Wnt Secretion Proteins Modulate RANKL-Induced Expression of Aire in Thymic Epithelial Cells

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Abstract

Thymic epithelial cells (TEC) are essential for a proper adaptive immune response by regulating thymocyte development and establishing central tolerance. In the thymus, TECs differentially express Wnt proteins, which activate canonical and non-canonical Wnt signaling pathways. Wnt signaling is thought to regulate cell survival, proliferation, and development although the direct molecular mechanisms in TECs have yet to be elucidated. The inducible inhibition of canonical Wnt signaling with Dkk1 leads to a rapid loss of TEC progenitors as well as a decline in mature Aire-expressing mTECs. Therefore, we explore the role of Wnt ligands potentially responsible for stimulating and/or regulating Wnt signaling in TECs by analyzing the genetic expression of various TEC populations in TCF/Lef:H2B/GFP Wnt reporter mice. Here we show that previously uncharacterized populations of postnatal mTECs undergoing canonical Wnt signaling differentially express Wnt proteins and we further identify Wnt-5a and Wnt-5b as possible candidates for regulating Wnt signaling in mTECs. It is well known that developing thymocytes indirectly affect TEC development—in order to study the effects of Wnt stimulation on TECs, we generate fetal thymic organ cultures (FTOC) in which we eliminate the developing thymocytes and instead use exogenous RANKL to drive TEC specific maturation, while simultaneously culture the TECs with different Wnt proteins. Our results show that the Wnt antagonist, Sfrp-1, and the Wnt ligand, Wnt-5b, interfere with RANKL-induced maturation as demonstrated by a reduced frequency of Aire$^+$ fetal TECs. Furthermore, we show two proteins that are hallmarks of thymic development, FoxN1 and Dll4, regulate Wnt-5b genetic expression in vitro. However, further work is needed to determine if Wnt-5b is affecting TEC development through canonical or non-canonical Wnt signaling and whether the modulation is due to an
altering of cell-fate, increased apoptosis, or by the preservation of cells in an undifferentiated state.
Introduction

Overview of T-cell and Thymic Development

In humans, the thymus consists of two semi-identically sized lobes positioned behind the sternum and overlies the pericardium and the great vessels at the base of the heart [39]. The thymus serves as a specialized primary lymphoid organ that recruits T-cell precursors from the bone marrow and supports the differentiation and selection of T-cells that express a functional, self-tolerant T-cell antigen receptor (TCR). Well studied for their essential role in the thymic microenvironment, thymic epithelial cells (TEC) construct branching, 3-D architectural niches in the thymus and create appropriate microenvironments for T-cell development and selection to occur [38]. Most importantly, TECs are critical in the establishment of central tolerance—the immune system’s ability to distinguish self from non-self—for T-cell adaptive immunity [40-42]. In the thymus, expression of the TCR is initiated by the lymphoid specific RAG1/2 proteins in a process known as VDJ recombination [44] and this process results in the production of a diverse repertoire ($10^{14}$-$10^{18}$) of TCRs capable of recognizing the almost infinite array of protein peptides, or antigens, that foreign entities produce. Once in the periphery, following a rigorous selection process that removes cells expressing self-reactive TCRs, mature T-cells wield their TCRs to identify pathogens, like viruses and bacteria, and can stimulate an immune response [43].

Organogenesis of the murine thymus begins by E11.0 when the third pharyngeal pouch (3rd pp) of the endodermal gut begins to pocket outwards [45]. The thymus-specific transcription factor, forkhead box N1 (FoxN1), generally marks the emergence of the thymic epithelium and is essential for proliferation and differentiation of thymic epithelial progenitor cells (TEPC) [45, 46]. This is evidenced by the fact that mice with a genetic mutation in the FoxN1 gene, called
Nude mice, do not develop a competent thymus [45, 47]. Yet, other factors needed for TEC lineage development from the fetal endoderm are poorly understood, as are regulators of TEPC homeostasis and differentiation in the adult thymus [60]. In addition, an assortment of growth and developmental signals are expressed in the early thymus, revealing a complex molecular symphony of signals necessary for appropriate thymic function [38]. Some of these signals—tumor necrosis factor super family (TNFSF) and WNT—will be discussed in more detail.

T-cells develop from multipotent hematopoietic stem cells (HSC) that migrate to the thymus through the cardiovascular system [48]. During embryonic development, HSCs derive from the fetal liver; in adults, they derive from bone marrow [77, 38, 81, 79]. Inside the thymus, the T-cell developmental progress can be tracked through the expression of certain membrane-bound molecules, particularly CD4 and CD8 [49]. When describing thymic organogenesis and development, it is important to include developing thymocytes because their interaction with thymic epithelium is required for correct TEC development. In a process termed cross-talk, the signals thymocytes and TECs give and receive in cell-cell interactions are essential for the development and maintenance of both cell types. One without the other results in an abnormal thymus, interrupted thymocyte development, and increased risk for autoimmunity [50-53].

The thymus can be divided into three architectural compartments: the cortex, the medulla, and the loosely defined border between the cortex and medulla known as the cortico-medullary junction (CMJ). Following chemokine signals, early thymic progenitor (ETP) cells enter into the thymus at the CMJ from the circulatory system [77, 38, 81, 79]. Developing thymocytes are then trafficked through different thymic microenvironments during distinctive steps in their maturation program [38, 48, 49]. T-cell development in the thymus, is an intricate, multi-step process that includes T-cell commitment, proliferation, differentiation, selection, and migration.
Yet, most developing thymocytes (95%) are expunged and do not leave the thymus to become mature effector T-cells [48, 80].

Cortical thymic epithelial cells (cTEC) secrete chemokines to attract ETPs from the CMJ to the cortex. There, ETPs are first induced by Dll4 and IL-7 to differentiate into immature double-negative (DN) thymocytes, which do not express the TCR co-receptors CD4 or CD8 [82, 83, 48, 50]. DN thymocytes are classified by four progressing phases each characterized by the expression of surface markers CD44 and CD25, and during which VDJ recombination takes place [38, 48, 49, 81]. Only a small population of ETPs enters the thymus, so this population undergoes a process of extreme growth during the DN1 and DN2 stages of thymocyte development [17, 84, 85]. By the end of the DN3 stage, the immature thymocyte develops a pre-TCR [81]. Eventually, the thymocyte forms a functional TCR and expresses both TCR co-receptors, CD4 and CD8, known as a double-positive (DP) T-cell [48, 49]. In a process called major histocompatibility complex class (MHC) restriction, the cTECs positively select DP thymocytes by exposing their TCR to a peptide-MHC complex in the context of either MHCI, which is recognized by CD8, or MHCII, which is recognized by CD4 [48, 49, 78]. The positive selection event results in single-positive (SP) thymocytes that exclusively express either CD4 or CD8 [48, 49, 78]. Thymocytes that do not form a functional TCR, and therefore cannot interact with cTECs presenting self-antigens, eventually die off in a process called death by neglect [48, 87, 88].

After positive selection, SP thymocytes increase their expression of the chemokine receptor CCR7 [35]. This prompts their migration to the thymic medulla, which is facilitated by medullary thymic epithelial cells (mTEC) that express CCL21 and CCL19, the ligands for CCR7 [35, 48]. In the medulla, a specialized molecular process called promiscuous gene expression
(pGE) allows mTECs and thymic dendritic cells (tDC) to present tissue-restricted self-antigens (TRA)—antigens intrinsic to other organs, tissues, and cells in the organism—to SP thymocytes [89-93]. Collectively, mTECs are able to express almost all known protein-coding genes [89-93]. SP thymocytes with TCRs that respond too intensely with TRAs are either negatively selected by mTECs and deleted by directed suicide through apoptosis, or are induced to differentiate into regulatory T-cell subsets [89-93].

The responsibility of pGE in mTECs is essential in the establishment of central tolerance. One of the best-studied proteins directing pGE is the nuclear protein autoimmune regulator (Aire), which can up-regulate over a thousand genes in mTECS [89-93]. In humans, a mutation to the Aire gene results in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), a serious autoimmune disease [93]. Likewise, Aire KO mice failed to establish central tolerance and suffer multiorgan autoimmunity [93]. In addition to its critical role in mTEC TRA presentation, Aire is also involved in the production of regulatory T-cells and controls mTEC secretion of XCL1, a chemokine that contributes to the accrual of tDCs [95].

The cortex and medulla are functionally distinct compartments that express a composite of surface, cytoplasmic, and nuclear proteins. Individually, mTECs and cTECs employ unique genetic and molecular programs that effectively govern thymus homeostasis and thymocyte progression. Though, both mTECs and cTECs express many molecules in common (MHC-I, MHC-II, EpCAM-1, CD40, LTβR, FoxN1), they are recognized in flow cytometry and immunohistology by cell-specific protein expression [38]. In general, cTECs are often identified by the expression of K8, K18, β5t, CD205, Cathepsin-L, CCRL1, and Dll4 [48]. While K5, K14, MTS10, Aire, CD80, CD86, RANK, UEA1, Cathepsin-S, and Claudin3/4 are used to identify mTECs [38].
Expectedly, the levels of cell-surface-protein expression change during the development and maturation of TECs, and this has been well studied by flow cytometry analysis. In brief, TEC surface-protein expression by flow cytometer analysis of enzymatically digested thymus preparations routinely defines all TECs as CD45^EpCAM^+ [38]. cTECs and mTECs are further purified by the expression of Ly-51^+ or UEA-1^+ respectively [38]. mTECs are then subdivided into two populations based on the protein expression levels of MHC-II and CD80. Immature mTECs, or mTEC_{lo} cells express UEA-1^+CD80^0MHC-II_{lo} while more mature mTECs, or mTEC_{hi} cells express UEA-1^+CD80^0MHC-II_{hi}. Analysis of the mTEC compartment during embryonic thymus development using CD80 and MHC-II expression showed that mTEC_{lo} cells appear prior to the emergence of mTEC_{hi} cells, suggesting a possible precursor product relationship between these two cell populations [38, 96, 97]. Nonetheless, TEPC lineage to cTECs and mTECs remains unclear. In embryonic mouse thymus, one study identified a single bipotent TEC progenitor capable of generating cTECs and mTECs [60, 61], but other studies identified lineage specific mTEC and cTEC progenitor cells [62-64].

Aire is thought to be a defining marker in mTEC differentiation. Aire expression was only found in a rare population of mTEC_{hi} cells and 5-bromo-2’deoxyuridine (BrdU) experiments showed that Aire^+ mTECs were postmitotic, derived from cycling Aire^- precursors, and induced apoptosis of mTECs [96, 97]. Thus, some argue that Aire expression can also be viewed as a terminal differentiation marker for mTECs [96, 97]. On the other hand, Aire does not act alone in pGE and Aire-independent mature mTECs exist. For example, the transcriptional regulator Fezf2 was shown to drive pGE in mTECs through a pathway independent of Aire and the TRAs regulated by Fezf2 differ from those regulated by Aire [94].
Overview of NF-κB Signaling

TNFSF members have important roles in innate and adaptive immunity, including the generation and maintenance of the thymic microenvironment. Moreover, RANKL, TNFα, and αLTβR all activate NF-κB responsive genes in mTECs in unique and overlapping ways [98]. Briefly, Mammalian NF-κB transcription factors consist five homologous subunits (Rela/p65, c-Rel, RelB, p50/NF-κB1, and p52/NF-κB2), which exist in unstimulated cells as homo- and heterodimers that are held in the cytoplasm by IκB family proteins to prevent translocation to the nucleus [144]. A common feature to all NF-κB proteins is that they include a Rel-homology domain (RHD) that contains a nuclear localization sequence that is involved in dimerization and sequence-specific DNA binding [145, 146]. Immediately upstream from the IκB-bound NF-κB dimers is the IKK complex, which is comprised of two catalytic, IKKα and IKKβ, and one regulatory, IKKγ/NF-κB essential modulator (NEMO) subunits [144]. Several signaling pathways converge to activate the IKK complex, which then phosphorylates NF-κB-bound IκB proteins that target the IκB protein for ubiquitination and degradation by the 26S proteasome by creating a binding site for Skp1-Cullin1-F-box protein (SCF)/β-TrCP ubiquitin ligase complex [144]. The now free NF-κB dimers translocate into the nucleus and activate transcriptional target genes. NF-κB signaling is commonly described as two recognized pathways, the canonical pathway and the non-canonical pathway. The canonical pathway generally depends on NEMO, IKKβ activation, and nuclear translocation of the RelA/p50 dimers to induce target gene expression, while the non-canonical pathway generally depends on IKKα activation and nuclear localization of p52/RelB heterodimers [145].

