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*J Immunol* published online 13 April 2015
http://www.jimmunol.org/content/early/2015/04/11/jimmunol.1403158

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2015/04/11/jimmunol.1403158.DCSupplemental

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Young, Proliferative Thymic Epithelial Cells Engraft and Function in Aging Thymuses

Mi-Jeong Kim,*‡ Christine M. Miller,*†‡§ Jennifer L. Shadrach,*†‡§ Amy J. Wagers,*†‡§ and Thomas Serwold*†

The thymus reaches its maximum size early in life and then begins to shrink, producing fewer T cells with increasing age. This thymic decline is thought to contribute to age-related T cell lymphopenias and hinder T cell recovery after bone marrow transplantation. Although several cellular and molecular processes have been implicated in age-related thymic involution, their relative contributions are not known. Using heterochronic parabiosis, we observe that young circulating factors are not sufficient to drive regeneration of the aged thymus. In contrast, we find that resupplying young, engraftable thymic epithelial cells (TECs) to a middle-aged or defective thymus leads to thymic growth and increased T cell production. Intrathymic transplantation and in vitro colony-forming assays reveal that the engraftment and proliferative capacities of TECs diminish early in life, whereas the receptivity of the thymus to TEC engraftment remains relatively constant with age. These results support a model in which thymic growth and subsequent involution are driven by cell-intrinsic changes in the proliferative capacity of TECs, and further show that young TECs can engraft and directly drive the growth of inviolated thymuses. 

The Journal of Immunology, 2015, 194: 000–000.

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Received for publication December 18, 2014. Accepted for publication March 9, 2015.

This work was supported by a Mary K. Iacocca Foundation Research Fellowship (to M.-J.K.), a Harvard Stem Cell Institute seed grant and a Harvard Stem Cell Institute program project grant (to T.S.), the Peabody Foundation (to T.S.), the Alexander and Margaret Stewart Trust (to T.S.), The Fleischer Foundation (to T.S.), The Pittsburgh Foundation (to T.S.), the Glenn Foundation for Medical Research (to A.J.W.), the National Institutes of Health (Grants R01 AG033053, 1DP2 OD004345, and 5U01 HL100402 to A.J.W.), and the National Institutes of Health Diabetes Research Center (Grant P30DK03636).

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The online version of this article contains supplemental material.

Abbreviations used in this article: cTEC, cortical TEC; E, embryonic day; EpCAM, epithelial cell adhesion molecule; ETP, early thymic progenitor; HSC, hematopoietic stem cell; MHCI, MHC class I; MHCI, MHC class II; mTEC, medullary TEC; P, postnatal day; PFA, paraformaldehyde; RFP, red fluorescent protein; TEC, thymic epithelial cell; UEA, Ulex europaeus agglutinin I; WT, wild type.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1403158
These studies suggested, but did not directly show, that the thymic microenvironment becomes progressively more toxic with age, and that gradual accumulation of adipocytes may be at least partially responsible for age-related thymic decline.

Loss of function of TECs with age has been specifically implicated in thymic involution (24, 25). TECs play multiple essential roles in driving T cell development; they express stem cell factor (c-Kit ligand), IL-7, Delta-like 4 (Dll4), and high levels of MHC class I (MHCⅠ) and class II molecules (MHCⅡ), all of which are essential for normal T cell development (26–29). TECs also express several different chemokines that recruit T cell progenitors from the blood and direct their migration within the thymus (30). Several lines of evidence suggest that the proliferation and function of TECs dictates thymus size. The most striking evidence of the importance of TECs in determining thymus size is the phenotypes of mice in which TECs are aberrantly proliferative. Murine TECs that transgenically overexpress the cell cycle protein, Cyclin D1, as well as TECs that are deficient in the Retinoblastoma cell cycle regulator, are hyperproliferative (31, 32). The thymuses of these genetically modified mice are functionally and architecturally normal but grow continuously and eventually cause death by asphyxiation. From these studies, it is clear that regulation of TEC proliferation is critical for maintaining normal thymus size.

Expression of the transcription factor Foxn1 is required for TEC differentiation, and mice lacking Foxn1 completely lack thymus (33). Experiments using blastocyst chimeric mice containing mixtures of wild type (WT) and Foxn1-deficient embryonic stem cells found that thymus size was dependent upon the absolute number of functional thymic epithelial progenitors within the embryo (34). Foxn1 itself appears to be downregulated in TECs with age, and restoration of Foxn1 expression within the aging thymus can drive thymus regeneration (35, 36). This observation suggested that loss of TEC function with age plays an important role in thymic involution; however, it was unclear whether the loss of TEC function resulted from acute environmental signals that drove changes in TEC function, or whether TECs underwent programmed, intrinsic changes that led to their dysfunction.

