TRANSCRIPTION FACTOR AND GROWTH FACTOR REQUIREMENTS FOR THYMUS VASCULARIZATION

by

JERROD L. BRYSON

(Under the Direction of Nancy R. Manley)

ABSTRACT

Scientists and philosophers have marveled over the complex nature of the vascular system for many centuries. In this dissertation work, we were interested in unraveling the molecular mechanisms driving the structuring and organization of this elaborate network in the thymus. Specifically, our aim was to elucidate the possible contributions of a number of thymus-specific transcription factors and growth factors to vascular network formation. The thymus is a specialized microenvironment responsible for the development of self-tolerant and self-restricted T cells. In this dissertation, we used an allelic series of the thymic epithelial cell (TEC)-specific transcription factor Foxn1 to define normal thymic vascular development and to investigate a potential role for Foxn1-dependent TEC differentiation in the formation of the thymic vasculature.

We show that endothelial cells initially enter the wild-type thymus at E13.5, with PDGFR-β^+ mesenchymal cells following at E14.5. These events are delayed by 1-2 days in *Foxn1*$^{\Delta/\Delta}$ mice, and were accompanied by an apparent loss of capillaries. In *Foxn1*$^{\Delta/nu}$ mice, endothelial cells could not be detected in the thymus until E15.5, and never enter in *Foxn1*$^{nu/nu}$ null mutants. *VEGF-A* and *PDGF-B* expression are reduced at E13.5 and E15.5 in *Foxn1*$^{\Delta/\Delta}$ mice compared with controls. Further, empty collagen IV sleeves (collagen IV$^+$ CD31$^-$) were present throughout the mutant thymus. Additionally, we tested the requirement for TEC-derived Shh, VEGF-A, and BMP4 for thymus vascularization. Deletion of these genes in TECs using Foxn1Cre resulted in no observable delay in initial thymus vascularization suggesting that they may be dispensable for thymus vascularization. Together, these data suggest that Foxn1 is required in TECs to produce the cellular and molecular environment needed for normal thymic vascularization, and may mediate a novel TEC-mesenchyme-endothelial form of crosstalk required for fetal thymus organogenesis.

Keywords and Abbreviations List:

cTEC, cortical thymic epithelial cell; EpCAM, epithelial cell adhesion molecule; EPC, endothelial progenitor cell; Foxn1, forkhead box transcription factor 1; LPC, lymphoid progenitor cell; mTEC, medullary epithelial cell; NCC, neural crest cell; PDGF-B, platelet-derived growth factor beta; PDGFR-β, platelet-derived growth factor receptor beta; CD31, platelet endothelial cell adhesion molecule; TEC, thymic epithelial cell; CD144, vascular endothelial cadherin; VEGF, vascular endothelial growth factor; crosstalk.

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JERROD L. BRYSON

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JERROD L. BRYSON

Major Professor:	Nancy R. Manley
Committee:	Brian G. Condie
	Scott T. Dougan
	Kimberly D. Klonowski
	Lianchun Wang

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

I dedicate this dissertation to my family, especially...

to my mom, Lucinda M. Dorsey for her countless sacrifices and for always expecting the best from me;

to my brother Michael Williamson Jr. for his support, encouragement, and sacrifices;

to my sister Shana L. Bryson for her inspiration;

to my cousin Ronald N. Dorsey Jr. for his support and encouragement;

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CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

Origins and Ontogeny of the Vertebrate Vascular System

The vascular system is one of the most remarkable organs in vertebrates. Blood vessels, which are its major component, function to deliver oxygen, nutrients, and progenitor cells to target tissues. The circular nature and complex branching of blood vessels throughout the body was one of the most striking observations made by early physicians, anatomists, and histologists such as Florence Sabin, and Wilhelm His (Loukas et al., 2008; Sabin, 2002). Later, the seminal works of researchers such as Judah Folkman, Eric Jaffe, Werner Risau, and more recently, Rakesh Jain, Peter Carmeliet, and Napoleone Ferrara, to name a few, were pivotal to our current understanding of the molecular mechanisms driving development of the vascular system under normal and pathological conditions.

Establishment of a functional vascular network occurs through a combination of mechanisms, termed vasculogenesis and angiogenesis (Figure 1.1). Vasculogenesis describes a two-step process in which blood vessels form *de novo* from mesoderm-derived progenitors called angioblasts that are specified and later differentiate to an endothelial cell fate (Kassmeyer et al., 2009). Conversely, angiogenesis is the process of new vessels sprouting from pre-existing vessels via endothelial cell migration and concomitant proliferation and pruning. Initial vascularization of the mouse embryo occurs at very early stages of development in both intraembryonic and extraembryonic

tissues. In the embryo, vasculogenesis is initiated in the primitive streak, a structure that establishes bilateral symmetry in organisms and determines the site of gastrulation initiation (Downs, 2009). Angioblasts are marked by the expression of kinase insert domain receptor (KDR/Flk-1), a Vascular Endothelial Growth Factor (VEGF-A) receptor. Cells positive for both Brachyury and Flk-1 in the primitive streak migrate to extra-embryonic tissues where they acquire an arterial or venous fate and subsequently form blood islands (Adams et al., 1999; Coultas et al., 2005; Gerety et al., 1999; Herzog et al., 2005). Blood islands are angioblast aggregates that form ring-like structures with hematopoietic cells forming at the center, and arterial or venous endothelium at the periphery (Coultas et al., 2005) Shortly thereafter, blood islands form a primary vascular plexus in the yolk sac (Coultas et al., 2005). In the embryo proper, angioblasts form the dorsal aorta and cardinal vein though signaling cascades involving Sonic Hedgehog (Shh), VEGF-A, and Delta-Like 4 (Dll4) (Coultas et al., 2005; Duarte et al., 2004; Lawson et al., 2001). By the 5-8 somite stage the heart begins beating and extraembryonic vasculature connects to the intraembryonic vessels, resulting in a primitive functional vasculature (Ji et al., 2003).

During embryogenesis, blood vessel maturation culminates with the recruitment of mural cells, and secretion of extracellular matrix by endothelial and perivascular cells, and with blood vessels eventually acquiring the ability to regulate vascular tone (Jain, 2003). The platelet-derived growth factor (PDGF) family of signaling molecules is critical for growth and maturation of blood vessels. Endothelial cells express PDGF-B platelet -derived growth factor-B ligand a secreted molecule that primarily acts on mesenchyme, perivascular cells expressing the Platelet-derived growth factor-β receptor (PDGFR-

β)(Shinbrot et al., 1994; Zerwes and Risau, 1987). PDGF-B/PDGFR-β signaling results in the recruitment of perivascular support cells to the outer walls of blood vessels. In mice, epithelial cells, neurons, astrocytes, surface ectoderm, and myotome express PDGF-A (Andrae et al., 2008). Its cognate receptor, PDGFR-α is expressed in mesenchyme, which includes a subset of non-neuronal cardiac and cranial neural crest cells, interstitial cells, and astrocytes (Andrae et al., 2008). Endothelial cells recruit vascular smooth muscle actin (vSMA) positive cells to larger vessels via the Ang1 and 2/Tie 1 and 2 signaling pathway (Loughna and Sato, 2001; Yancopoulos et al., 2000).

Vertebrate Neural Crest, An Endothelium Support Structure

The vertebrate neural crest is a transient migratory cell population whose lineage initiates in the CNS at the dorsal most aspect of the neural tube. Neural crest cells migrate throughout the embryo and give rise to a diverse population of cells that adopt both neuroglial and skeletogenic fates (Chai et al., 2000; Donoghue et al., 2008). These include melanocytes, neurons, glia, and bone, cartilage, dentine, and connective tissue, respectively (Chai et al., 2000; Donoghue et al., 2008). Fate mapping studies in avian and mouse embryos initially revealed a contribution of neural crest to the heart, a highly vascularized organ, as well as craniofacial structures, tooth morphogenesis, pharyngeal arch artery derivatives, and the thymus (Bockman et al., 1990; Chai et al., 2000; Jiang et al., 2000; Le Douarin and Jotereau, 1975). NCC ablation experiments in chick have demonstrated that removal of the region of the neural tube giving rise to cranial neural crest results in persistent truncus arteriosus, a hypoplastic thymus, parathyroid, and thyroid (Bockman and Kirby, 1984; Bockman and Kirby, 1985; Bockman and Kirby, 1989; Bockman et al., 1989; Bockman et al., 1990; Bockman et al., 1987). PDGFR-α

null mice (Patch) have varying thymus specific defects including hypoplasia, the presence of a single lobe, and an apparent loss of thymus (Morrison-Graham et al., 1992). More recent studies in mouse support these conclusions, as removal of PDGFRα^+ neural crest-derived mesenchyme surrounding the thymus from E12.5 embryos followed by transplantation to the kidney capsule results in a decline in thymic epithelial cell proliferation, hypoplasia, but normal TEC differentiation and organization, and normal T-cell development, although the total number of T-cells was reduced (Jenkinson et al., 2003; Jenkinson et al., 2007). Pax3 null mice (Splotch), that have few or no NCCs, were originally reported to have thymic hypoplasia or aplasia (Conway et al., 1997; Copp et al., 1990). However, a more recent analysis showed that these mice have an ectopic thymus that is initially larger than normal, due to a patterning defect in the 3rd pharyngeal pouch (Griffith et al., 2009). This study established a role for NCC in patterning, by determining the size of the initial thymus domain. Therefore, it is well established that neural crest cells are an important early contributing cellular component to early thymus development (Foster et al., 2010; Griffith et al., 2009).

In recent years, there has been considerable debate as to whether neural crest serves any critical function in later thymus ontogeny. This controversy arose when results from a lineage-tracing study using the Wnt1Cre transgenic strain to activate a ROSA26R reporter suggested minimal, if any contribution of lacZ positive cells in the neonatal and postnatal thymus (Jiang et al., 2000). The contribution of neural crest cells to the thymus after initial organogenesis was recently settled when two independent groups performed neural crest-specific heritable genetic labeling studies using, Sox10Cre and Wnt1Cre crossed to ROSA26YFP mice (Foster et al., 2008; Jiang

et al., 2000; Muller et al., 2008). These reports revealed that neural crest cells in both embryonic and adult thymus function as pericytes and smooth muscle cells (Foster et al., 2008; Muller et al., 2008), consistent with previous reports showing that craniofacial perivascular cells were neural crest derived (Etchevers et al., 2001). Thymic neural crest cells also express the receptors for PDGF-A and PDGF-B and are therefore likely recruited to the thymus via classical PDGF-A/PDGFR-α and PDGF-B/PDGFβ signaling pathways.

Thymus organogenesis

The vertebrate thymus is a pharyngeal endoderm derived organ (Gordon et al., 2004; Le Douarin and Jotereau, 1975) composed of a diverse assortment of stromal (non-lymphoid) and lymphoid cells (Figures 1.2 and 1.3). The majority of the stromal compartment consists of thymic epithelial cells, but also includes the neural crest-derived mesenchyme and endothelial cells (Blackburn and Manley, 2004; Manley and Blackburn, 2003; Rodewald, 2008). The mature thymic architecture is complex, as thymic epithelial cells compartmentalize into cortical and medullary subsets that differentially express the cytoplasmic markers, keratin 8 and keratin 5, respectively, among other markers (Klug et al., 1998; Klug et al., 2000). Thymic epithelial cells engage in mutual signaling with thymocytes (T cell precursors), a phenomenon termed crosstalk (van Ewijk et al., 1994). Initial establishment of this complex architecture has been shown to be independent of TEC crosstalk with thymocytes (Jenkinson et al., 2005; Klug et al., 2002). The thymus has been the subject of intense study in recent years, as we've learned a great deal about TEC-thymocyte (T cell precursors) crosstalk during development (Hollander et al., 1995; Shores et al., 1991; van Ewijk et al., 2000;

van Ewijk et al., 1994). Manley et al. described the early events of thymus organogenesis in mice as three developmental stages. The first stage, embryonic day 9-11 (E9-E11) consists of early organogenesis, the second, E11.5-E15, late organogenesis, and the third stage, E15.5-birth, described as late fetal development (Manley, 2000). It is well established that the thymus is required to support T-cell development (Miller, 1961a; Miller, 1961b). Until recently, the molecular mechanisms governing interactions between thymic stromal and lymphoid progenitor cells were poorly understood.

Transcription factor and growth factor regulators of thymus organogenesis

Transcription factors and signaling molecules involved in the regulation of thymus organogenesis have been identified largely through gene expression and mutational analyses (Figure 1.2). To date, members of the homeobox (*Hox*), paired box (*Pax)*, eyes absent (*Eya)*, and sine oculis-related (*Six)* homebox gene families have been shown to be important regulators of early thymus organogenesis (Blackburn and Manley, 2004; Manley and Condie, 2010). During early thymus organogenesis the formation of pharyngeal pouches is dependent on the expression of *Pax1/Pax9, Tbx1* and the growth factor, *Fgf8* (Frank et al., 2002; Hetzer-Egger et al., 2002; Peters et al., 1998; Wallin et al., 1996; Xu et al., 2005). Analysis of *Hoxa3* mutants suggests that Hoxa3 is essential for positional identity of the third pharyngeal pouch (Manley and Capecchi, 1995; Manley and Capecchi, 1998). The initiation of thymus formation immediately follows. At this stage, outgrowth of the third pharyngeal pouch endoderm is initiated, which requires the Hox-Pax-Eya-Six transcription factor network that is expressed in the budding endoderm and surrounding neural crest (Hetzer-Egger et al.,

2002; Su et al., 2001; Wallin et al., 1996; Xu et al., 2002). Neural crest derived mesenchyme surrounds the rudiment and promotes the progression of early thymus development, in part via Fgf signaling (Jenkinson et al., 2007; Jiang et al., 2000; Owen et al., 2000; Revest et al., 2001; Suniara et al., 2000). The outgrowth and patterning of the thymic rudiment persists concomitant with the expression of Foxn1 and Gcm2 transcription factors, which define the thymus and parathyroid domains respectively (Gordon et al., 2001). Thymus separation from the pharynx and from the Gcm2 expressing parathyroid domain occurs by E13.5, and the organ continues migrating ventrally and posteriorly until it reaches its final anterior mediastinal anatomical location. Expansion of the epithelial compartment requires fibroblast growth factors 7/10 (Fgf7/Fgf10) from the mesenchyme, as mice deficient in the fibroblast growth factor receptor 2 IIIb (Fgfr2-IIIb) display thymus hypoplasia (Revest et al., 2001). Epithelial cell-thymocyte crosstalk is required for later stages of TEC differentiation (Hollander et al., 1995; Klug et al., 2002; Naspetti et al., 1997; Palmer et al., 1993; Shores et al., 1991; van Ewijk et al., 2000)

Organ-Specific vascular cross-talk

It has long been observed that tissues and the vascular system develop interdependently. Until recently, the molecular mechanism responsible for these intricate interactions has been largely unexplored experimentally. However, it is now well established that organs engage in bidirectional crosstalk with blood vessels and supporting cells to meet organ-specific needs (Cleaver and Melton, 2003; Nikolova and Lammert, 2003; Red-Horse et al., 2007). The pancreas is a good example of this phenomenon as it shares an endoderm lineage with the thymus (Edlund, 2002; Gordon

et al., 2004; Grapin-Botton and Melton, 2000). The pancreas is composed of exocrine and endocrine tissues. Endocrine cells of the pancreas are clustered in structures called islets consisting of insulin, glucagon, somatostatin, and pancreatic polypeptide secreting cells. Insulin secreting β-cells comprise the chief cellular component of the islet while non-β-cells cluster at the periphery of the islet (Oliver-Krasinski and Stoffers, 2008). Pancreatic islets are highly vascularized by a dense capillary network (Eberhard et al., 2010). The complex organization of this vascular network is vital for the pancreatic endothelium's ability to transport hormones such as insulin into the blood stream according to physiological demands, i.e. elevated blood glucose levels (Eberhard et al., 2010). In several elegant experiments, Nikolova G et al., used the pancreas specific Pdx1Cre to delete VEGF-A within the pancreatic islets, which resulted in a loss of capillaries and the basement membrane components, collagen IV and laminin normally secreted by endothelial cells (Nikolova et al., 2006). β-cells express the β1-integrin, which interacts with laminin. Disruption of this interaction within the islet resulted in a reduction in β-cell insulin production (Nikolova et al., 2006). This result suggests crosstalk between endothelial cells and β-cells (Nikolova et al., 2006). In an independent study, Yoshitomi H. et al., demonstrated that endothelial cells in the aorta induce the expression of the pancreas specific transcription factor Ptf1a and crosstalk between endothelial cells and pancreatic endoderm is required for maintenance of Pdx1 (Yoshitomi and Zaret, 2004). It is therefore evident that endothelial cells are involved in crosstalk with pancreas tissue.

The liver provides another example of an endoderm-derived organ that interacts with blood vessels in a very organ specific manner (Nikolova and Lammert, 2003).

Much like the pancreas, the liver functions as both an endocrine and an exocrine gland. The chief cellular component of the liver is the hepatocyte, which comprise ~70% of the organ's cells (Si-Tayeb et al., 2010). The main functions of hepatocytes include bile secretion, cholesterol, glucose, glycogen, and urea metabolism, and blood clotting. The liver has two specialized populations of endothelial cells. The first population forms the hepatic artery, which supplies oxygenated blood to the liver, and the hepatic portal vein, which transports blood from the stomach, spleen and intestines to the liver (Si-Tayeb et al., 2010). The second population is the sinusoidal endothelial cell (SEC), which makes up the structured liver microvasculature. SECs are specialized endothelial cells that secrete cytokines, present antigen, function in waste elimination, and in circulating blood throughout the organ (Si-Tayeb et al., 2010). SECs are fenestrated which allows molecules, proteins, and waste from serum to be presented to hepatocytes (Si-Tayeb et al., 2010). Therefore blood vessels in the liver perform very unique organ specific functions. The complex structure of the liver vasculature is the direct result of crosstalk between hepatocytes, hepatic stellate or smooth muscle cells, and the endothelium (Carpenter et al., 2005; LeCouter et al., 2003; Red-Horse et al., 2007; Si-Tayeb et al., 2010). Particularly, it has been shown that signals from the endothelium promote initial and subsequent stages of liver organogenesis (Matsumoto et al., 2001). LeCouter J et al., identified hepatocyte growth factor as an endothelial cell derived signal capable of promoting hepatocyte growth (LeCouter et al., 2003). Interestingly, another study demonstrated that hepatocyte growth factor expression in sinusoidal endothelial cells was increased in response to VEGF-A secreted by adjacent hepatocytes and this increase in HGF provided the liver some protection against hepatotoxin induced liver

damage (Carpenter et al., 2005). This report also suggested that hepatocyte derived VEGF-A is required for normal liver vascularization, generation of fenestrated endothelium, and liproprotein uptake by sinusoidal endothelial cells (Carpenter et al., 2005). Therefore, it is widely accepted that crosstalk between the endothelium and hepatocytes, hepatic stellate cells, is required for normal liver organogenesis.

In this dissertation, we were primarily interested in understanding how endothelial cells and nascent blood vessels fit into our current models of thymus organogenesis. More specifically, we were interested in defining some mechanisms of endothelial cell-thymic epithelial cell crosstalk. Previous studies on the thymic vascular system have been primarily ultrastructural rather than molecular in nature, relying heavily on light, scanning, and transmission electron microscopy (Anderson et al., 2000; Kato, 1997). These reports described the morphology and organization of endothelial cell-derived vessels in relation to lymphocytes and thymic stromal cells. Until recently, there have been very few reports elucidating the molecular interactions between endothelial cells and thymic epithelial cells. Stalmans, et al., reported a hypoplastic thymus phenotype in mice expressing a single isoform of VEGF-A, VEGF-$A^{120/120}$ (Stalmans et al., 2003). In a subsequent study Muller, et al., used a nude mouse blastocyst complementation method to specifically delete VEGF-A in thymic epithelial cells (Muller et al., 2005). This resulted in a loss in thymic capillaries, and revealed a contribution of VEGF-A from thymus mesenchyme (Muller et al., 2005). In a more recent study, Cuddihy et al. used VEGF-A Trap assay (neutralizing antibody) to inhibit VEGF-A systemically (Cuddihy et al., 2009). This resulted in a significant loss of capillaries within the thymus and a reduction in thymocytes (Cuddihy et al., 2009). Mori, et al has shown that VEGF-A

expressing cells are largely absent in nude mice (Mori et al., 2010). These data suggest a role for VEGF-A and Foxn1 in thymus vascularization. Blood vessels have been commonly considered to be a means of transporting oxygen and nutrients to tissues. It is becoming increasingly evident that endothelial cells play larger roles in development than formerly considered. Here, we investigate the possible contributions of endothelial progenitor cells, nascent blood vessels, Foxn1 and candidate growth factors to TEC-Endothelium crosstalk and subsequent thymus organogenesis.

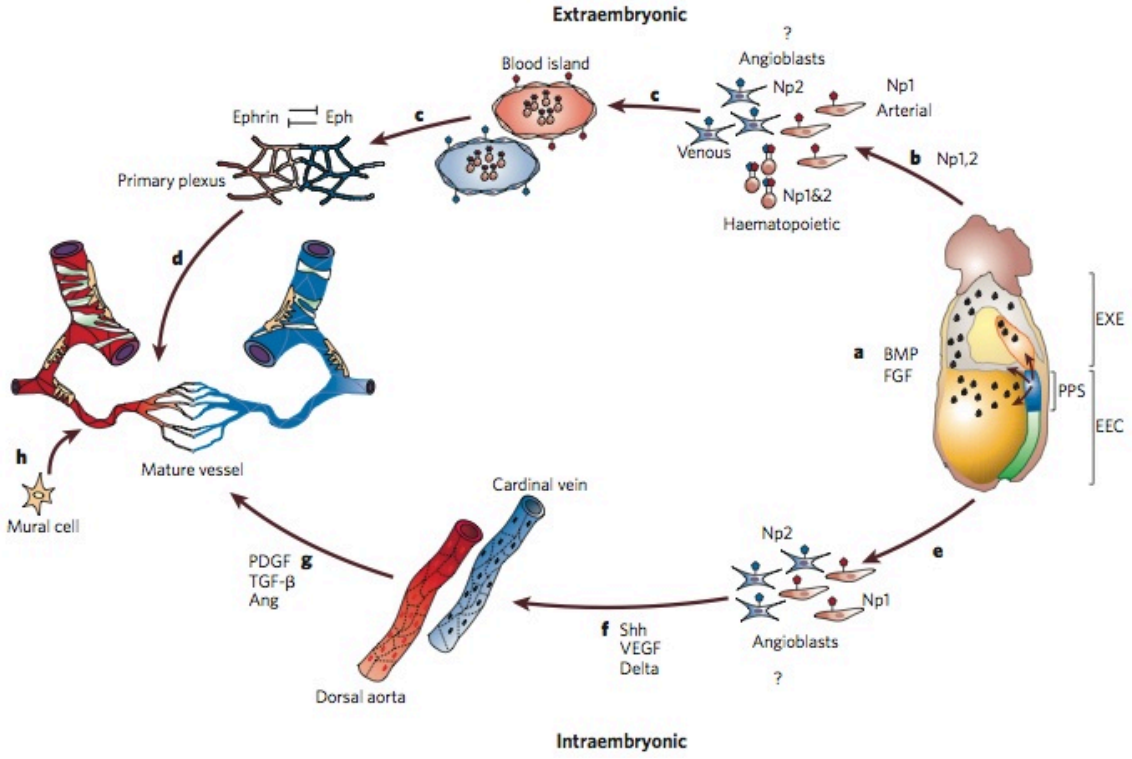

Figure 1.1 Adapted from (Coultas et al., 2005).

