MicroRNA profiling of the murine hematopoietic system
Silvia Monticelli, K Mark Ansel, Changchun Xiao, Nicholas D Socci, Anna M Krichevsky, To-Ha Thai, Nikolaus Rajewsky, Debora S Marks, Chris Sander, Klaus Rajewsky, Anjana Rao and Kenneth S Kosik

Addresses: *Department of Pathology, Harvard Medical School, and CBR Institute for Biomedical Research, Boston, MA 02115, USA. †Department of Biology and Genetics of Medical Sciences, Università degli Studi di Milano, 20133 Milan, Italy. ‡Computational Biology Center, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA. ¶Center for Functional Comparative Genomics, Department of Biology, New York University, New York, NY 10003, USA. §Department of Systems Biology, Harvard Medical School, Boston, MA 02115, USA. ¶Neuroscience Research Institute, University of California Santa Barbara, Santa Barbara, CA 93106, USA.

¤ These authors contributed equally to this work.

Correspondence: Silvia Monticelli. E-mail: monticel@cbri.med.harvard.edu

© 2005 Monticelli et al.; licensee BioMed Central Ltd.
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Background: MicroRNAs (miRNAs) are a class of recently discovered noncoding RNA genes that post-transcriptionally regulate gene expression. It is becoming clear that miRNAs play an important role in the regulation of gene expression during development. However, in mammals, expression data are principally based on whole tissue analysis and are still very incomplete.

Results: We used oligonucleotide arrays to analyze miRNA expression in the murine hematopoietic system. Complementary oligonucleotides capable of hybridizing to 181 miRNAs were immobilized on a membrane and probed with radiolabeled RNA derived from low molecular weight fractions of total RNA from several different hematopoietic and neuronal cells. This method allowed us to analyze cell type-specific patterns of miRNA expression and to identify miRNAs that might be important for cell lineage specification and/or cell effector functions.

Conclusion: This is the first report of systematic miRNA gene profiling in cells of the hematopoietic system. As expected, miRNA expression patterns were very different between hematopoietic and non-hematopoietic cells, with further subtle differences observed within the hematopoietic group. Interestingly, the most pronounced similarities were observed among fully differentiated effector cells (Th1 and Th2 lymphocytes and mast cells) and precursors at comparable stages of differentiation (double negative thymocytes and pro-B cells), suggesting that in addition to regulating the process of commitment to particular cellular lineages, miRNAs might have an important general role in the mechanism of cell differentiation and maintenance of cell identity.
Background

MicroRNAs (miRNAs) represent a recently discovered class of small, noncoding RNAs, found in organisms ranging from nematodes to plants to humans. Many individual miRNAs are conserved across widely diverse phyla, indicating their physiological importance. The primary transcript (pri-miRNA) is generally transcribed by RNA polymerase II; it contains a typical stem-loop structure that is processed by a nuclear enzyme complex including Drosha and Pasha, and releases a 60- to 110-nucleotide pre-miRNA hairpin precursor [1]. The premiRNA is further processed by Dicer to yield the 19- to 22-nucleotide mature miRNA product, which is then incorporated into the RNA-induced silencing complex (RISC) [2-4]. RISC-bound miRNAs direct the cleavage and/or translational repression of messenger RNAs, thus providing post-transcriptional control of gene expression.

Like many transcription factors, miRNAs are important determinants of cellular fate specification. One of the most prominent and genetically best-studied examples is given by miRNAs involved in neuronal fate determination in Caenorhabditis elegans, where a cascade of several miRNAs and transcription factors regulate each other’s activity to induce a different spectrum of putative chemoreceptors in the two main taste receptor neurons in C. elegans [5]. Furthermore, many miRNA genes are located at fragile sites, minimal loss of heterozygosity regions, minimal regions of amplification, or common breakpoints in human cancers, suggesting that miRNAs might play an important role in the pathogenesis of human cancer [6,7].

Hundreds of miRNAs have been identified in plants and animals, either through computational searches, RT-PCR-mediated cloning, or both. More than 200 human and rodent miRNAs have been reported and tabulated in the miRNA Registry [8], accounting for an estimated 1-2% of expressed human genes. Recent evidence suggests that the actual number of miRNAs is likely to be even larger [9,10]. MiRNAs have been implicated in biological processes ranging from cell proliferation and cell death during development to stress resistance, fat metabolism, insulin secretion and hematopoiesis [11]. However, for the most part, the regulation and function of most mammalian miRNAs are unknown. The bulk of the existing data on miRNA expression in mammalian cells has been derived from studies on whole tissues, which contain many heterogeneous cell types, or on transformed or established cell lines that may have diverged significantly from the primary cell types that they are assumed to represent [7,12-15]. To understand the role of miRNAs in mammalian development and differentiation, an important starting point is a systematic compilation of miRNAs expressed in individual cell types, especially those derived by differentiation from a common precursor.

The cells of the immune system originate from hematopoietic stem cells in the bone marrow, where many of them also mature. The hematopoietic stem cells give rise to both myeloid and lymphoid progenitors. The myeloid progenitor is the precursor of granulocytes, macrophages, dendritic cells, and mast cells of the innate immune system. Mast cells, whose blood-borne precursors are not well defined, terminate their differentiation in the body tissues, where they are widely distributed and where they orchestrate allergic responses and play a part in protecting mucosal surfaces against pathogens [16]. The common lymphoid progenitor gives rise to B and T lymphocytes and to natural killer cells. B lymphocytes differentiate in the bone marrow and T lymphocytes in the thymus; the stages of B and T cell development are defined by sequential rearrangement and expression of heavy- and light-chain immunoglobulin genes and TCR α and β chains, respectively. Mature B and T lymphocytes that have emigrated to the peripheral lymphoid organs, including the spleen and lymph nodes, but have not yet encountered their specific antigen are called ‘naïve’. In the event of an infection, T lymphocytes that recognize the infectious agent are arrested in the lymphoid organs, where they proliferate and differentiate further into effector cells capable of combating the infection.

