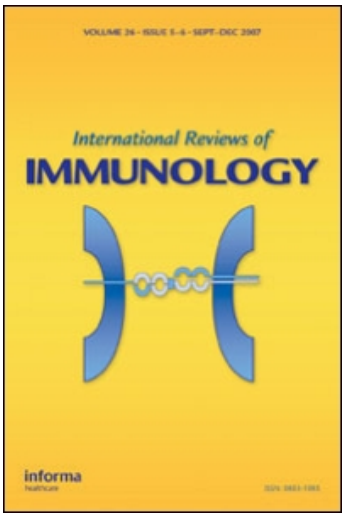

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Logic and Extent of miRNA-Mediated Control of Autoimmune Gene Expression

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Logic and Extent of miRNA-Mediated Control of Autoimmune Gene Expression

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Over the past few decades, multiple mechanisms have emerged that operate to prune the lymphocyte repertoire of self-reactive specificities and maintain immunological tolerance. Multiple families of small noncoding RNAs known as microRNAs (miRNAs) target immune transcripts to fine-tune gene expression and turn on negative feedback loops. Both of these actions are crucial to limit co-stimulation, set precise cellular activation thresholds, curtail inflammation, control lymphocyte growth, and maintain regulatory T cell homeostasis and suppressive function. Analysis of predicted miRNA-mediated regulation of 72 lupus susceptibility genes in humans and mice reveals most contain numerous target sites for over 140 miRNAs conserved in mammals. MECP2, ROQUIN/RC3H1, BCL2, BIM, and PTEN contain over 50 miRNA target sites each, highlighting the need to control their final protein products with enormous precision to maintain the balance between immunity and tolerance. Overlap among targets of individual miRNAs is considerable, with each miRNA targeting a median of nine autoimmune genes. Three miRNAs—miR-181, miR-186, and miR-590-3p—together are predicted to target over 50% of all lupus genes. Also, a single miRNA cluster located at 14q32.31 containing 11 miRNAs is predicted to regulate 48 lupus susceptibility genes. Dysregulation of single or a few miRNAs or miRNA clusters can result from genetic variation, hormonal influences, or environmental triggers including EBV infection. In the light of this vast and promiscuous miRNA-mediated regulation of autoimmune genes it is anticipated that changes in miRNA levels or their target sequences will help explain susceptibility to complex autoimmune diseases.

Keywords microRNA (miRNA), autoimmunity, lupus, SLE, tolerance

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INTRODUCTION: REGULATION OF IMMUNE TOLERANCE AND miRNAs

The adaptive immune system is capable of recognizing the myriad of life-threatening pathogens that an individual can encounter. This is achieved through a random recombination of T- and B-cell receptor genes that occurs during lymphocyte development. Further receptor diversification can also occur during responses to foreign antigens, in which B cells can acquire point mutations in their Ig V region genes during the process of somatic hypermutation. Given that these processes are stochastic, self-reactive specificities emerge and need to be silenced or eliminated from the mature peripheral repertoire so that autoimmunity does not occur.

One of the greatest advances in immunology over the last two decades has been to unravel the major mechanisms of immunological tolerance. Numerous tolerance checkpoints have been described that operate from lymphocyte development, through selection into the mature T- and B-cell repertoire, to lymphoid cell activation and death after antigen encounter, to ensure immune responses are not directed to self [1]. Most, if not all, strategies employed to maintain tolerance require changes in expression of key molecules that determine cell death vs. survival decisions, changes in B cell receptor (BCR) or T cell receptor (TCR) activation thresholds, expression of inhibitory receptors, regulation of T- and B-cell co-stimulatory signals or growth factors, sensitivity to innate pro-inflammatory signals, or active suppression by regulatory cells.

Until recently, regulation of these determinants of immunological tolerance was thought to occur mostly at the level of transcription, with some contribution from ubiquitin-dependent degradation and genomic DNA modification. But in the last three years, a new level of regulation of protein expression, namely microRNA (miRNA)-mediated inhibition of gene expression, has emerged as a powerful way of preventing autoimmune responses and switching off potentially harmful inflammatory responses. This capacity to fine-tune the extent of gene expression in a fast and very precise way posttranscriptionally to prevent self-reactivity contributes to the delicate balance between effective immunity and autoimmunity. A picture is emerging in which autoimmunity may often be a result of failing to control the expression of proteins that normally exert their physiological functions over a very narrow concentration range [2, 3]. These insights have also been gained from the study of mutant strains of mice in which autoimmune phenotypes are caused by less than a 2-fold change in expression of critical immune regulators such as BIM, PTEN, ICOS, and TLR7 [4–6].

miRNAs generally modulate expression of proteins that in a normal setting have a narrow range of concentrations. The net effect is usually repression of target transcripts, although in exceptional cases, the opposite may be true. The physiological consequences of this control span most aspects of animal and plant biology, from control of cellular differentiation, survival, or proliferation, to maintaining lineage integrity and shutting off responses [7–9]. Mature miRNAs are usually ~22 nucleotides in length and single stranded. The genes encoding the primary transcripts are usually located in intergenic regions of the genome, but can sometimes be located within introns of protein-coding genes [10]. Often, several microRNAs cluster together in the genome and are co-transcribed as part of the same primary transcript [8]. Precedents have been described for these clustered miRNAs functioning in the same biological pathways, so that their collective action is greater than that of the individual miRNAs. To achieve this, miRNAs in the same cluster sometimes share identical seed regions and therefore target the same transcript, or have different seed regions but target proteins in interrelated pathways [4, 8, 11]. Repression of different proteins that work in the same pathway by a single miRNA has also been described, and appears to be important to set TCR signaling thresholds and influence thymocyte selection [12].

After processing by the nuclear RNAse III endonuclease, Drosha, primary microRNAs (pri-miRNA) are cleaved to yield an ~60–70 bp precursor miRNA (pre-miRNA) that adopts a hairpin structure and is transported to the cytoplasm. Cleavage by a different RNAse III endonuclease, Dicer, produces the mature miRNA [8]. The miRNA directs the RNA-induced silencing complex (RISC) to its target mRNA. Recognition of the target transcript usually occurs through perfect pairing of 6–8 nucleotides at the 5′ end of the microRNA [7, 8]. Individual miRNAs can repress the expression of hundreds of proteins frequently through a combination of inhibi-tion of translation and mRNA degradation [13, 14]. Exceptionally, miRNAs can increase target mRNA translation [15] or impair gene transcription [16].

Although to date there is only experimental evidence for a handful of immune transcripts being regulated by a small number of miRNAs, current predictions estimate ~30% of the genes are regulated by miRNAs. Over 700 miRNAs have been identified in humans (miRBase [http://microrna.sanger.ac.uk]) but this number is predicted to rise as all the functional elements of the genome are revealed [17].

miRNAs INFLUENCE IMMUNOLOGICAL TOLERANCE

Regulation of gene expression in eukaryotic cells has many aspects, including chromatin remodelling, transcription factors, splicing factors,

and miRNAs among others. Is there a unique regulatory role for miRNAs, or do they behave like other repressors of gene expression? miRNAs and transcription factors both appear to apply a common regulatory logic including pleiotropy (one miRNA regulating many targets), combinatorial activity (several miRNAs regulating one target), accessibility (availability of *cis* regulatory elements in targeted genes for miRNA recognition), regulation (miRNA subject to the cell type/cellular status-dependent regulation within the processes of transcription, maturation, and RNA editing) and regulatory network motifs (feedback and feedforward regulation) [18]. Despite such similarities, systemic miRNA loss-of-function studies in *Caenorhabiditis elegans* revealed a more restricted phenotypic spectrum resulting from miRNA deficiencies compared to those resulting from loss of transcription factors: less than 10% of miRNA knockouts result in clear developmental or morphological defects [19] whereas ~30% of transcription factor-silenced lines have obvious phenotypes [http://www.wormbase.org/].

Although there is probably more redundancy in miRNA-mediated regulation than transcription factor-mediated regulation, it is becoming obvious that miRNAs act to fine-tune gene expression: recent analysis of the impact of miRNAs on protein output has shown miRNAs repress gene expression to a modest degree [13]. There are other distinguishing features of miRNA-mediated regulation, including the ability to regulate the speed of translation and compartmentalize protein synthesis [20, 21].

Fine-Tuning Gene Expression by miRNAs Prevents Autoimmunity

Regulation of Co-stimulation: Roquin, miR-101, and miR-103

The first evidence linking miRNA-mediated gene regulation with autoimmunity was provided by studies in the lupus-prone strain of mice, *sanroque,* homozygous for a hypomorphic allele of the *Roquin* gene (Roquin$^{san/san}$) [22]. Lymphoproliferation in these mice is caused by overexpression of the inducible T cell co-stimulator (ICOS), due to decreased miR-101 and miR-103-mediated *Icos* mRNA degradation [5]. Aberrant ICOS expression on naive T cells breaks the two-signal mechanism regulating T cell responses in secondary lymphoid tissues. TCR engagement by antigen/MHC on an antigen-presenting cell only triggers T cell accumulation and effector functions when a-stimulatory second signal is delivered through CD28 [23–25]. In *sanroque* mice, T cell activation, differentiation, and antibody responses can occur independent of CD28, and therefore independent of danger-induced CD28-ligands, but dependent on ICOS, whose ligands are expressed

constitutively in the absence of infection [26]. Roquin^{san} also acts T cell-autonomously to induce the accumulation of T follicular helper cells, which mediate aberrant positive selection of self-reactive germinal center B cells [22, 26].

