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Mechanisms of thymus organogenesis and morphogenesis

Julie Gordon and Nancy R. Manley*

Summary

The thymus is the primary organ responsible for generating functional T cells in vertebrates. Although T cell differentiation within the thymus has been an area of intense investigation, the study of thymus organogenesis has made slower progress. The past decade, however, has seen a renewed interest in thymus organogenesis, with the aim of understanding how the thymus develops to form a microenvironment that supports T cell maturation and regeneration. This has prompted modern revisits to classical experiments and has driven additional genetic approaches in mice. These studies are making significant progress in identifying the molecular and cellular mechanisms that control specification, early organogenesis and morphogenesis of the thymus.

Key words: T cell, Migration, Morphogenesis, Organogenesis, Parathyroid, Thymus

Introduction

The thymus is a bilobed organ that is located in the central compartment of the thoracic cavity, on top of the heart and behind the sternum. It is the primary site of T-lymphocyte (T cell; see Glossary, Box 1) development; it is essentially an epithelial organ, containing many developing lymphocytes (thymocytes; see Glossary, Box 1), that is surrounded by a mesenchymal capsule. Histologically, the thymus can be broadly divided into two subcompartments, the cortex and the medulla (Fig. 1; see Glossary, Box 1), each of which contains distinct populations of thymic epithelial cells (TECs; see Glossary, Box 1), as well as mesenchymal cells, endothelial cells and dendritic cells. The thymus thus provides a unique microenvironment for the efficient production of a diverse T cell repertoire (see Box 2). Briefly, thymocytes are first specified to the T cell fate and they then proliferate and differentiate, undergoing positive selection for the ability to recognize self-MHC (the specific alleles of major histocompatibility complex proteins present in the individual) and negative selection to eliminate T cells that are potentially autoreactive. This process ultimately gives rise to a diverse repertoire of peripheral T cells. The differentiation of T cells occurs along a stereotypical migratory route (Fig. 1C). Thymocytes enter the thymus via large vessels at the boundary between the cortex and the medulla, and travel through the cortical and medullary regions until their exit from the thymus, again through the vasculature (Lind et al., 2001). This migration is directed by molecular, rather than structural, cues and the TECs form a complex 3D network (Fig. 1D) rather than the more 'typical' 2D epithelial structures (van Ewijk et al., 1999). Thus, the correct patterning and organization of

500 D.W. Brooks Drive, 270 Coverdell Building, Department of Genetics, University of Georgia, Athens, Georgia 30602, USA.

*Author for correspondence (nmanley@uga.edu)

Box 1. Glossary of terms

Chick-quail chimera studies. Cell marking system in which transplantation of cells from one species (chick or quail) to another allows ontogeny to be studied based on the ability to distinguish tissue differences between species.

Cortex. 'Outer' region of the thymus; supports early stage thymocyte development, including positive selection for recognition of self-MHC (major histocompatibility complex).

Involution. Natural shrinking of the thymus with age. Accompanied by structural changes and decreased T cell output. Sometimes used to refer to any reduction of thymus size and function, e.g. from irradiation or chemotherapy.

Kidney capsule transplantation. Transplantation of fetal thymus or re-aggregated thymus cells to an ectopic site known to be permissive for thymus growth; classical technique used in immunology.

Lymphocytes. White blood cells, i.e. T cells, B cells and NK (natural killer) cells.

Medulla. 'Inner' region of the thymus; location of later stages of thymocyte differentiation to $CD4^+$ and $CD8^+$ stages, including negative selection against tissue-specific antigen recognition.

NCCs (neural crest cells). Ectodermal cell population that migrates from the hindbrain giving rise to many different cell types including neurons, cartilage, dermis and smooth muscle; comprises the capsule and pericytes of the thymus.

Pharyngeal clefts. Transient bilateral invaginations of surface ectoderm in the neck region; form between pharyngeal arches at E8.5-11 in mice.

Pharyngeal ectoderm. Surface epithelial layer covering the neck region in the embryo.

Pharyngeal endoderm. Epithelial cells of the gut tube in the neck region (pharynx).

Pharyngeal organs. Organs derived from anterior foregut (pharyngeal) endoderm, including thymus, parathyroids, thyroid and ultimobranchial bodies.

Pharyngeal pouches. Transient bilateral paired outpockets of endoderm on lateral surfaces of pharynx; form in opposition to ectodermal clefts.

Pharynx. Throat; most anterior region of the gut tube (endodermal epithelium).

PTH (parathyroid hormone). Hormone secreted by parathyroid glands; required for Ca^{2+} regulation.

T cells. Lymphocytes that develop within the thymus; types are $CD4^+$ helper, $CD8^+$ killer or $NK1.1^+$ natural killer cells.

Thymocytes. Immature lymphoid cells developing within the thymus.

Thyroid gland. Endocrine gland found in the neck; produces thyroid hormone and calcitonin.

Thyroid diverticulum. Precursor of thyroid; arises from ventral midline of foregut endoderm at the level of the second pharyngeal arch.

TECs (thymic epithelial cells). Major component of thymic stroma; required for all stages of thymocyte differentiation; classified as cortical TECs (cTECs), which reside in the cortex region, and medullary TECs (mTECs), which reside in the medulla.

Ultimobranchial bodies. Origin of the calcitonin-producing cells of thyroid; derived from the fourth pharyngeal pouches, then fuse completely with thyroid diverticulum-derived primordium during late embryogenesis.

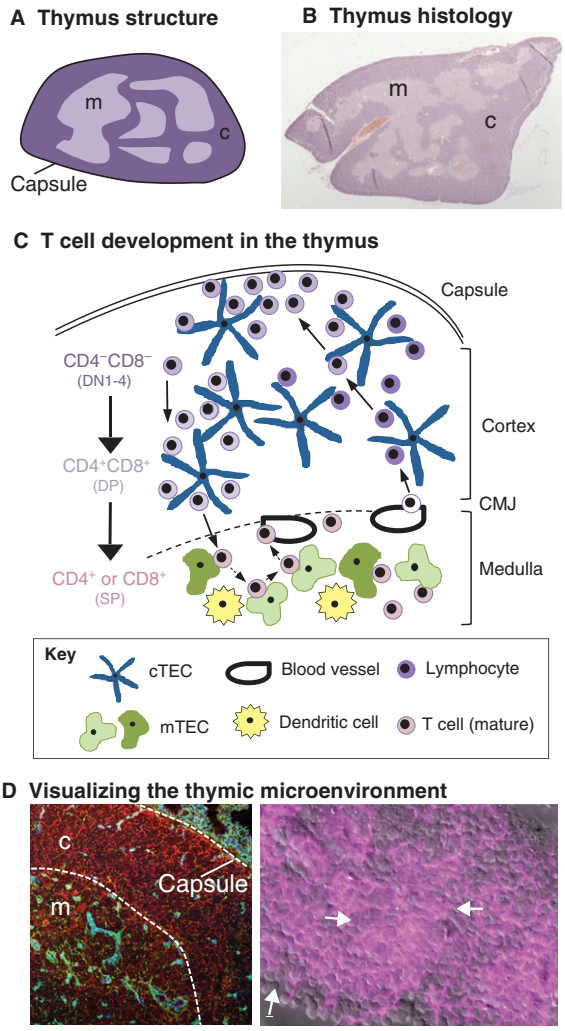

Fig. 1. Thymus structure. (**A**) Structure of the thymus**.** The thymus is an epithelial organ surrounded by a mesenchymal capsule. It can be divided into a central medulla (m) region, which contains medullary thymic epithelial cells (mTECs), and an outer cortex (c), which contains cortical thymic epithelial cells (cTECs). (**B**) Histology of the thymus. Hematoxylin and eosin-stained sagittal section of an adult mouse thymus, highlighting the thymus subcompartments. (**C**) T cell development in the thymus. The migratory route of thymocytes through the thymus, and their differentiation steps are shown. The outer mesenchymal capsule is indicated by solid lines, the cortico-medullary junction (CMJ) is indicated by a dashed line. Thymocytes (white) enter the thymus via large blood vessels at the CMJ and commit to the T cell fate. In the cortex, thymocytes differentiate through $CD4^-CD8^-$ double-negative 1-4 (DN1-4) stages to the $CD4^+CD8^+$ double-positive (DP) stage and undergo positive selection (indicated by a gradual change from dark to light purple); these events are mediated by interactions with cortical thymic epithelial cells (cTEC; blue). DP cells then migrate back through the cortex and differentiate into either $CD4^+$ or $CD8^+$ single-positive (SP) T cells. The SP cells then cross the CMJ and enter the medulla, where they interact with multiple medullary TECs (mTECs; green) during negative selection and then leave via the vasculature. Dendritic cells in the medulla (yellow) also participate in the process of negative selection. (**D**) Immunostaining showing cellular organization in the postnatal thymus. In the left panel, pan-keratin (red) highlights the epithelial network; CD31 (blue) labels endothelial cells; green is collagen IV, which labels perivascular matrix; the dotted lines show the cortico-medullary boundary and the capsular boundary of the cortex. The right panel shows the epithelial network (labeled with keratin-8, purple) in the cortical region. Differential interference contrast imaging allows visualization of thymocytes (arrows) and of epithelial cells 'wrapping' around them.

thymus stromal components (the TECs, the vasculature and the mesenchymal cells) are crucial for optimal T cell development and, hence, thymus function (see Box 2). Understanding the developmental processes that build correct thymus structure is important because defects in thymus structure and function can result in serious health consequences, including immunodeficiency or autoimmunity.

