DEVELOPING A NEW PARADIGM FOR THYMUS ORGANOGENESIS

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The mature thymic epithelium is complex, with two major compartments — the cortex and the medulla — each containing several functionally distinct epithelial-cell types. There is considerable debate as to the embryonic origins of these different thymic epithelial-cell subpopulations. The textbook view is a dual origin, with cortical thymic epithelium arising from the ectoderm and medullary thymic epithelium originating in the endoderm. However, the literature has been divided on this issue since it was first considered. In this review, we discuss recent embryological, functional, genetic and molecular data that collectively support a new model of thymus organogenesis and patterning.

STROMA
Cells that comprise the non-lymphocytic component of the thymus.

LINEAGE
Embryonic origin and fate of cells during normal development.

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The thymus has a central role in the immune system, as it is crucially required for T-cell differentiation and repertoire selection[1]. These processes are mediated by the thymic STROMA, which, correspondingly, has a complex cellular composition[2] (FIG. 1). The unique functions of the thymus, however, reside mainly in the thymic epithelium, which forms the major sub-compartment of the stroma. The stroma itself is commonly divided into two main regions on histological grounds, the cortex and the medulla, and each of these regions contains several ultrastructurally and phenotypically distinct types of thymic epithelial cell (TEC)[3,4].

A long-held view is that the different TEC subsets generate discrete intrathymic microenvironments, each specialized for mediating a particular aspect of thymocyte development[2,5,6]. Consistent with this model, T-cell development is characterized by the progression through several phenotypically distinct stages, defined as double negative (DN), double positive (DP) and single positive (SP) based on expression of the co-receptors CD4 and CD8; the DN subset is further subdivided into four stages (DN1–3 and DN4/pre-DP) by differential expression of CD44 and CD25. Thymocytes at different stages of development occupy distinct spatially restricted domains in the adult thymus[7–9] (FIG. 1), indicating that differentiation occurs concomitantly with a highly ordered migration; T-cell precursors enter the thymus at the cortico–medullary junction, then migrate progressively to the subcapsular zone of the outer cortex, back through the cortex and into the medulla, from where they egress to the periphery (FIG. 1). Thymocytes and TECs are in close contact throughout this differentiation programme.

The 'microenvironment model' is now supported by functional studies, which have shown essential roles for the cortical epithelium in regulating directional migration of thymocyte precursors[9] and in mediating positive selection[10], and have implicated the medullary epithelium in driving the final stages of thymocyte maturation[11]. In addition, several recent studies indicate that promiscuous gene expression by epithelial cells in the thymic medulla has a crucial role in tolerance induction[12–16]. Current understanding of the functions of the different TEC subsets, shown in FIG. 1, has been well reviewed elsewhere[2,12,17,18] and is not further discussed here.

The demonstration of the functional importance of the thymic epithelium, in combination with insights into TEC biology resulting from the analysis of mouse mutants generated by gene-targeting technology, has led to a renewed interest in the molecular basis of TEC function, and in the origins and LINEAGE relationships of the different TEC types. These areas are not only important from an embryological standpoint, but also have significant implications for *in vitro* organogenesis and T-cell generation, transplantation and regenerative medicine. In particular, the heterogeneity of the thymic epithelium

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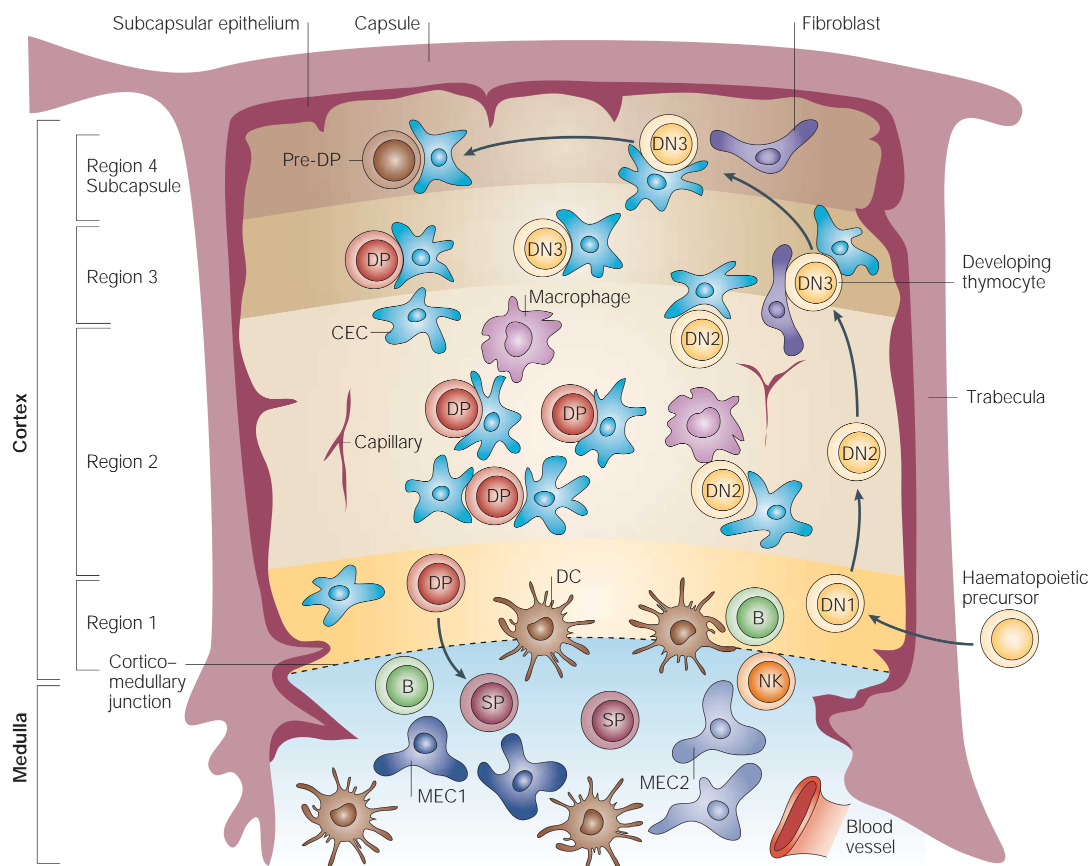

Figure 1 | **Thymus structure and function.** The thymus is broadly divided into two histologically defined regions, the cortex and the medulla, each of which contains several different thymic epithelial cell (TEC) subtypes. In adults, T-cell precursors enter the thymus at the cortico–medullary junction, and then begin a highly ordered differentiation programme, which is linked to migration through the thymic stroma. Different thymocyte subsets are therefore found in spatially restricted regions of the thymus. The thymic cortex has been separated into four regions by Lind and colleagues[8]: region 1, the cortico–medullary junction, is the site of entry into the thymus and contains uncommitted progenitors, $CD4^-CD8^-$ double-negative 1 (DN1) cells; in region 2, cells differentiate to the DN2 stage, undergo a proliferative clonal expansion, and lose B- and natural killer (NK)-cell potential; T-cell lineage commitment and the onset of T-cell receptor (TCR) β-chain rearrangement occurs in DN3 cells in region 3; and the transition from DN to $CD4^+CD8^+$ double-positive (DP) status occurs in region 4. DP cells then migrate back through the cortex and, having differentiated into either $CD4^+$ or $CD8^+$ single-positive (SP) cells, into the medulla. Positive selection occurs mainly in the cortex, and requires cortical TECs, whereas negative selection occurs mainly in the medulla, and is mediated by medullary TECs and thymic dendritic cells (DCs). SP cells that have completed the differentiation programme egress from the medulla to the periphery. CEC, cortical epithelial cell; MEC, medullary epithelial cell.

has proved a major impediment to attempts to recapitulate T-cell differentiation and selection efficiently *in vitro*. Although commitment of mouse fetal liver haematopoietic progenitor cells to T-cell lineages and their subsequent differentiation to the $CD4^+CD8^+$ DP stage can be achieved using OP9 bone-marrow stromal cells transfected with the Notch-ligand delta-like 1 (REF. 19), efficient generation of mature SP T cells is still only possible in cultures based on *ex vivo* thymic tissue[20,21].

