CHARACTERIZATION OF MURINE AND HUMAN THYMIC EPITHELIAL PROGENITOR CELLS

Karakterisatie van muis en humane epitheliale thymus voorloper cellen

by

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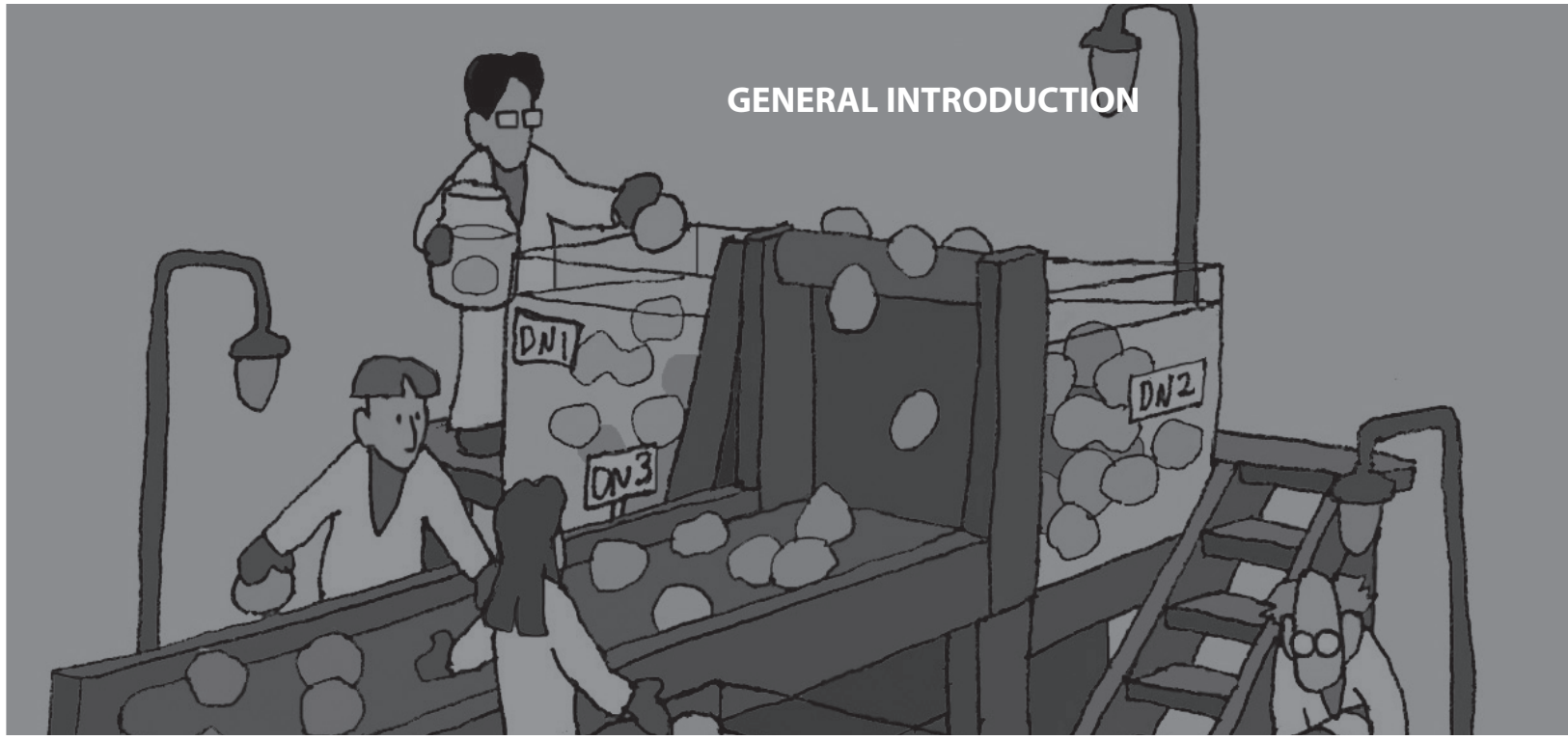
Voor mijn papa en mama

The most important thing we learn at school is the fact that the most important things can't be learned at school.

-Haruki Murakami, What I talk about when I talk about running

CONTENTS

GENERAL INTRODUCTION



The physiological role of the thymus

The mammalian immune system contains many features in terms of different cell subsets with their individual function. All these different cell subsets work together as a system to prevent and combat infections caused by bacteria, viruses and parasites. One major subset of the immune system, the hematopoietic system, can be subdivided in many branches. The hematopoietic system can be regarded as a hierarchical system in terms of stem cells and differentiated mature cells, once mature cells become less in number, stem cells will be activated and start to divide to replenish the mature cells. The mature pool of hematopoietic cells can roughly be divided in macrophages, dendritic cells, granulocytes, basophils, mast cells, megakaryocytes, erythrocytes, neutrophils, eosinophil, natural killer cells and B and T cells. All mature hematopoietic cells develop in the bone marrow, except for T cells. T cells use a specialized epithelial organ, the thymus, where they can differentiate from early thymic progenitors (ETPs) towards mature functional T cells.

In this thesis we will summarize literature of thymus biology, underline the problems that occur once the thymic function becomes impaired and explore strategies that have been undertaken to repair or rejuvenate the thymic function.

Thymic development, epithelial organization and function

The thymic anlage originates from the 3rd paryngeal pouch and is solely derived from the endodermal germ layer (Gordon et al., 2004) (Fig 1). At embryonic day (E)10.5 upregulation of the forkhead transcription factor Foxn1 in epithelial cells marks the specification of cells that contribute to the development of the thymus and Gcm2 the cells of the parathyroid (Balciunaite et al., 2002; Gordon et al., 2001; Nehls et al., 1996; Nehls et al., 1994). From E11.5 onwards, the first hematopoietic progenitors (ETPs) (Fig 1) enter the thymic primordium, providing signals contributing to the specification of thymic epithelial cells (TECs) (Itoi et al., 2001) (Fig 1). By E12.5, neural crest derived mesenchyme, surrounding the developing thymus, provides growth factors like fibroblast growth factor (Fgf)7 and Fgf10 which cause proliferation of the epithelium (Jenkinson et al., 2003; Jenkinson et al., 2007; Revest et al., 2001). During expansion of TECs, the formation of a three dimensional (3D) (non-polarized) network is established, allowing TECs to efficiently interact with thymocytes (Itoi et al., 2001). The parathyroid and thymus separate around E13.5 and the thymus descents from the pharyngeal region towards the chest cavity (Foster et al., 2010; Manley and Capecchi, 1998). At the same time, epithelial cells in the cortex and medulla become phenotypically distinguishable (Bennett et al., 2002; Gill et al., 2002; Klug et al., 2002; Shakib et al., 2009) (Fig 1). Both celltypes are derived from a single precursor TEC (Bleul et al., 2006; Rossi et al., 2006).

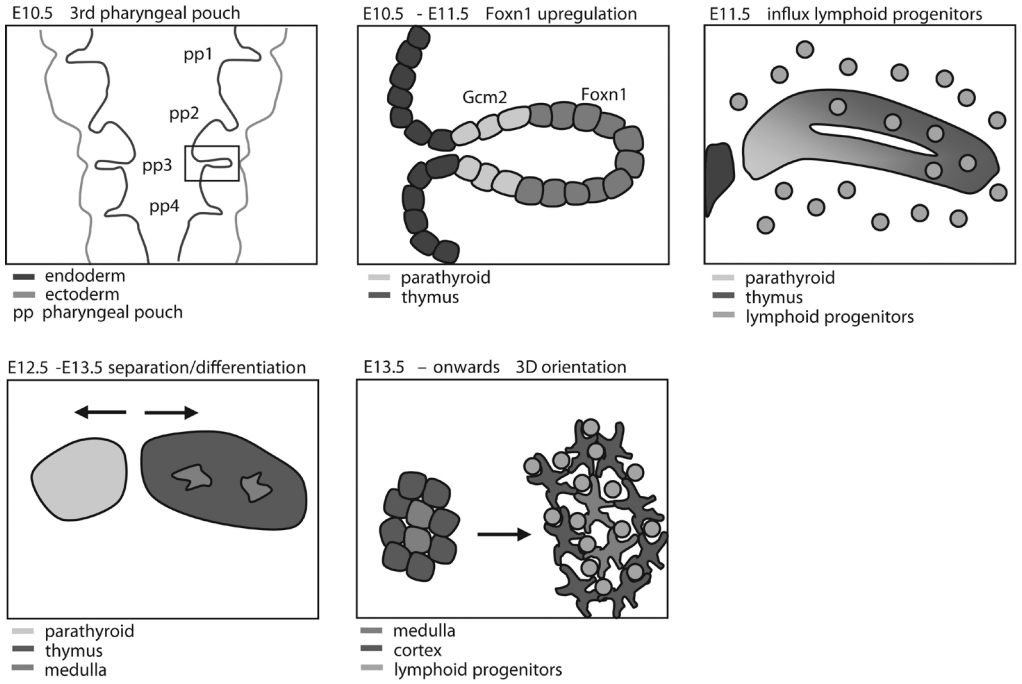
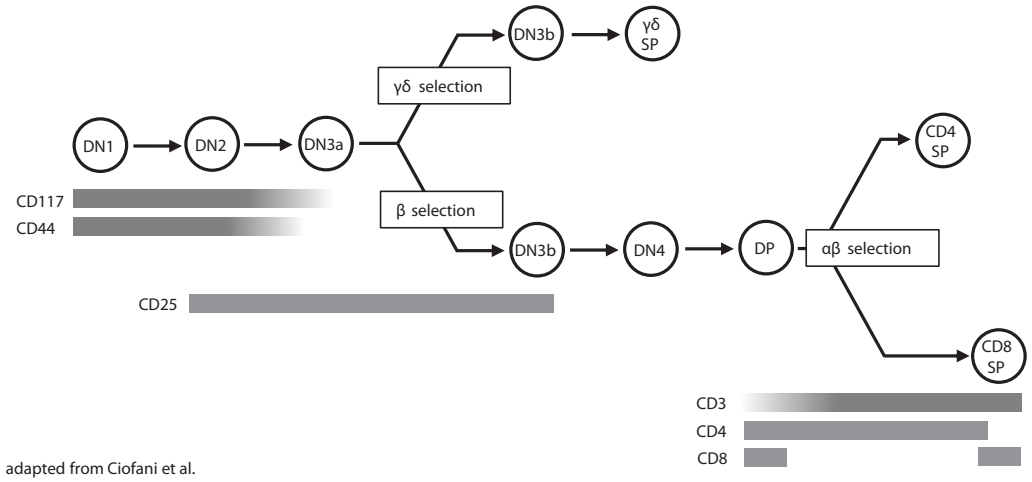


Figure 1: Early thymic development involves multiple steps. Development of the thymus starts at E10.5 and the thymic anlage is solely derived from the third pharyngeal pouch endoderm. The third pharyngeal pouch endoderm gives rise to the parathyroid, which can be identified by the transcription factor Gcm2, and the thymic primordium in which the crucial transcription factor Foxn1 becomes upregulated. At E11.5 of development the first hematopoietic progenitors will migrate into the thymic primordium and start to interact with the developing TECs which will be necessary for their development into SP T cells. From E13.5 the thymus will separate from the parathyroid and starts to descent from the pharyngeal region towards the chest cavity. The interaction between the hematopoietic progenitors and TECs is bi-directional as the hematopoietic cells will provide differentiation signals like TRANCE and Lt2β which will be important for the development of medullary TECs and the 3D orientation of TECs.

The first ETPs or thymocytes enter the thymus through the mesenchyme, while ETPs at the adult stage enter the thymus through vessels in the cortico-medullary junction (Lind et al., 2001). The phenotype of the thymic seeding progenitor that migrates from the bone marrow towards the adult thymus is still controversial as this cell is still capable to differentiate into different cell lineages. T cell progenitors can be isolated from the bone marrow, blood and thymus but retain myeloid, B cell, erythroid lineage potential. So far, the lack of proper surface markers limits the isolation of the true T cell progenitor. Alternatively, the thymic environment might be the only place where T lineage commitment is enforced and other lineages become excluded (reviewed in: (Bhandoola et al., 2007).

Influenced by cellular interactions (MHC/peptide, Delta/Notch) and soluble molecules (e.g. IL-7, SCF, Flt3L) provided by the thymic stroma, thymocytes embark on several differentiation steps, culminating in the appearance of functional CD4 or CD8 T lymphocytes (reviewed in: (Taghon and Rothenberg, 2008). One of these steps includes the gene rearrangement of the T cell receptor (TCR)β, TCRγ and TCRδ gene locus at the double negative (DN)3 stage. TCRγδ cells undergo negative selection based on their TCR receptor which will lead to their proliferation and

further maturation. TCRαβ thymocytes undergo TCRα gene rearrangement at the double positive (DP) ($CD4^+CD8^+$) stage. The rearrangement occurs random and contributes to increased receptor diversity. DP thymocytes are positively selected through interactions with cTECs after the production of a functional TCR. Then they will downregulate their CD4 or CD8 co-receptor and become single positive (SP) thymocytes (Fig 2) (reviewed in: (Ciofani and Zuniga-Pflucker, 2010). SP thymocytes will enter the medulla and become negatively selected based on their TCR specificity and affinity. Negative selection of SP thymocytes can only occur through tightly regulated interactions with mTECs that express self antigens on their Major Histocompatibility Complex (MHC)I and MHCII receptors (Fig 3) (reviewed in: (Klein et al., 2009).



adapted from Ciofani et al.

Figure 2: Thymocyte differentiation is a multi-step process. Thymic seeding progenitors enter the thymus as DN1 thymocytes. They will soon upregulate the SCF-receptor CD117 (Ckit). The most immature thymocytes (DN1 and DN2) can be identified by the markers CD44 and CD117 (CKit). They will gradually downregulate these receptors as they reach the DN3 stage. TCRβ, TCRγ and TCRδ gene rearrangement occurs at the DN3 stage. The IL-2 receptor (CD25) marks DN2 and DN3 thymocytes and becomes downregulated after TCRβ, TCRγ and TCRδ gene rearrangement. TCRαβ thymocytes undergo TCRα gene rearrangement at the DP stage and will downregulate either the CD4 or CD8 co-receptor. CD3 becomes gradually upregulated in mature thymocytes, together with the TCRα and TCRβ chain CD3 forms the TCR complex that can recognize antigens bound on MHC.

The communication between thymocytes and epithelial cells during T cell development works in a bi-directional manner. Such "thymic crosstalk" regulates TEC development and takes place from the moment when ETPs enter the developing thymus (reviewed in: (van Ewijk et al., 1994; van Ewijk et al., 1999). Mice with hampered development of thymocytes (e.g. $Rag1^{-/-}$, $Rag2^{-/-}$, huCD3εtg and $Rag2^{-/-}C\gamma^{-/-}$ mice) have a severe reduction in thymic size, while epithelial cells in the cortex and medulla are arrested at a very early stage in their development. The 3D arrangement remains absent and instead polarized cell structures (e.g. cysts, lined by secreting goblet cells and ciliated cells) that are normally not present in the thymus can be found. The 3D environment can be re-introduced once wild type hematopoietic cells are reintroduced in the deficient thymus (Hollander et al., 1995; Roberts et al., 2009; van Ewijk et al., 2000).

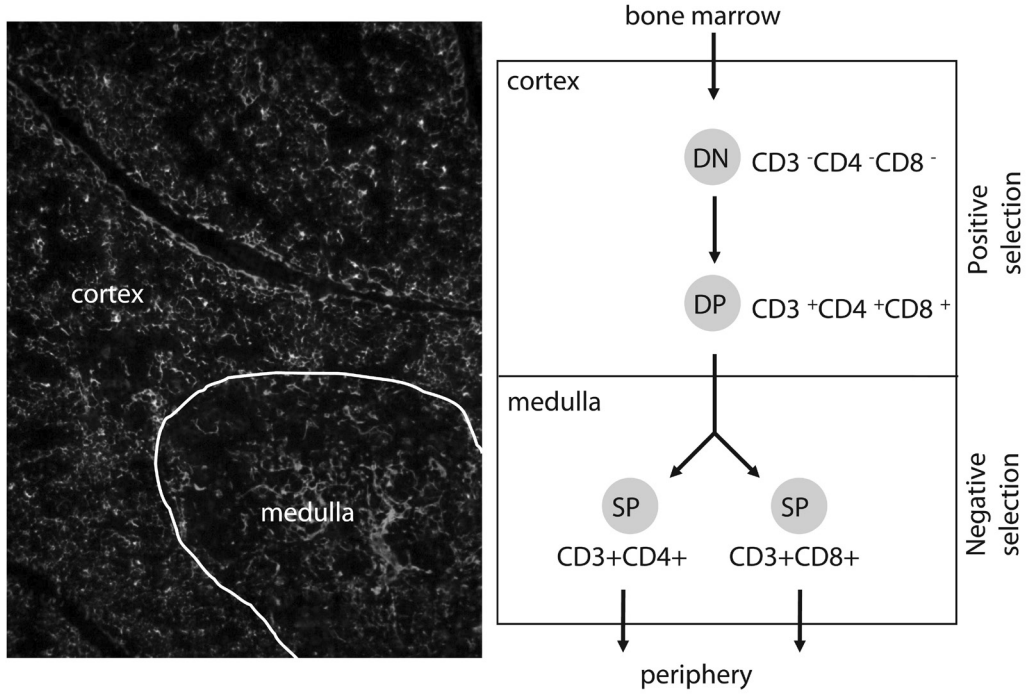


Figure 3: Thymocytes go through different developmental stages before they leave the thymus. Early thymic progenitors enter the thymus as double negative thymocytes (DN) at the cortico-medullary junction and migrate towards the cortex. Here they expand under the influence of different growth factors provided by cTECs and upregulate their CD3, CD4 and CD8 receptor to become double positive (DP) thymocytes. In the cortex thymocytes become selected for their T Cell Receptor (TCR) functionality, this process is called positive selection. From here the DP thymocytes will migrate towards the medulla where they will be downregulate their CD4 or CD8 receptor to become a single positive (SP) thymocyte. In the medulla SP thymocytes will become selected for their TCR specificity and affinity. If the specificity or affinity is incorrect the SP thymocytes will be negatively selected. When SP thymocytes successfully overcome negative selection they will leave the thymus and set for the periphery where they will exert their function as immune cells (adapted from Haynes et al.).

Cortical thymic epithelial cells

Cortical TECs are the first TECs that are involved in the education of thymocytes. Not only do they provide growth factors like Flt3, IL-7 and SCF for the expansion of thymocytes, they also fulfill an important role in positive selection of DP thymocytes. During this selection the affinity of the T cell receptor (TCR) for antigens expressed on cTECs is being checked. A narrow window for the right affinity allows DP thymocytes to towards the medulla to go into negative selection. Several mechanisms are responsible for antigen presentation of cTECs to developing thymocytes. Cathepsin is involved in the cleavage of the invariant chain from the MHCII protein. The invariant chain prevents binding of newly synthesized MHCII proteins with peptides or unfolded proteins in the cytoplasm of TECs and it is involved in the delivery of the MCHII proteins to the endosomes where loading with peptides occurs. Most antigen presenting cells, including mTECs, use Cathepsin-S to cleave the invariant chain from MHCII. However, cTECs

only express Cathepsin-L, enabling them to present a unique set of peptides on the MHCII receptor. Mice deficient for the gene encoding Cathepsin-L, $Ctsl^{-/-}$ mice, have a severely reduced number of CD4 SP thymocytes, indicating the importance of Cathepsin-L in cTECs to fulfill their role in positive selection (Cresswell, 1998; Honey et al., 2002; Nakagawa et al., 1998). The thymus specific serine protease (TSSP) Prss16 is a cTEC specific gene. The localization of Prss16 is in the endosomal/lysosomal compartment indicating a role in the presentation of peptides in the MHCII (Bowlus et al., 1999; Carrier et al., 1999). Experiments with $Prss16^{-/-}$ deficient mice showed severe reduction of CD4 SP thymocytes and CD4 T cells in the spleen and lymph nodes. This indicates that Prss16 is involved in the positive selection of thymocytes (Gommeaux et al., 2009). Peptide fragments of all proteins that are produced within the cell are presented on MHCI molecules, so called self-antigens. Through MHCI almost all the cells in the mammalian body give immune cells a possibility to distinguish native cells from foreign cells. The proteasome is responsible for the loading of MHCI molecules. The thymoprotease β5t is a regulator of positive selection of CD8 thymocytes. β5t expression can only be found in cTECs and it enables cTECs to present a unique diversity of self-antigens. Deficiency of the gene Pssmb11, encoding β5t, results in a severe reduction of CD8 T cells (Murata et al., 2007). Moreover, the diversity of the TCR repertoire of CD8 T cells was highly diminished resulting in a defective viral and allogeneic immune response (Nitta et al., 2010).

The developmental hierarchy and differentiation signals in cTECs are more obscure than mTECs. Different stages of cTECs can be defined by CD40 and MHCII. While $CD205^{+}CD40^{-}MHCII^{-}$ cells represent an immature cTEC state, $CD205^{+}CD40^{+}MHCII^{+}$ are thought to be the mature cells expressing high levels of β5t and Cathepsin-L. This mature cTEC subset is present in $RAG^{-/-}$ mice but not in huCD3ε tg mice, indicating that their differentiation signals are influenced by crosstalk signal provided by DN thymocytes (Fig 4) (Shakib et al., 2009). The adult cTEC compartment has some regenerative potential, as upon ablation the cTECs have a regenerative capacity. This capacity is independently influenced by sex hormone levels. Whether the cTECs are derived from a common mTEC/cTEC or committed cTEC precursor is unclear (Rode and Boehm, 2012).

The functional role of cTECs in positive selection has been known for many years. Still, exact mechanisms enabling cTECs to check the affinity of the TCR on developing thymocytes is largely uncovered. Lack of markers that can distinguish different subsets makes it more difficult to look at specific roles for cTEC subsets, including signals that allow their development.

Medullary thymic epithelial cells

mTECs have an important role in presenting tissue restricted antigens (TRAs) to developing SP thymocytes. mTECs cover a large amount of the TRAs, the exact mechanism behind the processing of the diverse array of TRAs remains largely unknown (Derbinski et al., 2001; Gotter et al., 2004). The responsible autoimmune regulator (Aire) gene for TRA expression via MHCII was first detected in patients with autoimmune polendocrinopathy candidiasis ectodermal dystrophy (APECED), a disease characterized by autoimmune reactions towards multiple organs

(Finnish-German, 1997; Nagamine et al., 1997). Aire is expressed in the nucleus of mTECs and enables these cells to present a large repertoire of TRAs (Anderson et al., 2005; Anderson et al., 2002). When MHCII is conditionally deleted under control of the Aire promoter mTECs have an impairment in presenting TRAs, resulting in an increase in the amount of CD4 SP thymocyt indicating the role of these cells in negative selection (Hinterberger et al., 2010). Not all TRAs are expressed by Aire expressing mTECs.

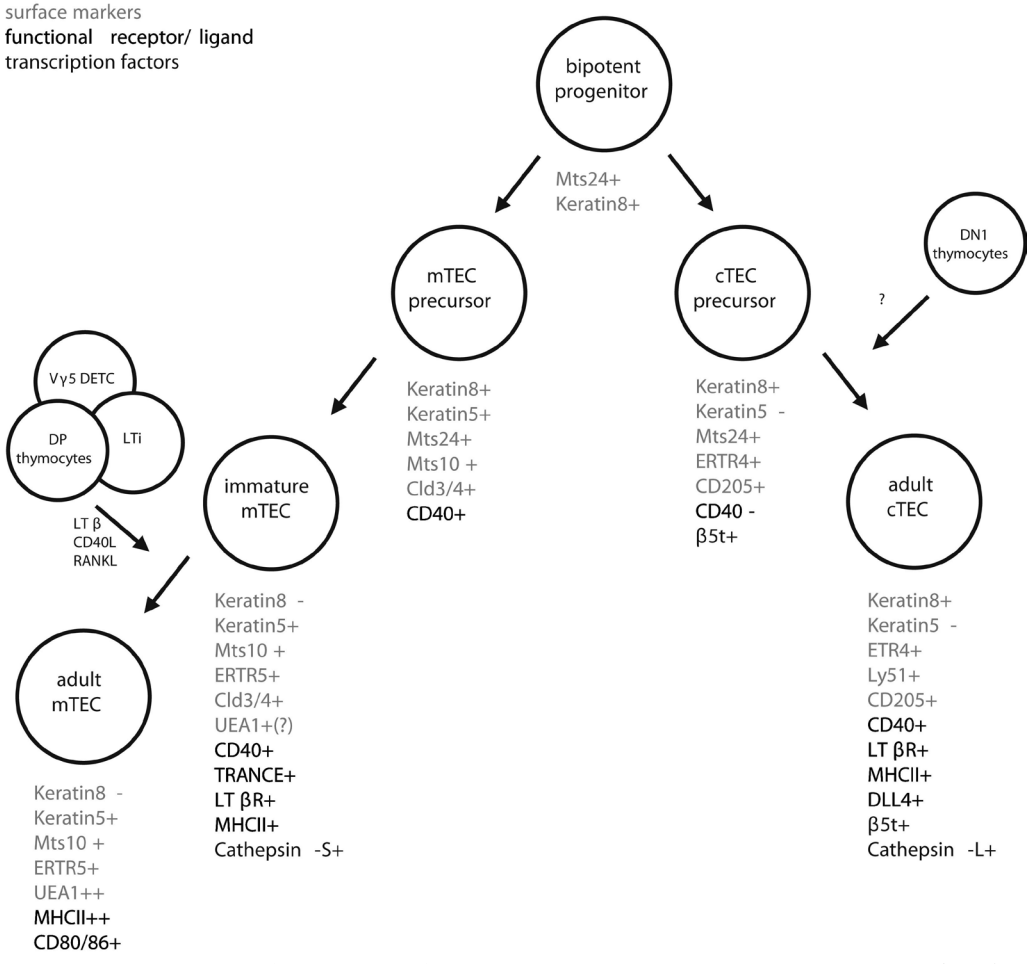


Figure 4: TECs go through different steps of differentiation and can be identified by a variety of markers. The earliest TEC that has been isolated, the bipotential progenitor, has the potential to develop into precursor cTECs and mTECs. The bipotential progenitor has only been identified at the fetal stage of thymic development (Rossi et al., 2006). Under the influence of hematopoietic cells mTECs and cTECs mature into TECs that efficiently present antigens on their MHC. mTECs need signals (RANKL, CD40L and LTβ), that are provided by LTi cells, Vγ5 dendritic epidermal T cells and thymocytes to upregulate Aire expression (Akiyama et al., 2008; Hikosaka et al., 2008; Irla et al., 2008; Lane et al., 2012; Roberts et al., 2012; Rossi et al., 2007c). **cTECs cannot develop beyond the precursor stage in the absence of DN1 tymo**cytes, however the signals involved in this process is unclear (Shakib et al., 2009).

Aire expressing cells are also located in the secondary lymphoid organs expressing a different set of TRAs compared to the thymus to naïve CD8 T cells (Gardner et al., 2008; Lee et al., 2007). Another process that enables TECs to present antigens via MHCII is macro-autophagy. Macro-autophagy is used by cells to recycle their peptides in times of nutrient-deprivation (reviewed in: (Mizushima and Klionsky, 2007) or by immune cells to process and present endogenous antigens (reviewed in: (Munz, 2009). TECs use macro-autophagy as a mechanism to load their MHCII receptor. Deletion of Atg5, a responsible gene for autophagy, leads to a diminished selection of CD4 T cell pool, resulting in colitis and multi-organ inflammation (Nedjic et al., 2008). This at least indicates a role for macro-autophagy in mTECs, however cTECs also use macro-autophagy. The functional role for macro-autophagy in cTECs still has to be addressed (reviewed in:(Nedjic et al., 2009).

The different factors that drive the differentiation of mature mTECs have mostly been revealed over the past years. Activation of the non-canonical nuclear factor (NF)-κB pathway directs immature mTECs towards a functional mature mTECs, expressing CD80, MHCII and AIRE (Fig 4). Mice deficient for molecules involving the NF-κB signaling cascade have severe defects in medullary compartments and develop autoimmunity (Akiyama et al., 2005; Boehm et al., 2003; Burkly et al., 1995; Kajiura et al., 2004; Kinoshita et al., 2006; Weih et al., 1995; Zhang et al., 2006; Zhang et al., 2007; Zhu et al., 2007). CD40/CD40L interactions are involved in development and maintenance of $CD80^{lo}MHCII^{lo}Aire^{-}$ mTECs which is a precursor stage for mature mTECs (Fig 4). CD40L first appears on thymocytes around E17 of thymic development (Akiyama et al., 2008). Signalling via receptor activator of nuclear factor κ B (RANK (TRANCE-receptor) allows $CD80^{lo}MHCII^{lo}Aire^{-}$ mTECs to mature towards $CD80^{hi}MHCII^{hi}Aire^{+}$ mTECs (Hikosaka et al., 2008; Irla et al., 2008; Rossi et al., 2007c). In the adult thymus, the ligand for RANK is expressed by positively selected CD4 and CD8 thymocytes. During the fetal stage it is believed that Lymphoid Tissue Inducer (LTi) cells deliver the RANK signal (Anderson et al., 2007; Rossi et al., 2007c) towards immature mTECs. Ligation of lymphotoxin β receptor (LTβR) also increases Aire levels in mTECs, but less efficient when stimulated via RANK and CD40 (reviewed in: (Zhu et al., 2010). mTECs develop even further as the $CD80^{hi}MHCII^{hi}AIRE^{+}$ phenotype is not the end-point for mTECs. Lineage tracing studies have revealed an mTEC population that had past Aire expression. These cells expressed involucrin and were $CD80^{int}Aire^{-}$ (Nishikawa et al., 2010; Yano et al., 2008). The functional role of $CD80^{int}Aire^{-}$ mTECs has not been established yet.

mTECs play a crucial role in negative selection of SP thymocytes, but the thymocytes itself have an important role in the activation of CD40, RANK and LTβR that allows immature mTECs to mature. Both celltypes are dependent on each other, if one of the two is absent, thymic development becomes impaired.

Mesenchymal cells

During fetal development, mesenchymal cells derived from neural crest cells colonize the branchial and pharyngeal arches and surround the developing thymic primordium, forming the thymic capsule (Le Douarin and Jotereau, 1975). The presence of these mesenchymal cells is

very important for the initial development of the thymus, as their absence results in thymi that are severely reduced in size or completely absent (Bockman and Kirby, 1984). ETPs can enter the thymic primordium at E11.5 but functional molecules like SCF, Dll4 and MHCII remain absent on these premature TECs (Itoi et al., 2007). Depletion of platelet derived growth factor (PDGF)-α from the thymic primordium at E12 results in a reduction in thymic size. However, thymic function remains in these mice as SP CD4 and CD8 T cells are formed. That indicates that even though the necessary factors for epithelial differentiation are already provided before E12, growth factors like Fgf7 and Fgf10 remain important for the outgrowth of a normal thymus (Jenkinson et al., 2007). Neural crest derived mesenchymal cells are also involved in the migration of the fetal thymus from the cervical region towards the thoracic cavity. Interaction via EphB-ephrin-B2 and BMP4 signalling are of crucial importance for this process (Foster et al., 2010; Gordon et al., 2010). In fate mapping experiments, in which Sox10 or wingless (Wnt)1 positive cells are lineage traced, neural crest derived mesenchymal cells were traced to the mesenchyme between the epithelial and endothelial cells around all of the thymic vasculature. These were associated with smooth muscle cells on large vessels and pericytes on capillaries, contributing to support and maintenance of the vessels (Foster et al., 2008; Muller et al., 2008). The role of neural crest derived mesenchymal cells in the developing thymus is clear. Presence of these cells can always be noticed in the adult thymus and even become more prominent upon aging (reviewed in: (Dixit, 2010), but the functional role of mesenchymal cells in the adult thymus is still puzzling.

Stem cells and their niche

Most organs in the mammalian body find their origin during embryogenesis. During this period, undifferentiated cells differentiate towards specialized cells which eventually form the functional part of an organ. Some organs in the adult body retain the undifferentiated cells, the so-called stem cells. Stem cells can be reactivated and divide to replenish the pool of differentiated cells if the function of the organ is hampering. For the thymus it is still unknown whether it contains stem cells for the rest of an individual's life. In this introduction we will review the available information of other epithelial organs (e.g. skin and intestine) and reflect it to the current knowledge of the thymic field.

Stem cells are generally characterized by two features; the ability to self-renew following mitosis, and the life-long capacity to produce progeny that differentiates into mature tissue with specialized function. A characteristic example of a stem cell is the hematopoietic stem cell that is well known for the capacity to continuously replenish all adult blood cells during an individual's life (Morita et al., 2010; Osawa et al., 1996). In elderly individuals the pool of hematopoietic stem cells is still sufficient, as evidenced by the unaltered ability to reconstitute recipients upon allogeneic transplantation (reviewed in: (Gazit et al., 2008; Rossi et al., 2008). Most known stem cells reside in a niche, that can be defined as the micro-environment that maintains and ensures the dividing non-proliferative capacity of a stem cell. The niche protects stem cells from possible injuries and exhausting differentiation of the stem cell compartment, thereby plays a pivotal role in maintaining self-renewal (Morrison and Spradling, 2008). In the hair-follicle, skin epidermis,

intestine, and the hematopoietic system recent advances have elucidated several local molecular requirements for stem cell maintenance. For example, it has become clear that all lineages of hematopoietic cells can be traced back towards one slow-dividing stem cell that was termed the long term hematopoietic stem cell (LT-HSC). The LT-HSC niche is found in close proximity to the inner surface of the trabecular bone, where a small subset of osteoblastic cells provides a docking station within the niche. Growth factors and cytokines like osteopontin, Jagged1, thrombopoietin, Ang1, bone morphogenic protein (BMP)4, SDF-1, FGFs, sonic hedgehog (Shh), annexin II and Wnts play a role in the maintenance and outgrowth of the LT-HSC (Li and Xie, 2005; Lo Celso and Scadden, 2011; Lymperi et al., 2010).

Epithelial stem cells of the skin

In the skin, Leucine rich G-protein coupled receptor (Lgr)6 has been identified as a marker for progenitor cells that can differentiate into different epithelial lineages (hair follicle, sebaceous gland and interfollicular epidermis). These cells, located in a region above the bulge, play a major role in wound-regeneration and repair of the adult murine skin (Snippert et al., 2010a). Resting stem cells, residing in the bulge region, appear to reside in a Wnt restricted environment. When these cells enter the growing phase, nuclear β-catenin and lymphoid enhancer binding factor-1 (Lef1)/β-catenin expression can be detected, indicating that a tightly regulated balance in the Wnt signalling cascade is necessary between the rest and growing phase (Blanpain et al., 2007; DasGupta and Fuchs, 1999). The Notch pathway also has a major role in hair follicle stem cells. Absence of recombination binding protein for immunoglobulin Kappa J region (RBP-J) or Hairy and enhancer of split 1 (Hes1) (both downstream targets of Notch signalling) leads to disruption in stem cell maintenance and hair fate determination (Blanpain et al., 2006; Moriyama et al., 2008; Yamamoto et al., 2003). Bone morphogenic protein (BMP) inhibition and Sonic hedgehog (Shh) activation also seem to play a role in stem cell maintenance and proper hair-follicle morphogenesis (reviewed in:(Blanpain et al., 2007).

Epithelial stem cells of the intestine

The gut consists of 5 different epithelial cell types that are all derived from a shared epithelial stem cell. Paneth cells, goblet cells, enteroendocrine cells, M cells and the enterocytes are known to be derived from one stem cell (Barker and Clevers, 2010; Barker et al., 2007). The quiescent stem cell is located in the +4 location of the crypt and expresses the transcriptional repressor Bmi (Sangiorgi and Capecchi, 2008). The direct progeny of these Bmi+ cells is located at the base of the crypt and these cells are characterized by the marker Lgr5 and are in direct physical contact with Paneth cells (Barker et al., 2007). The Paneth cells provide Wnt3a, Epithelial growth factor (EGF), Tissue growth factor (TGF)-α and the notch ligand Delta-like (Dll)4 to maintain the stem cells in a non-differentiating, but proliferative state (Sato et al., 2011). In contrast to Lgr6+ bulge cells, the Lgr5+ crypt cells are constantly activated by Wnt3a. The Lgr4 receptor is a relative of Lgr5 and often co-expressed in the small intestine. Together these receptors are responsive to the Wnt agonist R-spondin 1, which fine-tunes the Wnt3a signal. Both receptors are important

for maintenance of the intestinal crypt (de Lau et al., 2011). The Lgr5+ crypt cells symmetrically divide and as a result cells constantly exit the niche via neutral competition. Once Lgr5+ cells are pushed out of the niche, they no longer receive the inhibitory signals provided by Paneth cells, start to differentiate and become part of the villus structure (Sato et al., 2011; Snippert et al., 2010b).

Thus, Wnt, Notch and BMP signalling seem to play an important role in maintaining stem cells in their immature proliferative phenotype in both skin and gut niche structures. These signals are usually not provided via an autocrine fashion but by cell types derived from the stem cells themselves or by non-epithelial cells (e.g. mesenchymal cells). Inhibition or overexpression of Wnt, Notch or BMP signalling results in a loss of self-renewal capacity, impaired differentiation of progeny, and may even contribute to malignant transformation (He et al., 2004; Pear et al., 1996; Varnum-Finney et al., 2000), (reviewed in; (Reya and Clevers, 2005). These findings indicate that maintenance of stem cell self-renewal capacity by specialized cells within the stem cell niche is a highly ordered and tightly regulated process that is essential throughout an individual's life.

Wnts, Notch and BMP in thymic epithelial cells

It is well established that epithelial stem cells are at the basis of repair and maintenance of the skin and intestine. For the thymus it remains unresolved whether epithelial stem cells reside in adult thymic tissue. Thymic epithelial stem cells (TESCs) with the capacity to regenerate differentiated thymic tissue upon transplantation can be identified during embryogenesis in mice with the marker Plet-1 (Mts24) (Bennett et al., 2002; Depreter et al., 2008; Gill et al., 2002). However, that capacity seems to disappear after day 17 of embryonic development, disqualifying the stem cell marker Plet-1 in adult thymic tissue as unique TESC marker (Rossi et al., 2007a). So far, no likely candidates for adult TESCs have been found and its existence has become questionable. Nevertheless, many studies describe functional properties of the adult thymus which could be ascribed to TESCs. Therefore, we will compare the current knowledge on thymic development and function with well known properties of stem cells in the gut and hair follicle.

Both morphological and functional studies with fetal murine thymi have shown that Wnts and BMPs may play an important role in the development of the mouse thymus (reviewed in: (Hollander et al., 2006). However, their role in the maintenance or regenerating damaged thymic epithelium during adulthood is unclear. While Notch is known for its role in T lineage decision via expression of Dll4 on cTECs and Notch1 on thymocytes, the role of Notch signaling in TECs is unresolved.

Wnts

Wnts play a pivotal role in the development of the epithelial and lymphoid compartment of the thymus, including a regulatory role in Foxn1 expression (Balciunaite et al., 2002). Zuklys et al. showed that overexpression of β-catenin leads initially to normal TEC commitment in the endodermal epithelium. However, prolonged overexpression of β-catenin leads to loss of thymic phenotype, loss of Foxn1 expression and absence of mature TECs (Zuklys et al., 2009). This

observation was confirmed by Osada et al., who studied the elimination of the negative regulator of canonical Wnt signaling, Kremen1, with respect to thymic morphology. In the absence of Kremen1, most TECs were double positive for the markers K5/K8 which is considered as an immature phenotype in the fetal thymus. Additionally, a disruption of thymic architecture was observed, indicating that abundant Wnt signals are harmful for the proper development of the fetal thymic stroma (Osada et al., 2006). Inhibition of Adenomatous polyposis coli (Apc), a member of the Wnt signalling pathway, under the control of the K14 promoter resulted in impaired growth of the postnatal thymus. Thymocyte numbers were minimal, Hassal's Corpuscles enlarged and increased in number. Additionally, K8+/K14+ epithelial cells resembling basal cells of the skin were most prominent (Kuraguchi et al., 2006). Overexpression of Dickkopf-related protein 1 (DKK1) in K5+ TECs, another inhibitor of Wnt signalling, led to the degeneration of the adult thymus, decreased Foxn1+ and Aire+ TECs, induction of cysts and disappearance of K5/K8 double positive cells. The latter effect was reversible by removing DKK1 from the DKK1-transgenic thymus, indicating that continuous Wnt signalling plays a role in epithelial maintenance of the postnatal thymus (Osada et al., 2010). The role of Wnt signalling was further corroborated by Kvell et al, who showed that low levels of Wnt4 and high levels of Lamina associated protein 2 (LAP2), a pre-adipocyte marker, are correlated to epithelial to mesenchymal transition and pre-adipocyte differentiation in the mature thymus (Kvell et al., 2010).

Collectively, these reports show that Wnt signalling is important for specification towards a thymic fate in the embryonic phase. Later stages (after E13.5) of embryonic development require lower levels of Wnt to ensure the thymic specification program. During postnatal life a constant active state of Wnt is necessary to enable survival and growth of the mature medullary subsets to maintain proper thymic function.

Notch

The Notch1 ligand, Dll4, is highly expressed on cTECs and less on mTECs. Signalling via the Notch-pathway is essential for T lineage specification of lymphoid progenitor cells. Notch 1 is already expressed on early thymic progenitors and thymocytes of the DN1-4 stage, but not on the DP and SP stage. Deletion of Dll4 on TECs or Notch1 on hematopoietic progenitor cells resulted in the loss of T cells (Feyerabend et al., 2009; Hasserjian et al., 1996; Hozumi et al., 2008; Koch et al., 2008; Radtke et al., 2010; Radtke et al., 1999; Robey et al., 1996; Washburn et al., 1997)

Is Notch also involved in thymic epithelial development? The receptors Notch1 and Notch3 together with Hes1, a target gene of Notch, can be detected in E17 cTECs and mTECs. The ligands Jagged2 and Dll1 are expressed on thymocytes (Masuda et al., 2009). Notch and Hes1 expression suggests that signaling via Notch might be important for development and maintenance of thymic epithelial cells. However, in mice in which RBP-J was deleted under control of the Foxn1 promoter thymus development is normal (Hiroshi Kawamoto, personal communication).

Signaling via Notch requires members of the A Desintegrin And Metalloprotease (ADAM) family, ADAM10 and ADAM17 (TACE (TNF-α converting enzyme), which are known for their role to cleave Notch upon activation (Brou et al., 2000). ADAM enables the transmembrane do-

main of Notch being cleaved by γ-secretase, allowing subsequent transportation to the nucleus (Radtke et al., 2010). ADAM17 can be found in purified fetal and adult thymic epithelial cells (Black et al., 1997; Haidl et al., 2002; Kelly et al., 2005; Li et al., 2007). Disruption of ADAM17 in the non-hematopoietic fraction of the thymus resulted in impaired thymopoiesis at the DN4:DP transition (Li et al., 2007) and decreased Aire expression in TECs, however T cell function was not affected (Gravano et al., 2010). Although Notch receptors and downstream targets are present in TECs, an essential functional role for the Notch pathway in TEC development has yet to be established.

Bone morphogenic protein

During embryogenesis, BMP4 expression is localized in the cells in which (at a later time point) Foxn1 will become upregulated (Patel et al., 2006). Inhibition of BMP4 by overexpressing its antagonist Noggin results in the formation of thymic cysts in the fetal and postnatal thymus. Absence of BMP4 is also correlated to hampered expression of Foxn1 (Bleul and Boehm, 2005; Soza-Ried et al., 2008). By adding BMP4 to stromal cell cultures or dGuo treated fetal thymic organ cultures (FTOC) increased levels of Foxn1 transcripts and chemokine expression can be observed (Tsai et al., 2003). These experiments suggest that BMP4 (in)directly regulates the expression of Foxn1 during fetal development. However, the question which role BMP4 fulfills with respect to maintenance of adult stromal tissue remains obscure.

The role of Foxn1 as a key regulator during thymic development is well established. Foxn1 remains expressed after birth, but during thymic involution the Foxn1 expression levels decrease dramatically (Ortman et al., 2002). New evidence shows a role for Foxn1 during TEC maintenance and thymopoiesis in the embryonic and postnatal thymus (Corbeaux et al., 2010). Experimental downregulation of Foxn1 in the postnatal thymus results in decreased thymopoiesis and TEC degeneration, similar to thymic involution. The UEA1^{hi}MHCII^{hi} compartment is most sensitive to Foxn1 suppression, as this population is first lost in transgenic mice that are partially deficient for Foxn1 (Chen et al., 2009). Others confirmed that mature mTECs are affected by deletion of Foxn1and not cTEC subsets (Cheng et al., 2010). Specific deletion of Foxn1 in the K14 mTEC subset results in the formation of 2D polarized epithelial cysts, which increase in number with age (Guo et al., 2011). Deletion of Foxn1 in all TECs results in accelerated thymic involution with 3-6 months old thymi resembling 18-22 months old thymi. When Foxn1-cDNA was intrathymically reintroduced into involuted thymi a modest increase in thymic function was established (Sun et al., 2010). The role of BMP4 in correlation with Foxn1 in the fetal TECs is settled, however its role in maintenance of adult TECs in unclear. Foxn1 is necessary for the continuous function and maintenance of adult TECs, but how Foxn1 is maintained remains unknown. BMP4 can be produced by TECs themselves and DN thymocytes in the human thymus, suggesting that it is a potential maintenance factor in thymic crosstalk (Cejalvo et al., 2007)

Collectively, while Wnt, Notch, and BMP play a continuing, essential role in maintenance of adult epithelial stem cells of the skin and intestine, the role of these signaling pathways is less

clear in the development and maintenance of TESCs at the adult stage. Wnt and BMP play a role in thymic epithelial cell fate decision during embryogenesis and maintenance during adulthood. Conditional distortion in the Wnt signaling pathway results in aberrant development of the stromal organization, indicating its involvement in TEC development and maintenance of proper stromal organization. Foxn1 levels especially play an important role in the maintenance of TEC function during adult life. Deletion of the gene during adulthood results in a severely disrupted thymic architecture. Whether the function of Foxn1 and Wnts are intertwined and how the fine-tuning of these signals is orchestrated during maintenance of adult precursor TECs remains largely unknown.

Adult TEPC

There is so far no evidence that the TESC in the adult murine thymus exists. Experimental work indicates that the TEC compartment possesses a hierarchical system in which TESC are present during embryonic life but do not persist in adult life. Experiments in which growth factors enhance the thymic size and output for a brief period only suggests plasticity of the differentiated TEC subsets and not replenishment via TESCs. Many groups investigated the developmental hierarchy of the different TEC populations in the adult thymus (Bennett et al., 2002; Gill et al., 2002; Gray et al., 2007; Gray et al., 2006; Hamazaki et al., 2007; Klug et al., 1998; Klug et al., 2002; Shakib et al., 2009). During the earliest phase of thymic development TECs posses the ability to become either mTEC and/or cTEC. This bipotential TEC exists in the fetal thymus before a clear medulla/cortex separation occurs. However, it is unknown whether such bipotential progenitor cells are still present after birth (Bleul et al., 2006; Rossi et al., 2006). A study by Jenkinson et al. showed that the initial size of the progenitor pool during fetal development is decisive for the size of the postnatal organ (Jenkinson et al., 2008). It indicates that TEC expansion occurs in the fetal period and is lost in the postnatal life. This has previously been shown in pancreas development as well (Stanger et al., 2007). However, this is in contradiction with results from van Gent et al. in which patients that underwent thymectomy at early age showed recurrence of thymic tissue on a long term (van Gent et al., 2011). Whether this thymic growth is caused by expansion of differentiated TECs or outgrowth of TESC is unknown. One might postulate that the self-renewal capacity of TESCs is lost after the specialization towards cortex and medulla (Anderson et al., 2007) and therefore the true bipotential TESC no longer exist in the adult thymus.

Regenerative therapy

Involution of the thymus coinciding with a decline of thymopoiesis and narrowing of the T-cell repertoire in adulthood is a well known problem in aged men and mice (reviewed in: (Aspinall et al., 2010; Lynch et al., 2009; Taub and Longo, 2005). In mice, thymic cellularity declines over age, coinciding with epithelial to mesenchymal transition of the epithelial compartment (Youm et al., 2009). In humans, the overall thymic size might remain the same as assessed by radiographic imaging, but the cellular content changes, including increase of adipose tissue and perivascular

space. Thymic involution in humans is linked with impaired immunocompetence, predisposing for opportunistic infections and an increase of autoimmune disorders. Impaired immune competence in elderly is characterized by drastic reduction of the DP population, resulting in a decrease in the T cell repertoire diversity and less T cell receptor excision circle (TRECs) in the periphery (reviewed in: (Lynch et al., 2009). Patients, acquiring an immune deficiency as a result of infections (e.g. HIV-disease) or as a result of intensified treatment for leukaemia, may experience an even more severe immunocompromised condition. In these patients, thymopoiesis has already declined or is unable to compensate for the loss of T-cells, as may occur in elderly patients. As discussed below, several groups have investigated whether thymopoiesis may be improved by the exogenous administration of cytokines and growth factors, which at one hand directly exert a stimulatory effect on the development of the thymocytes themselves or promote the survival and/or proliferation of thymic epithelial cells, thereby indirectly stimulating thymopoiesis. These studies were performed in experimental animals as well as in early clinical trials in immunocompromised patients (reviewed in: (Chidgey et al., 2008; Hollander et al., 2010; Wils and Cornelissen, 2005).

