SMALL RNAs PLAY BIG ROLES IN HEMATOPOIETIC DEVELOPMENT

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ABSTRACT

The relatively recent identification of microRNAs (miRNAs) has added a new layer of complexity to our understanding of gene regulation. These small noncoding RNAs cause the downregulation of target mRNAs, leading to downstream effects. We have studied how particular miRNAs can impact hematopoietic development in mice and correlated these findings with miRNA deregulation in human disease. Specifically, we studied the role of miR-155 in myeloid cells, finding that it promoted myeloid cell expansions when induced by lipopolysaccharide and a myeloproliferative disease when constitutively expressed. Interestingly, miR-155 is overexpressed in acute myeloid leukemia, a human disease that has the proliferation of myeloid cells in common with our mouse model. miR-155 is a growth-promoting factor in cells and acts in early hematopoiesis by repressing the inositol phosphatase, SHIP1. We have also studied the role of miR-34a, a putative growth-suppressing miRNA, in hematopoietic development, finding a specific perturbation in B-cell development by gain- and loss of function analyses. In this case, the majority of the findings are attributable to repression of Foxp1, a transcription factor involved in regulation of immunoglobulin gene V(D)J recombination. Our findings show how miRNAs are integrated into developmental pathways that control hematopoiesis, and suggest that they may act as nodes of regulation during specific hematopoietic developmental processes.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

CHAPTER 1: INTRODUCTION

Overview of Thesis

The first chapter will deal with background information on the history of miRNA discovery, miRNA biogenesis and function, and the roles that miRNAs play in hematopoietic development. The next three chapters represent the bulk of the experimental work during my time at Caltech. In chapter 2, we describe the features of bone marrow in which miR-155 is constitutively expressed. My contributions to this work included the analysis of the histologic data, enabling a collaborative research project to profile acute myeloid leukemia (AML) samples at UCLA, analysis of the AML miRNA expression data along with the clinical data, and contributing to the organism-level understanding of the phenotype induced by constitutive miR-155 expression. In chapter 3, we describe a specific target of miR-155, the inositol phosphatase SHIP1, which is responsible for a large part of the phenotype induced by miR-155. Here, I contributed to the target analysis and to the histologic assessment of the animals in the experiment. In the chapter 4, I describe a B-cell developmental phenotype induced by constitutive expression of miR-34a. In this chapter I detail a novel target of miR-34a, Foxp1, and use loss of function and gain-of-function approaches to demonstrate specific targeting. In addition, I describe the utilization of retroviral strategy to induce miRNA loss of function, which can greatly aid in studying the physiological roles of miRNAs in general. In chapter 5, I will describe some of the conclusions based on this body of work, including implications for hematopoiesis and for our understanding of how miRNAs may be integrated into developmental networks. I will close chapter 5 with a brief summary of future directions of the work.

A brief history of the discovery of microRNA

The first animal microRNA (miRNA) was discovered in *C. elegans* in the early 1990s (Lee et al. 1993). This early work described a small noncoding RNA that controlled the timing of several events during postembryonic development. The gene, *lin-4*, was known from genetic studies to control the levels of the LIN-14 protein; in its absence, LIN-14 was unregulated. To further study this, the *lin-4* gene was cloned but found not to have an open reading frame. Further analyses revealed that there were two small RNAs in *C. elegans*, 61 nucleotides long and 22 nucleotides long, respectively, that were products of *lin-4*. These RNA species, remarkably, showed regions of complementarity to the 3' untranslated region (3'-UTR) of the *lin-14* mRNA. The principle that a small RNA acting in an antisense fashion could regulate an mRNA was first presented by these authors.

Concurrently, a colleague of Victor Ambros, Gary Ruvkun, had been working on this idea from the other "end" of the problem. His earlier work had shown that Lin-14 was a nuclear protein that controlled a developmental switch in *C. elegans* (Ruvkun and Giusto 1989). Upon careful examination of the gene, negative regulatory elements were discovered within the 3'-UTR of *lin-14* that were required to generate the above-mentioned developmental switch (Wightman et al. 1993). Further analysis revealed seven of these regulatory elements with complementarity to *lin-4*. These regulatory elements were both necessary and sufficient to confer *lin-4*-mediated temporal posttranscriptional regulation. This early work established much of the framework for our understanding of how miRNAs interact with their targets.

At the time of this discovery, it was speculated that this novel mechanism may represent an unusual mechanism unique to nematodes. However, this view changed dramatically with the description of RNA interference by Andrew Fire and Craig Mello (Fire et al. 1998). By injecting double-stranded RNA into *C. elegans*, they were able to induce specific and potent interference of gene expression. Although antisense approaches had been used previously to induce partial repression of gene expression, and some effects were specific (Fire et al. 1991; Izant and Weintraub 1985; Nellen and Lichtenstein 1993), the contribution of Fire and Mello was especially significant in recognizing the importance of double-stranded transcripts and in finding that the non-stochiometric inhibition of targets implied a catalytic mechanism. This study established RNA interference as a specific and powerful way of altering gene expression but also raised the possibility of the existence of a cellular pathway that utilizes RNA interference. It should also be mentioned that concurrently, David Baulcombe and colleagues had discovered a small noncoding RNA that mediated some aspects of innate immunity in plants (Hamilton and Baulcombe 1999). This, too, appeared to work by RNA interference.

The discovery of RNA interference was followed shortly by the discovery of the let-7 miRNA in *C. elegans*, and shortly thereafter by the description of a conserved mechanism in other species (Pasquinelli et al. 2000; Reinhart et al. 2000; Slack et al. 2000). The term microRNA was coined in 2001 in a series of articles that described the existence of additional miRNAs and their conservation across species (Lagos-Quintana et al. 2001; 2002; Lau et al. 2001; Lee and Ambros 2001). These discoveries established

miRNAs as significant and widespread in biology, with RNA interference as their putative mode of action.

Starting very early in the field, a major theme of research in miRNAs has involved mechanism of action. One line of research has involved the prediction of miRNA targeting based on sequence information-the idea being that small RNAs should have predictable targets in trans, unlike promoter/enhancers and transcription factors. In this effort, the sequencing of the genomes of multiple organisms has allowed for analyses that were simply not possible previously. Indeed, the mapping of miRNA genes and interspecies comparisons is central to miRNA discovery and target prediction. Several algorithms have been generated over the years to predict miRNA-target interactions, with varying degrees of success (Chen and Rajewsky 2006; Enright et al. 2003; Lewis et al. 2005; Rajewsky 2006). Of these algorithms, the one that has met with fairly good success in prediction utilizes a seed match that aligns nucleotides 2-7 of the miRNA with the 3' UTR (Lewis et al. 2005).

Finally, the biogenesis and mechanism of action of miRNAs has been an area of intensive research (the current state of knowledge will be discussed in the subsequent section). Major milestones included the discovery of the miRNA processing enzymes, Drosha and Dicer (Bernstein et al. 2001; Ketting et al. 2001; Lee et al. 2003). In addition, the description of the RISC complex and the proteins that comprise it, including the Argonaute proteins (which were first discovered in plants), has illuminated the mechanism of action of miRNAs to a large extent (Hammond et al. 2001; Tabara et al. 1999). However, significant work remains to be done, as the biochemistry, stochiometry and other aspects of miRNA function remain to be fully understood.

miRNA Biogenesis and Mechanism of Action

miRNAs are encoded by cellular genes and are generally transcribed by RNA polymerase II, which allows for their regulation by transcription factors much like protein-coding genes (reviewed in Kim et al. 2009). In the genome, miRNAs can exist as unique transcriptional units, or in other cases, reside within the intron of a gene. Certain miRNAs, like the miR-17-92 family, are grouped in clusters on a single mRNA transcript and are expressed together (He et al. 2005). miRNAs are processed from their primary transcripts (pri-miRNAs) by the enzymes Drosha and DiGeorge syndrome critical region gene 8 (DGCR8), and recent data suggest that this occurs cotranscriptionally (Ballarino et al. 2009). The resulting pre-miRNA is a stem-loop structured RNA that is approximately 60 nucleotides in length. This pre-miRNA is then exported to the cytoplasm where it is further acted on by the cytoplasmic RNAse Dicer, which cleaves the loop structure, leaving a double-stranded miRNA precursor. Following processing by Dicer, the double stranded miRNA-miRNA* duplex (which is now the "stem" without the "loop") is unwound concurrently with loading into the RNA Induced Silencing Complex (RISC). This complex, whose exact composition remains unknown, is thought to consist of multiple proteins and RNA, the most important of which is a member of the Argonaute (AGO) family of proteins (Liu et al. 2004). In miRNA-mediated regulation, Ago2 is the most important protein component of the RISC complex. The miRNA then guides the RISC complex to bind a target mRNA, leading to downregulation of protein expression. This can occur by several mechanisms, including degradation of the target mRNA and translational inhibition (reviewed in Nilsen 2007).

Regulation of miRNA expression and function occurs at four levels: transcription, processing, subcellular localization and turnover. The expression of miRNAs is controlled at the transcriptional level by transcription factors that regulate the production of miRNA-containing pri-miRNA, conferring cell type specificity and inducibility. For example, c-Myc, p53 and NF-κB have all been shown to induce specific miRNAs in different cell types (Bommer et al. 2007; Chang et al. 2007; He et al. 2007; 2005; Taganov et al. 2006). Several posttranscriptional regulatory mechanisms that affect miRNA processing have recently been identified. Arsenate resistance protein 2 (ARS2) is expressed by proliferating cells and is a component of the RNA cap-binding complex that promotes processing of pri-miRNA transcripts (Gruber et al. 2009). Adenosine deaminase acting on RNA (Adar) can regulate pri-miRNA processing through mutation of the double-stranded miRNA stem sequence (Yang et al. 2006). The tumor suppressor protein p53, in addition to regulating the transcription of specific miRNAs, also has an important role in miRNA processing following the onset of DNA damage (Suzuki et al. 2009). p53 can induce increased processing of pri-miRNAs to pre-miRNAs, by forming a complex with Drosha. The miRNA loop also mediates cis-regulatory control over miRNA processing, and the KH-type splicing regulatory protein (KSRP) has been implicated in this process (Ruggiero et al. 2009; Trabucchi et al. 2009).

After transport into the cytoplasm, posttranscriptional modifications can further regulate miRNA abundance. Some pre-miRNAs are inhibited from undergoing further processing by TUTase4, which is recruited by LIN28 to mediate uridylation of pre-let7 miRNA (Hagan et al. 2009; Heo et al. 2009). This modification prevents further processing of pre-let7 miRNA, thus abolishing its function. In addition, LIN28 itself

inhibits processing of the primary let-7 RNA, indicating a coordinated regulation of the abundance of this miRNA (Piskounova et al. 2008). Levels of canonical miRNA processing enzymes themselves can be regulated during certain conditions, such as the regulation of Dicer during inflammatory responses (Wiesen and Tomasi 2009). Another protein, importin-8, is thought to be important in the delivery of the processed miRNA to the RISC complex (Weinmann et al. 2009). It remains an important question as to whether subcellular localization plays an important role in regulation of miRNA function. miRNAs have been shown to associate with stress granules following the onset of cell stress (Leung et al. 2006). However, the relevance of this localization to the regulation of miRNA function is unknown.

The final aspect of miRNA regulation is turnover. In general, it is thought that miRNAs are highly stable, as evidenced by the long half-life of miRNAs in cells following siRNA-mediated inhibition of essential miRNA processing enzymes (Denli et al. 2004; Gregory et al. 2004). Some miRNAs, however, can be rapidly depleted, e.g., miR-122 in hepatocytes following treatment with interferon (Pedersen et al. 2007). This implies an active turnover mechanism for miRNAs, which is beginning to be studied, with early work coming from plants and nematodes. Specifically, a family of exoribonucleases in *Arabidopsis thaliana* that degrade miRNAs has recently been described (Ramachandran and Chen 2008). In *C. elegans*, recent work has highlighted the importance of Xrn-2 by gain- and loss of function approaches (Chatterjee and Grosshans, 2009). In this model system, siRNA-mediated Xrn-2 depletion led to the accumulation of many miRNAs, including let-7. It is unknown if this function is conserved in higher animals, as the homologous protein in man, XRN2, is thought to play

a role in transcriptional termination by causing cotranscriptional cleavage via its 5'-3' exoribonuclease activity (West et al. 2004). Mammalian regulation of miRNA turnover remains an aspect of miRNA biology that demands further examination.

The mechanism of miRNA action has been an area of active investigation in recent years. A canonical siRNA-type mechanism seems to be operant when the miRNA and its target share a high degree of complementarity. Here, the target mRNA is downregulated via degradation, an action thought to involve the RNAse catalytic activity of AGO2 (also called the "slicer function") (Joshua-Tor, 2004; Liu et al. 2004; Song et al. 2004). In general, it is thought that the target sites reside within the 3'UTR of the target gene, similar to the initially discovered interaction between *lin-4* and *lin-14*. In most of these target sites, the complementarity is far from perfect; in fact, there is usually a 6-8 nucleotide match at the 5' end of the miRNA, a bulged region without complementarity, and a 3'-complementary region of variable length. In these cases, a variety of translational inhibition mechanisms have been suggested to be responsible for the observed repression of the target protein. These mechanisms include deadenylation and destabilization of translational complexes, proteolysis of the nascent target peptide, repressed initiation of translation via decreased mRNA cap recognition or 60S ribosomal recruitment, and translation elongation block (reviewed in Nilsen (2007)). However, more recent studies seem to indicate that the level of target mRNA repression is a very good measure of the effects of miRNA action, calling into question the relative contribution of the modulation of these various translational mechanisms (Baek et al. 2008).

Central to studying the mechanism of miRNAs has been the development of target prediction algorithms. As alluded to in a previous section, several algorithms have been developed. Briefly, TargetScan utilizes a seed match which aligns nucleotides (nt) 2 to 7 of the miRNA with 3' UTR. Once this has been done, conservation of a 7 or 8 nt sequence is assessed between species, with additional weight given to a 5' flanking adenosine residue (Lewis et al. 2005). PicTar uses a more complex algorithm using an 11 nt-core "nucleus," following which the thermodynamics of binding of the entire miRNA is assessed. Again, conservation between species is used to identify "true" targets (Chen and Rajewsky 2006; Rajewsky 2006). miRanda utilizes a simpler algorithm assessing complementarity between the entire miRNA and the 3'UTR, with additional weight given to complementarity amongst the 11 residues at the 5' end (Enright et al. 2003). We have tested several of these algorithms against experimentally generated data sets, finding that they can be useful in predicting if a given repressed mRNA is likely to be a direct target of the miRNA under study. Unfortunately, none of the algorithms provide a completely fool proof, *a priori* method of target prediction.

It should be noted here that new and unexpected twists to our understanding of miRNA function are beginning to be reported; these include the upregulation of targets by miRNAs in certain cellular contexts, "seedless" target sites, and the presence of target sites outside of the 3'UTR (Lal et al. 2008, 2009; Vasudevan et al. 2007). Hence, while enormous progress has been made in understanding the biochemical underpinnings of miRNA biogenesis and action, important aspects remain incompletely understood. The major challenges, in my opinion, will be to understand the posttranscriptional processing and turnover of miRNAs in greater detail, to completely describe the mechanism of

miRNA repression and to concurrently develop high-fidelity algorithms to accurately predict miRNA targeting. This is in addition to the broader goal of integrating miRNAs into our knowledge of gene regulation and development.

Role of miRNAs in hematopoietic development.

Hematopoiesis in mammals involves a complex interplay of extracellular growth factors/cytokines and cellular contacts, transcription factor activation of gene expression programs, and DNA remodeling and epigenetic changes. The differentiation of cells in the hematopoietic lineage involves a progressive loss of multipotency from a pluripotent hematopoietic stem cell, with specific transcription factors leading to terminal lineage commitment. This has been best described in the differentiation of lymphocytes (reviewed in Medina and Singh (2005) and Rothenberg (2007)), but the general principles apply to other hematopoietic lineages as well. It stands to reason that mRNA transcripts coding for any of the molecular players in this developmental process could be regulated at the posttranscriptional level by miRNAs. In fact, a significant body of work has examined the roles of miRNA in hematopoietic development and cancer. Since the research in the current work focuses on myeloid and B-cell development, I will focus the next section on miRNA roles in hematopoiesis and aspects of development of these two lineages. For a broader review of the topic that includes all hematopoietic lineages, the reader is referred to a recent review on miRNAs in hematopoiesis that I coauthored fo*r* *Nature Reviews Immunology* and is presented as an appendix (see appendix 2).

Regulation of early hematopoietic development by miRNAs

Hematopoietic stem cells (HSCs) reside primarily in the bone marrow and maintain a balance between self-renewal and differentiation into multipotent progenitors. Several groups have performed global miRNA expression profiling of human $CD34^+$ stem and progenitor cells, and have identified certain miRNAs as being enriched in this cell population (Georgantas et al. 2007; Merkerova et al. 2009). Some studies have shown that deregulation of factors responsible for miRNA processing leads to major deficits in hematopoiesis. Mice deficient in Ars2, which contributes to pri-miRNA processing, exhibit bone marrow failure possibly due to defective HSC function (Gruber et al. 2009). Conditional deletion of Ago2 in the hematopoietic lineage leads to disruptions in hematopoiesis, including B-lymphocyte development and erythroid development (O'Carroll et al. 2007). Specific miRNAs are also thought to play important roles in HSC homeostasis. These include miR-126, miR-10 and miR-196b, which repress Hox genes; as well as miR-221 and miR-222, which target c-Kit (Argiropoulos and Humphries 2007; Garzon et al. 2008; Mansfield et al. 2004; Shen et al. 2008; Yekta et al. 2004).

The differentiation of hematopoietic stem cells into the various lineages has been an area of active investigation. The first study to address this idea utilized retroviral vectors to constitutively express miRNAs that were found to be enriched in particular hematopoietic populations (Chen et al. 2004). miR-181, miR-223 and miR-142, which are enriched in B-cells, myeloid cells, and T-cells, were studied. When miR-181 was overexpressed in hematopoietic stem and progenitor cells *in vitro* and *in vivo*, increased CD19+ cells were found, indicating a skewing of development toward a B-cell fate.

These experiments set an important precedent for the field and for much of the work presented here, as they showed the importance of miRNAs in lineage choice during hematopoiesis. Numerous additional studies have followed, and it is becoming increasingly clear that hematopoietic development is significantly modulated by miRNA expression.

Regulation of myeloid development by miRNAs

Myeloid development from hematopoietic stem and progenitor cells is driven by sequential activation of transcription factor networks, producing the specialized immune effector cells of this lineage. Classically, granulocytes and monocytes are the circulating cells that are derived from the bone marrow myeloid progenitors. Some of the key transcriptional regulators of monocyte and granulocyte differentiation are now known to be regulated by miRNA expression; in addition, many of the known transcription factors regulate miRNA gene expression (reviewed in Appendix 2). Of these miRNAs, miR-223 has been studied extensively, and it seems to have an important role in myeloid development.

miR-223 has been studied in the context of normal myelopoiesis and myeloid malignancy (Fazi et al. 2007; Johnnidis et al. 2008). This miRNA is expressed specifically in cells of the granulocytic lineage. Its expression changes during maturation, becoming incrementally higher as granulocytes mature (Johnnidis et al. 2008). miR-223 expression is lower in a subtype of acute myeloid leukemia, known as acute promyelocytic leukemia, that has a block in differentiation (Garzon et al. 2007). Granulocytic differentiation is restored by constitutive expression of miR-223 in leukemic blast cells; this suggests a physiological function for miR-223 in this process

that is disrupted in disease (Fazi et al. 2007). The mechanisms regulating miR-223 expression have been a chief focus of study, given its apparent importance in granulocytic differentiation.

Expression of miR-223 is regulated by a combination of factors. Initially, a circuit consisting of C/EBPα (a member of the CCAAT enhancer-binding protein family), NFI-A (a transcription factor related to the CCAAT family) and miR-223 was described (Fazi et al. 2005). In this scenario, C/EBPα activates transcription of miR-223, whereas NFI-A represses it; miR-223 itself targets NFI-A, thereby turning off its repressor once it is expressed. This positive-autoregulatory circuit was postulated to be critical in granulocytic maturation, causing commitment to this lineage. Further work has shown that the situation is probably more complex (Fukao et al. 2007). Analysis of conserved proximal cis-regulatory elements has shown that the putative binding sites for the transcription factors mentioned above do not overlap the promoter elements defined for the gene encoding miR-223. Instead, these analyses indicate that miR-223 expression may be driven by myeloid transcription factors such as PU.1 and C/EBP, similar to many protein-encoding genes involved in granulopoiesis. In disease, the AML1-ETO fusion oncoprotein targets the miR-223 promoter for epigenetic silencing, abrogating the ability of myeloid transcription factors to transcribe this miRNA precursor.

The precise physiological function of miR-223 remains elusive. Initial loss of function approaches focused on *in vitro* assays, where approaches based on “antagomirs” (cholesterol-linked single-stranded antisense RNA) disrupt induced granulocytic differentiation in leukemic blasts (Fazi et al. 2007). However, studies of a miR-223-knockout mouse rather unexpectedly showed that these mice had a two fold *increase* in

granulocytes (Johnnidis et al. 2008). These granulocytes were morphologically hypermature and hypersensitive to activating stimuli and demonstrated more fungicidal activity. The mice also had inflammatory lung lesions and develop more tissue destruction after endotoxin challenge. The relevant target in this context seems to be *Mef2c*, which encodes a transcription factor involved in promoting myeloid progenitor differentiation, as demonstrated by correction of the miR-223-null phenotype in mice lacking both miR-223 and Mef2c. These results collectively suggest that miR-223 is involved in regulation of granulocytic maturation but is not absolutely required for the production of granulocytes *in vivo*.

The miR-223 story highlights several important points about miRNAs. First, the field of miRNA research is relatively young, and there are many results which seem contradictory. As the methods and approaches to research mature, some of the technical issues responsible for discrepant results will likely be resolved. The second issue is a recurring theme in hematopoietic development in general-that miRNAs can be induced by transcriptional regulators of lineage development and can in turn influence the levels of developmental regulators. This finding with miR-223 and other miRNAs (reviewed in appendix 2) integrates miRNAs into the regulatory networks that control hematopoietic development. Last, these studies allude to the importance of specific regulation of single or small numbers of critical regulators of miRNA development in the control of developmental processes.

miRNAs play vital roles during inflammatory responses.

The interest of the Baltimore laboratory in miRNAs was initiated by a study of miRNAs regulated by a study of miRNAs that were responsive to endotoxin in a human

monocytic cell line (Taganov et al. 2006). Three miRNAs emerged from this analysis-miR-146, miR-155, and miR-132. Subsequently, NF-κB has been implicated in transcriptional activation of both miR-146a and miR-155. These miRNAs have now been found to be crucial in the regulation of the immune response, albeit in very different, almost opposing, ways. These findings began several important lines of research, including those presented in chapters 2 and 3 of this thesis.

The inflammatory response is an intensively studied aspect of mammalian biology and involves the coordinated action of the two arms of the immune system-innate and adaptive immunity. The innate system, mainly consisting of granulocytes and monocyte/macrophage system is important in the early response to foreign antigens. Simplistically, this involves the identification of these antigens by pattern-recognition receptors followed by activation of innate immune cells and killing of the invading pathogen (Janeway 2005). The adaptive immune system, consisting of B- and T-lymphocytes is important in the provision of long-term immunity, with the net effect of enabling a prophylactic response against a pathogen that has previously been encountered. It is now recognized that such a dichotomy is somewhat artificial, in that the innate and adaptive systems interact with each other at multiple levels. It has long been known that Th1-skewed T-lymphocytes, for example, can activate the innate immune system (Cherwinski et al. 1987; Mosmann et al. 1986). Similarly, the role of dendritic cells in antigen uptake and presentation to T-lymphocytes is central to their activation (Janeway 2005).

At the cellular level, activation of immune cells is controlled by pathways that share some features across the innate and adaptive immune systems. Distinctive features

include the cell surface receptors and adapter proteins that initiate the activation of different subsets of immune cells. For example, lymphocytes have antigen receptors (T-cell receptors and membrane antibody) while monocyte/macrophages have pattern recognition receptors (e.g., the Toll-like receptors) (Janeway 2005). Downstream of these receptors, many immune cells activate NF-κB in response to antigenic challenges. The details of this activation are an area of intensive study in the Baltimore lab and elsewhere, but the net effect is a transcriptional sea change in immune cells, generally leading to effector functions of the particular cell type. This occurs in a temporally regulated manner, with functionally distinct subsets of transcripts activated in a sequence of short-acting, intermediate-acting, and long-acting genes (Hoffmann et al. 2003). This sequential transcriptional activation is likely accompanied by posttranscriptional regulation that confers temporal specificity. In this regard, it is interesting to note that the 3'-UTRs of these genes may regulate their expression (Hao and Baltimore 2009).

miRNA-mediated regulation of mRNA/protein expression may have several very important advantages over transcription factor-mediated repression of gene expression (Hobert 2008). For the latter to occur, the transcription factor may need to be transcribed, translated, and then translocated to the nucleus so as to repress the transcription of the target gene. In addition, the chromatin state of the target gene may have to be modified in order to provide access to the transcriptional machinery. Following repression, the stability of the target mRNA greatly influences how quickly a gene can be downregulated. Since miRNAs are not translated, the time sequence for their production could be shorter. Second, they directly repress target mRNAs, and therefore may be able to more rapidly induce downregulation of a protein. Hence, in highly time-sensitive

processes (such as in controlling a bacterial infection, where the number of bacteria can double every 20 minutes or so), miRNA-mediated gene regulation may represent a preferred mode of action.

As examples of miRNAs important in inflammatory reposes (potentially in a time-sensitive fashion), our lab has shown that miR-155 is a positive regulator of the inflammatory response, while miR-146a negatively regulates the immune response ((O'Connell et al. 2007; Taganov et al. 2006) and data not shown). miR-155 is upregulated soon after LPS treatment, seems to promote B-cell activation, and also has important effects on early myeloid development, with constitutive expression leading to a myeloproliferative disease (Chapters 2 and 3). The latter miRNA may be important in the termination of inflammatory responses by targeting key adapter proteins that are involved in transducing the signal from the cell surface receptor, in this case TRAF6 and IRAK1 (Taganov et al. 2006). Overall, the study of miRNAs in the inflammatory response has uncovered these previously unknown molecules as integral components of cellular pathways controlling inflammation.

miRNAs in B-cell development.

The production of antigen-specific antibodies is a central component of mammalian immunity, and occurs through the development and activation of B-lymphocytes (Hardy and Hayakawa 2001). Following antigen-independent development in the bone marrow, B-lymphocytes further refine their antigen-specificity in secondary lymphoid organs under inflammatory conditions. MiRNAs play important roles in antigen-independent and antigen-dependent B-cell development, and are dynamically regulated during these processes. Profiling studies in early B-cell development are

lacking, but several groups have profiled the expression of miRNA in naïve, germinal center, and postgerminal center lymphocytes (Basso et al. 2009; Tan et al. 2009; Zhang et al. 2009). Dynamic developmental regulation of several different miRNAs has been observed, and putative new cell-type specific miRNAs have also been identified, suggesting that new regulatory pathways remain to be discovered in B-cell development.

The control of early B-cell development relies on the commitment of progenitor cells to the B-cell lineage by activation of transcription factor networks, rounds of V(D)J DNA rearrangement, and selection for effective antigen receptors. The roles of miRNA in controlling the early development of B-cells are now thought to relate to the modulation of critical protein factors that control development (Xiao and Rajewsky 2009). In a study examining the global role of miRNA in B-cell development, the conditional deletion of the essential miRNA processing RNAse Dicer in B-cells leads to a complete block in B-cell development (Koralov et al. 2008). This block is thought to relate to deregulated expression of Bim, an important regulator of cellular apoptosis. When this block was overcome by transgenic expression of Bcl-2, mature B-cells with a strange distribution of V(D)J rearrangements were observed, indicating that the defect lies in the regulation of antigen receptor selection. Of interest, the changes noted in gene expression profiling of Dicer-deficient B-cell precursors were largely similar to that observed for the knockout of the highly conserved miRNA cluster, miR-17-92 (Ventura et al. 2008). This cluster of miRNAs is plays a role in B-cell development and its overexpression is associated with pathological lymphoproliferative conditions in humans and mice (Xiao et al. 2008). It is likely then that the major effects of conditional deletion of Dicer in B-cells are largely due to the loss of these critical miRNA in early B-cells.

In addition to effects on antigen receptor selection, miRNAs can regulate critical transcription factors in early B-cell development, as evidenced by overexpression of miR-150. Constitutive expression of this miRNA, which shows a dynamic expression profile in the early B-cell lineage, causes a block at an early stage of B-cell development, namely at the pro-B to pre-B-cell transition, and this block is dependent on the dysregulation of c-Myb (Lu et al. 2008). Mice deficient in miR-150 show an accumulation of B-1 B-cells (B-cells with a limited repertoire of relatively broad-specificity antibodies) in the spleen and the peritoneum, with a relative decrease in the number of B-2 B-cells (Xiao et al. 2007).

During inflammation, T-cell-dependent somatic hypermutation and class-switch recombination occur in the germinal center, and again rely on specific transcriptional events, class-switch recombination and somatic hypermutation, and selection for high-affinity antibodies. MiRNAs are now thought to play important roles in these antigen-specific adaptive immune responses. The above-mentioned miR-150-deficient mice show exuberant antibody secretion both at baseline and following T-dependent antigenic stimulation, although the mechanism of the latter is not understood (Xiao et al. 2007). Perhaps the best-characterized miRNA during this stage of B-cell development is miR-155, which is upregulated upon B-cell activation. miR-155 deficient B-cells show defective class switching and differentiation into plasma cells, resulting in a blunted humoral response to T-dependent antigenic stimulation (Thai et al. 2007; Vigorito et al. 2007). These effects are likely to be mediated by multiple targets, as both Pu.1 and Aid are targeted by miR-155 (Dorsett et al. 2008; Teng et al. 2008; Vigorito et al. 2007). The targeting of Aid was investigated by germLine mutation of the miR-155 seed sequence in

the murine *Aicda* gene in an elegant genetic demonstration of direct targeting of a 3'UTR by miRNA. Based on these previous studies it seems safe to say that miRNAs play important and critical roles in normal B-cell development. In Chapter 4, I will describe a novel role for miR-34a in the regulation of B-cell development.

CHAPTER 2: Constitutive expression of miR-155 causes a myeloproliferative disorder in the bone marrow.

 Originally published as: RM O'Connell, DS Rao, AA Chaudhuri, MP Boldin, KD Taganov, J Nicoll, RL Paquette, and D Baltimore (2008). Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. *J Experimental Medicine*, 205(3):585-594. doi:10.1084/jem.20072108.

Abstract

Mammalian microRNAs (miRNAs) are emerging as key regulators of the development and function of the immune system. Here, we report a strong but transient induction of *miR-155* in mouse bone marrow after injection of bacterial LPS correlated with granulocyte/monocyte (GM) expansion. Demonstrating the sufficiency of *miR-155* to drive GM expansion, enforced expression in mouse bone marrow cells caused GM proliferation in a manner reminiscent of LPS treatment. However, the *miR-155*-induced GM populations displayed pathological features characteristic of myeloid neoplasia. Of possible relevance to human disease, *miR-155* was found to be overexpressed in the bone marrow of patients with certain subtypes of acute myeloid leukemia (AML). Furthermore, *miR-155* repressed a subset of genes implicated in hematopoietic development and disease. These data implicate *miR-155* as a contributor to physiological GM expansion during inflammation and to certain pathological features associated with AML, emphasizing the importance of proper *miR-155* regulation in developing myeloid cells during times of inflammatory stress.

Introduction

Mammalian hematopoiesis involves the generation of blood cells from a common hematopoietic stem cell (HSC) through many intermediate stages, each of which can give rise to various types of malignancies upon their dysregulation. However, the molecular mechanisms that govern this process are incompletely understood. In particular, the quantitative decisions regarding how many cells take which pathway of maturation remain obscure. Most studies of this process have focused on cytokines and transcription factors, which can control cellular proliferation and differentiation decisions (Rosenbauer and Tenen 2007). MicroRNAs (miRNAs) are a novel class of small, regulatory RNA molecules that play evolutionarily conserved roles in cellular development and function, and mediate target gene repression through 3' untranslated region (UTR) interactions (Ambros, 2004; Bartel and Chen 2004; He and Hannon 2004). Recently, a growing body of evidence has implicated specific miRNAs in the modulation of mammalian hematopoiesis during both physiological and pathological conditions (Georgantas et al. 2007; Kluiver et al. 2006b).

Among miRNAs expressed by hematopoietic cells, *miR-155* has emerged as having significant impact on the biology of lymphocytes (Costinean et al. 2006; Rodriguez et al. 2007; Thai et al. 2007). *MiR-155* is upregulated to high levels in response to B or T cell receptor engagement (Haasch et al. 2002; Van den Berg et al. 2003), and plays a B-cell intrinsic role in germinal center formation and subsequent antibody production *in vivo* following antigen challenge (Rodriguez et al. 2007; Thai et al. 2007). Furthermore, *miR-155* deficient T cells exhibit a Th2 bias, likely through repression of c-Maf. Beyond its apparent contribution to the humoral immune response,

the need to properly regulate *miR-155* levels is suggested by its dramatically elevated expression in several types of human B-cell lymphomas (Eis et al. 2005; Kluiver et al. 2005; Van den Berg et al. 2003), and its reported sufficiency in triggering B-cell lymphoma when overexpressed in a B-cell-restricted manner in mice (Costinean et al. 2006).

In contrast to the emerging picture of *miR-155* functions in lymphocytes, the role or consequences of *miR-155* expression in hematopoietic cells of myeloid origin have been largely uncharacterized. Our group recently reported high levels of *miR-155* expression in cells of the innate immune system, such as monocytes and macrophages, following exposure to inflammatory stimuli (O'Connell et al. 2007; Taganov et al. 2006). As in lymphocytes, *miR-155* expression is tightly regulated in myeloid cells, suggesting a specialized function during times of inflammatory stress. Interestingly, the inflammatory process is known to have a significant impact on hematopoiesis by enhancing production of granulocyte/monocyte (GM) populations in order to replenish those that become depleted while combating infection (Shortman and Naik 2007; Ueda et al. 2005). This developmental reprogramming is driven in part by cytokines and growth factors produced during inflammation, and by direct recognition of pathogen associated molecular patterns such as LPS by mammalian Toll-like receptors (TLRs) expressed on hematopoietic stem and progenitor cell populations (Nagai et al. 2006). Because *miR-155* is part of the TLR-induced gene program, we have examined its potential role in regulating GM expansion in the bone marrow during inflammation.

In the present study, we demonstrate that *miR-155* expression is greatly increased in mouse bone marrow cells after LPS injection and is sufficient to drive

granulocyte/monocyte (GM) expansion when constitutively expressed in mouse HSCs *in vivo*. However, sustained expression of *miR-155* also leads to several features characteristic of pathological myeloid proliferations, correlating with its overexpression in samples from human AML patients. Finally, *miR-155* directly repressed a broad range of target mRNAs implicated in myeloid hyperplasia and/or hematopoiesis. These data suggest an important physiological role for *miR-155* in GM expansion during times of inflammation, yet underscore the importance of its proper regulation for maintaining the balance between an efficient immune response and the potential for inducing malignant disease.

Results

LPS induces bone marrow expression of *miR-155* prior to myeloid expansion *in vivo*.

Although *miR-155* is expressed at low levels in mice under steady-state conditions, we examined whether its expression is elevated in the bone marrow compartment following the onset of inflammation *in vivo*, as seen in cultured macrophages (O'Connell et al. 2007). Mice were injected intraperitoneally with a sub-lethal dose of LPS (50 μg), or PBS control, and their bone marrow cells were analyzed for *miR-155* expression. Strong induction of *miR-155* levels were observed following 24 hours of LPS treatment, which returned to control levels by 72 hours (figure 2-1A). Upregulated *miR-155* expression was also detected upon direct LPS or GM-CSF stimulation of isolated bone marrow cells from Wt or Rag1-/- mice, demonstrating that cells other than mature B and T lymphocytes contribute to this response (figure 2.1B). Furthermore, both populations enriched in mature cells (Mac-1, B220 and Ter119 positive) and those enriched in immature cells (Mac-1, B220 and Ter119 low to negative) responded to LPS by upregulating *miR-155*, with the immature population having a distinctly stronger response than the mature (Supplemental figure 2.S1).

In addition to *miR-155* expression, we also monitored bone marrow cell dynamics in response to LPS *in vivo*. While there was little change in bone marrow GM (Mac1+Gr1+), B-cell (B220+) and erythroid precursor (Ter119+) populations by 24 hours postLPS treatment (Supplemental figure 2.S2), substantial expansion of GM cells and reductions in B-cells and erythroid precursors was evident by 72 hours (figure 2.1C),

consistent with a previous study (Ueda et al. 2005). Histological analyses also showed myeloid preponderance and hyperplasia, with relative erythroid hypoplasia, after 72 hours of LPS treatment (figure 2.1D). Together, these data indicate that LPS-induced *miR-155* expression in the bone marrow precedes GM cell expansion.

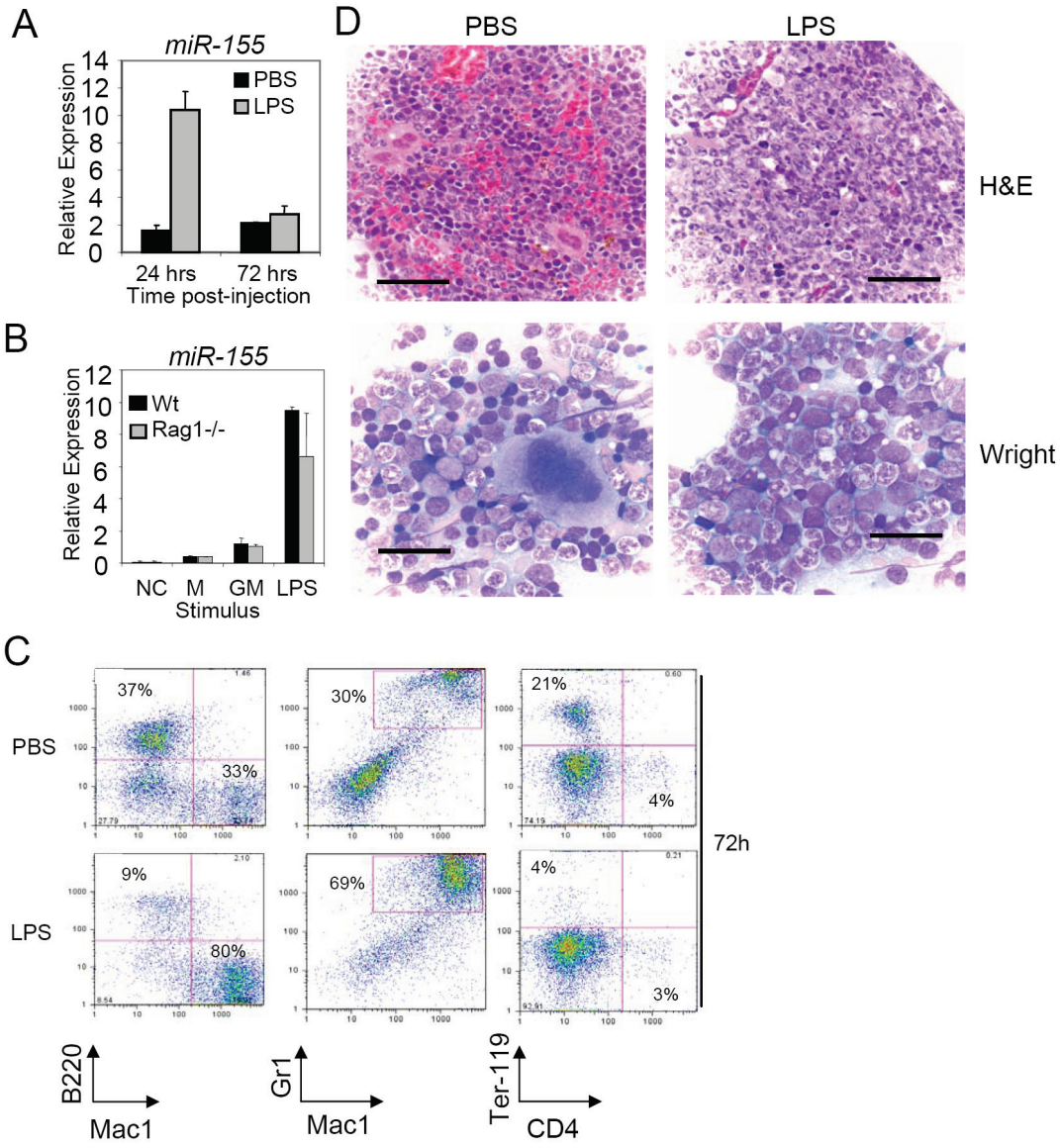


Figure 2.1. LPS treatment induces bone marrow expression of miR-155 prior to GM expansion. (A) Wt mice (n=3 per group) were injected *i.p.* with 50 μg of LPS (dissolved

in PBS), or PBS alone. RNA was collected from total bone marrow cells and *miR-155* expression assayed by qPCR (mean +/-SD). (B) Bone marrow was flushed out of femurs and tibias of Wt and Rag1-/- mice, stimulated with LPS (100 ng/mL), GM-CSF (GM) (100 ng/mL) or medium (M) for 24 hours, and RNA was then assayed for *miR-155* expression levels (mean +/-SD). NC: no template control. (C) BM cells collected from mice in A at the 72 hour time point were stained with antibodies against Mac1, Gr1, B220, Ter-119 or CD4 and analyzed by FACS. (D) Wright-stained bone marrow smears (top, scale bar=60 μm) or H&E-stained bone marrow sections (bottom, scale bar=25 μm) from Wt mice injected with LPS or PBS for 72 hours.

Enforced expression of *miR-155* in hematopoietic stem cells causes a myeloproliferative disorder in the bone marrow.

We next investigated whether *miR-155* is sufficient to mediate GM expansion in the mouse bone marrow *in vivo*. Retroviral-mediated gene transfer was used to force expression of GFP and *miR-155* in HSCs (figure 2.2A), followed by engraftment of these cells into lethally irradiated C57BL6 mouse recipients. By two months postreconstitution, mice were sacrificed and coexpression of *miR-155* and GFP was detected in various hematopoietic tissues, including the bone marrow (figure 2.2B), thymus, spleen, and lymph nodes (Supplemental figure 2.S3). Control mice only expressed GFP but not *miR-155*.

Gross analyses of femurs and tibias from mice expressing *miR-155* revealed a white-tan bone marrow coloration unlike the vibrant red seen in controls (figure 2.2C). Upon microscopic inspection of H&E-stained bone marrow sections and Wright-stained

bone marrow smears (figure 2.2D), *miR-155*-expressing bone marrow was dominated by GM cells at a variety of either normal or abnormal developmental stages based upon their morphology (figure 2.2E). Indeed, many of the cells that appeared to be granulocytic precursors showed irregular segmentation of their nuclei and lacked condensation of nuclear chromatin. Conversely, *miR-155* expression also led to a reduction in erythrocytes, megakaryocytes and lymphocytes in the bone marrow (figures 2.2D and 2.2E).

Flow cytometry identified approximately twice as many CD11b+GR1+ GM cells, very few Ter119+ erythroid precursors and a reduction in B220+ B-cells in the bone marrow of mice expressing *miR-155* versus the control vector (figure 2.2F). When gated on GFP+ cells (expressing *miR-155*), there was a dramatic increase in large, granular cells, as defined by having high forward scatter (FSC) and side scatter (SSC), respectively (figure 2.2G). Back gating confirmed that these cells were Mac1+, with a majority also positive for Gr1. Furthermore, the cells responsible for the overall GM, B and erythroid precursor differences were largely GFP+ (figure 2.2H). These observations reveal profound myeloid proliferation with dysplastic changes in the bone marrow of mice expressing *miR-155* compared to controls.