In this study, we sought to determine the relative importance of systemic, circulating, and thymus intrinsic factors in determining thymus size during aging, and we further sought to directly test whether the involuted thymus could be induced to grow by transplanted, functional TECs. We used heterochronic parabiosis to assess whether age-related changes in circulating factors or hematopoietic cells impact endogenous thymus size. We also used intrathymic transplantation and in vitro colony-forming assays to evaluate age-related changes in TEC proliferative and engraftment capacity, as well as age-related changes in the thymic microenvironment. These experiments establish the utility of intrathymic transplantation of TECs as a means of evaluating the thymic microenvironment, characterizing TEC developmental potential, and as a means of modulating T cell development. Intrathymic transplantation of TECs reveals that the involuted and young intrathymic environments are equally receptive to TECs, and it also reveals that TECs incur cell-intrinsic reductions in engraftability potential with age; moreover, resupplying young, engraftable TECs to involuted thymuses of middle-aged mice leads to renewed thymic growth and T cell production.

Materials and Methods

**Animals**

All the mice used in this study were derived from the C57BL/6 background. Retired breeder female mice (middle-aged) were purchased from Taconic. GFP-transgenic mice contained a GFP transgene under the control of the β-actin promoter (37). Red fluorescent protein (RFP)-transgenic mice contained a CAG promoter driving RFP inserted into the Rosa locus (38). Aged mice for parabiosis experiments (21–23 mo) were obtained from the National Institute of Aging. Young CD45.1 and CD45.2 C57BL/6 mice, as well as MHCI−/− mice, were obtained from The Jackson Laboratory. All mice were housed in the Joslin Diabetes Center animal facility. The Joslin Institutional Animal Care and Use Committee approved all experimental protocols involving mice.

**Parabiosis**

Parabiosis was performed as previously described (39, 40). Sex-matched C57BL/6 mice were used for parabiosis. Young C57BL/6 (2 mo) or aged C57BL/6 mice (21–23 mo) were parabiosed to young C57BL/6, SJL mice (2 mo, CD45.1) to enable detection of donor hematopoietic chimerism. For isochronic old pairs, CD45 distinction was not possible because aged CD45.1 mice were not available.

**TEC isolation**

The thymus was removed from the thoracic cavity and put into DMEM/F12 (Life Technologies) medium, where it was minced into small pieces using a scalpel, followed by rocking for 3 min. The released thymocytes were removed, and fresh DMEM/F12 was added to the remaining thymic tissue fragments. Tissue fragments were incubated with collagenase and Dispase (5 mg/ml; Roche Diagnostics) or collagenase IV (0.25 mg/ml) and papain (0.25 mg/ml; Worthington Biochemical Corporation) and DNase I (0.1 mg/ml; Roche Diagnostics) for 30 min at 37°C. Digested tissue samples were pipetted up and down using a wide-bore pipet tip and filtered through 70-μm nylon mesh to remove any undigested fragments of tissue. Dispersed cells were washed with PBS containing 2% bovine calf serum (HyClone).

**Flow cytometry**

TECs were stained with Abs to CD45, epithelial cell adhesion molecule (EpCAM), Ly-51, Ulex europaeus agglutinin I (UEA), MHCI, and CD80. In some experiments, as noted in the text, single thymic cells in suspension were stained with allophtocyanin/Cy7 anti-mouse EpCn (Biolegend) and anti-Cy7 MicroBeads (Miltenyi), and EpCAM+ TECs were positively selected by using a magnetic column (Miltenyi). For analysis of thymocytes and splenocytes, tissues were dispersed by grinding between frosted slides, and RBCs were removed by lysis in ACK solution (150 mM NH₄Cl, 10 mM Tris, 0.1 mM KHCO₃; 0.1 mM EDTA). Abs for all stains are listed in Supplemental Table 1. Stained cells were analyzed on either an LSRII flow cytometer (BD Biosciences) or sorted on a FACSAria cell sorter (BD Biosciences). The flow-cytometry data were analyzed using FlowJo (Tree Star).

**Intrathymic transplantation**

Total dispersed thymic cells or EpCAM+ or EpCAM− cells from fetal or postnatal mice were prepared as described in the earlier TEC Isolation and Flow Cytometry sections. Cells were kept on ice until mice were ready for the injection. Intrathymic injections were performed as previously described (41). Mice were anesthetized by i.p. injection of 250 mg/kg body weight 2,2'-dibromothanol (Avertin; Sigma) in saline. The unconscious mice were then placed in supine position, and a small incision was made by cutting 0.5 cm of the skin and manubrium to expose thymus within the thoracic cavity. Five to 10 μl of cells in suspension were drawn into a Hamilton syringe equipped with a 30-gauge needle (Fisher Scientific) and then injected into one thymic lobe. One or two wound clips (Fisher Scientific) were applied to close the incision. The surgery survival rate was >99%. The recipient thymuses with donor GFP+ or RFP+ cells were recovered from mice at the specified time points (e.g., 1 mo postintrathymic transplantation), and each thymus was examined for the presence of donor GFP+ or RFP+ cells, using an Olympus IX51 inverted fluorescence microscope (Olympus) attached with D727 camera with cellSens software (Olympus).

