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Conflict of Interest
The authors declare no potential conflicts of interest.

Abbreviations
Bcl, B cell CLL/lymphoma; CXCR, C-X-C chemokine receptor; Dgcr8, DiGeorge syndrome critical region 8; Fox, forkhead box protein; ICOS, inducible costimulatory; IRF, interferon-regulatory factor; miRNA, microRNA; Socs, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; TFH, follicular T helper; TFR, follicular regulatory T; Th, T helper; Treg, regulatory T cell; UTR, untranslated region

MicroRNA-mediated Regulation of the Development and Functions of Follicular Helper T cells

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ABSTRACT

The germinal center reaction is a key event of humoral immunity, providing long-lived immunological memory. Follicular helper T (T_{FH}) cells are a specialized subset of CD4^{+} T cells located in the follicles, which help B cells and thus control the germinal center reaction. T_{FH} cell development is achieved by multi-step processes of interactions with dendritic cells and B cells along with the coordination of various transcription factors. Since the T helper cell fate decision program is determined by subtle changes in regulatory molecules, fine tuning of these dynamic interactions is crucial for the generation functional T_{FH} cells. MicroRNAs (miRNAs) have emerged as important post-transcriptional regulatory molecules for gene expression, which consequently modulate diverse biological functions. In the last decade, the miRNA-mediated regulation network for the germinal center reaction has been extensively explored in T cells and B cells, resulting in the identification of several key miRNA species and their target genes. Here, we review the current knowledge of the miRNA-mediated control of the germinal center reaction, focusing on the aspect of T cell regulation in particular. In addition, we highlight the most important issues related to defining the functional target genes of the relevant miRNAs. We believe that the studies that uncover the miRNA-mediated regulatory axis of T_{FH} cell generation and functions by defining their functional target genes might provide additional opportunities to understand germinal center reactions.

Keywords: MicroRNAs; Follicular helper T cells; Development; Germinal center; Function

INTRODUCTION

Humoral immunity plays an important role in host defense by conferring the sophisticated antibodies required to fight against foreign targets. The production of antibodies largely depends on the generation of plasma cells that are differentiated from naïve B cells. Upon antigenic stimulation, naïve B cells begin to proliferate and undergo a cognate interaction with primed CD4^{+} T cells at the T–B cell border in the secondary lymphoid tissues. Some of the primed B cells are then further differentiated into extrafollicular plasmablasts. Although these plasmablasts are short-lived, they are responsible for the first wave of antigen-specific antibody production, and mainly secrete IgM-type low-affinity antibodies; these instantly

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secreted antibodies are very important in providing the first line of defense against invading pathogens (1,2). Subsequently, these low-affinity antibodies are gradually replaced with class-switched high-affinity antibodies produced by the experienced plasma cells of the germinal center to ultimately fortify host immunity.

The germinal center is a transient structure formed in the follicles of the secondary lymphoid tissues during infection or vaccination. A small number of specific antigen-primed B cells migrate toward the B cell follicle area from the T–B cell border, proliferate massively, and begin to form the germinal center. During the proliferation stage, the germinal center B cells undergo a class-switch and somatic hypermutation, and selected germinal center B cells are further differentiated into class-switched memory B cells or plasma cells. These reactions waned as the pathogens or antigens are cleared from the system, and the germinal centers also eventually disappear. The size, numbers, and duration of germinal centers must be tightly regulated for maintaining immune homeostasis (3); indeed, dysregulation of the germinal center reaction can result in many immunological disorders, including autoimmunity, immunodeficiency, and lymphoma (3,4).

