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Review

Insights on *Foxn1* Biological Significance and Usages of the "Nude" Mouse in Studies of T-Lymphopoiesis

Zhijie Zhang*, Preston Burnley, Brandon Coder, and Dong-Ming Su✉

Department of Molecular Biology and Immunology, University of North Texas Health Science Center at Fort Worth, Fort Worth, TX, 76107, USA.

*Current address: Affiliated Tumor Hospital of Guangzhou Medical College, Guangzhou city, Guangdong Province, 510095, China.

✉ Corresponding author: Dong-Ming Su, Department of Molecular Biology and Immunology, University of North Texas Health Center at Fort Worth, 3500 Camp Bowie Blvd. Fort Worth, TX, 76107, USA. Tel. 1-817-735-5186, Fax: 1-817-735-2118, E-mail address: dong-ming.su@unthsc.edu

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Abstract

Mutation in the "nude" gene, i.e. the *FoxN1* gene, induces a hairless phenotype and a rudimentary thymus gland in mice (nude mouse) and humans (T-cell related primary immunodeficiency). Conventional *FoxN1* gene knockout and transgenic mouse models have been generated for studies of *FoxN1* gene function related to skin and immune diseases, and for cancer models. It appeared that FoxN1's role was fully understood and the nude mouse model was fully utilized. However, in recent years, with the development of inducible gene knockout/knockin mouse models with the *loxP*-Cre(ER^T) and diphtheria toxin receptor-induced cell abolished systems, it appears that the complete repertoire of FoxN1's roles and deep-going usage of nude mouse model in immune function studies have just begun. Here we summarize the research progress made by several recent works studying the role of *FoxN1* in the thymus and utilizing nude and "second (conditional) nude" mouse models for studies of T-cell development and function. We also raise questions and propose further consideration of *FoxN1* functions and utilizing this mouse model for immune function studies.

Key words: *FoxN1* gene, T-Lymphopoiesis

Introduction

The nude mutation [1] in the gene (*FoxN1,* forkhead box N1) [2-4], which encodes a transcriptional factor for the family of forkhead proteins, is responsible for this defect and has been known for a long time. The *FoxN1* (former name: *Whn* or *Hfh11*) gene, located on chromosome 11 in mice and chromosome 17 in humans [5-9], is mainly expressed in thymic epithelium, distinct keratinocyte populations in the epidermis, and hair follicles. *FoxN1* in rodents and *FOXN1* in humans are highly conserved in sequence and function [5, 9]. Mutations in *FoxN1* cause inborn dysgenesis of the thymus (thymic rudiment and lack of lymphocytes) [10-13] and hairless skin (short and bent hair shafts inside the skin) [9, 13-15], which happen in mice, rats, and humans. The *FOXN1* mutation in humans causes human nude (alopecia and nail dystrophy) and results in a primary T-cell deficiency [13, 16-18] related to severe infections, whereas the *FoxN1* mutation in mice results in the generation of nude mice, which have been widely used as a model [19, 20] for experimental oncological, immunological, dermatological, and transplantation studies due to their immune deficiency in T-cell development and failure in hair follicle development (nude skin).

However, in recent years, comprehensive understanding of the nude gene in the thymus and utility of nude mouse models for immunology and cancer studies are just now emerging. For example, with molecular technology moving forward, such as the development of the *loxP*-Cre/-CreER^T system [21-23], it appears that the precise roles of *FoxN1* are just beginning to be unveiled. In this review, we summarize recent findings in ongoing attempts to determine the functions of *FoxN1* in the thymus and to utilize nude and "second (conditional) nude" mouse models for studies of T-lymphopoiesis and T-cell function.

1. General roles of *FoxN1* in the thymus, skin, and possibly, the neuronal system.

Generally, *FoxN1*, a transcription factor, acts through its target genes in order to regulate the differentiation of epithelial cells. Specifically, *FoxN1* regulates keratinocytes to differentiate under proliferating conditions [24, 25]. The typical phenotypes resulting from an inborn null mutation of *FoxN1* are developmental failures in the skin and thymic epithelium [15]. Maturation of the thymic epithelial meshwork during thymic organogenesis occurs in two genetic stages [12, 26] – the first stage involves *FoxN1*-independent induction and outgrowth of the thymic epithelial anlage from the third pharyngeal pouch, controlled by genes such as the *Eya1* and *Six* [27], *Hoxa3* [28], and *Tbx1* [29, 30]. The second genetic step involves epithelial patterning and differentiation, which is *FoxN1*-dependent differentiation of the immature epithelial cells into functional cortical TECs (cTECs) and medullary TECs (mTECs). Recent reports emphasize *FoxN1* as a powerful regulator that promotes differentiation in both the cTECs and mTECs during thymus organogenesis [31]. *FoxN1* expression in the thymus is ambiguously believed to be expressed in all fetal TECs but not in all adult TECs [32, 33], while FoxN1-negative TECs are reported to be derived from FoxN1$^+$ TECs [33]. Therefore, which TEC subsets lose FoxN1 with age, and why these subsets lose FoxN1 with age has yet to be clearly identified. An inborn null mutation in *FoxN1* [3] causes a differentiation failure in TECs thereby halting thymic development at a rudimentary stage—the thymic lobe is still present but thymic lymphopoiesis is completely blocked [12, 34]. This causes an alymphoid thymus and severe primary T-cell immunodeficiency in nude mice and humans [8, 35, 36] with congenital alopecia and defective immunity, resulting in death in early childhood from severe infections [37, 38]. Therefore, the *FOXN1* mutation is a severe human primary immunodeficiency disease [13, 16-18, 39, 40].

In the skin, *FoxN1* is required for normal hair follicle development regulating the initiation of keratinocyte terminal differentiation, which has been well reviewed [9, 15]. *FoxN1* expression, mainly in the hair shaft cortex, was reported to peak during anagen—hair growth period, then fall during catagen (destruction) and telogen (rest) [9, 14, 15]. Recently, *FoxN1* was also reported to regulate pigmentation in the skin (related to skin darkness), demonstrated by using an engineered—keratin-5-driven *FoxN1* (K5-*FoxN1*) transgenic (Tg) mouse [41]. These authors found that while the *FoxN1*-null nude mouse completely lacks pigmentation in the hair cortex, K5-*FoxN1* Tg confers ectopic acquisition of pigmentation in hair cortical cells. This is said [41] to be due to regulation via the FoxN1-Fgf2 regulatory axis on pigment transfer from melanocytes to keratinocytes.

