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REVIEW
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MicroRNA Functions in Thymic Biology: Thymic Development and Involution

Minwen Xu[1], *Tao Gan*[2], *Huiting Ning*[2] *and Liefeng Wang*[2]*

[1] First Affiliated Hospital of Gannan Medical University, Ganzhou, China, [2] Department of Biotechnology, Gannan Medical University, Ganzhou, China

During the entire processes of thymus organogenesis, maturation, and involution, gene regulation occurs post-transcriptionally via recently discovered microRNA (miRNA) transcripts. Numerous reports indicate that miRNAs may be involved in the construction of a normal thymic microenvironment, which constitutes a critical component to support T lymphocyte development. MiRNAs are also expressed in thymic stromal cells including thymic epithelial cells (TECs) during maturation and senescence. This review focuses on the function of miRNAs in thymic development and involution. A better understanding of these processes will provide new insights into the regulatory network of TECs and further comprehension of how genes control TECs to maintain the thymic microenvironment during thymus development and aging, thus supporting a normal cellular immune system.

Keywords: microRNA, thymic epithelial cells, thymic development, thymic involution, thymic microenvironment, thymus aging, regulatory network

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***Correspondence:**
Liefeng Wang
469730795@qq.com

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INTRODUCTION

The thymus plays a critical role in the cellular immune system by generating T lymphocytes, which are involved in anti-tumor immunity, anti-viral, and anti-intracellular infections, as well as the establishment of self-tolerance to avoid autoimmune disorders. During the entire process of thymus organogenesis, maturation, and involution, gene regulation not only occurs at the transcriptional level via transcription factors, but is also effected at the post-transcriptional level by microRNA (miRNA) transcripts. The ubiquitous and abundant existence of such small, non-protein-coding miRNAs in worms, plants, and animals plays an important role in the regulation of gene expression primarily at the post-transcriptional level by cleavage and/or translational repression of messenger RNAs. It has become evident that miRNAs control a wide range of developmental and physiological pathways including cell proliferation, differentiation, and apoptosis. Additionally, the deregulation of miRNAs can cause developmental blockage, dysregulation, or disease. Although many phenomena during thymic development and aging are unable to be simply explained by known protein-coding genes, many novel miRNAs have been identified within recent years that are expressed in the thymus. As the systemic miRNA gene profile and their functional characterization during thymic development and aging are gradually elucidated, we have adequate reason to infer that miRNAs may be involved in the construction of the normal thymic microenvironment that supports T lymphocyte development. In this review, we focus on the specific miRNAs that are involved in the thymic stroma, and how these play a role in thymic epithelial cell (TEC) development. Through understanding these roles, we can obtain new insights regarding the

regulatory network in TEC maturation and senescence, and further understand how genes control TECs to maintain the thymic microenvironment during thymus development and aging. Our review aims to reveal potential genetic targets and identify possible therapeutic tools for patients with thymic developmental diseases, which may lead to novel strategies to rejuvenate the functions of an aged thymus or delay thymic aging.

MIRNA IDENTIFICATION AND CHARACTERIZATION

MiRNAs comprise a large group of conserved, single-stranded, non-coding, abundant, short (∼21–25 nucleotide) RNAs (1, 2). They differ from small interfering RNAs (siRNAs) as they have molecular origins that derive from genomic loci whereas siRNAs are generated from exogenous RNA, such as viral infection, artificial RNA interference (RNAi), and endogenous transposon activity. A miRNA binds to a target mRNA through imperfect complementarity, generally at multiple sites, whereas a siRNA binds to a target mRNA to form an almost perfect duplex at only one site. However, the maturation of both miRNAs and siRNAs utilizes the common RNase-III processing enzyme, Dicer (3), prior to becoming single-stranded anti-sense RNA.