The development of the thymus is linked to that of the parathyroid glands, which are small endocrine glands in the neck that produce parathyroid hormone (PTH; see Glossary, Box 1) and are responsible for calcium homeostasis. The parathyroids are named for their proximity to the thyroid gland (see Glossary, Box 1), although the organs do not share the same function. In contrast to the thymus, the parathyroids have little structural organization that is critical for their function. Despite these obvious structural and functional differences, the embryonic development of the thymus and the parathyroid glands is intimately linked; both develop from single primordia that give rise to two different organs. Thus, mechanisms must exist to pattern this primordium into organ-specific domains, and then separate the thymus from the parathyroids during embryonic development. The details of these patterning and morphogenetic events are not fully understood and, therefore, not surprisingly, the molecular mechanisms regulating these tightly coordinated processes remain, for the most part, poorly defined.

This review summarizes the historical literature that, using the chick and mouse as model systems, has provided insight (and some misdirection) into the mechanisms underlying thymus organogenesis. Although the major focus of this review is thymus organogenesis, it is impossible to describe the morphogenetic events associated with thymus development without also discussing the parathyroids. We then review recent progress, made primarily using mouse genetics, that has increased our understanding of the origin of the thymus, as well as the cellular interactions and morphogenetic events that underpin thymus organogenesis. We also highlight what is currently known about the molecular players that control these events.

An overview of thymus and parathyroid development

The thymus and the parathyroid glands are derived from the endodermal gut tube, which consists of a single layer of epithelial cells and gives rise to all gut-derived organs via the formation of evaginating buds (Grapin-Botton and Constam, 2007; Zorn and Wells, 2009). The pharyngeal organs – the thyroid, thymus, parathyroids and ultimobranchial bodies (see Glossary, Box 1) – in particular, derive from outpockets of the most anterior region of the foregut, the pharynx (see Glossary, Box 1), which includes the ventrally located thyroid diverticulum (see Glossary, Box 1) and a series of paired transient outpockets of the lateral foregut called the

Box 2. The thymus as a niche for T cell development

T cells (T-lymphocytes) are an essential component of the adaptive immune system. The thymus provides a permissive environment for the development of T cells from hematopoietic progenitor cells, generating a functional and self-tolerant peripheral T-cell repertoire. The thymus consists of cells of stromal and hematopoietic origin, and includes thymic epithelial cells (TECs), neural crest-derived mesenchymal cells, endothelial cells and dendritic cells. TECs in the cortex and medulla form a complex 3D network that is the primary functional component of the microenvironmental niches that support T cell differentiation. Lymphocyte progenitor cells (LPCs) enter the postnatal thymus via blood vessels at the corticomedullary junction. Initial contact with notch ligands presented on TECs specifies LPCs that enter the thymus to the T cell fate; after specification, differentiating thymocytes undergo a stereotypical migration through the cortex and medulla, presumably encountering multiple functionally distinct microenvironments. The key components of this differentiation process are a dramatic expansion in numbers, positive selection in the cortex for T cell receptors (TCRs that recognize self-MHC molecules) and negative selection in the medulla to delete cells that react to strongly to self-antigens (and could therefore mediate autoimmunity). The end product is a highly selective collection of $CD4^+$ 'helper' and $CD8^+$ 'killer' T cells, which are exported to the peripheral immune system, once again via the vasculature. Although the existence and general locations of these diverse microenvironments have been inferred by numerous studies, the precise composition and structure of these niches remain to be defined and this is a major focus of research in the field.

pharyngeal pouches (see Glossary, Box 1; Fig. 2). In mice, the third pair of these pharyngeal pouches each forms a single epithelial organ primordium surrounded by a mesenchymal capsule, which will later develop into one thymus lobe and one parathyroid gland (Fig. 2). In humans, an additional pair of parathyroids develops from the fourth pharyngeal pouches. In contrast to the more posterior endoderm-derived organs (e.g. trachea/lungs, liver, pancreas), which follow a more 'typical' budding/branching model and maintain an attachment to the gut tube, the development of the pharyngeal organs differs such that the buds detach from the gut tube, and the developing organs migrate from their site of origin to their final locations. In the case of the third pharyngeal pouches, the paired primordia detach from the pharynx and then separate into individual thymus and parathyroid organs while migrating to their final positions in the body (Fig. 2). The parathyroids locate adjacent to, or sometimes embedded within, the thyroid gland. The thymus migrates further caudally into the chest cavity, where the two lobes meet at the midline just above the heart.

Historical perspectives and models of thymus development

The thymus has been a subject of study and speculation for centuries – speculation because, unlike most other organs, its location and morphology do not provide strong clues into its function. Experiments identifying the thymus as a site of T cell development were not published until the early 1960s (Miller, 1961b; Miller, 1961a), making the thymus possibly the last organ to be associated with a specific physiological function. There is a handful of papers from the 1960s and 1970s that investigated various aspects of early thymus organogenesis, most of which focused on the embryonic tissue of origin of the various cellular components of the thymus in mouse and chick. Although some of these resulted in important insights, others led the field astray; as discussed below, these various models are being resolved into the current view of the origins of thymus development.

Early experiments on the developmental origin of the thymus

The developmental origins of both the epithelial and lymphocytic components of the thymus have been sources of controversy within the field. Lymphocytes were proposed to originate either from transformation of epithelium within the rudiment or from immigration from extrathymic sources. In an early experimental study of thymus organogenesis (Auerbach, 1960), embryonic thymus lobes from mice were dissociated into the epithelial primordium and the surrounding mesenchymal capsule, then cultured alone or combined with mesenchyme from various embryonic sources. The epithelial primordium failed to develop in the absence of mesenchyme, but development was induced after co-culture with mesenchymal cells. Importantly, this was not only true for thymic mesenchyme but was also observed with mesenchyme from lung, salivary gland and kidney, suggesting that the epithelial cells might be instructing the mesenchyme to participate in thymus development. Because lymphoid cells developed only in the epithelial rudiment, Auerbach concluded that they developed from the epithelial cells. Although this conclusion was flawed (as the lymphoid cells had already immigrated into the thymus by the time of dissection), these experiments did reveal the crucial role of epithelial-mesenchymal interactions in the growth and morphogenesis of the embryonic thymus. The signaling pathways mediating these interactions are of great interest, and are still a matter of investigation (see below). Experimental studies using tissue recombination and grafting in chick and mouse subsequently demonstrated that the lymphocytes in the thymus were hematopoietic in origin (Moore and Owen, 1967; Le Douarin and Jotereau, 1975; Le Douarin, 1977). These later transplantation studies further suggested that lymphocytes are attracted to the thymus by factors secreted by the thymic epithelium; the chemokines responsible have since been identified as Ccl21 and Ccl25 in mice (see below).

The thymic epithelium, which comprises the primary stromal component of the thymus, was described quite early on as being derived from pharyngeal endoderm (see Glossary, Box 1), specifically from the third (and sometimes fourth) pharyngeal pouches (Schrier and Hamilton, 1952). Histological and histochemical analysis of mouse thymus development concluded that the epithelial portion of the thymus derived entirely from pharyngeal pouch endoderm (Smith, 1965). Two chick-quail chimera studies (see Glossary, Box 1) in 1975 by Le Douarin further showed that the mesenchymal capsule and the pericytes in the thymus are derived from neural crest cells (NCCs; see Glossary, Box 1) (LeLievre and LeDouarin, 1975). This latter study also demonstrated that transplanted pharyngeal pouch endoderm from chick embryos could result in a fully functional thymus, which further implied that thymus fate is specified prior to NCC migration, and that thymic epithelium can recruit heterologous mesenchyme to participate in thymus organogenesis. Together, these early studies suggested an 'endoderm-centric' model, with a single, endodermal origin for thymic epithelium, which subsequently recruits heterologous mesenchyme and hematopoietic-derived lymphoid progenitors to compose a functional thymus.