Although skin and thymus phenotypes resulting from the inborn *FoxN1*-null mutation are well-known, central nervous system deterioration, such as anencephaly, during the organogenesis resulting from this mutation has only recently been reported [36]. The Amorosi group found that *FoxN1* is expressed in the brain choroid plexus of murine embryos by using a *FoxN1* heterozygous mouse, in which one copy of *FoxN1* bears an inserted β-galactosidase (*LacZ*) reporter gene [12]. However, this leads us to ask why *FoxN1*-null gene knockout mice do not show a neural tube defect, and why do not all *FoxN1* deficient human fetuses have a neural tube defect [42]? Consequently, whether *FoxN1* mutation really causes congenital brain developmental abnormalities remains to be confirmed.

2. Introduction of conditional gene/cell manipulated system into *FoxN1* studies.

FoxN1 gene-manipulated mouse models, such as loss-of-function [12, 43] and gain-of-function [41, 44] models, have been available in the studies of *FoxN1* gene function. However, with molecular and cellular technology moving forward, many new methodologies have been developed. For example, the *loxP*-Cre/CreER^T-mediated conditional *FoxN1* gene "loss-resumption or revert" [31, 45] and the conditional *FoxN1* gene knockout [46] mouse models have been developed in recent years. This facilitates the determination of the precise roles of *FoxN1*.

These systems have been used to artificially (conditionally) control gene expression (conditional knockout or over-expression) in the mouse for a couple of decades [21-23]. The *loxP*-Cre/CreER^T system centered on the *Cre* gene, short for **c**yclization **re**combination [47]. The *Cre* gene encodes a site-specific DNA recombinase, which can recombine DNA at specific sites, which are 34-base pairs long, known as

loxP (locus of X-over P1) sequences. These sequences act as magnets for Cre to recombine the DNA fragment in between the two *loxP* sites, resulting in recombination-excision (deletion) of the *loxP*-flanked DNA fragment. If this excised DNA fragment is a functional part of a gene, its deletion will cause this gene to become dysfunctional. For example, in the conditional *FoxN1* gene knockout mouse (this mouse is now available at the Jackson Laboratory, #012941, http://jaxmice.jax.org/strain/012941.html) [46], the DNA binding domain, i.e. functional domain of transcription factor *FoxN1*, located on exons 5 and 6 of the *FoxN1* locus [48, 49], is flanked by two *loxP* genes (Fig. 1A). When this domain is deleted (termed ΔE5&6) by Cre or CreER^T, the *FoxN1* gene loses its function [46]. If this DNA fragment is a *loxP*-flanked *STOP* cassette (*STOP*^{flox}), a roadblock sequence positioned upstream of a functional gene or cDNA, the deletion of this *STOP*^{flox} will cause the gene to be re-expressed (Fig. 1B) or to resume (Fig. 1C). For example, in our unpublished novel *STOP*^{flox} –*FoxN1* transgenic mouse (Fig. 1B, this mouse is available by request), the flag-*FoxN1* cDNA (kindly provided by Dr. Brissette [25]) carried by a composite of CMV-immediate early gene enhancer/chicken β-actin promoter (pCAG) (kindly provided by Dr. McMahon [50, 51]) was inserted into a backbone of the *Rosa26* locus. In the front and the end of this fragment a *STOP*^{flox} cassette and IRES-GFP reporter gene were inserted, respectively. This makes conditional expression of the *FoxN1* transgene controlled by Cre/CreER^T. Furthermore, in conditional *FoxN1* gene "loss-resumption" or reversible mouse models [31, 45], a *STOP*^{flox} cassette (including two splice acceptors and a hygromycin or neomycine cassette) flanked by *loxP* sites is inserted into a normal *FoxN1* gene, which destroys and silences normal *FoxN1* transcription, resulting in an inborn mutant phenotype during organogenesis. After this *STOP*^{flox} cassette is depleted by introduction of Cre/CreER^T, the endogenous *FoxN1* expression resumes and the phenotype is reversed (Fig. 1C is an example).

Since *Cre* can be driven by different tissue-specific promoters, it can be uniquely expressed in certain tissues but not in others. Therefore tissue-specific promoter-driven *Cre* can achieve tissue-specific *loxP*-flanked DNA fragment deletion. This is one mechanism of conditional gene expression. The other mechanism is temporally-controlled gene expression in somatic cells rather than in germline cells. This can be achieved by *Cre-ER^T* gene [52, 53], which is the Cre-recombinase fused to a mutated ligand binding-domain of the human estrogen receptor (ER). The estrogen receptor binding-domain represses Cre in an inactive state until de-repressed by Tamoxifen (TM), because the ER binds TM but not estrogens. Therefore, deletion of the *loxP*-floxed DNA fragment is induced by administration of TM but not mouse or human estrogens [53, 54]. By combining a tissue-specific promoter with CreER^T, the *loxP*-flanked DNA fragment deletion can be controlled in a spatio-temporal fashion, thereby facilitating the introduction of a somatic mutation in a given gene, at a chosen time, in a selected cell type [21-23]. Particularly, this system benefits the study of the later roles of genes whose mutations cause early embryonic lethal phenotypes. Although a mutation of *FoxN1* will not cause lethality in embryos, its roles in the developed postnatal thymus and in different keratin-type epithelial cells would have largely remained unknown without the *FoxN1*^{flox} mouse model.

A new system for cell lineage ablation, based on transgenic expression of a diphtheria toxin receptor (DTR) carried by cell lineage specific gene and induced cyto-ablation via injection of diphtheria toxin (DT) has been developed in recent years [55-58]. Recently, this approach was used in the study of a *FoxN1*-positive thymic epithelial cell lineage [33]. Dr. Boehm's group clearly showed that after specific FoxN1$^+$ TEC lineage was killed (cytoablation) by induction with DT in early embryogenesis, the orthotopic thymus becomes aplastic, and these TECs cannot fully regenerate.

3. Identical or distinct roles of *FoxN1* in the skin and thymus.

Although the general role of *FoxN1* is to regulate the differentiation of epithelial cells in the thymus and skin, it was largely unknown whether the roles of FoxN1 in the thymus and skin are identical. If not, then how might they differ? The overt differences in *FoxN1*'s roles in the thymus and skin were revealed in a recently published paper [59]. One important difference is that *FoxN1* is involved in morphogenesis and maintenance of the three-dimensional (3D) thymic micro-structure, which is important for a functional thymus. As we know, two-dimensional (2D)-monolayer (non-Notch ligand transformed [60]) stromal cells cannot support T-cell development in culture. However re-aggregated stromal cell-constituted 3D pseudo-thymic lobes can fully support T-cell development in a fetal thymic organ culture (FTOC) setting. This is, at least in part, due to the alteration of certain key molecules. For example, dissociated thymic stromal cells lost the Notch ligand Delta-like expression, while re-aggregated thymic stromal cells (3D) regained its expression [61]. However, the normal micro-structure in the skin is

two-dimensional or polarized, i.e. the epithelial layer (basal layer) on one side expresses keratin (K)5 and K14, and the epithelial layer (apical layer) on the other side expresses K8 and K18. The other important difference is that *FoxN1* determines the pigmentation pattern in the skin [41], but this is inapplicable in the thymus.

