differ from mTECs in terms of ontogenic dynamics, and, unlike mTECs, at least one-third of cTECs in aged mice (~80%–50%) are not EGFP labeled by embryonic or neonatal Dox administration and, thus, may be generated de novo in adult mice.

Characteristics of mTECs Derived from Embryonic and Neonatal β5t$^+$ Progenitors

The mTECs include several distinct subpopulations that are best characterized by the expression of MHC class II and Aire (Gray et al., 2006; Villaseñor et al., 2008). The mTECs that express high amounts of MHC class II include Aire-expressing cells, which promiscuously express tissue-restricted self-antigens, whereas mTECs that express low amounts of MHC class II include CCL21-expressing cells, which attract positively selected thymocytes to the medullary region (Lkhagvasuren et al., 2013). We found that, among embryonically and neonatally labeled EGFP$^+$ mTECs, the frequencies of MHC class II^{high} cells and MHC class II^{low} cells modestly decreased and increased, respectively, during ontogeny by 10 weeks old and remained nearly unchanged thereafter during aging (Figures 4E and 4F). The frequency of Aire$^+$ cells among labeled mTECs modestly and gradually decreased during postnatal ontogeny in a pattern similar to that observed for MHC class II^{high} cells among mTECs (Figure 4G). These results indicate that embryonic and neonatal β5t$^+$ progenitors are similarly capable of forming mTEC subpopulations defined by MHC class II and Aire expression.

Immunofluorescence analysis of thymic sections demonstrated the broad distribution of EGFP$^+$ cells in the K5-negative cortical area and K5-positive medullary area in both embryonic and neonatal Dox mice, even at 45 weeks old (Figure 4H). Within the medulla, EGFP$^+$ cells were distributed in both the peripheral and middle regions (Figure 4H), indicating that mTECs derived from embryonic and neonatal β5t$^+$ progenitors localize indistinguishably in the medulla.

It is known that sex hormones affect the cellularity of thymic cells, including TECs (Sutherland et al., 2005; Dumont-Lagacé et al., 2015). Nonetheless, the contribution of embryonic and neonatal β5t$^+$ progenitors to the development, maintenance,

