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## **The role of microRNAs in lymphopoiesis**

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Review Article

## **Abstract**

The immune system is composed of a diverse range of cell types, each with a distinct function. It can be broadly divided into the lymphoid (T, B, NK, etc) and myeloid (monocyte, granulocyte, etc) arms. Lymphopoiesis, the development and differentiation of lymphoid lineages, has been studied extensively for decades. For example, the influence of extracellular signals, signaling pathways and transcription factors has already been well documented. However, the importance of microRNAs is only now been highlighted by a surge of studies in recent years. In this review, we will discuss what is currently known about the role of microRNAs in lymphopoiesis, from the hematopoietic stem cell through to the differentiation of mature lymphocytes including thymic development, helper and regulatory T cells, fate determination of B cells and dendritic cells.

## **Introduction**

MicroRNAs (miRNAs) are ~22 nucleotide small non-coding RNA molecules. Discovery of the first miRNA, *lin-4*, followed from a genetic screen for developmental mutants in the embryo of the nematode *C. elegans* [1,2]. MiRNAs have since been identified in all plants and animals, and are also encoded by some viruses. As of 2014, over 24 thousand miRNAs have been discovered across 206 species [3].

MiRNAs affect gene expression via complementary base-pairing with target messenger RNAs (mRNAs). Due to their small size, multiple miRNAs can simultaneously bind to a single target mRNA, while each miRNA can potentially bind to multiple different mRNAs [4]. It is predicted that up to 60 % of human mRNAs can be recognized by miRNAs [5]. Thus, mRNA-miRNA interactions have an enormous impact on the regulation of gene expression and ultimately the function of any cell. This realization led to the rapid development of an array of animal models and other tools for studying the miRNA pathway. Mouse models harboring mutations in common pathway components implicated roles for miRNAs in the immune system early on. Investigations have since focused on the function of individual miRNAs. It is now clear that the development and function of the immune system is highly dependent on miRNA-mediated gene regulation.

## **The microRNA pathway**

MiRNAs recognize mRNAs as part of the RNA-induced silencing complex (RISC). At the core of this complex is one of four members of the Argonaute family of proteins, Ago1 to 4 [6,7]. MiRNAs primarily interact with 3' untranslated regions of mRNAs [8,9]. As little as seven base pairs at the 5' end of the miRNA (known as the seed sequence) are sufficient for targeting in animals [10]. Once bound to the mRNA, miRNAs can affect expression via multiple

mechanisms, such as by interfering with initiation or ribosome processivity, or causing mRNA decapping and deadenylation [11].

miRNAs are derived from short stem-loop structures imbedded within long primary transcripts (pri-miRNA). Like other genes, most pri-miRNAs are transcribed as independent units from DNA. However, up to 40% miRNAs are derived from introns spliced out from mRNAs. Maturation of miRNAs from either independent transcriptional units or introns requires the activity of two enzyme complexes (Figure 1). This initial long primary miRNA (pri-miRNA) is first processed by the nuclear “microprocessor” complex, containing the RNase III endonuclease Drosha and a dsRNA-binding protein Dgcr8 (also known as Pasha in *D. melanogaster* and *C. elegans*) [12-14]. This complex cleaves both strands near the base of the stem-loop to release a pre-miRNA intermediate. The pre-miRNA is then exported to the cytoplasm, where it is processed by a complex containing another RNase III enzyme, Dicer, and the dsRNA-binding protein Tarbp2, to remove the loop [15,16]. This leaves a ~22bp miRNA:miRNA\* duplex. Tarbp2 then facilitates the loading of one of these strands into the RISC, while the other strand is degraded.

### **Regulation of hematopoietic stem cell function**

The delicate balance between self-renewal and differentiation of hematopoietic stem cells (HSCs) is critical for maintaining proper hematopoiesis. This is primarily sustained by transcription factors that act as master regulators of gene expression. However, miRNAs constitute another critical layer of regulation.

A likely role for miRNAs in the regulation of hematopoiesis was realized quite early, when it was shown that overexpression of miR-181a in bone marrow promotes the differentiation of B cells [17]. In fact, this was one of the first evidences of a function for miRNAs in mammals. Disruption of the miRNA pathway in HSCs, by deletion of machinery components, impairs the repopulation capacity of these cells, leading to bone marrow failure [18,19].

