MICRORNAS 155 AND 125B PHYSIOLOGICALLY AND PATHOLOGICALLY REGULATE HEMATOPOIESIS AND IMMUNITY

Thesis by

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Abstract

MicroRNAs are a class of ~ 22 nucleotide RNA molecules with roles in diverse biological processes. Here I focus on two microRNAs, miR-155 and miR-125b, and reveal pathways by which their dysregulation leads to myeloproliferative disorder (MPD) and leukemia, respectively. I begin by searching for miR-155 target genes relevant to MPD. By writing an algorithm to search microarray data for predicted microRNA target genes, I identified 89 candidate target genes for miR-155 in myeloid cells. Literature search whittled this list down to 11, and one gene among them, SHIP1, turned out to be largely responsible for miR-155's ability to cause MPD. My focus shifted to miR-125b when I noticed that miR-125b was enriched in macrophages and thus might play important roles in that cell type. Indeed, gain- and loss-of-function experiments indicated that miR-125b is a potent activator of macrophage activation, and I identified IRF4 as the primary target gene in this process. Finally I asked whether miR-125b plays pathophysiological roles in the development of the hematopoietic system. Thus I overexpressed miR-125b in the hematopoietic system and, to my surprise, observed a very aggressive myeloid leukemia capable of infiltrating peripheral organs including the lungs, liver, kidneys and brain. To determine whether miR-125b is physiologically necessary for normal hematopoietic development, I designed a loss-of-function sponge vector that acts as a decoy, attracting the microRNA away from its normal targets. Use of the sponge in the mouse hematopoietic system led to significantly decreased overall hematopoietic ouput, indicating that miR-125b is physiologically required for normal hematopoiesis. Next, I assayed in vitro a panel of miR-125b target genes and saw that one, Lin28, was superior to the rest. Indeed, Lin28 gain- and loss-of-function in vivo recapitulated major aspects of miR-125b loss- and gain-of-function, respectively. Thus I identified Lin28 as a primary target of miR-125b in the hematopoietic system. In summary, my work shows that two

microRNAs, miR-155 and miR-125b, physiologically and pathologically control hematopoietic development. I also identify important target genes for each of these microRNAs in their respective disease processes. Indeed, therapeutic targeting of these pathways may prove useful in the treatment of cancer.

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Chapter 1: Introduction

Overview of MicroRNAs

MicroRNAs (miRNAs) are a class of ~ 22 nucleotide RNA species that are generated from Drosha and Dicer-mediated cleavage of endogenous PolII-generated transcripts[1]. The first microRNA, lin-4, was discovered in 1993 by Victor Ambros and Gary Ruvkun and was shown to regulate early *C. elegans* larval development by mediating gene suppression[2-4]. Since that seminal finding, hundreds of other microRNAs have been discovered and sequenced in eukaryotic organisms, indicating that microRNAs are ubiquitous regulators of gene expression[5,6]. Indeed, it is thought that over half of all mammalian genes are regulated by microRNAs[7,8].

The mammalian immune system is a complex developmental system in which cells of hematopoietic origin differentiate and proliferate in response to environmental cues. The immune system is also in a constant state of renewal as hematopoietic stem cells give rise to new lineage-specific cells while memory lymphocytes wait in quiescence until they receive the proper signals to rapidly proliferate into effector cells[9]. In many ways, the development and function of the immune system is as dynamic as early embryonic development; thus, it is not surprising that microRNAs, such as in the case of lin-4 in *C. elegans* development, help orchestrate the development and function of the immune system.

MicroRNA Biogenesis and Function

MicroRNAs are transcribed from the genome by RNA polymerase II, initially as long transcripts called pri-miRNAs. Pri-miRNAs are cleaved by the enzyme Drosha into ~ 70 nucleotide pre-miRNAs, which are exported out of the nucleus by the exportin 5 transporter. Dicer cleaves each pre-miRNA into a 21-25 nucleotide, double-stranded RNA. The two RNA strands are separated and the strand with the thermodynamically

less stable 5' end becomes the mature miRNA, which is finally loaded onto a RISC complex. The miRNA-RISC complex mediates translational repression by partially complementing with sites in the 3' UTR of its target mRNA transcript[1,10-12].

The mechanism by which a microRNA selects and suppresses a particular mRNA transcript is not exactly known[13]. As result we can only predict which genes a particular miRNA targets. Prediction algorithms rely on conservation of target sites among multiple species, and the observation that a microRNA represses its target when at least 6-8 consecutive nucleotides in its 5' end (known as the seed region) perfectly complement a site in the target gene's 3' UTR[14,15]. Via this site, miRNAs downregulate expression of their targets by translational repression and in some cases by mRNA degradation[1,16].

Regulation of innate immunity by MicroRNAs

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 defense against infection. Emerging data have identified an important contribution of miRNAs to the development and function of innate immune cells (Figure 1). Furthermore, studies investigating myeloid development and function have identified a recurring theme of a dynamic interplay between lineage specific transcription factors (TFs) 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[17]. Furthermore, overexpression of miR-21 and miR-196 in lineage (LIN) depleted bone

marrow cells blocked granulopoiesis in vitro, leading to a decrease in neutrophil development, as is also observed in *Gfi1*$^{-/-}$ mice[18]. On a related note, we found that sustained expression of miR-155 can increase immature granulocyte numbers in vivo, and that several targets including Src homology-2 domain-containing inositol 5-phosphatase 1 (SHIP1) are likely involved in this process[19,20].

In addition to regulating granulocyte development, miRNAs also regulate their function. In particular, myeloid-specific miR-223 is important for modulating neutrophil proliferation and activation[21]. miR-223, which is induced by the myeloid-relevant transcription factors PU.1 and CEBPβ[22], negatively regulates proliferation and activity of neutrophils. Cultured miR-223$^{-/-}$ neutrophils had increased oxidative bursts and more effectively killed *Candida albicans* than wild-type cells[21]. Indicative of hyperactivity, miR-223$^{-/-}$ neutrophils had increased nuclear lobularity and hypersegmentation. MEF2C was shown to be an important target of miR-223 because mice lacking both miR-223 and MEF2C had normal neutrophil proliferation[21]. Still, these neutrophils displayed histological signs of hyperactivion, suggesting that other uncharacterized miR-223 targets regulate activation. In short, miR-223, miR-155, miR-21, miR-196b, and likely a plethora of other microRNAs, form vital components of the molecular network regulating granulocyte biology (Figure 1).

Monocytes, macrophages and DCs

Both macrophages and myeloid-derived DCs have key roles in the innate immune response to infection. Macrophages and DCs are derived from monocytes, which themselves arise from granulocyte/monocyte progenitors. Like granulocytes, several studies have shown that transcription factors involved in monocytopoiesis[23] are regulated by and/or regulate specific miRNAs, again demonstrating a connection between these molecular species during development.

The expression levels of miRNAs 17-5p, 20a and 106a, members of the miR-17-92 and related miR-106a-92 polycistrons, have been shown to decrease during the differentiation of human haematopoietic progenitor cells into monocytes, leading to a lack of repression of their target gene runt-related transcription factor 1 (RUNX1; also known as AML1)[24]. The resulting increase in RUNX1 expression promoted monocyte differentiation, presumably by increasing CSF1R 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 a transcription factor that is crucial for monocyte and macrophage differentiation and has been shown to upregulate miR-424 levels during TPA (12 *O*-tetradecanoyl phorbol 13-acetate)-mediated monocyte differentiation of NB4 cells[25]. Enforced expression of miR-424 in NB4 cells promoted monocytic differentiation, increasing 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 (NFI-A), which is important for myeloid cell differentiation and had been shown previously to be targeted by miR-223 during granulocyte differentiation in vitro[26].

The macrophage inflammatory response to infection involves the upregulation of several microRNAs, such as miR-155, miR-146 and miR-9[27-29]. Indeed, miR-155 expression is induced in mouse bone marrow-derived macrophages in response to a variety of different Toll-like receptor (TLR) ligands, and by the proinflammatory cytokines tumor necrosis factor (TNF) and interferon-β[28]. Interferons do not directly induce miR-155 expression by macrophages, but rather do so indirectly through TNF autocrine or paracrine signaling. The primary transcript encoding miR-155, known as the B-cell integration cluster (BIC), was shown to be transcriptionally activated by the transcription factors activator protein 1 (AP1) and nuclear factor-kB (NF-κB)[28,30,31]. Certain TLR

ligands and ligands for the cytoplasmic sensor retinoic acid-inducible gene I (RIG-I) also induce miR-146 expression in a NF-kB-dependent manner[29,32]. 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[27]. Therefore, certain inflammatory miRNAs are regulated transcriptionally like other inflammatory genes that encode protein.

The inflammatory response to infection must be robust enough to eradicate microbial pathogens, yet resolved in a timely manner to avoid excess damage to the host or the promotion of neoplasia. MicroRNAs have been shown to impact both of these aspects of inflammation. Both miR-146 and miR-155 are induced by TLR ligands and there is evidence that they can negatively regulate the activation of inflammatory pathways in myeloid cells[10,29,32-35]. MiR-146 directly represses several signaling molecules that are downstream from TLRs, including IL-1R-associated kinase 1 (IRAK1), IRAK2 and TNFR-associated factor 6 (TRAF6), all of which promote inflammation[29,32,34,35]. In human myeloid-derived DCs, knockdown of miR-155 expression significantly increased expression of the pro-inflammatory cytokine interleukin-1b (IL-1b)[36]. Furthermore, addition of a miR-155 mimic decreased the expression of a NF-κB reporter plasmid in cell lines[36,37]. MiR-155 has also been shown to repress the expression of the pro-inflammatory signaling molecule TAK1-binding protein 2 (TAB2), suggesting a mechanistic basis for its anti-inflammatory impact in certain settings[36]. By contrast, it has been shown that in certain settings miR-155 can positively regulate inflammatory pathways, enhancing rather than diminishing them**.** Indeed as we show here, enforced expression of miR-155 in the bone marrow compartment of adult mice leads to a myeloproliferative phenotype that is reminiscent of what occurs transiently after LPS injection[20]. We also show here that miR-155 negatively regulates SHIP1, an

important negative regulator of phosphoinositide 3-kinase (PI3K) and the downstream AKT pathway[19,38]. SHIP1 is a negative regulator of TLR4 signaling[39], and therefore repression of SHIP1 by miR-155 may counter this negative regulation and increase downstream AKT signaling.

Interestingly, AKT signaling has recently been shown to repress miR-155 expression in macrophages[40], 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 compared with 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 suppressor of cytokine signaling 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 reduced tolerance to systemically delivered LPS compared with wild-type mice. Taken together, it is clear that specific microRNAs 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 genes and the cellular contexts in which it is expressed.

There are also reports indicating that miRNAs regulate distinct aspects of DC biology, and thus 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 Jagged1 and WNT1[41]. Myeloid-derived DCs from BIC$^{-/-}$ (miR-155-deficient) mice displayed defects in antigen presentation to T cells, despite expressing normal levels of MHC class II and co-stimulatory molecules; thus, the mechanism behind the defect remains elusive[42]. In addition, another study found that miR-155 downregulates expression of DC-specific ICAM3-grabbing non-integrin (DC-

SIGN) by human monocyte-derived DCs (MDDCs) through suppression of PU.1[43]. DC-SIGN is a cell surface C-type lectin that binds pathogens, implicating miRNAs in the regulation of pathogen uptake by DCs.

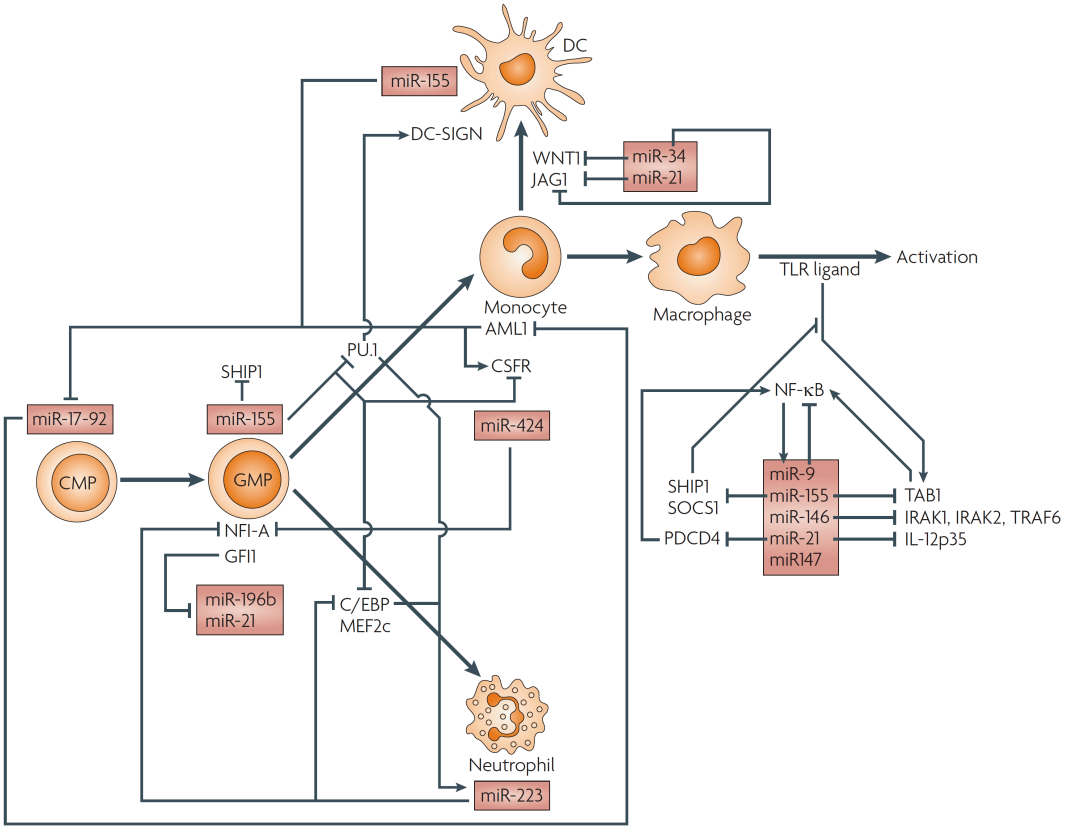

Figure 1: MicroRNAs in the network of myeloid development and function.
(© O'Connell et al., Nature Reviews Immunology, 2010.)

NK and NKT cells

NK cells are important components of immune surveillance against cancer and viral infection and microRNAs have been implicated in their development and function. NK cells express the receptor natural killer group 2, member D (NKG2D), which recognizes MHC class I polypeptide-related sequence A (MICA) and MICB, ligands that are

expressed by cells undergoing stress triggered by events such as viral infection or transformation. Engagement of NKG2D by NK cells leads to direct killing of the target cell. A recent study showed that a set of miRNAs, many of which are overexpressed in various cancers, could downregulate MICA and MICB expression in human cell lines[44]. Treatment of cells with antagomirs (antisense oligodeoxynucleotides) that inhibit the function of specific miRNAs led to increased cell surface expression of MICA and MICB and increased NK cell-dependent killing. Overexpression of these miRNAs had the opposite effect. Interestingly, 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. Rather, 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 virus, produce miRNAs that target the MICB mRNA, and this has been proposed as a mechanism of immune evasion by these viruses[45,46].

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[47,48]. Both studies observed a near complete loss of iNKT cells in the thymus and periphery in the absence of Dicer. Developing NK cells from conditional Dicer knockout animals exhibited increased apoptosis and decreased differentiation, whereas mature NK cells had major activation defects.

The studies described above clearly implicate miRNAs in the regulation of various aspects of innate immunity. This appears to include direct microbial killing, the production of cytokines and the presentation of antigens in the context of MHC. Here we will focus on just two microRNAs—miR-155 and miR-125b—and attempt to obtain a

mechanistic understanding of their roles in myeloid development and disease. We begin by showing that miR-155 overexpression in the hematopoietic system causes a myeloproliferative disorder[49], and show that miR-155 exerts its pathology via repression of the SHIP1 phosphatase[50]. Following this we focus on the microRNA, miR-125b, which we observed was enriched in hematopoietic stem cells[51]. When we overexpressed miR-125b in the hematopoietic compartment, we saw a very aggressive myeloid leukemia 4-5 months post-reconstitution[51] that was capable of infiltrating peripheral organs including the brain. We set out to identify the mechanism underlying miR-125b's function in the hematopoietic system and identified the stem cell gene, Lin28, as a primary target. Gain- and loss-of-function of lin28 mirrored important aspects of loss- and gain-of-function of miR-125b, respectively. Thus we integrate miR-125b and miR-155 and their target genes into the broader network of myeloid development, and importantly, we present a new set of pathways that could be therapeutically manipulated in the treatment of hematopoietic diseases and immune disorders.

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Chapter 2: MiR-155 overexpression causes a myeloproliferative disorder

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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. Extending possible relevance to human disease, miR-155 was found to be overexpressed in the bone marrow of patients with certain subsets 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 (Kluiver, Kroesen et al. 2006; Georgantas, Hildreth et al. 2007).

Among miRNAs expressed by hematopoietic cells, miR-155 has emerged as having significant impact on the biology of lymphocytes (Costinean, Zanesi et al. 2006; Rodriguez, Vigorito et al. 2007; Thai, Calado et al. 2007). *MiR-155* is upregulated to high levels in response to B or T cell receptor engagement (Haasch, Chen et al. 2002; van den Berg, Kroesen et al. 2003), and plays a B-cell intrinsic role in germinal center formation and subsequent antibody production in vivo following antigen challenge (Rodriguez, Vigorito et al. 2007; Thai, Calado 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 (van den Berg, Kroesen et al. 2003; Eis, Tam et al. 2005; Kluiver, Poppema et al. 2005), and its reported sufficiency in triggering B-cell lymphoma when overexpressed in a B-cell-restricted manner in mice (Costinean, Zanesi 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 (Taganov, Boldin et al. 2006; O'Connell, Taganov et al. 2007). 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 (Ueda, Kondo et al. 2005; Shortman and Naik 2007). 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, Garrett 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

a portion of 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 potentiality 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, Taganov et al. 2007). Mice were injected i.p. with a sub-lethal dose of LPS (50 ug), 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 (**Fig. 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 (**Fig. 1B**).

In addition to miR-155 expression, we also monitored bone marrow cell dynamics in response to LPS. While there was little change in bone marrow GM (Mac1+Gr1+), B-cell (B220+) and erythroid precursor (Ter119+) populations by 24 hours post-LPS treatment (Supplemental Fig. 1), substantial expansion of GM cells and reductions in B-cells and erythroid precursors was evident by 72 hours (**Fig. 1C**), consistent with a

previous study(Ueda, Kondo et al. 2005). Histological analyses also showed myeloid preponderance and hyperplasia, with relative erythroid hypoplasia, after 72 hours of LPS treatment (**Fig. 1D**). Together, these data indicate that LPS-induced miR-155 expression in the bone marrow precedes GM cell expansion.

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 (**Fig. 2A**), followed by engraftment of these cells into lethally irradiated C57BL6 mouse recipients. By two months post-reconstitution, mice were sacrificed and coexpression of miR-155 and GFP was detected in various hematopoietic tissues, including the bone marrow (**Fig. 2B**), thymus, spleen, and lymph nodes (Supplemental **Fig. 2**). 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 (**Fig. 2C**). Upon microscopic inspection of H&E-stained bone marrow sections and Wright-stained bone marrow smears (**Fig. 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 (**Fig. 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 lead to a reduction in erythrocytes, megakaryocytes and lymphocytes in the bone marrow (**Figs. 2D and 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 (**Fig. 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 (**Fig. 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+ (**Fig. 2H**). These observations reveal profound myeloid proliferation with dysplastic changes in the bone marrow of mice expressing miR-155 compared to controls.

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

Splenomegaly was observed in miR-155-expressing compared to control mice (**Fig. 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 (**Fig. 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 in control spleens (**Fig. 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 (**Fig. 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 (**Fig. 3D**). Furthermore, miR-155-expressing splenocytes contained overall higher numbers of Mac1+ cells that expressed GFP compared to controls (**Fig. 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.

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 post-reconstitution, FACS detected significantly elevated numbers of Mac1+ cells (**Fig. 4A**), and Wright stained blood smears revealed the presence of morphologically abnormal GM cells in miR-155 expressing mice (**Fig. 4B**). Complete blood cell counts showed a significant reduction in red blood cell, hemoglobin, and platelet levels (**Fig. 4C**), FACS found decreased B220+ B-cells and CD4+ T lymphocytes (**Fig. 4c**), and Wright staining identified several immature erythrocytes demonstrating polychromatophilia in miR-155-expressing animals (**Fig. 4D**).

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 (**Fig. 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 (**Fig. 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 (**Fig. 5B**). These observations demonstrate that miR-155 expression in the bone marrow is significantly elevated in a subset of patients suffering from AML.

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 Fig. 3). 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 Fig. 4). 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 (Lewis, Shih et al. 2003; Grimson, Farh et al. 2007). Finally, genes with reported roles in myeloid hyperplasias and/or hematopoiesis were identified through literature searching. Using these criteria, our attention was drawn to 10 candidate targets: Bach1, Sla, Cutl1, Csf1r, Jarid2, Cebpb, PU.1, Arntl, Hif1a and Picalm (**Fig. 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 (**Fig. 6A**). We also observed strong repression of Cebpb, PU.1, Cutl1 and Picalm protein levels in RAW 264.7 cells expressing miR-155 (**Fig. 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 Cebpb) 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. From 22% to 73% repression of luciferase expression was observed depending upon the 3' UTR tested (**Fig. 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 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 (**Fig. 7**). These results provide strong evidence that miR-155 can directly regulate several genes with relevance to hematopoiesis and the myeloproliferative phenotype.

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 (Zhan, Lieschke et al. 1998; Ueda, Kondo et al. 2005;

Nagai, Garrett et al. 2006). 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, Taganov 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, Hildreth et al. 2007). However, defective myeloid populations in miR-155 deficient mice were not observed when analyzed under steady-state (non-inflammatory) conditions (Rodriguez, Vigorito 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 pre-leukemic 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 miR181 expression positively correlates with M1 and M2 subtypes of AML, but not M4 or M5. 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 (Debernardi, Skoulakis et al. 2007).

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, Scumpia et al. 2007). The inflammatory response in the bone marrow involves profound myeloid proliferation, and through factors such as miR-155, may prove to 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, Zanesi 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, Kondo 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, Dakic et al. 2006; Okada, Jiang et al. 2006), or myeloproliferative conditions, as is true for Cutl1 and Csf1r (Sinclair, Lee et al. 2001; Fontana, Pelosi et al. 2007). 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 Cebpb (Hirai, Zhang et al. 2006), Bach1 (Toki, Katsuoka et al. 2005), Arntl (Scortegagna, Morris et al. 2003), Sla (Dragone, Myers et al. 2006), Jarid2 (Kitajima, Kojima et al. 1999), and Hif1a (Yoon, Pastore 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 multi-target regulation has recently been described for T-cell receptor signaling (Li, Chau 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 contributions of individual targets to the myeloproliferative phenotype and AML in the context of miR-155 repression remains an area of 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, Pelosi 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 Cebpb, they have been shown to transcriptionally regulate expression of miR-223, a myeloid-specific microRNA (Fukao, Fukuda et al. 2007). miR-223 is subsequently involved in unleashing CEBPa function, a central transcription factor in myelopoiesis, through the direct repression of its inhibitor NFI-A (Fazi, Rosa 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, Zheng 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, Rajewsky et al. 2005). Because miR-155 knockout mice display few detrimental phenotypes in the absence of infection (Rodriguez, Vigorito 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. 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, Tam 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 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). 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, Boldin 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 lines and bone marrow reconstitution. To generate VsVg pseudotyped retroviruses containing the miR-155 expression cassette, $2x10^6$ 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 $5x10^5$ RAW 264.7 cells for 8 hours in the presence of polybrene at 10 ug/ml. Following 48 hours, stably transfected cells were selected using puromycin at 7 ug/ml, 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 ug 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 uM beta-marcaptoethanol) 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 ug/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 hrs 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, Taganov et al. 2007). Gene-specific primer sequences used for qPCR are located in Supplemental Table 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 was analyzed using Rosetta Resolver Software.

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

Flow cytometry. 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 FACSCaliber flow cytometer and further analyzed with FloJo software.

Luciferase reporter assays. 8 x 10^4 293T cells were plated in DMEM media containing 5% FBS for 12 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 -80C 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, and required written consent by the participating patients.

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 were made of the cut surface of the spleen. Peripheral blood smears were obtained from 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=0.000018 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.

Online supplemental material. Supplemental Figure 1 provides FACS data showing the percentage of Mac1+, Gr1+, B220+, Ter119+ or CD4+ cells in the bone marrow following 24 hours of LPS versus PBS treatment. Supplemental Figure 2 presents the coexpression of miR-155 and GFP in several different immune organs from reconstituted mice. Supplemental Figure 3 compares miR-155 expression in Raw 264.7 cells and primary macrophages mediated by retroviral overexpression versus LPS stimulation. Supplemental Figure 4 outlines the scheme used to identify miR-155 targets with relevance to hematopoiesis. Supplemental Table 1 provides the primer sequences used for cloning different 3' UTRs into pmiReport. Supplemental Table 2 provides sequence information regarding mutations introduced into the 3' UTRs used for reporter assays. Supplemental Table 3 provides the primer sequences used to assay miR-155 target mRNAs by qPCR.

Figure Legends

Figure 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 ug 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 timepoint 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 mM) or H&E-stained bone marrow sections (bottom, scale bar=25 mM) from Wt mice injected with LPS or PBS for 72 hours.

Figure 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 % 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 mM) (**E**) Wright-stained bone marrow smears from mice reconstituted with MG155 or control vector HSCs (scale bar=25 mm). 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.

Figure 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 mm) or Wright stained touch preps (bottom, scale bar=25 mm) 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.

Figure 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.

Figure 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.

Figure 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 % expression of control, and are the average of 3 independent experiments. (**B**) Western blotting was performed to assay Cebpb, PU.1, Cutl1, Picalm or aTubulin 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 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 the 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 co-transfection with a control b-galactosidase expression plasmid, and a miR-155 expression vector (FUW-155) or empty vector control (FUW). Control vectors containing tandem miR-155 binding sites or the 3' UTR from Irak1 Traf6 which lack miR-155 sites, were also included. 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 % luciferase expression of control (mean +/- SD). All data are a triplicate set representing at least two independent experiments.

Figures

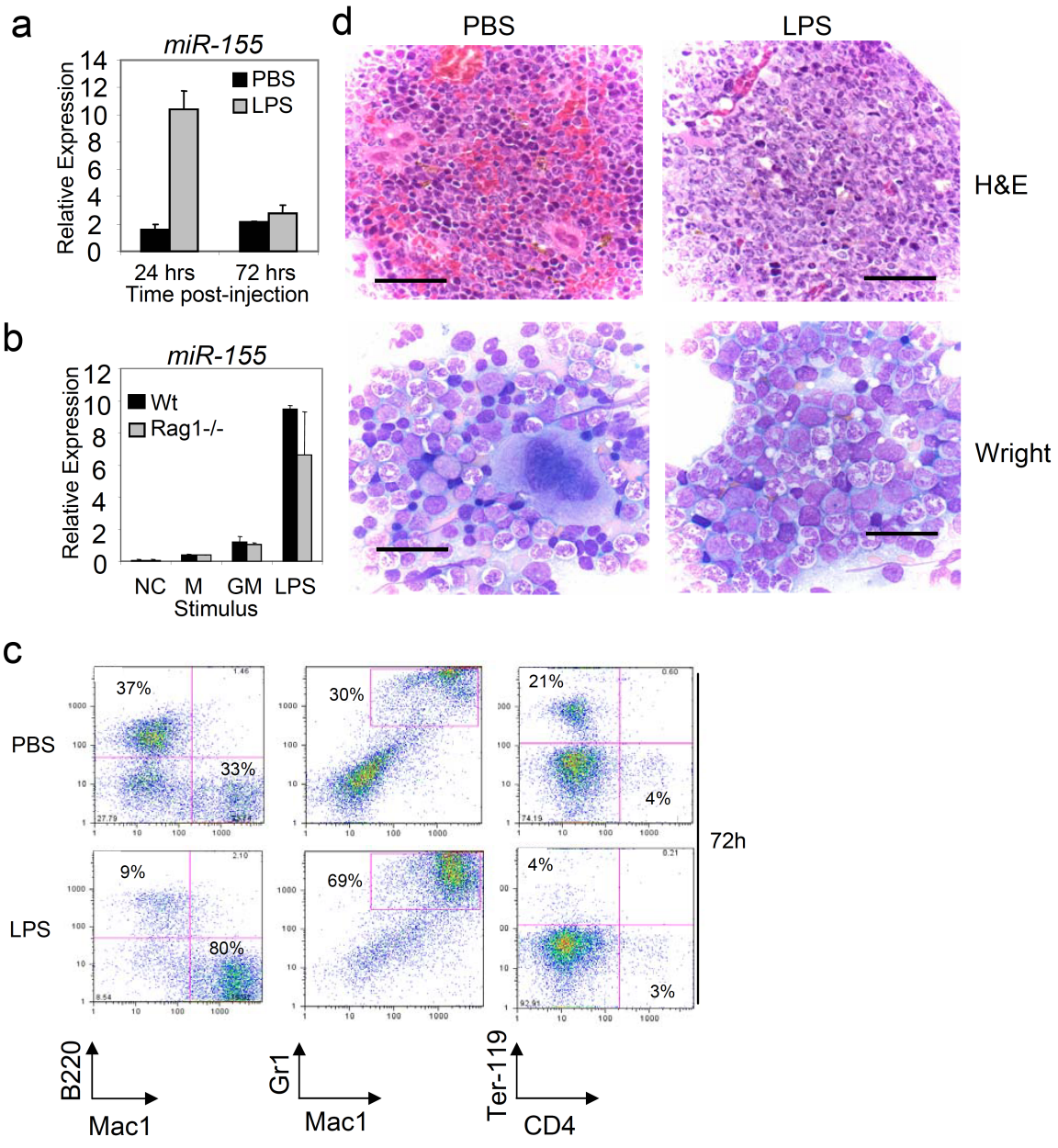

Figure 1. LPS treatment induces bone marrow expression of miR-155 prior to GM expansion

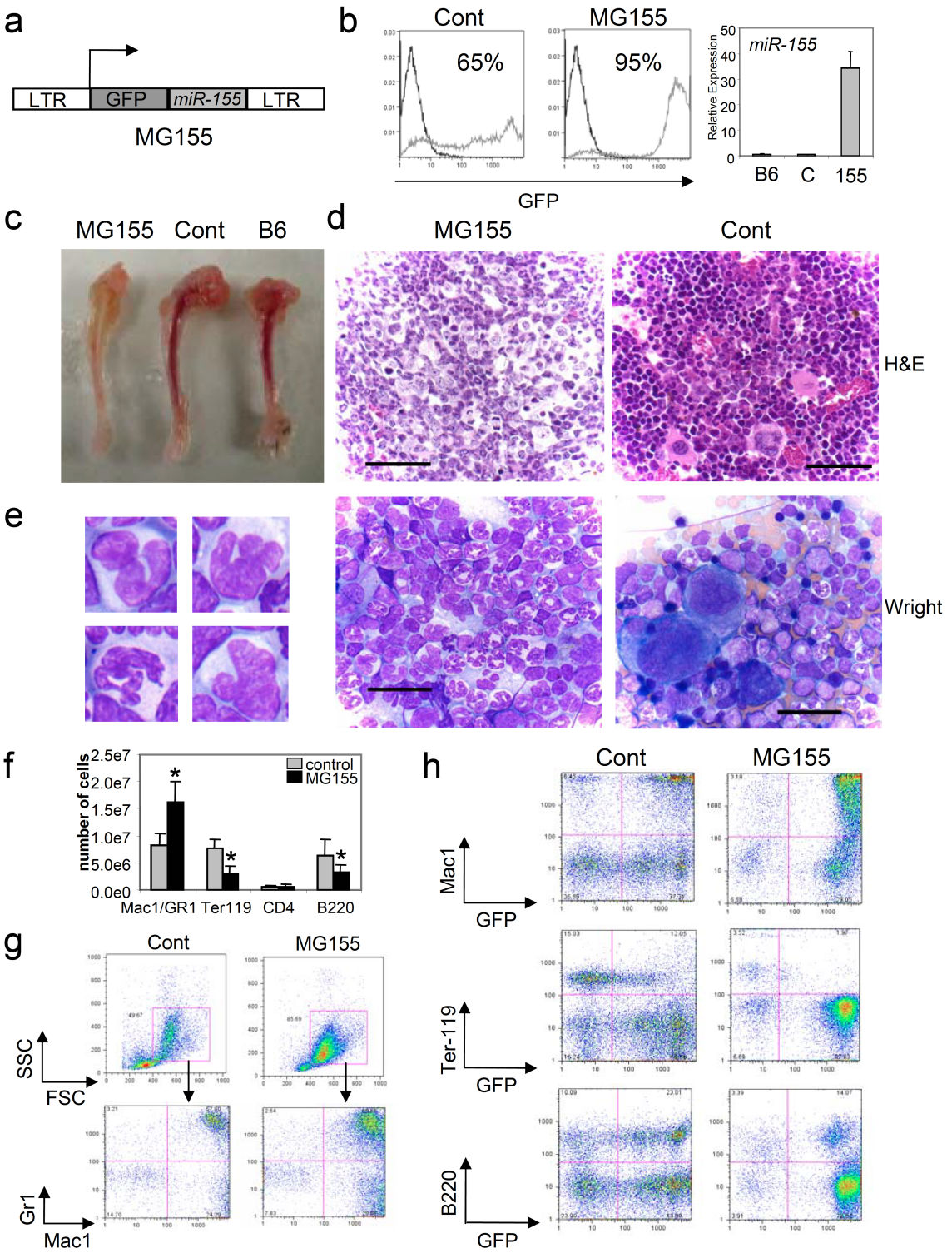

Figure 2. Expression of miR-155 causes a myeloproliferative disorder in the bone marrow

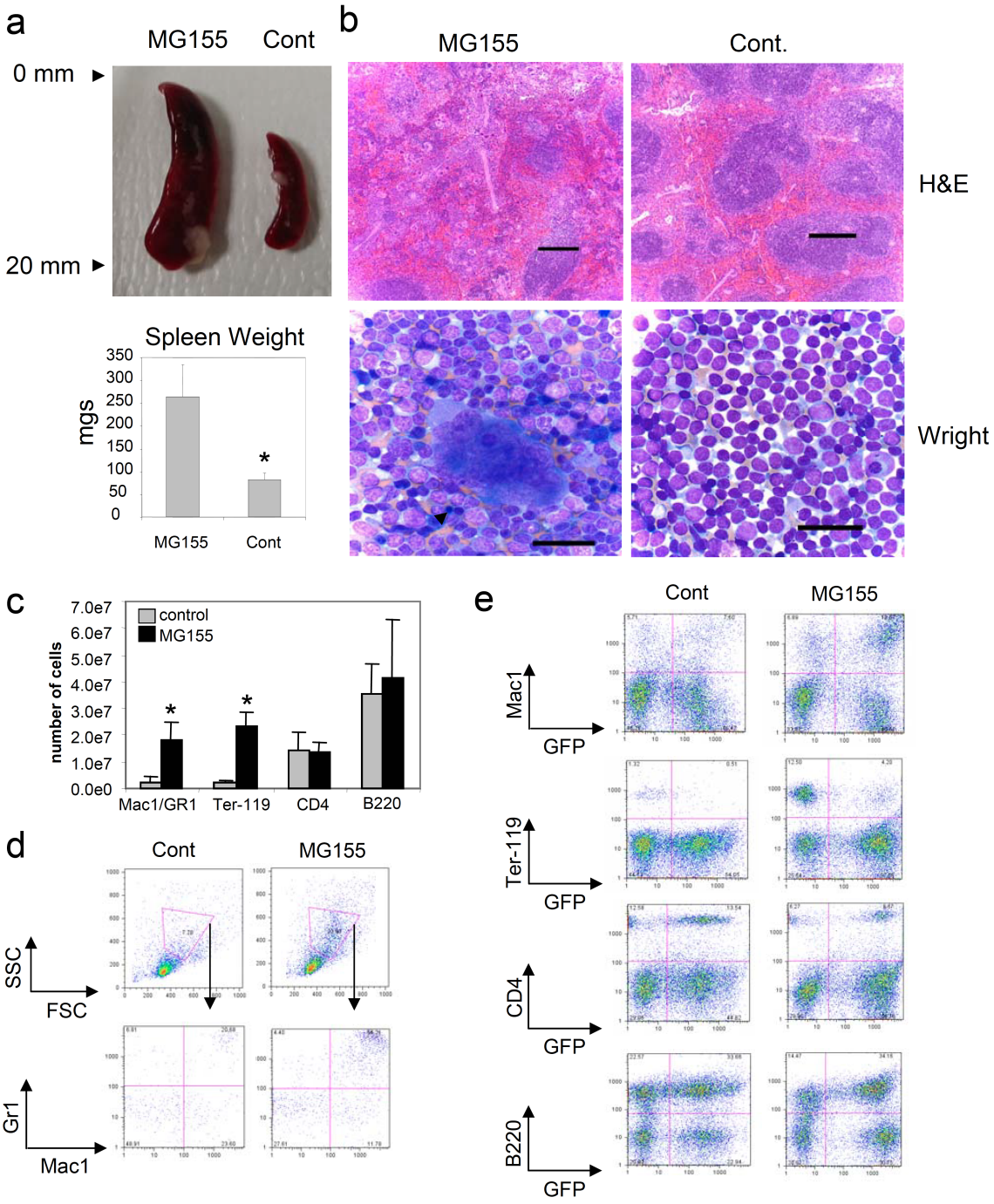

Figure 3. Expression of miR-155 in HSCs triggers extramedullary hematopoiesis in the spleen

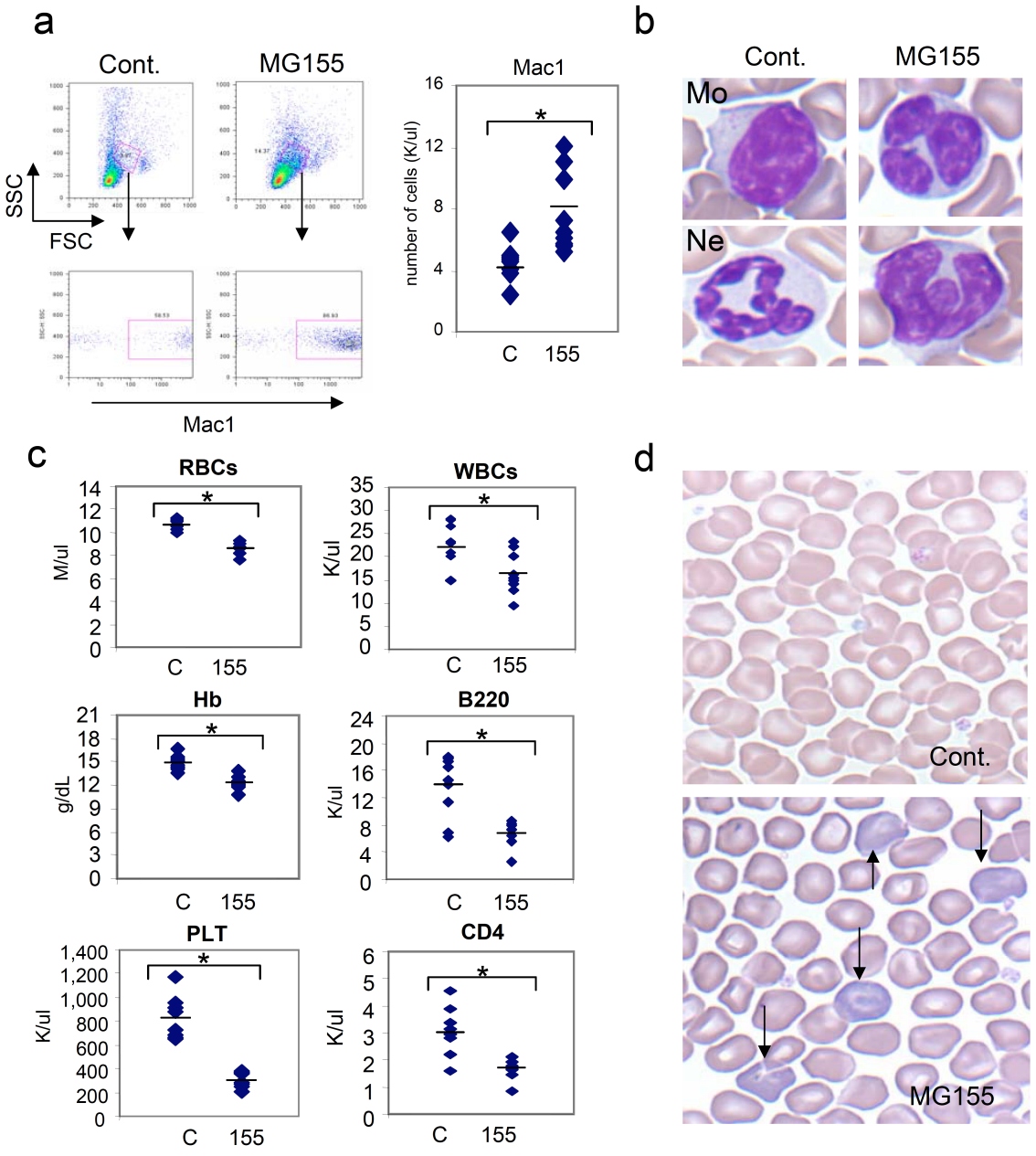

Figure 4. Expression of miR-155 in HSCs perturbs peripheral blood cell populations

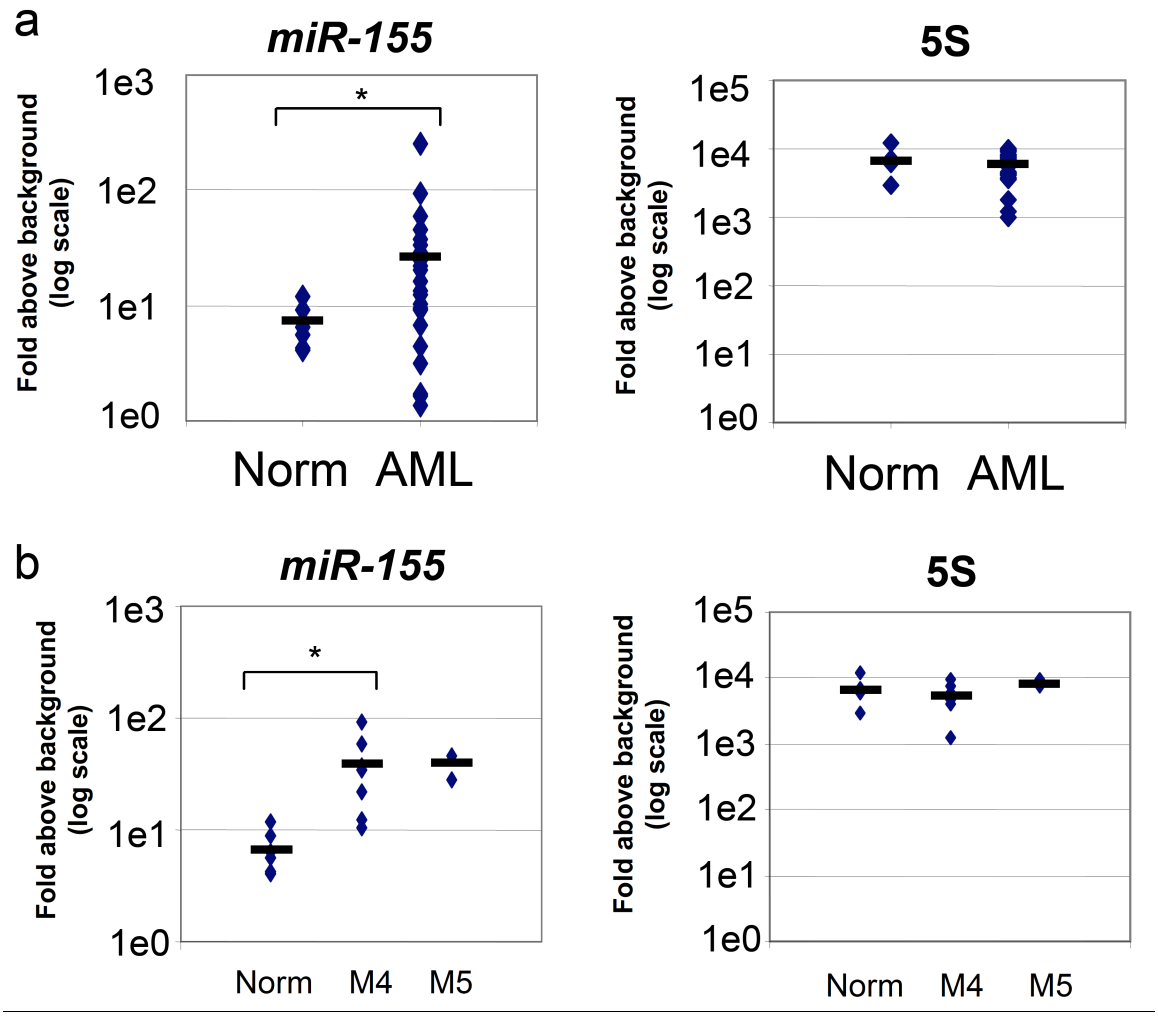

Figure 5. Overexpression of miR-155 in a subset of human AML patients

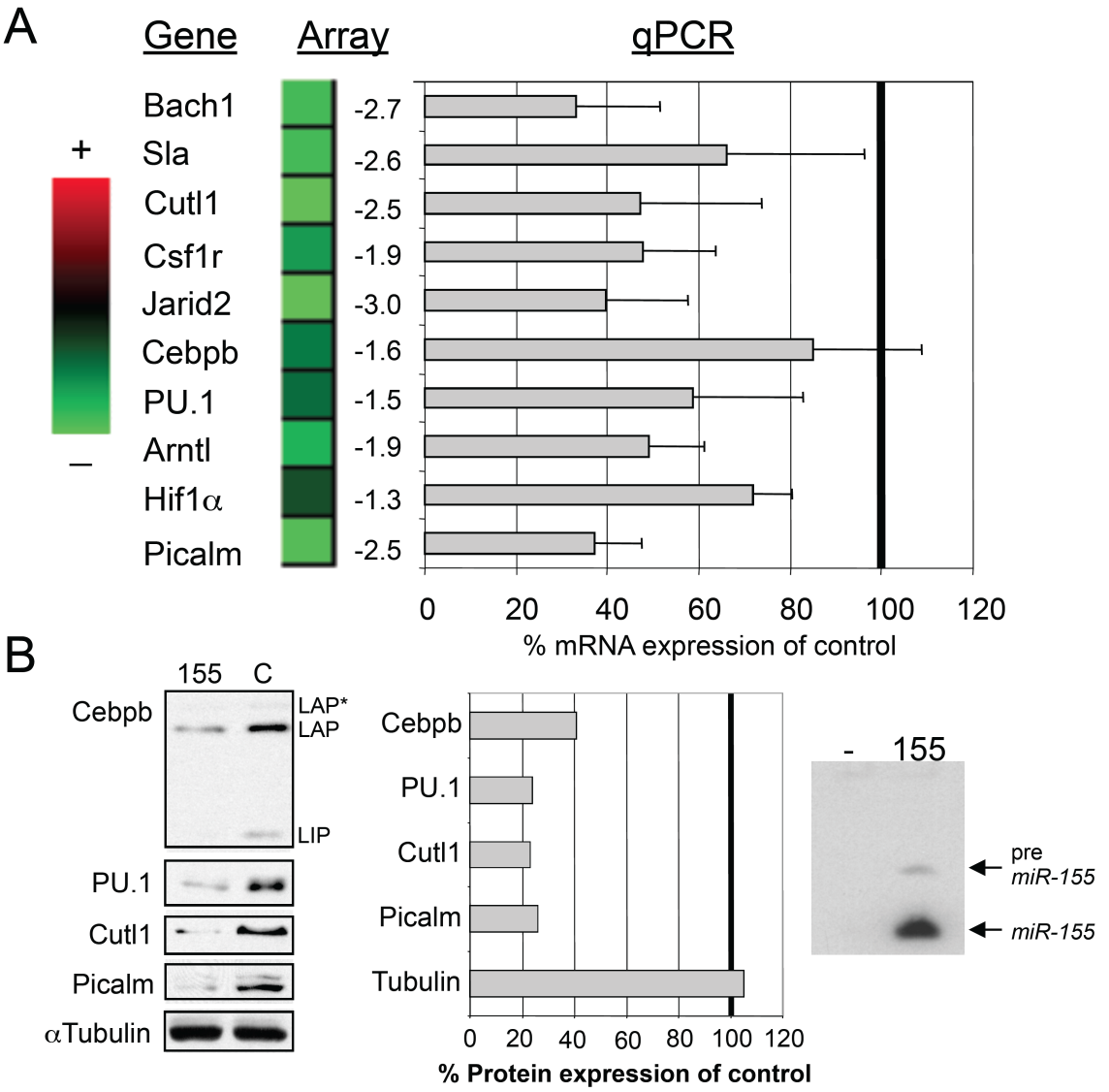

Figure 6. Repression of specific target genes involved in myeloid hyperplasia and/or hematopoiesis by miR-155