Growth factors

Fibroblast growth factor (Fgf)7 (or keratinocyte growth factor; KGF) plays an important role during thymic development. Fgf7 is produced by the thymus surrounding mesenchyme and is believed to play a role in the expansion of epithelial cells. Mice deficient for the KGF receptor, FgfR2-IIIb, have thymic hypoplasticity, resembling the size of a fetal E12 thymus (Revest et al., 2001). The NF-κB and p53 pathways and several transcription factors including BMP2, BMP4, Wnt5b and Wnt10b become activated through signalling via KGF receptor (Rossi et al., 2007b). FgfR2IIIb is expressed by most UEA1+ TECs, almost all Plet1+ TECs in the medulla and TECs in the subcapsular space in the cortex of the adult thymus (Erickson et al., 2002; Rossi et al., 2007b). Thymi of KGF deficient mice are more vulnerable to sublethal irradiation as thymopoiesis and T cell recovery were compromised after experimental bone marrow reconstitution (Kelly et al., 2010; Kelly et al., 2008; Min et al., 2002; Panoskaltsis-Mortari et al., 1998; Panoskaltsis-Mortari et al., 2000; Rossi et al., 2002). Administration of KGF to aged mice results in an expansion of the TEC compartment and increase of thymic output (Alpdogan et al., 2006; Min et al., 2007; Min et al., 2002; Rossi et al., 2002; Rossi et al., 2007b). The beneficial effects of KGF after autologous stem cell transplantation have also been studied in non-human primates. Also here, KGF enhanced thymopoiesis, induced accelerated haematological recovery, an improved recovery of naïve T cells and a preserved thymic architecture after autologous stem cell transplantation (Seggewiss et al., 2007; Wils et al., 2011).

Although promising results are obtained with KGF in experimental models, clinical applications of KGF administration are so far rather disappointing. The short term and long term effects of human recombinant KGF after hematopoietic stem cell transplantation seem to have a minor effect on graft versus host disease, thymopoiesis and survival (Blazar et al., 2006; Levine et al., 2008). Still, human recombinant KGF is used as a drug to reduce the incidence, duration and

severity of oral mucositis after irradiation therapy of haematological cancers (Langner et al., 2008; Spielberger et al., 2004), indicating a beneficial effect of KGF on mucosal epithelial cells.

The different effects of KGF treatment on thymopoiesis in men and mice can be explained by several factors. First, the dose applied in experimental mice is 800-fold higher when compared to humans (50mg/kg/day i.p. for mice vs 60ug/kg/day i.v. for humans). Second, most mouse studies were performed in relatively young mice, while human studies were confined to adult patients. It might well be that patients are too old and their thymus is devoid of cells that are responsive to KGF.

The presence of the FGFRII receptor on UEA1+Plet1+ TECs might indicate that the cells responsible for this plasticity are located in the mature mTEC subset. Because the effect of KGF is not permanent and diminishes after several weeks it suggests that it causes an expansion of differentiated mTECs instead of activation of the TESC compartment. Therefore the beneficial effects of KGF on thymopoiesis in mice cannot be ascribed to TESCs.

Sex steroid ablation

The role of sex-steroid ablation has been studied with respect to thymic recovery after chemotherapy and has been evaluated for a possible role in reversal of thymic involution (reviewed in; (Hince et al., 2008) The principle for these studies comes from the observation that the thymic size decreases as the levels of sex steroids are increasing upon aging. That inverse correlation provided the rationale to manipulate the hypothalamus-pituitary-gonadal axis in order to prevent or reverse thymic involution. Studies in rats showed a recovery of thymic size and peripheral lymphoid pool in aging rats upon sex-steroid ablation (Fitzpatrick et al., 1985; Greenstein et al., 1986). Studies in mice revealed that sex steroid ablation has no direct effect on TEC numbers and the expansion is mainly caused by mature thymocytes themselves (Gray et al., 2006).

Following these encouraging experimental studies, early clinical studies were performed in elderly males that underwent sex steroid therapy as part of therapy of prostate carcinoma. Those patients exhibited a moderate increase of circulating T cells and TREC+ T cells (Sutherland et al., 2005). Furthermore, sex steroid therapy was also studied in recipients of hematopoietic stem cell transplantation experiencing a severe immune deficiency. During a treatment period of several months with luteinizing hormone-releasing hormone agonist a significant increase of T-cell numbers, TRECs, T cell repertoire and T cell function was observed. Moreover, in the case of autologous transplantation an increase in the disease-free survival was seen (Sutherland et al., 2008).

So far, experiments in which the hypothalamus-pituitary-gonadal axis is manipulated seem promising. The exact mechanism of sex steroid ablation on thymopoiesis remains unclear. Receptors for LHRH, androgen and oestrogen are expressed in different organs and can have a systemic effect that can go beyond thymopoiesis. The presence of LHRH receptor on thymocytes indicates that there can be a direct effect of the ligand on thymocytes (Batticane et al., 1991). The role of sex steroids on TECs is less clear, however increased proliferation in the TEC fraction after 7 days of treatment has been documented. The observation that it takes 7 days for cTECs

and 14 days for mTECs to expand (Gray et al., 2006) might indicate that TEC expansion is driven by "indirect" effects. More ETPs can be mobilized from the bone marrow under the influence of change in sex-steroids levels. These ETPs will require more growth factors that need to be produced by TECs and therefore TECs need to expand to match up with the hunger of the developing thymocytes for growth factors. Whether this TEC expansion is driven from TESCs or TECs that were already committed towards cTEC and mTEC is unknown.

Cellular regenerative therapy

Transplantation of thymic tissue has been succesfull in patients with a DiGeorge syndrome (Kobrynski and Sullivan, 2007) or FOXN1 deficiency (Frank et al., 1999). This group of patients partially or completely lacks functional thymic tissue resulting in a severely compromised immune system. These patients are susceptible to opportunistic infections throughout their life. The group of Markert et al. successfully treated these patients by transplanting third-party, mismatched cultured postnatal thymic tissue into their quadriceps muscle. The patients developed functional immunity resulting in increased T cell counts and T cell proliferative responses (Markert et al., 1999; Markert et al., 2007; Markert et al., 1997; Markert et al., 2011; Markert et al., 2003). This therapy only works in patients that lack a residual immune system that can reject the transplanted thymic tissue. For patients that still have a residual immune system other thymic therapies need to be developed.

Several groups addressed the question whether TEPC could be generated ex-vivo using embryonic stem (ES) cells. Recently the group of Lai et al. successfully differentiated murine ES cells towards Foxn1 positive thymic epithelium by using the growth-factors Ffg7, Fgf10, BMP4 and EGF (Lai and Jin, 2009). The differentiated cells contributed to the generation of naïve T cells in a murine bone marrow transplantation setting upon transplantation of the ex-vivo differentiated cells into a thymic environment via intra-thymic injection (Lai et al., 2011).

Alternatively, induced pluripotent stem (iPS) cells might provide us with a source of autologous progenitor cells that are completely matched and also provide the advantage of using mature cells from the patient which is not restricted by ethical boundaries. With the introduction of iPS cells it became possible to induce stem cells from somatic cells by introducing the factors Klf4, Oct3/4, c-myc and Sox2 (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). iPS cells have the same characteristics as ES cells. The method may now enable researchers to create TECs from either autologous or HLA-matched donor tissue, optimizing acceptance of the graft after HSCT.

Aim of the thesis

One of the major issues in thymus biology that remains unsolved is the putative presence of a true thymic epithelial stem cell (TESC), that is able to regenerate TEC and continuously replace degenerating or dying TECs by newly developed TECs during an individual's life. The observation in men and mice that the thymic size and T cell output decreases with age suggests that this TESC is absent. However, involution can be partially intervened by KGF treatment and/or sex steroid ablation therapy in murine models (summarized in **chapter 1**), indicating a plasticity of

the TEC compartment which might possibly be ascribed to the presence of TESC. The overall aim of this thesis is to investigate whether thymic epithelial progenitor cells of murine and human origin can be identified and characterized and whether these progenitor cells may exhibit true stem cell properties.

Most information that is currently available on thymic development has been obtained from experimental models like the mouse. The murine model allowed researchers to closely study the developmental events that govern thymic development. The developemental program in humans is, however, still unclear. Intensive molecular and histological examinations of human thymic organogenesis have yet not been reported and therefore the extent to which mechanisms governing mouse thymus organogenesis is conserved in human remains to be elucidated. Therefore, we conduct a detailed analysis of early human thymus organogenesis in **chapter 2**. Whole human embryos and thymic samples of different gestational ages were collected and analyzed for the presence of known markers that govern thymic development in mice. Additionally, early entry of hematopoietic cells in the human fetal thymus is identified as well as key events in the developement of the human medulla and cortex.

Foxn1 is the sole transcription factor that is known to be the crucial in the development of the thymic epithelium. Initiation of a 3D microenvironment is inhibited in mice that are deficient for Foxn1, instead primitive 2D structures like cysts can be found within the TEC compartment. However, these structures can also be found in normal aged thymi at the cortico-medullary junction. Earlier, it was suggested that TECs in these structures failed to express Foxn1 during thymic development, because these structures are overrepresented in Foxn1 deficient mice. In **chapter 3** we hypothesize that thymic cysts are a normal product of thymic development and express normal levels of Foxn1 during embryogenesis. We use Fox1-Cre x Stop^{flox}-GFP reporter mice. This enables us to visualize all the cells that actively express Foxn1 and have expressed Foxn1 in the past. We also investigate whether downregulation of Foxn1 expression is the cause of cyst formation by using Foxn1:GFP reporter mice and a polyclonal antibody directed against the Foxn1 protein.

Epithelial stem cells in the intestine and skin can be found at a specific location, the so-called 'niche'. The niche does not only harbour stem cells but also cells that support the survival and maintenance of stem cells. A marker that clearly identifies stem cells in the niche of the intestine is Lgr5. When Lgr5 positive cells are isolated and cultured in-vitro, these cells can expand and develop similar structures as can be observed in the adult intestine. The thymus and intestine are both derived from the endoderm. Presence of Lgr5 positive epithelial cells in the thymus could possibly identify thymic epithelial progenitor cells that harbour stem cell properties. Therefore, the research questions addressed in **chapter 4** were: 1.Are Lgr5 positive TECs present in the murine thymus? 2. If so, do Lgr5 positive cells possess stem cell features? We conducted a thorough analysis of Lgr5 reporter mice and bred Lgr5 deficient mice to study the role of Lgr5 in thymus development. Eventually, we use a lineage tracing system that enabled us to look at the progeny of the cells that expressed Lgr5 during embryogenesis.

Using ES or iPS cells as a source to generate TECs is an interesting approach which may eventually be used for clinical applications. While several studies (Inami et al., 2011; Lai et al., 2011; Lai and Jin, 2009) reported on the generation of thymic epithelium from ES or iPS cells, the cultured cells were loosely characterized in these studies. In **chapter 5** we address the question whether thymic epithelium can efficiently be generated in-vitro from ES or iPS cells? We analyze genes which play an important role during thymic organogenesis and subsequently test the presence of proteins which are necessary for thympoiesis. Moreover, advantage is taken of a new iPS cell line created from fibroblasts of Foxn1:GFP reporter mice.

Lastly, the major conclusions of this thesis are summarized and discussed again in **chapter 6**, especially from a translational perspective and with an outlook to future directions for thymic research.

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DYNAMICS OF THYMUS COLONIZATION AND ORGANOGENESIS IN EARLY HUMAN FETAL DEVELOPMENT

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ABSTRACT

The thymus is the central site of T cell development and thus is of fundamental importance to the immune system, but little information exists regarding molecular regulation of thymus development in humans. Here we demonstrate, via spatial and temporal expression analyses, that the genetic mechanisms known to regulate mouse thymus organogenesis are conserved in humans. In addition, we provide molecular evidence that the human thymic epithelium derives solely from the 3rd pharyngeal pouch as in the mouse, in contrast to previous suggestions. Finally, we define the timing of onset of hematopoietic cell colonization and epithelial cell differentiation in the human thymic primordium, showing, unexpectedly, that the first colonizing hematopoietic cells are $CD45^{+}CD7^{+}CD34^{-}$. Collectively, our data provide essential information for translation of principles established in the mouse to the human, and are of particular relevance to development of improved strategies for enhancing immune reconstitution in patients.

Key words: thymus, thymus organogenesis, epithelium, progenitor, hematopoietic progenitor, thymocyte, mesenchyme, colonization, human, comparative analysis

INTRODUCTION

T cell development occurs in the thymus and depends on a diverse array of functionally distinct epithelial cell types within the thymic stroma. These thymic epithelial cell (TEC) types originate from the endoderm of the 3rd (and in some species 4th) pharyngeal pouches (PP), transient bilateral endodermal structures that generate both the thymus and parathyroid glands (Gordon et al., 2004; Le Douarin and Jotereau, 1975). The molecular and cellular processes that govern thymus organogenesis are best understood in the mouse (Blackburn and Manley, 2004; Manley and Condie, 2010). In contrast, knowledge of human thymic organogenesis is based primarily on histological studies, with molecular insight currently limited to analysis of genetic abnormalities affecting thymus development; principally DiGeorge Syndrome, the human *nude* syndrome and APECED, which are caused by mutations in *TBX1*, *FOXN1* and *AIRE* respectively (Baldini, 2005; Frank et al., 1999; Villasenor et al., 2005).

The human thymus is thought to develop from either the 3rd PP (Norris, 1938) or both the 3rd and 4th PP (Van Dyke, 1941). As in the mouse, the bilateral human primordia initially each comprise thymic and parathyroid domains, and are encased in a neural crest-derived mesenchymal capsule from early in development. From week 7 (Carnegie stage [CS] 18-19) to mid-week 8 (CS20-21) the thymic component of this primordium migrates ventrally, such that the leading tip of the migrating thymic rudiment remains attached to the parathyroid via a thin, elongated, highly lobulated, cord-like structure. This structure then resolves, separating the parathyroid and thymic primordia (Norris, 1938), and the two thymic primordia meet and attach at the pericardium by mid-week 8 (Norris, 1938). It is well documented that an accessory thymus can exist in the cervical region in humans (Norris, 1938; Van Dyke, 1941), and similarly, the occurrence of cervical thymi in the mouse has recently been reported (Dooley et al., 2006; Terszowski et al., 2006).

The early human thymic primordium is proposed to contain undifferentiated epithelial cells, as reported for the mouse (Bennett et al., 2002; Lampert and Ritter, 1988; Rossi et al., 2006). Medullary development has been observed from week 8 and distinct cortical and medullary compartments by week 16. The presence of other intrathymic cell types, including mesenchymal, vascular and lymphoid cells, has been reported from mid-week 8 (Haynes and Heinly, 1995; Haynes et al., 1984). Thymic seeding progenitors (CD34+CD45RA+CD7+) can be found in cord blood and still possesses T, B, NK and GM fate (Haddad et al., 2004). Others have found CD34+lin-CD24-CD10+ progenitors in cord blood, bone marrow but also blood throughout life. These cells lost myeloid potential but retain T, B, NK and potential (Six et al., 2007). Early thymic progenitors (CD34+CD1a-) retain T, B, NK and myeloid precursor activity and resemble the cells that are found in the bone marrow (Weerkamp et al., 2006). Whether the cells found in the bone marrow, cord blood or blood also colonize the thymus or go through and intermediate developmental stage is unknown. Between 14 and 16 weeks, mature lymphocytes begin to leave the thymus to seed the peripheral immune system (Lobach and Haynes, 1986; Van Dyke, 1941). However, while the morphological events in human thymus organogenesis are well documented

and some information exists regarding TEC differentiation, molecular regulation of these processes remains poorly understood. In particular, only very limited information exists regarding the extent to which mechanisms known to govern thymus development and function in the mouse are conserved in human. Furthermore, neither the time of onset of TEC differentiation, nor the identity of markers defining thymic epithelial progenitor cells, has been determined in the human fetal thymus. Similarly, neither the timing of initial hematopoietic colonization nor the identity of the colonizing cells has been reported. Improved understanding is urgently required, due to the clinical need for improved strategies for enhancing or replacing thymus function in patients (Awong et al., 2010; Lynch et al., 2009; Wils and Cornelissen, 2005).

Here we demonstrate that the major mechanisms currently known to underpin thymus organogenesis are conservation between mouse and human. We further establish the timing of initiation during human fetal thymus development of overt TEC differentiation and of expression of known mediators of TEC function, including the Autoimmune Regulator, AIRE, and the chemokines CCL21 and CCL25, thereby identifying the stage in thymus development at which the thymus is comprised principally of undifferentiated thymic epithelial progenitor cells. Finally, we determine the stage of thymus organogenesis at which colonization by hematopoietic, endothelial and mesenchymal cells first occurs - identifying the first thymus-seeding hematopoietic progenitors as $CD45^{+}CD7^{+}CD34^{-}$ cells, in contrast to expectations from previous studies (Haddad et al., 2006). These data, to our knowledge, constitute the first detailed analysis of human thymus organogenesis and TEC development that is not based solely on morphological and histological data, and thus provide essential information for the translation of principles established in the mouse to the human.

RESULTS

Spatial and temporal expression of essential regulators of mouse thymus organogenesis is conserved in human thymus development

To determine whether molecular mechanisms that regulate thymus development are conserved between mouse and human, we investigated the detailed spatial and temporal expression profiles of known regulators of mouse thymus organogenesis in human embryos. We first confirmed the relative staging of human and mouse thymus development. The earliest human tissue available for this study was early week 6 (CS16). At this stage, the 3rd PP had formed, and morphological criteria indicated that 3rd PP/thymic primordium development was at an equivalent stage to that at E10.5 in CBAxC57BL/6 F1 mouse embryos. By early week 7 (CS18), outgrowth of the 3rd PP to form the common thymus/parathyroid primordium (Gordon et al., 2001) had occurred, equivalent to approximately E11.5 in the mouse (Fig. 1A). Migration of the thymus component of the primordium along the carotid artery was observed by mid-week 8 (CS21), as described, and at this and subsequent stages the thymic primordium was surrounded by a mesenchymal capsule (Fig. 1B). By week 10, the thymic primordium comprised numerous lobules (Fig. 1C;

note that the mid-week 8 thymus is not lobulated), and lobules could also be seen within the elongated cord connecting the thymus and parathyroid primordia (Fig. 1). Of note is that from week 8, the mesenchyme surrounding the human thymic primordium was less closely associated with the primordium than the mesenchymal capsule in the mouse (Fig. 1C, arrow).

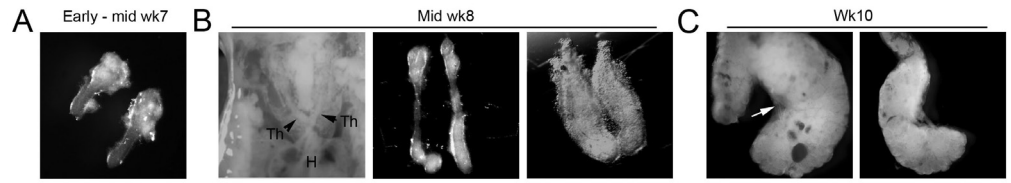

Figure 1: Morphological features of early human thymus development. (A-C) Images show microdissected human fetal thymic primordia at the ages shown, except for left hand image in (B) which shows anatomical details of the exposed chest cavity to indicate relative locations of thymic primordia, carotid artery and heart. Right hand image in (B) shows detail from lower part of thymic primordium (i.e. the leading edge during migration). Arrowheads indicate thymic primordia; white arrow in (C) indicates mesenchymal capsule. Images are representative of at least two independent analyses. Wk, week; Th, thymus; H, heart.

We then investigated the expression pattern in week 6 human embryos of *TBX1*, *HOXA3*, *PAX1* and *PAX9*, genes which have genetically defined roles in patterning of the PE and/or formation of the 3rd PP. *Tbx1* is required for development of the mouse foregut endoderm, where it regulates segmentation of the PE and PP patterning (Xu et al., 2005). Conserved stage-specificity and localization of *TBX1* expression between mouse and human embryos was demonstrated (ISH; Fig. 2A). In the early week 6 (CS16) human embryo, *TBX1* expression was detected in the core mesenchyme of the 1st, 2nd and 3rd pharyngeal arch (PA) and in the 3rd PP endoderm (Fig. 2A). Expression was also detected in the epithelium of the caudal half of the otic vesicle (Fig. 2A). *Hoxa3* is expressed in the mouse from E9.5 in the 3rd pharyngeal cleft ectoderm, 3rd and 4th PA neural crest cells, and in the 3rd PP endoderm, and is required for thymus and parathyroid formation (Chisaka and Capecchi, 1991; Manley and Capecchi, 1995). *HOXA3* expression was also conserved between mouse and human; the anterior boundary of *HOXA3* expression in the week 6 (CS16-17) human embryo was the 3rd PA and, as in the E10.5 mouse embryo, expression was observed throughout the 3rd PP and surrounding mesenchyme (Fig. 2A).

In the mouse, both *Pax1* and *Pax9* are expressed in the foregut endoderm from E8.5 (Neubuser et al., 1995; Wallin et al., 1996) and are then expressed in all four PP and in the thymus throughout organogenesis, becoming restricted to subpopulations of TEC postnatally (Neubuser et al., 1995; Wallin et al., 1996). Here, *PAX1* expression was investigated in a week 6 human embryo; expression was observed in all four PP (Fig. 2A). *PAX9* expression was also examined in human embryos at week 6; expression was evident in the 3rd and 4th PP but was not detected in the 1st and 2nd pouches (Fig. 2A). Thus, while the expression pattern of *PAX1* is conserved between mouse and human, *PAX9* expression in the human PE is more restricted than in the mouse at the equivalent developmental stage.

Foxn1 is required cell autonomously for development of mouse TEC but is not required for initiation of thymus organogenesis (Blackburn et al., 1996; Nehls et al., 1996; Nowell et al., 2011).

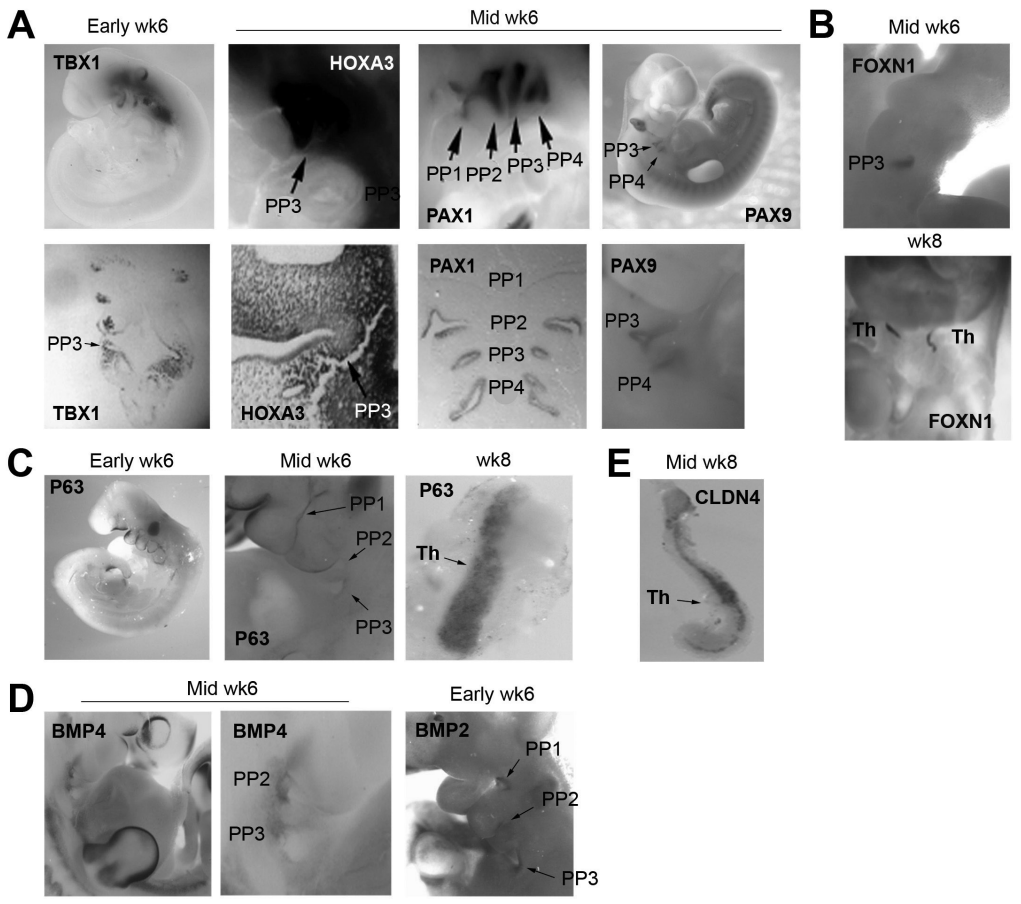

Figure 2: Expression profile of known regulators of mouse thymus organogenesis in human thymus development. Images show whole or sectioned human embryos or microdissected thymic primordia after ISH with the probes indicated. Ages of embryos or dissected thymus lobes are as shown. (A) *TBX1*, *HOXA3*, *PAX1* images show side view of whole embryos (top) and coronal sections (bottom). *PAX9*, images show side view of whole embryo; lower panel shows detail of upper panel. (B) *FOXN1*, images show ventral view of whole embryos (C) *P63,* images show side view of whole embryos (left and middle) and dissected thymic lobe. (D) *BMP4*, *BMP2,* images show side view of whole embryos. (E) *CLDN4,* images show dissected thymic lobe. Images are representative of at least two independent analyses. Th, thymus; ov, otic vesicle; PP1, 1st PP; PP2, 2nd PP; PP3, 3rd PP; PP4, 4th PP, pharyngeal pouch; Wk, week.

Null mutations in *Foxn1* result in athymia and hairlessness in mice, rats and humans (Festing et al., 1978; Flanagan, 1966; Frank et al., 1999; Nehls et al., 1994). In the mouse, *Foxn1* expression initiates at the ventral tip of the 3rd PP at E11.25 (in CBAxC57BL/6 embryos) and spreads to the entire thymus domain by E11.5 (Gordon et al., 2001). We could not detect *FOXN1* expression in the 3rd PP of early week 6 (CS16) human embryos (not shown) but observed strong expression throughout the thymus domain of the 3rd PP by mid-week 6 (CS16-17; Fig. 2B), suggesting that timing of initiation of high-level *FOXN1* expression occurs at the equivalent developmental stage in human and mouse. *FOXN1* expression was also seen throughout the thymic lobes at week 8 (CS20-21; Fig. 2B). The human thymus has been suggested to develop from both the 3rd and 4th

PP (Norris, 1938; Van Dyke, 1941). However, we did not detect *FOXN1* expression in the 4th PP at any stage of development (Fig. 2B), suggesting that in the human as in the mouse the thymus arises solely from the 3rd PP. Of note is that *GCM2*, which delineates the parathyroid domain within the pouch, is expressed in both the 3rd and 4th PP in human (Liu et al., 2010) in keeping with the proposition that human parathyroids derive from both the 3rd and 4th PP.

We also determined the expression pattern of *P63*, since in the mouse *p63* has a pivotal role in many stratified epithelia including the thymus (Candi et al., 2007; Senoo et al., 2007). In the early week 6 (CS16-17) human embryo, *P63* was expressed by the ectoderm surrounding the pharyngeal arches (Fig. 2C), consistent with its expression in the pharyngeal ectoderm in the mouse from E9.5 (Mills et al., 1999). By mid-week 8, *P63* expression was observed in the endoderm of the 3rd PP (Fig. 2C) and by late week 8, it appeared to be expressed by all TEC (Fig. 2C). Thus, *P63* expression in the pharyngeal region is also conserved between mouse and human.

Finally, we analyzed the expression of *BMP2* and *BMP4*, since BMP signaling has been implicated both in thymus development (Gordon et al., 2010) and as a possible regulator of *Foxn1* (Senoo et al., 2007; Tsai et al., 2003). *BMP4* expression was observed in the 2nd and 3rd PP endoderm and adjacent mesenchyme in the mid-week 6 (CS16-17) human embryo (Fig. 2D), consistent with its expression pattern at E10.5 in the mouse (Patel et al., 2006), while *BMP2* was expressed in the 3rd and 4th PP endoderm of the early week 6 (CS16) embryo (Fig. 2D), as observed in the mouse from E9.5 (Lyons et al., 1990).

Collectively, these data demonstrate that the spatial and temporal expression of transcription factors and signaling molecules involved in mouse thymus organogenesis is highly conserved in human thymus development, albeit with some minor regional differences. This strongly suggests that the genetic mechanisms controlling thymus development are conserved between mouse and human. Our previous studies have shown that *Rhox4* and *Plet1*, specific markers of the mouse 3rd PP/early thymus primordium, are not expressed in this region during human thymus organogenesis (Depreter et al., 2008; Morris et al., 2006). Thus, our data indicate that while the expression patterns of all genes implicated in thymus organogenesis by functional studies are conserved between mouse and human, not all gene expression patterns in this region are conserved between species.

Onset and progression of epithelial cell differentiation in the human thymic primordium

We next investigated whether patterning of the human thymic primordium into cortical and medullary compartments occurred in a similar manner to in the mouse, and at what stage the onset of TEC differentiation occurred. In the adult mouse thymus the structural proteins cytokeratin 8 (K8) and cytokeratin 5 (K5) are broadly restricted to cortical TEC (cTEC) and medullary TEC (mTEC) respectively, and additionally are co-expressed by a minor TEC population found at the cortico-medullary junction and scattered throughout the cortex (Klug et al., 1998). These markers are also co-expressed by most TEC in the E11.5 mouse thymic primordium (Bennett et al., 2002); by E12.5, high level K5 expression marks a medullary thymic epithelial progenitor cell population that also expresses high levels of Claudin 4 (Cldn 4) and, from ~E13.5, co-stains with the lectin UEA1 (Hamazaki et al., 2007).

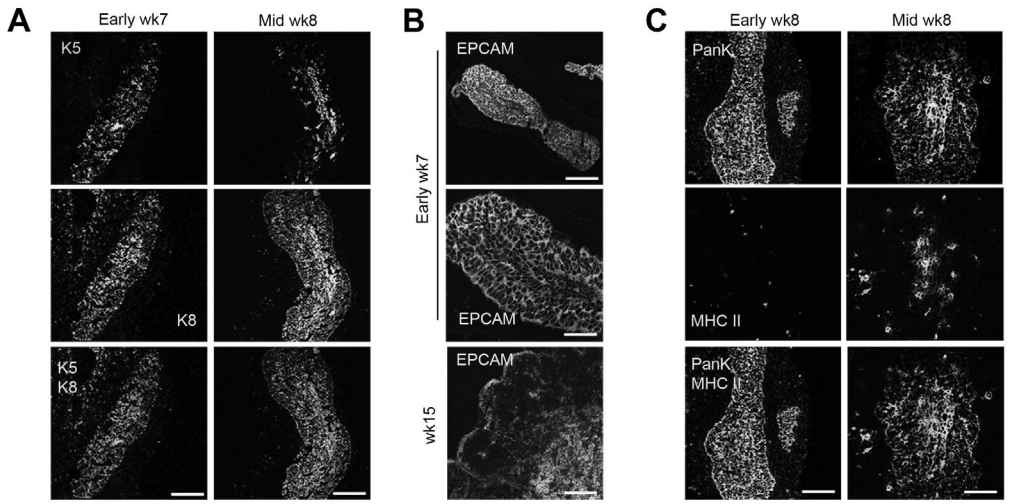

Figure 3: Onset of TEC differentiation in the human thymic primordium. Immunohistochemical staining of thymic primordia at the developmental stages shown with (A) α-Keratin 5 (K5), α-Keratin 8 (K8), (B) α-EPCAM and (C) α-HLA-DR/DP/DQ (MHC II) and α-pancytokeratin (PANK). Scale bars: A-C 150μm, EPCAM and MHC II Mid wk8 75μm. Images are representative of at least two independent analyses. Wk, week.

At early week 7 (CS18), K5 and K8 were co-expressed by most if not all TEC in the human thymic primordium, consistent with the expression pattern seen at the equivalent stage (E11.5) in mouse (Fig. 3A). By mid-week 8 (CS21), these markers had begun to segregate such that while most TEC expressed K8, K5 was more highly expressed by cells along the mid-line of the thymic lobe and in some scattered cells (Fig. 3A). Expression of EPCAM, a pan-epithelial marker in the mouse thymus that is expressed at higher levels in mouse mTEC than cTEC (Farr and Anderson, 1985), was also similar in the mouse and human thymus. EPCAM was expressed by all TEC in the week 7 human thymic primordium (Fig. 3B) and by week 15 was expressed strongly by mTEC and weakly by cTEC (Fig. 3B). These data suggested that the onset of overt TEC differentiation in human thymus development occurs around mid-week 8 (CS21), demonstrating that as in mouse human TEC development is initiated after the initiation of Foxn1 expression (Nowell et al., 2011). Consistent with this conclusion, expression of a functional marker of TEC maturation MHC Class II (HLA-DR/DP/DQ), expression of which is Foxn1-dependent in the early mouse thymic priordium (Nowell et al., 2011), was not detected in human TEC until mid-week 8 (Fig. 3C).

We next determined the pattern of UEA1 staining in human thymus development, since in the mouse UEA1 binding identifies mTEC in the thymic primordium from E13.5 (Hamazaki et al., 2007) and, subsequently, most postnatal mTEC (Farr and Anderson, 1985). Surprisingly, since no UEA1 staining is observed at the equivalent stage in the mouse, UEA1 bound most if not all TEC within the week 7 human thymus (Fig. 4A). By mid-week 8, UEA1 staining on TEC was restricted to the central area of the thymic lobes (Fig. 4A), suggesting that medullary epithelial progenitor cells had arisen by mid-week 8, and a subpopulation of mTEC binding UEA1 at high intensity were present by week 15 of human fetal development. Of note is that some non-

epithelial UEA1$^+$ cells were also observed in the human fetal thymus, unlike in the mouse. These cells were initially seen lining week 8 thymic lobes (Fig. 4A) and were found throughout the thymus at week 10 and week 15, possibly indicating UEA staining of vasculature-associated cells (Fig. 4A).

We also investigated the expression of CLDN4, since the Cldn4$^+$Uea1$^+$ population in the fetal mouse thymus contains progenitors that give rise to Aire$^+$ mTEC (Hamazaki et al., 2007). By week 8, CLDN4 was expressed by TEC in the central area of the thymic lobes (Fig. 2E, Fig. 4B). These CLDN4$^+$ cells also expressed MHC class II (HLA-DR/DP/DQ; Fig. 4C), suggesting that, as in the mouse **(Hamazaki et al., 2007), these are the first cells to differentiate into mature medul**lary TECs. CLDN4 expression remained restricted to a subpopulation of mTEC and, by week 18, identified both small clusters of nucleated mTEC and swirls of cells surrounding an enucleated region that were strongly suggestive of Hassall's corpuscles (Fig. 4B).

The Autoimmune Regulator, Aire, is essential for induction of central tolerance in mice and humans (Mathis and Benoist, 2009) and therefore we analyzed AIRE expression during human thymus development. AIRE$^+$ TEC were not present in the week 10 human thymic primordium (not shown), but were observed in medullary areas by week 13 (Fig. 4D) and had increased in number by week 17 (Fig. 4D).

To investigate differentiation towards the cTEC fate, we determined the expression pattern of CD205 (Shakib et al., 2009) and CDR2 (Rouse et al., 1988). CD205 has recently been shown to mark the onset of cTEC differentiation in the mouse and is expressed as early as E12.5 in mouse thymus development (Shakib et al., 2009). CDR2 is restricted to cTEC in the human adult thymus (Rouse et al., 1988). We were unable to detect CD205 or CDR2 expression in the early or mid-week 7 thymic primordium by immunohistochemistry (Fig. 4F,G). However by mid-week 8, extensive expression of both markers was observed throughout the thymic lobes (Fig. 4F,G). CDR2$^+$ TEC within the mid-week 8 thymus did not express K5, suggesting they may represent cortical sub-lineage restricted epithelial progenitor cells. Again, these data are consistent with the onset of TEC differentiation occurring around mid-week 8 in the human thymic primordium.

The chemokine CCL21, which plays a role in hematopoietic colonization of the thymus in mice (Liu et al., 2006), was detected in scattered cells in the human thymic primordium at week 11. The number of CCL21$^+$ TEC was substantially increased by week 17, and appeared to be largely restricted to medullary regions (Fig. 4E) consistent with its localization in the mouse (Kwan and Killeen, 2004). CCL25, a chemokine expressed by mouse TEC in a Foxn1-dependent manner which also plays a role in hematopoietic colonization of the thymus (Liu et al., 2006; Nowell et al., 2011), was first detected at mid-week 8 (Fig. 4H). CCL25 expression was initially detected in scattered cells throughout the primordium. By week 17, CCL25 expression was detected only in cortical TEC (Fig. 4H,I), as shown by co-staining with CDR2 and EPCAM (Fig. 4I, see also Fig. 3B), which mark cortical and medullary regions respectively by immunohistochemistry (Farr and Anderson, 1985; Rouse et al., 1988).

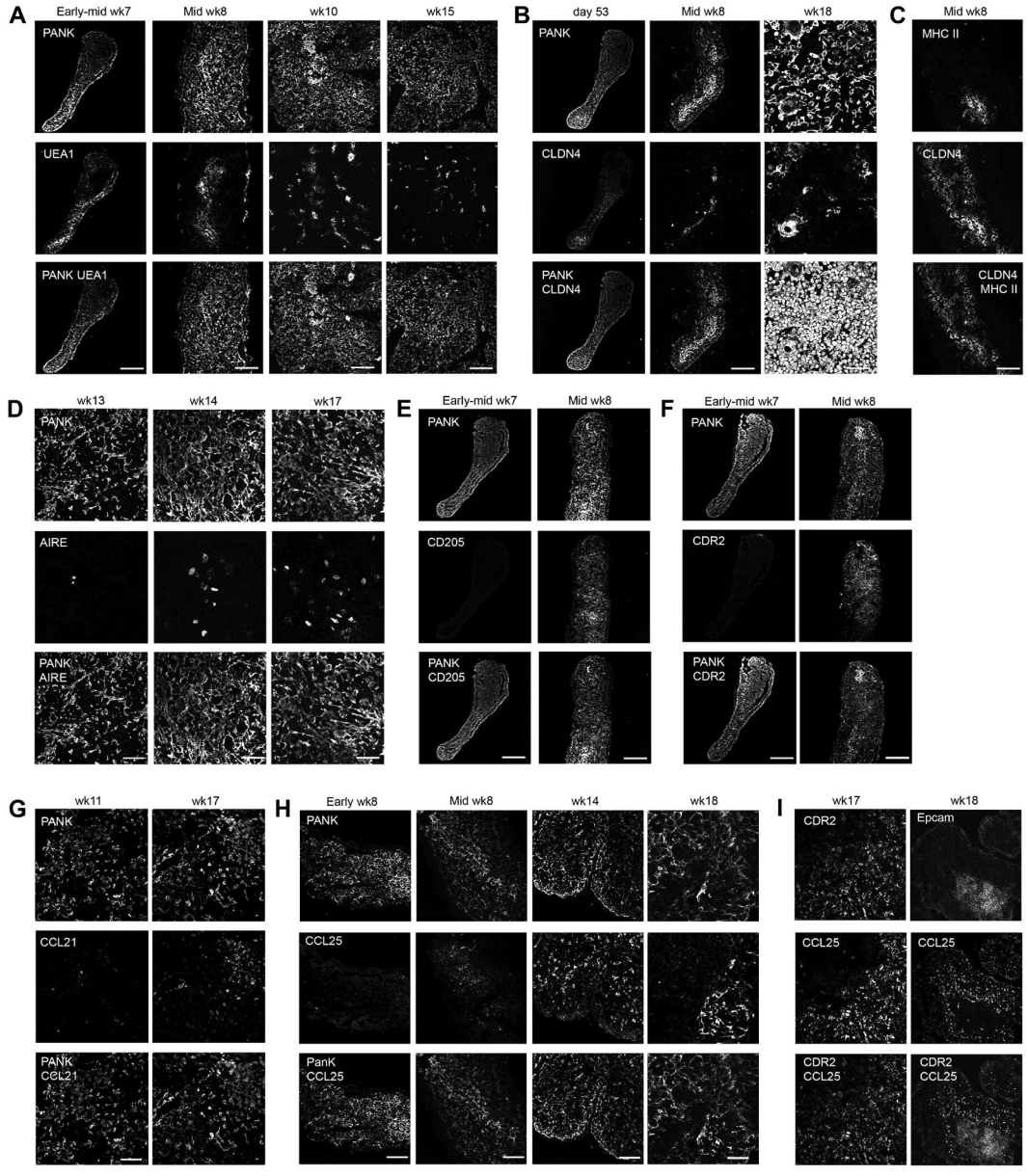

Figure 4: Expression of markers of cortical and medullary TEC in human thymus development. (A-D) Medullary TEC differentiation. Images show staining with (A) UEA1, (B) α-CLDN4, (C) α-CLDN4 and α-HLA-DR/DP/DQ (MHC II) and (D) α-AIRE. (E) Chemokine expression. Images show staining with (G) α-CCL21, (H, I) α-CCL25. (F,G) Cortical TEC differentiation. Images show staining with (E) CD205 and (F) CDR2. Co-stains are as indicated. Developmental stages are as shown. α-pancytokeratin (PANK) co-stain reveals the entire thymus primordium. Scale bars: (A,B,F,G) 150μm, except (A) wk10 - 75μm and (B) wk18 – 47.6μm; (D) wk13 - 50μm, wk14 and wk17 - 55μm; (E) 50μm. Images are representative of at least two independent analyses. Wk, week.

Immigration of mesenchymal cells and endothelial progenitors into the human thymic primordium

Neural-crest-derived mesenchymal cells form the capsule and trabeculae of the fetal thymus and also generate the intrathymic pericytes (Foster et al., 2008). It is well established that thymic mesenchyme is at least partly responsible for regulating expansion (Anderson et al., 2006; Auerbach, 1960; Jenkinson et al., 2003) and migration (Foster et al., 2010; Griffith et al., 2009) of the early thymic primordium. However, the timing of the initial immigration of mesenchymal cells into the human thymic primordium has not been determined. TE7 is a marker of human thymic mesenchymal cells which has been reported to stain the mesenchymal cells surrounding the thymic lobes at week 10, the earliest time point examined, and to mark interlobular fibrous septae, vessels and thymic fibrous capsule at week 15 (Haynes et al., 1984). At early week 7, we observed cells staining with TE7 surrounding the thymic primordium, but none were seen within the thymic epithelium (Fig. 5). By mid-week 7, formation of trabeculae was apparent and TE7 expression was also seen in the anterior end of the thymic/parathyroid primordium, where it appeared to be in the luminal spaces. However, mesenchymal cells were not evident within the thymic epithelium region itself until mid-week 8 (Fig. 5). At subsequent stages, $TE7^+$ thymic mesenchyme divided the thymus into discrete lobules, and $TE7^+$ cells were also present throughout the epithelial regions (Fig. 5) (Haynes et al., 1984). Thus, the timing of onset of migration of mesenchymal cells into the human thymic primordium closely follows the onset of FOXN1 expression (Figure 2), consistent with the timing of this event in the mouse (Foster et al., 2008).

Immigration of vascular progenitors into the mouse thymus is closely linked to that of neural-crest-derived cells, and both of these processes are Foxn1-dependent (Mori et al.; Nowell et al., 2011). However, again, the timing of vascular endothelial progenitor entry into the human thymus has not been determined. Our analysis identified the presence of $CD34^+CD31^+$ cells in the connective tissue between the carotid artery and the thymus primordium at mid-week 7; occasional cells at this stage appeared poised to enter the epithelial component of the primordium (Fig. 6A). By early to mid-week 8, $CD34^+$ cells were found within the thymic lobes (Fig. 6A) and all co-expressed both CD31 and VE cadherin, identifying them as endothelial progenitors (Fig. 6B). Blood vessels *per se* were present by mid-week 9 (note long blood vessel like structures at mid-week 9) (Fig. 6B).

Some variation in timing of colonization with endothelial progenitors was observed: in the thymus primordium from a 48-51 day old fetus (mid-late week 7; CS19), several $CD34^+$ cells were present within the epithelial compartment, and were localized to one discrete region (Fig. 6A). However, in a 53-54 day-old fetus (mid-week 8; CS21) $CD34^+$ cells were present in the mesenchymal capsule but were not detected within the epithelial component of the thymus (Fig. 6A). These differences in kinetics of colonization between different fetal thymi most likely reflect the effects of genetic variation within the human population on fetal thymus development; in this context, it is well established that thymus organogenesis proceeds at different rates in different inbred mouse strains.

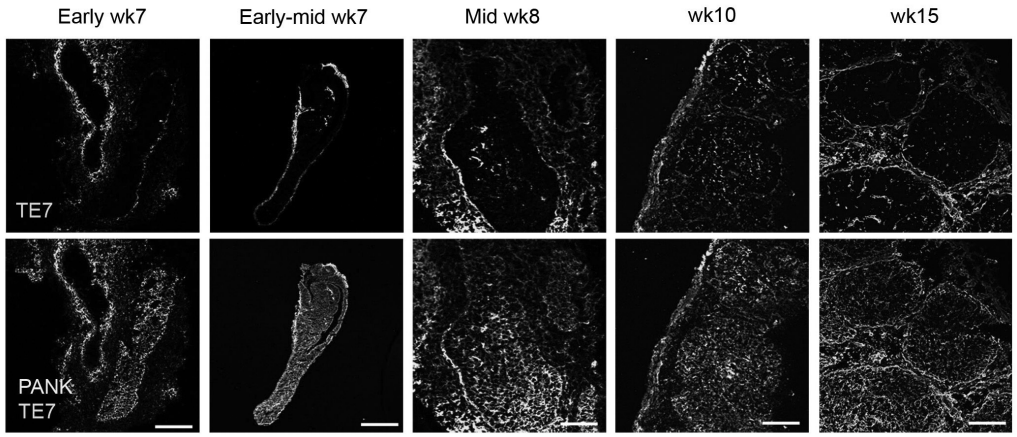

Figure 5: Colonization of the human thymic primordium by mesenchymal cells. Images show staining with TE7 at the developmental stages shown. α-pancytokeratin (PANK) reveals the entire thymus primordium. Images are representative of at least two independent analyses. Scale bars: 150μm, Mid wk8 75μm. Wk, week.

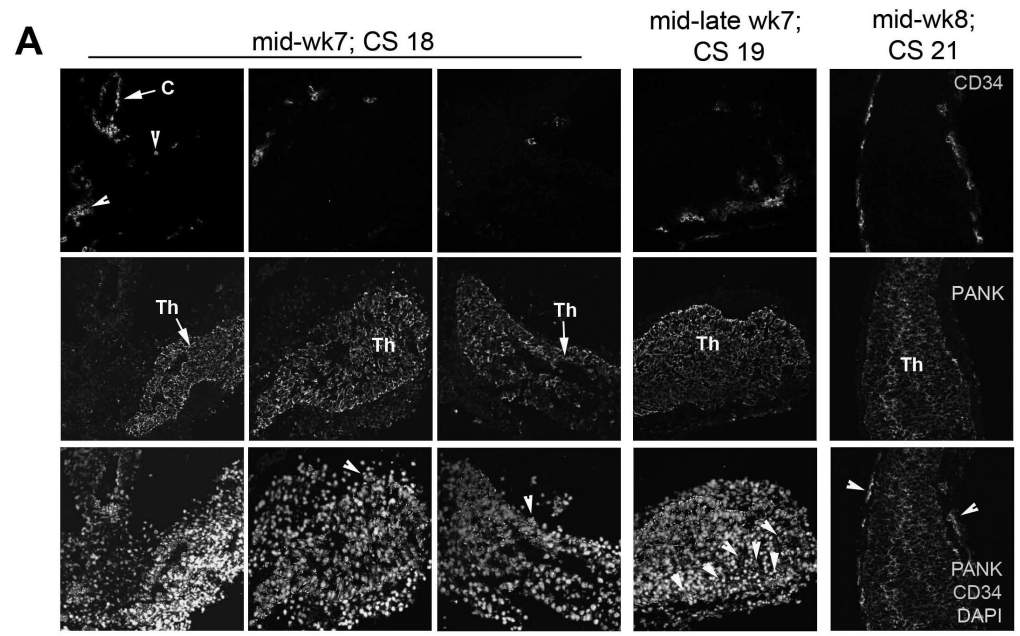

Figure 6: Colonization of the human thymic primordium by vascular endothelial cells. (A, B) Images show staining with α-CD34 at the developmental stages shown. α-panCytokeratin (PANK) reveals the entire thymus primordium. CD31 and VEcadherin (VEcad) staining reveals vascular endothelial cells (B). Scale bars: (A) 75μm. C, carotid artery; Th, thymus; white arrowheads in (A) indicate hematopoietic cells. Images are representative of at least two independent analyses.

