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Medullary Thymic Epithelial Stem Cells Maintain a Functional Thymus to Ensure Lifelong Central T Cell Tolerance

Miho Sekai,[1,2] Yoko Hamazaki,[1,*] and Nagahiro Minato[1]
[1]Department of Immunology and Cell Biology, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan
[2]Graduate School of Biostudies, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan
*Correspondence: hamazaki@imm.med.kyoto-u.ac.jp
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SUMMARY

Medullary thymic epithelial cells (mTECs) are crucial for central T cell self-tolerance. Although progenitors of mTECs have been demonstrated in thymic organogenesis, the mechanism for postnatal mTEC maintenance remains elusive. We demonstrate that implantation of embryonic TECs expressing claudin-3 and claudin-4 (Cld3,4) in a medulla-defective thymic microenvironment restores medulla formation and suppresses multiorgan autoimmunity throughout life. A minor SSEA-1^{+} fraction within the embryonic Cld3,4^{hi} TECs contained self-renewable clonogenic TECs, capable of preferentially generating mature mTECs in vivo. Adult SSEA-1^{+}Cld3,4^{hi} TECs retained mTEC reconstitution potential, although the activity decreased. The clonogenicity of TECs also declined rapidly after birth in wild-type mice, whereas it persisted in *Rag2*$^{-/-}$ adult mice with defective thymopoiesis. The results suggest that unipotent mTEC-restricted stem cells that develop in the embryo have the capacity to functionally reconstitute the thymic medulla long-term, thus ensuring lifelong central T cell self-tolerance.

INTRODUCTION

The thymus is a central lymphoid organ that is responsible for the production of self-restricted and self-tolerant T cells. It has a characteristic three-dimensional meshwork structure of epithelial cells and consists of two distinct anatomical regions, the cortex and the medulla, each containing distinct types of thymic epithelial cells (TECs) as major stromal components (Anderson and Jenkinson, 2001). Medullary TECs (mTECs) play a crucial role in establishing central T cell tolerance by promiscuously expressing tissue-specific self-antigens (TSAs), in part via an autoimmune regulator (Aire) (Anderson et al., 2002). The generation and maintenance of a thymic microenvironment requires complex interplay between the lymphoid and stromal compartments, and Aire^{+} mTEC development crucially depends on the signaling involving tumor necrosis factor receptor (TNFR) superfamily members (Akiyama et al., 2012; Anderson and Takahama, 2012). Defects in this pathway, for example the *Nik* mutation in *aly/aly* mice, result in impaired generation of Aire^{+} mTECs and development of multiorgan autoimmunity (Kajiura et al., 2004).

The TECs of both compartments develop from common progenitors during thymic organogenesis (Bleul et al., 2006; Rossi et al., 2006). On the other hand, the existence of unipotent TEC progenitors for each lineage has also been demonstrated (Bleul et al., 2006; Hamazaki et al., 2007; Rodewald et al., 2001; Shakib et al., 2009). The thymic medulla has been shown to develop as small islets of TECs, with each islet arising from a single progenitor cell (Rodewald et al., 2001). We previously identified embryonic TEC progenitors highly expressing claudin-3 and claudin-4 (Cld3,4^{hi}) that exclusively give rise to mature mTECs within 3 weeks after implantation (Hamazaki et al., 2007). These results suggest the derivation of unipotent mTEC progenitors from common progenitors, and recent studies demonstrate that mTECs derive from progenitors initially expressing cTEC lineage markers during thymic organogenesis (Baik et al., 2013; Ohigashi et al., 2013; Peterson and Laan, 2013). However, the mechanism for the maintenance of functional thymic medulla at the postnatal stage remains unknown. Tissue cell turnover might occur either via stem-cell-based regeneration in the tissues bearing high turnover rates, such as hematopoietic, skin, and intestinal tissues, or mainly by duplication of differentiated cells in those showing slower turnover rates, such as liver and pancreas (Barker et al., 2010; Manley et al., 2011). Recent studies have revealed that the thymus structure is much more dynamic than previously thought; specifically, mTECs have turnover rates in a matter of weeks, comparable with keratinocytes (Gray et al., 2006). Although the results might imply the stem-cell-based regeneration of mTECs, their putative stem cells have not been identified.

In the current study, we found that embryonic Cld3,4^{hi} TECs are sufficient for maintaining functional mTEC regeneration and assuring lifelong central T cell tolerance. We further showed that a minor SSEA-1^{+} fraction of the embryonic Cld3,4^{hi} TECs contains the self-renewable TECs with the capacity to generate mature mTECs, consistent with the presence of mTEC stem cells (mTECSCs). The clonogenic activity of SSEA-1^{+}Cld3,4^{hi} TECs was rapidly decreased after birth in wild-type (WT) mice but was sustained in adult *Rag2*$^{-/-}$ mice, suggesting that concomitant thymopoiesis profoundly affects the mTECSC activity.

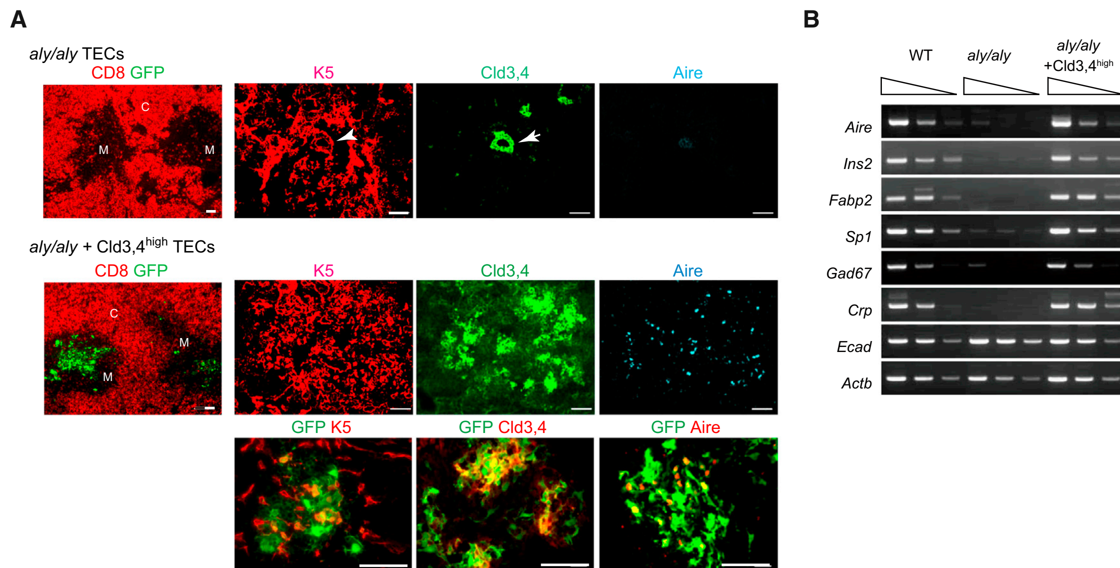

Figure 1. Embryonic Cld3,4^{hi} TECs Restore and Maintain the Thymic Medulla throughout Life in an mTEC-Defective *aly/aly* Thymic Microenvironment

(A) Immunostaining of the ectopic thymi formed by *aly/aly* TECs alone (top) and *aly/aly* plus Cld3,4^{hi} TECs (middle) at 12 months after implantation. Abbreviations are as follows: M, medulla; C, cortex. An arrow indicates a cystic structure of Cld3,4^{+} TECs. Bottom panels indicate two-color staining with the indicated antibodies of the medullary region of ectopic thymus formed by *aly/aly* plus Cld3,4^{hi} TECs. Scale bars represent 50 μm.

(B) Expression of *Aire*, *Aire*-dependent (*Ins2*, *Fabp2*, *Sp1*), and *Aire*-independent (*Gad67*, *Crp*) tissue-specific self-antigen (TSA) genes with semiquantitative RT-PCR at 4 months after implantation. *Ecad* and *Actb* served as controls. Representative results of at least three independent experiments are shown.

See also Figure S1.