In contrast to the above model, it had been suggested that the pharyngeal ectoderm (see Glossary, Box 1), which touches the pharyngeal pouches during embryonic development, also

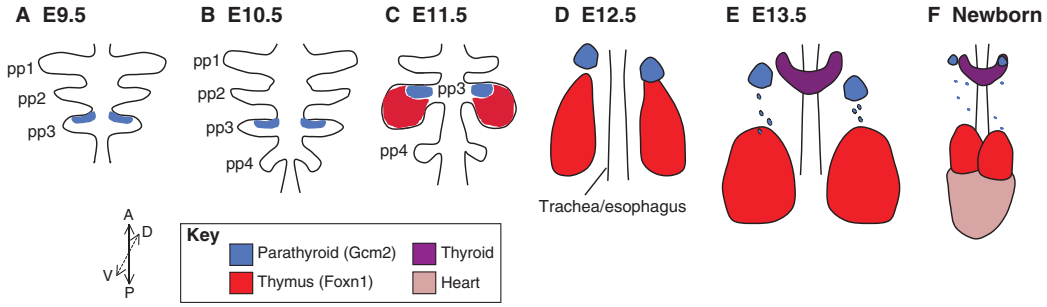

Fig. 2. An overview of thymus development. (**A**) The pharyngeal pouches (pp) form sequentially on the lateral surfaces of the foregut, with the third pouches (pp3) appearing at E9.5. Gcm2 expression marks the parathyroid domain (blue) from E9.5 onwards. (**B**) The fourth pouch appears by E10.5. (**C**) By E11.5, third pouches have developed into primordia that are ready to detach from the pharynx. At this stage, the primordia are patterned into thymus (red) and parathyroid (blue) domains as indicated by Foxn1 and Gcm2 expression, respectively. (**D**) At E12.5, the primordia have detached from the pharynx, and the parathyroids (blue) have begun to separate from the thymus lobes (red). (**E**) By E13.5, the parathyroids (blue) have separated from the thymus (red) and remain adjacent to the thyroid (purple). Some parathyroid cells detach from the main organ, and a few remain attached to the thymus. (**F**) By the newborn stage, the organs are in their final positions, shown here relative to the thyroid and the heart (pink). In panels B and C, note that the pouch and primordium on the embryo's right are slightly advanced relative to the left (see Box 3). Anterior (A), posterior (P), dorsal (D) and ventral (V) axes are indicated by double-headed arrows.

contributed to the thymic stroma (Hammond, 1954). This possibility was formalized into a model in two papers by Cordier (Cordier and Heremans, 1975; Cordier and Haumont, 1980), that used a histological approach to propose that TECs are derived from both ectoderm and endoderm, with ectoderm giving rise to cortical TECs (cTECs) and endoderm generating medullary TECs (mTECs). This conclusion was cited both by other scientists (Kingston et al., 1984; Owen and Jenkinson, 1984; Jenkinson et al., 1985; van Vliet et al., 1985) and by immunology textbooks (e.g. Parham, 2000; Janeway et al., 2001), as a 'dual-origin' model for TECs. A further modification of the prevailing model of thymus organogenesis came following observations that NCC ablation in chick resulted in a variable reduction in thymus size, including apparent athymia (absence of a thymus) (Bockman and Kirby, 1984). This paper was instrumental in popularizing the concept that thymus organogenesis, and possibly the establishment of thymus fate, requires inductive interactions between endoderm and NCCs. However, these studies did not assay the initial stages of thymus organogenesis and, in the absence of markers, could not have identified very hypoplastic thymi. An essental role for NCC in initial organogenesis was supported further by reports that mouse mutants with few or no NCCs (e.g. the *Pax3*-null 'Splotch' mutant, and platelet derived growth factor receptor alpha knockout mice) were athymic (Franz, 1989; Conway et al., 1997; Soriano, 1997; Epstein et al., 2000). Thus, by the mid-1980s and into the early 2000s, the prevailing model of thymus organogenesis held that NCCs were required for initial thymus organogenesis, and that both ectoderm and endoderm contributed to the thymic epithelium.

Recent studies provide support for the endoderm-centric model

In the last ten years, many studies have explored further the earliest stages of thymus organogenesis and morphogenesis. These studies have provided new information about the mechanisms underlying the patterning of the third pharyngeal pouch, the ontogeny of cells contributing to the thymus and how these early events set the stage for later organ morphogenesis and function. These studies have generally supported the 'endoderm-centric' model of thymus organogenesis.

The idea that cortical and medullary regions formed via juxtaposition of two epithelial layers was challenged by the experimental demonstration that the medulla comprises clonal clusters of cells, which was interpreted to support a stem or progenitor cell mechanism for TEC differentiation (Rodewald et al., 2001). A more direct experimental test of the dual origin hypothesis for thymic epithelium based on the experimental approach of Le Douarin assessed the developmental potential of the third pharyngeal pouch endoderm in mice (Gordon et al., 2004). Kidney capsule transplantation (see Glossary, Box 1) of early prospective pouch endoderm at E9 in mice resulted in a functional thymus that contained both cortical and medullary regions, and that could export $CD4^+$ and $CD8^+$ T cells to colonize the peripheral lymph nodes. Labeling of the surface ectoderm, coupled with whole-embryo culture, demonstrated that ectodermal cells do not contribute physically to the developing thymus. Furthermore, the cells at the contact points between the primordium and the pharynx, and the primordium and surface ectoderm, were shown to undergo apoptosis, precipitating the separation of the thymus primordium from both structures. These experiments mirrored and extended the results from the Le Douarin studies in chick (Le Douarin and Jotereau, 1975) and clearly supported the endoderm-only model for the origin of the thymic epithelium. The single-origin model was supported further by studies in which cultured epithelial cells from embryonic day (E) 12 mouse thymi (Rossi et al., 2006) or in vivo postnatal thymi (Bleul et al., 2006) had both cortical and medullary potential, supporting a common origin for both types of TECs. These and other studies (Bennett et al., 2002; Gill et al., 2002) have established the concept that both cTECs and mTECs have an endodermal origin, and might arise from and be maintained by a single TEC stem cell type. The existence and identity of this TEC stem cell, and whether it is present in both fetal and postnatal thymus, remains a topic of much debate and investigation in the field.

Molecular mechanisms establishing thymic fate

In recent years, much progress has been made in identifying the transcription factors and signaling pathways that play a role in thymus organogenesis. Many of these molecular insights have

Table 1. Factors implicated in thymus development

Factor	Gene family	Relevant expression pattern	Relevant function	Reference(s)
Signaling molecules				
Bmp4 (bone morphogenic protein 4)	Transforming growth factor-beta secreted signal superfamily	Distal-posterior (presumptive thymus) domain of third pouch; prior to Foxn1	Epithelial-mesenchymal interactions involved in morphogenesis	(Bleul and Boehm, 2005; Gordon et al., 2010; Patel et al., 2006)
Fgf8 (fibroblast growth factor 8)	Fibroblast growth factor, secreted signal	Distal-posterior (presumptive thymus) domain of third pouch; prior to Foxn1	Early pouch formation, possible role in patterning	(Frank et al., 2002)
Shh (sonic hedgehog)	Hedgehog family, secreted signal	Pharyngeal endoderm, but excluded from third pouch	Initial parathyroid fate	(Moore-Scott and Manley, 2005)
Wnt5b (wingless-int 5b)	Wingless homolog family, secreted signal	Distal-posterior (presumptive thymus) domain of third pouch; prior to Foxn1	No functional evidence to date	(Balciunaite et al., 2002)
Transcription factors				
Eya1 (eyes absent homolog 1)	Eyes absent family of transcriptional co-activators	Pharyngeal endoderm, mesenchyme and ectoderm	Early pouch formation and patterning	(Xu et al., 2002)
Foxn1 (forkhead box protein n1)	Winged-helix transcription factor	Ventral domain of third pouch from E11.25; mature TECs	TEC differentiation	(Gordon et al., 2001); (Blackburn et al., 1996)
Hoxa3 (homeobox protein a3)	Hox-class homeobox transcription factor	Pharyngeal endoderm and NCC-derived mesenchyme; E10.5	Early pouch patterning and initial organ formation	(Manley and Capecchi, 1995; Su et al., 2001)
Pax1 (paired box protein 1)	Pax transcription factor containing a paired domain only	Pharyngeal pouches (endoderm)	Early pouch formation and parathyroid development; minor role in thymus size	(Wallin et al., 1996)
Pax9 (paired box protein 9)	Pax transcription factor containing a paired domain only	Pharyngeal pouches (endoderm)	Pouch and initial organ formation; TEC differentiation	(Hetzer-Egger et al., 2002)
Six1/4 (sine oculis homolog 1/4)	Homeobox transcription factor	Pharyngeal endoderm, mesenchyme and ectoderm	Early organ formation and patterning	(Zou et al., 2006)
Tbx1 (T-box 1)	T-box transcription factor family	Dorsal third pouch and mesodermal core of pharyngeal arches	Pouch formation and patterning, might establish parathyroid fate	(Jerome and Papaioannou, 2001; Liu et al., 2007)

come from gene expression data and from genetic studies in mice. Although a number of transcription factors and signaling pathways have been implicated in thymus fate specification and initial organ development (Table 1), definitive functional data identifying the molecular mechanisms responsible for specification of thymus fate remain elusive.