Figure 1.1 Embryonic blood vessel development from angioblasts

(**a**) Endothelial cell progenitors in the primitive streak appear as Flk-1 positive cells in response to Bmp and FGF signaling. (**b**) Angioblasts acquire and arterial or venous fate and express NP1 or NP2, respectively. Cells that express both NP1 and NP2 differentiate to the hematopoietic lineage. (**c**) Angioblasts form blood islands with endothelial cells at the periphery and hematopoietic cells at the center. Endothelial cells within blood islands form tubes that branch forming a primary vascular plexus. (**d**) The vascular plexus branches into arteries, veins, arterioles, and venules. (**e-f**) In the intraembryonic tissue, angioblasts form the cardinal vein and dorsal aorta via a mechanism involving Shh, VEGF, and Notch signaling. (**g**) Extraembryonic and intraembryonic blood vessels connect and secret PDGF-, and TGF-β. (**h**) Vessel maturation culminates in the recruitment of perivascular cells to the vessel walls. Posterior primitive streak (**PPS**), extraembryonic ectoderm (**EXE**), and embryonic ectoderm (**EEC**)

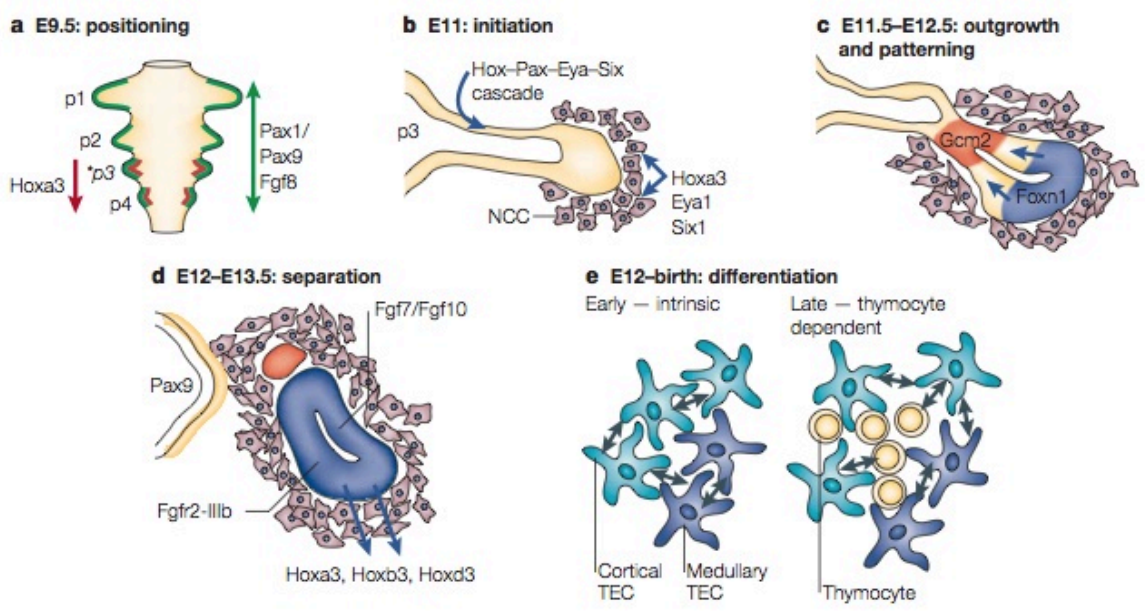

Figure 1.2 Adapted from (Blackburn and Manley, 2004).

Figure 1.2 Thymus Organogenesis

(**a**) Formation of the pharyngeal pouches at E9.5 requires the transcription factors Pax1/Pax 9, and the growth factor Fgf8. Hoxa3 is essential for positional identity of the third pharyngeal pouch. (**b**) Initiation of thymus begins at E11 and involves the outgrowth of the third pharyngeal pouch endoderm, which requires the Hox-Pax-Eya-Six transcription factor network that is expressed in the budding endoderm and surrounding neural crest (**c**) The outgrowth and patterning of the thymic rudiment persists concomitant with the expression of Foxn1 and Gcm2 transcription factors, which define the thymus and parathyroid domains respectively. (**d**) Neural crest derived mesenchyme surrounds the rudiment and promotes the expansion of the epithelial cell compartment and progression of early thymus development, in part via Fgf 7/10signaling. Thymus separation from the pharynx and from the Gcm2 expressing parathyroid domain occurs by E13.5, and the organ continues migrating ventrally and posteriorly. (**e**) Epithelial cell-thymocyte crosstalk is required for later stages of TEC differentiation.

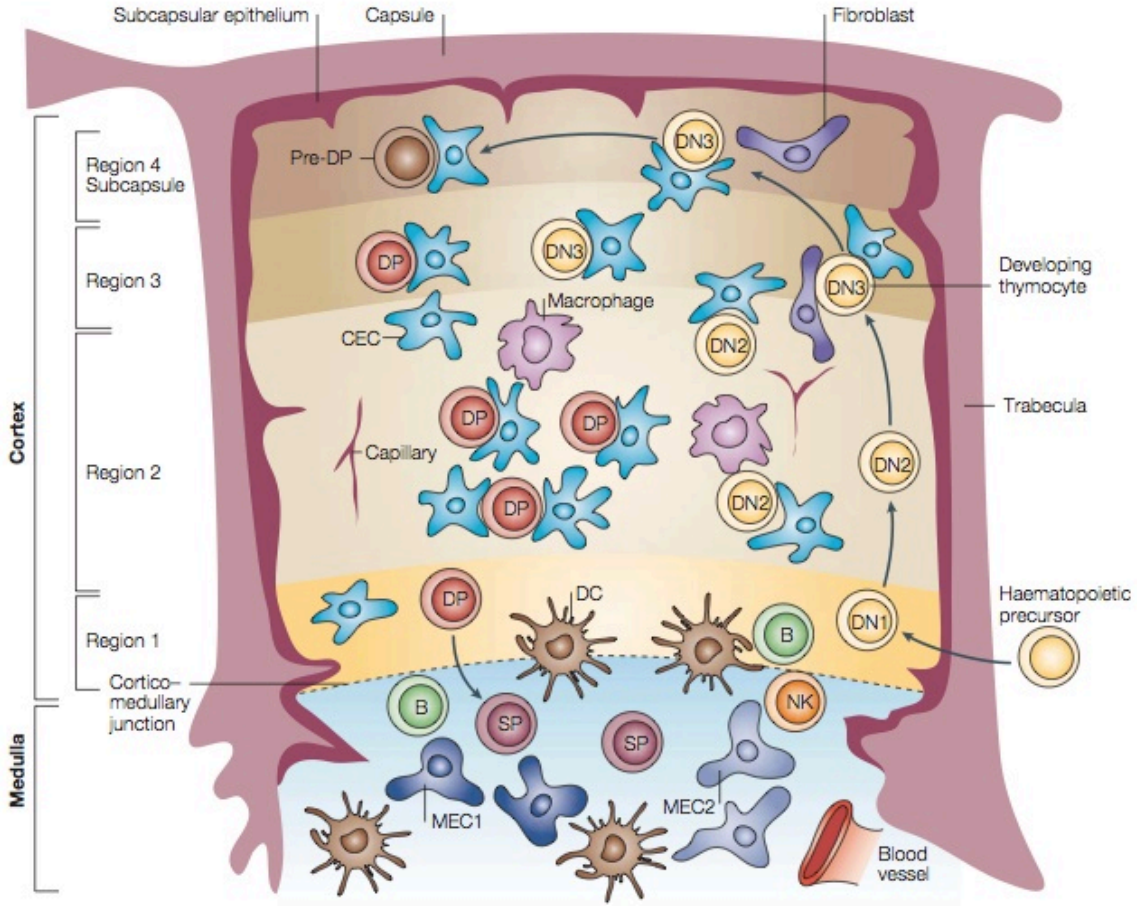

Figure 1.3 Adapted from (Blackburn and Manley, 2004).

Figure 1.3 Thymus Architecture

The primary function of the thymus is the generation of mature, single positive (SP) T cells. The organ can be divided into cortical (cTEC) and medullary epithelial cell (mTEC) compartments. Negative selection occurs in the cortex and positive selection of T-cells occurs in the medulla. The cortex is subdivided into function regions where thymocytes undergo specific stages of development. A dense capillary network is characteristic of the cortex, while larger vessels are predominantly localized to the medulla and the cortico-medullary junction. Cortical capillaries provide oxygen and nutrients to TECs. However larger vessels in the medulla and at the cortico-medullary junction mainly serve as an entry point for T cells immigrating into the organ and as an exit route for T cells that are transported to the periphery. Most, if not all perivascular mesenchyme and mesenchyme within the thymic capsule is neural crest derived.

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CHAPTER 2

CELL-AUTONOMOUS DEFECTS IN THYMIC EPITHELIAL CELLS DISRUPT ENDOTHELIAL - PERIVASCULAR CELL CROSSTALK

To be submitted to *Blood.* Jerrod L Bryson, Ann V Griffith, Bernard Hughes III, Fumi Saito, Yousuke Takahama, Ellen R Richie, and Nancy R Manley.

Abstract

The thymus is a specialized microenvironment responsible for the development of self-tolerant and self-restricted T cells. Here, we used an allelic series of the TEC-specific transcription factor Foxn1 to define normal thymic vascular development and to investigate a potential role for Foxn1-dependent TEC differentiation in the formation of the thymic vasculature. We show that endothelial cells initially enter the wild-type thymus at E13.5, with PDGFR-β^+ mesenchymal cells following at E14.5. These events are delayed by 1-2 days in *Foxn1*$^{\Delta/\Delta}$ mice, and at subsequent stages there is a loss of capillaries, blood vessels are leaky, tight association between the endothelium and perivascular cells is disrupted, vessel walls are indistinct, some vessels display vacuolated endothelium, and there is an overall failure of vessel modeling into a stereotypical thymus vasculature. In *Foxn1*$^{\Delta/nu}$ mice, endothelial cells could not be detected in the thymus until E15.5, and never enter in *Foxn1*$^{nu/nu}$ null mutants. *VEGF-A* and *PDGF-B* expression are reduced at E13.5 and E15.5 in *Foxn1*$^{\Delta/\Delta}$ mice compared with controls. Further, empty collagen IV sleeves (collagen IV$^+$ CD31$^-$) were present throughout the mutant thymus. Together, these data suggest that Foxn1 is required in TECs to recruit endothelial cells into the thymus and for endothelial cells to communicate with thymic mesenchyme and form a characteristic thymus vasculature with a dense cortical capillary network and large to medium sized vessels within the medulla and cortico-medullary junction. These data suggest that Foxn1 is required for the cellular and molecular environment needed for normal thymic vascularization, and may mediate a novel TEC-mesenchyme-endothelial form of crosstalk required for fetal thymus organogenesis.

Introduction

Organ vascularization is essential for the delivery of oxygen and nutrients to developing tissues and is required for normal tissue growth and homeostasis. The circulatory system is accordingly the first functional organ in the developing mammalian embryo, and is primarily comprised of endothelial, blood and perivascular support cells (Jin and Patterson, 2009). During embryogenesis and in adults, vascular ontogeny is driven by vasculogenesis, the de novo formation of blood vessels from mesoderm-derived precursors, and angiogenesis, the sprouting of endothelium from pre-existing vessels (Jain, 2003; Jin and Patterson, 2009). In addition to their essential role in providing oxygen and nutrients to tissues, recent reports have highlighted the versatility of the developing endothelium in organs (Red-Horse et al., 2007). Several groups have demonstrated that the vascular endothelium serves unique organ-specific functions in the early organogenesis of the liver, lung, pancreatic islets, and kidney (Lammert et al., 2001; Lammert et al., 2003; Matsumoto et al., 2001), and initial formation of vascular networks has been described in a number of organ systems.

In the thymus, the vascular network plays a critical role in organ function, and has also been implicated in organ development. The thymus is a specialized microenvironment that supports the development of self-MHC restricted and self-tolerant T cells.

Lymphoid progenitor cells (LPCs) enter the thymus and $CD4^+$ and $CD8^+$ single positive T cells exit to the periphery via specialized blood vessels at spatially defined regions of the organ (Lind et al., 2001; Petrie and Zuniga-Pflucker, 2007). The postnatal thymus is comprised predominantly of hematopoietic-derived cells, (mainly thymocytes, but also dendritic cells and macrophages) in close association with a complex network of non-hematopoietic-derived stromal cells (epithelial, mesenchymal, and endothelial cells) (Rodewald, 2008).

The predominant functional stromal cell population is the thymic epithelial cell (TEC) compartment. TECs are broadly subdivided into cortical (cTEC) and medullary (mTEC) subsets, which have specific roles in the positive and negative selection, respectively, of developing T cells (Ladi et al., 2006). The organization of the thymic stroma into cortical and medullary compartments is critical for correct and efficient production of developing T cells, and the thymic vasculature has been implicated in regulating this organization (Anderson et al., 2000). In spite of these essential functional roles, cellular and molecular mechanisms regulating the initial establishment of a functional thymic vasculature are poorly understood.

As in all organs, thymic vascularization takes place during fetal organ development. The fetal thymus and parathyroid glands develop from an outgrowth of the third pharyngeal pouch endoderm (Gordon et al., 2004; Manley, 2000). Between E10.5-11.5, neural crest cell (NCC)-derived mesenchymal cells condense around the third pharyngeal pouch and contribute to the progression of thymic organogenesis by providing patterning and positioning cues to the developing epithelium (Griffith et al., 2009). LPCs first immigrate into the thymus at E11.5, by direct migration across the

developing capsule in response to chemokines made by the endodermally-derived primordium (Liu et al., 2006). NCC mesenchyme contributes to later organogenesis and fetal development by forming the thymic capsule, which provides growth signals to the developing thymic rudiment (Revest et al., 2001) and perivascular support cells that stabilize the developing vasculature (Foster et al., 2008; Muller et al., 2008). The thymic vasculature is connected to the peripheral vasculature by E15.5 (Liu et al., 2006). In the postnatal thymus, LPCs enter the thymus via postcapillary venules (PCV) positioned at the cortical medullary junction (CMJ), while mature single positive (SP) T cells typically co-opt larger vessels positioned at the CMJ as a route of thymic egress to the periphery (Kurobe et al., 2006; Lind et al., 2001). Correct formation and patterning of the thymic vascular network is therefore critical for postnatal thymic function.

Two recent studies have reported on the role of VEGF in the thymus of neonatal (Cuddihy et al., 2009) and adult mice (Cuddihy et al., 2009; Muller et al., 2005). In one study, VEGF-A was deleted in the thymic epithelium using a nude mouse blastocyst complementation strategy (Muller et al., 2005). Analysis of these postnatal thymi showed altered thymic vascular network formation, but normal CD4:CD8 ratios in the thymus and periphery (Muller et al., 2005). Thymic cortical mesenchymal cells were also identified as a source of VEGF-A, suggesting that the coordinated expression of VEGF-A in TECs and mesenchyme contributes to the formation of thymic blood vessels (Muller et al., 2005). In another study, the thymic vascular networks were compared in neonatal and adult mice. The neonatal thymus expressed increased levels of VEGF-A relative to adult thymus, and contained dense, highly branched capillary networks lacking significant perivascular cell coverage, characteristic of immature vascular

structures (Cuddihy et al., 2009). In contrast, adult thymi expressed low levels of VEGF-A, exhibited less vessel branching, and increased expression of perivascular cell markers, suggestive of a mature vascular network (Cuddihy et al., 2009).

These reports therefore emphasize the importance of VEGF-A expression in the establishment of the postnatal thymic vasculature. However, neither of these studies investigated fetal thymus phenotypes, and the molecular and cellular control of initial thymic vascularization remains to be determined.

Thymic epithelial cells express the forkhead box transcription factor Foxn1 at E11.25, just before colonization of the thymus by the first wave of thymocytes (Gordon et al., 2001; Moore and Owen, 1967). Foxn1 is absent in nude mice and is required cell-autonomously for both cTEC and mTEC differentiation (Blackburn et al., 1996; Nehls et al., 1996). In the absence of Foxn1, lymphoid progenitor immigration is not supported (Itoi et al., 2001; Liu et al., 2006), and endothelial progenitor and NCC-derived mesenchymal cells fail to enter the thymic rudiment (Mori et al.). However, given the general failure of both TEC differentiation and of recruitment of cells into the nude thymic rudiment, it is not possible to determine from analysis of the null allele alone what role TECs play in the formation of the intrathymic vasculature. We previously reported the generation of a hypomorphic allele of *Foxn1*, referred to as *Foxn1*$^{\Delta}$ (Su et al., 2003). Mice homozygous for this mutation display defects in TEC differentiation that are less severe than those observed in nude mice.

However, TEC differentiation is largely arrested at an immature stage, and organization of defined cortical and medullary epithelial cell compartments fails. *Foxn1* therefore plays a central role in multiple aspects of TEC development and organization.

To test whether Foxn1-dependent TEC differentiation is required for vascular development during thymus organogenesis, we investigated thymus vascularization in an allelic series including *Foxn1*$^{+/\Delta}$, *Foxn1*$^{\Delta/\Delta}$, *Foxn1*$^{\Delta/nu}$ and *Foxn1*$^{nu/nu}$ embryos. We show that initial attraction of endothelial progenitor cells (EPCs), lymphoid progenitor cells (LPCs), and neural crest cells (NCCs) to the thymus is normal until E12.5 in *Foxn1*$^{+/\Delta}$ and *Foxn1*$^{\Delta/\Delta}$ mice. However, we demonstrate a specific delay in endothelial and neural crest-derived perivascular cell entry into the thymus in *Foxn1*$^{\Delta/\Delta}$ mice. We show a progressive exacerbation of this vascular phenotype in *Foxn1*$^{\Delta/nu}$ and *Foxn1*$^{nu/nu}$ mice, demonstrating that the process of thymus vascularization is sensitive to *Foxn1* dose. We further demonstrate that the thymic blood vessel network is connected to the fetal vascular network at E14.5. However, by E18.5 blood vessel integrity was compromised in *Foxn1*$^{\Delta/\Delta}$ *thymi* with an apparent loss of capillaries, leaky vessels, loss of tight association between the endothelium and perivascular cells, indistinct, vessel walls, vacuolated endothelium, and an overall failure of vessels to model into a stereotypical thymus vascular network. Further, collagen IV was broadly expressed throughout the postnatal mutant thymus. These results demonstrate that Foxn1-expressing thymic epithelial cells are required to orchestrate an epithelial-endothelial-mesenchymal cell crosstalk process that is necessary for vascularization of the thymus.

Results

Vasculature ontogenesis in the wild-type thymus

To identify the initial stages of thymic vascularization, we assayed E12.5-E14.5 *Foxn1*$^{\Delta/\Delta}$ embryonic thymi for the presence of the endothelial cell markers PECAM-1 (CD31) and VE-Cadherin (CD144). CD31^{+} and CD144^{+} endothelial cells surrounded the thymic epithelial rudiment by E12.5 (Figure 2.1A, D-E). These endothelial cells were in contact with the PDGFR^{+} neural crest-derived mesenchymal capsule that encompasses the thymic rudiment (Foster et al., 2008; Griffith et al., 2009; Kurobe et al., 2006; Muller et al., 2008). By E13.5, a network of nascent CD31^{+} vascular structures is present within the thymus in addition to the endothelial cells that remain associated with PDGFR-β^{+} cells in the perithymic mesenchymal capsule (Figure 2.1F, H, and I.) (Liu et al., 2006). Many of the intrathymic CD144^{+} endothelial cells were associated with the centrally located Keratin 5^{+} (K5) subset of thymic epithelial cells (Figure 2.1J). At E14.5, the vascular network was markedly more complex, and an increased number of CD31^{+} and CD144^{+} cells were present within the thymus (Figure 2.1K-O). At this stage PGDFR-β^{+} cells had migrated into the thymus and were in close association with the vascular network (Figure 2.1N). Note that the PGDFR-β^{+} cells remaining in the capsule had also begun to form invaginations into the TEC network at E14.5 (Figure 2.1L, N). These data suggest that PDGFR-β^{+} and endothelial cells first encapsulate the E12.5 thymus, and that EPCs (endothelial progenitor cells) subsequently enter the thymus, where they recruit PDGFR-β^{+} mesenchymal cells into the thymic rudiment.

Endothelial progenitor and NCC immigration into Foxn1$^{\Delta/\Delta}$ thymus is delayed

To test whether Foxn1-dependent TEC differentiation is required for initial vascular development; we analyzed mice homozygous for the hypomorphic *Foxn1*$^{\Delta}$ allele. At E12.5, the *Foxn1*$^{\Delta/\Delta}$ thymic rudiment resembled the stage-matched *Foxn1*$^{+/\Delta}$ control littermate thymus (Figure 1a-e), with endothelial cells and neural crest-derived cells clustered around the periphery of the thymic epithelial rudiment (Figure 2.1D, d, E and e). However, at E13.5 CD31^{+} and CD144^{+} EPCs remained localized at the periphery of the *Foxn1*$^{\Delta/\Delta}$ thymic epithelial primordium (Figure 2.1f, h-j). By E14.5, CD31^{+} and CD144^{+} EPCs in Foxn1$^{\Delta/\Delta}$ mice were observed in the thymus (Figure 2.1k, m-o), but in reduced numbers compared to control littermates (Figure 2.1K, M-O). Moreover, we observed only occasional PDGFR-β^{+} cells in E14.5 *Foxn1*$^{\Delta/\Delta}$ thymi (Figure 2.1l and n) compared to E14.5 controls, and most endothelial cells in the mutant thymus were not associated with mesenchyme at this stage (Figures 2.1L, N; P, Q). Overall, the localization of endothelial and neural crest cells in E14.5 *Foxn1*$^{\Delta/\Delta}$ mutant thymuses appeared similar to E13.5 controls, indicating a one-day delay and structural differences in initial thymic vascularization.

Initial thymic vascularization is sensitive to Foxn1 levels in TECs

We recently demonstrated that the postnatal thymic microenvironment is exquisitely dependent on *Foxn1* dose to maintain postnatal thymic architecture and function (Chen et al., 2009). To determine whether the initial establishment of thymic vasculature was sensitive to *Foxn1* dose, we combined our Δ hypomorphic allele and the null allele, nude (*nu*). We assayed EPC and NCC markers in *Foxn1*$^{\Delta/\Delta}$, *Foxn1*$^{\Delta/nu}$, and *Foxn1*$^{nu/nu}$ mice (Figure 2.2A-E). The delay in EPC immigration seen in the *Foxn1*$^{\Delta/\Delta}$

thymus was more pronounced in *Foxn1*$^{\Delta/nu}$ mice, in which both EPCs and NCCs remained restricted to the capsule at E14.5, and not detected in the primordium (Figure 2.2A-E). In the total absence of *Foxn1*$^{nu/nu}$, CD144^{+} EPCs failed to invade the thymic epithelial rudiment and remained localized at the periphery at E17.5 (Figure 2.2E). These data suggest that Foxn1 is required in a dose-dependent manner for thymic epithelial cells to regulate vascularization of the thymus primordium during organogenesis.

Timing of initial LPC immigration into Foxn1$^{\Delta/\Delta}$ thymi is normal

We previously reported that *Foxn1*$^{\Delta/\Delta}$ mice have a significant decrease in total thymocytes at both fetal and adult stages (Su et al., 2003). In nude mice (*Foxn1*$^{nu/nu}$), bone marrow derived-hematopoietic precursor cells (HPC) migrate to, but fail to colonize the thymus rudiment (Liu et al., 2006). We therefore tested whether the *Foxn1*$^{\Delta}$ mutation affected the timing of initial HPC infiltration of the thymus, and thus might contribute to these early endothelial defects in *Foxn1*$^{\Delta/\Delta}$ mice. We assayed for CD45^{+} cells in *Foxn1*$^{\Delta/\Delta}$ and heterozygous control thymi at E11.5, when thymocytes initially infiltrate the thymus (Moore and Owen, 1967). CD45^{+} cells were present in the E11.5 thymus rudiment of both *Foxn1*$^{\Delta/\Delta}$ and control littermates (Figures 2.3A and B). At later stages (E12.5-E14.5; Figure 2.3I-N), CD45^{+} cells are significantly reduced in the mutant thymus. These data suggest that although CD45^{+} cells in the thymus are noticeably reduced after E11.5, the timing of initial HPC colonization of the thymus is normal in *Foxn1*$^{\Delta/\Delta}$ mice.

We further tested whether CCL21 and CCL25, two key chemokines required for initial HPC recruitment to the shared thymus/parathyroid primordia, were reduced in

Foxn1$^{\Delta/\Delta}$ mice at E11.5 (Liu et al., 2006). Our recent data suggests that CCL25 is predominantly *Foxn1*-dependent, whereas CCL21 is *Gcm2*-dependent (Liu et al., 2006). Furthermore, embryos double deficient for CCL21 and CCL25 receptors, CCR7/CCR9, respectively, exhibited significant defects in early thymocyte colonization of the thymus (Liu et al., 2006). As expected, CCL21 expression in the Gcm2 domain was normal in E11.5 *Foxn1*$^{\Delta/\Delta}$ thymi compared to heterozygous controls (Figure 2.3C and D). In contrast, CCL25 expression in the thymus domain was reduced (Figure 2.3E, G and F, H). By qRT-PCR, CCL25 expression at E13.5 and E15.5 in *Foxn1*$^{\Delta/\Delta}$ (2.3O-P) and E15.5 in the *Foxn1*$^{\Delta/nu}$ (2.3Q) thymus was significantly reduced. As mutation of the CCL25 receptor, CCR9, exhibits only a ~40% reduction in the number of initially immigrating thymocytes, but does not affect timing of initial immigration, this result was consistent with our conclusion that the timing of initial LPC immigration was normal in *Foxn1*$^{\Delta/\Delta}$ mice (Liu et al., 2006). This result further indicates that the delay in EPC and mesenchymal immigration was not due to a general delay in thymus development.