The view that ICOS protein levels require precise tuning is supported by the observation that while expression is essential for humoral immunity, excessive expression leads to autoimmunity. ICOS deficiency in humans is a cause of common variable immunodeficiency (CVID) [27]. CVID is characterized by a profound hypogammaglobulinemia and an increased susceptibility to bacterial infections of the respiratory and gastrointestinal tracts due to impaired germinal center reactions and a severe reduction in memory B cells. In addition to the ICOS-driven lupus phenotype of *sanroque* mice, higher expression of ICOS has been detected on T cells from SLE and rheumatoid arthritis patients compared to healthy controls [28–30]. Expression of miR-101 inversely correlates to ICOS expression in human tonsillar T-cell subsets with naive cells expressing the highest levels of miR-101 and lowest levels of ICOS, and T follicular helper (T_{FH}) cells expressing the lowest levels of miR-101 but highest levels of ICOS [5].

Regulation of Thymic Selection: Phosphatases and miR-181a

In addition to fine-tuning co-stimulatory signals to maintain T-cell peripheral tolerance, miRNAs, notably miR-181a, have also been implicated in adjusting TCR signalling thresholds during T cell development in the thymus [12]. By targeting and downregulating multiple phosphatases, miR-181a leads to elevated steady-state levels of phosphorylated TCR signalling intermediates and a reduced TCR signalling threshold. miR-181a expression is dynamically regulated during T cell development, with immature double-negative cells having a three- to ten-fold higher expression of miR-181a than CD4 or CD8 single positive cells, which confers a two- to five-fold increase in sensitivity toward peptide-MHC complex ligands in double positive cells [12]. Modulating TCR thresholds to allow weak binding to peptide-MHC complex during positive selection and strong binding to peptide-MHC complex during negative selection is critical for the production of a self-tolerant T-cell repertoire.

Regulation of Treg Homeostasis: FoxP3 and miR-155

An important mechanism employed by the immune system to shut off potentially harmful anti-pathogen immunity or inappropriate activation of self-reactive lymphocytes is suppression mediated by regulatory T cells (Tregs), a mechanism often referred to as dominant

tolerance. The importance of Treg cells in the maintenance of tolerance is exemplified by the lethal autoimmune syndrome observed in mice and humans with mutations in FoxP3, the master Treg transcriptional regulator [31–33].

A key role for miRNAs in Treg differentiation was first described in mice subjected to T cell-specific deletion of the *Dicer* gene [34], in which Treg formation was reduced by more than 50%. These mice developed colitis and lung and liver inflammation from four months of age. A comparable phenotype was found in mice lacking T cell expression of *Drosha* [35]. Furthermore, generation of Treg -specific *Dicer* or *Drosha* conditional knockout mice revealed that functional miRNA machinery is not only essential for Treg homeostasis, but also for suppressive function: mice bearing Dicer- or Drosha-deficient Tregs succumbed to autoimmunity in a manner indistinguishable from mice lacking FoxP3 [35–37]. These data demonstrate the power of miRNAs in maintaining the 'functional identity' of a differentiated effector cell.

Multiple miRNA deficiencies are likely to contribute to defective Treg homeostasis and function in Treg Dicer or Drosha conditional knockout mice. Lack of miR-155, which is directly regulated by FoxP3, appears to account for the defect in Treg homeostasis observed in mice with Dicer-deficient Tregs [38]. MiR-155 represses suppressor of cytokine signalling 1 (SOCS1) by two- to three-fold, increasing sensitivity to IL-2 and conferring on Tregs competitive fitness for homeostatic proliferation. MiR-155 expression, and the targeting and suppression of SOCS1 [38], are required to maintain Treg proliferative activity. Nevertheless, miR-155-deficient Tregs had intact suppressive functions, suggesting that deficiencies in additional miRNAs account for the more profound Dicer-dependent Treg dysfunction [36, 38]. All the molecules identified to date as important for Treg development and function, including IL-2, IL-10, TGF-beta, CTLA-4, granzyme B, perforin, IFN-gamma, IL-9, heme oxygenase-1 (HO-1), cAMP, CD25, CD39, galectins, and IL-35 are not unique to Tregs, but simply expressed at higher levels. This suggests that Tregs are subject to a high degree of miRNA-mediated fine-tuning of gene expression.

miRNA-Mediated Negative Feedback Moderates the Immune Response to Prevent Autoimmunity

In order to maintain immunological tolerance, it is as important to prevent recruitment of self-reactive lymphocytes into immune responses as it is to turn off superfluous inflammatory responses. The importance of switching off immune responses has been underscored by

revelations that deficient activation-induced cell death, failure to negatively regulate the Toll-like receptor family of receptors that sense pathogen-associated molecular patterns, and inability to control immune responses via Tregs can all lead to autoimmunity [1]. Indeed, the mammalian immune system counts with a powerful network of negative regulators. These inhibitory pathways allow control of both the intensity and the duration of inflammation, and there are multiple examples to show that disruption of inhibitory regulatory elements, such as CTLA-4 or Fas signalling, leads to autoimmunity [1]. Recent data have suggested that miRNAs are particularly important in "regulating the regulators," acting themselves as negative regulators of the inhibitory regulatory elements. The following section highlights a number of areas in which miRNAs have a critical role in the regulation of the immune response.

Curtailing Inflammation: TLRs, TNF, Type I IFN, and miR-146a

Cells of the innate immune system, including macrophages and neutrophils, have a central role in autoimmune disease by virtue of the tissue damage they cause. Their responses are mainly controlled by signals from stimulatory or inhibitory receptors or cytokines. Pioneering studies on the role of miRNAs in modulating cellular responses to pro-inflammatory cytokines and microbial components revealed that miR-146a is induced by various bacterial-associated stimuli including LPS (through TLR4), peptidoglycan (through TLR2), and flagellin (through TLR5), as well the pro-inflammatory cytokines TNFα and IL-1β [39]. MiR-146a suppresses IL-1 receptor-associated kinase 1 (IRAK1) and TRAF6 (Fig. 1). Furthermore, miR-146a also appears to turn off type I IFN receptor signalling through repression of STAT1 and interferon regulatory factor 5 (IRF5), which serves as a co-adaptor protein of both IRAK1 and TRAF6 downstream of TLR signalling, and as a transcription factor for type I IFNs as well as pro-inflammatory cytokines [40]. A recent study on miRNA expression in a large cohort of systemic lupus erythematosus (SLE) patients revealed decreased expression of miR-146a in peripheral blood mononuclear cells, and a negative correlation between miR-146a levels and disease activity [40]. Thus, restoration of miR-146a expression may provide a powerful and novel avenue for therapeutic intervention particularly if targeted to those patients with low miR-146a expression.

Controlling Cell Growth and Survival: miR-17-92a

The human genomic locus encoding the polycistronic cluster miR-17–92, 13q31.3, has been found to be amplified in several types of

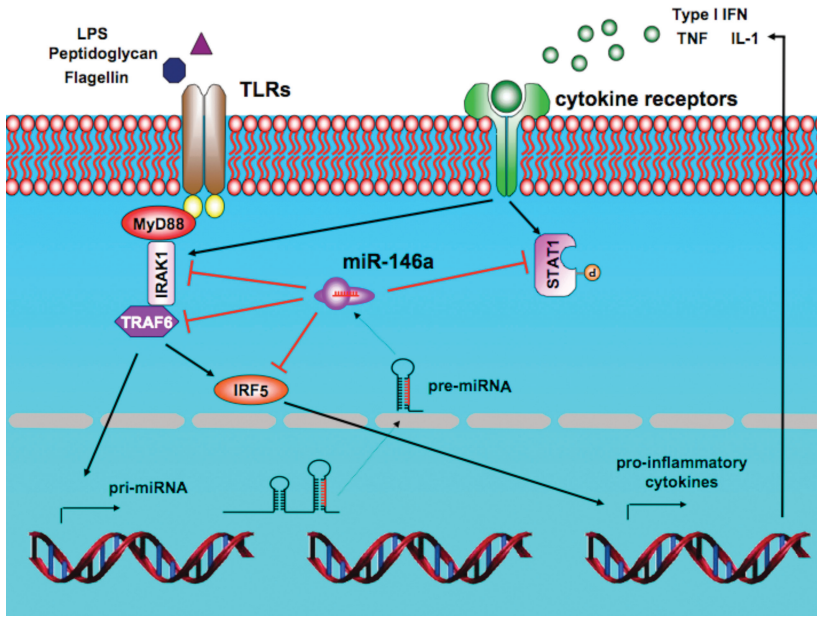


FIGURE 1 Mir-146a establishes negative feed-back loops to dampen pro-inflammatory signals downstream of Toll-like and cytokine receptors. Bacterial-associated stimuli and pro-inflammatory cytokines signal trough TLRs and cytokine receptors, respectively, to activate the NF-kB pathway and induce miR-146a expression. MiR-146a suppresses IL-1 receptor-associated kinase 1 (IRAK1), TRAF6, STAT1 and interferon regulatory factor 5 (IRF5) to turn off TLR signalling, TNF, IL-1, and type I IFN receptor signalling pathways.

lymphoma and solid tumors [41]. Enhanced expression of this miRNA cluster accelerates tumor development in the well-studied *Eμ-myc* transgenic mouse model of B cell lymphoma [42]. In this model, c-Myc and the cell cycle-regulating transcription factor E2F1 induce each other reciprocally, but c-Myc also induces miR-17-92 expression to inhibit E2F1 translation [43], revealing a negative feedback loop that dampens the positive feedback in order to control proliferation.

