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MicroRNAs are expressed and processed by human primary macrophages

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Abstract

Macrophages are crucial to host defense, functioning in innate and cell-mediated immunity. MicroRNAs (miRNAs) are small non-coding RNA molecules that repress transcription and protein production. Little is known about miRNA expression in primary human macrophages, or about how macrophage miRNAs contribute to both normal macrophage function and to the pathogenesis of disease in humans. Using western blot analyses, we demonstrated the production of miRNA machinery proteins by human primary macrophages. Using two different miRNA array techniques, we identified 119 miRNAs expressed by human primary macrophages, including hsa-let-7a, miR-16, -23a, 30b, -103, -146a, -212, and -378 and validated them by quantitative RT-PCR. Our findings provide a knowledge base to which macrophage miRNA expression in organ-specific macrophages or disease processes may be compared in humans.

Keywords

miRNA machinery; monocyte derived macrophage; Dicer; Drosha; Argonaute2; miRNome; miRNA variability

1. Introduction

MicroRNAs (miRNAs) are a subset of small non-coding RNA molecules of approximately 22 nucleotides (nt) in length, that function in both normal cellular and disease processes (1) and (2). Transcribed primary-miRNAs are processed by multi-protein complexes including Drosha, Dicer, DiGeorge Critical Region 8 (DGCR8), and TAR-RNA Binding Protein (TRBP) into pre-miRNAs and escorted into the cytoplasm by Exportin-5 (Exp5). MicroRNAs are cleaved by Dicer to produce mature miRNAs and associate with Argonaute2 (Ago2)-containing complexes, which limit the production of the proteins targeted by the miRNA through miRNA-facilitated degradation of target mRNAs, translation inhibition in P-bodies, or transcriptional gene silencing in the nucleus (3). Macrophages function in both innate and cell-mediated immunity. Monocyte differentiation/

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maturation into macrophages requires the expression of miR-17-5p, miR-20a, and -106a, which decreases as monocytes become macrophages (4). Most of what is known about miRNA expression in macrophages has been described in specific subpopulations of macrophages, and describes one, two, or a subset of highly expressed, or differentially expressed, miRNAs. For example, the miRNome of splenic macrophages in individuals with hypersplenism has been reported (5). A limited number of miRNAs, such as miR-146a and -155, have been described in mouse primary macrophages and human macrophage cell lines (6-7). This burgeoning field lacks a common knowledge base that would enable comparison of macrophage miRNA expression data accrued from studies of different types of macrophages and their function in various diseases. Further, the expression of proteins of the miRNA machinery, Dicer specifically, has only been described in human primary monocytes and human macrophage cell lines (8). In this study, we demonstrate that primary human monocyte-derived-macrophages (macrophages) produce Ago2, DGCR8, Dicer, Drosha, Exp5, and TRBP, all essential components of the miRNA processing machinery. We also demonstrate that total RNA from fully differentiated macrophages possesses fractions of small non-coding RNA molecules containing miRNAs. Using two different microarray-based miRNA detection methods, we detected 119 miRNAs expressed by human primary macrophages. Using quantitative Real Time-PCR (RT-PCR) we validated the presence of miRNAs at different levels of expression, including hsa-let-7a, miR-16, -23a, -30b -103, -146a, -212, and -378. Our data provide a baseline for the study of miRNA expression by macrophages during normal macrophage immune function and during disease pathogenesis.

2. Materials and methods

2.1 Cell Culture

PBMC from healthy donors were separated from Leukopaks (New York Blood Center) by gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare). "Donor #s" were assigned from abbreviated Leukopak serial numbers. CD14⁺ monocytes were isolated using CD14⁺ MACS Cell Separation Microbeads (Miltenyi Biotec), plated at 10⁷ cells per 100mm dish and cultured as we previously described (9) to mature macrophages in RPMI supplemented with 10% FBS, 5% Human AB serum (Lonza), 1% Pen/Strep (Invitrogen), 1% HEPES, and 10ng/ml Macrophage-Colony Stimulating Factor (M-CSF) to differentiate the monocytes into macrophages. These macrophages have been characterized by morphological changes such as adherence, expression of CD14 and CD11b by Western Blot, and phagocytotic function. Medium was replaced on days 3 and 6 post-isolation. Macrophages were used on day 9 post-isolation. HeLa cells were obtained from the ATCC and cultured according to vendor instructions.