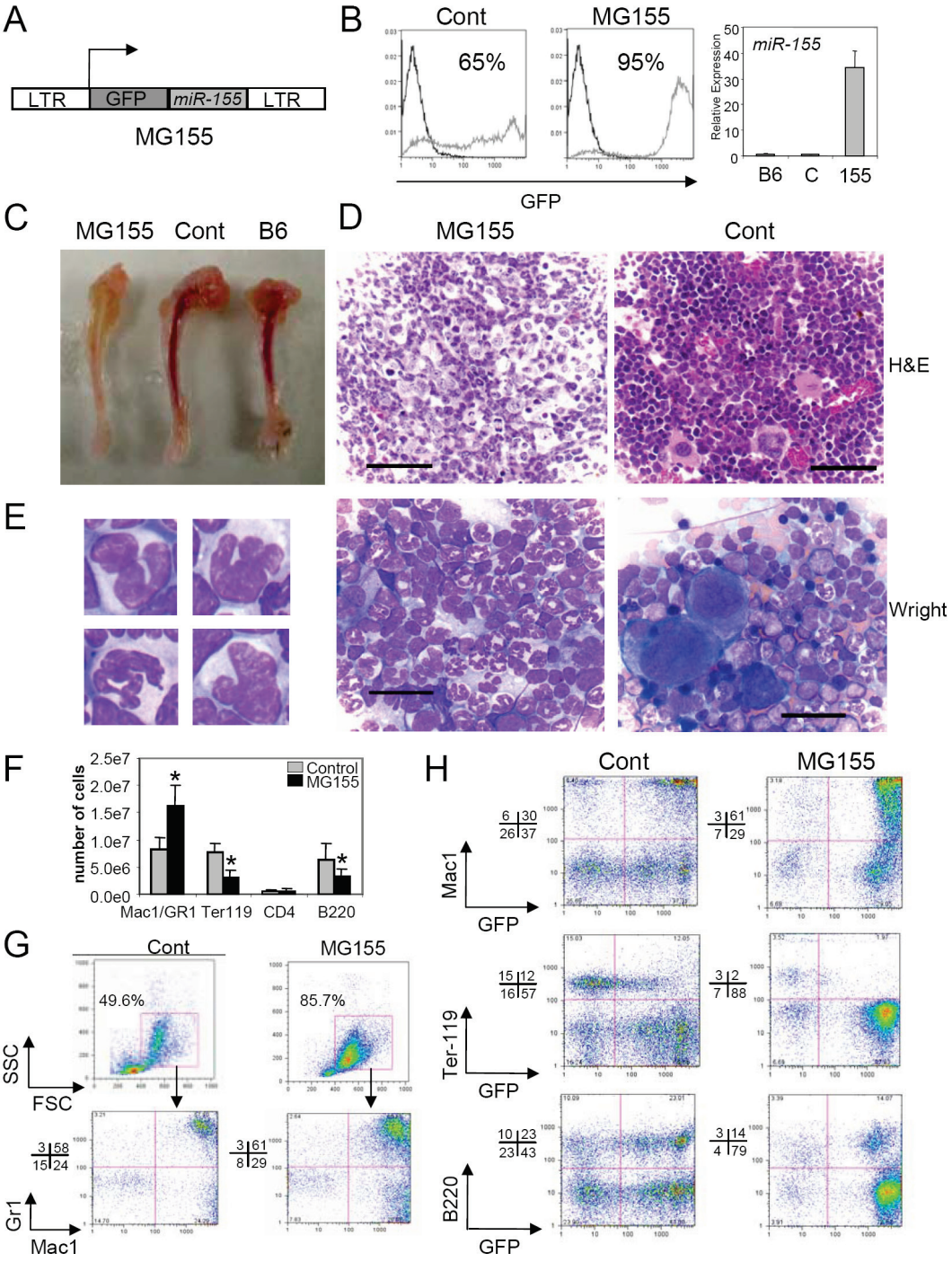


Figure 2.2. Expression of miR-155 in HSCs causes a myeloproliferative disorder in the bone marrow. (A) Depiction of the retroviral construct used to enable both miR-155 and GFP expression in HSCs. (B) Bone marrow cells of mice reconstituted with MG155-

or control vector-infected HSCs were analyzed for their expression of GFP by FACS, where the percentage of GFP positive cells is indicated; black line:C57BL6 control; grey line:MG155 or control vector. Cells from the same compartments were analyzed for miR-155 expression using qPCR (mean +/- SD). (C) Tibias were removed from mice reconstituted with MG155 or control vector HSCs for 2 months, or untreated C57BL6 (B6) mice and photographs were taken. (D) H&E-stained bone marrow sections from miR-155-expressing or control mice (scale bar=60 μm) (E) Wright-stained bone marrow smears from mice reconstituted with MG155 or control vector HSCs (scale bar=25 μm). Examples of dysplastic myeloid cells observed in miR-155-expressing bone marrow are enlarged on the left. (F) Number of specified cell types found in the bone marrow (1 femur+1 tibia) of mice reconstituted with MG155 or control vector HSCs (mean +/- SD). (G) GFP-gated bone marrow cells from mice reconstituted with MG155 or control vector HSCs were analyzed for forward (FSC) and side (SSC) scatter counts and expression of Mac1 and Gr1. (H) Bone marrow cells from mice reconstituted with MG155 or control vector HSCs were analyzed for expression of Mac1, Ter-119 or B220 on both GFP positive and negative cells by FACS. Data represent at least 6 independent animals in each group and *p values of <0.05 were considered significant following a student's 2 tailed t-test.

MiR-155 **expression in hematopoietic stem cells causes splenomegaly and extramedullary hematopoiesis.**

Splenomegaly was observed in *miR-155*-expressing compared to control mice (figure 2.3A). H&E staining of splenic sections from *miR-155*-expressing mice revealed

expanded interfollicular regions containing various hematopoietic elements, as well as constricted and disrupted B-cell follicles compared to control spleens (figure 2.3B). Upon analyses of Wright-stained splenic touch preparations, we observed a large number of erythroid precursors, megakaryocytes and some developing GM cells in the spleens of *miR-155*-expressing mice, while very few of these cell types were observed in control spleens (figure 2.3B). FACS analyses corroborated these observations: we saw elevated numbers of Mac1+Gr1+ myeloid cells and Ter119+ erythroid cells, with little change in CD4+ T cells and B220+ B-cells in *miR-155*-expressing compared to control spleens (figure 2.3C). When gated on GFP+ cells (expressing *miR-155*), there were elevated numbers of large, granular cells, as defined by having high FSC and SSC, respectively, with a majority coexpressing Mac1 and Gr1 (figure 2.3D). Furthermore, *miR-155*-expressing splenocytes contained overall higher numbers of Mac1+ cells that expressed GFP compared to controls (figure 2.3E). Conversely, the Ter-119+ cell population from *miR-155*-expressing spleens was largely negative for GFP, possibly arising from non-transduced HSCs. These results clearly demonstrate the presence of splenic extramedullary hematopoiesis in *miR-155*-expressing mice, likely compensating for the bone marrow defects.

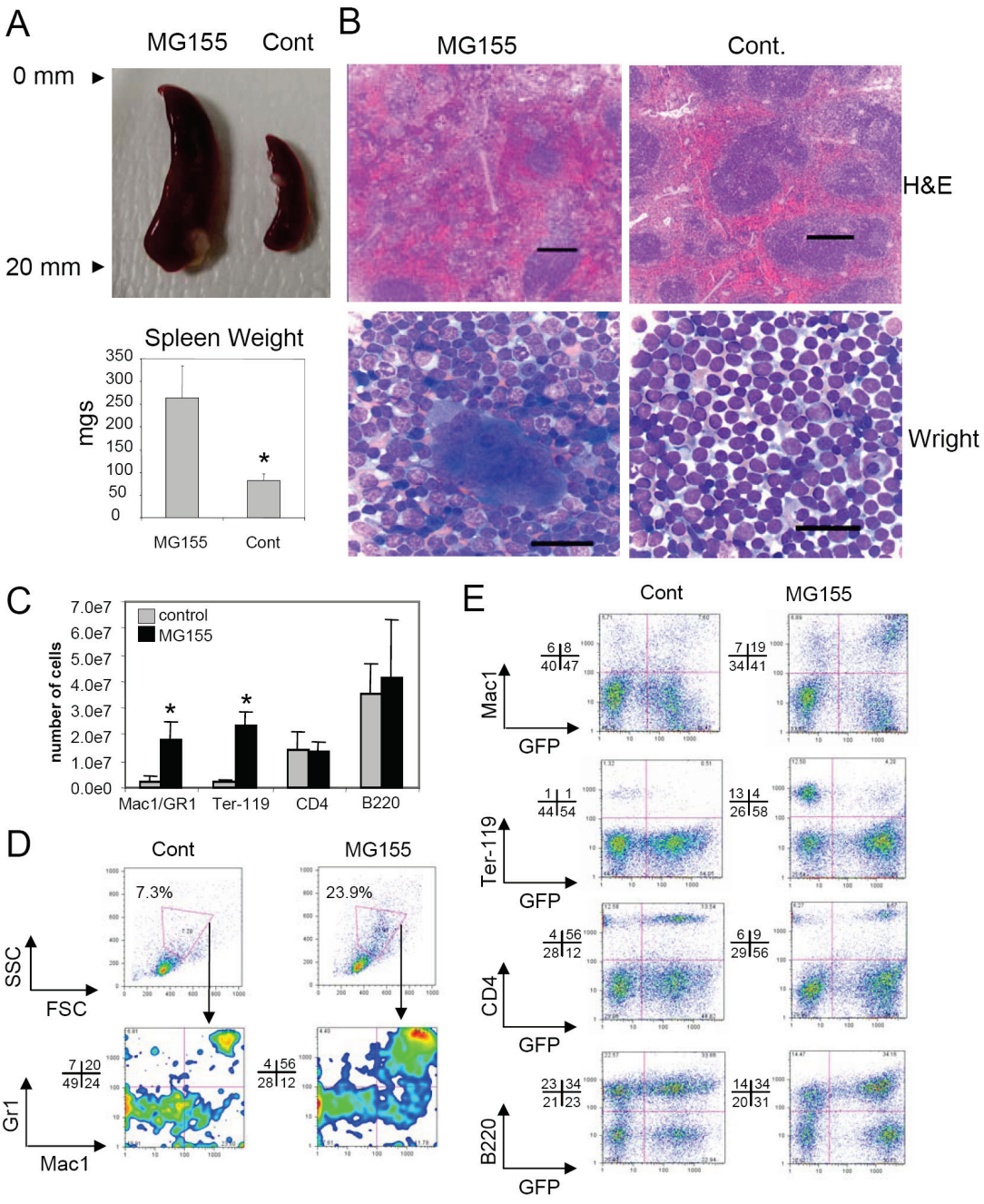


Figure 2.3. Expression of miR-155 in HSCs triggers extramedullary hematopoiesis in the spleen. (A) Spleens were removed from mice reconstituted with MG155 or control vector HSCs for 2 months and photographs were taken (top). Spleen weight was also determined in the two groups (bottom, mean +/- SD). (B) H&E stained sections (top,

scale bar=200 μm) or Wright stained touch preps (bottom, scale bar=25 μm) from mice reconstituted with MG155 or control vector HSCs. (C) Number of specified cell types found in the spleens of mice reconstituted with MG155 or control vector HSCs (mean +/- SD). (D) GFP-gated spleen cells from mice reconstituted with MG155 or control vector HSCs were analyzed for forward (FSC) and side (SSC) scatter counts and expression of Mac1 and Gr1. (E) Splenocytes from mice reconstituted with MG155 or control vector HSCs were analyzed for expression of Mac1, Ter-119, CD4 or B220 on GFP positive and negative cells by FACS. Data represent at least 6 independent animals in each group and *p values of <0.05 were considered significant using a Student's 2 tailed t-test.

Expression of *miR-155* in hematopoietic stem cells perturbs peripheral blood cell populations.

Consistent with the disrupted hematopoiesis observed in *miR-155*-expressing mice, their peripheral blood demonstrated several distinct abnormalities compared to controls. By two months postreconstitution, FACS detected significantly elevated numbers of Mac1+ cells (figure 2.4A), and Wright stained blood smears revealed the presence of morphologically abnormal GM cells in *miR-155* expressing mice (figure 2.4B). Complete blood cell counts showed a significant reduction in red blood cell, hemoglobin, and platelet levels (figure 2.4C), FACS found decreased B220+ B-cells and CD4+ T lymphocytes (figure 2.4C), and Wright staining identified several immature erythrocytes demonstrating polychromatophilia in *miR-155*-expressing animals (figure 2.4D).

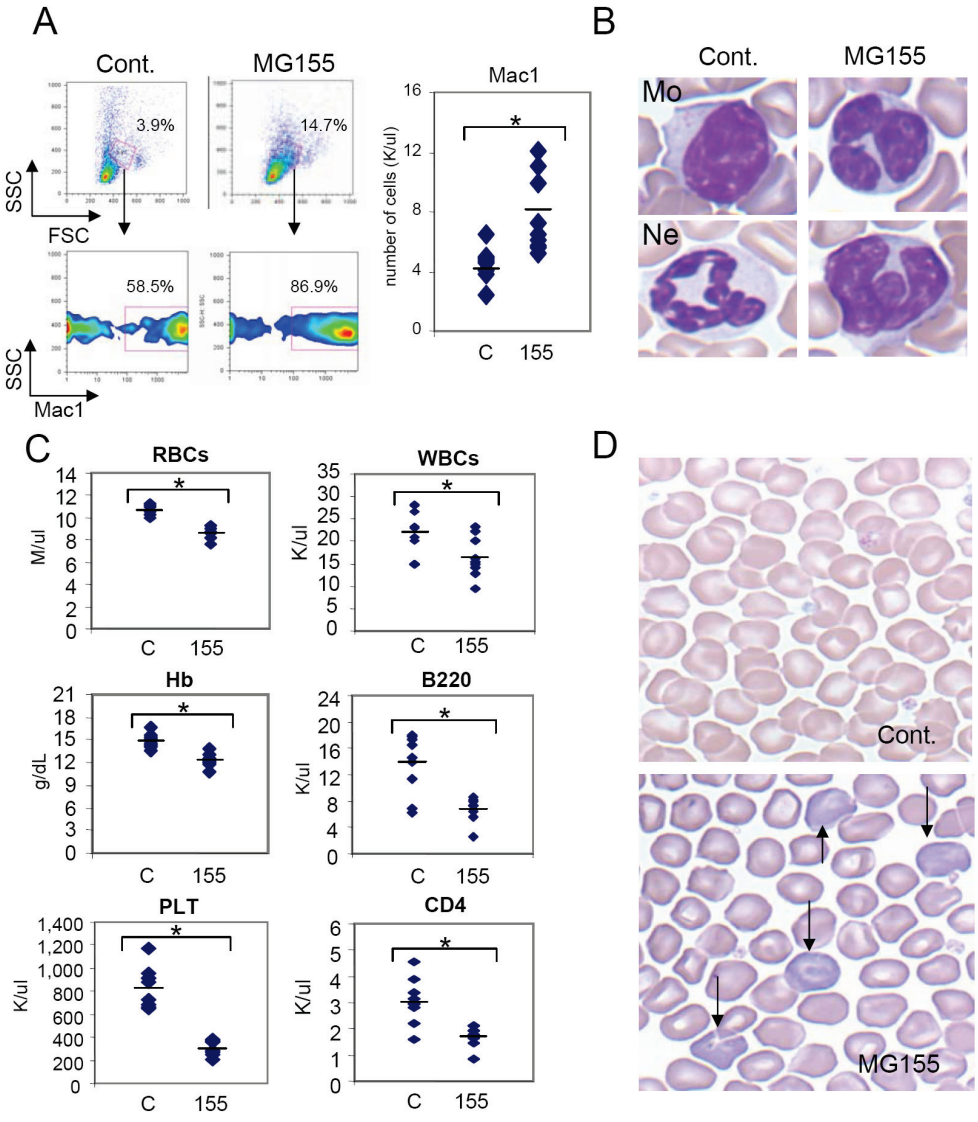


Figure 2.4. Expression of miR-155 in HSCs perturbs peripheral blood cell populations. (A) Peripheral blood was collected from mice reconstituted with MG155 or control vector HSCs for 2 months and analyzed by FACS to determine Forward (FSC) and Side (SSC) Scatter Counts and expression of Mac1. Total Mac1 cells were also determined. (B) Photomicrographs of a normal Wright stained monocyte (Mo) and neutrophil (Ne) from the blood of mice reconstituted with control vector HSCs, and 2 examples of the Wright stained irregular myeloid cells found in MG155 HSC

reconstituted animals. (C) RBC, Hemoglobin (Hb), platelet, WBC, B220 B-cell and CD4 T cell levels in the blood of mice reconstituted with MG155 or control vector HSCs. (D) Microscopic photographs of Wright stained peripheral blood RBCs from mice reconstituted with MG155 or control vector HSCs. Data represent at least 9 independent animals in each group and *p values of <0.05 were considered significant following a student's 2 tailed t-test.

A subset of human AML patients overexpress *miR-155*

Several of the pathological features observed in *miR-155*-expressing mice are associated with human myeloid malignancies, including acute myeloid leukemia (AML). Therefore, bone marrow samples from 24 AML patients and 6 normal people were assayed for *miR-155* and 5S expression levels by quantitative PCR. On average, the AML samples significantly overexpressed *miR-155* compared to healthy donors, with a level approximately 4.5 times higher (figure 2.5A). A few AML samples had *miR-155* levels that were lower than the normal samples, while the overall AML sample distribution had a wide variance. In contrast, no significant difference in the average expression levels of 5S RNA was observed between the groups (figure 2.5A). *MiR-155* levels in different subtypes of AML were next ascertained using the WHO classification system. Patients with acute myelomonocytic leukemia and acute monocytic leukemia, corresponding to FAB-AML-M4 and FAB-AML-M5, respectively, exhibited significant overexpression of *miR-155* compared to normal samples (figure 2.5B). These observations demonstrate that *miR-155* expression in the bone marrow is significantly elevated in a subset of patients suffering from AML.

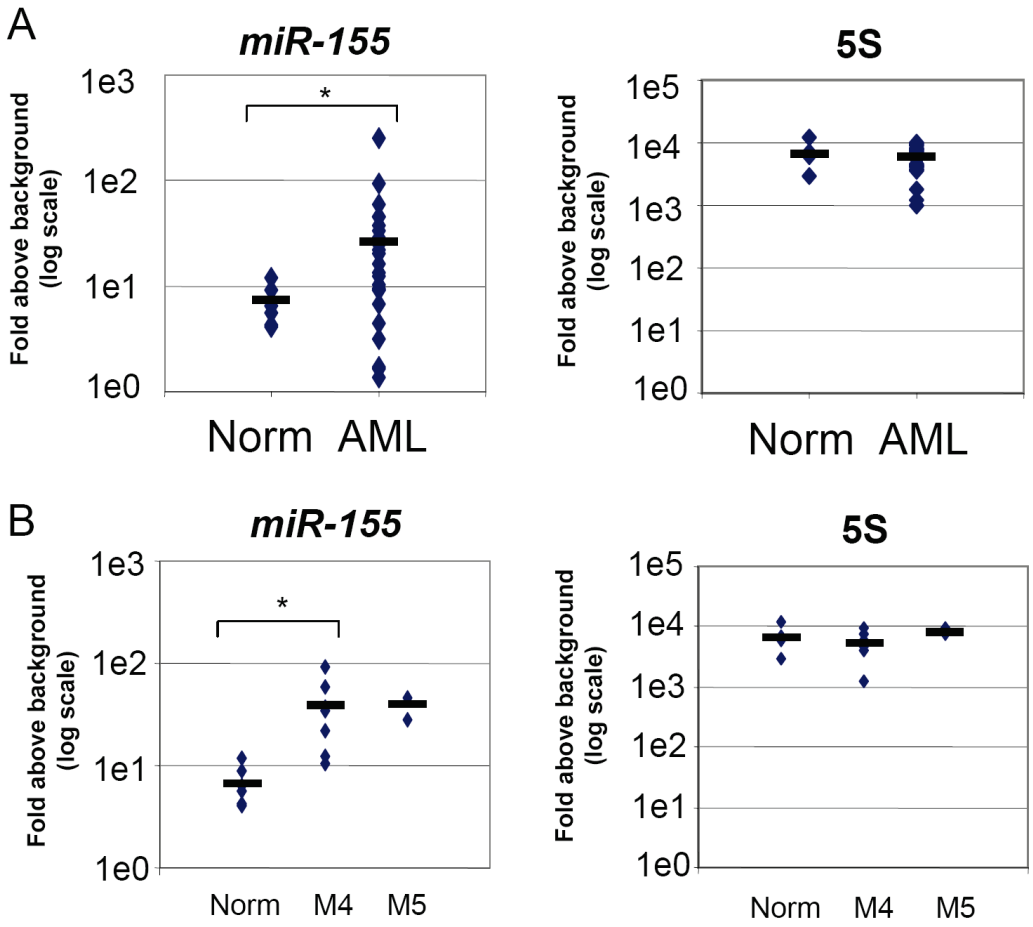


Figure 2.5. Overexpression of miR-155 in a subset of human AML patients. (A) RNA was collected from the bone marrow of 6 normal patients and 24 patients diagnosed with AML. *MiR-155* (left) and 5S RNA (right) expression levels were assessed using qPCR. (B) *MiR-155* and 5S RNA expression data were compared between normal subjects and AML patients of the FAB subtypes M4 and M5. *Group differences were considered statistically significant when the p value was <0.05.

MiR-155 can directly repress genes implicated in hematopoietic development and disease.

MiRNAs exert their biological functions through the degradation and/or translational repression of target mRNAs. To identify *miR-155* target genes that may be involved in the observed myeloproliferative phenotype, we first transduced RAW 264.7 myeloid cells with a *miR-155*-expressing retrovirus that increased *miR-155* cellular levels within 2-fold of those observed following LPS stimulation (Supplemental Figure 2.S4). A mRNA microarray analysis was next performed on RNA samples collected from *miR-155* expressing and control cells to identify genes regulated by *miR-155* (Supplemental Figure 2-S5). Some 1080 transcripts were downregulated more than 1.2 fold with a p value of < 0.08 and 89 of the repressed mRNAs contained conserved (human and mouse) *miR-155* binding sites with 7- or 8-mer seeds in their 3' UTRs according to published lists of computationally predicted target genes found on the TargetScan 4.0 website (Grimson et al. 2007; Lewis et al. 2003). Finally, genes with reported roles in myeloid hyperplasia and/or hematopoiesis were identified through literature searching. Using these criteria, our attention was drawn to 10 candidate targets: Bach1, Sla, Cutl1, Csf1r, Jarid2, Cebpβ, PU.1, Arntl, Hif1α and Picalm (figure 2.6A). To confirm the microarray results, qPCR was performed using gene specific primers: it showed that the mRNAs encoding these proteins were downregulated approximately 20% to 70% in RAW 264.7 cells expressing *miR-155* versus empty vector control (figure 2.6A). We also observed strong repression of Cebpβ, PU.1, Cutl1 and Picalm protein levels in RAW 264.7 cells expressing miR-155 (figure 2.6B).

Next, we tested whether *miR-155* could directly repress the identified mRNA targets through 3' UTR interactions. Each full length 3' UTR, or in 2 cases (Bach1 and Cebpβ) the region of the UTR containing the *miR-155* binding site(s), was cloned into a reporter vector downstream from luciferase. These vectors were then used to assess whether *miR-155* could repress luciferase gene expression in 293T cells. Luciferase expression was repressed between 35% to 78% depending upon the 3' UTR tested (figure 2.7). There was even a rough correlation between the qPCR results in RAW 264.7 cells and the luciferase repression in 293T cells. To demonstrate a direct interaction between *miR-155* and the 3' UTRs tested, we systematically mutated each conserved *miR-155* 7- or 8-mer seed region and found that a majority of the miR-155-mediated repression was abolished (figure 2.7). As a control, *miR-155* repressed a reporter construct containing tandem *miR-155* sites approximately 80%. However, luciferase levels were relatively unaffected when the Traf6 or Irak1 3' UTRs were tested, consistent with their lack of *miR-155* binding sites (figure 2.7). These results provide strong evidence that *miR-155* can directly regulate several genes with relevance to hematopoiesis and the myeloproliferative phenotype.

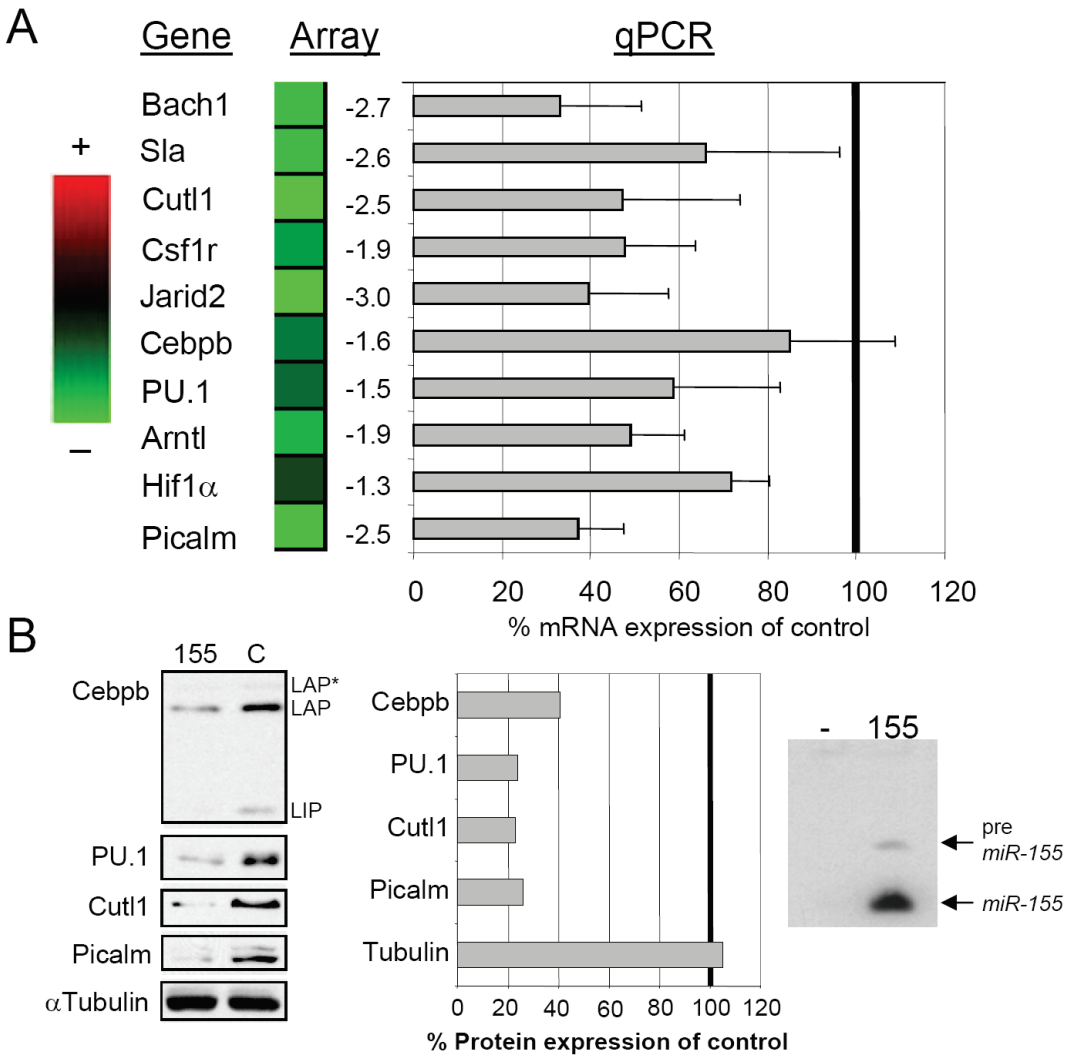


Figure 2.6. Repression of specific target genes involved in myeloid hyperplasia and/or hematopoiesis by miR-155. (A) Messenger RNA from Raw 264.7 cells infected with MSCVpuro-155 or empty vector control was subjected to a microarray analysis and results indicate expression changes mediated by *miR-155*. The intensities of red and green correlate with increased or decreased mRNA levels, respectively, and numerical repression values for each mRNA are listed. RNA from the same cell types were

converted to cDNA and used to assay expression of these genes by qPCR (mean +/- SD). All values have been normalized to L32 mRNA levels, are displayed as percent expression of control, and are the average of 3 independent experiments. (B) Western blotting was performed to assay Cebpβ, PU.1, Cutl1, Picalm or αTubulin using extract from Raw 264.7 cells stably expressing *miR-155* or empty vector, and data are representative of at least 3 independent experiments (left). Expression of *miR-155* in Raw 264.7 cells infected with MSCVpuro-155 or empty vector control was assayed by Northern blotting to ensure proper expression of mature *miR-155*.

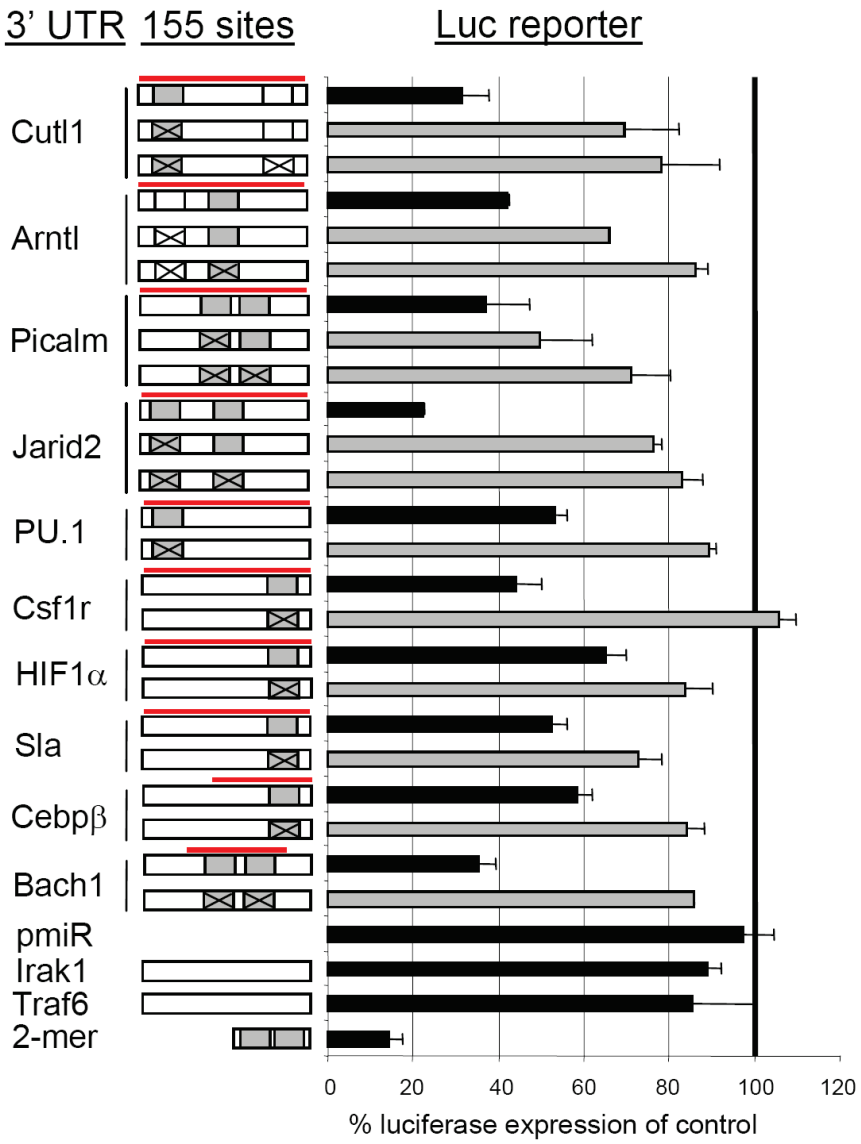


Figure 2.7. MiR-155 repression of specific target genes occurs through direct 3' UTR interactions. (**A**) The 3' UTR regions from identified *miR-155* target mRNAs containing *miR-155* binding site/s with conserved 7- or 8-mer seeds (grey boxes), non-conserved 7-mer seed (Arntl, white box) or conserved 6-mer seed (Cutl1, white box), were cloned downstream from luciferase (pmiReport vector). Mutations to these specific seed regions are marked with an X. The region of the 3' UTR cloned is designated with a

red line, and the cartoon schematics of the UTRs are not drawn to scale. These constructs were used for reporter assays in 293T cells by cotransfection with a control β-galactosidase expression plasmid, and a *miR-155* expression vector (FUW-155) or empty vector control (FUW). A positive control vector contained tandem *miR-155* binding sites, while negative controls contained no 3' UTR or the 3' UTR from Irak1 or Traf6 which lack *miR-155* sites. Data using wt 3' UTRs are in black, while mutant UTRs are in grey. All luciferase values have been normalized to β-galactosidase, and are represented as percent luciferase expression of control (mean +/- SD). All data are a triplicate set representing at least three independent experiments.

Discussion

As cells of the innate immune system combat infectious pathogens, their numbers are often depleted and must be replenished. This process is characterized by an increased production of GM populations, a response shown to involve GM-CSF- and LPS-mediated signaling events (Nagai et al. 2006; Ueda et al. 2005; Zhan et al. 1998). Our present findings demonstrate that *miR-155* is induced by GM-CSF and LPS in the bone marrow compartment, and sufficient to increase the relative and absolute numbers of GM cells when expressed in HSCs and throughout hematopoietic development. This expansion appears to be at the expense of B lymphocytes and erythroid precursors in the bone marrow, as also observed following LPS treatment. Thus, *miR-155* may play a regulatory role in hematopoietic cell fates during times of inflammatory stress when factors like LPS or GM-CSF are present. Because stimulation of most Toll-like receptors induces *miR-155* in macrophages (O'Connell et al. 2007), our results with LPS can probably be generalized to many conditions of microbial invasion.

It remains unclear whether *miR-155* participates in hematopoiesis under steady-state conditions. A recent study detected *miR-155* expression in human CD34+ cells, a population containing HSCs and early progenitors of lymphoid and myeloid lineages (Georgantas et al. 2007). However, defective myeloid populations in *miR-155* deficient mice were not observed when analyzed under steady-state (non-inflammatory) conditions (Rodriguez et al. 2007).

Whether *miR-155* is only sufficient or in fact required for increased myelopoiesis during inflammation, our findings demonstrate that its unregulated expression triggers a myeloproliferative disorder, exhibiting many preleukemic aspects. Because frank

myeloid leukemia was not observed in any of our mice analyzed within 2 months of reconstitution with *miR-155*-expressing HSCs, such a transition may require additional mutations. However, the relevance of our observed phenotype in mice is substantiated by the elevated expression levels of *miR-155* seen in human patients with AML. Interestingly, the two AML subgroups found to be overexpressing *miR-155* are characterized as myelomonocytic (M4) and monocytic (M5), both thought to be derived from cells demonstrating aspects of GM cell differentiation, similar to the expanded GM cells in our *miR-155*-expressing mice. It is of note that *miR-155* may also be elevated in other subtypes of AML where we did not have enough samples to make such a conclusion. If *miR-155* does prove to be dysregulated in specific subtypes of AML, it might complement the recent finding that miR-181 expression positively correlates with M1 and M2 subtypes of AML, but not M4 or M5 (Debernardi et al. 2007). Furthermore, as the full spectrum of miRNAs that become dysregulated during AML is defined, these small RNAs may prove to have utility as diagnostic indicators of AML subtypes.

Despite the similarities mentioned above, certain aspects of the *miR-155*-induced myeloproliferative phenotype were not observed following LPS-mediated acute inflammation, including extramedullary hematopoiesis, GM dysplasia, peripheral blood leukopenia and polychromatophilic RBCs, and reduced megakaryocyte and platelet levels (data not shown). These observations may reflect a differential effect of sustained *miR-155* expression in cell types that require strictly regulated levels of this miRNA. However, chronic inflammation (which may also sustain high *miR-155* expression levels) might trigger some of these pathological events given enough time. For instance, following months of polymicrobial sepsis in mice, there is reported extramedullary

hematopoiesis in the spleen and significantly increased numbers of morphologically heterogeneous GM cells in both the spleen and bone marrow compartments (Delano et al. 2007). The inflammatory response in the bone marrow involves profound myeloid proliferation, and through factors such as *miR-155*, may create a microenvironment suitable for cancer formation and development if not resolved in a timely manner.

Unlike a previous report, which found that B-cell restricted, transgenic expression of *miR-155* triggers B-cell lymphoma in mice (Costinean et al. 2006), we did not observe a B-cell malignancy in our model. This may be explained by differences in the systems used, because our model allows for *miR-155* expression beginning in adult hematopoietic stem cells, which precedes formation of pro-B-cells during hematopoietic development (Rosenbauer and Tenen 2007). These observations suggest that *miR-155* may trigger unique phenotypes when expressed at different stages or in distinct cell type/s during hematopoiesis. There is also evidence that developing B-cells and GMs may occupy an overlapping bone marrow niche. Based upon our current findings, *miR-155* expression may allow for GM progenitors to dominate this compartment and inhibit B-cell development, which has been proposed to occur during inflammation (Ueda et al. 2005). This mechanism might also block events required for *miR-155*-dependent B-cell transformation.

In an effort to explore the mechanistic basis for the myeloproliferative phenotype caused by HSC expression of *miR-155*, we identified several mRNA targets that were directly repressed by *miR-155* according to 3' UTR reporter assays. Of note, the reduced expression or altered function of some of these targets has been linked to AML, as in the case of PU.1 and Picalm (Metcalf et al. 2006; Okada et al. 2006), or myeloproliferative

conditions, as is true for Cutl1 and Csf1r (Fontana et al. 2007; Sinclair et al. 2001). Other identified targets have been implicated in control of various aspects of hematopoiesis involving many of the cell types that are perturbed in mice expressing *miR-155* in HSCs. These include Cebpβ (Hirai et al. 2006), Bach1 (Toki et al. 2005), Arntl (Scortegagna et al. 2003), Sla (Dragone et al. 2006), Jarid2 (Kitajima et al. 1999), and Hif1α (Yoon et al. 2006). Thus, *miR-155* could mediate its overall biological affects, both physiological and pathological, through the combinatorial repression of a broad range of targets in a variety of cell types. Such a multitarget regulation has recently been described for T-cell receptor signaling (Li et al. 2007). Therefore, it is possible that complete rescue of this phenotype will not be achieved through replacing any one of the specific *miR-155* target genes. However, the specific spatial and temporal contribution of individual targets to the myeloproliferative phenotype and AML in the context of miR-155 repression remains an area for future investigation.

There is emerging evidence that individual miRNAs are part of a more complex regulatory network involving other miRNAs and transcriptional regulators that cooperate to govern myelopoiesis. For example, Csf1r is important for monocyte development and has recently been reported to be regulated indirectly by miRNAs 17-5p-20a-106a (Fontana et al. 2007). miRNAs 17-5p-20a-106a repress the transcriptional regulator AML1 required for Csfr1 transcription. Therefore, both miRNAs 17-5p-20a-106a and *miR-155* can influence Csfr1 expression through different mechanisms. In the case of *miR-155* targets PU.1 and Cebpβ, they have been shown to transcriptionally regulate expression of myeloid specific miR-223 (Fukao et al. 2007). miR-223 is subsequently involved in unleashing C/EBPα function, a central transcription factor in myelopoiesis,

through the direct repression of its inhibitor NFI-A (Fazi et al. 2005). Such dynamic systems require appropriate miRNA expression levels and kinetics to carefully orchestrate hematopoietic development, as has been recently described during T cell development in the thymus (Neilson et al. 2007). However, it is easy to see how this delicate process would be vulnerable to dysregulated miRNA expression leading to pathological outcomes. This concept is exemplified by the dysplastic features observed in many GM cells from our *miR-155*-expressing mice. It is possible that while initial *miR-155* expression expands GM numbers, its timely downregulation is necessary for these cells to complete their developmental programs. Such a model would be consistent with the transient expression of *miR-155* that precedes expansion of morphologically normal GM populations in the bone marrow after LPS treatment.

Based upon our current study, *miR-155* appears to play a role in promoting GM cell expansion during inflammatory responses, while initiating pathological processes under forced expression. Due to the enhanced expression of *miR-155* in a subset of AML patients, and its ability to repress several genes relevant to myeloid malignancies, therapeutic targeting of *miR-155* with such agents as antagomirs may provide a beneficial option (Krutzfeldt et al. 2005). Because *miR-155* knockout mice display few detrimental phenotypes in the absence of infection (Rodriguez et al. 2007), decreasing *miR-155* function in human patients suffering from myeloid, lymphoid or other malignancies correlated with enhanced *miR-155* expression may provide more benefits than harm.

Materials and Methods

Cell culture and Reagents

RAW 264.7 and 293T cells were both cultured in complete DMEM containing 10% FBS, 100 units/mL penicillin, and 100 units/mL streptomycin with 5% C02. Murine bone marrow-derived macrophages were made using M-CSF containing media. LPS from *E. coli* strain 055:B5 was purchased from Sigma Aldrich, and recombinant mouse GM-CSF from eBioscience.

DNA constructs

A *miR-155* expression cassette containing the human *miR-155* hairpin sequence and flanking regions was cloned from a B-cell cDNA library into pcDNA3 as described (Eis et al. 2005). This cassette was subcloned into pMSCVpuro, FUW, or pMG. pMG155 is a modified MSCV vector whereby GFP was placed downstream from the 5' LTR, and the *miR-155* expression cassette was cloned downstream from the GFP stop codon (detailed cloning strategy available upon request). For reporter assays, the 3'UTRs of the respective mRNAs were cloned into pmiReport (Ambion) after amplification from a mouse macrophage cDNA library. Primer sequences are described in Supplemental Table 2.1. The Bach1 3' UTR region was amplified from a human B-cell library. Site directed mutagenesis was used to change specific nucleotides found within the *miR-155* seed regions (Supplemental Table 2.2). The 2-mer Control insert consists of a tandem repeat of the complimentary sequence to the mature mouse *miR-155* sequence. Cloning of the TRAF6 and IRAK1 3' UTR into pmiReport was previously described (Taganov et al. 2006).

Mice

Wt C57BL6 mice were purchased from the Jackson Laboratories and Rag1-/- mice were bred in house. All experiments involved female mice and were carried out according to IACUC approved protocols.

Retroviral infections, stable cell lines and bone marrow reconstitution

To generate VsVg pseudotyped retroviruses containing the *miR-155* expression cassette, 2 x 10^6 293T cells were transfected with pMSCVpuro-*miR-155*, pGag-Pol, and pVsVg using a standard calcium phosphate protocol. After 48 hours, viral supernatant was harvested and used to infect 5 x 10^5 RAW 264.7 cells for 8 hours in the presence of polybrene at 10 μg/mL. Following 48 hours, stably transfected cells were selected using puromycin at 7 μg/mL for 7 - 10 days, and *miR-155* expression was assessed at the same time as experiments were performed by Northern blotting or QPCR for all batches made.

To obtain HSC-enriched bone marrow cells, mice were injected intraperitoneally with 5 μg of 5-Fluorouracil for 5 days prior to bone marrow harvest (Yang and Baltimore, 2005). Cells were collected from the bone marrow and RBCs were removed using an RBC lysis solution (Invitrogen). Cells were cultured for 24 hours in IL-3 (20 ng/mL), IL-6 (50 ng/mL) and SCF (50 ng/mL), all from eBioscience, containing complete RPMI (10% FBS, 100 units/mL penicillin, 100 units/mL streptomycin, and 50 μM beta-mercaptoethanol) before initial retroviral infection. To generate retroviruses for infecting HSC-enriched bone marrow cells, 293Ts were transfected with pMG155 and pCL-Eco. After 48 hours, polybrene (8 μg/mL) was added to culture supernatant containing retroviruses and this was used to spin infect 10^6 HSC-enriched cells per donor for 1.5 hours at 2500 RPM and 30 degrees Celsius. This procedure was repeated 3 times

once daily, followed by injection of 10^6 retrovirally infected HSC-enriched cells per lethally irradiated (1100 Rads from Cesium 137 source at 50 Rads/minute) recipient. Recipients were maintained on Septra throughout the reconstitution period.

RNA quantification

Northern blotting and qPCR were used to assay *miR-155* and other mRNAs as described (O'Connell et al. 2007). Gene-specific primer sequences used for qPCR are located in Supplemental Table 2.3. For the microarray study, total RNA was collected from 5 RAW 264.7 stably infected clones expressing *miR-155* or empty vector using the RNeasy Mini Kit per manufacturer's instructions (Qiagen). The microarray analysis was then carried out using pooled RNA from each group by the Millard and Muriel Jacobs Genetics and Genomics Laboratory at Caltech according to their detailed protocols (http://mmjggl.caltech.edu). Data were analyzed using Rosetta Resolver Software.

Western blotting

Western blotting was performed using standard protocols and the following antibody clones from Santa Cruz Biotechnology: Cebpβ (C-19), PU.1 (T-21), Cutl1 (M-222), Picalm (C-18) and αTubulin (AA12). Protein expression differences were determined using Scion Image software.

Flow cytometry and cell separation

Fluorophor-conjugated monoclonal antibodies specific for either Mac1, Gr1, Ter-119, B220, and CD4 (all from eBiosciences) were used in various combinations to stain RBC-depleted splenocytes, bone marrow or peripheral blood mononuclear cells and fixed

after washing using paraformaldehyde (1% final). Stained cells were assayed using a BD FACSCalibur flow cytometer and further analyzed with FloJo software. Cell separation was performed using biotinylated monoclonal antibodies against Mac-1, Ter119 and B220 (eBioscience), streptavidin-conjugated magnetic beads (Miltenyi) and MACS LS Separation Columns (Miltenyi).

Luciferase reporter assays

8×10^4 293T cells were plated in DMEM media containing 5% FBS for 18 hours, followed by transfection of relevant plasmids using Lipofectamine (Invitrogen) per manufacturer's instructions. Luciferase assays were performed 48 hours later using a dual luciferase kit (Promega). A β-gal expression plasmid was cotransfected and β−gal levels were assayed and used to normalize the luciferase values.

Human AML sample collection and analysis

Bone marrow biopsy samples collected from patients with acute myeloid leukemia were flash frozen and stored at -80 degrees Celsius after the completion of diagnostic work in a tissue bank at UCLA. For this study, 24 samples were rapidly thawed and subjected to TRIzol purification of RNA. In addition, 6 RNA samples were isolated from normal volunteers. AML cases were categorized according to the World Health Organization "Classification of Tumors" using anonymous clinical reports. All work performed on these tissues was approved by the Institutional Review Board (IRB) at UCLA.

Morphologic assessment of hematolymphoid tissues

For histologic sectioning, organs were placed into 10% neutral buffered formalin immediately after necropsy, fixed for 12-18 hours, washed and transferred to 70% ethanol prior to standard paraffin embedding, sectioning and staining with H&E. Bones were also decalcified. For cytologic assessment, touch preparations of the cut surface of the spleen were performed. Peripheral blood smears were obtained from the tail vein bleeds or from the heart at necropsy. Bone marrow smears were prepared from extracted bone marrow of reconstituted mice. All cytologic preparations were air dried, and stained with Wright's stain. Both histologic and cytologic preparations were examined on an Olympus BX-51 microscope, and photographed using a Spot Digital Camera and software. Complete blood cell counts were performed at UCLA's Department of Laboratory Animal Medicine.