The first microRNA, lin-4 RNA, was identified in 1993 (4). It encodes a 22-nucleotide non-coding RNA that is imperfectly or partially complementary to seven conserved sites located in the 3′-untranslated regions of *lin-14*, a nuclear protein gene in *Caenorhabditis elegans*. Although this small RNA was overlooked for seven years as these short non-coding RNAs were considered to be non-existent beyond nematodes, this was completely changed by the discovery of the *let-7* gene in 2000. Notably,*let-7* was present not only in *C. elegans* but also in human and fly genomes. Currently, miRNAs are accepted as phylogenetically conserved genes and have been found in all metazoan genomes, with close to 1,000 miRNAs having been identified in *C. elegans*, *C. briggsae*, *Drosophila melanogaster*, *Arabidopsis thaliana*, rice, mouse, rat, and human to date (5–7).

MiRNAs are considered to represent novel biological regulators, as they regulate gene expression in a sequence-specific manner. Their primary role is to function as a negative genetic switch, which is involved in post-transcriptional regulation by targeting mRNAs for cleavage, translational repression, or chromatin modification (1, 8, 9). Recently, additional miRNA functions have been discovered including the control of developmental stages (10–12), hematopoietic cell lineage decisions (13–15), cellular proliferation, cell death/apoptosis (16–19), fat metabolism (20–22), neuronal patterning in nematodes (23–25), asymmetric expression in chemosensory neurons, and involvement in oncogenesis (26–29).

To effect such functions, the expression of miRNA is temporal and spatial in specific tissues. This implies the existence of different miRNAs that are expressed in the various thymus compartments, cell types, and developmental stages, and that expression patterns may differ between fetal and adult thymi.

Abbreviations: Aire, autoimmune regulator; cTEC, cortical thymic epithelial cell; DP, double positive; iNKT, invariant natural killer T cell; IR, ionizing radiation; miRNA, microRNA; mTEC, medullary thymic epithelial cell; TEC, thymic epithelial cell.

MIRNAS IN THYMOCYTE DEVELOPMENT

The thymus constitutes one of the most active organs in animal life. It undergoes organogenesis (cell migration, proliferation, and differentiation), development (proliferation, differentiation, and cell apoptosis), and involution (cell senescence and apoptosis). The thymus also generates T lymphocytes to support the cellular immune system. Generally, there are two main processes that interact and regulate each other during thymus development: T lymphocyte development to generate functional T cells, and stromal cell development to build up and maintain the thymic microenvironment for supporting T cell maturation, largely through TECs. Both of these processes represent stepwise or sequential pathways in development (30, 31).

Thymic involution results in marked morphological and functional changes; these mechanisms include TEC-driven programmed thymic involution and thymocyte apoptosis. Thymic involution results from multiple causes, which can easily be grouped into those arising from normal physiology including pregnancy and aging, and those from various pathophysiological mechanisms, such as infection, malnutrition, disease, and surgery. In particular, thymic aging involution comprises a physically progressive process that can be sped up by infections, autoimmune diseases, or cancer. In addition, a large category of pathophysiological changes can also lead to thymic involution, with infection representing a notable example (32–35).

Because miRNAs are involved in many important development events, it is not difficult to infer that numerous miRNAs are likely involved in regulating the many activities of TECs and thymocytes. Moreover, recent studies have shown that some miRNAs are present in the total thymus and are involved in T or B lymphocyte lineage determination (36–38), as well thymocyte survival. Moreover, deletion of the Dicer processing enzyme has an effect on thymocyte survival (39). Dicer promotes the development of regulatory $CD4^+$ T (T reg) cells in the thymus and the efficient induction of *Foxp3* by TGF-β, whereas deletion of Dicer decreases T reg cell numbers and results in immune pathology (40). Natural T reg cells share partial overlap of miRNA expression with conventional $CD4^+$ T cells. In turn, conventional $CD4^+$ T cells can express CD25, CTLA4, and GITR, markers, which are also constitutively expressed by T reg cells during activation (41). Dicer deletion can also result in a distinct reduction of invariant natural killer T (iNKT) cells in the thymus and other organs with immune functions, which indicates that the Dicer-dependent miRNA pathway plays a critical role in iNKT cell development, function, and homeostasis (42–44).

