**Methods**

**Cell isolation**

Murine bone marrow was obtained by flushing the femurs and tibias with bone marrow washing medium (DMEM, 2% FBS, 10 mM Hepes pH 7.2). A single-cell suspension was made by repeated pipetting of bone marrow and filtering through a 70 μm nylon cell strainer (Falcon). Magnetic-assisted cell sorting was carried out using Streptavidin microbeads (Miltenyi Biotec) and a biotinylated mouse lineage antibodies (Pharmingen) according to manufacturers’ instructions. Lineage antibodies included: anti-CD11b (M1/70); anti-Gr-1 (RB6-8C5); anti-B220 (RA3-6B2); anti-CD3ε (145-2C11); and anti-Ter119 (Ter119).

**Construction of miRNA expression vectors**

A retrovirus vector was developed using the murine stem cell virus backbone (Clontech). Approximately 270-bp miRNA gene segments containing the miRNA hairpin were amplified from each genomic locus and placed in the U3 region of the 3’ LTR under the control of the human H1 pol III promoter. High-titer retroviral supernatant was generated by co-transfection of miRNA expression vector and the pCLeco viral packaging construct into 293T cells (S1). The mir-181 gene used in this study was amplified from mouse chromosome 1.
Northern analyses

Total RNA from the indicated tissues (Fig 1) and cell lineages (Fig 2) was probed for the indicated miRNAs as previously described (S2).

S17-stromal culture assay.

C57Bl/6J or B6/SJL mice were primed by treatment with 150 mg/kg 5-fluorouracil (5-FU) for 3 to 4 days. Lin− cells from 5-FU treated bone marrow were enriched using MACS to deplete cells positive for CD3e, B220, Gr1, Mac1 and Ter119 surface markers. Lin− cells were infected with the miRNA expression vectors, then seeded onto S17 stromal cells (50,000 infected Lin− cells/well in a 24-well plate), and cultured in medium containing 10 ng/ml IL-3, 10 ng/ml IL-6, 10 ng/ml IL-7, and 50 ng/ml stem cell factor. For each infection, twelve culture replicates were conducted. Cells were fed with fresh growth medium every five days. After 10 days of culture, both suspended and adherent cells were harvested, stained with the indicated lineage markers, and analyzed for the lineage profiles using FACS (Fig. 4). Allophycocyanin-conjugated anti-Thy-1.2 antibody (Thy-1.2 APC) and phycoerythrin-conjugated anti-CD-19 antibody (CD-19 PE) were used to detect Thy-1.2 and CD-19 expression on differentiated lineages. Typically over 50% of the progenitor cells were infected with the miRNA vector, as indicated by GFP expression, and this percentage did not substantially change during the 10-day assay.

Bone marrow transplantation assay.

Lin− cells (5 x10^5) isolated from 5-FU primed B6/SJL mice were infected with miRNA retroviruses (at least 10^6 cfu/ml) by spinoculation. Following 24 hours of culture in medium containing 10 ng/ml IL-3, 10 ng/ml IL-6, and 100 ng/ml stem cell factor, a
second round of spinoculation was performed, followed by an additional 24 hours of culture. Then $2.5 \times 10^4$ infected cells were mixed with $4 \times 10^5$ “supportive” B6/SJL bone marrow cells that had been depleted by MACS of all Scal-1$^+$ cells (and thus of HSCs) and injected, via the retro-orbital route, into lethally irradiated C57/Bl6J (10 Gy) recipient mice (S3). For each miRNA construct or control construct to be tested, 30 to 50 mice were transplanted and analyzed. Peripheral blood cells were collected from transplanted recipients by retro-orbital bleeding at 4.5 weeks post-transplantation. Red blood cells were lysed with NH$_4$Cl. Nucleated cells were stained with combinations of antibodies against lineage-specific antigens and analyzed on a Becton Dickinson FACS Calibur.