As thymus organogenesis is a developmental process, similar to that of other organs, it is useful to think of it in terms of commonly defined stages of early organogenesis. Organogenesis can be divided into several distinct stages: positioning, or determining precisely where the organ rudiment will develop in the embryo; initiation, the overt development of the organ rudiment; outgrowth and patterning, resulting in the generation of regional differences in the growing rudiment; and differentiation, in which this spatial information is translated into distinct cell types. These steps must be carefully regulated and coordinated to ensure correct organ formation. A complete model of organogenesis will therefore include both morphological and molecular aspects of all of these stages.

In the case of the thymus, recent advances made in each of these areas not only provide a new framework for understanding thymus organogenesis as a developmental process, but also provide important insights into the precise origins of TECs and set the stage for understanding the mechanisms that control their differentiation. In this review, we discuss the origins and patterning of the cortical and medullary thymic epithelial compartments in the context of these developments, arguing the need for a new model of thymus organogenesis. The implications of this model for therapeutic purposes are also discussed.

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Cellular basis of thymus organogenesis

The early organogenesis of the thymus is closely tied to that of the parathyroid glands. Both organs develop from bilateral organ primordia that arise from the third pharyngeal pouch ENDODERM at around embryonic day 11 (E11) in mice[22,23]. At this stage, each endodermal primordium contains the precursors to one thymus lobe and one parathyroid gland[24], which seem, on the basis of gene-expression studies, already to be partitioned into discrete thymus and parathyroid domains[25]. Each primordium is also surrounded by a condensing mesenchymal capsule derived from NEURAL CREST CELLS (NCCs)[26,27], which support the growth and development of the primordium[28] and might also influence TEC differentiation. At about E12.5, the shared primordia separate from the pharynx and begin to migrate towards the anterior chest cavity, possibly under the guidance of NCCs[29,30]. By E13.5, the parathyroid and thymus-specific domains have resolved into physically separated organs. Soon after, they reach their respective approximate adult positions in the embryo; the thymus at the midline and the parathyroids at the lateral margins of the thyroid gland.

Origin of thymic epithelial cells

Two models for thymus organogenesis. The contributions of the third pharyngeal pouch endoderm and NCCs to the developing thymus are undisputed, although the extent to which NCCs or their progeny contribute to the adult thymus requires clarification[23,27,31]. However, considerable controversy exists as to whether the third pharyngeal cleft also contributes to the mature organ.

The most widely accepted model of thymus organogenesis suggests that both the third pharyngeal cleft ECTODERM and the third pharyngeal pouch endoderm contribute physically to the thymus during organogenesis, such that the epithelial component of the cortical compartment is generated from ectodermally derived cells, whereas cells of endodermal origin generate the medullary epithelium[24,32–34]. Support for this model is drawn from several morphological studies that date back to the early 1900s[23,35], the most convincing of which used a histological sectioning and reconstruction approach to compare thymus organogenesis in NUDE and wild-type embryos. Cordier and Heremans[32] reported that the endodermal and ectodermal germ layers made physical contact at E9.5, after which a strong proliferation of the third pharyngeal cleft derivative, the cervical sinus, occurred, such that ectodermal cells covered the third pouch endoderm between E10.5 and E11.5. The resulting compound structure detached from both the ectoderm and endoderm by E12.5, giving rise to the thymus primordium. Markedly diminished proliferation of the ectoderm was reported for nude embryos, leading to the conclusion that the primary nude defect affects ectodermal cells. Based on these observations, Cordier and colleagues[24,32] proposed the 'dual-origin' model of thymus development. Support for this model was provided by several studies in nude mice[36–38], and the scenario was further developed in terms of the anatomical and functional dichotomy between the cortex and medulla. Alternative morphological studies in mice and other mammals, however, concluded that the thymic epithelium is solely derived from the third pouch endoderm[23,35], indicating the requirement for functional studies to resolve this issue.

Remarkably, strong functional evidence supporting the 'single-origin' model has existed since 1975, when Le Douarin and Jotereau[39] reported their demonstration of the extrathymic origin of intrathymic T cells. In this seminal study, chick–quail chimaeras were generated by transplantation of pharyngeal endoderm isolated from quail embryos before colonization by lymphocytes (or development of the third pharyngeal pouch) into the body cavity of 3-day-old chick embryos. The grafted tissue was shown to develop into a thymus in which the T cells were of chick origin. Importantly, the 'reticular cells' in both cortical and medullary regions of these thymi were shown to be of quail origin, providing a clear indication that purified pharyngeal endoderm is sufficient to generate the epithelial component of both compartments. These data also showed that, at least in birds, cells in the developing endoderm are fated to adopt TEC lineages before formation of the third pharyngeal pouch.

Although these experiments did not test cell lineage directly, they provide a stringent assessment of the developmental potential of the pharyngeal endoderm. Arguably, most evidence has therefore favoured the single-origin model since the publication of this study. However, the controversy has persisted due to a lack of rigorous studies that are designed specifically to address cell lineage in the mammalian thymus.

Direct evidence for a single endodermal origin. Recent work from our laboratories has addressed this issue directly[40]. We initially repeated the histological analysis of thymus organogenesis. From this study, we concluded that although the third pouch endoderm and third cleft ectoderm certainly make contact between E10.5 and E11.5, these tissues subsequently separate, with concomitant apoptosis of cells in the contact region. The thymus primordium then seems to develop solely from the third pharyngeal pouch. We therefore assessed the fate and POTENCY of the pharyngeal ectoderm and endoderm directly.

Fate of the pharyngeal ectoderm. The fate of the pharyngeal ectoderm in the developing mouse embryo was investigated by lineage-tracing analysis in a whole embryo culture system established for this purpose[41]. In these experiments, the pharyngeal ectoderm of E10.5 mouse embryos was labelled specifically using a cell tracker dye. The embryos were then cultured for a further 30 hours, by which time they had developed to the equivalent of a normal E11.5 embryo: thymus development proceeded normally during this time, as assessed by morphological criteria and marker analysis[41]. The fate of the labelled pharyngeal ectoderm was subsequently analysed by histological and fluorescence analysis of sectioned embryos. These studies found no evidence of labelled cells in the thymic primordia of

ENDODERM
The epithelial tube inside the embryo, which gives rise to the small and large intestines, stomach, organs such as the liver and pancreas, and glands, including the thyroid and parathyroid glands.

NEURAL CREST CELLS
Migratory cells derived from the neural tube ectoderm.

ECTODERM
The epithelial sheet that covers the outside of the embryo, which gives rise to skin and hair, for example.

NUDE
A recessive mutation in the forkhead box N1 (*Foxn1*) gene that causes hairlessness and congenital athymia in mice, rats and humans. Nude individuals lack T cells as a secondary effect of athymia; nude bone marrow is normal.

POTENCY
The differentiative capacity of cells, which might be more extensive than is apparent in normal development.

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more than 100 embryos that were analysed. Importantly, the developmental period covered in this experiment spans the stage at which the ectodermal contribution to the thymus has been proposed to occur (E10.5 to E11.5). Therefore, this study found no evidence for a physical contribution of the ectoderm to the thymus, and specifically failed to support the dual-origin model of thymus organogenesis proposed by Cordier and colleagues[24].