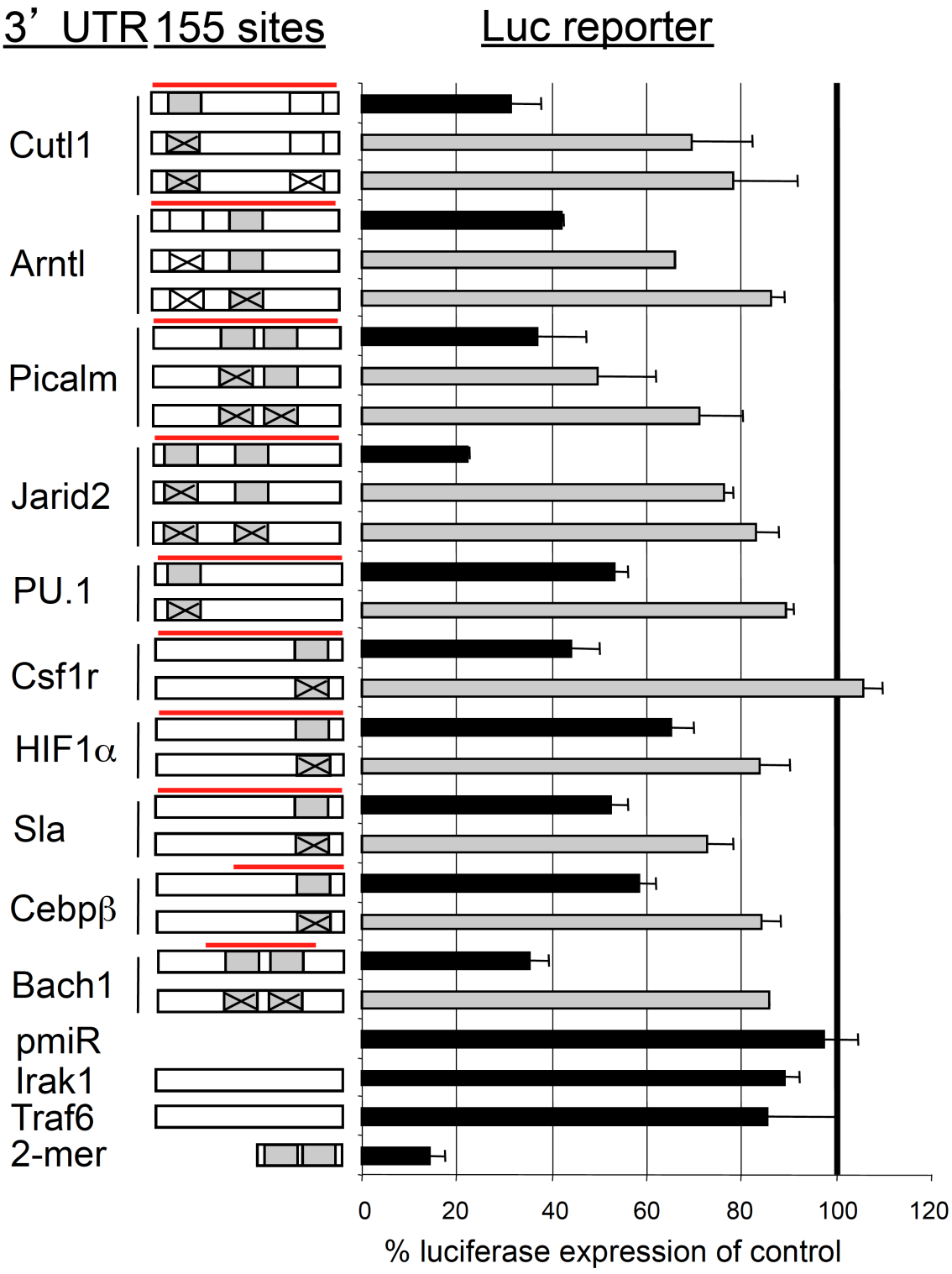

Figure 7. MiR-155 repression of specific target genes occurs through direct 3’ UTR interactions

Supplemental Figures

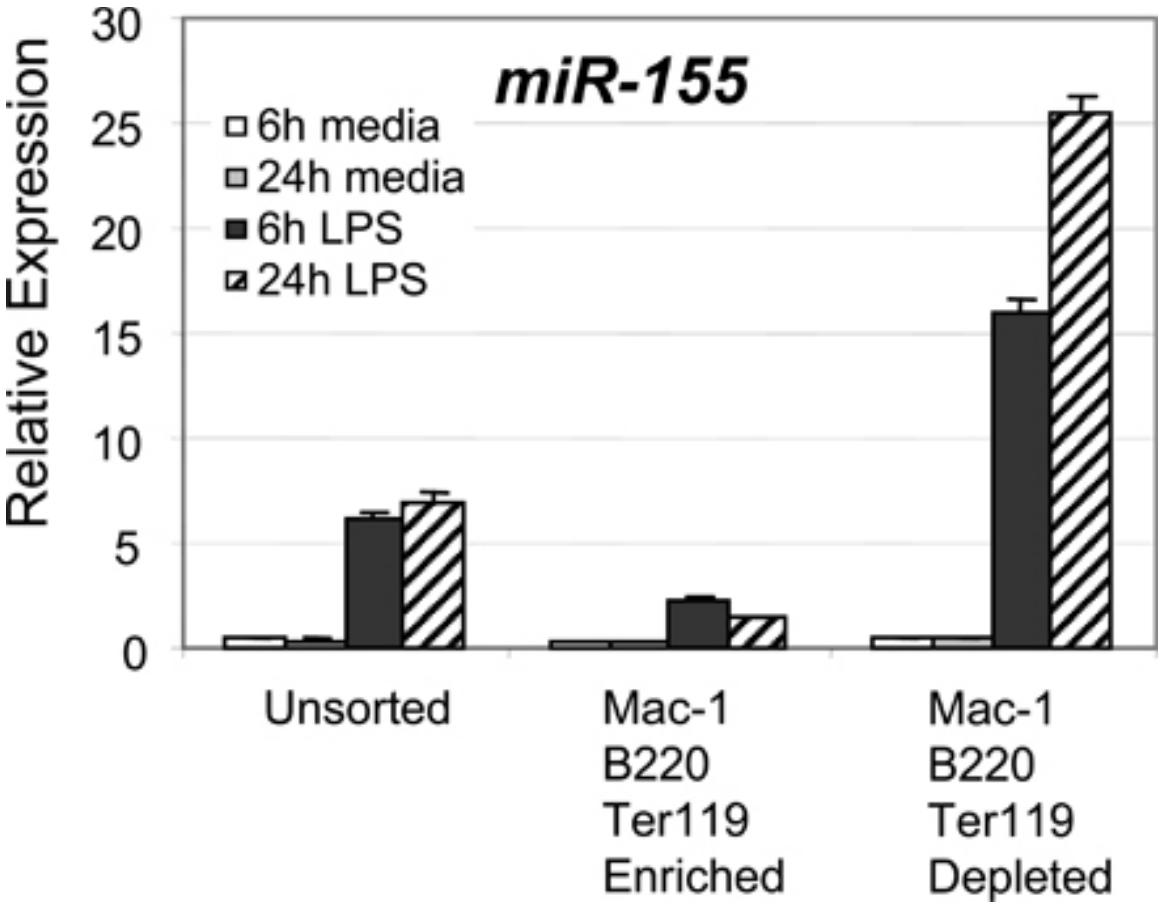

Figure S1. Mature cell–depleted bone marrow populations upregulate miR-155 to higher levels in response to LPS than mature cell–enriched populations. Mature cell–enriched populations were positively selected from mouse bone marrow using biotinylated antibodies against Mac-1, B220, and Ter119, and streptavidin-conjugated 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.

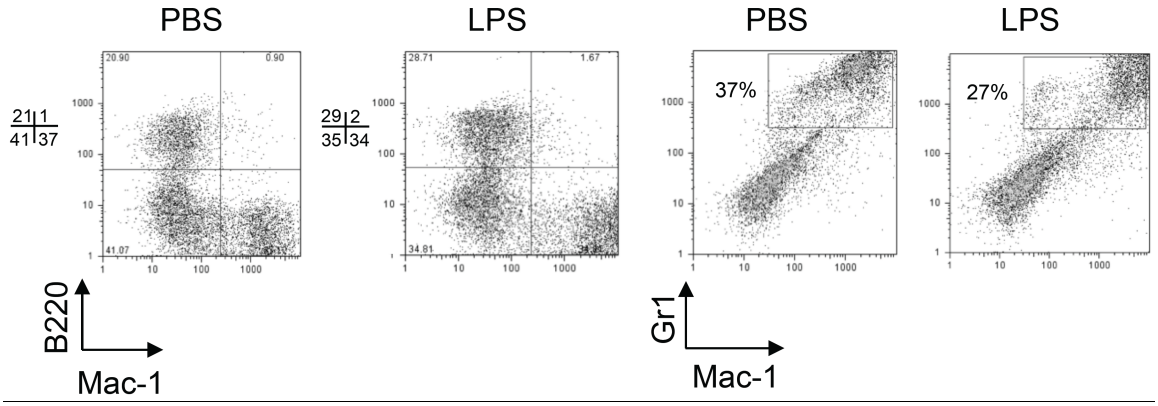

Figure S2. Bone marrow cell dynamics after 24 h of LPS treatment. C57BL6 mice were injected i.p. with 50 μg LPS or PBS for 24 h. B220, Gr1, or Mac1 surface expression was assayed on RBC-depleted bone marrow cells using FACS.

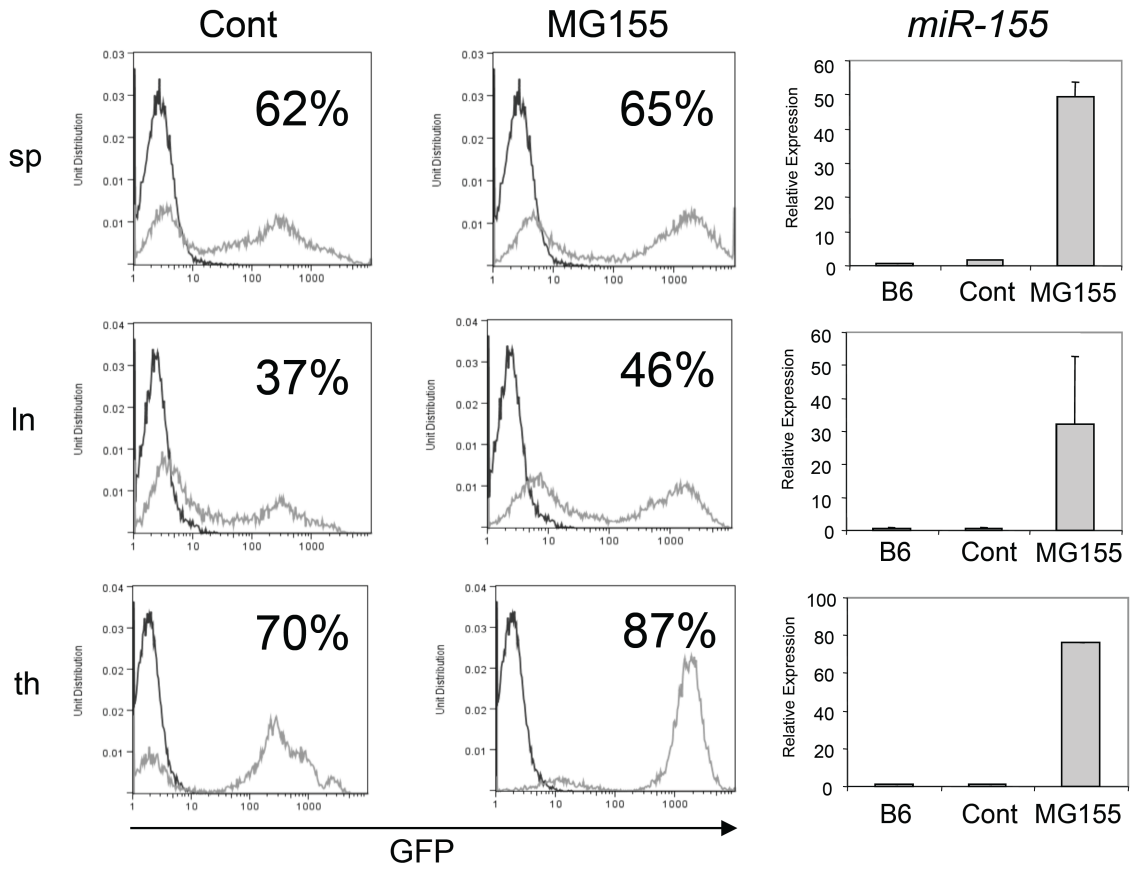

Figure S3. Coexpression of GFP and miR-155 in several lymphoid organs.

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.

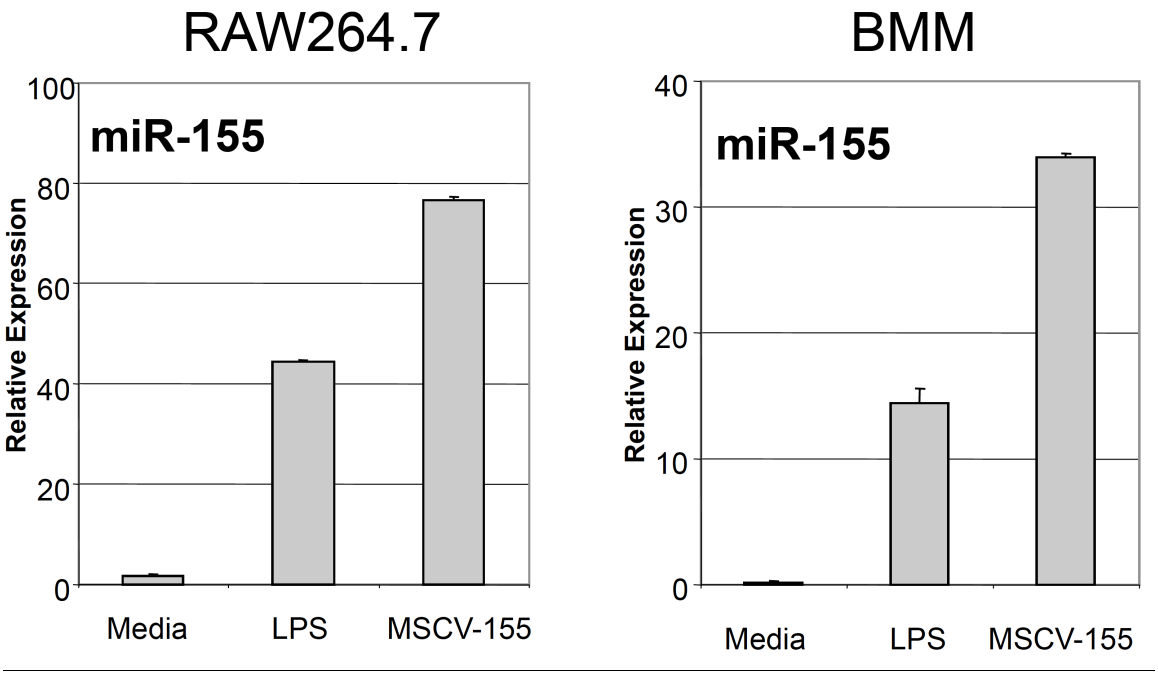

Figure S4. Comparative expression of miR-155 induced by LPS or produced from MSCV-155 in myeloid cells. MiR-155 expression was assayed in (left) Raw264.7 cells stably transfected with MSCV and treated with media or 50 ng/ml LPS, stably infected with MSCV-155 or (right) bone marrow–derived macrophages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

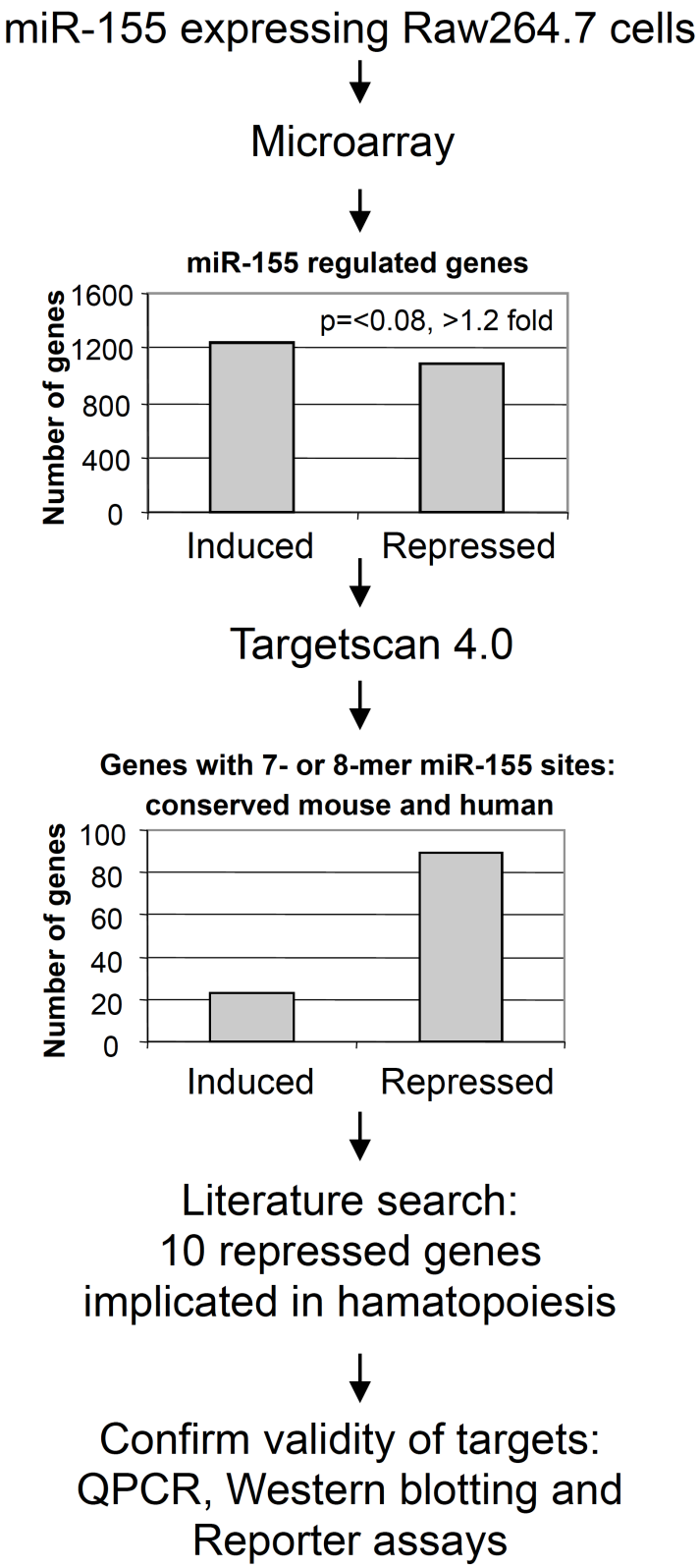

Figure S5. MiR-155 target identification and analysis scheme

Supplemental Tables

Table S1. Primer sequences used for cloning

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 S2. MiR-155 3' UTR seed sequences and introduced mutations

(Mutated nucleotides are in red)

miR-155 seed	Wt	Mutant
Bach1 1	AGCATTAA	AGGTAAAA
Bach1 2	AGCATTA	AGGTAAA
PU.1	AGCATTAA	AGGTAAAA
Cutl1 1	AGCATTAA	AGGTAAAA
Cutl1 2	GCATTA	GCTAAA
Picalm 1	GCATTAA	GGAGTGA
Picalm 2	AGCATTA	AGCTAAA
Arntl 1	GCATTAA	GCAAATA
Arntl 2	GCATTAA	GCTAAAA
Csf1r	AGCATTAA	AGCAAATA
Sla	AGCATTA	AGCAAAT
Arntl 1	GCATTAA	GCAAATA
Jarid2 1	AGCATTAA	ACGTAATA
Jarid2 2	AGCATTAA	AGCAAATA
Hif1a	AGCATTA	AGCAAAT

Table S3. Primer sequences used for qPCR

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

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Chapter 3: Inositol Phosphatase SHIP1 is a primary target of miR-155

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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 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 (Bartel and Chen 2004; Baltimore, Boldin et al. 2008; Lodish, Zhou 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 (He, Thomson et al. 2005; Costinean, Zanesi et al. 2006; Rodriguez, Vigorito et al. 2007; Thai, Calado et al. 2007; Xiao, Calado et al. 2007; Johnnidis, Harris et al. 2008; O'Connell, Rao et al. 2008; Ventura, Young et al. 2008; Xiao, Srinivasan 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 (Tam, Ben-Yehuda et al. 1997; van den Berg, Kroesen et al. 2003; Eis, Tam et al. 2005; Kluiver, Poppema et al. 2005; Fulci, Chiaretti et al. 2007; Rodriguez, Vigorito et al. 2007; Thai, Calado et al. 2007), T cells (Haasch, Chen et al. 2002), macrophages (Taganov, Boldin et al. 2006; O'Connell, Taganov et al. 2007), dendritic cells (Rodriguez, Vigorito et al. 2007) and progenitor/stem cell populations (Georgantas, Hildreth et al. 2007; O'Connell, Rao 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, Chen et al. 2002; Taganov, Boldin et al. 2006; O'Connell, Taganov et al. 2007; Rodriguez, Vigorito et al. 2007; Thai, Calado et al. 2007). Consistent with its expression pattern, miR-155 appears to function in hematopoiesis and the immune response (Rodriguez, Vigorito et al. 2007; Thai, Calado et al. 2007; O'Connell, Rao et al. 2008).

For example, defective germinal center formation and antibody isotype class switching have been observed in miR-155$^{-/-}$ mice following infection or vaccination (Rodriguez, Vigorito et al. 2007; Thai, Calado et al. 2007; Vigorito, Perks et al. 2007). In T cells, miR-155 promotes skewing towards the Th1 subset (Rodriguez, Vigorito et al. 2007; Thai, Calado et al. 2007). In dendritic cells, miR-155 is necessary for proper activation of responder T cells in the context of antigen presentation (Rodriguez, Vigorito et al. 2007).

Enhanced expression of miR-155 occurs constitutively in a subset of cancer cells of lymphoid (Tam, Ben-Yehuda et al. 1997; van den Berg, Kroesen et al. 2003; Eis, Tam et al. 2005; Kluiver, Poppema et al. 2005; Fulci, Chiaretti et al. 2007) and myeloid origin (Garzon, Garofalo et al. 2008; O'Connell, Rao 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, Rao 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 (Costinean, Zanesi et al. 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, Rao 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 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, Burge 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 (Helgason, Damen et al. 1998; Liu, Sasaki et al. 1999; Helgason, Antonchuk et al. 2003; Harder, Quilici et al. 2004; O'Connell, Rao 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 co-transfected 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 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 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 (Figures 1C and 1D). The wt miR-155 was overexpressed in cells receiving the wt miR-155 vector (Figure 1E) and the mature miR-155 seed mutant was produced in cells receiving the miR-155 seed mutant vector (Figure 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 1G). Demonstrating conservation of function, mouse miR-155-expressing RAW 264.7 cells also had reduced SHIP1 expression (Figure 1H) and increased LPS-mediated Akt activation compared to control cells (Figure 1I). The data show that enforced expression of human or mouse miR-155 can directly repress expression of endogenous SHIP1 and lead to increased Akt activation in response to cellular activation.

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, Taganov 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 (Figures 2A and 2B), as previously seen with poly (I:C) treated BMMs (O'Connell, Taganov et al. 2007). We also noticed that while BIC levels fall considerably by 24h, miR-155 expression peaks at this timepoint. 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 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 timepoint (Figure 2C). SHIP1 mRNA levels reflected similar differences between wt and miR-155$^{-/-}$ BMMs following 24h of LPS treatment (Figure 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.

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, Rao et al. 2008). Although this phenotype is predicted by the MPD observed in SHIP1$^{-/-}$ mice (Helgason, Damen et al. 1998; Liu, Sasaki et al. 1999; Helgason, Antonchuk et al. 2003), we wanted to perform a specific

reduction in SHIP1 using the same retroviral vector and bone marrow reconstitution context 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 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 3A). Knockdown of other genes in vitro has been shown using this approach (Chung, Hart 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 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 3C).

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 4 and 5). Gross analysis revealed a miR-155 or siSHIP1 dependent splenomegaly and pale

coloring of the bone marrow (Figure 5A and unpublished observations). Flow cytometry (FCM) detected an increase in CD11b+ (Mac1+) myeloid populations in the bone marrow and spleen (Figures 4A and 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 4A and 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 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. FCM 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 4C).

H&E staining of fixed spleen sections from miR-155 or siSHIP1 mice revealed expanded inter-follicular regions containing developing myeloid populations, erythroid precursors and megakaryocytes compared to control mice (Figure 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.

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, Tarasenko 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 PLCg (Lioubin, Algate et al. 1996; Backers, Blero et al. 2003; Sly, Rauh 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, Sly et al. 2003; Leung, Tarasenko et al. 2008). Furthermore, knockout of SHIP1 in B lymphocytes causes spontaneous GC formation and antibody class switching (Liu, Oliveira-Dos-Santos et al. 1998; Helgason, Kalberer et al. 2000; Leung, Tarasenko et al. 2008), while a SHIP deficiency in T cells skews peripheral T lymphocytes towards Th1 and away from Th2 in response to an immune challenge (Tarasenko, Kole 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 leukemias where miR-155 has been shown to be overexpressed (Garzon, Garofalo et al. 2008; O'Connell, Rao et al. 2008). Of note, SHIP1 is mutated in some AML patients, where loss of function has been implicated in the oncogenic process (Luo, Yoshida et al. 2003; Luo, Liu et al. 2004). As miR-155 levels are elevated in certain B cell lymphomas (Tam, Ben-Yehuda et al. 1997; van den Berg, Kroesen et al. 2003; Eis, Tam et al. 2005; Kluiver, Poppema et al. 2005; Fulci, Chiaretti et al. 2007), and because SHIP1 is a negative regulator of B cell activation and survival (Liu, Oliveira-Dos-Santos et al. 1998; Helgason, Kalberer et al. 2000; Leung, Tarasenko et al. 2008), 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 L 2008; Pedersen IM 2008). It is also plausible that virally encoded orthologs of miR-155 (Gottwein, Mukherjee et al. 2007; Skalsky, Samols et al. 2007; Zhao, Yao et al. 2009), or miR-155 induction by viruses such as Epstein-Bar virus (Yin, McBride 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, Schwanhausser 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 SHIP 1 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, Perks et al. 2007), AID (Dorsett, McBride et al. 2008; Teng, Hakimpour et al. 2008), SOCS1 (Lu, Thai 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, Perks 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, McBride et al. 2008; Teng, Hakimpour 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, Thai et al. 2009). Similar observations have been made for specific targets of miR-150 (Xiao, Calado et al. 2007; Lu, Guo et al. 2008), miR-223 (Johnnidis, Harris et al. 2008) and miR-17-92 (Ventura, Young et al. 2008; Xiao, Srinivasan 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/s sequences within the 3'UTR regions of specific targets, as has been done for AID (Dorsett, McBride et al. 2008; Teng, Hakimpour 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, 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, Taganov 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, Burge et al. 2005).

DNA constructs. Retroviral constructs MG and MG-155 (human sequence) were described previously (O'Connell, Taganov et al. 2007). Oligonucleotide sequences used to generate new constructs are provided as supplemental data (Table 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, m155, h155mut and siSHIP1 oligos, which produce mature human miR-155, mouse 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, Rao 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, Rao 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, Rao 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-m155, 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 ug/ml. After 48 hours, stably transduced cells were selected using puromycin at 10 ug/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, Rao 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 realtime PCR (qPCR) was conducted using the 7300 Realtime PCR system (Applied Biosystems, Foster City, CA) to assay BIC, miR-155, 5s, SHIP1 mRNA and L32 mRNA levels as described previously (O'Connell, Rao 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 S1). Northern blotting was performed as described (O'Connell, Taganov 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 semi-dry transfer apparatus (Bio-Rad, Hercules, CA). Western blotting was performed using the following antibodies: SHIP1 V-19 (sc-1963), SHIP1 M-14 (sc-1964), aTubulin 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); αActin (A2066), βActin (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, Rao et al. 2008).

Statistical tests. All statistical tests were performed using Microsoft Excel.

Figure Legends

Figure 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, aTubulin 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 timecourse and Ser-473-phosphorylated-Akt (p-Akt), total Akt and b-Actin were assayed by Western blotting. **H.** SHIP1 levels in Raw 264.7 cells expressing mouse miR-155 or control vector were assayed by western blotting. **I.** p-Akt was assayed in these cells following LPS treatment (200 ng/ml). Data represent at least 2 independent experiments.

Figure 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)**. bActin 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.

Figure 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. aTubulin 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.

Figure 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 mm). **C.** Representative FCM plots from control, MGP-155 and siSHIP1 vector-containing mouse bone marrow analyzing GFP and CD11b expression.

Figure 5. Knockdown of SHIP1 or expression of miR-155 in the hematopoietic compartment causes spenomegaly 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 mm).

Figures

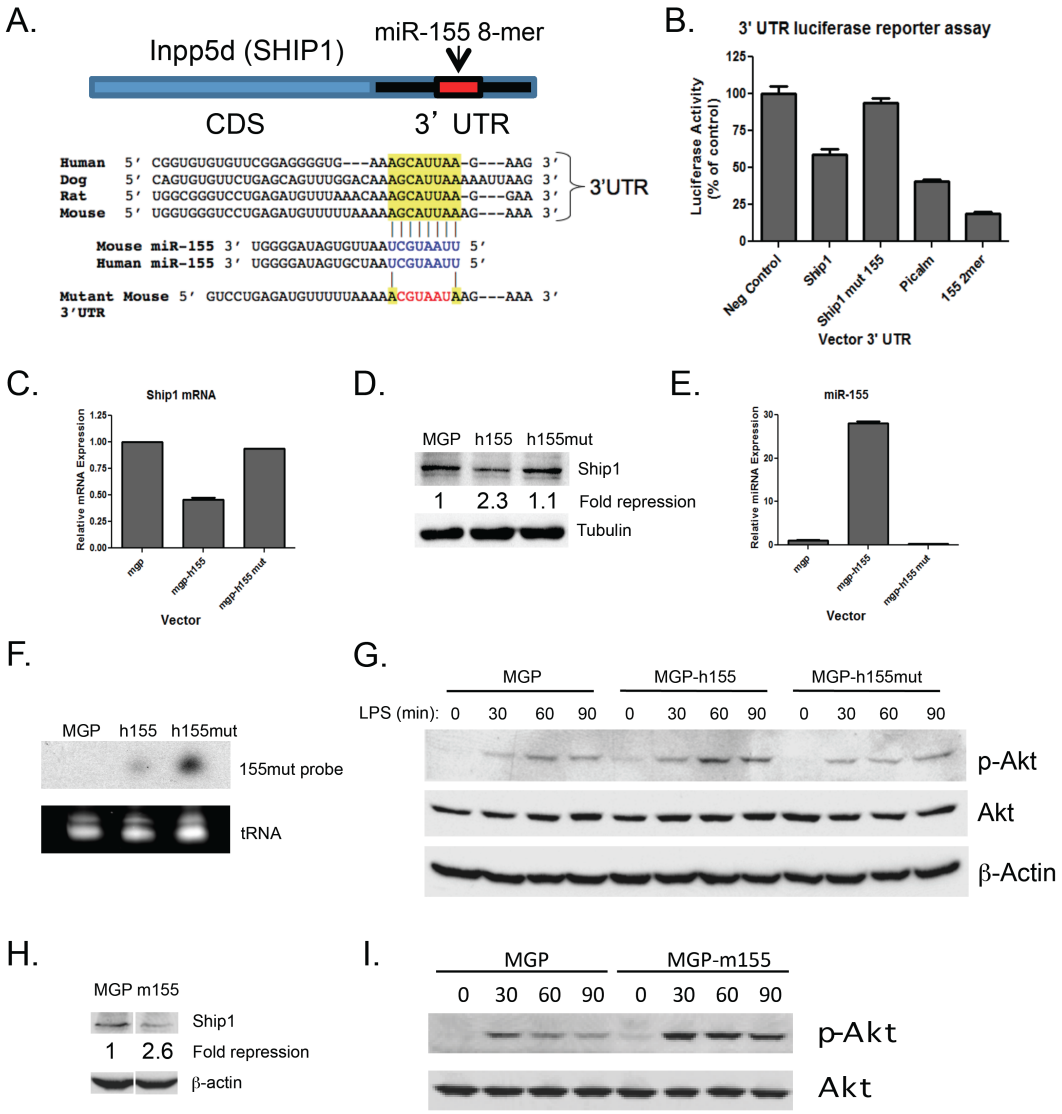

Figure 1. MicroRNA-155 represses SHIP1 expression through 3'UTR interactions

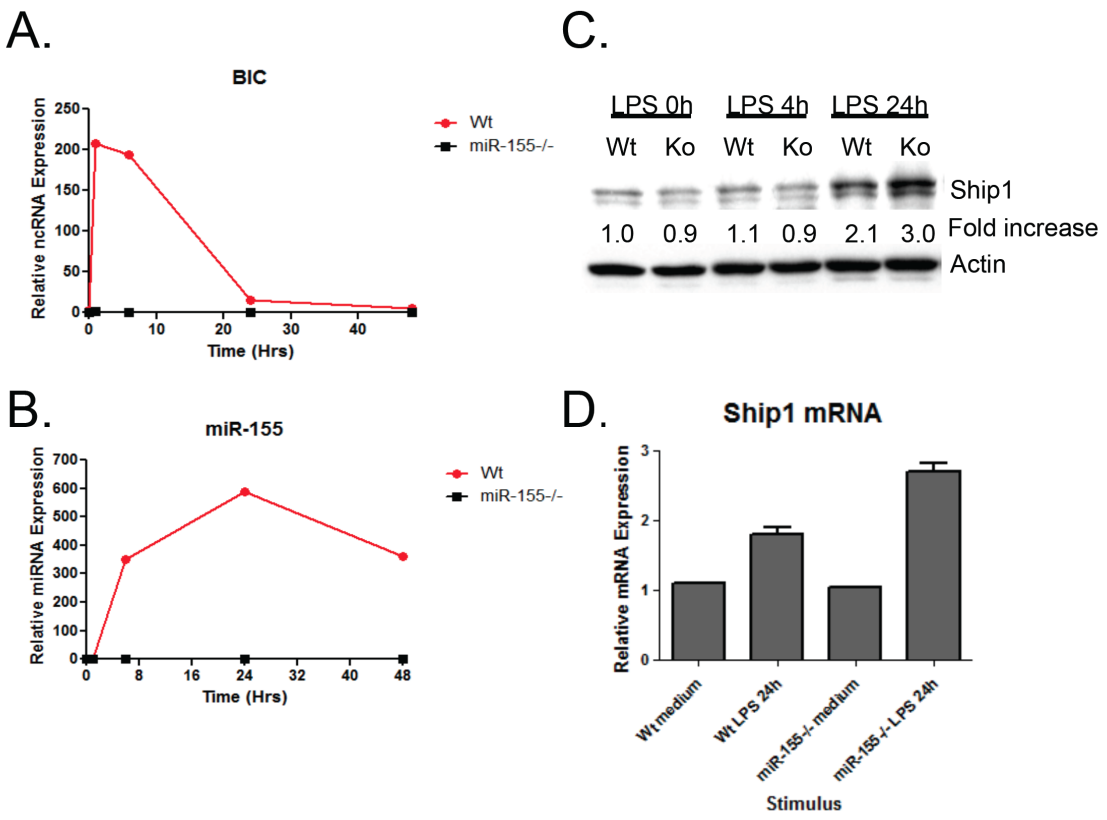

Figure 2. Enhanced expression of SHIP1 in miR-155$^{-/-}$ macrophages following LPS treatment

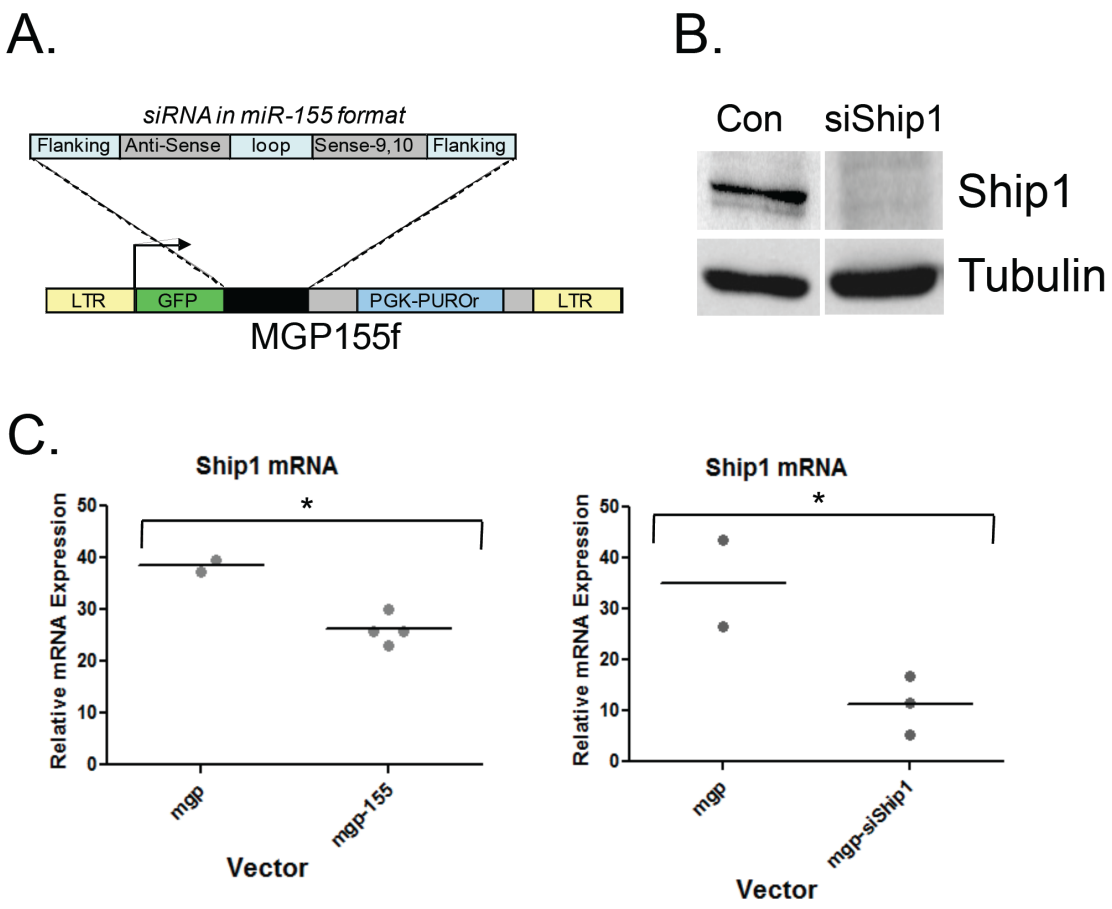

Figure 3. Knockdown of SHIP1 in vivo using siRNA in the context of miR-155 processing

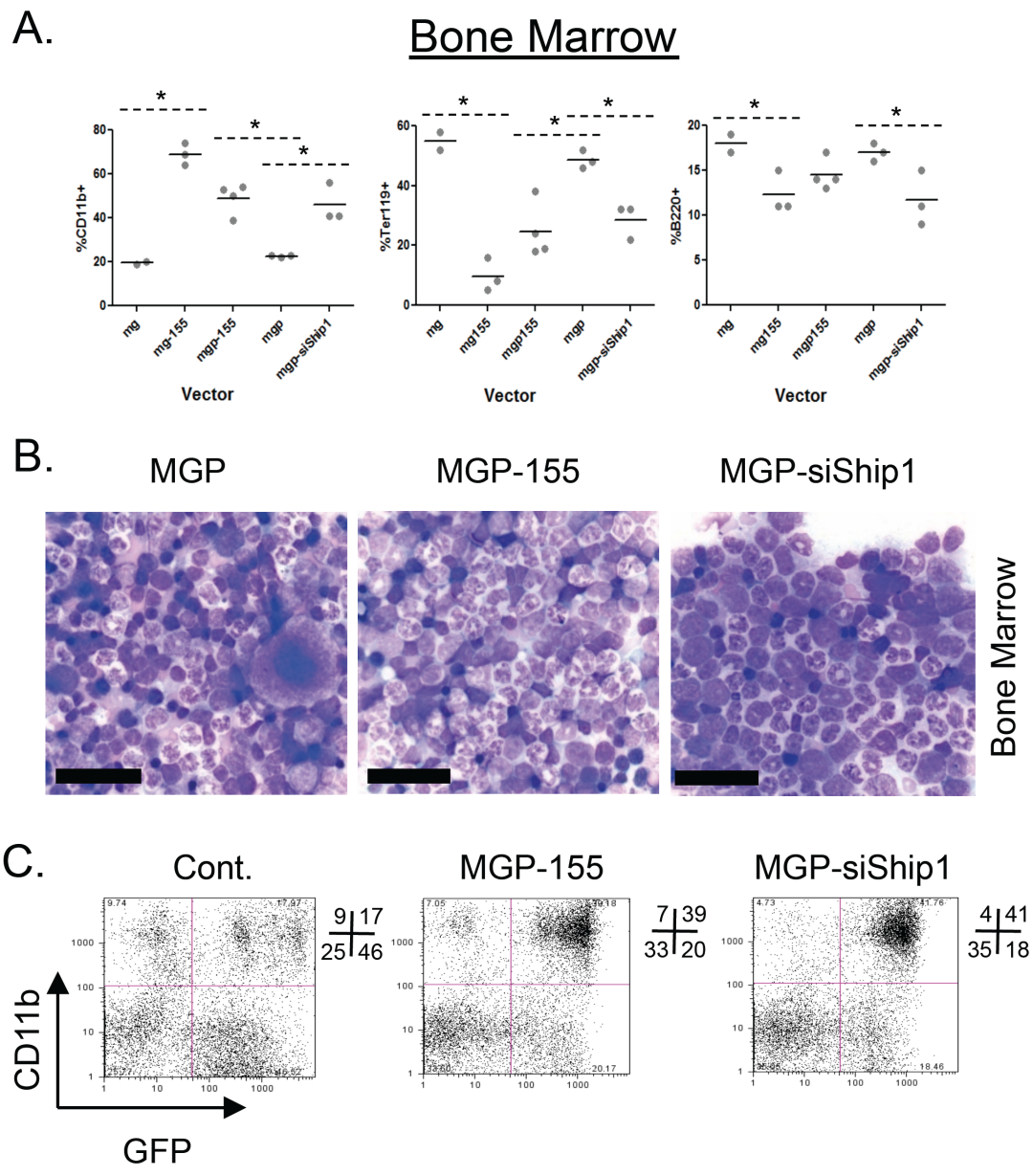

Figure 4. Knockdown of SHIP1 or expression of miR-155 in the hematopoietic compartment cause similar MPDs in the bone marrow

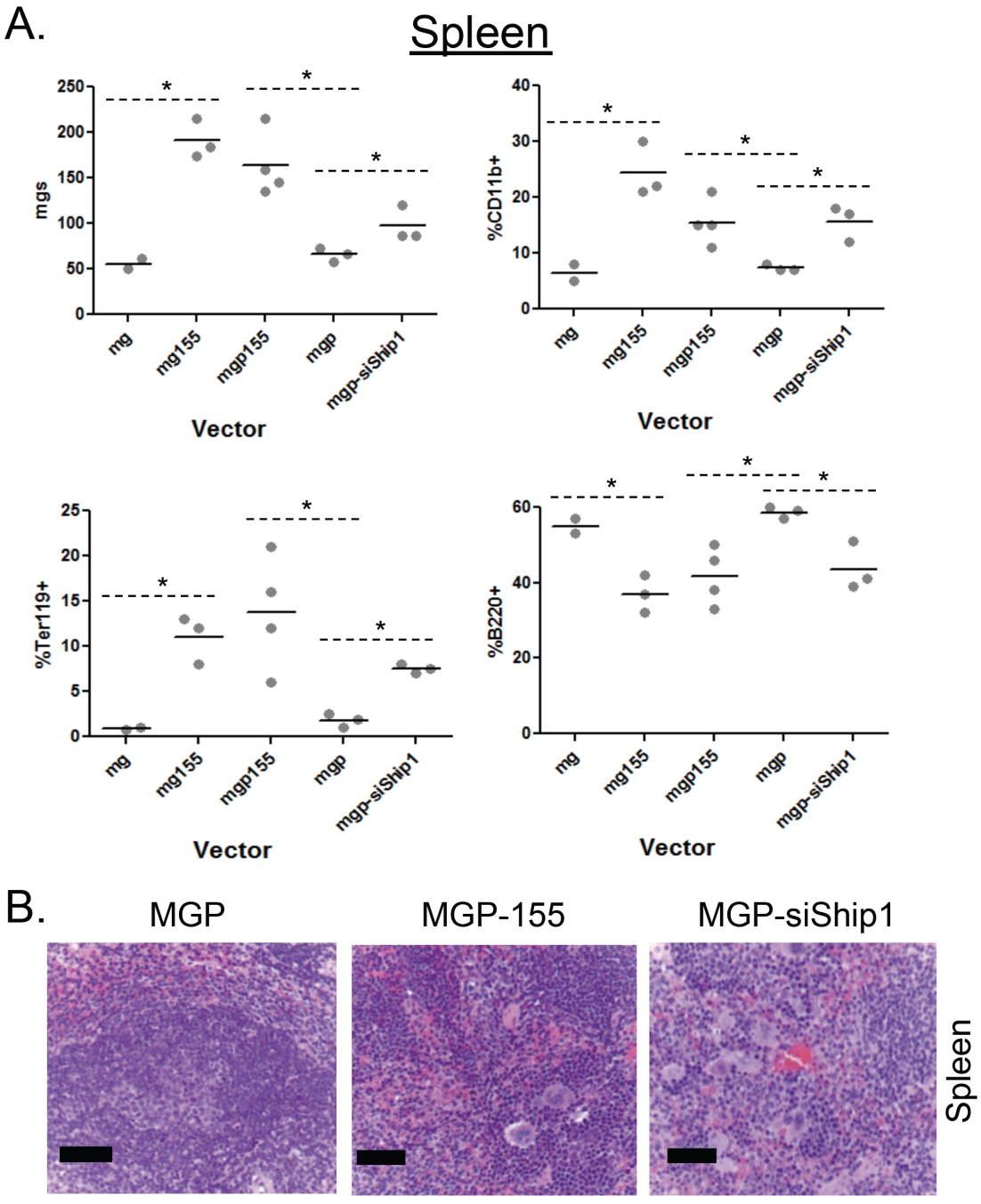

Figure 5. Knockdown of SHIP1 or expression of miR-155 in the hematopoietic compartment causes spenomegaly and extramedullary hematopoiesis in the spleen

Supplemental Table

Table S1. Cloning primers and template oligos

Primer	Sequence
m155 from LPS BMM Fw	AAACCAGGAAGGGGAAGTGT
m155 from LPS BMM Rev	ATCCAGCAGGGTGACTCTTG
m155 BlockiT Template oligo	GAAGGCTGTATGCTGTTAATGCTAATTGTGATAGGGGTTTT GGCCACTGACTGACCCCTATCAATTAGCATTAACAGGACAC AAGGCCTG
h155 BlockiT Template oligo	GAAGGCTGTATGCTGTTAATGCTAATCGTGATAGGGGTTTT GGCCACTGACTGACCCCTATCAATTAGCATTAACAGGACAC AAGGCCTG
h155mut BlockiT Template oligo	GAAGGCTGTATGCTGTATTACGTAATCGTGATAGGGGTTTT GGCCACTGACTGACCCCTATCAATTACGTAATACAGGACAC AAGGCCTG
siShip1 BlockiT Template oligo	GAAGGCTGTATGCTGTTAAATGTCATGCTGGTGACTGTTTT GGCCACTGACTGACAGTCACCAATGACATTTAACAGGACAC AAGGCCTG
m155 arms NotI Fw	ATCGGCTGAGTCGACGCGGCCGCCTGGAGGCTTGCTGAAG GCTGTATGCTG
m155 arms XhoI Rev	ATCGCAATTGCTCGAGTGGGCCATTTGTTCCATGTGAGTGC TAGTAACAGGCCTTGTGTC
mShip1 3'UTR SpeI Fw	TTCACTAGTGCTGCTGGTGATCGGAGCCTG
mShip1 3'UTR HindIII Rev	TTCAAGCTTTAGAAAGGCACAATTTTATTGGTCACTCAG
mShip1 UTRmut fragment 1 Rev	GGTTAGCTCTAAGAGACCGATGGTTTTCTTATTACGTTTTTA AAAACATCTCAGGACCCACC
mShip1 UTRmut fragment 2 Fw	GTGGGTCCTGAGATGTTTTTAAAAACGTAATAAGAAAACCAT CGGTCTCTTAGAGCTAACC

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Chapter 4: MicroRNAs enriched in hematopoietic stem cells differentially regulate long-term hematopoietic output

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Abstract

The production of blood cells depends upon a rare hematopoietic stem cell (HSC) population, but the molecular mechanisms underlying HSC biology remain poorly understood. Here, we identify a subset of microRNAs (miRNAs) that are enriched in HSCs compared to other bone marrow cells. An in vivo gain-of-function screen found that three of these miRNAs conferred a competitive advantage to engrafting hematopoietic cells, while other HSC miRNAs attenuated production of blood cells. Overexpression of the most advantageous miRNA, miR-125b, caused a dose-dependent myeloproliferative disorder (MPD) that progressed to a lethal myeloid leukemia in mice, and also enhanced hematopoietic engraftment in human immune system (HIS) mice. Our study identifies an evolutionarily conserved subset of miRNAs that are expressed in HSCs and that function to modulate hematopoietic output.

