

MicroRNA Modulation during the *In vitro* Culture of Hematopoietic Stem Cells Prior to Transplantation

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What's Known

- HUCB contains various stem progenitor cells with the ability to differentiate into both hematopoietic and non-hematopoietic cells.
- Transplantation of cord blood cells may offer an attractive route for cell therapy.
- miRNAs play a key role in hematopoiesis and new evidence on specific miRNA expression in this process is available.

What's New

- The precise function of miRNAs in hematopoiesis is relatively unknown and many of the individual targets of miRNA within the mentioned mechanism have not yet been identified.
- Despite evidence implicating miRNAs in miscellaneous physiological mechanisms, alterations of expression profiles *in vitro* have not been well characterized.
- Assessment of miRNA profiles is helpful for the recognition of changes that may cause unfavorable functions.

Abstract

Background: Human umbilical cord blood (HUCB) is an acceptable and readily accessible source of stem cells. There is an ongoing interest in cord blood stem cell therapies; however, little is known about the possible unfavorable effects of laboratory modifications on the isolated HUCB cells. The involvement of miRNAs in several biological processes has been shown. The aim of this study was to evaluate the possible changes in miRNA expression profiles in CD133⁺ hematopoietic cells after *in vitro* culture.

Methods: HUCBCD133⁺ hematopoietic stem cells were isolated by magnetic-activated cell sorting, and then the cells were counted using flow cytometry. The cells were divided into 2 groups. In the first group, RNA was extracted and the cells of the second group were cultured *in vitro* for 12 days and then these cells were used to assay miRNAs expression using real-time qPCR.

Results: The results showed that the expression of 349 out of 1,151 screened miRNAs was upregulated following a 12-day *in vitro* culture of CD133⁺ cells, whereas the expression of 293 miRNAs was downregulated. In addition, the expression of 509 miRNAs was not significantly altered. Another *in-silico* analysis involving the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways related to the selected miRNAs was also conducted.

Conclusion: Based on our results, the *in vitro* expansion of HUCB resulted in altered expression levels of miRNAs. This study provides information on the effects of 2-dimensional culture of hematopoietic cells prior to transplantation for more successful transplantation.

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Keywords • Hematopoietic stem cells • Umbilical cord blood • MicroRNAs • CD133 • Signal transduction

Introduction

Molecular and genetic studies carried out in the past 2 decades have shown different regulatory processes of hematopoiesis.¹⁻³ MicroRNAs (miRNAs) are small endogenous noncoding RNAs containing 20–23 nucleotides that operate post-transcriptionally and have emerged as a new mode of gene regulation. They are encoded at different genomic regions and bind to the 3'untranslated part of their target genes via base pairing for tuning numerous pathways that relate to the development of diseases or function as master switches that turn genes on and off.⁴ It is predicted by bioinformatic analysis that about 5% of transcriptome is for miRNAs and that the translation of more than one-third of human messenger RNAs (mRNAs) is regulated by these miRNAs.^{5,6} The epigenetic effects of miRNAs during multiple biological functions such as

differentiation and development have long been studied.⁷⁻⁹ miRNAs are usually expressed in a tissue-specific manner and sometimes during certain developmental stages.¹⁰ Generally, one miRNA can target several mRNAs, often in combination with other miRNAs. This indicates that miRNAs function in highly complex regulatory networks. Although the functions and target genes of most miRNAs are still unknown, they have been implicated in many diverse processes, including differentiation and organogenesis, developmental timing, growth control, apoptosis, patterning and embryogenesis, viral infections, and cancer.^{7,11} For example, mir-140 has been expressed more in the development of mouse cartilage,¹² miR-145 has been reported to regulate adipocyte differentiation,¹³ miR-133 regulates skeletal differentiation,¹⁴ miR-206 regulates muscle differentiation,¹⁵ miR-1 regulates cardiac morphogenesis and the cardiac cell cycle,¹⁶ and miR-155 is associated with immune system development,¹⁷ while mir-221 has been identified as a regulator in osteogenic differentiation.⁹ Despite evidence implicating miRNAs in miscellaneous physiological mechanisms, the alterations of expression profiles *in vitro* have not been well characterized and are poorly understood. An assessment of miRNA profiles is helpful for the recognition of changes that may cause unfavorable functions. Human umbilical cord blood (HUCB), a byproduct of childbirth, is an acceptable and readily accessible source of stem cells.¹⁸ Human leukocyte antigen (HLA) typing of cord blood for storage has raised hope for patients to access an HLA-matching cell source. Recently, it has been observed that HUCB contains various stem progenitor cells with the ability to differentiate into both hematopoietic and non-hematopoietic cells.¹⁹⁻²³ Thus, the transplantation of cord blood cells may offer an attractive route for cell therapy. However, the current dearth of knowledge regarding the probable alterations during laboratory processes has limited their use in practical applications (i.e., hematological diseases). We sought to study the possible changes in miRNA expression profiles of CD133+ hematopoietic cells *in vitro*. An assessment of miRNA profiles is helpful for the recognition of changes that may cause unfavorable functions.