RESULTS

Embryonic Cld3,4^{hi} TECs Can Contribute to the Lifelong Maintenance of Functional mTECs

We first investigated the durability of embryonic Cld3,4^{hi} TECs for sustaining the generation of mature mTECs in an *aly/aly* thymic microenvironment in vivo. The *aly/aly* mice with a mutation in *Nik* show a selective defect in mTEC development, particularly Aire^{+} mTECs (Boehm et al., 2003; Kajiura et al., 2004). Total thymic cells (1×10^6) from *aly/aly* mice at embryonic day 14.5 (E14.5) were reaggregated with or without Cld3,4^{hi} TECs (1×10^4) sorted from E14.5 enhanced green fluorescent protein (EGFP) transgenic (Tg) mice and then implanted under the renal capsules of nude (*nu/nu)* mice (Figure S1A available online). The ectopic thymi formed by *aly/aly* TECs alone showed poorly developed medulla with sparse Keratin-5 (K5)$^{+}$ reticular mTECs; Cld3,4^{+} mTECs with no Aire expression were detected only scarcely, often forming cystic structures (Figure 1A, top). In contrast, ectopic thymi developed from *aly/aly* plus Cld3,4^{hi} TECs revealed the presence of GFP^{+} TECs selectively in the medulla even at 12 months after implantation, including both K5^{+} reticular and Cld3,4^{+}Aire^{+} globular mTECs (Figure 1A, middle and bottom). Similar results were obtained from 3 months to 18 months after implantation. In agreement with the findings, the ectopic thymi of *aly/aly* plus Cld3,4^{hi} TECs expressed both Aire-dependent and Aire-independent TSA genes in similar amounts to WT ectopic thymus, whereas the same genes were hardly expressed by *aly/aly* thymi alone (Figure 1B). The same results were obtained by reaggregation of the Cld3,4^{hi} TECs with E14.5 WT total thymic cells (Figure S1B), eliminating the possibility that the long-term reconstitution by implantation of embryonic Cld3,4^{hi} TECs was attributable to the mTEC-defective microenvironment. Thus, embryonic Cld3,4^{hi} TECs are sufficient for maintaining lifelong functional mTEC regeneration.

Embryonic Cld3,4^{hi} TECs Prevent the Development of Autoimmunity Caused by *aly/aly* Thymic Stoma

The ectopic implantation of *aly/aly* thymi alone and *aly/aly* plus Cld3,4^{hi} TECs in *nu/nu* recipients supported the T cell development similar to WT thymi, including generation of regulatory T (Treg) cells, and proportions of peripheral T cells were also indistinguishable (Figure S2). However, the recipients with *aly/aly* thymi alone eventually developed marked lymphocytic infiltrations in multiple organs, which were reminiscent of the original *aly/aly* mice (Figure 2A, top, and Figure 2B; Tsubata et al., 1996). Moreover, these mice had development of anti-nuclear as well as various tissue-cell-reactive autoantibodies, which are rarely observed in the original *aly/aly* mice because of the additional effects of the *Nik* mutation on B cell function (Figure 2C, top; Shinkura et al., 1996). In contrast, the mice bearing the ectopic thymi of *aly/aly* plus Cld3,4^{hi} TECs showed minimal cellular infiltrations in these organs until 18 months after implantation, with no detectable autoantibody production (Figure 2A, bottom, Figure 2B, and Figure 2C, bottom). The results demonstrate that embryonic Cld3,4^{hi} TECs can rescue the effects of *aly/aly* mutation in thymic stroma and ensure lifelong central T cell self-tolerance.

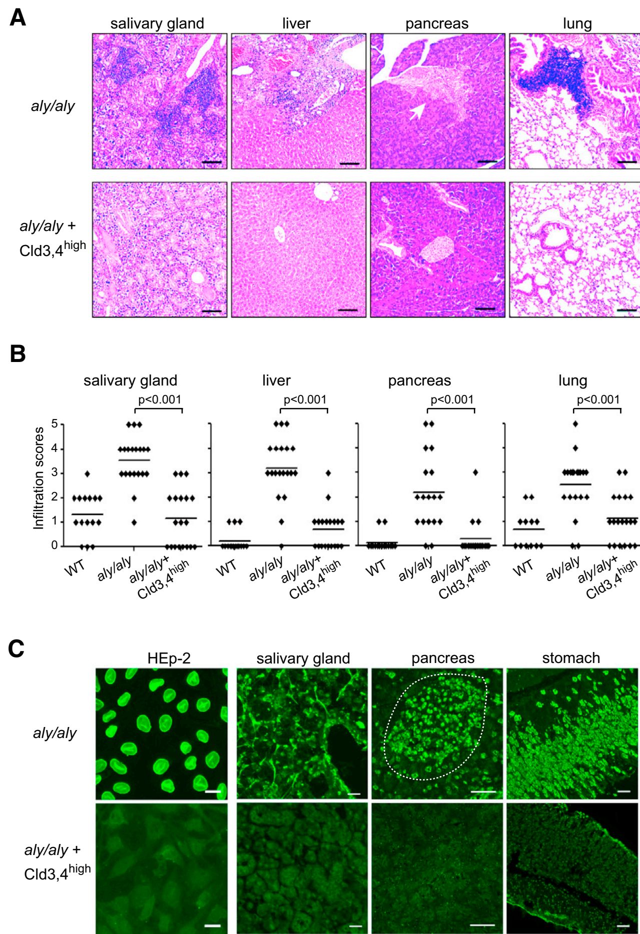

Figure 2. Inclusion of Embryonic Cld3,4^{hi} TECs in an *aly/aly* Thymic Microenvironment Prevents the Development of Multiorgan Autoimmunity

(A) Organs from the *nu/nu* mice with ectopic thymi of *aly/aly* (top) and *aly/aly* plus Cld3,4^{hi} TECs (bottom) at 3 months after implantation were stained with hematoxylin and eosin. An arrow indicates β-islet with cellular infiltration. Scale bars represent 100 μm.

(B) Extent of cellular infiltration in organs was scored at 3 to 18 months after implantation. The recipients bearing ectopic thymi with *aly/aly* TECs became ill during varying periods before 8 months and were euthanized when they were moribund. Individual scores (n = 15 for WT; n = 20 for *aly/aly*; n = 19 for *aly/aly* plus Cld3,4^{hi} TECs) and means (bars) are indicated.

(C) Anti-nuclear and tissue-reactive autoantibodies in the recipient sera at 3 months after implantation of *aly/aly* (top) and *aly/aly* plus Cld3,4^{hi} thymi (bottom) were examined using fixed HEp-2 cells (scale bars [left] represent 20 μm) and various tissue sections of *Rag2*$^{-/-}$ mice (scale bars [others] represent 50 μm), respectively. Dotted line indicates a β-islet.

See also Figure S2.

An Embryonic SSEA-1^{+}Cld3,4^{hi} Fraction Contains mTEC Stem Cells

Lifelong regeneration of mTECs prompted us to investigate whether the embryonic Cld3,4^{hi} TECs display mTEC stem cell activity. Because Cld3,4 is expressed through the mature mTECs (Hamazaki et al., 2007), we sought to identify a more restricted marker to define the putative stem cell population. We found that a minor proportion of the E14.5 Cld3,4^{hi} TECs expressed SSEA-1, but not SSEA-3 (Figure 3A). In mice, SSEA-1 is expressed in embryonic stem cells, whereas SSEA-3 expression is induced on their differentiation (Solter, 2006). The SSEA-1^{+} cells were UEA-1^{-} and rarely expressed mTEC-related maturation markers such as CD40, major histocompatibility complex class II (MHC-II), and CD80, although they exhibited lymphotoxin-β receptor (LTβR) expression that is crucial for early mTEC development (Figure 3A; Mouri et al., 2011). We then compared the reconstitution capacity of SSEA-1^{+} and SSEA-1^{-} fractions of E14.5 Cld3,4^{hi} TECs in vivo by using a reaggregation/transplantation system. At 8 weeks after implantation, the SSEA-1^{+} fraction showed remarkably greater reconstitution efficiency than SSEA-1^{-} fraction (12% versus <1% of the input cells) (Figure 3B). Moreover, the GFP^{+} TECs derived from the SSEA-1^{+}Cld3,4^{hi} TECs contained MHC-II^{hi} mature TECs comparable to the GFP^{-} host TECs in the same graft (Figure 3B), indicating that mTEC regeneration capacity was highly enriched in the minor SSEA-1^{+} fraction of Cld3,4^{hi} TEC population.