Introduction

Long-term production of blood cells depends upon proper hematopoietic stem cell (HSC) function (Orkin and Zon 2008). This involves a delicate balance between HSC self-renewal and differentiation into progenitor populations. The molecular networks that control these processes have begun to be elucidated in recent years, and several regulatory proteins have been shown to manage stem cell physiology (Ramalho-Santos, Yoon et al. 2002; Park, Qian et al. 2003; Zhang, Grindley et al. 2006; Deneault, Cellot et al. 2009; Santaguida, Schepers et al. 2009). Additionally, there is a substantial overlap between the proteins that regulate HSC biology and those that are dysregulated in cancers of immunological origin or other hematopoietic diseases underlying the importance of these factors in maintaining homeostasis.

Mammalian non-coding miRNAs have recently been identified as important repressors of gene expression through direct interactions with specific mRNA targets, and thus act to modulate gene function (Bartel and Chen 2004; Winter, Jung et al. 2009). Several reports have found that miRNAs regulate the development of specific hematopoietic lineages (O'Connell, Rao et al.). Conditional deletion of Dicer, which disrupts miRNA processing, has revealed critical roles for miRNAs during development of different hematopoietic lineages, including both B and T lymphocytes (Cobb, Nesterova et al. 2005; Muljo, Ansel et al. 2005; Koralov, Muljo et al. 2008). Specific miRNAs have also been shown to impact development of distinct blood cell lineages. Early B cell development is modulated by miR-150 (Xiao, Calado et al. 2007), miRNAs 17-5p-20a-106a direct human monocytes development (Fontana, Pelosi et al. 2007), and miR-223 functions to restrict the granulocytic compartment in vivo (Johnnidis, Harris et al. 2008).

Recent studies have demonstrated a causative role for miRNAs in mediating malignant diseases in the hematopoietic system. For example, sustained hematopoietic expression of miR-155 or miR-29a in the mouse hematopoietic system causes a myeloproliferative disorder (O'Connell, Rao et al. 2008) or leukemia (Han, Park et al.), respectively, while a B cell malignancy is triggered upon constant expression of miR-155 in a B cell restricted manner (Costinean, Zanesi et al. 2006). On the other hand, tumor suppressor miRNAs like miR-15/16 are deleted in a subset of lymphomas (Calin, Dumitru et al. 2002), which has been shown to cause chronic lymphocytic leukemia in mice (Klein, Lia et al.).

Despite their being clearly linked to hematopoietic development and cancer, there is little known regarding the expression patterns and function of mammalian miRNAs in HSC populations. One study found that mice deficient in Ars2, which is required for proper miRNA processing, suffer from bone marrow failure suggesting a functional role for miRNAs in HSCs (Gruber, Zatechka et al. 2009). Other groups have profiled miRNA expression in human CD34+ cells (Georgantas, Hildreth et al. 2007). However, many of the identified miRNAs are broadly expressed outside of HSCs, and their functional relevance in stem cells was not investigated *in vivo*. In the present study, we sought to both find specific miRNAs that are enriched in long-term HSCs and investigate their impact on long-term hematopoietic reconstitution.

Results

Identification of miRNAs enriched in Hematopoietic Stem Cells (HSCs)

To identify miRNAs enriched in hematopoietic stem and progenitor cells (HSPCs), we isolated lineage negative (lin-) cKit+Sca1+ (LKS) cells from adult C57BL6 mouse BM using FACS (**Fig. 1A** (top)). Total RNA was isolated from LKS cells, and also

from RBC-depleted total BM, and used to perform a microarray analysis to identify expression of mouse miRNAs. Some 137 miRNAs were expressed at detectable levels in the BM and/or LKS populations. Of these, 67 were expressed at higher levels in total BM versus LKS cells, 59 were expressed at similar levels in the two cellular populations, and 11 were expressed at higher levels in the LKS compartment versus total BM (**Fig. 1A** (bottom) and **Table S1**). Corroborating the microarray results, qPCR detected enrichment of these 11 miRNAs in LKS cells. They are miR125a-5p, miR-125b-5p, miR-155, miR-130a, miR196b, miR-99a, miR-126-3p, miR-181c, miR-193b, miR-542-5p and let7e (**Fig. 1B**). A more extensive cellular fractionation of mouse BM further confirmed enrichment of most of these miRNAs in the LKS subset (**Fig. 1C** and **Fig S1**). Expression of miR-223, known to be enriched in mature myeloid cells, was measured as a control (**Fig 1C**). The LKS population is comprised of both hematopoietic stem cells (HSCs), capable of establishing long-term multi-lineage hematopoietic engraftment, and multipotent progenitors, with reduced capacity to reconstitute the hematopoietic system. LKS cells expressing the HSC marker EPCR (Balazs, Fabian et al. 2006) were first analyzed and a higher relative expression of many of the miRNAs under study was found in EPCR+ versus EPCR- LKS cells (**Fig. 1C**). Long-term HSCs have also been shown to be CD150+CD48- while other LKS progenitor populations are negative for CD150 (Kiel, Yilmaz et al. 2005). Again, qPCR analysis found that most of the HSPC miRNAs were further enriched in CD150+CD48- long-term HSCs compared to CD150- LKS cells (**Fig. 1D**). These data indicate, using multiple criteria to define HSCs, that this subset of miRNAs is largely enriched in long-term HSCs.

Gain-of-function approach to assess the impact of HSC miRNAs on long-term hematopoietic engraftment

The expression patterns of these particular miRNAs suggest they have a specialized role in regulating hematopoiesis at the stem cell level. To ascertain the functional impact of these HSC miRNAs on long-term hematopoietic reconstitution, we constructed a series of murine stem cell virus (MSCV)-based miRNA expression vectors that carry each of the 11 miRNAs under study. The vectors were formatted with mouse miR-155 arms and stem loop sequences, with each miRNA duplex encoded by the sense and antisense stem regions (**Fig. 2A**). This format was chosen due to its established ability to be processed in hematopoietic cells (O'Connell, Chaudhuri et al. 2009), including HSCs where we have found mature miR-155 to be expressed (**Fig. 1**). Production of the different mature miRNAs using this format was confirmed in 293T cells by qPCR (**Fig. S2**).

Competitive repopulation experiments were performed by combining equal numbers of HSC-enriched CD45.1+ bone marrow cells transduced with retroviruses encoding a specific HSC miRNA with HSC-enriched CD45.2+ bone marrow transduced with a control retrovirus. This cellular mixture was used to reconstitute lethally irradiated CD45.2+ C57BL6 recipients. Because the retrovectors coexpress GFP in addition to a miRNA, the ability of the CD45.1+ miRNA expressing bone marrow to compete with CD45.2+ control vector-containing bone marrow during hematopoietic engraftment could be tracked by FACS analysis of peripheral blood cells. Mice were bled at 2 and 4 months post-reconstitution. When both vectors lacked a miRNA, there was similar representation of the two markers in the bone marrow cells (**Fig. S3**). In three cases, including miR-125b-5p, miR-126-3p and miR-155, the miRNA conferred an evident and statistically significant ($p < 0.05$) competitive advantage to the engrafted bone marrow (**Fig. 2B** and **Fig. S3**). In contrast, overexpression of several of the miRNAs caused a significant ($p <$

0.05) disadvantage to the engrafted bone marrow, with miR-196b, miR-181c, let7e and 542-5p having the largest impact (**Fig. 2B** and **Fig. S3**). FACS analysis of the peripheral blood was also used to determine the distribution of different hematopoietic lineages to assess whether engraftment was being skewed towards a single lineage or was multi-lineage at 4 months post reconstitution. The effects of the different miRNAs on engraftment were largely multi-lineage, consistent with these miRNAs regulating stem cell homeostasis (**Fig. 2C**).

MiR-125b causes a dose-dependent MPD that progresses to a myeloid leukemia

We next focused on further characterization of miR-125b because it had the largest positive impact on competitive reconstitution among the miRNAs tested, and is overexpressed in certain types of AML suggesting it plays an active role in human malignancies (Klusmann, Li et al. ; Bousquet, Quelen et al. 2008). miR-125b is expressed from two loci in the genome, and these sequences are referred to as miR-125b1 and miR-125b2. To assess the impact of expressing miR-125b with its native arms and loop sequences, we cloned segments of the miR-125b1 and miR-125b2 pri-miRNAs into our retroviral expression system (**Fig. 3A**). We found that these cassettes led to substantially increased expression levels of mature miR-125b in K562 cells compared to the miR-125b cassette formatted with miR-155 arms and loop sequences (used above and now referred to as 125b(f)), likely a result of more efficient processing (**Fig. 3A**). Furthermore, miR-125b1 expression was higher than miR-125b2, providing three vectors with conveniently graded expression of miR-125b.

Mice were subsequently reconstituted with bone marrow expressing either miR-125b1, miR-125b2 or control vector, and a separate cohort of mice expressing miR-125b(f) or its vector control was also established. All mice from both groups were bled following 2 months of reconstitution and complete blood cell counts (CBCs) were

recorded (**Fig. 3B**). Mice expressing miR-125b, regardless of the construct used, had subtly elevated total WBC counts compared to control mice. Reconstitution of mice with HSPCs expressing the lowest levels of miR-125b, miR-125b(f), resulted in a general increase in all WBC compartments analyzed including myeloid, lymphoid, and platelets, but did not impact RBCs. However, mice with the more highly expressed endogenous miR-125b1 or b2 vectors exhibited a clear myeloproliferative disorder (MPD) that was characterized by increased absolute numbers of monocytes and neutrophils, and decreased levels of lymphocytes, RBCs and platelets. Peripheral blood from miR-125b-1 and -2 expressing mice, compared to control mice, also had dramatic increases in the percentage of CD11b+ myeloid cells, many of which were also Gr1+ as assayed by FACS (**Fig. 3C**). We also found that enforced expression of endogenous miR-125a, which has the same "seed" sequence as miR-125b, also caused a competitive advantage to engrafting BM (RO, AC and DB unpublished observations). Expression of endogenous miR-125a was higher than the formatted miR-125a cassette used in our gain-of-function screen, which likely accounts for the difference in functional impact between the two vectors.

Analysis of Wright stained bone marrow smears from miR-125b(f) mice two months post reconstitution revealed a mild expansion of myeloid cells in the bone marrow, while H&E stained spleen sections were similar to those from control mice (**Fig. S4**). However, miR-125b1 and miR-125b2 expressing mice exhibited myeloid-dominated BM and spleens as well as myeloid cell infiltration into the liver (**Fig. S4**). By 3.5 months post-reconstitution miR-125b1 and miR-125b2 mice had substantially increased WBCs in their peripheral blood consisting mainly of immature granulocytic and monocytic cells (**Fig. S4**). Shortly after, the condition became lethal for most of the mice expressing the highest levels of miR-125b with tumor infiltration evident in the spleen and liver (**Fig. 4A** and **4B**). The surviving miR-125b2 mice also had progressively increasing WBC counts

and higher levels of leukemic blasts in their peripheral blood from 3.5 to 4.5 months post reconstitution, and this also became lethal (**Fig. 4C** and **4A**). Furthermore, transfer of malignant BM from moribund miR-125b1 expressing mice to immunodeficient Rag2-/-gc-/- secondary recipients resulted in death within weeks, while transfer of BM from control mice had no impact on survival (**Fig. 4D**). These findings demonstrate a dose-dependent sufficiency of miR-125b to drive pathological myeloid cell expansion, a condition that progresses to an aggressive myeloid leukemia.

Evolutionarily conserved miRNA expression and function in human CD34+ HSPCs

We investigated whether the subset of miRNAs enriched in mouse HSCs was also enriched in human CD34+ HSPCs. RNA was prepared from CD34+ and CD34- umbilical cord blood (CB) PBMCs and analyzed by qPCR. With the exception of miR-193b, all of the miRNAs showed significant enrichment in the CD34+ HSPC cellular subset compared to mature CD34- PBMCs (**Fig. 5A**). To determine whether miR-125b could also impact human hematopoietic engraftment, CD34+ CB cells were transduced with control or miR-125b1-expressing lentiviral vector (**Fig. 5B**), and subsequently injected intrahepatically into newborn Rag2-/-gc-/- mice on a Balb/c genetic background generating the previously described human immune system (HIS) model (Traggiai, Chicha et al. 2004)**.** After 10 weeks, peripheral blood was collected and analyzed for the presence of human CD45+ WBCs by FACS, and a dose-dependent enhancement in multi-lineage engraftment was again observed in miR-125b1-expressing versus control HIS mice (**Fig. 5C, 5D** and **5E**). At 12 weeks post-reconstitution, the high dose (MOI 40) groups were harvested and miR-125b-expressing HIS mice had significantly elevated levels of hCD45+ cells and hCD34+ HSPCs in their bone marrow (**Fig. 5F** and **5G**). These data provide evidence that miR-125b also promotes hematopoietic engraftment of human HSCs.

Discussion

In this work, we have identified an evolutionarily conserved subset of miRNAs that are enriched in HSCs and have provided evidence that these miRNAs function to properly manage hematopoietic output. Because some miRNAs promoted while others diminished hematopoietic engraftment, it is possible that these HSC miRNAs work in a concerted manner to ensure proper output of blood cells. A similar paradigm has recently been shown for embryonic stem (ES) cells, where opposing miRNA families modulate ES cell self-renewal and differentiation (Melton, Judson et al.). This suggests that regulatory networks involving multiple miRNAs participate in the control of both embryonic and adult stem cells.

Owing to their important developmental roles and ability to target known oncogenes and tumor suppressors (O'Connell, Rao et al.), miRNAs are a key part of the intricate molecular networks that drive or suppress cancer development and progression. Certain hematopoietic cancers, such as CML, are sustained by rare cancer stem cell populations that have significant mRNA expression overlap with normal HSCs despite producing malignant progeny (Savona and Talpaz 2008). Our newly identified set of HSC miRNAs might prove to be useful as novel biomarkers of such cancer stem cell populations. In the case of miR-125b, it will also be of interest to assess whether it transforms HSCs or a committed myeloid progenitor, and to characterize secondary mutations that may assist in this process.

Many of the miRNAs identified in our study, including miR-125b, miR-155, miR-126, miR-196b, and miR-99a, have been shown to be dysregulated in different subsets of AML (Cammarata, Augugliaro et al.). Because we have demonstrated functional relevance for many of these HSC miRNAs *in vivo*, including the abilities of miR-155 (O'Connell, Rao et al. 2008) and now miR-125b to trigger aggressive MPDs and myeloid

leukemia, respectively, therapeutic targeting of this subset may be effective in combating hematopoietic cancers.

Materials and Methods

Isolation of bone marrow cell subsets and FACS. Hematopoietic stem and progenitor cells (HSPCs) were isolated from C57BL6 bone marrow by first depleting lineage positive cells using specific biotinylated antibodies (Table S2), magnetic beads and a MACS column (Miltenyi). Viable (7-AAD negative) Lin- cells were next stained with fluorophore-conjugated antibodies (Table S2) and separated using a FACS Aria (BD) into cKit-, cKit+Sca1-, and LKS subpopulations. Hematopoietic stem cells were further purified from among the LKS population by sorting out EPCR+ or CD150+CD48- LKS cells. CD11b+ and B220+ cells were fractionated from total BM using a MACS column. RBC-depleted PBMCs were stained with the indicated antibodies (Table S2), washed and analyzed using a FACSCalibur (BD).

Microarray analysis. For the microarray, total RNA was collected from equal numbers of LKS cells and total RBC-depleted BM using the miRNeasy kit (Qiagen) and subsequently utilized for a low-input microarray with Agilent mouse miRNA 10.1 chips (Asuragen). Data represent miRNA expression levels in LKS and total bone marrow taken from 10 mice and pooled before RNA purification. Two biological replicates were analyzed in this way and both identified the 11 miRNAs described in this study.

Quantitative PCR. ABI Taqman was utilized to quantitate microRNA levels in different BM cell populations using gene-specific primers according to the manufacturer's instructions.

Competitive bone marrow reconstitutions. C57BL6 mice expressing the congenic WBC markers CD45.1 or CD45.2 were used as bone marrow donors and CD45.2 mice were used as recipients. Equal numbers of HSPC enriched bone marrow cells from each group (obtained from mice treated with 5-fluorouracil for 5 days) were infected with a miRNA expressing (CD45.1 cells) or control vector (CD45.2 cells) and a 1:1 cell mixture was injected i.v. into lethally irradiated mice.

Retroviral and lentiviral vectors. miRNA expression vectors formatted with miR-155 loop and arms were constructed as described previously (O'Connell, Chaudhuri et al. 2009) (Table S3). Endogenous miR-125b-1 and miR-125b-2 sequences were PCR cloned into MG and MGP retrovectors (O'Connell, Rao et al. 2008; O'Connell, Chaudhuri et al. 2009) or a third generation, replication-deficient lentiviral vector with an EF1a promoter. Retroviral transduction of mouse HSPCs and bone marrow transplantation were performed as previously described (O'Connell, Rao et al. 2008; O'Connell, Chaudhuri et al. 2009). K562 cells were cultured in complete RPMI and infected with retrovectors in the presence of polybrene (10 mg/ml). Lentiviral transduction of CD34+ cells was achieved using an MOI of 4 or 40, Retronectin (Fisher Scientific) coated plates and StemSpan serum free medium (Stem Cell Technologies). Transduced CD34+ cells were washed and injected 24 hours after infection.

Human Immune System (HIS) mouse model. HIS mice were generated by injecting $2x10^5$ lentivirus transduced CD34+ CB cells i.h. into irradiated newborn Rag2-/-gc-/- mice on a Balb/c genetic background as described (Traggiai, Chicha et al. 2004). Human hematopoietic cell engraftment was assessed by collecting blood and subjecting PBMCs to FACS (Table S2).

Histopathology and complete blood cell counts. These procedures were performed as described (O'Connell, Rao et al. 2008; O'Connell, Chaudhuri et al. 2009).

Statistics. For statistical analysis a Student's two-tailed t-test was used.

Figure Legends

Figure 1. Identification of miRNAs enriched in Hematopoietic Stem Cells (HSCs). **A**. Microarray experiment comparing miRNA expression in the Lineage-cKit+Sca1+ (LKS) compartment (FACS plot on top, subset boxed in red) to total bone marrow from C57BL6 mice. Number of miRNAs significantly enriched in total BM versus the LKS compartment, unchanged between the two groups, or significantly enriched in LKS versus total BM cells are shown (bottom). BM cells were pooled from 10 mice, sorted and RNA was extracted for the microarray. **B**. qPCR was used to determine the fold enrichment of the 11 miRNAs preferentially expressed in LKS cells versus total BM. Data represent two independent experiments. **C.** Expression levels of the 11 HSPC miRNAs were measured in hematopoietic cells from the different bone marrow compartments shown in Fig. S1 and represent different stages of blood cell development. **D.** LKS-gated cells were further sorted by FACS into three groups according to their expression of the SLAM markers CD150 and CD48. Total RNA was collected from each group and the relative expression levels of miRNAs from each LKS subpopulation were assayed by qPCR. Data represent two independent experiments, each starting with pooled BM from 20 mice. All data were normalized to sno202 and represent the mean + SEM.

Figure 2. Gain-of-function approach to assess the impact of HSC miRNAs on long-term hematopoietic reconstitution. **A**. HSC miRNAs were cloned into an MSCV-

based retroviral vector (MG) using a miR-155 arms-and-loop format. **B**. Competitive BM reconstitutions were performed using C57BL6 mice to assess the engraftment potential of BM expressing each of the 11 HSC miRNAs (CD45.1 cells) compared to control BM (CD45.2 cells). GFP was used to identify cells containing a vector. A four month time course showing the percentage of CD45.1+GFP+ cells in the peripheral blood of each group of mice is shown. The experiment was broken into two batches (top and bottom), each with its own negative control. **C.** Lineage analysis of the CD45.1+GFP+ peripheral blood cells at the 4 month time point was assessed by determining the percentages of B220+ (B cell), CD3+ (T cell) and CD11b+ (myeloid cell) in each group. Data represent the mean + SEM. Experiment had 4 mice per group.

Figure 3. MiR-125b causes a dose-dependent MPD. A. The three constructs used to enforce expression of miR-125b are shown. K562 cells were transduced with each retrovector and miR-125b levels were assayed by qPCR **B**. Blood concentrations of the indicated cell types are shown for mice expressing MG, MG-125b1, MG-125b2, and in a separate experiment (separated by a dashed line), MG and MG-125b(f) 2 months post-reconstitution. **C**. FACS plot showing a representative FACS plot of Gr1+CD11b+ cells in the peripheral blood of mice overexpressing miR-125b2, miR-125b1 or control vector 2 months post-reconstitution. * denotes a p value of < 0.05.

Figure 4. MPDs caused by miR-125b progress to myeloid leukemia in a dose-dependent manner. A. Survival of mice expressing different levels of miR-125b. Group sizes for the experiment performed were MG=7, MG-125b1=8, MG-125b2=8 and MG-125b(f)=4. **B.** Liver and spleen from miR-125b2-expressing mice with leukemia 4.5 months after reconstitution. Representative H&E stained tissue sections of each respective organ taken from miR-125b2 expressing mice are shown on the right. **C.**

Percentage of leukemic blasts in the peripheral blood of control or miR-125b2 expressing mice 3.5 and 4.5 months post-reconstitution, and representative Wright stained blood smears from miR-125b2 mice at each timepoint. Examples of blasts are indicated by red arrows. **D.** Survival of mice following intravenous transfer of malignant MG-125b1 (4 different donors) or MG control bone marrow to Rag2-/-gc-/- recipients (n=3-6 mice per group). * denotes a p value of < 0.05.

Figure 5. Evolutionarily conserved miRNA expression and function in human CD34+ HSPCs. **A**. Human CB was sorted into CD34+ (HSPC+) and CD34- (HSPC-) fractions. Expression of the miRNAs enriched in mouse HSCs was measured by qPCR. Data are normalized to RNU48 and presented as mean + SEM (n=3 different donors). **B.** Human CB CD34+ cells were transduced with a control or miR-125b1 expressing lentiviral vectors at an MOI of approximately 4 or 40 and miR-125b levels were assayed by qPCR. **C.** Representative FACS plots showing an increased ratio of human to mouse CD45+ WBCs in the peripheral blood of miR-125b expressing HIS mice 10 weeks after CD34+ cell injection. **D.** A gray dot represents each mouse. **E.** Human cells in the peripheral blood of high dose (MOI 40) HIS mice were analyzed by FACS for the expression of different lineage markers including CD19 (B cells) and CD33 (myeloid). Data are represented as the mean +/- SEM (n=7). **F.** A representative FACS plot of hCD45+ and hCD34+ cells in the BM of miR-125b-expressing and control vector HIS mice 12 weeks post CD34+ injection. **G.** A gray dot represents each mouse. * denotes a p value of < 0.05.

FIGURES

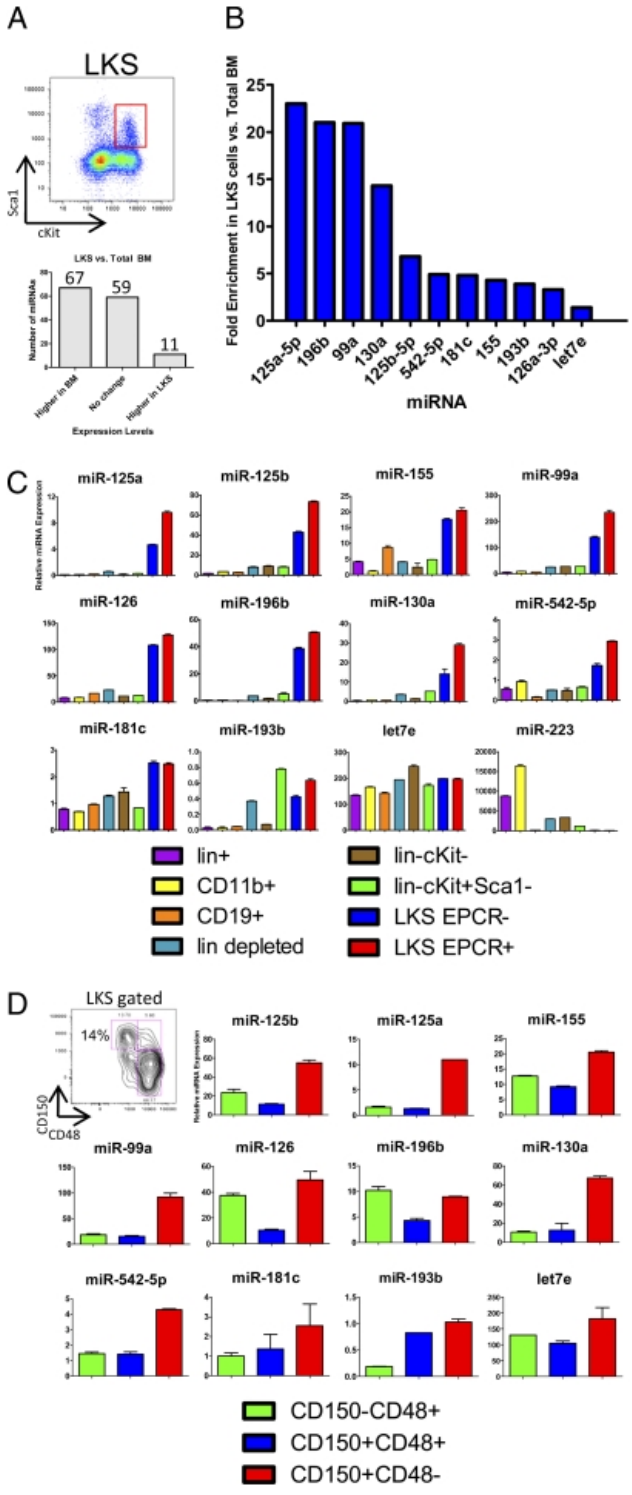

Figure 1. Identification of miRNAs enriched in Hematopoietic Stem Cells (HSCs)

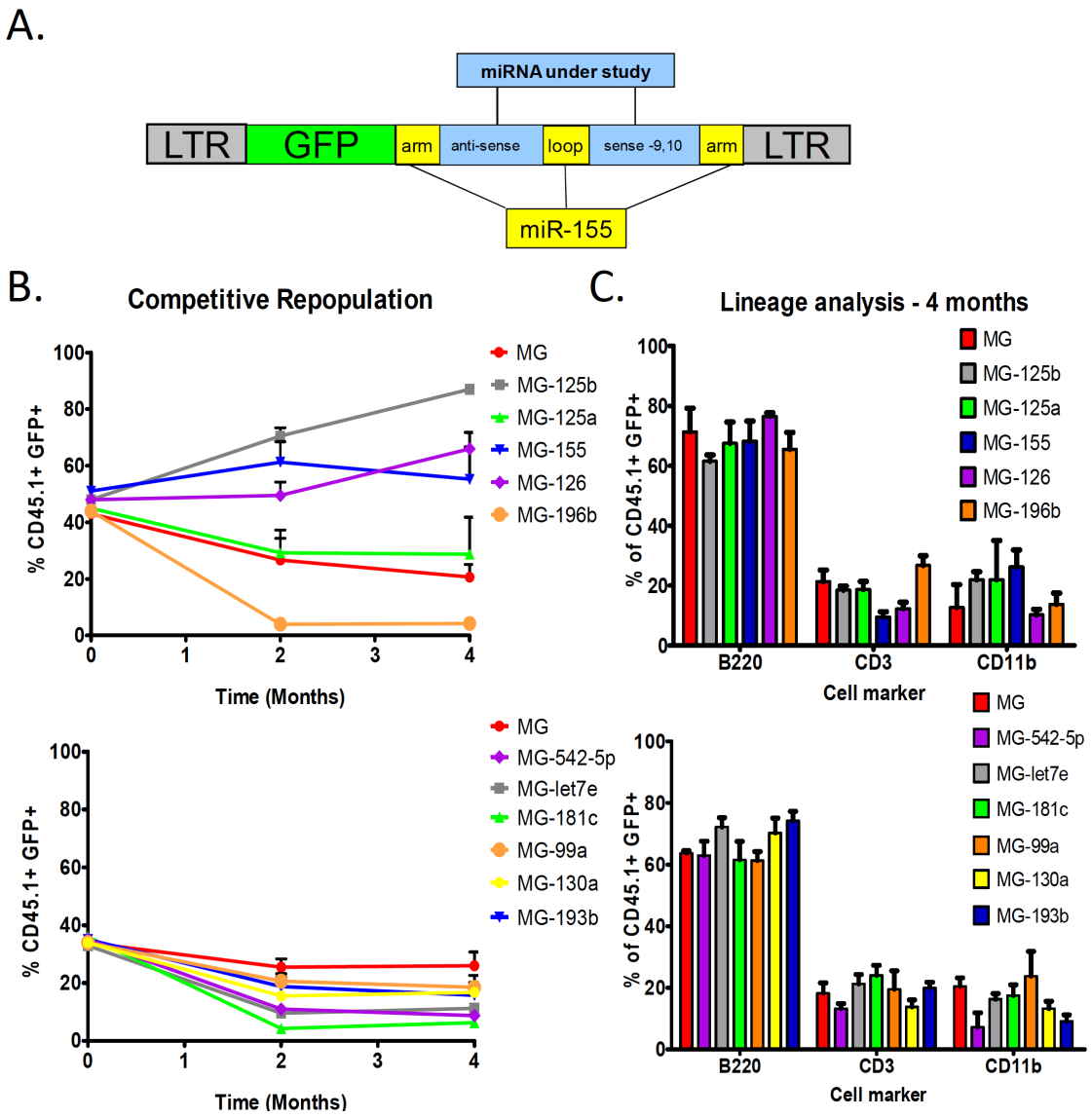

Figure 2. Gain-of-function approach to assess the impact of HSC miRNAs on long-term hematopoietic reconstitution

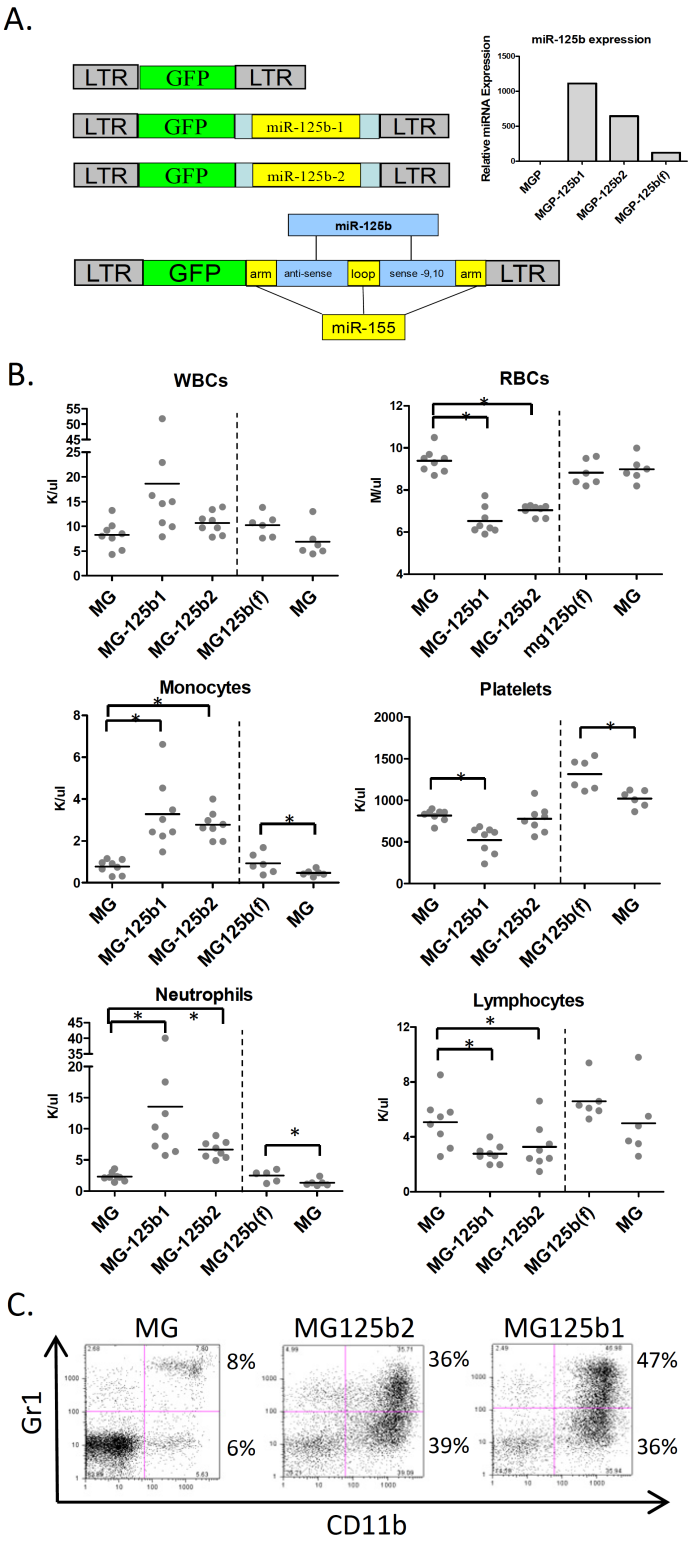

Figure 3. MiR-125b causes a dose-dependent MPD

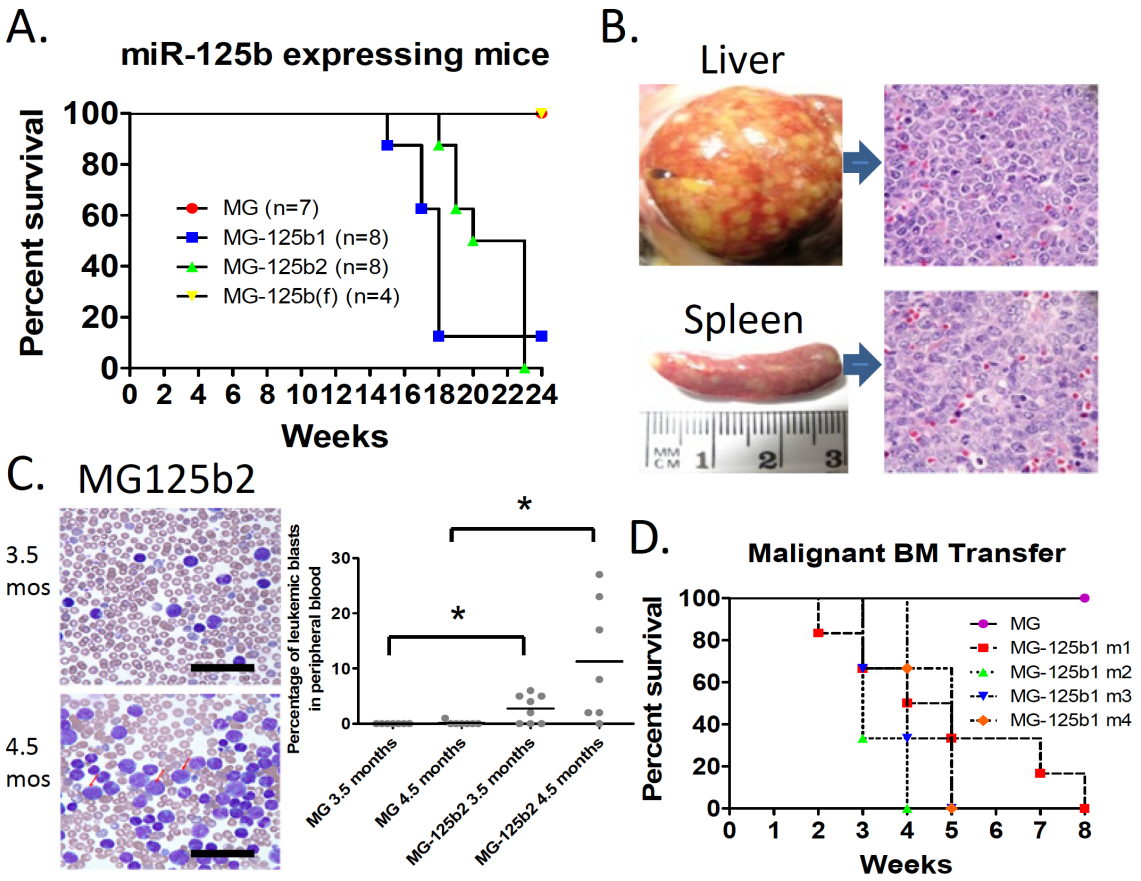

Figure 4. MPDs caused by miR-125b progress to myeloid leukemia in a dose-dependent manner

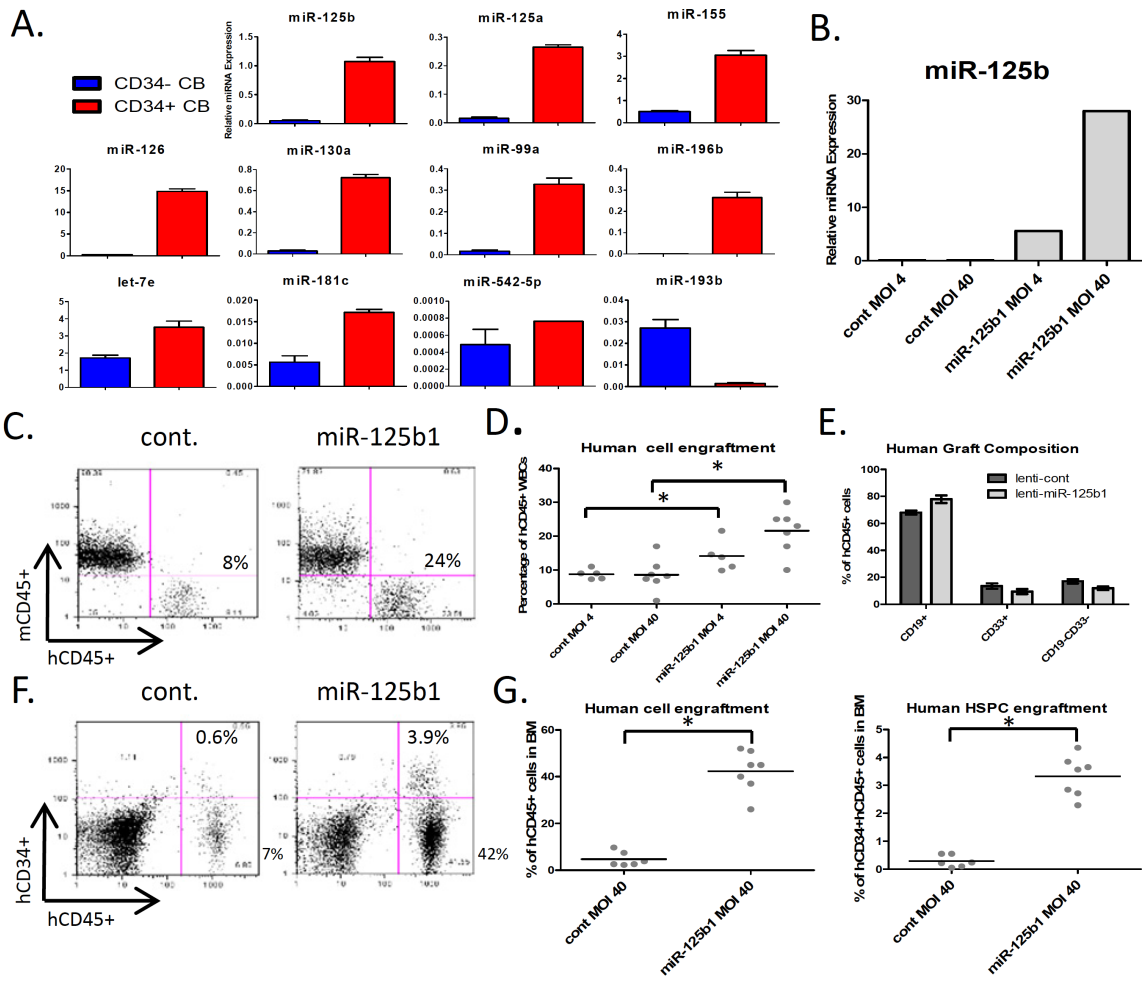

Figure 5. Evolutionarily conserved miRNA expression and function in human CD34+ HSPCs

Supplementary Figures

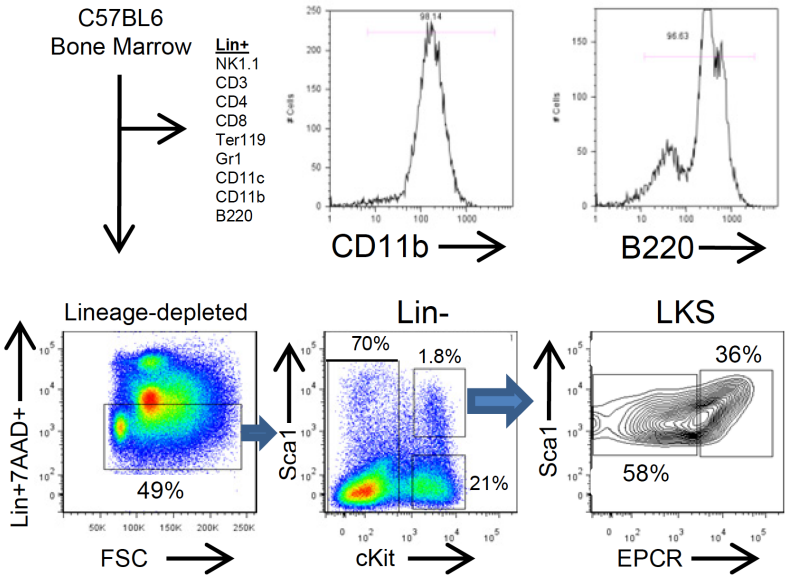

Figure S1 - A more extensive cellular fractionation scheme was utilized to further assess the expression profiles of the 11 miRNAs enriched in LKS cells.