There is a broad overlap between the genes and molecular pathways underpinning immune tolerance checkpoints and those involved in tumor suppression [44]. It is, therefore, not surprising that transgenic mice overexpressing miR-17-92 in lymphocytes develop lymphoproliferative disease and die prematurely [4]. This study also revealed two more targets suppressed by miR-17-92; the tumor suppressor PTEN and the pro-apoptotic molecule Bim. Bim is transcriptionally regulated by c-Myc and E2F1 [45], in part explaining how c-myc overexpression

induces apoptosis [46]. PTEN encodes a phosphatase that inactivates the P13-K/AKT pathway of lymphocyte growth and survival and is also important in maintaining chromosomal stability [47]. E2F1, PTEN, and Bim are important in maintaining central and peripheral tolerance [1]; downregulation of their expression can cause the breakdown of immune tolerance, particularly in certain genetic backgrounds [48]. Thus, it is conceivable that miR-17-92 helps to integrate extracellular or intracellular signals to maintain the balance between proliferation and apoptosis that helps prevent autoimmunity.

ANALYSIS OF PREDICTED miRNA-MEDIATED REGULATION OF AUTOIMMUNE GENES

Most Autoimmune Genes Are Predicted to Contain miRNA Target Sites

We have described several examples where dysregulation of individual miRNAs or miRNA clusters, targeting genes associated with autoimmunity, have been shown to lead to autoimmune manifestations. To gain some insight into the potential for miRNA-mediated regulation of autoimmunity susceptibility genes, we chose to focus on systemic lupus erythematosus (SLE), the prototypic systemic autoimmune disease. There is unequivocal evidence that SLE has a genetic component. Rare but catastrophic deletions in complement genes, 25–60% concordance rates in monozygotic twins, numerous confirmed gene associations from genome-wide association studies, and both mono- and polygenic lupus mouse models all contribute to this evidence.

Genes predisposing to SLE were compiled from two recent studies. Kono and Theofilopoulos recently reviewed the genes shown to contribute to laboratory or clinical manifestations of systemic autoimmunity, identified in either spontaneous mouse lupus models or genetically manipulated mice [49]. In a separate recent review, Harley and colleagues summarized the genes reported to be conclusively associated with human SLE in genome-wide association studies [50]. C1q, C4, and ICOS were added to the analysis due to their reported contribution to SLE in a gene-dose-dependent fashion in humans and/or mice [5, 51].

The potential for miRNA-mediated regulation of 72 genes, chosen based on the above studies and their presence in the Targetscan database, and designated henceforth “autoimmune genes,” was investigated. For all types of analysis, the miRNA target sites of the human genes were studied regardless of whether the existing association with lupus had been reported only in mice, for three reasons. First, these genes will only be of interest if they are relevant for human molecular

mechanisms of autoimmunity, and if this is the case, it is crucial to understand their regulation in humans. Second, the 3′UTRs of most genes are, to date, better annotated in the human genome, with many mouse transcripts remaining incomplete. Third, our analysis only includes miRNAs that are themselves completely conserved between humans and mice.

MiRNAs predicted to target the 3′UTR regions of autoimmune genes were identified using TargetScan Human (release 5.0) [13, 52, 53]. Bartel's Team recently compared the correspondence between computational target predictions and observed protein changes, concluding that Targetscan predictions are generally the most accurate when taking into account the "total context score" for 7mer or 8mer sites matching the cognate miRNA [13]. Context scores are calculated as a function of site type, site number, and site context [53]. We therefore performed searches for miRNAs conserved in either all vertebrates or only in mammals, with a context score cut-off of -0.1, which excluded $\sim$20–30% of all miRNAs predicted to target the 3′UTRs of interest.

All except one autoimmune gene, BAFF, were found to contain at least 1 target site for conserved miRNAs, with a median of 14 target sites per gene (Fig. 2a). Five genes contained more than 50 miRNA target sites each: *MECP2, ROQUIN, BCL2, BIM,* and *PTEN* had 70, 59, 55, 53, and 52 sites, respectively. It is striking that four of the five genes act as negative regulators of their respective signaling pathways: *ROQUIN, BIM,* and *PTEN* repress co-stimulation, survival, and cell growth, respectively, and *MECP2* encodes for methyl-CpG-binding protein 2, important for epigenetic transcriptional repression of methylation-sensitive genes [54], suggesting that miRNAs play important roles in regulating the negative regulators. As mentioned above, increased repression of BIM and PTEN in mice overexpressing the miR-17-92 cluster is responsible for at least part of the autoimmune phenotype these mice develop [4]. Of interest, our analysis predicts that this particular miRNA cluster regulates 11 other autoimmune genes besides the reported *PTEN* and *BIM*, including *ROQUIN, ATG5,* and *P2RX7*.

The bias towards negative regulators being extensively regulated by miRNAs was only observed among the genes containing the largest number ($>$40) of miRNA-binding sites: Besides the negative regulators described above, *MITF* and *RASGRP1* have also been described to repress cellular growth [55, 56]. Therefore, six of the top seven genes behave as negative regulators. Consideration of the polarity of effect—i.e., whether the gene product acts in a positive or a negative way to promote the disease phenotype—of the rest of the targets containing

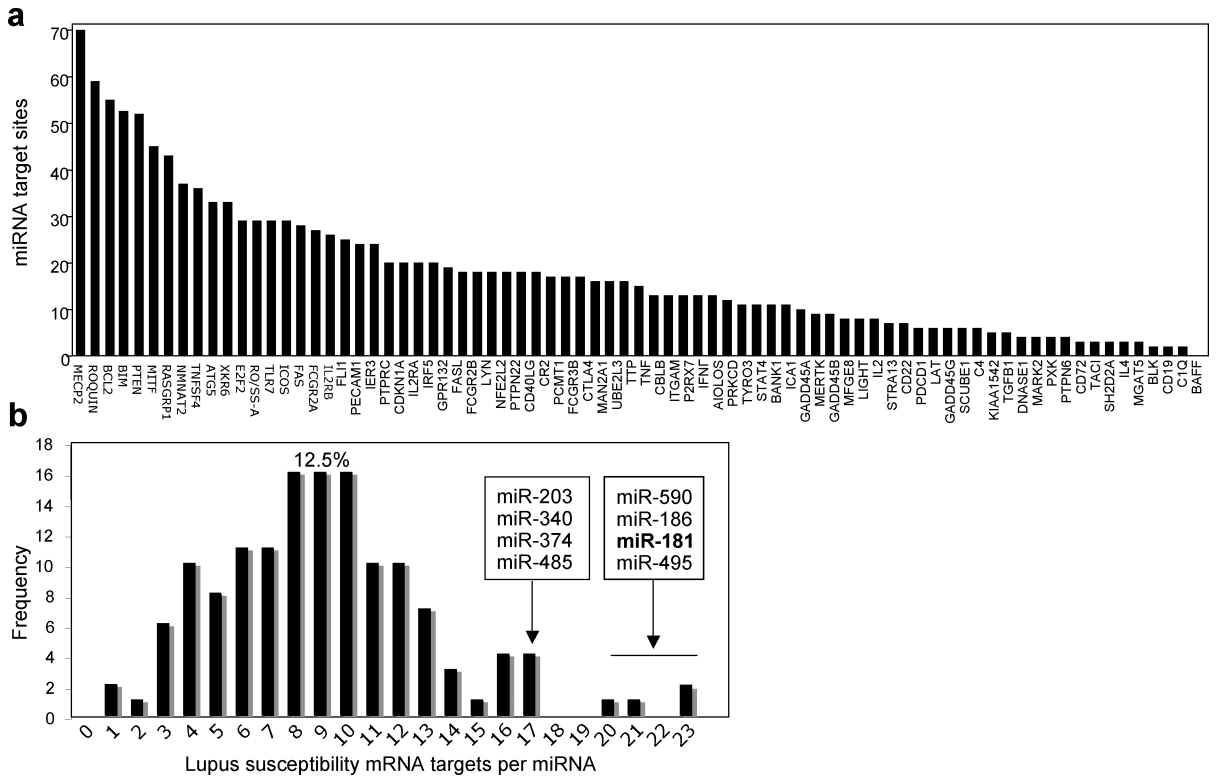


FIGURE 2 Most autoimmune transcripts are targeted by several miRNAs. (a) Graph illustrating the number of conserved miRNA-binding sites predicted to target the known lupus susceptibility genes described in mice and/or humans. (b) Histogram showing the frequency of miRNAs targeting the indicated numbers of SLE genes target genes per miRNA. The median proportion of SLE genes targeted by a miRNA is 12.5%. The miRNAs in the boxes correspond to those with the values in the indicated bars. Predictions have been generated by TargetScan Human 5.0 and only include miRNAs conserved in vertebrates or only in mammals, with a total context score cut-off of −0.1.

less than 40 predicted miRNA binding sites, did not reveal a bias among the targets to negative regulators.