We next intended to examine the self-renewal activity in vitro with the use of a newly developed clonogenic culture system under serum-free conditions (Figures S3A and S3B). The E14.5 SSEA-1^{+}Cld3,4^{hi} TECs developed discrete colonies (1.3% of the input cells) densely packed with small cells (Figure 3C). The single-cell-derived colonies could be propagated up to three passages (Figure 3C). The SSEA-1^{-}Cld3,4^{hi} TECs developed much fewer colonies (0.4% of the input cells), most of which were smaller (<2 mm) and propagated only once (Figure 3C). The replating efficiency of SSEA-1^{+} TEC-derived colonies appeared rather low compared with that previously reported for rat TEC colonies of undefined phenotype (Bonfanti et al., 2010). Because the Cld3,4 expression profiles in the embryonic and adult thymi were identical between rats and mice (Figures S3C and S3D), we also used rat TECs to confirm the self-renewal activity of embryonic Cld3,4^{hi} populations in our culture condition. The Cld3,4^{hi} TECs from E17.5 rat thymus developed much greater numbers of colonies than the corresponding mouse TECs (Figures S3E and S3F); moreover, the large colonies could be propagated at least five passages with nearly 80% efficiency (Figure S3G). Hence, seemingly low replating efficiency of the colonies appears to be an intrinsic property of murine TECs in culture. Nonetheless, the results of clonogenic assay in both rats and mice are consistent with the notion that TECs with self-renewal activity are present in the embryonic Cld3,4^{hi} TECs; the self-renewable activity is highly enriched in the SSEA-1^{+} fraction in mice.

The colony cells derived from the embryonic SSEA-1^{+}Cld3,4^{hi} TECs largely retained the original phenotype, with negligible expression of maturation-related markers (CD40, CD80, MHC-II) (Figure S3H). They also expressed genes characteristic of immature TECs, including *Foxn1* and *Plet1* (Corbeaux et al., 2010; Depreter et al., 2008) with no *Aire* expression (Figure S3I). We then examined the differentiation potential of the colony cells in vivo. The pooled colony cells (1 × 10^{4}) derived from a single colony were reaggregated with E14.5 WT thymic cells (1 × 10^{6}) and implanted. At 6 weeks after implantation, the GFP^{+} cells were integrated exclusively in the medulla, and a portion of them strongly expressed MHC-II and Aire (Figure 3D). Because the reconstitution efficiency was rather low, partly because of the inefficient incorporation of colony cells into the reaggregates, we also

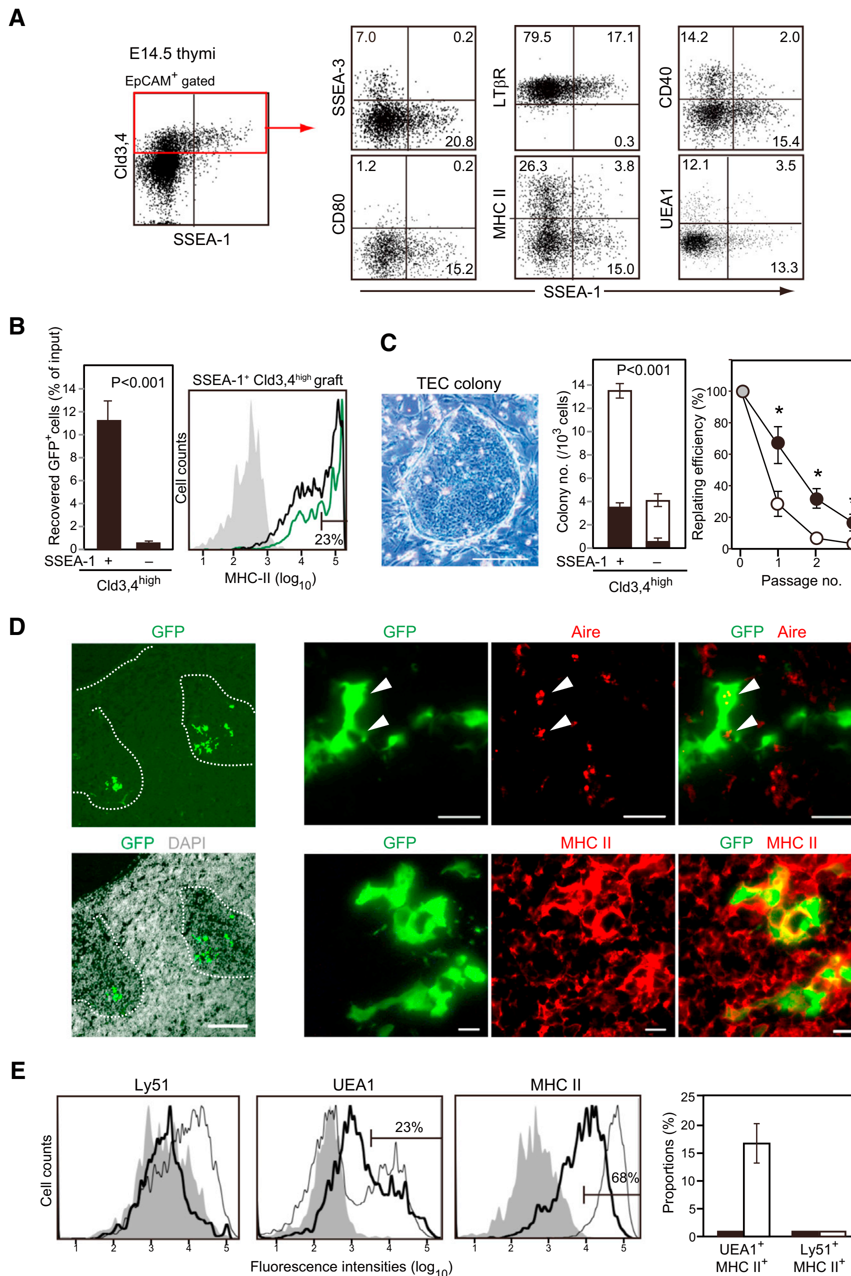

Figure 3. A Minor SSEA-1$^+$ Fraction of Embryonic Cld3,4^{hi} TECs Contains Self-Renewable TECs Capable of Generating Mature mTECs

(A) Multicolor flow cytometric analysis of E14.5 WT TECs with the indicated antibodies in EpCAM$^+$ (left) and EpCAM$^+$Cld3,4^{hi} (the rest) gates.

(B) Freshly isolated SSEA-1$^+$ or SSEA-1$^-$ Cld3,4^{hi} TECs (2 × 10^3) from E14.5 EGFP Tg mice were reaggregated with E14.5 WT thymic cells (1 × 10^6), followed by implantation under the capsule of the same kidney. Two months later, all TECs from the ectopic thymi were analyzed with flow cytometry. The means and standard error (SE) of GFP$^+$ cell recovery in three experiments and the expression of major histocompatibility complex class II (MHC-II) in the former ectopic thymus are shown. Shaded region indicates control staining of both groups, and black and green lines indicate anti-MHC-II staining in GFP$^-$ (internal control of host TECs) and GFP$^+$ cells (SSEA-1$^+$Cld3,4^{hi} input TECs), respectively.

(C) SSEA-1$^+$ and SSEA-1$^-$ fractions of Cld3,4^{hi} cells were sorted from E14.5 WT thymi and cultured in a defined clonogenic culture system for 10 days. A representative picture of the colony from the SSEA-1$^+$Cld3,4^{hi} TECs (left; scale bar represents 500 μm) and the means and SE of colony numbers per 10^3 cells in nine independent experiments are shown (middle). Filled and open columns indicate the colonies of >2 mm diameter and <2 mm diameter, respectively. The primary large colonies (>2 mm) from SSEA-1$^+$ (filled circles) and SSEA-1$^-$ (open circles) Cld3,4^{hi} fractions were isolated individually and replated serially in the clonogenic culture. At least six individual colonies (initial input colonies) were plated in an experiment, and the replating efficiencies (proportions of passaged colony numbers per initial input colony numbers) are shown (right). The means and SE of five independent experiments are indicated.

