

MicroRNAs Modulate Hematopoietic Lineage Differentiation

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Abstract MicroRNAs (miRNAs), an abundant class of ~22 nucleotide non-coding RNAs, are thought to play an important regulatory role in animal and plant development at the posttranscriptional level. Many miRNAs cloned from mouse bone marrow cells are differentially regulated in various hematopoietic lineages, suggesting that they might influence hematopoietic lineage differentiation. Some human miRNAs are linked to leukemias: the miR-15a/miR-16 locus is frequently deleted or down-regulated in patients with B-cell chronic lymphocytic leukemia and miR-142 is at a translocation site found in a case of aggressive B-cell leukemia. miR-181, a miRNA upregulated only in the B cell lineage of mouse bone marrow cells, promotes B cell differentiation and inhibits production of CD8⁺ T cells when expressed in hematopoietic stem/progenitor cells. In contrast miR-142s inhibits production of both CD4⁺ and CD8⁺ T cells and does not affect B cells. Collectively, these results indicate that microRNAs are components of the molecular circuitry controlling mouse hematopoiesis and suggest that other microRNAs have similar regulatory roles during other facets of vertebrate development.

Index Terms microRNA, B cells, T cells, leukemia, lymphoma, stem cells

INTRODUCTION

MicroRNAs (miRNAs) are ~22-nt non-coding RNAs that can play important roles in development by

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targeting the messages of protein-coding genes for cleavage or repression of productive translation [1]-[3]. Examples include *lin-4* and *let-7* miRNAs, which control the timing of *C. elegans* larval development [4]-[6], *Bantam* miRNA, which regulates *Drosophila* tissue growth by stimulating cell proliferation and preventing apoptosis [7], and miR-14 miRNA, which affects fat metabolism and prevents apoptosis [8]. Humans have between 200 and 255 genes that encode miRNAs, an abundance corresponding to almost one percent of the protein-coding genes [9]. Based on the evolutionary conservation of many miRNAs among the different animal lineages, it is reasonable to suspect that some mammalian miRNAs might also have important functions during development [10]-[14]. Moreover, genes for miR-142, miR-15, and miR-16 are at sites of translocation breakpoints or deletions linked to human leukemias [15]-[18]. However, few mammalian miRNAs have established functions [19].

RESULTS AND DISCUSSION

As a first step towards testing the idea that miRNAs might play roles in mammalian development, and more specifically that some might regulate mammalian hematopoiesis, we cloned about 100 unique miRNAs from mouse bone marrow using the protocol of Lau *et al.* [20]. Most had already been identified as vertebrate miRNAs, but their expression in bone marrow had not been examined. miR-181 [9], [12], [21], miR-223 [9], and miR-142s [18], were carried forward for further analyses because they, unlike miR-16 and most of the other miRNAs cloned, were differentially or preferentially expressed in hematopoietic tissues.

miR-181 was very strongly expressed in thymus, the primary lymphoid organ, which mainly contains T-lymphocytes. It was also strongly expressed in brain and lung, and detectable in bone marrow and spleen. miR-223 was nearly exclusively expressed in bone marrow, the primary hematopoietic organ, which consists of hematopoietic stem cells and myeloid, erythroid and lymphoid cells at various differentiation stages. miR-142s, whose gene is at the site of a translocation

associated with an aggressive B-cell leukemia [16], was highly expressed in all hematopoietic tissues tested, with little or no expression in non-hematopoietic tissues. Expression in E13 fetal liver, an embryonic hematopoietic organ, suggests that miR-142 might also function in early hematopoietic development.

