FOXN1 in thymus organogenesis and development

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Title: FOXN1 in thymus organogenesis and development

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Key words: FOXN1, thymus, thymic epithelium, ontogeny, cTECs, mTECs, nude mice, transcription factor, forkhead.

Abbreviations: TECs, thymic epithelial cells; cTECs, cortical thymic epithelial cells; mTECs, medullary thymic epithelial cells; ETPs, early thymocyte progenitors; DN, double negative; DP, double positive; SP, single positive; TEPcs, thymic epithelial progenitor cells; MEFs, mouse embryonic fibroblasts; PP, pharyngeal pouch; FOX, forkhead.

Abstract

Development of the primary T-cell repertoire takes place in the thymus. The linked processes of T-cell differentiation and T-cell repertoire selection each depend on interactions between thymocytes and thymic stromal cells; in particular, with the epithelial cells of the cortical and medullary thymic compartments (cortical and medullary thymic epithelial cells; cTECs and mTECs, respectively). The importance of the thymic epithelial cell lineage in these processes was revealed in part through analysis of nude (nu/nu) mice, which are congenitally hairless and athymic. The nude phenotype results from null mutation of the forkhead transcription factor FOXN1, which has emerged as a pivotal regulator both of thymus development and homeostasis. FOXN1 has been shown to play critical roles in thymus development, function, maintenance, and even regeneration, which positions it as a master regulator of thymic epithelial cell (TEC) differentiation. In this review, we discuss current understanding of the regulation and functions of FOXN1 throughout thymus ontogeny, from the earliest stages of organogenesis through homeostasis to age-related involution, contextualising its significance through reference to other members of the wider Forkhead family.
FOXN1 is a master regulator of thymus function, orchestrating thymic epithelial cell (TEC) differentiation and function in thymus development and homeostasis.

FOXN1 expression in TEC is downregulated with age.

TEC-specific restoration of high-level FOXN1 expression rejuvenates the fully involuted thymus.

Expression of FOXN1 converts some other cell-types into functional TEC.

Introduction
The thymus is a central organ of the adaptive immune system due to its obligatory role in T-lymphocyte differentiation and repertoire selection [1]. These functions depend on the thymic stroma, which comprises a variety of cell types including mesenchymal cells, vascular endothelium, macrophages, dendritic cells and, importantly, a highly specialized epithelial compartment, which confers both structural and functional attributes to the organ. The thymus is divided into two broad regions, the cortex and the medulla (Figure 1). The epithelial cells in each of these compartments are functionally distinct, with cortical and medullary thymic epithelial cells (cTECs and mTECs, respectively) mediating different aspects of T-cell development [2-7]. T-cell development has been reviewed extensively elsewhere (see references [2-7]) and is not revisited in detail herein. Briefly, haematopoietic progenitors enter the thymus at the junction between cortex and medulla. Commitment to the T-cell lineage and differentiation as far as the CD4⁺CD8⁺ ‘double positive’ (DP) stage of T-cell development occurs in the cortex. As discussed in detail below, cTECs are required for commitment of haematopoietic cells to the T-cell lineage, and also mediate both the β selection and positive selection stages of T cell lineage development. Developing T cells (called thymocytes) that successfully undergo positive selection can then enter the medulla, the site of central tolerance induction, with tolerance induction being mediated by both mTECs and thymic dendritic cells (Figure 1). Not surprisingly, in view of their functional differences, cTECs and mTECs are also phenotypically distinct. These differences are discussed in further detail below but in brief, expression of Bp-1 (En-pep, the Ly-51 antigen) by cTECs, and binding of the lectin *Ulex europeaus* agglutinin 1 (UEA1) by mTECs identifies these TEC sub-lineages and permits their isolation and subsequent analysis. In the adult thymus, Ly-51⁺ cTECs and UEA-1⁺ mTECs each constitute heterogeneous populations that can be subdivided into a number of different subsets based on expression of additional surface markers, including MHC Class II [8-12].
The epithelial component of the thymus arises from the endoderm of the pharyngeal pouches (PPs). These structures are bilateral outpocketings of the foregut endoderm. The number of PPs varies between species; in mouse and human it is the third PPs (3PPs) that generate the thymus, while other PPs also contribute in some species [13, 14]. In mice, the 3PPs form at around day 9 of embryonic development (E9.0). This initial budding is followed by outgrowth and patterning stages, such that each 3PP forms a shared primordium for two organs – the thymus and the parathyroid glands. These organ primordia can be distinguished on the basis of marker expression by E10.5 in mouse, when transcription factor Glial cells missing 2 (Gcm2) mRNA specifically delineates the parathyroid domain, and eventually separate from the pharyngeal endoderm and resolve into discrete organ primordia by about E12.5 [15]. In humans, the thymus domain within the 3PP is evident by week 6 of gestation [16]. The endodermal thymic rudiment within the 3PP is sufficient to direct thymus development, even after transplantation to an ectopic site [17], and appears to contain bipotent TEPCs that can generate both cortical and medullary TECs [18-21]. However, the normal process of thymus organogenesis involves interplay between a number of different cell types – including 3PP endoderm, neural crest-derived mesenchyme, endothelial progenitors and haematopoietic progenitors – all of which are components of the mature organ (reviewed in [22-26]) (Figure 2).