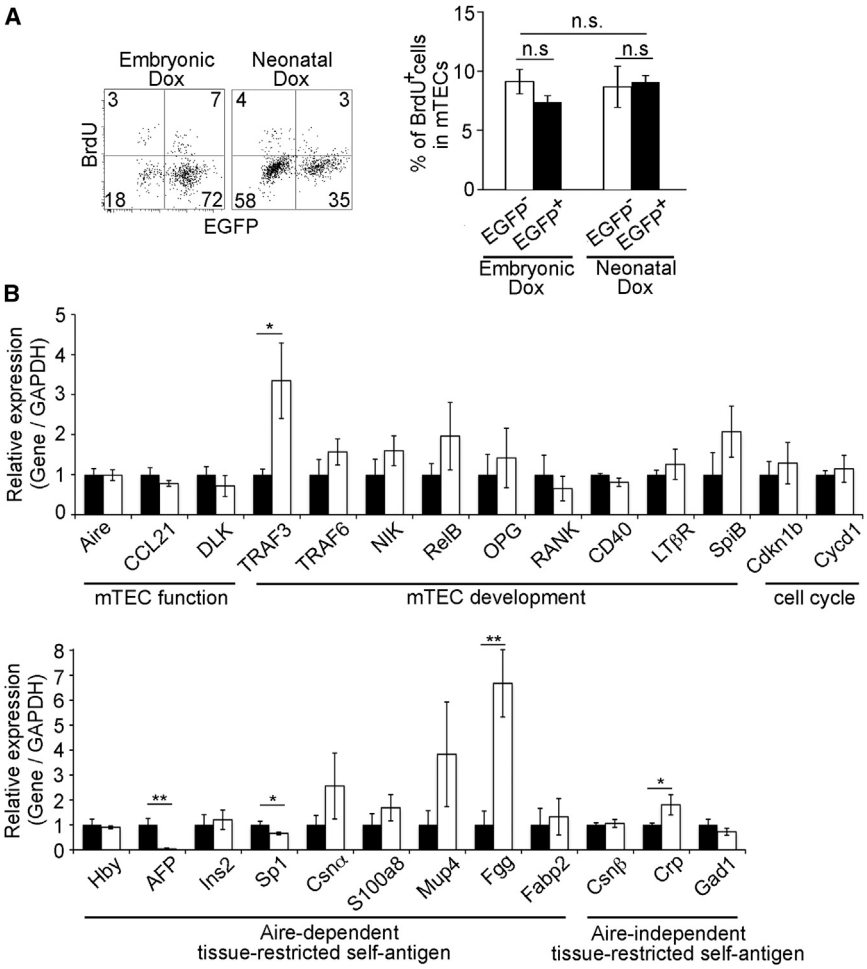

Figure 5. Characterization of Embryonically and Neonatally Labeled mTECs

(A) Flow cytometric analysis of BrdU incorporation in EGFP$^+$ and EGFP$^-$ mTECs in embryonic and neonatal Dox mice at 5 weeks old. Representative profiles are shown with the frequencies of cells within the indicated area (left). Bar graphs show the frequencies (means and SEs, n = 3) of BrdU$^+$ cells among the indicated mTECs (right). n.s., not significant.

(B) Relative mRNA expression of indicated genes among EGFP$^+$ mTECs isolated from embryonic Dox mice (filled bars) and neonatal Dox mice (open bars) at 2 weeks old. Expression levels (means and SEs, n = 3) measured by qRT-PCR were normalized to those of GAPDH and are shown relative to the level of embryonically labeled mTECs. *p < 0.05, **p < 0.01.

and subpopulation composition of mTECs was comparable between female and male mice (Figure S3).

We then compared the proliferation capabilities of embryonically and neonatally EGFP-labeled mTECs in adult mice. Mice were treated with a single intraperitoneal injection of 5-bromo-2′-deoxyuridine (BrdU) at 5 weeks old. One day after treatment, the frequencies of BrdU$^+$ cells were 7% ± 1% (n = 3) in embryonically EGFP-labeled mTECs and 9% ± 1% (n = 3) in neonatally EGFP-labeled mTECs, with no significant difference (Figure 5A). Therefore, in adult mice, mTECs derived from embryonic and neonatal β5t$^+$ progenitors are similarly capable of proliferation.

We further isolated embryonically and neonatally EGFP-labeled mTECs and compared their mRNA expression profiles. As shown in Figure 5B, many genes that were functionally relevant in mTECs were expressed similarly in embryonically and neonatally labeled mTECs. However, TNF receptor-associated factor 3 (TRAF3) was more strongly expressed in embryonically labeled mTECs than in neonatally labeled mTECs (Figure 5B). On the other hand, a fraction of tissue-restricted self-antigens, including α-fetoprotein (AFP) and salivary protein 1 (Sp1), were more strongly expressed in embryonically labeled mTECs than in neonatally labeled mTECs, whereas fibrinogen gamma chain (Fgg) and C-reactive protein (Crp) were more strongly expressed in neonatally labeled mTECs than in embryonically labeled mTECs (Figure 5B). Therefore, mTECs derived from embryonic and neonatal β5t$^+$ progenitors exhibit considerable overlap in gene expression profiles, but are not exactly identical with respect to the spectrum of promiscuously expressed self-antigens, including the fetal antigen AFP.

Injury-Triggered Regeneration of the Adult Thymic Medullary Epithelium Is Mediated by Cells Derived from Embryonic and Neonatal β5t$^+$ Progenitors

We next examined how β5t$^+$ progenitors may contribute to adult thymic medullary regeneration, which can be triggered following either irradiation-induced thymic injury (Huiskamp and van Ewijk, 1985; Huiskamp et al., 1985) or polyinosinic-polycytidylic acid (poly I:C) treatment (Démoulins et al., 2008; Papadopoulou et al., 2011). Following sublethal X-ray irradiation at 5.5 Gy, the numbers of total thymic cells and mTECs decreased to ~30% of those in control mice by 1 week after irradiation and subsequently recovered almost completely by 2 weeks after irradiation (Figure 6A). We traced mTECs that were EGFP labeled during the embryonic and neonatal periods, as well as during the recovery period after irradiation (Figure 6B). The frequencies of embryonically EGFP-labeled cells among mTECs were 67% ± 4% (n = 3) in the regenerated thymus of irradiated mice and 67% ± 3% (n = 3) in age-matched control mice, whereas the frequencies of neonatally EGFP-labeled mTECs were 22% ± 3% (n = 3) in irradiated mice and 21% ± 4% (n = 3) in control mice (Figure 6C). The frequency of EGFP$^+$ mTECs labeled during regeneration was <5% and was comparable to that in control mice (Figure 6C). Immunofluorescence analysis of thymic sections confirmed that EGFP$^+$ cells in the regenerated thymic medulla were readily detectable in embryonically and neonatally Dox-treated mice, but were only sparse in adult Dox-treated mice (Figure S4A). In addition, in adult Dox-treated β5t-rtTA-knockin tetO-Cre-transgenic

