LOSS OF FOXG1 IN THYMIC EPITHELIAL CELLS LEADS TO THYMUS HYPOPLASIA IN FETAL AND ADULT MICE AND A DECREASE IN THE EXPRESSION OF THE ESSENTIAL CYTOKINE IL-7

by

B. RODNEY JARVIS

(Under the Direction of Brian G. Condie)

ABSTRACT

The thymus is responsible for providing the necessary microenvironment for the production of a self-restricted, self-tolerant T-cell pool. These T-cells are necessary for the function of an adaptive immune system. Thymic epithelial cells (TEC) are the major component of the microenvironment and are essential for providing signals to the developing thymocytes. However, the genetic networks and signaling pathways responsible for proliferation, differentiation, and survival of TEC are still being uncovered. The transcription factor, *Foxn1*, has been called the master regulator of TEC and is essential for thymus development. Although, some evidence has shown that there are some aspects of thymus development which are not dependent on *Foxn1*. Our lab previously identified expression of the transcription factor, *Foxg1*, in thymus and showed it to be necessary for normal thymus development by controlling *Foxn1*-independent mechanisms of TEC differentiation, survival, and regulation of the essential cytokine *IL-7*.

This dissertation presents further analysis of the phenotypes caused by loss of *Foxg1*. Specifically, I utilized a conditional loss of function model by deleting *Foxg1* in TEC

(*Foxg1CKO*). I examined the phenotypes of these mutants and show that the defects present in the *Foxg1* null mice are also present in the *Foxg1CKO* at embryonic and postnatal stages, namely thymic hypoplasia, defective differentiation, and increased apoptosis. I also show that *Foxg1* is not necessary for initiation of *IL-7* but that it is necessary for normal expression levels of this cytokine in TEC. Furthermore, I provide preliminary evidence that suggests the regulation of *IL-7* during early thymus organogenesis is due to interactions between *Foxg1* and the transcription factor *Hoxa3*. Finally, I discuss possible interactions between *Foxg1* and several signaling pathways present in the thymus that *Foxg1* has been shown to directly interact with in the developing telencephalon.

INDEX WORDS: Thymus, *Foxg1*, *IL-7*, *Foxn1*, Thymic Epithelial Cells, Differentiation, Apoptosis, Thymus microenvironment

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DEDICATION

I dedicate this dissertation to my wife, Katherine Jarvis, who has given me all the love a man could ask for. You are an amazing mother to our beautiful daughters, Addison and Lillian Jarvis, and to my daughter, Kyra Jarvis. This has been a long and sometimes difficult journey for our family and I am eternally grateful to you for taking it with me. Thank you for believing in me and keeping me focused on what is most important in this life. I also dedicate this to my children for being patient with me being, sometimes, too busy to play and for bringing me more joy then I thought possible. Also to our newest family member that is still developing, we are excited to meet you and look forward to getting to know you.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

My dissertation research was to further investigate the role of the transcription factor, Foxg1, during thymus development. Development and function of this organ requires the interaction of many different signaling pathways and transcriptional networks. The following Literature Review attempts to cover much of the essential aspects of both the development of and the function of the thymus. Furthermore, it relates information about the known functions of Foxg1 in the central nervous system (CNS) and what is known so far about its functions in the thymus. Discussion of potential similarities of Foxg1 function between these two organs is presented in Chapter 3 of this dissertation instead of here.

Evolution and Function of the Thymus

Evolution

Adaptive immunity is common to all vertebrates, although the production of antigens by lymphocytes is solved differently in jawed and jawless vertebrates (Cooper and Alder 2006). Agnathosomes produce diversified variable lymphocyte receptors (VLR) by recombination of leucine-rich-repeat regions, while gnathosome lymphocytes produce either T-cell receptors (TCR) or B-cell receptors (BCR) through rearrangement of (V)ariable-(D)iversifying-(J)oining segments. TCR have also been detected in cartilaginous fish, such as shark (Criscitiello et al. 2010).

The thymus is the primary site for T-cell development and is an evolutionary conserved organ amongst all jawed vertebrates (Boehm and Bleul 2007) and potentially jawless vertebrates (Bajoghli et al. 2011). However, the thymus is highly variable from shark to mammal. For example, the first six branchial pouches produce thymus in shark, while in most bony fish and mammals the thymus is restricted to the third and/or fourth pouch (Ge and Zhao 2013). Indeed in mice, the thymus originates from the third pharyngeal pouch endoderm and migrates away from the pharynx towards the midline (Gordon and Manley 2011) while in birds and fish the thymus never separates from the pharynx. The similarity in morphology and genetic programs in thymus between mice and humans, as well as the genetic tractability of mice, makes the murine thymus a good model for investigation of human thymus function and development (Ge and Zhao 2013).

Lymphopoiesis

Lymphocyte progenitors are produced in the bone marrow from common hematopoietic stem cells (HSC) (Lai and Kondo 2008). In mice, HSC originate first from the yolk sac at E8.25, reside in the fetal liver by E11.0, and finally home to the fetal bone marrow (BM), which provides the necessary microenvironment for their self-renewal (reviewed in (Coskun et al. 2014)). Here they reside in postnatal stages as well. These HSC first differentiate into lineage restricted multi-potent progenitors, either common myeloid progenitors (CMP) or common lymphoid progenitors (CLP). The CMP is capable of producing any of the myeloid cells which includes erythrocytes, macrophages, and granulocytes. The CLP gives rise to natural killer (NK), T, and B-cells. These later two cell types are the cells responsible for adaptive immunity, while NK cells distinguish and destroy abnormal cells without the use of somatically recombined receptors similar to innate immune cells. While the HSC in bone marrow associate with osteoblasts, which provide the niche for self-renewal, B lymphoid progenitors are found

dispersed in the marrow where they associate first with CXCL12 expressing cells and then Interleukin 7 (IL-7) expressing cells (Tokoyoda et al. 2004). CXCL12 has been shown to be necessary for chemotaxis of hematopoietic progenitors (Bleul et al. 1996) and IL-7 is required for proliferative expansion of pro-B cells (von Freeden-Jeffry et al. 1995). Indeed, the CXCL12 associating cells were found to be pre-pro-B-cells, while the IL-7 associating cells were pro-B-cells (Tokoyoda et al. 2004).

T-cells are also produced from BM inhabiting HSC. However, there is some evidence that they may not originate from CLPs but may be derived from an earlier MPP that expresses CCR9 and is capable of circulating in the bloodstream (Schwarz et al. 2007). However, this discrepancy is likely due to the *in vitro* culture assays used that showed CLP still have myeloid potential (Richie Ehrlich, Serwold, and Weissman 2011). These T-cell progenitors do not mature in the BM like B-cells, but instead home to the thymus. This homing is partially controlled by CCL21/CCL25 chemokine signals in the pre-vascular thymus during embryonic development (Liu et al. 2006; Liu et al. 2005). After vascularization at around embryonic day 15.5 (E15.5), the mechanism for seeding of T-lymphoid progenitor cells (T-LPC) into the thymus involves P-selectin on vasculature and its ligand PSGL-1 which is expressed by T-LPCs (Rossi et al. 2005). These cells have no cell renewal capacity and therefore must periodically seed the thymus throughout the life of the organism (Foss, Donskoy, and Goldschneider 2001).

Thymus organization

The thymus is composed of two distinct regions, the cortex and the medulla, each with specialized functions. The stromal component of the organ is comprised of thymic epithelial cells (TEC), dendritic cells, macrophages, fibroblasts, and endothelial cells which are all responsible for different aspects of thymic function (Anderson, Lane, and Jenkinson 2007; Boyd et al. 1993;

Blackburn and Manley 2004; Anderson and Jenkinson 2001). The TEC are located in both the cortex (cTEC) and medulla (mTEC). These epithelial cells are responsible for much of the cell-cell interaction and signaling with developing thymocytes. Cortical TECs are characterized by expression of K8 (Klug et al. 2002), the thymoproteasome β5t (Xing, Jameson, and Hogquist 2013), CD205 (Shakib et al. 2009), and Ly51 (Gray, Chidgey, and Boyd 2002). Medullary TECs, however, express Keratins 5 and 14 (K5/K14) predominately (Klug et al. 2002). Subsets of these medullary TEC also express the autoimmune regulator *Aire* (Anderson et al. 2002), tight junction proteins *Claudin 3/4* (Hamazaki et al. 2007), and K14(-) mTEC that bind the lectin *Ulex europeus* (Farr and Anderson 1985; Klug et al. 2002). These cellular components of the thymus are responsible for providing the necessary microenvironment for the development of a self-restricted, self-tolerant T-cell repertoire.

T-cell maturation in the thymus

After homing to the thymus, T-LPCs enter via the vasculature located at the cortico-medullary junction (CMJ) (reviewed in (Petrie and Zuniga-Pflucker 2007)). All thymocytes express the cell surface marker CD45 and the transcription factor *Ikaros* (Wang et al. 1996), the latter is required for development of all T-cell lineages. These thymic immigrants are further characterized by expression patterns of CD4, CD8, CD44, and CD25, with this initial population being CD4-CD8-CD44+CD25- (DN1). After entry into the thymus, these DN1 cells undergo massive proliferation (Lind et al. 2001). This proliferation, survival and the subsequent differentiation of thymocytes is mediated by TEC produced signaling molecules that include *Kit* ligand (Rodewald et al. 1997) , *Interleukin-7* (IL-7) (Peschon et al. 1994), and Notch ligand *Dll-4* (Hozumi et al. 2008). After expansion of the DN1 population, thymocytes begin migrating outward toward the subcapsular zone (SCZ) via interactions with V-CAM1+ stromal cells and

most likely the chemokine CCL25 (reviewed in (Petrie and Zuniga-Pflucker 2007)). These migrating lineage restricted thymocytes (DN2) are CD44+CD25+ and have lost the potential to give rise to NK and B cells, but are still capable of giving rise to either αβ or γδ T-cells (Lind et al. 2001). This T-cell lineage commitment is due to interactions through the Notch1 receptor and TEC expressed Notch ligand, *Dll-4* (Hozumi et al. 2008). This Notch signaling along with TEC secreted IL-7 signals, which induce expression of the anti-apoptotic molecule *Bcl-2*, leads to survival of these DN2 cells (Boudil et al. 2015). At this stage of development, some of the thymocytes commit to the γδ T-cell lineage through recombination of the γδ T-cell receptors (TCR) (Livak et al. 1999) and this recombination is due to expression of two recombinase activating genes, *Rag*-1 and *Rag-2* (Wilson, Held, and MacDonald 1994). In general, Rags initiate the cleavage in the immunoglobin (Ig) genes by creating nicks in specific sites which lead to hairpin structures that get resolved imperfectly through non-homologous end joining thereby leading to a diversity of V(D)J rearranged TCR (Nishana and Raghavan 2012).

Continued migration of developing thymocytes towards the SCZ leads to the progression of DN2 cells to the next stage of development, DN3 (CD44-CD25+). This stage of thymocyte development is marked by irreversible commitment to the αβ-T-cell lineage due to recombination at the TCRβ locus (Petrie et al. 1995). Recombination of the TCRβ elements involves sequential rearrangement of the variable (V), diversity (D), and joining (J) gene elements (Muegge, Vila, and Durum 1993) which are linked together by site specific recombination (Nishana and Raghavan 2012). Accessibility of the TCRβ locus was shown by Muegge et al. (1993) to be dependent on *IL-7,* although the exact mechanism for this is not yet clear. IL-7 signaling to thymocytes occurs through the IL-7R and common γ chain receptor (γ_c) that are expressed by developing DN cells at different stages. As mentioned above, IL-7

signaling is important for survival of early DN1-DN2 thymocytes due to its regulation of the anti-apoptotic factor *Bcl-2* (Boudil et al. 2015). In addition to this and allowing access to the TCRβ locus, IL-7 is necessary for DN3 cells that have successfully rearranged the TCRβ locus (β-selection) and that express the pre-TCR, CD3, and Notch1 to undergo clonal expansion and further differentiation through these receptors and Notch ligands expressed by cTEC (Boudil et al. 2015; Petrie and Zuniga-Pflucker 2007).

Successful signaling through the pre-TCR on these DN cells initiates rearrangement at the α locus and expression of both CD4 and CD8 (DP) (Starr, Jameson, and Hogquist 2003). These DP cells begin migrating back through the cortex towards the medulla. During this migration, DP cells become quiescent after they successfully rearrange the TCRα locus. This exit from the cell cycle occurs only after successful binding of the TCR to self-antigens presented through major histocompatability complex I and II (pMHC), which is displayed by cTECs. Cortical TECs express distinct lysosomal proteases; cathepsin L and thymus-specific serine protease (TSSP) which process antigens in the MHC II presenting pathway (reviewed in (Xing, Jameson, and Hogquist 2013). The cTECs also express a unique thymoproteasome subunit, β5t, that is utilized in the MHC I antigen presenting pathway. The peptides presented by the cTEC differ from other antigen presenting cells (APC) in the thymus. Specifically, β5t, allows for presentation of antigens that are bound by the TCR with low affinity. This is crucial to the cTEC function in positive selection (Xing, Jameson, and Hogquist 2013). Positive selection in the cortex leads to mature thymocytes that become either MHC I or MHC II restricted CD4+ or CD8+ SP cells, respectively (reviewed in ((Xing, Jameson, and Hogquist 2013; Starr, Jameson, and Hogquist 2003; Petrie and Zuniga-Pflucker 2007). This selection is mediated by functional TCR expressed on DP thymocytes that can successfully bind to self-antigen MHC

complexes presented by the cTEC, as well as IL-7/IL-7R signaling (Yu et al. 2003). The cells that fail to engage MHC with the required affinity by their TCR do not upregulate IL-7R and do not therefore receive survival signals through IL-7 and undergo apoptosis (Carrette and Surh 2012).

After positive selection and differentiation into either CD4+SP helper T-cells or cytotoxic CD8+SP T-cells, these cells then enter the medulla where they interact with mTEC and other APC, such as dendritic cells (DC). The main function of the thymic medulla is to promote central tolerance through negative selection of self-reactive thymocytes. Negative selection is able to occur due to the presentation of tissue specific antigens (TSA) either directly by mTEC or by antigen transfer to (DC) (reviewed in (Klein et al. 2014)). mTEC are capable of producing TSA that represent all peripheral organs due to promiscuous gene expression partially under the control of the autoimmune regulator (*Aire*) (Derbinski et al. 2005). Therefore, thymocytes with TCR that bind with high affinity to any of the TSA are signaled for clonal deletion or potentially becoming CD4+FOXP3+ regulatory T-cells (reviewed in (Takahama 2006; Klein et al. 2014)). This process is necessary so that no potentially auto-reactive T-cells escape the thymus and enter the periphery where they can potentially cause autoimmune diseases.

From beginning to end, the purpose of thymocyte migration through the thymus is to produce functional T-cells, that can bind antigens through their TCR, from a pool of multi-potent progenitors derived from HSC. These cells receive all the necessary instructive signals either through direct interaction with TEC and other cells in the thymus or through secreted signals by these cells. The end result is a highly effective population of immune cells that can recognize foreign antigens and eliminate the invading body, all while being self-tolerant.

Specification and Patterning of the Thymus

Early Signals in the Third Pouch

The thymus consists of two bilateral lobes surrounded by a neural crest cell (NCC) derived mesenchymal capsule and originates solely from third pharyngeal pouch endoderm (Gordon et al. 2004). In mice there are four pharyngeal pouches which form as an out-pocketing of the pharyngeal endoderm during embryogenesis. The cells at the ventral end of the pouch, which will become thymic epithelium, are already specified by embryonic day 9.0 (E9.0). The earliest known signaling pathways involved in patterning and differentiation in the parathyroid and thymus are Sonic hedgehog (Shh) and Bmp4/Fgf8, respectively. Loss of *Shh* causes loss of parathyroid and expansion of Bmp4 (Moore-Scott and Manley 2005) while both loss of and overexpression of *Fgf8* causes thymic and parathyroid hypoplasia (reviewed in (Gardiner et al. 2012)). At E11.5, the pouch consists of an anterior-dorsal *GCM-2*+ parathyroid domain and a ventral-posterior *Foxn1*+ thymus domain (Gordon et al. 2001). By E12.5 the pouch lumen closes, the shared primordium separates from the pharynx, the NCC mesenchyme condenses around the thymus domain inducing separation from the parathyroid , and the thymus and parathyroid begin to migrate towards their final positions (Gordon and Manley 2011). The two thymic lobes both migrate towards the midline where they meet just above the heart and reside in this location throughout adulthood, while the parathyroids migrate to the thyroid gland.

Transcriptional Regulation of Thymus Organogenesis

The exact transcriptional network required for correct formation of the thymus has not been completely detailed, although there are many genes implicated in the process. One of the earliest genes implicated in the patterning and organogenesis of the thymus is *Hoxa3*. *Hoxa3* is expressed in the pharyngeal endoderm, neural crest cells (NCC), and ectoderm prior to pouch

formation (Manley and Capecchi 1995; Chisaka and Capecchi 1991). It has an anterior limit of expression in these cells at the 3rd pharyngeal arch. Normal expression of *Hoxa3* in the mouse embryo shows comparable levels between the third pouch endoderm and the neural crest mesenchyme at E10.5 (Chojnowski et al. 2016). Expression was undetectable by *in situ* hybridization in either cell type by E13.5, with a decrease in mRNA levels first seen within the endoderm at E11.0-E11.5. *Hoxa3* null mice initially form the pharyngeal arches and pouches but their further development is severely defective which results in loss of the thymus primordium by E13.5 (Chojnowski et al. 2016), as well as loss of the parathyroids and other developmental consequences (Chisaka and Capecchi 1991; Manley and Capecchi 1995). *Hoxa3* expression by both the endoderm and the NCC is necessary for organogenesis of the thymus as well as regulation of other genes necessary for differentiation and survival of the thymus (Chojnowski et al. 2016).