(D) Secondary colonies derived from a single primary colony from SSEA-1$^+$Cld3,4^{hi} TECs of E14.5 EGFP Tg mice were isolated, pooled (1 × 10^4), and reaggregated with E14.5 WT total thymic cells (1 × 10^6), followed by implantation. Six weeks later, the ectopic thymus was examined for the expression of GFP (left; scale bars represent 100 μm) and the indicated markers with immunostaining (right; scale bars represent 10 μm). Dotted circles indicate medullary regions and arrowheads indicate Aire$^+$GFP$^+$ TECs. Representative results of three recipients are shown.

(E) Colonies from SSEA-1$^+$Cld3,4^{hi} TECs of E14.5 EGFP Tg mice were pooled and directly injected into newborn (NB) WT thymic lobes in the organ culture. Five days later, the lobes were harvested and analyzed with flow cytometry using the indicated antibodies in the EpCAM$^+$ gate. Shaded regions indicate the profiles of colony cells before injection, solid lines indicate the GFP$^+$ cells in the cultured thymic lobes, and fine lines indicate primary NB TECs. The means and SE of the proportions of UEA-1$^+$MHC-II$^+$ and Ly51$^+$ cells in GFP$^+$ TECs of five independent experiments are shown.

See also Figure S3.

injected the colony cells directly into the thymic lobes in organ culture. Fluorescence-activated cell sorting (FACS) analysis revealed that as much as 15% of the GFP$^+$ cells retained in the thymic lobes were UEA-1$^+$MHC-II$^+$ after 5 days, whereas Ly51$^+$ TECs were negligible (Figure 3E). Essentially similar results were obtained by injecting the colony cells derived from rat embryonic Cld3,4^{hi} TECs into the adult thymus in vivo (Figure S3J). Altogether, these results strongly suggest that the embryonic SSEA-1$^+$Cld3,4^{hi} cell population contains the self-renewable clonogenic TECs generating mature mTECs in the thymic microenvironment, which is consistent with the presence of mTECSCs.

SSEA-1$^+$Cld3,4^{hi} mTECSCs Persist but Show Reduced mTEC Generation Activity in the Adult Thymus

We sought TECs potentially equivalent to the embryonic mTECSCs in the adult thymus. The proportions of SSEA-1$^+$ cells

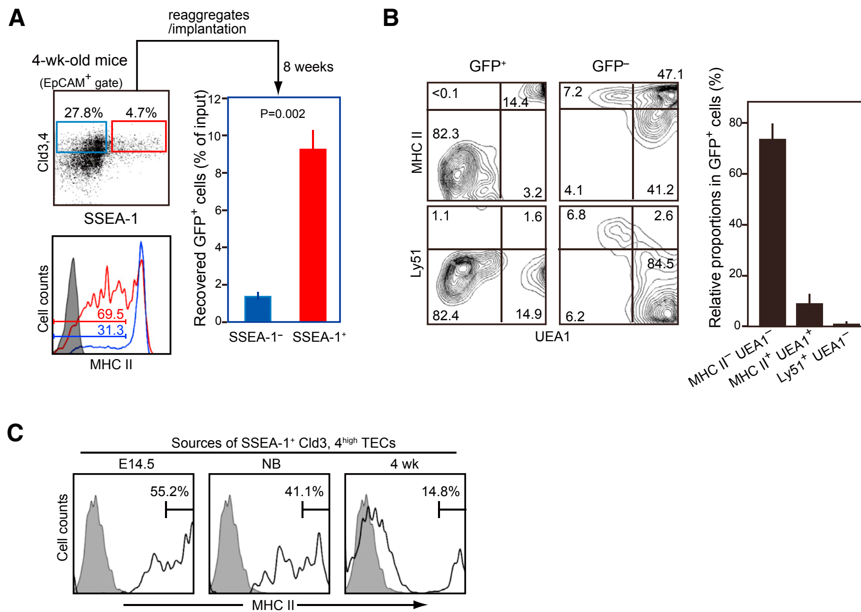

Figure 4. SSEA-1$^+$Cld3,4^{hi} mTECSCs in Adults Show Markedly Reduced Potential of Generating Mature mTEC Progenies

(A) SSEA-1$^+$ (boxed in red) and SSEA-1$^-$ (boxed in blue) Cld3,4^{hi} TECs were sorted from 4-week-old EGFP Tg mice and subjected to reaggregation/transplantation assay system. The MHC-II expression in SSEA-1$^+$ (red) and SSEA-1$^-$ (blue) in the Cld3,4^{hi} TECs are shown in the histogram. Shaded regions indicate control staining of C-CPE^{hi} TECs. Eight weeks after the implantation, all TECs from the ectopic thymi were analyzed with flow cytometry. The means and SE of GFP$^+$ cell recovery in three experiments are shown.

(B) The expression profiles of the indicated markers in GFP$^+$ (implanted) and GFP$^-$ (host) populations recovered from the ectopic thymus implanted with the SSEA-1$^+$ fraction are shown (left). The relative proportions of UEA-1$^-$MHC-II$^-$ TECs, UEA-1$^+$MHC-II$^+$ mature mTECs, and Ly51$^+$UEA-1$^-$ cTECs in the GFP$^+$ fraction recovered from the ectopic thymus are shown. The means and SE of three independent experiments are indicated (right).

(C) The expression profiles of MHC-II in the GFP$^+$ TECs recovered from the ectopic thymus implanted with E14.5, NB, and adult SSEA-1$^+$Cld3,4^{hi} TECs (4 weeks old) are shown (solid line). Shaded regions indicate control staining of host TECs in the graft of adult SSEA-1$^+$Cld3,4^{hi} TECs. The proportions of MHC-II^{hi} TECs are shown. Representative results in three experiments are shown. The analysis was also performed at 10 days, 2 weeks, and 6 weeks after implantation and yielded almost identical results.

See also Figure S4.

in the Cld3,4^{hi} TEC fraction tended to increase after birth (Figure S4A). However, approximately 70% of them in 4-week-old mice were MHC-II$^{lo/-}$ (Figure 4A) and often expressed keratin 10 (Figure S4B), resembling post-Aire mTECs (White et al., 2010; Yano et al., 2008), whereas MHC-II$^-$SSEA-1$^+$Cld3,4^{hi} TECs were rare (Figure S4A). We sorted the SSEA-1$^+$ and SSEA-1$^-$ Cld3,4^{hi} TECs from the pooled thymi of 4-week-old EGFP Tg mice, reaggregated them with E14.5 WT total thymic cells, and implanted them in *nu/nu* mice. At 8 weeks after implantation, the recovery of GFP$^+$ TECs in the grafts of the SSEA-1$^+$ fraction was again remarkably greater than that in the grafts of the SSEA-1$^-$ fraction (9.2% versus 1.3% of the input cells on average) (Figure 4A). The GFP$^+$ cells in the former grafts contained a significant proportion of UEA-1$^+$MHC-II^{hi} mature mTECs (14.4%) with minimal UEA-1$^-$Ly51$^+$ cTECs (1.1%). The MHC-II^{lo} TECs corresponding to the majority of TECs in the initial fraction, most of which might be terminally maturated, post-Aire mTECs, were negligible (Figure 4B). Most notably, the majority of the GFP$^+$ TECs were MHC-II$^-$UEA-1$^-$, the same phenotype as the embryonic mTECSCs (Figure 3A); such a population was detected at a much lower proportion (4.1%) in the GFP$^-$ TECs derived from the embryonic host TECs in the same grafts (Figure 4B). We then compared the potential of mTECSCs for generating mature progenies at different ages. We sorted the SSEA-1$^+$Cld3,4^{hi} TECs from the thymi of E14.5, newborn (NB), and 4-week-old EGFP Tg mice and examined the reconstitution profiles via a reaggregation/implantation system. At 8 weeks after implantation, the GFP$^+$ population in the grafts of adult SSEA-1$^+$Cld3,4^{hi} TECs contained a smaller proportion of MHC-II^{hi} TECs (14.8%) with a much greater proportion of MHC-II$^-$ TECs (72.6%) than that of E14.5 (MHC-II^{hi}, 55.1%; MHC-II$^-$, 2.8%) and NB (MHC-II^{hi}, 41.1%; MHC-II$^-$, 3.7%) SSEA-1$^+$Cld3,4^{hi} TECs (Figure 4C). These results suggest that the TECs bearing the equivalent surface phenotypes and properties to the embryonic mTECSCs persist in the adult thymus, but the potential of generating mature mTECs is remarkably compromised.

