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MicroRNA expression dynamics during murine and human erythroid differentiation

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Abstract

Objective— microRNAs (miRNAs) are an abundant class of small non-coding RNAs that regulate diverse cellular functions by sequence-specific inhibition of gene expression. We determined miRNA expression profile during erythroid differentiation and putative roles in erythroid differentiation.

Methods— The expression profile of 295 miRNAs before and after their erythroid differentiation induction was analyzed using microarray. FACS analysis was used to isolate mouse spleen erythroblasts at different differentiation stages. Human cord blood CD34+ progenitors were differentiated in vitro. Real time RT-PCR was used to confirm the results of miRNA microarray. Synthetic oligonucleotides for miR-451 overexpression or knockdown were transfected into MEL cells.

Results— More than one hundred miRNAs were found to be expressed in erythroid cells. The majority of them showed changes in their expression levels with progression of erythroid differentiation. Further analysis revealed that the overall miRNA expression levels are increased upon erythroid differentiation. Of the miRNAs analyzed, miR-451 was most significantly upregulated during erythroid maturation. Functional studies using gain of function and loss of function approaches showed that miR-451 is associated with erythroid maturation.

Conclusions— Dynamic changes in miRNA expression occurred during erythroid differentiation, with an overall increase in the levels of miRNAs upon terminal differentiation of erythroid cells. MiR-451 may play a role in promoting erythroid differentiation.

Introduction

MiRNAs are endogenous 18 ~ 25nt RNAs that control many aspects of cellular function including cell growth, cell differentiation, cell death, metabolism and development through regulation of gene expression [1–8]. MiRNAs may play important roles in normal hematopoiesis and hematological malignancies [9,10]. For example, miR-221 and miR-222 were shown to regulate erythropoiesis and angiogenesis through targeting c-kit [11,12]. MiR-223 forms a regulatory network with transcription factors NFI-A and C/EBP α to regulate human granulopoiesis [13]. MiR-181 is preferentially expressed in B-lymphoid cells of mouse bone marrow and its overexpression in hematopoietic stem/progenitor cells increased the

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number of B-lineage cells in vitro and in vivo with a decrease in the number of CD8⁺ T cells [14]. It was thus suggested that miR-181 might play a role in the normal development of B-cell and T-cell lineages in the mouse. MiRNA expression profiling of in vitro differentiated megakaryocytes derived from CD34⁺ hematopoietic progenitors revealed dynamic expression of miRNAs during megakaryocytic differentiation. For example, the expression of miR-223 and the miR-15a-miR-16-1 cluster was initially down-regulated during megakaryocytic differentiation, but after 14 days in culture, their expression returned to the levels comparable to that of CD34⁺ progenitors, whereas miR-181b, miR-155, miR-106a, miR-17 and miR-20 were down-regulated during megakaryocytopoiesis [15]. Conditional dicer knockout in T cells also demonstrated a general role of miRNAs in T cell differentiation [16,17]. Therefore, evidence is accumulating that miRNAs may play important roles in hematopoiesis.

Although the stages of erythroid cell differentiation are well characterized [18], the molecular mechanisms that orchestrate the coordinated changes from erythroid lineage commitment to terminal maturation remain poorly understood. As a new class of sequence-specific regulators of gene expression, miRNAs may form a regulatory network with growth factors and transcription factors to control erythroid lineage commitment and differentiation. In this study, we explored the expression profile of miRNAs in erythroid cells at different stages of differentiation using miRNA microarray analysis. Our studies show that, of the 295 miRNAs assayed, more than a hundred are expressed in erythroid cells with varied abundances. Of interest, the overall expression levels of miRNAs are increased during erythroid differentiation. A single miRNA, miR-451, was most significantly upregulated in differentiating erythroid cells. Functional studies revealed that increased level of miR-451 may promote erythroid differentiation.

Materials and methods

Cell culture

MEL cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 100 units/ml of penicillin and streptomycin, in a humidified incubator at 37 °C in the presence of 5% CO₂. Erythroid differentiation of MEL cells was induced by adding dimethyl sulfoxide (DMSO, 2% final concentration, Sigma, St. Louis, MO USA), or N, N' hexamethylene bisacetamide (HMBA, 5mM final concentration; Sigma) to the culture media. The cells were collected at 96 and 120 hours after DMSO or HMBA treatment respectively. Cord blood (CB) was obtained from healthy, full-term placentas at the obstetrical unit of the University of Washington Medical Center. CB CD34⁺ cell isolation and erythropoietic (E) culture of CB CD34⁺ progenitor cells was as described [19,20]. Experiments using human CB cells have been reviewed and approved by the IRB and appropriate Committees of the University of Washington.