Other factors in the transcriptional network controlling thymus development include *Eya1* (Xu et al. 2002; Zou et al. 2006), *Six1* (Zou et al. 2006), *Pax1* (Su and Manley 2000; Su et al. 2001), *Pax9* (Peters et al. 1998) and *Foxn1* (Cordier and Heremans 1975). Mutations in *Eya1*, which is a homolog of the *Drosophila eyes absent* (*eya*) gene, causes complete failure of thymus and parathyroid organ formation (Xu et al. 2002), although pouch formation initiates normally. It is also required for normal expression of *Six1/Six4* in the 3rd pharyngeal pouch endoderm. Loss of expression of these also leads to loss of thymus and parathyroid, although initial formation of these organs is normal (Zou et al. 2006). The thymus in these mutants forms and expresses *Foxn1*, however this expression fails to be maintained and the tissue undergoes apoptosis. Both *Eya1* and *Six* appear to be downstream of *Hoxa3* based on severity of phenotypes (Manley and Condie 2010) and genetic data (Zou et al. 2006), although the placement of Eya and Hoxa3 may

be more complicated as shown by recent analysis of *Hoxa3* mutants (Chojnowski et al. 2014). *Pax1* loss leads to thymic hypoplasia and this gene interacts with and is downstream of *Hoxa3* (Su et al. 2001). Its expression is also dependent on *Eya;Six* as its expression is lost in double homozygous mutants but is normal in either single mutant, although this effect could be complicated by the severity of the double mutant phenotype (Zou et al. 2006). *Pax9* causes a more severe phenotype then its homolog, *Pax1*, although their expression patterns overlap (Manley and Condie 2010). Loss of *Pax9* causes severe hypoplasia of the thymus and failure of the organ to separate from the pharynx. It does not require *Eya* or *Six* expression given that it is normally expressed in both *Eya/Six* single and double mutants (Zou et al. 2006). This suite of genes form what is referred to as the Hox-Eya-Pax-Six cascade, although based on different evidence it is unclear exactly the relationship between *Eya1* and *Pax9* (Zou et al. 2006; Manley and Condie 2010). This transcriptional cascade appears to control initial patterning and development of the thymus primordium, although pouch initiation and initial specification of thymic epithelium precedes even the earliest gene, *Hoxa3* (Chojnowski et al. 2016). Another gene, *Tbx1*, is required for proper pouch formation but does not seem directly required for thymus fate in the pouch (Manley and Condie 2010). Although, it could be required in the pouch to restrict the thymus domain given that it is expressed throughout the endoderm of the parathyroid domain (Manley and Condie 2010; Reeh et al. 2014).

TEC differentiation and proliferation

After initial organogenesis and specification of thymus fate, the earliest TEC progenitors (TECP) can be identified by the marker Plet-1 (Depreter et al. 2008). This marker is expressed in the pharyngeal endoderm from E8.5 and becomes progressively restricted to the pharyngeal pouches. By E11.5 the pouch is fully formed and the TECP express both Plet-1(Depreter et al.

2008) and Keratin-8 (K8) (Klug et al. 2002). The transcription factor FOXN1, which is required for TEC differentiation and function, also begins being expressed in the TECP. *Foxn1,* formerly identified as a *winged-helix* (*whn*) transcription factor, when mutated leads to hairlessness and athymia in mice (Nehls et al. 1996).

By E12.5, TEPC in the central region of the thymic primordium up regulate K5 (Klug et al. 2002). As development continues, the thymus begins forming its two distinct regions, cortex and medulla. The mTEC develop from the K8+K5+ cells in the central region of the organ and become K8-K5+, while the cTEC are K8+K5-. Epithelial cells, that continue to express both markers persist at the cortico-medullary junction (CMJ), are presumed to be capable of producing both mTEC and cTEC. Indeed, a bipotent progenitor population has been reported to persist through out development (Rossi et al. 2006; Klug et al. 2002) and in the adult thymus (Ulyanchenko et al. 2016). Generation of the K8+K5+ TEC during early development are independent of both *Foxn1* and thymocyte interactions, which further supports the idea that specification of thymus occurs prior to expression of *Foxn1* or homing of thymocytes to the organ between E11 and E12 (Klug et al. 2002).

In the *Foxn1* null, the very earliest stages of TEC formation and differentiation occurs normally and contains TECP that are Plet-1+K8+K5+ (Nowell et al. 2011; Blackburn et al. 1996) but the cells never progress past this earliest stage of development and the thymic anlage becomes highly cystic. Also, *Foxn1* is required for initial and continued differentiation of both TEC lineages from the progenitor population as illustrated by a hypomorphic *Foxn1* allele (Su et al. 2003). Further analysis with another *Foxn1* hypomorphic allele revealed that *Foxn1* is required in a dosage dependent manner for progressive differentiation of both cTEC and mTEC, but not for initiation of the earliest mTEC progenitors from the common TEPC (Nowell et al.

2011). *Foxn1* levels above a specific threshold are also required to induce the expression of genes necessary for TEC function as well. Therefore, *Foxn1* is required for generation of cTEC that express MHCII, Dll4, and Cathepsin L but not β5t, as well as functional mTEC that produce MHCII, AIRE, K14 and bind UEA-1.

TEC differentiation and proliferation also requires input from developing thymocytes. Arrest of T-cell development at an early DN1 stage caused the the loss of medulla formation, although some early mTEC were present (van Ewijk et al. (1999). This arrest at the DN1 stage also caused loss of the necessary 3 dimensional thymic stroma in the cortex and the thymus was only about 1% the size of a wild-type thymus. This reliance of TEC on thymocyte signals is further supported by observations in RAG2-/-, which have a T-cell arrest at the DN3 stage (Hollander et al. 1995). In these mice, the cortex takes on its normal structure with the cTECs lined up perpendicular to the capsule while the medulla is small but distinguishable from cortex. Furthermore, mTECs require signals from positively selected thymocytes that express members of the tumor necrosis factor super family (TNFSF) (Hikosaka et al. 2008), RANKL and CD40L specifically. These studies have shown that, while some mature Aire+ mTECs are present in the fetal thymus, expansion of these cells requires input through TNFSF receptors found on the mTECs. The end result of this TEC-thymocyte crosstalk, as well as instructive *Foxn1* levels, is the formation of a functional thymus 3D microenvironment comprised predominately of cTEC and mTEC by E17.5 (Klug et al. 2002).

Foxn1 Induces TEC from Fibroblasts

A recent study sought to determine the ability of Foxn1 to reprogram mouse embryonic fibroblasts (MEF) into functional TEC (Bredenkamp et al. 2014). To do this, the authors created a transgenic mouse line that would constitutively express full length *Foxn1* transcripts after Cre

mediated excision of a stop cassette. These mice were crossed to a strain with an inducible CreER. Primary MEFs were derived from the progeny of this mating and cultured with Tamoxifin to induce excision of the stop cassette thereby causing ectopic *Foxn1* levels comparable to that seen in fetal TEC. Some of the induced *Foxn1* MEFs (iFoxn1 MEF) began expressing TEC markers such as K8 and EpCAM. Isolation of EpCAM+ iFoxn1 MEFs and expression analysis of FOXN1 regulated genes showed that these iFoxn1 MEFs exhibited expression of TEC specific genes but not cutaneous epithelium specific genes. These induced TEC (iTEC) were also cultured with immature DN1 thymocytes and were capable of producing DP, CD4+ SP, and CD8+ SP thymocytes. Furthermore, transplantation of these iTEC was capable of forming thymic grafts that capable of forming cortical and medullary regions that resembled a normal thymus and was capable of functioning to produce a normal T-cell repertoire. This study illustrates the importance of Foxn1 as a major regulator of TEC specific transcription factors and that it is sufficient to drive TEC differentiation. It also is a promising step towards potential therapeutic applications.

Foxg1 is Required for Normal Thymus Development

Besides its well characterized function in telencephalon development, *Foxg1* expression has been reported in the pharyngeal pouches (Hebert and McConnell 2000). A more detailed examination of the expression pattern of *Foxg1* in the pharyngeal region showed that it is expressed in the foregut endoderm prior to pouch formation (Wei and Condie 2011). After formation of the 3rd pharyngeal pouch by E10.5, *Foxg1* expression was detected in the endoderm at the ventral end (thymus domain) and a dorsal-posterior domain which consists of an unspecified region between the presumptive thymus and parathyroid domains. This expression coincides with *Gcm2* expression in the parathyroid domain but is prior to *Foxn1* expression in

the thymus domain (Gordon et al. 2001). After *Foxn1* initiation in the ventral region at E11.25, *Foxg1* expression becomes restricted to the same domain. Given the timing of *Foxg1* expression and the overlap with *Foxn1* in the 3rd pouch endoderm it was reasoned that perhaps *Foxg1* was necessary for initiation of *Foxn1*. However, *in situ* hybridization analysis in both the *Foxg1*$^{-/-}$ and *Foxn1*$^{-/-}$ showed that both are expressed independently of the other (Wei et al., unpublished).

The *Foxg1*$^{-/-}$ mutants do show many different defects in thymus development. Specifically, the thymus is severely hypoplastic at newborn stages and defects in size and morphology became apparent by E14.5 (Wei et al., unpublished). The thymus appears disorganized, shown by a less distinct boundary between the medulla and cortex regions in histology samples; as well as smaller and less numerous medullary islets. Furthermore, flow cytometry analysis at E16.5 showed a 3-fold reduction in the UEA1+EpCAM+CD45- TEC and a 3-fold reduction in the ratio of MHCII high/MHCII low cells, suggesting a defect in TEC differentiation. This defect in differentiation is certainly more apparent in the mTEC compartment since the cTEC showed no real difference in levels of CD205, although β5t expression did seem reduced in the mutant. Analysis of Plet1+, Clad3/4+ TECP, and p21+ cells in the *Foxg1*$^{-/-}$ suggests that its loss in the thymus does not mimic the phenotype seen in the telencephalon, specifically the reduction of proliferation and premature depletion of progenitors. In addition to the decrease in the total number of TEC, there was a 5-fold reduction in the total thymocyte number; although no defect in T-cell differentiation was detected at embryonic stages. This is consistent with *Foxg1* expression being restricted to TEC in the thymus. Given the hypoplasia apparent in both TEC and T-cell populations but no apparent defect in proliferation, it reasoned that there may be an increase in apoptosis. Indeed, there was an approximately 5-fold increase in the number of apoptotic cells at E12.5 and this increase was seen in both TEC and T-

cells. In addition, Wei et al. (unpublished) showed that at early stages of development, E11.5 – E16.5, *IL-7* expression was undetectable by *in situ* hybridization. IL-7 proteins role in preventing apoptosis in T-cells (Boudil et al. 2015) suggested that perhaps its loss in *Foxg1* mutants may be the cause of the increase in apoptosis. However, the increase in apoptosis seen in the *Foxg1* null was not seen in *IL-7* mutants (Wei et al., unpublished). This study (Wei et al., unpublished) was limited to fetal and newborn analysis given the perinatal lethality of *Foxg1* mutants, so the requirements for *Foxg1* in postnatal thymus maintenance still remains to be determined.

Foxg1 regulates telencephalon patterning and repression of Cajal-Retzius cell fate in cortical neuronal progenitors

The vertebrate telencephalon develops from the anterior neural tube. As the telencephalon develops, it is partitioned into distinct structural domains. This partitioning occurs as the result of a multitude of transcription factors and signaling molecules that are subsequently expressed. The telencephalon is patterned along the dorsal-ventral axis leading to the dorsal telencephalon (pallium), which gives rise to the cerebral cortex, and the ventral telencephalon (subpallium), which gives rise to basal ganglia (Danesin and Houart (2012). One of the very first markers expressed by cells fated to become telencephalon is the *winged helix* transcription factor *Foxg1* (formerly *BF-1*) (Tao and Lai 1992). *Foxg1* is expressed highest in the ventral region but is also present in slightly lower levels in the dorsal telencephalon. The dorsal-ventral patterning is specified by interactions between *Gli3*, *Bmp/Wnt*, *Shh*, *Foxg1*, and *Fgf* (reviewed in (Hebert and Fishell 2008)). Gli3 promotes expression of BMP and WNTS that along with the EMX1/2 proteins lead to dorsalization of the telencephalon. *Shh*, which is expressed in a graded manner and is highest in the ventral region, represses *Gli3* (Aoto et al. 2002) and along with *Foxg1* and *Fgf8* promote the adoption of ventral identity in telencephalon progenitor cells (Hebert and

Fishell 2008). Another important transcription factor is *Pax6* (Hebert and Fishell 2008; Toresson, Potter, and Campbell 2000), which along with *Gsh2* sets up the sharp boundary between the dorsal and ventral compartments and its expression is at least partially dependent upon FOXG1 (Manuel et al. 2011). At this boundary, PAX6 expressing cells are directly next to NKX2.1 expressing cells. *Nkx2.1* is induced in these cells by SHH signaling. Slightly later, *Gsh2* is induced in the *Nkx2.1*+ cells that are directly bordering the PAX6+ cells and *Pax6* expression represses *Gsh2* expression thus setting the sharp boundary that occurs in this region between ventral and dorsal identity (Toresson, Potter, and Campbell 2000). *Foxg1*'s role in this seems to be as a downstream effector of SHH signaling in ventral progenitors and to directly repress WNT ligands and thus limit the expansion of dorsal identity into the ventral domain (Danesin et al. 2009).

Along with aiding in the overall patterning of the telencephalon, FOXG1 is absolutely required for specification of ventral identity and proliferation of progenitor neurons (Xuan et al. 1995). Furthermore, lack of FOXG1 protein leads to the expansion of the dorsal compartment and reduction of apoptosis (Martynoga et al. 2005). Loss of *Foxg1* is also associated with an expansion of BMPS and WNTS (Hanashima et al. 2002), which may be responsible for the expansion of the dorsal compartment in these mutants. Loss of *Foxg1* also leads to an increase in the cell cycle time and loss of direct repression of BMP's leads to premature differentiation. In *Foxg1*$^{-/-}$ mutants, *Fgf8* expression is reduced in the telencephalon suggesting that *Foxg1* and *Fgf8* may be involved in a positive feedback loop and together may be acting cooperatively to induce proliferation of ventral telencephalic progenitors (Martynoga et al. 2005).

In the cortex, progenitors in the ventral zone produce intermediate progenitors that transition through the intermediate zone and undergo further proliferation and final differentiation before

finally settling in the cortical plate (Hanashima et al. 2004). The progenitors give rise to different neural subtypes in a temporally regulated manner. The first-born cells give rise to the Cajal-Retzius (CR) cells of layer 1. Later born cells populate different layers of the cortex in an inside out manner and their fate is dependent solely on their birth order (reviewed in Hanashima et al 2004). *Foxg1* is not expressed during production of the CR cells but it is activated in all later progeny and it acts to repress the CR fate (Hanashima et al. 2004). Inactivation of *Foxg1* causes the progenitor cells to continuously give rise to CR cells at the expense of all later born cell types. The combination of this, the reduction in proliferation (Manuel et al. 2011), and the early depletion of progenitors due to ectopic differentiation (Xuan et al. 1995) causes the severe reduction in the size and function of the neocortex. Furthermore, *Foxg1* in pyramidal neuron precursors is down regulated to allow expression of *Unc5D* which promotes their transition through the multipolar phase of differentiation and is then up regulated to allow the pyramidal neurons to enter the cortical plate and assume their correct laminar identity (Miyoshi and Fishell 2012). This dynamic regulation of *Foxg1* is also seen in other cortical neurons, specifically GABAergic interneuron precursors. Interestingly, it was shown that ectopic activation of *Wnt* in neocortex leads to the premature differentiation of intermediate precursor cells to mature neurons (Munji et al. 2011). This is analogous to the results seen in *Foxg1* mutants. Given the dynamic regulation of *Foxg1* in these neuron precursors (Miyoshi and Fishell 2012) and the fact that *Foxg1* has been shown to directly repress *Wnt* (Danesin et al. 2009), it may be that *Foxg1*'s regulation is restricting the timing that WNT is allowed to act in neurons and thus tightly controlling the differentiation of intermediate progenitors into mature neurons in the neocortex.

Significance and Aims of this Dissertation

The aim of my PhD research is to further investigate the regulation of TEC development by *Foxg1*. Specifically, I aimed to uncover how *Foxg1* was involved in the regulation of *IL-7* and to determine if *Foxg1* is required for maintenance of the thymus postnatally. To this end, I designed genetic crosses that would allow for TEC specific conditional deletion of *Foxg1*. I analyzed both fetal and postnatal thymus defects using quantitative and qualitative techniques. Mostly, I generated new RNA probes for *in situ* hybridization analysis of both *Foxg1* and *IL-7* and used qPCR to validate the expression levels of these genes. I collaborated with a colleague to perform flow cytometric analysis of both the TEC and thymocyte populations at E18.5 in both *Foxg1*$^{-/-}$ and a *Foxg1* conditional LOF mutant and that the conditional mutant has a different TEC profile than the null. The significance of my findings were that I identified a potential target gene of FOXG1 and show that *IL-7* expression is not completely lost at fetal stages as previously shown. I also show that the embryonic defects are not recovered from in postnatal stages which suggests that *Foxg1* expression in early embryonic TEC is required for formation of the adult thymus.

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CHAPTER 2

ANALYSIS OF *FOXG1* CONDITIONAL LOSS OF FUNCTION MUTANT PHENOTYPES[1]

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Abstract

The thymus is composed of many different stromal cell types with thymic epithelial cells (TEC) comprising the major component responsible for thymus function. The genetic networks responsible for the proper development of this organ are still being determined. However, much is known about many of the different transcription factors and signaling events that must occur for normal TEC specification and differentiation. Although, how many of these interact still remains unknown. Recently our lab showed that *Foxg1* is necessary for normal TEC differentiation and survival independently of the transcription factor *Foxn1*, which is absolutely required for TEC differentiation. In this report, we utilize a TEC specific conditional loss of function model for deletion of *Foxg1* in mouse. We show that the severe hypoplasia and decrease in *IL-7* expression in the *Foxg1* conditional LOF is the same as that seen in the *Foxg1* null and that these defects persist at postnatal stages in the conditional *Foxg1* mutant. Our results suggest that the decreased cellularity in the thymus is due to increased apoptosis in the developing thymus and possibly throughout postnatal stages as well. We conclude that *Foxg1* is necessary to allow normal initiation of *IL-7* at E11.5 and that it is required to maintain normal levels of *IL-7* expression throughout development.