**In vitro culture of TECs**

Sorted GFP+ TECs were plated onto irradiated murine embryonic fibroblasts (60 Gy) and cultured in MCDB153 medium (Sigma) supplemented with 10% FBS (HyClone), 50 U/ml penicillin and 50 μg/ml streptomycin (Life Technologies), 2 mM l-glutamine (Life Technologies) and 1 mM sodium pyruvate (Life Technologies), and 10 μM p16ROCK inhibitor, Y-27632 (Tocris Bioscience). TEC colonies were imaged using an inverted fluorescence microscope (Olympus) or by colony staining. For colony staining, cultured TECs were fixed in 4% paraformaldehyde (PFA) for 10 min, washed in PBS, and then stained with 1% Rhodamine B (Sigma) in PBS at 60°C for 10 min. Images were taken with a Stylus Verve 5.0 camera (Olympus).

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Histology
Recipient thymuses that received GFP<sup>+</sup> or RFP<sup>+</sup> donor cells by intrathymic injection were removed and immediately fixed in 4% PFA at 4°C over-night. The fixed tissues were immersed in graded sucrose solutions and then embedded in OCT compound (Sakura). Frozen tissues were sectioned (5–10 μm) using a cryostat (Microm HM 550; Thermo Scientific). Sections were fixed in cold acetone, washed in PBS, and mounted in Fluoromount-G (Southern Biotech) medium, and were examined to locate sections containing donor GFP<sup>+</sup> or RFP<sup>+</sup> cells. For some experiments, sections were stained with the lectin UEA, or with Abs to Psmb11 or I-A/I-E (MHCI). Nuclei were visualized using DAPI (Sigma). Epifluorescence images were taken using an Axioplan 2 imaging microscope (Zeiss) equipped with a SPOT digital camera and software (SPOT Imaging), and confocal images were taken with an LSM 710 Duo (Zeiss) confocal microscope with an AxioCam camera (Zeiss). Serial images were compiled using the Z-stack function. The Adobe Photoshop CS4 program was used to convert color images to grayscale. Tile and binary images were generated using BZ-X700 microscope (Keyence).

Results

Parabiosis of young and old mice does not cause regeneration of the aged thymus
To evaluate globally whether circulating factors and cells that change with age control thymus size, we analyzed the thymuses of young and old C57BL/6 mice that had been joined in heterochronic parabiosis, a surgical intervention that produces a shared circulation between two animals. The parabiotic model has been used extensively to investigate the influence of bloodborne signals on aging phenotypes, and such studies have identified both cellular (42) and molecular mechanisms underlying the aging and rejuvenation of a variety of tissues (40, 43–46). Parabiotic mice begin sharing a circulatory system by 2–3 d after joining and achieve a steady-state of ~50% chimerism for circulating hematopoietic cell lineages in 7–10 d. Thus, we reasoned that if an age-variant cell type or factor circulating in the blood contributes substantially to the age-related decline of thymus size, then animals joined in heterochronic parabiosis should show changes in thymus size when compared with isochronically joined (either young-young or aged-aged) control pairs. Mice were joined at 2 mo of age (young) or 21–23 mo of age (aged), and their thymuses were analyzed 1 mo after joining. The young-isochronic and heterochronic pairs were congenic at their CD45 loci, enabling determination of the contribution of partner-derived thymus seeding cells using Abs to CD45.1 and CD45.2. We could not generate CD45 congenic pairs of old-isochronic mice because of the unavailability of aged CD45.1 animals; however, prior experience with this model indicates that old-isochronic animals develop peripheral chimerism at a rate that is indistinguishable from young-isochronic and heterochronic pairs (42).

The thymuses of all heterochronic and isochronic parabiotic mice became chimeric with their partner’s cells, to varying degrees (Fig. 1A, 1B). Furthermore, partner-derived thymocytes contributed to each major thymocyte subset, including all the immature double-negative subsets that are distinguished by c-kit and CD25 (DN1–DN4), as well as the CD4<sup>+</sup>CD8<sup>−</sup> subset, and the more mature single-positive subsets. These results are consistent with previous parabiotic experiments showing that blood-derived thymus seeding cells cross the parabiotic anastomoses and seed the partner’s thymus, where they undergo apparently normal T-lineage differentiation (47). Isochronic young parabionts averaged 9–13% thymus chimerism (range 1–50%; Fig. 1B). In contrast, heterochronic parabionts showed significantly increased contribution of young CD45.1 cells to the aged thymuses (average chimerism = 57%, range 6–72%) and a significantly decreased contribution of aged cells into the young thymuses (average chimerism = 3%, range 0–12%). These dramatic differences in thymocyte contribution between the heterochronic parabionts are unlikely to be attributable to the congenic CD45 alleles, because congenic pairs of young-isochronic parabionts showed equivalent cross-seeding, and instead suggest a significant competitive advantage of young thymus seeding cells and/or thymocytes in comparison with analogous populations of aged cells.