A small CD4$^+$ T cell population has been observed in the germinal centers, and these cells are believed to play important roles in antibody production in humans and mice (5,6) because they control the Ig class-switch, survival, selection, and differentiation of germinal center B cells via cytokine secretion and ligand-mediated interactions (7). Recently, this specialized subset of CD4$^+$ T cells, termed follicular helper T (T$_{FH}$) cells, has been proposed as a key regulator of the magnitude of the germinal center reaction (8). For instance, strong functional activity and/or increased numbers of T$_{FH}$ cells are strongly linked with autoimmune diseases such as systemic lupus erythematosus (9). By contrast, low numbers of T$_{FH}$ cells cause impaired humoral immunity that eventually leads to immunodeficiency (8,10). Therefore, a key to understanding humoral immunity regulation mechanisms might lie in elucidating the detailed mechanisms underlying the development of T$_{FH}$ cells.

The correct positioning of T$_{FH}$ cells in the B cell area is one of the most important steps in generating functional T$_{FH}$ cells in the germinal centers. Therefore, it is not surprising that the earliest sign of the T$_{FH}$ cell fate decision is upregulation of C-X-C chemokine receptor (CXCR) 5 (11), which is positively controlled by the transcriptional factor achaete scutelike 2 (Ascl2) (12). Interestingly, the CXCR5$^+$ antigen-primed T cells are repositioned at the T–B cell border by EBI2 and its ligand 7α,25-dihydroxycholesterol, which mediates chemotactic activity (13). These pre-T$_{FH}$ cells then interact with CD25$^+$ dendritic cells to confer inducible costimulator ligand (ICOSL)-inducible costimulator (ICOS)-mediated signals, which are responsible for the expression of B cell CLL/lymphoma (Bcl) 6 and sustained CXCR5 expression on pre-T$_{FH}$ cells (13). In addition, the CD25$^+$ dendritic cells result in an IL-2-deprived environment that is favorable for T$_{FH}$ cell development, because IL-2-mediated signal transducer and activator of transcription (STAT) 5 activation can suppress T$_{FH}$ cell development (13-15). Finally, the pre-T$_{FH}$ cells move across the T–B border and reside in B cell follicles where they fully differentiate into T$_{FH}$ cells. Surprisingly, crossing of the border does not rely on CXCR5; instead, the interaction of ICOS-ICOSL with bystander B cells is the crucial event for this step (16). In addition to the migratory control of T$_{FH}$ cell differentiation, networks of various transcriptions factors are also critical for the development and functions of T$_{FH}$ cells. The transcription factors Bcl6, interferon-regulatory factor (IRF) 4, B cell-activating transcription factor (BATF), c-Maf, Ascl2, and STAT3 have been revealed as positive drivers, while B lymphocyte induced maturation protein 1 (Blimp-1), forkhead box protein O (FoxO) 1,

forkhead box protein P (FoxP) 1, Fosl2, Krüppel-like factor (KLF) 2, and STAT5 are known as repressors of the development and functions of T_{FH} cells (17).

Because of the fundamental impact of the amount of lineage-specific transcription factors for the commitment of the fate and plasticity of CD4$^+$ T helper (Th) cells, even subtle changes of the expression of transcription factors and other regulatory genes have large impacts on T cell fates and functions (17,18). Therefore, exploration of the mechanisms that underlie the delicate control of the expression of these genes might shed new light on understanding the T cell differentiation program and related immune modulation. MicroRNAs (miRNAs), which are endogenously expressed small (up to 22 nucleotides) single-stranded RNA species, have emerged as important post-transcriptional gene regulatory factors in various types of cells and species (19). MiRNAs integrated in an RNA-induced silencing complex recognize their target mRNAs through partial sequence complementarity and then bind to the targets to suppress their translation or result in their decay; perfect base pairing to the target sequence mediates the degradation of target mRNAs. Although the miRNA-mediated suppression of gene expression is not substantial in terms of magnitude, recent research has revealed an association between the dysregulation of miRNAs and many pathological conditions, including immunological disorders (20). In the past few years, extensive studies have been conducted to identify roles of miRNAs in the regulation of the germinal center reaction. Since the miRNAs directly interact with several target mRNAs, revealing the miRNA species that regulate the germinal center reaction and their functional target genes might shed light on the underlying molecular mechanisms. In this review, we discuss the state of the current knowledge on the miRNA-mediated regulation of T_{FH} cell differentiation for the germinal center reaction, and highlight the current topics of debate in understanding of the molecular mechanisms of miRNA-mediated gene regulation.

