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Journal of Cellular Biochemistry 107:548–556 (2009)

Journal of Cellular Biochemistry

MicroRNAs Are Involved in Erythroid Differentiation Control

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ABSTRACT

MicroRNAs (miRNAs) are a class of 17–25 nucleotides non-coding RNA molecules that regulate gene expression by either translational inhibition or mRNAs degradation. We used miRNA array to characterize miRNA variation of K562 cells before and after hemin treatment. The differential expression of five miRNAs was validated by Northern blot analysis. Among them, miR-126 exhibited up-regulation while miR-103, miR-130a, miR-210, and miR-18b exhibited down-regulation after hemin induction. The same expression tendency of the five miRNAs was observed following erythroid induction of CD34+ cells derived from human cord blood. miR-103 was selected and examined for its role in erythroid differentiation. Over-expression of miR-103 in K562 could inhibit hemin-induced K562 erythroid differentiation, which suggests this miRNA may take part in erythropoiesis. We confirmed that miR-103 targeting mRNA of forkhead box J2 (FOXJ2), a transcription factor that was involved in the development of many tissues. Our results delineated the expression of miRNAs during erythroid differentiation and suggested regulatory roles of miRNAs in this process by targeting mRNAs related to erythropoiesis. J. Cell. Biochem. 107: 548–556, 2009.

KEY WORDS: microRNAs (miRNAs); ERYTHROID DIFFERENTIATION; miR-103; FORKHEAD BOX J2 (FOXJ2)

MicroRNAs (miRNAs) are recently discovered small (approximately 22 nucleotides), non-coding RNAs that recognize target sequences of imperfect complementarity in cognate mRNAs resulting in either translation repression or mRNA degradation.

Currently, 695 human miRNAs have been reported and tabulated in the miRNA Registry (Sanger miRBase sequence database ver. 12.0, http://microrna.sanger.ac.uk). By estimation, more than 5,000 human genes (30% of the tested gene set) are as candidate miRNA targets [Berezikov et al., 2005]. Their function is ubiquitous including cell proliferation, development, apoptosis, insulin secretion, and oncogenesis [Calin et al., 2002, 2004; Poy et al., 2004; Xu et al., 2004; Cheng et al., 2005; He et al., 2005; Karp and Ambros, 2005; Lu et al., 2005]. Computational algorithms predict up to hundreds of putative targets for an individual miRNA and a single transcript may be regulated by multiple miRNAs. MiRNAs may either eliminate target gene expression or serve to fine tune of transcript and protein levels.

Some miRNAs show universal expression, while others are limited to certain stages in development or to certain tissues and cell types. So the study of development and tissue specific miRNAs will provide important clues for identifying new sets of genes and regulatory circuitries involved in the control of cell-specific differentiation.

Hematopoiesis is a highly regulated multistage process that consists of two integral aspects, the self-renew of hematopoietic stem cells (HSCs) and the differentiation of hematopoietic stem/progenitor cells. It has been hotspot for many years that how HSCs undergo linage commitment events and develop into various mature blood cells. There is now strong evidence that miRNAs modulate not only hematopoietic differentiation and proliferation but also activity of hematopoietic cells [Chen and Lodish, 2005; Fazi et al.,

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: National Nature Science Foundation of China; Grant numbers: 30871249, 30570389, 30721063; Grant sponsor: National Basic Research Program of China; Grant number: 2007CB946902.

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Received 24 August 2008; Accepted 2 March 2009 • DOI 10.1002/jcb.22156 • 2009 Wiley-Liss, Inc.

Published online 6 April 2009 in Wiley InterScience (www.interscience.wiley.com).

2005; Fatica et al., 2006; Georgantas et al., 2007; Liao et al., 2008]. The expression profiles of murine hematopoietic-specific miRNAs (miR-142, miR-181, and miR-223) have been described in B cells, T cells, monocytes, granulocytes, and erythroid cells. Over-expression of these miRNAs in murine HSC dramatically alters the proportion of differentiated linkages, which suggests that they are important factors to murine linage differentiation [Chen et al., 2004; Ramkissoon et al., 2006]. Additional work found that miR-223 appears to be a key player in a simple regulatory circuit of transcription factors that control granulopoiesis. The replacement of NFI-A by C/EBPα could promote the expression of miR-223 and the increased miR-223 reversely repressed NFI-A. With the appearance of C/EBPα, miR-223 expression is activated, resulting in repression of its NFI-A mRNA target and promoting granulocyte differentiation [Fazi et al., 2005]. The over-expression of miR-223 also exhibited a suppressive effect on erythroid differentiation but an accelerative effect on megakaryocytic differentiation of K562 cells and via down-modulation of LMO2 [Yuan et al., 2008].

MiR-155 was discovered as an oncogenic miRNA that was critical for B-cell maturation and immunoglobulin production in response to antigen [Vigorito et al., 2007; Yin et al., 2007]. MiR-10a, miR-126, miR-106, miR-10b, miR-17, miR-20, and miR-130a were down-regulated in the differentiated megakaryocytes compared with CD34+ hematopoietic progenitors and further experiment testified transcription factor MAFB was the target of miR-130a in megkaryocyte differentiation [Garzon et al., 2006]. Whereas miRNAs that are involved in erythroid differentiation were known very little and most experiments were limited to changes of miRNAs expression. MiR-221 and miR-222 were the first discovered miRNAs related to erythropoiesis [Felli et al., 2005]. The two miRNAs declined during erythropoietic culture of umbilical cord blood (UCB) CD34+ progenitor cells and exerted their function by unblocking kit protein production. Correlation analysis on UCB-derived CD34+ cells showed that miR-15b, miR-16, miR-22, and miR-185 have strong positive correlation to the appearance of erythroid surface antigens (CD71, CD36, and CD235a) and hemoglobin synthesis, while miR-28 has an inverse relationship to the expression of those markers [Choong et al., 2007]. Recent report showed that miR-155 decreased greatly during the differentiation of purified normal human erythroid progenitors in a liquid culture system while miR-451 and miR-16 increased obviously in the same culture [Bruchova et al., 2007; Masaki et al., 2007; Zhan et al., 2007]. MiR-24 was the second miRNA whose function in erythroid differentiation was elucidated. It could repress the activin-mediated accumulation of hemoglobin, an erythroid differentiation marker, in K562 cells and decrease erythroid colony-forming and burst-forming units of CD34+ hematopoietic progenitor cells by influencing the expression of human activin type I receptor ALK4 (hALK4) [Wang et al., 2007].

