The Role of the Transcription Factor Sox9 for Thymic Epithelial Cell Differentiation and Function

Inauguraldissertation zur Erlangung der Würde eines Doktors der Philosophie

vorgelegt der Philosophisch-Naturwissenschaftliche Fakultät der Universität Basel

von

Tatjana Žalac

Zagreb, Kroatien

Basel, 2011

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von

Fakultätsverantwortlicher &

Dissertationsleiter: Prof. Dr. Georg A. Holländer

Korreferent: Prof. Dr. Antonius Rolink

Basel, den 24. Mai 2011

Dekan: Prof. Dr. Martin Spiess

creative commons

Namensnennung-Keine kommerzielle Nutzung-Keine Bearbeitung 2.5 Schweiz

---

Sie dürfen:

das Werk vervielfältigen, verbreiten und öffentlich zugänglich machen

Zu den folgenden Bedingungen:

Namensnennung. Sie müssen den Namen des Autors/Rechteinhabers in der von ihm festgelegten Weise nennen (wodurch aber nicht der Eindruck entstehen darf, Sie oder die Nutzung des Werkes durch Sie würden entlohnt).

Keine kommerzielle Nutzung. Dieses Werk darf nicht für kommerzielle Zwecke verwendet werden.

Keine Bearbeitung. Dieses Werk darf nicht bearbeitet oder in anderer Weise verändert werden.

- Im Falle einer Verbreitung müssen Sie anderen die Lizenzbedingungen, unter welche dieses Werk fällt, mitteilen. Am Einfachsten ist es, einen Link auf diese Seite einzubinden.
- Jede der vorgenannten Bedingungen kann aufgehoben werden, sofern Sie die Einwilligung des Rechteinhabers dazu erhalten.
- Diese Lizenz lässt die Urheberpersönlichkeitsrechte unberührt.

Quelle: http://creativecommons.org/licenses/by-nc-nd/2.5/ch/ Datum: 3.4.2009

SUMMARY

The thymus provides throughout life the specialized stromal microenvironment required for the lifelong generation of naïve T cells. This vital function is dependent on the regular composition and organization of its microenvironment, where specialized stromal cells promote thymocytes development and selection to functionally mature T cells. The thymic stroma is heterogeneous and mainly composed of thymic epithelial cells (TEC) which can be broadly subdivided according to their anatomical location, morphology, specific antigenic properties and function into two specialized subsets, i.e. cortical (c) and medullary (m) TEC. Although it is well established that appropriate Wnt signalling activity is required for normal TEC development and maintenance, the cellular and molecular mechanisms responsible for their differentiation and homeostasis remain largely undefined. The canonical Wnt signalling engaging *via* β–catenin directly regulates Foxn1 expression in TEC and a precise regulation of intracellular β-catenin protein levels is required for normal thymus development and function. Transcription factor Sox9 has been reported to physically interact with β-catenin to regulate Wnt/β-catenin transcriptional activity in chondrocytes. While the role of Sox9-Wnt interactions in development and maintenance of various organs has been acknowledged, no such association has yet been demonstrated in thymic epithelia and Sox9 target genes in TEC have not yet been identified.

In the present study, we demonstrate that Sox9, which is differentially expressed in the thymus by non-hematopoietic stromal cells, negatively regulates TEC proliferation and inversely correlates with the expression of the transcription factor Foxn1. The TEC-targeted loss of Sox9 disturbs thymus genesis and results in a hypoplastic thymus with a phenotypically altered epithelial compartment. Mice with a TEC-targeted Sox9 deficiency display subset-specific changes in TEC composition and proliferation, a phenotype which correlates with an upregulation of Foxn1 expression. Despite these alterations, thymopoiesis remains unaffected indicating that Sox9 expression is not required for the TEC`s capacity to support T cell development. *In vitro* studies revealed that Sox9 regulates Foxn1 transcription indirectly by binding and regulating the amount of intracellular β-catenin protein. This correlation suggests a crosstalk between Sox9 and the canonical Wnt pathway to occur in thymic epithelia.

To our best of knowledge, this study provides the first functional evidence that Sox9 controls TEC proliferation and differentiation in a dose-sensitive and subset-specific manner and negatively regulates Foxn1 expression in TEC. Although, the loss of Sox9 expression in TEC is not sufficient to jeopardize T cell development, differential Sox9 expression is critical for the establishment and maintenance of a regular thymic microenvironment.

TABLE OF CONTENTS

1. INTRODUCTION

The thymus is the primary lymphoid organ responsible for the lifelong generation of new T lymphocytes. This process is controlled by a unique stromal microenvironment which enables the attraction of hematopoietic precursors from the blood, foster their survival, and promote their expansion, maturation and eventual selection to functionally mature T cells (Anderson *et al.* 1996). A key feature of the thymic stroma is a highly organized network of specialized thymic epithelial cells (TEC) which exist as phenotypically and functionally different cortical (c) and medullary (m) TEC subsets (Anderson *et al.* 2006). In the mouse, approximately one to two millions of newly generated T cells are exported every day from the thymus into the peripheral circulation where they contribute to the maintenance of the peripheral T cell pool.

1.1. Fetal thymus organogenesis

The development of a diverse and functionally competent thymic epithelial compartment is a prerequisite for regular thymopoiesis. Thymus organogenesis is a highly dynamic process and comprises a cascade of developmental events involving coordinated interactions between all three cell types: epithelial, mesenchymal and hematopoietic (Manley 2000); reviewed in (Blackburn and Manley 2004; Rodewald 2008). There are two main temporal phases in TEC development. The early organogenetic stage (i.e. until embryonic day (E) 12.5) is marked by events that result in the positioning, induction, outgrowth and initial pattering of the thymic rudiment. This stage is also characterized by epithelial–mesenchymal interactions in the absence of thymocytes. Late stage organogenesis (i.e. E12.5-E15) critically depends on the concurrent presence of thymocytes and results in further epithelial differentiation into distinct cortical and medullary subsets. At completion of organogenesis, a fully developed primary lymphoid organ has been formed that is competent to generate mature thymocytes.

1.1.1. Formation of the thymic rudiment

Mouse thymus organogenesis initiates between E9-E11 with the out-budding of endoderm-derived epithelium of the third pharyngeal pouch (3^{rd}pp) (Figure 1.1) (Manley 2000). The epithelial cells in the ventral aspect of 3^{rd}pp adopt a thymic cell fate as early as E10.5. This lineage commitment is marked by the expression of the transcription factor Foxn1, a key regulator of TEC differentiation (Boehm and Bleul 2006; Boehm 2008; Rodewald 2008; Corbeaux *et al.* 2010). During development, thymus-committed

epithelia grow into the underlying neural crest mesenchyme of the third and fourth pharyngeal arches. This epithelial-mesenchymal interaction creates at E10.5 a first visible, common thymus-parathyroid anlage although the signals initiating this formation may occur as early as E9.5 (Cordier and Heremans 1975; Cordier and Haumont 1980). The molecular and cellular mechanisms responsible for forming the epithelial lining of the foregut and how these cells are induced to differentiate and acquire the competence to establish a thymus rudiment still remain largely unknown.

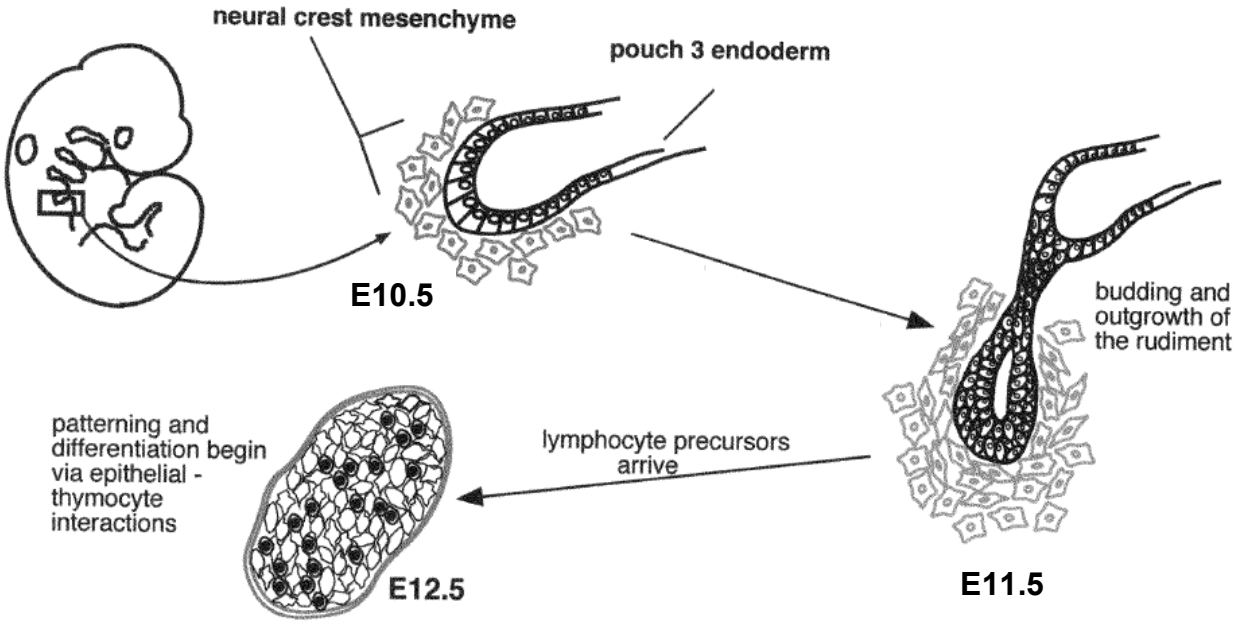


Figure 1.1 Formation of the thymic rudiment.

Neural crest cells migrate to the 3rd pharingeal pouch, transform to mesenchymal cells and surround the expanding epithelium. At E10.5 the epithelial cells in the ventral aspect of the 3rd pouch commit to the TEC fate. The budding and eventual outgrowth of these committed cells forms a thymic rudiment that is morphologically detectable by E11.5. Subsequently, lymphoid precursors (shown as round cells with large nucleus) colonize the thymic rudiment. Around E12.5, late stage organogenesis begins, with the onset of thymocytes-TEC interactions which further pattern the epithelial rudiment. Neural crest-derived mesenchymal cells are shown in gray (taken from Manley, 2000).

Chick-quail chimera experiments showed that ectopically transplanted prospective pharyngeal endoderm of quail isolated prior to the pattering of the 3rdpp and the homing of pro-thymocytes to this anatomical region was already sufficiently primed to induce the formation of a normal thymic microenvironment able to support T cells of host origin (Le Douarin and Jotereau 1975). These findings were later confirmed in mice where purified pharyngeal endoderm of E8.5-E9 embryos transplanted under the kidney capsule of nude mice generated a functional thymus with a regular cortex/medulla organization, independent of physical contribution from the pharyngeal ectoderm (Gordon *et al.* 2004). Together, these experiments concluded that TEC derive solely from endoderm and established the "single origin" model of thymus development (Gordon *et al.* 2004). This contention refuted the “dual-

origin" model that claimed cortical and medullary epithelium are derived from the 3rd pharyngeal cleft ectoderm and 3rdpp endoderm, respectively (Cordier and Heremans 1975). Importantly, these studies also conveyed that at this early stage, cells in the pharyngeal endoderm are already specified to enter the TEC lineage well before the formation of the 3rdpp and overt signs of thymus organogenesis (Le Douarin and Jotereau 1975). However, molecular markers have not yet been identified that distinguish endodermal cells specified to give rise to TEC from cells with other fate.

Budding and outgrowth of the common anlage at about E11.5 is coincident with separation into ventral and dorsal domains identified by the expression of transcription factors Foxn1 and Gcm2 (Gordon *et al.* 2001) which are essential for the development of thymus (Nehls *et al.* 1994; Nehls *et al.* 1996) and the parathyroid tissue (Liu *et al.* 2007), respectively. At this point in time, the prospective thymus epithelium is arranged in a two-dimensional bilayer containing phenotypically and morphologically homogenous cell populations that uniformly express cytokeratin (CK) 8, epithelial-cell-adhesion molecule 1 (EpCam1) and MTS24 (Plet-1) but lack the expression of CK5 and markers used later to differentiate cortical and medullary TEC subsets (Klug *et al.* 2002; Gill *et al.* 2003). Subsequent to E11.5, the organization of TEC changes markedly with the epithelium converting by E12 to a clustered organization and by E13.5, TEC form a typical three-dimensional scaffold (Itoi *et al.* 2001). High level of Foxn1 expression begins at E11.25 and is essential for subsequent TEC differentiation including the generation of distinct cortical and medullary epithelial subsets and the ability to attract T cell precursors (Nehls *et al.* 1996). In the absence of functional Foxn1, TEC fail to progress beyond an immature phenotype and to attract lymphoid precursors (Nehls *et al.* 1996; Itoi *et al.* 2001; Boehm *et al.* 2003).

First signs of the heterogeneity within the epithelial compartment are apparent by E12.5 when CK5 is upregulated in a discrete subset of TEC resulting in the emergence of a prominent $CK5^+CK8^+$ subset of stromal cells that is centrally located and surrounded by cortical $CK5^-CK8^+$ TEC. The $CK5^+CK8^+$ clusters contain $MTS24^+$ progenitors and subsequently are primarily associated with mature mTEC (Klug *et al.* 1998; Klug *et al.* 2000; Gill *et al.* 2002). The reciprocal interactions between TEC and thymocytes are required to achieve complete maturation of both cell types and are referred to as thymic crosstalk (van Ewijk *et al.* 1994). The formation and initial pattering of the TEC compartment during early stages in thymus development however, do not require inductive signals from hematopoietic cells (Klug *et al.* 2002; Jenkinson *et al.* 2005). This was demonstrated by analysing fetal thymi from Rag2/γc-deficient and Ikaros mutant mice in which, despite an early block in T cell development, the epithelial compartment organizes into a three-dimensional structure containing a predominant cortical $CK5^-CK8^+$ TEC subset and centralized $CK5^+CK8^+$ clusters of immature TEC progenitors (Klug *et al.* 2002). Blood-borne lymphoid precursors seed to the avascular thymus anlage at E11.5 (Cordier and Haumont 1980; Fontaine-Perus *et al.* 1981; Suniara *et al.* 1999) and are indispensable during late fetal stages for continued development and maintenance of TEC subsets in the postnatal thymus.

Late stage thymus organogenesis begins at around E12.5 with the onset of TEC–thymocyte interactions, which further pattern the epithelial rudiment. At E13.5 TEC begin to demonstrate further phenotypic heterogeneity, as determined by the expression of markers typically associated with cortical and medullary TEC. Binding to the Ulex europaeus agglutinin (UEA-1) lectin (Surh *et al.* 1992) and the expression of MTS10 (Klug *et al.* 1998), Claudin (Cld)-3 and 4 (Hamazaki *et al.* 2007) can be used to identify medullary, whereas the expression of Delta-like 4 (Dll4) (Tsukamoto *et al.* 2005), Ly51 (Gray *et al.* 2006; Gray *et al.* 2007a), β5t (Murata *et al.* 2007) and CD205 (Shakib *et al.* 2009) to characterize cortical TEC subsets present at this stage. Beginning of E12.5 the common thymus/parathyroid primordium detaches from the pharyngeal lining and migrates under the influence of neural crest cells towards the anterior chest cavity eventually joining at the midline above the heart (Bockman 1997). A day later, the parathyroid and thymus domains physically separate and resolve into distinct organs.

1.1.2. The role of mesenchyme in thymus development and function

Studies have shown that most of the mesenchymal cells surrounding the early thymus anlage are derived from neural crest cells (NCC) and not from adjacent pharyngeal arch mesoderm (Jiang *et al.* 2000). NCC represent a developmentally plastic population that migrates ventrolaterally from their origin in the dorsal lip of the neural tube. During their migration, NCC proliferate and some aggregate to produce distinct neural tissues, such as the dorsal root ganglia and sympathetic ganglia, while others become mesenchymal cells (Bockman 1997). NCC that migrate to the third pharyngeal arches and there transform to mesenchymal cells are referred to as ectomesenchyme because of the ectodermal origin of the constituent cells, though they are indistinguishable from mesoderm-derived mesenchymal cells (Le Lievre and Le Douarin 1975; Gordon *et al.* 2004). These cells surround the emerging thymus primordium and support growth and development of thymus-committed epithelia (Bockman 1997).

NCC contribute to the capsule, the septae of the fetal thymus (Jiang *et al.* 2000) and eventually establish the intrathymic network of fibroblasts (Suniara *et al.* 2000). Although most mesenchymal cells in the E12 thymus uniformly express platelet-derived growth factor receptor α (PDGFRα), cells with such a phenotype represent a heterogeneous population that changes dynamically during development (Rossi *et al.* 2007b). Their numerical contribution to the thymic stromal composition declines with subsequent thymus development, a correlation that mirrors the decrease in TEC proliferation (Yamazaki *et al.* 2005; Rossi *et al.* 2007b). Therefore, the physical contribution of mesenchymal cells beyond organogenesis had been questioned and several studies suggested that once epithelial cells have fully gained the competence to support lymphocyte differentiation, and the vasculature of the thymus is complete, the contribution of NCC in the thymus is greatly reduced or absent (Jiang *et al.* 2000; Yamazaki *et al.* 2005).

However, more recent studies using genetic labelling of NCC and their derivatives clearly demonstrate an ongoing role for these cells in thymus function (Foster *et al.* 2008; Muller *et al.* 2008). NCC that associate with the thymic capsule at E12.5, enter the thymus before E13.5 and differentiate into pericytes and smooth muscle cells associated with vessels. These cells persist in the adult thymus and provide structural support to the thymic blood vessels and possibly regulate endothelial cell function, suggesting a switch from their role in supporting TEC proliferation to a role in the vasculature development (Foster *et al.* 2008).

Mesenchymal cells of the thymus can be characterized using different markers, including ERTR-7 (Van Vliet *et al.* 1986) and MTS15 (Gray *et al.* 2007a). These provide largely overlapping staining patterns in distinct thymus structures such as the capsule, the septae, thymic fibroblasts, and notably vessel-associated pericytes (Van Vliet *et al.* 1986; Gray *et al.* 2007a). The majority of $MTS15^+$ stromal cells (75%) also express the cortical TEC marker Ly51 (Gray *et al.* 2007a), a phenotype proposed to represent “cortical mesenchyme” (Muller *et al.* 2005). Though in the adult thymus, virtually all $MTS15^+$ cells also co-express PDGFR-α, the phenotype of mesenchymal cells is still poorly characterized. Moreover, the turnover and the thymus-specific functions of these cells are largely unknown.

The functional role for NCC in the formation of the thymus has initially been inferred from the different experimental observations. Deficiencies in thymus development are detected in chick embryos with the ablation of NCC (Kuratani and Bockman 1990), in spontaneous Pax3-deficient (splotch) mice, which display a severe defect in NCC migration (Conway *et al.* 1997) and in humans with the DiGeorge syndrome (Greenberg 1993). Several studies have indicated a role for the mesenchyme in the regulation of embryonic TEC proliferation. For example, NCC interact with the epithelial cells of the pharyngeal endoderm through the production of fibroblast growth factors (Fgf) 7 and Fgf10 which induce the proliferation of FgfR2-IIIb (isoform IIIb of Fgf receptor 2)-expressing TEC (Revest *et al.* 2001; Jenkinson *et al.* 2003; Jenkinson *et al.* 2006; Itoi *et al.* 2007b), an epithelial–mesenchymal interaction that has already been observed in the formation of other organs. A typical example is the limb bud, where the mesenchymal production of Fgfs stimulates the growth and differentiation of Fgf receptor-bearing epithelial cells (Xu *et al.* 1999). In mice lacking either Fgf10 (Ohuchi *et al.* 2000) or FgfR2-IIIb (Revest *et al.* 2001), thymus organogenesis proceeds normally until about E12.5, after which the organ fails to increase in size. However, cTEC and mTEC development occurs normally and a phenotypically regular thymocytes maturation is supported in this hypoplastic thymus. These findings are consistent with experimental studies in which the removal of the E12 perithymic mesenchyme prevents normal thymus growth, though the initial specification of thymic progenitors into the cTEC and mTEC lineages remains undisturbed (Jenkinson *et al.* 2003). Thus, the mesenchyme regulates the proliferation of developing TEC, but does not appear to be required for the commitment to a TEC fate and the subsequent cell`s

differentiation. Whether the mesenchyme influences TEC proliferation in the adult thymus (Gray *et al.* 2006) is not clear, although it is interesting to note that adult thymic mesenchyme continues to express Fgf7 and Fgf10 which influence survival of adult TEC (Rossi *et al.* 2007a).

A role for mesenchymal cells in early T cell development has also been demonstrated. Experiments involving the generation of reaggregate thymus organ cultures (RTOC) from defined stromal components found that mesenchymal fibroblasts are necessary for the maturation of thymocyte precursors beyond the $CD4^-CD8^-CD25^+CD44^+$ (DN2) immature stage (Anderson *et al.* 1993; Anderson *et al.* 1997; Suniara *et al.* 2000). Although the precise mechanism by which mesenchymal cells influence thymus development and function is unclear, based on the current experimental data a two-stage mechanism for mesenchymal involvement has been proposed (Figure 1.2) (Anderson and Jenkinson 2001). In the initial stages of thymus formation, mesenchymal cells surround and later enter the thymus anlage, where they provide Fgfs and thus, directly stimulate the growth and development of TEC. Mesenchymal fibroblasts present in cortical regions of the thymus may directly influence the survival, proliferation or differentiation of immature $CD4^-CD8^-$ T cell precursors by providing an extracellular framework to present and concentrate essential soluble growth factors and cytokines (Banwell *et al.* 2000). Collectively, these studies show that the signals provided from cells of mesenchyme origin are necessary but not sufficient for the development of a regularly structured and normally functioning thymus which also depends on the inductive interactions with developing hematopoietic elements.

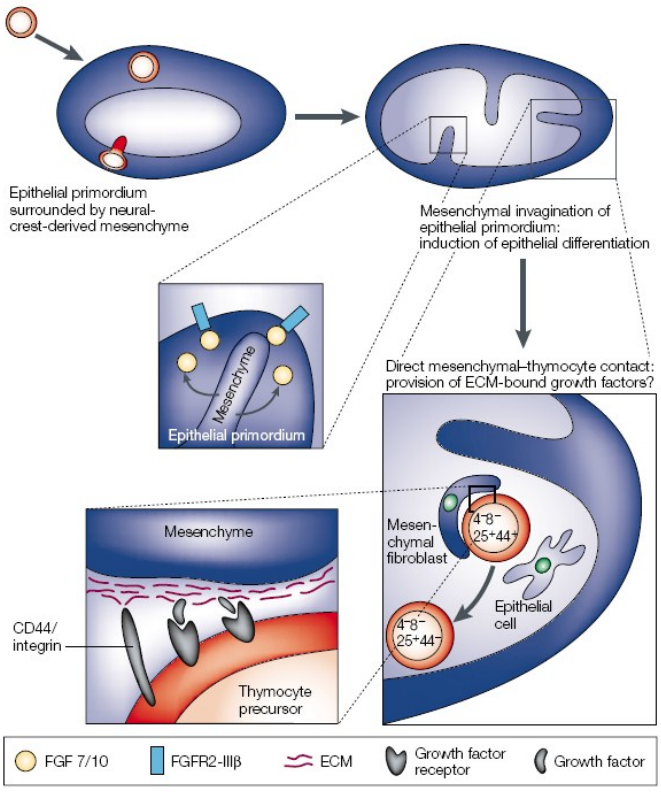


Figure 1.2 Model of mesenchymal involvement in thymus development and function.
Neural crest-derived mesenchymal cells surround and eventually invaginate the epithelial rudiment. These cells are proposed to have a dual role in the thymus. First, they produce Fgfs and directly influence the growth of early thymic epithelium. Second, mesechymal cells in cortex form an extracellular scaffold and present soluble growth factors essential for the development of immature thymocytes (ECM, extracellular matrix) (taken from Anderson, 2001).

1.1.3. Molecular control of early organogenesis

The initiation of thymus organogenesis is dependent on the correct initial formation of the pharyngeal region. Therefore, gene mutant mice with defective patterning of the pharyngeal region and defective pouch formation will consequently demonstrate a failure in the development of pouch-derived structures, including thymus. The analyses of mutant phenotypes and gene expression patterns have identified a transcription factor regulatory network that is required for the establishment and early patterning of the thymus primordium. At present Tbx1, Hoxa3, Eya1, Six1, Pax1, Pax3 and Pax9 are part of this network. Mutations in any of these genes lead to thymus aplasia, hypoplasia, or a complete failure of the thymus lobes to migrate caudally and centrally to their regular intrathoracic location (reviewed in (Manley 2000; Blackburn and Manley 2004; Hollander *et al.* 2006)). These genes are expressed in multiple cell lineages during development, and hence their loss of function causes also pleiotropic defects in embryonic development. It is therefore, often difficult to distinguish a direct role of these genes in thymus organogenesis, e.g. in TEC progenitors, from an upstream function, e.g. in formation or patterning of pharyngeal apparatus or in NC migration. Hence, experimental systems with TEC-specific and time-controlled gene deletions may be required to identify precisely the function of individual transcription factors in thymus development

The transcription factor T-box (Tbx1) is related to DiGeorge syndrome (DGS) (Greenberg 1993; Packham and Brook 2003; Naiche *et al.* 2005) which is caused genetically by heterozygous deletions within chromosome 22q1. The typical clinical features are cardiac outflow tract and heart pathologies due to malformations of pharyngeal arch arteries, parathyroid hypoplasia, and the absence or ectopic location of the hypoplastic thymus (Scambler 2000; Naiche *et al.* 2005). Hallmarks of this phenotype are recapitulated in mice homozygously lacking Tbx1, which display agenesis of pharyngeal pouches 2–4 and a concomitant loss or malformation of pharyngeal pouch-derived organs and tissues (i.e. thymus, parathyroid gland, cardiac outflow tract) (Jerome and Papaioannou 2001; Lindsay *et al.* 2001). Tbx1 is expressed in the pharyngeal pouch endoderm but also in the core arch mesoderm and the pharyngeal ectoderm but not in NCC-derived mesenchyme (Chapman *et al.* 1996; Garg *et al.* 2001). Hence, Tbx1 expression may play distinct roles in different anatomical sites during development. Pharyngeal pouches fail to develop in mice in which a Tbx1 deficiency is restricted to endoderm and they recapitulate the defects known from the constitutive null mice, including the absence of the thymus (Arnold *et al.* 2006). Similarly, mice lacking Tbx1 selectively in the pharyngeal mesoderm, have a hypoplastic pharynx with impaired pharyngeal endoderm and lack a thymus. A conditional reversion in pharyngeal mesoderm but not endoderm, from a defective to a functional Tbx1 allele, is sufficient to rescue the major pathologies observed in the Tbx1-deficient mice but fails to restore regular development of the thymus (Zhang *et al.* 2006). Therefore, expression of Tbx1 in both pharyngeal core mesoderm and endoderm is a prerequisite

for normal thymus development. Tbx1 expression in the pharyngeal region is regulated by sonic hedgehog (Shh) signalling (Garg *et al.* 2001). Analysis of gene expression patterns in wild type and Tbx1-deficient mice and experiments *in vitro* have identified several downstream targets of Tbx1 including Fgf8 and Fgf10 (Vitelli *et al.* 2002).

During development, Fgf8 is secreted by the pharyngeal pouch endoderm and provides survival, mitogenic and patterning signals to adjacent mesenchyme (Abu-Issa *et al.* 2002; Frank *et al.* 2002). Reduction of Fgf8 signalling by expressing a hypomorphic Fgf8 allele results either in athymic embryos (possibly secondary to severe defects in the formation of the 3rd and 4th pharyngeal arch and pouch) or in embryos with a hypoplastic, sometimes ectopic thymus that nevertheless supports a phenotypically normal thymopoiesis (Frank *et al.* 2002). The hypoplastic phenotype observed in these animals is similar to the one reported for mice deficient either for Fgf10 or FgfR2IIIb. The later is a specific Fgf receptor 2 variant expressed by TEC as early as E13 and transduces signals from its cognate ligands Fgf7 and Fgf10, which are secreted by the surrounding mesenchyme (Revest *et al.* 2001; Jenkinson *et al.* 2003). It is therefore thought that reciprocal Fgf signalling between the endoderm and the mesenchyme may be required at an early stage of thymus formation. Such interdependence by reciprocal Fgf signalling is also observed during the initial placement and induction of limb bud formation and during early lung organogenesis (Hogan and Yingling 1998; Martin 1998).

Signalling *via* the bone morphogenetic proteins (Bmp) has been implicated in thymus organogenesis (Ohnemus *et al.* 2002; Bachiller *et al.* 2003; Bleul and Boehm 2005). Bmps belong to the transforming growth factor beta (TGFβ) family of cytokines that encompasses multiple ligands and receptors. Upon ligand binding, two transmembrane receptor serine/threonine protein kinases (receptor types I and II) activate specific receptor-regulated Smad (R-Smad) proteins. Activated Smads form a multi-subunit complex with a common partner, Smad4. These complexes then translocate from the cytoplasm to the nucleus where they interact with additional nuclear factors to regulate gene transcription. Several proteins have been identified, which physiologically antagonize Bmp signalling by either blocking ligand binding to cognate receptor (Noggin or Chordin) or by altering the cytoplasmic signal transduction by competing with Smad4 (Smad7). Disruption of the canonical TGFβ/Bmp signalling in TEC by loss of Smad4 expression from E12.5 causes a progressive structural disorganization of the thymic microenvironment and a loss of TEC competence to attract early T lineage progenitors (ETP) resulting in the extensive thymic and peripheral lymphopenia. This findings place Smad4 within the signalling events in TEC that determine total thymus cellularity by controlling the number of ETP (Jeker *et al.* 2008). At E10.5 Bmp4 is expressed in the ventral aspect of the 3rdpp epithelia and at E12 and beyond also by the surrounding mesenchyme. Blocking of Bmp signalling in premigratory NCC by transgenic expression of the decoy receptor Noggin leads to a failure in the NCC migration and consequently affects thymus development indirectly

(Ohnemus *et al.* 2002). A TEC-relevant role of BMP-mediated signalling has been demonstrated in transgenic mice where Noggin is expressed as early as E11.5 in TEC (Bleul and Boehm 2005). These mice display a severely impaired thymus development and a reversion of TEC to a phenotype characteristic of foregut epithelium (Bleul and Boehm 2005; Soza-Ried *et al.* 2008). Together these studies demonstrated that in addition to having role in NCC, Bmp signalling also affects TEC biology directly (Bleul and Boehm 2005). Mice deficient for Chordin, another Bmp signalling antagonist, display an extensive array of malformations that encompass most of the DGS features including the lack of thymus, absent parathyroid glands and outflow tract defects (Bachiller *et al.* 2003). In addition, there is a major reduction in Tbx1, Pax9 and Fgf8 expression in the pharyngeal endoderm of Chordin-deficient mice. These data suggest that Chordin acts upstream of Tbx1 and Fgf8. In turn, Tbx1 relays the autocrine effect of Chordin in the pharyngeal endoderm necessary for a proper development of the thymus, parathyroid and thyroid glands. Fetal thymic organ culture (FTOC) experiments have recently provided evidence that at least some of the effects conferred by Fgfs may be mediated downstream by Bmp4 signalling. Indeed, the addition of Bmp4 to FTOC affects T cell differentiation along the αβ-lineage and leads to an abnormal accumulation of immature double negative (DN1) thymocytes (Hager-Theodorides *et al.* 2002), an effect that is suppressed Fgfs (Tsai *et al.* 2003). It was further proposed that Bmp4 upregulates Foxn1, which in turn increases FgfR2IIIb expression thus, rendering TEC more susceptible to Fgf7 and Fgf10 signals provided by different cells including mesenchymal cells as well as double or single positive thymocytes (Erickson *et al.* 2002). In addition, the thymic phenotypes of p63-deficient thymi (Candi *et al.* ; Senoo *et al.* 2007) and FgfR2IIIb-deficient (Revest *et al.* 2001; Dooley *et al.* 2007) are quite similar displaying thymic hypoplasia caused by a reduced proliferation. p63 has been implicated in TEC differentiation by promoting the survival of progenitor/transit-amplifying cells and, like Foxn1, acts upstream of FgfR2IIIb. The expression of p63 expression in the thymus is independent of Foxn1 (Senoo *et al.* 2007) and a possible genetic interaction of Foxn1 with Tbx-p63-FgfR2IIIb pathway is yet to be established.

Hoxa3 (Manley and Capecchi 1998), Pax1 (Wallin *et al.* 1996), and Pax9 (Hetzer-Egger *et al.* 2002) are expressed by thymic epithelial cells. The exact identity of signal(s) determining the site of the thymic anlage is still unknown. Since Hox genes control axial position identity during embryogenesis and since the anterior boundary of Hoxa3 expression is along the 3rdpp, it has been hypothesized that the expression of Hoxa3 determines the positioning and identity of the thymus rudiment (Krumlauf 1994). Moreover, the Pax–Eya–Six pathway together with Hoxa3 might also control the separation of the thymus/parathyroid primordium from the pharynx and its subsequent migration. Indeed, thymic rudiments do not detach from the pharynx of Pax9 mutant mice and the normal separation is delayed In Hoxa3$^{+/-}$ Pax1$^{-/-}$-deficient animals (Su *et al.* 2001; Hetzer-Egger *et al.* 2002). Pax1 expression is detected in virtually all cells in the early endodermal epithelium of the 3rdpp and is downstream of Eya1 and Six1 (Zou

et al. 2006). The proportion of Pax1-expressing cells declines with age and in the adult mouse only a small fraction of cortical thymic cells remains strongly Pax1 positive. Pax1 is necessary for the establishment of thymus microenvironment required for normal T cell maturation as naturally occurring mutations in Pax1 observed in the so called undulated mice, disturb the maturation of $CD4^+C8^+$ and $CD4^+$ thymocytes (Wallin *et al.* 1996). Pax9 is expressed in the entire pharyngeal endoderm at E9.0, and its function is required for the development of those organs that derive from the pharyngeal pouches. In mice deficient for Pax9 expression, a thymus anlage develops ectopically in the larynx. The TEC of this ectopic thymus do express Foxn1, a marker of thymic epithelium, but the entire anlage however, fails to migrate caudo-ventrally to the upper mediastinum (Hetzer-Egger *et al.* 2002).

These studies indicate that at least some molecular mechanisms operational in the patterning of the thymus primordium are intrinsic to the endoderm. However, the identity of the regulatory pathways that occur concurrently in the adjacent mesenchyme and the molecular nature of the ensuing epithelial–mesenchymal interactions are less well established. In this context, signals from NC-derived mesenchymal cells are essential for normal thymus organogenesis (Anderson *et al.* 1993; Suniara *et al.* 2000; Jenkinson *et al.* 2003) as the absence or specific defects in NCC formation, migration or survival correlate with athymia or thymic hypoplasia (Bockman and Kirby 1984; 1989; Conway *et al.* 1997; Soriano 1997; Ohnemus *et al.* 2002). Interestingly, Hoxa3, Eya1 and Six1 are also expressed in NCC and thus may be required indirectly for a proper development of the thymus. In the absence of Hoxa3 expression, NCC do densely populate the 3rd pharyngeal arches but the intrinsic capacity of these cells to differentiate and/or to induce a correct patterning of 3rdpp is impaired (Manley and Capecchi 1995). Eya1, a transcriptional co-activator, is expressed early in the pharyngeal endoderm, mesenchyme and ectoderm. The importance of Eya1 for thymus organogenesis is revealed by analysing knock out mice as these animals have no thymus or parathyroid and fail to express Wnt5b in the endoderm (Xu *et al.* 2002; Zou *et al.* 2006), which regulates Foxn1 expression in TEC (Balciunaite *et al.* 2002). Six1 expression is markedly reduced in the pharyngeal region of Eya1-deficient embryos, indicating that Six1 expression is Eya1 dependent. Six1-deficient mice and mutations in Pax genes display similar phenotypes to mice lacking Eya expression and suggests a regulatory network where Eya, Six and Pax control early inductive events in the thymus morphogenesis (Laclef *et al.* 2003).

To define specific Hoxa3, Eya1 and Six1 function separately for neural crest and endodermal cells during thymus organogenesis, the tissue-specific and time-controlled gene ablation studies will be required. So far, Pax3 is the only transcription factor known to affect thymus development and to be expressed exclusively by NCC. Mutations in Pax3 cause in mice the splotch phenotype (Franz 1989) characterized by thymus ectopia, hypoplasia or aplasia (Franz 1989; Conway *et al.* 1997; Griffith *et al.* 2009). However, this phenotype appears to be secondary to NCC death or their failure to migrate.

1.1.4. Bipotent thymic epithelial progenitors

While a single endodermal origin for cTEC and mTEC has been demonstrated in birds (Le Douarin and Jotereau 1975) and later confirmed in the mouse (Gordon *et al.* 2004), it was unclear for a long time whether these two morphologically and functionally distinct types of epithelial cells arise from a common, i.e. bipotent progenitor population or, alternatively, from separate lineage-committed, i.e. unipotent progenitors, and if such progenitors still exist in the postnatal period. A characteristic feature of progenitors is that a small number of cells generates a large pool of progeny as a consequence of proliferation and differentiation. This developmental process is concurrent with a progressive loss of differentiation potential and once completed is believed to be irreversible. Resident tissue-specific progenitor cells have been described for several somatic tissues, and their asymmetric self-renewal has been linked to homeostatic tissue maintenance (Sharma *et al.* 2006; Fuchs 2007; Xu *et al.* 2008).

An accurate phenotypic identification and assays to reveal functions of progenitor cells at a clonal level are major facilitators in elucidating progenitor biology. The existence of bipotent TEC progenitors (bTECp) was first suggested by the cytokeratin (CK) staining pattern that uniformly recognizes a large $CK5^+CK8^+$ TEC population in the early thymus whereas these cells become progressively restricted in either $CK5^-CK8^+$ cTEC or $CK5^+CK8^-$ mTEC during further development (Ritter and Boyd 1993; Ropke *et al.* 1995; Klug *et al.*). Moreover, the co-expression of cTEC- and mTEC-specific markers by a single cell population in the thymus anlage has further contributed to the notion that both TEC lineages may arise from a common progenitor pool. MTS24 (Plet-1) is expressed by all TEC at E12 whereas in the adult thymus its expression is limited to rare medullary TEC (Depreter *et al.* 2008). Although initial reports suggested that MTS24 could be used to identify and purify TEC progenitors able to generate a functional epithelial environment (Bennett *et al.* 2002; Gill *et al.* 2002) subsequent studies showed that also $MTS24^-$ embryonic TEC, though at a lower efficiency, can form the epithelial scaffold required for thymopoiesis (Swann and Boehm 2007; Rossi *et al.* 2007b).

Two groups using different experimental systems demonstrated that single thymus epithelial precursors are capable of generating a functional thymic microenvironment with defined cortical and medullary areas. Using a cellular approach, Rossi *et al.* established a clonal assay allowing TEC fate mapping (Rossi *et al.* 2006). Single $EpCam^+$ and genetically marked $eYFP^+$TEC isolated from an E12 thymus anlage were microinjected into wild-type 'foster' E12 thymus lobes and then transplanted under the kidney capsule of recipient mice. Subsequent immunohistological analysis revealed that single fetal TEC contributed to both cTEC and mTEC lineages whereas the contribution to a single TEC lineage was absent demonstrating the presence of bTECp in early thymus anlage. Using a genetic approach, Bleul and colleagues reported similar findings for postnatal thymus (Bleul *et al.* 2006). Reversion of conditional Foxn1 mutant allele to wild-type function in single epithelial cells led to the development of a structurally and functionally normal thymus tissue providing evidence for the continued persistence of bTECp beyond

the embryonic period (Bleul *et al.* 2006). The existence of mTEC-committed progenitors was also revealed in lineage tracing experiments demonstrating that the thymus medulla is comprised of individual epithelial 'islets' each arising from a single progenitor during TEC ontogeny (Rodewald *et al.* 2001). Recently, separate progenitors for cTEC lineage have also been identified (Shakib *et al.* 2009). However, the identity of the thymus epithelial stem cells remains elusive, and presently bTECp have only been identified on the basis of their functional properties. Despite evidence for their persistence after birth, the virtual lack of appropriate markers makes it impossible to track and localize these cells in the postnatal thymus. Moreover, any capacity for self-renewal of thymic epithelial progenitors is currently unknown and it is not clear whether the development and maintenance of TEC within the adult thymus occurs in the same way as in the embryonic thymus. Two models of TEC development have been proposed both addressing the question whether in the adult steady-state thymus cTEC and mTEC are derived from a population of bipotent or lineage-committed cells (Figure 1.3) (Anderson *et al.* 2007). In the first model a self-renewing pool of bipotent progenitors remains existent throughout life and generates transient amplifying populations of cTEC- and mTEC-committed precursors that have lost a self-renewing capacity. In a second model, there is a fixed number of bipotent progenitors in the thymus that lack the capacity for self-renewal and generates separate lineage-committed precursors with self-renewing capacity which give rise to cTEC and mTEC. Clearly, further studies are required to address the self-renewal capacity of TEC and the stages in TEC development at which it may occur. The identification of TEC stem cells remains an important goal not least in view of possible future prospects to reconstitute thymic function by transplanting such tissue-specific stem cells (Swann and Boehm 2007).

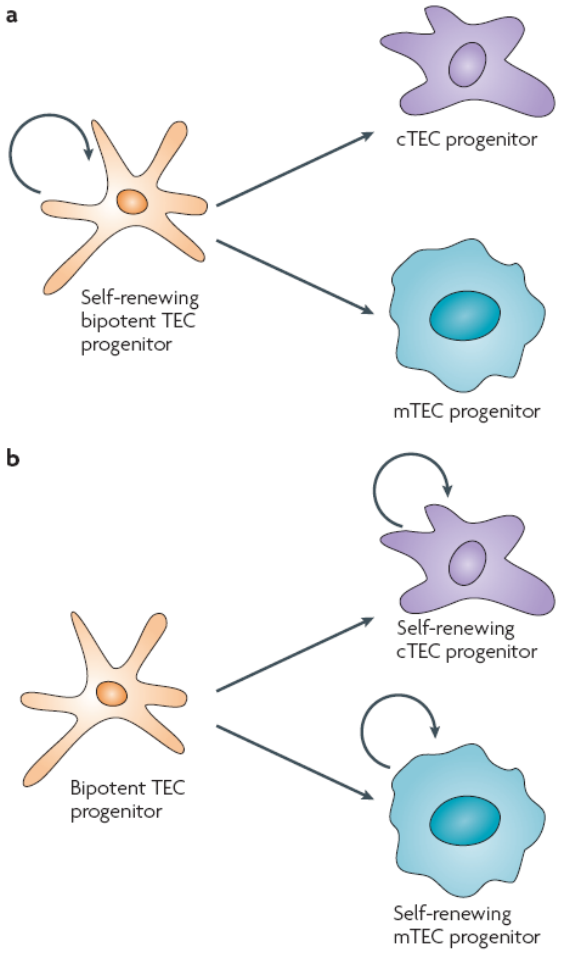


Figure 1.3 Models of TEC development and self-renewal.

a Bipotent TEC progenitors persist throughout life by continued self-renewal and produce lineage-committed TEC progenitors. **b.** There is a fixed number of bipotent progenitors in the thymus that lack the capacity to self-renew. The continued production of mature cTEC and mTEC is achieved by the generation of lineage-committed TEC progenitors with the capacity to self-renew (taken from Anderson, 2007).

1.2. Generation of a functional thymic epithelial compartment

Epithelia of the 3rdpp endodermal lining acquire a TEC fate and generate a population of progenitors that subsequently differentiate into phenotypically diverse cortical and medullary epithelial TEC (Bleul *et al.* 2006; Rossi *et al.* 2006). For a long time, TEC were considered as postmitotic, end-stage cells that, once generated during ontogeny, are maintained in their mature state. It is now clear that TEC can be generated from bipotent or committed progenitors in ontogeny (Bennett *et al.* 2002; Gill *et al.* 2002; Rossi *et al.* 2006), that stages of immature and mature TEC are phenotypically separable, and that TEC undergo a rapid renewal within a few weeks (Gray *et al.* 2006). These findings suggest that the generation and maintenance of steady state TEC compartments is likely to be a result of bTECp activity together with subsequent expansion of the cTEC- and mTEC-committed progeny. Identification of precursor-product relationships within the cTEC and mTEC lineage represents the first step in elucidating the stages and checkpoints in the formation of mature thymic microenvironments and is a major focus of current studies.

1.2.1. Cortical microenvironment

Cells of the cTEC lineage are typically identified by flow cytometry based on their $CD45^-$ $EpCam1^+Ly51^+$ cell surface phenotype (Derbinski *et al.* 2001). In tissue sections, intracytoplasmic markers are used to identify the major cortical subset of $CK5^-CK8^+$ and the minor subset of $CK5^+CK8^+$ cells located at the corticomedullary junction (Klug *et al.* 1998). Distinct cTEC subsets can be further defined based on the expression of other cell markers including DII-4 (Tsukamoto *et al.* 2005), CD205 (Shakib *et al.* 2009), β5t (Murata *et al.* 2007), CD40 (Akiyama *et al.* 2008a) and MHCII (Jenkinson *et al.* 1981; Yang *et al.* 2006). Of functional significance is the expression of both MHC class I and class II molecules on cTEC as this restriction elements regulate thymic selection (McDuffie *et al.* 1987; Kaye *et al.* 1989; Marrack *et al.* 1989; Bowlus *et al.* 1999; Takahama 2006; Yang *et al.* 2006).

Formation of the thymic cortex

The steps in cTEC differentiation downstream of bipotent progenitors and the molecular clues driving their genesis and maintenance are just beginning to be identified. During early embryogenesis, TEC with an immature ($CK5^+CK8^+$) or cortical ($CK5^-CK8^+$) phenotype are already present before the colonization of the anlage by lymphoid progenitors at E11.5 (Klug *et al.* 1998; Klug *et al.* 2002; Hamazaki *et al.* 2007). Indeed, the emergence of these TEC subpopulations is not impaired in Rag2/γc-deficient mice, which have a complete block in T, B, and NK cells development suggesting that the generation of cTEC does

not require hematopoietic-derived signals (Klug *et al.* 2002). However, later in ontogeny, the cortical microenvironment of these and other mice with an early block in T cell development displays a disturbed cortical architecture and an arrest in TEC differentiation at immature $CK5^+CK8^+$ stage due to a lack of thymocyte-mediated signals (Hollander *et al.* 1995; Klug *et al.* 1998; van Ewijk *et al.* 2000). The defective cTEC development can be rescued by regular thymopoiesis following hematopoietic stem cells transplantation as well as the generation of all T cell subsets up to the DP stage. However, this rescue is successful during a short window of time after birth suggesting that the induction of cTEC differentiation by thymocytes can only occur in a developmentally restricted manner (Hollander *et al.* 1995). Unlike mice with a very early block in T cell development, mice deficient for Rag1 or Rag2 expression, where thymocyte development is arrested at the DN3 stage, have an almost normal cTEC development (Klug *et al.* 1998; van Ewijk *et al.* 2000) indicating that cTEC differentiation depends on interactions with thymocytes committed to the T lineage.

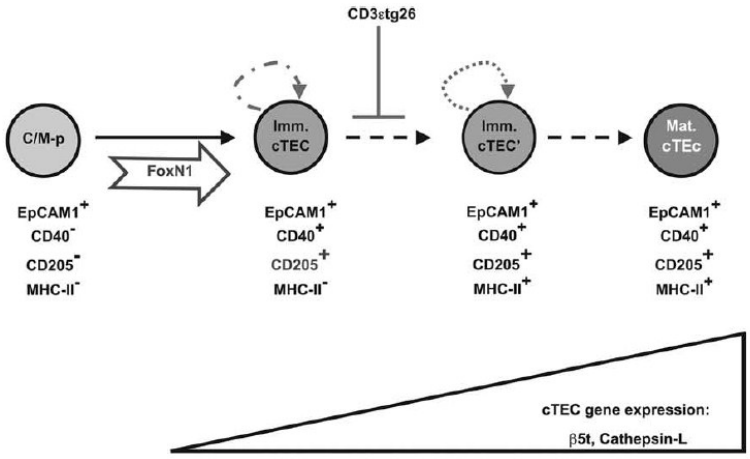


Figure 1.4 A model of cTEC development
cTEC develop from cTEC/mTEC bipotent progenitors *via* cTEC-commited progenitors to mature MHCII-expressing cTEC. The requirements for Foxn1 expression and thymocyte-TEC crosstalk are depicted. Circular arrows represent proliferation. Imm denotes immature, mat denotes mature (taken from Shakib, 2009).

Analysis of the precursor–product relationships for the cTEC lineage has been hampered by the lack of appropriate markers to stage the developmental progression of its cells. Only recently, committed cTEC progenitors (cTECp) and distinct stages in cTEC maturation have been identified (Shakib *et al.* 2009) based on the differential expression of CD205, CD40 and MHCII molecules (Figure 1.4) (Shakib *et al.* 2009). cTEC with an $EpCam1^+CD205^+CD40^-$ phenotype are proposed to represent a population of progenitor cells that is developmentally positioned between bipotent progenitors and mature $EpCam1^+CD205^+CD40^+MHCII^+$ cTEC. These intermediate cTEC progenitors express, in comparison with mature cTEC, lower levels of cTEC-specific transcripts including β5t and cathepsin L and display higher proliferation rate.

The requirement for Foxn1 expression and thymocyte-epithelial crosstalk in cTEC development has been investigated by analysis of nude (Foxn1-deficient) and tgε26 (block at the DN1 stage of T cell development) embryonic thymus, respectively. Foxn1 but not thymocyte–derived signals is required for the initial generation of $CD205^+$ cTEC from a bipotent progenitor as early as E12 and agrees with the

notion that Foxn1-deficient TEC may represent bipotent progenitors that have not yet undergone commitment to the cTEC and mTEC lineages (Bleul *et al.* 2006). Further developmental progression such as the acquisition of CD40 and MHCII expression on cTEC is however, critically dependent on the presence of early stage (DN1–3) thymocytes. Thus, a stage-specific requirement for thymocyte crosstalk exists for development of cTEC lineage. How exactly DN thymocytes influence cTEC development remains unresolved, though they have been proposed to, in addition to providing signals for differentiation, also play a role in remodelling developing cTEC and in the formation of the typical three-dimensional reticular cortical network (van Ewijk *et al.* 2000). The present work is focusing on studying the molecular mediators regulating the cTEC development and maintenance. A possible role in these processes has been assigned to Stat3 signalling as its loss in TEC causes severe postnatal thymic hypoplasia including alterations of the cortical architecture (Sano *et al.* 2001). Similarly, the loss of Kremen1, a negative regulator of Wnt signalling leads to a severe defect in cTEC architecture (though positive selection still occurs) (Osada *et al.* 2006) indicating the importance of an appropriate Wnt activity for cTEC development. To further investigate specific signalling pathways acting in a cTEC-lineage specific fashion, target gene deletion models will be needed.

Function of cortical thymic epithelial cells

The regular development of cortical TEC is a prerequisite for the generation of a functionally normal thymic microenvironment as cTEC provide essential signals and restriction elements during early stages of T cell development and positive selection of T cell antigen receptor, respectively. cTEC have unique antigen processing and presenting capacities that are distinct from other thymic stromal cells including mTEC and dendritic cells (DC) (Bowlus *et al.* 1999). For example, cTEC specifically express the thymus-restricted proteasome catalytic subunit β5t, which is a molecule linked to cTEC ability to generate low affinity peptides for MHC class I presentation (Murata *et al.* 2007). Consequently, mice lacking this subunit exhibit the selective reduction of $CD8^+$ T cells. However, the absence of β5t does not affect cortical or medullary architecture or overall thymus size, indicating that β5t may not be essential for the overt development and architectural organization of TEC (Murata *et al.* 2007). Positive selection of $CD4^+$ T cells requires the expression of the lysosomal protease cathepsin L in cTEC (Nakagawa *et al.* 1998; Honey *et al.* 2002). This enzyme mediates the invariant-chain degradation in cTEC, a key step in MHC class II restricted antigen presentation (Nakagawa *et al.* 1998; Honey *et al.* 2002). Similarly, the putative thymus-specific serine protease (TSSP) (Bowlus *et al.* 1999) which is exclusively expressed in the endosomal compartment of cTEC plays an important role in MHC class II restricted positive selection of $CD4^+$ T cells (Gommeaux *et al.* 2009). Together, these studies established that the unique protein degradation and self-peptide presentation by cTEC is pivotal for the positive selection of MHC class I and

II restricted thymocytes (Murata *et al.* 2008; Takahama *et al.* 2008). How possible heterogeneity among cTEC may reflect on their functional specialization remains unknown. The identification of cell surface and cytoplasmic markers together with the use of genetic models for *in vivo* cell tracing studies should help to uncover key regulators in the generation of cTEC and will be useful tools to study cTEC heterogenity.