Materials and Methods

Sample Preparation

With informed consent of the mother, an umbilical cord blood sample of a normal full-term delivery was collected in blood bags containing sodium citrate and then diluted with hydroxyethyl starch in a ratio of 1:4 in order

to deplete red blood cells. Mononuclear cells (MNCs) were separated from the diluted sample using Ficoll-Paque (Inno-Train) density gradient centrifugation for 30 minutes at 400×g at 20°C. The MNC interface layer was collected and washed twice with PBS/EDTA.

Isolation and Expansion of CD133⁺ Cells

The MNCs were incubated with magnetic CD133 antibody-conjugated microbeads (Miltenyi Biotec) according to the manufacturer's instructions. The adherent CD133⁺ cells were diluted in 1 mL of Hank's Buffered Salt Solution (HBSS); the cells were counted and their purity was assessed using flowcytometry (Sample 1). Then, the cells were cultured in 24-well plates with a Stem-Span culture medium (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with Flt3, TPO, and SCF for 12 days. On day 12, the expanded population was evaluated by flowcytometry again (Sample 3). Subsequently, for the second time, the CD133⁺ cells were isolated from the expanded cells by magnetic microbeads and the purity of the isolated population was determined by flowcytometry (Sample 2).

Flowcytometry Analysis

A total of 2×10^5 cells were divided into aliquots in amber-tinted 5-mL centrifuge tubes, and 3% human serum was added. The cells were incubated for 30 minutes, resuspended in 5 mL of PBS, and pelleted by centrifugation for 10 minutes at 300×g. Thereafter, the cells were resuspended in 100 µL of PBS and stained with PE-conjugated anti-human CD133 (Miltenyi Biotec) at 4°C for 30 minutes. Some cells were stained with MultiMix mouse IgG1 RPE (Dako) and were used as negative controls. The cells were then pelleted, washed twice with PBS, and fixed with 1% paraformaldehyde in PBS. After fixation, flowcytometry analysis was performed on a FACSCalibur cytometer (Becton Dickinson) using CellQuest software. Win MDI 2.8 software was used to create histograms.

RNA Isolation

RNA was isolated using TRIzol (Invitrogen, USA) according to the manufacturer's protocol. The synthesis of cDNA was carried out with an miRNA cDNA synthesis kit G269 (Applied Biological Materials, Richmond, BC, Canada).

Quantitative Evaluations of MicroRNA Expressions

Real-time PCR for miRNA assay was carried out using an Applied Biological Materials miRNA profiling kit (MA003, Richmond, BC, Canada) and the StepOnePlus Real-Time RT-PCR system

(Applied Biosystems, USA). All the reactions were run in triplicate, and the threshold cycle average was used for data analysis. The relative expressions of the miRNAs were normalized using the 4 endogenous miRNA controls on each plate for each sample.

qPCR Data Analysis

The resulting threshold cycle values for all the wells were exported to an Excel Spreadsheet. The miRNA qPCR data were analyzed with the $2^{-\Delta\Delta CT}$ method using the miRNA qPCR Array Data Analysis Excel, provided by the Applied Biological Materials website (<http://www.abmgood.com/>).

miRNA Target Prediction

Putative targets of the candidate miRNAs that showed highly modulated expression after 12 days' culture of the CD133⁺ cells compared to the fresh isolated cells were found using TargetScan (<http://genes.mit.edu/targetscan>) and miRBase (<http://www.mirbase.org>) online software.

