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Review

MicroRNA Control in the Immune System: Basic Principles

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MicroRNA (miRNA) control has emerged as a critical regulatory principle in the mammalian immune system. Genetic ablation of the miRNA machinery, as well as loss or deregulation of certain individual miRNAs, severely compromises immune development and response and can lead to immune disorders like autoimmunity and cancer. Although individual miRNAs modulate protein output from hundreds of target genes, they may impact physiological processes by regulating the concentrations of just a few key cellular proteins that may be components of a single or of functionally interrelated pathways in a given cellular context.

Introduction

The generation of the mammalian immune system from hematopoietic stem cells (HSCs) involves ordered events of lineage commitment, differentiation, proliferation, and cell migration. This includes developmental programs of ordered immunoglobulin (Ig) and T cell receptor (TCR) gene segments to equip each lymphocyte with a single antigen receptor—BCR in the case of B cells, and TCR in the case of T cells. It also includes the differentiation of the cells into distinct cellular subsets with distinct effector functions, and innate and adaptive responses to antigens, in which cell interactions between lymphocytes of different subsets and of lymphocytes with other cells of the hematopoietic system such as dendritic cells play a critical role and which may be accompanied by the generation of immunological memory. Because of its unique experimental accessibility, this system is perhaps the best-characterized developmental system in mammals, in terms of surface markers of cell subsets and of signaling pathways and transcriptional programs underlying developmental progression, cellular selection, and cell migration. The immune system has also been well characterized with respect to differentiation processes and effector mechanisms involved in acquired and innate immune responses.

Given the detailed conceptual and experimental framework of our present understanding of the immune system, it came as a surprise that there is a previously unrecognized layer of control impacting at least some of the salient features of the development and physiology of the system, namely control exerted by microRNAs (miRNAs). miRNAs are endogenously encoded single-stranded RNAs of about 22 nt in length that play essential roles in animals and plants, in a large variety of physiological contexts (Ambros, 2004; Bartel, 2004; Bushati and Cohen, 2007). Hundreds of miRNAs, many of them evolutionarily conserved, have been identified in mammals (miRBase, http://microrna.sanger.ac.uk/), and many of these molecules exhibit highly specific, regulated patterns of expression (Chen et al., 2004; Landgraf et al., 2007; Merkerova et al., 2008; Monticelli et al., 2005; Neilson et al., 2007), which can be transcriptionally or posttranscriptionally controlled. Many miRNAs derive from independent transcription units, but miRNA genes can also be located in the introns of protein-coding genes (Rodriguez et al., 2004). A large fraction of miRNAs are clustered in the genome, with arrangement and expression patterns suggesting that they are transcribed as polycistronic primary transcripts (Bartel, 2004). The primary transcript of a miRNA gene, the pri-miRNA, is cleaved by the RNAase III endonuclease Drosha in the nucleus, to release a 60–70 nt stem-loop intermediate, known as the pre-miRNA. The pre-miRNA is subsequently exported to the cytoplasm, where it is further cleaved by Dicer, another RNase III endonuclease, to produce a double-stranded RNA duplex, which contains the mature miRNA and its antisense strand. The Dicer cleavage process is coupled with the integration of the mature miRNA into the RNA-induced silencing complex (RISC), whose core components are the Argonaute family proteins (Ago1–4). The miRNA then directs the RISC to its target mRNA, which it recognizes through partial sequence complementarity. A major determinant in this recognition process is a perfect match in the so-called seed region of 6–8 nt at the 5′ end of the miRNA. The usual consequence of miRNA:mRNA interaction is the downregulation of protein expression by translational repression, mRNA cleavage, or promotion of mRNA decay (Kim, 2005). Recent proteomic experiments in mammalian cells have demonstrated that single miRNAs can directly repress the production of hundreds of proteins, that repression is typically mild, and that it is mostly, but not always, due to both downregulation of mRNA levels and translation inhibition (Baek et al., 2008; Selbach et al., 2008). There is some evidence that under certain conditions miRNAs can also upregulate the translation of target mRNAs (Vasudevan et al., 2007) or even directly interfere with gene transcription (Kim et al., 2008). The physiological impact of such modes of regulation remains to be determined.

Below, we discuss studies of the immune system, exemplifying that this multitarget, subtle mode of control turns out to be highly efficient and versatile in mammalian cells, through the targeting of multiple components of regulatory networks inside the cell. In this context, a special aspect of miRNA control is of particular interest, namely its rapid evolvability. Because of their simple structure and mode of target recognition, miRNAs as well as their targets can evolve rapidly (Chen and Rajewsky, 2007; Gardner and Vinther, 2008; Lu et al., 2008; Niwa and Slack, 2007). As rapid evolution is a characteristic feature and essential element of the interplay of the immune system with pathogens, one might thus predict that miRNAs may play a prominent role in the control of host-pathogen interactions and that the immune system may thus offer particularly rich opportunities to study mechanisms of miRNA control. Emerging experimental evidence supports this view.