Some of the earliest insights into the function of the thymus came from studies on nude (nu/nu) mice, which carry an autosomal recessive mutation leading to congenital hairlessness and athymia [27, 28]. Nu/nu mice are correspondingly immunocompromised as they lack normal T-cell populations [27, 28]. The functional athymia in nu/nu mice results from a severe developmental block early in thymus organogenesis. The common thymus-parathyroid primordium forms normally and thymus organogenesis proceeds until E11.5-
E12.0. However, a maturational arrest in thymic epithelial progenitor cells occurs at around E12.0 [29], such that the nude thymic epithelium never becomes competent to support T-cell development. Indeed, the nude thymic rudiment is never colonized by haematopoietic or vascular progenitors; instead, these remain in the perithymic mesenchyme [30, 31]. Adult nu/nu mice retain a small, cystic, alymphoid thymic rudiment, which does not support T-cell development at any stage in ontogeny.

**Identification of Foxn1 as the nude gene**

Foxn1 was originally identified as the gene mutated in nu/nu mice using genetic approaches [32, 33]. Following localization of nu to chromosome 11 in mice and subsequent fine-mapping, a member of the forkhead or winged helix superfamily, originally named winged helix nude (whn; later renamed Foxn1), was identified as the nude gene by positional cloning [32, 34]. Initial studies showed the whn transcript in nu/nu mice carried a single base pair deletion in its third exon, resulting in the absence of Whn mRNA due to nonsense-mediated decay [32]. RT-PCR analysis revealed that Whn was expressed in the developing mouse embryo from E9.5, and was restricted to skin and thymus in adult tissues [32]. Subsequently, a targeted null allele of Whn was generated by inserting a lacZ M2-neo cassette into exon 3 of Whn, close to the site of the spontaneous mutation in nu. Mice homozygous for this allele phenocopied nu/nu mice, confirming Whn as the nude gene [33].

**The Forkhead family of transcription regulators**

FOXN1 was one of the first members of the forkhead (FOX) superfamily of transcription factors (TFs) to be implicated in a specific developmental defect in vertebrates [35]. It is now known that this large family of TFs has important roles in the development, homeostasis,
function and aging of a variety of organs and tissues - including the immune system [36, 37].

As examples, FOXP3 is needed for the development and function of regulatory T cells (Treg cells) [38-40], FOXJ1 for suppression of T-cell activation [41], and FOXO3 for lymphocyte proliferation and apoptosis [42, 43], while FOXN1 itself is essential for production and maintenance of a functional thymus and is also required for hair production [29, 33, 44-48].

The FOX family is evolutionarily ancient. Its canonical member is the *Drosophila melanogaster* gene fork head (*fkh*) which, when mutated, causes a spiked head phenotype in adult flies [49]. Identification of the rat gene hepatocyte nuclear factor 3 alpha (*HNF3α*) in 1990 revealed an approximately 100 amino acid region of high homology between the HNF3α and fkh proteins, that was suggested to be a DNA binding domain (DBD) [35, 50]. TFs containing this ‘winged helix’ or ‘forkhead’ DBD (Structural Classification of Proteins (SCOP) classification n°46785) were subsequently identified in Eubacteria, Archaea, and Eukaryota, and classified as a new superfamily. More than 2000 FOX family members have now been identified in 108 species of animals and fungi, with numbers differing between species [36][51-53]. FOX proteins are currently classified based on phylogenetic analysis of their DBDs (forkhead domains), which are highly conserved and represent the only regions of peptide sequence that can be confidently aligned across all FOX proteins [34, 54]. Nineteen subclasses of FOX protein have now been identified - FOXA to FOXS - and of these, FOXQ, FOXR, and FOXS are vertebrate-specific [54].