Introduction

The thymus is the primary lymphoid organ in jawed vertebrates (Boehm and Bleul 2007) responsible for providing the necessary microenvironment to support the development of self-restricted, self-tolerant thymocytes. The thymus consists of both medulla and cortical regions, comprised of cortical and medullary thymic epithelial cells (cTEC and mTEC, respectively) that play important functional roles in the development of thymocytes (Petrie and Zuniga-Pflucker 2007). Thymocytes commit to the T-cell lineage (Hozumi et al. 2008) and undergo positive selection in the cortex which leads to the production of MHC restricted CD4+ or CD8+ T-cells (Starr, Jameson, and Hogquist 2003). This commitment and differentiation are controlled in part by NOTCH (Hozumi et al. 2008) and IL7 (Boudil et al. 2015) signals between the cTEC and migrating thymocytes. These CD4 and CD8 single positive (SP) thymocytes migrate into the medulla where they undergo negative selection due to interactions with mTEC and dendritic cells (DC) (Klein et al. 2014). This selection is mediated through T-cell receptor (TCR) binding with tissue restricted antigens (TSA) which are predominately produced through the action of the AIRE protein produced by the gene, *autoimmune regulator* (*Aire)* (Derbinski et al. 2001) in mTEC. This selection leads to self-tolerant T-cells that should not cause autoimmunity.

The thymus is derived from the third pharyngeal pouch endoderm in mice (Gordon et al. 2004) and its specification is due, at least in part, to a Hox-Pax-Eya-Six transcriptional cascade (Manley and Condie 2010). At embryonic day 11.5 (E11.5), *Foxn1* expression begins to be detected in the ventral region of the pouch which will become thymus. *Foxn1* null mice exhibit hairlessness and athymia (Nehls et al. 1996). This transcription factor has been shown to be essential for TEC proliferation and differentiation (Blackburn et al. 1996; Su et al. 2003). The cTEC are generally characterized as being K8+CD205+Ly51+β5t+ (Klug et al. 2002; Shakib et

al. 2009; Xing, Jameson, and Hogquist 2013; Gray, Chidgey, and Boyd 2002) while mTEC are generally characterized by expression of Keratins 5 and 14 (K5/14) (Klug et al. 2002). Some mTEC also express *Aire* (Anderson et al. 2002) and bind the lectin *Ulex europeus* (UEA-1) (Farr and Anderson 1985). After specification and initiation of *Foxn1* in the thymus domain of the third pouch, the thymus consists of a population of progenitor TEC that first express K8 (Klug et al. 2002) as well as the progenitor TEC marker PLET-1 (Depreter et al. 2008). The medulla begins to develop in the central region of the thymus primordium at around E12.5, when TEC in this region begin expressing K5 in addition to K8 (Klug et al. 2002). Some of these K8+K5+ cells will lose expression of K8 and differentiate into mTEC, while others will retain expression of both even into adulthood and are considered to be a progenitor population that gives rise to both TEC subsets (Rossi et al. 2006; Ulyanchenko et al. 2016).

Another transcription factor, *Foxg1*, was reported to be expressed in the pharyngeal endoderm at the time of thymus specification (Wei and Condie 2011; Gordon et al. 2004) as well as throughout development of the thymus (Wei and Condie 2011). Analysis of *Foxg1* null mice revealed that this transcription factor is necessary for normal thymus development (Wei et al. unpublished). Indeed, loss of *Foxg1* resulted in a severely hypoplastic thymus with reduced numbers of both TEC and thymocytes. *Foxg1* is best known for being required in the patterning of the telencephalon (Hebert and Fishell 2008) partially by suppressing WNT signaling (Danesin et al. 2009). It also controls aspects of neuronal cell proliferation and differentiation in part by antagonizing TGFβ signaling and inhibiting p21 expression (Dou et al. 2000; Hanashima et al. 2004; Rodriguez et al. 2001; Seoane et al. 2004). However, in the thymus there was no evidence that *Foxg1* was affecting proliferation or preventing the premature differentiation of TEC (Wei et al. unpublished). Loss of *Foxg1* did however show a reduction in UEA-1+ and AIRE+ mTEC

and a loss of the essential cytokine *IL-7*. It was also shown that *Foxg1* acts independently of *Foxn1* in TEC and that *Foxg1* loss resulted in increased apoptosis of both TEC and thymocytes, which is likely the cause of the observed hypoplasia. The analysis by Wei et al. (unpublished) was however limited to embryonic and newborn stages due to the perinatal lethality of *Foxg1* deletion.

The present study attempts to by-pass this lethality by utilizing TEC specific deletion of *Foxg1*. Here we report that conditional deletion of *Foxg1* results in similar embryonic phenotypes in the thymus and that these defects persist in the postnatal thymus. We further show that these defects are primarily due to loss of *Foxg1* after thymus specification has occurred, although there was a slight delay in specification observed. Interestingly, we conclude that *Foxg1* is required for normal expression levels of *IL-7* but that *IL-7* expression is not completely dependent on *Foxg1*.

Methods

Mice

All mice work conformed to the stipulations of the University of Georgia Institutional Animal Care and Use Committee. $Foxg1^{+/Cre}$ (Hebert and McConnell 2000) were purchased from Jackson Laboratory (stock # 006084) and maintained by crossing to C57Bl6/J mice. $Foxg1^{Cre/Cre}$ mice have the coding region of *Foxg1* replaced by Cre and act as a null (Kawauchi et al. 2009), hereafter $Foxg1^{-/-}$. Generation of $Foxg1^{-/-}$ mice was performed by crossing $Foxg1^{+/Cre}$ males to Swiss Webster (SW) females and then interbreeding heterozygous F1 mice. *Foxn1Cre* mice were generated by and imported from Dr. Nancy Manley's lab (Gordon et al. 2007). Conditional LOF $Foxg1^{C:Flpe}$ mice were generously donated by Dr. Gord Fishell (Miyoshi and Fishell 2012) and contain a *loxp* flanked coding region of *Foxg1* (hereafter, $Foxg1^{fl/fl}$). These

mice were maintained as a homozygous *Foxg1$^{fl/fl}$* colony by breeding within the colony. *Foxg1CKO* mice were generated by crossing *Foxn1Cre* mice with *Foxg1$^{fl/fl}$* to obtain *Foxg1$^{+/fl}$;Foxn1$^{+/Cre}$*. Male double heterozygous mice were then backcrossed to *Foxg1$^{fl/fl}$* females to generate *Foxg1CKO* and control littermates.

Kidney capsule grafting

Dissected thymic lobes from E15.5 *Foxg1$^{-/-}$* and littermate controls were grafted under the kidney capsule of 6 week old *Foxn1$^{nu/nu}$* male mice. Both lobes from three different mutant and control donors were grafted. Grafts were left for 21 days before dissection of the kidney from recipient mice followed by analysis.

Histology and Immunohistochemistry

For H&E, thymus from 4-5 week old mice were fixed in 4% Para formaldehyde (PFA) at 4^0C overnight. The tissue was then washed 3 times in PBS and dehydrated through a methanol and xylene series and then embedded into paraffin blocks. 10 µm transverse sections were cut on a Leica RM2155 microtome, dewaxed in xylene, and rehydrated through a decreasing methanol series. Standard hematoxylin and eosin staining was then performed.

For the thymic grafts, IHC was done on OCT (Sakura Tissue-Tek) embedded frozen tissue by cutting 10 µm sections on a Leica CM3050 S cryostat. Sections were fixed in acetone for 2 minutes, washed twice in 0.05% PBST (0.05% Triton X-100) and blocked in 10% donkey serum in PBST at room temperature (RT) for 30 minutes. Primary antibodies were mixed in 1% serum/PBST and slides were incubated overnight at 4^0C and then washed 3 times in PBST. Secondary antibodies were mixed in PBST and slides were incubated in the mix for 1-2 hours at RT. Slides were washed, stained with DAPI (1:10,000) in PBST, and mounted in FluoroGel (EMS). Images were taken using an Axioplan (Zeiss) microscope and AxioVision software.

For adult thymus, IHC was done on paraffin embedded dissected thymus prepared as mentioned above for H&E, except they were fixed in 4% PFA for only 30 minutes. Staining was performed as described for the thymic grafts with the following exceptions. PBST (0.10% Tween20) was used instead of Triton X-100 in the antibody mixes. Also, prior to staining antigen retrieval was performed by boiling the slides in AR buffer (10mM Na_3Citrate pH2, 0.05% Tween20) for 30 minutes and then allowed to cool.

Primary antibodies consisted of: goat anti-*Foxn1* (1:200, Santa Cruz, G-20), biotinylated UEA-1 (1:200, Vector Laboratories), rabbit anti-β5t (1:200, MBL, PD021), rabbit anti-cleaved Caspase3 (1:200, Cell Signaling), rat anti-Aire (1:100, clone: 02-5H12-2). Secondary antibodies were either Alexa (Invitrogen) or DyLight (Jackson Immunoresearch).

Riboprobe design and in situ hybridization

Foxg1 gBlock® Gene Fragment Design

We designed a double stranded cDNA gene fragment that contained a 5' primer region containing the T7 promoter sequence (*underlined*) as well as 7 random bases preceding the T7 sequence (5' – ATGCATCTAATACGACTCACTATAGGG – 3'). The 3' end of the fragment contained the anti-sense sequence for the T3 promoter (*underlined)* as well as 7 random bases at the most 3' end (5' – CTTTAGTGAGGGTTAATTGTCCATA – 3'). These sequences flanked the cDNA sequence of *Foxg1* from which the anti-sense RNA probe will be transcribed from (5'–GGCACGACCGGCAAGCTGCGGCGCCGCTCCACCACGTCTCGGGCCAAGCTGGC CTTTAAGCGCGGGGCGCGCCTCACCTCCACCGGCCTCACCTTCATGGACCGCGCCGG CTCCCTCTACTGGCCCATGTCGCCCTTCCTGTCCCTGCACCACCCCCGCGCCAGCAGC ACTTTGAGTTACAACGGGACCACGTCGGCCTACCCCAGCCACCCCATGCCCTACAGC TCCGTGTTGACTCAAAACTCGCTGGGCAACAACCACTCCTTCTCCACCGCCAACGGG

CTGAGTGTGGACCGGCTGGTCAACGGGGAGATCCCGTACGCCACGCACCACCTCAC
GGCCGCTGCGCTCGCCGCCTCGGTGCCCTGCGGCCTGTCGGTGCCCTGCTCCGGGAC
CTACTCCCTCAACCCCTGCTCCGTCAACCTGCTCGCGGGCCAGACCAGTTACTTTTTC
CCCCACGTCCCGCACCCGTCAATGACTTCGCAGACCAGCACGTCCATGAGCGCCCGG
GCCGCGTCCTCCTCTACGTCGCCGCAGGCCCCCTCGACCCTGCCCTGTGAGTCTTTA
AGACCCTCTTTGCCAAGTTTTACGACAGGACTGTCCGGGGGACTGTCTGATTATTTC
ACACAT – 3'). The PCR reaction protocol was as follows; initial denaturation 94 degrees Celsius for 5 minutes, [94^{0}C for 30s, annealing 56^{0}C for 45s, elongation 72^{0}C for 60s] x 33 cycles, final elongation 72^{0}C 10 minutes.

Interleukin 7 (IL-7) gBlock® Gene Fragment Design

We next designed a double stranded cDNA fragment to use to generate an anti-sense RNA probe against the cytokine *IL-7*. This fragment contained the same general design as the *Foxg1* gene fragment with the exception that both the 5' and 3' ends contained longer regions of random bases (5'- ATGCATCGCGTGCTGCTGGCCTGGCACTGTAATACGACTCACTA TAGGG – 3' – (*IL-7* cDNA sequence) – 5' – CCTTTAGTGAGGGTTAATTGTCCAGAGTA GCGGACGGTGACGCCGTCGAGTCAG – 3'). The *IL-7* cDNA a sequence was flanked by these promoter regions (*IL-7* cDNA sequence 5' – TCTGCTGCCTGTCACATCATCTGAGT GCCACATTAAAGACAAAGAAGGTAAAGCATATGAGAGTGTACTGATGATCAGCATC GATGAATTGGACAAAATGACAGGAACTGATAGTAATTGCCCGAATAATGAACCAAA CTTTTTTAGAAAACATGTATGTGATGATACAAAGGAAGCTGCTTTTCTAAATCGTGC TGCTCGCAAGTTGAAGCAATTTCTTAAAATGAATATCAGTGAAGAATTCAATGTCCA CTTACTAACAGTATCACAAGGCACACAAACACTGGTGAACTGCACAAGTAAGGAAG AAAAAAACGTAAAGGAACAGAAAAAGAATGATGCATGTTTCCTAAAGAGACTACTG

AGAGAAATAAAAACTTGTTGGAATAAAATTTTGAAGGGCAGTATATAAACAGGACA
TGTAGTAACAACCTCCAAGAATCTACTGGTTCATATACTTGGAGAGGTTGAAACCCT
TCCAGAAGTTCCTGGATGCCTCCTGCTCAAATAAGCCAAGCAGCTGAGAAATCTACA
GTGAGGTATGAGATGATGGACACAGAAATGCAGCTGACTGCTGCCGTCAGCATATA
CATATAAAGATATATCAACTATACAGATTTTTGTAATGCAATCATGTCAACTGC – 3').

The PCR reaction protocol was as follows; initial denaturation 94 degrees Celsius for 5 minutes, [94^0C for 30s, annealing 71^0C for 30s, elongation 72^0C for 60s] x 33 cycles, final elongation 72^0C 10 minutes.

In vitro transcription of RNA probe and In Situ hybridization of sectioned tissue

Digoxygenin(DIG)- labeled RNA probes were generated by standard protocols. For the *Foxg1* probe, we used 50% DIG labeled UTP to compensate for the high GC content of the probe sequence. For the *IL-7* probe, we used the standard 35% DIG labeled UTP concentration. Both anti-sense probes were synthesized using the T3 RNA polymerase.

Embryos were collected from pregnant females at different stages of development with noon of the day of plug identification being Embryonic day 0.5 (E0.5). Embryo's were then staged more accurately based on somite count or morphological features. Samples were fixed in 4% Paraformaldehyde (PFA) overnight, washed in 1xPBS(DEPC), and dehydrated in a series of washes in 70%, 80%, 90%, 95% Ethanol/DEPC-H2O, and 100% Ethanol. Samples were then paraffin embedded for sectioning by standard procedures and sectioned at a thickness of 12μm. Sectioned samples were collected on glass slides (Fisher-Scientific) and rehydrated. Paraffin sectioned ISH was performed as previously reported (Zamisch et al. 2005). Samples were then counterstained in 20% Nuclear Fast Red (NFR).

Flow cytometry

Total thymocytes were isolated from E18.5 *Foxg1*$^{-/-}$ and control embryos or 4 week old postnatal *Foxg1CKO* and control mice and counted using a hemacytometer. Equal numbers of thymocytes from each was then used for staining. Fc receptor blocking was done using anti-CD16/32 antibodies followed by staining with anti-CD45-APC, anti-CD19-PE, anti-CD4-APC-Cy7, anti-CD8-Percp (or -PE-Cy7), anti-CD25-APC (or -Percp), anti-CD44-PE (or -FITC). Data were then collected using a Beckman Coulter FACS CyAn ADP Analyzer and analyzed using FlowJo software.

For stromal cells, dissected lobes were digested in 1mg/mL collagenase/dispase (Roche) and 1mg/mL DNaseI (Sigma) in RPMI-1620/2%FBS as described in (Gray, Chidgey, and Boyd 2002). Staining was performed as stated above with the following antibodies: anti-CD45-PE-Cy7, biotinylated UEA-1 (Vector Labs) with secondary antibody (SA-FITC), anti-MHCII-Percp, anti-EpCAM-PE, and anti-Ly51-APC.

RNA extraction and qPCR

Total mRNA was extracted from dissected thymus (n=2 mutants and controls) or whole third pouches (n=3 mutants and controls), depending on stage, using a Qiagen microRNeasy kit. This was followed by reverse transcription using Superscript II (Invitrogen). cDNA was then used for quantification of *Hoxa3* (Mm01326402_m1), *Foxn1* (Mm00433948_m1), *IL-7* (Mm01295803_m1), relative to the reference gene *Gapdh* (Mm99999915_g1) using standard Taqman assay reagents which are commercially available (Invitrogen). All samples were run in triplicate using a Applied Biosystems 7500 Real Time PCR System. Relative quantification to the reference gene was done using the comparative C_t method.

Aire+ cell counting

Images were analyzed using Cell Profiler software to quantify the number of Aire+ cells in every fourth 10 μm section through the entire middle half of 4-week postnatal thymus from 2 mutants and controls. Sections were cut and stained as mentioned above.

Statistics

All data from cell counting, FACS, and qPCR were entered into Excel spreadsheets and statistics were done using the students two-tailed T-test. Significance was considered to be $p \leq 0.05$.