Because of the wealth of information available about the transcriptional and cellular networks involved in hematopoietic differentiation, the hematopoietic system is ideal for studying cell lineage specification. Many of the common progenitors of hematopoietic cells can be obtained as primary cells from humans and mice, and expanded and differentiated in vitro. Here we have performed a detailed analysis of miRNA expression in diverse hematopoietic cell types from the mouse, using a high-throughput system that allows analysis of many samples with minimal manipulation of the samples themselves. This has allowed us to identify miRNAs that are highly expressed in the hematopoietic system. Our results are consistent with a model of hematopoiesis in which transcriptional regulators act in concert with differentially expressed miRNAs to modulate the levels of mRNAs that control cell differentiation pathways.

Results

Microarray design

To probe the expression of miRNAs in a variety of different related and unrelated cell types, we chose to use miRNA arrays in preference to time-consuming Northern analysis that cannot be used efficiently with many different probes and samples. In the past year, several microarray methods have been developed [7,12,13,15,17-19]. Some of these groups [7,12,13,15,17] used cDNA or cRNA generated from total cellular RNA to apply to their microarrays. Other methods [18,19] rely heavily on several enzymatic steps, such as RNA ligation [18], or Klenow synthesis and exonuclease I degradation of ssDNA [19]. Instead, we chose a technique that does not involve reverse transcription of RNA and relies on only one enzymatic step ([20]; see Methods) thus reducing RNA manipulation to a minimum. In designing the arrays, we
expanded the array dataset already developed by Krichevsky et al. [20]. The new generation of arrays contains 181 gene-specific oligonucleotide probes, corresponding to human, rat, and mouse miRNAs as reported in the miRNA Registry [8].

Data from the arrays
Figure 1a shows a typical array experiment comparing miRNA expression in bone marrow-derived mast cells (BMMC) and a hematopoietic progenitor cell line (Pu.1−/−) derived from mice lacking the Ets-family transcription factor PU.1 [21]. This cell line differentiates efficiently into mast cells when rescued with PU.1 under conditions where GATA2 expression is maintained; expression of PU.1 in the absence of GATA2 results in commitment to the macrophage lineage instead [22]. Visual comparison of three arrays performed with each RNA sample shows the high reproducibility of the arrays, and emphasizes the difference in miRNA expression relative to hippocampal RNA (Figure 1a). This high degree of reproducibility was maintained over a total of nine arrays, each performed in triplicate using three independent RNA samples (not shown). Statistical analysis confirmed the high level of reproducibility (Figure 1b): when the standard deviation...
tion over replicates was plotted versus the mean of each replicate, 86% of the spots were considered good (see legend to Figure 1b for details). RNA loading for all arrays and Northern blot experiments was evaluated by ethidium bromide staining of a denaturing acrylamide gel as shown in Figures 1a and 2d.

The arrays were repeated using cells from several stages of lymphocyte differentiation (Figure 2a). Among the cell types compared were pro-B cells, which are in the process of rearranging the heavy-chain immunoglobulin locus; mature splenic B cells, which express IgM and IgD B cell receptors and are competent to respond to antigen; double-negative thymocytes (DN T), which are just beginning to rearrange their T cell receptor chains and lack surface expression of the CD4 and CD8 co-receptors; naïve CD4 T 'helper' cells, which have exited the thymus, bear the CD4 co-receptor and a mature T cell receptor, and are fully capable of recognizing and responding to antigen; and Th1 and Th2 T helper subsets, which are derived by differentiation from a common precursor, the naïve CD4 T cell, and are characterized by selective expression of the cytokines IFNγ and IL4, respectively. Figure 2a shows representative array data for pro-B cells, mature splenic B cells, naïve T cells, and Th1 and Th2 clones. Note that miR-150 is highly expressed in B cells purified from mouse spleen, but not in pro-B cells isolated from bone marrow of Rag2-/- mice; it is also expressed in naïve T cells but is down-regulated in the Th1 and Th2 T cell clones (Figure 2a, arrow).

Validation of array data by Northern analysis

Before analyzing the entire dataset from the microarrays, we validated the array results by Northern blot analysis. Single-stranded DNA oligonucleotides complementary to over 40 different miRNAs were used as probes; they were chosen because they were expressed in at least one cell type in the hematopoietic lineages and/or were highly expressed in neurons. Several of these Northern blots were already published with the first description of the microarray methodology [20] to confirm the array specificity. We performed several other Northern blots that included hematopoietic cells and tissues. These data are shown in Figures 2, 3 and 4, and summarized in Tables 1 and 2. With minor exceptions as discussed below, the results of Northern analysis were consistent with the array data for most miRNAs, by simple visual inspection (Table 2), and when the hybridization signal intensity was quantified by phosphorimager (Figure 3 and Table 1). For example, Northern analysis of miR-150 expression confirmed its expression in spleen B but not pro-B cells (Figure 2b, lanes 6 and 7), and in naïve T cells but not the Th1 and Th2 clones, D5 and D10 (Figure 2c, lanes 5-7). Figure 2b also shows that miR-150 is expressed in thymocytes and splenic T cells (lanes 9 and 10), but not in ES cells, mouse embryo fibroblasts or hippocampus (lanes 11-13). The lack of expression in RAG2-/- spleen and thymus (lanes 5 and 8) confirms that expression in these organs is confined to T and B lymphoid-lineage cells, and that within these lineages, miR-150 expression is restricted to cells that have developed beyond the DN T and pro B stages of development. Figure 2c confirms that naïve T cells show high level expression of miR-150 (lane 7) whereas the precursor cell line Pu.1-/- and BMMC, which are of the myeloid lineage, do not (lanes 1-4). Equivalent RNA loading in all lanes was confirmed by ethidium bromide staining of a denaturing acrylamide gel as shown in Figure 2d.

