EXPLORING THE ROLE OF NOTCH1 IN FETAL AND POSTNATAL DEVELOPMENT OF THYMIC EPITHELIAL CELLS

by

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(Under the Direction of Nancy Manley)

ABSTRACT

Notch1 signaling is known required for thymocyte development in the thymus, but little is known for its role in the development of thymic epithelial cells (TECs). In my study, I used immunostaining of DL4 and NOTCH1 antibody to determine their expression patterns in the embryonic thymus. I found that NOTCH1 is expressed in a putative progenitor population of TECs marked by PLET-1. To determine its role in the development of TECs, I used tissue-specific Cre recombinase to knock out *Notch1* gene from all TECs. My results show that Notch1 signaling is required for the embryonic differentiation of medullary TECs as well as the maintenance of PLET-1+ progenitor population of TECs. However, *Notch1* deletion from TECs does not affect postnatal development of TECs or thymocyte development.

INDEX WORDS: Notch1, DL4, Thymus, Thymic epithelial cells, Foxn1-Cre, Progenitor TECs, medullary TEC differentiation

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TABLE OF CONTENTS

Page

CHAPTER 1

INTRODUCTION

Thymus structure and function

The thymus is the primary lymphoid organ for the attraction, survival, expansion, migration and differentiation of T cell precursors and continuously feed the peripheral T cell pool (Rodewald 2008). The thymus stromal cells lacking pan-hematopoietic marker CD45 can be divided into keratin+ cells which are thymic epithelial cells (TECs) and keratin- cells called mesenchymal cells. TECs are composed of cortial TECs and medullary TECs which constitute the two histologically distinct regions, the outer cortical region and the inner medullary region, respectively. Mesenchymal cells include all keratin- cells, which are fibroblasts, nonfibroblastic mesenchymal cells, capsule- and septae-forming connective tissue cells, and endothelial cells forming the thymus vasculature. The thymus stroma also includes the dendritic cells and macrophages, which are CD45+ hemotopoietic cells (Manley et al. 2011, Rodewald 2008).

The embryonic thymus primordium is colonized by waves of lymphoid progenitor cells as early as E11.5, even before the thymic vasculature is established (Gordon and Manley 2011, Owen and Ritter 1969). The lymphoid progenitor cells migrate through the perithymic mesenchyme in response to chemoattractant factors until E14.5, when the establishment of the thymic vasculature is completed and provides direct lymphocyte trafficking between the blood circulation and the

thymus. The early fetal thymus colonization by lymphoid progenitor cells is divided into two steps: the Foxn1-independent recruitment of bloodborne hematopoietic cells to the vicinity of the thymus primordium, and the Foxn1-dependent accumulation of these cells within the thymus (Blackburn and Manley 2004, Gordon and Manley 2011, Gorg Hollander 2006, Le Douarin and Jotereau 1975, Owen and Ritter 1969).

In the postnatal thymus, T lymphocyte progenitors that enter the thymus through the thymic vasculature at the cortical medullary junction (CMJ) undergo stepwise maturation inside the thymus and finally exit the thymus through the thymic vasculature to the peripheral circulation. As T lymphocyte progenitors progress through distinct stages of differentiation in the thymus, defined as double negative (DN), double positive (DP) and single positive (SP) cells according to their expression of the co-receptors CD4 and CD8, they undergo highly ordered migration and occupy distinct regions in the adult thymus (Blackburn and Manley 2004, Lind et al. 2001, Prockop S. and Petrie 2000). T lymphocyte precursors enter the thymus at the cortico-medullary junction as DN cells, then migrate to the subcapsular zone of the outer cortex while they proliferate and undergo differentiation. These DN cells can be phenotypically and functionally divided into four subsets (DN1: CD44+CD25-, DN2: CD44+CD25+, DN3: CD44-CD25+, DN4: CD44-CD25-) by CD44 and CD25 expression (Godfrey et al. 1993). The DN cells that have successfully rearranged their T cell receptor (TCR) β go through a quick transition into DP cells and migrate back through the cortex and into the medulla. During this backward migration, DP cells undergo further selection and differentiate into either CD4 or

CD8 SP cells. Mature CD4 or CD8 SP cells exit to the peripheral circulation through vasculature in the medullary region. In this highly ordered development of thymocytes, the thymic epithelium plays an essential role both in regulating the directional migration and selection of the thymocytes and providing structural pathways for the migration of the thymocytes (Petrie 2002, Prockop S. E. et al. 2002).

Thymus organogenesis

The thymus epithelium is originated from the third pharyngeal pouch endoderm. The third pharyngeal pouch forms at the anterior endoderm gut tube during embryonic day (E)9-10.5 and is patterned into thymus and parathyroid domains. The fate of the thymus domain is already specified at this time (Gordon et al. 2004, Le Douarin and Jotereau 1975). The third pharyngeal pouch then proliferates and forms bilateral common primordia of the thymus and parathyroid domains, which is surrounded by the neural crest mesenchyme. The thymus and parathyroid domains separate from each other at about E12 while they migrate to their distinct final locations. Thymus domain endoermal cells then undergo thymus-specific cyto-differentiation into different subsets of TECs (Blackburn and Manley 2004, Gordon and Manley 2011, Manley et al. 2011).

Foxn1 (or *Whn*, Forkhead box protein N1) is the most important transcription factor known for TEC differentiation so far, which was identified from nude mice (Nehls et al. 1994). FOXN1 protein has a highly conserved forkhead DNA bind domain, C-terminal acidic activation domain, a nuclear localization signal and an N-terminal domain with no unidentified function. FOXN1 is expressed in skin

epithelial cells, hair follicles, and thymic epithelial cells. FOXN1 expression can be detected in all cells with the thymus-specific domain as early as E11.5. FOXN1 is shown essential for the normal proliferation and differentiation of thymic epithelial cells at multiple developmental stages of TEC differentiation (Chen et al. 2009, Su et al. 2003, Xiao and Manley 2010), and for the attraction of hematopoietic progenitor cells (Bryson et al. 2013, Itoi et al. 2001). Nude mice that are homozygotes for null alleles of *Foxn1* are hairless and athymic, and no mature T cells can be found in peripheral lymphoid organs in these mice (Nehls et al. 1996). However, thymus rudiment forms at the right position with correct identity, which indicates that FOXN1 is not required for the specification of thymus organ identity (Blackburn et al. 1996, Nehls et al. 1996). The upstream regulators and downstream targets of *Foxn1* remain to be identified.

Notch signaling

Notch receptors are highly conserved proteins through invertebrates and vertebrates that are key regulators of a lot of developmental processes. In mammals, there are four Notch receptors including Notch1-4 and five Notch ligands that include Delta-like 1(DL1), Delta-like 3 (DL3), Delta-like 4 (DL4), Jagged1, and Jagged2 (Dumortier et al. 2005). Notch receptors are transmembrane proteins with an extracellular domain containing epidermal growth factor (EGF)-like repeats, a transmembrane domain that gets cleaved upon activation, and an intracellular domain that contains the transactivation domain. Notch ligands are also tranmembrane proteins that all possess an N-terminal DSL (Delta, Serrate, and Lag2) domain followed by EGF-like repeats in their extracellular domain. Notch

signaling depends on cell-to-cell contact for activation. Binding of Notch ligand with receptor triggers two sequential cleavages that results in the release of intracellular domain of Notch receptor (NICD). NICD translocates into the nucleus where it binds to RBP-Jκ/CSL and acts as a transcriptional factor together. (Dumortier et al. 2005)

Notch signaling has been shown important for the maintenance of uncommitted progenitor cells in both invertebrates and mammals. In Notch1-deficient or RBP-Jκ mutant mice, the expression of neuronal differentiation markers are increased, indicating a premature up-regulation of neuronal differentiation (de la Pompa et al. 1997). In mouse intestine, Notch activation impairs the differentiation of the stem cells in the crypt, and expands the population of the proliferating intestinal progenitors (Fre et al. 2005).

Another important function of Notch signaling lies in its role in specifying cell fate decision. In the thymus, Notch1 signaling has been shown critical to T cell lineage commitment. Loss of Notch1 during postnatal development results in a significant decrease of T cells and ectopic B cells in the thymus (Radtke et al. 1999). Specifically, DL4 expressed by thymic epithelial cells is the essential ligand for interacting with Notch1 receptor during the T cell lineage commitment. Deletion of DL4 from thymic epithelial cells results in a complete block in T cell development accompanied by ectopic B cell development in the thymus (Hozumi et al. 2008, Koch et al. 2008).

Notch1 has also been reported to be required in late differentiation of murine keratinocytes (Rangarajan et al. 2001). Keratinocyte-specific deletion of

Notch1 leads to deregulated expression of differentiation markers by regulating WAF1, whereas activation of Notch1 causes growth suppression.

It has been reported that Notch1, 2 and 3 receptors are all expressed in postnatal thymus (Felli et al. 1999). Furthermore, Jagged1 and 2, DL1 and DL4 are also found in the postnatal thymus (Felli et al. 1999, Koch et al. 2008). DL1 is found to be expressed by blood vessels in the thymus examined by DL1-LacZ knock-in mice. DL4-LacZ mice show that DL4 is expressed by thymic epithelial cells (Koch et al. 2008). Northern blot results show that Notch1, 2 and Notch3 are all expressed by thymocytes, while Notch2 is relatively lower expressed. These three Notch receptors are also expressed by thymic epithelial cells. RNA in situ hybridization using sense- or anti-sense-specific probes shows that Notch1 expression is located in the thymic cortex region, highest in the subcapsular region (Felli et al. 1999).

In my study, I first examined the expression patterns of NOTCH1 protein. Based on its expression in progenitor TEC population, I hypothesized that Notch1 signaling may play a role in the maintenance of progenitor TECs and the differentiation of TECs. By conditionally knock out *Notch1* gene in all TECs using Foxn1-Cre mouse strain, I found that the number of progenitor TEC were reduced in the fetal thymus through newborn stage, and that the differentiation of mTECs were also impaired during late thymus organogenesis. However, postnatal phenotypes suggested redundancy with other *Notch* genes. These results suggest that Notch1 signaling is indispensable for the maintenance of progenitors TECs at fetal stage, and may also be required for the fetal differentiation of mTECs. With this study, I

can begin to understand the function of Notch1 signaling specifically in the development of thymic epithelial cells .