Despite the substantial engraftment of young thymocytes into the aged thymuses of heterochronic parabionts, the thymuses of the aged partners did not show an increase in total cellularity (Fig. 1C). In fact, the aged partners within the heterochronic pairs had significantly smaller thymuses than the aged-isochronic parabionts. These results indicate that the younger mice contain more efficient thymus seeding progenitors than old mice, but that the young circulatory system and progenitors are not, by themselves, capable of driving regeneration of the aged thymus. Furthermore, there was no correlation between donor chimerism and thymus cellularity; even the aged parabionts with the highest percentage of young thymocyte chimerism did not show increased thymic cellularity (Supplemental Fig. 1). The failure of the young circulating system to regenerate the aged thymus and the inability of the aged circulatory system to shrink the young thymus suggest that the major determinants of thymus size in aged mice are not circulating, and instead are likely intrinsic to the thymus.

TECs engraft and function after intrathymic injection

Thymus intrinsic components include stromal cells such as mesenchymal, epithelial, and endothelial cells, as well the extracellular matrix. To directly evaluate the role of thymus intrinsic components in age-related thymic decline, we established a new system for intrathymic injection of TECs, as well as other stromal cells. Intrathymic injection of hematopoietic progenitors is a well-established technique for studying the differentiation of T cell progenitors, but this method has not been widely applied to the study of thymic stromal components (48). To test whether intrathymically injected thymic stem cells could engraft and function, we isolated fetal thymic cells from embryonic days (E) 15–17 embryos that ubiquitously expressed GFP under the β-actin promoter (37). The fetal thymuses were proteolytically dissociated into single cells and injected intrathymically into MHCI<sup>−</sup><sup>−</sup> mice, which fail to efficiently drive positive selection of CD4<sup>+</sup> T cells and have both thymic and peripheral deficiencies of CD4<sup>+</sup> T cells (49). Thirty days after intrathymic injection of WT fetal thymic cells, positive selection of CD4<sup>+</sup> T cells was partially restored in the thymuses of MHCI<sup>−</sup><sup>−</sup> mice (Fig. 2A, 2B). This recovery of CD4<sup>+</sup> T cell selection was also reflected in the periphery, where the frequency of naive CD4<sup>+</sup> T cells increased significantly (Fig. 2C). The ability of intrathymically injected fetal thymic cells to rescue CD4<sup>+</sup> T cell development in MHCI<sup>−</sup><sup>−</sup> mice suggested that WT MHCI<sup>+</sup> donor cells had engrafted in the recipient thymuses. Moreover, because MHCI-expressing TECs are the major drivers of T cell positive selection (26), these results suggested that donor TECs functionally engrafted in the recipient thymuses after intrathymic injection.

To visualize TEC engraftment after intrathymic injection of GFP<sup>+</sup> thymic stromal cells into MHCI<sup>−</sup><sup>−</sup> mice, we analyzed the thymuses of recipient mice by immunofluorescence. Intrathymic injection of dissociated thymic cells from GFP-transgenic donors resulted in the engraftment of networks of donor-derived cells that strongly resembled TECs (Fig. 2D). In addition, injection of purified EpCAM<sup>+</sup> cells from GFP-transgenic donors into MHCI<sup>−</sup><sup>−</sup> recipients resulted in a similar pattern of engrafted GFP<sup>+</sup> donor cells, clearly indicating that purified EpCAM<sup>+</sup> TECs can engraft and establish typical TEC networks after intrathymic injection (Fig. 2E). Furthermore, donor TECs maintained MHCI expres-
sion (Fig. 2E), consistent with their ability to rescue T cell development in MHCII−/− mice. Thus, after intrathymic transplantation, TECs form networks and maintain their ability to drive T cell development.

Intrathymically transplanted TECs proliferate within the recipient thymus

Total dispersed thymic cells derived from postnatal day (P) 1 GFP-transgenic mice were intrathymically injected into MHCII−/− mice (3 mo old) and thymuses were analyzed at days 3 and 30 by fluorescence to understand TEC dynamics after transplantation. At day 3 after transplantation, donor TECs appeared as disorganized clusters of cells (Fig. 3A). In contrast, by 30 d posttransplantation, TECs were organized into large spherical clusters containing hundreds to thousands of highly interconnected cells that resembled the architecture of normal TECs (Fig. 3A). This extensive, patterned engraftment of TECs after intrathymic transplantation indicated that transplanted TECs either proliferated or reorganized within the recipient thymus.

