Development and developmental potential of cortical thymic epithelial cells

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Summary

The thymic cortex provides a microenvironment for the development and positive selection of immature T cells. Cortical thymic epithelial cells (cTECs), which structurally and functionally support the thymic cortical microenvironment, originate from endodermal epithelial progenitors that arise in the third pharyngeal pouch. Recent studies have revealed that thymic epithelial progenitors pass through a stage where the cells express cTEC-associated molecules prior to lineage separation into cTECs and medullary TECs (mTECs). Here we review the molecular signatures of cTECs and highlight the development and developmental potential of cTECs.

Keywords

Thymus, cortex, cortical thymic epithelial cell, positive selection, thymic epithelial progenitor

Running title

Cortical thymic epithelial cells

Introduction

The thymus is a primary lymphoid organ that supports the development and repertoire selection of T cells. The thymic architecture is mostly divided into two distinct microenvironments, the cortex and the medulla, which are characterized by the presence of cortical thymic epithelial cells (cTECs) and medullary thymic epithelial cells (mTECs), respectively. The cortical and medullary thymic microenvironments differently contribute to T cell development; i.e., the cortex supports early T cell development and positive selection of immature thymocytes, whereas the medulla supports the establishment of self-tolerance in T cells.

T-lymphoid progenitors that migrate into the thymus parenchyma are induced to differentiate into T cells through the signals through Notch ligand DLL4 and γc-cytokine IL-7, which are highly expressed in cTECs (1-4). Immature thymocytes are primarily detectable in the thymic cortex (5, 6), where the thymocytes are induced by DLL4 and IL-7 to express T-cell antigen receptor TCRαβ as well as co-receptors CD4 and CD8. The V(D)J rearrangement in the TCRα and TCRβ genomic loci in cortical thymocytes is responsible for the diversity in TCR recognition specificities carried by the pool of T cells. cTECs are also reported to contribute to the development of γδ□T cells (7).

Newly generated thymocytes that express TCRs carrying individual recognition specificities are selected for life or death according to their TCR recognition specificities, initially in the cortical microenvironment through the interaction with cTECs that express self-peptide-associated class I and class II MHC molecules (8). Only thymocytes that are signaled with low-affinity TCR engagement are selectively induced for cell survival. This process is termed positive selection. Positively selected

thymocytes begin expressing chemokine receptor CCR7 and migrate to the medullary parenchyma where mTECs abundantly produce CCR7 ligand chemokines (9, 10). The migration to the medulla is important for T cells to establish tolerance to self-components, including tissue-restricted self-antigens (11-15). Only thymocytes that survive multiple layers of positive and negative selection in the cortical and medullary microenvironments are entitled to export to the circulation.

Thus, cTECs are chiefly responsible for the early induction of T cell generation and the positive selection of newly generated T cells. Here we will initially provide a brief summary of molecular signatures expressed by cTECs, focusing on the functions and heterogeneity of cTECs. We will then discuss the development of cTECs as well as their developmental potential to give rise to mTECs.

cTECs provide microenvironment for early T cell development

cTECs express various molecules that support T cell lineage specification and regulate early T cell development in the thymic cortical microenvironment. As these aspects of cTEC functions have been reviewed previously (10, 15-17), here we only briefly list several molecules in this regard.

DLL4

The pioneering study by Schmitt and Zuniga-Pflücker unveiled the ability of Delta-mediated Notch signals to induce the lineage specification of early lymphoid progenitors to become T cells (18). It was later identified that among five mammalian Delta-like ligands, DLL4 is abundant in cTECs and responsible for T cell lineage commitment of early lymphoid progenitors and subsequent development to the

$CD4^+CD8^+$ stage (1, 2). The expression of DLL4 in cTECs is negatively correlated with ontogeny, so that DLL4 expression in the thymic cortex decreases with age (19). Low levels of DLL4 may be sufficient for the maintenance of T-lymphopoiesis in the adult thymus.

Cytokines

Interleukin-7 (IL-7) is a γc cytokine essential for the survival and differentiation of immature lymphoid cells, including immature thymocytes. IL-7 is produced by cTECs and mTECs, but is more abundant in cTECs than mTECs (20). Another cytokine kit-ligand (KL), also known as stem cell factor (SCF), which promotes the survival and proliferation of immature thymocytes, is also more highly expressed in cTECs than mTECs (21).

Transforming growth factor (TGF) β proteins, which are abundant in cTECs, contribute to regulating the rate of the generation of $CD4^+CD8^+$ thymocytes from intermediate $CD8^{low}$ precursor cells (22).

Chemokines

cTECs highly express chemokines CCL25 and CXCL12 as well as chemokine-binding protein CCRL1 (15, 23, 24).

During embryonic development, CCR9 ligand CCL25 produced by TECs in the thymus primordium critically regulates the colonization of lymphoid progenitors, in coordination with CCR7 ligand CCL21 produced by the neighboring parathyroid primordium, particularly before the vascularization of the thymus (25). The role of chemokine signals through CCR9 and CCR7 ligands in the thymus seeding of T cell

progenitors can also be detected in postnatal mice when lymphoid progenitor cells are competitively transferred in radiation bone marrow chimera experiments (26, 27).

CXCR4 ligand CXCL12 is detectable in cTECs throughout the cortex, and is most abundantly expressed in the outer cortex (23). CXCL12 critically regulates early thymocyte development by promoting the survival of immature thymocytes (28). CXCL12 also plays a role in the appropriate positioning of immature thymocytes in the thymic cortex (23).

CCRL1, also known as Ccx-ckr1, is a non-signaling receptor for chemokines CCL19, CCL21, and CCL25, and is more abundant in cTECs than mTECs (24). It was reported that CCRL1 regulates thymus colonization before vascularization in fetal mice (29) as well as optimal thymus homeostasis and normal thymocytes development in adult mice (30).

cTECs organize microenvironment for T cell positive selection

cTECs provide the microenvironment for not only generating TCR-expressing $CD4^+CD8^+$ thymocytes but also inducing positive selection of newly generated $CD4^+CD8^+$ thymocytes. In addition to expressing self-peptide-associated class I and class II MHC molecules for TCR recognition by $CD4^+CD8^+$ thymocytes, cTECs carry unique protein degradation machineries that provide MHC-associated self-peptides that optimize positive selection of thymocytes. These functions of cTECs have been extensively reviewed elsewhere (14, 15, 31-34). Here we briefly provide an update of the molecules involved in self-antigen presentation in cTECs.

