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Interplay between Follistatin, Activin A, and BMP4 Signaling Regulates Postnatal Thymic Epithelial Progenitor Cell Differentiation during Aging

Graphical Abstract

Highlights

- TEPC quiescence is initiated at the onset of puberty
- Adult TEPC quiescence leads to depletion of lineage-committed cTEC and mTEC precursors
- Follistatin antagonism of activin A signaling inhibits medullary TEC differentiation
- An imbalance in follistatin, activin A, and BMP4 signaling drives thymic involution

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In Brief

Mechanisms underlying age-associated thymic involution remain elusive. Here, Lepletier et al. show that an imbalance in activin A and BMP4 signaling drives thymic involution. This occurs in two major stages: impairment of medullary TEC differentiation and initiation of TEPC quiescence, followed by the broader, negative impact of reduced lineage committed precursors.
Interplay between Follistatin, Activin A, and BMP4 Signaling Regulates Postnatal Thymic Epithelial Progenitor Cell Differentiation during Aging

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SUMMARY

A key feature of immune functional impairment with age is the progressive involution of thymic tissue responsible for naive T cell production. In this study, we identify two major phases of thymic epithelial cell (TEC) loss during aging: a block in mature TEC differentiation from the pool of immature precursors, occurring at the onset of puberty, followed by impaired bipotent TEC progenitor differentiation and depletion of Sca-1lo cTEC and mTEC lineage-specific precursors. We reveal that an increase in follistatin production by aging TECs contributes to their own demise. TEC loss occurs primarily through the antagonism of activin A signaling, which we show is required for TEC maturation and acts in dissonance to BMP4, which promotes the maintenance of TEC progenitors. These results support a model in which an imbalance of activin A and BMP4 signaling underpins the degeneration of postnatal TEC maintenance during aging, and its reversal enables the transient replenishment of mature TECs.

INTRODUCTION

Thymic epithelial cells (TECs) are essential for the establishment of the specialized microenvironment that orchestrates the development of naive, self-tolerant T cells from hematopoietic precursors (Anderson et al., 1993). They are supported by non-epithelial thymic stromal cells (TSCs), such as fibroblasts and endothelial cells, in an extracellular matrix-rich three-dimensional (3D) scaffold structure (Hun et al., 2017). TECs can be broadly divided into functionally and spatially distinct cortical (cTEC) and medullary (mTEC) subsets. cTECs provide key inductive interactions for T lineage commitment through Notch signaling (Koch et al., 2008), and are responsible for the positive selection of thymocytes that express a functional T cell receptor with appropriate affinity for self-antigens (Ohigashi et al., 2016). mTECs are specialized for self-tolerance induction and express ubiquitous and peripheral self-antigens in an autoimmune regulator (AIRE)-dependent and -independent manner (Anderson et al., 2002; Derbinski et al., 2001). In the adult, mature mTECs express high levels of the major histocompatibility complex II (MHCII; mTEC\(^\text{hi}\)), have a high turnover frequency with a half-life of \(\sim 14\) days (Gray et al., 2007), and are maintained by a reservoir of lineage-committed mTEC precursors distinguished by low levels of MHCII (mTEC\(^\text{lo}\)) (Fletcher et al., 2009; Ohigashi et al., 2015). Lineage tracing experiments demonstrated that the mTEC\(^\text{lo}\) compartment can also contain post-AIRE mature mTECs (Metzger et al., 2013).

Bipotent thymic epithelial progenitor cells (TEPC) support the development of both cTECs and mTECs during thymus organogenesis (Alves et al., 2014; Bennett et al., 2002; Gill et al., 2002; Rossi et al., 2006, 2007). Accumulating evidence suggests that mTECs are derived from cells that express markers that are common to the cTEC lineage during development, such as the thymoproteosome subunit \(b\text{m}\) (Mayer et al., 2016; Ohigashi et al., 2013) and CD205 (Baik et al., 2013). Bipotent TEPC also exist in the postnatal thymus (Bleul et al., 2006) and reside in the cortical epithelial compartment (Dumont-Lagacé et al., 2017; Meireles et al., 2017; Ulyanchenko et al., 2016; Wong et al., 2014), with label retention and enriched colony-forming capacity found within the Sca-1hi cTEC subset expressing low levels of MHCII (cTEC\(^\text{lo}\)) (Dumont-Lagacé et al., 2017; Meireles et al., 2017; Wong et al., 2014). In contrast, another study demonstrated bipotent potential in a PLET1+ cTEC\(^\text{lo}\) population (Ulyanchenko et al., 2016). Thus, further resolution of the postnatal bipotent epithelial progenitor phenotype and the extent of its role in postnatal TEC maintenance and aging is needed.

Deterioration of thymus function occurs naturally during aging and ultimately constrains the host immune repertoire. It is characterized by a reduction in total thymic cellularity and naive T cell...
Figure 1. Aging Impairs mTEC and TEPC Differentiation
(A) Schematic model for TEPC progression toward cTECh.
(B) Representative flow cytometry plots used to identify thymocytes, TECs, and non-TECs. cTEClo, cTEChi, mTEClo, and mTEChi subsets were subsequently identified via UEA-1 and MHCII expression.
production (Ortman et al., 2002). Reduced TEC turnover (Gray et al., 2006; Ki et al., 2014) and diminished levels of transcription factor forkhead-box N1 (FOXN1), a master regulator of TEC lineage specification (Chen et al., 2009; Nowell et al., 2011; Ortman et al., 2002), have been observed in the aged thymus. An increase in steroidal hormone production at puberty has also been implicated in age-related thymus involution, with androgen deprivation (AD) inducing the recovery of naïve T cell production and bone marrow function in aged male mice and in humans (Khong et al., 2015; Marchetti et al., 1989; Olsen et al., 1991; Sutherland et al., 2005). However, the mechanisms and signaling pathways causing the post-pubertal loss of specific TECs and underpinning AD-induced thymocyte regeneration remain unclear.

In this study, we examined numeric, phenotypic, and transcriptomic alterations in TEC and non-TEC (non-epithelial stromal cells, fibroblasts, and endothelial cells) stromal subsets during age-related thymic involution, and following transient thymic recovery via AD. We identified cTEClo accumulation with aging, which became increasingly restricted to the 6-integrinhiSca-1hi subpopulation described by Wong et al. (2014). Given the loss of Sca-1lo cells within this cTEClo subset during aging, its poor colony-forming efficiency in comparison to our putative 6-integrinhiSca-1hi cTEClo postnatal bipotent TEPC, and its return during AD-induced thymus regeneration, we propose the Sca-1lo cTEClo population represents a downstream, single lineage immature cortical precursor (Figure 1A). Our data revealed two major stages of TEC loss: an initial block in the differentiation of mTEClo precursors to mature mTEC and the initiation of TEPC quiescence at the onset of puberty, followed by a loss in cortical and medullary epithelial precursors. We identified follistatin (FST), a potent antagonist of activin A signaling, as a putative candidate for post-pubertal modification of TEC differentiation. Our data demonstrate that activin A plays a role in TEC maturation, in contrast to the effects of bone morphogenetic protein 4 (BMP4), which enhances the progenitor or precursor state. We propose that increased production of FST from the onset of puberty quenches activin A signaling, resulting in impaired TEPC and/or TEC differentiation. Reversal of this effect underpins de novo mature TEC generation following AD. Therefore, we propose a TEC aging model whereby an imbalance in signaling by these transforming growth factor β (TGF-β) superfamily members alters post-pubertal TEC maintenance and leads to thymus involution.