CHAPTER 2

METHODS AND MATERIALS

Generation of mutant mouse and genotype analysis

Notch1$^{fx/fx}$ mouse strain has been previously reported (Yang et al. 2004)and was purchased from Jackson Laboratory. Foxn1-Cre mouse strain was also previously reported (Gordon et al. 2007). Homozygous *Notch1*$^{fx/fx}$ females were crossed with *Foxn1*$^{Cre/+}$ heterozygous males and double heterozygous male offspring was back-crossed with *Notch1*$^{fx/fx}$ females to generate *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutants and *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ controls. Embryonic age was calculated by considering the noon of the vaginal plug as E0.5.

Immunohistochemistry

For frozen sections, embryos were embedded in OCT compound, and sections were cut at 10μm, and stained with antibodies for KERATIN-8, KERATIN-5, UEA-1, PLET-1, CLAUDIN-3, KERATIN-14, UEA-1, AIRE or CD31. For PFA-fixed paraffin sections, embryos were dehydrated and embedded in wax, and sections were cut at 6μm, and stained with antibodies for NOTCH1, FOXN1, IKAROS, PDGFR-β or PLET-1. Images were obtained by a Zeiss Axionplan 2 Apotome microscope or a Zeiss LSM510 confocal microscope.

Fluorescent Activated Cell Sorting (FACS) analysis

Thymocytes were freshly prepared and counted for the total thymocyte numbers. 1×10^6 thymocytes were used. Anti-CD4–APC, anti-CD8–FITC, anti-CD44–

PE, and anti-CD25–biotin followed by streptavidin-PerCP were used. For analyzing the subpopulation profile in CD4-CD8- double negative (DN) T cells, phycoerythrin (PE)– conjugated lineage markers (anti-CD3, CD4, CD8, CD11b, CD19, B220, Gr-1, TER-119, NK1.1) combined with anti-CD25 and anti-CD44 antibodies were used. All antibodies were from either BD Pharmingen or Biolegend (San Diego, CA) if not indicated.

Generation of the panorama picture of postnatal thymus

Overlapping pictures of the immunostaining results on frozen sections of postnatal thymi were taken by a Zeiss Axionplan 2 Apotome microscope at one fixed exposure time for each color. All the partly overlapping pictures were organized and stitched into a single thymus panorama by the software "PTGui".

Size comparisons of epithelial-free-zones in postnatal thymi

Panorama pictures of K8/K5/UEA-1 immunostainings were done on four parallel sections from one *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant thymus and one *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ control thymus respectively, both of which were at one month age. Sections were selected from parallel positions in each quarter of each thymus. Epithelial-free-zones and whole sections were outlined manually and measured for areas in pixels by AxioVision Rel. 4.8 software. The size of epthelial-free-zones in each section was calculated by dividing total area of epithelial-free-zones by that of the whole section. Epithelial-free-zone sizes were compared between parallel sections from *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant and *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ control thymus.

CHAPTER 3

EXPRESSION PATTERNS OF DL4 AND NOTCH1 IN EMBRYONIC THYMUS

Introduction

Notch1 signaling has been well known for its role in specifying the T cell fate commitment (Radtke et al. 1999), but little is known for its role in thymic epithelial cells. Although it is shown that *Notch1* mRNA is present in thymic epithelial cells at postnatal ages (Felli et al. 1999), it is not clear whether fetal thymic epithelial cells express NOTCH1 protein and if yes, what subset of thymic epithelial cells are expressing NOTCH1 prenatally. Our hypothesis is that NOTCH1 is expressed in thymic epithelial cells from embryonic stages, and may have a role in their development. So I used a NOTCH1 antibody that recognized NOTCH1 intracellular domain (NICD) for immunostaining and tested its expression patterns in embryonic thymus. At the same time, I also want to test the expression patterns of DL4 ligand in the embryonic thymus to see whether it is expressed in adjacent to NOTCH1 receptor.

Results

DL4 protein is expressed by cortical thymic epithelial cells at E16.5.

I first tested the presence of Notch ligand DL4 in the embryonic thymus. To test this, I used anti-DL4 antibodies to do immunostaining of DL4 proteins in E16.5 thymus of wild-type mouse embryos. I found that the expression of DL4 co-localizes with that of KERATIN-8 (K8), a keratin protein largely expressed by cTECs at this

age in the embryonic thymus, whereas in the medullary region marked by presence of KERATIN 14, DL4 is absent (Fig. 1). This indicates that Notch ligand DL4 is abundantly present in the cortical region of embryonic thymus at E16.5.

NOTCH1 protein is expressed by developing lymphocytes and blood vessels in the embryonic thymus.

I then examined the expression patterns of NOTCH1 receptor in the embryonic thymus. Anti-NOTCH1 antibody is co-immunostained with anti-IKAROS and anti-PDGFR-β respectively in embryonic thymus. IKAROS is a lymphocyte marker, which is expressed by all lymphocytes in the thymus. PDGFR-β is expressed by neural crest cells, which is supporting endothelial cells in blood vessel structures.

NOTCH1 is first found expressed by lymphocytes. NOTCH1 is co-stained with IKAROS and K8 at E14.5(Fig 2A-E). Most NOTCH1+ cells are also positive for IKAROS, indicating that they are lymphocytes. Several NOTCH1+ cells co-localizes with K8, which is only expressed by epithelial cells in the thymus.

NOTCH1 is also found lining blood vessels. At E13.5, Notch1 is co-stained with PDGFR-β and K8 (Fig 2F-J). It shows that the neural crest cells in blood vessel structures are also expressing NOTCH1. And again, several NOTCH1+K8+ epithelial cells are found in the thymus too.

NOTCH1 protein is expressed by progenitor TECs in the embryonic thymus.

NOTCH1 is also expressed by thymic epithelial cells at embryonic stages. Since FOXN1 is a transcription factor expressed only by epithelial cells in the thymus, I then stained NOTCH1 with FOXN1 to test the identity of the NOTCH1+ cells that are not IKAROS-positive or PDGFR-β-positive. At E12.5 and E14.5, several

NOTCH1+FOXN1+ cells are found (Fig. 3). NOTCH1 staining was not only found in the cytoplasm of these cells, but they also localized in the nuclei of these cells (Fig. 3E-H, M-P, shown by arrows), which indicated that intracellular domain of NOTCH1 receptor had translocated into the nuclei to actively take effect on downstream genes. At E16.5, similar results are obtained that a small number of NOTCH1+FOXN1+ TECs are found in the thymus.

Since the number of NOTCH1+FOXN1+ TECs is relatively very small regarding to the total number of FOXN1+ TECs, I wondered whether these cells are progenitor population among all TECs. PLET-1 had been reported to be a marker protein for the progenitor population among all TECs (Bennett et al. 2002, Depreter et al. 2008, Gill et al. 2002, Rossi et al. 2006), so I did immunostaining of NOTCH1, FOXN1 and PLET-1 together at E13.5. The results showed that at E13.5, NOTCH1+FOXN1+ TECs are also positive for PLET-1 (Fig. 4), which indicated that they were the progenitor TEC population.

Discussion

Our immunostaining results showed that from E12.5 to E16.5, NOTCH1 is expressed by at least three cell populations, which are lymphocytes, neural crest cells and thymic epithelial cells. Among them, most of NOTCH1-expressing cells are lymphocytes, whereas a small number of thymic epithelial cells are also receiving active Notch1 signaling. These NOTCH1-positive TECs are progenitor TECs that are also expressing PLET-1, indicating that active Notch1 signaling is going on in progenitor TECs at embryonic stages. This suggests that Notch1 signaling could have a critical role for TEC progenitor identity. Since Notch signaling is required for

the embryonic thymus. On the other hand, since Notch1 signaling has been reported to regulating cell differentiation (Rangarajan et al. 2001), it is also possible that Notch1 signaling may be playing a similar role in TECs and regulating their differentiation in the embryonic thymus.

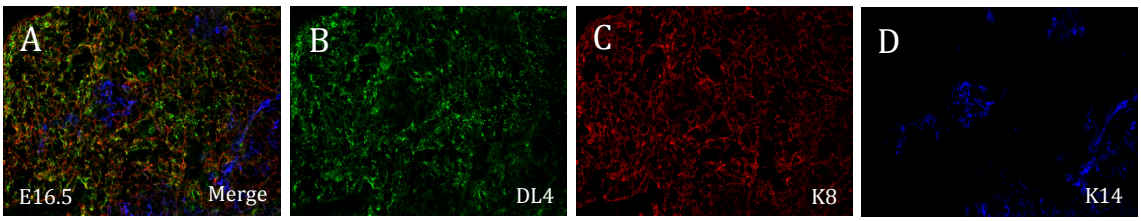


Fig. 1: DL4 is expressed by cTECs at age E16.5 in embryonic thymus. **A.** Immunostaining of DL4 antibody (**Fig. 1B**, in green) colocalizes with Keratin8 (**Fig. 1C**, in red), which is a keratin marker for cTECs in embryonic thymus. The immunostaining pattern of DL4 does not colocalize with Keratin 14 (**Fig. 1D**, in blue), which is a keratin marker of a subset of mature mTECs in embryonic thymus.