Thymoproteasome

The thymoproteasome is a cTEC-specific form of the proteasome, which cytoplasmically produces class I MHC-associated peptides. The thymoproteasome is specifically expressed in cTECs, as its β5 subunit, β5t encoded by *Psmb11*, is exclusively abundant in cTECs and not in any other cells (35). Cells that express thymoproteasomes display a unique repertoire of class I MHC-associated peptides (36). An analysis of β5t-deficient mice suggested that thymoproteasome-dependent peptides associated with class I MHC displayed by cTECs are enriched with low-affinity TCR ligands, so that thymoproteasome-expressing cTECs are capable of inducing optimal positive selection of functionally competent $CD8^+$ T cells (36, 37). A recent study using monoclonal TCR-transgenic mice further revealed a novel aspect of thymoproteasome-dependent positive selection, in which thymoproteasome-expressing cTECs are crucial for not only shaping an immunocompetent TCR repertoire but also fine-tuning TCR responsiveness in positively selected $CD8^+$ T cells (38).

Cathepsin L and thymus-specific serine protease

Cathepsin L is a ubiquitously expressed lysosomal endopeptidase. In the thymus, cTECs abundantly express cathepsin L, whereas other antigen-presenting cells, including mTECs, predominantly express cathepsin S rather than cathepsin L. In addition to its role in the degradation of invariant chain Ii, which is assembled with class II MHC molecules (39, 40), cathepsin L in cTECs is involved in the generation of class II MHC-associated self-peptides for positive selection of $CD4^+$ T cells (31, 41).

Thymus-specific serine protease (Tssp), which is encoded by *Prss16*, was initially reported in the human genome for its association with the susceptibility to type I diabetes (42). Tssp is highly expressed in cTECs (43). Tssp-deficient mice exhibit a

decrease in class II MHC expression in cTECs and partial impairment in positive selection of $CD4^+$ T cells (44-46). The role of Tssp in tumor regulation is also noted (47).

Autophagy

Autophagy, or macroautophagy, is an intracellular protein degradation system that is activated by nutrient starvation (48, 49). However, autophagy is constitutively active in the thymus, especially among TECs including many cTECs, even without the starvation (49, 50). As autophagosomes fuse with lysosomes for proteolysis, autophagy contributes to providing cytosolic protein antigens to the class II MHC presentation pathway. Indeed, autophagy in cTECs has been shown to contribute to the optimal positive selection of $CD4^+$ T cells (50).

Heterogeneity in cTECs and thymic nurse cells

Considering that cTECs play multiple roles in T cell development by promoting the early induction of T cell generation and by supporting positive selection, it is tempting to speculate that cTECs consist of functionally distinct subpopulations that individually play different roles in T cell development. It was reported that mTECs consist of at least two clearly distinct and functionally potent subpopulations, namely, Aire-expressing self-antigen-producing mTECs and CCR7-ligand-expressing thymocyte-attracting mTECs (51). However, it has been shown that the majority of cTECs express DLL4, IL-7, class II MHC, β5t, and CD205 (1, 4, 52, 53), suggesting that T cell development-inducing cTECs and positive selection-inducing cTECs are overlapped with each other.

cTECs are defined as epithelial cells localized in the cortex of the thymus. Thanks to recent progress in the molecular biology of thymic non-hematopoietic cells, as outlined above, cTECs can now be identified and isolated on the basis of the expression of CD205, Ly51 (CD249), and EpCAM (CD326). Other classical markers, such as class II MHC, keratin 8, and ER-TR4, as well as more recently identified functional molecules, such as DLL4, IL-7, CCRL1, and β5t, are additionally useful for the identification and characterization of cTECs. The undetectable expression of mTEC markers, such as keratin 5, keratin 14, MTS-10, ER-TR5, and Aire, as well as the reactivity to the lectin *Ulex europaeus* agglutinin 1 (UEA-1), can also offer clues for the identification of cTECs. Measuring the expression of these molecules has inspired studies of the heterogeneity in cTECs on a single cell basis. By detecting the expression levels of these molecules, it has been shown that cTECs are heterogeneous most obviously in class II MHC expression, consisting of class II MHC^{low} and class II MHC^{high} populations (54). Heterogeneity in the expression levels of DLL4, IL-7, CD205, and other molecules has also been noted (4, 11, 52, 55).

It is interesting to note that thymic nurse cells (TNCs) represent a functionally distinct subpopulation of cTECs. TNCs are large TECs that envelop many thymocytes (56). Since their discovery more than 30 years ago (57, 58), many researches have tried to uncover their functions. There have been suggestions that TNCs provide the microenvironments that support the proliferation and differentiation of cortical thymocytes (59-63) as well as positive and negative selection of thymocytes (64-66). Recently, Nakagawa, et al. (67) reported that approximately 10% of β5t-expressing cTECs in adult mouse thymus can be defined as TNCs that completely envelop many CD4$^+$CD8$^+$ thymocytes. It was shown that TNCs are not necessary for thymocyte

positive and negative selection, as TNCs were hardly detected in the thymus of TCR-transgenic mice in that positive or negative selection was readily detectable (67). Rather, thymocytes confined within TNCs were enriched with long-lived $CD4^+CD8^+$ thymocytes that underwent secondary TCRα rearrangement (67). Therefore, it was suggested that TNCs, which represent a subpopulation of cTECs, provide a microenvironment for the optimal TCR repertoire selection of $CD4^+CD8^+$ thymocytes through the secondary TCRα rearrangement. Heterogeneity in gene expression profiles between TNCs and non-TNC cTECs has also been noted (67).