K562 is a human erythroleukemia (HEL) cell line derived from a patient with chronic myelogenous leukemia (CML) characterized by aneuploidy and dysregulated constitutive expression of *BCR-ABL* due to the presence of the Philadelphia chromosome (t9; 22) [Liu et al., 2003]. The cells are bipotential and can be induced to express erythroid or megakaryocytic properties depending on the inducers [Lockhart et al., 1996]. Therefore, K562 cells induced by hemin and phorbol myristate acetate (PMA) have been used as representative models for the studies of erythroid and megakaryocytic differentiation for many years. In order to identify the miRNAs that may take part in erythroid differentiation, we first obtained information of miRNAs expression before and after hemin-induced erythroid differentiation of K562 cells by miRNA microarray analysis. Then, from the five differentially expressed miRNAs reconfirmed by Northern blot hybridization, miR-103 was selected out to examine its roles in erythroid differentiation and its targets.

MATERIALS AND METHODS

CELL LINES AND CULTURE

K562 (chronic myelogenous leukemia cell line), HEL (human erythroleukemia cell line), HL-60 (human promyelocytic leukemia cell line), Jurkat (human acute T cell leukemia cell line), HUT-78 (human T lymphoma cell line), CMK (human megakaryoblastic leukemia cell line), 3D5 (human B cell line), Raji (human B-non-Hodgkin's lymphoma cell line), and THP-1 (human promyelocytic leukemia cell line) used in our study were bought from the Cell Center of institute of Basic Medical Science, Chinese Academy of Medical Science. Cells were grown in RPMI 1640 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco BRL) and were cultured at 37°C in an incubator with controlled humidified atmosphere containing 5% CO_2.

INDUCTION OF K562 CELL WITH HEMIN

On the day of induction, cells were seeded at a density of 5×10^4/ml in the RPMI 1640 medium supplemented with or without hemin (Sigma-Aldrich, Deisenhofen, Germany) at a dosage of 50 μmol/L. Cells were harvested at each desired time point (0, 24, 48, and 72 h). The erythroid differentiation of K562 cells was assessed by benzidine cytochemical test and the benzidine-positive cells stand for more matured erythroid cells. Gamma-globin mRNA was also detected by real-time PCR and the mRNA level serves an indicator of undifferentiated and more matured erythroid cells.

ERYTHROID INDUCTION CULTURE OF HUMAN CD34+ CELLS

UCB samples were obtained from normal full-term deliveries after informed consent. Mononuclear cells from UCB were separated by density gradient centrifugation using percoll (Amersham Pharmacia Biotech, Freiburg, Germany). Magnetic Activated Cell Sorting (MACS) (Miltenyi Biotec, Bergisch-Gladbach, Germany) and the CD34+ cells were isolated by positive selection using anti-CD34-tagged magnetic beads and a VarioMacs magnet (Miltenyi Biotec, Auburn, CA). The purity of the recovered cells was typically more than 90%. The CD34+ cells were cultured in liquid medium using IMEM media (Gibco BRL), supplemented with 30% FBS, 100 U/ml penicillin/streptomycin, and a cocktail of cytokines (R&D Systems) containing 10 ng/ml interleukin-3 (IL-3), 10 ng/ml stem cell factor (SCF), and 2 U/ml EPO. Cells were harvested at each desired time point (at day 0, day 4, day 7, and day 9).

EXTRACTION OF TOTAL RNA AND miRNA

Total RNA was extracted from the cells using Trizol reagent (Invitrogen, Carlsbad, CA), an agent of single-step isolation. Small

RNAs (<200 nt) were extracted from cell pellets using the mirVana miRNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer's instructions.

MicroRNA MICROARRAY ANALYSIS

The uninduced and hemin-induced K562 cells were disrupted with TRIzol regent (Invitrogen) and sent to CapitalBio Corporation (CapitalBio Corp., Beijing, China) for miRNA expression analysis. Total RNA was isolated and miRNAs were further purified from 50 to 100 μg of total RNA by Ambion's miRNA Isolation Kit (Ambion). MiRNA microarray including labeling, hybridization, scanning, normalization and data analysis was carried out by CapitalBio. Briefly, RNA labeling was performed using RNA ligase as described [Thomson et al., 2004] and hybridized on the CapitalBio array (V1.0). Two independent hybridizations for each sample were performed on chips with each miRNA spotted in triplicate. Labeling efficiency was evaluated by analyzing the signals from control spike-in probes. Probes corresponding to human, mouse, and rat mature sense miRNA sequences (total 509 miRNAs, including 435 human miRNAs) were spotted on chemical modified slides using a robotic spotter named SmartArray™ (CapitalBio Corp.). Slides were hybridized for overnight at 42°C in 3 × SSC, 0.2% SDS, 15% formamide, and 50 × Denhardt's. After hybridization, slides were washed once in 0.2% SDS, 2 × SSC at 42°C for 4 min and then in 0.2 × SSC for 4 min at 25°C. Slides were scanned with LuxScan 10K/A (CapitalBio Corp.) and the raw pixel intensities extracted and analyzed with GenePix Pro 4.0 image manipulation software (Axon Instruments). Data were analyzed by first subtracting the background and normalized to per-chip median values and then used to obtain geometric means and standard deviations for each miRNA. Double arrays were performed under same treatment condition. Significantly expressed miRNAs were identified by significance analysis of microarrays (SAM, version 2.1) using two class-unpaired comparison in the SAM procedure. For each sample, two hybridizations were carried out and each miRNA probe has three replicate spots on a microarray.

PAGE–NORTHERN OF miRNAs

Twenty micrograms of denatured total RNA were loaded onto 15% polyacrylamide TBE gel and separated in 1 × TBE running buffer, followed by transfer onto N^+ membrane (Amersham) at 200 mA for 2 h in electro-transferring system and cross-linking under ultraviolet radiation for 150 s. Briefly, the miRNA-specific oligonucleotides was 5′ end labeled with γ-^{32}P-ATP through T4 polynucleotide kinase (Takara, Dalian, PR China) according to the manufacturer's protocol. The oligo probes were designed based on individual miRNA sequence information deposited in miRBase (http://microrna.sanger.ac.uk). An antisense oligo of U6 snRNA was used to detect U6 snRNA from each sample as a loading control. After prehybridization using hybridizing buffer (BioDev), blots were hybridized with ^{32}P-labeled DNA probes (2 μmol/ml) overnight at 37°C. After washing, the hybridized membranes were exposed to Kodak X-omat BT Film. After the hybridization with each miRNA probe was obtained, the blots were reprobed with U6 snRNA for an indication of equal RNA loading.

