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MicroRNAs Play a Role in the Development of Human Hematopoietic Stem Cells

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Abstract MicroRNAs (miRNAs) are a class of recently discovered noncoding RNA genes that post-transcriptionally regulate gene expression. It is becoming clear that miRNAs play an important role in the regulation of gene translation during development. However, in mammals, expression data are principally based on whole tissue analysis and are still very incomplete. We isolated $CD34^+CD38^-$ hematopoietic stem cells (HSCs) from human umbilical cord blood, on the basis of cell-surface markers using fluorescence-activated cell sorting (FACS). Also, $CD34^+$ subpopulation was FACS isolated as the control. Next, using microarray containing oligonucleotides corresponding to 517 miRNAs from human, mouse, and rat genomes, we obtained miRNA gene expression profiles of both subpopulations. We focused on the HSCs correlative miRNAs with comparison to the control. The miRNAs of particular interest were confirmed by real-time RT-PCR. HSCs-overexpressed hsa-miR-520h and underexpressed hsa-miR-129 were selected for target prediction and analysis. The result showed that EIF2C3 and CAMTA1, genes related to miRNAs processing or transcription regulation, were proved to be real targets for hsa-miR-129. And ABCG2, involved in stemness maintaining, a real target for hsa-miR-520h. Finally, we chose hsa-miR-520h, enriched in HSCs but low in $CD34^+$ cells, for functional characterization, because of its possible role in differentiation of HSCs by regulating ABCG2. As a result, hsa-miR-520h transduction into $CD34^+$ cells greatly increased number of different progenitor colonies in Colony-Forming-Cell assays, suggesting that hsa-miR-520h may promote differentiation of HSCs into progenitor cells by inhibiting ABCG2 expression. This study paves the way for identifying HSC-specific miRNAs and their roles in HSC development. J. Cell. Biochem. 104: 805–817, 2008.

Key words: hematopoietic stem cell; development; microRNA; microarray; real-time RT-PCR

MicroRNAs (miRNAs) are endogenously expressed, noncoding RNAs, 19–25 nucleotides in length that interact with native coding mRNAs to inhibit translation. MiRNAs are important determinants of cellular fate specification [Hobert, 2004]. MiRNAs have been implicated in many different cellular processes including metabolism, apoptosis, differentiation, and development [Ambros, 2003]. MiRNAs might also play an important role in the pathogenesis of human cancer [Calin et al., 2002, 2004]. Embryonic stem cells were found to express a set of miRNAs, suggesting a close relationship between miRNAs and stem cell development [Houbaviy et al., 2003; Ishii and Saito, 2006].

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It remains essential to define the roles of miRNAs in hematopoiesis. The hematopoietic stem cells (HSCs) give rise to both myeloid and lymphoid progenitors. However, to date, only a few studies have addressed the role of miRNAs in HSCs biology, and hematological disease. The expression profiles of murine hematopoietic-specific miRNAs (miR-142, miR-181, and miR-223) have been described in HSCs, B, T lymphocytes, monocytes, granulocytes, and erythroid cells; in vitro and in vivo ectopic expression of these miRNAs in murine HSCs, which show low expression level of miR-142, miR-181, and miR-223, dramatically altered the proportion of differentiated lineages [Chen et al., 2004]. These data suggest that miRNAs are lineage-specific and important components in murine hematopoietic lineage differentiation. In humans, several miRNAs were reported to be involved in hematological diseases. High expression of precursor miR-155/BIC was measured in pediatric Burkitt lymphoma [Metzler et al., 2004]. Additional work using miRNA microarrays revealed significant differences between B cell chronic lymphocytic leukemia and normal $CD5^+$ B cells [Calin et al., 2004]. Little was known about the role of miRNAs in human HSCs development. The intriguing questions are: What are the HSCs development-related miRNAs expression profiles? Which miRNAs play a role in multi-directional differentiation of HSCs? What are the mechanisms of miRNAs' involvement in the development of HSCs?

To further elucidate regulatory mechanisms of miRNAs in normal human hematopoiesis, an important starting point is to examine expression profiles of miRNAs in HSCs. We report here miRNAs expression profile of HSCs-enriched $CD34^+CD38^-$ subpopulation and further investigate the role of hsa-miR-520h in multi-directional differentiation of HSCs.

MATERIALS AND METHODS

Isolation and Purification of Hematopoietic Lineage Cells

Samples of umbilical cord blood (CB) were, after obtaining informed consent from all patients, collected from Haidian obstetrics and gynecology hospital (Beijing). Within 4 h after collection, mononuclear cells (MNCs) were separated from the samples over a Ficoll paque™ Plus (StemCell Technologies, Inc., Canada), and $CD34^+$ cells were enriched using immunomagnetic columns (Mini-Macs, Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany). A concentration of 1×10^4 $CD34^+$ cells were cultured in X-Vivo 15™ (BioWhittaker) serum-free medium. The following cytokines were added to the medium at a final concentration of 100 ng/ml: FL, SCF, TPO, and IL-3 (Sigma) for cell amplification. FACS (MoFlo, Dako-Cytomation, Inc.) sorting was performed to isolate CD34-PE^+ cells and CD34-PE^+CD38-$FITC^-$ HSCs cells.

Colony-Forming Cell (CFC) Assays

To test the pluripotency of the FACS-isolated CD34-PE^+CD38-$FITC^-$ HSCs, CFC assays were performed by using complete methylcellulose medium with recombinant cytokines (MethoCult™, StemCell Technologies, Inc.). $CD34^+$ cells were the control. After incubation at 37°C, 5% CO_2, with $\geq$95% humidity for 12 days, the colony-forming unit-erythroid (CFU-Es), burst forming unit-erythroid (BFU-Es), colony-forming unit-granulocyte, macrophage (CFU-GMs) and colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMMs) in every dishes were classified and counted under a high-quality inverted microscope (Leica, Heidelberg, Germany). The steps were repeated for six dishes for the test group and six for the control as well.