In our discussion, we mainly focus on experiments in which genetic evidence obtained in vivo provides unequivocal evidence for the impact of miRNA control.

miRNAs Control Development and Responses of the Immune System

After a first demonstration that overexpression of a miRNA in HSCs affected B lymphopoiesis in vivo upon HSC transplantation (Chen et al., 2004), it became apparent from experiments in which Dicer, the key enzyme in miRNA biogenesis, was conditionally inactivated in T or B lymphocytes of the mouse that miRNAs are critical for lymphocyte development and differentiation. In the case of T cell development, deletion of *Dicer* in immature thymocytes (at the so-called double negative 3 [DN3] stage) led to a 10-fold reduction in total thymocyte numbers, with a drastic reduction of the more mature double-positive ($CD4^+CD8^+$) and single-positive ($CD4^+$ or $CD8^+$) thymocytes, probably because of increased cell death. Although very few peripheral T cells were detectable in these animals (Cobb et al., 2005), the peripheral $CD4^+$ T cells were only reduced 2-fold when Dicer was deleted at the double-positive stage of thymocyte differentiation. The mutant cells exhibited reduced proliferation and increased cell death upon activation in vitro. When cultured under conditions that favor differentiation toward T helper 1 (Th1) and Th2 lineages, they showed a bias toward Th1 differentiation, reflecting the failure of these cells to repress interferon γ (IFNγ) expression (Muljo et al., 2005). The T cell subset most affected by the deletion of *Dicer* at the double-positive stage are the T regulatory cells, whose 6-fold reduction in both thymus and periphery likely resulted in the severe immunopathology developing in the mutant mice, characterized by splenomegaly, enlarged intestinal lymph nodes, and colitis (Cobb et al., 2006). That miRNAs are essential for the homeostasis and suppressor function of T regulatory cells was corroborated by *Dicer* and *Drosha* deletion in those cells and the ensuing fatal autoimmunity indistinguishable from that caused by deficiency in Foxp3, the master transcription factor controlling regulatory T cell differentiation (Chong et al., 2008; Liston et al., 2008; Zhou et al., 2008). Regulatory T cells require stronger signals from their TCR to be positively selected than do T cells of other subsets (Zheng and Rudensky, 2007), hinting at the possibility that miRNAs may be involved in the control of the sensitivity of T cells to signals from the TCR. As discussed further below, this seems indeed to be the case.

That a deficiency in miRNAs also affects B cell development was first suggested by the conditional deletion of Ago2, a component of RISC, in hematopoietic cells, which resulted in a partial deficiency in miRNAs and compromised development of B and erythroid cells (O'Carroll et al., 2007). When *Dicer* was deleted in the B cell lineage from the earliest stage of B cell development, an almost complete block at the pro- to pre-B transition resulted, which was at least in part due to apoptosis of *Dicer* deficient pre-B cells (Koralov et al., 2008). In an attempt to elucidate the molecular basis of this phenotype, gene expression profiles of *Dicer* deficient and proficient pro-B cells were established, and a bioinformatic search was conducted for conserved nucleotide hexamers complementary to miRNA seed regions in the 3′ untranslated regions (UTRs) of genes upregulated in *Dicer* deficient pro-B cells. Surprisingly, only a few such motifs were identified, with a corresponding small group of miRNAs predicted to be critical players at the pro-B cell stage. Most of these miRNAs belonged to three interrelated miRNA clusters, one of which, miR-17∼92, has emerged as a critical regulator of cellular development in a variety of cellular contexts, as will be further discussed below. The six miRNAs encoded in the miR-17∼92 cluster share four distinct seed regions, and when the annotated 3′ UTRs of genes in the mouse were grouped according to the number of corresponding conserved target sites, two genes on top of the list were immediately apparent as potentially causally involved in the induction of apoptosis in *Dicer* deficient pre-B cells: the genes encoding the proapoptotic Bcl2 family member Bim and the tumor suppressor Pten, which can positively control Bim expression. When this bioinformatic prediction was tested, Bim and Pten were indeed found to be strongly upregulated in *Dicer*-deficient B cell progenitors, and, most importantly, B cell development could be partially rescued by transgenic Bcl2 or Bim deficiency (Koralov et al., 2008).

These experiments exemplify a way to identify miRNAs and their targets in a particular biological context by combining bioinformatic and experimental approaches and also reveal that targeting of multiple components of a regulatory network as one of the emerging principles of miRNA control. This latter notion will be discussed in more detail below. In a broader sense, however, the inactivation of components of the miRNA machinery in lymphocytes has made us realize that although miRNA control does not seem to affect lymphocyte development in every respect—V(D)J recombination, for example, appears to function properly in *Dicer* deficient B cells in a first approximation—it operates at multiple levels in lymphocyte development and physiology and will have to be studied separately for individual miRNAs. It is important to keep in mind in this context that *Dicer* ablation by conditional gene targeting may well fail to uncover miRNA control in certain situations because of the persistence of Dicer protein or of miRNAs (whose decay in cells is known to vary substantially between individual miRNA species) after *Dicer* gene deletion (Cobb et al., 2005; Koralov et al., 2008; Muljo et al., 2005; Ramachandran and Chen, 2008). On the other hand,