It was important to distinguish whether the organized structures formed by donor TECs resulted from proliferation or from delayed organization of the transplanted cells. The low yield of donor TEC recovery from recipient mice precluded BrdU incorporation assays. Therefore, we performed an in vivo colony-forming assay. In this
FIGURE 2. Intrathymically injected TECs engraft and function in vivo. Thymic cells were prepared from E15 or E17 GFP-transgenic fetuses and injected into the thymuses of MHCII$^{-/-}$ mice (1–3 mo old). After 1 month, thymocytes and splenocytes of transplanted mice were analyzed. WT and nontransplanted MHCII$^{-/-}$ mice were analyzed as controls. (A) Thymocytes were stained with Abs to CD4 and CD8, and analyzed by flow cytometry. Plots are derived from representative mice analyzed in (B). (B) The plot shows the percentages of thymic CD4$^{+}$ T cells from control mice or mice intrathymically injected with E15/E17 dispersed thymic cells. Each point represents the percentage of CD4$^{+}$ T cells from one animal. Mean values are shown as a bar. (C) Splenocytes from the control and transplanted animals were stained with CD4, CD8, TCR$\beta$, CD44, and CD62L. Cells were gated on CD4$^{+}$TCR$\beta^{+}$ T cells and analyzed for CD62L and CD44 expression. Each point represents splenocytes from one animal. Mean values are shown as a bar. (D) Total thymic cells from P1 GFP-transgenic mice were intrathymically injected into 1-mo-old WT mice. After 1 month, recipient thymuses were fixed in PFA, sectioned, and examined for engraftment of GFP$^{+}$ cells by confocal microscopy. Scale bar, 20 $\mu$m. The image is representative among sections from five mice in this experiment. (E) Thymic cells derived from P3 and P5 GFP-transgenic mice were column purified to select EpCAM$^{+}$ TECs and then injected into 1-mo-old MHCII$^{-/-}$ male mice. After 1 month, thymuses were removed and PFA fixed, and thymic sections were analyzed for donor cell engraftment and MHCII expression. GFP$^{+}$ donor TECs are shown in the top panels. MHCII expression was detected by Ab staining (bottom panels). Both GFP and MHCII signals are shown in grayscale. Scale bar, 100 $\mu$m. Enlarged areas are shown at the right. The experiment was repeated >10 times, and a representative section is shown here. The $p$ values were determined using the unpaired Student $t$ test.

Transplantation of fetal thymic cells into involuted thymuses drives thymus growth and renewed T cell production

The finding that fetal thymic transplantation could rescue the defective immune function in MHCII$^{-/-}$ mice suggested the possibility that the transplantation of fetal TECs could rejuvenate involuted WT thymuses and subsequently restore robust T cell production. The parabiosis experiments were performed with 21- to 23-mo-old mice (Fig. 1); however, the fragile state of these mice precluded intrathymic injections. Therefore, to test the possibility of cell-mediated regeneration of the aged thymus, we used 9- to 12-mo-old, “middle-aged” recipient mice, which also have involuted thymuses. Thymuses from GFP-transgenic E14.5 or E15.5 fetuses were proteolytically dispersed into single cells and intrathymically injected into middle-aged female mice. After 45 d, the thymuses of the recipient mice were removed and analyzed. Thymic lobes transplanted with fetal cells were significantly larger than the nontransplanted lobes, clearly indicating that the injected fetal thymic cells drove thymic growth (Fig. 4A). The GFP$^{+}$ donor cells engrafted over a large area of the recipient lobes, as revealed by GFP fluorescence of the whole lobes (Fig. 4B, 4C, 4D).

Histological sections revealed that the engrafted cells were primarily composed of TECs that were present in stereotypical TEC networks (Fig. 5A). Immunostaining revealed that the engrafted TECs expressed MHCII and were composed of both Psmb11-expressing cortical TECs (cTECs) and UEA-expressing medullary TECs (mTECs; Fig. 5A). To further confirm donor TEC engraftment, recipient thymuses were proteolytically dispersed and analyzed by flow cytometry, which revealed a distinct population of donor-derived, CD45$^{+}$EpCAM$^{+}$GFp$^{+}$ TECs (Fig. 5B).
Thymic epithelial cells (TECs) are vital to thymus growth and development. In our study, we investigated whether engraftment of fetal thymic cells into middle-aged thymuses could drive growth and enhance the production of naive T cells. We observed substantial engraftment of donor TECs, induction of thymus growth, and increases in the production of naive T cells from endogenous, aged T cell progenitors.

FIGURE 3. Intrathymically transplanted TECs proliferate in vivo. (A) Thymic cells were isolated from P1 GFP transgenic mice and injected into 3-mo-old MHCII−/− mice. After 3 (top panels) or 30 d (bottom panels), recipient thymuses were fixed in PFA, sectioned, and analyzed for GFP fluorescence (grayscale, scale bar, 100 μm). Boxed areas are enlarged and shown on the right. Thymuses from five recipient mice were analyzed at each time point, and images from one engrafted mouse at each time point are shown. (B) Drawings show predicted outcomes of injections of mixed GFP+ and RFP+ TECs that distinguish between TECs that both form networks and proliferate (left) and TECs that form networks without proliferating (right). (C) Purified TECs from P5 GFP-transgenic and RFP-transgenic mice were co-injected into 6-mo-old MHCII−/− recipients. After 1 mo, thymuses were removed and PFA fixed, and tissue sections were examined by fluorescence microscopy (scale bar, 100 μm; n = 5 intrathymically transplanted mice).