Two prominent examples of miRNAs expressed in the thymus are miR-181 and miR-150. MiR-181 is highly expressed in double positive (DP) thymocytes and controls the development of early thymocyte cells by targeting CD69 and TCR (45, 46). MiR-181a, a member of the miR-181 family, controls the development of early thymocyte cells by regulating and controlling the negative

feedback loops that establish the NOTCH1 and TCR signaling pathway thresholds (47, 48). In particular, these thresholds play important roles in thymic T-cell positive and negative selection, with deletion of miR-181a leading to a decrease of the early thymic progenitor cells, DN3, DP, and single positive (SP) (47). MiR-181a deletion also impairs the development of invariant αβ NK-T cells, which are agonist-selected at the DP stage (49, 50); however, miR-181a-1/b-1 is not critically required for the innate development of γδ NKT cells or any other γδ T cell subtypes (51, 52).

In comparison, miR-150 can target *c-Myb* and plays an important role in lymphocyte development and physiology (53). In human T lymphocytes, miR-150 is obviously up-regulated during T cell maturation after the DP stage and targets Notch3, which plays an important role in T cell development (54). Over-expression of miR-150 can reduce the number of T cell lines *in vitro* by impacting their proliferation and survival. MiR-150 is also expressed in iNKT cells and targets *c-Myb* (55). MiR-150 over-expression increases iNKT maturation whereas deletion of the miRNA results in an interruption of iNKT cell final maturation in both the thymus and the peripheral space (56).

In addition, some other miRNAs, such as miR-155 (57–59), miR-19b (60), let-7 (61), and miR-17 (62), have been reported to play important roles in lymphocyte maturation, differentiation, development, and survival. The roles of certain miRNA candidates in thymocyte biology are listed in **Table 1**.

MIRNAS IN TEC BIOLOGY

As described above, TECs have three maturation stages, which can be segregated according to cell surface molecules (63). In wild type thymus, TECs completely differentiate into the three-dimensional cortical and medullar network TEC system. However, in the various stages of lymphocyte development identified by mutating the thymus, TECs themselves are arrested at different stages, indicating that TEC differentiation is tightly dependent on T-cell development. For example, in the thymus of mice with an Ikaros-null mutation (64) or the *RAG2*/common chain compound gene knock-out mutant thymus (64–66), which display distinct defects in the development of fetal and adult lymphocytes, the TECs are arrested during the early two-dimensional cortical TEC stage (67), whereas in the *RAG* null thymus, TECs are arrested in the middle three-dimensional stage.

As the expression of miRNAs is tightly regulated during tissue differentiation (68) and miRNAs can function to prevent cell division and drive terminal differentiation (69), miRNAs are therefore likely to be involved in thymic differentiation. Consistent with this supposition, a role of miRNAs in TEC biology has been demonstrated. In particular, miRNA microarray analysis of cortical thymic epithelial cells (cTECs) along with immature medullary thymic epithelial cell $(mTEC)^{low}$ and mature $mTEC^{high}$ cells indicated that miRNA expression differs among thymic cell subsets and fluctuates during TEC maturation (70). When Dicer was conditionally deleted in all TECs, thymus cellularity was decreased and the thymus failed to maintain a regular microenvironment (71). Moreover, mTEC apoptosis was enhanced in these mice, in which cTEC failed to impose efficient positive selection, T cell phenotypes were changed, and T lymphopoietic activity was decreased (71, 72). To further clarify the function of canonical miRNAs in TECs, *DGCR8*, encoding a component of the miRNA-specific microprocessor complex, was deleted (73). DGCR8 is critical for maintaining the proper expression of Aire, the gene for which is specifically expressed in the TEC compartment and affects TEC function, along with the overall architecture of the thymic medulla. Furthermore, miRNA deficiency in TECs causes a breakdown in central tolerance (73).