Fabrication of miRNA Microarray

Our miRNA microarray includes 517 mature miRNA sequences. Among them, 395 mature miRNA sequences from humans, mice and rats were downloaded from the miRNA Registry (http://microrna.sanger.ac.uk; miRBase Release 7.0, accessed June, 2005). We also integrated 122 published predicted miRNA sequences in 3′untranslated regions (3′UTRs) of human, mouse, rat, and dog genomes [Xie et al., 2005]. The probe corresponding to the highly conserved and consistent expressed small nuclear RNA U6 was spotted as an internal control. In addition, we designed eight short oligos possessing no homology with all existed RNA sequence and produced their corresponding synthetic miRNAs by in vitro transcription using Ambion's miRNA Probe Construction kit. We spike various amounts of synthetic miRNAs into the interrogated RNA samples. All miRNA probe sequences were designed to be complementary to the full-length

mature miRNA. To facilitate probe immobilization on aldehyde modified glass slides, the probe sequence were concatenated up to 40 nt and modified with 5′-amino-modifier C6. Oligonucleotide probes were synthesized (MWG Biotech. Co.) and dissolved in EasyArray™ spotting solution (CapitalBio Corp.) at 40 μM concentration. Each probe was printed in triplicate using a SmartArray™ microarrayer (CapitalBio Corp.).

Amplification and Labeling of Target RNAs

Total RNA was extracted from $CD34^+$ cells and $CD34^+CD38^-$ HSCs with TRIZOL reagent and the low-molecular-weight RNA was isolated using miRNA Isolation Kit (Ambion). The NCode™ miRNA Amplification System (Invitrogen) was used for amplifying sense RNA molecules from purified miRNA. Then the amplified RNA was labeled with 5′-phosphate-cytidyl-uridyl-cy3-3′ (Dharmacon, Lafayette, CO) with 2 units T4 RNA ligase (New England Biolabs, Ipswich, MA) according to Thomson' protocol [Thomson et al., 2004].

Microarray Hybridization, Imaging, and Data Extraction

Hybridization was performed under LifterSlip™ (Erie Company). The hybridization chamber was laid on a three-phase tiling agitator BioMixer™ II (CapitalBio Corp.) to prompt the microfluidic circulation under the coverslip. The array was hybridized at 42°C overnight and washed with two consecutive washing solutions (0.2% SDS, 2 × SSC at 42°C for 5 min, and 0.2% SSC for 5 min at room temperature). Arrays were scanned with a confocal LuxScan™ scanner (CapitalBio Corp.). Then data normalization was performed based on mean array intensity for inter-array comparison. Significance Analysis of Microarrays (SAM, version 2.1) was performed 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.

Real-Time RT-PCR

All primers were designed using Primer Express version 2.0 (Applied Biosystems, Foster City, CA). We followed Chen's protocol for primer design and real-time RT-PCR [Chen et al., 2005]. Oligonucleotide primers were 5′-ctcgcttcggcagcaca-3′ and 5′-aacgcttcacgaatttgcgt-3′ for the internal control, U6 small nuclear RNA. The analyzed miRNAs included hsa-miR-129, hsa-miR-526b*, hsa-miR-127, hsa-miR-520h, hsa-miR-452, hsa-miR-365. Real-time RT-PCR Oligonucleotide primers were hsa-mir-129: 5′-gtcgtatccagtgcagggtccgaggtattcgcactggatacgacgcaagc-3′ and 5′-ctttttgcggtctgggct-3′, hsa-mir-526b*: 5′-gtcgtatccagtgcagggtccgaggtattcgcactggatacgacgcctct-3′ and 5′-acgcaaagtgcttccttttaga-3′, hsa-mir-127: 5′-gtcgtatccagtgcagggtccgaggtattcgcactggatacgacagccaa-3′ and 5′-tcggatccgtctgagcttg-3′, hsa-mir-520h: 5′-gtcgtatccagtgcagggtccgag gtattcgcactggatacgacactcta-3′ and 5′-acgacaaagtgcttccctttag-3′, hsa-mir-452: 5′-gtcgtatccagtgcagggtccgaggtattcgcactggatacgacgtctca-3′ and 5′-cgtgtttgcagaggaaactga-3′, hsa-mir-365: 5′-gtcgtatccagtgcagggtccgaggtattcgcactggatacgacataagg-3′ and 5′-cgttaatgcccctaaaaatcc-3′. The miRNAs whose preliminary real-time RT-PCR result was concordant with that of the microarray were selected for two more repeated PCR reactions for data verification.

Target Prediction

Three online softwares, that is, picTar, miRanda 3.0 and targetscan 3.1, were used for target prediction for miRNAs. HSCs-overexpressed hsa-miR-520h and underexpressed hsa-miR-129 were selected for target verification. Two potential targets for hsa-miR-129, EIF2C3 and CAMTA1, and two for hsa-miR-520h, ABCG2 and SMAD6, were chosen for verification.

Transient Transfection and Reporter Gene Assay

To verify a potential target, we used Ambion's pMIR-REPORT™ miRNA Expression Reporter Vector System [Greco and Rameshwar, 2007]. For analysis of predicted miRNA binding sites, we synthesized 50–60 mer DNA oligonucleotides consisting of the test sequence flanked by single-stranded overhangs encoding *Spe*I and *Hind*III sites in the MCS. The synthesized double-stranded DNA fragments were as follows. EIF2C3: 5′-AGCTTCCTCAAGTTGCTTGGCAGCACAACTATCTTTGCAAAAAAAA-3′ and DNA 5′-CTAGTTTTTTTTGCAAAGATAGTTGTGCTGCCAAGCAACTTGAGGA-3′; CAMTA1: 5′-AGCTTAACTGTGAATCCTAGGCGAGGCAAAAATGCACTTA-3′ and 5′-CTAGTAAGTGCATTTTTGCCTCGCCTAGGATTCACAGTTA-3′; ABCG2: 5′-AGCTTTTATCCTCACATAAAAAAGAAGCACTTTGATTGAA-3′ and