Staining of the TECs with Abs to Ly-51 and CD80, as well as the lectin UEA, clearly identified donor-derived cTECs, as well as immature and mature mTECs subsets. Thus, injection of dispersed fetal thymic cells into thymuses of middle-aged mice results in substantial engraftment of TECs and significant growth of engrafted thymic lobes.

The transplantation of fetal thymic cells into the thymuses of middle-aged mice also led to increased production of naive T cells, as measured by the frequency of CD62L+CD4+ T cells in the spleens of recipient mice (Fig. 5C). However, the increase in splenic naive T cell frequencies did not correlate with the levels of donor-cell engraftment in recipient mice (Supplemental Fig. 2). There was also a trend toward increased naive CD8+ T cells in the transplanted mice. Overall, these data show that transplantation of fetal thymic cells into thymuses of middle-aged mice leads to substantial engraftment of donor TECs, induction of thymus growth, and increases in the production of naive T cells from endogenous, aged T cell progenitors.

Multiple cell types are present in the young thymus, including TECs, other stromal cell populations, developing thymocytes, and other hematopoietic CD45+ lineages. To distinguish whether engrafted TECs or, alternatively, a non-TEC population drives thymic growth, we sorted and transplanted into thymuses of middle-aged mice either TECs (CD45-EpCAM+) or the remaining non-TECs (CD45-EpCAM−), including all cells of the hematopoietic, mesenchymal, and endothelial lineages. We found that thymuses that received TECs exhibited growth specifically in the transplanted lobes (Fig. 6A). Interestingly, thymuses that received non-TECs, although showing no lobe-specific regeneration, also trended larger than the control recipient thymuses (Fig. 6B). Given the large degree of variability in thymus sizes between mice in this study, it is possible that the trend toward larger thymuses in the recipients of non-TECs was the result of statistical noise. In contrast with the large variability in thymic size between mice, intramouse variability in thymic lobe size was remarkably small; thus, the lobe-specific growth induced by transplanted TECs shows that these cells are uniquely potent at driving growth of involuted thymuses.

Thymus receptivity to TEC engraftment remains constant with age

With age, the thymus loses some of its characteristic organization and becomes increasingly populated with adipocytes. It has been hypothesized that the adipogenic thymic environment of the involuted thymus is hostile to thymic stromal cells and plays a role in thymic shrinkage with age (21). To test this hypothesis, we transplanted TECs from P1 GFP+ or RFP+ mice into recipient mice that ranged in age from 1 to 10 mo. At 1-mo posttransplantation, thymuses were screened for TEC engraftment. Remarkably, donor-derived TECs were equally abundant in recipient thymuses from mice of all ages (Fig. 7A, Fig. 7B). Thus, the thymic microenvironments of both young and middle-aged mice are receptive to TEC transplantation and proliferation. This result is consistent with the finding that fetal TECs engraft and drive growth of the middle-aged thymus (Figs. 4–6). Moreover, together with the results from the heterochronic parabiosis (Fig. 1), these data indicate that the middle-aged thymus remains receptive to the proliferation and development of both thymocytes and TECs.

TEC engraftment potential diminishes rapidly with donor age

Because the thymic microenvironments of both young and middle-aged mice appeared to be equally receptive to TEC transplantation and proliferation, we next asked whether TECs undergo intrinsic proliferative changes with age that might account for the small size of the aged thymus. TECs isolated from the thymuses of mice ranging in age from 1 d to 4 mo were injected into 2-mo-old recipient mice. At 1 mo posttransplantation, thymuses were harvested and donor TEC engraftment was evaluated. Thymuses transplanted with P1 TECs contained large networks of engrafted cells, whereas thymuses transplanted with TECs from 1-, 2.5-, or 4-mo-old mice contained only sparse donor cells and no network formation (Fig. 7C, Fig. 7D). These data clearly indicate that the in vivo engraftment and proliferation capacity of TECs declines rapidly with age.

The number of TEC colony-forming cells decreases rapidly with age

The striking age-related decline in TEC engraftment potential could result from diminished proliferative potential of TECs with age. Phenotypically, TECs undergo dramatic changes over the course of life (24). In the embryo, and shortly after birth, Ly-51+ cTECs predominate, whereas by 9 d after birth, UEA+ mTECs predominate (Fig. 8A). To determine whether these age-related
phenotypic changes correlate with changes in TEC proliferative capacity with age, we developed an in vitro colony-forming assay that efficiently promoted the survival and growth of TECs. This assay used a base medium optimized for epithelial cells, as well an inhibitor of rho-associated kinase (p160ROCK inhibitor Y-27632). ROCK inhibitor has been previously shown to enhance the survival of embryonic stem cells during passaging and cell sorting (50). We found that inhibition of ROCK also enhanced the survival and proliferation of sorted primary TECs in culture (Fig. 8B).

