Molecules and Cells

Minireview

The Roles of RUNX Family Proteins in Development of Immune Cells

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The Runt-related transcription factors (RUNX) transcription factors have been known for their critical roles in numerous developmental processes and diseases such as autoimmune disorders and cancer. Especially, RUNX proteins are best known for their roles in hematopoiesis, particularly during the development of T cells. As scientists discover more types of new immune cells, the functional diversity of RUNX proteins also has been increased over time. Furthermore, recent research has revealed complicated transcriptional networks involving RUNX proteins by the current technical advances. Databases established by next generation sequencing data analysis has identified ever increasing numbers of potential targets for RUNX proteins and other transcription factors. Here, we summarize diverse functions of RUNX proteins mainly on lymphoid lineage cells by incorporating recent discoveries.

Keywords: development, immune cells, Runx family, transcription factors

INTRODUCTION

The Runt-related transcription factors (RUNX) transcription factors are present in all metazoans, and there are three members of the RUNX family proteins in mammals that play pivotal roles in multiple developmental processes. Initial genetic studies in mice have characterized the essential roles of RUNX1, RUNX2 and RUNX3 in hematopoiesis (Okuda et al., 1996; Wang et al., 1996a), osteopoiesis (Komori et al., 1997; Mundlos et al., 1997; Otto et al., 1997) and neurogenesis (Levanon et al., 2002), respectively. However, as we comprehensively discuss in this review, recent studies have revealed that the functions of the RUNX family proteins in regulating development are much more complex than previously thought. Moreover, these transcription factors have additional functions in regulating cellular function beyond development. For instance, RUNX proteins are shown to be a functional regulator of tissue-resident T cells (Milner et al., 2017), and they have been reported to exert either oncogenic or tumor suppressive roles in the development of hematopoietic cancer as well as solid tumors such as gastric and colon cancers (Ito et al., 2015). One of the mechanisms for their diverse roles is the generation of multiple isoforms from each *RUNX* locus by alternative splicing events of RUNX transcripts (Nieke et al., 2017) and using two alternative promoters, a distal and proximal promoter. In addition, RUNX proteins undergo various post-translational modifications such as phosphorylation (Guo and Friedman, 2011), acetylation (Jin et al., 2004), methylation (Zhao et al., 2008) and sumoylation (Kim et al., 2014) (Fig. 1). All of these modifications are predicted to contribute to the functional complexity of RUNX proteins.

RUNX proteins contain a well-conserved DNA-binding domain (Runt domain). Heterodimerization with an essential binding partner protein, core binding factor beta (CBFβ), increases the DNA binding affinity of RUNX proteins (Wang et

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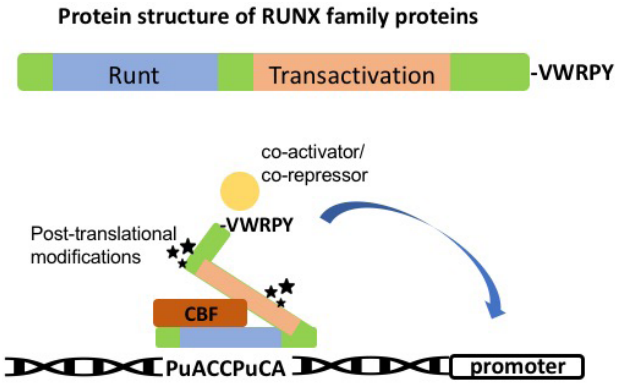

Fig. 1. Protein structure of RUNX family proteins. All RUNX proteins have evolutionarily conserved Runx domains with DNA binding capability. Transactivation domain is required for the transcriptional activity of RUNX proteins by interacting with multiple proteins such as P300 and SMADS. The C-terminal penta-peptide sequences, VWRPY, is responsible for the recruitment of transcriptional co-repressors such as Groucho-TLE and HDACs. In addition, RUNX proteins are reported to be post-translationally modified by phosphorylation, acetylation, methylation and sumoylation.