Several miRNAs have been implicated in the regulation of HSC self-renewal. miR-29a [20] and miR-196b [21] are both highly expressed by HSCs and downregulation is associated with differentiation. They are thought to regulate self-renewal, because overexpression induces various leukemias. miR-125a/b, the mammalian homologs of *lin-4*, are also highly expressed in HSCs, and downregulated with differentiation [18,22]. Both appear to promote HSC self-renewal by regulating apoptotic targets. Bak is regulated by miR-125a [18], while Bmf and Klf13 are regulated by miR-125b [22].

Indicative of the breadth of influence that miRNAs exert early in hematopoiesis, many of these miRNAs that regulate HSC self-renewal also influence hematopoietic differentiation. Forced expression of miR-125a promotes B lymphopoiesis [18], whereas forced expression of miR-29a [20] or miR-125b [22] promote myelopoiesis. How these miRNAs preference lymphopoiesis versus myelopoiesis is unclear, but forced expression of the latter two ultimately lead to myeloid leukemia as a result of imparting HSC-like self-renewal properties.

### **Regulation of T cell development in the thymus**

T cells develop in the thymus from progenitors that arrive from the bone marrow. Early thymocytes first progress through a series of four stages termed double negative (DN), as they lack expression of the CD4 and CD8 coreceptors. The DN3 stage is where  $\gamma\delta$ T cells bifurcate from the  $\alpha\beta$ T cell lineages. DN4 cells then upregulate both CD4<sup>+</sup>8<sup>+</sup> thus becoming double positive (DP). Selection then leads to the differentiation of CD8 single positive (SP) cytotoxic T cells, CD4 SP helper T cells, NKT T cells or one of several other possible lineages depending on the specificity and affinity of the expressed T cell receptor (TCR). miRNAs appear to be important at many stages throughout T cell development (Figure 2).

Initial characterization of mice in which the *Dicer1* gene was deleted in early thymocytes using an Lck-cre transgene, suggested that the miRNA pathway might not be required [23]. This was despite an apparent 10-fold reduction in thymocyte numbers. However, a later study confirmed that the miRNA pathway is indeed necessary for T cell development. Deletion of the genes encoding either Dicer or Drosha early in T cell development results in a developmental block at the DN3 stage [24]. This corresponds to the TCR $\beta$  rearrangement checkpoint, and productive rearrangement is required for progression to DN4. On the other hand, there was an accumulation of  $\gamma\delta$ T cells in these mice, indicating a requirement of miRNAs only for  $\alpha\beta$ T cell development. Deletion of either *Dicer1* or *Drosha* late in T cell development has a less severe impact, resulting in a 2-fold reduction in mature cell numbers [25,26].

The specific miRNAs that are necessary for T cell development are still not clear, but it is likely that multiple miRNAs are involved at different stages. miR-150 may be one that is important for the early checkpoint. The Notch pathway is important for T cell development, playing important functions throughout the lineage, but particularly in DN thymocytes [27]. Notch3 is a target of miR-150 in T cells [28], and the interplay between the two may be important. Transgenic expression of miR-185 impairs the progression to the DP stage by targeting Mzb1 [29], but whether endogenous miR-185 is important for T cell development remains to be determined.

miR-181a and other miR-181 members are very abundant at the DP stage, comprising as much as half the miRNA molecules expressed [26,30]. Overexpression of miR-181a results in an increase DP thymocyte numbers [31], whereas deficiency results in a mild decrease [32]. Thus, the upregulation miR-181a is important for the DN4 to DP transition. miR-181a is thought to regulate the metabolic status of thymocytes by targeting Pten [32], a necessary determinant for the increased cell proliferation that accompanies the progression to the DP stage.

mir-181a has also been shown to modulate TCR signaling by targeting multiple negative regulators of signaling, including Dusp5, Dusp6 and Shp-2 [31]. Regulation of TCR signaling is critical for positive and negative selection, and can also affect lineage decision. miR-181a deficient mice display a severe loss in NKT cells [32]. Disruption of NKT cell development had previously been observed when Dicer was deleted late in T cell development [33,34]. This suggests the NKT cell defect caused by Dicer deficiency is entirely due to miR-181a deficiency. Like the earlier DN4 to DP transition, the function of miR-181a at this latter stage appears to involve the targeting of Pten and the regulation of metabolic status [32].