In the thymus, Aire expression in mTECs depends on cross talk with thymocytes. T-cells that form successful TCRs receive survival signals from the thymic stroma and are induced to
express TNFSF cytokines (RANKL, CD40L, TNFα, and αLTβR) [34, 98]. TNFSF receptors expressed by TECs interact with the cytokines expressed by thymocytes to drive TEC proliferation and differentiation [34, 97, 98]. Double KO transgenic mice for RANK and CD40 resulted in the onset of T-cell mediated autoimmune diseases [99]. RANK and RANKL KO mice exhibited no mature mTECs in the embryonic thymus and a severely reduced number of postnatal mature mTECs [97, 100]. Recently, microarray gene profiles of fetal thymic organ cultures (FTOC)—FTOCs are in-vitro assays used to study the development of thymocytes and/or TECs—individually stimulated with different TNFSF cytokines revealed that RANKL alone was directly and specifically responsible for the expression of Aire in mTECs [98]. The exact molecular mechanisms surrounding RANKL induced Aire expression is incomplete and evidence suggests that both canonical and non-canonical NF-κB signaling pathways are involved; though recent data argues that the canonical NF-κB pathway is responsible for the induction of Aire expression [98, 101].

**Overview of Wnt Signaling**

Wnt proteins play diverse but a crucial role during the development, differentiation, cell migration, and proliferation in vertebrate and invertebrates, and the misregulation of Wnt signaling often leads to cancer and metastasis [29]. In vertebrates specifically, Wnt signaling is known to regulate cell fate, establishment of the dorsal axis, limb development, asymmetric cell division, progenitor-cell proliferation and survival, and long-term memory formation [17]. Moreover, Wnt signaling has also been shown to regulate the maintenance of hematopoietic and epithelial stem cells [17]. Presently, there are 19 known Wnt genes in the human genome and they all encode lipid-modified secreted glycoproteins that usually bind to 10 known, seven-transmembrane receptors of the Frizzled (Fzd) family [17, 139]. It is widely accepted that the
Wnt signaling is separated into three different intracellular pathways: the canonical Wnt or β-catenin-dependent pathway, the planar cell polarity pathway (PCP) or Wnt-Jun N-terminal kinase pathway, and the Wnt-Ca$^{2+}$ pathway. Pathway specificity seemingly comes from a particular combination of Wnt ligands, Fzd receptors, co-receptors, and even intracellular signaling components [29]. Yet, this is poorly understood since the combinations are frequently context, organ, and species specific, and because multiple Wnt ligands or Fzd receptors have redundant roles in activating multiple Wnt pathways.

The most well-known and understood pathway is called the canonical Wnt pathway and involves β-catenin and members of the T-cell factor (TCF)/lymphocyte-enhancer-binding factor (LEF) family [17]. The canonical Wnt signaling cascade exists in two main phases, active and inactive. In the absence of a Wnt ligand binding to its Fzd receptor complex, or inactive phase, cytoplasmic and nuclear β-catenin levels are minimal since it is targeted for degradation by the proteasome through the actions of a destruction complex [17]. The destruction complex is composed of two scaffolding and tumor-suppressor proteins adenomatous polyposis coli (APC) and axis inhibition protein 1 (AXIN1), and the serine/threonine kinase 1 (CSNK1A1) and glycogen synthase kinase 3β (GSK3β) [17]. CSNK1A1 phosphorylates β-catenin on Ser45, and then GSK3β phosphorylates β-catenin on Ser33, Ser37 and Thr41, creating recognition sites for β-transducin-repeat-containing protein (βTRCP) [23], and tagging β-catenin for ubiquitination-dependent proteolysis [19]. Meanwhile, in the nucleus, TCF assembles a transcriptional repressor complex comprised of Groucho/Transducin-like enhancer (GRG/TLE), C-terminal binding protein (CTBP), and histone deacetylases (HDACs) in order to silence Wnt target genes [20].

The canonical Wnt activation is initiated by a Wnt ligand binding to its Fzd receptor complex and results in the cytoplasmic stabilization and accumulation of β-catenin, which
subsequently translocates to the nucleus and coordinates transcriptional activity [17]. Wnt ligands bind to its Fzd receptor and its co-receptor, low density lipoprotein receptor related protein 5 (LRP5) or 6 (LRP6), at the cell membrane to activate the signaling cascade [17]. Dishevelled (DVL) then forms a complex with Fzd/LRP and promotes the phosphorylation of LRP5/6 by CSNK1A1 or GSK3β [21], which recruits AXIN1 to the plasma membrane and away from the destruction complex in the cytoplasm [22]. Thus, the inhibition of β-catenin phosphorylation results in the accumulation of β-catenin in the cytoplasm, which then translocates into the nucleus and associates with the TCF/LEF family of transcription factors to activate β-catenin-dependent responsive genes [24]. Furthermore, both TCF/LEF and β-catenin interact with additional proteins such as bcl9, Pygopus (PYGO), Pontin, and CBP/p300, to initiate transcription [17].

Regulation of the canonical Wnt pathway occurs at many points to ensure proper signaling. Some proteins, such as the secreted Frizzled related protein 1 (Sfrp-1), can act as a decoy receptor and prevent Wnt signaling initiation by binding to Wnt ligands in the extracellular membrane, this results in the unspecific inhibition of both canonical and non-canonical Wnt signaling [140]. Other proteins like, Wnt inhibitory factor 1 (Wif-1) and Dickkopf homologue 1 (Dkk1), specifically inhibit canonical Wnt signaling by binding and blocking the LRP5/6 co-receptors [17, 140]. Negative regulation also occurs in the nucleus by the cell autonomous inhibitor of β-catenin and TCF (ICAT), which can inhibit the nuclear interaction between β-catenin with TCF [25].

Less understood are the two non-canonical Wnt signaling pathways. The PCP pathway results in Jun N-terminal kinase (JNK) activation, which mediates cell position and shape [17]. It is β-catenin-independent and does not include the Fzd co-receptors LRP5/6 or TCF molecules
In this pathway, DVL associates with Disheveled-associated activator of morphogenesis (DAAM), which activates the RAS homologue gene-family member A (RHOA) and Rho-associated coiled-coil-containing protein kinase (ROCK) to facilitate cytoskeletal reorganization [26]. RAC1 is also activated by DVL and both RAC1 and RHOA activate JNK, which leads to changes in cell adhesion and motility [17]. Keeping in mind that individual pathway activation is often context specific, the Wnt ligands Wnt-5a and Wnt-11 are known to activate the PCP signaling pathway through Fzd and DVL in early mouse embryos and likely involves G protein mediation [141, 142].

A more recently recognized non-canonical Wnt pathway, the Wnt-Ca\textsuperscript{2+} pathway, is hypothesized to influence both non-canonical and canonical Wnt signaling [17]. Through a G protein, the Wnt-Fzd complex activates phospholipase C (PLC), leading to the cleavage of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)\textsubscript{P}_2) to inositol triphosphate (IP\textsubscript{3}) and diacylglycerol (DAG), which then activates protein kinase C (PKC) [17]. IP\textsubscript{3} binding to its receptor on intracellular calcium stores leads to an increase of Ca\textsuperscript{2+} ion levels in the cytoplasm [17]. Ca\textsuperscript{2+} ion levels are normally kept low and the increased Ca\textsuperscript{2+} concentration activates the phosphatase calcineurin and several calcium-dependent kinases, including PKC [17]. Increased calcineurin and PKC have a wide variety of effects but often activates the nuclear factor of activated T cells (NFAT) [17]. Interestingly, two well-characterized partners in Wnt-Ca\textsuperscript{2+} pathway initiation are Fzd-2 and Wnt-5a [27, 28], both of which are expressed in TECs.

Currently, Wnt signaling is understood on the assumption that there are three individual pathways, others however, argue against this divisive approach and instead view all three pathways as a single Wnt signaling network [29]. They observed that, \textit{in vivo}, different Wnt pathways simultaneously regulated each other in the same cell type at the same time—PCP
signaling activation of JNK has been reported to inhibit the canonical Wnt signaling pathway in addition to regulating cell movement [17, 29, 143]. They also observed that multiple proteins were repeatedly involved in different Wnt signaling pathways [29]. For example, Wnt-5a, thought to be a well-defined stimulator of the non-canonical Wnt-Ca\(^{2+}\) signaling has been shown to also activate JNK [30]; Wnt-5a also activates β-catenin when in the presence of human Fzd-5 in Xenopus embryos [31] or through mouse Fzd-4 in HEK293 cells [32]. In another study, Wnt-3a has been shown to simultaneously activate canonical and non-canonical pathways in the same cell type [33]. Additionally, the intracellular signaling pathway mediator DVL is involved in β-catenin-dependent signaling, as well as PCP and Wnt-Ca\(^{2+}\) signaling [29]. What is clear is that Wnt signaling is extremely context specific and, stimulation with a certain Wnt ligand does not necessarily produce the same results for different cell types in the same species, or the same cell type in different species, or even in the same cell type in same species but at different time points. Therefore, it is important to view Wnt signaling as a vast, interconnecting network rather than individual pathways because making assumptions about specific roles for certain proteins can lead to invalid conclusions.

**Wnt Signaling and Developing Thymocytes**

Wnt signaling controls cell fate, self-renewal, proliferation, and survival of HSCs in addition to the development and homeostasis of adult epithelial tissues. In mouse and human, a complex patterning of Wnt ligands and Fzd receptors are found in HSCs, lymphocytes, and the accompanying stromal cells of the HSC microenvironments (the bone marrow and thymus). This indicates that numerous immune cell types participate in Wnt signaling and that it occurs in both a paracrine and autocrine fashion. Furthermore, the Wnt pathway plays a major role in the development, proliferation, and survival of lymphocytes [1, 17]. Although some Wnt ligands
have been shown to modulate thymocyte development, there are even more with no known functions detected in thymus.

Thymocytes receive the majority of their Wnt ligands through cross talk with the thymic epithelium [7]. Though thymocytes themselves express a host of Wnt ligands, albeit at low levels [1]. Specific thymocyte responsive Wnt ligands and Fzd receptor combinations have yet to be established. Instead, through a myriad of gain-of-function and loss-of-function transgenic mice, a greater understanding has been made into the regulation of thymocytes by differential expressions of intracellular Wnt proteins [1, 17]. Especially the Wnt-responsive transcription factors TCF1 and LEF1, which are regulated by β-catenin [17, 103, 104]. Transcriptional activation of TCF1 and LEF1 require protein-protein interaction with β-catenin and this mechanism is necessary for full thymocyte development [17, 103, 104].

Intrathymic signals regulate the abundance of β-catenin in a developmentally significant manner and β-catenin directed signaling is highly active in DN proliferation and important for the transition from DN to DP stages [1, 17]. DN thymocytes naturally increase β-catenin expression and decrease expression of inhibitory factors like AXIN1, a hallmark of canonical Wnt signaling activation [1]. In addition, Fzd-6 is predominantly expressed in DN thymocytes while Fzd-5 is predominantly expressed during DP stages, suggesting that differential expression of Wnt receptors may aid in proper thymocyte development. [6]. An inhibitor of the β-catenin TCF/LEF nuclear interaction, ICAT, incompletely blocks thymocyte transition from DN to DP stages [105]; and a dose-dependent inhibition of canonical Wnt signaling by Dkk1 decreases thymocyte growth and ultimately prevents development past the DN1 stage [1, 17]. Furthermore, T-cell specific deletion of β-catenin results in abnormal T-cell development at the DN-DP stage and reduces the number of peripheral T-cells [108]. By the same token, T-cell specific
overexpression of β-catenin upregulates proliferation-associated genes in immature thymocytes and presumably drives their expansion [106, 107]. These studies indicate a prominent role for the β-catenin-Wnt signaling pathway in early thymocyte development and proliferation.

