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Review

Regulatory mechanisms of thymus and T cell development

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ABSTRACT

The thymus is a central hematopoietic organ which produces mature T lymphocytes with diverse antigen specificity. During development, the thymus primordium is derived from the third pharyngeal endodermal pouch, and then differentiates into cortical and medullary thymic epithelial cells (TECs). TECs represent the primary functional cell type that forms the unique thymic epithelial microenvironment which is essential for intrathymic T-cell development, including positive selection, negative selection and emigration out of the thymus. Our understanding of thymopoiesis has been greatly advanced by using several important animal models. This review will describe progress on the molecular mechanisms involved in thymus and T cell development with particular focus on the signaling and transcription factors involved in this process in mouse and zebrafish.

Contents

1. Introduction

The thymus, as the central hematopoietic site for making T cells which act as major players of the adaptive immune system in vertebrates, has been studied extensively since the 1960s by Miller and many others (Miller, 1961; Gordon and Manley, 2011). The

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thymus is comprised of two main components, the thymic epithelial cells (TECs) and the lymphoid thymocytes (T cells). Together with the surrounding mesenchymal cells, thymic epithelial cells, originally derived from the third pharyngeal endodermal pouch (anterior foregut), form an epithelial microenvironmental niche (the outer cortex and the inner medulla) that supports T cell differentiation. Several lines of evidence suggest that the medullary and cortical TECs originate from one common germ layer in the third pharyngeal pouch endoderm (Le Douarin et al., 1989; Rodewald et al., 2001; Gordon et al., 2004), and then undergo a series of differentiation and proliferation steps to form the functional TECs. The first phase in TEC development occurs during early embryonic gestation in a thymocyte-independent manner and the second thymocyte-dependent phase initiates in later phases of gestation while the thymus continues to develop and produce the compartmentalized structures which are finally organized after birth (Alves et al., 2009). Once specified into the T lymphocyte lineage, immature T cells undergo a successive and dynamic differentiation, including positive selection for T cell receptors (TCRs) in the cortex and negative selection to remove the self-immune responsive cells in the medulla. The postnatal thymus consists of three cell types, i.e., hematopoietic-derived cells ($CD45^+$), Foxn1-dependent cells including medullary and cortical TECs ($Keratin^+CD45^-$), and Foxn1-independent cells including mesenchymal cells, endothelial cells, and fibroblasts ($Keratin^-CD45^-$) (Rodewald, 2008).

Although this process has been extensively studied, the precise cellular origins and components of the thymus, the interaction between the TEC niches and the thymocytes, and the detailed molecular mechanisms employed during embryogenesis and immune system development are still largely unclear. Here we review recent progress on the current understanding of thymus organogenesis in vertebrates (mouse and zebrafish), mainly focussing on genetic control of TEC and T cell differentiation during early thymus development.

2. Thymus development in vertebrates

2.1. Thymus organogenesis in mouse

The thymus, which supports the development of self-tolerant T cells expressing a diverse repertoire of antigen receptors, is one of the essential components of the adaptive immune system (Rodewald, 2008). Besides morphological studies, cell biology and genetic approaches provide us with more detailed information on thymus organogenesis. The thymic anlage, adjacent to the developing primordium of the parathyroid, emanates from the third pharyngeal pouch endoderm and surrounding neural crest cells (NCCs) (Manley, 2000; Rodewald, 2008). At E11.5 in mice, the pouch endoderm is patterned into separate parathyroid and thymus-fated domains by the mutually exclusive expression of two transcription factors, *glial cells missing homolog 2* (*gcm2*) and *forkhead box N1* (*foxn1*) (Gordon et al., 2001). During further development, the common thymus/parathyroid primordia split at about E12, undergo a series of patterning, differentiation and morphological changes, and then migrate to their final positions (Manley and Condie, 2010). The parathyroid associates with the developing thyroid gland, while the thymus moves in a ventrocaudal direction to a location above the heart to form the orthotopic thymus (also called thoracic thymus) (Corbeaux et al., 2010). Recently, it is indicated that a second thymus emerges from the neck of mouse embryos, and expression of *foxn1* in cervical thymic epithelial cells indeed occurs later than that in the orthotopic thymus (Dooley et al., 2006; Terszowski, 2006; Corbeaux et al., 2010). Although small, the cervical thymus in mouse is functional and produces T cells only after birth (Dooley et al., 2006; Terszowski, 2006; Rodewald, 2008).

2.2. Thymus organogenesis in zebrafish

The zebrafish (*Danio rerio*) has recently become an important genetic model for the study of thymus and T cell development since blood development and the adaptive immune system are highly conserved throughout vertebrate evolution and there are many advantages to the zebrafish model. Zebrafish embryos are transparent at early stages with external fertilization and rapid development, which allows the visualization of hematopoietic stem cell (HSC) colonization and homing of lymphoid progenitors to the thymus *in vivo*. To facilitate this, several hematopoietic zebrafish transgenic lines expressing green fluorescent protein (GFP) or red fluorescent protein (RFP) have been developed, including, *cd41:*eGFP, *runx1:*GFP, *ikaros:*GFP, *lck:*GFP, *rag1:*eGFP, *rag2:*eGFP, and *rag2:*dsRed (Jessen et al., 1999, 2001; Langenau, 2004; Bertrand et al., 2008; Kissa et al., 2008; Lam et al., 2010).

In zebrafish, the thymic anlage arises from the pharyngeal endoderm, which develops from the epithelium between the third and fourth brachial arches, and can be detected around 48 h post-fertilization (hpf) by whole-mount RNA *in situ* hybridization (WISH) using a *foxn1* probe (Willett et al., 1999; Soza-Ried et al., 2008). After 48hpf, hematopoietic precursor cells colonize the thymus region, and then T lymphopoiesis is initiated following the expression of *rag1* at 78hpf (Langenau and Zon, 2005; Soza-Ried et al., 2008). By 1–2 weeks of age, the thymic epithelial cells are arranged into two distinct regions, the cortex and the medulla, both of which persist into adulthood (Lam et al., 2002; Langenau, 2004).

3. Genetic control of thymus and TEC development

3.1. Signaling pathways involved in thymus organogenesis

The thymic microenvironment, mainly composed of the thymic epithelial cells and other stromal cells, must be tightly controlled by extrinsic and intrinsic signals to support T cell differentiation and maturation. Several signaling pathways have been implicated in thymus and TEC development during vertebrate embryogenesis as discussed below.