CD45⁺CD7⁺ T lineage progenitors are first present at week 8 and lack CD34 expression

Hematopoietic colonization of the human fetal thymus has previously been proposed to occur during early week 8 and the week 7 human primordium was reported as being devoid of hematopoietic cells (Haynes and Heinly, 1995). However, as acknowledged by the authors, this study was compromised by the limited material available (Haynes and Heinly, 1995) and thus, although the timing at which subsequent stages in T cell development are first observed has been determined (Res and Spits, 1999), the stage at which the earliest hematopoietic cells colonize the human thymus remains unknown. We therefore sought to determine the kinetics of hematopoietic colonization of the human thymus using markers associated with human hematopoietic progenitor cells, including early T-lineage precursors. Of note is that the earliest T-lineage restricted precursors in human are thought to be CD45⁺CD34⁺CD7⁺ cells (Haddad et al., 2006) and CD7 expression is regarded as indicative of entry into the T-cell lineage.

We observed CD45⁺ hematopoietic cells in the perithymic space at early week 7 (Fig. 7A) and by early to mid-week 7, detected occasional CD45⁺ cells within the thymic primordium (Fig. 7A, green arrow). By early to mid-week 8, CD45⁺ cells lined the perithymic space and were also distributed throughout the thymic lobes, and by week 10 the numbers of CD45⁺ cells within the thymus had increased dramatically (Fig. 7A). However, no CD34^{hi}CD45⁺ cells could be detected prior to week 9 (Figure 7B-D). Co-staining with additional markers indicated that at mid-week 8 a proportion of the CD45⁺ cells were MAC1⁺ macrophages (Figure 7B).

Surprisingly, given the lack of CD45/CD34 co-staining at this stage, CD45⁺CD7⁺ cells were observed within the thymic lobes from early week 8, when they comprised a minor subpopulation of intrathymic CD45⁺ cells (Fig. 7C; CD45⁺CD7⁺Mac1⁻; CD45⁺CD7⁻Mac1⁺ and CD45⁺CD7⁻Mac1⁻ cells were all present at this stage). Consistent with previous reports that CD7 identifies extra-thymic hematopoietic precursor cells with T-lineage potential (Haynes and Heinly, 1995; Haynes et al., 2000), we observed some CD7^{lo} cells located in the perithymic space. These cells were sometimes juxtaposed to CD7^{hi} cells that appeared to be actively entering the thymus, suggesting that CD7 up-regulation may occur upon migration into the thymic epithelium (Fig. 7C). However, intrathymic CD34^{hi}CD7⁺ cells were not detected until mid-week 9 (also the first stage at which CD45⁺CD34⁺ cells were detected), when they were observed closely associated with the blood vessels (Fig. 7D, E). These data therefore pinpoint the stage of human thymus development at which colonization by both hematopoietic progenitors and T-lineage restricted hematopoietic progenitors occurs.

Subsequent analyses confirmed previous studies indicating the appearance of CD4 and CD8 single positive and gamma delta TCR⁺ thymocytes, and CD4⁺CD25⁺ T regulatory cells in the human fetal thymus by week 15 (Cupedo et al., 2005; Galy et al., 1993; Haynes and Heinly, 1995; Lobach and Haynes, 1986)(data not shown). Low numbers of conventional dendritic cells (cDC) were detected at week 15, consistent with a previous report indicating their presence at week 12 (Janossy et al., 1986). CD11c⁺CD11b⁺ cDC increased proportionally by week 16 (not shown), together with a numerical increase in the entire cDC population. Of note is that plasmacytoid dendritic cells were already evident at week 15 (Fig. 7F).

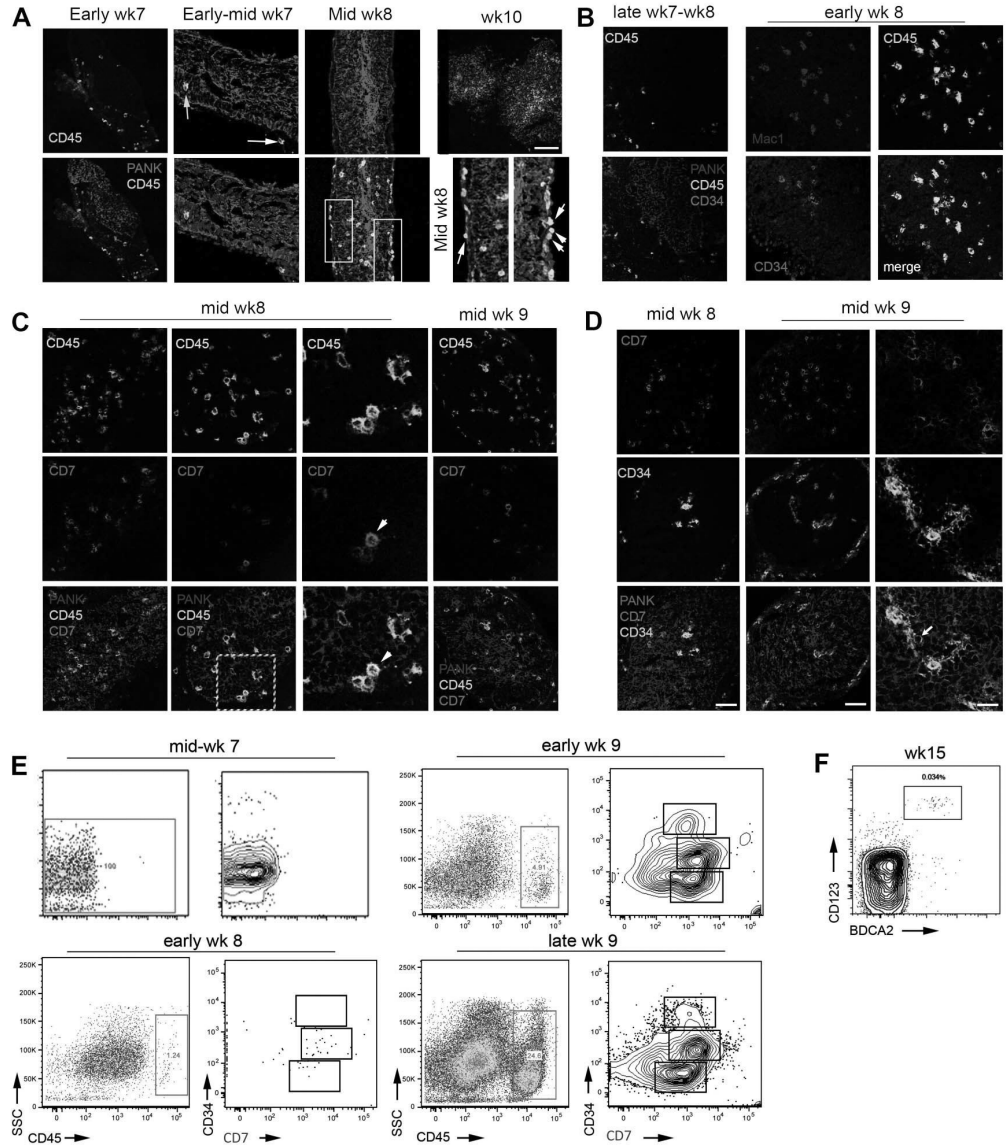

Figure 7: Colonization of the human thymic primordium by hematopoietic cells. (A-D) Images show staining of human thymic primordia with α-CD45, α-CD34 and α-CD7 as indicated, at the developmental stages shown. α-panCytokeratin (PANK) reveals the entire thymus primordium. Green arrow in A indicates CD45⁺ cell within the thymic epithelial compartment; white arrows indicate CD45⁺ cells present in the perithymic mesenchyme. The three bottom right hand images in (A) show detail from boxed regions on mid-week 8 image. Right hand panels in (C) show boxed area from middle panel. Scale bars: (A) early-mid wk7 and wk10 - 150μm, early-mid wk7 and mid wk8 75μm; Images are representative of at least two independent analyses. (E, F) Plots show flow cytometric analysis of dissociated human thymic primordia at the developmental stages shown. (E) Plots show staining with α-CD45 and α-CD34 and α-CD7 after gating on live cells (i.e. against DAPI). Right hand panel at each age shows cells after gating on CD45⁺ cells (left hand panel). Boxes on right hand plots at each age indicate CD34hi, int and lo/neg cells. (E) n = 1 for each age shown. (F) Plot shows staining with markers for (plasmacytoid (CD123⁺BDCA2⁺) dendritic cells after gating on CD45⁺CD19⁻CD3⁻ cells and is representative of at least two independent analyses. Wk, week.

DISCUSSION

The data presented above address the current profound gap in understanding of human thymus development. Collectively, they demonstrate that the principal genetic mechanisms currently known to regulate thymus organogenesis in the mouse are conserved in humans, and that key cellular processes including the developmental origin of the thymus in the 3rd pharyngeal pouches are also conserved. They establish that overt differentiation of cortical and medullary TEC occurs from mid-week 8 in the human thymic primordium and closely mirrors the progression seen in mouse in terms of phenotypic and functional marker expression; and that colonization of the human thymic primordium by endothelial and mesenchymal cells occurs from early and mid-week 8, respectively. Finally, they define the timing of onset of hematopoietic cell colonization of the human thymic primordium, and reveal, in contrast to expectations from previous studies, that the first colonizing hematopoietic cells are $CD45^{+}CD7^{+}CD34^{-}$.

Conservation of mechanisms of thymus organogenesis between mouse and human

The mouse is the principal model organism used to determine regulatory mechanisms operating to control thymus development and function, and therefore interrogation of its utility for predicting mechanisms that critically control these processes in the human is essential. The data presented above establish that the spatial and temporal expression pattern of all of the genes tested, that have been demonstrated by genetic analyses to have functional roles in thymus development, are conserved between mouse and human. They therefore validate the mouse as a model for understanding human thymus organogenesis. Of note is that while some variation in expression between mouse and human was observed in the genes analyzed herein, this did not affect expression in the thymus domain; for instance, PAX9 is expressed throughout the pharyngeal pouches in mouse but was detected only in pouches 3 and 4 in human. However it is of interest that two genes, *Rhox4* and *Plet1*, whose expression in mouse is restricted to the pharyngeal endoderm and the thymus domain of the 3rd pharyngeal pouch respectively at this developmental stage, are not expressed in the human pharyngeal pouches or thymic rudiment, indicating that not all gene expression patterns are conserved. In this regard, we note that neither of these genes has been shown to be important for thymus organogenesis by functional studies, and indeed, both are currently of unknown function.

Differentiation of the earliest progenitor cells in the human thymic primordium

The data presented above demonstrate that overt differentiation of cortical and medullary TEC occurs at around mid-week 8. However, the thymic rudiment itself is present from early week 6 and therefore, our data further indicate that the week 6 and week 7 thymic primordia consist mainly of undifferentiated TEC. Thus, cell surface markers expressed by most TEC at these developmental stages will be useful for isolating and characterizing the differentiative potential of early progenitor TEC. Similarly, the expression patterns demonstrated here for CD205 and

CLDN4 are consistent with these markers identifying early medullary and cortical sub-lineage restricted progenitors in the human, as well as mouse, thymus.

We note that FOXN1, a master regulator of TEC differentiation, is expressed in the human thymic primordia from mid-week 6. In the mouse, high-level Foxn1 expression is initiated at embryonic day 11.25 (E11.5) and the onset of overt TEC differentiation closely follows this up-regulation. The interval between up-regulation of FOXN1 and the appearance of differentiating cTEC and mTEC is therefore much longer in human than in mouse. This likely reflects differences in cell cycle, however it is also possible that other, as yet unknown mechanisms, limiting the time of onset of TEC differentiation are initiated or repressed with different kinetics to Foxn1 in the human.

Collectively, the data presented pave the way for prospective isolation and functional testing of defined human fetal TEC subpopulations, which will be essential if progress is to be made in defining conditions for *in vitro* propagation of human TEPC or for generating these cells from embryonic stem cells for use in cell replacement therapies.

Kinetics of initial mesenchymal and vascular endothelial cell entry into the human thymus

Our data indicate the time of onset of entry of vascular endothelial progenitor cells into the human thymus as early-mid week 8, with migrating cells observed in the connective tissue between the carotid artery and the thymus from early-mid week 7. They further show that, although formation of trabeculae is observed from mid-week 7, the time of entry of neural crest-derived mesenchymal cells into the epithelial regions of the thymus is mid-week 8. These processes are thus closely linked, as in the mouse. Notably, both events occur after initiation of high-level Foxn1 expression in the human thymus, consistent with the demonstration in mouse that thymic-colonization with both mesenchymal and vascular endothelial cells is Foxn1-dependent. However, our data further suggest that entry of vascular endothelial progenitors into the epithelial region of the thymic primordium precedes its colonization with mesenchymal cells, in contrast to the widespread assumption that immigration of vascular endothelial cells follows that of neural-crest-derived mesenchyme.

Identity of the first thymus-seeding hematopoietic cells

The data presented in Figure 7 establish that hematopoietic cell colonization of the human thymus primordium occurs from early-mid week 7. As in mouse, hematopoietic colonization of the thymus therefore closely follows up-regulation of high level Foxn1 expression - consistent with Foxn1 regulation of CCL25 and Dll4 which are required for migration and T lineage commitment of thymus seeding progenitors, respectively (Hozumi et al., 2008; Koch et al., 2008; Liu et al., 2006).

The earliest human thymus seeding T-lineage restricted progenitors have previously been proposed to be $CD45^{+}CD34^{hi}CD7^{+}$ cells, and indeed CD7 is regarded as indicative of commitment to the T cell lineage in humans (Galy et al., 1993; Haddad et al., 2006; Haynes and Heinly, 1995).

Our data indicate that the hematopoietic population of the early human fetal thymus is heterogeneous, and that all intrathymic hematopoietic cells are $CD34^{lo/-}$ until mid-week 9. Indeed, the first colonizing hematopoietic cells appearing at early week 7 are $CD45^{+}CD34^{-}CD7^{-}$, and some $CD45^{+}$ at least as early as early week 8 are $Mac1^{+}$ macrophages. We cannot rule out that the $CD45^{+}CD34^{-}CD7^{-}$ cells are LTi cells that are involved in the development of the medulla. From murine studies it is known that LTi cells are involved in upregulation of Aire in mTECs (Rossi et al., 2007). However, we find the first Aire positive mTECs at week 13, considering the time frame it makes it highly unlikely that the earliest hematopoietic immigrants are LTi cells. Surprisingly, $CD45^{+}CD34^{lo/-}CD7^{+}$ cells are present from early week 8. Although we cannot exclude that these cells mature within the thymic primordium to generate $CD45^{+}CD34^{hi}CD7^{+}$ cells, we note that $CD45^{+}CD34^{hi}CD7^{+}$ cells first appear within the thymus at mid-week 9 coincident with establishment of blood vessels, and furthermore that cells of this phenotype are initially found closely associated with blood vessels. Thus, we favor the idea that the thymus-seeding population changes once the vasculature has been established as the primary route of entry, and comprises $CD45^{+}CD34^{hi}CD7^{+}$ cells only after this point. The $CD45^{+}CD34^{lo/-}CD7^{+}$ population present in the pre-vascular thymus is therefore a novel intrathymic hematopoietic population, that is probably T-lineage committed based on its expression of CD7. In this regard, it is of interest that $CD7^{hi}$ cells were never detected in the connective tissue associated with the pre-vascular thymus or in the perithymic space, but were sometimes observed interspersed with thymic epithelial cells adjacent to the mesenchymal capsule suggesting that in the pre-vascular thymus, up-regulation of CD7 may occur upon contact with thymic epithelial cells. Based on these data, we therefore suggest that the pre-vascular thymus is initially seeded by $CD45^{+}CD34^{lo/-}CD7^{-}$ cells and that up-regulation of CD7 in these $CD45^{+}CD34^{lo/-}$ cells occurs dynamically as part of the thymus seeding process. We further suggest that this population is superseded by the previously described T-lineage restricted $CD45^{+}CD34^{hi}CD7^{+}$ population (Haddad et al., 2006) only following establishment the thymic vasculature.

MATERIALS AND METHODS

Human Tissue

First and second trimester human fetuses were obtained following elective medical terminal of pregnancy, and all were morphologically normal. Ethical approval for use of human fetal tissue in these studies was granted by the Lothian Research Ethics Committee or the Ethics Committee of the Erasmus University Medical Center. Consent was obtained from all women in writing, and the tissue was anonymized before being made available for research. All experiments using human tissue were performed at the University of Edinburgh or the Erasmus University Medical Center. Embryos were aged according to the standard head/rump measurement and Carnegie stage was determined (Gasser, 1975; O'Rahilly and Muller, 1987). Embryos used in this study were from mid to late week 6 (CS17), week 7 (CS18-19), early to mid-week 8 (CS20-21), week 15 to 16, and week 17 with days/weeks indicating days/weeks post fertilization. Note, Carnegie staging does not apply to second trimester fetuses.

In situ hybridization

Whole-mount *in situ* hybridizations were performed as described (Gordon et al., 2001). All probes used were generated by PCR amplification from human fetal tissue and were directed to the 5' UTR. NBT/BCIP (Roche) was used to localize the hybridized probe. The following primers were used to make probes; FOXN1F 5′GAAACCTGTGGGAACAGTTG3′, FOXN1 5′ACTTCCAGACCAGGCAAACG3′, HOXA3F 5′CTGGAATGAAAGAGTCGCCACC3′, HOXA3R 5′CCAGCGAATGCATAGAGTTC3′, PAX1F 5′CCAACGTGGTCAAGCACATC 3′, PAX1R5′CGAAGGCAGGTTTCTCTAGC3′, PAX9F 5′CCAGCAGGGTCAGGACGACT3′, PAX9R 5′ATGCTGGATGCTGACACAAA3′, BMP4F5′GAAGCTAGGTGAGTGTGGC3′, BMP4R 5′AGACCAGTGCTGTGGATCTG3′, BMP2F 5′GTTGTGTGTCAGCACTTGGC3′, BMP2R5′TGCGATACAGGTCTAGCATG3′, TBX1F5′TGCAGCTAGAGATGAAGGCG 3′, TBX1R 5′CAATCTTGAGCTGCGTGATA 3′, CLDN4 F 5′CCCCAGCGCTTGGAATCC3', CLDN4R5′TGGATGATGTTGTGGGCCG3′, P63F 5′AGGAAGGCGGATGAAGATAGC3′, P63R 5′AGGAATGGTTGTAGGAGTGAG3′.

Antibodies

The following antibodies were used for immunofluorescence as described (Gordon et al., 2004): α-cytokeratin (rabbit polyclonal anti-keratin, Dako); α-CLDN4 (3E2C1, Zymed); α-CD45 (BD Biosciences); α-Keratin 5 (AF138 rabbit IgG, Covance); α-Keratin 8 (CAM 5.2, Becton Dickinson); α-Keratin 14 (AF64 Covance); TE7 (DSHB); UEA1 (Biotinylated, Vector Labs); α-AIRE (D-17, Santa Cruz); α-CD205 (MG38, Serotec); CDR2 (from Kyewski, Heidelberg); α-HLA-DR/DP/DQ (clone TU39, BD Pharmingen); α-EPCAM (Ber-EP4, DakoCytomation);); α-CCL21 (α-6Ckine, R&D Systems); α-Green fluorescent protein Alexa488 (BD Biosciences). Unconjugated Abs were detected by using Goat α-mouse Alexa488, Goat α-rabbit Alexa647, Goat α-rat Alexa488, Chicken α-goat Alexa488, Chicken α-rabbit Alexa594, Donkey α-goat IgG Alexa488,

Streptavidin Alexa647 (all Invitrogen). Appropriate isotype-control antibodies (BD PharMingen) provided negative controls in all experiments. DAPI was used as a nuclear counterstain.

For flow cytometry the following antibodies were used: mouse α-CD19 DY590 (Exbio Praha), mouse α-BDCA2 APC (Miltenyi Biotech), mouse α-CD11c FITC (Immunotools), rat α-CD7 (Serotec, MCA344), mouse α-human CD45 (clone HI30), mouse α-human CD34 (clone 581) mouse α–CD3 Alexa700, mouse α-CD11b APC, mouse α-CD45 PerCP-Cy5, mouse α-CD27 PE-Texas Red, mouse α-HLA-DR APC-Cy7, mouse α-CD123 PE (BD Pharmingen).

Microscopy

Isolation of thymic tissue from embryos was carried out under an Olympus SZH dissecting microscope. Wholemount *in situ* images were captured using a ZEISS stemi SVII stereomicroscope and images of wholemount in situ paraffin sections were captured using an Olympus BX61 stereomicroscope. Fluorescent images were captured using a Leica AOBS confocal microscope or Leica DMRXA microscope under the appropriate excitation conditions. Images were processed using Adobe Photoshop CS2.

Flow cytometry

Thymic single cell suspensions were generated by mincing thymic fragments through a 100μm filter (BD). Flow cytometric analysis was performed on a LSRII Flow Cytometer (BD) and analyzed using FlowJo software (Tree Star Inc).

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CHAPTER 3

Thymic cysts originate from Foxn1 positive thymic medullary thymic epithelium

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ABSTRACT

Thymic epithelial cells (TECs), derived from polarized two-dimensional (2D) **oriented endo**-dermal cells, are distinguished from other epithelial cells by their unique three-dimensional (3D) phenotype. However, some polarized epithelial cells remain present in the normal thymus, forming thymic cysts at the cortico-medullary junction. Here, we analyse the dynamics, origin and phenotype of such thymic cysts. In time-course experiments, we show a reverse correlation between thymic cyst expansion and the presence of thymocytes, suggesting a default pathway for the development of TECs in absence of thymocytes. By transplanting isolated TEC populations into E15 fetal thymic lobes, we provide evidence that medullary thymic epithelial cells (mTECs), rather than cortical thymic epithelial cells (cTECs) contribute to the formation of thymic cysts. Finally, thymi of reporter mice reveal that the cysts originate from epithelia committed to a thymic fate, as indicated by the expression of Foxn1. The 2D-phenotype of cyst-lining TECs is not caused by a downregulation of Foxn1 expression, since a significant proportion of these cells in the embryonic and adult thymus continues to express Foxn1 at the protein level.

Keywords: thymic cysts , thymic epithelial cells, thymic crosstalk, Foxn1

INTRODUCTION

The formation of a functional thymic environment in the mouse is initiated around E10.5, when the thymic 'anlage' is induced by invagination of polarized 2D endodermal cells that line the third pharyngeal pouch (Gordon et al., 2004). At the same time, epithelial cells in this region start to express the thymus-specific transcription factor Foxn1 (Balciunaite et al., 2002; Gordon et al., 2001). Foxn1 is an important regulator in TEC development, since TECs of mice lacking this transcription factor are arrested at an immature stage of development, unable to attract T cell precursors (Nehls et al., 1996). Hematopoietic immigrants begin to colonize the thymic anlage from E11 onwards (Itoi et al., 2001). At E12, neural-crest derived mesenchymal cells surrounding the thymic primordium provide inductive signals that promote TEC proliferation and differentiation (Jenkinson et al., 2007). From E13 onwards the 3D architecture of the thymic epithelium is induced, and at the same time cTECs and mTECs can be discriminated by their phenotype (Bennett et al., 2002; Gill et al., 2002; Klug et al., 2002; Shakib et al., 2009; Van Vliet et al., 1984). Both subsets of differentiated TECs have recently been shown to arise from a single bi-potential TEC progenitor during fetal development (Bleul et al., 2006; Rossi et al., 2006).

In the adult thymic environment, the cortex and medulla cooperate as specialized TEC compartments, supporting defined steps in T cell development (Anderson et al., 2006; Gill et al., 2003). Within both thymic compartments, the typical non-polarized phenotype of TECs is thought to provide a meshwork architecture, facilitating thymocyte migration and lympho-stromal interaction (van Ewijk, 1988).

Lympho-stromal interaction in the thymus acts in a bi-directional manner as TECs promote the differentiation of thymocytes and, conversely, thymocytes are instrumental in the development of TECs. This interaction, known as "thymic cross-talk", is essential for the creation and maintenance of correctly organized thymic microenvironments (Boehm et al., 2003; Hikosaka et al., 2008; Irla et al., 2008; Klug et al., 2002; van Ewijk et al., 1994; van Ewijk et al., 1999). However, a minority of TECs fails to adopt a non-polarized phenotype and forms cysts, usually found at the cortico-medullary junction (Khosla and Ovalle, 1986). The frequency of thymic cysts dramatically increases in mice with an early block in T cell development, such as CD3ε Tg and Ikaros ^{null} mutant mice (Hollander et al., 1995; van Ewijk et al., 2000) or an early block in TEC development (Blackburn et al., 1996; Dooley et al., 2005a). Thymic cysts are also found under pathological conditions, and the idea has been put forward that they represent extra-thymic derived entities arising under either congenital, inflammatory or neoplastic conditions (Rieker et al., 2005). Cyst-lining epithelial cells are polarized and comprise cell types with a phenotype normally found in the respiratory and gastrointestinal tracts, like ciliated cells, absorptive cells and goblet cells (Dooley et al., 2005b; Gillard et al., 2007; Khosla and Ovalle, 1986; van Ewijk, 1988; van Ewijk et al., 2000). Interestingly, these TECs have recently been reported to express tissue specific antigens of the lung (Dooley et al., 2005a; Dooley et al., 2005b), and it has been suggested that these cells might play a role in T cell selection.

The origin of polarized TECs has not unequivocally been established. At the one hand, these cells might have differentiated from the thymic epithelial reticulum itself. At the other hand, polarized epithelial cells might have an extrathymic origin, following a Foxn1 independent differentiation program. The latter notion suggests the possibility that epithelial cells from adjacent endodermal derivates are included in the thymic environment during thymic development, or that non-thymic epithelial cells are incorporated in the thymic environment during the migration of the thymus from the pharyngeal region towards its final location under the sternum (Dooley et al., 2005a; Dooley et al., 2005b; Farr et al., 2002).

In the present paper we have analysed the dynamics of cyst formation and the phenotype and origin of cyst-lining TECs. Collectively, our data indicate that (i) cysts expand in the absence of crosstalk signalling, (ii) cyst-lining TECs follow a Foxn1 regulated developmental pathway, while downregulation of the Foxn1 protein is not instructive for cyst formation and (iii) cyst-lining TECs originate from the medullary thymic epithelium.

RESULTS

Thymic cysts expand in absence of thymocytes.

We have previously shown that incubation of fetal thymic lobes with deoxyguanosin (dGuo) for 6 days results in the formation of thymic cysts (Germeraad et al., 2003). To reveal the dynamics of cyst formation in fetal thymi (E15), we treated lobes with dGuo during a period of 5 days. Every 12 hours, lobes were removed from the culture and analysed by immunohistochemistry for the presence of thymocytes and for morphology and distribution of TECs. As a marker for thymocytes, a polyvalent antiserum directed to the lymphoid specific transcription factor IKAROS was applied. Cryosections incubated with anti-IKAROS showed that the frequency of thymocytes already started to decrease during the first 12 hours of dGuo treatment and that the large majority of thymocytes was removed from the thymic lobes after 60 hours (Fig. 1A). When serial cryosections were incubated with anti-pan-cytokeratin antibodies, small cysts were noted from 24 hours of treatment (Fig. 1B). From this time point, cysts expanded in size and frequency, indicating that a loss of crosstalk signalling promotes their development.

To determine whether expansion of thymic cysts was caused by proliferation of cyst-lining TECs, BrdU (5-bromodeoxyuridine) was added during the final 48 hours of dGuo treatment of the lobes in FTOC. Immunohistochemistry showed that several TECs lining the cysts had incorporated BrdU (Fig. 2; see also SI Fig. 7). BrdU labelled cells occurred in groups, separated from each other by non-dividing cells. Thus, these data suggest that thymic cyst expand in size by the proliferation of cyst lining TECs.

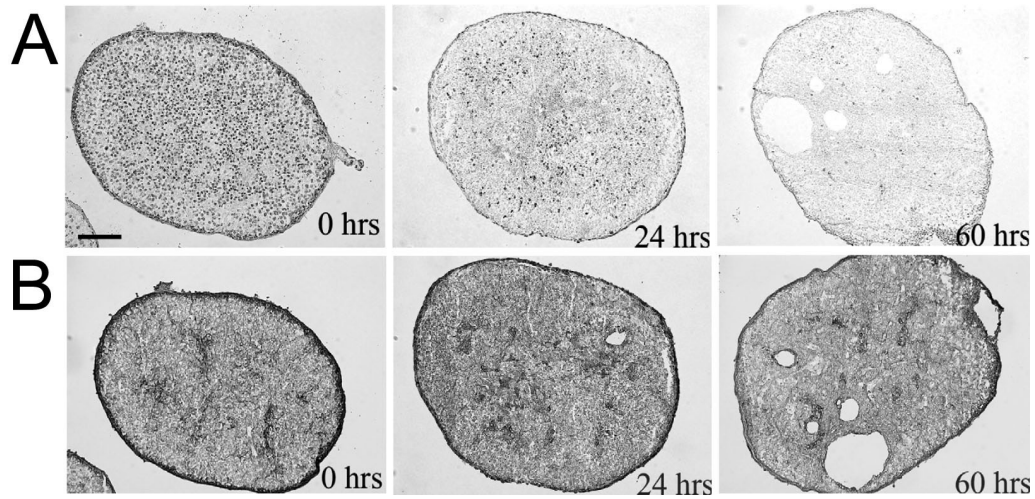

Figure 1.: Kinetics of thymic cyst formation in E15 fetal thymic lobes during dGuo treatment. Fetal thymic lobes were cultured in the presence of dGuo, and at 12-hour interval lobes were removed from the culture and snap-frozen in OCT compound. Serial cryosections of the time-course experiment were analyzed and stained with anti-IKAROS (*A*) and anti-pancytokeratin antiserum (*B*), respectively. Data are shown from the time points 0 hrs, 24 hrs, and 60 hrs. The scale bar indicates 200μm.

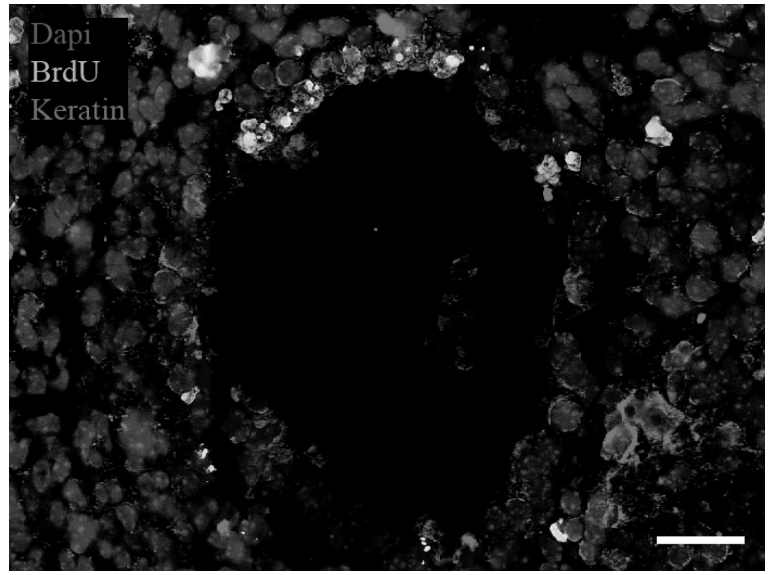

Figure 2: TECs proliferate during thymic cyst expansion. BrdU was added during the final 48 hours of dGuo treatment. Thymi were snap frozen in OCT compound, and stained with anti-BrdU and anti-keratin antibody. Groups of cyst-lining TECs incorporate BrdU, indicating that a subpopulation of cyst-lining TECs induces enlargement of thymic cysts. The scale bar indicates 20μm.

Epithelial cells in natural occuring thymic cysts are derived from Foxn1 positive TECs.

In Foxn1 reporter embryos it has recently been shown that cells committed to a thymic fate are able to contribute to cyst formation once Foxn1 expression becomes abrogated by the inhibition of BMP (Soza-Ried et al., 2008). The notion that thymic cysts are lined by ciliated epithelial cells also occurring in bronchioles (see SI Fig. 7) supports the non-engagement of Foxn1 during the development of these cells (Dooley et al., 2005b). However, thymic cysts also occur in the thymus of wild type mice, albeit at a low frequency (Dooley et al., 2005a; Germeraad et al., 2003; Khosla and Ovalle, 1986), suggesting that absence of Foxn1 in cyst-lining TECs is not instructive for the development of cysts. To confirm this notion, we decided to analyse Foxn1 expression in cyst-lining TECs. To identify TECs that previously transcribed, or still actively transcribe, the Foxn1 locus we took advantage of a transgenic mouse line, designated Foxn1-Cre: STOP^{flox}–GFP (Hauri-Hohl et al., 2008; Hozumi et al., 2008; Kawamoto et al., 2000; Rossi et al., 2007b). In these mice, Cre-mediated recombination and the irreversible expression of the green fluorescent protein (GFP) occurs in the thymus only in cells in which the Foxn1 locus is actively transcribed, which is the case for all TECs (SI Fig. 8). We then studied Foxn1 expression in situ in the thymus of 40 weeks old Foxn1-Cre: STOP^{flox} –GFP transgenic mice. Such thymi showed a few cysts at the cortico-medullary junction, and at higher magnification all cyst-lining TECs expressed GFP (Fig. 3A). This observation univocally demonstrates that naturally occurring cyst-lining TECs had, once during their development, committed to a thymic epithelial cell fate.

Foxn1 expression levels are not instructive for the formation of cysts.

Our findings so far did not reveal whether or not active Foxn1 transcription is involved in the induction of thymic cysts. It remains possible that down regulation of Foxn1 expression at the protein level induces the polarized TEC phenotype during an early phase of thymic development (Soza-Ried et al., 2008).

Since expression of GFP in TECs that once expressed Foxn1 during their differentiation is irreversible, it is technically impossible to discriminate between TECs that expressed Foxn1 in the past and TECs that still continue to do so at the time of analysis. We first investigated whether cyst-lining TECs continue Foxn1 protein expression in thymus sections of 40 weeks old Foxn1-Cre: STOP^{flox} –GFP transgenic mice, by incubation of frozen sections with a polyclonal antibody directed against Foxn1. As demonstrated in Fig. 3B, Foxn1 protein was absent in cells lining the cysts. To confirm this observation, another Foxn1 reporter mouse strain was introduced, namely Foxn1-EGFP (Terszowski et al., 2006), in which EGFP is directly under the control of Foxn1. By studying 23 wks old Foxn1-EGFP mice we noticed that Foxn1 protein expression still occurs in a majority of cyst-lining TECs (Fig. 3C). However, at 56 wks of age, Foxn1 protein expression was severely down-regulated (Fig. 3D). Importantly, decreased expression of Foxn1 seems not an exclusive phenomenon for cyst-lining TECs, since Fig. 3C and Fig. 3D also show absence of Foxn1 in 3D TECs surrounding thymic cysts. This finding confirms that permanent Foxn1 expression at the protein level is not required to maintain the typical 3D architecture of the TEC reticulum. At the same time, this experiment does not reveal a possible down-regulation of Foxn1

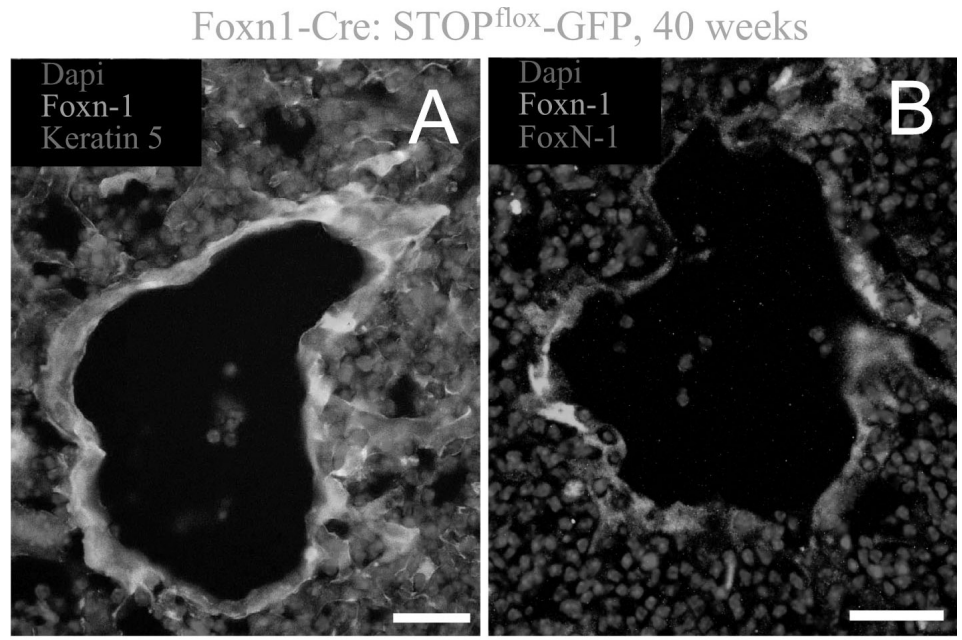

Figure 3: Foxn1 expression in natural occurring thymic cysts. Cyst-lining TECs in 40 weeks old Foxn1-Cre: STOP^{flox}–GFP express GFP (Fig. 3A), indicating that these epithelial cells have followed a Foxn1 regulated developmental pathway. Protein expression of Foxn1 was not detected in 40 weeks old cyst lining TECs (Fig. 3B). Fig. 3C shows that a majority (88%) of cyst-lining TECs (n = 79) in 23 wk old Foxn1-EGFP mice actively expresses Foxn1 protein, however, only 12 % of cyst-lining TECs (n = 58) expresses Foxn1 protein in 56 wks old Foxn1-EGFP mice (Fig. 3D). The scale bar represents 25μm (A, C and D) and 50μm for B.

expression during induction of thymic cysts. To address this issue more specifically, we studied Foxn1 expression during the induction of thymic cysts in fetal thymi. To this purpose, thymic lobes of Foxn1-Cre: STOP^{flox} –GFP E15 embryos were cultured for 7 days in the presence of dGuo. Fig. 4A and 4B show that after 7 days of culture the polarized cells lining cysts still expressed Foxn1 at the protein level. These results confirm that also during ontogeny, cyst-lining TECs are derived from the thymic epithelium, and argue against the idea that cysts are formed from epithelial cells downregulating Foxn1 during initial TEC specification. This notion was confirmed by studying dGuo treated fetal thymic lobes derived from Foxn1-EGFP mice (Fig. 4C), which also showed presence of Foxn1 protein in cyst-lining TECs.

Together, these data show that thymic cysts have derived from Foxn1 expressing epithelial cells and that maintenance of thymic cysts is not influenced by Foxn1 protein expression. Moreover, Foxn1 expression was also downregulated in the surrounding 3D stroma, indirectly demonstrating that Foxn1 levels are not instrumental for the maintenance of TECs within the adult thymus.

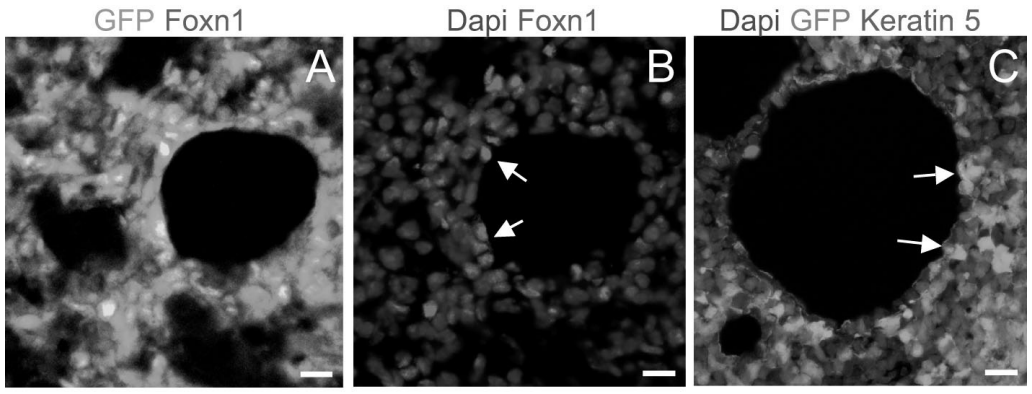

Figure 4: Clusters of cyst lining TECs in dGuo treated fetal thymic lobes synthesize Foxn1 protein. A polyclonal antibody directed against Foxn1 was applied to identify Foxn1 at the protein level. Fig. 4A shows that Foxn1 protein was variably expressed in 47% cyst-lining TECs (n = 214) in dGuo treated fetal thymic lobes derived from Foxn1-Cre: STOP^{flox} –GFP E15 (B; arrow heads indicate cells showing strong Foxn1 expression). Likewise, 81 % of cyst-lining TECs (n = 182) in dGuo treated thymic lobes from E15 Foxn1-EGFP embryos show groups of cells expressing Foxn1 protein (Fig. 4C; arrow heads show cells with strong Foxn1 expression). The scale bars indicate 25µm.

Cyst-lining TECs express K5.

To further characterize cyst-lining TECs in the thymus of normal mice, we phenotyped these TECs for markers expressed by thymic stromal cell types, such as cytokeratin (K8) for cTECs, K5 and ER-TR5 for mTECs, and ER-TR7 for thymic fibroblasts in mice of different ages. In 4 weeks old wild-type mice, small cysts were observed (data not shown), while in 8 months old mice larger thymic cysts were detected at the cortico-medullary junction, albeit still at low frequency. A majority (94 %) of cyst-lining TECs in the thymus of 8 months old mice exclusively expressed K5 (SI Fig. 9A), whereas a minority (6%) of cyst-lining TECs co-expressed K5 and K8 (SI Fig. 9B). Cells exclusively reactive with anti-K8 antibodies were not detected. Most cyst-lining TECs also expressed the medullary marker ER-TR5 (data not shown). Cyst-lining TECs lacked expression of functional molecules expressed on professional non-polarized TECs, such as MHC II (SI Fig. 9C), UAE-1 and Aire (SI Fig. 9D). Cyst-lining TECs did not react with ER-TR7, a marker for fibroblasts.

Taken together, these data indicate that cyst-lining TECs correspond to the thymic medullary epithelium rather than the cortical epithelium. Interestingly, a minority of cyst-lining TECs also co-expresses K5 and K8 (SI Fig. 9B, red arrows), suggesting the presence of immature TECs in thymic cysts.

We also examined the phenotype of cyst-lining TECs in the fetal thymus. Fig. 5A shows that a few small cysts lined by K5 expressing cells are indeed present in the normal fetal thymus, already from E14 (SI Fig. S11). Similarly, in dGuo treated fetal thymi cysts were lined by K5+ TECs (Fig 5B). Many cysts varying in size occur in dGuo treated thymic lobes (Fig. 5B and SI Fig. 10). We noted that a majority (86%) of cyst-lining TECs in frozen sections expressed K5, while 14% of cyst-lining TECs co-expressed K5 and K8. Cells exclusively expressing K8 were not detected. Similar to thymic cysts in aged mice, cyst-lining TECs induced in dGuo treated fetal thymic lobes lacked expression of functional markers (SI Fig. 10).

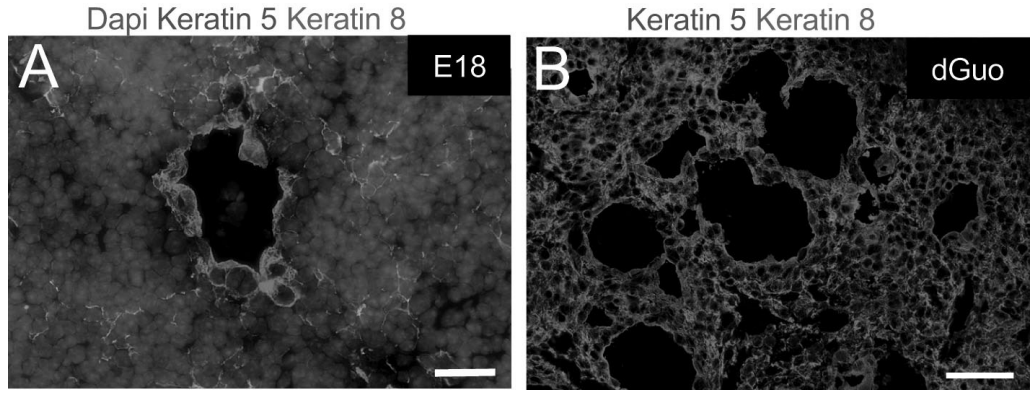

Figure 5: Thymic cysts in the fetus are lined by mTECs. K5 and K8 expression was analyzed in E 18 fetal thymic lobes of normal mice (A; see also SI Fig. 11) and in dGuo treated FTOC (B; see also SI Fig. 10). At all gestational ages, including dGuo treated fetal thymic lobes, cysts are lined by K5 expressing TECs. The scale bar indicates 25µm in A and 50µm in B.

mTECs but not cTECs contribute to thymic cyst formation.

To provide evidence that mTECs contribute to the generation of thymic cysts, we isolated different fractions of TECs from E16 GFP-Tg embryos, and analysed their ability to differentiate into cyst-lining TECs during FTOC (see SI Fig. 12 for schematic procedure). TECs were first isolated by flow-cytometry, using the pan-epithelial marker EpCAM (Fig. 6A). Next, EpCAM+ TECs were mixed with unfractionated E15 thymocytes and grown as re-aggregates. After 4 days of culture, the re-aggregates were placed into E15 'PACMAN' lobes and cultured for 2 days. Next, these PACMAN lobes were transferred to dGuo containing tissue culture medium. After six days cryosections were stained with an anti-K5 antibody. Microscopical analysis revealed that EpCAM+ GFP+ GFP/K5 positive cells were traced back as K5+ cyst-lining cells (Fig. 6C), indicating that individually sorted TECs indeed contribute to cyst formation in this in vitro experimental system.

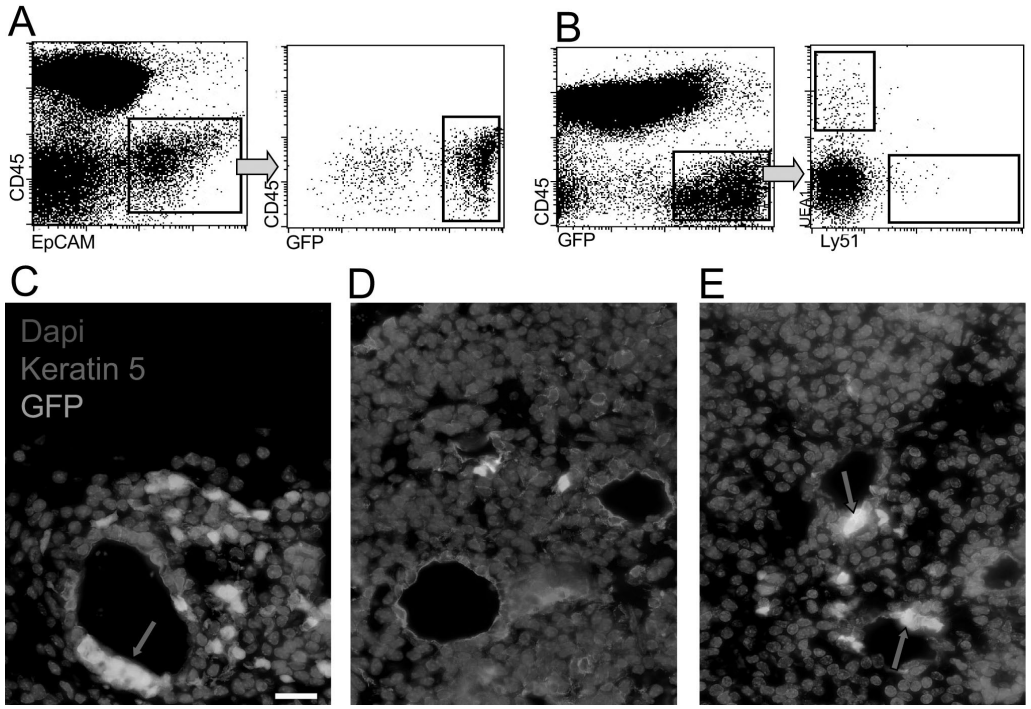

Figure 6: Cyst-lining cells are derived from mTECs. Flow-cytometry profiles of sorted EpCAM+, GFP+ TEC fraction (A), Ly51+, GFP+ (cTECs) and UEA1+, GFP+ (mTECs) (B) were seeded into E15 'PACMAN' lobes and analysed for their commitment in thymic cyst formation upon dGuo treatment. Cryosections were analysed for distribution of GFP+ seeded cells and stained with antibodies to K5. EpCAM+ sorted cells could be traced back as cyst-lining TECs (Fig. 6C; arrows). In a total of 10 frozen sections harbouring thymic cysts, 120 GFP+ cells were analysed for their location in the re-aggregated lobes. From these cells, 32 EpCAM+ were found back as cyst-lining TECs, the remaining cells were situated in the stroma surrounding the cysts. Ly51+GFP+ sorted cells (Fig 6 D) were found back as cyst-lining TECs only at low frequency (17 out of 111 cells in 15 sections). In contrast, 37 out of 74 GFP+, UEA1+ sorted cells (located in 8 sections) were traced back as cyst-lining TECs (Fig. 6 E; arrows). The scale bar indicates 20μm.

Since the EpCAM+ sorted TEC fraction contains both cTECs and mTECs, we decided to isolate both epithelial populations in order to analyse their individual contribution to cyst formation. To this purpose, Ly51+ (specifically expressed by cTECs harbouring both K8+ cTECs, and K5+K8+ TECs) cells and UEA1+ (expressed by mTECs) cells were isolated by flowcytometry (Fig. 6B) and cultured using PACMAN lobes, as described above. In three individual experiments we found that Ly51+ cells can be traced back as GFP+ TECs in the thymic environment, but these cells are largely absent from thymic cysts (Fig. 6D). In contrast, sorted UEA1+GFP+ cells were detected both in the thymic environment, and in thymic cysts (Fig. 6E). This observation supports the notion that mTECs rather than cTECs contribute to cyst formation in FTOC.