The transcriptional partners of β-catenin also impact normal thymocyte cellularity and differentiation. TCF1 and LEF1 double deficient mice have a complete block in T-cell development at the CD8+ immature single positive (ISP) stage [109]. TCF1 deficient mice diminish DP thymocyte survival and impair T-cell development from the CD8+ ISP stage to DP stage [109, 110]. The interaction between β-catenin and TCF1 regulates the expression of CD4 and both DP and CD4+ SP thymocytes in TCF1 deficient mice express lower levels of CD4 [111]. CD4 expression in thymocytes restores to normal levels β-catenin stabilizes [111]. Furthermore, only wild type TCF1, and not truncated isoforms of TCF1 (which lack the domain necessary for β-catenin interaction), restores CD4 expression in developing thymocytes and improves DP thymocyte survival [111, 112]. In addition, TCF1 deficient mice develop lymphomas, attaching an additional role to Wnt signaling proteins as tumor suppressor genes [114]. Taken together, these reports suggest a significant role for β-catenin-Wnt signaling at multiple stages of thymocyte development.

Intracellular stabilization of β-catenin in thymocytes depend on the paracrine Wnt signals TECs secrete and early on, this was determined in two ways. First, DN thymocytes cultured in the presence of TECs do not show evidence of β-catenin phosphorylation [6]. Once removed from the thymic stromal environment, however, Wnt signaling deactivates and β-catenin is phosphorylated and degraded [6]. Second, soluble Fzd receptors used in FTOCS as decoys to bind to and inactivate Wnt ligands, inhibits thymocyte differentiation by negatively affecting their proliferation [102]. Together this implies that TEC secretion of Wnt ligands is an important
event for thymocyte stabilization of β-catenin, but determining the role of specific signals has been a challenge.

Specific Wnt secreted proteins modulate thymocyte development. Wnt-3a deficient mice mirrors the phenotypes discovered in TCF1 deficient mice [3]. Wnt-3a often activates β-catenin signaling and is thought to mediate TCF dependent transcription in developing T-cells [3, 113]. Wnt-3a deficient FTOCs have fewer thymocytes when compared to WT FTOCs [3]. After long culture periods, the percentage of DP cells in Wnt-3a deficient FTOCs diminishes and there is a developmental block at the CD8+ ISP stage [3]. Additionally, Wnt-3a signaling affects T-cell development in a dose-dependent manner, increasing Wnt-3a expression fivefold in transduced BM stromal cells accelerates T-cell development when compared to control conditions—larger doses inhibit or block development [113]. Similarly, slightly overexpressing a purified human WIF1 (a Wnt secreted antagonist) in FTOCs enhances thymocyte cellularity but larger concentrations decrease cellularity [116]. This suggests that thymocyte sensitivity to certain Wnt secreted proteins is fine-tuned and likely depends on the developmental stage and the intensity of the signal.

Wnt-1 deficient or Wnt-4 deficient mice die at birth [56]. Wnt-1 and Wnt-4 double deficient mice display extremely low fetal thymic cellularity [56]. BM stromal cells transfected with Wnt-4 expands ETPs and DN thymocytes independently of the thymic microenvironment [57]. Consistent with this, the tetracycline-induced loss of Wnt-4 in adult TECs decreases thymic cellularity and disproportionately affects ETPs and DN thymocytes [57]. These data indicate that the differential expression of specific Wnt ligands have an important role in the survival and development of thymocytes in both young and adult thymus.
Finally, non-canonical Wnt signaling also influences the development and survival of thymocytes. Expressed by the thymic epithelium, Wnt-5a is known to frequently stimulate non-canonical Wnt pathways [2, 113]. Wnt-5a deficient mice die at birth and Wnt-5a deficient FTOCs increase the percent and total number of SP T-cells. [2]. The loss of Wnt-5a prevents apoptosis at several stages in thymocyte development through down-regulation of the apoptosis inducing protein—BCL2-Like Protein 4 (BAX)—and the up-regulation of survival signals like the apoptosis regulator BCL-2 (an outer mitochondrial membrane protein that blocks apoptotic death) [2]. The addition of exogenous Wnt-5a reverses thymocyte survival events and promotes apoptosis in immature DP and mature SP thymocytes [2, 113]. Thus, Wnt-5a modifies thymocyte survival via the non-conical calcium Wnt pathway and implies that several types of Wnt signals have significant roles in the establishment of central tolerance [2]. In summary, these manipulations suggest that Wnt signals, of all kinds, have both profound and subtle effects on the expansion, survival, and development of thymocytes.

Wnt Signaling and TECs

TECs are the primary source of Wnt secreted proteins in the thymus, which regulates thymocyte survival and development [7]. Nevertheless, since TECs also express membrane-bound Fzd receptors, many suspect that they can stimulate Wnt signaling cascades in themselves [7]. It is difficult, however, to study the effect of Wnt signaling in TECs independent of cross talk and the indirect influence of developing thymocytes. Few studies have aimed to describe direct relationships between individual Wnt ligands and their Fzd receptors or the transcriptional targets Wnt signaling stimulates in TECs. Some studies suggest an important role for canonical-\(\beta\)-catenin-Wnt signaling in the thymus but seldom considered is the potential impact non-canonical Wnt signaling has on TECs. The expression of several Wnt secreted proteins have
been identified in multiple TEC populations but the results vary (in part due to differing experimental techniques as well as the age, the sex, and the mice used) and most Wnt proteins detected in the thymus have unknown roles.

Wnt-4, however, has an important role in proper TEC development. Wnt-4 expression in TECs has been identified in several studies and at multiple time points during thymus organogenesis, postnatal expansion, and adult homeostasis. Furthermore, Wnt-4 staining co-localizes with EpCAM staining in both human and mouse embryonic TECs suggesting that Wnt-4 expression is evolutionary conserved [162]. In fact, the abundant expression of Wnt-4 in the early thymus highlights the significance of its absence in the aging thymus. By E10.5, the dorsal and ventral aspects of the 3rd pp already both express the Wnt ligands, Wnt-4 and Wnt-5b, but differentially express the Wnt inhibitors Wif-1, Nkd1, Sfrp-2 [14, 116]. This implies that Wnt signaling is spatiotemporally regulated within the 3rd pp and has potential roles in proper patterning and/or differentiation of the epithelium in early thymus organogenesis [116]. In line with this, Wnt-4 transgenic mice have been demonstrated to play a role in embryonic thymus development and migration [134, 56]. The transgenic mice that overexpress Wnt-4 in TECs disrupted TEC differentiation and resulted in abnormal thymus migration; NLK and β-catenin were not required to mediate the Wnt-4-induced signaling pathway that disrupted thymus morphogenesis and suggests that the Wnt-PCP pathway might be involved [134]. Interestingly, Wnt-4 predominantly affected cTECs and reduced fetal cTEC frequency and cell numbers; fetal mTEC numbers were similar to controls (though both cell populations were decreased at later time points when compared to controls) [134]. This implies that Wnt-4 disrupts fetal TEC differentiation primarily through a cTEC dependent mechanism.
Wnt-4 is able to trigger both canonical-\(\beta\)-catenin-Wnt and non-canonical-Wnt singling in a context-dependent manner, and it is the highest expressed Wnt ligand in the 1-month mouse thymus, expressed in both cTECs and mTECs [7]. Furthermore, Wnt-4 stimulates multiple cell populations in the thymus as well as prethymic precursors [117]. Through a PCP-like pathway involving Fzd-6, RAC1, and JNK, Wnt-4 expands hematopoietic progenitors cells derived from bone marrow and fetal liver [117]. Both conventional and conditional Wnt-4 KO mice suppress fetal and postnatal thymic expansion and this mutation causes a disproportionate loss of TEPCs, decreases overall TEC numbers, and alters the medullary-to-cortical TEC ratio [57]. This suggests Wnt-4 can act through a TEC-dependent mechanism [57]. In another study, Wnt-4 deficient mice substantially decrease the number of thymocytes and, Wnt-4 and Wnt-1 double deficient mice reduce the number of thymocytes by as much as 50% without the affecting their pattern of maturation [56]. Wnt-4 transfected BM cells cultured with thymocytes expands ETPs and DN thymocytes and suggests Wnt-4 is also capable of regulating thymocytes independent of TECs [57]. Together these data suggest that Wnt-4 dynamically regulates thymic cellularity and promotes thymopoiesis in multiple cell populations [57].

\(\beta\)-catenin also has an important role in TEC development and its been shown that Wnt-4 and Wnt-1 can each activate the canonical \(\beta\)-catenin-Wnt signaling pathway in TECs [14]. Indeed, canonical \(\beta\)-catenin-Wnt signaling in TECs is a complex and tightly regulated process. Overexpression or inhibition of \(\beta\)-catenin disrupts normal thymus development. Fetal TECs transfected with ICAT, inhibits of \(\beta\)-catenin and reduces the expressions of FoxN1, MHC-II, and IL-7 [162]. A stabilized form of \(\beta\)-catenin, specifically introduced to TECs by a FoxN1 promoter, blocks the early commitment of endodermal epithelia to a thymic fate, the TECs are unable to recruit thymocytes, and this results in abnormal formation and function of the thymus.
Mice with a FoxN1 specific deletion of APC stabilized canonical β-catenin-Wnt signaling and are athymic [118]. Kremen1 (a Wnt antagonist) deficient mice increase canonical β-catenin-Wnt signaling in TEC derived cell lines, reduce the frequency of cTECs and mTECs in the thymus, and alter the thymic epithelial architecture, which lack defined cortical and medullary regions [119]. These data suggest that the stabilization of canonical β-catenin-Wnt signaling in TECs disrupts the thymus harmfully.

Yet, inhibiting canonical β-catenin-Wnt signaling also disrupts the thymus architecture. Double transgenic (tetO-Dkk1) mice in the presence of doxycycline produce high levels of Dkk1 in both mTECs and cTECs, this leads to the inducible inhibition of canonical Wnt, a decrease in TEC proliferation, the reduction of all TEC subsets (including K5+K8+DP TECs thought to contain TEPCs), and premature thymic degeneration [4]. Consequently, because of their role in the creation of developing thymocyte niches, Dkk1 inhibition of β-catenin-Wnt signaling also decreases the number of thymocytes [4]. Taken together, these studies suggest that a precise regulation of canonical β-catenin-Wnt signaling in TECs is indispensable for proper thymus development, and enhancing or diminishing canonical β-catenin-Wnt signaling compromises the integrity of the thymic epithelia.

Mice with a FoxN1 conditional knockout for GPR177, a Wnt-specific cargo receptor required for secretion of Wnt ligands, loose the capability of all Wnt secretion in TECs, including both canonical and non-canonical signals. [7]. Without GPR177, TEC cellularity and thymus size reduces, thymus hypotrophy sets in close to birth, and the number of T-cells in the periphery reduces [7]. These mice increase the ratio of mature mTEC\textsuperscript{hi} to immature mTEC\textsuperscript{lo} cell populations and increase the rate of apoptosis in TECs [7]. Besides that, the mutation does not affect TEC development, chemokine production, or the differentiation of Aire\textsuperscript{+} mTECs [7]. It
does not alter the mTEC/cTEC ratio or thymocyte development, only the thymocyte frequency, which was less due to the reduced TEC population [7]. Other cell populations in the thymus do not compensate for the loss of Wnt secretion [7]. This, along with the phenotypes observed in Wnt-4 deficient mice, argues for a stronger role for Wnt signaling in TEC proliferation and survival rather than in TEC development and differentiation.