Using this colony-forming assay, we measured the proliferation of TECs sorted from mice of different ages. During 5 d in culture, TECs of E14.5 as well as P4 mice rapidly proliferated to form large colonies (Fig. 8C, Fig. 8D; plating efficiency mean = 58% for E14.5, and 45% for P4). TECs from P9 gave rise to similar colonies, but at a slightly lower plating efficiency (mean = 31%). In contrast, TECs from mice ≥1 mo were much less efficient at forming colonies in culture (plating efficiency = 2–13%), although the colonies that did arise were similar in size to the colonies derived from P4 and P9 mice. These results indicate that the frequency of highly proliferative TEC colony-forming cells declines rapidly in postnatal life (between P9 and P30), and indicate that an age-related loss of a subset of highly proliferative TECs, rather than a deficient proliferative potential of all TECs, likely underlies the rapid decrease in TEC engraftment potential with donor age seen in our in vivo studies (Fig. 7).

Interestingly, the colony-forming frequency of the TEC population closely tracks with the frequency of cTECs within the TEC population (Fig. 8E), suggesting that the colony-forming cells may derive from a subset of cells within the cTEC population. This finding is consistent with a recent study that identified a TEC progenitor within this population (51). In both male and female mice, the thymus grows rapidly for the first 4 wk of life, reaches a maximum, and then begins to shrink (Fig. 8F). Remarkably, the loss of TEC colony-forming cells exactly matches this timeline, suggesting a role for the colony-forming TECs in early thymus growth.

Discussion
In this study, we systematically evaluated three possible contributors to the small size of the aged thymus: age-related changes in circulatory factors, changes within the thymic stromal environment, and changes in TEC proliferative potential. We found that engraftable, colony-forming TECs are only abundant in the young, growing thymus and are rare in the thymuses of adult and aged mice. We also find that intrathymic transplantation of these young, engraftable TECs leads to substantial donor TEC engraftment, thymus growth, and increased T cell production.
FIGURE 5. TECs engraft and maintain functional phenotypes upon intrathymic transplantation. (A) Sections from recipient thymuses from Fig. 5 were fixed in PFA and analyzed for GFP+ engrafted cells (first column). Sections were analyzed for coexpression of GFP and Psmb11 (top row), UEA (middle row), and MHCII (bottom row), and Psmb11+", UEA+", and MHCII+ cells are shown in grayscale (second column). In merged images (third and fourth columns), GFP+ cells are shown in green, and Psmb11+, UEA+, or MHCII+ cells are in red. Boxed areas are magnified and shown in the fourth column. Scale bar, 100 μm. Images are representative of five recipient thymuses. (B) Thymuses from transplanted mice shown in Fig. 5A and 5B were enzymatically dissociated into single cells. Isolated cells were stained for CD45, EpCAM, MHCII, Ly-51, UEA, and CD80 and analyzed by flow cytometry. Plots show one well-engrafted thymus, from 10 similar experiments. The gating strategy to identify donor TECs is shown (CD45-"GFP+", and EpCAM+). Donor-derived TECs and endogenous recipient TECs were further analyzed for the expression of Ly-51 (cTECs) and UEA (mTECs). (C) Splenocytes from transplanted (Transpl.) and control (Ctrl.) mice shown in Fig. 5A and 5B were prepared into single cells and stained for CD4, CD8, CD44, and CD62L to distinguish naive and memory T cells. The percentages of naive CD62L CD4+ cells and naive CD62L CD8+ cells within the total CD4+ and CD8+ populations, respectively, are shown in the graph. Mean values for each group are shown as bars. The p values were determined using the unpaired Student t test.
Heterochronic parabiosis revealed that the thymuses of aged mice, when exposed to the circulating factors and cells of younger mice, became disproportionately colonized by young T cell progenitors. Conversely, aged circulating progenitors are either less abundant or less adept at colonizing the thymuses of young mice. Previous studies have observed that HSCs in aged mice have reduced lymphoid potential, especially in regard to B cell potential (52). The loss of T cell potential of aged HSCs, although apparent in some studies (15, 53), has been less consistently observed (54).

This inconsistent observation of T cell deficiencies in aged progenitors has been attributed to the use of irradiation in transplantation studies (15). Our results may help to explain some of the inconsistencies in measurements of T cell potential of aged HSCs. In the heterochronic parabionts, there was clearly a loss of T lineage potential within the aged hematopoietic system. However, once the thymus seeding cells from aged mice arrived at the thymuses of young mice, they proliferated and differentiated proportionately to their younger counterparts, suggesting that aged thymocytes are as capable as young thymocytes to proceed through the basic steps of T cell development.