Strikingly, miR-150 expression in naïve T cells is rapidly down-regulated upon TCR engagement, regardless of whether T cells are stimulated under Th1 or Th2 conditions (Figure 2c, lanes 8-12). The levels of expression of miR-150 were already reduced by ~50% after 12 h of stimulation with plate-bound αCD3 and αCD28 (lane 8), and by ~90% after 25 h (lanes 9 and 11), indicating a rapid and highly inducible mechanism of down-regulation. Expression was barely detectable after 49 h (lanes 10 and 12) and remained undetectable 3 days after stimulation (data not shown). Furthermore, miRNA expression was extinguished in fully committed Th1 and Th2 T cell clones (lanes 5 and 6). Together, these results suggest a role for miR-150 either in maintaining the undifferentiated status of naïve T cells or in promoting early steps in T cell differentiation.
Figure 2 (see legend on previous page)
MiR-142s and miR-26a were expressed at low levels in the Pu.1-/- precursor cells and the fully-differentiated BMMC; miR-26a and 27a, which are expressed at low levels in the Pu.1-/- precursor cells and at three- to fourfold higher levels in fully differentiated BMMC; and miR-223, which is most highly expressed in the Pu.1-/- precursor cells and is barely detectable in the differentiated BMMC. The results of Northern analysis for these two cell types show an overall good concordance with the values obtained from the arrays (Figures 2, 3 and Table 1).

The right panels of Figure 3 compare expression of the same miRNAs in naïve T cells and fully-differentiated Th1 and Th2 cells (the D5 and D10 clones). Several expression patterns are evident: miR-146 is highest in Th1 cells and low in naïve T cells and Th2 cells; miRNAs 142s and 26a are expressed at higher levels in the precursor naïve T cells; miR-27a is equivalently expressed in both the precursor naïve T cells and the differentiated Th1 and Th2 cells; and miR-223 is very poorly expressed in all these T cell types. The relative expression of these miRNAs in naïve versus differentiated T cells was confirmed in primary cultures of Th1 and Th2 cells (see Table 1).

There was full concordance of the Northern analysis with the miRNA array data for miRNAs 146, 142s, 26a and 223 (Figure 3, Table 1); however, as discussed further below, the signal for 27a and a handful of other miRNAs expressed at low levels in naïve T cells fell below the limit of detection on the microarrays.

Table 1 summarizes the results from Northern analysis of the miRNAs shown in Figures 2 and 3, as well as showing data for two additional miRNAs, let7d (let7 family) and miR-222. The miRNA expression pattern of D5 and D10 T cell clones was comparable with that of differentiated primary Th1 and Th2 cells respectively, validating the use of D5 and D10 cells as models for fully differentiated Th1 and Th2 cells. Like miR-150, the expression of miR-142s, miR-26a and let7d showed a rapid decline during differentiation of naïve T cells into Th1 or Th2 effectors. miR-27a was expressed at equivalent levels in naïve and differentiated T cells. miR-146 showed a Th1-skewed expression pattern: it’s levels increased in Th1 cells and decreased in Th2 cells relative to it’s expression in naïve T cells. We have not yet detected an miRNA with the converse expression pattern of high expression in Th2 cells relative to Th1. miR-223 was detectably expressed in BMMC, Pu.1-/- precursor cells and fully differentiated Th1 cells, but it’s
expression was not detectable in the other cell types tested (Table 1); in contrast, miR-16 was expressed in all cell types analyzed, but its expression was relatively variable both in arrays and Northern blots, so quantification was not attempted (data not shown). Some of our data confirm published reports. For example, miR-223 is reported to be expressed in myeloid cells [7,23]; miR-125 and 128 are highly expressed in the brain [13,14]; and miR-16 is expressed in a wide variety of tissues [7,14,23] (see also heat map of expression in Figure 5a).

Figure 4 shows Northern blot data for additional miRNAs. Most of the data from Northern blots correlated at least qualitatively with the expression data from the arrays (Table 2;
also compare data in Figures 3 and 4 to the heat map in Figure 5. Some exceptions were noted. For some of the miRNAs (miR-129, 151, 184, 185, 202, 212 and 351), we could not obtain any hybridization signal on Northern blots, so we were unable to compare Northern and array data. MiR-223 and miR-206 showed poor correlation between the arrays and the Northern blots: for miR-223, we detected a higher level of expression in pro-B in the arrays compared with what we detected on Northern blot, while for miR-206, the arrays showed high expression in pro-B and DN T that was undetectable by Northern blot. In a few other cases, the hybridization signal was lower in the arrays compared with Northern blots, but the relative expression levels between different cell types was similar. It is unclear at this point why the expression of some miRNAs appears different depending on the method used to detect them.

In a few cases, the probes used in Northern analysis hybridized to a cross-reacting band with a molecular weight higher than the mature or pre-miRNA molecules. In these cases the correlation between Northern blots (which use total RNA) and arrays (which use the low-molecular weight RNA) was only partial. Even though our system is designed to exclude RNA molecules bigger than ~300 nucleotides, we effectively obtained exclusion of molecules bigger than 60-80 nucleotides (as shown in [20]). Thus, the changes observed mainly reflect changes in mature miRNA levels, as also shown by the correlation with Northern blots. However, it remains possible that a strong expression of cross-reacting RNA close to this size might partially alter the array results; we observed such bands for miR-186, miR-188 and miR-321. Of note, miR-321 has been removed from the microRNA Registry because it was identified as a fragment of an Arg tRNA and not a miRNA.