Though, the involvement of Wnt signaling in TEC differentiation cannot be ruled out completely. Wnt signals are still present in the thymus of GPR177 deficient mice; tDC, t-fibroblasts, and even T-cells all express a wide array of low-level Wnt secreted proteins [1, 5, 56]. One can argue, that to some degree, these small amounts of Wnt signals contributed and facilitated normal T-cell and TEC development in GPR177 deficient mice [1, 5, 56]. Moreover, Wnt-ligand production by TECs is altered in Aire deficient mice [89]. Wnt-5a, Wnt-7b, and Wnt-8b are all downregulated in the mTEC\textsuperscript{hi} cells of Aire deficient mice when compared to WT mTEC\textsuperscript{hi} cells [89]. Though more conclusive evidence is still needed, this suggests a relationship between Aire and Wnt signaling. Furthermore, this represents possible roles for Wnt signaling in the differentiation of mature mTECs, the late processes of thymocyte development, and/or the emigration of thymocytes from the thymus [89].

Several papers have shown that potential downstream Wnt target genes impact thymus development. K5 driven expression of CCND1/2, a proposed target gene of Wnt signaling and FoxN1 [121, 9], in TEPCs causes thymic hyperplasia, expands TEC numbers, and promotes thymus development [122, 123]. Wnt proteins can regulate STAT3 in ESCs and retinal epithelium [124, 125] and STAT3 signaling promotes the survival and maintenance of mTECs as well as the postnatal development of the thymic medullary regions [126, 127]. RAC1 deficient mice upregulate the expression of c-MYC, another Wnt target gene (though c-MYC is frequently
regulated by other pathways), which results in thymic atrophy [128]. Together, these data suggest that potential downstream genetic targets of Wnt signaling influences TEC development.

**Wnt Signaling, Thymic Senescence and Age-Related Adipose Involution**

Age associated thymic involution, which affects both mice and humans, is characterized by reduced thymic cellularity, size, and weight [65, 66]. The thymus reaches its maximum size and T-cell output during adolescence; afterwards, it shrinks and T-cell production sharply declines [65, 66]. This negatively impacts the function of peripheral T-cell populations, severely compromises the immune system’s response to newly encountered antigens, and increases the risk of autoimmunity [36, 9]. Attractively, sex-steroid signaling was believed to have an important impact on thymic involution since castration studies of male mice display rapid regrowth of the thymus [8]. Influential findings, however, demonstrate that castration induced regeneration of the thymus is temporary and that immune dysfunction persist [11].

Instead, FoxN1, the crucial transcription factor required for proper TEC development, has an undeniable impact on thymus involution. A proportional amount of FoxN1+ TECS and lymphoid progenitor cells decrease with age; this results in the degeneration of the thymus, the disruption of normal TEC architecture, and adipocyte development within the thymic and hematopoietic niches [36, 65, 66]. Retinoblastoma (RB) inactivation prevents thymus involution through the upregulation of FoxN1 via the E2F transcription factors [37]. Conclusively, inducible FoxN1 expression in aging TECs vigorously regenerates the thymus so that it mimics the adolescent in both thymic stromal architecture and gene expression profiles, and increases thymopoeisis and T-cell output [9].

This is significant because Wnt signaling has been proposed to induce the expression of FoxN1 in the thymus [14, 162]. E15 murine embryonic lobes, following thymocyte-depletion
achieved by deoxiguansine-treatment, and incubated in the presence of a Wnt-4 secreting transgenic cell line increases the expressions of FoxN1, MHC-II, and IL-7 [167]. cTEC and mTEC cell lines transfected with Wnt-4 and Wnt-5b upregulates FoxN1 but transfection with Wnt-1 in the same cell lines do not [14]. Though frequently cited, this relationship is still poorly understood in vivo, and it is not known if the regulation is direct or indirect. For instance, a recent publication reports that Wnt-4, along with several other Wnt signaling proteins—AES, Kremen1, and Fzd-5—are possible FoxN1 target genes [15]. In line with this, induced FoxN1 expression in the aged thymus appears to rescue the expression of Wnt-3a, Wnt-4, and Wnt-5b in cTECs; and the expression of Wnt-7a, Wnt-10a, and Wnt-11 in mTECs [9]. This suggests that FoxN1 induces Wnt signaling and/or there are multiple feedback loops or undefined mechanisms involved in the regulation of FoxN1 and Wnt-4. Nevertheless, the loss of FoxN1 expressing TECs reduces the thymic functional capacity and there is a well-reported correlation between Wnt-4 and FoxN1 decreased expression in aged TECs [5, 9-10, 54-59].

Wnt-4 and Wnt-5b might regulate, or be regulated by, FoxN1 in early TECs but they also have additional roles in postnatal expansion, adult TEC homeostasis and age-related thymus involution [54-59, 9, 11]. Data sets of transcriptional profiles of purified thymocyte and thymic stromal subsets from mice 1, 3, and 6 months of age reveal that Wnt-3a and Wnt-4 is downregulated with age in mTEC\textsuperscript{lo} cells and cTECs, while Wnt-10a is upregulated with age in mTEC\textsuperscript{hi} cells [5]. FoxN1 is downregulated 2-fold when comparing 1month to 6month old mTEC\textsuperscript{lo} cells and cTECs [5]. Wnt-5b, which may contribute to age-related thymic involution and adipogenesis, increases expression with age in t-fibroblasts, mTECs, and cTECs [5, 9, 11]. One drawback to this investigation is that they did not look at late stages of thymic involution (past 6
months) [5]. Still, these data suggest that certain Wnt signaling members, along with FoxN1, are deregulated early in thymic involution [5].

In line with this, several studies implicate canonical β-catenin-Wnt signaling in thymic involution. First, postnatal disruption of TECs by inducible overexpression of the Wnt inhibitor Dkk1 exhibits reversible thymic atrophy [4]. Second, the production of extracellular Wnt inhibitors Sfrp-1 and Sfrp-2 increases with age in humans [55]. Third, Wnt receptors Fzd-4, Fzd-6, and Fzd-8 have all been shown to increase expression in the aged thymus, though their exact role is unclear [10, 55]. Finally, overexpressing Wnt-4 prevented the upregulation of adipose-related differentiation markers like LAP2a in dexamethasone induced thymus senescence [58-59].

Wnt signaling is an important regulator of adipogenesis and accordingly, aged-related adipogenesis of thymic stromal cells likely involves Wnt signaling. In support of this the treatment of pre-adipocytes with Wnt signaling inhibitors Sfrp-1, Sfrp-2, Sfrp-3, Dkk1, and Wif-1 trigger spontaneous differentiation into adipocytes [76]. Furthermore, Wnt4 expression, which is weakened in aged TECs, is inversely correlated with LAP2a expression [54-59]. LAP2a, strongly expressed in aged thymic fibroblasts and stromal cells, is thought to initiate epithelial-mesenchymal-transition (EMT), a primary cause for cellular transformation into pre-adipocytes [54]. Wnt-5a/Wnt-5b signaling through the Fzd-2 receptor also regulates EMT in numerous metastatic cancers [75]. Moreover, LAP2a is synthesized in the cytoplasm before being transported to the nucleus by a PKC–dependent mechanism [74]. PKC, involved in non-canonical Wnt signaling, also increases expression in the aged thymus [10] and several studies show that Wnt-5a regulates PKC expression and function in lung development and cancer [70-73].
PPARg, a key transcription factor for pre-adipocyte differentiation is upregulated by LAP2a, and PPARg expression also increases in the thymus with age [69, 54]. Indeed, constitutive PPARg activation induces ectopic adipogenesis and promotes age related thymic involution [36]. This is especially significant because adipocyte differentiation assays show regulation of PPARg by Wnt-5a, Wnt-4, and Wnt-5b [67, 68]. This suggests that Wnt signaling has a key part in the age-related upregulation of LAP2a, PPARg, and consequent adipogenesis [5, 9, 11]. Combined with evidence that the expressions of Wnt-4 and Wnt-5b are deregulated with age in the thymus, this suggests that Wnt signaling has a significant, if still obscure, role in thymic senescence and age-associated involution.
**Background and Summary**

Prior publications in our lab have demonstrated that proper regulation of Wnt signaling is important to the maintenance of the postnatal thymus [4, 119]. The inducible overexpression of Dkk1, which inhibits canonical Wnt signaling, in K5-expressing TECs resulted in rapid thymic degeneration marked by decreased frequencies of all TEC subsets, including that of Aire$^{+}$ mTECs [4]. In addition, Kremin1 is required for Dkk1 mediated inhibition of Wnt signaling and Kremin1 KO mice displayed both, increased canonical Wnt signaling in TECs and reduced frequencies of cortical and medullary epithelial subsets [119]. Taken together, these data suggest that the inhibition or overexpression of canonical Wnt signaling both result in the disruption of the thymic stromal architecture [4, 119]. Thus, our aim was to further explore the role and regulation of Wnt signaling in the postnatal thymus using TCF/Lef:H2B/GFP transgenic reporter mice to identify, isolate, and study TECs undergoing active canonical Wnt signaling. We reported the genetic expression patterns of mTECs undergoing active canonical Wnt signaling and identified Wnt-5a and Wnt-5b—both of which were regulated by the essential protein for thymus development, FoxN1—as potential players in this process. TECs are the primary source of the Wnt secretion proteins expressed in the thymus and we reasoned that the Wnt proteins expressed by TECs would be able to regulate their development. To this end, we examined hematopoietic depleted FTOCs stimulated with RANKL to induce Aire expression, in addition to stimulation with various Wnt proteins to regulate Wnt signaling. Stimulation of FTOCs with Wnt-5b and Sfrp-1 resulted in reduced mTEC maturation and decreased frequency of Aire$^{+}$ fetal TECs. Our results suggest that regulation of Wnt signaling induced by Wnt-5b, which was expressed by the mature medullary thymic epithelium, can operate in the thymus to govern TEC differentiation by modulating RANKL-induced expression of Aire$^{+}$ mTECs.
Methods

Ethics Statement:

All mice used in this study were bred and maintained at the City college of New York animal facility and all experiments were performed with approval from the City College of New York Institutional Animal Care and Use Committee. The animal care facility at the City College of New York is certified at both the State and Federal levels. The CCNY animal assurance number is A3733-01.

Mice:

C57BL/6 mice were obtained from the Jackson Laboratory (Bay Harbor, ME). Dr. Anna-Katerina Hadjantonakis (Sloan-Kettering Institute) generously provided TCF/Lef:H2B/GFP mice which have been previously described [138].

Thymic Stromal Cell Preparation and CD45 Depletion:

To generate single cell suspensions for TEC staining, thymi were removed by surgical dissections. The thymic lobes were cleaned; all the blood and fat were removed under a dissection microscope and washed cold PBS. Thymi were cut into 3-4 pieces per lobe and placed in a 50mL falcon tube with 20mL PBS. Thymi were mechanically agitated by being repeatedly passed through a glass pipette until the PBS solution was cloudy and the thymic tissue moved freely through the pipette. The thymic tissue were then digested with a cocktail of DNaseI (50 µg/mL) (7900; StemCell) and collagenase D (1.5 mg/mL) in HBSS media + 5% FBS for 3x15min at 37°C with agitation. Collected supernatants after each round of digestion were washed with HBSS media + 5% FBS + EDTA (EDTA was added to a final concentration of 5mM and the pH was readjusted to 7.3-7.4) and centrifuged at 1500RPM for 5min at 4°C. Resuspended cells were passed through a 100µm mesh filter before being counted with a
hemocytometer. CD45 purified anti-mouse (103102; Biolegend) was then added to the cell suspension at a concentration of 0.5µL per 1x10⁶ cells and incubated on ice for 10 minutes before being washed and centrifuged at 1500RPM for 5min at 4°C. The cell pellet was resuspended in 1mL for every 1x10⁷ cells HBSS media + 5% FBS + EDTA for every 1x10⁷ cells and sheep anti-rat dynabeads (11035; LifeTech) was added at a concentration 25µL per 1x10⁷ cells. Mixture was gently agitated at 32°C for 20min. CD45 positive cells were removed with a magnet and the remaining cells were centrifuged at 1500RPM for 5min at 4°C. Cells were resuspended in 1mL HBSS media + 5% FBS + EDTA, counted, and adjusted to a concentration of 100µL per 1x10⁶ cells in preparation of staining for flow cytometry.