Results

Survival of Foxg1$^{-/-}$ Thymus

Mice bearing a deletion of *Foxg1* die perinatally (Xuan et al. 1995). To test the ability of the thymus to survive past this point of development, whole thymic lobes were dissected from E15.5 *Foxg1Cre* and control embryos (n=2 each). The *Foxg1Cre* mice have the entire coding region of *Foxg1* replaced by Cre-recombinase coding sequence (Hebert and McConnell 2000), hereafter referred to as *Foxg1$^{-/-}$*. These tissues were transplanted under the kidney capsule of *nude* male hosts. Grafts were recovered 21 days after transplantation and analyzed by immunohistochemistry (Figure 2.1). Grafts were visible to the naked eye and showed visible vascularization (Figure 2.1 A,E). Comparison of control and mutant grafts showed a continued reduction in thymus size in mutants comparable to the phenotype seen at embryonic stages (Wei et al. unpublished). Markers for cTEC (Figure 2.1 C,G) and mTEC (Figure 2.1 B,F) also showed similar phenotypes to that seen *in vivo* at E16.5. This data shows that the hypoplasia seen in the *Foxg1$^{-/-}$* embryonic thymus persists into a postnatal time point and that there is also no recovery in the number of UEA1+ mTEC. This reduction in thymus size is likely due to the increase in

apoptosis seen between E12.5-E14.5 in mutant embryos (Wei et al. unpublished) and that increased apoptosis of some TEC may still be occurring undetected. We also furthered the analysis of the relationship between *Foxg1* and *Foxn1* that Wei et al. (unpublished) did by creating *Foxg1;Foxn1* double mutants. We thought that, given the persistence of the thymus rudiment in *nude* mice (Blackburn et al. 1996) and the apparent role of *Foxg1* in apoptosis in TEC (Wei et al. unpublished), loss of *Foxg1* in *nude* mice might cause the rudiment to undergo apoptosis. However, no difference was seen at E12.5 between double mutants and *Foxn1* single mutants (data not shown).

Thymus Specific Conditional Deletion of Foxg1

To circumvent the perinatal lethality of *Foxg1* loss, a conditional loss of function approach was utilized. Mice harboring a loxp flanked allele of *Foxg1* (Miyoshi and Fishell 2012) were crossed with mice expressing Cre recombinase under the control of endogenous *Foxn1* (Gordon et al. 2007). The resulting phenotypes at both embryonic and postnatal stages were analyzed. The thymus in $Foxg1^{fl/fl};Foxn1^{+/Cre}$ (hereafter referred to as *Foxg1CKO*) four-week postnatal mice showed comparable phenotypes to that seen in the $Foxg1^{-/-}$ (Figure 2.2). The thymus was severely hypoplastic and histology showed similar reductions in the number and size of medullary islets (Figure 2.2 A,B). Immunohistochemistry analysis of TEC markers (Figure 2.2 C-F) also showed the reduction in mTEC numbers seen in *Foxg1* null (Wei et al. unpublished) and at embryonic stages in the *Foxg1CKO* (data not shown), specifically a reduction in UEA1+ and Aire+ mTEC subsets. Interestingly, when the Aire+ TEC were quantified there was no significant difference in the relative numbers of these cells compared to the control (Figure 2.3E). This was true whether we looked at the Aire+/Foxn1+ TEC ratio (Figure 2.3E) or Aire+ cells per area (mm^2) of medulla (not shown). This suggests that, although there is a decrease in

total Aire+ mTEC in the *Foxg1CKO*, this is a result of a decrease in total TEC number and not due to a defect in the production or survival of Aire+ mTEC in the mutant thymus. The thymus of the postnatal *Foxg1CKO* thymus also showed reduced thymic stromal cells (Figure 2.3A) (2.97 ±0.38 (x10^6) Cre- compared to 0.48 ±0.055 (x10^6) Cre+, *p=0.007*) and thymocyte numbers (Figure 2.3B) (37.56 ± 5.01 (x10^6) Cre- compared to 5.70 ± 0.49 (x10^6) Cre+, *p=0.003*), reduced percentage of total TEC (Figure 2.3C), and reduced total UEA-1+ mTECs (Figure 2.3D) (0.89 ±0.23 (x10^6) Cre- compared to 0.066 ±0.019 (x10^6) Cre+, *p=0.17*).

To quantify any differences in TEC or thymocyte subsets between the *Foxg1*$^{-/-}$ and the *Foxg1CKO*, we performed flow cytometry on E18.5 thymi. The total number of thymocytes was significantly reduced in both the null and CKO thymuses (Figure 2.4 A,D). The null thymus showed no difference in the percentage of CD4+ or CD8+ SP thymocytes but did show a slight but significant reduction in DP cells and an ~5% increase of DN cells (Figure 2.4B). The CKO showed no difference in any of these thymocyte subsets compared to controls (Figure 2.4E). Further analysis of the DN subsets showed a reduction in DN1-3 subsets and an increase in DN4 thymocytes in both the null and CKO mutants (Figure 2.4 C,F). The reduction in total thymocyte number due to defective proliferation and survival is similar to that seen in *IL-7*$^{-/-}$ mice (von Freeden-Jeffry et al. 1995; Crompton et al. 1998), however the differences in the DN subsets do not seem to be a result of *IL-7* deficiency alone (Boudil et al. 2015). Flow cytometry of the CD45-EpCam+ epithelial cells showed an overall decrease in the total number of TEC at E18.5 (Figure 2.5) consistent with previous reports (Wei et al. unpublished). This reduction in TEC number was identical between the *Foxg1*$^{-/-}$ and the *Foxg1CKO* (Figure 2.5 A,C). Surprisingly, analysis of total TEC subsets shows that in the null we see similar decreases in both cTEC and mTEC with a slightly more severe reduction in mTEC numbers (Figure 2.5B). However, the

Foxg1CKO mice at this same stage show a more severe reduction in cTEC relative to mTEC numbers. This suggests a skewing in the differentiation of TEC progenitors into either mTEC or cTEC.

IL-7 Expression Levels are Reduced in Foxg1CKO

To further examine the loss of *IL-7* expression in *Foxg1* deficient mice (Wei et al. unpublished), we performed an *in situ* hybridization analysis in *Foxg1CKO* embryos (Figure 2.6). We first examined the loss of *Foxg1* transcripts to validate loss of expression of this gene in the *Foxg1CKO* (Figure 2.6 I,J & M,N). Since Cre expression is being driven by *Foxn1,* we examined *Foxg1* expression 1 and 2 days after initial Cre expression at E11.25. The expression of *Foxg1* was robustly detected in controls at both E12.5 and E13.5 (Figure 2.6 I,M, respectively). While the CKO embryos showed only a few cells with *Foxg1* mRNA transcripts still present at these stages (Figure 2.6 J,N). Indeed, analysis at E14.5 showed no detectable *Foxg1* expression (data not shown). This verifies that Cre mediated recombination of the *Foxg1* conditional alleles is highly efficient and results in nearly complete loss of *Foxg1* mRNA expression within 1 day of initial Cre expression. The expression of *IL-7* in the controls (Figure 2.6 A,E) showed the expected pattern of being restricted to the ventral thymus domain at E11.5 and assuming a more punctate pattern throughout the thymus anlagen at E12.5 (Zamisch et al. 2005). The *Foxg1CKO* embryos showed a delay in expression of *IL-7*, with expression only being detectable by E12.5 (Figure 2.6 B,F). Later stages of development continued to show *IL-7* expression, although it was visibly reduced compared to controls even at stages prior to obvious thymus defects (Wei et al. unpublished) in the mutant (data not shown).

In an attempt to recapitulate the *IL-7* phenotype seen in *Foxg1* null (Wei et al. unpublished), we crossed the *Foxg1* conditional mice to *Foxg1*$^{-/-}$ mice, which expresses Cre

(Hebert and McConnell 2000) instead of *Foxg1* under the control of the endogenous *Foxg1* promoter. This strategy allowed us to utilize the conditional loss of function allele of *Foxg1* over a *Foxg1* null allele, that also expresses Cre, prior to *Foxn1* expression in the third pouch endoderm. The gross morphological phenotypes in this conditional mutant were identical to the *Foxg1CKO* (data not shown). *In situ* hybridization against *Foxg1* mRNA in the $Foxg1^{fl/Cre}$ showed a complete loss of *Foxg1* transcripts at E11.5 and E12.0 (Figure 2.6 L,P, respectively), while the controls showed normal expression patterns (Figure 2.6 K,O). Hybridization with the *IL-7* riboprobe showed a delay in *IL-7* at E11.5 (Figure 2.6D), consistent with that seen in the *Foxg1CKO* (Figure 2.6B), with expression being detectable at E12.0 (Figure 2.6H). These results show that FOXG1 is necessary for normal *IL-7* expression at E11.5 (Figure 2.6 A,C). Furthermore since *Foxg1* is expressed at E10.5 in third pouch endoderm (Wei and Condie 2011), the $Foxg1^{fl/Cre}$ result suggests that *Foxg1* expression is required aound E11.5 for regulation of *IL-7* but is dispensable prior to this.

Quantification of IL-7 Expression

Given the delay and visible reduction in *IL-7* expression in both the *Foxg1* null and CKO models, we performed quantitative RT-PCR on $Foxg1^{-/-}$ (Figure 2.7A) and *Foxg1CKO* (Figure 2.7B) thymi. We dissected both third pharyngeal pouches from *Foxg1* mutants and controls and extracted total RNA for qPCR. As shown in Figure 2.7A, the mutant pouches showed an ~60% reduction in *IL-7* expression relative to controls. We also observed a reduction in *Foxn1* levels and an increase in *Hoxa3* levels at E11.5. The reduction in *Foxn1* and *IL-7* at this stage suggests that loss of *Foxg1* expression in the endoderm prior to E11.5 causes a general delay in thymus fate specification. The higher levels of *Hoxa3* expression seen in the $Foxg1^{-/-}$ are likely not due to increased expression but instead a delay in the down regulation of *Hoxa3* in the third pouch

endoderm (Chojnowski et al. 2014). Quantitative analysis of RNA levels in the *Foxg1CKO* was done by dissecting both lobes of the thymus from E14.5 and E16.5 mutant and control embryos. Total RNA was extracted for qPCR analysis (Figure 2.7B). At E14.5 and E16.5, *IL-7* levels were reduced by 60% in the conditional mutants relative to levels in the controls. This is consistent with the levels detected in the *Foxg1*$^{-/-}$ at E11.5 (Figure 2.7A). There was no difference in the levels of *Foxn1* at E14.5 or E16.5 in the *Foxg1CKO* (Figure 2.7B) which is consistent with findings that *Foxn1* and *Foxg1* act independently of each other after TEC specification has occurred (Wei et al. unpublished) and the idea that loss of *Foxg1* is only causing a general delay in this specification prior to E11.5.

Discussion

We show further evidence here that *Foxg1* is essential for the development of a normal thymus. TEC specific deletion of *Foxg1* with either *Foxn1Cre* or *Foxg1Cre* results in nearly identical phenotypes at embryonic stages, regardless of the difference in timing of Cre expression, and to *Foxg1* null embryos (Wei et al. unpublished). In the *Foxg1CKO*, we also show that these phenotypes persist in postnatal thymus (Figure 2.2 and 2.3). Indeed, *Foxg1CKO* mice show a decrease in both TEC and thymocyte cellularity at embryonic and 4 week postnatal stages. The proportions of DN1-4, DP, and SP thymocytes (Figure 2.4) are mostly normal although there was a slight increase in some of the DN subsets. This suggests that, while cellularity is greatly decreased overall, the thymus is still capable of producing naïve T-cells. Whether these T-cells in the *Foxg1* mutants are autoreactive was not directly tested, however, so it is not clear whether negative selection is defective or not.

The TEC compartments show interesting differences between the *Foxg1*$^{-/-}$ and the *Foxg1CKO* at E18.5 (Figure 2.5) with the null showing a greater decrease in the percentage of

mTEC and the *Foxg1CKO* showing a more severe reduction in the number of cTEC by flow cytometry. Interestingly, immunohistochemistry at embryonic and postnatal stages (Figure 2.2) with the cortical marker, β5t, and the mTEC marker, UEA-1, do not seem to confirm this loss of cTEC in the *Foxg1CKO*. Also, postnatal examination of the TEC compartments by flow cytometry in the *Foxg1CKO* shows a similar, although not significant, decrease in mTEC numbers comparable to that seen in the *Foxg1*$^{-/-}$ at E18.5. However, there is apparently new information which suggests that *Foxn1Cre* is not expressed in bipotent TEC progenitors (personal communication with Dr. Nancy Manley). This and other data suggests that in the thymus there exists a *Foxn1*(-) progenitor which gives rise to lineage restricted mTEC or cTEC progenitors (Ucar et al. 2014; Ulyanchenko et al. 2016). What this suggests is that in this Foxn1(-) bi-potent progenitor, Foxg1 has some role in controlling the choice of which lineage restricted TEC progenitor will be produced during differentiation. This model (Figure 2.8) suggests that when Foxg1 is absent in the bi-potent progenitor, the cell skews its differentiation towards the cTEC lineage. However, if Foxg1 is present in the progenitor it skews differentiation towards the mTEC lineage. This model further shows that the apoptosis phenotype is due to loss of *Foxg1* after *Foxn1* expression begins in the lineage restricted cells and their progeny. Further examination, such as detailed analysis of cortex and medullary volumes in H&E stained *Foxg1CKO* embryonic and postnatal thymus, is necessary to fully verify if this difference between conditional and null is accurate or merely an artifact of FACS.

Future Directions

Studies of *Wnt* signaling in the thymus show similar phenotypes to those of *Foxg1* mutants. Specifically, deletion of β-catenin in K5 expressing adult TEC led to a severely hypoplastic thymus, increased apoptosis of both TEC and thymocytes, reduced proliferation of

TEC, and fewer UEA-1+ mTEC (Liang et al. 2013). The authors also noted a decrease in *IL-7* expression levels similar to that which we report here. Constitutive activation of β-catenin in TECs at E11.5, however, causes TEC to shift their fate to that of other cell types such as skin epithelium (Zuklys et al. 2009). Indeed, many studies have shown that canonical Wnt signaling is essential for TEC development and function and that thymic epithelial cells are the source for production of these signals in the thymus (Kvell et al. 2014; Brunk et al. 2015; Zuklys et al. 2009; Liang et al. 2013). *Foxg1* has been shown to directly repress *Wnt* ligands (Danesin et al. 2009) in zebrafish telencephalon, as well as regulating Hedgehog, BMP, FGF, and TGF-β signals (Martynoga et al. 2005; Dou et al. 2000; Carlin et al. 2012). Therefore, while direct evidence is lacking in thymus, our results suggest that *Foxg1* may be regulating these same signaling pathways that are essential for normal thymus development (Hauri-Hohl et al. 2014; Zuklys et al. 2009; Kvell et al. 2014; Gordon et al. 2010; Moore-Scott and Manley 2005; Gardiner et al. 2012) and that perhaps this regulation is temporal given the difference in TEC phenotypes seen between the *Foxg1*$^{-/-}$ and the *Foxg1CKO*.

Studies have also shown that Foxg1 is required to promote survival in cultured neurons (Dastidar, Landrieu, and D'Mello 2011). This survival function was shown to require interaction of Foxg1 with the transducing-like enhancer of split-1 (TLE1) (Dastidar, Narayanan, et al. 2012) and that together these act upstream of Mecp2 to regulate survival in post-mitotic neurons (Dastidar, Bardai, et al. 2012). This function of Foxg1 in the regulation of apoptosis in neurons was further supported by a recent study that looked at Foxg1 localization to the mitochondria in both cultured neurons and mouse cortex (Pancrazi et al. 2015). The authors show that localization and transport of Foxg1 fractions into the mitochondria controls aspects of cellular differentiation and apoptosis. Indeed, apoptotic control of cells intricately involves the

mitochondria (reviewed in (Fuchs and Steller 2011)). Foxg1 has also been shown to be both cytosolic and nuclear and that its function in is dependent on this localization (Regad et al. 2007). In fact, in the nucleus it was shown to be a transcriptional repressor of p21 by binding FoxO/Smad complexes which activate p21 (Seoane et al. 2004) and in the cytoplasm it interferes with Tgf-β signaling by binding Smad (Rodriguez et al. 2001). It therefore stands to reason the Foxg1 may be acting in the cytoplasm to affect the cell cycle and survival of TEC by repressing Tgf-β and localizing to the mitochondria, respectively. These pathways need to be further analyzed in Foxg1 mutants and controls to determine if Foxg1 is acting as a mediator of these signaling pathways in the thymus thereby controlling cell decisions in apoptosis and survival.

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Figures and Figure Legends

Figure 2.1 Survival of *Foxg1*-deficient thymus grafts. Kidney capsule grafts of *Foxg1*$^{+/-}$ (**a**) and *Foxg1*$^{-/-}$ (**e**) E15.5 thymic lobes 3 weeks after transplantation. (**b,c,d,f,g,h**) Frozen sagittal sections of ctrl (b-d) and mutant (f-h) stained green with anti-UEA-1 (b,f), red with anti-β5t (c,g), and merged (d,h) with nuclei stained blue with Dapi.

Figure 2.2 Postnatal analysis of *Foxg1CKO* thymus. (**a,b**) Paraffin embedded transverse sections stained with hematoxylin and eosin of ctrl (a) and mutant (b). (**c,d**) Transverse sections of 4-week postnatal ctrl (c) and mutant (d) thymus stained with anti-UEA-1 (green) and β5t (red) or (**e,f**) anti-Foxn1 (blue) and anti-Aire (red).

Figure 2.3 Flow cytometry analysis of 4-week postnatal thymocytes and stromal cells (**a-d**). Graphs showing (a**)** total (±SE) CD45- thymic stromal cells (TSC) (*p=0.007)* (b) Total (±SE) thymocyte (CD45+) (*p=0.003)*. (c) % EpCam+ TEC from total TSC (*p=0.12)*. (d) Total UEA-1+ mTEC (*p=0.17*). Data set represents thymic lobes from ≥2 mice per genotype in two independent experiments. (**d**) Quantification of AIRE expressing TEC using Cell-Profiler analysis of paraffin embedded sections from 4-week postnatal thymus. Representative of total AIRE+ cells from both lobes from one ctrl and mutant mice (n=1)

Figure 2.4. Comparison of thymocyte numbers and DN, DP, and SP subsets in E18.5 *Foxg1* null and *Foxg1CKO* embryos. (**a,d**) Total thymocyte numbers (CD45+) in null and conditional mutants. (**b,c** and **e,f**) Percentages of DN, DP, CD4+, and CD8+ (b,e) and DN1-4 (c,f) in null (b,c) and conditional (e,f) *Foxg1* mutant models. All data is representative of FACS data from ≥3 embryos from two independent experiments. (*) denotes level of significance.