In mice, the third pharyngeal pouch is patterned into organ-specific domains concurrent with pouch formation and prior to primordium formation, as indicated by regionalized molecular markers (reviewed by Manley and Condie, 2010). The key events in this process are highlighted in Fig. 3. The earliest and truly 'thymus-specific' marker that is expressed regionally is Foxn1 (forkhead family transcription factor), which is required for TEC proliferation and differentiation and does not begin to be expressed until E11. The parathyroid-specific marker glial cells missing homolog 2 (Gcm2; the mouse homolog of the *Drosophila* transcription factor Glial Cells Missing), which is essential for parathyroid survival and differentiation (Gunther et al., 2000; Liu et al., 2007), is expressed in a discrete anterodorsal domain within the third pouch endoderm starting at E9.5 (Gordon et al., 2001; Patel et al., 2006). The current data, however, suggest that, despite these early and localized expression patterns, neither Gcm2 (Liu et al., 2007) nor Foxn1 (Blackburn et al., 1996; Zamisch et al., 2005) specifies initial organ fate; they are instead each required for organ-specific differentiation. A number of other transcription factors, including those in the Hoxa3 (homeobox A3)-Eya1 (eyes absent homolog 1)-Pax1/9 (paired box protein 1/9)-Six1/4 (sine oculis homolog 1/4) network and Tbx1 (T-box 1), are also thought to act during the early stages of pouch formation (Manley and Condie, 2010). However, although the Hox-Eya-Pax-Six network appears to be required early in thymus development, it has not yet been linked directly to thymus fate or to Foxn1 expression. Furthermore, because Foxn1 does not establish thymus fate, there appears to be a 'missing link' that establishes thymus fate between this earliest patterning network and Foxn1-driven organogenesis and TEC differentiation. Similarly, the Shh (sonic hedgehog), Bmp (bone morphogenic protein), Wnt (wingless-int) and Fgf (fibroblast growth factor) signaling pathways are involved in the initial patterning of the thymus. However, the links between these pathways and the transcription factors responsible for establishing initial thymus fate have still not been defined. Furthermore, the importance of these pathways in thymus organogenesis has not yet been confirmed with clear functional in vivo tests. For example, Wnt5b (Balciunaite et al., 2002), Fgf8 (Frank et al., 2002) and Bmp4 (Bleul and Boehm, 2005; Patel et al., 2006) are all expressed in the distal-posterior presumptive thymus domain prior to Foxn1

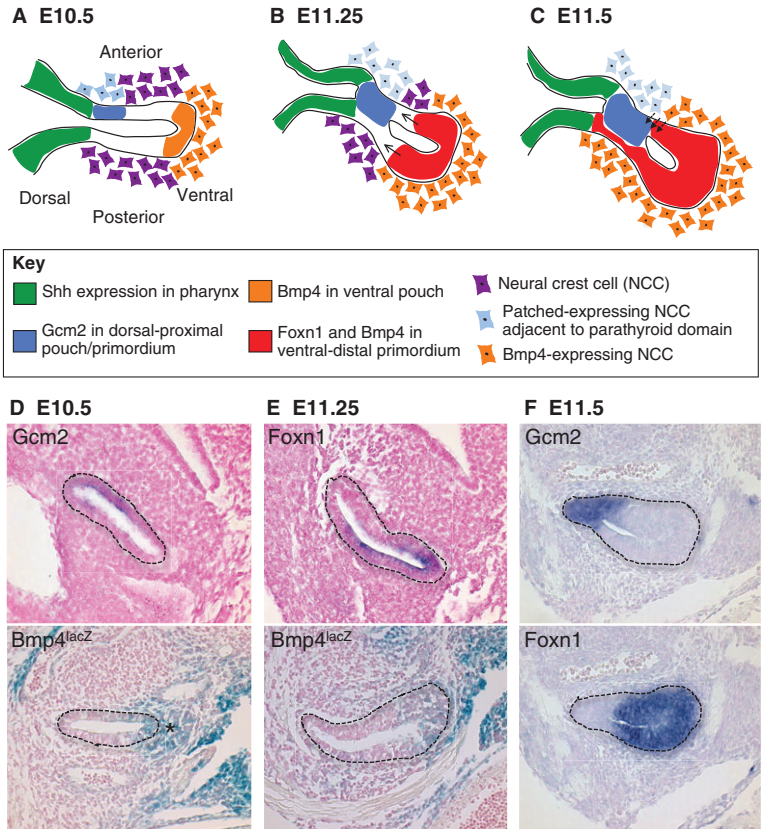

Fig. 3. Patterning the third pharyngeal pouch. (**A**) At E10.5, Gcm2 expression (blue) marks the parathyroid domain within the third pouch, Bmp4 (orange) is expressed at ventral tip of the pouch and adjacent mesenchyme, and Shh (green) expression is detected in the pharynx but is excluded from the pouch. Neural crest cells (NCCs; purple/light blue/orange) surround the pouch and some express regionalized markers (Bmp4, orange; patched, light blue). (**B**) By E11.25, Foxn1 and Bmp4 expression (red) spreads dorsally (arrows) towards the Gcm2-expressing parathyroid domain (blue). (**C**) By E11.5, the third pouch epithelial cells express either Foxn1 and Bmp4 (red) or Gcm2 (blue). Signals from adjacent NCCs refine the position of the border between domains (arrows). (**D**) In situ hybridization for Gcm2 (top) demonstrates its proximal-dorsal-anterior-restricted expression domain at E10.5. Analysis of $Bmp4^{lacZ}$ mice (bottom) demonstrates Bmp4 expression at the ventral tip of the pouch endoderm and in the adjacent mesenchyme (asterisk) at this stage. (**E**) Foxn1 in situ hybridization (top) and $Bmp4^{lacZ}$ expression (bottom) at the distal-ventral tip of E11.25 primordium. (**F**) In situ hybridization for Gcm2 (top) and Foxn1 (bottom) on adjacent sections show non-overlapping expression at E11.5. In D-F, third pouch endoderm is outlined by dashed line. All data panels are sagittal sections oriented with dorsal on the left and ventral on the right. Panels in F included with permission (Foster et al., 2010).

expression, but none of these proteins has yet been shown to specify thymus fate or directly control Foxn1 gene expression in vivo, although all three have been shown to be involved in later stages of thymus organogenesis and/or maintenance of the differentiated organ (Bleul and Boehm, 2005; Osada et al., 2006; Kameda et al., 2009; Zuklys et al., 2009; Gordon et al., 2010; Osada et al., 2010). The most convincing evidence comes from a transgenic mouse study in which noggin, a global suppressor of Bmp signaling, was expressed in TECs (Bleul and Boehm, 2005; Soza-Ried et al., 2008). This study provided some evidence that maintenance of Foxn1 in mice requires Bmp signaling, although it did not determine whether initial thymus fate in mice requires Bmp signaling. This study also suggested that Bmp signaling might be required in zebrafish for initial expression and maintenance of Foxn1. Finally, the Shh pathway has been proposed to be a negative regulator of thymus fate, and to promote parathyroid fate, based on the absence of parathyroid specification and expansion of the thymus domain in *Shh*-null embryos (Moore-Scott and Manley, 2005).

Thus, although not conclusive, the current data are generally consistent with a Shh-Bmp4 opposing gradient-based model for patterning the organ domains in the third pouch in mice, in which Shh promotes parathyroid fate and Bmp4 promotes thymus fate, although the role of other signaling pathways remains to be fitted into this model. Much remains to be discovered, however, with respect to the mechanisms driving both initial thymus and parathyroid organ specification in the third pouch endoderm, and induction of Gcm2- and Foxn1-driven organ-specific differentiation.

NCC contributions to thymus development

NCCs are an ectodermal cell population that originate in the hindbrain and migrate extensively during development, giving rise to many different cell types including neurons, cartilage, dermis and smooth muscle (Garcia-Castro and Bronner-Fraser, 1999; Nelms and Labosky, 2010). The NCCs that migrate into the pharyngeal region and surround the third pouch during early development constitute the mesenchymal cells that eventually form the thymic mesenchymal capsule and become associated with the thymic vasculature (LeLievre and LeDouarin, 1975; Manley and Capecchi, 1995; Jiang et al., 2000; Foster et al., 2008; Muller et al., 2008). Interactions between NCC-derived mesenchyme and thymic epithelial cells have long been recognized as being essential for thymus organogenesis (Auerbach, 1960; Jenkinson et al., 2003), and defects in thymus organogenesis resulting from experimental or genetic deletion of NCCs in chick and mice clearly implicates them in this process (Bockman and Kirby, 1984; Griffith et al., 2009). However, the precise functions of NCCs in the thymus and the molecular mechanisms driving these functions are still being discovered. The most recent data show that NCCs play multiple roles in early organogenesis and morphogenesis (Fig. 3).