Potency of the pharyngeal endoderm. To complement this analysis, the differentiative potential of mouse third pharyngeal pouch endoderm was assessed directly using an ectopic transplantation model. In these experiments, the third and fourth pharyngeal pouch endoderm of E8.5 to E9.0 embryos was isolated by enzymatic and manual dissection, and transplanted under the kidney capsule of nude mice (FIG. 2), which provides a permissive environment for thymus organogenesis[42–44]. Subsequent analysis of the grafted tissue showed that purified endoderm was sufficient to generate a functional thymus organized into cortical and medullary regions that could fully support thymus function. Analysis of mice grafted with E9.0 whole third and fourth pharyngeal arch showed that this tissue was less efficient than stage-matched endoderm either at generating thymi or conferring thymus function to recipients. This study therefore recapitulated the results of the classic chick–quail chimaera experiments in mice, indicating that purified pharyngeal endoderm can generate both cortical and medullary TEC compartments. It further showed that the presence of ectoderm provides no apparent advantage for thymus development. These experiments also indicate that some cells in the pharyngeal endoderm are specified to enter the TEC lineage before overt signs of organogenesis, as previously reported in birds[40].

Collectively, these histological, lineage and functional analyses provide compelling evidence in favour of a single-origin hypothesis, in which the thymic epithelium derives solely from the third pharyngeal pouch endoderm. Further evidence consistent with this conclusion has come from studies investigating the origins of the different TEC types during thymic ontogeny.

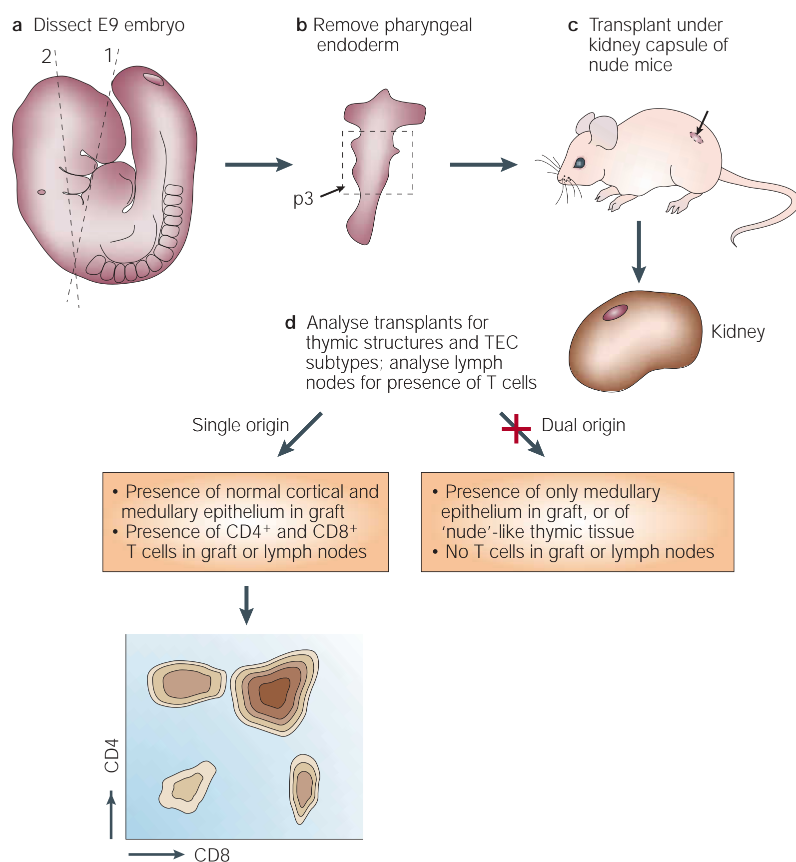

Figure 2 | **Design of transplantation experiments showing that endodermal cells can generate a functional thymus. a** | Mouse embryos are dissected at embryonic day 8.5 (E8.5)–E9, before or at the initial formation of the third pharyngeal pouch. **b** | The pharyngeal endoderm is dissected free from the ectoderm and surrounding mesenchyme. **c** | Endoderm containing the presumptive third pouch region (p3) is transplanted under the kidney capsule of nude mice. **d** | After two weeks, the transplant site is dissected and any tissue is removed for histological and immunohistochemical analysis. Lymph nodes are also removed and analysed by flow cytometry for the presence of T cells. The dual-origin model would predict the formation of either the medullary epithelium only, or thymic tissue similar to a nude thymus, as this model claims that the nude phenotype is due to endoderm developing in the absence of an ectodermal contribution. It would also predict an absence of T cells in the lymph nodes. By contrast, the single-origin model predicts the formation of both cortical and medullary thymic epithelial cells (TECs), and the ability to generate peripheral T cells. The data obtained fit the single-origin model predictions[40].

A putative common thymic epithelial progenitor cell. The existence of a common thymic epithelial stem cell has previously been suggested by several authors, based on evidence from marker studies of normal thymus and the observation that some thymomas can give rise to both cortical and medullary TEC types[35]. This area of research has been hampered by a lack of functional studies, largely because of the paucity of cell-surface markers available for the purification of candidate TEC subtypes. However, an indication of the probable phenotype of thymic epithelial progenitor cells (TEPCs) was provided by analyses of thymi in nude–wild-type aggregation chimaeras[45] and transplantation chimaeras[46].

The first of these indicated the cell autonomous requirement for the nude gene product, forkhead box N1 (Foxn1), for the development of all main TEC types[45]. This study also showed that nude cells apparently committed to TEC lineages were phenotypically similar in nude–wild-type chimaeras and in the thymic remnant of nude mice. These cells lacked markers associated with mature TEC subtypes, including MHC class II molecules, but expressed the determinants recognized by monoclonal antibodies MTS20 and MTS24 (REF. 45), which, in the adult mouse thymus, each recognize a rare subpopulation of medullary TECs[45,47]. Collectively, these data indicated that in the absence of functional Foxn1, TECs are arrested at an immature progenitor stage that is characterized phenotypically by expression of the MTS20 and MTS24 determinants[45].

In another study, Klug and colleagues[46] showed that differential expression of cytokeratin 5 (K5) and K8 distinguishes TEC subpopulations in the normal adult mouse thymus. The main cortical and medullary subsets are $K8^+K5^-$ and $K8^-K5^+$, respectively, with a minor subpopulation found mainly at the cortico–medullary junction that expresses both K5 and K8. The thymi of

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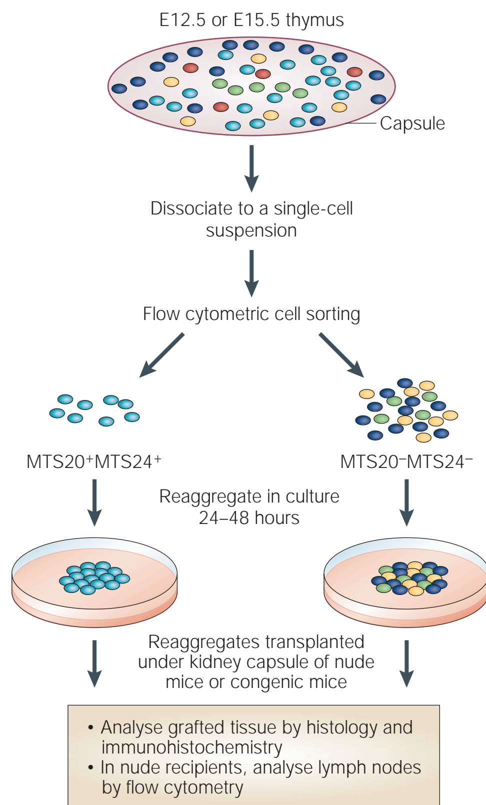