**Flow cytometry:**

Purified TEC suspensions were incubated with CD16/CD32 Fc block (BD Bioscience) to block Fc receptors prior to primary staining. Staining of primary antibodies were performed as indicated in Table 1 on ice, in the dark, for 30min with gentile agitation. Secondary antibodies were added and cells were incubated for an additional 20min on in the dark with gentile agitation. After washing, the cells were resuspended in HBSS without FBS for FACS analysis at a concentration of 2 million cells per 1mL.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Conjugation</th>
<th>Working Dilution</th>
<th>CAT# &amp; Company</th>
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<tbody>
<tr>
<td>CD45</td>
<td>APC Cy-7</td>
<td>(1:1)</td>
<td>557659; BD Bioscience</td>
</tr>
<tr>
<td>MHCII</td>
<td>APC</td>
<td>(1:40)</td>
<td>17532182; eBioscience</td>
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<tr>
<td>UEA1</td>
<td>Biotin</td>
<td>(1:5)</td>
<td>B-1065; Vector Labs</td>
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<tr>
<td>EpCAM</td>
<td>PE Cy-7</td>
<td>0.5 µl</td>
<td>25579180; eBioscience</td>
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<tr>
<td>CD80</td>
<td>Pacific Blue</td>
<td>7µl</td>
<td>104725; Biolegend</td>
</tr>
<tr>
<td>Strepavidin</td>
<td>Per CP Cy-5.5</td>
<td>1µl</td>
<td>45431782 eBioscience</td>
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Data acquisition and cell sorting was performed using a FACS Aria (BD Bioscience). Correct gating was determined by Fluorescence Minus One (FMO) controls. FACS data was analyzed using Flow Jo software (Tree Star).

**RNA Isolation:**

Total RNA was isolated from sorted EpCAM⁺ medullary thymic epithelial cells or cultured cells using TRIzol LS reagent (10296010; Invitrogen) according to manufacture’s guidelines. Further purification of RNA was achieved according to RNAeasy plus micro kit (74034; Qiagen) protocol and was subjected to gDNA eliminator spin columns. RNA was eluted with preheated (55°C) RNASE-free water and was analyzed by nanodrop. RNA was either, stored and combined in -80°C for up to one month for ethanol precipitation or directly synthesized into cDNA for qRT-PCR.

**qRT-PCR:**

Equal amounts of RNA were denatured in the presence of random hexamers (N808-0127; Applied Biosystems) and dNTPs at 65°C for 5 minutes. The denatured RNA was then used to synthesize cDNA using 5X buffer (y02321; Invitrogen), DTT (y00147; Invitrogen), SuperScriptIII (18080-044; Invitrogen), and RNase inhibitor (1077-019; Invitrogen) at 25°C for 10 minutes followed by 50°C for 50 minutes and 85°C for 5 minutes. The synthesized cDNA was subsequently used to preform quantitative real-time PCR using TaqMan universal master mix (4369016; Applied Biosystems) using the following TaqMan assay probes obtained from Applied Biosciences: 18srRNA (4333760), Foxn1 (Mm00433948_m1) EpCAM (Mm00493214_m1), DKK1 (Mm00438422_m1), Aire (Mm00477461_m1), Dll4 (Mm00444619_m1), Wnt-5a (Mm00437347_m1), Wnt-5b (Mm01183986_m1). Applied
Biosystems instrument default cycling protocol was used to perform qRT-PCR using a 7500 RT-PCR system.

**RNA Precipitation:**

RNA samples were defrosted on ice and measured by nano-drop before being pooled together. The total RNA sample volume was measured and 10% of total RNA volume of sodium acetate was added to the sample. 100% ethanol at 2.5x the sample volume and 1-2 µg/µl glycogen for visualization of RNA pellet were also added to the sample. The sample was vigorously mixed and placed in -80°C overnight. The next day, the sample was centrifuged at full speed for 20min in 4°C. The sample was aspirated and the pellet was washed twice in 250µl of 70% ethanol. The ethanol was aspirated and the RNA pellet was air dried in an Rnase-free environment for 10min. Rnase-free water was added to the pellet and incubated for 10min. The RNA was mixed by micropipette and analyzed by nanodrop.

**PCR Arrays:**

Changes in expression of Wnt singling pathway-related genes were detected by using RT² Profiler Mouse Wnt Signaling Pathway PCR Array (PAMM-043Z; Qiagen). Each 96-well plate contained 84 Wnt signaling related genes, one genomic DNA quality control, three reverse transcription controls, three PCR controls and five housekeeping genes including Actb, B2m, Gapdh, Gusb, and Hsp90ab1 (B2m was excluded from analysis because of variability between replicates). Total RNA was extracted from the previously described four distinct mTEC cell populations (mTEC<sub>lo</sub>Wnt<sup>-</sup>, mTEC<sub>lo</sub>Wnt<sup>+</sup>, mTEC<sub>hi</sub>Wnt<sup>-</sup>, and mTEC<sub>hi</sub>Wnt<sup>+</sup>) and RNA from at least three independent experiments (where two mice were used in each experiment) were isolated, pooled together, and precipitated, as explained above. Two PCR Array Wnt plates were generated for each mTEC cell population. For each plate, 500ng of RNA was synthesized into
CDNA with RT² First Strand Kit (Qiagen). RT² SYBR Green qPCR Master Mix (Qiagen) was used for the reaction following manufacture’s instructions. Amplification and real-time analysis were performed with Applied Biosystems instrument 7500 RT-PCR system and Ct values were cutoff after cycle 35. Each plate used for analysis passed all three built-in quality controls demonstrating efficiency of transcription, that no PCR contaminants were detected, and that no genomic DNA was detected. Analysis of PCR Array results and quality control was performed using the Qiagen GeneGlobe Data Analysis Center web resource (http://www.qiagen.com/us/shop/genes-and-pathways/technology-portals/qrt-pcr-for-mrna-expression/data-analysis/~link.aspx?_id=93C80B99536C4FCDBD1E5FC0759F1324& _z=z).

**Fetal Thymic Organ Culture:**

Fetal thymic lobes were harvested from E14.5 fetal C57BL/6J mice according to the protocol previously described (132). Lobes were cultured with RPMI 1640 (GIBCO) supplemented with 10% FBS (GIBCO), 2mM L-glutamine, 1% glutamax, 1% gentamycin, 1% nonessential amino acids, 1% sodium pyruvate, and 0.1% 2-ME, and depleted of hematopoietic cells with 1.35 mM 2`-deoxyguanosine (2-dG) for five days in 6-well transwell culture plates on polycarbonate isopore filter membranes (0.8µm 13mm) (Fisher Scientific) at 37°C in a humidified atmosphere of 5% CO₂ in air. On the sixth day, fetal thymic lobes were transferred to fresh medium and cultured for four days with either complete RPMI alone, RANKL (1 µg/mL) alone (Biolegend), RANKL (1 µg/mL) + Recombinant Mouse sFRP-1 (1 and 0.5 µg/mL), RANKL (1 µg/mL) + Recombinant Mouse Dkk-1 (1 and 0.5 µg/mL), RANKL (1 µg/mL) + Recombinant Mouse Wnt-5a (1 and 0.5 µg/mL), RANKL (1 µg/mL) + Wnt-5b (1 and 0.5 µg/mL), RANKL (1 µg/mL) + Wnt-4 (1 and 0.5 µg/mL) (R&D Systems). Fetal lobes were replenished with media + RANKL + recombinant proteins, where appropriate, every two days.
After the nine-day incubation period, fetal thymic lobes were embedded in OCT and snap frozen in preparation of cryostat sections. At least three lobes were used for each experimental condition.

**Plasmid Transformation, Transfection, and Transduction into TMSC-710 Cells:**

Lentiviral plasmid constructs of Dll4 (EX-mM21581-LV215; GeneCopeia) and FoxN1 (EX-mM03018-Lv214; GeneCopeia) were transformed into competent GCL-L3 E. coli cells (SK300-10) and purified DNA plasmids were isolated from the GCL-L3 E. coli cells according to company instructions with a Plasmid Midi Kit (Qiagen). The Lenti-Pac HIV Expression Packaging Kit (HPK-LvTR-20; Genecopia), which included a GFP positive control, and the purified DNA plasmid constructs containing Dll4 and FoxN1 were used to co-transfect HEK-293 cells according to company guidelines. Infectious lentiviral particles were harvested from the supernatant of HEK-293 cells 48hrs after transfection and immediately added to semiconfluent monolayers of TMSC-710’s. After a few days of culture, successfully transduced GFP+ and mCherry+ cells were isolated with FACS, and stable TMSC-710-CoGFP (empty vector transduced), TMSC-710-FoxN1 (FoxN1-transduced), and TMSC-710-Dll4 (Dll4-transduced) cell lines were generated. Cell lines were cultured with thymic stromal culture medium, MEM alpha (GIBCO) containing 10% FBS, penicillin-streptomycin, and gentamycin supplemented with recombinant human LIF (10 ng/mL), recombinant mouse EGF (50 ng/mL) and recombinant bFGF (20 ng/mL) (Invitrogen).

**Immunofluorescence:**

FTOC lobe tissues were embedded in OCT medium (Fisher); snap frozen and sectioned (7µm) using a Leica CM1950 Cryostat. Sections were air dried on bond-rite slides and then fixed in 4% paraformaldehyde followed by 100% ice-cold acetone. Sections were washed with 1%
PBS and blocked with blocking buffer (5% normal donkey serum in 1% BSA, 0.1% Triton-X) for 10 minutes. Sections were washed after blocking buffer was applied. Sections were incubated with appropriately diluted primary antibodies: AIRE (Santa Cruz Laboratories), DEC 205 (138202; Biolegend), and UEA-1 Fluorescein (Vector Laboratories) in blocking buffer in a 4 degree chamber overnight at 4°C. Then, slides were washed with 1% PBS followed by incubation with secondary reagents diluted in blocking buffer in humidified chamber for 60 minutes at 37°C: TRITC Donkey anti-rat (712-025-150) and Alexa 647 Donkey anti-rabbit (711-605-152) (Jackson Immuno Research). The slides were then mounted with ProLong gold anti-fade reagent with DAPI (Invitrogen).

**Quantification of number of Aire\(^+\) TECs using confocal microscopy:**

Frozen sections of fetal thymic tissue were prepared and stained as described above. Images were acquired using Zeiss LSM710 confocal microscope and analyzed using LSM software (Zeiss). Confocal images were prepared by scanning each fluorochrome independently with a 20X lens. For each image, a representative region overlay was manually drawn around the thymic tissue and the area for this overlay was automatically calculated using LSM software. The number of Aire\(^+\) UEA-1\(^+\) nuclei was manually counted within each measured representative region overlay for each image. DAPI staining was used to confirm that each fluorescent spot counted was indeed a nucleus. Sections were prepared from throughout the entire lobe to ensure a comprehensive overview of each lobe. At least 3 lobes were analyzed for each condition and a minimum of 15 independent calculations derived from 45 sections was used to calculate the mean and pooled standard deviation of the number Aire\(^+\) UEA-1\(^+\) TECs for each condition (i.e. 5 independent calculations derived from 15 sections was used to calculate the mean and pooled standard deviation of the number Aire\(^+\) UEA-1\(^+\) TECs for each lobe).
Statistical Analysis:

Statistical analysis was performed as indicated where there were at least three independent experiments using Student T-test.
Results

Characterization of mTECs derived from TCF/Lef:H2B/GFP Wnt reporter mice demonstrate canonical Wnt signaling

β-catenin-dependent or canonical Wnt signaling in the thymus of TCF/Lef:H2B/GFP Wnt transgenic reporter mice have previously been identified [138]. The TCF/Lef:H2B/GFP Wnt transgenic reporter mouse has six copies of a TCF/Lef responsive element and a hsp68 minimal promoter in front of a fluorescent protein fusion comprising human histone H2B to GFP [138]. Nuclear-β-catenin and its transcriptional co-activators, TCF/Lef, interacts with the TCF/Lef responsive elements to initiate GFP expression and this model is used to visualize cells undergoing active canonical Wnt signaling at the single-cell level in vivo. Since proper β-catenin-dependent signaling in TECs is crucial for proper thymus development [134], we aimed to characterize the cell populations undergoing canonical Wnt signaling pathway in the murine adult thymus of three-month-old male TCF/Lef:H2B/GFP Wnt reporter mice.