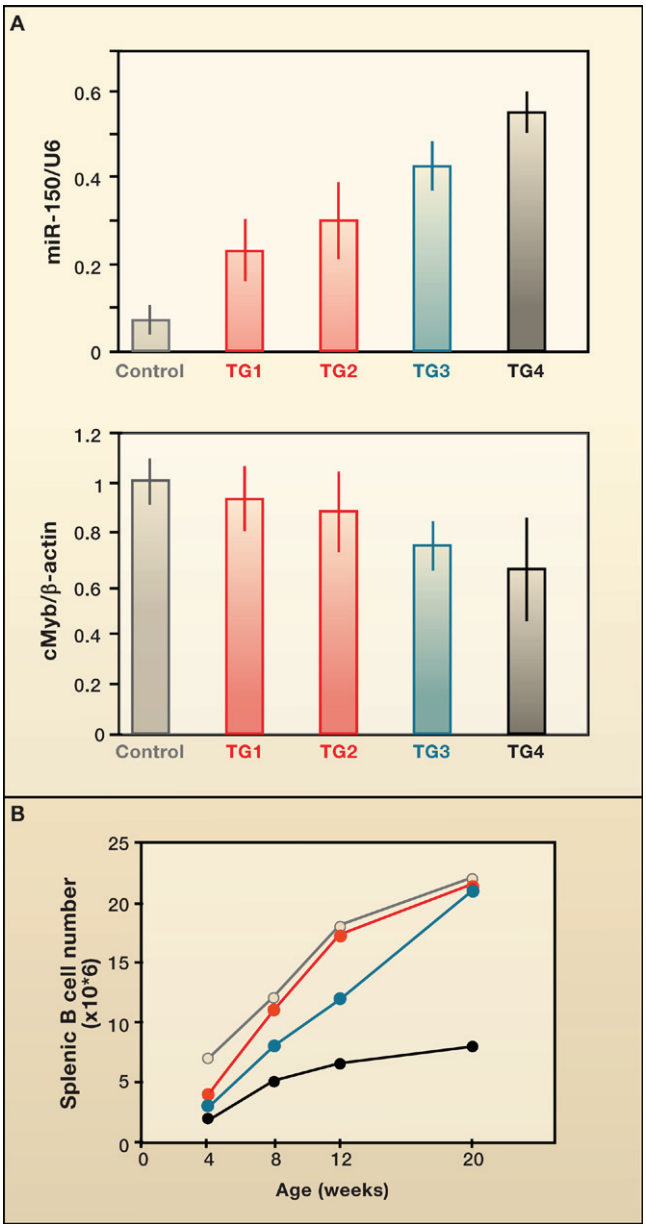

Figure 1. The miR-150-c-Myb Interaction

(A) A primary target of miR-150 is the proto-oncogene c-Myb. Ratios of miR-150/U6 and c-Myb/β-actin are shown for thymocytes of miR-150 transgenic mice. TG1–4 are four transgenic mouse strains with different miR-150 expression levels. The miR-150/U6 ratio in wild-type $CD4^+$ T cells and c-Myb/β-actin ratio in wild-type thymocytes are set as 1.

(B) The splenic B cell numbers in miR-150 transgenic mice. The transgenic mice exhibited a dose-dependent developmental block at the pro- to pre-B transition and a reduction of splenic B cell numbers at young age. Each dot represents the average of multiple mice at similar age. Adapted from Xiao et al. (2007).

Dicer deficiency may reveal functions of this enzyme outside of miRNA control, such as transcriptional gene silencing through the production of endogenous small interfering RNAs (siRNAs). Silencing of centromeric repeats through such a mechanism has been demonstrated in yeast (Verdel et al., 2004; Volpe et al., 2002), and there was indeed an indication in *Dicer*-deficient B cells for a derepression of a set of D elements in the *IgH* locus, which exhibit a similar repeat structure (Chakraborty et al., 2007; Koralov et al., 2008). Whether Dicer dependent mechanisms of epigenetic gene silencing play a significant role in mammalian cells remains to be elucidated.

That the genetic inactivation of individual mammalian miRNAs can produce discernible phenotypes became apparent early in 2007 from four papers (Rodriguez et al., 2007; Thai et al., 2007; van Rooij et al., 2007; Zhao et al., 2007), two of which addressed a miRNA specifically expressed in the hematopoietic system. Like most other cells, cells in the hematopoietic system express a multitude of miRNA species, which may or may not be shared between different cell types or developmental stages, but only a few of which are specific for the hematopoietic system (Merkerova et al., 2008; Monticelli et al., 2005; Neilson et al., 2007; Wu et al., 2007). Knockout of a miRNA of the latter kind, miR-155, affected a large spectrum of immune reactions, ranging from cytokine production by T and B cells to antigen presentation by dendritic cells and the germinal center B cell response (Rodriguez et al., 2007; Thai et al., 2007). There is also evidence that miR-155, together with others, is involved in the control of innate immune reactions. Expression profiling showed that stimulation of monocytes with lipopolysaccharide (LPS) induced the expression of miR-132, miR-146, and miR-155 (O'Connell et al., 2007; Taganov et al., 2006). miR-146 targets Traf6 and Irak1, components of the Toll-like receptor (TLR) signaling pathway that is activated by LPS, suggesting a negative feedback loop (Taganov et al., 2006). The functional importance of LPS-induced miR-155 expression in myeloid cells was further supported by the work of Tili et al. (2007), and the myeloproliferative disorder that develops in mice reconstituted with bone marrow progenitors that had been retrovirally transduced to express miR-155 (O'Connell et al., 2008). Induction of miR-155 expression by LPS could thus be a mechanism through which the immune system rapidly expands the myeloid cell population during inflammatory responses (O'Connell et al., 2008).

Other miRNAs control additional processes in the immune system. Thus, genetic ablation of miR-150 led to the expansion of the B1 B cell subset, involved in natural defense against pathogens, in spleen and peritoneal cavity, increased steady-state serum immunoglobulin levels, and enhanced T-dependent immune responses (Xiao et al., 2007). miR-181 seems to control TCR signaling thresholds in T lymphocytes, as discussed further below, and may also participate in the control of the antibody response (Li et al., 2007; de Yébenes et al., 2008). miR-223 is a myeloid-specific miRNA whose deletion leads to an increase in the numbers of neutrophil progenitors and mature neutrophils, as well as an increased neutrophil response to fungal infection, demonstrating that this miRNA plays important roles in the development and function of the myeloid lineage and innate immunity (Johnnidis et al., 2008).

Taking these findings together, miRNA control has emerged as a general regulatory mechanism in immune development and the acquired and innate immune response. In the sections below, we focus on what is known about mechanisms by which miRNAs exert their function.