Figure 3 | **Evidence for a common progenitor/stem cell for thymic epithelial cells (TECs).** Embryonic thymi containing MTS20⁺MTS24⁺ cells (turquoise), presumptive medullary epithelial cells (green), presumptive cortical epithelial cells (dark blue), mesenchymal cells (yellow) and haematopoietic cells (red) are partitioned into MTS20⁺MTS24⁺ and MTS20⁻MTS24⁻ fractions by flow cytometry. The fractions are then tested for their ability to differentiate into a functional thymus using the strategy outlined. The data show that thymi are generated only by the MTS20⁺MTS24⁺ cell fraction, indicating that this contains all thymic epithelial progenitor cell activity[44,52].

transgenic mice that express human CD3ε under the control of its endogenous promoter (CD3ε line 26, CD3ε26) and have a secondary block in TEC differentiation, resulting from an early block in thymopoiesis at the DN1 (CD44⁺CD25⁻) stage[48,49], were found to have an aberrant, mainly K5⁺K8⁺ epithelial-cell phenotype. Transplantation of CD3ε26 thymi into recombination-activating gene 1 (*Rag1*)$^{-/-}$ recipients resulted in a partial correction of this defect, indicated by the development of a marked K5⁻K8⁺ TEC population[46]. This indicated a precursor–progeny relationship, in which K5⁺K8⁺ progenitors give rise to K5⁻K8⁺ cortical epithelial cells[46]. Although a subsequent study has shown that the T-cell development blockade in CD3ε26 transgenic mice results from an insertion effect that affects the T/B-cell fate choice[50], and so raises the possibility that, in these mice, the K5⁺K8⁺ TEC phenotype might predominate as a direct consequence of increased intrathymic B-cell development, Klug's conclusion is consistent with subsequent ontological studies that show the presence of K8⁺K5⁺ TECs in the earliest thymic rudiments[44,51], including stages before the appearance of cortical- or medullary-specific markers[44]. The combined results from these studies indicated that a common progenitor cell might exist, with a marker phenotype of MTS20⁺MTS24⁺K8⁺K5⁺.

As both MTS20 and MTS24 recognize cell-surface determinants, it has been possible to test this hypothesis directly through functional assessment of the potency of MTS20⁺MTS24⁺ cells purified from the embryonic mouse thymus. Two recent papers have reported the findings of such analyses (FIG. 3). These studies show that reactivity with MTS20 (REF. 44) and MTS24 (REFS 44,52) defines a population of embryonic TECs that, when purified and grafted ectopically, is sufficient to generate a thymus containing organized medullary and cortical compartments that both supports normal thymopoiesis[44,52] and is sufficient to establish a peripheral T-cell compartment in nude mice[44]. This population constitutes ~50% of epithelial cells in the E12.5 thymus primordium[44], in which these markers are co-expressed[44], and persists at least until E15.5 (REF. 52). Intriguingly, although at both E12.5 and E15.5 the MTS24⁻ populations contain cells that express markers of cortical and medullary TECs and so apparently are differentiating into mature cortical and medullary TECs, these populations were unable to reconstitute thymic function as assessed by any of the parameters mentioned earlier. So, in the E12.5 and E15.5 mouse thymus, all TEPC activity is contained in the MTS24⁺ fraction[44,52].

Phenotypic analysis of MTS20⁺MTS24⁺ cells in both E11.5 and E12.5 thymic primordia shows that they co-express K5 and K8 (REF. 44). At E12.5, this population appears homogeneous with respect to presently available markers[44] and, importantly, is negative for differentiation markers that are indicative of mature cortical and medullary epithelial-cell types, including MHC class II molecules[44]. At E15.5, MTS24⁺ cells mainly express MHC class II molecules and the population can be divided on the basis of reactivity with UEA-1 (REF. 52), which marks medullary TECs in the postnatal thymus. These phenotypic changes are consistent with MTS24 reactivity describing a progenitor population that is maturing over time; thereby, the earliest progenitor phenotype will be seen at E11.5 or earlier stages of primordium development.

Taken together with the demonstration of a single origin for the thymic epithelium, these data indicate that a common endodermal progenitor cell gives rise to all cortical and medullary TEC types. However, in the absence of clonal analysis of the differentiative potential of MTS20⁺MTS24⁺ cells from the embryonic thymus, it remains possible that a common MTS20⁺MTS24⁺ phenotype describes distinct cortical and medullary progenitors.

Development of the medullary compartment. In light of these findings, it is interesting that the thymic medulla seems to arise as a series of clonal islets, which coalesce as the thymus matures[53]. This unexpected finding was demonstrated using chimeric mice, generated by

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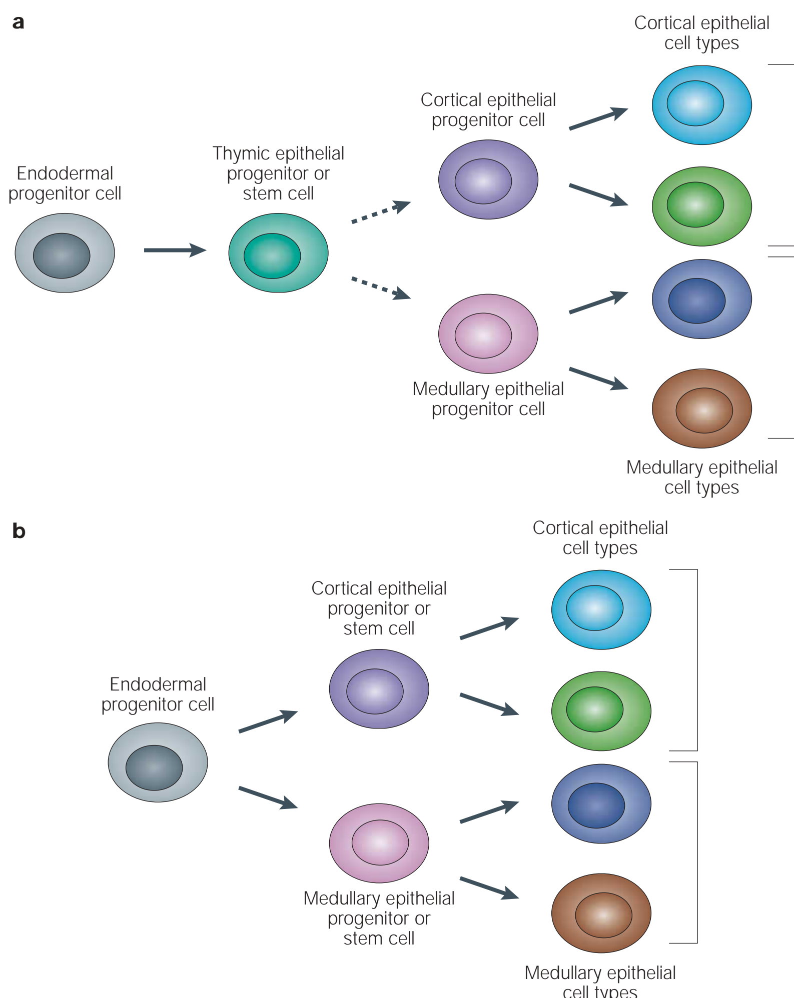

Figure 4 | **Two possible models of thymic epithelial-cell (TEC) development.** **a** | An endodermal progenitor cell might give rise to a committed common, thymic epithelial progenitor cell, which gives rise to all cortical and medullary TEC types either directly or through intermediate cortical and medullary epithelial progenitor cells. **b** | An endodermal progenitor might give rise directly to separate cortical and medullary epithelial progenitor cells. In either case, the progenitor population(s) might also contain stem cells, the potency of which is likely to be restricted to thymic epithelial, or possibly endodermal, lineages.

