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

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http://dx.doi.org/10.1016/j.celrep.2015.10.012
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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 (Kwekowsk and Klein, 2006; Anderson and Takahama, 2012). Following positive selection, newly generated TCRβ5t+ thymocytes migrate from the thymic cortex to the thymic medulla via CCR7-mediated attraction by medullary thymic epithelial cells (mTECs) (Julen 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...
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 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 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+TCRhigh 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

See also Figure S1.
Most mTECs Are Derived from Embryonic and Neonatal \( \beta \text{St}^+ \) Progenitors

The mTECs, most of which do not express \( \beta \text{St} \), are derived from \( \beta \text{St}^+ \) progenitors (Ohigashi et al., 2013), so that the EGFP expression detected in the majority of mTECs in \( \beta \text{St} \text{-rtTA} \times \text{Cre} \times \text{EGFP} \) mice (Figure 1F) reflected previous \( \beta \text{St}^+ \) expression in progenitors that later differentiated into \( \beta \text{St}^- \) mTECs (Figure 1A). Consequently, via periodic Dox administration to \( \beta \text{St} \text{-rtTA} \times \text{Cre} \times \text{EGFP} \) mice, we examined the dynamics of mTEC generation from \( \beta \text{St}^+ \) progenitors during postnatal ontogeny (Figure 2A). As shown in Figure 2B, 83% \( \pm \) 5% (\( n = 6 \)) of mTECs were EGFP \(^+\) after Dox treatment from 0 days to 0.3 weeks old. The frequency of EGFP \(^+\) mTECs was 2% \( \pm \) 1% (\( n = 4 \)) by Dox treatment from 1 week old to 2 weeks old, 3% \( \pm \) 0.3% (\( n = 3 \)) by Dox treatment from 2 weeks old to 3 weeks old, and 6% \( \pm \) 1% (\( n = 3 \)) by Dox treatment from 3 weeks old to 4 weeks old (Figure 2B). Therefore, the majority (\( \sim 95\% \); i.e., \( \sim 79\% \) among \( \sim 83\% \)) of mTECs in 4-week-old mice were derived from cells that transcribed \( \beta \text{St} \) by the first week of age, whereas the contribution of \( \beta \text{St}^+ \) expressing cells after 1 week old was minor (\( \sim 5\% \)). This result indicates that most mTECs are derived from embryonic and neonatal \( \beta \text{St}^+ \) 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 (\( \sim 5\% \)), indicating that the contribution of \( \beta \text{St}^+ \) progenitors to mTECs decreases rapidly during postnatal ontogeny.

Unlike mTECs, most cTECs express \( \beta \text{St} \) (Ripen et al., 2011). Accordingly, the labeling efficiency with 1-week Dox treatment in \( \beta \text{St} \text{-rtTA} \times \text{Cre} \times \text{EGFP} \) mice (number of mice examined).

### Figure 2. Tracing the Fates of \( \beta \text{St}^+ \)-Expressing TECs at Specific Time Periods

(A) Scheme shows Dox treatment in \( \beta \text{St} \text{-rtTA} \times \text{Cre} \times \text{EGFP} \) mice (number of mice examined).

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

![Figure 2](image-url)
embryonic and neonatal metric results to demonstrate that most mTECs are derived from cortical region (Figure 3). These results reinforced the flow cytodetectable with Dox treatment after 1 week old, even though EGFP labeling in K5-positive mTECs was barely detected by the expansion of cells that had passed through the β5t tran

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 increased to ~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 × 10³ per mouse at 0 days old to ~4 × 10⁴ 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 × 10³ per mouse at 0 days old to ~3.4 × 10⁴ 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 × 10⁵ per mouse at 1 week old to ~1.5 × 10⁶ 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 × 10⁴ per mouse at 6 weeks old to ~5 × 10² per mouse at 45 weeks old (Figure 4B), reflecting age-related involution of the thymus (Hironaka 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
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 $\beta$5t+ 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$^{\text{high}}$ cells and MHC class II$^{\text{low}}$ 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$^{\text{high}}$ cells among mTECs (Figure 4G). These results indicate that embryonic and neonatal $\beta$5t+ 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 $\beta$5t+ 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 $\beta$5t+ progenitors to the development, maintenance,
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.

Injury-Triggered Regeneration of the Adult Thymic Medullary Epithelium Is Mediated by Cells Derived from Embryonic and Neonatal 5t+ 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
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
mTEC-restricted progenitors/stem cells, which can be defined among EpCAM+ CD45−/CD326+ 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-Cre3loxP-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 βst-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.

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−SSEA1− 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 βst-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.
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, \(\sim 80\%\) 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 \(\beta\)St but originate from \(\beta\)St\(^{+}\) progenitors.

### DISCUSSION

This study has established a new method for tracing cells that express \(\beta\)St during a given time period in vivo. \(\beta\)St 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, \(\beta\)St 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 \(\beta\)St-expressing progenitors (Ohigashi et al., 2013; Alves et al., 2014). Therefore, the use of \(\beta\)St-rtTA \(\times\) Cre \(\times\) EGFP mice enabled a study of the time period when \(\beta\)St-negative mTECs pass through the \(\beta\)St-expressing progenitor stage. Our results indicate that the majority of adult mTECs, including those in aged mice, are derived from embryonic and neonatal \(\beta\)St\(^{+}\) progenitors rather than adult \(\beta\)St\(^{+}\) progenitors. Unexpectedly, we found that the contribution of adult \(\beta\)St\(^{+}\) 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 \(\beta\)St\(^{+}\) progenitors during the embryonic and neonatal periods rather than via continuous supply from adult \(\beta\)St\(^{+}\) 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 \(\beta\)St\(^{+}\) progenitors. Our results further indicate that mTEC-restricted progenitors in embryonic mice still contain \(\beta\)St 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 \(\beta\)St\(^{+}\) 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 \(\beta\)St\(^{+}\) 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 \(\beta\)St-expressing progenitors along the ontogeny may be due to various cellular mechanisms, including the quantitative reduction of \(\beta\)St-expressing progenitors and/or the qualitative reduction of developmental potential in \(\beta\)St-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 \(\beta\)St\(^{+}\) progenitors and those derived from neonatal \(\beta\)St\(^{+}\) progenitors. These results suggest that mTECs derived from embryonic and neonatal \(\beta\)St\(^{+}\) 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 IIhigh subpopulation (Figure S6C). In addition, β5t mRNA and protein expression were detectable in ~3% of MHC class IIhigh 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 stem 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 (Schöning 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 (Madsen 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 (H-irradiation) or intraperitoneally injected with 250 mg 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**


**ACKNOWLEDGMENTS**

We thank Drs. Kensuke Takada, Mina Koizai, and Bongju Kim for reading the manuscript. This work was supported by grants from Ministry of Education, Culture, Sports, Science and Technology (MEXT)-Japan Society for the Promotion of Science (JSPS) (24111004 and 23249025 to Y.T. and 23659241 and 23860361 to I.O.) and the Naito Foundation (to Y.T. and I.O.).

Received: May 18, 2015
Revised: August 24, 2015
Accepted: October 2, 2015
Published: November 5, 2015

**REFERENCES**