2.2 Western Blotting

Macrophages and HeLa cells (used as positive control) were lysed using 500µl Mammalian Protein Extraction Reagent (ThermoFisher/Pierce). Lysates were sonicated and then centrifuged at 13,000×g and 4°C for 10 minutes. Proteins were quantified by Bio-Rad Protein Assay (Bio-Rad). 100 µg lysate were used to perform SDS-PAGE. Proteins were transferred onto nitrocellulose filter at 4°C for 90 min. The following antibodies were used at the stated dilutions or concentrations: α-Tubulin, 1:10,000 (Sigma #T-9026); Ago2, 5µg/ml (Abcam #57113); DGCR8, 1:500 (Abnova #H00054487-A01); Dicer, 1:100 (Abcam #14601); Drosha, 1:500 (Upstate #07-717); Exp5, 1:500 (Abnova #H00057510-M01); and TRBP, 6 µg/ml (Imgenex #IMG-6071A).

2.3 MicroRNA Arrays

Total RNA was harvested from human primary macrophages and breast cancer tissue (used as positive control) using TRIzol LS (Invitrogen) following manufacturer instructions. For fractionated RNA used on spotted arrays, 20µg of total RNA were isolated from the macrophages of two donors, fractionated to separate molecules less than 40 nt in length using FlashPAGE, column purified using the FlashPAGE Reaction Clean-Up Kit (Ambion), and analyzed for quality using Small RNA Chips on a Bioanalyzer 2100 (Agilent). The miRNA fraction was amino-allyl labeled with Cy3 or Cy5 (GE Healthcare) using the mirVana miRNA Labeling Kit (Ambion), and hybridized to array chips that were spotted at the Albert Einstein College of Medicine (Einstein) microarray facility (<http://microarray1k.aecom.yu.edu>). The 1280 antisense probes from Ambion recognizing mature miRNAs (1564V2), miRNA precursors used by Liu et al (10-11), and positive and negative controls were spotted three times. MicroRNA expression signals were extracted and analyzed using GenePix Pro 6.0 (MDS Analytical Technologies). The correlation of the controls between the two Einstein arrays was 0.99, calculated from 7 controls spotted in triplicate per array, ranging from 1700 to 25,000 light units. Additional microarray experiments were performed using total RNA from two more macrophage donors and analyzed by LC Sciences for miRNA expression profiles using 925 probes developed from version 13.0 of miRBase, and 67 positive and negative controls, which included perfect match, mismatch, and spots that contained no probe. Each probe was spotted 4 times on the array chips. The correlation of the controls between the two LC Sciences arrays was 0.97, calculated from 67 controls spotted in quadruplicate per array, ranging from 0 to 45,000 light units. These extremely sensitive arrays have a dynamic range of 3.5 logs with a lower limit of detection of 10 attomoles (http://www.lcsciences.com/products/genomics/miRNA/miRNA_microarray/mirna_detail.html).

2.4 Quantitative RT-PCR

For each reaction, 10ng of total RNA, from the four donors used for the miRNA microarrays and one additional donor, were used to assay hsa-let-7a, hsa-miR-16, -23a, 30b, -103, -146a, -159a, -212, -224, -378, -486, -654, and cel-lin-4 (as a negative control), by quantitative RT-PCR. “No template controls” were reactions run with no RNA sample added. For quantification of each miRNA, a Taqman miRNA Assay (ABI, Foster City, CA) was utilized in two steps, following manufacturer’s instructions. A cDNA synthesis step (16°C for 30 minutes, 42°C for 30 minutes, and 85°C for 5 minutes) and a quantitative PCR step (Fast Cycle program: Denaturation for 10 min at 95°C, and 40 cycles of Denaturation, 15 sec at 95°C, and Amplification, 1 min at 60°C) were performed on a StepOne Plus Thermocycler (ABI). HL-60 total RNA (Stratagene) was the reference sample, and Rnu44 was the endogenous control for all samples to calculate relative expression (fold change) using the $\Delta\Delta C_T$ method, as described in the legend of figure 3 (ABI).