ROLE OF MIRNAS IN T_{FH} CELL DIFFERENTIATION

Among the thousands of miRNAs that have been identified in many species to date, only a few hundreds of these miRNA species are expressed in any given cell type (21,22). CD4$^+$ T cells are not an exception to this general pattern, as the expression profiles of miRNAs in CD4$^+$ T cells are dramatically changed during T cell activation and effector T cell differentiation (23). For instance, the expression levels of many miRNA species are down-regulated upon T cell activation. Only a handful of miRNAs, including miR-19b, miR-106b, miR-155, and miR-146a, are expressed at higher levels in activated T cells than in naïve CD4$^+$ T cells (23,24). A comprehensive study on the roles of miRNAs in T cells was performed using mice deficient in *Dicer1* or DiGeorge syndrome critical region 8 (*Dgcr8*), which are key enzymes of miRNA biogenesis. *Dicer1* or *Dgcr8*-deficient T cells showed severe defects in activation, proliferation and survival; surprisingly, however, the production of functional cytokines of Th1-like interferon-gamma was not impaired in Dicer-deficient CD4$^+$ T cells (25,26). Moreover, *Dgcr8*-deficient CD4$^+$ T cells are preferentially differentiated into Th1 and Th2 cells, but show impaired generation of T_{FH} cells (27). These results strongly suggest the specific regulatory roles of miRNAs in Th differentiation together with their general roles in T cell proliferation and survival. These findings have prompted researchers to explore the more detailed molecular mechanisms of individual miRNA species on T_{FH} cell differentiation. To date, the potential roles of miR-17–92, miR-155, and miR-146a on T_{FH} cell differentiation and functions have been most extensively studied. Interestingly, these miRNAs show different expression patterns with respect to T_{FH} differentiation. Although all of these miRNAs are

induced upon T cell receptor stimulation, miR-155 and miR-146a sustain high expression levels during the further progression of T$_{FH}$ differentiation, whereas induced miR-17–92 species waned out at this stage (24,28,29).

Six different miRNAs, miR-17, miR-18a, miR-19a, miR-20a, miR19b, and miR-92a, are encoded by a single miR-17-92 transcript, and are expressed and processed together as a cluster. Initially, miR-17–92 was identified as a negative regulator of T$_{FH}$ cell function through CXCR5 suppression (30). However, more careful studies with loss- or gain-of-function approaches revealed that miR-17–92 expression in CD4$^+$ T cells is essential for a functional germinal center reaction. Upon antigen priming in T cells, miR-17–92 induces the expression of the target gene *Rora*, which in turn suppresses the expression of inappropriate genes for T$_{FH}$ cells such as *Ccr6*, *Il1r1*, *Ilr2*, and *Il22* that are positively regulated by *Roar* (27). In addition, miR-17–92 allows for antigen-primed CD4$^+$ T cells to migrate further into the B cell follicles and become functional T$_{FH}$ cells through regulating the strength of ICOS-mediated phosphoinositide 3-kinase (PI3K) activity by targeting the negative regulators of PI3K signaling pathway *Pten* and *Phlpp2* (28). Interestingly, fully differentiated T$_{FH}$ cells retain miR-17–92 expression at low levels, which suggests that tight regulation of the miR-17–92 expression level is an important regulatory mode for ensuring an appropriate germinal center reaction. Indeed, overexpression of the miR-17–92 cluster in CD4$^+$ T cells leads to the generation of an excess number of T$_{FH}$-like cells and activated B cells, ultimately leading to lymphoproliferative disease and death (28).

