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Fig. S1

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MicroRNAs Modulate Hematopoietic Lineage Differentiation

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MicroRNAs (miRNAs) are an abundant class of ~22-nucleotide regulatory RNAs found in plants and animals. Some miRNAs of plants, *Caenorhabditis elegans*, and *Drosophila* play important gene-regulatory roles during development by pairing to target mRNAs to specify posttranscriptional repression of these messages. We identify three miRNAs that are specifically expressed in hematopoietic cells and show that their expression is dynamically regulated during early hematopoiesis and lineage commitment. One of these miRNAs, miR-181, was preferentially expressed in the B-lymphoid cells of mouse bone marrow, and its ectopic expression in hematopoietic stem/progenitor cells led to an increased fraction of B-lineage cells in both tissue-culture differentiation assays and adult mice. Our results indicate that microRNAs are components of the molecular circuitry that controls mouse hematopoiesis and suggest that other microRNAs have similar regulatory roles during other facets of vertebrate development.

MicroRNAs (miRNAs) are ~22-nucleotide (nt) noncoding RNAs that can play important roles in development by targeting the messages of protein-coding genes for cleavage or repression of productive translation (*1–3*). Examples include the *lin-4* and *let-7* miRNAs, which control the timing of *Caenorhabditis elegans* larval development (*4–6*); *Bantam* miRNA, which regulates *Drosophila* tissue growth by stimulating cell proliferation and preventing apoptosis (*7*); and miR-14, which affects *Drosophila* fat metabolism and prevents apoptosis (*8*). Humans have between 200 and 255 genes that encode miRNAs, an abundance corresponding to almost 1% 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, no mammalian miRNAs have established functions (*19*).

As a first step toward testing the idea that miRNAs might play roles in mammalian development, and more specifically that some might regulate mammalian hematopoiesis, we cloned ~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 (Fig. 1).

miR-181 was very strongly expressed in the thymus, the primary lymphoid organ, which mainly contains T lymphocytes. It was also strongly expressed in the brain and lung and was detectable in bone marrow and the 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*, *18*), was highly expressed in all the hematopoietic tissues tested, with little or no expression in nonhematopoietic tissues. Expression at embryonic day 13 in fetal liver, an embryonic hematopoietic organ, suggests that miR-142 might also function in early hematopoietic development.

Because the bone marrow, spleen, and thymus each have specialized functions in adult hematopoiesis and comprise largely different cell types, the differential expression of the miRNAs in these complex tissues sug-

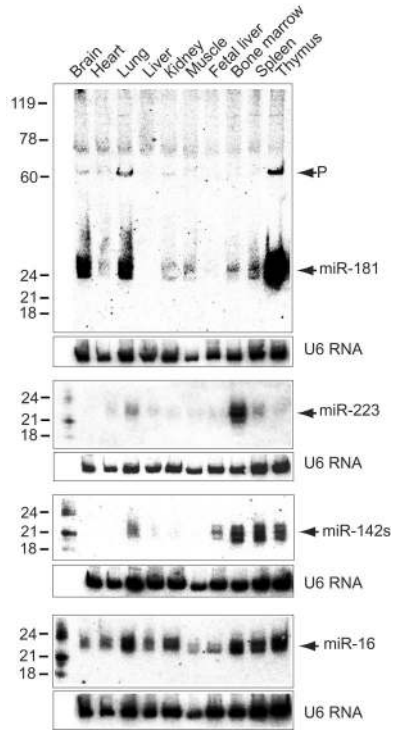

Fig. 1. Northern blots showing tissue expression of four miRNAs cloned from mouse bone marrow (*25*) (fig. S1). As loading controls, blots were also probed for U6 small nuclear RNA. The lengths (in nucleotides) of RNA markers are indicated, as are the bands that represent the mature miRNAs (miR) and presumed hairpin precursors (P).

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gested that individual hematopoietic cell types might differentially express the miRNAs. When cells within bone marrow were sorted based on lineage markers, they were found to differentially express the hematopoietic miRNAs (Fig. 2). In contrast, expression of miR-16, an miRNA seen in a broad range of tissues, was more constant.