RESULTS

Reduction in Medullary Epithelia and TEPC Differentiation with Aging

To better understand the impact of puberty and aging on TEC subset homeostasis, we examined the major epithelial cell populations that constitute the thymic microenvironment (Figure 1B; Seach et al., 2012), in conjunction with our previously identified TEPC phenotype (CD45 EpCAM+UEA-1 Ly51hiMHCIihi6-integrinhiSca-1hi; Wong et al., 2014). We analyzed pre-pubertal (4-week-old), post-pubertal (7-week-old), and middle-aged (8-month-old) male mice. A severe reduction in thymocytes evident at 7 weeks was not accompanied by a loss of total TECs or non-TECs (Figures 1C–1E), which led to a reduction in the thymocyte:TEC ratio (Figure 1F). Differential TEC loss was subsequently observed at 8 months (Figure 1D), resulting in a 2-fold increase in the non-TEC:TEC ratio between 7 weeks and 8 months (Figure 1G). This TEC loss occurred predominantly in the medullary compartment (Figure 1H).

Analyzing individual immature and mature TEC subsets highlighted interesting phenotypic changes from the onset of puberty (Figure 1). At 7 weeks, there was a major numerical loss of mature mTEC cells, including AIRE+ mTEC (Figure 1J), which marks the first stage of mature TEC diminution. The accompanying increase in mTEC precursors may demonstrate impaired mature TEC differentiation or an increase in post-AIRE cells. Thereafter, an overall reduction in mTEC precursors became evident, with further loss of mature mTEC by 8 months (Figure 1L). This translated to a 3-fold increase in the mTEClo:mTEC ratio at 7 weeks, which was increased 4-fold by 8 months (Figure 1K). A 2-fold decrease in the mTEC:cTEC ratio was observed at 7 weeks, and reached 5-fold by 8 months (Figure 1L). Thus, a disproportionate loss in mTEC marks the first stage of thymus involution at the onset of puberty, with a consequent loss in mTEC precursors.

Ki67 analyses of total proliferating TECs revealed a reduction from 12% at 4 weeks to 6% at both 7 weeks and 8 months of age (Figure 1M). This decline was mainly due to changes in the cTEClo subset at the onset of puberty (Figure 1N). Specifically, the proportion of proliferating cTEClo decreases from ~40% at 4 weeks to ~25% at 7 weeks and 8 months of age, which is slightly more than the proliferation seen in all of the other TEC subsets from 4 weeks of age (Figure 1N). These results further suggest that a block in mTEC to mTEC differentiation is
the PLET-1 +cTEChi cells expressed intermediate levels of an additional proportional shift—the accumulation of cTEClo (Figure 1I) with a 5-fold increase in the cTEC\textsuperscript{lo}:cTEC\textsuperscript{hi} ratio evident by 8 months (Figure 1P).

Further subdivision of cTEC\textsuperscript{lo} based on \textalpha6-integrin and Sca-1 expression (Figure 1R; Wong et al., 2014) revealed a proportional and numerical increase in TEPCs (\textalpha6\textsuperscript{hi}Sca-1\textsuperscript{hi}; Figure 1Q) at 7 weeks, despite a transient proportional drop in proliferating TEPCs (Figure 1S). This suggests that the accumulation of TEPCs may be due to a reduction in their rate of differentiation. The progressive proportional loss of Sca-1\textsuperscript{lo} cTEC\textsuperscript{lo} (\textalpha6\textsuperscript{lo}Sca-1\textsuperscript{lo}) from 7 weeks further supports diminishing TEPC differentiation into downstream Sca-1\textsuperscript{lo} cTEC\textsuperscript{lo} during aging (Figure 1T). Moreover, the increase in Ki67 expression by the few Sca-1\textsuperscript{lo} cTEC\textsuperscript{lo} remaining at 8 months suggests that there is an increased reliance on proliferation to maintain this downstream cTEC precursor during aging, rather than de novo TEPC differentiation (Figure 1U). Therefore, TEPCs appear to maintain self-renewal capabilities, but suffer from an impairment in differentiation.

These data portray two major stages of TEC loss with aging: stage 1 occurs at the onset of puberty, when there is a block in mTEC\textsuperscript{lo}-to-mTEC\textsuperscript{hi} differentiation, a reduction in cTEC\textsuperscript{lo} proliferation, and an accumulation of cTEC\textsuperscript{lo} by 7 weeks of age. The cTEC\textsuperscript{lo} cells become increasingly restricted to a TEPC phenotype. In stage 2, the impediment of TEPC differentiation results in a loss of downstream, lineage-specific Sca-1\textsuperscript{lo} cTEC\textsuperscript{lo} and mTEC\textsuperscript{lo} precursors. The loss of mature TECs continues. Thus, the post-pubertal adult thymus appears to rely mainly on lineage-specific precursors for mature TEC maintenance, with TEPCs entering a state of quiescence. These trends were also evident in BALB/c male (Figures S1A–S1H) and C57BL/6 female mice (Figures S1I–S1P). Females had more proliferating cTEC\textsuperscript{lo} (Ki67\textsuperscript{+}; Figure S1M) and maintained the cTEC\textsuperscript{lo} population for longer compared to male counterparts (Figures S1J and S1O).

Given the reduced proliferation of cTEC\textsuperscript{lo} with aging (Figure 1N), we investigated whether this decline was associated with the PLET-1-cTEC\textsuperscript{lo} subset described in Ulyanchenko et al. (2016). PLET-1 predominantly marks mTEC\textsuperscript{lo} cells; however, a small proportion of PLET-1\textsuperscript{+} cells are evident in other TEC subsets (Figure S2A). There was a proportional and numerical accumulation of PLET-1\textsuperscript{+} cells in the cTEC\textsuperscript{lo} subset with aging (Figures S2B and S2C), while the number of PLET-1\textsuperscript{+} cTEC\textsuperscript{lo} cells was reduced between 7 weeks and 8 months. The reduction in proliferating cTEC\textsuperscript{lo} cells at 7 weeks was not due to the PLET-1\textsuperscript{+} cTEC\textsuperscript{lo} population; in contrast, there was a slight increase in their Ki67\textsuperscript{+} expression at 8 months (Figure S2D). Almost half of the PLET-1\textsuperscript{+} cTEC\textsuperscript{lo} cells expressed intermediate levels of AIRE; while these expanded in number at 7 weeks, they reduced 4-fold by 8 months (Figures S2E and S2F). These data open the possibility that PLET-1\textsuperscript{+} cTEC\textsuperscript{lo} cells are downstream of cTEC\textsuperscript{lo} TEPC and are a precursor to AIRE\textsuperscript{+} mTECs. Their poor colony-forming efficiency compared to Sca-1\textsuperscript{lo}cTEC\textsuperscript{lo} TEPC (Figure S2G) would support this concept.

**cTEC\textsuperscript{lo} Mobilization, mTEC Proliferation, and TEPC Differentiation Are Induced in Middle-Aged Mice following AD**

We investigated an endocrine-based transient model of thymus regeneration to identify the mechanisms of post-pubertal TEPC quiescence and the potential for its reversal. Previously, we demonstrated the reconstitution of the middle-aged male mouse thymus post-AD by either surgical or chemical castration (Goldberg et al., 2009; Sutherland et al., 2005). These regenerative effects were found to be transient following surgical castration (Cx) (Griffith et al., 2012) and are also demonstrated in Figure S3A, with the thymocyte number reaching young adult (5–6 weeks old) levels between day 14 (D14) and D28 post-Cx in middle-aged (9–12 months old) male mice, and returning to pre-Cx levels by D84. Although total TEC cellularity did not significantly increase, except during the peak of thymus regeneration (Figure S3B), dramatic mTEC regeneration is evident post-Cx, as seen by the 4-fold increase in the mTEC:cTEC ratio at D28, when young adult levels are reached (Figure S3C).