**The FOXN subfamily**

The FOXN genes cluster separately from other FOX sub-classes, and the gene most closely related to *FOXN1* is its parologue *FOXN4*. The evolutionary history of the FOXN subclass as currently understood is depicted in Table 1 [55, 56]. *Foxn4* first appeared in
Cephalochordates (amphioxus) and is found in all higher organisms. Cephalochordates also contain a more ancient parologue Foxn4b, which is present in Echinodermata and Cnidaria but absent from urochordates and all vertebrates. Jawless fish possess a gene very similar to Foxn4, termed Foxn4-like (Foxn4L). Foxn1 is thought to be an orthologue of Foxn4L, based on protein sequence and short-range synteny relationships. The expression patterns of these genes further support this genealogy: Foxn4 (or Foxn4a) being expressed in the pharyngeal endoderm and other sites in amphioxus; FOXN4L, in the epithelium lining the gill basket in lamprey; and Foxn1 in the pharyngeal pouches giving rise to the thymus in cartilaginous fishes and all other jawed vertebrates [48]. The expression of Foxn4 in the pharyngeal endoderm in amphioxus suggests that this gene contributed to the evolutionary emergence of thymopoiesis [48]. Indeed in evolution, the emergence of FOXN4L and thymus-like function preceded the pinching off of pharyngeal pouches into a distinct organ, as observed in the lamprey gill basket. Both Foxn1 and Foxn4 are expressed in the thymi of catshark, zebrafish, and medaka; however Foxn4 is either not expressed or expressed at very low levels in higher order organisms [57]. Indeed, Foxn1 appears to have a unique role in the thymus in jawed vertebrates, which cannot be completely substituted by Foxn4 [57].

Transcriptional regulation by FOX proteins

The classical ‘forkhead’ DBD consists of three N-terminal α-helices (H1, H2, H3), three β-sheets (S1, S2, S3) and two C-terminal ‘wing’ regions/loops (W1, W2), arranged in the order H1-S1-H2-H3-S2-W1-S3-W2 [58]; an additional α-helix is present in some FOX proteins [59, 60]. The term ‘winged helix’ was coined to reflect the butterfly-like winged structure adopted by DNA-bound FOX proteins [59], which resembles structures formed during DNA interactions with linker histones such as H1 and H5 [59]. The DNA binding specificity of the forkhead domain depends on the variable region at the junction of the α-helices and wing
loops, which interact with bases in minor groove of DNA [61]. Whilst all FOX proteins share
the forkhead domain, their specific functions are thought to reside in their transactivation or
repression domains, which show almost no sequence homology between superfamily
members [37]. Functional diversity is also determined by differences in interaction partners
and spatio-temporal expression patterns such that, while FOX superfamily members have
largely distinct functions, some functional overlap exists between members of the same sub-
group [54].

Most FOX factors appear to bind to DNA as monomers [59], however cases of homodimers
[62] and heterodimers [63] have also been documented. FOX proteins also interact with non-
transcription factor proteins such as co-activators, co-repressors, and enzymes. Some FOX
proteins also have posttranslational modifications - including phosphorylation, acetylation,
methylation, and ubiquitination - which affect their binding affinity and specificity, nuclear
localization, and stability [36]. Finally, FOX proteins act as effector molecules for several
signaling pathways, coupling extra-cellular signals to changes in gene expression [36].

Recently, much interest has focused on the capacity of some TFs to act at the level of
chromatin organization, opening regions of previously compacted chromatin thus enabling
their transcription. TFs with this activity are called ‘pioneers’. Pioneer factors are defined as
being able to access their target sequence on nucleosomes and certain forms of compacted
chromatin, to bind nucleosomes stably and before the binding of other TFs or initiation of the
target gene expression, and to posses chromatin-opening capabilities [64]. Forkhead TFs –
specifically the FOXA proteins FOXA1 and FOXA2 required for hepatic development [65] -
were among the first to be identified as having ‘pioneer’ function. Detailed investigation of
the interaction of the FOXA proteins with chromatin showed they could stably bind their
target sequences on in vitro assembled nucleosomes, and could open the local nucleosomal domain through the activity of their C-terminal domain [66, 67]. The similarities between the ‘winged-helix’ structure of the forkhead domain and structures of linker histones is thought to explain how these TFs can displace linker histones from compacted chromatin, even in the absence of the SWI-SNF chromatin remodeling complex. Whether FOXN1 has a similar pioneer activity within the TECs remains to be determined. Such activity could explain the broad range of functions regulated by FOXN1 in TECs (see below). However, while the FOXA factors are required for liver specification, FOXN1 does not appear to required for specification of the TEC lineage (see below), indicating the potential for different functional requirements from these two classes of FOX proteins.