Pathway Analysis

In order to indicate the cellular pathways related to the putative targets of the most modulated miRNAs (mir-106a, mir-16, mir-146a, mir-222, mir-223, and mir-125a), we used the online *in-silico* software of DIANA-miRPath (<http://diana.cslab.ece.ntua.gr/pathways/>).

Statistical Analysis

The data are shown as mean \pm SD for the independent measurements. The statistical analyses were performed using the Student *t*-test by SPSS, version 13.0. A *P* value <0.05 was considered significant.

Results

Isolation and Evaluation of Isolated CD133⁺ Cells

The ratio of cell isolation was 1 million CD133⁺ cells per 85 million total nucleotide HUCB

cells. The first isolation of the CD133⁺ cells by microbeads resulted in spherical cells on day one. Flow cytometry analysis of this population (Sample 1) showed 98% purity (figure 1). After 12 days' culture, flow cytometry analysis indicated that 36% of the expanded cells were CD133⁺ (Sample 3). These cells were passed through a microbead column in order to isolate the CD133⁺ cells for the second time (Sample 2): the purity of the isolated cells was 97% (figure 2).

Evaluation of miRNA Expression Profiling

The microRNA expression profiling of the CD133⁺ cells on day 1 and day 12 (Sample 1 and Sample 2, respectively) showed modulation of a broad cohort of miRNAs, which predicted hematopoietic targets. Among 1,151 screened miRNAs in Sample 1 versus Sample 3, 349 miRNAs were upregulated, while the expression of 293 miRNAs was downregulated; the expression of 509 miRNAs was not significantly changed. Some miRNAs were chosen because of their reported roles in hematopoiesis mechanisms (table 1). Their expression levels and also their expression trends after *in vitro* culture are illustrated in table 2. In addition, their chromosomal locations and overlapping transcripts are shown in table 3.

Pathway Analysis

Our investigation of the most modulated miRNAs using available online software indicated that the majority of the putative targets of the mentioned miRNAs were involved in the chronic myeloid leukemia (CML) pathway.

Discussion

miRNAs are small 19–22 nucleotide non-coding, post-transcriptional regulatory RNA molecules which regulate gene expression and different biological processes, including development, proliferation, differentiation, and apoptosis, in various cells.²⁴ A better understanding of

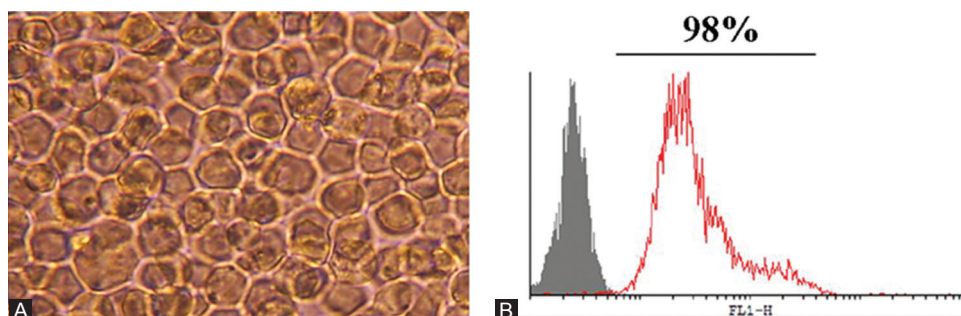


Figure 1: Phase contrast microscopy of the isolated CD133⁺ cells (A) and the flow cytometry analysis of the microbead isolated CD133⁺ cells (Sample 1) compared to the whole population (B) are illustrated. The first isolation of the CD133⁺ cells by microbeads resulted in spherical cells on day one. Flow cytometry analysis of this population showed 98% purity. Colored areas: isotype; white areas: CD133⁺ cells.