Statistical tests

All statistical analyses were performed using Microsoft Excel statistical software module. For patient samples, an F-test determined that the distributions of *miR-155* expression in normal samples versus AML samples were heteroscedastic ($p=4.5x10^{-6}$ for F-test). Similarly, the distributions of *miR-155* expression in normal versus AML-M4 was determined to be heteroscedastic ($p=1.8x10^{-5}$ for F-test). Following this, a two-tailed T-test was performed assuming heteroscedastic distributions for both comparisons. For all other data, a student's two-tailed T-test was used.

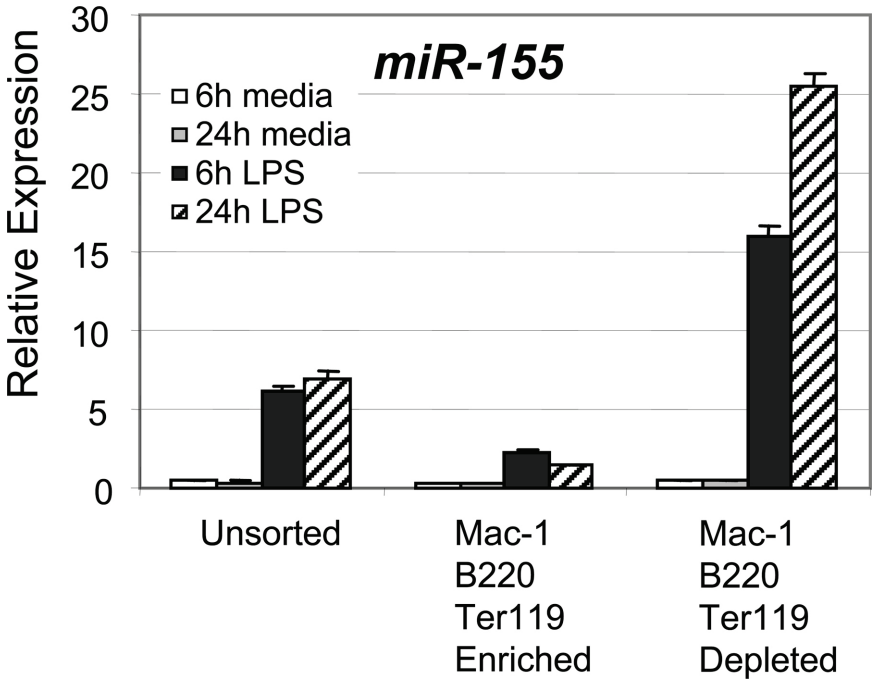


Figure 2.S1. Mature cell–depleted bone marrow populations up-regulate miR-155 to higher levels in response to LPS than mature cell–enriched populations. Mature cell–enriched populations were positively selected from mouse bone marrow using biotinylated antibodies against Mac-1, B220, and Ter119, and streptavidin-conjugated magnetic beads and a MACS column. Mature cell–depleted populations were negatively selected using the same column. FACS was used to verify the presence or absence of lineage marker expression. Cells were stimulated with medium or LPS (100 ng/mL) for 6 or 24 h, followed by miR-155 expression quantification by quantitative PCR.

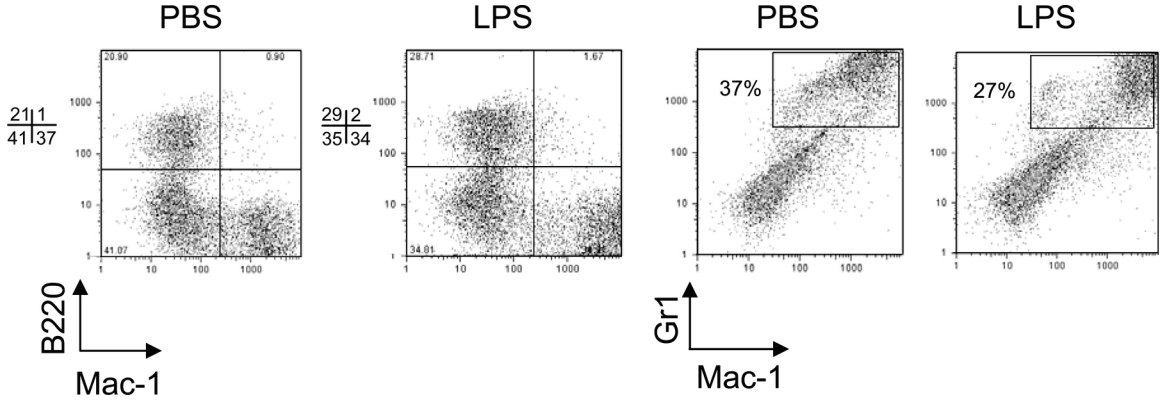


Figure 2.S2. Bone marrow cell dynamics after 24 h of LPS treatment. C57BL6 mice were injected i.p. with 50 μg LPS or PBS for 24 h. B220, Gr1, or Mac1 surface expression was assayed on RBC-depleted bone marrow cells using FACS.

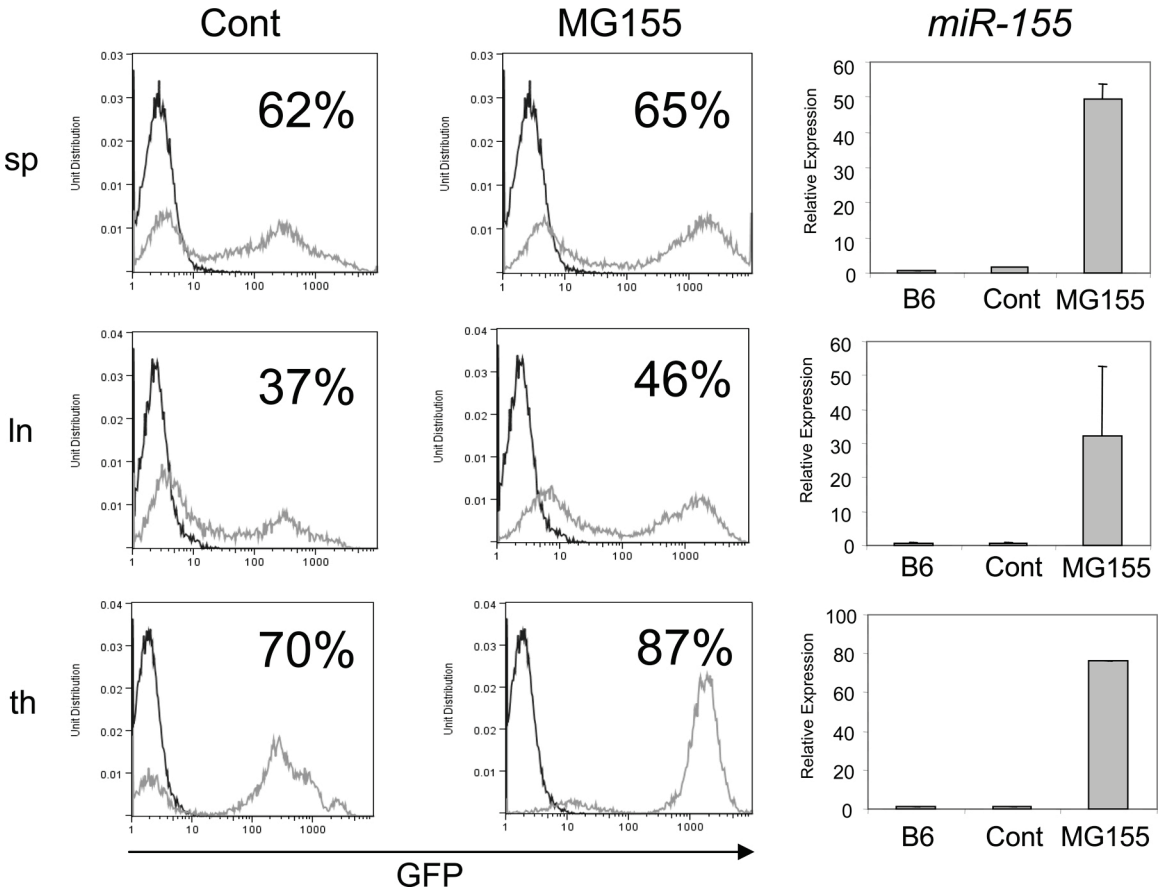


Figure 2.S3. Coexpression of GFP and miR-155 in several lymphoid organs. RBC-depleted cells from the spleen (sp), lymph nodes (ln), and thymus (th) of mice reconstituted with MG155- or control vector–infected HSCs were analyzed for their expression of GFP by FACS, where the percentage of GFP+ cells is indicated. Black line, C57BL6 control; gray line, MG155 or control vector. Cells from the same compartments were analyzed for miR-155 expression using quantitative PCR.

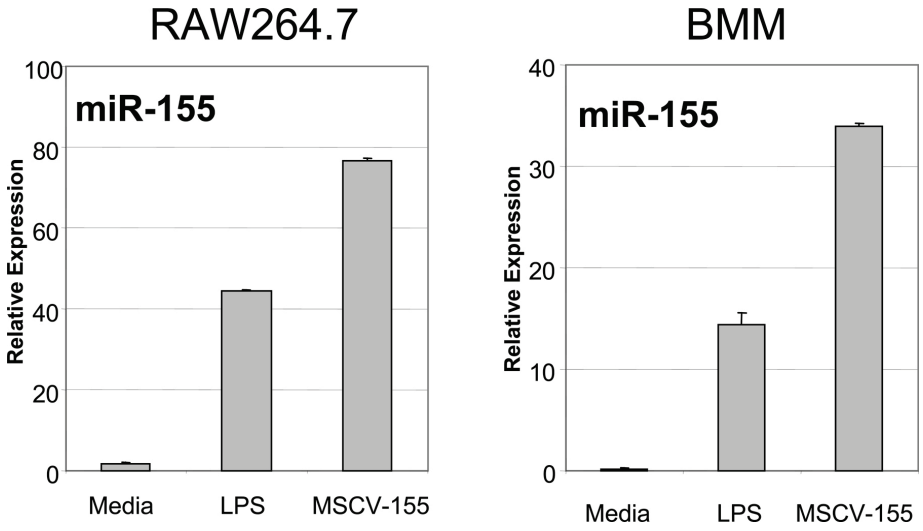


Figure 2.S4. Comparative expression of miR-155 induced by LPS or produced from MSCV-155 in myeloid cells. MiR-155 expression was assayed in (left) Raw264.7 cells stably transfected with MSCV and treated with media or 50 ng/mL LPS, stably infected with MSCV-155 or (right) bone marrow–derived macrophages (BMMs) generated from MG-infected bone marrow treated with media or LPS (5 ng/mL), or generated from MG155-infected bone marrow.

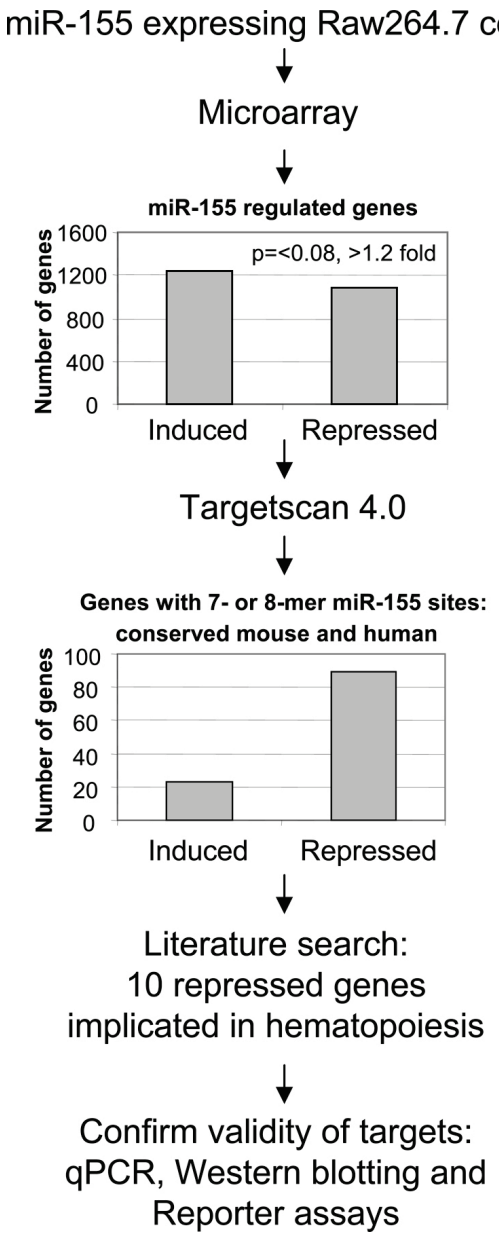


Figure 2.S5. miR-155 target identification and analysis scheme.

Table 2.S1. Primer sequences used for cloning each respective 3'UTR into pMIR-Report.

Primer Name	Sequence
Bach1 F	CCAGAGCTTAAATATAATTTGTAAAGC
Bach1 R	ACATTGAGAAGGCCAGTTCATAA
Sla F	GTAACTAGTTGACCTGGCTTGTACACACAC
Sla R	GTTAAGCTTTAAATACATGATTTGGCAAAGTGTAA
Cutl1 F	TCAAGAGCTCGGCAAAATCGCCATAGGC
Cutl1 R	AGCTACGCGTCCCTTCCTAACAATCAGATTAATAAAAT
Csf1r F	GTAACTAGTTCCTGCCGCTCTCTACGT
Csf1r R	GTTAGCTTCTGGCTGTGTTAATGCTGTTAGTT
Jarid2 F	GTAACTAGTAGATGCCGAACCCATGGT
Jarid2 R	GTTAAGCTTATGAAGAGAAAAAATAGACAAGAGGA
Cebpb F	GTAACTAGTTGCAATCCGGATCAAACG
Cebpb R	GTTAAGCTTGGCTTTTAAACATTCTCCAAAAAA
PU.1 F	GTAACTAGTCCGGCCATAGCATTAACC
PU.1 R	GTTAAGCTTGGGAGAATAGCTGTCAATAATTTTACT
Arntl F	GTAACTAGTACACTACATTTGCTTTGGCAAC
Arntl R	AGCTACGCGTAGAACAAGGGAAACATTTATTAAAAAT
Hif1a F	TCAAGAGCTCCTGAGCGTTTCCTAATCTCATTC
Hif1a R	AGCTACGCGTCCTGGTCCACAGAAGATGTTT
Picalm F	TCAAGAGCTCATGGAAGAGAATGGAATTACTCCA
Picalm R	GTTAAGCTTTGTTTTGTGGAAGCTGCATT

Table 2.S2. MiR155 seed sequences found in target gene 3'UTRs and subsequent mutations induced to disrupt miR-155-mediated repression

miR155 seed	Wt	Mutant
Bach1 1	AGCATTAAAG	**GTAA**AA
Bach1 2	AGCATTA	AG**GTAA**A
PU.1	AGCATTAAAG	**GTAA**AA
Cutl1 1	AGCATTAAAG	**GTAA**AA
Cutl1 2	GCATTA	GC**TAA**A
Picalm 1	GCATTAA	G**G**A**G**T**G**A
Picalm 2	AGCATTA	AGC**TAA**A
Arntl 1	GCATTAA	GCA**AAT**A
Arntl 2	GCATTAA	GC**TAA**AA
Csf1r	AGCATTAAAGCA	**AAT**A
Sla	AGCATTA	AGCA**AAT**
Arntl 1	GCATTAA	GCA**AAT**A
Jarid2 1	AGCATTAAA	**CGTAAT**A
Jarid2 2	AGCATTAAAGCA	**AAT**A
Hif1a	AGCATTA	AGCA**AAT**

Mutated nucleotides are in bold.

Table 2.S3. Primer sequences used for quantitative PCR

Primer Name	Sequence
Bach1 F	TGAGTGAGAGTGCGGTATTTGC
Bach1 R	GTCAGTCTGGCCTACGATTCT
Sla F	ATGGGGAATAGCATGAAATCCAC
Sla R	GGAGATGGGTAGTCAGTCAGC
Cutl1 F	CGCAGAGAACTGTTCATTGAGG
Cutl1 R	GAGCTGAAGGTGAGTCGCT
Csf1r F	TGTCATCGAGCCTAGTGGC
Csf1r R	CGGGAGATTCAGGGTCCAAG
Jarid2 F	GAAGGCGGTAAATGGGCTTCT
Jarid2 R	TCGTTGCTAGTAGAGGACACTT
Cebpb F	GACAAGCACAGCGACGAGTA
Cebpb R	AGCTGCTCCACCTTCTTCTG
PU.1 F	ATGTTACAGGCGTGCAAAATGG
PU.1 R	TGATCGCTATGGCTTTCTCCA
Arntl F	ACCACAGGAACTTCTAGGTACAT
Arntl R	GGACATTGGCTAAAACAACAGTG
Hif1a F	ACCTTCATCGGAAACTCCAAAG
Hif1a R	ACTGTTAGGCTCAGGTGAACT
Picalm F	GTCTGTCCACGCCATGTCG
Picalm R	TAGCAGAGAAAGGATCTCCCC

CHAPTER 3: Discovering a primary target of miR-155: The SHIP1 inositol phosphatase

 Published as: RM O'Connell, AA Chaudhuri, DS Rao, and D Baltimore (2009). Inositol phosphatase SHIP1 is a primary target of miR-155. *Proceedings of the National Academy of Sciences USA*. **106**(17):7113-7118. Epub 2009 Apr 9.

Abstract

MicroRNA-155 (miR-155) has emerged as a critical regulator of immune cell development, function and disease. However, the mechanistic basis for its impact on the hematopoietic system remains largely unresolved. Because miRNAs function by repressing expression of specific mRNAs through direct 3'UTR interactions, we have searched for targets of miR-155 implicated in the regulation of hematopoiesis. In the present study, we identify Src homology 2 domain-containing inositol 5-phosphatase 1 (SHIP1) as a direct target of miR-155, and show using gain and loss of function approaches that miR-155 represses SHIP1 through direct 3'UTR interactions that have been highly conserved throughout evolution. Repression of endogenous SHIP1 by miR-155 occurred following sustained overexpression of either mouse or human miR-155 in hematopoietic cells both *in vitro* and *in vivo*, and resulted in increased activation of the kinase Akt during the cellular response to LPS. Furthermore, SHIP1 was also repressed by physiologically regulated miR-155, which was observed in LPS treated wild-type (wt) versus miR-155$^{-/-}$ primary macrophages. In mice, specific knockdown of SHIP1 in the hematopoietic system following retroviral delivery of a miR-155 formatted siRNA against SHIP1 resulted in a myeloproliferative disorder (MPD) with striking similarities to that observed in miR-155 expressing mice. Our study unveils a molecular link between miR-155 and SHIP1 and provides evidence that repression of SHIP1 is an important component of miR-155 biology.

Introduction

In recent years, microRNAs have emerged as critical regulators of gene expression in a variety of mammalian cell types, including cells of the immune system (Baltimore et al. 2008; Bartel and Chen, 2004; Lodish et al. 2008). Through their ability to repress expression of specific target genes via direct 3'UTR interactions, several miRNAs have been shown to impact both physiological and pathological immune processes (Costinean et al. 2006; He et al. 2005; Johnnidis et al. 2008; O'Connell et al. 2008; Rodriguez et al. 2007; Thai et al. 2007; Ventura et al. 2008; Xiao et al. 2007; Xiao et al. 2008). Among the most prominent and well-studied immune system miRNAs to date, miR-155 clearly is involved in protective immunity when properly regulated, yet contributes to malignant conditions upon its dysregulated expression.

MiRNA-155 is expressed in a variety of immune cell types including B-cells (Eis et al. 2005; Fulci et al. 2007; Kluiver et al. 2005; Rodriguez et al. 2007; Tam et al. 1997; Thai et al. 2007; Van den Berg et al. 2003), T cells (Haasch et al. 2002), macrophages (O'Connell et al. 2007; Taganov et al. 2006), dendritic cells (Rodriguez et al. 2007) and progenitor/stem cell populations (Georgantas et al. 2007; O'Connell et al. 2008). Interestingly, miR-155 is found at low levels in most of these cells types until their activation by immune stimuli such as antigen, Toll-like Receptor ligands and/or inflammatory cytokines, which rapidly increase miR-155 expression (Haasch et al. 2002; O'Connell et al. 2007; Rodriguez et al. 2007; Taganov et al. 2006; Thai et al. 2007). Consistent with its expression pattern, miR-155 appears to function in hematopoiesis and the immune response (O'Connell et al. 2008; Rodriguez et al. 2007; Thai et al. 2007). For example, defective germinal center (GC) formation and antibody isotype class switching

have been observed in miR-155$^{-/-}$ mice following infection or vaccination (Rodriguez et al. 2007; Thai et al. 2007; Vigorito et al. 2007). In T cells, miR-155 promotes skewing toward the Th1 subset (Rodriguez et al. 2007; Thai et al. 2007). In dendritic cells, miR-155 is necessary for proper activation of responder T cells in the context of antigen presentation (Rodriguez et al. 2007).

Enhanced expression of miR-155 occurs constitutively in a subset of cancer cells of lymphoid (Eis et al. 2005; Fulci et al. 2007; Kluiver et al. 2005; Tam et al. 1997; Van den Berg et al. 2003) and myeloid origin (Garzon et al. 2008; O'Connell et al. 2008). We and others have recently demonstrated that sustained expression of miR-155 in the hematopoietic system leads to pathological outcomes. Our group expressed miR-155 ubiquitously in the hematopoietic compartment via bone marrow transfer of HSCs infected with a retroviral vector. This caused a myeloproliferative disorder (MPD) characterized by increased granulocyte/monocyte (GM) populations in the bone marrow, peripheral blood and spleen, impaired erythropoiesis, and severe splenomegaly due to extramedullary hematopoiesis (O'Connell et al. 2008). Costinean et al. found that transgenic expression of miR-155 from a B-cell specific promoter can trigger a B-cell malignancy (2006).

While much has been learned about miR-155 expression patterns and functions, the molecular basis underlying its biology is relatively uncharacterized. As a result, we have been searching for novel targets of miR-155 implicated in overlapping biological processes. Using microarray technology, bioinformatics, and an extensive review of the literature we have identified the inositol phosphatase SHIP1 as a target of miR-155. Through both gain and loss of function approaches we demonstrate that miR-155

represses SHIP1 through direct 3'UTR interactions during both sustained and physiological expression of miR-155. Furthermore, specific knockdown of SHIP1 in the hematopoietic system using a miR-155 formatted siRNA against SHIP1 largely recapitulated the MPD phenotype we previously described in miR-155 expressing mice. Together, our data demonstrate a novel molecular link between miR-155 and SHIP1 in the immune system, and suggest that repression of SHIP1 is a critical aspect of miR-155 function.

Results

MicroRNA-155 represses SHIP1 through 3'UTR interactions

We previously performed a mRNA microarray analysis using RAW 264.7 macrophages stably expressing miR-155 to identify possible targets of miR-155 (O'Connell et al. 2008). Among the targets was SHIP1 (INPP5D), a gene that is repressed by miR-155 and which has a conserved 8-mer target "seed" in its 3'UTR (figure 3.1A). SHIP1 was of particular interest because miR-155 is the only miRNA with a highly conserved binding site located in the SHIP1 3'UTR according to the TargetScan algorithm (Lewis et al. 2005), and because mice deficient in SHIP1 suffer from a myeloproliferative condition resembling that which we previously described for mice expressing miR-155 (Harder et al. 2004; Helgason et al. 2003, 1998; Liu et al. 1999; O'Connell et al. 2008).

To directly test whether miR-155 can repress SHIP1 through direct 3'UTR interactions, we cloned the 3'UTR of SHIP1 into a reporter plasmid downstream from luciferase and performed reporter assays using 293T cells. While miR-155 produced from a cotransfected plasmid repressed expression of luciferase fused to the wild-type (wt) SHIP1 3'UTR, it failed to repress the SHIP1 3'UTR containing a mutated miR-155 seed sequence (figure 3.1B). As controls, miR-155 repressed the Picalm 3'UTR and 2-mer control constructs, but not the control UTR without miR-155 sites (figure 3.1B). These data reveal that miR-155 directly targets the SHIP1 3'UTR leading to repressed expression.

To determine whether miR-155 can repress endogenous SHIP1, we assayed SHIP1 expression in RAW 264.7 cells expressing wt human miR155, human miR-155 containing a mutated seed region, or vector control. SHIP1 was measured at the mRNA and protein levels by qPCR and Western blotting, respectively. Wt miR-155 repressed SHIP1 mRNA and protein below control levels, while the miR-155 seed mutant had little impact on SHIP1 expression compared with the vector control (figure 3.1C and 3.1D). The wt miR-155 was overexpressed in cells receiving the wt miR-155 vector (figure 3.1E) and the mature miR-155 seed mutant was produced in cells receiving the miR-155 seed mutant vector (figure 3.1F), indicating that the specificity of the repression was determined by the seed region of miR-155.

SHIP1 is well known to be a negative regulator of the kinase Akt, a downstream target of the phosphatidylinositol-3-kinase (PI3K) pathway. Therefore, we assayed Akt activation following LPS treatment of the different RAW 264.7 derivatives. Consistent with reduced SHIP1 levels, cells expressing wt human miR-155 exhibited increased activation of Akt following LPS treatment, while Akt activation was similar in the control and human miR-155 seed mutant expressing cells (figure 3.1G).

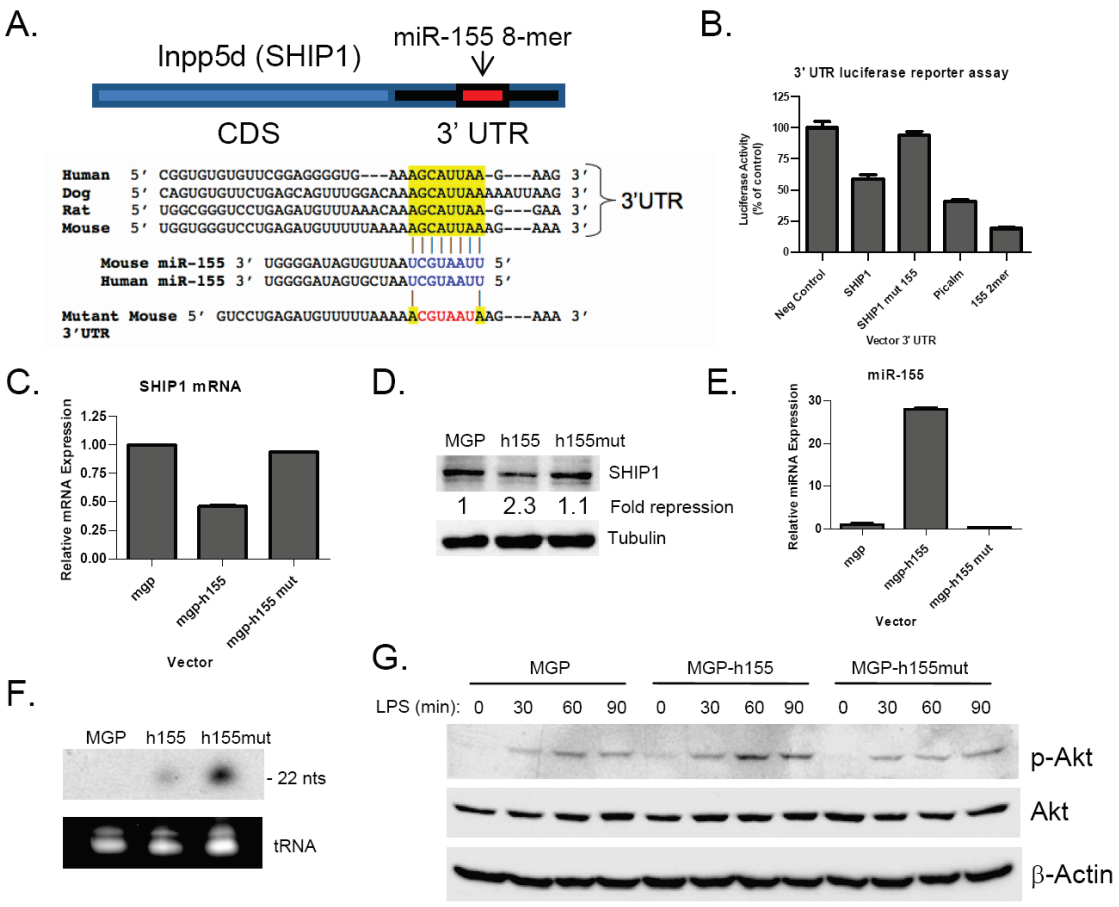


Figure 3.1: MicroRNA-155 represses SHIP1 expression through 3'UTR interactions

A. Schematic layout of the SHIP1 mRNA coding sequence (CDS) and 3'UTR, with the relative location of the miR-155 binding site. Depiction is not to scale. Sequence of mouse and human miR-155 and predicted interaction with conserved 8-mer miR-155 seeds found within the SHIP1 3'UTRs from different species (highlighted) are shown. The sequence of the SHIP1 3'UTR seed mutant used for reporter assays and predicted disruption of the miR-155 interaction is also shown. B. Luciferase reporter assays were performed by transiently transfecting 293T cells with an empty plasmid (FUW) or miR-155 expressing plasmid (FUW-155), the indicated 3'UTR

luciferase reporter plasmids, and a plasmid producing beta-galactosidase. Luciferase values have been normalized to beta-galactosidase, and the percent repression in cells transfected with miR-155 is presented. Raw 264.7 cells stably infected with a retroviral vector expressing wt human miR-155 (MGP-h155), mutant seed human miR-155 (MGP-h155mut), or control (MGP) were assayed for SHIP1 levels by qPCR (C) and Western blotting (D). As a loading control for the Western blot, αTubulin was also assayed. The fold repression of SHIP1 by the different constructs is shown. E. Levels of mature human miR-155 in the different cell types were assayed by qPCR with primers that detect the wt mature miR-155 sequence. F. RNA from the different cell types and a probe specific for the human miR-155 seed mutant sequence was used for Northern blotting. G. The different cell types were stimulated with LPS (200 ng/mL) over the indicated time course and Ser-473-phosphorylated-Akt (p-Akt), total Akt and β-Actin were assayed by Western blotting. Data represent at least two independent experiments.

Enhanced expression of SHIP1 in miR-155$^{-/-}$ macrophages following LPS treatment

We next evaluated whether SHIP1 is regulated by miR-155 under physiological conditions. To achieve this we generated bone marrow derived macrophages (BMMs) from either wt or miR-155$^{-/-}$ mice. As LPS has been shown to be a potent inducer of miR-155 in macrophages (O'Connell et al. 2007), we stimulated these cells with LPS over a time course. In wild-type cells, rapid induction of the miR-155 precursor, BIC, was followed shortly after by elevated expression of mature miR-155 (figure 3.2A and 3.2B), as previously seen with poly (I:C) treated BMMs (O'Connell et al. 2007). We also noticed that while BIC levels fall considerably by 24h, miR-155 expression peaks at this time point. Protein levels of SHIP1 were assayed at 0h, 4h and 24h after LPS treatment of both wt and miR-155$^{-/-}$ BMMs. We observed no change in SHIP1 protein levels up to 4h

following LPS treatment in cells of both genotypes (figure 3.2C). After 24h, both Wt and miR-155$^{-/-}$ BMMs demonstrated an increase in SHIP1 expression compared with earlier time points. However, miR-155$^{-/-}$ BMMs had an enhanced level of SHIP1 protein compared to wt control cells at this time point (figure 3.2C). SHIP1 mRNA levels reflected similar differences between wt and miR-155$^{-/-}$ BMMs following 24h of LPS treatment (figure 3.2D). These results are consistent with miR-155 repressing SHIP1 expression after their induced coexpression by LPS, and demonstrate that this can occur under physiologically relevant conditions in primary cells.

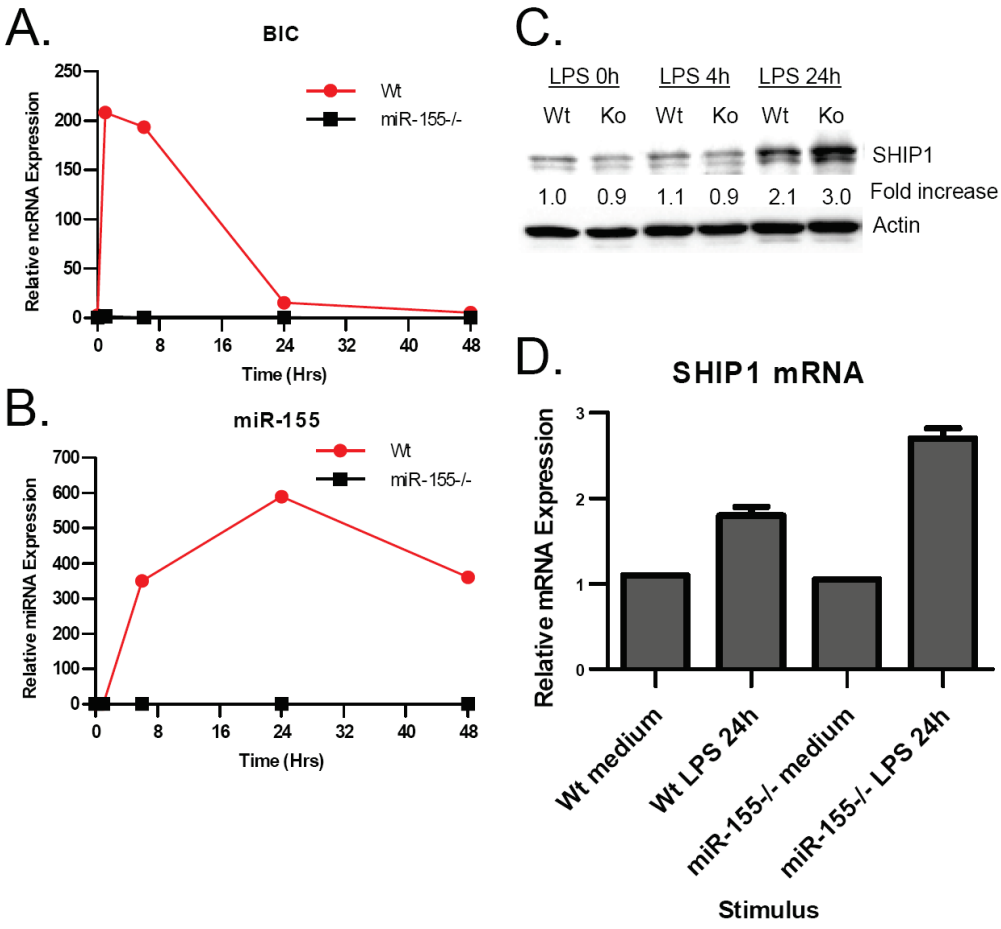


Figure 3.2: Enhanced expression of SHIP1 in miR-155-/- macrophages following LPS treatment

Bone marrow derived macrophages from Wt or miR-155$^{-/-}$ mice were stimulated with 10 ng/mL LPS from *E.coli* for the indicated periods of time. Expression of BIC (A) or mature miR-155 (B) was assayed by qPCR. Expression of SHIP1 was assayed in BMMs by Western blotting (C) and qPCR (D). β−Actin was assayed as a loading control for the Western blot, while qPCR data was normalized to L32. The fold increase in SHIP1 expression versus the wt 0h sample is shown. Data represent at least two independent experiments.

Knockdown of SHIP1 *in vivo* using siRNA in the context of miR-155 processing

Having identified SHIP1 as a direct target of miR-155, we next determined if specific knockdown of SHIP1 levels could recapitulate the miR-155 MPD phenotype in mice that we recently described (O'Connell et al. 2008). Although this phenotype is predicted by the MPD observed in SHIP1$^{-/-}$ mice (Helgason et al. 2003; 1998; Liu et al. 1999), we wanted to perform a specific reduction in SHIP1 using the same retroviral vector and bone marrow reconstitution context as we used to promote sustained miR-155 expression in the hematopoietic system. To accomplish this, we built a retroviral vector that expresses a miR-155 formatted SHIP1 siRNA cassette (figure 3.3A). The cassette is driven by a RNA Polymerase II promoter and the hairpin arms and loop are comprised of mouse miR-155 sequences, while the stem structure contains an antisense sequence designed to target the SHIP1 coding sequence (figure 3.3A). Knockdown of other genes *in vitro* has been shown using this approach (Chung et al. 2006). Following construction

of this vector, we stably infected RAW 264.7 cells and assayed SHIP1 expression. SHIP1 protein levels were markedly reduced in cells expressing the siRNA cassette, compared to the control vector (figure 3.3B).

We next tested whether we could achieve knockdown of SHIP1 expression *in vivo* by expressing miR-155 or the siRNA against SHIP1. To this end, HSC enriched bone marrow cells were infected with retroviral vectors encoding miR-155, siSHIP1 or controls and used to reconstitute lethally irradiated mice as we previously described for delivery of miR-155. Following 2 months of reconstitution, we analyzed SHIP1 expression in the total bone marrow by qPCR. We observed a reduction in SHIP1 mRNA levels in mice expressing miR155 or siSHIP1 compared to control vectors (figure 3.3C).

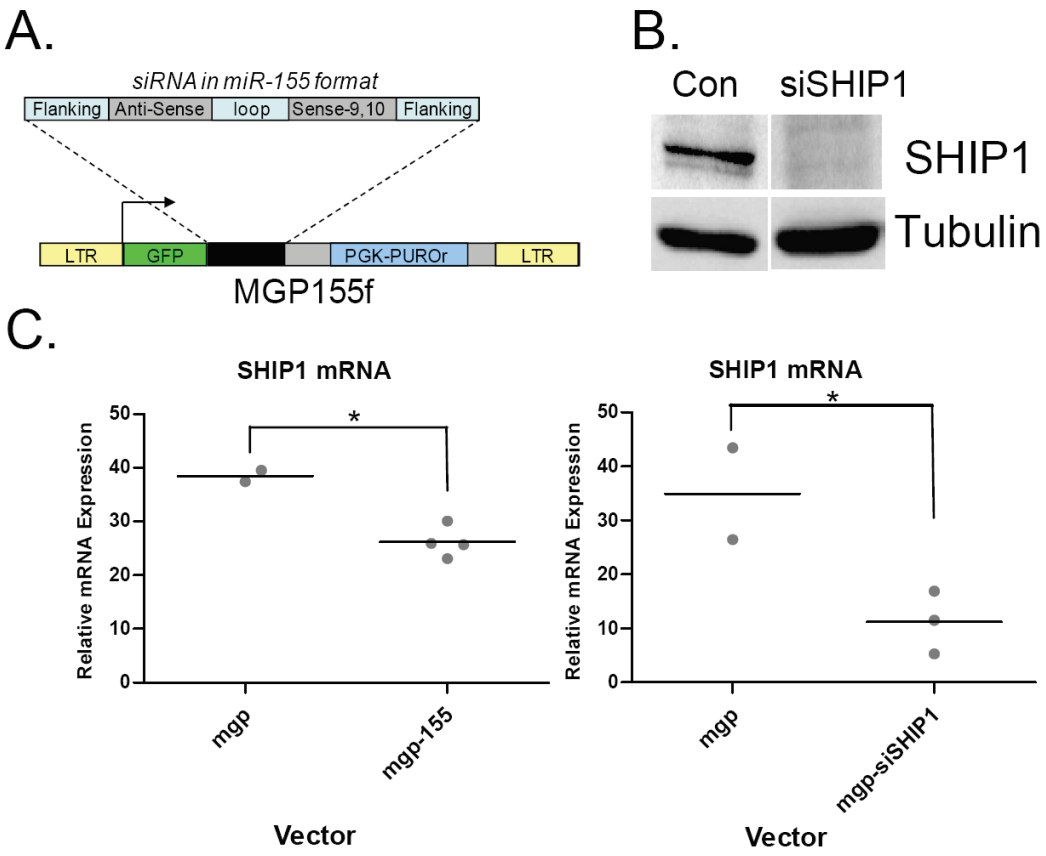


Figure 3.3: Knockdown of SHIP1 *in vivo* using siRNA in the context of miR-155 processing

A. Schematic of the retroviral vector (MGP-155f) used to deliver siRNA against SHIP1 in miR-155 format. B. Knockdown of SHIP1 was assayed in Raw 264.7 cells infected with MGP-siSHIP1 or control vector by Western blotting. αTubulin was assayed as a loading control.

C. Knockdown of SHIP1 *in vivo* by retroviral expression of miR-155 (MGP-155, n=4 mice) or siSHIP1 (MGP-siSHIP1, n=3 mice) in the hematopoietic compartment was assayed by qPCR using RNA isolated from total bone marrow following two months of hematopoietic reconstitution. Relative expression values have been normalized to L32 mRNA. A p-value of 0.05 or less using a Student's t-test was considered statistically significant and indicated with an asterisk.

Knockdown of SHIP1 in the hematopoietic compartment causes a MPD similar to that observed in mice expressing miR-155

Mice expressing miR-155, siSHIP1 or control vectors were next studied to determine their impact on hematopoietic populations after two months of reconstitution. Both miR-155 (human and mouse sequences) and siSHIP1 caused similar MPD phenotypes in the bone marrow and spleen compared to control vectors (figures 3.4 and 3.5). Gross analysis revealed a miR-155 or siSHIP1 dependent splenomegaly and pale coloring of the bone marrow (figure 3.5A and unpublished observations). Flow cytometry detected an increase in CD11b+ (Mac1+) myeloid populations in the bone marrow and spleen (figures 3.4A and 3.5A). The percentage of Ter119+ erythroid precursor cells was

increased in the spleen and decreased in the bone marrow, while the percentage of B220+ B-cells was decreased in both the spleen and the bone marrow (figures 3.4A and 3.5A).

Histological analyses of Wright stained bone marrow smears confirmed the presence of pathological myeloproliferative conditions in miR-155 and siSHIP1 expressing mice, characterized by elevated numbers of GM progenitors at various stages of development compared to controls (figure 3.4B). There was also a reduction in developing erythroid precursors and megakaryocytes in both miR-155 and siSHIP1 mice. Of note, miR-155 mice did exhibit a subtle increase in the number of dysplastic granulocytic cells compared with siSHIP1, possibly due to an additional miR-155 target. Flow cytometry also identified that both miR-155 and siSHIP1 expressing cells, which are GFP positive, are responsible for the increased myeloid populations (CD11b+) in the bone marrow (figure 3.4C).

Hematoxylin and Eosin staining of fixed spleen sections from miR-155 or siSHIP1 mice revealed expanded interfollicular regions containing developing myeloid populations, erythroid precursors and megakaryocytes compared to control mice (figure 3.5B). The normal follicular architecture of the spleen was disrupted by these expanded myeloid populations in both cases. Thus, miR-155 expression and specific SHIP1 knockdown in the hematopoietic system triggers marked extramedullary hematopoiesis, a likely consequence of the dysregulated blood cell development in the bone marrow.

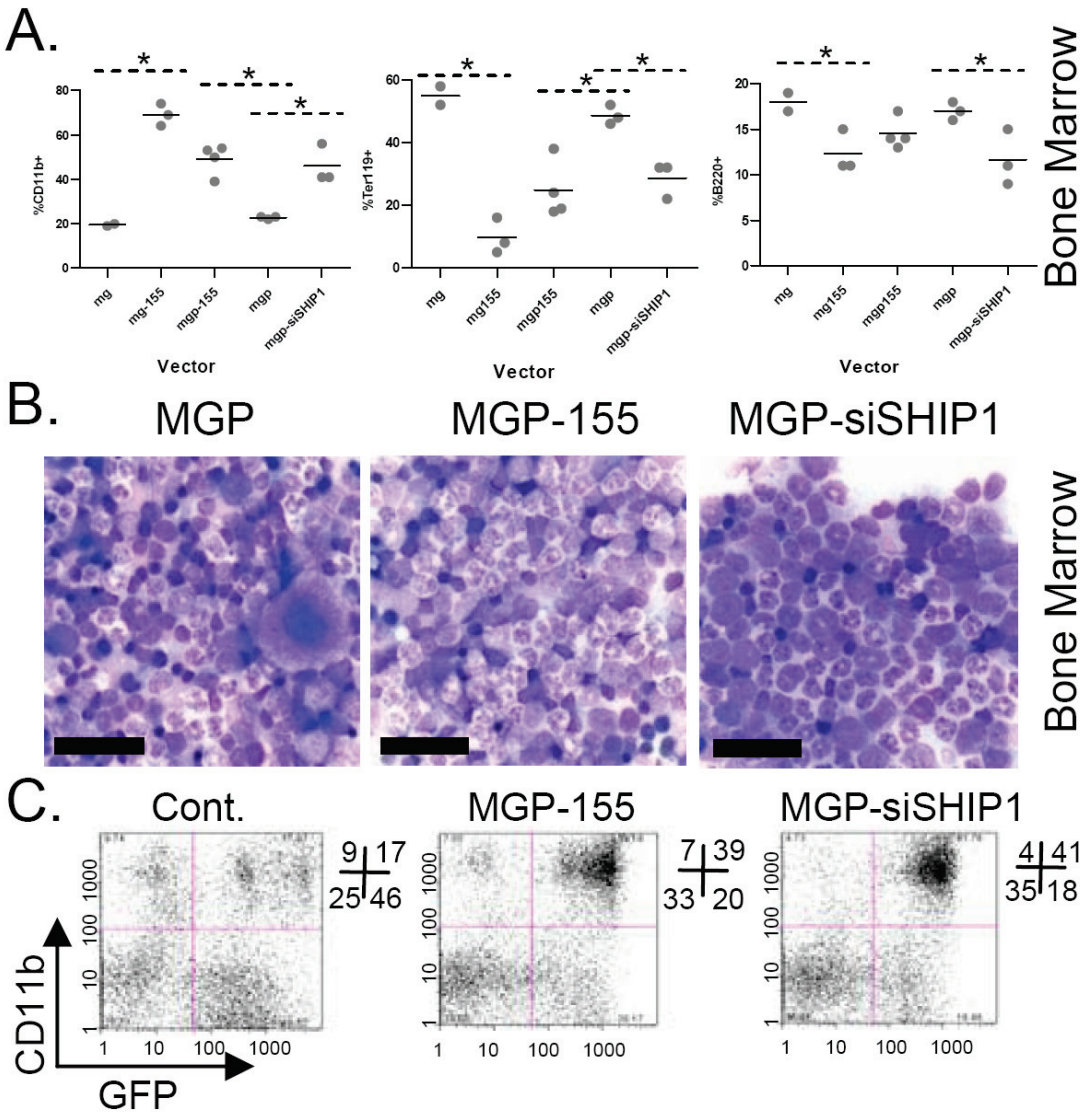


Figure 3.4: Knockdown of SHIP1 or expression of miR-155 in the hematopoietic compartment cause similar MPDs in the bone marrow.