al., 1996b). Transcripts generated from the *Cbfb* gene is also subjected to alternative splicing and yield at least two functional isoforms, thus adding another layer to the complexity of RUNX proteins (Tenno et al., 2018). The Runt domain binds to a target locus by recognizing the specific sequence of 5'-PuACCPuCA-3' (Fig. 1). The other evolutionarily conserved motif in the RUNX protein family is the VWRPY-motif located at the C-terminal end. This penta-peptide appears to serve as a platform to recruit transcriptional corepressors such as Tle/Groucho (Xing et al., 2018), thereby significantly contributing to RUNX mediated gene repression. RUNX proteins also interact with repressive chromatin modifiers including mSin3A, HDAC (histone deacetylases) and PRC (polycomb repressive complex). On the other hand, to initiate transcriptional activation, RUNX proteins also recruit many transcription coactivators such as p300, ETS-1, NERF-1/2, c-Fos, and AP1. With these structural complexity and numerous interaction partners, RUNX proteins have been suggested to exert multiple functionality and regulate cell fate decision at many developmental branches of hematopoietic lineages. In this short review, we will focus on the roles of RUNX proteins in regulating development and function of immune cells, mainly T lymphocytes, by incorporating recent findings. As the readers will find out, it is very interesting that RUNX proteins are heavily involved in the generation and/or maintenance of many T cell subsets.

RUNX AND THYMOCYTE DEVELOPMENT

T cell development occurs in the thymus and it begins with early T cell progenitors that are negative for CD4 and CD8 coreceptor expression, thereby referred to as double-negative (DN) thymocytes. A RUNX1-dependent differentiation process leads DN cells to become CD4 and CD8 double-positive (DP) thymocytes that express T cell receptor (TCR) (Fig. 2) (Egawa et al., 2007; Kim et al., 1999). It has been shown that RUNX1 mediates DN3 to DN4 transition by promoting the proliferation of thymocytes that have passed the selection process, known as β-selection (Fig. 2). During β-selection, DN3 cells that failed to express a functional TCRβ chain are eliminated. RUNX1 protein also facilitates activation of *Tcrb* locus by specifically binding to a transcriptional enhancer, known as Eβ, located on the 3' side in the *Tcrb* locus (Seo et al., 2017) (Fig. 2). Therefore, ablation of RUNX1 in early stages of T cell development by using *Lck-cre* results in the blocks of T cell development before the DP stage. DP thymocytes choose their fate to become either MHC Class II-restricted $CD4^+$ helper T cells or MHC Class I-restricted $CD8^+$ cytotoxic T cells. This CD4 helper versus CD8 cytotoxic lineage choice is controlled by two signature transcription factors, ThPOK and RUNX3, respectively. Ectopic expression of ThPOK redirects MHC Class I-restricted cells to $CD4^+$ T cell lineage, whereas loss of ThPOK activity results in the redirection of MHC class II-restricted cells to $CD8^+$ T cell lineage. This illustrates that ThPOK is an essential factor for the commitment of MHC class II-restricted cells to $CD4^+$ helper lineage. Similarly, overexpression of RUNX3 redirects a proportion of class II-restricted cells to $CD8^+$ lineage, even though ablation of RUNX3 alone does not result in significant redirection of MHC class I-restricted cells to $CD4^+$ T cell lineage. Importantly, ThPOK and RUNX3 repress the expression of each other, and thus reciprocal interplay between ThPOK and RUNX3 is the hallmark in the transcriptional regulation of helper versus and cytotoxic T cell lineage choice.