Mature miR-181 expression in bone marrow cells was detectable in undifferentiated progenitor cells (Lin^-) and up-regulated in differentiated B lymphocytes, which are marked by the B220 surface antigen. In other differentiated lineages, miR-181 expression did not increase over that seen in Lin^- cells. Sorted lineage cell populations are ~85% pure; thus, some miRNA signals in the other lineages might be caused by residual $B220^+$ cells. miR-142s expression was lowest in the erythroid ($Ter\text{-}119^+$) and T-lymphoid ($CD3e^+$) lineages and highest in B-lymphoid ($B220^+$) and myeloid ($Gr\text{-}1^+$ and $Mac\text{-}1^+$) lineages, consistent with its ubiquitous expression in bone, spleen, and thymus tissues (Figs. 1 and 2). miR-223 expression was confined to myeloid ($Gr\text{-}1^+$ and $Mac\text{-}1^+$) lineages, with barely detectable expression in T- and B-lymphoid and erythroid lineages ($CD3e^+$, $B220^+$, and $Ter\text{-}119^+$, respectively) (Fig. 2). This observation is consistent with miR-223 expression in bone marrow but not in the spleen or thymus (Fig. 1). As observed for miR-181, expression of miR-223 and miR-142s was low in Lin^- 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. 2).

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 (pol III) expression cassette was developed to efficiently express miRNAs in primary hematopoietic

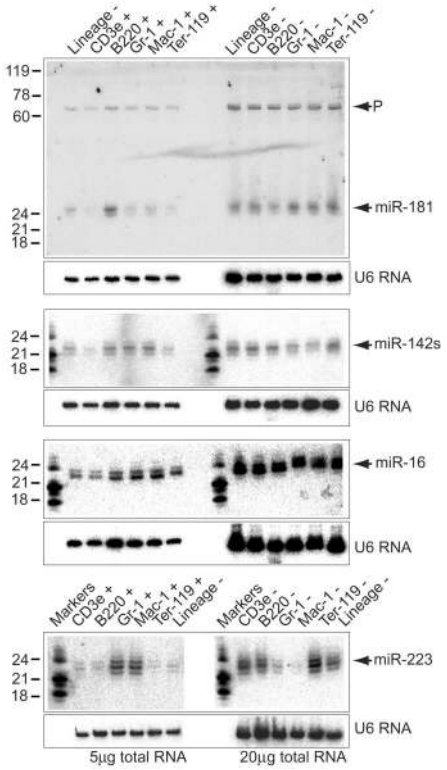

Fig. 2. Northern analysis of miRNA expression in hematopoietic lineages from mouse bone marrow (fig. S2). Antibodies to surface antigens CD3e, B220, Gr-1, Mac-1, and Ter-119 were used to purify mouse bone marrow cells of the T, B, granulocyte, macrophage, and erythroid lineages, respectively, with magnetic-assisted cell sorting. [Sorted cells were at least 85% pure by subsequent fluorescence-activated cell sorting (FACS) analysis.] Both total RNA (5 μg per lane) from the purified lineages (**left**) and total RNA (20 μg per lane) from the cells remaining after depletion of specific lineages [$CD3e^-$, $B220^-$, $Gr\text{-}1^-$, $Mac\text{-}1^-$, and $Ter\text{-}119^-$ cells (**right**)] were analyzed. Total RNA from a cell population depleted in Lin^+ cells and thus enriched for undifferentiated hematopoietic stem/progenitor cells was also analyzed ($Lineage^-$). The lengths (in nucleotides) of RNA markers are indicated, as are the bands that represent the mature miRNAs and presumed hairpin precursors. For the loading control, blots were reprobed for U6 small nuclear RNA.