In contrast to miR-17–92, induced miR-155 and miR-146a expression upon T cell receptor-mediated stimuli was found to be sustained at high levels on fully differentiated T$_{FH}$ cells. The B cell integration cluster (*bic*) gene encodes miR-155, and *bic*-null mice show impaired T cell-dependent antibody production resulting in failure to protect against virulent *Salmonella typhimurium* infection, suggesting an important role of miR-155 in humoral immunity (31). miR-155-deficient T cells are activated normally but are prone to become IL-4-producing Th2 cells via the de-repression of c-Maf *in vitro*, which is reminiscent of the phenomenon by which miR-17–92 deficiency relieves *Rora* expression and causes the induction of non-T$_{FH}$ cell-related genes (27,31). Indeed, recent studies revealed a miR-155-mediated specific role for functional T$_{FH}$ cell generation via targeting *Peli1* and *Fosl2* in CD4$^+$ T cells (29,32). *Peli1* is an important regulator of c-Rel protein, a member of the NF-κB family, by means of the ubiquitination in T cells, thus protecting against T cell intrinsic autoimmunity in mice (33). In line with previous results, miR-155 deficiency was shown to give rise to a low level of c-Rel expression due to the de-repression of *Peli1* during T$_{FH}$ cell development. Interestingly, the low level of c-Rel expression does not affect T$_{FH}$ cell lineage commitment but rather leads to depletion of T$_{FH}$ cells in the draining lymph node, mainly due to the impaired proliferation of pre-T$_{FH}$ cells during development (29). *Fosl2* binds to Jun and compete with BATF-containing activating (AP-1) complexes for DNA binding on AP-1-IRF composite elements (AICEs), which is necessary for T$_{FH}$ cell generation with IRF4 recruitment. Therefore, the miR-155-mediated repression of *Fosl2* is important for determining T$_{FH}$ cell fate commitment (32). Taken together, these results suggest that miR-155 acts as a driver of T$_{FH}$ cell fate commitment and as an inhibitor of Th2 cell differentiation by regulating several genes concurrently.

miR-146a shows a similar expression pattern to miR-155 during T$_{FH}$ cell development, which indicates that miR-146a might also play important roles in T$_{FH}$ cell generation and functions. However, the ablation of miR-146a results in the accumulation of both T$_{FH}$ cells and germinal center B cells with increased expression of ICOS on T cells, which represents a restrictive role of this miRNA on T$_{FH}$ cell functions (24). Interestingly, the T$_{FH}$ cell-driven regulation of the

germinal center reaction might occur through a regulatory interaction between miR-155 and miR-146a in T cells. In 7–10-month-old miR-146a-deficient mice, T cell-driven spontaneous germinal centers are formed followed by autoantibody production in the serum, and miR-155 knockout almost completely restored this aberrant activity of miR-146a-deficient T cells to that of wild-type T cells (32). These findings indicate the opposing roles of miR-146a and miR-155 as the brake and accelerator pedals for the function of T_{FH} cells, respectively.

IL-2 mediated STAT5 signaling attenuates T_{FH} cell fate commitment at early stages of the differentiation program. One study demonstrated that miR-182 is induced by IL-2 and regulates the late phase of expansion by the post-transcriptional regulation of FoxO1 (34). Inactivation of FoxO1 through the ICOS-mediated signaling pathway was also shown to be an important aspect in regulation of T_{FH} cell differentiation at late stages (35), indicating a potent role of miR-182 in T_{FH} cell generation. However, genetic approaches of miR-182 deficiency in the context of the *miR-183/96/182* cluster have revealed a dispensable role of miR-182 in generating functional T_{FH} cells (29).