Because bone marrow, spleen, and thymus, each have specialized functions in adult hematopoiesis and

comprise significantly different cell types, the differential expression of the miRNAs in these complex tissues suggested that individual hematopoietic cell types might differentially express the miRNAs. Indeed, when cells within bone marrow were sorted based on lineage markers, they were found to differentially express the hematopoietic miRNAs (Fig. 1). In contrast, expression of miR-16, an miRNA seen in a broad range of tissues, was more

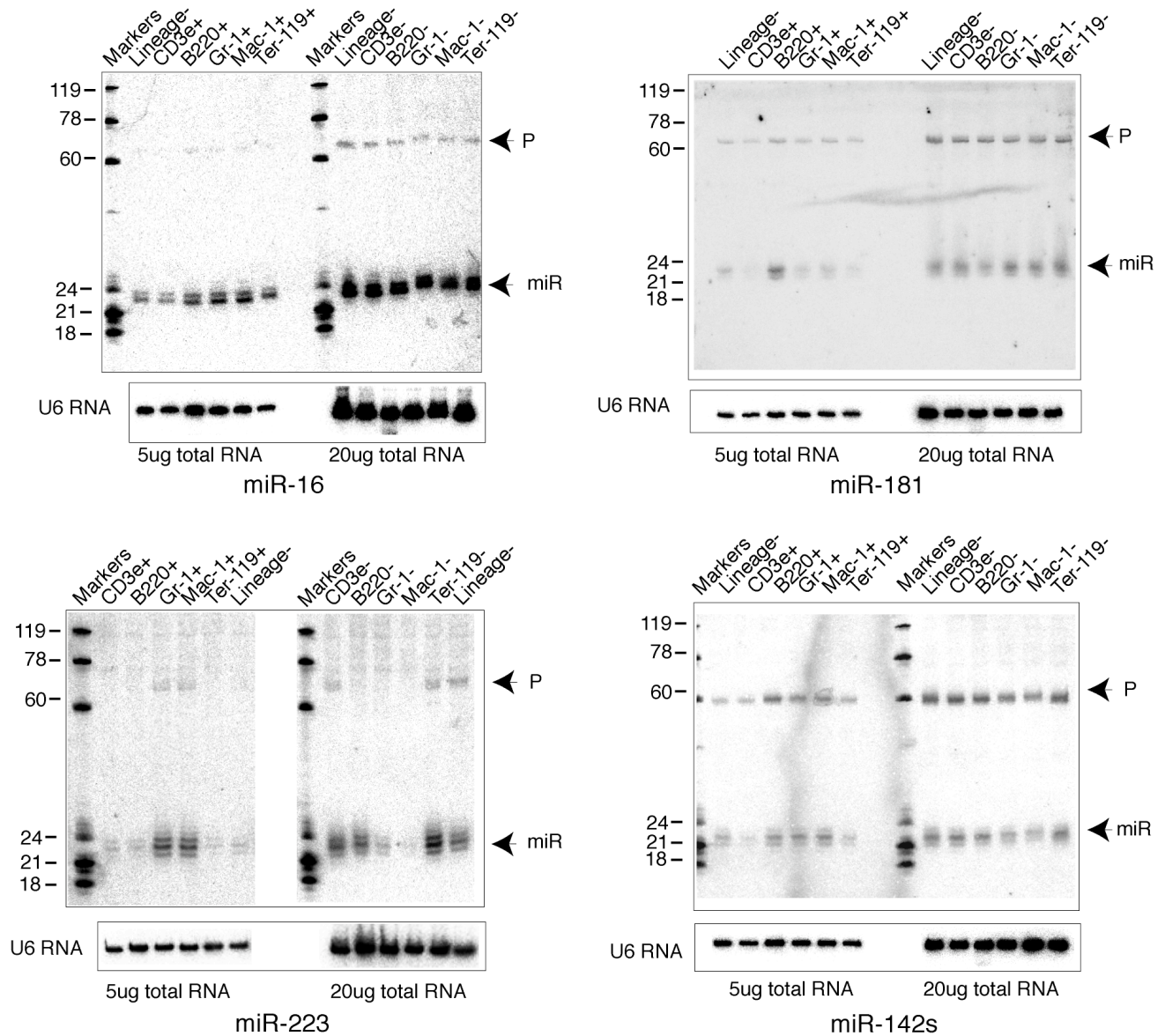


Figure 1. Northern analysis of miRNA expression in hematopoietic lineages from mouse bone marrow [29]. Antibodies against surface antigens CD3e, B220, Gr-1, Mac-1, and Ter119 were used to purify mouse bone marrow cells of the T, B, Granulocyte, Macrophage, and erythroid lineages, respectively, using magnetic-assisted cell sorting (>85% pure by subsequent FACS analysis). Total RNA (5 µg per lane) from the purified lineages was analyzed on the left half of each panel. Total RNA (20 µg per lane) from the cells remaining after depletion of specific lineages (CD3e⁻, B220⁻, Gr-1⁻, Mac-1⁻ and Ter119⁻ cells) was analyzed on the right half of each panel. Total RNA from a cell population depleted in Lin⁺ cells and thus enriched for undifferentiated hematopoietic stem/progenitor cells was also analyzed (Lin⁻, both halves of each panel). The lengths (nt) of RNA markers are indicated, as are the bands representing the mature miRNAs (miR) and presumed hairpin precursors (P). For the loading control, blots were reprobed for U6 snRNA.

constant.

Mature miR-181 expression in the bone marrow cells was detectable in undifferentiated progenitor cells (Lin^-) and up-regulated in differentiated B-lymphocytes, marked by the B220 surface antigen. In other differentiated lineages, miR-181 expression did not increase over that seen in Lin^- cells. Note that sorted lineage cell populations are about 85% pure; thus some miRNA signal in the other lineages might be caused by residual B220⁺ cells. miR-142s expression was lowest in