TABLE 1 | The role of candidate miRNAs in thymocyte biology.

miRNAs	Cell type	Biologic role	Targets	References
miR-150	T cell	Maturation of T cells	NOTCH3	(54)
	NKT/iNKT	Development of NKT ↑ iNKT ↓	C-Myc	(55, 56)
miR-155	iNKT	Maturation and differentiation of iNKT ↓	Ets1, ITK	(57)
	Treg	Development of Tregs ↑	Foxp3	(58, 59)
miR-181a-1/b-1	T cell Leukemia cell	Development	NOTCH1	(47)
	iNKT	Development of iNKT ↑	Ptpn22, Shp-2, Dusp6	(49)
miR-181	NKT	Maturation of NKT	PTEN	
miR-181a	T cell	T cell sensitivity and selection		(48)
miR-181d	$CD4^+CD8^+$	Immature $CD4^+CD8^+$ ↓	Foxo4, Myc	(52)
miR-19b	Th17	Development of Th17	Tslp	(60)
let-7	NKT		Zbtb16	(61)
miR-17	T cell	Survival	Jak1	(62)

MIRNAS IN THYMIC INVOLUTION

Although the mechanism of thymic involution remains unclear, certain miRNAs have been reported to be involved in thymic aging involution. Microarray data analysis shows that some microRNAs are significantly changed in aged thymuses, with quantitative polymerase chain reaction (qPCR) data confirming these changes (74). In particular, miR-181a-5p has been hypothesized to be associated with thymic aging involution as its expression is obviously decreased in TECs from aged mice. To test this hypothesis, a miR-181a-5p mimic was used in a mouse mTEC cell line (MTEC1). The miR-181a-5p mimic could induce cell proliferation of MTEC1 whereas its inhibitor reversed this effect. MiR-181a-5p was shown to target transforming growth factor beta receptor (*Tgfbr1*) gene using a luciferase reporter assay (75). Furthermore, the miR-181a-5p mimic could decrease Tgfbr1 protein expression as well as that of p-Smad3, is a key node of the TGF-β signaling pathway, *in vitro*. Tgfbr1 expression increases with age in mice, which is consistent with the decreased level of miR-181a-5p in addition to the ability of TGF-β to decrease the proliferation of mTECs.

In comparison, FoxN1 constitutes a pivotal transcription factor for TEC survival and differentiation, which decreases with age. WNT signaling in thymic epithelia is essential for normal thymus development and function (76) but is suppressed in the senescent human thymus (77). WNT4 can directly up-regulate FoxN1, indicating that miRNAs that target *FoxN1* or the WNT signaling pathway may be involved in thymic aging involution (78). Consistent with this, a study comparing the difference in miRNA expression between old and young thymi (from 70-year-old men vs. <10-month-old newborns, respectively) found that some miRNAs that act as modulators of the WNT pathway, such as miR-25, miR-7f, and miR-134, were among those altered (79).

Moreover, in a previous study from our laboratory, we compared changes in miRNA expression profiles between young and aged TECs using miRBase-V20 arrays (containing 1,892 unique probes), which clearly identified and validated that at least one miRNA, miR-125a-5p, was increased in aged thymi (80). In addition, the application of a miR-125a-5p mimic was able to inhibit FoxN1 expression (as indicated using 3′UTR luciferase activity) in a 293T cell line and suppress *FoxN1* expression in TEC Z210 cells (80).

The thymus represents an organ that is hyper-responsive to stress in the form of infections, radiation exposure, trauma, and drugs. Infection can induce a rapid yet transient atrophy, which is distinct from thymic aging involution. Such atrophy can recur after exposure to pathogen-associated molecular patterns (PAMPs) (81).

There is mounting evidence that miRNA expression is associated with stress. Some miRNAs might serve as potential biomarkers of stress specifically in the thymus: for example, the expression of miR-21 is increased during radiation-induced thymic lymphoma and its expression could be induced by the TGF-β (82) and by the TLR4 pathway (83). MiR-23a/b is also up-regulated in radiation-induced thymic lymphoma (84). MiRNAs may also play significant roles in protective mechanisms for counteracting stress. In particular, miR-34a and miR-7 may counteract radiation cytotoxicity by respectively targeting *NOTCH1*, *MYC*, *E2F3*, cyclin D1, and lymphoid-specific helicase (*LSH*) (85).