Ontogeny of cTECs

Like mTECs, cTECs originate from the endodermal epithelium of the third pharyngeal pouch (68, 69). Bipotent thymic epithelial progenitors (pTECs) that give rise to cTECs and mTECs have been detected in embryonic and postnatal thymus (70-72). In mouse, TECs are detectable as early as embryonic day 11 (E11) by the landmark expression of Foxn1, a member of the forkhead family of transcription factors that specify TECs and hair follicle cells (68, 73-75). Spontaneous mutations in Foxn1 lead to congenital thymic hypoplasia accompanied by severe T cell deficiency in mouse and human (74-77). Even without Foxn1, keratin-expressing epithelial cells are detectable in the third pharyngeal pouch, although thymic architecture supported by cTECs and mTECs is not subsequently formed (75), indicating that Foxn1 is indispensable for the development of TECs rather than the formation of the third pharyngeal pouch or its epithelial layers. The differentiation of cTECs from bipotent progenitors is initiated as early as embryonic day 12 (E12) in mouse (52, 53). Two recent papers have described the ontogenetic development of cTECs.

Shakib, et al. (52) reported the successive developmental stages of cTECs defined by the expression of CD205 and co-stimulatory molecule CD40 during mouse ontogeny. In Foxn1-deficient mice, the thymus primordium expressed neither CD205 nor CD40. $CD205^+$ TECs emerged at E12 without detectable CD40 and gradually acquired CD40 expression along with the increase in CD205 expression level during embryogenesis. Isolated $CD205^+$ $CD40^-$ embryonic TECs expressed β5t and cathepsin L genes, but were heterogeneous in the expression of class II MHC. Neither CD40 nor class II MHC was expressed in $CD205^+$ cTECs in hCD3ε tg26 mice, in which early thymocytes development was defective (52, 78). These results suggest that cTEC development occurs initially through the $CD205^+$ $CD40^-$ stage and the subsequent elevation of CD40 and class II MHC to give rise to $CD205^+$ $CD40^+$ class II MHC^{high} cTECs, and that thymocyte development influences the late phase of cTEC development (*Fig. 1*).

Mat Ripen, et al. (53) reported the ontogeny of β5t-expressing cTECs in mouse. β5t-expressing cells were detectable as early as E12.5, specifically in the thymus and in CD205-expressing cTECs. The expression levels of CD205 and CD249 as well as class II MHC were gradually elevated in β5t-expressing TECs during ontogeny, suggesting that β5t is expressed by cTECs at both immature and mature stages. In support of this finding, β5t expression in cTECs was detectable even in hCD3ε tg26 mice. β5t was undetectable in the thymus primordium of Foxn1-deficient mice, whereas β5t was present in abundance in relB-deficient mice that lacked mTECs. Thus, like CD205, β5t is expressed at the initial appearance stage of cTECs in Foxn1-dependent manner, but independent of thymocytes or mTECs (*Fig. 1*).

The molecular mechanisms regulating cTEC development are vague, in

contrast to the roles of TNFSF cytokine receptors, including RANK, CD40, and lymphotoxin β receptor, and the downstream signaling pathways for the activation of NF-κB transcription factors, which have been extensively documented in mTECs (79-85). Experiments conducted in hCD3ε tg26 mice, in which thymocyte development is arrested at the very early DN1 stage, have shown that the thymic cortex is disorganized and cTECs are arrested at the $CD40^-$ MHC II^{low} stage (52, 86, 87). In contrast, cTECs are fully capable of giving rise to the $CD40^+$ class II MHC^{high} stage even in Rag1-deficient mice, in which T cell development is arrested at the DN3 stage (52). Thus, cTECs require signals from developing thymocytes beyond the DN1 stage for optimal development (*Fig. 1*).

In addition to developing thymocytes, mesenchymal cells in the thymus contribute to the development of cTECs. Fibroblast growth factor (FGF)-7 (also known as KGF), FGF-10, and insulin-like growth factor 1 (IGF-1) produced by mesenchymal cells promote the proliferation of cTECs and mTECs (88-92). In contrast, mesenchymal cell-derived retinoic acid (RA) negatively affects the cellularity of cTECs and mTECs, whereas the blockade of RA signaling increases the cellularity of cTECs and elevates the expression of DLL4, β5t, and Tssp in fetal thymus organ culture (93) (*Fig. 1*). It remains unclear how RA affects cTECs.

Cells that express cTEC-associated molecules give rise to cTECs and mTECs

Through further analysis of cTEC development, recent studies have independently and unexpectedly reported that mTECs are derived from bipotent progenitors that express cTEC-associated molecules.

Baik, et al. (94) examined the developmental potential of embryonic TECs

expressing cTEC-associated molecule CD205. They cultured fetal thymuses isolated from E11 and E12 mouse embryos in the presence or absence of agonistic anti-RANK antibodies that were capable of promoting the development of mTECs, and analyzed the expression of TEC maturation markers CD40 and MHC II in $CD205^{negative}$, $CD205^{low}$, and $CD205^{high}$ TEC populations. All of these populations responded to RANK stimulation by expressing CD40 and MHC II in 1-day culture of E12 thymuses, and the frequency of $CD40^{+}$ MHC II^{+} cells progressively increased with the elevation of CD205 expression levels. On the other hand, E11 thymus cells did not respond to RANK stimulation; the cells failed to express CD40 and MHC II in the 1-day culture experiments. These results suggest that $CD205^{+}$ embryonic TECs serially acquire the potential to give rise to $CD40^{+}$ MHC II^{+} mTECs. They further showed that highly purified $CD205^{+}$ $CD40^{-}$ embryonic TECs, which expressed a set of cTEC-associated molecules (52), could differentiate into both Aire-expressing mTECs and β5t-expressing cTECs in *in vitro* reaggregate thymus organ culture followed by transplantation of the aggregates under mouse kidney capsules (94). Thus, $CD205^{+}$ embryonic TECs, which resemble cTECs, carry bipotent progenitor capability that gives rise to both cTECs and mTECs.