Benzidine staining

MEL cells were smeared on glass slides and air-dried. Then they were fixed in 100% Methanol for 5 minutes, stained in 1% Benzidine solution for 5 minutes and subsequently incubated in 30% H₂O₂ for 2 minutes. Benzidine positivity was indicated by golden brown color.

Phenylhydrazine Treatment

Phenylhydrazine hydrochloride solution in PBS was injected into mouse peritoneal cavity (60 mg/kg body weight, Sigma) on each of days 0, 1, and 3. On the first day after the third injection, mice were euthanized by cervical dislocation and spleens were removed under sterile conditions for further analysis. Animal experiments were performed in compliance with the University of Washington Institutional Animal Care and Use Committee and NIH guidelines.

Flow cytometry

Flow cytometry analysis of mouse spleen erythroblasts was carried out as described [21]. Briefly, spleens were mechanically dissociated by pushing with a syringe plunger through a 70 μm strainer in the presence of phosphate-buffered saline and 0.5% bovine serum albumin (PBS/0.5% BSA). The viability of the cells was determined by trypan blue exclusion and was always greater than 95%. Cells (1×10^6) were incubated with anti-CD16/CD32 antibodies (BD Biosciences) to block Fc receptors prior to staining for flow cytometry. Then they were immunostained with phycoerythrin (PE)–conjugated anti-TER119 (1:200) (BD Pharmingen, San Diego, CA USA) and fluorescein isothiocyanate (FITC)–conjugated anti-CD71 (1:200) (BD Pharmingen) antibodies. Propidium iodide was added to exclude dead cells from analysis. Erythroid cells at different stages of differentiation were sorted using a FACS Moflo machine (Cytomation, Fort Collins, CO USA).

RNA isolation and miRNA microarray

Total RNA was isolated using Trizol reagent (Invitrogen, CA, USA) and further purified using RNeasy mini kit (QIAGEN Valencia, CA USA). Purified RNAs were sent to EXIQON (Denmark) for miRNA expression analysis. MiRNA microarray including labeling, hybridization, scanning, normalization and data analysis was carried out by EXIQON. Briefly, RNA Quality Control is performed using Bioanalyser 2100. The samples were labeled using the mercuryTM Hy3TM/Hy5TM labeling kit and hybridized on the miRCURYTM LNA (locked nucleic acid) Array (v.8.0). Three independent hybridizations for each sample were performed on chips with each miRNA spotted in quadruplicate. Labeling efficiency was evaluated by analyzing the signals from control spike-in capture probes. LNA-modified capture probes corresponding to human, mouse, and rat mature sense miRNA sequences were spotted on slides. This set of LNA-modified oligonucleotides was designed to have uniform melting temperatures (T_m) of 72°C against their complementary targets. Spotted microarray slides were processed using an automated hybridization station (Lucidea Slide Processor; GE Healthcare). The resulting signal intensity values were normalized to per-chip median values and then used to obtain geometric means and standard deviations for each miRNA. Triplicate arrays were performed under each treatment condition.

Real-Time RT-PCR quantification of miRNAs

Real-time RT-PCR quantification of miRNA expression was carried out using TaqMan MicroRNA Assay kits according to the manufacturer's protocol (Applied Biosystems, Foster City, CA USA). The real-time RT-PCR assay is based on a stem-loop RT primer design [22]. Briefly, cDNAs were synthesized from total RNA using gene-specific primers. Reverse transcriptase reactions contained 10 ng of RNA samples, 50 nM stem-loop RT primer, 1 $\times$ RT buffer, 0.25 mM each of dNTPs, 3.33 U/ μl MultiScribe reverse transcriptase and 0.25 U/ μl RNase Inhibitor. The 15 μl reactions were incubated for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and then held at 4°C. Real-time PCR was performed using an Applied Biosystems 7300 Sequence Detection system. The 20 μl PCR included 1.33 μl RT product, 1 $\times$ TaqMan Universal PCR master mix and 1 μl of primers and probe mix of the Taq-Man MicroRNA Assay kit. The reactions were incubated in a 96-well optical plate at 95°C for 10 min, followed by 45 cycles of 95°C for 15s and 60° for 10 min. The threshold cycle (Ct) was determined using default threshold settings. The Ct value is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. All experiments were done in triplicates each, and were repeated three times. U6 small nuclear RNA was used as an internal control to normalize RNA input in the real-time RT-PCR assay. The assay was carried out using the TaqMan® Endogenous Control, RNU6B kit.