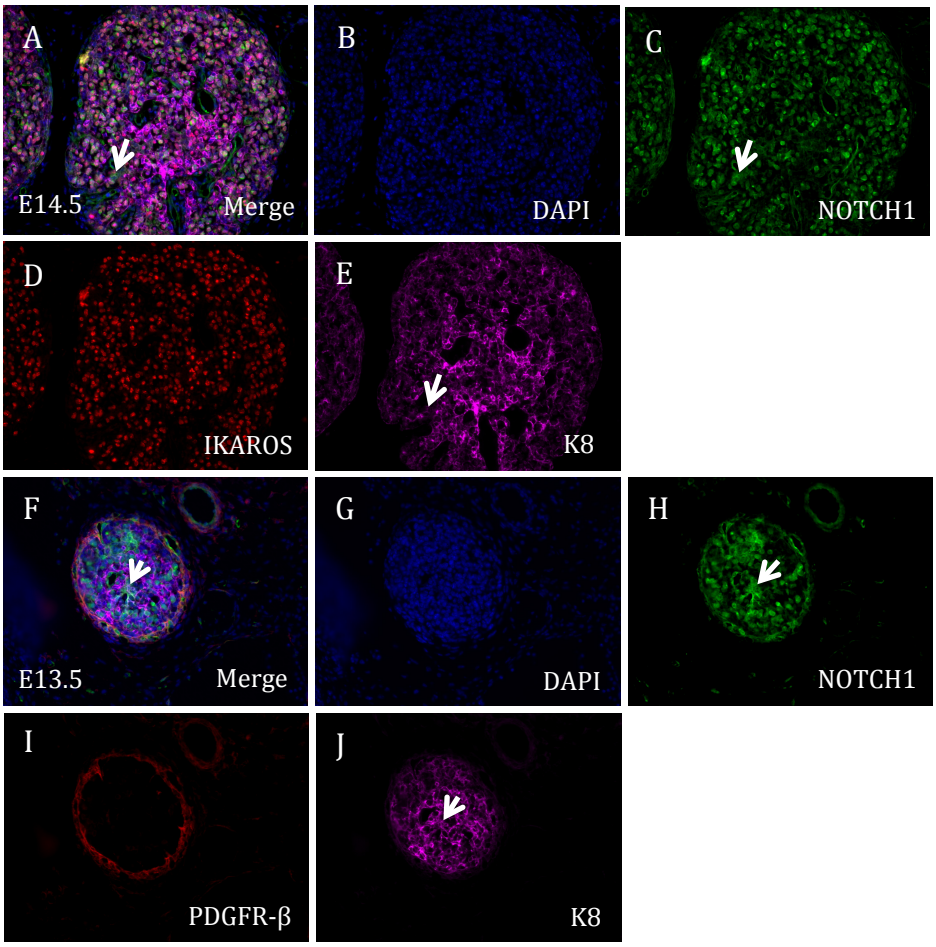


Fig 2: NOTCH1 is expressed both by thymocytes and by neural crest cells of blood vessel structures in embryonic thymus. A-E. Most of NOTCH1+ cells (Fig. 2C, in green) are thymocytes that are Ikaros+ (Fig. 2D, in red) at E14.5. Several NOTCH1+ cells colocalizes with K8 (Fig. 2E, in purple), which indicates they are epithelial cells (shown by arrows). F-J. At E13.5, NOTCH1 (Fig. 2H, in green), PDGFR-β (Fig. 2I, in red), and K8 (Fig. 2J, in purple) co-immunostaining shows that PDGFR-β+ neural crest cells in blood vessel structures are also expressing NOTCH1 receptors. And as at E14.5, several NOTCH1+K8+ cells can be found at this age too (shown by arrows).

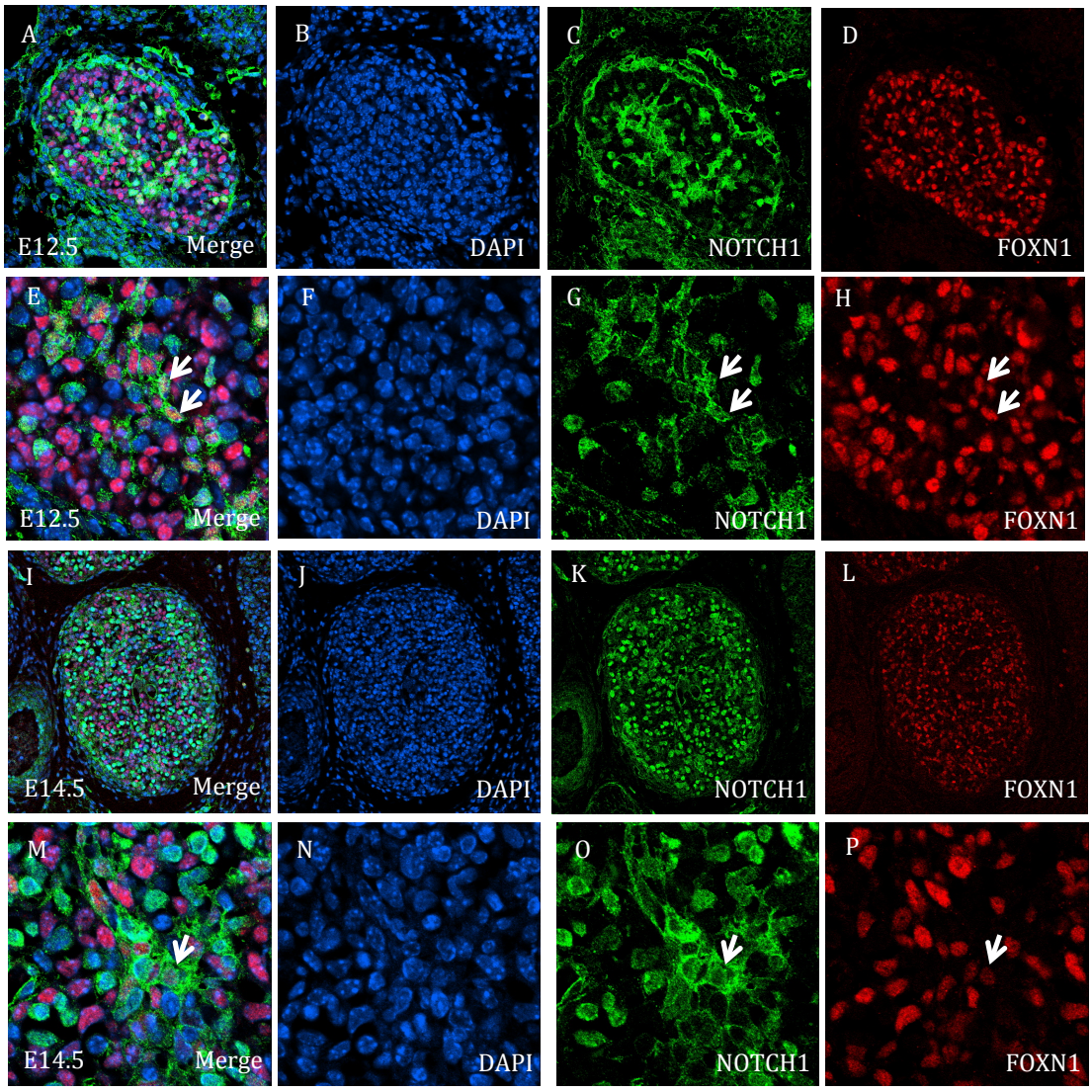


Fig 3: NOTCH1 is expressed by several FOXN1+ thymic epithelial cells in embryonic thymus. Immunostaining of DAPI (Fig. 3B, F, J, N, in blue), NOTCH1 (Fig. 3C, G, K, O, in green) and FOXN1 (Fig. 3D, H, L, P, in red) shows that several NOTCH1+ cells are positive for FOXN1, which is a key regulator specifically expressed in epithelial cells in the thymus. At E12.5 (A-D) and E14.5 (I-L), most of NOTCH1+ cells are negative for FOXN1, whereas only several cells are co-expressing NOTCH1 and FOXN1. Higher magnification pictures show that at E12.5 (E-H) and E14.5 (M-P), NOTCH1 proteins in NOTCH1+FOXN1+ cells are localized both in cytoplasm and in nuclei.

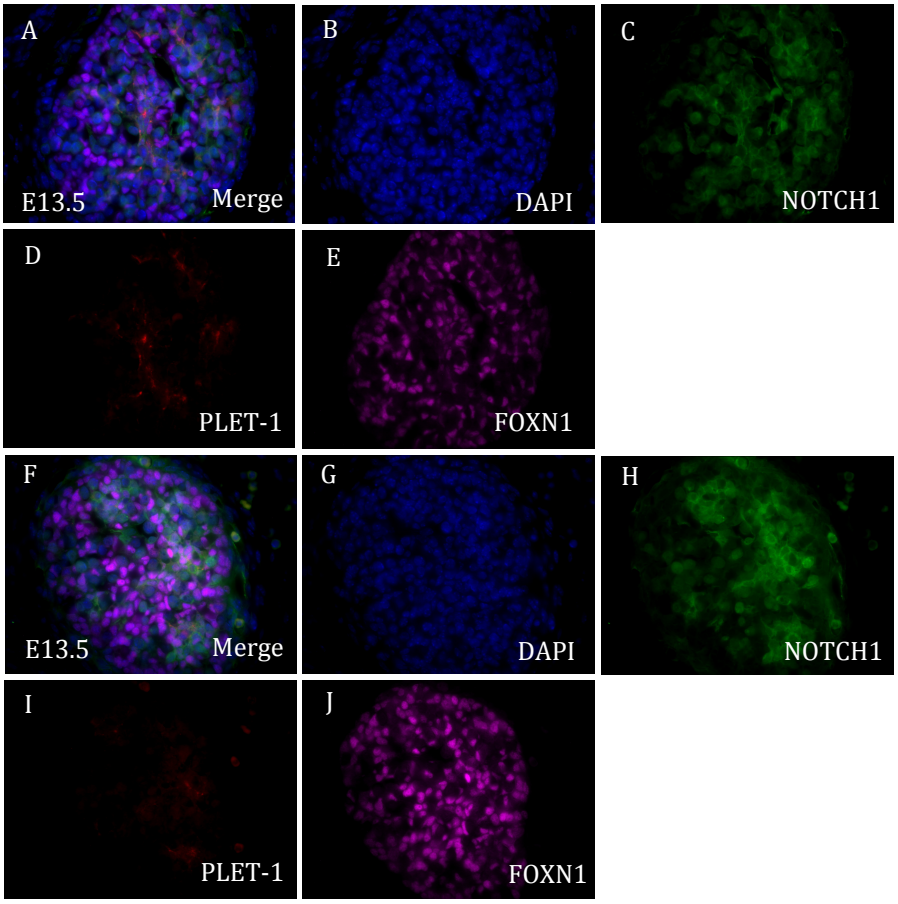


Fig 4: At E13.5, NOTCH1+FOXN1+ cells are progenitor TECs marked by PLET-1. Immunostainings of DAPI (B, G. in blue.), NOTCH1 (C, H. in green), PLET-1 (D, I. in red) and Foxn1 (E, J. in purple) show that the NOTCH1+FOXN1+ TECs are also positive for PLET-1, indicating that they are the progenitor TECs in the embryonic thymus.

CHAPTER 4

TISSUE-SPECIFIC DELETION OF NOTCH1

Introduction

Based on the results that NOTCH1 is expressed by progenitor TECs marked by PLET-1 in the embryonic thymus, we hypothesized that Notch1 signaling may play a role in maintaining the identity of PLET-1+ progenitor TECs or regulating cell differentiation in TECs. To test this hypothesis, I wanted to abolish Notch1 signaling in all thymic epithelial cells from embryonic stages. By crossing Foxn1-Cre mouse strain with Notch1 floxed mice, *Notch1* was specifically deleted from all thymic epithelial cells as early as E11.5 (Gordon et al. 2007). To test the changes of TEC differentiation by this deletion of Notch1 signaling from all TECs, I compared expression patterns of specific TEC differentiation markers between mutants and controls at fetal stages and postnatal stages by immunostaining. To test the effect of Notch1 deletion from TECs on the progenitor TEC population, I compared the expression patterns of PLET-1 and CLAUDIN-3, which marks the mTEC progenitors that give rise to Aire-positive mTECs (Hamazaki et al. 2007).