REVERSE TRANSCRIPTION AND REAL-TIME PCR

Total RNA was extracted from cell samples harvested at different time points using TRIzol reagent (Invitrogen) and quantified. DNA was removed by treatment with 5 units of DNase I (Promega, Madison, WI) at 37°C for 45 min followed by inactivation at 65°C for 10 min. Then first-strand of cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instruction. The expression of targeted miRNAs and mRNA in cultured erythroid cells was quantified using real-time PCR on ABI PRISM 7500 real-time PCR System (Applied Biosystems, Foster City, CA) with the SYBR Premix Ex Taq kit (Takara). PCR amplification was performed in a 20 μl volume containing 1 × SYBR Green PCR Master mix, 0.2 μM of forward and reverse primer and 2 μl product of reverse transcription. Each PCR reaction was performed in triplex tubes with GAPDH as an endogenous control to standardize the amount of sample RNA. The quantification data were analyzed with the sequence detection system (SDS) software (Applied Biosystems). For each standard curve, the linear correlation coefficient R was defined as $R^2 \geq 0.99$. The comparative CT method (number of cycles at which the fluorescence crosses the threshold) was used to quantify target genes relative to GAPDH. The following primers were used for real-time PCR: γ-globin FW, 5′-GCAGCTTGTCACAGTGCAGTTC-3′; γ-globin RW, 5′-TGGCAAGAAGGTGCTGACTTC-3′; GAPDH FW, 5′-TCAACG ACCACTTTGTCAAGC TC A-3′; GAPDH RW, 5′-GCTGGTGGTCCAGGGGTCTTACT-3′; FOXJ2 FW, 5′-CCACAAGCTCCCCACCTCTA3′; FOXJ2 RW, 5′-CTGATGGCCGAGGGACCATA-3′.

REAL-TIME QUANTIFICATION OF miRNAs BY STEM-LOOP RT-PCR

Real-time PCR for miR-126, miR-103, miR-130a, miR-18b, and miR-210 were executed and modified using standard procedure [Chen et al., 2005]. Specifically, reverse transcription reaction contained particularly following stem-loop RT primers: miR-126 RT, 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCATTAT-3′; miR-103 RT, 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATAC GACTCAGCC-3′; miR-130a RT, 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACT GGATACGACATGCCCT-3′; miR-18b RT, 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATT CGCACTGGATACGACCTAACTG-3′; miR-210 RT, 5′-GTCGTATCCAGTGCAGGGTCCGA GGTATTCGCACTGGATACGACTCAGCC-3′; U6 RT, 5′-AAAATATGGAACGCTTCACGA ATTTG-3′. The following primers were used for real-time PCR amplification of the reversed miRNAs: 126 FW, 5′-GCGCTCGTACCGTGAGTAAT-3′; miR-130a FW, 5′-CAGTGCAATGTTAAAAG-3′; miR-18b FW, 5′-GCGCTAAGGTGCATCTAGTGC-3′; miR-210 FW, 5′-GCCTGTGCGTGTGACAGC-3′; NR, 5′-GTGCAGGGTCCGAGGT-3′; U6 FW, 5′-CTCGCTTCGGCAGCACATATAC T-3′; U6 RW, 5′-ACGCTTCACGAATTTGCGTGTC-3′. NR was used as common RW primer for the six miRNAs. The amplification of U6 snRNA was used as internal control in the system and miRNA expression can be detected through relative quantification of CT method.

TRANSFECTION OF K562 CELL WITH OLIGONUCLEOTIDE

Pre-miR-103

K562 cells were transfected with oligonucleotides pre-miR-103 (Pre-miR™ miRNA Precursor Molecules) and scramble dsRNA (Pre-

miR™ miRNA Precursor–Negative Control) that does not target any known mRNA within the human or mouse transcriptome respectively at 60 pmol/ml using siPORT™NeoFx™ system (Ambion). The pre-miR-103 and scramble dsRNA were bought from Ambion company.

TARGET IDENTIFICATION OF miR-103

MiRNA target prediction was performed using PICTR (http://pictar.bio.nyu.edu), TARGETSCAN (www.targetscan.org) and MIRANDA (www.microRNA.org). The 3′-UTR segments of probable targets containing the target site of miR-103 were amplified by PCR from the cDNA and insert into *Xba* I and *Not* I sites of pRL-TK (Promega). To test the effect of the over-expressed miR-103 in increasing miRNA activity on reporter gene bearing miRNA-binding site, the synthetically double strand DNA molecules including complete complement sequence to miR-103 were annealed and insert into the two sites just down the coding region of luciferase. The recombinant plasmid was named as pRL-TK-103. The recombinant plasmid pRL-TK-103-*mut* that carrying the mutant miR-103-binding site (AGCAGCATTGTACAG***TTAACGT***A) was used as the negative control. Human Hela cells were cotransfected with the reporter plasmid and pre-miR-103 or scramble dsRNA respectively using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Firefly (pGL-3 served as internal control) and renilla luciferase (pRL-TK) activities were measured consecutively by using the dual luciferase assays (Promega).

RECOMBINANT LENTIVIRUS GENERATION AND INFECTION OF K562 CELLS

The lentiviral vector pLVTHM (Addgene, South San Francisco, CA) was used for direct cloning of shRNAs under the control of H1 promoter. The pLVTHM was digested with *Mlu*I and *Cla*I and two complementary oligonucleotides were annealed and cloned into the pLVTHM. The siRNA targeting sequence of FOXJ2 was: 5′-AAGGGTTCCTATTGGACAATT-3′. This recombinant vector expressing siRNA of FOXJ2 was denoted as pLVTHM-FOXJ2-RNAi. Recombinant lentiviruses were prepared by calcium phosphate-mediated cotransfection of 293T/17 cells. Briefly, 293T/17 cells were plated on poly-L-lysine-coated 10-cm plates at 3×10^6 cells per plate in DMEM with 10% FBS and allowed to grow for 24 h before transfection with 10 μg of recombinant vector plasmid, 10 μg of packaging plasmid pSPAX2 and 2 μg of envelope plasmid pMD.2G (VSV-G). Eight hours after application of the DNA precipitate, the cells were rinsed three times with phosphate-buffered saline (PBS), and then cultured in fresh DMEM containing 10% FBS. Virus supernatants were collected 48 h after application of fresh medium and then filtered through a 0.2 μm syringe filter and stored at −80°C.

For gene transduction into K562 cells, approximately 1×10^5 K562 cells and 1×10^6 viral particles were added to a RetroNectin-coated plate (Takara) with growth medium, and the plate was incubated at 37°C. Twenty-four hours after virus infection, the K562 cells were washed with PBS and induced to erythroid differentiation by hemin. The cells were collected at desirable induction time.