Despite differences in methods as well as in the number of microRNAs analyzed, there is good agreement between our results and those of others, with regard to specificity and sensitivity, when array and Northern blot analyses are examined for similar cell types [13,14]. Similar to the findings and discussion of Miska et al. [13], we do not expect our microarray technique to provide sufficient specificity to distinguish reliably between hybridizing sequences that have only one or few nucleotide mismatches. Although hybridization signals from several control probes containing three staggered nucleotide mismatches were lower than that for the corresponding miRNA probes (see also Material and methods), our method cannot efficiently discriminate between close miRNA paralogs. This limitation is alleviated somewhat by the fact that for most miRNAs, the most closely related paralogs differ by five mismatches or more [13]. The sensitivity of the arrays is similar to that of Northern blots. Synthetic RNA oligonucleotides ‘spiked’ into cellular RNA samples prior to array hybridization were detected at a 2-20 fmole range. Northern blot allowed detection of as little as 1-10 fmole of synthetic oligonucleotides (data not shown). In summary, therefore, we saw substantial concordance between arrays and Northern blots, allowing us to identify cell type-specific differences in miRNA expression as well as differences between miRNAs expressed by precursor cells and their differentiated progeny. This led us to analyze the array data more extensively using computational methods.

### Analysis of miRNA arrays

To identify patterns of miRNA expression among the cell types tested, we arranged the array data for miRNAs that were expressed at least three times over the background for at least one of the samples in a heat map (Figure 5a). Brown to white colors indicate increasing levels of miRNA expression in arrays. This analysis revealed a cluster of miRNAs that were preferentially expressed in the hippocampus compared with hematopoietic cells, as indicated by the blue bar in the left panel. MiRNAs expressed at higher levels in the hematopoietic system are indicated by the purple bar in the right panel.

**Table 1**

| miR  | BMMC | Pu. 1-/- | Naive | Th1 (49 h) | D5 Th1 | Th2 (49 h) | D10 Th2 |
|------|------|---------|-------|------------|--------|-----------|--------|
| 150  | n.d. | n.d.    | 39.4  | 3.5        | n.d.   | 1.0       | n.d.   |
| 146  | 1.7  | 1.0     | 2.8   | 3.7        | 6.8    | 1.3       | 1.0    |
| 142s | 2.0  | 1.0     | 32.0  | 7.5        | 3.8    | 4.7       | 3.9    |
| 26a  | 3.1  | 1.0     | 13.8  | 1.4        | 3.0    | 1.2       | 2.5    |
| 27a  | 3.9  | 1.0     | 1.3   | 0.9        | 1.4    | 0.8       | 1.5    |
| 223  | 1.0  | 45.0    | n.d.  | n.d.       | n.d.   | n.d.      | n.d.   |
| Let7d| 2.3  | 1.0     | 4.7   | 2.1        | 2.5    | 1.7       | 1.7    |
| 222  | 1.0  | 1.2     | n.d.  | n.d.       | 1.1    | n.d.      | n.d.   |

Northern blots for miRNA expressed in mast cells, precursor cells, and T cells at various stages of differentiation were quantified by phosphorimager. BMMC, bone marrow-derived mast cell; DN T, double-negative thymocyte; n.d., not detectable.
### Table 2

**Correlation between arrays and Northern blot data**

|     | DNT       | Pro-B     | Spleen B  | BMMC   | Hippocampus |
|-----|-----------|-----------|-----------|--------|-------------|
| MiR 7 | Arrays    | +         | ++        | ++     | +/-         | ++          |
|      | Northernns| +         | +++       | ++     | n.a.        | n.a.        |
| MiR 24 | Arrays    | +++       | ++++      | ++++   | ++          | ++          |
|      | Northernns| ++        | ++++      | ++++   | n.a.        | n.a.        |
| MiR 26a | Arrays   | +         | +         | ++     | +++         | +++         |
|      | Northernns| +         | ++        | ++++   | n.a.        | n.a.        |
| MiR 29a | Arrays   | +         | +         | ++     | ++          | +++         |
|      | Northernns| +         | +         | ++     | nd          | +++         |
| MiR 93 | Arrays    | ++        | ++        | +/-   | ++          | +/-         |
|      | Northernns| +         | ++        | +     | n.a.        | n.a.        |
| MiR 99a | Arrays   | n.s.s.    | n.s.s.    | n.s.s. | n.s.s.      | n.s.s.      |
|      | Northernns| +/-       | +/-       | -     | nd          | nd          |
| MiR 101 | Arrays   | ++        | +++       | +     | +++         | ++          |
|      | Northernns| +         | +         | +     | nd          | ++          |
| MiR-107 | Arrays   | +++       | ++++      | ++++   | ++          | ++          |
|      | Northernns| +         | ++        | +     | n.a.        | n.a.        |
| MiR-127 | Arrays   | +/-       | +         | +     | +/-         | +           |
|      | Northernns| -         | -         | -     | n.a.        | n.a.        |
| MiR-142-3p | Arrays | +++       | +++       | ++++   | +++         | +           |
|      | Northernns| ++        | +++       | ++++   | ++          | n.a.        |
| MiR-142-5p | Arrays | ++        | +         | ++++   | ++          | +           |
|      | Northernns| +         | ++        | ++++   | +           | n.a.        |
| MiR-144 | Arrays    | n.s.s.    | n.s.s.    | n.s.s. | n.s.s.      | n.s.s.      |
|      | Northernns| -         | -         | -     | n.a.        | n.a.        |
| MiR-148 | Arrays    | +         | +         | +     | +/-         | +           |
|      | Northernns| +         | +         | +     | n.a.        | n.a.        |
| MiR-150 | Arrays    | -         | +         | ++++   | +           | +/-         |
|      | Northernns| -         | -         | ++++   | n.a.        | -           |
| MiR-181b | Arrays   | +         | +         | +     | ++          | ++          |
|      | Northernns| +         | +         | -     | n.a.        | +           |
| MiR-191 | Arrays    | +/-       | +         | +     | +           | +           |
|      | Northernns| +         | ++        | +     | n.a.        | n.a.        |
| MiR-199 | Arrays    | +         | +         | +     | +           | +/-         |
|      | Northernns| -         | +         | -     | n.a.        | n.a.        |
| MiR-206 | Arrays    | ++        | +++       | +     | +/-         | +/-         |
|      | Northernns| -         | -         | -     | -           | n.a.        |
| MiR-213 | Arrays    | n.s.s.    | n.s.s.    | n.s.s. | n.s.s.      | n.s.s.      |
|      | Northernns| +/-       | -         | -     | n.a.        | n.a.        |
| MiR-223 | Arrays    | +         | +++       | +     | +/-         | +           |
|      | Northernns| +/-       | -         | +     | +           | n.a.        |
| MiR-342 | Arrays    | +         | +         | +     | +           | +           |
|      | Northernns| +         | +++       | ++     | n.a.        | n.a.        |