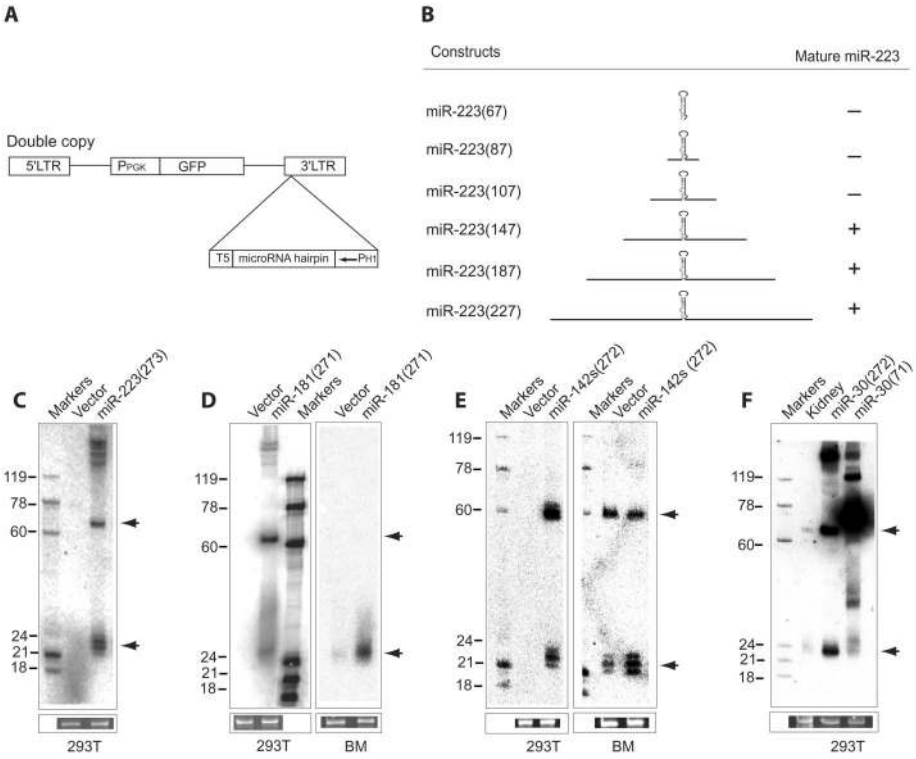

Fig. 3. A general strategy for the ectopic expression of miRNAs. (**A**) The retroviral construct for miRNA expression. A pol III expression cassette containing the human H1 promoter (P_{H1}), the miRNA hairpin, and the T5 termination signal was placed in the U3 region of the viral 3′ long terminal repeat (LTR). As a marker for infection, the vector also expressed the gene for *GFP* under control of the constitutive murine 3-phosphoglycerate kinase promoter (P_{PGK}). This vector configuration, which improves stable gene expression in primary cells, was termed "double copy," because the process of retroviral reverse transcription and integration causes two copies of the miRNA expression cassette to be integrated into the host genome (*29*). (**B**) Summary of miR-223 expression and maturation from vectors designed to express successively longer miR-223 genomic fragments, each containing the miR-223 predicted hairpin and flanking sequences. miR-223(67), (87), (107), (147), (187), and (227) transcripts included the 67-nt miR-223 predicted hairpin at their center, with 0, 10, 20, 40, 60, or 80 nt of genomic sequence flanking each side of the hairpin, respectively. Expression and maturation of miR-223 in transfected 293T cells was examined by Northern analysis. High expression of the primary transcript was seen for each of the three constructs that did not generate mature miRNA. (**C** to **E**) Expression of miR-223, miR-181, and miR-142s from ~270-nt primary transcripts that included the ~22-nt mature miRNA and 125 nt of genomic sequence flanking each side of the miRNA, in transfected 293T cells or viral-transduced mouse bone marrow cells (BM). (**F**) Expression of miR-30 from the 71-nt predicted miR-30 hairpin or a 272-nt fragment with 125 nt of genomic sequence flanking each side of the miRNA. Ethidium staining of 5S ribosomal RNA served as the loading control.

progenitor cells (Fig. 3A). 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 (Fig. 3B). 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 transcript. To include the sequences needed for proper processing, constructs with increasing genomic sequence flanking the 67-nt predicted hairpin were generated. All constructs with at least 40 nt on each side of the 67-nt hairpin were efficiently processed into the mature miRNA (Fig. 3B), with a heterogeneity pattern of 21- to 24-nt RNAs similar to that seen for the mature miR-223 in bone marrow cells (Figs. 1 and 2). In light of the recent report that Drosha is responsible for the nuclear processing of miRNA primary transcripts (*23*), our results can be explained by the idea that elements needed for Drosha recognition reside within the sequences that flank 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 miRNA expression 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 express, including the three hematopoietic miRNAs (Fig. 3, C to E). Mature miRNAs ectopically expressed in 293T cells or bone marrow cells had length distributions indistinguishable from those of the endogenous miRNAs, as shown for miR-142s (Fig. 3E). miR-30 is unusual in that it can be expressed from transcripts in which its 71-nt hairpin is flanked by heterologous sequence (*24*). Nonetheless, when expressed from our vector, miR-30 was much more efficiently processed when presented in the context of its native flanking sequence (Fig. 3F).