5′-CTAGTTCAATCAAAGTGCTTCTTTTTTATGTGAGGATAAA-3′; SMAD6: 5′-AGCTTATGGACAAAACAAGAAAGACGCACTTTGGCTTATA-3′ and 5′-CTAGTATAAGCCAAAGTGCGTCTTTCTTGTTTTGTCCATA-3′. All constructs were sequenced and the sequences in all the constructs were confirmed to be 100% correct. Transfection was performed using siPORT^{TM} XP-I kit (Ambion). For the test group, transfections of HeLa cells were performed with 200 ng recombinant construct and 50 nM Pre-miRNAs (Ambion). For control group 1 (blank control), transfections of HeLa cells were performed with 200 ng recombinant construct but no Pre-miRNAs (Ambion). For control group 2 (negative control), transfections of HeLa cells were performed with 200 ng recombinant construct and pre-miR-negative, miRNAs without homology to human genome. For control group 3 (anti-control), transfections of HeLa cells were performed with 200 ng recombinant construct and 50 nM anti-miRNAs (Ambion). For all groups, cells were also cotransfected with 200 ng pMIR-REPORT β-gal (pMIR-β-gal) for transfection efficiency. Seventy-two hours after transfection, cell-free lysates were prepared. Lysates were analyzed for luciferase and β-gal activity by using the Luciferase Assay System and β-Galactosidase assay kit (Promega), respectively. All transfection data were expressed as luciferase activity normalized by β-galactosidase activity. Experiments were performed in three replicates and were repeated at least twice. Data represent mean ± SEM.

Function Analysis for hsa-miR-520h

We selected hsa-miR-520h (GenBank accession no. NT_011109) for function analysis. FACS isolated $CD34^+$ hematopoietic cells were seeded in X-Vivo 15^{TM} medium at a concentration of 1×10^4 cells/ml. Cell amplification was performed by adding cytokines in X-Vivo 15^{TM} as above described. Transfections of $CD34^+$ hematopoietic cells were performed by using Ambion's siPORT NeoFX [Boughan et al., 2006]. At 24 h after transfection, 10^4 cells were collected from the well for CFC assays, and colony classification and counting was performed 12 days later. The rest of the cells in the well were collected for FACS analysis at 72 h after transfection. Experiments were performed in six replicates and were repeated at least twice. To make sure that the observed effects—if any—are specific to hsa-miR-520h, the control groups were set as follows: in control group 1, $CD34^+$ hematopoietic cells were treated in the same way as the test group but without any miRNA (mock). In control group 2, $CD34^+$ hematopoietic cells were transfected with pre-miR-129, a miRNA found to be underexpression in $CD34^+CD38^-$ cells compared to $CD34^+$ cells. In control group 3, $CD34^+$ hematopoietic cells were transfected with an anti-miR-520h oligonucleotide.

RESULTS

CFU From Isolated Hematopoietic Stem Cells

At the 12th day after incubation, both the cells in the test group and the control group generated four types of colonies, suggesting their ability to develop different progenitor cells (Fig. 1). The number of $CD34^+$ cells-formed colonies, CFU-E, BFU-E, CFU-GM, and CFU-GEMM were 29.17 ± 4.07, 38.50 ± 4.68, 58.67 ± 4.32, and 1.67 ± 0.52, respectively, indicating a total number of colonies of 148.00 ± 12.33. While the number of $CD34^+CD38^-$ HSCs-formed colonies, CFU-E, BFU-E, CFU-GM, and CFU-GEMM were 39.17 ± 7.55, 48.17 ± 4.62, 70.33 ± 7.23, and 2.17 ± 0.41, respectively, indicating a total number of colonies of 179.83 ± 15.72. The number difference between $CD34^+$ cells-formed CFU-E, BFU-E, CFU-GM and total colonies and their respective counterparts formed by $CD34^+CD38^-$ HSCs were statistically significant ($P < 0.05$). Compared with the $CD34^+$ cells, $CD34^+CD38^-$ HSCs showed greater colony-forming ability (Table I).

Data From the Arrays

For each of the cell types, we show one array of the two hybridizations (Fig. 2). The internal control U6 snRNA spots on all microarrays showed consistent signal strength and the signal of all the detected spots in the replicate microarrays demonstrated a high correlation efficiency ($R = 0.9616 \pm 0.0244$), indicating the repetitiveness and reproducibility of the microarrays. Our miRNAs expression profile of $CD34^+$ hematopoietic cells was in high concordance with the literature [Georgantas et al., 2007]. The differentially expressed miRNAs between the two cell types fall into two groups. (1) Nine miRNAs that have a expression level four times higher in HSCs than in $CD34^+$ cells, including hsa-miR-365, hsa-miR-526b*, hsa-miR-520h, hsa-miR-452, hsa-miR-127,

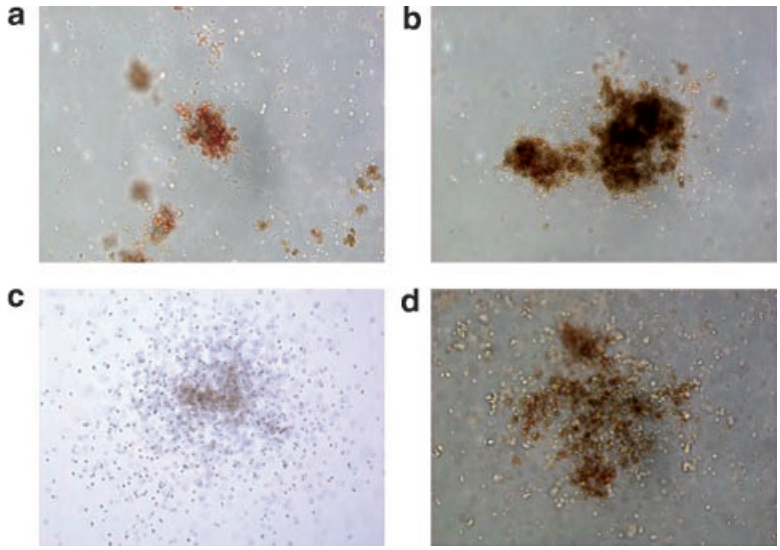

Fig. 1. CFC assay of $CD34^+$ and the $CD34^+CD38^-$ hematopoietic cells. observation under inverted microscope (×100) showed that both the $CD34^+$ hematopoietic cells and the $CD34^+CD38^-$ HSCs generated the following colonies: CFU-E (**a**), BFU-E (**b**), CFU-GM (**c**), and CFU-GEMM (**d**).