3.1.1. BMP signaling

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor (TGF-β) superfamily and are known to be involved in cell-fate determination and patterning of the embryo, early thymus and parathyroid morphogenesis, and maintenance of a normal thymic microenvironment (Hauri-Hohl et al., 2008; Jeker et al., 2008; Gordon et al., 2010). Using two lacZ transgenic mouse strains, it has been shown that *bmp4* is expressed in the ventral region of the third pharyngeal pouch endoderm at E10.5 and E11.5, in the cells that will express *foxn1* and form the thymus, while *noggin*, encoding a BMP4 antagonist, is expressed in the dorsal region of the pouch and primordium, and thus appeared to be co-expressed with *gcm2* in the parathyroid domain (Patel et al., 2006). It is also found that some TECs from cortical and medullary areas express BMP receptors. *bmpr1a* and *bmpr2* are mainly expressed by cortical thymocytes while *bmpr1b* is expressed in most human thymocytes (Cejalvo et al., 2007). In addition, specific expression of *noggin* in TECs under the control of a *foxn1* promoter shows that BMP signaling is crucial for thymic stromal development rather than developing thymocytes (Bleul and Boehm, 2005). In the 427 thymic stromal cell line and purified stroma, BMP4 treatment up-regulates *foxn1* expression (Tsai et al., 2003), while the expression of *noggin* can block BMP signaling in TECs and lead to down-regulated expression of *foxn1* in *foxn1::Xnoggin* mice (Soza-Ried et al., 2008). Recently, it has been shown that BMP4 signaling promotes the expression of *foxn1* in the

epithelial-mesenchymal interactions that takes place during early thymus development in chick embryos (Neves et al., 2011). In order to test whether BMP signaling is an evolutionarily conserved requirement for the maintenance of *foxn1* expression in thymic epithelium, a transgenic zebrafish line expressing the BMP antagonist noggin under the control of a heat-shock promoter element has been generated. It is found that the expression of *foxn1* is abolished in zebrafish larvae when BMP signaling is blocked (Soza-Ried et al., 2008). Similar results are observed when using the BMP inhibitor dorsomorphin to treat zebrafish embryos from 82 to 108hpf (Soza-Ried et al., 2008). Taken together, this suggests that the BMP signaling involved in thymus development might act by regulating the expression of *foxn1* in thymic epithelium, thus controlling the differentiation and proliferation of TECs.

3.1.2. Wnt signaling

Wnt genes encode small secreted proteins that are required for cell-fate specification, progenitor-cell proliferation and asymmetric cell division (Staal et al., 2008). There are three different Wnt pathways: the canonical Wnt/β-catenin cascade, the non-canonical planar cell polarity (PCP) pathway, and the Wnt/$Ca2^{+}$ pathway (MacDonald et al., 2009). Among them, the canonical Wnt/β-catenin pathway is best characterized during thymus development. In the canonical Wnt pathway, when Wnt binds to its Frizzled receptor complex, the co-receptor low density lipoprotein receptor-related protein 5 (LRP5) or LRP6 becomes phosphorylated, the β-catenin degradation complex is inhibited, and β-catenin is released and translocated into the nucleus. By interacting with factors of the TCF/LEF HMG domain family (TCFs), β-catenin promotes the expression of a series of target genes.

wnt4, *wnt5b*, and *wnt10b* are expressed in the thymus at E13 and during adulthood as detected by RT-PCR. Immunohistology and *in situ* hybridization indicates that *wnt4* and *wnt5b* can be detected in pharyngeal pouches II to IV and are co-expressed with *foxn1* in pharyngeal pouch III (Balciunaite et al., 2002). A negative regulator of Wnt, Kremen1 (Krm1), is detected in both cortical and medullary TEC subsets. Krm1 is also involved in proper development of thymic epithelium, as $krm1^{-/-}$ mice exhibit a severe defect in thymic cortical architecture and many epithelial components remain at an immature Keratin 5^{+} (K5) Keratin 8^{+} (K8) stage, with a loss of defined cortical and medullary regions (Osada et al., 2006). Using a luciferase reporter assay in TEC lines, it is found that both Wnt1 and Wnt4 are able to signal to TECs. In addition, Wnt-mediated signaling can induce *foxn1* transcription in both an autocrine and paracrine fashion (Balciunaite et al., 2002). Overexpression of a downstream target of Wnt, β-catenin, is in itself sufficient to

Table 1
Transcription factors involved in T cell development in vertebrates.

Transcription factor	Gene family	Expression pattern	Function	References
Aire	PHD	Thymus, brain	"Education" of the T cells, accumulation of thymic DCs for Treg development	Mathis and Benoist (2009) and Lei et al. (2011)
Bcl11b	KLF	Thymus, brain, liver, etc.	Early T-cell commitment and T-cell identity maintenance	Liu et al. (2010)
c-Myb	MYB	Thymus, liver, etc.	Development of DN3 thymocytes, survival and proliferation of DP thymocytes, differentiation of SP T cells, proliferation of mature T cells	Bender et al. (2004) and Lieu et al. (2004)
E2A	bHLH	Tonsil, central nervous system, weak in the thymus, etc.	T cell progenitor proliferation at DN stage	Naito et al. (2011)
Ets1	ETS	Thymus, central nervous system, etc.	CD8 lineage differentiation by up-regulating Runx3, repression of Th17 differentiation	Moisan et al. (2007) and Zamisch et al. (2009)
Foxp3	FOX	Thymus, retina, etc.	Treg development and function	Marson et al. (2007), Mitra et al. (2010) and Quintana et al. (2010)
Foxo1	FOX	Heart, outflow tract, blood vessel, weak in the thymus, etc.	Development of $Foxp3^{+}$regulatory T (Treg) cells, survival and homeostasis of mature T cells	Carpenter and Bosselut (2010) and Kerdiles et al. (2010)
Gata3	GATA	Thymus, spinal cord, etc.	Early T cell survival and lineage determination, the expression of T cell receptor subunits, CD4 T cell and Th2 cell development	Taghon et al. (2007) and Wei et al. (2011)
HEB	bHLH	Widely expressed, not specific	T-cell lineage commitment, survival, β-selection, NKT cells development	Braunstein and Anderson (2011)
Ikaros	IKAROS	Thymus, AGM, liver, etc.	Repression of the expression of Notch target genes, required for the development of earlier lymphoid progenitors and the differentiation and expansion of T cells at early stage	Yoshida et al. (2006) and Kleinmann et al. (2008)
Klf2	KLF	Lung, retina, very weak in the thymus	T cell maturation from DP to the terminal development stage, help T cells emigrate from the thymus	Carlson et al. (2006) and Carpenter and Bosselut (2010)
Lef1	HMG	Thymus, nervous system, blood vessels, etc.	Required for the development of T cells and the expression of TCRα	Okamura et al. (1998)
LRF1	IRF	Thymus, brain, midgut, etc.	Development of NK cells, CD8 T cells, Treg	Ko et al. (2002) and Fragale et al. (2008)
RBPJ	TIG	Uterus, prefrontal cortex, weak in the thymus, etc.	Lineage commitment of T cells versus B cells	Tanigaki et al. (2003)
Runx1	RUNT	AGM, thymus, nervous system, etc.	Definitive hematopoiesis, T cell differentiation at the DN2/DN3 transition, CD4 repression, and Treg development	Okuda et al. (1996), Taniuchi et al. (2002) and Talebian et al. (2007)
Runx3	RUNT	Thymus, rib, central nervous system, etc.	CD8 T cell lineage determination and NK cell development	Ohno et al. (2008) and Setoguchi et al. (2008)
Sox13	HMG	Pons, placenta, spinal cord, weak in the thymus, etc.	Tαβ VS Tγδ cell differentiation	Melichar et al. (2007)
TCF1	HMG	Thymus, intestine, renal/urinary system, etc.	Upregulation of *gata3* and *bcl11b* and the T-cell receptor components, necessary for T cell expansion, survival, β-selection	Yu et al. (2010) and Weber et al., (2011)
THpok	BTB/POZ	Stomach, midgut, weak in the thymus, etc.	Required for CD4 T cell lineage determination	Wang and Bosselut (2009)
Tox	HMG	Thymus liver, spleen, etc.	Necessary for CD4 T cell leneage determination, partially through upregulate ThPOK	Aliahmad et al. (2011)