Peripheral circulation connects to the thymus at E14.5

A critical event during thymic organogenesis is the connection of the peripheral vasculature and circulation to the developing vessels in the thymic anlage. This developmental time point likely indicates a switch in the route of LPC entry into the thymus, from initial trans-capsular direct immigration to vascular extravasation at late fetal and postnatal stages (Haynes and Heinly, 1995; Itoi et al., 2001; Lind et al., 2001; Moore and Owen, 1967). The mammalian embryonic circulatory system becomes functional at about E10 (McGrath et al., 2003). As endothelial cells initially immigrate into the fetal thymus at E13.5 (Figure 2.1) (Foster et al., 2008; Liu et al., 2006), the

external connection must occur after this point. To identify the developmental time point at which the peripheral circulation connects to the thymus, we performed FITC-dextran facial vein injections at E14.5. FITC-dextran was detected in *Foxn1*$^{+/\Delta}$ thymi associated with CD31^{+} vasculature (Figure 2.4A, B, and I). The timing of intrathymic FITC-dextran detection was similar in *Foxn1*$^{\Delta/\Delta}$ mutants (Figure 2.4C, D, and J), although fewer vessels were labeled in the mutants. This result was consistent with previous data showing the connection is established by E15.5, and suggested that timing of this connection to the embryonic vasculature was regulated by Foxn1-independent mechanisms.

While the timing of vessel connection was similar, vessel patterning was considerably different. Control thymi showed a network of mostly small vessels throughout the thymus (Figure 2.4A and B), while mutant thymi had large, centrally localized vessels with few small vessels between them and the periphery of the rudiment (Figure 2.4C, D, and J). We observed similar results at E18.5 (Figure 2.4E-H and K-L), in which a dense network of branched blood vessels was present throughout control thymi (Figure 2.4E, F and K) and a less dense and branched blood vessel network was characteristic of the E18.5 *Foxn1*$^{\Delta/\Delta}$ thymic vasculature (Figure 2.4G, H and L). Furthermore, at both stages mutants displayed a ‘leaky vessel’ phenotype, in which FITC-dextran within the thymus was not restricted to the CD31^{+} and PDGFRβ^{+} vasculature.

The accumulation of FITC-dextran in the subcapsular region (Figure 4L) is consistent with this phenotype extending to the VE-Cadherin^{+} endothelium in the subcapsular region of E17.5 *Foxn1*$^{nu/nu}$ mice (Figure 2.2E). This phenotype was confined to the thymic and perithymic vasculature, and was not seen in any other regions of the embryo (Figure 2.4C, D, G, H, J and L, and data not shown). These experiments reveal that the peripheral circulation connects to the thymus at E14.5, within 24 hours of initial colonization by endothelial cells. Furthermore, the timing of this process was normal in *Foxn1*$^{\Delta/\Delta}$ mutants, but the patterning and maturation of developing vessels was dramatically altered.

Vascular modeling is defective in late embryonic and postnatal Foxn1$^{\Delta/\Delta}$ ***thymus***

Because the E18.5 mutant thymus exhibited a 'leaky' vasculature phenotype (Figure 2.4E-H) we assessed whether mature vessels with perivascular cell coverage were capable of forming in the *Foxn1*$^{\Delta/\Delta}$ thymus. We assayed for the presence of PDGFR-β^{+} cell coverage on CD31^{+} thymic vasculatures (2.5A-D). On E18.5, we observed PDGFR-β^{+} cells associated with most vascular structures in the thymus (2.5A-B). However, modeling of the thymus vascular network was defective resulting in reduced cortical capillaries in *Foxn1*$^{\Delta/\Delta}$ thymi (2.5A-B). Medium-large sized blood vessels were detected throughout the mutant thymus compared to controls, which exhibited a cortical capillary network with larger blood vessels localized to the medulla and cortico-medullary junction, as previously reported (2.5A-B). This phenotype persisted in the postnatal thymus (2.5C-D). However, the lack of a dense cortical capillary network and the presence of medium to large sized vessels associated with

PDGFRβ^+ cells was more pronounced in the *Foxn1*$^{\Delta/\Delta}$ thymus compared to stage matched littermates (2.5C-D). There was some variability associated with this vascular phenotype in *Foxn1*$^{\Delta/\Delta}$ thymi and examples of this are shown according to increasing severity (2.6B-D), compared to control littermates. Analysis of *Foxn1*$^{\Delta/\Delta}$ thymi by electron microscopy show vacuolated endothelium, edema, indistinct vessel walls, and an overall loose arrangement of cells due to separation and cellular swelling (2.7C-D), compared to *Foxn1*$^{+/\Delta}$ thymi which show normal endothelial morphology and a compact arrangement of endothelium and pericytes (2.7A-B). These results indicate that vasculatures in the *Foxn1*$^{\Delta/\Delta}$ thymus are competent to recruit perivascular cells required for vessel maturation. However, endothelium-pericyte association was altered and vascular modeling was defective in *Foxn1*$^{\Delta/\Delta}$ thymi.

VEGF-A and PDGF-B levels are reduced in Foxn1$^{\Delta/\Delta}$ mice

Vascular endothelial growth factor (VEGF) is a potent inducer of vascular development during embryogenesis and in adults (Carmeliet et al., 1996; Ferrara et al., 1996; Keck et al., 1989; Leung et al., 1989). In the thymus, it has been reported that VEGF-A is expressed by TECs and NCCs (Cuddihy et al., 2009; Muller et al., 2005), endothelial cells, and from a subset of immature thymocytes (Cuddihy et al., 2009). PDGF-B is primarily expressed by endothelial cells and functions in the recruitment of mesenchyme to developing vasculatures, thus providing structural support and necessary growth/survival factors required for vessel homeostasis (Andrae et al., 2008; Gaengel et al., 2009; Hellstrom et al., 2001). We tested whether defects in Foxn1-dependent TEC differentiation affect VEGF-A and PDGF-B expression in the thymus, and could thus contribute to the observed vascular defects.

We measured VEGF-A and PDGF-B mRNA expression by qRT-PCR in $CD45^+$ cell depleted thymi of *Foxn1*$^{\Delta/\Delta}$, *Foxn1*$^{\Delta/nu}$ and control littermates (Figure 2.8C-H). Hematopoietic cell depletion was monitored by qRT-PCR for EpCAM, expressed by TECs, and CD45, expressed by hematopoietic cells, before and after depletion (Figure 2.8A, B). VEGF-A (Figure 2.8C-E) and PDGF-B (Figure 2.8F-H) expression were significantly reduced ($p<0.05$) at both E13.5 and E15.5 in *Foxn1*$^{\Delta/\Delta}$ mice compared to control littermates. VEGF-A protein was predominantly expressed in thymic endothelial cells, mesenchyme, and possibly perivascular TECs with detectable, but lower expression in most TECs (Figure 2.9A-H). In E13.5 *Foxn1*$^{\Delta/\Delta}$ thymi, detectable VEGF-A was higher in the capsule mesenchyme and endothelial cells with low VEGF-A present in TECs (Figure 2.9C-D). However, VEGF-A could be detected throughout the *Foxn1*$^{\Delta/\Delta}$ thymus by E15.5 (Figure 2.9G-H), although VEGF-A was present at lower levels in the endothelium, mesenchyme, and in TECs. These results show that VEGF-A and PDGF-B expression is reduced at E13.5 and E15.5 in *Foxn1*$^{\Delta/\Delta}$ and *Foxn1*$^{\Delta/nu}$ thymic stromal cells by qRT. Further in *Foxn1*$^{\Delta/\Delta}$ thymi VEGF-A levels were reduced in TECs, mesenchyme and the endothelium.

Collagen IV is Present Throughout the Foxn1$^{\Delta/\Delta}$ thymus

Collagen IV is a major component the mammalian basement membrane/extracellular matrix (ECM) and is critical for cell migration, adhesion, proliferation, and differentiation (Khoshnoodi et al., 2008). The perivascular localization of collagen IV in the thymus has been well documented as it delineates the perivascular space (PVS). It has been postulated that thymocytes and mature T-cells accumulate in the PVS prior to immigration into the thymus and mature T-cell

traverse the PVS prior to egress from the thymus, to the periphery (Mori et al., 2007). Previous reports suggest that an increased number of immature T-cells populate the periphery of adult *Foxn1*$^{\Delta/\Delta}$ mice (Xiao et al., 2008). Since, vascular modeling is altered in these thymi, this raises the possibility that thymocytes exit the thymus prematurely through blood vessels positioned throughout the organ. Given that the extracellular matrix serves key roles in T-cell trafficking, we examined ontogeny of collagen IV deposits in wild-type thymus to investigate whether the cell autonomous defects in *Foxn1*$^{\Delta/\Delta}$ TECs resulted in subsequent defects in collagen IV deposition and perivascular space formation (Savino et al., 2004). On E12.5, we observed collagen IV surrounding the CD31^{+} cells in the capsule, and was predominantly present in the capsule where CD31^{+} cells were noticeably absent and within the prevascularized thymic rudiment (2.10A-B). After initial thymic vascularization at E13.5, collagen IV deposits were detected adjacent to intrathymic blood vessels, in the capsule, and dispersed throughout the epithelium in the control (2.10C) while collagen deposition in mutants (2.10D) resembled E12.5 thymi (2.10A-B). On E15.5 collagen IV was also detected in the capsule and associated with thymic vascular structures, however CD31^{-} collagen beds were present in both control (2.10E) and mutant thymi (2.10F) compared to earlier stages. In newborn thymi, collagen IV deposits were mostly restricted to cortical capillaries and medullary blood vessels in the *Foxn1*$^{+/\Delta}$ thymus (2.10G), but collagen IV was very broadly localized throughout the newborn *Foxn1*$^{\Delta/\Delta}$ thymus (2.10H). In particular, we observed CD31^{-} collagen IV^{+} structures throughout the mutant thymus compared with littermate controls.

Discussion

In this study, we have defined the initiation and early stages of thymic vascularization during organogenesis, and demonstrated a functional requirement for *Foxn1* in this process. Previous reports demonstrated the significance of *Foxn1* in TEC differentiation (Nehls et al., 1996; Nehls et al., 1994; Su et al., 2003). This investigation establishes that Foxn1-dependent TEC differentiation is required for thymus vascularization. Specifically, our data demonstrate that Foxn1 function in TECs is required in a dose-dependent manner for TECs to regulate the initial formation of the thymic vasculature, and that part of this function may be mediated by regulation of VEGF-A expression in TECs. Although the number of LPCs in the thymus of *Foxn1*$^{\Delta/\Delta}$ mice was significantly reduced (Su et al., 2003), we found that the timing of initial LPC immigration into the fetal thymus was normal. EPCs normally initially infiltrate the thymus at E13.5, followed closely by PDGFR-β^{+} mesenchyme; these immigration events were delayed by one day in *Foxn1*$^{\Delta/\Delta}$ mice. We have also shown that the peripheral circulation connects to the thymic rudiment as early as E14.5, and that the timing of this process is normal in *Foxn1*$^{\Delta/\Delta}$ mutants. Since not all events during early thymus organogenesis are delayed, these defects likely reflect specific functions for Foxn1 in TECs, and not a general delay in thymus development. Further, we have demonstrated that organization and structure of the thymic vasculature in Foxn1$^{\Delta/\Delta}$ mice is abnormal, with changes in vessel size and integrity. These data therefore provide evidence for a novel TEC-endothelium-mesenchyme crosstalk mechanism that plays a key role in thymus organogenesis and the organization of the thymic architecture.

Taken together, our current results and those of previous studies suggest the following model for initial thymic vascularization. Neural crest cells are first recruited to the periphery of the thymus at E10.5-E11.5 (Jiang et al., 2000; Muller et al., 2008). Subsequently, EPCs are attracted to the pericapsular region of the thymus at E12.5. Between E12.5-E13.5, EPCs infiltrate the thymus ahead of NCCs (Figure 2.1F-J). One day later at E14.5, capsular NCCs form invaginations in the TEC network and migrate along the path of EPCs and nascent blood vessels to form perivascular support cells (Figure 2.1F-J) (Foster et al., 2008). This is the same time at which the developing intrathymic vasculature becomes physically connected to the rest of the fetal vasculature, further supporting the conclusion that E14.5 is the earliest stage at which the fetal thymic vasculature becomes functional. Thus, the thymic vasculature likely forms by the coordination of intrathymic vasculogenesis (controlled in part by signals downstream of Foxn1), and extrathymic angiogenesis (the timing of which is independently regulated).

This cellular order of vascular development, the *Foxn1*$^{\Delta/\Delta}$ phenotype, and the known expression and function of PDGF and VEGF further suggest a molecular model for the mechanisms controlling this process. Both NCCs (Mukouyama et al., 2005) and TECs (Cuddihy et al., 2009; Muller et al., 2005) express proangiogenic factors including VEGF-A that could facilitate recruitment of EPCs to the capsular region of the thymus, and subsequently into the thymic anlage. As initial EPC recruitment is not affected in the *Foxn1*$^{\Delta/\Delta}$ mutants, this process may be regulated by NCC-derived signals, while immigration into the rudiment itself may require TEC-derived signals. Once inside the rudiment, EPCs form the intrathymic vasculature via vasculogenesis.

The structure of this network is patterned by the level of intrathymic VEGF-A, which at early stages is primarily produced by TECs and under the control of Foxn1. Since blood vessels require perivascular cell recruitment for maturation, the reduction in PDGF-B, which is primarily expressed in EPCs, may be responsible for the leaky vessel phenotype observed in *Foxn1*$^{\Delta/\Delta}$ mice. This intrathymic network is connected to the peripheral vasculature at E14.5 via invasion of smaller vessels from outside the thymus; the timing of this angiogenic process is independent of Foxn1-dependent TEC-derived signals, and may be regulated by mesenchymal capsule-derived angiogenic factors. Earlier injections were ambiguous due to high background; this result may have a biological rather than technical origin, as the NCC-derived mesenchyme does not become fully associated with the intrathymic vasculature until E14.5. Thus, earlier time points would not likely have a functional vasculature based on this feature alone.

The delayed immigration of NCC-derived mesenchyme in the *Foxn1*$^{\Delta/\Delta}$ mutants is likely indirect, via reduced endothelial-dependent signals including PDGF. It is well documented that endothelial cell-mural cell contact is predominantly mediated by PDGFR-β/PDGF-B ligand/receptor signaling (Andrae et al., 2008; Bjarnegard et al., 2004; Gaengel et al., 2009; Leveen et al., 1994). During normal fetal thymus development and in adult thymi, greater than 90% of NCCs express PDGFR-β (Foster et al., 2008), which suggests that thymic endothelial cell PDGF-B expression facilitates NCC recruitment into the organ. Our data suggest that NCC-derived mesenchyme “follow” the EPCs into the thymus, supporting a previous report indicating that nearly all NCC-derived mesenchyme within the adult thymus is

associated with blood vessels (Foster et al., 2008). In the *Foxn1*$^{\Delta/\Delta}$ mutants, both the delay in mesenchymal cell immigration and the reduced intrathymic PDGF-B expression are likely secondary to the delayed and reduced number of intrathymic EPCs. PDGF-B expression in *Foxn1*$^{\Delta/\Delta}$ was significantly reduced, coincident with delayed PDGFR-β^+ NCC immigration into the thymus. This result is consistent with the established model for EPC-mesenchyme paracrine signaling, in which PDGFR-β^+ mesenchymal cells respond to PDGF-B secreted by EPCs and tip cells on angiogenic sprouts (Lindahl et al., 1997). PDGF-B may also be expressed in TECs, although previous reports support the former interpretation (Andrae et al., 2008; Gaengel et al., 2009; Hellstrom et al., 2001); alternatively, TECs could provide other factors that promote mesenchymal migration and/or differentiation that contribute to these phenotypes. Interestingly, it has been shown that the thymic vasculature in *PDGFR$\beta^{-/-}$* thymi is normal, although the integrity of the vasculature and initial timing of thymus vascularization were not examined (Foster et al., 2008). These findings suggest that reduced PDGF-B levels in the thymus may contribute to delayed pericyte recruitment to blood vessels, but redundant signaling pathway(s) may compensate in the absence of PDGF-B.

Our findings suggest that VEGF-A expression in TECs may be Foxn1-dependent. Interestingly, E15.5 *Foxn1*$^{\Delta/nu}$ mice had a similar level of VEGF-A as *Foxn1*$^{\Delta/\Delta}$ mice, but show more severe vascular defects. The low level of VEGF-A expressed in the *Foxn1*$^{\Delta/\Delta}$ mutant thymi could reflect a complete loss of TEC-derived VEGF, with the residual amount coming from non-TEC thymic stroma, although vascular-associated VEGF also appeared to be reduced. This would explain the lack

of further reduction in VEGF-A in the *Foxn1*$^{\Delta/nu}$ mutants, but not the more severe vascular phenotypes in these mice. There are several possible explanations for this result. TEC-derived pro-angiogenic factors other than VEGF-A that are regulated by Foxn1 might be more severely reduced in the *Foxn1*$^{\Delta/nu}$ mutants. Alternatively, the more severe phenotype may reflect other changes in TEC differentiation that could affect the accessibility of the thymic epithelial rudiment to EPC immigration.

Our finding that collagen IV was expressed broadly in the newborn thymus is reminiscent of tumor vasculature following treatment with VEGF-A inhibitors. It has been previously demonstrated in normal thymus and in mouse tumors that inhibition of VEGF-A or low-VEGF-A availability to the developing vasculature results in defects in angiogenesis (Cuddihy et al., 2009; Inai et al., 2004). Specifically, angiogenesis is initiated, but terminated at an early stage, resulting in empty (collagen IV^{+} CD31^{-}) collagen IV sleeves observed in our mutants (Figure 10H) (Inai et al., 2004). Therefore, reduced VEGF-A expression in TECs likely contributes to these observed defects in angiogenesis and vascular basement membrane deposition.

The defects in TEC differentiation and mTEC organization phenotypes in *Foxn1*$^{\Delta/\Delta}$ mice were observed as early as E13.5 (Su et al., 2003) coincident with the initial EPC immigration delay reported here. A previous study suggested that the thymic vasculature may play a role in organizing mTECs. They demonstrated that mTECs are frequently positioned adjacent to and interact with intermediate size thymic vascular structures in wild-type mice, and that this spatial arrangement between the thymic vasculature and mTECs could be observed in Rag2-/- mice, which lack TCR-expressing thymocytes previously shown to mediate TEC-thymocyte

crosstalk (Anderson et al., 2000). The combination of vascular and stromal organization defects during early thymus organogenesis in the *Foxn1*$^{\Delta/\Delta}$ mutants raises the possibility that some of the thymic stromal organization defects observed in *Foxn1*$^{\Delta/\Delta}$ thymi may, in part, be secondary to the early defects in initial thymic vascular development.

The paradigm of TEC-thymocyte crosstalk informs much of our understanding of the mechanisms underlying the organization and function of the thymus. The identification of further crosstalk interactions between TECs, endothelial cells, and mesenchymal cells expands the network of intercellular interactions required for the proper formation of the complex thymic architecture. Our current and previous data suggest that Foxn1 plays a central role in TECs to orchestrate these diverse crosstalk pathways during fetal thymus organogenesis. This novel TEC-EPC-NCC crosstalk may also be required for the cortico-medullary organization, and for formation of blood vessels at spatially defined regions of the thymus that facilitates the immigration of LPCs into the thymus and egress of LPCs to the periphery.

Materials and Methods

Mice

Foxn1$^{\Delta}$ mice were previously described (Su et al., 2003). *Foxn1*$^{\Delta}$ mice are on a mixed 129v/C57Bl6/J background that has been backcrossed to C57Bl6/J for 5 to 7 generations. *Foxn1*^{nu} mice on a C57Bl6/J background were purchased from The Jackson Laboratories (Bar Harbor, ME). *Foxn1*$^{\Delta/nu}$ mice were generated from *Foxn1*$^{\Delta/\Delta}$ X *Foxn1*$^{+/nu}$ crosses.

Immunofluorescence

Embryos were isolated at E13.5-E18.5, flash frozen in liquid nitrogen and cryosectioned at (10µm). The following primary antibodies were used for the embryonic thymus immunofluorescence analysis (Liu et al., 2006; Su et al., 2003): monoclonal rat CD31 and CD144 (BD Pharmingen, 1:100), goat PDGFR-b (1:50), goat VEGF (15µg/mL), rat CCL21, and rat CCL25 (R&D Systems), rat CD45 (eBioscience, 1:100), mouse polyclonal Keratin 5 (Covance, 1:500), mouse cytokeratin and α-SMA (Sigma, 1:400), rabbit Collagen IV (1:400). The following secondary antibodies were purchased from Jackson Immunology: α-mouse CY5, α-rat FITC, α-goat Texas Red, α-rabbit CY3, and SA-FITC. α-rat Alexa 488 and α-goat 633 were purchased from Invitrogen. For CD144 staining (Fig. 1), sections were acetone fixed and washed in TNT. After blocking with 10% donkey serum in TNB for 30 min, sections were incubated with αCD144 (1:100) overnight, washed in TNT and incubated with donkey anti-rat HRP (Jackson ImmunoResearch, 1:100) for 30 min at room temperature, followed by wash in TNT. We used the TSA Biotin System (PerkinElmer) for signal amplification, followed by incubation with streptavidin-FITC (Jackson Immunology, 1:100). After final wash, Gel/Mount mounting medium (biomeda corp.) and cover slips were added.

CD31, PDGFR- β, and cytokeratin fluorescence images in Fig. 1 and Fig. 3 were collected using a confocal microscope (LSM 510 Meta, Zeiss) and captured using a Plan-Apochromat 20x/0.8 lens (512 X 512 Pixels; 110us/pixel; pinhole 170µm, 158µm, and 156µm) with 488-, 543-, and 633-nm laser lines. CD144 fluorescence images in Fig. 1 were captured using a Plan-Apochromat 20x/0.8 lens (512 X 512 Pixels; 110us/pixel; pinhole 138µm and 128µm) with 488- and 543nm laser lines. For Fig 1P-T, images were collected and processed using a Zeiss Axioplan microscope and AxioVision 4.8 software.

Thymic stromal cell isolation

Thymi from E13.5 and E15.5 embryos were dissected and digested in 0.25% trypsin. Mutant and control littermates were pooled separately after genotyping yolk sac DNA. Following digestion, cells were incubated with purified mouse anti-mouse CD45 (BioLegend) for 30 min and washed three times with PBS. $CD45^+$ cells were depleted using Dynabeads® sheep anti-mouse and following manufacture's protocol for magnetic bead depletion (Invitrogen, USA). CD45 purity and EpCAM expression was determined by real-time quantitative polymerase chain reaction (qRT-PCR).

RNA isolation, cDNA synthesis and real-time quantitative reverse-transcriptase – polymerase chain (qRT-PCR)

RNA was isolated from embryonic thymi using the RNeasy Micro kit (QIAGEN). In each litter, mutant thymi were pooled together, as well as control thymi. First-strand cDNA was synthesized using a cDNA synthesis kit (Bio-Rad, USA). qRT-PCR was performed using TaqMan Universal Master Mix and TaqMan probes for VEGF-A, PDGF-B, EpCAM, CD45 and 18S rRNA (endogenous control) on an ABI 7500 thermocycler.

Controls are set to a value of 1 in each experiment relative to mutants. All experiments were performed in duplicate.

In vivo embryonic thymic vasculature labeling (Bryson et al., 2011)

FITC 50 µg/ml FITC dextran (Sigma) was injected into embryos via the facial vein. After 5 minutes, embryos were flash frozen in liquid nitrogen. Embryos were then sectioned at 10 µm, followed by immunofluorescence with CD31 and Cytokeratin. For whole mount FITC-dextran experiments, thymi were isolated following injection, washed in PBS, and placed on slides with Gel/Mount mounting medium (Biomeda Corp.) and cover slips. For Fig. 3I-L, fluorescence images were collected using a confocal microscope (LSM 510 Meta, Zeiss) and captured using a Plan-Neofluar 10X/0.3 lens (512 X 512 Pixels; 110us/pixel; pinhole 98µm) with 488nm laser line. 10µm z-stacks in Fig. 3B, H, F, H and I-L were processed using AxioVision 4.8 Software.

Statistics

Values are expressed as means plus or minus Standard Deviation (SD). Student's t test was performed to determine whether the difference between the means of mutants compared to control groups were statistically significant.

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Abbreviations List

cTEC, cortical thymic epithelial cell; EpCAM, epithelial cell adhesion molecule; EPC, endothelial progenitor cell; Foxn1, forkhead box transcription factor 1; LPC, lymphoid progenitor cell; mTEC, medullary epithelial cell; NCC, neural crest cell; PDGF-B, platelet-derived growth factor beta; PDGFR-β, platelet-derived growth factor receptor beta; CD31, platelet endothelial cell adhesion molecule; TEC, thymic epithelial cell; CD144, vascular endothelial cadherin; VEGF, vascular endothelial growth factor.