DISCUSSION

It is generally accepted that TECs influence the generation of a functional T cell repertoire (Anderson et al., 2006; Gill et al., 2003). While knowledge about T cell development has rapidly expanded over the last decades (Rothenberg and Taghon, 2005; Schmitt and Zuniga-Pflucker, 2006), the differentiation and dynamics of different TEC populations is still incompletely understood. Moreover, the origin and function of specialized epithelial microenvironments within the thymus, like thymic cysts, Hassal's Bodies and Thymic Nurse Cells has remained obscure.

Here, we have analysed the dynamics and the origin of thymic cysts using three different experimental approaches. First, our immunohistochemical analysis revealed that upon depletion of thymocytes from the thymic environment cysts expand in size, indicating that this phenotypical conversion is dependent on crosstalk signals derived from developing thymocytes (Germeraad et al., 2003; van Ewijk et al., 2000). In addition, we found that cyst expansion was caused by proliferation, since a proportion of cyst-lining TECs incorporated BrdU.

Second, by analysing the thymi of Foxn1-Cre: $STOP^{flox}$ –GFP transgenic mice, we observed that cyst-lining TECs once expressed the transcription factor Foxn1 in a physiological situation. Because Foxn1 expression is limited to all TECs, and to some epithelial cells in the skin (Lee et al., 1999), the present finding strongly suggests that cyst-lining TECs originate from the thymic environment itself. The 2D phenotype of these cells seems not to be caused by a downregulation in Foxn1 expression, since a large proportion of cyst-lining TECs in the fetal thymus continues to synthesize Foxn1 protein. Moreover, active Foxn1 expression was detected in cyst lining cells of dGuo treated fetal thymi and 23 wk old adult thymi, again indicating that Foxn1 protein expression in cyst lining TECs does not regulate cyst formation in the thymus. In contrast to our findings, a recent publication studying Foxn1:Xnoggin embryos reports that fetal Foxn1-expressing TECs become polarized upon downregulation of Foxn1 expression (Soza-Ried et al., 2008). However, a pitfall of this transgenic mouse line could be a short moment of Foxn1 expression that becomes directly abrogated during fetal development. To complete TEC specification prolonged Foxn1 signals might be required. Hence, the possibility remains that Foxn1 expression levels were sufficient for Cre-lox recombination (and therefore GFP expression) but not for final TEC maturation, explaining the presence of GFP expressing polarized epithelial cells in thymic cysts. Our observations at the one hand, confirm the notion that cyst lining cells once expressed Foxn1 during development. At the other hand, we could not find arguments that polarization of TECs per se is a consequence of downregulation of Foxn1 protein expression since in contrast to Soza-Ried's data we show here active Foxn1 expression within cyst lining cells in fetal and young adult mice. However, we did also confirm that, at advanced age, all cyst lining TECs lose GFP expression in Foxn1:EGFP reporter mice (Chen et al., 2009; Ortman et al., 2002). Importantly, downregulation of Foxn1 was also observed in professional non-polarized TECs, including $MHCII^{int}/UEA1^{int}$ mTECs (data not shown), indicating that the role of Foxn-1 in the maintenance ot the thymic epithelial stroma at prolonged age is still unclear.

Third, our data show that the phenotype of most cyst-lining TECs in the fetus, as well as in adult mice, corresponds to that of mTECs most clearly defined by the expression of K5 and ER-TR5. The medullary origin of cyst-lining TECs was confirmed by our cell separation-transfer experiments, where UEA1+ mTECs rather than Ly51+ flowsorted cTECs contributed to cyst development.

Next to K5+ mTECs, we and others (Dooley et al., 2005a) also noticed a minority of K5+K8+ TECs in thymic cysts. TECs with this phenotype are enriched in the fetal thymus (Klug et al., 2002), indicating an immature state for this subpopulation. Others have shown that cyst lining TECs express MTS20 and MTS24 (Blackburn et al., 1996; Dooley et al., 2005a), another marker for immature TECs (Depreter et al., 2008) with progenitor capacity in the fetal thymus (Bennett et al., 2002; Gill et al., 2002; Rossi et al., 2007a). The general absence of UEA1 and MHCII in cyst lining cells of dGuo treated FTOCs and the adult thymus indirectly confirms an immature stage in development for cyst lining cells.

Together with our present observation that a subset of cyst-lining TECs has the capacity to proliferate, these data argue that thymic cysts may harbour TECs with progenitor activity. Progenitor capacity has also been suggested previously by (Bleul et al., 2006) revealing immature TECs residing in the thymic rudiment of Foxn1 deficient mice. Once Foxn1 is re-supplied, these cells are able to contribute to the outgrowth of a functional thymus. Of great interest is, of course, to determine the developmental potential of cyst-lining TECs within the wild type mice. However, a major problem in here is the lack of markers specifically expressed at the cell surface of cyst-lining TECs, hampering their isolation by cell separation techniques.

How do polarized TECs arise within the thymic environment? We would like to suggest two different models explaining the development of TECs within the thymic medulla. A first model postulates that, in absence of crosstalk signals, polarized TECs arise by de-differentiation of non-polarized 3D mTECs. Thus, under these conditions, TECs withdraw their long cytoplasmic processes, polarize and reposition onto a basal lamina, suggesting that in absence of thymic crosstalk, TECs follow a “default” (i.e. crosstalk independent) differentiation pathway, as occurring in other epithelia. However, a problem with this model is the observed presence of cysts both in the fetal thymus, and in the thymus of aged mice ((Dooley et al., 2005a); our present study). In both types of mice, crosstalk signalling most likely takes place, yet thymic cysts are present.

A second model may explain this discrepancy in a different way. In this model we suggest the presence of two different developmental stages of TECs in the thymic medulla, as defined by their state of polarization (i.e. 3D type and 2D type) and markers (MHCII, UEA1 and AIRE). The first more advanced mTEC type is for its development dependent on thymic crosstalk signals derived from developing thymocytes (Hikosaka et al., 2008; Irla et al., 2008). In the presence of crosstalk signals, such progenitor TECs differentiate into adult non-polarized TECs, associating as professional TECs into a network configuration. The other mTEC type is more primitive in its developmental stage, is less dependent on crosstalk signalling and orients itself as polarized cells in thymic cysts. Both subsets may co-exist in the fetal thymus, explaining the presence of

small cysts already early in ontogeny. Under the influence of increased crosstalk signalling, as a consequence of increasing T cell production during thymic development, the first subset would expand at cost of the second subset. In contrast, under conditions of reduced crosstalk signalling, the second TEC population would find its chance to expand, also explaining why in the aged thymus, with a reduced level of T cell production (Gray et al., 2006; Ortman et al., 2002; Taub and Longo, 2005), thymic cysts are readily detected. Future research enabling cell tracing of cyst-lining cells and their precursor TECs is required to provide further insight into this issue.

In summary, our present work has revealed that cysts are an intrinsic part of the thymic environment of normal mice at all ages. The in vitro data indicate that cysts expand due to lack of crosstalk signals, once thymocytes have disappeared from the thymic environment. Moreover, both our in-vivo and in-vitro data support the notion that thymic cysts originate from mTECs that have developed through a Foxn1 dependent differentiation pathway, but that Foxn1 expression per se does not regulate the initiation and maintenance of this epithelial phenotype.

MATERIALS AND METHODS

Mice

C57BL/6 (B6) mice were purchased from CLEA Japan Inc. (Tokyo, Japan). Foxn1-Cre transgenic (Hauri-Hohl et al., 2008; Hozumi et al., 2008), STOP ^{flox} –GFP transgenic mice (Kawamoto et al., 2000), Foxn1-GFP transgenic mice (Terszowski et al., 2006) and actin-GFP transgenic (Okabe et al., 1997) mice were maintained in our animal facility. Embryos at various stages of gestation were obtained from time-mated pregnant mice.

Antibodies

For immunohistochemistry, the following antibodies were used: rabbit anti-pancytokeratin (DAKO, Glostrup, Denmark), rabbit anti-IKAROS, rat anti-ER-TR7 (Van Vliet et al., 1984), biotinylated anti-keratin8 (Progen, Heidelberg, Germany), rabbit anti-keratin5 (Covance, Denver, PA, USA), mouse anti-BrdU, biotinylated I-A/I-E (2G9) (both BD Pharmingen, San Diego, CA, USA), biotinylated UEA1 (Vector Laboratories, Burlingame, CA, USA), goat anti-AIRE-1 (D-17) (Santa Cruz Biotechnology, CA, USA) polyclonal anti-Foxn1 (Itoi et al., 2007) as primary antibody, followed by Alexa Fluor488 goat anti-rat IgG, Alexa Fluor488 goat anti-mouse IgG, Alexa Fluor488 goat anti-rabbit IgG, Alexa Fluor488 chicken anti-goat, Alexa Fluor594 streptavidine, Alexa Fluor594 donkey anti-rabbit IgG (all obtained from Molecular Probes, Eugene, OR, USA), goat anti-rabbit HRP IgG, rabbit anti-rat HRP IgG (both from Vector Laboratories) as secondary reagents. Nuclei were counterstained with DAPI (Molecular Probes).

For flow-cytometry, the following reagents were applied: biotinylated UEA-1 lectin and fluorescein isothiocyanate (FITC) UEA-1 lectin (both obtained from Vector), FITC-EpCAM (G8.8), phycoerythrin (PE)-EpCAM, PE-cychrome (cy)7-rat anti-CD45, biotinylated Ly51 mAb (clone 6C3/BP1, Pharmingen), rat anti-Ly51 (clone 6C3/BP1) (Rouse et al., 1988). As detecting reagents, PE-goat-anti rat IgG and Allophycocyanin (APC)-streptavidine (both Pharmingen) were applied.

Immunohistochemistry

Immunohistochemistry was performed as described before (Germeraad et al., 2003). To prevent the loss of GFP signal in Foxn1/GFP tissue, lobes were fixed for 5 minutes (fetal lobes) or 15 minutes (adult lobes) in 4% paraformaldehyde before sectioning. When BrdU staining was performed, slides were fixed in cold 70% acetone for 10 minutes, incubated for 30 minutes in 2N HCl and thereafter dipped in $Na_2B_4O_7$.

For cell counting of cyst-lining TECs the nuclei were electronically marked in JPEG files based on DAPI expression. From each marked cell the reactivity with various antibodies with subsets of TECs was assessed.

Fetal Thymic Organ Culture

To induce thymic cysts FT lobes from 15 dpc embryos were cultured on a polycarbonate membrane (pore size 8 μm) (Nucleopore Co., Pleasanton, CA, USA) floating on culture medium containing 1.35mM dGuo for a period of 6 days in a 6-well plate (Corning Inc., NY, USA).

Thymic stromal cell preparation

Thymic lobes were dissected from 15 dpc embryos and trimmed free from fat and connective tissue. Small fragments of the lobes were made and incubated for 30 minutes on a rotator at 4°C in RPMI-1640 supplemented with 10% FCS. Next, supernatant was taken out in order to remove most thymocytes. This procedure was repeated twice. Subsequently, thymic fragments were incubated in RPMI-1640 supplemented with 10%FCS, collagenase (Sigma, St Louis, MO, USA) (1mg/ml) and DNase (Sigma) (10mg/ml) 3 times, for 15 minutes at 37°C. At the first two steps the supernatant was removed, during the final step single cell suspensions were obtained by flushing through a 21G needle followed by a 23G needle. Cells were washed twice and finally passed trough a 40μm nylon mesh.

Flow Cytometry

Thymic stromal cells were stained as described above and analysed on a FACSCalibur using Cell Quest software (BD). Cell sorting was performed with a FACSVantage using Cell Quest software (BD).

Re-aggregate culture and PACMAN culture

10^4 sorted cells were recombined with $3.5x10^5$ wild type cells (single cell suspension obtained from dpc 15 fetal thymic lobes), washed in 100% FCS and centrifuged. The supernatant was removed and the pellet was placed on a polycarbonate membrane (pore size 0.8 μm; Nucleopore Co.) in RPMI-1640 supplemented with 10% FCS. After 4 days of incubation re-aggregate cultures were co-cultured with thymic lobes from 15dpc embryos to which an incision was made (so called 'PACMAN' lobes). These PACMAN lobes were cultured for two days on a floating membrane (pore size 8 μm) and further cultured as FTOC.

Conflict of interest

The authors have no competing financial interest.

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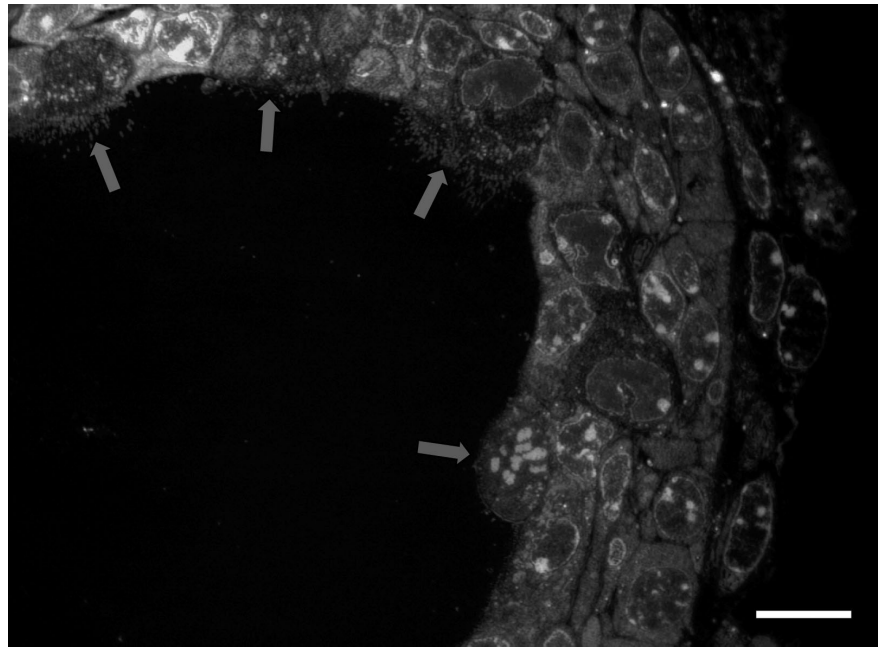
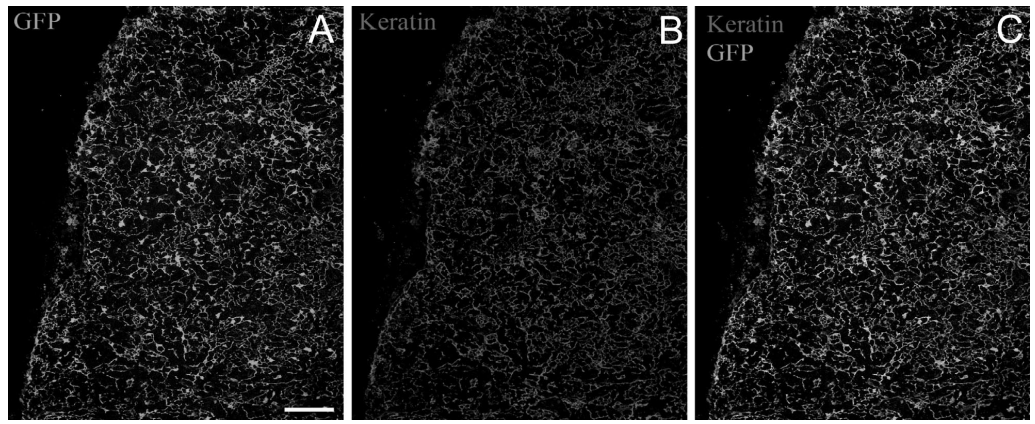
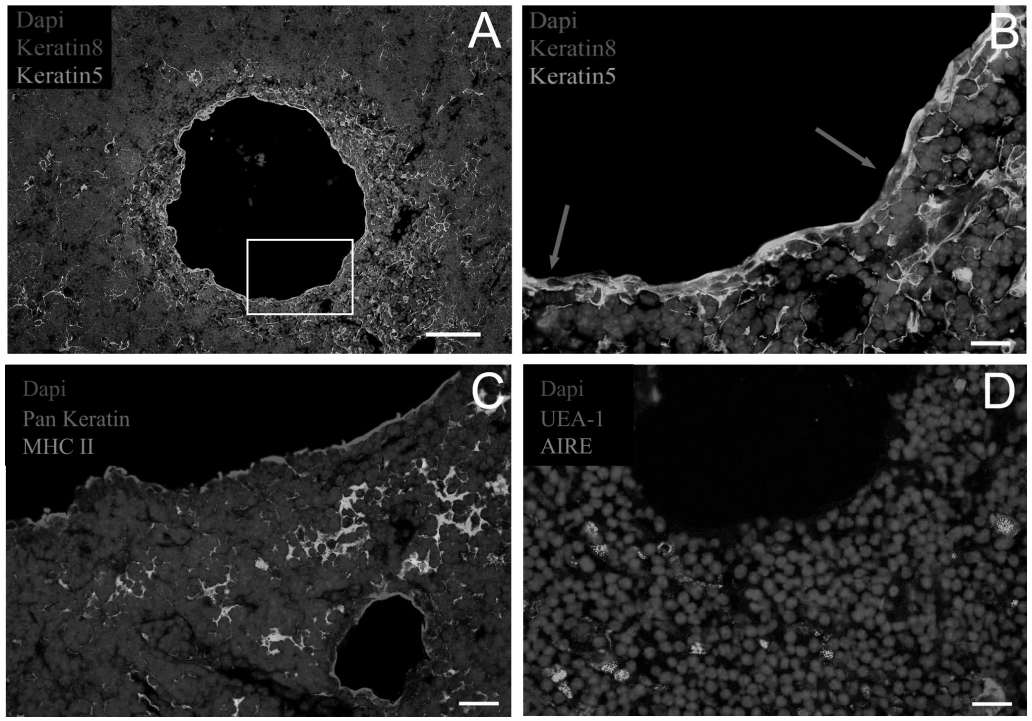
SUPPLEMENTARY FIGURES



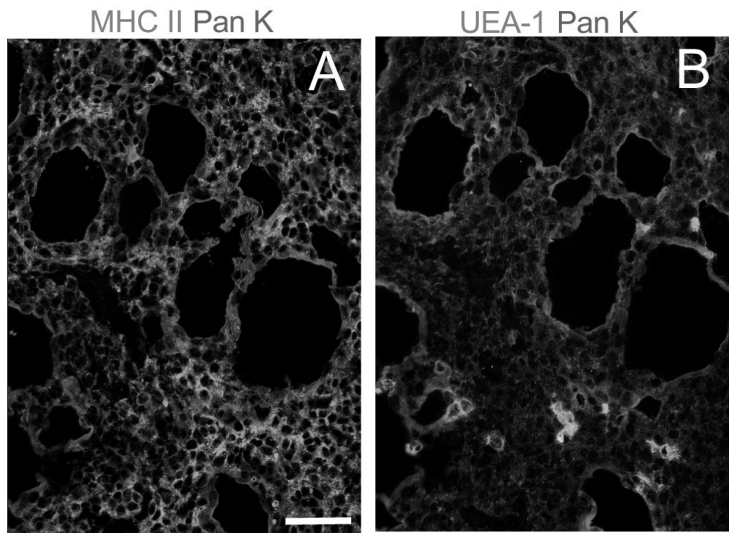
SI Figure 7: Reflection contrast microscopy of a thymic cyst in an E15 dGuo treated fetal thymic lobe. Note ciliated cyst lining epithelial cells (red arrows), and a proliferating cyst lining epithelial cell, showing chromosomes (blue arrow). Thymic lobes were prepared for high resolution light and transmission electron microscopy, using standard procedures. The scale bar indicates 10μm.



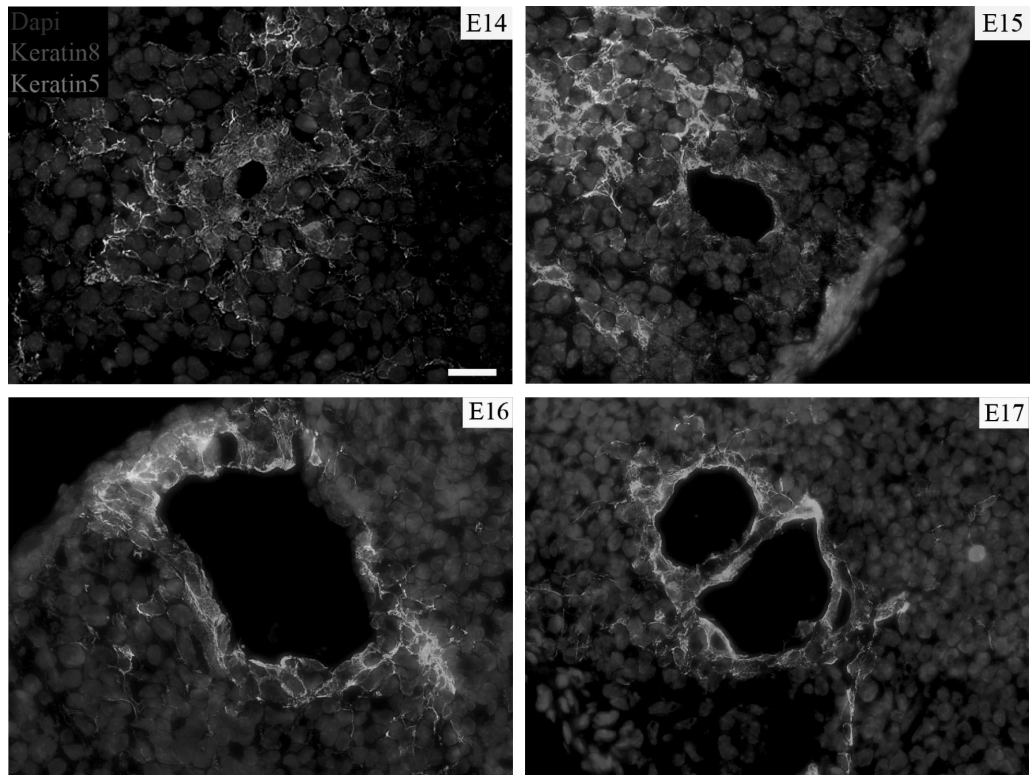
SI Figure 8: Cryosections of thymic lobes derived from 10-months old Foxn1-Cre: $STOP^{flox}$ –GFP mice were analyzed by immunohistochemistry. Comparison of Figs. 8 A, B and C show that the Cre/Lox system worked efficiently, since all pan cytokeratin expressing cells also express GFP. However, Foxn1 expression levels vary between individual TECs, as can also be observed in the thymus of Foxn1-EGFP mice (see Fig. 3C,D). The scale marker indicates 100μm.



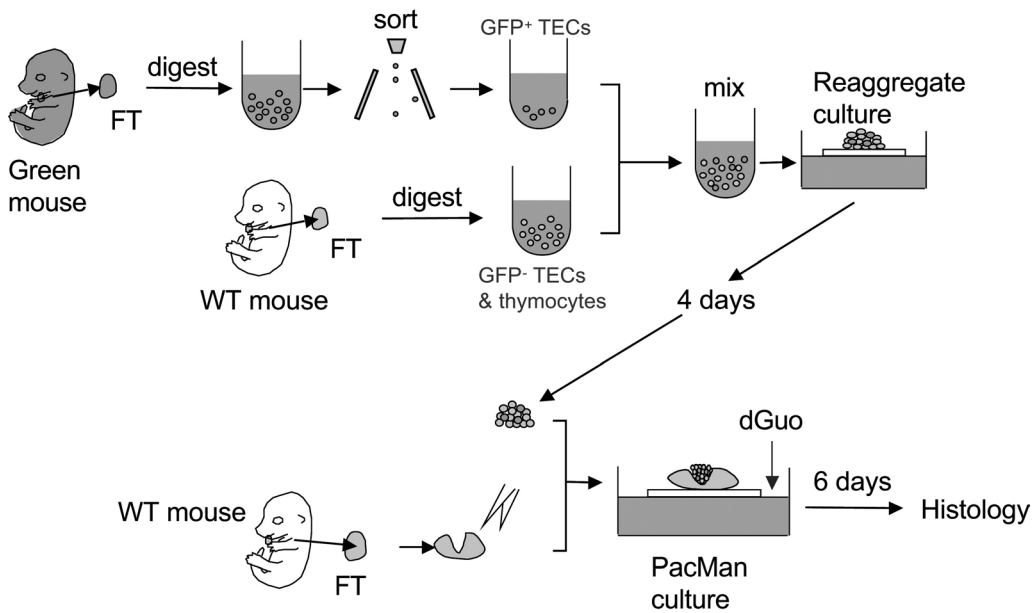
SI Figure 9: Thymic cysts in the aged thymus relate to medullary epithelium. A large majority of cyst lining TECs expresses the mTEC marker K5 (A). At higher magnification (B, see also boxed area in A), a few cells co-expressing K5 and K8 are present (B, red arrows). Cyst lining TECs do neither express MHC II (C), AIRE, nor UEA-1 (D) Scale markers indicate 100μm in (A) and 25μm in (B, C and D).



SI Figure 10: Thymic cysts in d-Guo treated lobes are lined by cells that generally lack mature markers such as MHC II (*A*) and UEA-1 (B). The scale marker represents 50 μm.



SI Figure 11: Thymic cysts in the normal developing fetal thymus are detectable from E14. The scale marker indicates 20 μm.



SI Figure 12: Experimental procedure to analyze the contributionof TEC subpopulations in the formation of thymic cysts.

CHARACTERIZATION OF LGR5 POSITIVE EPITHELIAL CELLS IN THE MURINE THYMUS

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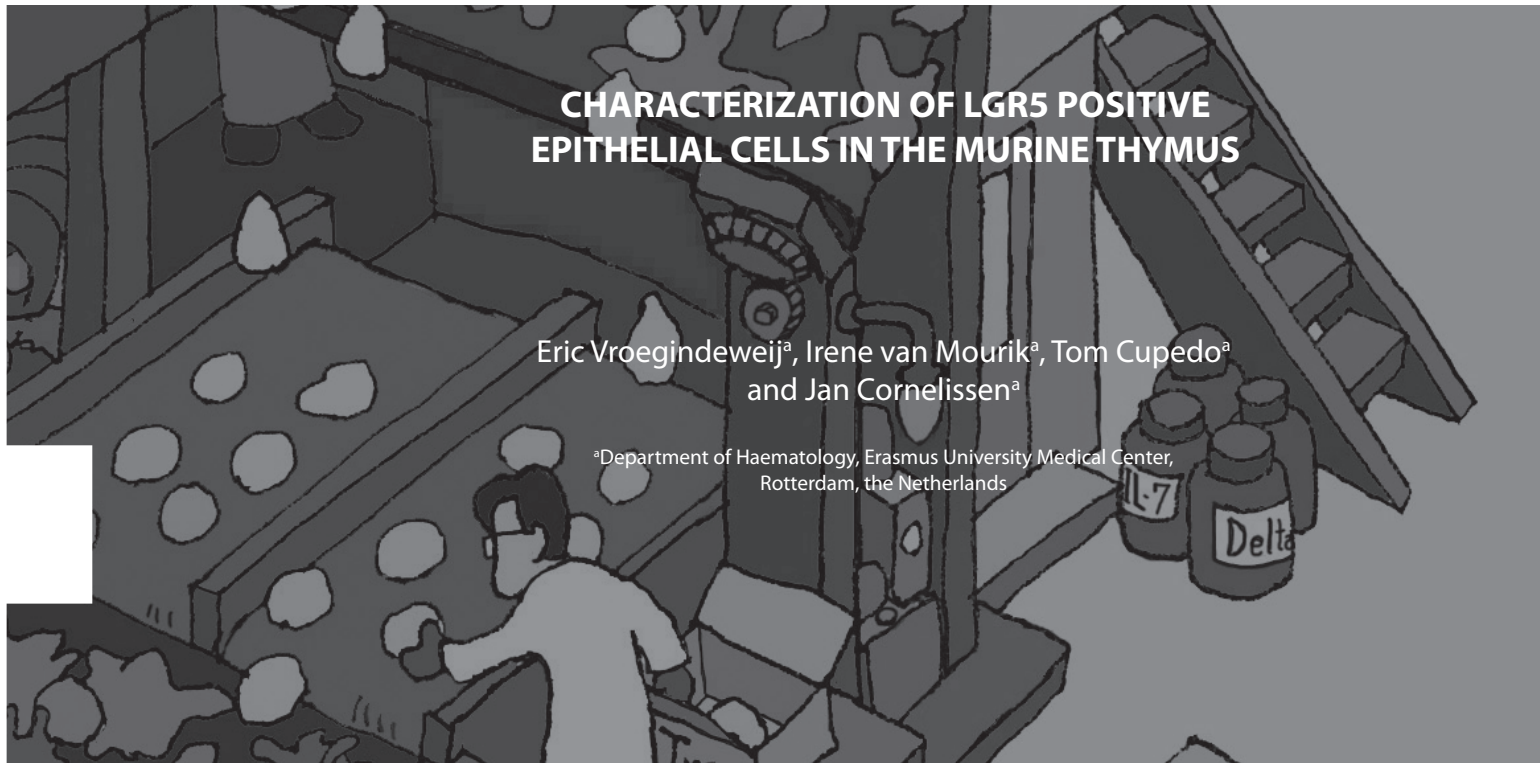

Submitted for publication

ABSTRACT

Leucine-rich repeat-containing G protein-coupled receptor (Lgr)5 is a marker for epithelial stem cells in the adult intestine of mice. Lgr5 transcripts have also been detected in the developing murine thymus, leading to speculations on Lgr5 as a marker for the long sought stem cell of the thymus. Identification of a thymus-specific stem cell would be a major step towards designing regenerative therapy for patients with impaired thymic function. To address the nature of the Lgr5 expressing thymic epithelial cells (TECs) we used Lgr5-GFP reporter mice. We show that epithelial cells expressing Lgr5 protein are present in the fetal thymus during a specific developmental window yet are no longer detectable at birth. To analyze the function of the Lgr5 protein during thymus development we generated Lgr5$^{-/-}$ mice. Since Lgr5 deficiency leads to postnatal mortality we also allowed fetal thymi from Lgr5$^{-/-}$ mice to mature under the kidney capsule of wild type mice. These experiments unequivocally defined that thymus development is not perturbed in the absence of Lgr5, that all TEC subsets develop and that T cells are produced in the expected ratios. Finally, to determine the differentiation potential of fetal Lgr5+ TECs we used an inducible lineage tracing system to track the progeny of Lgr5+ fetal TECs in-vivo. However, lineage tracing experiments revealed that Lgr5+ fetal TECs have no detectable progeny in the later fetal thymus. This indicates that the Lgr5 positive TEC population in the murine fetal thymus does not contain cells with stem cell properties.

Keywords: thymic epithelial cells, Lgr5, lineage tracing

INTRODUCTION

Thymic epithelial cells (TECs) form a 3 dimensional network that is essential for the proper proliferation, differentiation, and selection of developing thymocytes. Epithelial derived factors include growth factors, differentiation signals and self antigens expressed via MHCI and MHCII (reviewed in (Klein et al., 2009). Presentation of self-antigens on TECs (Anderson et al., 2005; Anderson et al., 2002; Murata et al., 2007; Nedjic et al., 2008), the exact timing, affinity and co-factors for the physiological contact between TECs and developing T cells (reviewed in (Gascoigne and Palmer, 2011) is fundamental for proper T cell development.

The murine thymus originates from the third pharyngeal pouch at E9.5 of embryonic development and is solely derived from the endoderm (Gordon et al., 2004). Specification of the thymus involves the sequential upregulation of important transcription factors (Hoxa3, Pax-9, Pax-1, Eya1, Rae2, chordin and BMP; reviewed in (Hollander et al., 2006) eventually leading to the expression of the thymic specific transcription factor Foxn1 (Balciunaite et al., 2002; Nehls et al., 1994). From E11.5 onwards, the first precursor T cells migrate into the thymic anlage and non-canonical NF-κB signaling becomes important for full differentiation of the medullary microenvironment, culminating in the upregulation of autoimmune regulator (Aire) (Hikosaka et al., 2008; Irla et al., 2008; Rossi et al., 2007b) which enables mTECs to express self-antigens (Anderson et al., 2005; Anderson et al., 2002). In the adult thymus crosstalk remains important, as the process of differentiation but also maintenance of medullary TECs, via ligation of RANK and CD40 by ligands expressed on thymocytes (Akiyama et al., 2008; Hikosaka et al., 2008; Irla et al., 2008).

Mature cortical and medullary TEC originate from a common thymic epithelial progenitor cell (TEPC) (Bleul et al., 2006; Rossi et al., 2006). Although full differentiation of mature TECs from a clonal precursor population has been demonstrated, the precise phenotypical characterization of that precursor as well as its genotype are still lacking, making it difficult to identify this TEC in the adult thymus. Despite this, expression of Plet-1 does identify a subset of TEPCs with the ability to generate differentiated progeny. Especially, fetal Plet-1+ TECs are able to give rise to a functional thymus when transplanted under the kidney capsule (Bennett et al., 2002; Depreter et al., 2008; Gill et al., 2002). However, although present on TECs in the adult thymus, Plet-1+ cells seem to lose their precursor potential after E15 of embryonic development (Rossi et al., 2007a). So far, no exclusive marker for TEPCs has been identified in the adult thymus Still, the regenerative capacity of the involuted thymi has been revealed in different murine models (reviewed in (Lynch et al., 2009), suggesting the presence of an adult TEPC population.

Leucine-rich repeat-containing G protein-coupled receptor (Lgr)5 is a marker for stem cells in the adult intestine of mice (Barker et al., 2007). Single Lgr5+ cells from adult murine intestine were able to expand and form a new crypt/villus structure in-vitro (Sato et al., 2011; Sato et al., 2009). Although Lgr5+ cells in the crypt are a transient state of the BMI+ stem cells, they still give rise to epithelial cell subsets of the intestine (Sangiorgi and Capecchi, 2008; Tian et al., 2011). Lgr5 together with Lgr4 responds to the Wnt agonist R-spondin, together these receptors

fine-tune Wnt signaling (Carmon et al., 2011; de Lau et al., 2011). Mice with a targeted deletion of Lgr5 die immediately after birth due to fusion of the tongue with the floor of the oral cavity (Morita et al., 2004). In addition, Lgr5-deficient embryos tend to have premature paneth cell differentiation in the small intestine (Garcia et al., 2009). Lgr5+ transcripts have been reported in the E13.5 fetal thymus when β-catenin is overexpressed in TECs, however the role and function of these cells remains unknown (Zuklys et al., 2009).

In this study, we determined the fate and function of Lgr5-expressing cells during thymic development. We show that TECs transiently express Lgr5 during fetal development and specifically marks a subset of TECs at E11.5. However, presence of Lgr5 is not essential for proper thymic development. Finally, lineage tracing confirmed that fetal Lgr5+ TECs do not generate detectable progeny in-vivo.

RESULTS

Lgr5 marks a subset of fetal TECs in early thymus development

The presence of Lgr5 transcripts has been reported at E13.5 of thymic development in mice with a TEC-specific overexpression of β-catenin (Zuklys et al., 2009). We first set out to determine the temporal regulation of these lgr5 transcripts in the fetal thymus. Fetal thymi of different ages were evaluated for presence of Lgr5 transcripts. Low levels of Lgr5 message were detected in the fetal thymus at E13.5 and E14.5 by RT-PCR. With increasing gestational age, the levels of lgr5 transcripts gradually decreased and were undetectable from E19.5 onwards. (Fig. 1A).To determine whether the observed lgr5 transcripts lead to Lgr5 protein expression and to identify the cells expressing Lgr5, individual fetal thymi from Lgr5-EGFP- IRES-CreERT2 reporter mice in which EGFP marks cells expressing Lgr5, were collected and single cell suspensions were made. First the hematopoietic (CD45+) fraction was analyzed for the presence of Lgr5 positive cells; however at early embryonic age no considerable amount could be detected (Fig. 1B). Next, the epithelial fraction (CD45-EpCAM+) was analyzed by flow cytometry for EGFP expression (Fig. 1C). In agreement with our transcript analysis we found that the percentage of EGFP positive TECs was highest at E13.5 (1.51%). At E14.5 0.50% of the EPCAM+ cells still expressed Lgr5 while Lgr5+ cells could no longer be detected at E19.5.

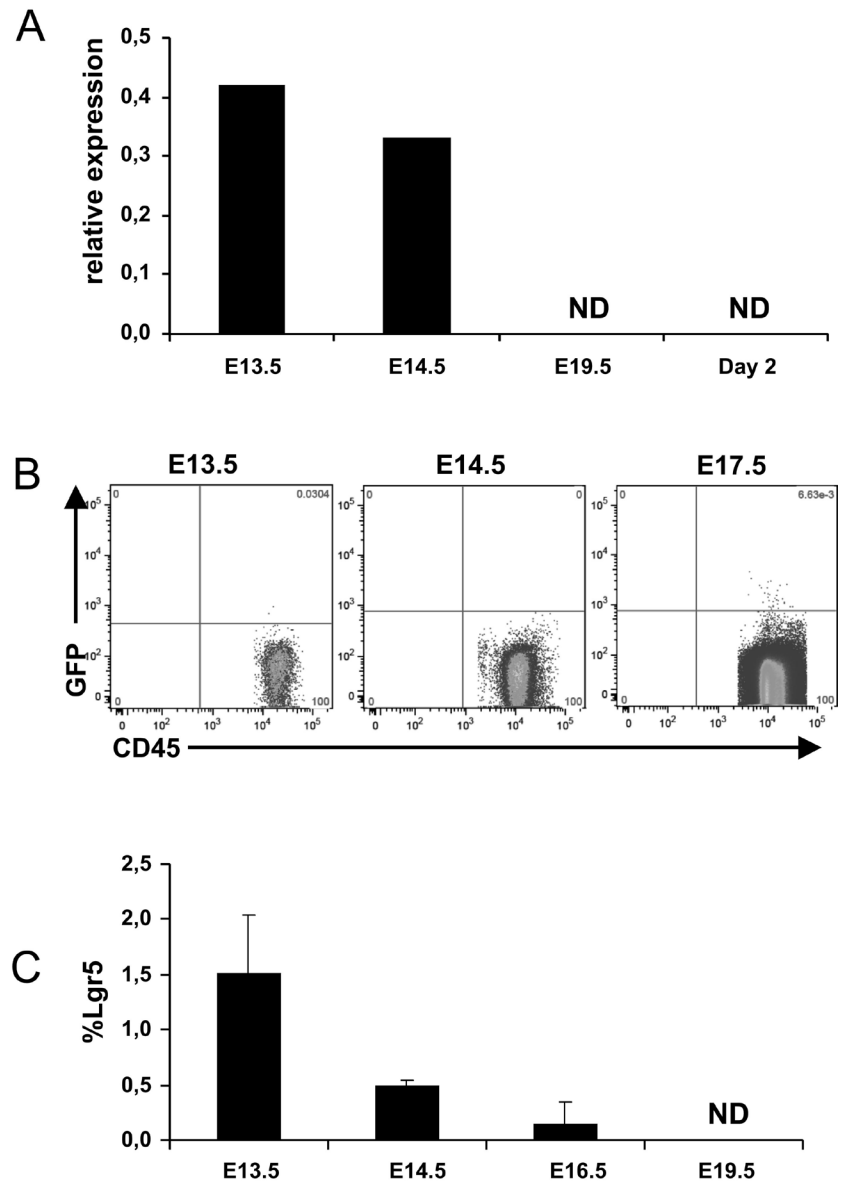

Figure 1: Lgr5 expression in the thymus is restricted to early embryonic development. Thymi of different ages were analyzed for the presence of Lgr5 mRNA. E13.5 and E14.5 were directly analyzed; E19.5 and day 2 neonatal thymi were first purified for EpCAM+ cells (A). CD45+ of different embryonic ages were analyzed for the presence of Lgr5:EGFP cells (B). CD45-/EpCAM+ cells from individual fetal thymic lobes from Lgr5:EGFP embryos were analyzed for the expression of Lgr5. The highest Lgr5 positive fraction was detected at E13.5 (C). For figure C E13.5 n=3, E14.5 n=3, E16.5 n=4 and E19.5 n=3

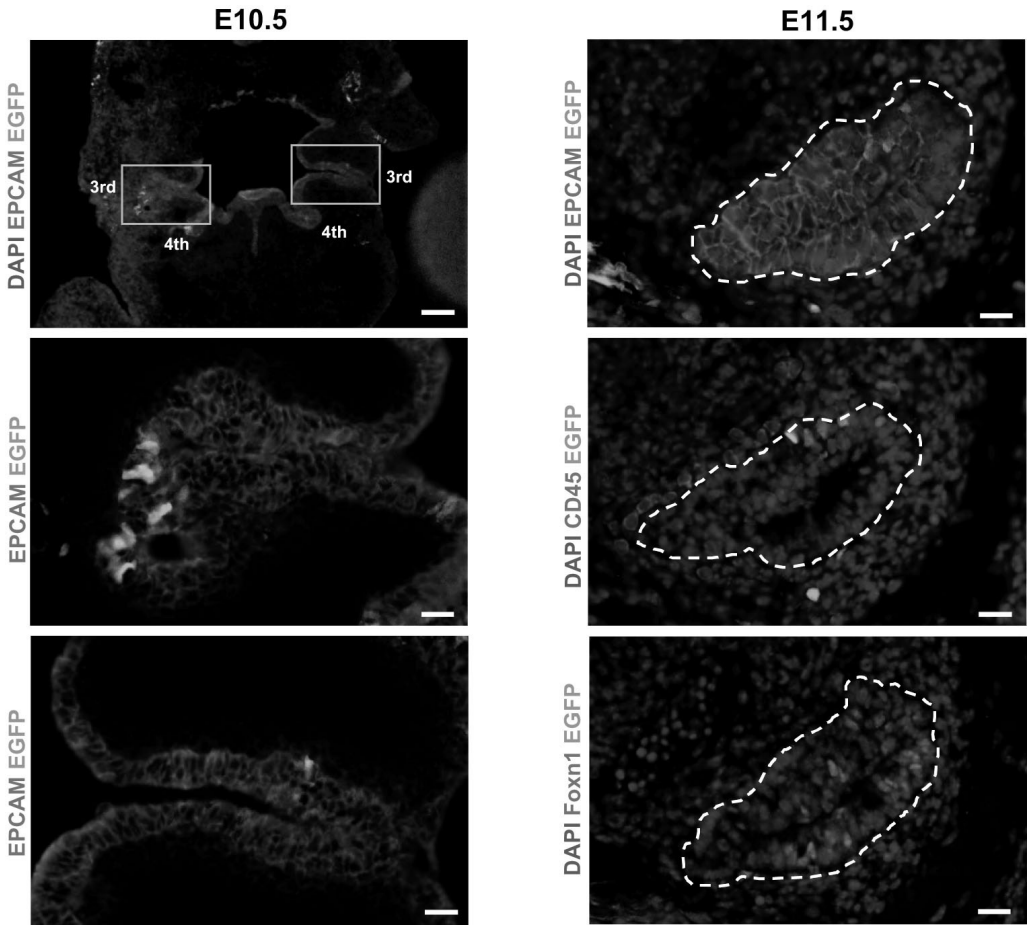

Figure 2: Lgr5 is expressed on a specific subset of TECs in the early fetal thymus. Whole E10.5 and 11.5 Lgr5:EGFP embryos were sectioned and analyzed by immunohistochemistry. The left panel shows a clear expression of Lgr5 in the third pharyngeal pouch endoderm which can be marked with EpCAM. The right panel shows serial sections of the E11.5 fetal thymus. The top panel indicates that the Lgr5+ cells are in the epithelial region. In the middle panel a staining for CD45 was performed on a serial section. Most thymocytes are still at the border of the thymic primordium. In the bottom panel Foxn1 was visualized to indicate that the Lgr5+ TECs were present in the thymic region and not the parathyroid region. Scale bars indicate 125μm in top panel of figure A and 25μm in middle and bottom panel of A and figure B.

In order to confirm that the Lgr5+ cells are indeed located within the thymus and to determine their in-situ localization, fetal thymi of lgr5-reporter embryos were analyzed by immunohistochemistry. E10.5 complete embryos were sectioned and analyzed for the presence of Lgr5+ cells in the thymic anlage. The 3rd pharyngeal pouch at E10.5 clearly showed EGFP+ cells within the thymic primordium and these cells co-expressed the epithelial marker EpCAM (Fig 2A). At the right side of the pharyngeal region the number of EpCAM+EGFP+ cells appeared to be higher, consistent with earlier observations that there is asymmetry in developmental timing between the two sides of the embryo (Gordon and Manley, 2011). Next, sections of whole E11.5 embryos were analyzed. Also at E11.5, EpCAM+EGFP+ cells were clearly detectable within the

thymic priordium and marked a subpopulation of fetal TECs. Serial sections were stained with anti-CD45 and anti-Foxn1 to confirm the thymus location (Fig 2B). Later time points (E13.5, E14.5 and E15.5) of thymic development were also analyzed for the presence of EGFP+ TECs, however no cells could be observed by fluorescence microscopy (data not shown). This is in accordance with the results obtained by flow-cytometry and RT-PCR. In sum, our results clearly show that Lgr5+ TECs are present in the thymus during fetal development. Lgr5 marks a distinct subset of fetal TECs and its expression is initiated prior to E10.5 and declines in time, until it is undetectable at E19.5.

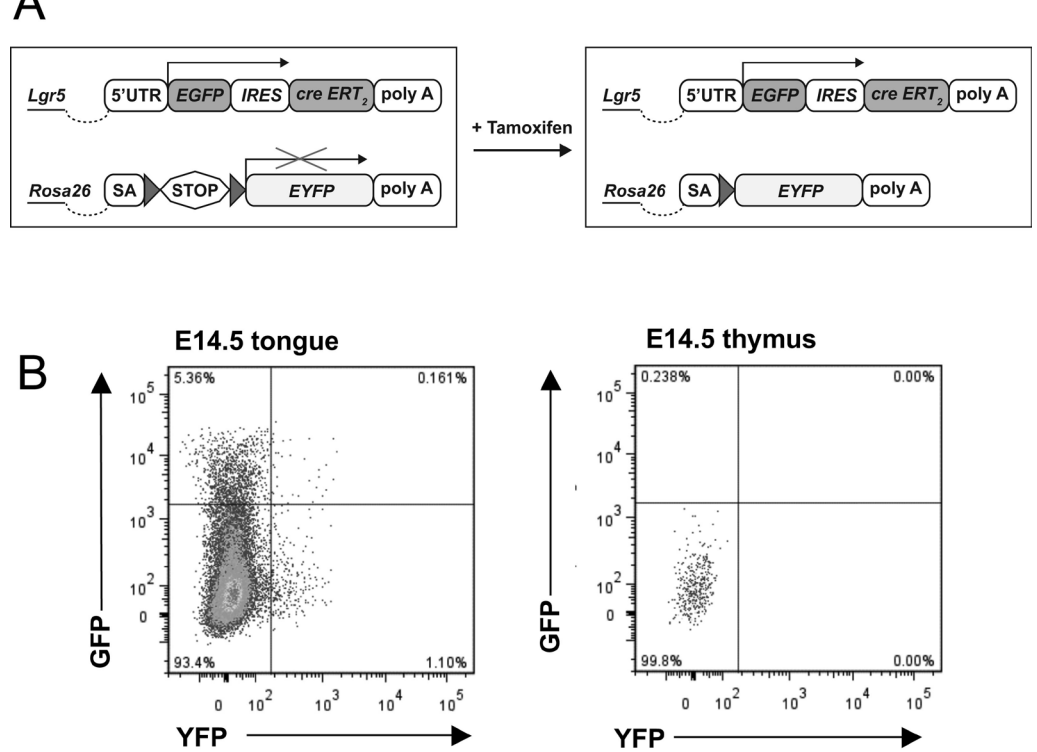

Figure 3: Lgr5 positive TECs do not give progeny. To follow the progeny of the Lgr5 expressing cells Lgr5-EGFP-IRES-CreERT2 mice were mated with Rosa26-Stop^{flox}-YFP mice (A). At E10.5 pregnant mice were pulsed with 0.10mg/g 4OH-tamoxifen. At E14.5 the pregnant mice were sacrificed and the fetal thymus and tongue was analyzed. In the tongue EGFP/EYFP double positive and EYFP single positive cells were clearly detected indicating that cre recombination occurred (B). The E14.5 fetal thymus does not contain any YFP positive cells.

Lgr5+ TECs do not generate detectable progeny

In order to evaluate the fate of fetal Lgr5+ TECs Lgr5-EGFP-IRES-CreERT2 females were time-mated with Rosa26-Stop-EYFP males to generate 4-Hydroxytamoxifen-inducible lineage tracer mice. Pregnant lineage tracer mice were i.p. injected at 10.5 dpc with 0.1mg/g 4-Hydroxytamoxifen to induce creERT2-mediated expression of EYFP (Fig. 3A). 4 days after EYFP induction, the embryos were harvested and the thymus isolated and analysed. As a positive control for intra-embryonic recombination in Lgr5+ cells we co-isolated from the same embryo the tongue region which always showed high levels of Lgr5 expression in sections of the complete Lgr5:EGFP embryos. For the tongue region total CD45- cells were analyzed and for the thymus the EpCAM+CD45- population was analyzed. As shown in figure 3B, left panel, the tongue region contained a large proportion of EGFP+ cells, a small proportion of EYFP+ cells and a minor population of EGFP+EYFP+ double positive cells, indicating the induction of CreERT2 . However, the E14.5 fetal thymus did not contain any detectable EYFP+ or EGFP+EYFP+ epithelial cells (Fig 3B, right panel). These data show that the Lgr5 expressing TECs in the E10.5 thymic primordium do not give rise to detectable numbers of progeny in the E14.5 fetal thymus.