3. Results

3.1 Human primary macrophages produce the proteins required for miRNA maturation and miRNA machinery function

We isolated CD14⁺ monocytes, differentiated them into macrophages as described in the methods (9), and harvested lysates for SDS-PAGE to identify the presence of miRNA machinery proteins in human primary macrophages. We used whole cell lysates from eight separate macrophage donors to perform western blot experiments testing for the major proteins of the miRNA machinery: Ago2, DGCR8, Dicer, Drosha, Exp5, and TRBP (Supplement Fig. 1). Each western blot experiment was performed in triplicate. All eight donors tested were positive for all proteins examined; four donors are shown in Fig. 1A.

HeLa whole cell lysate was included on each western blot as a positive control for each protein. Drosha, known to range in size between 130-160kDa (12), appeared at 160kDa in macrophages. The relative quantity of each protein in each sample was calculated by dividing its pixel density by the pixel density of its own α -Tubulin control band. The expression of Ago2, DGCR8, Drosha, Exp5, and TRBP was consistent among the donors as evidenced by the small error bars and a Standard Deviation (SD) of ± 0.58 , 0.47 , 0.37 , 0.94 , and 0.61 , respectively (Fig. 1B). Interestingly, the expression of Dicer varied among the donors when compared to its tubulin signal, with a SD of ± 3.34 (Fig. 1B).

3.2 Small non-coding RNAs are present in total RNA extracted from human primary macrophages

The presence of the miRNA machinery in macrophages led us to analyze the small non-coding RNA molecules present in freshly isolated, intact total RNA from macrophages, to identify the presence of mature miRNAs. We size fractionated intact total RNA (that had an RNA Integrity Number of 9.2 on a scale of 1-10, Agilent RNA Nano Chip, data not shown) from macrophage donors to obtain the small non-coding RNA fraction, and analyzed the product on an Agilent Bioanalyzer using the Small RNA Chip, which measures RNA molecules between 20 to 150 nt in length (Fig. 2A, Ladder). As a positive control, intact total RNA isolated from fresh breast cancer tissue obtained from Stratagene (that had an RNA Integrity Number of 9.9, data not shown), known to express miRNAs (10), was size fractionated to enrich the miRNA fraction (Fig. 3A, lanes 1 and 2, red arrowhead). The electropherogram of the breast tissue shows enrichment of RNA molecules overlapping in size with mature miRNAs at ~ 22 nt (Fig. 2B: total RNA, red; fractionated RNA, green; miRNA, green arrowhead). Lanes 3 and 4 of the gel image (Fig. 2A) show total macrophage RNA and fractionated RNA (Donor # 006). Although the breast tissue and macrophage RNA have different migration patterns, the electropherogram overlay demonstrates significant enrichment of mature miRNA at ~ 22 nt in the fractionated macrophage RNA (Fig. 2C: total RNA, red; fractionated RNA, blue; miRNA, blue arrowhead). These data demonstrate that human primary macrophage total RNA contains fractions in the size range of miRNA.

3.3 Detection of 119 miRNAs in human primary macrophages using two different miRNA microarray methods

To identify miRNAs expressed by macrophages, we used two miRNA microarray methods. For the first method, macrophage miRNAs from two donors were enriched by fractionation as described above, and analyzed using the Einstein miRNA microarrays. The probe-set used for these arrays included spiked positive control RNA, used to evaluate hybridization efficiency. These “non-MicroRNA” RNA controls indicated that efficient hybridization occurred at approximately 700 light units above background. Spots that did not contain probe were used to determine the background level, and had a value of approximately 300 light units. Therefore, for these arrays, a value of 1000 light units was used as the threshold above which miRNAs were identified as “detectable.” Seventy-four mature miRNA probes generated an average signal of 1000 light units or greater in all three spots and were identified as human primary macrophage signature miRNAs (Table 1), demonstrating the presence of mature miRNAs in the small RNA fraction collected.