Alternatively, some miRNAs may play a reverse role. For example, miR-467a directly targets *Fas* and/or *Bax* and may have oncogenic functions in radiation-induced thymic lymphoma (86). Together, the evidence suggests that some miRNAs might serve as new biomarkers of stress-induced thymic injury or as novel therapeutic targets of stress-induced thymic injury. Moreover, some miRNAs might be suitable for use as drugs to treat stress-induced thymic injury. The potential roles of candidate miRNAs in thymic biology are listed in **Table 2**.

TABLE 2 | The role of candidate miRNAs in thymic biology.

miRNAs	Organism status/biologic role	Targets	References
miR-181a-5p	Aging involution	smad3	(75)
miR-125a-5p	Aging involution	FoxN1	(80)
miR-25	Aging involution	WNT	(79)
miR-7f			
miR-134			
miR-29a	Infection	Ifnar1	(73)
miR-205	Inflammatory	FoxN1	
miR-182	Toxicity	AhR, CYP1A1, Fas, FasL	
miR-31			
miR-23a			
miR-18b			
mmu-let-7e			
miR-34a	IR-inducible involution	NOTCH1, MYC, E2F3, Cyclin D1	(85)
miR-7		LSH	
miR-21	IR-inducible involution	Big-h3	(82, 83)
miR-27b	IR-inducible involution	Cyclin A2	
miR-23a/b	IR-inducible thymic lymphoma	Fas	(84)
miR-23a/b	IR-inducible thymic lymphoma	Fas/Bax	(86)

MIRNAS DIRECTLY TARGET SIGNAL PATHWAY GENES OR *VICE VERSA*

The adult thymic microenvironment consists of epithelial cells, fibroblastoid cells, dendritic cells, and macrophages. Epithelial cells represent the resident cell type of the thymic microenvironment. Their development and differentiation depend on a variety of signaling pathways, such as WNT (87), tumor necrosis factor receptor (TNFR) and the downstream NF-κB (88), BMP (89), IFNAR1 pathway (72), and TGF-beta (90) signaling. Interferon (IFN)-α, a critical molecular mediator of pathogen-induced thymic involution, mediates rapid and transient involution by binding IFNAR1, which is expressed on the thymic stroma (81). The Dicer-dependent miRNA network, and specifically miR-29a, is critical for reducing the sensitivity of the thymic epithelium to simulated infection signals, protecting the thymus against infection-associated thymic involution. Loss of Dicer or the miR-29a cluster in the thymic epithelium results in IFNAR1-dependent hypersensitivity to pathogen-related signals, thereby allowing suboptimal signals to trigger the rapid loss of thymic cellularity (91).

TGF-β signaling might also play an important role in controlling thymus development and maintenance (92), especially by increasing the size of the mTEC compartment and enhancing negative selection and functional maturation of medullary thymocytes (93). The TGF-β pathway components, such as receptors or transcription factors, might thus serve as targets of miRNAs. Consistent with this, TGF-β receptor 1 was confirmed as a direct target of miR-181a-5p by luciferase assay (75). Over expression of miR-181a-5p down-regulated the phosphorylation of Smad3 and blocked the activation of TGF-β signaling. In turn, Smad7, which functions as a regulator of the TGF-β signaling pathway by preventing the phosphorylation of Smad2/3, was confirmed as a direct target of miR-195a-5p. Notably, miR-195a-5p is up-regulated in mouse TECs and over-expression of miR-195a-5p inhibits the expression of TEC cell cycle-related genes including those encoding cyclin D1,

cyclin E1, Cdk4, and C-myc by down-regulating the expression of Smad7 (94).