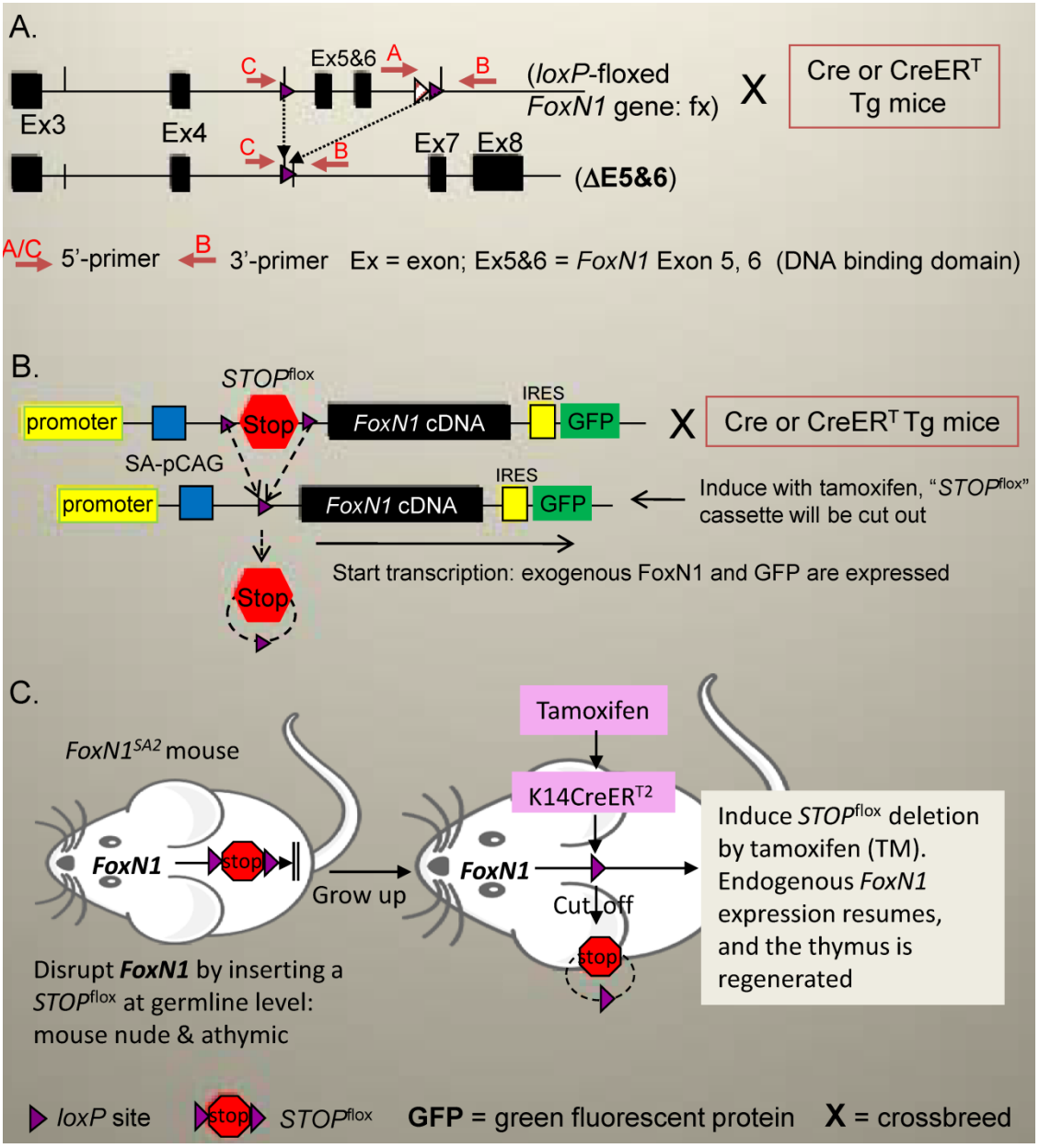


Figure 1. Schematic diagram of Cre/CreER^T-mediated *loxP*-deletion system in the *FoxN1* gene in mice. (A) *FoxN1* conditional gene knockout system [46]: *FoxN1* functional domain (exons 5 and 6, a DNA binding domain) is flanked by two *loxP* sites (termed "fx"). After introduction of Cre or CreER^T transgene (**Tg**) into these mice by crossbreeding (termed as "**X**"), and induction with tamoxifen (TM, only for CreER^T Tg), the *loxP*-flanked exons 5 and 6 are cut out (termed "ΔE5&6"), and the *FoxN1* gene loses its function (knockout). **(B)** *FoxN1* conditional transgenic system (under development): *FoxN1* cDNA (exogenous *FoxN1*) driven by an enhanced promoter and followed by a GFP reporter gene will be targeted into a housekeeping gene, such as *Rosa26*. Meanwhile, a *loxP* flanked "*STOP*" cassette (*STOP*^{flox}), a roadblock sequence, is placed upstream of *FoxN1* cDNA to block *FoxN1* expression initially. As soon as tamoxifen is administrated (for CreER^T Tg), the roadblock *STOP*^{flox} is deleted and transcription of exogenous *FoxN1* cDNA is turned on, and accompanied by GFP expression. **(C)** *FoxN1* resumption (loss- resumption) system [45]: A *STOP*^{flox} cassette (including two splice acceptors and a hygromycin cassette flanked by *loxP* sites) is inserted into the middle of the normal *FoxN1* gene, for example, just after exon 6, which destroys normal *FoxN1* splicing and silences *FoxN1* transcription. After the introduction of CreER^T Tg, such as K14-CreER^T, into these mice, and induced activation of K14-CreER^T with tamoxifen, the *STOP*^{flox} is cut out, and endogenous *FoxN1* expression resumes.