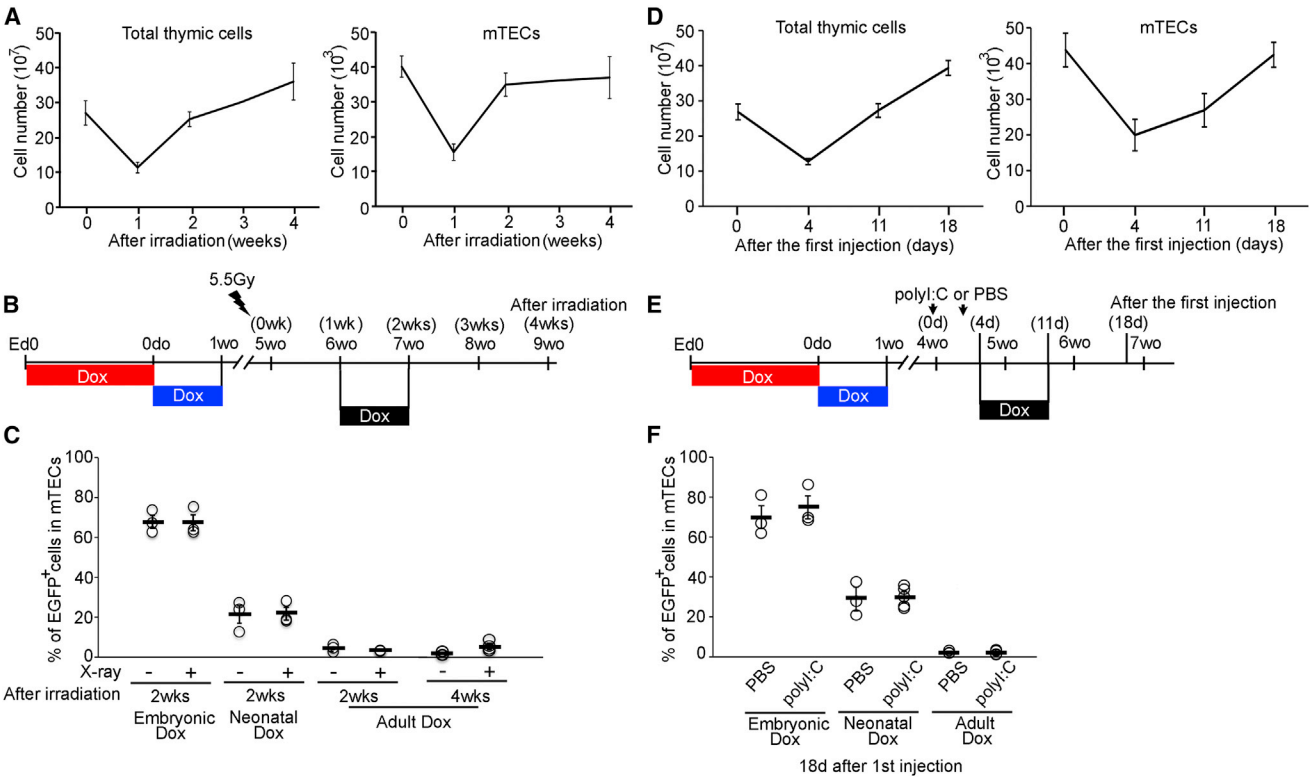

Figure 6. Contribution of β5t⁺ Progenitors to Injury-Triggered mTEC Regeneration

(A) Mice were sublethally irradiated with a 5.5-Gy X-ray dose at 5 weeks old. Graphs show the numbers of total thymus cells and mTECs at the indicated weeks post-irradiation.

(B) Schematic diagram shows X-ray irradiation and Dox treatment. wk, week.

(C) Frequencies (means and SEs) of EGFP⁺ cells among mTECs from control and irradiated mice are plotted. Measurements from individual mice also are plotted (open circles).