To verify these data, total RNA from two additional donors was sent to LC Sciences for microarray analysis. The probes used in the LC Sciences total RNA arrays were derived from version 13.0 of miRBase and included both perfect match and mismatch controls of 20 bases in length. The perfect match probe/sample set generated in the range of 1000 – 5000 light units per spot. In contrast, the mismatch controls, which contained a single base difference between the probe and spiked sample, consistently generated less than 10 light

units per spot. This very large difference between the perfect and mismatch controls indicated the extremely high degree of specificity and stringency of this array technique. Therefore, miRNAs with a signal intensity of greater than 80 light units (8 times higher than background) in an aggregate number of six or more out of eight spots between the two LC Sciences arrays, were identified as miRNAs expressed by macrophages. The data from these chips were normalized by dividing the average signal intensity for each miRNA by the average signal intensity of all the miRNAs. Seventy-four miRNAs from *both techniques* were reproducibly detected among the four donors, including miR-16, -21, -23a, -23b, -24, -26a, -99b, -103, -191, and -222, as detailed in Table 1. Because the LC Sciences probe-set was derived from a newer version of miRBase and was proven to be highly specific, these arrays identified 45 miRNAs that had not been spotted on the fractionated miRNA arrays from Einstein and we included these miRNAs in the list of macrophage miRNAs in Supplement Table 1. We also included the mature miRNA sequences targeted by the probes that detected all the miRNAs in the list (Supplement Table 1). Our analyses generated a list of 119 miRNAs that were consistently and reproducibly detected in human primary macrophages.

3.4 Quantitative RT-PCR validation of miRNAs expressed by human primary macrophages

To confirm the sensitivity of the miRNA microarray data, miRNA expression was assayed using Taqman miRNA quantitative RT-PCR experiments, the “gold standard.” For all quantitative RT-PCR experiments, the human promyelocytic monocyte cell line HL-60 was used as the reference. The small nucleolar box RNA C/D 44 (Rnu44) was used as the endogenous control to calculate the Relative Expression for each miRNA tested. The lower the Cycle Threshold (C_T) value of the miRNA tested, the more abundant the RNA species was in the sample. The curve of the amplification plot and a C_T value between 17 and 32 indicated significant expression of a miRNA when using the StepOne Plus Thermocycler and its software (ABI).

The mean C_T for HL-60 expression of Rnu44 is 22.75 with an SD of 0.10 (Supplement Fig. 2A, representative amplification plot), indicating the internal consistency of the endogenous control. The average of the mean C_T of Rnu44 for all macrophage samples tested is 25.57 with an SD of 0.73 (Supplement Fig. 2B), indicating consistent amplification of the endogenous control among donors and experiments. These values were used to calculate the relative expression of the miRNAs tested by quantitative RT-PCR in Figure 3. The *C. elegans* miRNA cel-lin-4, not expressed in human cells, was used as a negative control; therefore, no C_T value could be determined (Supplement Fig. 2D and Fig. 3C). The “no template control” (NTC) reactions were performed without an RNA input and verified the absence of contamination across all experiments (Fig. 3A and 3B, Supplement Fig. 2, Supplement Fig. 3, and Supplement Fig. 4).

To confirm the expression of miRNAs detected by the two microarray techniques, miR-146a, found on all of our microarrays to be strongly expressed by human primary macrophages, was assayed. Quantitative RT-PCR results indicate that macrophage expression of miR-146a was 670 to 3100 times greater than an HL-60 promyelocytic monocyte reference control (Fig. 3A and 3B), as the mean C_T for miR-146a was 24.58 (SD ± 1.19) for the macrophage samples tested, and 32.09 for the HL-60 reference (Fig 3A), thus indicating its high expression in macrophages. Further, we differentiated HL-60 promyelocytic monocytes into macrophages using 0.1nM PMA treatment for 24 hours, and found an 18 fold increase (Fig. 3B) in miR-146a expression as compared to the untreated control. These data are in agreement with a previous finding that miR-146a is strongly upregulated when HL-60 cells are matured into macrophages using PMA (13).

To validate further our miRNA microarray data, we performed quantitative RT-PCR on seven miRNAs that were suggested as expressed by both miRNA microarray methods used. Three miRNAs are depicted as amplification plots (Fig. 3C) and four are depicted in relative expression graphs (Supplement Fig. 3). MicroRNA-23a and -30b were chosen for their relatively high expression levels detected by miRNA array, whereas miR-212 was chosen to test its relatively low expression on both arrays with an average of 80 light units per spot on the LC Sciences arrays. For each donor tested, miR-23a, -30b, and miR-212 were expressed consistently, as depicted by their amplification plots (Fig. 3C). The mean C_T value for Rnu44 was 25.69 ($SD \pm 0.59$), for miR-23a was 26.29 ($SD \pm 1.13$), for miR-30b was 26.43 ($SD \pm 1.03$), and for miR-212 was 31.41 ($SD \pm 2.22$). These results not only support the lower limits of the cut-off values that we used for our arrays, but they also indicate that even miRNAs with a low level of detection can be consistently expressed by macrophages.