Cell Transfection with oligonucleotides

Stability-enhanced miR-451 oligonucleotides and control nontargeting scrambled oligonucleotide were purchased from Dharmacon (Lafayette, CO, USA). MiR-451 antisense oligonucleotides and scrambled control oligonucleotides were purchased from (Exiqon, Vedbaek, Denmark). One day before transfection, the MEL was seeded into 24-well plates. Transfection of miR-451 mimic oligonucleotides, miR-451 antisense oligonucleotides (ASO) or scrambled control oligonucleotides was carried out in triplicate using X-tremegene (Roche, Indianapolis, IN USA) according to the manufacturer's instructions.

RT-PCR

Total RNA was isolated as described above. Semi-quantitative RT-PCR was carried out using the SuperScript. III One-Step RT-PCR System (Invitrogen, Carlsbad, California, USA). Each PCR cycle included 94 °C for 30s, 60 °C for 30s and 72 °C for 1 min. GAPDH was used as control for RNA quality and quantity.

Results and discussion

MiRNA expression array and design

Unlike conventional mRNA expression profiling, accurate miRNA profiling is technically challenging due to the small size of mature miRNAs (about 22nt) and the sequence similarity between miRNA family members. For example, the short nature of miRNAs imposes limitation on the design of hybridization probes and optimization of hybridization conditions for their detection. Currently, hundreds of human and mouse miRNAs have been discovered. Their calculated melting temperatures (T_{ms}) toward the complementary DNA strand vary from 45 °C to 74 °C due to differences in their GC content. For example, miR-324-3p is 73% G-C and miR-369 is 24% G-C. Consequently, very different hybridization conditions are required for accurate detection of their expression using conventional microarray platform.

Recently, a new microarray platform (miChip) using LNA-modified capture probes showed increased sensitivity and specificity [23]. LNA modification increases the binding affinities for complementary RNA and DNA sequences [24]. In this microarray platform, the capture probes were designed to consist of a defined combination of unmodified and LNA-modified nucleotide analogs [25]. The LNA modification raises the thermal stability of nucleic acid duplex 3 °C to 8 °C per nucleotide depending on the sequence context and whether the complementary strand is DNA or RNA [24,26]. By achieving uniform hybridization conditions, LNA-modified probes offer higher sensitivity, specificity and equality for miRNA detection. Therefore, the miRNA expression analysis was carried out using the mercury™ LNA array, which has a normalized 72°C T_m. Three pairs of RNA samples from independent cultures with or without DMSO treatment were subjected to miRNA expression profiling analysis. Normalization involved background subtraction and normalization with a global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm. This within-slide normalization was performed to minimize differences between the colors in an intensity-dependent manner. Median Normalized Data contain normalized data where replicated measurements of quadruplicate spots of the same miRNA probe on each slide have been averaged. Median of ratio Hy5/Hy3 (sample/common reference) was median ratios of quadruplicate spots within each slide. Fold changes in miRNAs expression levels from three independent arrays under each experimental condition were calculated and presented as mean ± SD.

MiRNA expression profile during erythroid differentiation

MEL cells are derived from Friend virus transformed mouse spleen erythroid precursors that are blocked at about the pronormoblast stage of differentiation [27,28]. Terminal erythroid differentiation into cells resembling orthochromatophilic normoblasts can be induced by treatment of MEL cells with several chemical inducers such as DMSO. MEL cells have been used extensively as a cell model for studying erythroid differentiation. Therefore, analysis of miRNA expression status in MEL cells with or without induction of erythroid terminal differentiation should reveal a miRNA expression profile associated with erythroid differentiation by identifying miRNAs that are up- or down-regulated during this process. The identification of erythroid- and differentiation stage-specific miRNAs may provide important clues about their putative roles in erythropoiesis.