PREDICTED_MIR105, PREDICTED_MIR149, PREDICTED_MIR100 and PREDICTED_MIR209 (Table II). These miRNAs may have functions specific to HSCs. (2) Twenty-two miRNAs that have a expression level four times lower in HSCs than in $CD34^+$ cells, including hsa-miR-19a, hsa-miR-142-3p, hsa-miR-15a, hsa-miR-19b, hsa-miR-16, hsa-miR-27a, hsa-miR-20a, hsa-miR-142-5p, hsa-miR-21, hsa-miR-27b, hsa-miR-18a, hsa-miR-106b, hsa-miR-144, hsa-miR-451, hsa-miR-20b, hsa-miR-181a, hsa-miR-106a, hsa-miR-101, hsa-miR-129, hsa-miR-130a, hsa-miR-17-5p, and hsa-miR-93 (Table III).

Real-Time RT-PCR of miRNAs

In this case, we selected 5 HSCs-highly-expressed miRNAs, that is, hsa-miR-520h, hsa-miR-452, hsa-miR-127, hsa-miR-526b* and hsa-miR-365 and 1 lowly expressed miRNA, that is, hsa-miR-129, for verification. And our results demonstrated that 3 of 6 real-time RT-PCR data were concordant with the microarray data, that is, hsa-miR-520h, hsa-miR-129 and hsa-miR-526b* (Fig. 3 and Table IV). We showed amplification curves in PCR reactions of hsa-miR-520h (Fig. 4a), which was 5.6 times higher in $CD34^+CD38^-$ HSCs, hsa-miR-526b* (Fig. 4b), which was 2.3 times higher in $CD34^+CD38^-$ HSCs, and hsa-miR-129 (Fig. 4c), which was 6.3 times lower in $CD34^+CD38^-$ HSCs, here.

Potential Targets for hsa-miR-520h, hsa-miR-129 and hsa-miR-526b*

By using online softwares, we predicted potential targets for hsa-miR-129, hsa-miR-526b* and hsa-miR-520h. Our results demonstrated that (1) 33 potential targets of hsa-miR-129, including EIF2C3, CAMTA1, SH3KBP1, TGIF2, ING3, and DLGAP2, exist reservedly in human, mice, dogs, chimps, and chickens. And the potential targets include genes that are transcription factors, or related to miRNA processing and signal transduction. (2) hsa-miR-526b* only exists in human, with 771 potential targets, including ABCG2, EIF2C3, CAMK4, and CSF2RA, genes involved in signal transduction, transcription regulation, development of hematopoiesis, apoptosis regulation,

TABLE I. Colony-Forming Ability of $CD34^+$ Cells and $CD34^+CD38^-$ HSCs ($\overline{X} \pm S$, n = 6)

Groups	CFU-E	BFU-E	CFU-GM	CFU-GEMM	CFC-total
$CD34^+$ cells	29.17 ± 4.07	38.50 ± 4.68	58.67 ± 4.32	1.67 ± 0.52	148.00 ± 12.33
$CD34^+CD38^-$ HSCs	$39.17 \pm 7.55^*$	$48.17 \pm 4.62^*$	$70.33 \pm 7.23^*$	2.17 ± 0.41	$179.83 \pm 15.72^*$

Compared with the control, $P < 0.05$.

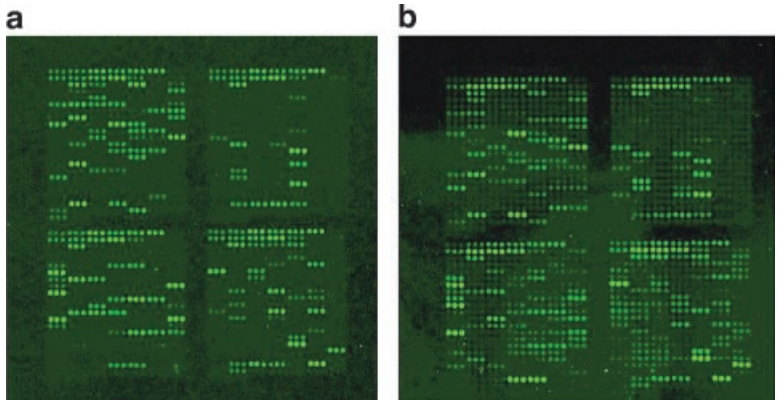

Fig. 2. Microarrays of $CD34^+$ hematopoietic cells and HSCs. The arrays demonstrated miRNA expression profiles of $CD34^+$ hematopoietic cells (**a**) and HSCs (**b**). Nine miRNAs have an expression level four times higher in HSCs than in $CD34^+$ cells. Twenty-two miRNAs that have an expression level four times lower in HSCs than in $CD34^+$ cells.

maintaining of stem cell function and miRNAs maturation. (3) hsa-miR-520h have 30 potential target genes reserved between human, mice and troglodyte. Among them are ABCG2, ID 1 (inhibitor of DNA binding 1), SRP19 and SMAD6, which are regulatory proteins related to signal transduction, transcription and maintaining of stem cell function [Krishnamurthy et al., 2004; de Paiva et al., 2005; Jurga et al., 2006]. Table V shows some of the potential targets.

Verified Targets for hsa-miR-520h and hsa-miR-129

For target verification of hsa-miR-129, as shown in Figure 5a, cotransfection of pre-miR-129 and pMIR-Luc-EIF2C3 plasmid decreased 41% of the luciferase activity in comparison to the blank control. Cotransfection of pre-miR-129 and pMIR-Luc-CAMTA1 plasmid decreased 47% of the luciferase activity. There was no significant difference of luciferase activity between three control groups. Taken together, these data indicate that both EIF2C3 and CAMTA1 are real targets for hsa-miR-129.