1.2.2. Medullary microenvironment

Cells of the mTEC lineage can be identified by flow cytometry on the basis of their $CD45^-$ $EpCam1^+Ly51^-$ phenotype and the concurrent expression of MHC class I and class II molecules (Derbinski *et al.* 2001). Immunohistochemistry identifies major stellate $CK5^+/14^+CK8^-$ population and a minor subset of $CK5^-/14^-CK8^+$ cells that is distinguished from the cortical subset by globular morphology (Klug *et al.* 1998). The minor subset can be further separated in two subpopulations defined by their reactivity with UEA-1, i.e. $CK5/14^-CK8^+UEA\text{-}1^+$ mTEC and $CK5/14^-CK8^+UEA\text{-}1^-$ mTEC. In contrast, the stellate mTEC do not bind UEA-1 lectin, but are marked by MTS10 expression (Klug *et al.* 1998). The population of mTEC displays further heterogeneity not least with respect to differential cell surface expression of MHCII, CD80, CD86 and CD40, the presence of Aire and consequently tissue-specific antigens (Kyewski and Klein 2006).

Function of medullary thymic epithelial cells

The thymic medulla serves two main functions, i.e. the completion of T cell maturation and the establishment of self-tolerance by promoting the elimination of autoreactive SP thymocytes carrying TCRs with high affinity for self-antigen–MHC complexes. A unique property of mTEC is their ability to express tissue-restricted self-antigens (TRAs) representing almost all peripheral tissues, irrespective of developmental or spatio-temporal expression patterns (Kyewski and Klein 2006). This promiscuous gene expression (PGE) program generates a large repertoire of TRAs that are either directly presented to SP thymocytes by mTEC or are shed and taken up by medullary DC for antigen presentation. This latter mechanism is referred to as cross-presentation (Gallegos and Bevan 2004; Koble and Kyewski 2009). Thymic mTEC and DC have also been implicated in the development of $Foxp3^+CD4^+CD25^+$ regulatory T cells, which are involved in the maintenance of self-tolerance in the periphery (Aschenbrenner *et al.* 2007; Proietto *et al.* 2008; Spence and Green 2008).

Of key importance to understanding PGE and its relevance for central tolerance was the identification of a mTEC subset that expresses the autoimmune regulator Aire, a transcription factor that is defective in the human autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) syndrome (Anderson *et al.* 2005). Aire protein is localized in the nucleus and regulates, at least in part, PGE aiding

to the establishment of central T cell tolerance to peripheral antigens. Aire deficiency in mice results in a reduction of specific TRAs available for negative selection and consequently leads to defective tolerance induction and organ-specific autoimmunity (Anderson *et al.* 2002; Liston *et al.* ; Derbinski *et al.* 2005). In addition, Aire also regulates mTEC differentiation (Gillard *et al.* 2007; Dooley *et al.* 2008; Yano *et al.* 2008). Aire-deficient thymus displays changes in mTEC composition and organization, including an absence of UEA-1^{hi} mTEC (Dooley *et al.* 2008). Moreover, the contracted CK14^{+} medullary compartment lacks the confluent stellate mTEC and has an increased representation of globular mTEC. As in the normal thymus, globular CK5^{-}/14^{-}CK8^{+} mTEC constitute only a minor subset (Klug *et al.* 1998; Klug *et al.* 2002; Gillard and Farr 2006) these cells are suggested to represent the cellular target of Aire-mediated apoptosis accounting for the accumulation of these terminally differentiated mTEC in the Aire-deficient thymus (Gillard *et al.* 2007).

Formation of the thymic medulla

The differentiation of bipotent progenitors into mTEC has been proposed to occur *via* committed mTEC progenitors (mTECp) (Rodewald *et al.* 2001). While we know little about the developmental pathway(s) linking bTECp to the earliest committed mTEC, a more detailed picture emerged on differentiation events within the mTEC lineage. The availability of well-defined markers to identify and isolate cells within this lineage together with the observation that dissociated fetal thymus stroma is able to form a functional thymus in RTOC settings allowed examination of lineage potential and function of different mTEC subsets (Rossi *et al.* 2007b).

Along this line, TEC progenitor population that give rise specifically to mature Aire^{+} mTEC was identified based on the expression of the tight junction components Claudin (Cld)-3 and 4 (Hamazaki *et al.* 2007). In early ontogeny, Cld-3,4^{+} TEC are found in the most apical layer of the bilayered TEC rudiment. By E13.5, the heterogeneity of TEC populations becomes evident as Cld-3,4^{+} cells begin to express UEA-1 ligand and MTS10 indicating the commitment to a medullary epithelial fate (Hamazaki *et al.* 2007). A developmental step in the mTEC lineage that involves maturation of Aire^{-}MHCII^{-}CD80^{-} mTEC progenitors into Aire^{+}CD80^{+}MHCII^{+} mTEC (Rossi *et al.* 2007c) and culminates in the appearance of Involucrin^{+} mTEC (Yano *et al.* 2008) has also been identified in the embryonic thymus. The developmental potential of E13.5 Cld-3,4^{+} TEC has been examined in grafting RTOC studies. While Cld-3,4^{lo} cells gave rise to both mTEC and cTEC, Cld-3,4^{hi} cells gave only rise to mTEC thus, inferring that these cells represent committed mTEC progenitors. This conclusion is in keeping with earlier studies demonstrating the thymus medulla to be composed of aggregates of clonally derived islets (Rodewald *et al.* 2001) and further suggests that commitment to the mTEC lineage may occur as early as E13.5 (Hamazaki *et al.* 2007). Whether the heterogeneity of mTEC reflects distinct mTEC lineages or various

stages of differentiation within a single lineage (Gillard and Farr 2005; Kyewski and Klein 2006) still remains to be determined.

Two opposite models of mTEC differentiation

Two models have been proposed to explain the heterogeneity and lineage relationships between mTEC subsets (Derbinski *et al.* 2005; Gillard and Farr 2005). The terminal differentiation model (Figure 1.5) postulates that mTEC development proceeds along distinct successive maturational stages and culminates in the generation of most mature Aire^{+} mTEC that are characterized by high levels of MHCII, CD80, CD86, CD40 expression (Derbinski *et al.* 2005). As these terminally differentiated mTEC are non-proliferating and also short lived they are continuously replenished *via* one or more rapidly cycling Aire^{-} intermediates that may express either low or high levels of MHCII, CD80, CD86 and CD40 expression

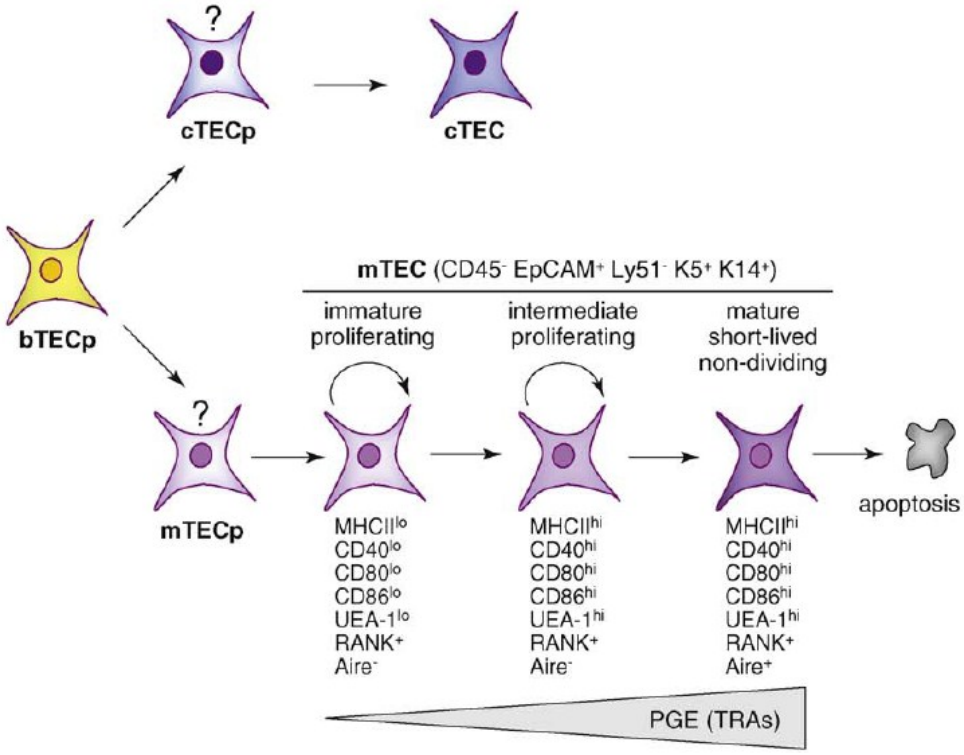


Figure 1.5 Terminal differentiation model of mTEC development.

cTEC and mTEC compartments of the thymus are derived from common bipotent progenitors (bTECp), possibly via committed cTEC (cTECp) and mTEC (mTECp) progenitors. Cells within the mTEC lineage can be divided into distinct subsets – believed to correspond to different maturation stages – based on various levels of MHCII, CD40, CD80, CD86 and Aire expression. mTEC also express RANK, and are bound differentially by the UEA-1 lectin. Mature mTEC (MHCII^{hi}Aire^{+}) are thought to be terminally differentiated, post-mitotic, short-lived and destined to die by apoptosis. The rapidly turning-over mature mTEC population is replenished continuously by cells differentiating from proliferating (circular arrow) immature precursors (MHCII^{lo} or MHCII^{hi}Aire^{-} cells). mTEC maturation is accompanied by a progressive increase in PGE leading to the ectopic synthesis of numerous TRAs (taken from Irla, 2010).

(Gabler *et al.* 2007; Gray *et al.* 2007b). The transition from immature to mature mTEC is in this model accompanied by a progressive expansion in the repertoire of promiscuously expressed TRAs. Apoptosis of the terminally differentiated mTEC has been postulated to be induced by Aire itself or to be a nefarious consequence of PGE (Gray *et al.* 2007b).

The developmental (also known as progressive restriction) model (Figure 1.6) suggests as an alternative, that high level of mitotic activity detected in the MHCII^{hi}CD80^{hi} mTEC (Gray *et al.* 2007b) is indicative of a transit-amplifying population that is less mature then the MHCII^{lo}CD80^{lo} population to which it gives rise, but express the highest levels of Aire and the most diverse TRAs repertoire (Gillard and Farr 2005). Supporting this model is the developmental sequence where MHCII^{hi} emerge before MHCII^{lo} mTEC inferring precursor-product relationship. Identification of lineage relationships between mTEC subsets paves the way to study molecular mediators and cell types regulating mTEC development and homeostasis.

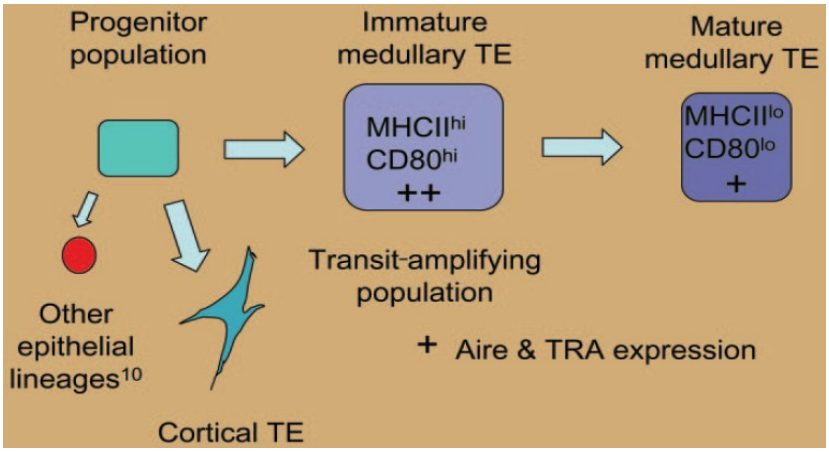


Figure 1.6 Developmental model of TEC differentiation.
The MHCII^{hi}CD80^{hi} mTEC have high mitotic activity and thus represent the immature mTEC that differentiate into more mature MHCII^{lo}CD80^{lo} mTEC. According to this scenario Aire and TRAs expression are properties of the immature mTEC compartment (taken from Gillard, 2006).

Control of mTEC development by NF-κB activation

The heteromeric nuclear factor (NF)-κB is activated by two signal transduction pathway (Figure 1.7) (Bonizzi and Karin 2004; Hoffmann *et al.* 2006). The canonical pathway activates the p50/RelA heterodimer and the noncanonical pathway results in the formation of the p52/RelB heterodimer that translocate into the nucleus to activate gene expression. The importance of the canonical pathway for mTEC development was demonstrated by the phenotype of mice lacking TRAF6, a key mediator in the upstream signalling events leading to NF-κB activation. The medulla of these mice exhibit an altered composition of mTEC subsets and an absence of UEA-1^{+} and mature Aire^{+} mTEC (Akiyama *et al.* 2005a). However, the exact contribution of the canonical NF-κB pathway to the medulla formation is not clear as TRAF6 has also been implicated in the signal transduction of other pathways (Darnay *et al.* 2007) including the NF-κB noncanonical pathway. Hence, the thymic phenotype of TRAF6 mice is likely caused by a combined abrogation of several signalling cascades. On the other hand, mice lacking individual components of the noncanonical pathway- such as NIK, IKKα and RelB – display disturbed medullary

architecture and a marked reduction in UEA-1$^+$ and Aire$^+$ mTEC (Burkly *et al.* 1995; Weih *et al.* 1995; Kajiura *et al.* 2004; Kinoshita *et al.* 2006; Zhang *et al.* ; Lomada *et al.* 2007). As a consequence of structural impairments to the medulla and its function in inducing central tolerance, mice deficient in regular NF-κB activation display also various signs of autoimmunity.

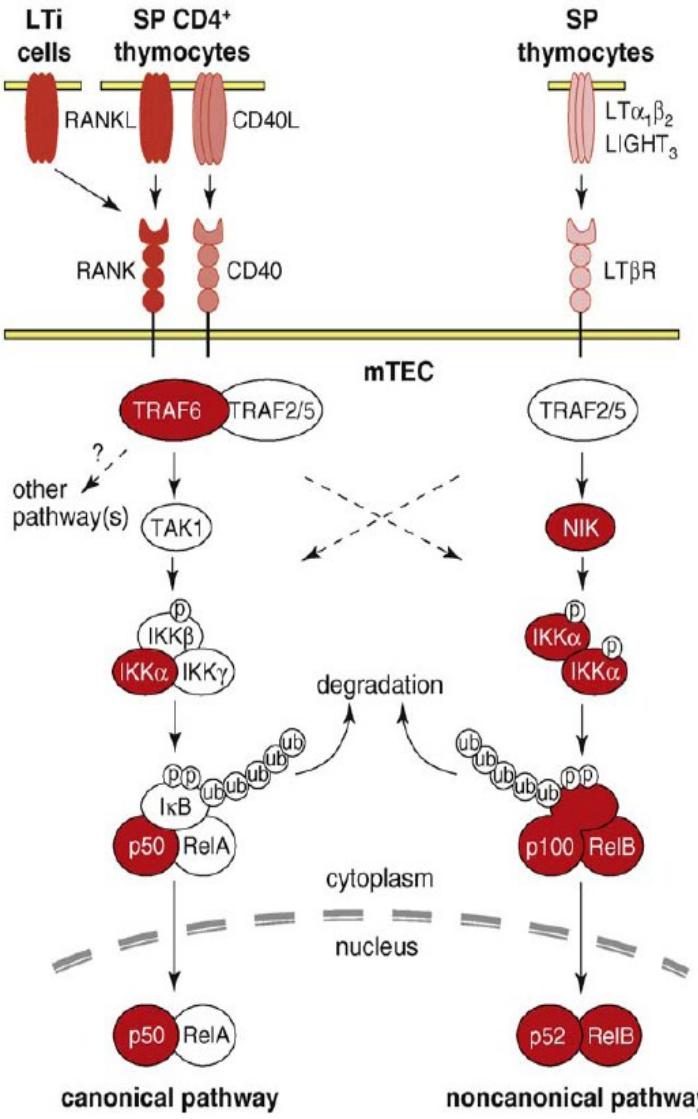


Figure 1.7 NF-kB signalling pathways governing mTEC development.
Members of the NF-kB family of transcription factors can be activated by the canonical (TRAF6) and noncanonical (NIK and RelB) pathways. The canonical and noncanonical pathways are coupled to TNF family of receptors which are implicated in mTEC development and include LTβR, RANK and CD40. In the postnatal thymus, the ligands of RANK (RANKL) and CD40 (CD40L) are provided by SP CD4$^+$ thymocytes. In the fetal thymus, RANKL is provided by LTi cells. LTbR ligands (LTa1b2 and LIGHT3) are expressed by both CD4$^+$ and CD8$^+$ SP thymocytes (taken from Irla, 2009).

Receptors and ligands of the TNF superfamily govern mTEC development

Tumor necrosis factor (TNF) signalling to mTEC is essential for the generation and maintenance of a correctly organized and functioning medulla. mTEC express lymphotoxin β receptor (LTβR), receptor activator of NF-κB (RANK) and CD40 receptor. Though RANK and CD40 engagement is linked to the canonical pathway *via* TRAF6 activation, and LTβR stimulates the noncanonical pathway (Basak and Hoffmann 2008), each of these three receptors may also activate the alternative NF-κB pathway but induces different biological effects in mTEC.

The heterotrimer LTα1β2 and the homotrimer LIGHT3 (Tumanov *et al.* 2003; Irla *et al.* 2008; Irla *et al.* 2009) are expressed by SP thymocytes and bind to LTβR. Activation of signalling *via* LTβR regulates

the mature mTEC in terms of size and organization, but does not affects the expression of Aire and its target genes or the differentiation and maintenance of Aire$^+$ mTEC as revealed in mice that lack LTβR signalling (Venanzi *et al.* 2007; Gray *et al.* 2008; Martins *et al.* 2008). Importantly, signalling through LTβR does not involve TRAF6 (Ishida *et al.* 1996). In contrast, signalling *via* RANK (Rossi *et al.* 2007c) engages TRAF6 (Akiyama *et al.* 2005a) and is directly responsible for Aire$^+$ mTEC differentiation and the transcriptional regulation of Aire and Aire-dependent TRAs. This signalling axis is essential for the initial emergence of mature mTEC as RANK-deficient embryos lack Aire$^+$ mTEC. However, Aire$^+$ mTEC are detected, albeit in reduced numbers, in adult RANKL- or RANK-deficient mice. This change in phenotype inferred that additional signal(s) may be relevant for the terminal development of mature mTEC in the postnatal thymus (Akiyama *et al.* 2008a). Indeed, subsequent analysis identified the CD40L/CD40 signalling axes as an additional pathway controlling postnatal mTEC development. Mice deficient for CD40 have a marked reduction in Aire$^+$ mTEC (Hikosaka *et al.* 2008; Irla *et al.* 2008; Akiyama *et al.* 2008a), while double deficiency of CD40 and RANKL results in the absence of Aire$^+$ mTEC altogether and exacerbated symptoms of autoimmunity demonstrating a cooperative role for RANK and CD40 in development of mTEC to establish self-tolerance (Akiyama *et al.* 2008a).

Furthermore, FTOC confirmed that the development of mature Aire$^+$ mTEC and PGE are, in addition to stimulation by CD40 or RANK, also dependent on signalling *via* TRAF6, NIK and RelB and verified that each receptor activates both the canonical and noncanonical NF-kB pathways in mTEC (Akiyama *et al.* 2008a). Given the heterogeneity and dynamic nature of the thymic microenvironment it remains to be determined whether the signals transmitted *via* LTβR, RANK and CD40 act concomitantly or sequentially during specific stages of embryonic and postnatal mTEC development.

Cellular interactions driving mTEC development

mTEC are not only promiscuous in their choice of genes they express but also in their predilection for cellular partners they interact with, reflecting a degree of complexity in the control of their differentiation that was not anticipated by the original concept of thymic crosstalk. During fetal thymus organogenesis, Aire$^+$ mTEC emerge before SP thymocytes are formed (White *et al.* 2008). In keeping with this observation, small numbers of Aire$^+$ mTEC are also detected in Rag2-deficient mice that lack SP thymocytes (Derbinski *et al.* 2001; Rossi *et al.* 2007c; Hikosaka *et al.* 2008). Aire$^+$ mTEC can thus, be generated also independent of mature SP thymocytes. On the other hand, the appearance of Aire+ mTEC in ontogeny coincides with the presence of lymphoid tissue inducer (LTi) cells. These cells represent a population of fetal liver derived hematopoietic cells and are characterized by the expression of CD4, the retinoic acid receptor related orphan receptor γt (RORγt) and the transcriptional repressor Id2 but lack CD3. LTi cells also express RANKL on their cell surface allowing for direct cell–cell interactions

with mTEC (Anderson *et al.* 2007; Rossi *et al.* 2007c; White *et al.* 2008). However, these developmental signals most likely do not contribute to the development of Aire^{+} mTEC in the postnatal thymus (Hikosaka *et al.* 2008; White *et al.* 2008) since Aire^{+} mTEC are not reduced in adult mice that lack LTi cells due to a deficiency in the expression of Id2 or RORγt (Hikosaka *et al.* 2008). Furthermore, LTi cells are exceedingly rare in the postnatal thymus and are hence unlikely to deliver signals that are sufficiently abundant to control mTEC maturation in a view of their high turnover rate.

Several studies have established the role of positively selected thymocytes for the expansion and organization of the medulla in the postnatal thymus. Given their relative late appearance during ontogeny, these cells are thought to take over the role of LTi in the adult thymus and contribute to the postnatal mTEC homeostasis. This is evident in mice that lack positive selection (TCRα$^{-/-}$, Zap70$^{-/-}$ and Rag2$^{-/-}$) or in mice that have deficiencies in CCR7 or CCR7 ligands and hence defective thymocytes migration and consequently display severely defective or reduced numbers of mTEC (Ueno *et al.* 2004; Hikosaka *et al.* 2008; Irla *et al.* 2008). Reconstitution of T cell-deficient severe combined immunodeficient (SCID) mice with mature T cells or TCR bearing thymocytes was sufficient to restore mTEC development and hence the formation of a correctly organized medulla (Shores *et al.* 1991; Surh *et al.* 1992). Similarly, the numbers of mature CD80^{hi}, CD40^{hi}, Aire^{+} mTEC are strongly reduced in mice that lack MHCII (IAα) expression and thus, lack positively selected CD4^{+} thymocytes (Nasreen *et al.* 2003; Irla *et al.* 2008). Conversely, aborted positive selection of CD8^{+} thymocytes in mice that lack MHCI (b2m) expression does not lead to a significant reduction in mature mTEC (Nasreen *et al.* 2003; Irla *et al.* 2008). Thus, these studies establish a specific role of positively selected CD4^{+} thymocytes in controlling mature mTEC cellularity in the postnatal thymus.

The specificity for positively selected CD4^{+} thymocytes is conferred by their selective upregulation of CD40L and RANKL and their capacity to induce the development of Aire^{+} mTEC and TRA in RTOC (Hikosaka *et al.* 2008; Irla *et al.* 2008; White *et al.* 2008). The relative contribution of LTi and CD4^{+} thymocytes to the development of medulla has now been reconciled in a model where RANKL signals provided by LTi cells promote the initial emergence of mature mTEC during embryogenesis and RANKL and CD40L signals delivered by CD4^{+} thymocytes contribute to the maintenance of these cells in the postnatal thymus (White *et al.* 2008; Anderson *et al.* 2009). This model emphasizes the fact that the molecular and cellular mechanisms governing the genesis and function of the thymus are dynamic, and that the processes operating in the fetal and postnatal thymus are not necessarily identical.

The cellularity of mature mTEC is also controlled by the TCR specificity of positively selected CD4^{+} thymocytes as negative selection of these cells provides to mTEC the signals required for their maturation and Aire expression (Irla *et al.* 2008). Experiments using OT-II transgenic mice which express an MHCII-restricted TCR specific for ovalbumin (OVA) show that positively selected CD4^{+} thymocytes can drive

mature mTEC development only if they can recognize their cognate antigen (i.e. transgene Rip-mOVA) on mTEC (Irla *et al.* 2008). Similarly, $CD4^+$ thymocytes in Marilyn mice, which express an MHCII-restricted TCR specific for the male-specific H-Y antigen, can stimulate $CD80^{hi}$ and $Aire^+$ mTEC development in male, but not female, mice (Irla *et al.* 2008). Thus, mature mTEC development is controlled by antigen specific, TCR–MHCII mediated contacts between autoreactive $CD4^+$ thymocytes and mTEC. These contacts presumably stabilize the cellular interactions permitting efficient transmission of signals *via* the RANKL/RANK and CD40L/CD40 signalling axes. Future work will concentrate on defining what is the nature of the biological processes (proliferation, survival and differentiation) that are regulated in thymic medulla by the canonical and noncanonical NF-kB pathways and by interactions with $CD4^+$ thymocytes and LTi cells.

1.3. T cell development in thymus

The thymus carries out two functions essential for a properly functioning adaptive immune response. These are the generation of new T cells from hematopoietic stem cells (HSC) and the selection of T cells expressing a functional and self-tolerant T cell receptor (TCR) repertoire (Figure 1.8). These critical processes are guided by the thymic stroma where different TEC subsets house distinct stages of T cell development. cTEC foster the development of $CD4^-CD8^-$ cells into $CD4^+CD8^+$ thymocytes and their subsequent positive thymic selection. The cells that carry TCRs capable of interacting with self-peptides bound to MHCI or MHCII molecules expressed by cTEC survive and differentiate into $CD4^-CD8^+$ or $CD8^-$ $CD4^+$ thymocytes, respectively. Positively selected thymocytes relocate *via* chemokine receptor 7 (CCR7)-mediated chemotaxis to the medulla, where the cognate CCR7 ligands (CCL19 and CCL21) are produced by mTEC (Kwan and Killeen 2004; Ueno *et al.* 2004). In the medulla, mTEC together with DC, mediate negative selection of thymocytes bearing high affinity self-reactive TCRs (Hayday and Pennington 2007) establishing central tolerance before thymus emigration (Kyewski and Klein 2006).

A continual supply of blood borne hematopoietic stem cells is crucial for thymopoesis as the thymus lacks self-renewing lymphoid precursors. At E11.5 intrathymic vessel formation has not yet occurred and hematopoietic precursors must enter the thymus anlage through the capsule (Itoi *et al.* 2001).The chemokine receptors CCR7 and CCR9 are important for this migration, as CCR7- and CCR9- deficient mouse embryos exhibit an impaired thymopoesis (Uehara *et al.* 2002; Benz *et al.* 2004; Liu *et al.* 2005). After establishment of the blood circulation, high endothelial venules (HEV) at the cortico-medullary junction (CMJ) provide the site of entry for these precursors (Lind *et al.* 2001). Whether the thymus emigration in adult mice relies upon the same chemokines that control this process in the embryo remains to be determined.

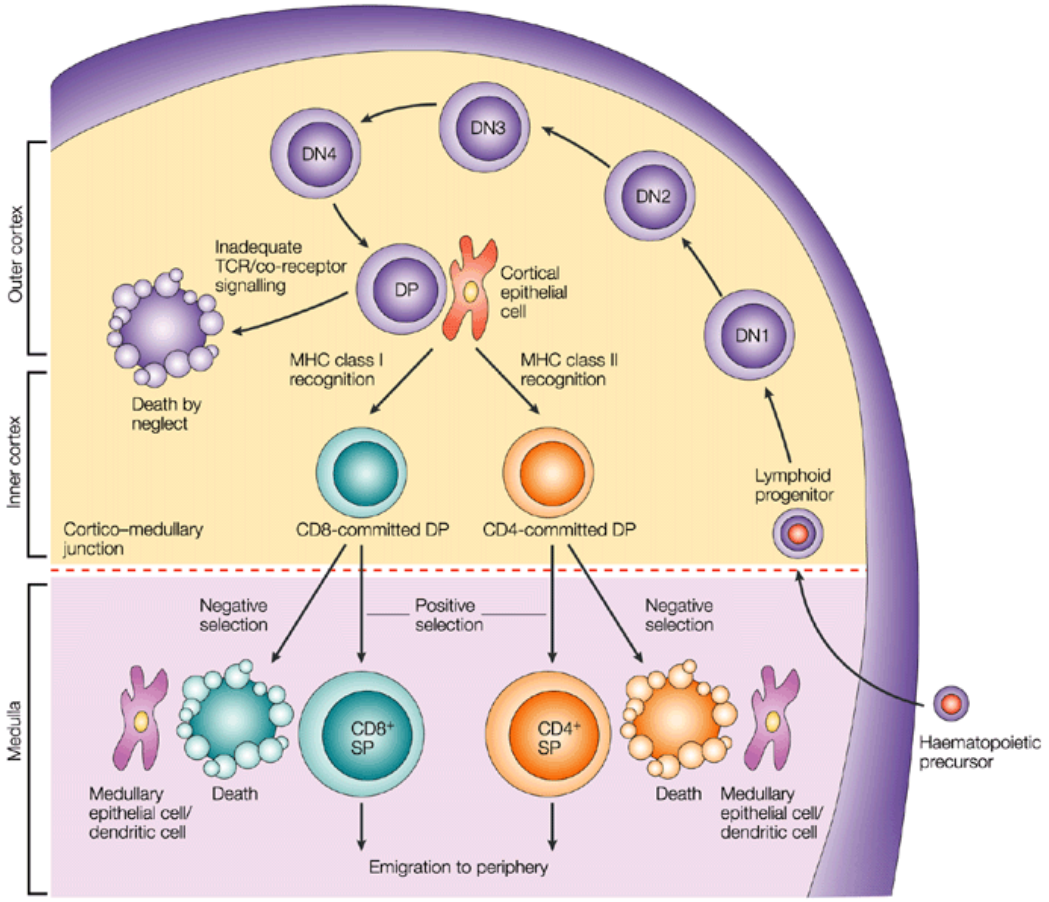


Figure 1.8 T cell development in the thymus.

Lymphoid progenitors arise in the bone marrow and enter the thymus through blood vessels at the CMJ junction. They migrate through the cortex to the subcapsular zone as DN ($CD4^-CD8^-$) thymocytes which can be subdivided into four developmental stages DN1-DN4. As cells progress through the DN2 to DN4 stages, they express the pre-TCR. Signalling through pre-TCR leads to substantial cell proliferation during the transition of DN4 to DP and replacement of the pre-TCR α-chain with a newly rearranged TCR α-chain forming a complete αβTCR. The αβTCR-expressing DP thymocytes interact with cTEC that express a high density of MHCI and MHCII molecules associated with self-peptides. The fate of the DP thymocytes depends on signalling mediated by interaction of the TCR with these self-peptide–MHC ligands. Too little signalling results in delayed apoptosis (death by neglect). Too much signalling can promote acute apoptosis (negative selection); this is most common in the medulla on encounter with strongly activating self-ligands on mTEC and DC. The appropriate, intermediate level of TCR signalling initiates effective maturation (positive selection). Thymocytes that express TCRs that bind self-peptide–MHCI complexes become $CD8^+$ T cells, whereas those that express TCRs that bind self-peptide–MHCII ligands become $CD4^+$ T cells. These cells eventually emigrate from the medulla to peripheral lymphoid sites (taken from Germain, 2002).

Once within the thymic environment, CCR7-, CCR9- and CXCR4-mediated signals separately influence the directional movement of developing thymocytes through the distinct regions of the thymus (Plotkin *et al.* 2003; Benz *et al.* 2004; Misslitz *et al.* 2004). The commitment to the T cell lineage occurs as a consequence of Notch 1 activation *via* engagement of Delta 1 that is expressed by TEC at the CMJ (Jaleco *et al.* 2001; Lind *et al.* 2001; Allman *et al.* 2002). The earliest intrathymic precursors committed to the lymphoid lineage express neither CD4 nor CD8 and are thus designated double negative (DN)

thymocytes. Using the expression profile of additional cell surface markers, CD44 (Lesley *et al.* 1985) and CD25 (Ceredig *et al.* 1985), DN murine thymocytes can be further separated into four developmental stages called DN1–DN4, where expression of CD117 (c-kit, tyrosine kinase receptor for stem cell factor) is used to exclude committed cells from non-T lineages (Balciunaite *et al.* 2005). The most immature population of thymocytes, known as DN1, is defined by the concomitant expression of CD44 (Pgp-1, phagocyte glycoprotein-1) and CD117 in the absence of CD25 (IL-2 receptor α chain) (Wu *et al.* 1991; Boyd *et al.* 1993). The expression of CD25 marks the progression from a DN1 to a DN2 ($CD44^+CD25^+$) stage (Godfrey *et al.* 1994) and the start of TCRβ locus rearrangement (von Boehmer and Fehling 1997). DN2 cells also begin to express the surrogate α-chain of the pre-TCR (pTα, also known as gp33). The subsequent loss of CD44 and CD117 expression defines the DN3 ($CD44^-CD25^+$) stage of early T cell development. DN3 cells have completed their rearrangement of the β-chain locus and if successfully express on their cell surface the complete pre-TCR consisting of a β-chain and a surrogate and invariant TCRα-chain. This immature form of the TCR allows cells to receive survival signals, a process referred to as β-selection, and to progress to the DN4 ($CD44^-CD25^-$) stage of thymocytes development, phenotypically characterized by the downregulation of the CD25 expression. This stage is short and followed within hours by the acquisition of CD4 and CD8 cell surface expression (reviewed in (Rodewald and Fehling 1998; Ceredig and Rolink 2002), a phenotype referred to as double positive (DP). During mouse embryonic development, DN1 cells first appear at E12 (Douagi *et al.* 2000). By E13, DN2 cells evolve and by E15.5 both DN3 and DN4 thymocytes can be detected. At E15.5 the first DP appear in small numbers.

The stage of DP thymocytes which holds ~80% of all thymocytes is reached *via* a transitional intermediary phenotype referred to as immature single positive (ISP) cells. These ISP express either CD4 or CD8 but not both co-receptors at the same time, and can be distinguished from more mature SP thymocytes by a lower cell surface expression of their TCRβ and a lack of TCRα-chain expression (Paterson and Williams 1987; Yu *et al.* 2004). The transition of ISP to DP is an actively regulated differentiation step that leads to the generation of a large pool of DP thymocytes (Yu *et al.* 2004). At the ISP stage of maturation, the rearrangement of the TCRα locus is initiated which still coincides with cell's active phase of proliferation. Hence, no rearrangement of the α loci occurs until the proliferative phase has ended. This sequence of events ensures that each successful rearrangement of the TCRβ-chain gives rise to many DP thymocytes. Each of these can independently rearrange their α-chain genes once the cells stop dividing, so that a single functional β-chain can be associated in the progeny of these cells with many different α-chains maximizing the chance of forming a useful TCR.

Following the period of α-chain gene rearrangement, a complete and functional α/β TCR is formed on the cell surface of DP (Lucas and Germain 1996; Sant'Angelo *et al.* 1998). Cells at this developmental

stage are now susceptible to be positively selected according to the specificity of their TCR. Positive selection occurs when the TCR of the thymocytes engages a self-peptide-MHC complex on cortical cTEC with a sufficient affinity, resulting in the transduction of a survival and differentiation signal (von Boehmer 1994; Bevan 1997; Palmer 2003). In addition to selection signals delivered *via* the TCR/MHCII interactions, TEC also provide survival, proliferation and differentiation signals in form of soluble factors such as Interleukin-7 (IL-7), stem cell factor (SCF). (Rodewald *et al.* 1997; Boursalian and Bottomly 1999) and Wnts (Raff 1992; Sprent and Kishimoto 2001; Pongracz *et al.* 2003). Thymocytes that fail to express a TCR or express TCR with an insufficient affinity for self MHC molecules complexed to self-antigens undergo death by neglect. Thus, developing T cells are destined to die by default unless they are rescued by life-sustaining signals.

Following positive selection and the migration to the medulla, thymocytes that express a TCR with a high affinity for self-peptide-MHC complex will be negatively selected by mTEC and DC and die by apoptosis resulting in the removal of thymocytes with a self-reactive TCR specificity (Smith *et al.* 1989; Nossal 1994; Sprent and Kishimoto 2002; Palmer 2003; Starr *et al.* 2003). Fewer than 5% of the developing thymocytes are positively selected and survive negative selection leaving the thymus as mature T cells bearing now a correctly selected TCR able to recognize foreign antigens in the context of self MHC molecules, i.e. they are functional and self-tolerant. Thymic selection occurs during a relatively short window of time as the life span of DP cells is limited to approximately 3-4 days. For autoreactive T cells that escape negative selection there are additional regulatory mechanisms in place in the periphery that control the functionality of these “forbidden” cells and prevent autoimmunity to occur (Sakaguchi *et al.* 2001).

The maturational transition from DP to single positive (SP) thymocytes is gradual and consists of various intermediate stages, and is paralleled by the physical translocation from the cortex to the medulla. Positively selected DP thymocytes transiently downmodulate both CD4 and CD8 (i.e., $CD4^{low}CD8^{low}$ $TCR^{intermediate}$) only to re-express CD4 attaining a $CD4^{high}CD8^{low}$ $TCR^{intermediate}$ phenotype. This expression pattern does not yet infer the TCR`s restriction to either MHC class I or II molecules. Subsequently, a lineage specific program is activated that results in the suppression of either CD4 or CD8 synthesis dependent on the MHC restriction of the selected TCR. $CD4^{high}CD8^{low}TCR^{intermediate}$ thymocytes that bear a TCR specific for antigens presented by MHC class II molecules stop the expression of CD8, upregulate the cell surface expression of their antigen receptor and gain the $CD4^{high}CD8^{-}TCR^{high}$ phenotype of mature thymocytes. In contrast, thymocytes which have successfully engaged the antigen in the context of MHC class I, maintain and eventually increase their CD8 expression while turning off CD4 synthesis and gain the cell surface phenotype of $CD4^{-}CD8^{high}TCR^{high}$ mature thymocytes. Further interactions of SP thymocytes with a diverse set of self-peptides, including TRAs displayed by mTEC and DC, is essential

for establishing central self-tolerance (Kyewski and Klein 2006; McCaughtry *et al.* 2007; Weinreich and Hogquist 2008). Completely selected, medullary SP thymocytes undergo a series of maturational stages including the upregulation of homing receptors (CD62L) and the downregulation of the heat-stable antigen (HSA) CD24 and the early activation marker CD69 (Lucas *et al.* 1994; Ernst *et al.* 1995; Gabor *et al.* 1997; Hare *et al.* 1998; Anderson and Jenkinson 2001). The release of naive T lymphocytes with a diverse yet self-tolerant TCR repertoire from the thymus to the circulation is regulated *via* chemotaxis driven by activation of the sphingosine-1-phosphate receptor 1 (S1P1) (Schwab and Cyster 2007).

1.4. Role of Wnt signalling and Foxn1 in thymus development and function

Wnt (Wingless) signalling is involved in virtually every aspect of embryonic development as it controls cell fate specifications, proliferation, migration, polarity and death of cells (Clevers 2006; Scheller *et al.* 2006). Wnts constitute a highly conserved family of 19 secreted glycoproteins in humans and 18 in mice. Within the thymus, both TEC and thymocytes of all developmental stages express various Wnt proteins and their receptors and established TEC lines are able to respond in autocrine and paracrine fashion to Wnt-mediated signals (Balciunaite *et al.* 2002; Pongracz *et al.* 2003; Osada *et al.* 2006; Weerkamp *et al.* 2006). Appropriate regulation of Wnt signalling activity is required for both normal TEC development and function (Kuraguchi *et al.* 2006; Osada *et al.* 2006; Zuklys *et al.* 2009; Osada *et al.* 2010) and early thymocyte development (Mulroy *et al.* 2003; Weerkamp *et al.* 2006). Wnt signalling engaging *via* β-catenin also regulates expression of Foxn1 (Balciunaite *et al.* 2002), which is a transcription factor critical for thymic epithelial development and responsible for the athymic nude phenotype when mutated in mice and humans (Blackburn *et al.* 1996; Nehls *et al.* 1996).

1.4.1. The canonical Wnt/β-catenin signalling pathway

Wnt proteins can stimulate distinct intracellular transduction pathways. Non-canonical Wnt signalling refers to a collection of pathways that either activate small GTPases of the Rho family to regulate the actin cytoskeleton (i.e. the planar cell polarity pathway) or stimulate the calcium flux and activate protein kinase C (PKC) modulating cell adhesion and motility during gastrulation (i.e. the Wnt/Ca^{2+} pathway) (reviewed in (Habas and Dawid 2005; Kohn and Moon 2005)). The most well characterized canonical Wnt pathway is mediated by β-catenin (Figure 1.9) (Cadigan and Liu 2006; Semenov *et al.* 2007). Most of the β-catenin in cells constitutes a structural component of the cytoskeleton, and is bound to the cytoplasmic domain of type 1 cadherins linking them *via* α-catenin to the actin cytoskeleton (Nelson and Nusse 2004).

However, a small dynamic pool of β-catenin shuttles between the cytoplasm and the nucleus to transduce canonical Wnt signals (Kimelman and Xu 2006).

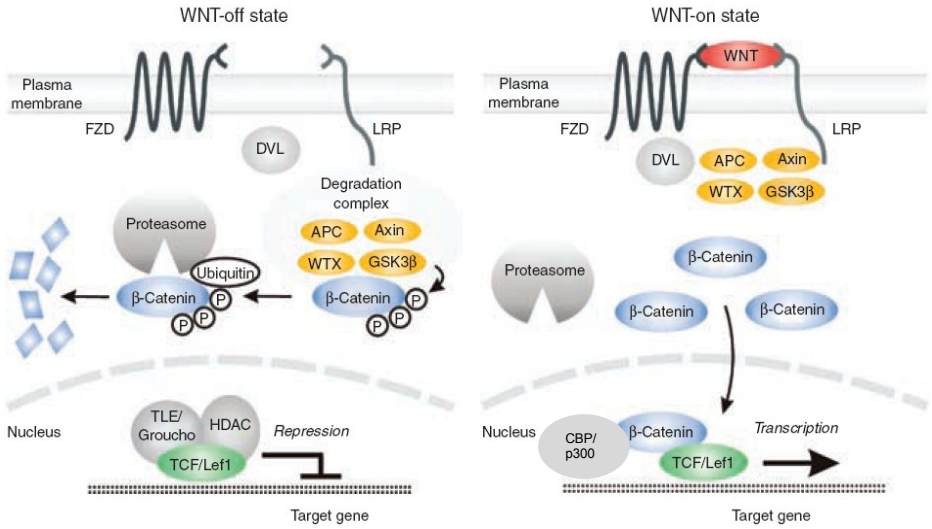


Figure 1.9 The canonical Wnt signalling.

Wnt-off state: In the absence of a Wnt signal, the destruction complex phosphorylates β-catenin. This leads to ubiquitin-mediated proteasomal degradation of β-catenin and keeps intracellular levels low. Meanwhile, Tcf/Lef transcription factors recruit Tle/Groucho and histone deacetylases (HDAC) to repress Wnt target genes. **Wnt-on state**: Wnt signalling inactivates the destruction complex, as a result β-catenin accumulates intracellularly, translocates to the nucleus where it interacts with Tcf/Lef factors and co-activators such as CBP/p300. This interaction promotes the transcription of Wnt target genes (taken from Schmidt-Ott, 2008).

In the absence of Wnt signalling, cytosolic β-catenin is captured by a large group of proteins known as the destruction complex. This complex is composed of the scaffold proteins, Axin and adenomatous polyposis coli (Apc), and the serine/threonin kinases, glycogen synthase kinase 3β (GSK3β) and casein kinase 1α (CK1α) which phosphorylate serine residues in the N-terminus of β-catenin. Phosphorylated β-catenin is recognized by the E3 ubiquitin ligase, the β-transducin repeat containing protein (β-TrCP) and targeted for proteasome degradation. Thus in the absence of Wnt signals, intracellular β-catenin levels are kept low (Kimelman and Xu 2006). Binding of Wnt ligands to the serpentine receptor Frizzled (Fz) and the single-span transmembrane co-receptor, low-density lipoprotein receptor−related proteins 5 and 6 (Lrp5/6) leads to the inactivation of the destruction complex through recruitment of its components to the Fz/Lrp/Dvl (Dishevelled) complex (Schmidt-Ott and Barasch 2008). As a result, free dephosphorylated cytosolic β-catenin is stabilized and translocates into the nucleus.

Nuclear β-catenin controls the transcription of Wnt target genes in concern with the members of T cell factor/lymphoid enhancer factor (Tcf/Lef) family of transcription factors (Logan and Nusse 2004; Clevers 2006; Xu and Kimelman 2007). In the absence of Wnt signalling, Tcf/Lef are bound to Wnt target gene promoters in a complex with transcriptional co-repressors such as Tle/Groucho and histone deacetylases (HDAC) and mediate repression of target genes (Roose *et al.* 1998; Brannon *et al.* 1999; Daniels and Weis 2005; Hoppler and Kavanagh 2007). As a result of Wnt signalling engaging the

Fz/Lrp5/6 complex, β-catenin interacts with Tcf, displaces these co-repressors and mediate the recruitment of co-activators such as the histone acetyltransferase CBP/p300, so that the DNA bound complex can now stimulate the transcription of Wnt target genes (Arce *et al.* 2006; Stadeli *et al.* 2006). Thus, the amount of freely available β-catenin and its post-translational modification are key factors that determine the outcome of canonical Wnt signalling (Miller 2002).

The Wnt activity is also regulated at the level of Wnt ligand binding to its cognate receptor. Different secreted proteins have been classified that modulate Wnt signalling by blocking access to receptors and signalling *via* Fz. These proteins include Wnt inhibitory factor (Wif)-1, soluble frizzled proteins (sFz), Dickkopf proteins (Dkk1-4) (Niehrs 1999) and Kremen proteins (Krm 1 and 2) (Wodarz and Nusse 1998). Wif-1 and sFz act as competitive inhibitors of Fz by directly binding available Wnts (Kawano and Kypta 2003; Logan and Nusse 2004). In contrast, Dkk1 functions as an antagonist of the canonical Wnt pathway as it binds to Lrp5/6 and prevents its interaction with Wnt-Fz complex (Bafico *et al.* 2001), a prerequisite for the successful transduction of signal. Krm1, acts as an additional negative regulator as it binds to Dkk and triggers the internalization and clearing of the Dkk/Lrp5/6 complex from the cell surface (Bafico *et al.* 2001; Mao *et al.* 2002; Devoss *et al.* 2008).

The interception of Wnt signalling pathway with diverse factors that induce the biochemical changes subsequent to Fz/Lrp5/6 engagement constitute another mechanism to alter the outcome of Wnt signalling. In this context, members of the Sox transcription factor family have been reported to fine tune the spatial and temporal activity of the canonical Wnt signalling pathway.

1.4.2. Modulation of Wnt signalling activity in thymic epithelia

Several studies have demonstrated that a precise regulation of Wnt pathway activity is required for normal TEC architectural organization, differentiation and function. Deletion of the negative Wnt signalling regulator, the adenomatous polyposis coli (Apc) in TEC (and other epithelia) results in a hypoplastic non-functional thymus characterized by loss of proper cortico-medullary organization and an increase in $CK14^{+}CK8^{+}$ TEC (Kuraguchi *et al.* 2006). Similarly, increased canonical Wnt signalling in TEC, as a consequence of a lack of Kremen1 expression, leads to a loss of defined cortical and medullary compartments and an abundance of $CK5^{+}CK8^{+}$ TEC progenitor population (Osada *et al.* 2006). The thymus phenotype observed in these mice however, cannot be exclusively attributed to the increased Wnt signalling in TEC, as the general loss of Kremen1 expression also affects thymocytes and mesenchymal cells. A precise regulation of intracellular β-catenin protein levels in TEC is also essential for regular thymus development and function. Constitutive activation of the canonical Wnt signalling in TEC following the expression of a stabilized form of β-catenin alters cellular identity of TEC and leads to an impaired

thymic microenvironment unable to support T cell development (Zuklys *et al.* 2009). Although these studies demonstrate the importance of appropriate Wnt signalling for normal thymus organogenesis, the early block(s) in thymus organogenesis or the premature death of mutant mice, prevented the analysis of the impact of Wnt signalling on TEC biology in the adult mice.

In a study by Osada et al, an inducible model was used to directly (i.e. without affecting the initial development of the thymus or other epithelial organs requiring Wnt signals during development) assess the contribution of Wnt signalling for the maintenance of adult epithelial microenvironments. Inhibition of canonical Wnt signalling in TEC by induced expression of Dkk1 results in a rapid degeneration of the thymus microenvironment as characterized by reduced TEC proliferation, loss of $CK5^+CK8^+$ progenitor population and disappearance of immature cycling $\Delta Np63^+$TEC and mature $Aire^+$mTEC subsets (Osada *et al.* 2010). Removal of Dkk1 expression results in a full recovery of the thymus phenotype, including a return to normal thymic size, a re-appearance of the $CK5^+CK8^+$ TEC progenitors and a normal frequency of both immature and mature TEC subsets. The reversibility of the pathology suggests that inhibition of Wnt signalling does not lead to death of the TEC progenitors but rather reduces their ability to cycle and/or give rise to progeny. The concomitant loss of immature $\Delta Np63^+$ TEC and $CK5^+CK8^+$ progenitors implies a role for canonical Wnt signalling in the development or expansion of TEC progenitor population with the capacity to maintain properly organized adult thymic microenvironment. Identification of the Wnt target genes that regulate biological activity of TEC progenitors may provide useful targets for the design of therapies aimed at counteracting either age-associated processes that lead to thymic involution or mechanisms resulting in premature thymic degeneration secondary to radio/chemo therapy of the thymus.

1.4.3. Role of Foxn1 in TEC development and function

The Foxn1 (Nehls *et al.* 1994) is the earliest identified transcription factor in the pharyngeal region that is specifically associated with, and has an obligate role in TEC development (Blackburn *et al.* 1996). Foxn1 is cell-autonomously required for initial pattering of the epithelial rudiment into thymus-specific domain (Gordon *et al.* 2001), is sufficient to induce both cortical and medullary differentiation (Blackburn *et al.* 1996; Bleul *et al.* 2006) and has been implicated in mediating crosstalk-dependent differentiation of TEC (Su *et al.*).

Low levels of Foxn1 expression in the 3rd pharyngeal pouch thymic epithelium can be first detected by qRT-PCR at E10.5. High Foxn1 expression is present at E11.25 in the prospective thymus domain of the common thymus/parathyroid anlage just before lymphoid progenitor infiltration (Nehls *et al.* 1996; Gordon *et al.* 2001; Itoi *et al.*). Since transplantation experiments suggest that the endoderm already at

E8.5-E9 commits to a thymus fate, which is well before an obvious anlage or Foxn1 expression can be detected, and because Foxn1-deficient (nude) mice only start to display morphological alternation by E11.5, it is unlikely that Foxn1 is responsible for specifying the TEC identity during the initial stages of thymus organogenesis. Moreover, cells with a $CK5^+CK8^+$ progenitor phenotype are generated despite the absence of Foxn1 function. As such, although often referred to as athymic, nude mice do undergo the initial thymus development, the rudiment forms, but fails to differentiate beyond an immature $CK5^+CK8^+$ stage and it is not colonized by lymphocyte progenitors (Nehls *et al.* 1996). Hence, the timing of Foxn1 expression divides thymus development into an early Foxn1-independent phase when the endodermal cells in 3rd pouch commit to the thymic fate and a subsequent Foxn1-dependent phase marked by the expansion and differentiation of TEC into defined cortical and medullary phenotypes. A domain of Foxn1 necessary for TEC differentiation has been identified in mice expressing a mutant form of Foxn1 that acts as a hypomorph (Su *et al.* 2003). The thymus of these mice displays an abnormal thymic architecture, lacking mature cortical and medullary compartments and harbours specific defects at the DN and DP stage of T cell development. In contrast to thymi of nude mice, TEC differentiation is blocked later at the intermediate $CK5^+CK8^+MTS10^+$ TEC progenitor stage that is formed independent of the interaction with maturing thymocytes. The allele responsible for the phenotype has a large deletion in its N-terminal aspect but harbours an intact DNA binding, nuclear localization and C-terminal domain (Brissette *et al.* 1996; Schuddekopf *et al.* 1996). Hence, the N-terminal domain of Foxn1 is required for crosstalk-dependent TEC differentiation. Importantly, the coat of these animals is not affected indicating a thymus-specific role for the N-terminal domain.

Despite well-established role of Foxn1 for TEC differentiation, it is still unknown whether the epithelial progenitors express Foxn1. However, circumstantial evidence suggests they do as first, early TEC all express Foxn1 (Nehls *et al.* 1996) and have precursor activity (Bleul *et al.* 2006; Rossi *et al.* 2006; Corbeaux *et al.* 2010); second, cells in the nude thymic anlage can be induced to differentiate normally upon re-supply of this gene (Bleul *et al.* 2006); and third, TEC compartment can be completely destroyed using Foxn1-directed cell ablation (Corbeaux *et al.* 2010). Furthermore, fate mapping of TEC using a Foxn1-Cre allele showed that most, if not all TEC arise from $Foxn1^+$ progenitors, or at least transit through a stage of ubiquitous Foxn1 expression (Gordon *et al.* 2007; Corbeaux *et al.* 2010).

Whether Foxn1 remains constitutively expressed in the adult thymus is also controversial. Although a $Foxn1^{lacZ}$ allele is expressed in most, if not all embryonic and adult TEC (Nehls *et al.* 1996), Foxn1 protein expression as characterized by immunohistology using a specific antibody suggests however, that Foxn1-negative TEC are already apparent at E13 and that many as 80% of adult TEC lack Foxn1 expression (Itoi *et al.* 2007a). Based on this finding the possibility was raised that once a functional thymic microenvironment is established, Foxn1 might no longer be required for TEC to sustain thymopoiesis in

the postnatal period. This issue was resolved in a transgenic model where adult Foxn1-expressing cells were specifically ablated to create a thymic microenvironment composed solely of Foxn1-negative TEC (Corbeaux *et al.* 2010). The finding that thymopoiesis ceases prematurely in these mice indicates that Foxn1-negative cells do not contribute to thymopoietic function of the adult thymus. Lineage tracing studies further established that Foxn1-negative TEC arise postnatally from Foxn1^{+} progenitors and hence do not represent a TEC lineage that develops independently of Foxn1 expression. The Foxn1-negative TEC may rather correspond to cells at the end of their lifespan. Hence, Foxn1 in addition to regulating TEC differentiation, also marks the functionally competent TEC both in embryonic and adult thymic tissues (Corbeaux *et al.* 2010).