We observed significant canonical Wnt signaling in the thymic medulla by immunofluorescent microscopy (Fig. 1A) and wanted to quantify the GFP reporter expression by flow cytometer analysis (Fig. 1B). Briefly, thymi harvested from three-month-old male TCF/Lef:H2B/GFP Wnt reporter mice were enzymatically digested into a single cell suspension before being partially depleted of CD45<sup>+</sup> cells by magnetic beads. The remaining cell suspension was stained with fluorescent antibodies (CD45, EpCAM, MHC-II, UEA1, and CD80) to separate TECs from remaining thymocytes and to purify mature and immature TEC populations. CD45<sup>-</sup> EpCAM<sup>+</sup>MHC-II<sup>+</sup> cells are defined as TECs (Fig. 1B). After gating on TECs, further analysis with UEA-1 allowed separation of EpCAM<sup>+</sup>UEA1<sup>+</sup> mTECs. The mTEC population can be further subdivided, immature mTECs (mTEC<sub>lo</sub>) are defined as MHC<sub>lo</sub>CD80<sup>-</sup> and more mature
mTECs (mTEC\textsuperscript{hi}) are defined as MHC\textsuperscript{hi}CD80\textsuperscript{+} (Fig. 1D). TECs that were CD45\textsuperscript{+}EpCAM\textsuperscript{+}MHC-II\textsuperscript{+}UEA-1\textsuperscript{−} contain a population of cTECs (Fig. 1C).

TECs that expressed canonical Wnt signaling are defined as Wnt\textsuperscript{+} and TECs where canonical Wnt signaling was undetected are defined as Wnt\textsuperscript{−}. The GFP reporter activity in mTECs showed that canonical Wnt signaling is differentially expressed in both mTEC\textsuperscript{hi} and mTEC\textsuperscript{lo} cell populations. This revealed previously uncharacterized subsets of mTEC populations that were undergoing active canonical Wnt signaling and allowed for the identification and analysis of four unique mTEC populations—mTEC\textsuperscript{lo}Wnt\textsuperscript{−}, mTEC\textsuperscript{lo}Wnt\textsuperscript{+}, mTEC\textsuperscript{hi}Wnt\textsuperscript{−}, and mTEC\textsuperscript{hi}Wnt\textsuperscript{+} (Fig. 1E). The fluorescent intensity of the GFP reporter and the proportion of cells that were Wnt\textsuperscript{+} were consistently greater in the mTEC\textsuperscript{hi}Wnt\textsuperscript{+} subset when compared to mTEC\textsuperscript{lo}Wnt\textsuperscript{+} (Fig. 1E). Most of the immature mTEC\textsuperscript{lo} population, ~73%, were Wnt\textsuperscript{−} and roughly 15% were Wnt\textsuperscript{+}. In contrast, only ~48% the mTEC\textsuperscript{hi} population was Wnt\textsuperscript{−} and over 43% were Wnt\textsuperscript{+} implying a greater frequency of canonical Wnt signaling activity in the mature subset (Fig. 1E). To a lesser degree, we also observed differential expression of canonical Wnt signaling in the cell population that contains cTECs (Fig. 1C). To substantiate these results, thymi isolated from C57BL/6 mice, which do not express endogenous GFP in any cell type, were used as a negative control for the TCF/Lef:H2B/GFP Wnt reporter activity. Using the same single cell suspension and antibody staining protocols, the mTECs derived from C57BL/6 mice were found negative for GFP reporter activity (Fig. 1F).

We sought to collect and study the mTEC populations described above for downstream experiments by a florescent activated cell sorter (FACS). To assess the accurate collections of mTECs in three-month-old male TCF/Lef:H2B/GFP Wnt reporter mice, we isolated RNA from mTEC\textsuperscript{lo} and mTEC\textsuperscript{hi} populations for qRT-PCR analysis of known mature mTEC markers: Aire,
Dkk1, FoxN1 and EpCAM (Fig. 1G). As expected, genetic expression of all four mature mTEC markers was detected in both cell populations. The genetic expression levels of Aire and Dkk1 were found to be significantly upregulated (P < 0.05) in mTEC\textsuperscript{hi} when compared to mTEC\textsuperscript{lo} and this is consistent with previous findings [4, 5]. The genetic expression levels of FoxN1 and EpCAM trended higher in mTEC\textsuperscript{hi} when compared to mTEC\textsuperscript{lo} and while this is also consistent with previous reports [137], it was not statistically significant. These qRT-PCR results validate the accurate collection of mTEC\textsuperscript{hi} and mTEC\textsuperscript{lo} cells for downstream applications.

**mTECs derived from TCF/Lef:H2B/GFP Wnt reporter mice differentially express Wnt signaling proteins**

To investigate which genes in the Wnt signaling pathway was active in mTEC Wnt\textsuperscript{+} cells, we used a Wnt signaling PCR array that simultaneously measures the genetic expression of 84 genes in the pathway. RNA from sorted mTEC\textsuperscript{lo}Wnt\textsuperscript{−}, mTEC\textsuperscript{lo}Wnt\textsuperscript{+}, mTEC\textsuperscript{hi}Wnt\textsuperscript{−}, and mTEC\textsuperscript{hi}Wnt\textsuperscript{+} populations from at least three independent experiments were isolated, pooled together, and precipitated. Equal amounts of RNA were then used to synthesize cDNA and two Wnt PCR array plates were generated for each mTEC population.

We discovered that Wnt ligands and Fzd receptors were differentially expressed in mTEC\textsuperscript{hi} and mTEC\textsuperscript{lo} when these populations were separated by canonical Wnt signaling. In mTEC\textsuperscript{lo}, we found that mTEC\textsuperscript{lo}Wnt\textsuperscript{−} was associated with increased expression of Fzd-2, Fzd-3, Fzd-7, and Wnt-4 as compared to mTEC\textsuperscript{lo}Wnt\textsuperscript{+} (Fig. 2B). The increase of Wnt-4 expression in mTEC\textsuperscript{lo} compared to mTEC\textsuperscript{hi} has been reported before [5, 6, 7], but here we show that within the mTEC\textsuperscript{lo} population Wnt-4 is differentially expressed based only on canonical Wnt activity. Additionally, we found that mTEC\textsuperscript{lo}Wnt\textsuperscript{+} was associated with increased expression of Fzd-1, Fzd-6, and Wnt-5a as compared to mTEC\textsuperscript{lo}Wnt\textsuperscript{−} (Fig. 2B). In mTEC\textsuperscript{hi}, we discovered that
mTEC^{hi}Wnt^{−} was associated with increased expression of Fzd-1, Fzd-6, and Fzd-7 as compared to mTEC^{hi}Wnt^{+} (Fig. 2A). mTEC^{hi}Wnt^{+}, on the other hand, was associated with increased expression of Fzd-3, Fzd-5, and Wnt-5b as compared to mTEC^{hi}Wnt^{−} (Fig. 2A). Together, these results argue that within both the mTEC^{lo} and mTEC^{hi} population there is differential expression of secreted Wnt ligands and membrane-bound Fzd receptors.

In addition to secreted Wnt ligands and their membrane-bound Fzd receptors, the Wnt signaling pathway involves numerous cytoplasmic, nuclear, and secreted proteins. We report the results for these other genes tested by the PCR Array Wnt plate for mTEC^{lo} (Fig. 3A) and for mTEC^{hi} (Fig. 3B). Surprisingly, and despite sorting the cells based on canonical Wnt signaling activity, β-catenin expression levels did not vary greatly between Wnt^{+} and Wnt^{−} cell populations (underlined in Fig. 3). In fact, β-catenin expression levels were dwarfed by the expression levels of Jun and similar to the expression levels of rhoA, both of which are major factors in the non-canonical Wnt-PCP signaling pathway. This suggests that mTECs, in addition to actively undergoing canonical Wnt signaling, could also be actively undergoing non-canonical Wnt signaling.

We also report the expression of AES, CCND2, and CSNK1A1 in mTECs and this is noteworthy because these proteins can potentially influence TEC homeostasis. AES is capable of inhibiting NF-κB target gene transcription though its interaction with p65 and suggests possible avenues of cross-regulation between the NF-κB and Wnt singling pathways in mTECs [135]. Mice with K5 driven CCND2 overexpression developed mild thymic hyperplasia that reversed at 4 months [122]. Finally, CSNK1A1 is capable of regulating various proteins in the canonical-β-catenin-Wnt signaling pathway by phosphorylation in both an inhibitory and activating manner, including that of β-catenin itself [129].
**qRT-PCR confirms the expression of Wnt-5b and Wnt-5a in mTECs**

Wnt-5a expression was increased in mTEC£©Wnt+ when compared to mTEC©Wnt− (Fig. 2B), and Wnt-5b expression was increased in mTEC©Wnt+ when compared to mTEC©Wnt− (Fig. 2A). Therefore, we chose to further study Wnt-5a and Wnt-5b in the thymus due to their differential expression levels in Wnt+ cell populations. Wnt-5a has a known function in the thymus and drives apoptosis in DP thymocytes [2], while Wnt-5b is thought to be capable of driving FoxN1 expression in the thymus [7]. Furthermore, both Wnt-5a and Wnt-5b have been implicated in stimulating adipogenesis and thus could have a possible role in thymic senescence and age-related adipose involution [67, 68]. In addition, Wnt-5a and Wnt-5b are capable of working in unison—they can coordinate chondrocyte proliferation and differentiation in bone development by differentially regulating gene transcription [136]. We hypothesized that a similar mechanism might be present in the thymus to coordinate TEC proliferation and differentiation.

We used qRT-PCR to confirm the results from the Wnt PCR array analysis regarding Wnt-5a and Wnt-5b expression. RNA derived from sorted EpCAM+ cells was used as a positive control and qRT-PCR analysis of the four unique mTEC cell populations revealed that Wnt-5a was significantly upregulated (~4-fold) in the mTEC©Wnt+ subset when compared to the other mTEC subsets (Fig. 4A). Moreover, the genetic expression level of Wnt-5b was also found to be significantly upregulated (~6-fold) in the mTEC©Wnt+ cell population when compared to the other mTEC subsets (Fig. 4B). These results are consistent with the results reported in the Wnt PCR array analysis and suggest a possible role for these ligands in the regulation of Wnt signaling in mTECs.
**Wnt secretion proteins modulate RANKL-induced expression of Aire in FTOCs independent of thymocytes**

As freshly isolated TECs are not suited to manipulations in vitro due to their rapid loss of TEC-specific features, we used E14.5 fetal thymic epithelial cultures to investigate the role of Wnt secretion proteins in TEC development [160]. FTOCs were prepared with 2′-dG to remove hematopoietic cells while leaving the TECs relatively unharmed. RANKL directly and specifically induces the expression of Aire in fetal TECs and fetal TECs were cultured with both RANKL (to induce TEC maturation) and different Wnt signaling inhibitors or Wnt ligands (Table 1) [91]. We then measured how Wnt secretion proteins influenced maturation by assessing the frequency of Aire⁺UEA-1⁺DEC-205⁻ TECs by immunofluorescent microscopy. Lobes cultured with only RPMI media after hematopoietic cell depletion were used as negative controls. We did not detect the presence of any Aire⁺ cells in the negative controls, indicating that all hematopoietic cells were successfully removed from the fetal lobes (Fig. 6). With the absence of hematopoietic cells, all induced Aire expression would be initiated by exogenous RANKL and lobes treated with RANKL alone were used as positive controls (Fig. 6).

<table>
<thead>
<tr>
<th>Wnt Inhibitors and Ligands</th>
<th>Description</th>
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<tr>
<td>Sfrp-1</td>
<td>Inhibitor of canonical and non-canonical Wnt signaling</td>
</tr>
<tr>
<td>Dkk1</td>
<td>Specific inhibitor of canonical Wnt signaling</td>
</tr>
<tr>
<td>Wnt-4</td>
<td>Regulates thymic cellularity and possibly FoxN1 expression; decreased expression with age</td>
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<tr>
<td>Wnt-5b</td>
<td>Increased expression with age; possible role in age-related adipogenesis</td>
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<tr>
<td>Wnt-5a</td>
<td>Induces apoptosis in thymocytes; possible role in age-related adipogenesis</td>
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Table 1. Wnt inhibitors and ligands used in FTOCs.