ROLE OF MIRNAS IN REGULATORY T_{FH} CELLS

FoxP3⁺ regulatory T cell (Treg)-mediated immune modulation is a widely accepted concept. Initially, it was considered that homogenous FoxP3⁺ T cells suppress several distinct subsets of effector T cells (36). However, recent growing evidence has shifted this concept toward a mechanism by which FoxP3⁺ Tregs employ distinct regulatory pathways to restrict the responses of different subsets of the effector T cells (37,38). The function of T_{FH} cells is also controlled by a specialized subset of Bcl6⁺ FoxP3⁺ T cells located in the germinal centers, named follicular regulatory T (T_{FR}) cells (39,40). Moreover, T_{FR} cells have been shown to suppress the numbers of T_{FH} cells and germinal center B cells and consequently the amount of antibody produced. T_{FR} cells are distinguished by T_{FH} cells according to the expression of IL-10, glucocorticoid-induced tumor necrosis factor family receptor (GITR), and cytotoxic T lymphocyte antigen (CTLA)-4. More importantly, T_{FR} cells share features in common with T_{FH} cells, including high expression of CXCR5, programmed cell death protein (PD)-1, and ICOS in addition to Bcl6. Beyond the location and expression profiles of T_{FH} cell signature molecules, the differentiation pathway of T_{FR} cells has also been co-opted from that of T_{FH} cells (41). Therefore, it is highly possible that several of the miRNAs described above would control the germinal center reaction through regulation of T_{FR} cell generation and function. Indeed, the miR-17–92 dosage is positively correlated with the number of antigen-specific T_{FR} cells, but not with that of polyclonal Tregs in the peripheral lymph nodes (27). In the absence of miR-146a in T cells, the numbers of both T_{FR} and T_{FH} cells are increased compared to those of wild-type T cells (24). However, miR-146a⁻/⁻ T_{FR} cells may lose their suppressive capacity due to the overexpression of STAT1, which can partially account for the spontaneous development of germinal centers over time, even in the presence of a high number of T_{FR} cells (42).

FoxP3 is responsible for the elevated expression of miR-155 in Tregs, indicating that miR-155 exerts certain important roles in FoxP3⁺ Tregs (43,44). Indeed, miR-155 has been shown to be responsible for maintaining the Treg pool in competitive settings via suppressing suppressor of cytokine signaling (Socs) 1 (44). Downregulation of *Socs1* expression confers Tregs with survival and proliferation ability because of the enhanced STAT5 activity through IL-2. IL-2-mediated STAT5 activation is a well-known inhibitory axis for T_{FH} cell generation (14,15). Therefore, it is possible that miR-155 may suppress the generation of T_{FR} cells from FoxP3⁺

Tregs to consequently enable adequate germinal center reactions. However, there has been no detailed study on the roles of miR-155 in T$_{FR}$ cells; thus, it would be interesting to address this issue in the future.

miR-10a is induced by transforming growth factor-beta and retinoic acid in induced Tregs, and is known to prevent the conversion of Tregs to T$_{FH}$ cells in Peyer's patches (45). Since miR-10a is also expressed in naturally occurring Tregs (46) and directly targets *Bcl6* and its co-repressor, *Ncor2* (45), it is plausible that miR-10a restrains the conversion of naturally occurring Tregs to Bcl6$^+$ T$_{FR}$ cells. In that regard, T$_{FR}$ cell generation might be accompanied by a decrease in the miR-10a expression level, representing another possible regulatory axis for the germinal center reaction. Although there is no direct evidence to date, it is plausible that such regulation would occur during T$_{FR}$ cell generation, because T$_{FH}$ cells reduce the copy number of miR-10a during T$_{FH}$ cell differentiation (29). However, it is still worthwhile to explore the role of miR-10a in humoral immunity using T cell-specific loss-of-function or gain-of-function approaches.

Overall, it is clear that several miRNA species regulate T$_{FR}$ cell generation and function. However, this subject has received relatively less attention than the regulation of T$_{FH}$ cells, mainly because of the prevailing concept that regulatory mechanisms for T$_{FR}$ cells might be common with those of T$_{FH}$ cells. Nevertheless, the ontology of T$_{FR}$ cells is different from that of T$_{FH}$ cells (conversion from Tregs vs. differentiation from naïve T cells) and their functions are also in opposition. Therefore, there might be a specific regulatory axis for T$_{FR}$ cell generation and function, which is also fine-tuned by certain miRNAs. Defining these mechanisms would be an interesting and important task toward gaining a comprehensive understating of regulation of the germinal center reaction.