the erythroid (Ter-119^+) and T-lymphoid (CD3e^+) lineages and highest in B-lymphoid (B220^+) and myeloid lineages (Gr-1^+ and Mac-1^+), consistent with its ubiquitous expression in bone, spleen and thymus (Fig. 1). miR-223 expression was confined to myeloid lineages (Gr-1^+ and Mac-1^+), with barely detectable expression in T- and B-lymphoid and erythroid lineages (CD3e^+ , B220^+ , and Ter119^+ , respectively; Fig. 1). This observation is consistent with miR-223 expression in bone marrow but not spleen and thymus. As observed for miR-181, expression of miR-223 and miR-142s was low in Lin^-

Figure 2. Infection of hematopoietic stem and progenitor cells by retroviruses expressing specific microRNAs alters hematopoietic lineage differentiation, as monitored by *in vitro* culture.

cells relative to their preferred Lin^+ cell populations, suggesting that these miRNAs are also induced during lineage differentiation. For each of the miRNAs, specific expression was validated by the reduction of correspondent miRNA expression in the reciprocal lineage-depleted cell populations (Fig. 1, right panels).

Differential expression of three miRNAs in specific hematopoietic lineages suggested that they might influence hematopoietic lineage differentiation. To test this possibility, we set out to ectopically express these miRNAs in hematopoietic progenitor cells. A vector with the murine stem cell retrovirus backbone and a polymerase III expression cassette was developed to efficiently express miRNAs in primary hematopoietic progenitor cells. We first tried expressing a 67-nt miRNA transcript that included the predicted miR-223 hairpin precursor. The miR-223(67) transcript was highly expressed, but no mature ~22-nt miRNA could be detected. Previous studies of miR-30 biogenesis indicate that miRNA primary transcripts are first cleaved in the nucleus to generate the hairpin precursor, which is exported to the cytoplasm, where it is cleaved by Dicer to generate the mature miRNA [22]. We reasoned that the miR-223(67) transcript was not cleaved by Dicer because it did not derive from a properly processed primary transcript and that sequences flanking the hairpin precursor were needed for nuclear processing of the primary