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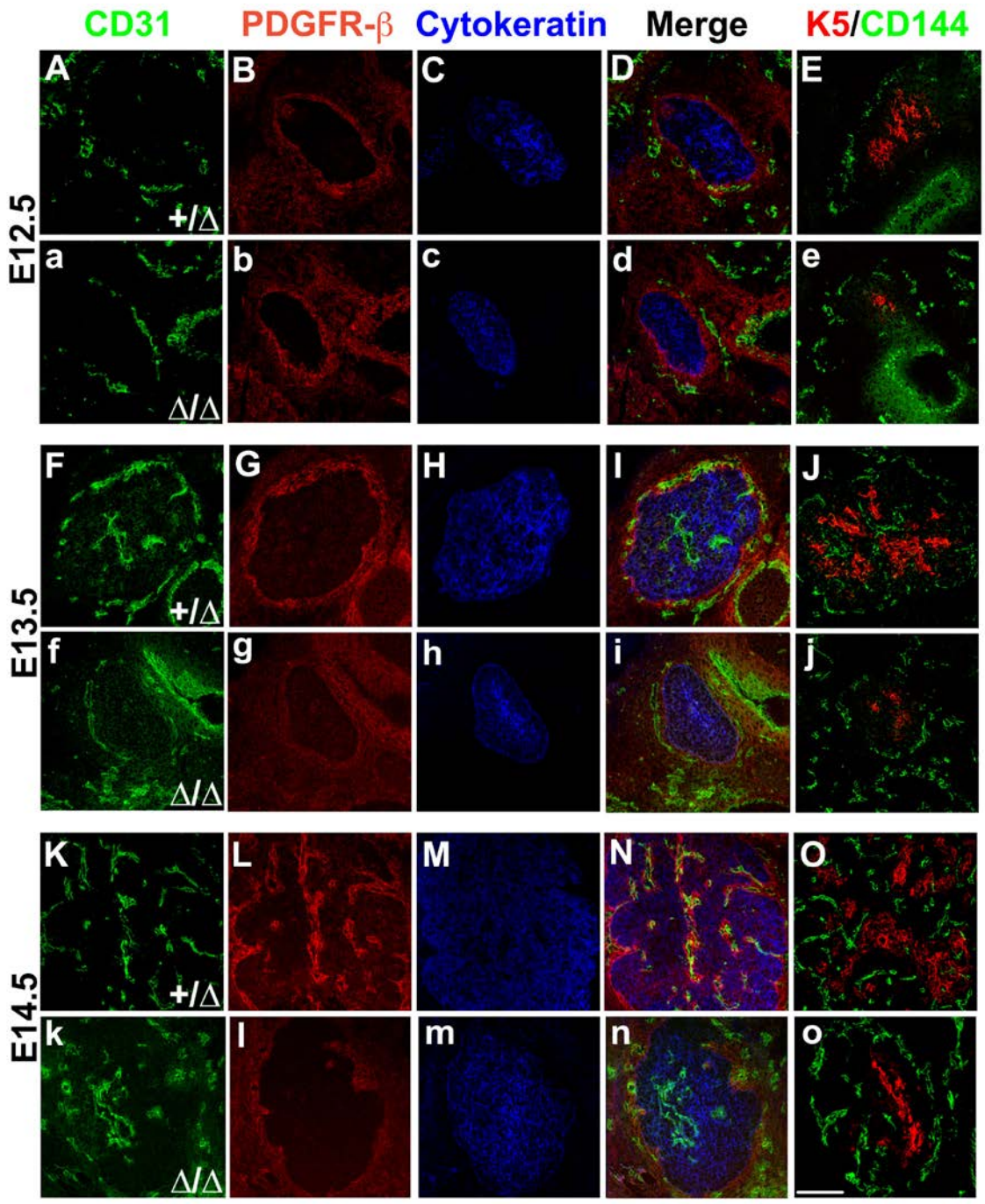
CD31
PDGFR-β
Cytokeratin
Merge
K5/CD144
E12.5
E13.5
E14.5
A
B
C
D
E
+/Δ
a
b
c
d
e
Δ/Δ
F
G
H
I
J
+/Δ
f
g
h
i
j
Δ/Δ
K
L
M
N
O
+/Δ
k
l
m
n
o
Δ/Δ

Figure 2.1

Figure 2.1: Initial embryonic thymic vascularization is defective in *Foxn1*$^{\Delta/\Delta}$ Mice. Immunostaining on frozen transverse sections of fetal thymus E12.5-E14.5. Endothelial and stromal cell markers used are listed above each column in the corresponding color: CD31$^+$/CD144$^+$ for endothelial cells (**green**); PDGFR-β^+ for neural crest mesenchyme (**red**); Cytokeratin (**blue**) or Keratin 5 (**red, K5**) for epithelial cells. Embryonic stages in the first column and genotypes to the left apply to the entire row unless otherwise labeled. (**A-e**) CD31$^+$/CD144$^+$ endothelial cells and PDGFR-β^+ neural crest cells are present in the thymic capsule region in *Foxn1*$^{+/\Delta}$ (**A-E**) and *Foxn1* $^{\Delta/\Delta}$ mice (**a-e**) at E12.5. (**F-o**) CD31$^+$/CD144$^+$ cells followed by PDGFR-β^+ cells initially immigrate into the thymus at E13.5 in heterozygotes (**F-J**) and at E14.5 in homozygotes (**k-o**).

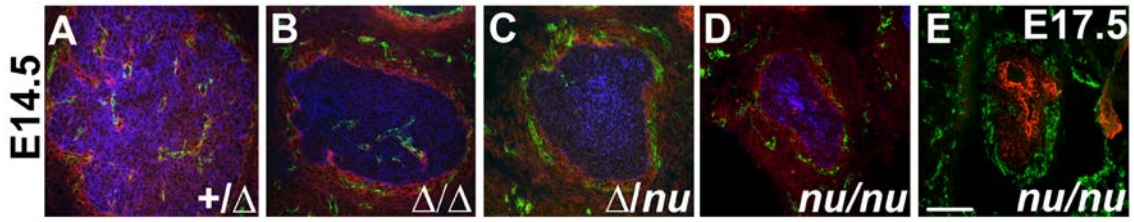

Figure 2.2

Figure 2.2: Initial embryonic thymic vascularization is sensitive to *Foxn1* levels.

(**A-E**) $CD31^+/CD144^+$ endothelial cells and PDGFR-β^+ neural crest mesenchyme can be detected in the thymic capsule and inside the keratin-positive thymus in E14.5 $Foxn1^{+/\Delta}$ (**A**) and $Foxn1^{\Delta/\Delta}$ mice (**B**), and in the capsule only in $Foxn1^{\Delta/nu}$ (**C**), and $Foxn1^{nu/nu}$ mice (**D**). $CD144^+$ endothelial cells are present at the periphery of the thymus at E17.5 in nude homozygotes, but remain absent in the rudiment (**E**). Scale bar = 100μm

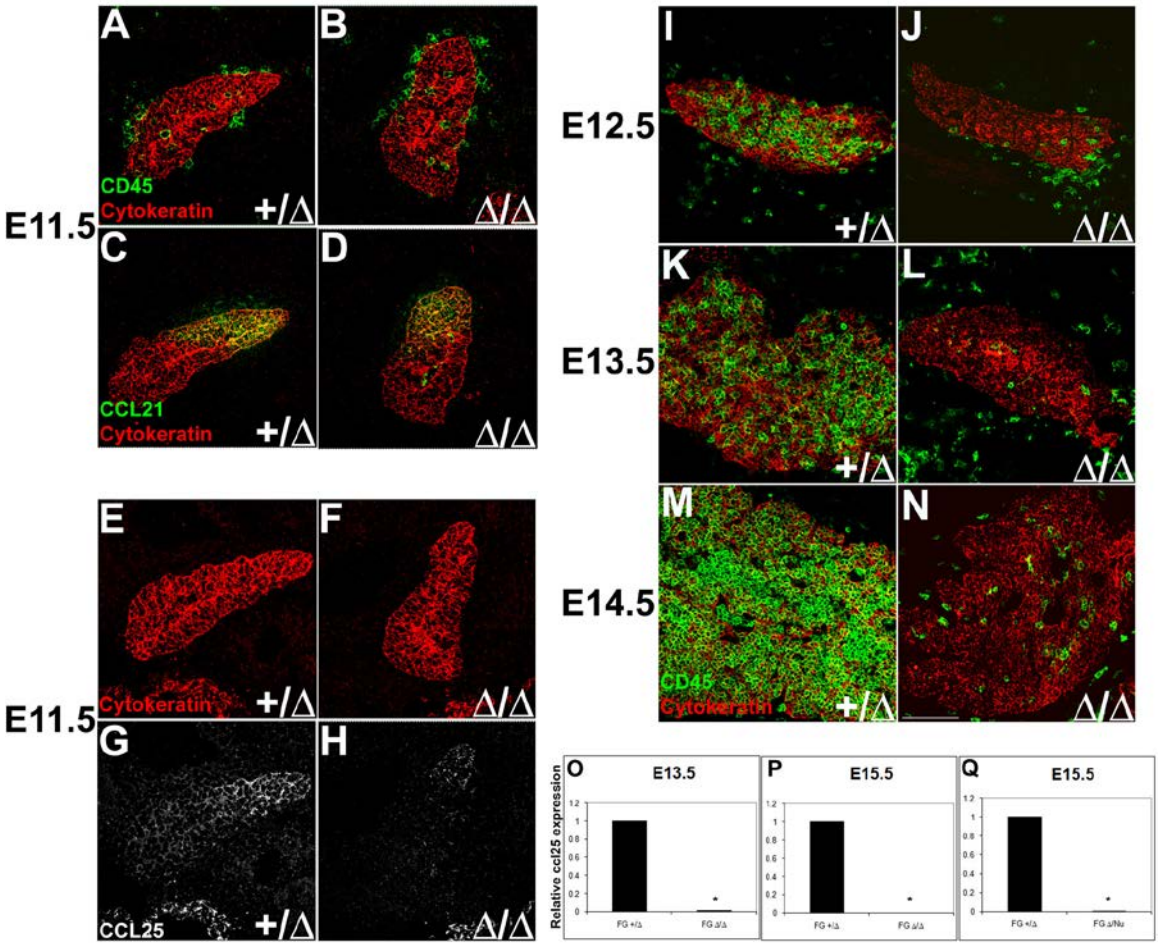
A
B
E11.5
CD45
Cytokeratin
+/Δ
Δ/Δ
C
D
CCL21
Cytokeratin
+/Δ
Δ/Δ
E12.5
I
J
+/Δ
Δ/Δ
E13.5
K
L
+/Δ
Δ/Δ
E14.5
M
N
CD45
Cytokeratin
+/Δ
Δ/Δ
E
F
E11.5
Cytokeratin
+/Δ
Δ/Δ
G
H
CCL25
+/Δ
Δ/Δ
O
E13.5
P
E15.5
Q
E15.5
Relative ccl25 expression
1.2
1
0.8
0.6
0.4
0.2
0
FG +/Δ
FG Δ/Δ
FG Δ/Nu

Figure 2.3

Figure 2.3: Initial LPC thymic immigration normal in *Foxn1$^{\Delta}$* mice. (**A**) CD45^{+} LPCs (green) colonize the *Foxn1$^{+/\Delta}$* and (**B**) *Foxn1$^{\Delta/\Delta}$* thymus at E11.5. (**C**) Immunostaining for CCL21 (**green**) expression is similar in *Foxn1$^{+/\Delta}$* and (**D**) *Foxn1$^{\Delta/\Delta}$* mouse thymus. (**E-G**) CCL25 (**white**) staining is normal in *Foxn1$^{+/\Delta}$* and reduced in (**F-H**) *Foxn1$^{\Delta/\Delta}$* thymus at E11.5. Cytokeratin (**red**). CD45^{+} cells (**green**) reduced in (**I-J**) 12.5, (**K-L**) 13.5, and (**M-N**) 14.5 *Foxn1$^{\Delta/\Delta}$* thymus reduced compared to control littermates. CCL25 expression in E13.5 *Foxn1$^{\Delta/\Delta}$* **(O,** n =4), E15.5 *Foxn1$^{\Delta/\Delta}$* (**P,** n=3), and E15.5 *Foxn1$^{\Delta/nu}$* (**Q,** n=6), compared to *Foxn1$^{+/\Delta}$* control thymi. Scale bar = 100µm. qRT experiments represent relative RNA expression of pooled thymi. Controls were set to 1. Asterisks denote statistical significance (P< .05).

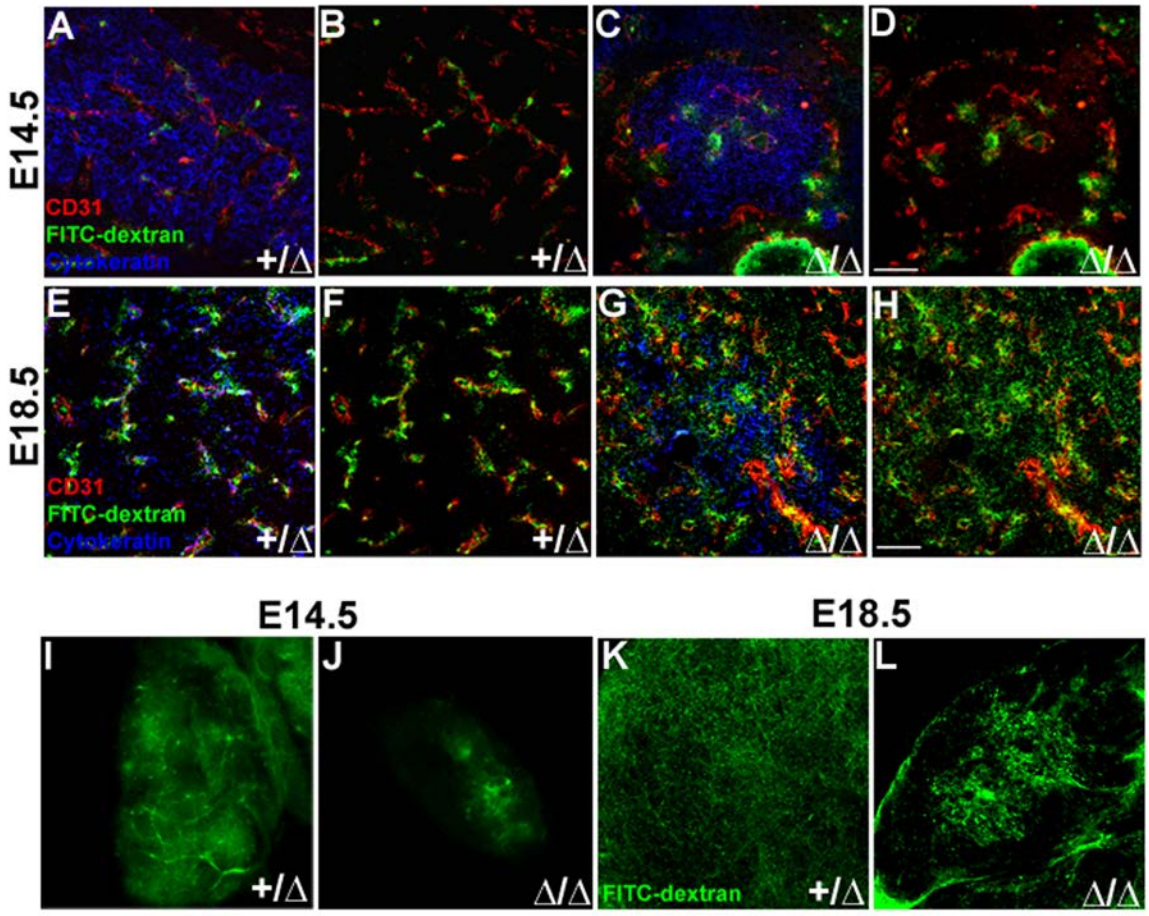
A
B
C
D
E14.5
CD31
FITC-dextran
Cytokeratin
+/Δ
+/Δ
Δ/Δ
Δ/Δ
E
F
G
H
E18.5
CD31
FITC-dextran
Cytokeratin
+/Δ
+/Δ
Δ/Δ
Δ/Δ
E14.5
E18.5
I
J
K
L
+/Δ
Δ/Δ
FITC-dextran
+/Δ
Δ/Δ

Figure 2.4

Figure 2.4: Peripheral circulation is connected to the thymus at E14.5 FITC-dextran (**green**) facial vein injections and immunostaining for CD31 (**red**) and cytokeratin (**blue**) on frozen sagittal sections of fetal mouse thymus. **(A-B)** FITC-dextran is detected in E14.5 *Foxn1*$^{+/\Delta}$ thymi, tightly associated with CD31^{+} blood vessels. **(C-D)** In *Foxn1*$^{\Delta/\Delta}$ embryos, FITC-dextran is also present, but the signal is more diffusely associated with vessels. **(E-F)** FITC-dextran is present throughout E18.5 *Foxn1*$^{+/\Delta}$ thymi tightly associated with branched blood vessels. (**G-H)** FITC-dextran is more diffusely present in the thymus of E18.5 *Foxn1*$^{\Delta/\Delta}$ mice. Panels **B, D, F,** and **H** are z-stacks of serial confocal images. (**I-L)** Whole-thymus confocal images of thymi dissected from E14.5 and E18.5 FITC-dextran injected embryos. FITC-dextran reveals a dense, highly branched vessel network throughout the thymus in control embryos (**I, K**). In *Foxn1*$^{\Delta/\Delta}$ mutants, FITC-dextran is more diffuse, and does not clearly delineate individual vessels, but is concentrated in the center of the lobes; at E18.5, FITC-dextran is accumulating in the capsular region, possibly due to leakage from peri-thymic vessels in the capsule. Dashed line outlines thymic rudiment (**G, J)**. Scale bar = 100μm

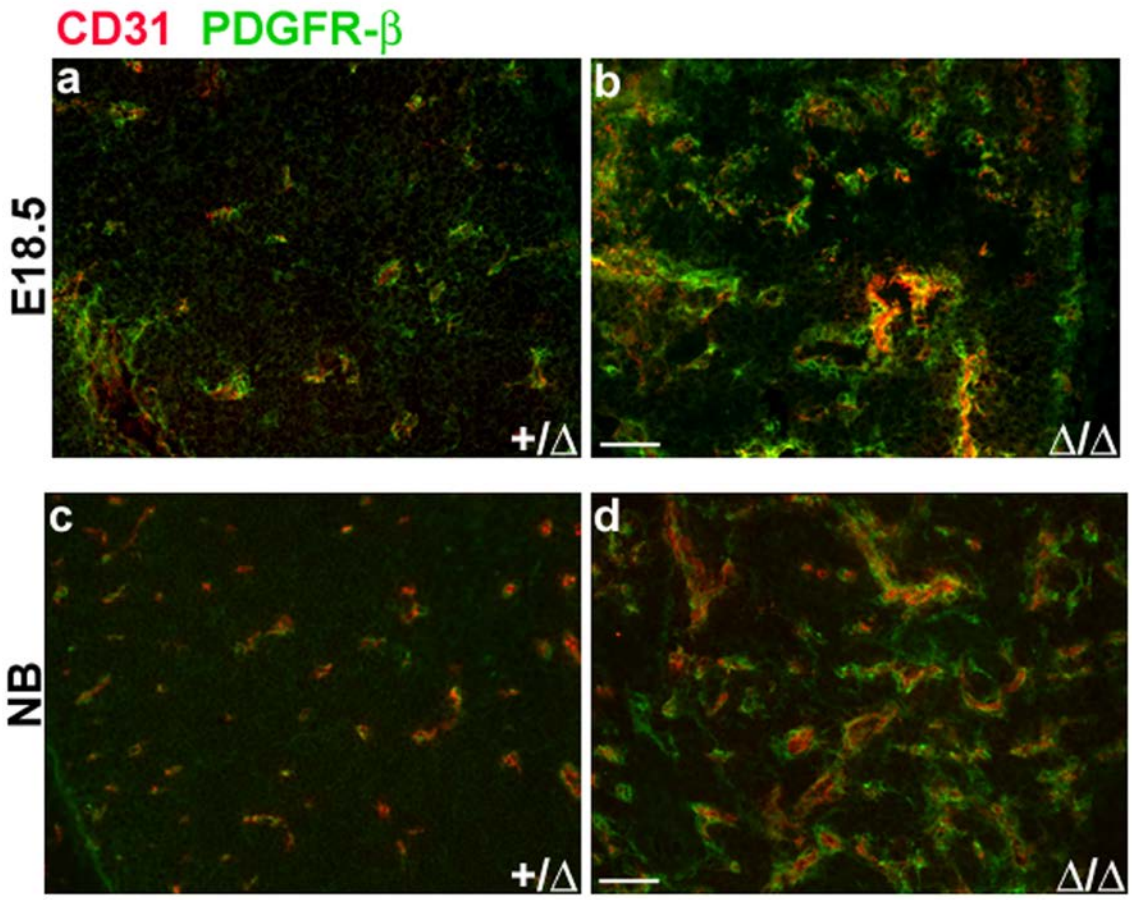

Figure 2.5

Figure 2.5: Thymus vascular modeling altered in Foxn1Δ mice

Immunohistochemical analysis on frozen sagittal sections of WT and Foxn1Δ thymus at E18.5 and newborn (**NB**) for $CD31^+$ (**red**) and PDGFR-β^+ (**green**) cells in (**A**) E18.5 *Foxn1*$^{+/\Delta}$, (**B**) E18.5 *Foxn1*$^{\Delta/\Delta}$, (**C**) NB *Foxn1*$^{+/\Delta}$, (**D**) NB *Foxn1*$^{\Delta/\Delta}$ Scale bar = 100µm

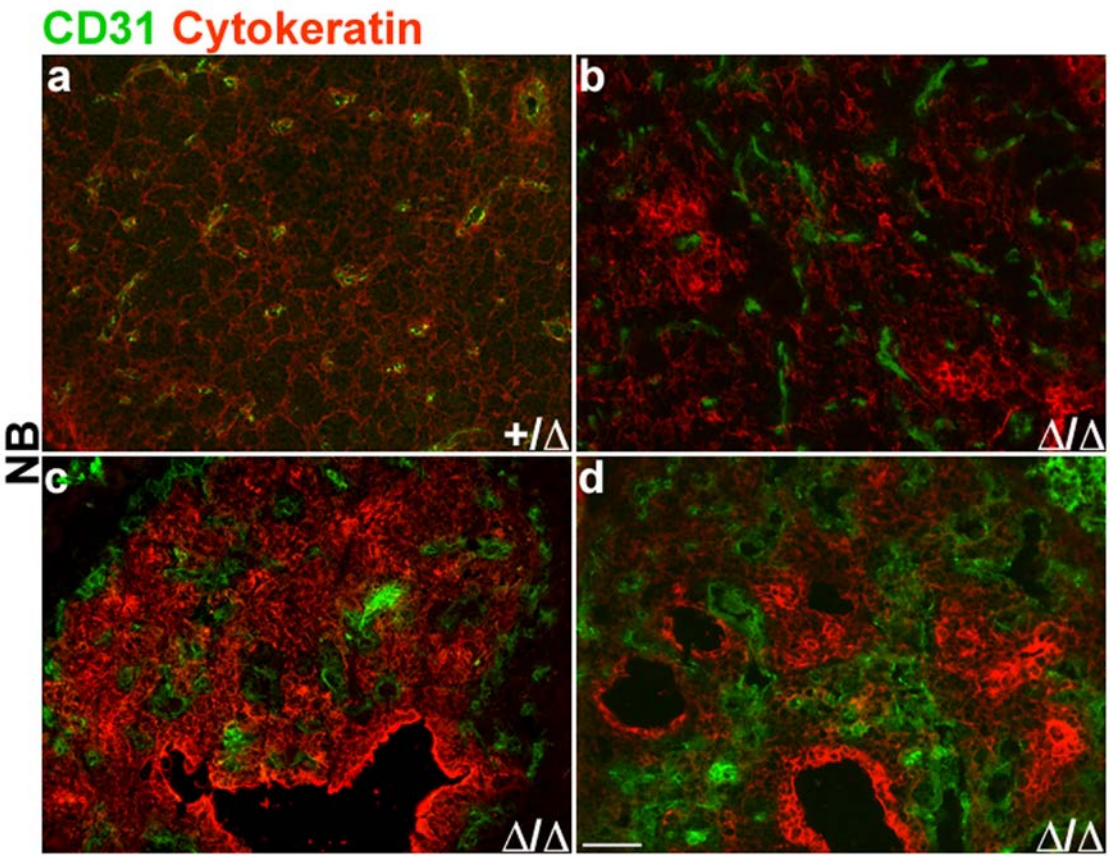

Figure 2.6

Figure 2.6: Variable severity of postnatal Foxn1Δ vasculature

Immunohistochemical analysis of newborn Foxn1Δ thymus vasculature show variable thymic epithelial and vascular structures. **(A)** *Foxn1*$^{+/\Delta}$ thymus,(**B**) *Foxn1*$^{\Delta/\Delta}$ mild thymus vascular defect, (**C**) *Foxn1*$^{\Delta/\Delta}$ intermediate vascular thymus defect, (**D**) *Foxn1*$^{\Delta/\Delta}$ severe thymus vascular defects. CD31 (**green**) and Cytokeratin (**red**) Scale bar =100µm