Studies using NCC ablation in chick suggested that thymus organogenesis depends on signals from NCCs (Bockman and Kirby, 1984). However, transplantation data from chick and mouse suggest that initial organ patterning is not dependent on specific signals from NCC-derived thymic mesenchyme, because pharyngeal endoderm transplanted prior to NCC migration was capable of establishing normal thymus organogenesis in an ectopic site (LeLievre and LeDouarin, 1975; Gordon et al., 2004). Analysis of NCC-deficient Splotch embryos showed further that the initial establishment of thymus and parathyroid cell identity occurs normally in the absence of NCCs in vivo (Griffith et al., 2009). Therefore, although epithelial-mesenchymal interactions are required for initial organogenesis in the development of many other organs, there is little evidence so far for a specific role for

epithelial-mesenchymal interactions, particularly for NCC, in either pouch formation or initial organ fate induction. It is possible that other mesenchymal cells, such as pharyngeal arch mesoderm or locally recruited mesenchyme at transplant sites, could provide the instructional cues needed to drive specification, although those cues have yet to be identified.

There is genetic evidence for a specific role for NCCs in the process of establishing the organ-specific domains in the third pouch endoderm. During mouse development, the initial thymus and parathyroid organ domains within the third pharyngeal pouches at E10.5 are restricted to small regions of the pouch, separated by regions of the pouch epithelium that, based on marker gene expression, do not appear to be specified to an organ fate. However, by E11.5, most or all cells in the developing primordium express the organ-specific markers Foxn1 or Gcm2 (Fig. 3B) (Gordon et al., 2001; Patel et al., 2006; Griffith et al., 2009). Because apoptosis of epithelial cells within the developing primordia between E10.5 and E11.5 is almost non-existent (Su et al., 2001; Manley et al., 2004; Griffith et al., 2009), the endoderm in the pouch that is not initially fated to a specific organ must decide whether to assume a thymus or parathyroid fate. The implicit cell-cell communication required for this 'domain spreading' clearly suggests the involvement of signaling pathways, although which signals, and in what direction, are unclear. This process could be an extension of the same mechanisms that establish the primary organ domains at E9.5-10; alternatively, this spreading could represent secondary fate specification, utilizing a second series of inductive interactions, possibly induced downstream of the primary mechanisms that establish initial cell fate. Furthermore, as this process occurs concurrently with formation and expansion of the shared organ rudiment (see below), these processes might also be linked.

Recent data indicate that domain spreading depends on epithelial-mesenchymal interactions between the pharyngeal endoderm and NCCs. Splotch mutant (*Pax3*-null) mice have a severe NCC deficiency, with few or no NCCs present in the vicinity of the third pharyngeal pouch during its formation and patterning. Splotch mutants were reported to have partially penetrant thymic agenesis, with defects ranging from ectopia to hypoplasia to complete agenesis (Franz, 1989; Conway et al., 1997; Soriano, 1997; Epstein et al., 2000). However, a recent analysis of Splotch mutant mice showed that the initial patterning of the third pharyngeal pouches into organ-specific domains occurs normally and that by E11.5 the shared organ primordium is of normal size and is entirely patterned into Gcm2- and Foxn1-expressing domains (Griffith et al., 2009). Importantly, however, the position of the border between the thymus and parathyroid domains is shifted in these mutants, such that the thymus domain, and subsequently the fetal thymus, is larger and the parathyroids correspondingly smaller. A further consequence of the lack of NCCs is delayed separation from the pharynx, resulting in ectopic thymi (see below). This result suggests that, whereas initial organ fate is NCC-independent, the positioning of the organ border (and therefore the mechanism of 'domain spreading') and separation from the pharynx are dependent on signals from the surrounding NCCs. The organ size difference is maintained through subsequent steps of morphogenesis, suggesting that, for these organs, size is determined in part by the number of cells originally specified to that organ fate. This conclusion is also supported by studies using mouse embryo fusion chimeras that indicated that thymus size is restricted by the initial size of the epithelial progenitor cell pool (Jenkinson et al., 2008).

The identity of the signals involved in this domain-spreading event has not been conclusively determined, although the phenotype of *Shh* mouse mutants provides some clues. In mice, Shh is required for specification of the parathyroid domain, and the thymus domain extends into the pharynx in the absence of Shh (Moore-Scott and Manley, 2005). Bmp4, by contrast, is expressed in the opposite, ventral domain of the pouch, and expands with Foxn1 expression in the wild-type pouch (Fig. 3A,B). This situation is reminiscent of the opposing Shh and Bmp gradients that pattern the dorsal-ventral axis in the developing neural tube (Litingtung and Chiang, 2000). Although *Bmp4* deletion does not result in athymia with expanded parathyroids (Gordon et al., 2010), other data from transgenic mice suggest that Bmp2 might be upregulated following *Bmp4* deletion, and thus might provide compensatory function (Bleul and Boehm, 2005). As Bmp4 is also expressed in the surrounding NCCs (Fig. 3A,B) (Bleul and Boehm, 2005; Patel et al., 2006; Gordon et al., 2010), and because expression of the Shh receptor patched indicates that Shh signaling is also active in the NCCs surrounding the developing pouches (Jeong et al., 2004; Moore-Scott and Manley, 2005), these two pathways could somehow mediate the epithelial-mesenchymal interactions involved in this process of domain spreading. Alternatively, other signals might be involved, either independent of or downstream of Bmp4 and/or Shh signaling; both Fgf (Frank et al., 2002) and Wnt (Balciunaite et al., 2002) proteins have also been suggested as candidates. Indeed, an explant culture study suggested that Bmp4 signaling to TECs could induce the expression of the Fgf10 receptor, Fgfr2IIIb, thus linking these pathways (Tsai et al., 2003). Other pathways, such as notch and ephrin signaling, cannot be excluded, although there are no studies that implicate them directly. The interactions between these different pathways remain to be determined; as the location of the thymus-parathyroid interface is a major determinant of future organ size, this process is significant to the formation of functional organs.

NCC mesenchyme also has clear roles at later stages of thymus organogenesis. Some of the earliest studies that attempted to identify specific mesenchyme-derived signals that could influence TEC differentiation found that epidermal growth factor (EGF), transforming growth factor alpha (TGFα) or insulin-like growth factor (IGF) signals could substitute for thymic mesenchyme to support TECs in culture, although an in vivo role was not identified (Shinohara and Honjo, 1996; Shinohara and Honjo, 1997). A primary role after E12.5 is to support the proliferation and differentiation of TECs. For this role, Fgf7 and Fgf10 from the NCC mesenchyme in the thymic capsule signaling to Fgfr2IIIb in TECs has been shown to be required after E12.5, primarily for TEC proliferation (Suniara et al., 2000; Revest et al., 2001), and possibly for differentiation (Dooley et al., 2007). More recent studies have also established a clear contribution of NCC-derived mesenchyme to the thymic vasculature (Muller et al., 2005; Foster et al., 2008; Muller et al., 2008) although their specific role there has not been defined.

Morphogenesis: the thymus-parathyroid connection

As with the processes of specification, the processes of thymus and parathyroid morphogenesis in the mouse (and in humans) are intimately linked. At E11.5, following patterning of the primordia into thymus and parathyroid domains, the paired primordia detach from the pharynx via apoptosis (Fig. 4A). The primordia then begin to migrate caudally into the chest cavity (Fig. 4B). Soon after

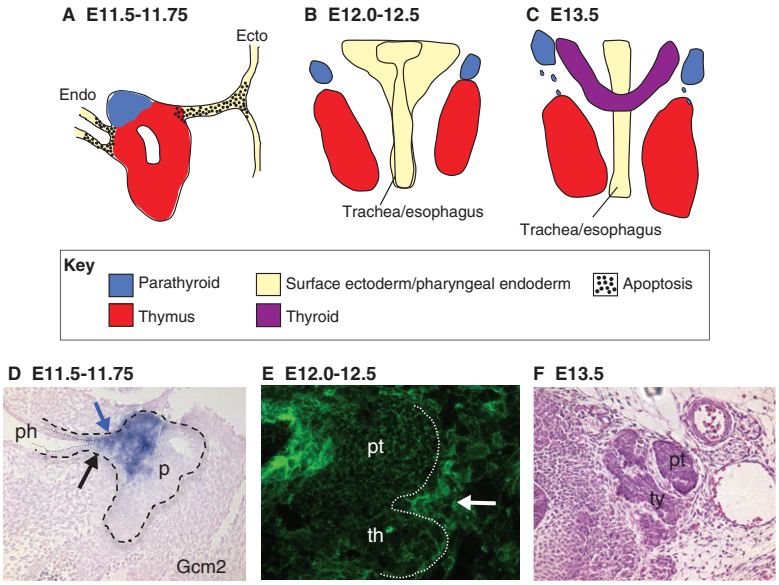

Fig. 4. Thymus-parathyroid morphogenesis.
(**A**) Between E11.5 and E11.75, the third pouch-derived primordia, containing the thymus (red) and parathyroid (blue) domains, detach from the endoderm of the pharynx (endo) and the surface ectoderm (ecto) via apoptosis (black dots), with pharyngeal separation initiated a few hours before ectodermal separation. (**B**) At E12.5, the parathyroids (blue) have separated from thymus lobes (red). Note the slight temporal difference between the left and right sides (see Box 3). (**C**) At E13.5, the thymus lobes have migrated caudally, whereas the parathyroids are lateral to the thyroid (purple), with small parathyroid remnants between the parathyroids and thymus lobes. (**D**) The primordial attachment point to the pharynx at E11.5 spans Gcm2-positive (blue) and Gcm2-negative cells (blue and black arrows, respectively). The primordium (p) and pharynx (ph) are indicated. Dashed line outlines the primordium and its attachment to the pharynx. (**E**) At E12.0, a mesenchymal 'wedge' (arrow; labeled with phalloidin in green) adjacent to the thymus-parathyroid (th-pt) border of the primordium (outlined by dotted line) can be observed. (**F**) By E13.5, a transverse hematoxylin and eosin-stained paraffin section shows that the parathyroid (pt) is located lateral to the thyroid (ty).