Although known for long time, Foxn1 function has mostly been studied in the fetal thymus. Mutant mice with a regular Foxn1 expression during fetal development but a gradually decreased Foxn1 expression postnatally (i.e. from 50% down to 20% of the normal Foxn1 levels) have however shed light on the functional role of Foxn1 for the postnatal thymus. This downregulation of Foxn1 expression causes a progressive degeneration of the thymic compartment culminating in a specific loss of mature MHCII^{hi}UEA-1^{hi} mTEC and reduced T cell production providing evidence that the maturation and maintenance of the postnatal thymic epithelium requires continuous Foxn1 expression (Chen *et al.* 2008a). As the mature MHCII^{hi}UEA-1^{+} mTEC express high Foxn1 levels and are most sensitive to its downregulation they are thought to represent a cellular target for Foxn1 function to maintain the postnatal thymus microenvironment. The progressive TEC phenotype directly correlates with the reduced Foxn1 expression and thus, suggests that there may be a threshold of Foxn1 expression levels required to maintain thymus. That the Foxn1 dose is critical for thymus development has been recognized long time ago as mice with 50% reduction of Foxn1 expression display mildly thymus size reduction, despite normal thymic stromal organization (Scheiff *et al.* 1978; Kojima *et al.* 1984). Decreasing further Foxn1 expression to 40-30% of normal levels affects the maintenance of MHCII^{+} TEC and reduction to 20% causes more rapid degeneration of thymic epithelium. These findings indicate that TEC are extremely sensitive to the modulation of Foxn1 expression, with small changes in Foxn1 levels of only 10 -20%, having large effects on thymus phenotypes.

The phenotype of these mutant mice is strikingly similar to that of older wild type mice undergoing normal age-related involution and suggests that changes in Foxn1 expression in TEC contribute to thymus senescence. This contention is in keeping with the observation that Foxn1 expression is progressively decreased in wild type TEC with aging (Ortman *et al.* 2002). However, it remains to be determined whether this change drives involution or is a consequence of the normal mechanisms underlying this physiological process. Independent of the sequence of events, this experimental model clearly shows that a TEC-intrinsic mechanisms are responsible for the phenotypic features of aging-

related involution and that a change in the expression level of a single critical transcription factor in TEC is sufficient to induce involution (Su *et al.* 2003; Chen *et al.* 2008a). Hence, the function of Foxn1 extends beyond its role in the initial TEC differentiation, playing a role in functional maintenance of the differentiated mature epithelium and perhaps also during thymus involution. Investigation of the mechanisms by which Foxn1 expression and activity are regulated in TEC during thymus development and aging will help to understand the molecular control of thymus homeostasis. Identification of Foxn1 target genes involved in crosstalk-dependent TEC and thymocytes differentiation may help to maintain or boost postnatal T cell production.

1.4.4. Role of Foxn1 in proliferation

Current evidence indicates that Foxn1 regulates both TEC differentiation and proliferation (Blackburn *et al.* 1996; Nehls *et al.* 1996; Itoi *et al.* 2001). Consequently, TEC that fail to express Foxn1 are arrested at an early undifferentiated $CK5^{+}CK8^{+}$ developmental stage and display only a limited capacity for proliferation (Blackburn *et al.* 1996; Nehls *et al.* 1996; Itoi *et al.* 2001). A role of Foxn1 in differentiation and proliferation has also been assigned to the integument, where Foxn1 is believed to promote the proliferation of progenitor cells of the hair shaft and the inner root sheath (Lee *et al.* 1999). Several studies further demonstrate a positive impact of Foxn1 expression on thymic growth. The re-introduction of Foxn1 transgene into the nude thymus (Cunliffe *et al.* 2002; Bleul *et al.* 2006) and/or intrathymic administration of exogenous Foxn1 cDNA in aged wild type mice (Sun *et al.* 2010) restore thymic size and function. Conversely, a downregulation of Foxn1 expression in postnatal TEC consequent to expression of a mutant Foxn1 allele reduces TEC proliferation and causes changes of the thymic compartment marked by specific decrease of $MHCII^{hi}UEA\text{-}1^{+}$ and concomitant increase of $MHCII^{lo}UEA\text{-}1^{+}$ mTEC (Chen *et al.* 2008a). Interestingly, the proliferation is specifically decreased in the immature $MHCII^{lo}$, but not in the mature $MHCII^{hi}$ mTEC, even though $MHCII^{hi}$ cells are decreased in number and $MHCII^{lo}$ increased. Similarly, reduced Foxn1 expression levels achieved by a transgenic expression of Wnt signalling inhibitor Dkk1 correlates with a specific reduction in proliferation of immature $MHCII^{lo}$ mTEC subset. Although the mechanism by which Foxn1 controls TEC proliferation remains to be determined, these studies provide clear evidence that Foxn1 affects both differentiation and proliferation in a subset-specific manner.

1.4.5. Regulation of Foxn1 expression in thymic epithelium

The identification of Foxn1-specific interaction partners and/or DNA binding sites that mediate its function has been hampered by the fact that only few primary TEC can be obtained from the nude thymus

and that these cells lose their functionality upon *in vitro* culture. However, several studies demonstrated a role of Bmp, Shh and Wnt signalling in the transcriptional control of Foxn1 expression in TEC (Figure 1.10) (Bleul and Boehm 2005; Patel *et al.* 2006).

In the 3rd pharyngeal pouch, Foxn1 expression marks the prospective thymic cells in the ventral region from E11.25, while Gcm2 is expressed in the dorsal parathyroid domain from E9.5 (Patel *et al.* 2006). Bmp4 is strongly expressed in the thymus domain of the 3rd pouch at E10.5 (Bleul and Boehm 2005; Moore-Scott and Manley 2005) and appears to upregulate directly Foxn1 expression (Patel *et al.* 2006). In contrast, Sonic Hedgehog (Shh) is expressed in the parathyroid domain, opposes Bmp4 expression and restricts the size of the thymic field during early thymus development (Moore-Scott and Manley 2005). In the absence of Shh, the pattern of Bmp4 and hence that of Foxn1 expression are broadened and encompass at E11.5 the entire pouch (Moore-Scott and Manley 2005), while Gcm2 expression is absent. These findings are consistent with a previous *in vitro* report suggesting a role for Bmp4 in the induction of Foxn1 in TEC (Tsai *et al.* 2003). In contrast, at E10.5 expression pattern of Noggin, a Bmp antagonist overlaps with that of Gcm2 in the parathyroid domain of 3rd pouch (Gordon *et al.* 2001). Given that Noggin expression is initiated after and independently of Gcm2 expression (Liu *et al.* 2007) but concurrent with Bmp4, it is conceivable that Noggin acts in a negative feedback loop inhibiting Bmp4 activity and thus, allowing the specification of the Gcm2-expressing parathyroid domain (Bleul and Boehm 2005). In this scenario, Foxn1 expression is the default pathway for the pouch endoderm and its active suppression is required to allow the establishment of an alternative parathyroid fate (Soza-Ried et al. 200Soza-Ried *et al.* 2008). Alternatively, Foxn1 could prevent the activation of a default pathway,

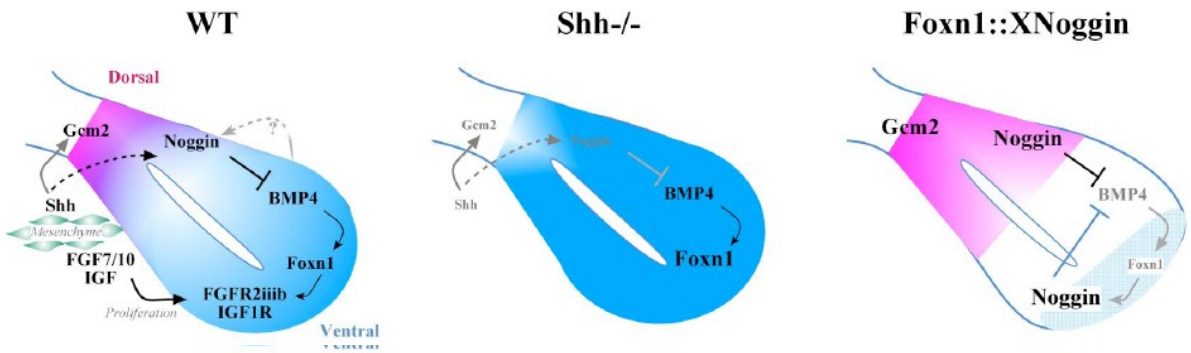


Figure 1.10 Regulation of the Foxn1 expression domain in the third pharyngeal pouch (3rdpp).
Signalling through Bmp within the ventral region of the 3rdpp endoderm at E10.5-11.5 results in expression of Foxn1 and downstream products such as FgfR2IIIb. Ligation of FgfR2IIIb by specific mesenchyme-derived ligands, Fgf7 or 10 results in TEC proliferation. Expression of the Bmp antagonist Noggin in the dorsal region, possibly under regulation of Shh, restricts the BMP signalling domain and allows for the maintenance of Gcm2 expression which is essential for the parathyroid development. In the mice lacking Shh, loss of Noggin expression results in broadened expression of Foxn1. In the Noggin transgenic mouse, decreased Bmp signalling results in the loss of Foxn1 expression and impaired thymus development (taken from Gill, 2009).

which imposes a respiratory cell fate on epithelia of the ventral aspect of the 3rd pouch (Dooley *et al.* 2005). Here, Foxn1 would also take part in the specification of the 3rd pharyngeal endoderm beyond a mere role as a differentiation factor for epithelial cells already committed to a thymus fate. Inhibition of Bmp signalling in TEC from E11.5 by a transgenic expression of Noggin results in abnormal TEC development characterized by TEC subsets failing to maintain Foxn1 expression, loss of differentiated TEC phenotype and reversion of thymic epithelium to a basal state of foregut epithelium (Bleul and Boehm 2005; Soza-Ried *et al.* 2008). Thus, Bmp signalling is also required from E11.5 onward for the maintenance of Foxn1 expression.

Wnt expression relevant for the thymus organogenesis is detected as early as E10.5 in the developing 3rd pharyngeal pouch, and Wnt receptors and downstream signalling components can be detected in both TEC and developing thymocytes (Balciunaite *et al.* 2002). Wnt4 and Wnt5b but not other Wnts induce Foxn1 expression in cultured TEC lines. Blocking of Wnt-mediated activation- either at the level of its specific receptor, Fz, or at distinct intracellular sites within the canonical signalling pathway- inhibits Foxn1 expression (Balciunaite *et al.* 2002; Osada *et al.* 2010). However, no single or compound Wnt mutant mouse has been reported that resembles the nude thymus phenotype and further clarification of the role of Wnt family members upstream of Foxn1 is needed. Loss of Wnt negative regulator Apc in TEC results in a hypoplastic non-functional thymus with aberrant architecture of TEC which exhibit increased β-catenin localization in the nucleus, a hallmark of canonical Wnt signalling (Kuraguchi *et al.* 2006). Whether these changes result in an increased Foxn1 expression in TEC was however not reported. A direct effect of Wnt signalling on Foxn1 expression has been confirmed in transgenic mice in which the inhibition of Wnt signalling by Dkk1 expression results in a reduction of Foxn1 expression in both cTEC and mTEC and correlates with thymic degeneration (Osada *et al.* 2010). Taken together these findings indicate that Wnt signalling is important for the correct TEC development and maintenance and, at least in part, this effect may be mediated *via* the regulation of Foxn1 expression.

1.5. The Sox transcription factor family and its interaction with Wnt signalling

The Sox (Sry-Box) transcription factors have emerged as modulators of canonical Wnt/β-catenin signalling in different developmental contexts. They are essential for the biological processes as diverse as cell fate specification, stem/progenitor cell proliferation and differentiation and tissue homeostasis (Wilson and Koopman 2002; Dong *et al.* 2004; Kiefer 2007). In this capacity, Sox transcription factors interact with the canonical Wnt pathway (Akiyama *et al.* 2004b; Blache *et al.* 2004; Kan *et al.* 2004; Mansukhani *et al.* 2005; Yano *et al.* 2005; Bastide *et al.* 2007; Iguchi *et al.* 2007; Sinner *et al.* 2007;

Tamashiro *et al.* 2008; Topol *et al.* 2008). Conversely, Wnt signalling also regulates sox gene expression establishing a regulatory feedback loop that fine tunes Wnt signals. The interactions between Sox members and Wnt signalling are evolutionarily well conserved and are for example, already described in fruit fly. *Drosophila* SoxF restricts Wnt signalling to a narrow stripe of cells in the developing wing imaginal disc, and elevated Wnt signalling in SoxF mutants causes an over-proliferation of the wing disc epithelium (Dichtel-Danjoy *et al.* 2009). Though in the thymus, a precise control of canonical Wnt signalling activity is critical for normal TEC development, function and maintenance a direct association between Sox family, including its member Sox9, and the Wnt/β-catenin pathway has so far not been established in these cells. The identification of precise mechanism(s) how Sox proteins regulate β-catenin/TCF activity during tissue development and maintenance of different organs constitutes a focus of intense interest.

Sox9, a member of the Sox family of transcription factors

The vertebrate genome encodes over 20 different members of the Sox transcription factor family. These molecules have so called Sry-box, a 79 amino acid motif encoding a high mobility group (HMG) DNA binding domain. This sequence was first described for the founding member of this gene family, the mouse Sry (sex-determining region on the Y chromosome) (Gubbay *et al.* 1990; Sinclair *et al.* 1990) and has characterized this family of transcription factors. Based on their sequence homologies, Sox molecules are classified into eight separate subfamilies (SoxA-H) (Bowles *et al.* 2000). Sox9, together with Sox8 and Sox10, belongs to the SoxE subfamily, and is composed of N-terminal HMG domain and a C-terminal transactivation (TA) domain (Sudbeck *et al.* 1996; Pevny and Lovell-Badge 1997; Wegner 1999). All Sox factors recognize a relatively loosely defined sequence, (A/T)(A/T)CAA(A/T)G (Pevny and Lovell-Badge 1997), resulting in DNA bending that is critical for bringing distal control elements to the proximal transcriptional start sites (Weiss 2001). Most of Sox proteins contain also nuclear import and nuclear export sequences and exhibit the shuttling between the two subcellular compartments, an ability that is apparently important for their function (Gasca *et al.* 2002; Rehberg *et al.* 2002; Smith and Koopman 2004).

Sox9 was first identified as a key regulator of cartilage and male gonad development (Wagner *et al.* 1994; Foster *et al.* 1994a; Kent *et al.* 1996; Sudbeck *et al.* 1996). Heterozygous Sox9 mutations are responsible for the campomelic dysplasia, a skeletal dysmorphology syndrome characterized by cartilage and endochondral bone malformations and by male-to-female sex reversal in humans (Wagner *et al.* 1994; Foster *et al.* 1994a). Sox9 has also been implicated in the development of cranial neural crest derivatives (Spokony *et al.* 2002; Sakai *et al.* 2006), in switch from neurogenesis to gliogenesis (Stolt *et al.* 2003) and the organogenesis of the intestine (Blache *et al.* 2004; Moniot *et al.* 2004; Bastide *et al.*

2007), the pancreas (Seymour *et al.* 2007), the hair (Vidal *et al.* 2005), and the heart (Akiyama *et al.* 2004a). Not surprisingly, Sox9 regulates distinct sets of target genes in the different tissues.

Sox9 and several others Sox proteins have two main biological properties that determine their function during tissues development and maintenance; they have the capacity to modulate canonical Wnt signalling and the ability to regulate in a dose-dependent manner stem/progenitor cell proliferation and differentiation (Taranova *et al.* 2006; Que *et al.* 2007; Formeister *et al.* 2009). The precise mechanism(s) by which Sox proteins modulate the canonical Wnt pathway is still unknown, thought several models have been proposed including: protein–protein interactions, the binding of Sox factors to Wnt target gene promoters, the recruitment of co-factors, modulation of protein stability and nuclear translocation (Figure 1.11). Several of these mechanisms may operate in parallel whereas some Sox proteins, including Sox9, employ in distinct cellular contexts different mechanisms. Many Sox proteins physically interact with β-catenin to regulate transcription of Wnt target genes either in an

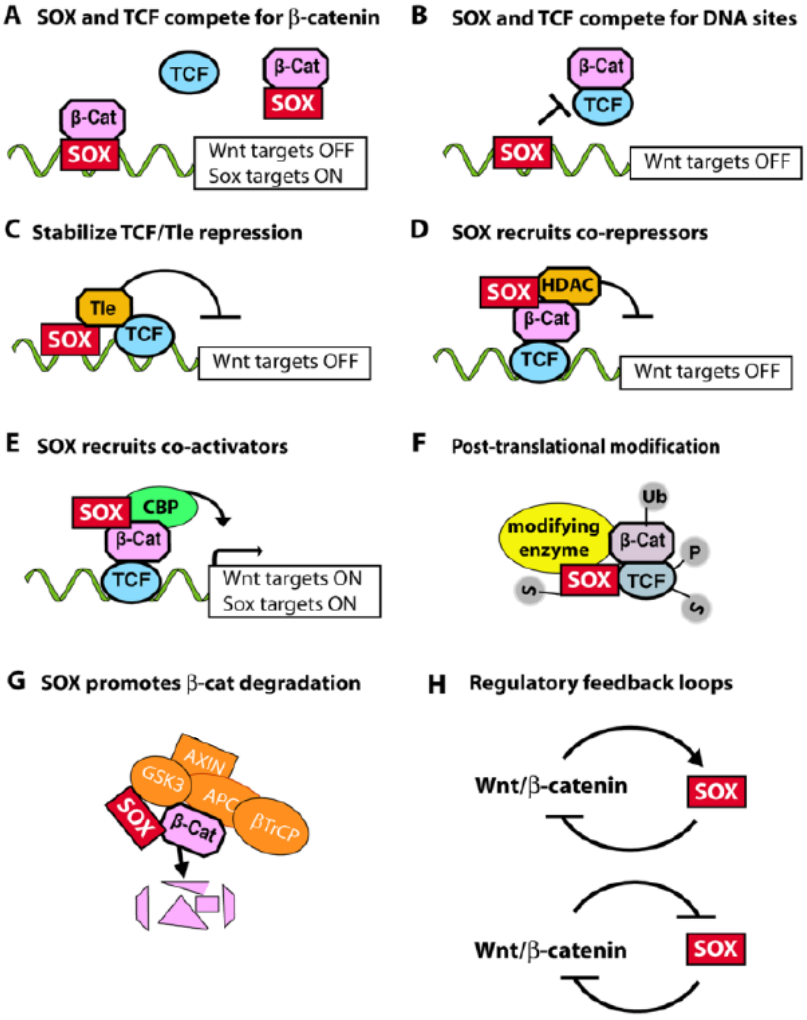


Figure 1.11 Models of Sox-Wnt regulation.

(A) Sox and Tcf may compete for a limited pool of β-catenin. This may occur on DNA or independent of DNA binding. (B) Sox and Tcf may compete for DNA binding to the same sites in target gene promoters. (C) Sox may stabilize Tcf/Tle repressor complexes and/or exclude β-catenin and co-activators. This could occur through Sox DNA binding or by a direct interaction between Sox and Tcf (not shown). Sox might recruit (D) transcriptional co-repressors or (E) co-activators to Wnt target gene promoters, either through DNA binding or by an association with β-catenin/Tcf. (F) Sox proteins may recruit post-translational modifying enzymes to β-catenin and/or Tcf. The resulting phosphorylations (P), ubiquitination (Ub), or sumoylation (S) impact protein stability, activity, and/or subcellular localization. (G) Some Sox proteins promote the degradation of β-catenin. (H) Sox and Wnt signalling can functionally interact in negative feedback loops (top) or in mutually repressive associations (taken from Kormish, 2009).

activating or repressive fashion (Zorn *et al.* 1999; Takash *et al.* 2001; Akiyama *et al.* 2004b; Kan *et al.* 2004; Mansukhani *et al.* 2005).

The central region of β-catenin consists of 12 armadillo (i.e. a 42 amino acid sequence motif) repeats which form a helical groove that mediates the interaction with over 20 different β-catenin binding partners (Figure 1.12A) (Xu and Kimelman 2007). Sox9, together with Sox17 and Sox6 directly binds in a region of the armadillo repeats that also interacts with Tcf (Zorn *et al.* 1999; Akiyama *et al.* 2004b; Iguchi *et al.* 2007; Sinner *et al.* 2007). Thus, Sox9 can sterically hinder Tcf binding to β-catenin which is a similar mechanism used by Inhibitor of β-catenin and TCF-4 (ICAT) to displace Tcf/Lef from β-catenin (Xu and Kimelman 2007). The β-catenin binding regions in various Sox proteins have also been mapped and demonstrate that β-catenin contacts several regions depending on the cell type (Figure 1.12B). Sox9, Sox7 and Sox17 all bind β-catenin *via* their C-terminal regions (Zorn *et al.* 1999; Takash *et al.* 2001; Akiyama *et al.* 2004b; Sinner *et al.* 2007), Sry binds through its N-terminal domain (Bernard *et al.* 2008) and Sox6 interacts via a centrally located leucine zipper (LZ/Q) element (Iguchi *et al.* 2007). The formation of a Sox9:β-catenin complex leads to the degradation of β-catenin by the proteasomal pathway and determines intracellular β-catenin protein levels (Akiyama *et al.* 2004b). This interaction may constitute a common mechanism to specify a cell's response to

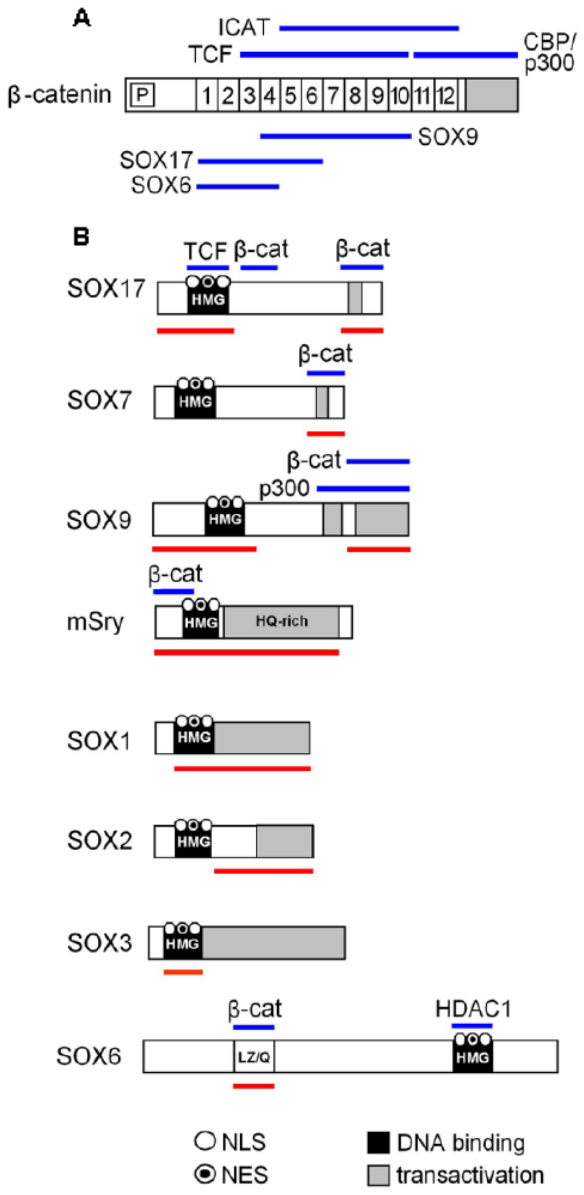


Figure 1.12 Schematic representation of Sox9, Tcf and β-catenin interaction domains.
(A) β-catenin has a central domain of 12 armadillo repeats, which can bind many proteins including Sox, Tcf and the inhibitor ICAT. The N-terminal domain harbors Serine residues that are phosphorylated by GSK3β, whereas the C-terminal region binds to CPB/p300. (B) The regions of various Sox proteins that have been shown to bind and/or inhibit β-catenin/Tcf. The nuclear import signal (NLS), nuclear export signal (NES) and DNA-binding HMG domains are indicated (taken from Kormish, 2009).

Wnt signals (reviewed in (Kormish *et al.* 2009)). Alternatively, Sox proteins may associate with promoters of Wnt target genes (Zhang *et al.* 2003; Bastide *et al.* 2007; Chao *et al.* 2007; Iguchi *et al.* 2007; Chen *et al.* 2008b). Indeed, Sox proteins bind similar DNA sequences as Tcf proteins and thus, may suppress Wnt-induced transcription by competing with Tcf for the same promoter sites. However, *in vitro* DNA binding studies argue rather against this model as Sox proteins bind very poorly, if at all, to an optimized Tcf consensus sequence (Harley *et al.* 1994; Zorn *et al.* 1999; Zhang *et al.* 2003; Akiyama *et al.* 2004b). Whether Sox factors bind other regulatory sequences of Wnt target genes that are independent of TCF sites remains to be determined.

Structure function studies aimed at determining which Sox protein domains are involved in the regulation of β-catenin/Tcf transcriptional activity have come to varying conclusions. In some reports, the β-catenin interaction domain is the most critical (Kenny *et al.* 2003; Mansukhani *et al.* 2005; Iguchi *et al.* 2007; Guo *et al.* 2008), whereas in other the HMG domain appears to be essential (Zhang *et al.* 2003; Tamashiro *et al.* 2008; Zhang *et al.* 2009) and in some studies both the HMG domain and β-catenin binding motif are required (Kan *et al.* 2004; Sinner *et al.* 2007). These apparent contradictions indicate that Sox can activate different mechanisms depending on the cellular contexts. For example, in colorectal cells Sox9 requires the HMG domain to repress Wnt signalling (Bastide *et al.* 2007), whereas in chondrogenesis it utilizes the β-catenin interaction domain (Akiyama *et al.* 2004b). Conversely, Wnt activity controls sox gene expression (Figure 1.11H) (Blache *et al.* 2004; Yano *et al.*2008 ; Bastide *et al.* 2007). Thus, a reciprocal Sox–Wnt interaction is in place that fine tunes Wnt activity in cells expressing Sox9. An illustrative example is the feedback loop engaging Sox9 and canonical Wnt signalling in the mouse intestine as the latter promotes Sox9 expression which in turn represses Wnt gene transcription restricting the epithelial proliferation (Blache *et al.* 2004; Bastide *et al.* 2007; Mori-Akiyama *et al.* 2007).

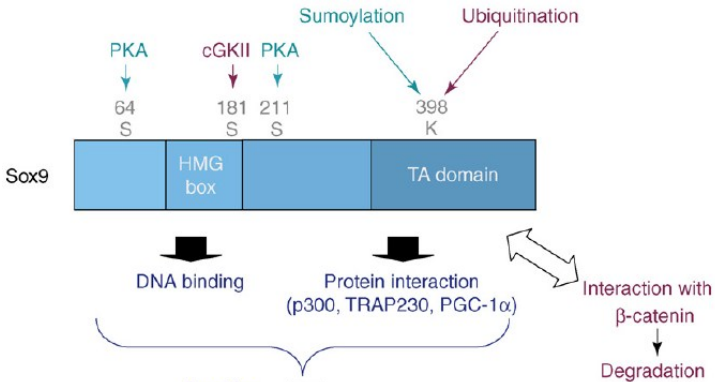


Figure 1.13 Schematic representation of Sox9 regulation.
Sox9 molecular domains, the N-terminal HMG and the C-terminal transactivation (TA) domain are represented as boxes. Regulators of Sox9 activity on specific amino acid residues are represented above the box, with blue representing positive regulators and red representing negative regulators. Interaction between Sox9 and other factors is represented under the boxes (taken from Kawakami, 2006).

Post-translational modifications determine also Wnt signalling activity as Sox, Tcf and β-catenin can be modified by ubiquitin and small ubiquitin-like modifier (sumo) moieties (Figure 1.13) (Gillard and Farr 2005; Savare *et al.* 2005; Taylor and Labonne 2005; Hattori *et al.* 2006; Pan *et al.* 2006). These modifications influence the molecular stability, activity, and sub-cellular localization of these transcription

factors and signal transducer, respectively (Arce *et al.* 2006; Hoppler and Kavanagh 2007). E3 ligase-mediated sumoylation of Sox9 at the Lysin (K) residue 398 enhances the stability and, thus transcriptional activity of Sox9 (Hattori *et al.* 2006), whereas ubiquitination of the same residue promotes Sox9 degradation (Figure 1.13) (Akiyama *et al.* 2005b). The parathyroid hormone related peptide-protein kinase A (PHTRP–PKA) regulates Sox9 activity *via* phosphorylation of the Serine (S) residues 64 and 211 (Huang *et al.* 2000). This post-translational change enhances DNA binding affinity of Sox9 and transcriptional activity at least in chondrocytes (Huang *et al.* 2000). In contrast, phosphorylation of Sox9 by the cGMP dependent protein kinase type II (cGKII) inhibits the nuclear entry and thus the activity of Sox9 (Chikuda *et al.* 2004). Because Sox9 can act either as a transcriptional activator or as repressor, the various cofactors that bind Sox9 *via* its C-terminal domain also determine its transcriptional activity (Zhou *et al.* 2002; Tsuda *et al.* 2003; Arnold *et al.* 2006).

In addition to their role in modulating Wnt signalling, Sox proteins have a capacity to act in dose-sensitive fashion during organ development and maintenance (Wagner *et al.* 1994; Kist *et al.* 2002; Fantes *et al.* 2003; Taranova *et al.* 2006; Formeister *et al.* 2009). Precise Sox9 expression levels are critical for cell fate specification and regular cell proliferation and differentiation since subtle changes in Sox9 dosage result in severe malformations. For example, an increase in Sox9 levels by 20% causes dwarfism (Akiyama *et al.* 2004b), whereas a 50% decrease in mice heterozygous for Sox9 null allele and in patients with campomelic dysplasia results in chondrodysplasia (Bi *et al.* 2001). Aberrant maintenance and differentiation of the progenitor cell compartment are thought to be the cellular cause of these pathologies.

1.5.1. Sox9 interacts with the Wnt /β-catenin pathway during development

The interaction of Sox9 with Wnt signalling plays important role both in the development as well as maintenance of the intestinal epithelium (Pinto *et al.* 2003; Blache *et al.* 2004; Bastide *et al.* 2007; Mori-Akiyama *et al.* 2007) and in cartilage formation (Akiyama *et al.* 2004b; Yano *et al.* 2005; Akiyama 2006; Zhou *et al.* 2006; Akiyama *et al.* 2008b). Canonical Wnt signalling controls Sox9 expression, as Sox9 cannot be detected in intestinal epithelia of Tcf4 null mice, but it is strongly identified in the presence of constitutive β-catenin expression (Blache *et al.*). Conversely, Sox9 overexpression strongly decreases β-catenin transcriptional activity (Akiyama *et al.* 2004b; Blache *et al.* 2004; Bastide *et al.* 2007) highlighting the reciprocal influence these two factors have on each other. The mechanism(s) by which this interdependence is achieved has not been elucidated thought it is has been hypothesised that Tcf may bind to and regulate the Sox9 promoter or, alternatively one or several intermediate Wnt-dependent transcription factors may control Sox9 transcription (Blache *et al.* 2004).

Sox9 interacts with and regulates Wnt signalling activity at least at two levels. It specifies the cell`s response to Wnt signals, which potentially trigger very diverse cellular behaviours, including proliferation and differentiation, and it also fine-tunes the transcriptional output of the canonical Wnt pathway. Conditional deletion of Sox9 at E10.5 from the mouse intestine leads to a complete absence of Paneth cells but only to a reduction of the goblet cells indicating different Sox9 requirements for the differentiation of these two distinct cell lineages (Bastide *et al.* 2007; Mori-Akiyama *et al.* 2007). Because the same phenotype is observed in mice in which the Wnt pathway is impaired either by deletion of Tcf (Korinek *et al.* 1998) or overexpression of the inhibitor Dickkopf (Pinto *et al.* 2003; Kuhnert *et al.* 2004), a role for Sox9 in specifying this particular aspect of the Wnt pathway is suggested. Sox9 also mediates the branch of Wnt signalling involved in the maintenance cells in an undifferentiated state by repressing the transcription of differentiation genes, including the homeobox gene Cdx2 and the Muc2, normally expressed in the mature villus cells of the intestinal epithelium (Blache *et al.* 2004). Hence, although Sox9 has generally been described as a transcriptional activator (Sudbeck *et al.* 1996; Bell *et al.* 1997; Lefebvre *et al.* 1997; Ng *et al.* 1997; De Santa Barbara *et al.* 1998; Liu *et al.* 2000; Sekiya *et al.* 2000; Bafico *et al.* 2001; Panda *et al.* 2001) it can also act as repressors depending on the cellular context and it associated interacting proteins. Recently, a model how differential Sox9 expression influences proliferation and lineage specification has been suggested for intestinal epithelium where two discrete steady-state levels exist (Formeister *et al.* 2009). Low Sox9 levels have been detected in proliferative crypt-based columnar (CBC) stem cells, whereas high Sox9 levels are present in non-cycling, mature enteroendocrine cells (Formeister *et al.* 2009). Moreover, overexpression of Sox9 in crypt cells represses proliferation and induces neuroendocrine-like morphologies. These data support a dose-dependent model for Sox9 activity, where low levels support and high levels of Sox9 suppress proliferation allowing for terminal differentiation of enteroendocrine cells.

A generalized role for Sox9 in regulating cell proliferation through the Wnt/β-catenin pathway has become clear (Akiyama *et al.* 2004b; Blache *et al.* 2004; Bastide *et al.* 2007; Mori-Akiyama *et al.* 2007). The molecular mechanisms responsible for the Sox9-mediated control of cell proliferation have been established by overexpressing Sox9 in several cell types (Panda *et al.* 2001; Afonja *et al.* 2002; Drivdahl *et al.* 2004; Bastide *et al.* 2007). High Sox9 levels decrease the transcription of Wnt/β-catenin target genes that positively regulate proliferation, c-myc, cyclin-D1 and increase inhibitors of β-catenin/Tcf proliferative capacity such as ICAT (inhibitor of β-catenin and Tcf) and TLE2–4 (T cell factor transducin-like enhancer of splits 2–4). *In vivo* deletion of Sox9 in intestinal epithelium also correlates with an upregulation of c-myc and cyclin-D1 causing an increase in crypt proliferation (Bastide *et al.* 2007). Similarly, enhanced Wnt signalling due to the loss of the negative regulator Apc leads to the expansion of the crypt (Sansom *et al.* 2004; Andreu *et al.* 2005). Thus, Sox9 inhibits Wnt/β-catenin signalling and its target genes involved in cell proliferation contributing to intestinal homeostasis (Blache *et al.* 2004;

Bastide *et al.* 2007; Mori-Akiyama *et al.* 2007; Formeister *et al.* 2009). This observation is in keeping with a perceived role of Sox9 as a gatekeeper preventing overactivity of Wnt-dependent transcriptional programs (Bastide *et al.* 2007).

In chondrocytes, Sox9 has been shown to inhibit the canonical Wnt signalling by promoting degradation of stabilized β-catenin (Akiyama *et al.* 2004b; Topol *et al.* 2008). According to the current model, β-catenin degradation occurs in the cytoplasm (Peifer and Polakis 2000) and is regulated by Wnt signalling which controls GSK3β- and CKIα- mediated phosphorylation of β-catenin in the destruction complex and degradation by proteasomes, keeping the β-catenin cellular levels low (Ikeda *et al.* 1998; Hart *et al.* 1999). A small increase in the amount of GSK3β results in a significant decrease in β-catenin protein levels and signalling activity (Ikeda *et al.* 1998; Dajani *et al.* 2003). The exact molecular mechanism by which Sox9 promotes β-catenin degradation is however, still poorly understood. The study by Akyama *et al.* demonstrated that the physical interaction of Sox9 with β-catenin *via* its C-terminal TA domain results in β-catenin degradation by the proteasomal pathway. In addition, this interaction may also result in Sox9 protein turnover and the relative levels of Sox9 and β-catenin have been suggested to govern proliferation and differentiation of chondrocytes (Akiyama *et al.* 2004b). Recently, a novel degradation mechanism taking place in the nucleus was demonstrated in study by Topol *et al.*, (Topol *et al.* 2008). Sox9 was shown to bind, in addition to β-catenin, components of the destruction complex, including the GSK3β and the ubiquitin ligase βTrCP and translocates them to the nucleus where more efficient phosphorylation and degradation of β-catenin occurs. It was further proposed that this rapid elimination of β-catenin in the nucleus may represent a general mechanism by which transcription factors effectively antagonize Wnt/β-catenin signalling during cell fate determination and maintenance. It is important to point out that these findings are derived from *in vitro* context which do not always correlate with the activity of transcription factors *in vivo*. Thus, the significance of these findings remains to be confirmed in cell-specific contexts.

Sox9 has also been involved in the sex determination and development of the hair and the pancreas. Sox9 is expressed in an initially bipotent gonad and together with Sry regulates mammalian testis determination (Foster *et al.* 1994a). During development cells are sensitive to Sox9 gene dosage and threshold levels of Sox9 expression exist to control the entry into male or female pathway (Wegner 1999; Chaboissier *et al.* 2004; Jakob and Lovell-Badge 2011). Sox9 expression is sufficient to initiate testis formation in xx individuals whereas mutations in Sox9 cause a sex reversal in xy individuals (Koopman *et al.* 1991; Clarkson and Harley 2002; Polanco and Koopman 2007). Homozygous deletion of Sox9 in xy gonads interferes with the sex cord development, the activation of the male-specific markers and leads to the expression of the female-specific markers. These effects are at least in part believed to be caused by the inhibition of canonical Wnt signalling *via* Sox9 (Bernard *et al.* 2008; Tamashiro *et al.* 2008) which

under physiological conditions promotes ovarian fate and blocks testis development (Chassot *et al.* 2008; Maatouk *et al.* 2008; Tomizuka *et al.* 2008).

Sox9 has also been identified as a crucial transcription factor required for the hair development. Its expression is first detected in the epithelial component of the hair placode but later becomes restricted to the hair stem cell compartment (bulge) and the outer root sheath (ORS) which is functionally implicated in the migration of hair stem cells from the stem cell niche toward the hair bulb (Vidal *et al.* 2005). Tissue-specific inactivation of Sox9 demonstrates that although dispensable for hair induction, Sox9 directs differentiation of the ORS cell lineage and is required for the formation of the bulge. Consequently, Sox9-deficient skin lacks external hair secondary to the severe proliferative defects of matrix cells in the hair bulb and the stem cell niche never forms (Vidal *et al.* 2005).

During pancreogenesis, Sox9 is required for the maintenance of early pancreatic progenitors and also governs their commitment to the endocrine cell fate (Seymour *et al.* 2008). Mice with 50% reduced Sox9 gene dosage display a specific decrease of endocrine cells caused by reduced generation of endocrine progenitors whereas formation of the exocrine compartment is unaffected. Thus, Sox9 dosage determinates cell fate assignment in multipotential progenitors. Since Sox9 marks progenitors in a variety of tissues, it is of great interest to elucidate the molecular mechanisms which control Sox9 expression in time and space as this knowledge would help in our understanding of how different developmental processes are regulated.

2. AIM OF THE THESIS

The aim of the thesis was to characterize the role of Sox9 in TEC differentiation and function. Our previous gene expression analysis by microarray detected Sox9-specific transcripts in epithelia of the third pharyngeal pouch at the time mouse thymus organogenesis is typically initiated. Also supporting a possible role for Sox9 in thymus formation and function are the findings from studies in zebrafish, *Danio rerio* where Sox9 dysfunction results in the displacement of pharyngeal arches and severely impaired thymus and T cell development (communicated by N. Trede). Further interest to investigate a role for Sox9 in thymus development was prompted by findings that Sox9 activity influences cell fate specification, differentiation and proliferation *via* control of the canonical Wnt signalling pathway (Akiyama *et al.* 2004b; Blache *et al.* 2004; Yano *et al.* 2005; Bastide *et al.* 2007; Mori-Akiyama *et al.* 2007; Topol *et al.* 2008; Formeister *et al.* 2009). Previous studies have demonstrated that a precise regulation of Wnt signalling activity is required for normal TEC development and maintenance, including Foxn1 expression (Balciunaite *et al.* 2002; Osada *et al.* 2006; Zuklys *et al.* 2009; Osada *et al.* 2010). Since a role for Sox9 in murine thymus organogenesis and function had not yet been examined, we hypothesized that Sox9 may impact TEC development and function either independently or, at least in part, *via* the interaction with Wnt signalling and thereby may modulate the expression of Wnt target genes. To explore this possibility, the temporal and spatial expression pattern of Sox9 during regular thymus development and tissue maintenance was first investigated. Subsequently, the sox9 gene was inactivated specifically in TEC as early as E12.5 using Foxn1-Cre activity and the consequences of an absence of Sox9 expression on the formation of structurally and functionally normal thymic microenvironment and transcriptional activity of Wnt/β-catenin pathway examined. Finally, the mechanism by which Sox9 may intersect with canonical Wnt/β-catenin signalling in thymic epithelia was investigated by overexpression of wild type and mutant form of Sox9 molecule in established TEC line. With this study, we sought to uncover the role for Sox9 in thymus organogenesis and function and to gain further clues in the cellular and molecular mechanisms governing the differentiation and life-long homeostatic maintenance of TEC.

1. Sox9 expression profiling in fetal and adult thymus
2. The effect of the TEC-specific ablation of Sox9 on thymic microenvironment
3. Defining the role for Sox9 in thymus organogenesis and function
4. Mechanism of Sox9-mediated regulation of Foxn1

3. RESULTS

3.1. Temporal and spatial expression pattern of Sox9 in thymus

3.1.1. Sox9 expression is detected at E10.5 in the 3rd pharyngeal pouch, a site of prospective thymus development

The role of Sox9 in thymus development and function has so far not been identified thought its importance in the formation and function of other organs has been well documented (Foster and Graves 1994; Kent *et al.* 1996; Akiyama *et al.* 2002; Stolt *et al.* 2003; Akiyama *et al.* 2004a; Blache *et al.* 2004; Moniot *et al.* 2004; Vidal *et al.* 2005; Bastide *et al.* 2007; Seymour *et al.* 2007; Scott *et al.* 2010; Furuyama *et al.* 2011). To detect Sox9 expression at the earliest time point of murine thymus development, both the ventral and dorsal aspects of the third pharyngeal pouch (3rdpp) were isolated from embryonic day (E) 10.5 wild type mice using laser-capture microscopy (Figure 3.1A). The microdissected tissue was subsequently analysed for the expression of Sox9-specific transcripts using qRT-PCR. At this stage of development, the endoderm-derived epithelial cells in the ventral aspect of the 3rdpp have already adopted a thymic fate and form a population of progenitors able to give rise to all cortical and medullary epithelial cells types. Sox9 transcripts were differentially expressed at E10.5, with lower Sox9 mRNA levels detected in the ventral epithelial lining of the 3rdpp circumference when compared to its dorsal aspect, known to develop into the parathyroid gland (Figure 3.1B).

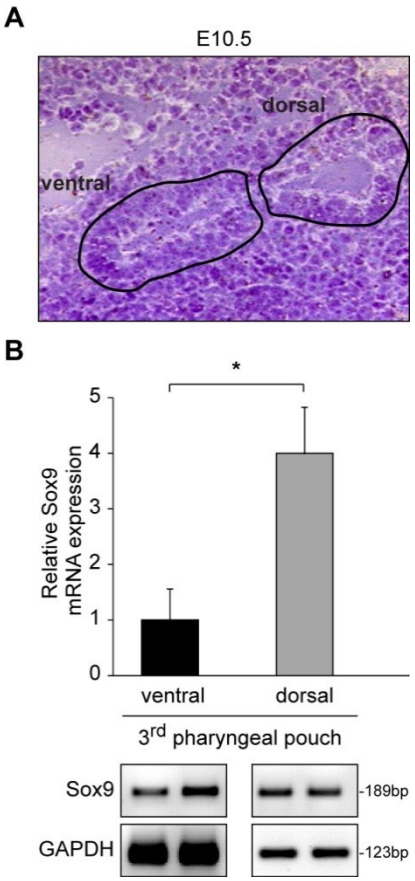


Figure 3.1 Sox9 is detected as early as E10.5 in the endodermal epithelia of the 3rd pharyngeal pouch.
(A) Ventral and dorsal areas were isolated separately from 3rd pharyngeal pouch of C57BL/6 embryos for (B) Sox9 expression analysis by qRT-PCR. The data represents the mean ± standard deviation (SD) of triplicate analyses; the ventral Sox9 expression was arbitrary set to a value of 1. Size of Sox9-specific transcripts was controlled by gel electrophoresis and visualized by EthBr. The expression of GAPDH was used as an internal control. The data is representative of two independent experiments. P values were calculated

3.1.2. Sox9 is expressed in thymus stromal but not in lymphoid cells

Whether Sox9 expression by TEC is maintained throughout thymus patterning and the formation of functionally competent microenvironment was next investigated. For this purpose, primary fetal TEC ($CD45^-EpCam^+$) were isolated from mice of E13.5 to E17.5 and analysed by qRT-PCR for Sox9 expression. As demonstrated in Figure 3.2, TEC continue to express Sox9 transcripts throughout the thymus organogenesis. To define the type of stromal cells that expresses Sox9 during steady-state conditions of the postnatal thymus, epithelial and mesenchymal cells were isolated by flow cytometry using the differentiation markers EpCam, Ly51 and MTS15. Adult primary TEC ($CD45^-EpCam^+$) were subdivided into cells with a cortical ($Ly51^+$) and a medullary ($Ly51^-$) phenotype. Non-epithelial stroma was defined by a $CD45^-EpCam^-Ly51^+$ phenotype and further characterized by the presence or absence of MTS15 expression, which marks a subpopulation of mesenchymal cells (Figure 3.3A). These cells were also analysed for the expression of the Epithelial V-like antigen (EVA) to exclude possible contamination by TEC. Sox9-specific transcripts were detected in epithelia with either a cortical or a medullary phenotype, and also in $MTS15^+$ mesenchymal cells isolated from adult thymus (Figure 3.3B). In contrast, thymocytes at any developmental stage (DN, DP, SP4 and SP8) were consistently negative for Sox9 expression (Figure 3.3C).

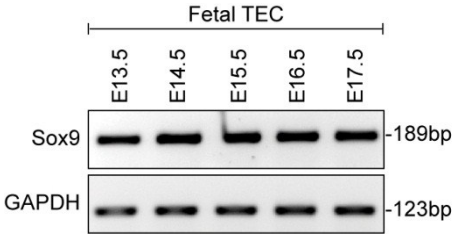


Figure 3.2 Sox9 expression is maintained throughout embryonic TEC development.
Primary fetal TEC ($CD45^-EpCam^+$) of the indicated developmental ages were isolated by cell sorting from C57BL/6 embryos and Sox9 expression analysed by PCR. Amplification products were separated by gel-electrophoresis and visualized by EthBr.

To verify Sox9 expression at the protein level, sections of embryonic and adult thymic tissue were investigated by immunohistochemistry. At E10.5, Sox9 protein was detected in the nucleus of $CK8^+$ epithelia in the ventral aspect of the 3rdpp (Figure 3.4, upper two panels). Strong Sox9 expression was also observed in the mesenchyme of the 3rd pharyngeal arch (Figure 3.4, upper left panel) correlating with the detection of Sox9 transcripts in adult thymic $MTS15^+$ mesenchymal cells (Figure 3.3B). By E13.5, Sox9 protein was detected in virtually all TEC. This expression persisted at later developmental stages and after birth (Figure 3.4, middle and bottom panels) corresponding with the detection of Sox9 transcripts in TEC (Figure 3.2 and 3.3B). Altogether, Sox9 expression was detected at the initiation of thymus organogenesis, was maintained throughout fetal thymus development and could be easily detected in the thymus stromal but not lymphoid compartment of adult mice.

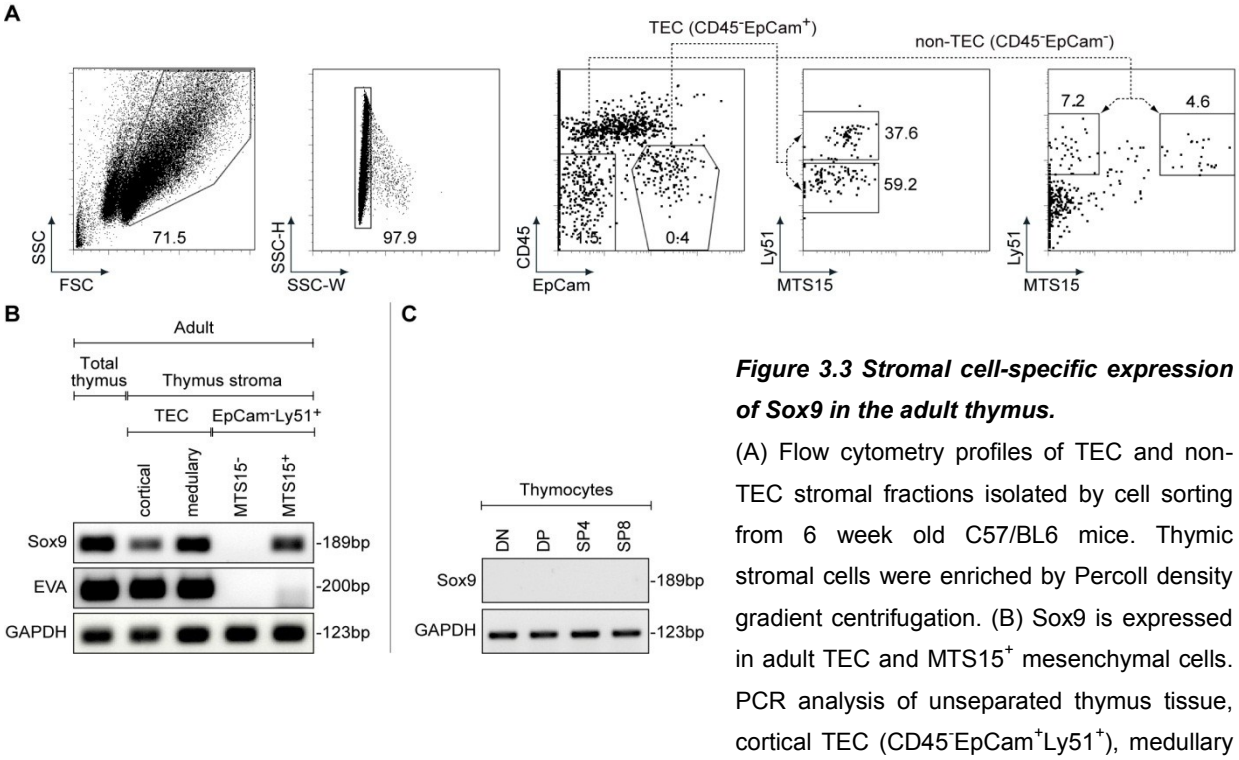


Figure 3.3 Stromal cell-specific expression of Sox9 in the adult thymus.

(A) Flow cytometry profiles of TEC and non-TEC stromal fractions isolated by cell sorting from 6 week old C57/BL6 mice. Thymic stromal cells were enriched by Percoll density gradient centrifugation. (B) Sox9 is expressed in adult TEC and MTS15$^+$ mesenchymal cells. PCR analysis of unseparated thymus tissue, cortical TEC (CD45$^-$EpCam$^+$Ly51$^+$), medullary TEC (CD45$^-$EpCam$^+$Ly51$^-$) and non-epithelial thymic stromal cells (CD45$^-$EpCam$^-$Ly51$^+$) that either express or lack MTS15 for Sox9 expression. The expression of GAPDH and the TEC marker Epithelial V-like antigen (EVA) were used as controls. (C) Thymocytes do not express Sox9. B220$^-$CD3$^-$CD4$^-$CD8$^-$ (DN), CD4$^+$CD8$^+$ (DP), CD4$^+$CD8$^-$ (SP4), CD4$^-$CD8$^+$ (SP8) thymocytes from 4 week old C57BL/6 mice were analysed for Sox9 expression. PCR amplification products were separated by gel-electrophoresis and visualized by EthBr. Data are representative of two independent experiments.