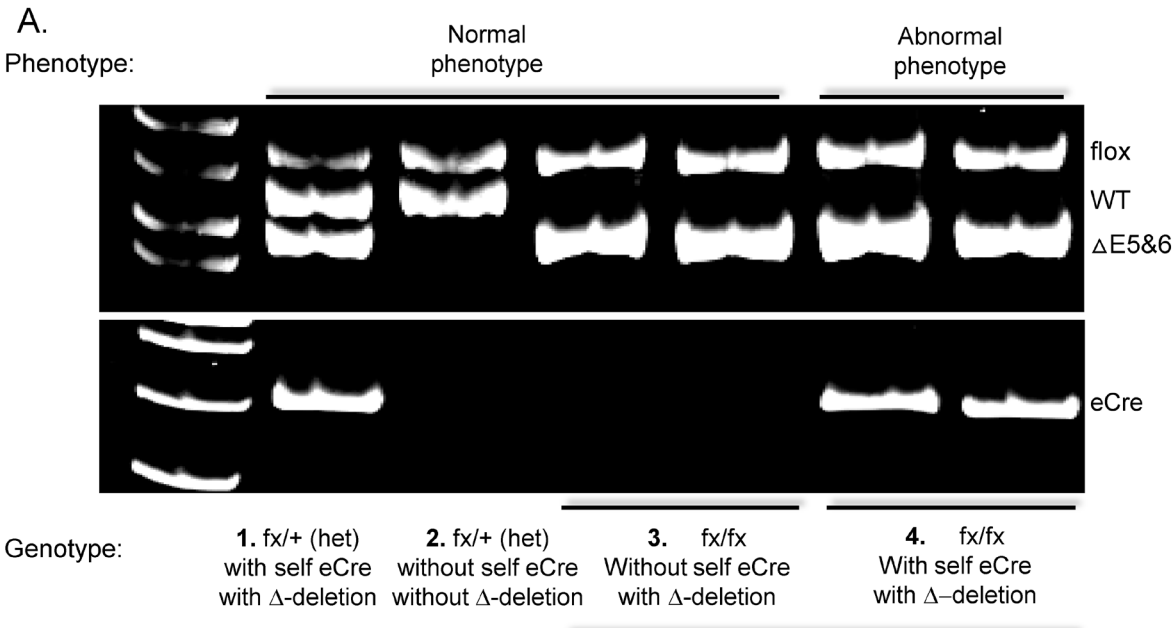
Additionally, using K14Cre transgenic mice [62] to delete *FoxN1*^{flox} in K14 promoter-driven epithelial cells seems to have a larger impact on the skin than on the thymus [59]. Deletion of *FoxN1*^{flox} in K14 epithelial cells is sufficient to cause a hair follicle defect resulting in a nude phenotype, similar to that of the natural *FoxN1*-null mutant mice, but does not induce an alymphoid thymic rudiment, thus differing from the thymic phenotype of the natural *FoxN1*-null mutant mice. It is unclear whether this phenotype is a result of low versus high expression of K14Cre in the thymus versus the skin. By using a K14Cre-mediated *LacZ* expression mouse model, which was generated by crossing K14Cre mice with *R26-STOP*^{flox}*-LacZ* reporter mice, in which the *STOP*^{flox} cassette is deleted upon K14Cre expression, thereby subjecting *LacZ* expression to be controlled by K14 promoter. Jackson Laboratory confirmed that the K14Cre-mediated *LacZ* is strongly expressed in the postnatal thymus, particularly in the thymic medulla (several images are posted in Jackson Laboratory web site: http://cre.jax.org/Krt14/Krt14-creNano.html). Therefore, the difference observed by Guo et al. should be due to the different impacts of *FoxN1* on K14 epithelial cells in the skin and thymus, rather than a result of lower expression of K14Cre in the thymus.

4. Roles of *FoxN1* in the prenatal only or both prenatal and postnatal thymus during thymic epithelial cell development and homeostasis.

As mentioned previously, there are *FoxN1*-independet and –dependent genetic stages, during thymic organogenesis and TEC differentiation [12, 26]. Owing to the lack of suitable genetic tools to address it, there was a long-running argument centered on whether *FoxN1* continues to maintain a functional thymus following the second genetic stage of thymic organogenesis, especially in the adult thymus. Gordon et al. generated a *FoxN1-LacZ* mouse model [63], in which a *LacZ* cDNA cassette was inserted into the 3'UTR of the *FoxN1* locus. Chen et al. observed that *LacZ* has an adverse effect on *FoxN1* expression with age, via a supposed methylation mechanism, to induce thymic postnatal involution [64]. Therefore, *FoxN1* was experimentally demonstrated to be required in the postnatal thymus. Because this mouse model cannot be spatio-temporally controlled, precise information of defects in timing and TEC subsets is not available, whereas, the inducible *FoxN1*^{flox} gene knockout mouse model can be used for addressing these question [46].

Recently, using *FoxN1*^{flox}-K14Cre mice, the Guo et al. [59] demonstrated that under certain circumstances the postnatal role of *FoxN1* may be even more important than its prenatal role. They found that homozygous *FoxN1*$^{flox/flox}$ mice without the *Cre* gene have *FoxN1*^{flox} deletion (Fig. 2A, genotype case #3) when their mother has the *Cre* gene. These mice have completely normal phenotypes in the thymus and skin. This deletion record comes from a historic Cre-mediated *FoxN1*^{flox} deletion and should happen in their prenatal life inside the mother's uterus. However, homozygous *FoxN1*$^{flox/flox}$ mice carrying their own *Cre* gene have a *FoxN1*^{flox} deletion (Fig. 2A, genotype case #4) and display mutant phenotypes in the thymus and skin. This *FoxN1*^{flox} deletion should happen in both prenatal and postnatal life. This finding demonstrated that *FoxN1* deletion happened inside the mother's uterus, driven solely by the parent's K14Cre (no Cre gene in offspring), which does not induce mutant phenotypes in the thymus and skin of the offspring. Instead, only when *FoxN1*^{flox} is deleted in both prenatal (mediated by mother's Cre) and postnatal (mediated by self Cre) are the mutant phenotypes in the thymus and skin induced. This confirmed the importance of postnatal *FoxN1*. However, this phenotype could not have been revealed without the *loxP*-Cre system because in the naturally occurring *FoxN1*-null mutation the *FoxN1* gene cannot be deleted separately in prenatal and postnatal life. Furthermore, this phenotype is not only found in *FoxN1*^{flox}-K14Cre mice, but also in other *FoxN1*^{flox}-Cre (resulting in germline deletion) mice, such as in ubiquitous *FoxN1*^{flox}-Cre mice (EIIa-Cre, Jackson Lab #003724) (Fig. 2). Therefore, *FoxN1* is required not only for prenatal epithelial patterning (previously known) but also crucial for postnatal epithelial homeostasis. Prenatal deletion (mediated by Cre inside the mother's uterus) of *FoxN1* alone cannot induce mutant phenotypes, but both prenatal and postnatal deletion (via offspring's own Cre) are able to induce thymic and skin mutant phenotypes.