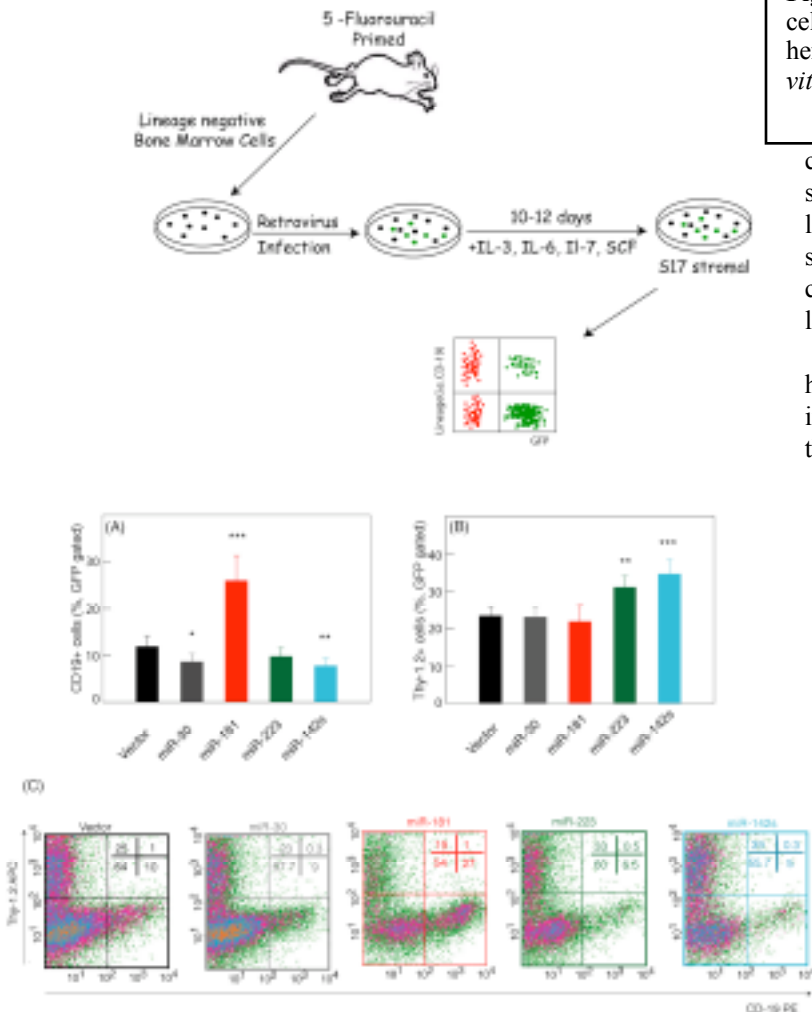
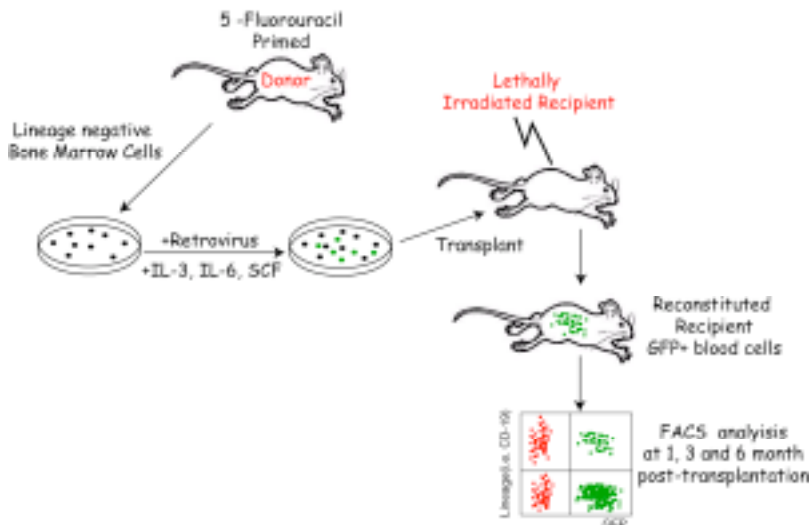


Figure 3. Effect of miRNA ectopic expression on hematopoietic lineage differentiation *in vitro*. **(A)** Percentage CD-19⁺ cells and **(B)** Percentage Thy-1.2⁺ cells among the differentiating hematopoietic progenitor cells ectopically expressing no miRNA (vector), a non-hematopoietic miRNA (miR-30), or one of three hematopoietic microRNAs (miR-181, miR-223, or miR-142s). The average of 12 culture replicates for each construct is shown, with error bars indicating the standard deviation. Statistically significant differences from the vector control, as determined by the Student's *t*-test, are indicated (* $P < 0.01$, ** $P < 0.0001$, *** $P < 10^{-7}$). **(C)** Representative FACS analyses of Thy-1.2 and CD-19 lineage marker expression, using antibodies conjugated to allophycocyanin (APC) and phycoerythrin (PE), respectively, for the experiment summarized in panels A and B. FACS plots were gated on GFP expression, which indicated the cells descending from infected progenitor cells. For each quadrant, the fraction of cells relative to the total number of GFP⁺ cells is shown as a percentage.