mTECSC Activity Decreases Rapidly after Birth due to Active Thymopoiesis

Because it was suggested that the potential of SSEA-1$^+$Cld3,4^{hi} mTECSCs changes remarkably after birth, we investigated the TEC clonogenic activity in vitro at various developmental stages. The total TEC colony numbers per thymus were rapidly diminished after birth in WT mice, and macroscopic large colonies became undetectable after 4 weeks of age (Figure 5A, left). Age-dependent decrease of the TEC clonogenic activity was similarly observed in rats (Figures S5A and S5B). To investigate whether the effect was TEC autonomous, we examined the clonogenic activity in the thymi of *Rag2*$^{-/-}$ mice, which lack mature thymocytes and have poor development of medulla (Holländer et al., 1995) with minimal Cld3,4$^+$ and Aire$^+$ mTECs (Figure S5C). The total TEC colony numbers in E14.5 *Rag2*$^{-/-}$ thymi were comparable with those in the WT thymi; however, the colony numbers per thymi were sustained at much higher frequency than in WT thymi at least until 8 weeks of age (Figure 5A, left). As in WT mice, the clonogenic activity in *Rag2*$^{-/-}$ thymi was enriched in the SSEA-1$^+$ fraction in the Cld3,4^{hi} TECs throughout the adult stage, and the colony number per thymus derived from the SSEA-1$^+$Cld3,4^{hi} TECs of *Rag2*$^{-/-}$ thymi at 12 weeks old was sustained at higher frequency comparable with that of WT NB stage (Figure 5A, right), in agreement with the increased proportion of MHC-II$^-$SSEA-1$^+$Cld3,4^{hi} TECs compared with WT mice (Figure S4A). We then sorted SSEA-1$^+$Cld3,4^{hi} TECs from NB WT and *Rag2*$^{-/-}$ EGFP Tg mice and compared the

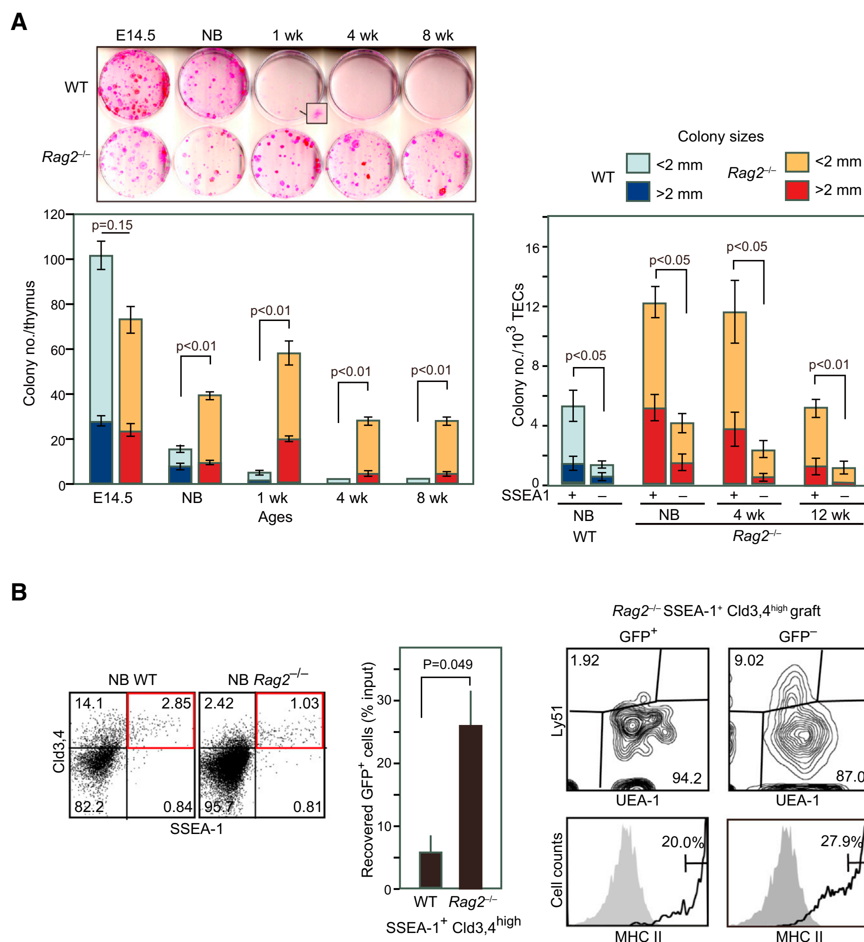

Figure 5. Clonogenic Activity of mTECSCs Rapidly Declines after Birth in WT Mice but Is Sustained in Adult $Rag2^{-/-}$ Mice

(A) Clonogenic TEC activity in the total thymi from WT and $Rag2^{-/-}$ mice at varying ages (left). The colonies were stained with Rhodamine-B and counted. Solid and pale columns in blue (WT) and red ($Rag2^{-/-}$) indicate the colonies of >2 mm diameter and <2 mm diameter, respectively (left). SSEA-1$^+$ and SSEA-1$^-$ Cld3,4^{hi} TECs were sorted from WT and $Rag2^{-/-}$ mice at the indicated ages and subjected to the clonogenic culture system (right). The mean colony numbers and SE of four to six dishes are shown.

(B) SSEA-1$^+$Cld3,4^{hi} TECs (1 × 10^3) sorted from NB WT and $Rag2^{-/-}$ EGFP Tg mice (boxed regions) were reaggregated with E14.5 WT thymic cells (1 × 10^6), followed by implantation under the capsule of the same kidney (left). Four weeks later, the ectopic thymi were analyzed with flow cytometry. The means and SE of GFP$^+$ cell recovery in two independent experiments are shown (middle). The representative expression profiles of the indicated markers in GFP$^+$ and GFP$^-$ populations (right) are shown (solid lines). Shaded regions indicate control staining of both groups. The analysis was also performed at 8 weeks after implantation and yielded essentially identical results. See also Figure S5.

reconstitution capacity via a reaggregation/implantation system. At 4 weeks after implantation, the SSEA-1$^+$ TECs from $Rag2^{-/-}$ mice revealed much greater reconstitution efficiency than the SSEA-1$^+$ TECs from WT mice (25% versus 6% of the input cells) (Figure 5B). The GFP$^+$ TECs in the former grafts were mostly UEA-1$^+$MHC-II$^{+/hi}$ mature mTECs with negligible Ly51$^+$ cTECs (Figure 5B), confirming that the SSEA-1$^+$Cld3,4^{hi} TECs in $Rag2^{-/-}$ mice had the same functional property as the embryonic SSEA-1$^+$Cld3,4^{hi} TECs in WT mice. Essentially similar results were obtained at 8 weeks after implantation. The results suggest that the rapid decrease of mTECSC activity after birth in WT mice is closely linked to the concomitant active thymopoiesis.

DISCUSSION

It has long been an issue of argument whether TEC maintenance in adults depends on a stem-cell-based or a stem-cell-independent mechanism (Manley et al., 2011; Swann and Boehm, 2007). Embryonic common TEC progenitors that give rise to both mTECs and cTECs during thymic organogenesis are suggested to have restricted self-renewal capacity, if any (Corbeaux et al., 2010; Jenkinson et al., 2008; Peterson and Laan, 2013). Although it has been shown that embryonic TECs are detectable for as long as 4 months after transplantation as medullary islets (Rodewald et al., 2001), evidence for mTEC stem cell activity has been missing (Swann and Boehm, 2007). In the current study, we demonstrated that the embryonic Cld3,4^{hi} TECs implanted in a medulla-defective microenvironment are capable of specifically maintaining mTECs and ensuring central T cell tolerance nearly throughout life. We previously reported that the generation of mature mTECs from the embryonic Cld3,4^{hi} TECs is accompanied by their proliferation (Hamazaki et al., 2007). It has also been reported that mTECs show relatively high turnover rates of several weeks (Gray et al., 2006). Thus, it seems quite unlikely that mature mTECs differentiated from the implanted immediate progenitors persisted for more than 18 months. Rather, our current results strongly suggest the presence of mTECSCs in the embryonic Cld3,4^{hi} TEC population, continuously generating mature mTECs almost throughout life.