increase the expression of *foxn1*. It is also shown that Wnt-induced signaling through phosphatidylinositol 3-kinase (PI3K) contributes to *foxn1* expression, but the precise mechanism is unclear (Balciunaite et al., 2002). Recently, inhibition of canonical Wnt by Dickkopf-1 (DKK1) in adult mice results in rapid thymic degeneration, decreased TEC proliferation, and the development of cystic structures, with phenotypes similar to an aged thymus (Osada et al., 2010). Thus, canonical Wnt signaling within TECs is required for the maintenance of epithelial microenvironments in the postnatal thymus (Osada et al., 2010). In purified TECs, an age-related down-regulation of Wnt4 (and subsequently foxn1) and a prominent increase in LAP2α expression have been demonstrated. Therefore, Wnt4 and LAP2alpha are considered to be pacemakers of thymic epithelial senescence (Kvell et al., 2010).

3.1.3. Other signaling pathways

Sonic hedgehog (Shh) is expressed in the pharyngeal arches and is necessary for normal craniofacial development. Studies also show that Shh expression is excluded or highly reduced in the posterior/caudal pouches but present at high levels in the more anterior pouches in chicken and mice (Garg et al., 2001; Grevellec et al., 2011). T-box transcription factor 1 (Tbx1) is downstream of Shh signaling in mouse embryos, and Shh is sufficient to induce *tbx1* expression when misexpressed in selected regions of chick embryos (Garg et al., 2001). Tbx1 might be regulated by Shh signaling through the winged helix/forkhead box (Fox) transcription factors Foxa2, Foxc1 or Foxc2, which can bind to a single *cis*-regulatory element located in the promoter region of *tbx1* to maintain *tbx1* expression in the pharyngeal endoderm and the head mesenchyme (Yamagishi et al., 2003; Hu, 2004; Hollander et al., 2006).

TGF-β plays a regulatory role in most biological processes including control of somatic tissue development, cell proliferation, differentiation, and cell death. The expression of TGF-β and its receptors can be detected during thymic organogenesis at E10.5 in mice (Schmid et al., 1991). It is also found that TGF-β increases transcription of leukemia inhibitory factor *LIF* and *IL-6*, slightly decreases *IL-1β* transcription, but has little effect on *IL-1α* transcription, in cultured human TECs (Schluns et al., 1997). These data demonstrate that TGF-β can modulate cytokine production in TECs. Another study on the function of TGF beta signaling in TECs shows that it regulates thymic involution and post irradiation reconstitution (Hauri-Hohl et al., 2008).

Although several signaling pathways have been reported to regulate thymus development, the precise molecular mechanisms involved need to be studied further. Whether there are other signaling pathways that can regulate this process remains to be elucidated.

3.2. Transcription factors involved in organ patterning and initial organogenesis of the thymus

Studies on thymopoiesis have been carried out for several decades, and more recent studies have focused on the molecular mechanisms controlling cell differentiation, proliferation, and migration during thymopoiesis. So far, the involvement of many transcription factors have been identified (Table 1). In the organ patterning and initial organogenesis of thymus, the earliest player *hoxa3*, the *pax–eya–six* gene network, and *tbx1* are all considered to be essential (Manley and Condie, 2010). Soon after, the thymus-specific transcription factor *foxn1* is induced by early patterning events (Nehls et al., 1994) and *foxn1*-dependent TEC development is activated to establish a functional thymic rudiment.

The earliest regulator of organ patterning, *hoxa3*, is expressed in both the third pouch endoderm and surrounding neural crest cells (NCCs). In *hoxa3* mutants, development of all pharyngeal-derived organs is either abnormal or absent, initial thymus and parathyroid fates are not specified, and the shared organ primordium fails to form (Manley and Capecchi, 1995; Kameda et al., 2004). It has been shown that the *hoxa3–pax1* genetic pathway is required for both growth and differentiation of the epithelial cells (Su et al., 2001). In order to study the conservation of *hoxa3* function across species, a new *hoxa3* allele (*hoxa3*^{zf}) was generated by knocking zebrafish *hoxa3a* (*zfhoxa3a*) into mice. *zfhoxa3a* can substitute for mouse *hoxa3* in the development of thyroid, ultimobranchial body, tracheal epithelium, and soft palate. However, these mice have distinct or null phenotypes in the development of cranial nerve, thymus, parathyroid and NCCs (Chen et al., 2010). Therefore, *hoxa3* might act first to specify the identity of the third pouch, laying the ground work for induction of organ-specific identities (Manley and Condie, 2010).

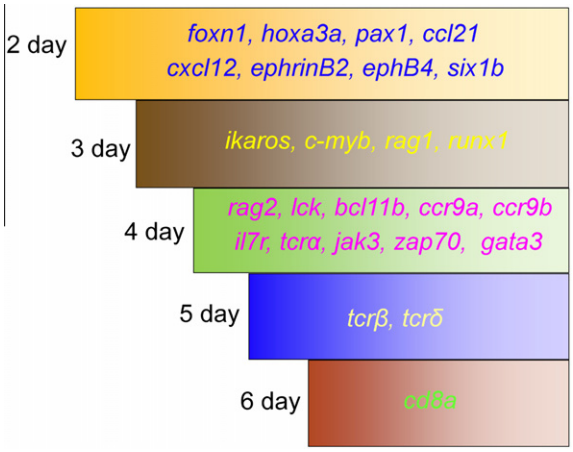

Fig. 1. Time-course of the expression of important genes in TEC and T cell development in zebrafish. Note that *tcrβ* expression at day 5 is from published literature, its earlier expression has not been reported yet.