(D) Mice were injected at 4 weeks old with 250 μg poly I:C twice at a 3-day interval. Graphs show the numbers of total thymus cells and mTECs at the indicated weeks after poly I:C injection.

(E) Schematic diagram shows poly I:C injection and Dox treatment. d, day.

(F) Frequencies (means and SEs) of EGFP⁺ cells among mTECs from PBS-injected and poly I:C-injected mice are plotted. Data from individual mice also are plotted in open circles.

See also Figure S4.

mice crossed with a different reporter strain that carried the CAG-loxP-stop-loxP-Zoanthus green (ZsGreen) gene knocked into the Rosa26 locus, the frequency of ZsGreen⁺ mTECs remained <5% among the regenerated mTECs 4–8 weeks after 5.5-Gy X-ray irradiation (Figure S4B). Therefore, adult β5t⁺ progenitors do not contribute considerably to the adult mTEC population, even during X-ray-triggered thymic regeneration.

Poly I:C mimics viral double-stranded RNA and directly induces interferon-α-mediated injury in TECs (Papadopoulou et al., 2011; Dooley and Liston, 2012). Two poly I:C administrations at a 3-day interval reduced the numbers of total thymic cells and mTECs to ~30% of those in control mice, with near-complete recovery by 18 days after the first injection (Figure 6D). Similar to the irradiation-triggered regenerated thymus and untreated control thymus, the frequencies of embryonic, neonatal, and adult EGFP-labeled cells among mTECs (Figure 6E) were ~70%, 20%, and 3%, respectively, with or without poly I:C treatment (Figure 6F). Immunofluorescence analysis of thymic sections supported these frequencies of EGFP⁺ cells in the regenerated thymic medulla (Figure S4A). Taken together, these results indicate that adult mTEC regeneration triggered by either X-ray irradiation or poly I:C treatment is mediated by cells derived from embryonic and neonatal β5t⁺ progenitors, without triggering a de novo contribution from adult β5t⁺ progenitors.

We noticed no differences between female and male mice in the contribution of embryonic, neonatal, and adult β5t⁺ progenitors during the regeneration of mTECs triggered by X-ray irradiation or poly I:C treatment (Figures S4C and S4D).

mTEC-Restricted Progenitors Are Derived from β5t⁺ Progenitors

Thus far, the results indicate that mTECs in adult mice are maintained and regenerated by cells that passed beyond the stage of β5t⁺ progenitors during early ontogeny rather than by adult β5t⁺ progenitors. To better characterize the cells that maintain and regenerate adult mTECs, we finally examined whether