Surprisingly, although the thymuses of aged mice became heavily colonized by younger thymocytes in heterochronic parabionts, the aged thymuses did not grow larger, indicating that young thymus seeding cells are not sufficient to drive regrowth of the aged thymus. This finding is consistent with a previous study that noted a failure of heterochronic parabiosis to induce aged thymic growth (55). Our parabiosis data also suggested that the circulatory system of 2-mo-old mice might contain factors that actually promote thymic involution. In this regard, it is worth noting that the thymuses of 2-mo-old mice, although larger than those of 18-mo-old mice, are undergoing more rapid involution; our data indicate that this rapid involution may be partly driven by circulating factors. However, in the absence of the identification of involution-driving factors in young mice, multiple possible interpretations of this observation exist. For example, we have not ruled out the possibility that stress levels may be higher in the heterochronous parabionts, and that the old thymuses might be more sensitive to stress-mediated thymus toxicity. The observation of thymus-inhibitory factors in the 2-mo-old circulatory system also reveals a limitation to the parabiosis model in the study of thymic growth factors. The murine thymus reaches maximal size over the course of the first 4 wk of life and involutes thereafter. Given that parabiosis requires mice of roughly similar size and feeding abilities, all heterochronous parabiosis experiments between mice are necessarily done in mice with involuting thymuses. Therefore, it remains unexplored whether circulatory factors drive thymus growth in mice younger than 4 wk. Nevertheless, the parabiosis experiments performed in this study suggest that circulatory factors and cells do not directly determine age-related thymus changes in size throughout adult life.

We used intrathymic transplantation of TECs to evaluate the young and middle-aged thymic environments. TEC transplantation showed that middle-aged thymuses are highly permissive for TEC proliferation. Previous studies have suggested that the aged thymic
environment, which accumulates significant numbers of adipocytes, may become hostile to TECs and impede thymocyte growth (21). Nevertheless, intrathymic transplantation indicated that middle-aged and young thymus environments were equally permissive for donor TEC proliferation.

Transplantation of fetal thymic cells into middle-aged thymuses drives thymus growth and increases naive T cell production. The increases in naive T cells detected in the spleens of recipient mice were modest, however, and did not correlate with engraftment levels. The lack of correlation between engraftment level and splenic naive T cell frequency may have been because of the large natural variation in the frequencies of naive T cells in the control middle-aged mice, together with the fact that the WT mice used in this study already had “full” T cell niches, which may have decreased the efficiency with which new thymic emigrants survived in the periphery. Furthermore, it is possible that different classes of TECs engrafted in different mice, leading to additional variability in naive T cell output.

We have not yet determined precisely which fetal TEC subset drives thymic growth in this model; however, both mTECs and cTECs were abundant in the engrafted lobes. Fetal TEC progenitors that give rise to both mTECs and cTECs have been conclusively identified in the fetal thymus (56), and bipotent progenitors have recently been identified in the adult thymus (51). Our findings indicate that engraftable, colony-forming TECs are abundant in the fetal and newborn thymus. Whether these postnatal TECs are bipotent progenitors, unipotent transient amplifying cells, or some mixture of the two is not known; however, the profound and rapid decrease in transplantable, colony-forming TECs that occurs during the first 4 wk of life suggests that these TECs are required...
for growth of the early thymus and that their loss spurs thymic involution.

In this study, we demonstrate that transplanted fetal TECs and progenitors can drive growth of the middle-aged thymus. Although further investigation will be required to identify the exact TEC subset that drives the thymic growth, this study provides proof-of-principle that intrathymic transplantation of TECs, either isolated from growing thymuses or generated de novo from pluripotent stem cells (57), can be used for immune regeneration.

Acknowledgments

We thank Stephan Kessler, Aldo Rossini, and Martin Thelin for input on the manuscript. We gratefully acknowledge the Joslin Diabetes Center Flow Cytometry Core, the Joslin Microscopy Core, and the Harvard Stem Cell Institute. We also thank Matt Levin for the use of the Keyence Microscope and software. We acknowledge the excellent support of the Joslin Animal Facility.

Disclosures

The authors have no financial conflicts of interest.

References


### Supplemental Table 1. List of antibodies used in the study.

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Supplemental Figure 1. Contribution of parabiotic partner thymocytes does not correlate with thymus cellularity in heterochronic parabionts. The contribution of partner cells to the thymus of each heterochronic parabiont (data from Figure 1B) was graphed against the total cellularity of that thymus (data from Figure 1C).
Supplemental Figure 2. Donor cell engraftment level does not correlate with percentage of naïve splenic T cells. Percentages of splenic naïve T cells shown in Figure 5C were compared to the engraftment levels of donor GFP+ thymic cells within recipient thymuses (examples in Figure 4B). Each recipient thymus was manually scored to determine the engraftment level.