Lgr5 protein is dispensable for normal thymic development

To assess whether there is a functional role for the Lgr5 protein during thymic development we analyzed E19.5 fetal thymi of individual Lgr5+/- and Lgr5-/- mice for the distribution of DN, DP and SP (Fig.4A) and DN1-DN4 thymocytes (Fig. 4B). As shown in Fig. 4B and C thymocyte subsets were distributed normally, suggesting that the absence of Lgr5 did not grossly affect thymopoeisis. Next, we compared the epithelial fractions of the E19.5 Lgr5+/- and Lgr5-/- thymic lobes by immunohistochemistry. The distribution of TECs and mesenchymal cells appeared normal in Lgr5-/- mice (Fig. 4C). In addition, medullary and cortical subsets were present as demonstrated by expression of cytokeratin5 and cytokeratin8 (Fig. 4D). Moreover, no difference in expression or distribution of MHCII, UEA1 and AIRE was found (Fig. 4E and F), suggesting that embryonic development of the thymus occurs independent of Lgr5.

Because lgr5-/- mice die perinatally, we could not analyse thymi from adult Lgr5-/- mice. To overcome this, fetal thymic Lgr5+/- and Lgr5-/- lobes were isolated at E19.5 and transplanted under the kidney capsule of wild type adult mice (Hoffmann et al., 1992). Grafts were allowed to mature for 9 weeks and subsequently analyzed for the distribution of different thymocytes subsets (Fig. 5A and B). No differences could be detected in numbers and percentages of DN1-DN4 or DN, DP and SP thymocytes in Lgr5+/- and Lgr5-/- thymi. In addition, the epithelial fractions of the transplanted thymi also appeared normal (Fig.5 C-F) and all the epithelial subsets were present. Collectively these data indicate that Lgr5 protein expression is not essential for normal thymic development.

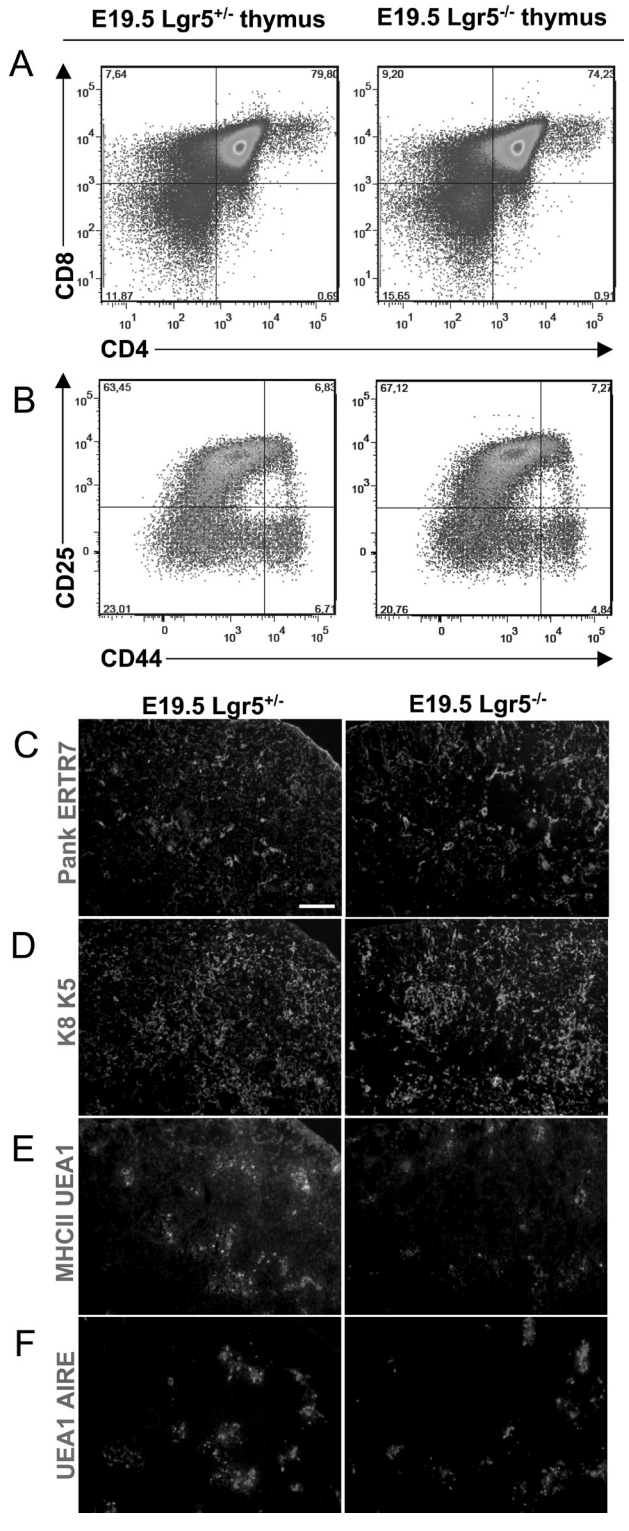

Figure 4: Lack of Lgr5 expression during thymic development shows no aberrant phenotype in the fetal thymus. The lymphoid fraction in fetal E19.5 Lgr5+/- and Lgr5-/- thymi was analyzed by flow cytometry for the DN, DP and SP populations (A) and DN1-DN4 populations (B). Cryosections of E19.5 fetal Lgr5-/- and Lgr5+/- thymi were compared for the expression of pancytokeratin/ERTR-7 (C), keratin 5 /keratin 8 (D), MHCII/UEA1 (E) and AIRE/UEA1 (F). Scale bar indicates 100µm.

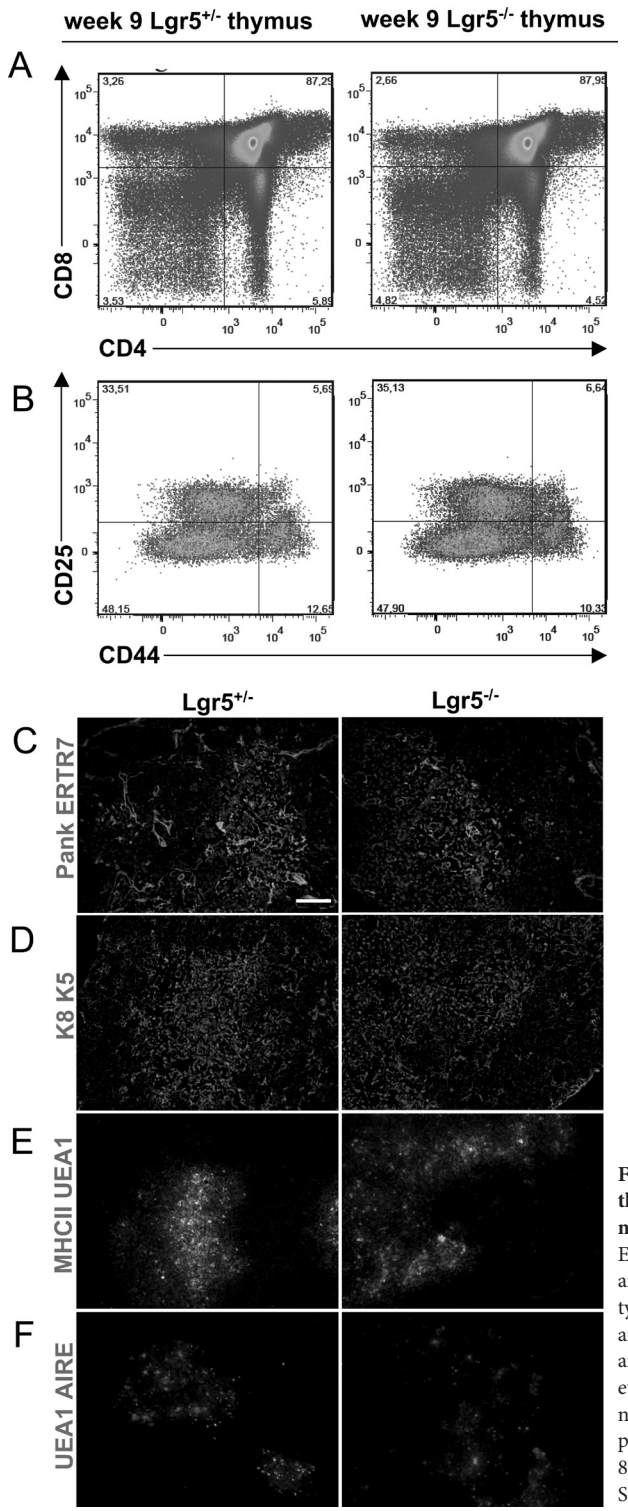

Figure 5: Lack of Lgr5 expression during thymic development shows no aberrant phenotype in the adult thymus. Fetal thymi from E19.5 Lgr5+/- and Lgr5-/- embryos were isolated and put under the kidney capsule of adult wild type mice. After 9 weeks the kidney grafts were analyzed for the DN, DP and SP populations (A) and DN1-DN4 populations (B) by flow cytometry. Cryosections of Lgr5-/- and Lgr5+/- kidney grafts were compared for the expression of pancytokeratin/ERTR-7 (C), keratin 5 /keratin 8 (D), MHCII/UEA1 (E) and AIRE/UEA1 (F). Scale bar indicates 100µm.

DISCUSSION

Expression of Lgr5 marks stem cells in several organs (e.g. small intestine, colon and stomach (Barker et al., 2007). A close relative of Lgr5, Lgr6, marks stem cells in the hair follicle that give rise to all the cell types in the skin (Snippert et al, Science, 2010). Here we asked what cells express lgr5 during fetal development, whether lgr5 protein expression has a role in thymopoesis and whether Lgr5+ TECs might represent the elusive thymic epithelial stem cells. We report the presence of Lgr5+ TECs in the fetal thymus starting from E10.5, extending earlier observations of lgr5 transcripts by Zuklys et al. (Zuklys et al., 2009). With increasing gestational age, Lgr5+ TECs disappear from the thymus and are no longer detectable at E19.5 of gestation. In-vivo lineage tracing experiments established that the E10.5 lgr5+ TECs do not give rise to detectable progeny after 4 days, making it highly unlikely that Lgr5+TECs are a major progenitor/stem cell population. Moreover, expression of lgr5 in TECs is not crucial for development of the thymus as all the stromal and lymphoid compartments appear normal in mice lacking Lgr5. Taken together, we have identified lgr5 as a marker of a subset of early TECs, that as yet have an unknown function.

The analysis of the E10.5 and E11.5 thymi of Lgr5-EGFP-IRES-CreERT2 reporter embryos unexpectedly indicated heterogeneity among TECS during early thymic development (Fig 2A and B). The only marker known so far to mark a subset of E10.5 TECs is Cld3/4. This protein identifies TECs at the apical side of the thymic rudiment. When sorted at E13.5 these cells exclusively contribute to medulla formation (Hamazaki et al., 2007), if this also holds true for E10.5 purified Cld3/4 positive TECs remains unknown.

During our in-vivo lineage tracing experiments, no EGFP/EYFP double positive TECs or YFP single positive TECs were retrieved from the fetal thymus. This indicates that Lgr5 TECs do not give rise to detectable numbers of daughter cells. Still, we cannot rule that progeny is generated, but that the number of YFP+ cells is below our detection limits with flow cytometry. Experiments in which Lgr5:EGFP cells are sorted and reaggregated with recipient thymic stroma or mouse embryonic fibroblast might provide definite prove on the potential of Lgr5+ TECs. However, these experiments will be technically challenging considering the low number of Lgr5+ TECs that can be obtained from an early fetal thymus.

Thymi from lgr5-deficient mice presented a normal phenotype. The stromal architecture developed normal and all the different stages of lymphoid development were present, indicating that the Lgr5 protein itself is not essential for maturation and survival of developing TECs or for generation of thymic stroma. When we started this study Lgr5 was the only stem cell marker and not much was known about Lgr4 and Lgr6. However, recently it has been shown that Lgr4 and Lgr6 fulfill similar roles as Lgr5 in the small intestine (Carmon et al., 2011; de Lau et al., 2011). We analyzed the transcription level of Lgr4 in thymic epithelial cells and found Lgr4 transcripts in the fetal and adult thymus which were comparable with Lgr4 levels in crypts (data not shown). It is possible that Lg4 has an overlapping function with Lgr5 in the thymus. Therefore, the true phenotype of Lgr5 deficient TECs might only be observed in combination with Lgr4 deficiency.

What can be the physiological role of Lgr5 in fetal thymic development? Wnt signaling plays an important role in the development of the thymus and is involved in the regulation of Foxn1 expression (Balciunaite et al., 2002). Zuklys et al showed that overexpression of β-catenin leads initially to normal TEC commitment in endodermal epithelium. Overexpression coincided with an increase in Lgr5 expression at 13.5 dpc of thymic development. However, prolonged Wnt-signalling in the fetal and adult thymus induced a loss of the thymic phenotype, characterized by reduced Foxn1 expression and loss of normal TEC markers (Osada et al., 2006; Osada et al., 2010; Zuklys et al., 2009). This indicates that Wnt signaling need to be tightly regulated throughout thymic specification and maintenance in the adult period. Lgr5 could be involved in regulating the narrow window of optimal Wnt signals that secure the thymic specification program, which mainly involves upregulation of Foxn1. Once Foxn1 expression is secured and the thymus program continues, regulators of Wnt signaling (Lgr4 or Lgr6) might come into play. At least for maintenance of the adult thymus Apc, Kremen and DKK1 seem to play important roles (Kuraguchi et al., 2006; Osada et al., 2006; Osada et al., 2010).

In summary, our current work uncovered the presence of Lgr5 positive TECs during a brief window in thymic development. However, Lgr5 positive TECs did not show any progeny at later stages of thymic development. Moreover, the protein Lgr5 is not important for proper development of the adult thymus. These data rule out that Lgr5 is a TEC marker for a bona fide epithelial stem cell in the embryonic thymus.

MATERIAL AND METHODS

Mice

Lgr5-EGFP-ires-CreERT2 (Barker et al, Nature, 2007) were obtained from Hans Clevers (Hubrecht Institute, Utrecht), Rosa26-EYFP (Srinivas et al., 2001) were provided by Ivo Touw (Erasmus MC, Rotterdam) and C57BL/6 mice were maintained in our animal facility. On the day that the vaginal plug was detected embryos were designated as E0.5 of gestation.

Antibodies

For immunohistochemistry the following antibodies were used: rabbit anti-pancytokeratin (DAKO, Glostrup, Denkmark), rat anti-ERTR-7 (Van Vliet et al., 1984), rabbit anti-keratin-5 (Covance, Denver, PA, USA), biotinylated anti-keratin-8 (Progen, Heidelberg, Germany), biotinylated anti-I-A/I-E (BD Pharmingen, San Diego, CA, USA), FITC conjugated anti-UEA-1, biotinylated anti-UEA-1 (both Vector Laboratories, Burlingame, CA, USA) and goat anti-AIRE-1 (Santa Cruz Biotechnology, CA, USA) as primary antibody, followed by chicken anti-rabbit Alexa594, donkey anti-rat Alexa488, chicken anti-goat Alexa488, streptavidine Alexa594 (all from Molecular Probes, Eugene, OR, USA).

For flowcytometry the following antibodies were used: rat anti-EpCAM Alexa647 (Biolegend, San Diego, CA, USA), rat anti-CD45 PerCP-Cy5, rat anti-CD3 Alexa700 (both Ebioscience), rat anti-CD4 APC-Cy7, rat anti-CD8a PE-Cy7, rat anti-CD44 FITC, rat anti-CD25 APC (all from BD).

Immunohistochemistry

Immunohistochemistry was performed as described previously (Germeraad et al., 2003). To maintain the EGFP and EYFP signals, tissues were fixed in 4% paraformaldehyde and submerged in a sucrose gradient prior to freezing. Sections were made on a Cryostat Jung CM3050. Pictures were made by a Leica DMRXA microscope and Leica FW4000 software.

Flow Cytometry

Flowcytometric analysis was performed on a LSRII Flow Cytometer (BD) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA). Cell isolations were performed on a FACSAria Cell sorter (BD).

PCR

mRNA was isolated with an RNA easy kit (Qiagen) and reverse transcription was done with random hexamer primers. An F-415L DyNAmo Flash SYBR Green qPCR kit (Finnzymes) and 7500 Fast Real-Time PCR system (Applied Biosystems) were used for qPCR.

Primers

Lgr5 (FW5'-TCCAGGCTTTTCAGAAGTTTA-'3 , REV: 5'-GGGGAATTCATCAAGGTT A-3') Cyclo (FW: 5'-AACCCCACCGTGTTCT-3', REV: 5'-CATTATGGCGTGTAA AGTCA-3')

Thymocyte preparation

Thymocytes were obtained by grinding thymic fragments trough a 100µm filter (BD). The collected cells were washed and subsequently stained for flow cytometry.

Tamoxifen treatment

4OH-Hydroxytamoxifen (Sigma) was dissolved in 1 part 99% ethanol and 9 parts sunflower-oil at 55° C in a stock concentration of 20mg/ml. At 10.5 dpc pregnant females were i.p. injected with 0.1mg/g 4OH-Hydroxytamoxifen to induce creERT2 recombination. Subsequently, the pregnant mice were sacrificed at the day of analysis and fetal thymi were isolated from the embryos.

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INTRODUCING A THYMIC EPITHELIAL FATE IN MURINE EMBRYONIC AND INDUCED PLURIPOTENT STEM CELLS

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ABSTRACT

Recent studies in fetal mice have identified common thymic epithelial progenitor cells (TEPC) that are capable to differentiate into medullary and cortical TECs when injected into a host fetal thymus. These TEPC arise exclusively from the anterior foregut endoderm during embryogenesis. Differentiation of thymic epithelium from Embryonic Stem (ES) or induced Pluripotent Stem (iPS) cells would be a major step towards designing regenerative therapy for patients with impaired thymic function.

Murine ES cells were differentiated in the presence of thymic specific growth factors. We established a 70-fold increase in the expression of Foxn1, the pivotal thymic transcription factor, upon differentiation. Other thymic transcription factors like Bmp4, Pax9, keratin8, Dll4 and CCL25 were also upregulated. However, we were unable to isolate an enriched fraction of differentiated ES cells from bulk cultures. Therefore, iPS from Foxn1:EGFP mouse embryonic fibroblasts were generated using the 4 transcription factors originally described by Takahashi et al. (Klf4, Sox2, Oct4, c-Myc) (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). iPS cells from Foxn1 reporter mice allowed us to evaluate the efficacy of differentiation protocols. An increasing percentage up to 15% EGFP+ cells within the epithelial fraction was observed, using a differentiation protocol containing the growth and differentiation factors BMP4, EGF, and FGF 7/10. GFP positivity appeared largely confined to differentiated cells with epithelial features in culture.

These data indicate that differentiation towards a thymic epithelial precursor fate, as evidenced by the expression of Foxn1, can be established in iPS cells using specific culture conditions. However, only a subset of cultured cells express GFP, which positivity appeared largely confined to a subset with morphological features of epithelial differentiation. Additionally, the EpCAM+/GFP+ population sorted from the culture showed the highest upregulation of Foxn1 transcripts compared to other cell populations in the culture. The differentiation protocol applied will allow us to purify Foxn1:EGFP+ cells from cultures for further functional analysis.

Keywords: embryonic stem cells, induced pluripotent stem cells, thymic epithelium, Foxn1

INTRODUCTION

The thymus is a lympho-epithelial organ consisting mainly of developing T cells (thymocytes) and thymic epithelial cells (TECs). All T cells develop in the thymus, which development is essentially facilitated by close interaction with TECs in the cortical and medullary regions. Communication between thymocytes and epithelial cells works in a bi-directional manner (thymic crosstalk) and takes place from the moment when thymocytes enter the developing thymus (reviewed in: (van Ewijk et al., 1994; van Ewijk et al., 1999). During the earliest phase of thymic development, precursor TECs posses the ability to differentiate either into medullary TEC or cortical TEC. The bi-potential TEC (bTEC) progenitor was shown in the fetal thymus before a clear medulla and cortex separation occurs (Bleul et al., 2006; Rossi et al., 2006). However, it is unknown whether such bTEC progenitor is still present after birth.

Involution of the thymus coinciding with a decline of thymopoiesis and narrowing of the T-cell repertoire in adulthood is a well known problem in aged men and mice. In mice, thymic cellularity declines with increasing age, which coincides with epithelial to mesenchymal transition (Youm et al., 2009). In humans the overall thymic size might remain the same as assessed by radiographic imaging, but the cellular content changes, including increase of adipose tissue and perivascular space. Impaired immune competence in the elderly is characterized by drastic reduction of thymocyte populations, resulting in a decrease in T cell repertoire diversity and less recent thymic emigrants in the periphery (reviewed in (Aspinall et al., 2010; Lynch et al., 2009; Taub and Longo, 2005). Patients that acquire an immune deficiency as a result of infections (e.g. HIV-disease) or as a result of intensified treatment for leukaemia may experience an even more immunocompromised condition than the elderly. Moreover, failure to recover thymopoiesis after hematopoietic stem cell transplantation strongly predicts for a higher incidence of opportunistic infections and treatment related mortality (Wils et al., 2011). These patients might benefit from thymic regenerative therapy.

Thymic therapy is already successful in patients with congenital diseases in which thymic development is affected. A mutation in the FOXN1 locus causes the rare nude/severe combined immune deficiency (SCID) syndrome. These patients have congenital alopecia, nail dystrophy and a hypo plastic thymus (Frank et al., 1999). The DiGeorge syndrome is another group of patients with a congenital disease; these patients lack functional thymic tissue due to a microdeletion in chromosome 22, resulting in a deficiency in TBX1. TBX1 is important for normal development of the thymus, parathyroids and formation of facial and cardiac tissue (Kobrynski and Sullivan, 2007). From birth nude/SCID and DiGeorge patients suffer from opportunistic infections as their immune system is severely hampered. Transplantation of thymic tissue has already been explored and was shown to enhance immune competence and quality of life (Hudson et al., 2007; Markert et al., 1999; Markert et al., 2007; Markert et al., 1997; Markert et al., 2011; Markert et al., 2003).

However, transplantation of mismatched, third party, thymic tissue may not be an option for patients with residual immunity capable of rejection. It has fuelled more basic research into the

field of regenerative medicine focused on epithelial stem cells. Nevertheless, such cells, while known to be present during embryogenesis, remain elusive, in terms of their persistence and possible isolation from adult thymus.

An alternative approach would be to create TECs from Embryonic Stem (ES) and/or induced Pluripotent Stem (IPS) cells. Theoretically the amount of ES and iPS cells is not limited as these cells can easily be expanded in-vitro. In the setting of allogeneic hematopoietic stem cell transplantation, donor iPS cells are attractive, because these cells will never suffer from graft versus host disease and therefore, may support thymopoiesis for a prolonged period. Recently the group of Lai et al. successfully differentiated murine ES cells towards Foxn1 positive thymic epithelium (Lai and Jin, 2009) by using the growth-factors Ffg7, Fgf10, Bmp4 and EGF. When injected into a thymus, the differentiated cells were shown to contribute to the generation of naïve T cells in a murine bone marrow transplantation setting (Lai et al., 2011). Whether the injected differentiated ES cells contributed actively to thymic selection or produced growth factors that enhanced the output of the thymus remains unknown. Others have explored the possibility to use IPS cells as a source to create an artifical thymus (Inami et al., 2011). In here, a more controlled differentiation was assessed. First, definitive endoderm was created by adding activin A and LiCl to the culture. Further treatment with Fgf8 followed by Fgf7, Fgf10 and BMP4 led to enhanced levels of Pax1, Pax9, Hoxa3 and Foxn1.

Here we explore the possibilities to use ES/iPS cells as a source for in-vitro manipulation, with the aim to reconstitute thymic function. First ES cells were differentiated in the presence of different thymic specific growth factors. Although we consistently found an increase in transcripts involved in thymic development, we were unable to isolate a purified population with a definitive TEC phenotype only. Therefore, we generated iPS cells from Foxn1:EGFP reporter mice and differentiated them in the same growth conditions as ES cells. By visualizing Foxn1 expression via GFP, it allowed us to monitor iPS cells that entered into a thymus-specific differentiation program.

RESULTS

The Sox17 reporter ES cell line demonstrates upregulation of Foxn1 upon differentiation

Differentiation of ES cells towards epithelial cells expressing Foxn1 levels that are comparable to levels in thymic epithelial cells have been reported (Lai and Jin, 2009). Upon transplantation into allogeneic recipients, enhanced thymopoiesis was observed. Although thymic function was rescued, the fate and functional role of the differentiated ES cells remained obscure (Lai et al., 2011). Based on these reports we set out to apply a similar differentiation protocol supplemented with a more thorough analysis of the differentiated ES cells. Cell lines R1, IB-10 and Sox17 were all tested for their capacity to upregulate transcripts for Foxn1 upon differentiation. Cell lines were harvested and transcription levels of Foxn1 were compared with that of expansion cultures (Fig 1.). Of the three ES cell lines that were tested, the Sox17 line showed the highest induction of

Foxn1. However when the levels were compared with complete thymi from E15.5 embryos the level of Foxn1 was still 88-fold lower (data not shown).

Next to Foxn1 we tested other transcription factors that are known to be important for thymic specification. Pax9 is required for normal development of organs derived from the whole pharyngeal region (Hetzer-Egger et al., 2002). Bmp4 is known for its early role in thymic development, as thymi lacking Bmp4 are reduced in size (Bleul and Boehm, 2005; Patel et al., 2006). We analyzed the levels of these two factors by RT-PCR. As shown in figure 2A, Pax9 and Bmp4 were both upregulated upon differentiation, suggesting differentiation towards the thymic lineage. The functional molecules Dll4 (Feyerabend et al., 2009; Hozumi et al., 2008; Koch et al., 2008) and Ccl25 (Liu et al., 2006; Liu et al., 2005; Wilkinson et al., 1999) form an important part of the initial communication with developing thymocytes. We tested whether the expression of the molecules becomes upregulated during the differentiation of ES cells. Indeed, we saw a small increase in the levels of Dll4 (6.3 fold) and Ccl25 (10.1 fold) upon differentiation (Fig 2B). However, when these transcription levels were compared with fetal TECs, Dll4 was still 54.8 times lower and Ccl25 28.8 times lower. These data indicate that different thymic markers become upregulated in ES cells upon differentiation in the presence of different thymic growth factors, but they do not reach the levels of normal TECs.

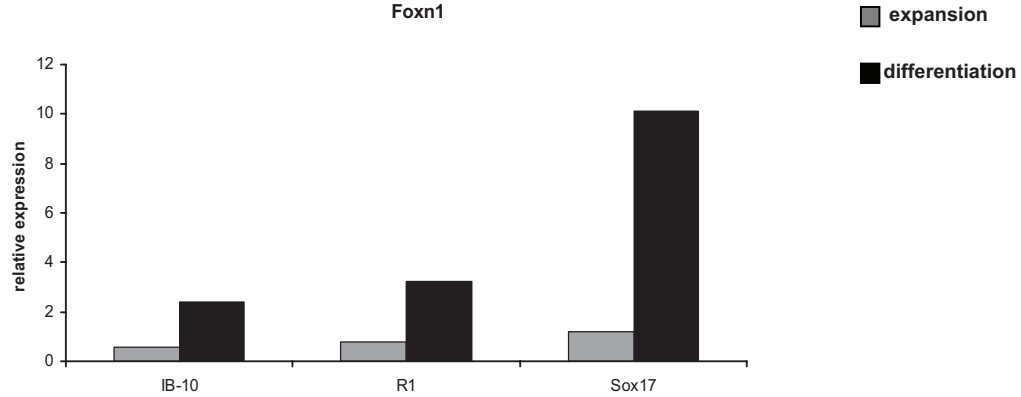

Figure 1: The ES cell line Sox17 gives the highest Foxn1 induction upon differentiation. Different ES cell lines were tested for their capacity to upregulate Foxn1 upon differentiation. The Sox17 reporter ES cell line gives the highest induction of Foxn1 transcription levels compared to IB-10 and R1. The levels in the fetal thymus were 884.6 (data not shown).

Induction of eGFP in a Foxn1:eGFP reporter IPS cell line

The results so far indicate that a thymic specific program is initiated during differentiation, however it is still unclear whether all cells express low levels of the tested thymic markers or that a fraction of the cells within the culture is responsible for the increase of transcription levels. For these reasons we created an iPS cell line (TC-11-01) which is derived from Foxn1:EGFP reporter MEFs. These cells allow to visualize Foxn1 expression upon differentiation and might enable to purify cells by flow-cytometric cell sorting.

Embryoid bodies of TC-11-01 were differentiated according to the Lai protocol as described in the previous experiments. After 10 days of culture, cells were harvested and compared with undifferentiated TC-11-01 iPS cells. Colonies of the undifferentiated TC-11-01 cells still retained their IPS like morphology that resembles ES cells during their expansion culture. The differentiated TC-11-01 cells formed colonies that resembled epithelial sheets (Fig 3A). Next we assessed the amount of EGFP positive cells within the EpCAM fraction. As shown in Figure 3B and 3C an EGFP+ population was present in the differentiation culture when compared with the undifferentiated iPS cells. It clearly indicates that upon culture in the presence of EGF, Fgf7, Fgf10 and Bmp4, EGFP is induced.

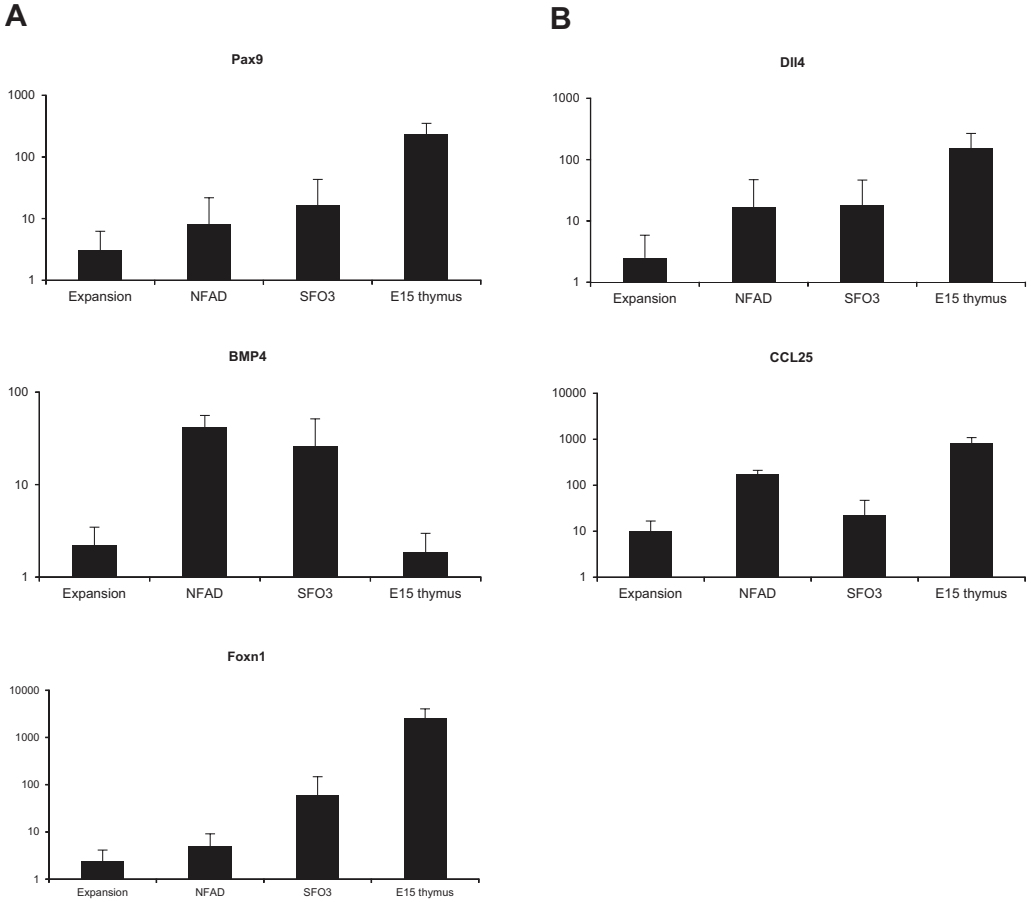

Figure 2: Upregulation of different thymus specific markers. Sox17 cells were differentiated in DMEM and SFO3 medium and compared with undifferentiated Sox17 cells for the upregulation of thymus specific markers. (A) Transcription levels of Pax9, Bmp4 and Foxn1 as marker for thymic specification were analyzed upon differentiation. (B) Differentiated Sox17 ES cells were analyzed for the functional molecules Dll4 and CLL25. Whole E15 murine fetal thymi were taken as a positive control. The average of n=3 is shown.

Next to the Lai protocol, we also examined the efficiency of the Inami protocol (Inami et al., 2011). With that protocol the definitive endodermal lineage is first established with activin A and LiCl. Further differentiation was done with Fgf8 followed by Fgf7, Fgf10 and Bmp4. Two time points were taken for analyses. At day 12 most colonies resembled embryoid bodies based on morphology (Fig 4A, left panel). When the differentiated cells were analyzed by flow cytometry an EGFP+ population could be observed in the EpCAM+ population (Fig 4B and C, left panel). Addition of RANKL was reported to enhance the Foxn1 levels. We added RANKL at the 12th day of differentiation and analyzed the culture after 4 days. The morphology of the colonies did not change (Fig 4B, right panel). However, the percentage of EGFP+ cells within the EpCAM fraction slightly enhanced by using additional growth factors (Fig B and C, right panel).

Recently Green et al. (Green et al., 2011) reported that it is important to inhibit BMP4 and TGF-β signalling to secure the differentiation toward anterior foregut endoderm. For this reason we adapted the Lai protocol by first including a culture step with activine A to secure the endodermal pathway, followed by Noggin and SB431542 (inhibitors of BMP and TGF-β) for 1 day. Subsequently the we followed the Lai protocol again by using BMP4, Fgf7, Fgf10 and EGF. Although the increase in the number of cells expressing GFP was less efficient, we isolated different populations from the bulk culture and analyzed the fold change of Foxn1 transcripts after differentiation (Fig 5). The EpCAM+/GFP+ population showed the highest increase in Foxn1 expression, indicating that GFP is a good marker to purify differentiated iPS cells committed towards a thymic fate.

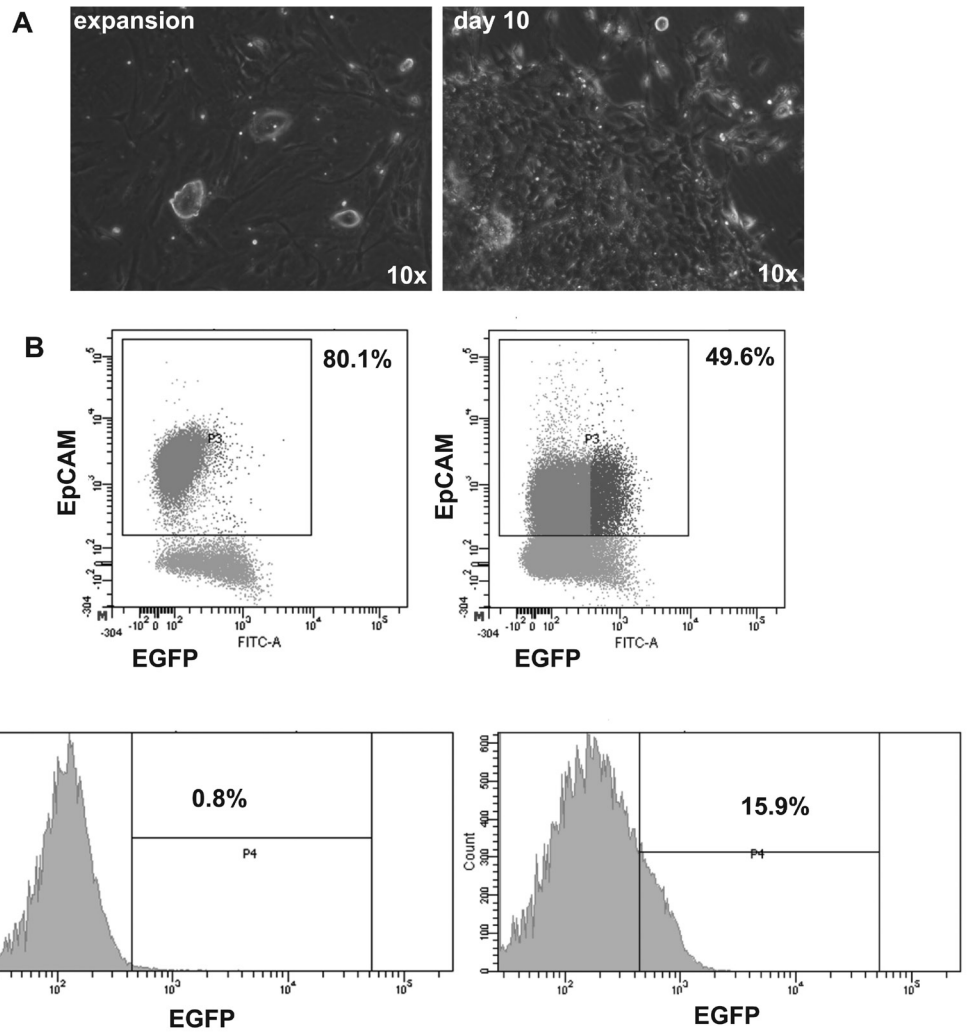

Figure 3: Upregulation of eGFP expression in Foxn1:EGFP IPS cells upon differentiation with the Lai protocol. TC-11-01 IPS cells derived from Foxn1:EGFP mice were differentiated towards thymic epithelium, after 10 days of culture the cells were analyzed. (A) Colonies were analyzed for their morphology. Differentiated colonies grew like epithelial sheets upon differentiation. (B) Colonies were harvested and the amount of EpCAM positive cells was examined by flow cytometry. Undifferentiated IPS cells already contained a major fraction of EpCAM positive cells. Differentiated cells had lower EpCAM expression and more cells that were EpCAM negative. (C) EGFP expression of the EpCAM positive fractions was analyzed. The differentiated IPS cells showed an increase in EGFP expression indicating that Foxn1 becomes upregulated upon differentiation.

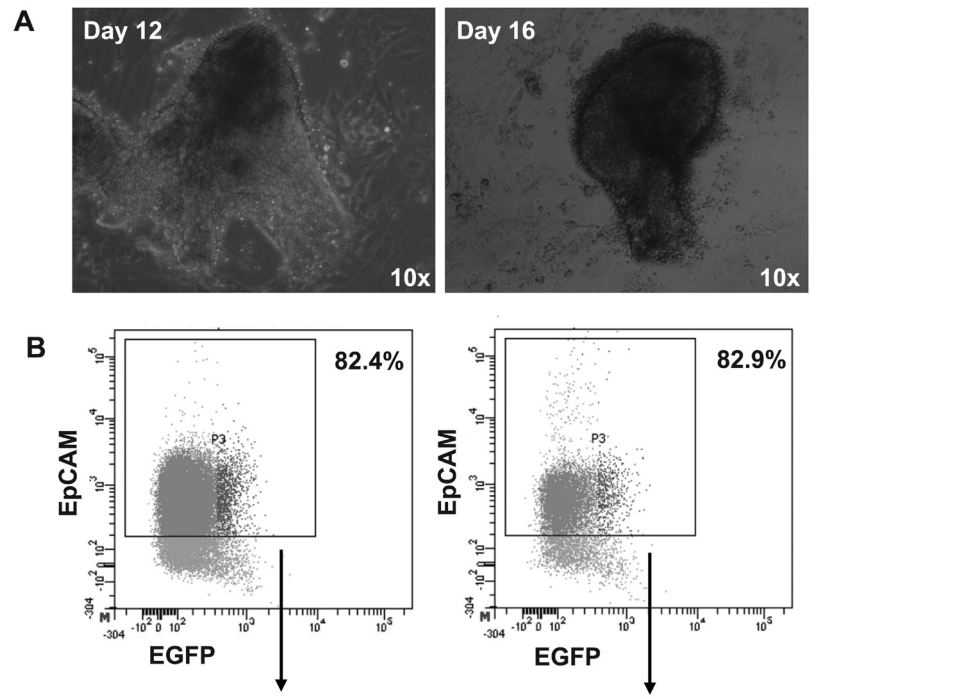

Figure 4: Upregulation of eGFP expression in Foxn1:EGFP IPS cells upon differentiation with the Inami protocol. TC-11-01 IPS cells were differentiated toward thymic epithelial according to the Lai protocol. The cultures were analyzed at day 12 and day 16 of differentiation. The morphology colonies still resembled embryoid bodies, as the cells did not grow like epithelial sheets (A). The EpCAM positive fraction was analyzed for the expression of EGFP (B). The culture at day 12 of differentiation contained 4.2% of EGFP positive cells (left panel). By adding RANKL to the culture this percentage was increased to 7.2% (right panel).

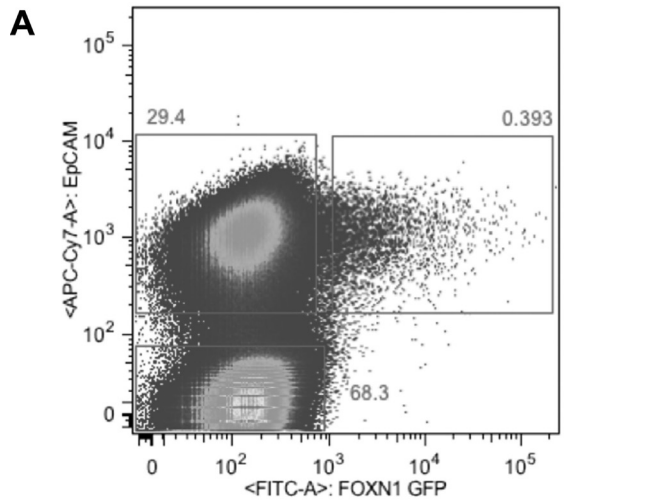

Figure 5: Sorted EpCAM+ GFP+ cells express most Foxn1 transcripts. TC-11-01 embryoid bodies were differentiation towards a thymic fate. After 13 days three different populations were isolated from the bulk culture based on their EpCAM and GFP expression (A). The different populations were analyzed for the presence of Foxn1 transcripts by RT-PCR. The EpCAM+/GFP+ population showed the highest fold change when compared with the EpCAM-/GFP- population or the EpCAM+/GFP- population. The expansion culture TC-11-01 was taken as the value 1 in this comparison (B)

DISCUSSION

The exact signals needed to secure thymic specification in-vivo are still incompletely understood, as a result of which current ES and iPS differentiation protocols are still inefficient and largely based on “trial and error”. The protocols that have been established in the past few years to differentiate pluripotent ES/iPS cells towards defined lineages have been very elaborate and many conditions were tested (Green et al., 2011).

Moreover, studies with non-marked cell lines has hampered the assessment in terms of yield and fold-increase of specific cell populations aimed at. In addition, it also hampered the isolation and harvest of a specific cell population. For example, Lai et al did not use reporter cell lines, as a result of which the differentiated ES cells, which were used for in-vivo studies, were rather loosely characterized. The differentiation culture was not monitored for alternative lineages nor was the efficiency reported. Therefore, the exact identity of the EpCAM+ cells that were used for in-vivo studies remains unclear. An assay in which different stages of development may be monitored would be highly desirable. In such a case, ES or iPS cells first need to be differentiated towards definitive endoderm, followed by patterning into pharyngeal and anterior foregut endoderm and finally TEC specification via pharyngeal pouch endoderm (Green and Snoeck, 2011). Generation of anterior foregut endoderm from human ES and iPS cells has been closely monitored in-vitro. The generation of definitive endoderm from ES cells can be established with high levels of activin A. After the generation of definitive endoderm it becomes important that TGF-β and BMP signaling are inhibited to obtain an enriched population of anterior foregut endoderm (Green et al., 2011). By using the Inami protocol we tried to establish a stepwise differentiation. Unfortunately, a higher efficiency in the differentiation towards TECs was not obtained. In future experiments, the differentiation state and quality of the cells after each step have to be assessed, which may subsequently enhance the culture efficiency. We added inhibitors of TGF-β and BMP signaling for one day to the culture, however, this did not result in an increase in the number of EpCAM+/GFP+ cells. By adding the factors EGF, Fgf7, Fgf10 and Bmp4 the protocol seems robust as most factors are known to play a role in thymic development once Foxn1 is already upregulated. Moreover, when the EpCAM+/GFP+ cells were sorted and tested for Foxn1 transcripts it became clear that this population contained the cells that were committed towards a thymic fate.

The role of Foxn1 during thymic development is clear, without transcription of Foxn1; thymic development is severely hampered (Nehls et al., 1996). In the adult thymus Foxn1 remains important for the maintenance of TECs and to secure thymopoiesis (Chen et al., 2009; Cheng et al., 2010; Sun et al., 2010). In our study we mainly focused on Foxn1 as readout for thymic commitment. Whether the Foxn1 transcripts detected in our assays represent physiological levels in-vivo remains unknown, as we did not test the functionality of the differentiated cells. Pax9 and BMP4 were also investigated in our study. The levels of Pax9 and Bmp4 increased upon differentiation. Pax9 levels were still a fraction of normal E15 TECs, but Bmp4 levels were higher than normal E15 TECs. Pax9 is involved in the development of pharyngeal pouch derivatives (Peters et al., 1998), whether Pax9 is expressed in older TECs is not known. Bmp4 has a role in

the determination of cell fate in the third pharyngeal pouch (Bleul and Boehm, 2005; Patel et al., 2006) and is involved in the maintenance of Foxn1 expression in TECs (Soza-Ried et al., 2008). However, Green et al. reported in a study with human ES/iPS cells that inhibition of BMP is important to secure differentiation towards anterior foregut endoderm (Green et al., 2011). The high levels of Bmp4 in our differentiated cells might reflect the presence of other lineages that are not derived from anterior foregut endoderm. We also looked at the functional molecules Dll4 (Feyerabend et al., 2009; Hozumi et al., 2008; Koch et al., 2008) and Ccl25 (Liu et al., 2006; Liu et al., 2005; Wilkinson et al., 1999) which are known to be controlled independently from Foxn1 (Itoi et al., 2007). Upregulation of Dll4 and Ccl25 was clearly detected upon the differentiation of ES cells, however, the levels were still low when compared to fetal TECs. This could be explained by the bulk culture that also contained cells that differentiated into an alternative lineage.

Although we consistently found an increase in transcripts involved in thymic development, we were unable to isolate a purified population with a definitive TEC phenotype only. Therefore, we generated iPS cells from Foxn1:EGFP reporter mice and differentiated them in the same growth conditions as ES cells. It allowed us to focus on the cells that express Foxn1 within the differentiation culture. When TECs from adult Foxn1:EGFP mice were directly analyzed by flow cytometry the intensity of EGFP was always much higher (data not shown). This could partially reflect the lower amount of Foxn1 transcripts found in differentiated ES cells compared to TECs. However, it is clear that the EGFP signal increases upon differentiation, making selection of cells that committed towards a thymic fate possible. Whether these cells have the characteristics of progenitor TECs or committed mTECs and cTECs in-vivo needs to be tested in future experiments. The undifferentiated state of the starting population prior to initiating the differentiation culture is important. Pluripotent ES/iPS cells need to be maintained properly on conditions that keep them in their pluripotent state, LIF should ensure this in our assays. However, that capacity was never tested after a couple of passages. Checking the quality of the iPS cells before differentiation and avoiding high passage numbers could contribute to a higher efficiency in inducing cells that committed into a thymic specific program. Adding GSK-3 inhibitor to the expansion culture of the IPS cells may even enhance this effect (Sato et al., 2004; Ying et al., 2008). Addition of extra factors that play a role in thymic specification might be interesting in the differentiation of pluripotent cells towards a thymic fate. Wnts are known to have an important function in the thymic primordium as they are involved in upregulation of Foxn1. In the thymus Wnts are produced in a paracrine and autocrine fashion by TECs and thymocytes (Balciunaite et al., 2002). Addition of Wnts at a certain stage of the differentiation culture might enhance Foxn1 levels. Continuous presence of thymocytes is important for differentiation and maintenance of TECs. Most factors involved in this process are unclear, however many experimental murine models proved the value of the presence of hematopoietic cells (van Ewijk et al., 2000; van Ewijk et al., 1999). Once Foxn1 positive iPS cells are purified the cells can be replated or reaggregated together with thymocytes, this could induce further differentiation and upregulation of markers known to be present on adult mTECs and cTECs. A recent study reported a role for IL-22 producing innate lymphoid cells in damage repair of thymic tissue. Upon sub-lethal irradiation

intrathymic IL-22 levels were increased and thymic recovery enhanced (Dudakov et al., 2012). Addition of IL-22 to the differentiation culture might enrich the number of Foxn1 positive cells and needs to be explored in future experiments.