FETAL THYMIC ORGAN CULTURE (FTOC). Experimental model for the analysis of T-cell development, typically based on *in vitro* culture of embryonic day 16.5 mouse fetal thymi.

REAGGREGATE FETAL THYMIC ORGAN CULTURE (RFTOC). A variation of FTOC used to investigate the role of particular stromal subsets in T-cell development: stromal-cell types purified from fetal thymi are mixed with mesenchymal cells and T-cell progenitors, allowed to reaggregate either on a filter or in a hanging drop, and then cultured *in vitro*, as for FTOC, before analysis of T-cell development.

injection of CBA (H–2^k) or BALB/c (H–2^d) embryonic stem (ES) cells into C57BL/6 (H–2^b) blastocysts, as a means to probe the cellular origins of the thymic epithelium. Thymi from balanced chimaeras were analysed using monoclonal antibodies specific for the MHC class II haplotype of either the ES cell or the blastocyst. This indicated that, in young mice, the medullary compartment consisted of discrete islets of cells, the epithelial-cell component of which was apparently clonal; most islets contained cells that express MHC class II molecules of one or other haplotype, but not both. Notably, no correlation was found between the haplotype of a given medullary islet and that of the surrounding cortical epithelium, indicating the existence of medullary TEPCs. A variation on REAGGREGATE FETAL THYMIC ORGAN CULTURE (RFTOC) was used to test the existence of this putative cell type in the embryonic thymus; reaggregates were grafted under the kidney capsule of recipient mice, as proper cortical and medullary architecture had previously been shown to develop in grafted, but not cultured, RFTOCs[54]. These experiments showed the persistence of a putative medullary epithelial progenitor cell until at least E16.5 (REF. 53).

In the context of a single endodermal-cell origin for both cortical and medullary TEC compartments, these studies can be integrated into either of two basic models that are outlined in FIG. 4. A common endoderm-derived TEPC might give rise directly to all TEC types, or separate cortical and medullary progenitors might exist. In either case, the progenitor population(s) might also contain stem cells, the potency of which is likely to be restricted to TEC lineages, or possibly to less restricted endodermal-lineage fates.

Although at present no lineage data exist that could discriminate between these possibilities, Rodewald's data[53] on the clonal origin of medullary islets seem to favour a model in which intermediate progenitor cells, or possibly stem cells, exist for the cortical and medullary epithelial-cell compartments. These might arise early in thymus organogenesis either from a common progenitor/stem cell that is committed to the thymic epithelial lineage, or from a less committed endodermal progenitor cell. However, Rodewald's findings would also be consistent with a model in which the cortical and medullary progenitors arise directly from within a field of apparently equivalent progenitor cells in the thymic primordium, for example, through a lateral inhibition mechanism similar to that responsible for assigning neural versus glial fates in *Drosophila*[55]. Observations regarding the early patterning of the thymic primordium into cortical and medullary compartments, which indicate the development of a central focus of presumptive medullary epithelial cells by E12.5 (REFS 44,51), would be consistent with either model. The close association of the adult medullary compartment with the vasculature has indicated that vascularization might have a subsequent role in establishing the mature medulla[56]. This could be direct, in the form of vascular endothelium-derived factors, or indirect, in the form of factors carried through the circulation.

Genetic control of early thymus development

The existing data on TEC development are therefore consistent with a model in which endodermal cells in the developing primordium acquire a thymic epithelial fate(s), forming a progenitor cell type(s) that subsequently differentiates into the diverse array of TECs. Given this framework, we are now in a position to identify the molecular mechanisms that control the initial formation of these progenitor cells, and direct their subsequent differentiation and organization into cortical and medullary domains. Such molecular data can be placed in the model for organogenesis described earlier, to generate an integrated model of thymus organogenesis (FIG. 5).

Transcription factors. Analyses of mutant phenotypes and gene-expression patterns have identified a transcription-factor network that is required for the initial formation

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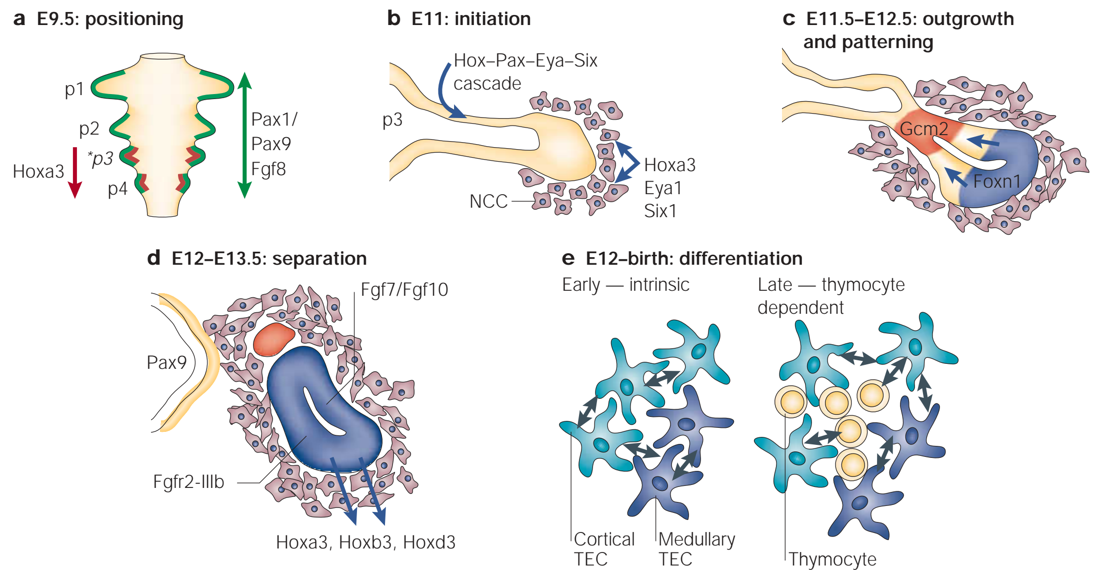

Figure 5 | **A new model of thymus organogenesis. a** | Embryonic day 9.5 (E9.5): positioning. Paired box gene 1 (Pax1)/Pax9 and fibroblast growth factor 8 (Fgf8; green) are required for pharyngeal pouch formation. Homeobox A3 (Hoxa3; red) is required for third pouch (p3) axial identity, possibly through the Pax–Eya–Six cascade. **b** | E11: initiation. Rudiment outgrowth begins at this stage. The Hox–Pax–Eya–Six cascade is required in the endoderm (yellow); Hoxa3 and Eya1 might also be required in neural crest cells (NCCs). **c** | E11.5–E12.5: outgrowth and patterning of the rudiment. Regionalization of the rudiment into thymus- and parathyroid-specific domains. This patterning actually begins at E10 with the expression of glial cells missing homologue 2 (Gcm2; red) in the third pouch, controlled at least in part by the Hox–Pax–Eya–Six cascade. High-level expression of forkhead box N1 (Foxn1; blue) begins at E11.25. Lymphoid progenitors (not shown) also begin to arrive at this time, entering the thymus through the capsule by a chemoattractive mechanism. **d** | E12–E13.5: separation from the pharynx and migration of the rudiment. Pax9 is required for separation from the pharynx. Migration might be controlled by *Hox3* genes expressed by NCCs. Separation of the parathyroid from the thymus might be regulated by Gcm2. **e** | E12–birth: differentiation. Foxn1 is required for the generation of all thymic epithelial-cell (TEC) subtypes — cortical and medullary. Initial differentiation is thymocyte independent. Final differentiation requires thymocyte-derived signals, and depends on the Foxn1 amino-terminal domain (Foxn1$^{\Delta}$). Wnt signalling (through the regulation of Foxn1$^{\Delta}$) has been implicated in both autocrine- (TEC–TEC) and paracrine- (TEC–thymocyte) mediated differentiation. The NCC mesenchyme (not shown) might support growth and differentiation of TECs, possibly through fibroblast growth factors, whereas a lymphotoxin-receptor-dependent signalling pathway seems to control late-stage differentiation and maintenance of medullary TECs. Eya1, eyes absent 1 homologue; Six1, sine oculis-related homeobox 1 homologue.