**FOXN1 in thymus development**

Specification of the TEC lineage is independent of FOXN1

As discussed above, low-level Foxn1 expression is evident in the pharyngeal endoderm as early as mouse E9.5 - the time of the initial outpocketing of the 3PP [32]. However, high-level expression is evident only from E11.25 [15]. This strong expression initiates in the most ventral tip of the 3PP and subsequently expands to encompass the entire thymus domain (see Figure 2). Histological analysis has established that FOXN1 is not required for formation of the 3PP or the thymic primordium itself [30, 68] and in keeping with this, several lines of evidence indicate that FOXN1 does not specify the thymic epithelial lineage [17, 29, 48, 68]. Following ectopic transplantation, the E9.0 3PPs (which do not yet express Foxn1) can generate an intact and functional thymus containing both cortical and medullary thymic epithelial compartments, indicating that 3PP cells are already committed to the TEC lineage [17]. Furthermore, both forkhead transcription factor g1 (Foxg1) and interleukin 7 (Il7) specifically mark the thymus domain of the 3PP and for each, this expression occurs
independently of FOXN1 [69, 70]. Additionally, analyses of revertible null or severely hypomorphic alleles of Foxn1 have shown that thymic epithelial progenitor cells (TEPC) that lack Foxn1 expression undergo a developmental arrest that can be reversed in neonatal and adult mice [21, 29, 71]. Thus, the fetal TEPC state appears to be extremely stable in vivo, strongly suggesting the presence of a stable transcriptional network upstream of Foxn1 that confers TEPC identity and thus thymic epithelial lineage specification. Overall, these studies indicate that TEC lineage commitment does not depend solely on FOXN1, implicating an as yet unidentified genetic network in this process.

FOXN1 in TEC differentiation

As discussed above, the absence of functional FOXN1 arrests fetal TEPC in a bipotent progenitor cell state, and normal functioning in these developmentally arrested fetal TEPCs can be restored by permitting normal FOXN1 expression. This was initially demonstrated using a revertible Foxn1 allele, Foxn1SA2 [21]. Using a Cre-ERT2 system that exhibited low-level activity in the absence of tamoxifen induction, reactivation of Foxn1 in a single cell in the thymic rudiment of Foxn1 null mice was shown to result in the generation of miniature thymis, each containing well-defined cortical and medullary areas [21]. How these findings relate to in vivo development is however still open to question, since Foxn1−/− thymi contain cytokeratin 5hi, claudin 4hi (K5hiCldn4hi) and K5Cldn4lo regions. Since mTEC-restricted progenitors in the early fetal thymus are Cldn4hi [72, 73], this suggests that the emergence of the mTEC sub-lineage may be Foxn1-independent and thus that the divergence of the cTEC and mTEC sub-lineages may occur earlier than implied by this particular genetic analysis [21, 48]. Current understanding is therefore that thymus organogenesis can be considered as two stages: early Foxn1-independent development which results in generation of the undifferentiated thymic primordium containing specified TEPCs, and later Foxn1-dependent
development in which FOXN1 expression in TEPCs results in their differentiation and the concomitant orchestrated development of the fully patterned and functional thymus [17, 21, 33, 70, 71].

How does FOXN1 effect these later stages of development, and what are its subsequent roles in thymus maintenance and function? Studies on FOXN1 function in early thymus development have revealed a role in TEC proliferation [31] and have also demonstrated its essential role in conferring competence to attract haematopoietic and endothelial progenitors upon TECs in the thymic rudiment [31, 74-76]. Furthermore, FOXN1 has been shown to regulate the maturation and migration of the neural crest cells that will form the thymic mesenchyme [48]. Interestingly, the expression of Vegf-a and Pdgf-b in TECs, thymic vasculature-associated mesenchyme, and endothelium, was severely reduced under conditions of low Foxn1 expression, with the thymic rudiment showing fewer capillaries, leaky blood vessels, disrupted endothelium-perivascular cell interactions, endothelial cell vacuolization, and an overall failure of vascular organization at later stages of organ development [76, 77]. Thus, FOXN1 appears to regulate not only the initial colonization of the thymus with endothelial progenitors, but also normal vascularization of the organ [77].

Further insight into the role of Foxn1 in TEC differentiation has been provided by studies in which Foxn1 is either under- or over-expressed specifically in TECs. For example, mice homozygous for a hypomorphic Foxn1 allele, Foxn1Δ, whose transcript lacks the N-terminal domain of FOXN1, develop severely hypoplastic and cystic thymi that lack distinct cortical and medullary regions [44]. T-cell development appears relatively normal in the fetal Foxn1Δ/Δ thymus. However, the adult Foxn1Δ/Δ thymus supports only aberrant thymopoiesis, characterized by the absence of CD25+ thymocytes (DN3 cells) and reduced expression of
TCRβ at the DP stage. Analysis of Foxn1Δ/Δ mice is consistent with TEC differentiation being initiated, but then blocked at an intermediate stage of development, such that the Foxn1Δ/Δ thymus has impaired functionality compared with that of the wild type [44]. A second severely hypomorphic Foxn1 allele, Foxn1R, allowed investigation of FOXN1 function in TEC differentiation independently of its role in proliferation [48]. Foxn1R generates only around 15% of WT levels of normal Foxn1 transcripts, which results in development in Foxn1R/R mice of a hypoplastic thymus which can only sub-optimally support T-cell development such that fewer thymocytes are generated during differentiation. Analysis of an allelic series based on the Foxn1R, Foxn1 and wild-type Foxn1 alleles revealed strong dose-dependent effects of Foxn1, in brief showing that increasing levels of Foxn1 expression are required for progression through multiple intermediate stages of TEC development – from exit from the earliest progenitor cell state(s) through to terminal differentiation, in both cTEC and mTEC sub-lineages in the fetal and adult thymus [48].