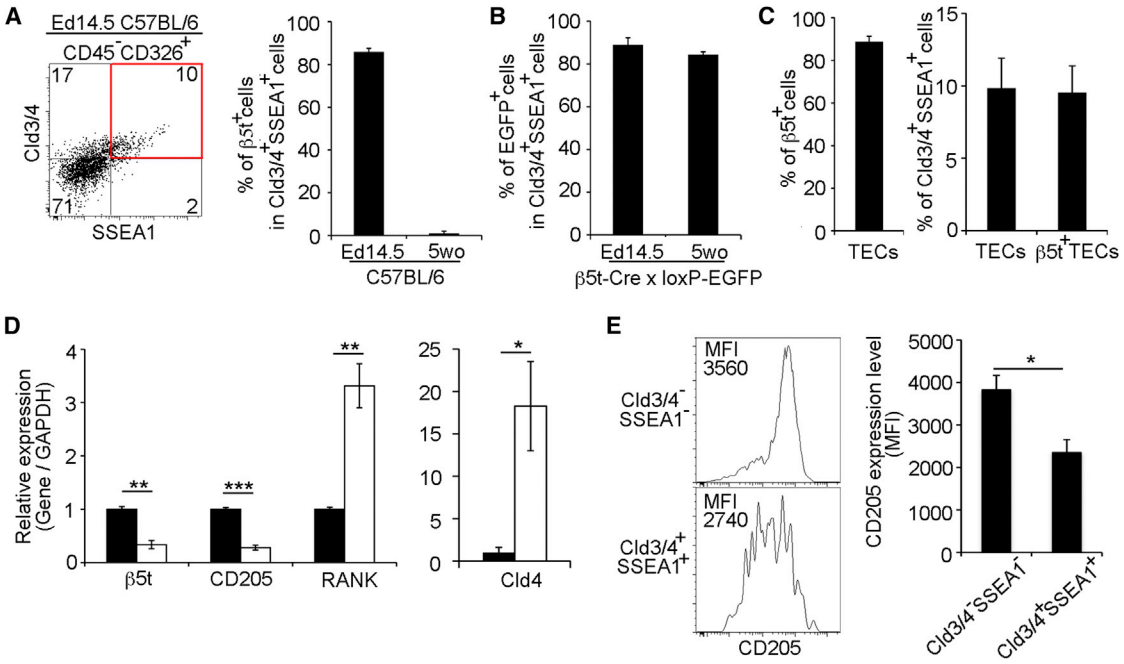

Figure 7. mTEC-Restricted Progenitors Are Derived from β5t⁺ Progenitors

(A) Representative flow cytometric claudin-3/4 (Cld3/4) and SSEA1 expression profiles in CD45⁻CD326⁺ TECs from an E14.5 C57BL/6 fetal thymus (left). Red box indicates Cld3/4⁺ SSEA-1⁺ mTEC-restricted progenitors. Bar graph shows frequencies (means and SEs, n = 3) of β5t⁺ cells among Cld3/4⁺SSEA1⁺ TECs at the indicated ages (right).

(B) Frequencies (means and SEs, n = 3) of EGFP⁺ cells within Cld3/4⁺SSEA1⁺ TECs in β5t-Cre × loxP-EGFP mice are shown.

(C) Frequencies (means and SEs, n = 3) of β5t⁺ cells among TECs (left) and of Cld3/4⁺SSEA1⁺ cells among TECs and β5t⁺ TECs (right) from an E14.5 C57BL/6 fetal thymus are shown.

(D) Relative mRNA expression of indicated genes in Cld3/4⁻SSEA1⁻ TECs (filled bars) and Cld3/4⁺SSEA1⁺ TECs (open bar) isolated from an E14.5 C57BL/6 fetal thymus. Expression levels (means and SEs, n = 3) measured by qRT-PCR were normalized to those of GAPDH and are shown relative to the levels in Cld3/4⁻SSEA1⁻ TECs. *p < 0.05, **p < 0.01, ***p < 0.001.

(E) Representative CD205 expression profiles in Cld3/4⁻SSEA1⁻ and Cld3/4⁺SSEA1⁺ TECs from an E14.5 C57BL/6 fetal thymus (left). Bar graphs show the mean fluorescence intensities of CD205 expression (means and SEs, n = 3) in indicated populations (right).

See also Figures S5 and S6.

mTEC-restricted progenitors/stem cells, which can be defined among EpCAM⁺ CD45⁻ TECs by the co-expression of claudin-3/4 and SSEA-1 (Sekai et al., 2014), were derived from β5t⁺ TEC progenitors. We found that 86% ± 2% (n = 3) and 1% ± 1% (n = 3) of claudin-3/4⁺ SSEA-1⁺ TECs expressed β5t protein at E14.5 and 5 weeks old, respectively (Figure 7A), whereas claudin-3/4⁺ SSEA-1⁺ TECs in β5t-Cre × loxP-EGFP mice (Ohigashi et al., 2013) were ~85% EGFP⁺ both at E14.5 and 5 weeks old (Figure 7B). These results indicate that mTEC-restricted progenitors, which are the most immature cells within the mTEC lineage as far as reported, express β5t protein at E14.5, but not in adult mice, and adult mTEC-restricted progenitors previously transcribed β5t and are thus derived from β5t⁺ progenitors.

We then examined how embryonic and neonatal β5t⁺ progenitors contribute to the generation of adult mTEC-restricted progenitors. In β5t-rtTA × Cre × EGFP mice, the frequencies of EGFP-labeled cells by embryonic and neonatal Dox administrations in claudin-3/4⁺ SSEA-1⁺ TECs were 68% ± 8% (n = 3) and 31% ± 5% (n = 3), respectively (Figure S5A). These frequencies were statistically not significant from, and therefore essentially identical to, those in total mTECs (Figure S5B). These results indicate that, like other mTEC-lineage cells, mTEC-restricted progenitors in adult mice are derived from cells that transcribed β5t during embryogenesis and the newborn period by the first week of age.