To uncover the effects of ectopic expression of miRNAs on hematopoietic lineage differentiation, Lin$^-$ hematopoietic progenitor cells from mouse bone marrow were infected with viral vectors that expressed either miR-181, miR-223, miR-142s, miR-30 (a control miRNA), or no miRNA (*25*). miR-30 was selected as a control because its expression was detectable in lung and kidney but not hematopoietic tissues. The cells were then seeded onto S17 bone marrow stromal cells and supplemented with a cocktail of cytokines and growth factors (*25*, *26*). Cells descending from infected progenitor cells were distinguished on the basis of the green fluorescent protein (GFP) marker carried by the vector, and differentiation of Lin$^-$ cells to Lin$^+$ cells was characterized by the expression of lineage-specific surface antigens (*25*).

Ectopic expression of the hematopoietic miRNAs substantially altered lineage differentiation (Fig. 4). Expression of miR-181 resulted in a doubling of cells in the B-lymphoid lineage with no significant change in the T-lymphoid lineage, as measured by the fractions of cells that express the Thy-1.2 or CD19 cell surface antigens, which are markers for the T- and B-lymphoid lineages, respectively (Fig. 4, A and B). Ectopic expression of miR-142s or miR-223 had opposite effects—a 30 to 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. 4) (when miR-181 and miR-142s were expressed, respectively). Modest effects were also seen when analyzing cells for myeloid lineage markers (fig. S3). 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 either the retrovirus that expressed miR-181 or the control vector that expressed no miRNA and were then transplanted into lethally irradiated mice, where they reconstituted all blood lineages. After 4.5 weeks, the lineage composition of peripheral blood cells descending from infected stem/progenitor cells (GFP$^+$ cells) was examined (*25*). As seen in vitro, miR-181 expression in vivo led to a substantial increase in B-lymphoid (CD19$^+$) cells, 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 a substantial (~88%) decrease in T-lymphoid (Thy-1.2$^+$) cells, particularly the CD8$^+$ T cells, for which the median percentage decreased from 16% to 1.2% (Fig. 5, A and B). There were relatively small or insignificant decreases in CD4$^+$ T cells and myeloid lineage cells (Fig. 5, B and C).

Hematopoietic lineage differentiation, the process of continuous development of hematopoietic stem cells into at least eight different blood lineages, is known to be controlled