and early patterning of the thymus/parathyroid rudiment. At present, this consists of five factors: homeobox A3 (*Hoxa3*)[29,57], paired box gene 1 (*Pax1*)[58], *Pax9* (REFS 59,60), eyes absent 1 homologue (*Eya1*)[61] and sine oculis-related homeobox 1 homologue (*Six1*)[61] (listed in order of action from early to late) (TABLE 1). This indicates a cascade that is reminiscent of the Pax–Eya–Six network that operates cell-autonomously to control development of the eye in *Drosophila*[62]. In mice, these transcription factors are co-expressed only in the pharyngeal endoderm (although, with the exception of Pax1 and Pax9, they are all also expressed in the NCC-derived mesenchyme), and all have been shown by mutation studies to be required for thymus organogenesis[29,58–61,63–65]. So, if the Pax–Eya–Six regulatory network is conserved in vertebrate thymus development, these genes must act specifically in the endoderm. Mechanistically, the phenotypes of these mutants are consistent with this network controlling the initiation of thymus organogenesis[23]. Moreover, as the anterior boundary of Hoxa3 expression is the third pharyngeal pouch, an attractive hypothesis is that this pathway might control positioning of the initial rudiment formation (that is, determine the identity of the third pouch to form the thymus/parathyroid rudiment), based on the model of *Hox* gene control of axial position identity during embryogenesis[66]. The Hoxa3–Pax– Eya–Six pathway might also control separation of the primordia from the pharynx and their subsequent migration, as separation of the rudiments from the pharynx does not occur in *Pax9* mutants[60] and is delayed in *Hoxa3*$^{+/-}$*Pax1*$^{-/-}$ mutants[64].

Following initiation of organogenesis, two processes must occur: the patterning of the rudiment into thymus- and parathyroid-specific domains, and the initiation of TEC differentiation. Insights into these processes have come from analysis of two transcription factors, Foxn1 and glial cells missing homologue 2 (Gcm2). Gcm2 and Foxn1 are expressed in complementary domains by the developing thymus/parathyroid primordium at E11.5, indicating the existence of prospective-parathyroid and prospective-thymus regions[25]. *Gcm2* was identified as a homologue of the *Drosophila* gene *gcm*[67], and its mutation results in failure of parathyroid development[68]. Gcm2 is also expressed in a discrete domain in the third pouch from E9.5, considerably earlier than Foxn1 expression is detectable by *in situ* hybridization (see later)[25]. So, establishment of the Gcm2-expression

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Table 1 | **Genes affecting thymus organogenesis and embryonic patterning**

Gene	Fetal expression pattern	Phenotype of knockout mice/ functional analysis	References
Hoxa3	Early: E9.5–E10.5: third cleft surface ectoderm, third and fourth arch NCCs, third pouch endoderm Late: thymic rudiment until adult	Failure of initial thymus and parathyroid organogenesis	29,76
Pax1	Early: E9.5–E10.5: all pharyngeal pouch endoderm Late: progressively restricted to a minor population of cells in the adult cortex	Thymic hypoplasia and mild thymocyte defects; *Pax1*/*Pax9* double mutants have defective pouch formation	58
Pax9	Early: E9.5–E10.5: all pharyngeal pouch endoderm	Thymus ectopic and hypoplastic, possible effect on γδ-TCR+ T-cell development	60
Eya1	Early: E9.5–E10.5: all pharyngeal pouch endoderm, cleft ectoderm and NCC mesenchyme Late: N.D.	Failure of initial thymus and parathyroid organogenesis	61
Foxn1	Early: E11.25: the thymus domain of third pouch (high level of expression); hair follicles and the epidermis from ~E14.5 Late: all TECs	Thymic primordium forms but arrests between E11.5 and E12.5; no colonization of primordium by lymphocytes	25,45, 69,72

E, embryonic day; *Eya1*, eyes absent 1 homologue; *Foxn1*, forkhead box N1; *Hoxa3*, homeobox A3; NCC, neural crest cell; N.D., not determined; *Pax*, paired box gene; TEC, thymic epithelial cell; TCR, T-cell receptor.

domain might define the future thymus–parathyroid boundary. Gcm2 expression seems to be controlled in the endoderm by the Hox–Pax–Eya–Six pathway, as it is not initiated in either *Hoxa3*$^{-/-}$ (N.R.M., unpublished observations) or *Eya1*$^{-/-}$ (REF. 61) mice, and is downregulated in *Hoxa3*$^{+/-}$*Pax1*$^{-/-}$ compound mutants[64].

The Foxn1 transcription factor[69], encoded by the gene that is mutated in nude mice[70,71], is crucial for development of the mature thymus[72]. However, although often referred to as athymic, nude mice do undergo the initial stages of thymus organogenesis — the primordium forms, but fails to differentiate or be colonized by lymphocyte progenitors. This observation fits a 'two-step model'[72], in which expression of Foxn1 divides thymus development into a Foxn1-independent early organogenesis phase that culminates in TEPC formation, and a Foxn1-dependent late phase that includes TEPC differentiation. At present, it is not clear which factors regulate the expression of Foxn1. Although Foxn1 expression was not detected by *in situ* hybridization in the *Hoxa3, Eya1* or *Six1* mutants, high-level Foxn1 expression begins only after E11.25 (REF. 25), which is well after the block to thymus organogenesis in these mutants. However, Foxn1 expression has been detected at E10.5 in the third pharyngeal pouch by RT-PCR[73], and this earlier expression has not been tested in the other mutants.

As no phenotype associated with expression before E11.25 has been found in *Foxn1* mutants, and cells with an apparent TEPC phenotype are generated in the absence of Foxn1 function[45], it is unlikely that Foxn1 is responsible for specifying thymic identity during initial organogenesis. However, the transplantation experiments in chicks provide functional evidence that the endoderm is specified to a thymus fate before organ formation[39]. One possibility is that thymus is the 'default' identity for the third pouch, established by the Hox–Pax–Eya–Six pathway and/or an alternative mechanism (possibly involving Wnt signalling, see later), and that Gcm2 expression in the pouch suppresses this identity, replacing it with a parathyroid fate. Alternatively, an as-yet-unidentified thymus-specific determinant that acts in the third pouch, with a role comparable to that of Gcm2 in specifying parathyroid fates, might exist.