The table summarizes and compares the Northern blot data shown in Figure 4 and the array data shown in the heat map in Figure 5. Northern blot and array data were scored independently using an arbitrary scale from undetectable (-) to strongly detected (++++) to indicate relative signal intensity in each case. BMMC, bone marrow-derived mast cell; DNT, double-negative thymocyte; n.s.s., non statistically significant hybridization signal; n.a., not analyzed.
To achieve a better understanding as to how miRNA expression patterns correlate with hematopoietic cell differentiation, we performed a hierarchical clustering of the normalized array data for hematopoietic cell types (Figure 5b). The subset of miRNAs detected in at least one hematopoietic cell sample was used to compute the distance function from the Pearson correlation between samples (Table 3). Standard hierarchical clustering with average linkage was used, and bootstrap resampling was employed to assess the robustness of the clustering results. This analysis showed that fully differentiated effector cells (Th1, Th2 and BMMC) are more closely related to each other in their miRNA expression pattern than to their respective precursor cells (DN T and Pu.1-/- precursor cells).

The miRNA expression patterns of pro-B and DN T, precursor cells for the B and T lymphocyte lineages respectively, were also very closely related. Although the detected miRNA expression pattern of naïve T cells most closely resembled that of splenic B cells (Table 3), naïve T cells were excluded from the clustering analysis. This was because RNA isolated from naïve T cells yielded much lower overall array hybridization signals compared with RNA from the other cell types examined, causing the signal for a handful of expressed miRNAs to fall below the limit of detection for the microarrays (for example, miR-27a, see Figure 4 and Table 1), and making it impossible to accurately normalize the array data for naïve T cells relative to the signal obtained from other cell types.

**Discussion**

In summary, pairwise comparisons of the expression of 181 mature miRNAs in selected highly purified hematopoietic cell types at immature, mature, and effector stages revealed specific differences between related cell types (see also Additional Data Files 1, 2, 3). As described above, the differences were confirmed by Northern analyses (Figures 2, 3 and 4, and Tables 1 and 2) and revealed a subset of miRNAs expressed at
higher levels in the hematopoietic system compared with neuronal tissue (Figure 5a). Figure 6 shows a schematic hematopoietic lineage tree, where lymphoid and myeloid cells derive from a common lymphoid and common myeloid progenitor, respectively. Both these cell types derive from a common precursor further upstream in the differentiation process and we used the Pu.1-/− cells as a model for such precursors. The figure also shows a summary of some confirmed changes in miRNA expression in the different stages of cell differentiation superimposed on the diagram, showing precursor-progeny relationships in lymphocyte and mast cell differentiation. Even though only selected hematopoietic cell lineages were analyzed, each differentiation step was characterized by changes in miRNA expression, with some miRNAs showing increased and some showing decreased expression. MiR-150 expression is of particular interest: this miRNA is up-regulated during the developmental stages of B and T cell maturation, but down-regulated again during the further differentiation of naïve T cells into effector Th1 and Th2 cells. miR-146 is also notable, since it is upregulated in Th1, but not Th2 cells. We therefore predict that these miRNAs probably play a role in establishing and/or maintaining cell identity in lymphocytes.

Differentiation of naïve T cells into Th1 and Th2 effector cells is a particularly tractable system for studying cell lineage specification. Northern blot analyses indicated that several miRNAs are rapidly down-regulated following activation of naïve T cells under both Th1 and Th2 differentiation conditions. miR-146 was a clear exception to this pattern, being up-regulated in Th1, but not Th2 cells. In this respect, miR-146 joins a group of Th1-associated genes that include cytokines (Ifnγ, Tnfa), chemokine receptors (Cxcr3, Ccr5), and transcription factors (Tbx21, Hlx) [6,24,25]. Our results suggest two hypotheses for further testing. Firstly, the transcription of pri-miRNAs may be controlled by the same transcription factors known to control specific cell differentiation events. For example, T-bet directs Ifnγ expression, and may also activate transcription of pri-miR-146. Conversely, miRNAs that are regulated during cell differentiation may target one or more of the miRNAs known to be differentially expressed between Th1 and Th2 cells. Although the predicted targets of conserved miRNAs represent a diverse array of gene products [26-30], targeting of key transcription factors would represent a particularly efficient means for miRNA participation in cell fate decisions.