Dose-Dependent Modulation of Key Targets by miRNAs

Studies are now revealing that miRNAs modulate the concentration of key target proteins over a narrow range in a dose-dependent manner. In general, the net outcome of miRNA binding to its target mRNAs is a reduction of the amounts of protein produced from these mRNAs (Baek et al., 2008; Selbach et al., 2008). Addressing this issue quantitatively, Xiao et al. (2007) studied the interaction between miR-150 and its chief target, c-Myb, in the context of lymphocyte development in vivo. miR-150 is highly expressed in mature B and T cells but not in their progenitors, whereas c-Myb is highly expressed in lymphocyte progenitors but downregulated upon maturation. Genetic studies had demonstrated that c-Myb plays a critical role at multiple stages of lymphocyte development, including the transition from pro- to pre-B cells (Bender et al., 2004; Thomas et al., 2005). When miR-150 was ectopically expressed at graded concentrations in lymphocyte progenitors in vivo, with the highest concentration at about 55% of that in mature wild-type $CD4^+$ T cells, a dose-dependent downregulation of c-Myb protein levels was observed, with a 35% reduction caused by the highest concentration of the transgenic miRNA. The transgenic mice exhibited a dose-dependent developmental block at the pro- to pre-B transition and a reduction of splenic B cell numbers at young age (Figure 1). That the developmental block was mainly caused by the reduction in c-Myb protein levels was supported by a similar phenotype in c-Myb heterozygous mice, which displayed a similar reduction of c-Myb levels in B cell progenitors.

These experiments suggested a few basic principles of the relationship between miRNAs and their target genes. First, the cellular concentration of a miRNA dictates the protein output of its target genes and is therefore of key importance in miRNA-mediated control. Second, small changes in the concentration of key cellular proteins as effected by miRNA control can have significant biological consequences, in line with the many examples of pathogenic hemizygous null mutations in man and mouse (Santarosa and Ashworth, 2004; Seidman and Seidman, 2002; Smilenov, 2006). miRNAs may have evolved to modulate the concentrations of such key cellular proteins. Third, although a single miRNA can repress protein production from hundreds of genes, only a few of these proteins may be critical for a particular biological process.

In studies in *Caenorhabditis elegans* and *Drosophila melanogaster*, the control of single key target genes also appeared to largely explain the functions of individual miRNAs (Karres et al., 2007; Lee et al., 1993; Teleman et al., 2006; Varghese and Cohen, 2007; Wightman et al., 1993). As in the case of miR-150 in mice, these claims were largely based on the similarity between the phenotypes caused by loss- and/or gain-of function of individual miRNAs and manipulating the protein levels of target genes to similar degrees. This clearly leaves room for a contribution of additional target genes to the observed phenotypes. Targeted mutagenesis of miRNA binding sites in target mRNAs is ultimately required to directly address this issue, as exemplified further below for miR-155 and its target gene AID.

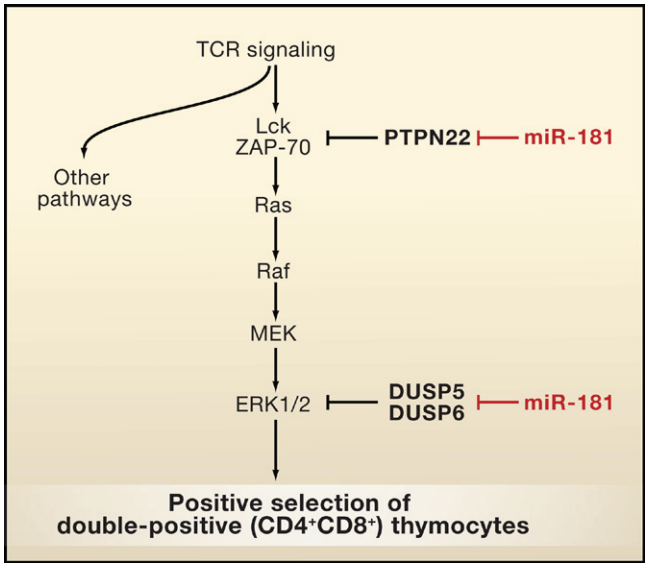

Figure 2. miR-181 Sets Signaling Thresholds by Modulating Multiple Negative Regulators

miR-181 is highly expressed in double positive ($CD4^+CD8^+$) thymocytes and downregulated upon T cell maturation. By downregulating the concentrations of several negative regulators of TCR signaling, miR-181 changes the outcome of signaling triggered by TCR engagement by peptide-MHC complexes: double-positive thymocytes, but not mature T cells, respond to self-antigens and are positively or negatively selected.

Setting Signaling Thresholds by Modulating Negative Regulators: miR-181

To accommodate the differential needs of developing and mature T cells in their response to antigen (the former being positively and negatively selected by self-antigens, the latter responding to foreign antigens), thymocytes exhibit a higher sensitivity than mature T cells to the signal triggered by TCR engagement of peptide-MHC complexes. This is despite the fact that the TCR uses the same set of molecules for signaling at different developmental stages (Germain and Stefanova, 1999). Recent experiments indicate that miR-181 is critically involved in this developmental control, through its predominant expression in thymocytes and its ability to downregulate the protein levels of multiple phosphatases that are negative regulators of distinct steps of the TCR signaling pathway (Li et al., 2007) (Figure 2). The protein tyrosine phosphatase PTPN22 dephosphorylates and inactivates Lck and ZAP70, two crucial components of proximal TCR signaling (Wu et al., 2006). Dual specificity phosphatases DUSP5 and DUSP6 dephosphorylate and inactivate the kinase ERK, with the former acting in the nucleus and the latter acting in the cytoplasm (Owens and Keyse, 2007; Tanzola and Kersh, 2006). ERK1 and ERK2 are essential for the positive selection of double-positive thymocytes, and this event is very sensitive to the dosage of ERK activity. Targeted deletion of both ERK1 and ERK2 leads to a complete block of positive selection, whereas retroviral expression of a dominant-negative mutant of DUSP6, which increases ERK activity, enhances positive selection (Bettini and Kersh, 2007; Fischer et al., 2005). Overexpression of miR-181 in mature T cells similarly increases ERK activity, and this con-

verts antagonist peptides into agonists. Treatment of thymocytes with antagomir-181, which degrades its endogenous counterpart, reduces ERK activity and impairs positive selection of thymocytes in a fetal thymic organ culture system (Li et al., 2007).