Ribeiro, et al. (20) studied the developmental potential of TECs expressing IL-7 in IL-7-reporter transgenic mice, in which the IL-7 promoter drove the gene encoding yellow fluorescence protein (YFP). They found that the majority of YFP^{+} cells were enriched in $CD205^{+}$ $Ly51^{+}$ cTECs through the ontogeny, whereas $CD80^{+}$ mTECs were predominantly detectable in YFP^{-} cells. In addition to those cell-surface molecules, YFP^{+} cells expressed other cTEC-associated genes, including DLL4, β5t,

and Tssp, whereas YFP$^-$ cells contained other mTEC-associated genes, including Aire and RANK. Thus, YFP$^+$ and YFP$^-$ thymic cells predominantly contained cTECs and mTECs, respectively. They further looked into the developmental potential of YFP$^+$ and YFP$^-$ TECs isolated from embryonic thymus in *in vitro* reaggregate thymus organ cultures, and found that both YFP$^+$ and YFP$^-$ TECs gave rise to Ly51$^+$ cTECs and CD80$^+$ mTECs. These results indicate that embryonic TECs that express high levels of IL-7 and so resemble cTECs retain the differentiation potential into mTECs. Subsequently, they also examined the developmental potential of embryonic cTECs by detecting another cTEC-associated molecule, CCRL1, using CCRL1-EGFP-knockin mice, in which EGFP is expressed under the control of CCRL1 gene expression (95). The expression of CCRL1-dependent EGFP in the thymus was detectable as early as E13.5, and gradually increased during ontogeny. *In vitro* reaggregate thymus organ culture experiments demonstrated that CCRL1-EGFP$^+$ TECs gave rise to UEA1$^+$CD80$^+$ mTECs in the presence of RANK and CD40 stimulation (95). Therefore, CCRL1$^+$ embryonic TECs, which resemble cTECs, retain developmental potential to give rise to mTECs.

Those studies by Baik, et al. (94) and Ribeiro, et al. (20, 95) examined the developmental potential of embryonic cTECs essentially by *in vitro* cell culture experiments with or without subsequent *in vivo* transplantation in mice. In contrast, in our recent study, we examined the developmental potential of cTECs by *in vivo* fate mapping experiments, which enabled the characterization of normally developed mTECs without employing *in vitro* cell cultures or invasive transplantation surgeries. To do so, we engineered mice in that the coding sequence of the cTEC-specific gene,

β5t, was replaced with Cre recombinase, and crossed those mice with CAG-loxP-stop-loxP-EGFP-transgenic reporter mice, in which EGFP would be driven under the control of the CAG promoter only when the loxP-flanked stop sequences were excised by Cre expression (96). In those mice, EGFP expression reflected present and/or past expression of β5t in cells. We found that β5t-Cre-mediated EGFP expression could be detected in TECs but not in other cells in the thymus or other organs. Among TECs, β5t-Cre-mediated EGFP was expressed in almost all mTECs as well as in almost all cTECs throughout the ontogeny. As mTECs do not presently express β5t, these results indicate that the majority of mTECs originate from cells that express β5t (96).

The expression of β5t-Cre-mediated EGFP is detectable in TECs as early as E12.75, approximately half a day after the first detection of β5t protein in TECs (53, 96). However, β5t protein is no longer detectable in $EGFP^+$ mTECs localized in the central region of E12.75 thymus. These results suggest that mTECs derived from $β5t^+$ TEC progenitors lose the ability to express β5t soon after the commitment to become mTECs (96). Perinatal $β5t^+$ TECs, which resemble cTECs, are indeed bipotent, giving rise to cTECs and mTECs as shown in the reaggregate organ culture and kidney transplantation experiments (97).

These studies from at least three independent laboratories have collectively proposed a novel concept for the TEC differentiation pathways, particularly the mechanisms of how cTECs and mTECs are diversified from their common progenitors; i.e., bipotent TEC progenitors progress through the stage that exhibits the molecular signatures of cTECs, including the expression of CD205, IL-7, and β5t, prior to

commitment to the cTEC and mTEC lineages (98) (*Fig. 2*). As these bipotent TEC progenitors express cTEC-associated molecules, these progenitors can be viewed by definition as a fraction of cTECs. Previously reported embryonic cTECs may contain, or even represent, the bipotent TEC progenitors expressing cTEC-associated molecules. In addition, it can be interpreted that a fraction of cTECs carry developmental potential to give rise to mTECs. The concept of "serial progression" of cTECs and mTECs, agrees with the earlier development of cTECs than mTECs in ontogeny and with the necessity for mTECs to establish medullary self-tolerance only when cTECs are functionally competent to induce and positively select T cells.

Perinatal and postnatal mTEC progenitors that express cTEC molecules

Several studies have reported the existence of bipotent TEC progenitors in the adult thymus (71, 72, 99). Accordingly, Ohigashi, et al. (100) and Mayer, et al. (97) examined the developmental potential of β5t^{+} bipotent TEC progenitors in a given period by employing β5t-rtTA knock-in mice, in which reverse tetracycline transactivator (rtTA)-encoding sequence was inserted in the β5t locus. β5t-rtTA knock-in mice were crossed with Tet operator-driven Cre transgenic mice along with loxP-dependent EGFP or ZsGreen reporter mice, which allowed *in vivo* tracing of cells that transcribed β5t during a given period by tracing fluorescent cells labeled by doxycycline (Dox) administered during that period (97, 100). The tracing of fluorescence-labeled mTECs in adult mice revealed that approximately 60% of cells were embryonically labeled, and approximately 30% of mTECs were fluorescence-labeled during the first week of life (100). In sum, at least 90% of mTECs in the adult thymus are derived from progenitors that transcribe β5t during

embryogenesis and the neonatal period up to 1 week of age. These frequencies of embryonically and neonatally labeled cells within mTECs remained unchanged at least up to 45 weeks of age (100). The fluorescence-labeled mTECs included class II MHC^{high} mTECs, which contained Aire^{+} cells, and class II MHC^{low} mTECs, which contained CCR7-ligand-expressing cells (51, 100). Embryonically and neonatally labeled mTECs similarly expressed genes that were functionally relevant in mTECs, but were not identical with respect to the spectrum of promiscuously expressed self-antigens, including the fetal antigen, α-fetoprotein (100). These results indicate that embryonic and neonatal β5t^{+} progenitors are capable of forming functional mTEC subpopulations.