WESTERN BLOT

The cultured cells were harvested and lysed in 200 μl ice-cold lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM EDTA). The samples were boiled for 5 min and subsequently spun down at 14,000*g* for 10 min at 4°C. After total protein extraction, FOXJ2 were determined by Western blot immunoassay with anti-FOXJ2 antibody using the Western blot kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) according to the manufacturer's instructions. Twenty micrograms of extracts were resolved on a 10% SDS–PAGE gel and the proteins were transferred to polyvinylidene difluoride membranes (Amersham) using Bio-Rad's Transblot for 1.5 h at 80 A at room temperature. When finished, the membrane was immersed in blocking buffer (5% degrease milk powder) more than 2 h at room temperature. Then the membrane was incubated with anti-Fox J2 antibody (Abcam, Cambridge, United Kingdom) and anti-GAPDH antibody (Abgent, San Diego) respectively for 2 h at room temperature. After incubating with goat-anti-rabbit antibody for 1 h at room temperature, the membranes were exposed to ECL hyper film (Amersham, Life Sciences International) for 5 s–15 min and then exposed to Kodak X-omat BT Film.

RESULTS

miRNA EXPRESSION CHANGES BETWEEN UN-INDUCED AND HEMIN-INDUCED K562 CELLS

Erythroid differentiation of K562 cells after hemin induction was evidenced by benzidine cytochemical test and γ-globin mRNA level examined by real-time PCR (data not shown). Using microarray (SAM, version 2.1) containing oligonucleotides corresponding to 509 miRNAs from human, mouse, and rat genomes, we compared miRNAs expression of hemin-treated K562 cells with untreated cells. Thirty-one miRNAs with different expression were detected (supplementary Table I). We then analyzed expression of theses miRNAs by Northern blot and confirmed the increase of miR-126 and the decrease of miR-103, miR-130a, miR-210, and miR-18b during hemin-induced K562 erythroid differentiation (Fig. 1). Their expression in 10 leukemia cell lines was also examined (Fig. 1) and most of them exhibited enrichment in erythroleukemia cell lines (K562 or/and HEL).

EXPRESSION OF THE miRNAs DURING ERYTHROID DIFFERENTIATION OF CD34+ CELLS

We examined the expression of miR-126, miR-103, miR-130a, miR-210, and miR-18b during the erythroid differentiation of purified cord blood CD34+ progenitors in a liquid culture system by real-time PCR. An obviously increased miR-126 expression whereas a significantly decreased expression of miR-103, miR-130a, miR-210, and miR-18b was detected during erythroid differentiation of CD34+ cells (Fig. 2). The results were in coincidence with hemin-induced K562 cell differentiation. These data indicate that hemin-induced erythroid differentiation of K562 cells could reflect normal erythroid differentiation in a certain extent and miR-126, miR-103, miR-130a, miR-210, and miR-18b may take part in normal erythropoiesis.

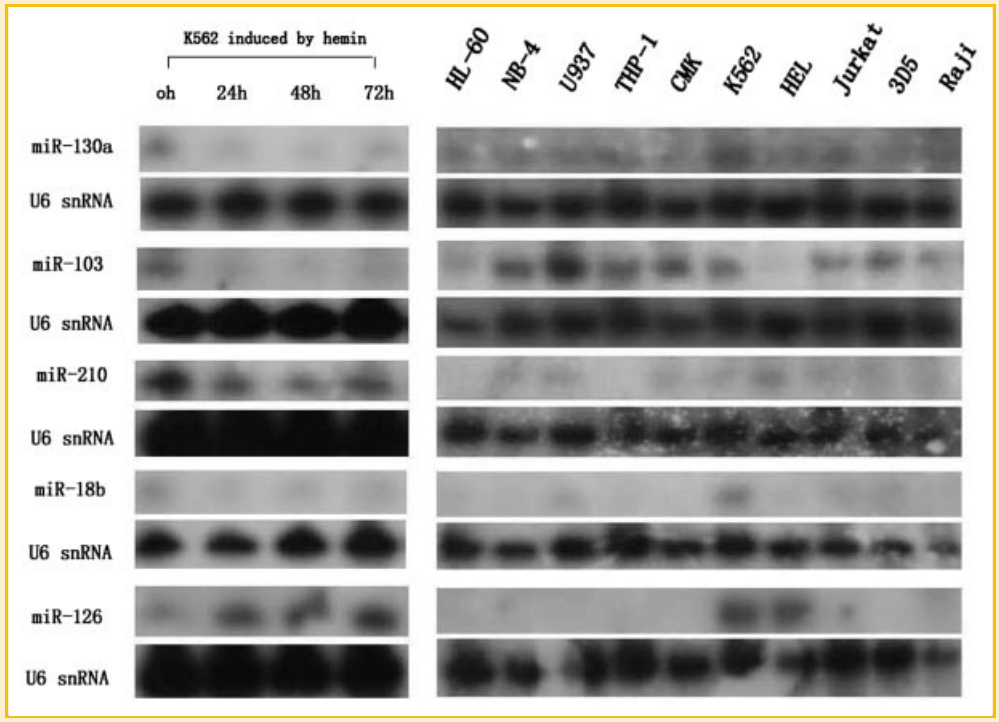

Fig. 1. Northern blot analysis of the selected miRNAs. RNA was extracted from K562 cells treated with hemin for 12, 24, 48, and 72 h and probed with γ-^{32}P-labeled DNA oligoneucleotide complementary to each mature miRNAs. After hybridization with each probe, the blots were stripped and reprobed with sequence complementary to U6 snRNA for loading control.

ENFORCED EXPRESSION OF miR-103 INHIBITS HEMIN-INDUCED K562 ERYTHROID DIFFERENTIATION

Before testing the role of miR-103 in erythroid differentiation, the efficacy of oligonucleotides pre-miR-103 was evaluated by using a luciferase reporter (pRL-TK-103) bearing target site that directly matches to miR-103. The Significant reduction of luciferase intensity was observed in K562 cells cotransfected with pre-miR-103 compared with the K562 cells cotransfected with the scramble dsRNA or the mutant miRNA binding sites (Fig. 3A) and untransfected K562 cells, indicating miR-103 was over-expressed in K562 cells with oligonucleotides pre-miR-103 transfection.