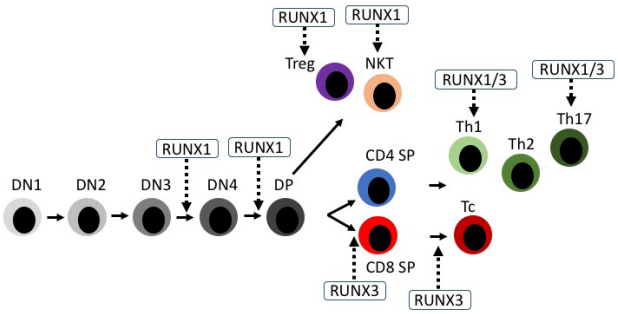

Fig. 2. The function of RUNX proteins during T cell development. Thymic T cell progenitors which are $CD4^-CD8^-$ double-negative (DN) progress in 4 stages (DN1 to 4) until becoming $CD4^+CD8^+$ double-positive (DP) expressing TCR on the cell surface. TCR expression requires TCR beta chain rearrangement which requires activation of a transcriptional enhancer, known as Eb, within *the Tcrb* gene by RUNX1. RUNX1 is also essential for the proliferation of DN4 cell during transition to the DP stage. RUNX proteins also play important roles in other lineages of T cells such as Treg, NKT, Th1, Th17, cytotoxic T cells, etc.

For many years, we have been trying to understand how TCR signaling in DP progenitors is translated into the exclusive expression of ThPOK versus RUNX3 in MHC class II-restricted and MHC class I-restricted cells, respectively (Fig. 2). The most widely-spread notion on this subject is that the strength and/or duration of TCR signaling during thymocyte differentia-

tion must play a key component. The kinetic signaling model proposed by Alfred Singer group suggest the following. The duration of TCR signaling is translated into the lineage choice. DP thymic progenitors terminate *Cd8* gene transcription after positive selection regardless of MHC restriction of their TCR (Zeidan et al., 2019). While TCR signaling is prolonged in MHC-class II restricted DP thymocytes with continuous CD4 expression, it is temporally terminated in MHC-class I restricted cells due to termination of *Cd8* transcription. Thus, the kinetic signaling model suggests that long and short duration of TCR signaling is translated into transcriptional program for helper and cytotoxic T cell development, respectively. In addition, MHC Class II-selected thymocytes intrinsically receive stronger TCR signals, because the Src family tyrosine kinase Lck associates more strongly with the cytoplasmic tail of CD4 than that of CD8 coreceptor (Ha-Lee et al., 2000). This stronger TCR signaling has been shown to be linked to ThPOK induction (Sun et al., 2005). Interestingly, after the termination of *Cd8* expression the transcriptional induction of *Runx3* gene in MHC-class I restricted cells was shown to be regulated by interleukin 7 (IL-7) signaling (Park et al., 2010). Thus, the duration and strength of TCR signaling (continued and strong signaling for $CD4^+$ lineage and switching to IL-7 signaling for $CD8^+$ lineage after the termination of *Cd8* transcription) is utilized to translate extracellular information into the helper/cytotoxic lineage choice.

RUNX proteins continue to function after the T cell lineage decision since continued repression of *Thpok* and *Cd4* by RUNX3 is a critical event for the differentiation of the cytotoxic T cell lineage. RUNX proteins repress *Thpok* transcription (Setoguchi et al., 2008) by binding to the transcriptional silencer in the *Thpok locus* together with another transcription factor MAZR (Sakaguchi et al., 2010; 2015; Setoguchi et al., 2008). RUNX proteins also directly repress *Cd4* expression by binding to the intronic transcriptional silencer of *Cd4* locus which contains RUNX recognition sites (Taniuchi et al., 2002). Recent studies showed that the activity of this *Cd4* silencer is required for DNA methylation of the *Cd4* locus (Sellars et al., 2015; Tsagaratou et al., 2014), indicating that DNA methylation is one critical epigenetic tool that RUNX utilizes to regulate the expression of target genes. DNA methylation on CpG island is one of the main epigenetic ways to control gene expression, and it is mechanistically generated or removed by DNA methyltransferases (DNMTs) and ten-eleven translocation (TET) proteins, respectively. Further studies, however, are required to fully understand the mechanism of how the interactions between RUNX proteins and the *Cd4* silencer control the recruitment of DNMTs and TETs and hence promote the epigenetic regulation of *Cd4* gene.