Despite 20 years having elapsed since confirmation of its identity as the nude gene product, the molecular functions of FOXN1 have not yet been determined in full, and indeed no direct targets have yet been verified in TECs by chromatin immunoprecipitation. However, FOXN1 has been shown to be required for the expression in TECs of proteins with essential roles in promoting thymocyte development, including Chemokine (C-C Motif) Ligand 25 (CCL25), C-X-C motif chemokine 12 (CXCL12; also known as stromal cell-derived factor 1 (SDF1), Delta-like ligand 4 (DLL4), Stem cell factor (SCF; also known as SCF, KIT-ligand, KL, or steel factor), Cathepsin L (CTSL), the 20S proteasome subunit beta-5t (β5t; also known as Psmb11) and MHC Class II [45, 48, 78]. CCL25 and CXCL12 are chemokines which are required for attracting thymic seeding cells into the developing thymic rudiment and the adult thymus [74, 79, 80]. DLL4 is a Notch ligand required for TECs for commitment of
haematopoietic progenitors to the T-cell lineage [81, 82], and SCF is required for thymocyte survival and proliferation [83]. Cathepsin L and \( \beta \)-tubulin regulate the production of peptides required in TECs to effect optimal positive selection of CD4\(^+\) and CD8\(^+\) thymocytes, respectively [84-87], while MHC Class II expression is critical for positive and negative selection of CD4\(^+\) T cells. Notably, transgenic expression of Dll4, Ccl25, Cxcl12 and Scf conferred some capacity to support production of CD4\(^+\) and CD8\(^+\) SP T cells to the Foxn1\(^+\) thymic rudiment, although the TEPCs within the rudiment remained in an undifferentiated state [78]. Since the Foxn1\(^-\) thymus expressing transgenic Dll4, Ccl25, Cxcl12, and Scf lacked functional TECs and normal thymus architecture, this study established that Foxn1 must regulate additional genes that are required for TEC differentiation and function. In keeping with this observation, in addition to the genes discussed above, the genes encoding transformation related protein 63 (Trp63), Paired Box 1 (Pax1), Fibroblast growth factor receptor 2 isoform IIIB (Fgfr2Iib), Autoimmune regulator (Aire), Cluster of differentiation 40 (CD40), Cluster of differentiation 80 (CD80), and Cyclin D1 (Ccnd1), which have known roles in TEC differentiation, proliferation, or function, and of several genes involved in Wnt signaling, are all FOXL1 responsive in TECs [48, 88]. Thus, although further insights, including delineation of which of these genes are direct FOXN1 targets, are undoubtedly required, the range of genes and breadth of functions known to be affected by FOXN1 expression already provides an indication of how this single TF can orchestrate thymus organogenesis and function.

**Foxn1 in thymus homeostasis and involution**

TECs in the adult thymus continue to express Foxn1 [33], with cTECs expressing higher levels than mTECs, and MHC Class II\(^+\) cells expressing higher levels of Foxn1 than MHC Class II\(^-\) TECs in each compartment [48, 89-91]. Several studies have demonstrated the
importance of FOXN1 in maintenance of the adult thymus [45-47]. Interestingly, down-regulation of Foxn expression in the thymic stroma is one of the earliest events in the age-associated degeneration of the thymus [92], suggesting that FOXN1 could play an important role in postnatal thymus homeostasis and subsequent thymic involution. This hypothesis was supported by analysis of a Foxn allele (Foxn\textsuperscript{lacZ}), which expresses normal levels of Foxn in the fetal and newborn thymus, after which Foxn expression declines to 20-30% of wild type levels by 5 weeks after birth [45]. Foxn\textsuperscript{lacZ/LacZ} mice exhibit a premature loss of thymus homeostasis, correlating with Foxn downregulation, that phenocopies many of the hallmarks of age-related involution [45]. The TEC subsets most affected were those that normally express high levels of Foxn, indicating their continued FOXN1 dosage sensitivity [45]. This study provided the first functional evidence linking FOXN1 down-regulation with age-related thymic involution. Consistent with this, ubiquitous deletion of Foxn or of FOXN1\textsuperscript{+} cells in postnatal mice, resulted in rapid thymic atrophy, further supporting a role for FOXN1 in thymus homeostasis [46, 47, 93].