Results

NOTCH1 is effectively deleted from thymic epithelial cells in embryonic thymi.

Notch1$^{fx/fx}$ homozygous females were crossed with *Foxn1*$^{Cre/+}$ heterozygous males to generate the double heterozygous F1 offspring with the genotype *Foxn1*$^{Cre/+;}$*Notch1*$^{+/fx}$. F1 males were backcrossed with *Notch1*$^{fx/fx}$ females and the

embryos were obtained at E14.5. *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant thymi were compared with *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ control thymi. To examine whether NOTCH1 proteins were completely deleted from all thymic epithelial cells, I did immunostaining of NOTCH1 with FOXN1 in E14.5 mutant and control thymi (Fig. 5). In the *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant thymi, NOTCH1 expression is excluded from all FOXN1+ TECs at E14.5 (Fig. 5A-C), while in the *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ littermate controls, NOTCH1+FOXN1+ TECs were found in the thymi at this stage (Fig. 5D-F).

Notch1 signaling is required for the maintenance of the number of progenitor TECs in the embryonic thymus.

To test whether the population of PLET-1+ progenitor TECs is affected by deletion of Notch1 signaling from thymic epithelial cells, I compared the staining of PLET-1, CLAUDIN-3 and PAN-CYTOKERATIN (PANK) between the *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant and *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ control thymi (Fig. 6) at E13.5, E16.5 and newborn stages. For each stage, one control and two mutant thymi were examined. At E13.5, the number of PLET-1+ progenitor TECs (Fig. 6B, F. in green) and CLAUDIN-3+ immature TECs (Fig. 6C, G, in red) were both reduced in the mutant thymi (Fig. 6E-H) compared with that in the control thymi (Fig. 6A-D). Similarly, reduced number of PLET-1+ cells and CLAUDIN-3+ cells were shown at both E16.5 (Fig. 6I-P) and newborn stage (Fig. 6Q-X).

mTEC differentiation is impaired by deletion of Notch1 signaling in the embryonic thymus.

To examine the cortical and medullary organization after deleting Notch1 signaling from all thymic epithelial cells, I compared the staining of KERATIN-8

(K8), KERATIN-5(K5), and UEA-1 in the *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant thymi and *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ control thymi at E13.5, E16.5 and newborn ages (Fig. 7). K5 is expressed largely by mTECs, but is also extended at corticol-medullary junction and into cortex region colocalizing with K8, which may represent a probable precursor subset (Klug et al. 1998). UEA-1 is a marker specifically expressed by a subset of mature medullary TECs (Farr and Anderson 1985). One control and two mutant thymi were examined through each stage and representative images were shown. At E13.5, the number and the distribution of UEA-1+ mature medullary TECs was comparable between mutant thymi (Fig. 7F-J) and control thymi (Fig. 7A-E). At E16.5, the cluster of UEA-1+ mature medullary TECs in the *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant thymi was distributed only in the center (Fig. 7O, P), whereas several clusters of UEA-1 mature medullary TECs could be found in the *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ control thymi and the clusters were distributed more evenly in the thymi (Fig. 7K, L). At the same time, the total number of UEA-1+ cells was greatly reduced in the mutant thymi. At newborn age, similar phenotypes were found. Mutant thymi had fewer UEA-1+ mature medullary TECs (Fig. 7S-U) and restricted localization of these cells, compared with UEA-1 cells in the control thymi (Fig. 7V-X).

Analysis of postnatal phenotypes after Notch1 deletion.

I then tested whether Notch1 signaling is required for the postnatal development of thymic environment. I examined the development of several TEC populations by immunostaing. At one month age, the expression of CLAUDIN-3 on immature mTEC was not obviously affected in the mutant thymi (Fig. 8C, D). The expressions of the markers for subsets of mature mTECs, KERATIN-14 (K14) and

UEA-1, appear both comparable between the mutants (Fig. 8C) and controls (Fig 8A). The expression of the functional marker of a subset of mature mTECs, AIRE, was not obviously changed either (Fig 8B, D).

I also looked at the phenotyeps with PLET-1+ progenitor TECs at four-month age. I immunostained PLET-1 with m-TEC marker UEA-1 and β5t, which is a cTEC marker (Ripen et al. 2011, Takahama et al. 2012) (Fig. 9). In both *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant (Fig. 9A-E) and *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ control (Fig. 9F-J) thymi, PLET-1+ progenitor TECs were detected and the number of PLET-1+ progenitor TECs appeared comparable between *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant and *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ control thymi. However, the staining of β5t did not show up very well, and further quantifications of PLET-1+ cells need to be done before drawing any conclusion.

At both one-month and four-month ages, control and mutant thymi showed such regions where no epithelial cells or endothelial cells marked by CD31 were found (Fig. 10, shown by arrows). To find out what are the DAPI+ cells in such epithelial-free zone, I used anti-IKAROS antibody, which labels all lymphocytes in the thymus to do immunostaining with cTEC marker CD205 and mTEC marker K14 (Fig. 10). The immunostaining results showed that in both *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant (Fig. 10G-L) and *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ control (Fig. 10A-F) thymi, epithelial-free regions were filled with IKAROS-positive lymphocytes.

To test whether these epithelial-free zones were normal patterns, I looked at TEC distributions in both *Notch1*$^{fx/fx}$ thymi and *wild-type* thymi, both at one-month age (Fig. 11). The results showed that in one-month-old *Notch1*$^{fx/fx}$ thymi, epithelial-

free zones were found (Fig. 11A-D) and were also filled with IKAROS-positive lymphocytes (Fig. 11A, B). In one-month-old *wild-type* thymi, such epithelial-free zones were also present (Fig. 11E-K).

To test whether postnatal development of cortex and medulla were affected by deletion of Notch1 signaling and epithelial-free zones, I compared cortex/medulla area ratio with epithelial-free zones excluded between *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant thymus and *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ control thymus at one-month age (Fig. 12). Panoramas of two sets of immunostainings (shown in Fig. 8) were generated from one mutant and one control thymus at one-month age. Total panorama of each thymus, medulla regions and epithelial-free zones were outlined and measured for respective areas with units of pixels. The area of cortex region was calculated by subtracting medulla regions and epithelial-free zones from the whole panorama. With two seperate cortex/medulla ratio for the mutant thymus and the control thymus, average cortex/medulla ratios and standard deviations for both mutant and control thymus were calculated (Fig. 12A). A chart was generated based on these data (Fig. 12B). It showed a trend of increase in cortex/medulla ratio in *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant thymus compared with *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ control thymus. However, additional N values are required to draw definitive conclusions.

To evaluate whether the size of epithelial-free zones were affected by *Notch1* deletion from all TECs, I calculated and compared the ratio of total epithelial-free zone area versus whole section area at one month age between *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant thymus and *wild-type* control thymus (Fig. 13). One mutant and one control thymus were used and each thymus was divided into four quarters at ventral-dorsal

plane. Sections from parallel positions in each quarter in both mutant and control thymus were immunostained with anti-K8, K5 and PANK antibodies and panorama figures of each section were obtained (Fig. 14). The size of epithelial-free zone in each section was calculated by dividing the total area of epithelial-free zones by the area of the whole thymu. Sizes of epithelial-free zones of each pair of parallel sections from mutant and control thymus were compared in each quarter 1, 2, 3 and 4. This comparison showed a tendency of increase in the size of epithelial-free zones in the mutant thymus compared with the *wild-type* control, but additional N values are required to draw a definitive conclusion.

Furthermore, to test whether T cell development was affected by deletion of Notch1 signaling from all TECs, I looked at the T cell profile at one-month age (Fig. 15). Thymocytes were freshly obtained and counted from three $Foxn1^{Cre/+;}Notch1^{fx/fx}$ mutant thymi and three $Foxn1^{+/+;}Notch1^{fx/fx}$ control littermates. Total thymocyte numbers was not significantly changed in $Foxn1^{Cre/+;}Notch1^{fx/fx}$ mutant thymi compared with $Foxn1^{+/+;}Notch1^{fx/fx}$ littermate controls (Fig. 15A). 10^6 thymocytes were used to stain with antibodies of each thymocyte subpopulation. Gated CD45+Epcam- thymocytes were plotted into CD4-CD8- DN cells, CD4+CD8- or CD4-CD8+ SP cells and CD4+CD8+ DP cells (Fig. 15B). Proportions of each subpopulation were comparable between mutant thymi and control thymi. DN cells were further gated and plotted into DN1, DN2, DN3, and DN4 subpopulations by different expressions of CD44 and CD25 (Fig. 15C). The results showed that DN1 through DN4 subpopulations were also comparable between mutant and control thymi. Gated CD44+CD25- DN1 cells were further plotted into DN1a, DN1b, DN1c, DN1d,

and DN1e subpopulations by different expressions of c-kit and HSA (Fig. 15D). The results showed that the proportions of each subpopulation were also comparable between *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutants and *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ controls. Representative plots were shown (Fig. 15E) for both *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutants and *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ controls.

Discussion:

By examining expression of TEC progenitor and TEC differentiation markers at embryonic stages in the *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutants, our results show that the number of progenitor TECs were reduced in the mutants, indicating that deletion of Notch1 signaling impaired the maintenance of progenitor population of thymic epithelial cells during late thymus organogenesis. This result suggests that Notch1 signaling may be critical for maintaining epithelial stem cells in the fetal thymus. However, at postnatal stages, the number of PLET-1+ progenitor TECs showed no obvious changes in the *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutants compared with *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ controls, although more careful quantification of PLET-1+ progenitor TECs need to be done at both embryonic and postnatal stages. One possible explanation for the less obvious change in the number of postnatal PLET-1+ progenitor TECs could be that other Notch family members compensate the loss of Notch1, regarding that both Notch2 and Notch3 are expressed by thymic epithelial cells at postnatal stages (Felli et al. 1999).

We also showed that the expression of a mature mTEC marker, UEA-1, was decreased in the *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutants at E16.5 and newborn stages, which indicates that fetal differentiation of mTECs was also impaired by deletion of Notch1

signaling from all TECs. This could suggest a direct role of Notch1 signaling in the fetal differentiation of mTECs, or the phenotypes may be secondary defects due to reduced number of progenitors.

At one-month age, we found epithelial-free zones in both *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant thymi, *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ control thymi, *Notch1*$^{fx/fx}$ thymi and *wild-type* thymi, which were all filled by IKAROS-positive lymphocytes. By comparing the size of such epithelial-free zones in the *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant thymus and *wild-type* thymus, we found a tendency of size increase in the mutants. Such results suggest that deletion of Notch1 signaling may expand the epithelial-free zones in the postnatal thymus. However, with not enough n number and lack of *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ and *Notch1*$^{fx/fx}$ control thymi, we could not draw definitive conclusions.