The importance of *FoxN1*'s role in the postnatal thymus, beyond the second genetic stage [12] of thymic organogenesis in the fetal thymus is indisputable. Furthermore, this raises two intriguing issues: 1) FoxN1's probable role in the postnatal thymus is to regulate epithelial cell homeostasis. 2) Since postnatal TECs continue to undergo homeostasis, this process should be supported by tissue-specific stem/progenitor cells *in situ*. Therefore, adult thymic epithelial stem/progenitor cells probably exist in the postnatal thymus. This is a glaring issue and is discussed in the following section.



B.

Total litters observed	Total pups observed	fx/fx (all with Δ-deletion, their parents carried eCre)		
		Total fx/fx pups	Normal phenotype (without self eCre)	Abnormal phenotype (with self eCre)
21	123	45	26 (57.8%)	19 (42.2%)

Figure 2. Observe mutant phenotypes in mice with prenatal (mediated by parents' Cre, without self Cre) deletion of *FoxN1*, or with both prenatal and postnatal (mediated by parents' and self Cre) deletion of *FoxN1*. (A) A representative result of genomic DNA PCR using primers shown in Fig. 1A. Linking this result with the phenotype, we found that homozygous fx/fx (*FoxN1*$^{flox/flox}$) mice possessed ΔE5&6 recombination band but did not carry self eCre (EIIaCre – transgenic mouse from Jackson Lab #003724) – genotype #3 in the figure, did not show abnormal phenotypes in the skin and thymus. Therefore, the genotype #3 animals' ΔE5&6 recombination band came from their parents' eCre (their mother carried an eCre transgene). However, homozygous fx/fx mice carrying self eCre with a ΔE5&6 recombination band – genotype #4 in the figure, possessed abnormal phenotypes. **(B)** Table showing observed total fx/fx mice with/without self eCre Tg, linked to abnormal/normal phenotypes. Although they all have a ΔE5&6 recombination band, the abnormal phenotypes can only be seen in the mice with self eCre Tg.

5. Are all TEC subsets equally *FoxN1*-dependent in the postnatal thymus?

Based on anatomic, keratin type, and functional criteria, thymic epithelial regions can be divided into the cortex and medulla. The TECs in the cortical region are called cTECs, while those in the medullary region are referred to as mTECs. Both epithelia provide different microenvironments that are responsible for distinct stages in thymocyte development [26, 65]. As we know, *FoxN1* is required for the development of both cTECs and mTECs in the fetal stage [12]. However, since *FoxN1* is expressed in almost all TECs of the embryonic thymus, but not in all TECs of the adult thymus [32, 33], *FoxN1*'s role in different TEC subsets in the prenatal and postnatal thymus does not seem to be identical. Therefore the question arises, is *FoxN1* equally required for homeostasis of both cTECs and mTECs in the postnatal thymus?

In the fetal stage, *FoxN1* mainly regulates TEC patterning. Since both cTECs and mTECs arise from the same bi-potential TEC progenitors [45, 66], and *FoxN1* regulates the process of differentiation, it is straightforward to conclude that both mTECs and cTECs are equally *FoxN1*-dependent during fetal thymic organogenesis [67]. However, in the postnatal thymus, mTECs with the keratin type K5$^+$ and K14$^+$, which are similar to epithelial stem cell markers and exhibit progenitor activity in the skin and mammary gland [68-70], were more sensitive to the loss of *FoxN1*. cTECs possess keratin type K8$^+$ and K18$^+$, with the same marker as mature epithelial cells and terminally differentiated epithelial cells in the apical layer of stratified squamous epithelium of the skin. Additionally, cTECs are not as sensitive as mTECs to the loss of *FoxN1* using K5- and K18-CreER^T-mediated *FoxN1*^{flox}-deletion mouse models [46]. This finding in the postnatal thymus may account for the fact that

$K8^+/K18^+$ cTECs and $K5^+/K14^+$ mTECs are not equally *FoxN1*-dependent in the postnatal thymus. Although it may be due to a long half-life in cTECs compared to that in mTECs, *FoxN1* may not be required for mature/differentiated epithelia, which have $K8^+/18^+$ markers in the thymic cortical region and skin epithelial apical layer. However, *FoxN1* should regulate the immature/undifferentiated TECs (epithelial progenitor cells), which may be a small subpopulation present in the $K5^+/K14^+$ TEC populations, located in the medullary region and/or the corticomedullary junction (CMJ) in the postnatal thymus. Specifically, *FoxN1* in the adult thymus is required for adult TEC progenitors [33], which express $K5^+/K14^+$ markers, and these progenitors support TEC homeostasis in the adult thymus. A recent report further confirms this, showing that thymopoiesis depends on a FoxN1-positive TEC lineage, while FoxN1-negative TECs are descendants of FoxN1-positive TECs. FoxN1-negative TECs do not contribute to thymopoietic function in the adult thymus [33]. Further support of the existence of postnatal TEC progenitors, which are dependent on FoxN1, was made in Osada et al 2010 [71], where premature thymic involution was observed after inhibition of Wnt signaling through conditional expression of Dkk1 resulting in a decline in FoxN1 expression and loss of TEC progenitors.

6. Thymus development is sensitive to *FoxN1* dosage: it can neither be insufficient nor excessive.

Further progress in the recognition of *FoxN1* function in recent years was made by two reports that determined whether thymus development is sensitive to the genetic dosage of *FoxN1* and the association with age-related thymic involution. They also determined if heterozygous *FoxN1* (a half genetic dose of *FoxN1*, i.e. $FoxN1^{nu/+}$), which is known to be sufficient to induce TEC patterning in the thymic organogenesis, is also sufficient to maintain homeostasis for a steady-state normal thymus in the postnatal life. One report [64] showed that the mutant phenotype is dependent on *FoxN1* genetic dosage. The thymus in wild type (WT) mice is completely normal, and it is completely abnormal in *FoxN1*-null mice (natural *FoxN1*-null nude mouse). The abnormality lies in between these two extremes for *FoxN1*-null heterozygote (*nu*/+) and *LacZ/nu* chimera mice. The degree of severity is: WT < *nu*/+ < *LacZ/nu* < null in a genetic dose-dependent manner. Another study [72] using a $FoxN1^{flox}$ mouse carrying a ubiquitous $CreER^T$ transgene ($uCreER^T$), that took advantage of a low-dose spontaneous Cre leakage due to incomplete ER blockage *in vivo* [73], found that spontaneous leakage of $uCreER^T$ caused $FoxN1^{flox}$ deletion accompanying a progressive loss of $FoxN1^+$ TECs with accelerated age-related thymic involution. This also occurred in heterozygous $FoxN1^{flox/+}$ mice (deletion of floxed-*FoxN1* in one copy of *FoxN1* gene), representing a haplo-insufficient phenotype but related to age, i.e. age-related haplo-insufficiency. This finding extends previous observations in adult natural *FoxN1*-nu/+ heterozygous mice [74, 75].