To examine the role of miR-103 in erythroid differentiation, K562 cells were transfected with pre-miR-103 oligonucleotides and the scramble dsRNA respectively. Real-time PCR assay confirmed significant over-expression of miR-223 in K562 cells transfected with pre-miR-103 oligonucleotides (Fig. 3B). Erythroid differentiation were evaluated by benzidine staining and γ-globin mRNA level after 12, 24, 48 h of hemin induction. Cells transfected with

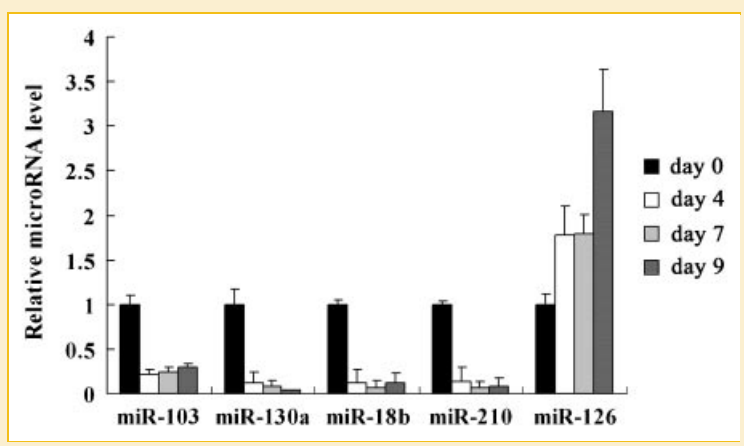

Fig. 2. Expression of the selected miRNAs during erythroid differentiation of CD34+ cells. Real-time PCR amplification of the microRNAs was performed in triplicate and expression level of each miRNA was normalized by U6 snRNA. The means of the normalized gene expression values for each time point were calculated and expressed as relative fold changes (mean $\pm$ SD).

pre-miR-103 showed a marked decrease of the benzide positive cells and γ-globin mRNA expression compared with cells transfected with the control oligonucleotides and untransfected K562 cells (Fig. 3C,D), suggesting that over-expression of miR-103 inhibits hemin-induced K562 erythroid differentiation.

TARGET IDENTIFICATION AND VALIDATION OF miR-103

By using three target prediction algorithms, PICTR (http://pictar.bio.nyu.edu), TARGETSCAN (http://www.targetscan.org), and MIRANDA (www.microrna.org), about 40 possible mRNA targets were identified. There are two miR-103 binding sites predicted in FOXJ2 3′-UTR and they are both highly conserved between different species (Fig. 4A). To detect whether the two sites function together or only one is true, we examined site 149 and site 2622 respectively. More than 25% reduction in luciferase level was observed in Hela cells cotransfected with pre-miR-103 together with FOXJ2-UTR-full or FOXJ2-UTR-2622 compared with Hela cells cotransfected with FOXJ2-UTR-full luciferase reporter and scramble dsRNA (Fig. 4B). Mutation in the second predicted site of FOXJ2 3′-UTR abolished this reduction. However, no obvious changes of luciferase activity was observed in Hela cells cotransfected with pre-miR-103 and FOXJ2-UTR-149 compared with Hela cells cotransfected with FOXJ2-UTR-full luciferase reporter and scramble dsRNA (Fig. 4B). The results indicated the second site that is close to poly(A) within FOXJ2 mRNA was the true target site of miR-103.

To confirm whether over-expression of miR-103 in vivo suppresses FOXJ2 expression, we measured FOXJ2 mRNA by real-time PCR and FOXJ2 protein by Western blot (Fig. 4C,D). As expected, FOXJ2 protein reduced greatly in Hela cells transfected with pre-miR-103 compared with the Hela cells transfected with the scramble dsRNA, while its mRNA level changed a little. Additionally a marked reduction in FOXJ2 expression was also observed in K562 cells transfected with pre-miR-103 compared with the K562 cells transfected with the scramble dsRNA (Fig. 4E). These data suggest that the FOXJ2 is a target for miR-103 in vivo.

To detect whether miR-103 regulates erythroid differentiation via targeting FOXJ2, we examined the effects of down-regulation of FOXJ2 on K562 cells. Real-time PCR and Western blot results showed that the expression of FOXJ2 was decreased in K562 cells infected with the recombinant lentivirus expressing siRNAs for FOXJ2 (Fig. 5A,B). The expression of γ-globin mRNA in these K562 cells was significantly decreased compared with the control at any indicated times (Fig. 5C). These results confirmed that the down-regulation of FOXJ2 had similar effects on K562 erythroid differentiation with the over-expression of miR-103. MiR-103 inhibited erythroid differentiation via down-modulation of FOXJ2.