The *pax–eya–six* gene network might act downstream of *hoxa3*. Several studies have shown that this network and its role in thymus and parathyroid development have been evolutionarily conserved in vertebrates (Heanue et al., 1999; Su and Manley, 2000; Su, 2001; Xu et al., 2002; Zou et al., 2006). *pax1* and *pax9* are both broadly expressed in the pharyngeal pouches and are required for normal thymus development in mice (Dietrich and Gruss, 1995; Wallin et al., 1996; Peters et al., 1998). *pax1* mutations result in a hypoplastic thymus deficient in thymocyte development (Dietrich and Gruss, 1995; Wallin et al., 1996), while *pax9* mutations result in a premature failure of thymus and parathyroid organogenesis (Peters et al., 1998). In addition, *eya1*, *six1*, and *six4*, which are also expressed in the third pouch and the surrounding mesenchymal cells, are necessary for early thymus organogenesis. Mutants of these genes all showed abnormal thymus and parathyroid development (Xu et al., 2002; Zou et al., 2006). *pax1*, *pax9*, *eya1*, and *six1* have all been identified in zebrafish. *In situ* hybridization indicated that *pax1* and *pax9* are expressed in the pharyngeal pouches (Nornes et al., 1996; Stock et al., 2006), while *eya1* is expressed in the inner ear sensory epithelial cell, neuromast, otic vesicle, and pharyngeal arch (Landgraf et al., 2010). Functional studies showed that zebrafish *eya1* and *six1* are required for lineage-specific differentiation of adenohypophyseal cells and are also involved in craniofacial development with another two cofactors, *sipl1* and *rbck1* (Nica et al., 2006; Lin et al., 2009; Landgraf et al., 2010). However, the detailed functions of the *pax–eya–six* gene cassette in zebrafish thymus and parathyroid organogenesis and other processes need to be explored (Fig. 1).

Another transcription factor involved in early thymus organogenesis is *tbx1*, which is associated with DiGeorge Syndrome (DGS) in human (Baldini, 2004). The *tbx1* gene is expressed throughout the third pharyngeal pouch during initial pouch formation, and

tbx1 mutation in mice results in phenotypes consistent with human DGS, including athymia and aparathyroidism (Jerome and Papaioannou, 2001; Xu et al., 2005). A specific time-dependent role of *tbx1* for the formation of the pharyngeal pouches and their derivatives is also reported in mouse (Xu et al., 2005). Zebrafish *tbx1* is expressed in the pharyngeal pouches, mandibular arch skeleton, and mesoderm. The zebrafish mutant *van gogh* (containing a mutation in the fish homolog of *tbx1*) displays defects in the pharyngeal arches and associated structures such as the thymus (Kochilas et al., 2003; Piotrowski, 2003; Hong et al., 2008). In addition, Tbx1 acts as a transcriptional activator invovled in head and pharyngeal arch development in *Xenopus laevis* (Ataliotis et al., 2005).

3.3. TEC-specific transcription factor Foxn1

The development of the epithelial component of the thymic stroma requires transcription factor Foxn1, as mostly illustrated by the phenotype of *nude* mice (Nehls et al., 1994). A single nucleotide deletion in the mouse *foxn1* gene causes a reading frame shift and thus loss of the DNA-binding domain of this FOX transcription factor. This recessive mutation in *foxn1* causes the hairless 'nude' phenotype in mice. More importantly, the same mutation results in a rudimentary thymus, with lack of T-cell development and defective thymic epithelial-cell differentiation and proliferation in mice, rats and humans (Nehls et al., 1994; Amorosi et al., 2008). *foxn1*-directed cytoablation further demonstrates that the epithelial progenitor cell expresses *foxn1* and thymopoiesis in mice depends on a *foxn1*-positive TEC lineage, whereas *foxn1*-negative TECs are descendants of *foxn1*-positive progenitors (Corbeaux et al., 2010). Boehm' team first cloned *foxn1* in zebrafish and found that it was also expressed in the epithelial compartment of the embryonic thymic rudiment in zebrafish embryos (Schorpp et al., 2002) (Fig. 1). Knock down of the expression of *foxn1* in zebrafish embryos using antisense morpholinos, significantly impairs T cell development partially because thymus homing was blocked (Bajoghli et al., 2009).

In addition to the essential role in regulating the growth and differentiation of all TECs, Foxn1 is also indispensable for vascularization of the murine thymus anlage. Moreover, a positive regulatory loop between FGFR3 and Foxn1 also plays a role in controlling keratinocyte (epithelial cells in the epidermis and hair follicles) differentiation (Mandinova et al., 2009; Mori et al., 2010).

Evolutionary analysis shows that *foxn1* is a paralog of *foxn4* in urochordates and all vertebrates, and a paralog of *foxn4l* in jawless fishes (Bajoghli et al., 2009). Although *foxn*1 occupies a central position in the genetic network(s) that establish a functional thymic rudiment, there is limited understanding of its downstream targets. Microdissection-based gene expression profiling has been carried out to compare wild-type and *nude* mice. A member of the B7 family, programmed death ligand 1 (PD-L1), was identified as a possible target of Foxn1 - its receptor, PD1, is thought to have a role in positive selection and prevention of autoimmunity (Bleul and Boehm, 2001). However, the relevance of this observation to the development of the athymic nude phenotype remains unclear.

The *nude* mice display failure of thymopoiesis owing to a non-functional epithelial microenvironment (Blackburn et al., 1996; Nehls et al., 1996), which lacks expression of chemokine genes and delta-like genes (Bleul and Boehm, 2000; Itoi et al., 2006). Chemokines are thought to be important for attraction of lymphoid progenitors (Bleul and Boehm, 2000), while delta-like genes are required for their specification toward the T cell lineage (Hozumi et al., 2008; Koch et al., 2008). Both *ccl25* and *dll4* genes are found to be down-regulated in TECs from *nude* mice, and similar phenomena are observed later in fish (Bajoghli et al., 2009). In the afore-mentioned work, *ccl25* and *dll4* are both up-regulated in transgenic medaka ectopically expressing mouse *foxn1*, while in the dominant negative *foxn1* transgenic line, the expression of these two genes are nearly abolished (Bajoghli et al., 2009). Although these genes are evolutionarily conserved and regulated by *foxn1*, detailed regulatory mechanisms remain unclear.