For target verification of hsa-miR-520h, as shown in Figure 5b, cotransfection of pre-miR-520h and pMIR-Luc-ABCG2 plasmid decreased 68% of the luciferase activity in comparison to the blank control. Cotransfection of pre-miR-129 and pMIR-Luc-SMAD plasmid decreased 27% of the luciferase activity. There was no significant difference of luciferase activity

TABLE II. miRNAs Overexpressed in $CD34^+CD38^-$ HSCs

miRNA name	Sequence (5′–3′)	Fold activation	Possible targets	Function
hsa-miR-452	uguuugcagaggaaacugagac	4.388826771	SREBF1	Transcription factor
			TAF1A	Transcription factor
			SRP54	Signal transduction
			TGFB3	Growth factor
hsa-miR-127	ucggauccgucugagcuuggcu	5.116001265	SSRP1	Signal transduction
			VSX1	Transcription factor
			MAPK4	Signal transduction
			POLR3A	RNA polymerase
hsa-miR-526b*	aaagugcuuccuuuuagaggc	7.1035433	ABCG2	Stemness maintaining
			EIF2C3	miRNA processing
			CAMK4	Signal transduction
			DDX1	apoptosis
hsa-miR-365	uaaugccccuaaaaauccuuau	9.339176013	NFE2_HUMAN	Transcription factor
			TBP	Transcription factor
			RASD1	Signal transduction
			POLR2A	RNA polymerase
hsa-miR-520h	acaaagugcuucccuuuagagu	46.49776722	Omitted	Omitted
PREDICTED_MIR209	uugugcuuugucuuuagaauua	4.205603093	/	
PREDICTED_MIR105	uagcccuuuaaugccggcagcu	6.75581198	/	
PREDICTED_MIR149	accugagagguaaauauugacc	6.796110884	/	
PREDICTED_MIR100	ucaguagcccugaaaauuucuc	14.56730945	/	

TABLE III. miRNAs Underexpressed in $CD34^{+}CD38^{-}$ HSCs

miRNA name	Sequence (5′–3′)	Fold reduction	Possible targets	Function
hsa-miR-129	cuuuuugcggucugggcuugc	0.086759858	Omitted	Omitted
hsa-miR-19a	ugugcaaaucuaugcaaaacuga	0.086854141	ATF7IP2	Transcription factor
			PPIL5	Signal transduction
			SOCS1	Signal transduction
			LIG4	DNA ligase
hsa-miR-142-3p	uguaguguuuccuacuuuaugga	0.092762397	BTF3	Transcription factor
			TCF12	Transcription factor
			POLI	DNA polymerase
			SRP19	Signal transduction
hsa-miR-15a	uagcagcacauaaugguuugug	0.103328677	DMRT1	Transcription factor
			TAF13	Transcription factor
			SRPR_HUMAN	Signal transduction
			POLR3D	RNA polymerase
hsa-miR-19b	ugugcaaauccaugcaaaacuga	0.111448325	SOCS1	Signal transduction
			TCF7L2	Transcription factor
			LIG4	DNA ligase
			IGF2R	Growth factor
hsa-miR-16	uagcagcacguaaauauuggcg	0.11857867	SALL1	Transcription factor
			TBP	Transcription factor
			SI1L2_HUMAN	Signal transduction
			NP_006324.1	DNA-binding protein
hsa-miR-27a	uucacaguggcuaaguuccgc	0.126781206	NR2F2	Transcription factor
			TBP	Transcription factor
			GATA2	Transcription factor
			SRP19	Signal transduction
hsa-miR-20a	uaaagugcuuauagugcagguag	0.15583554	VSX1	Transcription factor
			CDK7	Cell division
			E2F5	Transcription factor
			RGS10	Signal transduction
hsa-miR-142-5p	cauaaaguagaaagcacuac	0.163181298	RAD17	Cell cycle
			SOX30	Transcription factor
			SOCS1	Signal transduction
			LEO1	RNA polymerase
hsa-miR-21	uagcuuaucagacugauguuga	0.169529159	PDCD4	Apoptosis
			VSX1	Transcription factor
			NFIB	Nuclear factor
			PELI1	Signal transduction
hsa-miR-27b	uucacaguggcuaaguucugc	0.17434891	NR2F2	Transcription factor
			TBP	Transcription factor
			RGS17	Signal transduction
			SRP19	Signal transduction
hsa-miR-18a	uaaggugcaucuagugcagaua	0.179134917	VSX1	Transcription factor
			CDK7	Cell division
			CDK2	Cell division
			TNFAIP3	Tumor necrosis factor
hsa-miR-106b	uaaagugcugacagugcagau	0.184527923	E2F5	Transcription factor
			SNAPC4	Transcription factor
			POLQ	DNA polymerase
			C19orf2	Transcription regulation
hsa-miR-144	uacaguauagaugauguacuag	0.185278845	PRDM16	Transcription factor
			CDK7	Cell division
			TRAPPC4	Hematopoiesis
			PTF1A	Transcription factor
hsa-miR-451	aaaccguuaccauuacugaguuu	0.186075439	EEF1E1	Translation elongation factor
			MED12	Transcription regulation
			TCF21	Transcription factor
			IL5	Cell differentiation factor
hsa-miR-20b	caaagugcucauagugcagguag	0.19002495	C9orf78	Carcinoma-associated antigen
			POU3F2	Transcription factor
			NFIA	Nuclear factor
			SPIT1_HUMAN	Growth factor activator inhibitor
hsa-miR-181a	aacauucaacgcugucggugagu	0.192280992	TAF13	Transcription factor
			DIDO1	Transcription factor
			IL5	Cell differentiation factor
			ASCC1	Signal transduction
hsa-miR-106a	aaaagugcuuacagugcagguagc	0.196053622	E2F5	Transcription factor
			OPTN	Transcription factor
			EEF1E1	Translation elongation factor
			ING3	Growth Inhibitor
hsa-miR-101	uacaguacugugauaacugaag	0.196397708	SMARCA4	Transcription factor
			FOS	Proto-oncogene
			RGS1	Signal transduction
			FGFR3	Growth *factor*

(*Continued*)

TABLE III. (*Continued*)

miRNA name	Sequence (5′–3′)	Fold reduction	Possible targets	Function
hsa-miR-130a	cagugcaauguuaaaagggcau	0.204031916	BEX3_HUMAN	Cell death executor
			YY1	Transcriptional repressor
			MET	Growth factor receptor
			TAF9	Transcription initiation factor
hsa-miR-17-5p	caaagugcuuacagugcagguagu	0.218848197	VSX1	Transcription factor
			E2F5	Transcription factor
			NFX1	Nuclear transcription factor
			GABPAP	Transcription factor
hsa-miR-93	aaagugcuguucgugcagguag	0.226646473	E2F5	Transcription factor
			PDCD4	Apoptosis
			CHAF1A	Chromatin assembly factor
			NFX1	Nuclear transcription factor

between three control groups. Taken together, these data indicate that ABCG2 is real target for hsa-miR-520h, and hsa-miR-520h is relatively weak at inhibiting SMAD6.