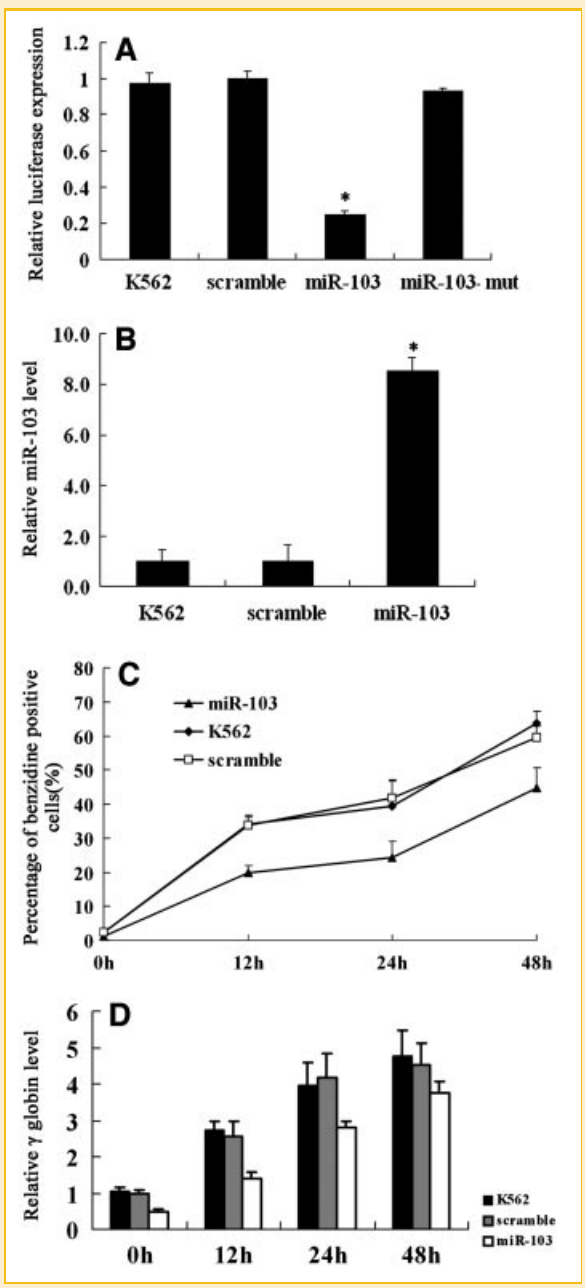

Fig. 3. Enforced expression of miR-103 inhibits erythroid differentiation of K562 cells. A: Over-expression of miR-103 reduced significantly luciferase activity. Luciferase activity was detected in K562 cells transiently cotransfected with the following groups: luciferase reporter vector containing complete complementary sequence to miR-103 and pre-miR-103; luciferase reporter vector containing complete complementary sequence to miR-103 and scramble dsRNA; and luciferase reporter vector containing the mutant miR-103 binding sequence and pre-miR-103. All experiments were performed respectively for three times. The luciferase activity was calculated and expressed as relative fold changes (mean ± SD). B: Real-time PCR amplification of the miR-103 in K562 cells transfected with pre-miR-103 and scramble dsRNA. The experiments were performed in triplicate and expression level of each miRNA was normalized by U6 snRNA. The means of the normalized gene expression values for each time point were calculated and expressed as relative fold changes (mean ± SD). Error bars represent one standard deviation. $^{*}P < 0.01$. C: Percentage of benzidine-positive cells in each group was counted at the indicated time points. Each assay was performed in triplicate and the data represent the mean ± SD of three experiments. D: The expression of γ-globin mRNA of K562 cells transfected with oligonucleotide pre-miR-103 and K562 cells transfected with the scramble dsRNAs before and after hemin induction. Comparative real-time PCR was performed in triplicate and expression level of γ-globin was normalized by GAPDH mRNA and expressed as relative fold changes (mean ± SD).

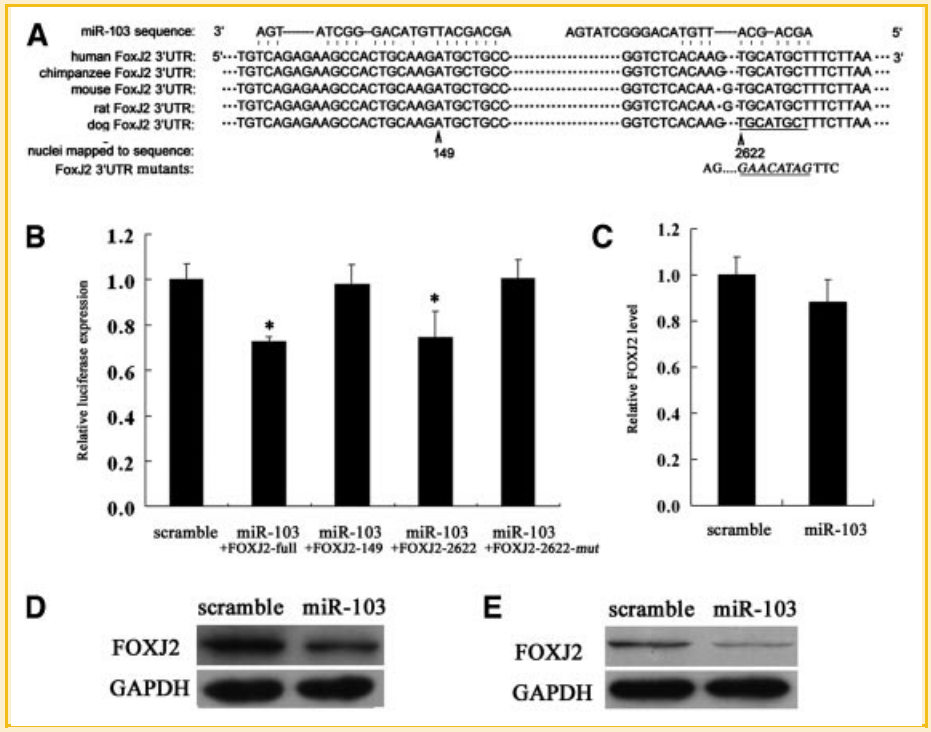

Fig. 4. FOXJ2 was confirmed as the target for miR-103. A: Sequence alignment between miR-103 and its putative binding sites in the FOXJ2 3′-UTR from different species. The sequences shown indicate the putative miR-103 target sites and the mutated derivative (FOXJ2 3′-UTR mutants). B: The enforced miR-103 expression reduced significantly luciferase level of FOXJ2-UTR luciferase reporter. Hela cells were cotransfected with the following groups: FOXJ2-UTR-full luciferase reporter and pre-miR-103; FOXJ2-UTR-149 luciferase reporter and pre-miR-103; FOXJ2-UTR-2622 luciferase reporter and pre-miR-103; FOXJ2-UTR-2622-*mut* luciferase reporter and pre-miR-103; and FOXJ2-UTR-full luciferase reporter and scramble dsRNA. The experiment was repeated for three times and luciferase activity was calculated and expressed as relative fold changes (mean ± SD). Error bars represent one standard deviation. $^{*}P < 0.05$. C: Relative FOXJ2 mRNA expression in Hela cells transfected with pre-miR-103 and scramble dsRNA respectively was detected by real-time PCR assay. D: Western blot analysis of FOXJ2 protein expression in Hela cells transfected with pre-miR-103 and scramble dsRNA respectively. The protein level was normalized with GAPDH. E: Western blot analysis of FOXJ2 protein expression in K562 cells transfected with pre-miR-103 or scramble dsRNA. The protein level was normalized with GAPDH. An obviously reduced FOXJ2 expression was observed in Hela cells (and K562 cells) transfected with oligonucleotide pre-miR-103 compared with Hela cells (and K562 cells) transfected with the control scramble dsRNA.

DISCUSSION

It is now well known that miRNAs are important components of gene regulation system. MiRNAs act as negative modulators operating at post-transcription level by knocking down their target protein-coding genes. This fine tuning regulation provides subtle changes of gene expression level required for development and cell fate determination. One appealing possibility, that miRNAs eliminate low expression of unwanted genes and hence refine unilineage gene expression, may be especially amenable to evaluation in models of hematopoiesis [Shivdasani, 2006].

To clarify the potential roles of miRNAs in human erythropoiesis, we compared the miRNA expression of hemin-treated K562 cells with untreated cells by Northern blot and found five changed miRNAs. Other studies also examined the expression changes of miRNAs during hematopoiesis. Georgantas et al. indicated miR-103 and miR-130a as hematopoietic stem-progenitor cell expressed miRNAs. We also showed that miR-103 and miR-130a was highly expressed in cord blood CD34+ cells but declined after EPO-induced erythroid differentiation. However, our observations were little conflicting with Choong et al., that may be due to erythropoietic induction and array step protocols were different. Here we only focused on miR-103, whereas miR-130a, miR-210, miR-126, and miR-18b may also have important roles in erythroid differentiation. So our data provided initiative information for the future study. The down-regulated miRNAs may unblock target genes that promote erythroid differentiation and our findings give important clues in their function in erythropoiesis.

Given to that the malignant K562 cell is similar but considerably different to normal human erythroid lineage in its inability to differentiate into mature erythrocytes and its CML origin, we also examined the expression of miR-103, miR-130a, miR-18b, miR-210, and miR-126 during the erythroid differentiation of purified cord blood CD34+ progenitors in a liquid culture system in vitro. The results were in coincidence with that obtained from hemin-induced K562 cell differentiation, which suggested that this model could reflect normal erythropoiesis in some extent.