transcript. To include the sequences needed for proper processing, constructs with increasing genomic sequence flanking the 67-nt predicted hairpin were tested. All constructs with at least 40 nt on each side of the 67-nt hairpin were efficiently processed into the mature miRNA, with a heterogeneity pattern of 21–23-nt RNAs similar to that seen for the mature miR-223 in bone marrow cells [29]. Considering the recent report that Drosha is

Figure 4. Infection of hematopoietic stem and progenitor cells by retroviruses expressing specific microRNAs alters hematopoietic lineage differentiation, as monitored by bone marrow transplantation.

responsible for the nuclear processing of miRNA primary transcripts [23], our results can be explained by elements needed for Drosha recognition residing within the sequences flanking the miR-223 predicted hairpin.

Having determined that sequences flanking the hairpin were needed for detectable miRNA expression, we speculated that a general strategy for expressing miRNAs would be to use ~270-nt primary transcripts that included the ~22-nt mature miRNA and 125 nt

of genomic sequence flanking each side of the miRNA. This strategy has proven successful for all 13 of the miRNAs that we have attempted to ectopically express, including the three hematopoietic miRNAs [29]. Mature miRNAs ectopically expressed in 293T cells or bone marrow cells had length distributions indistinguishable from those of the endogenous miRNAs.