Figure 2.5 Comparison of thymic epithelial cell number and cTEC/mTEC numbers in E18.5 *Foxg1* null and *Foxg1CKO* embryos by flow cytometry. (**a,c**) Total TEC (CD45-) numbers in

Foxg1 null (a) and *Foxg1CKO* (c) from FACS analysis. (**b,d**) Total cTEC (Ly51+) and mTEC (UEA-1+) numbers in *Foxg1* null (b) and *Foxg1CKO* (d) calculated using FACS analysis. Data representative of ≥2 embryos per genotype in two independent experiments. (*) denotes level of significance.

Figure 2.6 *IL-7* expression is delayed and reduced in two different *Foxg1* conditional LOF models. *In situ* hybridization analysis of *IL-7* on sagittal sections in $Foxg1^{fl/fl}$;*Foxn1Cre*+ and controls at E11.5 (**a,b**), E12.5 (**e,f**), and in $Foxg1^{fl/Cre}$ and controls at E11.5 (**c,d**) and E12.0 (**g,h**). *In situ* hybridization analysis of *Foxg1* on sagittal sections in $Foxg1^{fl/fl}$;*Foxn1Cre*+ and controls at E12.5 (**i,j**), E13.5 (**m,n**) and in $Foxg1^{fl/Cre}$ and controls at E11.5 (**k,l**) and E12.0 (**o,p**). Data representative of ≥2 embryos of each genotype from ≥2 independent experiments.

Figure 2.7 Quantitative RT-PCR analysis of TEC expressed genes in *Foxg1null* and *Foxg1CKO* embryos. (**a**) Relative mRNA expression levels of *IL-7*, *Foxn1*, and *Hoxa3* (*GAPDH* (ENDO)) at E11.5 from dissected 3rd pharyngeal pouches in *Foxg1null* (Three mutant and control embryos were run individually in triplicate in one qPCR experiment). (**b**) Relative expression of *IL-7* and *Foxn1* (*GAPDH* (ENDO)) from E14.5 and E16.5 *Foxg1CKO* dissected thymi (two mutant and control embryos at each stage were run individually in triplicate). The comparative C_t method was used against the reference gene *GAPDH* for both (a) and (b). Error bars are standard error. (*) denotes significance using unpaired T-test assuming unequal variances.

Figure 2.8 Model showing result of loss of or presence of Foxg1 in a Foxn1 negative bi-potent thymic epithelial cell progenitor. Foxg1 is controlling aspects of fate choice leading to reduction of either cTEC or mTEC lineage restricted progenitors depending on presence of absence of Foxg1 in bi-potent progenitor.

FIGURE 2.1

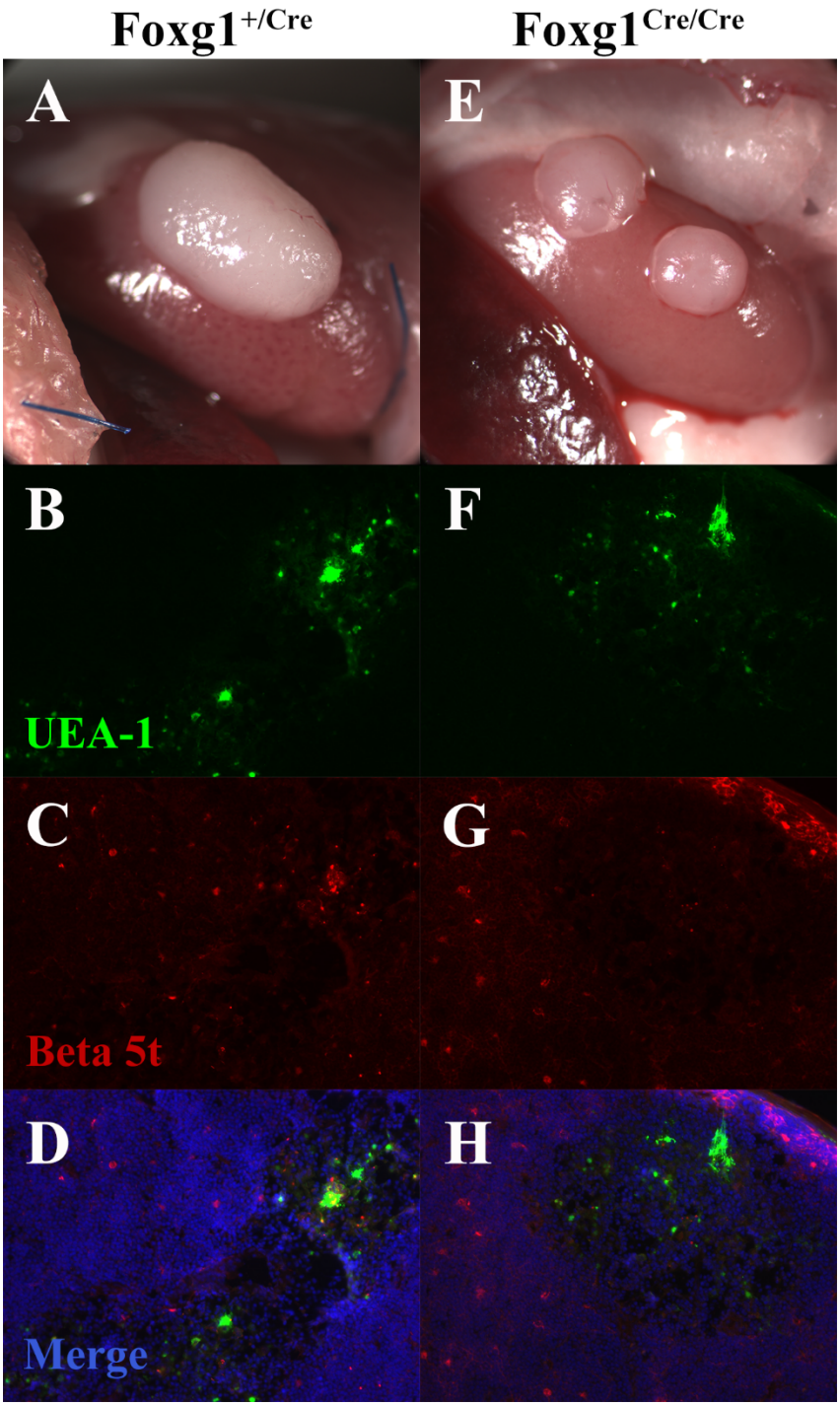


FIGURE 2.2

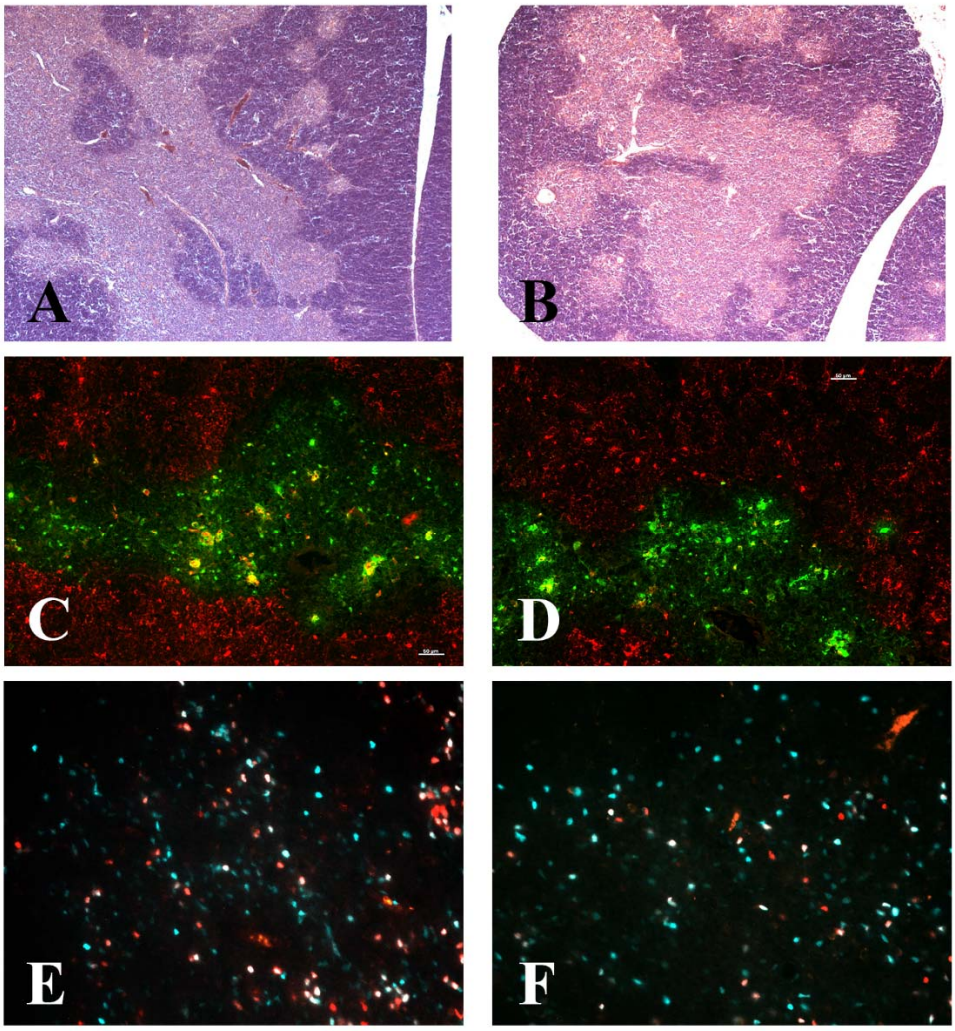


FIGURE 2.3

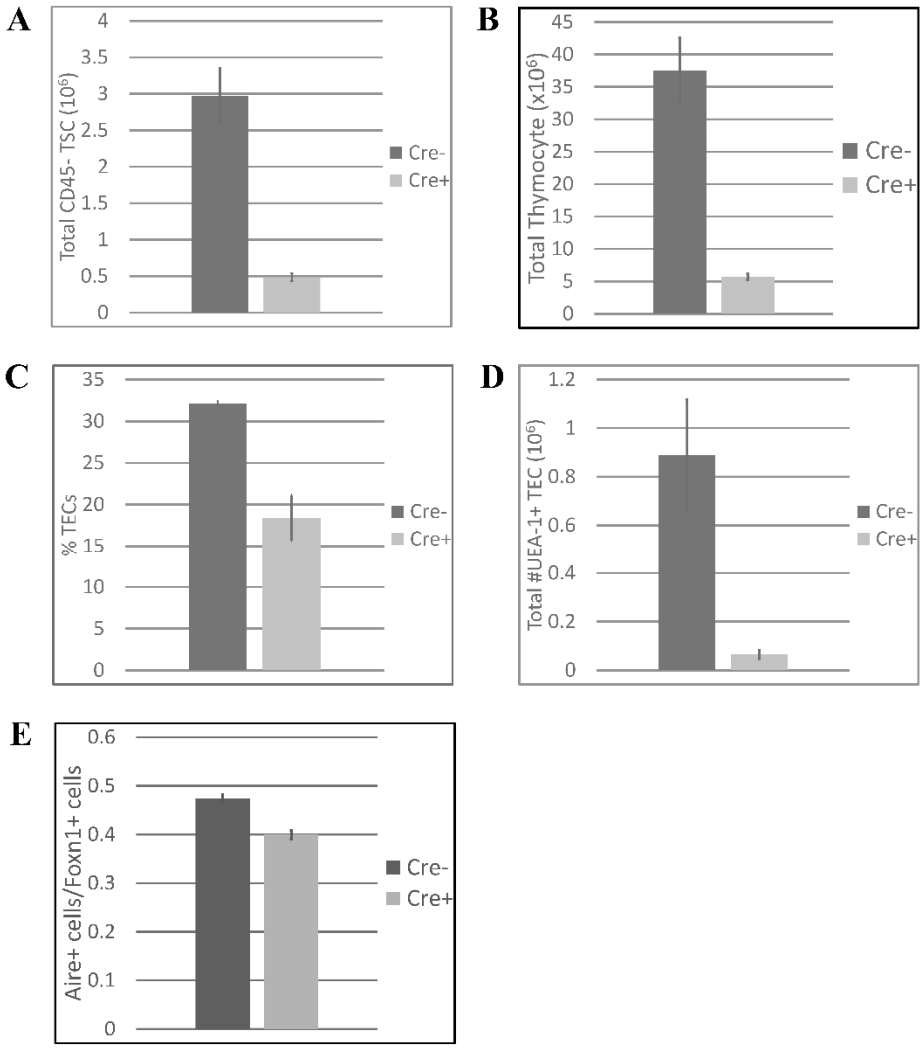

A
Total CD45- TSC (10⁶)
4
3.5
3
2.5
2
1.5
1
0.5
0
Cre-
Cre+
B
Total Thymocyte (x10⁶)
45
40
35
30
25
20
15
10
5
0
Cre-
Cre+
C
% TECs
35
30
25
20
15
10
5
0
Cre-
Cre+
D
Total #UEA-1+ TEC (10⁶)
1.2
1
0.8
0.6
0.4
0.2
0
Cre-
Cre+
E
Aire+ cells/Foxn1+ cells
0.6
0.5
0.4
0.3
0.2
0.1
0
Cre-
Cre+

FIGURE 2.4

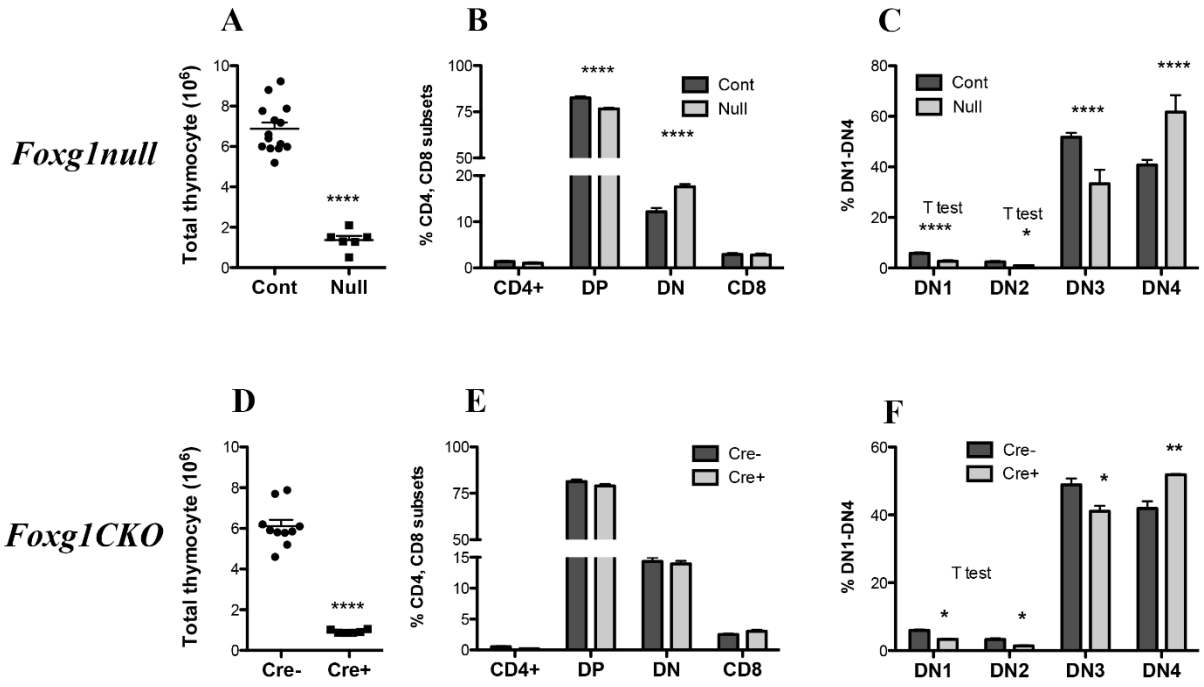

A
B
C
Foxg1null
Total thymocyte (10^6)
Cont
Null
% CD4, CD8 subsets
CD4+
DP
DN
CD8
% DN1-DN4
T test
DN1
DN2
DN3
DN4
D
E
F
Foxg1CKO
Cre-
Cre+

FIGURE 2.5

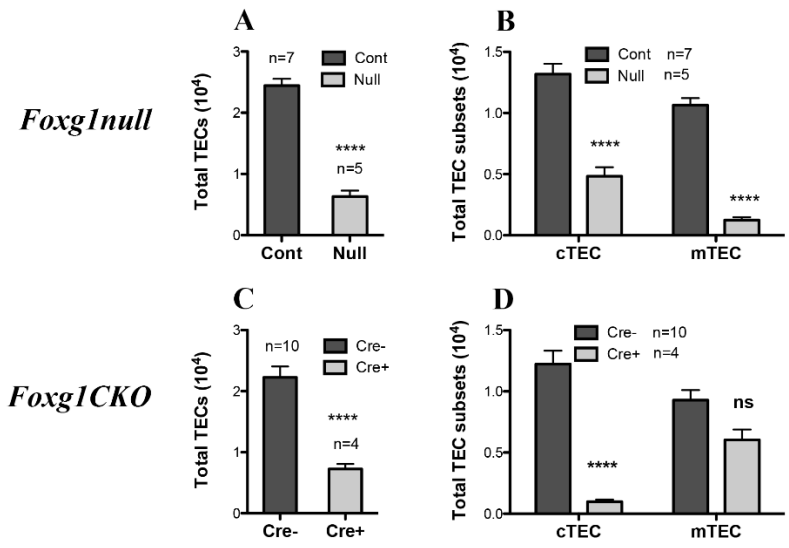

Foxg1null
Foxg1CKO
A
B
C
D
Total TECs (10^4)
Total TEC subsets (10^4)
n=7
Cont
Null
****
n=5
Cont
Null
Cont n=7
Null n=5
cTEC
mTEC
n=10
Cre-
Cre+
n=4
Cre- n=10
Cre+ n=4
ns