Significantly, the regulation of the response of T cells to signals from the TCR by miR-181 could not be fully recapitulated by knockdown of the protein levels of any one of its proposed targets with shRNAs, whereas restoration of the protein levels of individual targets partly or completely reversed the effects of miR-181 overexpression. This suggests that the repression of a given target was essential but not sufficient for the function of miR-181 (Li et al., 2007).

Although these experiments still await confirmation in vivo, they suggest that miRNAs may target multiple components in a common regulatory pathway, a concept that is also supported by the functional analysis of miR-155 and the miR-17∼92 cluster (see below). Conceptually similar to the additive effect on tumor development of double heterozygosity of functionally related, mutant tumor suppressor genes (Smilenov, 2006), this regulatory principle may contribute in a major way to the overall impact of miRNA control, despite moderate effects on the concentrations of individual target proteins.

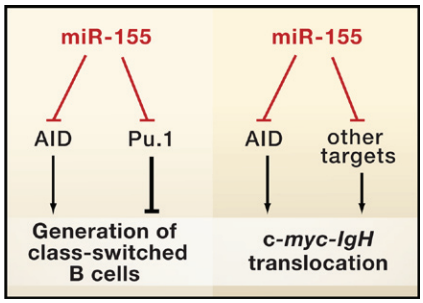

Figure 3. miR-155 Controls Distinct Regulatory Pathways

miR-155 controls the generation of class-switched B cells and c-*myc*-*IgH* translocations. In the former case, miR-155 downregulates AID, an essential enzyme for class switch recombination (CSR), and the transcription factor Pu.1, which counteracts the differentiation of class-switched cells. The net effect, possibly involving additional miR-155 targets, is the promotion of this process. As for c-*myc*-*IgH* translocations, miR-155 downregulates AID and other target genes, which synergize with AID in mediating these events.

Nonsynergistic Control of Distinct Regulatory Pathways: miR-155

Genetic gain- and loss-of function studies of miR-155, which is upregulated in B and T cells upon activation, have demonstrated that this miRNA plays a positive role in the control of the germinal center reaction in vivo, in addition to its manifold other regulatory roles (Rodriguez et al., 2007; Thai et al., 2007; Vigorito et al., 2007). Curiously, a potential target of this miRNA is the enzyme AID, a central player in the germinal center reaction, mediating class switch recombination (CSR) and somatic hypermutation (SHM) (Chaudhuri and Alt, 2004; Di Noia and Neuberger, 2007; Honjo et al., 2004). AID is also, however, causally involved in oncogenic reciprocal translocations between *IgH* and c-*myc* (c-*myc*-*IgH*), an unwanted byproduct of CSR in the course of the germinal center reaction (Muramatsu et al., 2000; Ramiro et al., 2004). This calls for a careful control of AID activity during this process. To analyze whether miR-155 is involved in this control, a knockin mouse strain carrying a mutation in the evolutionary conserved miR-155 binding site in the 3′ UTR of the AID gene was generated (Dorsett et al., 2008). AID protein levels were increased 2- to 3-fold in both activated miR-155$^{-/-}$ and AID^{155} B cells, demonstrating that AID is a direct target of miR-155 in vivo. The latter was also suggested by the work of Teng et al. (2008), who made use of a similarly mutated AID transgene. Interestingly, although SHM was affected by neither the AID gene mutation nor the miR-155 knockout, the generation of class-switched B cells was increased in the former and reduced in the latter case. Thus, in miR-155$^{-/-}$ B cells, the effect of increased AID protein levels on CSR must be offset by changes in other target genes. Indeed, the work of Vigorito et al. (2007) has shown that the protein level of Pu.1, another miR-155 target gene, is increased in miR-155$^{-/-}$ B cells and that this negatively affects the differentiation of cells undergoing CSR (Vigorito et al., 2007). In contradistinction, when c-*myc*-*IgH* translocation was analyzed, activated AID^{155} B cells exhibited a 3- to 6-fold increased translocation frequency compared to control cells, whereas the translocation frequency was increased 15-fold in *miR-155*-deficient B cells. Together, these data suggested that miR-155 regulates AID and some additional component(s) in a pathway minimizing AID-mediated oncogenic translocations, and in addition positively controls the generation of class-switched B cells in the germinal center reaction (Figure 3).

Paradoxically, the latter goes together with downregulation of the key mediator of CSR, AID. Perhaps miR-155 had originally evolved as a safeguard against AID-mediated oncogenic translocations and had to accommodate downregulation of AID acquired for this purpose in a separate functional context, namely the promotion of CSR. The particular risk associated with AID expression may also be the reason for its control by yet another miRNA, miR-181, perhaps at a different stage of B cell activation (de Yébenes et al., 2008).

Cooperation and Redundancy of Coexpressed miRNAs: miR-17∼92

According to bioinformatic predictions, the 3′ UTRs of miRNA target genes often contain binding sites for several different miRNAs (Krek et al., 2005; Lewis et al., 2005). On the other hand, miRNAs encoded by different genomic loci can have very similar mature sequences, and there are families of miRNAs sharing seed regions. Can coexpressed miRNAs regulate the expression of target mRNAs in a cooperative manner? Do members of the same miRNA family regulate different sets of targets, because of sequence differences outside the seed region, or are they functionally redundant? Recent studies of the miR-17∼92 cluster and related miRNA clusters begin to answer some of these questions.