Expression of *FoxN1* in the thymi of naturally middle-aged and aged WT mice is significantly reduced [76]. By increasing the dosage of *FoxN1* in these thymi via intrathymic administration of exogenous *FoxN1*-cDNA, a rapid gain-of-function approach, thymic involution and declining thymic function can be partially rescued [72]. Dr. Le's group confirmed this hypothesis at the genetic level with their *FoxN1* transgenic mouse model, which showed that an up-regulation of FoxN1 expression in the aged thymus can rejuvenate function of the atrophied thymus [44]. In their experiments, they ingeniously selected the *FoxN1* transgenic mice with low copy numbers for their observations. We found that highly over-expressed *FoxN1* induces adverse effects on thymus development (data unpublished), and even causes a lethal new-born phenotype (Fig. 3). In our newly generated $STOP^{flox}$-*FoxN1* transgenic mice (Fig. 1B), the *FoxN1* cDNA (kindly provided by Dr. Brissette [25]) is driven by the Rosa26 promoter and enhanced expression by a composite of CMV-immediate early gene enhancer/chicken β-actin promoter (pCAG) (kindly provided by Dr. McMahon [50, 51]). This results in high over-expression of FoxN1. In Figure 3, we show that K14Cre-mediated $STOP^{flox}$-*FoxN1* transgenic new-born mice died within 24 hours of birth. These neonatal mice share similar phenotypes with the involucrin promoter-driven *FoxN1* transgenic neonatal mice, which have ectopic and enhanced expression of FoxN1 [25], displaying abnormal skin and possessing open eyes at birth.

7. Utilizations of the nude or second nude mouse models in studying a T-cell developmental microenvironment and autoimmunity.

The nude mice or second nude (inducible *FoxN1* gene knockout) mice provide animal models to facilitate studies of T-cell development and postnatal T-cell function in immunity and autoimmunity related to human disease. Recently, Dr. Boehm's group designed elegant experiments by using a *FoxN1*-null (nude mouse) model to reveal that thymic epithelia

possess synergistic, context-dependent, and hierarchical functions in lymphopoiesis [77]. TECs of *FoxN1*-null mice were transformed by cDNAs of the chemokines Ccl25 and Cxcl12, the cytokine Scf, and the Notch ligand DLL4 carried by the *FoxN1*-promoter to generate *FoxN1-Ccl25, -Ccl12, -Scf, and –DLL4* transgenic mice under the *FoxN1*-null background. In these transgenic embryonic thymi, they found precise environmental components that can support mast cells, B progenitor cells, and T progenitor cells, respectively [77]. Another recent work using a tissue-specific *FoxN1*^{flox} gene knockout mouse model studied influenza infection in aging [78]. They found that K14Cre-mediated *FoxN1*^{flox} deletion-induced defects in the thymic medulla reduced antigen-specific CD8$^+$ T-cell and IgG responses to influenza virus, combined with increased lung injury, weight loss and mortality. These findings provided the first evidence that defects in the medulla directly causes changes in T-cell function that mimics aging defects during an immune response to an infectious agent [78]. A third recent work using the second nude (conditional *FoxN1*^{flox} gene knockout) mouse model addressed possible mechanisms of increased autoimmune susceptibility in the elderly [79]. Age-related disruption of steady-state thymic medulla caused by two-dimensional thymic epithelial cysts, primarily generated in the medulla, was found to perturb thymocyte negative selection. Negative selection is the main mechanism for the generation of central immune tolerance [80] necessary to prevent autoimmune diseases. This disruption was confirmed to provoke autoimmune phenotypes, such as inflammatory cell infiltration in multiple organs and the generation of anti-nuclear antibodies [79].

