

THYMUS: A ROLLER COASTER OF FOXN1

by

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(Under the Direction of Nancy R Manley, Douglas B Menke)

ABSTRACT

The thymus is a primary lymphoid organ housing various stromal cells, such as thymic epithelial cells (TECs), that are vital for proper T-cell development (thymopoiesis), shaping adaptive immune systems in vertebrates. Positively correlating thymic size and naïve T-cell export change dynamically across the lifetime. The thymus exponentially increases its size and function from organogenesis to the perinatal stage; and maintains homeostasis until it starts to undergo ageing-associated atrophy, defined as thymic involution. The molecular mechanisms behind each transition remain unidentified.

Forkhead Box N1 (FOXN1) is a transcription factor required for thymic organogenesis, TEC differentiation, proliferation, and maintenance. Despite its TEC-restricted expression, FOXN1 also determines the maintenance of non-TEC stroma, such as mesenchyme and endothelium. As a lynchpin of thymus biology, FOXN1 is the focal point of this dissertation.

In this dissertation, we are going to explore how each transition occurs. In Chapter 1, we will show the down-regulation of insulin-like growth factor 2 (IGF2) is a likely suspect behind the thymic transition from perinatal expansion to juvenile homeostasis. Using time-series single-cell RNA sequencing of murine total thymic stroma and TECs, we report mesenchyme- and endothelium-derived IGF2 likely promotes proliferation in the neonatal thymus, causing rapid

thymic expansion. Our time-series bulk RNA sequencing of human thymi further supports the hypothesis, suggesting an evolutionarily conserved role of IGF2 on perinatal thymic expansion. In Chapter 2, we will show a premature decrease in *Foxn1* is sufficient to cause aged-like TEC phenotypes and thymopoiesis. We also report an independent effect of thymic involution on premature aged-like peripheral T-cell phenotypes and reduced response to flu infection challenge. In Chapter 3, we will show a mis- and over-expression of *Foxn1* is sufficient to directly programme murine embryonic fibroblasts (MEFs) into TEC-like cells, iTECs, capable of supporting thymopoiesis. We will dissect the iTEC lineage reprogramming process with bulk- and single-cell RNA sequencing. We will also show how to improve iTECs with known *in vivo* mechanisms, further establishing the iTECs as an *in vitro* platform to study TEC biology.

INDEX WORDS: [Thymus, FOXN1, Development, Organogenesis, Thymic involution, *In vitro* platform, Murine embryonic fibroblast lineage reprogramming, Single-cell RNA sequencing, Intercellular network analysis, PhD Dissertation]

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DEDICATION

I dedicate this work to my best friend, companion, and wife, Émilie Elizabeth Gille. I truly appreciate our mutual understanding, respect, adoration, devotion, and love throughout my PhD journey. I love you to the end of the observable universe and back. From S to É.

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TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	v
LIST OF TABLES	xi
LIST OF FIGURES	xii
CHAPTER	
1 INTRODUCTION	1
1.1. Thymic organogenesis	1
1.2. Thymic microenvironment.....	2
1.3. T-cell generation in the thymus: Thymopoiesis.....	3
1.4. Ageing-associated thymic atrophy: Thymic involution.....	5
1.5. The keystone of TEC biology: Forkhead Box N1 (FOXP1).....	6
1.6. Post-thymic involution: Thymic rejuvenation and <i>in vitro</i> platforms	8
2 INSULIN-LIKE GROWTH FACTOR 2 (IGF2) AS A FOXP1-INDEPENDENT DRIVING FORCE OF EXPONENTIAL EXPANSION IN NEONATAL THYMUS .	
.....	11
Abstract.....	12
Introduction.....	13
Results.....	15
Discussion.....	27
Figures & Figure Legends	31

Materials & Methods	52
3 PREMATURE THYMIC INVOLUTION IN YOUNG FOXN1 ^{LACZ} MUTANT MICE CAUSES PERIPHERAL T CELL PHENOTYPES SIMILAR TO AGING- INDUCED IMMUNOSENESCENCE	58
Abstract	59
Introduction	60
Results	61
Discussion	73
Figures & Figure Legends	78
Materials & Methods	104
4 MECHANISMS UNDERLYING THE DIRECT PROGRAMMING OF MOUSE EMBRYONIC FIBROBLASTS TO THYMIC EPITHELIAL CELLS BY FOXN1	112
Abstract	113
Introduction	114
Results	117
Discussion	131
Figures & Figure Legends	135
Materials & Methods	153
5 CONCLUSIONS	159
5.1. Up- and down-stream of IGF2-CyclinD1-E2F-FOXN1 axis	160

5.2. Overcoming threshold: enhancing iTEC yield, mTEC and MHCII gene expressions.....	162
5.3. Figures & Figure Legends	164

APPENDICES

A BUILDING INTERCELLULAR SIGNALLING NETWORKS TO IDENTIFY THE POTENTIAL SIGNALLING PATHWAYS FOR THE THYMUS/PARATHYROID CELL FATE SPECIFIERS.....	166
Introduction.....	166
Results & Discussion	166
Figures & Figure Legends	171
Materials & Methods	179
B DELETION OF BOTH E2F BINDING SITES FROM FOXN1 PROXIMAL PROMOTOR RESULTS IN ENDOTHELIUM/PERICYTE REDUCTION	180
Introduction.....	180
Results.....	180
Discussion.....	181
Figures & Figure Legends	183
Materials & Methods	185
C NEONATAL MURINE INTRATHYMIC INJECTION PROTOCOL	186
Introduction.....	186
Methods.....	186

Discussion.....	189
Figures & Figure Legends	191
D DEFINING PERINATAL THYMIC TRANSITION POINT	193
Results.....	193
Methods.....	195
Figures & Figure Legends	197
BIBLIOGRAPHY	201

LIST OF TABLES

	Page
Table 2.1: Top 5 DEG of pseudo-bulked endothelium and mesenchyme	45
Table 2.2: Human thymus sample demographic.....	46
Table 2.3: Age-independent expression of <i>Igf1</i> , <i>Igf1r</i> , <i>Insr</i> in human thymus.....	47
Table 3.1: Summary of the T-cell phenotypes in young adult Z/Z and old wt.....	96
Table 4.1: Absolute number of the cells expressing MHCII genes per sample.....	150

LIST OF FIGURES

	Page
Figure 2.1: scRNAseq of total thymic stroma from <i>Foxn1^{lacZ}</i> shows dynamic stromal changes after the perinatal transition point	31
Figure 2.2: Dynamic differences in TECs, endothelium, and mesenchyme from subsampling of scRNAseq data.....	33
Figure 2.3: IGF2 and IGF signalling is increased during neonatal expansion of thymus	35
Figure 2.4: <i>Igf2</i> & <i>Igf</i> -related gene expression varies with ageing	37
Figure 2.5: Prominent capsular IGF2 expression during murine neonatal thymic expansion	39
Figure 2.6: IGF2 expression is increased during human neonatal thymic expansion	41
Figure 2.7: Hypothetical model: fibroblast-derived IGF2 interacts with IGF1R on cTECs, non-TEC stroma, and thymocytes to drive neonatal thymic expansion.....	43
Supplementary Figure 2.1: <i>Igfbp1</i> & <i>Igfbp2</i> expression in perinatal TECs.	48
Supplementary Figure 2.2: Thymocytes expressing IGF1R.....	49
Supplementary Figure 2.3: Cortical and medullary expression of IGF2	50
Figure 3.1: scRNAseq analysis of CD45- total thymic stroma and thymic epithelial cells revealed an aged TEC phenotype in 1-month-old <i>Foxn1^{lacZ}</i>	78
Figure 3.2: Thymic selection and maturation of CD4 and CD8 SP thymocytes in young <i>Foxn1^{lacZ}</i> mutants	80
Figure 3.3: The proliferative capability of thymocytes was slightly increased in young <i>Foxn1^{lacZ}</i> mutants similar to aged wild type mice	82

Figure 3.4: Premature thymic involution in young <i>Foxn1^{lacZ}</i> mice caused an increase in thymocyte apoptosis similar to aged wild type mice	84
Figure 3.5: Total splenocyte scRNAseq comparison showed decreased naïve CD4 & CD8 populations in young <i>Foxn1^{lacZ}</i> mice	85
Figure 3.6: Young <i>Foxn1^{lacZ}</i> mice showed age-related phenotypic changes in peripheral lymphocytes	86
Figure 3.7: Young <i>Foxn1^{lacZ}</i> mice displayed mild peripheral lymphopenia	88
Figure 3.8: T cells from young adult <i>Foxn1^{lacZ}</i> mutants and aged mice had reduced proliferative capability after TCR stimulation.....	90
Figure 3.9: T cells from young adult <i>Foxn1^{lacZ}</i> mutants and aged mice had decreased production of cytokines after TCR-CD3 stimulation.....	92
Figure 3.10: Premature involution in <i>Foxn1^{lacZ}</i> mice caused impaired response and function of activated antigen specific T cells after infection with influenza.....	94
Supplementary Figure 3.1: Down-regulation of CD24 was reduced in young <i>Foxn1^{lacZ}</i> mutants and old wt mice	97
Supplementary Figure 3.2: Percentage of splenic B cells, NK cells, and granulocytes were increased in 2.5-months-old <i>Foxn1^{lacZ}</i> mutants.....	99
Supplementary Figure 3.3: Peripheral lymphopenia promoted weak homeostatic proliferation in young <i>Foxn1^{lacZ}</i> mutants and aged wild type mice	100
Supplementary Figure 3.4: Peripheral CD4 T cells displayed weak activation upon stimulation of TCR-CD3 signaling <i>in vitro</i>	101

Supplementary Figure 3.5: Intracellular cytokine staining of medium and isotype control.....	103
Figure 4.1: Generation and characteristics of R26 ^{CAG-Foxn1-IRES-GFP/+} MEFs.....	135
Figure 4.2: Schematic diagram of RNA-seq experiment design and general transcriptome analysis.....	136
Figure 4.3: Detailed gene expression comparison analysis of different stage of iTECs	138
Figure 4.4: scRNAseq of MEF control, 3diTECs, and 12diTECs.....	140
Figure 4.5: Bulk RNAseq analysis of Notch pathway analysis and DAPT treatment experiment....	142
Figure 4.6: Cell cycle analysis of iTECs and cell cycle rescue using iMYC transgene	144
Figure 4.7: Transcriptome and differential gene expression analysis between iTEC and E14.5 Fetal TEC shows EpCam ⁺ iTECs are missing specific genetic signatures	146
Figure 4.8: Co-culturing with E16.5 DP T-cells increase relative <i>H2-Aa</i> (MHCII gene) expression	148
Supplementary Figure 4.1: Gene enrichment analysis of up/down regulated genes at different reprogramming stages	151
Supplementary Figure 4.2: scRNAseq reveals high TEC genes in the <i>Foxn1</i> ^{Hi} cell cluster	152
Figure 5.1: Transgenic Rosa26-CD8 α -IGF1R design	164
Figure 5.2: RT-qPCR of iTECs with RANKL treatment	165
Figure A.1: Signal interactions across all cells at E10.25.....	171
Figure A.2: IGF signal interaction at E10.25.....	173
Figure A.3: Signal interactions across all cells at E11.25.....	174

Figure A.4: IGF signal interaction at E11.25.....	176
Figure A.5: Signal interactions between epithelium and mesenchyme	177
Figure A.6: Signal interactions within epithelium.....	178
Figure B.1: Deletion of both E2F binding sites of the <i>Foxn1</i> promotor results in reduced endothelium and pericytes	183
Figure C.1: Custom-made Hamilton syringe and its tip	191
Figure C.2: Perinatal intrathymic injection.....	192
Figure D.1: Undetectable sexual dimorphism across the perinatal transition point	197
Figure D.2: Endothelial cells and fibroblasts exhibit major transcriptome shift across the perinatal transition point	198
Figure D.3: Fibroblast and endothelial cell distribution across perinatal stages	200

CHAPTER 1

INTRODUCTION

1.1. Thymic organogenesis

The thymus is a primary lymphoid organ located at the pericardial mediastinum whose function is to generate self-restricted and self-tolerant T-cells, shaping adaptive immunity in vertebrates (Boehm and Bleul, 2007). The thymus is of endodermal origin and develops from the evaginated epithelial layer, third pharyngeal pouch (PP) (Gordon and Manley, 2011). At embryonic day (E) 9.5, the antero-dorsal region expresses Glial cells missing homologue 2 (*Gcm2*), a marker for the parathyroid domain. At E11.25, the postero-ventral region expresses Forkhead Box N1 (*Foxn1*), a marker for the thymus domain (Gordon and Manley, 2011). 3rd PP patterning of the *Foxn1* and *Gcm2* is not an autonomous process but rather shaped by signals from surrounding cells. The endodermal pharynx attached to the dorsal-proximal 3rd PP expresses Sonic hedgehog (*Shh*), which is necessary for the *Gcm2* expression (Moore-Scott and Manley, 2005). Ectodermal neural crest cell (NCC)-derived mesenchyme is hypothesised to shape *Foxn1*+ thymus fate. Ventral-distal NCC-derived mesenchyme sends Bone morphogenetic protein 4 (*Bmp4*) (Gordon et al., 2010), which is shown to drive *Foxn1* expression in avian 3rd PP (Neves et al., 2012). By E11.5, thymus and parathyroid cell fates are determined and thymic lobes start to migrate caudally around E13.5 and further differentiate into full thymus (Gordon and Manley, 2011). Overall, thymic organogenesis is a complicated process requiring various signal interactions between an epithelial layer and surrounding cells.

1.2. Thymic microenvironment

The murine thymus is conventionally divided into three histologically and functionally distinct compartments: cortex, medulla, and cortico-medullary junction (CMJ) (Anderson and Takahama, 2012; Takahama et al., 2017). Surprisingly, developing T-cells of various stages, defined as thymocytes, comprise approximately 99% of total thymic cellularity. About 1% of the young adult thymus consists of thymic epithelial cells (TECs), mesenchymal cells, endothelial cells, dendritic cells (DCs), and B-cells (Manley et al., 2011).

The endoderm-derived TECs have been traditionally categorised into two based on their location and function in thymopoiesis: cortical thymic epithelial cells (cTECs) and medullary thymic epithelial cells (mTECs) (Anderson and Takahama, 2012). Originating from a common Placenta expressed transcript-1+ (PLET1+) bipotent progenitor (Bennett et al., 2002; Gill et al., 2002), cTECs and mTECs regulate positive and negative selection of T-cells, respectively (Anderson and Takahama, 2012). The indispensable roles of TECs in self-restricted and self-tolerant T-cell generation will be reviewed in Chapter 1.3. Various studies show heterogeneity of both cTECs and mTECs, indicating their differentiation/maturation steps (Alawam et al., 2020). However, recent single-cell RNA and single-cell ATAC studies of TECs revealed new subsets of mTECs further contributing to negative selection, mimetic TECs (Michelson and Mathis, 2022). Mimetic TECs are TECs that express canonical markers of peripheral cells with similar morphology to the peripheral cells they are mimicking, such as tuft, microfold, muscle, ciliated, and neuroendocrine cells (Michelson and Mathis, 2022). Two major non-TEC thymic stroma comprises mesenchymal cells and endothelial cells (Manley et al., 2011). NCC-derived

mesenchymal cells in the thymus are mainly categorised as fibroblasts and pericytes. Fibroblasts encapsulating the whole thymus are defined as capsular fibroblasts (cFbs); fibroblasts populating the medulla as medullary fibroblasts (mFbs) (Nitta and Takayanagi, 2020). Vascularisation of the thymic endothelial cells and mesenchymal cells occurs around E13.5 and is prominent at E15.5 (Liu et al., 2006). Endothelial cells surrounded by pericytes tightly regulate the extravasation of HSCs and the egress of naïve SP T-cells to the periphery (Nitta and Takayanagi, 2020).

An overarching theme of the thymic microenvironment is bi-directional intercellular interactions defined as thymic crosstalk (van Ewijk et al., 1994). TECs are necessary for thymocyte development (reviewed in the Chapter 1.3.). In return, developing thymocytes is necessary for the organisation and expansion of the thymic medulla (Lopes et al., 2015), showing their co-dependency. Similarly, TECs promote endothelium proliferation by expressing vascular endothelial growth factor (VEGF) (Bryson et al., 2013; Müller et al., 2005); endothelial cells upregulate BMP4 upon insult that subsequently initiates TEC regeneration (Wertheimer et al., 2018). Furthermore, the thymic mesenchyme is shown to regulate TEC proliferation and differentiation (Nitta et al., 2020; Sitnik et al., 2012; Sun et al., 2015); produce angiogenic factors to increase endothelial cell proliferation (Sitnik et al., 2016).

1.3. T-cell generation in the thymus: Thymopoiesis

Thymopoiesis begins with the thymus recruiting bone marrow-derived haematopoietic stem cells (HSCs) into the thymic cortex through blood vessels located near the CMJ (Lind et al., 2001). Endothelial cells (ECs) encapsulating the thymic blood vessels regulate the thymic entry of

HSCs and differentiation of HSCs through LT β R and Kit-L expressions (Buono et al., 2016; Shi et al., 2016). Following thymic entry, HSCs differentiate into early thymocyte progenitors (ETPs) that are also known as DN1 (double-negative 1) due to the absence of both CD4 and CD8 T-cell receptors (TCRs) on the cell surface (Godfrey et al., 1993; Rothenberg, 2011). DN1/ETPs undergo a series of differentiations (DN1-DN4) and T-lineage commitment in the thymic cortex to become CD4 and CD8 TCR-expressing double-positive (DP) T-cells (Godfrey et al., 1993; Sidwell and Rothenberg, 2021). Post-differentiation, CD4⁺ CD8⁺ DP T-cells need to receive a survival signal from cTECs through TCR-self-peptide major histocompatibility complex (pMHC) interactions. Approximately 10% of the DP T-cells express TCR that can recognise pMHC; the rest of the DP T-cells do not receive a survival signal and undergo “death by neglect” (Takada and Takahama, 2015). The self-restricted DP T-cells then migrate into the thymic medulla to encounter self-pMHC-expressing antigen-presenting cells (APCs), such as mTECs, DCs, and B-cells (Klein et al., 2014; Takada and Takahama, 2015). Based on the affinity and availability of TCR-self-pMHC interactions, DP T-cells undergo agonist or negative selection, shaping central tolerance (Ashby and Hogquist, 2024; Takada and Takahama, 2015). Of note, tissue-restricted antigens (TRAs) are promiscuously expressed on mTECs by transcription factors AIRE, FEZF2, and PRDM1 accountable for specific sets of TRAs (Nitta et al., 2020). Interestingly, thymic mFbs are also shown to express a different set of TRAs in an LT β R-dependent fashion (Nitta et al., 2020), showing the importance of the thymic mesenchyme beyond structural maintenance. Self-restricted and self-tolerant naïve single-positive (SP) T-cells then egress through thymic blood vessels near

the CMJ under tight control of endothelial cells and surrounding pericytes (James et al., 2018; Xia et al., 2021; Zachariah and Cyster, 2010).

1.4. Ageing-associated thymic atrophy: Thymic involution

Despite its critical role in shaping the adaptive immune system, the thymus undergoes an age-associated atrophy, defined as thymic involution (Shanley et al., 2009). Total murine thymic cellularity peaks around four weeks after birth, after which it starts to decline progressively (Aw et al., 2008; Gray et al., 2006). Ageing-related thymic involution is not a mere reduction in organ size; it results in a functional decline modelled to be closely associated with increasing infectious disease susceptibility and increasing autoimmunity and cancer prevalence in aged individuals (Palmer et al., 2018). Thymic involution results in a significant decrease in naïve SP T-cell export, which causes a decrease in peripheral T-cell diversity and proliferation, and an increase in memory T-cells and senescent markers (Chaudhry et al., 2016). Declining thymic function is mainly due to the altered thymic microenvironment: TEC-specific loss accompanying increased adipocytes and senescent cells was observed with ageing both in humans and mice (Aw et al., 2008; Bains et al., 2009a; Bains et al., 2009b; Sempowski et al., 2000). In the murine thymus, both cTEC and mTEC proportions decrease with age accompanying disruption of the CMJ with mesenchymal infiltration (Aw et al., 2008; Aw et al., 2009). The direct connection between the thymic microenvironment and thymopoiesis is demonstrated by a study transplanting young thymi under the kidney capsules of old mice (Zhu et al., 2007). However, further studies are required to determine whether the

physiological ageing and ageing of HSCs contribute to the ageing of the adaptive immune system, defined as immunosenescence.

Epithelial-to-mesenchymal transition (EMT), as the name suggests, is a biological process during which epithelial cells lose epithelial markers whilst acquiring mesenchymal markers (Kalluri and Weinberg, 2009). EMT is a vital process throughout the development and tissue repair but is also shown to cause metastasis and fibrosis of aged organs (Thiery et al., 2009). Organ fibrosis, albeit necessary for tissue repair, can lead to disruption of the organisation and function of the organs (Henderson et al., 2020). Decrement of TECs with accumulation of fibroblast and adipocytes in the ageing thymus (Aw et al., 2008; Aw et al., 2009; Bains et al., 2009a; Bains et al., 2009b; Gray et al., 2006) due to increase in EMT (Dixit et al., 2008) strongly suggests EMT as a potential therapeutic target of the ageing-associated thymic involution. In addition to the ageing-associated thymic involution, an acute thymic degeneration may occur due to stressors (Gruver and Sempowski, 2008), such as pregnancy (Laan et al., 2016), infection (Savino, 2006), chemotherapy, and steroid hormones (Chaudhry et al., 2016). The stressor-induced involuted thymus is capable of recovery once the insult is removed which results in an immune reconstitution (Gruver and Sempowski, 2008), whose molecular mechanism may elucidate a potential mechanism behind thymic rejuvenation.

1.5. The keystone of TEC biology: Forkhead Box N1 (FOXP1)

A murine model “nude” carries an autosomal recessive mutation, whose homozygosity disrupts hair follicle and thymic development (Flanagan, 1966; Pantelouris, 1968). The mutated

gene of the *nude* model was later identified: *Whn/Foxn1* (Nehls et al., 1994). As per chapter 1.1., *Foxn1* is a marker for the 3rd PP cells committed to the thymic epithelial fate. Although *Foxn1* is not the thymic epithelial fate specifier (Blackburn et al., 1996; Gordon et al., 2001; Nehls et al., 1996; Nowell et al., 2011), it is necessary for the thymic epithelial progenitor cells (TEPCs) to exit their progenitor cell state (Blackburn et al., 1996; Jin et al., 2014). Moreover, *Foxn1*-hypomorphic *Foxn1* ^{Δ / Δ} , *Foxn1*^{*lacZ*/*lacZ*}, and *Foxn1*^{*R/R*} models suggest that *Foxn1* is necessary for proper thymopoiesis, TEC proliferation, and multiple stages of TEC differentiation in a dosage-sensitive fashion (Chen et al., 2009; Nowell et al., 2011; Su et al., 2003). Despite its TEC-restricted expression, *Foxn1* also affect thymic mesenchyme and endothelium development (Bryson et al., 2013; Mori et al., 2010; Nowell et al., 2011; Su et al., 2003), further emphasising the importance of *Foxn1* on the total thymic microenvironment.

In TECs, *Foxn1* expression is regulated by the cell cycle G1 checkpoint pathway, CyclinD1-Retinoblastoma (RB)-E2F3 (Garfin et al., 2013; Robles et al., 1996). A transgenic murine model with overexpression of CyclinD1 under the Keratin 5 (K5) promotor exhibits an indefinite thymic growth accompanied by an increase in *Foxn1* expression (Robles et al., 1996). E2F-binding sites were identified in the *Foxn1* promotor, which was shown by the positive correlation between E2F3 activity and *Foxn1* expression (Garfin et al., 2013). A murine model with null mutations in 5 out of 6 RB protein families also exhibits thymic hyperplasia, whose phenotype is rescued by crossing with a *Foxn1*-hypomorphic model, *Foxn1*^{*lacZ*} (Chen et al., 2009; Garfin et al., 2013). Taken together, proliferation, differentiation, and maturation are determined

by *Foxn1* expression in TECs. *Foxn1* expression in TECs is regulated by Cyclin D1 activity, which hyper-phosphorylates RB and enables E2F3-mediated transcription.

Foxn1 decrease in TECs is one of the earliest perturbations of the ageing thymus (Ortman et al., 2002). Accumulation of *Foxn1*^{low} and *Foxn1*⁻ cTECs and their subsequent functional decline were observed with ageing (O'Neill et al., 2016; Rode et al., 2015), suggesting a role of *Foxn1* in the thymic homeostasis that is further supported by a *Foxn1*-hypomorphic model *Foxn1*^{lacZ} (Chen et al., 2009). *Foxn1*^{lacZ} expresses a comparable *Foxn1* level to wild-type during development, exhibiting normal thymic organogenesis. At postnatal day 7, the relative *Foxn1* expression to wild-type is decreased by ~60%, resulting in a premature onset and accelerated rate of the thymic involution of the animals (Chen et al., 2009). Two genetic models further showed a declining *Foxn1* is likely the cause of the thymic involution. Inducing *Foxn1* expression in 12- and 24-month-old thymus was sufficient for a comprehensive thymus rejuvenation (Bredenkamp et al., 2014a). A transgenic murine model overexpressing *Foxn1* under the K14 promotor exhibited increases in both the thymic size and function with the decelerated rate of the thymic involution (Zook et al., 2011). The aforementioned evidences strongly suggest that *Foxn1*, a quintessential thymic transcription factor, is a lynchpin of TEC biology.

1.6. Post-thymic involution: Thymic rejuvenation and *in vitro* platforms

Multiple strategies were proposed for the rejuvenation of the thymus and adaptive immune system. Inhibition of sex hormones, such as androgen and oestrogen, results in thymic regrowth and immune reconstitution after insults (Hince et al., 2008; Sutherland et al., 2005). Despite the

unknown molecular mechanism, targeted, transient inhibition of sex hormones is a probable strategy to rejuvenate the thymus. Insulin-like growth factor 1 (IGF1)/growth hormone is another appealing strategy. Exogenous IGF1 treatment increased TEC proliferation, resulting in increased thymopoiesis and T-cell export (Chu et al., 2008). Growth hormone, which is upstream of IGF1 (Laron, 2001), is shown to increase TEC proliferation and thymopoiesis (Chen et al., 2003; de Mello-Coelho et al., 1998; Tsuji et al., 1994; Welniak et al., 2002). Fibroblast growth factor 7 (FGF7), which promotes TEC proliferation and differentiation during organogenesis (Revest et al., 2001), is shown to protect TECs from insults (Min et al., 2002; Rossi et al., 2002) and improved thymopoiesis post-transplantation (Wils et al., 2012). However, the impact of FGF7 on the thymic microenvironment and thymopoiesis outside of insult-contexts needs further studies.

Another approach is establishing *in vitro* platforms to support thymopoiesis *ex vivo*. The establishment of an *in vitro* platform to study TEC biology has been rather challenging due to various reasons. For instance, isolated TECs on the culture plate rapidly lose their surface proteins, including MHCII, which is necessary for thymopoiesis (Larsson et al., 1991; Teh et al., 1988). Commonly used *in vitro* platforms are foetal thymus organ culture (FTOC) and reaggregated thymus organ culture (RTOC) (Anderson and Jenkinson, 2000). Using E15.0 thymus, FTOC and RTOC have been used to study thymopoiesis *in vitro* (Anderson and Jenkinson, 2000). However, FTOC and RTOC require foetal thymi, which limits its translational application. A translational *in vitro* platform, artificial thymic organoid (ATO) was developed that successfully supports thymopoiesis *ex vivo* (Bosticardo et al., 2020; Seet et al., 2017) supporting the feasibility of the platform. We and others developed another *in vitro* platform that mis- and over-expresses *Foxn1*

in murine embryonic fibroblasts (MEFs) (Bredenkamp et al., 2014b; Ma et al., 2022). Overexpression of *Foxn1* successfully reprogrammed MEFs into TEC-like cells, defined as induced TECs (iTECs), capable of forming a thymus to support thymopoiesis under a kidney capsule (Bredenkamp et al., 2014b; Ma et al., 2022). Further studies are required for mTEC differentiation and MHCII upregulation, but a further study will provide us with a platform to understand *Foxn1* downstream targets and a molecular mechanism behind TEC differentiation. With tools to increase transcriptional programme, such as CRISPR-dCas9-VPR (Chavez et al., 2015), iTECs will be a viable platform to grow autologous T-cells *ex vivo* for transplantation.

This dissertation will follow the lifespan of the thymus: in Appendix A, I will explore candidates of FOXN1 specifier during organogenesis with intercellular network analysis. In Chapter 2, using time-series bulk- and single-cell RNA sequencing, I will show fibroblast-derived IGF2 is a likely candidate that drives exponential perinatal thymic expansion; its down-regulation is a likely causing the thymus to enter homeostasis, both in mice and humans. In Chapter 3, we will explore how a decrease in *Foxn1* is sufficient to cause premature ageing of the thymic microenvironment, resulting in aged-like thymopoiesis and peripheral immune niches. In Chapter 4, we will dissect an iTEC lineage reprogramming process with bulk- and single-cell RNA sequencing, and how to induce mTEC maturation and MHCII upregulation with known *in vivo* mechanisms.

CHAPTER 2

INSULIN-LIKE GROWTH FACTOR 2 (IGF2) AS A FOXN1-INDEPENDENT DRIVING FORCE OF EXPONENTIAL EXPANSION IN NEONATAL THYMUS

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Abstract

Like all organs, the thymus grows in size and function rapidly during development, which comes to a halt after birth. However, the molecular mechanism behind such a transition in the thymus remains unknown. Using single-cell RNA sequencing of the total murine thymic stroma across the perinatal stages, we determined the major transcriptomic changes occur in endothelium and mesenchyme across the transition. Differentially expressed gene (DEG) analysis and intercellular network analysis of our time-series single-cell RNA sequencing data revealed fibroblast-derived insulin-like growth factor 2 (IGF2) is a likely candidate driving fetal and neonatal thymic expansion. Our additional time-series single-cell RNA sequencing of murine thymic epithelial cells (TECs) suggested tight regulation of IGF2 activity at the thymic growth transition and showed how IGF2 promotes a cTEC-specific proliferation. Finally, our bulk RNA sequencing of the human thymi across the transition point also yielded IGF2 as a likely candidate driving the thymic expansion, suggesting an evolutionarily conserved role of IGF2 in the early thymic expansion. This study highlights the role of fibroblast-derived IGF signal promoting cTEC proliferation, resulting in early thymic expansion, followed by down regulation to establish homeostasis.

Introduction

The thymus is a primary lymphoid organ and a major source of self-restricted and self-tolerant naïve T-cells that shape the adaptive immune system. Neonatal humans and mice are immunocompromised partially due to unique T-cell production by the neonatal thymus. T-cells exported in neonates travel to an underpopulated periphery, resulting in an effector memory T-cell (T_{EM}) phenotype (Adkins, 1991; Adkins, 1999; Adkins, 2003; Adkins and Hamilton, 1992; Adkins and Hamilton, 1994; Opiela et al., 2009; Xiao et al., 2008). Neonatal T_{EM} cells are capable of rapidly responding to antigen stimulation but are unable to establish long-term memory (Akue et al., 2012; Zens et al., 2017), resembling virtual memory-like T-cells (T_{VM}). This unique neonatal T_{EM} phenotype is likely shaped by a combination of neonatal hematopoietic intrinsic factors, underpopulated secondary lymphoid organs, and a unique neonatal thymic microenvironment (Cuddihy et al., 2009; Gray et al., 2006; Srinivasan et al., 2021; Yang et al., 2015) that prevent development of a fully functional and diverse T-cell repertoire. However, neonatal T-cells alongside T_{EM} and T_{VM} are suggested to play a unique role in the early response to infection (Kawabe et al., 2017; Li et al., 2005; Li et al., 2007). Additionally, both murine and human data suggest the neonatal thymus plays a vital role in generating unique regulatory T (T_{reg}) cells that confer life-long protection from autoimmunity (Mold et al., 2010; Yang et al., 2015), highlighting the importance of the neonatal thymic microenvironment in the proper development of the adaptive immune system.

The thymus, like other organs, must grow during fetal and early postnatal development to keep pace with increasing body size, yet this expansion must halt and transition into homeostasis

to maintain size and function (Lui and Baron, 2011). In murine thymus, such a transition occurs 10 days after birth (P10) (Chen et al., 2009), but the mechanism behind this transition is not well understood. The cell cycle G1 checkpoint pathway CyclinD1-Retinoblastoma (RB)-E2F axis regulates thymic growth (Garfin et al., 2013; Robles et al., 1996). Murine models with either null mutations in the RB family (Garfin et al., 2013) or with constitutive expression of Cyclin D1 under the control of Keratin 5 (K5) promotor (Robles et al., 1996) fail to undergo homeostasis. In these models, the thymi continue to grow with a concurrent increase in *Foxn1* expression, which has an E2F transcription factor binding site that is regulated by RB. FOXN1 is a transcription factor in the thymus regulating both fetal and postnatal TEC differentiation and proliferation whilst maintaining postnatal thymic size and function (Chen et al., 2009; Manley and Condie, 2010; Manley et al., 2011; Nowell et al., 2011; Su et al., 2003; Vaidya et al., 2016). Despite its TEC-restricted expression in the thymus, *Foxn1* also indirectly affects development of the thymic vasculature and mesenchymal capsule in the non-TEC thymic stroma (Bryson et al., 2013; Mori et al., 2010; Nowell et al., 2011). *Foxn1* overexpression is sufficient to cause thymic overgrowth (Bredenkamp et al., 2014; Zook et al., 2011), suggesting regulation of the CyclinD1-RB-E2F-*Foxn1* axis is a molecular switch regulating the transition from neonatal expansion to juvenile homeostasis. However, how this switch is triggered to enable this transition is not understood.

In this study, we used time-series single-cell transcriptomic analyses to identify the molecular mechanism behind the transition from neonatal expansion to juvenile homeostasis in the perinatal murine thymus at postnatal day 10. To investigate the role of *Foxn1* in this transition, we used the *Foxn1*^{lacZ} murine model, which exhibits normal thymic organogenesis and has

comparable *Foxn1* levels to wild-type until postnatal day 7 when *Foxn1* levels drop by ~60% (Chen et al., 2009). Surprisingly, we found that the major changes in the thymic microenvironment across the thymic growth transition occurred similarly in both *Foxn1*^{+/+} and *Foxn1*^{lacZ/lacZ}. Instead, using ligand enrichment analysis and intercellular signaling mapping, we identified IGF2 as a candidate factor in this transition. Additionally, our human bulk transcriptomic analysis also identified *Igf2* and its transcriptional regulator as the most differentially expressed genes across the thymic growth transition. IGF2 is well-categorized as a part of the imprinted gene network (IGN) and well-studied in cancer biology due to its highly mitogenic properties. However, its role in normal thymic development is not well understood. Here, we highlight the potential role of IGF2 in driving fetal and neonatal thymic growth prior to homeostasis both in mice and humans, with its down regulation as a critical factor in establishment of homeostasis.

Results

Age- and *Foxn1*-dependent transcriptome patterns in the total thymic stroma

Crosstalk between thymocytes and TECs is essential for their respective development (Kaneko et al., 2019; Lopes et al., 2015; Meireles et al., 2017; Su et al., 2003; White et al., 2022). Similarly, the thymic mesenchyme and TECs are also co-dependent pre- and postnatally (Balciunaite et al., 2002; Bleul and Boehm, 2005; Chu et al., 2008; Frank et al., 2002; Gordon et al., 2010; Kvell et al., 2014). Based on the unique neonatal T_{EM} phenotype (Yang et al., 2015; Zens et al., 2017), we hypothesized a TEC-restricted transcription factor *Foxn1* drives the transition from thymic neonatal expansion to juvenile homeostasis occurring at postnatal day 10 (Chen et

al., 2009). First, we used time-series single-cell RNA sequencing to identify differences in the total thymic stroma of wild-type and *Foxn1^{lacZ}*. We submitted 3, 7, 14, and 30-day-old CD45- total thymic stroma of males and females with multiple biological and technical replicates. Unsupervised clustering 206,271 CD45- total thymic stromal transcriptomes with Seurat (Butler et al., 2018; Hao et al., 2021; Satija et al., 2015; Stuart et al., 2019) revealed a complex non-hematopoietic thymic microenvironment. Using the archetypal markers, we identified *Epcam*⁺, MHC-II⁺ thymic epithelial cells, *Pecam1*⁺, *Kdr*⁺, *Cldn5*⁺ endothelial cells, *Pdgfrb*⁺, *Acta2*⁺, *Myl9*⁺ mural cells, *Pdgfra*⁺ fibroblasts, *Upk3b*⁺, *Lrrn4*⁺, *Nkain4*⁺ mesothelial cells (Huang et al., 2022), and *Gfap*⁺, *S100b*⁺ non-myelinating Schwann cells (Hu et al., 2018; Kousa et al., 2023) (Fig. 2.1A).

As an initial comparison, we superimposed the dataset using age and genotype to detect patterns (Fig. 2.1B). Thymic epithelial cells did not exhibit major differences across age and genotype, except for the appearances of previously described stress-responsive cTECs and ageing-associated TECs (aaTECs) (Chapter 3) in 30-day-old male *Foxn1^{lacZ/lacZ}*, located at the centre of the UMAP (magenta-colored cell clusters) (Fig. 2.1B). Major transcriptome differences, visualized by non-overlapping colors, were observed in non-TEC stroma, especially capsular and medullary fibroblasts located at the bottom-left side and endothelial cells located at the bottom-right side of the UMAP (Fig. 2.1B). Regardless of *Foxn1* level, 3- and 7-day-old mesenchymal and endothelial cells clustered together suggesting similar transcriptomic identities, whilst 14- and 30-day-old datasets clustered away from 3 and 7-day-old datasets (Fig. 2.1B). To take all the variables into consideration, we separated the merged dataset based on age and genotype with a red line

indicating the thymic growth transition at postnatal day 10. With the exception of 30-day-old *Foxn1*^{lacZ/lacZ}, every age-matched dataset exhibited a similar transcriptome regardless of *Foxn1* level (Fig. 2.1C).

To validate the major changes in endothelium and mesenchyme across the thymic growth transition point, we pseudo-bulked the age- and genotype-matched datasets and generated PCA plots to compare the overall structure (Fig. 2.1D-G). PCA plots of fibroblasts, pericytes, and endothelial cells unanimously showed age distribution across PC1 (x-axis), suggesting age mostly contributed to the variable transcriptomic identities, especially after the thymic transition to homeostasis. Postnatal day 3 and day 7 datasets exhibited overall similarities, whereas 14- and 30-day-old samples were unique (Fig. 2.1D-G). PC2 (y-axis) of the PCA plots revealed that decreased *Foxn1* affected the endothelium and mesenchyme as early as 14 days postnatally, but the largest effect on non-TEC stroma was seen in 30-day-old *Foxn1*^{lacZ/lacZ} (Fig. 2.1D-G). Taken together, our data suggest a major non-TEC stromal change across the thymic growth transition is *Foxn1*-independent. Although some *Foxn1*-dependent changes were observed in the total thymic stroma, these occurred after the thymic transition to homeostasis.

Large shifts in the transcriptomic profile of thymic endothelium and mesenchyme across the perinatal transition point

To have a higher resolution on the transcriptomic changes before and after the thymic growth transition at postnatal day 10, we subsampled major stromal cell types: thymic epithelial cells, endothelial cells, and mesenchymal cells. First, we subsampled *Epcam*⁺ TECs and identified

various TEC populations based on their canonical markers: cTECs with varying degrees of *Epcam* (*Prss16*, *Syng1*, *Ly75*, *Pax1*, *Tbata*), thymic nurse cells (*Prss16*, *Cd3e*, *Rag1*), mTECs with varying levels of *Aire* (*Aire*, *Cd52*, *Fzf2*, *Cd80*), proliferative cTECs and mTECs (*Top2a*, *Ccnd1*, *Mki67*), *Ccl21a*⁺ TECs (*Ccl21a*) (Baran-Gale et al., 2020), Post-Aire TECs (*Spink5*), tuft-like TECs (*Avil*, *Pou2f3*), microfold-like TECs (*Gp2*, *Spib*, *Ccl20*), neuroendocrine TECs (*Snap25*, *Stxbp5l*, *Foxa3*), ciliated TECs (*Spag8*, *Dnah12*, *Dynlrb2*), and muscle TECs (*Myog*, *Myl1*, *Actc1*) (Michelson et al., 2022) alongside stress-responsive cTECs (*Atf3*, *Igfbp5*, *Gas1*, *Apoe*, *Fosb*, *Jun*) (Chapter 3) and ageing-associated TECs (*Zeb2*, *Notch3*, *Prrx1*) (Kousa et al., 2023, Chapter 3) (Fig. 2.2A). When we separated the wild-type datasets based on age, we observed a decline in all of the cTEC populations over time, except for *Atf3*⁺ stress-responsive cTECs that increased with age (Fig. 2.2B). Due to varying absolute number of cells per dataset class, we designed proportional graphs to visualize TEC subset change across time per genotype (Fig. 2.2C, E). In wild-type littermate samples, we observed a decrease in the perinatal cTEC population and an increase in the *Aire*^{Lo} mTEC population accompanied by a decrease in the *Aire*^{Hi} mTEC population across the thymic growth transition, denoted by a white line (Fig. 2.2C). Similar patterns could be observed from *Foxn1*^{lacZ/lacZ} datasets (Fig. 2.2D, E). Interestingly, the proliferative cTEC population was drastically decreased after the transition to homeostasis and was not detectable in both 30-day-old *Foxn1*^{+/+} and *Foxn1*^{lacZ/lacZ}, whereas the proliferative mTEC population remained similar (Fig. 2.2C, E). In contrast, we observed a premature appearance of stress-responsive cTECs in 7-day-old *Foxn1*^{lacZ/lacZ} as well as an appearance of unique ageing-associated TECs in 30-day-old *Foxn1*^{lacZ/lacZ} (Fig. 2.2D, E). The appearance of these two TEC subsets was likely caused by

decreased *Foxn1* expression, which is decreased by ~60% at postnatal day 7 in *Foxn1*^{lacZ/lacZ} (Chen et al., 2009).

We then subsampled the *Pecam1*⁺ endothelial population to visualize endothelial cell (EC) changes across postnatal day 10. We determined 4 different endothelial subsets based on the conventional marker expression pattern: arterial (*Cxcl12*, *Gja4*, *Gja5*, *Edn3*), venous (*Vwf*, *Icam1*, *Vcam1*), capillary (*Car4*, *Kdr*, *Sgk1*, *Sparc*) (Kalucka et al., 2020), and proliferative population (*Top2a*, *Ccnd1*, *Mki67*) (Fig. 2.2F). We observed a loss of proliferative ECs (left side of the UMAP) with ageing, whereas there was an increase in *Vwf*⁺ venous ECs with ageing regardless of relative *Foxn1* level (Fig. 2.2G, I). Interestingly, we observed a major transcriptome shift in capillary and arterial ECs. Capillary ECs originally formed clusters 2, 4 before forming new capillary EC clusters 1, 3 across the thymic growth transition (Fig. 2.2G, H, I, J). Of note, new capillary EC cluster 3 was very prominent in 30-day-old *Foxn1*^{lacZ/lacZ} (denoted by an asterisk in Fig. 2.2I) compared to the small proportion found in 30-day-old *Foxn1*^{+/+} (Fig. 2.2I, J), which further supports that decreased *Foxn1* expression caused a late phenotype after the thymic growth transition.

Finally, we subsampled *Pdgfra*⁺ *Pdgfrb*⁺ mesenchymal populations comprised of *Pdgfra*⁺ fibroblasts (Fbs) and *Pdgfrb*⁺ mural cells encompassing pericytes and vascular smooth muscle cells (vSMCs). Based on the quintessential markers, we identified capsular Fbs (*Pdgfra*, *Dpp4*, *Sfrp2*, *Lrrn4cl*), medullary Fbs (*Pdgfra*, *Serpine2*), mature medullary Fbs (*Pdgfra*, *Serpine2*, *Mmp9*, *Ltbp1*, *Col6a5*) (Nitta et al., 2021), vSMCs (*Pdgfrb*, *Myh11*, *Acta2*, *Myl9*) (Sorokin et al., 2020), pericytes (*Pdgfrb*, *Myl9*), and proliferative populations (*Top2a*, *Ccnd1*, *Mki67*) (Fig. 2.2K).

Similar to cTECs and EC populations, proliferative mesenchyme subsets (middle of the UMAP) disappeared with age (Fig. 2.2L, N). Regardless of *Foxn1* level, both capsular (cFbs) and medullary Fibroblasts (mFbs) formed different clusters after the transition to homeostasis, suggesting a major change in the transcriptome. For cFbs, cluster 1 disappeared and cluster 2 was formed after the transition to homeostasis. For mFbs, the appearance of mature mFbs cluster 1, 2, and 3 accompanied the loss of mFbs clusters 1 and 2 across the thymic growth transition. Similar to the capillary ECs, new mature mFbs cluster 3 (denoted by an asterisk) was prominent in 30-day-old *Foxn1*^{lacZ/lacZ} compared to a much smaller presence in 30-day-old *Foxn1*^{+/+} (Fig. 2.2I, J, N, O), again suggesting a phenotype caused by decreased *Foxn1* expression after the transition to homeostasis. On the contrary, vSMCs and pericytes did not exhibit a major change after postnatal day 10 (Fig. 2.2L, M, N, O). Overall, the most striking transcriptomic changes occurred in the thymic endothelium and mesenchyme across the thymic growth transition at postnatal day 10. Intriguingly, these changes were *Foxn1*-independent. Combined, these data suggest a TEC-extrinsic signal driving the thymic transition from neonatal expansion to juvenile homeostasis.

IGF2 is a potential switch that regulates neonatal expansion to juvenile homeostasis in murine thymus

To further investigate this potential TEC-extrinsic signal, we utilized CellChat to build intercellular networks (Jin et al., 2021) to detect changes in intercellular signals before and after the thymic growth transition. Since the ligand-receptor pair database is utilized to build intercellular networks, we used the ligand enrichment analysis to compare (1) 7-day-old *Foxn1*^{+/+}

and 14-day-old *Foxn1*^{+/+} followed by (2) 7-day-old *Foxn1*^{lacZ/lacZ} and 14-day-old *Foxn1*^{lacZ/lacZ} total thymic stroma.