Promiscuous and Overlapping Regulation of Autoimmune Transcripts by miRNAs

An interesting observation emerging from this analysis is that most miRNAs identified target an average of 8–10 autoimmune genes (12.5% of all lupus susceptibility genes considered), with a surprising degree of overlap between their targets (Fig. 2b). There has been much effort to subset SLE patients according to pathogenic pathways, and there is good evidence to support the idea that there are different mechanisms that drive SLE. Indeed, both T-independent and T-dependent pathways have been described, and among the latter, either follicular or extrafollicular routes of autoantibody production can lead to SLE [57, 58]. In this context, it would not be unreasonable to expect that individual miRNAs or groups of miRNAs selectively regulate groups of genes critical for the different pathways. Nevertheless, the overlap in miRNA targets precludes identification of any such pattern. It is also true that while the cellular pathways have been defined relatively well, the molecular pathways explaining the different routes to SLE are less clearly understood. Furthermore, dysregulation of molecules that are clearly linked to one pathogenic pathway have been shown to cause cellular phenotypes typical of other pathways. An example is dysregulation of TLR-7 in T-independent or extrafollicular routes to autoimmunity [59], which also appears to cause accumulation of T-follicular helper cells, a typical feature of germinal-center-derived autoimmunity [22, 58]. Therefore, assignment of miRNAs to specific regulation of individual pathogenic pathways may prove very complex.

We were surprised to find 4 miRNAs—miR-590-3p, miR-186, miR-181, and miR-495—each targeting 20 or more autoimmune genes. Analysis of the targets of miR-590/590-3p, miR-186, and miR-181 revealed they are predicted to regulate over 50% of all autoimmune genes (Fig. 3). Furthermore, 20 autoimmune genes are predicted to be targeted by at least 2 of these 4 miRNAs. Four transcripts, *ROQUIN, BIM, PTEN,* and *TNFSF4,* were predicted to contain binding sites for the four miRNAs. Co-regulation of these four transcripts by numerous miRNAs shown to be highly expressed in T cells suggests they may form part of related biological pathways. Indeed, loss of function of Roquin, Bim, and Pten, and OX40L overexpression in T cells all lead to lymphoproliferative syndromes and anti-nuclear antibodies in mice [2, 60]. Roquin represses its targets through the miRNA

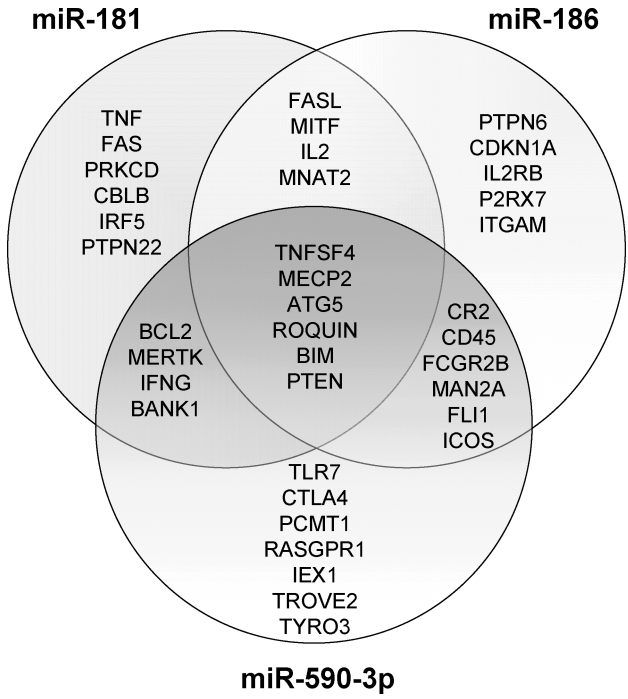
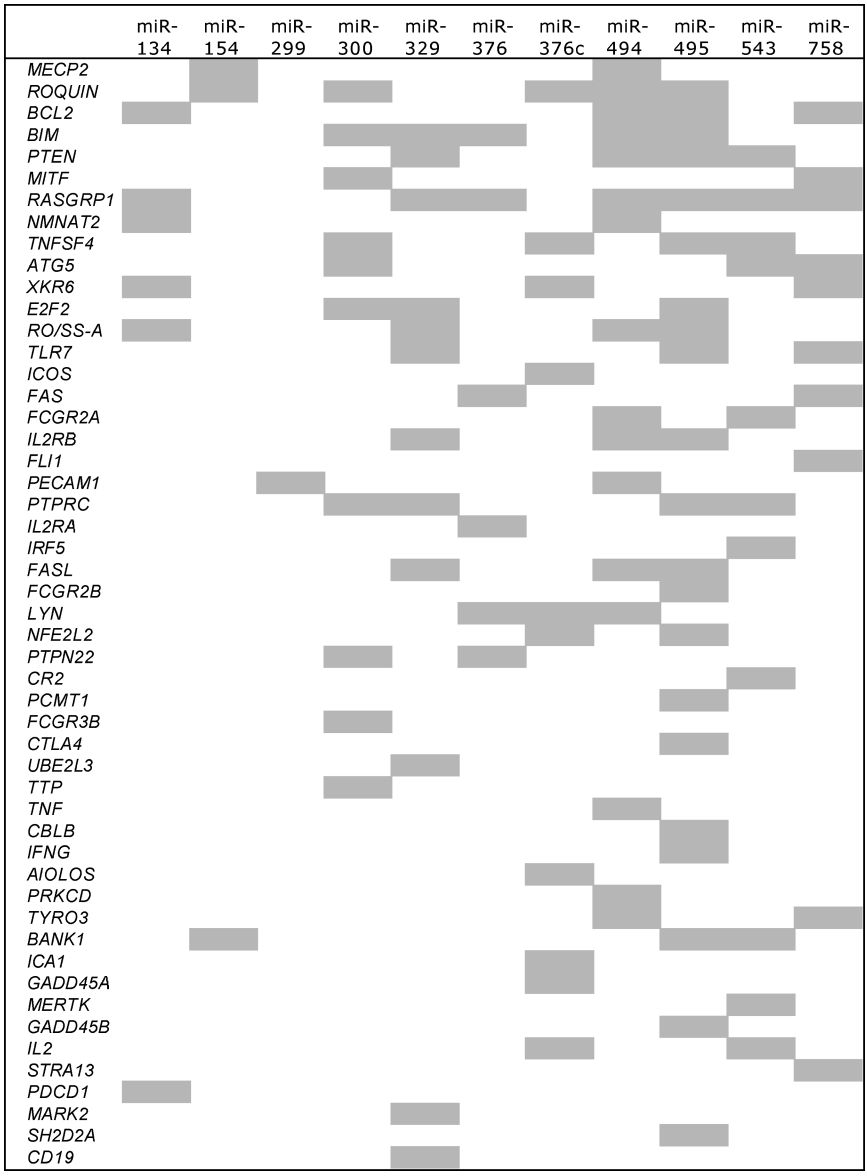


FIGURE 3 Three miRNAs are predicted to target over 50% of all SLE genes. Individual targets of the three miRNAs highlight the overlap in their targets. Predictions have been drawn as in Figure 2.

machinery; negative feedback loops in miRNA networks appear to be a common phenomenon. To date, only miR-181 has been reported to be expressed in hematopoietic cells, including T and B cells. Therefore, besides its reported role in regulating TCR activation thresholds during thymocyte development, it is possible it may contribute to the establishment of peripheral tolerance through targeting autoimmune genes.

MiR-495, predicted to regulate 20 lupus genes, belongs to the miR-329 gene cluster located at 14q 32.31 and is comprised of 11 miRNAs (miR-134, niR-154, miR-299, miR-329, miR-376, miR-376c, miR-494, miR-495, miR-543, and miR-758). These clusters typically are transcribed into a single polystronic primary-miRNA and, therefore, appear to be co-regulated and co-expressed. The mir-495 cluster alone is predicted to target a staggering 49 of the 72 (68%) autoimmune genes (Table 1), highlighting its potential to cause autoimmunity if dysregulated. Although miR-590-3p, miR-186, and miR-495 have all been

TABLE 1 Predicted SLE Targets of the miRNA Genes Comprising the miR-329 Gene Cluster in Chromosome 14.q 32.31



Shaded boxes indicate predicted miRNA targets for the indicated genes.

cloned from human and mouse tissues, their expression has not been reported in T cells [61], and as yet, there are no reports on their expression patterns in B cells or other hemopoietic cells. Thus, it is possible that they may not be expressed in cells where the relevant autoimmune genes need to be regulated.

Pervasive miRNA Mediated Regulation Is Not an Exclusive Feature of Immune Tolerance

This simple analysis showing miRNA-mediated regulation of 99% of autoimmune genes supports the notion that immunological tolerance is highly dependent on precise fine-tuning of protein expression levels, and is therefore likely to be one of the most heavily posttranscriptionally regulated lymphoid processes. A parallel and intriguing observation is that over 140 miRNAs are predicted to be involved in regulating this set of genes, and are likely to play a role in immunological tolerance. While this number is most likely an overestimate, given that a fraction of these miRNAs may not be expressed in the relevant cell types or immune tolerance checkpoints, we are still left with a very significant fraction of all miRNAs described to date to be conserved in mammals appearing to play a role in maintaining the delicate balance between immunity and autoimmunity.