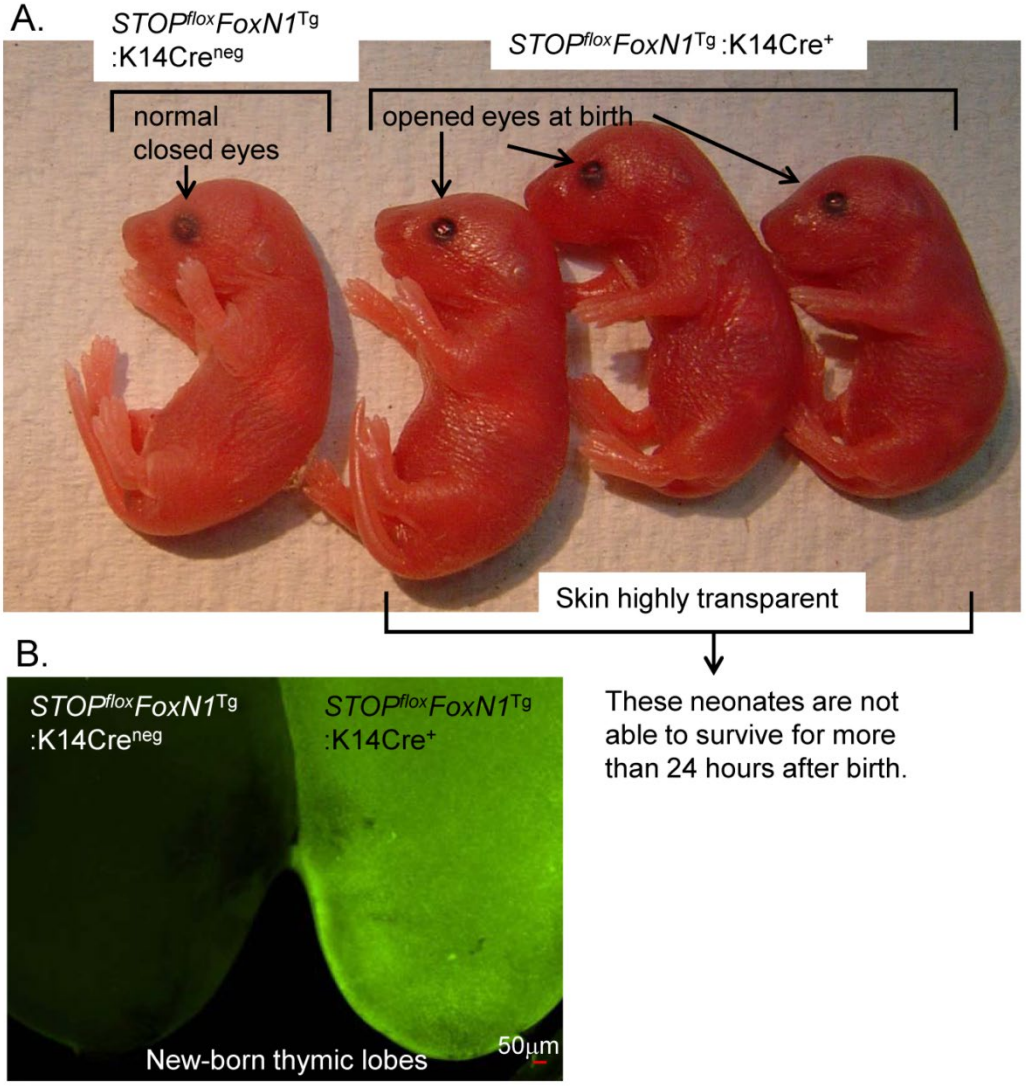


Figure 3. Over-expression of *FoxN1* in *STOP*^{flox}*-FoxN1* transgenic mice mediated by K14Cre results in neonatal lethality. (A) Image of eye opening can be seen in *STOP*^{flox}*FoxN1*^{Tg}*-K14Cre*$^+$ neonates (right three neonates), but not in control *STOP*^{flox}*FoxN1*^{Tg}*-K14Cre*^{neg} neonate (left). **(B)** Green fluorescence is shown in *STOP*^{flox}*FoxN1*^{Tg}*-K14Cre*$^+$ neonatal thymus (right), but not in control *STOP*^{flox}*FoxN1*^{Tg}*-K14Cre*^{neg} neonatal thymus (left).

8. Outstanding questions and future directions.

Although much progress has been made in recent years in unveiling the roles of *FoxN1* using advanced technology, quite a few questions in this field still exist. For example, if FoxN1 is involved in the regulation of adult thymic epithelial (TE) stem/progenitor cells, more experiments are required to understand the localization and characteristics of individual TE stem/progenitor cells from the postnatal thymus. There is an ongoing debate [81] about whether the adult thymic epithelial stem cells even exist and where they may be located, even though epithelial stem/progenitor cells have been implicated in the *FoxN1* "loss-resumption or revertible" (*FoxN1*^{SA2}) adult thymus [45]. In the *FoxN1*^{SA2} mice, a *loxP*-flanked hygromycin cassette was inserted into *FoxN1* introns 6-7 (Fig. 1C). This insertion destroys normal *FoxN1* splicing and silences the gene. The mice have a nude phenotype and a defective thymus. Upon hK14-CreER^{T} transgene activation in the adult thymus the insertion is deleted, and *FoxN1* is re-expressed in putative adult TE stem/progenitor cells, which can differentiate into normal cortical and medullary TECs and support normal thymic regeneration and function [45]. However, it can be argued that the TEC progenitors in the *FoxN1*^{SA2} defective thymus are dormant cells persisting from the fetal stage due to the *FoxN1* mutation, which may not represent TE stem/progenitors in the normal adult thymus. Further work is required to resolve the debate.

Another clue for TE stem/progenitors presenting in the natural adult thymus is provided by the *FoxN1*^{flox}-*K5CreER*^{T} mouse models, in which the *FoxN1* gene was conditionally knocked out in K5^{+} [46] epithelial cells after TECs fully developed in the adult thymus, resulting in acute thymic atrophy. This is probably due to the disruption of adult TEC homeostasis supported by TE stem/progenitor cells in the adult thymus. Since the K5 and/or K14^{+} promoters are active in epithelial stem/progenitor cells of the skin and mammary gland, the TE progenitors in the adult thymus may also be present within K5^{+} and/or K14^{+} TECs to support TEC homeostasis. However, the proportion and functional characteristics of adult thymic epithelial stem/progenitor cells that are K5^{+} and/or K14^{+} is largely unknown. Obtaining direct evidence by lineage-tracking changes in these adult thymic epithelial progenitors is a critical need.

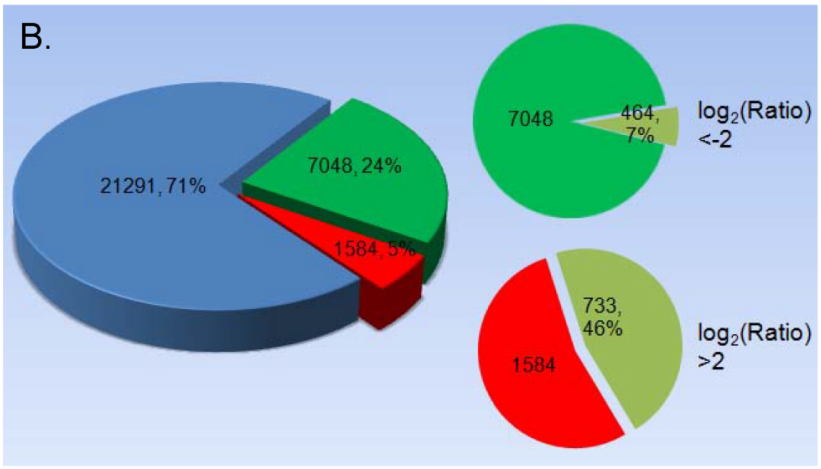
Regulation of TEC homeostasis is possibly co-regulated by *FoxN1* and other stem cell-related genes, such as *p63*. The transcription factor *p63*, which encodes for multiple isoforms (containing an N-terminal transactivation domain, termed as TAp63, and lacking this domain, termed as ΔNp63) [82], is pivotal for the development of stratified epithelial tissues, including the epidermis, breast, prostate, and thymus [83]. The role of *p63* in thymic development may be considered to be essential for the proliferation potential of thymic epithelial stem/progenitor cells [84, 85]. Specifically, thymic development is considered to be regulated by the ΔNp63 isoform through the maintenance of epithelial progenitor "stemness". By introducing ΔNp63 and TAp63 transgenes into a *p63* gene knockout background, ΔNp63, but not TAp63, is able to rescue defective thymus development [84]. Recently, we obtained a clue that the role of TAp63 in the thymus is probably associated with TEC senescence, since it was increased with thymic aging and associated with an age-related increase of senescent cell clusters (Manuscript under preparation). This phenotype may be accelerated by a blockade of TEC differentiation via conditionally knocking out the *FoxN1* gene. We suspect that p63 and FoxN1 may form a p63-FoxN1 regulatory axis in TEC homeostasis during aging. However, the mechanism controlling how the proliferation regulator *p63* and differentiation regulator *FoxN1* work collaboratively in the regulatory axis is still mysterious, and more studies are appreciated.