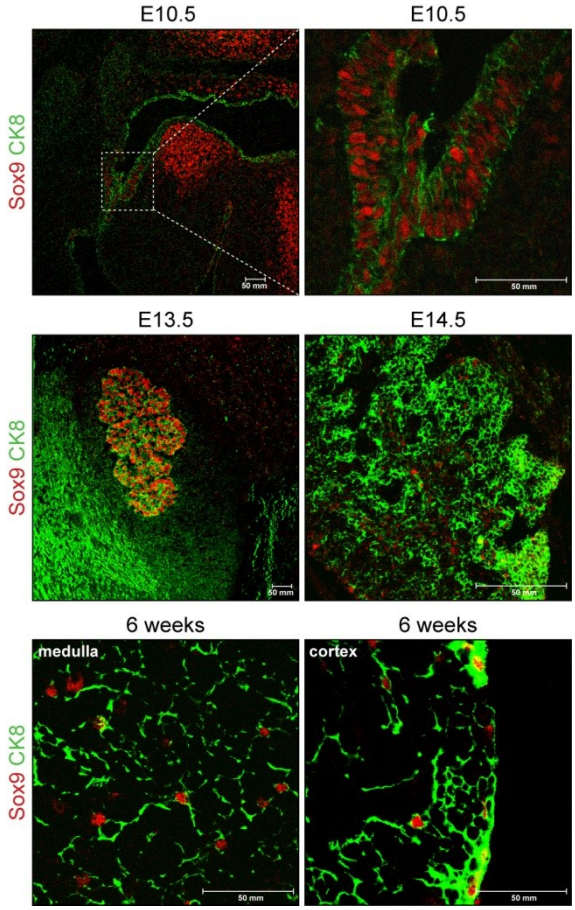


Figure 3.4 Sox9 protein is detected throughout thymus organogenesis and in the adults.

Immunofluorescence analysis of thymic tissue sections from embryonic and adult C57BL/6 mice for the detection of Sox9 (red) and cytokeratin CK8 (green) expression. The inset (upper right panel) represents a magnification of the CK8$^+$ epithelial cell lining in the ventral circumference of the 3rd pharyngeal pouch at E10.5 The data is representative of two independent experiments each analysing a minimum of 3 mice.

3.1.3. Sox9 is differentially expressed in adult thymic epithelia

Since Sox9 has been reported to effect intestinal epithelium biological activity in dose-sensitive fashion (Formeister *et al.* 2009), we next investigated whether Sox9 expression differ among the separate thymic epithelial subsets. For this purpose, DAPI negative (i.e. alive) TEC ($CD45^-EpCam^+$) were separated based on their Ly51 and UEA-1 expression into cortical (c) TEC ($UEA\text{-}1^-Ly51^+$) and medullary (m) TEC ($UEA\text{-}1^+Ly51^-$). The mTEC subset was further differentiated into immature (i.e. MHCII low expressing, designated $mTEC^{lo}$) and mature (i.e. MHCII high expressing, designated $mTEC^{hi}$) epithelia (Figure 3.5A). TEC purity was routinely assessed by re-analysing the sorted cell fractions using both flow cytometry and qRT-PCR. The integrity of isolated RNA was assessed by RNA 6000 Pico assay and the Agilent 2100 bioanalyzer (Appendix Figure 8.1). Distinct Sox9 transcript levels were detected among adult TEC dependent on the cells' developmental stage. $mTEC^{lo}$ expressed the highest Sox9 transcript levels, whereas cTEC contained significantly lower and $mTEC^{hi}$ the lowest levels of Sox9 transcripts (Figure 3.5B). As Sox9 mRNA and protein could also be detected in long-term established TEC lines (Figure 3.6 and Appendix Figure 8.3E), Sox9 expression appears to be cell autonomously regulated and thus, independent of continuous lymphoid-epithelial or lymphoid-mesenchymal crosstalk.

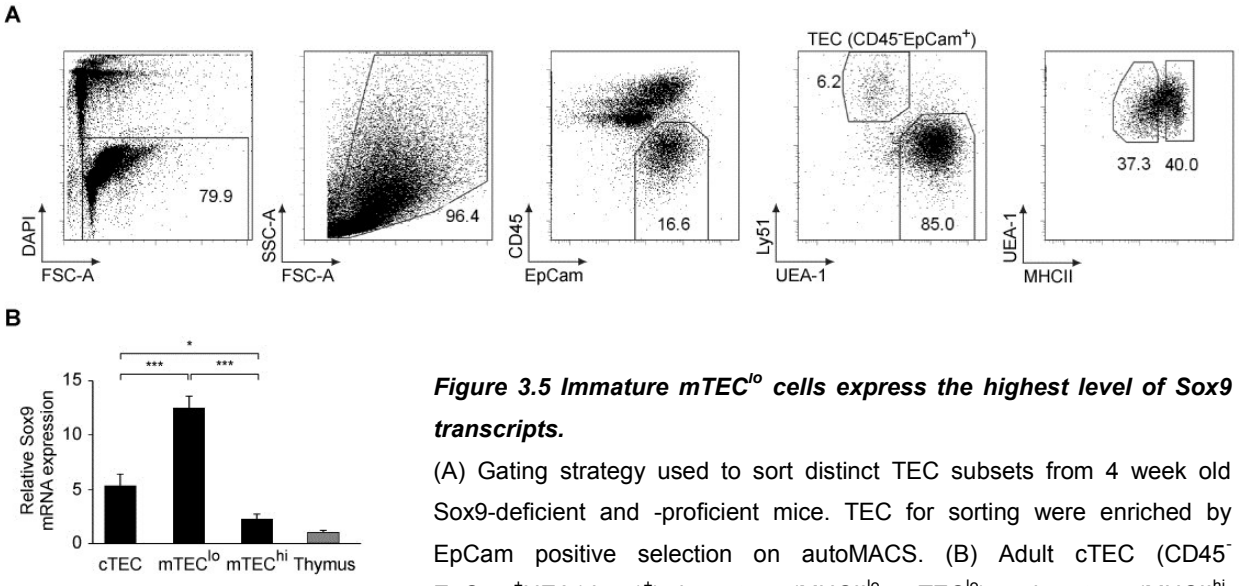


Figure 3.5 Immature $mTEC^{lo}$ cells express the highest level of Sox9 transcripts.

(A) Gating strategy used to sort distinct TEC subsets from 4 week old Sox9-deficient and -proficient mice. TEC for sorting were enriched by EpCam positive selection on autoMACS. (B) Adult cTEC ($CD45^-EpCam^+UEA1^-Ly51^+$), immature ($MHCII^{lo}$; $mTEC^{lo}$) and mature ($MHCII^{hi}$; $mTEC^{hi}$) mTEC ($CD45^-EpCam^+UEA1^+Ly51^-$) were analysed by qRT-PCR for Sox9 expression. Data represent the mean ± SD of triplicate analyses; Sox9 expression in thymus was set to an arbitrary value of 1. The data is representative of five independent experiments utilizing a minimum of 8 mice in each group and experiment. P values were calculated using the paired t-test; *p < .05, ***p < .001.

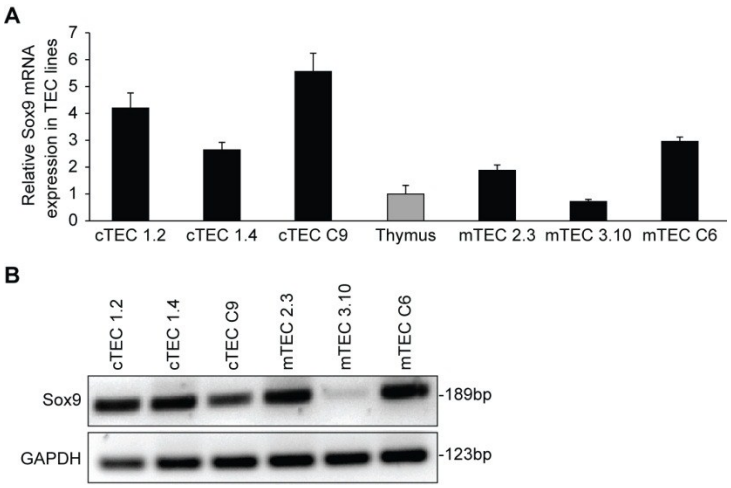


Figure 3.6 Sox9 expression in established thymic epithelial cell lines.
(A) Cortical (c) and medullary (m) TEC lines were analysed by qRT-PCR for Sox9 expression. The Sox9 mRNA data are the mean value ± SD of triplicate analyses where Sox9 expression in thymus was set arbitrary to a value of 1. (B) Size of Sox9-specific transcripts was controlled by gel electrophoresis and visualized by EthBr. The data is representative of two independent experiments.

3.2. Generation of mice deficient for Sox9 expression in thymic epithelium

3.2.1. TEC-targeted ablation of Sox9 expression

The expression pattern of Sox9 during fetal tymus development and in the adults suggested an important role for Sox9 in TEC differentiation and function. To define this role, Sox9 was conditionally ablated in the developing TEC, as mice with a general loss of Sox9 expression do not survive beyond E11.5 of gestation precluding the study of thymus development (Bi *et al.* 2001; Kist *et al.* 2002). Mice homozygous for a conditional Sox9 flox allele, designated [Sox9$^{f/f}$] have exon 2 and 3 of the Sox9 locus flanked by loxP sites (Kist *et al.* 2002) (Figure 3.7A). To generate mice with a TEC-restricted loss of Sox9

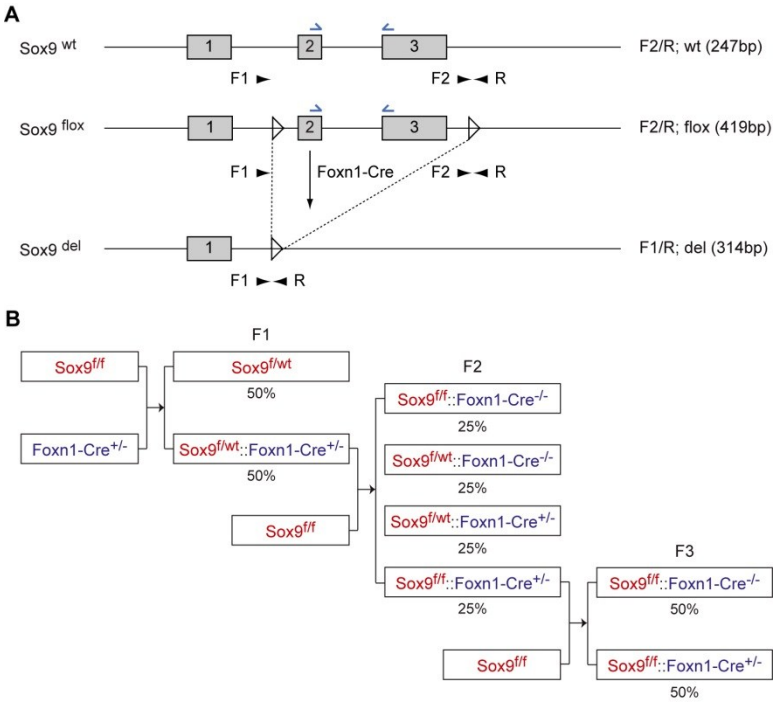


Figure 3.7 Generation of mice deficient for Sox9 expression in TEC.
(A) Schematic representation of Sox9 wild type (Sox9^{wt}) and conditional (Sox9^{flox}) locus and Cre-mediated deletion of exons 2 and 3 to obtain TEC-specific loss of Sox9 expression (Sox9^{del}). Grey boxes: Sox9 exons 1-3; triangles: loxP sites; arrowheads: location of genomic PCR primers (F1 and F2: forward primers; R: reverse primer). Primer combinations and expected amplicon sizes are indicated on the right. Location of qRT-PCR primers is indicated above the exons; adapted from (Kist *et al.* 2002). (B) Conditional Sox9 breeding: Sox9$^{flox/flox}$ x Foxn1-Cre.

expression, [Sox9$^{f/f}$] mice were crossed to the Foxn1-Cre transgenic line which expresses the Cre recombinase under the transcriptional control of the Foxn1 promoter (Zuklys *et al.* 2009). Recombination mediated by Foxn1-Cre results in the deletion of the last two exons, encoding part of the N`-terminal HMG DNA binding domain and the entire C-terminal transactivation (TA) domain of Sox9. Because the TA domain is essential for Sox9 function (Sudbeck *et al.* 1996) and because the HMG domain cannot bind DNA (Giese *et al.* 1991) this targeting strategy results in a complete loss of Sox9 function. Although it has not been excluded that a peptide might still be produced from the first exon, such a molecule, if at all stabile, does not contain any known functional domains.

As depicted in Figure 3.7B, heterozygous F1 animals [Sox9$^{wt/f}$::Foxn1-Cre] were established first and then backcrossed to [Sox9$^{f/f}$] mice to obtain mice homozygously deficient for Sox9 expression, designated [Sox9$^{f/f}$::Foxn1-Cre]. F2 males were backcrossed once again to [Sox9$^{f/f}$] to increase the frequency of [Sox9$^{f/f}$::Foxn1-Cre]. The (F3) generation showed a Mendelian distribution of genotypes, with 50% of animals expressing Cre recombinase.

3.2.2. Efficiency and specificity of Foxn1-Cre mediated deletion of Sox9

Sort-purified TEC (CD45$^-$ EpCam$^+$; >95%) derived from individual mice were next analysed by allele-specific PCR to verify that the targeted Sox9 locus was efficiently and completely recombined. As shown in Figure 3.8A, TEC from [Sox9$^{f/f}$::Foxn1-Cre] mice showed a complete recombination of the Sox9 locus,

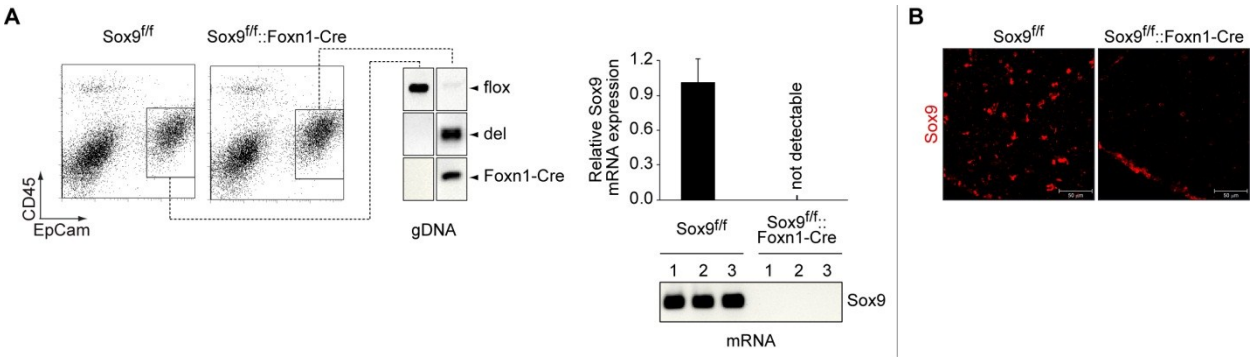


Figure 3.8 The floxed Sox9 alleles are efficiently and specifically recombined in Foxn1-Cre expressing mice resulting in a complete loss of Sox9 expression in TEC.

(A) Genomic PCR (middle panel) and qRT-PCR analysis (right panel) of TEC (CD45$^-$EpCam$^+$) isolated from 4 week old mice. Rectangles in dot plots (left panel) indicate the used sorting gates. The undeleted (flox) and the deleted (del) alleles are 419 and 314 bp, respectively, and the Foxn1-Cre allele is 300 bp. At mRNA level Sox9 deletion was confirmed by qRT-PCR using primers annealing in exons 2 and 3 on the both side of an intron giving 189 bp amplicon in control mice. (B) Sox9 is deleted specifically in thymic epithelia but not in mesenchymal stroma by Foxn1-Cre. Immunofluorescence analysis of thymus tissue sections from 4 week old [Sox9$^{f/f}$] and [Sox9$^{f/f}$::Foxn1-Cre] mice for the detection of Sox9 expression. Scale bar: 100µm. The data is representative of three independent experiments utilizing a minimum of 3 mice in each group and experiment.

indicated by the loss of the targeted “flox” allele and the presence of a smaller recombined allele, designated “del”. The efficiency of recombination was further confirmed at the transcriptional level using qRT-PCR (Figure 3.8A, right panel) and at the protein level using immunohistology (Figure 3.8A, right panel and 3.8B). As expected in mice with a TEC-restricted loss of Sox9 expression, Sox9 protein was still present in mesenchymal cells within the thymus stroma (Figure 3.8B), a finding that agrees with above described expression of Sox9 in fetal and adult mesenchymal cells (Figure 3.3B and 3.4). Thus, Foxn1-Cre mediated deletion of Sox9 results in a complete loss of Sox9 expression in TEC and provides an informative experimental system to study the role of Sox9 for thymus organogenesis and function.

3.2.3. [Sox9$^{f/f}$::Foxn1-Cre] mice are viable, fertile but show macroscopical changes of epidermal appendages

Given the TEC-restricted loss of Sox9 expression, [Sox9$^{f/f}$::Foxn1-Cre] mice developed regularly and were normally fertile (Figure 3.9). Previous studies have demonstrated the role for Sox9 in the formation of the hair stem cell compartment and differentiation of the outer root sheet (ORS) cell lineage (Vidal *et al.* 2005). Furthermore, Foxn1 expression was reported for the matrix, cortex and ORS of the hair during the anagen phase and the nail forming regions with a role in inducing keratin expression (Meier *et al.* 1999). In agreement with the documented activity of Sox9 and Foxn1 in the context of hair development, [Sox9$^{f/f}$::Foxn1-Cre] mice displayed an alopecia (Figure 3.9). This macroscopic lack of the hair was apparent as early as 12 days of life and persisted. Interestingly and contrary to the phenotype of nude mice which display a complete lack of the body hair, a normal coat was observed on the head region and the extremities in [Sox9$^{f/f}$::Foxn1-Cre] mice. This could be due to an inefficient Cre activity in Foxn1-Cre line. Moreover, the nails of the Sox9-deficient mice were longer correlating with the reported activity of Foxn1 in the nail forming region (Figure 3.9).

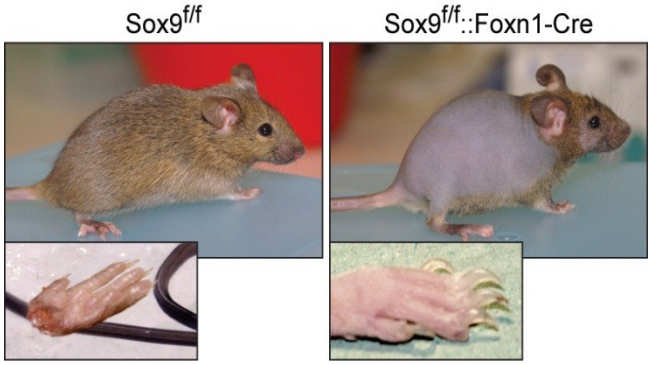


Figure 3.9 Sox9 deficient mice display alopecia and have longer nails.

Representative pictures of 4 week old [Sox9$^{f/f}$::Foxn1-Cre] and [Sox9$^{f/f}$] mice.

3.3. The effect of Sox9 deletion on the architecture and composition of the thymic microenvironment

3.3.1. Sox9 deficiency results in a hypoplastic thymus, with normal cortico-medullary segregation and regular T cell development

The gross anatomical appearance of the thymus from [Sox9$^{f/f}$::Foxn1-Cre] mice was normal and a regular segregation into separate cortical and medullary compartments was observed by histological analysis of the thymic sections using hematoxylin/eosin (HE) staining (Figure 3.10A). Thymus cellularity however, was significantly lower at all ages investigated (Figure 3.10B) when compared to control [Sox9$^{f/f}$] mice, though Sox9-deficient TEC remained responsive to age-related changes in cellularity showing typical increase during young adulthood and a decrease after the onset of sexual maturity.

The competence of Sox9-deficient TEC to support T cell development was next investigated. Cell suspensions of thymi at different postnatal stages were stained for the cell surface markers, CD4 and CD8, to characterize different maturational stages during T cell development. A normal progression from immature CD4$^-$CD8$^-$ to mature either CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$ T cells was observed in [Sox9$^{f/f}$::Foxn1-Cre] mice of all ages (Figure 3.10A). Also, early stages of thymocyte development as character-ized by the single or combined expression of CD44 and CD25 were not affected by the TEC-specific loss of Sox9 (Figure 3.11C). Developing thymocytes at later maturational stages normally upregulate expres-

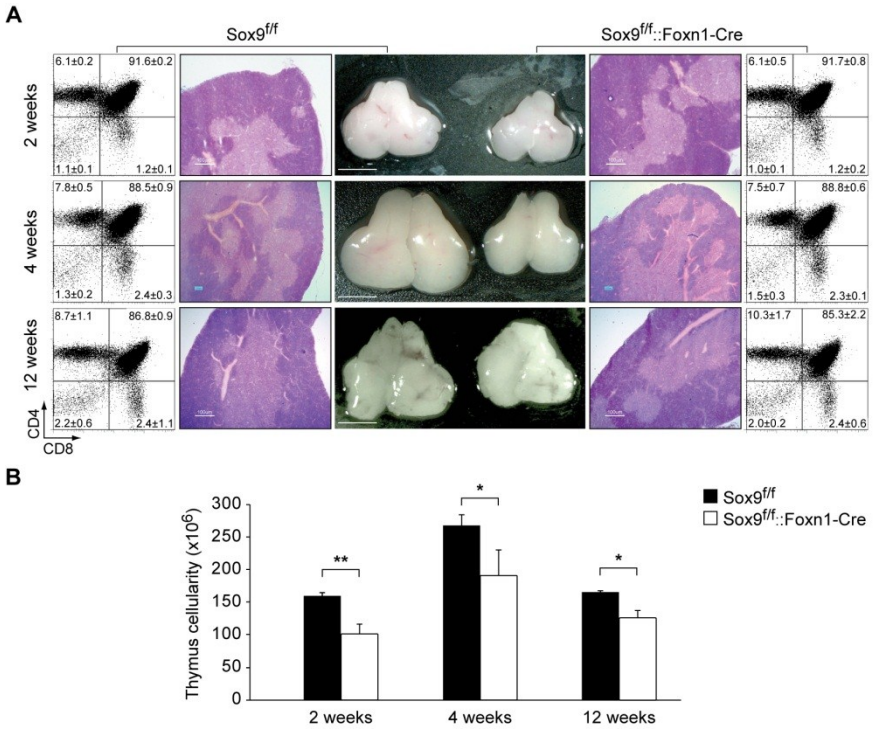


Figure 3.10 Mice with Sox9 deficiency in TEC develop hypoplastic thymus with regular cortex-medulla segregation and T cell development. (A) Gross anatomy, H&E staining of thymic sections (magnification 4x) and flow cytometric analysis of CD4 and CD8 expression in pre-pubescent (2wk), adolescent (4wk) and adult (12wk) mice either proficient [Sox9$^{f/f}$] or deficient [Sox9$^{f/f}$::Foxn1-Cre] for the Sox9 expression in TEC. The mean relative frequency ± SD for the different thymocytes subsets is represented in each quadrant. (B) Comparative analysis of thymus cellularity in [Sox9$^{f/f}$::Foxn1-Cre] and [Sox9$^{f/f}$] mice at indicated ages. A minimum of 3 mice per group was analysed at each time point in three independent experiments. Data represent the mean values ± SD and p values were calculated using the paired t-test; *p < .05, **p < .01.

sion of CD69 and TCR/CD3 expression upon positive thymic selection and SP thymocytes in the medulla downregulate CD69 and CD24 expression as a part of their post-selectional maturation (Lucas *et al.* 1994; Ernst *et al.* 1995; Gabor *et al.* 1997; Hare *et al.* 1998; Anderson and Jenkinson 2001). The expression pattern of CD69 and CD24 was normal in [Sox9$^{f/f}$::Foxn1-Cre] mice when compared to control [Sox9$^{f/f}$] mice suggesting regular thymic selection and post-selectional maturation of developing thymocytes in a Sox9-deficient microenvironment (Figure 3.11A and B). Moreover, the relative frequency of CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cells in the spleen was the same for adult [Sox9$^{f/f}$::Foxn1-Cre] and control [Sox9$^{f/f}$] mice (Figure 3.11D). Similar results were obtained by analysing wild type and mutant mice at 2

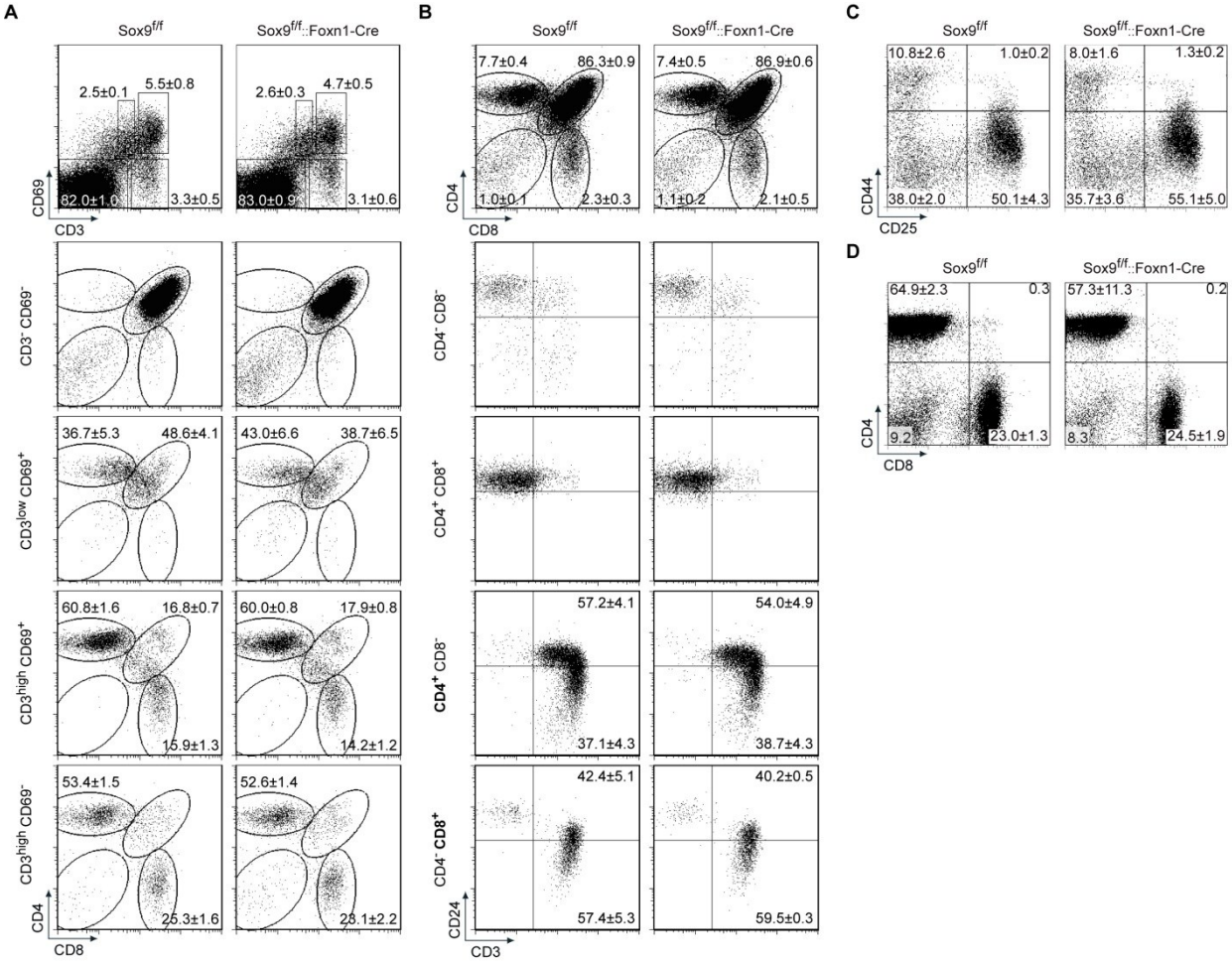


Figure 3.11 Sox9-deficient TEC support normal T cell maturation.

(A) Relative distribution of thymocyte subpopulations (i.e., DN-SP) based on the expression of CD3 and early activation marker CD69 in thymi of 4 week old [Sox9$^{f/f}$::Foxn1-Cre] and [Sox9$^{f/f}$] mice. B) Analysis of developmental progression from CD4$^-$CD8$^-$ to CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ T cells in 4 week old mice based on the expression of CD3 and cell surface marker CD24. CD24^{high} cells represent immature and CD24^{low} mature single positive CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ T cells. (C) Relative frequency of DN1 to DN4 thymocytes defined by the CD44 and CD25 expression in the Lin^{neg} thymocyte population of adult Sox9-deficient and -proficient mice. (D) Analysis of splenic T cells in 4 week old [Sox9$^{f/f}$::Foxn1-Cre] and [Sox9$^{f/f}$] mice. CD4 and CD8 T cells were gated on CD3$^+$ thymocytes. The mean relative frequency ± SD for the different thymocytes subsets is indicated in each quadrant. The data is representative of two independent experiments utilizing a minimum of 3 mice per group.

and 12 weeks of age (data not shown). Together, these findings demonstrate that a lack of Sox9 expression in TEC results in a moderately hypoplastic thymus that is able to support qualitatively normal T cell development.

3.3.2. Sox9 prevents cytokeratin 5 expression in cortical TEC

To determine the impact of Sox9 expression on TEC differentiation and organization, thymic tissue sections derived from mutant and control mice were analysed by immunofluorescence. Staining for the expression of cortical (CK8), medullary (MTS10, UEA-1, CK5, Aire) and mesenchymal (ERTR7) markers revealed a normal architectural organisation of the major epithelial and mesenchymal components in the thymus of [Sox9$^{f/f}$::Foxn1-Cre] mice (Figure 3.12). However, the proportion of cytokeratin 5 (CK5) positive epithelia was considerably increased since CK5^{+} TEC were not only restricted to the medulla marked by the MTS10 expression (Boyd *et al.* 1993), but could also be detected in the cortex (Figure 3.13A). Quantitative image analysis confirmed an increase in the frequency of single CK5^{+} and double CK5^{+}CK8^{+} TEC in mutant thymi (20% vs. 8.5% and 8% vs. 4.7%). Conversely, the frequency of single CK8^{+} TEC

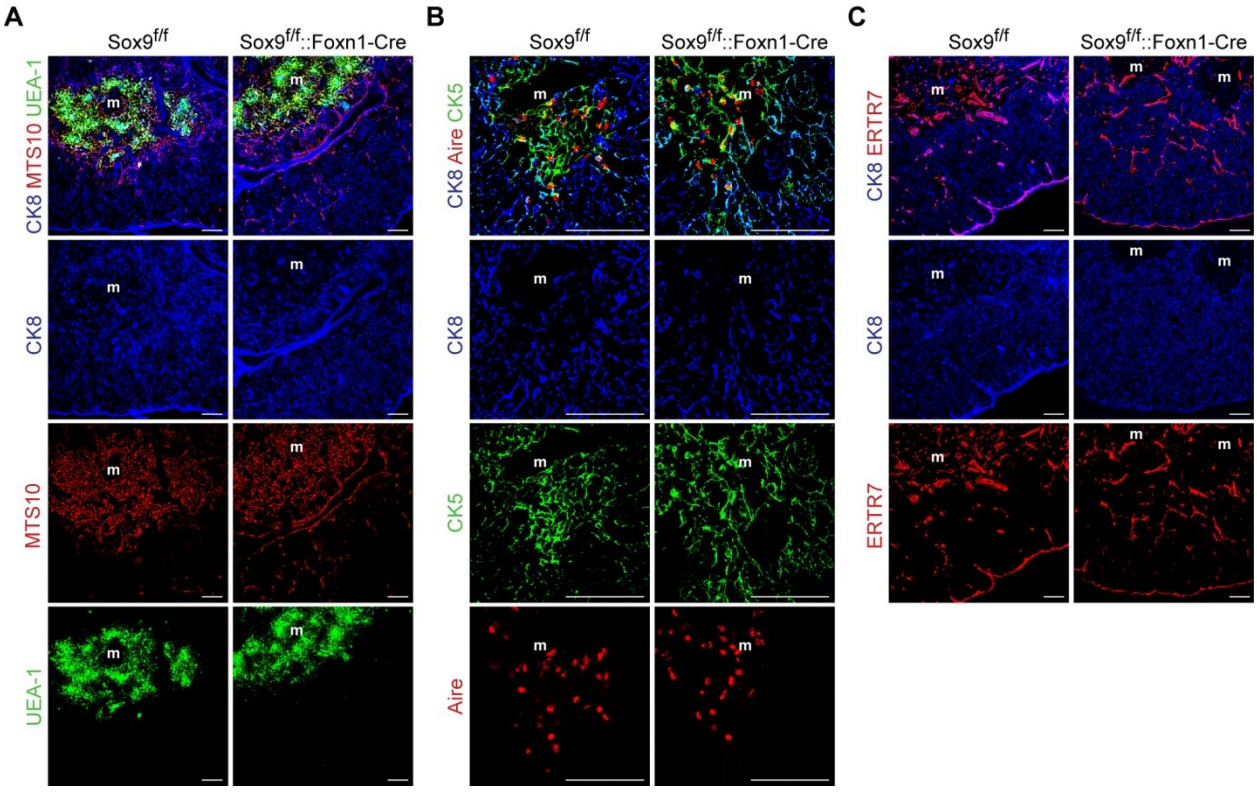


Figure 3.12 Normal stromal architecture of Sox9-deficient thymi.

Immunofluorescence analysis of thymus tissue sections from 4 week old mice for the expression of (A) CK8 (blue), MTS10 (red) and UEA-1 ligand (green), (B) CK8 (blue), CK5 (green) and Aire (red) or (C) CK8 (blue) and ERTR7 (red). Scale bar: 100µm. The data is representative of three independent experiments utilizing a minimum of 3 mice in each group and experiment.

was reduced in mutant when compared to control mice (23.7% vs. 35.2%) (Figure 3.13B). These changes in the relative abundance of the different TEC subpopulations were further confirmed by flow cytometric quantification of TEC subsets defined by differential levels of CK5 and CK8 expression. As demonstrated in Figure 3.13Ci, the frequency of $CK5^{hi}CK8^{lo}$ was increased at the expense of TEC with a $CK5^{lo}CK8^{hi}$ phenotype. The observed increase of $CK5^{+}$ TEC was however, not due to the change in the proliferation as the expression pattern of the cell cycle marker Ki67 was identical for the two mouse groups (Figure 3.13Cii). In keeping with a higher abundance of $CK5^{+}$ cells, transcripts specific for CK5 were also more frequent in TEC ($CD45^{-}EpCam^{+}$) isolated from Sox9-deficient mice (Figure 3.13D).

The $CK5^{+}$ TEC localized outside of the medulla also expressed other markers characteristic for cortical TEC including CK8, β5t, DEC205 and Ly51 (Figure 3.14) as assessed by immunochistochemistry. In contrast, these cells failed to display markers typical for mTEC such as Aire, MTS10 and UEA-1 (Figure 3.12A and B) suggesting that cTEC acquired the expression of CK5 in the absence of Sox9. To further characterize the thymic microenvironment in the absence of Sox9 expression, the localization of $CD25^{+}$ cells within the thymus was investigated (Figure 3.15). CD25 expression identifies DN2 ($CD44^{+}CD25^{+}$) and DN3 ($CD44^{-}CD25^{+}$) population of immature thymocytes. Physiologically, DN2 cells are located in the deeper cortex close to the CMJ, while the DN3 cells accumulate in the subcapsular region of the cortex (Lind *et al.* 2001; Petrie and Zuniga-Pflucker 2007). In Sox9-deficient microenvironment these immature cells were rather dispersed throughout the cortex implying a possible defect in migration of these cells (Figure 3.15).

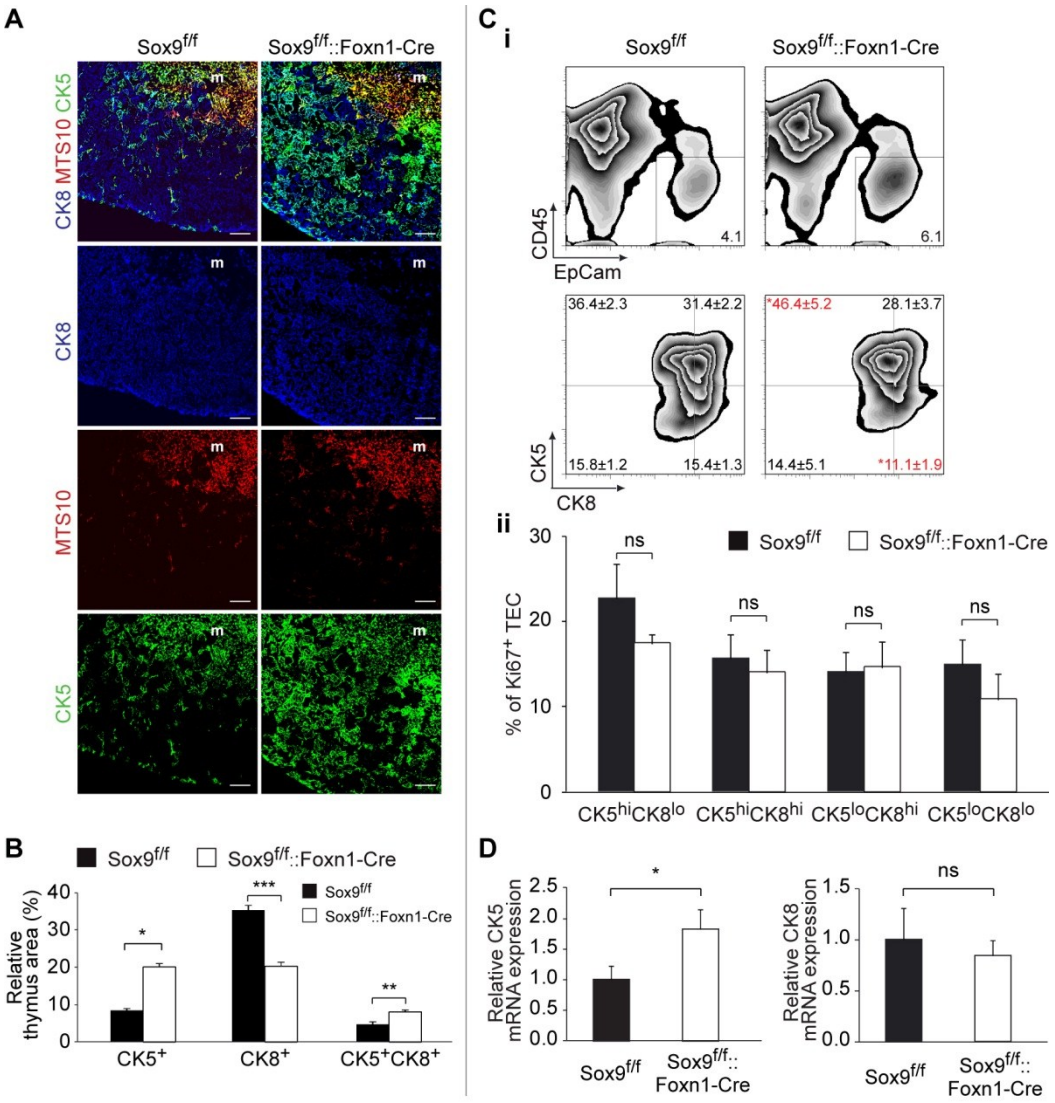


Figure 3.13 Increased proportion of CK5$^+$ TEC located in the cortex of Sox9-deficient mice.

(A) Immunofluorescence analysis of 4 week old [Sox9$^{f/f}$::Foxn1-Cre] and [Sox9$^{f/f}$] mice for the detection of cytokeratin CK8 (blue), MTS10 (red) and CK5 (green) expression. Scale bars: 100µm. (B) Quantification of CK5 and CK8 expression on thymic sections as measured by the NIH Image-J area analysis software. Data represent the proportion of area occupied in the thymus by TEC that express either CK5 (CK5$^+$), CK8 (CK8$^+$), or both (CK5$^+$CK8$^+$). Co-localization tool was used to determine the percentage of TEC co-expressing CK5 and CK8. The mean values ± SD were calculated using 3-4 sections from a minimum of 3 mice analysed in each group. Data in (A-B) are representative of three independent experiments. (C) Quantification of (i) TEC subsets defined based on the differential expression of CK5 and CK8 and (ii) their proliferative capacity in thymi of 4 week old [Sox9$^{f/f}$] and [Sox9$^{f/f}$::Foxn1-Cre] mice as assessed by flow cytometric analysis. The mean frequency ± SD of keratin subsets is provided as a percentage in each quadrant. (D) qRT-PCR analysis of CK5 and CK8 expression in TEC (CD45$^-$EpCam$^+$) isolated by cell sorting from 4 week old Sox9-proficient and -deficient mice. The data represents the mean ± SD of triplicate qRT-PCR analyses where cytokeratins expression in control [Sox9$^{f/f}$] mice was set to a value of 1 for comparison. The data in (C-D) are representative of two independent experiments utilizing a minimum of 3 mice per group. Statistical significance was calculated using the paired t-test; *p < .05, **p < .01, ***p < .001.

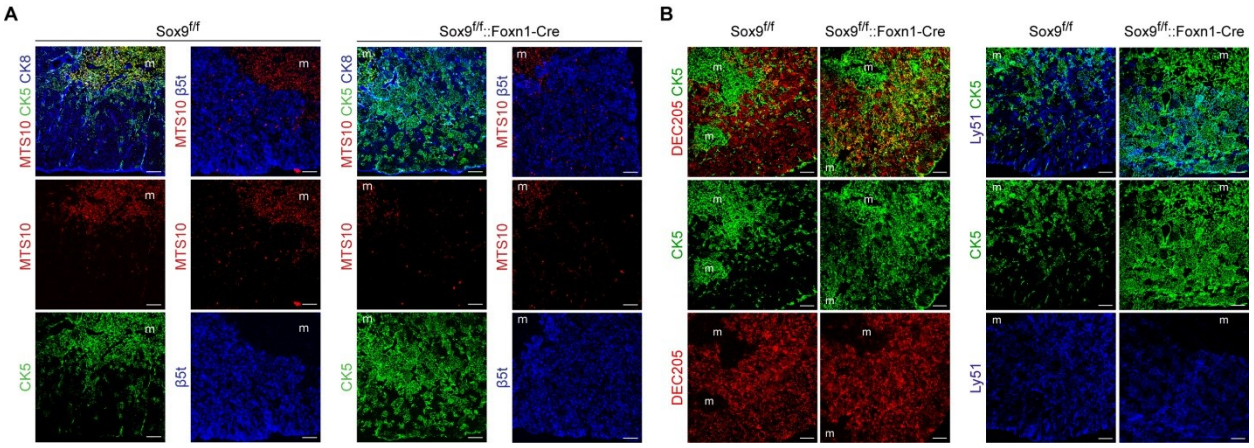


Figure 3.14 CK5^{+} cells detected in adult Sox9-deficient thymi display a cortical origin.

(A) Consecutive sections of adult thymus tissue stained by immunofluorescence for the expression of medullary markers MTS10 (red) and CK5 (green) and for cortical markers CK8 (blue) and β5t (blue), respectively and (B) for CK5 (green) and the cortical markers DEC205 (red) and Ly51 (blue), respectively. Data are representative of two independent experiments each analysing a minimum of 3 mutant and control mice. m: medulla; scale bars: 100μm.

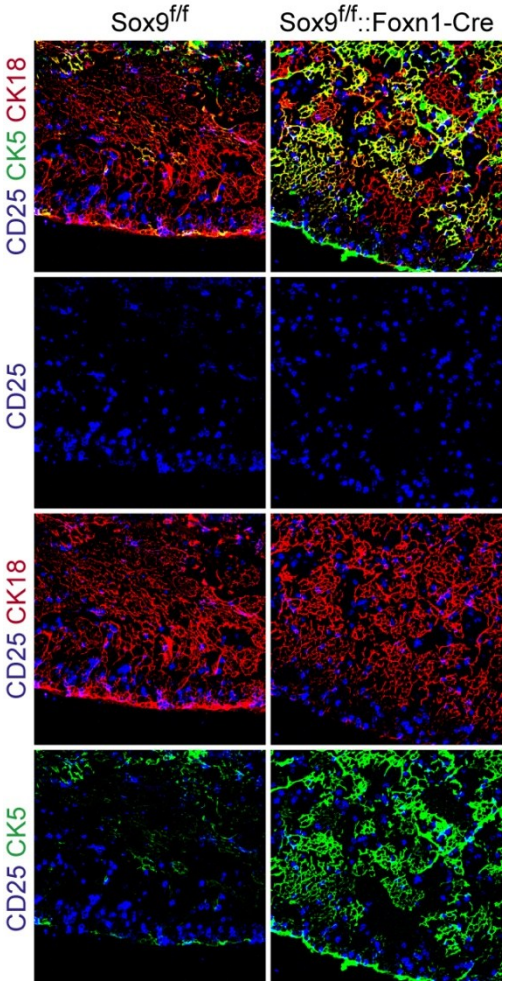


Figure 3.15 Altered distribution of CD25^{+} cells in Sox9-deficent microenvironment.

Immunohistofluorescence analysis of thymic sections from adult 10 week old [Sox9$^{f/f}$] and [Sox9$^{f/f}$::Foxn1-Cre] mice for the expression of CK18 (red), CK5 (green) and CD25 (blue). Scale bars: 100μm.

3.3.3. Composition, phenotype and proliferation of the thymic epithelia proficient and deficient for Sox9 expression

Differential Sox9 expression has been linked to the control of proliferation and terminal differentiation of intestinal epithelia (Formeister *et al.* 2009). To assess if Sox9 plays a similar role in physiology of thymic epithelia and given that distinct Sox9 expression levels exist among TEC, the cellularity, phenotype and proliferation of different TEC subsets in the absence and presence of Sox9 expression were investigated.

3.3.3.1. Changes in the mTEC composition in [Sox9$^{f/f}$::Foxn1-Cre] mice

Whether the thymic hypocellularity observed in [Sox9$^{f/f}$::Foxn1-Cre] mice is consequence of a decrease in the number of all TEC, only a specific TEC subpopulation or is independent of a change in thymic epithelial cellularity was first investigated. Absolute TEC (CD45$^-$EpCam$^+$) cellularity was consistently diminished in pre-pubescent (2 weeks), adolescent (4 weeks) and adult (12 weeks) [Sox9$^{f/f}$::Foxn1-Cre] mice (Figure 3.16Ai). Thus, the epithelial scaffold was reduced in size due to the fewer cells. The composition of thymic microenvironment was further analysed using the cortical marker Ly51 and differential expression of MHCII molecule (Appendix Figure 8.2). Thymic epithelial subsets changed in older (i.e 4 and 12 week old) [Sox9$^{f/f}$::Foxn1-Cre] mice demonstrating an increase in the mTEC^{lo} and decrease in the mTEC^{hi} frequency. This gradual change in the mTEC^{hi} to mTEC^{lo} ratio is a function of age and was independent of an absence of Sox9 expression as control [Sox9$^{f/f}$] mice showed identical changes with aging (Gray *et al.* 2006). A detailed quantification however, revealed that specific TEC subpopulations were differentially affected by the loss of Sox9 expression. mTEC with an immature phenotype (i.e. mTEC^{lo}) were diminished both in absolute and relative numbers (Figure 3.16Ai+ii) but the relative frequency of mTEC with a mature phenotype (i.e. mTEC^{hi}) was increased at all time points investigated (Figure 3.16Aii). In absolute numbers mTEC^{hi} were however decreased when compared to control [Sox9$^{f/f}$] mice of the same age (Figure 3.16Ai). The ratio of TEC to total thymus cellularity was only unchanged in 2 week old [Sox9$^{f/f}$::Foxn1-Cre] mice but significantly decreased at any time point thereafter (Figure 3.16B). In conclusion, the loss of Sox9 expression in TEC affects the composition of the epithelial microenvironment as marked by an altered representation of mTEC with an immature and mature phenotype.

3.3.3.2. Sox9 controls specifically proliferation of immature mTEC^{lo} cells

Since the lack of Sox9 expression changed the cellularity and representation of TEC within the thymic microenvironment, TEC proliferation in [Sox9$^{f/f}$::Foxn1-Cre] mice was next assessed using Ki67 as

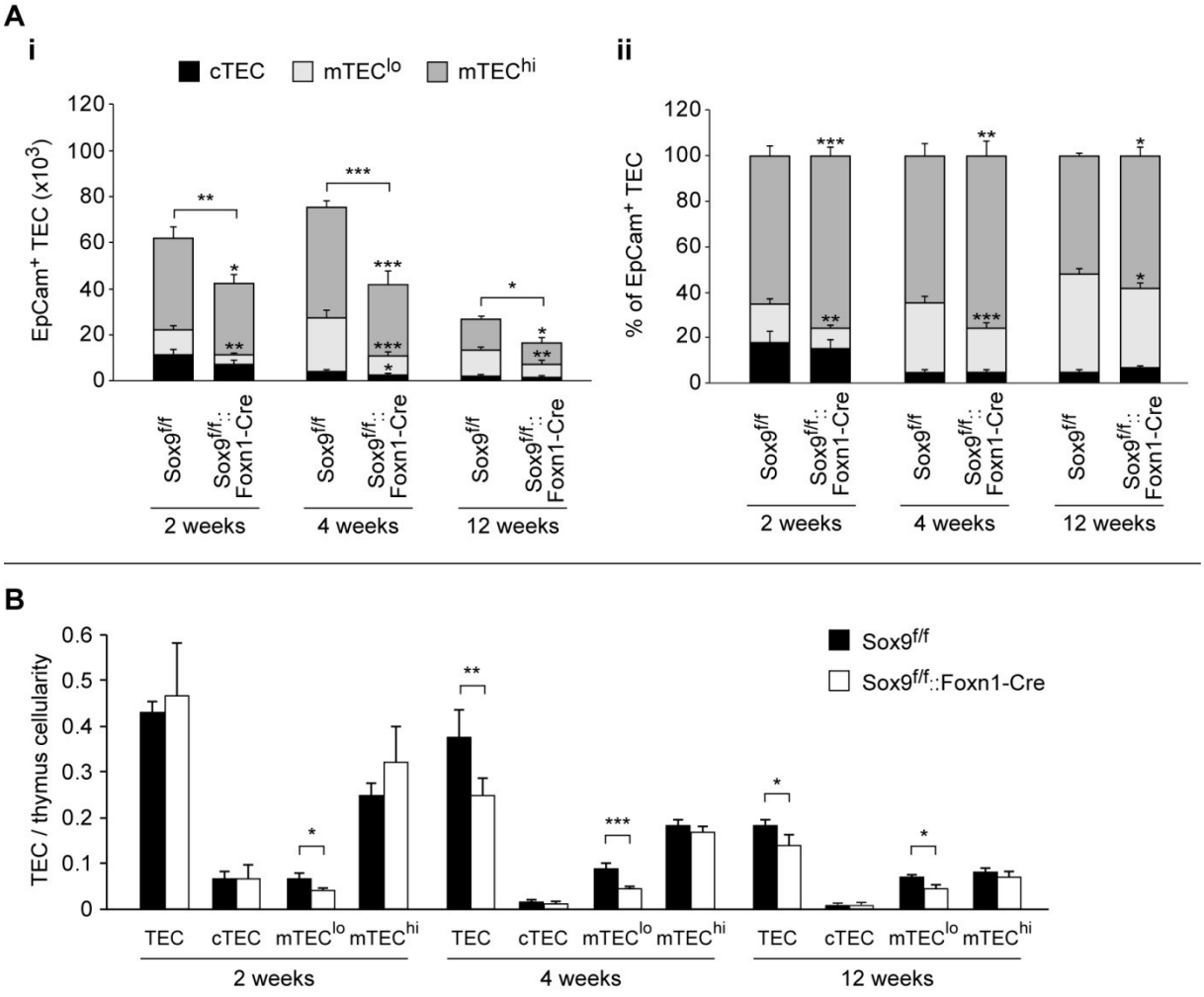


Figure 3.16 Specific reduction of mTEC^{lo} and relative expansion of mTEC^{hi} in Sox9-deficient thymus.
(A) Quantitative analysis of the (i) absolute cellularity and (ii) relative frequency of cortical (MHCII^{+}Ly51^{-}; cTEC), immature medullary (MHCII^{lo}Ly51^{-}; mTEC^{lo}) and mature medullary (MHCII^{hi}Ly51^{-}; mTEC^{hi}) thymic epithelia (CD45^{-}EpCam^{+}). (B) Ratio of TEC to thymus cellularity in [Sox9$^{f/f}$] and [Sox9$^{f/f}$::Foxn1-Cre] mice at different ages. The data (mean values ± SD) are representative of three independent experiments utilizing a minimum of 3 mice per group and time point. Statistical significance was calculated using the paired t-test; *p < .05, **p < .01, ***p < .001.

a marker of cell division (Gerdes *et al.* 1984). Previous studies showed that adult mTEC^{hi} proliferate the most among thymic epithelia (Yang *et al.* 2006; Gray *et al.* 2007b). In 2 week old Sox9-proficient mice 10% of mTEC^{lo} and 21% of mTEC^{hi} cells were actively dividing (Figure 3.17). Only 10% of the mTEC^{hi} cells were still dividing at 4 weeks, whereas mTEC^{lo} showed minor decrease in the proliferation rate. By 12 weeks of age, the proportion of dividing cells further fell and both mTEC^{lo} and mTEC^{hi} displayed a low steady state rate of cell division (6% and 7 %, respectively). The TEC-targeted loss of Sox9 expression resulted in an increased proliferation of mTEC^{lo} (Figure 3.17), which further correlated with an increased representation of mature mTEC^{hi} cells at all time points investigated (Figure 3.16Aii). These changes in mTEC composition and proliferation inversely correlated with distinct Sox9 expression levels normally detected among TEC subsets (Figure 3.5B) suggesting that Sox9 dosage may indeed have an impact on

mTEC growth. In contrast, a lack of Sox9 expression in cortical epithelia had no significant impact on proliferation and consequently, absolute and relative numbers of cTEC remained unchanged (Figure 3.16 and 3.17). Together these results revealed that Sox9 acts in a dose-sensitive and subset-specific manner in the regulation of TEC proliferation and differentiation.