In order to have some insight into the significance of this apparently profound miRNA-mediated regulation of autoimmune genes, we performed a parallel analysis of another complex disease, Type 2 diabetes (T2D). This disease was chosen because: (i) it is known to have a strong genetic component, (ii) many novel T2D susceptibility genes have been identified recently through large genome-wide association studies, (iii) there is no overlap in known susceptibility genes between SLE and T2D, and (iv) while T1D is autoimmune in origin, T2D is not thought to be a direct result of immune dysregulation. Nevertheless, there is accumulating evidence that T2D is a chronic inflammatory state accompanied by dysregulation of pro-inflammatory cytokines [62] and, therefore, it is important to bear in mind there is likely to be overlap in the pathogenesis between these two diseases. Indeed, T2D susceptibility genes NOTCH2 and AKT2 influence B and T cell development respectively [63, 64].

Forty-one T2D susceptibility genes were considered [65]. We found a very similar extent and pattern of miRNA-mediated regulation of SLE and T2D genes: all but one gene (INS) was targeted by miRNAs, with a median of 12 miRNA target sites per gene (Fig. 4a). Most individual miRNAs targeted a median of 5 T2D genes, which represents 12.2% of

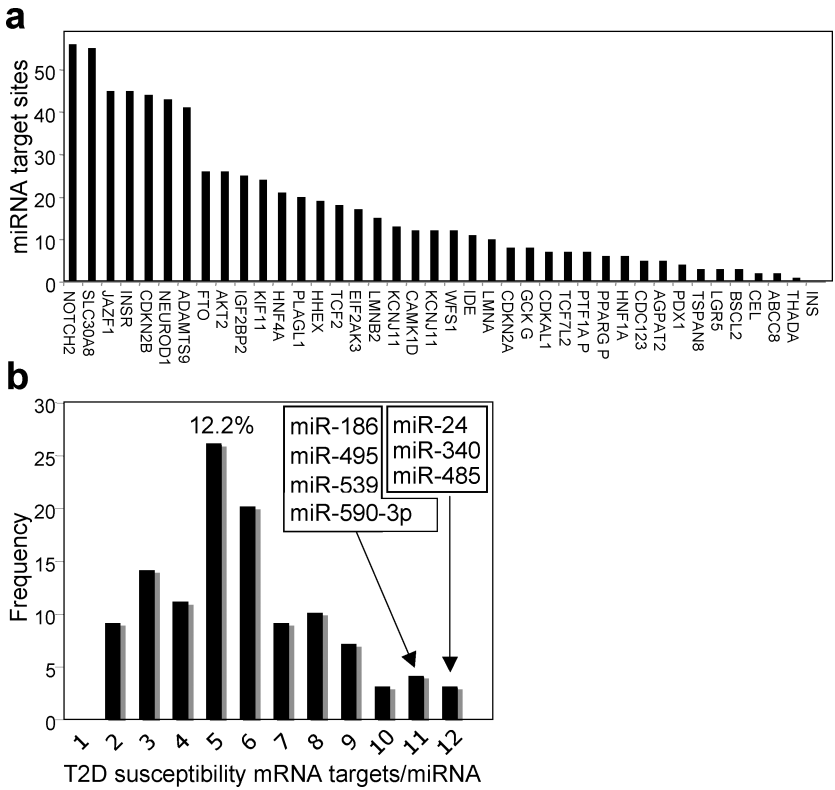


FIGURE 4 Pervasive miRNA-mediated regulation is not an exclusive feature of SLE and is comparable in type 2 diabetes (T2D). (a) Graph illustrating the number of conserved miRNA-binding sites predicted to target the known T2D susceptibility genes described in mice and/or humans. (b) Histogram showing the frequency of miRNAs targeting the indicated numbers of T2D genes target genes per miRNA. The median proportion of T2D genes targeted by a miRNA is 12.2%. The miRNAs in the boxes correspond to those with the values in the indicated bars. Predictions have been generated by TargetScan Human 5.0 and only include miRNAs conserved in vertebrates or only in mammals, with a total context score cut-off of −0.1.

the genes considered (Fig. 4b), a proportion identical to that described for SLE (Fig. 2b). Two of 41 genes contained more than 50 miRNA target sites. Furthermore, miR-186, miR-495, and miR-490, shown to target the largest number of autoimmune genes, were also among the top 7 miRNAs targeting the larger number of T2D genes. miR-181 behaved differently, targeting more autoimmune genes (29.1%) compared with T2D genes (14.6%).

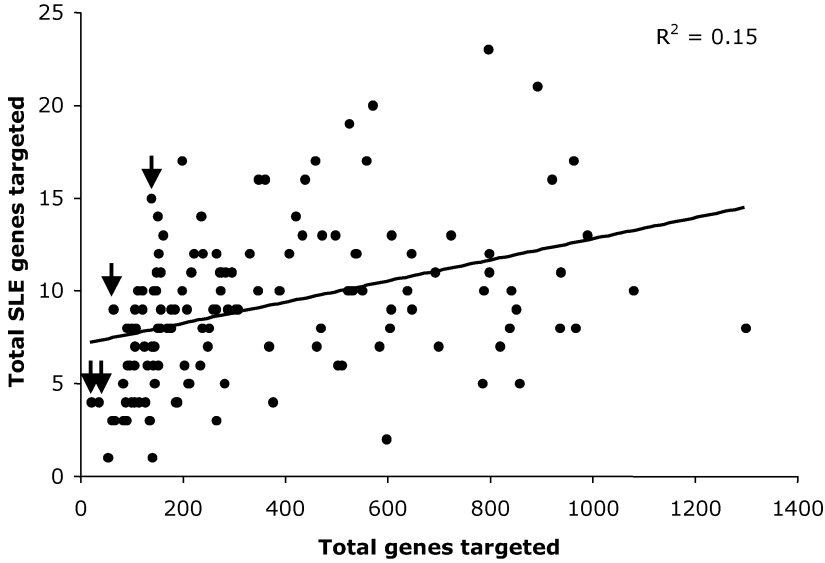


FIGURE 5 Weak correlation between total gene targets and SLE targets for individual miRNAs. Linear regression analysis between the number of total gene targeted and number of SLE targets for individual miRNAs indicates ~15% of the variance of the number of SLE genes targeted can be accounted for by changes in the number of total genes targeted.

A possible explanation for the observation that several miRNAs appear to target numerous genes that function in different biological process is that these miRNAs simply target a large number of genes across the genome, perhaps due to common recurrence of their specific binding sequence within 3′ UTRs. We found a weak linear relationship between the total number of predicted gene targets and the number of predicted SLE gene targets: only 15% of the variance of the number of SLE genes targeted can be accounted for by changes in the number of total genes targeted (Fig. 5). For miRNAs, miR-216, miR-411, miR-296-3p, and miR-361 5p (Fig. 5, symbols under arrows), SLE targets represented more than 10% of all genes targeted.

Another explanation for the observed overlap between miRNA regulating SLE and T2D susceptibility genes is that there is fundamental common pathogenic mechanism in these two diseases. As mentioned before, chronic inflammation is likely to be a feature of both conditions, and some of the miRNAs regulating both pathways may play physiological roles in inflammatory reactions. It must also be emphasized that finding that both SLE and T2D appear to be highly controlled by

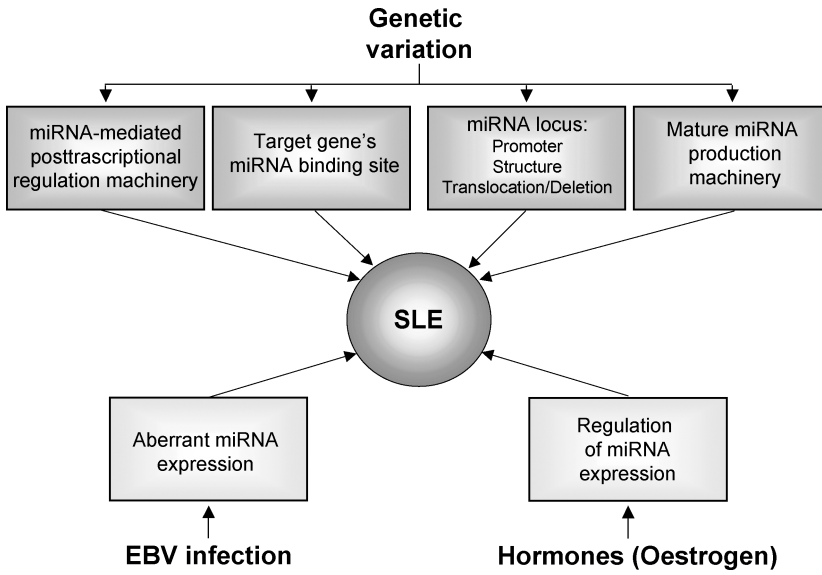


FIGURE 6 Causes of miRNA dysregulation that can contribute to SLE disease.

miRNAs does not necessarily mean that either of these diseases is more likely to be the outcome of miRNA dysregulation than other diseases. It would be interesting to be able to compare these findings with a disease, if it exists, in which miRNAs are not involved or with a random set of genes, to ascertain whether there is a true bias for autoimmune diseases to be more dependent on an intact miRNA-mediated silencing machinery.

CAUSES OF miRNA DYSREGULATION LEADING TO AUTOIMMUNE DISEASES

The pathogenesis of SLE is complex, but it is clear that the disease results from a combination of genetic, hormonal, and environmental factors (Fig. 6). The genetic contribution is generally believed to be the result of multiple common genetic variants combining to alter the bioavailability of self-antigen, disrupt tolerance, and lead to autoantibody formation and immune complex-mediated tissue damage. We have discussed how miRNAs have considerable potential to perturb tolerance, with the potential to result in autoimmunity. The following section will discuss some probable pathways by which miRNAs may contribute to the pathogenesis of SLE and other autoimmune diseases.