### **Regulatory T cells**

While the NKT lineage was most dramatically affected by a lack of miRNAs late in T cell development, other  $\alpha\beta$ T cell lineages were also affected to a varying extent. Foxp3<sup>+</sup> regulatory T cell (Tregs) numbers were reduced with either Drosha or Dicer deficiency [26,35]. The miRNA signature of Tregs is distinct from other T cells [35,36]. Whether specific miRNAs are responsible for thymic Treg differentiation or whether differentiation is simply a product of overall TCR regulation by miRNAs is still to be determined. One Treg-enriched miRNA that appears to contribute to this thymic differentiation is miR-155. miR-155 deficient mice display a mild reduction in Foxp3<sup>+</sup> cells in the thymus [37,38]. miR-155 exerts its effect on Foxp3 expression, and Treg development by inhibiting Suppressor of cytokine signaling - 1 (SOCS1) [38], a negative regulator of JAK-STAT signaling.

Conventional CD4<sup>+</sup> T cells can also differentiate into Foxp3<sup>+</sup> induced Tregs, such as in response to transforming growth factor -  $\beta$  (TGF $\beta$ ). This is also partially disrupted by Drosha or Dicer deficiency [26,35] (Figure 3). This induction of Foxp3 is at least partly dependent on miR-126 and miR-155. Regulation SOCS1 by miR-155 appears to be involved again [39], while miR-126 is thought to act by inhibiting p85 $\beta$  and modulating the PI3K/AKT pathway [40].

Not only is the miRNA pathway important for Treg differentiation, but it is also critical for the functional program of these cells. Tregs are necessary for maintaining immune homeostasis, and Treg deficiency, such as that caused by Foxp3 mutations, leads to lethal lymphoproliferation and inflammation in both mice and humans. *Dicer1* or *Drosha*

gene inactivation specifically in Foxp3<sup>+</sup> cells causes this same devastating lymphoproliferative disease [26,41,42].

However, unlike in Foxp3 deficient mice, the Foxp3<sup>+</sup> cells are still present in these animals, but are essentially non-functional.

Several studies have since revealed the contribution of specific miRNAs for Treg function. miR-146a is enriched in Tregs, and deficiency causes a partial loss of Treg function [43]. Interestingly, it is the control of T helper 1 (Th1) responses that is specifically affected. STAT1 was found to be a target of miR-146a. miR-10 is another miRNA that is expressed in Tregs associated with Th1 responses, whereas miR-182 is expressed in Th2-associated Tregs [44]. Computational predictions suggest that miR-10 and miR-182 cross-regulate opposing networks of genes to promote the Th1 or Th2-associated phenotype.

In addition to its role in Treg differentiation, miR-155 is important for Treg fitness [38], but not for suppressor activity [37]. The miR-17~92a cluster of miRNAs has also been shown to be important for Treg fitness. Several miRNAs derived from this polycistronic precursor are enriched in Tregs compared with other T cells [45]. Deletion of this gene specifically in the Tregs of mice causes a number of mild immunological perturbations. The cells display increased apoptosis and decreased proliferation [45], and impaired differentiation into IL-10-producing effector Tregs [46]. A primary function of this cluster is thought to be the regulation of lymphocyte survival [47], which is consistent with a phenotype of reduced fitness.

### **Differentiation of Helper T cell subsets**

The differentiation of mature helper T cells into effector subsets is also modulated by specific miRNAs (Figure 3). CD4<sup>+</sup> helper T cells can differentiate into one of a range of effector subsets (e.g. Th1, Th2, Th9, Th17, Th22), characterized by the expression of specific cytokines and transcription factors. In helper T cells, the miR-17~92a cluster is required for efficient Th1 differentiation, with miR-17 and miR-19b being the specific miRNAs of importance [48]. miR-17 promotes Th1 differentiation via the inhibition of TGFβRII and CREB1, while miR-19b regulates Pten. This also prevents the differentiation of induced Tregs.

On the flip side, miR-155 is important for restraining Th1 differentiation by inhibiting IFNγRα expression [49]. Mice lacking this miRNA display a bias towards Th2 differentiation [49]. However a second report found that miR-155 deficient mice are resistant to experimental autoimmune encephalomyelitis (EAE), due to poor Th1 and Th17 differentiation [50]. The reason of this discrepancy is not clear, but may reflect different immunological contexts. Another autoimmune model that is dependent on Th17-mediated responses, collagen-induced arthritis, is also

suppressed in miR-155 deficient mice [51]. Thus, the poor Th1 differentiation in the context of EAE may be due to a dampened overall immune response.

miR-29a has also been found to inhibit Th1 differentiation and IFN $\gamma$  production. Deficiency results in unrestrained Th1 responses [52,53]. A redirection to Th2 differentiation was not reported. miR-29a inhibits expression of the transcription factors T-bet and Eomesodermin that regulate IFN $\gamma$  gene expression [52,54].

miR-21 promotes Th2 differentiation by inhibiting the expression of Spry1, a negative regulator of the MAP kinase pathway [55]. Increasing the expression of miR-21 enhances Gata3, IL-4, IL-5 and IL-13 expression by Th2 cells. Th2 differentiation is also influenced by miR-126. Blockade of miR-126 function with antagomirs impairs IL-5 and IL-13 by Th2 cells [56], but the mechanism is not known. Whether these two miRNAs promote Th2 differentiation at the expense of other lineages is also unknown.