A. Bone marrow was extracted from mice expressing human miR-155 (MG-155, n=3 mice), mouse miR-155 (MGP-155, n=4 mice), siSHIP1 (MGP-siSHIP1, n=3 mice), or control vectors (MG, n=2 mice or MGP, n=3 mice) 2 months following bone marrow reconstitution. Total bone

marrow cells were assayed for expression of CD11b (Mac1), Ter119 or B220 using flow cytometry. Each dot represents an individual mouse. A p-value of 0.05 or less using a Student's t-test was considered statistically significant and indicated with an asterisk. B. Bone marrow from MGP, MGP-155 or MGP-siSHIP1 mice was smeared and Wright stained. Photomicrographs are shown (1000x magnification, scale bar 20 μm). C. Representative flow cytometry plots from control, MGP-155 and siSHIP1 vector-containing mouse bone marrow analyzing GFP and CD11b expression.

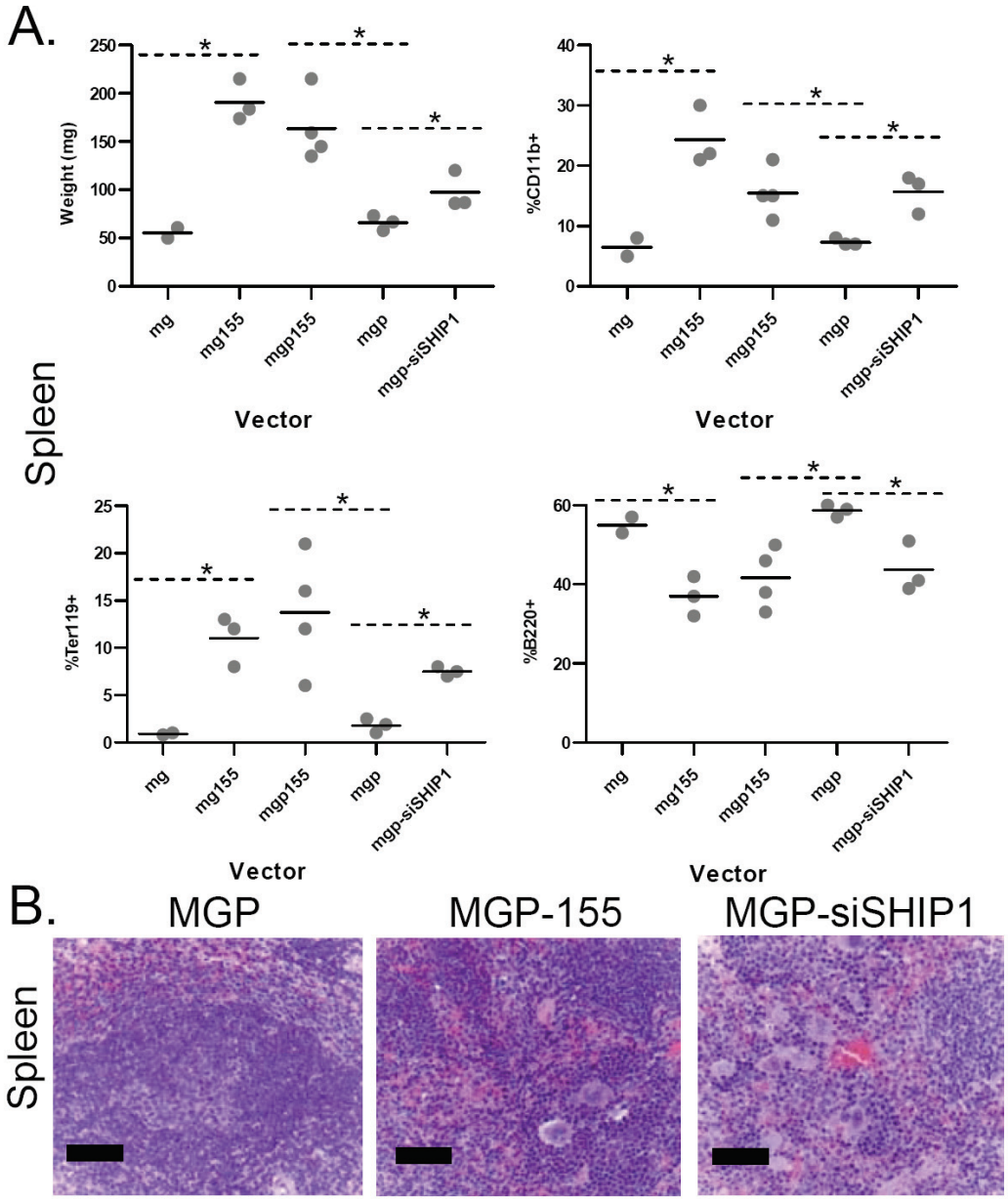

A.
Spleen
Weight (mg)
%CD11b+
%Ter119+
%B220+
mg
mg155
mgp155
mgp
mgp-siSHIP1
Vector
B.
MGP
MGP-155
MGP-siSHIP1
Spleen

Figure 3.5: Knockdown of SHIP1 or expression of miR-155 in the hematopoietic compartment causes splenomegaly and extramedullary hematopoiesis in the spleen.

A. Spleens were extracted from mice expressing human miR-155 (MG-155, n=3 mice), mouse miR-155 (MGP-155, n=4 mice), siSHIP1 (MGP-siSHIP1, n=3 mice), or control vectors (MG, n=2 or MGP, n=3 mice) 2 months following bone marrow reconstitution. Spleens were weighed and RBC-depleted splenocytes subsequently assayed for expression of CD11b, Ter119 or B220 by FACS. Each dot represents an individual mouse. A p-value of 0.05 or less using a Student's t-test was considered statistically significant and indicated with an asterisk. B. Spleens from MGP, MGP-155 or MGP-siSHIP1 mice were fixed, sectioned and H&E stained. Photomicrographs are shown (400x magnification, scale bar 50 μm).

Discussion

Similar to miRNAs like miR-155, many proteins have evolved to regulate immune cell function, and cause disease upon their dysregulated expression. Among such proteins, the inositol phosphatase SHIP1 is expressed in the hematopoietic system and has a broad impact on the biology of different hematopoietic cell types (Leung et al. 2008). SHIP1 functions at the molecular level by hydrolyzing the 5' phosphate of Phosphatidylinositol (PI)-3, 4, 5-P3 to generate PI-3,4-P2, a process that blocks PI3K-mediated membrane localization of certain PH domain containing signaling molecules such as Akt and PLCγ (Backers et al. 2003; Lioubin et al. 1996; Sly et al. 2003). Consequently, mice with a global SHIP1 deficiency develop a MPD characterized by increased GM populations, and decreased B lymphocyte numbers. This condition is thought to occur as a result of its role as a negative regulator of signaling by growth factors and other immune receptors (Kalesnikoff et al. 2003; Leung et al. 2008). Furthermore, knockout of SHIP1 in B lymphocytes causes spontaneous GC formation and antibody class switching (Helgason et al. 2000; Leung et al. 2008; Liu et al. 1998), while a SHIP deficiency in T cells skews peripheral T lymphocytes towards Th1 and away from Th2 in response to an immune challenge (Tarasenko et al. 2007). Thus, SHIP1 impacts the same cell types that express miR-155, and plays an opposing role in many cases. In the present study, we identify and characterize a direct link between miR-155 and SHIP1, whereby miR-155 can directly repress expression of SHIP1 and thereby impede its function.

The connection between miR-155 and SHIP1 has implications for normal immune physiology, as described above, as well as pathological conditions such as cancer. Our

present findings demonstrate a strong correlation between the MPDs caused by miR-155 expression or specific knockdown of SHIP1. Both perturbed the hematopoietic process, resulting in increased GM cell populations, reduced lymphocyte numbers, impaired erythropoiesis and extramedullary hematopoiesis in the spleen. Therefore, miR-155 repression of SHIP1 may prove to be a contributing factor to human MPDs and myeloid leukemia where miR-155 has been shown to be overexpressed (Garzon et al. 2008; O'Connell et al. 2008). Of note, SHIP1 is mutated in some AML patients, where loss of function has been implicated in the oncogenic process (Luo et al. 2004, 2003). As miR-155 levels are elevated in certain B-cell lymphomas (Eis et al. 2005; Fulci et al. 2007; Kluiver et al. 2005; Tam et al. 1997; Van den Berg et al. 2003), and because SHIP1 is a negative regulator of B-cell activation and survival (Helgason et al. 2000; Leung et al. 2008; Liu et al. 1998), the miR-155-SHIP1 axis should also prove to be of relevance to B-cell malignancies. Two recent abstracts suggest that this is the case (Chen et al. 2008; Pedersen et al. 2008). It is also plausible that virally encoded orthologs of miR-155 (Gottwein et al. 2007; Skalsky et al. 2007; Zhao et al. 2009), or miR-155 induction by viruses such as Epstein-Barr virus (Yin et al. 2008), can decrease SHIP1 expression en route to B-cell activation and transformation. Interestingly, the seed region of these orthologs is identical to that of miR-155, while the flanking regions have diverged dramatically from the mature mammalian miR-155 sequence. This would indicate that certain viral miRNAs have specifically evolved to repress seed-dependent targets of miR-155 including SHIP1.

An important question in the field is whether miRNAs function through repression of a single or a few targets, or via the cumulative impact of repressing large

sets of targets. To date, several putative targets of miR-155 have been predicted through bioinformatic and proteomic approaches (Selbach et al. 2008), indicating that there may be great complexity underlying miR-155 function. It may be that the MPD caused by miR-155 also involves other targets than SHIP1, but our studies show that SHIP1 alone can phenocopy the effects of miR-155. MiR-155 has various roles in different cell types and physiological situations and analysis of particular specific targets such as PU.1 (Vigorito et al. 2007), AID (Dorsett et al. 2008; Teng et al. 2008), SOCS1 (Lu et al. 2009) and now SHIP1 suggest that individual targets likely make significant contributions to miR-155 function in a context-dependent manner. For instance, PU.1 is repressed by miR-155 and its overexpression has been shown to recapitulate the immunoglobulin class switching defect observed in miR-155$^{-/-}$ B-cells (Vigorito et al. 2007). AID is repressed by miR-155 in B lymphocytes, which has been elegantly demonstrated via germLine mutation of the miR-155 seed in the 3'UTR of AID (Dorsett et al. 2008; Teng et al. 2008). The AID studies also provide evidence that miR-155 targeting of AID impacts Ig class switching and the rate of c-Myc translocations in B lymphocytes. SOCS1 targeting by miR-155 has just recently been shown to impact T-regulatory cell homeostasis (Lu et al. 2009). Similar observations have been made for specific targets of miR-150 (Lu et al. 2008; Xiao et al. 2007), miR-223 (Johnnidis et al. 2008) and miR-17-92 (Ventura et al. 2008; Xiao et al. 2008), suggesting a common theme of a few significant targets making dominant contributions to miRNA function. Ultimately, germLine mutation of miRNA target seed sequences within the 3'UTR regions of specific targets, as has been done for AID (Dorsett et al. 2008; Teng et al. 2008), will provide the strongest argument for relevance.

As both miR-155 and SHIP1 regulate critical and overlapping functions of a variety of cell types of the immune system, therapeutic manipulation of this novel molecular interaction may prove to be useful in the treatment of diverse pathological conditions including infection, cancer and autoimmunity.

Materials and Methods

Cell Culture

Raw 264.7 macrophage and 293T cells were cultured in complete DMEM with 10% FBS, 100 units/mL penicillin, and 100 units/mL streptomycin. For generation of BMMs, bone marrow cells were isolated from the tibias and femurs of mice as previously described (O'Connell et al. 2007). All cells were cultured in a humidified incubator with 5% CO_2 at 37°C. Primary macrophages were stimulated using fresh DMEM containing 10 ng/mL 055-B5 LPS (Sigma, St. Louis, MO), while Raw 264.7 cells were treated with LPS at 200 ng/mL.

Sequence alignments

SHIP1 3'UTR sequences from human, mouse, rat and dog were obtained and aligned with each other and with the miR-155 seed region using TargetScan (Lewis et al. 2005).

DNA constructs

Retroviral constructs MG and MG-155 (human sequence) were described previously (O'Connell et al. 2007). Oligonucleotide sequences used to generate new constructs are provided as supplemental data (Table 3.S1). The MGP-155 expression cassette containing the mouse miR-155 hairpin sequence and flanking regions was cloned from cDNA made from LPS treated BMMs. The cassette was subcloned into MGP. MGP is a modified pMSCV vector (Clontech, Mountain View, CA) where GFP was placed downstream of the 5' LTR, and the miR-155 expression cassette was cloned downstream of the GFP stop codon (detailed cloning strategy available upon request). The h155,

h155mut and siSHIP1 oligonucleotides, which produce mature human miR-155, human miR-155 seed mutant and siRNA against mouse SHIP1, respectively, were designed using the Invitrogen Block-iT pol II miR RNAi strategy and PCR amplified using Fw NotI- and Rev XhoI-containing primers. The Invitrogen Block-iT RNAi Designer was used to predict the siRNA sequence against mouse SHIP1. For reporter assays, the Picalm 3'UTR was cloned as described previously (O'Connell et al. 2008). The mouse SHIP1 3'UTR was amplified by PCR from cDNA derived from mouse RAW 264.7 cells. This PCR product was cloned into pmiReport (Ambion, Austin, TX) using SpeI and HindIII. Assembly PCR was used to mutate the 6 nucleotide miR-155 seed region. A 2-mer control insert and the IRAK1 3'UTR were described previously (O'Connell et al. 2008).

Luciferase-Beta Gal reporter assays

Experiments were performed as previously described using FUW, FUW-155, Beta-gal expression vector and pmiReport vectors transfected into 293T cells (O'Connell et al. 2008). Transfections were carried out with TransIT 293 (Mirus, Madison, WI). Data was normalized for transfection efficiency using a Beta-gal reporter and is represented as the ratio of luciferase activity of the transfection containing FUW-155 to that of the transfection containing FUW.

RAW 264.7 stable cell lines

To generate VSV-G-pseudotyped MSCV retroviruses, 2 x 10^6 293T cells were transfected with pGag-Pol, pVSV-G, and either MGP, MGP-h155, MGP-h155mut or MGP-siSHIP1. Transfection was performed with TransIT 293 as per manufacturer's

instructions. After 48 hours, viral supernatant was harvested and used to infect 5 x 10^5 RAW 264.7 cells for 8 hours in the presence of polybrene at 10 μg/mL. After 48 hours, stably transduced cells were selected using puromycin at 10 μg/mL for 7-10 days.

Mice

Wt mice on a C57BL/6 genetic background were bred and housed in the Caltech Office of Laboratory Animal Resources (OLAR) facility. Mice deficient in miR-155 and on a C57BL/6 genetic background were obtained from Alan Bradley at the Wellcome Trust Sanger Institute, Cambridge, UK. All experiments were approved by the Caltech Institutional Animal Care and Use Committee (IACUC).

Bone marrow reconstitution

Experiments were performed as previously described (O'Connell et al. 2008), with the following modifications: HSC enriched bone marrow was cultured for 48 hours before the first spin infection using the respective retroviral vector. Transfection of retroviral constructs was performed using TransIT 293. Cells were subjected to two spin infections, and transduced cells were delivered to recipient mice through retro-orbital injection.

RNA quantification

SYBR Green based quantitative real-time PCR (qPCR) was conducted using the 7300 Real-time PCR system (Applied Biosystems, Foster City, CA) to assay BIC, miR-155, 5s, SHIP1 mRNA and L32 mRNA levels as described previously (O'Connell et al. 2008). Mature miR-155 and 5s RNA were assayed using a mirVana miRNA detection kit as per manufacturer's instructions (Ambion). Mouse BIC, SHIP1 and L32 mRNA were detected

using specific primers (Table 3.S1). Northern blotting was performed as described (O'Connell et al. 2007) using a probe reverse complementary to the human miR-155 seed mutant (Table S1).

Western blotting

Cell extracts were size fractionated by SDS-PAGE and transferred to a nitrocellulose membrane using a semidry transfer apparatus (Bio-Rad, Hercules, CA). Western blotting was performed using the following antibodies: SHIP1 V-19 (sc-1963), SHIP1 M-14 (sc-1964), α–Tubulin B-7 (sc-5286), donkey anti-goat HRP-conjugated (sc-2020), goat anti-rabbit HRP-conjugated (sc-2004), goat anti-mouse HRP-conjugated (sc-2005) (Santa Cruz Biotechnology, Santa Cruz, CA); α-Actin (A2066), β-Actin (A1978) (Sigma, St. Louis, MO); SHIP1 (D1163), Akt1 (C73H10), Phospho-AKT (Ser473) (Cell Signaling, Boston, MA). Protein expression intensities were determined using Scion Image software.

Flow cytometry

Fluorophore-conjugated monoclonal antibodies specific to CD11b (Mac1), Ter-119 or B220 (eBioscience) were used to stain RBC-lysed splenocytes and RBC-containing bone marrow cells that were washed and fixed with paraformaldehyde (1% final). Stained cells were assayed using a BD FACSCalibur flow cytometer (BD, Franklin Lakes, NJ) and further analyzed with FlowJo software.

Morphological assessment of hematolymphoid tissues

Histological and cytological samples were prepared and analyzed as described previously (O'Connell et al. 2008).

Statistical tests

All statistical tests were performed using Microsoft Excel.

Supporting Information

O'Connell et al. 10.1073/pnas.0902636106

Table S1. Primers and oligonucleotides

Name	Sequence
m155 from LPS BMM Fw	AAACCAGGAAGGGGAAGTGT
m155 from LPS BMM Rev	ATCCAGCAGGGTGACTCTTG
h155 BlockIT Template oligo	GAAGGCTGTATGCTGTTAATGCTAATCGTGATAGGGGTTTTGGCC ACTGACTGACCCCTATCAATTAGCATTAACAGGACACAAGGCCTG
h155mut BlockIT Template oligo	GAAGGCTGTATGCTGTATTACGTAATCGTGATAGGGGTTTTGGCC ACTGACTGACCCCTATCAATTACGTAATACAGGACACAAGGCCTG
sIShip1 BlockIT Template oligo	GAAGGCTGTATGCTGTTAAATGTCATGCTGGTGACTGTTTTGGCC ACTGACTGACAGTCACCAATGACATTTAACAGGACACAAGGCCTG
m155 arms NotI Fw	ATCGGCTGAGTCGACGCGGCCGCCTGGAGGCTTGCTGAAGGCT GTATGCTG
m155 arms XhoI Rev	ATCGCAATTGCTCGAGTGGGCCATTTGTTCCATGTGAGTGCTAGT AACAGGCCTTGTGTC
mShip1 3'UTR SpeI Fw	TTCACTAGTGCTGCTGGTGATCGGAGCCTG
mShip1 3'UTR HindIII Rev	TTCAAGCTTTAGAAAGGCACAATTTTATTGGTCACTCAG
mShip1 UTRmut fragment 1 Rev	GGTTAGCTCTAAGAGACCGATGGTTTTCTTATTACGTTTTTAAAAA CATCTCAGGACCCACC
mShip1 UTRmut fragment 2 Fw	GTGGGTCCTGAGATGTTTTTAAAAACGTAATAAGAAAACCATCGG TCTCTTAGAGCTAACC
Blc qPCR Fw	AAACCAGGAAGGGGAAGTGT
Blc qPCR Rev	ATCCAGCAGGGTGACTCTTG
mSHIP1 qPCR Fw1	CCAGGGCAAGATGAGGGAGA
mSHIP1 qPCR Rev1	GGACCTCGGTTGGCAATGTA
mSHIP1 qPCR Fw2	GAGCGGGATGAATCCAGTGG
mSHIP1 qPCR Rev2	GGACCTCGGTTGGCAATGTA
mL32 qPCR Fw	AAGCGAAACTGGCGGAAAC
mL32 qPCR Rev	TAACCGATGTTGGGCATCAG
h155mut Northern probe	CCCCTATCACGATTACGTAATA

Supplemental Table S1. Primers and Oligonucleotides

CHAPTER 4: A novel role for miR-34a in B-lymphocyte development

Submitted as

DS Rao, RM O'Connell, AA Chaudhuri, Y Garcia-Flores, T Geiger and D Baltimore. "miR-34a perturbs B-cell development by targeting the Forkhead transcription factor Foxp1". *Immunity*, 2010

Summary

MicroRNAs (miRNAs) play important roles in hematopoietic development and in many cases, influence lineage choice or affect critical developmental checkpoints. The microRNA-34a (miR-34a) has recently been identified as a component of the p53 tumor suppressor network. In addition to its role in cancer, p53 also plays roles in hematopoietic development and specifically in lymphopoiesis. To search for a role of miR-34a in hematopoiesis, we performed a gain-of-function analysis in murine bone marrow. Constitutive expression of miR-34a led to a block in B-cell development at the pro-B-cell to pre-B-cell transition, leading to a reduction in mature B-cells in the bone marrow and peripheral blood. This block in B-cell development appears to be mediated primarily by inhibited expression of the Forkhead transcription factor, Foxp1. We demonstrate that Foxp1 is a direct target of miR-34a and that a conserved site in its 3'UTR mediates repression. Bone marrow transfer experiments with a siRNA directed against *Foxp1* recapitulated many aspects of the B-cell developmental phenotype induced by miR-34a. Cotransduction of miR-34a with *Foxp1* lacking its 3'UTR rescued the B-cell developmental phenotype. Last, knockdown of miR-34a resulted in an increased level of both Foxp1 and mature B-cells, indicating that Foxp1 is a major target of miR-34a during B-cell development. These findings identify an important role for miR-34a in connecting the p53 network with suppression of Foxp1, a known B-cell oncogene.

Introduction

Antigen-independent B-cell development from hematopoietic stem and progenitor cells is a complex process, closely coordinated with the generation of functional antigen receptors (Hardy and Hayakawa 2001). B-cells are the end product of an ordered series of developmental steps punctuated by checkpoints following the rearrangement of the immunoglobulin heavy and light chain loci. Defined stages of committed B-cell precursors include pro-B-cells, during which B-cells complete rearrangement of their heavy chain locus, pre-B-cells, during which rearrangement of the light chain locus occurs, and finally immature and mature B-cells expressing variable levels of surface immunoglobulin (IgM). A complex sequence of molecular events orchestrates successful V(D)J rearrangement in B-cells. It includes the activation of several transcription factors, notably PU.1, E2a, Ebf, and Pax5, the last of which is thought to result in the final commitment of lymphoid precursors to the B-cell lineage (Busslinger 2004). A recent addition to the list of transcription factors required for early B-cell development is Foxp1 (Hu et al. 2006). Foxp1 is a forkhead transcription factor with functions in tissue and cell-type specific gene expression and its targeted deletion is lethal in embryogenesis, mainly due to cardiac defects (Shu et al. 2001; Wang et al. 2004). By using a Recombination activating gene-2 (*Rag2*) complementation system, it was discovered that loss of this transcription factor results in a block in early B-cell development. Furthermore, a functional role in coordinating the development of B-cells was suggested by Foxp1's ability to bind to enhancer elements within the *Rag* gene loci (Hu et al. 2006).

During B-cell development, multiple checkpoints follow the rearrangement of the immunoglobulin loci, dependent on the proper assembly of the pre-B-cell receptor at the

pro-B to pre-B transition, and the B-cell receptor at the pre-B-cell to B-cell transition (Meffre et al. 2000). Based on the following observations, these checkpoints appear to be partially dependent on p53. First, *Trp53*$^{-/-}$ mice show decreased apoptosis of pro-B-cells, as well as accumulation of pre-B-cells and immature B-cells (Lu et al. 2000; Lu and Osmond, 2000). Second, in the context of decreased double-strand break repair in *scid* mice, deficiency of *Trp53* leads to a massive accumulation of pro-B-cells (Guidos et al. 1996). Presumably, such cells would normally undergo apoptosis as consequence of p53's action in coordinating the response of cells to DNA damage. The apoptosis of pro-B-cells depends on the ratio of pro-apoptotic Bax to the pro-survival Bcl-2 protein, and this ratio is disrupted in *Trp53*$^{-/-}$ mice.

Recently, the miR-34 family of miRNAs, was discovered to be transcriptionally induced by p53, and it is now thought to be an important component of the p53 tumor suppressor network (Chang et al. 2007; He et al. 2007; Raver-Shapira et al. 2007). These miRNAs act by suppressing a range of genes important in cell cycle progression, antiapoptotic functions, and regulation of cell growth. As might be expected, expression of these miRNAs is altered in a broad range of cancers, with the most common scenario being one in which both p53 and miR-34 are downregulated (reviewed in Hermeking (2009)). In cancers of the hematopoietic system, profiling studies have suggested a role for one member of the miR-34 family, miR-34a, in chronic lymphocytic leukemia and acute myeloid leukemia (Isken et al. 2008; Mraz et al. 2009; Zenz et al. 2009). In the case of chronic lymphocytic leukemia, miR-34a alterations have been described both in association with and independent of p53 deletions.

A role for miR-34a in normal hematopoiesis has not yet been defined. Such a role has been described for many miRNAs, with multiple recent studies describing the impact of miRNAs on lineage determinations during hematopoietic differentiation (reviewed in (Baltimore et al. 2008)). Indeed, these were some of the first functions described for miRNAs in mammalian systems, with miR-181 promoting B-lineage differentiation and miR-223 affecting myeloid differentiation (Chen et al. 2004). The functional effects of miRNAs may depend on the regulation of a few critical factors that control gene expression, thereby affecting lineage choice (Xiao and Rajewsky 2009). The range of targets ascribed to miR-34a suggests that it might have a role in the regulation of hematopoietic development. In fact, a role in B-cell development was hypothesized following the demonstration that Bcl-2 is a direct target of miR-34a regulation (Bommer et al. 2007). It was particularly striking that an imbalance between Bax and Bcl-2 could lead to a defect in pro-B-cell apoptosis in *Trp53*$^{-/-}$ mice.

Here, we describe the initial characterization of miR-34a in hematopoiesis. We found that miR-34a constitutive expression causes a block in B-cell differentiation. We have further characterized this block showing by a combination of gain-of-function and loss of function analyses that Foxp1 is a major target of miR-34a in this developmental context. These findings connect the p53-induced miR-34a with Foxp1, paradoxically a known B-cell oncogene as well as an important factor in B-cell development.

Results

Constitutive expression of miR-34a in the bone marrow leads to a significant decrease in B-lymphocytes

To explore the role of miR-34a in hematopoietic differentiation, we designed retroviral vectors that express the microRNA. The vector coexpresses miR-34a and GFP and exploits the miR-155 format vector that we have previously utilized successfully to give strong expression of microRNA and siRNA (figure 4.1A and (O'Connell et al. 2009)). The vector produced pre-miR-34a and mature miR-34a as demonstrated by Northern blotting (figure 4.1B) and the mature microRNA by RT-qPCR (supplemental figure 4.1A) in addition to GFP for labeling of transduced murine cells (supplemental figure 4.1B).

After transduction of murine bone marrow cells with the miR-34a and control vectors followed by reconstitution of lethally irradiated syngeneic C57/B6 mice, we analyzed the recipient's bone marrow 2 months later. GFP positive cells were readily observed in both the control and miR-34a-transduced marrow recipients (figure 4.1C). Expression of miR-34a was found to be elevated in primary bone marrow cells from the miR-34a recipients (figure 4.1D). The bone marrow was stained with a combination of antibodies to delineate the various hematopoietic lineages, including mature B-cells (defined as being CD19+IgM+), myeloid cells (CD11b+), T-cells (CD3ε+), and erythroid precursors (Ter119+). A significant reduction in mature B-cells, as a proportion of transduced cells (approximately 30% lower), was observed (figure 4.1E). We did not observe statistically significant changes in myeloid cells (figure 4.1F), erythroid

precursors (figure 4.1G) or T-cells (figure 4.1H). These differences were observed consistently across three experiments.

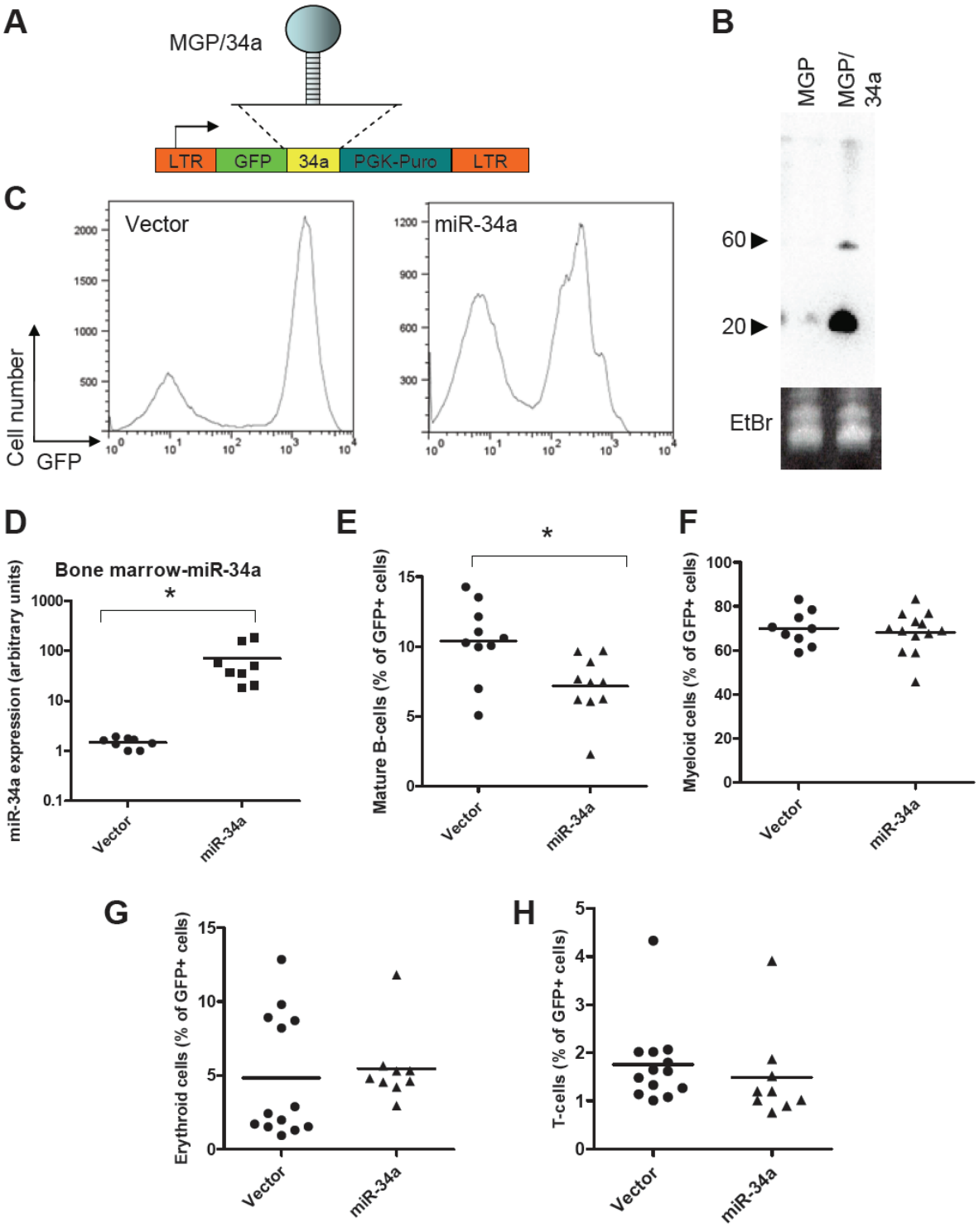


Figure 4.1. Constitutive expression of miR-34a in the bone marrow compartment leads to a decrease in mature B-lymphocytes.

A. Schematic representation of the MSCV-based retroviral vector used to express miR-34a, based on the previously described MGP vector (O'Connell et al. 2009). The expression of GFP and miR-34a is driven by the viral LTR.

B. Expression of mature the pre-miR-34a (~60nt) and the mature miR-34a (~20nt) was assayed by Northern blotting. The bottom panel shows ethidium bromide staining (EtBr) to confirm equal loading.

C. Analysis of GFP expression in bone marrow two months after retroviral transduction and transfer. Bone marrow was infected with either the vector control or with vector expressing miR-34a and delivered into lethally irradiated recipients.

D. The bone marrow from recipient mice was also analyzed for expression of miR-34a by RT-qPCR, and normalized by analysis of 5S. The overexpression of miR-34a was consistent in mice receiving MGP/34a transduced marrow (labeled miR-34a) as opposed to MGP (labeled vector) and was statistically significant (T-test, $p=0.009$).

E. Bone marrow mature B-cells (defined as GFP+CD19+IgM+) were detected by flow cytometry and their relative numbers were compared in mice expressing vector alone or miR-34a (T-test, $p=0.0093$).

F-H. Flow cytometric analyses for myeloid cells (defined as GFP+CD11b+), erythroid cells (GFP+Ter119+), and T-cells (GFP+CD3ε+) are shown.

miR-34a expression results in a block in B-cell development at the pro-B to pre-B-cell stage in the bone marrow

To determine whether the observed reduction in mature B-cells was the consequence of a specific developmental abnormality in B-cell ontogeny, flow cytometric analyses were performed to delineate the various stages of B-cell maturation. We found a significant (approximately two-fold) increase in pro-B-cells (CD19+ c-kit+ IgM-) in miR-34a mice (figure 4.2A). Conversely, there was a significant reduction in pre-B-cells (B220+CD43-IgM-) in the miR-34a mice (figure 4.2B). This pattern indicates a developmental retardation at the pro-B to pre-B transition when B-cells are passing through the pre-BCR checkpoint and beginning the process of light chain rearrangement. A similar trend was observed when alternate stains were used to delineate pro-B-cells (including CD19+CD43+AA4.1+ and CD43+B220+IgM-) and pre-B-cells (CD19+CD25+IgM-; data not shown). The numbers of pro- and pre-B-cells in miR-34a overexpressing marrow correlated inversely with the expression pattern of miR-34a in the normal B-lineage cells, where relatively high miR-34a was observed at the pro-B-cell stage with lower levels in pre-B-cells (figure 4.2C). A second miR-34a expressing vector, which uses the native stem-loop structure and flanking regions and produces mature miR-34a at lower levels, induced a similar phenotype (Supplemental Figure 4.2A, 4.2C and 4.2D). The decrease in bone marrow B-cells resulted in decreased circulating B-lymphocytes (figure 4.2D), but splenic populations of B-cells appear to be largely restored in miR-34a overexpressing mice (figure 4.2E). This likely represents homeostatic expansion of B-cells in the periphery by mechanisms that are independent of miR-34a expression.

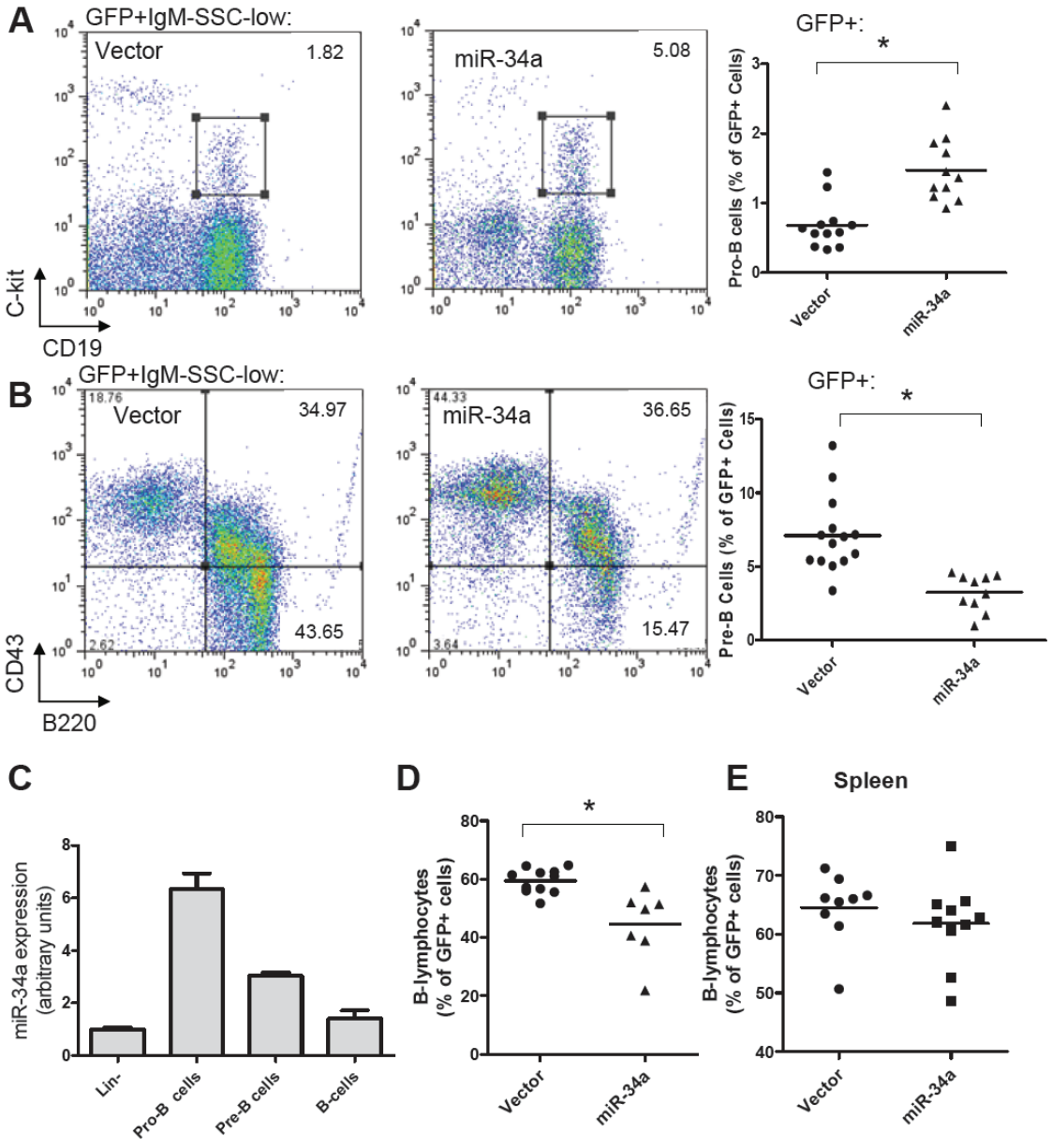

A
GFP+IgM-SSC-low:
Vector
1.82
miR-34a
5.08
C-kit
CD19
GFP+:
*
Pro-B cells (% of GFP+ Cells)
Vector
miR-34a
B
GFP+IgM-SSC-low:
18.76
Vector
34.97
43.65
2.62
44.33
miR-34a
36.65
15.47
3.64
CD43
B220
GFP+:
*
Pre-B Cells (% of GFP+ Cells)
Vector
miR-34a
C
miR-34a expression (arbitrary units)
Lin-
Pro-B cells
Pre-B cells
B-cells
D
*
B-lymphocytes (% of GFP+ cells)
Vector
miR-34a
E
Spleen
B-lymphocytes (% of GFP+ cells)
Vector
miR-34a

Figure 4.2. miR-34a expression causes an increase in pro-B-cells and a decrease in pre-B-cells.

A. Analysis of pro-B-cells. Bone marrow cells stained with CD19, c-kit and IgM, analyzed by flow cytometry. Left hand panels show representative histograms of the GFP+ IgM-negative compartment in control (vector) and miR-34a expressing mice. The right hand panel shows aggregate data enumerating pro-B-cells (CD19+IgM-c-kit+) as a percentage of total GFP+ cells for three experiments (n=12 for vector control and n=10 for miR-34a animals). All three experiments showed similar trends and overall differences are statistically significant (T-test, p=0.0001).

B. Analysis of pre-B-cells. Bone marrow cells stained with B220, IgM and CD43 were analyzed by flow cytometry. Left hand panels show representative histograms of the GFP+ IgM- compartment in control and miR-34a expressing mice. The right hand panel shows aggregate data enumerating pre-B-cells (B220+CD43-IgM-) as a percentage of the total GFP+ cells for three experiments (n=12 for vector control and n=10 for miR-34a animals). All three experiments showed similar trends and overall differences are statistically significant (T-test, p=0.0002).

C. Assessment of miR-34a expression in B-lineage cells at various stages of differentiation. miR-34a expression was assessed in the various cell populations collected by fluorescence activated cell sorting (FACS).

D. Peripheral blood B-lymphocytes are reduced in mice reconstituted with marrow that constitutively expresses miR-34a (data from 2 experiments, n=11 for vector control and n=7 for miR-34a; p=0.02).

E. Splenic B-lymphocyte numbers are similar in vector and miR-34a reconstituted mice.

Foxp1 is a bona fide target of miR-34a

To understand what targets of miR-34a might mediate the B-cell developmental block, we examined predicted targets using the TargetScan database (Grimson et al. 2007; Lewis et al. 2005). From this list of targets, we identified a novel target, Foxp1, which is predicted to have two 7-mer sites in the 3'UTR. Because *Foxp1* has previously been implicated in B-cell development and its downregulation essential to normal monocyte development (Shi et al. 2008, 2004), we examined whether this gene is a novel target of miR-34a. The 3'UTR of *Foxp1* is schematically represented in Figure 4.3A, showing the more 5' of the 2 conserved miR-34a sites. To determine if Foxp1 might represent a direct target of miR-34a, luciferase assays were performed as previously described (O'Connell et al. 2009, 2008). Cells cotransfected with pcDNA3-miR-34a and luciferase linked to the *FOXP1*-3'UTR showed repression of luciferase compared to control (figure 4.3B, FOXP1). When the conserved site in the *FOXP1* 3'UTR was mutated (as depicted in figure 4.3A), luciferase expression was derepressed (figure 4.3B; FOXP1mt). As controls, we examined repression of a UTR containing two repeats of the antisense sequence to miR-34a (2 repeats of antisense 22-mer), which showed the greatest repression (figure 4.3B, a.s. 2mer), as well as repression of a *BCL2*-3'UTR containing construct, a previously reported miR-34a target (figure 4.3B; BCL2 and BCL2mt; (Bommer et al. 2007)). As negative controls, we utilized the 3'UTR of two genes, CEBPβ and PU.1, which we have previously reported as being repressed by miR-155, as well as a control luciferase construct lacking a UTR (figure 4.3B, (O'Connell et

al. 2008)). We have also examined the second UTR site which does not show repression in a similar assay (data not shown).

To examine the effects of miR-34a expression on endogenous FOXP1, we utilized two cell lines at a similar stage of differentiation- NALM6, which is a human pre-B-cell line, and 70Z/3, which is a murine pre-B-cell line. First, we assayed NALM6 single cell clones that had been transduced with a lentivector expressing miR-34a (supplemental figure 4.1C). This vector expressed GFP as well as miR-34a by Northern blot analysis and RT-qPCR (supplemental figures 4.1D-F). Analysis of the single cell clones revealed repression of *FOXP1* and overexpression of miR-34a RNA (figure 4.3C and 4.3D, respectively). As expected, the protein level of FOXP1 was decreased in these clones (data not shown). Additionally, in murine 70Z/3 cells, we observed a similar repression of Foxp1 at the protein level within 4 days of transduction (figure 4.3E). Analysis of Foxp1 RNA levels in B-cell subsets showed an inverse correlation with miR-34a levels at the same stages (figure 4.3F; compare with figure 4.2C). Having observed repression of Foxp1 in cell lines, we also examined *Foxp1* mRNA expression in whole bone marrow from the bone marrow transfer recipient mice. *Foxp1* mRNA is significantly downregulated in the bone marrow of mice transduced with miR-34a (figure 4.3G), with no significant change observed in L32 control RNA (figure 4.3H). It is notable that this difference was observed in whole bone marrow, where only about half of the cells had been transduced with miR-34a. Hence, repression of Foxp1 by miR-34a is demonstrated in human and mouse cell lines, as well as in primary murine bone marrow, and is mediated through a conserved site in the 3'UTR of *Foxp1*.