EBV Infection, miRNAs, and SLE

There is a considerable body of evidence to indicate that Epstein-Barr virus (EBV) infection can contribute to the pathogenesis of SLE. EBV infection is more common in SLE patients [66], who seem unable to repress the virus, as indicated by both a higher viral load [67] and an increased number of EBV-infected peripheral blood cells compared to healthy controls [68]. The precise mechanism by which EBV may contribute to SLE pathogenesis is unknown, but molecular mimicry between EBV proteins and self-antigens may be a contributory factor. Recent studies have shown that latent membrane protein 1 (LMP-1) can mimic the function of TNF receptor family proteins, resulting in the activation of the transcription factor NF-κ B, and induction of miR-155 [69] and miR-146a [70].

MiR-155 has been shown to be a key regulator of the germinal center response, in which high affinity, class-switched autoantibodies are produced. These antibodies are the hallmark of SLE, and can be detected several years before the first clinical manifestations of the disease. Within germinal centers, B-cells undergo somatic hypermutation of the IgV region genes; subsequent selection of B-cells carrying advantageous, non-self-reactive mutations by T_{FH} cells results in the production of long-lived, high-affinity antibody-producing plasma cells and memory B cells. The precise defect in SLE that leads to the production of high-affinity autoantibodies from germinal centers is unknown. By studying mice deficient in miR-155/*bic* or overexpressing miR-155 in B cells, Thai et al. demonstrated that miR-155 is required for normal selection of high-affinity antibodies and that miR-155 overexpression leads to increased germinal center and antibody production [71]. The enzyme activation-induced cytidine deaminase (AICDA or AID), essential for somatic hypermutation (SHM), is a target of miR-155 repression [72]. MiR-155 can also regulate the expression of PU.1: in the absence of miR-155 the gene encoding PU.1, *Sfpi1*, is upregulated with a concurrent reduction in class-switched $IgG1^+$ cells, which is also seen upon PU.1 overexpression in vitro [73]. It is therefore possible that EBV infection-induced miR-155 overexpression may contribute to lupus pathogenesis through dysregulated SHM and selection of high-affinity self-reactive mutants in germinal centers.

A Link between Estrogen-Modulated miRNAs and SLE

There is considerable evidence indicating a role for estrogen in the etiology of SLE. SLE is more common in females, flares of disease

activity can be linked to pregnancy and the onset of menstruation, and both the use of oral contraceptives and hormone replacement therapy may increase disease activity. In lupus, mouse models the removal of estrogen increased survival and, conversely, addition of estrogen to nonautoimmune mice increased autoantibody production [74].

Two recent studies have provided a possible link between estrogen, miRNAs, and SLE. Estrogen treatment-induced miR-146a downregulation in mouse splenocytes [75] and SLE patients were found to have reduced expression levels of miR-146a in PBMCs compared to normal controls [40]. The latter was associated with increased levels of type 1 interferons, shown to play a key role in SLE pathogenesis (reviewed in [76]). There is, therefore, an intriguing potential for estrogen to contribute to lupus pathogenesis via miRNA dysregulation.

Genetic Polymorphisms in miRNA Genes and SLE

There is evidence from a number of studies across a wide range of diseases that genetic polymorphisms affecting different aspects of miRNA biology—mature miRNA production, miRNA-mediated post-transcriptional regulation, miRNA expression and structure, and binding to the target site—can be pathogenic. The production of miRNAs, from genomic locus to mature, functional molecule, and the subsequent miRNA-mediated post-transcriptional regulation involve a number of protein complexes and enzymes, reviewed in [77]. Functional mutations in the genes that encode these proteins can interfere with many aspects of the biological pathways that lead to miRNA-mediated post-transcriptional regulation and have been implicated in a number of cancers [78–80].

Although as yet undescribed, polymorphisms in miRNA promoters may also result in aberrant pri-miRNA production. There is also considerable potential for variation in the sequence of miRNAs to have phenotypic consequences [81]. Processing of pri-miRNAs and pre-miRNAs and the binding of miRNAs to the target locus are dependent on specific nucleic acid sequences. Polymorphisms in the seed region of miRNAs are rare and there is evidence that when they are present they can profoundly impair target repression. Furthermore, mutations in the seed region or adjacent loop-stem regions can also impair the production of the mature miRNA from the pre-miRNA by altering the secondary structure of the pre-miRNA [82]. In a recent report, a polymorphism (rs11614913) within *hsa-mir-196a2* pre-miRNA sequence was associated with increased mature miR-196a expression, reduced ability of the miRNA to bind to the target sequence, and

reduced survival rate from lung cancer [83]. With growing evidence of the important roles miRNA-mediated post-transcriptional regulation play in the immune system it is conceivable that functional SNPs in miRNA sequence may contribute to autoimmune pathology.

Dysregulation in miRNA-mediated gene expression may also occur due to polymorphisms in the 3′ UTR of autoimmune-susceptibility genes. Thousands of human genes are predicted to carry miRNA binding sites, and are, therefore, potentially post-transcriptionally regulated by miRNAs. SNPs in miRNA target sites could alter miRNA-mediated post-transcriptional regulation of gene expression in at least two ways: (i) the sequence-specific binding of the seed region of a miRNA to its corresponding nucleotides is critical for miRNA function, so SNPs in the region of the target mRNA 3′ UTR complementary to the seed region have the potential to impair post-transcriptional regulation, and (ii) a mutation in the 3′ UTR of a gene may create a miRNA-binding region, resulting in that transcript being aberrantly targeted by miRNAs. In a polygenic disease such as SLE, mutations in miRNA binding sites that alter or prevent the binding of the complementary miRNA have the potential to contribute to disease pathogenesis.

To date there have been no reports of a polymorphic miRNA binding site contributing to SLE; however, there is evidence from other diseases that this can occur, and has the potential to contribute to disease pathogenesis. A genome-wide study showed that there were 400 SNPs in miRNA binding sites, 17 of which were in sites that were biologically proven (the remainder being computationally predicted) [84]. In addition, there were 257 polymorphisms identified in 209 genes that have the potential to create a novel miRNA-binding site. Confirmed examples include a polymorphism in the miR-24 binding site of the dihydrofolate reductase gene resulting in increased enzyme production and subsequent resistance to the drug methotrexate [85], a SNP in the HLA-G gene linked to asthma that influences the binding of several miRNAs [86], and regulation of the human *AGTR1* gene by miR-155 shown to be profoundly regulated by a SNP in the miRNA-binding region previously associated with hypertension [87]. There are many similar studies, in different areas of biology, that show that polymorphic miRNA binding sites can have a phenotypic consequence, and it is surely just a matter of time before this is shown to contribute to the pathogenesis of SLE.

Finally, there may also be a role for loss or altered miRNA expression due to translocation or gene deletions, given their concentration in fragile sites of the genome [88]. An interesting example is the

location of miR-15 and miR-16 on a region of human chromosome 13q14 that is frequently found deleted in chronic lymphocytic leukemia (CLL) [89]. The fact that there appear to be no other protein-coding genes in this region suggests, together with the repressor effects of miR-15 and miR-16 on Bcl-2, that these miRNAs may prevent CLL through suppressing lymphoid growth [90]. It is possible that similar deletions may occur sporadically during T or B cell clonal expansion and contribute to the uncontrolled lymphoproliferation that accompanies autoimmune syndromes.

CONCLUSIONS

Evidence is accumulating that individual miRNAs or combinatorial miRNA activity regulate immune responses and prevent the development of autoimmunity. MiRNAs act predominantly to fine-tune gene expression and establish negative feed-back loops that turn off responses. Both of these processes are critical to maintain immune tolerance given the gene-dose-dependent effect of several autoimmune-susceptibility genes and crucial inhibitory feedbacks that normally curtail inflammatory responses. It is, therefore, not surprising to find that over one hundred miRNAs are predicted to target lupus genes, and some of these genes are predicted to be each regulated by over 50 miRNAs. It is now clear that numerous mechanisms can contribute to miRNA dysregulation. Genetic variation, EBV infection and oestrogen, all previously shown to contribute to SLE susceptibility, may well be capable of impairing miRNA-regulation of target genes. It is anticipated that the next few years will see exponential increases in our understanding of the roles of miRNAs in the immune system, and subsequently the potential for perturbations in all areas of miRNA biology to contribute to autoimmunity.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES

[1] C.C. Goodnow, J. Sprent, B. de St Groth, and C.G. Vinuesa, Cellular and genetic mechanisms of self-tolerance and autoimmunity. *Nature* **435**: 590–597, 2005.

[2] R.J. Rigby and C.G. Vinuesa, SiLEncing SLE: The power and promise of small noncoding RNAs. *Curr Opin Rheumatol* **20**: 526–531, 2008.

[3] C. Xiao and K. Rajewsky, MicroRNA control in the immune system: Basic principles. *Cell* **136**: 26–36, 2009.

[4] C. Xiao, L. Srinivasan, D.P. Calado, H.C. Patterson, B. Zhang, J. Wang, et al. Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes. *Nature Immunol* **9**: 405–414, 2008.

[5] D. Yu, A.H. Tan, X. Hu, V. Athanasopoulos, N. Simpson, D.G. Silva, et al. Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA. *Nature* **450**: 299–303, 2007.