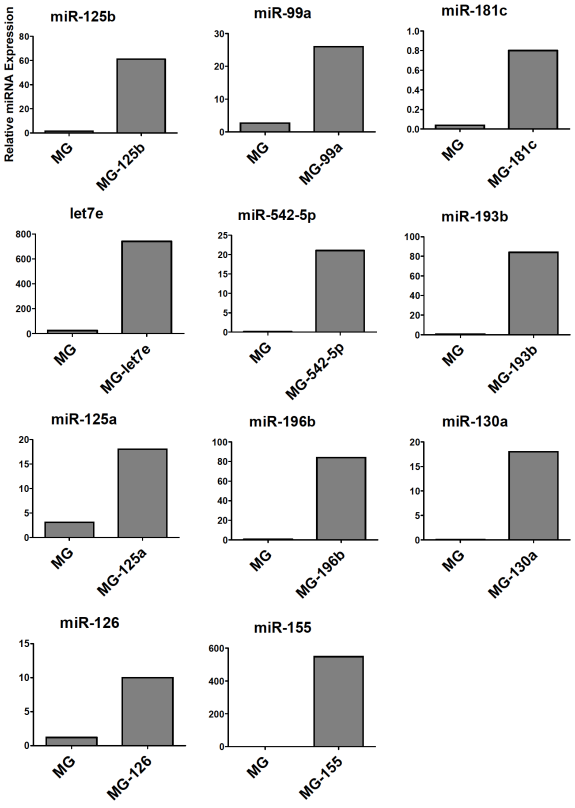

Figure S2 - Each respective miRNA expressing vector was transfected into 293T cells and microRNA levels were assessed by qPCR and normalized to RNU48.

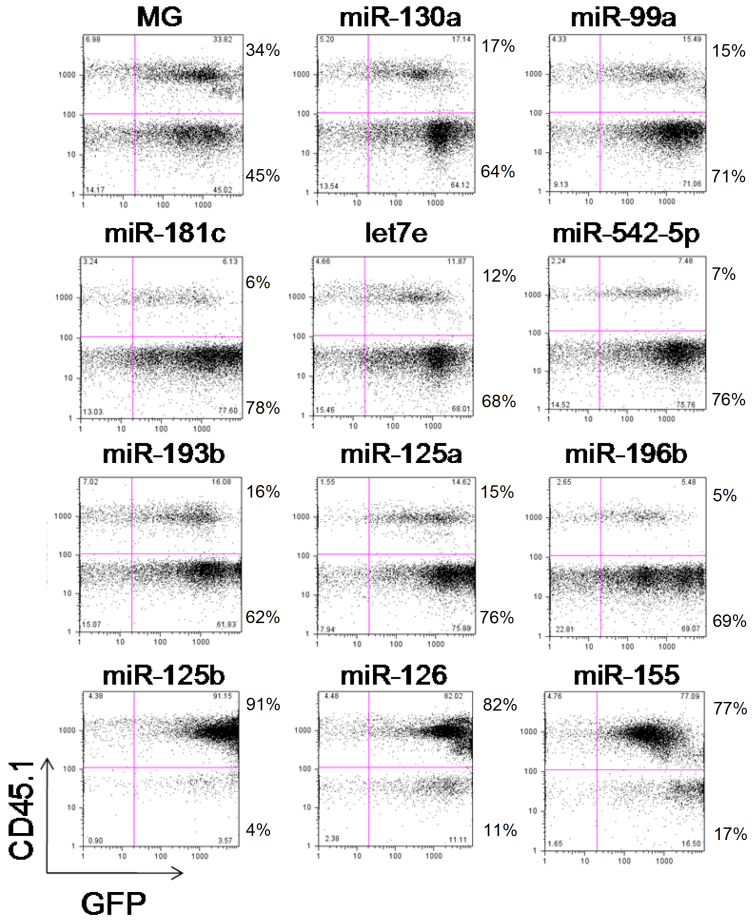

Figure S3 - Competitive BM reconstitutions were performed using C57BL6 mice to assess the engraftment potential of BM expressing each of the 11 HSC miRNAs (CD45.1 cells) compared to control BM (CD45.2 cells). A representative FACS plot of PBMCs from each mouse group stained with CD45.1 is shown (n=4, 4 months post-reconstitution). GFP was used to identify cells containing a vector.

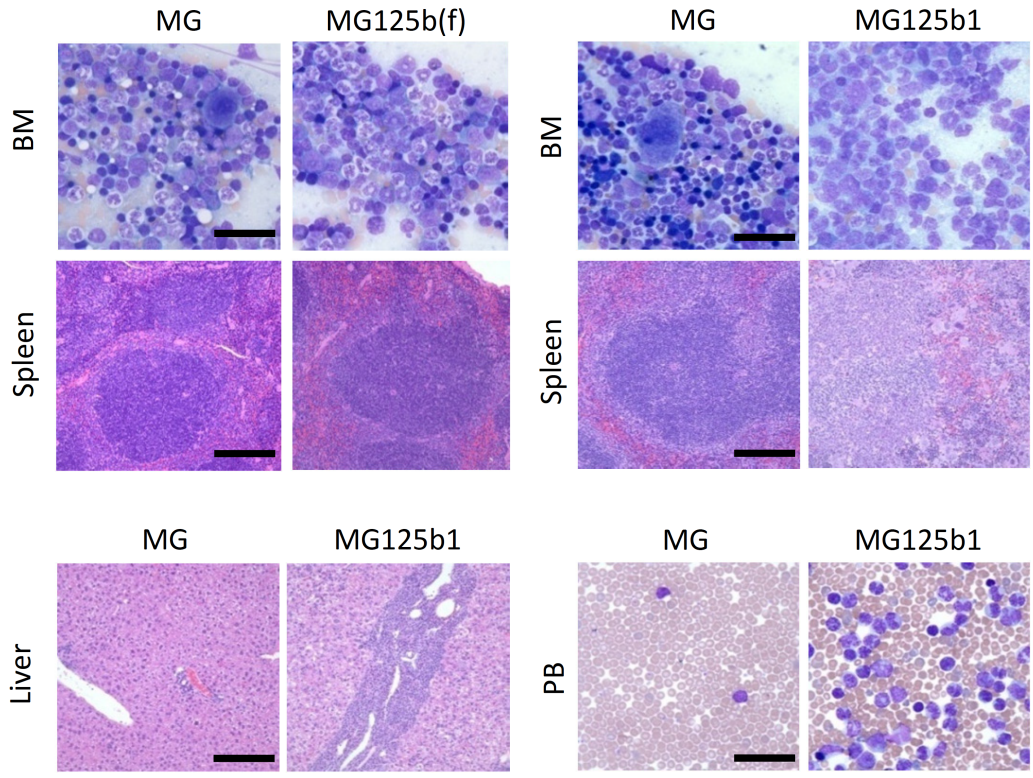

Figure S4 - Wright stained BM smears and H&E stained spleen sections from MG and MG-125b(f) expressing mice 2 months after reconstitution (Upper left) or the same tissues and liver from miR-125b1 expressing mice 2.5 months post-reconstitution (Upper right, Lower left). Wright stained blood smears from MG and MG-125b1 expressing mice 3.5 months after bone marrow reconstitution (Lower right). Data represent at least two independent experiments. Scale bars for the spleen and liver = 200 mm and for the blood and bone marrow = 40 mm.

Supplemental Tables

Table S1. Microarray Results

Probe_ID	miRNA_Accession	BM norm. exp.(log2)	LKS norm. exp.(log2)
mghv-miR-M1-1	MIMAT0001564	1.658388461	1.665290958
mghv-miR-M1-2	MIMAT0001565	5.256874189	4.223724501
mghv-miR-M1-3	MIMAT0001566	-1.708853573	-2.173177625
mghv-miR-M1-4	MIMAT0001567	-0.710358547	-0.013548819
mghv-miR-M1-5	MIMAT0001568	-1.969878595	-2.007085557
mghv-miR-M1-6	MIMAT0001569	1.633282789	0.589373906
mghv-miR-M1-7-3p	MIMAT0001571	2.829957102	3.036485454
mghv-miR-M1-7-5p	MIMAT0001570	-2.083392945	-2.351914656
mghv-miR-M1-8	MIMAT0001572	-1.419387204	-2.562143355
mghv-miR-M1-9	MIMAT0001573	2.759736162	3.0982055
mmu-let-7a	MIMAT0000521	8.570957416	8.679039209
mmu-let-7a*	MIMAT0004620	2.587261983	2.503677901
mmu-let-7b	MIMAT0000522	8.470202955	8.841128627
mmu-let-7b*	MIMAT0004621	3.35166403	3.11776057
mmu-let-7c	MIMAT0000523	7.612216945	8.326368152
mmu-let-7c-1*	MIMAT0004622	2.361676696	2.790416529
mmu-let-7d	MIMAT0000383	8.285647809	8.577517577
mmu-let-7d*	MIMAT0000384	2.091155024	2.255575826
mmu-let-7e	MIMAT0000524	2.161639523	5.283164186
mmu-let-7f	MIMAT0000525	8.24140687	8.296871861

mmu-let-7f*	MIMAT0004623	3.529436039	3.293028849
mmu-let-7g	MIMAT0000121	8.673913416	8.178103409
mmu-let-7g*	MIMAT0004519	0.265210602	-0.036937031
mmu-let-7i	MIMAT0000122	9.843133718	9.781146766
mmu-let-7i*	MIMAT0004520	0.767609973	0.500773655
mmu-miR-1	MIMAT0000123	-2.051914345	-2.145095925
mmu-miR-100	MIMAT0000655	0.700920794	1.858457828
mmu-miR-101a	MIMAT0000133	3.303812581	3.784572525
mmu-miR-101a*	MIMAT0004526	-1.334827797	-1.079448599
mmu-miR-101b	MIMAT0000616	2.177135233	2.646731597
mmu-miR-103	MIMAT0000546	7.278998257	6.699078213
mmu-miR-105	MIMAT0004856	1.062895698	0.4294107
mmu-miR-106a	MIMAT0000385	3.221552804	3.197113759
mmu-miR-106b	MIMAT0000386	9.210931934	8.982834438
mmu-miR-106b*	MIMAT0004582	-1.006368027	-1.565898396
mmu-miR-107	MIMAT0000647	7.280000094	7.887308234
mmu-miR-10a	MIMAT0000648	6.884504703	7.564515559
mmu-miR-10a*	MIMAT0004659	-0.46024917	-1.355543349
mmu-miR-10b	MIMAT0000208	2.082494391	2.261888041
mmu-miR-10b*	MIMAT0004538	-1.622835747	-1.389145991
mmu-miR-122	MIMAT0000246	-0.740334399	-0.916109473
mmu-miR-1224	MIMAT0005460	12.7606169	8.446866526
mmu-miR-124	MIMAT0000134	-2.000059207	-2.16854943
mmu-miR-124*	MIMAT0004527	-0.596132072	-0.326864855
mmu-miR-125a-3p	MIMAT0004528	5.177242285	0.735169955

mmu-miR-125a-5p	MIMAT0000135	1.352220013	3.294244495
mmu-miR-125b*	MIMAT0004529	0.047237385	-0.733528529
mmu-miR-125b-3p	MIMAT0004669	-0.879596767	-1.779341919
mmu-miR-125b-5p	MIMAT0000136	1.841177399	4.787841208
mmu-miR-126-3p	MIMAT0000138	4.499607206	6.300321724
mmu-miR-126-5p	MIMAT0000137	2.45076993	2.53113059
mmu-miR-127	MIMAT0000139	1.237437665	1.783334631
mmu-miR-127*	MIMAT0004530	-1.760682866	-2.353193503
mmu-miR-128	MIMAT0000140	5.035404172	5.104355573
mmu-miR-129-3p	MIMAT0000544	2.227605188	2.030993851
mmu-miR-129-5p	MIMAT0000209	-0.181649448	-0.808316819
mmu-miR-130a	MIMAT0000141	4.049473473	6.997191837
mmu-miR-130b	MIMAT0000387	7.705293666	7.820178624
mmu-miR-130b*	MIMAT0004583	2.382753309	2.864926957
mmu-miR-132	MIMAT0000144	-0.544875578	-0.527459329
mmu-miR-133a	MIMAT0000145	2.520005032	1.814748859
mmu-miR-133a*	MIMAT0003473	-0.873382446	-1.673773178
mmu-miR-133b	MIMAT0000769	2.346762852	1.547889843
mmu-miR-134	MIMAT0000146	4.833960157	-2.35622959
mmu-miR-135a	MIMAT0000147	2.725493305	2.769002398
mmu-miR-135a*	MIMAT0004531	3.7836828	0.821810284
mmu-miR-135b	MIMAT0000612	2.412313953	2.630017575
mmu-miR-136	MIMAT0000148	2.75740244	2.968331013
mmu-miR-136*	MIMAT0004532	1.862550274	2.060017043

mmu-miR-137	MIMAT0000149	-0.046888883	0.39198953
mmu-miR-138	MIMAT0000150	-1.096545113	-2.017948042
mmu-miR-138*	MIMAT0004668	1.530756215	-0.850505257
mmu-miR-139-3p	MIMAT0004662	4.018224922	0.983550583
mmu-miR-139-5p	MIMAT0000656	6.497248529	2.508369355
mmu-miR-140	MIMAT0000151	6.240944023	5.204460731
mmu-miR-140*	MIMAT0000152	6.449992011	5.254834451
mmu-miR-141	MIMAT0000153	2.415705785	-0.849415781
mmu-miR-141*	MIMAT0004533	-1.702043853	-1.676988782
mmu-miR-142-3p	MIMAT0000155	9.161768446	6.796280308
mmu-miR-142-5p	MIMAT0000154	7.581383502	5.164116308
mmu-miR-143	MIMAT0000247	-1.26427378	-1.733964582
mmu-miR-144	MIMAT0000156	4.120191326	-0.472270581
mmu-miR-145	MIMAT0000157	-0.153210571	-1.147551652
mmu-miR-145*	MIMAT0004534	0.924352865	1.494256717
mmu-miR-146a	MIMAT0000158	3.742598318	3.135766676
mmu-miR-146b	MIMAT0003475	2.639176537	2.881054651
mmu-miR-146b*	MIMAT0004826	-1.710814725	-1.428064795
mmu-miR-147	MIMAT0004857	-0.994965136	-1.098060866
mmu-miR-148a	MIMAT0000516	5.787231851	5.590756677
mmu-miR-148a*	MIMAT0004617	-1.302380866	-1.068369298
mmu-miR-148b	MIMAT0000580	2.725218546	1.768801264
mmu-miR-149	MIMAT0000159	4.409232295	3.358487225
mmu-miR-150	MIMAT0000160	4.125205905	1.282416944
mmu-miR-150*	MIMAT0004535	1.062115295	-2.55866226
mmu-miR-151-3p	MIMAT0000161	-2.002359842	-2.340844057

mmu-miR-151-5p	MIMAT0004536	3.909798203	4.042815192
mmu-miR-152	MIMAT0000162	-0.054644158	1.543063844
mmu-miR-153	MIMAT0000163	-1.468330644	-1.381496025
mmu-miR-154	MIMAT0000164	2.177288744	2.662636656
mmu-miR-154*	MIMAT0004537	1.755273878	2.202125843
mmu-miR-155	MIMAT0000165	4.422819897	5.768249292
mmu-miR-15a	MIMAT0000526	10.23390111	8.651660027
mmu-miR-15a*	MIMAT0004624	1.173171068	1.478342572
mmu-miR-15b	MIMAT0000124	11.47561437	10.28767092
mmu-miR-15b*	MIMAT0004521	2.500218212	2.517997125
mmu-miR-16	MIMAT0000527	9.19874769	7.436168072
mmu-miR-16*	MIMAT0004625	-0.370846328	-0.895292388
mmu-miR-17	MIMAT0000649	3.239623207	3.185039163
mmu-miR-17*	MIMAT0000650	4.412769463	4.836391266
mmu-miR-181a	MIMAT0000210	4.801976077	4.376353131
mmu-miR-181a-1*	MIMAT0000660	2.059528469	2.284338273
mmu-miR-181a-2*	MIMAT0005443	1.743116713	1.685219535
mmu-miR-181b	MIMAT0000673	5.057947445	5.045742966
mmu-miR-181c	MIMAT0000674	4.280591189	5.893362241
mmu-miR-181d	MIMAT0004324	3.294005289	4.175622359
mmu-miR-182	MIMAT0000211	-1.050156563	-1.493859796
mmu-miR-183	MIMAT0000212	0.078256258	-0.225907481
mmu-miR-183*	MIMAT0004539	-1.94483673	-2.530740598
mmu-miR-184	MIMAT0000213	-0.27532556	-1.760953352
mmu-miR-185	MIMAT0000214	3.630088193	1.669398165
mmu-miR-186	MIMAT0000215	2.232988751	2.329861338

mmu-miR-186*	MIMAT0004540	1.75291291	1.710618073
mmu-miR-187	MIMAT0000216	-2.188776372	-2.302653393
mmu-miR-188-3p	MIMAT0004541	-1.400770678	-1.444831809
mmu-miR-188-5p	MIMAT0000217	7.843171881	1.579374152
mmu-miR-18a	MIMAT0000528	7.939678386	7.405437282
mmu-miR-18a*	MIMAT0004626	2.217775477	2.232559728
mmu-miR-18b	MIMAT0004858	0.797327511	0.727389414
mmu-miR-190	MIMAT0000220	0.443652989	1.343690047
mmu-miR-190b	MIMAT0004852	1.110561455	0.954677247
mmu-miR-191	MIMAT0000221	-0.845089515	-1.105883172
mmu-miR-191*	MIMAT0004542	3.452748603	3.169133117
mmu-miR-192	MIMAT0000517	4.675931411	2.95792509
mmu-miR-193	MIMAT0000223	2.782529581	1.569438768
mmu-miR-193*	MIMAT0004544	-2.578498652	-2.953928263
mmu-miR-193b	MIMAT0004859	1.843034952	3.489505936
mmu-miR-194	MIMAT0000224	1.682782517	-0.672953815
mmu-miR-195	MIMAT0000225	2.969137293	1.398178221
mmu-miR-196a	MIMAT0000518	1.879684462	1.968028908
mmu-miR-196a*	MIMAT0004618	-1.645255355	-1.722601402
mmu-miR-196b	MIMAT0001081	3.476265536	7.162135464
mmu-miR-197	MIMAT0004860	2.715058309	2.630409308
mmu-miR-199a-3p	MIMAT0000230	1.840477398	1.430219551
mmu-miR-199a-5p	MIMAT0000229	1.848303397	1.788343381
mmu-miR-199b*	MIMAT0000672	2.350366793	2.855465299
mmu-miR-19a	MIMAT0000651	6.724914206	6.600309357

mmu-miR-19a*	MIMAT0004660	1.633819453	1.553399055
mmu-miR-19b	MIMAT0000513	9.710523812	10.17340111
mmu-miR-200a	MIMAT0000519	-0.301816008	0.122107874
mmu-miR-200a*	MIMAT0004619	-1.147725578	-1.86256567
mmu-miR-200b	MIMAT0000233	0.959697492	0.305107657
mmu-miR-200b*	MIMAT0004545	-1.663379465	-2.504404298
mmu-miR-200c	MIMAT0000657	4.131889218	-1.460538318
mmu-miR-200c*	MIMAT0004663	-0.735794111	-0.1467394
mmu-miR-201	MIMAT0000234	1.703561679	2.088463562
mmu-miR-202-3p	MIMAT0000235	6.687775808	5.501978128
mmu-miR-202-5p	MIMAT0004546	2.640725959	2.8560065
mmu-miR-203	MIMAT0000236	0.491668004	2.085646657
mmu-miR-203*	MIMAT0004547	1.306030495	1.814668824
mmu-miR-204	MIMAT0000237	2.586888005	3.008958105
mmu-miR-205	MIMAT0000238	1.03673243	0.232158035
mmu-miR-206	MIMAT0000239	1.387947102	-2.572455723
mmu-miR-207	MIMAT0000240	2.733329261	2.307126513
mmu-miR-208a	MIMAT0000520	-1.509493029	-1.712806626
mmu-miR-208b	MIMAT0004939	-0.855139847	-2.000846052
mmu-miR-20a	MIMAT0000529	10.36877283	10.69474225
mmu-miR-20a*	MIMAT0004627	0.599184036	1.541637123
mmu-miR-20b	MIMAT0003187	8.332200953	8.544882975
mmu-miR-20b*	MIMAT0004788	-1.046388241	-1.046779961
mmu-miR-21	MIMAT0000530	9.898689484	9.582774789
mmu-miR-21*	MIMAT0004628	0.075141825	-1.58768218
mmu-miR-210	MIMAT0000658	4.207419745	1.426015712

mmu-miR-211	MIMAT0000668	2.978999696	3.35678187
mmu-miR-212	MIMAT0000659	6.049774949	4.863992782
mmu-miR-214	MIMAT0000661	-1.88234125	-2.511237698
mmu-miR-214*	MIMAT0004664	0.265593297	0.634005822
mmu-miR-215	MIMAT0000904	1.006194936	0.405080732
mmu-miR-216a	MIMAT0000662	-1.444438118	-1.939866315
mmu-miR-216b	MIMAT0003729	-2.454137681	-2.552058149
mmu-miR-217	MIMAT0000679	-2.410287725	-2.765869889
mmu-miR-218	MIMAT0000663	1.116285369	1.159395757
mmu-miR-218-1*	MIMAT0004665	0.171686192	-0.119379179
mmu-miR-218-2*	MIMAT0005444	-1.556523082	-2.117326656
mmu-miR-219	MIMAT0000664	2.943723986	2.390491951
mmu-miR-22	MIMAT0000531	7.434630287	4.776519621
mmu-miR-22*	MIMAT0004629	1.296062306	0.840833637
mmu-miR-220	MIMAT0004863	-1.231172448	-0.7506906
mmu-miR-221	MIMAT0000669	4.261505693	4.981167266
mmu-miR-222	MIMAT0000670	2.039909462	3.010867136
mmu-miR-223	MIMAT0000665	12.50118568	9.443498237
mmu-miR-224	MIMAT0000671	1.132369414	1.734184404
mmu-miR-23a	MIMAT0000532	7.522132996	6.510298105
mmu-miR-23b	MIMAT0000125	5.902042877	5.943614826
mmu-miR-24	MIMAT0000219	7.981965747	7.702525826
mmu-miR-24-1*	MIMAT0000218	1.981751982	2.075154364
mmu-miR-24-2*	MIMAT0005440	-1.289708343	-1.661816579
mmu-miR-25	MIMAT0000652	8.569601536	8.054393963
mmu-miR-26a	MIMAT0000533	7.437286973	6.922694833

mmu-miR-26b	MIMAT0000534	6.404881732	5.880268364
mmu-miR-26b*	MIMAT0004630	2.159684471	2.338060832
mmu-miR-27a	MIMAT0000537	8.34395871	7.147290458
mmu-miR-27a*	MIMAT0004633	-1.327779917	-1.362619053
mmu-miR-27b	MIMAT0000126	4.869923068	4.401926709
mmu-miR-27b*	MIMAT0004522	-1.65399201	-1.515242407
mmu-miR-28	MIMAT0000653	3.728616144	3.385467861
mmu-miR-28*	MIMAT0004661	-0.25686275	-2.487928105
mmu-miR-290-3p	MIMAT0004572	3.298101834	2.858803147
mmu-miR-290-5p	MIMAT0000366	2.99444746	0.93436425
mmu-miR-291a-3p	MIMAT0000368	2.267744877	2.771238707
mmu-miR-291a-5p	MIMAT0000367	0.669770001	-1.565233523
mmu-miR-291b-3p	MIMAT0003190	2.21722441	2.719901416
mmu-miR-291b-5p	MIMAT0003189	2.306892202	1.389820097
mmu-miR-292-3p	MIMAT0000370	0.522906183	1.061132635
mmu-miR-292-5p	MIMAT0000369	1.057076981	-1.043947967
mmu-miR-293	MIMAT0000371	-0.22920919	0.222917244
mmu-miR-293*	MIMAT0004573	1.169131697	1.394372936
mmu-miR-294	MIMAT0000372	2.778531971	2.929921536
mmu-miR-294*	MIMAT0004574	0.020834441	-1.521020443
mmu-miR-295	MIMAT0000373	2.290450521	2.762753077
mmu-miR-295*	MIMAT0004575	-1.07261849	-1.829930058
mmu-miR-296-3p	MIMAT0004576	-1.750107888	-2.614857506
mmu-miR-296-5p	MIMAT0000374	7.071013822	4.492672982

mmu-miR-297a*	MIMAT0004864	0.957832791	0.773661326
mmu-miR-297b-5p	MIMAT0003480	-0.927298172	-1.230789068
mmu-miR-297c	MIMAT0004865	1.475204114	1.48071467
mmu-miR-298	MIMAT0000376	2.520554011	2.122699227
mmu-miR-299	MIMAT0004577	-2.059632344	-2.185803058
mmu-miR-299*	MIMAT0000377	2.142223924	2.289863617
mmu-miR-29a	MIMAT0000535	9.683604277	9.43547056
mmu-miR-29a*	MIMAT0004631	2.221658351	2.416427941
mmu-miR-29b	MIMAT0000127	6.465729808	6.391739525
mmu-miR-29b*	MIMAT0004523	1.491287588	1.461550223
mmu-miR-29c	MIMAT0000536	6.347390129	5.316474604
mmu-miR-29c*	MIMAT0004632	2.865640841	2.683015141
mmu-miR-300	MIMAT0000378	0.505734222	-0.239365496
mmu-miR-300*	MIMAT0004578	0.133684087	-0.086570165
mmu-miR-301a	MIMAT0000379	5.729233319	4.263606223
mmu-miR-301b	MIMAT0004186	1.136619573	1.100979145
mmu-miR-302a	MIMAT0000380	2.153555029	2.436370949
mmu-miR-302a*	MIMAT0004579	0.065564738	0.608842304
mmu-miR-302b	MIMAT0003374	2.300635929	2.223797035
mmu-miR-302b*	MIMAT0003373	-0.186369499	0.113240479
mmu-miR-302c	MIMAT0003376	1.693402104	2.031934025
mmu-miR-302c*	MIMAT0003375	-1.966154934	-1.938587373
mmu-miR-302d	MIMAT0003377	1.041903257	1.744703134
mmu-miR-30a	MIMAT0000128	4.699643595	4.384524502
mmu-miR-30a*	MIMAT0000129	-0.749010286	-0.434192407

mmu-miR-30b	MIMAT0000130	4.541239815	4.165520656
mmu-miR-30b*	MIMAT0004524	3.754281743	3.209747541
mmu-miR-30c	MIMAT0000514	4.817840148	4.770854246
mmu-miR-30c-1*	MIMAT0004616	0.688556741	-0.758332184
mmu-miR-30c-2*	MIMAT0005438	1.886562666	-1.18036058
mmu-miR-30d	MIMAT0000515	6.71978252	5.876613778
mmu-miR-30e	MIMAT0000248	6.042287113	5.373582361
mmu-miR-30e*	MIMAT0000249	1.527931525	1.208410627
mmu-miR-31	MIMAT0000538	-0.517818571	1.576330623
mmu-miR-31*	MIMAT0004634	1.118993532	1.825989417
mmu-miR-32	MIMAT0000654	1.470984924	1.956119872
mmu-miR-320	MIMAT0000666	4.063629973	0.581128823
mmu-miR-322	MIMAT0000548	7.076123846	7.66167582
mmu-miR-322*	MIMAT0000549	-1.196525163	-1.300491042
mmu-miR-323-3p	MIMAT0000551	1.206719081	1.01570116
mmu-miR-323-5p	MIMAT0004638	-2.234566894	-2.407612056
mmu-miR-324-3p	MIMAT0000556	3.180002342	2.988025597
mmu-miR-324-5p	MIMAT0000555	4.292625136	4.274899681
mmu-miR-325	MIMAT0004640	0.768033811	0.266307619
mmu-miR-325*	MIMAT0000558	-1.74761927	-1.654879197
mmu-miR-326	MIMAT0000559	2.788969138	2.972614248
mmu-miR-327	MIMAT0004867	4.01313231	-2.943221731
mmu-miR-328	MIMAT0000565	2.922188693	3.372306431
mmu-miR-329	MIMAT0000567	2.66925663	2.616411319
mmu-miR-33	MIMAT0000667	3.336779161	3.292651586
mmu-miR-33*	MIMAT0004666	-0.127515718	-0.667332308

mmu-miR-330	MIMAT0004642	-0.961083432	-1.352853783
mmu-miR-330*	MIMAT0000569	-0.064296153	-1.950341535
mmu-miR-331-3p	MIMAT0000571	4.869501564	4.654473368
mmu-miR-331-5p	MIMAT0004643	-1.12037785	-1.924076214
mmu-miR-335-3p	MIMAT0004704	3.040406892	3.00440183
mmu-miR-335-5p	MIMAT0000766	-1.90264476	-1.930265935
mmu-miR-337-3p	MIMAT0000578	1.617907362	1.758553047
mmu-miR-337-5p	MIMAT0004644	-1.758229153	-1.921587586
mmu-miR-338-3p	MIMAT0000582	4.083145513	2.159044868
mmu-miR-338-5p	MIMAT0004647	0.579561288	-1.250811373
mmu-miR-339-3p	MIMAT0004649	-1.771383779	-2.484097372
mmu-miR-339-5p	MIMAT0000584	-1.789969543	-2.497892065
mmu-miR-340-3p	MIMAT0000586	4.103118032	3.46300419
mmu-miR-340-5p	MIMAT0004651	4.256147243	2.534558017
mmu-miR-341	MIMAT0000588	3.718084395	-1.918968957
mmu-miR-342-3p	MIMAT0000590	7.990586605	5.231568983
mmu-miR-342-5p	MIMAT0004653	0.943001627	-0.626352469
mmu-miR-343	MIMAT0004868	1.352476363	0.910744544
mmu-miR-344	MIMAT0000593	-0.873271078	-1.155110301
mmu-miR-345-3p	MIMAT0004656	-0.129699546	-2.909411968
mmu-miR-345-5p	MIMAT0000595	4.526128484	4.148004071
mmu-miR-346	MIMAT0000597	0.150218706	-0.85608024
mmu-miR-34a	MIMAT0000542	6.114063807	5.801020131
mmu-miR-34b-3p	MIMAT0004581	0.597539522	1.198774658
mmu-miR-34b-5p	MIMAT0000382	1.381707917	0.883363401
mmu-miR-34c	MIMAT0000381	0.243985027	0.117839131

mmu-miR-34c*	MIMAT0004580	-0.693125968	-1.24480953
mmu-miR-350	MIMAT0000605	4.826754759	3.813329761
mmu-miR-351	MIMAT0000609	0.672085619	0.698779389
mmu-miR-361	MIMAT0000704	6.373503853	4.910143779
mmu-miR-362-3p	MIMAT0004684	4.629616935	3.834230842
mmu-miR-362-5p	MIMAT0000706	0.496724185	-1.983564586
mmu-miR-363	MIMAT0000708	2.833489847	2.548691952
mmu-miR-365	MIMAT0000711	3.541256436	3.226034253
mmu-miR-367	MIMAT0003181	2.057235684	2.150545508
mmu-miR-369-3p	MIMAT0003186	1.422130781	1.238364289
mmu-miR-369-5p	MIMAT0003185	1.933147912	2.364489982
mmu-miR-370	MIMAT0001095	6.787242731	-1.500324573
mmu-miR-374	MIMAT0003727	2.85414028	2.954729655
mmu-miR-374*	MIMAT0003728	1.212679725	1.634194605
mmu-miR-375	MIMAT0000739	-1.202063908	-1.342107049
mmu-miR-376a	MIMAT0000740	-1.976262496	-2.175824022
mmu-miR-376a*	MIMAT0003387	1.981685648	1.759241273
mmu-miR-376b	MIMAT0001092	-0.92207293	-1.116804283
mmu-miR-376b*	MIMAT0003388	1.890961488	1.950129725
mmu-miR-376c	MIMAT0003183	-0.689595781	-0.526097376
mmu-miR-376c*	MIMAT0005295	1.701197374	1.82372057
mmu-miR-377	MIMAT0000741	1.148312181	1.069113678
mmu-miR-378	MIMAT0003151	3.232228115	1.92641787
mmu-miR-378*	MIMAT0000742	1.879463913	1.998036894
mmu-miR-379	MIMAT0000743	-1.885251535	-1.986326047
mmu-miR-380-3p	MIMAT0000745	1.650270235	1.442055047

mmu-miR-380-5p	MIMAT0000744	-1.073629112	-1.68954064
mmu-miR-381	MIMAT0000746	-0.660974513	-0.804802988
mmu-miR-382	MIMAT0000747	-0.485197893	-0.97553247
mmu-miR-382*	MIMAT0004691	2.268506147	2.685944565
mmu-miR-383	MIMAT0000748	-1.629670515	-2.088843579
mmu-miR-384-3p	MIMAT0001076	1.802313222	1.1779027
mmu-miR-384-5p	MIMAT0004745	0.619398332	1.142050841
mmu-miR-409-3p	MIMAT0001090	-0.059173823	-0.357383475
mmu-miR-409-5p	MIMAT0004746	1.757754085	1.512966747
mmu-miR-410	MIMAT0001091	-0.318842633	0.585768695
mmu-miR-411	MIMAT0004747	-1.139840596	-0.995712884
mmu-miR-411*	MIMAT0001093	0.725737201	1.227354101
mmu-miR-412	MIMAT0001094	-1.103607361	-0.982302846
mmu-miR-421	MIMAT0004869	1.887362208	0.605019628
mmu-miR-423-3p	MIMAT0003454	-0.776151839	-1.868308923
mmu-miR-423-5p	MIMAT0004825	6.194049441	5.236998224
mmu-miR-425	MIMAT0004750	6.377121411	6.016649306
mmu-miR-425*	MIMAT0001342	-0.049989842	-0.055794443
mmu-miR-429	MIMAT0001537	1.871783878	1.988485624
mmu-miR-431	MIMAT0001418	-1.560844517	-1.292709481
mmu-miR-431*	MIMAT0004753	0.948625779	0.712166411
mmu-miR-433	MIMAT0001420	-1.473296767	-1.851602188
mmu-miR-433*	MIMAT0001419	1.494513346	1.667710392
mmu-miR-434-3p	MIMAT0001422	2.040616439	2.155893125
mmu-miR-434-5p	MIMAT0001421	0.558199756	0.286247162
mmu-miR-448	MIMAT0001533	0.462340558	-0.450014378

mmu-miR-449a	MIMAT0001542	0.685271571	0.78803702
mmu-miR-449b	MIMAT0005447	-0.60450622	-0.96540453
mmu-miR-449c	MIMAT0003460	-0.978285465	-1.173659785
mmu-miR-450a-3p	MIMAT0004789	1.821344374	0.779719862
mmu-miR-450a-5p	MIMAT0001546	2.11821949	2.617587165
mmu-miR-450b-3p	MIMAT0003512	1.358551759	1.508485551
mmu-miR-450b-5p	MIMAT0003511	1.651182313	1.808247237
mmu-miR-451	MIMAT0001632	7.71938396	3.061462669
mmu-miR-452	MIMAT0001637	4.110342831	-2.047833805
mmu-miR-453	MIMAT0004870	-0.450543831	-0.251091746
mmu-miR-455	MIMAT0003742	-0.212877633	-1.361733274
mmu-miR-455*	MIMAT0003485	1.204019058	1.309826675
mmu-miR-463	MIMAT0004758	-0.798814279	-1.043753736
mmu-miR-463*	MIMAT0002104	2.521524498	2.657769524
mmu-miR-464	MIMAT0002105	1.746393436	1.947614051
mmu-miR-465a-3p	MIMAT0004217	-0.066952062	0.215537754
mmu-miR-465a-5p	MIMAT0002106	-2.106553585	-2.375916786
mmu-miR-465b-5p	MIMAT0004871	-0.878803691	-1.475727464
mmu-miR-465c-5p	MIMAT0004873	-1.758387561	-1.779064239
mmu-miR-466a-3p	MIMAT0002107	0.902656131	0.816782749
mmu-miR-466a-	MIMAT0004759	-0.49565446	-0.482459958

5p			
mmu-miR-466b-5p	MIMAT0004875	-0.928895466	-0.987271867
mmu-miR-466c-5p	MIMAT0004877	3.423269611	1.988355934
mmu-miR-466d-3p	MIMAT0004931	-1.132151308	-1.077176029
mmu-miR-466d-5p	MIMAT0004930	-1.867320825	-1.880745326
mmu-miR-466f-3p	MIMAT0004882	1.563923008	1.373608292
mmu-miR-466f-5p	MIMAT0004881	-1.067558856	-1.650000682
mmu-miR-466g	MIMAT0004883	-0.552965501	-0.294476078
mmu-miR-466h	MIMAT0004884	1.15661067	0.407530664
mmu-miR-467a	MIMAT0003409	1.836333002	1.427897647
mmu-miR-467a*	MIMAT0002108	2.025403395	1.385790226
mmu-miR-467b	MIMAT0005448	2.315793721	2.167885533
mmu-miR-467b*	MIMAT0003478	0.465266856	-0.285644099
mmu-miR-467c	MIMAT0004885	1.771954119	1.63963448
mmu-miR-467d	MIMAT0004886	-1.138267545	-1.592034643
mmu-miR-467e	MIMAT0005293	-0.028176294	0.426007032
mmu-miR-467e*	MIMAT0005294	1.831928249	2.096003299
mmu-miR-468	MIMAT0002109	2.692323328	0.526672911
mmu-miR-469	MIMAT0002110	2.942035607	3.215554929
mmu-miR-470	MIMAT0002111	-1.423843237	-1.750720359
mmu-miR-470*	MIMAT0004760	1.614855034	1.864640269
mmu-miR-471	MIMAT0002112	1.645279907	1.636128189
mmu-miR-483	MIMAT0004782	6.51562285	-2.447984229
mmu-miR-483*	MIMAT0003120	3.75005955	3.188756058

mmu-miR-484	MIMAT0003127	3.205766806	2.320316803
mmu-miR-485	MIMAT0003128	-0.689757057	-0.349758767
mmu-miR-485*	MIMAT0003129	1.179976387	0.559317932
mmu-miR-486	MIMAT0003130	4.859086533	-0.80771241
mmu-miR-487b	MIMAT0003184	1.86898684	1.44175839
mmu-miR-488	MIMAT0003450	-0.05942876	0.56253181
mmu-miR-488*	MIMAT0003449	0.097308517	-0.220885905
mmu-miR-489	MIMAT0003112	-0.735539623	-0.925805061
mmu-miR-490	MIMAT0003780	-0.382038408	-1.657148105
mmu-miR-491	MIMAT0003486	-0.878045761	-0.951501854
mmu-miR-493	MIMAT0004888	-1.071972588	-1.966223199
mmu-miR-494	MIMAT0003182	10.10949515	5.987053374
mmu-miR-495	MIMAT0003456	1.543038523	1.616498479
mmu-miR-496	MIMAT0003738	0.533893282	0.686751906
mmu-miR-497	MIMAT0003453	3.155951215	1.579564888
mmu-miR-499	MIMAT0003482	0.54593222	0.563951662
mmu-miR-500	MIMAT0003507	5.720212156	4.536301688
mmu-miR-501-3p	MIMAT0003509	-2.131802467	-2.388983559
mmu-miR-501-5p	MIMAT0003508	-1.532709532	-2.071493069
mmu-miR-503	MIMAT0003188	3.93569992	3.356310351
mmu-miR-503*	MIMAT0004790	-0.509033262	-1.298251089
mmu-miR-504	MIMAT0004889	0.006892847	0.316355261
mmu-miR-505	MIMAT0003513	2.544898372	2.492359424
mmu-miR-509-3p	MIMAT0004891	1.275781545	1.640938677
mmu-miR-509-5p	MIMAT0004890	-1.349072372	-0.84062569
mmu-miR-511	MIMAT0004940	2.465797589	2.543205161

mmu-miR-532-3p	MIMAT0004781	0.041117084	-0.754167353
mmu-miR-532-5p	MIMAT0002889	3.982559465	2.680791699
mmu-miR-539	MIMAT0003169	0.613160326	0.996872391
mmu-miR-540-3p	MIMAT0003167	-1.847152893	-2.244477781
mmu-miR-540-5p	MIMAT0004786	1.407910865	1.220692805
mmu-miR-541	MIMAT0003170	-0.145110458	0.947450677
mmu-miR-542-3p	MIMAT0003172	1.129246693	2.613189074
mmu-miR-542-5p	MIMAT0003171	3.211739149	5.086370973
mmu-miR-543	MIMAT0003168	1.865024892	2.297631033
mmu-miR-544	MIMAT0004941	2.354578244	2.779898184
mmu-miR-546	MIMAT0003166	4.869891558	-2.496529131
mmu-miR-547	MIMAT0003173	-1.192068874	-0.529504421
mmu-miR-551b	MIMAT0003890	2.229749041	1.674180581
mmu-miR-568	MIMAT0004892	-0.051709285	0.129960117
mmu-miR-574-3p	MIMAT0004894	0.436730115	0.350750089
mmu-miR-574-5p	MIMAT0004893	8.104836175	4.632877086
mmu-miR-582-3p	MIMAT0005292	-1.062211027	-1.568540049
mmu-miR-582-5p	MIMAT0005291	2.148531164	2.399912573
mmu-miR-590-3p	MIMAT0004896	0.947696475	1.592731227
mmu-miR-590-5p	MIMAT0004895	0.898244503	1.057822295
mmu-miR-592	MIMAT0003730	1.646095146	-0.408239422
mmu-miR-598	MIMAT0004942	1.694893349	2.066414835
mmu-miR-615-3p	MIMAT0003783	0.995151624	0.757380504
mmu-miR-615-5p	MIMAT0004837	-1.926719049	-2.013241824
mmu-miR-652	MIMAT0003711	7.600822806	6.608058956
mmu-miR-653	MIMAT0004943	0.202331488	0.808710774

mmu-miR-654-3p	MIMAT0004898	1.114021548	0.999129498
mmu-miR-654-5p	MIMAT0004897	-1.364372899	-1.539048079
mmu-miR-665	MIMAT0003733	-1.113123435	-2.31102536
mmu-miR-666-3p	MIMAT0004823	-1.254053829	-0.884049522
mmu-miR-666-5p	MIMAT0003737	0.387382856	-1.902979354
mmu-miR-667	MIMAT0003734	0.820572937	-1.300495475
mmu-miR-668	MIMAT0003732	0.80595443	0.477230019
mmu-miR-669a	MIMAT0003477	3.222134735	2.802570483
mmu-miR-669b	MIMAT0003476	0.272808302	-1.872514343
mmu-miR-669c	MIMAT0003479	4.100681658	1.911714977
mmu-miR-670	MIMAT0003736	-1.556016837	-2.323365098
mmu-miR-671-3p	MIMAT0004821	-0.444147193	-1.557061511
mmu-miR-671-5p	MIMAT0003731	7.34674841	1.469139308
mmu-miR-672	MIMAT0003735	0.689332917	-1.747640427
mmu-miR-673-3p	MIMAT0004824	0.446844117	-0.257923679
mmu-miR-673-5p	MIMAT0003739	-0.945490272	-1.286728997
mmu-miR-674	MIMAT0003740	1.46456685	-2.835283756
mmu-miR-674*	MIMAT0003741	2.929659873	2.994063232
mmu-miR-675-3p	MIMAT0003726	0.236036424	0.723499632
mmu-miR-675-5p	MIMAT0003725	-1.980950405	-2.388535612
mmu-miR-676	MIMAT0003782	0.224920134	0.317183772
mmu-miR-676*	MIMAT0003781	-0.053944537	0.120534364
mmu-miR-677	MIMAT0003451	1.201898173	0.631407583
mmu-miR-678	MIMAT0003452	4.240000754	-1.987599011
mmu-miR-679	MIMAT0003455	-1.666960065	-1.759088458
mmu-miR-680	MIMAT0003457	5.093257629	-1.49429763

mmu-miR-681	MIMAT0003458	2.971135575	-1.967573056
mmu-miR-682	MIMAT0003459	0.873528133	-1.556447827
mmu-miR-683	MIMAT0003461	-0.48058979	-0.65310516
mmu-miR-684	MIMAT0003462	1.221700059	2.056500506
mmu-miR-685	MIMAT0003463	2.49452262	-2.615671678
mmu-miR-686	MIMAT0003464	-2.152071139	-2.774343134
mmu-miR-687	MIMAT0003466	-2.167599964	-2.507442682
mmu-miR-688	MIMAT0003467	0.258823457	0.596369031
mmu-miR-689	MIMAT0003468	9.726739807	5.210869472
mmu-miR-690	MIMAT0003469	5.420678522	3.407647344
mmu-miR-691	MIMAT0003470	0.70617163	-2.809452262
mmu-miR-692	MIMAT0003471	-0.490315993	-0.823923112
mmu-miR-693-3p	MIMAT0004189	-2.08977265	-2.063471207
mmu-miR-693-5p	MIMAT0003472	1.426767337	1.701489791
mmu-miR-694	MIMAT0003474	-1.582511905	-1.629072805
mmu-miR-695	MIMAT0003481	-2.34402177	-2.501798323
mmu-miR-696	MIMAT0003483	0.044165587	-1.570295187
mmu-miR-697	MIMAT0003487	4.715060256	3.178714499
mmu-miR-698	MIMAT0003488	2.102187095	2.610073065
mmu-miR-699	MIMAT0003489	-0.805705312	-1.413578357
mmu-miR-700	MIMAT0003490	1.603788317	-0.067643873
mmu-miR-701	MIMAT0003491	-2.239005445	-2.483786954
mmu-miR-702	MIMAT0003492	3.445071146	3.386831197
mmu-miR-703	MIMAT0003493	-1.070600626	-1.667620425
mmu-miR-704	MIMAT0003494	-0.030358196	-0.372394071
mmu-miR-705	MIMAT0003495	7.412657583	-0.751162155

mmu-miR-706	MIMAT0003496	3.136663208	1.119140491
mmu-miR-707	MIMAT0003497	-1.14080843	-0.688497804
mmu-miR-708	MIMAT0004828	-0.518290539	-1.602512569
mmu-miR-708*	MIMAT0003498	-0.681155897	-1.211122556
mmu-miR-709	MIMAT0003499	11.2903681	8.837902863
mmu-miR-710	MIMAT0003500	3.116663449	-2.942451696
mmu-miR-711	MIMAT0003501	2.090303443	-3.01830248
mmu-miR-712	MIMAT0003502	5.66109448	2.949098681
mmu-miR-712*	MIMAT0003743	4.44497163	-2.133835117
mmu-miR-713	MIMAT0003504	-2.348685259	-2.37520349
mmu-miR-714	MIMAT0003505	9.406839601	4.176204167
mmu-miR-715	MIMAT0003506	4.542114524	1.315087414
mmu-miR-717	MIMAT0003510	0.056142713	-0.097478839
mmu-miR-718	MIMAT0003514	0.712693786	-2.357071903
mmu-miR-719	MIMAT0003465	-0.977779468	-1.360571704
mmu-miR-720	MIMAT0003484	11.04883202	11.09337948
mmu-miR-721	MIMAT0003515	8.290759783	4.560899922
mmu-miR-741	MIMAT0004236	-0.241401107	0.31881022
mmu-miR-742	MIMAT0004237	-1.674106656	-1.413448489
mmu-miR-742*	MIMAT0004838	0.333766293	0.516142371
mmu-miR-743a	MIMAT0004238	-1.487059605	-2.001447034
mmu-miR-743b-3p	MIMAT0004840	0.010306123	-0.475734955
mmu-miR-743b-5p	MIMAT0004839	-1.091499999	-1.161749258
mmu-miR-744	MIMAT0004187	-2.159191948	-2.283935697
mmu-miR-744*	MIMAT0004820	1.888058504	1.571540064

mmu-miR-758	MIMAT0003889	-0.909594115	-0.973196472
mmu-miR-759	MIMAT0003897	0.881543937	0.127403106
mmu-miR-760	MIMAT0003898	4.666251402	-0.023303787
mmu-miR-761	MIMAT0003893	-2.087055121	-2.226832348
mmu-miR-762	MIMAT0003892	3.783791091	-2.74846252
mmu-miR-763	MIMAT0003896	-2.198099075	-2.457531752
mmu-miR-764-3p	MIMAT0003895	-1.192264167	-1.230734065
mmu-miR-764-5p	MIMAT0003894	1.159029667	1.29997425
mmu-miR-770-3p	MIMAT0003891	3.674632664	-2.60238728
mmu-miR-770-5p	MIMAT0004822	-1.538385739	-2.106602075
mmu-miR-7a	MIMAT0000677	2.455965976	1.661881292
mmu-miR-7a*	MIMAT0004670	1.946233186	2.15315134
mmu-miR-7b	MIMAT0000678	1.216867688	1.459788143
mmu-miR-801_v10.1	NA	8.71539989	2.585106601
mmu-miR-802	MIMAT0004188	1.433736656	1.343706708
mmu-miR-804	MIMAT0004210	1.46402044	1.344489583
mmu-miR-805	MIMAT0004211	-2.121875539	-2.498902496
mmu-miR-871	MIMAT0004841	-0.044368961	-0.733110154
mmu-miR-872	MIMAT0004934	2.128865836	2.543765753
mmu-miR-872*	MIMAT0004935	0.380951621	0.70159221
mmu-miR-873	MIMAT0004936	0.975218191	0.955851275
mmu-miR-874	MIMAT0004853	3.730630464	-0.099422265
mmu-miR-875-3p	MIMAT0004938	-1.403211621	-1.85224945
mmu-miR-875-5p	MIMAT0004937	2.027193548	1.927198946
mmu-miR-876-3p	MIMAT0004855	-0.193717862	0.323874124

mmu-miR-876-5p	MIMAT0004854	1.628394516	2.118435435
mmu-miR-877	MIMAT0004861	5.035930978	0.364372925
mmu-miR-877*	MIMAT0004862	3.605165028	3.396426006
mmu-miR-878-3p	MIMAT0004933	2.817789671	0.957953104
mmu-miR-878-5p	MIMAT0004932	-1.350981586	-1.729419693
mmu-miR-879	MIMAT0004842	0.209437555	0.13780115
mmu-miR-879*	MIMAT0004843	-0.05532006	0.514547868
mmu-miR-880	MIMAT0004844	1.817568569	1.519679311
mmu-miR-881	MIMAT0004846	2.017091911	2.303689989
mmu-miR-881*	MIMAT0004845	-0.869948224	-0.548231101
mmu-miR-882	MIMAT0004847	-1.364515877	-1.521337183
mmu-miR-883a-3p	MIMAT0004849	1.097572712	1.664724891
mmu-miR-883a-5p	MIMAT0004848	-2.108245969	-2.445372177
mmu-miR-883b-3p	MIMAT0004851	1.606834715	2.270051144
mmu-miR-883b-5p	MIMAT0004850	-2.180778495	-2.148255735
mmu-miR-9	MIMAT0000142	-0.376366927	-0.396352929
mmu-miR-9*	MIMAT0000143	-0.680622327	-0.693802823
mmu-miR-92a	MIMAT0000539	10.13288125	10.75573482
mmu-miR-92a*	MIMAT0004635	-0.850713721	-1.556307971
mmu-miR-92b	MIMAT0004899	1.48953729	1.505732696
mmu-miR-93	MIMAT0000540	7.381950317	6.531666564
mmu-miR-93*	MIMAT0004636	0.075135915	0.266502674
mmu-miR-96	MIMAT0000541	2.157092901	2.27894918
mmu-miR-98	MIMAT0000545	4.309986394	3.981152229

mmu-miR-99a	MIMAT0000131	4.114316795	7.134121736
mmu-miR-99b	MIMAT0000132	-0.503073082	1.139445639
mmu-miR-99b*	MIMAT0004525	2.566953382	-1.780003374