The TNFR and NF-κB pathway constitutes another important pathway for mTEC development, which is required to successfully establish the medullary microenvironment (88). Specifically, mice deficient for receptor activator of NF-κB (RANK) exhibit variable defects in mTECs (95). MiRNAs also participate in this signaling pathway and regulate mTEC differentiation. For example, RANK ligand and downstream canonical or non-canonical NF-κB can induce the expression of miR-449a. In turn, overexpression of miR-449a as well as miR-34a, which shares similar seed sequence with miR-449a, may induce TEC differentiation *in vitro* by targeting SATB2 (an epigenetic regulator identified as an miRN-449a target in colorectal tumor cells (96), as SATB2 was significantly decreased in a thymic epithelial progenitor cell line following miR-449a overexpression (91).

MIRNAS DIRECTLY TARGET TRANSCRIPTION FACTOR GENES OF THYMIC STROMAL CELL HOMEOSTASIS

Transcription factors Foxn1 and p63 also play crucial roles in thymic biology. Foxn1 has an important function in TEC survival and differentiation by promoting thymic epithelial progenitor cells to differentiate into functional mTECs and cTECs during organogenesis (97, 98) and for postnatal TEC homeostasis (99, 100). p63 is important for the development of the thymus (101) and is essential for the proliferative potential of thymic epithelial progenitor cells (101, 102). Several reports have shown that miRNAs participate in TEC development and differentiation by directly targeting the *Foxn1* gene. For example, Kushwaha et al., screened out two miRNAs, miR-18b and miR-518b, that directly targeted Foxn1 (103). Their results demonstrate that miR-18b and miR-518b act as upstream controllers of Foxn1 in epithelial cell development. Moreover, interfering with these miRNAs individually or together can up-regulate Foxn1 gene expression whereas their individual or combined over-expression can decrease Foxn1 protein levels. In turn, miR-22 also regulates epithelial cell development via direct inhibition of Foxn1 (104).

p63 also serves as a target of numerous miRNAs. 29MiR-203 has immediate and long-term impact on epidermal cell proliferation by directly regulating p63 (105–107). The regulation of p63 by IASPP, an inhibitory member of the apoptosis stimulating protein of p53 (ASPP) family, via miR-574-3p and miR-720 is required for epithelial homeostasis (108). Notably, p63-mediated cell cycle progression in epidermal cells occurs through the direct repression of miR-34a and miR-34c (109). Furthermore, several miRNAs, such as miR-192/215, miR-107, miR-96,132, and miR-145, are known transcriptional targets of p63. In particular, the role of the p63-FoxN1 regulatory axis in the regulation of postnatal TEC homeostasis has been revealed by Burnley et al. (110), Overall, miRNA function can be defined as having a fine-tuning effect by targeting the p63-FoxN1 regulatory axis.

Aire constitutes another transcription factor that controls peripheral tissue-restricted antigens in mTECs. miR-29a deletion resulted in a progressive decrease in expression of *Aire* and Aire-dependent genes in miR-29a null mutant mice (70). In addition, miR-220b may act as a possible regulatory factor for *Aire* gene translation as it could significantly reduce the expression of Aire protein (111).

PERSPECTIVES

MiRNAs play important roles in the processes of thymus organogenesis, maturation, and involution at a post-transcriptional level by targeting relevant mRNAs. Herein, we reviewed some of the miRNAs involved in thymocyte development, thymic architecture, thymic aging involution, and thymic involution during stress. We hope this review will help to deepen the appreciation of miRNA impact on thymic biology and facilitate the identification of potential candidates for therapeutic targeting. In addition, we checked miRNA profiles of serum-derived exosomes from young and aged mice with microarray of *Mus musculus* miRBase version-21 array chips and we found that young and old showed different miRNA expression profiles (112). These different spectrums of microRNAs in the young and old exosomes may generate a base for a potential epigenetic regulation and may play important roles in the processes of thymus organogenesis, maturation, and involution.

AUTHOR CONTRIBUTIONS

MX and LW wrote the paper. TG and HN reviewed and edited the manuscript. All authors read and approved the manuscript.

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