[6] P. Pisitkun, J.A. Deane, M.J. Difilippantonio, T. Tarasenko, A.B. Satterthwaite, and S. Bolland. Autoreactive B cell responses to RNA-related antigens due to TLR7 gene duplication. *Science* **312**: 1669–1672, 2006.

[7] V. Ambros, The functions of animal microRNAs. *Nature* **431**: 350–355, 2004.

[8] D.P. Bartel, MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* **116**: 281–297, 2004.

[9] N. Bushati and S.M. Cohen, microRNA functions. *Annu Rev Cell Dev Biol* **23**: 175–205, 2007.

[10] A. Rodriguez, S. Griffiths-Jones, J.L. Ashurst, and A. Bradley, Identification of mammalian microRNA host genes and transcription units. *Genome Res* **14**: 1902–1910, 2004.

[11] S.B. Koralov, S.A. Muljo, G.R. Galler, A. Krek, T. Chakraborty, C. Kanellopoulou, et al. Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. *Cell* **132**: 860–874, 2008.

[12] Q.J. Li, J. Chau, P.J. Ebert, G. Sylvester, H. Min, G. Liu, et al. miR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell* **129**: 147–161, 2007.

[13] D. Baek, J. Villen, C. Shin, F.D. Camargo, S.P. Gygi, and D.P. Bartel. The impact of microRNAs on protein output. *Nature* **455**: 64–71, 2008.

[14] M. Selbach, B. Schwanhausser, N. Thierfelder, Z. Fang, R. Khanin, and N. Rajewsky. Widespread changes in protein synthesis induced by microRNAs. *Nature* **455**: 58–63, 2008.

[15] S. Vasudevan, Y. Tong, and J.A. Steitz, Cell-cycle control of microRNA-mediated translation regulation. *Cell Cycle* **7**: 1545–1549, 2008.

[16] D.H. Kim, P. Saetrom, O. Snove, Jr., and J.J. Rossi, MicroRNA-directed transcriptional gene silencing in mammalian cells. *Proc Natl Acad Sci USA* **105**: 16230–16235, 2008.

[17] M. Pheasant and J.S. Mattick, Raising the estimate of functional human sequences. *Genome Res* **17**: 1245–1253, 2007.

[18] O. Hobert, Gene regulation by transcription factors and microRNAs. *Science* **319**: 1785–1786, 2008.

[19] E.A. Miska, E. Alvarez-Saavedra, A. Abbott, N. Lau, A. Hellman, et al. Most Caenorhabditis elegans microRNAs are individually not essential for development or viability. *PLoS Genet* **3**(12):, e215, doi:210.1371/journal.pgen.0030215, 2007.

[20] S.I. Ashraf, A.L. McLoon, S.M. Sclarsic, and S. Kunes, Synaptic protein synthesis associated with memory is regulated by the RISC pathway in Drosophila. *Cell* **124**: 191–205, 2006.

[21] G.M. Schratt, F. Tuebing, E.A. Nigh, C.G. Kane, M.E. Sabatini, M. Kiebler, and M.E. Greenberg. A brain-specific microRNA regulates dendritic spine development. *Nature* **439**: 283–289, 2006.
[22] C.G. Vinuesa, M.C. Cook, C. Angelucci, V. Athanasopoulos, L. Rui, K.M. Hill, et al. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature* **435**: 452–458, 2005.
[23] C.A. Janeway, Jr. and K. Bottomly, K. Signals and signs for lymphocyte responses. *Cell* **76**: 275–285, 1994.
[24] K.J. Lafferty, L. Andrus, and S.J. Prowse, Role of lymphokine and antigen in the control of specific T cell responses. *Immunolog Rev* **51**: 279–314, 1980.
[25] Matzinger, P. Tolerance, danger, and the extended family. *Annual Rev Immunol* **12**: 991–1045, 1994.
[26] M.A. Linterman, R.J. Rigby, R. Wong, D. Silva, D. Withers, G. Anderson, et al. Roquin differentiates the specialized functions of duplicated T cell costimulatory receptor genes CD28 and ICOS. *Immunity* **30**: 228–241, 2009.
[27] B. Grimbacher, A. Hutloff, M. Schlesier, E. Glocker, K. Warnatz, R. Drager, et al. Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. *Nature Immunol* **4**: 261–268, 2003.
[28] A. Hutloff, K. Buchner, K. Reiter, H.J. Baelde, M. Odendahl, A. Jacobi, T. Dorner, and R.A. Kroczek. Involvement of inducible costimulator in the exaggerated memory B cell and plasma cell generation in systemic lupus erythematosus. *Arthr & Rheuma* **50**: 3211–3220, 2004.
[29] J.H. Yang, J. Zhang, Q. Cai, D.B. Zhao, J. Wang, P.E. Guo, L. Liu, X.H. Han, and Q. Shen. Expression and function of inducible costimulator on peripheral blood T cells in patients with systemic lupus erythematosus. *Rheumatology* **44**: 1245–1254, 2005.
[30] M. Kawamoto, M. Harigai, M. Hara, Y. Kawaguchi, K. Tezuka, M. Tanaka, et al. Expression and function of inducible co-stimulator in patients with systemic lupus erythematosus: Possible involvement in excessive interferon-gamma and anti-double-stranded DNA antibody production. *Arthr Res & Ther* **8**: R62, 2006.
[31] S. Hori, T. Nomura, and S. Sakaguchi, Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299**: 1057–1061, 2003.
[32] H.J. van der Vliet, and E.E. Nieuwenhuis, IPEX as a result of mutations in FOXP3. *Clin & Dev Immunol* **2007**: 89017, 2007.
[33] M.E. Brunkow, E.W. Jeffery, K.A. Hjerrild, B. Paeper, L.B. Clark, S.A. Yasayko, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nature Genet* **27**: 68–73, 2001.
[34] B.S. Cobb, A. Hertweck, J. Smith, E. O'Connor, D. Graf, T. Cook, et al. A role for Dicer in immune regulation. *J Exp Med* **203**: 2519–2527, 2006.
[35] M.M. Chong, J.P. Rasmussen, A.Y. Rudensky, and D.R. Littman, The RNAseIII enzyme Drosha is critical in T cells for preventing lethal inflammatory disease. *JExp Med* **205**: 2005–2017, 2008.
[36] A. Liston, L.F. Lu, D. O'Carroll, A. Tarakhovsky, and A.Y. Rudensky, Dicer-dependent microRNA pathway safeguards regulatory T cell function. *J Exp Med* **205**: 1993–2004, 2008.
[37] X. Zhou, L.T. Jeker, B.T. Fife, S. Zhu, M.S. Anderson, M.T. McManus, and J.A. Bluestone. Selective miRNA disruption in T reg cells leads to uncontrolled autoimmunity. *J Exp Med* **205**: 1983–1991, 2008.
[38] L.F. Lu, T.H. Thai, D.P. Calado, A. Chaudhry, M. Kubo, K. Tanaka, et al. Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting SOCS1 protein. *Immunity* **30**: 80–91, 2009.

[39] K.D. Taganov, M.P. Boldin, K.J. Chang, and D. Baltimore, NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad SciU S A* **103**: 12481–12486, 2006.
[40] Y. Tang, X. Luo, H. Cui, X. Ni, M. Yuan, Y. Guo, et al. MicroRNA-146a contributes to abnormal activation of the type I interferon pathway in human lupus by targeting the key signaling proteins. *Arthritis Rheum* **60**: 1065–1075, 2009.
[41] J.T. Mendell, miRiad roles for the miR-17-92 cluster in development and disease. *Cell* **133**: 217–222, 2008.
[42] L. He, J.M. Thomson, M.T. Hemann, E. Hernando-Monge, D. Mu, S. Goodson, et al. A microRNA polycistron as a potential human oncogene. *Nature* **435**: 828–833, 2005.
[43] K.A. O'Donnell, E.A. Wentzel, K.I. Zeller, C.V. Dang, and J.T. Mendell, c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* **435**: 839–843, 2005.
[44] C.C. Goodnow, Multistep pathogenesis of autoimmune disease. *Cell* **130**: 25–35, 2007.
[45] T. Hershko and D. Ginsberg, Up-regulation of Bcl-2 homology 3 (BH3)-only proteins by E2F1 mediates apoptosis. *J Biolog Chem* **279**: 8627–8634, 2004.
[46] B. Hoffman and D.A. Liebermann, Apoptotic signaling by c-MYC. *Oncogene* **27**: 6462–6472, 2008.
[47] W.H. Shen, A.S. Balajee, J. Wang, H. Wu, C. Eng, P.P. Pandolfi, and Y. Yin. Essential role for nuclear PTEN in maintaining chromosomal integrity. *Cell* **128**: 157–170, 2007.
[48] D. Merino and P. Bouillet, MicroRNAs and lymphocyte homeostasis: Dangerous eggs in a single basket. *Immunol & Cell Biol* **86**: 387–388, 2008.
[49] D.H. Kono and A.N. Theofilopoulos, Genetics of SLE in mice. *Springer Semin Immunopathol* **28**: 83–96, 2006.
[50] J.B. Harley, M.E. Alarcon-Riquelme, L.A. Criswell, C.O. Jacob, R.P. Kimberly, K.L. Moser, B.P., et al. Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PXK, KIAA1542 and other loci. *Nature Genet* **40**: 204–210, 2008.
[51] R.I. Rynes, Inherited complement deficiency states and SLE. *Clin Rheum Dis* **8**: 29–47, 1982.
[52] B.P. Lewis, C.B. Burge, and D.P. Bartel, Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120**: 15–20, 2005.
[53] A. Grimson, K.K. Farh, W.K. Johnston, P. Garrett-Engele, L.P. Lim, and D.P. Bartel. MicroRNA targeting specificity in mammals: Determinants beyond seed pairing. *Mol Cell* **27**: 91–105, 2007.
[54] A.H. Sawalha, R. Webb, S. Han, J.A. Kelly, K.M. Kaufman, R.P. Kimberly, M.E. Alarcon-Riquelme, et al. Common variants within MECP2 confer risk of systemic lupus erythematosus. *PLoS ONE* **3**: e1727, 2008.
[55] N. Tsukiji, D. Nishihara, I. Yajima, K. Takeda, S. Shibahara, and H. Yamamoto. Mitf functions as an in ovo regulator for cell differentiation and proliferation during development of the chick RPE. *Dev Biol* **326**: 335–346, 2009.
[56] H. Schneider, H. Wang, M. Raab, E. Valk, X. Smith, M. Lovatt, et al. Adaptor SKAP-55 binds p21 activating exchange factor RasGRP1 and negatively regulates the p21-ERK pathway in T-cells. *PLoS ONE* **3**: e1718, 2008.
[57] E.A. Leadbetter, I.R. Rifkin, A.M. Hohlbaum, B.C. Beaudette, M.J. Shlomchik, and A. Marshak-Rothstein. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature* **416**: 603–607, 2002.