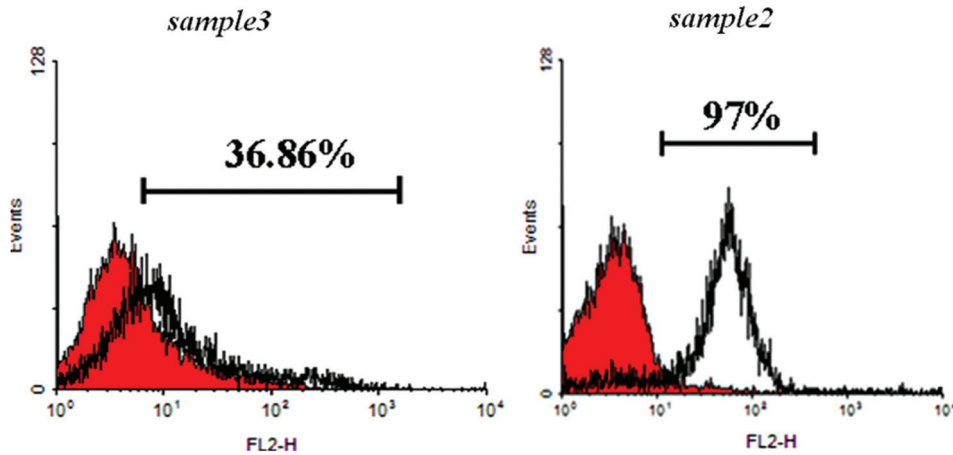


Figure 2: Flowcytometry analysis of the 12-day *in vitro* culture of the sample (Sample 3) and the second microbead isolated CD133⁺ cells (Sample 2) shows 36% and 97% purity, respectively. Colored areas: isotype; white areas: CD133⁺ cells.

Table 1: Selected hematopoietic miRNAs and their related putative targets		
microRNA (hsa)	Putative targets*	Functions
mir-10a	HOXA1	Megakaryocytic differentiation
mir-34a	CDK4, CDK6, MYB	Megakaryocytic differentiation
mir-223	LMO2, NFI-A	Regulation of erythroid and megakaryocytic differentiation
mir-24	activin type I receptor ALK4	Inhibition of erythropoiesis
mir-221, mir-222	KIT	Inhibition of erythropoiesis
mir-155	ETS-1, MEIS1	Inhibition of myelopoiesis and erythropoiesis
mir-16	MYB	Contribution in erythropoiesis
mir-451	GATA1	Contribution in erythropoiesis
mir-125b	BMF, KLF13, Lin28A	Regulation of hematopoiesis
mir-17-5p	BCL2	Promotion of hematopoietic cell expansion
mir-106a	BCL2	Promotion of hematopoietic cell expansion
mir-144	GATA1	Function in erythroid phenotype
mir-424	NFI-A	Regulation of monocyte/macrophage differentiation
mir-181	HOXA5	Inhibitory effect on hematopoietic differentiation
mir-29a	HBP1, FZD5, TPM1	Acquisition of self-renewal capacity by myeloid progenitors
mir-146	PROM1	Proliferation of CD133 ⁺
mir-196a	HOXA9	Related to pediatric acute lymphoblastic leukemia and participation in myeloid differentiation
mir-15	BCL2	Related to chronic lymphocytic leukemia
mir-150	MYB	Inhibition of leukemogenesis

*Selected putative targets are predicted using Target scan and miRBase online websites

miRNA regulatory mechanisms in the pattern of gene expression during hematopoiesis will not only increase our knowledge regarding this phenomenon but also provide relevant information for therapeutic intervention.²⁵ MiRNAs play a key role in hematopoiesis; however, the precise function of miRNAs in hematopoiesis is relatively unknown, and many of the individual targets of miRNAs within the mentioned mechanism have not been identified yet.