Alterations in TEC subset phenotypes occurred initially in the cortical compartments of Cx animals, with medullary effects observed thereafter (Figures 2A and S3D). Reversal of the middle-aged cTEC\textsuperscript{lo} accumulation began immediately, reaching young adult levels by D28, and was maintained until D135. The increase in cTEC\textsuperscript{lo} proportions at D7 and D10, with no change in their proliferation status (Figure S3E), suggests early mobilization of cTEC\textsuperscript{lo} precursors toward the mature cTEC\textsuperscript{hi} compartment. In contrast, a more sustained increase in mTEC\textsuperscript{lo} was seen from D10, yet depletion of mTEC\textsuperscript{lo} precursors was not obvious, despite an increase in the proportion of Ki67\textsuperscript{+} mTEC\textsuperscript{lo} from D7 (Figure 2B). This may reflect a fast transition rate of differentiation, with mTEC\textsuperscript{lo} precursors being replenished as quickly as they differentiate. An increase in Ki67\textsuperscript{+} mTEC\textsuperscript{lo} from D7 reveals that increased mature mTEC proliferation also contributes to medullary regeneration. Sequential recovery of the cTEC and mTEC compartments is evident in the reduced ratios of cTEC\textsuperscript{lo}:cTEC\textsuperscript{hi}, from D4, and mTEC\textsuperscript{lo}:mTEC\textsuperscript{hi}, from D7, respectively, reaching young adult levels by D10 (Figure S3D). We detected an increase in the number of AIRE\textsuperscript{+} mTEC\textsuperscript{lo} for self-tolerance induction at the height of thymic cellularity and mTEC\textsuperscript{lo} recovery (Figure 2C). These findings imply a normal sequence of thymopoiesis. A previous study by Dumont-Lagacé et al. (2015) also demonstrated a significant increase in AIRE-dependent and -independent genes in castrated 4-week-old male mice, even though the age-induced loss of AIRE\textsuperscript{+}mTECs is not yet evident at 4 weeks. A bioinformatics study of microarray data from microdissected castrated thymus tissue indicated no increase in tissue-restricted antigen (TRA) expression in castrated aged mice compared to aged mice (Griffith et al., 2012). Therefore, further investigations on purified mTEC\textsuperscript{lo} are needed before conclusions on restoration of self-tolerance induction can be reached.

We demonstrated the mobilization of the cTEC\textsuperscript{lo} population, which accumulated during aging, in Figure 2A. As TEPCs represent ~60% of this middle-aged cTEC\textsuperscript{lo} population (Figure 1O), and cTEC\textsuperscript{lo} FOXN1 levels are increased 10-fold by D4 post-Cx (Figure 2D), we were interested in determining the extent of TEPC involvement in this regenerative process. We detected...
an increase in TEPC frequency (D14 and D28) and mobilization of Sca-1int cTEClo (D14–D56), with subsequent increased proportions of Sca-1int cTEClo from D56 to D135 following Cx (Figures 2E and S3F). This delayed response may reflect a deep state of TEC quiescence or quiescent TEPCs may be triggered by depleting TEC precursors, rather than directly through AD. All cTEClo subsets returned to sham-castrated (ShCx) control or middle-aged levels by D180 (Figure 2A). From these data, we propose that a series of dynamic differentiation events follow AD, with TEPC reactivation involved in replenishing both Sca-1int cTEClo and mTEClo precursors.

In summary, our phenotypic analyses describe a dynamic series of events that immediately follow AD. We propose that TEPC re-activation and differentiation for the de novo generation of cTEC and mTEC precursors, occurs in addition to the differentiation of existing precursors and mTEC proliferation during AD-induced regeneration of cTEClo and mTEClo subsets, to support increased thymopoiesis.

**Transcriptome Analyses Identify Age-Associated Alterations in FST and BMP4, with Reversal Induced following Cx**

The signaling cascades that underlie TEC loss during aging and the early stages of AD-induced regeneration are poorly understood. Here, we attempt to elucidate this issue through transcriptome analyses on TEC and non-TEC samples collected from 5- to 6-week-old (young) and 9- to 12-month-old (middle-aged) male mice at day 4 post-Cx (CxD4) and CxD7 and ShCx. Principal-component analyses (PCAs), used to validate replicate consistency and identity population clusters, revealed a predominant effect of AD on the non-TEC global transcriptomic signature (Figures S4A and S4B). Three distinct clusters were evident in non-TECs (Figure S4B), representing young, Cx, and ShCx populations, while differences in the more heterogeneous TECs between the Cx and ShCx groups were less apparent (Figure S4A). Common differentially expressed genes (DEGs) from non-TEC analyses (Figure S4D). The majority of the DEGs identified in TECs were downregulated with aging and upregulated following Ca, while DEGs identified in non-TEC predominantly displayed an inverse trend (Figures S4E and S4F).

From our TEC and non-TEC transcriptome analyses, we identified TGF-β superfamily genes that demonstrated increased expression with aging and decline following Cx. FST and BMP4...
displayed this expression profile in both TEC and non-TEC samples (Figures 3A and 3B). The declining trend of FST expression post-AD was evident from Cx4 and continued to decrease in TECs to Cx20 (Figures S4G and S5A). Biological process Gene Ontology (GO) analysis identified pathways at Cx4 that are predominantly in cTECs, mTECs, and fibroblasts, with a substantial increase evident in each of these populations during aging (Figure 3C). AD immediately counteracted this increase in cTECs, downregulating cTEC FST expression for at least 56 days post-Cx (Figure 3D). No significant change in mTEC FST expression was observed (data not shown). Downregulation of FST was slightly delayed in fibroblasts, reaching significance by D10, but also persisting to D56 (Figure 3D). FST is the physiological antagonist of activin A, a homodimer composed of two inhibin (INHBA) subunits. The persistent downregulation of FST production by cTECs following Cx (hence increasing activin A availability and signaling) may underpin Cx-induced cTEC differentiation. Downregulation of FST by fibroblasts may also modulate TEC differentiation, given their broad spatial distribution.

Coincident with the increase in FST expression during aging, we found an increase in the activin receptor 2a (ACVR2A) transcript by cTECs (Figure 3E) and mTECs (data not shown), which may be a response to reduced activin availability. Analysis of a public database of TEC transcriptional profiling in 1-, 3-, and 6-month-old mice confirmed this finding (Ki et al., 2014). After an initial spike in ACVR2A at D4 following AD, receptor levels decreased to young levels for at least 56 days (Figure 3E). This may reflect homeostatic regulation of activin signaling, with increased activin A availability due to the loss of FST production.
Early studies have demonstrated significant suppression of the ACVR2A in the presence of high doses of exogenous activin (Di Simone et al., 1998; Trudeau et al., 1994).

No alteration in serum activin A was evident with aging or following AD (Figures S4H and S4I), which suggests that the upregulation of ACVR2A by TECs during aging is a response to a thymus-specific event, rather than to a pan reduction in activin A bioavailability. Thus, we propose that activin A signaling may be homeostatically regulated by cTECs in an autocrine fashion, via modulation of both FST and ACVR2A expression.