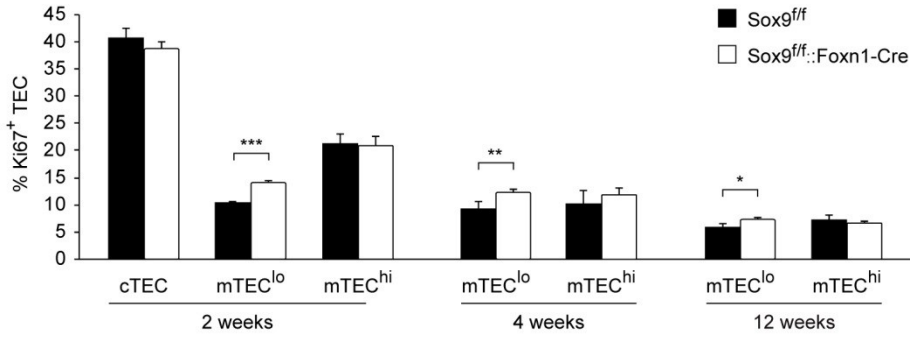


Figure 3.17 Increased proliferation of immature mTEC^{lo} cells in Sox9-deficient thymus.

Proliferation of distinct TEC subsets in [Sox9$^{f/f}$] and [Sox9$^{f/f}$::Foxn1-Cre] mice was assessed by analysis of Ki67 expression at the indicated ages. The data (mean values ± SD) are representative of three independent experiments utilizing a minimum of 3 mice per group and time point. Statistical significance was calculated using the paired t-test; *p < .05, **p < .01, ***p < .001. Note that Ki67 expression in cTEC at 4 and 12 weeks of age could not be accurately assessed, due to the too low frequency of Ly51^{+} cTEC obtained per individual thymus.

3.4. High Sox9 expression levels suppress the proliferation of a thymic epithelial cell line

To directly probe the effect of Sox9 expression on TEC proliferation, wild type Sox9 was overexpressed in an established cortical (c) TEC1.2 line using retroviral transduction (Appendix 8.3 A and B). An upregulation of Sox9 mRNA and protein levels was verified in transduced cells by qRT-PCR and immunochistochemistry analysis (Appendix 8.1 C and D). Subsequently, cell proliferation was assessed by BrdU incorporation and detection of Ki67 expression. Seventy and 81 percent of the control cells actively proliferated 16 and 24 hours after a BrdU pulse, respectively. Enforced Sox9 expression however, significantly reduced spontaneous TEC proliferation (Figure 3.18A+B). In parallel, the absolute cellularity of Sox9-transduced TEC had decreased by a third on day three of culture when compared to control cells (Figure 3.18C). This decrease was specific as the expression of a Sox9 mutant lacking the C-terminal transactivation (TA) domain, designated Sox9 M, failed to repress TEC proliferation as assessed by BrdU incorporation, Ki67 expression and the determination of the absolute cell number (Figure 3.18A-C). These results indicated that high Sox9 expression levels are able to suppress cell

proliferation, and agree with the *in vivo* data showing that high Sox9 expression in TEC correlates with an increased proliferation consequent to the loss of Sox9.

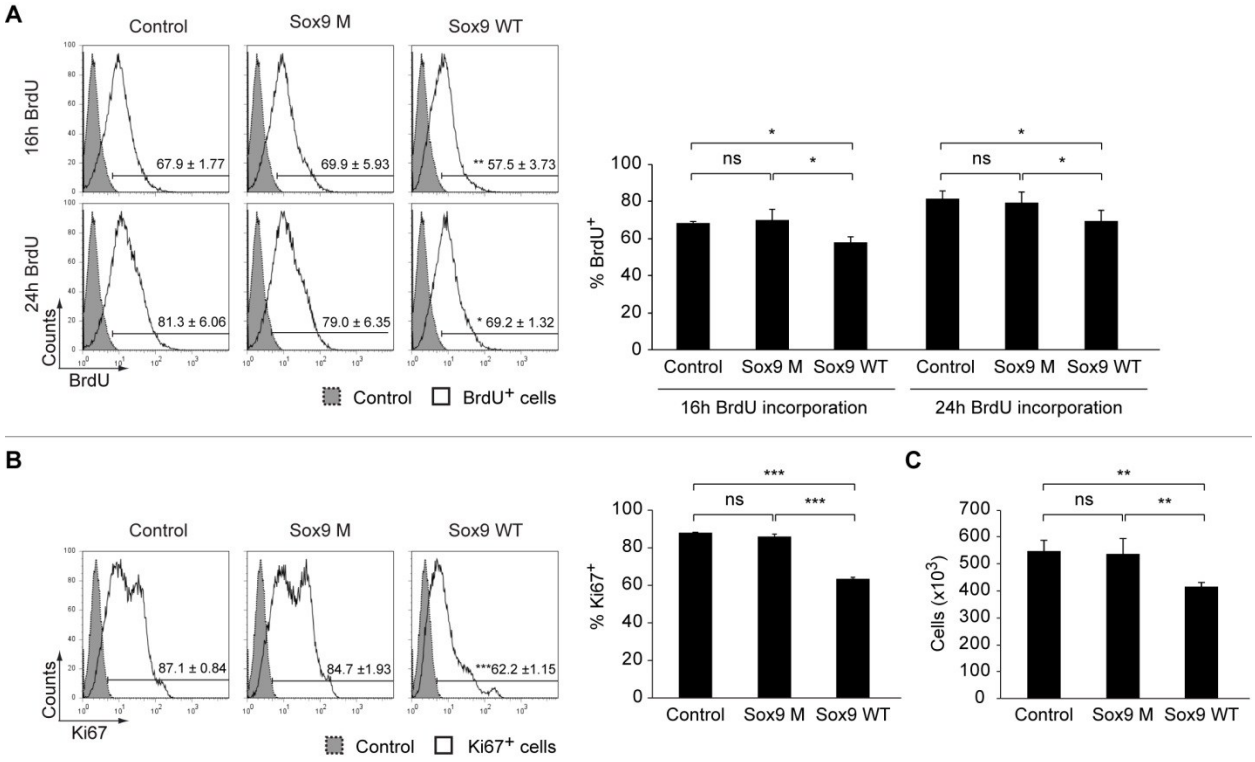


Figure 3.18 High Sox9 expression levels suppress TEC proliferation in vitro.

(A) BrdU incorporation and (B) Ki67 expression were measured in TEC lines stably transduced with an empty retroviral vector (control) or a retroviral vector recombinant for the expression of either Sox9 mutant (Sox9 M) or wild type (Sox9 WT). (C) The absolute number of transduced cells after three days culture. The values represent the mean ± SD of triplicate analyses. The data is representative of three independent experiments. Statistical significance was calculated using the paired t-test; *p < .05, **p < .01, ***p < .001.

3.5. Sox9 as a modulator of Wnt/β-catenin signalling in thymic epithelia

A functional and physical interaction between Sox9 and the Wnt/β-catenin pathway has been demonstrated in the context of tissue development and maintenance of different organs. Whether such a "cross-talk" exists during thymus organogenesis and maintenance was next investigated.

3.5.1. Sox9 negatively regulates Foxn1 expression in thymic epithelia

Sox9 has been identified to modulate "Wnt/β-catenin" transcriptional activity by controlling β-catenin protein levels (Akiyama *et al.* 2004b). A precise regulation of β-catenin amounts is essential for regular TEC development and function (Zuklys *et al.* 2009) and Wnt signalling engaging β-catenin regulates Foxn1 expression (Balciunaite *et al.* 2002). Therefore, the abundance of Foxn1 transcripts in adult thymic

epithelia was investigated in relation to Sox9 expression. Lower steady-state levels of Foxn1 transcripts were typically detected in wild type thymus and sorted TEC ($CD45^-EpCam^+$) when compared to Sox9 levels (Figure 3.19A i+ii). The TEC-targeted loss of Sox9 expression associated with a significant upregulation of Foxn1-specific transcripts in whole thymic tissue and sorted $EpCam^+$ TEC (1.5 and 2.9 fold, respectively) (Figure 3.19A i+ii). Given that a lack of Sox9 expression changed TEC composition and proliferation, Foxn1 transcripts were also investigated in separate thymic epithelial subsets. For this purpose, TEC ($CD45^-EpCam^+$) with cortical ($UEA\text{-}1^-Ly51^+$) and medullary ($UEA\text{-}1^+Ly51^-$) immature $mTEC^{lo}$ ($MHCII^{lo}$) and mature $mTEC^{hi}$ ($MHCII^{hi}$) phenotype (Appendix 8.1) were isolated from adult wild type and mutant mice. Wild type $mTEC^{lo}$ expressed fewer Foxn1 transcripts when compared to $mTEC^{hi}$ and cTEC (Figure 3.19A iii) revealing an inverse correlation between the steady-state levels of Sox9 and Foxn1 transcripts (Figure 3.19B). Similarly, an inverse correlation of Sox9 and Foxn1 expression was also noted in analysed TEC lines (Figure 3.20). Consequent to a loss of Sox9 expression, all TEC upregulated Foxn1 transcripts. The greatest increase was observed in $mTEC^{lo}$ subset but the differences in Foxn1

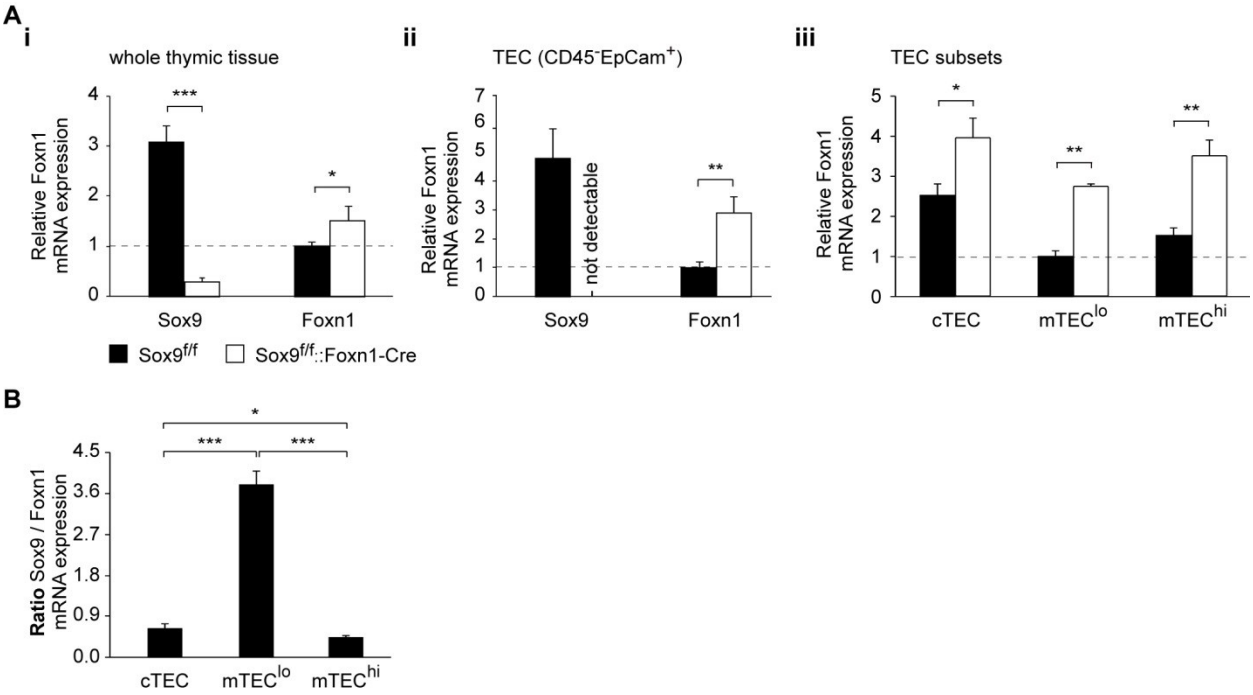


Figure 3.19 Foxn1 transcripts are upregulated in Sox9-deficient thymus.

(A) qRT-PCR analysis of Foxn1 expression in (i) whole thymic tissue, (ii) TEC ($CD45^-EpCam^+$), (iii) cortical (c: $UEA1^-Ly51^+$) and medullary (m: $UEA1^+Ly51^-$) immature ($MHCII^{lo}$) and mature ($MHCII^{hi}$) TEC isolated from 4 week old [$Sox9^{f/f}$::Foxn1-Cre] and [$Sox9^{f/f}$] mice. Foxn1 mRNA expression in tissue of control [$Sox9^{f/f}$] mice was set arbitrary to a value of 1, respectively. (B) Sox9 and Foxn1 expression in TEC subsets is inversely correlated. Ratio of Sox9 and Foxn1 mRNA expression in distinct TEC subsets of Sox9-proficient and -deficient mice. The Foxn1 and Sox9 mRNA data represent the mean ± SD of triplicate qRT-PCR analyses. Statistical significance was calculated using the paired t-test; *p < .05, **p < .01, ***p < .001. The data are representative of four independent experiments utilizing a minimum of 8 mice per group and experiment. Statistical significance was calculated using the paired t-test; *p < .05, **p < .01, ***p < .001.

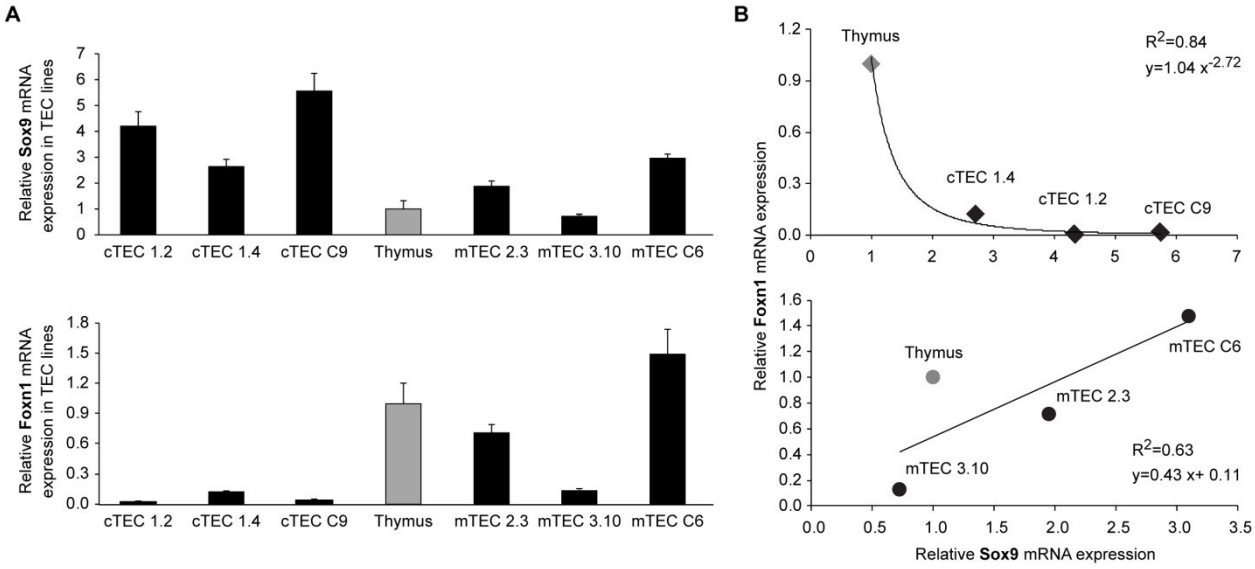


Figure 3.20 Sox9 and Foxn1 expression levels in established TEC lines.

(A) qRT-PCR analysis of cortical (c) and medullary (m) TEC lines for Sox9 and Foxn1 expression. (B) Correlation of Sox9 and Foxn1 expression in indicated TEC lines. The Sox9 and Foxn1 mRNA data are the mean value ± SD of triplicate analyses where Sox9 and Foxn1 expression in thymus was set arbitrary to a value of 1. The data are representative of three independent experiments.

expression levels typical observed between the separate subpopulation remained also in the gene targeted mice (Figure 3.19A iii).

The association of Foxn1 expression with Sox9 (Figure 3.19) was further confirmed at the protein level by quantitative expression analysis of thymic tissue sections derived from [$Sox9^{f/f}$] and [$Sox9^{f/f}$::Foxn1-Cre] mice using Image-J software. Foxn1 protein expression was significantly increased in both cortex and medulla of the mutant mice when compared to their wild type controls (Figure 3.21) and correlated with above described changes in Foxn1 mRNA expression (Figure 3.19). These results identified a specific role of Sox9 as a negative regulator of Foxn1 expression in TEC. In summary, the loss of Sox9 expression in TEC leads to subset-specific and dose-dependent changes in both TEC proliferation and composition which correlated with an increase in Foxn1 expression.

3.5.2. Sox9 physically binds and regulates β-catenin protein levels in thymic epithelia

Next, the underlying molecular mechanism by which Sox9 modulates Foxn1 expression in TEC was explored. Previous studies in chondrocytes have shown that Sox9 controls β-catenin protein levels through the formation of a physical complex with β-catenin. This interaction is mediated *via* the C-terminal domain of Sox9 and leads to the degradation of β-catenin and hence, inhibits Wnt/β-catenin controlled transcriptional activity (Akiyama *et al.* 2004b). To investigate whether in thymic epithelia Sox9 physically interacts with β-catenin and thus regulates its cellular availability, co-immunoprecipitations

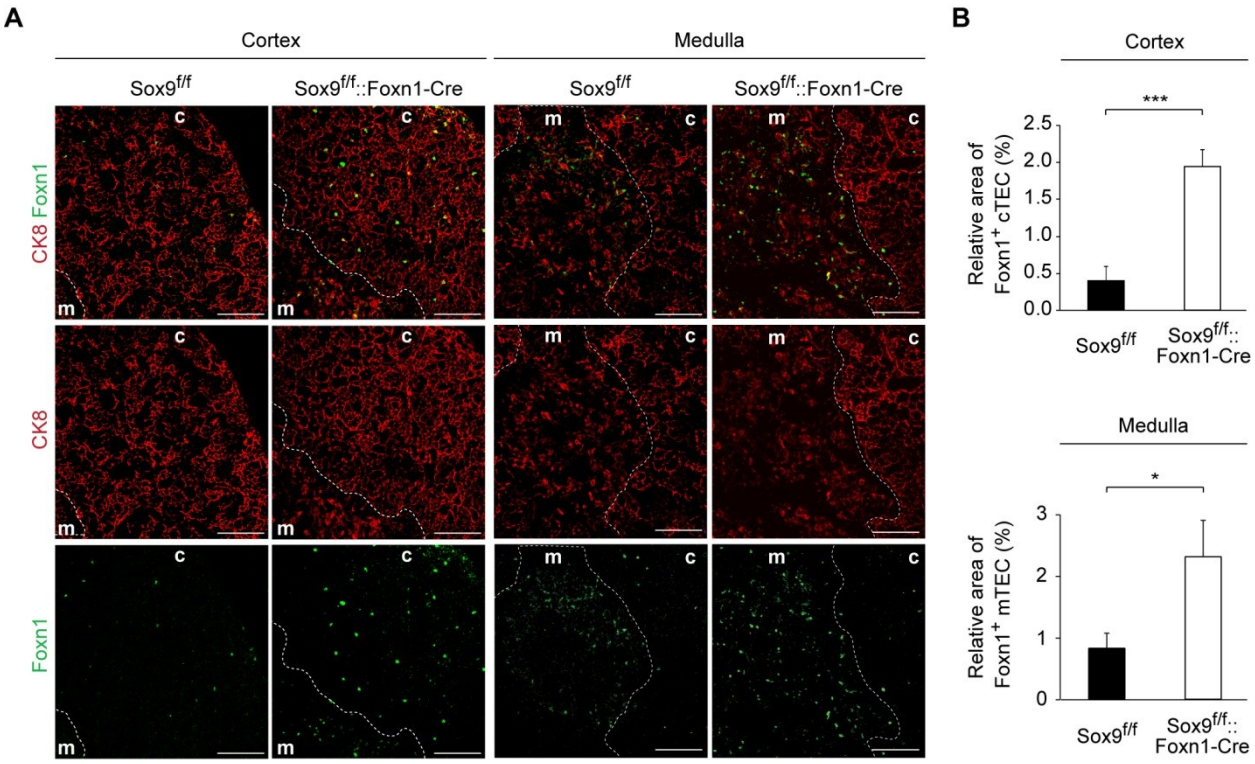


Figure 3.21 Foxn1 protein expression is increased in the thymus with Sox9-deficient TEC.

(A) Immunofluorescence analysis for thymic CK8 and Foxn1 expression in adult [Sox9$^{f/f}$::Foxn1-Cre] and [Sox9$^{f/f}$] mice. Scale bars: 100µm. (B) Quantitative analysis of Foxn1 protein expression on thymic sections of Sox9-proficient and -deficient mice as measured by NIH Image-J area analysis software. Cortical and medullary areas were defined by staining for CK8 and TEC morphology (i.e., globular *versus* satellite, respectively). Data represent the percentage of cortical and medullary area occupied by Foxn1^{+} cells. The mean values ± SD were calculated using 3-4 sections from a minimum of 3 mutant and control mice. P values were calculated using the paired t-test; *p < .05, ***p < .001. The data are representative of two independent experiments.

were carried out with TEC lines stably transduced to express either a HA-tagged Sox9 wild type (Sox9 WT) or Sox9 mutant (Sox9 M) molecule that lacked the C-terminal TA domain but contained N-terminal HMG domain (Appendix 8.3). Sox9 physically interacted in TEC with β-catenin in a fashion that was partially dependent on its C-terminal TA domain as mutant Sox9 could also formed a physical complex with β-catenin (Figure 3.22A, upper panel and gel). However, fewer β-catenin molecules were bound by mutant Sox9 as quantified by Image-J analysis (Figure 3.22A, lower panel) indicating that wild type Sox9 associated more efficiently with β-catenin, probably due to the engagement of both the C- and N-terminal domains. Moreover, the overexpression of wild type Sox9 markedly reduced the amount of β-catenin that could be immunoprecipitated by β-catenin antibody when compared to the mutant Sox9 molecule (Figure 3.22A, upper panel, lower gel).

To obtain direct evidence that Sox9 regulates β-catenin protein levels in thymic epithelia, lysates from TEC lines stably transduced to express either a control vector or a wild type and mutant Sox9 were analysed by Western blotting. The overexpression of wild type Sox9 strongly reduced the nuclear and to a lesser extent the cytoplasmic concentrations of β-catenin in TEC whereas the expression of a C-

terminal TA-deficient mutant Sox9 did not change the cellular β-catenin amounts (Figure 3.22B). These findings suggested that the C-terminal domain is involved in the regulation of β-catenin levels.

Though the efficiency in expressing wild type Sox9 in TEC was lower when compared to mutant Sox9 (Figure 3.22C, middle gel in upper panel and Appendix 8.3C), the ratio of β-catenin bound to Sox9 in relation to total cellular β-catenin was consistently higher for TEC overexpressing wild type Sox9 (Figure 3.22C, lower panel) indicating both more efficient binding and degradation of β-catenin in the presence of the C-terminal domain. Thus, in TEC both the C- and N-terminus of Sox9 can bind β-catenin, but only the interaction mediated *via* the C-terminal TA domain determines the cellular β-catenin levels and nuclear availability, probably by promoting β-catenin degradation.

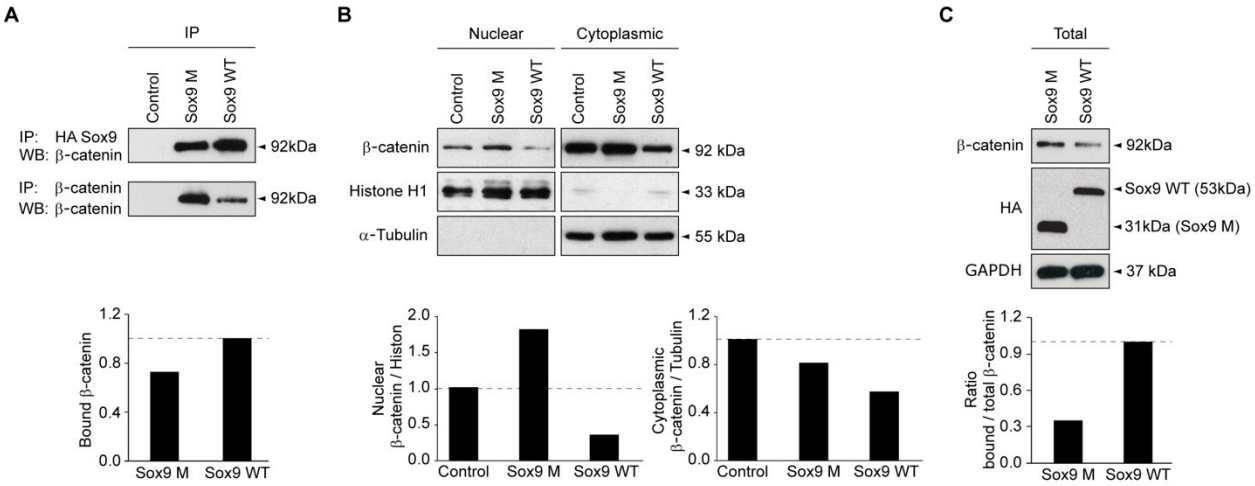


Figure 3.22 Sox9 binds and regulates β-catenin protein levels in thymic epithelia.

(A) Wild type Sox9 (Sox9 WT) binds β-catenin more efficiently than its C-terminal deletion mutant (Sox9 M). Upper panel: cell lysates of cTEC1.2 transduced to express a HA-tagged mutant (M) or wild type (WT) form of Sox9 were immunoprecipitated using either anti-HA or anti-β-catenin antibodies and bound β-catenin was detected by Western blotting (WB). Immunoprecipitation of cell lysates with mouse IgG served as a control. Lower panel: quantification of β-catenin bound by mutant and wild type Sox9 molecule as measured by densitometry using the NIH Image-J software. Sox9 WT was arbitrary set to a value of 1. (B) Wild type but not mutant Sox9 reduces β-catenin protein levels in nucleus and cytoplasm. Upper panel: subcellular fractions of cTEC1.2 transduced with an empty retroviral vector (control) or vectors recombinant to express either mutant or wild type Sox9 were analysed by WB for the expression of β-catenin. Histon H1 and α- tubulin expression were used to measure lysate purity and to control loading. Lower panel: nuclear and cytoplasmic β-catenin expression in indicated lysates was quantified by the NIH Image-J software. Cells transduced with an empty retroviral vector (control) were set to a value of 1 for comparison. (C) β-catenin and HA-Sox9 protein expression in total lysates of transduced cTEC1.2 was assessed by WB analysis (upper panel) and the ratio of bound β-catenin to total cellular β-catenin expression in indicated lysates calculated (lower panel). Sox9 WT was arbitrary set to a value of 1. The data are representative of two independent experiments.

3.5.3. Reduced β-catenin protein levels correlate with downregulation of Foxn1 expression in wild type Sox9-transduced cells

Finally, whether the reduction of β-catenin protein detected in TEC line overexpressing wild type Sox9 associates with changes of Foxn1 expression was investigated. Indeed, a significant decreases of Foxn1 transcripts (Figure 3.23A) and consequently protein (Figure 3.23B) were observed as a consequence of wild type Sox9 overexpression and thus, correlated with the reduced β-catenin availability in TEC (Figure 3.22B). In contrast, the stable expression of a TA-deficient Sox9 mutant did neither alter protein concentration of β-catenin nor Foxn1 expression (Figure 3.23 and 3.22B). Thus, Sox9 regulates Foxn1 expression indirectly *via* the control of β-catenin levels and an intact C-terminal domain is required for this effect. In conclusion, these *in vitro* findings established that Sox9 expression negatively modulates Foxn1 through the physical and functional interaction with β-catenin and suggest crosstalk between Sox9 and the Wnt canonical pathway in thymic epithelia (Figure 3.24).

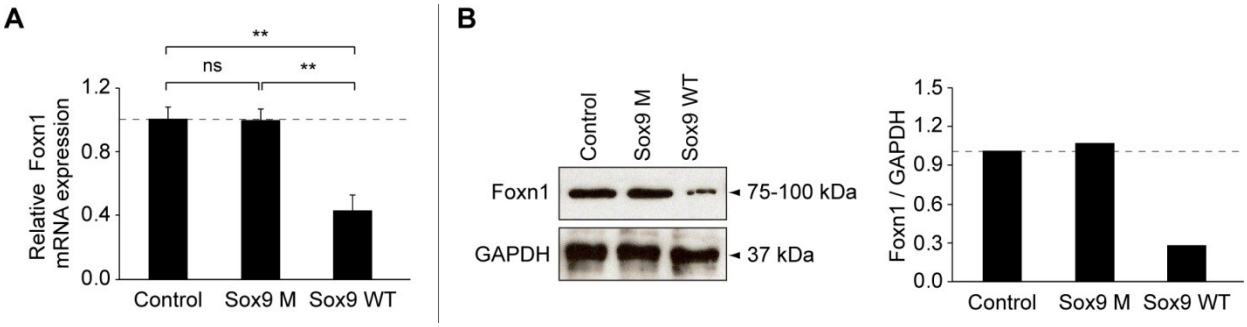


Figure 3.23 Wild type but not mutant form of Sox9 downregulates Foxn1 expression.

(A) Foxn1 mRNA levels in transduced cTEC1.2 were assessed by qRT-PCR. Data represents the mean ± SD of triplicate analyses where Foxn1 expression in cTEC1.2 transduced with an empty retroviral vector (control) was set to a value of 1. (B) Foxn1 protein expression in indicated lysates of transduced cTEC1.2 was detected by WB (left) and quantified by densitometry using Image-J software (right). The data are representative of two independent experiments. Sox9 WT was arbitrary set to a value of 1 for comparison.

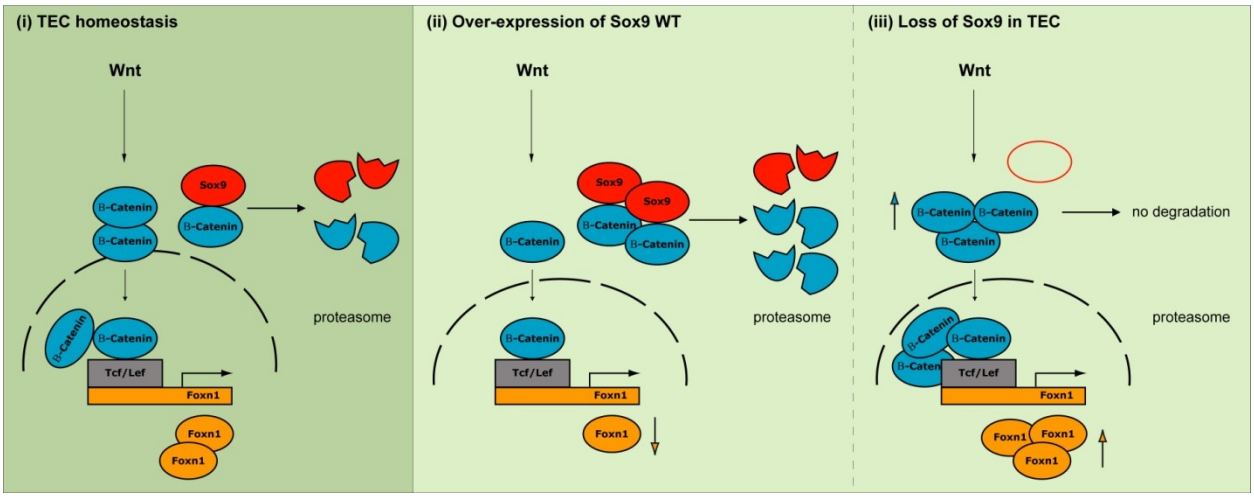


Figure 3.24 Hypothetical model of Sox9-mediated regulation of Foxn1 in thymic epithelia.

(i) The canonical Wnt signalling engaging via β-catenin directly regulates Foxn1 transcription in TEC (Balciunaite *et al.* 2002) and a precise regulation of intracellular β-catenin levels is required for normal thymus development and function (Zuklys *et al.* 2009). Sox9 determines intracellular β-catenin protein levels by promoting β-catenin degradation through formation of Sox9:β catenin complex. (ii) Overexpression of wild type Sox9 leads to β-catenin degradation inhibiting expression of Wnt/β-catenin target gene Foxn1. (iii) Loss of Sox9 expression in TEC results in increased β-catenin amounts and thus upregulation of Foxn1.

4. DISCUSSION

The interest to investigate a role for Sox9 in thymus development was prompted by findings that Sox9 activity may influence cell fate specification, differentiation and proliferation *via* control of the canonical Wnt signalling pathway (Akiyama *et al.* 2004b; Blache *et al.* 2004; Yano *et al.* 2005; Bastide *et al.* 2007; Mori-Akiyama *et al.* 2007; Akiyama *et al.* 2008b; Topol *et al.* 2008; Formeister *et al.* 2009). Though a role for Sox9 had not yet been examined, we and others have already demonstrated that an appropriate Wnt signalling activity is critically important for thymus organogenesis and tissue maintenance (Balciunaite *et al.* 2002; Kuraguchi *et al.* 2006; Osada *et al.* 2006; Pongracz *et al.* 2006; Weerkamp *et al.* 2006; Zuklys *et al.* 2009; Osada *et al.* 2010). As Sox9 has been demonstrated in mesenchymal and epithelial cells to inhibit Wnt/β-catenin signalling by promoting β-catenin degradation in the cytoplasm and by inhibiting β-catenin transcriptional activity in the nucleus (Akiyama *et al.* 2004b; Blache *et al.* 2004; Bastide *et al.* 2007; Topol *et al.* 2008), we first investigated the expression pattern of Sox9 during regular thymus development and subsequently examined the consequences of an absence or overexpression of Sox9 in thymic epithelia.

At the time mouse thymus organogenesis is typically initiated, Sox9 expression could readily be detected in both prospective thymic epithelia of the 3rd pharyngeal pouch and also mesenchyme of the 3rd pharyngeal arch suggesting an early role for Sox9 in the thymus formation and function. However, TEC-directed Sox9 expression was largely dispensable for the formation of a functional microenvironment because the deletion of Sox9 did not impact on the cell's fitness to support regular thymopoiesis. Since the formation of the early thymic primordium involves coordinated actions between cells of ectodermal, endodermal and mesodermal origin (Graham 2001), Sox9 may be required in thymus stromal cells other than TEC and thus may, *via* an involvement in the mesenchymal-epithelial crosstalk, indirectly impact on TEC development. Indeed, a role of Sox9 in epithelial-mesenchymal transformation (EMT) during development of various organs has been reported (Akiyama *et al.* 2004b; Moniot *et al.* 2004; Sakai *et al.* 2006; Scott *et al.* 2010). For example during gut development, Sox9 specifies the pyloric sphincter epithelium through mesenchymal-epithelial crosstalk (Moniot *et al.* 2004). Ectopic expression of Sox9 in the gizzard mesoderm transdifferentiates the adjacent epithelium into pyloric sphincter-like epithelium and contrary, the loss of Sox9 function in the mesoderm of the pyloric sphincter affects the differentiation of pyloric sphincter epithelium. In heart development, Sox9 expression is activated in endocardial endothelial cells and is required for EMT and subsequent proliferation and differentiation of endocardial mesenchymal cells that form endocardial cushions, primordia of valves and septa (Akiyama *et al.* 2004a). Sox9 is also required for NCC formation, differentiation and maintenance (Sakai *et al.* 2006; Scott *et al.* 2010). It is thus conceivable that mesenchymal Sox9 expression in the 3rd pharyngeal arch and adult

$MTS15^{+}Ly51^{+}$ stromal subset impacts indirectly on TEC development and maintenance though, the molecular signature of such a mesenchymal-epithelial crosstalk would still need to be defined. Alternatively, the lack of a pronounced phenotype may be explained by a relatively late deletion of Sox9 should the activity of this transcription factor be required only transiently during early thymus organogenesis. In [$Sox9^{f/f}$::Foxn1-Cre] mice, the Cre recombinase is active in TEC from E12.5 (Zuklys *et al.* 2009), resulting in the recombination of the Sox9 locus both after the commitment of endodermal cells to a TEC fate (as marked at E10.5 by Foxn1 expression) and after the formation of a separate thymus anlage by E12.5 (Gill *et al.* 2003). Thus, both the relatively late and TEC-restricted deletion of Sox9 may account for the absence of a gross phenotype during early thymus organogenesis.

Sox9 was differentially expressed at E10.5 in the epithelia of 3rd pharyngeal pouch. Low Sox9 levels were typically observed in the ventral aspect of the pouch correlating with the Foxn1 expression normally detected in this region and required for subsequent TEC differentiation. Contrary, high Sox9 expression levels were detected in the dorsal aspect which lacks Foxn1 and develops into the parathyroid gland. This reciprocal expression pattern may suggest a possible interdependence between these two transcription factors.

Sox9 expression persisted throughout the fetal TEC development and in the adult thymus distinct Sox9 expression levels were detected among TEC subsets with cortical ($Ly51^{+}UEA\text{-}1^{-}$) and medullary ($Ly51^{-}UEA\text{-}1^{+}$) immature $mTEC^{lo}$ ($MHCII^{lo}$) or mature $mTEC^{hi}$ ($MHCII^{hi}$) phenotype. The importance of discrete Sox9 levels in regulation of epithelial cell proliferation and differentiation has previously been demonstrated in the intestinum (Formeister *et al.* 2009). The low levels of Sox9 support proliferative capacity in the stem/progenitor cell compartment whereas high levels of Sox9 suppress proliferation of cycling precursors and allow terminal differentiation of enteroendocrine cells. These data suggested that Sox9 may also in thymic epithelia act in a dose-dependent fashion. Indeed, substantial numerical and compositional defects were detected in Sox9-deficient thymic microenvironment and the $mTEC^{lo}$ subset was most severely affected. Interestingly, this latter subset contained the highest steady-state Sox9 expression levels which correlated with its enhanced proliferation upon the loss of Sox9. This correlation indicated that high Sox9 levels present in $mTEC^{lo}$ normally suppress cell proliferation, a finding similar to what has been reported for the intestinal epithelium (Formeister *et al.* 2009). The repressive effect of high Sox9 expression levels on TEC proliferation was confirmed by *in vitro* studies where the enforced expression of wild type Sox9 in an established TEC line reduced the cell`s proliferation. Together, these results revealed that Sox9 dosage directly impacts on the cell proliferation and thus may influence cell differentiation.

The molecular mechanism responsible for the Sox9-mediated control of cell proliferation has been established by overexpressing Sox9 in several cell types (Panda *et al.* 2001; Afonja *et al.* 2002;

Akiyama *et al.* 2004b; Drivdahl *et al.* 2004; Bastide *et al.* 2007). High Sox9 levels decrease the transcription of Wnt/β-catenin target genes associated with cell cycle progression (i.e. c-myc and cyclin-D1) and increase inhibitors of β-catenin-mediated cell proliferation (i.e. ICAT: inhibitor of β-catenin and Tcf and TLE2-4: transducin-like enhancer of splits 2-4). *In vivo* deletion of Sox9 in the intestinal epithelium also correlates with an upregulation of c-myc and cyclin-D1 causing an increase in crypt proliferation (Bastide *et al.* 2007) whereas Sox9 overexpression in chondrocytes downregulates cyclin D1 and inhibits proliferation (Akiyama *et al.* 2004b). In Sox9-deficient TEC, the expression of c-myc and cyclin-D1 was only slightly increased however, the results were not reproducible. Nevertheless, the possible mechanism by which Sox9 controls TEC proliferation may relay on the same principle, but include other yet not identified Sox9 target gene and/or Wnt associated genes. In this regard, high Sox9 expression detected in mTEC^{lo} subset may suppress proliferation either indirectly by inhibiting transcription of genes that positively regulate proliferation or directly by controlling genes encoding inhibitors of cell proliferation. In either case, the lack of Sox9 expression would remove this suppression accounting for the enhanced proliferation of mTEC^{lo}. To identify Sox9 target genes and molecular mechanism by which Sox9 regulates TEC proliferation, a transcriptome analysis of mTEC^{lo} and mTEC^{hi} subsets isolated by cell sorting from Sox9-deficient and -proficient mice have been initiated.

The TEC-targeted loss of Sox9 expression affected the composition of the adult thymic compartment as marked by a specific reduction of immature mTEC^{lo} (MHCII^{lo}UEA-1^{+}) and concomitant increase of mature mTEC^{hi} (MHCII^{hi}UEA-1^{+}) subset indicating that the maturation and maintenance of the postnatal thymic epithelium requires Sox9 expression. Moreover, the numerical and proportional decrease of mTEC^{lo} and the proportional increase of mTEC^{hi} appeared to derive, at least in part, from the enhanced mTEC^{lo} proliferation suggesting that the transition of mTEC from a MHCII low to a MHCII high phenotype is Sox9 dependent. This phenotype further implied that Sox9 influences the dynamic process of mTEC proliferation and thereby affects their differentiation. Currently, two models of mTEC differentiation are proposed both addressing the lineage relationships between mTEC subsets (Derbinski *et al.* 2005; Gillard and Farr 2005). According to the terminal differentiation model (Derbinski *et al.* 2005), increased MHCII expression levels directly correlate with mTEC differentiation dissecting mTEC into immature MHCII^{lo} and mature MHCII^{hi} subsets. Alternatively to this view and based on the higher mitotic activity of MHCII^{hi} compared to the MHCII^{lo} subset (Gray *et al.* 2007a), the developmental model suggests that mTEC^{hi} may rather represent a transit-amplifying population and hence be less mature than and give rise to MHCII^{lo} population (Gillard and Farr 2005). The later is supported by the sequential developmental appearance of first MHCII^{hi} and then MHCII^{lo} mTEC suggesting a precursor-progeny relationship. However, there are still large gaps in our understanding of TEC differentiation and thus the maturation

state, developmental potential and homogeneity of these subsets is undetermined and open to speculation.

Relevant to the terminal differentiation model (Derbinski *et al.* 2005) and given the specific increase in the proliferation of immature mTEC^{lo} upon the loss of Sox9, the decrease in immature mTEC^{lo} may be due to their faster transition to mature mTEC^{hi} whose frequency is consequently increased. However, though the frequency was increased, the absolute cellularity of mTEC^{hi} was reduced. One explanation for the numerical decrease of mTEC^{hi} may be that mTEC^{hi} as terminally differentiated cells have a shorter half life resulting in a higher turnover rate of these cells in the absence of Sox9 expression. Alternatively, mTEC^{lo} as progenitors of mTEC^{hi} may, in addition to the increased proliferation, also die faster due to the lack of Sox9, accounting for the decrease of their numbers and consequently mTEC^{hi} progeny, two possibilities that were not addressed in this study. The fact that Sox9 deficiency affected specifically medullary compartment but had no discernable impact on cTEC biology, indicates different requirement for Sox9 in the development of these two cell lineages. These findings here strongly favor a terminal differentiation model for Sox9 function in medullary thymic epithelium as distinct Sox9 expression levels were associated with both the immature and terminally differentiated cells and affected cell proliferation. Moreover, the changes in mTEC composition and proliferation inversely correlated with endogenous Sox9 expression levels suggesting that Sox9 dosage plays an important role in the regulation of TEC homeostasis. Findings that all mTEC that express high levels of Sox9 are immature and their proliferation increases consequent to the loss of Sox9, combined with the *in vitro* data demonstrating that high Sox9 levels are able to suppress the proliferative capacity of a TEC line support a dose-sensitive model for Sox9 activity in thymic epithelia. In this model, high levels of Sox9 expression act to repress the proliferation of mTEC^{lo} progenitors allowing their maturation to terminally differentiated mTEC^{hi}. The loss of Sox9 expression, releases this repression leading to the enhanced proliferation and accelerated transition of mTEC^{lo} to mTEC^{hi} accounting for the relative increase of the mature mTEC^{hi} observed in Sox9-deficient hypocellular thymus. Thus, Sox9 expression controls TEC proliferation and influence the composition of the epithelial microenvironment in a subset specific and dose-sensitive manner.

Sox9 may, in addition to kinetics of mTEC maturation, also regulate the expansion of mTEC^{lo} compartment. A central role of CD40/CD40L signalling in the induction of mTEC^{lo} expansion and medulla maintenance has been established (Gray *et al.* 2006; Hinterberger *et al.* 2010; Metzger and Anderson 2011). This contention is supported by the finding that CD40L overexpression on thymocytes causes massive expansion of mTEC at the expense of cTEC (Dunn *et al.* 1997). An accelerated transition of mTEC^{lo} to mTEC^{hi} in the absence of Sox9, may reduce the permissive period for CD40 signalling and thus result in an inefficient expansion of mTEC^{lo} compartment. Indeed, the TEC subset distribution in

Sox9-deficient thymus is similar to that of mice lacking CD40L by exhibiting the numerical and proportional decrease in $mTEC^{lo}$ and proportional increase in $mTEC^{hi}$ subset (Gray *et al.* 2006). Alternatively, Sox9 may directly regulate the cell surface expression of CD40 on TEC. Consequently, its loss would result in a reduced CD40 expression and thus, a suboptimal $mTEC^{lo}$ expansion accounting for the contraction of $mTEC^{lo}$ compartment in the absence of Sox9 expression.

The inability of Sox9-deficient $mTEC^{lo}$ to expand efficiently may further account for the reduced size of the $EpCam^{+}$ TEC scaffold observed in mice at 2, 4 and 12 weeks of age. During TEC development, the population of $mTEC^{lo}$ expands significally after the second week of age becoming the predominant subset in the adult thymus and leading to a gradual decrease in the ratio of $mTEC^{hi}$ to $mTEC^{lo}$ with age (Gray *et al.* 2006). In 2 week old Sox9-deficient mice, though TEC cellularity was reduced, the ratio of TEC to thymus cellularity was not changed. This may be explained by the fact that at this age $mTEC^{lo}$ represent a minor population of TEC and thus, any changes in their number may not be sufficient to be reflected in the ratio. In contrast, at the age of 4 and 12 weeks, $mTEC^{lo}$ represent the major TEC subset, and consequently any decrease in their number due to the lack of Sox9 is directly reflected in the TEC to thymus cellularity ratio. It is likely that a smaller mTEC compartment would be less efficient in supporting negative selection of thymocytes, due to the relative rare expression of antigens. However, a regular thymic selection and post-selectional maturation of developing thymocytes was observed in the thymus with Sox9-deficient TEC indicating that Sox9 expression is not required for TEC capacity to support thymopoiesis. Furthermore, the fact that the epithelial scaffold, despite the size reduction, was able to support all stages in T cell development argues for its high efficiency in the absence of Sox9.

The high turnover rate of postnatal TEC with a replacement time of 10 to 14 days (Gray *et al.* 2006) indicates that the composition and organization of the thymic epithelial compartment changes dynamically and includes interplay between progenitor cells, transit-amplifying cells and loss of cells from the terminally differentiated compartment. The early and sustained Sox9 expression in TEC precursors and their committed progeny may suggest a possible role of Sox9 for both the generation of new TEC from a stem/progenitor pool or rather mTEC-committed progenitors, and for the maintenance of medullary TEC. Both of these events, if defective, cause a failure in TEC replacement after turnover and may account for the numerical and compositional changes of medullary compartment observed in the thymus lacking Sox9 expression. Supporting the possible role of Sox9 in the regulation of TEC progenitors biology are findings that Sox9 marks progenitors in a variety of tissues, and influences cell fate assignment, proliferation and differentiation. For example, Sox9 specifies an initially bipotent gonad to testis fate and triggers the differentiation of Sertolli cells (Chaboissier *et al.* 2004; Kim and Capel 2006; Jakob and Lovell-Badge 2011). During pancreogenesis, Sox9 maintains early pancreatic progenitors and

governs their commitment to the endocrine cell fate (Seymour *et al.* 2007; Seymour *et al.* 2008). Sox9 is also required for the formation of hair stem cells and their migration as well as for the formation of multipotent NCC, their differentiation and maintenance (Sakai *et al.* 2006; Scott *et al.* 2010). In the intestinum, Sox9 regulates proliferation of crypt stem/progenitor cell compartment and affects cell lineage specification (Akiyama *et al.* 2004b; Blache *et al.* 2004; Bastide *et al.* 2007; Formeister *et al.* 2009). However, defining the role of Sox9 in TEC progenitors is rather a difficult task as relatively little is known about developmental pathway(s) and lineage relationships of TEC stem/progenitor populations and presently, they are neither clearly defined nor technically assessable for isolation. Altered mTEC proliferation and composition observed in Sox9-deficient thymus further argue for a role of Sox9 in the maintenance of the medullary compartment, in particular the immature $mTEC^{lo}$ subset. Finding that this subset expresses the highest Sox9 levels and is most sensitive to the loss of Sox9 implays that it may represent a cellular target for Sox9 function to maintain the postnatal thymus microenvironment.

Though, a lack of Sox9 expression in TEC led to the development of a hypocellular thymus, regular cortico-medullary organization was observed and the major cortical (CK8), medullary (MTS10, UEA-1, CK5, Aire) and mesenchymal (ERTR7) populations were present and properly located. However, the frequency of $CK5^+$ TEC localized outside of the medulla was significantly increased. This increase of $CK5^+$ TEC was not due to the change in the proliferation as the TEC subsets defined by distinct CK5 and CK8 expression levels displayed normal proliferation activity in the absence of Sox9. They also do not represent a population of $CK5^+CK8^+$ TEC progenitors or $CK8^-CK5^+$ mTEC (Klug *et al.* 1998; Klug *et al.* 2000; Klug *et al.* 2002) that expanded in the cortex as detailed phenotypic analysis showed that they co-express several markers typically associated with differentiated cTEC, such as CK8, β5t, DEC205 and Ly51, but not mTEC-specific markers Aire, MTS10 and UEA-1. The altered thymic CK5 expression pattern rather suggests that cortical epithelia in the absence of Sox9 acquired CK5 expression. An *in silco* analysis of CK5 promoter region situated 10kb downstream of the CK5 transcriptional start site revealed 11 putative Sox9 binding sites suggesting that Sox9 may indeed directly regulate CK5 transcription in thymic epithelia. This contention was further supported by the upregulation of CK5-specific transcripts in Sox9-deficent TEC which also correlated with an increased CK5 protein expression as demonstrated by quantitative flow cytometric and image analysis. Thus, Sox9 deficiency results in a hypocellular thymus marked by the altered representation of immature and mature mTEC and the abundance of $CK5^+$ cells in the cortex.

For normal TEC differentiation, maintenance and function, a precise regulation of Wnt activity is essential (Kuraguchi *et al.* 2006; Osada *et al.* 2006; Pongracz *et al.* 2006; Weerkamp *et al.* 2006; Zuklys *et al.* 2009; Osada *et al.* 2010). A functional and physical interaction between Sox9 and the Wnt/β-catenin pathway has been demonstrated in the development and maintenance of various tissues but has yet not

been demonstrated in thymus. The canonical Wnt signalling engaging *via* β-catenin directly regulates Foxn1 expression in TEC as blocking of Wnt signalling at the cell surface or at distinct intracellular sites within the canonical pathway inhibits Foxn1 (Balciunaite *et al.* 2002). However, a precise mechanism by which Wnt signals control the transcription of Foxn1 *in vivo* is not yet fully established. Following the deletion of Sox9 in TEC, Foxn1 was increased in both cortex and medulla identifying Sox9 as a negative regulator of Foxn1 in TEC. This increase of Foxn1 expression may be due to a preferential expansion of a TEC subset with the high Foxn1 expression (i.e cTEC and/or mTEC^{hi}) or alternatively, due a single cell upregulation of Foxn1 in the absence of Sox9. Which of these contentions is true depends greatly on the sensitivity of Foxn1 antibody. If for example low Foxn1 levels are not detected, the Foxn1 increase could be easily misinterpreted as there are new and/or more cells expressing (high) Foxn1 (i.e increased number of Foxn1^{+} cells) although, it may as well be, that an individual cell upregulated Foxn1 from non-detectable to detectable (and high) Foxn1 levels. Given the general contraction of mTEC compartment in Sox9-deficient thymus combined with the Sox9 ability to modulate Wnt/β-catenin transcriptional activity suggest that enhanced Foxn1 expression is rather caused by a single cell upregulation of Foxn1 and affects all three major TEC subsets (i.e. cTEC, mTEC^{lo} and mTEC^{hi}) deficient for Sox9. Further intriguing observation from this study is that the strongest increase of Foxn1 expression was observed in the mTEC^{lo} subset which normally expresses the highest Sox9 and the lowest Foxn1 levels and displays an increased proliferation upon the loss of Sox9. Though, a direct correlation between Foxn1 and proliferation has yet not been clearly demonstrated in the postnatal thymus, the reintroduction of Foxn1 transgene into the nude thymus (Cunliffe *et al.* 2002; Bleul *et al.* 2006). and administration of exogenous Foxn1 cDNA in aged wild type mice (Sun *et al.* 2010) restore thymic size It is tempting to speculate that the increased Foxn1 expression in immature mTEC^{lo} contributed, at least in part, to their enhanced proliferation in the absence of Sox9. In turn, the possibility that the alternations of mTEC composition and proliferation observed in Sox9-deficient microenvironment also contributed to the changes in Foxn1 expression cannot be formally excluded.