The miR-17∼92 transcript encoded by mouse chromosome 14 is the precursor of six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92, not including miR-17*). Additionally, this cluster is homologous to the miR-106a∼363 cluster on the X chromosome and the miR-106b∼25 cluster on chromosome 5. Together, these three clusters contain 15 miRNA stem-loops, giving rise to 13 distinct mature miRNAs,

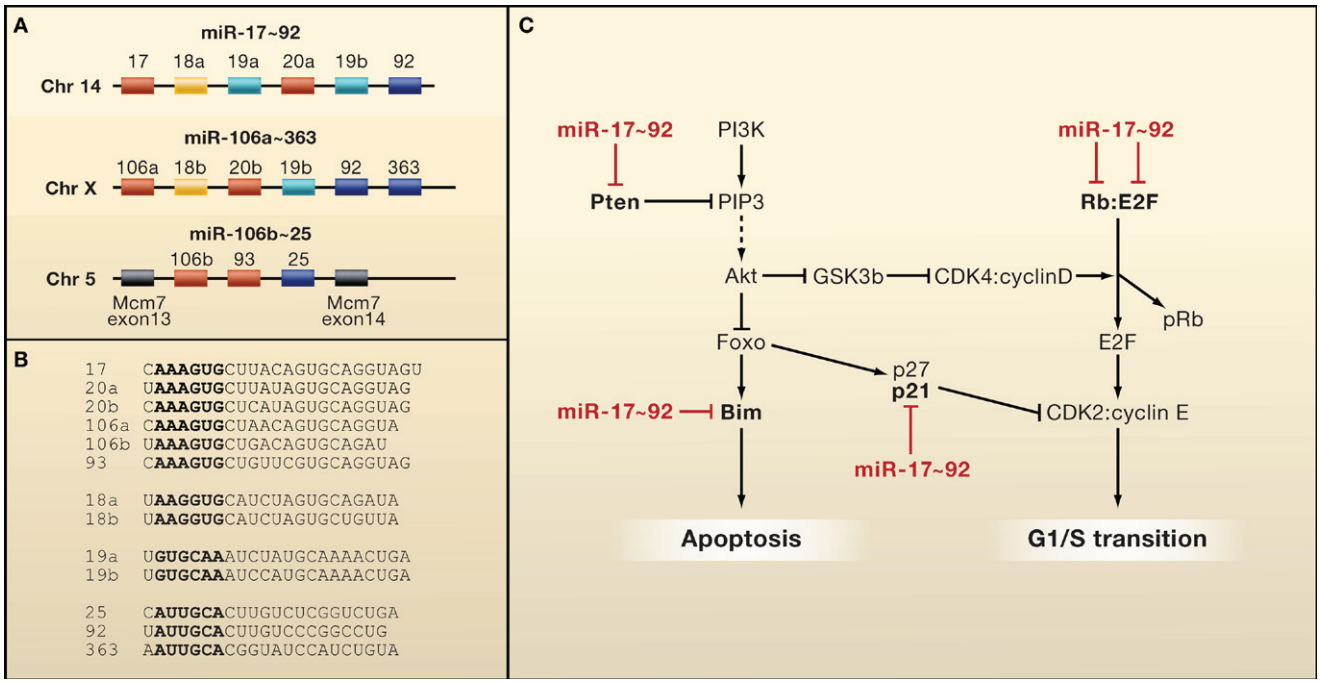

Figure 4. miR-17∼92 Regulates Functionally Interrelated Pathways

(A) The genomic organization of miR-17∼92 and homologous clusters. miRNAs of the same color have identical seed regions (nucleotides 2–7). The miR-106b∼25 cluster resides in an intronic region of the *Mcm7* gene.

(B) Alignment of mature miRNA sequences. Bold letters indicate seed regions.

(C) miR-17∼92 regulates components of functionally interrelated pathways. miRNA targets are indicated in bold. Pten is an inhibitor of the PI3K pathway, which promotes cell cycle progression, and inhibits apoptosis by negatively regulating the transcription of Bim, a proapoptotic factor and itself a miR-17∼92 target. p21 is an inhibitor of G1/S cell cycle transition. The miR-17∼92 cluster also targets Rbl2, a member of the Rb family of pocket proteins that negatively regulate cell cycle, and E2F1, E2F2, E2F3. Whereas a complete loss of the three E2Fs blocks G1/S transition, mice with compound mutations of E2F1 and E2F2 develop lymphomas.

which fall into four miRNA families (miR-17, miR-18, miR-19, and miR-25) (Figures 4A and 4B). This genomic organization is highly conserved in all vertebrates from which complete genome sequences are available (Tanzer and Stadler, 2004). The miR-17∼92 and miR-106b∼25 clusters are ubiquitously expressed, whereas expression of the miR-106a∼363 cluster was undetectable in the tissues examined (Ventura et al., 2008). During lymphocyte development, miR-17∼92 miRNAs are highly expressed in progenitor cells, with the expression level decreasing 2- to 3-fold upon maturation (Ventura et al., 2008; Xiao et al., 2008).

Addressing the biological functions of these miRNAs, Ventura and colleagues (2008) showed that although deficiency of miR-106a∼363 or miR-106b∼25 did not produce a detectable phenotype, mice deficient of miR-17∼92 were 40% smaller than their wild-type littermates and died within minutes after birth, because of lung hypoplasia and a ventricular septal defect of the heart. In the hematopoietc system, there was a severe block of B cell development at the pro- to pre-B transition, with increased apoptosis of pro-B cells. When both the miR-17∼92 and miR-106b∼25 cluster were deleted, the mice died before embryonic day 15, with the mutant embryos displaying severe cardiac developmental abnormalities and apoptosis in specific regions of the central nervous system and the fetal liver. The B cell developmental block also became more severe in the absence of these two miRNA clusters (Ventura et al., 2008). This demonstrates functional cooperation between the two miRNA clusters.