We have previously shown that BMP4 supplementation of in vitro 3D postnatal TEC cultures maintained cTEC<sup>b</sup> and mTEC<sup>b</sup> precursors at the expense of differentiation into their mature counterparts (Barsanti et al., 2017). Furthermore, endogenous regeneration following thymic damage was shown to involve TEC stimulation by endothelial cell-derived BMP4, which subsequently increased FOXN1 expression (Wertheimer et al., 2018). TEC expression of the BMP4 receptor BMPR2 was predominantly in TEPcs, Sca-1<sup>+</sup> cTEC<sup>b</sup> precursors, and mTEC<sup>b</sup> precursors of 7-week-old mice (Barsanti et al., 2017). Although the majority of BMP4 production in the adult thymus is derived from fibroblasts and endothelial cells (Barsanti et al., 2017), in addition to an increase in fibroblast BMP4 expression with aging, we identified increased BMP4 expression by TECs (Figure 3F). Thus, an increase in autocrine BMP4 signaling in aging TECs may also modulate TEPc and TEC precursor differentiation. Following AD, there is an immediate reduction in the fibroblast BMP4 transcript, which reaches statistical significance by 10 day post-Cx, with maximal reduction at D28 (Figure 3G). Reversal of this trend is seen by D56, coincident with the reduction in mTEC<sup>Hi</sup>.

These data suggest an important role for activin A signaling in the activation of TEPc and immature TEC precursor differentiation into mature TECs. This cascade appears to counterbalance BMP4 signaling, which maintains a pool of TEPcs and immature TECs. Thus, we propose that the age-associated increase in FST reduces activin A availability and signaling, and together with the increased production of BMP4 maintains TEPc and TEC precursors in their immature state, thereby disrupting the normal balance of differentiation. AD reverses these age-induced increases to reinstate the balance between activin A and BMP4 signaling and enables the generation of mature TECs.

As our model proposes that dysregulated FST, activin A, and BMP4 signaling influences mature TEC generation in middle-aged mice, we sought to determine whether changes in these pathways are also evident at the onset of puberty, when diminished mTEC<sup>lo</sup>-to-mTEC<sup>hi</sup> differentiation occurs. A substantial increase in the FST transcript was evident in cTECs and mTEC<sup>lo</sup> (Figure 3H) at 7 weeks, as was an increase in the BMP4 transcript in thymic fibroblasts (Figure 3I), implicating the involvement of these TGF-β signaling pathways.

**FST 288 Treatment in Young Mice Impairs TEPc and mTEC Differentiation and Attenuates AD-Induced mTEC Recovery in Middle-Aged Mice**

Recombinant FST288 was administered to 7- to 8-week-old and 9-month-old mice over 14 days to confirm the effects of increased FST on TEC differentiation. This isoform of FST binds and blocks activin A with markedly greater affinity than its counterparts (Sugino et al., 1997). An increase in TEPc number and proportion was evident in 7- to 8-week-old FST288-treated mice, with a concomitant reduction in Sca-1<sup>+</sup> cTEC<sup>b</sup> proportions (Figure 4A). No further change in TEPc number in 9-month-old-treated animals suggests the saturation of FST-induced TEPc quiescence has already been achieved, with the majority of cTEC<sup>b</sup> being of the TEPc phenotype. Treated 7–8 week-old mice demonstrated a significant decrease in both mTEC<sup>lo</sup> number (Figure 4B) and proportion (Figure 4C), with a concomitant proportional increase in cTEC<sup>b</sup>, similar to the effects of aging observed in middle-aged mice. The decrease in mTEC<sup>lo</sup> was not due to reduced proliferation (Figure 4B). These data suggest an influence of FST288 on TEPc differentiation and the generation of mTEC<sup>lo</sup> precursors.

Given that Fst was persistently downregulated in 9- to 12-month-old cTECs and fibroblasts following AD, we next examined how the re-administration of FST288 would affect the transient thymic regenerative process Cx7 and Cx14. At Cx7, FST288 treatment reduced the number of generated mTEC<sup>lo</sup> compared to PBS-treated Cx controls (Figure 4D). By CxD14, decreased mTEC<sup>lo</sup> and mTEC<sup>hi</sup> numbers were evident (Figure 4E). The reduction in cTEC<sup>b</sup>:cTEC<sup>lo</sup> ratio that was evident at CxD14 (Figure S3D) was also attenuated with FST288 treatment (Figure 4F). There was no change in K67<sup>+</sup> expression in mTEC<sup>lo</sup> or mTEC<sup>hi</sup> at Cx7 (data not shown) or CxD14 (Figure 4G). With no significant change in total thymic cellularity (data not shown), the TEC:thymocyte ratios were consequently reduced (Figure 4H). While there was no change in the CxD14 TEPc number with FST288 treatment, a clear impediment in TEPc differentiation toward a Sca-1<sup>+</sup> cTEC<sup>b</sup> phenotype was observed in FST288-treated Cx14 mice, evident both numerically and proportionally (Figure 4I).

These data reveal a role for activin A signaling in mediating AD-induced TEC re-establishment in middle-aged mice, via both TEPc mobilization and immature mTEC precursor differentiation.

**Activin A- and FST288-Deficient Mice Confirm a Role for Activin A Signaling in TEC Maturation**

Having demonstrated the diminishing effects of FST on TEPc and immature TEC precursor differentiation and given that FST is a strong antagonist of activin A blockade, we next investigated the impact of activin A and FST288 deficiency using genetically modified embryonic and adult mice. TEC subsets from activin A knockout (activin KO), activin A<sup>−/−</sup> heterozygotes (activin HET), and FST288 KO animals were analyzed.

Given that the activin KO phenotype is lethal postpartum, we assessed KO embryos at embryonic day 14.5 (E14.5; gating strategy shown in Figure 5A), and HET animals at 9–10 weeks of age. E14.5 activin KO embryos presented with reduced total TEC numbers (Figure 5B). This was attributable mainly to cTECs, with both cTEC<sup>lo</sup> and cTEC<sup>hi</sup> subsets showing a significant reduction in cellularity (Figure 5B), as did the number of early T cell progenitors (ETPs; Figure 5C). Activin A deficiency also had a negative impact on the minor mTEC population that develops at this early embryonic stage, significantly reducing mature mTEC<sup>Hi</sup> production (Figure 5B). This trend
was reflected as disseminated medulla regions in immunofluorescence staining of thymic lobes from E17.5 activin KOs in comparison to wild-type (WT) sections (Figure 5D). Activin HETs at 9–10 weeks of age displayed a significant reduction in total TEC and mTEC numbers (Figure 5E). This decline was mainly due to the reduced generation of mTEClo precursors, with a trend toward reduced mTEChi (Figure 5E). No significant change was evident in TEPC number (Figure 5F), early thymic progenitors (ETPs), or thymocyte subsets (Figure 5G). In contrast to activin KO mice, total TEC numbers were significantly increased in E18.5 FST288 KO mice (Figure 5H). This was attributable mainly to cTECs, with increases in both cTEClo and cTEChi subsets (Figure 5H). No significant change was evident in the expression of AIRE (Figure 6B, upper panel) and Foxn1 (Figure 6B, lower panel). We also investigated the periphery of recipient nude mice for alterations in de novo T cell production. Our results reveal a reduction in circulating CD4+ T cells (Figure 6C), with a

Activin A KO Grafts Present with an Altered Microenvironment and Reduced TEC Function

We directly assessed the functional impact of activin A deficiency on TECs through in vivo fetal thymic organ culture (FTOC) (Anderson and Jenkinson, 2007). Athymic nude mice were engrafted with thymocyte-depleted (via 2′-deoxyguanosine treatment) E14.5 thymi from WT and activin KO mice and were analyzed after 6 weeks. Immunofluorescence analysis of the grafts revealed disseminated medullary regions (identified by keratin 14 and UEA-1 staining) in activin KO grafts when compared to WT (Figures 6A and 6B), similar to our observations in activin KO mice (Figure 5D). Activin KO graft thymocyte analyses detected a small but significant increase in CD4+CD8+ double-positive (DP) thymocytes (data not shown).