The underlying molecular mechanism by which Sox9 modulates Foxn1 expression in thymic epithelia was explored in TEC lines stably transduced to express either wild type or mutant Sox9 molecule that lacks the C-terminal TA domain but contains N-terminal DNA binding domain. Sox9 has been demonstrated in mesenchymal and epithelial cells to inhibit Wnt/β-catenin signalling by promoting β-catenin degradation and/or by inhibiting β-catenin transcriptional activity in the nucleus (Akiyama *et al.* 2004b; Blache *et al.* 2004; Bastide *et al.* 2007; Topol *et al.* 2008). In TEC, the amount of β-catenin protein needs to be precisely regulated for regular thymus development and function (Zuklys *et al.* 2009). Studies aimed to determine which domains of Sox9 protein are required to inhibit Wnt/β-catenin activity have come to various conclusions, and it seems that Sox9 activates different mechanisms in distinct cell types

and several of these may also operate in parallel. The study by Akyama *et al.* demonstrated that the C-terminal TA domain of Sox9 is required for binding and subsequent β-catenin degradation. This interaction determines intracellular β-catenin protein levels and affects the transcription of Wnt/β-catenin target genes (Akiyama *et al.* 2004b). Contrary, Topol *et al.* showed that Sox9 promotes β-catenin degradation and inhibits β-catenin dependent transcription by two parallel and independent mechanisms that employ different domains of Sox9. In their study, the N-terminus of Sox9 binds and degrades β-catenin whereas the C-terminus is required to repress β-catenin mediated transcription (Topol *et al.* 2008). Results here demonstrate that in thymic epithelia Sox9 physically associate with β-catenin using both N- and C-terminal domains. However, the interaction mediated *via* N-terminus of Sox9 mutant molecule did not reduce β-catenin amount. Instead, the binding *via* the C-terminal TA domain was required for Sox9 to induce a decrease of β-catenin protein. These results agree with Akiyama *et al.* by showing that the C- and not N-terminal domain of Sox9 regulates the cellular β-catenin protein levels and its nuclear availability, probably by promoting β-catenin degradation. Whether in TEC, Sox9 uses the C-terminal domain also to repress directly β-catenin mediated transcription, as suggested by Topol *et al.*, cannot be excluded.

The Sox9-mediated degradation of β-catenin can take place in both the nucleus and/or cytoplasm (Akiyama *et al.* 2004b; Topol *et al.* 2008). While Akyiama *et al.* demonstrated efficient β-catenin degradation in the cytoplasm by the ubiqitination/26S proteasome pathway (Akiyama *et al.* 2004b), Topol *et al.* reported a novel degradation mechanism in the nucleus (Topol *et al.* 2008). Sox9 was shown to bind, in addition to β-catenin, components of the destruction complex, the GSK3β and the ubiquitin ligase βTrCP and translocates them to the nucleus where more efficient phosphorylation and degradation of β-catenin occurs. The latter mechanism could also be activated by Sox9 in thymic epithelia, given that the strongest reduction of β-catenin protein was detected in nucleus when compared to the cytoplasm of TEC stably overexpressing wild type form of Sox9, though both mechanisms may also act in parallel and at different efficiency. These *in vitro* findings also predict increased β-catenin protein levels in primary TEC after Sox9 removal as there is no Sox9 to interact with and degrade β-catenin. Assessment of β-catenin protein levels in total cell fraction of primary TEC lacking Sox9 expression by flow cytometry revealed only slight increase of β-catenin protein. This may be explained by the fact that most of β-catenin in the cell is associated with cytoskeleton and thus generates the high background hindering the detection of free, non bound β-catenin and changes in its amount. Therefore, and given the most pronounced reduction of β-catenin protein in the nuclear fraction of wild type Sox9-transduced TEC, β-catenin levels should be quantified separately in nucleus and cytoplasm of primary TEC. This however requires sorting of TEC and subsequent separation of cellular subfractions which is at the moment technically not feasible.

The finding that Sox9 to regulates β-catenin protein levels in TEC represents one possible mechanism by which Sox9 may control transcription of Wnt/β-catenin target gene, including Foxn1 in thymic epithelia. Indeed, the reduction of β-catenin protein detected in a TEC line overexpressing wild type Sox9 associated with a significant downregulation of Foxn1 expression whereas, the stable expression of a TA-deficient mutant Sox9 did neither alter β-catenin nor Foxn1 expression. These *in vitro* studies demonstrated that Sox9 regulates Foxn1 expression indirectly through the physical and functional interaction with β-catenin mediated *via* the C-terminal TA domain of Sox9. In keeping with Topol *et al.* it is also conceivable that the C-terminus of Sox9 directly inhibits Foxn1 transcription. The N-terminus of Sox9 however, does not take part in direct regulation as the TA-deficient Sox9 mutant did not modulate Foxn1 expression levels. The capacity of Sox9 to bind and control β-catenin levels in thymic epithelia may constitute a mechanism to fine tune the activity of Wnt signalling and thus the expression of Wnt target genes during thymus development and maintenance. Further, this interaction may also serve to control the extent of Wnt activity and prevent overactivity of Wnt/β-catenin dependent transcriptional program during normal TEC homeostasis.

Collectively, the present study demonstrated that the differential expression of Sox9 is important in the regulation of TEC proliferation and differentiation. Moreover, Sox9 *via* its association with β-catenin modulates Wnt signalling activity and acts as a negative regulator of Foxn1 expression in thymic epithelia. We propose that Sox9 plays a role in the establishment and maintenance of the normal adult thymic microenvironment, at least in part, by regulating β-catenin protein and thus level of Wnt activity through the physical and functional interaction with β-catenin (Figure 3.24). Sox9 may also influence TEC development and function independently of the association with β-catenin and identification of downstream targets of Sox9 during thymus development and maintenance is next important step.

5. CONCLUSIONS

The work described in this thesis leads to the following findings:

1.) Sox9 is expressed as early as E10.5 in the prospective thymic epithelial cells and also in mesenchymal but not in lymphoid cells, and persists into adulthood.

2.) Loss of Sox9 expression in TEC leads to the specific changes in TEC composition and proliferation which inversely correlate with the differential Sox9 expression levels normally present among TEC subsets. The immature MHCII^{lo} UEA-1^{+} mTEC express high Sox9 levels and are most sensitive to the loss of Sox9 and thus, may represent a cellular target for Sox9 function to maintain the postnatal thymus microenvironment.

3.) The high Sox9 expression in immature mTEC^{lo} cells correlates with their enhanced proliferation upon loss of Sox9 and indicates that high steady-state Sox9 levels act to suppress TEC proliferation. This conclusion is confirmed by the overexpression of Sox9 in an established TEC line which reduces the cell`s proliferation.

4.) In Sox9-deficient thymus, the contraction of mTEC^{lo} and the relative expansion of mTEC^{hi} compartment appear to derive, at least in part, from enhanced mTEC^{lo} proliferation suggesting that Sox9 expression controls TEC proliferation and thereby influences the composition of the thymic microenvironment.

5.) Despite the alterations in mTEC composition, thymopoiesis remains unaffected in the thymus with Sox9-deficient TEC indicating that Sox9 expression is not required for TEC capacity to support T cell development.

6.) The TEC-targeted loss of Sox9 expression correlates with a significant upregulation of Foxn1 in all TEC subsets and identifies Sox9 as a negative regulator of Foxn1 expression in thymic epithelia.

7.) *In vitro* studies established that Sox9 controls Foxn1 transcription indirectly through the formation of a physical complex with β-Catenin. Binding mediated *via* the C-terminal TA domain of Sox9 induces a decrease in β-catenin protein levels and consequently inhibits Wnt/β-catenin transcriptional activity on Foxn1. Hence, Sox9 negatively regulates Foxn1 by controlling amount of β-catenin.

Collectively, this study provides evidence that transcription factor Sox9 acts in dose-sensitive and subset-specific manner in the regulation of TEC proliferation and differentiation and negatively regulates Foxn1 expression in TEC. Thus, differential Sox9 expression is critical for the establishment and maintenance of a regular thymic microenvironment.

6. FUTURE PERSPECTIVES

Several issues still need to be addressed to understand the detailed function of Sox9 in thymus development.

1.) To achieve Cre-mediated recombination and Sox9 deletion both at an earlier time point during thymic organogenesis and in mesenchymal cells, $Sox9^{flox/flox}$ mice should be next crossed to Hoxa3-Cre line in which Cre expression occurs before E10.25 (Macatee *et al.* 2003) and is active not only in TEC but also in cells of mesenchymal and ectodermal origin. This is purposeful given that Sox9 expression was detected already at E10.5 in both prospective thymic epithelia as well as in the mesenchyme, and Foxn1-Cre used here acts only from E12.5 and solely in TEC.

2.) To further address the fate of immature $mTEC^{lo}$ and mature $mTEC^{hi}$ cells lacking Sox9 expression, the survival and turnover of these cells should be assessed. This may help to clarify the decrease of their absolute numbers in Sox9-deficient thymus.

3.) To explore the molecular mechanism and identify Sox9 target genes involved in the regulation of TEC proliferation and differentiation a transcriptome analysis of $mTEC^{lo}$ and $mTEC^{hi}$ subsets isolated by cell sorting from Sox9-deficient and -proficient mice have been initiated. In particular, it would be interesting to know whether CD40 is one of Sox9 downstream genes considering its role in the expansion of $mTEC^{lo}$ compartment and given the specific contraction of $mTEC^{lo}$ observed in Sox9-deficient thymus.

4.) Since Sox9 can act as a transcriptional activator or repressor, the identification of thymus-specific cofactors that interact with Sox9 and regulate its function in spatio-temporal fashion would contribute to our knowledge of how Sox9 transcriptional activity and thus developmental processes in which Sox9 participates are controlled.

5.) Also an important step in understanding TEC development is the identification of signalling pathways and mediators involved in the epithelial-mesenchymal crosstalk during thymus organogenesis and maintenance. Given Sox9 detection in the mesenchyme of the 3rd pharyngeal arch at the onset of thymus organogenesis and in the adult $MTS15^{+}$ $Ly51^{+}$ mesenchymal cells, defining the role of Sox9 in epithelial-mesenchymal communication represents an avenue worth further investigation.

Together, the ablation of Sox9 expression in both thymic epithelia and mesenchyme combined with gene expression profiling should help to dissect complex genetic network controlling TEC differentiation and normal thymus homeostasis.

7. MATERIAL AND METHODS

7.1. Materials

7.1.1. Mice

Conditional Sox9$^{flox/flox}$ mice, (Sox9$^{f/f}$) with loxP-sequences flanking Sox9 exons 2 and 3 (Kist *et al.* 2002) were a kind gift of Andreas Kispert and Gerd Scherer (Institute of Human Genetics, University of Freiburg, Germany). They were originally on a 129 x C57BL/6 x NMRI mixed genetic background. Foxn1-Cre mice were produced as P1 artificial chromosome (PAC) transgenic animals (Zuklys *et al.* 2009). Briefly, the CDS for iCre (i.e for eukaryotes codon usage optimized version of the bacteriophage p1 cre recombinase) was inserted into Foxn1 PAC clone RPCI21-436p24 and injected into pronuclei of fertilized B6D2F1 oocytes. Two independent transgenic lines were generated each displaying identical pattern of Cre activity. The line B6;D2-Tg (Foxn1-Cre)8Ghr, designated Foxn1-Cre was used for the present study and was maintained as a heterozygous colony. Foxn1-Cre mice were bred to [Sox9$^{f/f}$] mice to generate mice deficient for Sox9 expression specifically in TEC [Sox9$^{f/f}$::Foxn1-Cre] as described in section 3.2 and depicted in Figure 3.7. C57BL/6 mice were purchased from RCC Ltd, Füllingsdorf. For developmental staging, the day of the vaginal plug appearance was designated as embryonic day (E) 0.5. Mice were housed at the Center for Biomedicine`s animal facility in accordance with Institutional and Cantonal Review Board.

7.1.2. Standard buffers

Cell culture

Cell culture media

500ml Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen)

50ml Fetal Bovine Serum (FBS) (10%) (Perbio)

0.5ml 50mM 2-Mercaptoethanol (50µM) (Invitrogen)

Antibiotics: used only for PlatE cultures

Penicillin-Streptomycin solution (10 000U penicillin and 10mg streptomycin/ml; Sigma): used at final concentration of 100U/ml Penicillin and 100µg/ml Streptomycin

Puromycin (1mg/ml): used at final concentration of 1µg/ml

Blasticidin (10mg/ml): used at final concentration of 10µg/ml

Puromycin and/or Blasticidin were added from time to time for three to four days to re-select PlatE cells containing retroviral packaging construct.

Freeze medium

90% FBS and 10% Dimethylsulfoxid (DMSO)

Flow cytometry

FACS buffer: for staining of thymic stromal cells

Hank's Balanced Salt Solution (HBSS) containing 2% or 5% FBS (Perbio)

FACS buffer: for staining of lymphocytes

Phosphate buffered saline (PBS) pH7.4 containing 2% FBS (Perbio)

0.5M Ethylenediamine-tetraacetic acid (EDTA) (Ambion): for stromal cell preparation, used at final concentration of 2.5mM or 10mM

Collagenase/Dispase (Roche): stock (10mg/ml) used at final concentration of 1mg/ml

Dnase (Roche): stock (10mg/ml) used at final concentration of 50µg/ml

DAPI: stock (0.5mg/ml) used 1: 5000

Fixation/Permaebilization:

Fixation/Permaebilization kit (eBioscience): Diluent and Concentrate mixed at ratio 3:1

Immunohistochemistry

1% cresyl violet acetate: 0.5g per 50ml 100% EtOH

0.1% Dethylpyrocarbonate (DEPC): 1ml per 1L milliQ H_2O, autoclaved

0.5% Toluidine Blue: 0.25g per 50ml milliQ H_2O

Fixation:

4% PFA/PBS: 4g per 100ml PBS; dissolved at 55°C for 1 hour

4%PFA/4% sucrose/PBS: 4g sucrose per 100ml 4%PFA/PBS

Aceton

Permeabilization:

0.25% Triton X-100/PBS

Blocking:

5% normal goat serum (NGS)/PBS

10% bovine serum albumin (BSA)/PBS

Western blotting

Separating gel (80%, 10ml, separation range between 36-94kDa):

4.6ml milliQ H_2O

2.7ml 30% Acyrlamide/Bis Solution (Biorad)

2.5ml 1.5M Tris-HCl pH8.8 (Sigma)

100µl 10% Sodium Dodecyl Sulfate (SDS) (Eurobio)

100µl 10% Ammonium Persulfate (APS) (Sigma)

6µl Tetramethylethylendiamin (TEMED) (Eurobio)

Stacking gel (5%, 4ml):

2.7ml milliQ H_2O

0.67ml 30% Acrylamide/Bis Solution (Biorad)

0.5ml 1M Tris HCl pH6.8 (Sigma)

40µl 10% SDS (Eurobio)

40µl 10% APS (Sigma)

4µl TEMED (Eurobio)

RIPA buffer

5ml 1M Tris-HCl pH8 (50mM) (Sigma)

3ml 5M NaCl (150mM)

10ml 10% NP-40 (1%)

5ml 10% Deoxycholic Acid (0.5%)

Fill up to 100ml with milliQ H_2O

Complete Protease Inhibitor Cocktail Tablets (Roche): 1 tablet in 2ml H_2O; 40µl per 1ml Ripa buffer

SDS Loading buffer (2x):

1ml 1M Tris-HCl pH6.8 (100mM)

2ml 20% SDS (4%) (Eurobio)

0.02g Bromophenol blue (0.2%) (Sigma)

2ml Glycerol (20%) (Sigma)

142.85µl β-Mercaptoethanol (200nM) (Sigma)

Fill up to 10ml with miliQ H_2O

SDS Running buffer (5x)

15.1g Tris base (125mM) (Sigma)

94g Glycine (1.25M) (Biorad)

25ml 20% SDS (0.5%)

Fill up to 1L with milliQ H_2O

Transfer buffer (10x)

30.3g Tris Base (Sigma)

144.1g Glycine (Biorad)

Fill up to 1L with milliQ H_2O

Transfer buffer (1x)

100ml Transfer buffer (10x)

200ml Methanol (Fluka)

Fill up to 1L with milliQ H_2O

Washing:

TPBS: 1%Tween 20 (1ml) (Sigma) in PBS (1L)

Blocking:

Blotto: 5% skim milk (2.5g) in TPBS (50ml)

Antibody dilutions: 2.5% skim milk (1.25g) in TPBS (50ml)

Stock solution

PBS (10x)

80g NaCl

2g KCl

14.4g Na_2HPO_4

2.4g KH_2PO_4

Fill up to 1L with milliQ H_2O

1.5M Tris-HCl pH8.8

90.75g Tris-HCl per 500ml milliQ H_2O, adjust pH with HCl

1M Tris-HCl pH6.8

60.5g per 500ml milliQ H_2O, adjust pH with HCl

1M MgCl2

20.3g $MgCl_2$•6 H_2O per 100ml milliQ H_2O

5M NaCl

29.3g NaCl per 100ml milliQ H_2O

Tail lysis buffer

10ml 1M Tris pH 8.5 (100mM)

1ml 0.5M Na-EDTA (5mM)

1ml 20% SDS (0.2%)

4ml 5M NaCl (200mM)

Fill up to 1L with H_2O

Proteinase K added freshly at final concentration of 100µg/ml

Molecular cloning

Luria-Bertani medium (LB)

10g Bactotryptone (Difco)

5g Bacto-yeast extract (Difco)

10g NaCl

Fill up to 1L with deionized water, autoclave

SOB Medium

20g Tryptone

5g Yeast extract

0.5g NaCl

Fill up to 950ml with deionized water and shake it until dissolved

Add 10ml 250mM KCl and adjust pH to 7.0 with 5N NaOH (~ 0.2ml)

Fill up to 1L with deionized water, autoclave

Add 5ml of sterile 2M MgCl2 just before use

250mM KCl

1.86g in 100ml deionized water

2M $MgCl_2$:

19g in 100mL deionized water

CCMB80 buffer: for preparation of competent cells

10mM KOAc pH7.0 (10ml of a 1M stock/l)

80mM CaCl2xH20 (11.8g/l)

20mM MnCl2x4H20 (4g/l)

10mM MgCl2x6H20 (2g/l)

10% Glycerol (100ml/l)

Adjust pH down to 6.4 with 0.1N if necessary

Filter sterile and store at 4°C

7.1.3. Primers

All primer sequences are indicated in 5`→3` direction

Number	Gene	Forward primer	Reverse primer
Genotyping			
3514	wt/flox Sox9_F2	CCGGCTGCTGGGAAAGTATATG	
3516	del Sox9_F1	CTCCGGTAGCAAAGGCGTTTAG	
3515	Sox9_R		CGCTGGTATTCAGGGAGGTACA
1621/1626	Foxn1-Cre	CTCTCCTCCGAGTATCCAATCTG	CCCTCACATCCTCAG GTTCAG

qRT-PCR analysis			
3459/3460	Sox9	ACGGAACAGACTCACATCTC	CCTCTCGCTTCAGATCAACT
465/466	Foxn1	TCTACAATTTCATGACGGAGCACT	TCCACCTTCTCAAAGCACTTGTT
3971/3972	CK5	TCAACAAGCGTACCACGGC	GGCATCGACCCTGGCC
1268/1269	CK8	GCCACTGAAGTCCTTGCCAG-	GGTTGGCCAGAGGATTAGGG
490/491	Aire	TGTGCCACGACGGAGGTGAG	GGTTCTGTTGGACTCTGCCCTG
2156/2157	CCL21	AGCTATGTGCAAACCCTGAGGA	TTCCAGACTTAGAGGTTCCCCG
3476/3477	β5t		
3157/3158	CD45	AATTTCAGAGCATTCCACGG	GAAGTCATCAACTGTCTC ATCC
605/606	GAPDH	GGTGAAGGTCGGTGTGAACG	ACCATGTAGTTGAGGTCAATGAAGG
1108/1109	EVA	GTAAACTGTGCCGCCTGCTC	TCCCGTTGACTGCCTCCA
4134/4135	EpCam	TGAGGACCTACTGGATCATC	TATCGAGATGTGAACGCCTC
571/572	CyclinD1	GGAGACCATTCCCTTGACTG	GTTGTGCGGTAGCAGGAGAG
670/671	c-myc	CACAGCAAACCTCCGCACAG	TGTGTGTCCGCCTCTTGTCGT
Cloning of Sox9 wild type and mutant			
3914	*BamHI* Sox9WT/M	*cgcGGATCCatgtcggaggactcggctggttcgc	
3916	*NotI* Sox9 WT		*ataagaatGCGGCCGCtcagggtctggtgagctgtgt
3915	*NotI* Sox9 M		ataagaatGCGGCCGCTCAtgggtggccgttgggtggcaa
Sequencing of Sox9 plasmids (5`HA pPRIG)			
440	T7	(G)TAATACGACTCACTATAGGG	
3800	Sox9 WT/M	TCCAGCAAGAACAAGCCACA	
3801	Sox9 WT/M	CATGAGTGAGGTGCACTC	
3802	Sox9 WT	ACGTGTGGATGTCGA AGCA	
3803	Sox9 WT	TACAGCGAGCAGCAGCAGCA	
3918	Sox9 N`HA		TGTGGCTTGTTCTTGCTGGA

*linker RESTRICTION SITE Sox9 mRNA

TGA stop codon introduced in Sox9 M reverse primer

7.1.4. Antibodies

Antibody	**Clone**	**Manufacturer**
TEC analysis		
CD45 PECy7	30-F11	BioLegend
EpCam Cy5	G8.8	Our laboratory
EpCam bio	G8.8	Our laboratory
pan MHC class II (I-A/I-E) Fluos	M5/114.15.2	BD Pharmingen
Ly51 PE	6C3/BP1	BD Pharmingen
Ly51 A647	6C3/BP1	BioLegend
Ly51 FITC	6C3/BP1	eBioscience
UEA-1 Cy5.		Our laboratory
MTS15 Cy5		Our laboratory
BrdU PE	MOPC-21	BD Pharmingen

Ki67 PE	B56	BD Pharmingen
CD16/CD32	2.4G2	BD Pharmingen
Lymphocytes analysis		
CD3 Cy5	KT3	Biolegend
CD4 PE	GK1.5	Biolegend
CD8 PECy7	53-6.7	Biolegend
CD24 FITC	M1/69	Biolegend
CD69 FITC	H1.2F3	eBioscience
B220 FITC	RA3-6B2	eBioscience
ckit APC	2B8	eBioscience
CD44 PE	IM7	eBioscience
CD25 FITC	PC61	eBioscience
CD3 bio	KT3	BD Pharmingen
CD4bio	GK1.5	BD Pharmingen
CD8 bio	53-6.7	BD Pharmingen
TCRβ bio	H57-597	Biolegend
TCRγδ bio	GL3	eBioscience
NK1.1 bio	PK136	eBioscience
CD11b bio	M1/170	eBioscience
CD11c bio	N418, p150/90	eBioscience
CD19 bio	ID3	eBioscience
Immunohistochemistry		
Rabbit anti-CK5 pAb		Covance
Mouse anti-CK8 mAb		Progene
Mouse anti-CK18 bio mAb		Progene
Rat anti-CD25 Cy5		BD Biosciences
Rat anti-ERTR7 mAb		Our laboratory
Rat anti-MTS10 mAb		Our laboratory
Rat anti-CD25 Cy5		BD Biosciences
UEA-1 bio lectin		Vector Laboratories
Rabbit anti-β5t		
Rat anti-Ly51 A647		Biolegend
Rat anti-CD205 bio		Abcam
Rabbit anti-Foxn1 pAb		Kindly provided by T. Amagai
Rabbit anti-Sox9 pAb		Kindly provided by M. Wegner

Mouse anti-HA mAb		Covance
Goat anti- Rabbit A488		Invitrogen
Goat anti-Rabbit A555		Invitrogen
Goat anti-Rat A488		Invitrogen
Goat anti-Rat A555		Invitrogen
Goat anti-Rat A647		Invitrogen
Goat anti-Mouse A555		Invitrogen
Goat anti-Mouse A647		Invitrogen
IP and Western blotting		
Mouse anti-β-catenin mAb		BP Transduction Laboratories
Rabbit anti-Foxn1 pAb		Kindly provided by T. Amagai
Mouse anti-HA mAb		Covance
Rabbit anti-Sox9 pAb		Kindly provided by M. Wegner
Rabbit anti-GAPDH pAb		Abcam
Mouse anti-Histon H1 mAb		Santa Cruz
Mouse anti-Tubulin mAb		Sigma
Goat anti-Mouse Ab, HPR-conjugated		GE Healthcare
Goat anti-Rabbit Ab, HPR-conjugated		Southern Biotechnology

7.2. Methods

7.2.1. Genotyping of [Sox9$^{f/f}$] and [Sox9f/f::Foxn1-Cre] mice

Isolation of genomic DNA from mouse tissue

Small tissue piece (tip of tail, embryonic limb) was digested in 700µl lysis buffer containing Proteinase K overnight at 55°C in a Thermomixer shaker (Eppendorf). Undigested tissue was pelleted for 5 min at maximum speed and the supernatant poured into a new tube containing an equal volume of isopropanol. DNA was precipitated by inverting the tubes, thereafter pelleted by centrifugation at full speed, washed in 70% ethanol and pelleted again. The supernatant was then discarded and the DNA pellet air dried. TE buffer was added (200-400µl) and after incubation at 55°C for 30 min the pellet resuspended. DNA was stored at +4°C.

PCR reaction mix:

100ng genomic DNA (or 10ng cDNA)

0.5µl Forward primer (10µM)

0.5µl Reward primer (10µM)

0.5µl dNTPs (10mM)

2.5µl Buffer 10x (Sigma)

0.2µl Taq Polymerase (Sigma)

µl H_2O up to 25µl

PCR conditions:

94°C 2 min (initial denaturation)

32 cycles: 94°C 30 sec; 56°C 45 sec; 72°C 30 sec (denaturation; annealing; elongation)

72°C 5 min (final elongation)

Same reaction mix and conditions were used for PCR analysis of Sox9 expression in thymic stroma.

7.2.2. RNA isolation, cDNA synthesis and quantitative real time RT-PCR (qRT-PCR) analysis

Total RNA isolation

RNA isolation was carried out in an RNase free environment wearing disposable gloves to avoid contamination of the samples. Total RNA from whole thymic tissue and TEC lines was isolated according to the standard TRI-reagent protocol (Molecular Research Center Inc). Snap-frozen (using liquid nitrogen) tissue was suspended in 1ml of TRI-reagent homogenized with a Polytron homogenizer (Kinematica PT 1200) for 30-60 sec with increasing speed and incubated for 10 min at RT. To extract the aqueous phase, 100µl (1:10) Bromochloropropanol (Molecular Research Center Inc.) were added, the samples vortexed for 10 sec and incubated for another 10 min at RT. After centrifugation at 14`000 rpm for 10 min at 4°C the aqueous phase was transferred to a new Eppendorf tube into an equal volume of isopropanol (Sigma), mixed and incubated for 1 h at RT in order to precipitate the RNA. The samples were then centrifuged at 14000 rpm for 30 min at 4°C, the supernatant discard and the pellet washed with 1ml 75% ethanol (Fluka), vortexed and centrifuged at full speed for 10 min at 4°C. The supernatant was completely removed and the RNA pellet air dried for 5-10 min at RT, thereafter dissolved in 10-30µl of RNAase free water. The concentration of the total RNA was measured by optical density (OD) using a Gene-Quant machine II (Pharmacia) and the following formula: concentration (µg/ml) = 40 x OD_{260} x dilution. The OD_{260}/OD_{280} ratio of the samples was always equal or above 1.8. RNA integrity was analysed by gel electrophoresis on a 1% agarose/ethidium bromide (0.25µg/ml) gel. To extract RNA from sorted TEC and microdissected tissue the RNeasy Micro kit (Qiagen) (max. 500`000 cells) was used following the manufacturer's instructions. Here, the yield and integrity of RNA was routinely assessed by RNA 6000 Pico assay and the Agilent 2100 bioanalyzer. The samples were then stored at -80°C until further processed.

cDNA synthesis

For the generation of cDNA, 3-5µg of total RNA (dissolved in 10µl water) were added to 0.5ml microtubes containing the following master mix solution (8µl per sample):

4µl of 5x First strand buffer (Invitrogen)

2µl DTT 0.1M (Invitrogen)

1µl dNTP (10mM)

1µl RNAse-free DNase I (10U) (Roche).

The reactions were incubated in a thermal cycler for 30 min at 37°C to digest genomic DNA contaminants, cooled on ice for 1 min, and subsequently heated for 15 min at 70°C to inactivate the DNAse I and denature the secondary structure of the RNA. Next, 1µl of the oligo dT (50µM) and 1µl of random hexamers N6 (500ng/µl) were added and the reactions incubated for 5 min at 70°C, thereafter cooled on ice for 1 min. After addition of 1µl of Superscript III reverse transcriptase (200U/µl) (Invitrogen) the samples were incubated for 5 min at 25°C, 60 min at 50°C and finally 15 min at 70°C. As a negative control, reaction where the reverse transcriptase was omitted has been included. Water was added to all samples to achieve the final concentration of 50ng/µl and the samples stored at -20°C until further processed.

qRT-PCR

The primers for qRT-PCR were designed to be exon spanning excluding potential genomic contamination and to give the amplicons relatively short in size (<200bp) ensuring a maximal amplification efficiency. Intron-exon boundaries were viewed with Pearl Primer software. The sequence of the used primers can be found in section 5.1.3. qRT-PCR was performed in triplicates on a Rotor-Gene 3000 (Corbett Research) using SensiMix SYBR kit (Bioline) and the data acquired using Rotor-Gen 6 software (Corbett Research). 1µl of cDNA (1-10ng) was added to 0.1ml strip tubes (LabGene Scientific) containing the following master mix solution (11.5µl per sample):

6.25µl 2x SensiMix

0.25µl 50x Syber Green,

0.375µl Forward primer (10µM)

0.375µl Reverse primer (10µM)

µl H20 up to 12.5µL

The cDNA was amplified using following PCR conditions:

95°C 10 min

40 cycles: 95°C 10 sec; 60°C 15 sec; 72° 20 sec

Expression levels of specific mRNA were normalized to GAPDH expression ($2^{-\Delta Ct}$; ΔCt= Ct (gene of interest) - Ct (housekeeping gene)), averaged and mean values were compared between mutant and wild type cells using student T-test. Data were presented relative to the corresponding control value (i.e [$Sox9^{f/f}$] mice or cells transduced with the empty vector) which was arbitrary set to 1 for comparison. In case of the whole thymic tissue, amounts of specific mRNA were normalized to the expression of TEC-specific gene EpCam. PCR specificity was controlled by analyzing both the melting curves and the size of amplified products on a 1% agarose/ethidium bromide gel. Gel images were acquired on a Molecular Imager Gel Doc XR system using Quantity One 4.6.2 software (Bio-Rad).

7.2.3. Histology

Tissue embedding

Freshly isolated thymic lobes or whole embryos (E10.5-13.5) were embedded in OCT compound (Medite) in Tissue-Tek Cryomolds (Miles). Tissues were frozen by submerging in a mixture of dry ice and methyl-butane isopentyl (Fluka) and then stored at –80°C. Frozen samples were cut at 6-8µm thickness using a cryostat (Roche) and mounted either on normal glass slides for HE staining or on SuperFrost plus slides (Menzel-Gläser) for IHC. Tissues sections were air dried for at least 1 h at RT, then either frozen at -80°C wrapped in aluminum foil or processed directly.

Hematoxylin and eosin (HE) staining

For HE staining, tissue sections were fixed in Delaunay`s fixation solution for 1min, then rehydrated in a series of ethanol dilutions for 1min each (100%, 96%, 70%, 50%) and finally in water for 1min. Tissues were stained with Mayer`s Hämalaun for 2 min, thereafter washed with warm water 3x1 min and stained with 1% Erythrosin. After washing with water for 1 min, sections were dehydrated in increasing ethanol concentrations (50%, 70%, 96%) for 1 min each, then in 100% ethanol for 2 min. Slides were then air dried before mounting the cover slips with Pertex. Imaging was done on a Nikon Eclipse E600 microscope (4x/0.13 NA Plan Fluor objective), captured with a Nikon DXM 1200F camera, and processed with Nikon ACT-1 software (Nikon Instruments Europe).

Immunohistochemistry (IHC)

For immunohistochemical detection, tissue sections (6-8 µm) were circled by a PAN pen and fixed in a Kopplin Jar with cold acetone (-20°C) for 5 min at RT, washed in PBS and incubated with 5% goat serum/PBS for 10 min to block nonspecific binding prior incubation with primary antibodies. For nuclear stainings, the dissected tissues were fixed in 4% PFA/PBS overnight at 4°C, washed in PBS and sucrose gradient (5%, 10%, 20% sucrose/PBS) was run prior to cryopreservation in OCT compound containing

20% sucrose. For the detection of Sox9, tissue sections were fixed again (i.e. post cryopreservation) with acetone as described above whereas to detect Foxn1 protein antigen retrieval by heating was performed. Briefly, slides with dried sections were placed into a Kopplin jar containing 10mM Na citrate, pH6 and boiled twice in the microwave, allowing to cool down for 20 min in between. Subsequently, blocking was performed as describe above. Following primary antibodies diluted at the appropriated final concentration with 5% NGS/PBS were used: rabbit anti-cytokeratin (CK)5 polyclonal antibody (pAb), mouse anti-CK8 monoclonal antibody (mAb), rabbit anti-Foxn1 pAb (Itoi *et al.* 2007a), rat anti-MTS10 mAb (Godfrey *et al.* 1988), rabbit anti-Sox9 pAb (Stolt *et al.* 2003), rat anti-ERTR7 mAb, rat anti-CD25 as well as biotinylated Ulex europaeus agglutinin-1 (UEA-1) lectin. After 2 h incubation with primary antibodies, slides were washed 2x10 min and sections incubated with appropriate Alexa-Fluor Goat IgG conjugated secondary antibody for 30 min at RT. As a negative control, primary antibody was either replaced with corresponding isotype control or omitted on purpose. Care was taken to remove the liquid remaining after the washing and prior adding of the antibody solution and slides were always incubated in a closed humid chamber box with a paper soaked with water to avoid drying of tissue sections. Sections were mounted with fluorescent mounting medium (Hydromount, National Diagnostics) and images acquired on a Zeiss LSM 510 confocal microscope (10x/0.5 NA, 20x/0.5 NA, and 40x/0.5 NA objectives) using Zeiss LSM 510 acquisition software, version 3.2 (Carl Zeiss). Quantitative image analysis of protein expression was performed using the NIH Image-J analysis software. The relative area of specific protein expression was determined as a percentage of stained pixels in the defined area of the thymus. The mean relative area was calculated using 3-4 sections from a minimum of 3 mutant and wild type mice analysed. Statistical analyses were performed using student t-test.

IHC on coverslips

For the detection of exogenous Sox9 expression in stably transduced TEC lines, IHC was performed on coverslip according to the Sigma protocol for the detection of monoclonal ANTI-FLAG M2 antibody. Briefly, cTEC1.2 transduced either with HA-tagged wild type and mutant Sox9 or with empty retroviral vector (control) were seeded ($5x10^4$ cells/1ml medium) and grown on coverslips in a 12 well plate. Cells were fixed by incubation with 4% PFA/4% sucrose/PBS for 15 min at RT. Fixed cells were then washed 2x5 min with PBS, permeabilized by incubation with 0.25% TritonX-100/PBS for 5 min, washed again and blocked with 10% BSA/PBS for 30 min at 37°C. Next, mouse anti-HA mAb or rabbit anti-Sox9 pAb (Stolt *et al.* 2003) diluted in 3% BSA/PBS were added for 2 h at 37°C. After washing, cells were incubated with Goat anti-Mouse A647 and Goat anti-Rabbit A555, respectively and DAPI for 45 min at 37°C. Cells were washed and coverslips mounted on glass slides with the cell side down using fluorescent mounting

medium (Hydromount; National Diagnostics). Samples were examined by confocal microscopy as described above.

7.2.4. Laser capture microscopy (LCM)

Tissue sections (10µm) of C57BL/6 embryos at day E10.5 of gestation were mounted on glass slides covered with a 1.35µm thin polyethylene foil (PALM Microlaser Technologies) which were pre-treated first with RNaseZap (Ambion) and then UV-irradiated in a cell culture hood for 30-60 min to improve the adherence of frozen sections. The sections were air dried for 2 min, fixed in 70% ethanol (Fluka) (1 min), stained with 1% Cresyl violet acetate (1 min), dehydrated (1 min 75% EtOH and 1 min 100% EtOH), air dried and stored at -80°C until used for LCM. DEPC-treated water was used to dilute the ethanol and working space was cleaned with RNaseZap to avoid contamination with RNases.

The PALM Microlaser dissecting microscope (Carl Zeiss) was used to excise the specific cells from the ventral and dorsal aspect of the third pharyngeal pouch. All microdissected tissues were catapulted into the PALM Adhesive Caps (PALM Microlaser Technologies) by slightly increasing the energy (Δ5-10U) of the laser. At the end of each LCM session which lasted at maximum one hour, 350µL of cell lysis buffer RLT (Qiagen) was added to the laser captured cell, the cap was closed and tissue homogenized by vortexing for 20 sec. Total RNA was then extracted using the RNAeasy Micro Kit (Qiagen) and the manufacturer`s recommendation. The yield and integrity of isolated RNA was routinely assessed by RNA 6000 Pico assay and Agilent 2100 bioanalyzer. Subsequently, cDNA was synthesized and analysed for Sox9 expression.

7.2.5. Cell isolation and flow cytometry

To obtain thymic stromal cells, thymi were cleaned of fat and connective tissue and either single (for TEC enumeration and analysis of Ki67 expression) or maximal three pooled thymi (for enrichment and cell sorting) were sequentially digested 3x15 min at 37°C with HBSS containing Collagenase D (1mg/ml) and DNase I (50µg/ml) (both Roche Diagnostic) (2ml per thymus) until a single-cell thymic suspension was achieved. After each step, the thymic fragments were gently resuspended, allowed to settle, the supernatant was transferred into a tube containing 5ml cold HBSS/5% FBS/10mM EDTA and digestion repeated using remaining thymic fragments. Supernatants from all digestions were centrifuged at 1500 rpm for 5 min, resuspended in HBSS/5% FBS/10mM EDTA (2ml per thymus) and filtered through a 40µm mesh to remove clumps. Viable cells gated according to size were counted (total thymus cellularity) using a Z2 Coulter Counter (Beckman Coulter).

For phenotypic and numerical analysis of TEC, 20×10^6 cells from non-enriched single-cell suspension were stained to avoid selective loss of any TEC subset consequent to the enrichment procedure. Following combination of directly conjugated antibodies was used: CD45 PECy7, EpCam Cy5, pan MHCII (I-A/I-E) Fluos and Ly51 PE. Cells were incubated with 1ml of antibody mixture (i.e 100µl per $2x10^6$ cells) for 1 h on ice, thereafter washed with ice cold HBSS/ 2%FBS and harvested for flow cytometry. The labelled cells were acquired with a FACS Calibur flow cytometer (BD Bioscience) and data analysed using Cell Quest Pro Software (Becton Dickinson) or FlowJo 7.2.5 (Tree Star). For TEC quantification, forward/side scatter gates were set to include both T-cells and TEC and to exclude non-viable cells. TEC defined as $CD45^-EpCam^+$ were subdivided based on Ly51 and MHCII expression into three subsets: cTEC ($Ly51^+MHCII^+$); mTEClo ($Ly51^-MHCII^{lo}$) and $mTEC^{hi}$ ($Ly51^-MHCII^{hi}$). Absolute TEC numbers (i.e TEC cellularity) were derived from the percentage of TEC obtained in whole thymus (total thymic cellularity). The absolute numbers of different TEC subsets were then calculated as the percent of TEC cellularity which was set to 100%.

To obtain lymphocytes, thymi and spleens were placed between a 40µm nylon mesh in 1mL of IMDM/2% FCS and gently squeezed with a plunger until all cells were in suspension. After washing, cells were resuspended in 2ml PBS/2% FBS per thymus, filtered and counted.

For analysis of T-cell development, $1.5x10^6$ cells from the single cell suspuspension was stained with directly conjugated antibodies specific for CD3 Cy5, CD4 PE, CD8 PECy7 in combination with either CD24 FITC, or CD69 FITC. Development of lineage negative, double negative (DN) thymocytes was assessed by staining with directly conjugated antibodies specific for CD44 PE, CD25 FITC and c-kit APC combined with bio-conjugated lineage markers specific for CD3, CD4, CD8, TCRβ, TCRγδ, NK1, CD11b, CD11c, CD19 followed by Streptavidin (ST) PECy7. The frequency of splenic T and B cells was determined by staining cells with antibodies specific for B220 FITC, CD3 Cy5, CD4 PE and CD8 PECy7. For the staining, cells were distributed into a 96-well round bottom plate, spun down, resuspended in 100µL of antibody solution per 1x106 cells and incubated for 30-45 min on ice in the dark. Cells were then washed and either harvested for flow cytometry or incubated with appropriate secondary reagent, washed and then analysed by flow cytometry.

7.2.6. Thymic stromal cell (TSC) enrichment and sorting

Enrichment of TEC by autoMACS

TEC for sorting were enriched by EpCam-mediated magnetic separation on autoMACS (Miltenyi Biotec) according to the manufacturer's protocol. Briefly, thymic single-cell suspensions, each containing maximal three digested 4 week old thymi, were centrifuged at 1`500 rpm for 5 min and cells resuspended in HBSS/5% FBS at 100×10^6 cells per ml (approximately $600\text{-}800\times10^6$/6-8ml). Cells were stained with

biotinylated EpCam (G8.8) mAb for 15 min on ice, washed and again adjusted to 100x10^6 cells per ml. 35µl Anti-Biotin Microbeads (Miltenyi Biotec) was added and the mixture incubated for 15 min in the fridge, washed, filtered and resuspended once more at 100×10^6 cells per ml. Labelled cells were positively selected on an AutoMACS machine (Miltenyi Biotec) using the "Possel/QRinse program. EpCam-enriched cells were collected in tubes pre-coated and filled with 2ml IMDM/10% FBS/2.5mM EDTA, recovered by centrifugation (1`800 rpm, 5 min), washed, resuspended in 1ml HBSS/5% FBS, and counted (app. 6-12×10^6 per three 4 week old thymi).

Cells were stained with the following combination of primary antibodies diluted in HBSS/5% FBS: CD45 PECy7, EpCam bio, Ly51 PE, MHC class II (I-A/I-E) Fluos, UEA-1 Cy5. Briefly, 100µL antibody mixture was added per 1×10^6 enriched TEC and incubated for 1 h on ice. After washing, cells were incubated with ST APC-Cy7 for 15 min, thereafter washed, resuspended in 200µl HBSS/2% FBS and 2x concentrated DAPI solution (200µl) was added to final concentration of 0.1ng/µl. Different TEC populations (Figure 3.5 and Appendix 8.1) were sorted using a FACSAria (BD Biosciences) and subsequently proceeded for RNA isolation (RNAeasy Micro kit, Quigen) and qRT-PCR analysis. TEC purity was routinely assessed by re-analyzing sorted cell fractions and was always above 95%. DAPI and doublet discrimination by SSC-H/SSC-W were used to exclude the death cells. Thymi from mice of both sexes at 4 weeks of age were pooled to obtain sufficient TEC numbers for RNA isolation. Between 6-12 Sox9-deficient and -proficient thymi were used per sort.

Enrichment of TSC by Percol

For analysis of Sox9 expression in different thymic stromal subsets, thymi from 15 adult 6 weeks old C57BL/6 mice were enzymatically digested as described before. TSC were enriched by depleting most thymocytes with gradient density centrifugation in Percoll PLUS silica-based colloidal medium (GE Healthcare). Thymic single-cell suspension each containing maximal three digested 4 week old thymi were centrifuged for 5 min at 1500 rpm, resuspended in 3ml Percoll dense (1.115kg/m3) and carefully overlaid with 3ml Percoll light (1.065 kg/m3) followed by 2ml 1xPBS. Samples were centrifuged at 2500 rpm for 30 min, rotor radius 17.3, brake/acceleration off. Cells were collected from the interface Percoll light/PBS, into a fresh tube with 5ml PBS/2% FCS, washed, resuspended in 1mL PBS/2% FCS, filtered and counted. Usually, about 15-30x10^6 Percoll-enriched stromal cells from three thymi were recovered. Cells were stained with the following combination of primary antibodies using 100µl Ab mixture per 1-2x10^6 cells: CD45 PECy7, EpCam bio with ST-PE, Ly51 FITC and MTS15 Cy5. Different TEC ($CD45^-$ $EpCam^+$) and non-TEC ($CD45^-EpCam^-Ly51^+$) subsets (Figure 3.3) were sorted using a FACSAria (BD Biosciences).

7.2.7. Ki67 staining

To measure TEC proliferation, EpCam-enriched cells prepared as described above were stained for surface antigens for 30 min on ice using the combination of following primary antibody: CD45 PECy7, Ly51 A647 and MHC class II (I-A/I-E)-Fluos. After washing, TEC were fixed and permeabilized in Fixation/Permaebilization Buffer (Diluent and Concentrate mixed 3:1; eBioscience) for 30 min on ice, thereafter washed in 1x Permeabilization Buffer and stained with an anti-Ki67 PE mAb for 30 min on ice. After washing with 1x Permeabilization Buffer (eBioscience) cells were harvested for flow cytometry. Ki67 expression was assessed in distinct TEC subsets (Appendix 8.2). Non-viable cells and hematopoietic cells were excluded from the analysis based on their FSC/SSC scatter properties. As a negative control for Ki67, Mouse IgG-PE antibody (isotype control) was used. An FcR block was employed before applying the antibody stain. Ki67 expression in cTEC isolated from 4 and 12 weeks old mice could not be accurately assessed, as the frequency of $Ly51^+$ cTEC obtained per individual thymus was too low. The same protocol for Ki67 staining was used to analyse the proliferation of TEC lines retrovirally transduced with either wild type and mutant Sox9 or empty vector control.

7.2.8. Generation of Sox9 retroviral constructs

Full-length, wild type Sox9 (Sox9 WT) encodes amino acids 27-508 (76-1524bp, amplicon 1449bp) and contains the N-terminal (HMG) DNA binding domain and C-terminal transactivation (TA) domain. C-terminal truncated, mutant Sox9 (Sox9 M) encodes amino acids 27-304 (76-912bp, amplicon 837bp) and lacks the TA domain but retains the DNA binding domain (Lefebvre *et al.* 1997). The forth ATG (26. codon) of the Sox9 coding sequence (362-1885bp) was used as a start codon. Two sets of Sox9-specific PCR primers containing *BamHI/NotI* restriction sites were designed by hand. For the generation of mutant Sox9, reverse primer introducing an additional stop codon (TGA) and containing NotI site was designed to terminate the translation.

5`HA pPRIG_c (plasmid Polylinker Retroviral IRES GFP) (Albagli-Curiel *et al.* 2007) used in this study (Appendix 8.3) is an IRES-containing retroviral vector allowing expression of both the protein of interest (i.e Sox9 WT and Sox9 M) and a marker protein (eGFP) from a single bicistronic mRNA facilitating the selection of transduced cells based on eGFP detection. The hemagglutinin (HA) tag sequence is inserted in one (i.e c) of three reading frames 5' of the multiple cloning site (MCS) flanked by T7 and SP6 sequences and allows the detection of exogenous protein in target cells and pull down experiments. In contrast to many other retroviral vectors which harbour two complete LTR sequence, in this vector the 5`LTR in the U3 region has been replaced by CMV enhancer/promoter allowing more potent CMV-driven expression. Also the use of wild type IRES sequence enhances the translation of

eGFP about 10 fold without affecting any other important parameters (i.e viral titer or the cDNA expression).

7.2.8.1. Cloning of wild type and mutant Sox9

PCR amplification of Sox9

cDNAs for wild type and mutant mouse Sox9 were PCR amplified from the OP9 DL1 cell line with the high fidelity KOD Hot Start DNA Polymerase (Novagen) and Sox9-specific primers introducing BamHI and NotI restriction sites. Following PCR reaction was set up:

50ng cDNA (OP9 DL1)

1µl Forward primer (10µM)

1µl Reverse primer (10µM)

5µl dNTPs (2mM)

5µl KOD buffer

2µl $MgSO_4$ (25mM)

1.2µl KOD Polymerase

Add H_2O up to 50µl

PCR conditions

94°C 4 min

30 cycles: 94°C 30 sec; 58°C 30 sec; 68°C 2 min

68°C 10 min

The wild type (1449bp) and mutant (837bp) Sox9 fragments were precisely excised from a 1% agarose gel in order to avoid contamination with unspecific products that might be amplified by PCR. The subsequent purification of the amplicons from the piece of gel was done using the MinElute Gel Extraction kit (Qiagen) according to the manufacturer's instructions. The DNA fragments were eluted in 15µl of TE buffer. Since KOD Hot Start DNA Polymerase generates blunt ends, purified PCR products were next digested for BamHI/NotI to obtain sticky ends. As the enzyme`s buffers were not compatible, the sequential digestions were performed with intermediate purification of fragments using MinElute PCR purifications kit (Qiagen).

Restriction endonuclease digest of PCR products

Sequential *BamHI/NotI* digestion of PCR-amplified Sox9 WT and Sox9 M

(i) 15µl DNA

2µl *BamHI* Buffer (x10)

1µl *BamHI* (10U/µl)

2µl H_2O

V_{total} = 20µl; incubation for 1 h @ 37°C

(ii) PCR Purification

(iii) $V_{elution}$ = 15µl

2µl Orange Buffer (x10)

1µl *NotI* (10u/µl)

2µl H_2O

V_{total} = 20µl; incubation for 2 h @ 37°C

The digested DNA fragments were purified from the gel and eluted in 15µl of TE buffer. Wild type and mutant Sox9 were originally cloned into the retroviral vector LZRSpBMN-linker-IRES-eGFP (p166) (Heemskerk *et al.* 1997) (generously provided by H. Spits), but since for biochemical analysis we needed tagged-Sox9 they were cut out by BamHI/NotI digestion and re-cloned into 5`HA pPRIG (Albagli-Curiel *et al.* 2007) retroviral vector. For this purpose, 5`HA pPRIG was cut with the same restriction enzyme as following.

Sequential *BamHI/NotI* digestion of 5`HA pPRIG vector

(i) 2µl cDNA (3.9µg/µl)

1µl *BamHI* Buffer (x10)

1µl *BamHI* (10U/µl)

6µl H_2O

V_{total} = 10µl; incubation 1 h @ 37°C

(ii) PCR Purification:

(iii) $V_{elution}$ = 30µl

3.5 µl Orange Buffer (x10)

1µl *NotI* (10U/µl)

0.5µl H_2O

V_{total} = 35µl; incubation 2 h @ 37°C

(iv) Heat inactivation of *NotI* (20 min @ 80°C)

Dephosphorylation of DNA

The linearized vector backbone was dephosphorylated with calf intestine phosphatase (CIP) (Roche) to avoid self-ligation. Briefly, 1µl CIP was added to the vector and incubated for 30 min at 37°C followed

by another round of CIP addition and incubation. After dephosphorylation the retroviral backbone was gel-purified using the gel extraction kit (Macherey Nagel) and eluted in 15µl of TE buffer.

Ligations

The amount of vector and insert was estimated on a 1% agarose gel and ligation reactions were carried out in a final volume of 10-20µl using a molar ratio of 1:3 vector:insert. “Sticky ends” were ligated for 1 h at RT with 1µl T4 ligase (Invitrogen).

Ligation reaction:

xy µl Vector backbone

xy µl Insert

2µl 5x DNA Ligase Reaction Buffer

1µl T4 DNA Ligase (Invitrogen) (1U/µl)

µl H_2O up to 10-20µl

Generation of competent bateria

Competent bacteria for plasmid transformation were made in-house according to the TOP10 competent cells protocol (OpenWetWare). For the preparation of competent bacteria 10µL of DH5α E. coli bacteria were mixed with 100µl LB medium, streaked on an agar plate and grown overnight for single colonies at 37°C. A single colony was inoculated in 5ml SOB medium and grown overnight at RT on a shaker at 220 rpm (Vaudaux-Eppendorf). The 5ml starting culture were added to 200ml SOB medium and grown at RT with shaking at 220 rpm to an optical density (OD_{600}) of 0.5. The bacteria culture was then centrifuged at 3`000 g for 10 min at 4°C. The supernatant was removed and the pellet resuspended in 80ml of ice-cold CCMB80 buffer (see 5.1.2), incubated on ice for 20 min, thereafter centrifuged again and resuspended in 20ml of ice-cold CCMB80 buffer. After another 20 min of incubation on ice, 200µl of the suspension were aliquoted into chilled PCR strip tubes and frozen at -80°C.

Bacterial transformation

To transform bacteria, 5µl of the ligation mix were incubated with 100µl of DH5α competent cells in chilled 15ml polypropylene tubes for 30 min on ice. The mixture was then transferred into a waterbath at 42°C for 2 min and immediately placed back on ice for 2 min for the heat shock transformation. To recover bacteria, 500µl SOC medium was added and tubes were incubated at 37°C for 1 h shaking at 220 rpm. 100µl bacteria were streaked onto prewarmed and dried agar plates supplemented with Ampicilin (100mg/ml) (Sigma) and grown overnight at 37°C. The plates were stored at 4°C until further use.