The Introduction of hsa-miR-520h Results in an Increase of Colonies in CFC Assays

In the control groups, the numbers of CFU-E, BFU-E, CFU-GM, and CFU-GEMM derived from 10^3 $CD34^+$ cells were 28.50 ± 4.32, 37.83 ± 5.60, 57.67 ± 5.16, and 1.50 ± 0.55, respectively. In the test group, the numbers of CFU-E, BFU-E, CFU-GM, and CFU-GEMM derived from 10^3 Pre-miR-520h-transfected $CD34^+$ cells were 37.83 ± 7.70, 46.5 ± 4.85, 68.17 ± 7.73, and 1.67 ± 0.52, respectively ($n = 6$). The difference of the numbers of CFU-E, BFU-E, CFU-GM between the test group and the control groups was statistically significant ($P < 0.05$). Whereas the difference of the numbers of CFU-GEMM between the test group and the control groups was not statistically significant ($P < 0.05$; Table VI).

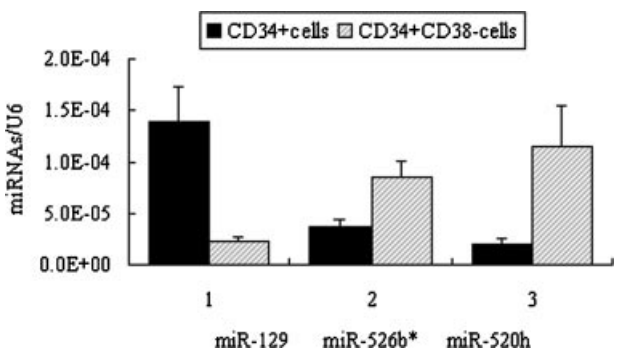

Fig. 3. The histogram of real-time RT-PCR results. The real-time RT-PCR data of has-miR-520h, hsa-miR-129 and hsa-miR-526b* were concordant with the microarray data. hsa-miR-129 expression level in $CD34^+$ cells was 6.3 times higher than in HSCs (1). has-miR-526b* expression level in HSCs was 2.3 times higher than in $CD34^+$ cells (2). has-miR-520h expression in HSCs was 5.6 times higher than in $CD34^+$ cells (3).

The result of FACS analysis at 72 h after Pre-miR-520h transfection showed that the proportion of $CD34^+$ cells and that of $CD34^+CD38^-$ HSCs were similar between the three control groups (Table VII). Among them, the proportion of $CD34^+$ cells and that of $CD34^+CD38^-$ HSCs in the blank control group are $22.91\% \pm 2.03$ and $12.57\% \pm 0.81$, respectively. However, the proportion of $CD34^+$ cells and that of $CD34^+CD38^-$ HSCs in the test group are $30.43\% \pm 2.34$ and $13.08\% \pm 1.08$, respectively. Analysis of variance revealed that, compared with the control groups, the proportion of $CD34^+$ cells in the test group was significantly higher ($P < 0.05$, $n = 6$), while that of $CD34^+CD38^-$ HSCs showed no significant difference ($P < 0.05$, $n = 6$). These data indicate that hsa-miR-520h plays a role in HSCs' maintaining $CD34^+$ progenitor phenotype without increasing the proportion of HSCs.

Discussion and Conclusion

In this study, we isolated $CD34^+CD38^-$ HSCs from human umbilical cord blood by FACS and confirmed that they display stem cell properties by CFC assays, in keeping with similar findings obtained with HSCs [Ponti et al., 2005]. We also isolated $CD34^+$ hematopoietic cells as the control to research on miRNA expression profile of $CD34^+CD38^-$ HSCs. $CD34^+$ subpopulation is a mixed population consisting in the great majority, almost 90%, of differentiated, committed hematopoietic progenitor cells (HPCs). We compared miRNA expression profiles of

TABLE IV. miRNAs in $CD34^+CD38^-$ Cells and $CD34^+$ Cells Verified by Q-RT-PCR

Name	E	CT (con)	CT (sam)	ΔCT	con/U6 RQ (10^{-4})	sam/U6 RQ (10^{-4})	sam/con RQ
U6	1.821 ± 0.042	16.047 ± 0.425	14.613 ± 0.375	1.433 ± 0.058	/	/	2.360 ± 0.036
miR-129	1.924 ± 0.013	28.297 ± 0.307	29.783 ± 0.329	-1.487 ± 0.0.025	1.392 ± 0.341	0.222 ± 0.050	0.160 ± 0.005
miR-526b*	1.855 ± 0.042	32.113 ± 0.822	29.390 ± 0.771	2.723 ± 0.085	0.372 ± 0.068	0.848 ± 0.160	2.276 ± 0.058
miR-520h	1.909 ± 0.022	31.610 ± 0.332	27.633 ± 0.532	3.977 ± 0.214	0.203 ± 0.051	1.152 ± 0.392	5.596 ± 0.861

Con (control): $CD34^+$ cells; sam (sample): $CD34^+CD38^-$ cells; RQ: relative expression rates.

these two hematopoietic cell subpopulations using microarrays, with the purpose of determining the miRNAs that are differentially expressed between the HSCs and $CD34^+$ subpopulation.

Recent study has shown miRNA expression among human hematopoietic cell lines as well as in human hematopoietic diseases [Ramkissoon et al., 2006]. However, there are no reports about expression profiles of miRNAs of human HSCs. In this case, we first investigated their expression profile in $CD34^+CD38^-$ HSCs subpopulation. By FACS each of two populations, $CD34^+CD38^-$ cells and $CD34^+$ cells, was extremely purified (98–99%). And we identified the miRNAs over- or underexpressed in HSCs. Our data support the use of the miRNA microarray for detection of patterns of miRNA expression, with the obvious advantage that the expression of several hundred genes can be identified in the same sample at once, and with relatively small amounts of total RNA.