MiRNA expression in MEL cells with or without DMSO treatment was assayed using the miRCURY™ LNA Array (v.8.0), which contains 295 known murine miRNAs. More than a hundred miRNAs were detected in MEL cells. Their expression levels, however, varied significantly with hybridization signals ranging from several ten thousands to a few hundreds (Figure 1A). Of the miRNAs on the array, miR-298 was the most abundant miRNA with signals of about 50,000 in uninduced MEL cells. MiR-320 was the second most abundant miRNA in uninduced MEL cells. Since MEL cells represent erythroid cells at pronormoblast stage of differentiation, the miRNA expression array using uninduced MEL cells should represent the miRNA expression profile of early pronormoblasts.

Parallel miRNA expression arrays were carried out using RNAs from MEL cells after their induction to terminal erythroid differentiation by DMSO treatment for 4 days. Terminal erythroid differentiation of MEL cells following DMSO treatment was confirmed by benzidine staining and β globin expression analyses (data not shown). The miRNA profile analysis showed that more than a hundred miRNAs are expressed in terminally differentiating erythroid cells with widely varied levels of expression (Figure 1A). There were changes in the levels of many miRNAs upon induction of erythroid differentiation (Figure 1A). For example, miR-29b, miR-140*, miR-193, miR-382 and miR-434-5p which were undetectable in untreated MEL cells, became detectable following induction of erythroid differentiation by DMSO. In contrast, the levels of both miR-298 and miR-320, the two most abundant miRNAs in untreated MEL cells, decreased upon induction of erythroid maturation with DMSO. The level of miR-451 is the most significantly increased (more than 7 fold), whereas the levels of several miRNAs, such as miR-29a, miR-26a, miR-22, miR-144, miR-15b, miR-292-5p and miR-30a-5p, increased more than 2 fold upon induction of erythroid differentiation. As similar increases in miRNA levels in other settings have been shown to have regulatory function, these miRNAs may have regulatory functions in erythroid differentiation. MiRNAs that generated hybridization signals below background level in MEL cells using this microarray platform are listed in table 1 of the supplementary data.

Recently, it was reported that miR-221 and miR-222 are down-regulated in E culture of human cord blood CD34+ progenitor cells [11]. Since miR-221 and miR-222 produced hybridization signals below the background level using the current array platform, we analyzed their expression using real-time RT-PCR. This assay showed that both miR-221 and miR-222 are expressed in MEL cells and mouse spleen primary erythroblasts (data not shown). However, the level of miR-221 and 222 in MEL cells is very low since the ct value of both miRNAs are higher than a control miRNA which generated a hybridization signal that is near the threshold level for detection using the microarray platform. Therefore, the failure to detect miR-221 and miR-222 in the microarray maybe due to their relatively low levels in MEL cells. In addition, the levels of miR-221 and miR-222 decreased following DMSO or HMBA induction of MEL cells and as primary spleen erythroblast maturation progresses from early to later stages (data not shown). These data are consistent with the observation that miR-221 and miR-222 levels

decreased during erythroid cell maturation of E cultures of cord blood CD34⁺ progenitor cells by Felli et al. [11].

Box plot analysis revealed that overall miRNA expression increased upon induction of erythroid differentiation (Figure 1B). The biological implications of overall increase in miRNA expression following erythroid differentiation remain to be determined. Nevertheless, it is likely that the elevated levels of miRNAs during erythroid differentiation may play a role in inhibiting target genes whose down-regulation is required for erythroid differentiation.

Confirmation of array data using real-time RT-PCR

We next performed real-time RT-PCR analysis on several selected miRNAs to verify the array results. As shown in Figure 2, the real-time RT-PCR assays validated the microarray results.

HMBA, another inducer of erythroid differentiation of MEL cells, induced similar to DMSO levels of erythroid differentiation of MEL cells, as judged by the percentage of benzidine-positive cells and the level of β globin expression (data not shown). As similar levels of up-regulation of miR-15b, miR-16, miR-22, miR-26a, miR-29a and miR-451 were also observed following HMBA treatment for 5 days (data not shown), they confirmed the fact that the changes in miRNA expression are erythroid differentiation-specific rather than inducer-specific.