PERSPECTIVES

Over the past few years, extensive studies have uncovered the miRNA-mediated regulatory mechanisms of the germinal center reaction at both the cellular and molecular levels (47). These studies mainly focused on the development of T$_{FH}$ cells from naïve CD4$^+$ T cells and their functions. Interestingly, most of the miRNAs identified to date, including the miR-17–92 cluster and miR-155, seem to share similar regulatory features by which they suppress non-T$_{FH}$ cell differentiation while concurrently facilitating the T$_{FH}$ cell development program (**Fig. 1**). This regulatory mode is fundamentally possible because even a single miRNA species is able to bind to numerous different genes and repress their expression simultaneously. Discovery of this interesting mode of regulation also raises the question as to whether or not miR-17–92 and miR-155 are specific miRNAs for T$_{FH}$ cell development and functions; however, this question remains to be clearly resolved. Moreover, several studies have demonstrated that miR-17–92 and miR-155 also regulate the Th1 responses against lymphocytic choriomeningitis virus infection and the generation of many other function T cell subsets (48-51), raising the general question as to how a single miRNA species is able to control so many different aspects of immune responses.

Recently, Lu et al. (52) elegantly explored this issue by generating *Socs1* 3′-untranslated region (UTR) mutant mice, which lack miR-155-mediated repression of only the *Socs1* gene among the validated miR-155 targets. Previous studies demonstrated that miR-155 regulates Th17/Th2/Treg/T$_{FH}$/natural killer (NK) cell generation and anti-viral immune responses using
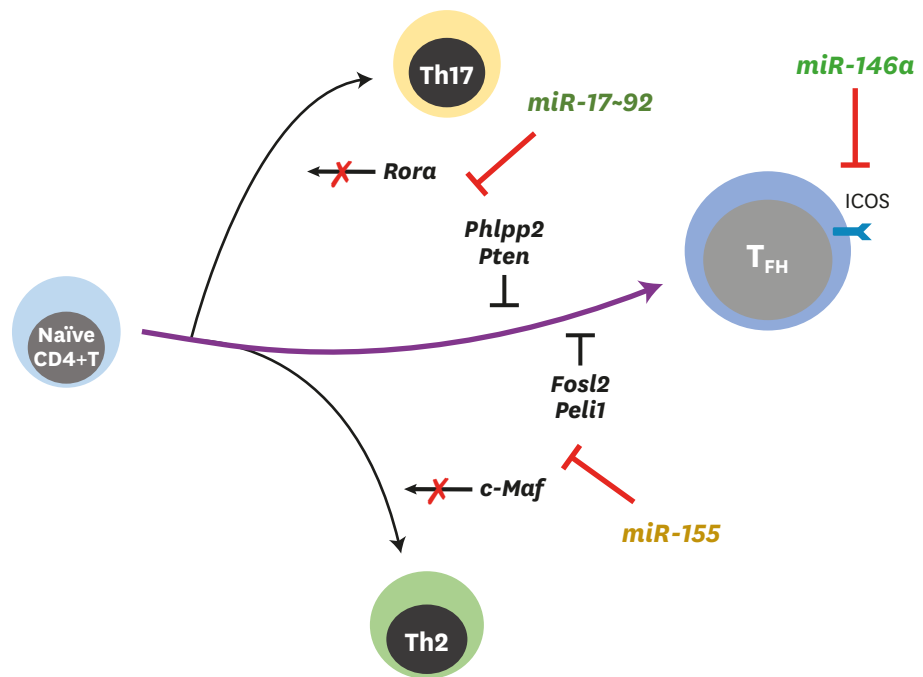
Figure 1. miRNA regulation of T_{FH} cell differentiation. During the priming of naïve CD4+ T cells, miR-17–92, miR-155, and miR-146a are induced upon T cell receptor mediated stimulation. miR-17–92 and miR-155 positively regulate T_{FH} cell differentiation via repression of negative regulators of T_{FH} cells, *Rora*, *Phlpp2*, *Pten*, *c-Maf*, *Peli1*, and *Fosl2*. However, miR-146a tones down T_{FH} cell functions by suppressing ICOS expression on the surface of effector T_{FH} cells.