As previously mentioned, mature $CD4^+$ T and $CD8^+$ T cells stably express ThPOK and RUNX3, respectively, to maintain their lineage identity. This all-or-none reciprocal expression pattern, however, is variable, as it has been reported that a proportion of intestinal $CD4^+$ intraepithelial lymphocytes (IELs) show much lower expression of ThPOK (Mucida et al., 2013). Instead, these cells acquire RUNX3 expression which is associated with the expression of CD8αα coreceptor ($CD8\alpha^+CD8\beta^-$) (Reis et al., 2013). This indicates that signals from the gut microenvironment control transcriptional program of intraepithelial $CD4^+$ T cells, which make them distinct from conventional $CD4^+$ helper T cells. In addition, the acquisition of RUNX3 expression by $CD4^+$ IELs correlates with the unique expression patterns of CD8αα IEL resembling features of cytotoxic $CD8^+$ T cells. Therefore, suppression of ThPOK and induction of RUNX3 by $CD4^+$ IELs are necessary steps for the acquisition of CTL-like features of CD8αα IELs.

RUNX AND EFFECTOR T CELL DIFFERENTIATION

Early works on RUNX proteins focused on primary development of conventional T cells. However, recent studies have given new insight on the importance of RUNX in the differentiation of effector and memory T cells. For example, RUNX3 functions beyond the development of naïve $CD8^+$ T cells and when naïve $CD8^+$ T cells encounter foreign antigens RUNX3 plays a critical role in driving transcriptional programs for their effector functions. In addition, the role of RUNX3 in promoting $CD8^+$ effector function is synergized with the help of T-box proteins such as T-bet (Cruz-Guilloty et al., 2009). Moreover, during the resolution of immune responses, RUNX3 is also required to epigenetically reprogram the surviving cytotoxic $CD8^+$ T cells into effector memory $CD8^+$ T cells. These epigenetic changes mediated by RUNX3 are based on the modification of chromatin accessibility of target gene loci (Wang et al., 2018).

Naïve $CD4^+$ helper T cells differentiate into effector Th1 or Th2 subset depending on the nature of environmental cues. This bifurcation is regulated by two transcription factors, T-box expressed in T cells (T-bet) and GATA-binding protein-3 (GATA-3), respectively. It was reported that RUNX1 negatively regulates GATA-3 and thus inhibits Th2 cell differentiation (Komine et al., 2003). Indeed, RUNX1 overexpression was shown to be enough to enforce Th1 cell differentiation even in Th2-stimulating culture condition *in vitro*. Interestingly, RUNX3, the mediator of the cytotoxic T lineage, was reported to be expressed during the differentiation of Th1 cells and help the establishment of Th1 subset (Djuretic et al., 2007). During the differentiation of Th1 subset, RUNX3 not only interacts with T-bet but also augments the activity of each other to attenuate IL-4 expression and enhances interferon gamma (IFNγ) expression. Therefore, RUNX1 and RUNX3 together contribute to drive Th1 program. A recently discovered transcriptional repressor Twist1 has been reported to negatively regulate Th1 differentiation by interfering with the expression and function of RUNX3 (Pham et al., 2012).

RUNX1 and RUNX3 are also reported to be involved in the differentiation of Th17 cells (Wang et al., 2014). Th17 cells produce IL-17, IL-21 and IL-22 to defend hosts from extracellular pathogens by recruiting macrophages and neutrophils to infected area. The differentiation of Th17 cells requires the transcription factor RAR-related orphan receptor gamma t (RORγt) as well as transforming growth factor beta (TGFβ)/IL-6 signaling pathways. RUNX1 contributes to the differentiation of Th17 cells by cooperating with RORγt to induce *Il17* transcription while suppressing *Foxp3* transcription which is inhibitory to Th17 differentiation process (Zhang et al., 2008). Recently, it has been known that some IL-17 producing cells also produce IFNγ, the signature cytokine for Th1 cells which

is mainly controlled by T-bet (Wang et al., 2014). This special $IL17^{+}IFN\gamma^{+}$ double-producers, so called Th1-like Th17 cells, require RUNX1 activity to enforce the expression of Th1-signature proteins from Th17 cells (Wang et al., 2014). More specifically, RUNX1 binds to *Ifng* locus in a T-bet-dependent manner to mediate IFNγ expression.