Among E14.5 embryonic TECs, 89% ± 3% (n = 3) were β5t⁺ and 10% ± 2% (n = 3) were claudin-3/4⁺ SSEA-1⁺ (Figure 7C). Approximately 85% of claudin-3/4⁺ SSEA-1⁺ TECs were confined to the β5t⁺ population (Figure 7A), whereas 9% ± 2% (n = 3) of β5t⁺ TECs were claudin-3/4⁺ SSEA-1⁺ (Figure 7C). Interestingly, claudin-3/4⁺ SSEA-1⁺ TECs expressed reduced levels of cTEC-trait mRNAs, including β5t and CD205, and increased levels of mTEC-trait mRNAs, including RANK and claudin-3/4, when compared with claudin-3/4⁻ SSEA-1⁻ TECs (Figure 7D). The surface expression of CD205 protein was lower in claudin-3/4⁺ SSEA-1⁺ TECs than in claudin-3/4⁻ SSEA-1⁻ TECs (Figure 7E). These results indicate that β5t⁺ TECs in the E14.5 thymus are heterogeneous and include claudin-3/4⁺ SSEA-1⁺ mTEC-restricted progenitors, which exhibit reduced expression levels of cTEC-trait molecules such as β5t and CD205, and suggest that claudin-3/4⁺ SSEA-1⁺ TECs represent an mTEC lineage-restricted progenitor stage proximal downstream of the bipotent TEC progenitors that express cTEC-trait molecules.

A recent study showed that, in the adult thymus, bipotent TEC progenitors that were capable of giving rise to cTECs and mTECs were enriched in UEA1$^-$ TECs expressing low levels of MHC class II molecules (Wong et al., 2014). We found that in mice that were continuously treated with Dox from E0 to 4 weeks old, ~80% of UEA1$^-$ MHC class II^{low} TECs were EGFP$^+$, although UEA1$^-$ MHC class II^{low} TECs were detectable not only in EGFP$^+$ cells but also in EGFP$^-$ cells (Figures S6A and S6B). On the other hand, in mice that were Dox treated from 4 weeks old to 5 weeks old, only 1%–2% of UEA1$^-$ MHC class II^{low} TECs were EGFP$^+$ (Figures S6C and S6D). These results indicate that the majority of UEA1$^-$ MHC class II^{low} TECs in adult mice do not actively transcribe β5t but originate from β5t$^+$ progenitors.

DISCUSSION

This study has established a new method for tracing cells that express β5t during a given time period in vivo. β5t is expressed in the majority of cTECs but is not detectable in the majority of mTECs or any non-TECs (Ripen et al., 2011). Among TECs, β5t is expressed not only in cTECs but also in bipotent TEC progenitors that are capable of giving rise to both cTECs and mTECs; indeed, almost all mTECs are derived from β5t-expressing progenitors (Ohigashi et al., 2013; Alves et al., 2014). Therefore, the use of β5t-rtTA × Cre × EGFP mice enabled a study of the time period when β5t-negative mTECs pass through the β5t-expressing progenitor stage. Our results indicate that the majority of adult mTECs, including those in aged mice, are derived from embryonic and neonatal β5t$^+$ progenitors rather than adult β5t$^+$ progenitors. Unexpectedly, we found that the contribution of adult β5t$^+$ progenitors remains minor even during thymic medullary regeneration after injury. Our results, therefore, reveal that mTECs in adult mice are maintained and regenerated by cells that have passed through the stage of β5t$^+$ progenitors during the embryonic and neonatal periods rather than via continuous supply from adult β5t$^+$ progenitors.

Our results also indicate that, in adult mice, mTEC-restricted progenitors/stem cells, which are the most immature cells within the mTEC lineage according to reports and are best characterized by the co-expression of claudin-3/4 and SSEA-1 (Sekai et al., 2014), are derived from β5t$^+$ progenitors. Our results further indicate that mTEC-restricted progenitors in embryonic mice still contain β5t and CD205 proteins, albeit at reduced levels, suggesting that mTEC-restricted progenitors represent a stage proximally downstream of the bipotent TEC progenitors that express cTEC-trait molecules. Collectively, these results suggest that mTECs in adult mice are maintained and regenerated by mTEC-lineage-restricted cells, including the recently identified mTEC-restricted progenitors as well as by various conventionally described mTEC subpopulations.