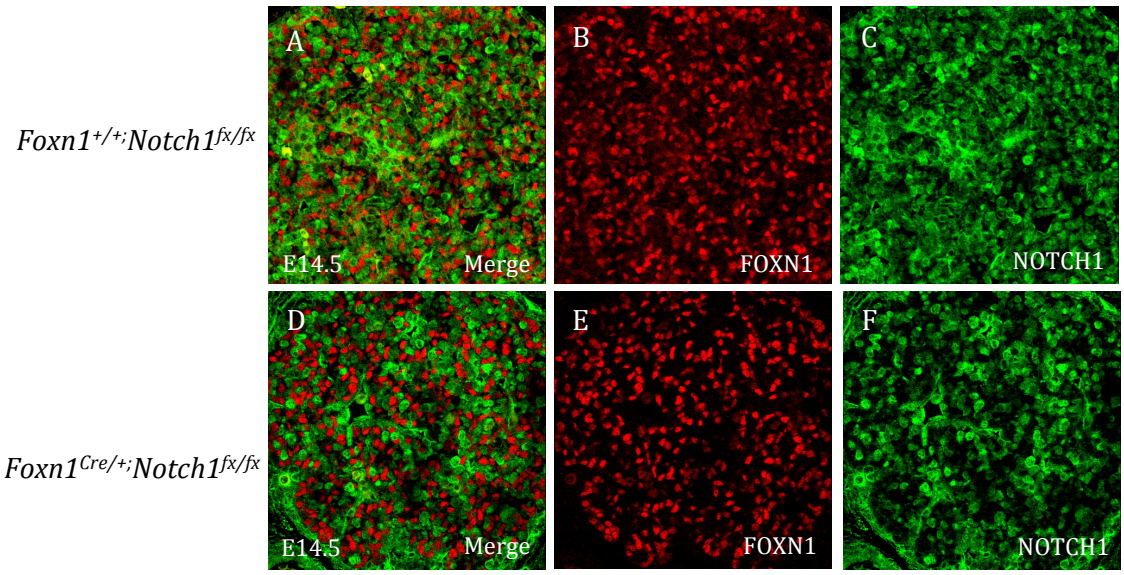


Fig 5: At E14.5, NOTCH1 is completely deleted from all thymic epithelial cells in *Foxn1*$^{Cre/+}$;*Notch1*$^{fx/fx}$ embryos.Immunostaining of NOTCH1 (C, F. in green) and FOXN1 (B, E. in red) shows that in the embryos genotyped *Foxn1*$^{Cre/+}$;*Notch1*$^{fx/fx}$ (D-F), NOTCH1 staining does not co-localize with FOXN1, whereas *Foxn1*$^{+/+}$;*Notch1*$^{fx/fx}$ control embryos (A-C) have NOTCH1+FOXN1+ cells in their thymi at E14.5 age. Images are taken by a Zeiss LSM510 confocal miscroscope.

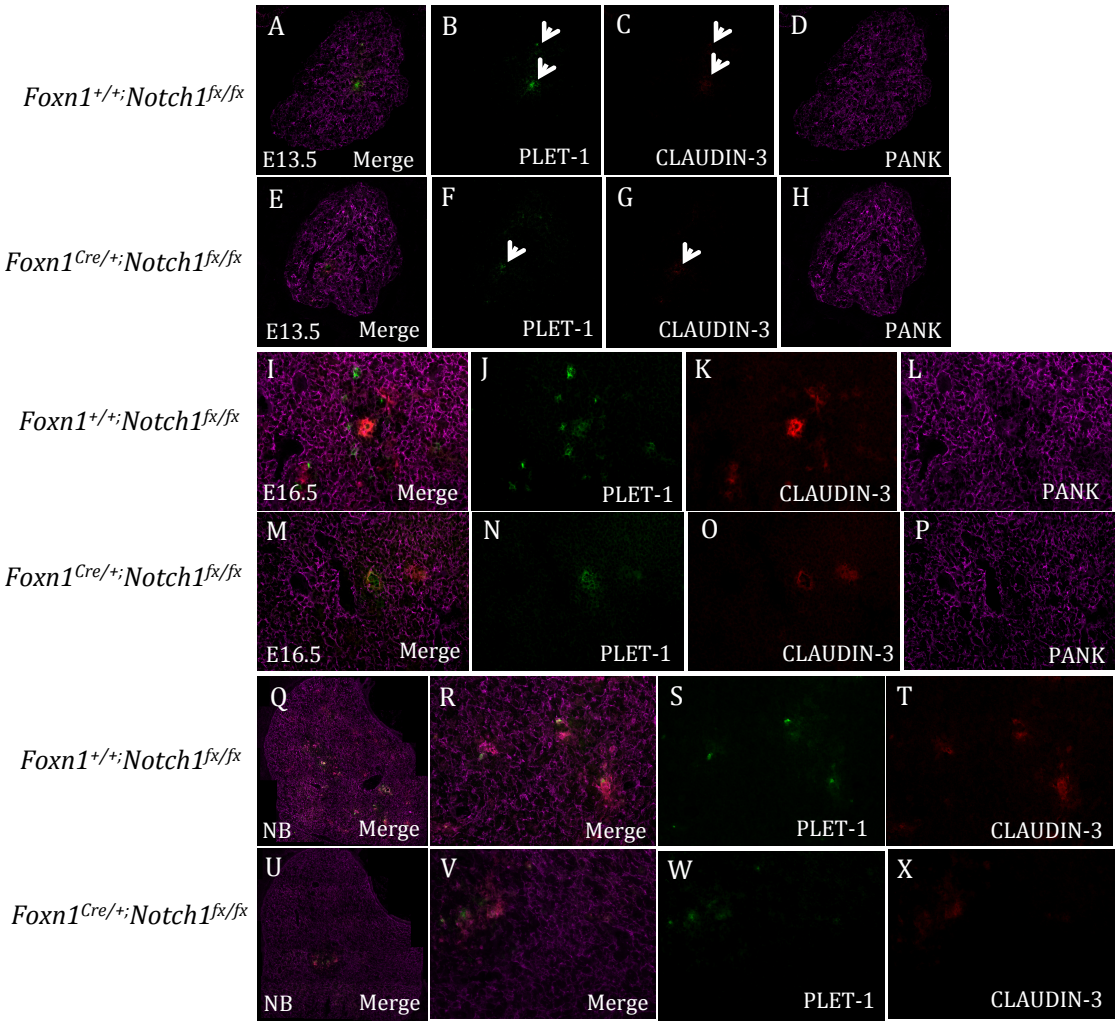


Fig 6: The number of progenitor TECs is reduced after deletion of Notch1 signaling in TECs from E13.5 through newborn.PLET1-1 (B, F, J, N, S, W. in green), a marker of progenitor TECs, and CLAUDIN-3 (C, G, K, O, T, X. in red), are immunostained with PAN-CYTOKERIN (PANK), which labels all TECs (D, H, L, P. in purple). At E13.5 stage, the *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant thymi (E-H) have fewer PLET-1+ progenitor TECs compared with *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ control thymi (A-D). Similar phenotypes are found at ages of E16.5 (I-P) and E13.5 (Q-X).

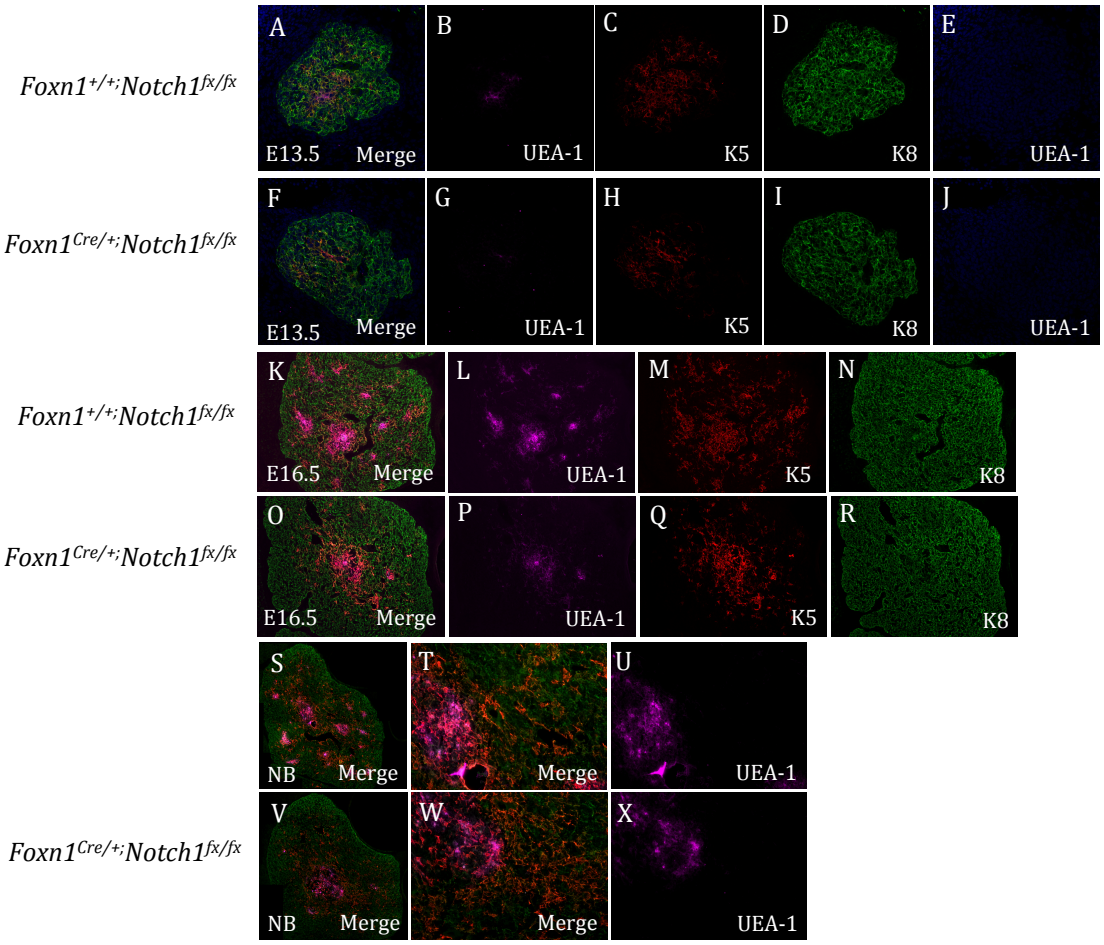


Fig 7: Differentiation of medullary TECs are impaired at both E16.5 and at newborn age by deletion of Notch1 signaling from thymic epithelial cells. UEA-1+ mature medullary TECs are stained in purple and compared between *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ control thymi and *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant thymi at E13.5 (A-J), E16.5 (K-R) and newborn ages (S-X). A, B, C. At E13.5, the organization and total number of UEA-1+ cells are comparable between mutant (F-J) and control thymi (A-E). In normal E16.5 thymi, UEA-1+ mature medullary TECs form several clusters of cells distributed in the thymus (K-N). However, the localization of UEA-1+ cells is centralized in the *Notch1* conditional knockout thymi, where they are only found in a much smaller central region (O-R). At the same time, the total number of UEA-1+ cells is also reduced in the mutant thymi compared to the littermate control. Immature medullary TECs, marked by K8 expression in red are comparable between mutant thymi and control thymi. Similar phenotypes are also found at newborn age (S-X).