Taken together, we observed an increase of transcription factors that are important for thymic specification upon differentiation of ES cells. This was accompanied by an increase in the levels of Dll4 and Ccl25, supporting the hypothesis that priming towards a thymic lineage took place. When we visualized Foxn1 by using IPS cells that were derived from Foxn1:EGFP reporter mice we could clearly detect a subset of EGFP+ cells within the EpCAM+ population.This population showed the highest increase in Foxn1 transcripts compared to other populations that were obtained from the culture. Using the Foxn1-EGFP iPS cells in thymic differentiation cultures will thus allow for isolation of cells that differentiated into TECs in-vitro and the functional analysis of these cells in future studies.

EXPERIMENTAL PROCEDURES

Cell culture

R1 (Nagy et al., 1993), IB-10 (Robanus-Maandag et al., 1998) and Sox17 ES cells (Yasunaga et al., 2005) were maintained on Glasgow Minimal Essential Medium (Invitrogen) supplemented with 10% knockout serum replacement (Invitrogen), 1% fetal calf serum (Hyclone) , 5ml non-essential amino acids (Invitrogen), 5ml sodium pyruvate (Invitrogen), 5ml penicillin/streptomycin (Invitrogen), 0.5mM β-mercaptoethanol (Sigma) and 1000U/ml Leukemia Inhibition Factor (Invitrogen) on 0.1% gelatin coated culture discs (Becton Dickenson). IPS cell line TC-11-01 was maintained on irradiated MEFs in DMEM supplemented with non-essential amino acids, ES qualified fetal calf serum, 1mM 2-Mercaptoethanol, 5ml penicillin/streptomycin and 1000U/ml Leukemia Inhibition Factor (Invitrogen).

The Lai (Lai and Jin, 2009) differentiation protocol was performed as followed: two days prior to different ES cells and IPS cells were cultured as embryoid bodies in expansion medium. At day 0 they were differentiated in NFAD medium (1 part Ham/F12, 3 parts DMEM) (Invitrogen) or SFO3 (Sanko Junyaku) supplemented with fibroblast growth factor 7 (20ng/ml), fibroblast growth factor 10 (20ng/ml), bone morphogenic protein 4 (20ng/ml) and epithelial growth factor (50ng/ml) (all R&D systems). The medium was replaced every 4 days. At day 10 the differentiated cells were harvested with trypsin/EDTA (Invitrogen).

The Inami (Inami et al., 2011) differentiation protocol was done as followed: embryoid bodies were cultured two days prior to the differentiation culture. At day 0, embryoid bodies were harvested and cultured in NFAD medium or SFO3 supplemented with activin A (10 ng/ml) (R&D systems) and LiCl (Sigma). At day 4, the medium was replaced with medium to which Fgf8 (4 ng/ml) (R&D systems) and LiCl (5 mM) was added. At day 7, the medium was changed and Fgf7 (20ng/ml), Fgf10 (5 ng/ml), BMP4 (10ng/ml) and LiCl were added to the culture. At day 12 cells were harvested and analyzed or cultured 4 days longer in the presence of RANKL (20ng/ml) (R&D systems) and LiCl.

In indicated experiments the Lai protocol was enhanced based on recent findings in literature. Embryoid bodies of TC-11-01 were cultured for 5 days in medium to which activine A was added. At day 5 the medium was changed and the embryoid bodies were cultured in the presence of Noggin and SB431542 (BMP and TGF-β inhibitors) for 1 day. From day 6 until day 13 the embryoid bodies were cultured with BMP4, Fgf7, Fgf10 and EGF.

Antibodies

For flowcytometry the following antibodies were used: rat anti-EpCAM APC-Cy7 (Biolegend, San Diego, CA, USA).

Flow cytometric analysis

Flowcytometric analysis was performed on a LSRII Flow Cytometer and analyzed using FACS-Diva software. Cell isolations were performed on a FACSAria Cell sorter (BD).

PCR

mRNA was isolated with an RNA easy kit (Qiagen) and reverse transcription was done with random hexamer primers (Invitrogen). An F-415L DyNAmo Flash SYBR Green qPCR kit (Finnzymes) and 7500 Fast Real-Time PCR system (Applied Biosystems) were used for qPCR.

Primers

The following primers were used: BMP4 (FW: 5'-TTCCATCACGAAGAACATC-'3, REV:5'-GGGCTTCATAACCTCATAAA'3), DLL4 (FW: 5'-GGGAACCTTCTCACTCAAC-'3, REV: 5'-GGAGCCTTGGATGATGATTT-3'), CCL25 (FW: 5'-CTGGTTGCCTGTTTTGTT-3', REV: 5'-AGGCAGCAGTCTTCAAAG-3'), EpCAM (FW: 5'-ATGCAAAACTCTTCTCAGAAA-3', REV: 5'-GTCCATGCTCTTAGAAGAATG-3'), PAX9 (FW: 5'-ATCCGACCTTGTGACATC-3', REV: 5'-GGTCCCTCTGCTTGTAAGT-3'), FoxN1 (FW: 5'-TGGGCTCACCTCACTATC-3', REV: 5'-AGGCTTCCGGTCTTACTG-3'), Cyclo (FW: 5'-AACCCCACCGTGTTCT-3', REV: 5'-CATTATGGCGTGTAA AGTCA-3')

Conflict of interest

The authors have no competing financial interest.

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6

GENERAL DISCUSSION

Thymic regenerative therapy

Although the thymus is the only site where T-cells are being generated and educated to ensure lifelong T-cell dependent immune competence, the thymus already starts to involute before adulthood. Involution of the thymus coincides with a gradual decline of thymopoiesis and with narrowing of the T-cell repertoire, which is a well known problem in aged men and mice (reviewed in: (Aspinall et al., 2010; Lynch et al., 2009; Taub and Longo, 2005). In mice, thymic cellularity declines over age, which coincide, with epithelial to mesenchymal transition (Youm et al., 2009). In humans the overall thymic size might change little in time as assessed by radiographic imaging, but the cellular content does change, including increase of adipose tissue and perivascular space. Thymic involution in humans is linked to an impaired immunocompetence, predisposing for opportunistic infections and an increase of autoimmune disorders. Impaired immune competence in the elderly is characterized by a reduction of various thymocyte populations, resulting in a decrease in T cell repertoire diversity and less recent thymic emigrants (RTE) in the periphery (reviewed in (Lynch et al., 2009). The presence of RTE in the peripheral blood reflects active thymopiesis and T-cells that contain so-called "T-cell receptor excision circles (TRECs)" are considered among the most immature peripheral T-cells that recently re-arranged their genes encoding the T-cell receptor and generated TRECs during that process. Therefore, TREC-positive T-cells are considered immature T-cells, which recently egressed from the thymus (Kong et al., 1998).

Older patients that acquire an immune deficiency as a result of infections (e.g. HIV-disease) or as a result of intensive cancer treatment may experience an even more immunocompromised condition. An extreme example of the latter category includes adult recipients of allogeneic hematopoietic stem cell grafts, pre-treated with myeloablative conditioning. Their (partially) involuted thymus may be additionally damaged by high dose chemo-radiotherapy and by graft versus host disease, initiated by donor T-cells that react with foreign minor and/or major histocompatibility antigens expressed by recipient thymic epithelial cells. As a result, T-cell recovery after allogeneic hematopoietic stem cell transplantation (HSCT) is extremely slow, which may extend up to two years after transplantation, while neutrophil recovery is established within 1-2 weeks after transplantation (Wils et al., 2011). A protracted T-cell recovery is especially evident in recipients of T-cell depleted hematopoietic stem cell grafts and in recipients of umbilical cord blood grafts, which grafts usually contain less than 10-5 T-cells per Kg bodyweight (Cornelissen and Lowenberg, 2000)

. While patients receiving T-cell repleted grafts may show better T-cell recovery, their T-cell repertoire may be restricted due to strong peripheral expansion of limited numbers of T-cell clones. During the period of T-cell lymphopenia, patients are highly susceptible for opportunistic infections and treatment related mortality after transplantation is largely accounted for opportunistic viral and bacterial infections. Patients exhibiting the highest susceptibility to opportunistic infections are those without signs of thymic recovery, as indicated by absence of peripheral T-cells that contain TRECs (Wils et al., 2011). Especially older recipients of hematopoietic stem cell grafts are highly susceptible to severe opportunistic infections and would be

candidates for thymic regenerative therapy. While translational research has been undertaken to improve thymopoiesis by cytokine therapy, including IL-7 and stem cell factor (SCF), results so far were not very encouraging (reviewed in (Wils and Cornelissen, 2005).

In contrast, thymic therapy has been succesfull in patients with the DiGeorge syndrome (Markert et al., 1997). Patients within this group have a microdeletion in chromosome 22 encoding for the gene TBX1 which is important for the normal formation of the face, cardiac tissue, parathyroid and thymus. These patients are partially or completely devoid of thymic tissue (Kobrynski and Sullivan, 2007) and have a severely hampered immune system due to the lack of adult T cells. Transplantation of cultured postnatal thymic tissue into the quadriceps muscle restored thymopoiesis with restoration of peripheral T-cell counts and T cell proliferative responses (Markert et al., 1999; Markert et al., 1997). Surviving patients showed a restored immune function and survived longterm up to > 10 years from transplantation. At present, 44 complete DiGeorge patients in total were treated by an allogeneic transplantation using completely mismatched third party thymic tissue. Currently, 33 patients out of these 44 are alive and well with restored immune function. Eleven patients died, including 5 deaths due to opportunistic infections, 5 deaths due to sepsis or progressive bronchopulmonary dysplasia and 1 from complications of calcium therapy during the first year after transplantation (Markert et al., 2007).

Successful transplantations could be characterized by diverse T cell repertoires, presence of an increased number of CD4 and CD8 T cells and a normal proliferative T cell function (Markert et al., 2007). Transplantation of cultured thymic tissue has been extended to patients with a FOXN1 deficiency as well. Also here, patients developed functional immunity (Markert et al., 2011). However, in both studies some subjects developed autoreactive T-cell clones after transplantation, which could be ascribed to host derived T cells, educated on donor derived, HLA mismatched, thymic epithelial cells (TECs). Collectively, transplantation of third party cultured thymic tissue to patients that completely lack a functional thymus is associated with favourable engraftment and restoration of thymopiesis and T-cell dependent immunity. However, it should be stressed that these studies were performed in patients without residual T-cell immunity, whereas the majority of elder patients with insufficient thymopoiesis still contain memory T-cells and even residual thymopoiesis that may initiate an alloreactive immune response towards transplanted tissue, especially if completely mismatched. Therefore, other approaches have to be developed for older patients developing thymic insufficiency as a result of intensive cancer treatment or infections

Future prospects for thymic regenerative therapy

Current approaches are focused on the development of regenerative cellular therapy directed at the epithelial compartment of the thymus and on improving thymic output. However, the field is still very much in a preclinical/fundamental phase with major challenges to be addressed. For example, assessing the efficacy of transplantation of epithelial tissue or cells in experimental in-vivo studies in animal models are complicated, because it necessitates to monitor engraftment

and recovery of transplanted epithelium apart from monitoring thymopoiesis. While recapitulation of thymopoiesis can be monitored in the peripheral blood by quantifying RTE's, imaging of transplanted tissue or cells in, for example, the residual thymus is extremely difficult. Residual thymic tissue might possibly recover itself and non-epithelial cells that reside in the thymus may possibly affect the survival and outgrowth of transplanted TECs (Bockman and Kirby, 1984; Jenkinson et al., 2007). Furthermore, the amount of TECs present in the thymus is important for the amount of naïve T cells that can be produced. Most thymocytes in the murine thymus can be found at 4 weeks, the time point at which the number of TECs is highest (Gray et al., 2006). Experiments focused on a higher thymic output by providing extra growth factors can work for a brief period (reviewed in: (Chidgey et al., 2008; Hollander et al., 2010; Wils and Cornelissen, 2005), but to establish a long term recovery of thymopoiesis the size of the TEC compartment has to be improved as well. Therefore it is of importance that future experiments are also focused on the retrieval of transplanted (progenitor) TECs and outgrowth of the TEC compartment.

Epithelial sources

Next to using thymic tissue from an unrelated donor as a source for thymic reconstitution, generating thymic tissue ex-vivo from adult thymi and other epithelial sources have been explored in a variety of experimental settings. Adult TECs are organized in a 3 dimensional (3D) architecture when present in the thymus. It provided the incentive to use matrices to provide a 3D backbone for mature murine TECs when cultured ex-vivo. Upon addition of bone-marrow derived hematopoietic stem cells (HSC) to the culture, functional mature T cells were generated (Poznansky et al., 2000). The use of cells from other epithelial organs as replacement for thymic tissue has been reported as well. Fibroblasts and epithelial cells from the skin were used as a source and seeded on a matrix. The skin epithelial cells were able to adapt towards a 3D phenotype and expression of different TEC markers like AIRE, Foxn1 and Hoxa3 could be detected. HSC that were cultured in the presence of the skin cells differentiated towards the T lineage, contained TRECs, had a diverse T cell repertoire and were tolerant to self MHC (Clark et al., 2005). Whether the epithelium within the matrices was able to support thymopoiesis for a prolonged period when transplanted into immunocompromised mice or patients remains unknown. These findings initially evoked enthusiasm for clinical applications, suggesting that skin cells of patients, who underwent hematopoietic stem cell transplantation (HSCT), may possibly be used as a source for thymic therapy, which could circumvent the problem of HLA mismatch. However, other researchers tried to repeat the protocol that was used by Clark et al. but failed to efficiently grow skin epithelium from primary material, and were therefore unable to differentiate HSCs towards the T cell lineage (Meek et al., 2011).

Alternatively, several research groups have explored whether mature TECs could be generated with different sources of progenitor cells as a starting population. Such approaches, however, heavily depend on detailed knowledge of the physiological development of thymic epithelium in the murine and human embryo. In Chapter 2 of this thesis we focused on the development of the human fetal thymus. The thymic anlage could first be identified at mid week 6 of embryonic

development by the expression of HOXA3, PAX1, PAX9 and FOXN1 in the third pharyngeal pouch endoderm. It means that also in humans the thymic primordium is solely derived from the third pharyngeal pouch endoderm. Immigration of early hematopoietic, thymic progenitors occurred early mid week 7 and the separation of a cortex and medulla was finished at mid week 8 of development. It suggests, like in mice, there is a period during thymic development that the thymus contains a bipotential TEC (bTEC) progenitor that can differentiate into cTECs and mTECs. If the human bTEC progenitor needs to be isolated from fetal thymic tissue the largest purity can be obtained before mid week 8 of development, which is comparable with E12 in murine development. Expansion of this cell type ex-vivo can be considered as a good source for epithelial progenitor cells to be studied further and characterized at the molecular level. Collectively, our study in human embryos and other studies in the murine setting have suggested, that 1. thymic epithelium exclusively originates from 3d pharyngeal pouch endoderm (Gordon et al., 2004), 2. essential transcription factors include HOXA3, PAX1, PAX9 (reviewed in (Hollander et al., 2006), 3. the ultimate expression of FOXN1 provides proof of commitment to a thymic epithelial cell fate (Balciunaite et al., 2002; Gordon et al., 2001; Nehls et al., 1996; Nehls et al., 1994). Based on these studies, transplantation studies in experimental animals were performed with embryonic thymic epithelial progenitor cells, harvested at E12.5 of development. These studies demonstrated that a single bipotential TEC progenitor can give rise to cTECs and mTECs (Rossi et al., 2006). Thereby, these studies also generated the hypothesis, for translational studies, that mature thymic epithelium might be obtained upon transplantation of thymic epithelial progenitor cells (TEPC) in-vivo.

Following the delineation of the development of thymic epithelium in the embryo, several groups addressed the question whether TEPC could be generated ex-vivo using embryonic stem (ES) or induced pluripotent stem (iPS) cells. While a complete picture of all the growth factors involved in thymic organogenesis is still lacking, several researchers set out to explore a variety of differentiation protocols. Recently the group of Lai et al. successfully differentiated murine ES cells towards Foxn1 positive thymic epithelium by using the growth-factors Ffg7, Fgf10, BMP4 and EGF (Lai and Jin, 2009). The differentiated cells contributed to the generation of naïve T cells in a murine bone marrow transplantation setting upon transplantation of the ex-vivo differentiated cells into a thymic environment via intra-thymic injection (Lai et al., 2011). Whether the injected differentiated ES cells contributed actively to thymocyte selection or produced growth factors that enhanced the output of the thymus remains unknown. Therefore, new studies have to monitor the state of the TEPC, differentiated from ES cells, that were transplanted in the thymic environment. It should be kept in mind that transplantation of ES/IPS cell derived TEPCs will be followed by a timelag period needed for the progenitor cells to differentiate into mature TEC. In murine development this process may take up to 1.5 weeks, in humans it may take more than 4 weeks (this thesis; Chapter 2), which should then also be followed by a period of at least 2 week for establishing thymopoiesis and egress of thymocytes into the peripheral blood. Therefore, from a clinical perspective, such an approach should selectively be developed for patients with a severe and long lasting deficiency of T-cell recovery. For example, older recipients of HSCT

without any evidence of thymopoiesis by 6-12 months after HSCT might be candidates for such thymic regenerative therapeutic approaches (Wils et al., 2011).

Use of human ES cells is still an ethical dilemma and in some countries human ES cells are forbidden for use in experimental settings, which impairs potential progress in this field. In addition, various human ES cell lines may differ with respect to growth and differentiation characteristics, including the differentiation efficiency towards endoderm derived tissues. Currently, it is unknown how efficiently available human ES cell lines differentiate towards TEPC and to what extent it may quantitatively differ between these lines. Differentiated cells or tissue would also need to be HLA-matched in order to prevent a host vs graft reaction (rejection) by the residual immune system of the patient. Both prerequisites may limit the number of available human ES cell lines that could potentially serve for thymic regenerative therapy.

Alternatively, iPS cells might provide us with a source of autologous progenitor cells that are completely matched and also provide the advantage of using mature cells from the patient which is not restricted by ethical boundaries. With the introduction of IPS cells it became possible to induce stem cells from somatic cells by introducing the factors Klf4, Oct3/4, c-myc and Sox2 (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). The method may now enable researchers to create TECs from either autologous or HLA-matched donor tissue, optimizing acceptance of the graft after HSCT. However, ES and IPS cells have the capacity to differentiate into all three primary germ layers. If ES and IPS cells are not properly differentiated in-vitro and still retain stem cell abilities, they might generate teratomas. Before such applications can be tested in clinical trials, exclusion of teratoma potential of the in-vitro differentiated ES or iPS cells is essential. Therefore, the identification of surface markers to purify differentiated ES and iPS cells that cannot form teratomas is crucial. In Chapter 5 of this thesis we repeated the Lai experiments in-vitro. We consistently observed upregulation of Foxn1 upon differentiation but as these differentiations were done in bulk cultures we were unable to use these cells for in-vivo experiments. With the use of Foxn1:EGFP reporter IPS cells it has become possible to observe the population that differentiated towards a thymic fate. And indeed, upon differentiation a population of 16% of EpCAM positive cells expressed EGFP. These results are currently followed by sorting of the EGFP-positive population and subsequent functional in-vivo studies, focussed on restoration of thymopoiesis.

Hematopoietic sources

Apart from thymic regenerative cellular therapy, an alternative approach to supplement lymphopenic patients with T-cells is to generate functional T cells in-vitro which might eventually be infused into the patient. In the last few years, researchers focussed on the minimal requirements that are necessary for T cell differentiation in-vitro. The initial idea came with the observation that the thymic microenvironment can be partially mimicked by stromal cell lines. The OP9 stromal cell line is derived from the calvaria (Nakano et al., 1994) of macrophage colony stimulating factor deficient newborn mice (Yoshida et al., 1990). When the Notch ligand Dll1 is transduced

into the OP9 stromal cells it supports T cell lymphopoiesis in-vitro until the double positive (DP) stage and less efficient to the CD8 single positive (SP) stage (Schmitt and Zuniga-Pflucker, 2002). Transplantation of OP9-Dll1 derived DN2 T cell precursors together with T cell depleted BM or purified HSC to lethally irradiated allogeneic mice resulted in an enhancement in T cell recovery (Zakrzewski et al., 2006). A similar observation was done using human hematopoietic progenitors cells from cord blood, that were differentiated in-vitro up to a differentiation stage of pro T cells. When co-cultured with OP9-Dll1, these cells were able to efficiently colonize the thymus of immunodeficient mice (Awong et al., 2009). The same results were obtained with mobilized peripheral blood stem cells that were co-cultured with the stromal cell line TSt-4 (Watanabe et al., 1992), expressing either Dll1 or Dll4 (Meek et al., 2010). These results indicate that T cell development can partially be reconstructed with the use of stromal feeders together with the ligand Dll1 or Dll4. Notch signaling is important for the initial steps of thymopoiesis facilitating survival and proliferation of DN1, DN2 and DN3 thymocytes (Sambandam et al., 2005; Tan et al., 2005) and commitment of early thymic progenitors towards the T lineage (Pui et al., 1999). At least for 'priming' towards the T cell lineage, the expedite recovery of thymopoiesis suggests interesting features for future regenerative therapy. However, whether all the necessary steps (including positive and negative selection) for T cell production can eventually be induced in-vitro remains an intriguing scientific challenge.

Future directions for thymic research

The search for the putative thymic epithelial stem cell that is at the basis of all TEC compartments in the fetal and adult thymus is still ongoing, but so far this search has been complicated. A bonafide stem cell should exhibit both differentiation potential to all adult epithelial progeny and stem cell renewal capacity. The fetal epithelial progenitor cell described in the murine setting by a number of investigators, does exhibit the potential to differentiate into all mature epithelial lineages, but that progenitor cell has not been demonstrated to renew itself in experimental settings (Bleul et al., 2006; Rossi et al., 2006). The human fetal progenitor cells decribed by us in Chapter 2 seems largely similar to its murine counterpart and self-renewal capacity of that progenitor cell also remains to be established. With respect to the thymic epithelium after birth, so far progenitor cells as were demonstrated in fetal life have not been demonstrated. Thus, evidence is currently lacking, that a thymic epithelial progenitor cell is present in the adult thymus and it might well be that an adult progenitor cell that is able to regenerate a whole thymus does not exist. Circumstantial evidence for the absence of such a progenitor cell also comes from the observation that thymic involution seems to be an irreversible process without intrinsic regenerative capacity. Involution of the thymus also occurs in humans although the process takes much longer. The onset of involution starts from the first year of life and in elderly the thymus mainly consists of fat and perivascular space, filled by mesenchymal cells (Steinmann et al., 1985). However, a recent study performed by Gent et al. shows that thymic tissue may regenerate after thymectomy, if performed at an early age (before 1.5 years). Thymic regeneration coincided with restoration of the naïve CD4 and CD8 T cell pool and restoration of TREC numbers (van Gent et al., 2011).

That observation could indicate that the TEPC are still present in humans after birth until the start of thymic involution. Once this pool of TEPCs is 'exhausted' it can no longer replenish the adult TECs and involution may start. Such a time frame may differ from that in mice, as the size of the progenitor pool in the murine embryo is decisive for the thymic size of the postnatal murine thymus (Jenkinson et al., 2008). Still, more information about fetal TEPC should be obtained in order to exclude its presence in the adult murine thymus.

Current knowledge of thymic development does not allow elegant studies in which single thymic epithelial cells can be studied ex-vivo. The field of thymus biology is restricted to in-vivo experiments as there is no available TEC cell line that truly represents the characteristics of TECs in-vivo. Moreover, once TECs are isolated from their natural environment and put into culture they lose their functional capacities and start to transdifferentiate towards mesenchymal cells by the process called epithelial-mesenchymal-transition (Youm et al., 2009). In-vivo experiments that are focused on TECs remain complicated because of non-epithelial cells (e.g. hematopoietic cells and mesenchymal cells) that influence the TECs. Therefore, there is an urge for finding new ways to address fundamental research questions within thymus biology. A major challenge in the field of thymus biology is the development of a culture system in which TECs can be functionally maintained and expanded. Several key issues should be resolved: 1. which factors retain bipotential (b)TEC progenitors in their proliferative non-differentiating state? 2. Does the availability of these factors decline over time and thereby promote thymic involution? 3. Which signals are involved in the lineage choice of bTEC progenitors towards medulla and cortex? 4. Which factors influence the homeostatic hierarchical balance between the different types of mTECs and cTECs? The successful protocol of the Clevers lab (Sato et al., 2009) clearly shows that it is possible to culture epithelial stem cells of the gut without the presence of mesenchymal cells as support. However, the difference between the thymus and gut is the high turnover of gut epithelium that is maintained throughout an individual's life, the physical presence of a known niche for stem cells in the gut and the plethora of available information of epithelial stem cells of the gut. The TEC population that is taken as a starting point of the culture is important for the growth factors that will be necessary to maintain TECs ex-vivo. The factors that are involved in the continuation of bTEC progenitors and adult TECs are largely uncovered and this will be a major challenge for designing a culture system for TECs. Collectively, the still incompletely understood differentiation process of immature progenitor cells towards mature medullary and cortical thymic epithelium, the absence of a bona-fide thymic epithelial stem cell, and the absence of a robust culture system to propagate both immature and mature thymic epithelial cells in-vitro preclude a swift transition of experimental studies towards translational studies with a clinical perspective on the horizon. Therefore, it is expected that research endeavours in the coming decade will still be focussed on unravelling the complete picture of proliferation and differentiation of thymic epithelial (progenitor) cells.

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SUMMARY

In vertebrates, including humans, the immune system takes care of the defence against harmful pathogens (like bacteria, viruses and parasites) that infect the body. The immune system can be divided in two major subsets, the innate immune system, which is active once we are born and is able to quickly recognize and kill pathogens and the adaptive immune system that is slower but more specific and eventually more specific. The adaptive immune response also makes a memory of the last infection in order to make a quicker response once the same pathogen tries to infect the body again. The adaptive immune response contains many cell types with individual functions that are important for the whole system. The T cells comprise one important subset of the adaptive immune response. Patients with a severe defect or absence of T cells will be more prone to opportunistic infections which can be resembled with patients who suffer from the acquired immune deficiency syndrome (AIDS). All cell populations of the immune system develop in the bone marrow, except T cells, they develop in the thymus. The precursors of T cell is derived from the bone marrow and migrates towards thymus where it will mature in about 3 weeks to eventually leave the thymus and leave to the peripheral organs. The development of T cells in the thymus (thymocytes) can only occur through interactions with epithelial cells that reside in the thymus.

In newborn only the innate immune system is active and the adaptive immune system still needs to develop. In this period the thymus is most active because all the T cells that are necessary for the adaptive immune system need to be produced. Until puberty the thymus will be most active, from that moment it will only decrease in size and less T cell will be produced, this process is called thymic involution. In elderly the size of the thymus is very small and the organ itself is not very active anymore. This can give problems as their immune system weakened and is not as active as in young people. The problem can even become worse in leukemic patients that receive bone marrow or cord blood transplantation. Prior to the transplantation, these patients receive chemo- and irradiation therapy. This does not only kill the leukemic cells, but also the healthy cells of their immune system. The immune cells that are produced in the bone marrow will recover within weeks, however T cells that need to develop in the thymus remain absent for a prolonged period. There are enough progenitor cells in the bone marrow ready to colonize the thymus, but the thymic function itself remains impaired due to thymic involution. It might take up to two years before the T cells return to their normal levels. Within this period the patients have a severely hampered immune system and are very prone to opportunistic infections. For this group of patients it is important that a therapy becomes available that can restore the thymic function.

A lot of information about the thymus became available over the past years. The epithelial cells in the thymus make sure that every T cell that leaves the thymus recognizes a pathogen derived antigen and not a self antigen of the host. If T cells specific for self antigens have a chance to leave the thymus they can play a role in the development of auto immune diseases.

In this thesis we focused on the epithelial cells of the thymus. Over the recent years a lot has been published on thymus development and functionality, we summarized the most important advances in **chapter 1** of this thesis. The thymus is solely derived from the third pharyngeal pouch endoderm. The transcription factor FOXN1 is crucial for thymic development and function of thymic epithelium, mice and humans deficient for FOXN1 lack a functional thymus. The epithelium can be divided in two major compartments, the cortex and the medulla. Both cell types are derived from the same precursor cell, which is only present in the fetal thymus. The cortex and medulla develop soon after the first thymocytes enter the thymus. Thymocytes produce factors that are important for the development of thymic epithelial cells, indicating that the two populations important for each other's development. Most knowledge about thymic development has been obtained from thorough analysis on mouse embryos. There is not much information available on human thymic development. In **chapter 2** of this thesis we conducted a detailed analysis of early human thymus organogenesis. We showed that the expression pattern of important regulators in mouse organogenesis also occured in humans. The key regulator FOXN1 was expressed in the 3rd pharyngeal pouch and not the 4th pharyngeal pouch. This indicated that also the human thymus was solely derived from the 3rd pharyngeal pouch endoderm. Colonization of the first hematopoietic cells started from week 7 of gestation. Furthermore, we established the presence of a separate cortex and medulla at week 8 of gestation. Our results indicated that thymic organogenesis in humans showed the same molecular and cellular events as in the mouse, confirming that the mouse is a good model to validate human thymic organogenesis

The epithelial cells of the thymus have in comparison with epithelial cells of other organs (like the skin or intestine) a 3-dimensional arrangement. This arrangement allows thymic epithelial cells to form a sponge like network. In this way, thymocytes that migrate through the thymus can contact as many epithelial cells as possible in a short period. The thymus also harbours epithelial cells that do not contribute to a 3-dimensional network; instead they form 2-dimensional structures that can be found in the skin and intestine. These structures have been described as Hassle Corpuscle's and thymic cysts. For a long period researchers thought that the 2-dimensional structures followed a 'default' differentiation program during thymus organogenesis which was different from the thymic differentiation program which included expression of FOXN1. In **chapter 3** of this thesis we focussed on the development of thymic cysts. In our studies we took advantage of the Fox1-Cre x Stop^{flox}-GFP mouse model. This model enabled us to visualize all the cells that expressed FOXN1 in the past. This taught us that epithelial cells that followed a thymus specific differentiation program were present in thymic cysts. Morever, when we looked at active expression of Foxn1 in cyst lining cells with an antibody directed against Foxn1 of the Foxn1:GFP reporter mouse model we could clearly detect epithelial cells that actively expressed the Foxn1 protein. This indicated that thymic epithelial cells took part of thymic cysts regardless of their Foxn1 expression. In chapter 3 we have shown that even if thymic epithelial cells do not have a 3-dimensional conformation, they still belonged to the thymus microenvironment. The physiological role of thymic cysts remains unknown.

Thymic involution is a major problem for elderly and patients undergoing treatment for leukemia. This group could benefit from therapy in which thymic tissue would be regenerated. For several epithelial organs like the skin and intestine it is known that there are stem cells present that replace the old cells for young cells. It has been shown in mouse models that these cells can be found in a protective environment, the so called niche, and which markers stem cells express. A couple of years ago Lgr5 was identified as a marker for epithelial stem cells of the intestine. Researchers were able to isolate the Lgr5 positive stem cells from the murine intestine and culture them. It turned out that also in-vitro these cells had the capability to grow as their original structure as in the mouse.

In the adult thymus it is unknown whether epithelial stem cells exist and whether there is a niche that harbors them. In **chapter 4** of this thesis we have looked whether Lgr5 was expressed in thymic epithelial cells of the mouse and had the same stem cell capacity as in the intestine. It is known that epithelial stem cells are present during the fetal stage of thymic development. Therefore we first looked into the fetal thymus of Lgr5-GFP-IRES-CreERT2 reporter mice for the presence of Lgr5 positive epithelial cells. We could detect Lgr5 on 2% of all the thymic epithelial cells in the early fetal thymus, but when we looked at later stages we could no longer detect the Lgr5 positive cells. Next, we looked whether expression of the protein Lgr5 on early thymic epithelial cells had an important role for thymic development. We bred mice that were not able to produce the protein Lgr5, but as it turned out the epithelial and T cell compartment of the thymus developed normal. We finally looked whether the small population of thymic epithelial cells that was positive for Lgr5 during the embryonic phase of thymic development gave rise to a subset of thymic epithelial cells in the adult thymus. We bred Lgr5-GFP-IRES-CreERT2 together with Rosa26:YFP reporter mice. In the offspring of these mice we could lineage trace all the offspring of epithelial cells that expressed Lgr5 in the embryonic thymus. After careful analysis we concluded that the Lgr5 positive cells could not give rise to a specific thymic epithelial cell population in the fetal and adult thymus. In chapter 4 we have described that the stem cell marker Lgr5 is present in the fetal thymus but as the foetus ages the Lgr5 positive cells disappear. Therefore, Lgr5 is not a stem cell marker in the mouse thymus.

Another approach for thymic therapy is differentiation of embryonic and induced pluripotent stem cells (ES and iPS cells) towards thymic epithelium. ES and iPS cells can be inevitably cultured in-vitro and when put in the right conditions differentiated toward the three different germ layers (endoderm, ectoderm and mesoderm). In **chapter 5** of this thesis we have focused on the use of ES and iPS cells as a source for thymic stem cell therapy. We cultured different ES cell lines in the presence of thymic specific growth factors (BMP4, Fgf7, Fgf10 en EGF) and took FOXN1 as the readout for differentiation towards a thymic fate. We established an increase in FOXN1 expression with the Sox17 cell line, but it was still a 100-fold lower when we compared it with freshly isolated thymic epithelial cells. We found other important genes to become up regulated as well (PAX9, BMP4, Dll4 en CLL25), indicating that the ES cells differentiated towards a thymic fate. However, the low FOXN1 expression indicated that a majority of the cells in the culture differentiated towards a non-thymic fate. With these differentiation cultures we were not

able to isolate a pure fraction of ES cells that differentiated towards a thymic fate. Therefore we made a new iPS cell line from the Fox1:GFP reporter mouse. In these mice all cells that expressed FOXN1 also expressed the green fluorescent protein (GFP). We used this Foxn1:GFP iPS cell line in our differentiation culture and we could distinguish a GFP positive population from the rest of the cells in the culture. Further analysis is necessary to identify the functionality of this population.

The most important findings of this thesis indicate that the early event that occur during thymic development in the mouse strongly resemble the early events that occur in human thymic development. This makes the mouse a valid model to study thymic organogenesis. We have shown that thymic cysts, which have been regarded as an aberrant structure of the murine thymus, has a thymic origin and belongs to the normal cell population of the thymus. It remains unclear what the specific role they fulfil in the thymus. The stem cell marker Lgr5 was detected at an early stage of thymic development, however its existence did not indicate that it was the long sought thymic epithelial stem cell.

More studies with the aim to gain fundamental knowledge of thymus organogenesis, to understand whether there is a regenerative potential that can be explained by the presence of stem cells exist in the murine thymus, will be an easier way instead of testing the presence of stem cell marker of other organs in the thymus.

NEDERLANDSE SAMENVATTING

In gewervelde dieren, waaronder de mens, zijn er vele systemen, die het individu in zijn of haar omgeving laten gedijen. Een van deze systemen is het immuunsysteem wat van groot belang is voor de afweer tegen pathogene micro-organismen, waaronder bacteriën, virussen en parasieten, die het lichaam binnendringen en ziektes kunnen veroorzaken. Het immuunsysteem kan ingedeeld worden in twee onderdelen: de aangeboren immuniteit, wat het lichaam in staat stelt om indringers snel te identificeren en op te ruimen, en de verworven immuniteit, wat langzamer is maar specifieker en uiteindelijk efficienter. De laatstgenoemde tak maakt ook een geheugen aan, wat bij een herinfectie met hetzelfde micro-organisme een versnelde immuunrespons oplevert en het infectieuse agens nog sneller opruimt. De verworven immuniteit omvat vele celpopulaties met allemaal een eigen functie, die van groot belang zijn voor het afweersysteem als geheel.

T lymfocyten vormen een belangrijk onderdeel van de verworven immuniteit. Patienten met afwijkingen of afwezigheid van T lymfocyten hebben een ernstige deficiëntie van het immuunsysteem en zijn vatbaar voor opportunistische infecties. Een voorbeeld hiervan zijn patiënten, die na infectie met het humane immuundeficientie-virus (HIV) een zogenaamd Aquired Immune Deficiency Syndrome (AIDS) kunnen ontwikkelen, waarbij zij vatbaar zijn voor vele micro-organismen. Alle celpopulaties van het immuunsysteem ontstaan initieel in het beenmerg, maar B-cellen en T-cellen ondergaan verdere uitrijping en selectie elders in het lichaam. Voorloper lymfocyten kunnen zich alleen ontwikkelen tot rijpe T-cellen in de thymus (zwezerik). De onrijpe voorloper T cellen migreren vanuit het beenmerg via het bloed naar de thymus, alwaar ze binnen 3 weken verder uitrijpen tot volwassen T cel. Deze ontwikkeling gaat gepaard met een intensieve interactie tussen de ontwikkelende T cellen (thymocten) en de epitheelcellen van de thymus. De volwassen T cellen migreren tenslotte vanuit de thymus naar de verschillende organen in het lichaam om uiteindelijk een rol te spelen in de afweer tegen pathogene micro-organismen.

Bij pasgeborenen is met name het aangeboren immuunsysteem belangrijk, maar moet het verworven immuunsysteem zich nog ontwikkelen. Dit is het moment dat de meeste activiteit in de thymus plaatsvindt. Tot aan de puberteit is de thymus het meest actief, waarna de thymus in grootte afneemt en ook minder T cellen aanmaakt. Dit proces heet involutie. Bij ouderen is de thymus bijna niet meer actief en is vitaal thymus-epitheel zo goed als afwezig. Dit kan infectieuze problemen geven bij ouderen, met name als het T-cel repertoire ernstig versmalt is en er bovendien contact is met virulente micro-organismen. Het probleem wordt nog groter als volwassen patienten een beenmerg of navelstrengbloed transplantatie krijgen als behandeling voor leukemie. Voorafgaand aan de transplantatie krijgen deze patienten bestraling en chemotherapie, wat niet alleen de kankercellen doodt, maar ook gezonde cellen van het immuunsystem. De meeste cellen van het immuunsysteem kunnen binnen enkele weken na de bestralingstherapie weer in het beenmerg worden aangemaakt, maar de productie van nieuwe T cellen is ernstig vertraagd, met name doordat de thymus tijdens de involutie in grootte is afgenomen of geheel afwezig is. Het beenmerg bevat genoeg voorlopercellen die naar de thymus kunnen migreren, maar het epitheliale compartiment van de thymus is onvoldoende in staat de jonge thymocyten uit te laten

rijpen. Het kan tot wel twee jaar duren voordat patienten weer voldoende T cellen aanmaken en het immuunsysteem weer normaal functioneert. Tot die tijd zijn deze patiënten afhankelijk van de rijpe T-cellen, die met de beenmergtransplantatie werden meegegeven. Vaak zijn deze patienten zeer vatbaar voor opportunistische infecties. Voor deze groep patienten is het van groot belang dat er een therapie komt waarmee de funtie van hun thymus zo snel mogelijk weer herstelt. Er is al veel kennis over de thymus vergaard door verschillende wetenschappers, waarbij met name veel kennis ten aanzien van de T-cel ontwikkeling beschikbaar is gekomen, maar de biologie van de epitheelcellen in de thymus is een nog relatief onbekend terrein.

In dit proefschrift wordt onderzoek beschreven dat betrekking heeft op het epitheliale compartiment van de thymus. Een groot deel van wat er tot nu toe bekend is, wordt samengevat in **hoofdstuk 1** van dit proefschrift. Het thymusepitheel ontstaat uit 1 van de 3 kiembladen en wel uit het endoderm en heeft daarmee een strikt endodermale oorsprong, zoals ook bijvoorbeeld de darm en de longen. Thymusepitheel onstaat al relatief vroeg tijdens de embyogenese. Het gen FOXN1 is van cruciaal belang voor de ontwikkeling en functie van thymusepitheel, bij afwezigheid van dit gen ontstaat er geen thymus. Het epitheel kan opgedeeld worden in twee compartimenten, namelijk de cortex en de medulla. Beide populaties zijn afkomstig van 1 voorlopercel, waarvan verondersteld wordt dat die alleen aanwezig is tijdens de embryonale ontwikkeling. De cortex en medulla ontstaan tijdens de embryogenese, nadat de eerste thymocyten in de thymus arriveren. De thymocyten produceren ook factoren die van belang zijn voor de ontwikkeling van het thymusepitheel, waarmee er dus sprake is van wederzijdse beïnvloeding. De meeste kennis aangaande de ontwikkeling van thymusepitheel werd vergaard door zorgvuldige analyse van embryo's van muizen. Over de ontwikkeling van de humane thymus tijdens de embryogenese was nog weinig bekend. In **hoofdstuk 2** van dit proefschrift hebben we een nauwkeurige studie beschreven over de ontwikkeling van thymusepitheel in humane embryo's. Zo werd gevonden, dat het gen FOXN1 selectief tot expressie komt in het endoderm van de derde kiemboog, en dat ook in de mens thymusepitheel afkomstig is van endoderm. De aanwezigheid van thymocyten kon op week 7 worden aangetoond en de scheiding van de cortex en medulla vond plaats tijdens week 8 van de embryogenese. Deze studie laat zien, dat de humane thymus ontwikkeling grote overeenkomsten vertoont met de ontwikkeling in muizen. Dit geeft aan, dat de muis waarschijnlijk een relevant model is om vroege thymusorganogenese te bestuderen.

De epitheelcellen in de thymus hebben in tegenstelling tot epitheelcellen van andere organen (zoals de huid en darm) een speciale, 3-dimensionale vorm. Alle epitheelcellen samen vormen op die manier een netwerk waar de thymocyten doorheen migreren tijdens hun ontwikkeling. Op deze manier kunnen de thymocyten binnen een korte tijd veel interacties aangaan met zo veel mogelijk verschillende epitheelcellen. De thymus herbergt ook epitheelcellen die niet in staat zijn om een 3-dimensionale vorm aan te nemen. Deze cellen hebben een 2-dimensionale rangschikking en vormen structuren die ook aangetroffen worden in de huid en darm. Deze structuren zijn lang geleden al beschreven als Hassle's Corpuscles en thymale cysten. Eerder werd gesuggereerd, dat deze structuren een "fout" differentiatie-programma hadden doorgemaakt in plaats van een thymus-specifiek differentiatieprogramma tijdens de thymus organogenese. In **hoofdstuk 3** van dit proefschrift wordt ons onderzoek gepresenteerd naar de oorsprong van thymale cysten. Door gebruik te maken van het Fox1-Cre x Stop^{flox}-GFP muizenmodel waren we in staat om alle epitheelcellen te identificeren die een thymus-specifieke rijping ondergingen. Cellen die in het verleden Foxn1 tot expressie hadden gebracht produceren een groen fluorescerend eiwit (GFP) in dit model. De eptiheliale cellen die we terugvonden in de cysten brachten GFP tot expressie, wat erop duidde dat ze een thymus-specifieke rijping hadden ondergaan. Vervolgens hebben we gekeken of de epitheliale cellen in de cysten actief Foxn1 tot expressie brachten met een antilichaam tegen het eitwit Foxn1 en in een Foxn1:GFP verklikker muis. In beide gevallen zagen we dat sommige cellen nog actief Foxn1 tot expressie brachten, hetgeen aangeeft dat het niet uitmaakt of Foxn1 aan of uit moet staan om deel te nemen aan een cyste. In hoofdstuk 3 hebben we derhalve laten zien dat ook de epitheelcellen, die geen 3-dimensionale configuratie hebben, toch thuis horen in een thymus omgeving. De functie van dergelijke epitheelcellen is vooralsnog echter onbekend.

Involutie van de thymus blijft een groot probleem voor ouderen en patiënten, die een behandeling voor kanker ondergaan. Voor deze groep mensen zou een therapie gericht op het herstel van de thymusfunctie een uitkomst kunnen bieden. Van verschillende epitheliale organen (zoals de huid en darm) is bekend, dat er stamcellen aanwezig zijn, die met enige regelmaat jonge cellen aanmaken, die de oudere cellen kunnen vervangen. Deze stamcellen bevinden zich veelal in een zogenaamde "niche" of stamcelnis: een gespecialiseerde micro-omgeving, die cellen herbergt, die de stamcellen ondersteunen in hun functie en overleving. Met name is kennis vergaard ten aanzien van darmstamcellen, de darmstamcelnis, en de daarbij betrokken ondersteunende cellen. Enkele jaren geleden is de marker Lgr5 beschreven voor epitheliale stamcellen in de darm. De cellen, die Lgr5 tot expressie brengen, bevinden zich in de nis, en deze cellen konden vervolgens ook geisoleerd worden. Na isolatie bleek het mogelijk om deze voorlopercellen in een kweekschaaltje te laten uitgroeien tot rijp darmepitheel met ook een volwassen darmstructuur. In de volwassen thymus is het onbekend of er epitheliale stamcellen aanwezig zijn en of er een nis is, waar deze stamcellen zouden kunnen gedijen. Daar thymusepitheel dezelfde endodermale oorsprong heeft als darmepitheel, werd door ons onderzoek gedaan naar de aanwezigheid van Lgr5 positieve epitheelcellen in de thymus. De resultaten daarvan worden beschreven in **Hoofdstuk 4**. Eerst hebben we gezocht naar Lgr5 positieve epitheelcellen in de embryonale thymus van Lgr5-GFP-IRES-CreERT2 verklikker muizen. Lgr5 positiviteit kon waargenomen worden in ongeveer 2% van alle thymusepitheelcellen in de vroege embryo's, maar Lgr5 positieve cellen werden niet meer teruggevonden in oudere embryo's. Dit betekent dat de Lgr5 positieve epitheelcellen alleen maar aanwezig zijn in een heel vroeg stadium van de thymusontwikkeling. Vervolgens is geanalyseerd of het eiwit Lgr5 belangrijk is voor de ontwikkeling van de thymus. Hiervoor werd de productie van het eiwit uit gezet in alle cellen van het embryo. De embryonale en volwassen thymus van deze Lgr5 deficiente muizen vertoonde een normale structuur en bleek goed in staat om T celontwikkeling te ondersteunen. Tenslotte is bekeken of Lgr5 positieve cellen in de embryonale thymus een voorloper populatie vormen voor een subpopulatie in de volwassen thymus. Door Lgr5-GFP-IRES-CreERT2 muizen te kruisen met Rosa26:YFP muizen konden we

alle nakomelingen van de cellen die tijdens de embryogenese Lgr5 tot expressie hadden gebracht zichtbaar maken. Na zorgvuldige analyses op embryonale en jong volwassen thymi konden we geen epitheelcellen terugvinden, die nakomelingen waren van cellen tijdens de embryogenese Lgr5 positief waren. Derhalve moet geconcludeerd worden, dat ofschoon de stamcelmarker Lgr5 wel voorkomt in de embryonale thymus, Lgr5 echter geen epitheliale stamcellen in de thymus identificeert en de aanwezigheid van het eiwit ook niet belangrijk is voor de ontwikkeling van epitheel in de thymus.

Cellulaire therapie met het oog op thymusregeneratie kan bewerkstelligd worden door bijvoorbeeld gebruik te maken van thymusweefsel van een donor, hetgeen al op zeer bescheiden schaal wordt toegepast. Een alternatieve mogelijkheid zou de toepassing van stamceltherapie kunnen zijn, waarbij epitheelcellen gekweekt worden vanuit embryonale stamcellen (ES cellen) of vanuit stamcellen, die verkregen kunnen worden door middel van genetische manipulatie van volwassen weefsels, de zogenaamde geinduceerde pluripotente stamcellen (iPS cellen). Deze ES en iPS stamcellen kunnen oneindig veel delen in een kweekschaal en zijn in staat om uit te rijpen naar alle drie de verschillende kiembladen (ectoderm, endoderm en mesoderm). Als het mogelijk zou zijn om op efficiënte wijze ES cellen of iPS cellen in een kweekschaal richting thymusepitheel te dwingen, dan zou het gekweekte epitheel vervolgens in de toekomst misschien gebruikt kunnen gaan worden voor thymus regeneratieve therapie.

In **hoofdstuk 5** van dit proefschrift worden de resultaten beschreven van onderzoek naar de mogelijkheid om thymusepitheel te kweken vanuit ES en iPS cellen. Verschillende ES cellijnen werden getest op hun capaciteit om Fox1 tot expressie te brengen in de aanwezigheid van thymusspecifieke groeifactoren (BMP4, Fgf7, Fgf10 en EGF). De Sox17 cellijn bleek na kweken een duidelijk Foxn1 signaal te geven, maar kwantitatief nog steeds 100 keer minder dan normaal thymusepitheel. Andere belangrijke eiwitten voor de thymusfunctie (PAX9, BMP4, Dll4 en CLL25) bleken ook tot expressie te komen tijdens deze kweekcondities. De lage Foxn1 expressie betekent mogelijk, dat maar een deel van de cellen richting thymusepitheel differentieert. Het nadeel van deze kweekmethode is dat de Foxn1 positieve cellen niet gemarkeerd zijn en derhalve niet zichtbaar worden tijdens het kweken. Het bleek dus ook niet mogelijk om selectief de Foxn1 positieve cellen op te zuiveren. Daarop werd besloten stamcellen te verkrijgen van Foxn1:GFP verklikker muizen. Hiertoe werd een nieuwe iPS cellijn gemaakt vanuit fibroblasten van deze verklikkermuizen. Cellen, die Foxn1 tot expressie brengen tijdens de ontwikkeling, worden dan GFP positief, hetgeen de mogelijkheid biedt om die cellen te zuiveren en daarnaast ook de mogelijkheid biedt om het ontwikkelingsproces beter te kwantificeren. Inderdaad kon op deze manier een populatie verkregen worden, die zich duidelijk onderscheidde van andere gekweekte cellen op basis van GFP expressie. Momenteel wordt deze populatie verder functioneel gekarakteriseerd in het laboratorium.