Changes in expression patterns such as gene expression alterations after *in vitro* cell culture have been reported in several studies. These changes include cell growth arrest due to an increased expression of transforming growth

factor beta (TGFβ1 and TGFβ2) followed by the inactivation of c-Myc through SMAD3 in human mesenchymal stem cells,²⁶ downregulation of cell-cycle quiescence and stemness-related genes and upregulation of signal transduction, cell adhesion, and cytoskeletal-related genes in human stromal stem cells.²⁷

There is also a significant alteration in gene expression profiles and a reduction in differences between myometrial and fibroid smooth muscle cells after *in vitro* cell culture. Downregulated genes include interleukin receptors IL10RB, IL11RA, and IL17R; TGFβR; platelet-activating factor receptor; tumor necrosis factor receptor superfamily 25; retinoic acid receptor-β; MYST3

Table 2: Expression levels of the selected miRNAs before and after *in vitro* culture

MicroRNA	Expression level in Sample 1	Expression level in Sample 2	Ratio of expressions in Sample 2 to Sample 1
mir-125a	2.67	0.23	0.1
mir-125b	0.05	0.01	0.2
mir-196b	0.1	0.12	1.2
mir-29a	0.01	0	0
mir-223	0.57	1.81	3.2
mir-221	0.09	0.07	0.8
mir-15	0.25	0.14	0.6
mir-155	0.03	0.05	1.7
mir-181	0.34	0.1	0.3
mir-146	0.46	1.81	3.9
mir-150	0.09	0.03	0.3
mir-34	0.03	0.02	0.7
mir-10a	0.01	0.012	1.2
mir-24	0.26	0.3	1.1
mir-222	4.4	0.58	0.1
mir-144	0.02	0.04	2
mir-16	0.03	0.12	4
mir-451	0.13	0.04	0.3
mir-424	0.2	0.25	1.2
mir-17-5p	2.04	2.05	1
mir-20a	0.99	2.59	2.6
mir-106a	0.05	0.57	11.4

and 4; Homeobox A10 (HOXA10 and D4); SOX13; and TGF β 1/4. Upregulated genes include cell adhesion-related genes (ADM9/10 and THBS1/2) and cellular metabolism-related genes (fatty acid and lipid, carbohydrate, protein, and amino acid metabolism).²⁸

Despite these studies, there are no published studies on miRNA expression profiles after *in vitro* cell culture. Our study is the first study on miRNA expression profiles in HUCB cells after *in vitro* culture compared with that in fresh isolated CD133⁺ hematopoietic cells.

In the present study, the possible alterations in the miRNA profile of CD133⁺ hematopoietic cells were investigated via *in vitro* culture and expansion. To address this issue and uncover the related targets and pathways, we compared the miRNA expression patterns of CD133⁺ hematopoietic cells after isolation and after 12 days' *in vitro* culture. The microbead isolated CD133⁺ hematopoietic cells from HUCB (Sample 1) were spherical, and their purity analyzed by flow cytometry was 98% (figure 1). The investigation of the 12 days' cultured cells by flow cytometry indicated that 36% of the population consisted of CD133⁺ cells (Sample 3, figure 2). This decrease in the CD133⁺ population percentage may be explained by the more rapid

proliferation of the other populations. Following a second isolation by beads, the purity of the CD133⁺ cells increased to 97% (Sample 2).

Based on our miRNA profiling data in general, the expression of about half of the analyzed miRNAs was significantly modulated in Sample 2 as compared to Sample 1.

We selected more than 20 miRNAs with the most expression alterations in Sample 2 and compared them to Sample 1 (table 2). The role of selected miRNAs in hematopoiesis has been reported in previous studies and is summarized in table 1 according to TargetScan and miRBase online websites.

Mature miRNAs are associated with the RNA-induced silencing complex for loading onto 3' UTR of targeted mRNAs. Because of imperfect complementarity with their target mRNA, the prediction of miRNA targets is difficult.^{29,30} Fortunately, significant progress in data mining has provided a wide range of bioinformatic analysis options to aid researchers in the interpretation of their data.³¹ *In-silico* identification of genes targeted by miRNAs is widely believed to be an important step toward understanding the role of miRNAs in gene regulatory networks. Using online software, we predicted some hematopoietic-related targets of selected miRNAs and summarized them in table 1.

The initiation and activation of multiple intracellular pathways is necessary for the alteration in cell fate.³² In addition to post-translational/translational regulations, miRNAs are another set of molecules for regulating the signaling pathways and play a role in physiological/pathological processes.³³ In this regard, we chose the 6 most modulated miRNAs to find the related pathways using the DIANA-miRPath. According to the classification of the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology in the KEGG databases (<http://www.genome.jp/kegg/>), the majority of the putative targets of these miRNAs were involved in the CML pathway.