Foxn expression in the involuting thymus has recently been investigated at the single cell level. Using a new antibody generated against the C-terminus of FOXN1 protein and a tagged version of FOXN1, Rode and colleagues showed that in aging mice, Foxn expression progressively decreases and there is an age-related accumulation of Foxn\textsuperscript{low} TECs [90]. Lineage tracing of Foxn-negative TECs has shown that these cells arise from Foxn-positive precursors [47, 91, 94]. A second study used a Foxn-eGFP reporter mouse line (in which eGFP was knocked into the Foxn locus) to investigate transcriptional changes in Foxn expression with age, and similarly showed that the emergence of Foxn\textsuperscript{+} TECs correlates with the onset of age-related thymic involution [91]. This study suggested down-regulation of FOXN1 in a subset of cTECs as a primary event in age-related thymic
involution, and further showed that this FOXN1 downregulation correlated with diminished cTEC functionality based on decreased expression of genes required in cTECs to promote T-cell differentiation. Together, these analyses suggest that both the onset and progression of involution are the result of declining Foxn1 expression.

The emerging evidence, discussed above, suggesting that down-regulation of FOXN1 might be a primary cause of age-related thymic involution has recently been tested in two studies, which have respectively determined the outcome of maintaining high-level FOXN1 expression throughout ontogeny [95], and of up-regulating FOXN1 function in the aged thymus [88]. The first used a strain of transgenic mice, hK14-Foxn1 (also known as Foxn1tg). In this strain the mouse Foxn1 cDNA, under control of the human K14 promoter, was introduced into the genome by random insertion, resulting in twenty-fold over-expression of Foxn1 in TECs due to multiple copies of the transgene [95]. These mice initially exhibited increased thymus size, with increased thymic output and numbers of early thymocyte progenitors (ETPs) [95]. However, although age-related thymic involution was delayed in this model, it was not prevented (Figure 3A,B) [95]. This suggested that FOXN1 is a target in age-related thymic involution, but that other targets might also exist.

The second study, from this laboratory, used a novel transgenic mouse strain, R26-Foxn1ERT2, which allows tissue-specific expression of a tamoxifen-regulatable form of FOXN1 (FOXN1ER) [88]. Using this model, we showed that increasing FOXN1 activity in TECs in 12- or 24- month old mice resulted in thymus regeneration, characterized by restoration of thymic architecture and functionality close to that found in young mice [88] (Figure 3C). This up-regulation of FOXN1 function led to increased expression of genes important for TEC biology and function, including Dll4, Ccl25, Kitl, Cxcl12, Ctsl, Cd40, Cd80,
Pax1, Trp63, Fgfr2IIIb, and Aire, to levels similar to those observed in the young thymus (Figure 4A) [88]. It also led to increased proliferation in immature TEC subsets, strongly suggesting that the observed thymus regeneration was instigated by a coordinated proliferation and differentiation of TEC progenitors [88]. These data show that up-regulation of FOXN1 function is sufficient to drive regeneration of the aged thymus, establishing FOXN1 as the primary target of the mechanisms driving age-related thymic involution. Of note is that uncontrolled differentiation of TEC progenitor/stem cells was not indicated in either the K14-Foxn1 or R26-Foxn1ERT2 models [76, 82], suggesting that other factors must interact with FOXN1 to regulate the balance between proliferation and differentiation.

**FOXN1: a master transcriptional regulator of TECs**

The evidence discussed above has established FOXN1 as a powerful mediator of TEC differentiation and maintenance.Remarkably, recent work from this laboratory has shown that overexpression of FOXN1 in an unrelated cell-type, mouse embryonic fibroblasts (MEFs), is sufficient to reprogramme the MEFs into functional TECs [96] (Figure 3B). These FOXN1-induced MEFs were shown to express genes indicative of TEC lineage identity, including Dll4, Ccl25, and Kitl (Figure 3B), and to provide a permissive environment for the maturation of ETPs to DP and SP thymocytes in vitro [96]. Furthermore, these ‘induced’ TECs (iTECs), upon transplantation under the kidney capsule of 5-6 week old nu/nu or syngeneic wild-type mice together with supporting thymic mesenchymal cells and immature thymocytes, went on to generate a fully functional thymus, with characteristic cortical and medullary architecture [96]. The iTECs were shown to express endogenous Foxn1, consistent with the positive auto-regulation of Foxn1 observed in the K14-Foxn1 transgenic mice, and iTECs recovered after transplantation expressed a range of genes required for TEC differentiation, proliferation and function (Figure 4B) [95, 96]. This study thus extends
previous understanding to establish that FOXN1 functions as a master regulator of TEC
differentiation, which is capable of initiating and maintaining the transcription factor network
required to promote TEC identity (Figure 4).