The activity of long-term mTEC reconstitution in vivo was associated with a minor SSEA-1$^+$ fraction of the E14.5 Cld3,4^{hi} TECs. An important property of stem cells that distinguishes them from committed progenitor cells is the self-renewing activity. To demonstrate this property, we developed a clonogenic assay culture system of mouse TECs; by using it, we found that the TECs with clonogenic activity were also enriched in the SSEA-1$^+$Cld3,4^{hi} fraction of embryonic TECs. Although the replating efficiency of the TEC colonies seemed to be rather low, we confirmed that rat TEC colonies derived from the embryonic Cld3,4^{hi} TEC fraction showed remarkably high replating efficiency and were propagated for many passages in the same culture condition. Thus, it appears that the clonogenic TECs of

mice are less adjustable and/or stable in the culture or have intrinsically lower self-renewing activity than those of rats. Nonetheless, the results in two systems were quite consistent, and we conclude that the embryonic SSEA-1$^+$Cld3,4^{hi} fraction includes the TECs bearing a self-renewing activity. Moreover, the TEC colony cells propagated in vitro were capable of generating mature mTECs, but rarely cTECs, in the thymic microenvironment in vivo, thus fulfilling the properties of stem cells specified for an mTEC lineage. Minimal estimates of the frequency of mTECSCs based on the clonogenic units were 1.0% to 1.5% of the E14.5 SSEA-1$^+$Cld3,4^{hi} TEC population. The reported frequency of keratinocyte stem cells in the skin is approximately 0.4% of the CD34$^+$ cells (Blanpain et al., 2004). Considering that the turnover rate of mTECs in situ is comparable with that of keratinocytes (Gray et al., 2006), the estimated frequency of mTECSCs might be reasonable. If the embryonic SSEA-1$^+$ Cld3,4^{hi} mTECSCs continue to support the mTEC regeneration and medullary function throughout the adult stage, then it would be expected that the TECs with similar phenotype and properties are present in the adult thymus as well. Our current results indicated that a minor SSEA-1$^+$Cld3,4^{hi} TEC fraction from 4-week-old mice showed efficient reconstitution capacity and were capable of preferentially generating MHC-II^{hi} mature mTECs in vivo. The results further support the notion that SSEA-1$^+$Cld3,4^{hi} mTECSCs generated in the embryonic stage are capable of supporting mTEC regeneration and medullary function throughout adult stages. Most recently, presence of progenitors (Wong et al., 2014) and stem cells (Ucar et al., 2014) with bipotency or multipotency have been reported in the adult thymus, although their lifelong reconstitution potential for maintaining mTECs and cTECs remains to be seen. These TEC populations were defined by markers and assay systems different from those of our mTECSCs, and the relationship among them is currently unclear.

It was recently reported that the cTEC lineage cells have their own potent regenerative capacity after the specific ablation of cTECs in the adult thymus, although it remains to be seen whether the activity depends on the cTEC stem cells or common TEC progenitors (Rode and Boehm, 2012). Thus, it might be possible that the maintenance and regeneration of cTECs and mTECs, which have quite different turnover rates (Dumont-Lagacé et al., 2014), are supported autonomously and independently mainly via respective lineage-restricted stem cells throughout the adult stage. Such a mechanism might be feasible and secure for assuring the distinct functions of the thymic cortex and medulla in T cell development. Multipotency is considered an important aspect of stem cells. However, it is suggested that specific epithelial cell lineages in certain adult tissues such as skin are maintained mainly by their own stem cells, although these restricted stem cells might retain multipotency and reveal their potential of plasticity to reconstitute all the lineages when tissue homeostasis is severely disrupted (Barker et al., 2010). Regarding the adult mammary gland, it was recently reported that the maintenance of different types of epithelial cells depends on unipotent stem cells for each cell type, rather than embryonic common multipotent progenitor/stem cells (Van Keymeulen et al., 2011). A developmental shift from multipotent to unipotent compartment-specific stem cells might be a common feature in certain organs, including the thymus.

The thymic organ shows progressive involution beginning early in adult life and is eventually replaced by fat tissues at a later stage (Manley et al., 2011). Although its exact mechanisms still remain elusive, it is implied that qualitative and quantitative changes in the thymic stroma including TECs play crucial roles and that such changes actually begin soon after birth, much before the recognizable thymic tissue involution (Gray et al., 2006; Shanley et al., 2009). Our current results indicate that mTEC clonogenic units decrease rapidly after birth. At the age of 4 weeks or older, the macroscopically visible large TEC colonies became undetectable, although smaller TEC colonies continued to be detected in very low numbers. The effect could be attributable to the loss of clonogenic TECs or their decreased self-renewal potential, or both. In this aspect, it is noteworthy that the SSEA-1$^+$Cld3,4^{hi} TECs isolated from 4-week-old mice showed remarkably lower potential for generating mature mTECs in vivo in a thymic microenvironment than the corresponding TECs in the embryonic thymi, and the majority of them retained immature phenotypes. The results suggest that the activity and/or rate of generating mature progenies of SSEA-1$^+$Cld3,4^{hi} mTECSCs is markedly reduced after birth, in concordance with the rapid decrease of their clonogenic activity in the culture. Age-dependent changes in stem cell activity have been reported in other tissue systems, even during early postnatal stages. For instance, it was shown that hematopoietic stem cells show extensive self-renewal until 3 weeks of age in mice to expand the stem cell population, but that these cells are switched to a more quiescent state at later stages (Bowie et al., 2006, 2007). Thus, in the adult stage, lower self-renewing activity and slower progeny-generating potential of the mTECSCs, which have robustly expanded during perinatal stage, might suffice for generating functional mTECs enough to maintain T cell self-tolerance.

The differentiation and maturation of mTECs crucially depend on mature T cells (Shores et al., 1991) and, accordingly, the thymus of *Rag2*$^{-/-}$ mice show poor development of mTECs (Holländer et al., 1995). Intriguingly, however, the mTEC clonogenic activities in *Rag2*$^{-/-}$ mice were sustained at much higher levels than in WT mice, even during the adult stage. Moreover, the SSEA-1$^+$Cld3,4^{hi} TEC fraction of NB *Rag2*$^{-/-}$ mice revealed much more efficient reconstitution and generation of mature mTECs in vivo in a WT thymic microenvironment than the counterpart TEC fraction of NB WT mice. Therefore, the rapid decrease of mTECSC activity after birth in WT mice should be closely linked to the concomitant active thymopoiesis. It could be a direct consequence of the strong and persistent drive toward mTEC differentiation by thymic T cells (Anderson and Takahama, 2012) or an indirect effect attributable to "unusual" microenvironmental stresses related to the robust thymocyte proliferation and massive cell death (Shortman et al., 1990). In either case, we propose that a rapid postnatal change in mTECSC activity might be an inevitable cost of highly active thymopoiesis occurring during the perinatal stage to establish the functional T cell immune system soon after birth. This might be also reminiscent of the long persistence of bipotent TEC progenitors in the adult FoxN1-deficient thymus with the complete defect of thymopoiesis (Bleul et al., 2006).

Because TECs, particularly mTECs, are highly susceptible to cytoablative treatments (Fletcher et al., 2009; Kelly et al.,

2008), thymic renewal by TEC regeneration is an important part of immune reconstitution after various cytoablative therapies such as bone marrow transplantation (Hakim et al., 2005; Mackall et al., 1995). Importantly, significant regenerative potential of the involuted or injured thymus has been reported (Alpdogan et al., 2006; Kelly et al., 2008; Min et al., 2007). Our results might provide a clue for novel strategies to restore immune function in various clinical settings and to control immune dysfunction and autoimmunity related to diseases and aging.