hsa-miR-520h, hsa-miR-526b* and 7 other miRNAs were high in $CD34^+CD38^-$ HSCs,

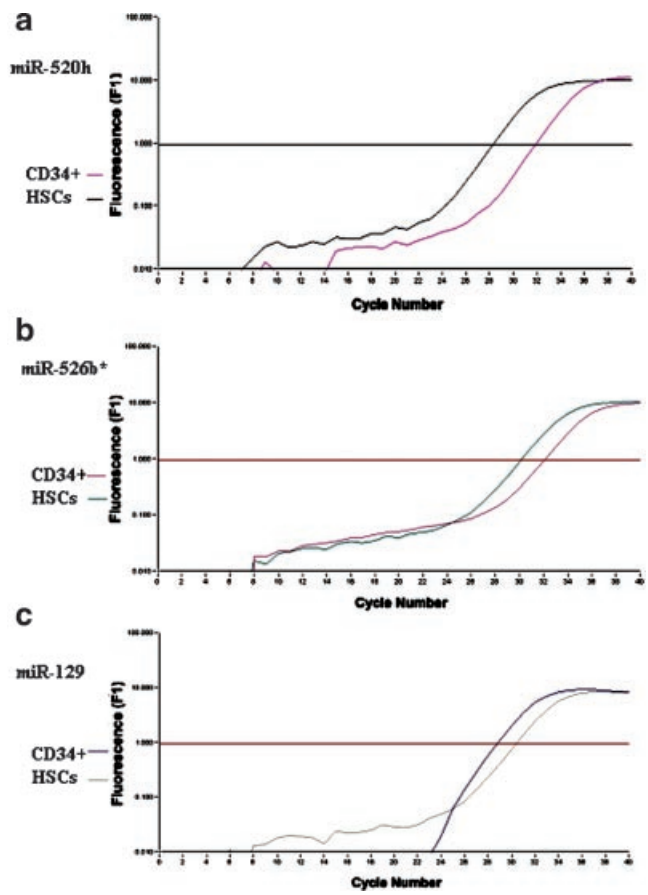

Fig. 4. Schemes of amplification curves in real-time RT-PCR. hsa-miR-520h and hsa-miR-526b* expression in HSCs was significantly higher than that in $CD34^+$ cells (**a**), (**b**). hsa-miR-129 expression in HSCs was significantly lower than that in $CD34^+$ cells (**c**).

TABLE V. Potential Targets for miR-129 and miR-520h

miRNAs	Softwares	Predicted target gene		
		Full name	Abbr.	Function
miR-129	PicTar	Homo sapiens eukaryotic translation initiation factor 2C, 3	EIF2C3	miRNA processing
		Homo sapiens calmodulin binding transcription activator 1	CAMTA1	Transcription factor
		Homo sapiens SH3-domain kinase binding protein 1	SH3KBP1	Signal transduction
		Homo sapiens TGFB-induced factor 2	TGIF2	Growth factor
miR-520h	miRanda 3.0	ATP-binding cassette, subfamily G	ABCG2	Stemness maintaining
	Targetscan 3.1	SMAD, mothers against DPP homolog 6	SMAD6	Transcription factor
		Inhibitor of DNA binding 1	ID 1	Signal transduction
		Signal recognition particle 19 kDa	SRP19	Signal transduction

but weak in $CD34^+$ cells. 22 miRNAs including hsa-miR-129 were low in $CD34^+CD38^-$ HSCs, but high in $CD34^+$ cells. Six of the 31 differentially expressed miRNAs were selected for real-time RT-PCR verification. The following three have a result of real-time RT-PCR concordant with that of microarray: hsa-miR-520h, hsa-miR-129, and hsa-miR-526b*. We focused on HSC enriched miRNAs, which may play a role specific to HSCs development. Of the two real-time RT-PCR verified HSC-enriched miRNAs, hsa-miR-526b*, 2.3 times higher in $CD34^+CD38^-$ HSCs, and hsa-miR-520h, 5.6 times higher in $CD34^+CD38^-$ HSCs, the latter was chosen for function characterization because of its much higher expression in HSCs than in the control groups. Moreover, according to target prediction and verification, ABCG2, a gene involved in stemness maintaining, is a real target of hsa-miR-520h. Probably, hsa-miR-520h may play roles in HSC development by inhibiting ABCG2 expression.

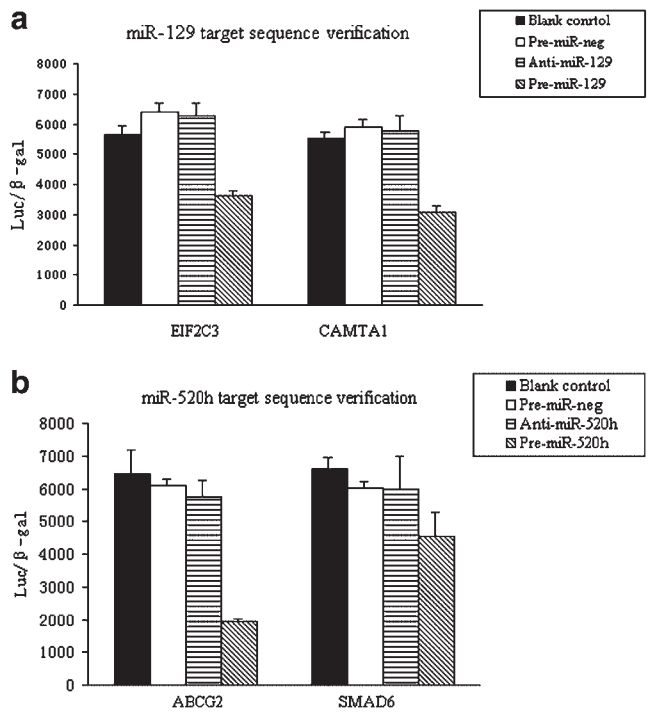

Fig. 5. Verification of the potential targets of miR-129 (**a**) and miR-520h (**b**). Cotransfection of pre-miR-129 and pMIR-Luc-EIF2C3 plasmid decreased 41% of the luciferase activity in comparison to the blank control. Cotransfection of pre-miR-129 and pMIR-Luc-CAMTA1 plasmid decreased 47% of the luciferase activity. There was no significant difference of luciferase activity between three control groups (a). cotransfection of pre-miR-520h and pMIR-Luc-ABCG2 plasmid decreased 68% of the luciferase activity in comparison to the blank control. Cotransfection of pre-miR-129 and pMIR-Luc-SMAD plasmid decreased 27% of the luciferase activity. There was no significant difference of luciferase activity between three control groups (b).