In contrast to these perinatal β5t^{+} progenitors, the contribution of adult β5t^{+} progenitors to the *de novo* generation of mTECs in adult thymus was minor. The frequency of fluorescence-labeled mTECs dropped to approximately 3-5% in mice that were Dox-treated after 1 week of age (100). These fluorescence-labeled mTECs might in part reflect the promiscuous expression of β5t gene in a small fraction of mTECs. However, the fluorescence in mTECs remained detectable even several months after Dox treatment and so possibly reflected the contribution of adult β5t^{+} progenitors in the long-term maintenance of mTECs in the adult period albeit at a low frequency (97). Nevertheless, these results indicate that unlike perinatal β5t^{+} progenitors, adult β5t^{+} progenitors play only a minor role in the maintenance of mTECs in the adult thymus (*Fig. 3*).

Considering the active proliferation and rapid turnover of mTECs in the adult thymus (101-103), it is conceivable that mTEC-lineage committed cells that exceed the

β5t$^+$ bipotent stage during early ontogeny, rather than postnatal bipotent TEC progenitors, mainly maintain adult mTECs via active proliferation throughout life (*Fig. 3*). It is even possible that mTECs are actually maintained by the continuous self-duplication of mTECs (*Fig. 3*), as observed in other epithelial tissues, such as pancreas and liver (104, 105).

In this regard, Sekai, et al. (106) recently reported that mTEC-lineage-restricted progenitor/stem cells, which are capable of maintaining functional mTECs, can be defined as claudin-3/4$^+$ SSEA1$^+$ cells. In collaborative experiments with their laboratory, we have shown that the majority of those claudin-3/4$^+$ SSEA1$^+$ mTEC-lineage-restricted progenitor/stem cells detectable in the adult thymus are derived from perinatal β5t$^+$ progenitors (100). Thus, it is possible that mTEC-lineage-restricted progenitor/stem cells contribute to the maintenance of mTECs in the adult thymus (*Fig. 3*).

Where do bipotent TEC progenitors localize in the thymus? Classically, it was shown that K5$^+$ K8$^+$ TECs, which were presumed to contain bipotent TEC progenitors, were enriched at the cortico-medullary junction in the adult thymus (87, 107). More recently, perinatally labeled β5t$^+$ TEC progenitors were detected at the cortico-medullary junction in the adult thymus (97). It is therefore possible that bipotent TEC progenitors localize at the cortico-medullary junction in the adult thymus, to supply cTECs to the cortex and mTECs to the medulla (*Fig. 4*). Alternatively, the localization of bipotent TEC progenitors in the adult thymus is not limited to the cortico-medullary junction in the thymus. Rather, they produce cTECs and mTECs to newly generate the microenvironments of the cortex and the medulla, respectively, in the areas neighboring the place where the progenitors localize, so that the

cortico-medullary junction is consequently formed wherever bipotent TEC progenitors are present in the thymus parenchyma (*Fig. 4*).

Postnatal maintenance of cTECs

The use of β5t-rtTA knock-in-dependent TetO-Cre-mediated fluorescence reporter mice enabled the analysis of the postnatal maintenance of cTECs with respect to the contribution and decay of cTECs that express β5t in a given time period. The fluorescence labeling of cTECs in these mice could reflect either the current expression of β5t in cTECs or the past expression of β5t during the differentiation from $\beta5t^{+}$ bipotent TEC progenitors. We found that the majority of cTECs in the adult thymus are fluorescence-labeled by Dox administered during either embryogenesis or the neonatal period (97, 100). The frequency of the embryonically and neonatally labeled cTECs gradually decreased to approximately 70% by 45 weeks old, suggesting that approximately two-thirds of cTECs in the adult thymus are maintained by cells that are derived from embryonic or neonatal β5t-expressing cells, whereas approximately one-third of adult cTECs are *de novo* generated in adult mice (100). Thus, the postnatal dynamics for the generation and maintenance of cTECs appears to differ from that of mTECs. Unlike mTECs, a considerable portion of cTECs in the adult thymus may be *de novo* generated in adult life.

Age-dependent damage in cTECs

The thymus is one of the most susceptible organs to age-dependent atrophy, or involution. Thymic involution leads to a decline in *de novo* T cell production and in the diversity of T cell repertoires, thereby resulting in the deterioration of the immune

system (108). The cortical compartment of the thymus, which is predominantly composed of cTECs and $CD4^+CD8^+$ thymocytes, is highly susceptible to the involution (108, 109). A recent study by Griffith, et al. (110) revealed that reactive oxygen species contribute to the early senescence of cTECs during age-dependent thymic involution. Through global transcriptome analysis and transgenic overexpression experiments, they showed that the expression of the antioxidant enzyme, catalase, is reduced in TECs, particularly in cTECs, and that either the transgenic overexpression of catalase or the administration of antioxidants diminishes thymic atrophy. These results suggest that metabolic damage in cTECs by catalase deficiency and thereby by the accumulation of reactive oxygen species plays an important role in the age-dependent loss of cTECs.

Age-dependent thymic involution is correlated with the decrease in Foxn1 expression in TECs (111). Genetic manipulation to reduce Foxn1 expression in the postnatal thymus leads to a decrease in TEC cellularity, whereas overexpression of Foxn1 in aged mice restores the number of TECs (112-115). Inactivation of retinoblastoma (RB) protein enhances Foxn1 expression by activating E2F transcription factors, so that the RB-E2F transcriptional pathway regulates Foxn1 expression. Inactivated RB protein in TECs decreases with age (116), whereas the decrease in E2F3 transcription activity in cTECs and MHC II^{low} mTECs is correlated with thymic involution (21). Age-associated decrease in Wnt4, which promotes Foxn1 expression, is also correlated with the decrease in Foxn1 expression (21, 117, 118).