When comparing 7- and 14-day-old *Foxn1*^{+/+}, *Igf2*, *Fn1*, and *Dlk1* were highly expressed ligands in 7-day-old *Foxn1*^{+/+}, whereas *C4b*, *C3*, *Wnt2*, *Fgf1*, and *Cxcl12* were highly expressed in 14-day-old *Foxn1*^{+/+}. The comparison of 7- and 14-day-old *Foxn1*^{lacZ/lacZ} datasets revealed a quite different pattern. *Igf2* and *Fn1* were still highly expressed ligands in 7-day-old *Foxn1*^{lacZ/lacZ}, but *Wnt5a*, *Wnt4*, *Tgfb3*, *Jag1*, *Cxcl12*, *Cxcl13*, and *Gas6* were highly enriched in 14-day-old *Foxn1*^{lacZ/lacZ} (Fig. 2.3A). Between *Foxn1*^{+/+} and *Foxn1*^{lacZ/lacZ}, *Igf2* was greatly enriched at 7 days, but expression was drastically reduced at 14 days. Due to our interest in a positive signal driving the initial exponential growth before the transition to homeostasis at postnatal day 10, we focused on the *Igf2*-mediated insulin-like growth factor (IGF) signal. This is concordant with the pseudo-bulked differentially expressed gene (DEG) analysis using a logistic regression test that revealed *Igf2* as the top 3 differentially expressed gene across the thymic growth transition (Table 2.1). Strikingly, the circle plots visualizing IGF signal comparison between 7- and 14-day-old datasets revealed IGF signal interaction strengths were decreased in 14-day-old thymi regardless of *Foxn1* level (Fig. 2.3B). In both 7-day-old *Foxn1*^{+/+} and *Foxn1*^{lacZ/lacZ}, capsular, mature medullary, and proliferative fibroblasts sent strong IGF signals to other stromal cells in both autocrine and paracrine fashion. On the contrary, in 14-day-old *Foxn1*^{+/+} & *Foxn1*^{lacZ/lacZ}, capsular fibroblasts were the sole source of IGF ligand and had substantially reduced overall relative signal strength (Fig. 2.3B). Heatmap analysis of IGF signal interaction probability further supported these observations (Fig. 2.3C). In 7-day-old *Foxn1*^{+/+} and *Foxn1*^{lacZ/lacZ}, capsular, mature medullary, and

proliferating fibroblasts sent IGF signals to thymic nurse cells, perinatal cTECs, *Aire*^{Hi} mTECs, and *Ccl21a*⁺ TECs (Fig. 2.3C, D). In 14-day-old *Foxn1*^{+/+} and *Foxn1*^{lacZ/lacZ}, only capsular fibroblasts were the major source of IGF signal. Although similar targets received IGF signals in 7- and 14-day-old samples, the interaction strength was much lower at 14 days (Fig. 2.3C, D). Together, our intercellular network analyses strongly suggest that IGF2-mediated proliferation drives the exponential growth and expansion of the neonatal murine thymus.

Dynamic IGF2 & IGF-related gene expression in total thymic stroma and TECs across perinatal transition point

IGF2 mediates murine embryonic growth during development through interactions with IGF1R that promote cell proliferation and prevent apoptosis. On the contrary, IGF2 interacting with IGF2R undergoes lysosomal degradation (Chao and D'Amore, 2008; Plasschaert and Bartolomei, 2014; Sélénou et al., 2022). IGF2-IGF1R interaction promotes CyclinD1 transcription (Hartmann et al., 2005; Preusser et al., 2018), which hyper-phosphorylates RB that enables E2F-mediated transcriptions (Chu et al., 2008). Additionally, IGF1 increases TEC numbers and proliferation, enhancing thymic function (Chu et al., 2008). To understand how *Igf2* and *Igf1r* expression changes over time, we calculated the average expression of each in our P3, P7, P14, and P30 scRNAseq datasets and plotted them as line graphs (Fig. 2.4A, D). We analyzed the expression of *Igf2* and *Igf1r* in TECs, endothelium, and fibroblasts as well as the total average expression at each time point. *Igf2* was highly expressed in fibroblasts prior to the thymic growth transition. However, from P7 to P14, *Igf2* expression decreased by ~85%. On the contrary, *Igf2*

expression in endothelial cells gradually dropped over time (Fig. 2.4A). *Igf2* expression in all of the subsets was not detectable at P30 (Fig. 2.4A). In the mesenchyme subsampled dataset, we observed high levels of *Igf2* expression in capsular, medullary, and proliferative fibroblasts in 3- and 7-day-old datasets, which disappeared after postnatal day 10 except for a capsular fibroblast subset in 14-day-old datasets (Fig. 2.4B, 2.2K). In the endothelium subsampled dataset, we observed high levels of *Igf2* expression in arterial, capillary, and proliferative endothelial cells in 3- and 7-day-old datasets. *Igf2* expression remained detectable in arterial ECs of 30-day-old datasets (Fig. 2.4C, 2.2F), suggesting a potential role in adult EC proliferation. For *Igf1r*, expression was decreased by approximately 50% in all of the major stroma at postnatal day 7, right before transition to homeostasis. At P14, *Igf1r* expression level in endothelium and fibroblasts was increased compared to P7, suggesting enhanced *Igf1r* during the thymic growth transition at postnatal day 10 (Fig. 2.4D). However, cTEC and *Ccl21a*⁺ TEC-restricted *Igf1r* expression in TECs continued to decrease during this time (Fig. 2.4D, E).

We analyzed the TEC-sorted wild-type time-series scRNAseq dataset to acquire a higher resolution of IGF-associated gene changes in TECs. *Igf1r* was highly expressed in cTECs (denoted by an asterisk) and *Ccl21a*⁺ TECs compared to mTECs (Fig. 2.4F), supporting our previous observations. In cTECs, *Igf1r* expression rapidly decreased until postnatal day 7 and transiently increased at postnatal day 10, after which it decreased again (Fig. 2.4F). IGF2-inhibitory *Igf2r* expression in cTECs, *Ccl21a*⁺ TECs, and *Aire*^{Hi} mTECs increased at postnatal day 10 (Fig. 2.4G), suggesting a tightly regulated IGF2 activity at the thymic growth transition. Interestingly, the *Igf2r* expression level in cTECs (denoted by an asterisk) was substantially lower than that in other TEC

subsets (Fig. 2.4G), further suggesting the role of IGF2 on cTEC proliferation. IGF-binding proteins (IGFBPs) bind to IGF1 and IGF2 to regulate their activities in a context-specific fashion (Allard and Duan, 2018). We surveyed the expression of murine IGFBP family genes (*Igfbp1-7*) in our TEC scRNAseq dataset (Fig. 2.4H-L, S. Fig. 2.1A, B). Anti-proliferative *Igfbp3* and *Igfbp7* expression (Jin et al., 2020) were rapidly increasing in cTECs over time (Fig. 2.4H, L), supporting a selective decrease in cTEC proliferation in aged thymi. Interestingly, IGF2-specific inhibitor *Igfbp6* (Bach et al., 2013) (Fig. 2.4K) was exclusively expressed in mTEC subsets, suggesting IGF2 activity inhibition in the thymic medulla. Additionally, expression of *Igfbp4* and *Igfbp5* that induces cellular senescence (Sanada et al., 2018) was increased in *Ccl21a*⁺ TECs across the perinatal stage (Fig. 2.4I, J), further supporting the IGF2 activity regulation in the thymic medulla. Of note, both *Igfbp4* and *Igfbp5* expression in cTECs were increased after postnatal day 15 (Fig. 2.4I, J), suggesting its role in aged cTECs. *Igfbp1* was exclusively detected in mTECs, but the overall expression was extremely low (S. Fig. 2.1A). Also, *Igfbp2*, which is highly expressed during development (Li et al., 2020), was highly expressed in neonatal cTECs and *Ccl21a*⁺ TECs, which immediately dropped at postnatal day 3 and maintained a plateau until it increased in cTECs at postnatal day 28 (S. Fig. 2.1B). Overall, our two independent time-series scRNAseq datasets strongly suggest IGF signaling via IGF2 is driving the exponential thymic expansion, and IGF2 activity regulation is causing the transition to thymic homeostasis.

Capsular IGF2+ fibroblasts may drive neonatal thymic expansion prior to homeostatic transition

Our scRNAseq data suggested mesenchyme, especially fibroblasts, is the main source of *Igf2* that could mediate the proliferation of cTECs. To validate our scRNAseq data, we used immunohistochemistry (IHC) to acquire *in situ* distribution of mesenchyme and soluble IGF2 protein. Due to CD3+ thymocytes expressing IGF receptors (S. Fig. 2.2A, B), measuring the proximity between IGF2 and IGF1R with IHC was not feasible. Age- and genotype-matched thymi to scRNAseq datasets were collected and processed for IHC-Frozen (Fig. 2.5, S. Fig. 2.3). Due to technical limitations, we chose Apolipoprotein D (APOD) as a fibroblast marker, identified from our scRNAseq data. There was an overall decrease in capsular fibroblast distribution with age, likely due to thymic expansion, and capsular fibroblasts were not prominent in 14-day-old *Foxn1*^{+/+} thymic sections (Fig. 2.5A-C). Strong IGF2 protein signal was observed throughout the subcapsular area in 3- and 7-day-old *Foxn1*^{+/+} thymi, whereas capsular IGF2 was not detected in 14-day-old *Foxn1*^{+/+} (Fig. 2.5D-F, S. Fig. 2.3A-C). IGF2 signal coincided with APOD+ fibroblast levels, further suggesting capsular fibroblasts were the source of IGF2 (Fig. 2.5A-F).

Similar to *Foxn1*^{+/+}, a decrease in capsular fibroblast distribution was observed with age in *Foxn1*^{lacZ/lacZ} thymi, (Fig. 2.5G-I). There was also a decrease in fibroblast frequency in 14-day-old *Foxn1*^{lacZ/lacZ} thymi compared to 3- and 7-day-old (Fig. 2.5I). Like in *Foxn1*^{+/+} thymi, IGF2 signal correlated with APOD+ fibroblast detection. There were strong IGF2 signals in the capsule of 3- and 7-day-old *Foxn1*^{lacZ/lacZ} thymi, whereas IGF2 was not detected in 14-day-old *Foxn1*^{lacZ/lacZ} (Fig. 2.5J-L, S. Fig. 2.3D-F). Additionally, we observed an overall greater IGF2 signal in the

thymic cortex (Fig. 2.5D-F, J-L, S. Fig. 2.3A-F). Moreover, there were high levels of IGF2 in the cortico-medullary junction (CMJ) and medulla in 3- and 7-day-old *Foxn1*^{+/+} and *Foxn1*^{lacZ/lacZ} thymi (S. Fig. 2.3G, H, J, K). Interestingly, we detected IGF2 in the 14-day-old *Foxn1*^{+/+} CMJ and medulla (S. Fig. 2.3I), but not in the 14-day-old *Foxn1*^{lacZ/lacZ} (S. Fig. 2.3I, L). Altogether, our IHC analyses strongly suggest capsular IGF2 from capsular fibroblasts drives exponential thymic expansion before postnatal day 10.

IGF2 may drive human neonatal thymic expansion

We mapped human thymic weight over time, which was obtained from an autopsy of 3,777 female and male subjects ranging from 16 weeks gestational age (shown as -24 weeks; birth as 0 weeks) to 52 weeks after birth (Archie et al., 2006; Pryce et al., 2014). In humans, the thymic transition from growth to homeostasis occurred between 12 and 16 weeks after birth (Fig. 2.6A). We then performed bulk RNA sequencing with 28 perinatal human thymi (Table 2.2) and stratified the datasets into two groups based on age: before and after three months. DEG analysis between the two groups yielded IGF2 and IGF2BP1 as the most differentially expressed genes. Mapping *Igf2* expression over time and separating into two groups before and after 3 months (0.25 years) showed a striking pattern. *Igf2* expression is highly maintained until three months when there is an immediate drop in expression after the transition to homeostasis with a strong negative correlation as ageing continues (Fig. 2.6B). *Igf2bp3*, which regulates *Igf2* transcription (Nielsen et al., 2003), exhibited a similar pattern with age, though the decrease in expression across the thymic growth transition was not as severe compared to *Igf2* (Fig. 2.6C). Overall, *Igf2* and its

transcriptional regulators were significantly decreased after the transition to homeostasis (Fig. 2.6D, F, G), suggesting an identical role of *Igf2* mediating neonatal thymic expansion in humans. Notably, the expression of *Igf2* receptors *Igf1r* and *Insr* did not change with age (Table 2.3), whereas the expression of *Igf2r*, a receptor sequestering IGF2, increased after the transition to homeostasis (Fig. 2.6E), which suggests increased regulation of IGF2 activity after three months. Also, expression of *Igfbp* family proteins (*Igfbp1-5*) was significantly reduced after three months (Fig. 2.6H-L), suggesting IGF2 activity regulation in the human thymus may differ from murine thymus (Fig. 2.4G-M). Additionally, our analysis of a published scRNAseq dataset (Heimli et al., 2022) revealed a similar pattern in human thymic fibroblasts. We also observed a substantial decrease in *Igf2* expression in human thymic fibroblasts after 3 months (Fig. 2.6M), supporting our hypothesis. Combined, our human RNAseq data strongly suggests IGF2 drives exponential expansion of the neonatal human thymus.

Discussion

Thymic size and function rapidly increase until 10 days (mice) (Chen et al., 2009) and 3 months (humans) (Fig. 2.6A) after birth, after which they reach a plateau and maintain homeostasis. However, the molecular mechanism behind this transition remained unknown. Transcription factor *Foxn1* is a lynchpin of TEC biology, for it determines TEC differentiation, proliferation, and maintenance as well as development of non-TEC thymic stroma (Bryson et al., 2013; Chen et al., 2009; Manley and Condie, 2010; Mori et al., 2010; Nowell et al., 2011; Su et

al., 2003; Vaidya et al., 2016). Here, using time-series single-cell RNA sequencing of *Foxn1*-hypomorphic model *Foxn1^{lacZ}*, we investigated the molecular mechanism/signaling pathway behind the thymic growth transition as well as the potential role of *Foxn1*. *Foxn1^{lacZ}* mice have an approximately 60% decrease in *Foxn1* expression beginning at postnatal day 7 (Chen et al., 2009). Rather intriguingly, this decrease did not have a significant impact on shaping the thymic microenvironment nor the thymic growth transition at postnatal day 10. Unlike our initial expectation, these results suggested that the transition from neonatal expansion to juvenile homeostasis was *Foxn1*-independent. Decreased *Foxn1* did however cause thymic changes at later time points such as a premature occurrence of *Atf3*⁺ stress-responsive cTECs and ageing-associated TECs in 30-day-old thymi (Fig. 2.2A-E). Furthermore, the premature decrease in *Foxn1* resulted in a rather late phenotype in the non-TEC stroma with unique clusters of endothelium and mesenchyme (Fig. 2.2F-O). Our results suggest regulation of murine neonatal thymic expansion itself is not downstream of the CyclinD1-RB-E2F-FOXN1 axis.

Our intercellular network analysis alongside unbiased DEG analysis with a logistic regression model strongly suggested the presence of *Igf2* and genes belonging to an imprinted gene network were the major source of distinct transcriptomes in endothelium and mesenchyme across the thymic growth transition (Fig. 2.3A). Thymic capsular and medullary fibroblasts were the main source of *Igf2*, which was mostly received by *Igf1r*-expressing thymic nurse cells, perinatal cTECs, and fibroblasts (Fig. 2.3C, D, Fig. 2.4A, B). IGF signaling regulates TEC proliferation during early thymic organogenesis likely via interactions between thymic mesenchyme-derived IGF1/2 and

TEC-derived IGF1R (Jenkinson et al., 2007). This hypothesis is further supported by dynamically regulated IGF receptors and IGF binding proteins in different subsets of TECs (Fig. 2.4E-M), even though it is possible IGFBPs can function independently (Allard and Duan, 2018). Of note, one of the major IGF interactions we observed was between fibroblasts and thymic nurse cells (Fig. 2.3B-D). Since thymocytes express IGF1R (S. Fig 2.2A, B), IGF2 in the neonatal thymus may also promote thymocyte proliferation, further contributing to neonatal thymic expansion. This is supported by a single-cell RNA sequencing study with thymocyte categorization of a murine model with a placental-specific deletion of *Igf2*, resulting in thymic hypoplasia. Placental *Igf2* deletion not only decreases the relative thymic cellularity but also alters thymopoiesis (Bacon et al., 2018), suggesting its impact on the TEC phenotype. Our human thymus bulk RNA sequencing data comparison before and after the thymic growth transition also yielded *Igf2* as a top differentially expressed gene (Fig. 2.6), suggesting an evolutionarily conserved role of IGF2 in driving neonatal thymic expansion.

With our immunohistochemistry data (Fig. 2.5, S. Fig. 2.3) suggesting a strong IGF2 presence in capsular fibroblasts before the transition to homeostasis, we generated schematic diagrams depicting our hypothetical model (Fig. 2.7). In neonatal thymus, capsular and medullary fibroblasts served as the main source of IGF2, sending signals to IGF1R-expressing thymic nurse cells, perinatal cTECs, thymocytes, and non-TEC stroma in a paracrine and autocrine fashion. Mitogenic quality of IGF2-IGF1R interactions would cause proliferation of the thymic nurse cells, perinatal cTECs, and thymocytes, resulting in a rapid expansion of the thymic cortex (Fig. 2.7A). This exponential expansion would come to a halt after postnatal day 10 due to significantly

decreased *Igf2* transcription in fibroblasts. Decreased IGF2 production would lead to reduced cell proliferation, causing the thymus to enter homeostasis (Fig. 2.7B). We propose fibroblast-derived IGF2 is the main source of IGF signaling in IGF1R-expressing cTECs during the neonatal period. This IGF signal would enable E2F-mediated *Foxn1* transcription in cTECs via phosphorylation of RB by CyclinD1 (Fig. 2.7C). Our hypothesis is concordant with an H₂K^b promotor-driven IGF2 overexpression murine model. This model exhibits a significantly larger thymic cortex compared to wild-type until 3 months of age, whereas the thymic medulla remains similar (Van der Ven et al., 1997). This IGF2 transgenic model poses an interesting question, however, as it is not the exact phenocopy of the K5.D1 model (Robles et al., 1996) or the RB-deficient model (Garfin et al., 2013), whose thymi grow indefinitely. This may be due to a decrease in *Igf1r* expression, but how this is regulated in cTECs remains a question. Overall, our study suggests IGF signaling via IGF2-IGF1R interaction promotes the proliferation of thymocytes, perinatal cTECs, endothelium, and fibroblasts, resulting in exponential neonatal thymic expansion prior to the transition to homeostasis at postnatal day 10. Comprehensive identification of the IGF family protein and receptor regulation in the thymus could be key to understanding thymic size expansion and maintenance.

Figures & Figure Legends

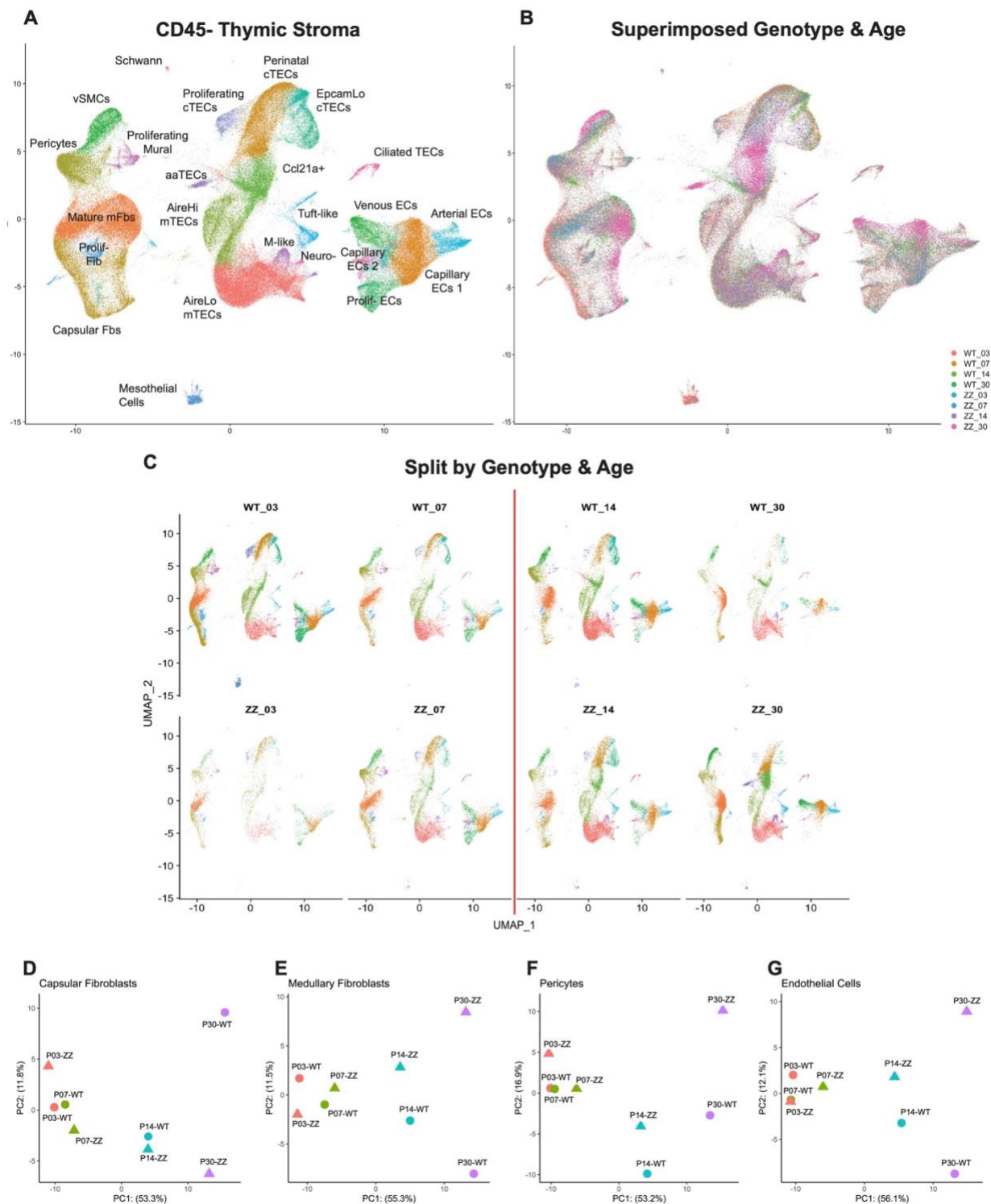


Figure 2.1. scRNAseq of total thymic stroma from *Foxn1^{lacZ}* shows dynamic stromal changes after the perinatal transition point.

(A) UMAP visualization of 206,271 CD45- total thymic stromal cells with annotations. (B) UMAP visualization of superimposed datasets split by age and genotype. *Foxn1^{lacZ}* wild-type littermates and *Foxn1^{lacZ/lacZ}* homozygotes are denoted by WT and ZZ, respectively. Numbers indicate the age of the datasets. (C) UMAP visualizations of merged dataset split by age and genotype. The red line indicates postnatal day 10, a thymic transition point from neonatal expansion to juvenile homeostasis (the perinatal transition point). (D-G) PCA plots of pseudo-bulked data classes split by age and genotype.

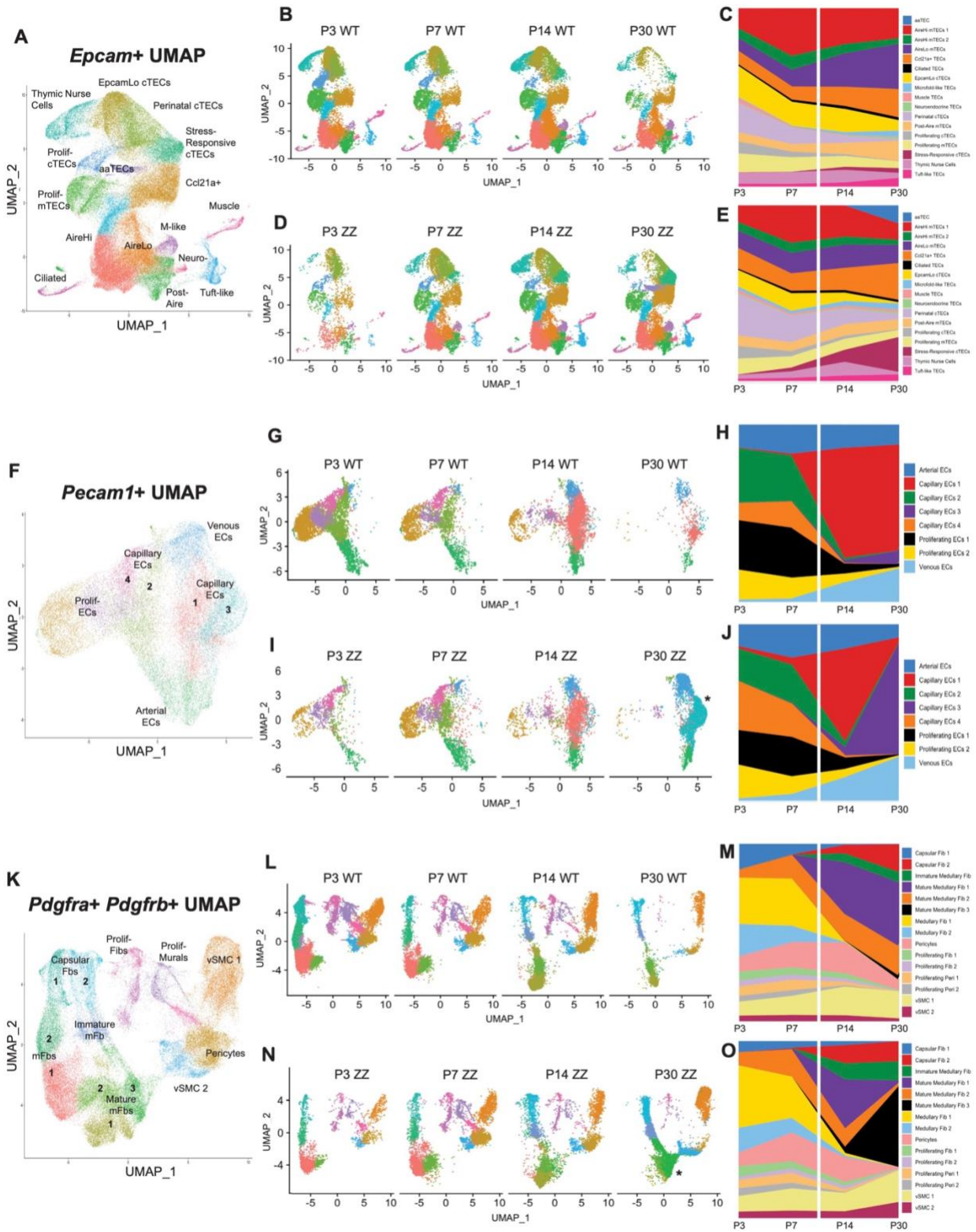


Figure 2.2. Dynamic differences in TECs, endothelium, and mesenchyme from subsampling of scRNAseq data.

(A, F, K) UMAP visualizations of TECs, endothelium, and mesenchyme based on known markers with annotations. Cluster numbers were ranked based on the number of cells per cluster. (B, D, G, I, L, N) UMAP visualizations of subsampled datasets split by their age and genotype. (C, E, H, J, M, O) Percent stacked charts showing the proportion of cell clusters across time. The white lines indicate the perinatal transition point. * indicates endothelium and mesenchyme clusters unique to P30 *Foxn1^{lacZ/lacZ}* dataset.

A



B



C



Figure 2.3. IGF2 and IGF signaling is increased during neonatal expansion of thymus.

(A) Ligand enrichment comparison of 7-day-old and 14-day-old *Foxn1*^{+/+} (top panels) and 7-day-old and 14-day-old *Foxn1*^{lacZ/lacZ} (bottom panels). The red line indicates the perinatal transition point. (B) Circle plots of IGF interactions amongst different cell types. The direction and thickness of arrows indicate interaction strengths that are deduced from the weight of ligand/receptor expressions per cell basis. The top panels are *Foxn1*^{+/+}, bottom panels are *Foxn1*^{lacZ/lacZ}. (C, D) Heat maps of IGF interactions amongst different cell types. The Y-axis indicates the clusters expressing ligands; the X-axis indicates the clusters expressing receptors.

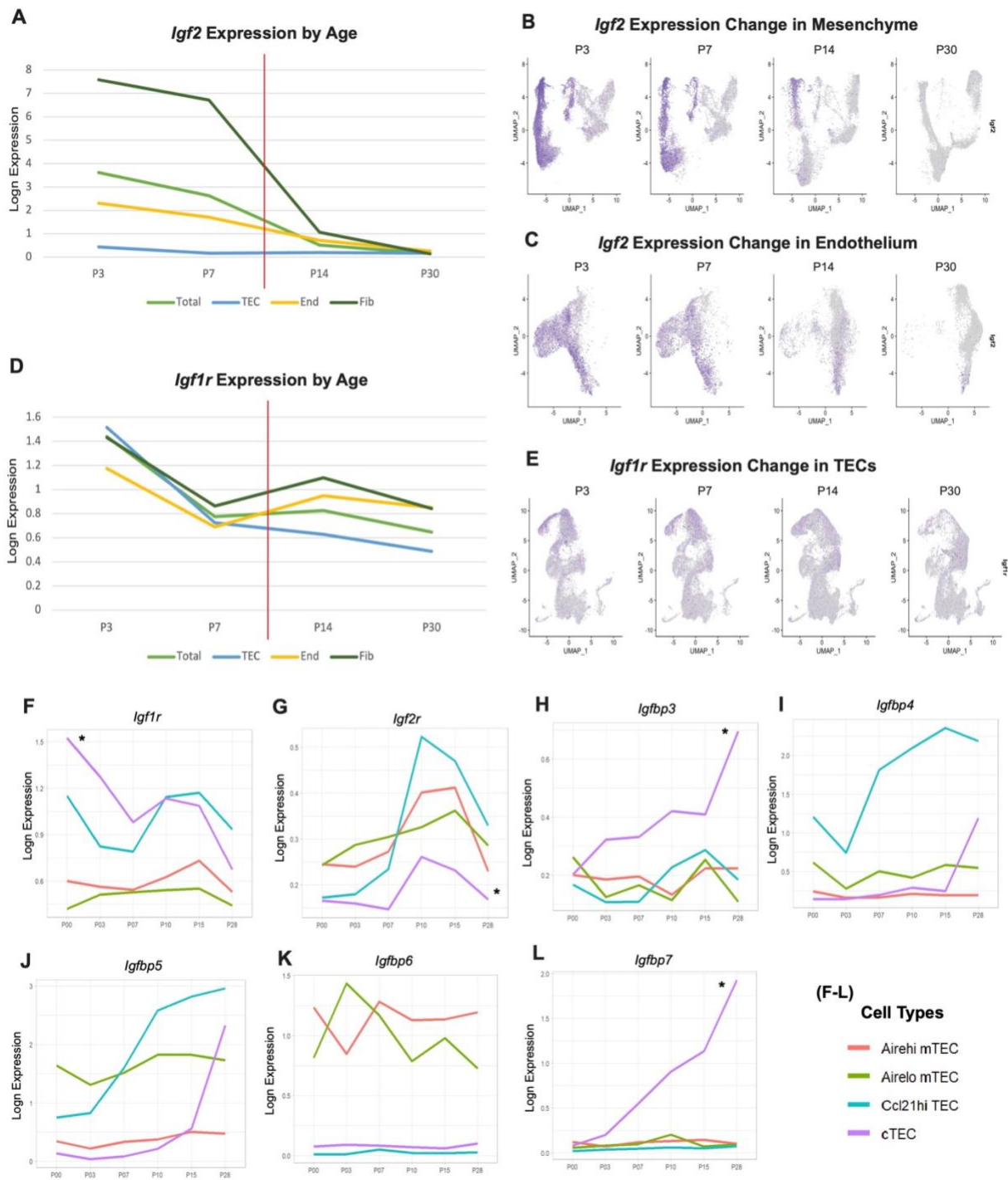


Figure 2.4. *Igf2* & *Igf*-related gene expression varies with aging.

(A, D) Average expression of *Igf2* & *Igf1r* calculated from total and subsampled scRNAseq across time. The red lines indicate the perinatal transition point. (B, C) Feature plot visualization of *Igf2* in *Pdgfra*⁺ *Pdgfrb*⁺ mesenchyme & *Pecam1*⁺ endothelium across time. (E) Feature plot visualization of *Igf1r* in *Epcam*⁺ TECs across time. (F-L) Data was acquired from a new time-series scRNAseq of TECs from 0-, 3-, 7-, 10-, 15-, and 28-day-old thymi. Line graphs show the average expression of IGF2 receptors and IGF binding proteins across time in different TEC subsets. Figure legends are located at the bottom right. TEC: thymic epithelial cells, End: endothelial cells, Fib: fibroblasts. Asterisks (*) are added for visualization purposes.

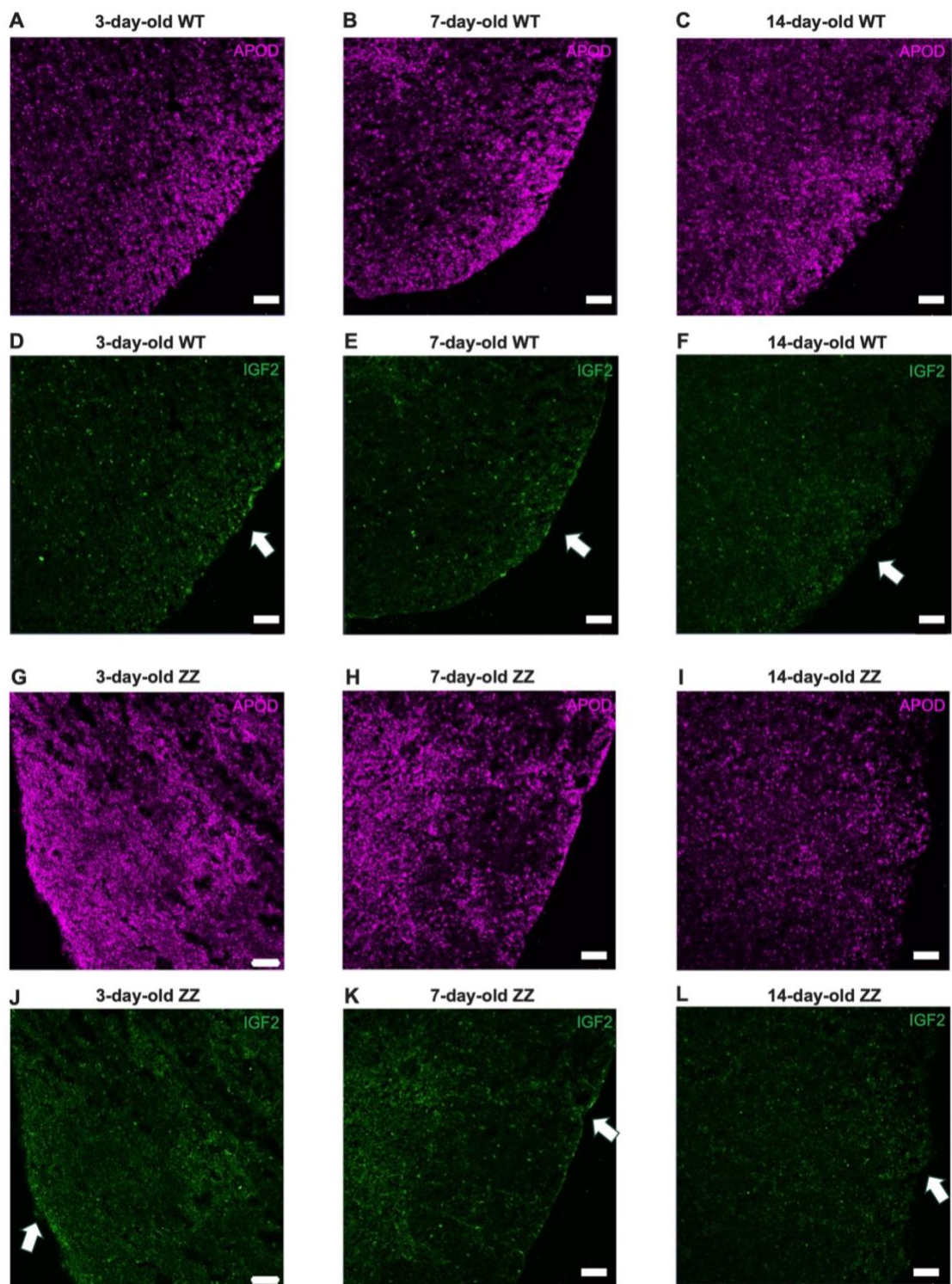


Figure 2.5. Prominent capsular IGF2 expression during murine neonatal thymic expansion.

Immunohistochemistry (IHC) of frozen thymic sections (10 μ m). (A-C) APOD+ fibroblasts in 3-, 7-, and 14-day-old *Foxn1*^{+/+} males. (D-F) IGF2 distribution of the identical sections to panel A-C. Arrows indicate APOD concentrated region. (G-I) APOD+ fibroblasts in 3-, 7-, and 14-day-old *Foxn1*^{lacZ/lacZ} males. (J-L) IGF2 distribution of the identical sections to panel G-I. Arrows indicate APOD concentrated region. WT: *Foxn1*^{+/+}, ZZ: *Foxn1*^{lacZ/lacZ}. N = 2 per category, Scale bars = 50 μ m.

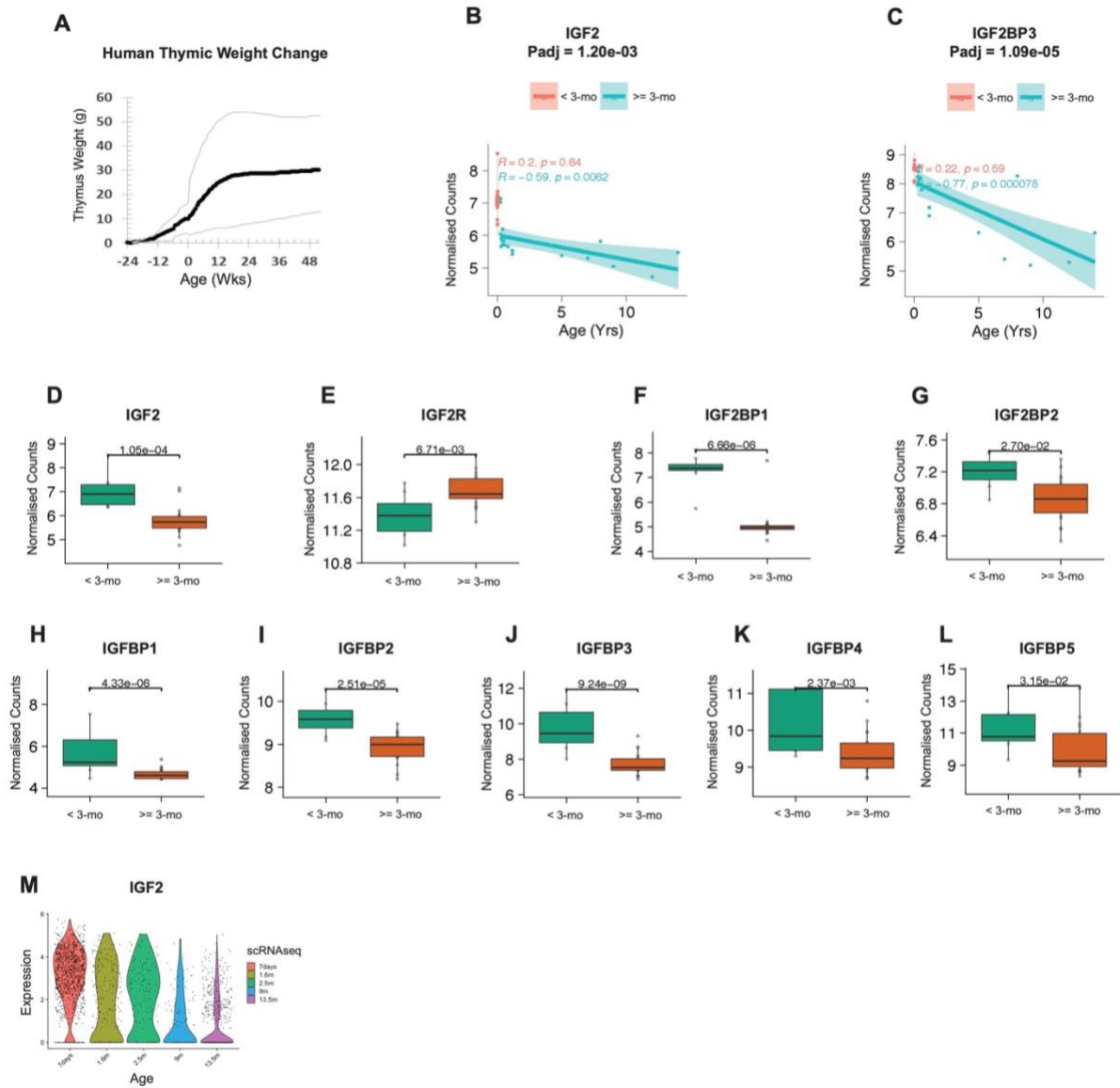


Figure 2.6. IGF2 expression is increased during human neonatal thymic expansion.

(A) Human thymus weights compiled from published sources (Archie et al., 2006; Pryce et al., 2014) were replotted as a function of age. Birth is defined as day/week 0. The 5th and 95th percentiles are indicated by grey lines. (B, C) Scatter plot visualizations showing *Igf2* and *Igf2bp3* across age. Pink indicates samples before 3 months, teal indicates samples after 3 months. (D-L) Boxes and whiskers plots visualizing IGF2-related genes before and after 3 months (0.25 years). (M) Violin plot visualizing *Igf2* expression in thymic fibroblasts, using published human thymus scRNAseq dataset (Heimli et al., 2022). R: correlation coefficient. p: adjusted p-values calculated from Wald test.

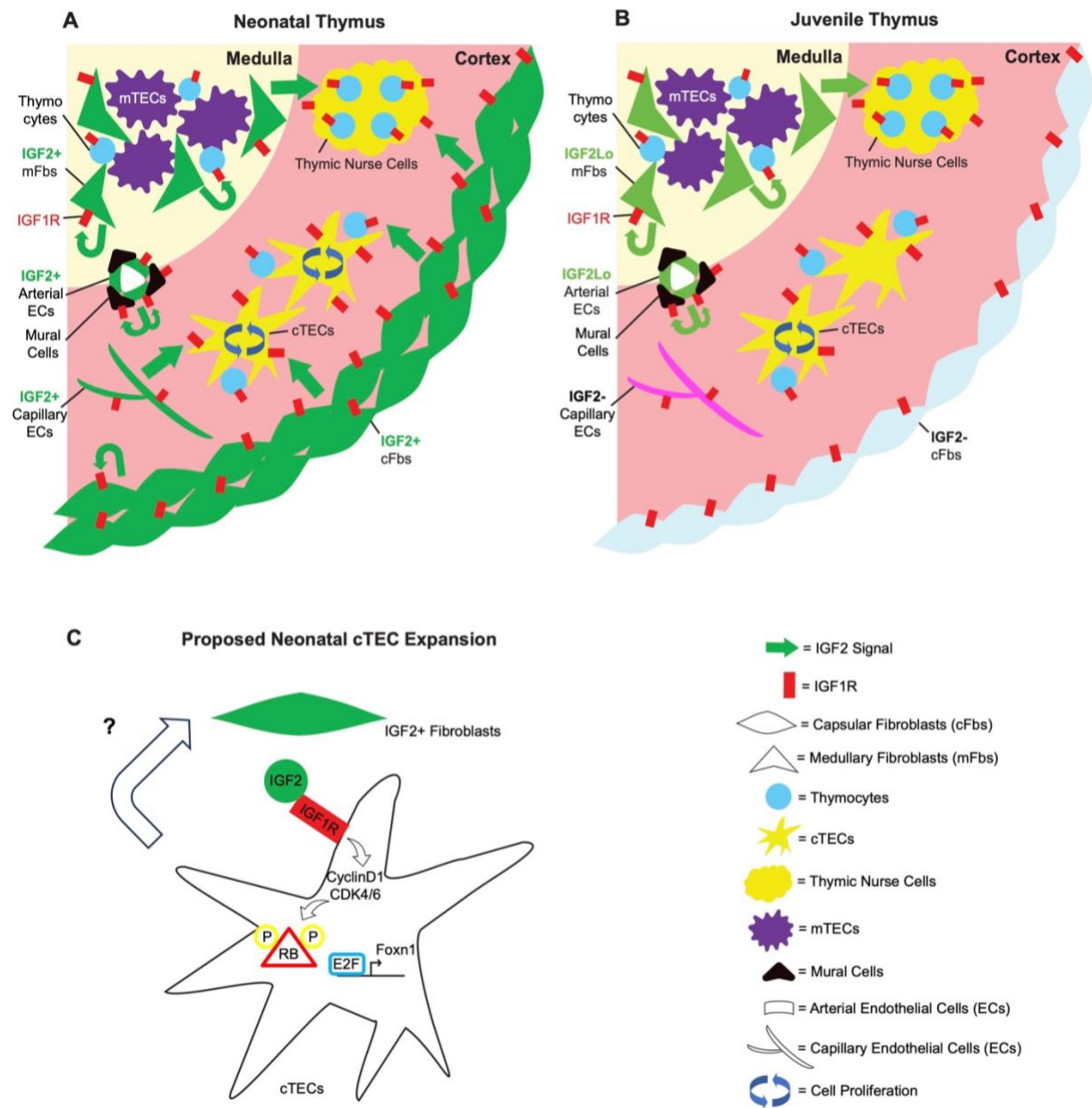


Figure 2.7. Hypothetical model: fibroblast-derived IGF2 interacts with IGF1R on cTECs, non-TEC stroma, and thymocytes to drive neonatal thymic expansion.

Schematic diagrams of our hypothetical model. (A) Strong IGF2 signal is coming from non-TEC stroma in an autocrine and paracrine fashion, promoting proliferation of cTECs, non-TEC stroma, and thymocytes. (B) After postnatal day 10, the IGF2 signal is diminished and the proliferation of cTECs and non-TEC stroma slows down, resulting in thymic homeostasis. (C) Fibroblast-derived IGF2 interacts with IGF1R. Through CyclinD1-CDK4/6, RB is hyperphosphorylated and detached from E2F. This enables E2F-mediated transcription of *Foxn1* in cTECs, promoting their proliferation.