To determine whether there are similar changes in miRNA expression during the differentiation of primary murine erythroblasts, we next analyzed the expression of the selected miRNAs in primary erythroid cells at different stages of their maturation. Erythroblasts at different stages of maturation including proerythroblasts (Ter119^{med}CD71^{high}), basophilic erythroblasts (Ter119^{high}CD71^{high}), late basophilic and polychromatophilic erythroblasts (Ter119^{high}CD71^{med}), and orthochromatic erythroblasts (Ter119^{high}CD71^{low}), were isolated from mouse spleen as described [21]. Figure 3A shows a representative flow cytometry density plot. Real-time RT-PCR analysis revealed that the levels of the selected miRNAs are also increased during erythroid differentiation (Figure 3B). The level of miR-451 increased more than 10 fold when erythroid differentiation proceeds from proerythroblasts to basophilic erythroblasts, and maintained at high levels throughout the subsequent stages of erythroid maturation. The real-time RT-PCR analysis of the expression of several miRNAs in both MEL cells and primary cells validates the array results.

Association of miR-451 expression with human erythroid maturation

The above analysis of miRNA expression in primary mouse spleen erythroid cells suggested that miR-451 upregulation is associated with primary erythroid cell maturation. Recently, it was reported that human miR-451 is abundant in human red blood cells [29]. The same study also found that miR-451 was not detectable using Northern blot analysis in the cell lines J2E, F4N, BW5147, J558L and 416B, representing progenitors of fetal liver erythroid, bone marrow erythroid, T cell, B cell and multipotent precursor stages, respectively. To determine the expression status of miR-451 during human erythroblast differentiation and maturation, we carried out E culture of CB CD34⁺ progenitor cells. Erythroblast differentiation and maturation during E culture of human CB CD34⁺ cells were monitored by FACS analysis of glycophorin A expression and cell size distribution using FACS forward scatter (Figure 4A). As shown in figure 4B, Glycophorin A⁺ cells increased to 60% at day 8 of E culture. MiR-451 is beginning to be expressed at very early stages of erythroid differentiation. MiR-451 level increased near 20 fold at day 8 of E culture. At day 15 and day 20 of E culture, erythroblasts mature into late erythroblasts as indicated by increases in the percentage of glycophorin A⁺ cells with reduced cell size (data not shown). MiR-451 remained at the peak level (near 20 fold)

throughout the assay period up to 20 days in which the majority of the erythroblasts are late erythroblasts (data not shown).

The significant up-regulation of miR-451 during erythroid cell maturation suggests that miR-451 may play a role in erythroid differentiation. MEL cells are blocked at about the pronormoblast stage of differentiation [27,28] and resume erythroid differentiation upon treatment with DMSO. The percentage of benzidine stain positive cells and the level of β globin reach maximum after 96 hours following DMSO treatment, recapitulating the normal process of erythroid differentiation from pronormoblast to later stages of erythrocytes. Real-time RT-PCR analysis of RNAs from MEL cells at different time points following DMSO treatment demonstrated that miR-451 level increases significantly at 12 hours, reaches a peak level at 24 hours, and remains at significantly high levels thereafter (Figure 5A, scrambled oligonucleotides (SO) transfected or mock transfected cells). These data signify that the level of miR-451 begins to rise at relatively early time points and remains elevated throughout terminal erythroid differentiation of MEL cells.

Mouse miR-451 and miR-144 genes are in a cluster [30]. Consistent with this, the levels of both miR-451 and miR-144 are significantly increased upon induction of erythroid differentiation, although the latter is not increased as significantly as the former. In agreement with the microarray results, RT-PCR analysis confirmed that the level of miR-144 increased in a similar pattern as that of miR-451 but at a lower magnitude, in MEL cells upon induction by DMSO or HMBA as well as in primary spleen erythroblasts at different stages of maturation (data not shown).

Regulation of erythroid maturation by miR-451

To determine whether the increase in miR-451 level plays a role in erythroid differentiation, we transfected MEL cells with miR-451 oligonucleotides. As shown in figure 5A, the levels of miR-451 were further elevated by transfection of miR-451 oligonucleotides, compared with SO or mock transfected cells. The transfected miR-451 sustains the elevated levels of miR-451 in MEL cells up to 96 hours post-transfection.