**Regulation of Foxn1 expression in TECs**

Given the importance of FOXN1 in thymus biology, there has been considerable interest in
its upstream regulation. However, information regarding transcriptional regulators of Foxn1 is
surprisingly scarce. Concrete evidence supports positive autoregulation of Foxn1 in TECs
[95, 96]. However, whether this is direct or indirect remains to be determined. Recently,
some members of the E2F family of transcription factors (E2Fs; specifically E2F3 and E2F4),
which mediate cell cycle progression among other functions and are negatively regulated by
Retinoblastoma tumour suppressor (Rb) proteins, have been shown to be able to bind to
their consensus binding site in the presumptive Foxn1 promoter in vitro [97]. Additionally,
increased activity of E2F3 in vivo was shown to correlate with increased expression of Foxn1
in TECs [97]. This link between E2F3 activity and Foxn1 expression was revealed by
analysis of compound transgenic mice that lack the Rb family genes Rb and p103, and carry
only a single copy of the third Rb family member p107 (Mx1-Cre;Rb\textsuperscript{lox/lox}; p130\textsuperscript{lox/lox}; p107\textsuperscript{-/-};
called Mx1-Cre p107-single mice). By 9 months old, these mice exhibit severe thymus
hyperplasia, characterized by increased cellularity and increased Foxn1 expression levels in
TECs. Further genetic analysis showed that reduced levels of Foxn1 expression (achieved
by breeding the Foxn1\textsuperscript{LacZ} allele onto the Mx1-Cre p107-single background) were sufficient
to reverse this hyperplastic phenotype, implicating RB and hence E2Fs in Foxn1 regulation
[97]. Another candidate transcriptional regulator of Foxn1 in TECs is the T box transcription
factor TBX1, mutation of which is thought to cause DiGeorge Syndrome [98, 99]: induced
expression of TBX1 in Foxn1 expressing cells of the E11.5 3PP resulted in down-regulation
of Foxn1 expression, indicating that TBX1 can repress Foxn1 transcription in TECs [100]. Consistent with its expected effect on Foxn1 repression, the forced TBX1 expression in Foxn1Cre::R26-iTbx1 thymi appeared to block TEC differentiation in an early progenitor state, evidenced by the accumulation of progenitor-phenotype cells (characterized by expression of Placenta expressed transcript 1; PLET1) and the absence of differentiated TECs in the fetal thymus [100]. However, it remains to be determined whether TBX1 regulates Foxn1 directly or indirectly, and in this light TBX binding sites have not yet been identified in the putative Foxn1 promoter regions [100]. Transcriptional regulation of Foxn1 in the hair follicle and skin may also provide some clues as to its regulation in TECs, and notably, a homeobox family member, Hoxc13, has been suggested to regulate Foxn1 in skin and hair follicle. However whether Hoxc13 is also involved in regulation of Foxn1 expression in TECs remains to be determined [101]. Finally, the BMP and WNT-signaling pathways have been implicated in regulating Foxn1 expression in TECs [102-111], although again, the molecular details have not been reported.

Surprisingly, the regulatory regions governing Foxn1 expression in TEPCs or TECs are also still only poorly characterized. Several studies have investigated whether a minimal genomic region surrounding the Foxn1 gene on mouse chromosome 11 can reproduce the wild-type Foxn1 expression pattern in skin and thymus. The largest region tested was 110kb, containing the entire Foxn1 locus with an additional 74kb of 5’-flanking sequence and 12kb of 3’-flanking sequence; this region rescued the nude phenotype in vivo, indicating that it contains all the regulatory elements required for normal expression of Foxn1 [112]. A 26kb region of genomic DNA encompassing the coding exons of Foxn1 plus 8.5kb of 5’-flanking sequence and 3kb of 3’-flanking sequence could rescue the hairless but not the athymic phenotype of nude mice, showing that it lacked at least some of the regulatory regions
required for Foxn1 expression in TECs [113]. However, a 30kb fragment containing the entire upstream sequence between the first coding exon of Foxn1 (exon-2) and the upstream gene Slc13a2 can recapitulate Foxn1 expression pattern in the developing thymus [114], although definitive characterization of its capacity to drive normal Foxn1 expression in the postnatal and adult thymus has not been provided. Within these regions, the promoter and enhancers governing the expression of Foxn1 in TEPCs and TECs remain to be definitely identified and similarly the identity of the tissue-restricted transcription factors important for its expression remains elusive.