DNA extraction

Single colonies were picked from plates and used to inoculate 2ml of LB medium containing Ampicillin (100mg/ml). Bacteria were grown overnight at 37°C in an incubator shaking at 220 rpm. Samples were then centrifuged at 4`000 rpm for 10 min at 4°C, the supernatant was discarded and the remaining pellet further processed for DNA extraction. The plasmids containing either no insert oder wild type and mutant Sox9 were purified using Miniprep or Maxiprep plasmid extraction kits (Macherey Nagel) according to the manufacturer's guidelines. Each of the purified plasmids was eluted in 40µl elution buffer. The concentration of the purified plasmids was measured by optical density (OD) using the following formula: concentration (µg/ml) = 50 x OD_{260} x dilution.

Restriction endonuclease digestions of plasmids

Plasmids were screened for the presence of Sox9 inserts by performing either single digestions with enzymes that cut exclusively within the Sox9 sequence (*SmaI, Sfi, BglI, AvaI*) or by double digestions where one enzyme cut within the Sox9 and other one within the retroviral backbone (*SfiI/BamHI, SfiI/SnaBI, Sfi/NotI*). Restriction endonucleases were bought either from MBI Fermentas or NEB and in general, digestion reactions were carried out in a total volume of 10µl according to the manafacture`s instructions. The size of the digested plasmid fragments was checked on a 1% agarose gel. Positive clones were further confirmed by sequencing.

DNA Sequencing

Sox9 sequencing primers were designed using Vector NTI (Invitrogen) to generate 400-600bp products (max. 700bp allowed for sequencing) and to cover the full length of wild type and mutant Sox9, including the N`HA-tag and part of the vector backbone. For the sequencing of the Sox9 plasmids, the following PCR reaction was prepared in a 0.2ml microtube:

500ng plasmid

2µl Ready Reaction Mix v1.1 (Applied Biosystems)

2µl Big Dye Terminator Sequencing Buffer (x5)

0.5µl DMSO

0.5µl Primer x (10µM) (see section 5.1.3 for its sequence)

µl H_2O up to 10µl.

The DNA was amplified using the following PCR conditions:

96°C 1min

25 cycles: 96°C 15s; 50°C 5s; 60°C 4min

The PCR products were purified using nucleoSEQ columns (Macherey-Nagel) and the manufacturer's instructions. For the sequencing, 4µl of the purified products were mixed with 16µl of fresh Formamide (Sigma) in a new 0.2ml microtube and the mixture was store at 4°C. Sequencing was performed in-house on an ABI 310 sequencing machine (Applied Biosystems). Trace files were first checked for quality and reliability by inspecting the obtained profiles of fluorescence peaks. Analysis was carried out using Vector NTI software for sequence alignments and reassembly of single sequencing reactions.

7.2.8.2. Production of Sox9 recombinant retroviruses and transduction of target cells

Cell lines

The retrovirus packaging cell line Platinum-E (Plat-E) (Morita *et al.* 2000) was a gift from T.Kitamura, (Tokyo, Japan). This cell line contains a packaging construct that utilizes the EF1α promoter which is 100 fold more potent than the conventionally used MuLV-LTR promoter, and in combination with the Kozak sequence upstream of the initiation codon results in high expression of virus structural proteins (gag-pol and env). The cortical thymic epithelial cell line cTEC1.2 was derived from young animals (Kasai *et al.* 1996) and was a gift from Dr. M. Kasai (Tokyo, Japan).

PlatE cells were cultured in IMDM containing L-glutamine, 25mM HEPES and 3,024mg/L sodium bicarbonate supplemented with 10% FCS, 2-mercaptoethanol (50µM) and Penicillin (100U/ml)/ Streptomycin (100µg/ml) solution. cTEC1.2 were cultured in the same medium but in the absence of antibiotics. Cells were grown at 37°C with 5% CO2 in an incubator. To detach the cells, Trypsin 0.25% /EDTA was added for 5-10min at 37°C, thereafter cells were washed with the culture medium to inactivate Trypsin and recovered by centrifugation (1`500 rpm for 5 min at 4°C).

Transfection of PlatE

PlatE cells were grown in 2ml culture medium in a 6-well plate to reach 1/3 confluency. Plasmids containing only eGFP (control) and either the HA-tagged wild type or mutant Sox9-ires-eGFP sequence were transfected into PlatE cells using FuGene6 (Roche diagnostics) transfection formulation according to the manufacturer. Briefly, 3µl of Fugene6 reagent were mixed with 97µl of serum free IMDM media and incubated for 5 min at RT. 1µg of plasmid DNA was added, tubes flicked and incubated for 15 min at RT. PlatE medium was replaced with 2ml fresh media and 100µl of the DNA/transfection mixture added dropwise to the cells of each well. The plate was then gently stirred in a circular and horizontal fashion to get a homogenous distribution. The transfection efficiency was evaluated by FACS and IHC analysis of eGFP expression (Appendix 8.3). The supernatants containing virus particles were collected 48-72 h

later, filtered through a 0.45µm filter using a syringe and polybrene was added to a final concentration of 10µg/ml.

Transduction of TEC

cTEC1.2 were grown in a 6-well plate to an approximate density of 30%. For the transduction, the medium was aspirated and 1ml of viral supernatant added. After 5 h incubation at 37°C 2ml fresh cell culture media were added to the cells. 72 h post-transduction $eGFP^+$ cells were sorted using a FACSAria to deplete the cultures of untransduced cells. Sorted cells were then expanded for subsequent qRT-PCR, WB and IP analysis. Transduction efficiency of wild type Sox9 was always lower when compared to mutant Sox9 as evaluated by qRT-PCR, FACS and WB analysis of Sox9, HA and eGFP expression.

7.2.9. Western blotting (WB)

Cell lysate preparations

For total lysate preparations, stably transduced cTEC1.2 with either HA-tagged Sox9 WT and Sox9 M or an empty retroviral vector (control) were lysed in RIPA buffer (200µl per well of a 6-well plate) containing Complete protease inhibitor cocktail (Roche). Nuclear and cytoplasimic fractions were prepared using the CelLytic NuCLEAR Extraction kit (Sigma-Aldrich) according to the manufacturer`s instructions. Protein concentration was measured using the Bradford assay (Biorad) in comparison to a previously established standard curve and equal protein amounts were subjected to SDS-PAGE or stored at -20° until further use.

SDS-Page Gel

Glass plates were assembled according to the Mini-Protean 3 Electrophoresis system (Biorad). The separating gel (see 5.1.2) was poured, overlayed with H_2O and left for 30 min at RT to polymerize. The water was removed, the stacking gel (see 5.1.2) poured onto the top of the separating gel and the comb inserted. Once the stacking gel has polymerized, the comb was removed and the glass plates with gels mounted in the electrophoresis apparatus. 1x Running buffer (see 5.1.2) was added to the system and the wells briefly washed using a syringe. Samples were prepared by heating them to 95°C for 5 minutes in 1x Loading buffer (see 5.1.2). Proteins (20-40µg per well) were loaded into the wells, one well was loaded with 2µl of a colored marker (Caleidoscope, Biorad) and the empty wells with 1x Loading buffer. The gel was run at 150V for appropriate time. The glass plates and stacking gel were removed and the separating gel rinsed with transfer buffer.

Transfer

The nitrocellulose membrane cut to the appropriate size was pre-wet in 1x Transfer buffer (see 5.1.2). The gel-membrane sandwich was assembled preventing air bubbles between the layers as following: **Cathode (-):** fiber pad - 2 Whatman papers - gel – membrane - 2 Whatman papers - fiber pad: **Anode (+).** The sandwich was inserted into a transfer plate and then put in the electrophoresis apparatus (BioRad) filled with 1xTransfer buffer. The electrophoris was run at 100V for 1h.

Western Blot

Once the proteins were transferred, the membrane was rinsed with TPBS (0.1% Tween 20 in PBS) and unspecific binding blocked with Blotto solution (5% skim milk in TPBS) for 1 h at RT. The membrane was then incubated overnight at 4°C on a shaking platform with following primary antibodies diluted in Blotto solution: mouse anti β-Catenin mAb, rabbit anti-Foxn1 pAb (Itoi *et al.* 2007a), mouse anti-HA mAb, and rabbit-anti Sox9 polyclonal Ab (Stolt *et al.* 2003). Rabbit anti-GAPDH polyclonal Ab, mouse anti-Histon H1 mAb and mouse anti-α-Tubulin mAb served as loading or purity controls. The membrane was then washed 2x10 min with TPBS and incubated with the appropriate HRP-conjugated secondary antibodies diluted in Blotto for 45 min at RT (on a shaking platform, followed by washing 2x10 min with TPBS. Finally, the membrane was incubated for 5 min with SuperSignal West Pico Chemiluminescent Substrate working solution (Pierce) (Luminol/Enhancer and Stable Peroxide solution mixed 1:1). The wet membrane was then wrapped in a plastic foil, an appropriately sized piece of Kodax BioMax Light film (Sigma) was put on the membrane in a dark room and exposed for 5 sec to 60 min.

7.2.10. Protein immunoprecipitation (IP)

For IP experiments, HA-tagged Sox9 WT and Sox9 M or alternatively β-catenin were immunopercipitated from total cell lysates using anti-HA or β-catenin antibodies, respectively and Protein-G GammaBind Plus Sepharose beads (GE Healthcare) according to the manufacturer`s instructions. Briefly, equal amounts of cleared lysates (1mg) of cTEC1.2 stably transduced to express HA-tagged Sox9 WT or Sox9 M were incubated overnight at 4°C with antibodies specific for HA, β-catenin or mouse IgG as a control (5µg per sample). Protein-G beads were then added and the mixture incubated for 1 h at 4°C. Immunoprecipitates were washed 3x with Ripa buffer, resolved by SDS-PAGE and analysed by WB analysis (as described above) using anti-β-catenin or Sox9 antibodies, respectively. The secondary reagents used were HPR-conjugated Goat-anti Mouse Ab and Goat-anti Rabbit Ab. Signals were detected with SuperSignal West Pico chemiluminiscent substrates (Pierce). The amounts of β-catenin and Sox9 protein were quantified using NIH Image-J software.

7.2.11. *In vitro* BrdU labelling of TEC

To measure proliferation of TEC stably transduced to express either Sox9 WT and Sox9 M or empty vector (control), cells were seeded one day before the BrdU (Sigma-Aldrich) addition in a 6-well plate at density of $1x10^5$ cells per well. Next day, cells were pulsed with BrdU at final concentration of 1.25µg/ml and BrdU incorporation assessed 16 h and 24 h later. The total cell numbers were determined at the end of the experiment using a Neubauer chamber. For the BrdU staining, cells were trypsinized, washed with medium and resuspended in 0.5ml ice cold 0.15M NaCl. The suspensions were then added dropweise to 1.2ml ice cold 95% ethanol in 5ml FACS tube while vortexing. Samples were incubated for 30 min on ice, spun down (2`000 rpm for 5 min at 4°C) and cells fixed with 0.5-1ml 1% PFA/0.01% Tween/PBS for 30 min at RT. After centrifugation, cells were incubated in 1x DNAse incubation buffer containing RNAse-free DNAse I (Roche Diagnostics) at 5U/ml for 10 min at RT, thereafter spun down and stained with anti-BrdU PE mAb diluted 1:1 with 1x PBS for 30 min at RT. The cells were then washed with 1xPBS, resuspended in 100µl PBS/2% FBS and BrdU incorporation analysed by flow cytometry. Experiments were always performed in triplicates for both a 16 h and 24 h BrdU pulse. As a negative control, cells not exposed but stained for BrdU were used. Also, an isotype control, i.e cells treated with BrdU and stained with Mouse IgG1-PE isotype antibody was included.

8. REFERENCES

Abu-Issa, R., G. Smyth*, et al.* (2002). "Fgf8 is required for pharyngeal arch and cardiovascular development in the mouse." Development 129(19): 4613-4625.

Afonja, O., B. M. Raaka*, et al.* (2002). "RAR agonists stimulate SOX9 gene expression in breast cancer cell lines: evidence for a role in retinoid-mediated growth inhibition." Oncogene 21(51): 7850-7860.

Akiyama, H. (2006). "[Sox family regulate chondrogenesis]." Clin Calcium 16(2): 368- 372.

Akiyama, H. (2008b). "Control of chondrogenesis by the transcription factor Sox9." Mod Rheumatol.

Akiyama, H., M. C. Chaboissier*, et al.* (2004a). "Essential role of Sox9 in the pathway that controls formation of cardiac valves and septa." Proc Natl Acad Sci U S A 101(17): 6502-6507.

Akiyama, H., M. C. Chaboissier*, et al.* (2002). "The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6." Genes Dev 16(21): 2813-2828.

Akiyama, H., T. Kamitani*, et al.* (2005b). "The transcription factor Sox9 is degraded by the ubiquitin-proteasome system and stabilized by a mutation in a ubiquitin-target site." Matrix Biol 23(8): 499-505.

Akiyama, H., J. P. Lyons*, et al.* (2004b). "Interactions between Sox9 and beta-catenin control chondrocyte differentiation." Genes Dev 18(9): 1072-1087.

Akiyama, T., S. Maeda*, et al.* (2005a). "Dependence of self-tolerance on TRAF6-directed development of thymic stroma." Science 308(5719): 248-251.

Akiyama, T., Y. Shimo*, et al.* (2008a). "The tumor necrosis factor family receptors RANK and CD40 cooperatively establish the thymic medullary microenvironment and self-tolerance." Immunity 29(3): 423-437.

Albagli-Curiel, O., Y. Lecluse*, et al.* (2007). "A new generation of pPRIG-based retroviral vectors." BMC Biotechnol 7: 85.

Allman, D., J. A. Punt*, et al.* (2002). "An invitation to T and more: notch signaling in lymphopoiesis." Cell 109 Suppl: S1-11.

Anderson, G., K. L. Anderson*, et al.* (1997). "Fibroblast dependency during early thymocyte development maps to the CD25+ CD44+ stage and involves interactions with fibroblast matrix molecules." Eur J Immunol 27(5): 1200-1206.

Anderson, G. and E. J. Jenkinson (2001). "Lymphostromal interactions in thymic development and function." Nat Rev Immunol 1(1): 31-40.

Anderson, G., E. J. Jenkinson*, et al.* (1993). "MHC class II-positive epithelium and mesenchyme cells are both required for T-cell development in the thymus." Nature 362(6415): 70-73.

Anderson, G., E. J. Jenkinson*, et al.* (2009). "A roadmap for thymic epithelial cell development." Eur J Immunol 39(7): 1694-1699.

Anderson, G., W. E. Jenkinson*, et al.* (2006). "Establishment and functioning of intrathymic microenvironments." Immunol Rev 209: 10-27.

Anderson, G., P. J. Lane*, et al.* (2007). "Generating intrathymic microenvironments to establish T-cell tolerance." Nat Rev Immunol.

Anderson, G., N. C. Moore*, et al.* (1996). "Cellular interactions in thymocyte development." Annu Rev Immunol 14: 73-99.

Anderson, M. S., E. S. Venanzi*, et al.* (2005). "The cellular mechanism of Aire control of T cell tolerance." Immunity 23(2): 227-239.

Anderson, M. S., E. S. Venanzi*, et al.* (2002). "Projection of an immunological self shadow within the thymus by the aire protein." Science 298(5597): 1395-1401.

Andreu, P., S. Colnot*, et al.* (2005). "Crypt-restricted proliferation and commitment to the Paneth cell lineage following Apc loss in the mouse intestine." Development 132(6): 1443-1451.

Arce, L., N. N. Yokoyama*, et al.* (2006). "Diversity of LEF/TCF action in development and disease." Oncogene 25(57): 7492-7504.

Arnold, J. S., U. Werling*, et al.* (2006). "Inactivation of Tbx1 in the pharyngeal endoderm results in 22q11DS malformations." Development 133(5): 977-987.

Aschenbrenner, K., L. M. D'Cruz*, et al.* (2007). "Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire+ medullary thymic epithelial cells." Nat Immunol 8(4): 351-358.

Bachiller, D., J. Klingensmith*, et al.* (2003). "The role of chordin/Bmp signals in mammalian pharyngeal development and DiGeorge syndrome." Development 130(15): 3567-3578.

Bafico, A., G. Liu*, et al.* (2001). "Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow." Nat Cell Biol 3(7): 683-686.

Balciunaite, G., R. Ceredig*, et al.* (2005). "The role of Notch and IL-7 signaling in early thymocyte proliferation and differentiation." Eur J Immunol 35(4): 1292-1300.

Balciunaite, G., M. P. Keller*, et al.* (2002). "**Wnt** glycoproteins regulate the expression of FoxN1, the gene defective in nude mice." Nat Immunol 3(11): 1102-1108.

Banwell, C. M., K. M. Partington*, et al.* (2000). "Studies on the role of IL-7 presentation by mesenchymal fibroblasts during early thymocyte development." Eur J Immunol 30(8): 2125-2129.

Basak, S. and A. Hoffmann (2008). "Crosstalk via the NF-kappaB signaling system." Cytokine Growth Factor Rev 19(3-4): 187-197.

Bastide, P., C. Darido*, et al.* (2007). "Sox9 regulates **proliferation** and is required for Paneth cell **differentiation** in the intestinal." J Cell Biol 178(4): 635-648.

Bell, D. M., K. K. Leung*, et al.* (1997). "SOX9 directly regulates the type-II collagen gene." Nat Genet 16(2): 174-178.

Bennett, A. R., A. Farley*, et al.* (2002). "Identification and characterization of thymic epithelial progenitor cells." Immunity 16(6): 803-814.

Benz, C., K. Heinzel*, et al.* (2004). "Homing of immature thymocytes to the subcapsular microenvironment within the thymus is not an absolute requirement for T cell development." Eur J Immunol 34(12): 3652-3663.

Bernard, P., H. Sim*, et al.* (2008). "Human SRY inhibits beta-catenin-mediated transcription." Int J Biochem Cell Biol 40(12): 2889-2900.

Bevan, M. J. (1997). "In thymic selection, peptide diversity gives and takes away." Immunity 7(2): 175-178.

Bi, W., W. Huang*, et al.* (2001). "Haploinsufficiency of Sox9 results in defective cartilage primordia and premature skeletal mineralization." Proc Natl Acad Sci U S A 98(12): 6698-6703.

Blache, P., M. van de Wetering*, et al.* (2004). "SOX9 is an intestine crypt transcription factor, is regulated by the Wnt pathway, and represses the CDX2 and MUC2 genes " J Cell Biol 166(1): 37-47.

Blackburn, C. C., C. L. Augustine*, et al.* (1996). "The nu gene acts cell-autonomously and is required for differentiation of thymic epithelial progenitors." Proc Natl Acad Sci U S A 93(12): 5742-5746.

Blackburn, C. C. and N. R. Manley (2004). "Developing a new paradigm for thymus organogenesis." Nat Rev Immunol 4(4): 278-289.

Bleul, C. C. and T. Boehm (2005). "BMP signaling is required for normal thymus development." J Immunol 175(8): 5213-5221.

Bleul, C. C., T. Corbeaux*, et al.* (2006). "Formation of a functional thymus initiated by a postnatal epithelial progenitor cell." Nature 441(7096): 992-996.

Bockman, D. E. (1997). "Development of the thymus." Microsc Res Tech 38(3): 209-215.

Bockman, D. E. and M. L. Kirby (1984). "Dependence of thymus development on derivatives of the neural crest." Science 223(4635): 498-500.

Bockman, D. E. and M. L. Kirby (1989). "Neural crest function in thymus development." Immunol Ser 45: 451-467.

Boehm, T. (2008). "Thymus development and function." Curr Opin Immunol 20(2): 178-184.

Boehm, T. and C. C. Bleul (2006). "Thymus-homing precursors and the thymic microenvironment." Trends Immunol 27(10): 477-484.

Boehm, T., C. C. Bleul*, et al.* (2003). "Genetic dissection of thymus development in mouse and zebrafish." Immunol Rev 195: 15-27.

Bonizzi, G. and M. Karin (2004). "The two NF-kappaB activation pathways and their role in innate and adaptive immunity." Trends in immunology 25(6): 280-288.

Boursalian, T. E. and K. Bottomly (1999). "Survival of naive CD4 T cells: roles of restricting versus selecting MHC class II and cytokine milieu." Journal of immunology 162(7): 3795-3801.

Bowles, J., G. Schepers*, et al.* (2000). "Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators." Dev Biol 227(2): 239-255.

Bowlus, C. L., J. Ahn*, et al.* (1999). "Cloning of a novel MHC-encoded serine peptidase highly expressed by cortical epithelial cells of the thymus." Cell Immunol 196(2): 80-86.

Boyd, R. L., C. L. Tucek*, et al.* (1993). "The thymic microenvironment." Immunol Today 14(9): 445-459.

Brannon, M., J. D. Brown*, et al.* (1999). "XCtBP is a XTcf-3 co-repressor with roles throughout Xenopus development." Development 126(14): 3159-3170.

Brissette, J. L., J. Li*, et al.* (1996). "The product of the mouse nude locus, Whn, regulates the balance between epithelial cell growth and differentiation." Genes & development 10(17): 2212-2221.

Burkly, L., C. Hession*, et al.* (1995). "Expression of relB is required for the development of thymic medulla and dendritic cells." Nature 373(6514): 531-536.

Cadigan, K. M. and Y. I. Liu (2006). "Wnt signaling: complexity at the surface." Journal of cell science 119(Pt 3): 395-402.

Candi, E., A. Rufini*, et al.* (2007). "DeltaNp63 regulates thymic development through enhanced expression of FgfR2 and Jag2." Proc Natl Acad Sci U S A 104(29): 11999-12004.

Ceredig, R., J. W. Lowenthal*, et al.* (1985). "Expression of interleukin-2 receptors as a differentiation marker on intrathymic stem cells." Nature 314(6006): 98-100.

Ceredig, R. and T. Rolink (2002). "A positive look at double-negative thymocytes." Nat Rev Immunol 2(11): 888-897.

Chaboissier, M. C., A. Kobayashi*, et al.* (2004). "Functional analysis of Sox8 and Sox9 during sex determination in the mouse." Development 131(9): 1891-1901.

Chao, A. T., W. M. Jones*, et al.* (2007). "The HMG-box transcription factor SoxNeuro acts with Tcf to control Wg/Wnt signaling activity." Development 134(5): 989-997.

Chapman, D. L., N. Garvey*, et al.* (1996). "Expression of the T-box family genes, Tbx1-Tbx5, during early mouse development." Dev Dyn 206(4): 379-390.

Chassot, A. A., F. Ranc*, et al.* (2008). "Activation of beta-catenin signaling by Rspo1 controls differentiation of the mammalian ovary." Human molecular genetics 17(9): 1264-1277.

Chen, L., S. Xiao*, et al.* (2008a). "Foxn1 is required to maintain the postnatal thymic microenvironment in a dosage-sensitive manner." Blood.

Chen, X., J. Yang*, et al.* (2008b). "Wnt signaling: the good and the bad." Acta Biochim Biophys Sin (Shanghai) 40(7): 577-594.

Chikuda, H., F. Kugimiya*, et al.* (2004). "Cyclic GMP-dependent protein kinase II is a molecular switch from proliferation to hypertrophic differentiation of chondrocytes." Genes & development 18(19): 2418-2429.

Clarkson, M. J. and V. R. Harley (2002). "Sex with two SOX on: SRY and SOX9 in testis development." Trends Endocrinol Metab 13(3): 106-111.

Clevers, H. (2006). "Wnt/beta-catenin signaling in development and disease." Cell 127(3): 469-480.

Conway, S. J., D. J. Henderson*, et al.* (1997). "Pax3 is required for cardiac neural crest migration in the mouse: evidence from the splotch (Sp2H) mutant." Development 124(2): 505-514.

Corbeaux, T., I. Hess*, et al.* (2010). "Thymopoiesis in mice depends on a Foxn1-positive thymic epithelial cell lineage." Proc Natl Acad Sci U S A 107(38): 16613-16618.

Cordier, A. C. and S. M. Haumont (1980). "Development of thymus, parathyroids, and ultimo-branchial bodies in NMRI and nude mice." Am J Anat 157(3): 227-263.

Cordier, A. C. and J. F. Heremans (1975). "Nude mouse embryo: ectodermal nature of the primordial thymic defect." Scand J Immunol 4(2): 193-196.

Cunliffe, V. T., A. J. Furley*, et al.* (2002). "Complete rescue of the nude mutant phenotype by a wild-type Foxn1 transgene." Mamm Genome 13(5): 245-252.

Dajani, R., E. Fraser*, et al.* (2003). "Structural basis for recruitment of glycogen synthase kinase 3beta to the axin-APC scaffold complex." EMBO J 22(3): 494-501.

Daniels, D. L. and W. I. Weis (2005). "Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation." Nature structural & molecular biology 12(4): 364-371.

Darnay, B. G., A. Besse*, et al.* (2007). "TRAFs in RANK signaling." Advances in experimental medicine and biology 597: 152-159.

De Santa Barbara, P., N. Bonneaud*, et al.* (1998). "Direct interaction of SRY-related protein SOX9 and steroidogenic factor 1 regulates transcription of the human anti-Mullerian hormone gene." Mol Cell Biol 18(11): 6653-6665.

Depreter, M. G., N. F. Blair*, et al.* (2008). "Identification of Plet-1 as a specific marker of early thymic epithelial progenitor cells." Proc Natl Acad Sci U S A 105(3): 961-966.

Derbinski, J., J. Gabler*, et al.* (2005). "Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels." J Exp Med 202(1): 33-45.

Derbinski, J., A. Schulte*, et al.* (2001). "Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self." Nat Immunol 2(11): 1032-1039.

Devoss, J. J., A. K. Shum*, et al.* (2008). "Effector mechanisms of the autoimmune syndrome in the murine model of autoimmune polyglandular syndrome type 1." J Immunol 181(6): 4072-4079.

Dichtel-Danjoy, M. L., J. Caldeira*, et al.* (2009). "SoxF is part of a novel negative-feedback loop in the wingless pathway that controls proliferation in the Drosophila wing disc." Development 136(5): 761-769.

Dong, C., D. Wilhelm*, et al.* (2004). "Sox genes and cancer." Cytogenet Genome Res 105(2-4): 442-447.

Dooley, J., M. Erickson*, et al.* (2008). "Alterations of the medullary epithelial compartment in the Aire-deficient thymus: implications for programs of thymic epithelial differentiation." J Immunol 181(8): 5225-5232.

Dooley, J., M. Erickson*, et al.* (2007). "FGFR2IIIb signaling regulates thymic epithelial differentiation." Dev Dyn 236(12): 3459-3471.

Dooley, J., M. Erickson*, et al.* (2005). "Nude thymic rudiment lacking functional foxn1 resembles respiratory epithelium." Dev Dyn 233(4): 1605-1612.

Douagi, I., I. Andre*, et al.* (2000). "Characterization of T cell precursor activity in the murine fetal thymus: evidence for an input of T cell precursors between days 12 and 14 of gestation." European journal of immunology 30(8): 2201-2210.

Drivdahl, R., K. H. Haugk*, et al.* (2004). "Suppression of growth and tumorigenicity in the prostate tumor cell line M12 by overexpression of the transcription factor SOX9." Oncogene 23(26): 4584-4593.

Dunn, R. J., C. J. Luedecker*, et al.* (1997). "Thymic overexpression of CD40 ligand disrupts normal thymic epithelial organization." J Histochem Cytochem 45(1): 129-141.

Erickson, M., S. Morkowski*, et al.* (2002). "Regulation of thymic epithelium by keratinocyte growth factor." Blood 100(9): 3269-3278.

Ernst, B., C. D. Surh*, et al.* (1995). "Thymic selection and cell division." The Journal of experimental medicine 182(4): 961-971.

Fantes, J., N. K. Ragge*, et al.* (2003). "Mutations in SOX2 cause anophthalmia." Nature genetics 33(4): 461-463.

Fontaine-Perus, J. C., F. M. Calman*, et al.* (1981). "Seeding of the 10-day mouse embryo thymic rudiment by lymphocyte precursors *in vitro*." J Immunol 126(6): 2310-2316.

Formeister, E. J., A. L. Sionas*, et al.* (2009). "Distinct SOX9 levels differentially mark stem/progenitor populations and enteroendocrine cells of the small intestine epithelium." Am J Physiol Gastrointest Liver Physiol 296(5): G1108-1118.

Foster, J. W., M. A. Dominguez-Steglich*, et al.* (1994a). "Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene." Nature 372(6506): 525-530.

Foster, J. W. and J. A. Graves (1994). "An SRY-related sequence on the marsupial X chromosome: implications for the evolution of the mammalian testis-determining gene." Proc Natl Acad Sci U S A 91(5): 1927-1931.

Foster, K., J. Sheridan*, et al.* (2008). "Contribution of neural crest-derived cells in the embryonic and adult thymus." J Immunol 180(5): 3183-3189.

Frank, D. U., L. K. Fotheringham*, et al.* (2002). "An Fgf8 mouse mutant phenocopies human 22q11 deletion syndrome." Development 129(19): 4591-4603.

Franz, T. (1989). "Persistent truncus arteriosus in the Splotch mutant mouse." Anat Embryol (Berl) 180(5): 457-464.

Fuchs, E. (2007). "Scratching the surface of skin development." Nature 445(7130): 834-842.

Furuyama, K., Y. Kawaguchi*, et al.* (2011). "Continuous cell supply from a Sox9-expressing progenitor zone in adult liver, exocrine pancreas and intestine." Nature genetics 43(1): 34-41.

Gabler, J., J. Arnold*, et al.* (2007). "Promiscuous gene expression and the developmental dynamics of medullary thymic epithelial cells." Eur J Immunol 37(12): 3363-3372.

Gabor, M. J., D. I. Godfrey*, et al.* (1997). "Recent thymic emigrants are distinct from most medullary thymocytes." Eur J Immunol 27(8): 2010-2015.

Gallegos, A. M. and M. J. Bevan (2004). "Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation." The Journal of experimental medicine 200(8): 1039-1049.

Garg, V., C. Yamagishi*, et al.* (2001). "Tbx1, a DiGeorge syndrome candidate gene, is regulated by sonic hedgehog during pharyngeal arch development." Dev Biol 235(1): 62-73.

Gasca, S., J. Canizares*, et al.* (2002). "A nuclear export signal within the high mobility group domain regulates the nucleocytoplasmic translocation of SOX9 during sexual determination." Proc Natl Acad Sci U S A 99(17): 11199-11204.

Gerdes, J., H. Lemke*, et al.* (1984). "Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67." J Immunol 133(4): 1710-1715.

Giese, K., A. Amsterdam*, et al.* (1991). "DNA-binding properties of the HMG domain of the lymphoid-specific transcriptional regulator LEF-1." Genes Dev 5(12B): 2567-2578.

Gill, J., M. Malin*, et al.* (2002). "Generation of a complete thymic microenvironment by MTS24(+) thymic epithelial cells." Nat Immunol 3(7): 635-642.

Gill, J., M. Malin*, et al.* (2003). "Thymic generation and regeneration." Immunol Rev 195: 28-50.

Gillard, G. O., J. Dooley*, et al.* (2007). "Aire-dependent alterations in medullary thymic epithelium indicate a role for Aire in thymic epithelial differentiation." J Immunol 178(5): 3007-3015.

Gillard, G. O. and A. G. Farr (2005). "Contrasting models of promiscuous gene expression by thymic epithelium." J Exp Med 202(1): 15-19.

Gillard, G. O. and A. G. Farr (2006). "Features of medullary thymic epithelium implicate postnatal development in maintaining epithelial heterogeneity and tissue-restricted antigen expression." J Immunol 176(10): 5815-5824.

Godfrey, D. I., D. J. Izon, *et al.* (1988). "Thymic stromal elements defined by M.Abs: ontogeny, and modulation *in vivo* by immunosuppression." Adv Exp Med Biol 237: 269-275.

Godfrey, D. I., J. Kennedy, *et al.* (1994). "Onset of TCR-beta gene rearrangement and role of TCR-beta expression during CD3-CD4-CD8- thymocyte differentiation." Journal of immunology 152(10): 4783-4792.

Gommeaux, J., C. Gregoire, *et al.* (2009). "Thymus-specific serine protease regulates positive selection of a subset of CD4+ thymocytes." Eur J Immunol 39(4): 956-964.

Gordon, J., A. R. Bennett, *et al.* (2001). "Gcm2 and Foxn1 mark early parathyroid- and thymus-specific domains in the developing third pharyngeal pouch." Mech Dev 103(1-2): 141-143.

Gordon, J., V. A. Wilson, *et al.* (2004). "Functional evidence for a single endodermal origin for the thymic epithelium." Nat Immunol 5(5): 546-553.

Gordon, J., S. Xiao, *et al.* (2007). "Specific expression of lacZ and cre recombinase in fetal thymic epithelial cells by multiplex gene targeting at the Foxn1 locus." BMC Dev Biol 7: 69.

Graham, A. (2001). "The development and evolution of the pharyngeal arches." J Anat 199(Pt 1-2): 133-141.

Gray, D., J. Abramson, *et al.* (2007b). "Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire." J Exp Med 204(11): 2521-2528.

Gray, D. H., A. L. Fletcher, *et al.* (2008). "Unbiased analysis, enrichment and purification of thymic stromal cells." J Immunol Methods 329(1-2): 56-66.

Gray, D. H., N. Seach, *et al.* (2006). "Developmental kinetics, turnover, and stimulatory capacity of thymic epithelial cells." Blood 108(12): 3777-3785.

Gray, D. H., D. Tull, *et al.* (2007a). "A unique thymic fibroblast population revealed by the monoclonal antibody MTS-15." J Immunol 178(8): 4956-4965.

Greenberg, F. (1993). "DiGeorge syndrome: an historical review of clinical and cytogenetic features." J Med Genet 30(10): 803-806.

Griffith, A. V., K. Cardenas, *et al.* (2009). "Increased thymus- and decreased parathyroid-fated organ domains in Splotch mutant embryos." Dev Biol 327(1): 216-227.

Gubbay, J., J. Collignon, *et al.* (1990). "A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes." Nature 346(6281): 245-250.

Guo, L., D. Zhong, *et al.* (2008). "Sox7 Is an independent checkpoint for beta-catenin function in prostate and colon epithelial cells." Mol Cancer Res 6(9): 1421-1430.

Habas, R. and I. B. Dawid (2005). "Dishevelled and Wnt signaling: is the nucleus the final frontier?" J Biol 4(1): 2.

Hager-Theodorides, A. L., S. V. Outram, *et al.* (2002). "Bone morphogenetic protein 2/4 signaling regulates early thymocyte differentiation." J Immunol 169(10): 5496-5504.

Hamazaki, Y., H. Fujita, *et al.* (2007). "Medullary thymic epithelial cells expressing Aire represent a unique lineage derived from cells expressing claudin." Nat Immunol 8(3): 304-311.

Hare, K. J., R. W. Wilkinson, *et al.* (1998). "Identification of a developmentally regulated phase of postselection expansion driven by thymic epithelium." J Immunol 160(8): 3666-3672.

Harley, V. R., R. Lovell-Badge, *et al.* (1994). "Definition of a consensus DNA binding site for SRY." Nucleic acids research 22(8): 1500-1501.

Hart, M., J. P. Concordet, *et al.* (1999). "The F-box protein beta-TrCP associates with phosphorylated beta-catenin and regulates its activity in the cell." Curr Biol 9(4): 207-210.

Hattori, T., H. Eberspaecher, *et al.* (2006). "Interactions between PIAS proteins and SOX9 result in an increase in the cellular concentrations of SOX9." J Biol Chem 281(20): 14417-14428.

Hayday, A. C. and D. J. Pennington (2007). "Key factors in the organized chaos of early T cell development." Nat Immunol 8(2): 137-144.

Heemskerk, M. H., B. Blom, *et al.* (1997). "Inhibition of T cell and promotion of natural killer cell development by the dominant negative helix loop helix factor Id3." The Journal of experimental medicine 186(9): 1597-1602.

Hetzer-Egger, C., M. Schorpp*, et al.* (2002). "Thymopoiesis requires Pax9 function in thymic epithelial cells." Eur J Immunol 32(4): 1175-1181.

Hikosaka, Y., T. Nitta*, et al.* (2008). "The cytokine RANKL produced by positively selected thymocytes fosters medullary thymic epithelial cells that express autoimmune regulator." Immunity 29(3): 438-450.

Hinterberger, M., M. Aichinger*, et al.* (2010). "Autonomous role of medullary thymic epithelial cells in central CD4(+) T cell tolerance." Nat Immunol 11(6): 512-519.

Hoffmann, A., G. Natoli*, et al.* (2006). "Transcriptional regulation via the NF-kappaB signaling module." Oncogene 25(51): 6706-6716.

Hogan, B. L. and J. M. Yingling (1998). "Epithelial/mesenchymal interactions and branching morphogenesis of the lung." Curr Opin Genet Dev 8(4): 481-486.

Hollander, G., J. Gill*, et al.* (2006). "Cellular and molecular events during early thymus development." Immunol Rev 209: 28-46.

Hollander, G. A., B. Wang*, et al.* (1995). "Developmental control point in induction of thymic cortex regulated by a subpopulation of prothymocytes." Nature 373(6512): 350-353.

Honey, K., T. Nakagawa*, et al.* (2002). "Cathepsin L regulates CD4+ T cell selection independently of its effect on invariant chain: a role in the generation of positively selecting peptide ligands." The Journal of experimental medicine 195(10): 1349-1358.

Hoppler, S. and C. L. Kavanagh (2007). "Wnt signalling: variety at the core." J Cell Sci 120(Pt 3): 385-393.

Huang, W., X. Zhou*, et al.* (2000). "Phosphorylation of SOX9 by cyclic AMP-dependent protein kinase A enhances SOX9's ability to transactivate a Col2a1 chondrocyte-specific enhancer." Molecular and cellular biology 20(11): 4149-4158.

Iguchi, H., Y. Urashima*, et al.* (2007). "SOX6 suppresses cyclin D1 promoter activity by interacting with beta-catenin and histone deacetylase 1, and its down-regulation induces pancreatic beta-cell proliferation." J Biol Chem 282(26): 19052-19061.

Ikeda, S., S. Kishida*, et al.* (1998). "Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3beta and beta-catenin and promotes GSK-3beta-dependent phosphorylation of beta-catenin." The EMBO journal 17(5): 1371-1384.

Irla, M., G. Hollander*, et al.* (2009). "Control of central self-tolerance induction by autoreactive CD4+ thymocytes." Trends Immunol 31(2): 71-79.

Irla, M., S. Hugues*, et al.* (2008). "Autoantigen-specific interactions with CD4+ thymocytes control mature medullary thymic epithelial cell cellularity." Immunity 29(3): 451-463.

Ishida, T., S. Mizushima*, et al.* (1996). "Identification of TRAF6, a novel tumor necrosis factor receptor-associated factor protein that mediates signaling from an amino-terminal domain of the CD40 cytoplasmic region." J Biol Chem 271(46): 28745-28748.

Itoi, M., H. Kawamoto*, et al.* (2001). "Two distinct steps of immigration of hematopoietic progenitors into the early thymus anlage." Int Immunol 13(9): 1203-1211.

Itoi, M., N. Tsukamoto*, et al.* (2007a). "Expression of Dll4 and CCL25 in Foxn1-negative epithelial cells in the post-natal thymus." Int Immunol 19(2): 127-132.

Itoi, M., N. Tsukamoto*, et al.* (2007b). "Mesenchymal cells are required for functional development of thymic epithelial cells." Int Immunol 19(8): 953-964.

Jakob, S. and R. Lovell-Badge (2011). "Sex determination and the control of Sox9 expression in mammals." FEBS J 278(7): 1002-1009.

Jaleco, A. C., H. Neves*, et al.* (2001). "Differential effects of Notch ligands Delta-1 and Jagged-1 in human lymphoid differentiation." J Exp Med 194(7): 991-1002.

Jeker, L. T., T. Barthlott*, et al.* (2008). "Maintenance of a normal thymic microenvironment and T-cell homeostasis require Smad4-mediated signaling in thymic epithelial cells." Blood 112(9): 3688-3695.

Jenkinson, E. J., W. E. Jenkinson*, et al.* (2006). "The thymus and T-cell commitment: the right niche for Notch?" Nature reviews. Immunology 6(7): 551-555.

Jenkinson, E. J., W. Van Ewijk*, et al.* (1981). "Major histocompatibility complex antigen expression on the epithelium of the developing thymus in normal and nude mice." J Exp Med 153(2): 280-292.

Jenkinson, W. E., E. J. Jenkinson*, et al.* (2003). "Differential requirement for mesenchyme in the proliferation and maturation of thymic epithelial progenitors." J Exp Med 198(2): 325-332.

Jenkinson, W. E., S. W. Rossi*, et al.* (2005). "Development of functional thymic epithelial cells occurs independently of lymphostromal interactions." Mech Dev 122(12): 1294-1299.

Jerome, L. A. and V. E. Papaioannou (2001). "DiGeorge syndrome phenotype in mice mutant for the T-box gene, Tbx1." Nat Genet 27(3): 286-291.

Jiang, X., D. H. Rowitch*, et al.* (2000). "Fate of the mammalian cardiac neural crest." Development 127(8): 1607-1616.

Kajiura, F., S. Sun*, et al.* (2004). "NF-kappa B-inducing kinase establishes self-tolerance in a thymic stroma-dependent manner." Journal of immunology 172(4): 2067-2075.

Kan, L., N. Israsena*, et al.* (2004). "Sox1 acts through multiple independent pathways to promote neurogenesis." Developmental biology 269(2): 580-594.

Kasai, M., K. Hirokawa*, et al.* (1996). "Difference in antigen presentation pathways between cortical and medullary thymic epithelial cells." Eur J Immunol 26(9): 2101-2107.

Kawano, Y. and R. Kypta (2003). "Secreted antagonists of the Wnt signalling pathway." J Cell Sci 116(Pt 13): 2627-2634.

Kaye, J., M. L. Hsu*, et al.* (1989). "Selective development of CD4+ T cells in transgenic mice expressing a class II MHC-restricted antigen receptor." Nature 341(6244): 746-749.

Kenny, A. P., D. W. Oleksyn*, et al.* (2003). "Tight regulation of SpSoxB factors is required for patterning and morphogenesis in sea urchin embryos." Developmental biology 261(2): 412-425.

Kent, J., S. C. Wheatley*, et al.* (1996). "A male-specific role for SOX9 in vertebrate sex determination." Development 122(9): 2813-2822.

Kiefer, J. C. (2007). "Back to basics: Sox genes." Dev Dyn 236(8): 2356-2366.

Kim, Y. and B. Capel (2006). "Balancing the bipotential gonad between alternative organ fates: a new perspective on an old problem." Dev Dyn 235(9): 2292-2300.

Kimelman, D. and W. Xu (2006). "beta-catenin destruction complex: insights and questions from a structural perspective." Oncogene 25(57): 7482-7491.

Kinoshita, D., F. Hirota*, et al.* (2006). "Essential role of IkappaB kinase alpha in thymic organogenesis required for the establishment of self-tolerance." J Immunol 176(7): 3995-4002.

Kist, R., H. Schrewe*, et al.* (2002). "Conditional inactivation of Sox9: a mouse model for campomelic dysplasia." Genesis 32(2): 121-123.

Klug, D. B., C. Carter*, et al.* (1998). "Interdependence of cortical thymic epithelial cell differentiation and T-lineage commitment." Proc Natl Acad Sci U S A 95(20): 11822-11827.

Klug, D. B., C. Carter*, et al.* (2002). "Cutting edge: thymocyte-independent and thymocyte-dependent phases of epithelial patterning in the fetal thymus." J Immunol 169(6): 2842-2845.

Klug, D. B., E. Crouch*, et al.* (2000). "Transgenic expression of cyclin D1 in thymic epithelial precursors promotes epithelial and T cell development." J Immunol 164(4): 1881-1888.

Koble, C. and B. Kyewski (2009). "The thymic medulla: a unique microenvironment for intercellular self-antigen transfer." J Exp Med 206(7): 1505-1513.

Kohn, A. D. and R. T. Moon (2005). "Wnt and calcium signaling: beta-catenin-independent pathways." Cell Calcium 38(3-4): 439-446.

Kojima, A., M. Saito*, et al.* (1984). "NFS/N-nu/ + mice can macroscopically be distinguished from NFS/N- +/+ littermates by their thymic size and shape." Exp Cell Biol 52(1-2): 107-110.

Koopman, P., J. Gubbay*, et al.* (1991). "Male development of chromosomally female mice transgenic for Sry." Nature 351(6322): 117-121.

Korinek, V., N. Barker*, et al.* (1998). "Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4." Nature genetics 19(4): 379-383.

Kormish, J. D., D. Sinner*, et al.* (2009). "Interactions between SOX factors and Wnt/beta-catenin signaling in development and disease." Dev Dyn.

Krumlauf, R. (1994). "Hox genes in vertebrate development." Cell 78(2): 191-201.

Kuhnert, F., C. R. Davis*, et al.* (2004). "Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1." Proc Natl Acad Sci U S A 101(1): 266-271.

Kuraguchi, M., X. P. Wang*, et al.* (2006). "Adenomatous polyposis coli (APC) is required for normal development of skin and thymus." PLoS Genet 2(9): e146.

Kuratani, S. and D. E. Bockman (1990). "Impaired development of the thymic primordium after neural crest ablation." The Anatomical record 228(2): 185-190.

Kwan, J. and N. Killeen (2004). "CCR7 directs the migration of thymocytes into the thymic medulla." J Immunol 172(7): 3999-4007.

Kyewski, B. and L. Klein (2006). "A central role for central tolerance." Annu Rev Immunol 24: 571-606.

Laclef, C., E. Souil*, et al.* (2003). "Thymus, kidney and craniofacial abnormalities in Six 1 deficient mice." Mech Dev 120(6): 669-679.

Le Douarin, N. M. and F. V. Jotereau (1975). "Tracing of cells of the avian thymus through embryonic life in interspecific chimeras." J Exp Med 142(1): 17-40.

Le Lievre, C. S. and N. M. Le Douarin (1975). "Mesenchymal derivatives of the neural crest: analysis of chimaeric quail and chick embryos." Journal of embryology and experimental morphology 34(1): 125-154.

Lee, D., D. M. Prowse*, et al.* (1999). "Association between mouse nude gene expression and the initiation of epithelial terminal differentiation." Developmental biology 208(2): 362-374.

Lefebvre, V., W. Huang*, et al.* (1997). "SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1(II) collagen gene." Mol Cell Biol 17(4): 2336-2346.

Lesley, J., R. Hyman*, et al.* (1985). "Evidence that the Pgp-1 glycoprotein is expressed on thymus-homing progenitor cells of the thymus." Cell Immunol 91(2): 397-403.

Lind, E. F., S. E. Prockop*, et al.* (2001). "Mapping precursor movement through the postnatal thymus reveals specific microenvironments supporting defined stages of early lymphoid development." J Exp Med 194(2): 127-134.

Lindsay, E. A., F. Vitelli*, et al.* (2001). "Tbx1 haploinsufficieny in the DiGeorge syndrome region causes aortic arch defects in mice." Nature 410(6824): 97-101.

Liston, A., S. Lesage*, et al.* (2003). "Aire regulates negative selection of organ-specific T cells." Nat Immunol 4(4): 350-354.

Liu, C., T. Ueno*, et al.* (2005). "The role of CCL21 in recruitment of T-precursor cells to fetal thymi." Blood 105(1): 31-39.

Liu, Y., H. Li*, et al.* (2000). "Identification of an enhancer sequence within the first intron required for cartilage-specific transcription of the alpha2(XI) collagen gene." The Journal of biological chemistry 275(17): 12712-12718.

Liu, Z., S. Yu*, et al.* (2007). "Gcm2 is required for the differentiation and survival of parathyroid precursor cells in the parathyroid/thymus primordia." Dev Biol 305(1): 333-346.

Logan, C. Y. and R. Nusse (2004). "The Wnt signaling pathway in development and disease." Annu Rev Cell Dev Biol 20: 781-810.

Lomada, D., B. Liu*, et al.* (2007). "Thymus medulla formation and central tolerance are restored in IKKalpha-/- mice that express an IKKalpha transgene in keratin 5+ thymic epithelial cells." J Immunol 178(2): 829-837.

Lucas, B. and R. N. Germain (1996). "Unexpectedly complex regulation of CD4/CD8 coreceptor expression supports a revised model for CD4+CD8+ thymocyte differentiation." Immunity 5(5): 461-477.

Lucas, B., F. Vasseur*, et al.* (1994). "Production, selection, and maturation of thymocytes with high surface density of TCR." J Immunol 153(1): 53-62.

Maatouk, D. M., L. DiNapoli*, et al.* (2008). "Stabilization of beta-catenin in XY gonads causes male-to-female sex-reversal." Hum Mol Genet 17(19): 2949-2955.

Macatee, T. L., B. P. Hammond*, et al.* (2003). "Ablation of specific expression domains reveals discrete functions of ectoderm- and endoderm-derived FGF8 during cardiovascular and pharyngeal development." Development 130(25): 6361-6374.

Manley, N. R. (2000). "Thymus organogenesis and molecular mechanisms of thymic epithelial cell differentiation." Semin Immunol 12(5): 421-428.

Manley, N. R. and M. R. Capecchi (1995). "The role of Hoxa-3 in mouse thymus and thyroid development." Development 121(7): 1989-2003.

Manley, N. R. and M. R. Capecchi (1998). "Hox group 3 paralogs regulate the development and migration of the thymus, thyroid, and parathyroid glands." Dev Biol 195(1): 1-15.

Mansukhani, A., D. Ambrosetti*, et al.* (2005). "Sox2 induction by FGF and FGFR2 activating mutations inhibits Wnt signaling and osteoblast differentiation." J Cell Biol 168(7): 1065-1076.

Mao, B., W. Wu*, et al.* (2002). "Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling." Nature 417(6889): 664-667.

Marrack, P., M. Blackman*, et al.* (1989). "T-cell repertoire and thymus." Cold Spring Harb Symp Quant Biol 54 Pt 1: 105-110.

Martin, G. R. (1998). "The roles of FGFs in the early development of vertebrate limbs." Genes Dev 12(11): 1571-1586.

Martins, V. C., T. Boehm*, et al.* (2008). "Ltbetar signaling does not regulate Aire-dependent transcripts in medullary thymic epithelial cells." J Immunol 181(1): 400-407.

McCaughtry, T. M., M. S. Wilken*, et al.* (2007). "Thymic emigration revisited." J Exp Med 204(11): 2513-2520.

McDuffie, M., N. Roehm*, et al.* (1987). "T cell receptor/MHC interactions in the thymus and the shaping of the T cell repertoire." Transplant Proc 19(6 Suppl 7): 111-116.

Meier, N., T. N. Dear*, et al.* (1999). "Whn and mHa3 are components of the genetic hierarchy controlling hair follicle differentiation." Mechanisms of development 89(1-2): 215-221.

Metzger, T. C. and M. S. Anderson (2011). "Control of central and peripheral tolerance by Aire." Immunol Rev 241(1): 89-103.

Miller, J. R. (2002). "The Wnts." Genome Biol 3(1): REVIEWS3001.

Misslitz, A., O. Pabst*, et al.* (2004). "Thymic T cell development and progenitor localization depend on CCR7." J Exp Med 200(4): 481-491.

Moniot, B., S. Biau*, et al.* (2004). "SOX9 specifies the pyloric sphincter epithelium through mesenchymal-epithelial signals." Development 131(15): 3795-3804.

Moore-Scott, B. A. and N. R. Manley (2005). "Differential expression of Sonic hedgehog along the anterior-posterior axis regulates patterning of pharyngeal pouch endoderm and pharyngeal endoderm-derived organs." Dev Biol 278(2): 323-335.

Mori-Akiyama, Y., M. van den Born*, et al.* (2007). "SOX9 is required for the **differentiation** of **paneth cells** in the **intestinal epithelium**." Gastroenterology 133(2): 539-546.

Morita, S., T. Kojima*, et al.* (2000). "Plat-E: an efficient and stable system for transient packaging of retroviruses." Gene therapy 7(12): 1063-1066.

Muller, S. M., C. C. Stolt*, et al.* (2008). "Neural crest origin of perivascular mesenchyme in the adult thymus." J Immunol 180(8): 5344-5351.

Muller, S. M., G. Terszowski*, et al.* (2005). "Gene targeting of VEGF-A in thymus epithelium disrupts thymus blood vessel architecture." Proc Natl Acad Sci U S A 102(30): 10587-10592.

Mulroy, T., Y. Xu*, et al.* (2003). "beta-Catenin expression enhances generation of mature thymocytes." Int Immunol 15(12): 1485-1494.

Murata, S., K. Sasaki*, et al.* (2007). "Regulation of CD8+ T cell development by thymus-specific proteasomes." Science 316(5829): 1349-1353.

Murata, S., Y. Takahama*, et al.* (2008). "Thymoproteasome: probable role in generating positively selecting peptides." Curr Opin Immunol 20(2): 192-196.

Naiche, L. A., Z. Harrelson*, et al.* (2005). "T-box genes in vertebrate development." Annual review of genetics 39: 219-239.

Nakagawa, T., W. Roth*, et al.* (1998). "Cathepsin L: critical role in Ii degradation and CD4 T cell selection in the thymus." Science 280(5362): 450-453.

Nasreen, M., T. Ueno*, et al.* (2003). "*In vivo* treatment of class II MHC-deficient mice with anti-TCR antibody restores the generation of circulating CD4 T cells and optimal architecture of thymic medulla." J Immunol 171(7): 3394-3400.