FIGURE 2.6

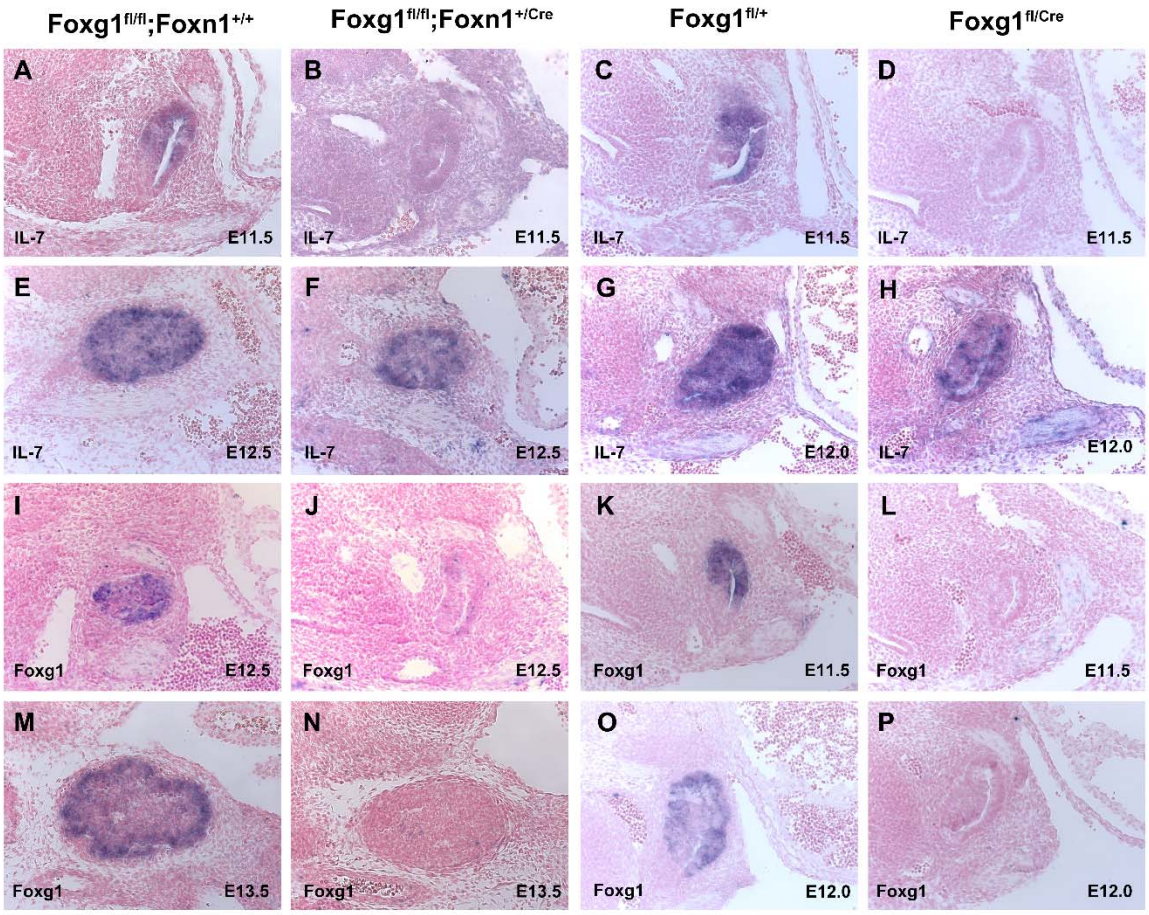
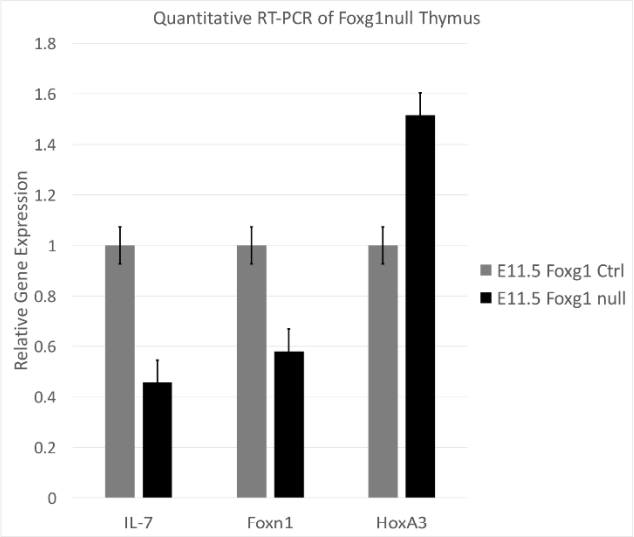

Foxg1fl/fl;Foxn1+/+
Foxg1fl/fl;Foxn1+/Cre
Foxg1fl/+
Foxg1fl/Cre
A
IL-7
E11.5
B
IL-7
E11.5
C
IL-7
E11.5
D
IL-7
E11.5
E
IL-7
E12.5
F
IL-7
E12.5
G
IL-7
E12.0
H
IL-7
E12.0
I
Foxg1
E12.5
J
Foxg1
E12.5
K
Foxg1
E11.5
L
Foxg1
E11.5
M
Foxg1
E13.5
N
Foxg1
E13.5
O
Foxg1
E12.0
P
Foxg1
E12.0

FIGURE 2.7

A


Quantitative RT-PCR of Foxg1null Thymus
Relative Gene Expression
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
IL-7
Foxn1
HoxA3
E11.5 Foxg1 Ctrl
E11.5 Foxg1 null

B

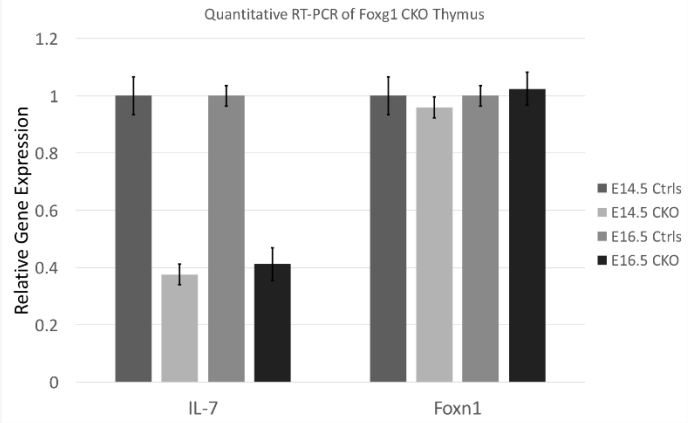
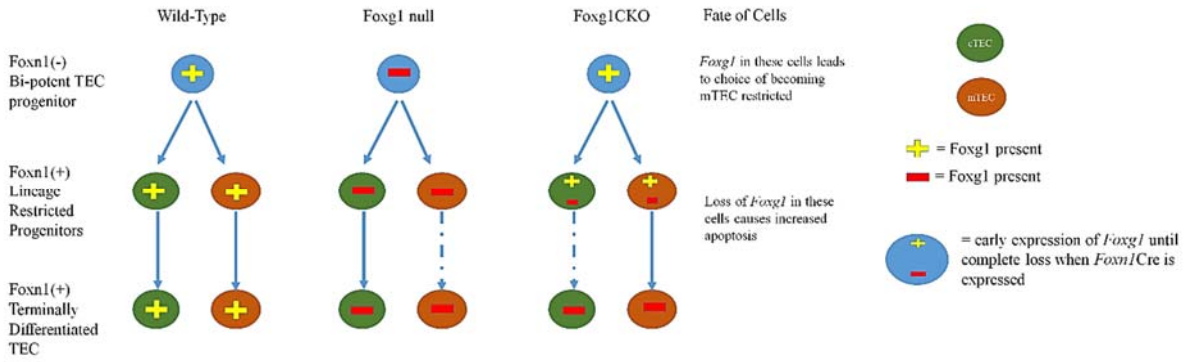

Quantitative RT-PCR of Foxg1 CKO Thymus
Relative Gene Expression
1.2
1
0.8
0.6
0.4
0.2
0
IL-7
Foxn1
E14.5 Ctrls
E14.5 CKO
E16.5 Ctrls
E16.5 CKO

FIGURE 2.8


Wild-Type
Foxg1 null
Foxg1CKO
Fate of Cells
Foxn1(-)
Bi-potent TEC
progenitor
Foxn1(+)
Lineage
Restricted
Progenitors
Foxn1(+)
Terminally
Differentiated
TEC
Foxg1 in these cells leads to choice of becoming mTEC restricted
Loss of Foxg1 in these cells causes increased apoptosis
cTEC
mTEC
= Foxg1 present
= Foxg1 present
= early expression of Foxg1 until complete loss when Foxn1Cre is expressed

CHAPTER 3

FOXG1 ALLEVIATES REPRESSION OF IL-7 IN THE EARLY THIRD POUCH ENDODERM BY DOWN REGULATION OF HOXA3

Introduction

The thymus is derived from the third pharyngeal pouch endoderm (Gordon et al. 2004) and is the primary lymphoid organ responsible for production of self-restricted, self-tolerant T-cells. The pouch itself develops as an outpocketing of the pharyngeal endoderm and is initially attached to the pharynx (Gordon et al. 2004; Blackburn and Manley 2004). Initially, the pouch consists of a ventral thymus domain and a dorsal parathyroid domain. These two regions are distinguishable at embryonic day 11.5 (E11.5) by expression of *Gcm2* in the parathyroid domain and *Foxn1* in the thymus domain (Gordon et al. 2001). *Foxn1* is essential for thymic epithelial cell (TEC) differentiation into the two main components of the thymus, the medulla (mTEC) and cortex (cTEC) (Nehls et al. 1996; Nowell et al. 2011; O'Neill et al. 2016). The genetic networks and signaling pathways that lead to the specification of thymus fate are still being investigated but some evidence exists that suggests that Bmp4 and Fgf8 are necessary to promote thymus fate while Sonic Hedgehog (Shh) promotes parathyroid fate (Gordon et al. 2010; Gardiner et al. 2012; Moore-Scott and Manley 2005; Bleul and Boehm 2005). The HOX-EYA-PAX-SIX transcriptional cascade has also been shown to be necessary for normal thymus development, although specification of thymus still occurs when any of these genes are mutated. (Manley and Condie 2010; Chojnowski et al. 2014).

Hoxa3 is required in the third pouch and surrounding mesenchyme for normal thymus development as shown by mice deficient in *Hoxa3*. Loss of *Hoxa3* in embryos leads to loss of thymus and other developmental defects (Chisaka and Capecchi 1991; Manley and Capecchi 1995). Also, Hoxa3 was shown to be necessary for sustained expression of *Pax1* as an upstream regulator (Manley and Capecchi 1995) and loss of *Pax1* expression leads to thymus hypoplasia (Dietrich and Gruss 1995). More recent analysis of *Hoxa3* in both the neural crest derived mesenchyme and third pouch endoderm showed that *Hoxa3* expression in the endoderm is required for patterning of the pouch and regulation of thymus specific gene expression (Chojnowski et al. 2014). Also, loss of *Hoxa3* causes an increase in expression levels of *Interleukin 7* (*IL-7*). Furthermore, loss of thymus due to apoptosis required deletion of *Hoxa3* in both endoderm and neural crest mesenchyme.

Another transcription factor, *Foxg1*, has been shown to be necessary for normal thymus development (Wei et al. unpublished and Chapter 2). Loss of *Foxg1* leads to a reduction in total thymocyte and TEC cellularity. It also causes a delay in and reduction of *IL-7* expression. Interestingly, TEC specific loss of *Foxg1* results in a reduction in cTEC proportions relative to mTEC while the *Foxg1* null has the opposite effect (Chapter 2). One possible explanation for this is that the conditional deletion of *Foxg1* using the *Foxn1Cre* (Gordon et al. 2007) mouse doesn't cause deletion of *Foxg1* in the most basal TEC stem cell and suggests that Foxg1 is acting to direct the production of mTEC lineage restricted progenitors in this stem cell population.

This study examines the interaction and overlapping expression of both *Foxg1* and *Hoxa3* during early thymus development. We show that *Foxg1* is first expressed in a region that doesn't express markers for either parathyroid or thymus. This region also has reduced *Hoxa3* expression (Chojnowski et al. 2014) and we suggest that Foxg1 is responsible for controlling the initial

down regulation of *Hoxa3* in the thymus domain of the pouch. We also show that this initial downregulation of Hoxa3 by Foxg1 is necessary for initiating *IL-7* expression at E11.5 but that after E12.5 Hoxa3 is dispensable.

Methods

Mice

All mouse work conformed with the stipulations of the University of Georgia Institutional Animal Care and Use Committee. All mice were described in Chapter 2 of this dissertation with the exception of *Hoxa3Cre* mice. Hoxa3IRES-Cre (Macatee et al. 2003) were generously donated by Dr. Anne Moon. Conditional mice were generated by mating $Foxg1^{C:Flpe}$ mice to *Hoxa3Cre* mice and then back crossing those mice heterozygous for both genes to homozygous $Foxg1^{C:Flpe}$ mice. *Foxg1;Hoxa3* compound mutants were generated by mating $Foxg1^{-/-}$ mice (previously described in Chapter 2) to *Hoxa3del* (Chojnowski et al. 2014) mice which were generated in our lab.

Immunohistochemistry

Embryonic and adult tissues were embedded in paraffin, sectioned, and stained as described in Chapter 2. Primary antibodies used were: goat anti-Foxn1(1:200, Santa Cruz, G-20), biotinylated UEA-1 (1:200, Vector Laboratories), rat anti-Aire (1:100, clone: 02-5H12-2), chicken anti-GFP (1:200, Abcam), rabbit anti-Keratin 5 (1:200, Biolegend, Clone: Poly19055), rabbit anti-Gcm2 (1:200, Abcam). Secondary antibodies were either Alexa (Invitrogen) or DyLight (Jackson Immunoresearch).

In situ Hybridization

Tissue preparation, *in situ* protocol, and riboprobe generation were all performed as described in Chapter 2.

Results

Foxg1 GFP reporter analysis

We first sought to examine the expression of *Foxg1* by utilizing the GFP reporter located in the *Rosa26* locus in the *Foxg1*$^{C:Flpe}$ (hereafter *Foxg1floxed*) mice. This conditional allele drives *Flipase* expression under the control of the endogenous Foxg1 promoter after Cre mediated recombination of the loxp flanked coding region of *Foxg1*. We utilized a Cre recombinase line that expresses Cre under the control of endogenous *Hoxa3* (Macatee et al. 2003*)*. GFP expression only occurs in cells that have expressed both *Hoxa3* and *Foxg1* and only after *Foxg1* expression is normally initiated. Furthermore, during early pouch development differing levels of GFP expression can be reasoned to be a proxy for which cells first begin expressing *Foxg1*. One caveat to this experiment is that GFP expression was nearly undetectable in mice heterozygous for the conditional allele at embryonic stages. Therefore, all examination in fetal stages was done in mice homozygous for *Foxg1floxed* and that also expressed Cre. To minimize complications most analysis was done at very early embryonic stages which do not show any major phenotypes due to loss of *Foxg1*.

At embryonic day 11.5 (E11.5), GFP expression is highest in the dorsal posterior domain that sits between the ventral thymus domain and the dorsal parathyroid domain (Figure 3.1A). These cells do not express either *Foxn1* or *GCM2* which mark the thymus and parathyroid, respectively. Also, GFP expression in the ventral domain is low but present suggesting that Foxg1 is normally expressed in the presumptive TEC later then in this "unspecified" region. At E12.5, GFP expression is present throughout the TEC (Figure 3.1B). This shows that *Foxg1* expression is initiated in most of the Foxn1+ TEC although there does appear to be some TEC that express either low or no *Foxg1* at this stage.

Postnatal thymus of *Foxg1*$^{+/fl}$*;Hoxa3Cre+* mice shows that fewer TEC express GFP compared to embryonic stages (Figure 3.2A). Interestingly, overlap of GFP and Foxn1 shows that there are many Foxn1+ TEC that have never expressed *Foxg1*. Alternatively, it is also possible that these TEC never expressed *Hoxa3* and therefore have not deleted *Foxg1*. However, based on examination of *Hoxa3* (Chojnowski et al. 2014) in wild-type mice it seems more likely that these cells just never turn on *Foxg1*. Although it is possible that the *Hoxa3Cre* mice do not drive Cre expression efficiently in all cells. GFP expression also doesn't overlap much with Aire+ cells (Figure 3.2B) suggesting that the most terminally differentiated mTEC came from a population of TEC that are negative for *Foxg1*. Although many cells seem to express GFP and therefore Foxg1 at early embryonic stages, this data shows that there is a large population of TEC that never express *Foxg1*. Also there are some TEC at this stage that have expressed *Foxg1* at some point in their lineage but that either never have or no longer do express *Foxn1* (Figure 3.2A).