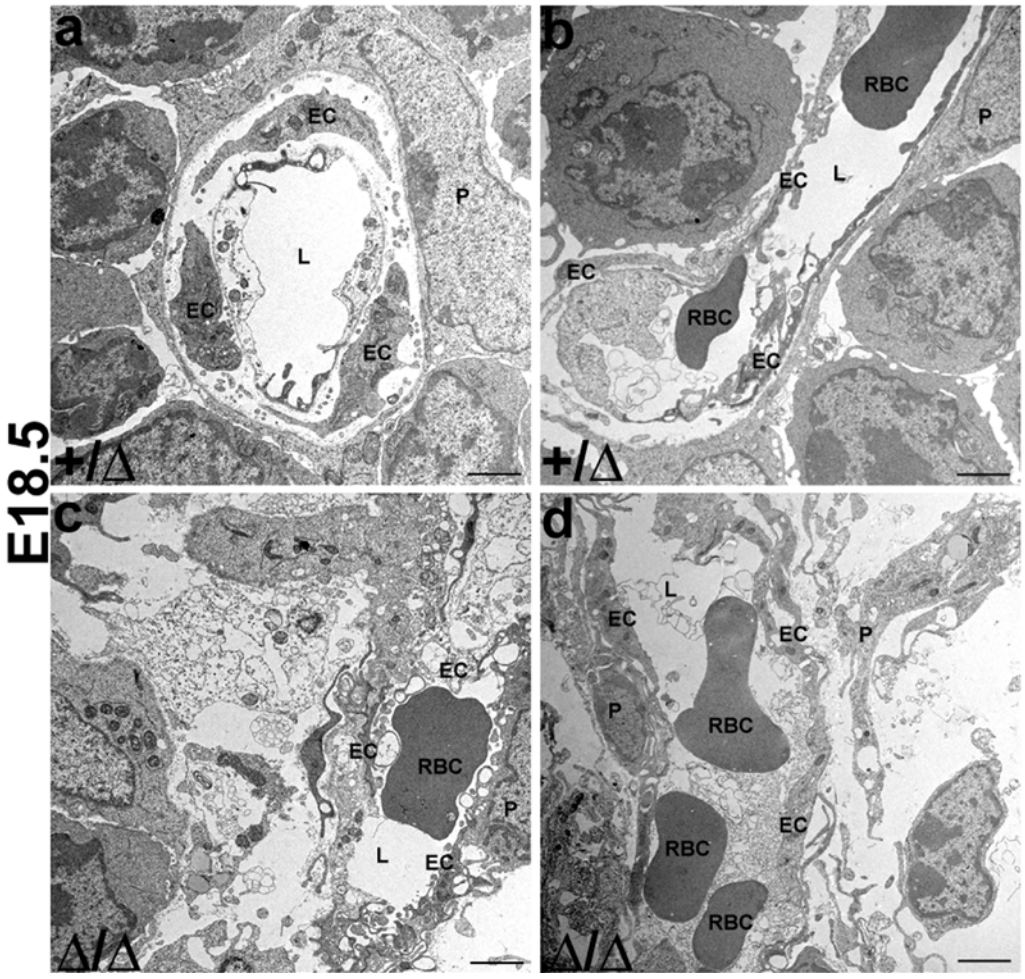
E18.5
a
EC
P
L
EC
EC
+/Δ
b
RBC
P
EC
L
EC
RBC
EC
+/Δ
c
EC
EC
RBC
P
L
EC
Δ/Δ
d
L
EC
EC
P
P
RBC
EC
RBC
RBC
Δ/Δ

Figure 2.7

Figure 2.7: Electron Microscopic Analysis of E18.5 thymus vascular defects

Electron microscopy analysis of **(A-B)** *Foxn1*$^{+/\Delta}$ thymus show compact arrangement of cells including endothelial cells and pericytes, while the **(C-D)** *Foxn1*$^{\Delta/\Delta}$ thymus display loose arrangement of cells, vacuolated endothelium, and indistinct vessel walls. Endothelial Cell (**EC**), Pericyte (**P**), Lumen of Blood Vessel (**L**), and Red Blood Cell (**RBC**)

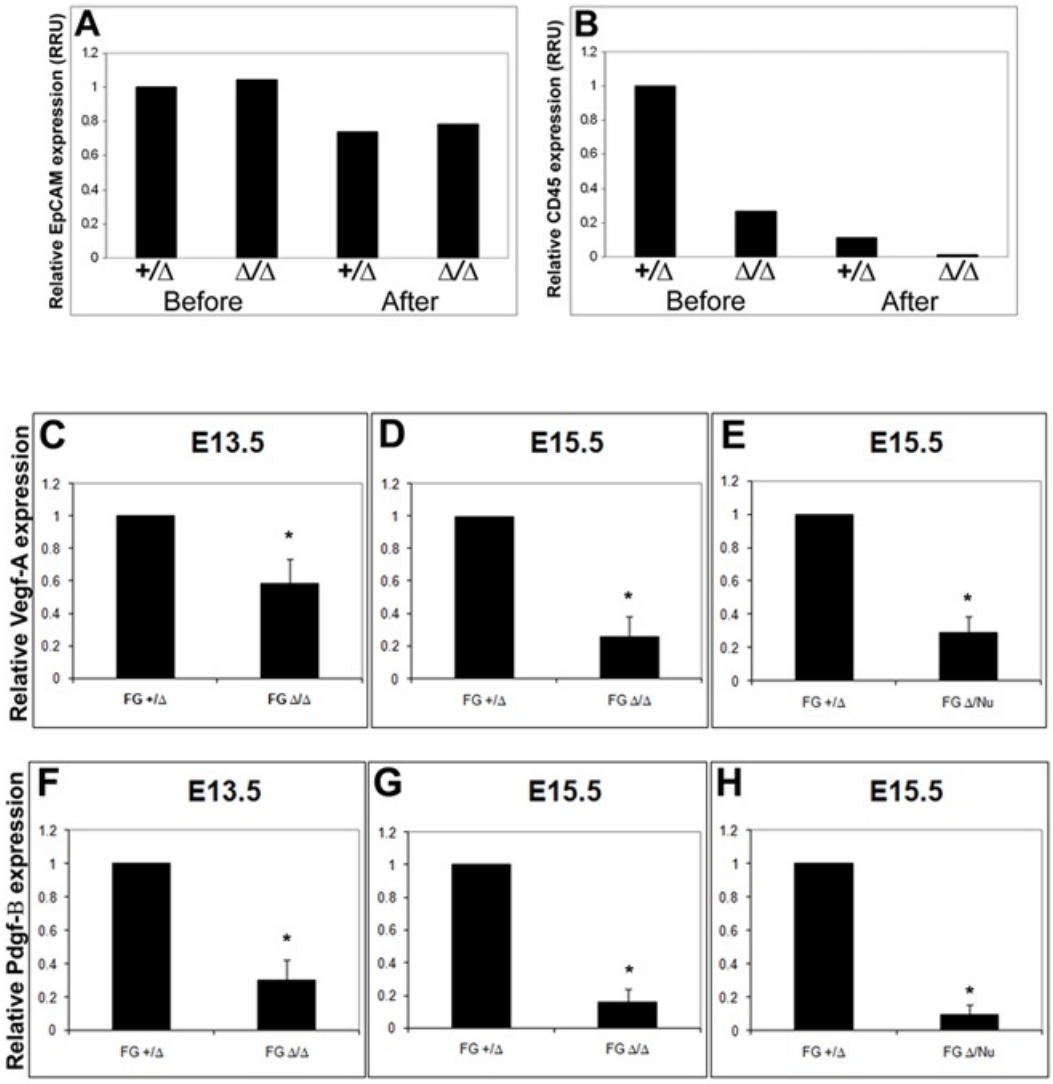
A
Relative EpCAM expression (RRU)
1.2
1
0.8
0.6
0.4
0.2
0
+/Δ
Δ/Δ
+/Δ
Δ/Δ
Before
After
B
Relative CD45 expression (RRU)
1.2
1
0.8
0.6
0.4
0.2
0
+/Δ
Δ/Δ
+/Δ
Δ/Δ
Before
After
C
E13.5
Relative Vegf-A expression
1.2
1
0.8
0.6
0.4
0.2
0
*
FG +/Δ
FG Δ/Δ
D
E15.5
1.2
1
0.8
0.6
0.4
0.2
0
*
FG +/Δ
FG Δ/Δ
E
E15.5
1.2
1
0.8
0.6
0.4
0.2
0
*
FG +/Δ
FG Δ/Nu
F
E13.5
Relative Pdgf-B expression
1.2
1
0.8
0.6
0.4
0.2
0
*
FG +/Δ
FG Δ/Δ
G
E15.5
1.2
1
0.8
0.6
0.4
0.2
0
*
FG +/Δ
FG Δ/Δ
H
E15.5
1.2
1
0.8
0.6
0.4
0.2
0
*
FG +/Δ
FG Δ/Nu

Figure 2.8

Figure 2.8: VEGF-A and PDGF-B expression is reduced in *Foxn1*$^{\Delta/\Delta}$ thymus. (A) EpCAM expression is normal in pooled E13.5 *Foxn1*$^{\Delta/\Delta}$ and control thymi before and after CD45^{+} cell depletion. **(B)** CD45 expression before and after CD45^{+} cell depletion in pooled E13.5 *Foxn1*$^{\Delta/\Delta}$ and control thymi. VEGF-A expression in E13.5 *Foxn1*$^{\Delta/\Delta}$ **(C,** n =4), E15.5 *Foxn1*$^{\Delta/\Delta}$ (**D,** n=3), and E15.5 *Foxn1*$^{\Delta/nu}$ (**E,** n=6), compared to *Foxn1*$^{+/\Delta}$ control thymi. PDGF-B expression in E13.5 *Foxn1*$^{\Delta/\Delta}$ (**F,** n=4), E15.5 *Foxn1*$^{\Delta/\Delta}$ (**G,** n=3), and E15.5 *Foxn1*$^{\Delta/nu}$ (**H,** n=6), compared to *Foxn1*$^{+/\Delta}$ control thymi. Experiments represent relative RNA expression of pooled thymi. Controls were set to 1. Asterisks denote statistical significance (P<.05).

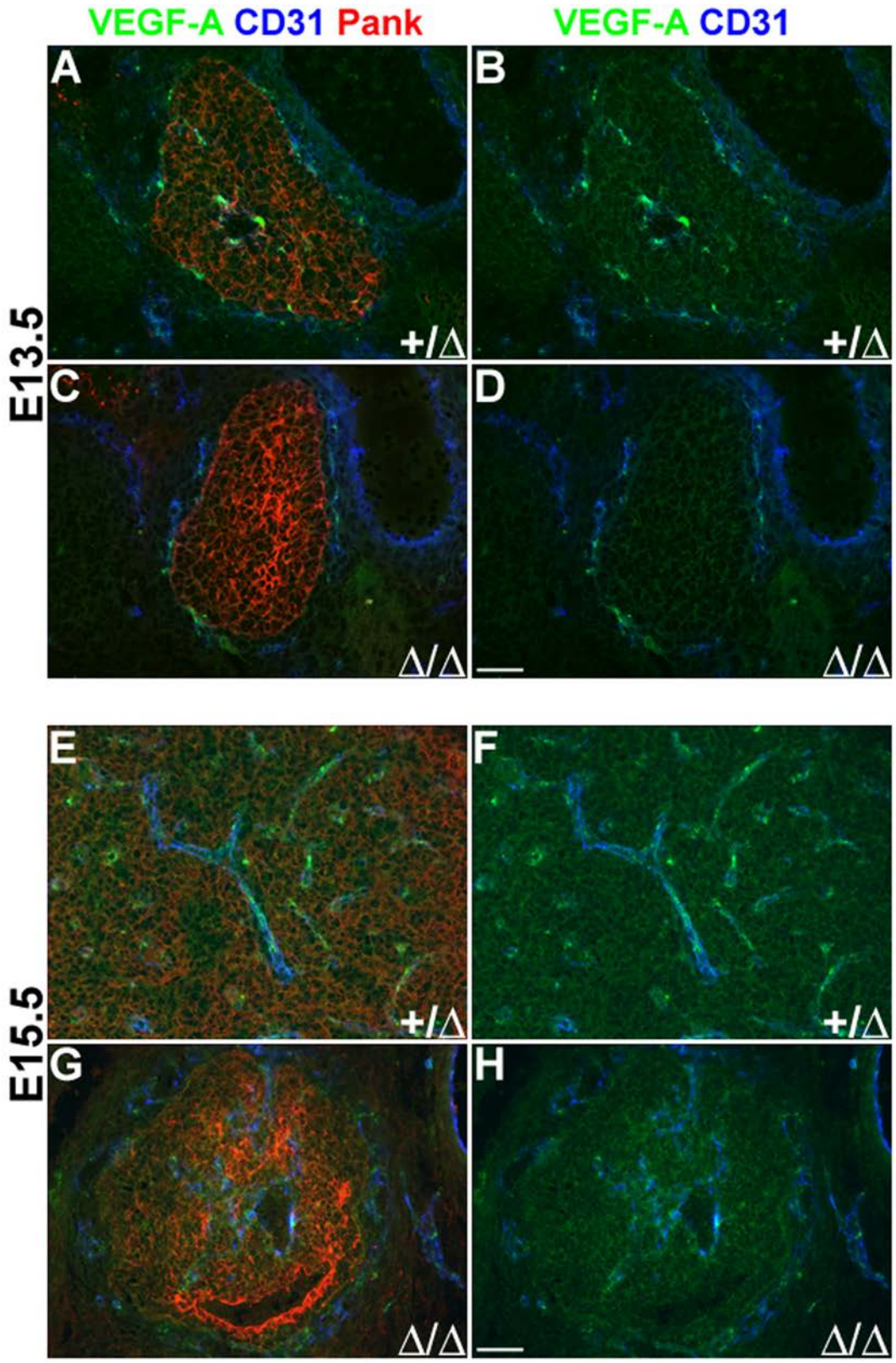

Figure 2.9

Figure 2.9: VEGF-A protein expression is reduced in *Foxn1*$^{\Delta/\Delta}$ thymus.

Immunohistochemical analysis of VEGF-A expression performed on frozen transverse sections of embryonic thymus for CD31^{+} (**blue**), VEGF-A (**green**) and Pan keratin (**red**). VEGF-A expression was detected in thymic endothelium, perivascular cells, and TECs in *Foxn1*$^{+/\Delta}$ and *Foxn1*$^{\Delta/\Delta}$ mice (**A-H**). VEGF-A expression is reduced in E13.5 (**C-D**) and E15.5 (**G-H**) *Foxn1*$^{\Delta/\Delta}$ thymus compared to E13.5 (**A-B**) and E15.5 (**E-F**) *Foxn1*$^{+/\Delta}$ controls, respectively. Scale bar = 100µm

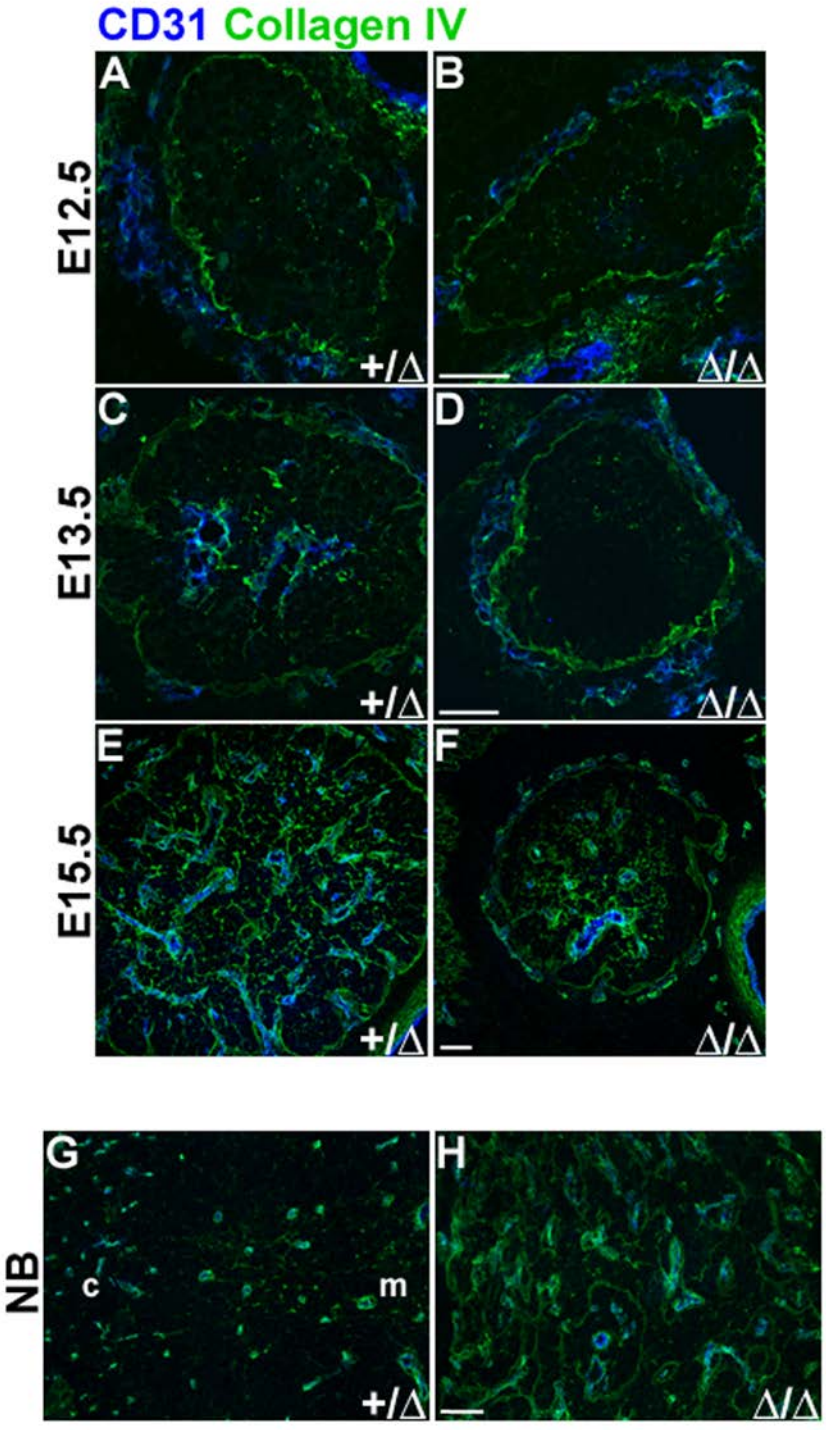
CD31 Collagen IV
A
B
E12.5
+/Δ
Δ/Δ
C
D
E13.5
+/Δ
Δ/Δ
E
F
E15.5
+/Δ
Δ/Δ
G
H
NB
c
m
+/Δ
Δ/Δ

Figure 2.10

Figure 2.10: Collagen IV is broadly expressed throughout NB *Foxn1*$^{\Delta/\Delta}$ thymus.

Immunohistochemical analysis of embryonic (**A-F**) and newborn transverse sections (**G-H**) of thymus for CD31^{+} (**blue**) and Collagen IV (**green**). Collagen IV deposits adjacent to CD31^{+} cells in E12.5 (**A**) *Foxn1*$^{+/\Delta}$ and (**B**) *Foxn1*$^{\Delta/\Delta}$, E13.5 (**C**) *Foxn1*$^{+/\Delta}$ and (**D**) *Foxn1*$^{\Delta/\Delta}$, E15.5 (**E**) *Foxn1*$^{+/\Delta}$ and (**F**) *Foxn1*$^{\Delta/\Delta}$, Collagen IV expression in newborn (**G**) *Foxn1*$^{+/\Delta}$ and (**H**) *Foxn1*$^{\Delta/\Delta}$ thymus. cortex (**c**) and medulla (**m**)

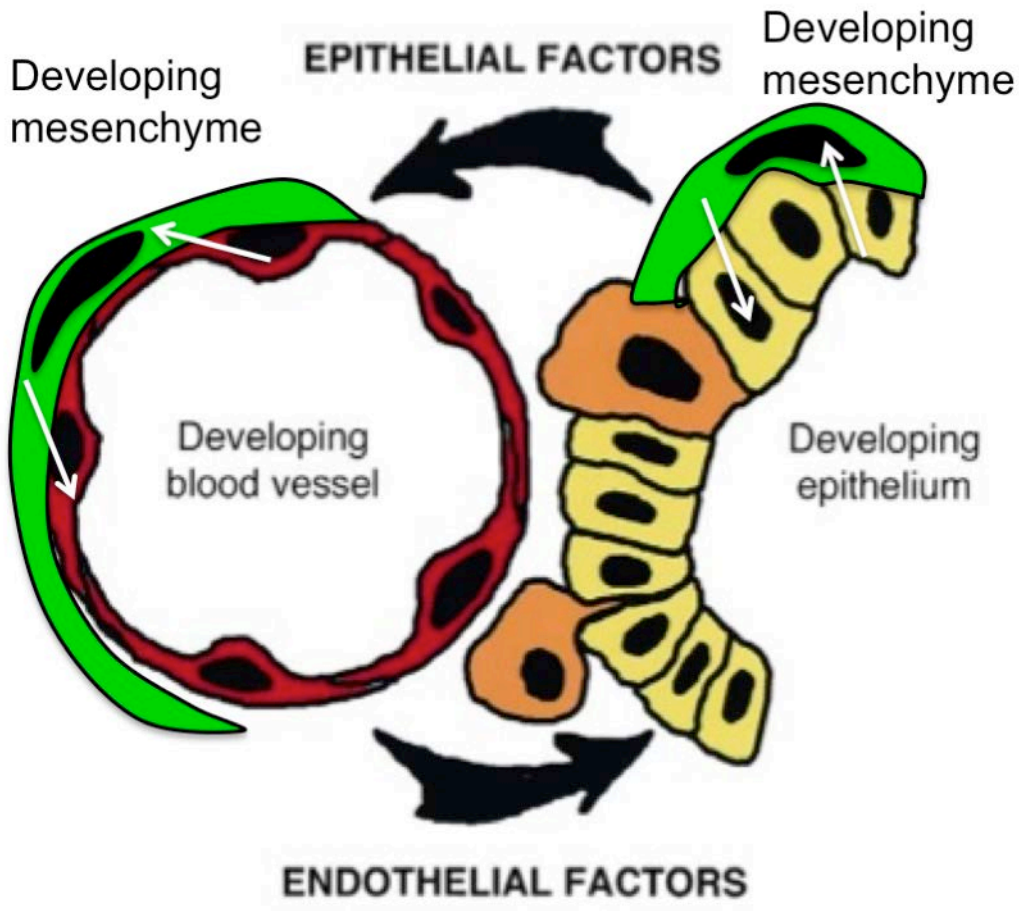
Developing mesenchyme
EPITHELIAL FACTORS
Developing mesenchyme
Developing blood vessel
Developing epithelium
ENDOTHELIAL FACTORS

Figure 2.11

Figure 2.11: Model for TEC-Endothelium-NCC Crosstalk

Our model suggests that TECs secrete proangiogenesis factors, which results in the recruitment of endothelial cells into the thymus. Endothelial cells are involved in crosstalk with thymic neural crest-derived mesenchyme and this interaction is required for blood vessel maturation. In addition TEC are involved in crosstalk with PDGFR-α/β^{+} thymic mesenchyme. Disruption of *Foxn1* in TECs disrupts NCC-Endothelium crosstalk, and may also disrupt TEC-NCC crosstalk in the thymus and in the capsule. Adapted from (Lammert et al., 2003**)**

Chapter 3

THYMIC EPITHELIAL CELL-DERIVED VEGF-A, SHH, AND BMP4 ARE DISPENSABLE FOR INITIAL THYMUS VASCULARIZATION

Introduction

The development of the thymus as a primary lymphoid organ requires the concomitant establishment of an intricate organ specific vascular network. Our current understanding of the thymus vascular biology suggests that blood vessels primarily function, in the transport of oxygen, nutrients, and molecules and in the recruitment and transport of T-cells progenitors. The later events involve T-cell progenitor colonization of the organ via the functional thymus blood vessel network, beginning at ~E14.5 (unpublished data, Bryson et al.), and egress of mature CD4+ and CD8+ T-cells to the periphery at later stages. The mechanisms responsible for T-cell progenitor recruitment to both the pre and post-vascularized thymus are well understood (Bleul and Boehm, 2000; Fontaine-Perus et al., 1981; Jenkinson et al., 2007; Liu et al., 2006; Liu et al., 2005; Wurbel et al., 2001). Similarly, the molecular mechanisms driving T-cell egress from the thymus via the vasculature have begun to be unraveled in recent years (Weinreich and Hogquist, 2008; Zachariah and Cyster, 2009; Zachariah and Cyster, 2010). These studies highlight the existence of endothelial cell-thymocyte crosstalk, as T-cells home from circulation to their target tissues, a process mediated by endothelial cell expression of CCL25, P-selectin, VCAM-1, and ICAM-1 ligands, with T-cells expressing corresponding receptors, CCR9, PSGL-1, $\alpha_4\beta_1$ integrin, and $\alpha_L\beta_2$ integrin, respectively (Cyster, 2009; Scimone et al., 2006).