Nehls, M., B. Kyewski*, et al.* (1996). "Two genetically separable steps in the differentiation of thymic epithelium." Science 272(5263): 886-889.

Nehls, M., D. Pfeifer*, et al.* (1994). "New member of the winged-helix protein family disrupted in mouse and rat nude mutations." Nature 372(6501): 103-107.

Nelson, W. J. and R. Nusse (2004). "Convergence of Wnt, beta-catenin, and cadherin pathways." Science 303(5663): 1483-1487.

Ng, L. J., S. Wheatley*, et al.* (1997). "SOX9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse." Developmental biology 183(1): 108-121.

Niehrs, C. (1999). "Head in the WNT: the molecular nature of Spemann's head organizer." Trends in genetics : TIG 15(8): 314-319.

Nossal, G. J. (1994). "Negative selection of lymphocytes." Cell 76(2): 229-239.

Ohnemus, S., B. Kanzler*, et al.* (2002). "Aortic arch and pharyngeal phenotype in the absence of BMP-dependent neural crest in the mouse." Mech Dev 119(2): 127-135.

Ohuchi, H., Y. Hori*, et al.* (2000). "FGF10 acts as a major ligand for FGF receptor 2 IIIb in mouse multi-organ development." Biochemical and biophysical research communications 277(3): 643-649.

Ortman, C. L., K. A. Dittmar*, et al.* (2002). "Molecular characterization of the mouse involuted thymus: aberrations in expression of transcription regulators in thymocyte and epithelial compartments." Int Immunol 14(7): 813-822.

Osada, M., E. Ito*, et al.* (2006). "The Wnt signaling antagonist Kremen1 is required for development of thymic architecture." Clin Dev Immunol 13(2-4): 299-319.

Osada, M., L. Jardine*, et al.* (2010). "DKK1 mediated inhibition of Wnt signaling in postnatal mice leads to loss of TEC progenitors and thymic degeneration." PLoS ONE 5(2): e9062.

Packham, E. A. and J. D. Brook (2003). "T-box genes in human disorders." Hum Mol Genet 12 Spec No 1: R37-44.

Palmer, E. (2003). "Negative selection--clearing out the bad apples from the T-cell repertoire." Nat Rev Immunol 3(5): 383-391.

Pan, X., H. Li*, et al.* (2006). "Ubc9 interacts with SOX4 and represses its transcriptional activity." Biochem Biophys Res Commun 344(3): 727-734.

Panda, D. K., D. Miao*, et al.* (2001). "The transcription factor SOX9 regulates cell cycle and differentiation genes in chondrocytic CFK2 cells." J Biol Chem 276(44): 41229-41236.

Patel, S. R., J. Gordon*, et al.* (2006). "Bmp4 and Noggin expression during early thymus and parathyroid organogenesis." Gene Expr Patterns.

Paterson, D. J. and A. F. Williams (1987). "An intermediate cell in thymocyte differentiation that expresses CD8 but not CD4 antigen." J Exp Med 166(5): 1603-1608.

Peifer, M. and P. Polakis (2000). "Wnt signaling in oncogenesis and embryogenesis--a look outside the nucleus." Science 287(5458): 1606-1609.

Petrie, H. T. and J. C. Zuniga-Pflucker (2007). "Zoned out: functional mapping of stromal signaling microenvironments in the thymus." Annu Rev Immunol 25: 649-679.

Pevny, L. H. and R. Lovell-Badge (1997). "Sox genes find their feet." Curr Opin Genet Dev 7(3): 338-344.

Pinto, D., A. Gregorieff*, et al.* (2003). "Canonical Wnt signals are essential for homeostasis of the intestinal epithelium." Genes & development 17(14): 1709-1713.

Plotkin, J., S. E. Prockop*, et al.* (2003). "Critical role for CXCR4 signaling in progenitor localization and T cell differentiation in the postnatal thymus." J Immunol 171(9): 4521-4527.

Polanco, J. C. and P. Koopman (2007). "Sry and the hesitant beginnings of male development." Developmental biology 302(1): 13-24.

Pongracz, J., K. Hare*, et al.* (2003). "Thymic epithelial cells provide WNT signals to developing thymocytes." Eur J Immunol 33(7): 1949-1956.

Pongracz, J. E., S. M. Parnell*, et al.* (2006). "Overexpression of ICAT highlights a role for catenin-mediated canonical Wnt signalling in early T cell development." Eur J Immunol 36(9): 2376-2383.

Proietto, A. I., S. van Dommelen*, et al.* (2008). "Dendritic cells in the thymus contribute to T-regulatory cell induction." Proc Natl Acad Sci U S A 105(50): 19869-19874.

Que, J., T. Okubo*, et al.* (2007). "Multiple dose-dependent roles for Sox2 in the patterning and differentiation of anterior foregut endoderm." Development 134(13): 2521-2531.

Raff, M. C. (1992). "Social controls on cell survival and cell death." Nature 356(6368): 397-400.

Rehberg, S., P. Lischka*, et al.* (2002). "Sox10 is an active nucleocytoplasmic shuttle protein, and shuttling is crucial for Sox10-mediated transactivation." Molecular and cellular biology 22(16): 5826-5834.

Revest, J. M., R. K. Suniara*, et al.* (2001). "Development of the thymus requires signaling through the fibroblast growth factor receptor R2-IIIb." J Immunol 167(4): 1954-1961.

Ritter, M. A. and R. L. Boyd (1993). "Development in the thymus: it takes two to tango." Immunol Today 14(9): 462-469.

Rodewald, H. R. (2008). "Thymus organogenesis." Annu Rev Immunol 26: 355-388.

Rodewald, H. R. and H. J. Fehling (1998). "Molecular and cellular events in early thymocyte development." Advances in immunology 69: 1-112.

Rodewald, H. R., M. Ogawa*, et al.* (1997). "Pro-thymocyte expansion by c-kit and the common cytokine receptor gamma chain is essential for repertoire formation." Immunity 6(3): 265-272.

Rodewald, H. R., S. Paul*, et al.* (2001). "Thymus medulla consisting of epithelial islets each derived from a single progenitor." Nature 414(6865): 763-768.

Roose, J., M. Molenaar*, et al.* (1998). "The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors." Nature 395(6702): 608-612.

Ropke, C., P. Van Soest*, et al.* (1995). "A common stem cell for murine cortical and medullary thymic epithelial cells?" Developmental immunology 4(2): 149-156.

Rossi, S. W., A. P. Chidgey*, et al.* (2007b). "Redefining epithelial progenitor potential in the developing thymus." Eur J Immunol 37(9): 2411-2418.

Rossi, S. W., L. T. Jeker*, et al.* (2007a). "Keratinocyte growth factor (KGF) enhances postnatal T-cell development via enhancements in proliferation and function of thymic epithelial cells." Blood 109(9): 3803-3811.

Rossi, S. W., W. E. Jenkinson*, et al.* (2006). "Clonal analysis reveals a common progenitor for thymic cortical and medullary epithelium." Nature 441(7096): 988-991.

Rossi, S. W., M. Y. Kim*, et al.* (2007c). "RANK signals from CD4+3- inducer cells regulate development of Aire-expressing epithelial cells in the thymic medulla." J Exp Med.

Sakaguchi, S., N. Sakaguchi*, et al.* (2001). "Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance." Immunol Rev 182: 18-32.

Sakai, D., T. Suzuki*, et al.* (2006). "Cooperative action of Sox9, Snail2 and PKA signaling in early neural crest development." Development 133(7): 1323-1333.

Sano, S., Y. Takahama*, et al.* (2001). "Stat3 in thymic epithelial cells is essential for postnatal maintenance of thymic architecture and thymocyte survival." Immunity 15(2): 261-273.

Sansom, O. J., K. R. Reed*, et al.* (2004). "Loss of Apc *in vivo* immediately perturbs Wnt signaling, differentiation, and migration." Genes & development 18(12): 1385-1390.

Sant'Angelo, D. B., B. Lucas*, et al.* (1998). "A molecular map of T cell development." Immunity 9(2): 179-186.

Savare, J., N. Bonneaud*, et al.* (2005). "SUMO represses transcriptional activity of the Drosophila SoxNeuro and human Sox3 central nervous system-specific transcription factors." Molecular biology of the cell 16(6): 2660-2669.

Scambler, P. J. (2000). "The 22q11 deletion syndromes." Hum Mol Genet 9(16): 2421-2426.

Scheiff, J. M., A. C. Cordier*, et al.* (1978). "The thymus of Nu/+ mice." Anat Embryol (Berl) 153(2): 115-122.

Scheller, M., J. Huelsken*, et al.* (2006). "Hematopoietic stem cell and multilineage defects generated by constitutive beta-catenin activation." Nature immunology 7(10): 1037-1047.

Schmidt-Ott, K. M. and J. Barasch (2008). "WNT/beta-catenin signaling in nephron progenitors and their epithelial progeny." Kidney Int 74(8): 1004-1008.

Schuddekopf, K., M. Schorpp*, et al.* (1996). "The whn transcription factor encoded by the nude locus contains an evolutionarily conserved and functionally indispensable activation domain." Proc Natl Acad Sci U S A 93(18): 9661-9664.

Schwab, S. R. and J. G. Cyster (2007). "Finding a way out: lymphocyte egress from lymphoid organs." Nature immunology 8(12): 1295-1301.

Scott, C. E., S. L. Wynn*, et al.* (2010). "SOX9 induces and maintains neural stem cells." Nat Neurosci.

Sekiya, I., K. Tsuji*, et al.* (2000). "SOX9 enhances aggrecan gene promoter/enhancer activity and is up-regulated by retinoic acid in a cartilage-derived cell line, TC6." The Journal of biological chemistry 275(15): 10738-10744.

Semenov, M. V., R. Habas*, et al.* (2007). "SnapShot: Noncanonical Wnt Signaling Pathways." Cell 131(7): 1378.

Senoo, M., F. Pinto*, et al.* (2007). "p63 Is essential for the proliferative potential of stem cells in stratified epithelia." Cell 129(3): 523-536.

Seymour, P. A., K. K. Freude*, et al.* (2008). "A dosage-dependent requirement for Sox9 in pancreatic endocrine cell formation." Dev Biol 323(1): 19-30.

Seymour, P. A., K. K. Freude*, et al.* (2007). "SOX9 is required for maintenance of the pancreatic progenitor cell pool." Proc Natl Acad Sci U S A 104(6): 1865-1870.

Shakib, S., G. E. Desanti*, et al.* (2009). "Checkpoints in the development of thymic cortical epithelial cells." J Immunol 182(1): 130-137.

Sharma, A. D., T. Cantz*, et al.* (2006). "The role of stem cells in physiology, pathophysiology, and therapy of the liver." Stem Cell Rev 2(1): 51-58.

Shores, E. W., W. Van Ewijk*, et al.* (1991). "Disorganization and restoration of thymic medullary epithelial cells in T cell receptor-negative scid mice: evidence that receptor-bearing lymphocytes influence maturation of the thymic microenvironment." European journal of immunology 21(7): 1657-1661.

Sinclair, A. H., P. Berta*, et al.* (1990). "A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif." Nature 346(6281): 240-244.

Sinner, D., J. J. Kordich*, et al.* (2007). "Sox17 and Sox4 differentially regulate beta-catenin/T-cell factor activity and proliferation of colon carcinoma cells." Mol Cell Biol 27(22): 7802-7815.

Smith, C. A., G. T. Williams*, et al.* (1989). "Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures." Nature 337(6203): 181-184.

Smith, J. M. and P. A. Koopman (2004). "The ins and outs of transcriptional control: nucleocytoplasmic shuttling in development and disease." Trends Genet 20(1): 4-8.

Soriano, P. (1997). "The PDGF alpha receptor is required for neural crest cell development and for normal patterning of the somites." Development 124(14): 2691-2700.

Soza-Ried, C., C. C. Bleul*, et al.* (2008). "Maintenance of thymic epithelial phenotype requires extrinsic signals in mouse and zebrafish." J Immunol 181(8): 5272-5277.

Spence, P. J. and E. A. Green (2008). "Foxp3+ regulatory T cells promiscuously accept thymic signals critical for their development." Proceedings of the National Academy of Sciences of the United States of America 105(3): 973-978.

Spokony, R. F., Y. Aoki*, et al.* (2002). "The transcription factor Sox9 is required for cranial neural crest development in Xenopus." Development 129(2): 421-432.

Sprent, J. and H. Kishimoto (2001). "The thymus and central tolerance." Philos Trans R Soc Lond B Biol Sci 356(1409): 609-616.

Sprent, J. and H. Kishimoto (2002). "The thymus and negative selection." Immunol Rev 185: 126-135.

Stadeli, R., R. Hoffmans*, et al.* (2006). "Transcription under the control of nuclear Arm/beta-catenin." Curr Biol 16(10): R378-385.

Starr, T. K., M. A. Daniels*, et al.* (2003). "Thymocyte sensitivity and supramolecular activation cluster formation are developmentally regulated: a partial role for sialylation." Journal of immunology 171(9): 4512-4520.

Stolt, C. C., P. Lommes*, et al.* (2003). "The Sox9 transcription factor determines glial fate choice in the developing spinal cord." Genes Dev 17(13): 1677-1689.

Su, D., S. Ellis*, et al.* (2001). "Hoxa3 and pax1 regulate epithelial cell death and proliferation during thymus and parathyroid organogenesis." Dev Biol 236(2): 316-329.

Su, D. M., S. Navarre*, et al.* (2003). "A domain of Foxn1 required for crosstalk-dependent thymic epithelial cell differentiation." Nat Immunol 4(11): 1128-1135.

Sudbeck, P., M. L. Schmitz*, et al.* (1996). "Sex reversal by loss of the C-terminal transactivation domain of human SOX9." Nat Genet 13(2): 230-232.

Sun, L., J. Guo*, et al.* (2010). "Declining expression of a single epithelial cell-autonomous gene accelerates age-related thymic involution." Aging Cell 9(3): 347-357.

Suniara, R. K., E. J. Jenkinson*, et al.* (1999). "Studies on the phenotype of migrant thymic stem cells." Eur J Immunol 29(1): 75-80.

Suniara, R. K., E. J. Jenkinson*, et al.* (2000). "An essential role for thymic mesenchyme in early T cell development." J Exp Med 191(6): 1051-1056.

Surh, C. D., E. K. Gao*, et al.* (1992). "Two subsets of epithelial cells in the thymic medulla." J Exp Med 176(2): 495-505.

Swann, J. B. and T. Boehm (2007). "Back to the beginning--the quest for thymic epithelial stem cells." Eur J Immunol 37(9): 2364-2366.

Takahama, Y. (2006). "Journey through the thymus: stromal guides for T-cell development and selection." Nat Rev Immunol 6(2): 127-135.

Takahama, Y., K. Tanaka*, et al.* (2008). "Modest cortex and promiscuous medulla for thymic repertoire formation." Trends Immunol 29(6): 251-255.

Takash, W., J. Canizares*, et al.* (2001). "SOX7 transcription factor: sequence, chromosomal localisation, expression, transactivation and interference with Wnt signalling." Nucleic Acids Res 29(21): 4274-4283.

Tamashiro, D. A., V. B. Alarcon*, et al.* (2008). "Ectopic expression of mouse Sry interferes with Wnt/beta-catenin signaling in mouse embryonal carcinoma cell lines." Biochimica et biophysica acta 1780(12): 1395-1402.

Taranova, O. V., S. T. Magness*, et al.* (2006). "SOX2 is a dose-dependent regulator of retinal neural progenitor competence." Genes Dev 20(9): 1187-1202.

Taylor, K. M. and C. Labonne (2005). "SoxE factors function equivalently during neural crest and inner ear development and their activity is regulated by SUMOylation." Dev Cell 9(5): 593-603.

Tomizuka, K., K. Horikoshi*, et al.* (2008). "R-spondin1 plays an essential role in ovarian development through positively regulating Wnt-4 signaling." Human molecular genetics 17(9): 1278-1291.

Topol, L., W. Chen*, et al.* (2008). "Sox9 inhibits Wnt signaling by promoting beta -catenin phosphorylation in the nucleus." J Biol Chem.

Tsai, A. D., L. C. Yeh*, et al.* (2003). "Effects of osteogenic protein-1 (OP-1, BMP-7) on gene expression in cultured medial collateral ligament cells." J Cell Biochem 90(4): 777-791.

Tsuda, M., S. Takahashi*, et al.* (2003). "Transcriptional co-activators CREB-binding protein and p300 regulate chondrocyte-specific gene expression via association with Sox9." The Journal of biological chemistry 278(29): 27224-27229.

Tsukamoto, N., M. Itoi*, et al.* (2005). "Lack of Delta like 1 and 4 expressions in nude thymus anlages." Cell Immunol 234(2): 77-80.

Tumanov, A. V., S. I. Grivennikov*, et al.* (2003). "Dissecting the role of lymphotoxin in lymphoid organs by conditional targeting." Immunol Rev 195: 106-116.

Uehara, S., K. Song*, et al.* (2002). "Characterization of CCR9 expression and CCL25/thymus-expressed chemokine responsiveness during T cell development: CD3(high)CD69+ thymocytes and gammadeltaTCR+ thymocytes preferentially respond to CCL25." J Immunol 168(1): 134-142.

Ueno, T., F. Saito*, et al.* (2004). "CCR7 signals are essential for cortex-medulla migration of developing thymocytes." J Exp Med 200(4): 493-505.

van Ewijk, W., G. Hollander*, et al.* (2000). "Stepwise development of thymic microenvironments *in vivo* is regulated by thymocyte subsets." Development 127(8): 1583-1591.

van Ewijk, W., E. W. Shores*, et al.* (1994). "Crosstalk in the mouse thymus." Immunol Today 15(5): 214-217.

Van Vliet, E., M. Melis*, et al.* (1986). "Reticular fibroblasts in peripheral lymphoid organs identified by a monoclonal antibody." J Histochem Cytochem 34(7): 883-890.

Venanzi, E. S., D. H. Gray*, et al.* (2007). "Lymphotoxin pathway and Aire influences on thymic medullary epithelial cells are unconnected." Journal of immunology 179(9): 5693-5700.

Vidal, V. P., M. C. Chaboissier*, et al.* (2005). "Sox9 is essential for outer root sheath differentiation and the formation of the hair stem cell compartment." Curr Biol 15(15): 1340-1351.

Vitelli, F., I. Taddei*, et al.* (2002). "A genetic link between Tbx1 and fibroblast growth factor signaling." Development 129(19): 4605-4611.

von Boehmer, H. (1994). "Positive selection of lymphocytes." Cell 76(2): 219-228.

von Boehmer, H. and H. J. Fehling (1997). "Structure and function of the pre-T cell receptor." Annu Rev Immunol 15: 433-452.

Wagner, T., J. Wirth*, et al.* (1994). "Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9." Cell 79(6): 1111-1120.

Wallin, J., H. Eibel*, et al.* (1996). "Pax1 is expressed during development of the thymus epithelium and is required for normal T-cell maturation." Development 122(1): 23-30.

Weerkamp, F., M. R. Baert*, et al.* (2006). "Wnt signaling in the thymus is regulated by differential expression of intracellular signaling molecules." Proc Natl Acad Sci U S A 103(9): 3322-3326.

Wegner, M. (1999). "From head to toes: the multiple facets of Sox proteins." Nucleic Acids Res 27(6): 1409-1420.

Weih, F., D. Carrasco*, et al.* (1995). "Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-kappa B/Rel family." Cell 80(2): 331-340.

Weinreich, M. A. and K. A. Hogquist (2008). "Thymic emigration: when and how T cells leave home." J Immunol 181(4): 2265-2270.

Weiss, M. A. (2001). "Floppy SOX: mutual induced fit in hmg (high-mobility group) box-DNA recognition." Molecular endocrinology 15(3): 353-362.

White, A. J., D. R. Withers*, et al.* (2008). "Sequential phases in the development of Aire-expressing medullary thymic epithelial cells involve distinct cellular input." Eur J Immunol 38(4): 942-947.

Wilson, M. and P. Koopman (2002). "Matching SOX: partner proteins and co-factors of the SOX family of transcriptional regulators." Current opinion in genetics & development 12(4): 441-446.

Wodarz, A. and R. Nusse (1998). "Mechanisms of Wnt signaling in development." Annu Rev Cell Dev Biol 14: 59-88.

Wu, L., R. Scollay*, et al.* (1991). "CD4 expressed on earliest T-lineage precursor cells in the adult murine thymus." Nature 349(6304): 71-74.

Xu, P. X., W. Zheng*, et al.* (2002). "Eya1 is required for the morphogenesis of mammalian thymus, parathyroid and thyroid." Development 129(13): 3033-3044.

Xu, W. and D. Kimelman (2007). "Mechanistic insights from structural studies of beta-catenin and its binding partners." J Cell Sci 120(Pt 19): 3337-3344.

Xu, X., J. D'Hoker*, et al.* (2008). "Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas." Cell 132(2): 197-207.

Xu, X., M. Weinstein*, et al.* (1999). "Fibroblast growth factor receptors (FGFRs) and their roles in limb development." Cell and tissue research 296(1): 33-43.

Yamazaki, H., E. Sakata*, et al.* (2005). "Presence and distribution of neural crest-derived cells in the murine developing thymus and their potential for differentiation." Int Immunol 17(5): 549-558.

Yang, S. J., S. Ahn*, et al.* (2006). "The quantitative assessment of MHC II on thymic epithelium: implications in cortical thymocyte development." Int Immunol 18(5): 729-739.

Yano, F., F. Kugimiya*, et al.* (2005). "The canonical Wnt signaling pathway promotes chondrocyte differentiation in a Sox9-dependent manner." Biochem Biophys Res Commun 333(4): 1300-1308.

Yano, M., N. Kuroda*, et al.* (2008). "Aire controls the differentiation program of thymic epithelial cells in the medulla for the establishment of self-tolerance." J Exp Med 205(12): 2827-2838.

Yu, Q., B. Erman*, et al.* (2004). "IL-7 receptor signals inhibit expression of transcription factors TCF-1, LEF-1, and RORgammat: impact on thymocyte development." J Exp Med 200(6): 797-803.

Zhang, C., T. Basta*, et al.* (2003). "The beta-catenin/VegT-regulated early zygotic gene Xnr5 is a direct target of SOX3 regulation." Development 130(23): 5609-5624.

Zhang, Y., S. Huang*, et al.* (2009). "SOX7, down-regulated in colorectal cancer, induces apoptosis and inhibits proliferation of colorectal cancer cells." Cancer Lett 277(1): 29-37.

Zhang, Z., T. Huynh*, et al.* (2006). "Mesodermal expression of Tbx1 is necessary and sufficient for pharyngeal arch and cardiac outflow tract development." Development 133(18): 3587-3595.

Zhou, G., Q. Zheng*, et al.* (2006). "Dominance of SOX9 function over RUNX2 during skeletogenesis." Proc Natl Acad Sci U S A 103(50): 19004-19009.

Zhou, R., N. Bonneaud*, et al.* (2002). "SOX9 interacts with a component of the human thyroid hormone receptor-associated protein complex." Nucleic Acids Res 30(14): 3245-3252.

Zorn, A. M., G. D. Barish*, et al.* (1999). "Regulation of Wnt signaling by Sox proteins: XSox17 alpha/beta and XSox3 physically interact with beta-catenin." Molecular cell 4(4): 487-498.

Zou, D., D. Silvius*, et al.* (2006). "Patterning of the third pharyngeal pouch into thymus/parathyroid by Six and Eya1." Dev Biol 293(2): 499-512.

Zuklys, S., J. Gill*, et al.* (2009). "Stabilized {beta}-Catenin in Thymic Epithelial Cells Blocks Thymus Development and Function." J Immunol 182(5): 2997-3007.

9. ACKNOWLEDGEMENTS

The present work was done in the laboratory of Pediatric Immunology at the Department of Biomedicine of University of Basel under the supervision of Prof. Dr. med. G.A. Holländer. First, I would like to thank Georg for giving me the opportunity to work in his lab on this interesting project, for his constant impulse, constructive criticism and continuous support till the very end of my thesis. What I will surly remember is the writing of weekly reports. Although at the beginning chaotic with all Georg`s corrections and patience they became more and more structured and helped me to develop the understanding of the subject with a critical point of view.

I am indebted to many people for their advice, encouragement and most of all for creating a great working and social environment. I enjoyed being part of this lab, where the work was mostly a pleasure and I felt always welcome. Special thanks go to Jason Gill who introduced me to the secrets of flow cytometry and immunohistology, who had many valuable ideas and suggestions and always found the time to answer my burning questions. I am also deeply grateful to Marcel Keller who supervised me at the very beginning of my PhD and for his help in the molecular cloning.

My sincere thanks go to many friends and colleagues for scientific discussion, advice and continuous support always so greatly appreciated, among them Kyung Na for showing me how to find the famous site of thymus development, the 3rd pharyngeal pouch and for the introduction in laser capture microscopy; Werner Krenger, Thomas Barthlott and Caroline Berkemeier for their professional help with TEC sorting; Noriko Shikama for her practical advices and ability to point to the important issues, also for her support during my writing period in the library and our early morning chats that I will miss; Gretel Nusspaumer for assisting me at my first i.p injection, for her open nature and readiness to help even if she had more than enough things of her own to do, and for sharing a cup of tea; Sebastian Löffler for his help with IP and WB and for our cute French talks; Katrin Hafen for teaching me the extraction of thymus primordium and BrdU staining advices; Annick Peter for taking care of cell cultures during my holidays; Mathias Hauri-Hohl, Saulius Zuklys, Werner Krenger for exciting tennis relays. Many thanks also to the rest of the lab members for the friendly lab atmosphere and additional help: Radhi Velayutham, Saule Zhanybekova, Elli Christen, Gabor Szinnai, Angela Bosch, Simone Dertschnig, Carlos Mayer, Sanjay Gawade and also Martha Gaio for administrative issues and supplying us so good with “Büro” equipment.

I like to thank Michèle Attenhofer for her assistance in sequencing; Pascal Lorentz for showing me Image-J program; Philippe Demougin from Biozentrum for performing DNA microarray.

I am grateful to the members of the animal facility, especially Rodrigo Recinos and Angelika Offinger for taking care of the mice.

I would like to thank the technical staff of the institute Peter Isler, Manfred Sieber, Daniel Wyniger for the fast and unproblematic help.

I owe thanks to several researchers who kindly provided reagents: Prof. Dr. Gerd Scherer (University of Freiburg, Germany) for the conditional Sox9 mice; Prof. Dr. Michael Wegner and Petra Lommes (Universität Erlangen-Nürnberg, Germany) for the Sox9 antibody; Toshio Kitamura (University of Tokyo, Japan) for PlatE cells and Nikolaus S. Trede (University of Utah, Salt Lake City, USA) for sharing his findings on the role of Sox9 in zebrafish thymus development.

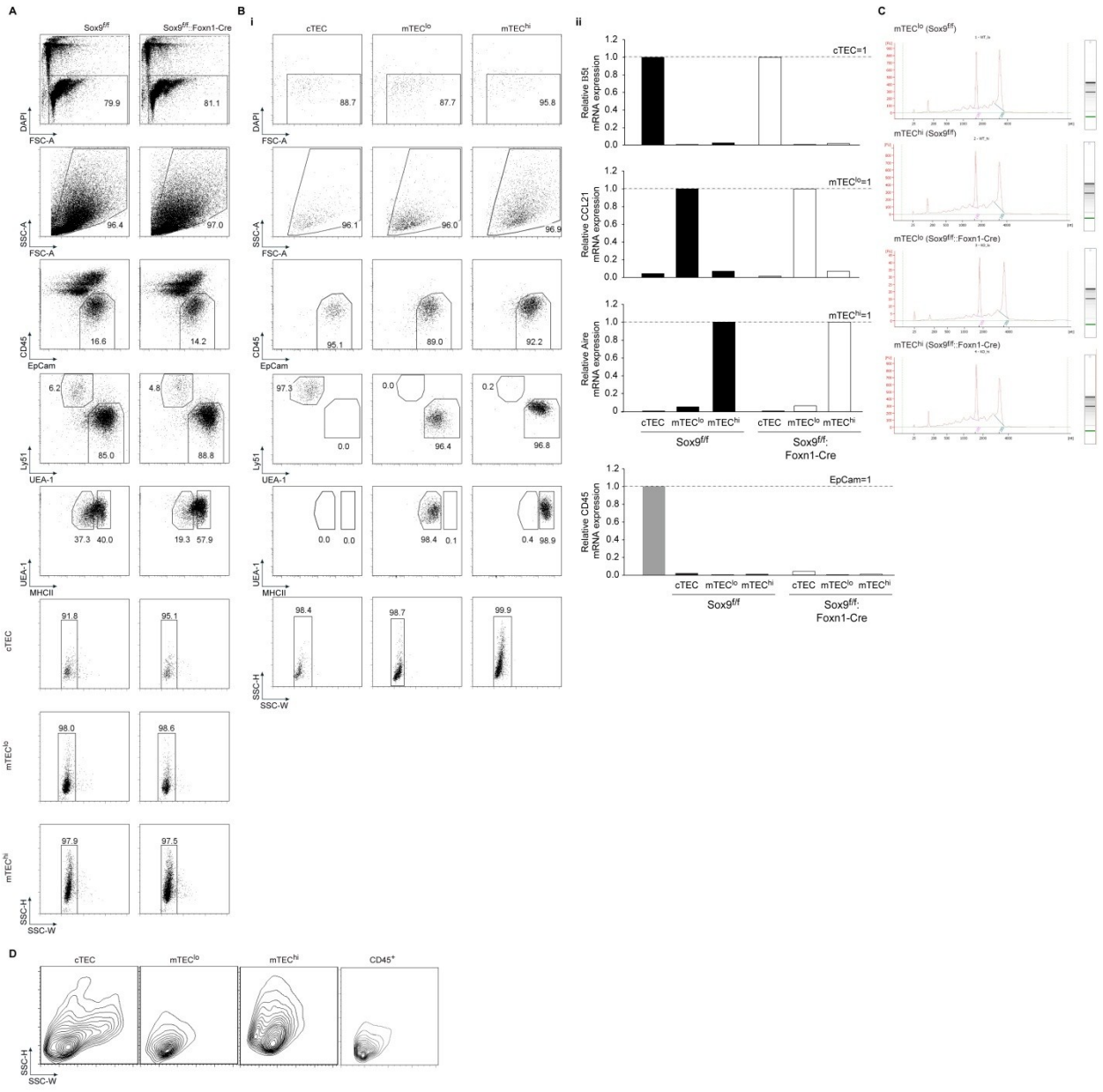
Additionally, I would like to thank the thesis committee Prof. Dr. A. Rolink and Prof. Dr. Ed Palmer for taking the time to read and evaluate my thesis and for the small talks about the dogs.

I am particularly grateful to my parents for their love, never-ending encouragement and support, always ready to help with their whole heart and soul.

Very special thanks go to my beloved fiancé Daniel who always found a word of comfort when I found myself lost in the "ivory tower" of science. He managed with such an easiness to find the simple and the understandable answer to the most complicated questions, not always related to science but to life in general and I am looking forward to our new chapter in life which is just about to start.

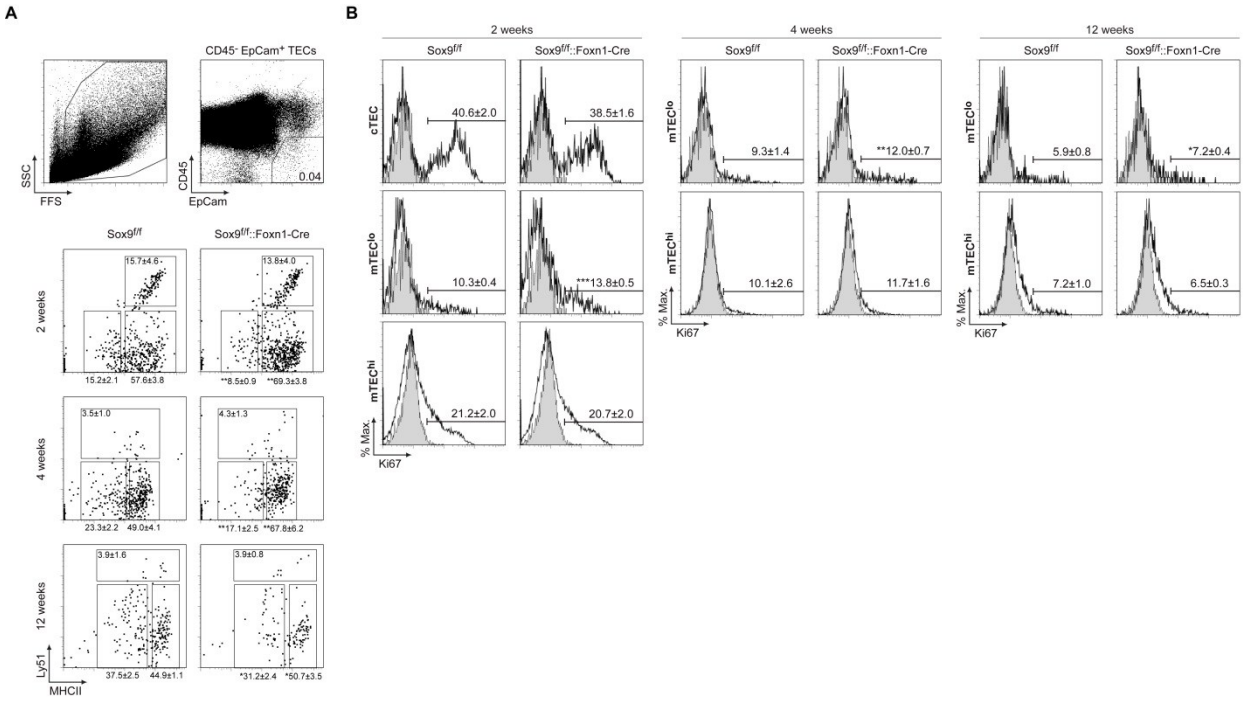
10. APPENDIX

10.1. Figures



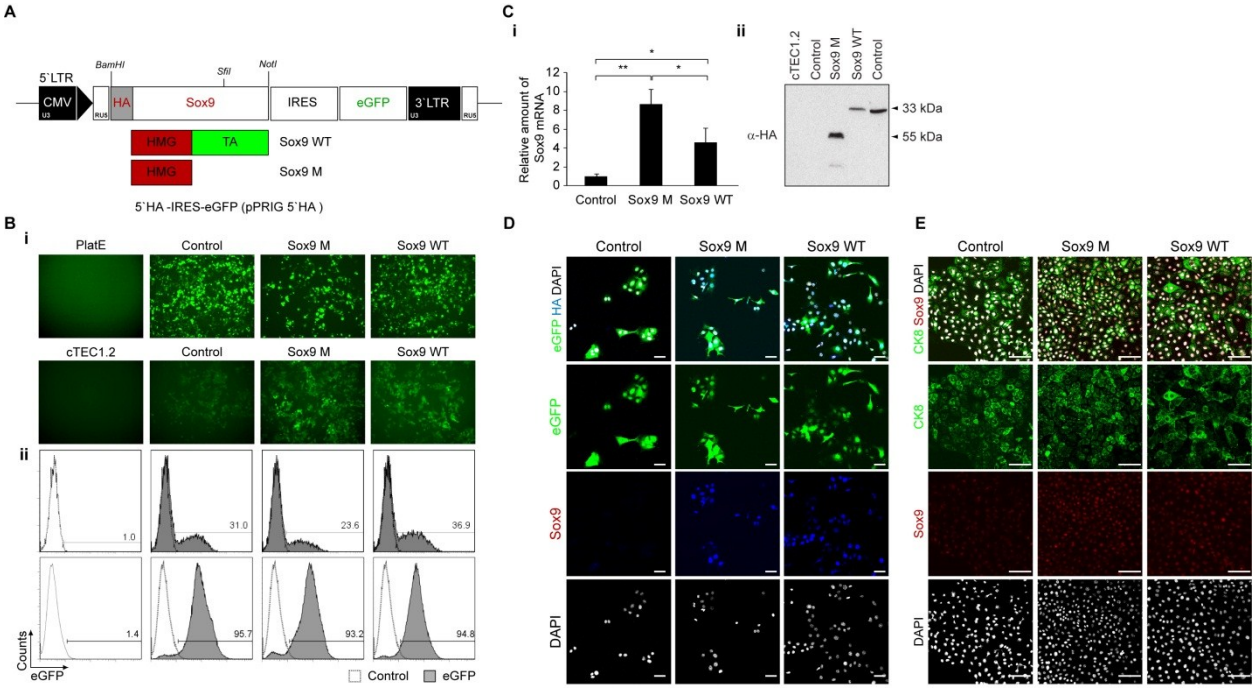
Appendix Figure 8.1. Cellular and molecular analysis of adult thymic epithelia.

(A) Gating strategy used to sort distinct TEC subsets from adult 4 week old Sox9 deficient and proficient mice. Alive ($DAPI^-$) TEC ($CD45^-EpCam^+$) were separated into cTEC ($UEA1^-Ly51^+$) and mTEC ($UEA\text{-}1^+Ly51^-$). The mTEC were further differentiated into immature ($MHCII^{lo}$; $mTEC^{lo}$) and mature ($MHCII^{hi}$; $mTEC^{hi}$) subsets. (B) Assessment of TEC purity by (i) re-analysing of sorted cell fractions and (ii) qRT-PCR analysis using ß5t as cTEC, Aire as $mTEC^{hi}$ and CCL21 as $mTEC^{lo}$ marker. The expression of each TEC subset-specific marker was set to an arbitrary value of 1 and compared with the signal detected in two other subsets, respectively. For assessment of possible contamination with the cells of hematopoietic origin, CD45 expression detected in distinct TEC subsets was normalized to GAPDH and compared to EpCam expression in the same subset which was arbitrary set to a value of 1. (C) RNA integrity of sorted TEC was assessed using RNA 6000 Pico assay and Agilent 2100 bioanalyzer. Electropherograms and corresponding gel picture are shown. (D) Sorted TEC subsets were plotted according to their size (FSC) and granularity (SSC).



Appendix Figure 8.2. Composition and proliferation of thymus with Sox9 proficient and deficient TEC.

(A) Representative dot plots show gates used to define and quantify three major TEC subsets present in whole thymic preparation of [Sox9$^{f/f}$] and [Sox9$^{f/f}$::Foxn1Cre] mice at the indicated ages. TEC defined as CD45^{-}EpCam^{+} cells were subdivided based on Ly51 and MHCII expression into: cTEC (MHCII^{+}Ly51^{+}), mTEC^{lo} (MHCII^{lo}Ly51^{-}) and mTEC^{hi} (MHCII^{hi}Ly51^{-}). Different gates were applied because levels of MHCII expression changed with age and the compensation needed to be accordingly adjusted. (B) Assessment of TEC proliferation in Sox9 deficient and proficient mice. Representative histograms of Ki67 expression in above defined TEC subsets with markers set according to isotype control are shown.



Appendix Figure 8.3. Retroviral transduction of cTEC1.2 line with mutant Sox9 (Sox9 M) and wild type Sox9 (Sox9 WT).

(A) Design of retroviral vector pPRIG 5'HA. CMV: cytomegalovirus promoter; LTR: long terminal repeat; HMG: high mobility group; TA: transactivation domain; IRES: internal ribosomal entry site; eGFP: enhanced green fluorescent protein; HA: hemagglutinin tag; Sox9 M: mutant Sox9; Sox9 WT: wild type Sox9. (B) Generation of retroviruses and transduction of target cells. (i) Transfection of Plat-E packaging cell line with an empty retroviral vector encoding eGFP (control) or a vector recombinant for either a HA-tagged Sox9 M- or a Sox9 WT- ires – eGFP (upper panels) and transduction of cTEC1.2 (lower panels). (ii) eGFP translated from IRES as a reporter gene served to assess transduction efficiency (upper panels) and to sort $eGFP^+$ cell populations which have stably integrated the viral construct into the genome (lower panels). (C) Assessment of Sox9 expression in stably transduced cTEC1.2. (i) Up regulation of Sox9 mRNA expression levels in cTEC1.2 transduced to express HA-tagged mutant (M) or wild type (WT) form of Sox9. The Sox9 data represents the mean ± SD of triplicate qRT-PCR analyses where Sox9 expression in cells transduced with an empty retroviral vector (control) was set arbitrary to a value of 1. The data is representative of four independent experiments. Statistical significance was calculated using the paired t-test; *p < .05, **p < .01. (ii) Detection of HA-tagged mutant and wild type Sox9 in transduced cells by WB analysis using anti-HA antibody. (D) Exogenous Sox9 protein expression in cTEC1.2 transduced with mutant or wild type Sox9 was assessed by immunofluorescence analysis using (i) anti-Sox9 and (ii) anti-HA antibodies.

10.2. Abbreviations

Aire	murine autoimmune regulator protein
APECED	autoimmune polyendocrinopathy candidiasis ectodermal dystrophy
bp	base pair
CD	cluster of differentiation
cDNA	complementary DNA
CDS	coding sequence
CK	cytokeratin
CMV	cytomegalovirus
cTEC	cortical thymic epithelial cell
DC	dendritic cell
DN	double negative T cells
DNA	deoxyribonucleic acid
DP	double positive T cells
E	embryonic day of gestation
ECM	extracellular matrix
eGFP =	enhanced green fluorescent protein
FACS	fluorescence activated cell sorting
H-2	mouse MHC (see also HLA and MHC)
HA tag	hemagglutinin tag
HLA	human leukocyte antigen (see also MHC and H-2)
HMG	high mobility group
HSC	hematopoietic stem cell
Ig	immunoglobulin
IRES	internal ribosomal entry site
ISP	immature single positive
LCM	laser capture microdissection
LTR	long terminal repeat
M	mutant
MHC	major histocompatibility complex (See also HLA and H-2)
mAb	monoclonal antibody
mRNA	messenger RNA
mTEC	medullary thymic epithelial cell
NCC	neural crest cells
ORF	open reading frame
NK	natural killer cell
NLS	nuclear localization signal
pAb	polyclonal antibody

PCR	polymerase chain reaction
PGA	promiscuous gene expression
pp	pharyngeal pouch
qRT-PCR	quantitative real time polymerase chain reaction
Rag	recombination activation gene
RNA	ribonucleic acid
RT	room temperature
RTE	recent thymic emigrant
SCID	severe combined immunodeficient
SOX9	human Sry-box containing protein 9
Sox9	murine Sry-box containing protein 9
sox9	murine Sry-box containing gene 9
SP	single positive T cells
TA	transactivation domain
TCR	T cell receptor
TEC	thymic epithelial cell
TSA	tissue specific antigen
WT	wild type

10.3. Figure and Table Index

10.4. Meetings and Posters

19.-20. April 2007 Annual Congress SGAI-SSAI, Basel, Switzerland

19.-22. May 2007 “Rolduc Workshop on Thymocyte and T cell Biology”, Rolduc Abbey, Kerkrade, Netherlands

10.-12. September 2009, “Second Basel Immunology Focus Symposium”, Basel, Switzerland

13.10.2009, “Biovalley Science Day”, Basel, Switzerland

15.-16. April 2010, “Translational Immunology”, Annual Congress SGAI-SSAI, St. Gallen, Switzerland

(see Poster next page)

Departement Biomedizin Basel

Sox9 controls Foxn1 expression in thymic epithelia and influences their differentiation and proliferation

Tatjana Žalac, Kyung Na, Marcel P. Keller, Jason Gill, Salius Zuklys, Nikolaus S. Trede*, Gerd Scherer#, Katrin Hafen, Werner Krenger, and Georg A. Holländer

*Pediatric Immunology, Center for Biomedicine, University of Basel and The University Children's Hospital (UKBB), Basel, Switzerland; * Huntsman Cancer Institute, Department of Pediatrics, University of Utah, Salt Lake City; # Institute of Human Genetics, University of Freiburg, Freiburg, Germany*

Introduction

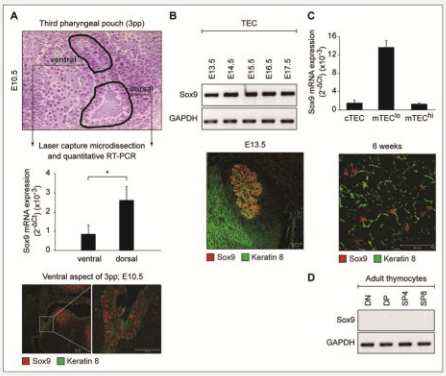
T-cell development is controlled in the thymus by a unique microenvironment largely composed of thymic epithelial cells (TEC). These stromal cells form the scaffold of thymus cortex and medulla and exist as different functional and phenotypic subpopulations. Recently, canonical Wnt signaling in TEC was identified to be essential for thymus organogenesis and the expression of Foxn1, a key regulator of TEC differentiation. β–catenin is a signaling molecule in the canonical Wnt signaling pathway and its precise regulation is required for normal TEC development. Canonical Wnt signaling is modulated by Sox9, a member of the SOX (Sry-Box) transcription factor family, via physical interaction with β-catenin.

Rationale of the study

The cellular and molecular mechanisms responsible for TEC differentiation and homeostatic maintenance remain incompletely defined.
Because Sox9 plays a role as both a transcriptional target and a regulator of Wnt-mediated signaling and since a precise Wnt signaling is required for normal TEC biology, we sought to investigate whether Sox9 expression impacts on TEC differentiation and function via its control of Wnt activity.

Results

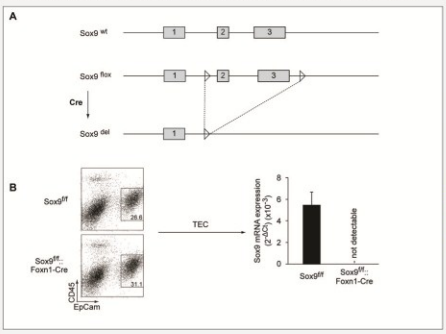
1. Stromal cell-specific expression of Sox9 in the thymus



(A) Sox9 expression in the third pharyngeal pouch (3pp), embryonic day 10.5 (E10.5).
(B) Sox9 expression during fetal TEC (CD45$^-$EpCam$^+$) development (PCR & IHC).
(C) Sox9 expression in cortical (cTEC) and medullary (mTEC) subsets isolated from adult wild type mice. mTEC cells were subdivided into immature MHCII^{lo} (mTEC^{lo}) and mature MHCII^{hi} (mTEC^{hi}) subpopulations.
(D) Sox9 expression in adult thymocytes.

Sox9 is expressed during thymus development as early as E10.5 in stromal but not lymphoid cells. mTEC^{lo} cells express the highest Sox9 mRNA levels.

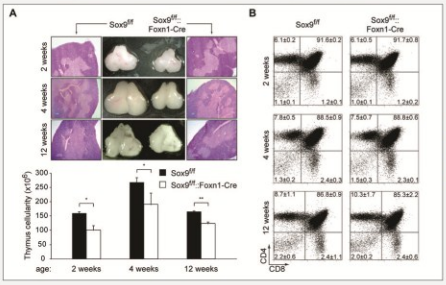
2. Generation of mice deficient for Sox9 expression in TEC



(A) Sox9 locus in wild type and gene targeted mice. Cre-mediated deletion of exons 2 and 3 in TEC.
(B) Quantification of Sox9 expression in TEC isolated by cell sorting from 4 week old Sox9$^{f/f}$ and Sox9$^{f/f}$::Foxn1-Cre mutant mice.

Foxn1-Cre mediated recombination of the Sox9 locus results in a complete loss of Sox9 mRNA in TEC isolated from Sox9$^{f/f}$::Foxn1-Cre mice.

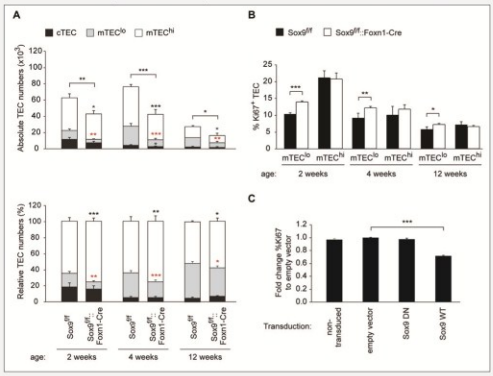
3. TEC-targeted loss of Sox9 leads to thymic hypoplasia



(A) Comparative analysis of thymic architecture and cellularity in mice with or without Sox9 expression in TEC.
(B) Flow cytometric analysis of T-cell development.

Sox9-deficient mice have a hypoplastic thymus with normal thymic architecture and T-cell development.

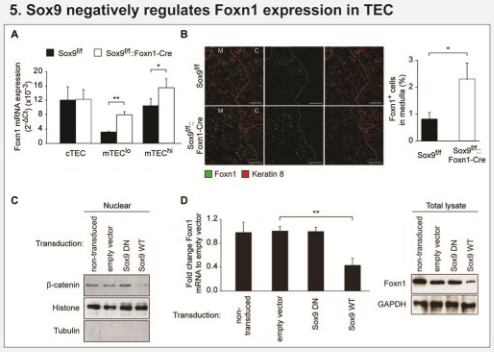
4. Altered composition and proliferation of Sox9-deficient TEC



(A) Absolute and relative TEC cellularity.
(B) Frequency of proliferating (Ki67$^+$) mTEC in 2 to 12 week old mice.
(C) Cell proliferation in a TEC line retrovirally transduced to over-express wild type (WT) or dominant negative form of Sox9 (Sox9 DN).

Loss of Sox9 leads to an alteration in the representation of different TEC subpopulations and their proliferation.
High Sox9 expression levels correlate with decreased TEC proliferation.

5. Sox9 negatively regulates Foxn1 expression in TEC



(A) Foxn1 mRNA expression in TEC subsets isolated from 4 week old mice.
(B) Foxn1 protein expression and Image J analysis of thymus tissue.
(C) β-catenin protein expression in a TEC line transduced to over-express Sox9 WT or Sox9 DN.
(D) Foxn1 expression in a retrovirally transduced TEC line.

Sox9 expression levels inversely correlate with β-catenin and Foxn1 expression.

Conclusion

Lack of Sox9 expression in TEC results in subset-specific changes in their representation and proliferation, a phenotype that correlates with an up-regulation of Foxn1 expression.

This study also provides evidence that Sox9 acts as a negative regulator of Foxn1 expression in TEC most likely via its interaction with β-catenin.

Poster presented in St.Gallen 2010

10.5. Curriculum Vitae

Personal information

Name	**Tatjana Žalac**
Address	Roggenburgstrasse 21, 4055 Basel
Phone	+41 61 321 54 09
Mobile	+41 76 545 13 47
E-Mail	tatjana.zalac@unibas.ch
Birth date/-place	8. March 1975, Baden, Switzerland
Citizenship	Croatian
Marital status	single
Work Address	Pediatric Immunology Center for Biomedicine University of Basel Mattenstrasse 28 4058 Basel
Phone	+41 61 695 30 77 +41 61 695 30 08

Education and Professional Experience

1981-1989	Primary school “Veceslav Holjevac“, Zagreb, Croatia
1989-1993	V. Gymnasium; Natural-scientific and mathematical Gymnasium, Zagreb, Croatia
1994-2001	University of Zagreb, Faculty of Natural Sciences, Department of Biology, Ecology
2000-2001	Practical work for the diploma thesis in the Laboratory for Parasitology at the Veterinary Faculty, University of Zagreb Degree: Bachelor of Science in Biological Engineering Diploma thesis: "Research on Endoparasite Fauna in Feces of Living Snakes."
2001	Voluntary work in the Laboratory for Molecular Medicine/Oncology at the Institute “Ruđer Bošković”, Zagreb (3 months)
2001	Summer student in the Laboratory for Molecular Immunology, Department of Medical Parasitology & Infection Biology, Swiss Tropical Institute, Basel (3 months)
2002-2003	ESKAS scholarship in the Laboratory for Molecular Immunology, Department of Medical Parasitology & Infection Biology, Swiss Tropical Institute, Basel
2004-2006	Diagnostic and Research Biologist in the Laboratory for Molecular Diagnostics, HIV Diagnostic Laboratory, InPheno AG, Basel
2006-present	PhD at the Center for Biomedicine in the group of Prof. G. A. Holländer in biological-medical research, University of Basel

Publication

Diaz, D., C. A. Daubenberger, *et al.* (2002). "Sequence and expression of MHC-DPB1 molecules of the New World monkey Aotus nancymaae, a primate model for Plasmodium falciparum." Immunogenetics **54**(4): 251-9.

Skills

Technical: molecular biology techniques (PCR, quantitative RT-PCR, cloning, sequencing), biochemical techniques (protein expression and purification), immunochemical techniques (ELISA, IP & Western blotting, IHC), FACS, BrdU-based proliferation assay, cell culture and others.

Permit to work with animals (LTK Module 1E corresponding to FELASA Cat. B).

EDV: MS Office programs (Word, Excel, Powerpoint)

Adobe Programs (Illustrator, Photoshop)

Endnote

Languages: Croatian (first language)

English (fluent)

German (fluent)

French (basic)

Hobbies

Tennis, Swimming, Jogging, Volleyball, Inline-skating, Dancing, Music, Reading

Miscellaneous

Licensed Volleyball Trainer

2002 Au-Pair in Ashford, UK (5 months)

10.6. Declaration

I declare that I wrote this thesis “The Role of the Transcription Factor Sox9 for Thymic Epithelial Cell Differentiation and Function” with the help indicated and only handed it in to the faculty of science of the University of Basel and to no other faculty and no other university.

Tatjana Žalac