Our results call to attention the ‘common logic’ and shared roles of transcriptional regulation by transcription factors and post-transcriptional regulation by miRNAs [5]. It is likely that both pathways of regulation are integral to successful regulation of hematopoietic cell differentiation. Decades of research have been devoted to the elucidation of the transcriptional networks involved in hematopoiesis. For example, the Ets family transcription factor, PU.1, is necessary for the generation of certain hematopoietic lineages but not others: it directs the differentiation of hematopoietic progenitors into macrophages, neutrophils, B lymphocytes and mast cells, but is not involved in erythroid or megakaryocytic differentiation [21,22]. Lineage specification is controlled by the level of PU.1 expression and by which partner transcription factors are co-expressed: for instance, low levels of PU.1 and co-expression of early B-cell factor (EBF) control differentiation to the B cell lineage, whereas high levels of PU.1 predispose to macrophage differentiation unless GATA2 is co-expressed, in which case differentiation is tilted to the mast cell lineage [22,31,32]. It will be informative to compare miRNA expression in Pu.1-/− progenitor cells that have been reconstituted to promote differentiation along these various lineages. Further study of the expression and function of miRNAs in hematopoiesis will probably uncover additional complexity and subtlety, as well as interconnections between miRNA and transcription factor networks [5].

Systematic analysis of miRNA expression patterns within our dataset indicates that besides influencing the process of com-

| Table 3 |
| --- |
| **Correlation coefficients (Pearson correlation)** |
|  | Naive | DN T | Pro-B | Pu.1-/− | Spleen B | BMMC | Th1 | Th2 |
| Naive | 1.00 |  |  |  |  |  |  |  |
| DN T | 0.50 | 1.00 |  |  |  |  |  |  |
| Pro-B | 0.47 | 0.94 | 1.00 |  |  |  |  |  |
| Pu.1-/− | 0.56 | 0.83 | 0.78 | 1.00 |  |  |  |  |
| Spleen B | 0.76 | 0.87 | 0.80 | 0.81 | 1.00 |  |  |  |
| BMMC | 0.63 | 0.74 | 0.68 | 0.78 | 0.79 | 1.00 |  |  |
| Th1 | 0.68 | 0.85 | 0.79 | 0.84 | 0.88 | 0.92 | 1.00 |  |
| Th2 | 0.67 | 0.83 | 0.80 | 0.84 | 0.87 | 0.90 | 0.96 | 1.00 |

The table represents a standard statistical correlation between the indicated samples, where 1 = perfectly correlated and 0 = uncorrelated. BMMC, bone marrow-derived mast cell; DN T, double-negative thymocyte.
mitment to a particular cellular lineage, miRNAs may play an important general role in the mechanism of cell differentiation and maintenance of cell identity. The highest degree of correlation in the expression pattern of miRNAs was observed 'horizontally' between hematopoietic cell types at a similar stage of differentiation. For instance, early B and T cell precursors are more closely related to each other in their miRNA expression patterns than to their more differentiated progeny, mature splenic B cells and naïve T cells. Most strikingly, fully differentiated effector cells, including the closely related Th1 and Th2 cells, but also the much more distantly related BMMC, are more closely related to each other in their miRNA expression pattern than to their respective precursor cells. The high degree of correlation in the miRNA expression patterns of these distantly related immune effectors suggests that a common set of miRNAs may be employed in both lineages to regulate similar effector functions, such as tissue homing and cytokine production. Alternatively, these findings may reflect a general role for some of these miRNAs in stabilizing gene expression and thereby lineage specification. This could be accomplished through the promiscuous targeting of many transcripts or by specific targeting of genes that regulate the plasticity of transcriptional states, such as chromatin-modifying proteins. Similarly, miRNAs shared among early precursors may regulate precursor cell self-renewal and maintenance of an undifferentiated state. The rapid loss of several miRNAs early in the process of differentiation of Th1 and Th2 effector cells from naïve T cell precursors is consistent with this concept.

**Conclusion**

We report miRNA expression patterns for diverse murine hematopoietic cell types, identify a subset of miRNAs prefer-
entially expressed in the hematopoietic system compared with neuronal cells, and identify individual miRNA expression changes that occur during cell differentiation. Our data support the use of the miRNA microarray for detection of patterns of miRNA expression and quantification of miRNA expression, with the obvious advantage that the expression of several hundred genes can be identified in the same sample at once, and with relatively small amounts of total RNA. Deciphering the miRNA expression status of cells under different conditions of development and activation and in different disease states will be useful to identify miRNA targets, and alterations in the pattern of miRNA expression may disclose new pathogenic pathways and new ways to target diseases.

**Materials and methods**

**Tissue preparation, cells differentiation and RNA extraction**

Hippocampi were dissected from 10-14 week old Balb/c mice. For pro-B cell preparation, bone marrow cells were isolated from femurs and tibias of 6-12 week old Rag2-/- C57BL/6 x 129 mice and pro-B cells were isolated using CD19 MACS beads. Spleen B cells were isolated using CD19 MACS beads from splenocytes obtained from 6-12 week old C57BL/6 mice. Double-negative thymocytes (DN T) were obtained from the thymi of 4-6 week old Rag2-/- C57BL/6 x 129 mice without purification; more than 90% of the cells were DN2 and DN3 (not shown). Due to the low amount of cells that can be obtained from one mouse, proB and DN T cells were purified from 20 Rag-/- mice, while the spleen B cells were obtained from 10 C57BL/6 mice, and the samples were pooled.

BMMC preparation and differentiation was as previously described [33,34]. Briefly, bone marrow cells were isolated from femurs and tibias of 6-12 week old BALB/c mice and maintained for 4-12 weeks in RPMI medium containing 50% WEHI-3 (American Type Culture Collection, VA, USA) conditioned supernatant as a source of IL-3.

**Pu.1 knock-out cells** were kindly provided by Dr Harinder Singh (University of Chicago), and were maintained in IMDM media supplemented with 10 ng/ml of recombinant IL-3 (Peprotech Inc., NJ, USA). Naïve CD4 cells were purified from spleen and lymph nodes of Tcrα/- Do11 TCR transgenic mice by magnetic bead selection (Dynal, Oslo, Norway) as previously described [33]. Primary Th1 and Th2 cells were differentiated in culture for 7 days as previously described [35]. Murine Th1 (D5) and Th2 (D10) clones were maintained as previously described [36]. RNA was prepared using Ultraspec or Trizol reagents following manufacturer’s instructions.