Endothelium-thymocyte crosstalk is required for chemoattraction, rolling adhesion, tight adhesion, and eventually entry of thymocytes into the vascularized thymus via trans-endothelial migration at post-capillary venules positioned at the cortico-medullary junction (Mori et al., 2007; Petrie and Zuniga-Pflucker, 2007; Scimone

et al., 2006). Studies on mature T-cell egress from the thymus have identified a novel form of crosstalk between T-cells and the thymic neural crest, a structural component of blood vessels in the organ (Zachariah and Cyster, 2010). It is therefore clear that vascular endothelium in the thymus and structural components of blood vessels, such as perivascular neural crest cells, play critical roles in organ development and communicate with their surrounding microenvironment.

Thymic epithelial cells, the chief cellular component of the thymus engage in bi-directional signaling with most, if not all cells in the thymus. We were therefore interested in investigating whether significant crosstalk exists between thymic epithelial cells and thymus vasculature. As shown previously, thymic epithelial cells are organized into cortical and medullary epithelial cell clusters and can be distinguished histologically using a suite of markers specific to cortical and medullary epithelial cell subsets. A study by Farr et al., suggested that the thymus vasculature might play a role in organizing medullary epithelial cells, which play a key role in negative selection (Anderson et al., 2000). These conclusions were based on the observations that medually thymic epithelial cells consistently associate with larger vessels known as post-capillary venules, and that this association persists in the absence of thymocytes (Anderson et al., 2000). However, no mechanism has been reported to elucidate the nature of these TEC-vasculature interactions.

Using wild-type, hypomorphic, and null alleles of Foxn1, we recently demonstrated that the initial stages of thymic vascularization, and later, modeling of the postnatal vasculature into a stereotypical thymus specific network, with a dense cortical capillary network and larger vessels in the medulla and at the cortico-medullary junction,

is Foxn1-dependent. In Foxn1$^{\Delta/\Delta}$ mutants, in which the N-terminal domain of Foxn1 is mutated, endothelial progenitor cells were not detected in the thymus until E14.5, as their colonization of the thymus was delayed 1-2 days, with subsequent defects in endothelial-neural crest cross-talk observed (Su et al., 2003). Most strikingly loss of Foxn1 function in Nude mice resulted in a complete failure of thymic epithelial cells to recruit endothelial cells into the rudiment. Vascular defects in Foxn1 mutants were associated with reduced VEGF-A expression.

Vascular endothelial growth factor (VEGF-A) is a potent endothelial cell-specific mitogen and chemo attractant involved in organ vascularization (Carmeliet et al., 1999). There are three known isoforms of VEGF-A (120, 164, 188). Mice with null and heterozygous VEGF-A mutations are embryonic lethal between E8.5-9.5 and E11-12, respectively (Carmeliet et al., 1996; Ferrara et al., 1996). Mice expressing a single isoform of VEGF-A, VEGF-A 120 experience perinatal lethality due to congenital birth defects, and myocardial ischemia (Carmeliet et al., 1999; Stalmans et al., 2003). A hypoplastic thymus phenotype has also been reported in VEGF-A 120 mice (Stalmans et al., 2003). Therefore conditional genetic analysis is often employed to circumvent embryonic lethality. In mice with targeted deletion of vascular endothelial growth factor A (Vegf-A) in thymic epithelial cells, generated using a nude mouse blastocycst complementation strategy, there were various defects in blood vessel formation (Muller et al., 2005). Muller et al. confirmed Vegf-A deletion in cortical and medullary thymic epithelial cell subsets by cell sorting and subsequent RT-PCR, but also identified a subset of cells in the thymus in which Vegf-A expression persisted. This subset of cells was identified as cortical mesenchymal cells (cMes). It is probable that these cMes

VEGF-A expressing cells are clones of the capsule population. Therefore, VEGF-A from the neural crest-derived mesenchymal capsule may be responsible for attracting endothelial progenitor cells to the periphery of the thymus at E11.5-E12.5.

Previous reports suggest that several molecules expressed in the thymus have pro-angiogenesis properties and their reduction in Foxn1Δ mice contribute to the observed defects. Shh plays a number of important roles in mammalian development including cell growth, specification, and patterning (Ingham and Placzek, 2006). Several lines of evidence suggest a role for Shh as an angiogenic stimulus. First, Hochman et al. demonstrated endothelial cell response to Shh by measuring increased expression of Shh target genes, gli1 and patched 1 expressed in endothelial cells (Hochman et al., 2006). Second, a number of groups have shown that Shh induces migration of endothelial cells in *in vitro* via Boyden chamber and scratch (wound) assays (Fu et al., 2006; Hochman et al., 2006; Kanda et al., 2003). In humans, Shh is expressed in subcapsular and medullary thymic epithelium and therefore may be involved in attracting endothelial cells into the thymus (Sacedon et al., 2003). Bmp4 is another important developmental signaling molecule, expressed by the early thymic primordium and implicated in blood vessel development. Lui et al. demonstrated a role for Bmp4 signaling in the outflow tract (OFT) myocardium in mice via conditional ablation of Bmp4 by using the Nkx2.5Cre (Liu et al., 2004). This resulted in defects in branching of the outflow tract and brachial arch artery morphogenesis.

Another recent study elucidated a role for Bmp4 signaling in endothelial cell maturation, pericyte recruitment and vessel remodeling by using the endothelial cell specific FLK1Cre to knockout the BMP4 receptor Bone morphogenetic protein receptor 1A signaling is dispensable for hematopoietic development but essential for vessel and atrioventricular endocardial cushion formation (Park et al., 2006). These results suggest that Bmp4 has a direct effect on endothelial cells.

Here we show that conditional deletion of *Vegf-A, Bmp4*, and *Shh* in the thymic epithelium, using our previous generated Foxn1Cre resulted in no obvious defects in initial thymus colonization by endothelial progenitor cells. Further, no subsequent defects in the formation of the thymus network in postnatal thymi were observed. Surprisingly, vascularization of the thymus in mice with both TEC and neural crest *Vegf-A* deleted produced similar results.

Results

Initial thymus vascularization is normal mice deficient in TEC and NCC VEGF-A

Our lab previously showed that initial thymic vascularization was *Foxn1* dependent. In these mice, which carry a hypomorphic allele of *Foxn1,* reduced *VEGF-A* expression coincided with the observed vascular defects. VEGF-A was expressed throughout the thymic compartment in thymic epithelial cells, neural crest cells, endothelial cells, and possibly in the hematopoietic cell population. We were therefore interested in testing the direct requirement for VEGF-A in the thymic epithelium during thymus ontogeny. To directly test whether VEGF-A in thymic epithelial cells was necessary for early and subsequent stages of thymus vascularization, we deleted VEGF-A in TECs using our previously described Foxn1Cre strain (Gordon et al., 2007).

We assayed for the endothelial cell marker CD31, and neural-crest marker PDGFR-β at E13.5, when vascularization initially occurs in mouse thymus and at E15.5. Initial vascularization of the thymus appeared normal, as CD31+ cells were detected within the thymus, (3.1b) and the organ was well vascularized with PDGFR-β^+ cells (3.1e-f) associated with most vessels by E15.5.

Since $CD31^+$ cells were detected in the pericapsular region in Foxn1Δ and control littermate thymi, we reasoned that neural crest mesenchyme in the capsule was involved in recruitment of endothelial cells and nascent blood vessels to the periphery of the thymus via VEGF-A. To test this hypothesis, we deleted *VEGF-A* in the neural crest. We observed normal distribution of endothelial cells at the periphery of the thymic capsule and within the rudiment in VEGF-$A^{flx/flx}$Wn1Cre+ mice (1c-g). Simultaneous deletion of VEGF-A in TECs and thymic mesenchyme resulted in no obvious thymus specific vascular defects (1d-h).

Basement membrane deposition is normal in mice deficient in TEC and NCC VEGF-A

Components of the basement membrane are expressed and deposited by endothelial cells in the thymus (Khoshnoodi et al., 2008). Collagen IV is a major constituent of the basement membrane and delineates the perivascular space, where mature T-cells accumulate during entry into and egress from the thymus (Khoshnoodi et al., 2008). Several reports suggest that reduced VEGF-A expression results in an increase empty collagen bed, described as CD31- Collagen IV^+ structures (Baluk et al., 2005; Mancuso et al., 2006). We observed normal collagen IV deposition at E13.5 in thymi with VEGF-A deleted in thymic epithelial cells (3.2b), neural crest (3.2c), as well

as thymi with VEGF-A deleted in both cells types (3.2d), when compared with stage matched littermate control thymi (3.2a). In all instances, in E13.5 thymi, most collagen IV was predominantly restricted to blood vessels and comparable to control thymus (3.2a). Collagen IV was more broadly expressed throughout the thymus at E15.5 in VEGF-A conditional knockouts, but comparable to control thymus (2e-h). These results suggest that VEGF-A deletions in TECs and NCCs did not result in noticeable vascular defects.

TEC-derived BMP4 is dispensable for initial and subsequent thymus vascularization

BMP4 is expressed in the early thymic epithelium and has been implicated in vascular development including, but not limited to endothelial cell tube formation, endothelial cell migration, and invasion of tissues (Gordon et al., 2010; Park et al., 2006; Patel et al., 2006; Rothhammer and Bosserhoff, 2007). We therefore performed a conditional deletion of *Bmp4* in TECs to test whether there was a requirement of TEC expression for colonization of endothelial cells into the thymus. We observed endothelial cells and some PDFGRβ^+ cells within the thymus and within the capsule the E13.5 thymus in Bmp4$^{ko/flx}$Foxn1Cre$^+$ mice (3.3b) and control littermates (3.3a). The distribution of CD31$^+$ cells with associated thymic mesenchyme was relatively normal (3.3c-d). To determine whether this mutation altered the postnatal thymus vasculature structure and TEC compartment formation, we assayed for the basement membrane structural component, collagen IV. These data show a stereotypical arrangement of highly branched cortical vascular structures with larger vascular support structures in the medulla of Bmp4$^{ko/flx}$Foxn1Cre$^+$ and control thymi (3.4a-c).

Thymus compartment formation was unaffected by TEC specific deletion of Bmp4 as a clear demarcation was observed between β-5t^{+} cTECs and UEA-1^{+} mTECs (5a-b). These data suggest that Bmp4 is dispensable for initial and subsequent thymus vascularization and organization of cortical and medullary epithelial cell subsets.

The morphogen Shh has numerous pleiotropic functions in development such as patterning of limbs, Several groups have demonstrated the presence of Hedgehog signaling pathway components in the thymus, the role of Shh in the thymus is controversial (Crompton et al., 2007; El Andaloussi et al., 2006; Gao and Aifantis, 2009; Gao et al., 2009; Hager-Theodorides et al., 2009; Hofmann et al., 2009; Varas et al., 2003). Recent reports suggest Shh functions in the development of the coronary vasculature (Lavine et al., 2008a; Lavine et al., 2008b; Lavine and Ornitz, 2007; Lavine and Ornitz, 2008; White et al., 2007). To directly test whether TEC-derived Shh is required for thymus vascularization, we deleted *Shh* from TECs with the Foxn1Cre strain. Our results show normal thymus vasculature in Shh$^{flx/flx}$;Foxn1Cre^{+} mice at E16.5 (3.6a-b) and 6 months (3.6c-d) was normal with CD31^{+} vascular structures throughout the thymus. TEC differentiation and compartment formation was normal as assessed by the cortical TEC marker β-5t, and the medullary TEC marker, UEA-1 in Shh$^{flx/flx}$;Foxn1Cre^{+} thymi at E16.5 (3.6a-b) and 6 months (3.6c-d). These results suggest that TEC-derived Shh is not required for thymus vasculature development.

Materials and Methods

Mice

VEGF-A$^{fx/flx}$ mice were provided by Napoleon Ferrara (Genentech Inc.). Brigid Hogan provided Bmp4LacZ and Bmp4$^{flx/flx}$ mice. Shh$^{fx/flx}$ and Wnt1Cre mice were purchased from The Jackson Laboratory (Bar Harbor, ME). VEGF-A$^{flx/flx}$, Bmp4LacZ, Bmp4$^{flx/flx}$, Shh$^{flx/flx}$ mice were genotyped as previously described in (Gerber et al., 1999; Lewis et al., 2001; Mukouyama et al., 2005).

To generate VEGF-A$^{fx/flx}$Foxn1Cre^{+}; Wnt1Cre^{+} mice, Foxn1Cre (Gordon et al., 2007) and Wnt1Cre mice (Danielian et al., 1998) strains were each crossed to the VEGF-A$^{fx/flx}$ strain. VEGF-A$^{fx/flx}$;Foxn1Cre mice and VEGF-A$^{fx/flx}$;Wnt1Cre mice were crossed together, resulting in the VEGF-A$^{fx/flx}$;Foxn1Cre^{+}; Wnt1Cre^{+} strain. Bmp4$^{ko/flx}$Foxn1Cre mice were generated as previously described (Gordon et al., 2010).

Immunofluorescence

Embryos were flash frozen in liquid nitrogen and cryosectioned at (10µm). The following primary antibodies were used for the embryonic thymus immunofluorescence analysis (Liu et al., 2006; Su et al., 2003): monoclonal rat CD31 goat PDGFR-b (1:50), mouse cytokeratin (Sigma, 1:400), rabbit Collagen IV (1:400). The following secondary antibodies were purchased from Jackson Immunology: α-mouse CY3, α-rat 649, α-rat 488 α-goat 488, α-rabbit CY3, and SA-649. After final wash, Gel/Mount mounting medium (biomeda corp.) and cover slips were added. Vibratome sectioned samples were processed as previously described in (Bryson et al., 2011). Images were collected and processed using a Zeiss Axioplan microscope and AxioVision 4.8 software.

Discussion

Here, we tested the requirement for several candidate growth factors in embryonic and postnatal thymus vascularization. These molecules were expressed in the early thymic epithelium and have been reported to play a role in both vasculogenesis and angiogenesis. We show that thymic epithelium-derived Vegf-A, Bmp4, and Shh, as well as neural crest mesenchyme derived VEGF-A are dispensable for initial and subsequent vascularization in embryonic and adult mouse thymus. Our data do slightly conflict with reports suggesting that VEGF-A is required for thymus blood vessel network formation (Muller et al., 2005). Muller et al., deleted VEGF-A in the epithelium using nude blastocysts chimeras (Muller et al., 2005). In this experiment, embryonic stem cells with wild-type Foxn1, and null for the VEGF-A gene were injected into blastocyts that were null for Foxn1, but wild-type for VEGF-A (Muller et al., 2005). This resulted in the generation of mice with a functional thymus, which lacked the VEGF-A gene (Muller et al., 2005). Therefore, VEGF-A was never expressed by the epithelium, which likely limited the number of endothelial cells in the thymus. This may have also limited the number of VEGF-A expressing cells, recruited into the thymus by the endothelium, i.e. neural crest and non-neural crest derived mesenchyme, which express high levels of VEGF-A (Muller et al., 2005). Given that our Foxn1Cre turns on in the third pouch pharyngeal pouch at E11.5, there may already be enough VEGF-A in the thymus to drive initial vascularization. Further, in postnatal thymi, it is probable that other VEGF-A expressing cells compensate for the loss of VEGF-A in TECs. In a recent study by Cuddihy, et al., VEGF-A was inhibited in the postnatal thymus by intraperitoneal injection of a VEGF-A Trap (Cuddihy et al., 2009). This treatment

resulted in the inhibition of VEGF-A throughout the neonatal thymus, and was therefore not restricted to a specific cell type, such as thymic epithelial cells (Cuddihy et al., 2009). Therefore, as expected, this systemic inhibition of VEGF-A resulted in more severe vascular defects, in addition to reduction in overall T-cell and thymus cellularity (Cuddihy et al., 2009).

We were a bit surprised by the lack of an initial delay in thymus vascularization in our neural crest specific *VEGF-A* deletions ($VEGF^{flx/flx}$; Wnt1Cre) given the dramatic delay in initial vascularization of dorsal root ganglion (DRG), and skin vascular defects in these mice (Mukouyama et al., 2005). This may be a result of the DRG being totally neural crest derived, so *VEGF-A* is deleted throughout the tissue, whereas in the thymus, only the thymic capsule and perivascular cells are neural crest-derived (Foster et al., 2008; Mukouyama et al., 2005; Muller et al., 2008). We were equally surprised that simultaneous deletion of *VEGF-A* in the thymic epithelium and neural crest failed to reveal vascular defects. Again, the timing of our Foxn1Cre deletion of *VEGF-A* in the epithelium may not be early enough to effectively delete *VEGF-A* before it acts in the initial recruitment of endothelial cell progenitors. In the future, it may prove useful to repeat these experiments using an endoderm specific Cre-recombinase strain such as the tamoxifen-inducible FoxA2Cre (Park et al., 2008). This deletion would theoretically result in loss of *VEGF-A* throughout the endoderm, including the third pouch and its derivatives, thymus and parathyroid. In the future, this strategy may be employed for *Shh*, *Bmp4*, and other TEC-derived molecules to elucidate the role of these genes in thymus vascularization

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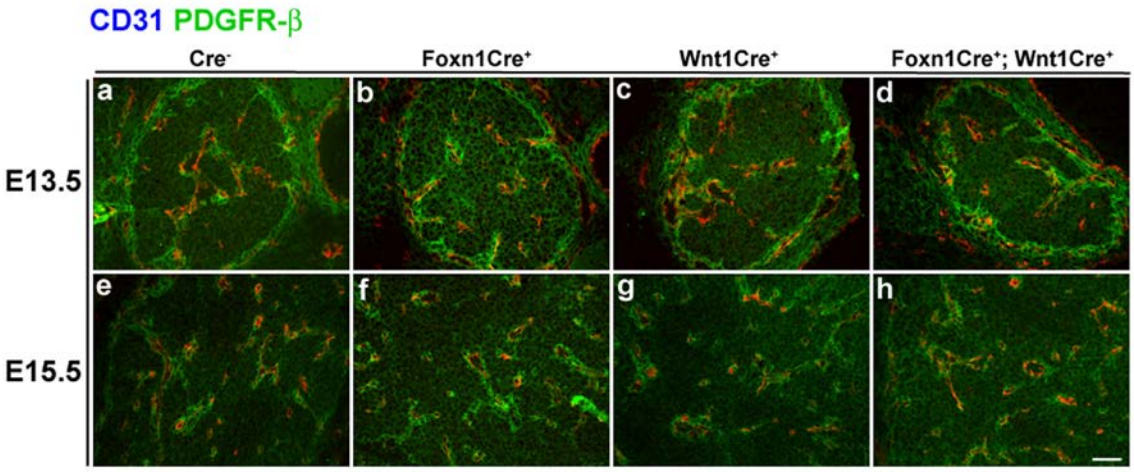
CD31 PDGFR-β
Cre-
Foxn1Cre+
Wnt1Cre+
Foxn1Cre+; Wnt1Cre+
E13.5
E15.5
a
b
c
d
e
f
g
h

Figure 3.1

Figure 3.1: Initial embryonic thymus vascularization is normal in TEC and NCC specific *VEGF-A* deletion.

Immunohistochemical analysis of frozen transverse sections on fetal thymus at E13.5 **(a-d)** and E15.5 **(e-h)**. Endothelial and NCC cell markers CD31 (blue) and PDGFR-β (green). **(a and e)** VEGF-$A^{flx/flx}$, **(b and f)** VEGF-$A^{flx/flx}$;$Foxn1Cre^{+}$, **(c and g)** VEGF-$A^{flx/flx}$;$WntCre1^{+}$, **(d and h)** VEGF-$A^{flx/flx}$;$Foxn1Cre^{+}$,$Wnt1Cre^{+}$ Scale bar = 50μm

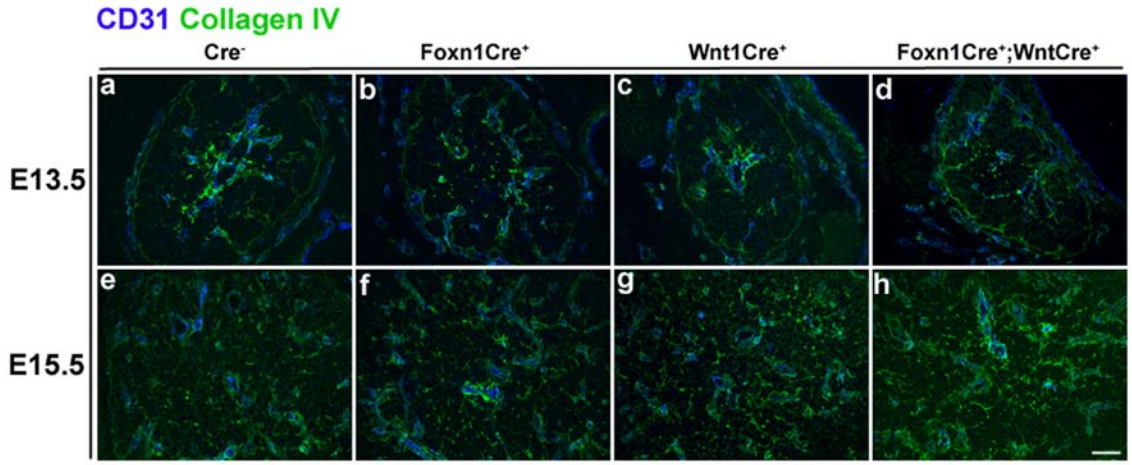
CD31 Collagen IV
Cre-
Foxn1Cre+
Wnt1Cre+
Foxn1Cre+;WntCre+
E13.5
E15.5
a
b
c
d
e
f
g
h

Figure 3.2

Figure 3.2: Collagen IV deposition is normal embryonic thymus of mice with. TEC and NCC specific *VEGF-A* deletion. Immunohistochemical analysis of frozen transverse sections on fetal thymus at E13.5 **(a-d)** and E15.5 **(e-h)**. Endothelial and NCC cell markers CD31 (blue) and Collagen IV (green). **(a and e)** VEGF-$A^{flx/flx}$, **(b and f)** VEGF-$A^{flx/flx}$;$Foxn1Cre^{+}$, **(c and g)** VEGF-$A^{flx/flx}$;$WntCre1^{+}$, **(d and h)** VEGF-$A^{flx/flx}$;$Foxn1Cre^{+}$,$Wnt1Cre^{+}$ Scale bar = 50μm

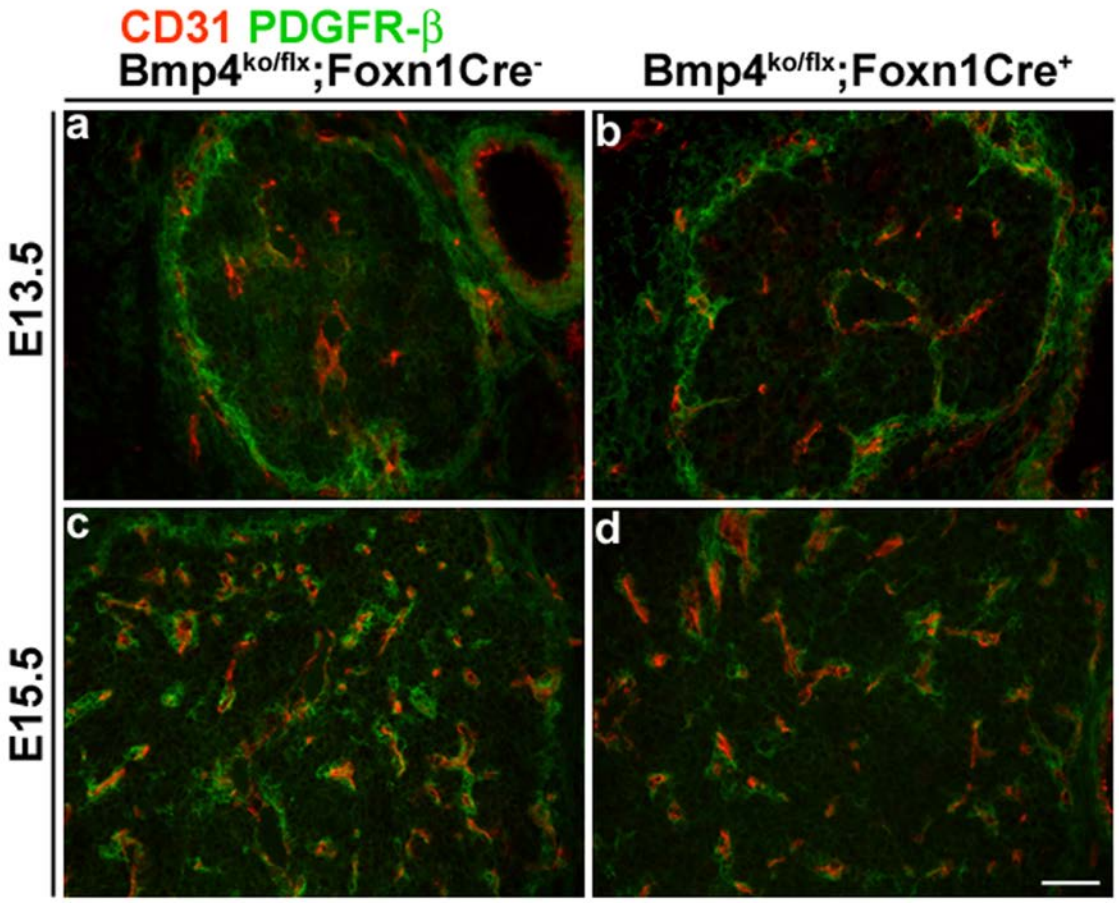
CD31 PDGFR-β
Bmp4ko/flx;Foxn1Cre-
Bmp4ko/flx;Foxn1Cre+
E13.5
E15.5
a
b
c
d