To uncover the effects of ectopic expression of

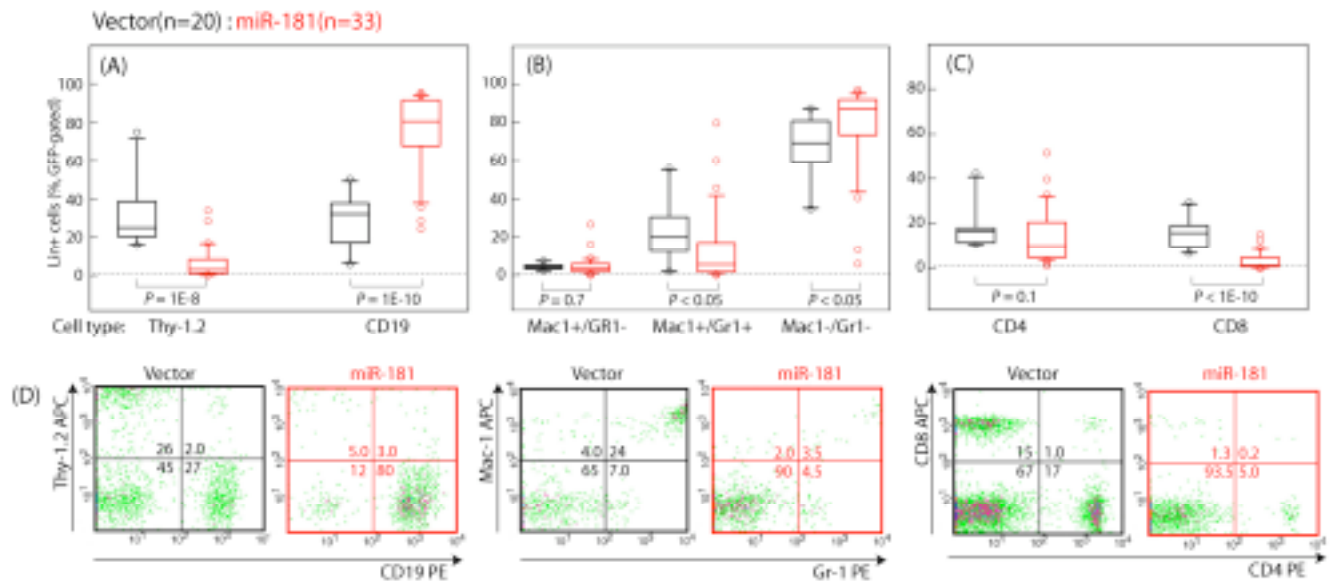


Figure 5. Effect of miR-181 ectopic expression on hematopoietic lineage differentiation *in vivo*. **(A)** Percentage of T-lymphoid (Thy-1.2⁺) and B-lymphoid (CD19⁺) lineage cells in GFP⁺ nucleated peripheral blood cells in mice reconstituted with bone marrow cells transduced with control (black) or miR-181 (red) retroviral vectors. Box-plots describe the distribution of individual lineage composition from all significantly reconstituted recipients (those with more than 1.0% GFP⁺ cells in peripheral blood). The ends of the boxes define the 25th and 75th percentiles, a line indicates the median, and bars define the 5th and 95th percentiles. Individual outliers are also shown. *P* values determined by using Mann-Whitney Rank Sum Test are indicated. **(B)** T-cell subtypes marked by CD4 and CD8 surface antigens. Box plots and color scheme are as in panel A. **(C)** Neutrophils and monocytes marked by Mac-1 and Gr-1 double-positive cells (Mac-1⁺ Gr-1⁺) and Mac-1 positive and Gr-1 negative-to-low cells (Mac-1⁺ Gr-1⁻), respectively. Mac-1 and Gr-1 double-positive cells (Mac-1⁻ Gr-1⁻) are non-myeloid cells. Box plots and color scheme are as in panel A. **(D)** Representative FACS analyses for the experiment summarized in panel A–C. Gating was on GFP⁺ cells. For each quadrant, the fraction of cells relative to the total number of GFP⁺ cells is indicated as a percentage.

miRNAs on hematopoietic lineage differentiation, Lin⁻ hematopoietic progenitor cells from mouse bone marrow were infected with viral vectors expressing either miR-181, miR-223, miR-142s, no miRNA (vector), or a control miRNA, miR-30. miR-30 was selected as a control because its expression was detectable in lung and kidney but not in hematopoietic tissues. The cells were then seeded onto S17 stromal cells and supplemented with a cocktail of cytokines and growth factors [25]. Cells descending from infected progenitor cells were distinguished based on the green fluorescent protein

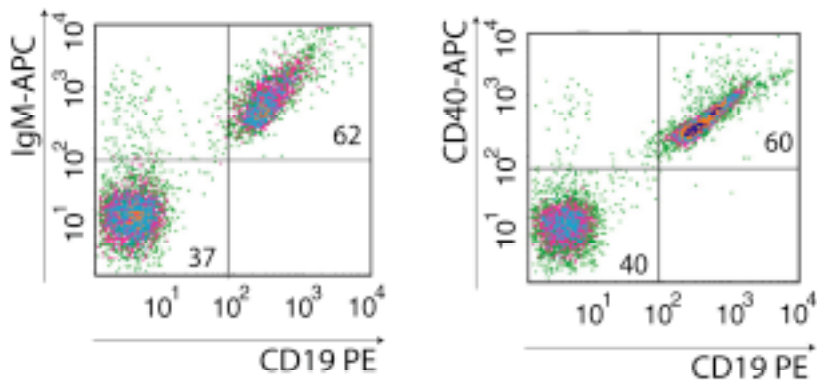


Figure 6. Immunophenotype of miR-181-expressing CD19-positive B-lymphoid cells

(GFP) marker carried by the vector, and differentiation of Lin⁻ cells to Lin⁺ cells was characterized by the expression of lineage-specific surface antigens (Fig. 2).

Ectopic expression of the hematopoietic miRNAs substantially altered lineage differentiation (Fig. 3). Expression of miR-181 resulted in more than a 2-fold increase in cells of the B-lymphoid lineage with no

significant change in the T-lymphoid lineage, as measured by the fractions of cells expressing the Thy-1.2 or CD-19 cell surface antigens, markers for T- and B-lymphoid lineages, respectively (Fig. 3A and B). Ectopic expression of miR-142s or miR-223 had opposite effects—a 30–40% increase in the T-lymphoid lineage with little or no reduction in the B-lymphoid lineage. At the two extremes, the ratio of T- to B-lineage cells ranged from about 1:1 to about 4:1 (Fig. 3, miR-181 and miR-142s, respectively). Modest effects were also seen in this *in vitro* assay when analyzing cells for myeloid lineage markers. In contrast,

ectopic expression of miR-30 had little or no effect on the output of lymphoid and

myeloid cells, indicating that merely expressing an arbitrary miRNA does not influence lymphoid differentiation.