References


Supplemental Figure Legends

**Supplemental Figure 1.** Northern blots showing tissue expression of four miRNAs cloned from mouse bone marrow. As loading controls, blots were also probed for U6 snRNA. The lengths (nt) of RNA markers are indicated, as are the bands representing the mature miRNAs (miR) and presumed hairpin precursors (P). Accumulation of the presumed miR-142 precursor (~60-nt band) was high and the ratio of mature to precursor 21-nt RNAs varied in different tissues, suggesting posttranscriptional regulation of miRNA expression at the levels of precursor processing or RNA stability.

**Supplemental Figure 2.** Northern analysis of miRNA expression in hematopoietic lineages from mouse bone marrow. 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. Expression of the miR-181 precursor was at similar levels in
all lineages, suggesting that the differential accumulation of the mature miR-181 during hematopoietic lineage commitment might be regulated at the level of miRNA processing or degradation.

**Figure 3.** Effect of miRNA ectopic-expression on myeloid lineage differentiation in S17 stromal culture. (A) Percentage Mac-1 and Gr-1 negative cells (Mac-1<sup>−</sup> Gr-1<sup>−</sup>), Mac-1 positive and Gr-1 negative-to-low (Mac-1<sup>+</sup> Gr-1<sup>−/low</sup>), Mac-1 and Gr-1 positive (Mac-1<sup>+</sup> Gr-1<sup>+</sup>) cells among the differentiating hematopoietic progenitor cells ectopically expressing no miRNA (vector), miR-30, miR-142s, or miR-181. The average of 12 culture replicates for each construct is shown, with error bars indicating the standard deviation. Neutrophils are Mac-1<sup>+</sup> Gr-1<sup>+</sup>, whereas monocytes are Mac-1<sup>+</sup> Gr-1<sup>−/low</sup>. Non-myeloid cells are Mac-1<sup>−</sup> Gr-1<sup>−</sup>; they mostly express Thy1.2 or CD-19 lymphoid markers. Statistically significant differences from the vector control, as determined by the Student’s t-test, are indicated (* P <0.01, ** P <0.0001). (B) Representative FACS analyses of Mac-1 (Mac-1 APC) and Gr-1 (Gr-1 PE) lineage marker expression for the experiment summarized in panel C. 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<sup>+</sup> cells is indicated as a percentage. We note that some cells in the culture remain progenitor cells and express both myeloid and lymphoid markers. Thus, our S17 stromal culture and differentiation conditions recapitulated limited aspects of hematopoietic lineage differentiation. In vivo, miR-181 has more pronounced effects on myeloid differentiation (Fig. 5), which might be recapitulated using other in vitro conditions.
Supplemental Figure 2

miR-16

miR-181

miR-223

miR-142s

U6 RNA

CD3e+ B220+ Mac-1+Ter-119+Lineage-Markers Gr-1+

CD3e- B220- Mac-1- Ter-119-Lineage-Markers Gr-1-

5ug total RNA 20ug total RNA

miR-16

miR-181

miR-223

miR-142s

U6 RNA

CD3e+ B220+ Mac-1+Ter-119+Lineage-Markers Gr-1+

CD3e- B220- Mac-1- Ter-119-Lineage-Markers Gr-1-

5ug total RNA 20ug total RNA

miR-16

miR-181

miR-223

miR-142s

U6 RNA

CD3e+ B220+ Mac-1+Ter-119+Lineage-Markers Gr-1+

CD3e- B220- Mac-1- Ter-119-Lineage-Markers Gr-1-

5ug total RNA 20ug total RNA

miR-16

miR-181

miR-223

miR-142s

U6 RNA

CD3e+ B220+ Mac-1+Ter-119+Lineage-Markers Gr-1+

CD3e- B220- Mac-1- Ter-119-Lineage-Markers Gr-1-

5ug total RNA 20ug total RNA
Supplemental Figure 3

(A) Mac1 Gr1 cells (% of GFP gated) for Vector, miR-30, miR-181, miR-223, and miR-142s.

(B) Mac1 Gr1 cells (% of GFP gated) for Vector, miR-30, miR-181, miR-223, and miR-142s.

(C) Mac1 Gr1 cells (% of GFP gated) for Vector, miR-30, miR-181, miR-223, and miR-142s.

(D) Flow cytometry plots showing Mac1 APC and Gr-1 PE for Vector, miR-30, miR-181, miR-223, and miR-142s with corresponding counts.