Despite disseminated K14/UEA-1 staining in the activin KO grafts, there were no obvious alterations in the expression of AIRE (Figure 6B, upper panel) and Foxn1 (Figure 6B, lower panel). We also investigated the periphery of recipient nude mice for alterations in de novo T cell production. Our results reveal a reduction in circulating CD4+ T cells (Figure 6C), with a
significantly reduced proportion of CD4<sup>+</sup> T cells also seen in the spleen (Figure 6D). This reduction in splenic CD4<sup>+</sup> T cells, however, did not reach numerical significance (Figure 6E).

Here, we demonstrate that TECs from activin A-deficient mice are functionally compromised, presenting with disseminated medullary formation and a reduced capacity to generate CD4<sup>+</sup> T cells.

**Activin A and BMP4 Orchestrate In Vitro Immature cTEC Differentiation**

Through in vitro 3D culture systems (Wong et al., 2014), we demonstrate that activin A signaling may directly induce TEPC differentiation. Supplementation of 7-week-old TEPC culture wells with the anti-activin A antibody over 7 days revealed a trend toward a more cortical phenotype, while treatment of 9-month-old TEPC cultures with 10 ng/mL activin A (Figure 7A) or anti-FST antibody (data not shown) enhanced mTEC differentiation. These findings led us to consider whether TEC development involves interplay between activin A and BMP4 signaling. We investigated this possibility through the supplementation of TEPC cultures with increasing concentrations of recombinant activin A or BMP4.

Immature cTECs (PI<sup>CD45</sup> EpCAM<sup>+</sup>UEA-1<sup>-</sup>MHCII<sup>+</sup>) from E14.5 mice (Figure 7B), which are predominantly intermediate (<i>b</i>5t+<i>b</i>bipotent cells (Takahama et al., 2017) that do not yet express Sca-1, and TEPC from 7-week-old mice were isolated for 3D co-culture with mouse embryonic fibroblasts (MEFs) over 7 days. BMP4 supplementation increased the colony-forming efficiency (CFE) (Figure 7C) in both E14.5 immature cTEC and 7-week-old TEPC 3D cultures, whereas a reduction in CFE was seen with activin A supplementation irrespective of BMP4 administration (Figure 7C) and increased proportion of UEA1<sup>+</sup> cells (data not shown). These observations support our model of interplay between these TGF-β signaling pathways, with activin A inducing TEPC differentiation and BMP4 supporting TEPC maintenance. Cultures without additional activin A or BMP4 formed a heterogeneous range of colony sizes and phenotypes, from least differentiated <i>b</i>5t TEPC colonies and <i>b</i>b core colonies that contain some differentiated outer keratin 14<sup>+</sup> (K14+) cells that mark mTEC precursors, to more differentiated colonies that contain mixed <i>b</i>5t/K14 cells or are predominantly K14<sup>+</sup>mTECs (Figures 7D–7F). Cultures supplemented with 80 ng/mL BMP4 demonstrated an increase in larger colonies, with predominantly undifferentiated <i>b</i>5t core-type colonies observed (Figures 7D–7F). In contrast, supplementation of colonies with 10 ng/mL activin A resulted in reduced colony size and an increase in K14<sup>+</sup> colonies. The combined supplementation of activin A and BMP4 to cultures presents an intermediary phenotype, indicating the presence of interplay between these factors in terms of TEPC self-renewal and differentiation.

These in vitro findings support a model of interplay between activin A and BMP4 signaling, with BMP4 promoting progenitor maintenance, while activin A (modulated by FST288) induces their differentiation toward an immature medullary phenotype (Figure 7G).

**DISCUSSION**

In this article, we sought to investigate sequential changes in the TEC compartment during aging, the role of TEPC in supporting postnatal TEC maintenance, and the mechanisms underpinning age-induced thymus involution using a transient model of thymus regeneration. To date, progress in understanding the basis for TEC loss during aging has been slow, despite their importance for the restoration of thymic function and T cell immunity.

Through phenotypic analyses of pre-pubertal, post-pubertal, and middle-aged mice, we identified two major stages of TEC diminution: progressive mTEC<sup>+</sup> loss from the onset of puberty, with a reduction in cortical and medullary precursors thereafter. We detected an impediment of TEPC differentiation, which we show through an endocrine-based, transient model of thymic regeneration to be caused by an imbalance in TGF-β superfamily signaling. We provide elucidation on the mechanisms underlying both involution and Cx-induced reconstitution of the thymus. Specifically, we show evidence of the interplay between FST, activin A, and BMP4 signaling in TEC regulation during age-related thymic involution.

We identified an accumulation of immature cTEC<sup>+</sup> during aging, which was increasingly restricted to the little Sca-1<sup>+</sup>cTEC<sup>+</sup> TEPC, first described by Wong et al. (2014). The increase in TEPC number and proportion and the reduced generation of downstream Sca-1<sup>+</sup>cTEC<sup>+</sup> from the onset of puberty imply diminished TEPC differentiation. Similar to previous reports, we also show the diminution of TEC turnover by middle age (Gray et al., 2006); however, our data demonstrate that this reduction occurs from the onset of puberty and is predominant in the cTEC<sup>+</sup> subset. This suggests that the pool of mature cTEC<sup>+</sup> cells is primarily established pre-puberty, with expansion due to both TEPC differentiation and cTEC<sup>+</sup> proliferation. At the onset of puberty, the initiation of TEPC quiescence and the reduced generation of Sca-1<sup>+</sup>cTEC<sup>+</sup> precursors, together with reduced cTEC<sup>+</sup> proliferation, may be a significant rate-limiting step for T cell lineage commitment and early T cell differentiation events. With the additional significant loss in mTEC<sup>+</sup> number, these TEC...
Figure 6. Altered Microenvironment and Reduced TEC Function in Activin A KO Grafts

(A) Representative IF images of 6-week-old thymic grafts derived from E14.5 WT and activin KO animals. Keratin 14 (red) and UEA-1 (green) stainings are shown. Dotted lines represent boundaries between cortical (c) and medullary (m) regions. Scale bars, 100 μm.

(B) Representative IF images of 6-week-old thymic grafts derived from E14.5 WT and activin KO animals. Top: pan cytokeratin (blue), UEA-1 (green), and AIRE (red) stainings are shown, followed by a higher magnification co-localization image. Bottom: pan cytokeratin (green), DEC205 (blue), and Foxn1 (red) stainings are shown, followed by a higher magnification co-localization image. Dotted lines represent boundaries between cortical (c) and medullary (m) regions. Scale bars, 100 μm.

(C and D) Proportion of CD4+ and CD8+ T cells in the blood (C) and spleen (D) of engrafted animals.

(E) Number of CD4+ and CD8+ splenic T cells.

*p < 0.05 and **p < 0.01. Data are represented as means ± SEMs. Three or more biological replicates; two or more independent experiments.
subset alterations would have a severe impact on T cell development, contributing to the dramatic loss of thymocytes evident from the onset of puberty.