Al met al laten onze bevindingen het toe om de volgende, meer algemene, conclusies te trekken. Ten eerste lijkt de muis een valide model voor thymusorganogenese, daar de ontwikkelingsstadia van de humane thymus grote overeenkomsten vertonen met die van de muis. Ten tweede moet aangenomen worden, dat thymale cysten, die eerder beschouwd werden als een

afwijkende structuur, toch behoren tot een normale celpopulatie in de thymus. Ten derde is gebleken, dat de stamcelmarker Lgr5, ofschoon aanwezig in de foetale thymus, hoogstwaarschijnlijk geen rol speelt bij de ontwikkeling van thymusepitheel. Ten vierde suggereren onze resulaten met pluripotente voorlopercellen, dat specifieke kweekcondities het inderdaad mogelijk maken om thymus epitheliale voorlopercellen te kweken, hetgeen van groot belang is voor de verdere ontwikkeling van thymus regeneratieve therapie. Tenslotte hebben ook onze resultaten, evenals die van vele collega's, het ontbreken van een bona-fide stamcel voor thymusepitheel aannemelijk gemaakt, maar andermaal gewezen op het (tijdelijke) bestaan van een voorlopercel tijdens de embryogenese, die de potentie heeft om uit te rijpen naar zowel medullair als corticaal thymusepitheel.

ABBREVIATIONS

2D	2 dimensional
3D	3 dimensional
ADAM	A Disintegrin and metalloproteinase
Aire	Autoimmune regulator
BMP	Bone metalloprotease
BRDU	Bromodeoxyuridine
bTEC	bipotential TEC
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
Cldn	Claudin
CS	Carnegie stage
cTEC	cortical TEC
dGUO	deoxyguanosine
DKK1	Dickkopf-related protein 1
Dll	Delta like ligand
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
E	Embryonic day
EGF	Epidermal growth factor
EpCAM	Epithelial cell adhesion molecule
ES	Embryoinic stem cell
ETP	Early thymic progenitor
Fgf	Fibroblast growth factor
Flt3L	fms-like tyrosine kinase receptor-3 ligand
Foxn1	Forkhead box protein N1
FTOC	Fetal thymic organ culture
Gcm	Glial cells missing
GFP	Green fluorescent protein
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
IL	Interleukin
iPS	Induced pluripotent stem cell
K	Keratin
KGF	Keratinocyte growth factor
Lgr	Leucine-rich repeat-containing G-protein coupled receptor

LiCl	Lithium chloride
LTi	Lymphoid tissue inducer
LTβR	Lymphotoxin β receptor
MHC	Major histocompatibility chain
mRNA	messenger Ribonucleic acid
mTEC	medullary TEC
NFκB	Nuclear Factor κB
PCR	Polymerase chain reaction
PE	Pharyngeal endoderm
Plet1	Placenta-expressed transcript 1
PP	Pharyngeal pouch
RAG	Reombination activating gene
RANK	Receptor activator of nuclear factor kappa-B
RT	Real time
RTE	Recent thymic emigrant
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SP	Single positive
TCR	T cell receptor
TEC	Thymic epithelial cell
TEPC	Thymic epithelial progenitor cell
TESC	Thymic epithelial stem cell
TRA	Tissue restricted antigen
TRANCE	TNF-related activation-induced cytokine
TREC	T cell receptor excision circle
UEA1	Ulex europaeus agglutinin-1
YFP	Yellow fluorescent protein

DANKWOORD

Eindelijk kan ik beginnen aan mijn dankwoord; het boekje is af. Na ruim 5 jaar kan ik met plezier terugkijken op mijn periode als AIO op de afdeling hematologie (onplezierige dingen vergeet je snel). Ik heb een hoop dingen kunnen leren en veel nieuwe mensen leren kennen. Ik wil alle mensen bedanken die zowel direct als indirect hebben bijgedragen aan de voltooiing van dit proefschrift. Graag wil ik de volgende mensen in het bijzonder bedanken.

Ten eerste wil ik **Tom** bedanken. Wat een toeval dat we elkaar vijf jaar geleden in Japan tegenkwamen. Ik kende je daarvoor al van een stage op de afdeling celbiologie en histologie op het AMC. Ik informeerde naar een promotieplek en twee maanden daarna kreeg ik een e-mail met de medeling dat er een vacature was vrijgekomen. Vrij snel waren de interviews via de telefoon of skype afgerond en boden jullie me een promotieplek aan. Bedankt voor de kennis die beschikbaar stelde en de coaching als co-promotor. Je deur stond altijd open als er problemen waren of een experiment een rare (en soms goede) uitkomst had. Je was altijd in voor een grap tijdens werkbesprekingen, ik hoop dat je dezelfde werkwijze blijft houden voor je toekomstige aio's.
Jan, bedankt voor de kans die je me hebt gegeven om me te bewijzen als promovendus. Onze solicitatieprocedure ging niet zoals gebruikelijk, maar toch heb je me een promotieplek aangeboden nav een skype-gesprek. De periode heeft pieken en dalen gekend, ik kan me goed herinneren dat je sommige dingen onacceptabel en waardeloos vond. Uiteindelijk heeft de samenwerking geleid tot een promotie dus er zijn ook veel dingen goed gegaan. Ik ben dankbaar voor de begeleiding die je me hebt gegeven gedurende de afgelopen vijf jaar. Vaak zat je vast in de kliniek maar toch probeerde je vaak tijd vrij te maken voor een discussie over experimenten of een hoofdstuk in dit proefschrift.
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Prof. dr. **Rudi Hendriks**, Prof. dr. **Ricardo Fodde** en Prof. dr. **Frank Staal**, bedankt voor het beoordelen van mijn manuscript en dat jullie zitting nemen in de kleine commissie. Prof. dr. **Joost Gribnau**, bedankt voor uw samenwerking op het iPS project en dat u zitting neemt in de grote commissie. Prof. dr. **Ivo Touw** wil ik bedanken voor de boeiende discussies tijdens de vrijdagochtend werkbespreking op de afdeling hematologie. Ik vind het leuk dat u als opponent

deelneemt in de grote commissie. Prof. dr. **Hergen Spits**, ik vind het een grote eer dat u als thymus-expert (samen met Willem van Ewijk) deelneemt aan de grote commissie.
Dear **Hiroshi**, thank you so much for your mentorship. It was not always easy to survive in your lab, but I have learned a lot and I will remember the time being in your lab as a great period. Even now we still have contact once in a while and you are still helping me out by giving experimental advise or by sending lab equipment. I should also mention the excellent drawing you made which is on the cover of this thesis. Good luck with your new lab in Kyoto and I hope we will meet each other again soon. I would also like to thank **Kyoko**, **Tomo (Ikawa-san)** and **Haruka (Wada-san)** for their help in the Kawamoto-lab. You guys taught me a lot and I am still using this knowledge. Hope to see you guys soon at a thymus meeting.
Aan wie ik ook veel te danken heb is mijn oud-stage begeleider **Eddy Wierenga**. Ik kwam bij je terecht als een groentje voor een master-stage op de afdeling celbiologie en histologie van het AMC. Mijn Elispot project verliep niet zoals verwacht maar ik vond je een enorm toffe begeleider. Ik had nog geen idee wat ik wilde met mijn opleiding, maar toen ik de afdeling verliet wist ik dat in de wetenschap verder wilde. Bedankt dat je me in die periode de juiste richting op hebt kunnen sturen.
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Bob Meek, wat ben je toch een fijne kerel. Ik heb je leren kennen in het RIKEN instituut in Yokohama. Helaas vertrok je snel na mijn aankomst maar je hielp me wel op weg in de eerste maanden. We hebben elkaar vaak genoeg nog in Nederland ontmoet, in Maastricht of Rotterdam. Ik wens je het allerbeste met je carrière bij Crucell in Leiden.
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Ik moet **Suming** natuurlijk ook nog bedanken. Je bent een enorme pessimist (volgens jezelf realist), zo erg dat het gewoon grappig is. We hebben er allemaal om kunnen lachen op de kopkamer. Met jou heb ik veel tijd doorgebracht bij de chinees onder het genot van een hoop dumplings. Hier konden we na het werk of in het weekend vaak stoom uitblazen en onze frustraties delen over promoveren. Ik heb er in ieder geval veel aan gehad, ik hoop jij ook. Ik zal nooit meer vergeten dat ik tegen jou en Menno dubbelde met tennis en jij Menno snoeihard op zijn oor serveerde. Veel sterkte met je promotie en je postdoc in Leiden.
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CURRICULUM VITAE

Eric Vroegindeweij is geboren op 19 Maart 1982 te Utrecht. Zijn jeugd heeft hij doorgebracht in Wijk bij Duurstede waar hij op de basisschool de Hoeksteen zat. Na het behalen van zijn HAVO diploma aan het Revium Lyceum in Doorn in 1999 heeft hij zijn studie voortgezet aan de Hogeschool van Utrecht waar hij Hogere Laboratorium Onderwijs heeft gevolgd. Als afsluiting van deze periode heeft hij stage gelopen in het Academisch Ziekenhuis van Paramaribo onder begeleiding van Drs. Kwok Wah Choy en het Rijks Instituut voor Volkgezondheid en Milieu (RIVM) te Bilthoven onder begeleiding van Drs. Titia Kortbeek en Dr. Joop Schellekens. Tijdens deze laatste stage heeft hij onderzoek gedaan naar immuunreacties van verschillende patienten samples op antigenen van verschillende stammen van de bacterie Borrelia, de verwekker van de ziekte van Lyme. Vervolgens is hij in 2003 gaan studeren aan de Vrije Universiteit van Amsterdam waar hij de master biomedische wetenschappen heeft gevolgd. Tijdens de colleges die hij hier kreeg is zijn interesse voor immunologie gewekt. Om meer ervaring in immunologisch onderzoek te krijgen besloot hij in januari 2006 aan de slag te gaan als research analist in het Research Center for Allergy and Immunology (RCAI) van het RIKEN instituut in Yokohama, Japan. Onder begeleiding van Dr. Hiroshi Kawamoto en Prof. Dr. Willem van Ewijk heeft hij onderzoek gedaan naar de oorsprong van thymale cysten. Vanaf juni 2007 werd vervolgens promotieonderzoek verricht op de afdeling Hematologie van de Erasmus Universiteit in Rotterdam onder begeleiding van Dr. Tom Cupedo en Prof. Dr. Jan Cornelissen. In Rotterdam heeft hij onderzoek gedaan naar thymusontwikkeling in de mens en muis en differentiatie van ES en iPS cellen naar thymusepitheel. Sinds April 2012 is hij werkzaam op de afdeling Immunolobiology aan het Joslin Diabetes Center, Boston,VS, in de groep van Dr. Thomas Serwold.

PUBLICATIONS

Albert Wölfler, Astrid A. Danen-van Oorschot, Jurgen R. Haanstra, Marijke Valkhof, Claudia Bodner, **Eric Vroegindeweij**, Paulette van Strien, Alexandra Novak, Tom Cupedo, Ivo P Touw.
Lineage-instructive function of C/EBPα in multipotent hematopoietic cells and early thymic progenitors. *Blood.* 2010 Nov 18;116(20):4116-25

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Mol Immunol. 2010 Feb;47(5):1106-13.

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Dynamics of thymus colonization and organogenesis in early human fetal development.
Submitted. ([*]) These authors contributed eaqually to the manuscript.

Eric Vroegindeweij, Irene van Mourik, Tom Cupedo and Jan J. Cornelissen.
Characterization of Lgr5 positive epithelial cells in the murine thymus.
Submitted.

PHD PORTFOLIO

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Research School: Molecular Medicine

PhD period: 25-06-2007 – 29-02-2012
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Courses		
• Course on Neuro-Immuno-Endocrinology(MM)	2008	1.8
• The Course Molecular Medicine (MM)	2008	1.8
• Article 9 Animal Course (Erasmus MC)	2007	4.2
Workshops and Seminars		
• 3rd Symposium & master classes on Mucosal Immunology (MM)	2008	0.5
• Guiding the Actions of the Immune System (NVVI)	2010	0.5
• Erasmus Hematology Lectures	2007-2012	3.0
Presentations		
• 9 Hematology Presentation	2007-2012	4.5
• 3 Journal Club Presentation	2007-2012	1.5
(Inter)national conferences		
• Rolduc Workshop on Thymocytes and T cell Biology, Kerkrade (oral)	2007	1.5
• Annual meeting of the Dutch Society for Immunology, Noordwijkerhout (poster)	2008	1.0
• Annual meeting of the Dutch Society for Immunology, Noordwijkerhout (oral)	2009	1.0
• The 3rd Dutch Hematology Congress, Papendal (oral)	2009	1.0
• 5th International workshop of Kyoto T Cell Conference, Kyoto, Japan (poster)	2009	2.0
• Annual meeting of the Dutch Society for Immunology, Noordwijkerhout (poster)	2010	1.0
• EUThyme-Rolduc Meeting, Noordwijkerhout (poster)	2011	1.5
• The 6th Dutch Hematology Congress, Papendal (oral)	2012	1.0
Teaching activities		
• Supervising HLO student	2009	7.0
• Organizing AIO-lunch	2010-2011	1.0
		35.8

CHAPTER 1

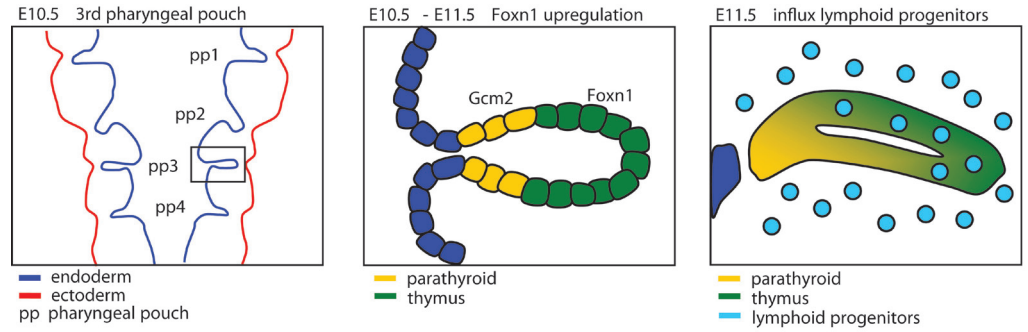


Figure 1: Early thymic development involves multiple steps. Development of the thymus starts at E10.5 and the thymic anlage is solely derived from the third pharyngeal pouch endoderm. The third pharyngeal pouch endoderm gives rise to the parathyroid, which can be identified by the transcription factor Gcm2, and the thymic primordium in which the crucial transcription factor Foxn1 becomes upregulated. At E11.5 of development the first hematopoietic progenitors will migrate into the thymic primordium and start to interact with the developing TECs which will be necessary for their development into SP T cells. From E13.5 the thymus will separate from the parathyroid and starts to descent from the pharyngeal region towards the chest cavity. The interaction between the hematopoietic progenitors and TECs is bi-directional as the hematopoietic cells will provide differentiation signals like TRANCE and Lt2β which will be important for the development of medullary TECs and the 3D orientation of TECs.

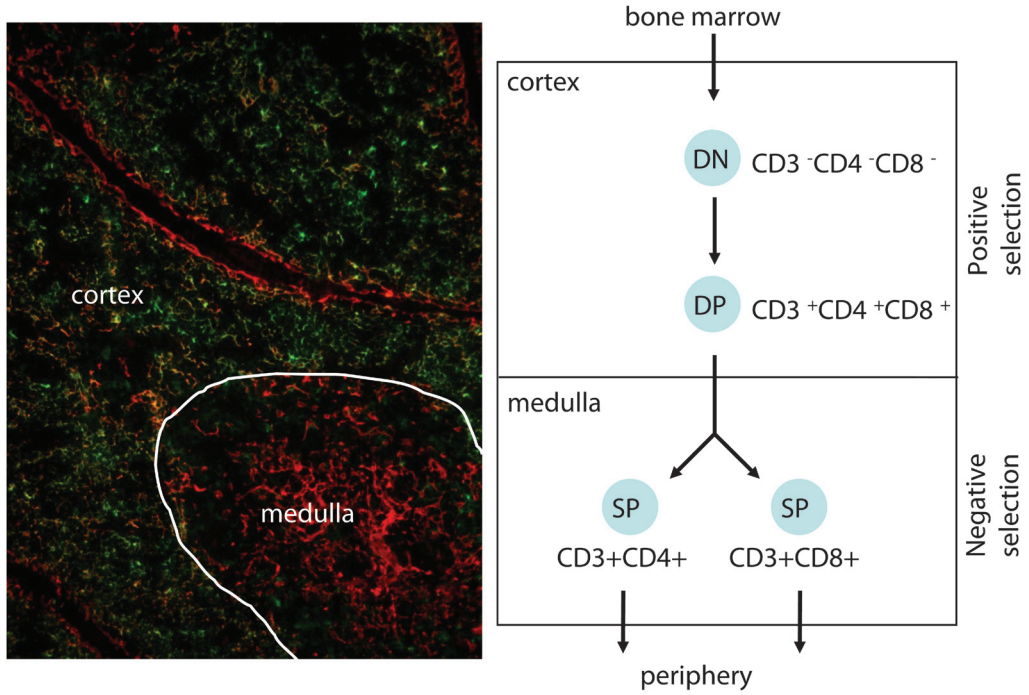


Figure 3: Thymocytes go through different developmental stages before they leave the thymus. Early thymic progenitors enter the thymus as double negative thymocytes (DN) at the cortico-medullary junction and migrate towards the cortex. Here they expand under the influence of different growth factors provided by cTECs and upregulate their CD3, CD4 and CD8 receptor to become double positive (DP) thymocytes. In the cortex thymocytes become selected for their T Cell Receptor (TCR) functionality, this process is called positive selection. From here the DP thymocytes will migrate towards the medulla where they will be downregulate their CD4 or CD8 receptor to become a single positive (SP) thymocyte. In the medulla SP thymocytes will become selected for their TCR specificity and affinity. If the specificity or affinity is incorrect the SP thymocytes will be negatively selected. When SP thymocytes successfully overcome negative selection they will leave the thymus and set for the periphery where they will exert their function as immune cells (adapted from Haynes et al.).

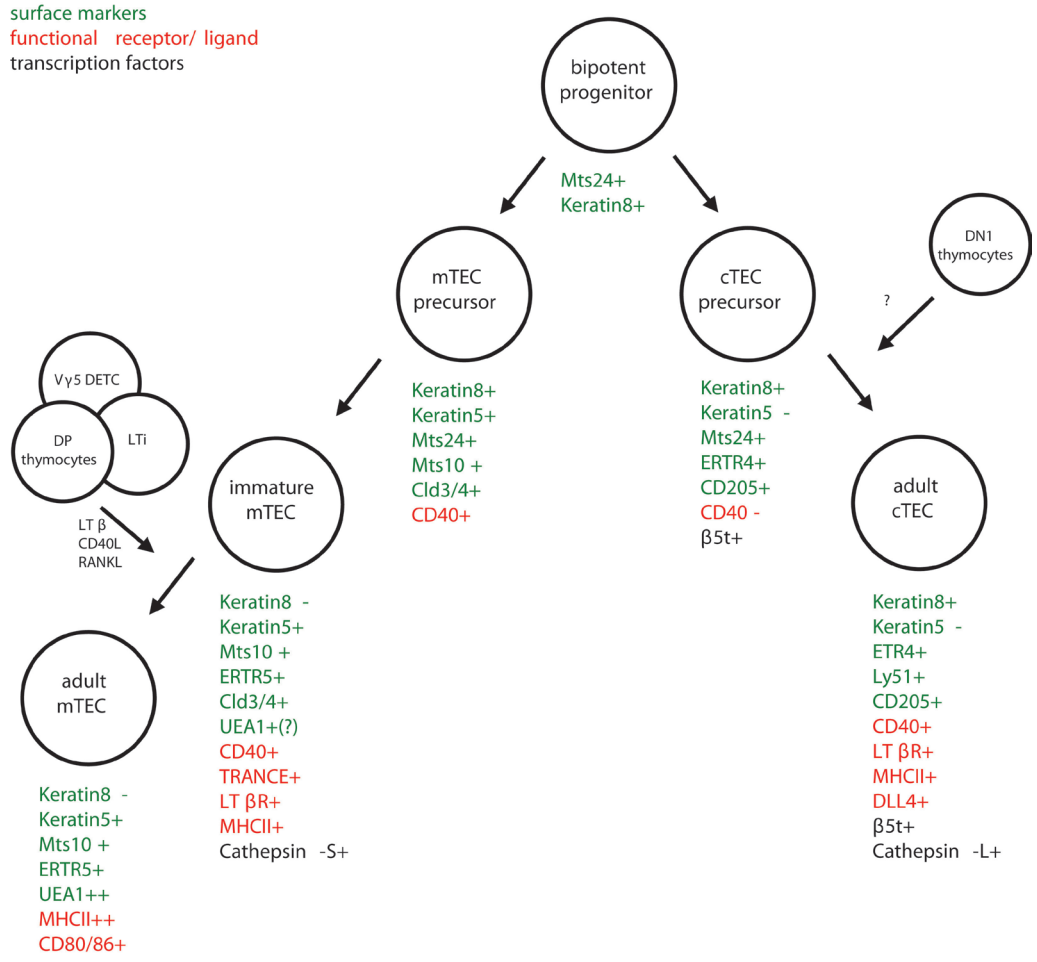


Figure 4: TECs go through different steps of differentiation and can be identified by a variety of markers. The earliest TEC that has been isolated, the bipotential progenitor, has the potential to develop into precursor cTECs and mTECs. The bipotential progenitor has only been identified at the fetal stage of thymic development (Rossi et al., 2006). Under the influence of hematopoietic cells mTECs and cTECs mature into TECs that efficiently present antigens on their MHC. mTECs need signals (RANKL, CD40L and LTβ), that are provided by LTi cells, Vγ5 dendritic epidermal T cells and thymocytes to upregulate Aire expression (Akiyama et al., 2008; Hikosaka et al., 2008; Irla et al., 2008; Lane et al., 2012; Roberts et al., 2012; Rossi et al., 2007c). **cTECs cannot develop beyond the precursor stage in the absence of DN1 tymo**cytes, however the signals involved in this process is unclear (Shakib et al., 2009).

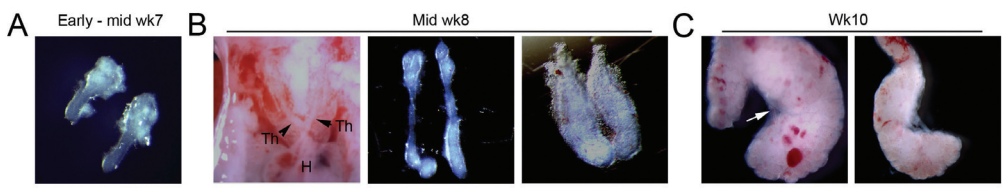


Figure 1: Morphological features of early human thymus development. (A-C) Images show microdissected human fetal thymic primordia at the ages shown, except for left hand image in (B) which shows anatomical details of the exposed chest cavity to indicate relative locations of thymic primordia, carotid artery and heart. Right hand image in (B) shows detail from lower part of thymic primordium (i.e. the leading edge during migration). Arrowheads indicate thymic primordia; white arrow in (C) indicates mesenchymal capsule. Images are representative of at least two independent analyses. Wk, week; Th, thymus; H, heart.

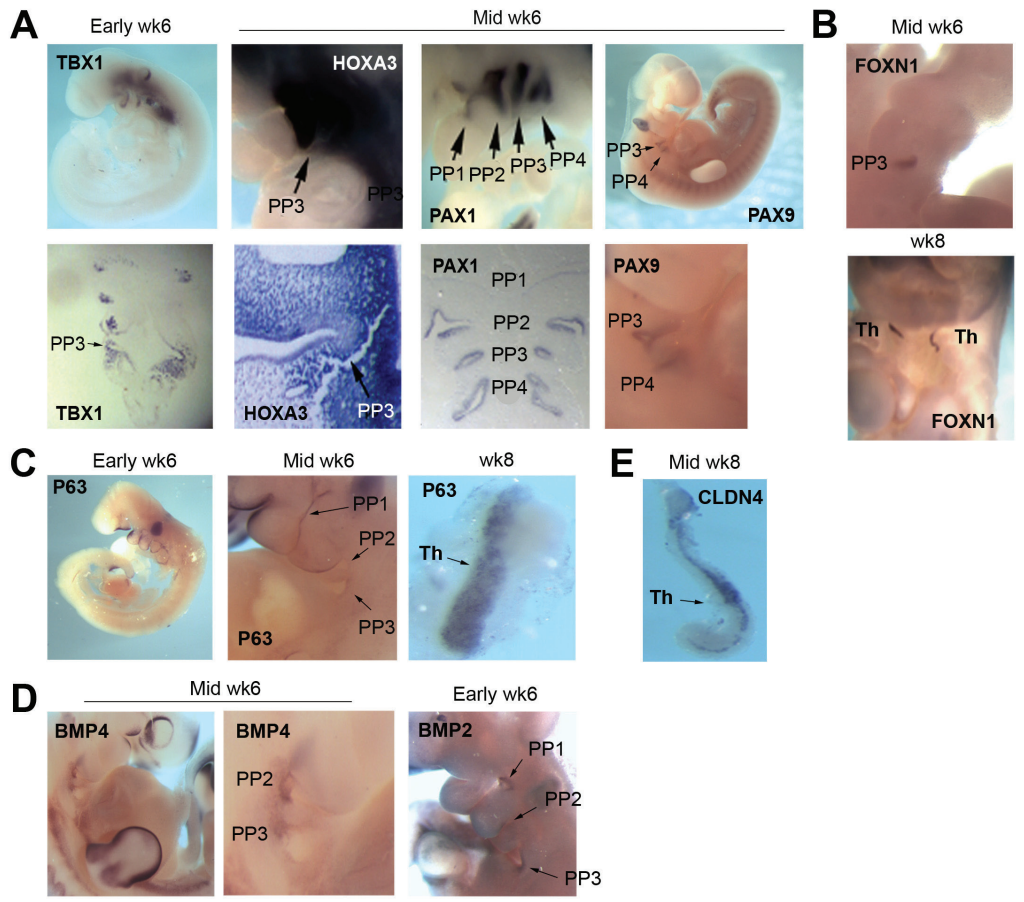


Figure 2: Expression profile of known regulators of mouse thymus organogenesis in human thymus development. Images show whole or sectioned human embryos or microdissected thymic primordia after ISH with the probes indicated. Ages of embryos or dissected thymus lobes are as shown. (A) *TBX1*, *HOXA3*, *PAX1* images show side view of whole embryos (top) and coronal sections (bottom). *PAX9*, images show side view of whole embryo; lower panel shows detail of upper panel. (B) *FOXN1*, images show ventral view of whole embryos (C) *P63,* images show side view of whole embryos (left and middle) and dissected thymic lobe. (D) *BMP4*, *BMP2,* images show side view of whole embryos. (E) *CLDN4,* images show dissected thymic lobe. Images are representative of at least two independent analyses. Th, thymus; ov, otic vesicle; PP1, 1st PP; PP2, 2nd PP; PP3, 3rd PP; PP4, 4th PP, pharyngeal pouch; Wk, week.

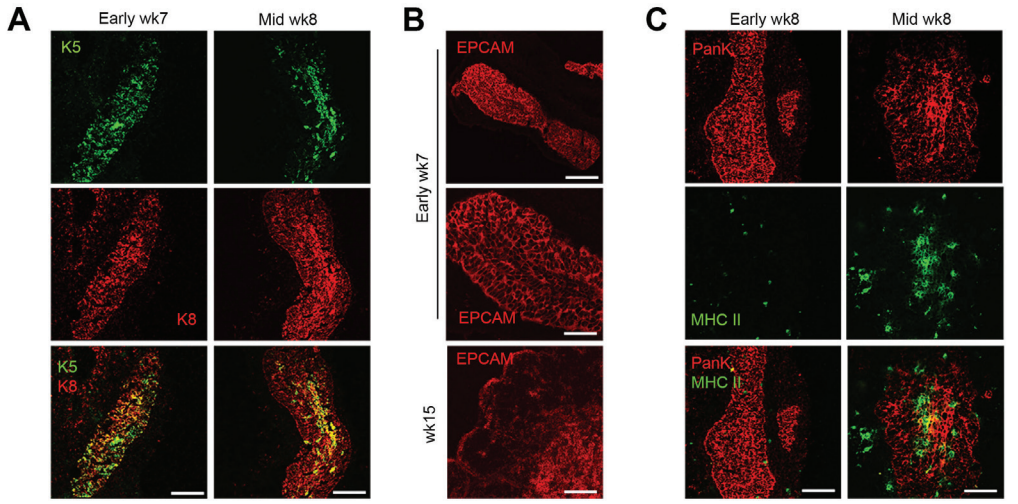


Figure 3: Onset of TEC differentiation in the human thymic primordium. Immunohistochemical staining of thymic primordia at the developmental stages shown with (A) α-Keratin 5 (K5), α-Keratin 8 (K8), (B) α-EPCAM and (C) α-HLA-DR/DP/DQ (MHC II) and α-pancytokeratin (PANK). Scale bars: A-C 150μm, EPCAM and MHC II Mid wk8 75μm. Images are representative of at least two independent analyses. Wk, week.

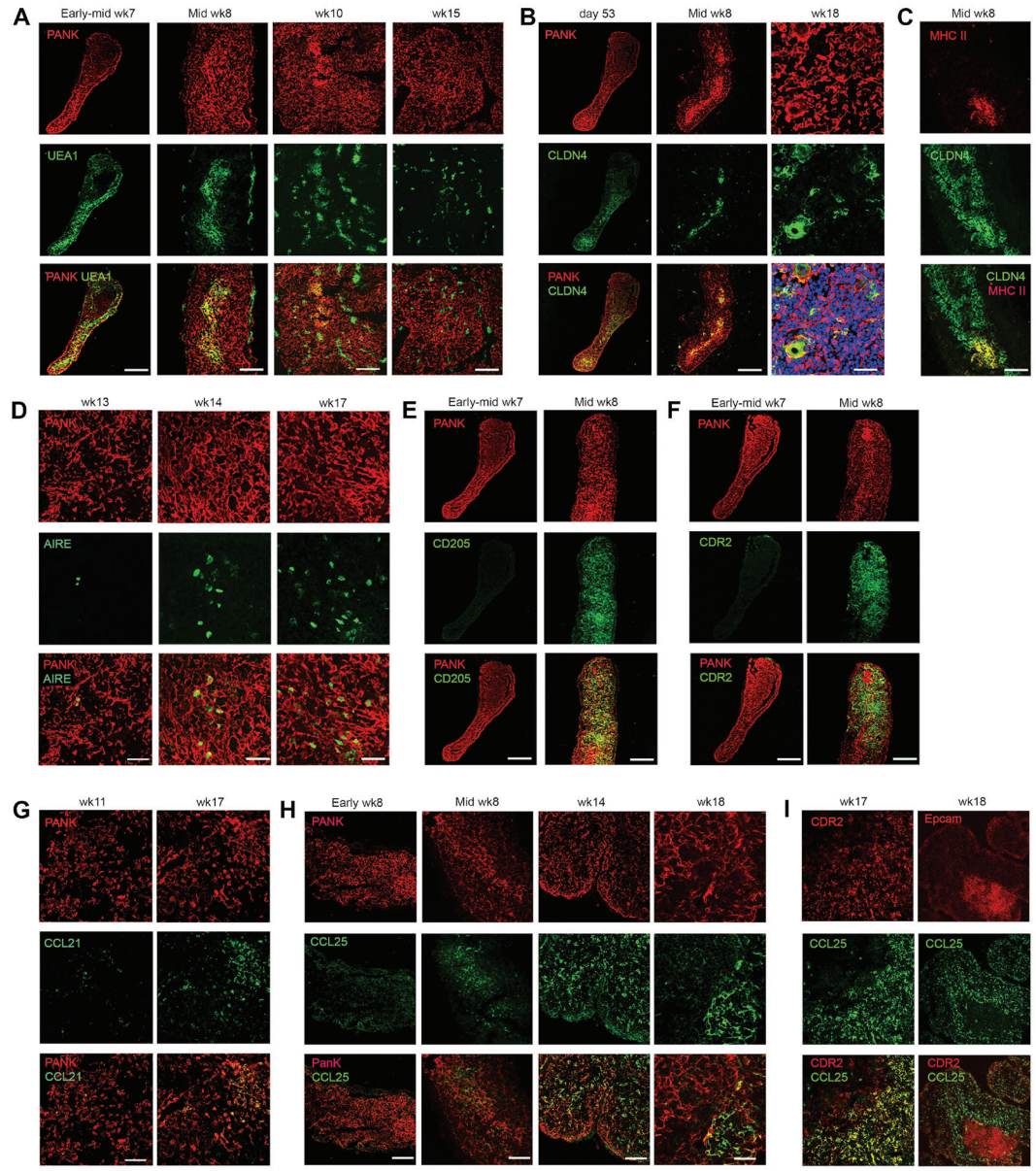


Figure 4: Expression of markers of cortical and medullary TEC in human thymus development. (A-D) Medullary TEC differentiation. Images show staining with (A) UEA1, (B) α-CLDN4, (C) α-CLDN4 and α-HLA-DR/DP/DQ (MHC II) and (D) α-AIRE. (E) Chemokine expression. Images show staining with (G) α-CCL21, (H, I) α-CCL25. (F,G) Cortical TEC differentiation. Images show staining with (E) CD205 and (F) CDR2. Co-stains are as indicated. Developmental stages are as shown. α-pancytokeratin (PANK) co-stain reveals the entire thymus primordium. Scale bars: (A,B,F,G) 150μm, except (A) wk10 - 75μm and (B) wk18 – 47.6μm; (D) wk13 - 50μm, wk14 and wk17 - 55μm; (E) 50μm. Images are representative of at least two independent analyses. Wk, week.

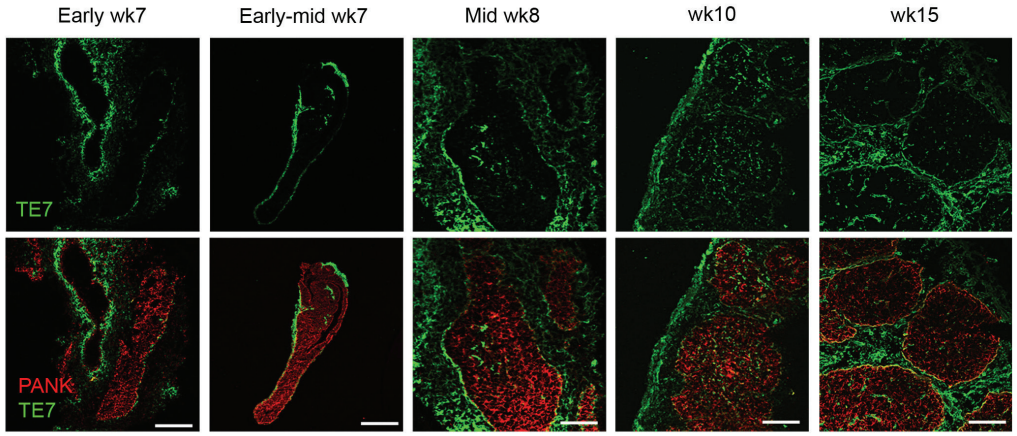


Figure 5: Colonization of the human thymic primordium by mesenchymal cells. Images show staining with TE7 at the developmental stages shown. α-pancytokeratin (PANK) reveals the entire thymus primordium. Images are representative of at least two independent analyses. Scale bars: 150μm, Mid wk8 75μm. Wk, week.

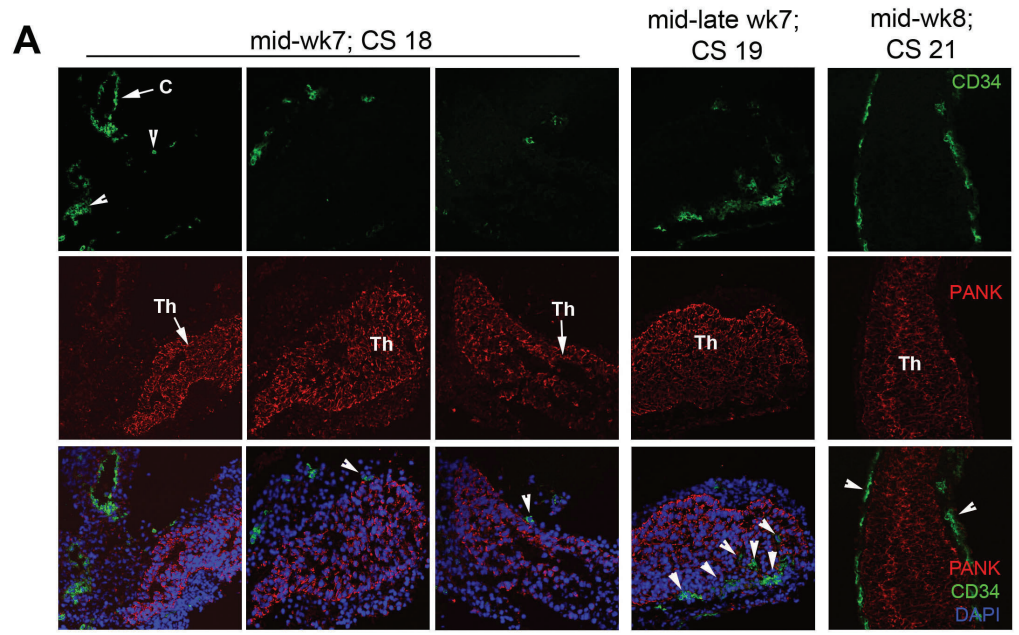


Figure 6: Colonization of the human thymic primordium by vascular endothelial cells. (A, B) Images show staining with α-CD34 at the developmental stages shown. α-panCytokeratin (PANK) reveals the entire thymus primordium. CD31 and VEcadherin (VEcad) staining reveals vascular endothelial cells (B). Scale bars: (A) 75μm. C, carotid artery; Th, thymus; white arrowheads in (A) indicate hematopoietic cells. Images are representative of at least two independent analyses.

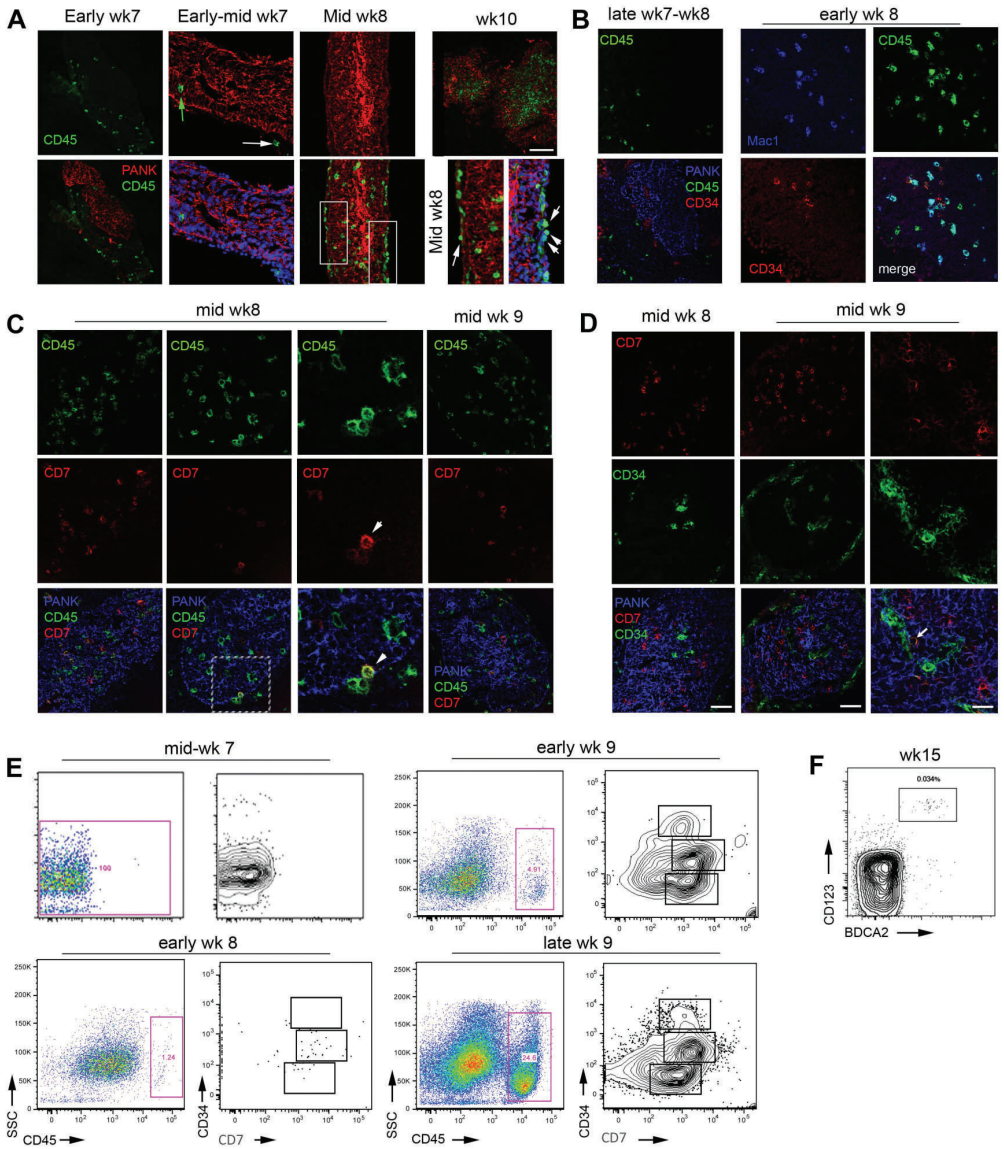


Figure 7: Colonization of the human thymic primordium by hematopoietic cells. (A-D) Images show staining of human thymic primordia with α-CD45, α-CD34 and α-CD7 as indicated, at the developmental stages shown. α-panCytokeratin (PANK) reveals the entire thymus primordium. Green arrow in A indicates $CD45^+$ cell within the thymic epithelial compartment; white arrows indicate $CD45^+$ cells present in the perithymic mesenchyme. The three bottom right hand images in (A) show detail from boxed regions on mid-week 8 image. Right hand panels in (C) show boxed area from middle panel. Scale bars: (A) early-mid wk7 and wk10 - 150μm, early-mid wk7 and mid wk8 75μm; Images are representative of at least two independent analyses. (E, F) Plots show flow cytometric analysis of dissociated human thymic primordia at the developmental stages shown. (E) Plots show staining with α-CD45 and α-CD34 and α-CD7 after gating on live cells (i.e. against DAPI). Right hand panel at each age shows cells after gating on $CD45^+$ cells (left hand panel). Boxes on right hand plots at each age indicate CD34hi, int and lo/neg cells. (E) n = 1 for each age shown. (F) Plot shows staining with markers for (plasmacytoid ($CD123^+BDCA2^+$) dendritic cells after gating on $CD45^+CD19^-CD3^-$ cells and is representative of at least two independent analyses. Wk, week.

CHAPTER 3

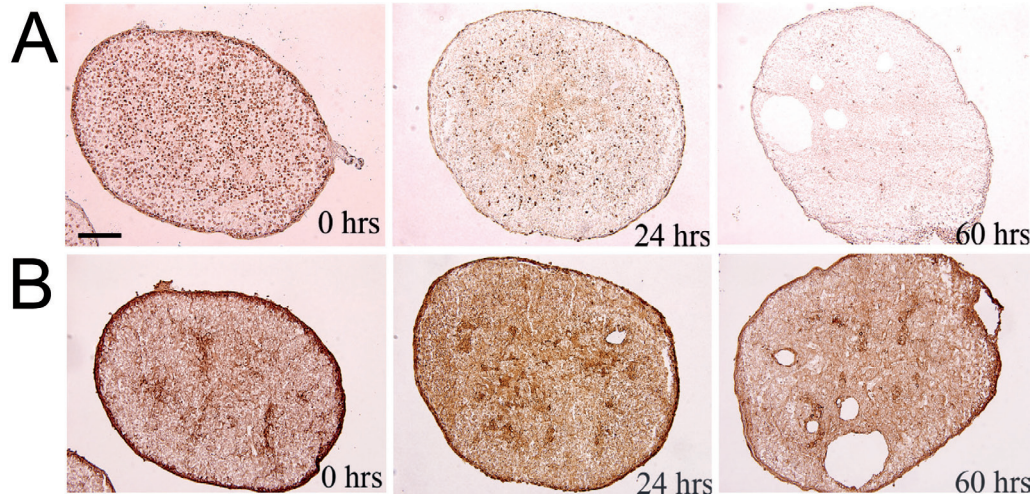


Figure 1.: Kinetics of thymic cyst formation in E15 fetal thymic lobes during dGuo treatment. Fetal thymic lobes were cultured in the presence of dGuo, and at 12-hour interval lobes were removed from the culture and snap-frozen in OCT compound. Serial cryosections of the time-course experiment were analyzed and stained with anti-IKAROS (*A*) and anti-pancytokeratin antiserum (*B*), respectively. Data are shown from the time points 0 hrs, 24 hrs, and 60 hrs. The scale bar indicates 200μm.

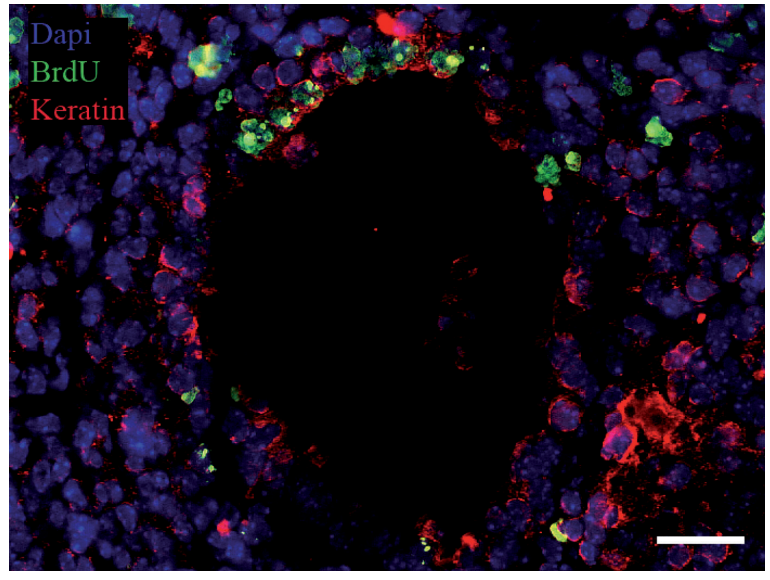


Figure 2: TECs proliferate during thymic cyst expansion. BrdU was added during the final 48 hours of dGuo treatment. Thymi were snap frozen in OCT compound, and stained with anti-BrdU and anti-keratin antibody. Groups of cyst-lining TECs incorporate BrdU, indicating that a subpopulation of cyst-lining TECs induces enlargement of thymic cysts. The scale bar indicates 20μm.