MiR-103 is down-regulated in the process of hemin-induced K562 cells erythroid differentiation. The enforced expression of miR-103 could inhibit erythroid differentiation of K562, suggesting that miR-103 may take part in tumorigenesis of erythroleukemia by arresting erythroid maturation. As our hypothesis, miR-103 and its paralog miR-107 have been found to be over-expressed in several tumor types. In a recent report, miR-103 and miR-107 are shown to

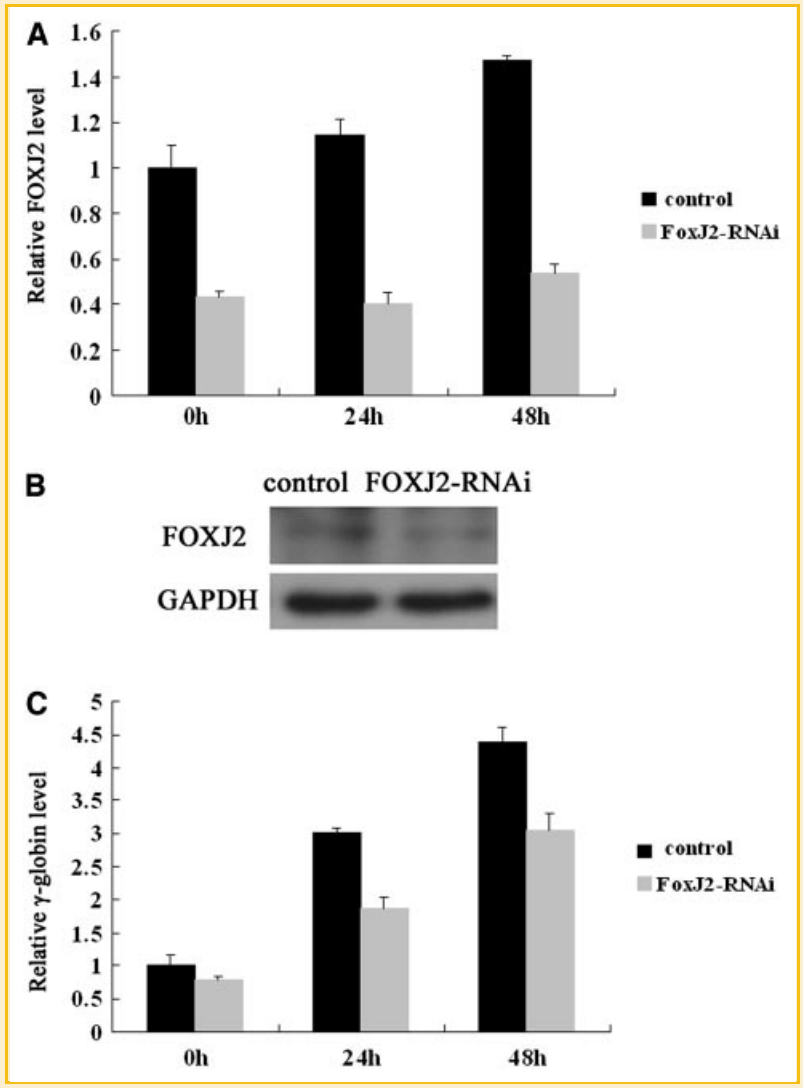

Fig. 5. Down-regulation of FOXJ2 inhibits erythroid differentiation of K562 cells. A: The expression of FOXJ2 mRNA of K562 cells infected with recombinant lentivirus pLV-FOXJ2-RNAi and control pLVTHM respectively before and after hemin induction. Comparative real-time PCR was performed in triplicate and expression level of FOXJ2 was normalized by GAPDH mRNA and expressed as relative fold changes (mean ± SD). B: Western blot analysis of FOXJ2 protein expression in K562 cells infected with recombinant lentivirus pLV-FOXJ2-RNAi and control pLVTHM respectively. The protein level was normalized with GAPDH. C: The expression of γ-globin mRNA of K562 cells infected with recombinant lentivirus pLV-FOXJ2-RNAi and control pLVTHM respectively before and after hemin induction. Comparative real-time PCR was performed in triplicate and expression level of γ-globin was normalized by GAPDH mRNA and expressed as relative fold changes (mean ± SD).

serve as discriminators for pancreatic tumors and normal pancreas [Roldo et al., 2006]. High expression of hsa-miR-103/107 correlated with poor patient survival in esophageal squamous cell carcinoma [Guo et al., 2008]. Our luciferase report experiment and Western blot analysis showed that FOXJ2 mRNA was the target of miR-103. FOXJ2 is a member of forkhead family of transcription factors that have been proven important in processes such as proliferation, differentiation and survival for embryonic development, hematopoietic T cell differentiation and neural systems. Even though the reports about the function of FOXJ2 in hematopoiesis are relative little, we believed it is necessary to study its effect in erythroid cell differentiation. Additionally, since it is possible that a single miRNA may target multiple transcripts and that an individual transcript may be subject to regulation by multiple miRNAs, so the function of miR-103 in erythroid differentiation may be exerted through other targets except for FOXJ2.

The same miRNAs may play different roles according to its temporal and spatial expression specificities. Here, miR-126 is a typical example. Previous report showed that the expression of miR-126 declined in vitro-differentiated megakaryocytes derived from CD34+ cells [Garzon et al., 2006]. In this study, we found miR-126 increased dominantly in K562 cells after hemin induction and in CD34+ cells after erythroid induction. So the function of miR-126 in erythropoiesis may be exerted through targets different from those in megakaryocyte differentiation.

It is now well accepted that miRNAs are expressed widely in developing and adult tissues and their functions are likely to be widespread, diverse and ranging from seminal to subtle depending

on the context. MiRNA function at post-transcriptional level makes up efficient complementarity to transcription regulation. Here, we identified five miRNAs with expression change during erythroid differentiation and analyzed the function and probable mechanism of miR-103 in erythroid differentiation. Our data may provide new insight into the molecular mechanism in human erythropoiesis and erythroleukemia tumorigenesis.

REFERENCES

Berezikov E, Guryev V, van de Belt J, Wienholds E, Plasterk RH, Cuppen E. 2005. Phylogenetic shadowing and computational identification of human microRNA. Genes Cell 120:21–24.

Bruchova H, Yoon D, Agarwal AM, Mendell J, Prchal JT. 2007. Regulated expression of microRNAs in normal and polycythemia vera erythropoiesis. Exp Hematol 35:1657–1667.

Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Adler H, Rttan S, Keating M, Rai K, Rassenti L, Kipps T, Negrini M, Bullrich F, Croce CM. 2002. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci 99: 15524–15529.