EXPERIMENTAL PROCEDURES

Thymic Reaggregation and Implantation

Thymic lobes were enzymatically treated in single-cell suspension, followed by staining with various antibodies, and the TEC fraction was defined as $CD45^{-}Ter119^{-}EpCAM^{+}$. TEC subpopulations were sorted with FACSAria II (BD Biosciences) or MoFlo Astrios (Beckman Coulter) as described (Hamazaki et al., 2007; Seach et al., 2012). Sorted TECs ($0.1–2 \times 10^4$ cells) were incubated together with total thymic cell suspensions (1×10^6 cells) from E14.5 WT or *aly/aly* mice on filters (Whatman) floated in RPMI-1640 medium with 10% fetal calf serum (FCS) for 24 hr, and the resulting cell reaggregates (organoids) were implanted under the kidney capsules of anesthetized nude mice. This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University (MedKyo14049).

Cell Culture

STO cells (obtained from Riken BRC) were cultured in DMEM supplemented with 10% fetal calf serum at a density of 2.5×10^4 cells cm^{-2} in a 60 mm dish and treated with 10 $\mu g\ ml^{-1}$ mitomycin-C (Kyowa Hakko Kirin) before being used as feeder cells.

TEC Clonogenic Assay

Single-cell suspensions of total $CD45^{-}$ thymic cells or TEC subpopulations were cultured in 60 mm dishes in the presence of STO feeder cells pretreated with 10 $\mu g\ ml^{-1}$ mitomycin C in serum-free medium, 3:1 mixture of DMEM, and Ham's F-12 and supplemented with 10% KnockOut Serum Replacement (KSR) (Invitrogen), 0.4 $\mu g\ ml^{-1}$ hydrocortisone, 10^{-10} M choleratoxin (Wako), 5 $\mu g\ ml^{-1}$ insulin, 2×10^{-9} M 3,3,5-triido-L-thyronine (Sigma), and 10 ng ml^{-1} recombinant mouse epidermal growth factor (Invitrogen). Unless otherwise specified, initial plating density of purified TECs was approximately 1,000 cells per 6 cm plate to make sure that each colony was derived from a single cell. Medium was changed every 3 days. To enumerate the colonies, the cultures were fixed with 3.7% formalin and stained with 1% Rhodamine-B. For further passages, individual large colonies (<2 mm) were isolated with cylinder rings, and the cells of single colonies were transferred to the secondary cultures. Replating of the colony was defined as appearance of at least five secondary large colonies. For the functional study, the cultures were treated with mild CTK solution containing 1 mg/ml collagenase, 0.25% trypsin, 20% KSR, and 1 mM $CaCl_2$ in phosphate-buffered saline to detach the feeder cells, and remaining colonies were collected with regular trypsin (0.25%)/EDTA (0.91 mM).

Intrathymic Injection in Organ Culture

Primary TEC colony suspensions from E14.5 EGFP Tg mice were injected into NB WT thymic lobes (5,000 cells in 2 μl per lobe), and the lobes were cultured on filters floated in RPMI-1640 medium with 10% FCS. Five days later, 12 to 16 lobes were pooled, digested in single-cell suspension, and analyzed with FACS CantoII (BD Biosciences) with FACS DIVA.

Histological Examination

Organs from *nu/nu* recipients bearing ectopic thymi were fixed and stained with hematoxylin and eosin, and the degrees of cell infiltration were scored as follows: 0, no detectable infiltration; 1, a focus of perivascular infiltration; 2, several foci of perivascular infiltration; 3, cellular infiltration in more than 50% of vasculature; 4, cellular infiltration in more than 80% of vasculature; and 5, massive infiltration throughout the interstitial regions.

Semiquantitative RT-PCR

Total RNA was isolated from freshly prepared or cultured TECs with TRIzol reagent (Invitrogen), reverse-transcribed with SuperScript III with random primers (Invitrogen), and amplified with Takara Ex Taq (Takara Bio).

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.10.011.

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REFERENCES

Akiyama, T., Shinzawa, M., and Akiyama, N. (2012). TNF receptor family signaling in the development and functions of medullary thymic epithelial cells. Front. Immunol. *3*, 278.

Alpdogan, O., Hubbard, V.M., Smith, O.M., Patel, N., Lu, S., Goldberg, G.L., Gray, D.H., Feinman, J., Kochman, A.A., Eng, J.M., et al. (2006). Keratinocyte growth factor (KGF) is required for postnatal thymic regeneration. Blood *107*, 2453–2460.

Anderson, G., and Jenkinson, E.J. (2001). Lymphostromal interactions in thymic development and function. Nat. Rev. Immunol. *1*, 31–40.

Anderson, G., and Takahama, Y. (2012). Thymic epithelial cells: working class heroes for T cell development and repertoire selection. Trends Immunol. *33*, 256–263.

Anderson, M.S., Venanzi, E.S., Klein, L., Chen, Z., Berzins, S.P., Turley, S.J., von Boehmer, H., Bronson, R., Dierich, A., Benoist, C., and Mathis, D. (2002). Projection of an immunological self shadow within the thymus by the aire protein. Science *298*, 1395–1401.

Baik, S., Jenkinson, E.J., Lane, P.J., Anderson, G., and Jenkinson, W.E. (2013). Generation of both cortical and Aire(+) medullary thymic epithelial compartments from CD205(+) progenitors. Eur. J. Immunol. *43*, 589–594.

Barker, N., Bartfeld, S., and Clevers, H. (2010). Tissue-resident adult stem cell populations of rapidly self-renewing organs. Cell Stem Cell *7*, 656–670.

Blanpain, C., Lowry, W.E., Geoghegan, A., Polak, L., and Fuchs, E. (2004). Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. Cell *118*, 635–648.

Bleul, C.C., Corbeaux, T., Reuter, A., Fisch, P., Mönting, J.S., and Boehm, T. (2006). Formation of a functional thymus initiated by a postnatal epithelial progenitor cell. Nature *441*, 992–996.

Boehm, T., Scheu, S., Pfeffer, K., and Bleul, C.C. (2003). Thymic medullary epithelial cell differentiation, thymocyte emigration, and the control of autoimmunity require lympho-epithelial cross talk via LTbetaR. J. Exp. Med. *198*, 757–769.

Bonfanti, P., Claudinot, S., Amici, A.W., Farley, A., Blackburn, C.C., and Barrandon, Y. (2010). Microenvironmental reprogramming of thymic epithelial cells to skin multipotent stem cells. Nature *466*, 978–982.

Bowie, M.B., McKnight, K.D., Kent, D.G., McCaffrey, L., Hoodless, P.A., and Eaves, C.J. (2006). Hematopoietic stem cells proliferate until after birth and

show a reversible phase-specific engraftment defect. J. Clin. Invest. *116*, 2808–2816.

Bowie, M.B., Kent, D.G., Dykstra, B., McKnight, K.D., McCaffrey, L., Hoodless, P.A., and Eaves, C.J. (2007). Identification of a new intrinsically timed developmental checkpoint that reprograms key hematopoietic stem cell properties. Proc. Natl. Acad. Sci. USA *104*, 5878–5882.

Corbeaux, T., Hess, I., Swann, J.B., Kanzler, B., Haas-Assenbaum, A., and Boehm, T. (2010). Thymopoiesis in mice depends on a Foxn1-positive thymic epithelial cell lineage. Proc. Natl. Acad. Sci. USA *107*, 16613–16618.

Depreter, M.G., Blair, N.F., Gaskell, T.L., Nowell, C.S., Davern, K., Pagliocca, A., Stenhouse, F.H., Farley, A.M., Fraser, A., Vrana, J., et al. (2008). Identification of Plet-1 as a specific marker of early thymic epithelial progenitor cells. Proc. Natl. Acad. Sci. USA *105*, 961–966.

Dumont-Lagacé, M., Brochu, S., St-Pierre, C., and Perreault, C. (2014). Adult thymic epithelium contains nonsenescent label-retaining cells. J. Immunol. *192*, 2219–2226.

Fletcher, A.L., Lowen, T.E., Sakkal, S., Reiseger, J.J., Hammett, M.V., Seach, N., Scott, H.S., Boyd, R.L., and Chidgey, A.P. (2009). Ablation and regeneration of tolerance-inducing medullary thymic epithelial cells after cyclosporine, cyclophosphamide, and dexamethasone treatment. J. Immunol. *183*, 823–831.

Gray, D.H., Seach, N., Ueno, T., Milton, M.K., Liston, A., Lew, A.M., Goodnow, C.C., and Boyd, R.L. (2006). Developmental kinetics, turnover, and stimulatory capacity of thymic epithelial cells. Blood *108*, 3777–3785.