Foxp3 is an indispensable transcription factor for the generation of regulatory T (Treg) cells, a subtype of $CD4^{+}$ T cells required for the negative regulation of T cell responses. *Foxp3* locus contains three conserved non-coding sequences (CNSs), two within the first intron and one on the second intron, all of which serve as transcriptional enhancers (Zheng et al., 2010). These three enhancers specifically work together to induce and maintain *Foxp3* mRNA expression at the specific developmental stages. Recently, a super-enhancer was also discovered at the 5' upstream area of the gene which regulates *Foxp3* and other related genes by the help of SATB1, a well-known chromatin organizer (Kitagawa et al., 2017). Many transcription factors are shown to associate on these enhancers to regulate *Foxp3* expression, one of those factors is RUNX protein. Treg cell-specific deletion of RUNX-CBFβ complexes showed that they are required for the maintenance of *Foxp3* transcription in Treg cells even though they are not necessary during the development of Treg cell (Bruno et al., 2009; Kitoh et al., 2009; Ono et al., 2007; Rudra et al., 2009). Coincidently, the maintenance of *FoxP3* transcription also requires CNS2 (the second CNS within the intron 1) even though CNS2 is not required for the induction of Foxp3 *in vitro*. Later it was revealed that the activity of CNS2 region is sustained by the auto-regulatory binding of FoxP3 itself and this binding of FoxP3 to the CNS2 requires RUNX1 activity.

NKT cells are a subset of non-conventional T cells expressing invariant TCR and exhibit properties of both T and natural killer (NK) cells. Similar to conventional T cells, NKT lymphocytes respond to glycolipid antigens presented by the nonpolymorphic MHC class I-like CD1d molecule. Upon activation, NKT cells produce variety of cytokines such as IFNγ, IL-4, tumor necrosis factor alpha (TNFα), GM-CSF, IL-3 and IL-10. Development of NKT cells from DP thymocytes in the thymus is dependent on RUNX1 (Egawa et al., 2005). Specifically, during the development into mature NKT cells, RUNX1 is required for the positive selection of NKT cells during the development of NKT cells. A recent study further discovered that differentiation of NKT17 cells, a special subtype of NKT cells expressing IL-17, also requires RUNX1 (Thapa et al., 2017).

RUNX AND OTHER LYMPHOID CELLS

Epidermis layer of skin contains a variety of special immune cells such as Langerhans cells, dendritic cells (DCs) specialized for skin immunity. RUNX3 has been reported to be essential for differentiation of Langerhans cells, especially with the CBFβ2 isoform (Tenno et al., 2017; Woolf et al., 2007). Another special type of cells in epidermis is dendritic epidermal T cells (DETCs), which are γδ T cells with a shape resembling DCs. Similar to NKT cells, DETCs express invariant TCRs with limited repertoire which recognize antigens in a classical MHC-independent manner. RUNX3 has been shown to regulate expression of CD103, an important molecule for the migration of DETCs, and IL-2Rβ, a receptor for IL-2 or IL-15 necessary for the proliferation of DETCs. Thus RUNX3 plays a crucial role in DETC maturation and maintenance in epidermal layer (Woolf et al., 2007).