In humans, the miR-17∼92 miRNA cluster is located at chromosome 13q31, a genomic region that is frequently amplified in lymphomas and solid tissue cancers, and the mature miRNAs encoded by this locus are expressed in high amounts in those cancer cells (Hayashita et al., 2005; Lu et al., 2005; Ota et al., 2004; Tagawa and Seto, 2005; Zhang et al., 2007). He and colleagues showed that retroviral expression of miR-17∼92 on the Eμ-Myc transgenic background accelerated c-Myc-mediated lymphomagenesis (He et al., 2005). To address whether miR-17∼92 overexpression by itself can reproduce some of these pathologies, we generated transgenic mice expressing the human miR-17∼92 cluster specifically in B and T lymphocytes, at levels comparable to those found in human lymphoma cell lines, to study the consequences of elevated miR-17∼92 expression (Xiao et al., 2008). The mutant mice exhibited spontaneous activation and pronounced expansion of B and T lymphocytes, as well as symptoms typical of lymphoproliferative and autoimmune disease, and died prematurely. Lymphoma development may have been precluded in the animals simply because of their short life span. Reporter assays in cell culture revealed that, as predicted bioinformatically, (see Koralov et al., 2008), Pten and Bim were functional targets of miRNAs in the

miR-17∼92 cluster. Indeed, mice doubly heterozygous for Pten and Bim partly reproduced the phenotype of the miR-17∼92 transgenics, demonstrating that downregulation of Pten and Bim did contribute to the transgenic phenotype, but deregulation of other targets must also be involved (Xiao et al., 2008). Such additional targets have been identified in other cellular contexts, as discussed below.

These studies demonstrated additional principles of miRNA control. First, homologous miRNAs with similar expression patterns can be functionally redundant, as exemplified by the lack of an obvious phenotype in mice deficient for miR-106b∼25, whose function is apparently largely compensated by miR-17∼92. (The phenotypic difference caused by deletion of miR-17∼92 and miR-106b∼25 may be due to the fact that only the miR-17∼92 cluster contains members of the miR-18 and miR-19 families). Previous studies of the let-7 family of miRNAs in *C. elegans* came to a similar conclusion (Abbott et al., 2005). Second, there is a dose effect among coexpressed homologous miRNAs, as demonstrated by the more severe phenotype produced by combined miR-17∼92 and miR-106b∼25 deficiency than produced by miR-17∼92 deficiency alone. Third, miRNAs organized in a cluster can regulate not only multiple components of a single pathway but also components of functionally interrelated pathways. Thus, miR-17∼92 miRNAs target Pten and Bim in the PI3K pathway (Xiao et al., 2008), and a few critical cell cycle regulators (such as p21, Rbl2, E2F1, E2F2, and E2F3), as suggested by studies in various cellular contexts (Lu et al., 2007; O'Donnell et al., 2005; Petrocca et al., 2008; Sylvestre et al., 2007; Wang et al., 2008) (Figure 4C). P21, Rbl2, E2F1, E2F2, and E2F3 regulate cell cycle progression at the G1 to S transition (Balomenos et al., 2000; Cobrinik, 2005; Field et al., 1996; Massague, 2004; Murga et al., 2001; Santiago-Raber et al., 2001; Yamasaki et al., 1996; Zhu et al., 2001). The PI3K pathway promotes cell cycle progression through at least two mechanisms: (1) AKT-mediated inactivation of GSK3β, which phosphorylates and destabilizes cyclin D, an essential cyclin for the G1 to S transition, and (2) AKT-mediated phosphorylation of FOXO transcription factors, and their subsequent translocation to the cytoplasm from the nucleus, where they drive transcription of p21 and p27, two negative regulators of the G1 to S transition (Manning and Cantley, 2007). The net outcome of regulating these targets is enhanced cell survival and proliferation. Finally, there was an indication that coexpressed miRNAs can regulate a common target in a cooperative manner, in accord with earlier work in cell culture systems (Doench and Sharp, 2004; Grimson et al., 2007; Krek et al., 2005). In the case of the control of Pten by miR-17 and miR-92, the upregulation of a reporter gene containing the Pten 3′ UTR caused by knockdown of both miR-17 and miR-19 was more pronounced than that caused by knockdown of either miR-17 or miR-19 alone (Xiao et al., 2008).

miRNAs Control Host-Virus Interactions

As mentioned, the ability of miRNAs and their targets to rapidly evolve makes miRNAs ideal candidates for the control of host-pathogen interactions, with virus control on the top of the list, as viruses entirely depend on the host cellular machinery for their survival and propagation. Accordingly, many viruses express miRNAs, and a plethora of regulatory mechanisms is emerging in which host- or virus-encoded miRNAs interact with virus or host mRNAs, respectively, in addition to their own mRNAs (Scaria et al., 2006).