We also found evidence of variation in the expression of some miRNAs in human primary macrophages. Amplification of hsa-let-7a, miR-16, -103, and -378 had an average C_T value of 24.85, 24.94, 26.19, and 29.69, respectively. Each of these miRNAs showed remarkable variability in their calculated relative expression: let-7a ($SD \pm 2.76$), miR-16 ($SD \pm 1.34$), miR-103 ($SD \pm 5.29$), and miR-378 ($SD \pm 4.60$) (Supplement Fig. 3). Donor #200 is a consistent outlier, although this donor's cells were cultured identically to the other donor macrophages and appeared similar in morphology (data not shown) at the time of RNA extraction. This variability does not impact our miRNA signature, as the quantitative RT-PCR data represent relative expression data, compared to the HL-60 control. This variability does, however, highlight the expression differences among some of the miRNAs expressed by macrophages.

We verified the sensitivity and specificity of our miRNA microarray data by performing quantitative RT-PCR on four miRNAs, miR-159a, -224, -486, and -654, that *were not* detected by microarray. MicroRNA-486 and -654 could be amplified from all 5 donors, but had very high mean C_T values of 35.32 and 35.68, respectively, indicating extremely low (therefore not reliable) expression of these two miRNAs (Supplement Fig. 4, left panels). MicroRNA-159a and -224 also showed minimal expression in 2 out of the 5 donors tested (Mean $C_T = 35.60$) and 3 out of the 5 donors tested (mean $C_T = 35.92$), respectively (Supplement Fig. 4, right panels). These data certified the sensitivity and specificity of the arrays used to determine the miRNA expression signature of human primary macrophages.

Altogether, the quantitative RT-PCR experiments support our miRNA microarray findings and demonstrate a range of miRNAs that show both consistent and variable expression in macrophages. Thus, our data demonstrate the presence of a reproducible miRNA signature in human primary macrophages.

4. Discussion

There are many subtypes of human primary macrophages, including splenic macrophages, alveolar macrophages, bone marrow stromal macrophages, Kupffer cells, resident peritoneal macrophages, Central Nervous System perivascular macrophages, and microglia. To date, there has not been extensive description of the miRNA expression profile of human primary macrophages. The few reports of miRNA expression in these critical immune cells mainly focus on a subset of organ-specific macrophages, such as splenic (5) and alveolar (14). There are also conflicting data about macrophage production of the machinery required for miRNA function. Klase et al (8) used U937, OM10.1, and THP-1 cell lines as models for pro-myelocytic cells and monocytes as well as *human primary peripheral blood monocytes*, and tested them for the presence of Dicer. The report found that Dicer, the protein required for miRNA maturation, could be detected in OM10.1 cells, a promyelocytic cell line, and

barely detected in human primary monocytes (8). Further, they differentiated U937 and THP-1 cells into a macrophage phenotype using PMA, and also found very low amounts of Dicer, but never tested human primary macrophages (8). This led us to test human primary monocyte-derived-macrophages for expression of the miRNA machinery.

As macrophages are essential to both innate and acquired immunity as well as to disease pathogenesis, we proposed that macrophages would utilize miRNAs as a regulatory mechanism for protein production, and thus would express the necessary miRNA processing machinery. Therefore, we performed western blot analyses on proteins extracted from human primary macrophages and demonstrated that these cells do produce Ago2, DGCR8, Dicer, Drosha, Exp5, and TRBP proteins, which participate in complexes required for miRNA maturation, transport, and function. Studies successfully used shRNA/siRNA knockdowns in macrophages (15-17), and so it is strongly inferential that Dicer is present in sufficient amounts to allow RNAi processes to occur in macrophages. Ours is the first direct demonstration that Dicer is produced by human primary macrophages. This may have been facilitated by the relatively large quantity of lysate we used for the detection of Dicer in our cells. We also found that the production of Dicer is variable among donors. This may be explained, in part, by a study showing that let-7 is capable of regulating the production of Dicer (18). We found that macrophages from our donors produce varying amounts of let-7, and this may account for the variability of Dicer production we detected. This may also explain the conflicting data in the literature. Additional work will be required to determine the basis for this variability. The copy number values of miRNAs that were successfully amplified from macrophage total RNA samples, inferred from the inverse of the comparative C_T data, indicate that mature miRNA production is not apparently affected by the comparatively low quantity of Dicer in these cells. For example, miR-146a, is produced in great quantity by human primary macrophages when compared to PMA-induced HL-60 cells (13).