[58] M. Linterman, R. Rigby, R. Wong, D. Yu, R. Brink, J. Cannons, et al. Follicular helper T cells are required for systemic autoimmunity. *J Exp Med* **206**: 561–576, 2009.

[59] S. Subramanian, K. Tus, Q.Z. Li, A. Wang, X.H. Tian, J. Zhou, et al. A Tlr7 translocation accelerates systemic autoimmunity in murine lupus. *Proc Natl Acad Sci USA* **103**: 9970–9975, 2006.

[60] K. Murata, M. Nose, L.C. Ndhlovu, T. Sato, K. Sugamura, and N. Ishii. Constitutive OX40/OX40 ligand interaction induces autoimmune-like diseases. *J Immunol* **169**: 4628–4636, 2002.

[61] H. Wu, J.R. Neilson, P. Kumar, M. Manocha, P. Shankar, P.A. Sharp, and N. Manjunath. miRNA profiling of naive, effector and memory CD8 T cells. *PLoS ONE* **2**: e1020, 2007.

[62] C.B. Guest, M.J. Park, D.R. Johnson, and G.G. Freund, The implication of proinflammatory cytokines in type 2 diabetes. *Front Biosci* **13**: 5187–5194, 2008.

[63] T. Saito, S. Chiba, M. Ichikawa, A. Kunisato, T. Asai, K. Shimizu, et al. Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. *Immunity* **18**: 675–685, 2003.

[64] M.M. Juntilla, J.A. Wofford, M.J. Birnbaum, J.C. Rathmell, and G.A. Koretzky, Akt1 and Akt2 are required for alphabeta thymocyte survival and differentiation. *Proc Natl Acad Sci U S A* **104**: 12105–12110, 2007.

[65] M. Ridderstrale and L. Groop, Genetic dissection of type 2 diabetes. *Molec & Cellu Endocrin* **297**: 10–17, 2009.

[66] B.D. Poole, A.K. Templeton, J.M. Guthridge, E.J. Brown, J.B. Harley, and J.A. James. Aberrant Epstein-Barr viral infection in systemic lupus erythematosus. *Autoimmun Rev* **8**: 337–342, 2009.

[67] I. Kang, T. Quan, H. Nolasco, S.H. Park, M.S. Hong, J. Crouch, E.G. Pamer, J.G. Howe, and J. Craft. Defective control of latent Epstein-Barr virus infection in systemic lupus erythematosus. *J Immunol* **172**: 1287–1294, 2004.

[68] A.J. Gross, D. Hochberg, W.M. Rand, and D.A. Thorley-Lawson, EBV and systemic lupus erythematosus: A new perspective. *J Immunol* **174**: 6599–6607, 2005.

[69] G. Gatto, A. Rossi, D. Rossi, S. Kroening, S. Bonatti, and M. Mallardo. Epstein-Barr virus latent membrane protein 1 trans-activates miR-155 transcription through the NF-kappaB pathway. *Nucleic Acids Res* **36**: 6608–6619, 2008.

[70] J.E. Cameron, Q. Yin, C. Fewell, M. Lacey, J. McBride, X. Wang, et al. Epstein-Barr virus latent membrane protein 1 induces cellular MicroRNA miR-146a, a modulator of lymphocyte signaling pathways. *J Virol* **82**: 1946–1958, 2008.

[71] T.H. Thai, D.P. Calado, S. Casola, K.M. Ansel, C. Xiao, Y. Xue, et al. Regulation of the germinal center response by microRNA-155. *Science* **316**: 604–608, 2007.

[72] Y. Dorsett, K.M. McBride, M. Jankovic, A. Gazumyan, T.H. Thai, D.F. Robbiani, et al. MicroRNA-155 suppresses activation-induced cytidine deaminase-mediated Myc-Igh translocation. *Immunity* **28**: 630–638, 2008.

[73] E. Vigorito, K.L. Perks, C. Abreu-Goodger, S. Bunting, Z. Xiang, S. Kohlhaas, et al. microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. *Immunity* **27**: 847–859, 2007.

[74] M. Petri, Sex hormones and systemic lupus erythematosus. *Lupus* **17**: 412–415, 2008.

[75] R. Dai, R.A. Phillips, Y. Zhang, D. Khan, O. Crasta, and S.A. Ahmed. Suppression of LPS-induced interferon-gamma and nitric oxide in splenic lymphocytes by select estrogen-regulated microRNAs: A novel mechanism of immune modulation. *Blood* **112**: 4591–4597, 2008.

[76] L. Ronnblom, M.L. Eloranta, and G.V. Alm, The type I interferon system in systemic lupus erythematosus. *Arthr & Rheuma* **54**: 408–420, 2006.
[77] M. Jinek, and J.A. Doudna, A three-dimensional view of the molecular machinery of RNA interference. *Nature* **457**: 405–412, 2009.
[78] Y. Horikawa, C.G. Wood, H. Yang, H. Zhao, Y. Ye, J. Gu, et al. Single nucleotide polymorphisms of microRNA machinery genes modify the risk of renal cell carcinoma. *Clin Cancer Res* **14**: 7956–7962, 2008.
[79] W.M. Merritt, Y.G. Lin, L.Y. Han, A.A. Kamat, W.A. Spannuth, R. Schmandt, et al. Dicer, Drosha, and outcomes in patients with ovarian cancer. *N Engl J Med* **359**: 2641–2650, 2008.
[80] S.A. Melo, S. Ropero, C. Moutinho, L.A. Aaltonen, H. Yamamoto, G.A. Calin, et al. A TARBP2 mutation in human cancer impairs microRNA processing and DICER1 function. *Nature Genet* **41**: 365–370, 2009.
[81] C. Borel and S.E. Antonarakis, Functional genetic variation of human miRNAs and phenotypic consequences. *Mamm Genome* **19**: 503–509, 2008.
[82] R. Duan, C. Pak, and P. Jin, Single nucleotide polymorphism associated with mature miR-125a alters the processing of pri-miRNA. *Hum Mol Genet* **16**: 1124–1131, 2007.
[83] Z. Hu, J. Chen, T. Tian, X. Zhou, H. Gu, L. Xu, et al. Genetic variants of miRNA sequences and non-small cell lung cancer survival. *J Clin Invest* **118**: 2600–2608, 2008.
[84] M.A. Saunders, H. Liang, and W.H. Li, Human polymorphism at microRNAs and microRNA target sites. *Proc Natl Acad Sci USA* **104**: 3300–3305, 2007.
[85] P.J. Mishra, R. Humeniuk, P.J. Mishra, G.S. Longo-Sorbello, D. Banerjee, and J.R. Bertino. A miR-24 microRNA binding-site polymorphism in dihydrofolate reductase gene leads to methotrexate resistance. *Proc Natl Acad Sci USA* **104**: 13513–13518, 2007.
[86] Z. Tan, G. Randall, J. Fan, B. Camoretti-Mercado, R. Brockman-Schneider, L. Pan, et al. Allele-specific targeting of microRNAs to HLA-G and risk of asthma. *Am J Hum Genet* **81**: 829–834, 2007.
[87] P. Sethupathy, C. Borel, M. Gagnebin, G.R. Grant, S. Deutsch, T.S. Elton et al. Human microRNA-155 on chromosome 21 differentially interacts with its polymorphic target in the AGTR1 3' untranslated region: A mechanism for functional single-nucleotide polymorphisms related to phenotypes. *Am J Hum Genet* **81**: 405–413, 2007.
[88] K. Huppi, N. Volfovsky, M. Mackiewicz, T. Runfola, T.L. Jones, S.E. Martin, et al. MicroRNAs and genomic instability. *Seminars Cancer Biol* **17**: 65–73, 2007.
[89] G.A. Calin, C.D. Dumitru, M. Shimizu, R. Bichi, S. Zupo, E. Noch, et al. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* **99**: 15524–15529, 2002.
[90] A. Cimmino, G.A. Calin, M. Fabbri, M.V. Iorio, M. Ferracin, M. Shimizu, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci USA* **102**: 13944–13949, 2005.