An alternative TEPC phenotype was recently proposed to exist in the PLET-1+cTEChi subset (Ulyanchenko et al., 2016). While the vast majority of PLET-1+ cells are in the mTEC5o subset, minor populations exist in other TEC subsets. The reduction of PLET-1+cTEChi by middle age with a concomitant increase in the proportion of PLET-1+ cells in the cTEC5o population, together with their poor CFE and our finding that half of this

Figure 7. In Vitro Orchestration of Immature cTEC Differentiation via BMP4 and Activin A
(A) Representative flow cytometry TEC plots from D7 in vitro 3D TEPC co-cultures, supplemented with anti-activin βA antibody (7-week-old TEPCs) or activin A (9-month-old TEPCs), cTEC (UEA-1+) and mTEC (UEA-1-) proportions are shown.
(B) Representative flow cytometry TEC plot used to isolate E14.5 immature cTECs for in vitro 3D co-culture.
(C) CFE of E14.5 immature cTEC and 7-week-old male mice TEPC in vitro 3D co-cultures, supplemented with BMP4 and/or activin A.
(D) TEC colony size at D7. E14.5 immature cTEC cultures were supplemented with BMP4 and/or activin A. Scale bars, 25 μm.
(E and F) Representative IF images of each TEC colony type observed in E14.5 immature cTEC cultures at D7. Scale bars, 25 μm.
(F) Proportion of TEC colony types observed was calculated from total colony counts for each condition.
(G) Schematic model depicting the interplay between FST288, activin A, and BMP4 signaling in TEPC differentiation.

**p < 0.01 and ****p < 0.0001. Data are represented as means ± SEMs. Three or more biological replicates; one or more independent experiments.
population expresses intermediate levels of AIRE, suggest that PLET-1+cTEC\textsuperscript{hi} may be downstream of the cTEC\textsuperscript{lo}TEPC population described herein. The possibility that PLET-1+cTEC\textsuperscript{hi} may be destined to become AIRE\textsuperscript{+} mTECs warrants further investigation.

Thymic involution has been attributed to the direct action of androgens on TECs (Olsen et al., 2001). In addition to confirming the transient reversal of thymus atrophy through AD, we reveal that this regenerative process is both systematic and dynamic; restoring cortical regions before the medulla, while promoting mTEC proliferation thereafter. We demonstrate that these events can be attributed to the generation of cTEC\textsuperscript{lo} from the initial mobilization of Sca-1\textsuperscript{int/lo} cTEC\textsuperscript{lo} and the rapid proliferation and transition of mTEC\textsuperscript{lo} to mTEC\textsuperscript{hi}, which regenerates the mature TEC populations that support enhanced thymocyte development. TEPC reactivation likely occurs concurrently or soon after to replenish these immature TEC precursors. The de novo generation of AIRE\textsuperscript{+} mTEC\textsuperscript{hi} suggests that central tolerance mechanisms accompany the increase in thymopoiesis. However, since a previous study did not find increased TRAs following AD in aged animals (Griffith et al., 2012), further investigations are required before the restoration of self-tolerance can be concluded. It has not been confirmed why thymic reconstitution is not sustained following AD; however, this may be the result of a compensatory mechanism against the effects of Cx, such as androgen receptor signaling through Wnt and β-catenin (Schweizer et al., 2008).

Using transcriptomic analyses, we identified two TGF-β signaling molecules, BMP4 and FST, that are upregulated in TSCs with age and downregulated immediately following Cx. Given that our analyses derive from whole TECs and total non-epithelial stroma, it is clear that only distinct differences in gene expression were detected. Further subdivision of these groups will allow for the study of DEGs in specific cell populations and potentially the identification of more subtle molecular alterations that are associated with thymic involution and regeneration.

We have previously shown that BMP4 enhances TEC precursor maintenance (Barsanti et al., 2017; Wertheimer et al., 2018). Together with the upregulation of BMP4 from puberty and the persistent decline in TEC and fibroblastic expression post-Cx, these data suggest that its modulation contributes to the observed increase in TEC differentiation with AD. Predominant TEC expression of its receptor, BMPR2, on TEPC, Sca-1\textsuperscript{lo}cTEC\textsuperscript{lo}, and mTEC\textsuperscript{lo} populations (Barsanti et al., 2017) further supports this model. Whether there is a bias toward TEC precursor maintenance or differentiation appears to be dose dependent (Barsanti et al., 2017).

FST competitively binds activin A to inhibit its signaling capacity and is increased during aging. The significant decrease in FST levels observed post-Cx in cTECs and fibroblasts from middle-aged mice imply a role for the activin A signaling pathway in inducing TEC differentiation. Activin KO mice were deficient in both cortical and medullary TEC and had disseminated medullary regions, which affected CD4\textsuperscript{+} T cell generation, whereas the analysis of mice deficient in FST288, the surface-bound isoform (Sugino et al., 1997), revealed an increase in TEC differentiation. In contrast, administration of this isoform diminished TEPC differentiation and mTEC development in young mice and attenuated TEC regeneration in middle-aged Cx mice. Activin A is a homodimer that comprises two Inhba subunits. Although it is essential for the differentiation of human embryonic stem cells into definitive endoderm (D’Amour et al., 2005), its role in TEC proliferation and differentiation has not been previously described. How this TGF-β signaling molecule interacts with other key members in its family during thymogenesis also remains poorly defined. Here, we examined the interplay between activin A and BMP4, two key TGF-β signaling molecules that are involved in thymus organogenesis. We hypothesized that activin A signaling counteracts BMP4, instead promoting overall TEC differentiation, with BMP4 maintaining TEC precursor populations. Through 3D TEC co-culture analyses, we provide evidence to support this model, with a combination of both signaling molecules producing an intermediate colony profile phenotype, while the more undifferentiated j15\textsuperscript{+} colonies and more K14\textsuperscript{+} differentiated colonies were associated with BMP4 and activin A supplementation, respectively. It is possible that the disruption of one pathway may allow for a more prompt and more prominent effect in the other since both cascades use SMAD4 for downstream signal transduction (Yoshimatsu and Watabe, 2011). Together with the compensatory increase in the expression of its receptor, ACVR2A, on aging cTEC\textsuperscript{lo} and mTEC\textsuperscript{lo} populations, the decrease in mature TEC populations observed with activin deficiency and via FST antagonism supports our model of reduced activin A signaling in age-related thymic involution.

These findings demonstrate a model in which the modulation of FST, activin A, and BMP4 axis contribute to the maintenance of mature TECs, their lineage-specific epithelial progenitors, and TEPC. The initiation of TEPC quiescence at the onset of puberty and subsequent reliance on lineage committed progenitors with an attenuated capacity for differentiation together affect the sustainability of maintaining mature TECs during aging. Our findings also suggest that it may be possible to release the age-associated block in TEPC differentiation to promote mature cTEC and mTEC recovery via an activin A-dependent mechanism.

**STAR METHODS**

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.05.045.

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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.

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R Development Core Team (2013). R: A language and environment for statistical computing.


**STAR METHODS**

**KEY RESOURCES TABLE**

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LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, A/Prof Ann Chidgey (ann.chidgey@monash.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

4wk, 7wk and 8mo old C57BL6/J and BALB/c male, and C57BL6/J female mice were analyzed to determine the effects of thymic aging, with surgical castration studies performed on 9-12mo old C57BL6/J males. The role of follistatin (FST) and activin A in TEC development was assessed using embryonic mFST288 knock-out mice (E18.5, 4mo old; Lin et al., 2008), adult mFST288 knock-out mice (hTgFST315; Matzuk et al., 1995b) and activin A deficient animals (E14.5, 9-10wk old; Matzuk et al., 1995a). In vitro 3D culture assays were performed on TECs isolated from E14.5 embryos, and TEPCs (Wong et al., 2014) isolated from 7wk and 9mo old C57BL6/J males. All animals were bred and housed at Monash Animal Research Platform and Laboratories (Monash University, Australia), according to ethical guidelines (SOBSA/ADB/2015/039). For surgical procedures, mice were anaesthetized with 90 mg kg⁻¹ ketamine + 4.5 mg kg⁻¹ xylazine i.p., and then treated with 10 mg kg⁻¹ carprofen for pain relief. Procedures were conducted in accordance with the National Health and Medical Research Council Australian Code of Practice for the Care of Scientific Purposes (1997), and were approved by the Monash University and Monash Medical Centre Animal Ethics Committees.