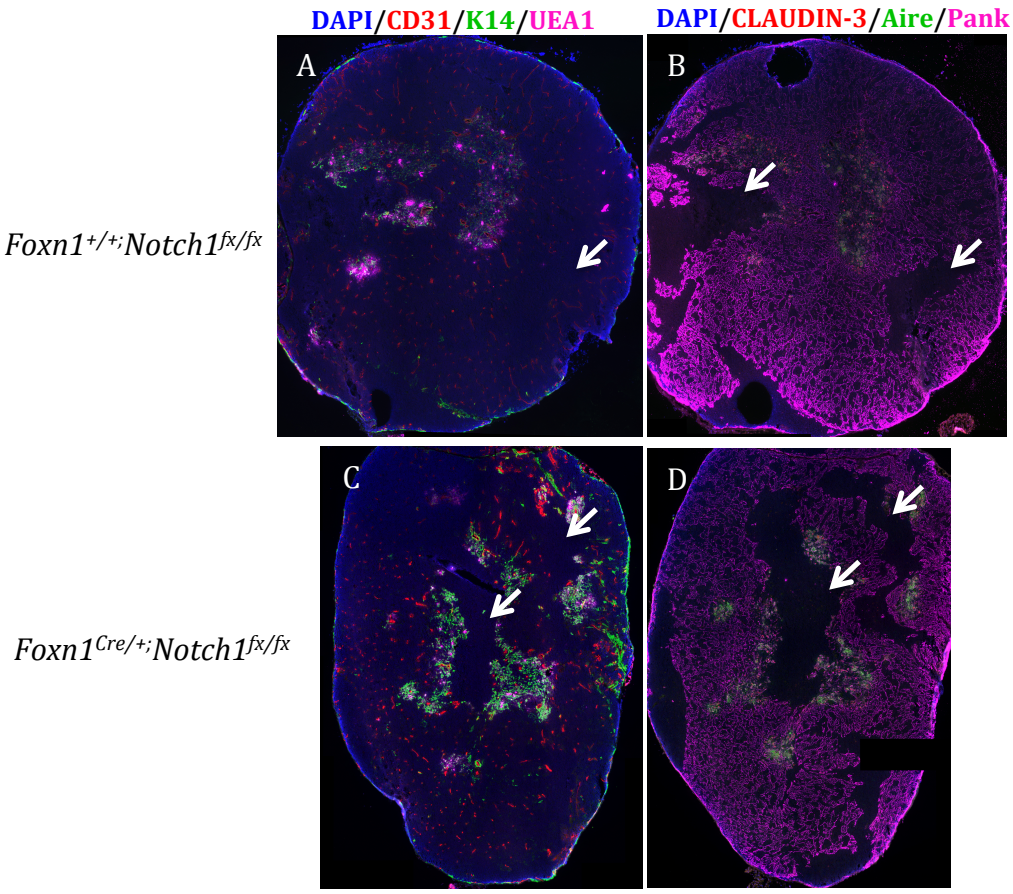


Fig 8: Deletion of Notch1 signaling from TECs does not affect postnatal thymic development.A, B. At 1 month age, the expression of endothelial marker CD31, mature mTEC subset markers K14 and UEA-1 are comparable between *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant thymi and *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ control thymi. C, D. At 1 month age, the expression of immature mTEC marker CLAUDIN-3 and the functional mature mTEC marker Aire are comparable between mutant and control thymi. Epithelial-free-zones are found in both mutants and controls (arrows).

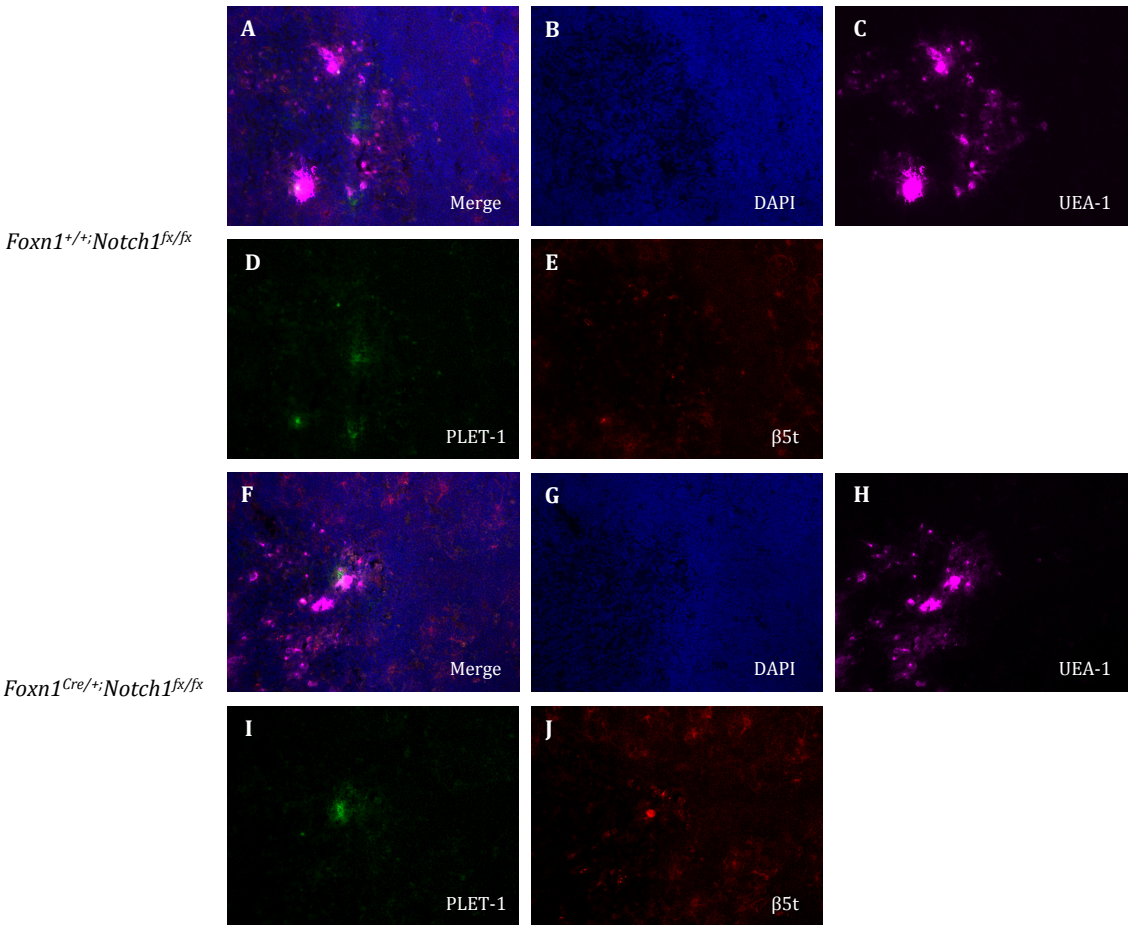


Fig 9: The number of PLET-1 progenitor TECs is not obviously changed at 4-month age. At 4-month age, progenitor TEC marker, PLET-1 is immunostained with cTEC marker β5t, and mTEC marker UEA-1 in *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant thymi and *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ control thymi. PLET-1+ progenitor TECs are found in both genotypes.

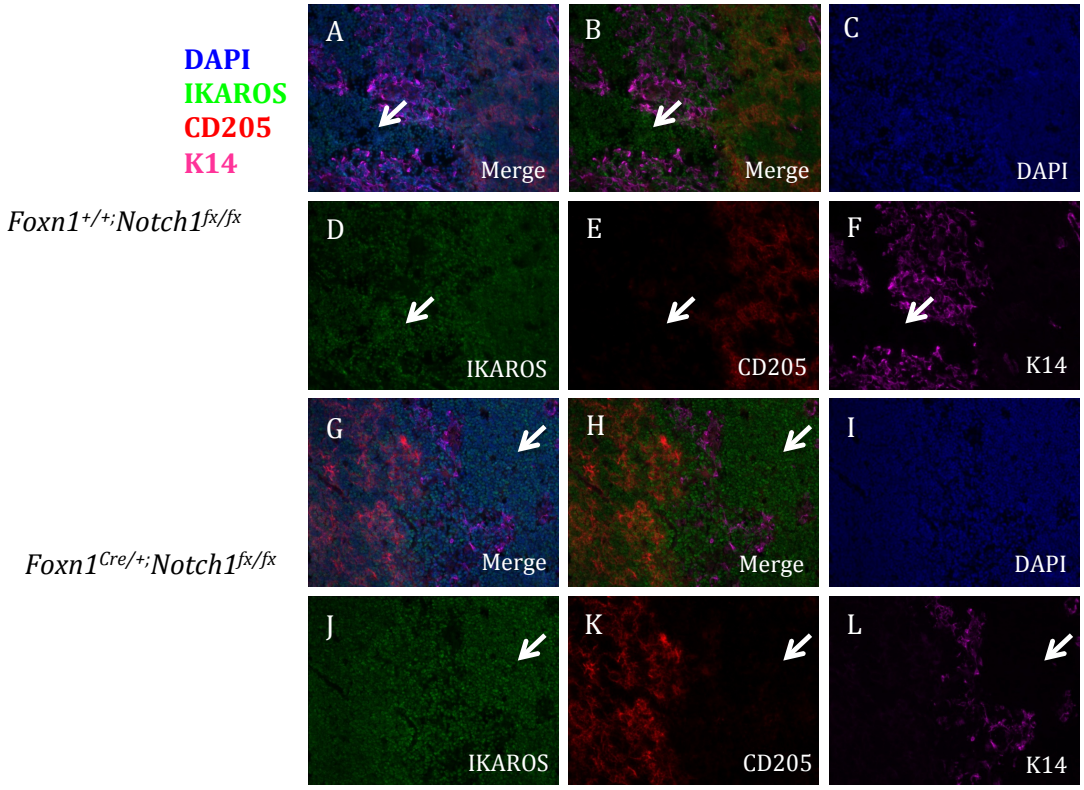


Fig 10: Epithelial-free zones are filled with IKAROS-positive lymphocytes in both *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant thymi and *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ control thymi at one month age. Both mutant and control thymi were immunostained with cTEC marker, CD205 (E,K, in red), mTEC marker K14 (F,L, in purple) and lymphocyte marker IKAROS (D, J, in green). Cells in epithelial-free zones that are negative for CD205 and K14 (shown by arrows) are positively stained by IKAROS, in both mutant and control thymi. Images are taken by a Zeiss Axionplan 2 Apotome microscope.