Hakim, F.T., Memon, S.A., Cepeda, R., Jones, E.C., Chow, C.K., Kasten-Sportes, C., Odom, J., Vance, B.A., Christensen, B.L., Mackall, C.L., and Gress, R.E. (2005). Age-dependent incidence, time course, and consequences of thymic renewal in adults. J. Clin. Invest. *115*, 930–939.

Hamazaki, Y., Fujita, H., Kobayashi, T., Choi, Y., Scott, H.S., Matsumoto, M., and Minato, N. (2007). Medullary thymic epithelial cells expressing Aire represent a unique lineage derived from cells expressing claudin. Nat. Immunol. *8*, 304–311.

Holländer, G.A., Wang, B., Nichogiannopoulou, A., Platenburg, P.P., van Ewijk, W., Burakoff, S.J., Gutierrez-Ramos, J.C., and Terhorst, C. (1995). Developmental control point in induction of thymic cortex regulated by a subpopulation of prothymocytes. Nature *373*, 350–353.

Jenkinson, W.E., Bacon, A., White, A.J., Anderson, G., and Jenkinson, E.J. (2008). An epithelial progenitor pool regulates thymus growth. J. Immunol. *181*, 6101–6108.

Kajiura, F., Sun, S., Nomura, T., Izumi, K., Ueno, T., Bando, Y., Kuroda, N., Han, H., Li, Y., Matsushima, A., et al. (2004). NF-kappa B-inducing kinase establishes self-tolerance in a thymic stroma-dependent manner. J. Immunol. *172*, 2067–2075.

Kelly, R.M., Highfill, S.L., Panoskaltsis-Mortari, A., Taylor, P.A., Boyd, R.L., Holländer, G.A., and Blazar, B.R. (2008). Keratinocyte growth factor and androgen blockade work in concert to protect against conditioning regimen-induced thymic epithelial damage and enhance T-cell reconstitution after murine bone marrow transplantation. Blood *111*, 5734–5744.

Mackall, C.L., Fleisher, T.A., Brown, M.R., Andrich, M.P., Chen, C.C., Feuerstein, I.M., Horowitz, M.E., Magrath, I.T., Shad, A.T., Steinberg, S.M., et al. (1995). Age, thymopoiesis, and CD4+ T-lymphocyte regeneration after intensive chemotherapy. N. Engl. J. Med. *332*, 143–149.

Manley, N.R., Richie, E.R., Blackburn, C.C., Condie, B.G., and Sage, J. (2011). Structure and function of the thymic microenvironment. Front Biosci (Landmark Ed) *16*, 2461–2477.

Min, D., Panoskaltsis-Mortari, A., Kuro-O, M., Holländer, G.A., Blazar, B.R., and Weinberg, K.I. (2007). Sustained thymopoiesis and improvement in functional immunity induced by exogenous KGF administration in murine models of aging. Blood *109*, 2529–2537.

Mouri, Y., Yano, M., Shinzawa, M., Shimo, Y., Hirota, F., Nishikawa, Y., Nii, T., Kiyonari, H., Abe, T., Uehara, H., et al. (2011). Lymphotoxin signal promotes thymic organogenesis by eliciting RANK expression in the embryonic thymic stroma. J. Immunol. *186*, 5047–5057.

Ohigashi, I., Zuklys, S., Sakata, M., Mayer, C.E., Zhanybekova, S., Murata, S., Tanaka, K., Holländer, G.A., and Takahama, Y. (2013). Aire-expressing thymic medullary epithelial cells originate from β5t-expressing progenitor cells. Proc. Natl. Acad. Sci. USA *110*, 9885–9890.

Peterson, P., and Laan, M. (2013). Bipotency of thymic epithelial progenitors comes in sequence. Eur. J. Immunol. *43*, 580–583.

Rode, I., and Boehm, T. (2012). Regenerative capacity of adult cortical thymic epithelial cells. Proc. Natl. Acad. Sci. USA *109*, 3463–3468.

Rodewald, H.R., Paul, S., Haller, C., Bluethmann, H., and Blum, C. (2001). Thymus medulla consisting of epithelial islets each derived from a single progenitor. Nature *414*, 763–768.

Rossi, S.W., Jenkinson, W.E., Anderson, G., and Jenkinson, E.J. (2006). Clonal analysis reveals a common progenitor for thymic cortical and medullary epithelium. Nature *441*, 988–991.

Seach, N., Wong, K., Hammett, M., Boyd, R.L., and Chidgey, A.P. (2012). Purified enzymes improve isolation and characterization of the adult thymic epithelium. J. Immunol. Methods *385*, 23–34.

Shakib, S., Desanti, G.E., Jenkinson, W.E., Parnell, S.M., Jenkinson, E.J., and Anderson, G. (2009). Checkpoints in the development of thymic cortical epithelial cells. J. Immunol. *182*, 130–137.

Shanley, D.P., Aw, D., Manley, N.R., and Palmer, D.B. (2009). An evolutionary perspective on the mechanisms of immunosenescence. Trends Immunol. *30*, 374–381.

Shinkura, R., Matsuda, F., Sakiyama, T., Tsubata, T., Hiai, H., Paumen, M., Miyawaki, S., and Honjo, T. (1996). Defects of somatic hypermutation and class switching in alymphoplasia (aly) mutant mice. Int. Immunol. *8*, 1067–1075.

Shores, E.W., Van Ewijk, W., and Singer, A. (1991). Disorganization and restoration of thymic medullary epithelial cells in T cell receptor-negative scid mice: evidence that receptor-bearing lymphocytes influence maturation of the thymic microenvironment. Eur. J. Immunol. *21*, 1657–1661.

Shortman, K., Egerton, M., Spangrude, G.J., and Scollay, R. (1990). The generation and fate of thymocytes. Semin. Immunol. *2*, 3–12.

Solter, D. (2006). From teratocarcinomas to embryonic stem cells and beyond: a history of embryonic stem cell research. Nat. Rev. Genet. *7*, 319–327.

Swann, J.B., and Boehm, T. (2007). Back to the beginning—the quest for thymic epithelial stem cells. Eur. J. Immunol. *37*, 2364–2366.

Tsubata, R., Tsubata, T., Hiai, H., Shinkura, R., Matsumura, R., Sumida, T., Miyawaki, S., Ishida, H., Kumagai, S., Nakao, K., and Honjo, T. (1996). Autoimmune disease of exocrine organs in immunodeficient alymphoplasia mice: a spontaneous model for Sjögren's syndrome. Eur. J. Immunol. *26*, 2742–2748.

Ucar, A., Ucar, O., Klug, P., Matt, S., Brunk, F., Hofmann, T.G., and Kyewski, B. (2014). Adult thymus contains FoxN1(-) epithelial stem cells that are bipotent for medullary and cortical thymic epithelial lineages. Immunity *41*, 257–269.

Van Keymeulen, A., Rocha, A.S., Ousset, M., Beck, B., Bouvencourt, G., Rock, J., Sharma, N., Dekoninck, S., and Blanpain, C. (2011). Distinct stem cells contribute to mammary gland development and maintenance. Nature *479*, 189–193.

White, A.J., Nakamura, K., Jenkinson, W.E., Saini, M., Sinclair, C., Seddon, B., Narendran, P., Pfeffer, K., Nitta, T., Takahama, Y., et al. (2010). Lymphotoxin signals from positively selected thymocytes regulate the terminal differentiation of medullary thymic epithelial cells. J. Immunol. *185*, 4769–4776.

Wong, K., Lister, N.L., Barsanti, M., Lim, J.M., Hammett, M.V., Khong, D.M., Siatskas, C., Gray, D.H., Boyd, R.L., and Chidgey, A.P. (2014). Multilineage potential and self-renewal define an epithelial progenitor cell population in the adult thymus. Cell Rep. *8*, 1198–1209.

Yano, M., Kuroda, N., Han, H., Meguro-Horike, M., Nishikawa, Y., Kiyonari, H., Maemura, K., Yanagawa, Y., Obata, K., Takahashi, S., et al. (2008). Aire controls the differentiation program of thymic epithelial cells in the medulla for the establishment of self-tolerance. J. Exp. Med. *205*, 2827–2838.