Endothelium					
	p_val	avg_log2FC	pct.1	pct.2	p_val_adj
Mest	0	1.915944891	0.811	0.251	0
Ccnd1	0	1.208513309	0.872	0.532	0
Igf2	0	1.081733948	0.55	0.146	0
Apln	0	0.986626533	0.446	0.089	0
Rbm3	0	0.982894688	0.909	0.639	0

Mesenchyme					
	p_val	avg_log2FC	pct.1	pct.2	p_val_adj
DIK1	0	3.036904601	0.836	0.121	0
Mest	0	2.244602716	0.844	0.204	0
Igf2	0	2.166934013	0.9	0.273	0
Peg3	0	2.162785689	0.882	0.383	0
Itm2a	0	2.154728963	0.914	0.569	0

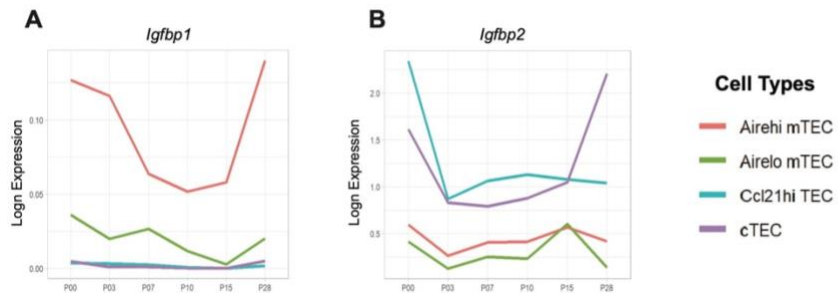
Table 2.1. Top 5 DEG of pseudo-bulked endothelium and mesenchyme

Sample ID	Age (yrs)	Sex
T2010	0.5	Male
T2012	0.008	Male
T2013	8	Male
T2015	0.25	Female
T2016	0.013	Female
T2018	0.3	Female
T2024	0.416	Female
T2025	0.25	Female
T2027	5	Female
T2033	9	Female
T2035	1.167	Female
T2036	7	Female
T2038	12	Male
T2039	14	Female
T2044	1.166	Female
T2046	0.008	Male
T2048	0.008	Female
T2050	0.328	Male
T2052	0.021	Male
T2055	0.328	Male
T2056	0.008	Male
T2057	0.024	0
T2059	0.833	Male
T2060	0.583	Male
T2063	0.333	Female
T2064	0.25	Male
T2065	0.4166	Female
T2067	0.016	Female

Table 2.2. Human thymus sample demographic

Gene_name	Mean	log2FoldChange	lfcSE	stat	pvalue	padj	weight
IGF1	9.934	-0.611314943	0.311	-1.963	0.0496	0.158	0.844
IGF1R	1238.4	-0.223700525	0.097	-2.299	0.0215	0.064	1.332
INSR	416.85	-0.292287167	0.135	-2.159	0.0308	0.097	1.058

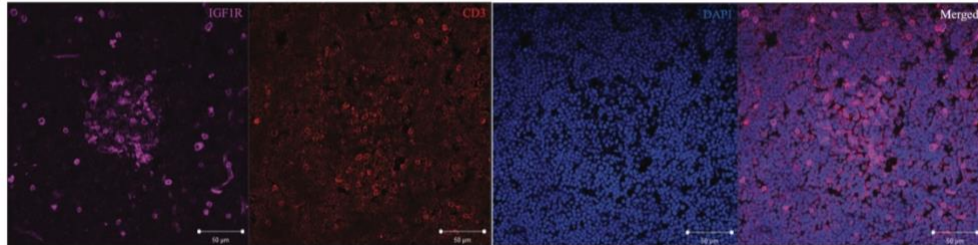
Table 2.3. Age-independent expression of *Igf1*, *Igf1r*, *Insr* in human thymus



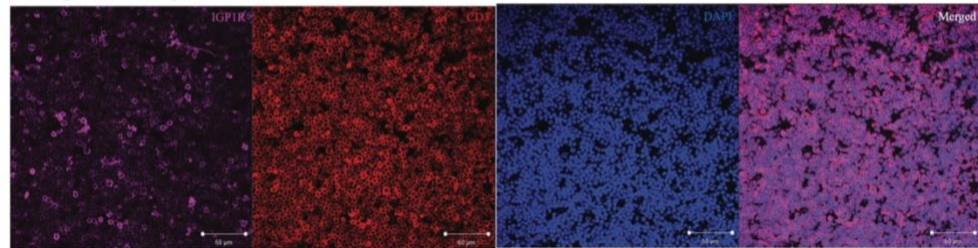
Supplementary Figure 2.1. *Igfbp1* & *Igfbp2* expression in perinatal TECs.

(A, B) Line graphs show average expression of *Igfbp1* and *Igfbp2* across time in different TEC subsets. Figure legends are located at the right.

A 7-day-old wild-type



B 14-day-old wild-type

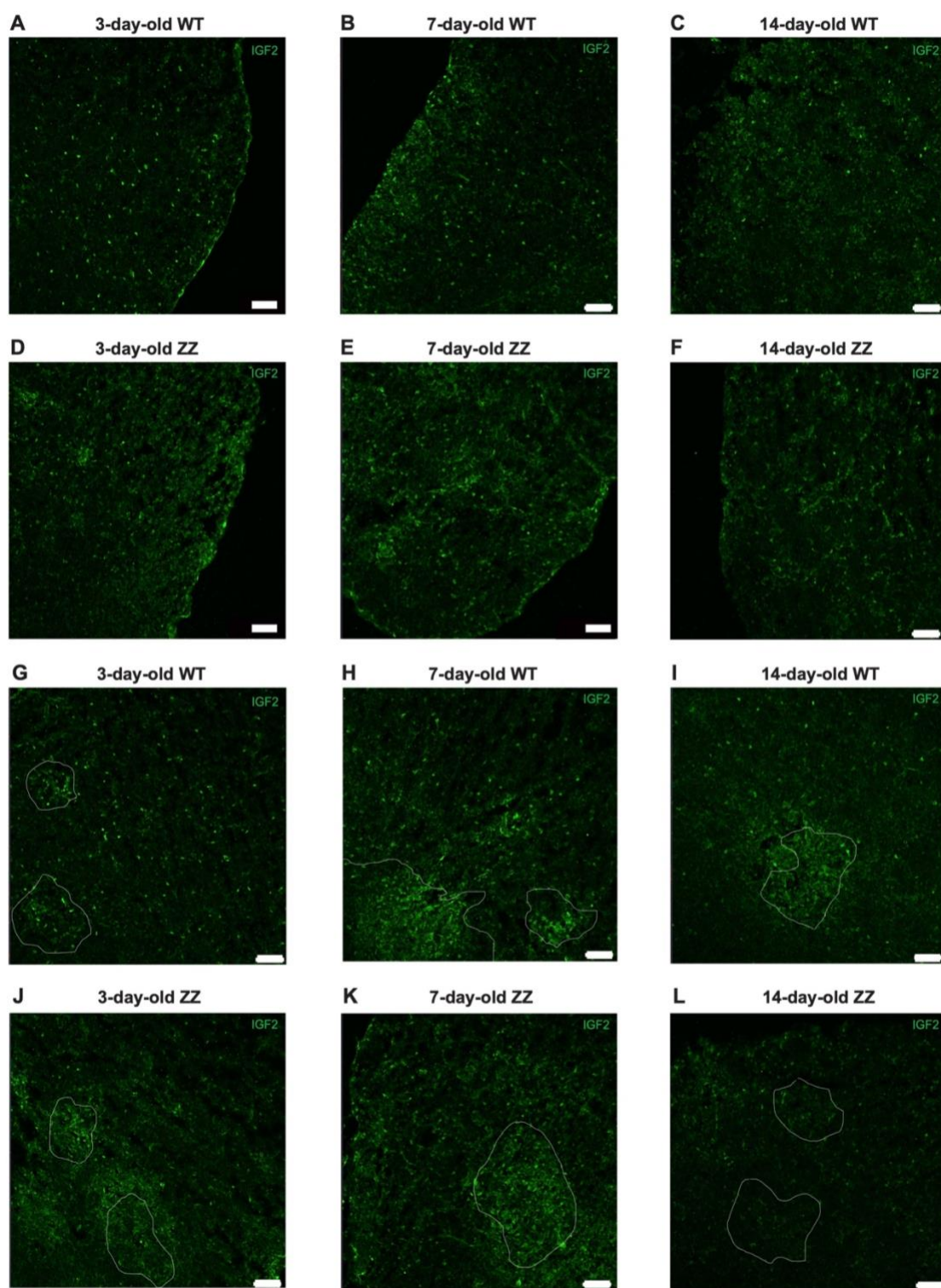


Supplementary Figure 2.2. Thymocytes expressing IGF1R.

(A) Immunohistochemistry of frozen 7-day-old *Foxn1*^{+/+} thymic sections (10 µm). The first panel is IGF1R (magenta), the second is CD3 (red), the third is DAPI (blue), and the fourth is merged.

(B) Immunohistochemistry of frozen 14-day-old *Foxn1*^{+/+} thymic sections (10 µm). The first panel is IGF1R (magenta), the second is CD3 (red), the third is DAPI (blue), and the fourth is merged.

Many CD3⁺ cells are IGF1R⁺. Of note, the CD3 protein level is visibly increased after postnatal day 10. Scale bars = 50µm.



Supplementary Figure 2.3. Cortical and medullary expression of IGF2.

Immunohistochemistry (IHC) of frozen thymic sections (10 μm). (A-C) IGF2 distribution in 3-, 7-, and 14-day-old *Foxn1*^{+/+} males. (D-F) IGF2 distribution in 3-, 7-, and 14-day-old *Foxn1*^{lacZ/lacZ} males. (G-I) IGF2 distribution in 3-, 7-, and 14-day-old *Foxn1*^{+/+} males. Medulla was circled based on CD205 and DAPI expression. (J-L) IGF2 distribution in 3-, 7-, and 14-day-old *Foxn1*^{lacZ/lacZ} males. Medulla was circled based on CD205 and DAPI expression. WT: *Foxn1*^{+/+}, ZZ: *Foxn1*^{lacZ/lacZ}. Scale bars = 50 μm .

Materials and Methods

Mice

The *Foxn1*^{lacZ} mice were generated and maintained on a C57BL6/J background by the Manley lab (Chen et al., 2009). All experiments involving animals were performed with approval from the University of Georgia Institutional Animal Care and Use Committee.

Single-cell suspension of the murine thymic stromal cells for scRNAseq

For our time-series single-cell RNA sequencing, we used 3 thymi per library. Our merged scRNAseq dataset contains 3-day-old (2 *Foxn1*^{+/+} males, 2 *Foxn1*^{lacZ/lacZ} males, 2 *Foxn1*^{+/+} females, 2 *Foxn1*^{lacZ/lacZ} females), 7-day-old (2 *Foxn1*^{+/+} males, 2 *Foxn1*^{+/+} females), 14-day-old (2 *Foxn1*^{+/+} males, 2 *Foxn1*^{lacZ/lacZ} males, 2 *Foxn1*^{+/+} females, 2 *Foxn1*^{lacZ/lacZ} females), 30-day-old (2 *Foxn1*^{+/+} males, 3 *Foxn1*^{lacZ/lacZ} males). Excess blood vessels, connective tissues, and adipocytes were carefully removed in ice-chilled 4°C sterile 1X PBS (w/o Ca²⁺, Mg²⁺). Thymi were finely cut into 1~2 mm³ fragments and transferred into digestion buffer (2% FBS/1640 RPMI (w/ 25mM HEPES), DNase I (20µg/µL), Collagenase/Dispase (1mg/mL)). Tubes containing the thymic fragments with digestion buffer were incubated in a 37°C water bath for 20 minutes, 3 times. The solution was gently mixed up and down with transfer pipettes in between the incubations, 100 times each. To remove undigested thymic fragments, the solution was passed into a new tube, through a 70µm cell strainer. Single-cell suspension was then centrifuged for 10 minutes at 300rcf, 4°C, a condition for centrifugation throughout this protocol. After decanting the supernatant, erythrocytes were lysed with a 2-minute-long incubation in 2mL RBC Lysis Buffer

(10X) (BioLegend 420301). After another centrifugation & decantation, cells were resuspended in MACS buffer (filtered and degassed 0.5% BSA/2mM EDTA/1X PBS (w/o Ca^{2+} , Mg^{2+})). Post cell counting with a haemocytometer, cells underwent CD45 separation as per MACS separation guidelines with mouse CD45 microbeads (Miltenyi Biotec 130-052-301) to collect CD45⁻ cells.

Single-cell suspension of the murine thymic epithelial cells for scRNAseq

The above methods with different digestion buffer (2mL digestion buffer containing 40ul of 0.5 mg/mL of LiberaseTM (Roche) and 20 units/mL of DNaseI (Roche) in PBS (pH 7.0)) were used to collect TECs from postnatal day 0, 3, 7, 10, 15, and 28 wild-type mice. Cells underwent MACS separation to collect CD45⁻ cells. After centrifugation & decantation, CD45 BV510 (BioLegend), EPCAM APC (BioLegend), MHCII APC-Cy7 (BioLegend), and CD11c PerCP-Cy5.5 (Tonbo Biosciences) were added and incubated for 15 minutes at 4°C. Cells were then washed and resuspended in FACS AriaTM Fusion for sorting. 125,000 ~ 150,000 TECs per time point were submitted for scRNAseq.

10X library preparation

Post thymic stromal cell isolation, cell concentration and viabilities were calculated with a haemocytometer & Countess 3 (ThermoFisher Scientific). Libraries were only generated using samples with >85% viability and minimal cell clumping. Due to thymic stromal cells' tendency to adhere to one another, the cells were gently pipetted up and down to avoid further cell clumping. 10,000 cells were loaded into the 10X genomics Chromium instrument, using a Single Cell 3'

Reagent Kit (v3). Post cDNA generation and quantity/quality check steps, the final DNA library was sequenced on Illumina NextSeq2000 P3 with 200 cycles. To minimise batch effects, samples of different conditions were sequenced together whenever possible.

Single-cell transcriptomic data analysis

As per the 10X guideline, CellRanger (v.6) was used to identify droplets containing cells and create gene-by-cell matrices. Filtered matrices underwent QC steps and downstream analyses were performed using Seurat (v.4.3.0) (Butler et al., 2018; Hao et al., 2021; Satija et al., 2015; Stuart et al., 2019) workflow in R (v.4.2.1) environment. Low information cells and possible doublets were removed based on thresholds (Number of genes > 500, UMI <50,000, percent of mitochondrial gene reads < 20%). Post-unsupervised clustering, cell clusters were identified using known genes. A logistic regression model was used to identify differentially expressed genes for each cluster. Intercellular network analysis was done with CellChat per Github guidelines (Jin et al., 2021).

Human thymus for RNAseq

Human thymus was obtained from anonymous patients undergoing corrective cardiac surgery where a portion of the thymus was removed in order to expose the operative field. This tissue was not excised for the purpose of this study and would otherwise have been discarded. The age and sex of the donor were provided, without any other potentially identifying information. The use of anonymous discarded thymus tissue for this research was approved by the Duke University Institutional Review Board (Duke IRB Exempt Protocols #474 and #Pro00103028). Portions of

each thymus were immediately placed in RNALater (ThermoFisher), incubated at 4°C overnight, and then stored in liquid nitrogen until used. Frozen tissues were homogenized in a Precellys soft tissue lysing tube (Bertin) and then RNA was extracted using an RNeasy kit (Qiagen). RNA was cleaned and concentrated using Turbo DNA-free (Invitrogen) and RNeasy MiniElute (Qiagen). Libraries were then prepared using a KAPA Stranded mRNA-seq kit (Kapa Biosystems) and sequenced by 100bp paired-end sequencing on a NovaSeq 6000 (Illumina). For most thymus donors, bulk RNAs derived from at least 2 separate tissue aliquots were sequenced and the data combined for analysis.

Bulk transcriptomic data analysis

Quality control on both raw reads and adaptor-trimmed reads was performed using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc). RNA reads were aligned to the reference genome hg38 using STAR (Dobin et al., 2013) and quantified by featureCounts (Liao et al., 2013). DESeq2 (Anders and Huber, 2010; Love et al., 2014) was used to detect differential expression between younger (< 3-month-old) and older ($\geq$ 3-month-old) samples, considering sex as a confounding factor. Benjamini-Hochberg correction was applied to adjust for multiple testing. Functional enrichment analysis was performed by GSEA (Subramanian et al., 2005). Principal component analysis was utilised to show variations across samples.

Immunohistochemistry

After the thymus was harvested from an animal, connective tissues and excess blood vessels were removed from the thymus in ice-cold sterile Ca^{2+} , Mg^{2+} -free 1X PBS. The thymus was then fixed in 4% PFA/1X PBS for 2 hours on ice. After washing away 4% PFA/1X PBS, the thymus was incubated in 20% sucrose (w/ 0.05% NaN_3) at 4°C, overnight. The following day, the thymus was then placed in an O.C.T. mould filled with O.C.T. to be snap-frozen with dry ice. Frozen sections (10 μm) of murine thymus on slides were prepared with a cryostat. ~100 μm of murine thymus was discarded to capture the whole thymus with proper cortical-medullary structure. The slides were then acclimatised at room temperature for 30 minutes. After removing residual O.C.T. on the slide with sterile Ca^{2+} , Mg^{2+} -free 1X PBS, the sections were blocked with 10% donkey serum diluted in (0.5% BSA, 0.2% Tween-20 in 1X PBS) for 30 minutes. Post-blocking, sections were incubated in a solution containing optimally diluted primary antibodies at 4°C, overnight. The following day, the sections were washed in sterile Ca^{2+} , Mg^{2+} -free 1X PBS. The sections then were incubated in fluorochrome-conjugated secondary antibodies for 1 hour at room temperature. Post-washing, the coverslips were applied and Zeiss LSM880 was used to acquire microscopic images.

Antibodies

The following antibodies were used: α -APOD (Invitrogen, Cat. # PA5-27386), α -VEGFR2 (Invitrogen, Cat. # MA5-15157), α -IGF2 (Invitrogen, Cat. # PA5-47946), α -IGF1R β (Invitrogen, Cat. # PA5-37601), α -CD3 (Invitrogen, Cat. # MA1-80783), α -CD205 (BioLegend, Cat. # 138201), Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488

(Invitrogen, Cat. # A-11055), Donkey anti-Rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594 (Invitrogen, Cat. # A-21209), Donkey anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 647 (Invitrogen, Cat. # A-31573).

CHAPTER 3

**PREMATURE THYMIC INVOLUTION IN YOUNG FOXN1^{LACZ} MUTANT MICE
CAUSES PERIPHERAL T CELL PHENOTYPES SIMILAR TO AGING-INDUCED
IMMUNOSENESCENCE**

Kang, Seung Woo*. Xiao, Shiyun*. Oliva, Kimberly. Zhang, Wen. Klonowski, Kimberly.
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Abstract

The thymus is a primary lymphoid organ generating self-restricted and self-tolerant naïve T-cells. Early in life the thymus starts to involute, resulting in decreased naïve T-cell output which may be more self-reactive, leading to an increased prevalence of autoimmunity. A decrease in the transcription factor FOXP1 is an early event in thymic involution. Using the *Foxp1^{lacZ}* model, we studied how premature thymic involution affects the thymic microenvironment, thymocytes, and peripheral T cell immunity. We found that early thymic involution led to aged-like thymic epithelial cells that resulted in aged-like thymocyte phenotypes, with a significant decrease in CD4⁺ single-positive T-cells. We also observed severe lymphopenia in *Foxp1^{lacZ}* mice caused by the premature decrease in T-cell production, resulting in a peripheral T-cell phenotype similar to *de novo* aged peripheral T-cells. Moreover, following T cell receptor stimulation, *Foxp1^{lacZ}* peripheral T cells had reduced IL-2 secretion and strong initial IFN- γ responses, resembling aged wild-type peripheral T-cell responses. Lastly, influenza response in *Foxp1^{lacZ}* had a reduction in some aspects of T cell responses to influenza infection, similar to thymectomized mice. Our study shows an independent and direct impact of premature thymic involution on both thymopoiesis and peripheral immune niches likely contributing to immunosenescence and inflammaging as observed in the elderly population.

Introduction

Thymus size and function peak early in life and then start to decline with age, defined as thymic involution. As a result, naive T cell (T_N) export declines and T cell receptor (TCR) diversity and self-tolerance is impaired over time (Bains et al. 2009; Shanley et al. 2009). These changes are regulated by thymic epithelial cell (TEC)-based mechanisms and crosstalk between TECs, non-TEC stroma, and developing thymocytes (Chinn et al. 2012). While hematopoietic stem cells (HSC) that seed the thymus functionally decline with age, thymic involution occurs prior to this decline and is stromal-intrinsic (Mackall et al. 1998; Zhu et al. 2007).

Reduced expression of Forkhead Box N1 (FOXN1) is an early event in thymic involution (Chen et al. 2009; Ortman et al. 2002). As a key regulator of TEC biology throughout life (Chen et al. 2009; Manley & Condie 2010; Nowell et al. 2011; Blackburn et al. 1996; Nehls et al. 1996; Zook et al. 2011; Su et al. 2003), over-expression of FOXN1 can preserve TECs and delay thymic involution (Zook et al. 2011; Bredenkamp et al. 2014). *Foxn1* is incredibly dosage-sensitive, and small decreases cause changes in TEC differentiation and function (Chen et al. 2009; Nowell et al. 2011; Bredenkamp et al. 2014). Although *Foxn1* expression in the thymus is restricted to TECs, it indirectly affects the maturation of thymic vasculature and mesenchyme (Nowell et al. 2011; Bryson et al. 2011), orchestrating nearly all aspects of thymic function and maintenance.

Our previous report of the *Foxn1*^{lacZ} mouse model showed that *Foxn1* expression in TECs is normal in fetal and early postnatal stages but declines after postnatal day 7 to about 40% of wild type levels, causing a progressive reduction in thymopoiesis and premature thymic involution (Chen et al. 2009). Since down-regulation of *Foxn1* expression occurs similarly in TECs ,

preceding aging-related involution (Chen et al. 2009), *Foxn1^{lacz}* models age-related thymic involution in young mice and allows investigation of how thymic involution contributes directly to peripheral immunosenescence.

In this study, we used single-cell RNA sequencing and flow cytometry to dissect the independent effect of premature thymic involution on thymopoiesis and peripheral immune function. Using *Foxn1^{lacz}* mice, we show that an early decrease in *Foxn1* leads to an aged TEC phenotype in young animals, resulting in *de novo* aged-like thymopoiesis. The premature decline in thymic output resulted in lymphopenia in *Foxn1^{lacz/lacz}* mice and formed an aged wild type-like peripheral immune system and *in vitro* T-cell responses suggesting immunosenescence and inflammaging in young *Foxn1^{lacz/lacz}*. Together, our findings provide insight into the direct impact of premature thymic involution on peripheral T-cells independent from hematopoietic stem cells or physiological aging.

Results

Single-cell RNA sequencing of CD45- total thymic stroma shows an aged TEC phenotype in 1-month-old *Foxn1^{lacz/lacz}*.

In order to study how the relative level of *Foxn1* affects the thymic stroma transcriptome at a cellular level, we submitted CD45- thymic stroma of 1-month-old *Foxn1^{+/+}* (+/+) males and *Foxn1^{lacz/lacz}* (Z/Z) males for single-cell RNA sequencing. We merged these datasets with a batch correction to a published 18-months-old C57BL6/J wild type (wt) female dataset (Kousa et al. 2023) to compare *de novo* aged thymus to thymus with low *Foxn1* expression. We detected all

previously described stromal subsets based on their signature marker expressions. We identified 10 TEC clusters [*Epcam*, MHC class II], 4 endothelial clusters [*Pecam1*, *Cdh5*], 3 fibroblast clusters [*Pdgfra*, *Col1a1*], 2 pericyte clusters [*Pdgfrb*, *Acta2*, *Myl9*], mesothelial cells [*Upk3b*, *Nkain4*], and rare non-myelinating Schwann cells [*Gfap*, *Ngfr*, *S100b*] (Kousa et al. 2023; Hu et al. 2018) (Fig. 3.1 A).

Major differences were observed in TECs between the three datasets. Two TEC populations (TEC-6-2, TEC-9) were absent in the 18-months-old wt sample compared to 1-month-old *+/+* and *Z/Z* (Fig. 3.1 A, B). A subset of TEC-6 and TEC-9 were identified as muscle TECs [*Myog*, *Myl1*, *Actc1*] and ciliated TECs [*Spag8*, *Dnah12*, *Dynlrb2*] (Michelson et al. 2022), respectively. Of note, ciliated TECs express high levels of *Cldn3*, *Cldn4*, and *Plet1* (Depreter et al. 2008), suggesting a decrease in TEC progenitor cell population in the 18-months-old wt). We also observed a TEC population (TEC-3) that was scarce in 1-month-old *+/+* samples but was highly enriched in both 1-month-old *Z/Z* & 18-month-old wt (Fig. 3.1 B).

We subsampled *Epcam*⁺ MHC Class II genes⁺ clusters to acquire a better resolution of TEC populations (Fig. 3.1 C). We identified 12 previously defined TEC populations (Brennecke et al. 2015; Miragaia et al. 2018; Bornstein et al. 2018; Baran-Gale et al. 2020; Park et al. 2020; Bautista et al. 2021; Michelson et al. 2022) and two unknown TEC populations (Fig. 3.1 D). To identify these new TEC populations, we used differentially expressed gene analysis (DEG) and gene set enrichment analysis (GSEA). GSEA of one of the unique cTEC population revealed IFN α response, P53 pathway, hypoxia, and epithelial to mesenchymal (EMT) transition, suggesting it was stress-responsive (Fig. 3.1 E). GSEA of the other unique TEC population revealed TNF α

signaling via NF- κ B and EMT. DEG analysis showed that this population was previously described as aging-associated TECs (aaTECs) (Kousa et al. 2023) (Fig. 3.1 F). Concordant to Kousa et al. (2023), the aaTEC population is undergoing EMT based on their loss of epithelial markers (*Epcam*, *Pax1*) and acquisition of mesenchymal markers (*Zeb2*, *Notch3*, *Prrx1*), which we confirmed (Fig. 3.1 G-I). However, aaTECs in 1-month-old Z/Z had a slightly different transcriptome compared to 18-month-old wt. One-month-old Z/Z aaTECs had 1.6 times higher *Zeb2* expression but 16.3 times less *Spp1* compared to 18-month-old wt aaTECs. aaTECs comprised 10.4% of 1-month-old Z/Z TECs, whereas this increased to 22.4% of 18-month-old wt TECs. Overall, our single-cell RNA sequencing data suggests a decrease in *Foxn1* resulted in the premature occurrence of stress-responsive cTECs and aaTECs, resembling aged TEC phenotypes.

Premature thymic involution alters thymic selection and accelerates CD4 and CD8 SP

thymocyte maturation in *Foxn1*^{lacZ} mutant mice

Down-regulation of *Foxn1* expression in TECs from postnatal day 7 causes premature thymic involution in young adult *Foxn1*^{lacZ} mice with a significant reduction of medullary thymic epithelial cells (mTECs), early thymic progenitors (ETPs), and total thymocyte number (Chen et al. 2009; Xiao et al. 2018). These changes are similar to the profiles of age-related thymic involution in naturally aging wt mice (Min et al. 2004). To identify differences in thymocyte developmental deficiency between premature thymic involution of young (2-months-old) Z/Z mice and age-related thymic involution of aged (18-months-old) wt mice, we compared their thymocyte phenotype to young (2-months-old) *Foxn1*^{+/-lacZ} control mice (+/Z). The percentages of both CD4

and CD8 SP thymocytes were significantly reduced in young Z/Z mutants, but in old wt mice, only CD8 SP thymocytes were significantly reduced compared to +/Z (Fig. 3.2 A). To determine the effects of thymic positive selection on the production of both CD4 and CD8 SP thymocytes, we measured the expression of positive selection initiative molecules CD69, CD5, and TCRb (Žuklys et al. 2016; Saini et al. 2010). Compared to +/Z control, the expression of CD69 (Fig. 3.2 B) and CD5 (Fig. 3.2 C) on TCRβ^{int} DP thymocytes and TCRβ^{hi} SP were reduced in both young Z/Z mutants and old wt mice, indicating decreased thymic positive selection.

We measured the negative selection process by analyzing the down-regulation of CD69, CD24, and CCR7 on post-selection of TCRβ^{hi} CD4 and CD8 SP thymocytes (Žuklys et al. 2016; Ueno et al. 2004). The percentages of CD69⁻ TCRβ^{hi} (Fig. 3.2 D) T cells were significantly reduced in young Z/Z but not in old wt mice. However, CD24^{lo}Qa2⁻ CD4 but not CD8 (Fig. 3.2 E), CD24^{lo}CCR7⁺CD4, and CD24^{lo}CCR7^{lo}CD8 (S-Fig. 3.1 A) T cells were significantly reduced in both Z/Z and old wt mice. These results indicated that negative selection was increased in both young Z/Z and old wt mice with some differences. After negative selection, CD4 and CD8 SP thymocytes stay in the thymic medulla for approximately 15 days for further function maturation, with one of the important changes being the up-regulation of Qa2 expression (Vernachio et al. 1989). The percentage of CD24^{lo/-}Qa2⁺ cells was significantly increased in both CD4 and CD8 SP thymocytes in young Z/Z mutants and only mildly increased in old wt mice compared to +/Z controls (Fig. 3.2 E). A BrdU incorporation test showed that the proliferation potential of Qa2⁺ cells was similar between mouse genotypes (S-Fig. 3.1B), suggesting that the increase in CD24^{lo/-}Qa2⁺ CD4 and CD8 SP thymocytes in Z/Z mutants was likely due to accelerated function

maturation rather than increased proliferation. Together, young Z/Z mutants and old wt mice showed similarly reduced thymic positive selection and maturation (Fig. 3.2 B, C) but differences in thymic negative selection, with young Z/Z mutants showing increased negative selection (Fig. 3.2 D, E, S-Fig. 3.1 A).

The proliferation of developing thymocytes is altered in young *Foxn1^{lacZ}* mutant mice

The size of the postnatal thymus is controlled by the expansion of early-stage thymocytes at CD4⁻CD8⁻ DN and CD4⁺CD8⁺ DP stages. DN proliferation is limited by the availability of stromal niches, but DP proliferation is limited intrinsically (Prockop & Petrie 2004). IL-7 and c-KitL are two key cytokines supporting the proliferation of early thymocytes at the DN stage (Peschon et al. 1994; von Freeden-Jeffry et al. 1995). We measured the receptors of IL-7 and c-KitL on DN thymocytes to determine if alteration of cytokine-mediated DN proliferation could be the cause of the reduced number of total thymocytes. Compared to +/Z control (1.5-months-old), both young Z/Z (1.5-months-old) and old wt (18-months-old) showed a significant increase of IL-7 receptor (IL-7R α / CD127) expression on DN1 subsets, whereas only young Z/Z mutants showed an increase on DN3 subsets. (Fig. 3.3A, B). c-Kit expression on DN1 and DN2 subsets was significantly reduced in young Z/Z and old wt (Fig. 3.3C-E). Additionally, there was a reduction in DN2 proliferation but an increase in DN3 proliferation in young Z/Z compared to controls (Fig. 3.3F, G). Old wt DN thymocytes displayed no changes in proliferation compared to controls (Fig. 3.3F, G). The proliferation capabilities of thymocytes in later stages were also significantly increased in DN, DP, CD4, and CD8 subsets in young Z/Z but not in old wt thymocytes (Fig. 3.3H,

I). These results suggest while premature thymic involution in *Foxn1^{lacZ}* altered thymocyte proliferation, it is not likely the reason behind the reduction in total cellularity of the Z/Z thymus.

We further tested the effect of thymic involution on thymocytes by measuring apoptosis with annexin V staining. Apoptotic cells increased in DN1, DN2, CD4 SP, and CD8 SP stages (Fig. 3.4A, B) in both young Z/Z and old wt mice compared to +/Z control. Together, these results indicate that although there are some differences in proliferation and apoptosis in thymocyte developmental stages of young Z/Z and old wt mice, they are quite similar at DN1 and DN2 stages. Apoptotic profile changes causing the reduction of ETPs could be a key factor contributing to thymocyte reduction in these mice (Xiao et al. 2018; Min et al. 2004).

Naïve CD4 and CD8 T-cells are decreased in young *Foxn1^{lacZ}* spleen

Since decreased *Foxn1* resulted in aging-like TEC phenotypes, we wanted to investigate how the peripheral immune niches were affected. We submitted total peripheral mononuclear cells (PMCs) from spleen of 1-month-old +/+ and Z/Z mutants with 18-months-old C57BL6/J wt for single-cell RNA sequencing. We detected 7 follicular B-cell clusters [*Cd19*, *Ighd*, *Cr2*, *Cd22*], 1 plasma cell/plasmablast cluster [*Jchain*, *Iglv1*, *Derl3*, *Eaf2*], 4 T-cell clusters [*Cd3e*, *Cd3g*, *Cd4*, *Cd8a*], 1 NK cluster [*Ccl5*, *Klra4*, *Klrd1*], 1 cDC cluster [*Clec9a*, *Xcr1*, *Clec10a*], 1 pDC cluster [*Siglech*, *Irf7*, *Irf8*], 1 monocyte cluster [*Fn1*, *Clec4a1*, *Sirpb1c*], 1 basophil cluster [*Prss34*, *Fcer1a*, *Cpa3*, *Mpo*], 1 macrophage cluster [*Ms4a2*, *Ccl9*, *Cyp11a1*], and 2 neutrophil clusters [*Wfdc21*, *Ltf*, *Ngp*, *Lcn2*] (Schulze et al. 2022) (Fig. 3.5 A). We observed a decrease in a follicular B-cell population accompanying an increase in plasma cell/plasmablast cluster in 18-month-old

wt samples compared to both 1-month old $+/+$ and Z/Z (Fig. 3.5 B). We also observed an increase in both CD4 and CD8 T cell populations in the 18-month-old wt sample compared to $+/+$ and Z/Z (Fig. 3.5 B).

We subsampled $Cd3^+$ clusters to have a higher resolution in T-cell subset changes (Fig. 3.5 C). The 18-months-old wt sample showed unique T-cell clusters, such as CD8 virtual memory-like T cells [$Cd8a$, $Cd44^{Hi}$, $Cd49d^{Lo}$] (Hussain & Quinn 2019), aged CD4 T cells [$Cd4$, $Tnfrsf4k$, $Tnfsf8$, $Ctla4$, $Nt5e$] (Yang et al. 2020), and CD8 exhausted T cells [$Cd8a$, $Pdcd1$, $Lag3$, $Havcr2$, $Prdm1$] (Jiang et al. 2015), reflecting their age and exposures during their lifetime (Fig. 3.5 D). To understand the direct effect of decreased *Foxn1* in the thymus on peripheral T cell changes, we compared 1-month-old $+/+$ and Z/Z . There was only a slight decrease in the proportion of CD4 naïve T cells in Z/Z , 35.6% in $+/+$ compared to 31.9% in Z/Z . Conversely, there was approximately a 45% decrease in the proportion of CD8 naïve T cells in Z/Z , 12.1% in $+/+$ compared to 6.8% in Z/Z . Additionally, there was a substantial decrease in the proportion of $\gamma\delta$ T-cells in Z/Z and old wt. 12.9% of $CD3^+$ T-cells were $\gamma\delta$ T in WT, 8.4% in ZZ, and 0.9% in old wt. Overall, these data suggest that the aging TEC phenotype in 1-month-old Z/Z led to decreased splenic naïve CD4 and CD8 T-cell populations compared to $+/+$ control, similar to 18-month-old wt.

Peripheral lymphocytes from young *Foxn1*^{lacZ} mice show an age-related phenotypic change comparable to aged wild type mice

To further evaluate the effects of premature thymic involution on peripheral lymphocytes in young *Foxn1*^{lacZ}, we used flow cytometry to compare the phenotypic changes of splenic

lymphocytes between +/Z controls (2.5-months-old), Z/Z mutants (2.5-months-old), and old wt mice (18-months-old). The percentage of CD4 T cells was significantly reduced in young Z/Z and slightly reduced in old wt mice, but no significant changes were observed in CD8 cells compared to +/Z controls (Fig. 3.6 A). However, the expression CD4 and CD8 were both downregulated on SP T cells from young Z/Z and old wt mice (Fig. 3.6 B, C). Resembling an aged wt lymphocyte profile, T cells from young Z/Z showed significantly increased effector memory T cells (T_{em}) and a reduction of naïve T cells (T_n) (Fig. 3.6 D). Consistent with this, young Z/Z and old wt mice had similar trends in the percentages of TCR β ⁺ cells and B cells (TCD19⁺) compared to +/Z controls (S-Fig. 3.2 A-C). The percentage of natural killer (NK) cells and granulocytes in the spleen were only increased in young Z/Z (S-Fig. 3.2 D-G), but the percentage of Treg cells had no significant difference between mice (data not shown).

Young *Foxn1*^{lacZ} mice show mild peripheral lymphopenia analogous to aged wild type mice

In wt mice, increased thymocyte production enlarges the pool of peripheral lymphocytes during ontogeny, which remains stable even with thymic involution (Surh & Sprent 2005; Mackall et al. 1997). However, in *Foxn1*^{lacZ} mice, the production of thymocytes dramatically decreases on postnatal day 7, prematurely affecting the pool of peripheral lymphocytes (Chen et al. 2009). To measure the size of the peripheral lymphocyte pool, we collected cells from the spleen and lymph nodes (LNs) of 2-3-months-old +/Z and Z/Z and 15-20-months-old wt mice. The total number of peripheral mononuclear cells (PMC) was significantly reduced in young Z/Z mutants and old wt mice compared to +/Z control (Fig. 3.7 A). The total number of CD4 and CD8 T cells was also

reduced in young Z/Z and old wt mice (Fig. 3.7 B, C). For both CD4 & CD8 T-cells in Z/Z and old wt, the most reduced subset was naïve T cells, and effector memory T cells (T_{em}) were the most abundant, especially in old wt mice (Fig 3.7 D, E). To test the peripheral lymphopenia status in *Foxn1^{lacZ}* mice, we transferred CFSE-labeled CD45.1⁺ CD4 and CD8 T cells into young +/Z, Z/Z, and old wt (CD45.2⁺) mice (S-Fig. 3.3 A). Although most CD45.1⁺ donor cells remained CFSE^{hi} in the host, there was a slight reduction in the percentage of CFSE^{hi} cells in young Z/Z and old wt mice compared to the +/Z mice (S-Fig 3.3 B-F). However, the total number of CD45.1⁺ donor cells recovered from young Z/Z and old wt after transfer was significantly reduced compared to +/Z control (S-Fig. 3.3 G). These results suggest that both young Z/Z and old wt mice show a slight peripheral lymphopenia status that drives homeostatic proliferation of lymphocytes; however, the peripheral environment does not support the survival of proliferated cells. Consistent with lymphopenia, proliferating CD4 and CD8 T cells increased (Fig. 3.7 F, G) and showed similar T-cell subset profiles in young Z/Z and old wt with the exception of CD8 T_{em} subset (Fig. 3.7 H, I), which are uniquely elevated in old wt mice. Also, the percentage of apoptotic cells was increased in both CD4 and CD8 T cells in young Z/Z and old wt mice (Fig. 3.7 J, K). Combined, these results indicate that peripheral lymphopenia in young Z/Z mutants and old wt mice led to homeostatic lymphocyte proliferation, and a concurrent increase in apoptosis significantly reduced the total T-cell number compared to +/Z controls.

T cells from young *Foxn1^{lacZ}* and aged mice display weak TCR signaling and impaired proliferation

Since young Z/Z mutants and old wt displayed lymphopenia, with a smaller peripheral lymphocyte pool than +/Z control, we analyzed the proliferative capability of these cells after TCR stimulation. To do this, we isolated splenic and LN-derived CD4 T cells and co-cultured them with or without aCD3+CD28 antibodies *in vitro*. Compared to +/Z mice, CD4 T cells from Z/Z and old wt had ~ 50% reduction in proliferation after stimulation (Fig. 3.8 A). Phenotypic analysis of co-cultured cells 48 hours after activation showed that both activation markers CD69 and CD25 were significantly reduced on CD4 T cells from both Z/Z and old wt compared to +/Z (S-Fig. 3.4 A-E). These results suggest that CD3-TCR signal-induced activation was impaired in both young Z/Z mutants and old wt mice, which is consistent with the down-regulation of TCR β expression in CD4 and CD8 T cells from both Z/Z and old wt mice (Fig. 3.8 B, C).

To test the proliferative capability of T cells *in vivo*, CD4 and CD8 T cells from +/Z, Z/Z, and old wt mice were isolated and transferred into sub-lethally irradiated CD45.1 wt mice (Fig. 3.8 D). Six days later, donor T cells (CD45.2) were analyzed in host (CD45.1) mice. There was an increased frequency of CFSE^{hi} cells CD4 T cells in Z/Z and old wt compared to +/Z control, but cell division was reduced (Fig. 3.8 E-G, H). In contrast, there was a decreased frequency of CFSE^{hi} CD8 T cells in Z/Z and old wt compared to +/Z control while cell division was increased (Fig. 3.8 E-G, I). The absolute number of recovered CD45.2 donor cells was much less in both young Z/Z and old wt mice than in +/Z control (Fig. 3.8 J, K). These results indicate that peripheral CD4 and

CD8 T cells from mice with premature or age-related thymic involution displayed weak TCR-CD3 stimulation that led to reduced activation and impaired homeostatic-driven proliferation *in vivo*.

T cell cytokine production is similar between young *Foxn1^{lacZ}* and aged wild type mice

To test T cell function after activation, peripheral CD4 and CD8 T cell cytokine production was analyzed 5 and 24 hours after activation with aCD3+CD28 mAbs *in vitro*. Consistent with weak TCR activation, IL2 production was greatly reduced in CD4 T cells from old wt mice and slightly reduced in CD4 T cells from young Z/Z mice after 5 hours (Fig. 3.9 A, S-Fig. 3.5 A, B). After 24 hours, IL2 production was substantially reduced in CD4 T cells from both young Z/Z and old wt mice (Fig. 3.9 A, B). IFN γ levels were higher in CD4 T cells from old wt mice compared to young +/Z and Z/Z mice after 5 hours, but no significant differences were found after 24 hours (Fig. 3.9 A, C). CD8 T cells from both young Z/Z mutants and old wt mice had increased IFN γ production after 5 hours compared to +/Z. However, after 24 hours, IFN γ levels were similar between +/Z and Z/Z, and both were increased compared to old wt (Fig. 3.9 D and E). Together, these results suggest that CD4 T cells from young Z/Z mutants and old wt mice have impaired IL2 production, but both CD4 and CD8 cells from these mice can quickly produce the inflammatory cytokine IFN γ in response to TCR signals.

The anti-influenza CD8 T cell response in the spleen is increased in young *Foxn1^{lacZ}* mutants compared to aged wild type mice

To investigate the effects of thymic involution on T cell immunity *in vivo*, we infected young +/Z, Z/Z, and old (16-months-old) wt mice with influenza and assessed morbidity (weight loss post-infection) and anti-viral T cell immunity. Overall, weight loss and cytokine production of CD4 T cells restimulated with HA peptide was similar (not significant) between all 3 groups except for a significant reduction of IFN- γ production in the spleen of old wt mice compared to +/Z control (data not shown). Next, we assessed the overall frequency and number of activated CD8 T cells reactive to the immunodominant NP (NP-tet⁺) and producing the inflammatory cytokines IFN- γ and TNF- α in the lung and spleen at 10 days post infection (dpi), which is the peak of CD8 T cell response in C57BL/6 mice (Flynn et al. 1998). We performed a similar analysis on lymphocytes extracted from bronchoalveolar lavage (BAL), but the variability in cell extraction inherent to the isolation procedure restricted our analysis to cell frequency.

The only statistically significant change in the frequency of activated CD44^{hi} NP-tet⁺ CD8 T cells was found in the spleen, where there was an increase in Z/Z mice but a decrease in old wt compared to +/Z control (Fig. 3.10 A and B). The total number of activated CD44^{hi} NP-tet⁺ CD8 T cells was similar in Z/Z mice but decreased in old wt mice compared to control. A significant reduction of IFN- γ production in the spleen was only observed in old wt compared to +/Z controls (Fig. 3.10 C). In the lung, there was a trend of decreased CD44^{hi} NP-tet⁺ CD8 T cells and IFN- γ production in Z/Z and old wt. From BAL, the frequency of CD44^{hi} NP-tet⁺ CD8 T cells was slightly decreased in Z/Z, but no changes were found in IFN- γ production. Finally, there were no

differences in TNF- α production between the three groups in any of the analyzed tissues (data not shown). Overall, these data demonstrate a similar CD8 T cell response to influenza in the respiratory tract of +/Z control mice, Z/Z mutants, and old wt mice. Interestingly, splenic T cells were significantly affected in old wt mice, and moderately affected in Z/Z mutants, suggesting that different subsets of responsive CD8 T cells were differentially affected by thymic involution.

Discussion

Establishing a direct impact of thymic involution on the peripheral immune niches has been challenging due to various components contributing to the ageing peripheral immune system. The results of this study show an independent effect of thymic involution on the peripheral immune system distinct from the aging of hematopoietic stem cells and secondary lymphoid organs. Using single-cell RNA sequencing, we provided evidence of aged thymic microenvironments, especially in TECs from 1-month-old Z/Z compared to 18-month-old wt, which further supports the previously reported *Foxn1*^{lacZ} model (Chen et al. 2009) as a genetic model of premature onset and accelerated rate of thymic involution. We identified new stress-responsive cTECs and previously reported aging-associated TECs that lose epithelial markers whilst acquiring mesenchymal markers, potentially undergoing EMT. Notably, aaTECs that are exclusive to 18-month-old wt samples (Kousa et al. 2023) were present in 1-month-old Z/Z but were absent in 2-month-old wt samples, further supporting that deletion of *Foxn1* is sufficient to cause premature thymic involution.

As expected, thymopoiesis was affected by premature thymic involution in young *Foxn1^{lacZ}* including reduced thymic positive selection, increase thymic negative selection, and accelerated thymocyte maturation. Although there were some differences between young Z/Z and old wt mice, their changing trends were similar in both mice. Young Z/Z mice also exhibited a similar thymocyte phenotype to old wt (Table 1). Although there were some differences in proliferative and apoptotic thymocyte populations between young Z/Z and old wt compared to young +/Z control, young Z/Z mice overall displayed aged-like thymopoiesis similar to old wt. In young Z/Z, there was an overall increase in DN proliferation, which supports that thymic size reduction is caused by a decrease in *Foxn1* and is stromal-intrinsic. Thymocytes exhibited increased proliferative capacity in young Z/Z thymus compared to old wild-type thymus, suggesting relatively younger hematopoietic stem cells that are capable of homeostatic-driven proliferation. The most striking difference in young Z/Z was the significant decrease in CD4⁺ SP thymocytes compared to young +/Z and old wt, which could be due to the previously reported decrease in MHCII^{hi} and UEA-1⁺ cells and decreased proliferation of MHCII^{lo} TECs in young Z/Z mice (Chen et al. 2009). Alternatively, given the decrease in MHCII^{hi} TECs in naturally aging mice (Gray et al. 2006; Aw et al. 2008), different mTEC subset changes in young Z/Z compared to *de novo* mTEC aging may have caused the disparity. Increased negative selection in young Z/Z compared to young +/Z and old wt supports different mTEC subset compositions in young Z/Z.

scRNAseq of total splenocytes did not reveal significant differences between 1-month-old +/+ and Z/Z. The major differences were observed in 18-month-old wt, further demonstrating antigen exposures, physiological ageing, and HSC ageing shape the peripheral adaptive immune

environment. Intriguingly, $\gamma\delta$ T-cells are substantially decreased both in young Z/Z and old wt. There seems to be a direct relationship between the level of *Foxn1* and $\gamma\delta$ T-cell production, which requires further investigation.

Decreased thymic output caused by premature thymic involution resulted in aged-like peripheral immune niches (Table 1). Reflecting a significant decrease in CD4 SP T-cells in the thymus, CD4⁺ cells in the spleen decreased significantly in young Z/Z. Further splenic T-cell analyses showed a decrease in naïve T-cell population with increased memory T-cell phenotypes in young Z/Z, closely resembling old wt. Moreover, we showed how premature thymic involution in *Foxn1^{lacZ}* mice resulted in lymphopenia, similar to *de novo* aged peripheral immune system. Due to prematurely decreased naïve T-cell production in the thymus, the absolute number of PMC, CD4, and CD8 were significantly decreased in Z/Z compared to +/Z control and old wt. With the decrease in naïve T-cell populations and increased T-cell proliferation and apoptosis, the peripheral immune profile of young Z/Z closely resembled that of old wt. However, we observed a huge disparity in the absolute number of CD4 and CD8 effector memory T-cell populations. These T cell populations were significantly decreased in young Z/Z, but were significantly increased in old wt compared to young +/Z control. Since the relative proportions of memory T-cells in young Z/Z is high akin to old wt, this disparity is likely caused by a more severe lymphopenia in young adult Z/Z with prematurely decreased naïve T-cell export from the thymus. We also observed a major difference in apoptosis between young Z/Z and old wt. While overall proliferation was similar between young Z/Z and old wt, apoptosis of CD4⁺ & CD8⁺ T-cells was significantly increased in young Z/Z compared to old wt and young +/Z control, further

contributing to lymphopenia. Our data suggests overall peripheral immune niches of young Z/Z resembled those of old wt, but some differences existed due to severe lymphopenia in young Z/Z and potential antigen exposure in old wt.