The mTECs comprise a dynamic, rather than dormant, cellular compartment with active proliferation and turnover (Gillard and Farr, 2006; Gray et al., 2006, 2007). BrdU pulse-chase experiments have estimated that mTECs turn over in ~2 weeks (Gray et al., 2006; Gäbler et al., 2007). It is, therefore, possible that mTECs in adult mice are maintained and regenerated by the continuous differentiation of mTEC-restricted progenitors. Alternatively, it is also possible that adult mTECs are replenished by mTEC self-duplication in a manner similar to other epithelial tissues, including the pancreas and the liver, which are maintained in adult mice by self-duplication rather than progenitor cell differentiation (Dor et al., 2004; Yanger et al., 2014).

Bipotent TEC progenitors were reported to be detectable in the adult mouse thymus (Bleul et al., 2006; Jin et al., 2014; Wong et al., 2014; Ucar et al., 2014). However, it was unknown whether and how those progenitors contributed to the maintenance and regeneration of the adult thymus. The developmental potential of those progenitors has been demonstrated in transplantation and in vitro culture experiments (Wong et al., 2014; Ucar et al., 2014) and in mice in that the thymus is congenitally and severely developmentally impaired by absent or reduced expression of Foxn1, the transcription factor essential for TEC development (Bleul et al., 2006; Jin et al., 2014). In contrast, our study employed in vivo fate-mapping experiments to examine normally developed mTECs without transplantation or cell culture, and it has demonstrated that bipotent TEC progenitors, which must pass through the β5t$^+$ progenitor stage before becoming mTECs, exhibit a minor contribution to the maintenance and injury-triggered regeneration of mTECs in adult mice. It was reported recently that the lung epithelium in adult mice is maintained and regenerated by the proliferation of differentiated cells under steady-state conditions or following mild injury, whereas the contribution of early progenitor cells to tissue regeneration becomes apparent following severe injury (Vaughan et al., 2015; Zuo et al., 2015). It is, therefore, possible that the recruitment of bipotent TEC progenitors in adult mice may be limited to situations in which the developing thymus is severely damaged.

Nonetheless, our results show that bipotent TEC progenitors, which must pass through the β5t$^+$ progenitor stage before becoming mTECs, are active in mice during embryogenesis as well as the newborn period up to 1 week old. The importance of bipotent TEC progenitors in embryonic thymus development has been noted previously (Corbeaux et al., 2010). The rapidly declining contribution of these progenitors after 1 week old suggests that postnatal thymus development switches off the recruitment of those bipotent progenitors. Indeed, the reduction in the contribution of β5t-expressing progenitors along the ontogeny may be due to various cellular mechanisms, including the quantitative reduction of β5t-expressing progenitors and/or the qualitative reduction of developmental potential in β5t-expressing progenitors. It is important to identify molecular and cellular mechanisms that regulate this developmental switch for the recruitment of bipotent TEC progenitors.

The mTECs promiscuously express tissue-restricted self-antigens that contribute to the establishment of self-tolerance in T cells (Kyewski and Klein, 2006). Among these self-antigens whose expression was examined in mTECs, AFP, Sp1, Fgg, and Crp were differently expressed between mTECs derived from embryonic β5t$^+$ progenitors and those derived from neonatal β5t$^+$ progenitors. These results suggest that mTECs derived from embryonic and neonatal β5t$^+$ progenitors contribute differently to the establishment of self-tolerance in T cells by differently expressing self-antigens, in agreement with the previous suggestion that mTECs temporally shift

through distinct pools of promiscuously expressed genes (Pinto et al., 2013).

Our results also show that the frequency of EGFP-labeled mTECs in β5t-rtTA × Cre × EGFP mice was <5% with Dox administration during adulthood. Even though this frequency was low, we noticed that the majority of these EGFP$^+$ mTECs were enriched among the MHC class II^{high} subpopulation (Figure S6C). In addition, β5t mRNA and protein expression were detectable in ~3% of MHC class II^{high} mTECs, in an Aire-dependent manner (C.E.M., unpublished data). Promiscuously expressed self-antigens have been detected in mTECs at frequencies of 1%–15% for mRNAs and 1%–3% for proteins (Cloosen et al., 2007; Derbinski et al., 2008; Sansom et al., 2014). Therefore, the detection of a small fraction of β5t-expressing cells (3%–5%) among mTECs may represent promiscuous expression of the β5t gene in a fraction of mTECs.