In the human thymus, the secretion of proinflammatory cytokines, including IL-6, is elevated in an age-dependent manner (119). In mouse, many proinflammatory cytokine genes, including *Il1a*, *Il1b*, *Cxcl2*, *Il6*, *Il12b*, *Il18*, and *Tnf*, are elevated in thymic dendritic cells in aged thymus, whereas cTECs and thymic fibroblasts express

IL-1 activating receptor gene *Il1r1* rather than IL-1 antagonists *Il1rn* and *Il1r2*, which are detectable in mTECs (21). In mice deficient in inflammasome Nlrp3, active IL-1β is reduced and cTECs are maintained in aged thymus (120). Thus, the elevated expression of proinflammatory cytokines may contribute to the age-dependent damage in cTECs.

Injury and regeneration of cTECs

Thymic involution is induced not only by the ageing but also by other stresses, including irradiation, infection, and chemotherapeutic drugs. In contrast to age-dependent thymic involution, stress-induced thymic injuries are transient and regenerable. Rode and Boehm (24) engineered Ccx-ckr1-diphtheria toxin receptor (DTR)-transgenic mice, in which the Ccx-ckr1 promoter drives DTR expression. In these mice, transient treatment with diphtheria toxin (DT) efficiently ablated cTECs accompanied by the decrease in thymocytes, and cessation of the DT treatment led to the recovery of both cTECs and thymocytes. These results indicate that cTECs carry regenerative potential to counter injury-triggered thymic involution. Upon irradiation, radio-resistant lymphoid tissue inducer cells promote the production of IL-22 in an IL-23-dependent manner (121). The IL-22 receptor is expressed in cTECs and mTECs, and IL-22 promotes the increase in the number of cTECs and MHC II^{low} mTECs in irradiated thymus, contributing to the repair of the thymic cortex and medulla (121).

During the post-injury thymic repair, cTECs and mTECs are regenerated from injury-resistant cells that could be either bipotent progenitors or lineage-restricted cells. We recently examined the contribution of $\beta5t^{+}$ bipotent progenitors during injury-triggered thymic regeneration, by employing β5t-rtTA x tetO-Cre x GFP-reporter mice. We found that embryonic and neonatal, rather than adult, $\beta5t^{+}$ progenitors

contributed to the majority of mTECs in the thymus regenerated from either total body irradiation or polyinosinic-polycytidylic acid treatment, which mimicked viral double-stranded RNAs and induced interferon-α-mediated injury in TECs. Similar to mTECs, the *de novo* generation of cTECs was not enhanced during the injury-triggered thymus regeneration (100). Therefore, the injury-triggered regeneration of mTECs is mainly mediated by cells that are derived from perinatal β5t$^+$ TEC progenitors, rather than by cells derived from adult β5t$^+$ TEC progenitors (*Fig. 5*). Self-duplication or lineage-committed progenitors likely contribute to the regeneration of most cTECs and mTECs.

Sex hormones affect cTECs

TECs are highly dynamic and exhibit continuous turnover. It was estimated that approximately 10% of TECs in the adult thymus are newly supplied daily, and TECs turnover occurs every 10 to 14 days (101). cTECs and mTECs proliferate at a similar rate in young mice, but the proliferation rate of cTECs become lower than that of mTECs in aged mice (122). The ablation of male sex hormones by castration promotes the regeneration of the thymus in aged mice by enhancing the proliferation of TECs and thymocytes (101).

Dumont-Lagacé, et al. (123) reported that the expression in cTECs of molecules associated with cTEC functions, including Foxn1, DLL4, CCL25, β5t, and cathepsin L, was lower in males than females or castrated males. The proliferation of cTECs was less active in males than females or castrated males, whereas mTEC proliferation was little affected by gender. They also reported that the cellularity of cTECs was higher in males than females or castrated males, which could be associated

with the higher expression of cell death inhibitors and the lower expression of cell death activators (123). However, we detected lower cellularity of cTECs in male mice than female mice (100). The regeneration of cTECs in DT-treated Ccx-ckr1-DTR-transgenic mice was reduced in males compared with females or castrated males (24). Nonetheless, the contribution of embryonic, neonatal, and adult β5t$^+$ progenitors in the generation, maintenance, and injury-triggered regeneration was essentially comparable between female and male mice (100). Thus, gender and sex hormones strongly affect cTECs.

Epithelial-mesenchymal transition in the thymus

Epithelial-mesenchymal transition (EMT) is a process that allows an epithelial cell to change into a mesenchymal cell, and contributes to various phases of development, tumor metastasis, and tissue repair fibrosis (124). In the thymus, EMT has been suggested to contribute to tissue adipogenesis associated with age-dependent involution (125-127).

The β5t-Cre x loxP-EGFP mice enabled efficient labeling of virtually all TECs (96) and were so useful for the quantitative analysis of EMT in young and aged mice. Immunofluorescence analysis of thymic sections showed that a fraction of MTS15$^+$ mesenchymal fibroblasts were EGFP$^+$ in 2-week-old and 11-month-old mice (*Fig. 6A*). Flow cytometric analysis indicated that the frequency of EGFP$^+$ cells in CD45$^-$PDGFRα$^+$ mesenchymal cells was approximately 10% and comparable between 2-week-old mice and 11-month-old mice (*Fig. 6B*). These results suggest that EMT is detectable in young mice and is not greatly elevated by age, at least up to 11 months old.