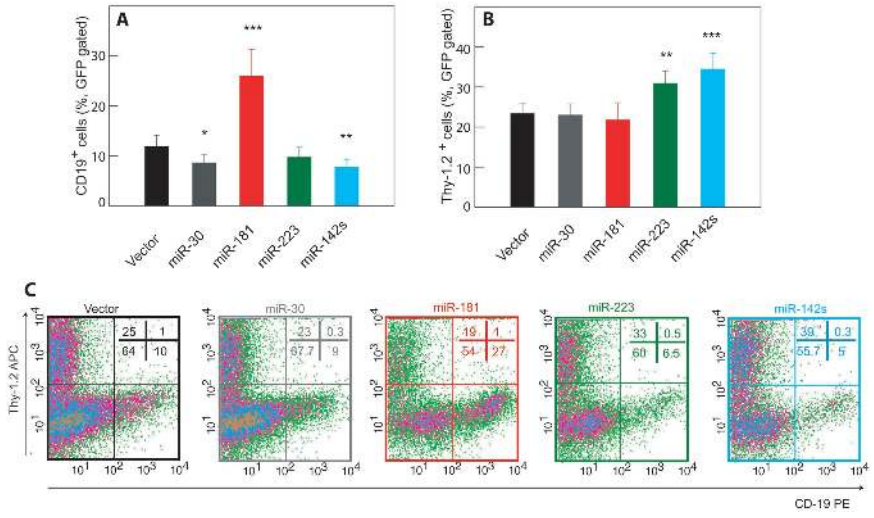

Fig. 4. Effect of miRNA ectopic expression on hematopoietic lineage differentiation in vitro. (**A**) Percentage CD-19$^+$ cells and (**B**) percentage Thy-1.2$^+$ cells among the differentiating hematopoietic progenitor cells ectopically expressing either no miRNA (vector), a nonhematopoietic miRNA (miR-30), or one of three hematopoietic microRNAs (miR-181, miR-223, and 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 (*, $P < 0.01$; **, $P < 0.0001$; ***, $P < 10^{-7}$). In independent repetitions of this experiment, analogous changes relative to the control vectors were observed with similar statistical significance; however, the absolute percentages of Thy-1.2$^+$ and CD-19$^+$ cells differed, perhaps because of the heterogeneity of the Lin$^-$ bone marrow cells from different groups of 5-fluorouracil–primed mice. (**C**) Representative FACS analyses of Thy-1.2 and CD-19 lineage marker expression, with antibodies conjugated to allophycocyanin (APC) and phycoerythrin (PE), respectively, for the experiment in (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.

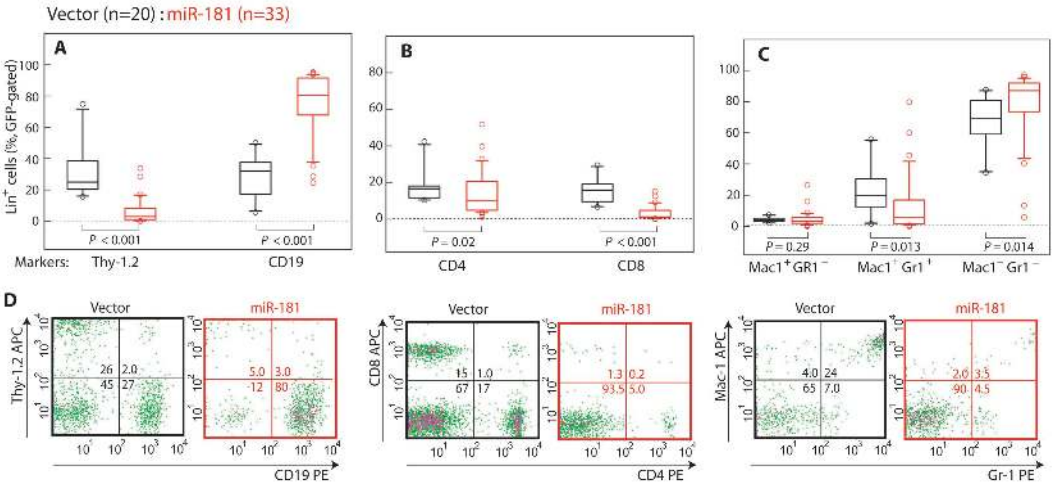

Fig. 5. Effect of miR-181 ectopic expression on hematopoietic lineage differentiation in vivo. (**A**) Percentage of T-lymphoid (Thy-1.2$^+$) and B-lymphoid (CD19$^+$) lineage cells in GFP$^+$ nucleated peripheral blood cells, in mice reconstituted with bone marrow cells transduced with control (black) or miR-181 (red) retroviral vectors. Box plots describe the distribution of individual lineage composition from all positively 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 were determined with the Mann-Whitney rank sum test. (**B**) T cell subtypes marked by CD4 and CD8 surface antigens. (**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-negative cells (Mac-1$^-$ Gr-1$^-$) are nonmyeloid cells. (**D**) Representative FACS analyses for the same experiment. 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.

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 (Figs. 4 and 5) coincides with its preferential expression in B-lymphoid cells in mouse bone marrow (Fig. 2), 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 (Fig. 1). 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 than as a switch.

In the known invertebrate examples, miRNAs repress the productive translation of their mRNA targets (*1*). To facilitate further exploration of the roles of hematopoietic miRNAs in modulating lineage differentiation, computational and molecular experiments are under way to determine their regulatory targets. If we assume 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 underappreciated. Studies on the gene expression profiles 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 coexpressed (*27*). 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.

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