Multiple miRNAs have been found to promote Th17 differentiation. As discussed above, miR-155 is necessary for the development of disease in Th17 animal models. miR-155 promotes Th17 differentiation by repressing the transcription factor Ets1 [57]. The miR-17~92a cluster is also required for effective Th17 differentiation. Like in Th1 cells, inhibition of Pten by miR-19b appears to be important, while miR-17 was found to regulate IKZF4 [58]. Given the ubiquitous expression of miR-17~92a miRNAs in all hematopoietic cells, it is not surprising to find a diverse range of functions for this cluster of miRNAs in T cells. miR-301 also participates in Th17 differentiation by repressing PIAS3, a negative regulator of STAT3 signaling [59].

### **Regulation of B cell development**

One of the first indications that miRNAs may have important biological functions in mammals was in B cell development. B cell development occurs in the bone marrow, and overexpression of miR-181a was found to promote the differentiation of the B lineage [17]. Like T cells, the stages of B cell development can be identified by the expression of various cell surface markers, including immunoglobulins. Recombination of the immunoglobulin heavy chain locus occurs at the pro to early pre-B cell stages, followed by light chain rearrangement at the late pre-B cell stage. Selection of cells with appropriate immunoglobulin expression then leads to the B cell maturation.

The first definitive proof that the miRNA pathway is necessary for the B lineage came when the *Dicer1* gene was deleted early in B cell development in mice. These mice exhibit a block at the pro-B to pre-B transition [60]. The terminal differentiation of mature B cells into antibody-producing cells or memory B cells is also dependent on Dicer



[61]. This suggests that like T cell development, multiple steps in B cell development are also dependent on miRNAs (Figure 4). The requirement of the other RNase III enzyme, Drosha, has yet to be reported.

The miRNAs from the miR-17~92a cluster are clearly important for early B cell development. miR-17~92a deficient mice display a greatly reduced B cell compartment, with a developmental block at the pre-B cell stage [47]. This cluster of miRNAs was found to regulate B cell survival, in part, by repressing the proapoptotic molecule Bim. The fact that miR-17~92a deficiency only causes a partial block indicates that the severe block caused by Dicer deficiency is dependent on multiple miRNAs. On the other hand, overexpression of miR-34a has been shown to block B cell development at the pro-B to pre-B transition [62]. It was found to inhibit B lymphopoiesis by repressing the expression of Foxp1 (a B cell oncogene that is required for the pro-B to pre-B transition) [63].

The latter stages of B cell development are regulated by a number of different miRNAs. The differentiation of B1 cells is regulated by the transcription factor c-Myb, which in turn is regulated by miR-150. miR-150 deficiency results in a loss of c-Myb control and an expansion of B1 cells [64]. The differentiation of mature B cells into germinal center B cells is regulated by miR-155. B cells from miR-155 deficient mice fail to express IgM or class-switch, resulting in impaired antibody responses [65,66].

### **Other innate lymphocytes**

Innate lymphocytes are an expanding family of cells, which do not have the exquisite specificity of T or B cells but nevertheless fight infections, maintain homeostasis with commensal bacteria, maintain epithelial integrity and help organize lymphoid tissues. They include NK cells, nuocytes, MAIT cells and lymphoid tissue inducer cells. The ontogeny of NKT cells (which have a restricted T-cell receptor and therefore appear to behave more like innate cells) is critically dependent on mir-181 [32]. Other than the differentiation of NKT cells, the role of miRNAs in the differentiation of innate lymphocyte populations is not as well understood. A handful of studies have indicated a potential role in NK cell differentiation. Deficiency in miR-150 causes a reduction in mature cells in the periphery due to a loss of c-Myb repression [67]. miR-181a regulation of NLK, an inhibitor of Notch signaling, also contributes to NK cell differentiation [68]. The role of miRNAs in innate lymphocyte differentiation clearly remains a key area for further investigation.