Increased expression of β -globin and hemoglobinization have been used as markers of erythroid differentiation of MEL cells. The levels of β -globin and hemoglobinization gradually increase in MEL cells during DMSO-induced erythroid differentiation and reach maximum by day 4 following DMSO treatment. We first determined whether increased levels of miR-451 affect globin expression. The levels of β globin were analyzed using RT-PCR at day 2 and day 4 following transfection of miR-451 oligonucleotides. As shown in figure 5B, increased level of β -globin was observed at day 2 and further increased at day after miR-451 transfection, indicating the progression of erythroid differentiation of the cells. This result demonstrates that forced increase in of miR-451 level MEL cells can partially remove the blockade of erythroid differentiation. At day 2 after DMSO treatment, 7% of MEL cells that are transfected with SO oligonucleotides or mock transfected are benzidine stain positive. However, 11% of the cells transfected with miR-451 oligonucleotides are benzidine stain positive (Figure 5C). This significant increase in hemoglobinization indicated that increased levels of miR-451 promote erythroid differentiation. The induction of erythroid differentiation is miR-451 specific since transfection with SO or mock transfection had no effects on β -globin expression and hemoglobinization. These results together demonstrated that miR-451 levels increased significantly during erythroid maturation and elevated level of miR-451 in MEL cells promotes erythroid differentiation.

If an increase in miR-451 level is required for erythroid differentiation, inhibition of its upregulation or activity should have inhibitory effects on erythroid differentiation. Therefore, we also tested the effects of inhibition of miR-451 on erythroid differentiation. Antisense

oligonucleotide (ASO) against miRNAs have been shown to be very effective inhibitors of miRNA activity [31]. MEL cells were treated with DMSO and transfected with different amounts of ASO targeting miR-451, SO control oligonucleotides or mock transfected for 2 days. MiR-451 ASO significantly and dose-dependently decrease in endogenous miR-451 levels in MEL cells (Figure 6A) and the inhibitory effects of transfected miR-451 ASO on endogenous miR-451 lasted throughout the experimental period (Figure 6B). This inhibition is highly specific since SO control oligonucleotides showed no effects on endogenous miR-451 levels (Figure 6A and 6B).

To determine whether inhibition of miR-451 using miR-451 ASO blocks erythroid differentiation, MEL cells were transfected with miR-451 ASO and treated with DMSO to induce erythroid differentiation. RT-PCR analysis showed increased levels of β -globin in cells transfected with SO and in mock transfected cells at day 2 following DMSO treatment (Figure 6C). The levels of β -globin further increased at day 4, indicating the progression of erythroid differentiation. However, no increase in the levels of β -globin was observed at day 2 following DMSO induction in cells transfected with miR-451 ASO (Figure 6C). The levels of β -globin increased to a lesser degree at day 4 after DMSO induction in cells transfected with miR-451 ASO (Figure 6C). Benzidine staining also demonstrated that inhibition of miR-451 using miR-451 ASO significantly reduced the levels of hemoglobinization of MEL cells (Figure 6D). These studies using gain of function and loss of function approaches demonstrated that miR-451 positively regulates erythroid differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1.

Dynamic changes in miRNA expression during erythroid differentiation. (A) Total RNAs were purified from MEL cells with or without DMSO treatment for 96 hours. MicroRNA profiling was carried out using miRCURYTM LNA array. Three independent arrays were performed under each treatment condition. Relative hybridization signals for microRNAs in MEL cells with and without induction of erythroid differentiation by DMSO are shown (mean $\pm$ SD). (B) Overall increase in miRNA expression level during erythroid differentiation. Box plot shows the distribution of hybridization signals in all capture probes on each array. RNAs from each sample (Hy5) and common reference pool (Hy3) are labeled using the miRCURYTM Hy3TM/Hy5TM labelling kit and hybridized on the miRCURYTM LNA Array (v.8.0). Capture probes with a Log2 median ratio of "0" on the Y-axis correspond to miRNAs that are equally expressed in MEL cells with or without DMSO induction of erythroid differentiation. Slide 1, 3 and 5 are data from untreated MEL cells. Slide 2, 4 and 6 are data from MEL cells that are treated with DMSO for 96 hours. The lower boundary of the box indicates the 25th percentile, the line within the box shows the median, and the upper edge of the box marks the 75th percentile. Whiskers above and below each box indicate the 95th and 5th percentiles. All data points that

lie outside the 5th and 95th percentiles are shown as symbols. This analysis revealed that there is an overall up-regulation of miRNA expression during erythroid differentiation.

Figure 2.