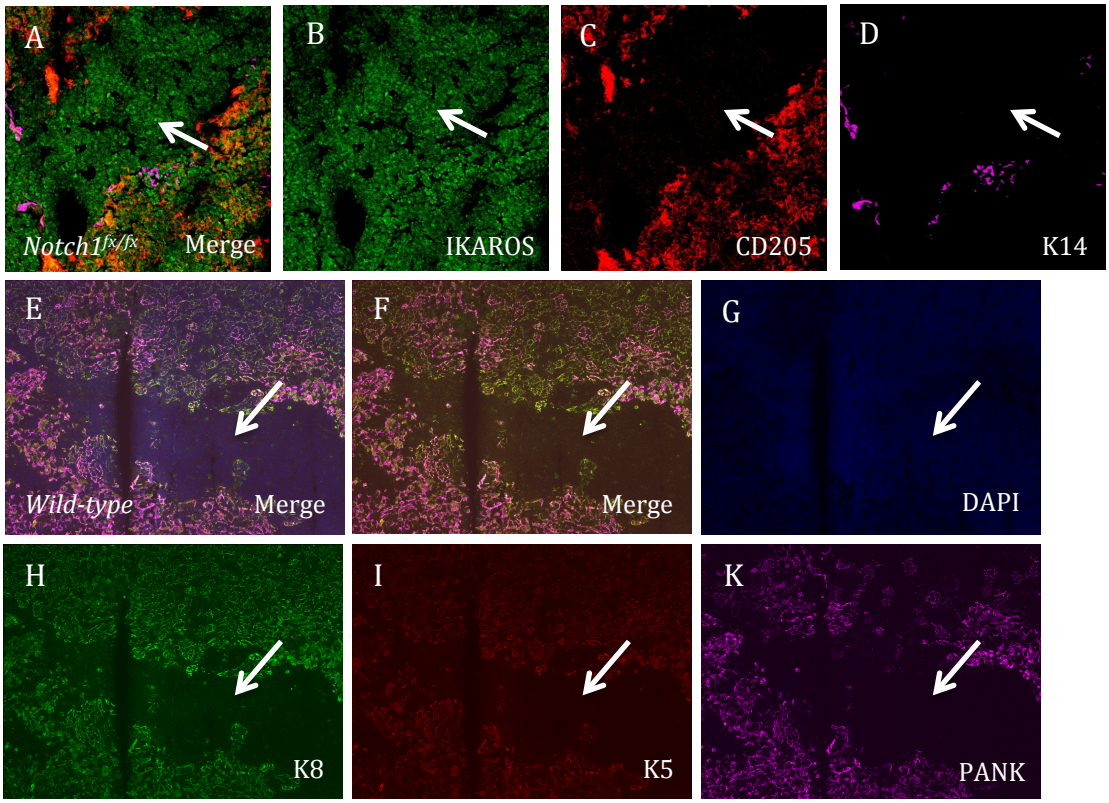
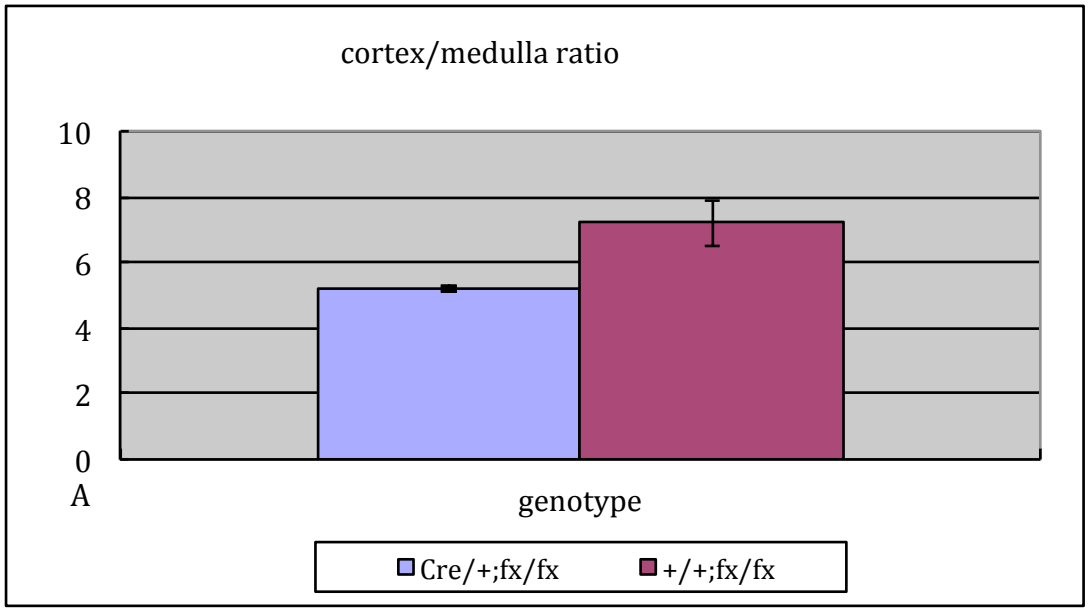


Fig 11: Epithelial-free zones are also found in *Notch1*$^{fx/fx}$ thymi and wild-type thymic at one month age, and are filled with IKAROS-positive lymphocytes too. Fig. A-D. At one month age, *Notch1*$^{fx/fx}$ thymi are immunostained with CD205, K14 and IKAROS, which are cTEC, mTEC and lymphocyte markers. Epithelial-free zones are found too and are filled with IKAROS-positive lymphocytes (shown by arrows). Fig. E-K. At one month age, *wild-type* thymi are immunostained with K8, K5, and IKAROS, which are cTEC, mTEC and lymphocyte markers. Again, epithelial-free zones are found and are filled with IKAROS-positive lymphocytes. Images are taken by a Zeiss Axionplan 2 Apotome microscope and a Zeiss LSM510 confocal microscope.



A

	c/m ratio-1	c/m ratio-2	Average	Standard Deviation	Deviation Square
Cre/+;fx/fx	5.2793	5.1139	5.1966	0.0827	0.01367858
+/+;fx/fx	6.606	7.7941	7.20005	0.59405	0.705790805

B

Fig 12: At one month age, the cortex/medulla area ratio suggests a trend of increase in *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant thymus compared with that in *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ control thymus. Panoramas of two sets of immunostaining of each thymus was used to calculate the cortex/medulla ratio. In total, two cortex/medulla ratios are generated for the two immunostainings for the mutant thymus and the control thymus. A. Raw data of cortex/medulla ratio statistics between the mutant and the control thymus. B. A chart is plotted from data in A. A trend of increase is shown in the mutant thymus. Images shown in Fig. 8 are used for calculation.

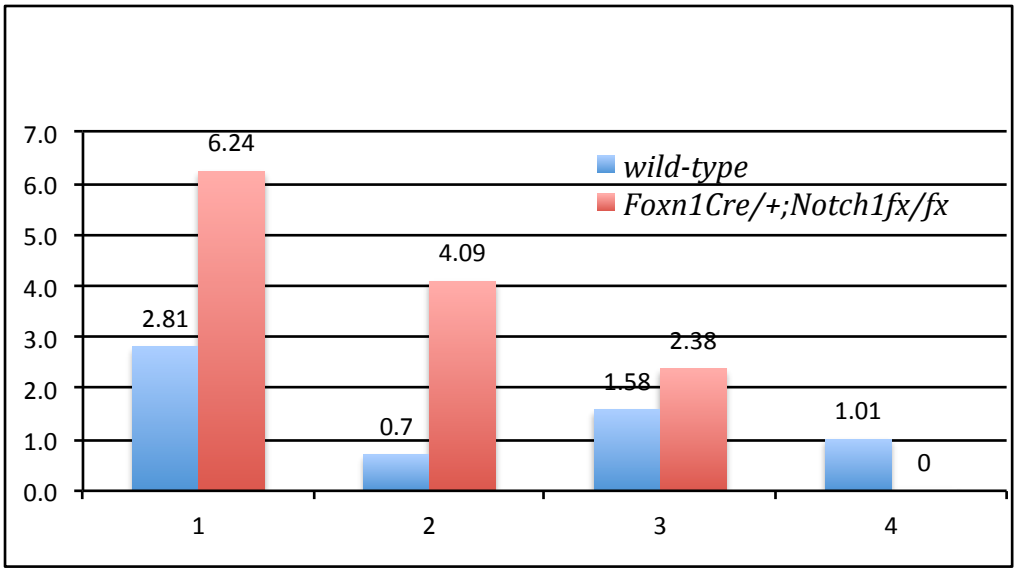


Fig 13: At one month age, the size of epithelial-free zone has a tendency of increase in *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant thymus compared with *wild-type* thymus. Parallels sections each from one quarter of the thymus were obtained from both thymi and immunostained with K8, K5 and PANK. Sizes of epithelial-free zones for each section were compared. Sections from *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant thymus tend to have a larger size of epithelial-free-zone in all three quarter parts of the thymus. The section from the bottom quarter of *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant thymus does not have epithelial-free zones.