### **Regulation of DC development**

Dendritic cells (DCs) are sentinel cells specializing in the capture and presentation of antigen to T cells, and can differentiate from both lymphoid and myeloid progenitors. DCs can be broadly divided into four major subsets viz.

tissue DCs (including epidermal Langerhans DCs, gut DCs, dermal DCs etc), lymphoid-resident conventional DCs, plasmacytoid DCs and inflammatory DCs (monocyte-derived DCs). Early investigations into the role of miRNAs in DCs involved conditionally deleting Dicer in mature DCs with CD11c-cre, which had little impact on most DC populations [69]. However, deletion was never fully achieved in these mice because the half-life of miRNAs is longer than that of most mature DCs [70]. Whereas splenic DCs live for 3 days, Langerhans DCs can live for many months and as such the role of miRNAs on Langerhans cell homeostasis can be revealed. Indeed such cells are dramatically depleted in CD11c-dependent ablation of Dicer or Drosha.

To definitively assess if the miRNA pathway is necessary for DC development (other than Langerhans DCs) will require approaches that delete *Dicer1* and *Drosha* earlier. Nevertheless, a number of miRNAs have been implicated by means of knockdown or overexpression studies. This includes miR-22 that represses the transcription factor IRF8 [71], miR-21 and miR-34a that coordinately repress JAG1 and WNT1 [72], and miR-155 that represses p27kip1, KPC1, and SOCS-1 [73,74]. Manipulating the expression of these miRNAs influences subset differentiation to varying degrees. The more rigorous studies of employing genetically modified mice to probe the roles of specific miRNAs are still somewhat lacking. One exception was the discovery that DC differentiation is partially disrupted in miR-142 deficient mice; this affected all DC subsets [75].

In contrast to differentiation, a number of studies have demonstrated the importance of miRNAs for DC function. In addition to defects in DC differentiation, miR-142 deficiency impairs the ability of conventional DCs to prime CD4<sup>+</sup> helper T cells [75]. A similar phenotype was observed in miR-155 deficient mice [76]. Antigen presentation by Langerhans cells is also regulated by miRNAs. Langerhans cells from miR-150 deficient mice have reduced capacity to present exogenous antigens to cytotoxic T cells [77], whereas miR-223 deficiency enhances this process [78]. The mechanisms by which these miRNAs regulate antigen presentation are not known. As with the differentiation of these cells, knockdown and overexpression have implicated many more miRNAs in the regulation of DC function.

### **Concluding remarks**

MiRNAs are without doubt critical regulators of cell differentiation, and lymphopoiesis pathways are prime examples where miRNAs exert important and diverse functions. However, we still only have a nominal understanding of how miRNAs regulate lymphopoiesis and much work remains, especially in regards to innate lymphocytes. There are several important considerations as we move forward. Many of the early studies investigating the potential role of the miRNA pathway relied on selective deletion of the *Dicer1* gene. Recent studies have revealed that the two RNase III enzymes central to miRNA biogenesis, Drosha and Dicer, have important functions beyond miRNA biogenesis

(reviewed in [79]). Thus, it is possible that some of the phenotypes caused by Dicer deficiency may in fact be unrelated to miRNAs. A re-evaluation of the role of miRNAs may be warranted in some cases, such as by analyzing the impact of deleting other key miRNA pathway genes. Understanding the specific miRNA-target networks involved will also be vital. However, because mismatching is an important feature of miRNA-mRNA interactions in animals, target prediction remain equivocal. Experimental validations will always to be essential, and the continued development of knockout mice for specific miRNAs will be particularly important.

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## Figure Legends

**Figure 1: MiRNA biogenesis.** The biogenesis of miRNAs in animals is dependent on processing of precursors by two RNase III enzyme complexes. Long primary transcriptions are first cleaved by Drosha to release the pre-miRNA stem-loop intermediate in the nucleus. This pre-miRNA is then processed by Dicer in the cytoplasm to release the mature miRNA for loading into the effector RISC.

**Figure 2: MiRNA regulation of T lymphopoiesis.** MiRNAs play important roles throughout the T cell development. Indicated are miRNAs and their targets that are known to contribute to control of this developmental pathway. Also shown are developmental blocks caused by Drosha or Dicer deficiency.

**Figure 3: MiRNAs and effector CD4+ T cell differentiation.** Mature CD4+ helper T cells can differentiate into various flavors of effector T cells or induced regulatory T cells. Differentiation into the differentiation subsets is dependent on specific miRNAs and the repression of specific targets. Also shown is the block caused by Drosha or Dicer deficiency.

**Figure 4: MiRNA regulation of B lymphopoiesis.** MiRNAs are also important regulators of B cell development. Indicated are miRNAs and their targets that have been found to be important. Also shown are developmental blocks caused by Dicer deficiency.