Validation of microarray data using real-time RT-PCR. The levels of miR-15b, miR-16, miR-22, miR-26a, miR-29a and miR-451 are significantly up-regulated upon erythroid differentiation using microarray analysis. Real-time RT-PCR analysis of these miRNAs using total RNAs isolated from MEL cells without and with DMSO treatment as described for the microarray assays was carried out to validate the array results. Total RNAs from three independent cultures under each treatment condition are used for the analysis. Triplicate assays were performed from each RNA sample. Data are normalized using U6 small nuclear RNA as an endogenous control for RNA input. Fold changes for these miRNAs following DMSO treatment from array and real-time RT-PCR are shown as mean $\pm$ SD.

Figure 3.

Expression of selected miRNAs in murine erythroblasts at different stages of erythroid differentiation. **(A)** flow cytometry density plot of spleen cells from PHZ treated mice. Spleen cells were immunostained with PE-conjugated anti-TER119 and FITC-conjugated anti-CD71 antibodies. X and y axes indicate the fluorescence units for PE and FITC respectively. Selected regions are proerythroblasts (P4), basophilic erythroblasts (P5), late basophilic and polychromatophilic erythroblasts (P6), and orthochromatic erythroblasts (P7). **(B)** Real-time RT-PCR assay of miR-15b, miR-16, miR-22, miR-26a, miR-29a and miR-451 levels in each erythroblast cell population as depicted. Data are shown in logarithmic scale as fold changes in miRNA levels relative to proerythroblasts (P4), which is set as 1 (mean $\pm$ SD).

Figure 4.

Up-regulation of miR-451 during erythroid differentiation in E cultures of human CB CD34+ progenitors. (A) Cells in E culture were incubated with a PE-conjugated anti-glycophorin A antibody. Erythroblast differentiation and maturation were analyzed by FACS. X-axis indicates relative fluorescence units for PE. Y-axis indicates the relative cell size. (B) MiR-451 levels were measured using Real-time RT-PCR at day 0, 4 and 8 of E culture. The percentage of Glycophorin A + cells in E culture at day 4 and day 8 was analyzed using FACS (as shown in A).

Figure 5.

Overexpression of miR-451 induced erythroid differentiation of MEL cells. (A) miR-451 levels increased following DMSO induction of erythroid differentiation. miR-451 level was further elevated following transfection of miR-451 oligonucleotides. MEL cells were treated with DMSO and transfected with miR-451 oligonucleotides (451), scrambled oligonucleotides (SO) as control or mock transfected as indicated. Total RNA was prepared at different time points as indicated after transfection and DMSO treatment. miR-451 levels were analyzed using real-time RT-PCR. Assays were carried out in triplicate for each RNA sample. Data are normalized using U6 small nuclear RNA as an endogenous control for RNA input. Fold changes in miR-451 levels are shown as mean $\pm$ SD. (B) MEL cells were transfected as in (A) without DMSO treatment. β globin expression was analyzed using RT-PCR at day 2 and 4 following transfection. GAPDH was used as a control for RNA input. (C) Hemoglobinization was analyzed by benzidine staining 48 hours after transfection and DMSO treatment as in (A). *, $p < 0.05$ compared with SO and mock transfection controls.

Figure 6.

Inhibition of miR-451 using miR-451 antisense oligonucleotides (ASO) blocks erythroid differentiation of MEL cells. (A) Dose-dependent decrease in miR-451 levels by miR-451 ASO (ASO). MEL cells were treated with DMSO and transfected with different amounts of miR-451 ASO or SO oligonucleotides as indicated for 48 hours. MiR-451 levels were analyzed using real-time RT-PCR. (B) Time course of miR-451 inhibition by ASO. MEL cells were treated with DMSO and transfected with 50 nM of ASO or SO control oligonucleotides as indicated. The levels of miR-451 were analyzed using real-time RT-PCR at different time points as depicted. (C) Inhibition of endogenous miR-451 blocks DMSO-induced β globin expression. MEL cells were treated with DMSO and transfected with ASO, SO oligonucleotides or mock transfected as indicated. β -globin levels were analyzed using RT-PCR at day 2 and day 4 post-transfection and induction as indicated. GAPDH was used as control for RNA input. (D) Inhibition of endogenous miR-451 blocks DMSO-induced hemoglobinization. Hemoglobinization was analyzed using benzidine staining on day 0, day 2 and day 4 following transfection as in C. *, $p < 0.05$ compared with SO and mock transfection controls.