TABLE VI. Colony-Forming Ability of $CD34^+$ Cells After miR-520h Transfection ($\overline{X} \pm S$, n = 6)

Groups	CFU-E	BFU-E	CFU-GM	CFU-GEMM	CFC-total
Control 1: no miRNAs	28.50 ± 4.32	37.83 ± 5.6	57.67 ± 5.16	1.50 ± 0.55	145.50 ± 14.35
Control 2: miR-129	26.50 ± 4.04	36.17 ± 4.58	56.50 ± 4.14	1.33 ± 0.52	140.50 ± 12.11
Control 3: anti-miR-520h	24.50 ± 4.72	33.17 ± 4.36	55.00 ± 5.44	1.17 ± 0.41	133.83 ± 13.49
Test group: pre-miR-520h	37.83 ± 7.7	46.50 ± 4.85	68.17 ± 7.73	1.67 ± 0.52	174.33 ± 17.82
F-value	7.15429	8.295827473	6.4663662	1.11111111	8.969048643
P-value [$F0.05\ (3,20) = 3.10$]	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$

Several algorithms for target prediction have been based on sequence similarity between targets and miRNAs [Bentwich, 2005]. Potent prediction softwares help to make target prediction rational and convenient. Prediction of potential target genes of miRNAs by bioinformatics can further provide guidance for analysis of miRNA binding sequence on particular targets and research on the accurate regulatory mechanism of miRNAs. Bioinformatic analysis and target verification showed that ABCG2, crucial for stemness maintaining, is a real target gene of hsa-miR-520h. $CD34^-$ HSCs show highest ABCG2 level. With the expression of CD34 on HSCs surface, ABCG2 is down-regulated and remains low in HPCs. During the development of $CD34^-$ earlier HSCs into $CD34^+CD38^-$ HSCs, ABCG2 is decreasing, consistent with the increase of hsa-miR-520h. Most likely, hsa-miR-520h may contribute to $CD34^+CD38^-$ HSCs differentiation into progenitor cells by inhibiting ABCG2 expression. However, due to temporal expression of miRNAs, their expression may decrease right after fulfilling their functions. In this case, in spite of the fact that ABCG2 expression keeps low in $CD34^+$ progenitor cells, hsa-miR-520h does not remain high.

To further study the role of hsa-miR-520h in HSCs and begin the process of identifying potential targets, we transfected hsa-miR-520h into FACS-isolated $CD34^+$ hematopoietic cells and observed the alteration in subsequent CFC assays. Note that the introduction of hsa-miR-520h resulted in an increase of number of different progenitor colonies in CFC assays but no significant increase of that of CFU-GEMM colonies. Also, FACS analysis at 72 h after transfection showed an remarkable increase of proportion of $CD34^+$ cells and little change of proportion of HSCs. Taken together, these data indicate that hsa-miR-520h plays a role in promoting differentiation of HSCs into progenitor cells. And the possible mechanism may be negative regulation of ABCG2 expression by hsa-miR-520h.

In contrast to hsa-miR-520h, hsa-miR-129 was low in $CD34^+CD38^-$ cells, but high in $CD34^+$ hematopoietic cells. Using PicTar, another software for target prediction, we found several notable potential targets. EIF2C3 (Homo sapiens eukaryotic translation initiation factor 2C, 3) is one. As a member of Argonaute family, EIF2C3 is thought to be involved in the development and maintenance of stem cells through the RNA-mediated gene-quelling mechanisms associated with DICER [Sasaki et al., 2003]. And DICER is implicated in maturation of miRNAs. Combined with miRNA's role of translation repression, the data may indicate a possible network consisting of hsa-miR-129, EIF2C3 and HSCs, which may play a role in stem cell biology. CAMTA1 (Homo sapiens calmodulin binding transcription activator 1) is another potential target gene of hsa-miR-129. CAMTA1 may participate in

TABLE VII. FACS Analysis at 72 h After miR-520h Transfection Into $CD34^+$ cells ($\overline{X} \pm S$, n = 6)

Groups	$CD34^+$ proportion	$CD34^+CD38^-$ proportion
Control 1: no miRNAs	22.91 ± 2.03	12.57 ± 0.81
Control 2: miR-129	23.95 ± 2.11	12.61 ± 0.82
Control 3: anti-miR-520h	24.37 ± 1.93	12.53 ± 0.79
Test group: pre-miR-520h	30.43 ± 2.34	13.08 ± 1.08
F-value	15.5685295	0.497210317
P-value [$F0.05\ (3,20) = 3.10$]	$P < 0.05$	$P < 0.05$

induction of cell differentiation and cell cycle regulation [Nakatani et al., 2004]. hsa-miR-129 may affect cell differentiation and cell cycle by inhibiting CAMTA1 translation.

Predication of potential targets for hsa-miR-129, hsa-miR-520h, and hsa-miR-526b* by using softwares indicate the overlapping of targets between different miRNAs. For example, ABCG2 is the potential target for both hsa-miR-520h and hsa-miR-526b*. EIF2C3 mRNA has binding sequences for both hsa-miR-129, and hsa-miR-526b*. Probably, $CD34^{+}CD38^{-}$ HSCs-overexpressed hsa-miR-520h and hsa-miR-526b* and underexpressed hsa-miR-129 may regulate common target genes and form a miRNAs gene network to regulate HSCs development by cooperating or rivaling with each other.

As for the predicted miRNAs, whose existence is yet to be proved, no accurate miRNA sequence can be used for synthesis of accurate primers, which makes real-time RT-PCR verification unavailable. Their target analysis is also unpractical.

In this study, we identified a subset of HSCs-enriched miRNAs, which may contribute to the development of HSCs. hsa-miRNA-520h could be a representative for the group. Our results may provide theoretical support and experimental basis for further research on regulating HSCs differentiation and miRNA-targeting therapy of HSCs damage-related diseases. Identification of miRNAs and gene networks specifying either stemness or commitment will not only be of major relevance for a fundamental understanding of developmental biology, but also for the emerging fields of tissue engineering and regenerative medicine [Haan et al., 2006]. The combined application of microRNA expression profiling and other approaches to study HSCs-specific genes is hopefully to uncover human hematopoietic mechanism as well as shed new light on regenerative medicine for human hematological disease.

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