That even a comparably small amount of Dicer is sufficient to produce to maturity both a rich array of intrinsically produced miRNAs and experimentally introduced siRNAs suggests that Dicer has important functions in differentiated cells of the monocytic lineage, such as human primary macrophages. Other intriguing possibilities include the potential presence of a protein that functions in support of the efficiency of Dicer in macrophages. Dicer is not the only miRNA machinery protein to show variability in its expression among our donors. This may indicate that, like let-7 regulation of Dicer, there may be an intrinsic regulation of all of the miRNA machinery, which may vary among people, and among primary cell types. Therefore, further work is required to examine this finding. It should be considered during the development of therapies targeting either the machinery or the miRNAs expressed by macrophages.

We also identified the consistent expression of miRNAs in human primary macrophages using miRNA microarrays. Human CD14⁺ monocytes were isolated from peripheral blood and differentiated into macrophages *in vitro* to obtain macrophages whose miRNA profile would enable comparisons across the many subsets of organ-specific macrophages. The reproducible presence of 74 miRNAs, as well as of 45 additional miRNAs newly added to miRBase (19), in our macrophages provides a signature of 119 miRNAs expressed by these cells.

The diversity of miRNAs we were able to detect suggests that miRNAs have an important role in fully differentiated macrophages, and such functions are being examined currently. MicroRNA-146a and -155, previously found to be expressed by macrophages, were consistently identified by our microarrays and miR-146a was confirmed using quantitative RT-PCR (6-7,13,20). We also verified the expression of hsa-let-7a, miR-16, -23a, -30b,

-103, -212, and -378 in human primary macrophages. We found consistent amplification of miR-23a, -30b, and -212, and determined that some miRNAs expressed by these cells are consistently high, such as miR-23a and -30b, while others are consistently low, such as miR-212. We confirmed the specificity and sensitivity of detection of our microarray experiments by testing miR-159a, -224, -486, and -654, which were not in the range of detection using quantitative RT-PCR. Thus, our data indicate that a number of miRNAs expressed by macrophages show high reproducibility among donors and may suggest important functions.

Our data also indicate the existence of miRNAs that are highly variable among donors, such as of hsa-let-7a, miR-16, -103, and -378. Some of these have been shown to be involved in the pathogenesis of cancer (21) and heart disease (22). The cause of this variability is unknown and will require examination as it may provide an understanding of susceptibilities, preventions, and therapeutic interventions. Further, we detected 69 of the 292 splenic macrophage miRNAs previously identified in a study of hypersplenism, including let-7a, miR-16, -23a, 30b, -103, -146a, -212, and -378 (5). The reported splenic macrophage array data, utilizing probes from miRBase version 8.1, showed expression of miR-487 and miR-654, both of which were not expressed in our primary macrophages. This difference suggests that macrophages will express a significant subset of miRNAs that are either involved in or affected by tissue differentiation and/or the pathogenesis for disease, marking an important area for further study. Comparison of our data with that from other types of macrophages would facilitate the identification of miRNAs that are specific to a particular macrophage subpopulation or expressed by macrophages during the pathogenesis of disease.

The roles of miRNAs in infectious disease have only recently begun to be examined. Our miRNA arrays also confirmed the presence of miR-20a, -23a, -26a, -27a, -29a, and -223, all of which have been shown to be differentially expressed in certain viral infections. MicroRNA-20a, -23a, -26a, and -27a, are dysregulated in the liver tissue of individuals infected with Hepatitis B and C (23). The source of these miRNAs could be the resident liver macrophages, Kupffer cells. Additionally, miR-29a has been shown to decrease the HIV replication cycle in T cells (24). Our finding of miR-29a expression in macrophages, cells that are also infected by HIV, warrants the examination of this miRNA during macrophage infection. Expression of miR-223 has been shown to decrease as monocytes differentiate into macrophages and may be associated with the ability of macrophages to become HIV infected (25). Thus, because the pathogenesis of many human diseases involve macrophages, and macrophages express miRNAs shown to be involved in the pathogenesis of these diseases, the examination of macrophage miRNAs in disease may provide novel approaches for the development of therapeutics for these and other pathologies.