detachment from the pharynx, the parathyroids separate from the thymus and remain in the proximity of the thyroid gland (Fig. 4C). The thymus lobes continue to migrate caudally and eventually meet at the midline, above the heart. Interestingly, there is a slight left-right asymmetry in all of these processes during mouse embryogenesis (Gordon et al., 2010), which is important to take into account when analyzing mutant phenotypes (see Box 3). The processes of pharyngeal detachment, organ separation and migration do not occur in birds and fish, as the thymus remains attached to the pharynx in these animals and does not migrate, and fish do not have discrete parathyroids at all (Le Douarin et al., 1984; Okabe and Graham, 2004; Grevellec and Tucker, 2010). Below, we discuss each of these morphogenetic processes in turn, highlighting the consequences of disrupted morphogenesis during mouse development.

Initial morphogenesis of common primordia

As discussed above, by E10.5 of mouse embryogenesis the third pharyngeal pouch comprises a single layer of columnar epithelial cells that is continuous with that of the pharynx (Fig. 3A). Between E10.5 and E11.5, each third pharyngeal pouch begins to form a single organ primordium, while maintaining an attachment to the pharynx. The cells proliferate to form a multilayered pseudo-stratified epithelial structure with a central lumen, which is a residual of the original pouch cavity. This lumen gradually closes during the initial stages of organ formation, although the mechanism by which this happens is unclear. By E11.5 of mouse embryogenesis, the thymus-parathyroid primordia are completely patterned into two organ domains (Fig. 3C, Fig. 4A) (Gordon et al., 2001) and are still attached to the lateral surfaces of the pharynx. The differentiation of organ-specific cell types is also initiated at this stage, as indicated in the thymus domain by the immigration of lymphocyte progenitors (Liu et al., 2006), and in the parathyroid domain by the expression of PTH (Liu et al., 2007). Differential proliferation within the common primordium (higher in the thymus domain relative to the parathyroid domain) exacerbates the size difference between these two domains (Griffith et al., 2009).

Detachment from the pharynx and surface ectoderm

Marker studies performed in mice, in which one or both of the organ domains are labeled, indicate that each primordium is attached to the pharynx near the border region between the thymus and parathyroid domains (Fig. 4A,D). In mouse mutants in which pharyngeal detachment is disrupted, the thymus, but not the parathyroid, is the physical site of attachment to the pharynx (e.g. Fig. 5A,B), suggesting that the thymus domain of the common primordium is the final connection point to the pharynx. By E11.5-11.75, the paired primordia become detached from the lateral surfaces of the pharynx (Fig. 4A). This detachment is mediated by apoptotic cell death (Gordon et al., 2004). Some clues into the molecular mechanisms controlling this apoptosis-mediated detachment event have come from studying mouse mutants in which the thymus is persistently attached to the pharynx. For example, Splotch mutants with a severe loss of NCCs exhibit persistent attachment, suggesting that signals from NCCs are required for pharyngeal separation (Griffith et al., 2009). *Shh*-null mutants also display persistent attachment, implicating the Shh pathway in separation (Moore-Scott and Manley, 2005). However, both of these mutants also have pouch-patterning defects, which could indirectly block or delay separation. Likewise, *Pax9* mutants show failed thymus separation, although earlier defects in pouch formation and patterning might underlie this phenotype (Peters et al., 1998). Mutation of the FGF pathway-associated docking protein Frs2alpha (Frs2 – Mouse Genome Informatics) resulted in failure of all pharyngeal organs to detach from the pharynx, suggesting that a common FGF-dependent pathway is required for this detachment (Kameda et al., 2009). A more selective defect in separation of the thymus and parathyroids from the pharynx is seen following NCC-specific deletion of the gene encoding the Hoxa3 transcription factor (Chen et al., 2010). In these mutants, organ patterning is normal, but the thymus-parathyroid primordia remain attached to the pharynx at least until birth, when the mice die. This phenotype clearly implicates a non-cell autonomous role for NCCs in inducing apoptosis and organ-pharynx separation, and this mutant might present a good mouse model for identifying the molecular mechanisms underlying this separation process.

Box 3. Left-right asymmetry during thymus development

The pharyngeal pouches are often described as bilaterally symmetrical. However, a subtle, transient left-right asymmetry in their developmental timing and morphology exists during normal embryogenesis in mice (Gordon et al., 2010). Development of the right side of the pharyngeal region is slightly more advanced than that of the left side: the right pouch/primordium is larger at E10.5 and E11.5 (Patel et al., 2006), and parathyroid-thymus separation happens earlier on the right side (Su et al., 2001; Gordon et al., 2010). This subtle asymmetry influences phenotypes that affect pharyngeal organs, making it essential to compare the same sides (right with right; left with left) in mutant and control animals. Left-right phenotypic differences might not have a mechanistic basis (e.g. differential gene expression), but might represent exaggeration of a natural developmental timing difference. Changes in a signal or transcription factor at a specific time in development could induce differential effects on the left and right sides because they are at slightly different developmental stages, revealing a tight molecular temporal control of pharyngeal organ development (Manley and Capecchi, 1998; Su et al., 2001; Moore-Scott and Manley, 2005; Rizzoti and Lovell-Badge, 2007). In humans, third pharyngeal pouch anomalies occur almost exclusively on the left side (Lin and Wang, 1991; Liberman et al., 2002). This supports further the existence of a natural left-right asymmetry within the pharyngeal region.

Pharyngeal detachment can either be delayed (e.g. Splotch mutants) (Griffith et al., 2009) or fail completely (e.g. NCC-specific *Hoxa3* mutants) (Chen et al., 2010); both cases result in ectopic thymus lobes. This is not surprising in the case of failed detachment, in which the lobes remain in the neck physically attached to the pharynx. However, delayed detachment does not result simply in delayed migration. The fact that the lobes fail to migrate suggests the existence of a time window during which the thymus is either exposed to a specific factor(s) or able to respond to such a factor(s), or after which there is a structural block to migration. Failure of thymus migration is rare in humans, and presents with a mass in the neck, generally in children (Saggese et al., 2002).

At E11.5 of mouse embryogenesis, the thymus-parathyroid primordia are also in transient contact with the surface ectoderm of the third pharyngeal cleft (see Glossary, Box 1); this contact point is retained from the initial endoderm-ectoderm contact point that is made during pouch formation, and in terrestrial vertebrates might be an evolutionary remnant of gill formation (Manley and Blackburn, 2004). Detachment of the primordia from the surface ectoderm occurs within roughly the same time window as separation from the pharynx at E11.5-11.75, and is also mediated by apoptosis (Gordon et al., 2004). To date, mutants that display a failure of separation from the ectoderm have not been identified. However, the existence of mutants that exhibit defects in pharyngeal detachment but not ectoderm detachment suggests that these two detachment processes are controlled by independent mechanisms, despite the shared (apoptotic) mechanism.

In summary, by E12.0 of normal mouse development, the paired primordia (Fig. 4B), have completely detached from both the pharynx and the surface ectoderm, and have begun their caudal migration into the chest cavity.