A possible candidate for this determinant is Ehox, a distant member of the paired-box family of homeodomain transcription factors[74] with a markedly restricted expression pattern that is consistent with a role in early thymus organogenesis and/or specification of the thymic epithelial lineage. Ehox is expressed throughout the foregut endoderm at E8.5, but by E9.5, its expression is limited to a ventral domain in the second and third pharyngeal pouches[75] (L.M. Morris, J.G. Gordon, N.R.M. and C.C.B., unpublished observations), and at E10.5, it is largely restricted to a domain that is complementary to the Gcm2-expressing, prospective-parathyroid domain of the third pharyngeal pouch. Notably, at E11.5, expression of Ehox in the pharyngeal pouches is not detectable by *in situ* hybridization, and the domain previously marked by Ehox now strongly expresses Foxn1 (L.M. Morris, J.G. Gordon, N.R.M. and C.C.B., unpublished observations). Ehox, therefore, seems to be a strong candidate for defining a region that is competent to form the thymus. However, functional analyses are required to test this possibility.

Although marked progress has been made in identifying the transcription factors that act in the endoderm during early thymus organogenesis, the molecular mechanisms that occur in the mesenchyme are less clear. As Hoxa3, Eya1 and Six1 are all also expressed in the mesenchyme, and are required for the formation of NCC-derived skeletal elements, additional functions for these genes in NCCs during thymus organogenesis cannot be ruled out. In fact, analysis of mutants of *Hoxa3* and its paralogues, *Hoxb3* and *Hoxd3*, indicates that these *Hox* genes have a redundant function in promoting migration of the primordia after they separate from the pharynx[76]. Evidence for any further roles for Hoxa3, Eya1 and Six1 in NCCs during thymus organogenesis will require tissue-specific genetic approaches to separate their role in the endoderm from any function in the mesenchyme. So far, the only transcription factor

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SPLOTCH MICE
Mice carrying a mutation in the transcription factor paired box gene 3 (*Pax3*), which have defects in derivatives of the somatic mesoderm and neural crest.

known to affect thymus development that, in this region, is expressed only by the NCCs is Pax3, which is encoded by the gene mutated in SPLOTCH MICE[77]. However, the thymus hypoplasia/aplasia in *Pax3* mutants is probably secondary to the death or failure of migration of NCCs in these mutants, rather than a defect in NCC function[78,79]. So, although this phenotype supports a role for NCCs in general in thymus organogenesis, it does not provide any molecular clues as to the function of NCCs in early thymus development.

Signalling molecules. The evidence presented above indicates that at least some of the patterning of the shared primordium might be intrinsic to the endoderm. However, in other systems, many of the initial steps in organogenesis are known or thought to involve cell–cell interactions, often between mesenchymal and epithelial cells. Therefore, signals from other cell types, such as the surrounding NCC mesenchyme, might also be required. Although it is widely accepted that NCCs contribute to the fetal thymus and provide signals that are important for thymus development, the precise role and nature of these signals remain unclear.

Ablation studies in chicks, and the phenotypes of mutant and transgenic mice with defects in NCC formation, migration or survival, have indicated that loss of NCCs results in variable loss or reduction in the size of the thymus[30,78,80–82], supporting the idea that NCCs provide growth and/or differentiation signals to the developing primordium. Comparisons with other endodermal organs would predict that a cascade of reciprocal signals between the endoderm and mesenchyme control positioning and outgrowth of the rudiment, and might also be involved in patterning. However, although all of the main developmental signalling pathways — pathways involving the fibroblast growth factors (Fgfs), Wnts, bone morphogenetic proteins (Bmps) and sonic-hedgehog homologue (Shh) — have been implicated in thymus development at some level, genetic evidence for involvement in early development exists only for Fgfs.

Two recent genetic studies have shown that decreasing Fgf signalling between the endoderm and mesenchyme results in thymus hypoplasia, but does not markedly affect thymocyte differentiation. Fgf8 is expressed at E10.5 in the pharyngeal pouch endoderm, where it presumably signals to the surrounding NCC mesenchyme. Reduction of this signal in a hypomorphic *Fgf8* allele[83] results in two phenotypes. Half of the embryos are athymic, possibly owing to a secondary effect of severe defects in formation of the third and fourth pharyngeal arch and pouch. The other half of the embryos have hypoplastic, sometimes ectopic, thymic lobes that support relatively normal thymocyte differentiation. This phenotype is similar to that reported for an isoform-specific knockout of Fgf receptor 2 isoform IIIb (*Fgfr2-IIIb*)[84]; Fgfr2-IIIb is expressed by TECs from about E13 (REFS 84,85), where it is thought to receive signals from Fgf7 and Fgf10, which are expressed by the surrounding mesenchyme[84]. Expression of Fgfr2-IIIb is also detectable in associated mesenchyme at E14 by RT-PCR[85]. In *Fgfr2-IIIb*$^{-/-}$ mice, thymus organogenesis proceeds apparently normally until about E12.5, after which time the organ does not increase further in size[84]. However, some TEC differentiation seems to occur after this point and, although thymocyte proliferation is severely impaired, the resulting, severely hypoplastic, thymus is functional with respect to its capacity to support T-cell differentiation[84]. As expression of Fgf10 is reduced in homozygotes for the *Fgf8* hypomorphic allele[83], it is possible that reciprocal Fgf signalling between the endoderm and mesenchyme is required at the early stages of primordium formation. This is reminiscent of the reciprocal Fgf signalling that is required for initial placement and induction of limb-bud formation[86], and of the requirement for Fgf10 expression in initial lung organogenesis[87]. However, because of the partial function of the *Fgf8* hypomorphic allele and the earlier role of Fgf8 in pouch formation[88], it is still not clear whether Fgf signalling is directly required for positioning or initial development of the primordium.

Recent organ-culture experiments have provided evidence that at least some of the role of Fgfs in the thymus might be downstream of Bmp4 signalling. Addition of Bmp4 to FTOCs affects thymocyte development in a stromal-cell-dependent manner, and this effect is suppressed by inhibition of Fgf signalling[89]. Bmp4 also upregulated both Foxn1 and Fgfr2-IIIb expression in these experiments. Taken together, this study supports a model in which Bmp4 upregulates Foxn1, which in turn upregulates Fgfr2-IIIb expression, increasing the sensitivity of TECs to Fgf7 and Fgf10 signals from the mesenchyme. However, it is unclear what relevance this pathway has to the initiation of Foxn1 expression.

Wnt signalling has also been implicated functionally in TEC–TEC or TEC–thymocyte interactions that promote TEC differentiation and thymocyte development[73,90]. Wnts are expressed by both TECs and developing thymocytes, and Wnt receptors and downstream signalling components are expressed by TECs. Transf ection and TEC–thymocyte co-culture studies showed that Wnts can induce Foxn1 expression by cultured TECs[73]. These studies indicated that Wnts might maintain Foxn1 expression by TECs by both autocrine and paracrine mechanisms, and could therefore promote TEC differentiation through the regulation of Foxn1 expression.

These studies show that many signalling pathways are involved in promoting proliferation, differentiation and function of developing TECs. It will be of particular interest to investigate the role of Bmp4 and Wnt signals *in vivo*, including determining whether they have a role in initiation of Foxn1 and/or Fgfr-2IIIb expression in the thymic rudiment. However, no functional data have yet indicated a role for any of these signalling pathways in events that occur before E12.5 — that is, in induction, outgrowth or early establishment of the thymic primordium. This might be due in part to functional redundancy between many pathway members or between pathways. So, it is unclear so far which signalling pathways are important in the earliest stages of thymus organogenesis.

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COMMON γ-CHAIN
(γc). A type I cytokine receptor chain that is shared by the receptors for interleukin-2 (IL-2), IL-4, IL-7, IL-9, IL-15 and IL-21. Mutant mice that lack both γc and recombination-activating gene 2 (Rag2) have a severe block in T-, B- and natural killer-cell development; thymocyte development in these mice is blocked at the $CD25^+$ double-negative 2 stage.