Figure 3.3

Figure 3.3: Initial embryonic thymus vascularization is normal in TEC and NCC specific *Bmp4* deletion. Immunohistochemical analysis of frozen transverse sections on fetal thymus at E13.5 **(a-b)** and E15.5 **(e-d)**. Endothelial and NCC cell markers CD31 (**red**) and PDGFR-β (**green**). **(a)** $Bmp4^{ko/flx}$;Foxn1Cre- **(b)** $Bmp4^{ko/flx}$;Foxn1Cre-$^{+}$ **(c)** $Bmp4^{ko/flx}$;Foxn1Cre^{-} **(d)** $Bmp4^{ko/flx}$;Foxn1Cre^{+} Scale bar= 50μm

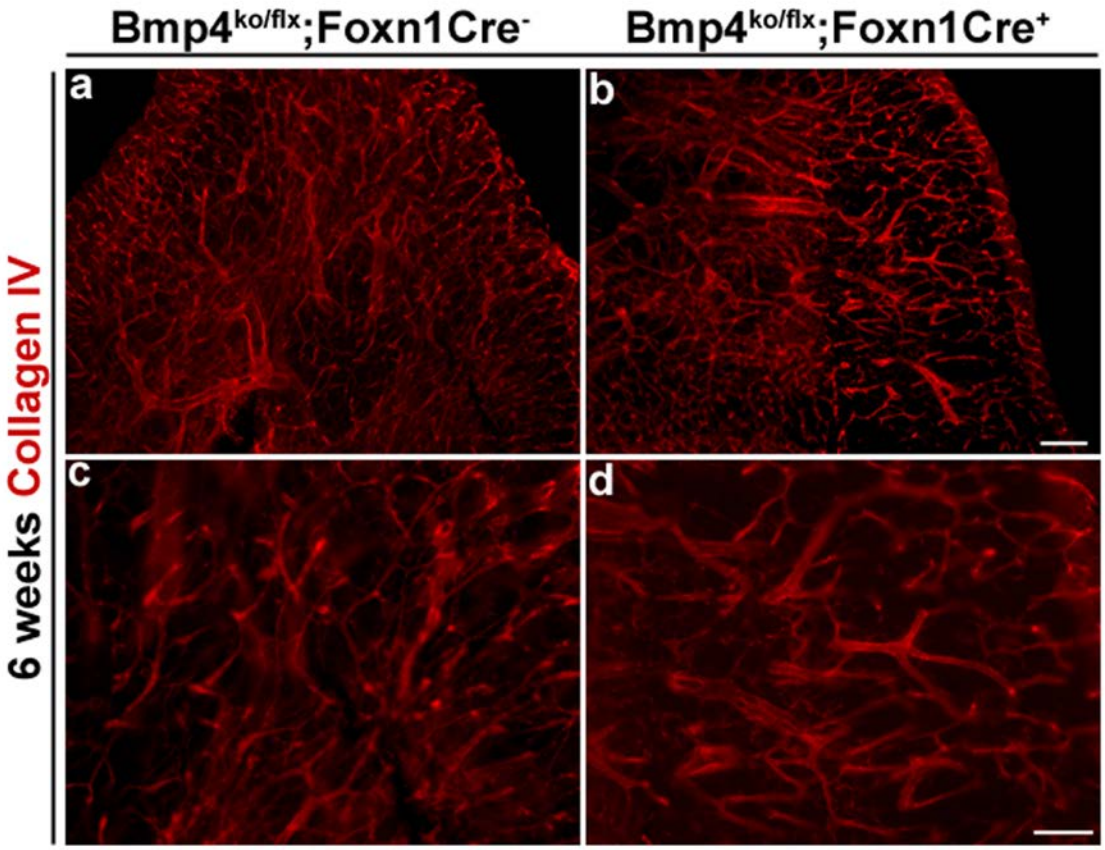
Bmp4ko/flx;Foxn1Cre-
Bmp4ko/flx;Foxn1Cre+
6 weeks Collagen IV
a
b
c
d

Figure 3.4

Figure 3.4: Collagen IV^{+} vascular structures normal in adult thymus of mice with TEC specific *Bmp4* deletion. Immunohistochemical analysis of 100μm transverse sections adult thymus at 6 weeks. Collagen IV (**red**) shows vascular basement membrane structure. **(a)** Bmp4$^{ko/flx}$;Foxn1Cre- **(b)** Bmp4$^{ko/flx}$;Foxn1Cre^{+}, **(c)** Bmp4$^{ko/flx}$;Foxn1Cre- **(d)** Bmp4$^{ko/flx}$;Foxn1Cre-$^{+}$ **(a-b)** Scale bar = 100μm **(c-d**) Scale bar = 50μm

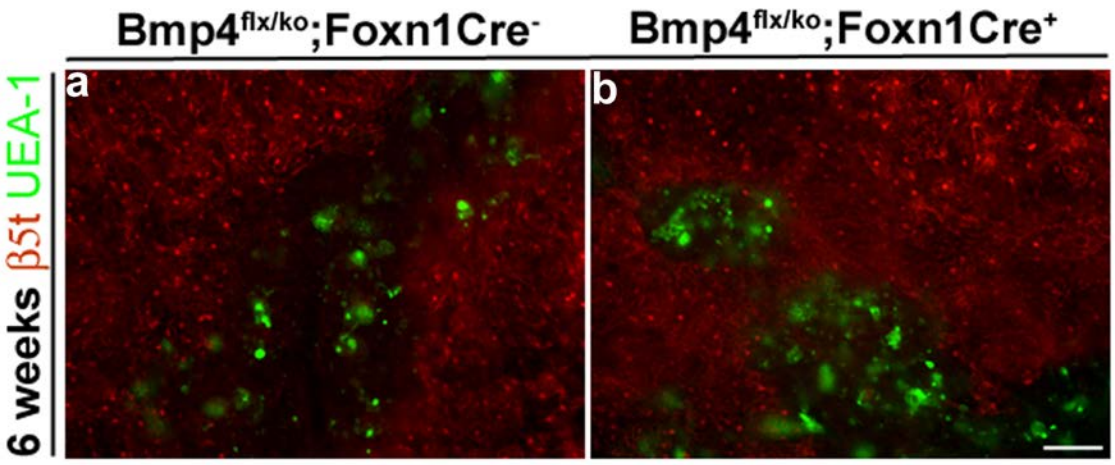

Figure 3.5

Figure 3.5: Thymic epithelial cell compartment formation of adult thymus is normal mice with TEC and NCC specific *Bmp4* deletion. Immunohistochemical analysis of 100μm transverse sections of adult thymus at 6 weeks. **(a)** $Bmp4^{ko/flx}$;Foxn1Cre- **(b)** $Bmp4^{ko/flx}$;$Foxn1Cre^{+}$, Scale bar = 50μm

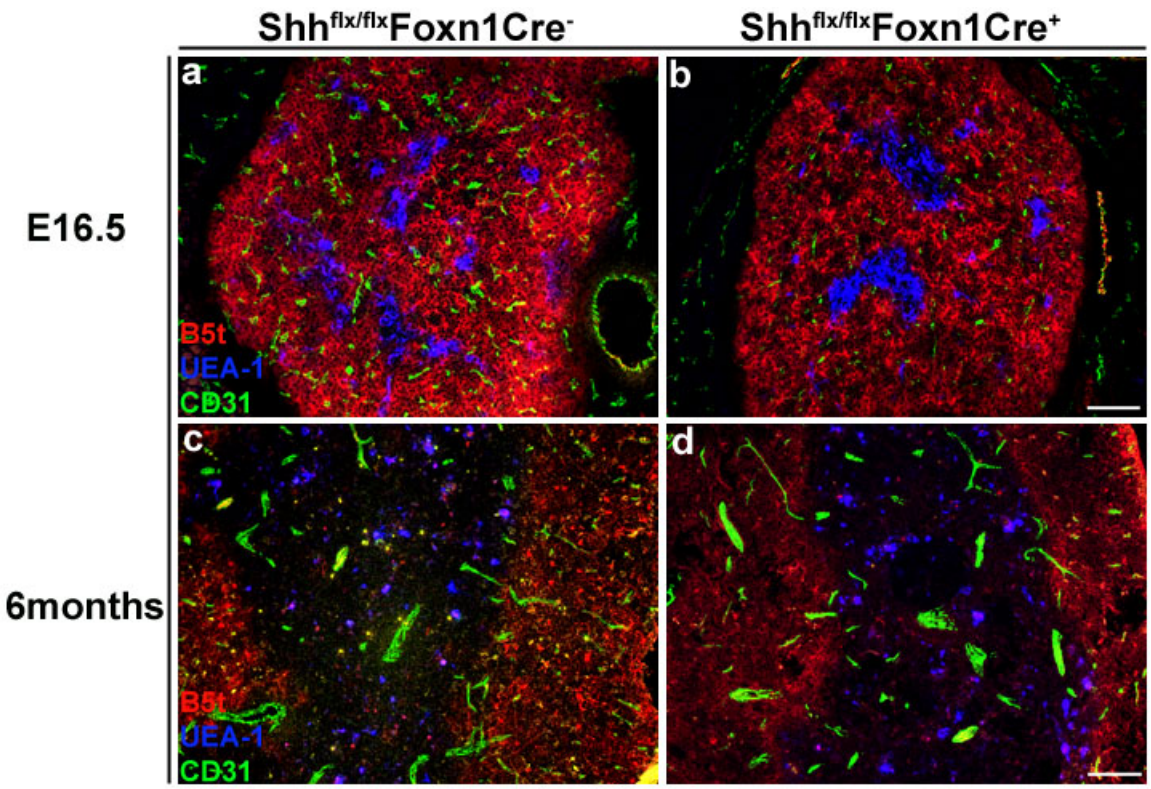
Shh^flx/flx Foxn1Cre-
Shh^flx/flx Foxn1Cre+
E16.5
6months
a
b
c
d
B5t
UEA-1
CD31
B5t
UEA-1
CD31

Figure 3.6

Figure 3.6: Thymus vascularization is normal in mice with TEC specific *Shh* deletion. Immunohistochemical analysis of frozen transverse sections for the endothelial cell marker, CD31 (green) and TEC markers β-5t (red) and UEA-1 (blue). **(a)** $Shh^{flx/flx}$;Foxn1Cre- **(b)** $Shh^{flx/flx}$;Foxn1Cre-$^{+}$ **(c)** $Shh^{flx/flx}$;Foxn1Cre^{-} **(d)** $Shh^{flx/flx}$;Foxn1Cre^{+} Scale bar= 50μm

CHAPTER 4

CONCLUSIONS AND DISSCUSSION

In this dissertation we have sought to define roles for the thymus and skin specific transcription factor, Foxn1 and candidate growth factors in thymus vascularization. We largely employed a genetic approach to dissect out the role of Foxn1 in this process. Our data show that *Foxn1* expression in TECs is necessary to attract both circulating endothelial cells and branching blood vessels into the embryonic thymus. By combining the wild-type, *Foxn1*Δ, and *Foxn*1 nude alleles, our data further established a dose specific requirement for Foxn1 in initial thymus vascularization. These data also infer a possible role for Foxn1 in regulating the expression of TEC-derived cross-talk factors that endothelial cells, nascent blood vessels, and mature vessels in the postnatal thymus presumably respond to. We identified VEGF-A as a candidate TEC derived growth factor involved in initial thymus vascularization, since its reduced expression correlated with reductions in *Foxn1*. Additionally, we selected BMP4 and Shh, as candidate molecules since their expression had been reported in the embryonic thymus and several studies have implicated them in vascular development.

Preliminary results from *Foxn1*$^{\Delta/\Delta}$ embryonic thymus vascular phenotypes suggested the following model for initial thymus vascularization (Figure 4.1-4.4): Neural crest-derived mesenchyme surrounding the embryonic thymus secretes signals that attract endothelial progenitor cells to the periphery of the thymus. Once at the periphery

of the thymus endothelial progenitor cells begin to immigrate into the thymus via thymic epithelial cell-derived signals. After entering the thymus, endothelial progenitor cells signal to both thymic epithelial and neural crest-derived cells. Signaling to thymic epithelial cells contributes to their differentiation, and signaling to neural crest-derived cells results in their immigration into the thymus. Neural crest-derived cells then surround nascent blood vessels thereby stabilizing them. Once blood vessels begin to mature, 'cross-talk' between thymic epithelial cells and nascent blood vessels is mediated via neural crest-derived cells surrounding blood vessels. Furthermore, we hypothesized that this crosstalk might be required for further differentiation of thymic epithelial cells. We tested specific aspects of this model using a thymic epithelial cell specific Cre recombinase to delete *VEGF-A*, *BMP4*, and *Shh*. Additionally, we employed a neural crest specific Cre to delete *VEGF-A*. We observed no obvious defects in initial or subsequent thymus vascularization in these mutants. These data therefore suggest that these genes, in the respective cellular subsets were dispensable for initial thymus vascularization. It is also possible that Foxn1 regulates the expression of a number of pro-angiogenesis factors and deletion of a single TEC derived molecule is not sufficient to result in dramatic vascular defects.

Several lines of evidence suggested that *VEGF-A* was essential for establishment and maintenance of blood vessels in the thymus during development and in adults. Carmeliet's group investigated the effects of expressing *single vascular endothelial growth factor-A* (*Vegf-A*) isoforms *120*, *164* and *188* in mice and reported a hypoplastic thymus phenotype in $Vegf^{120/120}$ mice, compared with wild-type littermates (Stalmans et al., 2003). This hypoplastic phenotype is suggestive of a failure in thymic

epithelial cell differentiation and subsequent proliferation. In a previous study by Muller et al. loss of *VEGF-A* in TECs resulted in reduced vessel branching, loss of the dense cortical capillary network, but thymus size was normal, and VEGF-A persisted in a subset of thymic neural crest mesenchyme.

In our model *VEGF-A* was deleted in TECs, mesenchyme, and in both cell populations in the same animal. We observed no defects in thymus microvasculature in our model of *VEGF-A* loss in TECs and NCCs. This result is not entirely surprising since *VEGF-A* is a ubiquitously expressed molecule and its expression is differentially regulated in various cell types. As it relates to thymus development, *VEGF-A* was strongly expressed in the pharyngeal endoderm prior to thymus formation (Miquerol et al., 1999). Therefore, Foxn1Cre which turns on at ~E11.25 likely deletes *VEGF-A* late, so dramatic defects in initial thymus vascularization were not observed (Gordon et al., 2007). This may also account for the lack of vascular phenotypes in our *Bmp4* conditional knockouts in TECs, although there may be redundancy in BMP signaling in the thymus. It may be useful to repeat these experiments with the an endoderm specific Cre strain such as *FoxA2Cre*, which will allow deletion of genes in cells fated to become thymic epithelial cells (Park et al., 2008). Since, cells colonizing the thymus express *VEGF-A*, in addition to TECs, deletion of *VEGF-A* in TECs alone may insufficient to observed vascular defects. An alternative interpretation is that TECs, and thymic stromal cells express a number of pro-angiogenesis molecules, and these other TEC and/or stromal cell derived pro-angiogenesis factors may compensate for the loss of *VEGF-A* in TECs. Although a recent study showed a requirement for neural crest derived VEGF-A in initial vascularization of the dorsal root glanglion, our conditional

deletion of *VEGF-A* in the thymic neural crest did not cause dramatic defects in thymus vascularization (Mukouyama et al., 2005). The key difference between thymus and dorsal root ganglion is that the thymus is an endoderm-derived organ with neural crest comprising the thymic capsule and perivascular cells, a minor percentage of the total thymus cell population (Foster et al., 2008; Gordon et al., 2004; Muller et al., 2008). Conversely, the dorsal root ganglion is entirely neural crest derived and was the main source of VEGF-A, which accounts for the disparity in phenotypes between these mice (Anderson, 2000).

The signals that induce the initial expression of *Foxn1* after *HOXA3* establishes positional identity of thymus in pharyngeal pouch endoderm are unknown. *Bmp4*, *Wnts*, and *Shh* have been implicated as possible upstream inducers of Foxn1 expression. However, the initial organ inducing signals have been elucidated for several other endoderm-derived organs, the liver and pancreas. In both instances, elegant experiments have revealed a requirement for signals from endothelial cells and nascent blood vessels in inducing pancreas bud formation and the expression of There have been reports demonstrating endothelial cell expression of a number of developmental signaling molecules such as BMP2, 4, and 7 for example. It is therefore possible that the pharyngeal arch arteries play a role in inducing Foxn1 for example.

As previously discussed the thymus vasculature has been proposed to be an organizer of medullary epithelial cells (Anderson et al., 2000). We have shown here numerous examples of thymic epithelial cells in close proximity to blood vessels. On occasion, we have observed TECs physically wrapped around vascular structures. These observations obviously support the notion of TEC-endothelium crosstalk, but also

raise the question of whether a vascular niche exists in the thymus. Further, if one exists, what cells are supported by it, and is it required for differentiation and/proliferation of any given resident, or migratory thymus cellular subset? In the thymus, several crosstalk mechanisms between T-cells and the vasculature, and between T-cells and thymic neural crest have been defined. Therefore, by Lammert et al.'s definition of a vascular niche, "a microenvironment that is generated by endothelial cells and/or mural cells and that affects adjacent cells" a vascular niche does indeed exist in the thymus. Post-capillary venules within the medulla, and at the cortical-medullary junction are likely the anatomical location of these proposed vascular niches.

In order to begin defining possible influences of the thymus vascular endothelium on TECs, we will likely have to incorporate a cell and organ culture system to complement our studies in mice. Here, in attempting to define TEC-endothelium crosstalk mechanisms, as they relate to TEC derived signals, we have relied heavily on a conditional genetic approach. However, there are considerable of drawbacks to applying this approach to define endothelium-derived signals influencing TECs. The most apparent drawback is that deletions in endothelial cells are systemic and may result in a variety of phenotypes, thereby complicating analyses. Inducible conditional deletions in endothelial cells may be employed as an alternative, but are best suited for defining the cell autonomous roles of a gene within endothelial cells themselves.

TECs display a tremendous amount of diversity in gene expression and function, which has prompted their classification into "seven functionally defined signaling zones" (Griffith et al., 2009; Lind et al., 2001; Petrie and Zuniga-Pflucker, 2007). The most obvious distinction among TECs is their demarcation into cortical and medullary

subsets. This compartmentalization of epithelial cells is functionally relevant as cortical and medullary epithelial cells differentially influence T-cell proliferation, differentiation, and lineage specification, and are critical for positive and negative selection, respectively. Several recent studies suggest that Foxn1 expression in these zones is both spatially and temporally regulated (Gordon et al., 2007). In *Foxn1LacZ* mice, which were generated by insertion of a LacZ cassette into the 3'UTR of *Foxn1*, and thereby provides a read out of endogenous *Foxn1* expression, *Foxn1* levels were high in the medulla and relatively low in the cortex. This observation is intriguing for several reasons. Firstly, our data and others have demonstrated that the thymic cortex is capillary dense, and its establishment and maintenance is VEGF-A dependent (Cuddihy et al., 2009; Kato, 1997; Muller et al., 2005; Raviola and Karnovsky, 1972). In addition, we've shown here, in a *Foxn1* allelic series, that *VEGF-A* expression is reduced with concomitant reductions in *Foxn1* dose. Therefore, it would be useful to determine how Vegf-A expression correlates with *Foxn1* expression in the postnatal thymus, using the *Foxn1LacZ* and *VEGFA-LacZ* strains. We predict, high VEGF-A expression in the cortex with lower expression in the medulla. This hypothesis is supported by previous studies in which VEGF-A was genetically or therapeutically ablated in the thymus (Cuddihy et al., 2009; Muller et al., 2005). In these experiments, disruption of *VEGF-A* resulted in loss of the dense cortical capillary network, reminiscent of our postnatal Foxn1Δ thymus vascular defects. These observations underscore a likely influence of TEC diversity in generating blood vessels of diverse morphology and function throughout the thymus.

Kato et al. previously described the thymus microvasculature in both normal and aged mice. The work presented here extends our current understanding of factors involved in the establishment of the elaborate network. However, it is worth revisiting the subject of thymus microvasculature during involution with modern tools. Given that our lab has several ongoing aging studies, it would be informative to add vascular markers to these analyses. For example, future studies on thymus microvasculature in *Foxn1Lacz* mice, which prematurely down-regulate *Foxn1*, and initiate involution 2 weeks after birth, in conjunction with modern techniques may be useful for elucidating vasculature function and its influence on the hematopoietic and stromal cell compartments during aging. Our current hypothesis is that the cortical thymic epithelial cell network collapses in these mice as a result of *Foxn1* reduction/loss, and subsequent reductions in TEC-derived crosstalk factors required for microvasculature. We propose several models for crosstalk in the thymus in Figures 4.1-4.4 and we are eager to test specific aspects of these models in the future.

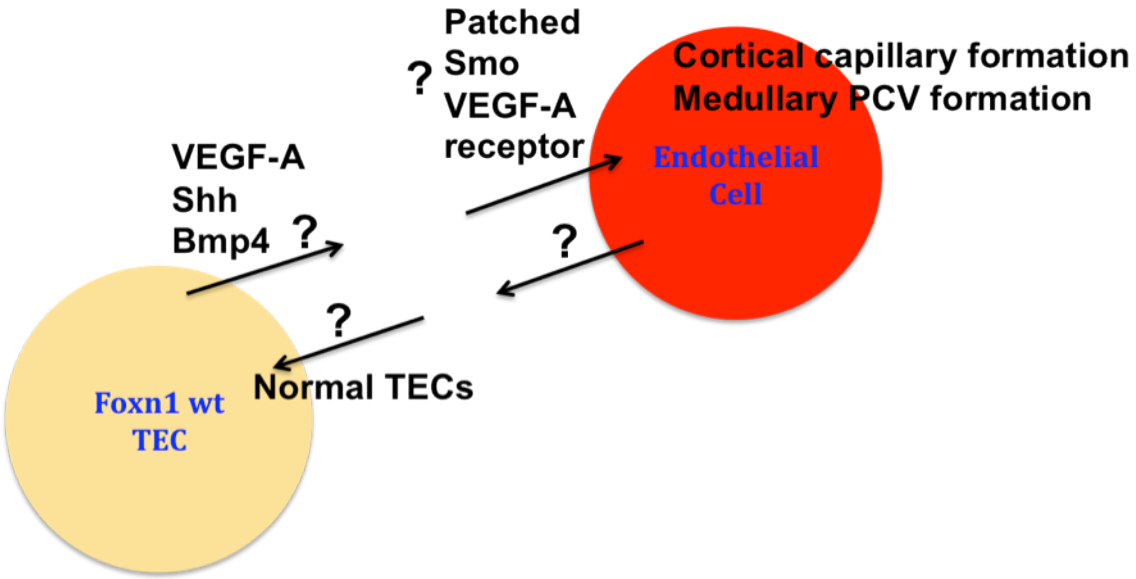
Patched
Smo
VEGF-A
receptor
?
Cortical capillary formation
Medullary PCV formation
Endothelial
Cell
VEGF-A
Shh
Bmp4
?
?
?
Normal TECs
Foxn1 wt
TEC

Figure 4.1

Figure 4.1: Model for TEC-Endothelial Cell Crosstalk

Our model suggests that TECs and thymic endothelium communicate via a number of signaling systems. In wild-type thymus, TECs engage in crosstalk with the endothelium, resulting in the recruitment and development of blood vessels adjacent to TECs. This results in a dense cortical vascular network with larger vessels in localized to the cortical medullary junction and medulla. Although, TECs secrete VEGF-A, Hh, and Bmp4, and the cognate receptors for these molecules are present on the endothelium, our data suggests that these factors may be dispensable for initial thymus vascularization. What signaling systems are involved in this process and whether endothelial cells signal back to TECs are open questions.