Peripheral T-cell function in young Z/Z was also similar to old wt due to premature thymic involution (Table 1). *In vitro*, CD4 T-cell proliferative capacity was reduced by 50% upon TCR stimulation. We also observed distinct cytokine profiles across T-cells from young +/Z, Z/Z, and old wt. IL-2 maintenance in CD4⁺ T-cells was impaired in both young Z/Z and old wt, suggesting immunosenescence. Compared to a strong initial IL-2 response that increased over time in +/Z control, CD4⁺ T-cells from Z/Z displayed a strong initial IL-2 response that rapidly decreased in the span of 19 hours. In comparison, old wt CD4⁺ T-cells had weak IL-2 secretion that decreased further with time. On the contrary, both CD4⁺ & CD8⁺ T-cells had stronger initial IFN- γ response in young Z/Z and old wt, suggesting inflammaging.

Unlike *in vitro* stimulation, we did not observe statistically significant differences in T-cell immune response to influenza challenge in young Z/Z. We observed no difference in CD4⁺ T-cell responses, but a decreasing trend in antigen-specific and antigen-responsive CD8⁺ T-cells in the lung and airways of young Z/Z and old wt. The similarity in total cell count accompanied by functional decline is concordant with a previous report that thymectomized mice exhibit decreased influenza-responsive CD8⁺ T-cells, similar to aged mice due to a decrease in diversity of CD8 T-cells (Yager et al. 2008). Collectively, our findings support a direct effect of premature thymic involution on thymocytes and peripheral T-cells, which may provide an insight into which components contribute to immunosenescence and inflammaging.

Acknowledgements

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Figures and Figure Legends

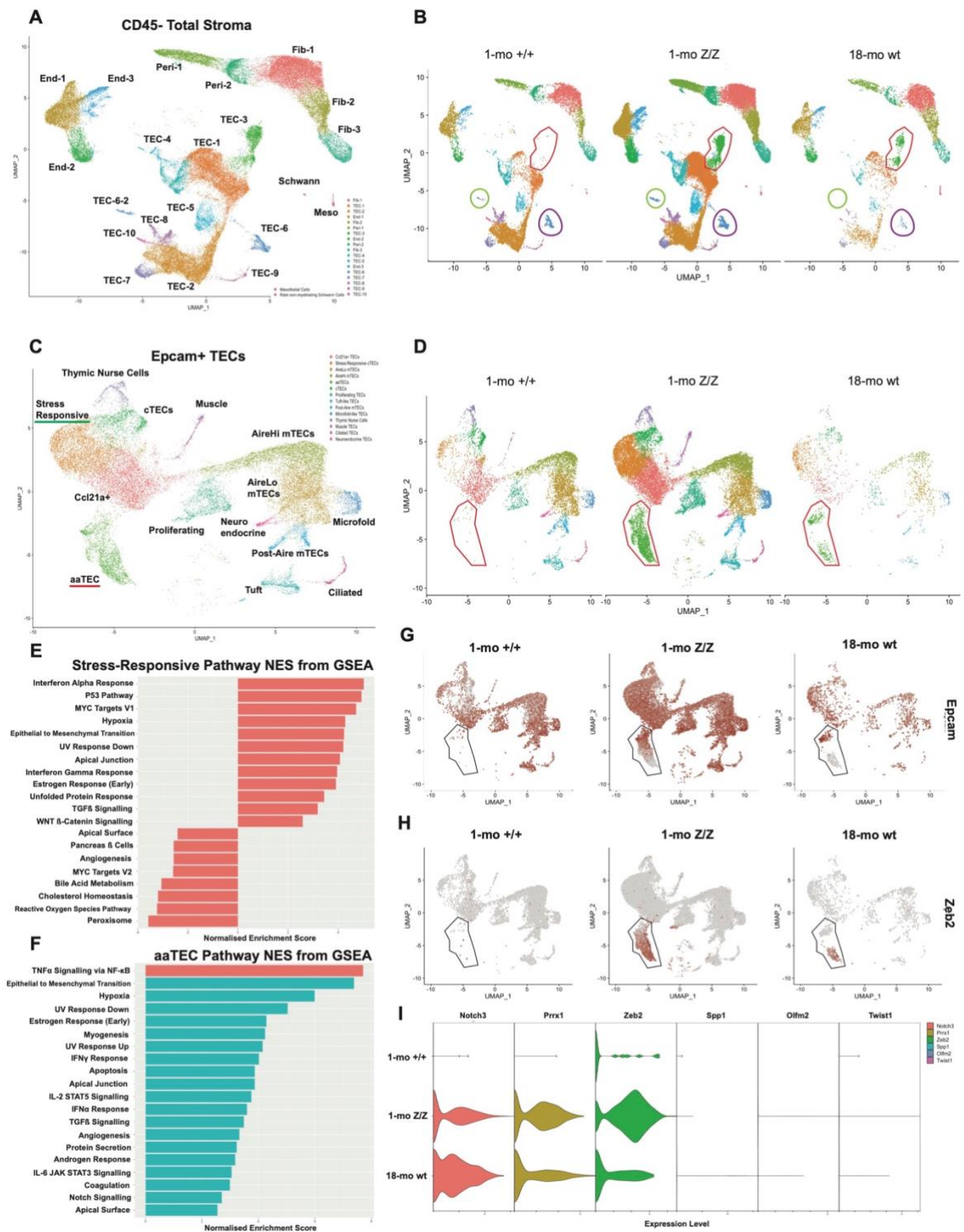


Figure 3.1. scRNAseq analysis of CD45- total thymic stroma and thymic epithelial cells revealed an aged TEC phenotype in 1-month-old *Foxn1^{lacz}*.

(A) UMAP plot visualization of the CD45- total stroma. (B) UMAP plot visualization of the CD45- total stroma split by age/genotype. Red circles indicate TEC-3 that were only largely present in 1-month-old (mo) *Foxn1* Z/Z and 18-mo wt. Lime green circles indicate TEC-6-2 and purple circles indicate TEC-6 that were absent in 18-mo wt. (C) UMAP plot visualization of the *Epcam*⁺ TECs. (D) UMAP plot visualization of the *Epcam*⁺ TECs split by age/genotype. Red circles indicate aaTEC that were only present in 1-mo *Foxn1* Z/Z and 18-mo wt. (E) Top 20 hallmark pathways ranked by normalized enrichment score for stress-responsive cTECs vs. rest of the TECs. (F) Top 20 hallmark pathways ranked by normalized enrichment score for aaTECs vs. rest of the TECs. (G) Feature plot of an epithelial cell marker, *Epcam*, split by age/genotype. (H) Feature plot of a mesenchymal cell marker, *Zeb2*, split by age/genotype. (I) Violin plot of an aaTEC cluster, split by age/genotype. Abbreviations: *mo*, months old; *wt*, wild type; Z/Z, *Foxn1^{lacz/lacz}*.

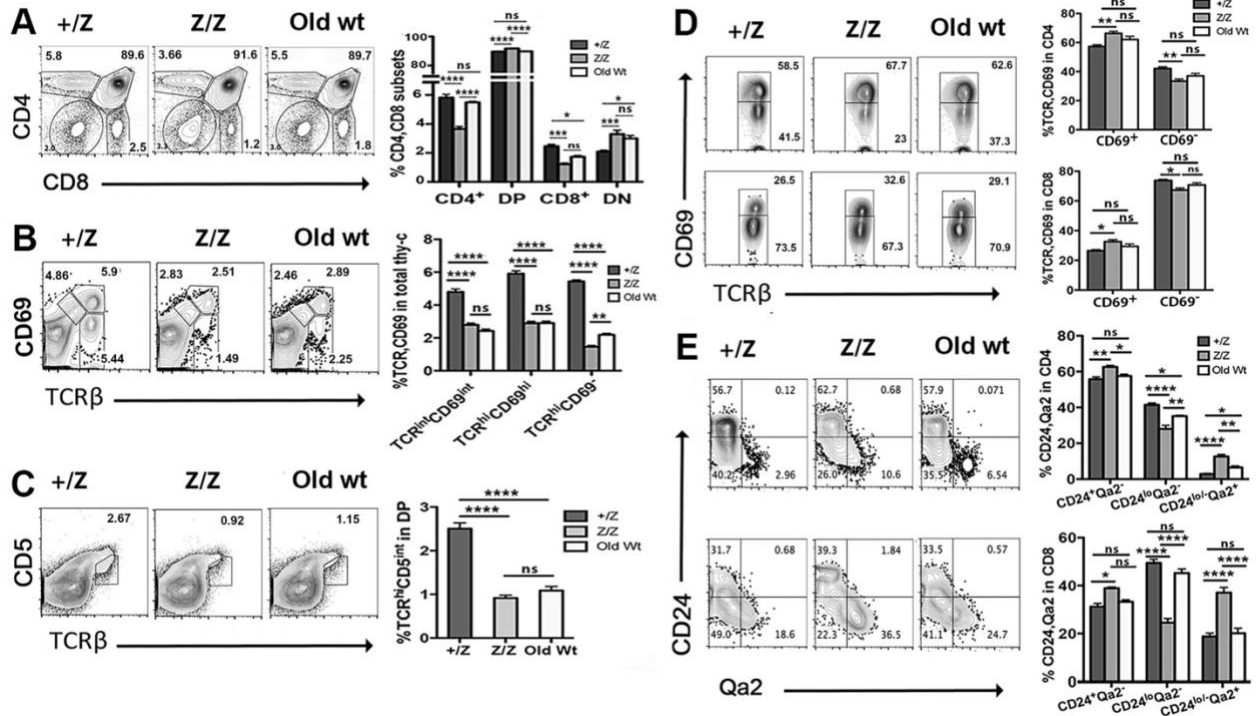


Figure 3.2. Thymic selection and maturation of CD4 and CD8 SP thymocytes in young *Foxn1^{lacZ}* mutants.

Flow cytometry analysis of thymocytes from +/Z (2-months-old), Z/Z (2-months-old) and old wt mice (18-months-old). **(A).** Expression of CD4 and CD8 on total thymocytes. Percentage of DN, DP, CD4, and CD8 subsets on the right. **(B).** Expression of CD69 and TCRβ on total thymocytes. Percentage of TCRβ^{int}CD69^{int}, TCRβ^{hi}CD69^{hi} and TCRβ^{lo}CD69^{lo} subsets on the right. **(C).** Expression of CD5 on DP thymocytes. Percentage of TCRβ^{hi}CD5^{int} on the right. **(D).** Expression of CD69 and TCRβ on gated CD4 (top) and CD8 (bottom) SP thymocytes. Percentages of TCRβ⁺CD69⁺ and TCRβ⁺CD69⁻ on the right. **(E).** Expression of CD24 and Qa2 on gated CD4 (top) and CD8 (bottom) SP thymocytes. Percentage of CD24⁺Qa2⁻, CD24^{lo}Qa2⁻ and CD24^{lo}Qa2⁺

on the right. (A, B, D and E): Two-way ANOVA; (C): One-way ANOVA. P values: **** ≤ 0.0001 , *** ≤ 0.001 , ** ≤ 0.01 , * ≤ 0.05 .

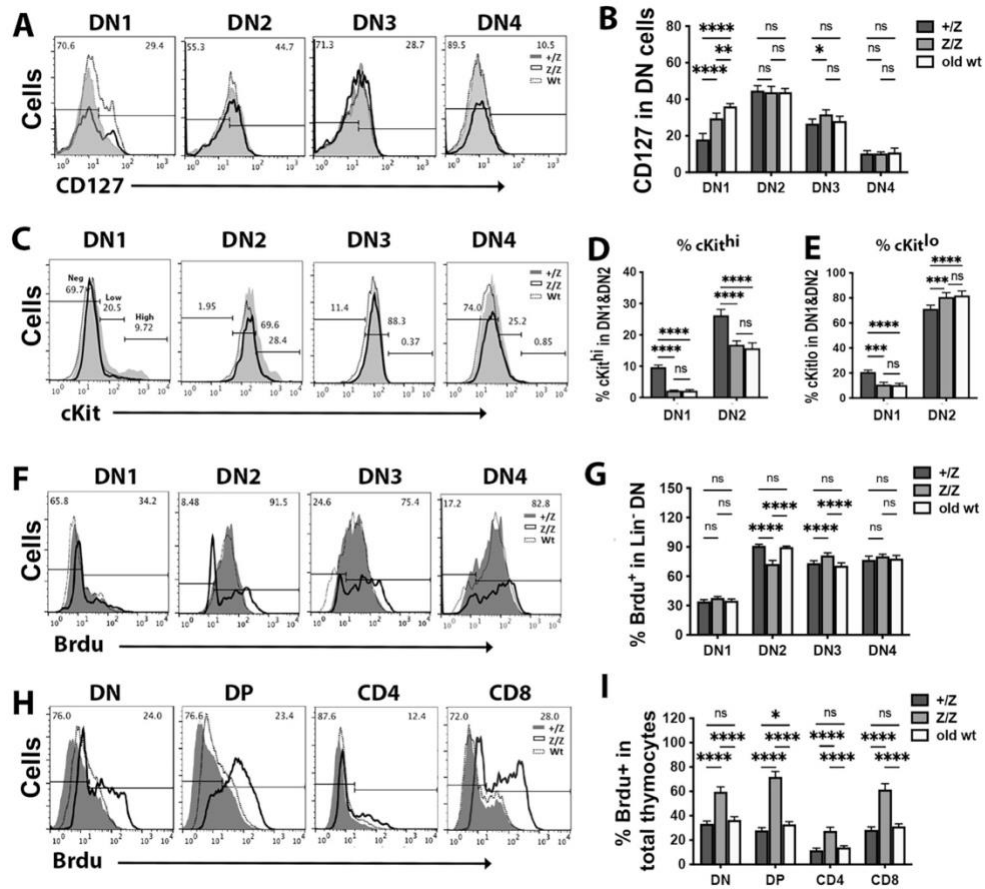


Figure 3.3. The proliferative capability of thymocytes was slightly increased in young *Foxn1^{lacZ}* mutants similar to aged wild type mice. (A-I).

Flow cytometric analysis of thymocytes from +/Z (1.5-months-old), Z/Z (1.5-months-old), and old wt (18-months-old) mice. **(A)**. Histogram of CD127 expression on subsets of DN thymocytes. **(B)**. Summary data of percentage of CD127 on DN1-4 subsets. **(C)**. Histogram of cKit expression on subsets of DN thymocytes. **(D)**. Percentage of cKit^{hi} on DN1 and DN2 subsets. **(E)**. Percentage of cKit^{lo} on DN1 and DN2 subsets. **(F)**. Histogram of BrdU staining on subsets of DN thymocytes. **(G)**. Summary data of percentage of BrdU⁺ cells on DN1-4 subsets. **(H)**. Histogram of BrdU staining on DN, DP, CD4, and CD8 thymocytes. **(I)**. Summary data of percentage of BrdU⁺ cells

in DN, DP, CD4, and CD8 thymocytes. Two-way ANOVA. P values: **** ≤ 0.0001 , *** ≤ 0.001 , ** ≤ 0.01 , * ≤ 0.05 .

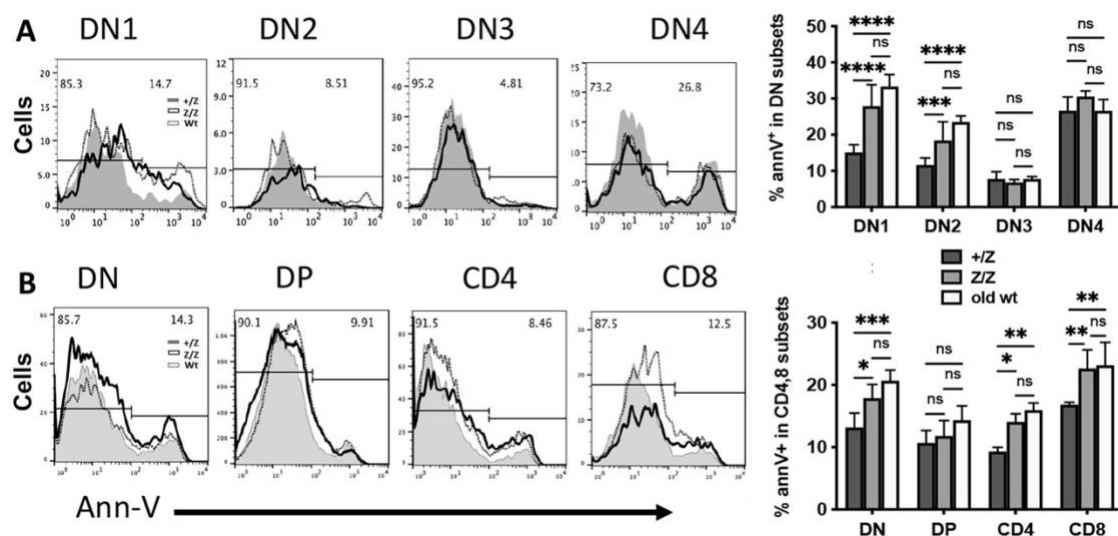


Figure 3.4. Premature thymic involution in young *Foxn1^{lacZ}* mice caused an increase in thymocyte apoptosis similar to aged wild type mice.

Flow cytometric analysis of thymocytes from +/Z (1-month-old), Z/Z (1-month-old), and old wt (15-months-old) mice by Annexin V staining. **(A).** Histogram of annexin V expression on subsets of DN thymocytes. Summary data of percentage of annexin V⁺ cells in DN1-4 subsets. **(B).** Histogram of annexin V expression on subsets of DN, DP, CD4, and CD8 thymocytes. Summary data of percentage of annexin V⁺ cells in DN, DP, CD4, and CD8 thymocytes. (A and B): Two-way ANOVA. P values: **** ≤ 0.0001 , *** ≤ 0.001 , ** ≤ 0.01 , * ≤ 0.05 .

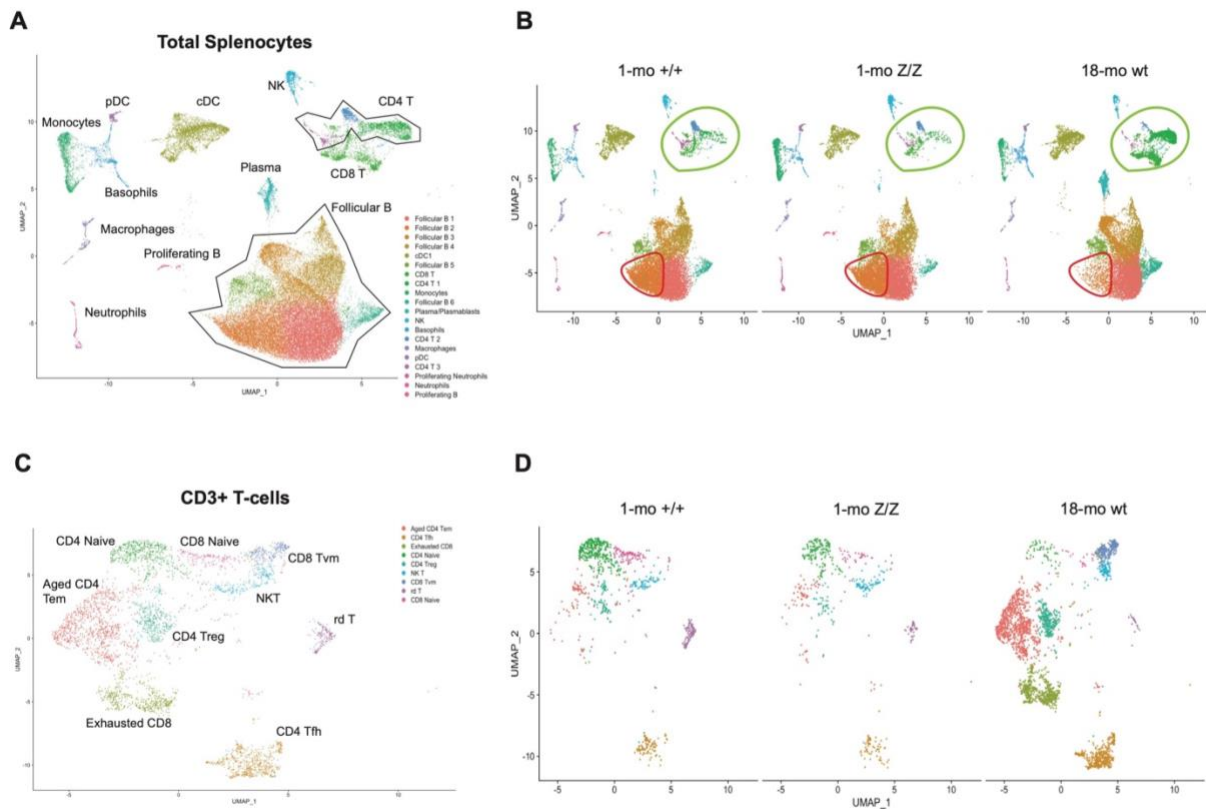


Figure 3.5. Total splenocyte scRNAseq comparison showed decreased naïve CD4 & CD8 populations in young *Foxn1^{lacZ}* mice.

(A) UMAP plot visualization of total splenocytes. (B) UMAP plot visualization of total splenocytes split by age/genotype. Red circles indicate a follicular B-cell cluster that is exclusively decreased in 18-mo wt. Lime circles indicate CD4 and CD8 single-positive (SP) T-cells. (C) UMAP plot visualization of CD3+ T-cells. (D) UMAP plot visualization of CD3+ T-cells split by age/genotype. Abbreviations: *mo*, months old; *Z/Z*, *lacZ*; *WT*, wild type.

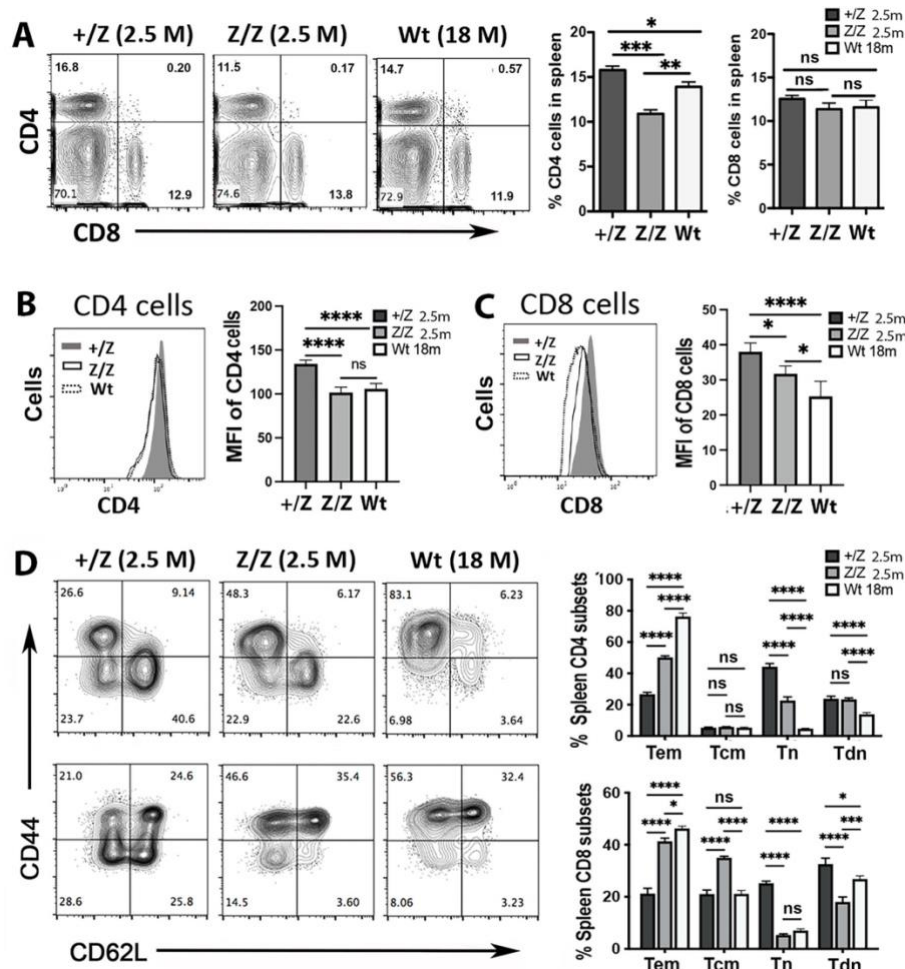


Figure 3.6. Young *Foxn1*^{lacZ} mice showed age-related phenotypic changes in peripheral lymphocytes.

Flow cytometric analysis of spleen cells from +/Z (2.5-months-old), Z/Z (2.5 months-old), and aged wt (18-months-old) mice. **(A)**. CD4- and CD8-expressing spleen cells. Right, percentage of CD4 and CD8 T cells of total spleen cells. **(B)**. Histogram of CD4 fluorescence density on gated CD4 T cells. CD4 mean fluorescence density (MFI) is shown to the right. **(C)**. Histogram of CD8 fluorescence density on gated CD8 T cells. CD8 mean fluorescence density (MFI) is shown to the

right. **(D)**. Expression of CD44 and CD62l on gated CD4 (top) and CD8 (bottom) spleen T cells. Percentages of effector memory (Tem), central memory (Tcm), naïve (Tn), and double negative (DN) (CD62L⁻CD44⁻) subsets of T cells are to the right. (A-C): One-way ANOVA; (D): Two-way ANOVA. P values: **** ≤ 0.0001 , *** ≤ 0.001 , ** ≤ 0.01 , * ≤ 0.05 . Abbreviations: *M*, months.

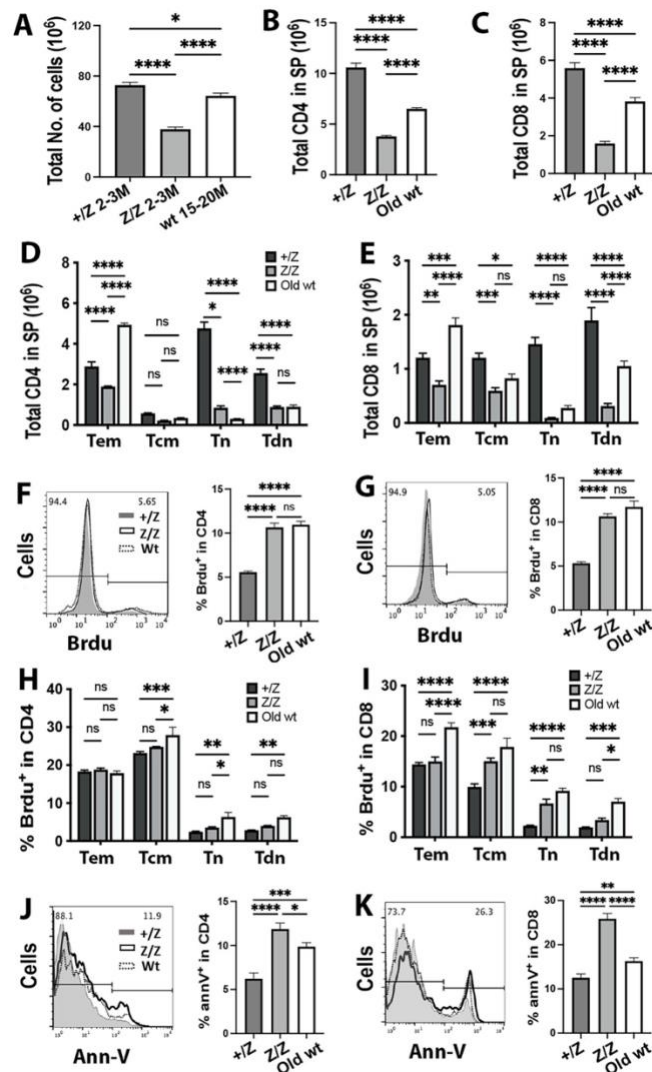


Figure 3.7. Young *Foxn1^{lacZ}* mice displayed mild peripheral lymphopenia.

(A). Histogram of the total number of peripheral lymphocytes prepared from spleen and lymph nodes (LNs) of +/Z (2-3 months-old), Z/Z (2-3 months-old), and old wt (15-21 months-old) mice. **(B and C).** Histogram of the total number of CD4 T cells (B) and CD8 T cells (C) in spleen. **(D and E).** Histogram of the total number of CD4 T cells subsets (D) and CD8 T cell subsets (E) in spleen. **(F and G).** Histogram of BrdU staining CD4 T cells (F) and CD8 T cells (G). Percentages

of Brdu⁺ CD4 and CD8 T cells shown to the right. **(H and I).** Histogram of Brdu⁺ CD4 T cell subsets (H) and CD8 T cell subsets (I). **(J and K).** Histogram of Annexin V staining CD4 T cells (J) and CD8 T cells (K). Percentages of Annexin V⁺ CD4 and CD8 T cells shown to the right. (B-K): Cells analyzed in graphs were prepared from +/-Z (2 mos), Z/Z (2 mos), and old wt (18 mos) mice. (A-C, J and K): One-way ANOVA; (D, E, H, and I): Two-way ANOVA. P values: **** ≤ 0.0001 , *** ≤ 0.001 , ** ≤ 0.01 , * ≤ 0.05 . Abbreviations: Tem, Effector memory T-cells; Tcm, Central memory T-cells; Tn; Naïve T-cells.

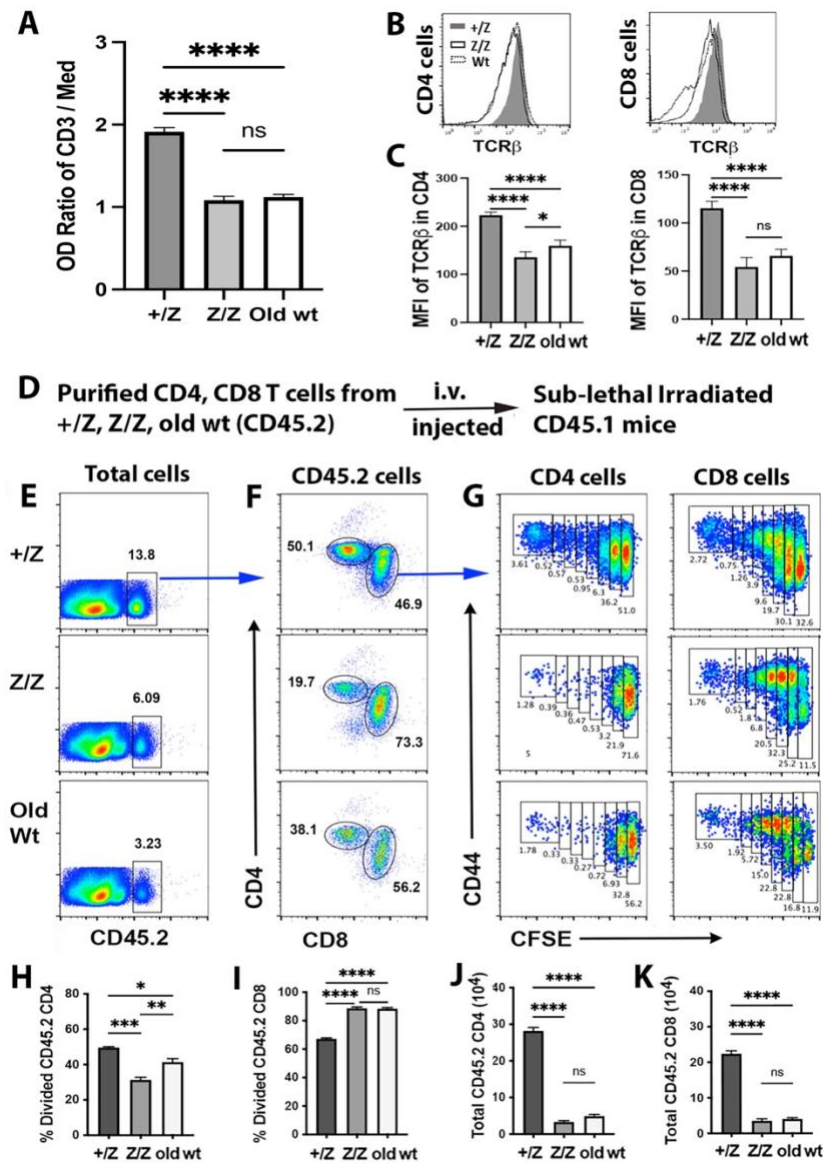


Figure 3.8. T cells from young adult *Foxn1^{lacZ}* mutants and aged mice had reduced proliferative capability after TCR stimulation.

(A). Purified CD4 T cells from +/Z (3-months-old), Z/Z (3-months-old) and old wt (18-months-old) were co-cultured with or without anti-CD3 + CD28 antibodies in vitro for 48 hours and tOD values were measured. The OD ratio of anti-CD3 to medium is shown. **(B).** Histogram of TCRβ

expression on gated CD4 (left) and CD8 (right) T cell. **(C)**. TCR β mean fluorescence density (MFI) on CD4 T cells (left) and **(C)**. CD8 T cells (right). **(D)**. Description of transfer experiment: 6×10^6 cells/mouse of B cell-deleted CD4 and CD8 T cells isolated from +/Z (3-months-old), Z/Z (3-months-old), and wt (18-months-old) mice (CD45.2) were transferred into sub-lethally irradiated CD45.1 (2-3 mons) mice. Six days later, donor cells (CD45.2⁺) were analyzed. **(E)**. Expression of CD45.2 on total spleen cells. **(F)**. Expression of CD4 and CD8 on gated CD45.2. **(G)**. The profiles of CFSE and CD44 on gated CD4 (left) and CD8 (right) T cells (CD45.2). **(H-I)**. Percentage of cells with at least one division in CD45.2⁺ CD4 (H) and CD8 (I) T cells. **(J-K)**. Total number of recovered CD45.2⁺ CD4 (J) and CD8 (K) T cells from spleen. (A): Two-way ANOVA; (C, H-K): One-way ANOVA. P values: **** ≤ 0.0001 , *** ≤ 0.001 , ** ≤ 0.01 , * ≤ 0.05 .

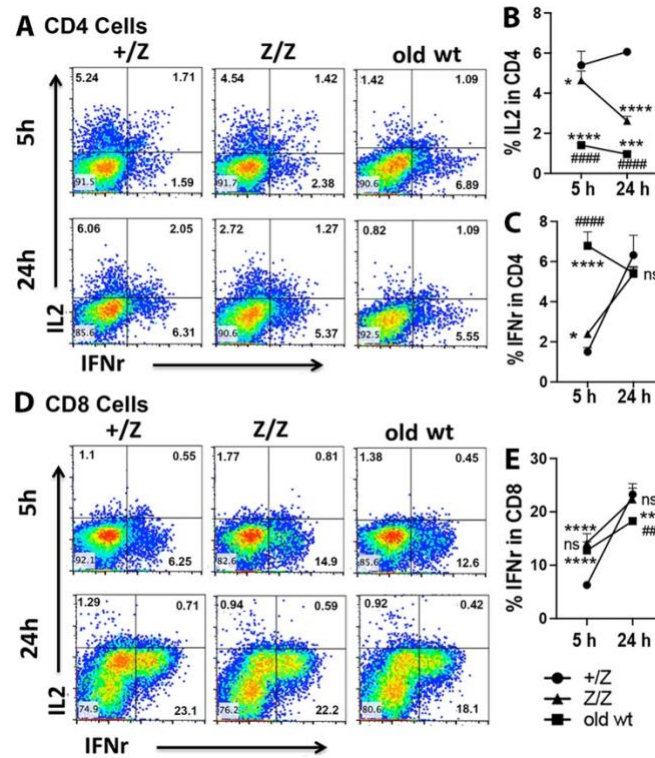


Figure 3.9. T cells from young adult *Foxn1^{lacZ}* mutants and aged mice had decreased production of cytokines after TCR-CD3 stimulation.

2x 10⁶ cells/well of B cell-deleted CD4 and CD8 T cells isolated from +/Z (3-months-old), Z/Z (3-months-old), and wt (18-months-old) mice (CD45.2) were co-cultured with or without anti-CD3 + CD28 in vitro for 5 hours (h) or 24 h. Cytokine production was analyzed for collected cells by intracellular staining. **(A)**. Production of IL2 and IFN γ in CD4 T cells at 5 h (top) and 24 h (bottom). **(B)**. Percentage of IL2 production in CD4 T cells at 5 h and 24 h. **(C)**. Percentage of IFN γ production in CD4 T cells at 5 h and 24 h. **(D)**. Production of IL2 and IFN γ in CD8 T cells at 5 h (top) and 24 h (bottom). **(E)**. Percentages of IFN γ production in CD8 T cells at 5 h and 24 h. (B, C, and E): Two-way ANOVA. #: comparisons between +/Z and Z/Z or aged wt. *:

comparisons between Z/Z and aged wt. P values: **** ≤ 0.0001 , #### ≤ 0.0001 , *** ≤ 0.001 , ** ≤ 0.01 , ## ≤ 0.01 , * ≤ 0.05 . ns: no significance.

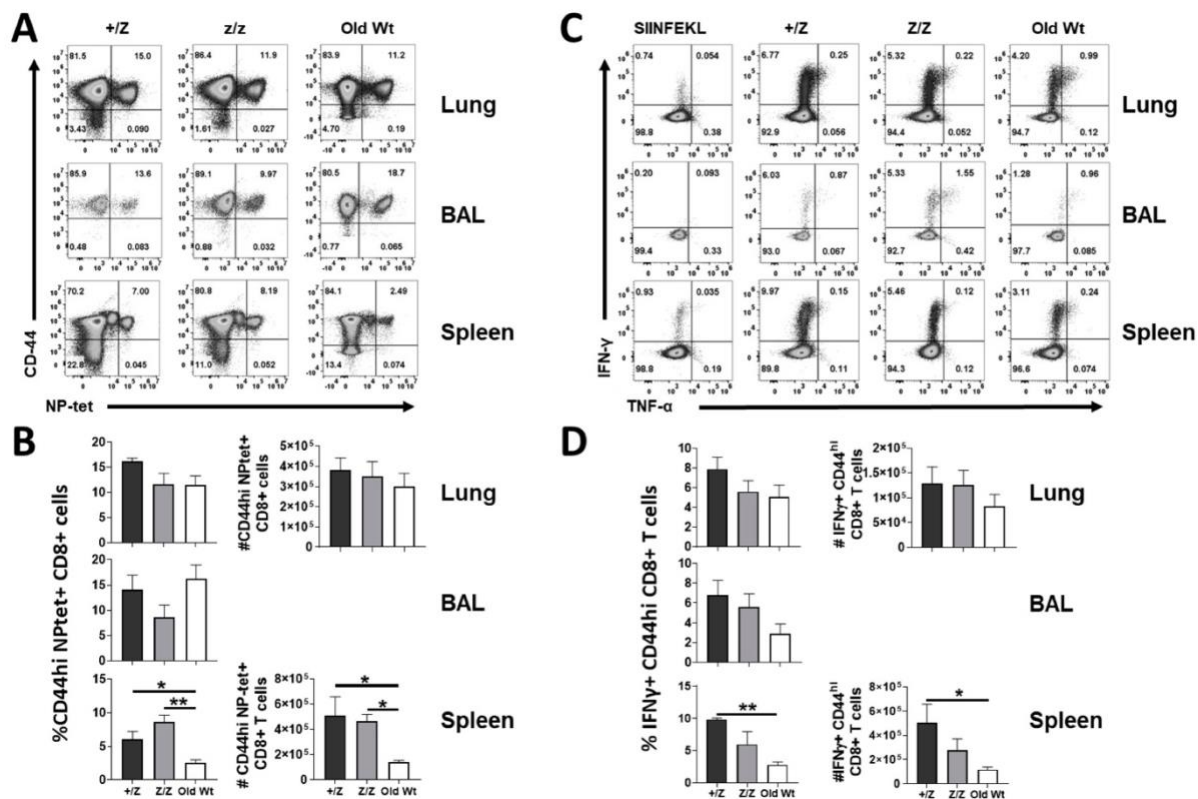


Figure 3.10. Premature involution in *Foxn1^{lac}* mice caused impaired response and function of activated antigen specific T cells after infection with influenza.

+/Z, Z/Z, and old wt (16-months-old) mice were infected intranasally with 10³ pfu of influenza (x31). Ten days later, the overall frequency and number of activated CD8 T cells that were NP-tetramer positive or producing IFN-γ and TNF-α were measured. Representative flow plots (A) and their corresponding frequency and cell number of NP-tet⁺CD44⁺ cells of total CD8⁺ T cells from the lung, BAL, and spleen (B). Representative flow plots of IFN-γ⁺ and/or TNF-α⁺ cells (C) and the corresponding IFN-γ⁺ cells of CD8⁺CD44⁺ T cells from the lung, BAL, and spleen (D). Data is representative of 3 independent experiments of at least N=3 mice per group. Statistics

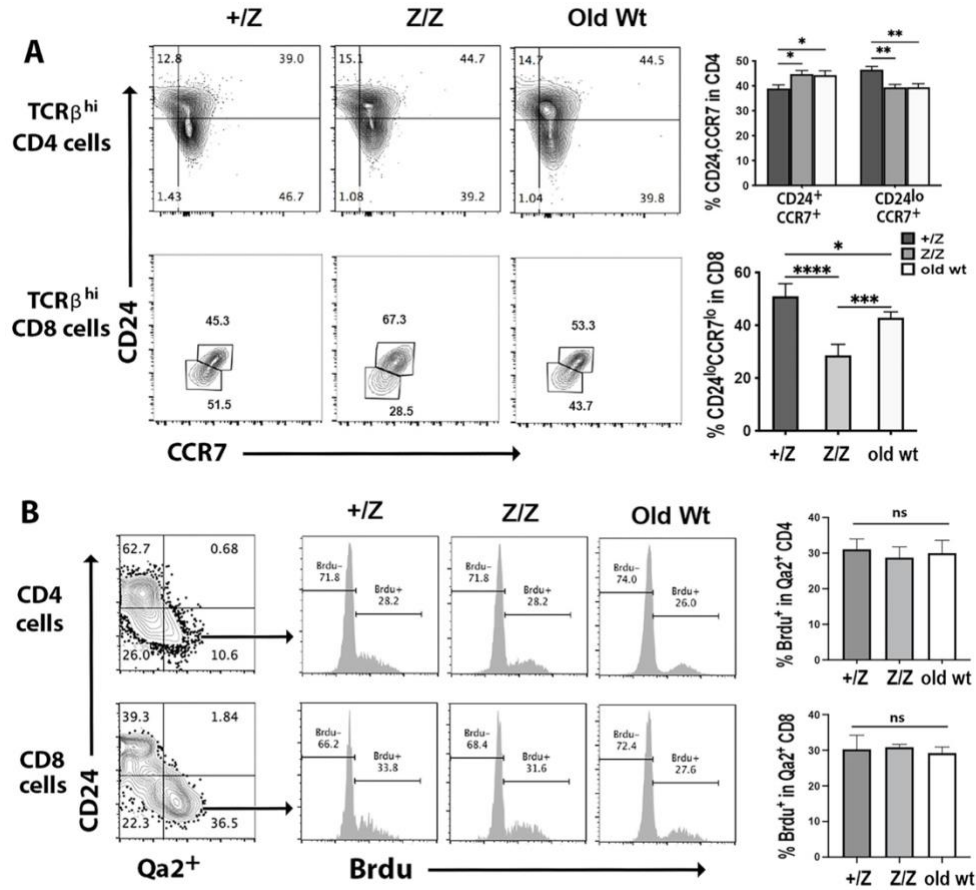
according to one-way ANOVA using Graph Pad Prism 8 software. * = $p \leq 0.05$, ** = $p \leq 0.01$.

Abbreviations: *BAL*, bronchoalveolar lavage.

Blue: Phenotype or trend shared between young Z/Z and old wt
Red: Phenotype unique to young adult Z/Z
Green: Phenotype unique to old wild-type
(*) indicates more severe phenotype

Samples	Phenotype	Comparison to young adult +/Z	
		Young adult Z/Z	Old wild-type
Thymocytes	% DN	Increased	Increased
	% DP	Increased	Similar
	% CD4 SP	Decreased	Similar
	% CD8 SP	Decreased	Decreased
	% Proliferative DN2	Decreased	Similar
	% Proliferative DN3	Increased	Similar
	% Proliferative DN	Increased	Similar
	% Proliferative DP	Increased (*)	Increased
	% Proliferative CD4	Increased	Similar
	% Proliferative CD8	Increased	Similar
	% Apoptotic DN1	Increased	Increased
	% Apoptotic DN2	Increased	Increased
	% Apoptotic DN	Increased	Increased
Splenocytes	% CD4	Decreased (*)	Decreased
	% CD4 Tem	Increased	Increased (*)
	% CD4 naïve T	Decreased	Decreased (*)
	% CD8 Tem	Increased	Increased
	% CD8 naïve T	Decreased	Decreased
	% CD8 Tcm	Increased	Similar
Spleen + lymph nodes	# PMC, CD4, CD8	Decreased (*)	Decreased
	# CD4 naïve T	Decreased	Decreased (*)
	# CD8 Tcm	Decreased	Decreased
	# CD8 naïve T	Decreased	Decreased
	% Proliferative CD4, CD8	Increased	Increased
	% Proliferative CD8 Tcm	Increased	Increased
	% Proliferative CD8 naïve T	Increased	Increased
	% Apoptotic CD4, CD8	Increased (*)	Increased
	# CD4 Tem	Decreased	Increased
	# CD8 Tem	Decreased	Increased
	% Proliferative CD4 Tcm	Similar	Increased
	% Proliferative CD4 naïve T	Similar	Increased
Influenza infection	% Proliferative CD8 Tem	Similar	Increased
	# % NP-tet+ CD8 T	Similar	Decreased
	# % IFN- γ + CD8 T	Similar	Decreased
In vitro stimulation	CD3 Proliferation	Decreased	Decreased
	% Initial IL-2 in CD4	Decreased	Decreased (*)
	% Prolonged IL-2 in CD4	Decreased	Decreased (*)
	% Initial IFN- γ in CD4	Increased	Increased (*)
	% Prolonged IFN- γ in CD4	Similar	Similar
	% Initial IFN- γ in CD8	Increased	Increased
	% Prolonged IFN- γ in CD8	Similar	Decreased
Slope Change Over Time			
	Young adult +/Z	Young adult Z/Z	Old wild-type
IL-2 in CD4	Increase	Decrease	Decrease
IFN- γ in CD4	Increase	Increase	Decrease
IFN- γ in CD8	Increase	Increase	Increase

Table 3.1. Summary of the T-cell phenotypes in young adult Z/Z and old wt.