different experimental settings with miR-155 knockout mice (29,31,32,44,50,53). These same studies indicated that *Jarid2*, *Socs1*, *Fosl2*, *Peli1*, *Il23r*, and *c-Maf* were functional targets, which are responsible for each of the observed phenotypes in the miR-155 knockout mice. Surprisingly, the *Socs1* 3′-UTR mutant mice phenocopied only a few phenotypes among those observed in the miR-155 knockout mice, such as NK cell expansion upon viral infection and enhanced Treg fitness in competitive settings (52). Considering all of these findings, Lu et al. (52) proposed that a single miRNA is capable of regulating several distinct immune responses by selecting a functional target in a cell type- and context-specific manner. This insightful conclusion can explain the variation observed within and between studies in 1 or 2 functional target genes even when using the same miR-155 knockout mice. However, this idea further brings forth a new and important question to resolve: how do miRNAs select the right target for the cell type and context?

Many research groups are currently pursuing answers to this abstruse question, including Xiao and colleagues (54,55). Recently, this group executed an elegant systemic transcriptome and translatome analysis of miR-17–92-deficient or overexpressed primary B cells (56). Although the conclusions of this study were not able to provide clear answers to this question, they nevertheless provided important insights on this issue. First, there are more putative miR-17–92-binding sites in the pool of mRNAs than the total copy number of miR-17–92 species at the single-cell level, indicating that the presence of conserved miRNA-binding sites in the 3′-UTR of mRNA is not a sufficient condition to be repressed by any given miRNA. This implies the existence of certain mechanisms by which miRNAs select their targets. Second, the authors found that the validated target genes of miR-17–92 show distinct repression sensitivity to miR-17–92 in primary B cells. In other words, certain miR-17–92 target genes are repressed only after miR-17–92 are up-regulated in the B cells; however, those genes are not necessarily de-repressed

when miR-17–92 are down-regulated. In contrast, another group of genes are responsive only when miR-17–92 are down-regulated; these 2 groups of genes rarely overlap with each other, indicating that functional target genes of miR-17–92 are probably selected on the basis of miRNA levels in B cells. Therefore, miRNAs might be able to select their appropriate target based on cell type and the context in accordance with the expression level of a given miRNA. Third, the secondary structure of mRNA was found to have a greater impact on its possibility of being selected as a target than other *cis* element criteria such as the length of the 5′-UTR, 3′-UTR, location, and numbers of miRNA-binding sites. Taken all together, these findings provide mechanistic insights into how the same miRNA species is able to choose different target genes in a cell type- and context-dependent manner.

The last few years has seen an explosion in research focused on defining the roles of miRNAs in the initiation and maintenance of the germinal center reaction. However, recent findings of the presence of a memory type of T_{FH} cells implies the existence of another level of regulation of the germinal center reaction by memory T_{FH} cells (57). Indeed, memory T_{FH} cells rapidly respond to the second challenged pathogen and acquire T_{FH} effector functions. This recall response of memory T_{FH} cells is considered as a key event in long-lasting protective humoral immunity. Thus, revealing the mechanisms of the generation and maintenance of memory T_{FH} cells might be crucial in the development of effective vaccines. Since CXCR5$^+$ T_{FH}-like cells are also characterized by plasticity with respect to their cell fate commitment upon stimulation, the roles of the memory type of T_{FH} cells in various aspects of immunology are actively being explored (58). Therefore, defining the specific miRNA species and deciphering the molecular mechanisms of the miRNA-mediated regulation of memory T_{FH} cell generation and functions will be of great interest, but remain to be elucidated.

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