Our studies identify miRNAs expressed by human primary macrophages. This report represents one of the many important first steps required to examine how miRNAs function in macrophages during normal and disease processes. Therefore, our findings underscore the importance of miRNA study in these cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Human primary macrophages express proteins of the miRNA machinery

(A) Representative western blots performed on macrophage whole cell lysate from 4 donors using antibodies to Ago2, DGCR8, Dicer, Drosha, Exp5, and TRBP. HeLa whole cell lysates served as positive controls. (B) To determine variability in miRNA machinery expression among individual donors, the relative quantity of each protein was calculated using densitometric analysis. To standardize the expression of each protein, the ratio of each protein to its tubulin control was calculated. The mean of these ratios was determined from the replicates of all the donors tested, and the standard deviation for the expression of Ago2, DGCR8, Dicer, Drosha, Exp5, and TRBP among the donors was calculated: $SD \pm 0.58$, 0.47, 3.34, 0.37, 0.94, and 0.61, respectively.

A

B

C

Figure 2. Small RNA profile of human primary macrophages

Agilent 2100 Expert Bioanalyzer Small RNA Chip quantification of RNA populations between 20 and 150 nts. Quantity is measured on the y-axis, in fluorescence units (FU). Size is measured on the x-axis, in nucleotides (nt). The expected size migration of miRNAs is indicated by colored arrowheads. (A) Gel image depicting size ladder, pre-, and post-fractionation of positive control breast cancer tissue RNA (lanes 1 and 2) and macrophage RNA (lanes 3 and 4). (B) Overlay of electropherograms depicting pre- (red), post-fractionation (green) RNA, and miRNA (green arrow) species of positive control tissue. (C) Overlay of electropherograms depicting pre- (red), post-fractionation (blue) RNA, and miRNA (blue arrow) species of macrophage RNA.

A

B

C

C

C

C

C

Figure 3. Quantitative RT-PCR validation of miRNAs consistently detected in human primary macrophages

(A) Amplification Plots depicting the normalized fluorescence values (ΔRn) during amplification of miR-146a ($C_T = 24.58$, $SD = 1.19$). (B) Relative quantity graph of miR-146a. The Cycle Threshold (C_T) is inversely proportional to the sample copy number. ΔC_T is the C_T of Rnu44 subtracted from the C_T of the miRNA of interest. The $\Delta\Delta C_T$ is the value of the test sample ΔC_T subtracted from the ΔC_T value of the HL-60 -PMA sample. Relative Expression (Fold Change) is the value of $2^{-\Delta\Delta C_T}$. (C) Amplification Plots depicting the normalized fluorescence values (ΔRn) during amplification of Rnu44 in five different donors ($C_T = 25.688$, $SD \pm 0.59$), miR-23a ($C_T = 26.29$, $SD \pm 1.13$), miR-30b ($C_T = 26.43$, $SD \pm 1.03$, and miR-212 ($C_T = 31.41$, $SD \pm 2.22$).

Table 1
Macrophage MicroRNAs consistently detected using two miRNA microarray techniques.

let-7a	miR-19b	miR-29a	miR-107	miR-181a	miR-324-5p
let-7b	miR-20a	miR-30a	miR-125a-5p	miR-181b	miR-328
let-7c	miR-21	miR-30b	miR-128	miR-185	miR-331-3p
let-7d	miR-22	miR-30c	miR-130b	miR-186	miR-342-3p
let-7d*	miR-23a	miR-30d	miR-132	miR-191	miR-345
let-7e	miR-23b	miR-34a	miR-133a	miR-197	miR-361-5p
let-7f	miR-24	miR-92a	miR-133b	miR-210	miR-362-5p
let-7g	miR-25	miR-93	miR-140-3p	miR-212	miR-378*
let-7i	miR-26a	miR-99b	miR-146a	miR-221	miR-425*
miR-15a	miR-26b	miR-103	miR-146b-5p	miR-222	miR-484
miR-15b	miR-27a	miR-106a	miR-151-3p	miR-223	miR-498
miR-16	miR-27b	miR-106b	miR-155	miR-320a	miR-500*
miR-17	miR-28-5p				

* The asterisk is a feature of miRNA nomenclature (14).