IKAROS
This gene encodes a member of a family of zinc-finger transcription factors that are required for the development of all lymphoid lineages, as well as lymph nodes and Peyer's patches. *Ikaros*-null mutant fetuses lack B cells and T-cell precursors; few *Ikaros*-null cells enter the fetal thymus, and these fail to develop to the $CD25^+$ double-negative 2 stage.

Regulation of TEC differentiation

Lymphocyte-dependent and -independent development. It is widely accepted that proper establishment of the cortical and medullary compartments, in particular the development of an organized medulla, requires interactions between immature TECs and normally differentiating thymocytes[5]. Recent advances have indicated molecular mechanisms that might at least partly explain this 'crosstalk', but have challenged the commonly held view that developing thymocytes are largely responsible for regulating initial development of the cortical and medullary thymic epithelium.

Evidence that the initial stages of TEC differentiation, including the formation of TECs with medullary phenotypes, can occur in the absence of thymocytes was provided by a recent study that compared the development of the cortical and medullary epithelial compartments in wild-type mice with that in Rag2/COMMON-γ-CHAIN-deficient and *IKAROS*-null mice[51], each of which have marked early blocks in thymocyte development[91,92]. These data show that the initial formation of both compartments is similar in all three strains, and therefore occurs through a lymphocyte-independent mechanism. TEC–thymocyte interactions were, however, required at later stages of fetal-thymus development (from about E15.5), and in the postnatal thymus to elaborate this initial patterning and to sustain normal organization of both the medullary and cortical thymic epithelial compartments. Although the mechanisms that regulate the initial and late-stage differentiation of medullary and cortical TECs remain largely undetermined, a recent study has shown that lymphocyte-dependent development of medullary TECs, and their maintenance in the medullary compartment, is controlled by signalling through the lymphotoxin β receptor[93].

Molecular regulation of TEC differentiation. As discussed earlier, the differentiation of all TEC subtypes requires the action of Foxn1. The phenotype of the presumptive TECs in nude mice indicates that these cells are not so much abnormal as they are arrested at an early progenitor stage. In addition, the proliferation of TECs in the rudiment of nude mice is reduced[94]. Therefore, in the thymus, Foxn1 seems to promote both initial TEC differentiation and proliferation. This role is similar to the role Foxn1 is thought to have in the skin and hair, in which Foxn1 promotes the proliferation of progenitor cells and the expression of early differentiation markers. However, in the skin, Foxn1 expression suppresses later stages of keratinocyte differentiation and is downregulated as keratinocytes differentiate[95]. By contrast, Foxn1 is reported to be expressed by most TECs in the adult thymus[72].

Analysis of a hypomorphic allele for *Foxn1* in a recent study showed that Foxn1 is required for both initial and subsequent stages of TEC differentiation[96] and, importantly, uncouples the function of Foxn1 in thymus and skin/hair development. This allele, designated *Foxn1*$^{\Delta}$, has most of an amino-terminal domain of unknown function deleted, while leaving the DNA-binding, nuclear-localization and acidic-activation domains intact. The truncated protein resulted in a milder effect on thymus development than the null allele, but had no effect on hair development, demonstrating a thymus-specific role for the amino-terminal domain. Analysis of TEC phenotypes during thymus development in these mutants indicated that initial TEC differentiation was delayed but relatively normal, and revealed a subsequent differentiation block before acquisition of competence to establish mature medullary and cortical compartments. The observed phenotype is similar to that reported for mice with early blocks in thymocyte development[91,92], consistent with the *Foxn1*$^{\Delta/\Delta}$ TECs having a defect in thymocyte-dependent TEC differentiation. Unlike *Foxn1*$^{nu/nu}$ TECs, *Foxn1*$^{\Delta/\Delta}$ TECs can support both thymus colonization and some thymocyte development. However, the *Foxn1*$^{\Delta/\Delta}$ TEC defects result in severely impaired thymocyte differentiation, with a 25-fold decrease in thymocyte numbers and partial blocks at both the DN1 and DP stages of thymocyte differentiation in the adult thymus, although SP cells are produced and exported to the periphery in reduced numbers. The mechanism by which the Foxn1 amino-terminal domain regulates TEC differentiation remains to be determined, as does the basis for its tissue-specific activity. The *Foxn1*$^{\Delta}$ and nude alleles constitute a unique allelic series for the *Foxn1* gene, providing a valuable genetic system to investigate Foxn1 function.

Directions for the future

We have argued here that a single, endodermal origin for all thymic epithelium should now be established as the paradigm for thymus organogenesis. Within this framework, the following issues might now be addressed with a high degree of stringency.

The point at which endodermal cells become committed to a thymic fate and the factors that are required for this process remain to be determined. Elucidation of the mechanisms that lead to the generation of committed TEPCs, and testing of the possible models for subsequent TEC differentiation will require rigorous lineage and potency studies. In particular, the potency of individual $MTS20^+MTS24^+$ TEPCs must be determined, both with respect to differentiation into thymic lineages and the ability to adopt other endodermal lineage fates. It is also important to determine whether true thymic epithelial stem cells exist in the $MTS20^+MTS24^+$ TEPC fraction and whether these exist in the adult thymus, as identification of an adult thymic epithelial stem cell would open up the potential for therapeutic reactivation of such cells in immunocompromised individuals. In this respect, the implication that $MTS20^+MTS24^+$ cells might be required for the maintenance/function of cortical and medullary epithelial cells, at least in fetal thymi, requires further investigation. Identification of the antigens that are recognized by MTS20 and MTS24 is also required to illuminate the role, if any, of these markers in TEPC biology.

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Although marked progress has been made in identifying the molecular regulators of thymus organogenesis, the signalling pathways that control early thymus organogenesis remain to be identified, as do the patterning mechanisms that link these signalling pathways to the Hox–Pax–Eya–Six pathway and Foxn1/Gcm2 transcription factors. Identification of the mechanism that is responsible for determining thymus identity in the third pharyngeal pouch and the issue of what gene(s) acts upstream of *Foxn1* to specify thymus fate are also of particular importance. In addition, as evidence from a Cre-based lineage analysis in mice indicates that the role of NCCs in thymus organogenesis might be restricted to the early stages of thymus organogenesis[27,31], the origins and role of mesenchymal cells in the adult thymus requires further investigation. Clearly, further study is needed to identify the mechanisms that underlie TEC differentiation and establishment/maintenance of the mature TEC compartments. In this respect, $MTS20^+MTS24^+$ TEPCs might provide an important *in vitro* model for the investigation of gene function during late thymus organogenesis and patterning.

The identification of a putative common TEPC also indicates the potential to use such cells for therapeutic purposes, as, at least in principle, cell lines corresponding to TEPCs could be propagated, and would differentiate into functional cortical and medullary TECs under appropriate conditions. Primary TEPCs or, feasibly, TEPC lines might be able to restore thymic function in athymic[97] or immunocompromised individuals, and could also provide a means of improving the outcome of bone-marrow and organ transplantation protocols. In this regard, one could envisage using TEPCs/TEPC lines to boost thymus function — required for reconstitution of the T-cell compartment[98] — in transplant recipients, or for the efficient *in vitro* generation of specific T-cell repertoires. Such repertoires could provide a means of 'patching' the recovery phase of transplantation protocols, which by necessity require T-cell depletion as part of the pre-transplantation conditioning regime[99,100], by adoptive immunotherapy. Definition of the conditions required to propagate undifferentiated TEPCs will therefore constitute a major advance, and is an important challenge for the future.

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