We aimed to demonstrate that secreted Wnt proteins would be able to impact the RANKL-induced development of TECs. Therefore, we chose to culture fetal TECs with Sfrp-1, an antagonist that directly binds to Wnt ligands that was also upregulated in the aged human
thymus as well as being implicated in thymic senescence and age-related adipose involution [55]. In FTOCs, Sfrp-1 should disrupt both canonical and non-canonical Wnt signaling by interfering with endogenous Wnt ligands produced by fetal TECs. However, the ability of Sfrp-1 to inhibit both canonical and non-canonical Wnt ligands means we cannot determine which pathway influenced TEC development. Yet, since Sfrp-1 is also capable of blocking the interaction of RANKL with its receptor directly, we anticipated Sfrp-1 would influence RANKL-induced Aire expression in some way and thus demonstrate that the secretion of Wnt proteins were capable of impacting TEC development [131]. Indeed, fetal lobes treated with RANKL and Sfrp-1 significantly reduced the frequency of Aire^{+}UEA-1^{+}DEC-205^{-} TECs at both concentrations (Fig. 5A). Sfrp-1 does not seem to interfere with RANKL-induced expression of Aire in TECs in a dose-dependent manner. Sfpr-1 negatively impacted RANKL-induced expression of Aire in fetal TECs and these results suggest that certain secreted Wnt proteins are capable of modulating the development and maturation of the thymus.

To determine whether the decrease in the frequency of Aire^{+}UEA-1^{+}DEC-205^{-} TECs may be attributed to canonical or non-canonical Wnt signaling, we tested the effect of co-culturing Dkk1 with RANKL in FTOCs. Dkk1 specifically inhibits canonical Wnt signaling by blocking the interaction between Fzd and its co-receptor LRP—it does not directly interfere with non-canonical Wnt signaling. A reduction in the frequency of Aire^{+}UEA-1^{+}DEC-205^{-} TECs in fetal lobes treated with RANKL and Dkk1 could suggest that the inhibition of canonical-Wnt signaling negatively effects the development and maturation of fetal TECs. However, we observed no difference with regards to the frequency of Aire^{+}UEA-1^{+}DEC-205^{-} TECs in samples treated with Dkk1 when compared to the RANKL-only positive controls (Fig. 5B). This
indicates that the inhibition of canonical Wnt signaling, independent of thymocytes, is indifferent to the process of RANKL-induced expression of Aire in fetal TECs.

Next, we asked if stimulating fetal TECs with secreted Wnt ligands were capable of affecting RANKL-induced maturation. Wnt-4 is highly active at multiple time points during thymus development and its absence in the aged thymus is thought to contribute to thymic senescence. Strong Wnt-4 expression in adult TECs has been demonstrated here (Fig. 3) and in other reports [5, 7]. Wnt-4 is also able to trigger both canonical-β-catenin-Wnt and non-canonical-Wnt singling in a context-dependent manner, and has previously been demonstrated to play a role in embryonic thymus development and migration [134, 56]. Here, we report that Wnt-4 did not lessen RANKL-induced expression of Aire in fetal TECs (Fig. 5C). Instead, the frequency of Aire\(^+\)UEA-1\(^-\)DEC-205\(^-\)TECs in fetal lobes treated with RANKL and Wnt-4 (for both concentrations) consistently trended higher than that in the RANKL-only positive controls—but it was not statistically significant (Fig. 5C). Nonetheless, FTOCs stimulated with Wnt-4 had the highest measured frequency of Aire\(^+\)UEA-1\(^+\)DEC-205\(^-\)TECs and this might suggest that Wnt-4 supports mTEC differentiation. At the very least, these results indicate that Wnt-4 does not negatively influence RANKL-induced Aire expression in fetal TECs.

Wnt-5b is thought to drive the expression of FoxN1 [14], its expression has been reported to increase in t-fibroblasts, mTECs, and cTECs with age [5, 9, 11], and we showed evidence that Wnt-5b is expressed at higher levels in the mature mTEC\(^{ht}\)Wnt\(^+\) population (Fig. 2A). Furthermore, keratinocyte growth factor (KGF) enhanced postnatal T-cell development, TEC proliferation and function, and increased TEC expression of Wnt-5b, suggesting that Wnt-5b might be important for the maintenance of the thymus [160]. Remarkably, FTOCs treated with Wnt-5b resulted in a severely reduced frequency of Aire\(^+\) UEA-1\(^+\) DEC-205\(^-\) TECs in fetal lobes.
in a dosage-dependent manner (Fig. 5D). After 4 days of co-culturing RANKL with 500ng/mL of Wnt-5b, the frequency of Aire$^+$ UEA-1$^+$ DEC-205$^-$ TECs dropped more than 35% when compared to positive controls (Fig. 5D). Increasing the concentration of Wnt-5b to 1µg/mL resulted in an even more profound decrease in the frequency of Aire$^+$ UEA-1$^+$ DEC-205$^-$ TECs, which was reduced ~70% when compared to positive controls (Fig. 5D). The decrease in the frequency of Aire$^+$ UEA-1$^+$ DEC-205$^-$ TECs in the absence of hematopoietic cells indicates that Wnt-5b exerts its effect directly on fetal TECs. These results strongly suggest that the secreted Wnt-5b ligand obstructs RANKL-induced Aire expression and that certain Wnt ligands can regulate thymus development and fetal TEC maturation.

In the mTEC$^{lo}$ Wnt$^+$ population we discovered strong Wnt-5a mRNA expression (Fig. 2B). Furthermore, Wnt-5a induces apoptosis in thymocytes [2] and we hypothesized if it could also reduce the frequency of Aire$^+$ UEA-1$^+$ DEC-205$^-$ TECs by inducing apoptosis in fetal TECs. Instead, FTOCs treated with Wnt-5a did not change the frequency of Aire$^+$ UEA-1$^+$ DEC-205$^-$ TECs at either concentration and were similar to positive controls (Fig. 5E). This suggests that Wnt-5a does not induce apoptosis in fetal TECs and does not influence RANKL-initiated TEC development.

Lastly, on average, samples treated with Wnt inhibitors Dkk1 and Sfrp-1 were measured at about the same area size as RANKL positive controls (Fig. 5F). This suggests that in the absence of thymocytes, Wnt inhibition did not alter TEC cellularity. However, samples treated with Wnt ligands (with the exception of Wnt-5b 500ng/mL) were, on average, reduced in measured area size. In particular, Wnt-4 treated samples were the lowest measured fetal tissue area sizes, yet induced mTEC maturation by RANKL appeared unaffected (Fig. 5F). This is consistent with the recent publication that described transgenic mice overexpressing Wnt-4.
reduced overall embryonic TEC numbers, particularly by disturbing fetal cTEC development, while indifferent to fetal mTEC development [134].

**FoxN1 and Dll4 regulate Wnt-5a and Wnt-5b genetic expression**

Once we confirmed the presence of Wnt-5a and Wnt-5b in Wnt⁺ mTECs in the adult male thymus and saw that Wnt-5b modulated RANKL-induced maturation of fetal TECs, we examined their possible role in response to two hallmark genes of thymic development: FoxN1 and Dll4. Mesenchymal stem cells derived from the thymus capable of differentiating into other cell linages—such as osteocytes, chondrocytes, and adipocytes—known as TMSC-710’s, have been described in a previous publication from our lab [130]. We transformed the TMSC-710 cells with lentivirus particles containing a FoxN1-mCherry gene vector, a Dll4-GFP gene vector, or a control-GFP (CoGFP) vector. TMSC-710 cells that successfully integrated the FoxN1 vector into its genome were visualized with mCherry and cells that successfully integrated the Dll4-GFP vector were visualized GFP. Both samples were then sorted and purified by FACS for cell culturing.

We quantified the relative expression of Dll4 and FoxN1 in TMSC-710-FoxN1, TMSC-710-Dll4, and in TMSC-710-CoGFP using qRT-PCR. TMSC-710-FoxN1 had ~7-fold higher expression of FoxN1 when compared with either TMSC-710-CoGFP or TMSC-710-Dll4 (Fig. 7A). TMSC-710-Dll4 had ~35-fold higher expression of Dll4 when compared with TMSC-710-CoGFP or TMSC-710-FoxN1 (Fig. 7B). Together, this demonstrated successful transformation of Dll4 and FoxN1 into these cell lines.

Next, we analyzed the genetic expression of Wnt-5a and Wnt-5b in these transformed cell lines. When compared to TMSC-710-CoGFP cells, TMSC-710-Dll4 cells significantly downregulated Wnt-5b genetic expression levels, while there was no change in Wnt-5a
expression (Fig. 7C). TMSC-710-FoxN1 cells significantly upregulated Wnt-5b 3-fold and simultaneously, significantly downregulated Wnt-5a genetic expression levels when compared TMSC-710-CoGFP cells (Fig. 7C). The FoxN1 driven upregulation of Wnt-5b in TMSC-710-FoxN1 argues that FoxN1 is capable of driving Wnt signaling. This finding is supported by previous publications showing that induced FoxN1 expression in the aged thymus rescued the expression of Wnt-5b in cTECs [9]. Together with the previous report that Wnt-5b upregulated FoxN1, it is conceivable that Wnt and FoxN1 are a part of a positive feedback loop regulating Wnt signaling in TECs [14]. These results suggest FoxN1 and Dll4 can significantly regulate the expression of Wnt ligands and thus regulate Wnt signaling in the thymus.
**Future Work**

Immediate future projects will investigate in more detail 2 important issues related to our findings in fetal TECs: (1) the regulation in the expression of differentiation and survival factors; and (2) the signal transduction pathway in TECs activated by Wnt secretion proteins. Specifically, we aim to address the molecular mechanisms involved in the Wnt-5b and Sfrp-1 modulation of RANKL-induced Aire expression in fetal TECs. Currently, it was not clear how the manipulation of Wnt signaling in FTOCs altered TEC development. In Wnt-4 transgenic mice, TEC profiles revealed that Wnt-4 altered proper TEC differentiation and increased the frequency of an undefined TEC population [134]. It is possible that our Wnt manipulation fetal TECs behaved similarly and augmented an atypical TEC population and, therefore, it is imperative to consider if and how the mTEC/cTEC ratio was altered. Likewise, our Wnt manipulation might have increased the rate of apoptosis in TECs, as was the case with Wnt-5a and thymocyte development [2]. Or rather, the manipulation of Wnt signaling in fetal TECs could have reduced TEC survival without increasing apoptosis, as was the case in FoxN1-Gpr177 KO mice where the secretion of Wnt proteins was blocked [7]. On the other hand, Wnt-5b might be driving mTEC differentiation, and when combined with RANKL, might be pushing the cells to terminal differentiation at increased rates.

Wnt signaling may have a direct affect on regulating the NF-κB pathway. AES, which we identified to be expressed in the mTEC subsets, was also revealed to be a potent inhibitor of NF-κB-dependent gene expression [157]. Specifically through a protein-protein interaction with the p65 (RelA) subunit, a transcription factor of NF-κB, AES repressed p65-driven gene expression and inhibited NFKB-dependent gene expression induced by TNFα, interleukin-1β, or MEKK1 [157]. However, RelA was also reported to be involved in the down-regulation of canonical Wnt
signaling and likely mediated β-catenin suppression after the binding of the β-catenin/TCF complex with target DNA since RelA did not disturb the nuclear import of β-catenin nor its DNA-binding ability [144, 158]. This suggests that there may be competition between the Wnt pathway and the NF-κB pathway for gene transcription in TECs.