It is well established that B lymphocytes develop in a stepwise progression from lymphoid progenitors by the cooperative programing of three core transcription factors, E2A, EBF1 and PAX5 (Mandel and Grosschedl, 2010). More specifically, the earliest step of B lymphoid specification from CLP (common lymphoid progenitors) to pre-pre B cells is regulated by E2A/EBF1. At the molecular levels, it was shown that E2A binds to the promoter of *Ebf1* and directly induces its transcription at the CLP stage. Once expressed, EBF1 cooperates with E2A to initiate B lineage program by inducing B cell-specific genes including *Igll* (λ5) and *Vpreb1*. Recent studies have proposed that these master transcription factors need to function in association with other transcription factors such as RUNX1, FOXO1 and BCL11a. Several studies have shown that RUNX1 protein has pivotal roles in priming B lymphoid lineage (Growney et al., 2005; Ichikawa et al., 2004), specifically by working along with EBF1 (Maier et al., 2004; Seo et al., 2012). First, RUNX1 appears to cooperate with E2A to activate *Ebf1* transcription. Then RUNX1 functions together with EBF1 to induces *mb-1* gene expression which encodes the pre-B cell receptor CD79a that is required for pro-B cell differentiation process. RUNX proteins continue to function beyond this progenitor stage and assist IgA class switching in mature B cells. When B-cell receptor signaling is induced in the presence of TGFβ-TGFβ receptor engagement, SMAD3/4 and RUNX3 binds on the TGFβ responsive element in the $I\alpha$ promoter of the *Igh* gene and induce IgA class switching (Hanai et al., 1999; Pardali et al., 2000; Shi and Stavnezer, 1998; Stavnezer and Kang, 2009; Watanabe et al., 2010; Zhang and Derynck, 2000).

NK cells are considered as innate lymphocytes because they exhibit features resembling both innate and adaptive immunity (Vivier et al., 2011). Initially, mice with reduced CBFβ expression showed defects in NK cell development, indicating an involvement of RUNX-CBFβ complexes during the NK cell differentiation (Guo et al., 2008). RUNX3 expression is initiated from NK precursors and is maintained throughout the whole developmental processes of NK cells (Ohno et al., 2008). It is elegantly shown that RUNX3 cooperates with ETS and T-box transcription factors to activate the transcription program of NK cells, which is dependent on IL-15 signaling (Levanon et al., 2014). RUNX3 ChIP-seq analysis in resting or IL-15 activated NK cells showed that around 1,000 genes are bound by RUNX3 specifically after IL-15 signaling and many of them were related to NK proliferation and function, indicating that RUNX3 plays pivotal roles in NK cell development. Interestingly, recent findings suggest that NK cells exhibit their lymphocyte-like function by undergoing clonal expansion and memory responses reminiscent of $CD8^{+}$ T cells (Cooper et al., 2009; Kamimura and Lanier, 2015; O'Sullivan et al., 2015; Sun et al., 2012). This clonal expansion and memory phenotype by NK cells require IL-12 signaling followed by STAT4 signaling. Since RUNX1 and RUNX3 are targets of STAT4, increased expression RUNX proteins after the clonal

expansion might be the result of the binding of STAT4 to the promoters of *Runx* genes (Rapp et al., 2017).

CONCLUDING REMARKS

RUNX1 and RUNX3 have originally been discovered to promote hematopoietic stem cell and cytotoxic T cell development. During the last decade, many studies have reported that RUNX1 and RUNX3 appear to play diverse roles in many other lymphoid lineage cells as we summarized in this review. Although the role of RUNX proteins in non-lymphoid lineage cells was not discussed in this short review, it should be appreciated that RUNX proteins do have important roles in DCs and macrophages function. ChIP-seq powered by next generation sequencing has identified numerous RUNX target genes in several types of immune cells. However, we still lack detailed mechanistic view on how RUNX transcription factors function after they associate with genomic regions in their target loci. Future studies by using biochemical and genetic studies will be required to answer the molecular mechanism of transcriptional regulation conducted by RUNX proteins in this post-genomic era.

Disclosure

The authors have no potential conflicts of interest to disclose.

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