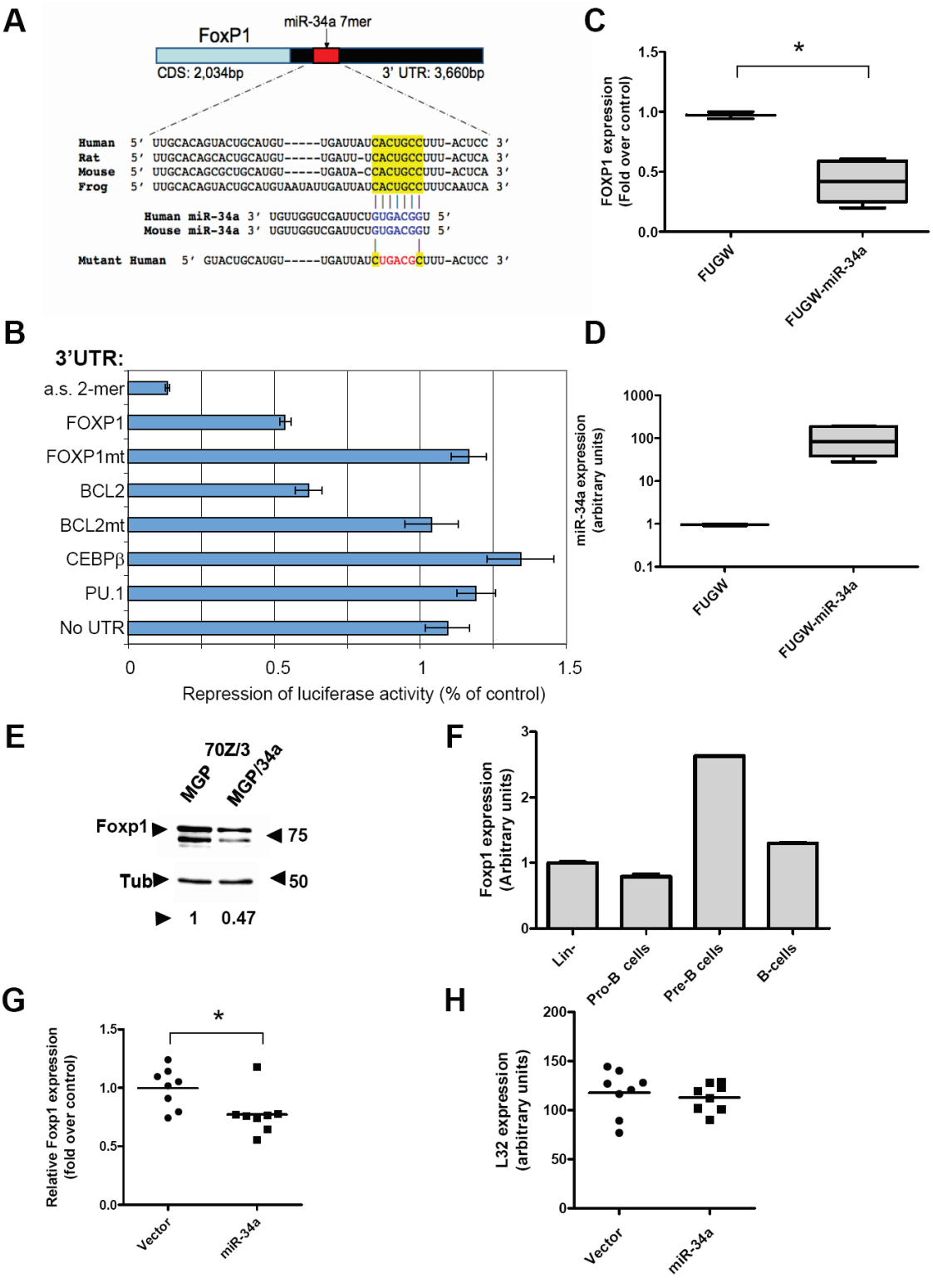

A
FoxP1
miR-34a 7mer
CDS: 2,034bp
3' UTR: 3,660bp
Human 5' UUGCACAGUACUGCAUGU-----UGAUUAUCACUGCCUUU-ACUCC 3'
Rat 5' UUGCACAGCACUGCAUGU-----UGAUU-UCACUGCCUUU-ACUCA 3'
Mouse 5' UUGCACAGCGCUGCAUGU-----UGAUA-CCACUGCCUUU-ACUCA 3'
Frog 5' UUGCACAGUACUGCAUGUAAUAUUGAUUAUCACUGCCUUUCAAUCA 3'
Human miR-34a 3' UGUUGGUCGAUUCUGUGACGGU 5'
Mouse miR-34a 3' UGUUGGUCGAUUCUGUGACGGU 5'
Mutant Human 5' GUACUGCAUGU-----UGAUUAUCUGACGCUUU-ACUCC 3'
C
FOXP1 expression (Fold over control)
FUGW
FUGW-miR-34a
B
3'UTR:
a.s. 2-mer
FOXP1
FOXP1mt
BCL2
BCL2mt
CEBPβ
PU.1
No UTR
Repression of luciferase activity (% of control)
D
miR-34a expression (arbitrary units)
FUGW
FUGW-miR-34a
E
70Z/3
MGP
MGP/34a
Foxp1
Tub
75
50
1 0.47
F
Foxp1 expression (Arbitrary units)
Lin-
Pro-B cells
Pre-B cells
B-cells
G
Relative Foxp1 expression (fold over control)
Vector
miR-34a
H
L32 expression (arbitrary units)
Vector
miR-34a

Figure 4.3. Foxp1 is a bona fide target of miR-34a.

A. Schematic representation of *Foxp1* cDNA and 3'UTR showing the conserved miR-34a seed region in its 3'UTR. Human and mouse miR-34a mature sequences with proposed base pairing to the 3'UTR are presented as is the mutant 3'UTR used in the luciferase assays depicted in B.

B. Luciferase assays demonstrate that the targeting of *Foxp1* by miR-34a is via direct 3'UTR interactions. Graphed is the relative luciferase activity of 293T extracts transfected with the designated luciferase-3'UTR construct and a miR-34a expressing vector compared to cells transfected with the luciferase-3'UTR and empty vector. All data is normalized for transfection efficiency by a β-galactosidase reporter. Designations are for the various 3'UTRs attached to the luciferase reporter: a.s.2-mer, two copies of the miR-34a anti-sense sequence; FOXP1mt, FOXP1 3'UTR with mutated mir-34a seed site; BCL2mt, BCL2 3'UTR with mutated mir-34a seed site; No UTR, luciferase gene without a 3'UTR.

C. *FOXP1* expression, measured by RT-qPCR in NALM6 clones that express control vector (FUGW) or miR-34a (FUGW/34a). These vectors were derived from FUGW (Lois et al. 2002). Differences are statistically significant (T-test, $p=0.0086$).

D. miR-34a expression, measured by RT-qPCR, in NALM6 clones as in (C).

E. Western blot analysis of murine Foxp1 in 70Z/3 cells infected with MGP/34a as opposed to control vector alone. The numbers below the blot indicate the relative expression level of the protein as compared with the control, as determined by using Scion software.

F. Foxp1 RNA levels in the same subsets of bone marrow cells as in figure 4.2C. Note that the expression levels are roughly inversely correlated, particularly at the pro-B to pre-B-cell transition.

G. and H. *Foxp1* (G) and L32 (H) expression measured by RT-qPCR in the bone marrow of mice reconstituted with either vector- or miR-34a (n=7 for vector, n=8 for miR-34a). T-test, p=0.0225 for comparison of *Foxp1* expression levels.

Direct repression of Foxp1 by siRNA mediated knockdown recapitulates many features of miR-34a expression in the bone marrow

Previous reports have indicated that the complete lack of Foxp1 results in a profound B-cell developmental block that generates a near-total lack of mature B-cells (Hu et al. 2006). In the experiments described above, expression of miR-34a resulted in a partial block at the same developmental stage, but mature B-cells were still produced. To examine whether the phenotype of miR-34a expression might be explained by the repression of Foxp1, we developed constructs to express a siRNA against *Foxp1* (Foxp1si) in the same format as the construct expressing miR-34a (figure 4.4A). Testing of this construct in 70Z/3 cells showed that *Foxp1* mRNA was repressed about 50%, similar to the repression observed in miR-34a-expressing cells (figure 4.4B). In addition, repression by Foxp1si was similar to that by miR-34a at the protein level, where Foxp1 expression was reduced by about 50% (figure 4.4B, right hand panel, compare to figure 4.3E, right hand panel). Retroviral transduction and bone marrow transfer showed a high degree of reconstitution of the marrow by Foxp1si-expressing cells (figure 4.4C). *Foxp1* RNA was reduced by about 50% in the marrow of mice reconstituted with this vector (figure 4.4D). With this degree of repression, a dramatic increase in pro-B-cells

(about 3.5-fold) was observed (figure 4.4E) along with a corresponding decrease in pre-B-cells (figure 4.4F). No changes were observed in the myeloid compartment by flow cytometric and morphologic analyses (data not shown). On the other hand, retrovirally mediated knockdown of Bcl-2 in the bone marrow by a similar vector failed to recapitulate the phenotype observed in the miR-34a overexpressing mice, despite the demonstration that Bcl-2 is a direct target of miR-34a (supplemental figure 4.3A and 4.3B). Instead, there were major reductions in all stages of B-cell differentiation and a mild reduction in myeloid cells in the bone marrow (supplemental figures 4.3C, D, E, and F). Hence, the block in B-cell development is largely recapitulated in a specific manner by the repression of Foxp1 using a siRNA in the miRNA-processing format.

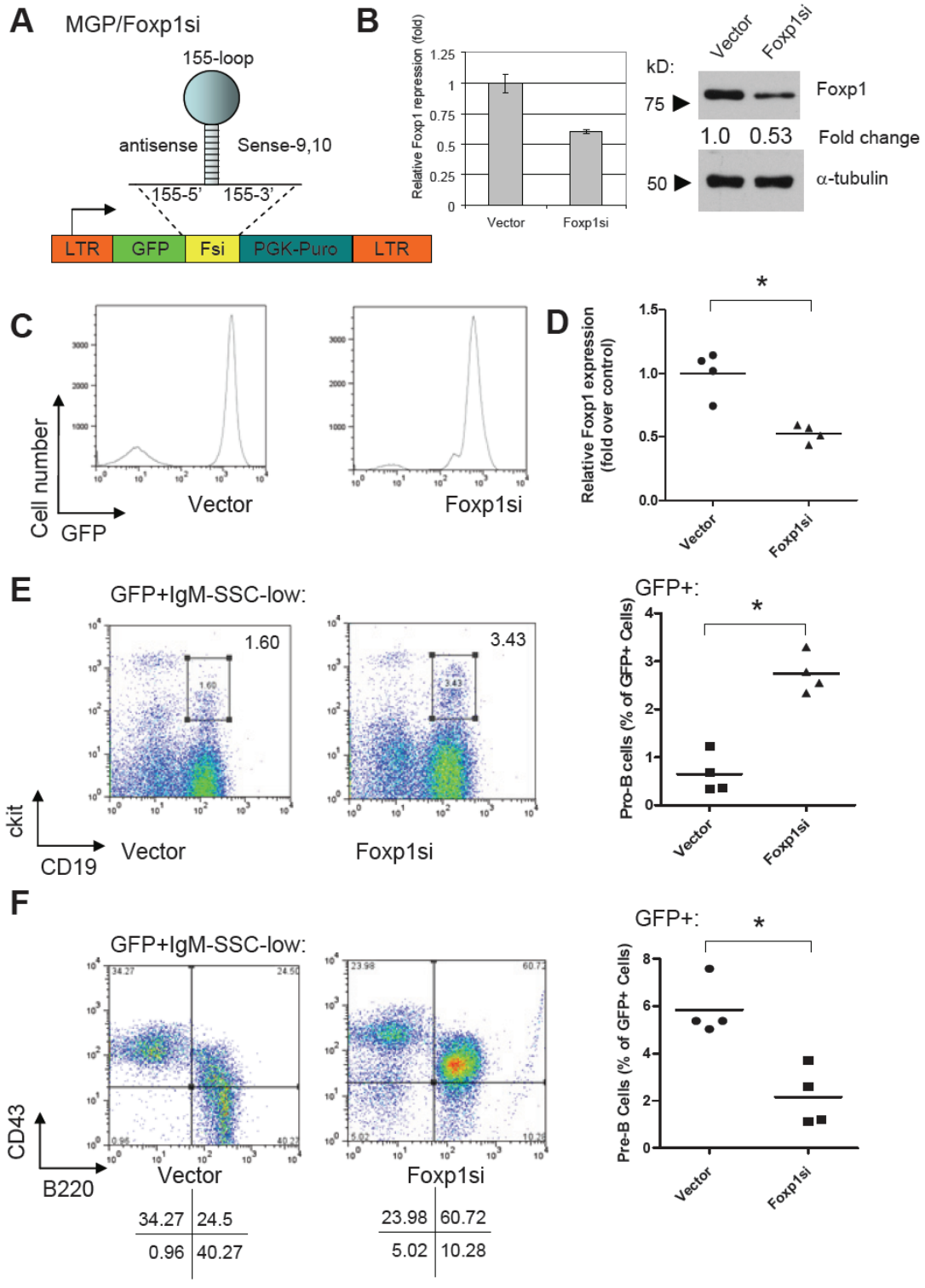

A
MGP/Foxp1si
155-loop
antisense
Sense-9,10
155-5'
155-3'
LTR
GFP
Fsi
PGK-Puro
LTR
B
Relative Foxp1 repression (fold)
1.25
1
0.75
0.5
0.25
0
Vector
Foxp1si
kD:
Vector
Foxp1si
75
Foxp1
1.0
0.53
Fold change
50
α-tubulin
C
Cell number
GFP
Vector
Foxp1si
D
Relative Foxp1 expression (fold over control)
1.5
1.0
0.5
0.0
*
Vector
Foxp1si
E
GFP+IgM-SSC-low:
1.60
3.43
ckit
CD19
Vector
Foxp1si
GFP+:
Pro-B cells (% of GFP+ Cells)
*
Vector
Foxp1si
F
GFP+IgM-SSC-low:
CD43
B220
Vector
Foxp1si
34.27
24.5
0.96
40.27
23.98
60.72
5.02
10.28
GFP+:
Pre-B Cells (% of GFP+ Cells)
*
Vector
Foxp1si

Figure 4.4. Knockdown of Foxp1 recapitulates miR-34a-induced B-lineage abnormalities.

A. Schematic diagram showing the MGP-based construct used to express the Foxp1 siRNA. Note that the format is identical to the format used to express miR-34a.

B. Left-hand panel shows *Foxp1* RNA, measured by RT-qPCR, in 70Z/3 cells infected with either empty vector (Vector) or MGP/Foxp1si (Foxp1si). Right hand panel shows Western blot analysis of Foxp1 in these cells. The numbers represent the fold expression of Foxp1 at the protein level compared to vector control, as determined by quantitation using Scion software.

C. GFP expression in the bone marrow of bone marrow recipient mice (vector and Foxp1si) 2 months after transplant.

D. *Foxp1* expression in the bone marrow of recipient mice (vector and Foxp1si), as measured by RT-qPCR. Shown is one representative experiment with 4 mice in each group. Experiments were repeated three times, with similar trends in all three experiments. (T-test, p=0.0026)

E. Bone marrow cells stained with CD19, c-kit and IgM, analyzed by flow cytometry. Left hand panels show representative histograms of the GFP+ IgM-negative compartment in control (vector) and Foxp1si expressing mice. The right-hand panel shows data enumerating pro-B-cells (CD19+IgM-c-kit+) as a percentage of total GFP+ cells for a representative experiment (n=4). All three experiments showed similar trends and the differences shown here are statistically significant (T-test, p=0.0004).

F. Bone marrow cells stained with B220, IgM and CD43 were analyzed by flow cytometry. Left hand panels show representative histograms of the GFP+ IgM- compartment in control and Foxp1si expressing mice, below which the percentage of cells in each of the four quadrants is shown. The right-hand panel shows data enumerating pre-B-cells (B220+CD43-IgM-) as a percentage of the total GFP+ cells for one representative experiment (n=4). All three experiments showed similar trends and the differences shown here are statistically significant (T-test, p=0.005).

Foxp1 cDNA lacking its 3'UTR is able to rescue the miR-34a mediated block in B-cell development

Next, we tested whether complementation of Foxp1 by an exogenous cDNA not responsive to miR-34a could correct the B-cell phenotype. MSCV based constructs containing the *Foxp1* coding sequence were generated with and without miR-34a as depicted in Figure 4.5A, and designated as the "rescue vector." These constructs contained the entire coding sequence of Foxp1, followed by an internal ribosomal entry sequence (IRES) to allow for GFP expression. The resultant constructs, when used to infect 70Z/3 cells, overexpressed Foxp1 at the protein level, at about 2-3 times endogenous levels (figure 4.5B), and also overexpressed miR-34a (figure 4.5C). Retroviral transduction and bone marrow transfer was completed as before, and we detected constitutive expression of *Foxp1* RNA by RT-qPCR (figure 4.5D). In addition, miR-34a was constitutively expressed in mice receiving the rescue vector, Foxp1+34a, in a quantitatively similar manner to that observed when miR-34a was expressed by itself (figure 4.5E). The resultant mice showed recovery of B-cell numbers in the periphery (data not shown). In the bone marrow, pro-B-cell numbers were significantly decreased

in animals receiving the rescue vector as compared to those receiving miR-34a alone, and similar to pro-B-cell numbers observed in the mice receiving the control vector (figure 4.5F). Similarly, pre-B-cell numbers were comparable to those in control mice, with a significant increase in animals receiving the rescue vector compared with those animals receiving miR-34a alone (figure 4.5G). These findings indicate that B-cell maturation was largely rescued by provision of *Foxp1* lacking its 3'UTR in murine bone marrow. In contrast, such an effect was not observed when *BCL2* was utilized in a similar "rescue experiment" (constructs depicted in Supplemental figure 4.5A). Despite BCL2 being a direct target of miR-34a and the detection of transduced *BCL2* in the bone marrow, the number of pro-B-cells remained elevated, and the number of pre-B-cells did not increase (Supplemental figure 4.5 B,C,D, and E). Hence, the dependence of the miR-34a mediated B-cell phenotype on Foxp1 is demonstrated in both loss of function and rescue contexts, indicating that Foxp1 is a highly specific target of miR-34a during B-cell differentiation.

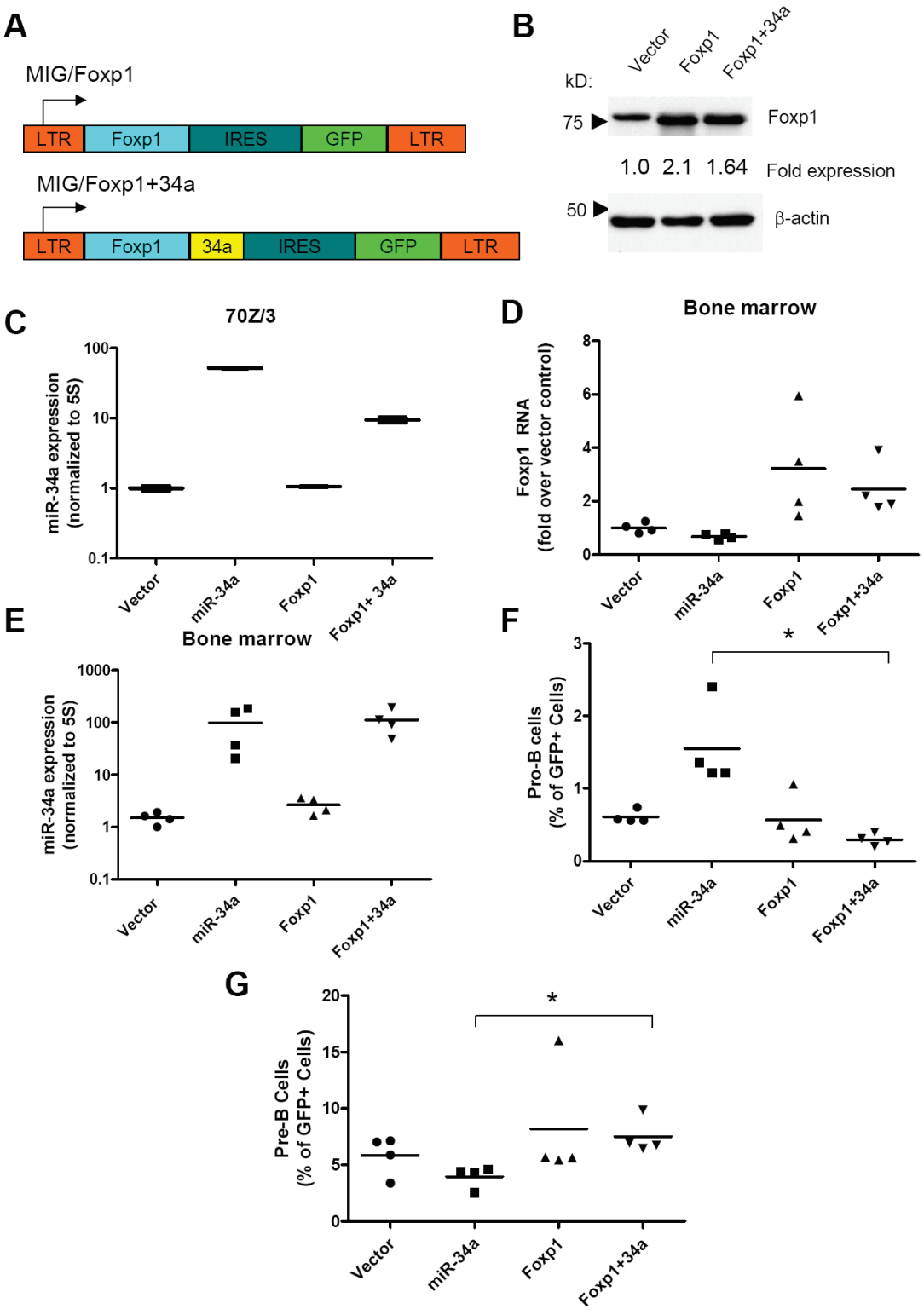

A
MIG/Foxp1
LTR
Foxp1
IRES
GFP
LTR
MIG/Foxp1+34a
LTR
Foxp1
34a
IRES
GFP
LTR
B
Vector
Foxp1
Foxp1+34a
kD:
75
Foxp1
1.0 2.1 1.64
Fold expression
50
β-actin
C
70Z/3
miR-34a expression (normalized to 5S)
100
10
1
0.1
Vector
miR-34a
Foxp1
Foxp1+ 34a
D
Bone marrow
Foxp1 RNA (fold over vector control)
8
6
4
2
0
Vector
miR-34a
Foxp1
Foxp1+34a
E
Bone marrow
miR-34a expression (normalized to 5S)
1000
100
10
1
0.1
Vector
miR-34a
Foxp1
Foxp1+34a
F
*
Pro-B cells (% of GFP+ Cells)
3
2
1
0
Vector
miR-34a
Foxp1
Foxp1+34a
G
*
Pre-B Cells (% of GFP+ Cells)
20
15
10
5
0
Vector
miR-34a
Foxp1
Foxp1+34a

Figure 4.5. Foxp1 expression rescues the miR-34a induced B-cell developmental abnormality.

A. Schematic representation of the constructs used to rescue miR-34a-mediated block in B-cell development.

B. Western blot analysis of extracts from 70Z/3 cells infected with retroviruses prepared from constructs depicted in A. The numbers represent relative expression of Foxp1, compared with control, as determined by using the Scion program.

C. Expression of miR-34a as assayed by RT-qPCR in 70Z/3 cells infected with retroviruses [vector control only, MGP/34a (miR-34a), MIG-Foxp1 (Foxp1), or MIG/Foxp1+34a (Foxp1+34a)].

D. Expression of *Foxp1* in bone marrow, as assayed by RT-qPCR, in mice receiving bone marrow transduced with the same constructs as in C. Data were normalized to mouse L32, which did not show significant differences between the various marrow samples, and represented as fold overexpression as compared with the control vector.

E. Expression of miR-34a in bone marrow, as assayed by RT-qPCR, in mice receiving bone marrow transduced with the same constructs as in C. Data were normalized to 5S levels, which did not show significant differences between the various marrow samples, and represented as fold overexpression as compared with the control vector.

F. Enumeration of pro-B-cells (CD19+IgM-c-kit+) as a percentage of total GFP+ cells for a representative experiment (n=4 for each group). Experiments were repeated twice and the difference shown here is statistically significant (T-test, p=0.0048).

G. Enumeration of pre-B-cells (B220+CD43-IgM-) as a percentage of total GFP+ cells for a representative experiment (n=4 for each group). Experiments were repeated twice and the difference shown here is statistically significant (T-test, p=0.0084).

Knockdown of miR-34a results in increased numbers of mature B-cells in the bone marrow

The evidence that miR-34a acts as a regulator of Foxp1, coupled with the previous evidence that Foxp1 is an activator of the Rag enzymes (Hu et al. 2006) led us to study whether miR-34a loss of function leads to an effect on B-cell development *in vivo*. We did this by attaching a so-called sponge sequence to a reporter (Gentner et al. 2009). In this approach, four consecutive synthetic miR-34a target sequences separated by four 4.6-nucleotide spacer sequences were cloned downstream of GFP in the previously described MGP vector (MGP/anti-34as; figure 4.6A and supplemental Table 4.1). Retrovirus generated from this construct was used to transduce 70Z/3 cells, which were immunoblotted for Foxp1. The cells expressing anti-34as showed an approximately 1.4-fold derepression of Foxp1 at the protein level and also showed a modest derepression at the RNA level (figure 4.6B and data not shown). Luciferase assays performed using synthetic 34a target sites (i.e., a perfectly complementary sequence; 34a-2mer) as well as the Foxp1 3'UTR in the reporter constructs demonstrated derepression as a consequence of expression of anti-34as (figure 4.6C and 4.6D, respectively). This derepression was seen both as an effect on endogenous miR-34a activity (left two bars in figures. 4.6C and 4.6D) and an effect on exogenously overexpressed miR-34a (right two bars in figures. 4.6C-6D). The derepression was specific in that no derepression was seen if there was no

UTR in the reporter plasmid or an irrelevant UTR (figure 4.6E and data not shown). Next, we utilized this construct in a bone marrow transfer experiment, and found reproducible GFP expression in the recipient mice 2 months following transfer (figure 4.6F). Examination of the bone marrow revealed an increase in the mature B-cell population in mice with miR-34a knockdown (figure 4.6G versus 4.6H). These differences were statistically significant (figure 4.6I). Minor differences were seen in the pro-B and pre-B-cell populations, but these were not significant. In the bone marrow of mice transduced with anti-34as, Foxp1 RNA levels were modestly, but significantly derepressed (figure 4.6J). Because Foxp1 RNA levels seem so important in determining the course of B-cell development, we analyzed pooled data from two experiments in which we overexpressed Foxp1 (see figure 4.5), finding that indeed, it is the mature B-cell pool (CD19+ IgM+) that is increased upon modest overexpression of Foxp1 (figure 4.6K).

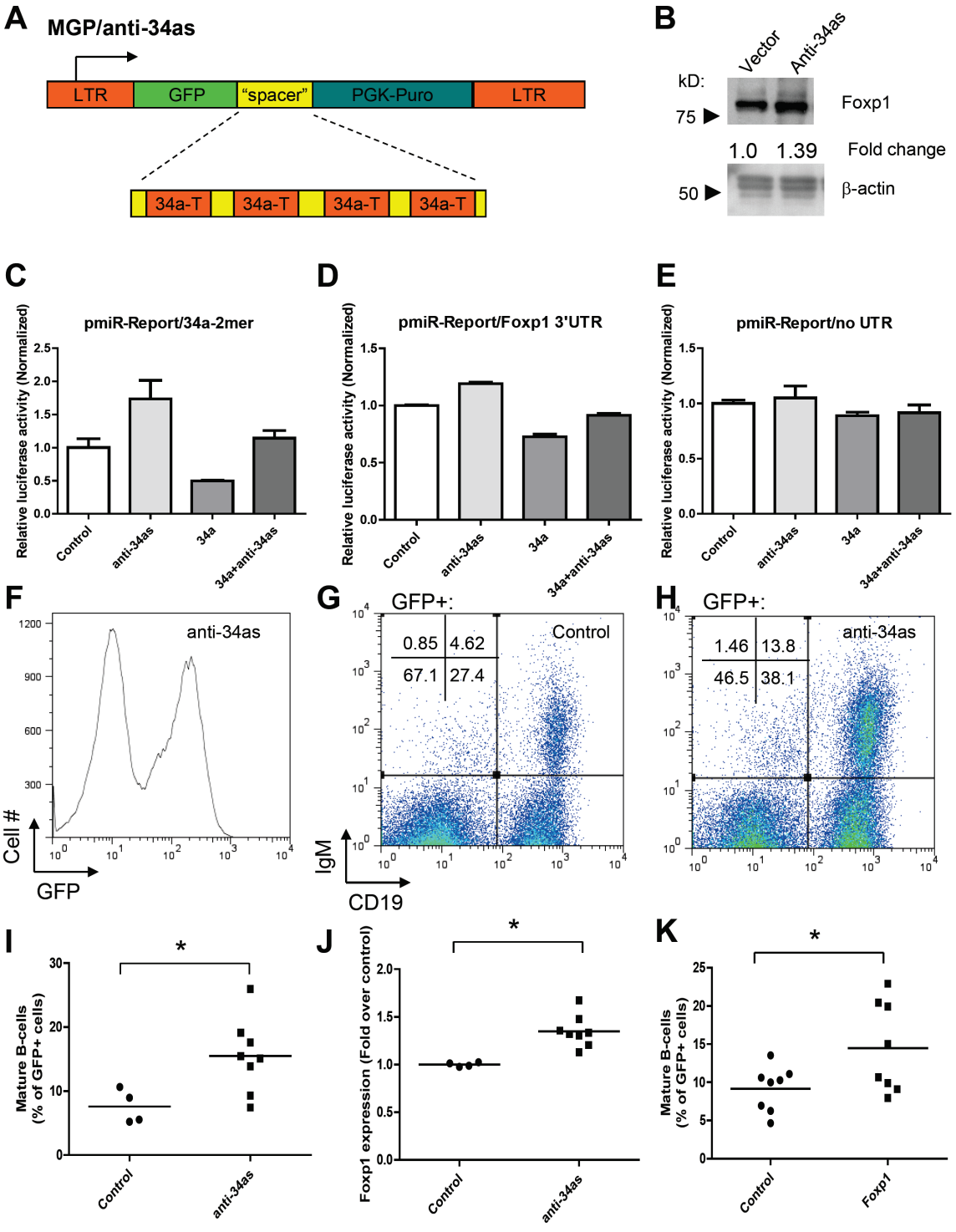

A
MGP/anti-34as
LTR
GFP
"spacer"
PGK-Puro
LTR
34a-T
34a-T
34a-T
34a-T
B
Vector
Anti-34as
kD:
75
Foxp1
1.0
1.39
Fold change
50
β-actin
C
pmiR-Report/34a-2mer
Relative luciferase activity (Normalized)
Control
anti-34as
34a
34a+anti-34as
D
pmiR-Report/Foxp1 3'UTR
Relative luciferase activity (Normalized)
Control
anti-34as
34a
34a+anti-34as
E
pmiR-Report/no UTR
Relative luciferase activity (Normalized)
Control
anti-34as
34a
34a+anti-34as
F
anti-34as
Cell #
GFP
G
GFP+:
Control
0.85
4.62
67.1
27.4
IgM
CD19
H
GFP+:
anti-34as
1.46
13.8
46.5
38.1
I
Mature B-cells (% of GFP+ cells)
Control
anti-34as
J
Foxp1 expression (Fold over control)
Control
anti-34as
K
Mature B-cells (% of GFP+ cells)
Control
Foxp1

Figure 4.6. Knockdown of miR-34a using a "sponge" strategy is effective and results in increased mature B-cells in the bone marrow.

A. Schematic representation of the construct used to knockdown miR-34a expression. The backbone is the MGP vector described previously with the insertion of "spacer miR-Target sites" downstream of the GFP. Abbreviation, 34a-T: miR-34a synthetic target site.

B. Western blot analysis of extracts from 70Z/3 cells infected with retroviruses prepared from construct depicted in A. The numbers represent relative expression of Foxp1, compared with control, as determined by the Scion program.

C-E. Luciferase assays demonstrate that the knockdown of miR-34a by the construct designated in A is specific for miR-34a target-containing 3'UTRs. For these assays, 293T cells were transfected with a β-galactosidase reporter, luciferase reporter constructs containing the designated 3'UTR along with combinations of a miR-34a expression plasmid (pcDNA3/34a) and MGP/anti-34as. All data is normalized for transfection efficiency by a β-galactosidase reporter. These experiments were repeated three times with similar results.

F. Bone marrow FACS plot showing that transduction results in the expression of GFP.

G-H. Bone marrow FACS plot showing GFP+ cells plotted for expression of CD19 and IgM. Bone marrow is from a mouse transduced with MGP (Control; G), and a mouse transduced with MGP/anti-34as (H).

I. Enumeration of mature B-cells (CD19+IgM+) as a percentage of total GFP+ cells in mice recieivng either control or the knockdown construct for miR-34a (n=4 for control

MGP; n=8 for anti-34as). Experiments were repeated twice and the difference shown here is statistically significant (T-test, p=0.0285).

J. Foxp1 expression in bone marrow transductant mice reciveing marrow with either MGP (Control) or anti-34as marrow (n=4 for control MGP; n=8 for anti-34as). The difference in expression is statistically significant (T-test, p=0.0022).

K. Enumeration of mature B-cells (CD19+IgM+) as a percentage of total GFP+ cells in mice recieivng either control or constitutive expression contruct for Foxp1 (n=8 for control MIG; n=8 for MIG/Foxp1). Experiments were repeated twice and the difference shown here is statistically significant (T-test, p=0.0383).

Discussion

We describe here a novel role for miR-34a in B-cell development that is largely explained by its repression of Foxp1. The block induced by miR-34a specifically involves Foxp1 because it is rescued by Foxp1 expression and not expression of another miR-34a target, BCL2. In early B-cell development, loss of Foxp1 is known to cause a block at the pro-B-cell to pre-B-cell transition, and a near complete absence of B-lymphocytes in the peripheral lymphoid tissues (Hu et al. 2006). Additionally, Foxp1 downregulation is essential to monocytic differentiation, indicating a role in directing hematopoietic progenitors towards a B-cell fate and away from the myeloid fate (Shi et al. 2008, 2004). By partial repression of Foxp1 in the bone marrow, constitutive miR-34a expression slows passage through the pro-B-cell to pre-B-cell transition, resulting in a lower number of circulating B-cells. Conversely, loss of miR-34a function results in increased numbers of mature B-cells, implying a less stringent or faster transit through this checkpoint. In differentiated B-cells, *FOXP1* appears to act as a B-cell oncogene, whose dysregulated expression can be a consequence of translocations to the *IGH* locus, in cases of marginal zone lymphoma, and through amplification in many cases of diffuse large B-cell lymphoma (Lenz et al. 2008; Streubel et al. 2005; Wlodarska et al. 2005). Thus, Foxp1 is both needed for B-cell differentiation and is a danger in mature B-cell life. We speculate that the effects of miR-34a on Foxp1 may be the mode through which p53 suppresses this potentially oncogenic protein in postgerminal center B-cells. Such an effect would parallel the previously described role of miR-34a as a direct connection between the tumor suppressor p53 and the oncogenic protein Bcl-2.

The effect of miR-34a on the B-cell developmental pathway is consistent with previously reported abnormalities seen with a deficiency of p53, namely an increased number of pre-B-cells as well as B-cells, with the latter finding also as a consequence of loss of miR-34a function (Lu et al. 2000; Lu and Osmond 2000). With miR-34a constitutively expressed, we find the opposite-an increase in pro-B-cells and a decrease in pre-B and B-cells. Our findings imply a connection between p53 and Foxp1 via the action of miR-34a. In early B-cell development, Foxp1 activates several B-cell factors, such as E2a and Pax-5 and binds to the enhancers of the *Rag* genes (Hu et al. 2006). This activity of Foxp1 may be dependent on the downregulation of miR-34a, releasing repression of Foxp1, and allowing B-cell development to proceed. There appears to be a differential requirement for *Rag* gene expression in D-J recombination as opposed to V-DJ recombination during pro-B-cell development, with the latter process requiring higher levels of Rag (Hu et al. 2006). The data we have presented indicates that miR-34a levels are inversely correlated with Foxp1 expression levels during the different stages of B-cell development. There is a downregulation of miR-34a at the pro- to pre-B-cell transition, which we interrupt by constitutive expression. With miR-34a loss of function, mature B-cells are increased, likely arising from the loss of a miR-34a dependent checkpoint. In both the gain-of-function and loss of function contexts, the changes in B-cell development are accompanied by changes in the levels of Foxp1. The idea that Foxp1 levels are important for the output of B-cells from the bone marrow is borne out by the finding that mature B-cells are increased in the marrow of mice that overexpress Foxp1 and that a partial arrest at the pro-B to pre-B transition is found in Foxp1si-expressing mice. We do not know if there is a role for regulation of p53 and/or miR-34a during the

pro-B to pre-B transition, but we speculate that it may be involved in the alternating generation and repair of double-stranded DNA breaks that occurs when heavy chain genes are being rearranged (Perkins et al. 2002).

The effects of miRNAs in B-cell development have been assessed globally as well as in terms of specific miRNAs. The deletion of Dicer at the earliest stages of B-cell development leads to a block in development at the pro-B to pre-B transition (Koralov et al. 2008). In this case, bioinformatic analysis showed that upregulated genes in Dicer-deficient B-lineage cells were likely those targeted by the miR-17~92 cluster, and that ablation of the pro-apoptotic protein Bim (a target of miR-17~92) or transgenic overexpression of Bcl-2 could partially rescue the developmental block. This target specificity is distinct from the block induced by miR-34a, because Bcl-2 increased survival of miR-34a expressing pro-B-cells but did not significantly promote their differentiation. B-cell developmental blocks have also been seen following the disruption of specific miRNAs. Early expression of miR-150 led to a block in B-cell development, while its deletion led to expansions of certain B-cell subsets (Xiao et al. 2007; Zhou et al. 2007). In this case, the relevant target appeared to be c-Myb, a transcription factor involved in many stages of lymphocyte differentiation. Overexpression of the miR-17~92 cluster led to a B-lymphoproliferative disease, likely as a consequence of suppression of the tumor suppressor gene PTEN and Bim (Xiao et al. 2008). Other miRNAs with effects of B-cell development include miR-181a and miR-155, which promote early B-cell development and the germinal center response, respectively (Chen et al. 2004; Rodriguez et al. 2007; Thai et al. 2007; Vigorito et al. 2007). Specific targeting by miR-155 in the context of B-cell development has been extensively

investigated and it appears that multiple targets are responsible for miR-155-induced phenotypes (Chen et al. 2008; Pedersen et al. 2008; Vigorito et al. 2007).

The specificity of miRNA targeting remains incompletely understood. On the one hand, miRNAs, including miR-34a, are predicted to target hundreds of genes by computational algorithms. On the other hand, an increasing number of reports suggest that in particular developmental contexts, one or a few genes are most important in the causation of a miRNA-mediated phenotype. Such observations have been made in both loss of function and gain-of-function contexts, indicating the general applicability of this principle for particular miRNAs. Our study extends these observations by demonstrating a specificity of miR-34a targeting for Foxp1 in early B-lymphoid development. This is based on both recapitulation and rescue of the phenotype by Foxp1 loss- and gain-of-function, respectively. The basis of such specificity is likely the fact that certain cell fate decisions hinge on small changes in the expression of critical lineage specification genes, and miRNAs can cause such changes. From this, it can be inferred that the study of miRNAs in hematopoietic development may illuminate critical points in cell fate decisions, and bring novel insights into this complex and dynamic developmental system.

Our findings may be of relevance in human disease as others have observed that miR-34a is functionally lost (either directly or as a consequence of p53 deletion) in chronic lymphocytic leukemia but is overexpressed in acute myeloid leukemia (Isken et al. 2008; Mraz et al. 2009; Zenz et al. 2009). In our study overexpression of miR-34a led to disruptions in B-cell development, but not myeloid development. We have confirmed independently that miR-34a is overexpressed in AML by RT-qPCR (data not shown) and that its overexpression does not correlate with that of miR-155, which we had examined

previously in the same set of samples (Isken et al. 2008). Since we did not observe a myeloproliferative disorder in miR-34a-overexpressing mice, any mechanism whereby miR-34a contributes to leukemia is likely to be distinct from that observed in miR-155 expressing mice (O'Connell et al. 2008). Our findings suggest one possibility-the expression of miR-34a may affect the phenotype of the leukemic cells by blocking cells transformed by other oncogenic lesions from differentiating into B- lineage cells. Indeed, some recent studies indicate that there may be a progenitor with lymphoid characteristics that gives rise to acute myeloid leukemia (Deshpande et al. 2006). Our results suggest that miRNA expression may be one mechanism whereby the leukemia assumes a distinctive lymphoid or myeloid phenotype. Such phenotypic specification certainly has clinical relevance, as acute myeloid leukemia in general remains the more difficult of the acute leukemias to treat. However, further work needs to be done to understand the role that miR-34a plays in leukemia and how its action or inhibition can be parlayed into novel therapeutic strategies.

Materials and Methods

Cell Culture

All cells were cultured in a sterile incubator maintained at 37°C with 5% CO_2. 293T cells were cultured in complete DMEM with 10% FBS, 100 units/mL penicillin and 100 units/mL streptomycin. WEHI-231 and 70Z/3 cells were cultured in complete RPMI with 10% FBS, 100 units/mL penicillin, 100 units/mL streptomycin and 50 μM 2-Mercaptoethanol.

DNA constructs

The MGP vector system has been described previously, and further detailed cloning strategies are available upon request (O'Connell et al. 2009). Table 4.S1 contains sequence information for all oligonucleotides used to build new constructs. MGP-34a expresses human miR-34a, MGP-FoxP1si expresses a siRNA against *FoxP1*, and MGP-BCL2si expresses a siRNA against *BCL2*. MiR-34a and siRNAs were built using the Invitrogen BlockiT pol II miR RNAi strategy as described previously, using the miR-155 arms for processing (O'Connell et al. 2009). SiRNA sequences were predicted using the Invitrogen BlockiT miR RNAi designer. In addition, a 300 base pair sequence of human genomic DNA encoding the native miR-34a mature sequence, endogenous stem-loop structure and flanking sequences was PCR-amplified using the 34a.NotI.f and 34a.XhoI.r primers and cloned into the MGP vector using the NotI and XhoI sites, as well as into pcDNA3 using 34a.EcoRI.f and 34a.XhoI.r primers and cloning into the EcoRI and XhoI sites. The MGP-based vector is referred to as MGP-miR-34a-lo; as the expression level of miR-34a from this vector was lower than that from the vector utilizing miR-155 arms. The last set of expression vectors, utilized for expression of miR-34a in human cell lines,

was developed from the FUGW vector system (Lois et al. 2002). For this purpose, miR-34a genomic sequence was cut out of pcDNA3-34a and blunt-ligated into the FUGW EcoRI site, and screened for by using standard recombinant DNA techniques. For reporter assays, two regions of the *FoxP1* 3' UTR were amplified by PCR, each containing a putative miR-34a binding site as determined by TargetScan (Lewis et al. 2005). The 5' segment was 637 base pairs and the 3' segment was 760 base pairs. 2000 base pairs of the *BCL2* 3' UTR was also PCR-amplified. These PCR products were cloned into pmiReport (Ambion, Austin, TX). The middle 6 nucleotides of the miR-34a seed regions in both the *FoxP1* 3'UTR 5' segment and the *BCL2* 3' UTR segment were then mutated using PCR, and the product was cloned into pmiReport to yield Foxp1mt and BCL2mt constructs. A positive control insert consisting of two miR-34a antisense sites (antisense 2-mer) was constructed by annealing oligonucleotides with SpeI and HindIII sticky ends and cloning this into pmiReport. Mouse *CEBPβ* and *PU.1* 3'UTRs cloned into pmiReport were described previously (O'Connell et al. 2008). T For the rescue vectors, we utilized the MSCV-IRES-GFP (MIG) vector as a backbone. The cDNAs for *Foxp1* and *BCL2* were PCR amplified from pCMV-Sport-Foxp1 (Open Biosystems) and pDNR-Dual-BCL2 (Harvard PlasmID Core Facility), and cloned between the BglII and XhoI sites in MIG, yielding MIG/Foxp1 and MIG/BCL2, respectively. The cDNAs contain the entire coding sequence for Foxp1 (listed in supplemental table 4.S1) and BCL2, respectively. miR-34a was cloned by PCR amplification from pcDNA3-34a and cloned between the XhoI and EcoRI sites in the MIG vectors, yielding MIG/Foxp1+34a and MIG/BCL2+34a vectors respectively. The MGP/anti-34as construct was generated by annealing the 34a-spacer-f and 34a-spacer-r

oligonucleotides (designed with XhoI/NotI sticky ends), digesting the parental MGP vector with XhoI and NotI, and ligating the annealed oligonucleotides downstream of GFP. The spacers between each miR-34a binding site were the same as those previously published (Gentner et al. 2009).

Mice and Bone marrow reconstitution experiments

C57BL/6 mice were bred and housed in the Caltech Office of Laboratory Animal Resources (OLAR) facility. The Caltech Institutional Animal Care and Use Committee (IACUC) approved all experiments related to mice. For reconstitution experiments, lethally irradiated mice were reconstituted via either tail-vein or retro orbital injection with 5-FU enriched bone marrow spin- infected with MSCV-based retroviruses (MGP-34a, MGP-siFoxP1, MGP-siBCL2, MGP, MIG/Foxp1, MIG/Foxp1+34a, MIG/BCL2, MIG/BCL2+34a, and MGP/anti-34as). Retroviruses were prepared by calcium phosphate based or TransIT-293 based-transient transfection as previously described (O'Connell et al. 2009, 2008). Experimental groups of 4-5 mice received each of the vectors described. Recipient mice were then monitored for health and a peripheral blood sample removed at 1 month for flow cytometric analysis. Mice were sacrificed 2 months after reconstitution and were examined by morphology, FACS and RT-qPCR as described below. All experiments were repeated at least twice and in most cases, 3 times, as described in figure legends.

Flow cytometry

Cells were isolated from the relevant tissue, homogenized, and red blood cells were lysed using RBC lysis buffer (Biolegend). Cells were stained with the following Fluorophore-conjugated antibodies (all from Ebioscience): CD3ε, CD11b, CD19, B220, CD43,

CD117 (c-kit), CD25, IgM, Gr-1, Ter-119, AA4.1, CD4, and CD8α, in various combinations to delineate the hematopoietic lineages and various stages of B-cell differentiation. Cells were sorted using a FACSCalibur (Becton Dickinson) and all data were analyzed using FloJo software (Treestar). Specific gating strategies to delineate various B-cell populations are available upon request. Data were presented as the percentage of GFP positive cells in the relevant hematopoietic compartment. These data were also compared against the GFP-negative cells within the same mouse.