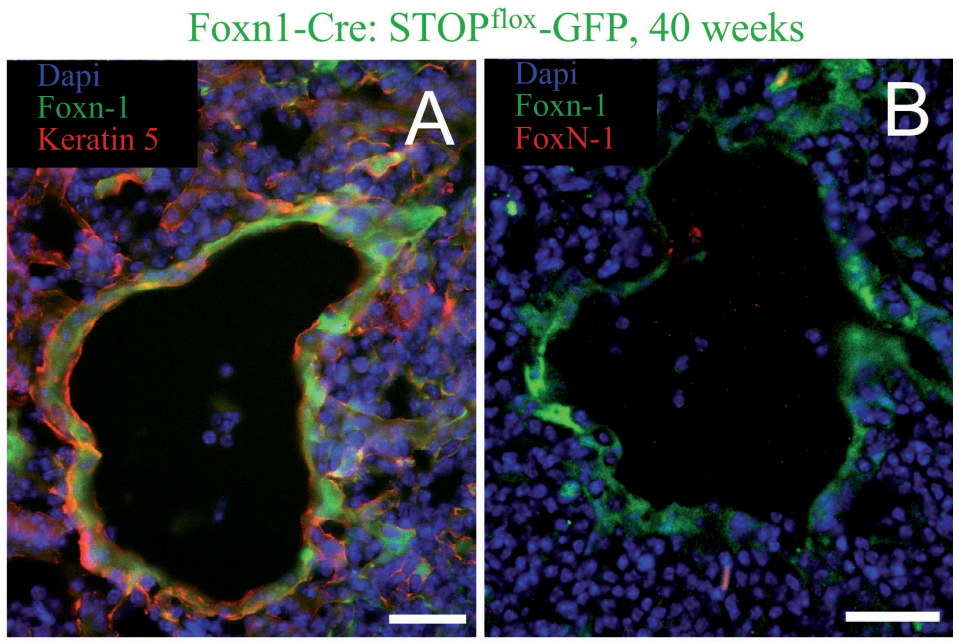


Figure 3: Foxn1 expression in natural occurring thymic cysts. Cyst-lining TECs in 40 weeks old Foxn1-Cre: STOP^{flox}–GFP express GFP (Fig. 3A), indicating that these epithelial cells have followed a Foxn1 regulated developmental pathway. Protein expression of Foxn1 was not detected in 40 weeks old cyst lining TECs (Fig. 3B). Fig. 3C shows that a majority (88%) of cyst-lining TECs (n = 79) in 23 wk old Foxn1-EGFP mice actively expresses Foxn1 protein, however, only 12 %

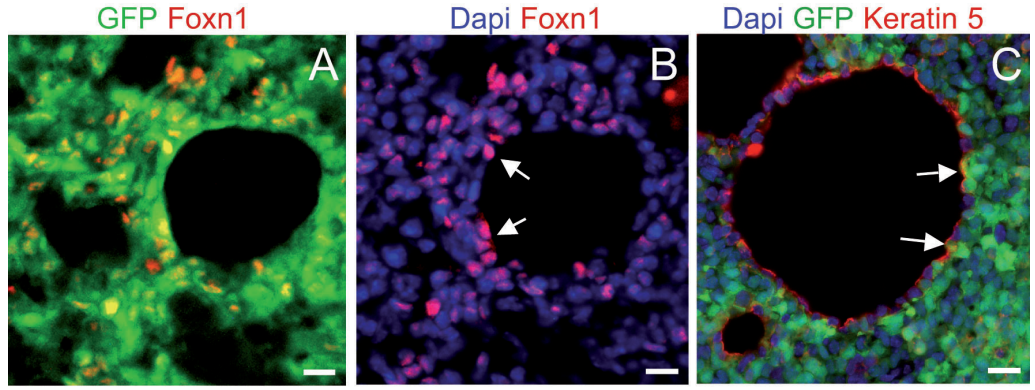


Figure 4: Clusters of cyst lining TECs in dGuo treated fetal thymic lobes synthesize Foxn1 protein. A polyclonal antibody directed against Foxn1 was applied to identify Foxn1 at the protein level. Fig. 4A shows that Foxn1 protein was variably expressed in 47% cyst-lining TECs (n = 214) in dGuo treated fetal thymic lobes derived from Foxn1-Cre: STOP^{flox} –GFP E15 (B; arrow heads indicate cells showing strong Foxn1 expression). Likewise, 81 % of cyst-lining TECs (n = 182) in dGuo treated thymic lobes from E15 Foxn1-EGFP embryos show groups of cells expressing Foxn1 protein (Fig. 4C; arrow heads show cells with strong Foxn1 expression). The scale bars indicate 25µm.

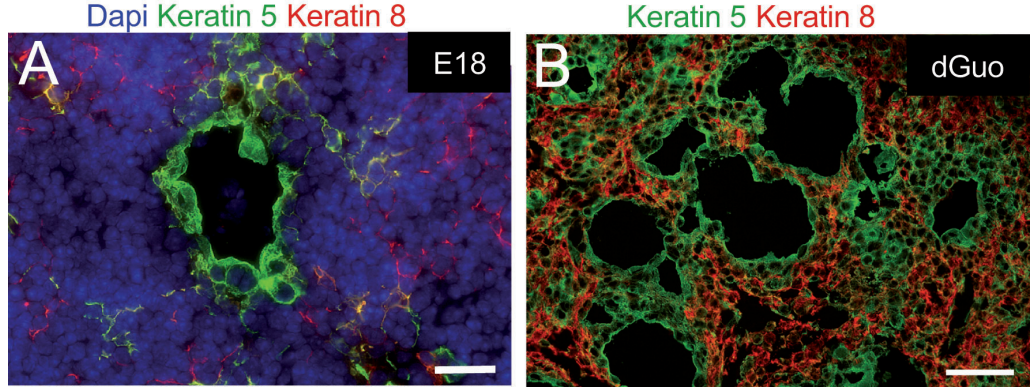


Figure 5: Thymic cysts in the fetus are lined by mTECs. K5 and K8 expression was analyzed in E 18 fetal thymic lobes of normal mice (A; see also SI Fig. 11) and in dGuo treated FTOC (B; see also SI Fig. 10). At all gestational ages, including dGuo treated fetal thymic lobes, cysts are lined by K5 expressing TECs. The scale bar indicates 25µm in A and 50µm in B.

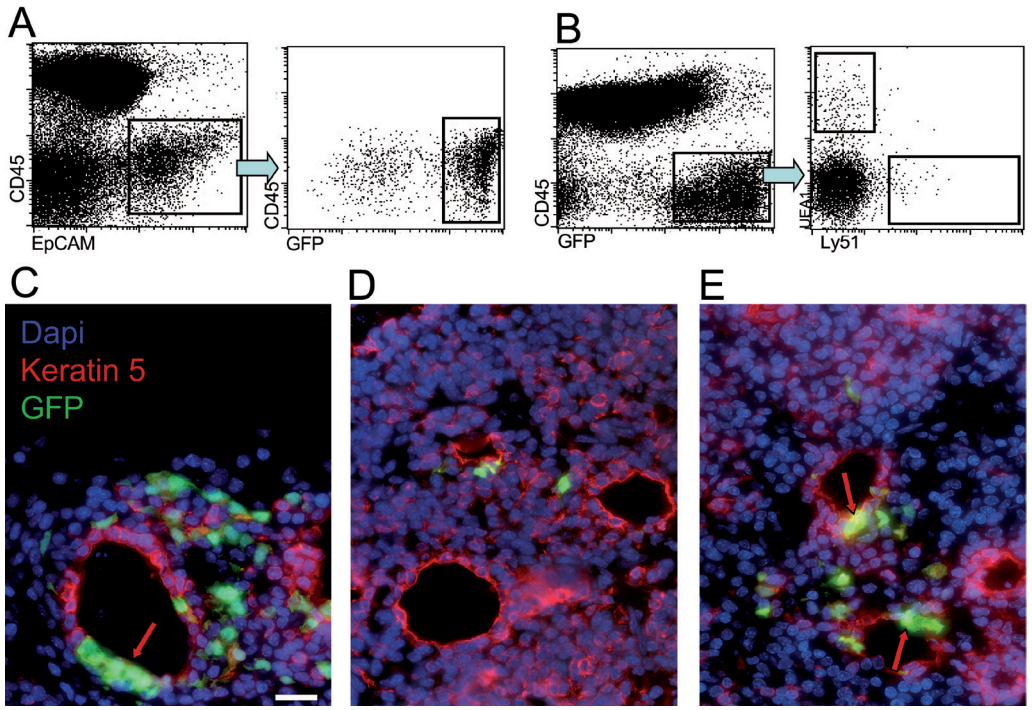
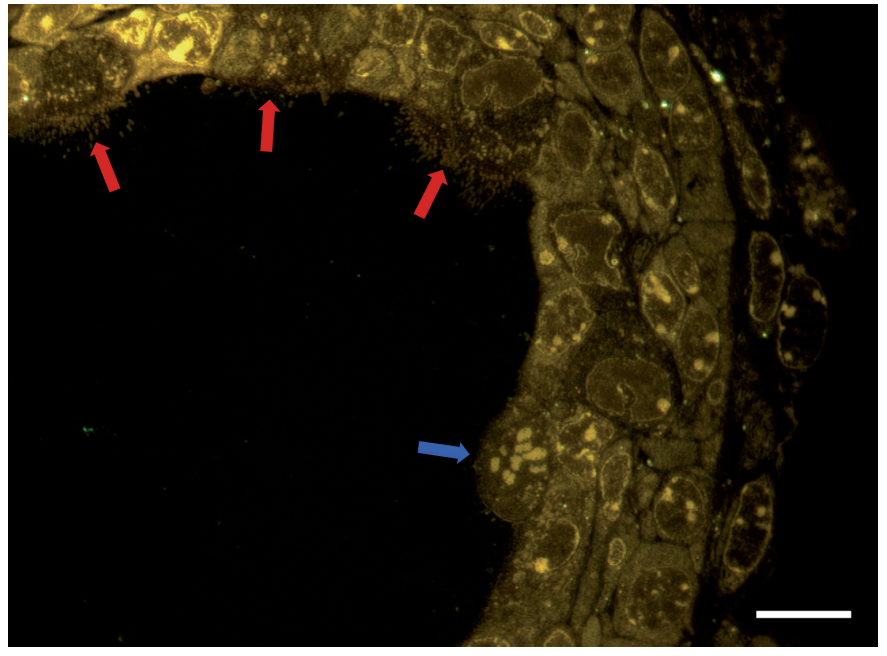
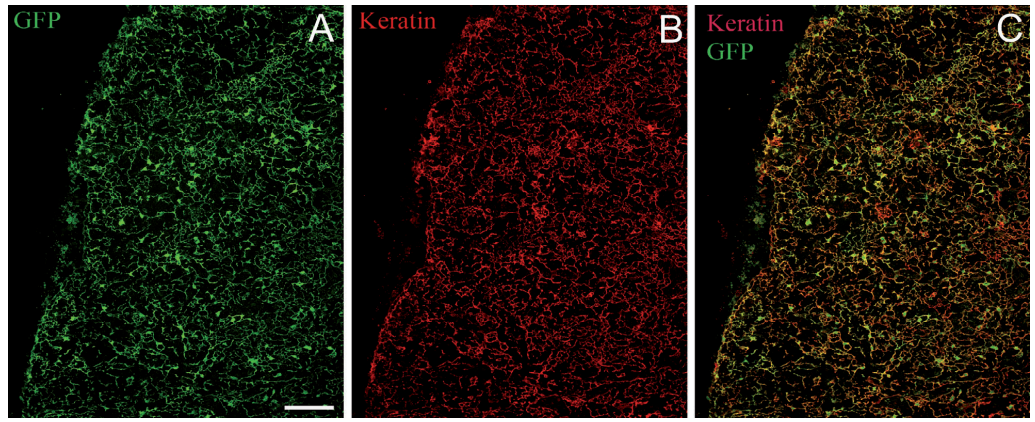


Figure 6: Cyst-lining cells are derived from mTECs. Flow-cytometry profiles of sorted EpCAM+, GFP+ TEC fraction (A), Ly51+, GFP+ (cTECs) and UEA1+, GFP+ (mTECs) (B) were seeded into E15 'PACMAN' lobes and analysed for their commitment in thymic cyst formation upon dGuo treatment. Cryosections were analysed for distribution of GFP+ seeded cells and stained with antibodies to K5. EpCAM+ sorted cells could be traced back as cyst-lining TECs (Fig. 6C; arrows). In a total of 10 frozen sections harbouring thymic cysts, 120 GFP+ cells were analysed for their location in the re-aggregated lobes. From these cells, 32 EpCAM+ were found back as cyst-lining TECs, the remaining cells were situated in the stroma surrounding the cysts. Ly51+GFP+ sorted cells (Fig 6 D) were found back as cyst-lining TECs only at low frequency (17 out of 111 cells in 15 sections). In contrast, 37 out of 74 GFP+, UEA1+ sorted cells (located in 8 sections) were traced back as cyst-lining TECs (Fig. 6 E; arrows). The scale bar indicates 20μm.

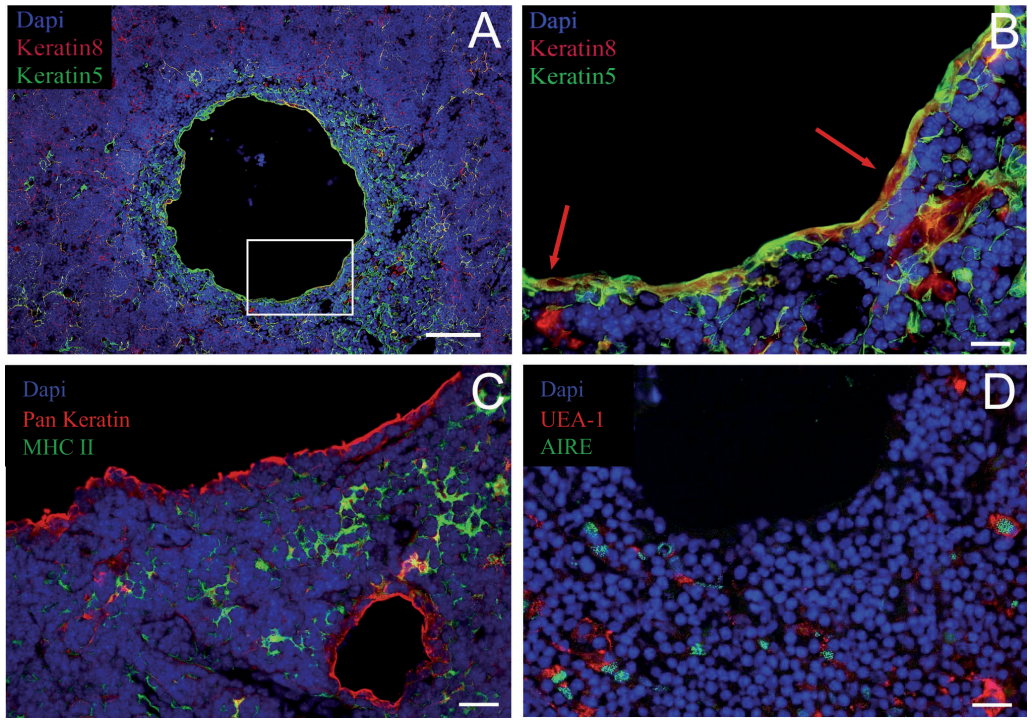
CHAPTER 3 SUPPLEMENTARY FIGURES



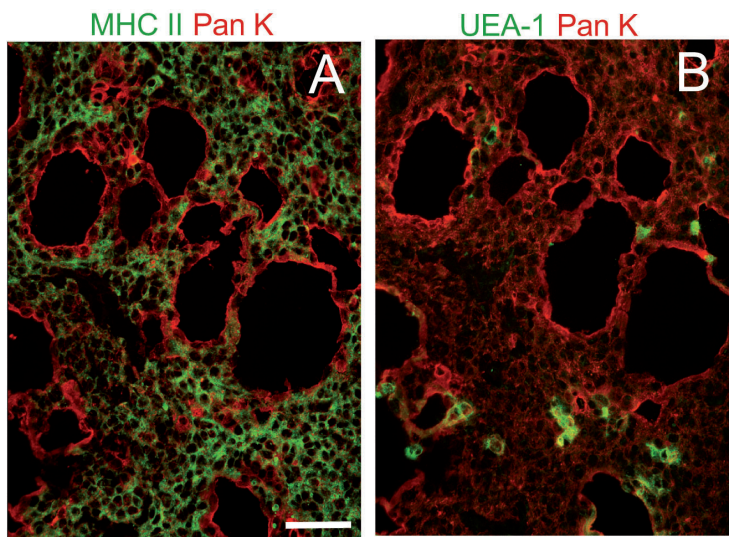
SI Figure 7: Reflection contrast microscopy of a thymic cyst in an E15 dGuo treated fetal thymic lobe. Note ciliated cyst lining epithelial cells (red arrows), and a proliferating cyst lining epithelial cell, showing chromosomes (blue arrow). Thymic lobes were prepared for high resolution light and transmission electron microscopy, using standard procedures. The scale bar indicates 10μm.



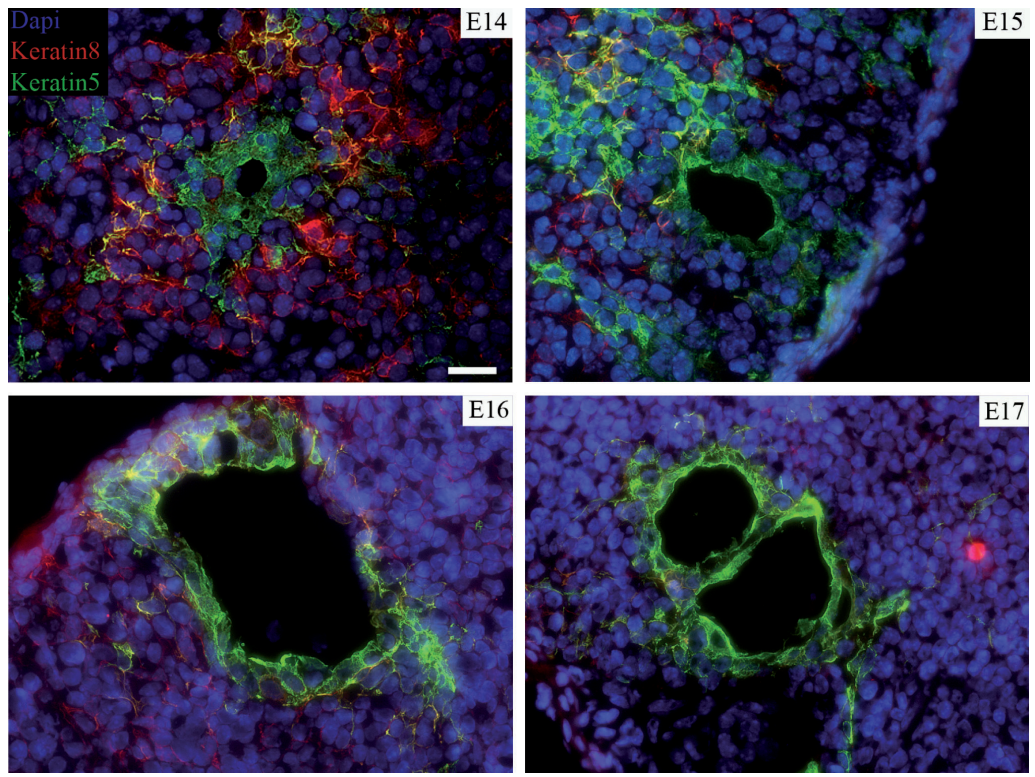
SI Figure 8: Cryosections of thymic lobes derived from 10-months old Foxn1-Cre: $STOP^{flox}$ –GFP mice were analyzed by immunohistochemistry. Comparison of Figs. 8 A, B and C show that the Cre/Lox system worked efficiently, since all pan cytokeratin expressing cells also express GFP. However, Foxn1 expression levels vary between individual TECs, as can also be observed in the thymus of Foxn1-EGFP mice (see Fig. 3C,D). The scale marker indicates 100μm.



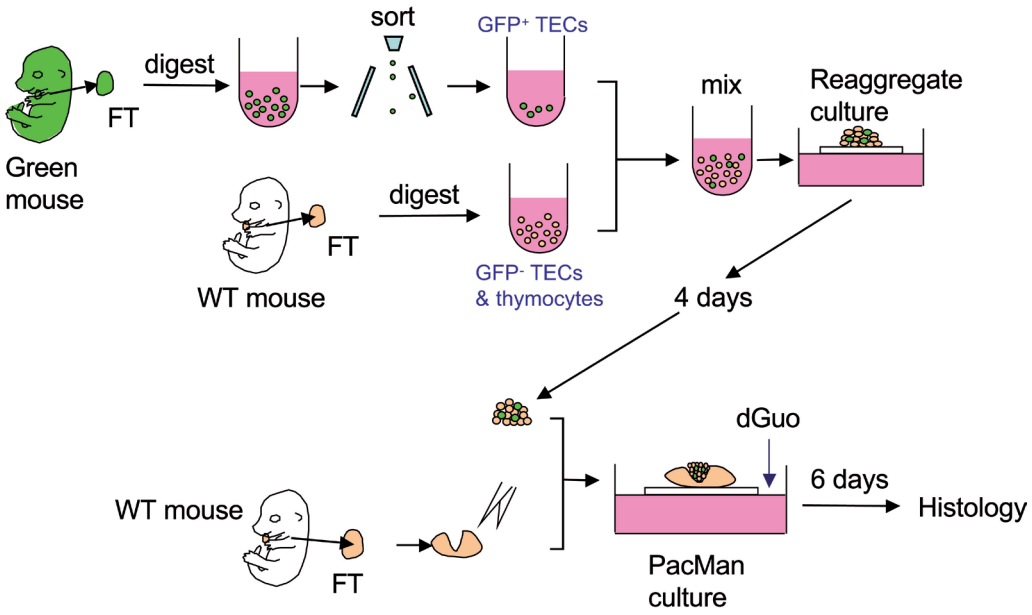
SI Figure 9: Thymic cysts in the aged thymus relate to medullary epithelium. A large majority of cyst lining TECs expresses the mTEC marker K5 (A). At higher magnification (B, see also boxed area in A), a few cells co-expressing K5 and K8 are present (B, red arrows). Cyst lining TECs do neither express MHC II (C), AIRE, nor UEA-1 (D) Scale markers indicate 100μm in (A) and 25μm in (B, C and D).



SI Figure 10: Thymic cysts in d-Guo treated lobes are lined by cells that generally lack mature markers such as MHC II (*A*) and UEA-1 (B). The scale marker represents 50 μm.



SI Figure 11: Thymic cysts in the normal developing fetal thymus are detectable from E14. The scale marker indicates 20 μm.



SI Figure 12: Experimental procedure to analyze the contributionof TEC subpopulations in the formation of thymic cysts.

CHAPTER 4

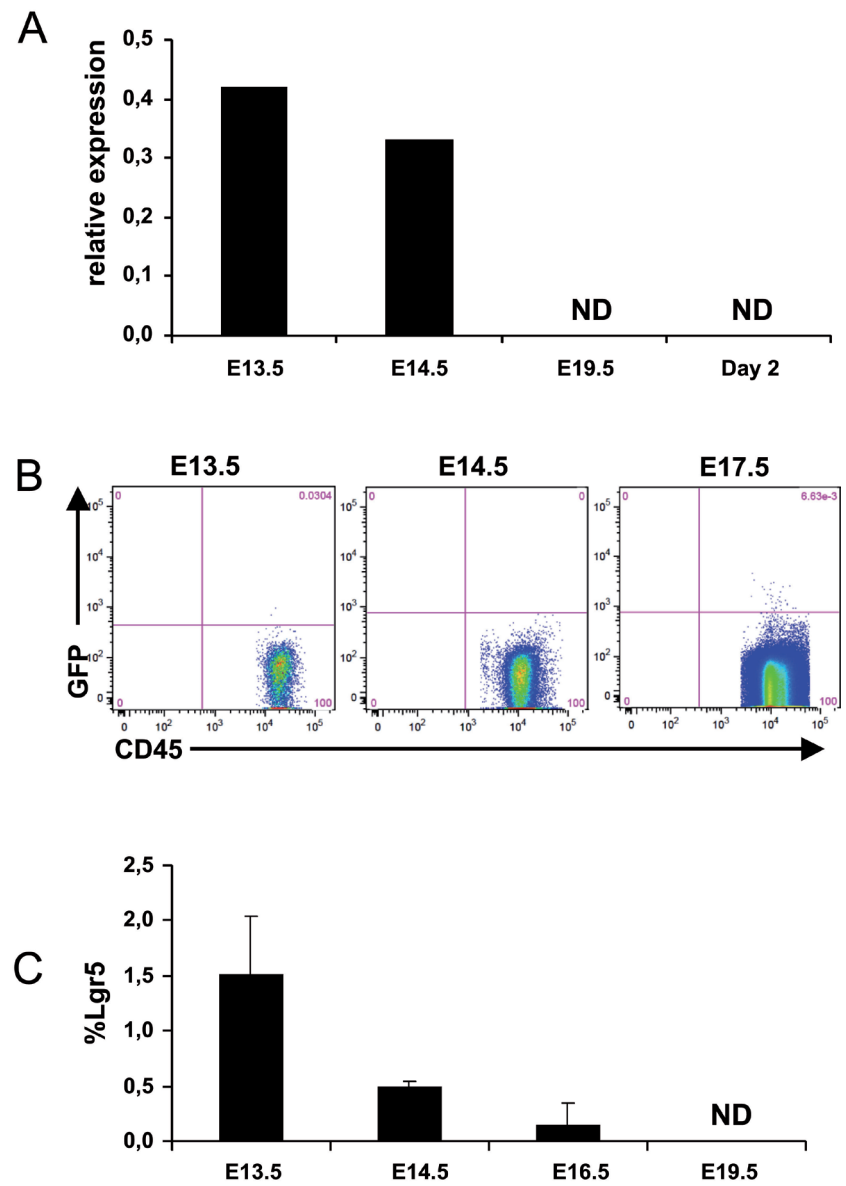


Figure 1: Lgr5 expression in the thymus is restricted to early embryonic development. Thymi of different ages were analyzed for the presence of Lgr5 mRNA. E13.5 and E14.5 were directly analyzed; E19.5 and day 2 neonatal thymi were first purified for EpCAM+ cells (A). CD45+ of different embryonic ages were analyzed for the presence of Lgr5:EGFP cells (B). CD45-/EpCAM+ cells from individual fetal thymic lobes from Lgr5:EGFP embryos were analyzed for the expression of Lgr5. The highest Lgr5 positive fraction was detected at E13.5 (C). For figure C E13.5 n=3, E14.5 n=3, E16.5 n=4 and E19.5 n=3

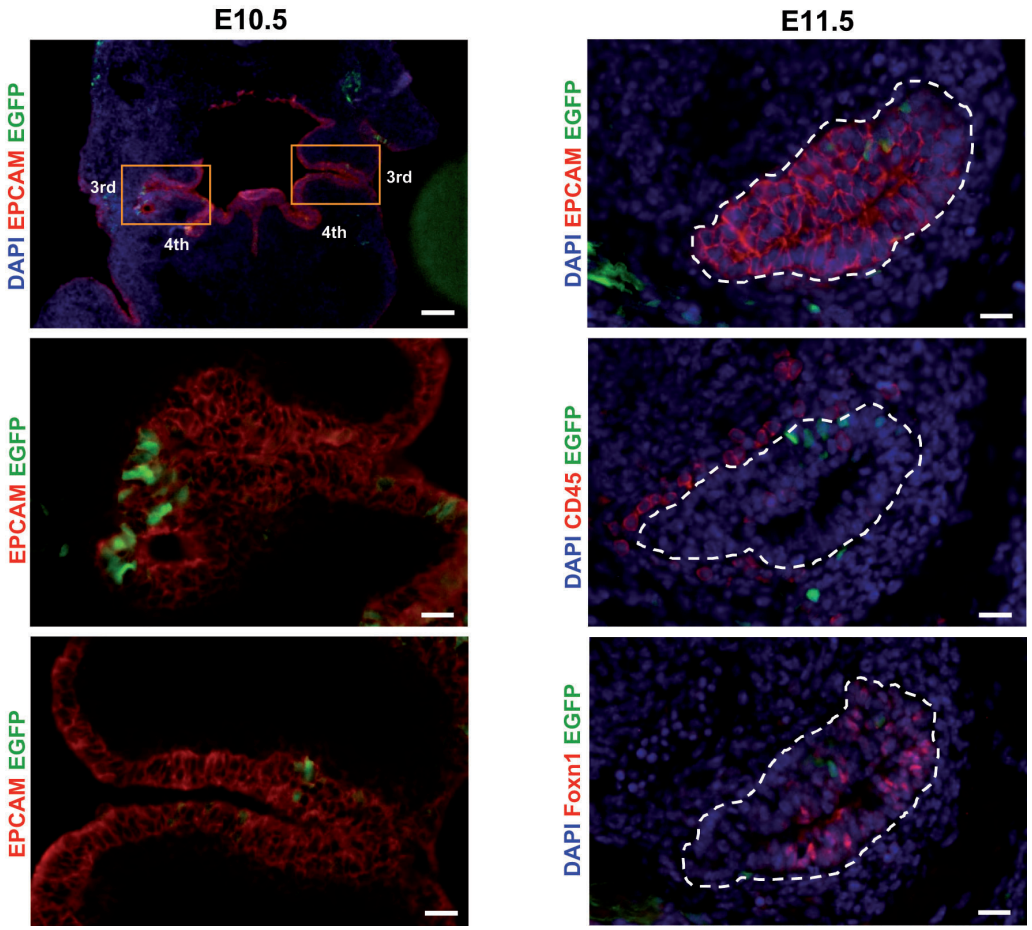


Figure 2: Lgr5 is expressed on a specific subset of TECs in the early fetal thymus. Whole E10.5 and 11.5 Lgr5:EGFP embryos were sectioned and analyzed by immunohistochemistry. The left panel shows a clear expression of Lgr5 in the third pharyngeal pouch endoderm which can be marked with EpCAM. The right panel shows serial sections of the E11.5 fetal thymus. The top panel indicates that the Lgr5+ cells are in the epithelial region. In the middle panel a staining for CD45 was performed on a serial section. Most thymocytes are still at the border of the thymic primordium. In the bottom panel Foxn1 was visualized to indicate that the Lgr5+ TECs were present in the thymic region and not the parathyroid region. Scale bars indicate 125µm in top panel of figure A and 25µm in middle and bottom panel of A and figure B.

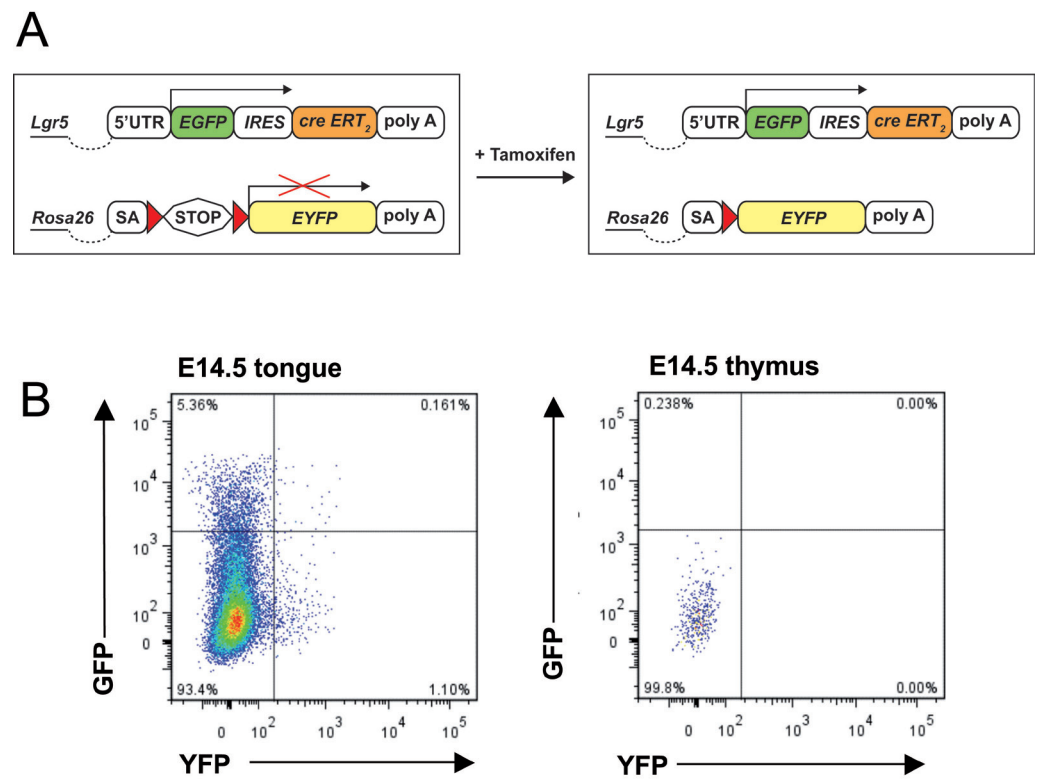


Figure 3: Lgr5 positive TECs do not give progeny. To follow the progeny of the Lgr5 expressing cells Lgr5-EGFP-IRES-CreERT2 mice were mated with Rosa26-Stop^{flox}-YFP mice (A). At E10.5 pregnant mice were pulsed with 0.10mg/g 4OH-tamoxifen. At E14.5 the pregnant mice were sacrificed and the fetal thymus and tongue was analyzed. In the tongue EGFP/EYFP double positive and EYFP single positive cells were clearly detected indicating that cre recombination occurred (B). The E14.5 fetal thymus does not contain any YFP positive cells.

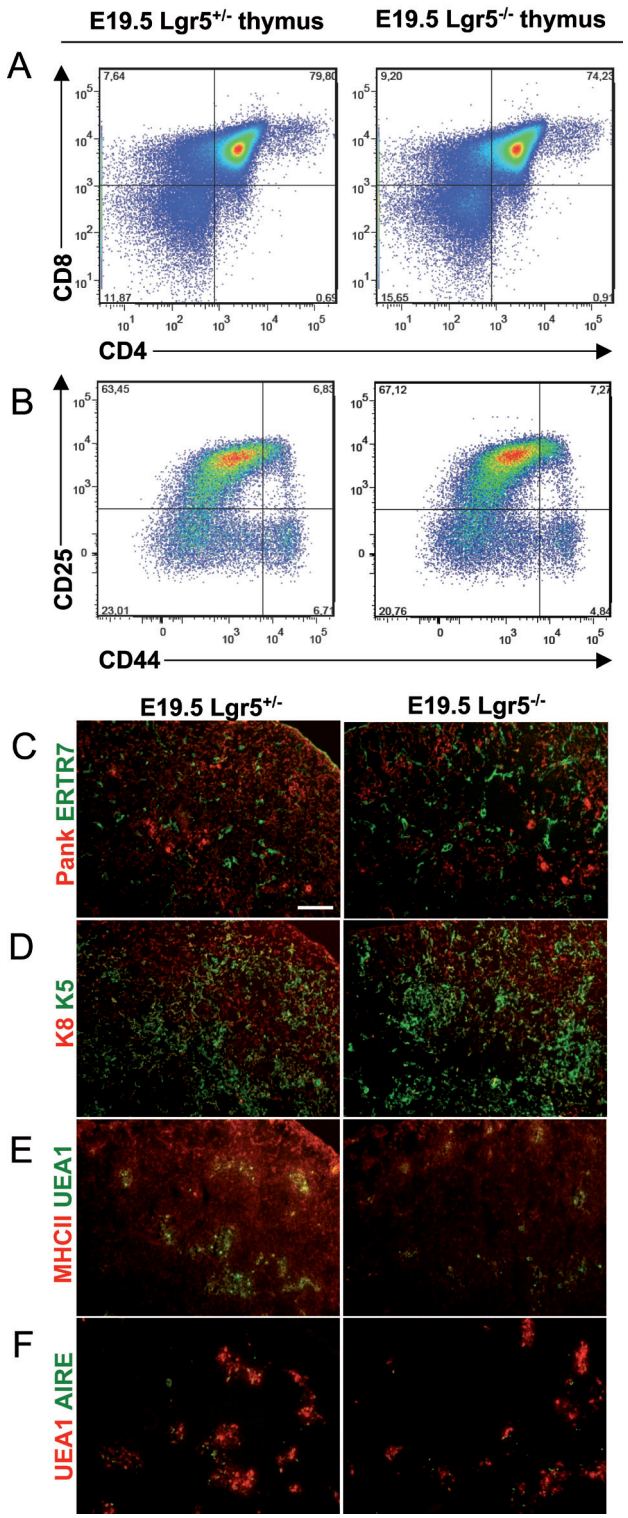


Figure 4: Lack of Lgr5 expression during thymic development shows no aberrant phenotype in the fetal thymus. The lymphoid fraction in fetal E19.5 Lgr5+/- and Lgr5-/- thymi was analyzed by flow cytometry for the DN, DP and SP populations (A) and DN1-DN4 populations (B). Cryosections of E19.5 fetal Lgr5-/- and Lgr5+/- thymi were compared for the expression of pancytokeratin/ERTR-7 (C), keratin 5 /keratin 8 (D), MHCII/UEA1 (E) and AIRE/UEA1 (F). Scale bar indicates 100µm.

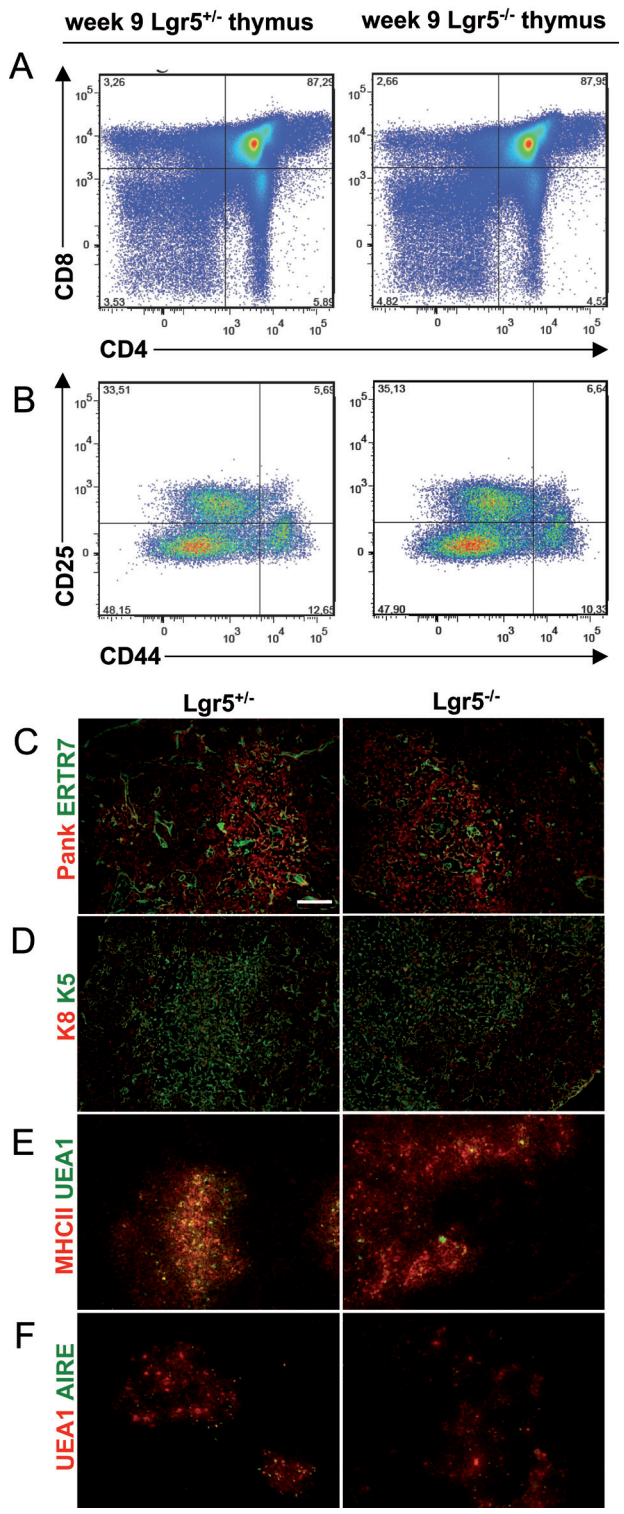


Figure 5: Lack of Lgr5 expression during thymic development shows no aberrant phenotype in the adult thymus. Fetal thymi from E19.5 Lgr5+/- and Lgr5-/- embryos were isolated and put under the kidney capsule of adult wild type mice. After 9 weeks the kidney grafts were analyzed for the DN, DP and SP populations (A) and DN1-DN4 populations (B) by flow cytometry. Cryosections of Lgr5-/- and Lgr5+/- kidney grafts were compared for the expression of pancytokeratin/ERTR-7 (C), keratin 5 /keratin 8 (D), MHCII/UEA1 (E) and AIRE/UEA1 (F). Scale bar indicates 100µm.

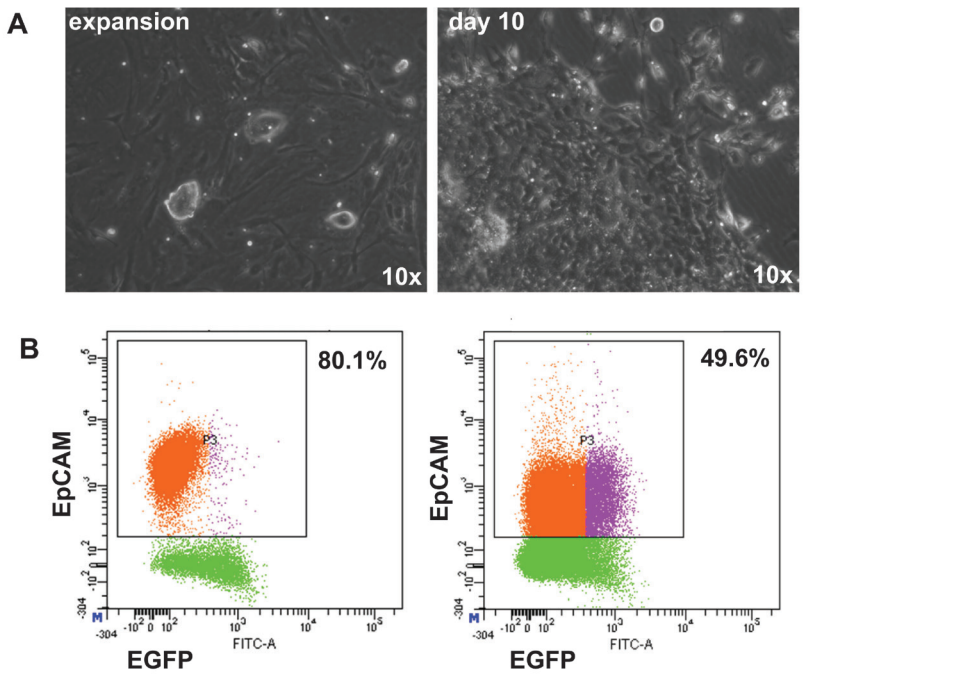


Figure 3: Upregulation of eGFP expression in Foxn1:EGFP IPS cells upon differentiation with the Lai protocol. TC-11-01 IPS cells derived from Foxn1:EGFP mice were differentiated towards thymic epithelium, after 10 days of culture the cells were analyzed. (A) Colonies were analyzed for their morphology. Differentiated colonies grew like epithelial sheets upon differentiation. (B) Colonies were harvested and the amount of EpCAM positive cells was examined by flow cytometry. Undifferentiated IPS cells already contained a major fraction of EpCAM positive cells. Differentiated cells had lower EpCAM expression and more cells that were EpCAM negative. (C) EGFP expression of the EpCAM positive fractions was analyzed. The differentiated IPS cells showed an increase in EGFP expression indicating that Foxn1 becomes upregulated upon differentiation.

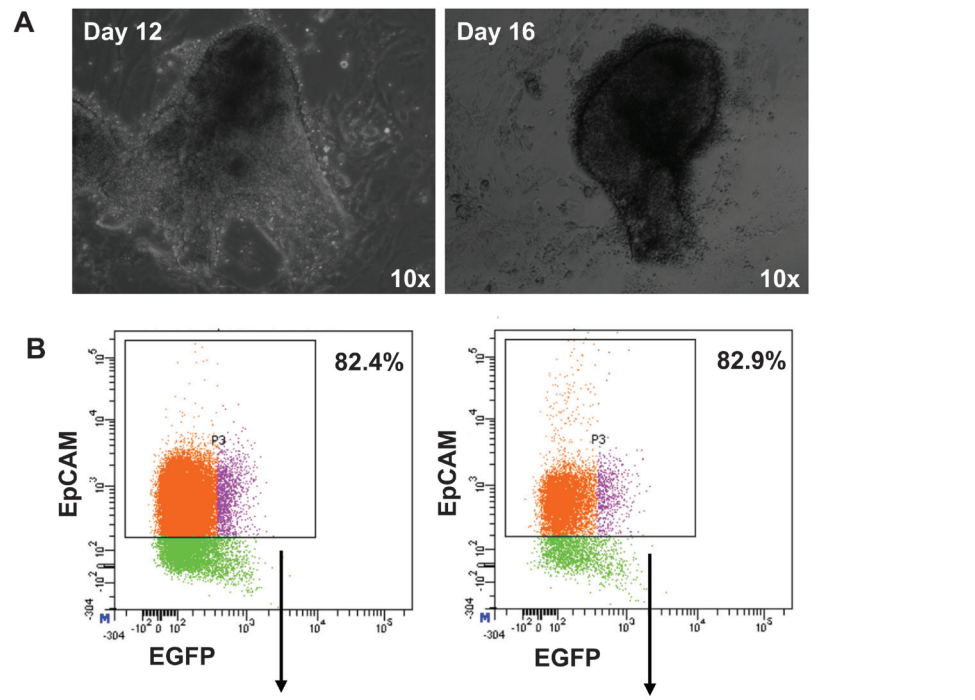
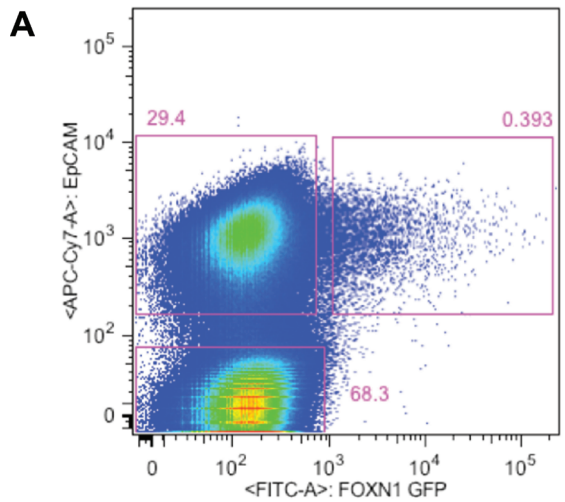


Figure 4: Upregulation of eGFP expression in Foxn1:EGFP IPS cells upon differentiation with the Inami protocol. TC-11-01 IPS cells were differentiated toward thymic epithelial according to the Lai protocol. The cultures were analyzed at day 12 and day 16 of differentiation. The morphology colonies still resembled embryoid bodies, as the cells did not grow like epithelial sheets (A). The EpCAM positive fraction was analyzed for the expression of EGFP (B). The culture at day 12 of differentiation contained 4.2% of EGFP positive cells (left panel). By adding RANKL to the culture this percentage was increased to 7.2% (right panel).



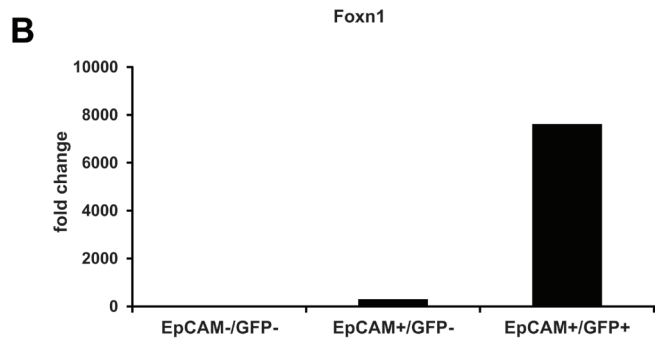


Figure 5: Sorted EpCAM+ GFP+ cells express most Foxn1 transcripts. TC-11-01 embryoid bodies were differentiation towards a thymic fate. After 13 days three different populations were isolated from the bulk culture based on their EpCAM and GFP expression (A). The different populations were analyzed for the presence of Foxn1 transcripts by RT-PCR. The EpCAM+/GFP+ population showed the highest fold change when compared with the EpCAM-/GFP- population or the EpCAM+/GFP- population. The expansion culture TC-11-01 was taken as the value 1 in this comparison (B)

STELLINGEN

1. De muis is een relevant diermodel om thymus-organogenese te bestuderen: de vroege ontwikkelingsstadia tijdens thymus-organogenese in de muis zijn immers goed vergelijkbaar met die van de mens (dit proefschrift).

2. Epitheelcystes in de thymus hebben geen extra-thymale oorsprong en behoren tot de normale celpopulaties van de thymus (dit proefschrift).

3. De in-vitro differentiatie van thymusepitheel vanuit pluripotente stamcellen is een ingewikkeld en nog grotendeels onbekend proces, waarbij kenmerkende tussenstadia, die geïdentificeerd kunnen worden aan de hand van specifieke oppervlakte antigenen, het onderzoek en de manipulatie van dit proces zullen vergemakkelijken (Green et al. Nat Biotech, 2011 en dit proefschrift).

4. Het aantonen van epitheliale stamcelkenmerken uit andere organen op thymusepitheel is een omweg naar de identificatie van een voorlopercel voor thymusepitheel. Bestuderen van de embryogenese en fysiologie van de thymus zelf, is waarschijnlijk rechter op het doel af. (dit proefschrift).

5. Geslachtshormonen spelen een belangrijke rol bij de start van de fysiologische thymusinvolutie en mogelijk ook bij de manipulatie van deze involutie (Rode et al. PNAS 2012).

6. Om de basisfunctie van thymusepitheel te ontrafelen zouden alle (bekende) factoren uitgezet moeten kunnen worden en vervolgens weer één voor één gereintroduceerd worden (Calderon et al. Cell 2012).

7. Zoeken naar de epitheliale stamcel van de thymus lijkt op het zoeken naar de “Holy Grail”. Een dergelijke stamcel past mooi binnen ons biologisch denkraam, zou klinisch van grote betekenis kunnen zijn, maar betreft waarschijnlijk een illusie.

8. De zogenaamde “crosstalk” ofwel reciproke cel-communicatie in de thymus is een dynamisch hiërarchisch proces wat het systeem van vraag en aanbod dirigeert bij de thymopoiese, ruimte voor ontwikkelende thymocyten creëert, en deze ruimte weer laat afnemen zodra dit minder nodig is (involutie).

9. De volwassen thymus bij primaten ontbeert een thymus epitheliale stamcel, wellicht omdat vanuit evolutionair perspectief stamcelfunctie voor thymusepitheel niet nodig is om een T-cel repertoire te genereren en te onderhouden, dat gedurende 40 jaar voldoende bescherming biedt tegen de meest voorkomende infectieuze micro-organismen.

10. Alleen die prestaties waar je extra werk voor hebt moeten verzetten, verdienen het om trots op te zijn. Prestaties, die komen aanwaaien, nopen meer tot bescheidenheid.