Table S2. Antibodies for Flow Cytometry and Western Blotting

Target Antigen	Antibody Clone
Gr1 (Ly-6G)	RB6-8C5
CD11b	M1/70
Ter-119	TER-119
B220 (CD45R)	RA3-6B2
CD45.1	A20
CD45.2	104
cKit	2B8
Sca1 (Ly6A/E)	D7
CD150	TC15-12F12.2
CD48	HM48-1
EPCR (CD201)	eBio1560
CD4 (L3T4)	GK1.5
CD8a (Ly-2)	53-6.7
CD11c (p150/90)	N418
NK-1.1 (Ly-55)	PK136
CD3e	145-2C11
CD19	MB19-1

Table S3. Oligonucleotide and PCR Primer Sequences

Primer	Sequence
miR-125a 155-formatted template	GAAGGCTGTATGCTGTCCCTGAGACCCTTTAACCTGTGAGTTTTGGCCACTGACTGACTCACAGGTAAGGGTCTCAGGGACAGGACACAAGGCCTG
miR-125b 155-formatted template	GAAGGCTGTATGCTGTCCCTGAGACCCTAACTTGTGAGTTTTGGCCACTGACTGACTCACAAGTGGGTCTCAGGGACAGGACACAAGGCCTG
miR-155 155-formatted template	GAAGGCTGTATGCTGTTAATGCTAATTGTGATAGGGGTTTTGGCCACTGACTGACCCCTATCAATTAGCATTAACAGGACACAAGGCCTG
miR-99a 155-formatted template	GAAGGCTGTATGCTGAACCCGTAGATCCGATCTTGTGGTTTTGGCCACTGACTGACCACAAGATGATCTACGGGTTCAGGACACAAGGCCTG
miR-126 155-formatted template	GAAGGCTGTATGCTGCATTATTACTTTTGGTACGCGGTTTTGGCCACTGACTGACCGCGTACCAAGTAATAATGCAGGACACAAGGCCTG
miR-196b 155-formatted template	GAAGGCTGTATGCTGTAGGTAGTTTCCTGTTGTTGGGGTTTTGGCCACTGACTGACCCCAACAAGGAAACTACCTACAGGACACAAGGCCTG
miR-130a 155-formatted template	GAAGGCTGTATGCTGCAGTGCAATGTTAAAAGGGCATGTTTTGGCCACTGACTGACATGCCCTTAACATTGCACTGCAGGACACAAGGCCTG
miR-542-5p 155-formatted template	GAAGGCTGTATGCTGCTCGGGGATCATCATGTCACGAGTTTTGGCCACTGACTGACTCGTGACAATGATCCCCGAGCAGGACACAAGGCCTG
miR-181c 155-formatted template	GAAGGCTGTATGCTGAACATTCAACCTGTCGGTGAGTGTTTTGGCCACTGACTGACACTCACCGAGGTTGAATGTTCAGGACACAAGGCCTG
miR-193b 155-formatted template	GAAGGCTGTATGCTGAACTGGCCCACAAAGTCCCGCTGTTTTGGCCACTGACTGACAGCGGGACTGTGGGCCAGTTCAGGACACAAGGCCTG
let7e 155-formatted template	GAAGGCTGTATGCTGTGAGGTAGGAGGTTGTATAGTTGTTTTGGCCACTGACTGACAACTATACCCTCCTACCTCACAGGACACAAGGCCTG
miR-125b-1 NotI Fw	TTCGCGGCCGCGAGTTTTCTCTGATGTACTCGTGATCGTATGT
miR-125b-1 XhoI Rev	TTCCTCGAGAACAGAAATCCAGGAGCTGCCACTC
miR-125b-2 NotI Fw	TTCGCGGCCGCGCCCTTGCTAGCGAAGCAGATTTT
miR125b-2 XhoI Rev	TTCCTCGAGAGTATTTTTGGGGATGGGTCATGGTG

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Chapter 5: MiR-125b potentiates macrophage activation

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Abstract

MicroRNA-125b expression is modulated in macrophages in response to stimulatory cues. Here we report a functional role of miR-125b in macrophages. We found that miR-125b is enriched in macrophages compared to lymphoid cells and whole immune tissues. Enforced expression of miR-125b drives macrophages to adapt an activated morphology that is accompanied by increased co-stimulatory factor expression and elevated responsiveness to interferon gamma, while anti-miR-125b treatment decreases CD80 surface expression. To determine whether these alterations in cell signaling, gene expression and morphology have functional consequences, we examined the ability of macrophages with enhanced miR-125b expression to present antigens and found that they better stimulate T cell activation than control macrophages. Further indicating increased function, these macrophages were more effective at killing EL4 tumor cells in vitro and in vivo. Moreover, miR-125b repressed IRF4 and IRF4 knockdown in macrophages mimicked the miR-125b overexpression phenotype. In summary, our evidence suggests that miR-125b is at least partly responsible for generating the activated nature of macrophages, at least partially by reducing IRF4 levels, and potentiates the functional role of macrophages in inducing immune responses.

Introduction

The mammalian innate immune system provides a critical first line of defense against pathogens. Macrophages are key components of this system, acting to release cytokines, kill pathogens directly and present antigens to the adaptive immune system. The macrophage surface contains sensing proteins, like Toll-like receptors and the interferon gamma receptor that, when engaged, lead to a rapid differentiation event termed “activation”, where the cell transforms from relative quiescence to an effector state characterized by far-heightened microbicidal ability. Macrophages also carry co-stimulatory proteins such as CD80 and CD86 for interacting with T cells, thus bridging innate immunity to adaptive immunity [1,2].

Recently microRNAs have been shown to be important mediators of the macrophage activation process. MicroRNAs-155, 146, 147, 9 and 21 are induced by ligands of the Toll-like receptors (TLRs) [3,4]. These microRNAs, in turn, inhibit expression of protein in the inflammatory signaling cascade, thus modulating immunity through feedback regulation [3,4]. MiR-125b, a homologue of the *C. elegans* microRNA lin-4, has been shown to be decreased in macrophages in response to TLR4 signaling [5-7].

We and others found that miR-125b is enriched in hematopoietic stem cells and that increased miR-125b enhances hematopoietic engraftment [8,9]. Further increased levels of miR-125b cause an aggressive myeloproliferative disorder that leads to leukemia [8]. Here we examine the role miR-125b plays in regulating macrophage activation. We find that macrophages express a particularly high concentration of miR-125b. When miR-125b is overexpressed in macrophages, it enhances surface activation markers both basally and in response to interferon gamma. By contrast, treatment of RAW264.7 macrophages with anti-miR-125b causes them to express decreased surface CD80 both basally and in response to interferon gamma. We demonstrate that

macrophages expressing increased miR-125b become more potent stimulators of immune responses as shown by increased antigen-specific T cell activation and anti-tumor immunity. Lastly, we find that IRF4 is an important target of miR-125b in macrophages and that IRF4 knockdown mimics the miR-125b overexpression phenotype.

Materials and Methods

Cell culture. 293T cells, RAW264.7 cells and BMMs were cultured at 37°C with 5% CO_2 in DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 units/ml streptomycin. For IFNγ treatment, cells were treated overnight with 200 units/ml of recombinant mouse IFNγ (eBioscience).

Mice. C57Bl/6 and OTI Ovalbumin TCR-transgenic Balb/c mice were bred in the Caltech Office of Laboratory Animal Resources (OLAR) facility or purchased from Jackson Laboratories. The Caltech Institutional Animal Care and Use Committee (IACUC) approved all mouse experimental protocols.

Isolation of immune cells and tissues. T cells and B cells were purified from the spleens of C57Bl/6 mice using magnetic beads (Miltenyi). Peritoneal macrophages were isolated four days after injecting mice with 3% Thyoglycollate.

DNA constructs. The MG, MGP, MG-125b-1 and MGP-125b-1 vector systems have been described previously [10-12]. The human miR-125b-1 sequence was also cloned into the pcDNA3 vector downstream of the CMV promoter. The IRF4 shRNA sequence was predicted and cloned into MGP as described previously [11,13]. NC1 is a negative control shRNA sequence predicted not to target any protein coding genes in the mouse genome (Invitrogen). For reporter assays, pMIR-REPORT vector (Ambion) containing Picalm and

Cutl1 3'UTRs were constructed previously [10]. A 3 kb region of the human IRF4 3'UTR, which includes the miR-125b putative binding site, was cloned into pMIR-REPORT downstream of luciferase. A positive control 2-mer containing two tandem sites complementary to miR-125b was also cloned. Primer sequences are listed in Table S1.

Retrovirally transduced bone marrow derived macrophages. To generate retrovirus for infecting bone marrow, 293T cells were transfected with pCL-Eco and MG, MGP, MG-125b-1 or MGP-125b-1 vectors. After 36 h, 10 ug/ml polybrene (Millipore) was added to retrovirus-containing culture supernatant, which was used to spin-infect bone marrow from C57Bl/6 mice. Cells were counted and 1 million were plated per well in a 6 well plate with 10 ng/ml M-CSF (eBioscience), and differentiated for 6 days to yield retrovirally transduced BMMs [12].

Stable cell lines. RAW264.7 cells were stably transduced with VSV-G-pseudotyped MGP or MGP-125b-1 retrovirus and puromycin selection was subsequently performed as described previously [11].

Electroporation of anti-miRs. RAW264.7 cells were co-electroporated with anti-miR-125b or a mismatched control (Regulus Therapeutics) and pmaxGFP vector (Lonza) using an Amaxa Nucleofector. 36 hours post-electroporation, GFP positive cells were analyzed by FACS. Anti-miR-125b or mismatched control compound were chimeric 2′-fluoro/2′-O-methoxyethyl-modified oligonucleotides with a completely modified phosphorothioate backbone [14] (Regulus Therapeutics). The exact chemistry is available on request.

T-cell macrophage co-culture. 50,000 BMMs stably expressing either MG or MG-125b-1 were co-cultured with 150,000 T cells harvested from OTI Ova TCR-transgenic Balb/c mouse in a 48 well flat-bottom plate in the absence or presence of ovalbumin

protein. Flow cytometry and ELISAs to assess T cell activation were performed 72 hours later. ELISAs were performed with an IL-2 detection kit from eBioscience and carried out according to the manufacturer's instructions.

EL4 tumor cell experiments. 500,000 BMMs stably expressing either MG or MG-125b-1 were generated as described above in 6 well plates. One million EL4-Fluc cells were added to each well, supplemented with 20 ng/ml LPS. EL4-Fluc apoptosis was measured 94 hours later by staining cells in suspension with AnnexinV antibody (BD Pharmingen). **For the in vivo experiments**, 2 million EL4-Fluc cells were co-injected with 400,000 BMMs subcutaneously into albino C57Bl/6 mice. Mice were closely monitored over the next 12 days. Tumor luminescence was measured using a Xenogen imager. At the experimental endpoint, animals were euthanized and tumors were removed and weighed. Tumor surface area was assessed using a caliper— tumor length and width were measured in cm, and the product was taken to determine surface area.

Sequence alignment. The miR-125b seed region and IRF4 3'UTR sequences from human (Homo sapien), mouse (Mus musculus), cat (Felis catus) and armadillo (Dasypus novemcinctus) were obtained and aligned using Targetscan [15-17].

Luciferase reporter assay. 293T cells were co-transfected with pcDNA-125b, β-gal expression vector, and the pMIR-REPORT vectors containing 3'UTRs of Cutl1, Picalm, IRF4 or 2-mer. The luciferase activity was quantified 48 hours later and normalized to β-gal activity as previously described [11,13].

RNA preparation and quantification. RNA was isolated using TRIzol (Invitrogen), RNEasy (Qiagen) or miRNEasy (Qiagen) as per manufacturer's instructions. Quantitative real-type PCR (qPCR) was conducted using a 7300 Real-time PCR machine (Applied Biosystems) or a Realplex Real-time PCR machine (Eppendorf).

SYBR green was used to assay IRF4 and L32 expression. PCR with previously published primer sequences for mouse pri-miR-125b-1 and pri-miR-125b-2 were used to assay levels of miR-125b primary transcripts [18]. Taqman-based qPCR was conducted to assay miR-125b, miR-125a and snoRNA-202 (Applied Biosystems). Primer sequences are listed in Table S1.

Flow cytometry. Cells were stained with the following fluorophore-conjugated antibodies: CD80, CD86, CD40 (Biolegend); MHC II (eBioscience); AnnexinV (BD Pharmingen). Cell surface receptors were measured using a FACSCalibur (Becton Dickinson) and all data was analyzed with FloJo (Treestar). Data was gated on GFP positive events when retrovirally transduced cells were analyzed.

Results

MiR-125b expression is enriched in macrophages

To investigate the expression of miR-125b in different immune cells and tissues, we harvested RNA from total splenocytes, thymocytes, splenic T cells, splenic B cells, and peritoneal macrophages from C57Bl/6 mice. Levels of miR-125b were assessed by reverse transcription followed by quantitative PCR. The expression of miR-125b was much higher in macrophages compared to the other immune cells and tissues (Fig. 1A). Within macrophages, miR-125b levels were also significantly higher than its homologue, miR-125a, indicating that miR-125b is the dominant isoform in these cells (Fig. S1). Also, miR-125b is expressed from two loci in the mouse genome, each encoding a different primary transcript. We performed RT-PCR for each of these primary transcripts and determined that macrophages express primarily miR-125b-1 (Fig. 1B). Because miR-125b-1 is enriched in macrophages, we set out to determine the functional role of miR-125b-1 (referred from here on as miR-125b) in these cells.

Enforced expression of miR-125b enhances macrophage activation status

To examine the response of macrophages to miR-125b, we utilized a miR-125b overexpression system based on the MG vector (MG-miR-125b), which was originally derived from the murine stem cell virus [10] (Fig. 2A). Bone marrow cells isolated from C57Bl/6 mice were spin-infected with either MG-miR-125b or MG control vector. These cells were then differentiated into bone marrow derived macrophages (BMMs) by treatment with macrophage-colony stimulating factor (M-CSF). Using this system, miR-125b was overexpressed 15-fold above endogenous levels in BMMs (Fig. 2B). Interestingly, miR-125b overexpressing BMMs acquired a spread morphology with extensive pseudopods that resembled activated macrophages (Fig. 2C). We performed flow cytometric analyses and observed increased expression of MHCII and the co-stimulatory molecules CD40, CD86 and CD80 in these macrophages, indicating that these cells were indeed more activated (Fig. 2D). Ectopic expression of miR-125b in RAW264.7 macrophages gave similar results (Fig. S2 A), further emphasizing that this microRNA promotes activation of macrophages.

MiR-125b increases macrophage response to interferon gamma

Next, we assessed the effect of miR-125b on the responsiveness of macrophages by stimulating these cells with interferon gamma (IFNγ). IFNγ treatment increased the expression of MHCII, CD40, CD86, and CD80 activation markers in control macrophages (Fig. 3A) while miR-125b overexpressing macrophages expressed significantly higher levels of these markers (Fig. 3A). Similar results were obtained in RAW264.7 macrophages with enforced miR-125b expression (Fig. S2 A).

To examine whether reducing the concentration of miR-125b had an effect inverse to that of overexpression, RAW264.7 macrophages were treated with synthetic antisense oligonucleotides (anti-miRs) and surface CD80 levels were monitored as an

indication of the activation status of the cells. Anti-miR125b did cause a reduction of both basal and IFNγ-induced levels of CD80 compared to cells treated with a control anti-miR (Fig3 B). Thus, miR-125b appears to control CD80 expression in macrophages under normal, physiological conditions.

A likely reason for the heightened response to IFNγ in miR-125b-treated cells could be an increased expression of the interferon gamma receptor (IFNγR). Indeed, miR-125b overexpressing BMMs (Fig. 3C) and RAW264.7 macrophages (Fig. S2 B) had significantly higher levels of the receptor. Thus, in addition to potentiating macrophage activation, miR-125b promotes enhanced macrophage responsiveness to IFNγ and increases surface expression of its cognate receptor.

MiR-125b enhances macrophage-mediated function

Because we found that miR-125b drove macrophages to adopt an elevated activation status and become more responsive to stimulatory cues, we examined whether miR-125b would also potentiate macrophage-mediated immune function. To this end, we investigated whether miR-125b would increase the ability of macrophages to present antigens and induce activation of T cells. MiR-125b overexpressing macrophages were co-cultured with transgenic T cells that express a chicken ovalbumin-specific T cell receptor (OT1) in the presence of ovalbumin. Indeed, compared to control macrophages, miR-125b overexpressing cells were more effective at inducing T cell activation, which was indicated by increased CD25 expression and IL-2 secretion by the T cells in response to ovalbumin (Fig. 4A). Thus, enforced expression of miR-125b led to an elevated ability of macrophages to act as effective antigen presenting cells for stimulation of T cell responses.

In addition to serving as antigen presenting cells, another major function of macrophages is to eliminate aberrant cells, such as tumor cells. We therefore assessed whether miR-125b-stimulated macrophages were more effective at killing tumor cells. We used the EL4-Fluc thymoma tumor line [19], which was engineered to express luciferase, and co-cultured these cells with either control macrophages or macrophages overexpressing miR-125b. Consistent with augmented function, miR-125b expressing macrophages were better at inducing apoptosis of EL4-Fluc cells (Fig. 4B). Macrophages exposed to LPS gained the ability to induce apoptosis of EL4-Fluc cells, with miR-125b-overexpressing macrophages having superior effectiveness (Fig. 4B). To test whether miR-125b levels in macrophages affect tumor killing in vivo, we subcutaneously co-injected into mice equal numbers of LPS-activated control or miR-125b overexpressing macrophages with EL4-Fluc cells and tracked growth of the resulting tumor by measuring tumor surface area over time. Since EL4-Fluc cells were engineered to express luciferase, we also monitored tumor growth by measuring luminescence in vivo. Consistent with our *in vitro* data, macrophages with miR-125b ectopic expression suppressed the ability of EL4 cells to expand in vivo (Fig. 4C). At the endpoint of the experiment on day 12, animals injected with MG-125b macrophages had smaller EL4-derived tumors that were significantly less luminescent than those injected with control macrophages (Fig. 4 D-E). Thus, miR-125b expression in macrophages appears to aid them in preventing the expansion of tumorigenic cells, further demonstrating that miR-125b enhances macrophage function.

IRF4 is a miR-125b target in macrophages

To identify targets regulated by miR-125b that modulate macrophage activation, we used TargetScan 5.1 to identify transcripts in the mouse genome that contain conserved putative miR-125b binding sites in their 3' untranslated regions (UTRs).

Among these genes, the 3'UTR of IRF4 harbored a conserved miR-125b binding site (Fig. 5A) and has been previously validated as a miR-125b target in B cell lines [20-23]. We found that miR-125b indeed represses via the 3'UTR of IRF4 (Fig. 5B) and that miR-125b inhibits IRF4 expression in macrophages (Fig. 5C). Next, using the MGP retroviral vector system [11], we knocked down the expression of IRF4 using RNAi (Fig. 5D) and examined the effect in macrophages. MGP-125b led to a six-fold increase in miR-125b in BMMs. Similar to miR-125b overexpression, decreased IRF4 expression resulted in increased surface expression of MHCII, CD40, CD86, CD80 and IFNγR (Fig 5E). Thus IRF4 knockdown in macrophages enhances activation, mimicking the miR-125b overexpression phenotype. This data is consistent with previous reports demonstrating that IRF4 is a negative regulator of macrophage pro-inflammatory pathways[21,24]. Collectively, our data suggests that IRF4 is a primary target of miR-125b in regulating macrophage activation.

Discussion

In this study, we demonstrate that miR-125b is enriched in macrophages and that further elevation of miR-125b promotes greater activation, IFNγ response, and immune function in these cells. We also performed loss-of-function studies using synthetic antisense oligonucleotides designed to inhibit miR-125b, and found that this anti-miR-125b compound effectively decreased CD80 levels, supporting a physiological role for miR-125b in macrophage activation. Other groups have reported that miR-125b levels decrease in macrophages 3 hours post-inflammatory stimulation [5,7]. Thus, decreasing miR-125b may serve as a natural mechanism to limit the inflammatory response. In our studies, miR-125b overexpression also promoted the ability of macrophages to present antigen and induce T cell activation, demonstrating that miR-125b can enhance the

macrophage's role in mediating adaptive immunity. The increase in activated T cells would in theory result in more secretion of IFNγ, which in turn would further magnify the activation status of miR-125b-expressing macrophages. In this way, by affecting macrophage function alone, miR-125b could amplify both innate and adaptive immune responses by orchestrating positive feedback loops between macrophages and T cells.

In B cells, miR-125b inhibits differentiation of germinal center B cells into plasma cells, and does so via repression of the transcription factors IRF4 and BLIMP1 [23,25]. IRF4, a member of the Interferon Response Factor family of transcription factors, also has important functions in macrophages where it acts as an inhibitor of the inflammatory response [21,24]. We demonstrate here that IRF4 is a target of miR-125b and that IRF4 knockdown mimics the miR-125b mediated activation phenotype in macrophages.

MiR-125b is upregulated in certain leukemias and downregulated in many non-hematopoietic solid cancers [12,26-36]. We have shown here that increased miR-125b expression in tumor macrophages slows tumor growth. Our data suggests that supplementing tumor macrophages with miR-125b may be a useful strategy for treating certain cancers.

Figure Legends

Figure 1. MiR-125b expression is enriched in macrophages. A) Relative expression of miR-125b in immune tissues and cells assessed by quantitative PCR. Data represents the mean with SEM of 3 biological replicates per group. B) Expression of the miR-125b primary transcripts, pri-125b-1 and pri-125b-2, in bone marrow derived macrophages (BMMs). Data is representative of two independent experiments.

Figure 2. MiR-125b enhances basal macrophage activation. A) Retroviral vector design for overexpression of miR-125b-1. B) Relative expression of miR-125b in BMMs after transduction with MG or MG-miR-125b expressing vector. C) Morphology of control MG or miR-125b overexpressing BMMs. Data represent five independent experiments. D) Geometric mean fluorescence (GMF) of MHCII, CD40, CD86 and CD80 are shown. Representative plots obtained from flow cytrometric analyses are also shown for each marker. Data is the mean with SEM of 3-5 samples per group and is representative of two independent experiments.

Figure 3. MiR-125b increases macrophage response to IFNγ. A) Surface expression of MHCII, CD40, CD86, and CD80 in response to media alone or IFNγ. A representative flow cytometric plot of the IFNγ-treated samples is shown for each factor. B) Raw264.7 macrophages electroporated with control (NC) or anti-miR-125b were subjected to flow cytometry for the surface expression of CD80. A representative FACS plot of the media-treated samples shown. C) Surface expression of IFNγR in control (MG) versus miR-125b overexpressing macrophages. A representative FACS plot is shown. All data shown represents the mean expressed with SEM of three samples per group and is representative of two independent experiments.

Figure 4. MiR-125b enhances macrophage function. A) BMMs expressing the vectors MG or MG-125b were co-cultured with ovalbumin-specific OT1 T cells with or without ovalbumin for 72 hours. The percent $CD8^{+}CD25^{+}$ T cells are shown in the left panel. Concentration of IL-2 (pg/ml) produced by the T cells in the supernatant is shown in the right panel. Data represents the mean with SEM of 3 biological replicates per group. B) The percent $AnnexinV^{+}$ EL4-Fluc cells after 94 hours of co-culture with control or miR-

125 overexpressing macrophages in the presence of media alone or lipopolysaccharide (LPS). A representative flow cytometric plot of the LPS-treated group is shown. Data is expressed in mean with SEM of 1-3 experimental samples per group. C-E) EL4-Fluc cells were subcutaneously co-injected with LPS-activated control or miR-125b overexpressing macrophages into albino C57Bl/6 mice. Tumor surface area in cm^2 was monitored from day 9-12 (C). The relative intensity of luminescence (D) and weight (E) of the EL4 tumors were measured on day 12. Data represents the mean plotted with SEM of eight mice per group. Representative of two independent experiments.

Figure 5. IRF4 is a target of miR-125b in macrophages. A) IRF4 contains a conserved miR-125b target site. B) Luciferase reporters carrying the 3'UTR of IRF4, Picalm (negative control), Cutl1 (negative control) or 2-mer (positive control) were co-transfected into 293T cells with β-gal reporter and +/- miR-125b. The relative luciferase activity of each reporter in the presence of miR-125b is shown relative to the no microRNA control. C) RAW264.7 macrophages were transduced with either a control (MGP) or miR-125b expressing vector, or with D) control (NC1) or IRF4 shRNA expressing vector. RNA was harvested and L32-normalized IRF4 levels were determined by qPCR. E) BMMs expressing MGP, MGP-125b, or shRNA against IRF4 were measured for surface expression of the activation markers MHCII, CD40, CD86, CD80 and IFNγR. Geometric Mean Fluorescence (GMF) measured by flow cytometry is shown. All data represents the mean with SEM of 3 samples per group and is representative of two independent experiments.

Figures

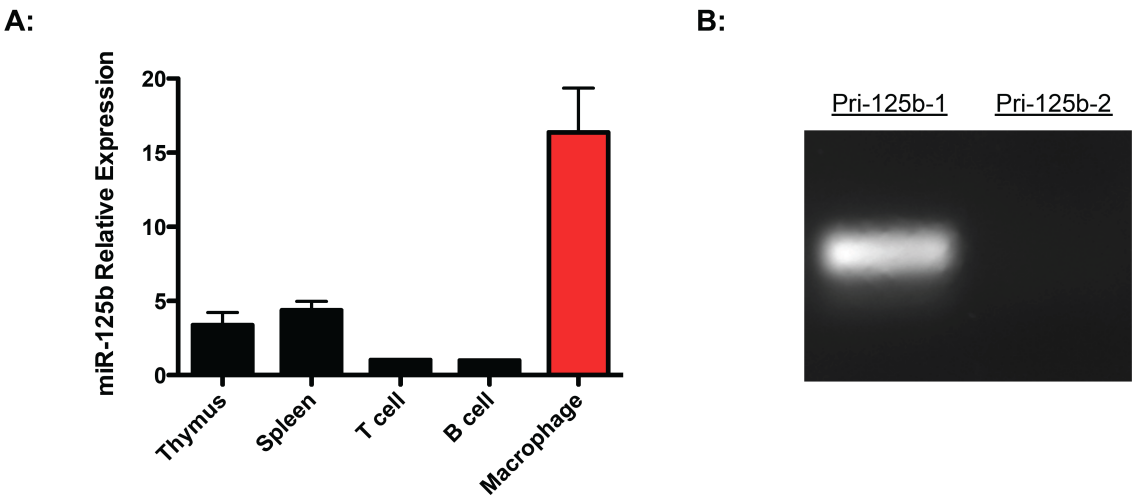

Figure 1. MiR-125b expression is enriched in macrophages

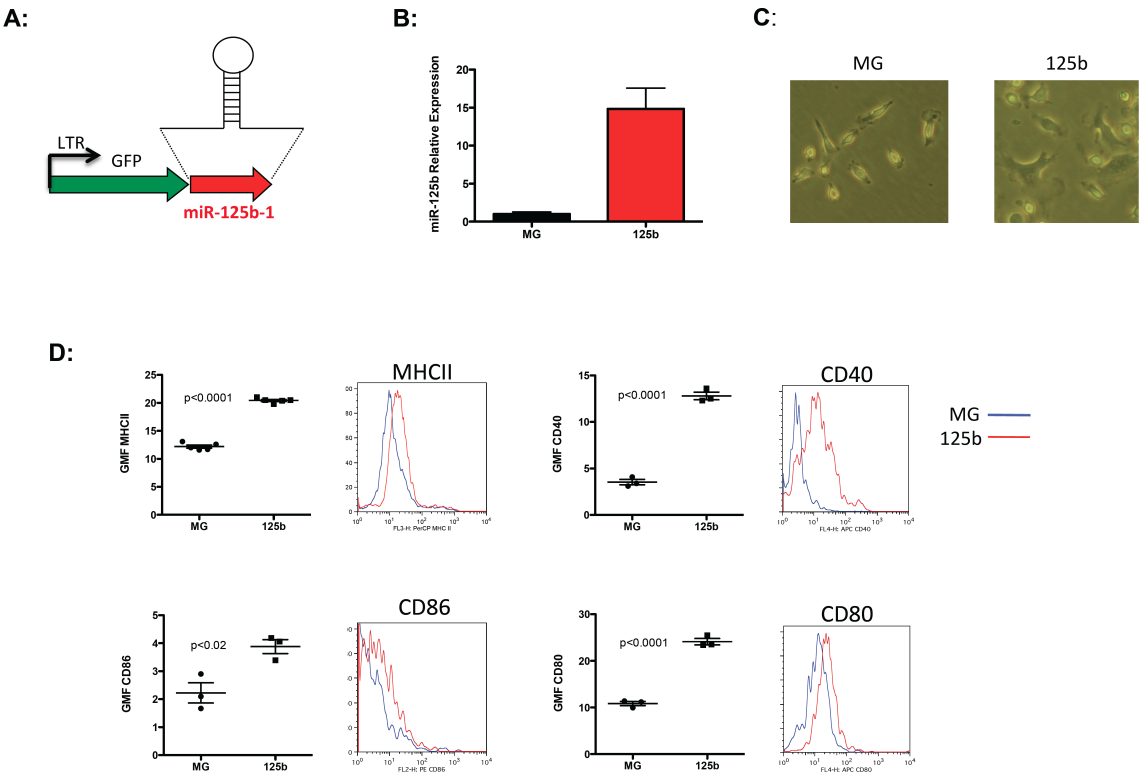

Figure 2. MiR-125b enhances basal macrophage activation

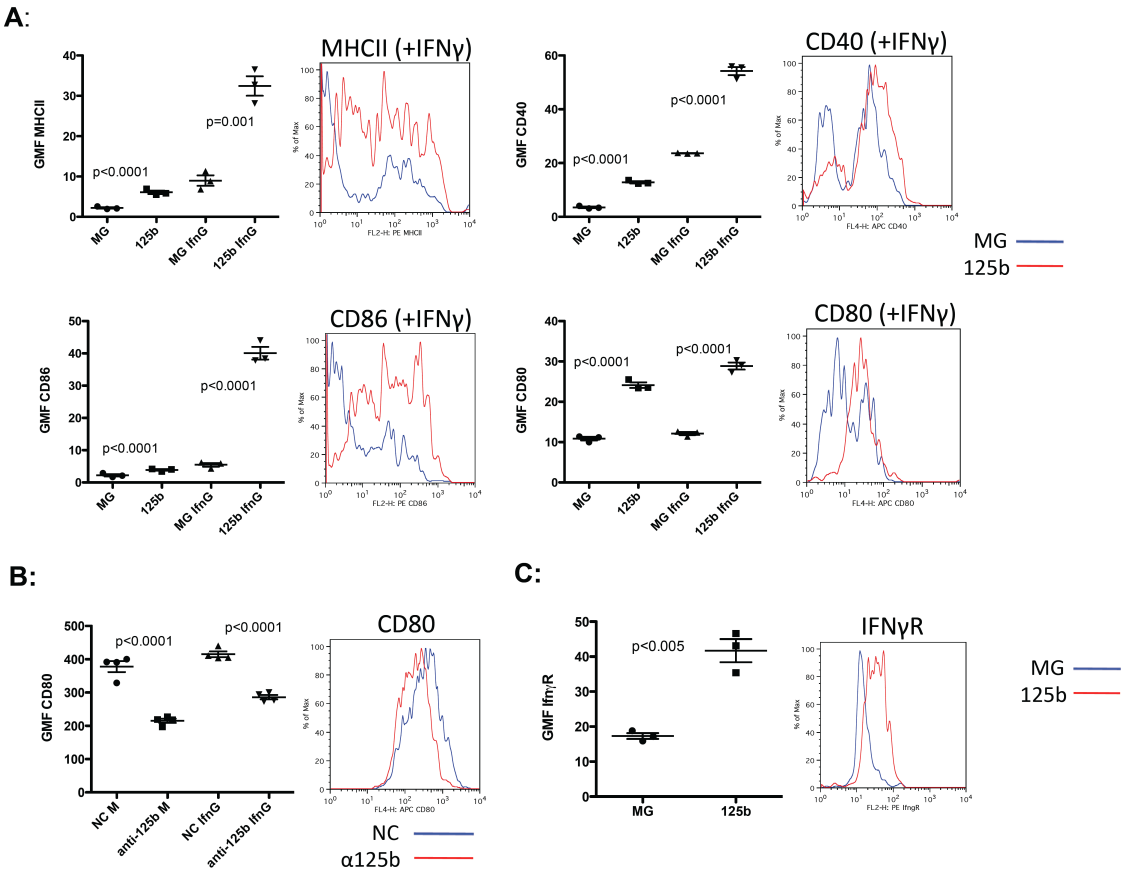

Figure 3. MiR-125b increases macrophage response to IFNγ

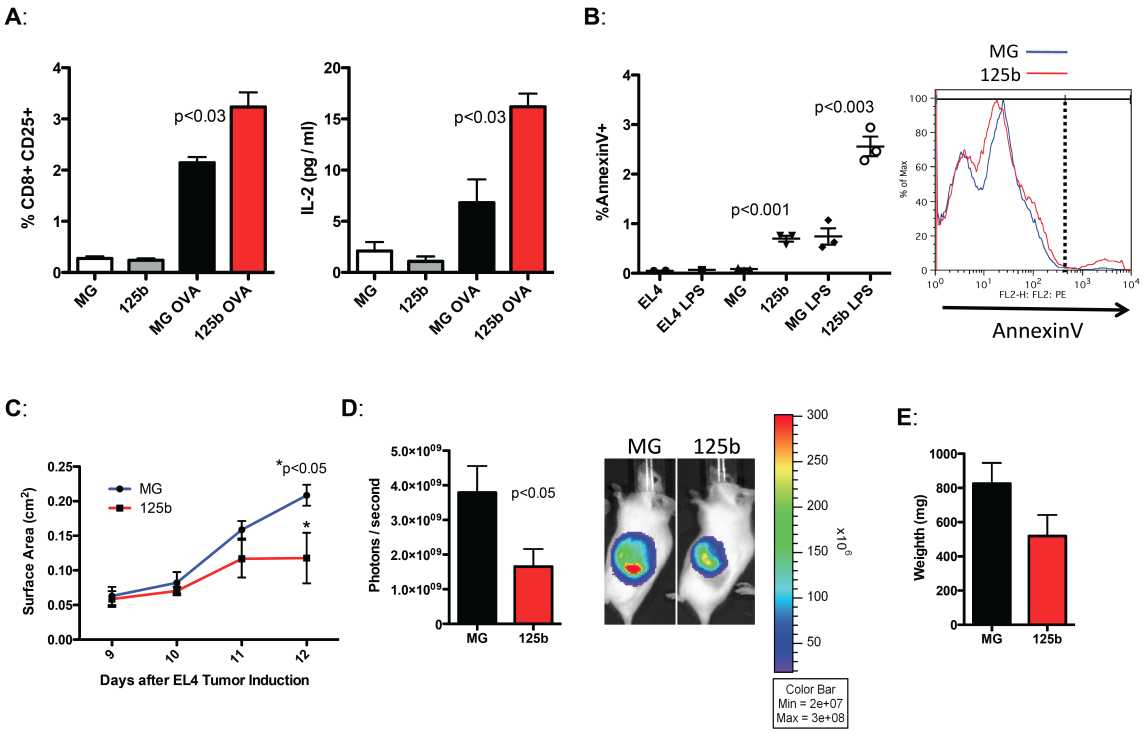

Figure 4. MiR-125b enhances macrophage function

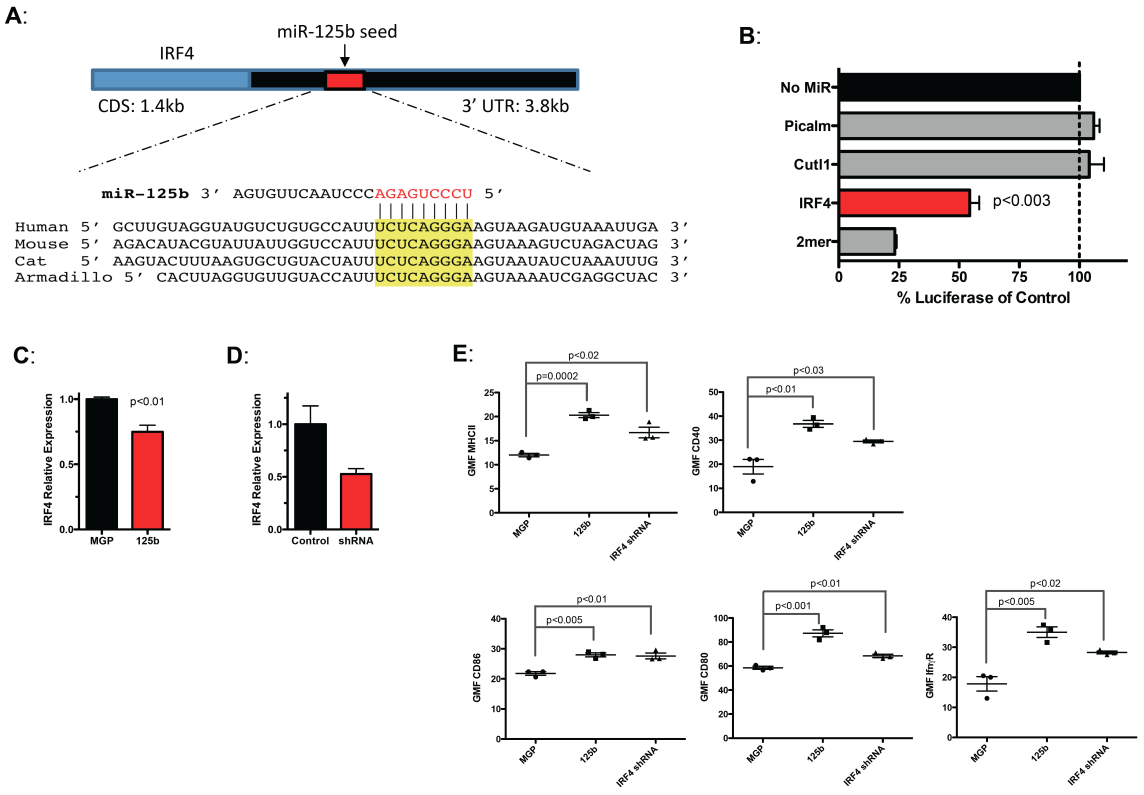

Figure 5. IRF4 is a target of miR-125b in macrophages

Supplemental Figures

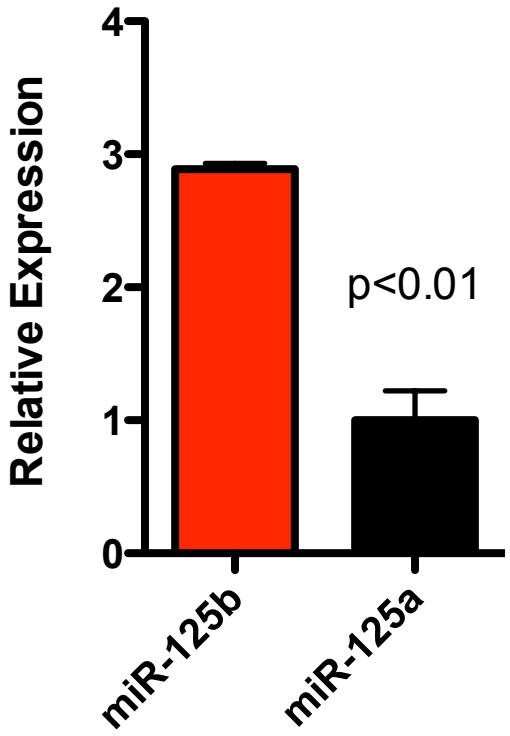

Figure S1. MiR-125b is expressed higher than miR-125a in macrophages. Relative expression of miR-125b and miR-125a in peritoneal macrophages. Data represents the mean and SEM of three biological replicates.