Cell sorting for RNA analysis at various stages of B-cell differentiation

Bone marrows were extracted from 2 mice, lysed in RBC lysis buffer, and spun down. Cells were then resuspended in lymphocyte FACS buffer (1X Hanks Buffered Salt Solution, 10mM HEPES, 2.5 mg/mL BSA), filtered through a 40μm mesh, blocked with FcBlock (Becton Dickinson), and depleted on a magnetic column for CD11b, Gr-1, Ter119, CD3ε, and NK1.1 using biotinylated antibodies and streptavidin-MACS beads, as per manufacturer's instructions (Miltenyi). Cells were then stained with c-kit, B220, CD43, IgM, Streptavidin, and CD138, and sorted on a BD Biosciences FACSVantage flow cytometer (Caltech Core facility). 4 populations of cells were collected: Lin- (B220-, IgM-, Streptavidin-, and CD138-); pro-B-cells (c-kit+B220+CD43+IgM-streptividin-), pre-B-cells (B220+CD43-c-kit-IgM-streptavidin-) and B-cells (B220+c-kit-CD43-IgM+strepatavidin-). RNA was isolated by TRIzol (Invitrogen) purification and subjected to RT-qPCR as described below. These sorts were repeated three times, with similar results.

Sequence alignments

The miR-34a seed region and Foxp1 3' UTR sequences from human (*Homo sapiens*), mouse (*Mus musculus*), rat (*Rattus norvegicus*) and dog (*Canis familiaris*) were obtained and aligned using TargetScan (Lewis et al. 2005, 2003).

Experiments with cell lines

VSV-G-pseudotyped FUGW lentiviruses were made using TransIT-293 based transient transfections of 293T cells as previously described (O'Connell et al. 2009). The viruses were then used to infect NALM6 human pre-B-cells by spin-infection at 1,200xg for 90 minutes at 30 degrees centigrade, supplemented with 10μg/mL polybrene (Chemicon). Single cell clones were derived by subjecting the cells to limiting dilution assays. Clones were maintained in RPMI supplemented with 10% FBS and antibiotics. For examination of Foxp1 levels in murine cells, MSCV-based retroviruses were prepared as described previously, pseudotyped with pCL-Eco, and used to infect 70Z/3 murine pre-B-cells. Cells were expanded for 3-4 days, and then utilized for RNA purification or protein extraction for Western blotting.

Luciferase Reporter assays

2 x 10^5 293T cells were cultured for 18 hours and subsequently transfected with relevant plasmids with TransIT 293. Transfected plasmids included pcDNA3, pcDNA3-34a, β-gal expression vector, and pmiReport vectors. For the derepression assays with the sponge construct, we added in MGP (empty vector) or MGP/anti-34as. After 48 hours, cells were lysed using Reporter Lysis Buffer (Promega, Madison, WI) and firefly luciferase levels were measured in an Optocomp I luminometer (MGM Instruments, Hamden, CT) using a dual luciferase reporter assay kit (Promega) as per manufacturer's

instructions. β-galactosidase expression was assayed using a β-gal reporter gene assay kit (Roche, Basel, Switzerland) and used to normalize luciferase values.

RNA preparation and quantitation

RNA was isolated using TRIzol as per manufacturer's instructions. SYBR Green based quantitative real-time PCR (qPCR) was performed using the 7300 real-time PCR system (Applied Biosystems, Foster City, CA) to assay miR-34a, 5s, *FoxP1* mRNA, *BCL2* mRNA and L32 mRNA levels as described previously (O'Connell et al. 2008). Mature miR-34a and 5s RNA were assayed using a miRVana miRNA detection kit as per manufacturer's instructions (Ambion). Human *FOXP1*, *BCL2* and L32 mRNA were detected using specific primers and utilizing the SybrGreen system (Applied Biosystems) (Table 4.S1).

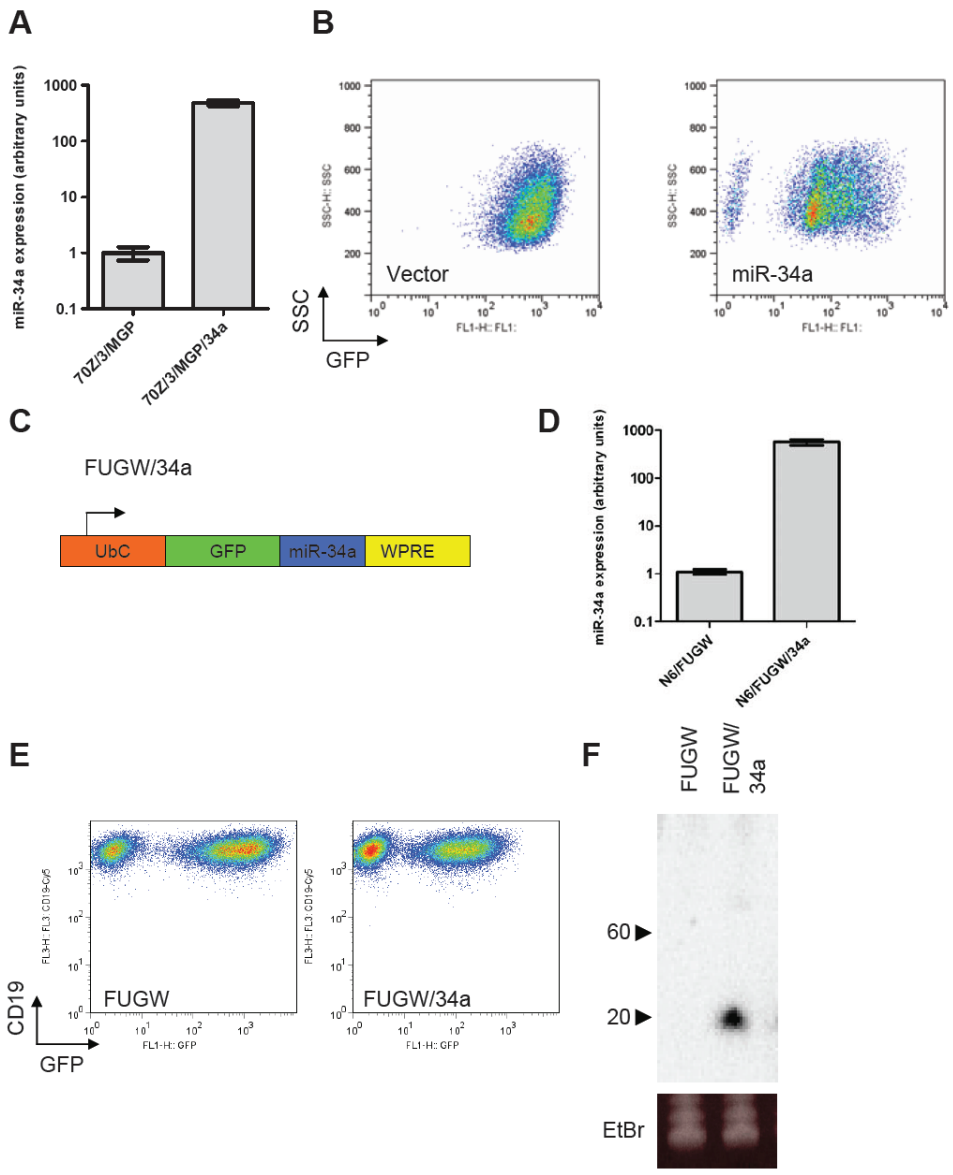
Western blotting

Total cell extracts were fractionated by electrophoresis on 10% SDS polyacrylamide gel and electroblotted to Trans-Blot nitrocellulose membrane (Bio-Rad, Hercules, CA) using a semi-dry transfer apparatus (Bio-Rad). Protein detection was performed using the following antibodies: FoxP1 (ab16645) (Abcam, Cambridge, MA), β-Actin (A1978) (Sigma, St. Louis, MO); GAPDH (sc-47724), α-Tubulin (sc-5286), goat anti-mouse HRP-conjugated secondary antibody (sc-2005), goat anti-rabbit HRP-conjugated secondary antibody (sc-2004) (Santa Cruz Biotechnology, Santa Cruz, CA). Expression intensities were determined using Scion Image software.

Statistical tests

All statistical analyses were performed using Microsoft Excel. Expression and flow cytometric data were first analyzed by an F-test to determine whether the distributions

were homo- or heteroscedastic. Then, the correct type of unpaired, two-tailed T-test was applied to determine if the distributions were statistically different. T-tests that returned p-values of less than 0.05 were considered to be statistically significant.



Supplemental figure 4.S1. ***In vitro*** **analyses of vectors used in this study.**

A. RT-qPCR analysis of expression of miR-34a from the vector described in Figure 4.2 shows overexpression following transduction of 70Z/3 cells.

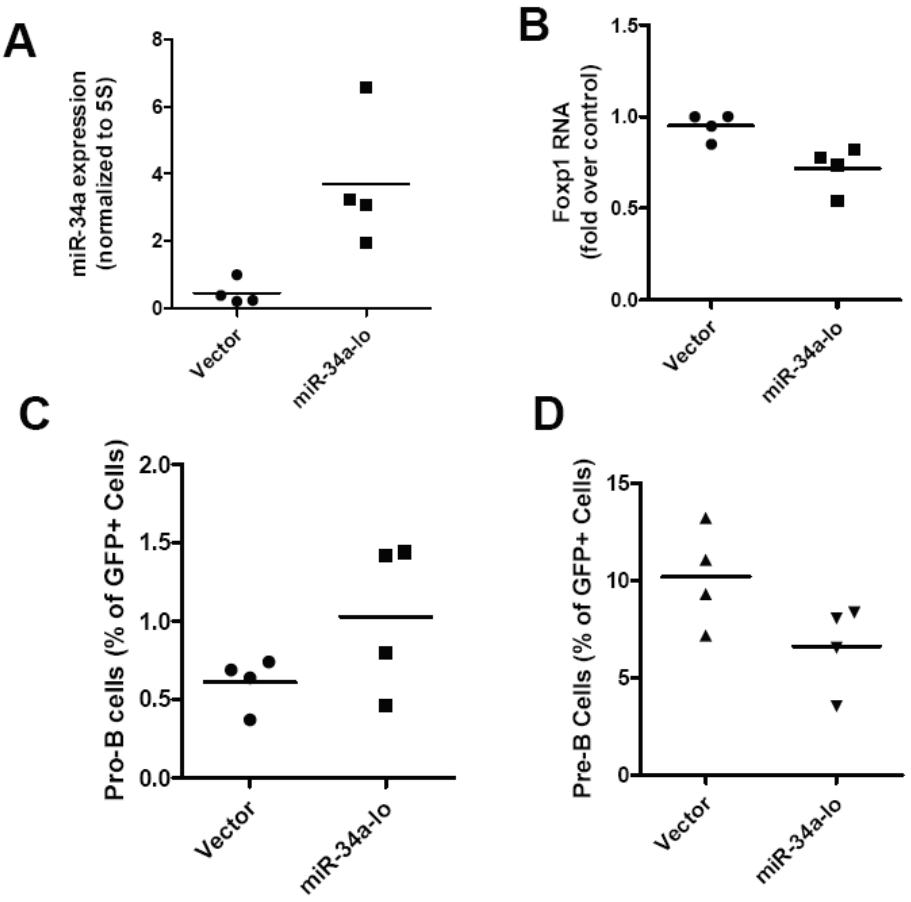
B. Flow cytometric analysis of GFP expression in 70Z/3 cells following transduction with either MGP(vector) or MGP/34a (miR-34a).

C. Schematic representation of lentiviral construct used to express miR-34a in human cell lines. A ubiquitin promoter drives expression of GFP and miR-34a (Lois et al. 2002).

D. miR-34a expression was measured by RT-qPCR in NALM6 human pre-B-cells following lentiviral transduction of FUGW or FUGW/miR-34a.

E. Flow cytometric analysis of GFP expression in NALM6 cells following lentiviral transduction of either FUGW or FUGW/miR-34a.

F. Northern blot analysis of miR-34a expression in NALM6 cells transduced with the lentiviral vectors, FUGW and FUGW/miR-34a.



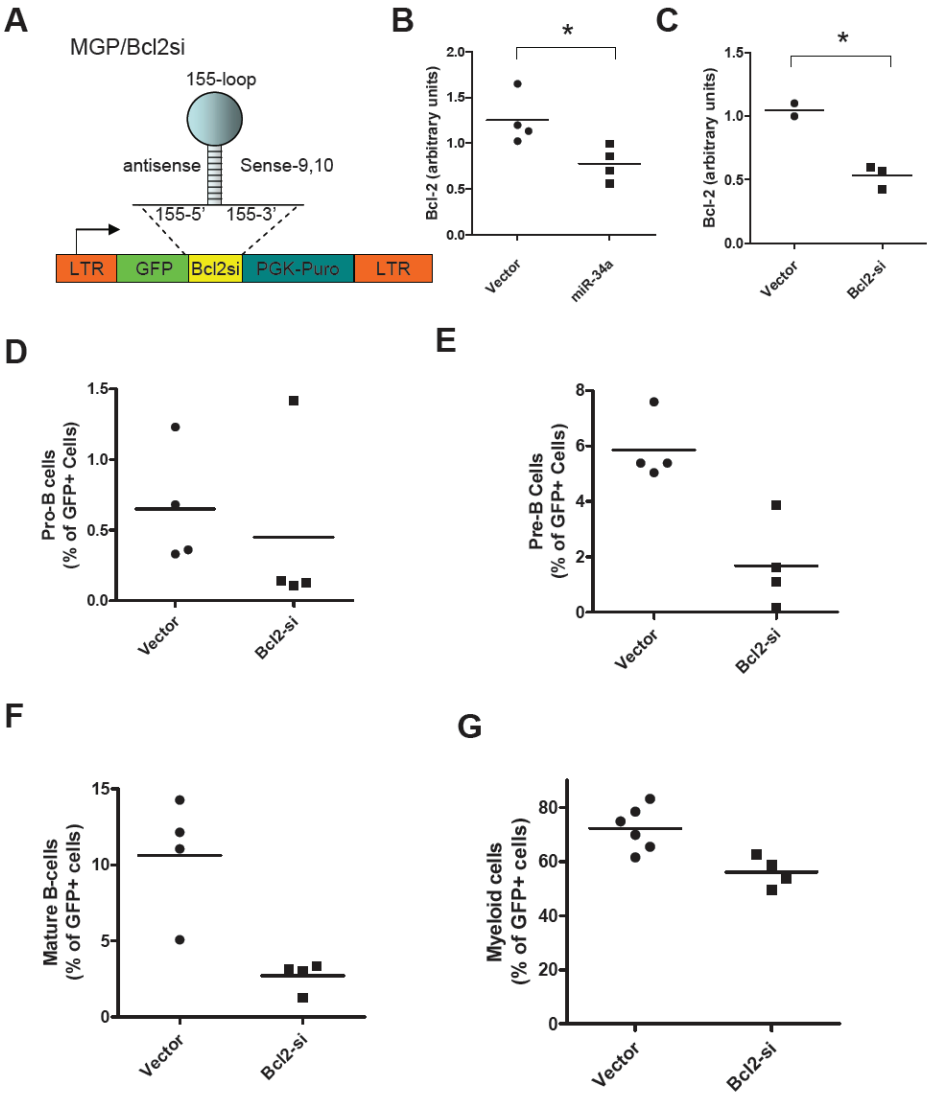
Supplemental figure 4.S2. Low-level miR-34a expression has subtle impacts on B-cell development.

A. miR-34a expression was measured in bone marrow samples of mice receiving MGP-transduced (vector) or MGP-miR-34a-lo (miR-34a-lo) vectors. As described in the methods section, miR-34a-lo contains the endogenous stem-loop structure and 5' and 3' flanking regions found in the genomic sequence of miR-34a. This results in production of lower amounts of mature miR-34a (n=4 for each group).

B. Foxp1 expression was measured in the bone marrow by RT-qPCR in the same mice as in A. A modest reduction in Foxp1 levels is seen, but less than that observed in mice overexpressing miR-34a at high levels.

C. Enumeration of pro-B-cells in mice described in A. There is an elevation of pro-B-cells, but results are not statistically significant. This experiment was repeated three times, with similar trends being observed.

D. Enumeration of pre-B-cells in mice described in A. There is a mild decrease in pre-B-cells, but the results are not statistically significant ($p=0.08$).



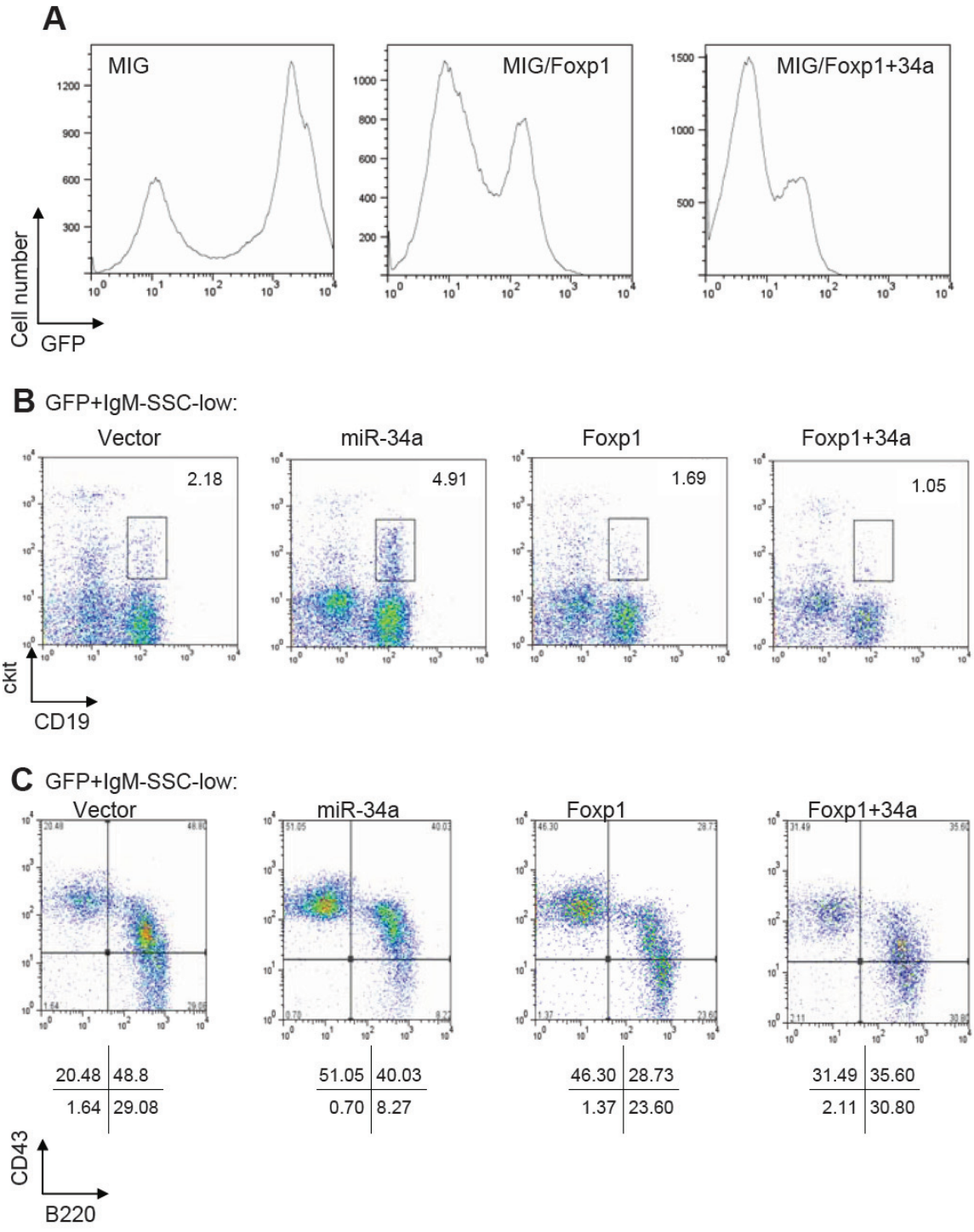
Supplemental figure 4.S3. Bcl2 inhibition does not recapitulate the B-cell phenotype induced by miR-34a.

A. Vector design for MGP/Bcl2si which produces a siRNA that targets Bcl-2.

B. Bcl-2 expression in murine bone marrow measured by RT-qPCR, in mice that receivied marrow transduced with MGP (vector) or MGP/miR-34a (miR-34a). This is the result from a representative experiment (n=4; T-test, p=0.03). A second experiment showed a similar trend.

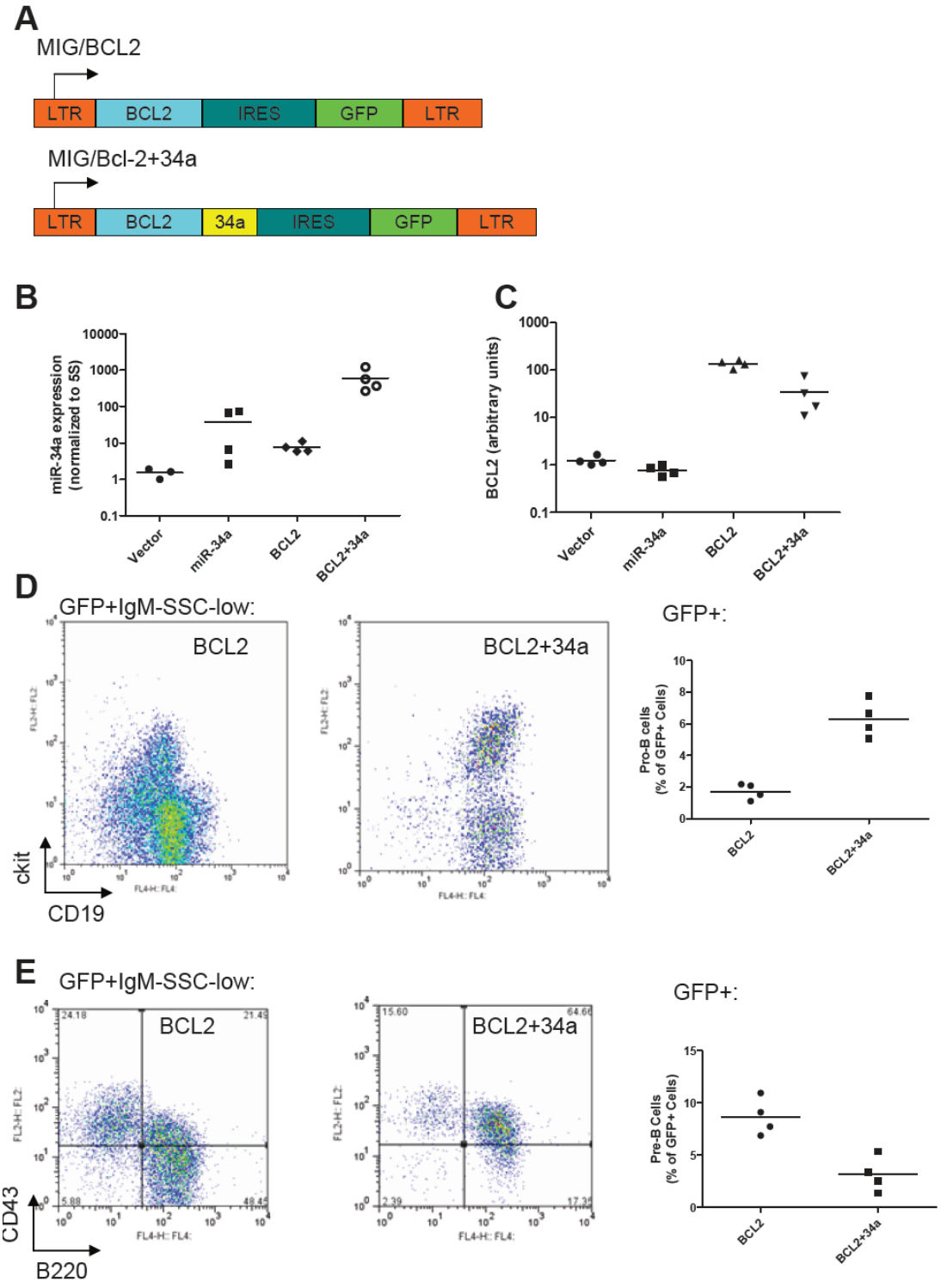
C. Bcl-2 expression in murine bone marrow, measured by RT-qPCR in MGP- or MGP-Bcl2si-transduced mouse bone marrow. As expected, there is knockdown of Bcl2.

D-G. Enumeration of pro-B-cells (D), pre-B-cells (E), mature B-cells (F), and myeloid cells (G) in mice receiving marrow transduced with MGP (Vector) or MGP-Bcl2si (Bcl2-si). Note that all B-cell lineages and myeloid lineage cells show a trend toward reduction in the marrows that express Bcl2-si.


A
MIG
MIG/Foxp1
MIG/Foxp1+34a
Cell number
GFP
B GFP+IgM-SSC-low:
Vector
miR-34a
Foxp1
Foxp1+34a
2.18
4.91
1.69
1.05
ckit
CD19
C GFP+IgM-SSC-low:
Vector
miR-34a
Foxp1
Foxp1+34a
20.48 48.8
1.64 29.08
51.05 40.03
0.70 8.27
46.30 28.73
1.37 23.60
31.49 35.60
2.11 30.80
CD43
B220

Supplemental figure 4.S4. Foxp1 cDNA rescues the phenotype caused by miR-34a.

A. Flow cytometric analysis of bone marrow from recipient mice transduced with MIG, MIG/Foxp1, or MIG/Foxp1+34a. Shown is a histogram of GFP fluorescence. Note that the mean fluorescence as well as the size of the positive population is lowered by the addition of Foxp1 to MIG and is further lowered by the addition of the miR-34a element. B. Flow cytometry histograms of GFP+ cells analyzed for pro-B-cell markers (B) and pre-B-cell markers (C). The increased number of pro-B-cells seen in miR-34a mice (CD19+ c-kit+) is not seen in Foxp1-expressing or Foxp1+34a expressing mice (B). The decreased number of pre-B-cells (CD43-B220+) observed in miR-34a mice is not seen in Foxp1-expressing or Foxp1+34a expressing mice (C).


A
MIG/BCL2
LTR
BCL2
IRES
GFP
LTR
MIG/Bcl-2+34a
LTR
BCL2
34a
IRES
GFP
LTR
B
miR-34a expression (normalized to 5S)
10000
1000
100
10
1
0.1
Vector
miR-34a
BCL2
BCL2+34a
C
BCL2 (arbitrary units)
1000
100
10
1
0.1
Vector
miR-34a
BCL2
BCL2+34a
D
GFP+IgM-SSC-low:
BCL2
BCL2+34a
ckit
CD19
GFP+:
Pro-B cells (% of GFP+ Cells)
BCL2
BCL2+34a
E
GFP+IgM-SSC-low:
BCL2
BCL2+34a
CD43
B220
GFP+:
Pre-B Cells (% of GFP+ Cells)
BCL2
BCL2+34a

Supplemental figure 4.S5. Bcl2 does not rescue the B-cell developmental block induced by miR-34a.

A. Schematic representation of constructs encoding BCL2 and BCL2+miR-34a.

B. miR-34a expression in MIG (vector), MGP/34a (miR-34a), MIG/BCL2(BCL2) and MIG/BCL2+34a (BCL2+34a)-transduced mice, as measured by RT-qPCR.

C. BCL2 expression, measured by RT-qPCR in the same mice as in (B).

D. Bone marrow cells stained with CD19, c-kit and IgM, analyzed by flow cytometry. Left hand panels show representative histograms of the GFP+ IgM-negative compartment in BCL2 and BCL2+34a expressing mice. The right-hand panel shows data enumerating pro-B-cells (CD19+IgM-c-kit+) as a percentage of total GFP+ cells (n=4). The number of pro-B-cells was not significantly different in BCL2 mice versus control, but was significantly increased in BCL2+34a mice (T-test, p=0.0003).

E. Bone marrow cells stained with B220, IgM and CD43 were analyzed by flow cytometry. Left hand panels show representative histograms of the GFP+ IgM- compartment in BCL2 and BCL2+34a expressing mice. The right-hand panel shows data enumerating pre-B-cells (B220+CD43-IgM-) as a percentage of the total GFP+ cells (n=4). The number of pre-B-cells was not significantly different in BCL2 mice versus control, but was significantly increased in BCL2+34a mice (T-test, p=0.004).

Supplemental Table 4.S1. Cloning primers, template oligos, cDNA qPCR primers and cDNA sequence of Foxp1.

Primer	Sequence
miR-34a BlockiT Template	GAAGGCTGTATGCTGTGGCAGTGTCTTAGCTGGTTGTGTTTTGGCC ACTGACTGACACAACCAGAAGACACTGCCACAGGACACAAGGCCTG
siFoxP1 BlockiT Template	GAAGGCTGTATGCTGAATATCTGCTGAAGAAATGGGGTTTTGGCCA CTGACTGACCCCATTTCCAGCAGATATTCAGGACACAAGGCCTG
siBCL2 BlockiT Template	GAAGGCTGTATGCTGTATCATACCCTGTTCTCCCGGGTTTTGGCCA CTGACTGACCCGGGAGAAGGGTATGATACAGGACACAAGGCCTG
FoxP1 3'UTR segment1 SpeI Fw	TTCACTAGTGGACACTGAGGACGCCCGACAGAGCTTGTGCACCTAA GCTGCAGACCAAGCCTTTGCCCAGAATTTAAGGATTCCA
FoxP1 3'UTR segment1 HindIII Rev	TTCAAGCTTCCCAATACTGCTGCGTGGAATGA
FoxP1 3'UTR segment1 Mut HindIII Fw	TTCACTAGTGGACACTGAGGACGCCCGACAGAGCTTGTGCACCTAA GCTGCAGACCAAGCCTTTGCCCAGAATTTAAGGATTCCAATGGACG ACCTATTTGCACAGTACTGCATGTTGATTATCTGACGGTTTACTCC
FoxP1 3'UTR segment2 SpeI Fw	TTCACTAGTTCAGGCTTTACAGCAAGCAAACTTCAC
FoxP1 3'UTR segment2 MluI Rev	TTCACGCGTGTTTAAGATAGCCAGGAAGGCAGTGGTAG
BCL2 3'UTR SpeI Fw	TTCACTAGTAGTCAACATGCCTGCCCCAAACA
BCL2 3'UTR HindIII Rev	TTCAAGCTTTCAGTGGCCAGTGACATCCAGGT

BCL2 3'UTR Mut fragment1 Rev	TGTCTTAAATAAATAAATCTTTTTTTCTTAATAATGTAAAAAATAAATG ATATTTCCCTTTCCGTCAAAATAGCTG
BCL2 3'UTR Mut fragment2 Fw	CAGCTATTTTGACGGAAAGGGAAATATCATTTATTTTTTACATTATTA AGAAAAAAAGATTTATTTATTTAAGACA
2mer positive control for Luc Assay oligo1	5'-P-CTAGTACAACCAGCTAAGACACTGCCAACAACCAGCTAAGAC ACTGCCAA
2mer positive control for Luc Assay oligo2	5'-P-AGCTTTGGCAGTGTCTTAGCTGGTTGTTGGCAGTGTCTTAGCT GGTTGTA
MIG.Foxp1.f	TTCAGATCTATGATGCAAGAATCTGGGTCTGAGACA
MIG.Foxp1.r	TTCCTCGAGTCACTCCATGTCCTCATTTACTGGTTC
MIG.Bcl2.f	GAAGATCTATGGCGCACGCTGGGAGAAC
MIG.Bcl2.r	CCGCTCGAGCTACTTGTGGCCCAGATAGGCACC
q.Foxp1.f	AAGTGTTTTGTGCGAGTAGAGAA
q.Foxp1.r	GGGAAGGGTTACCACTGATCTT
q.Bcl2.f	GTCGCTACCGTCGTGACTTC
q.Bcl2.r	CAGACATGCACCTACCCAGC
q.L32.f	AAGCGAAACTGGCGGAAA C
q.L32.r	TAACCGATGTTGGGCATCAG
34a.genomic.EcoR I.f	CGGAATTCGGGCTGGTCTTGAACTCCTG

34a.genomic.XhoI.r	CCGCTCGAGAAGTCCTGGCGTCTCCCACT
34a.genomic.XhoI.f	TTCCTCGAGGGGCTGGTCTTGAACTCCTGG C
34a.genomic.EcoRI.r	TTCGAATTCAGAAAGTCCTGGCGTCTCCCACTGG
34a.spacer.f	ggccgcACAACCAGCTAAGACACTGCCAcgatACAACCAGCTAAGACACTGCCA accggtACAACCAGCTAAGACACTGCCAtcacACAACCAGCTAAGACACTGCCAc
34a.spacer.r	tcgagTGGCAGTGTCTTAGCTGGTTGTgtgaTGGCAGTGTCTTAGCTGGTTGT accggtTGGCAGTGTCTTAGCTGGTTGTatcgTGGCAGTGTCTTAGCTGGTTGTgc
Foxp1 cDNA sequence	atgatgcaagaatctgggtctgagacaaaaagtaacggatcagccatccagaacgggtccagcggtggcaaccact tactagagtgcggggcacttcgtgacactcggtccaacggagaggcaccagcggtggacctgggggcagccgacc ttgcccacgtccagcagcagcaacagcagcagcaacaacagcagcagcagcaacagcagcagcagcagcagc aagttagtggattaaagtctcccaagaggaatgacaagcaaccagctcttcaggttcccgtgtcagtggctatgatgac acctcaggttatcactcctcaacaaatgcagcagatcctccagcagcaggtgctgagcccccagcagctccaggttct cctccagcagcagcaggccctcatgcttcaacagcagcttcaagaattttataaaaaacaacaggaacagttgcagc ttcaacttctccaacagcaacatgctggaaaacagccgaaagagcagcaggtggctacccagcagttggctttccag cagcagcttctccagatgcagcagctgcagcagcagcacctcttgtctctccagcgccaaggcctcctaacaattcag ccggggcagcctgcccttccctccaaccccctcgctcaaggcatgattccaacggaactgcagcagctctggaaaga agtgacaagtgcccacactgcagaggaaaccacaagcagcaaccacagcagcctagacctgaccagcacatgtg tctcgtcctcggcaccttccaagtcctccctaatcatgaacccgcatgcctctaccaatggacagctctcggtccacactc ccaaaagggaaagcttgtcccacgaggagcacccccacagccaccctctctatggacatggcgtatgcaagtggcc aggctgtgaggcggtttgtgacgacttcccagcctttctaaaacatctcaacagtgagcatgcgctggacgatagaagc acagctcaatgtagagtacaaatgcaggttgtacagcagttagagctacagcttgcaaaagacaaagagcgcctgc aagccatgatgacccacctgcatgtgaagtctacagaacccaaagctgcccctcagcccctgaatctggtatcaagtg tcaccctctccaagtctgcctcagaggcttctccacagagcttacctcatactccaacaacccccaccgccccctgact cctgtcacccaaggcccctccgtcatcaccaccaccagcatgcacacggtgggacctatccgcaggcggtactcag acaaatacaacgtgcccatttcttcagcagatattgcgcagaaccaagaattttataagaacgcggaagttagaccac catttacatatgcatctttaatcaggcaggccattctcgaatctccagaaaagcagctaacactaaacgaaatctataac tggttcacacgaatgtttgcttacttccgacgcaatgcagccacgtggaagaatgcagtgcgtcataatcttagtctccac aagtgttttgtgcgagtagagaacgttaaaggggcagtatggacagtggatgaagtagagttccaaaaacggaggcc acaaaagatcagtggtaacccttcccttattaaaaacatgcagagcagccacgcctactgcacacctctcaatgcagc tttacaggcttccatggctgagaatagtatacctctgtacactaccgcttccatgggaaatcccactctgggcagcctggc cagtgccatccgggaggagctgaacggggccatggagcacaccaacagcaacgagagtgacagcagtccaggc agatcccctatgcaagctgtgcaccccatacacgtcaaagaagaaccctcgaccccgaggaagctgaaggccctc tgtccttagtgacaacagccaaccacagtccagattttgaccatgacagagattacgaagacgaaccagtaaatgag gacatggagtga

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Summary of Thesis

The findings in the previous three chapters share the common theme of exploring the role of miRNAs in hematopoiesis and dissecting their connections with target mRNA/proteins *in vivo*. These studies indicate that miR-155 and miR-34a are important players in myeloid and B-cell development, respectively. In both of these cases, we can account for the majority of the phenotypic effects of the miRNA by downregulation of a single target. Hence, this body of work has important implications for our understanding of cellular pathways that control hematopoietic development, and suggests specific modulation of a single or a few targets may be the relevant mode of miRNA function in hematopoietic developmental processes. Additionally, we have developed a versatile methodology that will allow us to answer questions as to the role of particular miRNAs in hematopoiesis.

The experimental approach to assessment of miRNA function in hematopoiesis that emerges from these studies is as follows. In the hypothesis generation stage, particular hematopoietic developmental processes of interest can be assessed for miRNA involvement by profiling of cells at distinct developmental stages by array-based technologies. Alternatively, the profiling of leukemic cells compared to their normal counterparts can reveal miRNAs of interest in malignancy. In this regard, it is important to note that massively parallel sequencing techniques are beginning to reveal species-specific and cell-type-specific miRNAs based on the presence of a mature small RNA sequence and the appropriate genomic context, i.e., the presence of 5' and 3' flanking regions, and a predicted stem-loop RNA structure (Basso et al. 2009).

Following the identification of miRNAs of potential importance in a given developmental sequence, retrovirally based gain-of-function and loss of function approaches can be performed to assess the importance of a particular miRNA. For this purpose, the MGP constitutive expression and "sponge" sequence vectors can be extremely useful (Chapters 3 and 4). If these experiments reveal phenotypic changes in hematopoiesis, one can then begin to explore a molecular mechanism to explain the changes. In this effort, array-based mRNA profiling of cell lines combined with a bioinformatics approach can reveal putative targets of the miRNA. Further analyses to prove direct targeting (such as the luciferase-UTR assays described in the previous three chapters) can be followed by an assessment of the putative targets by gain- and loss of function analyses in hematopoiesis. If a particular miRNA seems of great importance, traditional genetic techniques of targeted deletion can be utilized to further assess the miRNA of interest.

miR-155 has gained tremendous importance as an "oncomiR" over the past several years, and such a general role is consistent with our discovery of miR-155 overexpression in acute myeloid leukemia (Eis et al. 2005; Habbe et al. 2009; Kluiver, Harakambieva et al. 2006; Kluiver et al. 2005; O'Connell et al. 2008; Pedersen et al. 2009; Tam and Dahlberg, 2006; Tili et al. 2007). In addition, we found a role in early hematopoietic development that seems to be connected to the inflammatory response. The critical distinction is that miR-155 is transiently upregulated from a very low baseline level during an inflammatory response, while it is constitutively active in cancer. It will be important to study the mechanisms whereby miR-155 is downregulated in cells following the acute phase of an inflammatory response. While transcriptional

downregulation may play an important role, it will be very interesting to determine if posttranscriptional mechanisms, including miRNA degradation, are necessary for miR-155 downregulation.

On the other hand, miR-34a is thought to be a tumor-suppressor miRNA, and it has been described to be a part of the p53-mediated transcriptional program. miR-34a regulates B-cell development by modulating Foxp1, which is a transcription factor that is known to upregulate the Rag recombinase enzymes. One curious reported finding is that miR-34a is upregulated in both acute lymphoblastic leukemia and acute myeloid leukemia (Isken et al. 2008; Zhang, Luo et al. 2009). In the former case, it is tempting to speculate that miR-34a may be involved in causing the differentiation block found in this neoplasm of early B-cell progenitors, as was demonstrated in Chapter 4. In the latter case, it has been postulated that a lympho-myeloid progenitor of AML exists (Deshpande et al. 2006); and miR-34a may play some role in modulating the lineage phenotype of a leukemia resulting from such a progenitor by blocking B-cell development. However, both of these ideas require further study to be validated.

As this work developed, I have often wondered about how important miRNAs really are. In fact, the majority of hematopoietic development seems to depend on transcription factor "switches," and the conventional thought about miRNAs was that they served more as "fine tuners." However, the work presented here and elsewhere (Fontana et al. 2007; Xiao et al. 2007; Xiao and Rajewsky 2009; Zhou et al. 2007) is changing that paradigm-notably in the idea that miRNAs may be important in certain stages of developmental processes where they impact the levels of a few critical targets.

The idea that miRNAs regulate large numbers of genes is based on several lines of evidence-(i) miRNA-knockout cells show small changes in putative target mRNA abundance; (ii) miRNA overexpression in cell lines shows small impacts on large numbers of genes; (iii) luciferase-based reporter assays show repression in the context of a given miRNA and numerous putative target mRNAs, and (iv) target-prediction algorithms, based on a seed-sequence and interspecies conservation of both miRNA and target UTR sequences, predict large numbers of interactions (reviewed in Bartel and Chen (2004)). However, these findings are based on models that fail to recapitulate a critical feature of hematopoietic development-that the cells are concurrently undergoing cell divisions and differentiating. In most profiling studies, including those conducted with primary cells derived from knockout animals, the cells are relatively static, or fixed, in a particular stage of differentiation. As such, it is difficult to capture what miRNA-mRNA interactions are occurring in a cell that is transitioning from one stage of development to the next with a rapidly changing transcriptional profile. In such a cell, a miRNA that regulates the abundance of a critical transcription factor may have dramatic effects on hematopoietic development, even if the effect on the transcription factor is small. Indeed, such has been the case with miR-150/c-Myb, where small alterations in the concentration of miR-150 with reciprocal changes in c-Myb had dramatic changes in the output of B-lymphocytes from the bone marrow (Xiao and Rajewsky 2009).

This is not to say that all miRNA-target interactions follow such a specific paradigm. There are many cells in the body that are quiescent and terminally differentiated. It may very well be that in such cells, miRNAs do act in a manner analogous to dampeners in electrical circuits, shutting down the effects of transcriptional

noise, and maintaining the cells in a stable state. This also has implications for cancer cells, where profiling studies have shown that there are fewer miRNAs expressed and that there are shorter 3'UTRs on the mRNAs that are expressed. Hence, it implies fewer miRNA-target interactions, perhaps making it possible to target crucial interactions in these cells.

In addition to these concepts on miRNA-target specificity, I take away one additional idea from my thesis work. miRNAs have shown new connections between cellular pathways that were previously considered disparate. For example, the repression of SHIP1 by miR-155 connects NF-κB activation with SHIP1 repression, tying together a growth promoting pathway with repression of a tumor suppressor/growth inhibitory gene. In another example from the literature, c-MYC activates the transcription of the miR-17-92 cluster, with resulting repression of the tumor suppressor PTEN by miRNAs from this cluster (He et al. 2005; Ventura et al. 2008). Such connections may prove to be critical in the malignant transformation of cells, and disruption of miR-155 or miR-17-92 may markedly decrease the growth advantage of constitutive NF-κB or c-MYC. Hence, there is an opportunity to more rationally understand how different growth-regulatory pathways are interconnected and to then parlay these discoveries into therapeutic possibilities for patients.

Future Directions

A mutually inhibitory regulatory circuit in myeloid differentiation?

Myeloid differentiation in the steady state and during an inflammatory response seems to differ significantly. In humans, for example, there is a so-called left-shift in the

peripheral blood during acute inflammation, which leads to a marked elevation of granulocytes in the circulation, as well as the release of some earlier myeloid forms from the bone marrow. In the bone marrow of human patients with acute and chronic inflammatory diseases, there is an increase in myeloid cells, similar to the bone marrows of mice treated with LPS (personal observations and Chapter 2). In surveying the literature in the past several years and from the work done in our laboratory, I have developed a hypothesis that may explain how two particular miRNAs may be important in regulating steady state and inflammatory myelopoiesis.

Finding a powerful myeloproliferative phenotype with miR-155 overexpression, it was somewhat surprising that miR-155-deficient mice show no impairment in granulocytic differentiation at steady state. Minor, but inconsistent, differences were seen during LPS-induced inflammatory myeloid development. miR-223, when overexpressed, fails to cause a significant hematopoietic phenotype (Chen et al. 2004). However, when it is knocked out, mice develop a severe granulocytosis, including features indicative of activation (Johnnidis et al. 2008). Indeed, some of the morphologic features of the granulocytes are similar to those seen in LPS-stimulated bone marrow. Also of interest, a recent study showed that miR-155 and miR-223 levels are inversely correlated in cases of acute myeloid leukemia (Wang et al. 2009).

We and others have reported that LPS stimulation leads to induction of miR-155 (O'Connell et al. 2008; Tili et al. 2007). In a pilot experiment, I have discovered that miR-223 is repressed in bone marrow samples from mice treated with LPS (unpublished observations). Hence it is possible that miR-155, by targeting transcription factors such as PU.1 and CEBP-β (known to enhance transcription of miR-223), can lead to a

downregulation of miR-223. In doing so, an inflammatory program of myeloid differentiation may be activated. Conversely, targets of miR-223 may represent activators of miR-155, although these factors have not yet been identified.

The resultant hypothesis is that miR-155 and miR-223 form a mutually inhibitory miRNA-based network in regulating steady-state versus inflammatory granulopoiesis and myeloid development. The predictions of this hypothesis lead to the formulation of broad specific aims as follows:

(i) To determine if miR-155 expression is sufficient or necessary for repression of miR-223

(ii) To determine if miR-223 expression is sufficient or necessary for repression of miR-155

(iii) To determine if myeloid development is compromised if both miR-155 and miR-223 are absent

It will be relatively straightforward to test these hypotheses in both the gain- and loss of function contexts. It should be noted that miR-155 and miR-223 knockout mice have been generated and can be used for these experiments (Johnnidis et al. 2008; Thai et al. 2007).