Concluding remarks

In this review, we summarized current knowledge of cTEC biology, focusing on the development and developmental potential of cTECs. Despite the importance of cTECs in the development and selection of T cells, little is known about the molecular mechanisms underlying the development of cTECs. Important issues to be addressed in this regard include the molecular mechanisms that specify cTEC lineage from bipotent progenitors and that induce thymocyte-dependent maturation of cTECs. The recent finding that bipotent TEC progenitors transiently express cTEC-associated molecules, such as β5t and CD205, may provide a useful clue useful to unravel the molecular mechanisms that regulate the bifurcation of cTECs and mTECs as well as the subsequent development of cTECs and mTECs.

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Figure legends

Fig. 1. **Phenotypic progression of cTECs during ontogeny.** cTEC expression of cell-surface molecules including MHC II, CD40, CD205, Ly51, and CCRL1 increases along the ontogeny, whereas EpCAM cell-surface expression declines. The expression of β5t and high levels of IL-7 is also detectable in cTECs throughout the ontogeny. The development of cTECs is regulated by signals provided by developing thymocytes. FGF-7, FGF-10, and IGF-1 produced by mesenchymal cells promote the proliferation of cTECs, whereas mesenchymal cell-derived RA negatively affects the cellularity of cTECs.

Fig. 2. **mTECs are derived from cells that express cTEC-associated molecules.** Bipotent TEC progenitors progress through the stage in that cells express cTEC-associated molecules, including β5t, CD205, CCRL1, and high levels of IL-7, prior to the lineage specification into cTECs and mTECs. cTECs retain the expression of these molecules, which is down-regulated in mTECs.

Fig. 3. **Adult mTECs are maintained by mTEC-lineage-restricted cells that pass beyond the bipotent stage during early ontogeny.** Adult mTECs are maintained by cells that pass through the $\beta5t^{+}$ bipotent stage rather than by bipotent progenitors. It is possible that adult mTECs are maintained by the continuous self-duplication of mTECs. Alternatively, mTEC progenitor/stem cells may contribute to the maintenance of mTECs in the adult thymus.

Fig. 4. **Two hypotheses regarding the localization of bipotent TEC progenitors.** (A) Bipotent TEC progenitors in the adult thymus localize at the cortico-medullary junction to supply cTECs into the cortex and mTECs into the medulla. (B) The localization of bipotent TEC progenitors in the adult thymus is not limited to the cortico-medullary junction but can be anywhere in the parenchyma. Bipotent TEC progenitors newly produce cTECs and mTECs, which generate the microenvironments of the cortex and the medulla, respectively. Consequently, the cortico-medullary junction will be formed in the area where bipotent TEC progenitors originally reside.

Fig.5. **Contribution of perinatal versus adult β5t$^+$ TEC progenitors in the development, maintenance, and regeneration of adult mTECs.** The majority of adult mTECs are maintained and regenerated by cells that pass beyond the β5t$^+$ bipotent stage during embryogenesis (red line) and neonatal period (blue line). The contribution of adult β5t$^+$ TEC progenitors is minor, even during injury-triggered mTEC regeneration (green line).

Fig. 6. **Epithelial-mesenchymal transition in the thymus.** (A) Immunofluorescence analysis of the thymus obtained from β5t-Cre-knockin mice crossed with CAG-loxP-stop-loxP-EGFP-transgenic reporter mice (abbreviated as β5t-Cre x loxP-EGFP mice). Thymus sections were examined in 2-week-old (wo) and 11-month-old (mo) mice for the expression of EGFP (green), K5 (blue), and MTS15 (red). Data are representative of at least three separate experiments. Scale bar = 25 μm. (B) Representative flow cytometry profiles of collagenase-digested thymus cells from β5t-Cre x loxP-EGFP mice. Cells were multi-color-stained for CD45, PDGFRα, and

propidium iodide (PI). Histograms show EGFP expression profiles in $PI^-CD45^-PDGFR\alpha^+$ viable thymic mesenchymal cells (left panels) in β5t-Cre x loxP-EGFP mice (solid lines) and littermate control β5t-Cre-knockin mice (shaded lines). Numbers in histograms indicate frequencies within the indicated area. Bar graph shows the frequency (means and SEs, n = 3) of $EGFP^+$ cells in $PI^-CD45^-PDGFR\alpha^+$ cells (right panel). n.s., not significant.

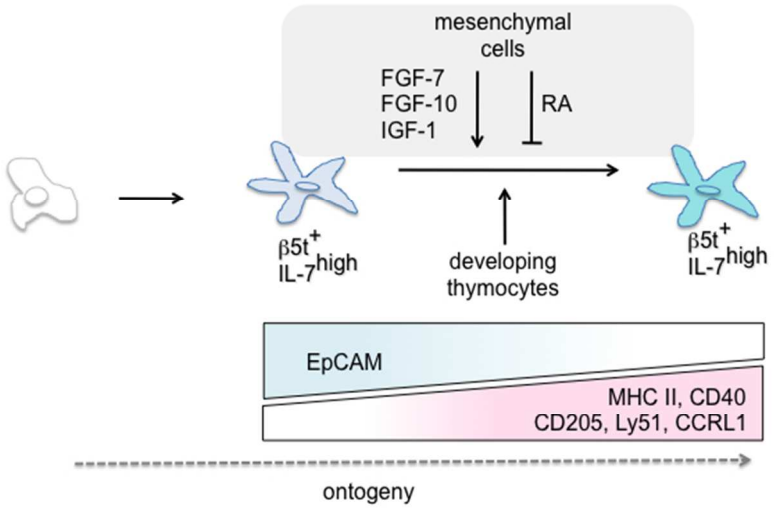

Figure 1
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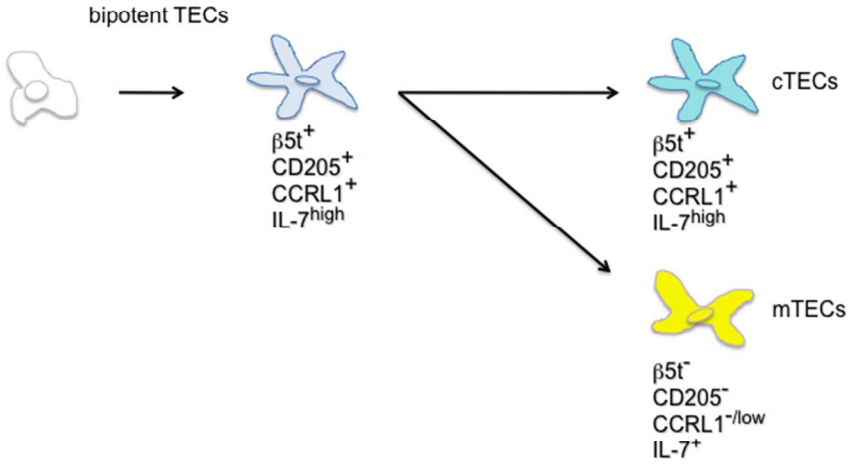