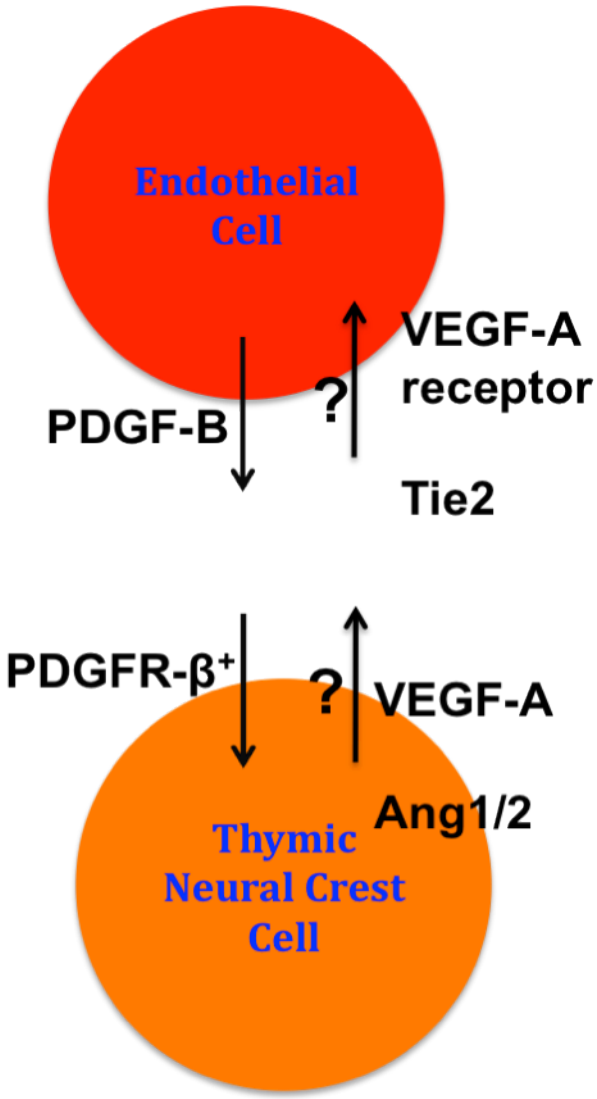
Endothelial Cell
VEGF-A receptor
?
PDGF-B
Tie2
PDGFR-β+
?
VEGF-A
Ang1/2
Thymic Neural Crest Cell

Figure 4.2

Figure 4.2: Model for Thymic NCC-Endothelial Cell Crosstalk

This model suggests that thymic neural crest and endothelial cells communicate via the classical VEGF-A/Flk-1, PDGF-B/PDGFR-β, and Tie2/Ang1/2 signaling systems. Endothelial cells express PDGF-B and the Tie2 receptor, and NCCs express PDGFR-β and Ang1/2 resulting in the modeling and maturation of a stereotypical thymus vascular network. NCCs further support the endothelium via VEGF-A signaling, which may function as a NCC-derived paracrine survival factor.

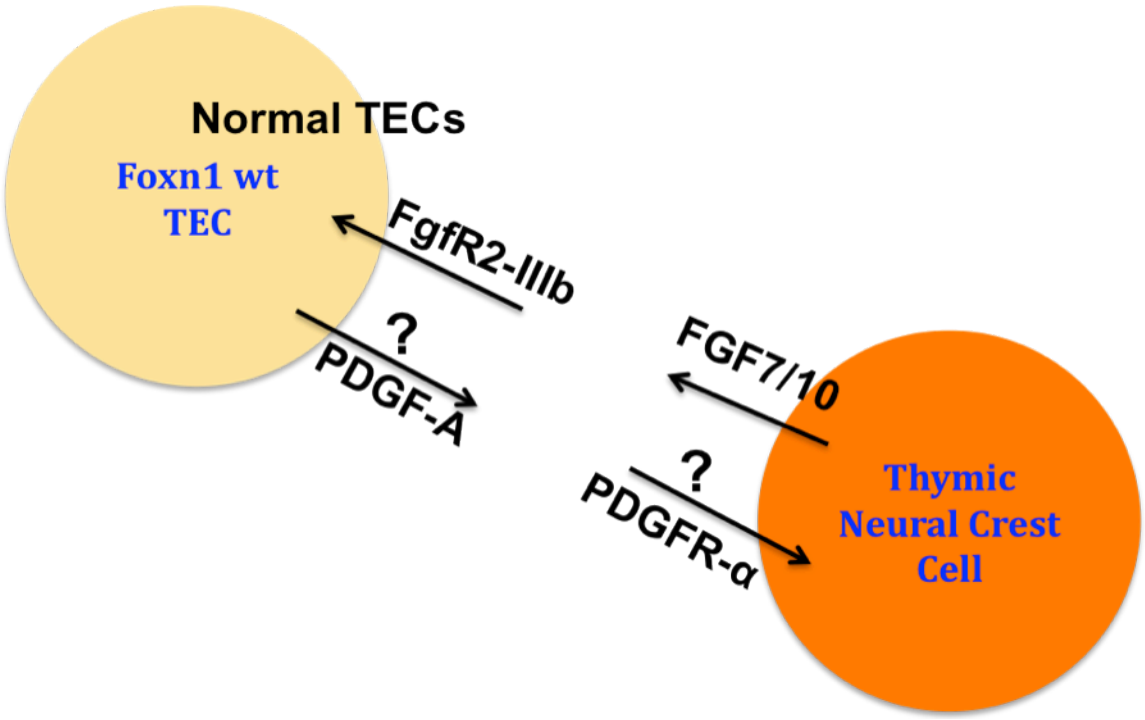
Normal TECs
Foxn1 wt
TEC
FgfR2-IIIb
?
PDGF-A
FGF7/10
?
PDGFR-α
Thymic
Neural Crest
Cell

Figure 4.3

Figure 4.3: Model for TEC-Thymic NCC Crosstalk

This model suggests that PDGFR-α^+ NCCs are recruited to the thymus via PDGF-A secreted by TECs. In addition NCCs secrete Fgf7/10, which are required for proliferation of FgfR2-IIIb$^+$ TECs. This crosstalk is initiated prior to thymus vascularization, and may be required for TEC homeostasis at later embryonic stages. In addition, a subset of PDGFR-α^+ NCCs function as perivascular mesenchyme. Whether PDGF-A is involved in TEC-NCC crosstalk is unresolved.

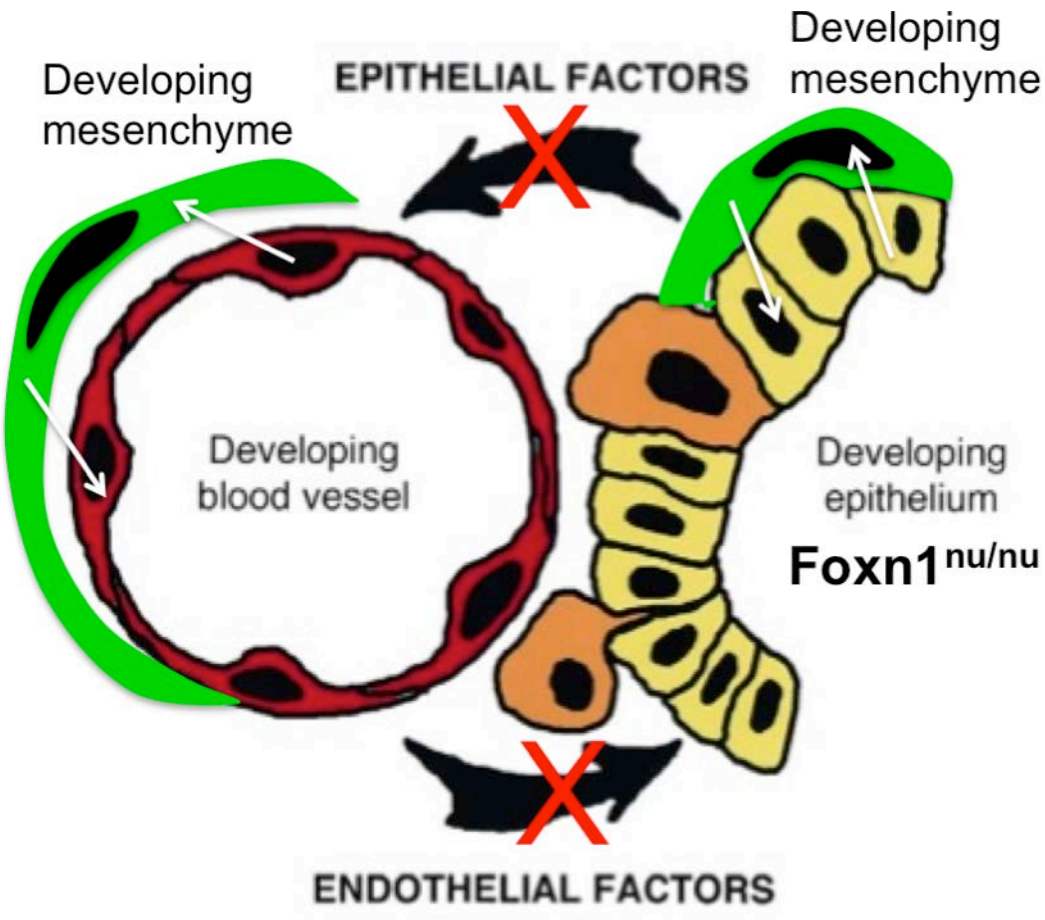
Developing mesenchyme
EPITHELIAL FACTORS
Developing mesenchyme
Developing blood vessel
Developing epithelium
Foxn1nu/nu
ENDOTHELIAL FACTORS

Figure 4.4

Figure 4.3 Model for Non Cell-Autonomous Defects in Foxn1 mutant thymi

This model suggests that loss or reduction in *Foxn1* levels in TECs results in a subsequent loss or reduction in TEC-derived crosstalk factors. This lack of TEC-derived signals disrupts NCC-endothelium crosstalk. Our data also suggests that TEC-NCC crosstalk may also be disrupted in *Foxn1* mutants.

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APPENDIX A

A METHOD FOR LABELING VASCULATURE IN EMBRYONIC MICE

Jerrod L. Bryson, Mark C. Coles, and Nancy R. Manley 2011. Submitted to the Journal of Visualized Experiments.

Short Abstract: This article describes a method for labeling embryonic skin and thymus blood vessels.

Long Abstract:

The establishment of a functional blood vessel network is an essential part of organogenesis, and is required for optimal organ function. For example, in the thymus proper vasculature formation and patterning is essential for thymocyte entry into the organ and mature T-cell exit to the periphery. The spatial arrangement of blood vessels in the thymus is dependent upon signals from the local microenvironment, namely thymic epithelial cells (TEC). Several recent reports suggest that disruption of these signals results in thymus blood vessel defects (Cuddihy et al., 2009; Muller et al., 2005). Previous studies have described techniques used to label the neonatal and adult thymus vasculature (Cuddihy et al., 2009; Muller et al., 2005). We demonstrate here a technique for labeling blood vessels in the embryonic thymus. This method combines the use of FITC-dextran or isolectin B4 facial vein injections and CD31 antibody staining to identify thymus vascular structures and PDGFR-β to label thymic perivascular mesenchyme (Foster et al., 2008; Liu et al., 2006; Muller et al., 2008). The option of using cryosections or vibratome sections is also provided. This protocol can be used to identify thymus vascular defects, which is critical for defining the roles of TEC-derived molecules in thymus blood vessel formation. As the method labels the entire vasculature, it can also be used to analyze the vascular networks in multiple organs and tissues throughout the embryo (Lavine et al., 2008; Lavine et al., 2006; Mukouyama et al., 2005; Mukouyama et al., 2002; Murphy et al., 2008).

Protocol

1. Fluorescein labeled dextran and GSL I-isolectin B4 facial vein injections to label embryonic vasculature. 2. Whole-mount analysis of skin vasculature. 3. Multi-color labeling of thymus vasculature and perivascular cells for cryosections. 4. Multi-color labeling of thymus vasculature and perivascular cells for vibratome sections. 5. Image Acquisition.

1. Fluorescein labeled dextran and GSL I-isolectin B4 facial vein injections to label embryonic vasculature.

1. Prepare FITC-dextran (50ug/mL) in PBS or GSL 1 – isolectin B_4 (20ug/200uL) in PBS in a 1.5mL Eppendorf tube and warm to 37^0C. Add 100uL of stock 1.25mM Fast Green/PBS to the FITC-dextran solution (total volume 1mL) and 180uL of stock 1.25mM Fast Green/PBS to the GSL 1 – isolectin B_4 (total volume 200uL), so that the solution is visibly blue.
2. Dissect E14.5-E18.5 embryos and yolk sac together, leaving the allantoic stalk (umbilical artery and vein) intact.
3. Transfer embryos to a new Petri dish (60 X 15 mm) and immerse them in PBS at room temperature.
4. Position the embryo to provide a sagittal view of the head/face. Use micro dissecting forceps (ROBOZ RS-5135) to gently grasp the embryo at the head.
5. Using a 30G needle, inject 50uL FITC-dextran (50ug/mL) or GSL 1 – isolectin B_4 (20ug in 200uL PBS) into the facial vein pointing the needle toward the back of the head.

6. When the dye is visible in the umbilical vein, remove the needle and separate the embryo from the allantoic stalk (umbilical artery and vein).
7. Following injections, allow embryo to remain in PBS at room temperature for 2-3 minutes so that the dye circulates throughout the embryo.

2. Whole-mount analysis of skin vasculature.

1. After allowing the dye to circulate throughout the embryo, remove skin samples from regions of the limbs, back, and stomach, etc. (Mukouyama et al., 2005; Mukouyama et al., 2002).
2. Wash skin sample in cold PBS, and fix in 4% PFA/PBS for 2 hours (Mukouyama et al., 2005; Mukouyama et al., 2002). Wash 3 times for 10 minutes each in 4mL clear vial (National Scientific) with 2mL cold PBS.
3. Place skin sample on a microscope slide (Superfrost) and add 100 μl of mounting media to each slide and a cover glass.
4. Allow slides to dry in a dark storage area.
5. Proceed to 'Image Acquisition' section.

3. Multi-color labeling of thymus vasculature and perivascular cells for cryosections.

1. 'Flash freeze' whole embryo in liquid nitrogen. Embryos can be and stored at -80^{0}C until analysis.
2. Alternatively, dissect out thymus, rinse in 4°C PBS, and fix in 2mL 4% PFA/PBS for 2 hours. Wash 3 times for 10 minutes in cold PBS, place thymi in OCT, and freeze and store until use at -80^{0}C.

3. For cryosectioning, spread OCT on a section 'block' and mount the embryo or dissected organs/tissues for sectioning.
4. Cut frozen tissue into 10 μm thick sections and collect on slides.
5. Fix sections in acetone for 5-10 minutes. Wash 3 times in cold TBS.
6. Block in 10% donkey serum/TBS in a humidity chamber at room temperature.
7. Incubate sections for 1 hour-overnight with 100 μl of primary antibodies in a humidity chamber at 4^0C: in this example, we use rat anti-mouse CD31 (1:100) to label endothelium, and goat anti-mouse PDGFR-β (1:100) to label perivascular cells. It is useful to cover slides with individually cut Parafilm strips to ensure that the antibody is uniformly spread across the section.
8. Following incubation with primary antibody, wash sections 3 times in cold TBS. Incubate with 100 μl of appropriate secondary antibodies for 30 minutes minimum.
9. Wash 3 times in cold TBS. Add 100 μl of mounting media to each slide and a cover glass.
10. Allow slides to dry in a dark storage area.
11. Proceed to 'Image Acquisition' section.

4. Multi-color labeling of thymus vasculature and perivascular cells for vibratome sections.

1. Dissect out thymus lobes from embryo and rinse in cold PBS.
2. Fix thymus in 4% PFA/PBS at room temperature for 2 hours.

3. Wash in PBS-Triton X (0.15%) 3 times, 10 minutes and place thymi in a small plastic cartridge and submerge in 4% low melt agarose/PBS (~40C). The thymus should be in contact with the bottom of the cartridge.
4. Allow agarose to solidify on ice (3-5 minutes). Use a razor blade cut off excess agarose. Add glue to the vibratome block and adhere sample to the block.
5. Add cold PBS to vibratome water bath until the sample and blade are immersed.
6. Set speed and amplitude (high amplitude and low-moderate speed is ideal for soft thymus sections). The amplitude should be reduced if sections break up due excess agitation.
7. Cut 50 um sections.
8. Using a paintbrush, collect sections in a 24-well microplate in cold PBS.
9. Block sections in 500 μl of 10% donkey serum in PBS-Triton X (0.15%) for 30 minutes.
10. Incubate sections for 8 hours to overnight with primary antibody, such as anti-CD31 and anti-PDGFR-β, in a humidity chamber at 4^0C.
11. Wash 3 times in PBS-Triton X (0.15%) over a total of 8 hours at 4°C.
12. Block sections in 10% donkey serum in PBS-Triton X (0.15%) for 30 minutes.
13. Incubate sections for 8 hours to overnight at 4°C with appropriate secondary antibodies.
14. Wash 3 times in PBS-Triton X (0.15%) over a total of 8 hours at 4°C.
15. Re-fix samples in 4% PFA/PBS for 30 minutes at room temperature.
16. Wash 3 times in PBS-Triton X (0.15%) over 30 minutes at room temperature.

17. Dehydrate samples through a graded MeOH/PBS-Triton X series: 25% MeOH, 50% MeOH, 75% MeOH, and 100% MeOH at 10 minutes for each step. Replace 100% MeOH with fresh MeOH after 10 minutes and incubate for 1 hour at room temperature.
18. In a glass container, mix BABB (Benzyl Alcohol:Benzyl Benzoate) in a 1:2 ratio. Combine BABB with MeOH for a final concentration of 50% BABB and 50% MeOH. Incubate the sample in BABB:MeOH for 10-15 minutes.
19. Transfer sample to a glass container with 100% BABB and incubate for 10-15 minutes or until cleared, at room temperature.
20. Fill depression slide (0.7mm depth) with fresh 100% BABB and transfer sample to the side. Add cover glass (No. 1.5) and seal with 2-3 coats of nail polish. Allow nail polish to harden in the dark at room temperature, then store sample at 4°C.

    Note: Slides must be completely sealed prior to confocal image acquisition. Images should be acquired within 12-24 hours, as fluorescent dyes can fade in BABB.
21. Proceed to 'Image Acquisition' section.

5. Image Acquisition

1. Image 10 μm frozen sections with a confocal microscope using the Plan-Apochromat 20X/0.8 objective (512 X 512 Pixels) with 488- (FITC-dextran/GSL 1 – isolectin B_4), 543-, and 633-nm laser lines.
2. Acquire confocal z-sections of 50 μm agarose-embedded sections using the Plan-Apochromat 10X/0.4 objective (512 X 512 Pixels) with 488- (FITC-

dextran/GSL 1 – isolectin B_4), 543-, and 633-nm laser lines. Serial Z-sections should be collected sequentially at 1-micron for each respective channel.

3. Reconstruct serial Z-sections using Zeiss Axiovision 4.6 or other image analysis software.

Representative Results

Efficient labeling of the embryonic vasculature is critical for assessing blood vessel defects in embryonic mice. Figure A-1 shows specific labeling of E16.5 thymus blood vessels (A1a-b) and co-labeling with CD31 (A1b), in addition to staining of the right and left ventricles (A1e-f), respectively. The GSL I-isolectin B4 protocol for cryosections as described in sections 1, 3, and 5 was used in these experiments. Whole-mount labeling of the skin blood vessel network on E16.5 mice, using the protocols described in sections 1, 2, and 5 is shown in Figure 1c-d.

Discussion

Embryos should be placed in PBS warmed to 37^0C PBS. A dye such as Fast Green can be added to the FITC-dextran/GSL 1 - isolectin B_4 solution in order to visualize the mixture as it is injected into the facial vein. Shortly after FITC-dextran injection, the dye can be observed in the umbilical vein and in blood vessels throughout the yolk sac and placenta. At this point, the umbilical cord should be removed from the embryo. In the event that dye is not detected in the allantoic stalk (umbilical artery and vein) the technique likely failed. We have observed that if the needle does not directly enter the facial vein, FITC-dextran/ Fluorescein labeled GSL 1 - isolectin B_4 accumulates in adjacent areas of the face. We have also observed that FITC-dextran labels the entire vasculature, while GSL 1 - isolectin B_4 labels most embryonic vascular

structures, but also non-vascular cells in the liver. A fluorescence-dissecting microscope can be used to test whether the injection was successful.

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Table A-1: Name of specific reagents and equipment.

Name of the reagent	Company	Catalogue number
FITC-dextran	Sigma	FD150S-1G
Fluorescein labeled GSL 1 – isolectin B_4	Vector Laboratories	FL-1201
Fast Green	MP Biomedicals	195178
PFA	Fluka	76240
Fetal Bovine Serum	Atlanta Biologicals	S11550
Optimal Cutting Temperature Compound (O.C.T.	VWR	25608-930
Acetone	JT Baker	9006-33
Donkey Serum	Jackson	017-000-121
rat anti-mouse CD31,	BD Pharmingen	558736
goat anti-mouse PDGFR-β	R&D Systems	AF1042
donkey anti-rat CD31 Alexa 647 (Invitrogen)	Biolegend	102516
donkey anti-goat Alexa 594 (Invitrogen)	Invitrogen	A11058
Triton X -100	Sigma-Aldrich	X-100
Low melt agarose/PBS	Sigma-Aldrich	A9414-25G
Methanol	Fisher Scientific	A413-4
Benzyl Alcohol	Acros Scientific	148390010
Benzyl Benzoate	Acros Scientific	105860010
Depression slides	Fisher Scientific	S175201
Fluorogel	Electron Microscopy Sciences	17985-10
Cover Glass (22X22)-1.5	Thermo Scientific	152222
Zeiss LSM 510 Meta Confocal Microscope	Zeiss	
Micro dissecting forceps	Roboz	RS-5135

Parafilm No. OM992	Fisher Scientific	13-374-16
12 and 24 well microplates	Evergreen Scientific	222-8044-01F
Superfrost/Plus Microscope Slides	Fisher Scientific	12-550-15
4mL clear vials	National Scientific	B7800-2

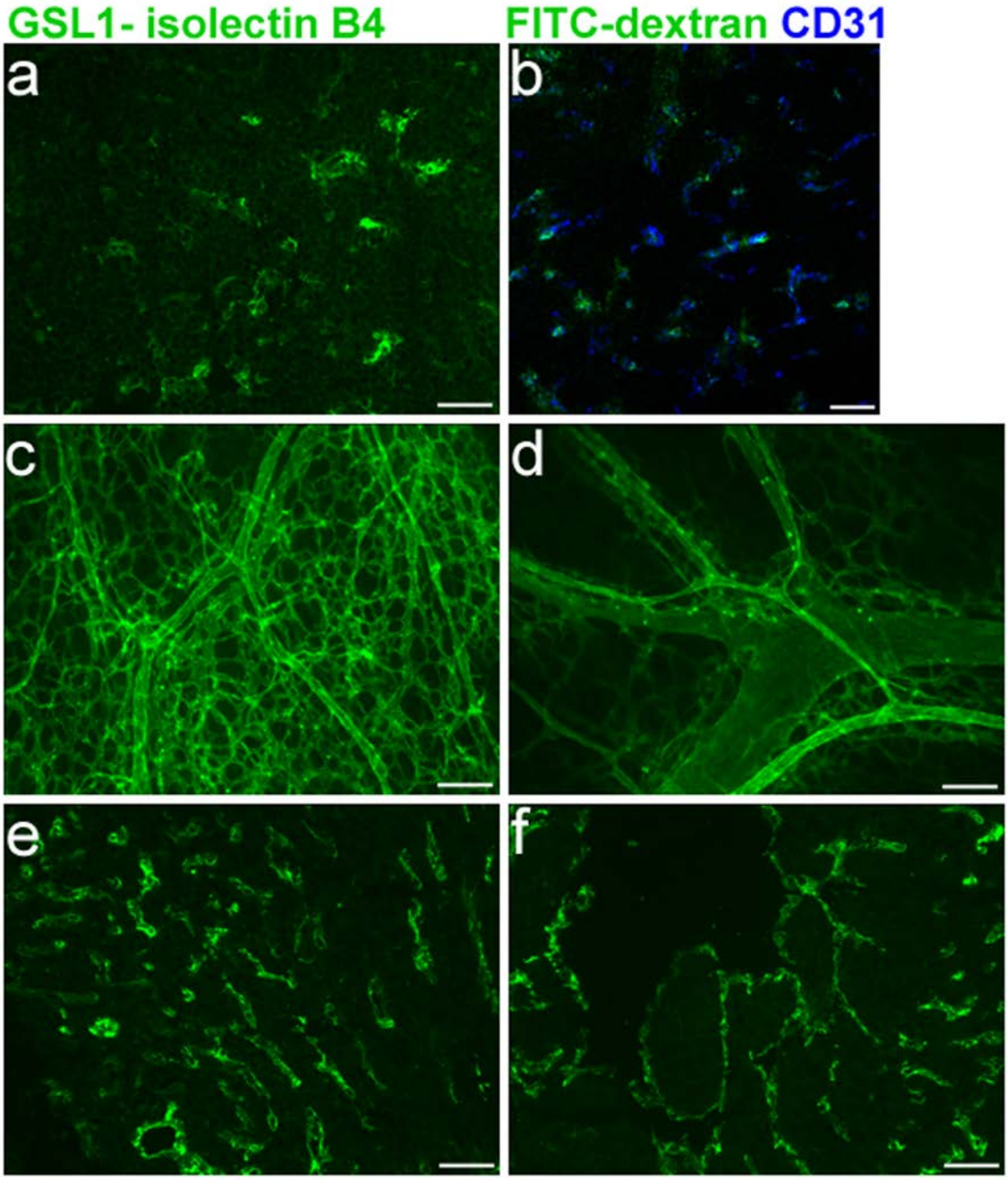

Figure A-1

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