IL-7 expression in Foxg1;Hoxa3 compound mutants

It has been shown in *Hoxa3*$^{-/-}$ embryos that the expression of *IL-7* is increased at E12.0 (Chojnowski et al. 2014). Since we also know that *IL-7* expression is altered in the *Foxg1*$^{-/-}$ (Wei et al. unpublished) and in *Foxg1CKO* (Chapter 2) mice, we examined the expression of *IL-7* in *Foxg1;Hoxa3* compound mutants. We crossed *Foxg1*$^{+/-}$ mice to *Hoxa3*$^{+/-}$ to generate double heterozygotes for both Foxg1 and Hoxa3 and then interbred these double heterozygotes to obtain compound mutants. *In Situ* hybridization for *IL-7* in these mice is shown in Figure 3.3. Expression of *IL-7* in *Foxg1*$^{+/-}$*;Hoxa3*$^{+/+}$ embryos at ~E13 -E13.5 (Figure 3.3A) shows a normal level of expression. Loss of one allele of Hoxa3 (Figure 3.3B) shows increased expression levels. This same pattern is seen in *Foxg1*$^{-/-}$ mice that either have normal amounts of Hoxa3 (Figure

3.3C) or loss of one allele (Figure 3.3D). These results are preliminary and data is only representative of 1 sample per genotype. It still remains to be seen what *IL-7* expression is in *Foxg1*$^{+/-}$*:Hoxa3*$^{-/-}$ or *Foxg1*$^{-/-}$*;Hoxa3*$^{-/-}$ because at this stage of development mice lacking *Hoxa3* expression do not have a thymus rudiment.

Discussion

This report shows that Foxg1 is expressed in the dorsal posterior "unspecified" region prior to being expressed in the ventral thymus domain. This evidence when combined with data which shows *Hoxa3* expression in this dorsal posterior region to be low (Chojnowski et al. 2014) suggests that Foxg1 is necessary to induce the down regulation of *Hoxa3* in the pouch. It further suggests that this down regulation is occurring in the "unspecified" region prior to E11.5 and that as *Foxg1* expression begins to increase in the ventral domain around E11.5 which causes the subsequent down regulation of *Hoxa3* in TEC progenitors. Furthermore, the prolonged expression of *Hoxa3* in the Foxg1 null mice shows that Foxg1 is necessary to regulate the down regulation of *Hoxa3* temporally.

The *IL-7* phenotype in the *Hoxa3*$^{-/-}$ (Chojnowski et al. 2014) and the data presented here further suggest that Hoxa3 is responsible for the temporal regulation of *IL-7* in the third pharyngeal pouch. In the Hoxa3 null mice, it was also shown that *Foxg1* expression is delayed in the ventral region but not the dorsal posterior region of the pouch endoderm. This suggests also that Hoxa3 may be regulating *Foxg1* expression in a temporal manner in TEC progenitors. All this evidence combined suggests a model (Figure 3.4) in which Hoxa3 represses IL-7 during early pouch patterning and that Foxg1 induction alleviates the repression of IL-7 by down regulating Hoxa3 in these early TEC progenitors. It also suggests that Hoxa3 is regulating its own repressor prior to TEC specification at E11.5. This could mean that Foxg1 is necessary to

shut down the early thymus organogenesis pathways and that this is necessary to allow the correct timing of TEC specification. However, the data presented here is preliminary and so further examination in *Foxg1;Hoxa3* compound mutants between E11.5 and E12.5 is necessary to elucidate its exact role in this process.

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Figures and Figure Legends

Figure 3.1 GFP reporter analysis by immunohistochemistry in *Foxg1fl;Hoxa3Cre* mice. (**a-c**) Staining of third pouch with Foxn1(red), GFP(green), GCM2(purple) in (a) E11.5, (b) E12.5, and (c) E13.5 *Foxg1$^{fl/fl}$;Hoxa3Cre*+ mice.

Figure 3.2 GFP reporter analysis by immunohistochemistry of postnatal 4 week thymus from *Foxg1fl;Hoxa3Cre* mice. (**a**) Staining of *Foxg1$^{+/fl}$;Hoxa3Cre*+ thymus with Foxn1(red), GFP(green), or K5(purple) (**b**) Staining of *Foxg1$^{+/fl}$;Hoxa3Cre*+ thymus with Aire(red), GFP(green), or UEA-1(purple)

Figure 3.3 *IL-7* expression is sensitive to reduction in *Hoxa3* levels in *Foxg1;Hoxa3* compound mutants. *IL-7 in situ* hybridization levels in (**a**) *Foxg1$^{+/-}$;Hoxa3$^{+/+}$*, (**b**) *Foxg1$^{+/-}$;Hoxa3$^{+/-}$*, (**c**) *Foxg1$^{-/-}$;Hoxa3$^{+/+}$*, and (**d**) *Foxg1$^{-/-}$;Hoxa3$^{+/-}$*.

Figure 3.4 Model showing Foxg1, Hoxa3, and IL-7 network. Model shows that Hoxa3 is a repressor of IL-7 and that Foxg1 represses Hoxa3 which allows expression of IL-7. Hoxa3 also promotes the expression of its own repressor, Foxg1.

FIGURE 3.1

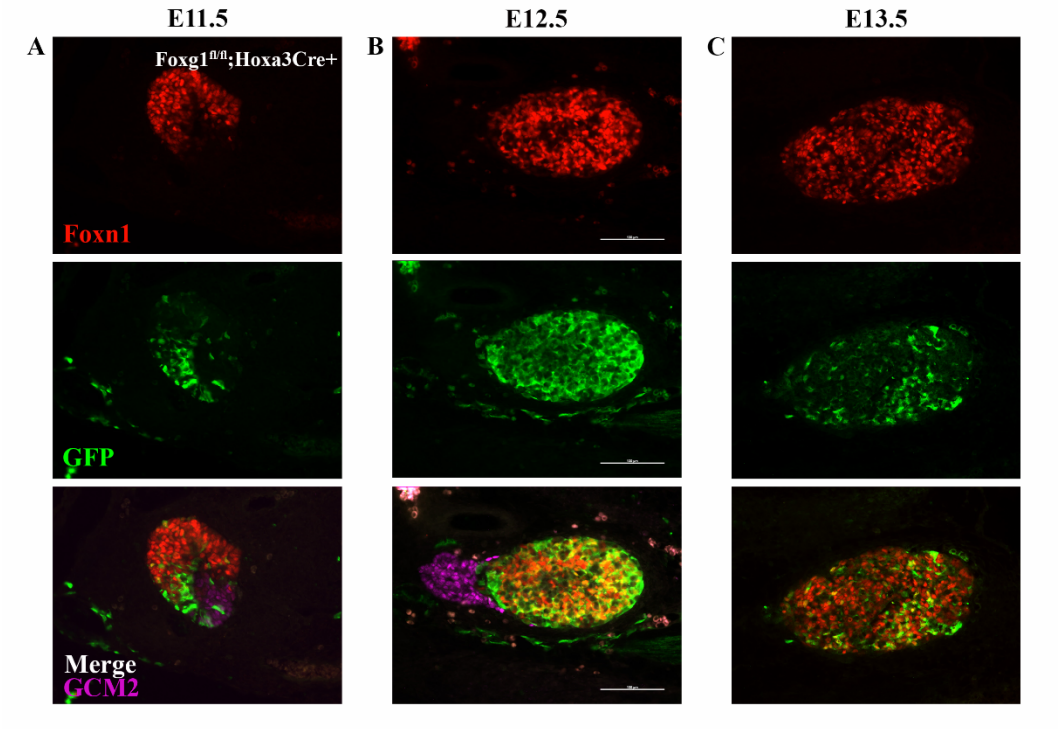

FIGURE 3.2

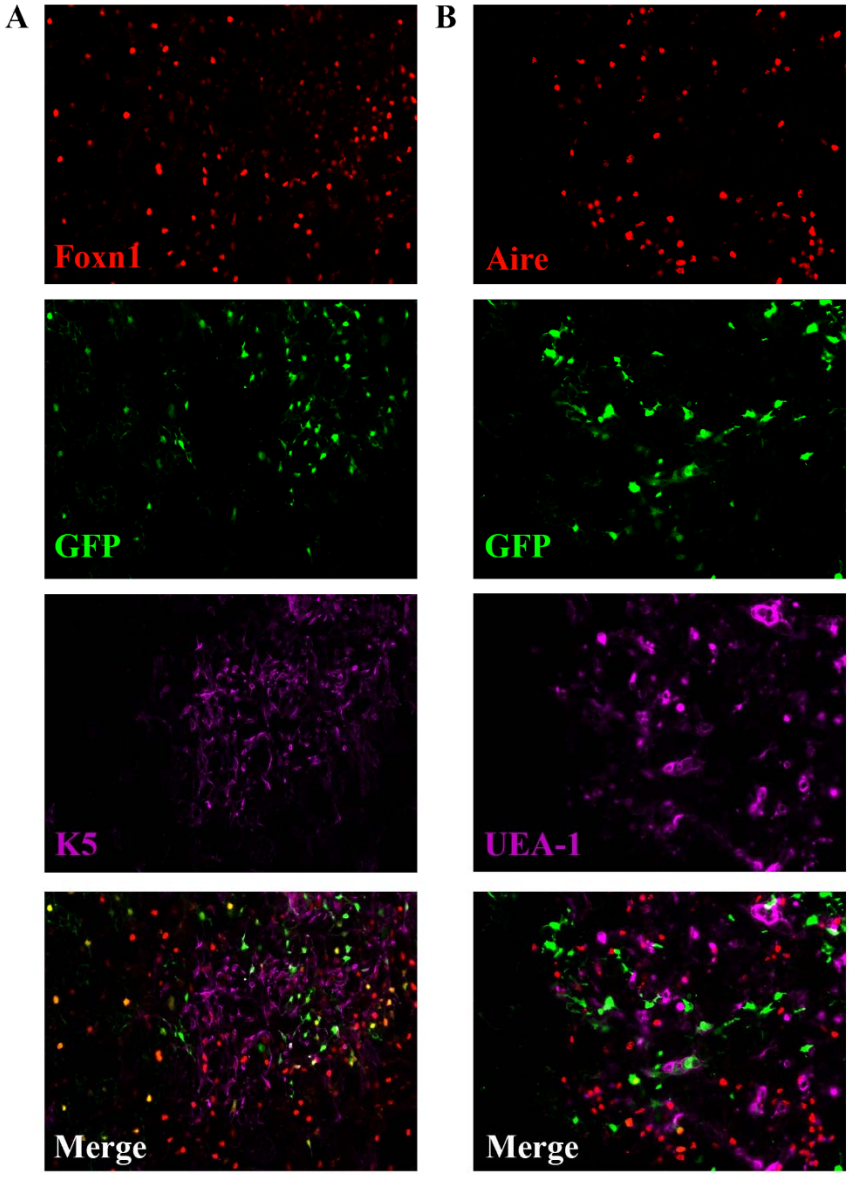

FIGURE 3.3

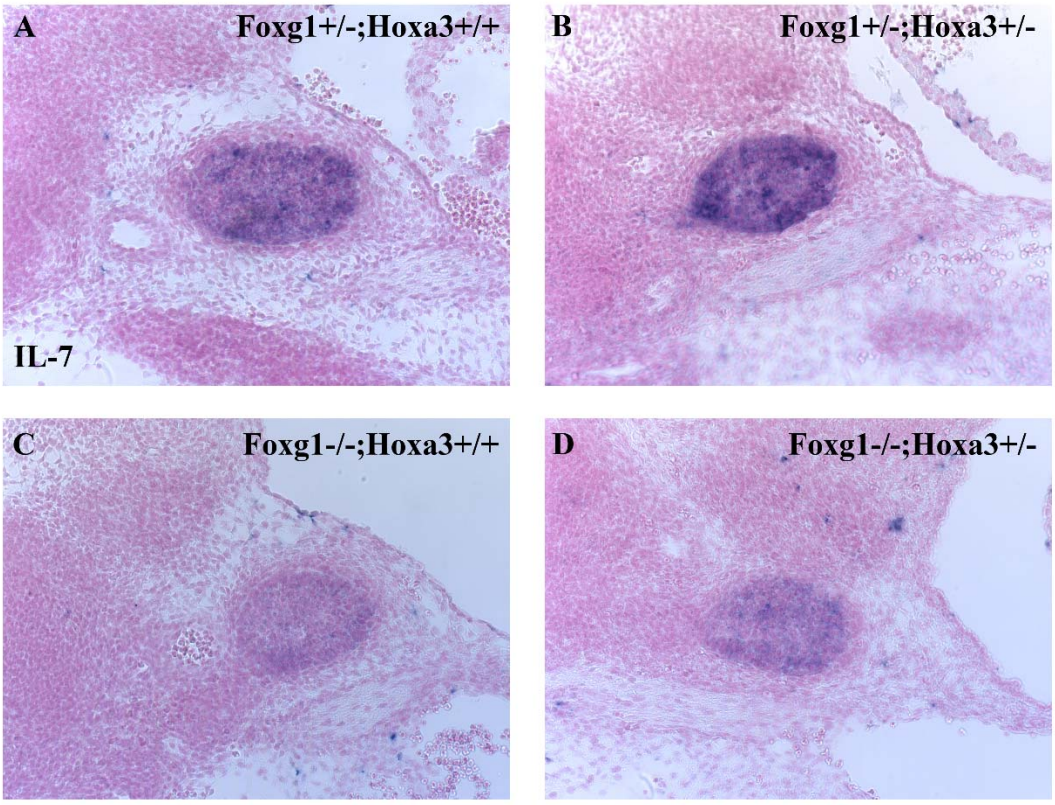

FIGURE 3.4

CHAPTER 4

DISCUSSION AND CONCLUSIONS

The thymus is an intricate network of epithelial cells who's only known function is to provide the necessary microenvironment for the production of self-restricted, self-tolerant T-cells which migrate to the periphery to provide organisms with an adaptive immune system. Therefore, understanding how these thymic epithelial cells (TEC) are specified to this fate and what controls their differentiation into the two main subsets, cortical (cTEC) and medulla (mTEC) is paramount if we are to try and unravel possible ways to prevent or replenish TEC due to age related involution. A *forkhead* transcription factor, *Foxn1*, has long been hailed as the master regulator of TEC differentiation and proliferation (Blackburn et al. 1996; Nehls et al. 1996). It has been shown that *Foxn1* expression is capable of driving TEC differentiation in an adult revertible *Foxn1* hypomorph; as well as being sufficient to reprogram cultured primary mouse fibroblasts into functional TEC (Jin et al. 2014; Bredenkamp et al. 2014). However, recent studies in our lab identified many different potential regulators that are expressed in thymus (Wei and Condie 2011). Of the genes identified in this screen, *Foxg1* was investigated and shown to be necessary but not sufficient for normal thymus development (Wei et al. unpublished). Indeed, it was shown that Foxg1 was necessary to control Foxn1 independent aspects of TEC differentiation and survival. The results of this analysis showed that loss of *Foxg1* leads to a severely hypoplastic thymus with reductions of both TEC and thymocytes and suggested that *Foxg1* deficient mice lacked *IL-7* expression during early thymus organogenesis.

This study was limited to embryonic development and was unable to characterize the necessity of *Foxg1* in the postnatal thymus.

The purpose of my Ph.D. research was to further examine the role of Foxg1 in postnatal thymus as well as to determine whether FOXG1 has specific functions before and after TEC specification at E11.5, as determined by *Foxn1* expression, and how this transcription factor might be regulating the expression of the essential non-redundant cytokine, *IL-7*. To accomplish this, I created TEC specific *Foxg1* loss of function mutants by crossing mice with *loxp* sites flanking the entire coding region of *Foxg1* (*Foxg1*$^{fl/fl}$) (Miyoshi and Fishell 2012) with mice expressing Cre recombinase under the control of endogenous *Foxn1* (*Foxn1Cre*) (Gordon et al. 2007). The resulting *Foxg1* conditional knock-out (*Foxg1CKO*) mice showed comparable phenotypes at embryonic stages as the null (Wei et al. unpublished) albeit with one striking difference at E18.5 (Chapter 3). I was also able to examine the postnatal necessity for Foxg1 and show that the embryonic defects in thymic cellularity persist postnatally which suggests the continued necessity of *Foxg1* to maintain normal cell numbers in the thymus.

Foxg1 and Foxn1 in Thymus

As mentioned above, *Foxn1* is sufficient to promote thymic epithelial cell progenitors (TECP) in a *Foxn1* revertible hypomorphic adult thymus to differentiate into both mTEC and cTEC (Jin et al. 2014). It has also been shown to be sufficient to reprogram embryonic fibroblasts into TEC that, when transplanted under mouse kidney capsule, led to the formation of an organized and functional thymus (Bredenkamp et al. 2014). However, our lab showed that *Foxn1* is necessary but not sufficient for normal thymus development *in vivo* and that *Foxg1* is necessary for *Foxn1* independent TEC differentiation and survival in the thymus as well as regulation of *IL-7* (Wei et al. unpublished), which, consistent with these findings, is also

independent of *Foxn1* (Zamisch et al. 2005). Furthermore, generation of *Foxg1;Foxn1* double mutants (Chapter 3) showed that *Foxg1* is not necessary for the survival of the TEPC population which persists in the *Foxn1* null (Blackburn et al. 1996). The defects in *Foxg1* and *Foxn1* single mutants and double mutants combined supports the idea that *Foxg1* is not necessary for TEC specification or survival prior to *Foxn1* expression but that after specification it is necessary for *Foxn1* independent TEC survival and/or differentiation during embryonic development and maintenance in postnatal thymus.