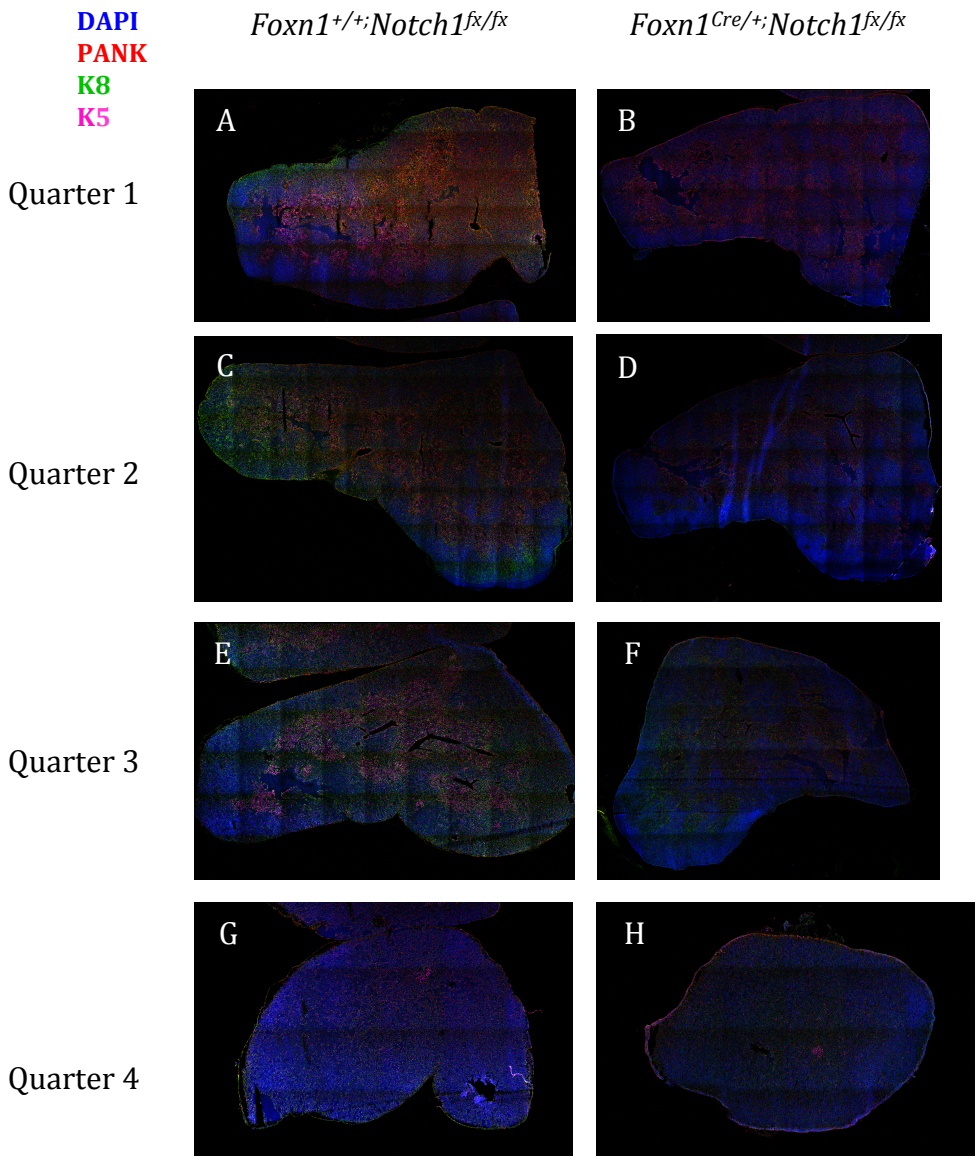


Fig 14: Original images used in the calculation in Fig. 13. At one month age, the size of epithelial-free-zone has a tendency of increase in *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant thymus compared with *wild-type* thymus. Parallels sections each from one quarter of the thymus were obtained from both thymi and immunostained with K8, K5 and PANK. Sizes of epithelial-free zones for each section were compared.

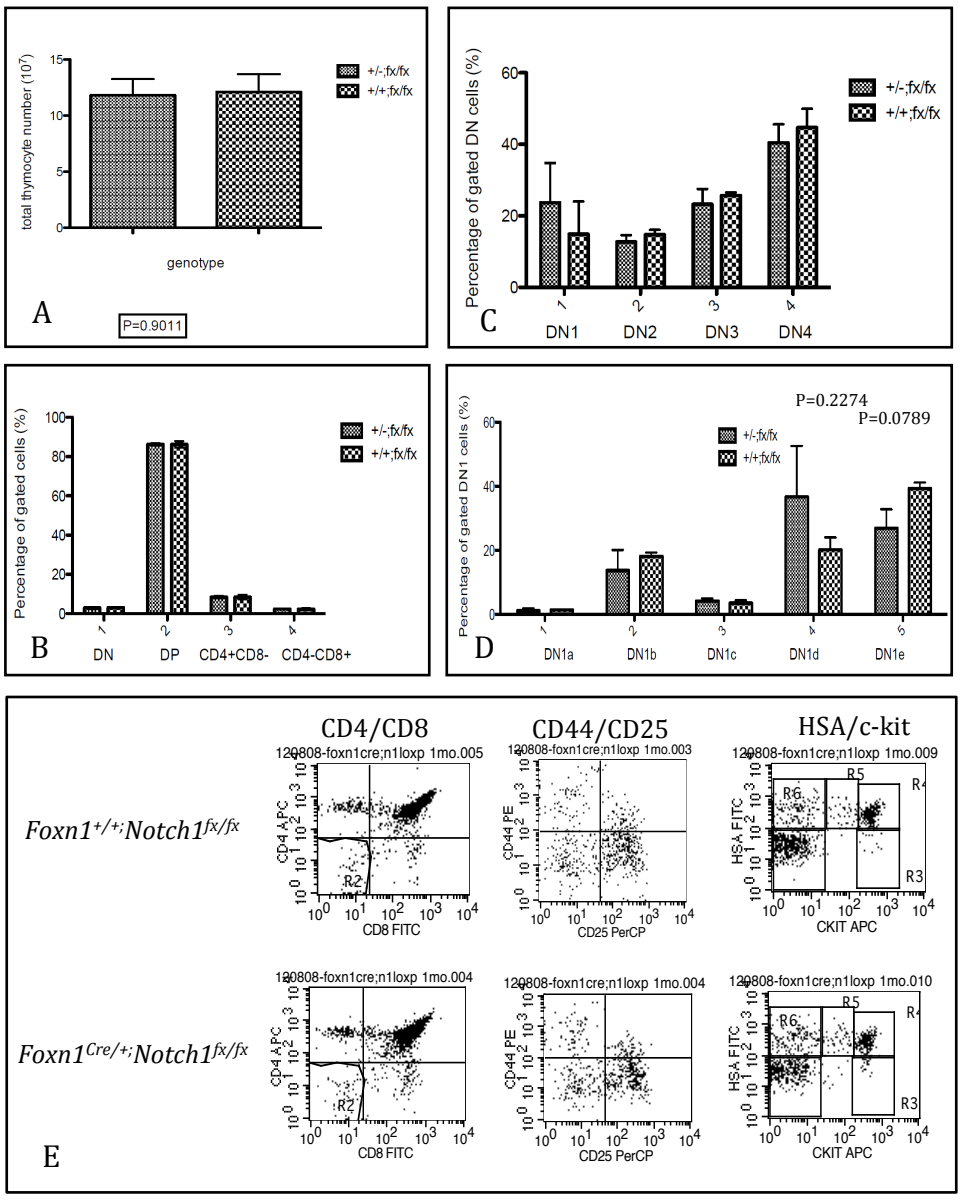


Fig 15: The ratios of CD4/CD8 T cells, the proportions of DN1 through DN4 cells and the proportions of DN1a through DN1e cells are comparable at one month age between *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutants and *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ controls. A. Total thymocyte numbers are comparable in mutant and control thymi. B-D. Subpopulations of thymocytes are compared. No significant differences are found between between *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutants and *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$ controls. E. Representative plots from mutant and control thymi are shown. +/+;fx/fx: *Foxn1*$^{+/+;}$*Notch1*$^{fx/fx}$. +/-;fx/fx: *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$. Three mutant and three control thymi were tested.

CHAPTER 5

FUTURE DIRECTIONS

Although it is quite obvious from the immunostaining that the numbers of PLET-1+ progenitor TECs are reduced in the *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant thymi, a more accurate quantification remains to be conducted. This can be achieved by FACS analysis of PLET-1 expression on sorted TECs from mutant and control thymi.

In the postnatal thymi, no significant differences either in the differentiation of TEC subsets or in the development of thymocytes were detected between *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutant and control thymi. It is possible that redundancy among Notch family members compensates the loss of NOTCH1 receptor in the thymic epithelial cells. To delete all Notch signalings from thymic epithelial cells, RBP-J$^{fx/fx}$ (Han et al. 2002) mouse strain can be used to cross our Foxn1-Cre strain. Phenotypic analysis will be informative whether Notch signaling is required in the postnatal development of thymic epithelial cells.

At embryonic stages, I have shown that Notch1 signaling is required for both the differentiation of mTEC and maintenance of progenitor TEC population, although it is possible that the fetal defects in mTEC differentiation may be secondary phenotypes due to the lack of progenitor TECs. To find out whether this phenotype is due to direct regulation of mTEC differentiation by Notch1 signaling or due to secondary effects of fewer progenitor TECs, the fold of reductions in UEA-1+

mTECs and PLET-1+ progenitor TECs should be carefully quantified and compared at each embryonic stage.

I am also interested in the mechanism or target genes that are involved in this regulation. One candidate is Wnt signaling pathway. Notch has been reported to regulate canonical Wnt signaling pathway by negatively titrating active β-catenin protein levels in stem and progenitor cells (Kwon et al. 2011). To test this, I have crossed a Wnt reporter strain TCF/LEF-GFP with *Notch1*$^{fx/fx}$ strain. By examining the GFP expression level in NOTCH1+FOXN1+PLET-1+ progenitors, I can test whether active Wnt signaling is going on in these progenitors. Furthermore, by comparing GFP expression levels in Foxn1$^{Cre/+;}$Notch1$^{fx/fx}$;TCF/LEF-GFP$^{GFP/0}$ mutants and their littermate controls, I can test whether Wnt signaling is affected by conditional knockout of Notch1 signaling from thymic epithelial cells. Gain-of-function modulation of Notch1 signaling by crossing Foxn1-Cre strain with Rosa26-StopFloxed-NICD (Murtaugh et al. 2003) strain, which will constitutively express NICD in all TECs, will also be informative to answer this question. If Wnt signaling levels are changed in these mice, it will suggest that Wnt signaling is regulated by Notch1 signaling. On the other hand, modulation of Wnt signaling by crossing Foxn1-Cre;Apc(CKO/CKO) strain (Kuraguchi et al. 2006) with *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mouse strain can also be informative. If the phenotypes in *Foxn1*$^{Cre/+;}$*Notch1*$^{fx/fx}$ mutants were rescued by activation of Wnt signaling level, it will be a strong evidence suggesting that Wnt signaling is a downstream target of Notch1 signaling involved in the regulation of maintenance of progentior TECs and differentiation of mTECs.

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