Most human miRNAs lie between protein-coding genes (intergenic region), whereas about one-third lie within the introns of annotated mRNAs.³⁴ Strongly-correlated expression patterns have been observed between miRNAs and their host genes, and miRNAs are processed from the same primary transcripts as their host genes.³⁵ An investigation of the location of these top modulated miRNAs showed that the majority were located within their host genes, while only 6 miRNAs were intergenic (table 3).

Given that miRNAs participate in the molecular regulation of cell differentiation, proliferation, and death, it could be concluded that some important changes occurred in the CD133⁺ cells during *in vitro*

Table 3: Accession numbers, chromosomal locations, and overlapping transcripts of the selected miRNAs

MicroRNA (has)	Accession number	Chromosome/Overlapping transcripts
mir-125a	MI0000469	Chr19/antisense AC018755.2-201; exon 1
mir-125b	MI0000446	Chr11/sense RP11-166D19.1-016; intron 1
mir-196b	MI0001150	Chr7/sense RP1-170O19.21-004; intron 1 Antisense HOXA-AS4-002; intron 1
mir-520h	MI0003175	Chr19/intergenic
mir-29a	MI0000087	Chr7/sense MIR29A-201; exon 1 antisense AC016831.7-001; intron 2
mir-223	MI0000300	chrX/sense AL034397.1-201; exon 2
mir-221	MI0000298	chrX/intergenic
mir-15	MI0000069	Chr13/sense DLEU2-001; intron 3
mir-155	MI0000681	Chr21/sense MIR155HG-001; exon 4
mir-181a	MI0000269	Chr9/sense MIR181A2HG-001; intron 1 antisense NR6A1-001; intron 2
mir-146	MI0000477	Chr5/sense CTC-231O11.1-001; exon 2
mir-150	MI0000479	Chr19/intergenic
mir-34	MI0000268	Chr1/intergenic
mir-10a	MI0000266	Chr17/sense HOXB3-003; intron 1 antisense RP11-357H14.7-012; intron 1
mir-24	MI0000080	Chr9/sense C9orf3-007; intron 4 antisense MIR24-1-201; exon 1
mir-222	MI0000299	chrX/intergenic
mir-144	MI0000460	Chr17/sense RP11-20B24.2-001; exon 1 MIR451B-001; exon 1
mir-16	MI0000070	Chr13/sense DLEU2-001; intron 3
mir-451	MI0001729	Chr17/sense RP11-20B24.2-001; exon 1 antisense RP11-20B24.9-001; exon 1
mir-424	MI0001446	chrX/sense AC004383.4-001; exon 1
mir-17-5p	MIMAT0000070	Chr13/sense MIR17HG-002; exon 2
mir-20a	MI0000076	Chr13/sense MIR17HG-002; exon 2
mir-106a	MI0000113	chrX/intergenic

Data from miRBase database (<http://www.mirbase.org>)

culture. Because of the therapeutic applications of these cells, even minor modifications or alterations during laboratory adaptation (or processing) are essential and require illustration. Considering the important role of cellular environment for cell culture, providing and altering cellular conditions similar to the *in vivo* environment, for example by culturing and expanding cells in 3-dimensional collagen matrix, may decrease differences between *in vitro* and *in vivo* gene expression profiles such as miRNA expression. These cell culture conditions may provide a sufficient and appropriate environment for CD133⁺ hematopoietic stem cells with their clonogenicity and pluripotency potential for differentiating to different lineages. Soufizomorrod et al.³⁶ cultured CD133⁺umbilical cord blood cells in collagen-coated 3-dimensional scaffold and demonstrated the high expansion of these cells compared to routine cell culture (2-dimensional microenvironment).

Conclusion

The present study is the first of its kind on miRNA profile modulations and their related pathway

in *in vitro* cultured CD133⁺ cells. However, further screening is needed to provide precise details regarding the relation between post-transplantation disorders and miRNA profile modulations. Taking the results into account, this study could provide a basis for further research on the effects of 2-dimensional culture on hematopoietic cells before transplantation and the subsequent side effects or disorders that may be identified and prevented.

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Conflict of Interest: None declared.

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