Calin GA, Liu CG, Sevignani C, Ferracin M, Felli N, Dumitru CD, Shimizu M, Cimmino A, Zupo S, Dono M, Dell'Aquila ML, Alder H, Rassenti L, Kipps TJ, Bullrich F, Negrini M, Croce CM. 2004. MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias. Proc Natl Acad Sci 101: 11755–11760.

Chen CZ, Lodish HF. 2005. MicroRNAs as regulators of mammalian hematopoiesis. Immunology 17:155–165.

Chen CZ, Li L, Lodish HF, Bartel DP. 2004. MicroRNAs modulate hematopoietic lineage differentiation. Science 303:83–86.

Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, Lao KQ, Livak KJ, Guegler KJ. 2005. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 33:179.

Cheng AM, Byrom MW, Shelton J, Frd LP. 2005. Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. Nucleic Acids Res 33:1290–1297.

Choong ML, Yang HH, McNiece I. 2007. MicroRNA expression profiling during human cord blood-derived CD34+ cell erythropoiesis. Exp Hematol 35:551–564.

Fatica A, Rosa A, Fazi F, Ballarino M, Morlando M, De Angelis FG, Caffarelli E, Nervi C, Bozzoni I. 2006. MicroRNAs and hematopoietic differentiation. Cold Spring Harb Symp Quant Biol 71:205–210.

Fazi F, Rosa A, Fatica A, Gelmetti V, De Marchis ML, Nervi C, Bozzoni IA. 2005. Minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBP alpha regulates human granulopoiesis. Cell 123:819–831.

Felli N, Fontana L, Pelosi E, Botta R, Bonci D, Facchiano F, Liuzzi F, Lulli V, Morsilli O, Santoro S, Valtieri M, Calin GA, Liu CG, Sorrentino A, Croce CM, Peschle C. 2005. MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation. Proc Natl Acad Sci 102:18081–18086.

Garzon R, Pichiorri F, Palumbo T, Iuliano R, Cimmino A, Aqeilan R, Volinia S, Bhatt D, Alder H, Marcucci G, Calin GA, Liu CG, Bloomfield CD, Andreeff M, Croce CM. 2006. MicroRNA fingerprints during human megakaryocytopoiesis. Proc Natl Acad Sci 103:5078–5083.

Georgantas RW 3rd, Hildreth R, Morisot S, Alder J, Liu CG, Heimfeld S, Calin GA, Croce CM, Civin CI. 2007. CD34+ hematopoietic stem-progenitor cell microRNA expression and function: A circuit diagram of differentiation control. Proc Natl Acad Sci 104:2750–2755.

Guo Y, Chen Z, Zhang L, Zhou F, Shi S, Feng X, Li B, Meng X, Ma X, Luo M, Shao K, Li N, Qiu B, Mitchelson K, Cheng J, He J. 2008. Distinctive microRNA profiles relating to patient survival in esophageal squamous cell carcinoma. Cancer Res 68:26–33.

He L, Thompsom JM, Hermann MT, Hermando-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe S, Hannon GJ, Hammond SM. 2005. A microRNA polycistron as a potential human oncogene. Nature 435: 828–833.

Karp X, Ambros V. 2005. Developmental biology. Encountering microRNAs in cell fate signaling. Science 310:1330–1333.

Liao R, Sun J, Zhang L, Lou G, Chen M, Zhou D, Chen Z, Zhang S. 2008. MicroRNAs play a role in the development of human hematopoietic stem cells. J Cell Biochem 104:805–817.

Liu G, Loraine AE, Shigeta R, Cline M, Cheng J, Valmeekam V, Sun S, Kulp D, Siani-Rose MA. 2003. NetAffx: Affymetrix probesets and annotations. Nucleic Acids Res 31:82–86.

Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS, Mittmann M, Wang C, Kobayashi M, Horton H, Brown EL. 1996. Expression monitoring by hybridization to high-density oligonucleotidearrays. Nat Biotechnol 14: 1675–1680.

Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. 2005. MicroRNA expression profiles classify human cancers. Nature 435: 834–838.

Masaki S, Ohtsuka R, Abe Y, Muta K, Umemura T. 2007. Expression patterns of microRNAs 155 and 451 during normal human erythropoiesis. Biochem Biophys Res Commun 364:509–514.

Poy MN, Eliasson L, Krutzfeldt J, Kuwajima S, Ma X, Macdonald PE, Pfeffer S, Tuschl T, Rajeswski N, Rorsman P, Stoffel M. 2004. A pancreatic islet-specific microRNA regulates insulin secretion. Nature 432:226–230.

Ramkissoon SH, Mainwaring LA, Ogasawara Y, Keyvanfar K, McCoy JP, Jr., Sloand EM, Kajigaya S, Young NS. 2006. Hematopoietic-specific microRNA expression in human cells. Leuk Res 30:643–647.

Roldo C, Missiaglia E, Hagan JP, Falconi M, Capelli P, Bersani S, Calin GA, Volinia S, Liu CG, Scarpa A, Croce CM. 2006. MicroRNA expression abnormalities in pancreatic endocrine and acinar tumors are associated with distinctive pathologic features and clinical behavior. J Clin Oncol 24:4677–4684.

Shivdasani RA. 2006. MicroRNAs: Regulators of gene expression and cell differentiation. Blood 108:3646–3653.

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

Vigorito E, Perks KL, Abreu-Goodger C, Bunting S, Xiang Z, Kohlhaas S, Das PP, Miska EA, Rodriguez A, Bradley A, Smith KG, Rada C, Enright AJ, Toellner KM, Maclennan IC, Turner M. 2007. MicroRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. Immunity 27: 847–859.

Wang Q, Huang Z, Xue H, Jin C, Ju XL, Han JD, Chen YG. 2007. MicroRNA miR-24 inhibits erythropoiesis by targeting activin type I receptor ALK4. Blood 111:588–595.

Xu P, Guo M, Hay BA. 2004. MicroRNAs and the regulation of cell death. Trends Genet 20:617–624.

Yin Q, Wang X, McBride J, Fewell C, Flemington EK. 2007. B-cell receptor activation induces BIC/MIR-155 expression through a conserved AP-1 element. J Biol Chem 283:2654–2662.

Yuan JY, Wang F, Yu J, Yang GH, Liu XL, Zhang JW. MicroRNA-223 reversibly regulates erythroid and megakaryocytic differentiation of K562 cells. J Cell Mol Med (Epub ahead of print, doi:10.1111/j.1582-4934.2008.00585.x).

Zhan M, Miller CP, Papayannopoulou T, Stamatoyannopoulos G, Song CZ. 2007. MicroRNA expression dynamics during murine and human erythroid differentiation. Exp Hematol 35:1015–1025.