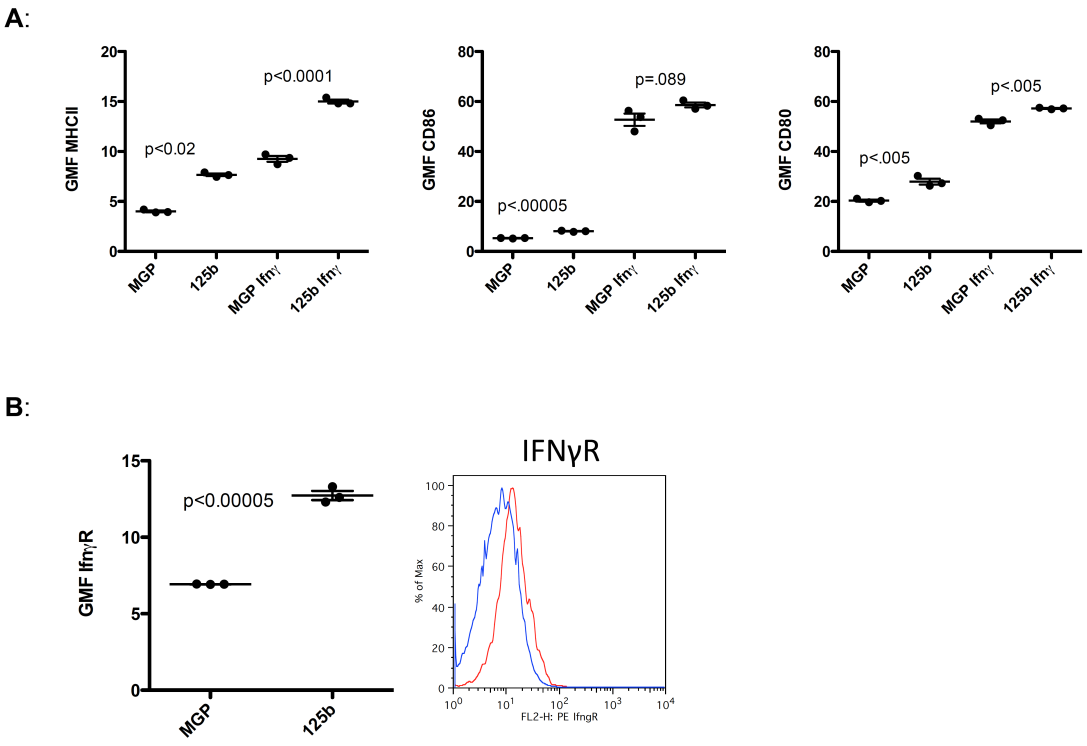

Figure S2. MiR-125b enhances basal and IFNγ mediated activation of RAW264.7 macrophages. A) Surface expression of the activation markers MHCII, CD86 and CD80 in media and IFNγ-treated samples. B) Surface expression of IFNγR in MGP control or miR-125b overexpressing RAW264.7 macrophages. All data shown is the mean with SEM of three samples per group, and is representative of two independent experiments.

Supplemental Table

Table S1. Primer Sequences

Primer	Sequence
IRF4 shRNA oligo	GAAGGCTGTATGCTGAAACAATGCCCAAGCCTTGAAGTT TTGGCCACTGACTGACTTCAAGGCGGGCATTGTTTCAGG ACACAAGGCCTG
IRF4 3' UTR NotI Fw	TTCGCGGCCGCGAGTTTTCTCTGATGTACTCGTGATCGT ATGT
IRF4 3' UTR XhoI Rev	TTCCTCGAGAACAGAAATCCAGGAGCTGCCACTC
2-mer SpeI Fw	CTAGTTCACAAGTTAGGGTCTCAGGGATCACAAGTTAGG GTCTCAGGGAA
2-mer HindIII Rev	AGCTTTCCCTGAGACCCTAACTTGTGATCCCTGAGACCC TAACTTGTGAA
IRF4 qPCR Fw	TCCGACAGTGGTTGATCGAC
IRF4 qPCR Rev	CCTCACGATTGTAGTCCTGCTT
L32 qPCR Fw	AAGCGAAACTGGCGGAAAC
L32 qPCR Rev	TAACCGATGTTGGGCATCAG

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Chapter 6: MicroRNA-125b targets Lin28 and physiologically and pathologically regulates hematopoiesis

Abstract

MicroRNA-125b (miR-125b) is upregulated in patients with leukemia. We show here that overexpression of miR-125b alone in mice is sufficient to promote a highly aggressive myeloid leukemia characterized by tumor cell infiltration into peripheral organs, including the brain, lungs, kidneys and liver. Prior to leukemia, mice with miR-125b overexpression have normal white blood cell counts but the hematopoietic compartment is rearranged such that myeloid cell numbers are dramatically increased at the expense of B cells. We reveal that miR-125b exerts this effect by upregulating the number of common myeloid progenitors while inhibiting the development of pro-B cells. Next we applied a miR-125b 'sponge' decoy loss-of-function approach to uncouple the role of miR-125b in tumorigenesis from its potential physiological function in hematopoiesis; indeed, we found that miR-125b physiologically regulates hematopoietic development. To investigate the mechanism by which miR-125b regulates hematopoiesis, we examined and found that the inducible pluoripotent stem cell (IPS) regulator Lin28 was the most repressed by miR-125b in mouse bone marrow cells among a panel of computationally derived candidate targets. Of importance, overexpression of Lin28 in the mouse hematopoietic system mimicked the phenotype observed upon inhibiting miR-125b function, leading to a decrease in hematopoietic development. Relevant to the miR-125b overexpression pre-cancer phenotype, we revealed that knockdown of Lin28 also led to a hematopoietic rearrangement with increased myeloid and decreased B cell numbers. Thus, Lin28 is an important regulator of hematopoiesis, and is a primary target of miR-125b in the hematopoietic system.

Introduction

MicroRNAs are a class of ~ 22 nucleotide RNA species that follow a canonical processing pathway generated by sequential cleavage of endogenous PolII-generated transcripts by Drosha and Dicer[1,2]. The first microRNA, lin-4, was discovered in *C. elegans* and was shown to regulate the transition from early larval L1 to later L4 stages by repressing its target genes, the transcription factor Lin-14 and the RNA-binding protein Lin-28[3-7]. Indeed, as lin-4 levels increase between larval stages L1 and L3, levels of its target genes Lin-14 and Lin-28 decrease[3]. Lin28 itself represses biogenesis of another microRNA, let-7[8-11], a microRNA necessary for late-stage larval development[12,13]. Thus, the lin-4:Lin-28:let7 cascade was shown to be critical for proper worm development. This signaling cascade is conserved in vertebrates as the mammalian homologue of lin-4, miR-125b, is also able to repress Lin-28. Previously, ectopic expression of miR-125b was shown to repress Lin28 expression in the mouse P19 embryonic carcinoma cell line[14]. MiR-125b was also shown to mediate repression via specific target sites in the Lin28 3' untranslated region (UTR)[14]. This highlights a potential critical developmental function of miR-125b:Lin-28 signaling in mammalian systems, which has yet to be explored.

In humans, miR-125b is enriched in hematopoietic stem cells[15,16] and is upregulated in a range of human leukemias, including acute myeloid leukemia (AML)[17,18], chronic myeloid leukemia (CML)[18], acute megakaryocytic leukemia (AMKL)[19], childhood acute lymphoblastic leukemia (ALL) with the ETV6/Runx1 fusion protein[20], and Philadelphia-chromosome positive B-cell precursor ALL[18]. Indeed, overexpression of miR-125b alone in the bone marrow of mice is sufficient to induce leukemia[16,18,21], showing that constitutive miR-125b upregulation can initiate and subsequently lead to cancer. Recent in vitro work has also demonstrated the role of miR-125b in the

development of plasma cells[22] and effector T cells[23], showing that miR-125b regulates development of immune cells as well.

Also demonstrating relevance in mammalian development, Lin-28 has been shown to be important for early embryogenesis in mice and humans[24]. The levels of Lin-28 are enriched in embryonic stem (ES) cells and decrease as these cells differentiate[24]. Indeed, ectopic expression of Lin-28 along with three other genes (Oct4, Sox2 and Nanog) causes de-differentiation of mature human cells into induced pluripotent stem (IPS) cells[25]. This demonstrates the power of Lin28 to endow cells with pluripotent qualities. Still, it remains to be seen whether Lin28 also plays developmental roles in biological systems arising from adult stem cells, such as hematopoiesis. Given the importance of miR-125b in leukemic development and hematopoiesis, it will be interesting to investigate whether Lin28 has overlapping functions with miR-125b in these events.

In this study we seek to better understand how miR-125b and Lin-28 regulate hematopoietic development. We began by characterizing that the cancer caused by miR-125b overexpression is a highly aggressive myeloid leukemia, which is further demonstrated by infiltration of myeloid cells into non-hematopoietic organs including the lungs, kidneys and brain. To investigate the cellular changes that lead to leukemia, we next examined the impact miR-125b has on the hematopoietic system prior to disease onset. We found that miR-125b overexpression dramatically enhances common myeloid progenitors (CMPs) and downstream mature myeloid cells. Interestingly, whereas common lymphoid progenitors (CLPs) were also modestly increased, downstream pro-B and pre-B cells were much lower in the miR-125b overexpressing animals, suggesting that miR-125b blocks the transition from CLPs to pro-B cells. In order to determine a physiological role for miR-125b in hematopoiesis, we showed through loss-of-function that miR-125b decreases output of all examined hematopoietic lineages, thus

establishing that miR-125b is necessary for proper hematopoietic output. Finally we demonstrate that Lin28 is a primary target of miR-125b in the hematopoietic system. We show that Lin28 is efficiently repressed by miR-125b in both mouse and human hematopoietic cells. We show that Lin28 knockdown and overexpression mimic miR-125b gain- and loss-of-function, respectively. Thus, lin-28 is a powerful regulator of hematopoiesis. In summary, we show here that miR-125b physiologically and pathologically regulates hematopoiesis, and that Lin28 is a primary target gene of miR-125b in the hematopoietic system.

Experimental Results

MiR-125b causes an aggressive and highly metastatic myeloid leukemia

We and others previously showed that overexpression of miR-125b in bone marrow transplanted recipient mice causes leukemia 4-6 months after bone marrow reconstitution[16,21]. Here we interrogated the precise types of cells that were neoplastic in the disease and investigated the aggressive nature of the disease. We began by performing bone marrow reconstitution experiments into lethally irradiated recipient mice with donor hematopoietic stem cell (HSC)-enriched bone marrow that were infected with retroviruses harboring the MG control or miR-125b-1 overexpressing vector (MG-125b)[16]. These vectors also express green fluorescent protein (GFP)[16,26], which provided the advantage of tracking cells that ectopically express miR-125b in the recipient animals (Fig S1A). Of note, these bone marrow reconstitution experiments allowed the study of miR-125b function specifically in the hematopoietic compartment, uncoupling it from its potential function in other tissues. In these experiments, the transduction efficiency of HSC-enriched bone marrow cells with retro-vectors at the time of transplantation into recipients was 40% and 25% GFP^+ in MG and MG-125b animals

(Fig S1B), respectively. The level of miR-125b overexpression in MG-125b transduced bone marrow was 25-fold over MG control (Fig S1C). Five months post-reconstitution, consistent with previous reports[16,21], MG-125b animals had severely enlarged spleens compared to control animals (596 mg compared to 85 mg) (Fig S1D-E) and the white blood cell count was four-fold increased (Fig S2A). When we performed flow cytometry on these spleens, we observed a dramatic increase in the percent of GFP+ myeloid cells, but a decrease in percent GFP^+ lymphoid cells, indicating that miR-125b leads to a myeloid type of leukemia (Figure S2B).

In our previous study, we suggested that miR-125b overexpressing mice get a myeloid-type of leukemia[16]. Here we confirmed this finding by showing that miR-125b overexpressing mice develop a 33-fold increase in myeloid white blood cells ($CD11b^+$ $CD45^+$) in the blood at 4.5 months post-reconstitution (Fig 1A), a time point at which the mice appear moribund and some had already died (Fig S3A). Further confirming that the leukemia was myeloid, we examined B and T cell numbers and observed those to be decreased or only modestly elevated, respectively (Fig S3B-C). Of note, a second cohort of ten animals (Fig S4A) delivered similar results with two notable exceptions. At five months post-reconstitution (Fig S4B), the seven surviving animals strongly upregulated myeloid-type leukocytes (32-fold increase) in their blood (Fig S4C), but one animal also showed an 8-fold increase of B cells (Fig S4D) and a second showed a 29-fold increase in T cells (Fig S4E). Blood from the other three animals was also measured within two weeks of their deaths, and revealed dramatic upregulation of myeloid but not lymphoid cells (Data not shown). Still this confirms previous studies that miR-125b is capable of producing lymphoid tumor cells[18,21] too, although the majority of our animals (86%) exhibited myeloid-only types of leukemia.

In order to probe further into the nature of the miR-125b-driven myeloid leukemia, we examined whether neoplastic myeloid cells infiltrate non-hematopoietic organs. The

blood-brain barrier normally prevents infiltration of white blood cells into the brain, an organ thus referred to as “immunoprivileged”. In miR-125b overexpressing mice, however, flow cytometry revealed dramatic infiltration of $CD45^+$ GFP^+ white blood cells and $CD11b^+$ GFP^+ myeloid cells into the brain 5 months post-reconstitution (Fig 1B). The kidneys of MG-125b mice were also heavily infiltrated with myeloid leukemic cells as indicated by histopathology (Fig 1B) and flow cytometry (Fig S5A). They were morphologically larger and pale colored compared to control animals (Fig S5B). Myeloid leukemic cells also infiltrated the lungs and livers of miR-125b cancer mice as evidenced by histopathology, FACS and gross pathology (Fig 1B, Fig S5C-F). Further indicative of miR-125b infiltrating cells in the lung, kidneys and liver, we observed 18-74 fold increase of miR-125b levels in these tissues compared to control animals (Fig S6A-C). Displaying increased survival, splenocytes taken from miR-125b overexpressing animals, when cultured for 204 hours, displayed decreased apoptosis compared to MG controls (Fig S7A-B). Interestingly, mice overexpressing miR-125a also developed an aggressive leukemia and died by six months post-reconstitution (Fig S8), indicating that the capability to cause leukemia is conserved between miR-125 homologues. In summary, miR-125b leads to an aggressive myeloid leukemia that efficiently metastasizes into non-hematopoietic organs including the brain.

MiR-125b ectopic expression rearranges the hematopoietic system prior to cancer onset

In order to understand the initial events that lead to miR-125b mediated leukemia that occurs 5 months post-reconstitution, we examined the spleen and bone marrow at seven weeks post-reconstitution, a time point that falls well before the onset of cancer as indicated by similar white blood cell counts in the spleen and bone marrow of MG and MG-125b animals (Fig 2A, Fig S10A-B). At this time point, MG-125b animals showed a

dramatic increase in all myeloid lineages including granulocytes, macrophages and dendritic cells, with T cell numbers being similar between MG and MG-125b animals (Figure 2A, Fig S10C-E). However, there was a dramatic decrease in B cell and plasmablast numbers (Fig 2A, Fig S10F). Importantly and consistently with decreased B and plasmablast cell development, MG-125b animals were severely impaired in the ability to produce anti-ovalbumin IgG antibody (Fig 2A). Thus, miR-125b overexpression at the pre-cancerous stage causes a rearrangement of the hematopoietic compartment that favors myeloid cell development at the expense of B cells and consequently impairs humoral immunity.

Next, we analyzed hematopoietic stem and progenitor cell numbers in the bone marrow at the 7 week time point following bone marrow transplantation. We observed that hematopoietic stem cell numbers were similar between MG and MG-125b animals (Fig 2C, Figure S11), showing that miR-125b overexpression does not increase HSC numbers early in the disease course. Progenitors directly downstream of HSCs, however, including multipotent progenitors (MPPs), common myeloid progenitors and granulocyte macrophage progenitors (CMP/GMPs), myeloid-erythroid progenitors (MEPs) and common lymphoid progenitors (CLPs) were all significantly up (Fig 2B-C). Of these, CMP/GMPs were the most dramatically increased, being up nine-fold in MG-125b animals compared to MG controls (Fig 2B). Notably, pro-B and pre-B cell numbers were severely decreased (Fig 2B), providing a developmental basis for the decreased B cell numbers we observed in MG-125b animals. This also demonstrates that miR-125b overexpression causes a developmental block between the CLP and Pro-B cell stages. In summary, we show here that miR-125b, even prior to the onset of leukemic disease, strongly enhances myeloid development at the expense of B cell development by regulating their precursor cells.

Physiological regulation of hematopoiesis by miR-125b

While overexpression studies are relevant in the context of understanding the role of miR-125b as an onco-miR capable of promoting hematopoietic cancer, we needed to develop a robust loss-of-function system to assess its potential physiological function in hematopoiesis. Thus we generated a “sponge” decoy system to competitively inhibit miR-125b binding to its natural targets (Fig 3A). We designed our sponge by placing four adjacent miR-125b antisense sites directly downstream of GFP in the MG vector (Fig 3A). Each site contained a region corresponding to nucleotides 9-12 of the microRNA that were mismatched. Expression in the MG vector system allowed us to monitor sponge-expressing cells by tracking GFP.

To test whether the sponge decoy is capable of de-repressing a miR-125b target sequence, we tested whether a reporter adjacent to an artificial 3’ UTR composed of two perfect miR-125b complementary sequences could be de-repressed by the sponge. We observed that co-expression of this 2-mer reporter with miR-125b and the sponge led to significant de-repression of the reporter compared to MG control in 293T cells (Fig 3B). An empty control reporter was not de-repressed by the sponge, demonstrating target specificity of our sponge decoy (Fig 3C).

In order to determine the hematopoietic effect of the miR-125b sponge, we sorted GFP^+ cells from MG and sponge transduced bone marrow and reconstituted recipient animals with equal numbers of cells (Fig 4A). At 1 month post-reconstitution we analyzed the blood of recipient mice by FACS and observed significant decreases in numbers of total white blood cells ($CD45^+$), myeloid cells ($CD45^+$ $CD11b^+$), granulocytes ($CD45^+$ $Gr1^+$), B cells ($CD45^+$ $CD19^+$ $CD3e^-$), T cells ($CD45^+$ $CD3e^+$ $CD19^-$) and pre-erythrocytes ($CD45^+$ $Ter119^+$) (Fig 4B). The blood was also noticeably thinner in the sponge mice compared to controls, indicating that mature erythrocytes were decreased as well. Of note, the sponge mouse with the thinnest-appearing blood and lowest $CD45^+$

count died at four weeks post-reconstitution, likely due to impaired hematopoiesis, while all the controls survived. Together our data shows a physiological role for miR-125b in the development of all blood lineages.

Lin28 is a primary target of miR-125b in the hematopoietic system

Next, we strove to identify miR-125b targets that also regulate hematopoietic development. We performed a nonbiased screen using Targetscan[27-29] to isolate putative miR-125b targets with $P_{\text{Conserved Targeting}} > 0.8$ ($P < 0.2$ that the putative microRNA site is maintained due to selective microRNA targeting rather than by chance[29]). Application of this screen yielded 192 genes. We focused on those that were pro-apoptotic or involved in the stem cell state, as these processes have been correlated with leukemic development. This yielded four genes, Bak1, Trp53inp1, BMF and Lin28, which independently have been previously validated as miR-125b targets by other groups[14,18,30,31]. To test whether miR-125b represses these genes in hematopoietic cells, we transduced hematopoietic progenitor-enriched bone marrow with MG-125b or MG control vectors and assessed levels of each of these genes by qPCR. Indeed, we saw efficient repression of all four targets, with Lin28 being most repressed by miR-125b (Fig 5A). Upon miR-125b overexpression, Lin28 expression was reduced to 20% of the control. Validation by luciferase reporter paralleled the qPCR results—the 3' UTRs of all the target genes were significantly repressed by miR-125b with that from Lin28 being the most repressed (Fig 5B). As a positive control we used an antisense 2-mer sequence complementary to miR-125b. However, a luciferase empty vector reporter or one harboring a non-target 3' UTR (Picalm) was not repressed by miR-125b expression, demonstrating that miR-125b specifically represses Bak1, Trp53inp1, BMF and Lin28 expression via their 3' UTRs. Of note, Lin28 was also more de-repressed in miR-125b

loss-of-function progenitor-enriched bone marrow than the other measured targets (Fig 5C).

Lin28 is known to be involved in maintaining pluripotency of embryonic stem cells but its role in hematopoiesis remains largely uninvestigated. To investigate the potential role of Lin28 in miR-125b-mediated hematopoiesis, we first investigated whether miR-125b can physiologically regulate gene expression via the Lin28 3' UTR. Lin28 contains one putative miR-125b site with a 9 nucleotide seed region complementary to the microRNA 5' end and a significant amount of complementarity to the 3' end as well (Fig 5D). Co-transfecting the miR-125b sponge decoy compared to MG control with a luciferase reporter containing the mouse Lin28 3' UTR led to five-fold de-repression (Fig 5E). Demonstrating that the miR-125b-Lin28 interaction exhibits species conservation between mouse and human hematopoietic cells, human myeloid K562 cells transduced with either MGP, MGP-125b-1, MGP-125b-2 or a seed mutant of miR-125b exhibited seed-dependent repression of Lin28 by both miR-125b species at both the RNA and protein level (Fig 5F,G). Thus miR-125b represses Lin28 in hematopoietic cells, an interaction conserved between mouse and human.

Lin28 regulates aspects of hematopoiesis that mirror those controlled by miR-125b

To determine whether miR-125b-mediated repression of Lin28 has a role in the hematopoietic system, we bypassed the repressive effect exerted by endogenous miR-125b by overexpressing Lin28 lacking its 3'UTR. We utilized the MSCV-IRES-GFP (MIG) vector system[32], which allowed the co-expression of Lin28 and GFP from the same vector. Western blot was performed to confirm expression of Lin28 from the MIG-Lin28 vector in transfected 293T cells (Fig 6A). Next we performed bone marrow transplant experiments with progenitor-enriched bone marrow cells transduced with

either MIG or MIG-Lin28. We achieved over 80% transduction efficiency as measured by GFP-positive cells (Fig 6B). Similar to mice reconstituted with the miR-125b sponge decoy (Fig 4), animals with Lin28 overexpression had significant decreases in total white blood cells ($CD45^+$), total myeloid cells ($CD45^+$ $CD11b^+$), granulocytes ($CD45^+$ $Gr1^+$), T cells ($CD45^+$ $CD3e^+$ $CD19^-$), NK cells ($CD45^+$ $NK1.1^+$) and erythrocytes ($Ter119^+$ $CD11b^-$) (Fig 6C). Also similar to the sponge mice, the blood was noticeably thinner in MIG-Lin28 animals compared to MIG controls, likely due to decreased erythrocyte numbers. Of importance, the Lin28 overexpressing mice with the thinnest blood (2 out of 15) died at five weeks post-reconstitution whereas none of the controls died, likely a consequence of impaired hematopoietic development. Thus, overexpressing Lin28 in the hematopoietic system mirrored the phenotypes observed upon miR-125b inhibition (Fig 4).

Next, we applied Lin28 loss-of-function studies to examine whether Lin28 physiologically regulates hematopoiesis. Also, loss-of-function assays allowed us to determine whether inhibiting Lin28 function would mimic specific aspects of hematopoietic development observed upon overexpressing miR-125b. Thus we transduced progenitor-enriched bone marrow with Lin28 shRNA and achieved ~ 70% transduction with two-fold knockdown of Lin28 (Fig 7A,B), less than the level of repression exerted by miR-125b overexpression. Strikingly, mice reconstituted with shLin28 exhibited similar but less dramatic features in hematopoietic development as those that overexpressed miR-125b in the pre-cancerous stage; knockdown of Lin28 led to an expansion of myeloid cells ($CD45^+CD11b^+$, $CD45^+Gr1^+$) and decrease of B cells ($CD19^+CD11b^-$) (Fig 7C). Thus Lin28 knockdown mimicked rearrangement of the hematopoietic system that we saw early post-reconsitution in miR-125b overexpressers (Fig 7D), namely an increase in myeloid cells at the expense of B cells, suggesting that Lin28 knockdown by miR-125b plays a role in setting the stage for myeloid leukemia. We

did not, however, observe obvious signs of leukemia in the shLin28 mice after ten weeks following bone marrow transplantation, suggesting that repression of other targets such as the pro-apoptotic genes Bak1, Trp53inp1 and BMF, likely collaborate with Lin28 during miR-125b mediated tumorigenesis.

Discussion

In this study, we show that: 1) MiR-125b overexpression causes a highly aggressive myeloid leukemia capable of infiltrating non-hematopoietic organs, including the brain. 2) Early in the disease course at the pre-cancerous stage, miR-125b ectopic expression induces a rearrangement of the hematopoietic system favoring myeloid development at the expense of B cell development. 3) Before terminal leukemic development, miR-125b does not affect HSC numbers but drastically increases CMP/GMP numbers while blocking pro-B cell development. 4) In addition to the role of miR-125b in pathological disease, miR-125b also physiologically regulates hematopoiesis. 5) Lin28 is a primary target of miR-125b in hematopoietic cells. 6) Lin28 overexpression and knockdown mimic important aspects of miR-125b loss-of-function and gain-of-function, respectively. We conclude that miR-125b physiologically regulates hematopoiesis, and constitutive overexpression of miR-125b leads to uncontrolled generation of myeloid progenitors that subsequently causes a myeloid leukemia. We also conclude that the miR-125b's direct target Lin28 is an important regulator of hematopoietic development that may be targeted by miR-125b in the pathogenesis of myeloid leukemia.

In our previous study, we observed that miR-125b overexpression induces leukemia[16]. In this study, we begin by further characterizing this phenotype and determined that 86% of the mice analyzed develop a highly aggressive form of myeloid

leukemia. Two mice out of the fourteen analyzed (14%) developed mixed myeloid-lymphoid leukemias, confirming the studies by Bousquet et al., Ooi et al. and Enomoto et al. that miR-125b is also capable of causing leukemias involving lymphoid cell types. Interestingly, restricted overexpression of miR-125b in B lineage cells was shown to be capable of inducing lymphoid leukemia[18], similar to the effect previously observed with miR-155 B lineage-specific overexpression[33]. Here, we show that upon overexpressing miR-125b in a mixed population of bone marrow progenitor cells, 86% of the transplanted mice exhibited myeloid-only leukemia characterized by a profound increase in the number of myeloid cells, which were capable of infiltrating into peripheral organs. Thus, it appears that miR-125b overexpression favors myeloid over lymphoid development in an environment where these different cell types may need to compete for survival. Potentially, miR-125b-overexpressing myeloid progenitors and mature myeloid cells have a greater capacity to proliferate and survive than miR-125b overexpressing lymphoid cells, giving these myeloid cells the capacity to outcompete their lymphoid neighbors. Still, Enomoto et al. confirms miR-125b's potency as an onco-miR, showing that B-cell restricted ectopic expression of miR-125b leads to lymphoid leukemia[18]. Of importance, miR-125b has been found to be overexpressed in a variety of human leukemias, including AML[17,18], CML[18], AMkL[19], and ALL[18,20], indicating that deregulated miR-125b expression in different contexts can lead to distinct cancer types.

In this study, we reveal that miR-125b overexpression does not induce an increase in HSC numbers after 7 weeks post-reconstitution, which is at the pre-leukemic stage. Interestingly, Ooi et al. showed that recipient mice reconstituted with miR-125b overexpression in purified HSCs resulted in elevated HSC numbers at later time points[15]. Guo et al. also showed that overexpressing the miR-125b homologue miR-125a increased HSC numbers after 4-5 months of reconstitution[34]. These mice were likely undergoing leukemic transformation, as suggested by the surface marker profile of their

HSCs being altered[34]. Indeed, we have shown here that constitutive overexpression of miR-125a causes leukemia and premature death, with the gross appearance of the disease being similar to miR-125b overexpression. Collectively, it appears that the impact of miR-125b and its homologues might have different influences on HSC numbers at varying stages of cancer development. Here we show that prior to the onset of proliferative disease, miR-125b overexpression does not affect HSC numbers but leads instead to a significant increase in early progenitors (MPP, CMP/GMP, MEP, CLP), with CMP/GMPs exhibiting the most profound magnitude of elevation. These findings have several important implications. First, it suggests that miR-125b likely does not initiate leukemic development by driving uncontrollable HSC proliferation, which is supported by our data that HSC numbers were not affected at the pre-cancerous stage. It is possible that miR-125b overexpression promotes HSC differentiation into downstream progenitors and mature cells, and through repression of its apoptotic target genes, gives these cells a survival advantage that eventually leads to cancer; indeed, the numbers of progenitors downstream of HSC were elevated. If HSC differentiation capacity alone was sufficient to drive leukemia, one might expect that miR-125b overexpressing mice would have similar frequency of myeloid and lymphoid leukemia. However, we found that the majority of these mice exhibited only myeloid leukemia. One possibility is that myeloid development is the default pathway for hematopoiesis since it has fewer negative checkpoints than lymphoid development[35]. Alternatively miR-125b might act intrinsically in the CMP to drive expansion of these cells. In the future, it will be interesting and important to distinguish between these possibilities and determine if myeloid progenitors and/or the mature myeloid cells that overexpress miR-125b can initiate tumorigenesis.

In searching for a mechanism by which miR-125b exerts its effects on the hematopoietic system, we tested Lin28, a gene we saw was potently regulated by miR-

125b in both mouse and human hematopoietic cells. In performing gain- and loss-of-function of Lin28 in the hematopoietic system, we further identified Lin28 as a novel regulator of hematopoietic development. Lin28 is an RNA binding protein that is capable of de-differentiating mature mammalian cells back to pluripotent stem cells when co-expressed with the genes Oct4, Sox2 and Nanog[25]. Expression of Lin28 has been shown to be stem cell specific[36-38] and to enhance stem cell growth and survival[39,40]; its downregulation in embryonic stem cells corresponds temporally with differentiation of these cells[37,38]. Our data here suggests that miR-125b might similarly promote differentiation of HSCs into downstream progenitors. This process might rely on Lin28, and it is conceivable that repression of Lin28 by miR-125b may promote HSCs to differentiate into downstream progenitors. Coordinated repression of other miR-125b target genes, such as those involved in the anti-apoptotic process, may then endow these cells with an enhanced survival advantage, providing them with the opportunity to increase in number and eventually transform into leukemia.

We show here that miR-125b is an important regulator of hematopoiesis both physiologically and pathologically. MiR-125b overexpression promotes early hematopoieic progenitors, especially myeloid progenitors, and eventually leads to myeloid leukemia. Our results indicate that the effects miR-125b has on the hematopoietic system are in part due to its ability to efficiently repress Lin28 expression. Indeed, we show that manipulating Lin28 expression levels in mice mimicked the hematopoietic phenotypes of those with modulated miR-125b function. Collectively, this report further advances our understanding of the mechanism by which miR-125b regulates hematopiesis and leukemic development. Indeed the interplay between miR-125b and Lin28 appears to be a powerful regulatory pathway in this process, and one that could be toggled therapeutically in order to ameliorate hematopoietic disorders such as leukemia and bone marrow failure.

Materials and Methods

Cell culture. 293T cells were cultured in complete DMEM with 10% FBS, 100 units/ml penicillin and 100 units/ml streptomycin. K562 cells and splenocytes from mice were cultured in complete RPMI with 10% FBS, 100 units/ml penicillin, 100 units/ml streptomycin and 50 μM 2-Mercaptoethanol.

DNA constructs. The retroviral vectors MG, MGP, MGP-125b-1 and MGP-125b-2 have been described previously. The miR-125b sponge decoy vector was constructed by cloning four miR-125b antisense sites downstream of GFP in MG, where each anti-sense site was separated by a 4-6 nucleotide spacer sequence, and each anti-sense site contained a binding mismatch with miR-125b corresponding to nucleotides 9-12 of the microRNA. Both Lin28 shRNA and MGP-125b-Mutant were cloned using a miR-155 arms-and-loop format, which was previously described. The Lin28 shRNA sequence was predicted using the Invitrogen BlockiT RNAi Designer. The 3' UTRs for mouse Lin28, Trp53inp1, Bak1 and BMF as well as the antisense 2-mer were cloned downstream of luciferase in the pMIR-REPORT vector. For the Lin28 overexpression vector, the mouse Lin28 coding sequence (Open Biosystems) was cloned downstream of the LTR and prior to the IRES-GFP segment of the MSCV Ires GFP (MIG) vector. All primers are listed in Table S1.

Retroviral transduction 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. Virus production and reconstitution experiments were performed as previously described (O'Connell et al., 2008). Experimental groups of four to ten mice received each of the vectors described. The health of recipient animals was closely monitored, and flow cytometry on the peripheral blood was performed

periodically. K562 cells were transduced with VSV-G pseudotyped MGP, MGP-125b-1, MGP-125b-2 and MGP-125b-Mut as described previously.

Cell counting. Cells were counted using a Coulter Counter (Beckman Coulter) or a MACSQuant flow cytometer (Miltenyi Biosciences). Absolute cell counts in the blood were obtained by FACS-staining equivalent volumes of blood, resuspending the stained samples in equivalent volumes of FACS buffer, and running equal volumes of each sample using the MACSQuant.

Flow cytometry. Cells were isolated from relevant tissues, homogenized, and red blood cells were lysed with RBC lysis buffer (Biolegend). Cells were stained with the following fluorophore-conjugated antibodies that were purchased from Biolegend, Ebioscience or BD Pharmingen in various colors and combinations: CD11b, CD45, CD3ε, Ter119, Gr1, CD19, CD11c, B220, CD138, cKit, Thy1.2, IL7Rα, Lin, CD34, Sca1, Flk2, CD43, CD48, CD150, CD45, F480 (Biolegend); IgM, CD34 (eBioscience); AnnexinV (BD Pharmingen). 7AAD (BD Pharmingen) was used in some stains to distinguish live from dead cells. Cells were sorted with a MACSQuant (Miltenyi Biosciences) or a FACSCalibur (Becton Dickinson) and all data was analyzed with FlowJo software (Treestar). Specific gating strategies are available upon request.

Cell sorting for sponge reconstitution. 5-FU enriched bone marrow was transduced with retroviral vectors as previously described. Prior to IV injection into mice, GFP-positive MG and MG-Sponge transduced samples were sorted using an iCyt cell sorter. Equal numbers of cells were then injected into lethally irradiated recipient animals.

Target analysis. Predicted miR-125b targets in *H. Sapien* were downloaded from Targetscan and rank-ordered by their Probability of Conserved Targeting (P_{CT}) scores. Repeated target genes were removed from this list using a PERL script (available upon

request). Literature search was performed on the resulting list in order to identify candidate target genes. For one target, Lin28, the 3' UTR for the mouse version of the gene was obtained and aligned with miR-125b using TargetScan.

Luciferase reporter assays. Luciferase reporter assays to measure microRNA-based repression of target 3' UTRs were performed as previously described. For de-repression assays with the sponge construct, we added MG (empty vector) or MG-Sponge to the transfection mix. After 24 hours, cells were lysed with Passive Lysis Buffer (Promega) and luciferase reporter assays were performed as previously described.

RNA preparation and quantitation. RNA was isolated with TRIzol (Invitrogen) as per the manufacturer's instructions. We performed SYBR Green (Quanta Biosciences) based quantitative real-time PCR (qPCR) with a 7300 Real-Time PCR machine (Applied Biosystems) to assay Lin28, BMF, Trp53inp1, Bak1, mutant miR-125b and L32 mRNA levels. A Taqman approach was used to quantitate levels of miR-125b and snoRNA-202 (Applied Biosystems). All genes measured using SYBR Green were normalized to L32. MicroRNA-125b levels measured by Taqman were normalized to snoRNA-202. Sequence specific primers are listed in Table S1.

Immunoblotting. Total cell extracts were fractionated by electrophoresis on a 12% SDS polyacrylamide gel and electroblotted onto a Trans-Blot nitrocellulose membrane with a semidry transfer apparatus. Protein detection was performed using the following antibodies: Lin28 (A177) (Cell Signaling); Beta-Actin (A1978) (Sigma); anti-rabbit HRP-conjugated secondary antibody and anti-mouse HRP-conjugated secondary antibody (Santa Cruz Biotechnology).

Statistical tests. Statistical tests were performed using Graphpad PRISM software or Microsoft Excel. The two-tailed student's T test was used to determine P values.

Figure Legends

Figure 1. MiR-125b overexpression causes an aggressive myeloid leukemia 4-5 months post-reconstitution. A) CD45+CD11b+ cells were analyzed in the blood of MG and MG-125b expressing mice 4.5 months post-reconstitution. (Left) A representative plot is shown. (Right) CD45+ CD11b+ (cells / ml) are shown for 8 MG and 4 MG-125b mice. B) Tissue was taken from the kidney, lung and liver from MG and MG-125b expressing mice 5 months post-reconstitution, and stained with hematoxylin and eosin. The normal structures of the MG-125b mouse kidney, lung and liver are effaced by a dramatic infiltrate of leukemic cells. A representative image for each tissue is shown. Scale bar, 40 microns. C) CD45+ GFP+ and CD11b+ GFP+ cells were analyzed in the brain of MG and MG-125b expressing mice 5 months post-reconstitution. (Top) A representative plot is shown. (Bottom) % GFP+ CD45+ and % GFP+ CD11b+ are shown for 3 MG and 2 MG-125b mice. Data shown graphically represents Mean + SEM. All data is representative of two independent experiments. Abbreviations: Inf, Infiltrate; G, Glomerulus; A, alveolar space; CV, central vein.

Figure 2. MiR-125b overexpression causes a rearrangement of the hematopoietic system at 7 weeks post-bone marrow reconstitution. A) Leukocyte and erythroid compartments in the spleens of MG and MG-125b overexpressing mice were measured by flow cytometry. B) GFP+ hematopoietic progenitor populations in the bone marrow of MG and MG-125b mice were measured by flow cytometry. C) The percent of GFP+ long-term HSCs (LT HSC) and multipotent progenitors (MPPs) were measured by flow cytometry in the bone marrow. Horizontal lines represent the mean and each dot represents an individual mouse. Data is representative of 2-3 independent experiments with 4-5 mice per group. Abbreviations: CMP/GMP, Common Myeloid

Progenitor/Granulocyte Macrophage Progenitor; MEP, Myeloid Erythroid Progenitor; CLP, Common Lymphoid Progenitor.

Figure 3. Design of the miR-125b "sponge" decoy system. A) MiR-125b sponge decoy was constructed by placing four miR-125b complementary sites downstream of GFP in MG, with 4-6 nucleotide spacers in-between each site and a region of mismatch corresponding to nucleotides 9-12 of the microRNA. B) Sponge de-represses a miR-125b reporter vector comprised of 2 perfect antisense sites placed downstream of luciferase. C) Sponge does not de-repress the pMiR-Report negative control vector. Luciferase values were measured and normalized to the loading control Beta-gal. Assays were performed with three samples per group. Results are representative of three independent experiments.

Figure 4. MiR-125b loss-of-function decreases hematopoietic output. A) GFP+ cells were FACS-sorted from MG and MG-Sponge transduced progenitor-enriched bone marrow. Equal numbers were injected into two groups of 10 recipient animals. (Left) Oval indicates gating on the primary population of cells. (Right) GFP+ cells within the primary population were FACS-sorted as indicated by the blue arrow. B) Total numbers of white blood cells (CD45+), myeloid (CD45+ CD11b+), granulocytes (CD45+ Gr1+), B cells (CD45+ CD19+ CD3-), T cells (CD45+ CD3+ CD19-) and pre-erythrocytes (CD45+ Ter119+ CD19-) were measured by flow cytometry. Data shown represent Mean + SEM and indicate number of cells/ml of blood. P values were calculated using a student's T test and are indicated for each graph. All results are representative of two independent experiments with 9-10 animals per group.

Figure 5. Lin28 is a primary target of miR-125b in the hematopoietic system. A) Relative expression of miR-125b candidate targets in miR-125b overexpressing progenitor-enriched bone marrow compared to MG control was measured by qPCR. B) Relative luminescence of miR-125b candidate targets in the presence of miR-125b and normalized to empty vector control. The Picalm 3' UTR is shown as a negative control since it contains no miR-125b putative binding sites. 2-mer consists of two adjacent miR-125b antisense sites and serves as a positive control. C) Relative expression of the genes Lin28, trp53inp1 and BMF were measured in sponge-transduced progenitor-enriched bone marrow and normalized to MG transduced controls. The percent expression relative to MG is shown. Broken line demarcates 100%. Data is Mean + SEM of five samples per group. D) Schematic layout of the Lin28 mRNA coding sequence (blue) and 3' UTR with relative location of the miR-125b binding site. Depiction is not to scale. Predicted interaction between the Lin28 3' UTR and miR-125b microRNA is shown below. E) Relative luminescence of a reporter vector containing the Lin28 3' UTR co-transfected into 293T cells with either MG or miR-125b sponge decoy vector. The relative luminescence is depicted graphically as the Mean + SEM of six samples per group. K562 cells were transduced with either MGP empty vector or MGP expressing miR-125b-1, miR-125b-2 or a miR-125b seed mutant. F) Relative RNA and G) protein expression of Lin28 are shown. β-actin is included as a loading control for Western blotting. L32 is used as a loading control for mRNA expression. All data shown represent Mean + SEM. * indicates $p < 0.02$; ** indicates $p < 0.01$. A, E, F and G are representative of 2-3 independent experiments. B depicts the Mean + SEM of three independent experiments with three technical replicates per sample.

Figure 6. Lin28 overexpression inhibits all hematopoietic lineages in the blood. A) MIG and MIG-Lin28 were transfected into 293T cells. Lin28 expression was measured

by Western blot. β-actin serves as a loading control. B) Progenitor-enriched bone marrow was transduced with MIG or MIG-Lin28. The % GFP+ cells in each sample were measured by flow cytometry and the results are indicated on each FACS plot. C) Five weeks post-reconstitution, the number of cells/ml in the blood of the following cell types were measured by flow cytometry: white blood cells (CD45+), myeloid (CD45+ CD11b+), granulocytes (CD45+ Gr1+), B cells (CD45+ CD19+ CD3-), T cells (CD45+ CD3+ CD19-), NK cells (CD45+ NK1.1+) and erythrocytes (Ter119+ CD11b-). Data shown graphically represents the Mean + SEM. P values are also shown. Data is pooled from two independent groups of MIG-Lin28 animals, each with 7-8 animals and compared to MIG (6 animals).

Figure 7. ShRNA knockdown of Lin28 increases myeloid cells at the expense of B cells. A) Flow cytometry of progenitor-enriched bone marrow transduced with MG or MG-Lin28 shRNA was performed and the percent GFP+ cells is indicated. B) Lin28 mRNA expression was performed on the samples shown in A, and results were normalized to L32. Representative of two independent experiments with 4 samples per group. C) (Left) Total myeloid (CD45+ CD11b+), granulocytes (CD45+ Gr1+) and B cells (CD19+ CD3-) were measured from MG and MG-Lin28 shRNA animals and (Right) total myeloid (CD45+ CD11b+ CD19-), granulocytes (CD45+ Gr1+) and B cells (CD45+ CD19+ CD3ε-) from MG and MG-125b animals. Data depicts the number of cells/ml of blood as Mean + SEM, and was collected 5 weeks post-bone marrow reconstitution with 8-10 animals per group. P values are indicated.