METHOD DETAILS

Surgical castration
Mice were anesthetized with 90 mg kg⁻¹ ketamine + 4.5 mg kg⁻¹ xylazine i.p., and a small scrotal incision (3-4mm) was made to reveal the testes. The testes were tied off and removed along with surrounding fatty tissue. The wound was subsequently closed using surgical staples. Sham castrations were performed using the same surgical procedure, without removal of the testes. Mice were treated with 10 mg kg⁻¹ carprofen for pain relief.

FST treatment
5-6wk old and 9mo old (9mo untreated, castrated or sham-castrated) animals were injected intraperitoneally every other day for 7 or 14 days with 0.3 mg/g body weight of recombinant human FST288 (Mark Hedger, Hudson Institute of Medical Research; Hardy et al., 2015) or PBS. Mice were subjected to phenotypic analyses on day 7 and day 14. Recombinant human FST288 was produced in HEK293E cells transfected with a FST-expressing plasmid (pSV2HF288), a gift from Professor Shunichi Shimasaki (University of California, San Diego, USA), and purified by successive rounds of chromatography: heparin-Sepharose affinity, size exclusion and RP-HPLC.

Antibodies and reagents
The following anti-mouse antibodies and reagents were obtained from BD PharMingen (San Diego, CA) unless otherwise stated: anti-EpCAM, CD45, Ly51, MHCII, Sca-1, integrin 𝛼6, PLET-1/MTS24 (gift from Richard Boyd), Ki67, CD31, TCR 𝛳, CD4, CD8, DEC205, Keratin 5 (K5; Abcam), pan cytokeratin (Dako), AIRE (eBioscience), FOXN1 (Bioss Inc.), jìSf (MBL), and K14 (Biolegend); UEA-1 (Vector Laboratories) and DAPI. Propidium iodide (PI) was used as a viability marker.
Flow cytometric analysis and cell sorting

Primary antibody cell surface staining was performed for 20 min at 4°C. Cells were subsequently twice washed with FACS buffer, and stained with secondary antibody (where applicable). PI was added to remove non-viable cells from analyses. For intracellular Ki67 staining, surface-stained cells were treated with Cytofix/Cytoperm (BD Biosciences), and incubated with antibodies for 40 min. Cells were acquired using a FACSCanto II flow cytometer (BD Biosciences). All FACS data were analyzed using FlowLogic software v400.1A (Inivai Technologies). TEC sorting was performed using an Influx I cell sorter (BD Biosciences), with a 100 μm nozzle at 20 psi. Adult TEC subsets were counted and used directly in functional 3D culture assays, or snap frozen in liquid nitrogen for RNA extraction.

Individual and pooled thymus digestion

Thymi were digested as previously described (Seach et al., 2012). Following digestion in RPMI-1640 containing 0.3% Liberase TM and 0.03% DNase I (Roche, Germany), the resulting suspensions were passed through a 100 μm mesh to remove debris, and counted using a Z2 Coulter Counter (Beckman Coulter, Fullerton, CA). Thymic digests were immediately stained with antibodies for FACS analysis. For TEC isolation, pooled thymic digests were incubated with CD45 MicroBeads and depleted of thymocytes using an autoMACS Pro Separator (Miltenyi Biotec).

FTOC and kidney graft

E14.5 thymi were treated with 1.35 μM of 2-deoxyguanosine (dGuo, Sigma-Aldrich) at 37°C (5% CO2) during 6 days for the selective removal of thymocytes and dendritic cells before engraftment under the kidney capsule of athymic nude mice. Grafts were harvested after 6 weeks and enzymatically digested for FACS analysis or snap frozen for immunofluorescence.

3D TEC cultures

in vitro 3D culture experiments were performed on E14.5 cTECs, and TEPCs isolated from 7-week and 9-month old C57BL6/J males. TEC cultures were supplemented with 10 ng ml⁻¹ activin A (saturated levels, data not shown), 80 ng ml⁻¹ Bmp4 (Barsanti et al., 2017), and/or 7.5 μg ml⁻¹ anti-activin α antibody (R&D Systems), 160 μg ml⁻¹ anti-FST serum (Mark Hedger, Hudson Institute of Medical Research) and cultured as previously described (Wong et al., 2014). 2 × 10⁵ purified cTECs/TEPCs were co-cultured with 2 × 10⁵ irradiated mouse embryonic fibroblasts (MEFs) in 50% Matrigel (356231; BD Biosciences). These cultures were allowed to set in 24-well 0.4 μm transwell inserts (Millipore) for 10 min at 37°C, and subsequently provided with TEC media [RPMI-1640 with 10% FBS, 55μM 2-mercaptoethanol (2-ME), 10mM HEPES, 1x10³U/mL penicillin, 1x10⁴M streptomycin, 2mM GlutaMAX, 1mM sodium pyruvate, 0.1mM non-essential amino acids, 5μg/mL insulin, 2.75μg/mL transferrin, 3.35ng/mL selenium, 2x10⁻⁵ M 3,3’,5-triodo-L-thyronine, 0.4μg/mL hydrocortisone, 24μg/mL adenosine, 1x10⁻¹⁰M cholera toxin, 0.0002% (v/v) heparin and 20ng/mL KGF (R&D Systems, U.S.A)]. Incubation was performed under low oxygen conditions (5% O₂, 10% CO₂, and 85% N₂) during 7 days. The resultant colonies were digested with 0.3% Liberase TM for FACS analysis or fixed for immunofluorescence.

RT-qPCR

Thymic stromal cell subsets purified through cell sorting were immediately treated with lysis buffer reagent (Molecular Research Center). Total RNA was recovered following standard protocols. cDNA synthesis was performed using Superscript III and oligo(dT) oligonucleotides (Invitrogen, San Diego, CA). Quantitative PCR was performed on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) in 10 μL reactions using SYBR Green Supermix and individual bioinformatically pre-validated primers (QIAGEN, Hilden, Germany: FOXN1, ACVR2A, FST, BMP4, BMPR2). Target gene expression levels relative to those of GAPDH were determined using the standard 2⁻ΔΔCT method (Wong et al., 2014).

TEC microarray and bioinformatics analysis

RNA was purified from TEC (CD45-EpCAM+) and non-TEC (CD45-EpCAM-) samples and total RNA was quality ascertained using the Agilent Bioanalyzer 2100 using NanoChip protocol. Samples were retro-transcribed to cDNA for microarray analysis using Illumina Mouse WG-6-V2 arrays, by the Australian Genome Research Facility (AGRF). Bioinformatics analysis was performed by the Monash Bioinformatics Platform. R statistical computing environment (R Development Core Team, 2013) and Bioconductor limma package (Ritchie et al., 2015) were used for processing and analysis.