Viral miRNAs can help viruses to evade the host immune response by regulating cellular and viral gene expression (Sullivan, 2008). Thus, miR-UL112 encoded by human cytomegalovirus (HCMV) downregulates the expression of major histocompatibility complex class I-related chain B (MICB), a stress-induced ligand of the natural killer (NK) cell activating receptor (NKG2D) that is critical for the NK cell killing of virus-infected cells (Stern-Ginossar et al., 2007). Interestingly, the same miRNA binding sites in MICB seem to be exploited by cellular miRNAs to maintain the expression of MICB protein under a certain threshold in normal tissues and to help tumor cells to escape immune attack (Stern-Ginossar et al., 2008). miRNAs encoded by simian virus 40 (SV40) accumulate at late stages of infection, are perfectly complementary to early viral mRNAs, and target these mRNAs for cleavage. This reduces the expression of viral T antigens encoded by the early viral mRNAs, enabling the infected cells to evade killing by cytotoxic T cells (Sullivan et al., 2005). miRNAs expressed by herpes simplex virus 1 (HSV-1) facilitate the establishment and maintenance of viral latency through a similar mechanism (Umbach et al., 2008).

In turn, cellular miRNAs, in addition to their normal regulatory roles, can participate in antiviral defense. Thus, miR-32 counteracts the accumulation of primate foamy virus type 1 (PFV-1) in human cells, by directly targeting the PFV-1 genome and causing translation inhibition (Lecellier et al., 2005). Some viruses overcome miRNA-mediated antiviral defense by encoding proteins that suppress RNA silencing, through mechanisms ranging from sequestering small RNAs to disrupting functions of key proteins involved in miRNA processing (Haasnoot et al., 2007). Viruses can also take advantage of host miRNAs. A liver-specific miRNA, miR-122, interacts with sequences in the 5′ noncoding region of the hepatitis C virus (HCV) RNA, and this interaction is required for viral replication and maintains high viral RNA abundance in liver cells. The tissue specificity of miR-122 expression also helps HCV to establish its tissue selectivity (Jopling et al., 2008; Jopling et al., 2005).

Although general rules of miRNA control are unlikely to emerge from the analysis of host-virus interactions, such studies should lead to major new insights into mechanisms of viral latency, tropism, and escape from innate and adaptive immune responses, and thus potentially new therapeutic approaches.

miRNAs and Hematopoietic Malignancies

Deregulation of miRNA expression has become a recurrent theme in the cancer field. Gene expression profiling of human cancer cells has revealed specific miRNA expression signatures in many human cancers (Calin et al., 2004a; Lu et al., 2005). A global downregulation of mature miRNAs was frequently observed in such experiments, often accompanied by upregulation of specific groups of miRNAs (Calin and Croce, 2006; Lu et al., 2005). In addition, miRNA genes are frequently located at fragile sites and genomic regions involved in carcinogenesis (Calin et al., 2004b), and recent evidence indicates that miRNAs can directly contribute to various aspects of this

process, including tumor metastasis and cancer stem cell maintenance (Huang et al., 2008; Kent and Mendell, 2006; Ma et al., 2007; Tavazoie et al., 2008; Yu et al., 2007).

miRNAs appear also to be heavily involved in the pathogenesis of leukemias and lymphomas. This topic has been recently reviewed (Garzon and Croce, 2008), and we limit our discussion in this context to the two cases in which genetic evidence in mouse models points to a causal role of miRNAs in these diseases. Thus, miR-155 and the miRNAs of the miR-17∼92 cluster (also called oncomir-1) are highly expressed in many kinds of human cancers, including lymphomas. Retroviral expression of miR-17∼92 in mice on the Eμ-myc transgenic background accelerated c-Myc-mediated lymphomagenesis (He et al., 2005), and transgenic mice with elevated miR-17∼92 expression in lymphocytes died prematurely from a lymphoproliferative and autoimmune disease, as discussed earlier (Xiao et al., 2008). In the case of miR-155, transgenic mice overexpressing this miRNA in B cells developed polyclonal pre-B cell proliferation followed by B cell malignancy (Costinean et al., 2006). In addition, a subset of human patients with acute myeloid leukemia (AML) have elevated miR-155 expression in their bone marrow, and retroviral expression of miR-155 in bone marrow progenitor cells caused a myeloproliferative disorder in mice (O'Connell et al., 2008).

Although this field is still in its infancy, the mechanistic study of the role of miRNAs in malignancies of the hematopoietic system promises not only to lead to a better understanding of miRNA control in normal physiology but also to allow the problem of hematopoietic malignancies to be approached from a new clinical angle.

Conclusions

miRNAs have emerged in the immune system as regulatory elements involved in the control of cellular development, homeostasis, and response in highly specific ways. Key features of this control are a dose-dependent regulation of target protein concentrations over a mostly modest range and the targeting of multiple functionally related proteins. Subtle changes of intracellular protein concentrations can have profound physiological effects as documented by the many pathologies arising from haploinsufficiency, and the targeting of several components of a functional network may further enhance the functional impact of miRNA control. It is therefore not surprising that the genetic ablation as well as ectopic or overexpression of individual miRNAs can have severe physiological consequences, ranging, in the immune system, from cell death and impairment of immune functions to autoimmunity, lymphoproliferation, and cancer.

A major challenge in the field is the identification of the critical miRNA targets in a particular biological in vivo context. This can ultimately only be done by combining bioinformatic, biochemical, and genetic approaches: (1) using bioinformatics to predict possible targets and to organize targets into functional networks, (2) using proteomic and gene expression analysis, as well as reporter assays and the (still challenging) isolation of miRNA:mRNA complexes, to identify "real" miRNA targets, and (3) using genetic experiments like the overexpression or inactivation of target genes or of miRNA target sequences within these genes to address the biological relevance of such targets in a particular biological context. Interdisciplinary approaches have thus become indispensable in the pursuit of the biological impact of miRNA control.

The unique ability of miRNAs and their target sequences to rapidly evolve seems to be reflected in a plethora of miRNA-based control mechanisms operating in host-virus interactions. In the medical context, this together with the notion that miRNAs control, in a dose-dependent manner, cellular states or responses rather than single or collections of functionally unrelated proteins, makes these molecules attractive candidates or targets for future medical therapies.

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