Several questions remain open for further studies. How does Foxn1 regulate thymus development through chemokines and delta-like genes? Can Foxn1 bind to the promoter region of these genes and directly regulate their expression? Are there other functional downstream targets of Foxn1 that need to be identified? Taken together, Foxn1 occupies a central position in the genetic network(s) that establish a functional thymic rudiment, but much remains to be learned about this network, including the precise molecular mechanisms involved in its regulation of thymus development.

4. An overview of T cell development

T cells, which develop in the thymus, are key players in cell-mediated immunity. The progenitors of T cells, the thymic settling precursors (TSPs) are derived from HSCs (Fig. 2). There are two waves of hematopoiesis in vertebrates, the primitive and the definitive. Only the definitive wave gives rise to HSCs which can differentiate into T cells and many other cell types (Orkin and Zon, 2008; Ciau-Uitz et al., 2010). In mammalian embryos, HSCs first emerge from the aorta-gonad-mesonephros (AGM), then colonize the fetal liver and eventually home to the thymus and bone marrow, where chemokine signaling is needed, for further differentiation or expansion (Calderón and Boehm, 2011). During thymopoiesis, T cell progenitors derived from the bone marrow, move through blood vessels and enter the cortical-medullary junction (CM junction) of the thymus. In zebrafish embryos, the first definitive HSCs are derived from the ventral wall of the dorsal aorta (VDA), and then migrate to the caudal hematopoietic tissue (CHT), eventually homing to the thymus and the kidney (Ciau-Uitz et al., 2010). In zebrafish, at the 3 days postfertilization (dpf) stage, T cell progenitors can be marked by *ikaros* in the area of the thymus which come from the kidney and some from the (CHT) (Murayama et al., 2006), but the exact migratory path is still unclear. Newly settled TSPs comprise only a very small proportion of blood cells, so identification is very difficult and has not yet been accomplished (Love and Bhandoola, 2011). It has been suggested that chemokine receptor 7 (CCR7), CCR9 (Schwarz et al., 2007; Zlotoff et al., 2010; Calderón and Boehm, 2011) and P-selectin glycoprotein ligand 1 (PSGL1) (Rossi et al., 2005) cooperatively regulate the process of thymic homing. After immigration, T cell progenitors begin to differentiate, expand and eventually mature in the thymus. In mice, distinguished by the cell surface markers, CD4 and CD8, T cells in the thymus can be divided into three successive development stages: double negative (DN), double positive (DP), single positive (SP)., However, CD4 and CD8 have not been identified in zebrafish making it difficult to investigate the late stage of T cell development in this model (Toda et al., 2011). Despite that, *rag1*, *ikaros*, *tcrα* and *tcrβ*, *tcrγ*, *tcrδ* are detected in the thymus area of zebrafish (Danilova et al., 2004; Langenau and Zon, 2005; Schorpp et al., 2006; Meeker et al., 2010), which indicates that there may be a similar mechanism driving T cell development as that in mouse. Based on the T cell markers emerging from the zebrafish thymus, we can roughly trace the process of T cell development in zebrafish as depicted in Fig. 1.

4.1. Positive selection

DN cells are derived from TSPs and further classified into four subsets based on the expression of CD44 and CD25: $CD44^{+}CD25^{-}$(DN1), $CD44^{+}CD25^{+}$ (DN2), $CD44^{-}CD25^{+}$(DN3) and $CD44^{-}CD25^{-}$ (DN4). DN1 cells, also termed early T cell progenitors

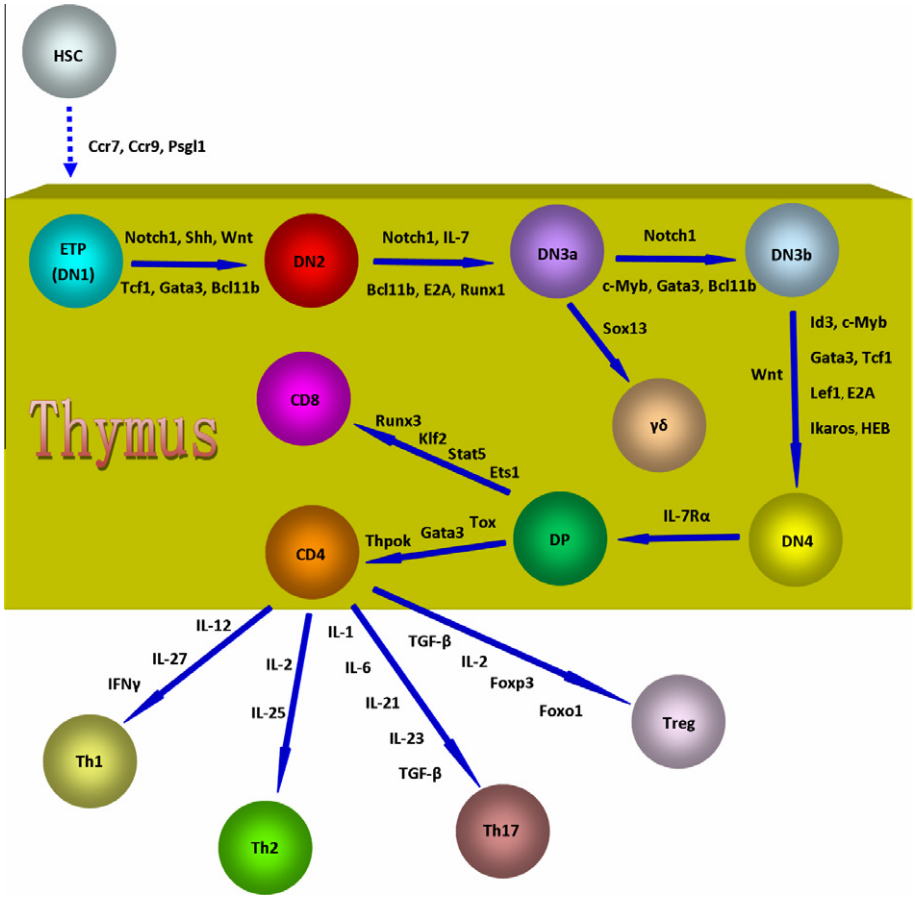

Fig. 2. Genetic regulation of early T-cell development. Distinct developmental stages during T-cell development are indicated by colored balls with essential signaling and transcription factors.