Figures

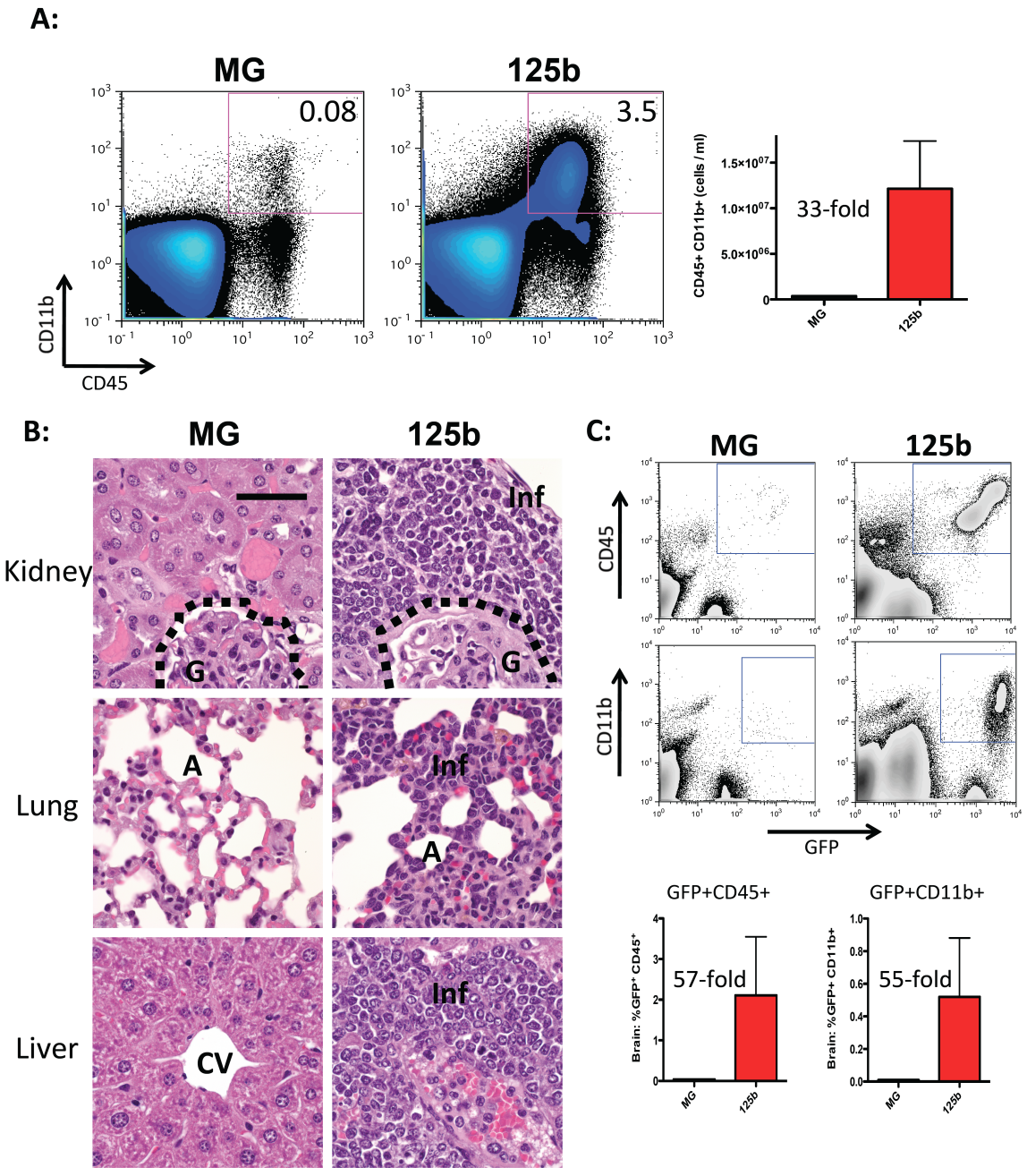

Figure 1. MiR-125b overexpression causes an aggressive myeloid leukemia 4-5 months post-reconstitution

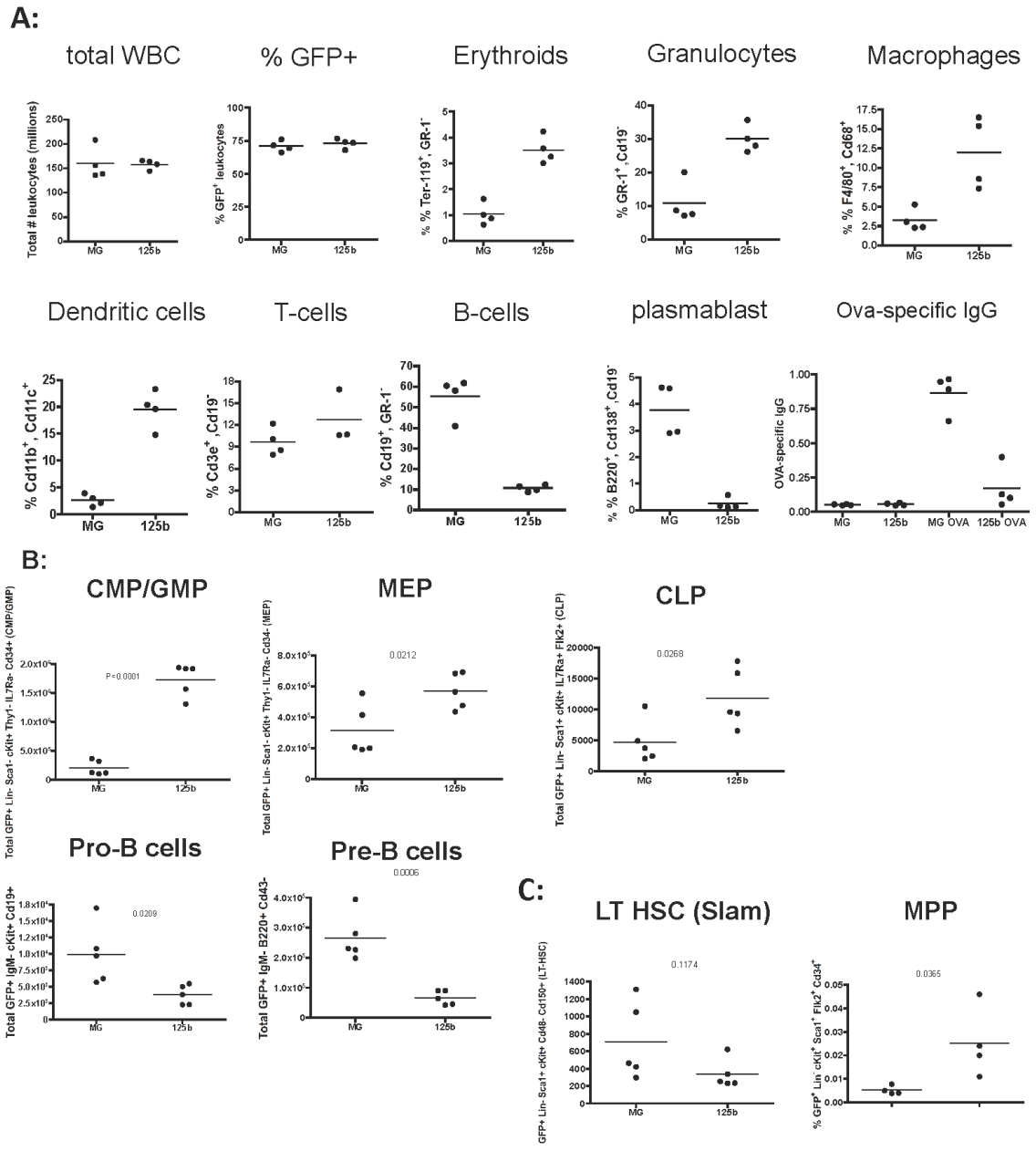

Figure 2. MiR-125b overexpression causes a rearrangement of the hematopoietic system at 7 weeks post-bone marrow reconstitution

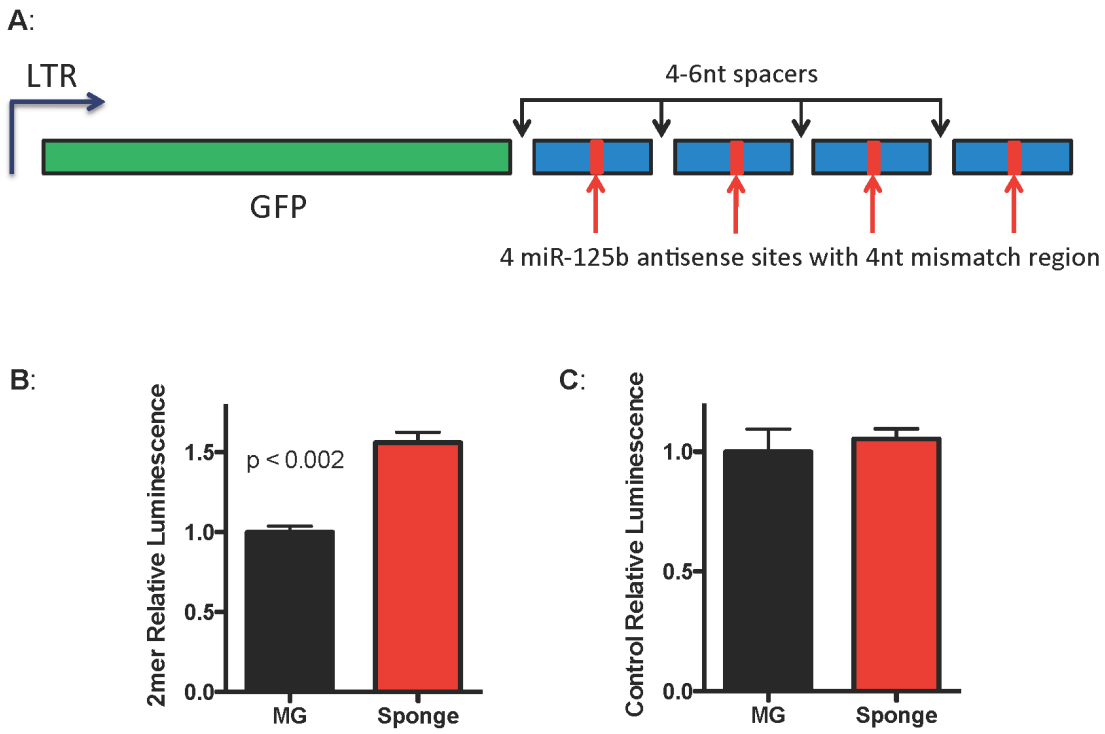

Figure 3. Design of the miR-125b "sponge" decoy system

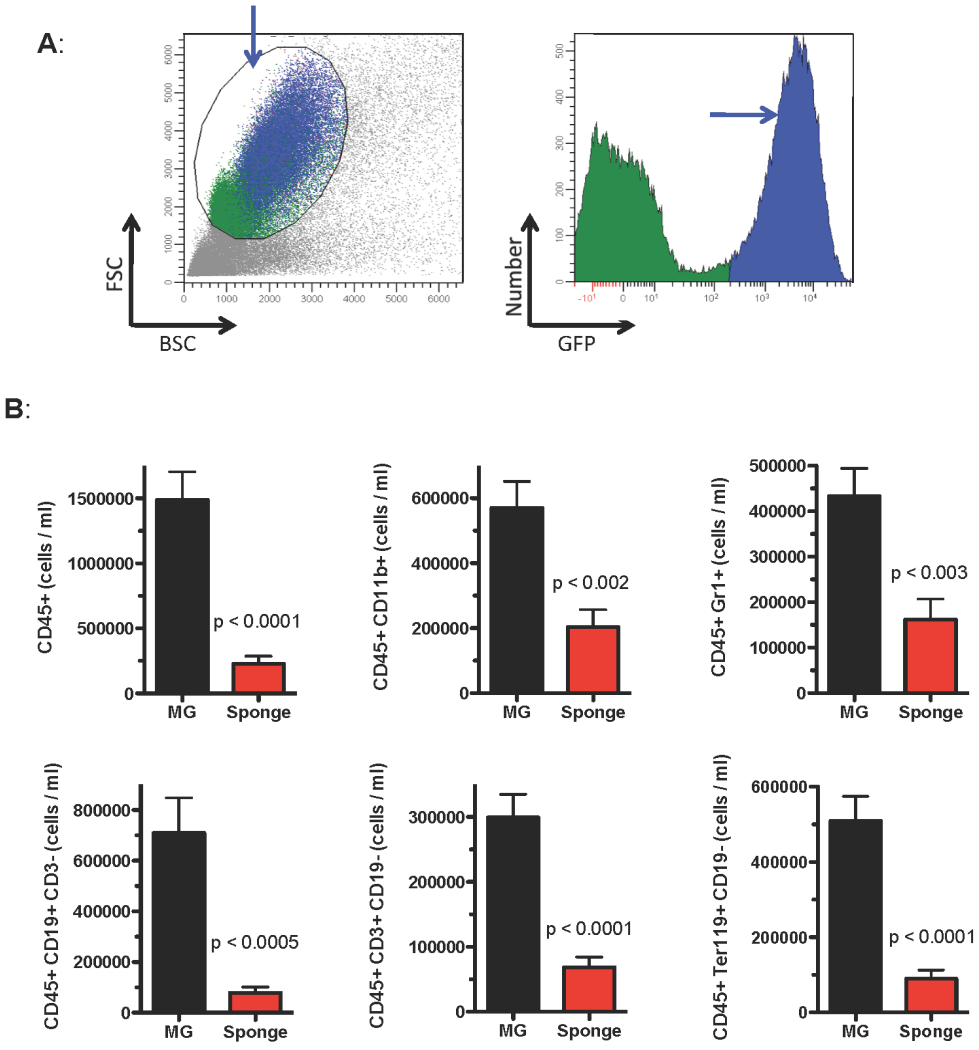

Figure 4. MiR-125b loss-of-function decreases hematopoietic output

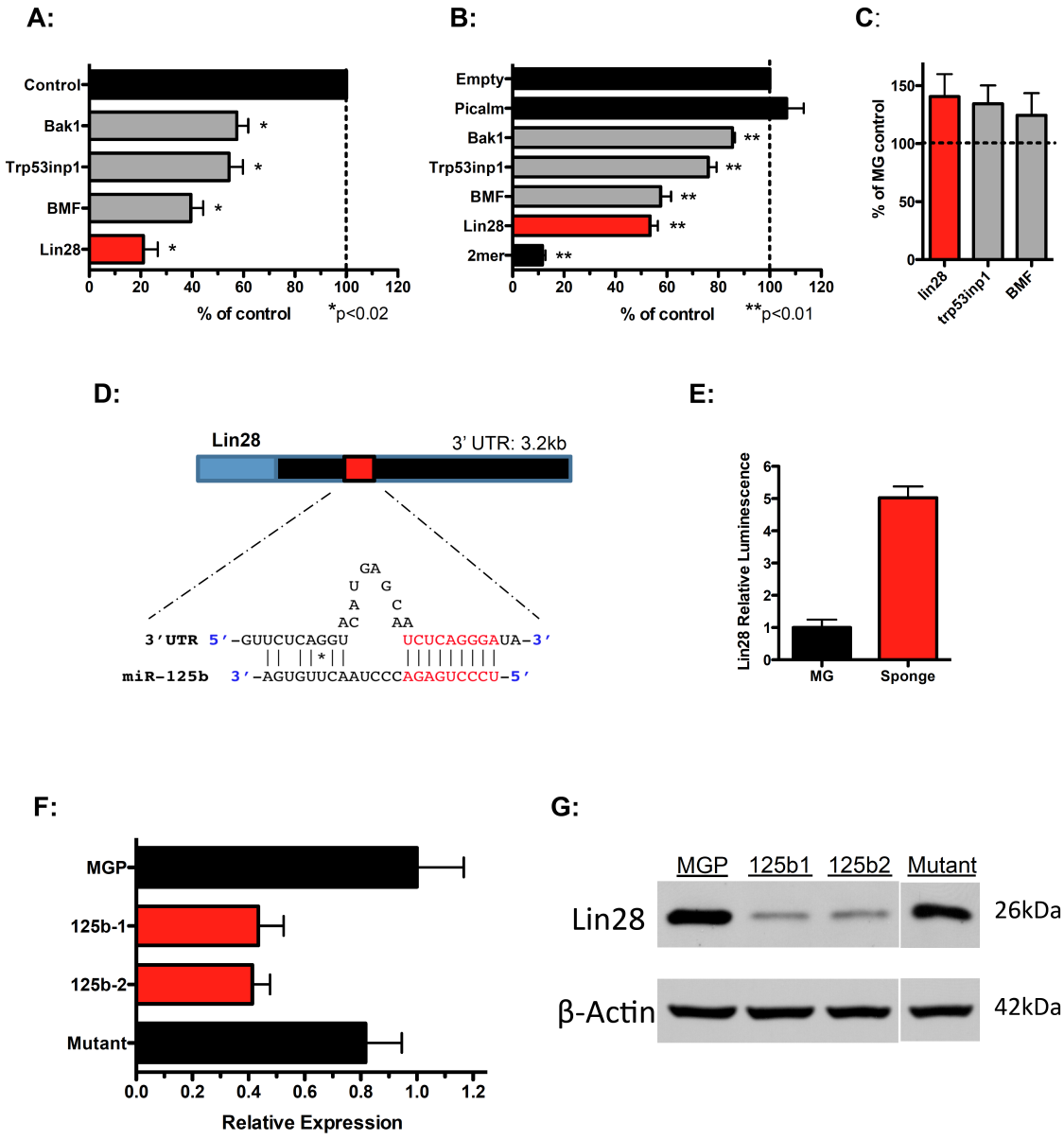

Figure 5. Lin28 is a primary target of miR-125b in the hematopoietic system

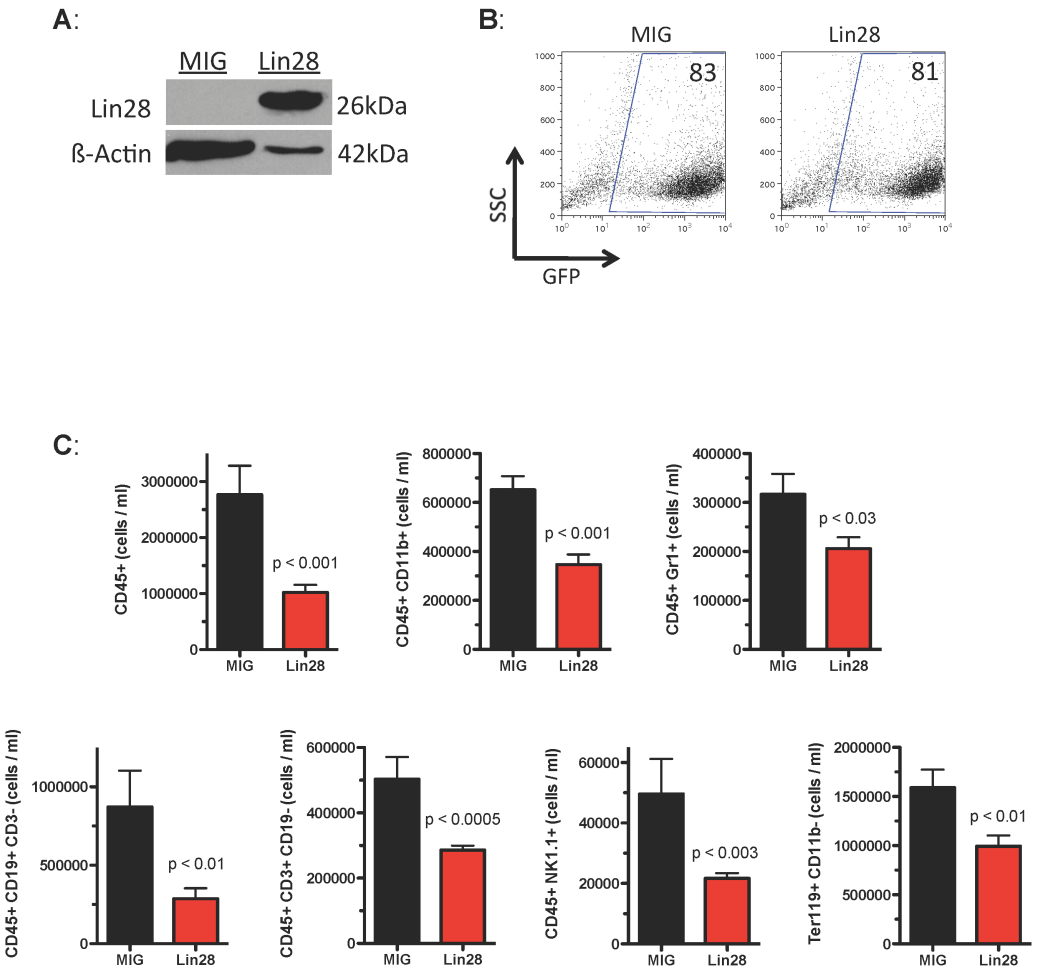

Figure 6. Lin28 overexpression inhibits all hematopoietic lineages in the blood

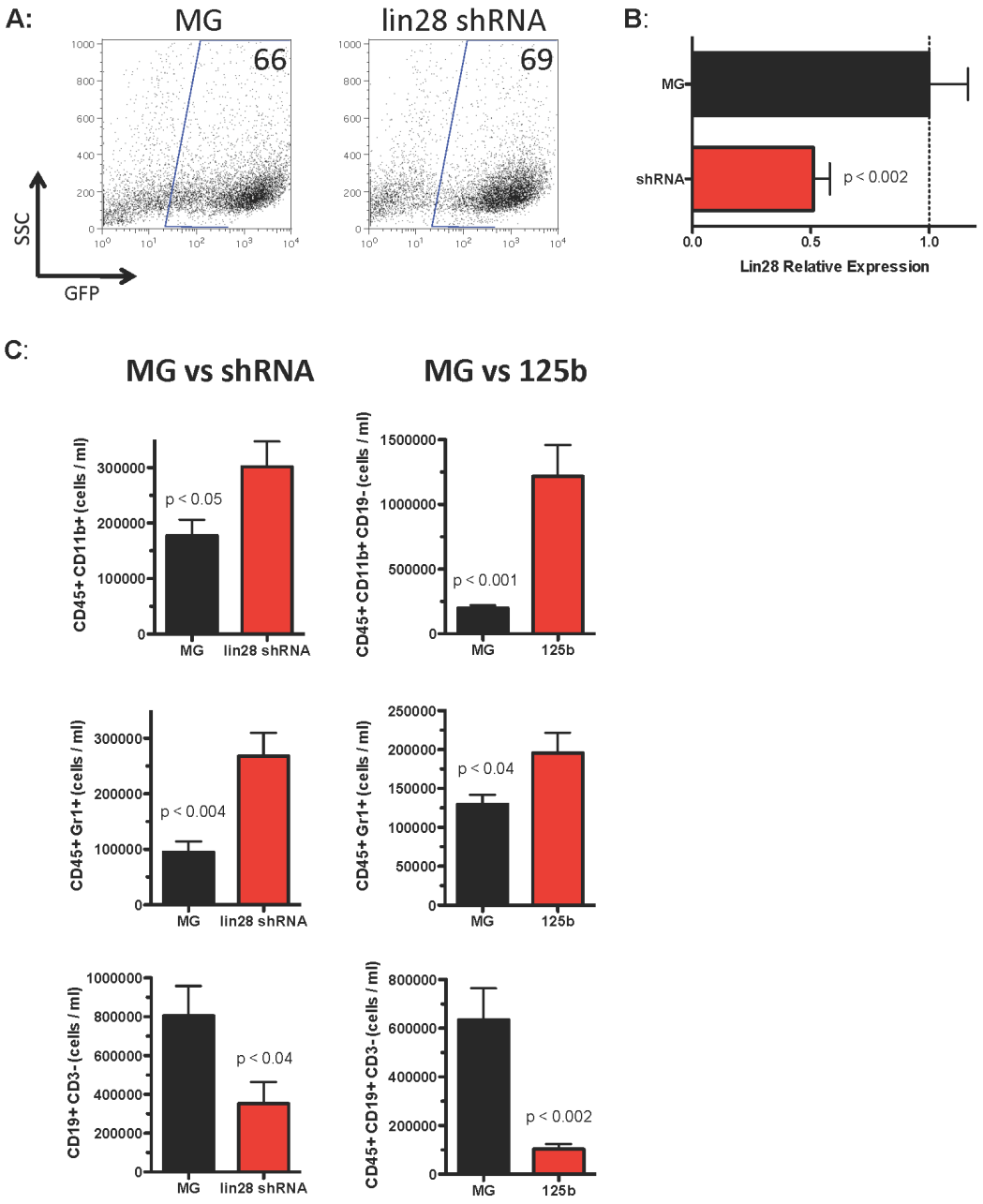

Figure 7. ShRNA knockdown of Lin28 increases myeloid cells at the expense of B cells

Supplemental Figure Legends

Figure S1. MG-125b animals develop large spleens 5 months post-reconstitution. A) Design of MG-125b, an MSCV-based retrovirus that expresses GFP and pri-miR-125b-1. B) Pre-transplant FACS showing transduction efficiency in progenitor-enriched bone marrow with MG and MG-125b. C) Quantitative PCR was performed to determine the relative expression of miR-125b in each sample. Fold-increase of miR-125b expression in the MG-125b transduced sample is indicated. D) By 5 months post-reconstitution, animals developed cancer. A representative image indicating spleen sizes of MG and MG-125b overexpressing mice at this time point is shown. E) Spleen weight in milligrams (mg) is indicated graphically. Data is from 11 MG and 11 MG-125b animals from three independent experiments harvested ~ 5 months post-reconstitution.

Figure S2. MiR-125b causes extramedullary myelopoiesis 5 months post-reconstitution. A) Relative leukocyte counts, as measured by flow cytometry, of MG and MG-125b spleens 5 months post-reconstitution were measured by flow cytometry. B) Representative FACS data showing the percent of GFP+ CD11b+, GFP+ CD19+, CD11b+ Gr1+ and GFP+ CD3+ in MG-125b animals 5 months post-reconstitution compared to MG controls. Data is representative of 7 MG and 7 MG-125b animals.

Figure S3. MG-125b overexpression leads to death by 5 months post-reconstitution. A) Survival curve showing percent survival of 8 MG and 4 MG-125b animals over a 5 month period. B) B cell (CD45+ CD19+ CD11b-) and T cell (CD45+ CD3+) numbers (number of cells/ml) were measured by flow cytometry 4.5 months post-reconstitution. Mean + SEM are indicated. Data are from 4 MG-125b and 8 MG animals.

Figure S4. Progression of MG-125b cohort #2 towards cancer. A) FACS to determine the percent GFP+ cells was performed on MG and MG-125b transduced donor bone marrow cells prior to bone marrow transplantation. B) Survival curve of miR-125b overexpressing animals compared to MG controls. The arrow just prior to 21 weeks indicates when these animals were bled for FACS. Data is from 4 MG and 10 MG-125b animals. C) The number of total myeloid cells (CD45+ CD11b+ CD19-), D) B cells (CD45+ CD19+ CD11b-), and E) T cells (CD45+ CD3ε+) was measured at the indicated time point. Values shown represent the number of cells/ml. C represents Mean + SEM. For D and E, each dot represents an individual mouse, and the horizontal bar represents the group average.

Figure S5. MiR-125b overexpressing animals exhibit myeloid infiltration into peripheral organs 5 months post-reconstitution. Moribund MG-125b animals were sacrificed and their organs harvested. Flow cytometry on GFP+ CD45+ and GFP+ CD11b+ populations were performed for the A) kidney, C) lung and E) liver. Gross pathology comparing MG and MG-125b B) kidney and D) lung are shown. F) A representative MG-125b liver is also shown. All data is representative of 8 MG animals and 5 MG-125b animals.

Figure S6. MiR-125b expression is increased in peripheral organs from MG-125b animals 5 months post-reconstitution compared to controls. RNA was harvested from moribund MG-125b animals after 5 months post-reconstitution. MiR-125b expression was measured by Taqman in A) kidney, B) lung and C) liver. Data shown is the Mean + SEM of 2-3 animals per group.

Figure S7. MiR-125a overexpression causes an aggressive leukemia 6 months post-reconstitution. Mice overexpressing miR-125a became moribund 6 months post-reconstitution and were thus harvested. Gross pathology from A) liver, B) spleen and C) kidneys are shown. Representative of two animals.

Figure S8. MG-125b splenocytes display less apoptosis than MG controls. A) Percent 7AAD+ splenocytes cultured in vitro for 204 hours is shown graphically on the left. A representative FACS plot is shown on right. B) % AnnexinV+ splenocytes cultured in vitro for 204 hours is shown on the left. A representative FACS plot is shown on right. Each dot represents a spleen culture taken from a separate mouse. Mean and SEM are shown for each set of samples, and P values are indicated.

Figure S9. Representative blood smear at 4.5 months post-reconstitution. MG-125b animals display a dramatic increase in myeloid white blood cells. Examples of this cell type are indicated by black arrows.

Figure S10. Bone marrow from MG-125b animals isolated 7 weeks post-reconstitution display hematopoietic rearrangement. A) Total white blood cell counts and B) total GFP+ white blood cells were measured in MG and MG-125b mouse bone marrows. C) Total number of GFP+ granulocytes (GFP+ Gr1+), D) dendritic cells (GFP+ CD11c+ CD19-), E) macrophages (GFP+ F480+) and F) B cells (GFP+ CD19+ CD11c-) were measured by flow cytometry. Each dot represents a separate mouse. Data is representative of two independent experiments.

Figure S11. Hematopoietic stem cell gating strategy. 1) The primary population of cells, as determined by forward scatter (FSC) and side scatter (SSC) is gated. 2) From

these cells, GFP+ Lineage- cells are gated. 3) From this population, cKit+ Sca1+ cells are subgated. 4) Within this population of cells, gating on CD150+ CD48- cells results in the long-term HSC compartment.

Figure S12. MiR-125b mutant vector expresses seed mutant version of miR-125b. A) MGP-125b-Mutant vector was designed to express a seed mutant version of miR-125b by utilizing a miR-155 arms-and-loop format (top). Sequences expressed by wild-type miR-125b and the mutant are shown below. B) Quantitative PCR shows relative expression of the mutant version of miR-125b (normalized to L32) in K562 cells transduced with MGP-125b-Mutant or MGP control.

Figure S13. Lin28 is efficiently expressed by MIG-Lin28 in progenitor-enriched bone marrow. Relative expression of Lin28 was measured in 5-FU treated bone marrow transduced with either MIG or MIG-Lin28. Each dot represents an independent sample. Horizontal lines represents the mean for each group and error bars represent SEM.

Supplemental Figures

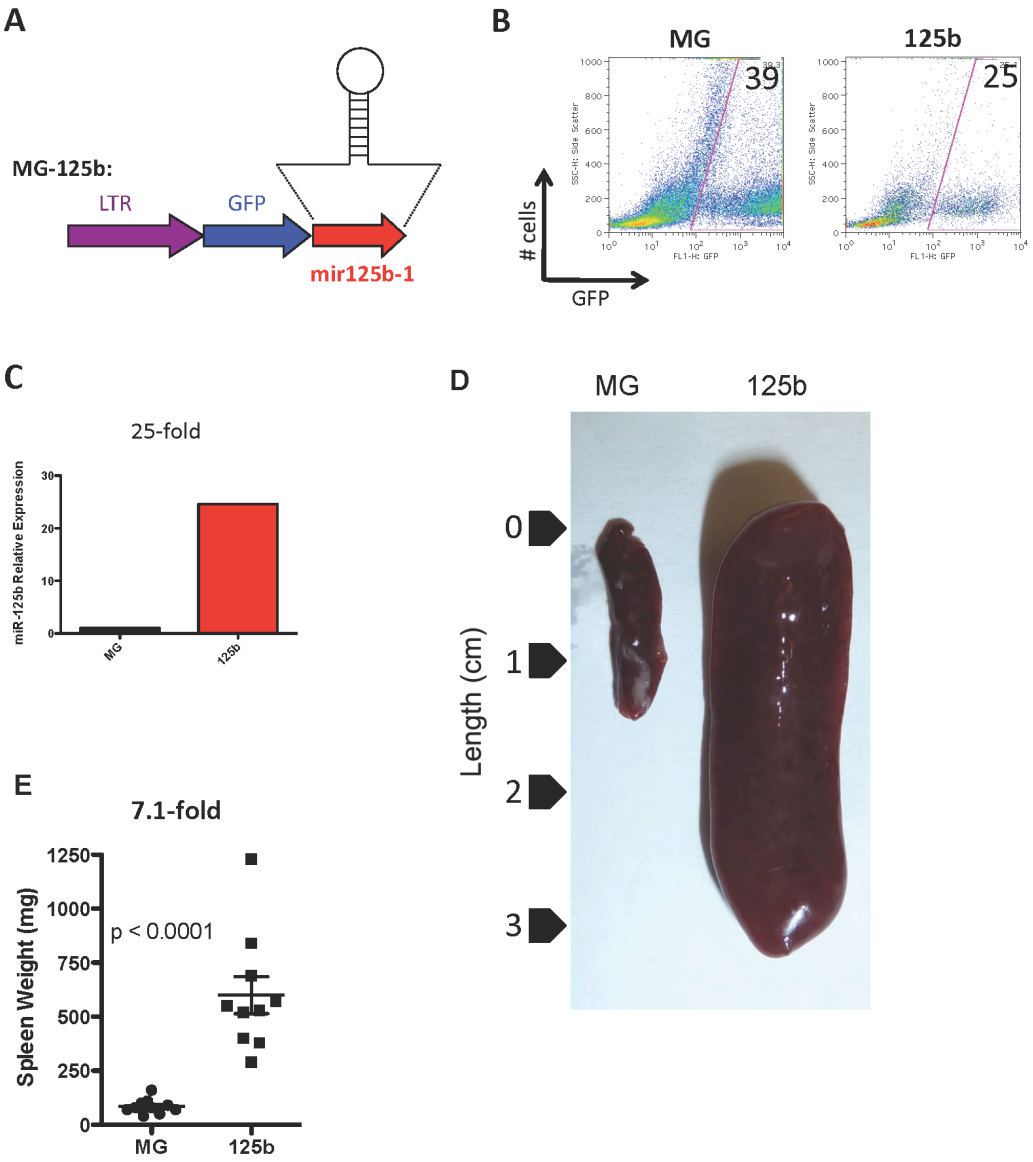

Figure S1. MG-125b animals develop large spleens 5 months post-reconstitution

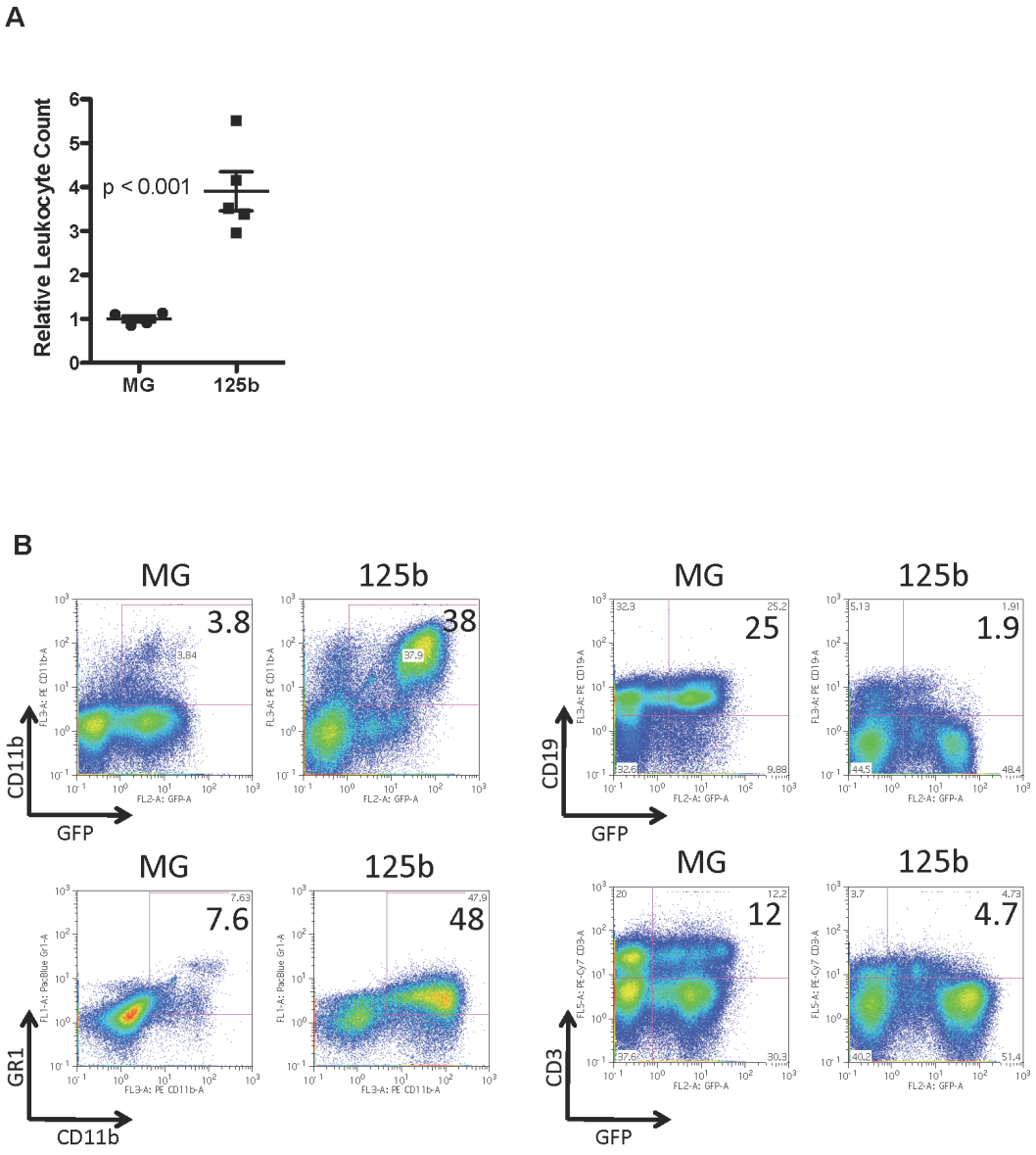

Figure S2. MiR-125b causes extramedullary myelopoiesis 5 months post-reconstitution

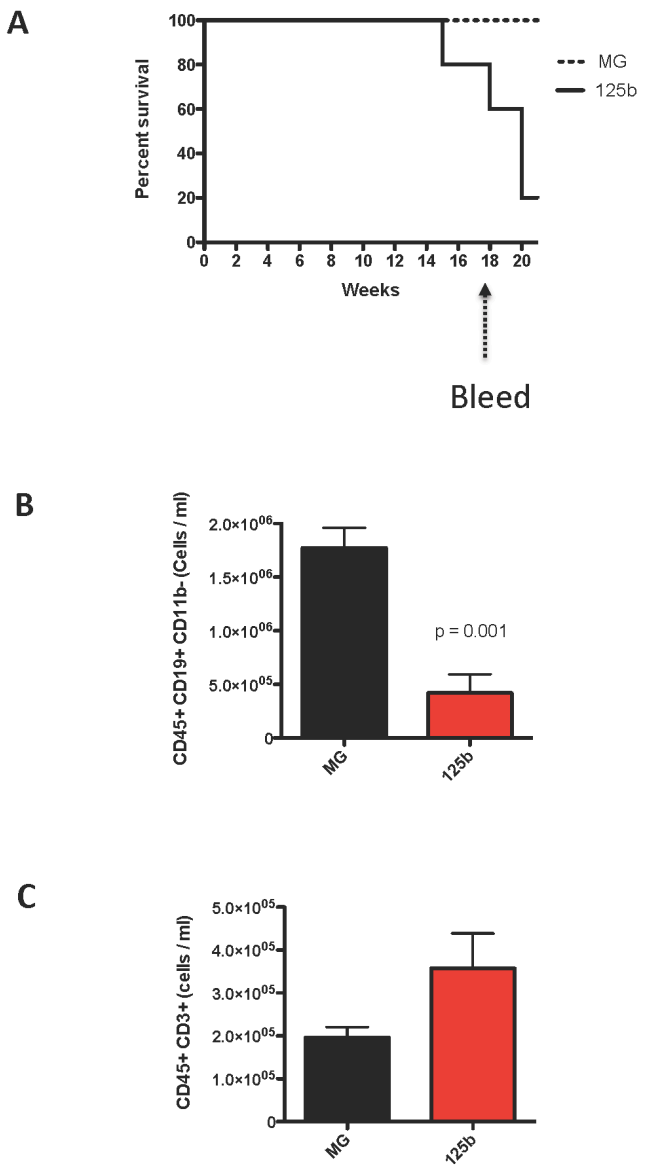

Figure S3. MG-125b overexpression leads to death by 5 months post-reconstitution

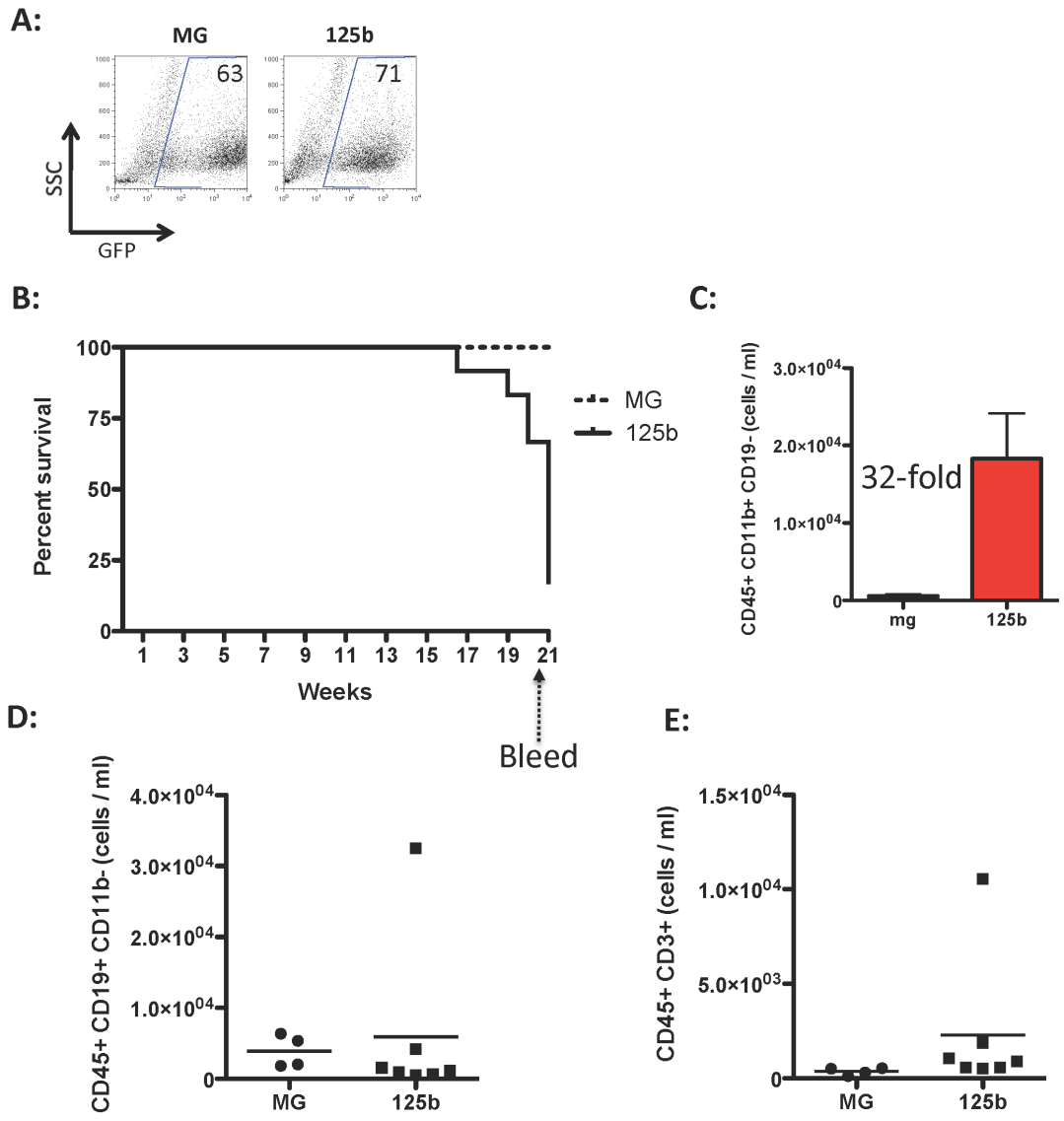

Figure S4. Progression of MG-125b cohort #2 towards cancer

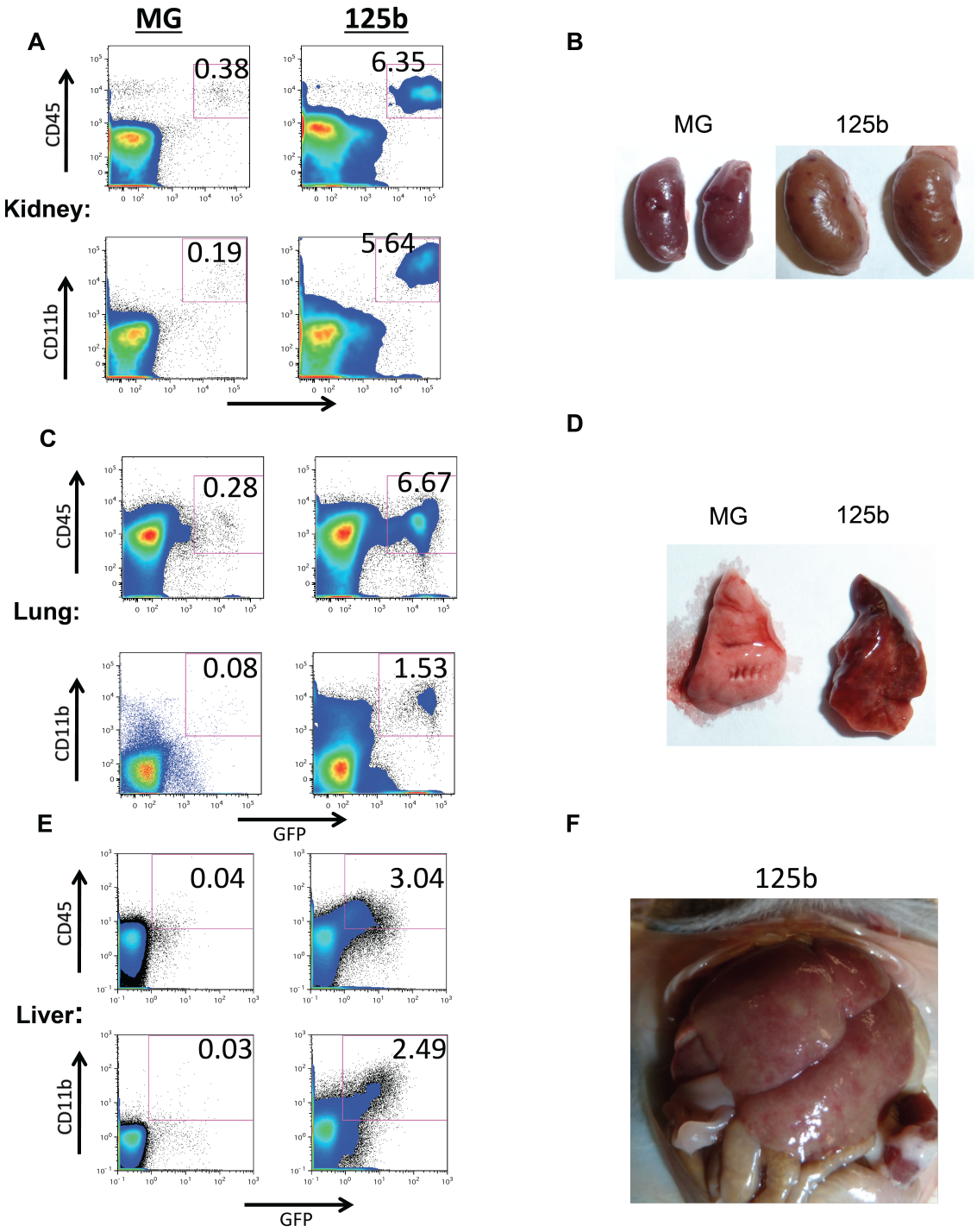

Figure S5. MiR-125b overexpressing animals exhibit myeloid infiltration into peripheral organs 5 months post-reconstitution

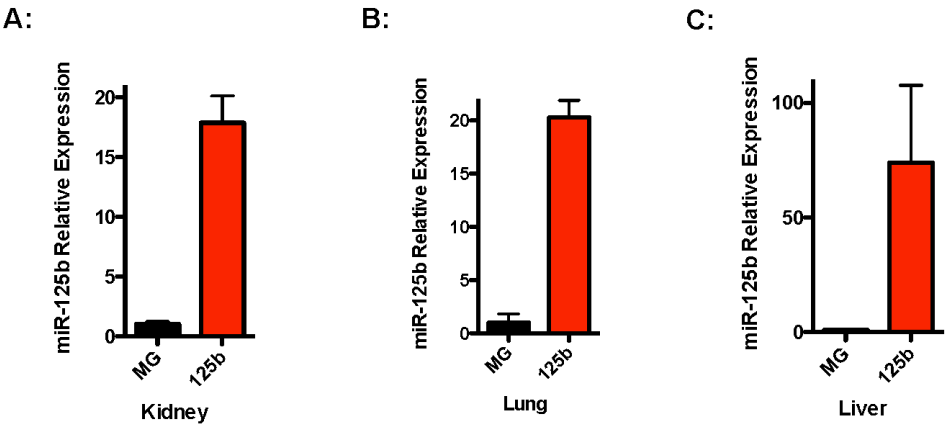

Figure S6. MiR-125b expression is increased in peripheral organs from MG-125b animals 5 months post-reconstitution compared to controls