Separation of the thymus and parathyroid organs

The parathyroid glands detach from the thymus lobes between E12.0 and E12.5 of mouse development, dividing each primordium into two discrete organs (Fig. 4B). There is no evidence for a role for apoptosis in this process of organ separation, however the exact molecular and cellular details are just beginning to be discovered. For this process to occur efficiently, epithelial cells within the primordium must be correctly organized prior to tissue separation. The expression patterns of Foxn1 (in the thymus domain) and Gcm2 (in the parathyroid domain) illustrate that cells within the primordium are arranged along a well-delineated border, creating two distinct organ domains (Fig. 3F). Histological evidence from mouse embryos also suggests that the physical separation of these organs is associated with a ‘wedge’ of condensing neural crest-derived mesenchymal cells that moves between the tissue domains and might help to push them apart (Gordon et al., 2010) (Fig. 4B). A similar mechanism exists in the classical somite tissue segmentation model (Takahashi and Sato, 2008). A role for neural crest-derived mesenchyme in parathyroid-thymus separation is supported by several mouse mutants in which this process is delayed, including Splotch mutants (Griffith et al., 2009) and NCC-specific knockouts of *ephrin B2* (Foster et al., 2010) and *Hoxa3* (Chen et al., 2010). Thymus-parathyroid organ separation is also delayed following *Bmp4* deletion from both the endoderm and NCC, but not from either cell type alone, implicating Bmp4-mediated epithelial-mesenchymal interactions in this process (Gordon et al., 2010).

Thymus migration

The thymus lobes originate in the pharyngeal region of the embryo; in mammals, and following organ detachment from the pharynx, the thymus lobes move caudally and medially to their final adult location above the heart. Migration is, therefore, an essential part of their embryonic development (at least in mammals), although the cellular and molecular mechanisms that control thymus migration are not well understood. E-cadherin (cadherin 1) expression is maintained on TECs during organ migration, indicating that an epithelial-mesenchymal transition does not play a role in this process (Gordon et al., 2010). Several lines of evidence, however, support a role for neural crest-derived mesenchyme during thymus migration. NCCs are themselves a migratory population, and it is therefore feasible to imagine that they ‘pull’ the epithelial thymus lobes along. In a recent study, NCC-specific deletion of ephrin B2 in mice resulted in failed thymus migration and the formation of ectopic thymi (Foster et al., 2010). Mutant NCCs from these mice were shown to have reduced motility in vitro, although their migration to and into the developing thymus lobes was normal. This suggests that the mechanisms controlling NCC migration into the thymus are independent of those that regulate thymus lobe migration, although the same cells are involved. At present, this mutant mouse study represents the only unambiguous evidence that NCCs drive thymus migration, and provides evidence that this process is controlled, at least in part, by ephrin signaling.

The ephrin B2 deletion is also the only mouse mutant described to date that has been shown conclusively to have a direct defect in organ migration per se, without a prior defect in organ separation from the pharynx. This is important, as it is possible to mistake delayed pharyngeal separation for a thymus migration defect. Ectopic thymus lobes can occur secondary to persistent attachment to the pharynx or delayed separation; however, this does not constitute a true migration failure. An ectopic thymus seen at later embryonic or postnatal stages could be interpreted as a migration defect, but only careful analysis at E11.5-12.5 can determine whether the defect is in migration or in pharyngeal detachment. On a similar note, without a careful

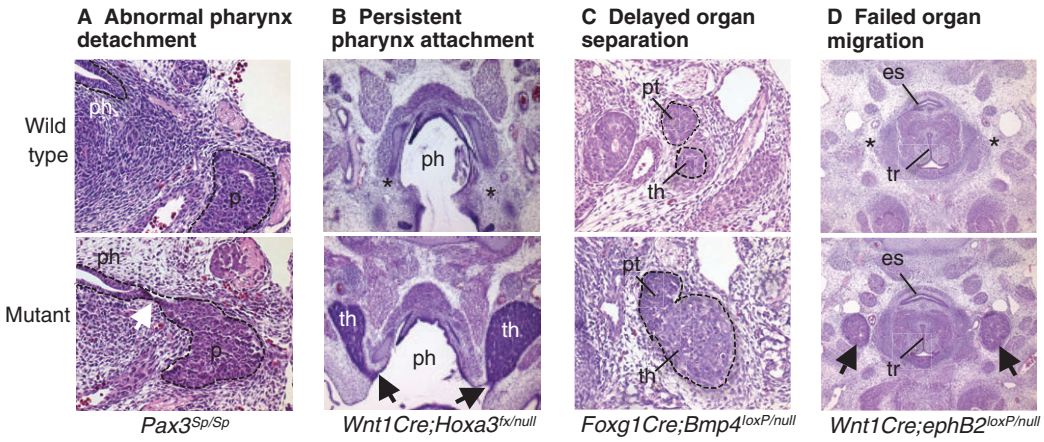

Fig. 5. Consequences of disrupted morphogenesis. Examples of mouse mutants with defects in organ detachment, separation or migration. All panels are transverse hematoxylin and eosin-stained sections. (**A**) Abnormal pharynx detachment. At E12.5 of normal development, detachment is complete (top), but in Splotch mutants (*Pax3*$^{Sp/Sp}$; bottom) the primordium remains attached by a thin cord of endoderm (arrow). (**B**) Persistent pharynx attachment. In newborn *Wnt1Cre;Hoxa3*$^{fx/null}$ mutants persistent attachment (arrows) is observed. A similar section level of a wild-type mouse is shown; the thymus is in a more posterior section. (**C**) Delayed organ separation. Separation of the parathyroid and thymus is complete by E12.5 of normal development, but not in *Foxg1Cre;Bmp4*$^{loxp/null}$ mutants. (**D**) Failed organ migration. In *Wnt1Cre;ephrinB2*$^{loxp/null}$ mutants, the thymus lobes (arrows) are ectopically located in a more anterior position than in wild type. A similar section level is shown for wild type; the thymus cannot be seen as it is in a more posterior section. es, esophagus; p, primordium; ph, pharynx; pt, parathyroid; th, thymus; tr, trachea. Images in A and B included with permission (Griffith et al., 2009; Chen et al., 2010).

search of the pharyngeal region for ectopic lobes, an absence of the thymus lobes from their normal location could be misconstrued as athymia (e.g. Fig. 5B,D).

What drives and controls the directionality of thymus migration? It is reasonable to propose that the thymus lobes follow a directional cue. Recent evidence from mouse and zebrafish (Alt et al., 2006) has demonstrated that thyroid migration follows the pharyngeal blood vessels (the ventral aorta in zebrafish; the carotid arteries in mice), and the direction of migration is disrupted if these vessels are perturbed. In *Shh* mutant mice, mislocation of the carotid arteries, in which the two vessels develop asymmetrically, is accompanied by failed bifurcation of the thyroid primordium, and relocation of the resulting single lobe such that it is in contact with the ectopic vessels (Fagman et al., 2004; Alt et al., 2006). Furthermore, ectopic endothelial cells can actually redirect thyroid migration in zebrafish (Alt et al., 2006). In mice, the thymus lobes migrate in close proximity to the carotid arteries, making it an attractive possibility that vessels also dictate the path along which the thymus lobes will migrate in mice, although a functional correlation has yet to be made.

After separation from the thymus at E12.5-13 of mouse development, the parathyroids do not move any further caudally; their movement appears to be secondary to their attachment to the migrating thymus lobes. The link between parathyroid 'migration' and attachment to the thymus is consistent with the absence of a well-defined mesenchymal capsule in the parathyroid glands, as the NCC in the thymic capsule drive thymus migration. The variable location of parathyroid glands in the neck is also consistent with a model in which the parathyroids remain at whatever position they are in when they release completely from the thymus. In the majority of cases, the parathyroids remain in close proximity to the lateral part of the thyroid and often embed into it, but always remain a discrete structure (Fig. 4C). To date, mouse mutants with truly ectopic parathyroids, other than those secondary to an ectopic thymus, have not been described.

Immigration and function of lymphoid progenitors during thymus organogenesis

The purpose of the thymus is to promote T cell differentiation and, at all but the very initial stages of thymus development, the majority of cells in the thymus are lymphocytes. Immigration of lymphoid progenitor cells (LPCs) begins at ~E11.5 in mouse development and is controlled by expression of the chemokines Ccl21 (produced by the parathyroid domain only) and Ccl25 (produced by both thymus and parathyroid domains) (Liu et al., 2005; Liu et al., 2007). LPC immigration is not a continuous process, but occurs at precise stages of organogenesis in successive discrete waves (Le Douarin, 1973; Le Douarin and Jotereau, 1975). In the mouse embryo, the first inflow of cells occurs at E11.5 (Owen and Ritter, 1969; Fontaine-Perus et al., 1981) and involves extremely low numbers of cells. As this immigration occurs prior to vascularization of the thymic primordium (Liu et al., 2005; Foster et al., 2008), LPCs must traverse the surrounding mesenchyme to enter through the basement membrane (Le Douarin and Jotereau, 1975). The first inflow of cells is followed by a refractory period during which very few additional cells enter the primordium; in the mouse embryo, this period corresponds roughly to the period of thymus organ migration. These cells then become completely replaced by a second wave, which contains significantly more T cell precursors than those from the first round of colonization (Jotereau et al., 1987; Douagi et al., 2000). This precisely controlled cyclic mechanism operates during both avian and mouse organogenesis and is controlled by the thymic stroma (Ritter, 1978; Jotereau and Le Douarin, 1982; Coltey et al., 1987). This property of periodic permissiveness for LPC entry is actually continued in the postnatal thymus (Foss et al., 2001; Donskoy et al., 2003; Goldschneider, 2006), although it is not clear whether the mechanisms controlling this process are precisely the same in the fetal and postnatal thymus.