Because miR-181 ectopic expression had the greatest effect *in vitro*, we examined its effect *in vivo*. Mouse Lin⁻ bone marrow cells were infected with the miR-181-expressing retrovirus or the control vector expressing no miRNA and transplanted into lethally irradiated mice, where they reconstituted all blood lineages (Fig. 4). After 4.5 weeks, the lineage composition of peripheral blood cells descending from infected stem/progenitor cells (GFP⁺) was examined. As seen *in vitro*, miR-181 expression *in vivo* led to a substantial

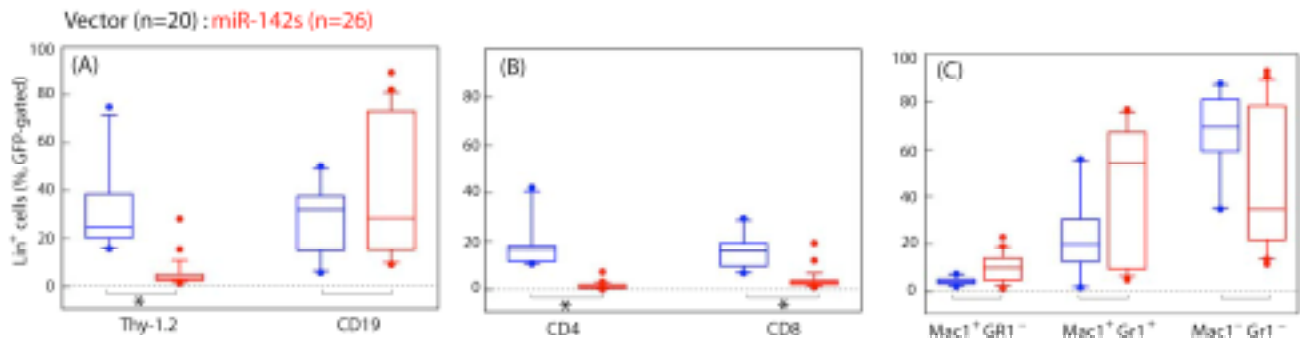


Figure 7. Effect of miR-142s ectopic expression on hematopoietic lineage differentiation *in vivo*. (A) Percentage of T-lymphoid (Thy-1.2⁺) and B-lymphoid (CD19⁺) lineage cells in GFP⁺ nucleated peripheral blood cells in mice reconstituted with bone marrow cells transduced with control (black) or miR-181 (red) retroviral vectors. (B) T-cell subtypes marked by CD4 and CD8 surface antigens. Box plots and color scheme are as in panel A. (C) Neutrophils and monocytes marked by Mac-1 and Gr-1 double-positive cells (Mac-1⁺ Gr-1⁺) and Mac-1 positive and Gr-1 negative-to-low cells (Mac-1⁺ Gr-1⁻), respectively. Mac-1 and Gr-1 double-positive cells (Mac-1⁺ Gr-1⁺) are non-myeloid cells.

increase in B-lymphoid cells (CD19⁺), with the median fraction of these cells in peripheral blood increasing to 80% from the control value of 32% (Fig. 5A). This increase was accompanied by an 8-fold decrease in T-lymphoid cells (Thy-1.2⁺), particularly the CD8⁺ T cells, for which the median percentage decreased 13-fold (Fig. 5A and 5C). There were relatively small or insignificant decreases in CD4⁺ T cells and myeloid lineage cells (Fig. 5B).

Furthermore, the B cells formed by overexpression of miR-181 appeared normal, as all of them expressed on their surface the CD 40 antigen as well as immunoglobulin (Fig. 6).