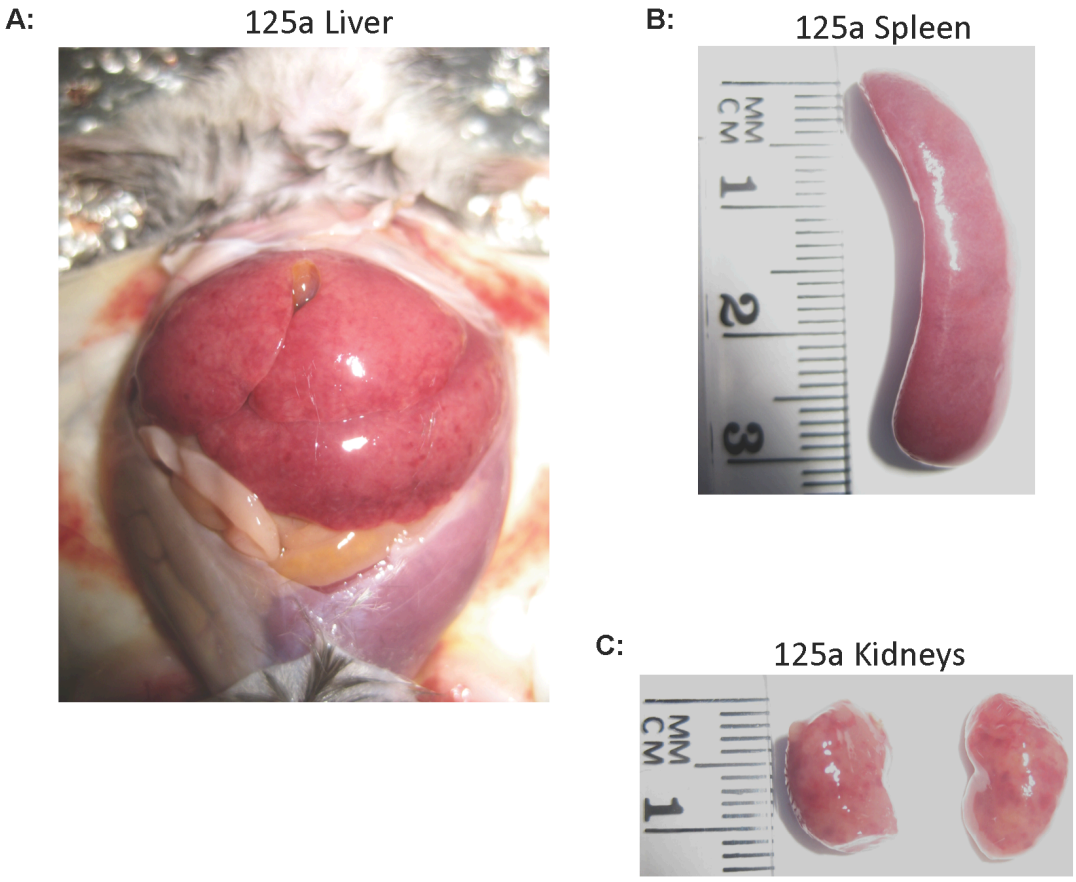

Figure S7. MiR-125a overexpression causes an aggressive leukemia 6 months post-reconstitution

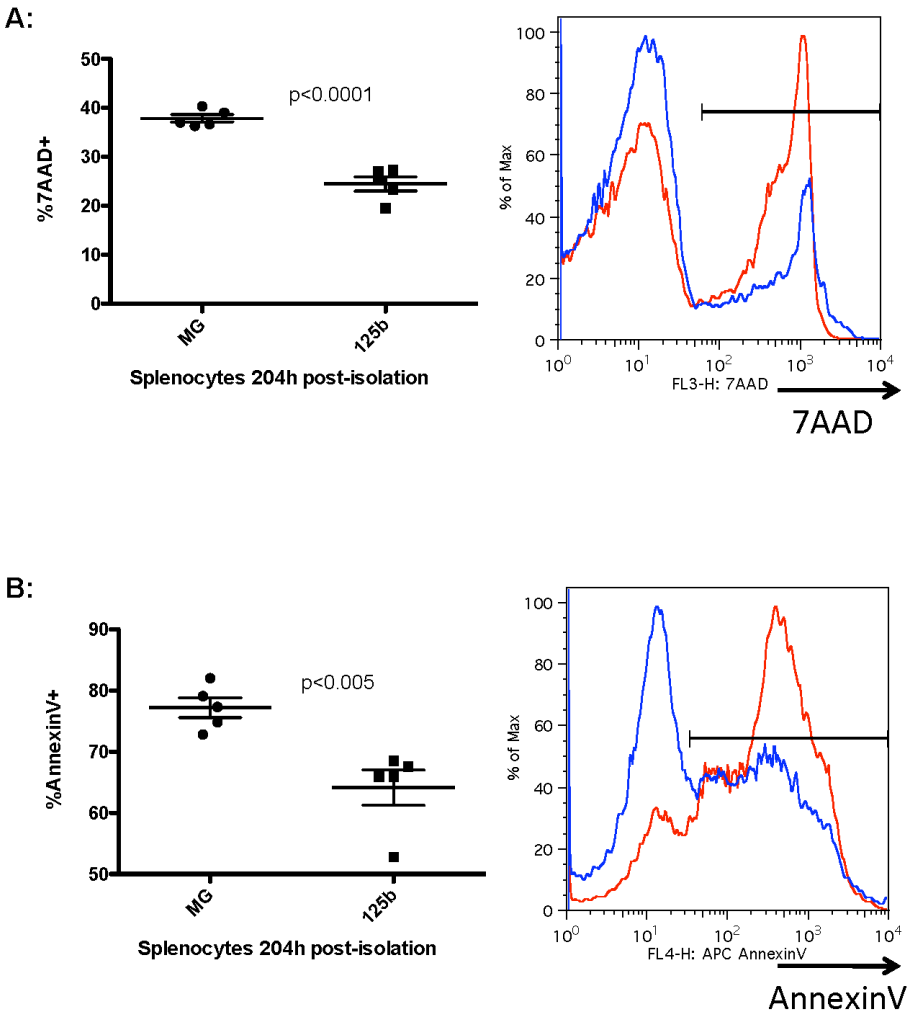

Figure S8. MG-125b splenocytes display less apoptosis than MG controls

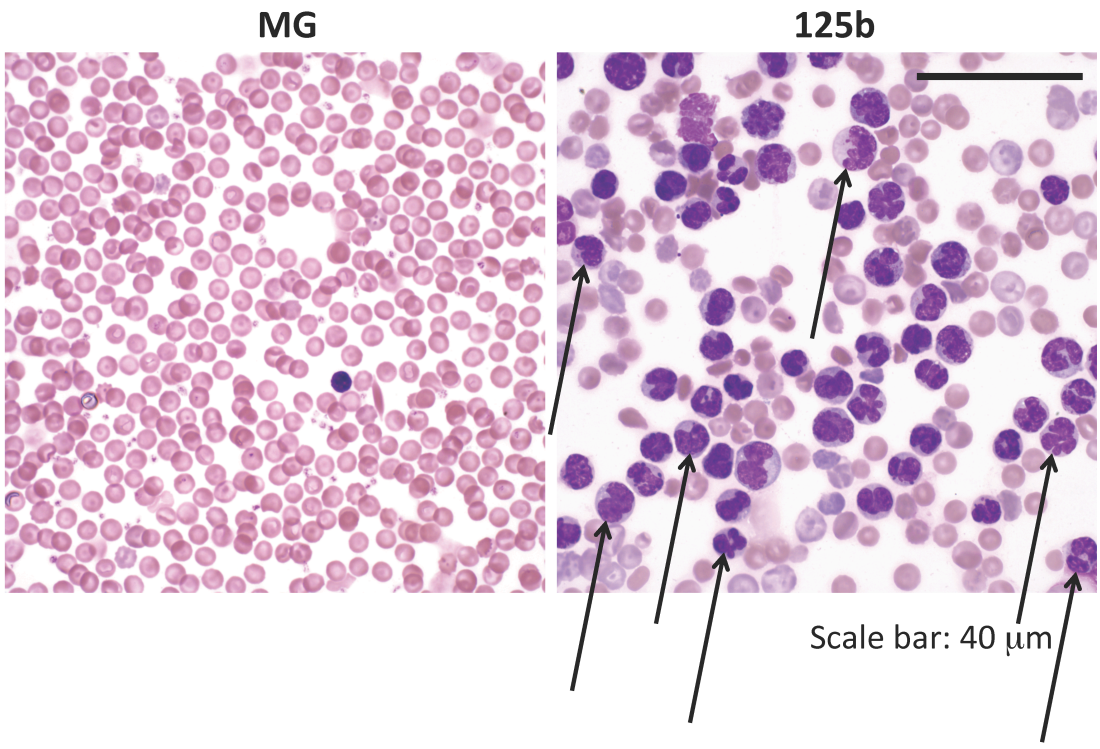

Figure S9. Representative blood smear at 4.5 months post-reconstitution

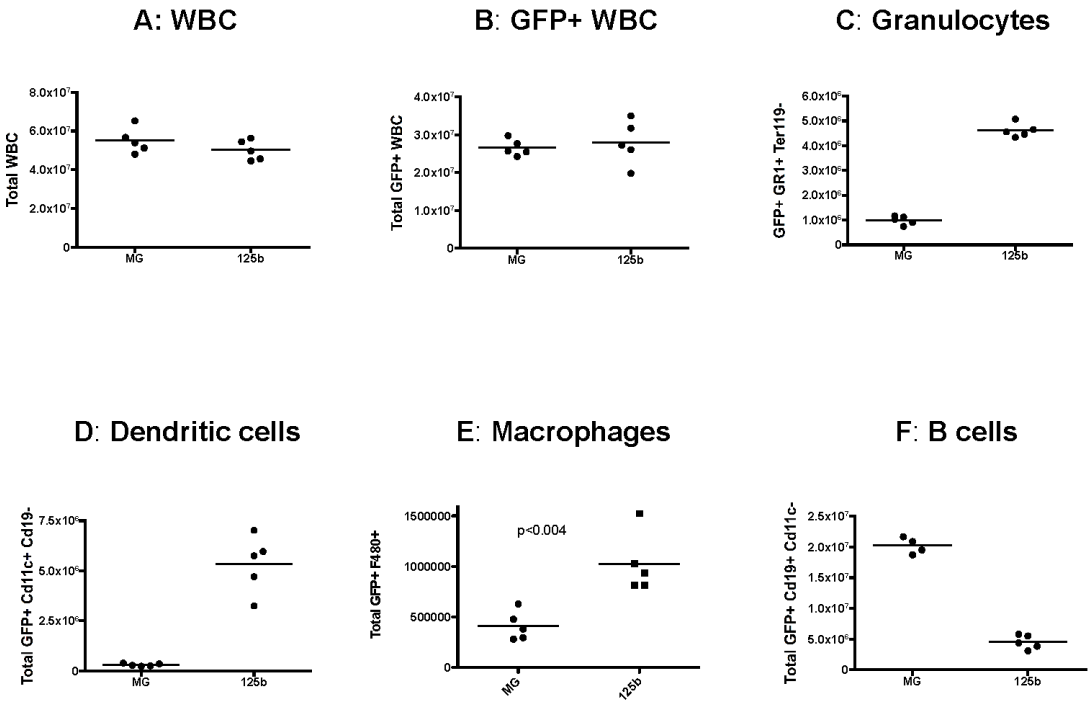

Figure S10. Bone marrow from MG-125b animals isolated 7 weeks post-reconstitution display hematopoietic rearrangement

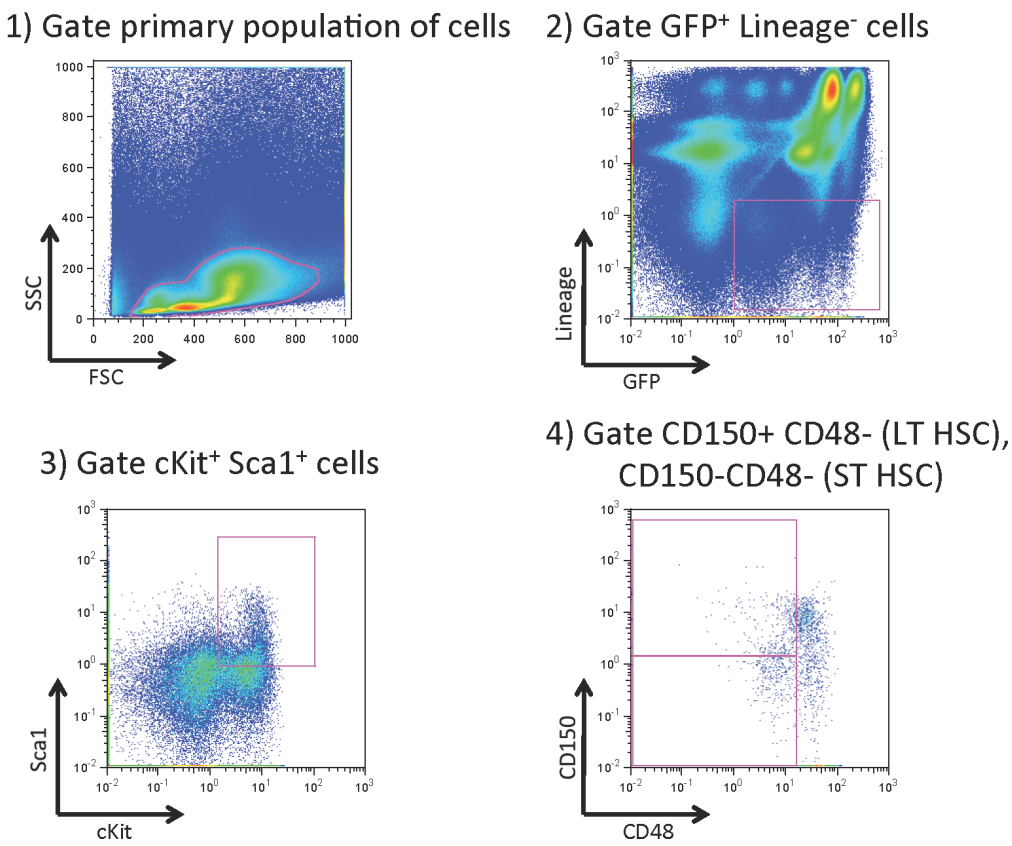

Figure S11. Hematopoietic stem cell gating strategy

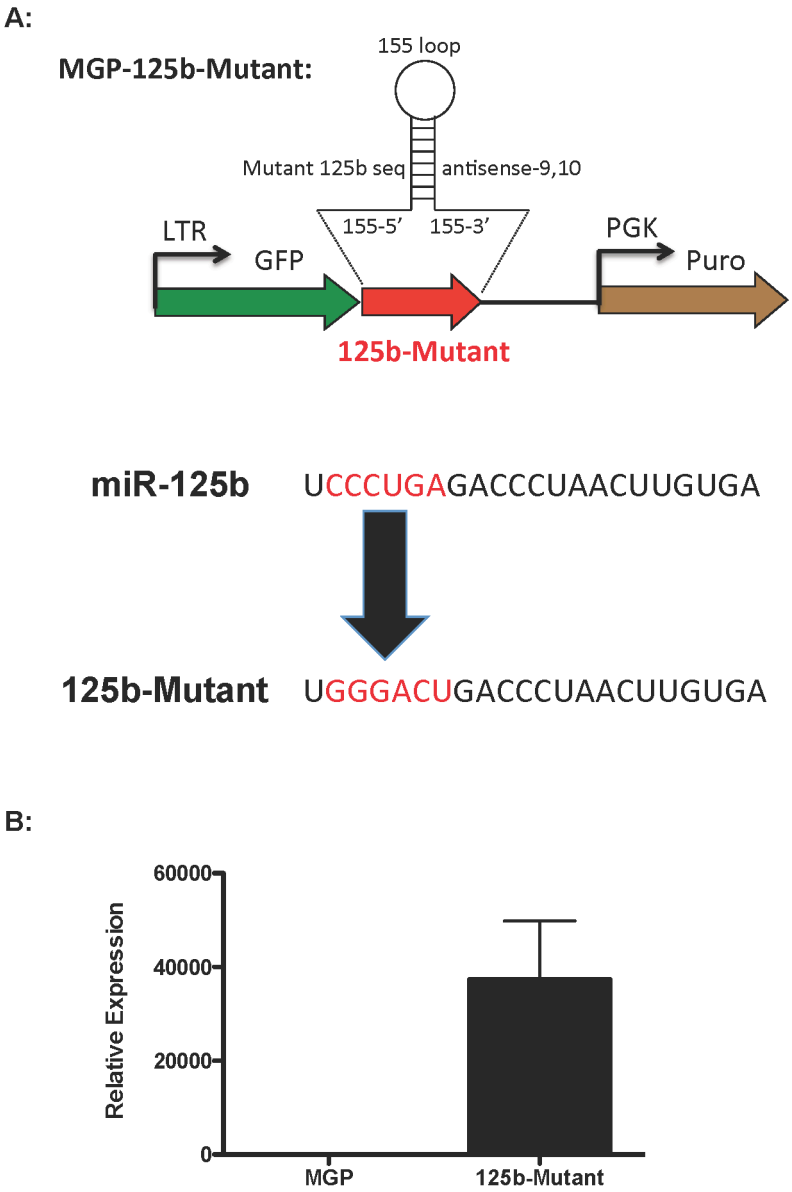

Figure S12. MiR-125b mutant vector expresses seed mutant version of miR-125b

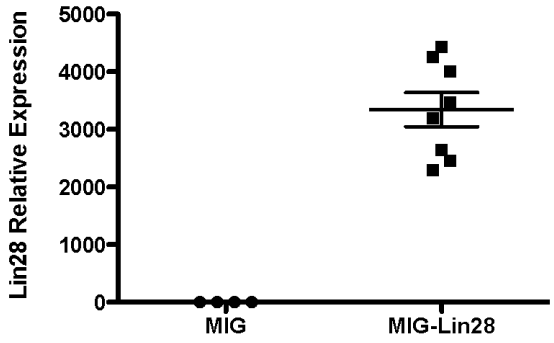

Figure S13. Lin28 is efficiently expressed by MIG-Lin28 in progenitor-enriched bone marrow

Supplemental Table

Table S1. Primer Sequences

Primer	Sequence
125b Sponge NotI 5' oligo	GGCCGCTCACAAGTTACCACTCAGGGACGATTCACAAGTTACCACTCAGGGAACCGGTTCACAAGTTACCACTCAGGGATCACTCACAAGTTACCACTCAGGGAC
125b Sponge XhoI 3' oligo	TCGAGTCCCTGAGTGGTAACTTGTGAGTGATCCCTGAGTGGTAACTTGTGAACCGGTTCCCTGAGTGGTAACTTGTGAATCGTCCCTGAGTGGTAACTTGTGAGC
Antisense 2-mer SpeI 5' oligo	CTAGTTCACAAGTTAGGGTCTCAGGGATCACAAGTTAGGGTCTCAGGGAA
Antisense 2-mer HindIII 3' oligo	AGCTTTCCCTGAGACCCTAACTTGTGATCCCTGAGACCCTAACTTGTGAA
mouse Lin28 3' UTR SpeI Fw	TTCACTAGTGGCCCAGGAGTCAGGGTTATTCTTT
mouse Lin28 3' UTR HindIII Rev	TTCAAGCTTTGAGCCTAAGTTTTCTTACGAGGGAGAA
mouse Trp53inp1 3' UTR SpeI Fw	TTCACTAGTAATGTTGAGTTATGTTGGTTTGTTTCCTTAGG
mouse Trp53inp1 3' UTR MluI Rev	TTCACGCGTTTGCTGATTTACAGGAACAAAACACTAAGC
mouse BAK1 3' UTR SpeI Fw	TTCACTAGTTCCCGGGGAGCCCCACTG
mouse BAK1 3' UTR HindIII Rev	TTCAAGCTTCCCTCTGCCTCCCCCTCTTCC
mouse Lin28 shRNA oligo	GAAGGCTGTATGCTGTTCCTTGGCATGATGGTCTAGGTTTTGGCCACTGACTGACCTAGACCAATGCCAAGGAACAGGACACAAGGCCTG
miR-125b seed Mutant oligo	GAAGGCTGTATGCTGTGGGACTGACCCTAACTTGTGAGTTTTGGCCACTGACTGACTCACAAGTGGGTCAGTCCCACAGGACACAAGGCCTG

mouse Lin28 CDS BglII Fw	TTCAGATCTATGGGCTCGGTGTCCAACCAG
mouse Lin28 CDS XhoI Rev	TTCCTCGAGTCAATTCTGGGCTTCTGGGAGCA
Mutant miR-125b qPCR Fw	TCACAAGTTAGGGTCAGTCCCA
Lin28 qPCR Fw	GTTCACCTTTAAGAAGTCTGCCA
mouse Lin28 qPCR Rev	TGTCTCCTTTGGATCTTCGCTT
human Lin28 qPCR Rev	CACCTGTCTCCTTTTGATCTGC
mouse L32 qPCR Fw	AAGCGAAACTGGCGGAAAC
mouse L32 qPCR Rev	TAACCGATGTTGGGCATCAG
human Actin qPCR Fw	CTGTGCTATCCCTGTACGCCTC
human Actin qPCR Rev	CATGATGGAGTTGAAGGTAGTTTCGT

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Chapter 7: Discussion and future directions

MicroRNAs in leukemia: Markers of disease and potential targets of therapy

Many groups are exploring the role of specific microRNAs in hematopoietic cell lineages. Georgantas III et al. found 33 miRNAs expressed in CD34+ hematopoietic stem progenitor cells (HSPCs) from normal human bone marrow and human peripheral blood stem cell harvests[1]. Bartel and colleagues showed that overexpression of mir-181 in the hematopoietic system leads to increased numbers of B cells at the expense of T cells[2]. Later, mir-150 was shown by the Rajewsky group to control B1 versus B2 B-cell differentiation via repression of c-Myb. Mir-150 was shown by luciferase assay to strongly repress via the c-Myb 3' UTR. Mir-$150^{-/-}$ mice had an expansion of B1 B cells in the spleen and periotineal cavity, likely due to de-repression of c-Myb[3].

Given the emerging role of miRNAs in controlling differentiation of hematopoietic progenitors into specific lineages, several groups performed screens on leukemic cells to reveal differences in miRNA expression compared to normal. Navarro et al. analyzed miRNA expression in the lymph nodes of patients with Hodgkin lymphoma. They revealed that 36 microRNAs were differentially expressed in nodular sclerosis versus mixed cellularity Hodgkin's lymphoma. Chromogenic in situ hybridization was used to show that Reed-Sternberg cells, the pathognomonic cell type of Hodgkin's lymphoma, preferentially express mir-21, mir-134 and mir-138 in their cytoplasm.[4]

Mi et al. showed that microRNA expression signatures can distinguish between two different forms of leukemia, acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML). ALL is the most common childhood cancer, while AML is the most common acute leukemia in adults. Mir-128a and mir-128b were significantly overexpressed in ALL compared to AML, while let-7b and mir-223 were significantly overexpressed in AML compared to ALL.[5]

The Croce and Falini groups looked at AML more extensively, specifically NPMc$^+$ NK-AML. NPMc$^+$ NK-AML is AML with a normal karyotype and a mutation in the nucleophosmin (NPM1) gene occurring on exon-12 and more rarely on exon-11. Several miRNAs were upregulated in NPMc$^+$ NK-AML versus NPMc- NK-AML including miR-155.[6] Our own studies show here that AML patients have 4.5 times higher levels of miR-155 in their bone marrow compared to normal. Also several groups have now shown that miR-125b is dysregulated in a plethora of human leukemias. By overexpressing miR-155 and miR-125b specifically in the mouse hematopoietic disease, we have been able, in some ways, to model what happens in human disease.

It will be interesting to use our robust mouse models of miR-155 and miR-125b overexpression as testbeds for microRNA-based therapies that attempt to ameliorate the profound symptoms we observed by targeting microRNA levels and related pathways following disease onset. Is the miR-125b cancer phenotype driven entirely by miR-125b? Are there secondary mutations that are required to ultimately push the mouse towards disease? Indeed it will be interesting to sequence the mouse cancer genome 5 months post-reconstitution, when the mice become moribund from the cancer, and see what genes are mutated. It is possible that miR-125b overexpression merely provides cells with enough of a growth advantage to acquire other secondary mutations, which then ultimately become responsible for pushing the mouse towards cancer. We can test this idea by performing loss-of-function on the miR-125b overexpression mice at different time points and determine whether miR-125b loss-of-function can A) prevent disease when applied during the pre-leukemic stage (4-7 weeks), B) stop disease progression during leukemic progression but prior to blast crisis (2.5-3.5 months), or C) actually halt blast crisis and instigate recovery when the mice are on the verge of death (4-5 months). Performing these experiments will be a critical step towards developing a miR-125b based therapy to treat human leukemia.

MiR-155 target identification: Can one target be the silver bullet?

MiR-155 is normally expressed at very low levels in the bone marrow compartment of mice. Expression is strongly but transiently induced after intraperitoneal (IP) injection of bacterial LPS. This injection correlates with a granulocyte/monocyte (GM) expansion[7]. Enforced high expression of miR-155 in mouse bone marrow cells leads to a similar GM expansion, but the GM cells have dysplastic characteristics reminiscent of myeloproliferative disorder (MPD) and pre-AML[7]. I was fascinated by this profound phenotype and also by its relevance to human disease. Thus I devoted the first half of my PhD to determining the mechanism by which miR-155 causes a myeloproliferative disorder in mice.

Since miRNA seed regions are small in size, targeting algorithms predict that gene regulation by microRNAs is promiscuous, with several genes targeted by the same microRNA, and several microRNAs targeting the same gene[8,9]. Here we show that miR-155 is capable of regulating several genes important for hematopoiesis by repressing expression by 22-73% via their 3' UTR regions[7]. Thus we hypothesized that sustained miR-155 expression leads to myeloproliferative disorder via combinatorial repression of several target genes. In some physiological contexts, however, a single target may account for a majority of a miRNA's physiological or pathological effects. For example, mir-150 targeting of c-Myb leads to a defect in B-cell maturation[3]. Indeed, of the 1080 genes downregulated by miR-155 overexpression in RAW cells, 89 had putative miR-155 target sites, 11 were implicated in hematopoietic development or disease, and knockdown of just one of these target genes, SHIP1, recapitulated the miR-155 overexpression phenotype in vivo. The ability for one target out of such a plethora of possible candidates to be responsible for a microRNA's phenotype is intriguing.

Similarly, for miR-125b, of the 604 candidate targets in the human genome[10], one, IRF4, appeared responsible for the macrophage activation phenotype and another, Lin28, appears to be more important than others in miR-125b's role as a regulator of hematopoiesis. It will be interesting to explore further in the future whether selective use of just a handful of targets in specific settings, similar to siRNA targeting, is a general theme in microRNA targeting. If this were the case, then why didn't microRNAs evolve to be like siRNAs? Indeed it seems on the surface that the reason microRNAs don't bind their targets perfectly, like siRNAs do, is to preserve the option to target many genes. It's the age-old quality over quantity debate; if microRNAs imperfectly bind several targets, rather than perfectly bind any one of them, then we would expect phenotypes arising from their dysregulation to be multi-target in nature, involving graded modulation of hundreds of target genes. While we did view reduction of 1,080 target genes by miR-155 overexpression in macrophages, it is nonetheless intriguing that just one of these targets, SHIP1, was primarily important in the context of hematopoietic disease.

MiR-125b as a macrophage activator

Here we show that miR-125b is enriched in macrophages and that elevating miR-125b promotes macrophage activation, IFNγ response, and immune function of these cells. Interestingly, miR-125b in macrophages promoted the ability of these cells to present antigen and induce T cell activation, demonstrating that miR-125b can enhance the macrophage's role in mediating adaptive immunity. In addition to its intrinsic function in macrophages, miR-125b likely further amplifies its role in these cells in vivo through a paracrine mechanism by enhancing their response to interferon γ.

We found that macrophages overexpressing miR-125b had increased surface expression of co-stimulatory molecules, allowing them to be more effective at activating

T cells. The increase in activated T cells would in theory result in more secretion of IFNγ, which in turn further magnifies the activation status of miR-125b expressing macrophages, given that these cells exhibit a higher response to IFNγ signaling. In this model, by affecting macrophage function alone, miR-125b could amplify immune responses by orchestrating positive feedback loops between macrophages and T cells. Breaking this positive feedback loop by inhibiting miR-125b in macrophages in vivo may be an effective strategy to treat autoimmune diseases such as Multiple Sclerosis and Macrophage Activation Syndrome that involve both macrophages and T cells in their pathogenesis.

We demonstrated here that IRF4 is a target of miR-125b and that IRF4 knockdown mimics the miR-125b overexpression phenotype in macrophages. MiR-125b's ability to potentiate macrophage activation is consistent with previously demonstrated ability of IRF4 to inhibit pro-inflammatory macrophage polarization. IRF4 is a competitive inhibitor of IRF5 [11], a transcription factor that upregulates macrophage inflammatory genes [12]. By inhibiting IRF4, miR-125b may de-repress IRF5, allowing IRF5 to more effectively undergo its function, thus heightening macrophage activation. Indeed, polymorphisms in the IRF5 gene associated with increased IRF5 mRNA levels have been reported in Macrophage Activation System [13], Multiple Sclerosis [14-16], Systemic Lupus Erythematosus [17-19], Rheumatoid Arthiritis [20,21] and Inflammatory Bowel Disease [22]. It will be interesting to examine whether dysregulated miR-125b levels are also associated with these autoimmune disorders.

MiR-125b enhances macrophage activation and appears to do so by inhibiting IRF4 expression. IRF4 has been shown to polarize macrophages towards the anti-inflammatory (M2) type [23], while the factor it inhibits, IRF5, polarizes macrophages towards the pro-inflammatory (M1) type [12]. Thus it will be interesting to investigate

whether miR-125b acts as a toggle switch that regulates macrophage polarization in response to situations such as acute infection, sepsis or cancer.

MiR-125b is upregulated in certain leukemias and downregulated in many solid non-hematopoietic solid tumors [24-35]. While tumor associated macrophages (TAMs) are known to infiltrate solid tumor cancers and help promote tumor development [36], to our knowledge none of these studies separated macrophages from the cancerous tissue prior to analyzing microRNA levels. Thus it is tempting to speculate that the results of these studies may be due to the presence of tumor infiltrating macrophages in the cancerous tissue. We have shown here that increased miR-125b expression in tumor macrophages slows tumor growth. TAMs that promote tumor growth may thus downregulate miR-125b; it will be interesting to measure miR-125b levels in TAMs and determine if this is indeed the case. Our data suggests that supplementing tumor macrophages with miR-125b may be a useful therapeutic strategy for treating solid tumor cancers.

MiR-125b as a positive regulator of myelopoiesis

We show here that miR-125b very strongly promotes early myeloid progenitors, and promotes all downstream myeloid lineages in vivo even prior to the onset of hematopoietic disease. Thus we suggest that miR-125b causes an aggressive myeloid leukemia by strongly driving development of early myeloid progenitors. Consistent with this result, we saw that miR-125b overexpression dramatically increased numbers of myeloid progenitors even prior to the onset of overt disease. Indeed, it has been suggested that certain leukemias may arise from committed progenitor cells that have acquired stem cell capacity for self renewal[37,38]. While miR-125b has been shown to have anti-apoptotic effects in multiple cell types[28,39,40], it is possible that miR-125b

endows myeloid progenitors with a particularly potent survival advantage, giving them the opportunity to transform into cancer stem cells.

While miR-125b loss-of-function leads to a decrease in all downstream hematopoietic lineages suggesting a physiological role in hematopoietic stem cells, our overexpression data suggests that miR-125b has different roles in different hematopoietic lineages. MiR-125b enhanced all lineages immediately downstream of HSCs including multipotent progenitors (MPPs), common lymphoid progenitors (CLPs) and myeloid progenitors (MPs). Enhancement of myeloid progenitors was much greater than that of other lineages. On the contrary, pro-B cells were significantly decreased in both the bone marrow and spleen in mice overexpressing miR-125b. Indeed, miR-125b overexpressing animals displayed decreased numbers of B cells in the blood, spleen and bone marrow. Interestingly, miR-125b sponge mice also displayed decreased B cell numbers. We believe this is because miR-125b loss-of-function decreases CLP numbers, which is upstream of the pro-B cell stage of hematopoiesis; thus all lymphoid cells, including B cells, become subsequently decreased.

The differential effects of miR-125b on different hematopoietic progenitors may be explained by regulation of different sets of targets. Our data here suggests that Lin28 is a primary target of miR-125b in the context of hematopoietic stem cell output. Indeed, Lin28 overexpression in vivo led to a significant decrease in all downstream hematopoietic lineages examined, mimicking the miR-125b loss-of-function phenotype. Other groups have shown Lin28 is an embryonic stem cell specific gene[41-43]. Our data suggests that it also has important roles in hematopoietic stem and progenitor cells, and that its interaction with miR-125b controls hematopoietic output.

We observed a significant decrease in pro-B and pre-B cells in mice hematopoietically overexpressing miR-125b, suggesting that miR-125b represses genes necessary for early B cell development. We note that miR-125b has been shown in vitro

to repress the transcription factors IRF4 and BLIMP1 in B cell lines[44,45], and it has been suggested that these interactions are responsible for miR-125b's demonstrated in vitro ability to prevent the ability of these cell lines to acquire CD138, a marker for plasmablasts[44]. In our studies here, we observed decreased numbers of plasmablasts in vivo in MG-125b mice, as well as decreased antigen-specific antibody response in MG-125b mice compared to controls. We note that IRF4 is also an important transcription factor for early B cell development[46]. Thus miR-125b impedes in vivo the development of B cells and plasmablasts, and may do so via repression of the transcription factors IRF4 and BLIMP1.

MiR-125b may also interact with other targets to selectively enhance myelopoiesis. Indeed, we observed that miR-125b overexpression enhances myeloid lineages dramatically, and increases myeloid progenitor numbers more than any other progenitor type. We show here and others have shown previously, that miR-125b is capable of repressing a number of pro-apoptotic genes including BMF, BAK1 and Trp53inp1[39,47-49]. Efficient repression of these genes in myeloid progenitors may enhance survival of these cells, providing them with the opportunity to eventually transform into a cancer stem cell pool.

MiR-125b mediated cancer: Is this mouse CML?

The cancer disease process in MG-125b mice is interesting in the sense that the mice appear healthy and disease-free for 7 weeks post-reconstitution. White blood cell counts in the bone marrow and spleen are even during this time period. The mice continue to appear healthy and active until 3.5-4 months post-reconstitution, at which point they suddenly fall ill, rapidly deteriorate, and become moribund with the symptoms of an aggressive myeloid leukemia. Indeed, it appears that the disease goes through an

indolent phase, followed by a rapidly progressing phase and then death. In many ways the disease course resembles that of Chronic Myeloid Leukemia (CML)[38], a disease characterized by the BCR-Abl translocation[50]. Interestingly, IRF4/IRF8 double knockout mice develop a disease that also resembles CML[51]. IRF8-/- mice also develop cancer, but addition of IRF4-/- to the IRF8-/- background cause the mice to deteriorate much more rapidly after the onset of disease[51]. IRF4 is a published target of miR-125b[44,45], and its repression by miR-125b may play an important role in the overexpression leukemia phenotype.

In CML, the cancer stem cell during the indolent chronic phase strongly resembles a hematopoietic stem cell[38]. As the cancer progresses into the accelerated phase, the cancer stem cell pool appears to shift, resembling an early myeloid progenitor[38]. BCR-Abl inhibitors such as Imatinib effectively halt the disease, but do not cure it, since stopping therapy leads to a return of the disease[50]. It is thought that the reason for this is that the cancer stem cell pool does not require BCR-Abl for its survival even though downstream cells do[38]. Thus Imatinib appears to kill cancer cells, but not the cancer stem cell pool. It is possible that the cancer stem cells are instead addicted to miR-125b, a microRNA that we show here is enriched in HSCs[52] and which upregulates myeloid progenitors, thus leading to a disease in mice that appears on the surface to resemble CML. Treatment of CML using miR-125b antagonists, perhaps in addition to BCR-ABL inhibitors, may therefore prove therapeutically useful.

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Appendix: Review of physiological and pathological roles for microRNAs in the immune system

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

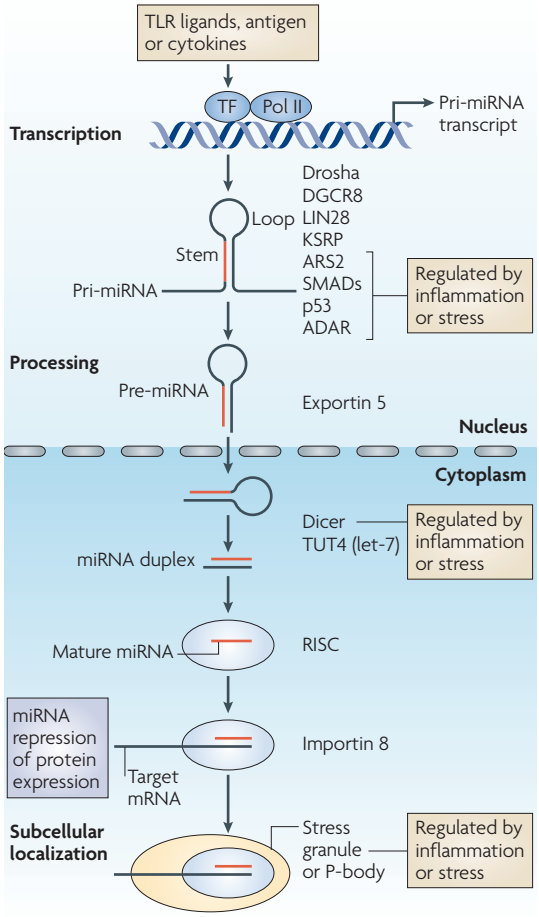

Figure 1 | **MicroRNA expression and function are regulated at three levels and influenced by inflammation and stress.** MicroRNA (miRNA) expression is first regulated by transcription factors (TFs) that can themselves be activated or repressed during immune responses by Toll-like receptor (TLR) ligands, 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.

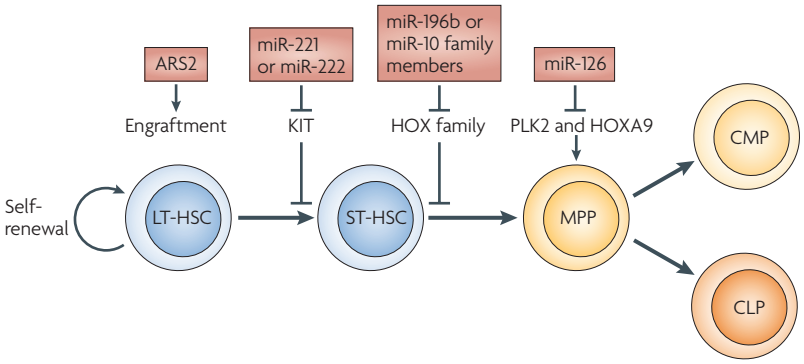

Figure 2 | **MicroRNA-mediated regulation of early haematopoietic cell development.** The microRNA (miRNA) pathway, which involves arsenate resistance protein 2 (ARS2), has a general role in haematopoietic stem cell (HSC) engraftment, which probably influences the reconstitution potential of long-term 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.

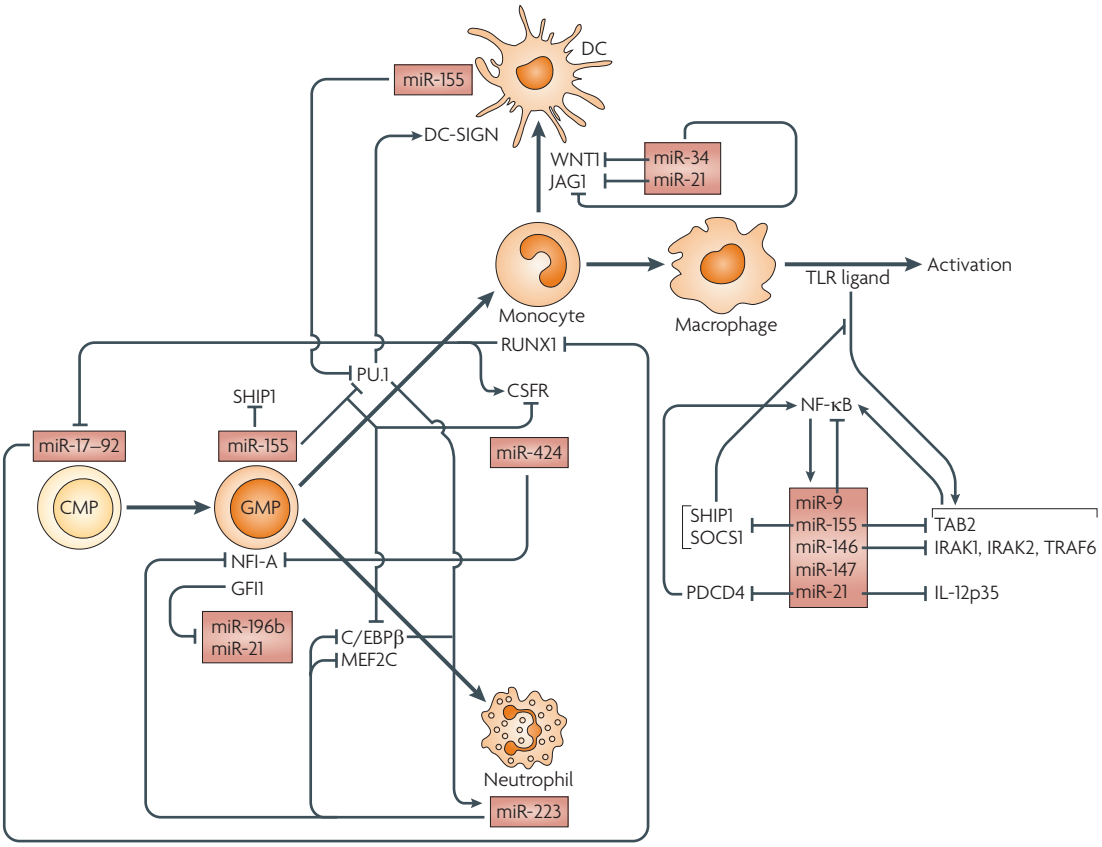

Figure 3 | **MicroRNA-mediated regulation of myeloid cell development and function.** The generation of different myeloid cell types is regulated by the interactions between microRNAs (miRNAs) and relevant transcription factors. Common myeloid progenitors (CMPs) give rise to granulocyte–monocyte progenitors (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

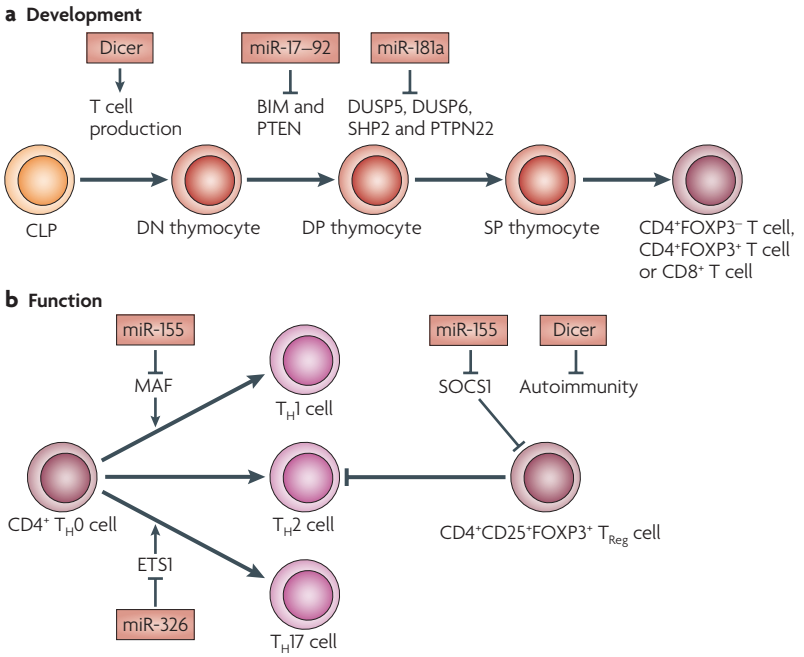

Figure 4 | **MicroRNA-mediated regulation of T cell development and function.** **a** | The production of microRNAs (miRNAs) by Dicer is required for efficient T cell development *in vivo*. T cells go through a stepwise developmental programme in the thymus. During this process, T cell survival and selection 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

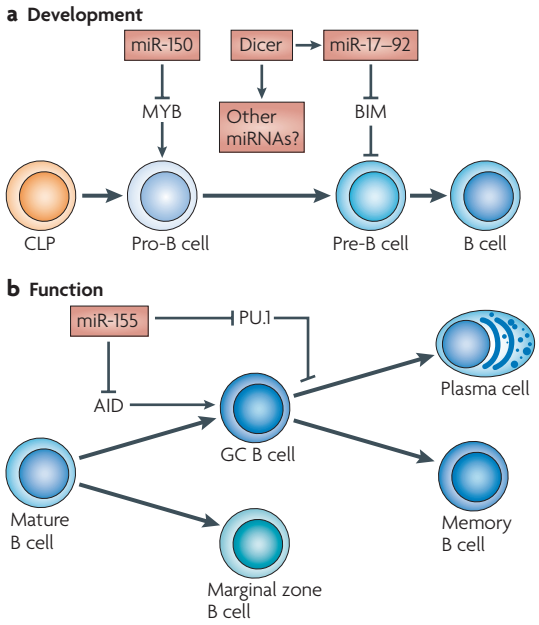

Figure 5 | **MicroRNA-mediated regulation of B cell development and function. a** | The survival and maturation of developing B cells requires microRNAs (miRNAs). Dicer-deficient animals have defective B cell development owing to a survival defect at the progenitor (pro) to precursor (pre) B cell transition. 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).

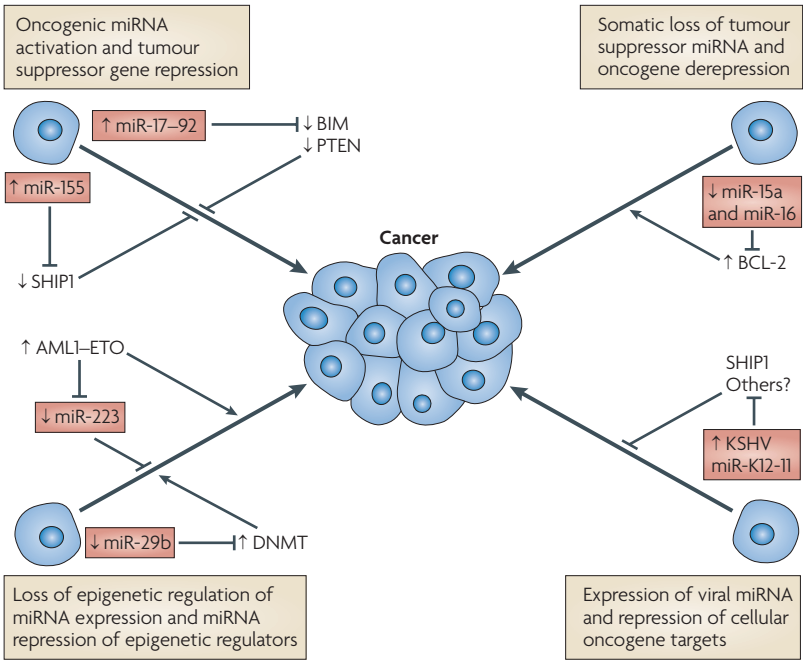

Figure 6 | **Mechanisms of microRNA contribution to cancer.** MicroRNAs (miRNAs) can influence immune cell-based malignant phenotypes through different mechanisms, including functioning as oncogenes (miR-155 and miR-17–92) or tumour suppressors (miR-15a and miR-16). miRNAs can also affect cancer by their 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
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.

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].

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.

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