Conclusions

FOXN1 plays a critical role in thymus biology, functioning as a master regulator of TEC differentiation, function and maintenance in the fetal and adult thymus and displaying remarkable potency as a regeneration and reprogramming factor. Further investigation of FOXN1 function will thus illuminate TEC biology during development, homeostasis and ageing, and contribute to a broader understanding of how master regulator TFs function to regulate and coordinate gene expression programs. Elucidation of the transcription factor networks responsible for regulating the initiation and maintenance of Foxn1 in different TEC subsets will also be crucial. This presents a major challenge, but should now become tractable in light of recent technological advances allowing interrogation of global gene expression and TF binding in single cells/small cell populations. In this regard, the recent identification of TBX1 and E2F as transcriptional regulators of Foxn1 should provide a tangible starting point for deciphering the molecular details of Foxn1 transcriptional regulation.

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Conflict of interest statement

CCB and ABL declare no financial or commercial conflict of interest. HJV is a consultant for a privately held company.

References


Table 1: *Foxn1* and its orthologues and paralogues through evolution (adapted from [46]). Table indicates the presence or absence of *Foxn1, Foxn4, Foxn4b and Foxn4l* in different subphyla or superclasses. Based on sequence and synteny homology, *Foxn1 and Foxn4* are thought to have arisen from a common ancestor gene, *Foxn4b*. Green denotes the presence of the gene in the genome.

Figure legends

Figure 1. Thymus structure and development. Schematic representation of a human thymus. Left panel shows location of the thymus, at the midline above the heart. Middle panel shows representation of a section through a young thymus, indicating the thymic cortex (c) and medulla (m). Right panel shows detail of stromal cells (thymic epithelial cells, TECs; dendritic cells, macrophages and blood vessels.) Note that mesenchymal cells and the vascular network are omitted for clarity, although the mesenchymal capsule bounding the thymus is shown. Haematopoietic progenitors enter the thymus at the junction between cortex and medulla. Commitment to the T-cell lineage and differentiation as far as the CD4⁺CD8⁺ ‘double positive’ (DP) stage of development occurs in the cortex. Thymocytes that successfully undergo positive selection can then enter the medulla, which is the site of central tolerance induction. CD4⁺ and CD8⁺ single positive (SP) T cells exit the thymus from the medulla (see references [2-7]). DN, CD4⁻CD8⁻ ‘double negative’ thymocytes.
Figure 2. Early events in thymus development. Schematic representation of early thymus development in the mouse. 3PP, third pharyngeal pouch. Grey ovals represent neural crest-derived mesenchymal cells. Red denotes region of GCM2 expression, marking the parathyroid primordium, blue denotes region of FOXN1 expression, indicating the thymus primordium. E10.5, E11.5 and E12.5 denote day 10.5, 11.5 and 12.5 of embryonic development, respectively.

Figure 3. Foxn1 expression levels and thymic involution. The level of expression of
*Foxn1* is correlated with and influences the age-related thymic involution. (A) During normal healthy aging, FOXN1 expression levels decrease concomitant with the decrease in size, organization and TEC functionality that characterizes age-related thymic involution. (B) Enforced high-level FOXN1 expression in TECs, as with the *Foxn1* tg transgene, delays but does not prevent age-related involution. (C) Induction of increased levels of functional FOXN1 in TECs in the fully involuted, aged thymus, leads to true thymus regeneration evidenced by increased thymus size, increased thymopoiesis and output of naïve T cells, and restoration of thymus architecture and TEC phenotype to close to those of the young thymus.
A. Young → Aged
   
   Foxn1 expression in TEC declines with age

B. Young → Aged
   
   Outcome: Age-related involution delayed but not prevented

   Enforced maintenance of FOXN1 expression

C. Aged → Aged regenerated
   
   Outcome: Regeneration, characterised by improved architecture and restored TEC phenotypes

   Levels of functional FOXN1 increased in TEC in old mice, following normal age-related decline
**Figure 4. FOXN1 – a master transcriptional regulator of TEC gene expression.** FOXN1 acts as a master transcription factor in TECs. It directly or indirectly regulates the expression of a number of genes in TECs during Foxn1-induced thymus regeneration (A) and transdifferentiation of MEFs to iTECs (B). The genes regulated by FOXN1 are responsible for a variety of functions in TECs, including differentiation, maintenance, and function, as well as including those with as yet unknown functions. Lower panel in (A) shows genes whose expression in TECs is down-regulated with age, and restored to close to juvenile expression levels when FOXN1 function is increased in aged TECs, grouped according to their known functions in TECs. Lower panel in (B), TEC identity and function is evidenced by expression of the genes shown. * denotes genes whose expression was demonstrated in MEF-derived iTECs recovered after transplantation but which were not present or not tested in iTECs prior to transplantation. Note that the cohort of genes regulated by FOXN1 in (A) and (B) is likely to be broader than depicted here.