(ETPs, $LIN^{low}SCA1^{+}KIT^{+}$) (Love and Bhandoola, 2011), are a group of heterogeneous cells characterized by differential expression of CD24, CD117, CD90 (Thy-1) or CD127 (IL-7Rα). These cells retain their myeloid potential, and can differentiate into dendritic cells (DCs) and natural killer cells (NKs) (Porritt et al., 2004; Bell and Bhandoola, 2008; Wada et al., 2008). After contact with a series of signals provided by the TECs, these cells lose their multipotency at the DN2 stage (Rothenberg et al., 2008). At the DN3 stage, driven by a component of the RAG complex, the locus of *tcrb* begins to rearrange and TCRβ chain expression is initiated upon the successful rearrangement. TCRβ can form a pre-TCR complex with CD3 and pre-Tα (pTα) to provide a signal for T cell differentiation. Except for cells chosen to develop into γδ T cells, those failing to express the TCRβ chain will undergo apoptosis, so this process is also referred to as β-selection (Carpenter and Bosselut, 2010). After the accomplishment of β-selection, the immature T cell will begin to express CD4 and CD8, as well as rearranged TCRα, and become DP cells with the fully functional TCRαβ complex (Fig. 2).

Newly generated DP cells are a pool of cells containing randomly rearranged TCRαβ complexes. To ensure restricted major histocompatibilty complex (MHC) presentation on the cell surface, only cells with low avidity for interaction with self-peptide-MHC I/II presented by cTECs or DCs in the cortex can be selected to survive, and this process is called positive selection (Jameson et al., 1995).

4.2. Negative selection

Pre-T cells in the cortex which have passed positive selection will, via the CCR7 signal, move into the medulla. The majority of cells that interact with self-antigen presented by TECs or DCs in the medulla with a high avidity will undergo apoptosis then removed, i.e. negative selection, through Aire-dependent and –independent mechanisms, thus preventing autoimmunity. The remaining cells will be induced to differentiate into single positive (SP) T cells. SP cells are characterized by the expression profiles of CD62 ligand (CD62L) and CD69. The newly generated SP thymocytes are $CD62L^{low}CD69^{hi}$ while mature SP thymocytes are $CD62L^{hi}CD69^{low}$ (Takahama, 2006). Qa2 could be another useful marker for SP maturation, in addition to CD69 and CD62L (Li et al., 2007).

4.3. Emigration of T cells

After residence in the medulla for about 12 days, the mature T cells begin to emigrate out of the thymus. This emigration is regulated by a novel pertussis toxin-sensitive signaling pathway involving a G protein (Chaffin and Perlmutter, 1991). S1P1, which is a widely distributed G protein-coupled receptor (GPCR), is upregulated in mature thymocytes and plays a critical role in the egress of T cells into the periphery (Chaffin and Perlmutter, 1991; Allende et al., 2004; Matloubian et al., 2004).

5. Genetic control of T cell development in mammals and zebrafish

5.1. Signaling pathways in T cell development

The thymus is a delicate niche that provides all the signals required for the survival, proliferation, differentiation, apoptosis

and maturation of T cells. Here we summarize some critical signals that are indispensable for T cell development.

5.1.1. Notch

The *notch* was first cloned from Drosophila in the 1980s (Wharton et al., 1985; Kidd et al., 1986). It codes for a transmembrane receptor which is activated when interacting with its ligand, Delta and Serrate. In mammals, there are 4 Notch receptors (Notch1–4) and 5 Notch ligands (Delta-like 1 (DL1), DL3,DL4, Jagged1 and Jagged2) (Bray, 2006). In zebrafish, according to the Uniprot database, 4 Notch receptors (Notch1a, 1b, 2 and 3) and 8 Notch ligands (Dla, Dlb, Dlc, Dld, Dll4, Jag1a, Jag1b, Jag2) have been identified (www.uniprot.org/). When activated, the Notch is cleaved by a γ-secretase mediated complex that releases the intracellular domain of Notch (NICD), which can translocate into the nucleus and form a transcription activation complex with the DNA-binding protein CSL and Mastermind (Bray, 2006). Of the 4 Notch receptors, only Notch1 acts on early T cell development (Ciofani and Zúñiga-Pflücker, 2007).

Early in T cell development, the expression level of *notch1* increases from the DN1 (aks earliest thymus progenitors, ETPs) to DN3 stage (Yashiro-Ohtani et al., 2009). Notch1 provides important signals for the differentiation, survival, proliferation and metabolism of T cells (Yashiro-Ohtani et al., 2010). As mentioned above, newly settled ETPs retain their potential to different into myeloid cell, B cell and NK cell etc., and the Notch signal helps early T cell to suppress the multiple cell fate potentials and establish T cell identity (Radtke et al., 1999; Wilson et al., 2001; Bell and Bhandoola, 2008; Feyerabend et al., 2009). In fact, constitutive activation of Notch1 pathways in HSCs and/or CLPs (common lymphoid progenitors) results in the ectopic development of DP T cells in the bone marrow or *in vitro* (Schmitt and Zuniga-Pflucker, 2006; Maeda et al., 2007). However, Nocth1 is not sufficient for T cell commitment under physiological circumstances, since an array of transcription factors including *runx1*, *gata3*, and *bcl11b* are also required (Carpenter and Bosselut, 2010; Di Santo, 2010). In addition to its role in T cell commitment, a low level of Nocth1 signal can promote β-selection (Pear et al., 2006; Taghon et al., 2009). Notch1 promotes survival of pre–T cells at the β-selection checkpoint by regulating cellular metabolism through the PI3K-Akt pathway (Ciofani and Zúñiga-Pflücker, 2005). Although Notch1 is very important for Tαβ development, the differentiation of the Tγδ lineage is not dependent on Notch signaling. In other words, Notch1 does not affect the lineage choice of TCRαβ vs TCRγδ (Wolfer et al., 2002; Tanigaki et al., 2004; Ciofani et al., 2006; Garbe et al., 2006; Taghon et al., 2006). At the β-selection checkpoint, the expression of *notch1* decreases significantly (Kee, 2009), suggesting that the Notch1 signal may fail to affect T cell differentiation beyond the DN3 stage (Fig. 2). E2A-dependent *notch1* transcription is negatively regulated by Id3, an antagonist of E2A, during thymocyte development (Radtke et al., 2010), and the abnormal expression of *notch1* can lead to T-cell acute lymphoblastic leukemia (Li and von Boehmer, 2011). Overexpression of the human *notch1* intracellular domain (NICD) in zebrafish driven by the promoter of *rag2*, leads to T-cell leukemia (Chen et al., 2007), implying that Notch1 may have a similar role during normal T cell development in zebrafish.