**Oligonucleotide array for miRNA**

This method has been previously described (see [20]). Briefly, trimer oligonucleotides (antisense to miRNAs) of 54-72 nucleotides at a final concentration of 7 µM were spotted on GeneScreen Plus (NEN) membranes with a 1536 pin plate replicator (V&P Scientific, CA, USA). Oligonucleotides were immobilized in 100 mM NaOH, after which the membranes were briefly neutralized in 5% SDS at room temperature and with 0.2% SDS at 72°C. Arrays were stored in 0.2% SDS at -4°C.

Total RNA (5-10 µg) from hippocampus tissue and various hematopoietic cell types was preheated at 80°C for 3 min, cooled on ice and filtered through Microcon YM-100 concentrators to obtain a low molecular weight (LMW) fraction of RNA enriched in molecules less than 60 nucleotides in size. The LMW RNA was end-labeled with 30 µCi of γ32P dATP (3000 Ci/m mole) with T4 polynucleotide kinase, and purified using the QIAgen Nucleotide Removal kit (QIAgen Inc., CA, USA).

For hybridization, membranes were first prehybridized in MicroHyb hybridization buffer (ResGen, AL, USA) at 37°C for at least 30 min, followed by an overnight hybridization in the same solution containing the RNA probe. Following hybridization, membranes were washed twice in 2 × SSC/0.5% SDS at 37°C and once in 1 × SSC/0.5% SDS at 37°C. Membranes were exposed to a phosphor storage screen, scanned using a Phosphor Imager, and signals were quantified using the ImageQuant software (Molecular Dynamics, CA, USA). For reuse, membranes were striped with 0.2% SDS at 72°C, tested again by exposure to phosphorimager screen, andrehybridized three to five times. Each experiment included two to three independent RNA samples and to ensure accuracy of the hybridizations, each RNA sample was hybridized with three membranes.

To confirm specificity, a series of oligonucleotides with three mismatches (G>C or C>A) were included on the array. These mismatches resulted in a significant drop in signal intensity as compared with their cognates. The melting temperature of oligo probe:miRNA pairs could affect the sensitivity and specificity of the arrays for different miRNAs. An analysis of this correlation showed that hybridization signals significantly above background were obtained for probes in a wide range of melting temperatures (Additional Data File 4). Also, three synthetic 21-nt RNA oligonucleotides with sequences that do not correspond to any known miRNA, but that are exact complements to randomly spotted sequences, were added to the RNA samples at a known concentration as a reference for normalization.

**Northern blot analysis**

Total RNA (20 µg) was loaded and separated on a denaturing 12-15% polyacrylamide gel and transferred electrophoretically to a GeneScreen Plus or Nytran SuPerCharge membrane (Scheicher and Schuell, NH, USA). Membranes were UV-crosslinked. Probes were prepared by T4 polynucleotide kinase labeling of antisense oligonucleotides with γ32P dATP.
Hybridization was performed with UltraHyb Hybridization buffer (Ambion, TX, USA) or Denhardt’s solution at 37-42°C. Blots were washed at the same temperature with 2 × SSC/0.1% SDS with a brief final wash with 0.1 × SSC/0.1% SDS. Radiolabeled Decade RNA markers (Ambion) were loaded as size markers. tRNA and 5S RNA stained with ethidium bromide served as a sample loading control. For reuse, blots were stripped by boiling in 0.1 × SSC/0.1% SDS twice for 10 min and reprobed.

Data analysis
Before analysis, the raw data needed to be processed in order to handle overall scaling differences between the individual scans and negative values arising from the background subtraction. Although these are common issues in array-based experiments, it is not obvious what the optimal preprocessing algorithm should be. For the data presented here, the Variance Stabilization Normalization method [37] was used. The method has a considerable advantage in that it uses a generalized log transformation that can deal directly with negative values, eliminating the need to artificially shift or truncate these data points.

Standard hierarchical clustering with average linkage was used to cluster the hematopoietic samples. A subset of the miRNAs was used to compute the distance function. These miRNAs had to have a signal level of three times the background standard deviation in at least one of the hematopoietic samples. The Pearson correlation was used to compute the distance function with dist = (1-p)/2 where p is the correlation. To assess the robustness of the results, bootstrap resampling was carried out using a parametric method to add noise to the data. Gaussian noise with zero mean and a standard deviation equal to that for each spot’s replicates was added to each point. One thousand resampled datasets were created and clustered with a consensus tree built from the results. The number at each node indicates how often that subtree appeared in the 1000 replica trees.

To identify miRNAs that were differentially expressed between the various sample subtypes, a variation of the standard t-test was used on the transformed expression values. To handle the low number of samples, a Bayesian correction method [38] was used to adjust the standard deviation. To account for the multiple testing problem, the False Discovery Rate (FDR) method was used and the lists were cut off at specific values of the FDR. Additionally, the results were filtered to include only those miRNAs that were expressed threefold above the background standard deviation in at least one sample.

Data availability
The primary microarray data is deposited in the ArrayExpress database with accession number E-MEXP-372.

Additional data files
The following additional data are available with the online version of this article: miRNAs differentially expressed between hippocampus and combined hematopoietic samples (Additional data file 1), miRNAs differentially expressed between BMMC and Th1, Th2 and Pu.1/-/- cells (Additional data file 2), miRNAs differentially expressed between spleen B versus pro-B (Additional data file 3), and an analysis of the probe melting temperatures (Tm) versus the average signal obtained in the arrays (Additional data file 4).

Acknowledgements
We thank Dr P Laslo and Dr H Singh for the Pu.1/-/- cells. K.M.A. is a fellow of the Damon Runyon Cancer Research Fund (DRG-1682). C.X. is a Cancer Research Institute postdoctoral fellow. A special thanks to Prof A Siccardi for help and support. This work was supported by NIH grants to A.R., K.R., and K.S.K., and a grant from the Sandler Program for Asthma Research to A.R.