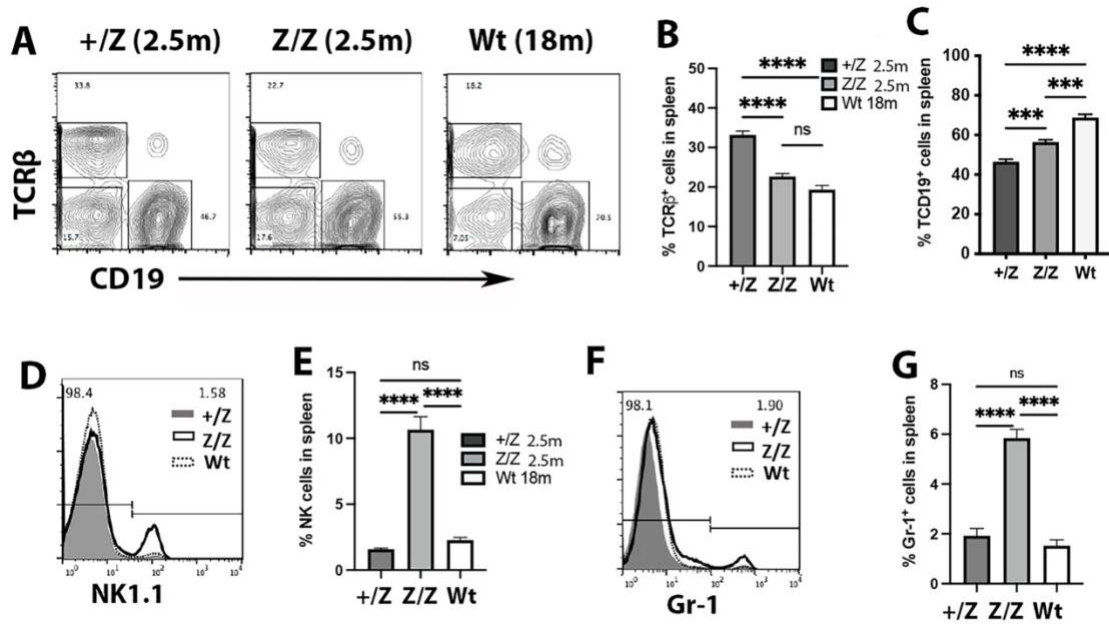


Supplementary Figure 3.1. Down-regulation of CD24 was reduced in young *Foxn1*^{lacZ} mutants and old wt mice.

Flow cytometric analysis of thymocytes from +/Z (2-months-old), Z/Z (2-months-old) and old wt mice (18-months-old). **(A)**. Expression of CD24 and CCR7 on TCRβ^{hi} CD4 and CD8 SP thymocytes. **(B)**. Expression of CD24 and Qa2 on gated CD4 (top) and CD8 (bottom) SP thymocytes (left). Histogram of Brdu staining on TCRβ⁺ Qa2⁺CD4 and TCRβ⁺ Qa2⁺CD8 SP thymocytes (middle). Summary data of percentage of Brdu⁺ in Qa2⁺ CD4 and CD8 SP thymocytes on the right. Data are representative of three individual experiments with at least 5 mice per group.

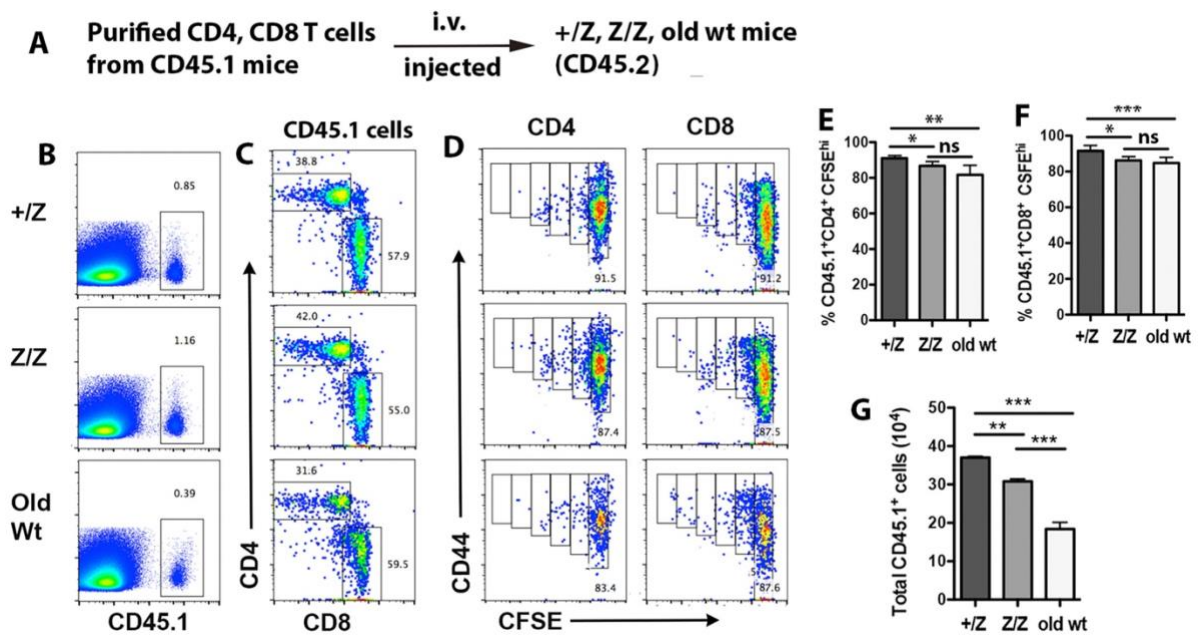
(A for CD4 cells): Two-way ANOVA; (A for CD8 cell and B): One-way ANOVA. P values:

**** ≤ 0.0001 , *** ≤ 0.001 , ** ≤ 0.01 , * ≤ 0.05 .



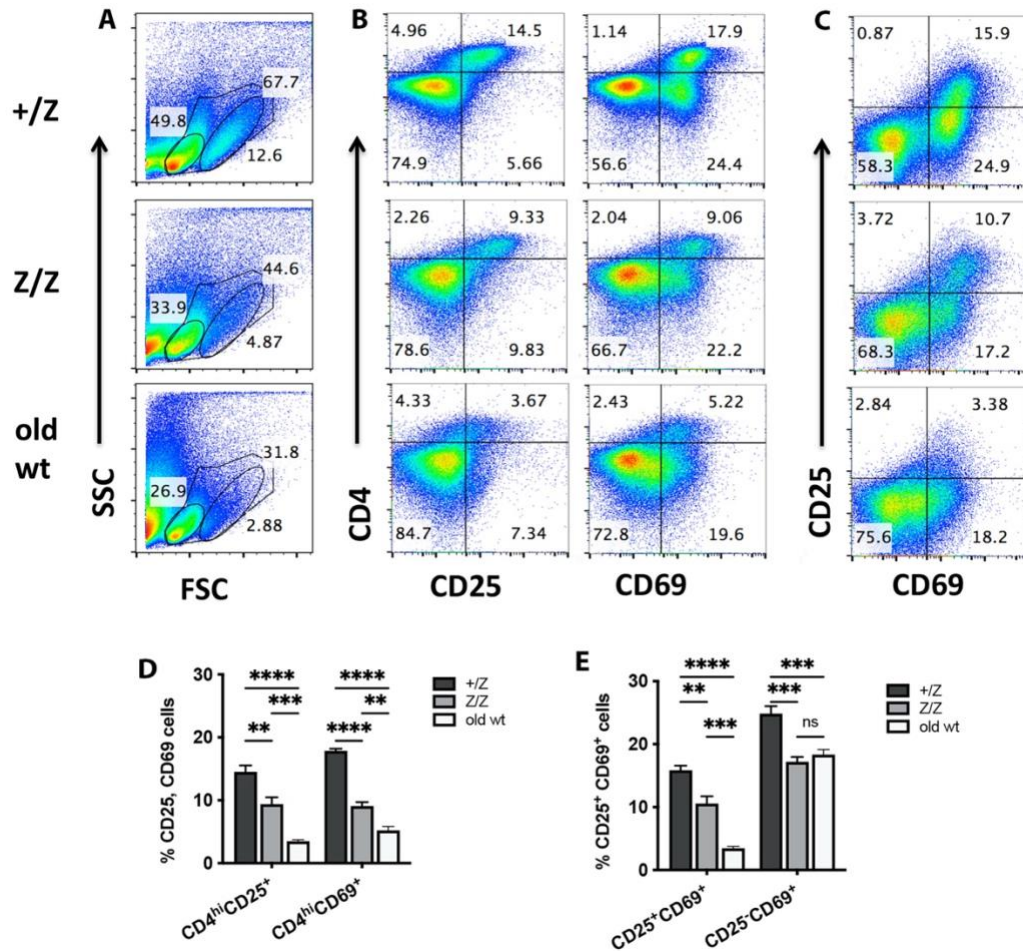
Supplementary Figure 3.2. Percentage of splenic B cells, NK cells, and granulocytes were increased in 2.5-months-old *Foxn1*^{lacZ} mutants.

Flow cytometric analysis of splenocytes from +/Z (2.5-months-old), Z/Z (2.5-months-old) and aged wt mice (18-months-old). **(A)** Histogram of TCRβ and CD19 total spleen cell expression. **(B).** Percentage of TCRβ⁺ spleen cells. **(C).** Percentage of CD19⁺ spleen cells. **(D).** Histograms of NK1.1-expressing spleen cells. **(E).** Percentage of natural killer (NK) cells in the spleen. **(D).** Histogram of Gr-1-expressing spleen cells. **(D).** Percentage of granulocyte in the spleen. Data are representative of three individual experiments with at least 5 mice per group. (B, C, E and G): One-way ANOVA. P values: ****≤0.0001, ***≤0.001, ns: No significance. Abbreviations: m, months.



Supplementary Figure 3.3. Peripheral lymphopenia promoted weak homeostatic proliferation in young *Foxn1*^{lacZ} mutants and aged wild type mice.

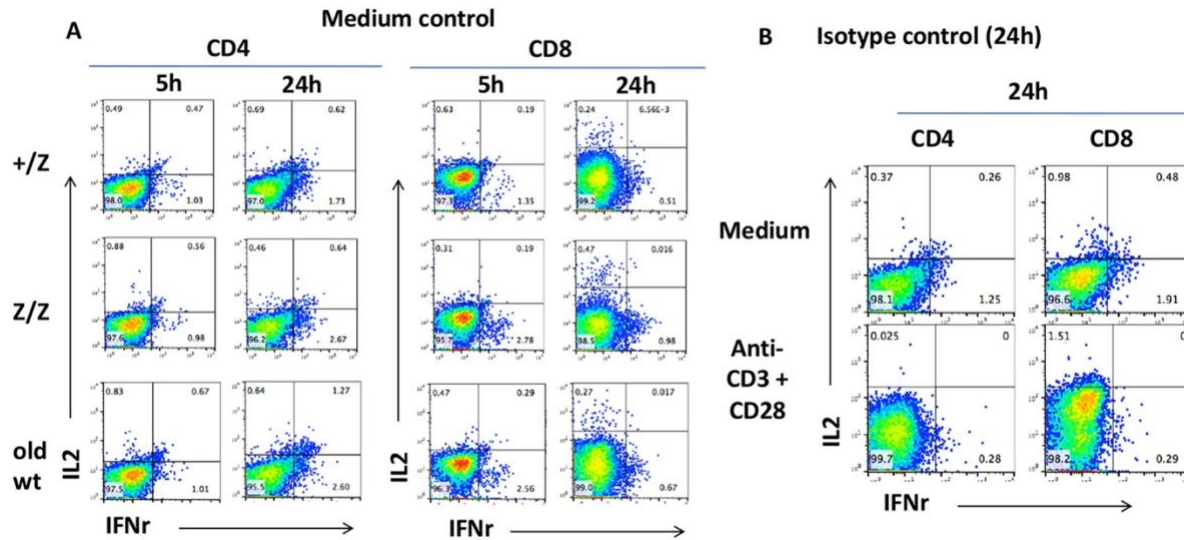
6 x 10⁶ cells/mouse of B-cell-deleted CD4 and CD8 T cells isolated from CD45.1 mice were transferred into +/Z (2-3 months-old), Z/Z (2-3 months-old) and old wt (18-months-old) mice (CD45.2). After 6 days, the donor cells (CD45.1⁺) were analyzed from hosts by showing the gate of CD45.1 cells. **(A)**. Description of cell transfer experiment. **(B)**. CD45.1 expression on total spleen cells. **(C)**. CD4 and CD8 expression on gated CD45.1 positive cells. **(D)** Profile of CFSE and CD44 expression on gated donor CD4 and CD8 T cells. **(E)**. Histogram of CFSE^{hi} percentage in CD45.1⁺ CD4 T cells. **(F)**. Histogram of CFSE^{hi} percentage in CD45.1⁺ CD8 T cells. **(G)**. Total number of donor lymphocytes (CD45.1) recovered from each host. Data are representative of two individual experiments with at least 5 mice per group. (E- G): One-way ANOVA. P values: ***≤0.001, **≤0.01, *≤0.05. ns: No significance.



Supplementary Figure 3.4. Peripheral CD4 T cells displayed weak activation upon stimulation of TCR-CD3 signaling *in vitro*.

Purified CD4 T cells from +/Z (3-months-old), Z/Z (3-months-old) and old wt mice (18-months-old) were co-cultured with or without anti-CD3 + CD28 antibodies for 48 hours *in vitro*. Activated markers CD25 and CD69 were analyzed on collected cells by flow cytometry. **(A)**. Profiles of FSC and SSC for cell ages. **(B)**. Expression of CD25 vs CD4 (left) and CD69 vs CD4 (right) on gated live cells. **(C)**. Expression of CD25 and CD69 on gated live cells. **(D)**. Percentage of CD4^{hi}CD25⁺ and CD4^{hi}CD69⁺ cells in gated live cells. **(E)**. Percentage of CD25⁺CD69⁺ and CD25⁻CD69⁺ cells

in gated live cells. Data are representative of two individual experiments with at least 5 mice per group. (D and E): One-way ANOVA. P values: **** ≤ 0.0001 , *** ≤ 0.001 , ** ≤ 0.01 , ns: No significance.



Supplementary Figure 3.5. Intracellular cytokine staining of medium and isotype control.

2x 10⁶ cells /well of B cell-deleted CD4 and CD8 T cells isolated from +/Z (3-months-old), Z/Z (3-months-old), and wt (18-months-old) mice (CD45.2) were co-cultured with anti-CD3 + CD28 and/or medium only *in vitro* for 5 hours (h) or 24 h. Cytokine production was analyzed for collected cells by intracellular staining. **(A)**. Production of IL2 and IFN γ at 5 h and 24 h in CD4 T cells (left) and in CD8 T cells (right). **(B)**. Profiles of isotype-FITC and isotype-PE reagents that matched IL-2-FITC and IFN γ -PE antibodies at 24 h post-activation of CD4 (left) and CD8 T cells (right) with medium (top) or in anti-CD3 + CD28 (bottom).

Material and Methods

Mice

Male and female C57BL6/J mice at 2-3 months and old mice at 15-18 months of age were purchased from National Institute on Aging. CD45.1 C57BL6/J mice were purchased from Jackson Laboratory. *Foxn1*^{+/*lacZ*} (+/Z) and *Foxn1*^{*lacZ*/*lacZ*} (Z/Z) mice were generated on a C57BL6/J background as described previously (Chen et al. 2009; Gordon et al. 2007). All mice were maintained in a specific pathogen-free facility at the University of Georgia. The experiment was approved by the Institutional Animal Care and Use Committee of the University of Georgia.

Isolation of the total thymic stroma

The thymus from three 1-month-old ZZ homozygotes and wild-type littermates were collected and pooled per single-cell dataset. Connective tissues, excess blood vessels, and adipocytes were carefully removed in 4°C sterile 1X DPBS. Clean thymuses were then transferred to digestion buffer (2% FBS/1640 RPMI (w/ 25mM HEPES), DNase I (20µg/µL), Collagenase/Dispase (1mg/mL)) to be thinly sliced into 1~2mm fragments. The digestion solution containing the thymic fragments was then incubated at 37°C for 1 hour. In between the incubation period, the solution was gently mixed up and down with transfer pipettes every 20 minutes, 100 times each. Post-digestion, the solution was passed through a 70µm cell strainer to remove undigested fragments. Cells were centrifuged at 300 x g, 4°C for 10 minutes. Red blood cells were then removed with a 2-minute incubation in RBC Lysis Buffer (10X) (BioLegend 420301). The solution was centrifuged again at 300 x g, 4°C for 10 minutes. Cells were then resuspended in 3mL MACS

buffer (filtered 0.5% BSA/2mM EDTA/1X DPBS). As per MACS separation guidelines, cells underwent CD45 separation with CD45 microbeads, mouse (Miltenyi Biotec 130-052-301).

10X library preparation and sequencing

Stromal cell viability was determined using a hemocytometer with Trypan Blue staining. Libraries were generated with samples with > 85% cell viability. Approximately 10,000 thymic stromal cells/splenocytes per sample were loaded into a 10X genomics Chromium instrument using a Single Cell 3' Reagent Kit (v3). Post cDNA QC, the final DNA library was sequenced on Illumina NextSeq2000 P3.

Single-cell RNA sequencing data analysis

As per the 10X guideline, we used CellRanger (v7) to generate and pre-process FASTQ files. CellRanger generated filtered_feature_bc_matrix files underwent QC steps and were analyzed using Seurat (v.4.3.0) in R (v.4.2.1). Logistic regression test was used for the differentially expressed gene analysis.

Flow cytometry

Freshly isolated thymocytes in suspension (1.0×10^6) were used for each sample. Cells were blocked by anti-CD16/32 (Clone:93) antibody before staining. For tracing the kinetic phenotypic profile and counting the numbers of thymocyte subsets, anti-CD4 APC-Cy7 or anti-CD4 violet 427 (GK1.5), anti-CD8 PerCp or anti-CD8 violet 570 (53-6.7), anti-CD44 FITC or PE (IM7), anti-

CD25 APC (3C7), Anti-CD19 PE-Cy7 (6D5), anti-NK1.1 PE (PK136), anti-CD69 APC, anti-TCR β PE-Cy7, and Qa2-biotin following PE-cy7 avidin were used to make a 6 or 8 color staining. For analysis of thymic progenitor Lin⁻ DN1a,b T cells in the total DN1 subpopulations, phycoerythrin (PE) conjugated lineage markers anti-CD3 (145-2C11), CD4, CD8, CD11c (N418), CD19, Gr-1 (RB68-C5), TER-119 (TER-119), NK1.1 antibodies were mixed and combined with anti-CD25 PerCp and anti-CD44 PE-Cy7, anti-CD117 APC (2B8), and CD24 FITC (M1/69) antibodies. For phenotypical analysis of spleen cells, anti-CD4 APC-Cy7 or anti-CD4 violet 427, anti-CD8 PerCp or anti-CD8 violet 570, anti-CD44 FITC, anti-CD62l PE-Cy7, anti-CD69 APC, anti-CD25 PerCp, anti-CCR7 PE, anti-CD45.1-PE, and anti-CD45.2-APC antibodies were used to make a 6 or 8 color staining. All antibodies were purchased from BioLegend (San Diego, CA). For AnnexinV staining (BioLegend, San Diego, Cat. # 640916), we followed the manufacturer instructions after surface antibodies staining. For antigen-specific CD8 T cell analysis, influenza nucleoprotein (NP) MHC class I tetramer [H-2D(b)/ASNENMETM], anti-CD8a $\square$ FITC (53-6.7), anti-CD4 PE (RM4-5) and anti-CD44 PE-Cy7 (IM7) were incubated with cells for 1 hour at room temperature. All antibodies were purchased from Tonbo Biosciences (San Diego, CA) and the NP tetramer was generously provide by the NIH Tetramer Core Facility at Emory University (Atlanta, GA). Phenotypical analysis was performed with a Cyan ADP Flow Cytometer (Beckman Coulter, Miami, FL). The data were analyzed by FlowjoTM Software (Tree Star, Ashland, OR).

Thymic epithelial cell sorting

For thymic epithelial cell (TEC) sorting, thymic lobes were cut into 1 mm³ pieces and gently washed in 2% FBS + RPMI1640 medium to partially remove thymocytes. The thymic pieces were then transferred to a digestion solution containing collagenase/dispase (Roche) at 1mg/ml and DNase I 20ng/ ml in 8ml of 2% FBS + PRMI1640 medium, and then placed into a 37 °C water bath to digest for 60 minutes. The thymic tissue was gently agitated by plastic pipette every 20 minutes. The cells were filtered by passing through a size 70µm cell strainer and then incubated with anti-CD45 APC (30-F11), anti-EpCAM PE (G8.8) and anti-MHCII FITC (M5/114.15.2). TECs were sorted as CD45-EpCAM+MHCII+ cells by MoFlo™ cell sorter.

Cell culture and intracellular staining of cytokines

Peripheral lymphocytes were prepared from spleen and LNs of 2-3 months of age +/Z, Z/Z mice and 15-18 months of age BL6 wt mice. The cells (2.0×10^6 /well) were plated in 24-well plates previously coated with 10 µg/ml anti-CD3ε (clone 145-2C11) and cultured in the presence of anti-CD28 at 2µg/ml for 5 or 24 h. As a control, cells were cultured in medium alone. To inhibit the secretion of newly synthesized cytokines, stimulations were conducted in the presence of 5 µg/ml brefeldin A (Sigma-Aldrich) in the last 5 hours of culture. After stimulation, cells were harvested and surface marker staining was performed with anti-CD4, anti-CD8, anti-CD44, CD25, and CD69. The cells were fixed in 0.5 ml/tube of fixation buffer (Cat. No. 420801) and permeabilized with permeabilization wash buffer (Cat. No. 421002) and then stained with anti-IL2 FITC, anti-IFNγ PE. Isotype of antibodies for each color was used as negative control. Stained cells were

washed twice with permeabilization wash buffer and kept in FACS stock solution for flow cytometry analysis. Foxp3 staining was performed according to the protocol of the Treg staining kit from eBioscience. T cells from influenza infection experiments were re-stimulated after pulsing tissue-resident antigen-presenting cells (APCs) in the isolated tissues with either NP [ASNENMETM] or HA [YVQASGRVTVSTRRS] peptides, or a non-specific peptide [SIINFEKL] in RPMI supplemented with 10% FBS, 10% supplementum completum (HEPES, pen/strep, L-glutamine, β -mercaptoethanol, gentamycin, FCS, and RPMI) and golgistop for 5 hours at 37°C. Splenocytes from naïve mice were added to the airway extracted cells as an exogenous APC source. Subsequently, cells were surfaced stained with anti-CD8a FITC and anti-CD44 PE-CY7 for 20 minutes at 4°C and fixed overnight in 2% paraformaldehyde. The following day, cells were permeabilized using eBioscience PermWash (San Diego, California) for 30 minutes at 4°C, stained with anti-IFN- γ APC (XMG1.2) and anti-TNF- α PerCP efluor 710 (MP6-XT22) for 20 minutes 4°C, and fixed in 2% paraformaldehyde for flow cytometry analysis. All antibodies purchased from Tonbo Biosciences (San Diego, CA) or eBioscience (Carlsbad, CA).

Cell culture and proliferation

Peripheral lymphocytes were isolated from the spleen and lymph nodes of 2-3 months-old +/Z and Z/Z mice and 15-18 months-old BL6 wild-type mice. CD4⁺ T cells were purified by incubation with anti-CD4 microbeads and passing through a magnetic column (Miltenyi Biotec). The purity of cells was higher than 95% as determined by flow cytometry. Purified CD4⁺ cells (2.0×10^5 /well) were then placed in 96-well plates previously coated with 10 μ g/ml anti-CD3 ϵ Ab. Anti-

CD28 was added at 2 µg/ml. Cells were cultured in a 37°C, 5% CO₂ incubator for 48 hours. For the last 4 hours, cultures were pulsed with CellTiter 96 Aqueous One Solution Reagent (Promega), 20 µl/well. As a control, cells were cultured in medium alone. We measured and recorded the absorbance at 490 nm using a 96-well plate reader. Some cells were also collected for phenotypic analysis with anti-CD4, CD8, CD25, CD69 antibodies.

Chimera generation

For analysis of peripheral lymphopenia, purified CD4 and CD8 T cells isolated from CD45.1 mice were transferred into Foxn1 Z/Z and old wt mice. For analysis of the proliferative capability of T cells, purified CD4 and CD8 T cells isolated from Foxn1 Z/Z and old wt (CD45.2) mice were transferred into sublethal irradiated (500 Rad) CD45.1 wt mice. CD4 and CD8 T cells isolated from spleen and lymph nodes were purified by incubating with anti-CD4 and CD8 microbeads (Miltenyi Biotec) and passing through LS magnetic columns following manufacturer instructions. The purity of cells was higher than 95%. Purified T cells were then incubated with CFSE (Molecular Probes) at final concentrate of 10 mM in PBS + 0.1% BSA, 37°C, for 10 min. The unlabeled CFSE was quenched by adding 10% FCS medium and then washed by 2% FBS medium. Depending on the experiment, Foxn1 Z/Z and old wt or sublethal irradiated BL6 CD45.1 wt mice were retro-orbitally injected with CFSE-labeled cells (4.0×10^6 /mouse). Six days after the transfer, peripheral lymphocytes collected from host lymph nodes and spleen were stained and analyzed by flow cytometry.

BrdU incorporation and staining

Mice were injected once intraperitoneally with 1 mg of 5-bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich) and then fed with BrdU-containing water (0.8 mg/ml) for five days. The thymocytes were harvested and stained with CD4, CD8, CD25, CD44 antibodies. The surface-stained cells were fixed and permeabilized in PBS containing 1% paraformaldehyde plus 0.01% Tween20 for 48 hours at 4°C and then submitted to the BrdU DNase1 detection technique. FITC-anti-BrdU Ab (clone 3D4; BioLegend) was used for BrdU staining.

Influenza infection of mice and single cell suspension preparation

All mice were intranasally infected with 10^3 pfu of influenza A virus A/HK-x31 (x31, H3N2) in 50 μ l of PBS. At 10 days post infection (dpi), mice were sacrificed to obtain single cell suspensions from the lung tissue, lung airways, and spleen. Briefly, an incision was made into the trachea to perform a bronchoalveolar lavage (BAL) by dispensing 1 mL of PBS into the lung and aspirating the same volume. Lungs were then perfused with PBS/heparin, excised, and collected into 5 mL RPMI. Lastly, spleens were excised and collected into 5 mL RPMI. For tissue digestion, spleens were mechanically disrupted and passed through 40 μ M nitex nylon mesh. Red blood cells were removed by incubation with tris ammonium chloride for 2 minutes at room temperature. Lungs were minced before incubation for 30 minutes at 37°C with 1.25 mM EDTA followed by incubation in 150 units/mL collagenase. Lungs were then passed through a 40 μ M cell strainer to obtain cells, resuspended in 44% Percoll, and underlaid with a 67% Percoll solution. After centrifugation at 2800 rpm for 20 minutes, lymphocytes were then collected at the cellular

interface.

Statistical analysis

All data were collected in a Microsoft Excel file and analyzed using Prism 8 software by one-way or two-way analysis of variance (ANOVA).

CHAPTER 4

MECHANISMS UNDERLYING THE DIRECT PROGRAMMING OF MOUSE EMBRYONIC FIBROBLASTS TO THYMIC EPITHELIAL CELLS BY FOXN1

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Abstract

Thymic epithelial cells (TECs) are a critical functional component of the thymus's ability to generate T cells for the adaptive immune system in vertebrates. However, no *in vitro* system for studying TEC function exists. Overexpressing the transcription factor FOXP1 initiates transdifferentiation of fibroblasts into TEC-like cells (iTECs) that support T cell differentiation in culture or after transplant. In this study, we characterized iTEC programming at the cellular and molecular level to determine how it proceeds and identified mechanisms that can be targeted for improving this process. These data showed that iTEC programming consisted of discrete gene expression changes that differed early and late in the process, and that iTECs upregulated markers of both cortical and medullary TEC (cTEC and mTEC) lineages. We demonstrated that promoting proliferation enhanced iTEC generation, and that Notch inhibition allowed induction of mTEC differentiation. Finally, we showed that MHCII expression was the major difference between iTECs and fetal TECs. MHCII expression was improved by co-culturing iTECs with fetal double-positive T-cells. This study supports future efforts to improve iTEC generation for both research and translational uses.

Introduction

The thymus is a primary lymphoid organ and is the major source of self-restricted, self-tolerant naïve T cells required for a robust adaptive immune system. However, through ageing, the thymus is one of the earliest organs that starts losing its function, also known as thymic involution. Thymic involution leads to decreased immune function, which significantly increases the risk of cancer and auto-immune diseases (Chinn et al., 2012; Hale et al., 2006; Palmer et al., 2018). Thus, finding an effective method to rescue loss of the thymus function caused by thymus involution and thymus abnormalities could have a significant translational impact.

Thymic epithelial cells, or TECs, comprise the main functional resident cell types in the thymus. TEC-thymocyte interactions are required for T lineage commitment and all stages of thymocyte differentiation, proliferation, selection, and survival (Takahama, 2006). TECs are divided into cortical (cTEC) and medullary (mTEC) lineages based on their location and function. There is evidence that both lineages derive from a common progenitor, and that mTEC lineages derive from cells that have initiated cTEC differentiation (the ‘cTEC first’ model) (Baik et al., 2013; Bornstein et al., 2018; Rossi et al., 2006; Takahama et al., 2017). The mechanisms inducing cTEC differentiation are not understood. Recent evidence indicates that Notch signalling is required for mTEC lineage commitment but must be suppressed for mTEC differentiation to proceed past the progenitor stage (Li et al., 2020; Liu et al., 2020).

Forkhead transcription factor FOXN1 is both necessary and sufficient for TEC differentiation and maturation and is the key transcription factor required for fetal TEC differentiation. FOXN1 is expressed by all cells specified for TEC identity within the 3rd

pharyngeal pouch by mouse embryonic day E11.5 (Vaidya et al., 2016). Null mutation of *Foxn1* (nude) in mice, rats, and humans disrupts both normal hair growth and thymus development, resulting in immunodeficiency (Blackburn et al., 1996; Nehls et al., 1994). *Foxn1* also plays a critical role in postnatal thymus function and maintenance. *Foxn1* is expressed in both cTECs and mTECs during postnatal stages but significantly decreases during ageing, which is considered the major cause of thymic involution (Bredenkamp et al., 2014a; Chen et al., 2009; O'Neill et al., 2016; Ortman et al., 2002; Rode et al., 2015; White et al., 2022).

Transplanting neonatal thymus or cytokine induction can partially rescue thymic function after involution, but these methods are all highly limited by resources and efficacy. Another approach is to use cellular programming to generate key cell types *in vitro* for functional studies or transplantation. Direct lineage programming is characterized by a dynamic conversion of cellular morphology and transcriptomes. We and our collaborators have shown that over-expression of *Foxn1* is sufficient to reprogram mouse embryonic fibroblasts (MEFs) into functional induced thymic epithelial cells (iTECs) (Bredenkamp et al., 2014b). These iTECs express FOXN1 downstream targets *Dll4*, *Ccl25*, and *Kit-l*, and exhibit epithelial cell-like morphology. However, iTECs cultured alone do not express MHC Class II (MHCII), a critical functional component of TECs, although upon co-culture with early thymic progenitors (ETPs) iTECs can support their maturation into single positive (SP) T cells. Finally, iTECs grafted with supporting mesenchyme under the kidney capsule of nude mice can generate an organ with distinct cortical and medullary regions that could generate and export T cells. These results suggest that iTECs placed in a cross-talk environment (upon co-culture and/or transplant) are competent to

upregulate MHCII and mature into both cortical and medullary lineages. They thus suggest the potential for both the generation of an *in vitro* system to study TEC function and TEC-T cell interactions, and for future therapeutic application of iTECs; however, there are significant challenges to overcome before the *in vitro* system can have broader utility.

In this study, we used both bulk and single-cell RNA-seq to dissect the *Foxn1*-induced direct reprogramming process (Treutlein et al., 2016). We sequenced iTEC transcriptomes at different stages of programming to characterize iTECs at the cellular and molecular levels. We showed that iTEC generation consisted of early and late programming stages characterized by discrete gene expressions. In the early stage, iTECs were characterized by activation of FOXN1 downstream targets, cell-matrix re-organization and cell morphology changes, and inhibition of cell cycle and cell proliferation. We also demonstrated that releasing the proliferation block with overexpression of Myc improved the programming efficiency. In the late programming stages, our data showed that iTECs not only up-regulate additional epithelial markers but also expressed both cTEC and mTEC markers, although only mTEC progenitor and not mature mTEC markers were observed. Our current data provide new evidence demonstrating that these mTEC progenitors are pre-existing in iTEC cultures. We further showed that Notch inhibition using DAPT in iTEC cultures induced mTEC terminal differentiation (albeit at a low level) as measured by the marker *Aire*. Lastly, we found that co-culturing iTECs with CD3^{Lo} double positive thymocytes increased MHCII expression in iTECs to similar levels found in fetal TECs. Together, this study provides novel insight into the mechanisms behind iTEC programming, which is critical for developing

iTECs as a useful experimental tool both for understanding *de novo* TEC biology and for potentially translational pre-clinical applications.

Results

Generation of iTECs using nucleofection-based methods

To generate samples representing different iTEC programming time-points, we first modified the originally published method of iTEC generation by using nucleofection-based direct transfection of a *Cre*-expressing plasmid, instead of 4-OHT treatment, to activate an inducible CreER transgene (Bredenkamp et al., 2014b). In brief, R26-CAG-STOP-Foxn1-IRES-GFP/+ (Fig. 4.1A, B; R26-iFoxn1) heterozygous MEFs from E13.5 embryos were transfected with a PGK-Cre plasmid on day 0. After 48hrs of culture, about a third of the MEFs removed the STOP cassette by Cre recombinase and activated the R26-iFoxn1 locus, which was identified by GFP expression (Fig. 4.1C). We termed these *Foxn1*- and GFP-expressing cells iFoxn1 MEFs. This method avoids the low level of “leaky” Cre expression from CreER, allowing us to precisely control the onset of iTEC programming and providing a clean starting point for downstream analyses.

As an initial characterization of the programming process, we analyzed the iFoxn1 MEFs for the epithelium-specific marker EpCam and the TEC maturation marker MHCII. Flow cytometry showed that more than 10% of iFoxn1 MEFs express EpCam 13 days after the initial activation of transgenic *Foxn1* expression; however, as in the previous report, MHCII protein was not detected (Bredenkamp et al., 2014b) (Fig. 4.1D). Also consistent with the previous report,

many of the iFoxn1 MEFs were Keratin-8 positive 10 days after transfection (Fig. 4.1F). No keratin-8 positive cells were observed in the mock-transfected MEF controls (Fig. 4.1E).

iTEC programming has progressive gene expression changes with a distinct intermediate cell step

We collected iFoxn1-MEFs at different time points after the initial iFoxn1 activation and used RT-qPCR to analyze the expression of several known FOXN1 target genes (Žuklys et al., 2016) in TECs (Fig. 4.2A, B). FOXN1 target genes were detected early after induction of *Foxn1* expression. *Dll4* and *Kit-l* significantly increased as early as 24hrs after transfection, and *Ccl25* was significantly up-regulated 2 days (2d) after transfection (Fig. 4.2A). The increase in these FOXN1 target genes over the course of the 12d iTEC induction period did not follow a linear pattern (Fig. 4.2B). Instead, these three genes underwent rapid expression increases starting at 7d after transfection, accelerating further after 10d (Fig. 4.2B). This pattern suggests that programming has two different stages: an early stage with initial low-level expression of target genes followed by a later stage in which FOXN1 target gene expression is dramatically accelerated.

To further understand the dynamic process of iTEC programming, we designed a time-point based bulk-RNA-seq experiment, choosing our time points based on the induction of FOXN1 target genes (Fig. 4.2C). We used mock-transfected R26-iFoxn1/+ MEFs as our control, representing the initial MEFs transcriptome status (No Cre MEFs). We induced R26-iFoxn1/+ MEFs to express *Foxn1* using Cre transfection, cultured for 2 days, and sorted cells that had

successfully induced *iFoxn1* expression as GFP⁺ using flow cytometry (as in Fig. 4.1C). These GFP⁺ *iFoxn1* MEFs represented initial programming and are referred to as iTEC2d. We also cultured iTECs for a total of 10days after transfection to represent the late stage of programming, termed iTEC10d. For the final stage, we cultured iTECs for a total of 12 days and used EpCam antibody staining to separate them into two distinct groups: EpCam⁻ & EpCam⁺. Each RNA-seq sample consisted of cells pooled from multiple independent iTEC cultures. RNA-seq was performed in duplicate (MEFs, iTEC2d, EpCam⁻ and EpCam⁺) or triplicate (iTEC10d).

To get an initial understanding of the progression of iTEC programming, we first analyzed MEF and variance stabilizing transformation (VSD) of the iTEC whole transcriptome profiles were performed followed by PCA analysis (Fig. 4.2D). We found that even 48hrs after the initial activation of ectopic *Foxn1*, iTEC gene expression was changed significantly compared to MEFs as indicated by the clear separation of iTEC2d and No Cre MEF samples. The iTEC10d sample was further separated from the iTEC2d sample but did not show a linear increase of variance from No Cre to iTEC10d through iTEC2d. This indicated that the iTEC programming process is not continuous at the gene expression profile level and instead has distinct gene expression patterns at the early and late stages. Finally, the EpCam⁺ samples were more distinct than EpCam⁻ samples compared to iTEC 10day. The whole transcriptome analysis showed a linear progression of differential gene expression in the order of 10d, EpCam⁻, and EpCam⁺, which suggests that EpCam⁺ cells represent a more advanced programming state (Fig. 4.2D). The sample distance count matrix also showed this progression from MEFs to EpCam⁺ iTECs with a significant difference between each step of programming (Fig. 4.2E). Furthermore, each programming stage

is generally reproducible, although two EpCam⁺ samples differed more from each other than did the replicates of earlier stages.

The gene count matrix of differentially expressed genes (DEG) (adjusted p-value < 0.01) results further showed that each stage of programming had a distinct signature with sets of genes turning off and on across the timeline (Fig. 4.2F). While many genes were gradually upregulated throughout the process, some genes were dynamically expressed. Other genes are downregulated in iTEC2d & iTEC10d compared to MEFs but then upregulated further in EpCam⁺ and EpCam⁻ samples. Similar to the global DEG analysis, our assessment of transcription factor changes revealed that specific sets of transcription factors were expressed dynamically at the early and late programming stages. While some transcription factors start to express in the early stage, many are not expressed until the late stage of programming (Fig. 4.2G), suggesting a time-dependent gene expression regulation.

Finally, we analyzed up-regulated & down-regulated genes at different stages by gene enrichment analysis (S. Fig. 4.1). We found that in early programming stages from MEFs to iTEC2d, iTECs were characterized by down-regulation of cell shape, extracellular matrix (ECM), and other fibroblast-related genes, and up-regulation of cytokine signaling and other immune-related categories (S. Fig. 4.1A, B). Between 2d and 10d, the cell cycle and DNA replication were inhibited and genes related to epithelial characteristics were upregulated, indicating that the iTECs are transitioning into epithelial cells during this second stage of programming (S. Fig. 4.1C, D). The distinctions between EpCam⁻ and EpCam⁺ 12d iTECs were also made more evident when compared to iTEC10d. EpCam⁻ iTECs upregulated programmed cell death and cell cycle

inhibition categories while epithelial-related genes were downregulated, suggesting that they constitute cells failing to maintain the transdifferentiation process and may undergo cell death (S. Fig. 4.1E-F). In contrast, EpCam⁺ cells upregulated differentiation and metabolism-related genes, while genes related to mesenchymal properties were downregulated (S. Fig. 4.1G-H).

Together, our results show that *Foxn1*-induced iTEC generation proceeds through intermediate steps that are significantly different from both MEF controls and the late programming stage. These data indicate that each programming stage is characterized by a unique gene expression profile, rather than a gradual accumulation of gene expression changes that build through the process.

cTEC and mTEC genes are activated at different stages of reprogramming

The initial iTEC report showed the formation of a well-organized organoid and differentiation of iTECs into both cTEC and mTEC upon transplantation under the kidney capsule (Bredenkamp et al., 2014b). However, it did not characterize cTEC and mTEC lineage markers in detail in iTECs in culture. We performed MA plot analysis to investigate TEC lineage specification and differentiation-related gene expression patterns during the programming process (Fig. 4.3A-E). Comparison of MEFs to 2d (Fig. 4.3A) showed up-regulation of FOXN1 target genes *Dll4*, *Kit-l*, and *Ccl25*. Strikingly, cTEC markers appeared very early, with *Krt18* and to a lesser extent *Krt8* already significantly upregulated at iTEC2d. *Psmb11* (β 5t), a cTEC marker and direct *Foxn1* target, was also upregulated but expressed at a lower level than *Krt8* and *Krt18*. In contrast, mTEC

markers such as *Krt5*, *Krt14*, and *Cldn4* were not detected at early stages (Fig. 4.3A), indicating that cTEC markers are expressed before mTEC markers during iTEC generation.

Between 2d and 10d, *FOXN1* target genes were further upregulated. More remarkable is very strong up-regulation of both cTEC (*Krt8* and *Psmb11*) and mTEC markers (Fig. 4.3B). mTEC markers first appeared at iTEC10d stage, where they were highly differentially expressed compared to 2d samples but still at much lower expression levels than the cTEC markers. Interestingly, *Foxn1* target genes and cTEC markers remained stable from iTEC10d to both EpCam- (Fig. 4.3C) and EpCam+ iTEC (Fig. 4.3D), but mTEC-related genes *Krt14* and *Krt5* were differentially upregulated specifically in EpCam+ iTEC at this latest stage of programming. (Fig. 4.3E). Notably, only mTEC markers were upregulated in EpCam+ iTECs compared to EpCam- iTECs. This progressive up-regulation of TEC differentiation genes with delayed mTEC marker up-regulation was further evident in a heat map analysis (Fig. 4.3F). However, EpCam+ iTECs had much lower expressions of these markers than fetal TEC, which could be due to lower expression of *Foxn1* in the iTEC system, or because this is a population analysis so far, and the proportions are very low (Fig. 4.3G). We also detected cTEC markers B5T (translated from *Psmb11*), *Krt8*, and *DLL4* at the protein level in iTECs (Fig. 4.3H).

A subset of *Foxn1*-receptive MEFs is successfully programmed and expresses TEC genes

To understand the iTEC programming process whilst understanding TEC lineage specification, we generated single-cell RNA-sequencing data of MEFs, iTEC3d, and iTEC12d. An unsupervised clustering of a batch-corrected merged dataset with Seurat (Hao* & Hao* et al.,

2021) yielded 9 high-quality cell clusters (Fig. 4.4A). We detected a *Foxn1*-high cell cluster located at the middle of the UMAP space that also expressed high levels of TEC markers, and a distinct set of proliferative cells. Despite having sorted iTECs based on their GFP expression (Fig. 4.1C), many of the iTECs retained MEF-like transcriptomes across these time points, suggesting *Foxn1*-susceptible & *Foxn1*-recalcitrant MEFs (Fig. 4.4B). The *Foxn1*-high cluster was detected at iTEC3d, but only appeared in large numbers in iTEC12d. We also detected a loss of the proliferative cell clusters 12 days post-programming, which we will explore later (Fig. 4.6).

Our RT-qPCR and RNA-sequencing data revealed an exponential increase in the relative expression of *Ccl25*, *Dll4*, and *Kit-l* (Fig. 4.2A, B, 4.3F). Violin plots showed that this increase correlated with high levels of *Foxn1* (Fig. 4.4C). Atop previously surveyed cTEC genes (S. Fig. 4.2), we also detected cells expressing a high level of *Prss16*, a thymus-specific serine protease (Fig. 4.4D-F). A small number of cells expressed high *Cldn4* within the *Foxn1*^{Hi} iTEC cluster in 12d iTECs (Fig. 4.4G-I), suggesting that mTEC progenitor (mTEPC) generation also requires a high level of *Foxn1*. When we surveyed relationships between a cTEC marker *Krt8* and an mTEPC marker *Cldn4*, it exhibited a Pearson correlation coefficient of 0.45, a moderate positive correlation between the two genes. Furthermore, while there are many *Krt8*⁺ *Cldn4*⁻ cells, the majority of *Cldn4*⁺ cells are also *Krt8*⁺, suggesting the developmental trajectory of *Cldn4*⁺ mTECs is consistent with the “cTEC first” model of TEC differentiation (Baik et al., 2013; Bornstein et al., 2018; Rossi et al., 2006; Takahama et al., 2017) (Fig. 4.4J). Of note, a small number of the iTEC12d expressed high levels of *Avil*, a tuft-like mimetic TEC marker (Fig. 4.4K-M).

Overall, our merged single-cell transcriptomic data validated our bulk-RNA-seq result showing different stages of iTEC programming and revealed that iTEC programming did not occur evenly across Cre-induced MEFs. There was a subset of MEFs that were predisposed to undergo transdifferentiation in response to *Foxn1* over-expression, whereas other MEFs were recalcitrant. Successfully programmed MEFs yielded iTECs with high levels of archetypal cTEC, mTEC, and tuft-like mimetic TEC markers. Only a small number of iTECs expressed high levels of canonical mTEC markers, which our results suggest could be further improved by sorting *Foxn1*-receptive MEFs prior to inducing *Foxn1* expression.

Notch signaling inhibits mTEC differentiation during iTEC programming.

The differential lineage-specific gene expression analysis showed that the mTEC progenitor marker *Cldn4* was one of the highest expressed mTEC markers at later iTEC stages, but the terminal differentiation marker *Aire* was not detected in our bulk-RNA-seq data (Fig. 4.3). In our single-cell RNA-seq data of 12-day-old iTECs, only 1 out of 8290 cells expressed *Aire*, (Fig. S4.2E). Given these data, we hypothesized mTEC differentiation is induced at the progenitor stage during programming, but further mTEC differentiation is inhibited or incomplete. Recently we and others showed that Notch signaling plays an essential role in mTEC proliferation and differentiation (Li et al., 2020; Liu et al., 2020). Most critically, too much Notch signaling after initial mTEC specification blocks mTEC terminal differentiation and mTECs arrest at *Cldn4*⁺ progenitor states (Goldfarb et al., 2016). These *in vivo* mechanisms are similar to what we observe

in iTEC generation and suggest the possibility that persistent Notch signaling may suppress mTEC differentiation during iTEC programming *in vitro*.

To test this hypothesis, we first analyzed the gene expression of the Notch signaling pathway. (Fig. 4.5A, B). We found that multiple Notch receptors, ligands, and downstream target genes were upregulated during early stages and maintained throughout late programming. To test whether this maintenance of Notch signaling blocked mTEC differentiation at the *Cldn4*⁺ stage, we designed a temporally controlled Notch inhibition experiment (Fig. 4.5C). We first allowed early programming to progress to establish initial mTEC identity. At day 5 after the initial transfection, we added the Notch signaling inhibitor DAPT daily for 7 days. DAPT-treated cultures reduced but did not eliminate Notch signaling as indicated by a 50% reduction in *Hes1* expression (Fig. 4.5D). *Cldn4* was also downregulated to a similar degree, indicating that iTECs had reduced mTEC progenitor characteristics under DAPT treatment. mTEC differentiation was analyzed by measuring *Aire* expression which is a critical marker for mTEC maturation and terminal differentiation. *Aire* expression was detected in 6 out of 11 experiments. In 5 out of those 6 experiments, *Aire* expression was significantly upregulated in the DAPT cultures compared to DMSO controls (Fig. 4.5D). To test whether this *Aire* expression was sufficient to induce AIRE-dependent promiscuous gene expression (PGE), we tested *Ins2*, *Chrna1*, and *Tpo* expression by qPCR. However, these assays were negative, suggesting that the levels of *Aire* are too low to efficiently induce PGE, or that additional conditions are required other than *Aire* expression alone that are not present in the iTEC cultures. As an independent assessment of mTEC differentiation,

we also measured *Fezf2*, an *Aire*-independent marker of mTEC differentiation. *Fezf2* was expressed, but not consistently different between DMSO and DAPT-treated cultures (Fig. 4.5D).