5.1.2. Wnt

Wnt signaling is required for normal thymocyte development, both at the pro-T-cell stage and at later stages of thymocyte differentiation (Gounari et al., 2001; Staal et al., 2001; Mulroy et al., 2002; Staal and Clevers, 2003). Of the three different Wnt pathways, the canonical and the Wnt/$Ca2^+$ pathway have been reported to affect T cell differentiation and proliferation (Staal and Clevers, 2003; Staal et al., 2008; Weber et al., 2011).

Wnt acts on T cells at as early as the DN1 stage (Verbeek et al., 1995; Okamura et al., 1998; Weerkamp et al., 2006) (Fig. 2). The Wnt target transcription factor TCF1 plays a critical role in the expansion of double-negative thymocytes by up-regulating the expression of many T-cell essential genes, including *gata3* and *bcl11b* and components of the T-cell receptor (Schilham et al., 1998; Weber et al., 2011). *tcf1*-deficient T cells fail to differentiate or expand at the DN stage (Schilham et al., 1998). In addition, another component of the Wnt pathway, LEF-1, a transcription factor with a HMG box DNA binding domain, shows some redundancy with TCF-1 in regulating T cell differentiation and TCRα expression. Double knock-down of *lef1* and *tcf-1* has a more severe effect on T cells than the single knock-out of TCF-1. During the DN to DP stage (Fig. 2), Wnt signaling provides a signal for T cell proliferation and facilitates β-selection. When overexpressing mouse DKK-1 (mDkk-1), the inhibitor of Wnt LRP co-receptor, T cells were blocked at the most immature DN1 stage (Weerkamp et al., 2006). However, overexpression of the inhibitor for β-catenin and TCF1 (*icat*) which disrupts β-catenin-TCF interactions, blocked T cells at the transition of DN to DP (Pongracz et al., 2006). Overexpression of *icat* also impairs the survival of thymocytes by reducing the expression of *bclxL* (an anti-apoptotic protein, a member of the Bcl-2 family) (Hossain et al., 2008). These results are consistent with a report that deletion of either Wnt-1 or Wnt-4 in mice led to a decrease of the number of thymocytes, and the double knock-out causes a more severe decrease of both immature and mature thymocytes (Mulroy et al., 2002). T cell–specific deletion of β-catenin can impair T cell development at the β-selection checkpoint (Xu et al., 2003). Also, loss of Adenomatous Polyposis Coli (APC), which deregulates β-catenin signaling, promotes the proliferation of T cells at the DN3 and DN4 stages and reduces gene rearrangements of the TCRβ, leading to aberrant thymocytes lacking pre-TCR and αβ TCR (Gounari et al., 2005). In contrast, conditional stabilization of β-catenin in immature thymocytes results in the generation of SP T cells that lacked the αβ TCR and develops in the absence of pre-TCR, but these T cells show reduced proliferation and survival capacity (Gounari et al., 2001). During the transition from DP to SP stage, β-catenin is up-regulated. It is demonstrated that in a transgenic mice (CAT-Tg), increased expression of stabilized β-catenin enhances positive selection of thymocytes and accelerates generation of CD8 SP thymocytes, which may be achieved through enhancing IL-7 signaling (Yu and Sen, 2007; Yu et al., 2007). Conditional stabilization of β-catenin promotes negative selection while *tcf-1* deficiency can inhibit negative selection. The β-catenin /TCF-1 cascade affects this process by modulating the intracellular strength of TCR signaling, leading to altered expression of mediators of thymocyte survival (Kovalovsky et al., 2009). In terms of non-canonical Wnt signaling, *wnt5a* deficiency down-regulates *bax* expression, promotes *bcl-2* expression, elevates β-catenin in thymocytes and inhibits apoptosis of DP thymocytes. In contrast, overexpression of *wnt5a* increases apoptosis of fetal thymocytes in culture, indicating that Wnt5a induction of the non-canonical Wnt/$Ca2^+$ pathway is important for normal T cell development (Liang et al., 2007). Taken together, Wnt signals exert multiple effects which guide T cell development.

In zebrafish, Wnt signaling has been demonstrated to be involved in HSC development by interacting with Prostaglandin E2 (PGE2) (Goessling et al., 2009). However, its role in T cell differentiation is still unknown.

5.1.3. IL-7

IL-7 is a critical cytokine essential for normal development of B cells and T cells (Fry and Mackall, 2005) and a key molecular marker for lymphoid progenitors. It is produced by nonhematopoietic stromal cells in multiple organs including thymus, lymphoid organs, skin, intestine, and liver (Schlenner et al., 2010). IL-7 deficient

mice display a significant reduction of thymocyte expansion and blocked *V–J* recombination at the TCRγ locus, indicating that IL-7 plays a crucial role in T cell development (Peschon et al., 1994; Maki et al., 1996; Puel et al., 1998; Roifman et al., 2000). With an IL-7 receptor α (Il7rα) Cre recombinase knockin mouse, Schlenner et al. demonstrates that Il7r labeled all T cells but few myeloid cells, indicating that IL-7 may have an effect on early T cell lineage determination (Schlenner et al., 2010). In addition, in a mouse fetal liver cell culture, Ikawa et al. reveals that reduced concentrations of IL-7 at the DN2 stage can up-regulate Lck, Tcf1, pTα, and Bcl11b to promote T cell lineage commitment (Ikawa et al., 2010). At the DN2 stage, IL-7 is important for recruiting histone acetylases to the TCRγ locus and facilitates V(D)J recombination. Moreover, the T progenitors expressing high levels of *il7* tend to become Tγδ (Maki et al., 1996; Huang et al., 2001; Kang et al., 2001; Ye et al., 2001). It is interesting to note that, in the thymus of mice, IL-7Rα is detected in $CD4^{-}CD8^{-}$ DN T cells and also in CD4 or CD8 SP cells but not in $CD4^{+}8^{+}$ DP cells (Sudo et al., 1993), while in human thymus, *il7* is expressed from ETP (DN1) to DP cells (Vicente et al., 2010), indicating a possible role for IL7 in positive selection. In fact, overexpression of stabilized *β-catenin* in T cell progenitors in mice can augment IL-7Rα-chain expression and promote the development of CD8 SP thymocytes during positive selection (Yu et al., 2007). However, the differentiation of B cells in mice but not human requires an IL-7 signal (Peschon et al., 1994; Puel et al., 1998; Roifman et al., 2000), indicating that the temporal function of IL-7 is not always identical in human and mouse. In summary, IL-7 exerts a crucial function on early T cell expansion, lineage determination, Tγδ vs Tαβ choice and may also affect positive selection. In zebrafish, three mutations affecting key components of the IL-7 receptor, *Il-7ra*, *Jak3*, and *Jak1*, lead to remarkably reduced *rag1* expression, revealing a conserved role of IL-7 signaling in T cell development (Iwanami et al., 2011).