Putative roles of miR-34a in normal and malignant B-cell development

The antigen-dependent stage of B-cell development that occurs in germinal centers of lymphoid organs and subsequent differentiation into plasma cells remains incompletely understood. It is thought that mutually inhibitory transcription factor networks can explain the transition between the activated B-cells in the germinal center

and terminally differentiated quiescent plasma cells that are their progeny (reviewed in Johnson et al. (2005) and Shapiro-Shelef and Calame (2005)). However, the mechanisms whereby the balance is shifted from the B-cell transcription factors to the plasma cell transcription factors is not completely understood. Here, I propose that miR-34a may be an important factor in shifting that balance.

By examining B-cell activation in tissue culture, we have discovered that miR-34a is upregulated following stimulation with LPS and IL-4, which results in the formation of activated B-cells and so-called plasmablasts that are partially differentiated antibody secreting cells. In such cells, B-cell factors, such as Bcl6, Mitf, BCL2 and Bcl2, are repressed, and plasma cell factors, such as Blimp1, Xbp1, and Irf-4, are upregulated (Shapiro-Shelef and Calame, 2005). One of the obfuscating points in the literature is the sometimes difficult distinction between activated B-cells and plasmablasts, the latter of which is thought to represent a later, more committed stage of differentiation. Indeed, many of the papers in the field equate the presence of class-switched B-cells with plasma cell differentiation, which are related but distinct processes, at least from the standpoint of a cell biologist. In this regard, the availability of a *Blimp1-gfp* reporter mouse, where GFP is expressed when the *Blimp1* locus is transcriptionally active, has helped to clarify the issue by demonstrating low- and high-GFP expressing cells as B-cells at different points along the differentiation spectrum to the quiescent plasma cell.

We plan to study miR-34a involvement in this process for the following reasons: (i) miR-34a is upregulated upon activation of B-cells; (ii) p53, the transcriptional activator of miR-34a, is repressed by BCL6 (He et al. 2007; Phan and Dalla-Favera, 2004); (iii) The predicted targets of miR-34a include Bcl6 and Bach2, which are both B-

cell factors (Diehl et al. 2008; Muto et al. 2004; Ochiai et al. 2006, 2008); and (iv) p53 itself was known to promote B-cell differentiation in some early work on this subject (Aloni-Grinstein et al. 1993). The connections between p53 and miR-34a also raise the possibility that miR-34a is downregulated in p53-deficient malignancies and that restoration of its function can be a useful therapeutic strategy in cancer.

To summarize future work on this topic, I plan to test the following specific hypotheses:

1. miR-34a is necessary and sufficient for plasma cell differentiation in cell culture models and following immunization *in vivo*.

2. Bcl6 and Bach 2 represent important targets of miR-34a in plasma cell differentiation.

3. miR-34a restoration can mitigate the development of p53-deficient hematopoietic tumors by repressing several oncogenic miR-34 targets.

Involvement of miRNAs and other noncoding RNA in human leukemia

As alluded to in several previous chapters, miRNAs are deregulated in several types of cancer. The functional characterization of these deregulated species, as in the case of miR-155, has led to a better understanding of normal hematopoiesis as well as the relationship between regulatory cellular pathways. Yet, most studies show that several miRNAs are deregulated in various hematopoietic disease states. An important question that arises from such profiling studies is whether multiple miRNAs that are deregulated in a particular disease state can synergistically have much larger effects on either normal or malignant development than a single miRNA alone. In this, the development of

retroviral vectors that can constitutively express or simultaneously knockdown multiple miRNAs will be critical. These can then be used in the bone marrow transfer system described in Chapters 2-4.

It is also becoming apparent that miRNAs may form just one small portion of the cellular repertoire of noncoding RNA. Indeed, some RNA biologists have estimated that the cell transcribes up to 20%-30% of its DNA sequence, with only 1% thought to represent protein coding genes (Cheng et al. 2005; Kapranov et al. 2002, 2007). Although the abundance of these noncoding transcripts (in comparison to protein coding transcripts and miRNA precursor transcripts) is only now being assessed, it is safe to say that there are likely additional classes of noncoding transcripts with gene regulatory functions. Indeed, a recently described class of noncoding transcripts, linc-RNA (long intergenic noncoding RNA), associates with chromatin-modifying complexes and can modify gene expression (Guttman et al. 2009; Khalil et al. 2009; Rinn et al. 2007). Newly described classes of small RNA, dubbed promoter associated small RNA (PASR) and termini-associated small RNA (TASR) have also been ascribed putative functions in gene regulation (ENCODE Project, 2009).

Hence, it is an exciting time to be a physician-scientist studying the roles of noncoding RNA in normal and malignant hematopoiesis. We have good questions to ask and robust experimental systems to use. The ultimate hope is to transform the knowledge thus gained into a more meaningful understanding of biology and disease.

APPENDIX 1: A review of miRNA in immune cell development

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REVIEW

nature immunology

MicroRNAs: new regulators of immune cell development and function

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Decades of research went into understanding immune cell development and function without awareness that consideration of a key element, microRNA (miRNA), was lacking. The discovery of miRNAs as regulators of developmental events in model organisms suggested to many investigators that miRNA might be involved in the immune system. In the past few years, widespread examination of this possibility has produced notable results. Results have shown that miRNAs affect mammalian immune cell differentiation, the outcome of immune responses to infection and the development of diseases of immunological origin. Some miRNAs repress expression of target proteins with well established functions in hematopoiesis. Here we bring together much of this work, which has so far only scratched the surface of this very fertile field of investigation, and show how the results illuminate many historic questions about hematopoiesis and immune function.

Thanks to work built on pioneering studies in plants and nematodes[1–3], the biogenesis and general cellular function of mammalian microRNA (miRNA) is now generally understood. First, miRNA is transcribed mainly by RNA polymerase II, which produces a primary transcript containing the mature miRNA sequence and a varying amount of flanking region[4,5]. Then, the Drosha nuclease cleaves that primary RNA into a precursor miRNA that is exported from the nucleus by exportin 5 (refs. 6,7). After reaching the cytoplasm, the precursor miRNA is further processed by the Dicer nuclease and is loaded into the RNA-induced silencing complex (which contains Argonaute and other proteins). Next, a specific single strand of the miRNA duplex is selected as a guide to direct sequence-specific targeting of mRNA 3′ untranslated regions (UTRs) by the RNA-induced silencing complex [8,9].

The overall importance of miRNA during hematopoiesis has been investigated by specific disruption of steps in miRNA biogenesis. For example, conditional deletion of Dicer in hematopoietic stem cells (HSCs) renders these cells unable to reconstitute the hematopoietic system, whereas knockout of Argonaute 2 in the hematopoietic system results in impaired B cell and erythroid differentiation that triggers the population expansion of immature erythroblasts[10]. Furthermore, conditional deletion of Dicer in T lymphocytes results in many fewer T cells in the thymus and periphery[11,12]. Dicer deficiency in B lymphocytes has been shown to diminish B cell survival and the antibody repertoire[13]. Such data indicate a critical function for miRNA in the biology of cells that constitute the immune system.

Investigators have made extensive use of microarray technology and high-throughput sequencing to assess miRNA expression globally in the hematopoietic system[14,15]. Human CD34$^+$ cell populations, known to be enriched for HSCs and progenitor cells, have high expression of 34 different miRNAs[16]. Expression of miRNA changes during the differentiation of HSCs along specific cell lineages, with alterations in miRNA abundance in each[17,18]. In mature immune cells, investigators have found unique yet partially overlapping miRNA expression profiles. Similarly, some but not all miRNA species are differentially expressed in resting versus activated immune cells[19–23]. Such approaches have delineated miRNAs that are differentially expressed, both spatially and temporally, in many types of immune cells. Cancerous immune cells show profoundly aberrant expression of miRNAs relative to that of their healthy counterparts, and in some cases, these miRNAs have been shown to function as either tumor suppressors or oncogenes[24].

With the general importance of hematopoietic miRNAs established and many of the specific miRNAs expressed by immune cells identified, the challenging task of defining the function(s) of individual miRNAs in immune cell development and function has now begun, often with gain- and loss-of-function approaches. Here we will describe advances in the knowledge of selected, immunologically relevant miRNAs that are among the most intensively investigated at this point and that are beginning to show how this new class of noncoding RNA participates in mammalian immunity. We have also provided an overview of where the miRNAs we will discuss have been linked to either a hematopoietic cellular lineage or a bifurcation in the 'differentiation tree' (**Fig. 1**).

miR-155

The miRNA miR-155 is processed from a primary transcript, called 'BIC', encoded by a gene originally isolated near a common retroviral integration site in avian leukosis virus–induced lymphomas[25]. Subsequent studies have shown that HSCs have moderate expression of miR-155 and mature hematopoietic cells have much lower miR-155 expression[16]. Notably, expression of the BIC transcript and of mature miR-155 is

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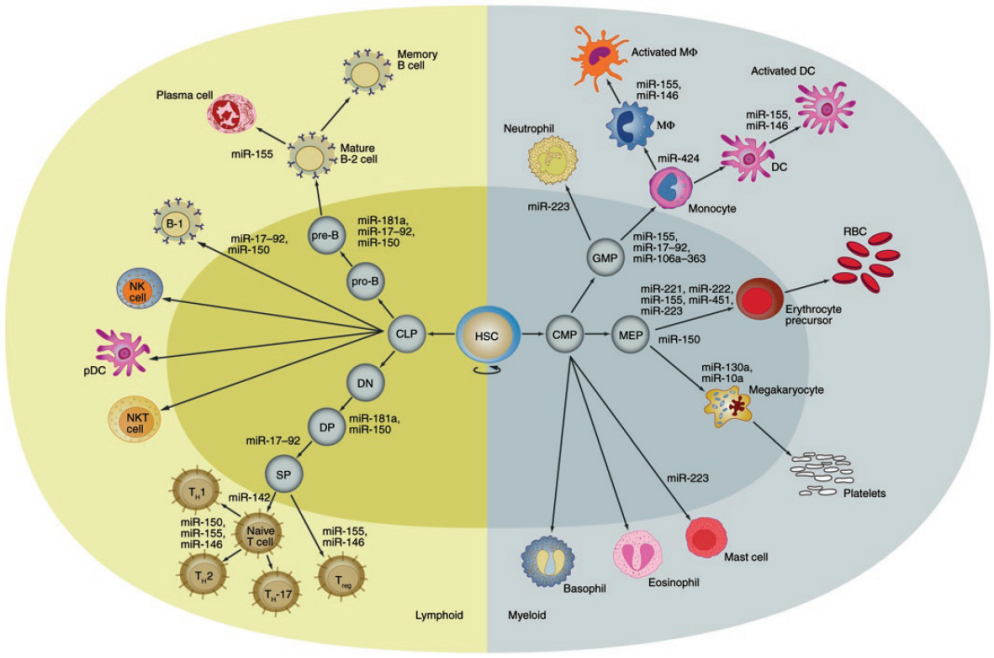

Figure 1 Involvement of miRNAs in hematopoiesis and immune system function. Overview of the involvement of the miRNAs discussed in this review in the differentiation and function of various hematopoietic lineages. The miRNAs near arrows are involved in but do not necessarily enhance the developmental transition indicated by the arrow (full explanation in text). B-1, B-1 type B cell; B-2, B-2 type 'conventional' B cell; pre-B, pre–B cell; pro-B, pro–B cell; CLP, common lymphoid progenitor; DN, double-negative T cell; DP, double-positive T cell; SP, single-positive T cell; NK, natural killer; pDC, plasmacytoid dendritic cell; NKT, natural killer T; T_H1, T helper type 1 cell; T_H2, T helper type 2 cell; T_H-17, interleukin 17–producing helper T cell; T_{reg}, regulatory T cell; Mφ, macrophage; DC, dendritic cell; GMP, granulocyte-macrophage progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte-erythrocyte progenitor; RBC, red blood cell. Created by Cici Koenig.

rapidly induced in B lymphocytes and T lymphocytes after engagement of the antigen receptor and in a variety of different leukocytes after exposure to inflammatory mediators[21,22,26,27]. Thus, the function of miR-155 seems to be 'unleashed' during an active immune response to infection. The importance of proper regulation of miR-155 expression is exemplified by its much higher expression in several types of hematopoietic malignancies[28–33] and its ability to cause a myeloproliferative disorder or B cell lymphoma when overexpressed in various cellular contexts in mice[31,34].

What is the physiological function of miR-155 during mammalian immune responses to infection? Two studies of mice lacking miR-155 have reported normal immune cell populations in the absence of miR-155 in steady-state conditions[27,35]. However, defective humoral responses have been found after immunization, consistent with a specialized function for miR-155 during infection. This defect has been shown to be B cell intrinsic, to involve impaired germinal center formation and to result in less antibody class switching to immunoglobulin G_1 (IgG_1; **Fig. 1**). Providing mechanistic insight, PU.1 has been identified as a functionally relevant target of miR-155, negatively regulating switching to IgG_1 (ref. 36). In addition, miR-155 targets the cytidine deaminase AID, a critical enzyme that mediates class-switch recombination and somatic hypermutation[37,38]. These AID studies have used mutant mice in which the miR-155 binding site in the 3′ UTR of AID is abolished. This approach provides a powerful method for assessing the contribution of a specific miRNA target to a given phenotype.

Although there is need for further investigation of the full function of miR-155 in T lymphocytes, it seems to regulate T cell lineage fate by promoting T helper type 1 versus T helper type 2 differentiation, possibly by targeting the transcription factor c-Maf[27,35] (**Fig. 1**). Furthermore, the regulatory T cell–specific transcription factor Foxp3 has been shown to bind to the promoter region of the gene encoding BIC, which correlates with higher miR-155 expression in this cell type than in conventional T cells[19,39] (**Fig. 1**).

Is miR-155 involved in innate immunity? Because mature macrophages are in the first line of defense against infectious microbes, their rapid induction of direct effector and immunomodulatory genes may be critical for pathogen clearance. In monocytes, macrophages and myeloid dendritic cells, miR-155 increases substantially after exposure to a variety of inflammatory stimuli[21,22,40] (**Fig. 1**). Direct recognition of microbial products by Toll-like receptors, particularly pathogen-associated molecular motifs derived from bacteria or viruses, leads to miR-155 upregulation. Furthermore, a plethora of immunoregulatory cytokines, such as tumor necrosis factor and interferons, also induce miR-155 upregulation in macrophages. Because miR-155 induction involves both the

transcription factor NF-κB and the kinase Jnk, it fits the profile of a typical immune-response molecule[21,41,42]. The function of miR-155 in macrophages remains an open issue. Further elucidation of miR-155 targets should show whether it is a positive or negative regulator of the macrophage inflammatory response or whether it has a more specialized task. For example, miR-155-deficient myeloid dendritic cells have an impaired ability to trigger T cell activation after antigen presentation, which indicates involvement in bridging innate and adaptive immunity[35].

The work described above suggests the importance of miR-155 in mature immune cells, but a separate issue is whether it influences hematopoiesis. It has been noted that miR-155 is upregulated in mouse bone marrow during systemic inflammation[31]. In an effort to test the influence of miR-155 on hematopoietic cell development, an effect that might be evident only when it is highly expressed during inflammation, miR-155 was constitutively expressed in the mouse hematopoietic system with retrovirus-mediated gene transfer into HSCs. Sustained expression of miR-155 had profound effects on hematopoietic populations, resulting in a myeloproliferative disorder (**Fig. 1**). Consistent with an ability of miR-155 to influence human myelopoiesis, its overexpression has been noted in certain subtypes of acute myeloid leukemia[31,43], whereas its enforced expression regulates the differentiation of human $CD34^+$ cells into myeloid lineages *in vitro*[16]. Notably, B cell–restricted expression of miR-155 can reportedly trigger a polyclonal pro–B cell leukemia, which suggests the sufficiency of this single miRNA in transforming B cells[34]. Furthermore, transforming B cell–tropic viruses can either express orthologs of miR-155, as noted with Kaposi's sarcoma virus[44,45], or induce expression of cellular miR-155, as in B cells latently infected with Epstein-Barr virus[42,46,47]. These observations collectively demonstrate that dysregulated miR-155 can affect myeloid or lymphoid development depending on the context of its expression. However, further studies are needed to determine the extent to which physiologically regulated miR-155 expression directs hematopoietic cell fate 'decisions' during infection.

The miR-146 family

The miRNA miR-146 was first identified as an immune system regulator in a systematic effort to find miRNAs that influence the mammalian response to microbial infection[22]. Exposure of human monocytic THP-1 cells to various bacterial components (such as lipopolysaccharide and peptidoglycan) and proinflammatory cytokines (such as interleukin 1 and tumor necrosis factor) results in rapid induction of the expression of mature miR-146 species (**Fig. 1**). In human primary dendritic cells, miR-146a production increases in response to lipopolysaccharide[40] (**Fig. 1**), whereas in B cells with latent infection with Epstein-Barr virus, miR-146a expression increases as a result of signaling of the virus-encoded latent membrane protein 1, which acts as a constitutively active homolog of the tumor necrosis factor receptor[48,49].

Many expression-profiling studies have demonstrated distinct patterns of miR-146 expression in various hematopoietic lineages, which suggests its involvement in the maintenance of lineage identity in lymphocyte subsets. For example, miR-146 abundance is higher in T helper type 1 cells but lower in T helper type 2 cells relative to that in mature naive T cells[50] (**Fig. 1**). In addition, miR-146 is among the most highly expressed miRNAs in regulatory T cells, and overall, the miRNA expression pattern of the regulatory T cell lineage (as well as the cells' constitutive expression of CD25) resembles the profile of activated rather than naive T cells[19] (**Fig. 1**).

Most vertebrates that have been examined have two copies of the gene encoding miR-146—*miR-146a* and *miR-146b*—located on separate chromosomes in the context of unrelated genes. These miRNAs differ in their mature sequence by just two nucleotides at the 3′ end, which suggests that they probably regulate the same group of mRNA targets[51]. However, we do not believe these two genes have a redundant biological function, given the complex post-transcriptional processing they undergo. Transcription of both *miR-146a* and *miR-146b* is upregulated after lipopolysaccharide stimulation, but only mature miR-146a is produced, which suggests that this miRNA family (perhaps resembling the regulation of the let-7 family of miRNAs or miR-138)[52,53] has a rather sophisticated mode of regulation of expression involving more than one point of control. Promoter analyses have shown that transcriptional upregulation of *miR-146a* induced by Toll-like receptor 4 and latent membrane protein 1 is NF-κB dependent[22,48]. Additionally, genome-wide mapping of binding sites for RelA (one of the five NF-κB subunits) by chromatin immunoprecipitation has shown its physical association with the *miR-146a* promoter in a lipopolysaccharide-treated human monocytic cell line[54]. The basal amount of *miR-146a* transcription and, to some extent, its induction by latent membrane protein 1 signaling, is under the control of Oct transcription factors[48], whereas the proto-oncogene product c-Myc might be involved in the transcriptional repression of the *miR-146a* locus during tumorigenesis[55].

Although the physiological functions of the mir-146 family are not yet fully defined, the potential scope of its functions in the immune system can be glimpsed in the growing list of its confirmed target genes. This list is topped by the genes encoding the IRAK1 and TRAF6 adaptor molecules, whose involvement in Toll-like receptor and interleukin 1 receptor signaling is well established[56]. Moreover, TRAF6 is crucial in NF-κB activation 'downstream' of antigen receptors in B cells and T cells. Both target genes (encoding IRAK1 and TRAF6) are downregulated by miR-146a coexpression, as demonstrated with 3′ UTR reporters[22] and by measurement of endogenous protein expression in THP-1 cells (M.P.B., K.D.T. and D.B., unpublished data). As miR-146a expression is induced in response to microbial infection and miR-146a targets signaling intermediates 'downstream' of innate immune receptors, we propose that this miRNA functions as a negative regulator of inflammation. Because both miR-146 targets (IRAK1 and TRAF6) act in the same linear signaling cascade, the cumulative effect of a drop in their protein abundance would probably have a considerable effect on Toll-like receptor and interleukin 1 receptor signaling and might be involved in regulation of the innate immune response.

In addition, a microarray survey of miR-146a targets after its enforced overexpression in Akata B cell line has demonstrated miR-146a-mediated inhibition of a group of interferon-responsive genes[48]. Whether their regulation by miR-146a is direct or indirect remains to be determined. Another confirmed target of miR-146a is ROCK1, a serine-threonine kinase that is activated through its interaction with the GTP-bound form of Rho[57]. There is considerable evidence to suggest that Rho-ROCK1 signaling becomes dysregulated in certain cancers and contributes to the metastatic and invasive activity of transformed cells. Relevant to that observation, miR-146a expression is downregulated in hormone-refractory prostate carcinomas relative to its expression in androgen-sensitive noncancerous prostate epithelium, and enforced expression of miR-146a in prostate tumor cells *in vitro* diminishes their proliferation, invasiveness and ability to metastasize[57].

Knowledge of miR-146's function in the development and function of the immune system should be advanced in the future through the phenotypic analyses of mice with targeted deletion of *miR-146a* and/or *miR-146b*.

miR-150

The miRNA miR-150 has a dynamic expression profile during lymphocyte development, being highly expressed in mature B cells and T cells but not in their progenitors; its expression is then extinguished after

further differentiation of naive T cells into the effector T helper type 1 and T helper type 2 subsets[50,58] (**Fig. 1**). A highly sensitive method that can profile miRNA expression in limiting numbers of primary cells has shown that miR-150 expression is higher in megakaryocytes and, conversely, lower in erythrocytes relative to its expression in megakaryocyte-erythrocyte progenitors[59] (**Fig. 1**).

A combination of gain-of-function and loss-of-function mouse models established in several laboratories has defined the physiological function of miR-150 in hematopoietic development[58,60] (**Fig. 1**). Overexpression of miR-150 in mouse HSCs results in a selective defect in B cell development that occurs at the transition from pro–B cell to pre–B cell. Similarly, transgenic mice with moderate ectopic but ubiquitous expression of miR-150 under the control of the *Rosa26* promoter have impaired development of many lymphoid lineages[60]. Mice with targeted deletion of the gene encoding miR-150 are morphologically normal and fertile but have more B-1 cells in the spleen and peritoneal cavity, accompanied by fewer conventional B-2 cells[58]. B-1 cells reside mainly in the pleural and peritoneal cavities and differ from B-2 cells phenotypically, functionally and developmentally[61]. B-1 cells often recognize self antigens and common bacterial components, and antibodies produced by this B cell subset tend to be of broad specificity and low affinity. B-1 cells are believed to function as innate-like immune effectors and, together with marginal zone B cells, are key participants in the early humoral response against invading pathogens. Unchallenged miR-150-knockout mice have much higher serum concentrations of various classes of immunoglobulins (with the most profound change in the IgA class), consistent with expansion of the B-1 subset. However, follicular B cells (B-2 subset) probably also contribute to these higher immunoglobulin titers, as immunization of the knockout mice with a T cell–dependent antigen elicits a stronger humoral response than that in the control mice[58].

The proposed mechanism of miR-150 function in B cell development involves its regulation of expression of the transcription factor c-Myb. Studies have confirmed the gene encoding c-Myb as an miR-150 target by 3′ UTR reporter assays and have shown that activated B cells deficient in miR-150 have more c-Myb, whereas B cells from transgenic mice overexpressing miR-150 have less c-Myb. Mice with haploinsufficiency for the gene encoding c-Myb have fewer mature B cells in the spleen and B-1 cells, as well as a block at the transition from pro–B cell to pre–B cell, which resembles the phenotype of miR-150-transgenic mice.

The function of miR-150 in the specification of megakaryocytic versus erythrocytic lineage commitment has been studied *in vitro* and *in vivo*[59]. Lentiviral transduction of human $CD34^+$ HSCs with a miR-150 -expressing construct leads to a substantial balance shift in favor of the megakaryocytic lineage. In addition, enforced expression of miR-150 in mouse HSCs in a bone marrow transplantation model results in considerable expansion of the megakaryocyte population at the expense of erythrocytes, as well as more circulating platelets. The lineage commitment of megakaryocyte-erythrocyte progenitor cells is directly affected by miR-150, as bone marrow cells overexpressing miR-150 produce many more megakaryocyte colony-forming units than do control bone marrow cells. Notably, control of the megakaryocyte-erythrocyte lineage commitment by miR-150 is also dependent on the negative regulation of the gene encoding c-Myb. Consistent with reports that mice lacking c-Myb have megakaryocytosis[62], knockdown of c-Myb expression promotes megakaryocyte development[59].

miR-181a

Originally identified in a seminal paper that examined the involvement of miRNA in hematopoiesis[63], miR-181a has been ascribed functions in hematopoietic differentiation and in T cell differentiation and function[63,64]. Other functions in hematopoiesis are suggested by expression-profiling studies showing upregulated miR-181a expression in common myeloid-erythroid progenitors[65]. A nonhematopoietic developmental function for miR-181a, as well as a second isoform, miR-1816, in myoblast differentiation has also been described[66].

The original study described tissue expression patterns of several hematopoietic miRNAs and used gain-of-function analyses to suggest their function in hematopoiesis[63]. The bone marrow, spleen and thymus have the highest expression of miR-181a. In the bone marrow, progenitor (lineage-negative) cells have low expression of miR-181a and B cells have higher expression of miR-181a. When overexpressed *in vitro* in culture conditions that promote multilineage differentiation, miR-181a induces a two- to threefold increase in $CD19^+$ B cell populations but does not affect T or myeloid cells (**Fig. 1**). *In vivo* transfer of bone marrow cells overexpressing miR-181a results in an increase in $CD19^+$ B cells, a decrease in Thy-1.2^+ T cells and no change in myeloid cells.

In addition to directing the lineage of bone marrow progenitor cells, miR-181a also influences T cell development and function[64]. In purified thymocyte subsets, miR-181a is dynamically regulated, with expression increasing in populations in double-negative stage 1 through double-negative stage 3 and then decreasing during the remainder of T cell development (**Fig. 1**). Overexpression of miR-181a augments T cell receptor (TCR) signaling strength. Conversely, knockdown of miR-181a results in lower TCR signal strength and in the inhibition of positive and negative selection in an *in vitro* fetal thymic organ culture model. MiR-181a seems to 'tune' TCR signal strength by downregulating expression of several protein tyrosine phosphatases, including SHP-2, PTPN22, DUSP5 and DUSP6. The net effect of this regulation, which seems to be dependent on the downregulation of many targets, is to enhance basal activation of the TCR signaling molecules Lck and Erk. These results indicate that miR-181a has a physiological function during thymic selection by regulating TCR signaling strength in a cell-intrinsic way and that miR-181a may regulate the activation of mature T cells by modulating TCR signal strength. Thus, miR-181a influences B cell lineage selection as well as T cell development and activation. Further work on how miR-181a itself is regulated during normal hematopoiesis will add substantially to the understanding of how this miRNA has distinct functions in these different cellular contexts.

The miR-17–92 cluster and its paralogs

The miR-17–92 cluster is a polycistronic miRNA gene residing on chromosome 13 in the human genome. It encodes six different miRNAs (miR-17, miR-18a, mir-19a, miR-20a, miR-19b and miR-92), some of which share identical 'seed' sequences. Several lines of evidence have established the importance of the miR-17–92 cluster in cell transformation and tumorigenesis[67]. Two existing paralogs of the miR-17–92 cluster (miR-106b–25 and miR-106a–363) are apparently the result of a series of ancient genomic duplications and deletions. The genomic organization of these three miRNA clusters is highly conserved in all vertebrates, and most of the encoded miRNAs are widely expressed in various mouse tissues[64,68,69]. In the lymphocyte lineage, the mir-17–92 cluster is highly expressed in B precursor cells and T precursor cells, and its expression diminishes somewhat after maturation. In myeloid development, expression of miR-17, miR-20a and miR-106a decreases during monocytopoiesis *in vitro*[70]. This enables expression of the gene encoding AML1, a direct target of these miRNAs, which is required for the initiation of monocyte maturation (**Fig. 1**).

Mice with targeted deletion of the miR17–92 cluster die within minutes of birth, most likely because of severe underdevelopment of the lungs and/or heart[68]. The livers of the knockout fetal mice have fewer pre–B cells. Reconstitution of the hematopoietic system of lethally irradiated

mice with progenitor cells from livers of miR-17–92-deficient fetal mice shows a selective defect in adult B cell development, with a block at the transition from pro–B cell to pre–B cell and excessive apoptosis in the pro–B cell fraction (**Fig. 1**).

In contrast, mice with ectopic expression of the miR-17–92 cluster in the lymphocyte compartment develop severe lymphoproliferative disease and autoimmunity and die prematurely[69]. Almost all lymphocyte populations in the periphery of miR-17–92-transgenic animals undergo expansion, but $CD4^+$ T cells show the greatest increase. The spleens and peritoneal cavities of these mice have more B-1a cells ($CD5^+$ B-1 cells[61]), whereas the number of marginal zone B cells in the spleen decreases with age. In addition, B lymphocytes and T lymphocytes from the transgenic mice show greater proliferation and survival after activation *in vitro*.

Bim, a proapoptotic member of the antiapoptotic Bcl-2 family, has been identified as a true target of the *miR-17–92* cluster[13,68,69]. Mice with targeted deletion of the miR-17–92 locus have more Bim in various tissues, whereas $CD4^+$ T cells from mice with enforced miR-17–92 expression have less Bim[68]. In addition, miR-17–92-mediated regulation of the expression of the gene encoding the tumor suppressor PTEN might also contribute to the lymphoproliferation in the miR-17–92-transgenic model[69]. Resembling the phenotype of miR-17–92-transgenic mice, mice with compound heterozygous mutations in the genes encoding Bim and PTEN develop an accumulation of antigen-experienced T cells and germinal center B cells that becomes evident at a later age. However, the number of single-positive T cells, marginal zone B cells and B-1a cells remains unaffected in this mouse model, which suggests that unknown miR-17–92 targets are involved in the control of proliferation of these lymphocyte populations.

Compound deletion of the miR-17–92 cluster and its paralog, miR-106b–25, in mice results in more severe developmental defects and exacerbated apoptosis relative to ablation of only the miR-17–92 locus, which emphasizes the possibility that these two miRNA clusters functionally 'cooperate' in regulating mouse embryonic development and cell survival[68].

miR-223

Initially identified as a hematopoietic miRNA expressed most highly in bone marrow, miR-223 has been studied in both normal and pathological situations[14]. This miRNA is expressed specifically in cells of the granulocytic lineage. Its expression changes during maturation, becoming incrementally higher as granulocytes mature[71]. Conversely, miR-223 expression decreases as cells mature during erythroid and mast cell differentiation[50,72]. In human disease, miR-223 expression is lower in some types of acute myeloid leukemia that have a block in differentiation[73]. Granulocytic differentiation is restored by enforced expression of miR-223 in leukemic blast cells; this suggests a physiological function for miR-223 in this process, which is disrupted in disease[73–75].

The mechanisms regulating miR-223 expression have been a chief focus of study, given its apparent importance in granulocytic differentiation. Expression of miR-223 is regulated by a combination of factors. Initially, a circuit consisting of C/EBPα (a member of the CCAAT enhancer-binding protein family), NFI-A (a transcription factor related to the CCAAT family) and miR-223 was described[74]. In this scenario, C/EBPα activates transcription of miR-223, whereas NFI-A represses it; miR-223 itself targets NFI-A, thereby turning off its repressor once it is expressed. This unique circuit was postulated to be critical in granulocytic maturation. Further work has shown that the situation is probably more complex and that miR-223 is in fact a 'myeloid gene', as shown by bioinformatics-based analysis of several miRNA genes[76]. Analysis of conserved proximal *cis*-regulatory elements has shown that the putative binding sites for the transcription factors mentioned above do not overlap the promoter elements defined for the gene encoding miR-223. Instead, these analyses indicate that miR-223 expression may be driven by myeloid transcription factors such as PU.1 and C/EBP, similar to many protein-encoding genes involved in granulopoiesis. In disease, the AML1-ETO fusion oncoprotein targets the miR-223 promoter for epigenetic silencing, which presumably abrogates the ability of myeloid transcription factors to activate this miRNA[73].

The precise physiological function of miR-223 remains elusive. Initial loss-of-function approaches focused on *in vitro* assays, where approaches based on 'antagomirs' (cholesterol-linked single-stranded antisense RNA) abrogate granulocytic differentiation in leukemic blasts[73,74]. Studies of a miR-223-knockout mouse have somewhat unexpectedly shown that these mice have twofold more granulocytes[71]. These granulocytes are morphologically hypermature and hypersensitive to activating stimuli and have more fungicidal activity. The mice also have inflammatory lung lesions and develop more tissue destruction after endotoxin challenge. The relevant target in this context seems to be *Mef2c*, which encodes a transcription factor involved in promoting myeloid progenitor differentiation, as demonstrated by correction of the miR-223-null phenotype in mice lacking both *miR-223* and *Mef2c*. These results collectively suggest that miR-223 is involved in regulation of granulocytic maturation but is not absolutely required for the production of granulocytes *in vivo*. It also seems to suppress activation of neutrophils and hence is important in linking differentiation with function in the granulocytic lineage during homeostatic granulopoiesis.

Other miRNAs of relevance to hematopoietic development

In addition to the miRNAs described above, many others have been linked to the modulation of immune cell development. Ectopic overexpression of the ubiquitously expressed miR-142 has been found to increase production of T lymphocytes *in vitro*[63]. In addition, miR-221 and miR-222 are downregulated during erythropoiesis, thus relieving repression of their target, which encodes the stem cell factor receptor c-Kit[77]; also, expression of several miRNAs, including miR-10a and miR-130a, is lower during megakaryocyte development[17]. In this scenario, miR-10a is thought to decrease expression of its target, the transcription factor HOXA1, whereas miR-130a probably decreases expression of the transcription factor MAFB. Other miRNAs increase in expression during the development of specific cell lineages. Expression of miR-424 is induced by the transcription factor PU.1 during monocyte-macrophage differentiation, and miR-424 subsequently downregulates expression of the transcription factor NFI-A[78]. Expression of several miRNAs, including miR-451, gradually increases during erythropoiesis[72]. Thus, it seems that specific miRNAs and their target proteins are reciprocally expressed throughout the hematopoietic differentiation process.

Conclusions

In a very short time, immunologists have substantially advanced understanding of the involvement of miRNA in the development and function of the immune system. However, as scientific understanding of miRNA continues to evolve, new challenges regularly present themselves. For example, a study of mature T lymphocytes has identified considerable sequence heterogeneity in individual mature miRNAs defined by nucleotide deletions, insertions and substitutions introduced by unknown mechanisms[23]. Such diversity is reminiscent of mRNA splicing and might considerably expand the task of defining which miRNA species are present in a given immune cell type.

Also critical to understanding miRNA function are the continuing efforts to link specific miRNAs to their biologically relevant targets, a process that will be enhanced as methods for identifying true targets

are refined. Developments in this area should show whether a given miRNA functions by repressing large sets of proteins or only a few key targets. Additionally, because some mRNAs contain binding sites for several different miRNAs in their 3′ UTRs, investigators must also consider the ability of miRNAs to 'collaborate' during their regulation of immune genes. Data obtained with knockout models suggest that miRNAs function as 'fine-tuners' of protein expression rather than as 'on-off' switches. However, those same studies have shown considerable functional consequences of these partial changes in target expression, which emphasizes the importance of maintaining precise cellular protein quantities at given moments in the ever-changing functional status of developing immune cells.

With the identification of several hundred mammalian miRNAs[14] along with many other putative noncoding RNAs[79], it is likely that the studies described here are but the tip of the iceberg in terms of the involvement of regulatory RNAs in orchestrating hematopoietic development and function.

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APPENDIX 2:

A review of the roles of miRNAs in normal and pathological immune cell development

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Physiological and pathological roles for microRNAs in the immune system

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Abstract | Mammalian microRNAs (miRNAs) have recently been identified as important regulators of gene expression, and they function by repressing specific target genes at the post-transcriptional level. Now, studies of miRNAs are resolving some unsolved issues in immunology. Recent studies have shown that miRNAs have unique expression profiles in cells of the innate and adaptive immune systems and have pivotal roles in the regulation of both cell development and function. Furthermore, when miRNAs are aberrantly expressed they can contribute to pathological conditions involving the immune system, such as cancer and autoimmunity; they have also been shown to be useful as diagnostic and prognostic indicators of disease type and severity. This Review discusses recent advances in our understanding of both the intended functions of miRNAs in managing immune cell biology and their pathological roles when their expression is dysregulated.

3′ untranslated region
The sequence of a messenger RNA that is located downstream of the stop codon.

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The recent discovery of microRNAs (miRNAs) has revealed a new layer of gene expression regulation that affects many biological systems, including the mammalian immune system[1,2]. miRNAs are small, single-stranded non-coding RNAs, many of which have been highly conserved throughout evolution. They function by directly binding to the 3′ untranslated regions (UTRs) of specific target mRNAs, leading to the repression of protein expression and the promotion of target mRNA degradation. So far, ~700 different miRNAs have been identified in the human genome. Each miRNA could have the potential to repress the expression of many, perhaps hundreds of, target genes, highlighting the extent of this form of regulation in mammals. More than 100 different miRNAs are expressed by cells of the immune system; they have the potential to broadly influence the molecular pathways that control the development and function of innate and adaptive immune responses (TABLE 1). The expression of miRNAs is also markedly dysregulated in cancers of immunological origin, in which they are thought to have tumour suppressive or tumour promoting activities depending on the nature of their specific target mRNAs[3].

In this Review, we describe newly discovered mechanisms that regulate miRNA biogenesis and so determine the spatial and temporal patterns of miRNA expression in the immune system. We then review relevant examples of how miRNAs influence both innate and adaptive immune cell development and function and highlight emerging paradigms. In the final sections, we discuss the connection between miRNAs and human diseases of immunological origin. We focus on examples in which the expression of specific miRNAs becomes dysregulated, leading to inappropriate target mRNA repression or derepression and the exacerbation of disease.

Regulating miRNA biogenesis

Our knowledge of miRNA biogenesis and mechanisms of target mRNA regulation by miRNAs is expanding in parallel with our understanding of their physiological roles in the immune system. Characterization of these processes is crucial for determining how, when and where miRNAs are produced and for clarifying the mechanistic activities that underlie their physiological functions in immune cells.

The processes of miRNA biogenesis and target mRNA repression have been intensively studied in recent years[4]. miRNAs are encoded by genomic DNA and are most commonly transcribed by RNA polymerase II. Some miRNA-containing primary transcripts produce a single miRNA, whereas other transcripts encode proteins in their exons and miRNAs in their introns. Alternatively, certain miRNAs, such as the miR-17–92 family, are grouped in clusters on a single unprocessed transcript and are expressed together. miRNAs are processed from their primary transcripts (known as pri-miRNAs) by the enzymes Drosha (also known as ribonuclease 3) and DiGeorge syndrome critical region gene 8 (DGCR8), and recent data suggest that this occurs co-transcriptionally[5]. Pre-miRNAs are then exported from the nucleus by exportin 5.

Table 1 | **Selected microRNAs with roles in the immune system**

MicroRNA	Cell type expressing microRNA	Target genes
miR-221 and miR-222	HSCs	*KIT*
miR-10	HSCs	HOX family
miR-196b	HSCs	HOX family
miR-126	HSCs	*HOXA9* and *PLK2*
miR-155	B cells, T cells, macrophages and DCs	*SHIP1*, *PU.1*, *AID*, *SOCS1*, *BACH1*, *CEBPB*, *CSFR*, *TAB2*, *MAF* and *JARID2*
miR-150	B cells and T cells	*MYB*
miR-17–92	B cells and T cells	*BIM* and *PTEN*
miR-181a	T cells	*DUSP5*, *DUSP6*, *SHP2* and *PTPN22*
miR-326	T cells	*ETS1*
miR-142-3p	T_{Reg} cells	*AC9*
miR-424	Myeloid cells	*NFIA* and *SPI1*
miR-21	Myeloid cells	*PTEN*, *PDCD4* and *IL12A*
miR-17-5p-20a-106a	Myeloid cells	*RUNX1*
miR-223	Myeloid cells	*MEF2C*
miR-146	Monocytes	*IRAK1*, *IRAK2* and *TRAF6*
let-7e	Macrophages	*TLR4*
miR-9	Myeloid cells	*NFKB1*
miR-34	DCs and B cells	*JAG1*, *WNT1* and *FOXP1*

AC9, adenylate cyclase 9; *AID*, activation-induced cytidine deaminase; *BACH1*, BTB-, CNC- and bZIP-domain-containing transciption factor; *BIM*, BCL-2-interacting mediator of cell death; *CEBPB*, CCAAT/enhancer binding protein-β; *CSFR*, colony-stimulating factor receptor; DC, dendritic cell; *DUSP*, dual-specificity protein phosphatase; *FOXP1*, forkhead box P1; HOX, homeobox; HSC, haematopoietic stem cell; *IL12A*, interleukin-12A; *IRAK*, IL-1R-associated kinase; *JAG1*, Jagged1; *JARID2*, jumonji, AT rich interactive domain 2; *MEF2C*, myeloid ELF1-like factor 2C; miR, microRNA; *NFIA*; nuclear factor I/A; *NFKB1*, nuclear factor-κB subunit 1; *PDCD4*, programmed cell death 4; *PLK2*, polo-like kinase 2; *PTEN*, phosphatase and tensin homologue; *PTPN22*, protein tyrosine phosphatase, non-receptor type 22; *RUNX1*, runt-related transcription factor 1; *SHIP1*, SH2-domain-containing inositol-5-phosphatase 1; *SHP2*, SH2-domain-containing protein tyrosine phosphatase 2; *SOCS1*, suppressor of cytokine signalling 1; *TAB2*, TAK1-binding protein 2; *TLR4*, Toll-like receptor 4; *TRAF6*, TNFR-associated factor 6; T_{Reg} cell, regulatory T cell.

On reaching the cytoplasm, further processing of the pre-miRNA is carried out by Dicer, resulting in the formation of a miRNA duplex. The duplex is unwound, and one strand, the guide strand, is packaged into the RNA-induced silencing complex (RISC), which comprises Argonaute and other proteins. The miRNA then guides the RISC to its target 3′ UTRs, leading to inhibition of translation and/or a decrease in mRNA stability as a result of accelerated uncapping and deadenylation[6,7]. There is evidence that the miRNA-silenced mRNA is directed to processing bodies, in which the targeted transcript is released from its inhibition or actively degraded[8]. Recent work in plants and worms has also identified proteins that regulate miRNA turnover[9,10]; however, a role for turnover control of miRNAs in mammals has yet to be shown.

Several recent studies indicate that the regulation of miRNA expression and function occurs at three levels: transcription, processing and subcellular localization (FIG. 1). There is emerging evidence that some of these processes are influenced by immune challenge, inflammation or other forms of cellular stress. The expression of miRNAs is initially controlled at the transcriptional level by transcription factors that regulate the production of miRNA-containing primary transcripts in specific cell types during development or in response to different environmental cues. For example, the transcription of certain miRNAs found in immune cells, such as miR-155 and miR-146a, is upregulated in response to inflammatory stimuli such as Toll-like receptor (TLR) ligands or pro-inflammatory cytokines[11,12].

In addition, several post-transcriptional regulatory mechanisms that affect miRNA processing have recently been identified, functioning at various stages from the initial primary transcript to the delivery of mature, single-stranded miRNAs to their target mRNAs. Among the factors involved are the SMAD proteins, which have been shown to regulate miR-21 processing in a ligand-dependent manner[13]. Arsenate-resistance protein 2 (ARS2; also known as SRRT) is expressed by proliferating haematopoietic cells and is a component of the RNA cap-binding complex that promotes processing of pri-miRNA transcripts[14]. Adenosine deaminase acting on RNA (ADAR; also known as DRADA) expression is upregulated during inflammation and can regulate pri-miRNA processing through mutation of the double-stranded miRNA stem sequence[15]. In addition, the tumour suppressor protein p53 has an important role in miRNA processing following the onset of DNA damage[16]. p53 forms a complex with Drosha and induces increased processing of pri-miRNAs to pre-miRNAs. The miRNA loop also mediates regulatory control over miRNA processing, and KH-type splicing regulatory protein (KSRP; also known as FUBP2) has been implicated in this process[17,18]. In an apparently specific form of regulation, LIN28 binds to the loop of the let-7 pre-miRNA in human stem cells and inhibits its processing[19].

After transport into the cytoplasm, post-transcriptional modifications of some pre-miRNAs are catalysed by terminal uridylyltransferase 4 (TUT4), which has been shown to be recruited by LIN28 to mediate uridylation of pre-let-7 miRNA[20,21]. This modification prevents further processing of pre-let-7 miRNA, thus inhibiting its function. The abundance of pri-miRNA processing factors themselves can be influenced during immune responses. For example, cytokines such as interferons have been shown to inhibit Dicer expression, decreasing the processing of pre-miRNAs[22]. On reaching maturation, delivery of the RISC containing a fully processed miRNA guide strand to a cognate target mRNA has been shown to involve importin 8 (REF. 23). miRNAs can also be regulated by subcellular localization mechanisms. For example, miRNAs have been shown to associate with stress granules following the onset of cell stress; however, the roles of these organelles in miRNA biology remain unknown[24].

Together, these recent findings show that there is great complexity underlying both the production and subcellular localization of mature miRNAs. Future studies will be required to determine if differential miRNA processing occurs in particular immune cell types or under specific inflammatory conditions as a means of controlling the spatial and temporal patterns of miRNA expression that have been observed in the immune system.

RNA-induced silencing complex
A multicomponent ribonucleoprotein complex, comprising miRNAs or siRNAs and Argonaute proteins, that silences the expression of proteins from target mRNAs by cleavage or other unknown mechanisms depending on the complementarity of mRNA sequences to the packaged small RNAs.