Immunocytochemistry (ICC) of iTECs12d treated with DMSO (Fig. 4.5E) or DAPT (Fig. 4.5F) shows AIRE only in Notch inhibited iTECs12d. AIRE expression in these cells resembled punctate *in vivo* AIRE expression (Fig. 4.5G). Inhibiting Notch signaling did not affect iTEC programming efficiency based on the percentage of the EpCam⁺ population, and it was also insufficient to induce MHCII protein expression (Fig. 4.5H). Taken together, these data suggest that the upregulation of *Aire* is a specific response to inhibiting Notch signaling during iTEC programming and that iTEC generation may follow the same mTEC differentiation mechanisms *in vivo*, which opens the possibility of establishing iTEC as a useful system for investigating mTEC differentiation and function.

Rescuing cell cycle arrest improved iTEC programming efficiency.

FOXN1 promotes TEC proliferation *in vivo* (Chen et al., 2009; Chojnowski et al., 2014; Nowell et al., 2011); however, we observed the disappearance of proliferative cells in 12-day-old iTECs *in vitro* (Fig. 4.4A, B). To investigate this question at the molecular level, we interrogated our RNA-seq data for changes in cell cycle-associated genes during the early and late stages of iTEC programming. Our expression data showed that cell cycle inhibitors including *Rb*, *Cdkn1a/p21*, and *Gadd45b* were upregulated beginning at the early iTEC2d stage, while cell cycle-promoting genes such as *Myc*, *Mcm7*, and *p53* were downregulated in the late stage (Fig. 4.6A, B).

Cell cycle arrest is common, and often required, in cell programming systems (Treutlein et al., 2016). This proliferation block prevents the expansion of iTECs in culture and limits their utility as an experimental system. To test if we could rescue cell cycle arrest without inhibiting the programming process, we utilized an inducible Rosa26 transgene for Myc that can be activated by Cre plasmid transfection. This transgene also expresses human CD2 antigen, which allows for the detection of MYC-positive cells by huCD2 staining (Calado et al., 2012). We generated double transgenic MEFs (iFoxn1/+;iMYC/+) in which Cre expression during initial iTEC induction should activate both Foxn1 and Myc gene expression. (Fig. 4.6C). Cells that have activated both alleles are GFP⁺ and CD2⁺. Using PI staining and Cell trace analysis, we found that Myc overexpression partially rescued cell cycle arrest in iTECs (Fig. 4.6D, E). iFoxn1+iMyc⁺ iTEC cultures had increased S phase frequency (Fig. 4.6D) and a dramatic shift to the left in Cell Trace analysis (Fig. 4.6E). This shift indicated dilution of the Cell Trace reagent consistent with cells undergoing proliferation. Activation of Myc alone was unable to induce iTEC generation or EpCam expression as detected by flow cytometry (Fig. 4.6F). Surprisingly, activation of the cell cycle did not block iTEC programming, but instead it further increased its efficiency as measured by EpCam expression. (Fig. 4.6G). These results suggest that cell cycle arrest is a side-effect of the programming process rather than a necessary step, which would enable the expansion of iTECs in culture and increase their utility.

EpCam+ iTECs are missing specific genetic signatures found in fetal TECs

We isolated and sequenced *in vivo*-derived total fetal TECs at E14.5 and compared this data to EpCam+ iTECs to determine their similarity at the transcriptome level. PCA analysis clearly demonstrated that all iTEC samples are still quite different from fetal TECs (Fig. 4.7A). The sample distance count matrix showed a similar result (Fig. 4.7B). GO term analysis indicated that fetal TEC overall had higher expression of cell adhesion molecules relevant to epithelial identity and TEC differentiation (including Claudins, CD40, and CD28) and a variety of immune function related gene categories (Fig. 4.7C). EpCam+ iTECs had higher expression of categories indicating retention of mesenchymal and/or fibroblast-like characteristics (Fig. 4.7D). MA Plot analysis of EpCam+ iTEC vs fetal TEC also showed that both mTEC and cTEC markers were higher in fetal TEC, but the degree of difference was less in 10d vs 2d iTEC (Fig. 4.7E, F).

To investigate the gene expression differences between iTEC and fetal TEC in more detail, we first used a count matrix heatmap (Fig. 4.7G). This analysis clearly showed that one of the two EpCam+ samples was clearly more similar to fetal TECs, indicating a more advanced stage of programming. There were four genes that were not significantly up-regulated in any iTEC samples compared to fetal TEC (blue/white in all iTEC samples, red/orange in fetal TEC). Three of these were MHCII genes (annotated as *H2-A* or *H2-E*), and the other was *Tbata*, which regulates TEC proliferation *in vivo* (Flomerfelt et al., 2010). The significant downregulation of *Col6a3* (white in fetal TEC, orange in iTEC) in fetal TEC compared to all iTEC samples is consistent with persistent retention of fibroblast characteristics in iTECs. We also performed heatmap analysis of 18 of the most highly DEGs (Fig. 4.7H). Ten of these genes associated with MHCII, including *Ciita*, which

encodes the major transcriptional regulator of MHCII expression (León Machado and Steimle, 2021). This analysis further highlighted the lack of MHCII-related genes as a defining feature in *in vitro* iTECs when compared to fetal TECs. In addition, 3 genes are associated with TEC function and/or proliferation (*Psem1*, *Prss16*, *Tbata*) and one with Notch signal modulation (*Ifng*). This heatmap analysis also demonstrated that the more differentiated EpCam⁺ sample shared some characteristics with fetal TEC, including upregulation of MHCII-related genes (*Psmc1*, *Rfxap*) and *Ifngr1* and downregulation of *Creb1*, *Rfx1*, *Nfya*, and *Ctsb*. Similar patterns were seen with cell cycle-related genes (Fig. 4.7I) and Notch pathway-related genes (Fig. 4.7J). In both cases, one of the two EpCam⁺ samples was more similar to fetal TEC while still retaining significant differences. These data are consistent with our results above and suggest that these pathways may be potential candidates to target for improving iTEC programming to generate cells more comparable to fetal TEC.

MHCII is upregulated by co-culture with fetal thymocytes

Expression of MHCII genes *H2-Aa*, *H2-Ab1*, and *H2-Eb1* was not detectable with our RNA-sequencing. However, we could detect a low, overall increase in their expression when we surveyed these genes with our single-cell RNA-sequencing dataset. About 2% of iTEC3d expressed low levels of *H2-Ab1*, which increased to 2.9% at the iTEC12d stage (Fig. 4.8A-C, Table 1). We calculated the average expression of *H2-Aa*, *H2-Ab1*, and *H2-Eb1* in each sample and made a graph with relative values per gene (Fig. 4.8D). The average expression of *H2-Ab1*

(2.4-fold), *H2-Aa* (2-fold), and *H2-Eb1* (2-fold) increased in iTEC12d compared to iTEC3d (Fig. 4.8D), suggesting an overall increase in MHC class II gene expression in iTEC12d.

Although we did not detect MHCII protein on the surface of iTECs after DAPT treatment (Fig. 4.5H), the increase in mTEC differentiation seen with DAPT could impact MHCII at the gene expression level. To assess the effects of Notch signaling on MHCII gene expression, we performed single-cell RNA-sequencing after treating iTEC12d with DAPT and measured expression changes in *H2-Aa*, *H2-Ab1*, and *H2-Eb1* (Fig. 4.8E-K). *H2-Ab1* expression, which exhibited the largest increase in iTEC12d compared to iTEC3d, did not change when Notch signaling was inhibited with DAPT (Fig. 4.8E-G, K). However, Notch-inhibited iTEC12d compared to iTEC12d control showed 2.3 and 2.6-fold increases in *H2-Aa* and *H2-Eb1* expression, respectively (Fig. 4.8H-K).

iTECs can support early T lineage progenitor (ETP) differentiation, which requires MHCII expression (Bredenkamp et al., 2014b). However, MHCII gene expression was not evaluated directly in those experiments. We designed a co-culture experiment to determine if TEC-thymocyte crosstalk can successfully induce expression of MHCII. At the beginning of late stage iTEC programming (5 days after transfection), we co-cultured 100,000 iTECs with 80,000 E16.5 double-positive thymocytes for 5 consecutive days before collecting them for single-cell RNA-seq. Co-culturing with fetal thymocytes increased MHCII gene expression: about 2-3 times more cells are expressing ~3 times higher MHCII genes compared to DAPT controls (Fig. 4.8L-N). Next, we compared co-cultured and DAPT-treated iTECs to MEF and iTEC10d samples by RT-qPCR (Fig. 4.8O-Q). CD80 expression was not different between any iTEC samples (Fig. 4.8O).

However, we found a significant increase in the relative *H2-Aa* expression in Notch-inhibited iTEC10d compared to all controls (Fig. 4.8P), supporting our previous observation with the single-cell RNA-seq data (Fig. 4.8H-K). Co-culturing with E16.5 DP thymocyte significantly increased the *H2-Aa* expression level even further compared to all samples (Fig. 4.8Q), showing that crosstalk with DP thymocytes is sufficient to induce MHCII gene expression. Combined, these data provide a stepping stone towards further understanding and dissecting the molecular mechanism behind MHCII gene expression, which will improve the iTEC *in vitro* platform.

Discussion

The previous iTEC study demonstrated that expression of *Foxn1* in MEFs induced a TEC-like differentiation state, with up-regulation of FOXN1 direct targets and induction of epithelial characteristics (Bredenkamp et al., 2014b). That study also showed that co-culture with fetal ETP or transplant under the kidney capsule could induce further differentiation and support T cell development. These results indicated that the iTEC system could represent a novel platform for studying FOXN1 function and TEC-thymocyte interactions, and could present a pathway to therapeutic thymus transplant. The current study builds on the initial report, and provides a significant advancement of our understanding of iTEC programming and open avenues for future improvement of this technique. We showed that iTEC programming is not linear, and defined characteristics of early and late stages. Although we cannot fully rule out the possibility other factors are necessary for the transdifferentiation, our data indicate that programming begins with immediate up-regulation of FOXN1 direct target genes that are not normally expressed in MEFs,

consistent with the role of Forkhead transcription factors as pioneer factors (Zaret and Mango, 2016). During later stages, we observed strong up-regulation of known FOXN1 targets, as well as gene expression and morphological changes not directly attributable to known FOXN1 *in vivo* functions. Our data are also generally consistent with iTEC programming following a path consistent with the “cTEC first” model, including arrest at the mTEC progenitor stage (Baik et al., 2013; Bornstein et al., 2018; Rossi et al., 2006; Takahama et al., 2017). We rescued this arrest by inhibiting the Notch signaling pathway, which suggests that mTEC differentiation in iTECs follows similar pathways as those of endogenous fetal mTECs. Additionally, we improved the efficiency of iTEC programming by rescuing cell cycle arrest. Lastly, we identified critical gene expression differences between iTECs and fetal TECs. Notably, expression of MHC-related genes was severely downregulated in iTECs, but we demonstrated this can be significantly improved by co-culturing iTECs with fetal DP thymocytes.

One persistent question is why iTEC differentiation is not uniform across iTEC cultures. In our current iTEC direct programming protocol, the EpCam⁺ population is always a subset of the cultures even though all iTECs are expressing the same *Foxn1* transgene for the same amount of time. One possibility is that the cell cycle plays a partial role in this process since rescuing the cell cycle block increased the percentage of Epcam⁺ iTECs (although it still was not 100%). Moreover, MEFs are a heterogeneous population (Shakiba et al., 2019), and our single-cell RNA-seq suggested that some MEFs are more susceptible to *Foxn1*-programming than others. Another variable is endogenous *Foxn1* expression. We consistently detect activation at the endogenous *Foxn1* locus at a low level during the late stage of programming (transgenic *Foxn1* levels were 2037-

fold higher than endogenous *Foxn1*; +/- 193 SEM, n=6), although it is unclear whether this occurs in all cells. While this additional low level of *Foxn1* expression is unlikely to have a significant impact on programming, it could indicate the degree to which programming is occurring at the epigenetic level. Additional analyses of heterogeneity during this process needs to be done to test these hypotheses.

We detected both cTEC and mTEC markers during iTEC programming, but mTECs were limited to the *Cldn4*⁺ progenitor stage. While we demonstrated that blocking Notch signaling increased mTEC differentiation, it is unclear how cTEC and mTEC differentiation occurs at the individual cell level. Following the “cTEC first” model, it is possible that all iTECs express cTEC markers before subsets of more differentiated cells start to express mTECs markers later in programming. Alternatively, it is possible that different subsets of cells initiate cTEC and mTEC differentiation directly. Our single-cell RNA-seq data suggests mTECs were rising from cTECs due to the co-expression of cTEC markers with *Cldn4*, but further studies are needed to test this model in the iTEC system.

Several previous studies show that cell cycle arrest is necessary for direct lineage reprogramming of some cell types such as neuron direct reprogramming (Treutlein et al., 2016). While cell cycle arrest occurs during iTEC direct programming, our data showed that this arrest is not necessary. In contrast, removing this cell cycle block by forced Myc expression resulted in higher efficiency of EpCam⁺ iTEC generation. It is still unclear what role the cell cycle has during iTEC programming and how proliferating iTECs compare to cell cycle-arrested iTECs. It is possible that Myc overexpression has a larger impact on iTEC programming than on cell cycle

regulation since Myc expression both alone and as a component of the retinoblastoma pathway is implicated in TEC differentiation *in vivo* (Cowan et al., 2019; Garfin et al., 2013). However, we found that Myc expression alone had no detected impact in the absence of *Foxn1* expression. Alternatively, this could be due to a technical artifact where Cre-induced gene damage caused reduced cell proliferation (Loonstra et al., 2001), which could be addressed with a control in which Cre is expressed without *Foxn1* induction. Further experiments are needed to analyze the impact of rescuing cell cycle arrest and to further understand the mechanisms underlying how the cell cycle affects iTEC programming.

iTEC cultures alone do not significantly express MHC Class II, which is a crucial functional hallmark of TEC differentiation. In the original iTEC report, co-culture or transplant of iTEC supported the development of single positive CD4⁺ T cells (Bredenkamp et al., 2014b). These data suggest that iTEC-thymocyte crosstalk induced MHCII gene expression, although the impact of crosstalk on iTEC phenotypes was not directly assessed. Our single-cell RNA-seq datasets suggest that *Foxn1* overexpression is sufficient to induce MHCII gene expression at low efficiency. Intriguingly, Notch-inhibited iTECs had increased expression of *H2-Aa* & *H2-Eb1*, suggesting a potential role of the Notch signaling pathway on MHCII expression. Co-culturing with fetal DP thymocytes was sufficient to increase relative expression of MHCII genes, *H2-Aa*, *H2-Ab1*, and *H2-Eb1*. Thus, future experiments to identify the crosstalk elements that increase MHCII expression will be essential to design a fully viable iTEC culture system that can be utilized as a novel experimental platform for understanding TEC biology and function and as an approach to generate human iTECs for *in vitro* or *in vivo* therapeutic uses.

Figures and Figure Legends

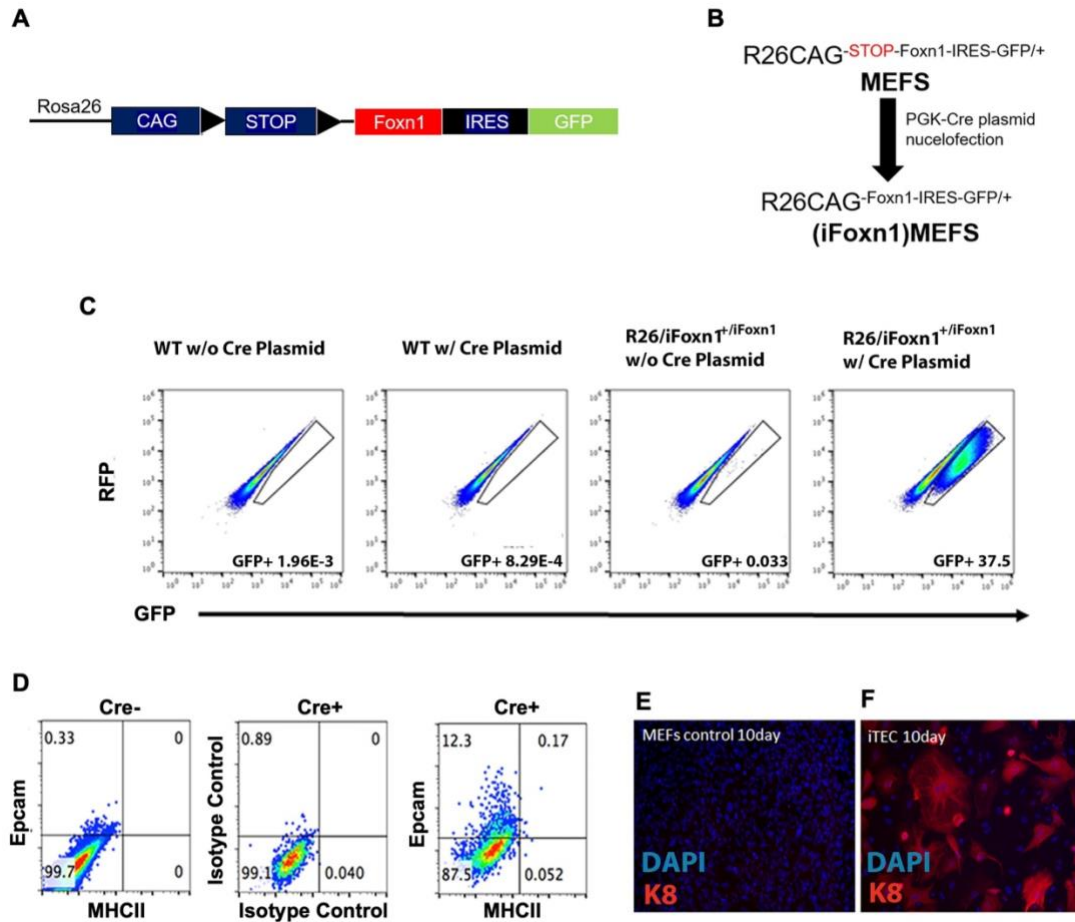


Figure 4.1: Generation and characteristics of R26^{CAG-Foxn1-IRES-GFP/+} MEFs.

A) Gene structure of R26CAG-STOP-FOXN1-IRES-GFP transgene. B) Workflow of generation (iFoxn1) MEFS USING Cre-plasmid based nucleofection. C) IRES-GFP reporter signal analysis by flow cytometry for R26-iFoxn1^{+/+} with Cre plasmid and three control groups. D) Epithelium marker EpCam/MHCII analysis of R26-iFoxn1^{+/+} and 2 control groups with Cre⁺ plasmid 13 days after transfection. E) Epithelial marker Keratin 8 staining and DAPI staining of control MEFs. F) Keratin 8 staining and DAPI staining of iTEC 10 day cells.

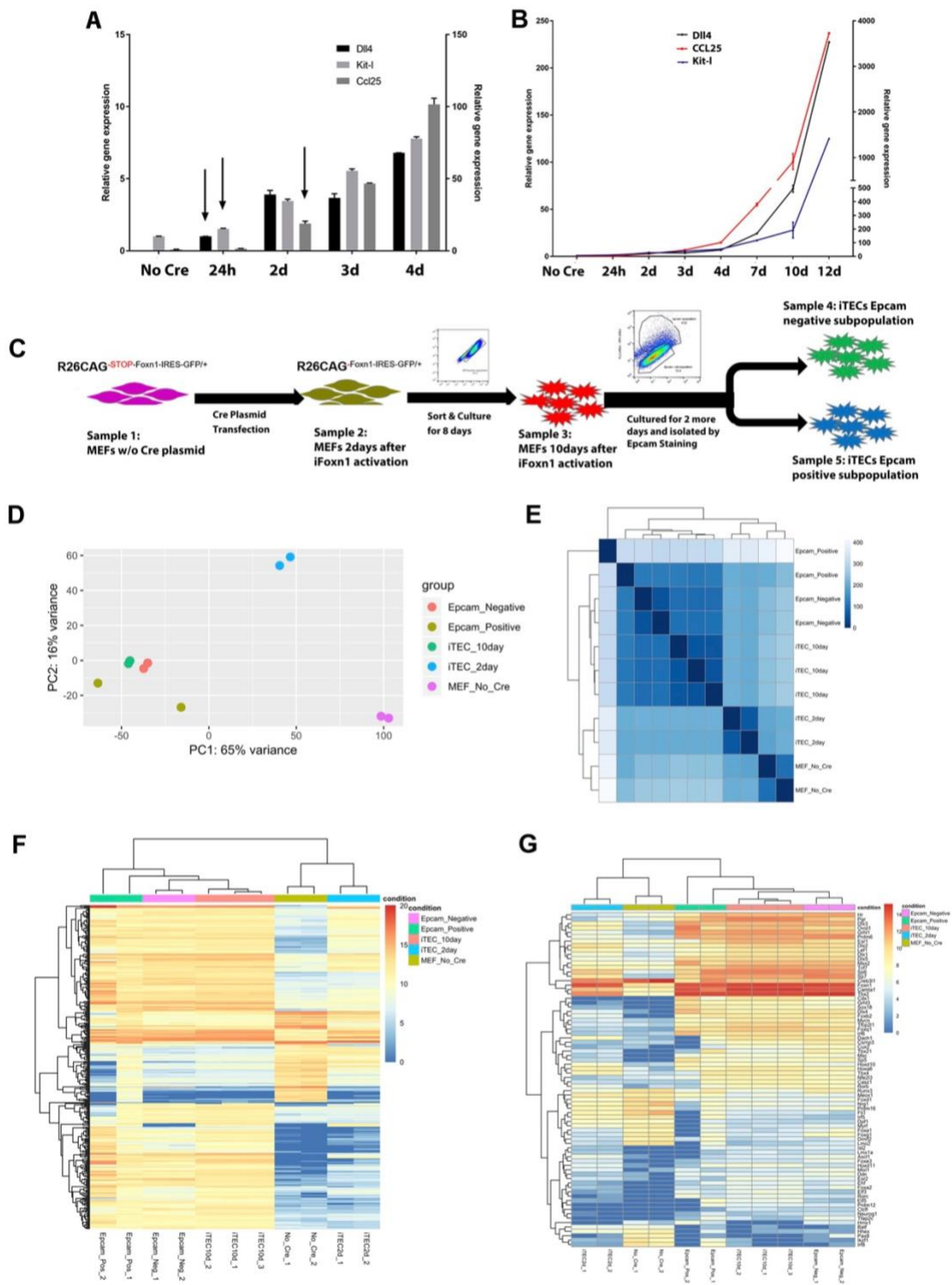


Figure 4.2: Schematic diagram of RNA-seq experiment design and general transcriptome analysis

A) Gene expression analysis of selected *Foxn1* target genes using qRT-PCR at different time-points after Cre transfection from 24 hours (h) to 4 days (d). B) Gene expression analysis using qRT-PCR at different time-point after Cre transfection from 24 hours to 12days. C) Schematic diagram and sample FACS sorting plot from generating iTEC samples for RNA-seq analysis. D) PCA analysis of all sample transcriptomes. E) Sample distance analysis of all sample transcriptomes. F) Top differentially expressed genes heatmap analysis of all samples. G) Top differentially expressed transcription factor heatmap analysis.

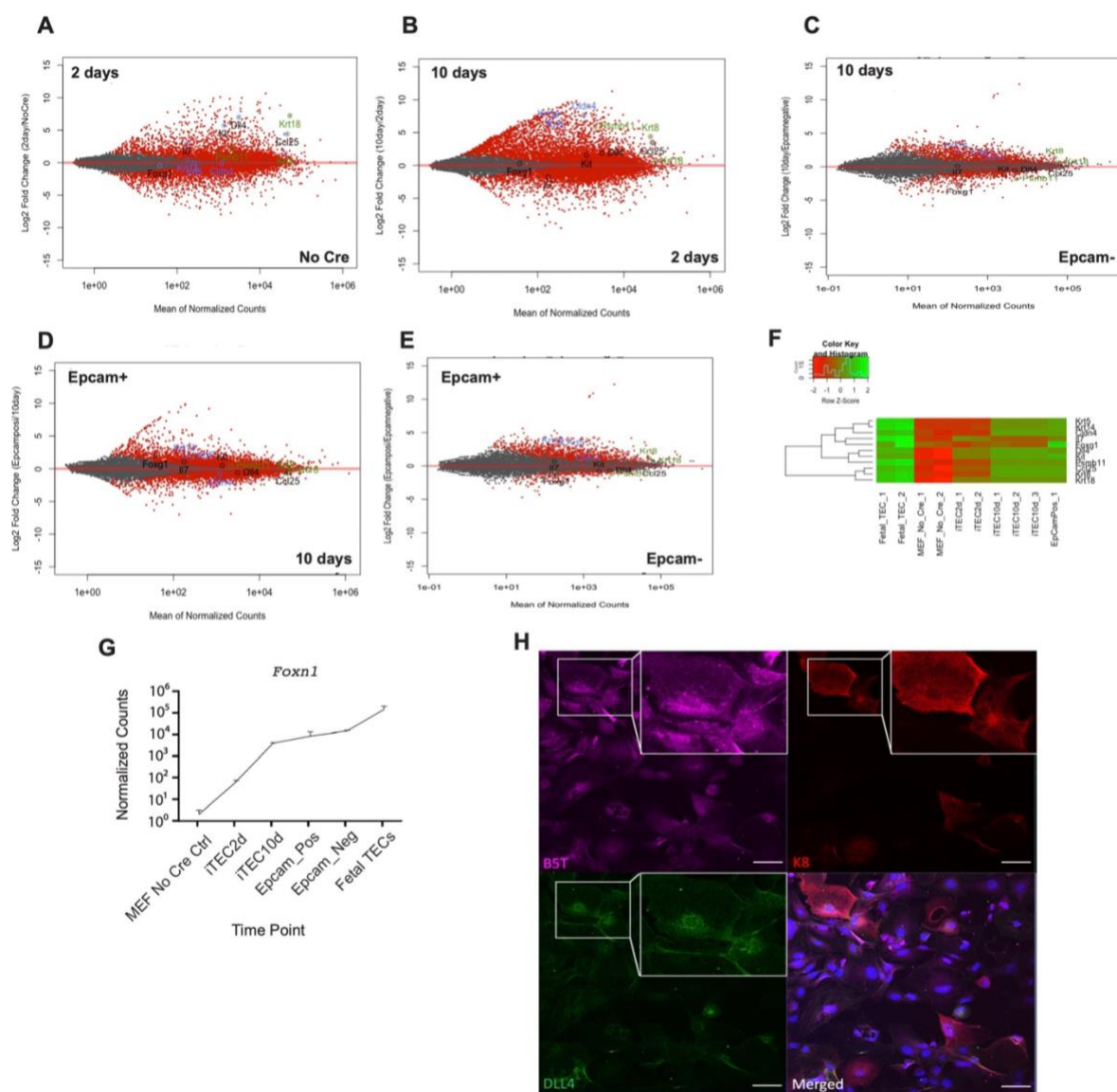


Figure 4.3: Detailed gene expression comparison analysis of different stage of iTECs.

A) - E) MA plot analyses comparing successively more advanced pairs of iTEC reprogramming stages with selected mTECs/cTEC markers highlighted. F) Heatmap analysis of different stages of iTECs reprogramming with important mTECs/cTEC markers. Fetal TEC is also presented in this figure for comparison. G) Normalized counts of *Foxn1*. H) Immunocytochemistry (ICC) of 12diTECs with cTEC markers. B5T (top-left), K8 (top-right), DLL4 (bottom-left) are shown. Inlaid images are equally corrected for visualization purposes. N=2 with technical replicates. Scale bar: 50µm.

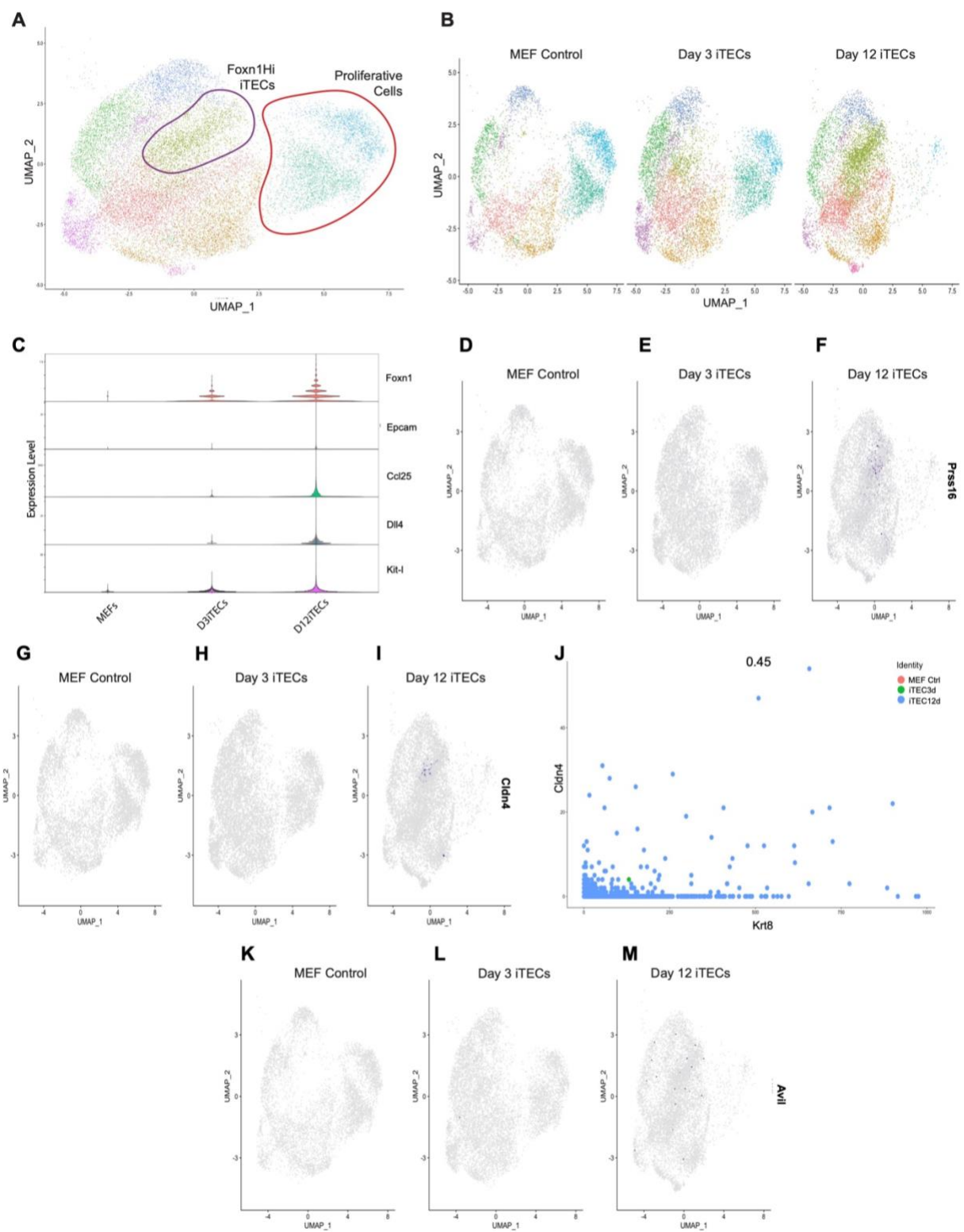


Figure 4.4: scRNAseq of MEF control, 3diTECs, and 12diTECs.

A) Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP) visualization of a merged object of MEF control, iTECs3d, and iTECs12d. B) UMAP visualization split by iTEC reprogramming process. C) Violin plot of *Foxn1* and its direct downstream target *Ccl25*, *Dll4*, *Kit-l*. Epithelial marker *Epcam* is also included. D-F) Feature plot visualization of *Prss16*, a thymus specific serine protease. G-I) Feature plot visualization of *Cldn4*, an mTEC marker. J) Scatter plot visualizing correlation between a cTEC gene *Krt8* and an mTEC gene *Cldn4*. Pearson correlation coefficient is 0.453, calculated after removing cells exhibiting 0 for *Krt8* and *Cldn4*. K-M) Feature plot visualization of *Avil*, a tuft-like mimetic TEC marker.

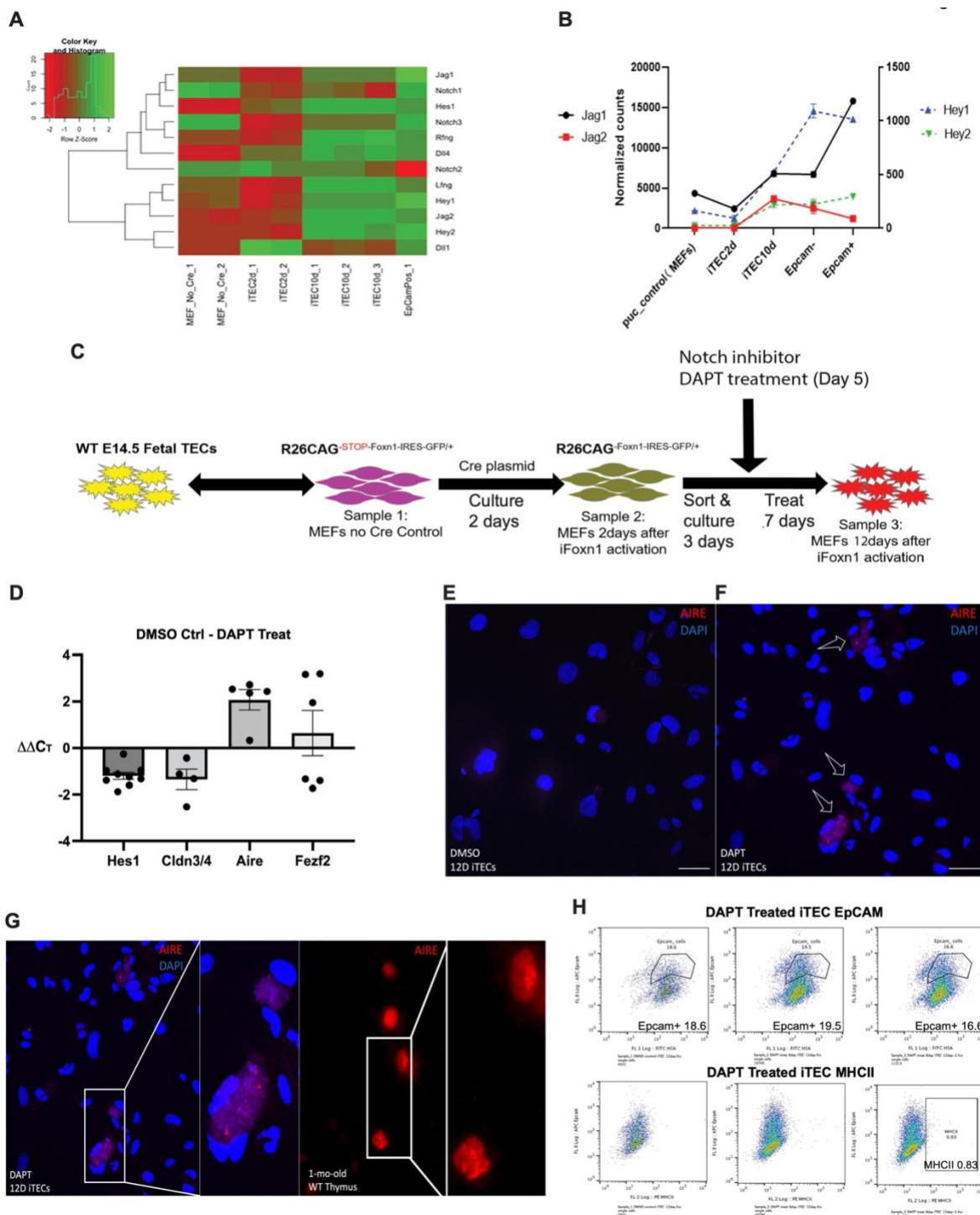


Figure 4.5: Bulk RNAseq analysis of Notch pathway analysis and DAPT treatment experiment.

A) and B) Gene expression heatmap & line plot of components of the Notch signaling pathway. C) Experimental design of DAPT treatment experiments for activating Notch signaling. D) RT-qPCR analysis of DAPT treatment effect on Notch pathway target gene *Hes1* and mTEC differentiation related genes *Cldn4*, *Aire*, and *Fezf2*. E, F) ICC of iTECs12d without and with Notch inhibition with α -AIRE. Arrows indicate AIRE+ iTECs. 2 biological replicates with 3 technical replicates per experiment. Scale bar: 50 μ m. G) ICC of iTECs12d with Notch inhibition and 1-month-old WT thymus with α -AIRE. AIRE protein expression pattern resembles *de novo* punctate AIRE in the nuclei. Inlaid images are for visualization purposes. H) EpCam/MHCII flow cytometry analysis of DAPT treated iTECs.

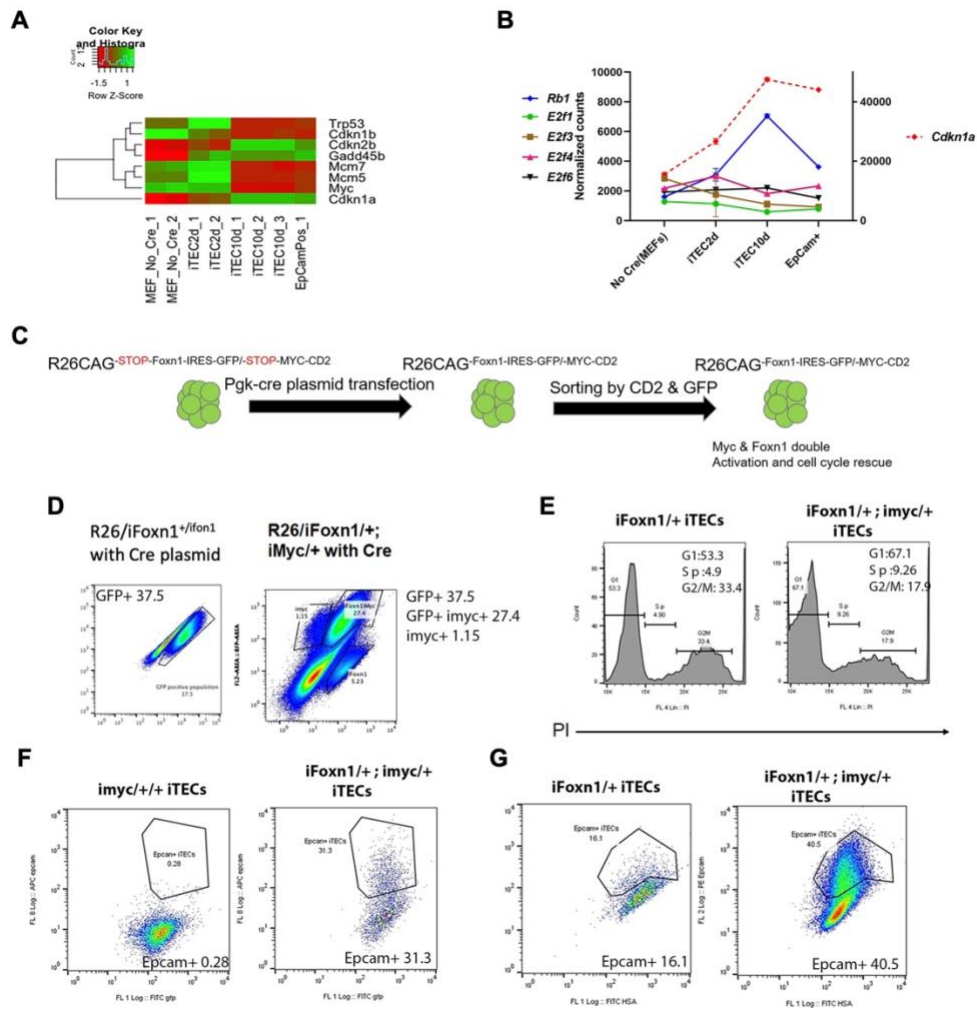


Figure 4.6: Cell cycle analysis of iTECs and cell cycle rescue using iMYC transgene.

A) and B) Gene expression analysis of cell-cycle related genes during iTEC reprogramming. C) Experimental design of inducing iTEC proliferation by overexpressing iMyc. D) Flow cytometric analysis showing that activation of the iMyc transgene leads to about 30% of iTECs expressing both iFoxn1 and iMyc. E) PI staining showing significant increase in S phase frequency indicating a rescue of the cell cycle by overexpressing iMyc during iTEC induction. F) EpCam staining showing that iMyc alone was unable to induce reprogramming or EpCam expression. G) Comparison of iFoxn1 alone and iFoxn1 with iMyc showing that Myc expression increases reprogramming efficiency.

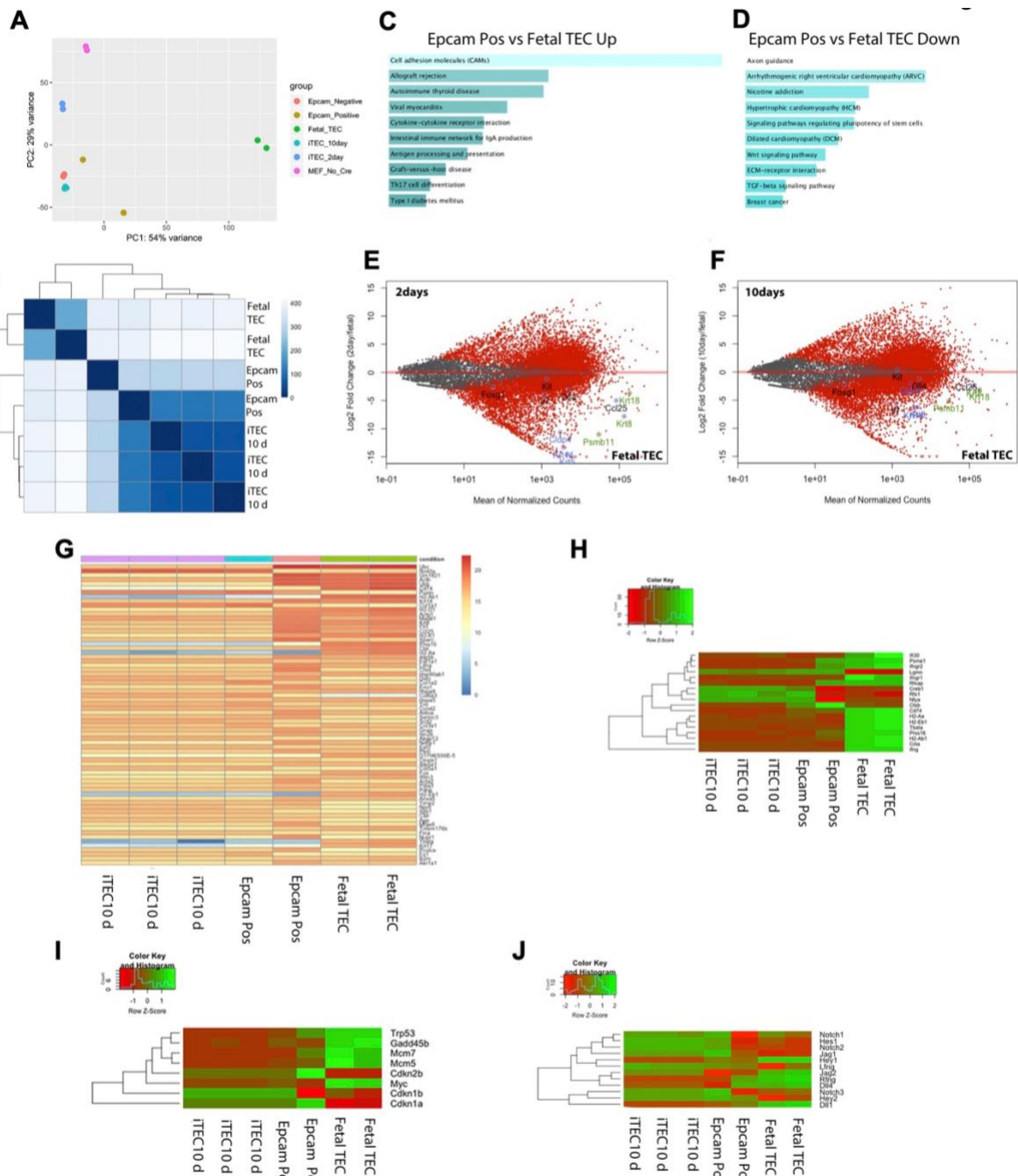


Figure 4.7: Transcriptome and differential gene expression analysis between iTEC and E14.5 Fetal TEC shows EpCam+ iTECs are missing specific genetic signatures.

A) PCA analysis of different time points of iTEC samples and fetal TECs. B) Sample distance analysis of iTEC 10day, Epcam+ iTEC, and fetal TECs. C, D) Enrichment analysis for Epcam+ iTEC vs Fetal TEC up & down regulated genes. E, F) Gene enrichment analysis comparing 2days and 10days iTEC stages to fetal TECs for select mTEC and cTEC markers. G) Top differentially expressed genes between different iTEC reprogramming stages and fetal TECs. H - J) Heatmap analysis of top differentially expressed genes between iTEC reprogramming and fetal TECs by category: H) MHCII related genes, I) cell cycle related genes, and J) Notch pathway related genes.