Foxn1 was also shown to be absent from an expanding population of TEC between 1 and 3 months postnatally and this expansion of *Foxn1*(-) TEC was correlated with reduced cTEC function, suggesting that this increase in *Foxn1*(-) TEC may be contributing to age related involution in the thymus (O'Neill et al. 2016). It would therefore be interesting to investigate the role of Foxg1 in these *Foxn1*(-) TEC using our *Foxg1CKO* to determine if Foxg1 is regulating the expansion of this population of cells. It has also shown that both cTEC and mTEC are derived from a common bi-potent progenitor (Klug et al. 2002; Ohigashi et al. 2013; Rossi et al. 2006; Bennett et al. 2002) and that mTEC are *Foxn1*^{low} expressing TEC (O'Neill et al. 2016) and are derived from TECP that have expressed the cortical markers, CD205 (Baik et al. 2013) and β5t (Ohigashi et al. 2013; Mayer et al. 2016). Therefore, the generation of UEA-1+ and AIRE+ mTEC from this TECP population could very well be due to the downregulation of *Foxn1* in these cells, as suggested by O'Neill et al. (2016). Given the reduction in UEA-1+ and AIRE+ mTEC in the *Foxg1*$^{-/-}$ embryos and the *Foxg1CKO* postnatal thymus, it seems possible that FOXG1 may be regulating this downregulation of *Foxn1* in the TECP which leads to reduced differentiation of these progenitors into mTEC. Alternatively, Foxg1 may be responsible for survival in the mature mTEC population, which at least for the AIRE+ mTEC have been shown

to be post-mitotic (Gray et al. 2007). Overall the data suggest that Foxg1 and Foxn1 do not have overlapping functions within the thymus. Furthermore, the increase in apoptotic cells within the *Foxg1* mutants suggests that it is this function of Foxg1 that leads to the reduction in mTEC and not regulation of *Foxn1* levels by FOXG1, although further experiments need to be performed to verify this.

Foxg1 and the Phosphatidylinositol 3-Kinase (PI3K)/Akt Pathway

In addition to regulating differentiation and cell cycle in the central nervous system (CNS) (Xuan et al. 1995; Dou et al. 2000; Hanashima et al. 2004; Miyoshi and Fishell 2012), Foxg1 has been linked to regulation of apoptosis in both the CNS (Shen et al. 2006; Martynoga et al. 2005) and cancers (Li et al. 2013). Foxg1 has also been shown to promote survival of post-mitotic neurons and that this effect is due to downstream phosphorylation of FOXG1 by the PI3K/Akt pathway (Dastidar, Landrieu, and D'Mello 2011) as well as the association of FOXG1 protein with the transducin-like enhancer of split-1 (TLE1) (Dastidar, Narayanan, et al. 2012). It was further shown that in these neurons FOXG1 and TLE1 are necessary to inhibit the methyl-CpG binding protein 2 (MeCP2) and that loss of *Foxg1* allows MeCP2 to promote neuronal death (Dastidar, Bardai, et al. 2012). FOXG1 has also been shown to inhibit FoxO/Smad complexes from inducing *p21Cip1* transcription in neurons (Seoane et al. 2004) thereby inhibiting the cytostatic TGFβ signals and allowing for neural progenitor proliferation. FoxO in these cells is also negatively regulated due to Akt phosphorylation of FoxO proteins which inhibits their entry into the nucleus and prevents them from interacting with target genes. Although FoxO proteins have been shown to be active in thymocyte survival downstream of PI3K/Akt (Coffer and Burgering 2004), they have not been reported to be necessary for TEC survival or differentiation.

The PI3K/Akt signaling pathway has been shown to be necessary for thymus and T-cell development (Garfin, Nguyen, and Sage 2016; Yang et al. 2005; Thien et al. 2010; Mao et al. 2007; Guo, Teng, and Ji 2011). In mouse, there are three *Akt* genes (*Akt1, Akt2,* and *Akt3*) and deletion of any one causes developmental defects in the brain and other organs and in some cases perinatal lethality (Yang et al. 2005). Compound mutants, $Akt1^{-/-}Akt3^{+/-}$, all showed perinatal lethality and exhibited severe thymus hypoplasia (Yang et al. 2005). Surprisingly, another recent study deleted a suppressor of PI3K signaling Phosphatase and Tensin Homologue (PTEN) in TEC and showed that mutants with constitutive activation of the PI3K pathway also have a hypoplastic thymus and that there is severe dysregulation of TEC differentiation which likely leads to disorganized thymus architecture (Garfin, Nguyen, and Sage 2016). Although these two reports seem to oppose each other, they show that temporally regulated PI3K/Akt signaling in the thymus is required for normal organ development and that much is still to be uncovered about the function of this pathway in TEC and T-cell development. In light of reports that *Foxg1* acts downstream of Akt (Dastidar, Landrieu, and D'Mello 2011), it needs to be determined if phosphorylation of FOXG1 by Akt in TEC is also occurring. The similarities in phenotypes, severe hypoplasia of both TEC and thymocytes, seen in *Foxg1* mutants and mice defective in PI3K/Akt signaling seems to suggest that Foxg1 may be acting downstream of this pathway to promote the survival of TEC. This would fit with the increase in apoptosis in the thymus reported in $Foxg1^{-/-}$ (Wei et al. unpublished) and in TEC specific *Foxg1* mutants (Chapter 3).

Foxg1 and Wnt Signaling

Canonical Wnt/β-catenin signaling is another important pathway necessary for normal thymus development. In this pathway, Wnt ligands activate Frizzled (Fz) receptors which along with axin, adenomatous polyposis coli (APC) protein, and glycogen synthase kinase 3β (GSK-

3β) lead to the stabilization of β-catenin, which locates to the nucleus and activates transcription of target genes (reviewed in (Balciunaite et al. 2002)). *Foxg1* has been shown to interact in the Wnt pathway in telencephalic development (Danesin et al. 2009; Aguiar, Sghari, and Creuzet 2014) as well as other structures during development (Fotaki et al. 2013). Indeed, examination of FOXG1 binding to *Wnt8b* in zebrafish, showed that FOXG1 is a direct repressor of this essential signaling ligand and the putative binding site in the *Wnt8b* promote*r* was reported to be conserved in mouse (Danesin et al. 2009). In the thymus, TEC have a non-redundant role in Wnt ligand production (Brunk et al. 2015). Loss of Wnt signals at embryonic stages (Brunk et al. 2015) or loss of β-catenin in adult K5+ TEC (Liang et al. 2013) leads to TEC hypotrophy and a decrease in the number, but not the proportions, of T-cell subsets within the thymocyte pool (Liang et al. 2013; Brunk et al. 2015), although loss of β-catenin in T-cells causes arrest of thymocytes at the DN3-DN4 stage (Xu et al. 2003). A gain of function mutant with constitutively active β-catenin in TEC also displayed severe thymus hypotrophy (Zuklys et al. 2009). Indeed, the authors suggested that the TEC downregulated *Foxn1* and switched their cell fate to simple epithelium. These results together suggest that precise Wnt signaling is necessary in TEC for survival and differentiation.

Interestingly, both of these Wnt/β-catenin deficient TEC examples show a more severe reduction in the mTEC compartment similar to that seen in *Foxg1*$^{-/-}$ (Wei et al. unpublished) and in *Foxg1CKO* (Chapter 3). In fact, most of the phenotypes between these Wnt signaling deficient mice and either of the *Foxg1* mutants discussed in this dissertation are very comparable including the decreased cellularity in TEC/thymocytes, more severe reduction in mature Aire+ and UEA-1+ mTEC, mostly normal proportions of T-cells, increase in apoptosis, and, in the case of the β-catenin mutants (Liang et al. 2013), a reduction in the levels of *IL-7*. One caveat to this

is the finding that, while the *Foxg1*$^{-/-}$ mice show a decrease in UEA-1+ mTEC at E18.5 by flow cytometry, the *Foxg1CKO* mice at the same stage show a reduction in LY51+ cTEC relative to UEA-1+ mTEC. This suggests that there may be different temporal requirements for Foxg1 in TEC before and after *Foxn1* expression at E11.5. Although, immunohistochemistry using a different cTEC marker, β5t, at both embryonic and postnatal stages does not support there being a decrease in cTEC relative to mTEC in the *Foxg1CKO* and flow cytometry on 4wk postnatal *Foxg1CKO* show the same reduction in mTEC that is seen in the null (see Chapter 3). This difference between the null and conditional could be due to some other difference that has not yet been discovered that leads to a reduction in the detection of Ly51+ cTEC by flow cytometry in the *Foxg1CKO* mice and this could be due to differential survival potential of the different TEC subsets.

However, the similarities in phenotypes between Wnt signaling deficient mice and *Foxg1* mutants seems to strongly suggest that loss of *Foxg1* is altering Wnt signaling in TEC and that it may not be as simple as FOXG1 repressing Wnt ligand transcription as it has previously been shown to do in the telencephalon (Danesin et al. 2009). It actually seems to suggest that FOXG1 may be required either to activate canonical Wnt signaling or perhaps to repress a repressor of Wnt ligands that act in the canonical pathway. Alternatively, *Foxg1* could be a downstream mediator of Wnt signals although presently there is no evidence that would support this. Another interesting paper showed through *in vitro* cell culture of cTEC and mTEC lineage cells, that canonical Wnt signaling controls the transcriptional regulation of *Foxn1* in TECs and that the PI3K pathway through Akt phosphorylation contributes to this (Balciunaite et al. 2002). It therefore seems plausible that FOXG1 is mediating this link between these pathways by being phosphorylated by Akt downstream of PI3K signals, as shown in neurons (Dastidar, Landrieu,

and D'Mello 2011), and that it then interacts in the Wnt/β-catenin pathway in TEC. These are all very compelling hypotheses which require further investigation to confirm. Although it seems very likely that the *Foxg1* phenotypes are due in part to defects in Wnt signaling, exactly where FOXG1 acts in this pathway still needs to be investigated in TEC.

Foxg1 Regulation of IL-7

It has been shown that *IL-7* expression is reduced in *Foxg1* null (Wei et al.unpublished & Chapter 3) and *Foxg1CKO* (Chapter 3). Also discussed above is the fact that *IL-7* expression was decreased in mice defective in Wnt signaling (Zuklys et al. 2009; Liang et al. 2013). A recent study examining thymus in *Hoxa3* mutants showed delayed expression of *Foxg1, Foxn1*, and increased expression of *IL-7* at E11.5 in the ventral thymus domain of the 3rd pharyngeal pouch (Chojnowski et al. 2014). However, *Foxg1* expression in the dorsal-posterior region between the parathyroid and thymus domains of the pouch was normal at E10.5 in the *Hoxa3*$^{-/-}$. Hoxa3 has been implicated as a regulator of patterning, survival, proliferation and differentiation in the 3rd pharyngeal pouch (Chisaka and Capecchi 1991; Manley and Capecchi 1995, 1998). It has also been shown to be upstream of PAX1 (Manley and Capecchi 1995; Su et al. 2001) in the HOX-EYA -PAX -SIX transcriptional cascade which is necessary for normal thymus development (Manley and Condie 2010; Zou et al. 2006). Given the increase in *IL-7* expression in *Hoxa3* null and its decrease in *Foxg1* mutants, it seemed reasonable to investigate whether Foxg1 and Hoxa3 were interacting to control the expression of *IL-7*. As reported in Chapter 3, analysis of *IL-7* by *in situ* hybridization in *Foxg1;Hoxa3* compound mutants suggests that Foxg1 may be necessary during early thymus development to downregulate *Hoxa3* which allows for normal expression of *IL-7*. Also, given the delay in *Foxg1* expression in *Hoxa3* null mice (Chojnowski et al. 2014), it may be that HOXA3 is regulating its own repressor. Aside from this potential role of Foxg1 in

regulating the initiation of *IL-7* by downregulation of *Hoxa3,* Foxg1 seems to be necessary for maintaining normal levels of *IL-7* through its interactions in the Wnt signaling pathway in TEC (discussed above).

Foxg1 May Serve Two Functions in the Third Pharyngeal Pouch Endoderm

The third pharyngeal pouch in mice consists of a dorsal parathyroid domain and a ventral thymus domain. Sonic hedgehog (*Shh*) has been shown to be necessary for specification of the parathyroid domain and to restrict expression of *Bmp4* to the ventral thymus domain (Moore-Scott and Manley 2005). Both *Bmp4* and *Fgf8* are expressed in the thymus domain prior to *Foxn1* expression and are implicated in the patterning and differentiation of the thymus (Gordon and Manley 2011). Loss of *Shh* in the parathyroid domain leads to loss of parathyroid and expansion of *Bmp4* into the dorsal region of the pouch (Moore-Scott and Manley 2005). It has therefore been suggested that Shh and Bmp4 form opposing gradients which allow each to promote the development of parathyroid and thymus, respectively. *Foxg1* was shown to be expressed in a dorsal-posterior region (Wei and Condie 2011) that does not express either parathyroid marker *Gcm2* or thymus specific *Foxn1*. *Foxg1* is first detected in this region at E10.5 and, unlike its expression in the ventral region, is unaffected by loss of *Hoxa3* (Chojnowski et al. 2014). This suggests that Foxg1 may have a different function in this "unspecified" region compared to any function it has in the ventral thymus domain of the pouch.

Foxg1 has been shown to prevent Bmp expansion into the telencephalon and loss of *Foxg1* also results in a large reduction in *Fgf8* expression in the telencephalon (Martynoga et al. 2005). Shh has also been shown to be important for development of ventral telencephalon fates along with Fgf8 and Foxg1 (reviewed in (Hebert and Fishell 2008)). In fact, SHH acts to repress *Gli3.* Gli3 normally represses *Fgf8* and is necessary to promote *Bmp* and *Wnt* expression both of

which promote dorsal telencephalon fates. Therefore, Foxg1 and Shh work together to promote ventral telencephalon fates by promoting *Fgf8* expression and repressing *Gli3*, respectively, which leads to restriction of dorsal telencephalon fates to their appropriate region of the telencephalon. It therefore seems likely that the function of FOXG1 in this "unspecified" region is to act as a signaling center which helps set up the boundary between parathyroid and thymus fates possibly by restricting expression of Bmp/Fgf to the thymus domain. Interestingly in the *Hoxa3* null, *Bmp4* expression is lost in the thymus domain and *Fgf8* expands throughout the pouch endoderm (Chojnowski et al. 2014). Since *Foxg1* expression remained unchanged in the region between the ventral and dorsal domains in *Hoxa3*$^{-/-}$, it would suggest that Foxg1 is acting to modulate these signals upstream of HOXA3. A study in neurons showed that Fgf8 acts to inhibit Bmp4 by activating Akt via the PI3K pathway which leads to cell survival instead of Bmp4 promoted apoptosis (Vantaggiato et al. 2011). FOXG1 is also known to act downstream of TGF-β signaling, of which Bmp is part of the TGF-β superfamily (Gordon et al. 2010), by interfering with Smad binding partners (Dou et al. 2000). One possibility then is that FOXG1 is acting downstream of Fgf8 via Akt phosphorylation and it then interferes with Bmp4 induced apoptosis thereby promoting survival of TEC in the ventral thymus domain. This scenario would explain the increase in apoptosis in the thymus domain of *Foxg1* mutant mice during early organogenesis, although loss of *Bmp4* in thymus also causes increased apoptosis (Gordon et al. 2010). It would therefore be interesting to investigate the expression of both *Bmp4* and *Fgf8* in *Foxg1* mutants and in *Foxg1;Hoxa3* compound mutants to determine the requirement, if any, for FOXG1 in their regulation and to determine if it serves dual functions in the pouch.

Foxg1 and Mitochondria

Programmed cell death requires the cleavage of executioner caspases by the apoptosome (reviewed in (Fuchs and Steller 2011)). For this to occur, the apoptosome first must be assembled which requires the release of cytochrome c from the mitochondria. This release is tightly regulated by both pro- and anti-apoptotic Bcl2 family members which act at the mitochondrial outer membrane. Therefore, the mitochondria are a crucial site for the regulation of apoptosis. Recently, Foxg1 was shown to localize to the mitochondria in mouse cortex and neuronal cell culture (Pancrazi et al. 2015). Different size fractions of FOXG1 were also observed to be localized to submitochondrial regions suggesting transport and post-translational modification of FOXG1. The increase in apoptosis in TEC deficient for *Foxg1* could therefore be a result of some function of FOXG1 at the mitochondria. A more detailed analysis of Foxg1 localization in TEC needs to be done to determine if its regulation of apoptosis in these cells may be occurring at the mitochondria.

Foxg1 in Foxn1 negative bi-potent TEC progenitors

We know that *Foxg1* expression precedes that of *Foxn1* in TEC progenitors (Wei et al. unpublished). There is also evidence of bi-potent progenitors that can give rise to both mTEC and cTEC lineage restricted TEC progenitors (Ulyanchenko et al. 2016) and that these are *Foxn1* negative (Ucar et al. 2014). I propose a model (see Chapter 2) where Foxg1 is responsible for affecting the fate choice of the bi-potent progenitors by biasing them towards production of the mTEC restricted lineage. This would explain why in the *Foxg1* null we see a greater reduction in the mTEC population compared to cTEC. Furthermore, in the *Foxg1CKO*, since these bi-potent progenitors are *Foxn1* negative, this model would explain the larger number of mTEC compared to cTEC. To fully validate this model, future studies could utilize the *Foxg1Cre* mice to perform

lineage tracing in the thymus by crossing them to a reporter mouse. Also, thorough H&E staining should be done at both embryonic and adult stages in the *Foxg1CKO* to examine the relative volumes of the cortex and medulla to determine if the numbers quantified by flow cytometry reflect real differences in the proportions of these thymic regions.

In summary, my investigations into the TEC specific loss of *Foxg1* in mice have shown that Foxg1 is necessary for the development of a normal thymus microenvironment and that the defects present during development persist in postnatal stages. Furthermore, I show that Foxg1 is not required for initiation of *IL-7* but that it is required for normal levels of *IL-7* expression and that this defect is most likely due to repression of *Hoxa3* during early thymus organogenesis. I also suggest that Foxg1 may be regulating lineage restriction choices in *Foxn1* negative bi-potent TEC progenitors. My results further suggest that many of the defects seen in the *Foxg1* mutant thymus are potentially due to loss of FOXG1 in multiple signaling pathways which include the Wnt/β-catenin pathway and the PI3K/Akt pathway, although more direct evidence needs to be collected. These results taken together further our understanding of Foxn1-independent TEC differentiation and survival and illustrate new avenues of exploration into the function of Foxg1 in the thymus.

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