Elisa

Serum samples for activin A determinations were obtained by cardiac puncture from 5-6wk, 9-12mo ShCx and Cx mice from day 4 (CxD4) - day 135 (CxD135) post-surgery. Blood was centrifuged for 10 min at 1,500 rpm, 25°C and serum was stored at –80°C until analyzed. Activin A concentration was evaluated by specific ELISA kit, according to the manufacturer’s specifications (R&D Systems, Minneapolis, USA).

Histology and immunofluorescence

Hematoxylin and eosin staining of paraffin-embedded samples was performed courtesy of the Monash Histology Platform (Monash University, Australia).
Sections from OCT (Tissue-Tek) -embedded thymi and FTOC graft samples were cut 8 µm thick, and stained as previously described (Hun et al., 2017), with minor alterations. For thymic samples, staining was performed at RT for 15 min with K5 (secondary: anti-rabbit Alexafluor 568) and UEA-1 (secondary: streptavidin Alexafluor 647). Sections were twice washed in Tris-buffered saline (TBS) between primary and secondary staining steps. Stained slides were subsequently mounted in fluorescent mounting media (Dako), and imaged on a Nikon inverted confocal microscope (Monash Micro Imaging, Australia). FTOC graft sections were stained with: i) pan cytokeratin (secondary: anti-rabbit Alexafluor 647), UEA-1 (secondary: streptavidin Alexafluor 488), and Aire (secondary: anti-rat Alexafluor 568); or ii) pan cytokeratin (secondary: anti-rabbit Alexafluor 488), DEC205 (secondary: streptavidin Alexafluor 647), and Foxn1 Alexafluor 555. Sections stained with cocktail i) utilized the protocol described above. Slides stained with cocktail ii) were instead fixed with 4% PFA, blocked with 1% FBS in TBS, and stained with pan cytokeratin and DEC205 (including secondaries) prior to 1% Tween in TBS washes (Barsanti et al., 2017). Foxn1 staining was then performed for 1 h, followed by 1% Tween in TBS washes. All FTOC graft sections were subsequently stained with DAPI for 5 min, and washed in TBS prior to mounting and imaging.

Immunofluorescence of 3D TEC colonies was performed following 4% PFA fixation of colony transwell inserts for 20 min, and antigen retrieval with sodium citrate buffer at 95°C for 30 min (Wong et al., 2014). After a 2 h rinse in 0.1% Triton-X in PBS (washing buffer) at RT, colony inserts were blocked with 1% BSA in washing buffer for 1 h, and washed for subsequent jist (secondary: anti-chicken Alexafluor 488) and K14 (secondary: anti-rabbit Alexafluor 647) staining. Inserts were washed in between 2 h staining periods, with a 15 min DAPI stain performed prior to mounting. Transwell membrane discs were cut from inserts using a scalpel, and placed in mounting media with colonies facing the coverslip.

QUANTIFICATION AND STATISTICAL ANALYSIS

All values are expressed as mean ± SEM, unless otherwise specified. Statistical analyses were performed using GraphPad Prism v7.02 software (GraphPad), with the appropriate tests utilized. A p value < 0.05 was considered statistically significant.

DATA AND CODE AVAILABILITY

Microarray Data reported in this paper is available on the GEO repository and the accession number is GEO: GSE132278.
Supplemental Information

Interplay between Follistatin, Activin A, and BMP4 Signaling Regulates Postnatal Thymic Epithelial Progenitor Cell Differentiation during Aging

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Figure S1 (Related to Figure 1). Aging impairs mTEC and TEPC differentiation regardless of species or gender

(A-B) Number of thymocytes (A) and TECs (B) per BALB/c male thymus.

(C) Proportion and number of TEPCs.

(D-F) Proportion of Sca-1lo cTEClo (D), Ki67+ TECs (E), and Ki67+ TEPCs (F).

(G) Representative flow cytometry plots; used to identify changes in TEC subsets during aging.

(H) Representative flow cytometry cTEClo plots; used to identify TEPC, Sca-1int cTEClo, and Sca-1lo cTEClo.

(I-J) Number of thymocytes (I) and TECs (J) per C57BL/6 female thymus.

(K) Proportion and number of TEPCs.

(L-N) Proportion of Sca-1lo cTEClo (L), Ki67+ TECs (M), and Ki67+ TEPCs (N).

(O) Representative flow cytometry plots; used to identify changes in TEC subsets during aging.

(P) Representative flow cytometry cTEClo plots; used to identify TEPC, Sca-1int cTEClo, and Sca-1lo cTEClo.

*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (three or more biological replicates; one or more independent experiments).

Data are represented as mean ±SEM.
Figure S2 (Related to Figure 1). PLET-1+ cTEC<sup>hi</sup> do not contribute to the reduced proliferation seen in cTEC<sup>hi</sup> during aging

(A) Representative flow cytometry plots; used to identify PLET-1<sup>-</sup> cTECs.

(B) Representative flow cytometry plots; used to identify changes in PLET-1<sup>-</sup> TEC subsets during aging.

(C) Proportion and number of PLET-1<sup>-</sup> cTECs.

(D) Proportion of Ki67<sup>+</sup> PLET-1<sup>-</sup> cTEC<sup>hi</sup>.

(E) Proportion and number of AIRE<sup>+</sup> PLET-1<sup>-</sup> cTEC<sup>hi</sup>.

(F) Representative flow cytometry plots; used to identify changes in AIRE expression within cTEC, mTEC, and PLET-1<sup>-</sup> cTEC<sup>hi</sup> populations during aging.

(G) CFE of 4-7wk old male mice TEPC, PLET-1<sup>-</sup> cTEC<sup>hi</sup>, and PLET-1<sup>-</sup> cTEC<sup>hi</sup> in vitro 3D co-cultures.

*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (three or more biological replicates; one or more independent experiments).

Data are represented as mean ±SEM.
Figure S3 (Related to Figure 2). Reestablishment of thymic function following AD
(A and B) Number of thymocytes (A) and TECs (B) per Cx and ShCx thymus. Dotted line represents 5-6wk old.
(C) mTEC/cTEC ratio.
(D) cTEC<sub>b</sub>/cTEC<sub>h</sub> and mTEC<sub>b</sub>/mTEC<sub>h</sub> ratio.
(E) Proportion of Ki67<sup>+</sup> TEC subset per thymus.
(F) Representative flow cytometry cTEC<sub>h</sub> plots; used to identify TEPC, Sca-1<sup>hi</sup> cTEC<sub>h</sub>, and Sca-1<sup>lo</sup> cTEC<sub>h</sub> post-Cx.
*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (three or more biological replicates; two independent experiments).
Data are represented as mean ±SEM.
Figure S4 (Related to Figure 3). Differential gene expression in young, aged, post-AD TECs and non-TECs

(A-B) Principal component analysis of TEC (A) and non-TEC (B) samples (two or more biological replicates; one or more independent experiments). Circled groups represent 5-6wk (orange), 9-12mo ShCx (light blue), 9-12mo Cx (green), and 9-12mo (dark blue) population clusters.

(C-D) Common DEGs between young vs middle-aged, and 9-12mo old Cx vs ShCx groups from TEC (C) and non-TEC (D) samples.

(E-F) Expression profiles for common DEGs between young vs middle-aged, and 9-12mo old Cx vs ShCx TEC (E) and non-TEC (F) groups.

(G) TEC expression profiles for FST and BMP4.

(H-I) Concentration of serum activin A in 5-6wk old and 9-12mo old male mice (H), and 9-12mo old ShCx and Cx male mice (I) (five biological replicates; two independent experiments). Data are represented as mean ±SEM.
A List of DEGs; p<0.05, logFC>1.5

B Biological processes: GO analysis; p<0.05