The present results additionally show that cTECs and mTECs exhibit unequal developmental dynamics, as suggested in previous reports (Rode and Boehm, 2012; Dumont-Lagacé et al., 2014). Unlike mTECs, in which the frequencies of embryonically and neonatally EGFP-labeled cells remained essentially unchanged in adult mice after 6 weeks old and up to 45 weeks old, the frequencies of those EGFP-labeled cTECs gradually and distinctly decreased from ~80% at 6 weeks old to ~50% at 45 weeks old. Therefore, unlike mTECs, approximately one-third of cTECs may be generated de novo in aged mice. However, the de novo generation of cTECs does not seem to be enhanced during thymus regeneration after X-ray irradiation or poly I:C treatment, as the frequencies of embryonically and neonatally EGFP-labeled cTECs were comparable between control and thymus-regenerated mice (data not shown).

In conclusion, this study demonstrates that the adult thymic medullary epithelium is maintained and regenerated by mTEC-lineage-restricted cells that pass beyond the bipotent stage during early ontogeny rather than by adult β5t$^+$ progenitors. The contribution of mTEC-lineage-restricted cells includes the possibility of mTEC self-renewal. These findings advance the understanding of the mechanisms by which mTECs are maintained and regenerated in adulthood. In particular, the finding that bipotent TEC progenitors do not continuously differentiate to contribute to maintenance and injury-triggered regeneration of the adult thymic medulla has important implications for a better understanding of adult mTEC dynamics and development of future therapeutic thymic epithelial manipulations in clinical situations.

EXPERIMENTAL PROCEDURES

Mice

β5t-rtTA knockin/knockout mice were generated by homologous genomic DNA recombination in embryonic stems cells (ESCs), according to the basic method previously described for the production of β5t-Cre knockin mice (Ohigashi et al., 2013). LC-1 tetO-Cre-transgenic mice (Schönig et al., 2002), CAG-loxP-stop-loxP-EGFP transgenic mice (Kawamoto et al., 2000), and Rosa26 knockin mice that were engineered to contain the CAG-loxP-stop-loxP-ZsGreen sequence (Madisen et al., 2010) were described previously. Mice were maintained under specific pathogen-free conditions and experiments were conducted under the approval of the Institutional Animal Care and Use Committee of University of Tokushima. The day of vaginal plug observation was designated as E0.5.

Dox Administration

Mice were exposed to Dox through drinking water, which contained 2 mg/ml Dox (Sigma-Aldrich) and 5% (g/vol) sucrose. Embryos and unweaned mice were exposed to Dox via the mother's drinking water.

Irradiation and poly I:C Treatment

Mice were either exposed to whole-body sublethal X-ray irradiation at 5.5 Gy (Hitachi) or intraperitoneally injected with 250 μg poly I:C (InvivoGen) twice at a 3-day interval.

Flow Cytometric Analysis and Isolation of Thymus Cells

Minced fragments of the thymus and other organs were digested with 0.125% collagenase D (Roche) in the presence of 0.01% DNase I (Roche), as described previously (Gray et al., 2006). Single-cell suspensions were stained for indicated surface molecules. For intracellular staining, cells were fixed in 2% (g/vol) paraformaldehyde, permeabilized with 0.1% saponin, and stained with either an AlexaFluor 647-conjugated anti-Aire antibody or rabbit anti-β5t antibody, followed by an AlexaFluor 488-conjugated anti-rabbit IgG antibody. For TEC isolation, CD45$^-$ cells were enriched using a magnetic bead-conjugated anti-CD45 antibody (Miltenyi Biotec). Multicolor flow cytometry and cell sorting were performed on a FACSAria II (BD Biosciences). The information on antibodies is provided in the Supplemental Experimental Procedures.

Immunofluorescence Analysis of Thymus Sections

Thymic tissues were fixed in 4% (g/vol) paraformaldehyde and embedded in optimum cutting temperature compound (Sakura Finetek). Frozen thymuses were sliced into 5-μm-thick sections and stained using antibodies specific for K5 and Aire, followed by AlexaFluor-conjugated anti-IgG antibodies. Images were analyzed with a TSC SP8 confocal laser-scanning microscope (Leica).

Antibodies used in this study and the methods for BrdU labeling and qRT-PCR analysis are described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.10.012.

AUTHOR CONTRIBUTIONS

I.O., S.Z., G.A.H., and Y.T. conceived the study. I.O. and Y.T. designed the experiments. I.O., M.S., S.Z., and C.E.M. performed the experiments. Y.H. and N.M. provided the reagents and their unpublished results regarding mTEC-restricted progenitors. I.O. and Y.T. wrote the paper.

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