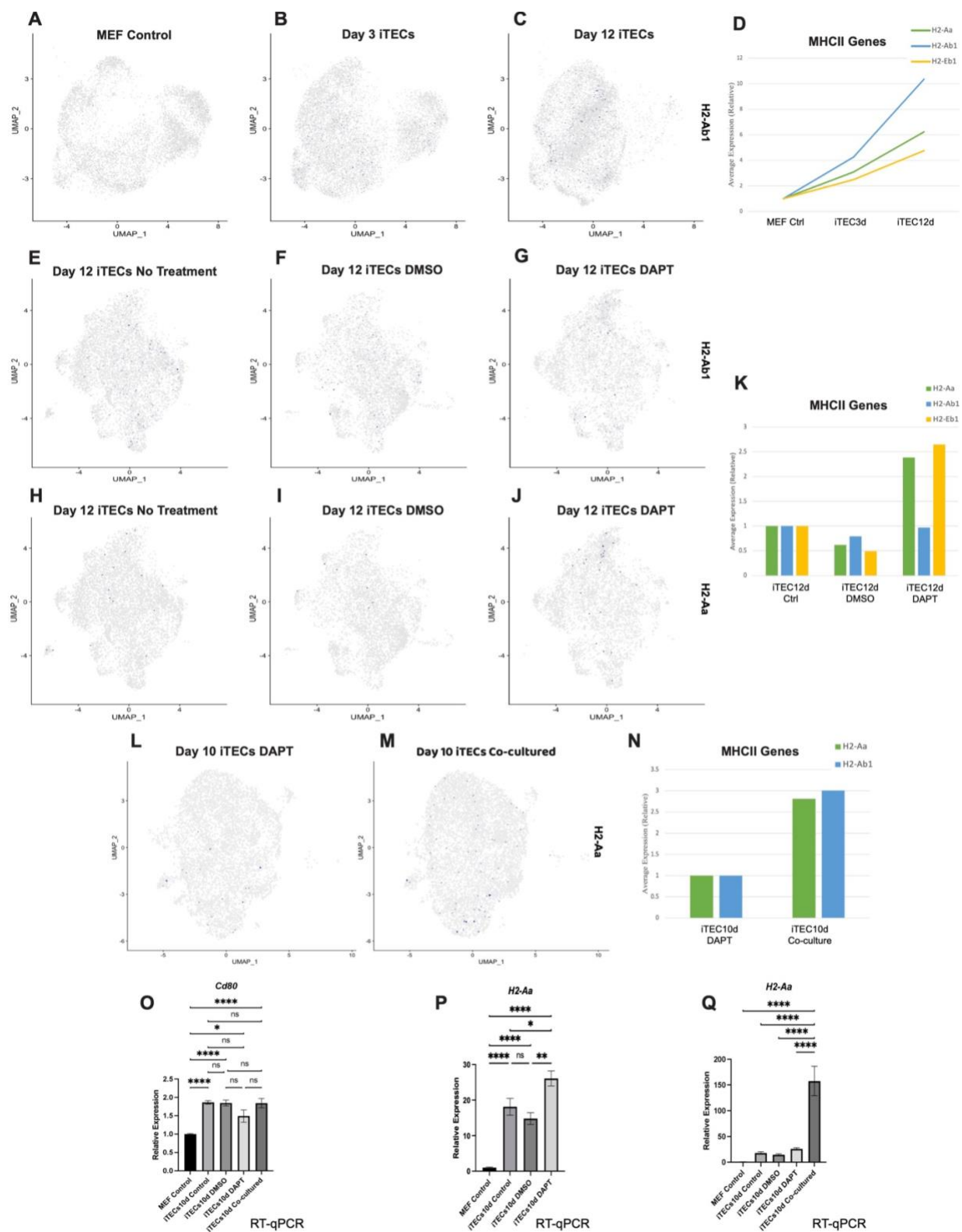


Figure 4.8: Co-culturing with E16.5 DP T-cells increase relative *H2-Aa* (MHCII gene) expression.

A-C) Feature plot visualization of an MHCII gene, *H2-Ab1* across the iTEC reprogramming process. D) Line graph showing the relative average expression of MHCII genes across the iTEC reprogramming process. E-G) Feature plot visualization of *H2-Ab1* in iTECs12d without treatment, with vehicle treatment (DMSO) and Notch inhibition (DAPT). H-J) Feature plot visualization of *H2-Aa* in iTECs12d without treatment, with vehicle treatment (DMSO) and Notch inhibition (DAPT). K) Bar graph showing the relative average expression of MHCII genes across the different treatments with iTEC12d. L, M) Feature plot visualization of *H2-Aa* in iTECs10d treated with DAPT or co-cultured with E16.5 DP T-cells. N) Bar graph showing the relative average expression of MHCII genes between the iTEC10d with DAPT treatment and iTEC10d co-cultured with E16.5 DP thymocytes. *H2-Eb1* was excluded from the analysis due to an outlier showing 790-fold increase in the co-culture group. O-Q) RT-qPCR of an mTEC marker *Cd80* and a MHCII gene *H2-Aa* across different conditions. MEF control indicates sham-transfected MEFs. RT-qPCR was done with three biological replicates with three technical replicates each. One-way ANOVA with pairwise comparisons were used with Prism 8 software.

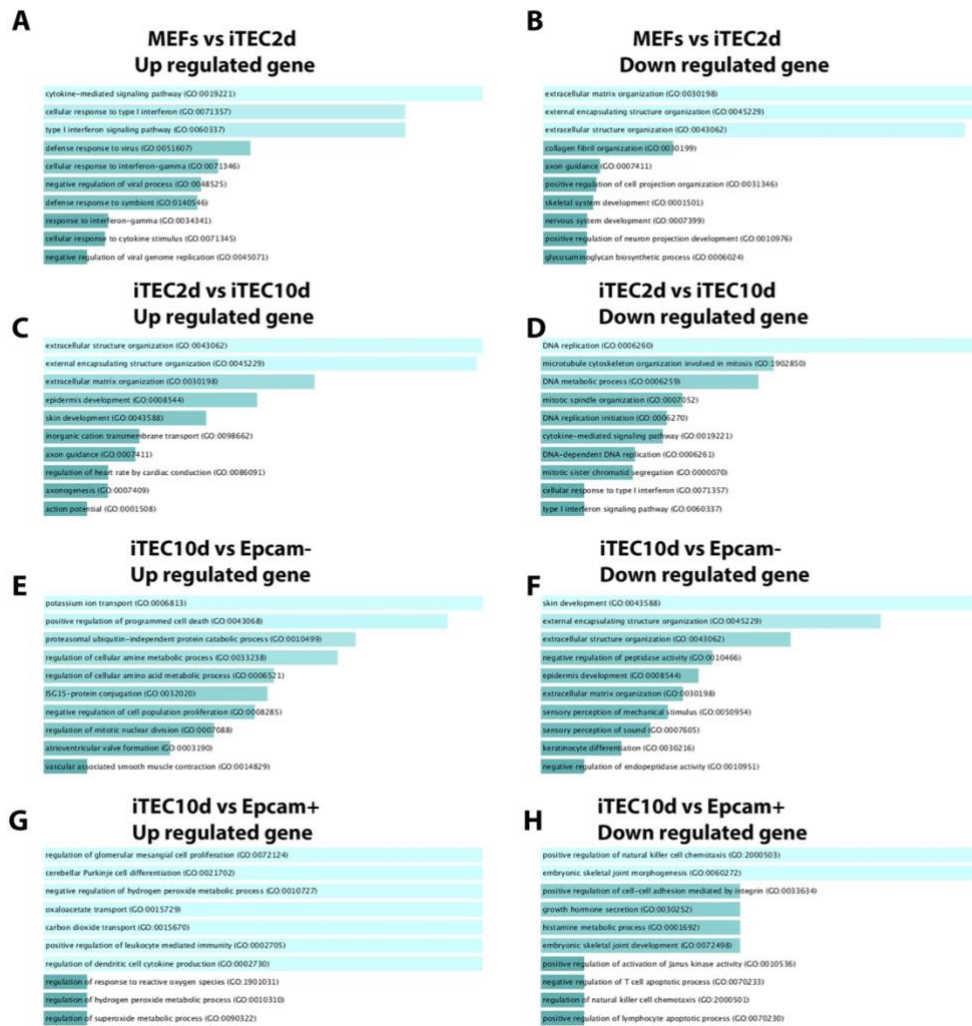
**** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$

Table 1

	<i>H2-Aa</i>	<i>H2-Ab1</i>	<i>H2-Eb1</i>
MEF Control	0.05%	0.61%	0.99%
iTEC3d	0.09%	2.03%	2.21%
iTEC12d	0.33%	2.88%	3.23%
iTEC12d Ctrl	0.28%	2.50%	2.69%
iTEC12d DMSO	0.17%	2.60%	1.62%
iTEC12d DAPT	0.60%	2.20%	4.05%
iTEC10d DAPT	0.31%	1.97%	1.42%
iTEC10d Co-culture	0.99%	3.69%	2.14%

Table 4.1: Proportion of the cells expressing MHCII genes per sample.

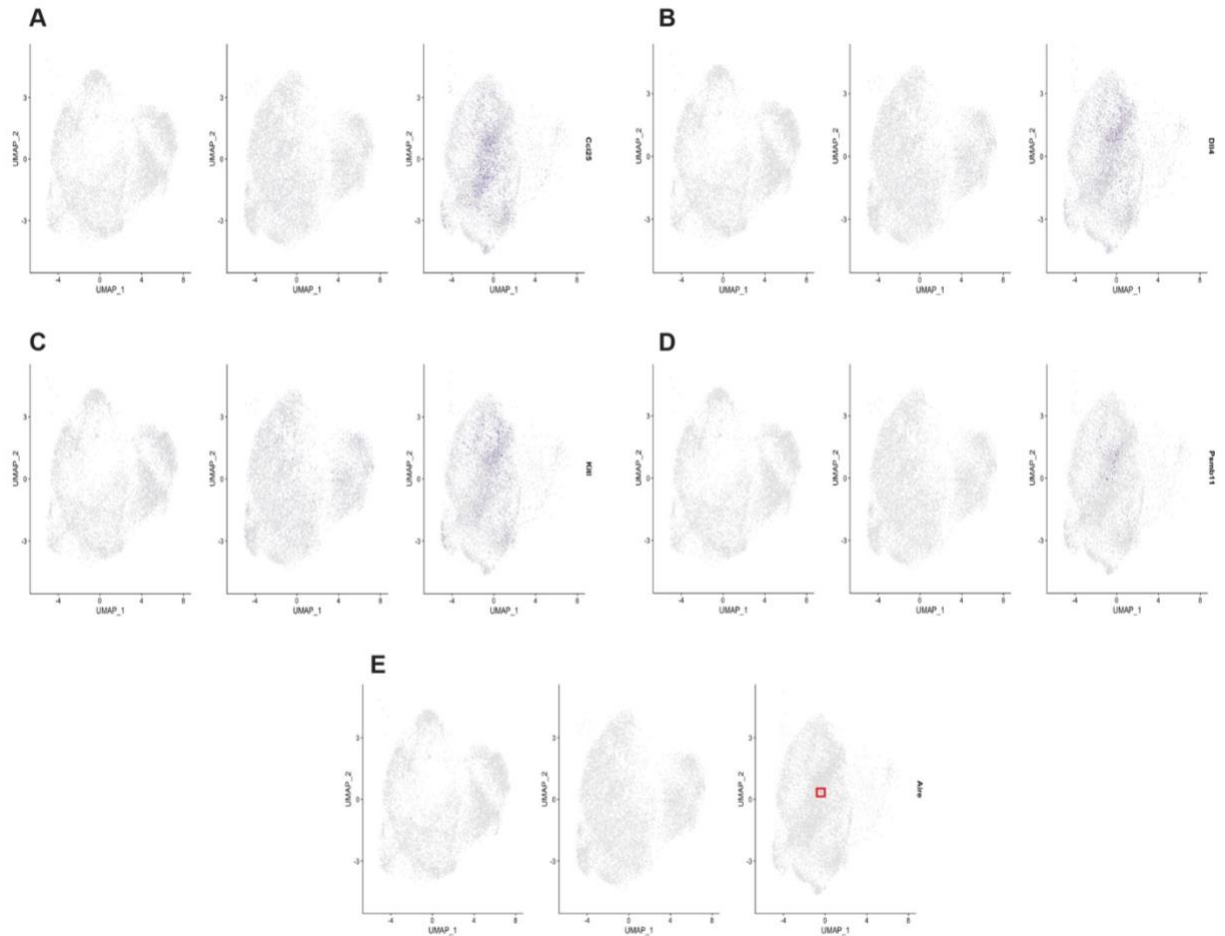
Supplementary Figure 1



Supplementary Figure 4.1: Gene enrichment analysis of up/down regulated genes at different reprogramming stages

A), B) Gene enrichment analysis for MEFs vs iTEC2d up (A) and down (B) regulated genes; iTEC2d vs iTEC10d up (C) and down (D) regulated genes; iTEC10d vs Epcam- up (E) and down (F) regulated genes; iTEC10d vs Epcam+ up (G) and down (H) regulated genes.

Supplementary Figure 2



Supplementary Figure 4.2: scRNAseq reveals high TEC genes in the *Foxn1*^{Hi} cell cluster.

A-D) Feature plot visualization of cTEC genes, *Ccl25* (A), *Dll4* (B), *Kit-l* (C), and *Psmb11* (D).

E) Feature plot visualization of mTEC gene *Aire*. Red square emphasizes one *Aire*⁺ cell in iTECs12d.

Materials and Methods

Mice

Rosa26^{CAG-STOP-Foxn1-IRES-GFP/+} mice (Bredenkamp et al., 2014b) were backcrossed onto the C57BL/6J genetic background for at least 5 generations then maintained by intercrossing. Each batch of mouse embryonic fibroblasts (iFoxn1+ MEFs) were pooled from embryos in a single litter of E13.5 embryos generated from a Rosa26^{CAG-STOP-Foxn1-IRES-GFP/+} female crossed to a C57Bl/6J male. For timed matings, noon of the day of the vaginal plug was taken as day 0.5. All experiments with animals were approved by the UGA Institutional Animal Care and Use Committee.

MEF isolation

MEFs were prepared from decapitated and eviscerated E13.5 embryos. The embryos were then finely minced and trypsinized into a single-cell suspension. Cells were plated in DMEM containing 10% fetal calf serum, 2 mM sodium pyruvate, 4 mM glutamine, 50 µg/ml streptomycin and 50 U/ml penicillin (DMEM/FCS). Each embryo was genotyped using the following primers to detect the iFoxn1 allele: iFoxn1 Forward Primer 5' – TGG AGT AGG CGG GGA GAA GG -3' iFoxn1 forward Primer Mid: 5'—TCG CCC TTC CCA ACA GTT GC –3' iFoxn1 Reverse Primer: 5' – GCC CAC ACA CCA GGT TAG CC—3'. MEFs were freshly isolated and initially cultured in 60mm plates until reaching 80% confluency and transferred to T-75 flasks until reaching 80~90% confluency. Then, MEFs were trypsinized and transferred into cryovials containing freezing medium (MEF medium with 10% DMSO). The cryovials in the isopropanol freezing container (to achieve -1°C/minute) were placed in -20°C for 1 hour, then -80°C overnight. Cryovials were then

transferred into liquid nitrogen tanks until usage. Once MEFs were thawed, they were immediately transferred to pre-warmed (37°C) MEF medium. MEF medium was replaced with fresh MEF medium the following day to remove residual DMSO. MEFs were expanded once or twice more for downstream experiments.

Cell Culture & Transfection

Primary iFoxn1/+ MEFs were thawed then cultured in DMEM containing 15% fetal calf serum, 2 mM sodium pyruvate, 4 mM glutamine, 50 µg/ml streptomycin and 50 U/ml penicillin (DMEM/FCS). Cells were passaged once at 70% ~ 80% confluency, then collected at 70% confluency for Nucleofection. Primary iFoxn1/+ MEFs for Nucleofection experiment were passaged no more than 5 times for all analyses in this research. Nucleofection experiments were performed following the manufactures' protocol for mouse embryonic fibroblasts: free program N-024 (Amaxa™ Nucleofector™, Lonza). To induce iFoxn1 expression, pPGK-Cre-bpA (Addgene # 11543) was used in the transfection according to the manufacturer's protocol. After Nucleofection, cells were further cultured for 48 hours until sorted by flow cytometry.

Flow cytometry & Sorting

Adherent MEFs or iTEC2d were collected by trypsinization using 0.05% trypsin for 2 minutes at 37°C. iTEC10d & iTEC12d were collected using 0.25% trypsin for 15 minutes at 37°C. For fetal TEC collection, E15.5 fetal stage thymi were dissected and digested in 1 mg/ml collagenase/dispase (Roche, Basel, Switzerland) and passed through a 100µm mesh to remove

debris. Thymi were processed individually before genotyping, then pooled. PE-Cy7 conjugated anti-CD45 (BioLegend, 30-F11, 1:150) and APC-conjugated anti-EpCam (BioLegend, G8.8, 1:150) were used to isolate TEC populations. Anti-MHCII (BioLegend, 1:150) was also used for TEC analysis. Cell sorting was performed using a BioRad-S3 cell sorter.

Notch Signal Inhibition Experiment

Exactly 60 hours (5 days) after transfection, 120,000 iTECs were treated with No Treatment, DMSO control, or 10 μ M of γ -secretase inhibitor, DAPT (GSI-IX) (Apex Bio, Catalogue A8200). The treatments were continued once every 24 hours for 7 consecutive days. Two or three technical replicates and at least 5 biological replicates were done for analyzing the effect of Notch signal inhibition on iTECs. Cells were then collected as below for RNA extraction.

Co-culture Experiment

Exactly 60 hours (5 days) after transfection, iTECs were treated with No treatment control, DMSO control, 10 μ M of γ -secretase inhibitor, DAPT (GSI-IX) (Apex Bio, Catalogue A8200), or co-cultured with E16.5 double-positive thymocytes. MEF medium, DMSO vehicle medium, and DAPT medium were replenished every 24 hours for 5 consecutive days before downstream analyses. Due to DP thymocytes' non-adhering nature, small quantities of MEF medium was added every day without removing existing MEF medium, restricting the duration of the experiment.

RNA extraction, Reverse transcription, and quantitative PCR (RT-qPCR)

RNA was prepared using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. All samples were DNase treated during the preparation. cDNA was synthesized from concentration-matched RNA using the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad) according to the manufacturer's instructions. Thermal cycling conditions for the cDNA synthesis were as follows: 25°C for 5 minutes; 46°C for 20 minutes; 95°C for 1 minute. Quantitative PCR was performed with Applied Biosystems™ 7500 Real-Time PCR System (ThermoFisher Scientific). Thermal cycling conditions were as follows: 50°C for 2 minutes; 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds, 60°C for 1 minute. Relative expressions were determined after normalization to the geometric mean of two reference genes (*Gapdh* and *Tbp*) and after normalization to *Gapdh* whenever *Tbp* was undetected. All samples were run with two or three technical triplicates and with no RT controls. The primers used for RT-qPCR were purchased from ThermoFisher Scientific, TaqMan Gene Expression Assays. All probes were designed with FAM-MGB Reporter-Quencher.

Immunocytochemistry (ICC)

Post-sorting, iTECs were placed on the gelatin coated glass coverslips (Electron Microscopy Science, 72293-01) placed in wells of 24-well-plate. On the day of ICC, iTECs were washed in sterile Ca^{2+} , Mg^{2+} free 1X PBS. iTECs were then fixed in 4% PFA (EM grade) (Electron Microscopy Sciences, 157-4) for 15 minutes at room-temperature (RT). Post-incubation, cells were washed in sterile Ca^{2+} , Mg^{2+} free 1X PBS 5 minutes, 3 times at RT (washing condition). To

permeabilize, iTECs were incubated in 1X PBS (w/ 0.1% Triton X-100) for 15 minutes at RT. Post-washing step, iTECs were then incubated in blocking solution (10% donkey serum) for 45 minutes at RT. iTECs were then incubated in primary antibodies at 4°C overnight. Post-washing step, iTECs were incubated in secondary antibodies conjugated with fluorophores for 1 hour at RT. Post-washing step, cells were incubated in DAPI for 5 minutes before being placed on the slides with fluorogel with TRIS buffer (Electron Microscopy Sciences, 17985-10). Primary antibodies: AIRE Monoclonal Antibody (5H12), eBioscience™ (Invitrogen, Cat. # 14-5934-82), Anti-mDLL4 (R&D Systems, Cat. # AF1389), MHC class II (ER-TR3) (Santa Cruz Biotechnology, sc-59318). Secondary antibodies: Donkey anti-Goat IgG (H+L), Alexa Fluor™ 488 (Invitrogen, Cat. # A-11055), Donkey anti-Rat IgG (H+L), Alexa Fluor™ 594 (Invitrogen, Cat. # A-21209), Donkey anti-Rabbit IgG (H+L), Alexa Fluor™ 647 (Invitrogen, Cat. # A-31573).

Bulk RNA sequencing data analyses

Bulk RNA sequencing was performed in Georgia genomics and bioinformatics core (GGBC) using an Illumina NextSeq 500 sequencing platform to generate single end sequencing data. Raw reads were pre-processed with sequencing grooming tools FASTQC, Trimmomatic and then further assembled by Hisat2, SAMtools, and StringTie. Differential gene expression analysis between samples and graphical display (including count matrix plot, PCA, heatmap and other plots) were performed using DEseq2 and R package ggplot2. Gene ontology enrichment analysis was performed using online EnrichR program (Chen et al.; Kuleshov et al.).

10X library preparation and Illumina sequencing

Viabilities of the iTECs (3 wells/24-well-plate) were confirmed with a hemocytometer with cells stained with Trypan Blue. Libraries were generated with cells with >97% viability. 10,000 iTECs with Single Cell 3' Reagent Kit (v3) were loaded into a 10X Chromium instrument. Quantity and quality of cDNA were examined before the final DNA library was sequenced on Illumina NextSeq2000 P3 with 200 cycles.

Single-cell RNA sequencing data analysis

As per the 10X guideline, we used CellRanger (v7) to generate and pre-process FASTQ files. CellRanger generated filtered_feature_bc_matrix files underwent QC steps with Seurat (v.4.3.0) (Hao* & Hao* et al., 2021) in R (v.4.2.1). Individual datasets were merged and batch corrected with top 3,000 features. The merged datasets were then analyzed with standard Seurat workflow.

CHAPTER 5

CONCLUSIONS

Our studies further emphasised the importance of FOXN1 as a lynchpin of the thymus biology throughout the organ's lifespan. In Chapter 2, I hypothesised TEC-derived signal orchestrating the thymic growth transition from neonatal expansion to juvenile homeostasis, based on unique perinatal T-cell phenotypes. However, my time-series single-cell RNA sequencing of murine thymic stroma & TECs alongside time-series bulk RNA sequencing of human thymic stroma suggested a TEC-extrinsic signal driving the transition. My datasets strongly suggested a thymic capsular fibroblast-derived IGF2 promoting the proliferation of cTECs. And downregulation of *Igf2* transcription of IGF2 activity causes the thymus to enter homeostasis. Due to the scope of Chapter 2, it could be difficult to see how a relative *Foxn1* level affects the total thymic microenvironment. A decrease in *Foxn1* level had an immediate effect on TECs with an appearance of stress-responsive cTECs in 7-day-old *Foxn1*^{lacZ/lacZ} (Fig. 2.2D, E) as well as ageing-associated TECs in 30-day-old *Foxn1*^{lacZ/lacZ} (Fig. 2.2D, E), which we explored more in Chapter 3. It is particularly interesting that cTECs, which tend to have the highest *Foxn1* level (Vaidya et al., 2016), were the most susceptible to a decrease in *Foxn1* level. Moreover, a decrease in *Foxn1* caused a stress-responsive thymic endothelium and mesenchyme (Fig. 2.2D, E), further suggesting the importance of *Foxn1* in maintaining the total thymic microenvironment. It is also important to remember that we cannot fully rule out a TEC-derived signal that could not be seen in our transcriptomic datasets, which we will explore in Chapter 5.1.

In Chapter 3, we showed a decrease in *Foxn1* was sufficient to cause premature ageing of TECs (Fig. 3.1C, D), supporting a decrease in *Foxn1* causing premature onset and accelerated rate of thymic involution (Chen et al., 2009). With the *Foxn1* hypomorphic model, *Foxn1^{lacZ}*, we dissected an independent effect of thymic involution on immune niches, apart from other variables, such as the ageing of haematopoietic stem cells. Naturally, a decrease in *Foxn1* impacted thymopoiesis with reduced positive selection, increased negative selection, and altered thymocyte maturation, proliferation, and apoptosis. Interestingly, this resulted in aged-like peripheral T-cell phenotypes with reduced T-cell responses to stimulation and flu infection challenge. Chapter 3 showed how a decline in *Foxn1* causes immunosenescence and inflammageing. With ever-declining thymic function, we need either a means to rejuvenate the thymus or an organoid/*in vitro* platform to support thymopoiesis. In Chapter 4, we dissected an *in vitro* platform, iTEC, to study TEC biology whilst providing a means to grow autologous T-cells *ex vivo* for transplantation. With bulk- and single-cell RNA sequencing, we dissected the programming process of iTECs, generated by overexpressing *Foxn1* in murine embryonic fibroblasts (MEFs). *Foxn1* expression is sufficient to programme MEFs into TEC-like cells capable of supporting thymopoiesis but needs further investigations, which will be addressed in Chapter 5.2. Overall, we explored the dynamic fluctuation of *Foxn1* throughout the lifespan of the thymus, closely resembling the roller-coaster.

5.1. Up- and down-stream of IGF2-CyclinD1-E2F-FOXN1 axis

In Chapter 2, I determined that IGF2-IGF1R interaction is a likely candidate driving the neonatal thymic expansion and its downregulation may cause the transition to thymic homeostasis.

In order to ascertain the role of IGF2 on the perinatal thymic transition, we are currently conducting additional experiments with two genetic models. First, we will cross *Foxn1*-Cre to *Igf1r*-cKO (loxP sites on either side of exon 3 of *Igf1r*) to delete *Igf1r* in a TEC-specific manner. If my hypothesis is correct, we will be able to observe an early cTEC phenotype. I expect to see a significantly smaller thymic cortex in neonatal mice. Next, I designed a constitutively active *Igf1r* model under Rosa26-CAG promotor (Fig. 5.1) based on previous literature (Carboni et al., 2005; Jones et al., 2006). If my hypothesis is correct, we will be able to observe perinatal cTEC phenotype. I expect to see a significantly large thymic cortex, but thymic medulla in 3-week-old mice. With loss- and gain-of-function genetic models, we are planning to determine their ability to expand exponentially and establish homeostasis, as well as how it impacts thymopoiesis.

In Chapter 2, there are a few questions that remain unanswered. Firstly, I did not fully explore if the fibroblast-derived IGF2 is accountable for the said unique perinatal T-cell phenotypes (T_{vm} , T_{reg}). More detailed experimental designs are required to determine if a decrease in IGF signal is orchestrating the unique thymopoiesis or through the proliferation/maturation of TECs. Another unanswered question is the regulation of *Igf2* transcription in the thymic stroma. A potential candidate is the Sonic Hedgehog (*Shh*) pathway. SHH pathway is shown to promote *Igf2* mRNA transcription *in vitro* by activating pluripotent mesenchymal cells with *Shh* (Ingram et al., 2002) and *in vivo* by showing *Igf2* overexpression in Patched (*Ptch*) deficient model (Hahn et al., 2000). Fascinatingly, several cTECs in adult murine thymus are shown to express *Shh* with an increased frequency towards the outer cortex (Outram et al., 2000). Moreover, TEC-specific *Shh*-deletion leads to a reduced number of cTECs and mTECs (Saldaña et al., 2016). If cTEC-

derived SHH indeed promotes IGF2 production in fibroblasts, the transition from neonatal expansion to juvenile homeostasis could simply be due to cell-to-cell distance. As the thymus expands, fewer SHH⁺ cTECs will be in close proximity to capsular fibroblasts, which results in reduced IGF2 production. This intercellular proximity hypothesis does not violate the small thymic size of *Foxn1*^{lacZ/lacZ}, for *Foxn1*-promoted TEC proliferation is a downstream target of IGF2-IGF1R interaction.

5.2. Overcoming threshold: enhancing iTEC yield, mTEC and MHCII gene expressions

In Chapter 4, we introduced an *in vitro* platform, iTECs. There are a few remaining questions that need to be addressed. I have yet to identify whether the *Foxn1*-transgene-induced recalcitrant MEFs are beneficial or not. We know thymic mesenchyme is vital for TEC proliferation and differentiation (Chapter 2, Bredenkamp et al., 2014b). It is possible that reducing *Foxn1*-recalcitrant MEFs could yield more functional iTECs. However, it is equally possible that *Foxn1*-recalcitrant MEFs are serving as the surrounding mesenchyme, providing necessary signals. Next, I could not increase the yield of AIRE⁺ mature mTECs. As observed with RT-qPCR and ICC, I consistently detected a small number of AIRE⁺ cells in our Notch signal inhibited iTECs. Notably, my additional experiments to increase AIRE⁺ iTECs with the known *in vivo* RANK pathway by treating RANKL did not increase *Aire* expression. Rather, my RT-qPCR results suggest it initiates an alternative differentiation pathway (Fig. 5.2), which needs further investigation. Identifying a molecular mechanism to increase the yield of AIRE⁺ iTECs, which is necessary for tissue-restricted antigen (TRA) expression to prevent autoimmunity, will be vital for

the platform to be used clinically. Furthermore, I demonstrated that co-culturing iTECs with DP thymocytes induced MHCII gene transcription in iTECs. However, further studies are required to fully understand which specific ligand (foetal thymocytes) – receptor (iTECs) interactions increased MHCII gene expression to identify the molecular mechanism behind MHCII gene transcription.

5.3. Figures

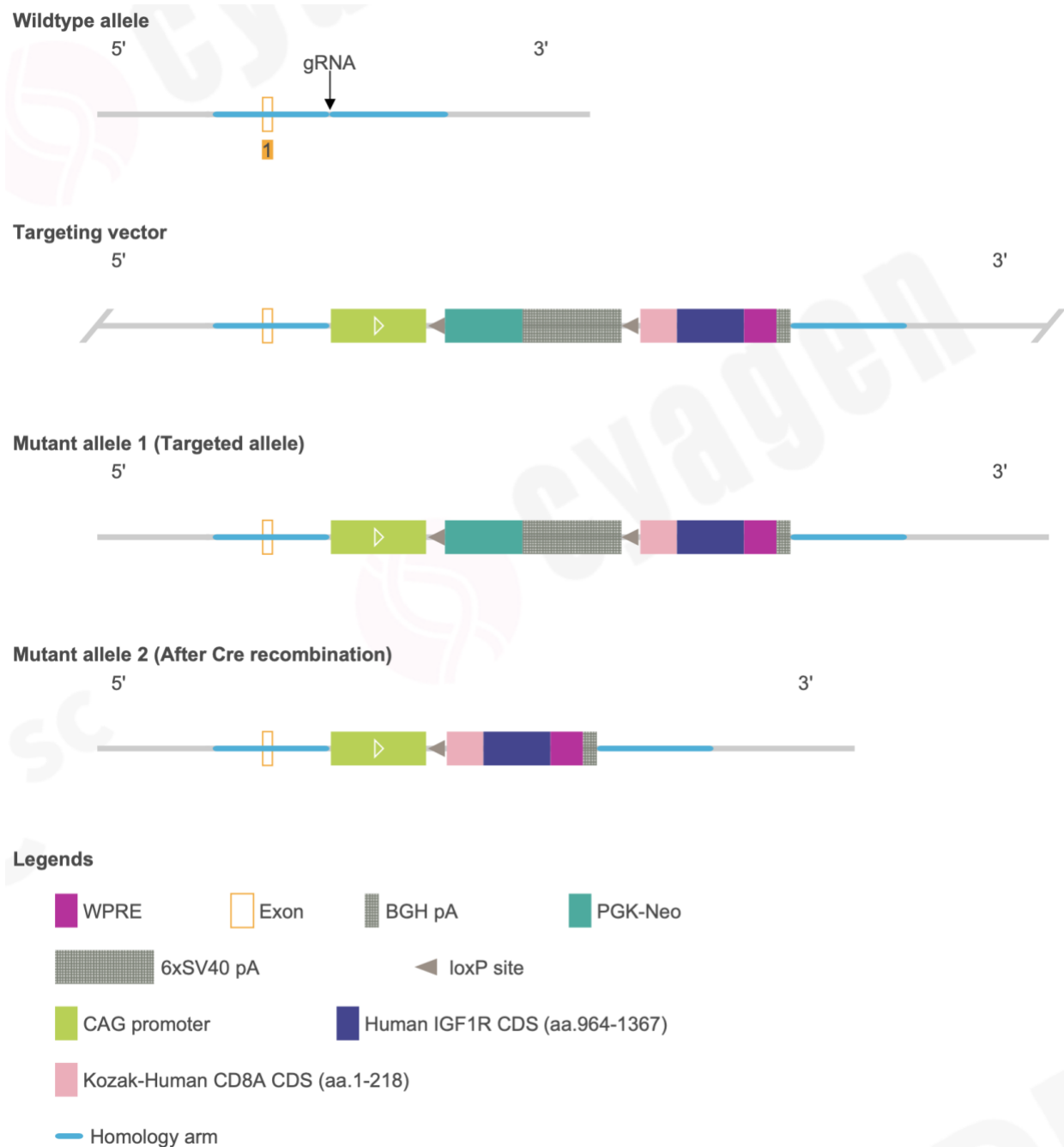


Fig. 5.1. Transgenic Rosa26-CD8 α -IGF1R design

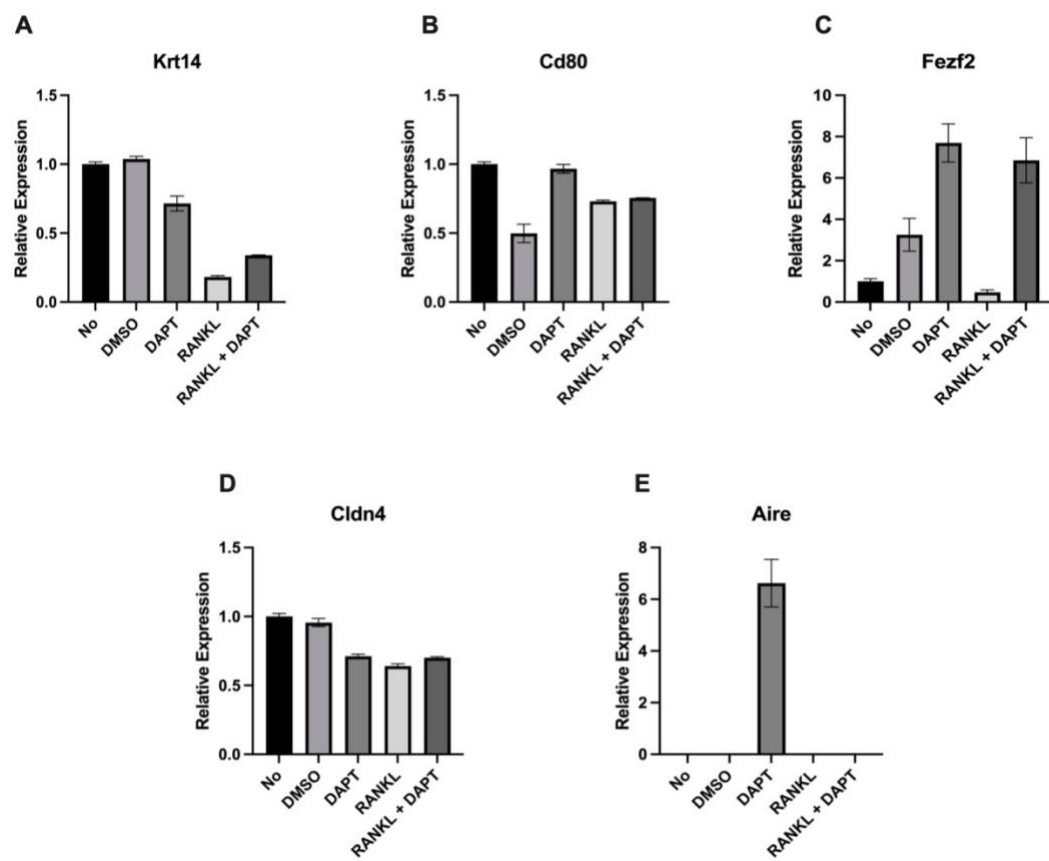


Fig. 5.2. RT-qPCR of iTECs with RANKL treatment

APPENDIX A

BUILDING INTERCELLULAR SIGNALLING NETWORKS TO IDENTIFY THE POTENTIAL SIGNALLING PATHWAYS FOR THE THYMUS/PARATHYROID CELL FATE SPECIFIERS

Introduction

The thymus and the parathyroid are vertebrate organs that have different functions. However, during development, the two organs develop from the same primordium, the third pharyngeal pouch (PP). Cells committed to the parathyroid fate start to express a transcription factor *Gcm2* at E9.5; the thymus fate start to express a transcription factor *Foxn1* at E11.5 (Gordon and Manley, 2011). However, neither of the transcription factors specify organ fates and the specifiers remain a mystery (Gordon et al., 2001; Liu et al., 2007; Nehls et al., 1996). Using single-cell RNA sequencing of E10.25 and E11.25 3rd PP and surrounding mesenchyme, we sought to determine a thymus specifier alongside a change in intercellular signals between E10.25 and E11.25. In this appendix, we built the intercellular signal networks with the scRNAseq data with CellChat (Jin et al., 2021).

Results & Discussion

All cells

First, we built intercellular signal networks with all of the cells except for low-information droplets/cells. For the E10.25 scRNAseq dataset, we identified 4 different mesenchyme, 3rd PP,

ectoderm, mesoderm, endothelium, neural crest cell, smooth muscle, cardiac muscle, neural, mast cell, satellite cell, and red blood cell/erythrocyte clusters. For the E11.25 scRNAseq dataset, we identified 4 different mesenchyme, parathyroid- and thymus-fate-bound endoderm, endothelium, neural crest cell, neural cells, and myoblasts. We used two different plots to visualise the signal interactions: circle plots and chord plots. Circle plots' arrows show the direction of the signal interactions. The thickness of the arrows in the circle plots signifies the weight of the signal interactions/signalling probabilities that are deduced from the ligand and receptor expression levels. On the contrary, chord plots are designed to show the proportions of signals sent and received. It is of importance to remember that the single-cell RNA sequencing data lacks accurate spatial information. Not all ligands are soluble proteins and a lot of signalling pathways require direct cell-to-cell contact. Further validations *in situ* are required with either fluorescent *in situ* hybridisation (FISH) and/or immunohistochemistry (IHC).

At E10.25, prominent BMP signal interactions were observed across different cell types (Fig.A.1A, B). Interestingly, major BMP signal interactions were observed across different mesenchymal cell clusters and the cardiac muscle cluster (Fig.A.1A, B). BMP signal that shapes the ventral patterning of the 3rd PP was predicted to be significantly weaker (Fig.A.1A), which is due to strong BMP interactions amongst mesenchymal cells. Ectoderm served as a sole source of *Wnt6*, sending WNT signal to all of the surrounding cells (Fig.A.1C). Non-canonical WNT signal occurred across mesenchyme, mesoderm, and satellite cells (Fig.A.1D, E). Rather interestingly, satellite cells are predicted to send ncWNT signal via *Wnt5a* to ectoderm and 3rd PP, which requires further investigation (Fig.A.1E). We observed strong FGF signal from 3rd PP, ectoderm, and

mesoderm to all of the surrounding cells except for mast cells and erythrocytes (Fig.A.1F, G). Prominent Notch signal interactions were observed across the majority of the major cell types except for ectoderm and 3rd PP (Fig.A.1H, I). *Igf2*-driven IGF signal interactions were observed across major cell types except for 3rd PP and ectoderm (Fig.A.2).

At E11.25, BMP signal interactions were observed across mesenchyme, myoblast, neural crest cells, neural cells, and thymus-specified 3rd PP (Fig.A.3A, B). Thymus-specified 3rd PP received the BMP signal from mesenchyme 3 (Fig.A.3A, B), which is likely located antero-ventrally. The source and ligand of the WNT signal changed into thymus-specified 3rd PP, which sent *Wnt4* to all of the surrounding cells except for endothelium (Fig.A.3C). Interestingly, strong non-canonical WNT interactions were observed at E11.25, except for thymus-specified 3rd PP (Fig. A.3D, E). The complete absence of ncWNT signal to the thymus-specified 3rd PP suggests a potential role of ncWNT signal on the 3rd PP patterning. Interestingly, at E11.25, the 3rd PP no longer sent or received the FGF signal to mesenchyme (Fig.A.3F, G). FGF signal from mesenchyme to TECs via FGF7/10-(FGF-R2IIIb) is shown critical for the early thymic development demonstrated with a murine model with FGF-R2IIIb deficiency exhibiting thymic development blocked at E12.5 (Revest et al., 2001). This could be either TECs expressing FGF-R2IIIb later in the development or a technical artefact with an annotation lacking FGF-R2IIIb and/or a database lacking FGF7/10-(FGF-R2IIIb) interaction. Strong Notch signal interactions were still observed at E11.25, but the 3rd PP remained Notch-free (Fig.A.3H, I). Thymus-specified 3rd PP started to receive IGF signal from mesenchymal cells (Fig.A.4A, B), concordant to our discovery in Chapter 2.

Epithelium and mesenchyme

Next, we investigated signal interactions between epithelium and mesenchyme to compare signals between E10.25 and E11.25. As previously observed, major signal interactions were occurring within mesenchyme and masked the known interactions between epithelium and mesenchyme. At E10.25, strong BMP signal interactions were observed amongst mesenchyme subsets. Ectoderm and pharynx sent BMP signal to mesenchyme subset 1; mesenchyme 1 sent BMP signal to both dorsal and ventral 3rd PP via *Bmp4* and *Bmp2* (Fig.A.5A). At E10.25, FGF signal was sent from both ventral and dorsal 3rd PP to all the mesenchyme through *Fgf8*-(*Fgfr1*+*Fgfr2*) interactions (Fig.A.5B). GDF signal interactions occurred within mesenchyme, but a weak interaction occurred between ventral 3rd PP and two mesenchyme subsets via *Gdf11*-(*Tgfr1*+*Acvr2a/b*, *Acvr1b*+*Acvr2b*) (Fig.A.5C). WNT signals were exchanged mainly from ectoderm to mesenchyme and 3rd PP. The ventral 3rd PP sent the WNT signal to the dorsal 3rd PP and mesenchyme through *Wnt4* and *Wnt6* (Fig.A.5D).

At E11.25, BMP & IGF signal interactions could be observed amongst mesenchyme subsets. However, mesenchyme sent BMP & IGF signals to unspecified central and thymus-specified 3rd PP, but parathyroid-specified 3rd PP (Fig.A.5E, F). On the contrary, non-canonical WNT signal interactions occurred within mesenchyme and between mesenchyme and parathyroid-specified 3rd PP (Fig.A.5G). The main source of the WNT signal in the E11.25 dataset was the thymus-specified 3rd PP, which sent the WNT signal to mesenchyme and unspecified-central 3rd PP only via *Wnt4* (Fig.A.5H).

Within epithelium

Our final survey of signalling pathways within epithelium revealed minimal signal interactions amongst epithelial subsets, both in E10.25 and E11.25. At E10.25, WNT, BMP, and HH signals were observed. Ectoderm-derived WNT signal was sent to dorsal and ventral 3rd PP through *Wnt4/6*-(*Fzd2/3*+*Lrp6*) interactions; pharynx and ectoderm-derived BMP signal was sent to dorsal 3rd PP with *Bmp4*-(*Bmpr1a*+*Acvr2a*); pharynx-derived HH signal was sent to dorsal and ventral 3rd PP via *Shh-Ptch1* interaction (Fig. A.6A-C). At E11.25, signal interactions amongst epithelial subsets were minimal & only WNT signal interaction from the thymus to a central via *Wnt4*-(*Fzd2*+*Lrp6*) was observed (Fig. A.6D).

Figures & Figure Legends

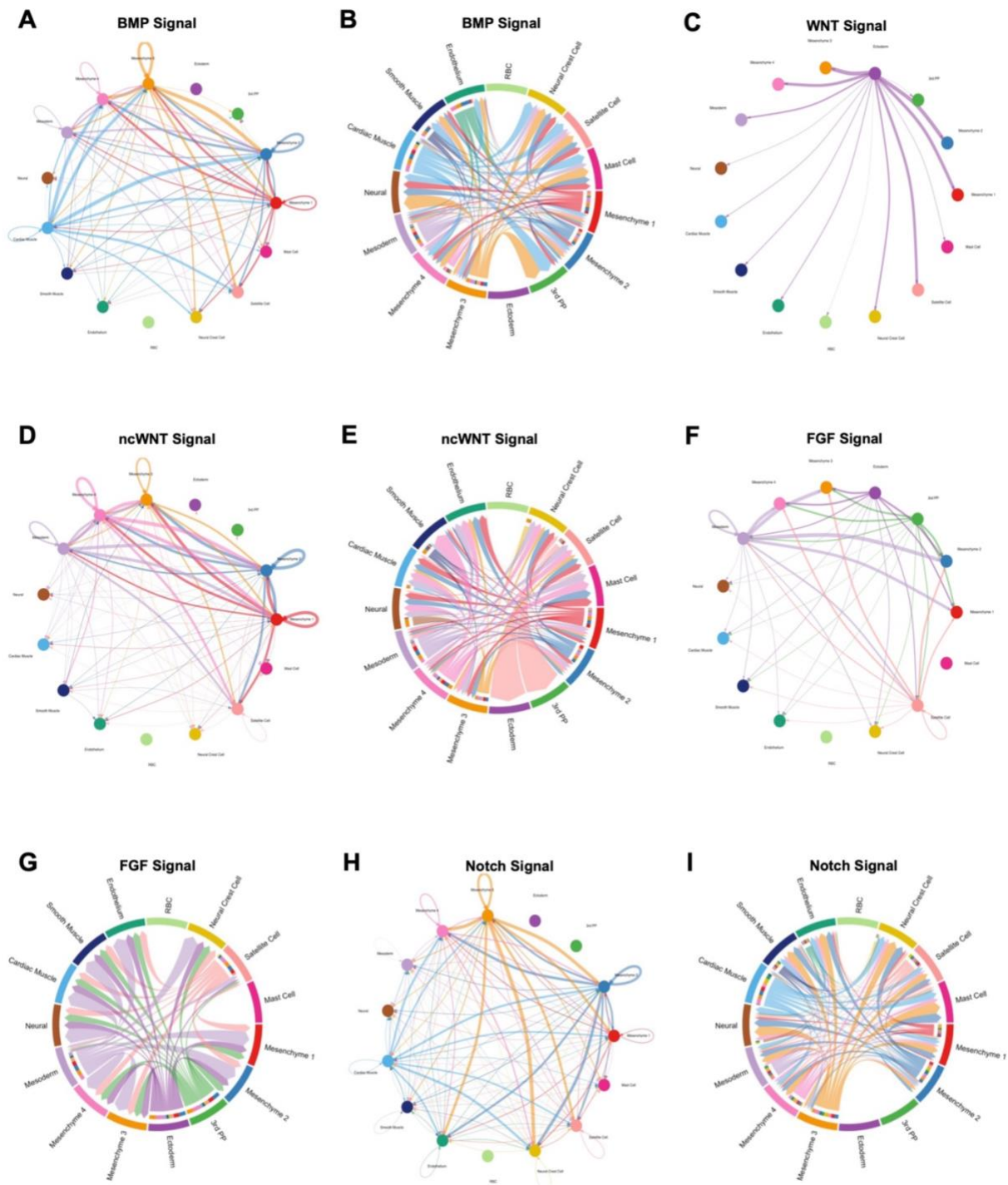


Figure A.1. Signal interactions across all cells at E10.25

(A, C, D, F, H) Circle plots showing signal interactions at E10.25. (B, E, G, I) Chord plots showing signal interactions at E10.25.