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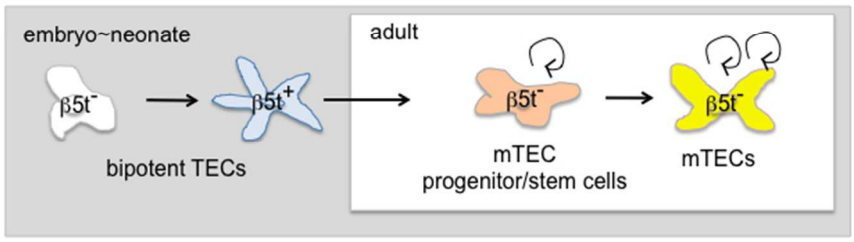

Figure 3
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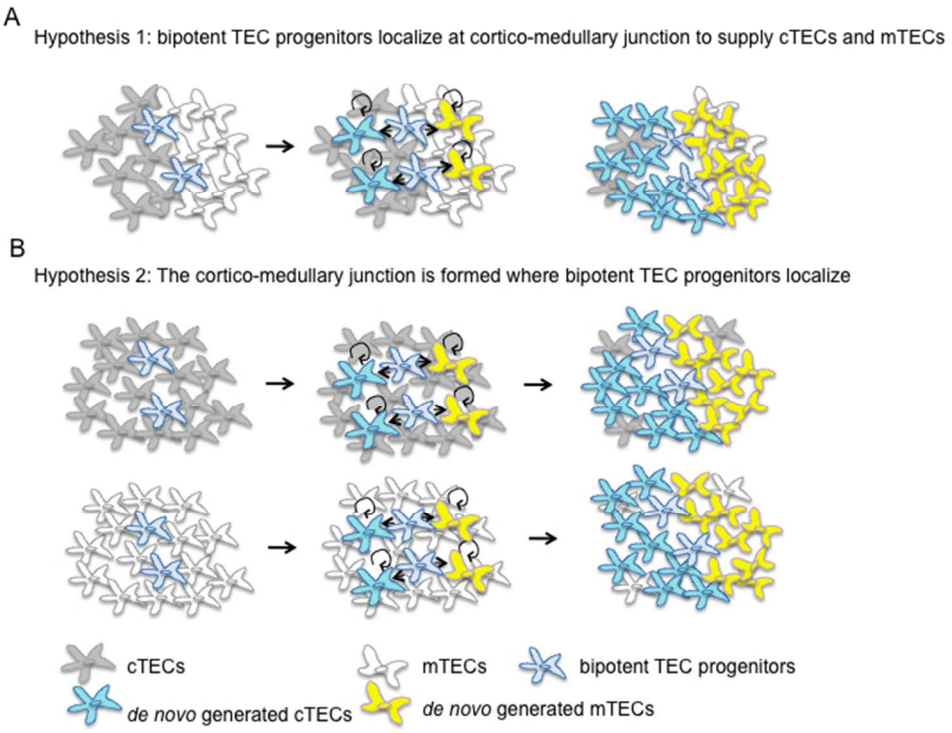

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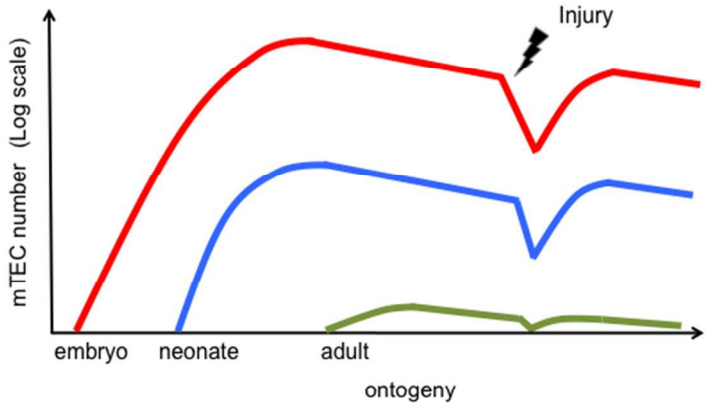

Figure 5
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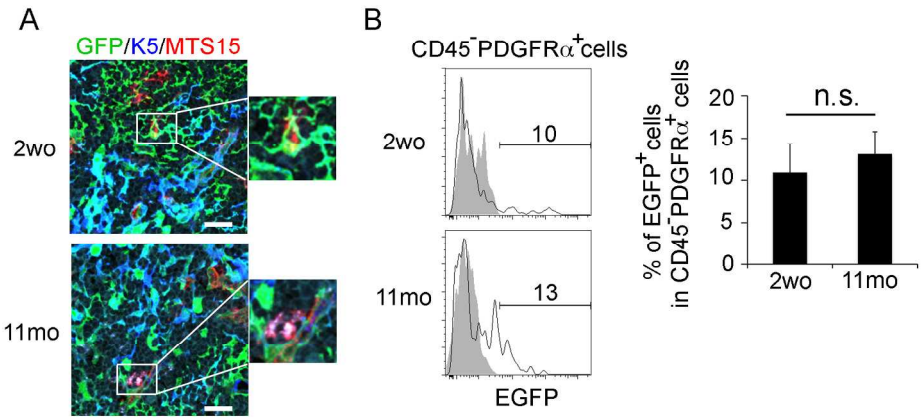

Figure 6
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