References
1. Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ: Processing of primary microRNAs by the Microprocessor complex. Nature 2004, 432:231-235.
2. Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004, 116:281-297.
3. Ambros V: The functions of animal microRNAs. Nature 2004, 431:350-355.
4. Meister G, Tuschl T: Mechanisms of gene silencing by double-stranded RNA. Nature 2004, 431:343-349.
5. Hobert O: Common logic of transcription factor and micro-RNA action. Trends Biochem Sci 2004, 29:462-468.
6. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, et al.: Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci USA 2002, 99:15524-15529.
7. Calin GA, Liu CG, Sevignani C, Ferracin M, Felli N, Dumitru CD, Shimizu M, Cimmino A, Zupo S, Dono M, et al.: MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias. Proc Natl Acad Sci USA 2004, 101:11753-117560.
8. Griffiths-Jones S: The microRNA Registry. Nucleic Acids Res 2004, 32:D109-111.
9. Berezikov E, Guruvy V, van de Belt J, Wienholds E, Plasterk RH, Cuppen E: Phylogenetic shadowing and computational identification of human microRNA genes. Cell 2005, 120:21-24.
10. Xie X, Lu J, Kubokas EJ, Golub TR, Mootha V, Lindblad-Toh K, Lander ES, Kellis M: Systematic discovery of regulatory motifs in human promoters and 3′ UTRs by comparison of several mammalians. Nature 2005, 434:338-345.
11. Ambros V: MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. Cell 2003, 113:673-676.
12. Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM: Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature 2005, 433:769-773.
13. Misra EA, Alvarez-Saavedra E, Townsend M, Yoshii A, Sestan N, Rakic P, Constantine-Paton M, Horvitz HR: Microarray analysis of microRNA expression in the developing mammalian brain. Genome Biol 2004, 5:R68.
14. Semperle LF, Freemantle S, Pitha-Rowe I, Moss E, Dmitrovsky E, Ambros V: Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. Genome Biol 2004, 5:R13.
15. Baskerville S, Bartel DP: Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. RNA 2005, 11:241-247.
16. Galli SJ, Kalesnikoff J, Grimbaldeston MA, Piliponsky AM, Williams CM, Tai M: Mast cells as ‘tunable’ effector and immunoreg-
laboratory cells: recent advances. Annu Rev Immunol 2005, 23:749-786.

17. Esau C, Kang X, Peralta E, Hanson E, Marcussen EG, Ravichandran LV, Sun Y, Koo S, Perera RJ, Jain R, et al.: MicroRNA-143 regulates adipocyte differentiation. J Biol Chem 2004, 279:52361-52365.

18. Thomson JM, Parker J, Perou CM, Hammond SM: A custom microarray platform for analysis of microRNA gene expression. Nat Methods 2004, 1:47-53.

19. Nelson PT, Baldwin DA, Scarece LM, Oberholtzer JC, Tobias JW, Moulrelatos Z: Microarray-based, high-throughput gene expression profiling of microRNAs. Nat Methods 2004, 1:155-161.

20. Krivchevsky AM, King KS, Donahue CP, Khrapko K, Kosik KS: A microRNA array reveals extensive regulation of microRNAs during brain development. RNA 2003, 9:1274-1281.

21. DeKoter RP, Walsh JC, Singh H: PU.1 regulates both cytokine-dependent proliferation and differentiation of granulocyte/macrophage progenitors. EMBO J 1998, 17:4456-4466.

22. Walsh JC, DeKoter RP, Lee H, Smith ED, Lancki DW, Gurish MF, Friend DS, Stevens RL, Anastasi J, Singh H: Cooperative and antagonistic interplay between PU.1 and GATA-2 in the specification of myeloid cell fates. Immunity 2002, 17:665-676.

23. Yeh EC, Li L, Lodish HF, Bartel DP: MicroRNAs modulate hematopoietic lineage differentiation. Science 2004, 303:83-86.

24. Murphy KM, Reiner SL: The lineage decisions of helper T cells. Nat Rev Immunol 2002, 2:933-944.

25. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB: Prediction of mammalian microRNA targets. Cell 2005, 120:15-20.

26. Rajewsky N, Socci ND: Computational identification of microRNA targets. Dev Biol 2004, 267:529-535.

27. Solymar DC, Agarwal S, Bassing CH, Alt FW, Rao A: A 3' enhancer in the IL-4 gene regulates cytokine production by Th2 cells and mast cells. Immunity 2002, 17:41-50.

28. Ansel KM, Greenwald RJ, Agarwal S, Bassing CH, Monticelli S, Interlandi J, Djuricic IM, Lee DU, Sharpe AH, Alt FW, et al.: Deletion of a conserved Il4 silencer impairs T helper type 1-mediated immunity. Nat Immunol 2004, 5:1251-1259.

29. Avni O, Lee D, Macian F, Szabo SJ, Glimcher LH, Rao A: A 3' enhancer in the IL-4 gene regulates cytokine production by Th2 cells and mast cells. Immunity 2002, 17:41-50.

30. Avni O, Lee D, Macian F, Szabo SJ, Glimcher LH, Rao A: T(H) cell differentiation is accompanied by dynamic changes in histone acetylation of cytokine genes. Nat Immunol 2002, 3:643-651.

31. Agarwal S, Rao A: Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. Immunity 1998, 9:765-775.

32. Huber W, von Heydebreck A, Sultmann H, Poustka A, Vingron M: Variance stabilization applied to microarray data calibration and to the quantification of differential expression. Bioinformatics 2002, 18:S96-S104.

33. Baldi P, Long AD: A Bayesian framework for the analysis of microarray expression data: regularized t-test and statistical inferences of gene changes. Bioinformatics 2001, 17:509-519.