FoxN1 is a transcription factor whose functions are executed by targeting other genes through its DNA binding domain. Therefore, to understand its functional mechanisms in determining its target genes is important. However, the precise target genes that are regulated by FoxN1 remain ill defined, mostly due to technical difficulties in precisely isolating enough physiologically intact TECs at certain developmental stages. One group performed laser-capture micro-dissection to capture TECs from the E12.5 *FoxN1*-null nude mouse thymi, and found five *FoxN1* target TEC genes in their microarray analysis, of which programmed cell death-1 ligand is the only gene of known function [86]. However, emerging studies via immuno-histological methods suggest that changes in these five genes in the *FoxN1*-null mutant thymus have been undervalued. For example, *FoxN1* target Fgf2 has been identified in the skin [41]. *FoxN1* may target the *Notch* ligands, DLL-1 and DLL-4 [87], and the chemokines, CCL25 and CXCL12 [88], in nude thymic anlages. It may also target Notch-1 receptor in the skin as demonstrated in a transgenic mouse model [89]. We also preliminarily analyzed FoxN1 targeting genes by using a microarray assay from FACS sorted CD45^{-} EpCAM^{+} TECs. One TEC group was derived from ubiquitous-CreER^{T}-mediated *FoxN1*^{flox} knockout induced by TM in the postnatal thymus [46], the other

group was WT mouse TECs. We found that at least 5 groups (Fig. 4A) in over 300,000 genes screened underwent significant changes, either increasing or decreasing. Changes of log2 ratio > 2 or < -2 were observed in 1197 genes (Fig. 4B). The most interesting gene family is toll-like receptor (TLR), in which TLR4 shows a significant increase (Fig. 4C). We are conducting further work on this gene family to determine its physiological significance. Chromatin immunoprecipitation (ChIP) on Chip and ChIP on Sequence approaches may be one of the best ways to determine FoxN1 target genes.

A.

Top Bio Functions	p-value	# of affected
Molecular and Cellular Functions		**Molecules**
Cellular Development	7.62E-07 - 2.69E-02	138
Cell Death	1.75E-06 - 2.77E-02	134
Cellular Function and Maintenance	3.97E-06 - 2.60E-02	62
Cellular Growth and Proliferation	3.97E-06 - 2.95E-02	122
Cellular Movement	8.99E-05 - 2.40E-02	75



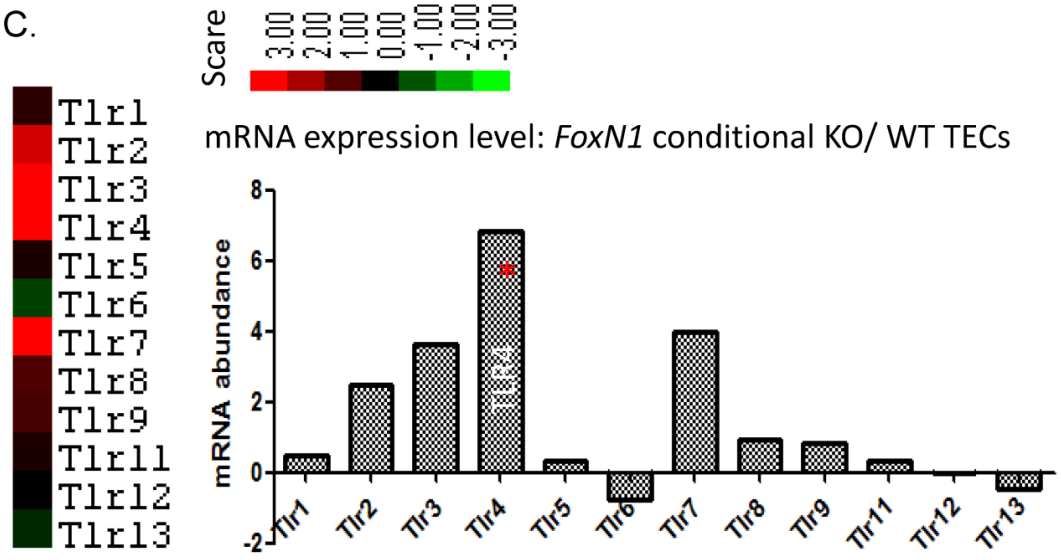


Figure 4. Microarray results of FoxN1 target genes. Total RNAs were isolated from flow cytometry sorted TECs ($CD45^-$ $MHC\text{-}II^+$) of young mice from ubiquitous-$CreER^T$-mediated *FoxN1*-deleted (tamoxifen x5 to conditionally delete *FoxN1* postnatally) thymic pool and WT thymic pool. **(A)** Number of affected molecules in top five bio-function groups; **(B)** Number and % of genes with upward (red) /downward (green) changes in total arrayed genes. Number and % of genes down to log2 ratio < -2 and up to log2 ratio > 2 are given; **(C)** One of the most involved genetic networks was the Toll-like receptor (TLR) pathway. As shown TLR4 was significantly increased after the *FoxN1* was conditionally knocked out.

Concluding remarks

Recent progress using advanced technology to study *FoxN1*'s roles in the thymus shows that *FoxN1* regulates not only TEC patterning in the fetal stage but also TEC homeostasis in the postnatal thymus. Comparing the thymus with the skin, *FoxN1* has its own distinct roles and impacts on organs in the generation and maintenance of three-dimensional microstructure and pigmentation, respectively. *FoxN1*'s role in the neuron has been brought up, but is still obscure. There is still plenty of room to apply nude and secondary nude (conditional *FoxN1* gene knockout) mouse models in studies of immunology, hematology, and tumorgenesis. The functional mechanisms of *FoxN1*'s collaborative roles with other genes during thymic development and aging remain to be further determined.

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Conflict of interest

We do not have conflict of competing financial interest.

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