In contrast miR-142s inhibits production of both

CD4⁺ and CD8⁺ T cells and does not affect B cells (Fig. 7). This result establishes that different lineage-specific miRNAs induce hematopoietic stem or early progenitor cells to undergo differentiation along different cellular pathways, and form different proportions of differentiated hematopoietic cells.

Hematopoietic lineage differentiation, a process of continuous development of hematopoietic stem cells into at least eight different blood lineages, is known to be controlled or modulated by complex molecular events that simultaneously regulate the commitment, proliferation, apoptosis, and maturation of hematopoietic stem/progenitor cells. The demonstration that certain miRNAs are differentially expressed in hematopoietic lineages *in vivo* and are able to alter lineage differentiation provides solid evidence that miRNAs represent a class of molecules that regulate mouse hematopoiesis, and more broadly, mammalian development.

The ability of ectopically expressed miR-181 to increase the fraction of B-lineage cells *in vitro* and *in vivo* (Fig. 3 and 5) coincides with its preferential expression in B-lymphoid cells in mouse bone marrow (Fig. 1), suggesting that miR-181 is a positive regulator for B-cell differentiation. One explanation for the effect of miR-181 expression on the differentiation of both B cells (CD19⁺) and cytotoxic T cells (CD8⁺), which are not developmentally linked during hematopoietic lineage commitment, is that miR-181 acts independently in the two lineages, perhaps through the repression of different target genes. Indeed, miR-181 is highly expressed in the thymus, supporting the idea that it also modulates T-cell development in this organ. The observation that the differentiation of myeloid and other lymphoid cell types was not totally blocked when the B-lymphoid lineage increased suggests that miR-181, at least when considered singly rather than in combination with other miRNAs, appears to function more as a lineage modulator rather than as a switch.

In the known invertebrate examples miRNAs repress the productive translation of their mRNA targets [1]. To facilitate further exploration of roles of hematopoietic miRNAs in modulating lineage differentiation, computational and molecular experiments are underway to determine their regulatory targets. Assuming a mode of regulation analogous to that observed in invertebrates, miRNA modulation of hematopoietic lineage differentiation supports the notion that the roles of translational regulation in hematopoiesis and, more broadly, vertebrate development might have been under-appreciated. Studies on the gene expression profile of uncommitted hematopoietic stem cells and intermediate progenitor cells reveal that stem cells exhibit a “promiscuous beginning”, a so-called “priming state” in which many lineage-specific genes required for subsequent unique lineages are co-expressed [26]. Thus selective gene silencing might be a key event during subsequent hematopoietic lineage differentiation events. Clearly,

progressive silencing of lineage-specific genes could be mediated by changes in the activation of “master” transcription factors or by chromatin remodeling. Our work adds to this list a set of hematopoietic-specific miRNAs that presumably act by pairing to the mRNAs of their target genes to direct gene silencing processes critical for hematopoiesis.

The study of miR-181 function in B-lymphoid lineage differentiation sheds light on the potential signaling pathways regulated by the miRNA and allows us to search for miR-181 target genes using a candidate gene approach by focusing on those that are known to play important roles in B-cell development. For example, the expression of miR-181 causes reduction in T-cell formation in parallel with enhancing B cell formation, and activated Notch causes inhibition of formation of B-lineage cells. This raises the possibility that miR-181 may promote B-cell differentiation through the repression of Notch signals [27]. Thus, we can try to search miR-181 target genes in the Notch signal pathway by examining those Notch signal molecules for miR-181 complement sites. Furthermore, knowing the miRNA function in lineage differentiation will also provide additional guidance to select biologically relevant target genes from multiple computationally predicted target genes [28]. The ability to regulate multiple target genes by each miRNA also suggests tremendous flexibilities in miRNA-mediated gene regulation. One interesting scenario is that a miRNA may exert distinct function in various cell types by regulating different target genes. For instance, we have noted that miR-181 is preferentially expressed in the B220 positive fraction of the mouse bone marrow cells, but is also high differentially expressed in the thymus that consists of primarily T-lymphoid cells. Thus, it will be of interest to determine whether miR-181 regulates different target genes in bone marrow B-cells and Thymus T-cells and exerts distinct function in bone marrow and thymus.

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