Processing bodies
Cytoplasmic foci that are thought to store and degrade translationally repressed RNA.

miRNA regulation of haematopoietic stem cells

Haematopoietic stem cells (HSCs) reside mainly in the bone marrow and give rise to all blood cell lineages, including cells that constitute the immune system[25]. HSCs must maintain a precise balance between self-renewal and differentiation into multipotent progenitors, which subsequently give rise to both the lymphoid and myeloid branches of the haematopoietic system (FIG. 2). Although miRNAs have been studied in other stem cell types, such as embryonic stem cells, there are currently limited data on the role of miRNAs in HSCs[26]. Several groups have carried out global miRNA expression profiling of human CD34$^+$ stem and progenitor cells and have identified certain miRNAs expressed by this cell population[27,28]. Mice deficient in ARS2, which contributes to pri-miRNA processing, have bone marrow failure possibly owing to defective HSC function[14]. These studies provide initial evidence that the miRNA pathway is important in HSC function.

Individual miRNAs have also been implicated in HSC biology. Homeobox (HOX) genes have important roles in regulating HSC homeostasis, and miRNAs from the miR-196 and miR-10 families were found to be located in the HOX loci; both could directly repress HOX family expression[29–32]. miR-196b is expressed specifically in mouse short-term HSCs, regulated by the HSC transcription factor family mixed lineage leukaemia (MLL), and has a functional role in modulating HSC homeostasis and lineage commitment, possibly through the regulation of expression of certain HOX genes[33]. miR-126 has also been shown to regulate expression of HOXA9 (REF. 34) and the tumour suppressor polo-like kinase 2 (PLK2)[35], through which miR-126 is thought to mediate its biological effects. Functional studies of bone marrow progenitors showed that miR-126 increased colony formation *in vitro*, suggesting that it may promote the production of downstream progenitors by HSCs[35]. miR-221 and miR-222 were also shown to inhibit KIT expression by stem and progenitor cells, leading to impaired cell proliferation and engraftment potential[36]. Although these studies suggest a role for specific miRNAs in HSC biology, additional work is needed to directly assess the influence of these and other miRNAs on the function of carefully sorted HSC populations regarding long-term, multilineage engraftment *in vivo*.

Regulation of innate immunity by miRNAs

Cells of the innate immune system, such as granulocytes, monocytes (which differentiate into myeloid-derived dendritic cells (DCs) or macrophages) and natural killer (NK) cells, provide an important first line of defence against infection. Emerging data have identified an important contribution of miRNAs to the development and function of innate immune cells (FIG. 3). Furthermore, studies investigating myeloid cell development and function have identified a common theme of a dynamic interplay between lineage-specific transcription factors and miRNAs.

Granulocytes. Granulocytes arise from granulocyte–monocyte progenitors under the influence of the transcription factor growth factor independent 1 (GFI1). GFI1 was recently shown to bind to the promoter regions of pri-miR-21 and pri-miR-196b and repress their expression[37]. Furthermore, overexpression of miR-21 and miR-196 in lineage-depleted bone marrow cells blocked granulopoiesis *in vitro*, a phenotype also observed in

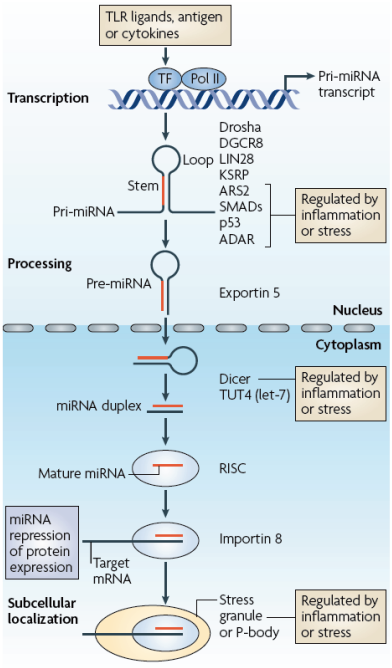

Figure 1 | **MicroRNA expression and function are regulated at three levels and influenced by inflammation and stress.** MicroRNA (miRNA) expression is first regulated by transcription factors (TFs) that can themselves be activated or repressed during immune responses by Toll-like receptor (TLR) ligands, antigens or cytokines. Following the production of a miRNA primary transcript (pri-miRNA) in the nucleus, its processing into a pre-miRNA is regulated by various factors, including Drosha, DiGeorge syndrome critical region gene 8 (DGCR8), LIN28, KH-type splicing regulatory protein (KSRP), arsenate resistance protein 2 (ARS2), SMADs, p53 and adenosine deaminase acting on RNA (ADAR), some of which are themselves influenced by inflammation. The pre-miRNA is then exported to the cytoplasm by exportin 5. On reaching the cytoplasm, the pre-miRNA is processed by Dicer (or modified by terminal uridylyltransferase 4 (TUT4) in the case of let-7). The miRNA duplex is unwound and the guide strand is loaded onto the RNA-induced silencing complex (RISC). This complex is then transported by importin 8 to its cognate mRNA, leading to repression of the target. Finally, miRNAs can be localized to specific organelles, such as stress granules or processing (P-) bodies, some of which are thought to be used during stress. Pol II, RNA polymerase II.

p53
A tumour suppressor protein that is mutated in ~50% of all human cancers. The p53 protein is a transcription factor that is activated by DNA damage, anoxia, expression of certain oncogenes and several other stress stimuli. Target genes activated by p53 regulate cell cycle arrest, apoptosis, cell senescence and DNA repair.

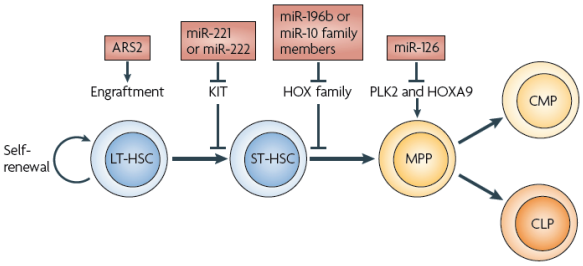

Figure 2 | **MicroRNA-mediated regulation of early haematopoietic cell development.** The microRNA (miRNA) pathway, which involves arsenate resistance protein 2 (ARS2), has a general role in haematopoietic stem cell (HSC) engraftment, which probably influences the reconstitution potential of long-term HSCs (LT-HSCs). Individual miRNAs have been shown to repress the expression of HSC-relevant genes and affect the production of haematopoietic progenitor and lineage-positive cells. Potential points of miRNA function during early haematopoiesis are indicated. miR-221 or miR-222 regulate KIT expression, which is thought to affect stem cell homeostasis. miR-196b is specifically expressed by short-term HSCs (ST-HSCs) and regulates mRNAs encoding the homeobox (HOX) family, in cooperation with miR-10 family members. The expression of miR-10 family members by HSCs is not as clearly defined. miR-126 represses the mRNAs encoding polo-like kinase 2 (PLK2) and HOXA9, and has been shown to promote expansion of progenitor cells. CLP, common lymphoid progenitor; CMP, common myeloid progenitor; MPP, multipotent progenitor.

Gfi1$^{-/-}$ mice[38]. Our group has found that sustained expression of miR-155 can increase immature granulocyte numbers *in vivo*, and that several of its targets, including SH2-domain-containing inositol-5-phosphatase 1 (*SHIP1*; also known as *INPP5D*) are probably involved in this process[39,40].

In addition to regulating granulocyte development, miRNAs also regulate granulocyte function. miR-223 is induced by the myeloid transcription factors PU.1 and CCAAT/enhancer-binding protein-β (C/EBPβ)[41], and it negatively regulates both the proliferation and activation of neutrophils[42]. miR-223-deficient neutrophils had increased capacity to undergo oxidative burst and more effectively killed *Candida albicans* than did wild-type cells[42]. Myeloid ELF1-like factor 2C (*MEF2C*) was shown to be a direct target of miR-223, although this was only responsible for the increased neutrophil proliferation observed in miR-223-deficient mice and not the higher neutrophil activation status. This indicates that different miR-223 targets regulate distinct aspects of neutrophil biology.

Monocytes, macrophages and DCs. Both macrophages and myeloid-derived DCs have key roles in the innate immune response to infection. Similar to granulopoiesis, several studies have shown that transcription factors involved in monocytopoiesis[43] are regulated by, and/or regulate, specific miRNAs, which indicates a connection between these molecular species during development.

The expression levels of miR-17-5p, miR-20a and miR-106a, members of the miR-17–92 and related miR-106a–92 families, have been shown to decrease during the differentiation of human haematopoietic progenitor cells into monocytes. This led to derepression of their target runt-related transcription factor 1 (*RUNX1*; also known as *AML1*)[44]. The resulting increase in RUNX1 expression promoted monocyte differentiation, presumably by increasing colony-stimulating factor receptor (CSFR) expression. RUNX1 was also found to bind the miR-17–92 and miR-106a–92 promoter regions and repress their expression, suggesting that miR-17–92 and RUNX1 are involved in a mutual negative feedback loop. PU.1 is another transcription factor that is crucial for monocyte and macrophage differentiation and has been shown to upregulate miR-424 levels during 12-*O*-tetradecanoyl phorbol 13-acetate (TPA)-mediated monocyte differentiation of NB4 cells[45]. Enforced expression of miR-424 in NB4 cells promoted monocytic differentiation, increasing the numbers of $CD11b^{+}CD14^{+}$ cells and inducing morphological changes consistent with monocytic maturation. miR-424 promoted monocytic differentiation by inhibiting the expression of nuclear factor I/A (*NFIA*), which is important for myeloid cell differentiation and had also been shown previously to be targeted by miR-223 during granulocyte differentiation *in vitro*[46].

The macrophage inflammatory response to infection involves the upregulation of several miRNAs, such as miR-155, miR-146, miR-147, miR-21 and miR-9 (REFS 11,12,47–49). Indeed, miR-155 expression is induced in mouse bone marrow-derived macrophages in response to various TLR ligands and by the pro-inflammatory cytokines tumour necrosis factor (TNF) and interferon-β[11]. Interferons do not directly induce miR-155 expression by macrophages but instead do so indirectly through TNF autocrine or paracrine signalling[11]. The primary transcript encoding miR-155, known as the B cell integration cluster (*BIC*)[50], was shown to be transcriptionally activated by the transcription factors activator protein 1 (AP1) and nuclear factor-κB (NF-κB)[11,51–53]. Certain cognate ligands for TLRs and the cytoplasmic sensor retinoic acid-inducible gene I (RIG-I; also known as DDX58) also induce miR-146 expression in a NF-κB-dependent manner[12,54]. In peripheral human monocytes and neutrophils, miR-9 is similarly upregulated by pro-inflammatory signals in a myeloid differentiation primary-response protein 88 (MYD88)- and NF-κB-dependent manner[49]. Therefore, certain inflammatory miRNAs are regulated transcriptionally in a similar manner to other inflammatory genes.

The inflammatory response to infection must be robust enough to eradicate microbial pathogens but resolved in a timely manner to avoid excessive damage to the host. miRNAs have been shown to influence both of these aspects of inflammation. There is evidence that many of the miRNAs induced by TLRs, such as miR-146, miR-9, miR-147, miR-21 and miR-155, can negatively regulate the activation of inflammatory pathways in myeloid cells[12,17,47,48,54]. For example, miR-146 directly represses several signalling molecules that are downstream of TLRs, including IL-1R-associated kinase 1 (*IRAK1*), *IRAK2* and TNFR-associated factor 6 (*TRAF6*), all of which promote inflammation[12,54]. miR-9 represses NF-κB subunit 1 (*NFKB1*) and helps to maintain a constant level of NF-κB1 protein expression during TLR4-mediated activation of monocytes and neutrophils.

Retinoic acid-inducible gene I
A cytoplasmic pathogen sensor that recognizes viral double-stranded RNA molecules and triggers an antiviral response.

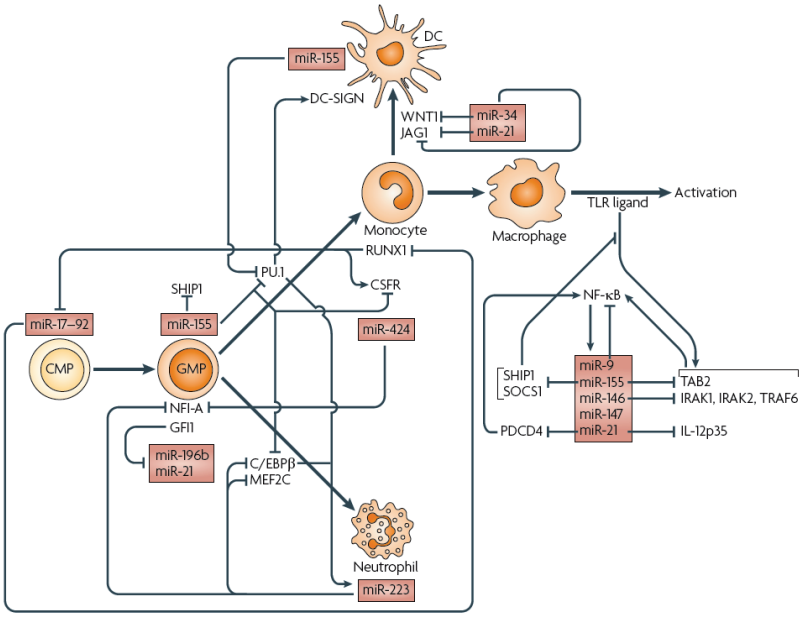

Figure 3 | **MicroRNA-mediated regulation of myeloid cell development and function.** The generation of different myeloid cell types is regulated by the interactions between microRNAs (miRNAs) and relevant transcription factors. Common myeloid progenitors (CMPs) give rise to granulocyte–monocyte progenitors (GMPs). GMPs produce both monocytes and neutrophils, and monocytes can further differentiate into macrophages and dendritic cells (DCs). The miR-17–92 cluster represses runt-related transcription factor 1 (RUNX1) production, which regulates colony-stimulating factor receptor (CSFR) expression during monocytopoiesis. Growth factor independent 1 (GFI1) represses miR-196b and miR-21 during granulocyte development. The myeloid transcription factors PU.1 and CCAAT/enhancer-binding protein-β (C/EBPβ) help to promote miR-223 expression. miR-223 and miR-424 promote monocyte and neutrophil differentiation, respectively, by repressing nuclear factor I/A (NFI-A) production. miR-223 also represses C/EBPβ and myeloid ELF1-like factor 2C (MEF2C) expression to modulate neutrophil differentiation and proliferation. miR-155 has roles in myeloid cell differentiation and activation. miR-155 represses SH2-domain-containing inositol-5-phosphatase 1 (SHIP1), PU.1, C/EBPβ and CSFR production; these interactions may influence myeloid differentiation. miR-155 also represses expression of dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) through the suppression of PU.1 expression to affect antigen presentation by DCs. miR-34 and miR-21 repress the mRNAs encoding WNT1 and Jagged 1 (JAG1) to promote DC differentiation. During Toll-like receptor (TLR) activation, activator protein 1 (AP1; not shown) and nuclear factor-κB (NF-κB) enter the nucleus and induce the expression of several miRNAs, including miR-9, miR-155, miR-146, miR-21 and miR-147. miR-155 represses the mRNA encoding TAK1-associated binding protein 2 (TAB2), miR-9 targets a mRNA encoding NF-κB and miR-146 represses the mRNAs encoding IL-1R-associated kinase 1 (IRAK1), IRAK2 and TNF-receptor-associated factor 6 (TRAF6), which are TLR signalling molecules. miR-21 negatively regulates macrophage activation by repressing expression of programmed cell death 4 (PDCD4) and interleukin-12 p35 subunit (IL-12p35). miR-155 also represses expression of suppressor of cytokine signalling 1 (SOCS1) and SHIP1, which are negative regulators of the TLR pathway.

In human myeloid-derived DCs, knockdown of miR-155 expression significantly increased protein expression of the pro-inflammatory cytokine interleukin-1β (IL-1β)[55]. Furthermore, addition of a miR-155 mimic decreased the expression of a NF-κB reporter plasmid in cell lines[55,56]. miR-155 has also been shown to repress the expression of the pro-inflammatory signalling protein TAK1-binding protein 2 (TAB2; also known as MAP3K7IP2), suggesting a mechanistic basis for its anti-inflammatory effect in certain settings[55]. By contrast, it has been shown that in some situations miR-155 can positively regulate inflammatory pathways, enhancing instead of diminishing them. Indeed, enforced expression of miR-155 in the bone marrow compartment of adult mice leads to a myeloproliferative phenotype that is similar to that which occurs transiently after lipopolysaccharide (LPS) injection[40]. miR-155 was shown to negatively regulate SHIP1, an important negative regulator of phosphoinositide 3-kinase (PI3K) and the downstream AKT pathway[39,57]. SHIP1 is a negative regulator of TLR4 signalling[58], and therefore repression of SHIP1 by miR-155 may counter this negative regulation and increase downstream AKT signalling.

Interestingly, AKT signalling has recently been shown to repress miR-155 expression in macrophages[59], suggesting the existence of a negative feedback loop. LPS-stimulated *Akt*$^{-/-}$ macrophages expressed higher levels of miR-155 and miR-125b and also lower levels of let-7e and miR-181c than wild-type controls. *Akt*$^{-/-}$ macrophages were more responsive to LPS, an effect mediated in part by the increased miR-155 and decreased let-7e levels. Notably, miR-155 directly repressed levels of suppressor of cytokine signalling 1 (SOCS1), which, similar to SHIP1, is a negative regulator of the TLR pathway. let-7e directly downregulated expression of the LPS receptor TLR4. The *in vivo* relevance of this pathway is supported by the finding that *Akt*$^{-/-}$ mice have a lower tolerance to systemically delivered LPS than wild-type mice. Taken together, it is clear that specific miRNAs are involved in regulating inflammation, presumably to help create a properly balanced response to pathogens and noxious stimuli. However, the role of miR-155 seems to be complex, possibly owing to its wide range of relevant target mRNAs and the cellular contexts in which it is expressed.

There are also reports indicating that miRNAs regulate distinct aspects of DC biology, and so are involved in the crucial connection between innate and adaptive immune responses. miR-34 and miR-21 were shown to be important for human myeloid-derived DC differentiation by targeting the mRNAs encoding Jagged1 and WNT1 (REF. 60). Myeloid-derived DCs from *Bic*$^{-/-}$ (miR-155-deficient) mice show defects in antigen presentation to T cells, despite expressing normal levels of MHC class II and co-stimulatory molecules; so, the mechanism behind the defect remains elusive[61]. In addition, another study found that miR-155 downregulates expression of DC-specific ICAM3-grabbing non-integrin (DC-SIGN; also known as CD209) by human monocyte-derived DCs through suppression of PU.1 expression[62]. DC-SIGN is a cell surface C-type lectin that binds pathogens, implicating miRNAs in the regulation of pathogen uptake by DCs.

NK and NKT cells. NK cells are important components of immune surveillance against cancer and viral infection, and miRNAs have been implicated in their development and function. NK cells express the receptor natural killer group 2, member D (NKG2D), which recognizes ligands — MHC class I polypeptide-related sequence A (MICA) and MICB — that are expressed by cells undergoing stress triggered by events such as viral infection or cell transformation. Engagement of NKG2D on NK cells leads to direct killing of the target cell. A recent study showed that a set of miRNAs, many of which are overexpressed by various cancer cells, could downregulate MICA and MICB expression in human cell lines[63]. Treatment of cells with antagomirs (antisense oligodeoxynucleotides) that inhibit the specific miRNAs being tested led to increased cell surface expression of MICA and MICB and increased NK cell-dependent killing; overexpression of these miRNAs had the opposite effect. Although heat shock-induced cell stress increased MICA and MICB expression in the HCT-116 cell line, expression of the relevant miRNAs did not concomitantly decrease. Instead, these miRNAs seem to set a threshold for MICA and MICB expression that prevents their expression during non-stress conditions. It is interesting to note that certain herpesvirus family members, namely cytomegalovirus, Epstein–Barr virus and Kaposi's sarcoma-associated herpesvirus (KSHV), produce miRNAs that target *MICB* mRNA, and this has been proposed as a mechanism of immune evasion by these viruses[64,65].

Invariant NKT (iNKT) cells are a class of innate-like T cells that express an invariant T cell receptor (TCR) that recognizes lipids presented by the MHC class I-like CD1d molecule. Two recent studies identified a requirement for Dicer in the development of iNKT cells[66,67]. Both studies observed a near complete loss of iNKT cells in the thymus and periphery in the absence of Dicer. Developing iNKT cells from conditional Dicer-knockout animals exhibited increased apoptosis and decreased differentiation, whereas mature iNKT cells had severe activation defects.

The studies described above clearly implicate miRNAs in the regulation of various aspects of innate immunity. This includes regulation of direct microbial killing, the production of cytokines and the antigen presentation by MHC molecules. All of these mechanisms are important for host defence and are instrumental in initiating antigen-specific responses by cells of the adaptive immune system.

Regulation of adaptive immunity by miRNAs

T cells. Similar to the development of innate immune cells, the development of T cells in the thymus and their activation in the periphery are also controlled by complex protein signalling networks that are subject to regulation by miRNAs (FIG. 4). Expression profiling of T cells has identified a broad range of expressed miRNA species and found that the expression patterns vary between T cell subsets and stages of development[68–70]. Adding to this complexity, several variants of a given miRNA species can be found in T cells, with the mature miRNAs varying in length at either the 3′ or 5′ end or containing mutated sequences[70]. Furthermore, proliferating T cells express genes with shorter 3′ UTRs than those in resting T cells[71], rendering these mRNAs less susceptible to regulation by miRNAs owing to the loss of miRNA binding sites. These findings suggest that miRNA-mediated regulation of mRNA targets in T cells, and probably other immune cells, is a dynamic process that is influenced by a broad range of factors.

T cell-specific deletion of *Dicer* has revealed a requirement for the miRNA pathway in the development of mature T cells, the total numbers of which are lower in the mutant mice than wild-type mice[72,73]. Two specific miRNAs have been implicated in T cell development, and probably account for some of the phenotype of Dicer deficiency in T cells. The miR-17–92 cluster impairs the expression of mRNAs encoding pro-apoptotic proteins, including BCL-2-interacting mediator of cell death (BIM; also known as BCL2L11) and phosphatase and tensin homologue (PTEN). This miRNA cluster is thought to increase T cell survival during development and is expressed during the double negative 2 stage of thymopoiesis[74]. Furthermore, the strength of TCR signalling influences whether thymocytes are positively

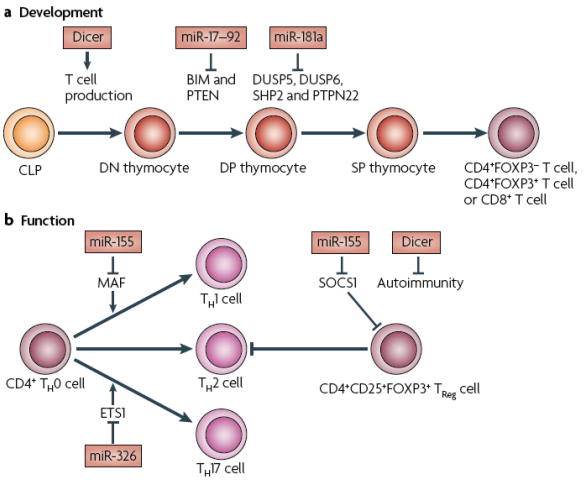

Figure 4 | **MicroRNA-mediated regulation of T cell development and function.** **a** | The production of microRNAs (miRNAs) by Dicer is required for efficient T cell development *in vivo*. T cells go through a stepwise developmental programme in the thymus. During this process, T cell survival and selection is influenced by the miR-17–92 cluster (which targets the mRNAs encoding BCL-2-interacting mediator of cell death (BIM), and phosphatase and tensin homologue (PTEN)) and miR-181a (which targets mRNAs encoding several phosphatases, including dual-specificity protein phosphatase 5 (DUSP5), DUSP6, SH2-domain-containing protein tyrosine phosphatase 2 (SHP2) and protein tyrosine phosphatase, non-receptor type 22 (PTPN22)). **b** | In the periphery, mature T cell differentiation is modulated by miRNAs, including miR-155, which promotes skewing towards T helper 1 (T_H1) cells through macrophage-activating factor (MAF) repression, and miR-326, which promotes skewing towards T_H17 cells by targeting the mRNA encoding ETS1. Regulatory T (T_{Reg}) cells also depend on miRNAs to maintain immune tolerance to self tissues, thereby preventing autoimmunity. miR-155 repression of suppressor of cytokine signalling 1 (SOCS1) expression has been implicated in T_{Reg} cell survival. CLP, common lymphoid progenitor; DN, double negative; DP, double positive; FOXP3, forkhead box P3; SP, single positive.

Germinal centre
A lymphoid structure that arises within follicles after immunization with, or exposure to, a T cell-dependent antigen. It is specialized for facilitating the development of high-affinity, long-lived plasma cells and memory B cells.

Affinity maturation
The somatic mutation process by which B cells are selected for survival and proliferation on the basis of their increased affinity for antigen.

Class switching
The somatic recombination process by which the class of immunoglobulin is switched from IgM to IgG, IgA or IgE.

or negatively selected during thymic development, and specific miRNAs have been implicated in this process. miR-181a, which is increased during early T cell development, enhances TCR signalling strength by directly targeting a group of protein phosphatases, including dual-specificity protein phosphatase 5 (*DUSP5*), *DUSP6*, SH2-domain-containing protein tyrosine phosphatase 2 (*SHP2*; also known as *PTPN11*) and protein tyrosine phosphatase, non-receptor type 22 (*PTPN22*)[75].

Recent data have also indicated a role for miRNAs in the differentiation of T cells into distinct effector T helper cell subsets. This is best exemplified by mice deficient in miR-155, in which T cells are biased towards T helper 2 (T_H2)-cell differentiation, indicating that miR-155 promotes differentiation into T_H1 cells[51,61]. Certain miRNAs, such as the miR-17–92 cluster, might also be involved in the development and function of T follicular helper (T_{FH}) cells, which are specialized T cells that are dedicated to supporting B cells in germinal centres and facilitating antibody affinity maturation and class switching[76,77]. T_H17 cells have been identified as important mediators of inflammatory disease. A recent study found that miR-326 promotes T_H17 cell development both *in vitro* and *in vivo* by targeting *ETS1* (REF. 78).

The generation of mice with a conditional deletion of *Dicer* or *Drosha* in regulatory T (T_{Reg}) cells has shown a requirement for the miRNA pathway in forkhead box P3 (FOXP3)$^+$ T_{Reg} cells[79–81]. These mice develop a lethal autoimmune inflammatory disease, consistent with impaired development or function of T_{Reg} cells. Specifically, it was shown that miR-155 is important for T_{Reg} cell homeostasis and overall survival, and this is thought to involve the direct targeting of *Socs1* (REFS 82,83). However, because the absence of miR-155 did not reproduce the severe disease that occurs in mice with a conditional deletion of *Dicer*, additional miRNAs are probably involved in T_{Reg} cell biology. Of note, the expression of miR-142-3p was recently shown to be repressed by FOXP3, leading to increased production of cyclic AMP and suppressor function of T_{Reg} cells[84]. Several other miRNAs are expressed by T_{Reg} cells and await functional assessment[85].

B cells. The generation of B cells that express high-affinity antigen receptors involves two main stages: antigen-independent development in the bone marrow and antigen-dependent selection in the secondary lymphoid organs; both of which are associated with dynamic regulation by miRNAs (FIG. 5). miRNA profiling studies during early B cell development have not been carried out but several groups have analysed the expression of miRNAs in naive, germinal centre and post-germinal centre B cells[86–88]. Temporal regulation of several different miRNAs was observed and putative new cell type-specific miRNAs were identified, suggesting the involvement of many, but undefined, regulatory pathways in B cell development and maturation.

The control of B cell development in the bone marrow depends on the commitment of progenitor cells to the B cell lineage by the activation of transcription factor networks, V(D)J recombination events and selection for effective antigen receptors. The roles of miRNAs in controlling the early development of B cells are now thought to involve the modulation of key protein factors that control these aspects of B cell development[89]. An initial study found that overexpression of miR-181 causes a skewing of haematopoiesis towards the development of B cells, leading to a two to threefold increase in the number of B cells and no increase in T cells or myeloid cells[90]. By contrast, mice with a conditional deletion of *Dicer* in B cells had a complete block in B cell development[91]. This block is thought to relate to dysregulated expression of the pro-apoptotic protein BIM, probably during the selection of effective antigen receptors. The block in B cell development in these mice could be overcome by transgenic expression of B cell lymphoma 2 (BCL-2), but the mature B cells had an abnormal distribution of V(D)J rearrangements, indicating that there is a defect in the regulation of B cell selection. Notably, the changes observed by gene expression profiling of Dicer-deficient B cell precursors were generally similar to those observed in B cells lacking the miR-17–92 family[92], which has been associated with

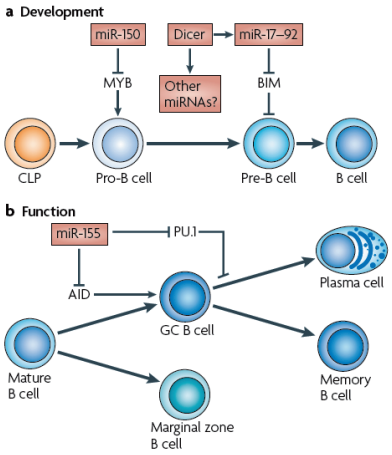

Figure 5 | **MicroRNA-mediated regulation of B cell development and function. a** | The survival and maturation of developing B cells requires microRNAs (miRNAs). Dicer-deficient animals have defective B cell development owing to a survival defect at the progenitor (pro) to precursor (pre) B cell transition. miR-150 and the miR-17–92 cluster influence early development through repression of MYB and BCL-2-interacting mediator of cell death (BIM) expression, respectively. **b** | In the periphery, further development of mature B cells is regulated by miR-155. This miRNA targets mRNAs encoding activation-induced cytidine deaminase (AID) and PU.1, which promotes antibody class switching and antibody production. CLP, common lymphoid progenitor; GC, germinal centre

Regulatory T (T_{Reg}) cells
A small population of $CD4^+$ T cells that naturally express high levels of CD25 (the interleukin-2 receptor α-chain) and FOXP3. They have suppressive regulatory activity towards other T cells that are stimulated through their T cell receptor. An absence of T_{Reg} cells or their dysfunction is associated with severe autoimmunity.

V(D)J recombination
Somatic rearrangement of variable (V), diversity (D) and joining (J) regions of the genes that encode antigen receptors, leading to repertoire diversity of both T cell and B cell receptors.

B-1 B cells
A subset of self-renewing B cells found mainly in the peritoneal cavity and the pleural cavity. They recognize self components, as well as common bacterial antigens, and they secrete antibodies that generally have low affinity and broad specificity.

B-2 B cells
Conventional B cells. These cells reside in secondary lymphoid organs and secrete antibodies with high affinity and narrow specificity.

RAG proteins
RAG1 and RAG2 are proteins that mediate V(D)J recombination in pre-B cells and thymocytes, which allows the production of antibodies and T cell receptors, respectively.

Activation-induced cytidine deaminase
(AID). An RNA-editing enzyme that is necessary for antibody affinity maturation and class switching.

Fragile site
A site in a chromosome that is susceptible to chromosome breakage and fusion with other chromosomes.

pathological lymphoproliferative conditions in humans and mice[74]. So, the key effects of conditional deletion of *Dicer* in B cells could be mainly due to the absence of these crucial, clustered miRNAs in early B cells.

In addition to effects on antigen receptor selection, miRNAs also regulate the transcription factors involved in early B cell development. Constitutive expression of miR-150, which has a dynamic expression profile in the early B cell lineage, causes a block at a proximal stage of B cell development, the pro-B to pre-B cell transition[93], and this inhibition depends on dysregulation of MYB expression. By contrast, mice deficient in miR-150 have an accumulation of B-1 B cells in the spleen and the peritoneal cavity, with a relative decrease in the number of B-2 B cells[94]. We have found that constitutive expression of miR-34a perturbs B cell development by causing an increase in cells at the pro-B to pre-B cell transition. This is mainly the result of miR-34a targeting of the transcription factor *Foxp1*, which regulates expression of the recombination-activating genes (*Rag1* and *Rag2*) (D.S.R. and D.B., unpublished observations). Because miR-34a is a p53-induced miRNA[95], this effect may link the regulation of DNA damage responses with regulation of RAG proteins.

An involvement for miRNAs in the antigen-driven stages of the humoral response in secondary lymphoid organs has also been described. Despite a decrease in total numbers of B-2 B cells (mentioned above), miR-150-deficient mice have increased levels of antibody secretion both at baseline and following T cell-dependent antigenic stimulation; the mechanism of the antigen-dependent defect is not understood[94]. Perhaps the best characterized miRNA during this stage of the B cell response is miR-155, which is upregulated following B cell activation in the germinal centre. miR-155-deficient B cells have defective antibody class switching and differentiation into plasma cells, resulting in an impaired humoral response to T cell-dependent antigenic stimulation[51,61,96]. These effects are likely to be the consequence of miR-155-mediated regulation of many targets: indeed, two proteins that are important for T cell-dependent antibody responses — PU.1 and activation-induced cytidine deaminase (AID) — are directly repressed by miR-155 (REFS 96–98). The repression of AID has been investigated by germline mutation of the miR-155 target sequence in the 3′ UTR of mouse *Aicda* (which encodes AID)[98]. From these studies, it is clear that miRNAs are important for normal B cell development, implying that defects in miRNA regulation could be associated with B cell-mediated diseases.

miRNAs in diseases of immunological origin

As discussed, miRNAs directly modulate the concentration of many regulatory proteins that are required for normal development and function of the immune system. These proteins have been linked to immunological diseases, in which the miRNAs are found to be mutated or their expression levels dysregulated, consequently triggering altered or impaired function. miRNA levels are also dysregulated in diseases of immunological origins and many are known to be encoded near fragile sites in the genome[99]. Emerging data indicate that this can have a causative effect during disease initiation and progression owing to inappropriate repression or derepression of crucial protein targets (FIG. 6).

Cancers with immune cell origins. Profiling studies of immune cell-associated cancers have revealed changes in expression patterns of many miRNA species in different types of malignancy compared with normal cells[3,100,101]. In many cases, miRNA expression patterns have been correlated with clinical and/or pathological parameters of the cancer, revealing useful and sometimes clinically relevant markers for disease. For example, miRNA 'signatures' can be used to distinguish some subclasses of acute myeloid leukaemia (AML) and to distinguish B cell lymphoma from follicular hyperplasia[102–104]. Functional characterization of these miRNAs has been carried out in cell culture model systems, as well as gain-of-function and loss-of-function analyses in model organisms. We discuss the best characterized miRNAs that have tumour suppressive or oncogenic properties in immune cell-based cancers and describe how this knowledge has provided insight into the causes and progression of cancer (FIG. 6).

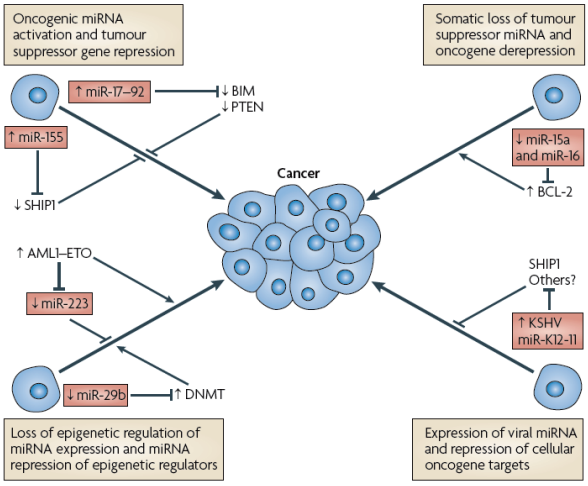

Figure 6 | **Mechanisms of microRNA contribution to cancer.** MicroRNAs (miRNAs) can influence immune cell-based malignant phenotypes through different mechanisms, including functioning as oncogenes (miR-155 and miR-17–92) or tumour suppressors (miR-15a and miR-16). miRNAs can also affect cancer by their involvement in epigenetic regulatory pathways. For example, miR-29b represses DNA methyltransferases (DNMTs), and AML1–ETO recruits chromatin remodelling factors to the miR-223 locus, leading to repressed miRNA expression. miRNAs, in the form of viral orthologues, might also modulate cancer-associated gene expression during viral infection. Kaposi's sarcoma-associated herpesvirus (KSHV) produces an orthologue of miR-155 known as miR-K12-11. BIM, BCL-2-interacting mediator of cell death; PTEN, phosphatase and tensin homologue; SHIP1, SH2-domain-containing inositol-5-phosphatase.

The first miRNAs identified as tumour suppressors, miR-15a and miR-16, were discovered by Croce and colleagues[105] in a region of chromosome 13 that was deleted in chronic lymphocytic leukaemia (CLL). These investigations subsequently showed that miR-15a and miR-16 negatively regulated the survival of cells *in vitro*, probably by targeting the pro-survival protein BCL-2 (REF. 106). More recently, the miR-34 family of miRNAs, consisting of miR-34a, miR-34b and miR-34c, were identified as transcriptional targets of p53 and shown to target mRNAs important in cell cycle progression and cell survival[95]. Profiling studies have revealed that miR-34a expression is downregulated in CLL[107]. In addition, the expression of the miR-34b and miR-34c primary transcript seems to be downregulated owing to promoter methylation in AML cell lines[108]. Other putative tumour suppressor miRNAs include miR-29ab and miR-181b, which are downregulated in CLL, and target *BCL2*, T cell lymphoma 1 (*TCL1*) and myeloid cell leukaemia sequence 1 (*MCL1*), which are all oncogenes[109]. In the context of miRNA-induced tumour suppression, it is important to note that the demonstration of tumorigenesis by genetic loss-of-function analysis has not yet been reported. However, our unpublished observations indicate that miR-146a-deficient mice develop a spontaneous malignancy (M. Boldin, K. Taganov and D.B., unpublished observations).

Epigenetic regulation of miRNAs and their targets also seems to be associated with cancer development and progression. For example, the expression of miR-223 is silenced following promoter methylation by the oncogenic fusion protein AML1–ETO[110]. In addition, miR-29b has been shown to repress specific DNA methyltransferases leading to global DNA hypomethylation and re-expression of tumour suppressor genes, thus implicating this miRNA as a tumour suppressor[111].

miR-17–92 and miR-155 have been found to cause oncogenesis. miR-17–92 is overexpressed by many human haematopoietic cell neoplasms, including acute lymphoblastic leukaemia (ALL), diffuse large B cell lymphoma (DLBCL) and chronic myeloid leukaemia (CML)[112]. In mouse B and T cells, miR-17–92 overexpression leads to a marked lymphoproliferative disease, characterized by lymphoid hyperplasia in the spleen, activated lymphocytes and lymphoid infiltration of peripheral tissues with autoimmune sequelae[74]. The mRNA targets in this context are thought to encode PTEN and BIM, both of which have tumour suppressive activities. A mechanism involving BIM is consistent with the findings that *Bim*$^{+/-}$ mice develop a phenotype similar to that of mice overexpressing miR-17–92 and that heterozygous deficiency of BIM, similar to overexpression of miR-17–92, cooperates with Eμ–*Myc* to induce oncogenesis[113].

miR-155 is also overexpressed in a diverse array of immune cell cancers, including CLL, DLBCL and AML[30,40,50,114–116]. Forced overexpression of miR-155 causes features of both lymphoid and myeloid oncogenesis, as shown by a low-grade B cell lymphoma that develops in mice that overexpress miR-155 in all B cells (Eμ–miR-155 transgenic mice) and by marked myeloproliferative disease in mice following adoptive transfer of bone marrow cells overexpressing miR-155 (REFS 40,117). In both of these cases, *SHIP1* mRNA is an important target of miR-155 (REFS 39,57). In the bone marrow adoptive transfer model, the myeloproliferative phenotype can be almost completely reproduced by knockdown of SHIP1 expression, providing *in vivo* evidence of target specificity[39,57]. In a similar manner, a viral orthologue of miR-155 encoded by KSHV (miR-K12-11) can have growth-promoting effects on cells and lead to KSHV-induced tumours, especially in patients with immunodeficiency[118].

Emerging roles for miRNAs in autoimmunity. The roles of miRNAs are only beginning to be explored in the context of autoimmunity, in which they may be involved in regulating immune responses against self tissues[119]. Interestingly, synovial tissue samples from patients with rheumatoid arthritis show some changes in the expression of miR-155 and miR-146a compared with control samples[120]. T cells isolated from such patients also show increases in the expression of miR-146a, miR-155 and miR-16. Several miRNAs were either upregulated or downregulated in rheumatoid fibroblast-like synoviocytes treated with LPS, and, among these, miR-346 was shown to modulate IL-18 production, albeit through an indirect mechanism[121].

Epigenetic regulation
The alteration of gene expression through transcriptional mechanisms (owing to promoter methylation) or post-transcriptional mechanisms instead of 'genetic' alteration of sequences of bases in genomic DNA.

AML1–ETO
The fusion protein that is generated by the t(8;21) translocation found in some acute myeloid leukaemias.

Another study identified three miRNAs, miR-18b, miR-599 and miR-96, that are differentially expressed by peripheral blood mononuclear cells from patients with multiple sclerosis compared with control cells[122]. A recent investigation identified miR-326 as being overexpressed in a cohort of patients with multiple sclerosis. Studies in mice showed that miR-326 could potentiate disease severity in the context of experimental autoimmune encephalomyelitis by promoting the development of T_H17 cells, which are important mediators of tissue damage during antigen-specific inflammation[78].

Further support for a causal relationship between specific miRNAs and the onset of autoimmunity has come from studies involving miR-17–92 overexpression in mice. miR-17–92 overexpression promoted marked lymphoproliferation, the presence of serum autoantibodies and tissue changes such as lymphoid infiltrates and antibody deposition[74]. T cells seem to develop normally in these mice, but the number of mature $CD4^+$ T cells was markedly increased and they had a highly activated profile, suggesting a failure of peripheral tolerance. There are also recent data showing that inhibition of miR-181a in T cells during thymic development converts endogenous positively selecting peptides into autoantigens[123]. Other evidence for a role of miRNAs in autoimmunity is provided by studies in T_{Reg} cells[79–81] (described above), by a report that the 3′ UTR of inducible T cell co-stimulator (*ICOS*) contains a binding site for miR-101 (REF. 124), and by the findings that expression of the anti-inflammatory protein IL-10 is regulated by miR-106a and the mRNA encoding the IL-12 p35 subunit (IL-12p35) is targeted by miR-21 (REFS 125,126). The relevance of these findings to human autoimmune diseases remains to be established, but should be addressed in future studies.

Concluding remarks and future perspectives

As described in this Review, miRNAs are being identified as key regulators of immune cell development and function, as well as disease pathogenesis. The observations that a single miRNA can have several effects on different cellular lineages indicate that miRNA targets in individual cell types may vary, allowing one miRNA to have many roles just as single transcription factors have unique functions in distinct immune cell lineages. There is also an emerging theme that miRNAs and transcription factors modulate one another in regulatory loops during development, and that this helps to establish a stable cellular identity.

It is now evident that aberrant miRNA expression in the immune system is sufficient to cause disease, and so proper regulation of miRNA expression seems to be crucial for disease prevention. Furthermore, it is instructive to note that knowledge of miRNA targets could provide a missing link between pathways that previously seemed separate. For example, miR-155 may connect NF-κB activation to repression of SHIP1 expression[11,39,56], and MYC-mediated miR-17–92 expression may be linked to suppression of PTEN[74,127]. As such, miRNAs may have an important role in the organization of gene regulation networks, and so it is possible that these systems are far larger than previously thought. Recognizing the interactions between these pathways may prove to be important for determining how cellular proliferation, differentiation and apoptosis are modulated, all of which are central to the development and function of the immune system and the initiation of cancer and autoimmunity.

Several areas of miRNA biology warrant future investigation both for our basic understanding and to enable translational utility. First, the ability of miRNAs to combinatorially regulate key mRNAs containing several 3′ UTR miRNA-binding sites should be assessed. This may lead to an even greater level of target mRNA repression than that mediated by a single miRNA, and could have important physiological and disease relevance. It may also identify miRNAs that commonly function together. Second, the importance of a single miRNA repressing many target mRNAs simultaneously needs to be further elucidated, and this may reveal new gene networks. Third, the mechanisms controlling miRNA levels and stability as cells differentiate or divide must be determined, including the processes by which mature miRNAs are degraded or cleared from the cell. Defining these cellular and molecular mechanisms will help us to further understand miRNA homeostasis in the context of dynamic cellular systems. Finally, the development of improved technologies for delivering miRNA antagonists or agonists to specific cell types *in vivo* would facilitate target mRNA identification and allow the exploitation of miRNAs as therapeutic entities for the treatment of disease.

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Competing interests statement
The authors declare competing financial interests: see Web version for details.

DATABASES
Entrez Gene: http://www.ncbi.nlm.nih.gov/gene
BIC | *ETS1* | *Foxp1* | *IRAK1* | *IRAK2* | *MEF2C* | *NFIA* | *NFKB1* | *Rag1* | *Rag2* | *RUNX1* | *SHIP1* | *TRAF6*
UniProtKB: http://www.uniprot.org
ADAR | AP1 | ARS2 | BIM | C/EBPβ | DGCR8 | Dicer | Drosha | exportin 5 | GFI1 | HOXA9 | importin 8 | KIT | KSRP | LIN28 | MYB | p53 | PLK2 | PTEN | PU.1 | RIG-I | SOCS1 | TUT4

FURTHER INFORMATION
David Baltimore's homepage:
http://baltimorelab.caltech.edu

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