Moreover, BMP (Hager-Theodorides et al., 2002; Tsai et al., 2003; Cejalvo et al., 2007; Varas et al., 2009), Hedgehog (El Andaloussi et al., 2006; Crompton et al., 2007; Hager-Theodorides et al., 2009; Outram et al., 2009; Rowbotham et al., 2009; Bommhardt, 2010; Drakopoulou et al., 2010; Hanson et al., 2010), FGF (Tsai et al., 2003), TGFβ (Do et al., 2010; Ouyang et al., 2010) and Retinoic acid (Mulder et al., 1998) are morphogens that regulate T cell progenitor development. They are all expressed in TECs and provide external signals for thymocytes in a cell non-autonomous way. Among these morphogens, BMP and Shh are extensively studied and accumulating evidence has demonstrated that they mostly exert negative effects on T cell development at multiple facets including differentiation (Outram et al., 2000; Hager-Theodorides et al., 2002), proliferation and survival (Tsai et al., 2003; Varas, 2003; El Andaloussi et al., 2006; Cejalvo et al., 2007), T cell subsets choice (Melichar and Kang, 2007; Drakopoulou et al., 2010), positive selection (Takagi et al., 2001; Crompton et al., 2007), and negative selection (Hager-Theodorides et al., 2009). In zebrafish, BMP is required for the maintenance of *foxn1* expression in the TECs, and blocking BMP signal causes a drastic decrease of *foxn1* and *rag1* (Soza-Ried et al., 2008). Whether BMP can directly regulate T cell development in zebrafish remains unclear.

5.2. Transcription factors

Gene regulatory networks (GRNs) describing key regulatory players during T cell development have been reviewed quite well recently (Rothenberg et al., 2008; Naito et al., 2011). Many transcription factors have been identified that are important for T cell development in mammals, involving cell fate specification, differentiation, survival, expansion, negative or positive selection and migration (Rothenberg et al., 2008; Naito et al., 2011). Here, we summarize what have been known about transcription factors involved in vertebrate T cell development below as well as in Table 1. However, very little is known about the roles of the transcriptional pathways required for T cell development in the zebrafish.

As mentioned before, Notch1 helps ETPs to get over multipotential and adopt T cell identity. TCF-1, a high-mobility group (HMG) box-containing transcription factor, is positively up-regulated by Notch1 in the very early T cell progenitors and plays a significant role in early T cell development. *tcf-1*$^{-/-}$ hematopoietic progenitors fail to acquire T cell fate when cocultured with OP9-DL1 stromal cells (Germar et al., 2011; Weber et al., 2011). *tcf-1*$^{-/-}$ progenitors also lose their ability to differentiate or expand when cultured within fetal thymic organ cultures or injected intrathymically into normal recipients (Schilham et al., 1998). Ectopic expression of *tcf-1* in long-term hematopoietic stem cells (HSCs) on OP9 stroma can drive the development of T-lineage cells (Weber et al., 2011). In spite of its important function, the detailed mechanism is still far from fully elucidation. Weber et al. reported that overexpression of *tcf-1* increases the expression level of *bcl11b*, *gata3* and *cd3e*, while Germar et al. observed that the expression of *notch1*, *hes1*, *gata*3, *bcl11b*, *runx1*, and *ikaros* are not affected in the *tcf-1*$^{-/-}$ DN1 thymocytes (Germar et al., 2011; Weber et al., 2011). As a component of canonical Wnt signal, TCF1 also exerts important role beyond the early DN stage as discussed above.

T cells lineage commitment is achieved until DN3 stage. Bcl11b, a two zinc finger containing transcription factor, increases at the DN2 to DN3 stage, suppresses the alternate lineage potential of and specifies T cell fate (Di Santo, 2010). *bcl11b*-deficient DN2 cells express elevated amounts of NK-promoting genes, *id2*, *Il2rb*, *nfil*3, and *plzf*, and cannot progress to DN3 stage but acquire a NK-like phenotype (Li et al., 2010a,b). Ikawa et al. reveal that Bcl11b may be a sensor to the down-regulation of IL-7 and helps T cell progenitors to establish T lineage fate (Ikawa et al., 2010). With ChIP assay, Li et al. reveal that *bcl11b* is also a direct target of Notch1 (Li et al., 2010a,b).

Besides lineage commitment, proliferation is also critical for the development of T cell. It is reported that E2A, HEB, and Gata3 all regulate the cell expansion and survival of T cell progenitors. Both E2A and HEB are HLH transcription factors. E2A deficiency leads to the reduced cell number from DN1 to DN3 stage (Dias et al., 2008). Loss of HEB at DP stage results in a greater proliferation but these cell show a less survival rate (D'Cruz et al., 2010). At DN3 stage, E2A and HEB can block IL-7-mediated proliferation to facilitate TCRβ gene rearrangement and preclude the accumulation of TCRβ negative cells beyond the pre-TCR checkpoint (Engel and Murre, 2004; Wojciechowski et al., 2007). Moreover, HEB is important for T cell lineage determination since *heb*$^{-/-}$ DN3 cells adopt a DN1-like phenotype and could be induced to differentiate into NK cells (Braunstein and Anderson, 2011). E2A and HEB also positively regulate the expression of pre-*tcrα* by directly binding to its promoter (Takeuchi et al., 2001; Tremblay et al., 2003) and promote the rearrangement at the loci of *tcrδ* and *tcrγ* (Langerak et al., 2001). Thymocytes lacking *heb* fail to launch *V–D–J* rearrangement and initiate *tcrα* expression (D'Cruz et al., 2010). Gata3 is required for the cell-autonomous development of the ETPs. In the absence of Gata3, the cell number of ETPs will be severely reduced (Hosoya et al., 2009). Proper level of Gata3 is important for T cell linage determination and its overexpression can induce T lineage precursors deviate and adopt to a mast cell fate instead (Taghon et al., 2007). The expression of *tcrβ*, as well as genes involved in CD4 T cell development and Th2 cell differentiation also require Gata3 (Naito et al., 2011; Wei et al., 2011). Moreover, many other transcription factors like Ikaros, Lef-1, Runx1 etc. all have critical functions during T cell development whose functions are summarized in Table 1.

6. Perspective

Substantial progress has been made during the past decades with the use of several animal models and *in vitro* cell culture systems, which greatly further our understanding of the molecular mechanisms of thymus and T cell development under normal and pathological conditions, providing more potential for the development of clinical therapies for immunological diseases, such as autoimmunity, etc. Importantly, with the rapid advancement of ES and iPS cell technologies, we may predict potential cell therapies using transplantable TEC and T cells generated *in vitro* in the near future.

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