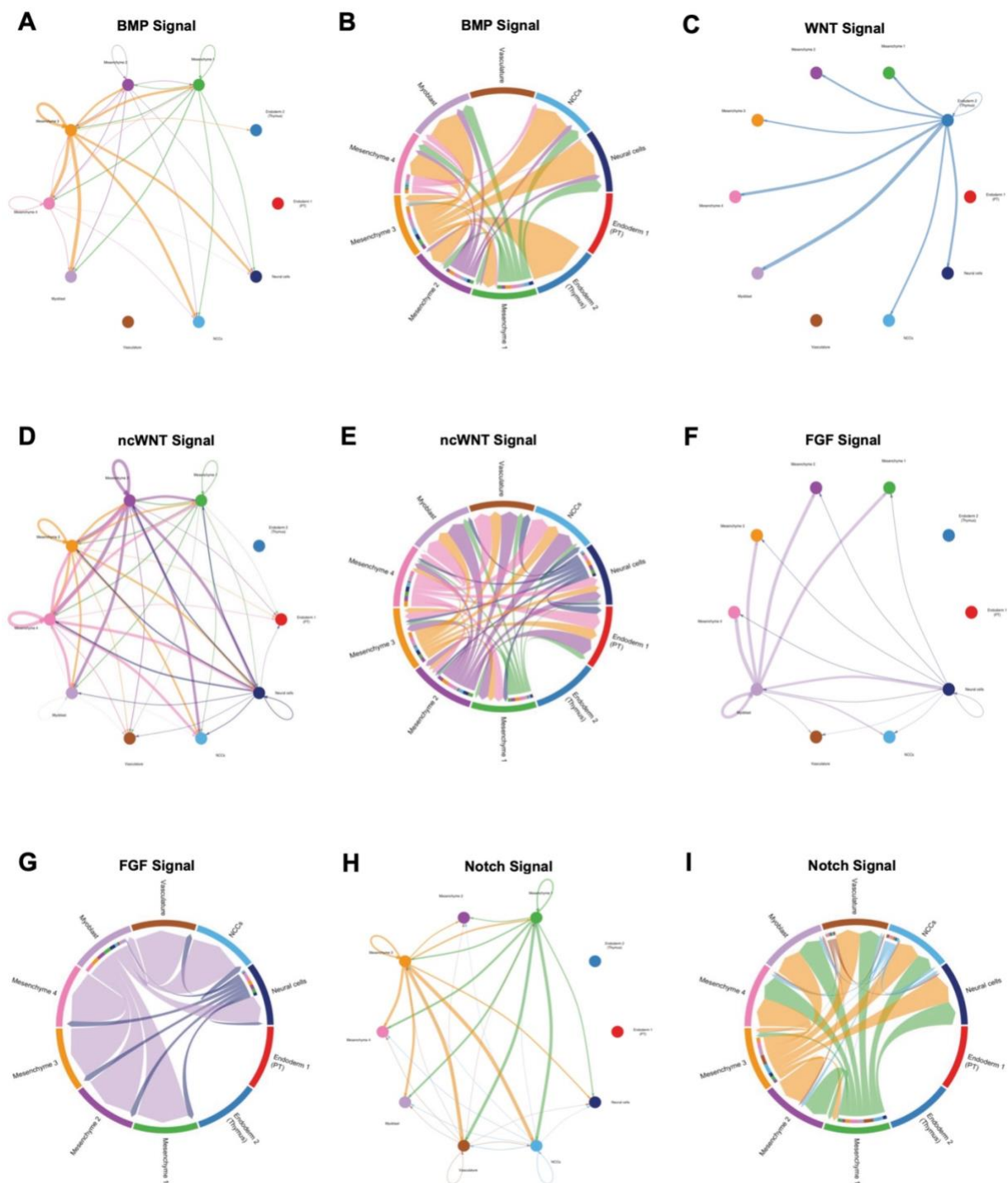


Figure A.3. Signal interactions across all cells at E11.25

(A, C, D, F, H) Circle plots showing signal interactions at E11.25. (B, E, G, I) Chord plots showing signal interactions at E11.25.

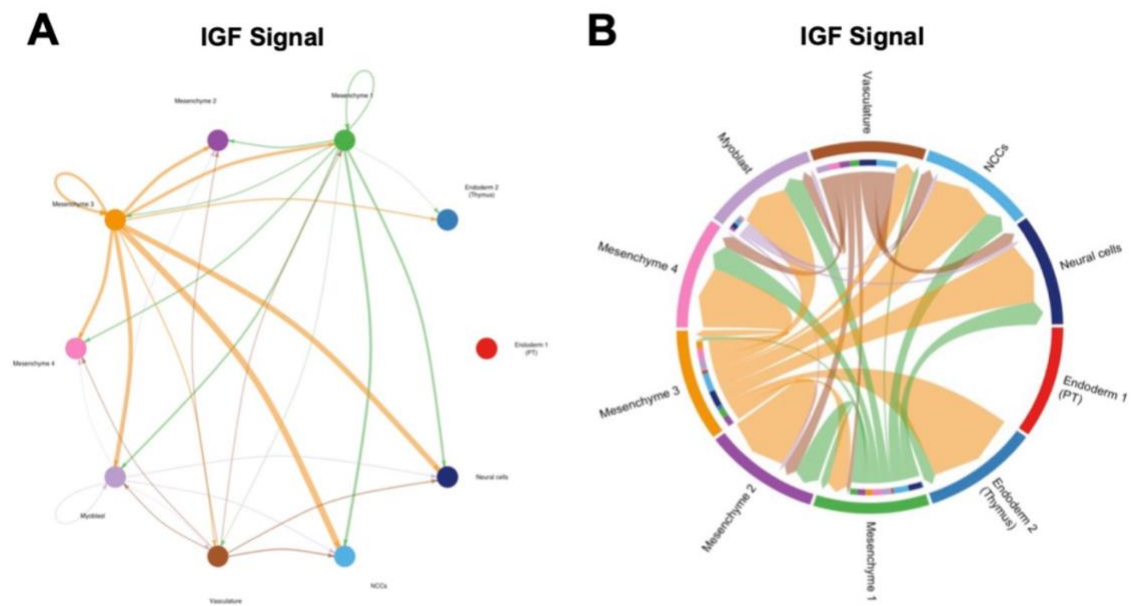


Figure A.4. IGF signal interaction at E11.25

(A) A circle plot showing IGF signal interactions at E10.25. (B) A chord plot showing IGF signal interactions at E11.25.

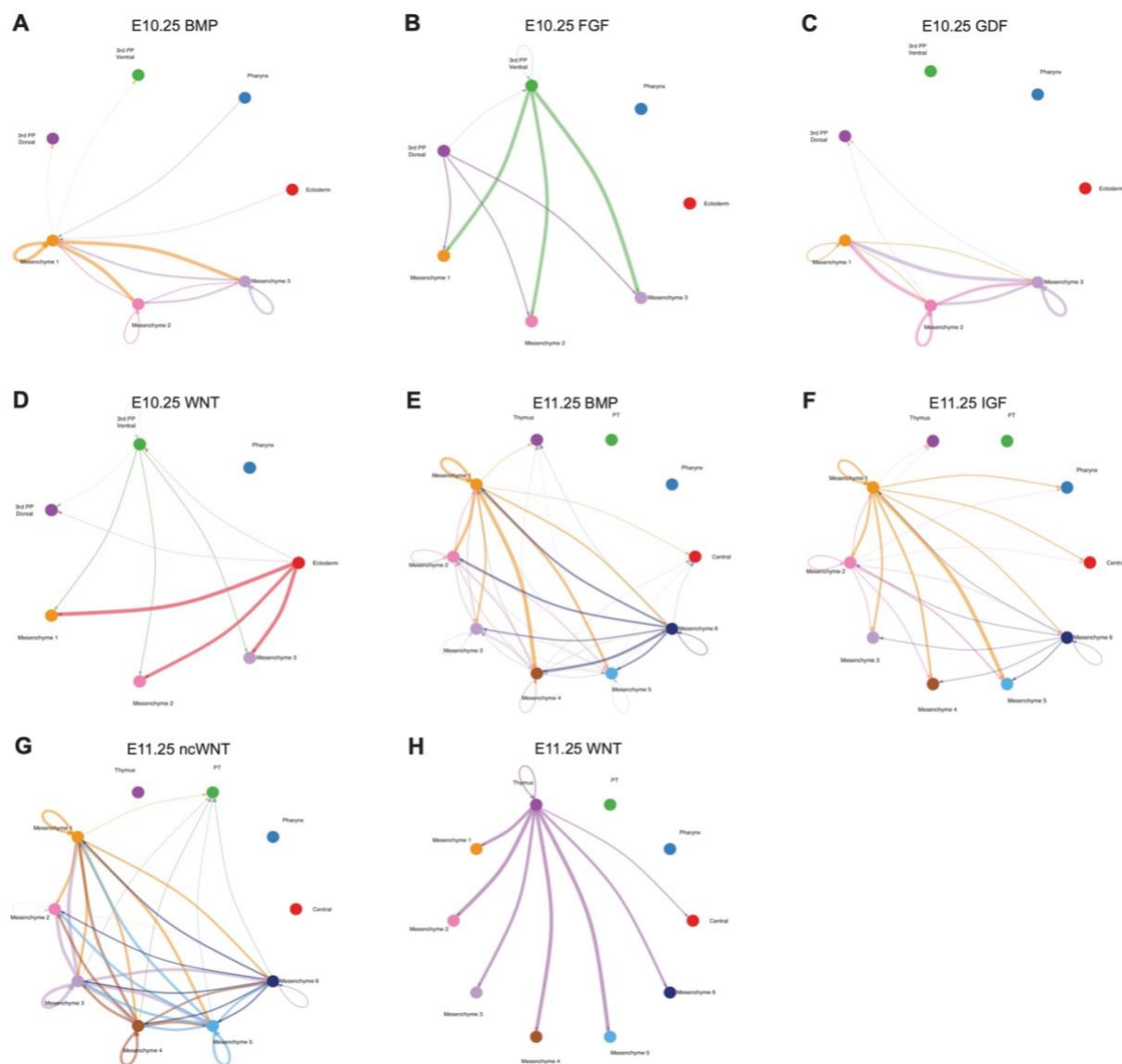


Figure A.5. Signal interactions between epithelium and mesenchyme

(A-D) Circle plots showing signal interactions in E10.25 scRNAseq data. BMP, FGF, GDF, and WNT signals are shown. (E-H) Circle plots showing signal interaction in E11.25 scRNAseq data. BMP, IGF, non-canonical WNT, and WNT signals are shown.

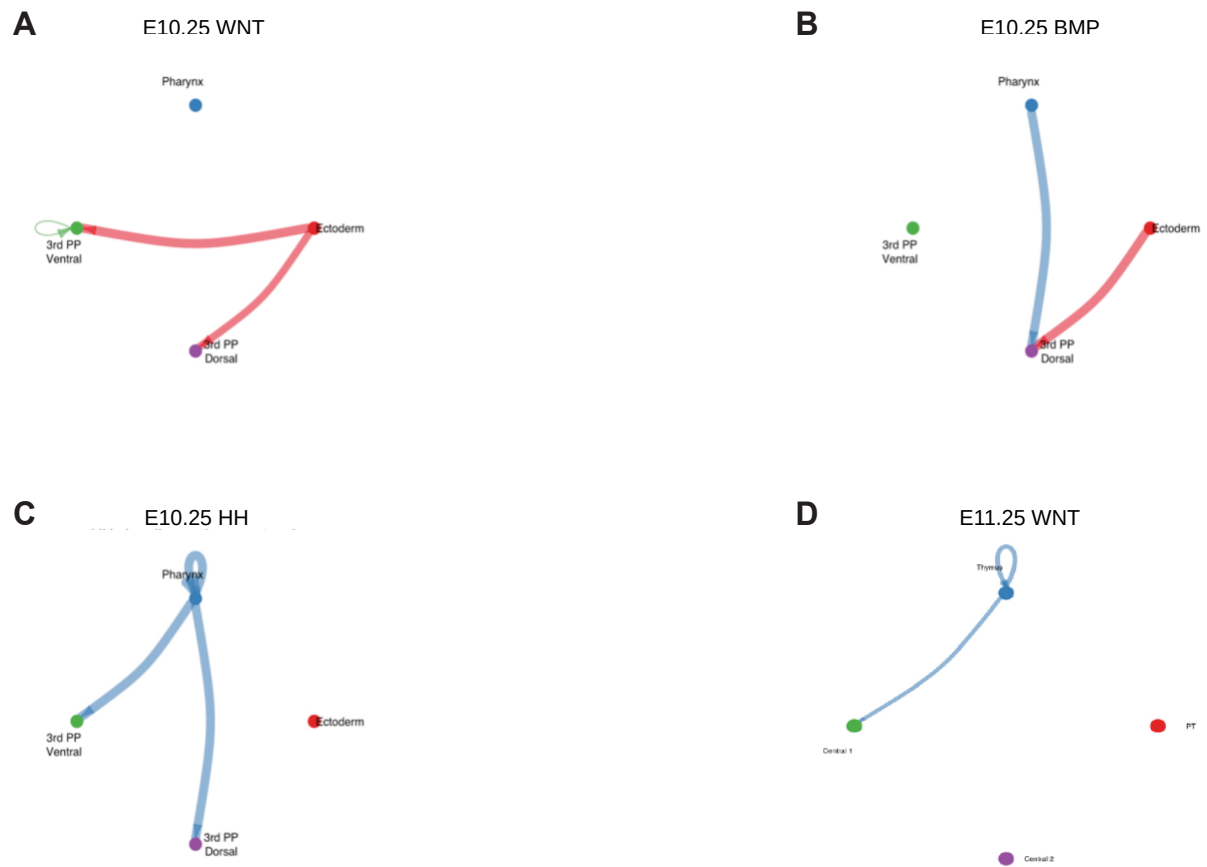


Figure A.6. Signal interactions within epithelium

(A-C) Circle plots showing signal interactions in E10.25 scRNAseq data. WNT, BMP, and HH signals are shown. (D) A circle plot showing WNT signal interaction in E11.25 scRNAseq data.

Materials & Methods

Intercellular network analysis

CellChat (Jin et al., 2021) was used to build intercellular networks of E10.25 & E11.25 Seurat datasets. The analyses were done as per guidelines on GitHub. Due to vastly different cluster identities, CellChat objects were generated from individual Seurat objects. We surveyed: secreted signalling, ECM-receptor, and cell-to-cell contact databases. Due to the different cell numbers of the Seurat objects, different thresholds determining the minimum cell numbers expressing ligands/receptors were assigned (E10.25 dataset = 50; E11.25 dataset = 10).

Appendix B

DELETION OF BOTH E2F BINDING SITES FROM *FOXN1* PROXIMAL PROMOTOR RESULTS IN ENDOTHELIUM/PERICYTE REDUCTION

Introduction

In the thymus, we and our collaborators have established that the CyclinD1-RB-E2F pathway regulates the thymic expansion and the homeostatic transition, based on analyses of mice with null mutations in 5 out of 6 alleles in the RB gene family (Garfin et al., 2013), and constitutive CyclinD1 under the Keratin 5 promotor (Robles et al., 1996). Both lines fail to establish homeostasis, resulting in constant thymic growth accompanying increasing *Foxn1* expression (Klug et al., 2000; Robles et al., 1996). We decided to use CRISPR-Cas9 to generate lines to delete previously identified E2F binding sites in the *Foxn1* proximal promotor (Garfin et al., 2013) to determine how E2Fs regulate *Foxn1* expression and the thymic microenvironment. Here, we submitted CD45- total thymic stromal cells of an E2F_M11 model with a deletion including both of the E2F binding sites (CTTTC, TTTGGCA) for single-cell RNA sequencing.

Results

We used single-cell RNA sequencing to determine the direct impact of E2F binding site deletion on each subset of the thymic stroma. We submitted 1-month-old male wild-type and E2F_M11 thymus for scRNAseq. Unsupervised clustering of CD45- total thymic stromal transcriptomes yielded a UMAP clustered by canonical markers: *Epcam*⁺ *H2-Aa*⁺ thymic

epithelial cells (TECs), *Pdgfra*⁺ *Apod*⁺ fibroblasts, *Pdgfrb*⁺ *Myl9*⁺ pericytes, and *Pecam1*⁺ *Kdr*⁺ endothelial cells (Fig. B.1A). Superimposing the dataset split by genotype revealed differences in *Ccl21a*⁺ TECs and fibroblasts (Fig. B.1B). However, further analysis revealed the differences are due to a technical artefact: ambient RNA contamination, likely caused by lysed erythrocytes during a sample processing. Splitting the dataset by genotype revealed a major difference between the two thymuses is the substantially decreased frequency of the endothelium and encapsulating pericytes (Fig. B.1C). Frequency of endothelial cells decreased from 0.11 (wild-type) to 0.03 (E2F_M11); pericytes from 0.085 (wild-type) to 0.007 (E2F_M11). Differentially expressed gene (DEG) analysis between wild-type and E2F_M11 with a logistic regression test yielded a few genes that are highly expressed in wild-type thymus, *Ccl25* and *Tbata* among them (Fig. B.1D).

Discussion

Rather surprisingly, a deletion of two E2F binding sites on the *Foxn1* proximal promotor did not result in major differences in TECs. The deletion resulted in a comparable *Foxn1* expression level between wild-type (0.26) and E2F_M11 (0.28), showing only a minor difference. Typically, RB proteins regulate “transcriptional activators” (E2F1, E2F2, E2F3), or “transcriptional repressors” (E2F4, E2F5) to regulate cell cycle genes (Henley and Dick, 2012). Indistinguishable relative *Foxn1* level is likely due to different E2F protein families binding to the different sites, which will be further investigated in our manuscript in preparation. Despite the comparable *Foxn1* expression level, however, its downstream target *Ccl25* and a functionally important cortical TEC marker *Tbata* were significantly downregulated in the E2F_M11 model.

The results suggest E2F binding site deletions did not result in *Foxn1* transcription itself, but may have affected FOXN1 function.

Instead of TECs, the deletion of two E2F binding sites on the *Foxn1* promotor significantly reduced the number and frequency of endothelial cells and pericytes. In the thymus, endothelial cells are important for the attraction, entry, and survival of the early thymic progenitor cells (Buono et al., 2018; Lepique et al., 2003; Rossi et al., 2005), whereas pericytes are vital for egress of the thymocytes (Zachariah and Cyster, 2010). Further thymocyte and peripheral T-cell analyses are required, but the substantial decrease in endothelial cells and pericytes will likely cause impaired haematopoietic stem cell entry and mature thymocyte egress.

Figures & Figure Legends

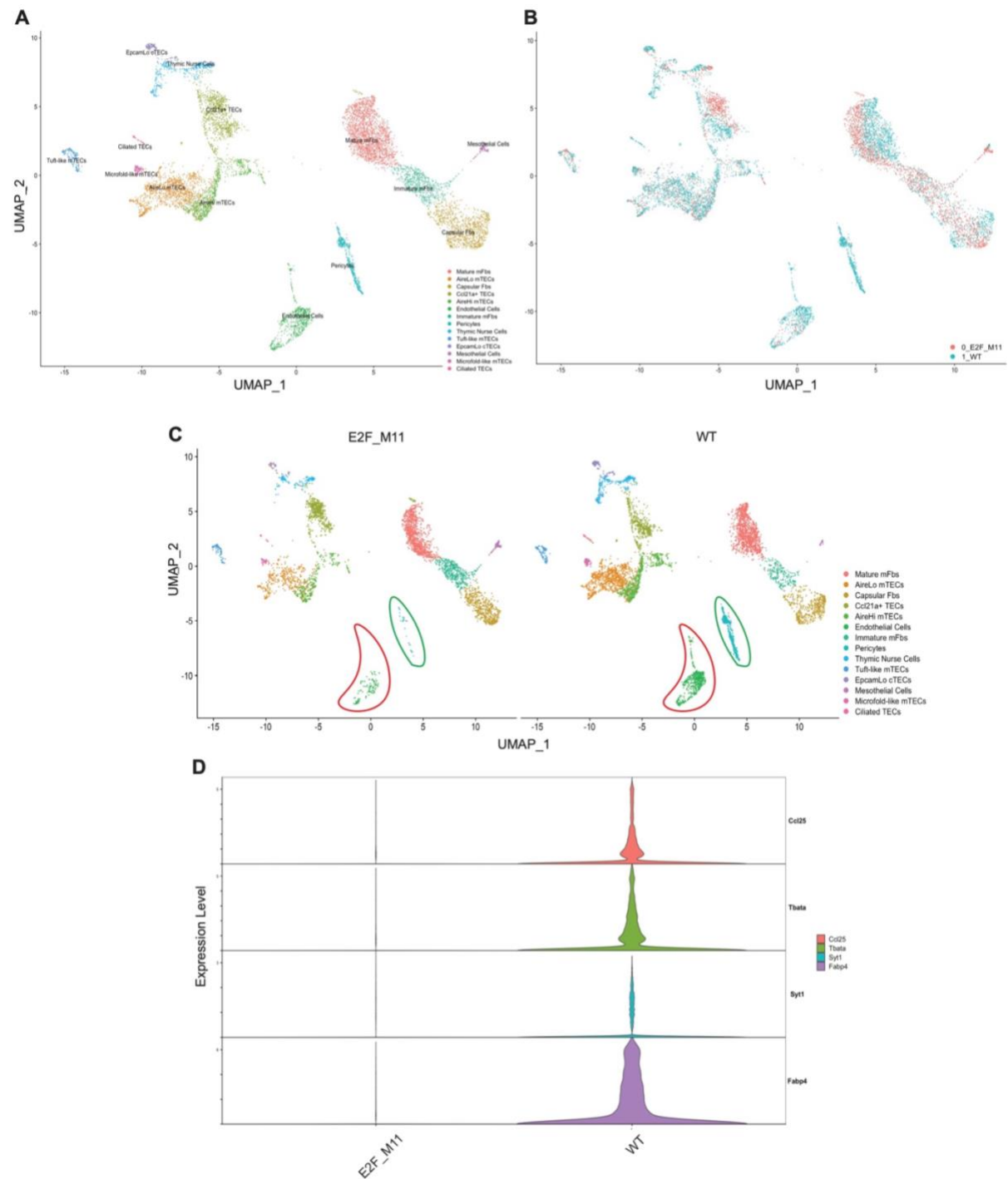


Figure B.1. Deletion of both E2F binding sites of the *Foxn1* promotor results in reduced endothelium and pericytes.

(A) UMAP visualisation of CD45- total thymic stromal cells with annotations. (B) UMAP visualisation of superimposed datasets split by genotype (age & sex-matched, 1-month-old wild-type and E2F_M11). (C) UMAP visualisation of merged dataset split by genotype. Red circles indicate *Pecam1*⁺, *Kdr*⁺ endothelial cells. Green circles indicate *Pdgfrb*⁺, *Myl9*⁺ pericytes. (D) Violin plot of top 4 genes highly expressed in wild-type thymus compared to E2F_M11 thymus. Differentially expressed gene analysis was performed with a logistic regression test.

Materials & Methods

Mice

The E2F_M11 mice were generated in collaboration with the Bi Lab at the University of Georgia. All experiments involving animals were performed with approval from the University of Georgia Institutional Animal Care and Use Committee.

Single-cell suspension of the thymic stromal cells for scRNAseq, 10X library preparation, single-cell transcriptomic data analyses

Refer to methods of Chapter 2, 3.

Appendix C

NEONATAL MURINE INTRATHYMIC INJECTION PROTOCOL

Introduction

For chapter 2, IGF2 manuscript, we initially decided to determine the role of IGF2-IGF1R interactions on the neonatal expansion of the thymus via intrathymic injection in young murine pups. The intrathymic injection is a well-established method (Manna and Bhandoola, 2016) enabling the reliable delivery of cells and drugs directly into the adult murine thymus. In order to minimise the physical stress and trauma that could lead to lethality, we decided to establish the intrathymic injection method on neonatal murine pups.

Perinatal Murine Intrathymic Injection Protocol (UGA IACUC Approved)

- Performed on the neonatal murine pups established from the *Foxn1^{lacZ}* & C57BL6/J.
- Per Jackson laboratory guidelines, the day the pups are born will be counted as day 0.

Hypothesis: Insulin-like Growth Factor (IGF) signalling via IGF2-IGF1R interaction is driving the exponential growth of the thymus during the neonatal stage until P10.

Experimental Plan I

- By injecting murine IGF-II protein, I expect the thymus to continuously grow after P10.
- Injecting mouse IGF-II protein, every day from P7 ~ P12.

- Recombinant mouse IGF-II protein (R&D Systems: 792-MG-050)
 - Constituted in 0.1% BSA/1X PBS (Sterile): 100µg/mL
 - 250ng (=2.5µL) per injection (Based on (Chen et al., 2011))
- Sham vehicle (0.1% BSA/1X PBS (Sterile))

Experimental Plan II

- By injecting an IGF1R inhibitor, I expect the thymus to stop growing prematurely before P10.
- Injecting IGF1R inhibitor, JB1 trifluoroacetate salt, every day from P5 ~ P10.
 - JB1 trifluoroacetate salt (Sigma-Aldrich: J3705-1MG).
 - Constituted in 1X PBS (Sterile): 100µg/mL
 - 333ng (=3.3µL) per injection (Based on (Brock et al., 2011))

1µg/injection in rat pups.

Mice pups are typically 1/3 of age-matched rat pups.

- Sham vehicle (1X PBS (Sterile))
- Other IGF1R inhibitors require cytotoxic DMSO as a vehicle.

For both of the experiment sets, quantity-matched sterile 1X PBS will be injected as a sham injection for one set of murine pups. The other set will be injected with IGF-II or JB1 trifluoroacetate salt. The animals injected with treatments will be tail-marked for distinction.

Considering there are no previously established protocols/studies, we expect complications. We expect ~200 pups will be used for the protocol.

For both of the experiment sets, pups will be euthanised by P28 for downstream analyses.

1. Thymus weight/body weight ratio
2. Thymus collection for Immunohistochemistry (IHC)
3. FACS analyses of thymic epithelial cell subsets & thymocyte subsets

Injection Method

- Practise with properly euthanised pups. Due to the relatively big size of the thymus in pups, we have been able to reliably inject into the thymus with a success rate of 90% (Fig. C.1).
 - Potential complication
 - Punctured thoracic cavity may cause infection and/or lung expansion that could cause difficulty in breathing.
 - Potential hypothermia for P5~P6 pups. We will try to maintain the temperature/end the protocol as soon as possible.
1. Gently scruff the pups of age and apply EMLA cream (Lidocaine/Prilocaine 2.5%) on the skin. Gently wrap EMLA cream and the pups to hold the cream.
 2. Wait for 10~15 minutes prior to the injection.
 - The standard duration is 20 minutes for the adults, but neonate lethality was reported.
 - We will check the response to avoid pain/stress on the pups.

3. Gently scruff the pups again. Using the Hamilton syringe with 33G, 10mm needle with 60° tip (Fig. C.2), inject < 4µL of the concoctions & vehicle control per lobe described in the Experimental Plans above, directly to the thymuses without opening up the chest cavity.
 - Needles will be injected between the 2nd and 3rd rib cages close to the sternum, with the tip pointing towards the neck with an angle of 20 ~ 30°, based on the age in order to hit the thymus consistently. The younger the age, the steeper the angle due to the pups' small size and closer proximity of vital organs.
4. Apply triple antibiotics (Neomycin Sulfate, Polymyxin B Sulfate, Bacitracin Zinc) on the punctures post-injection & monitor the pups for 10 minutes.
5. After monitoring, apply maternal faecal material on the pups to prevent parental cannibalism.
 - Faecal material will be applied widely whilst avoiding punctures.
6. Pups will be monitored the next day for their well-being.

Discussion

Our intrathymic injection method to neonatal murine pups is easy, and reliable with minimal pain, stress, and trauma to young murine pups. We expect the method will be of assistance to immunologists inclined to study perinatal murine thymus biology.

Acknowledgement

We thank Dr Stephen B. Harvey at the University Research Animal Resources, the University of Georgia for the invaluable insights about the neonatal murine pups.

Figures & Figure Legends

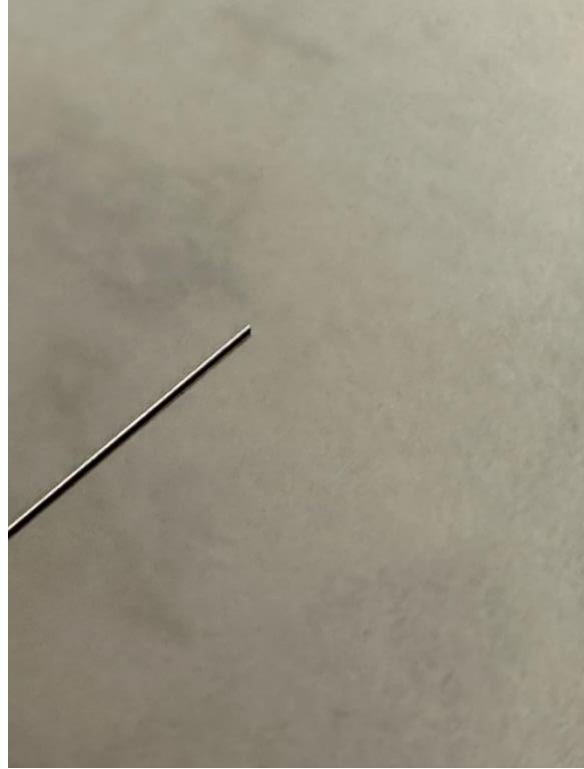


Figure C.1. Custom-made Hamilton syringe and its tip

(Left) Hamilton syringe. (Right) Tip.

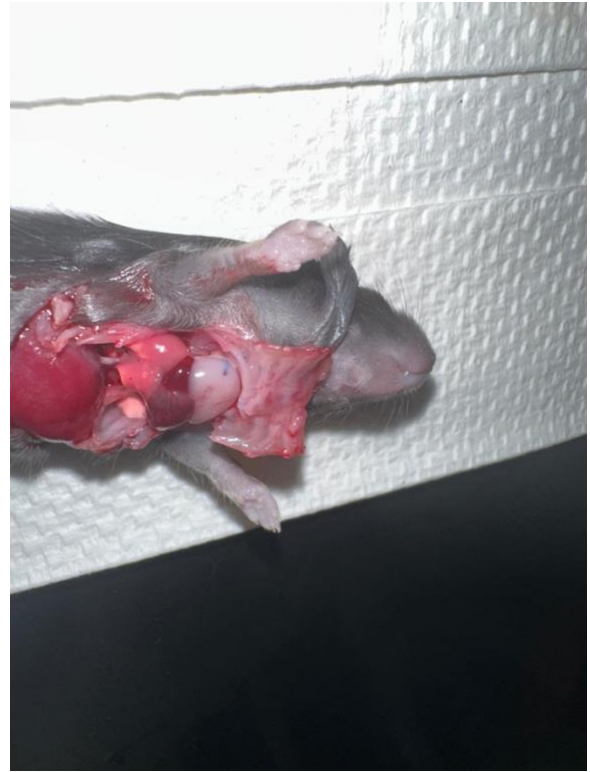


Figure C.2. Perinatal intrathymic injection

(Left) P7 murine pup intrathymic injection. (Right) P10 murine pup intrathymic injection.

Appendix D

DEFINING PERINATAL THYMIC TRANSITION POINT

Results

Major transcriptome changes in endothelium and mesenchyme across the perinatal transition point

To understand how non-TEC stroma changes across the perinatal transition point, we analysed a time-series single-cell RNA sequencing dataset of CD45- total thymic stroma. We did not observe sexual dimorphism across the perinatal transition point (Fig. D.1A, B). Only minor transcriptome differences were observed, which were due to erythrocyte contamination during sample processing. Unsupervised clustering of the CD45- total thymic stroma yielded major all of the major stromal populations: cTECs, mTECs, mimetic TECs, endothelial cells, fibroblasts, vascular mural cells, mesothelial cells, and rare non-myelinating Schwann cells (Fig. D.2A). The clusters were annotated with archetypal markers: *Epcam* and *H2-Aa* for the TECs, *Pecam1* and *Cdh5* for the endothelial cells, *Pdgfra* for the fibroblasts, *Pdgfrb*, *Acta2*, and *Myl9* for the vascular mural cells, *Upk3b* and *Nkain4* for the mesothelial cells (Huang et al., 2022), and *Gfap*, *Ngfr*, and *S100b* for the non-myelinating Schwann cells (Hu et al., 2018) (Fig. D.2B). To determine non-TEC stromal changes across the perinatal transition point, we surveyed proportion of each non-TEC stroma at 3-, 7-, 14-, and 30-day-old (Fig. D.2C). Interestingly, proportion of capsular fibroblast consistently decreased from 3- to 14-day-old. Also, the proportion of endothelial cells increased across the perinatal transition point (Fig. D.2C). To understand the

overall data structure, we pseudo-bulked age-matched datasets of endothelial cells, capsular fibroblasts, and medullary fibroblasts (Fig. D.2D-F). Our PCA analysis revealed age (x-axis) was the main factor determining the data variances. The transcriptomes of 3- and 7-day-old samples were similar, whereas 14- and 30-day-old samples were further distinct in all of the non-TEC stroma (Fig. D.2D-F).

We subsampled *Pecam1*⁺ endothelium, which yielded *Cxcl12*⁺ arterial, *Vwf*⁺ venous, *Kdr*⁺ capillary, and *Ccnd1*⁺ proliferative endothelial cells (ECs) (Fig. D.2G). Separating the endothelial dataset by age revealed a striking pattern. First, we observed a gradual disappearance of proliferative ECs with age (Fig. D.2H). Next, we observed a transcriptomic shift in both arterial and capillary ECs. Interestingly, capillary ECs formed different clusters after the perinatal thymic transition point whilst retaining the canonical marker expressions (Fig. D.2H). Moreover, *Vwf*⁺ venous ECs started to appear after the perinatal transition point (Fig. D.2H), suggesting a potential difference in thymic egress across the perinatal thymic transition point.

Subsampling mesenchyme, comprising *Pdgfra*⁺ fibroblasts and *Pdgfrb*⁺ mural cells, yielded *Dpp4*⁺ capsular fibroblasts, *Apod*⁺, *Serpine2*⁺ immature medullary fibroblasts, *Serpine2*⁺, *Mmp9*⁺, and *Ltbp1*⁺ mature medullary fibroblasts (Nitta and Takayanagi, 2020), *Pdgfrb*⁺, *Acta2*⁺, and *Myl9*⁺ vascular smooth muscle cells (vSMC), and *Pdgfrb*⁺, *Myl9*⁺ pericytes (Fig. D.2I). Splitting mesenchymal dataset by age revealed a pattern akin to endothelial dataset. Both capsular and medullary fibroblasts formed different clusters after the perinatal transition point alongside disappearing proliferative populations. Of note, immature medullary fibroblasts were completely missing after the perinatal transition point. Mural cells exhibited

more subtle changes. Besides the formation of a new pericyte cluster, proliferative mural cells and vSMC2 populations disappeared over age (Fig. D.2J). Overall, our time-series scRNAseq of CD45- total thymic stromal cells suggest there are major shifts in the transcriptome of endothelial cells and fibroblasts, shaping a distinct thymic microenvironment.

We then performed immunohistochemistry (IHC) to verify the scRNAseq data. Corroborating with Figure D.2C, we observed a substantial decrease in capsular fibroblasts. APOD+ capsular fibroblasts substantially decreased their density after the perinatal transition point (Fig. D.3A-C). Surprisingly, we did not see an increase in a number of endothelial cells nor their morphological changes across the perinatal transition point (Fig. D.3D-F).

Methods

CD45- thymic stroma isolation

3 thymi of 3, 7, and 14-day-old wild-type littermates of *Foxn1^{lacZ}* (Chen et al., 2009) were collected and pooled per library generation to minimise individual variabilities. The thymi were processed and sorted as per (lacZ Ref). Excess blood vessels and adipocytes were removed in 4°C sterile 1X PBS (w/o Ca²⁺, Mg²⁺). Thymi were transferred into digestion buffer (2% FBS/1640 RPMI (w/ 25mM HEPES), DNase I (20µg/µL), Collagenase/Dispase (1mg/mL)) to be thinly minced into 1~2mm fragments. They were then subsequently incubated at 37°C for one hour. The solution was gently mixed with the transfer pipettes every 20 minutes, 100 times each. After the incubation, the solution was passed through a 70µm cell strainer to remove undigested thymic fragments. Cells were centrifuged at 300g, 4°C for 10 minutes. Erythrocytes were lysed

with a 2-minute-long incubation in RBC Lysis Buffer (10X) (BioLegend 420301). After centrifuging (300g, 4°C for 10 minutes), cells were resuspended in MACS buffer (filtered 0.5% BSA/2mM EDTA/1X PBS (w/o Ca^{2+} , Mg^{2+})). As per the MACS separation guidelines, cells underwent CD45 separation with CD45 microbeads, mouse (Miltenyi Biotec 130-052-301) to collect CD45- cells.

10X library preparation and Illumina sequencing

Viabilities of the stromal cells were confirmed with a haemocytometer with a fraction of cells stained with Trypan Blue. Libraries were generated with cells with >85% viability.

Approximately 10,000 thymic stromal cells were loaded into a 10X genomics Chromium instrument using a Single Cell 3' Reagent Kit (v3). Post cDNA QC, the final DNA library was sequenced on Illumina NextSeq2000 P3 (200 cycles).

Single-cell RNA sequencing data analysis

As per the 10X guideline, we used CellRanger (v6) to generate and pre-process FASTQ files. CellRanger generated filtered_feature_bc_matrix files underwent QC steps and then analysed using Seurat (v.4.3.0) (Hao et al., 2021) in R (v.4.2.1).

Immunohistochemistry (IHC)

Refer to Chapter 2.

Figures & Figure Legends

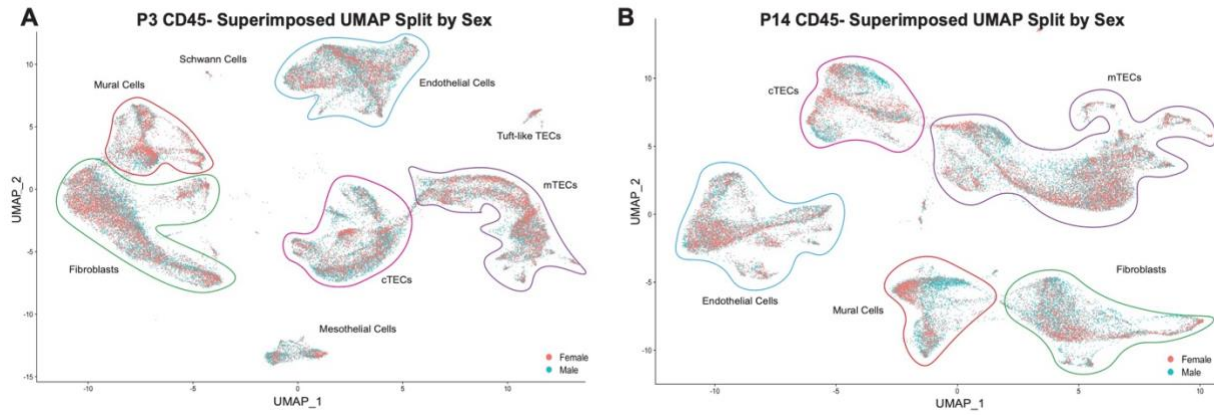


Figure D.1. Undetectable sexual dimorphism across the perinatal transition point.

(A, B) UMAP visualisation of the P3 and P14 datasets. Datasets were split by sex and superimposed.

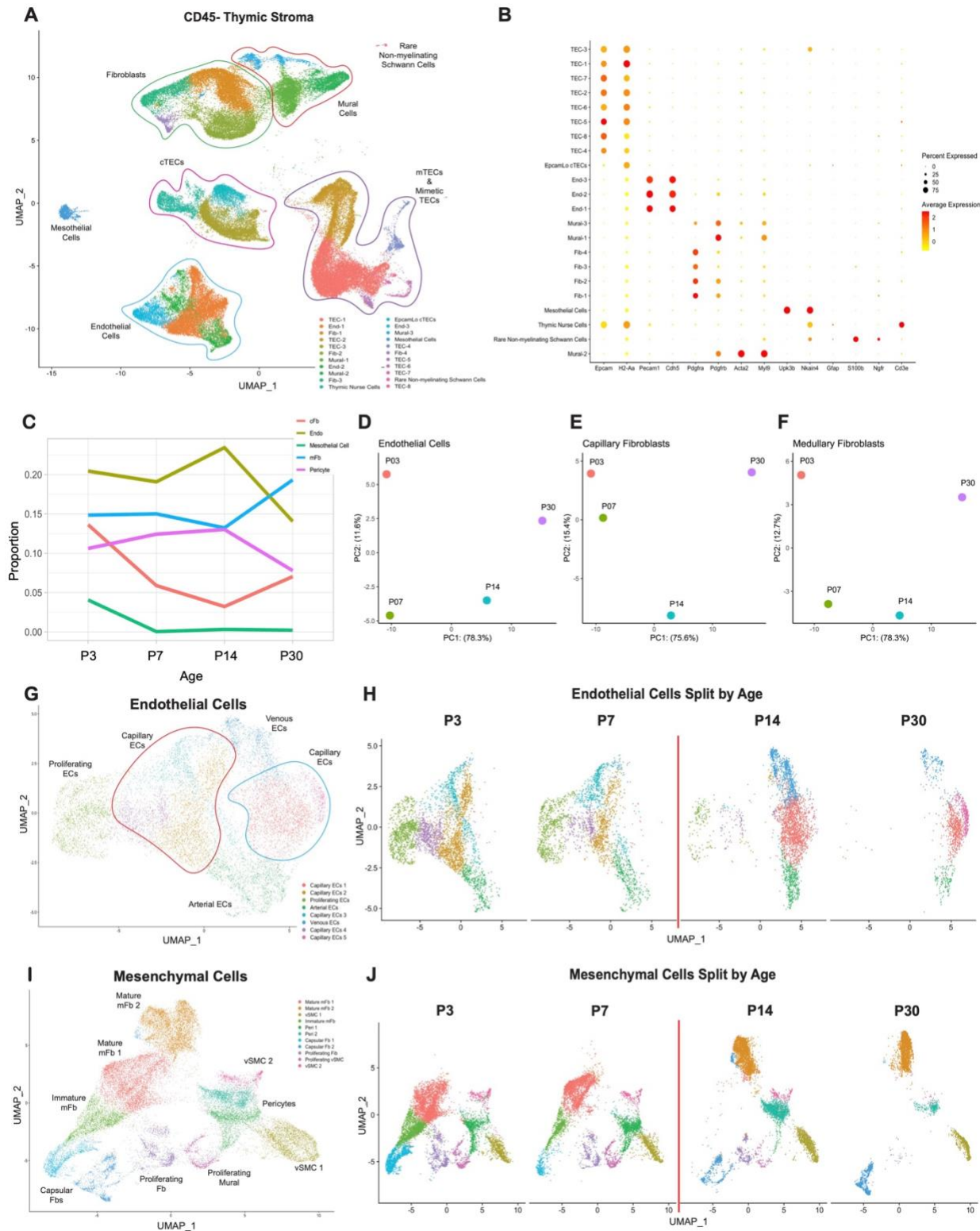


Figure D.2. Endothelial cells and fibroblasts exhibit major transcriptome shift across the perinatal transition point.

(A) UMAP plot visualisation of the CD45⁻ thymic stroma. (B) Dot plot visualisation of the CD45⁻ thymic stroma with canonical marker expressions. (C) Line graph showing proportions of major thymic stroma across time. (D-F) PCA plot visualisation of pseudo-bulked endothelial cells, capillary fibroblasts, and medullary fibroblasts. (G, I) UMAP plot visualisation of subsampled endothelium and mesenchyme. (H, J), UMAP plot visualisation of subsampled endothelium and mesenchyme split by age. P denotes postnatal day.

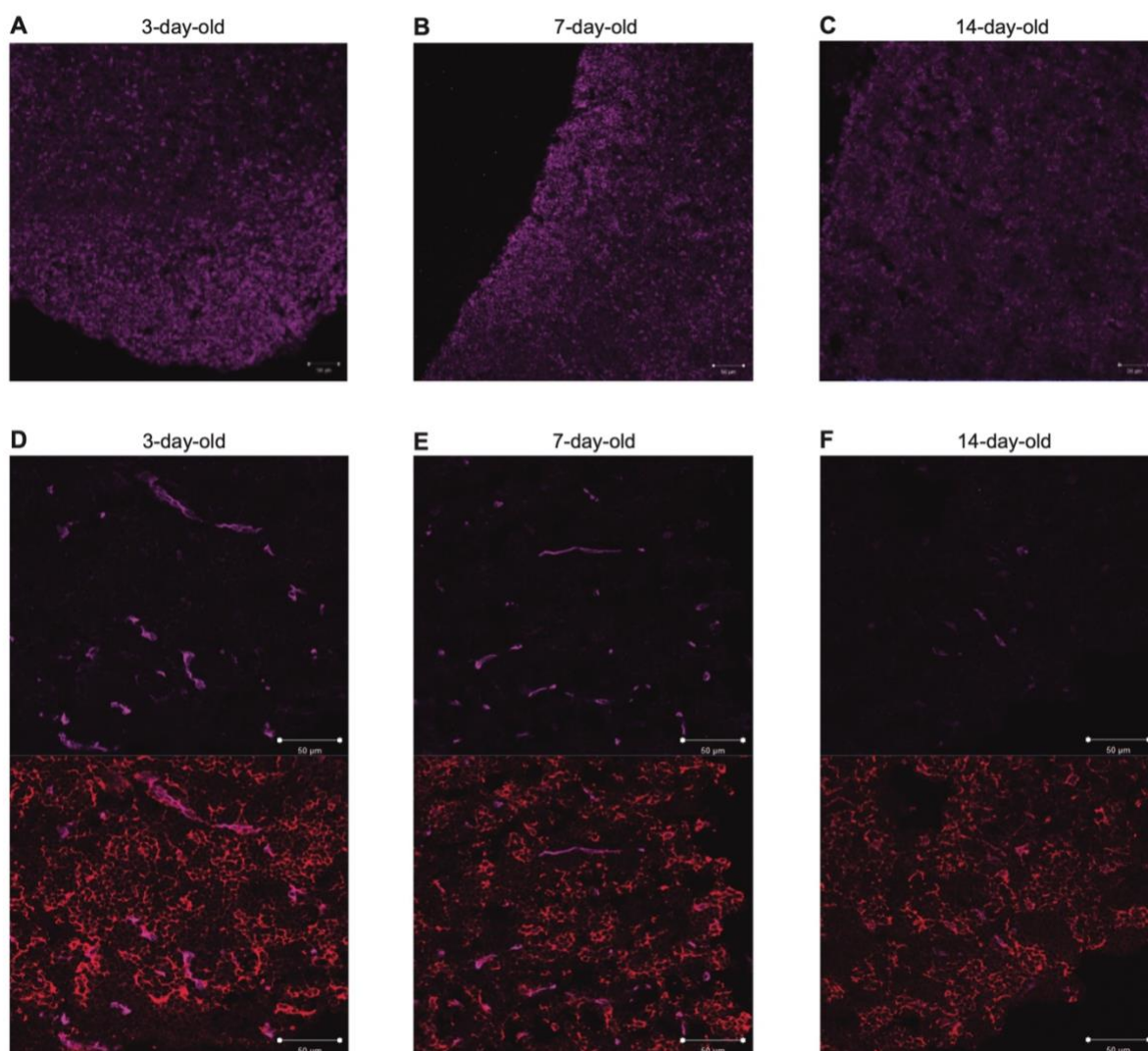


Figure D.3. Fibroblast and endothelial cell distribution across perinatal stages.

(A-C) Immunohistochemistry (IHC) of APOD+ fibroblasts. The density of capsular fibroblasts substantially decreases with age. (D-F) IHC of VEGFR2 (magenta) & CD205 (red). Scale bar = 50µm.

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