As initial LPC entry occurs before significant TEC differentiation or the morphogenetic events discussed above, it is reasonable to ask what role, if any, lymphocytes play in the early

events of thymus organogenesis. The most direct test of this question was performed by analyzing thymus organogenesis and TEC differentiation in two mouse mutants that have a block in fetal lymphocyte differentiation: recombination-activating gene 2/common cytokine receptor γ-chain-deficient (RAG 2/γc) and *Ikaros*-null mice (Klug et al., 2002). These mutants have normal TEC differentiation and organogenesis at least until E13.5. After this stage, further TEC differentiation and medullary compartment formation requires interactions with developing lymphocytes in a process termed 'crosstalk' (reviewed by Ritter and Boyd, 1993; Nitta et al., 2011). Thus, in spite of their early presence in the thymus, lymphocytes do not appear to be required for or influence any crucial events during early organogenesis or morphogenesis.

Thymus-parathyroid connections: shared functions?

Although the physiological functions of the thymus and parathyroids are different, their common embryonic origins and their intimately linked morphogenesis pathways have occasionally led to speculation that these organs can also have shared functions. This link has been demonstrated in the case of initial lymphocyte immigration, in which the expression of Ccl21 by the parathyroid domain at E11.5 participates in attracting lymphocyte progenitors to the shared organ primordium, thus assisting in early thymus organogenesis (Liu et al., 2005; Liu et al., 2006). Similarly, the thymus has been proposed to provide a 'back-up' function for the parathyroids, based on the initial analysis of *Gcm2*-null mouse mutants (Gunther et al., 2000). *Gcm2*-null mutants lack parathyroids (Gunther et al., 2000) owing to an early requirement for Gcm2 in survival of parathyroid-fated cells (Liu et al., 2007); however, ~50% of the mutants survive, despite the lack of PTH. Based on the common origin of the thymus and parathyroid, and on the lethality of the *Hoxa3* mouse mutants that lack both organs (Chisaka and Capecchi, 1991; Manley and Capecchi, 1995), the authors proposed that the thymus provided an independent auxiliary physiological source of PTH, proposed to be controlled by the related transcription factor Gcm1 (Gunther et al., 2000).

To determine the embryonic origins of this proposed endocrine role for the thymus, recent studies have investigated further the mechanism of organ separation during mouse embryogenesis. These studies have shown that the organ separation process is not perfect; the parathyroids tend to fragment, leaving small clumps of parathyroid cells either attached to the thymus or distributed throughout the neck between the main parathyroid gland and the thymus (Liu et al., 2010). These small clusters, and often single cells, are not identifiable by conventional histology and were only revealed by high resolution in situ hybridization studies. These cells are present in both mice and humans (Phitayakorn and McHenry, 2006; Liu et al., 2010), and can be located anywhere along the path of thymus migration as well as embedded within the thymus; these trailing parathyroid cells are excellent candidates for being the source of supernumerary or accessory parathyroid glands, which are frequently seen in humans (Weller, 1933; Gilmour, 1937; Norris, 1938; Van Dyke, 1959). The misplaced parathyroid cells that remain attached to the thymus were shown to be the source of thymus-associated secreted PTH in mice; the thymus itself was also shown to express PTH, but as a self-antigen only for negative selection (and not under the control of Gcm1 or Gcm2) and not contributing to serum PTH (Liu et al., 2010). These studies suggest that the appearance of parathyroid function in the thymus is actually due to the residual authentic parathyroid cells that remain attached to the thymus as a side effect of the process of organ separation, thus reflecting an anatomical, but not functional, connection between the thymus and parathyroid.

Another recent development in understanding thymus function is the identification of cervical thymus lobes in mice (Dooley et al., 2006; Terszowski et al., 2006). These are small accessory thymus organs located in the neck region in mice and humans. In contrast to the primary thoracic thymus, these secondary thymi appear after birth, but do have molecular and functional features of the thoracic thymus, including expression of Foxn1. Recent data suggest that these cervical thymi originate from cells that do not express Foxn1 until at least E12.5 (Corbeaux et al., 2010). The location and timing of the appearance of these cervical thymi is reminiscent of the trailing third pouch-derived cells that result from the normal, but inefficient, thymus-parathyroid organ separation process, suggesting that these cells might be the source of their development, although this remains to be proven.

Conclusions

The past decade has seen expanded interest in the mechanisms operating during early thymus development. Based on studies performed over the last decade, the prevailing view of thymus organogenesis has returned to the endoderm-centric model, in which thymic epithelium originates from endoderm alone. These studies have also identified multiple specific roles for epithelial-mesenchymal interactions, including epithelial cell differentiation, positioning of the organ border within the common primordium, separation of the thymus from the parathyroids, and organ migration. We are now beginning to have a detailed picture of the events that occur during normal early thymus organogenesis and morphogenesis, and with this model we can fully analyze mouse mutant phenotypes. The field is thus now in a position to propose and test hypotheses about the mechanisms underlying these processes. Identifying new mouse mutants, as well as revisiting those known to have thymus defects, as was performed in the recent Splotch mutant study, could provide significant information. Parathyroid organogenesis is rarely assayed, but relatively simple assessments at early stages using currently available markers could also provide further information.

Perhaps the most glaring gap in our knowledge of thymus organogenesis is that we have not identified the molecular mechanisms responsible for specifying thymus, and parathyroid, fate. Foxn1 and Gcm2 appear to be not required for initial organ fates, so what signaling pathways and transcription factors do establish these organ fates? A related question is how early are organ fates specified? Transplantation studies in both chick and mouse suggest that thymus fates, at least, are specified very early, concurrent with pouch formation. The recent identification of the cervical thymus has also opened up new avenues and has prompted new questions. For example, what is the cellular origin of the cervical thymus? Do the same mechanisms control thoracic and cervical thymus development and, if so, how are they induced and deployed at different times during development? Analysis of cervical thymus phenotypes in mouse mutants known to affect thoracic thymus development would be an obvious place to start.

Analysis of the earliest stages of TEC specification could also address the question of how early and by what mechanisms the cTEC and mTEC lineages are established, and whether a true, single thymic epithelial stem cell for all TEC lineages exists and is maintained in the fetal and postnatal thymus. As thymic epithelial stem/progenitor cells are also probably established quite early in organogenesis, identifying the factors that specify initial organ fate

Box 4. Generating a thymus de novo from embryonic stem cells

As is true for many organs, efforts are underway to generate the cellular components of the thymus from embryonic stem (ES) or induced pluripotent stem (IPS) cells. The potential clinical uses are clear (Legrand et al., 2007; Green and Snoeck, 2011) – production of transplantable cells to regenerate the thymus after aging-related involution or after hematopoietic stem cell transplant is the most obvious. The two key target cell types for this purpose are thymic epithelial cells (TECs) and T cells. To date, there is only a single report of the generation of TECs from mouse ES cells (Lai and Jin, 2009). A report of anterior foregut endoderm derived from human ES and IPS cells holds more promise for robust and controlled generation of TECs, although that goal has not yet been reached (Green et al., 2011). By contrast, substantial progress has been reported in generating T cells from both human and mouse hematopoietic stem cells or from ES cells in vitro (Schmitt et al., 2004; de Pooter and Zuniga-Pflucker, 2007; Awong et al., 2008; Martin et al., 2008) using an OP9 cell line expressing delta-like 1 (OP9-DL1) that can sustain most aspects of T cell differentiation in vitro (Schmitt and Zuniga-Pflucker, 2002; Holmes and Zuniga-Pflucker, 2009). So, why make TECs at all? The main arguments are efficiency, accuracy and persistence, i.e. making enough of the right cells to provide continuing immunosufficiency without autoimmunity. Given the potential benefits of this approach and the consequences of insufficient control of the process, there is considerable interest in successful de novo thymus formation.

could provide important information that could help develop these cell types from embryonic stem cells, which could be valuable in the production of T cells in vitro, or for transplantation (Box 4). In addition, such information could be used to promote or activate thymic regeneration after aging-related, irradiation-induced or chemotherapy-induced involution (see Glossary, Box 1).

The unique morphogenetic processes that drive thymus and parathyroid organ separation and migration also provide a fertile opportunity to study how patterning in general is translated into the cellular responses that drive morphogenetic processes. What cellular processes (e.g. adhesion, migration) and specific pathways are required? In this regard, there is a significant lack of organ culture-based systems for studying and manipulating these events, and few mouse mutants that have been identified and characterized with defects in these morphogenetic processes. The development of these tools in the future would enable targeted investigation of the molecular and cellular mechanisms driving morphogenesis in this system.

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Competing interests statement

The authors declare no competing financial interests.

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