Tnfrsf19 expression, a member of the TNFSF receptors, was shown to be a direct target of canonical Wnt signaling [159]. Tnfrsf19 induced NF-κB signaling through interaction with Traf6, which was shown to be essential for mature mTEC differentiation [161]. Furthermore, increased expression of Tnfrsf19 in the ventral aspect of 3rd pp of the developing thymus was reported and it was proposed that Tnfrsf19 is targeted by Wnt signaling in the fetal thymus to induce Traf6 via the NF-κB pathway to promote the differentiation of thymic epithelial progenitors into an mTEC cell fate [116]. If so, this process might be disrupted by Sfrp-1 or Wnt-5b ectopic expression leading to decreased TEC maturation. Interestingly, Wnt-4 and Wnt-5b are also highly expressed in the ventral aspect of 3rd pp at this time [116].

Keratinocyte growth factor (KGF), increased TEC expression of Wnt-5b and Wnt-10b in the adult thymus and enhanced TEC proliferation and function; it was also revealed that this was highly dependent on NF-κB signaling and thus, linking Wnt-5b to the NF-κB signaling pathway [160]. In contrast to the results in the postnatal thymus, KGF failed to drive the expression of Wnt-5b in the fetal thymus [160]. However, KGF was able to drive Wnt-10b expression in the fetal thymus where it increased TEC proliferation [160]. The divergence of KGF driving the expression Wnt-5b in the postnatal thymus but not the fetal thymus was attributed to the differences in maturation of the TECs [160]. This suggests that the regulatory relationship between NF-κB signaling and Wnt signaling could change based on the level of maturity of the TECs [160].
Wnt signaling in the thymus is very complex and likely involves both canonical and non-canonical pathways at the same time. We will investigate the signal transduction pathway activated by Wnt-5b and repressed by Sfrp-1 to determine if TEC development was modulated through canonical or non-canonical Wnt pathways or both. Our data indicates that Wnt-5b might be involved in the regulation of canonical Wnt signaling since its expression was localized to mTEC^{hi}Wnt^{+}. Despite this, Wnt-5b and Wnt-5a are commonly referred to as ‘classical’ non-canonical Wnt ligands and often work together in several cell types. In mice, Wnt-5b and Wnt-5a coordinated chondrocyte proliferation and differentiation by differentially regulating downstream target genes [136] and Wnt-5b was later shown to regulate mesenchymal and chondrocyte differentiation through the PCP signaling pathway [148]. In addition, Wnt-5b and Wnt-5a promoted osteogenic differentiation in Rat tendon-derived stem cells through the activation of the PCP-JNK signaling pathway [153]. Furthermore, Wnt-5a, Wnt-5b, and Sfrp-1 together with intracellular signaling via PCP proteins, polarized node cells of the mouse embryo along the anterior-posterior axis for breaking of left-right symmetry [154].

It is not always the case, however, that Wnt-5b always activates the PCP pathway. In zebrafish, gastrulation movement required the Wnt-Ca^{2+} signaling pathway, which was initiated by Wnt-5b involving the receptor tyrosine kinase (Ryk) and the Fzd-2 receptors [147, 151], while osteoclast differentiation was also mediated by Wnt-5b through Ryk [152]. Interestingly, Wnt-5b is also known to inhibit β-catenin-dependent transcription—in murine preadipocyte differentiation and adipogenesis Wnt-5b, through an unknown mechanism, impaired β-catenin nuclear translocation [68].

And yet, in other studies, Wnt-5b either stimulates canonical Wnt signaling or has been suggested to. In a human, lung derived fibroblasts cell line; Wnt-5a, Wnt-5b, RhoA, Fzd-2, and
\(\beta\)-catenin were all upregulated in response to bFGF in a concentration and time dependent manner [155]. Wnt-5b, Wnt-5a, Wnt-4, Wnt-11 and \(\beta\)-catenin are all expressed during bone fracture repair, which suggested that \(\beta\)-catenin-dependent transcription was activated [149]. Moreover, Wnt-5b triggered \(\beta\)-catenin signaling in triple negative breast cancer cells and increased TCF transcription activity by 30\%, while knocking-down Wnt-5b in these cells reduced TCF transcription activity by 50\% [150]. Finally, in zebrafish, lymphatic progenitors arise from angioblasts induced directly by Wnt-5b through the canonical Wnt pathway—and since Wnt-5b also induced the angioblast-to-lymphatic transition in human embryonic cells, implied that it did so through activating the canonical Wnt signaling pathway [156]. Thus, Wnt-5b is capable of activating the non-canonical Wnt signaling pathways—both the PCP and Wnt-Ca\(^{2+}\) pathways—and can also activate and inhibit canonical Wnt signaling.
**Figures**

**Legends**

**Figure 1:** Characterization of TCF/Lef:H2B/GFP Wnt activity in the postnatal thymus and representative TEC sorting gating hierarchy.

(A) TCF/Lef:H2B/GFP Wnt activity is localized to the medulla (UEA-1+) in the thymus and not the cortex (Dec-205+). (B) Strategy for sorting TEC populations by flow cytometer cell sorter in 3 month old male TCF/Lef:H2B/GFP Wnt reporter mice. mTECs are defined as EpCAM−MHC-II−UEA-1−CD45−. (C) EpCAM−UEA-1− cells contain the cTEC population which has low TCF/Lef:H2B/GFP Wnt activity. (D) mTECs are divided into two cell populations: CD80−MHC-II-lo describes mTEClo and CD80−MHC-IIhi describes mTEC hi. (E) GFP Wnt reporter mice allow for further separation of mTEClo and mTEC hi into mTEClo Wnt−, mTEClo Wnt+, mTEC hi Wnt−, and mTEC hi Wnt+. (F) TECs derived from three-month-old C57BL/6 mice were used as a negative control for properly gating GFP Wnt reporter activity. (G) qRT-PCR analysis of mature mTEC markers (EpCAM, FoxN1, Dkk1, and Aire) in FACS sorted mTECs. Data are normalized to the housekeeping control gene 18s and relative to mTEC hi. Equal starting amounts of RNA were synthesized into cDNA. Experiment was repeated 3 times and included NTC and NRT controls. Error Bars ±SEM. Asterisks indicate P < 0.05 (Students t test).

**Figure 2:** Relative mRNA expression of detected WNT ligands and FZD receptors in mTECs.

Differential genetic expression of Wnt ligands and FZD receptors in (A) mTEC lo and (B) mTEC hi, Wnt+ and Wnt− cell populations. RNA isolated from 3 independent experiments was pooled together and equal amounts (500ng) were used for each cDNA synthesis reaction. cDNA were then loaded onto Wnt PCR-Array plates and gene expression was assessed by Applied Biosystems instrument 7500 RT-PCR system. Ct values were cutoff at cycle 35 and the 2−ΔΔct relative quantification method was applied to calculate the expression values. Gene-of-interest expression levels are relative to the geometric mean of the expression of four housekeeping genes (Actb, Gapdh, Gusb, & Hsp90ab1) in the same sample. Graphs represent the mean of two independent PCR-Array experiments and only genes where trends were similar were included.

**Figure 3:** Relative mRNA expression of Wnt signaling proteins.

Differential genetic expression of Wnt signaling proteins in (A) mTEC lo and (B) mTEC hi, Wnt+ and Wnt− cell populations. RNA isolated from 3 independent experiments was pooled together and equal amounts (500ng) were used for each cDNA synthesis reaction. cDNA were then loaded onto Wnt PCR-Array plates and gene expression was assessed by Applied Biosystems instrument 7500 RT-PCR system. Ct values were cutoff at cycle 35 and the 2−ΔΔct relative quantification method was applied to calculate the expression values. Gene-of-interest expression levels are relative to the geometric mean of the expression of four housekeeping genes (Actb, Gapdh, Gusb, & Hsp90ab1) in the same sample. Graphs represent the mean of two independent PCR-Array experiments and only genes where trends were similar were included.
Figure 4: qRT-PCR of Wnt5a and WNT5b expression levels in sorted TECs. (A) WNT5a expression was relative to the housekeeping control gene 18s in mTEC^loWnt^, mTEC^hiWnt^, and mTEC^hiWnt^+. (B) WNT5b expression was relative to the housekeeping control gene 18s in mTEC^loWnt^, mTEC^loWnt^+, mTEC^hiWnt^, and mTEC^hiWnt^+. Data are normalized to the expression in the positive control EpCAM^+ sorted thymic cells. Equal amounts of starting RNA were synthesized into cDNA. Experiments were performed in triplicate repeats and repeated 3 times. Experiments included no-template negative controls and no-reverse transcriptase negative controls. * P < 0.05 (Students t test). Error Bars ±SEM

Figure 5: Secreted WNT proteins modulate RANKL-induced Aire expression in Fetal TECs. E14.5 fetal thymic lobes were isolated from pups and were cultured with 1.35mM 2-dG + RPMI medium for 5 days to deplete them of hematopoietic cells. Lobes were then treated with either RANKL (1ug/mL), (A) RANKL + Sfrp-1, (B) RANKL + Dkk1, (C) RANKL + Wnt-4, (D) RANKL + Wnt-5b, and (E) RANKL + Wnt-5a and analyzed for the mean number of Aire^+ UEA-1^+ DEC-205^- nuclei (confirmed by DAPI) per mm^2 of measured fetal thymic tissue. (F) Average measured area (mm^2) of imaged thymic tissue sections. At least 15 sections from 3 individual lobes were analyzed for each condition. Therefore, a minimum of 15 independent calculations derived from 45 sections was used to calculate the mean and pooled standard deviation (±SD) of the number Aire^+UEA-1^+DEC-205^- TECs for each condition. Asterisks indicate P < 0.001 (Students t test).

Fig 6: Representative FTOC images. RANKL-induced development of Aire^+UEA-1^+DEC-205^- fetal TECs in E14.5 FTOCs post hematopoietic depletion and subsequent co-culturing with Wnt secreted proteins.

Figure 7: FOXN1 and DLL4 influence WNT5a and WNT5b expression. (A) qRT-PCR analysis of FoxN1 expression in transformed TMSC-710 cell lines. (B) qRT-PCR analysis of Dll4 expression in transformed TMSC-710 cell lines. (C) qRT-PCR analysis of Wnt-5a and Wnt-5b in transformed TMSC-710 cell lines. For (A) and (B), experiments were performed on three occasions, representative experiments are shown. For (C), graph represents the average of three independent experiments performed in technical triplicates. Equal amounts of RNA (500ng) were synthesized into cDNA with reverse transcriptase. Data are normalized to TMSC-710-CoGFP and relative to the control housekeeping gene 18s. * P < 0.05; ** P < 0.01 (Students t test). Error Bars ±SEM
Figure 1
Figure 2

A

B
Figure 3

A

B
Figure 4

(A) WNT5A

(B) WNT5B

* indicates significant difference.
Figure 5

A

![Graph A](image1.png)

B

![Graph B](image2.png)

C

![Graph C](image3.png)

D

![Graph D](image4.png)

E

![Graph E](image5.png)

F

![Graph F](image6.png)
Figure 6

2-Deoxy NC
RANKL PC
SFRP1 500ng
SFRP1 1μg
Dkk1 500ng
Dkk1 1μg
WNT4 500ng
WNT4 1μg
WNT5b 500ng
WNT5b 1μg
WNT5a 500ng
WNT5a 1μg

AIRE
DAPI
DEC-205
UEA-1
Combined
Figure 7

A

B

C

Relative Fold Change

Sample

710-CoGFP  710-FOXN1  710-DLL4

Relative Fold Change

Sample

710-CoGFP  710-FOXN1  710-DLL4

Legend:

FOXN1

DLL4

WNT5B

WNT5A

710-CoGFP  710-FOXN1  710-DLL4

Relative Fold Change

0  0.5  1  1.5  2  2.5  3  3.5  4  4.5

710-CoGFP  710-FOXN1  710-DLL4

Relative Fold Change

0  0.5  1  1.5  2  2.5  3  3.5  4  4.5

710-CoGFP  710-FOXN1  710-DLL4

Relative Fold Change

0  0.5  1  1.5  2  2.5  3  3.5  4  4.5

710-CoGFP  710-FOXN1  710-DLL4

Legend:

WNT5B

WNT5A

*  **  *

Bibliography


