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Expression profiles and potential roles of microRNAs in erythrocytes during the aging process

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Abstract

Studies have shown that microRNAs (miRNAs) in red blood cells (RBCs) contribute most of the miRNAs in whole blood, and miRNAs in RBCs are closely related to storage lesions in vitro. However, the role of miRNAs in the process of RBC senescence in vivo remains unclear. We conducted a comprehensive miRNA expression analysis of RBCs collected from enriched mature RBCs in five density layers. The results showed that the type and number of RBC miRNAs changed with the aging of RBCs, the expression levels of 10 RBC miRNAs decreased markedly at the early stage of RBC aging and the levels of 5 RBC miRNAs increased significantly at the terminal stage of RBC senescence. The analysis identified 32 miRNAs whose changes in expression levels were correlated with the two selected aging indexes—pyruvate kinase (PK) activity and RBC indices. The differential expression amounts of the two selected miRNAs (miR-22-3p and miR-144-3p) were confirmed by real-time polymerase chain reaction (PCR) analysis. A bioinformatics analysis identified the potential targets and biological functions of these miRNAs. The experiment of miR-22-3p in the human erythroblast cell line K562 confirmed its negative effects on PK levels. Overall, our research demonstrates, for the first time, that changes in the expression levels of miRNAs during the RBC aging process, and RBC miRNAs thus have the potential to serve as markers of RBC aging in vivo. In addition, the expression of miR-22-3p may regulate RBC senescence by inhibiting PK levels.

Key Words: Aging process; Erythrocytes; MicroRNAs

1. INTRODUCTION

Cellular senescence as stable cell-cycle arrest plays an important role in multiple biological processes including organismal aging, tumorigenesis, embryonic development, etc.[1] The factors involved in the regulation of cellular senescence are diverse and constituted a large and fine regulatory network. Many studies have confirmed that the senescent cells have undergone significant metabolic changes, and various intermediate products of cell metabolism play crucial roles in cell senescence, such as pyruvate kinase (PK), which is a key rate-limiting metabolic enzyme.[2]

During the aging process, red blood cells (RBCs) are exposed to oxidative stress and affected by the following processes: the Band 3-based aging pathway, caspase signaling and calcium homeostasis; these processes lead to microvesiculation, eryptosis, and erythrophagocytosis. Moreover, PK enzyme, as an important regulator of glycolysis, changes along with RBC aging and is related with erythrocyte senescence.[3–8] However, the molecular participants and the signaling pathways involved have not yet been clearly described.

Though lacking large-sized mRNAs, mature human RBCs have an abundant amount of diverse microRNAs (miRNAs).[9,10] The miRNA expression pattern of the whole blood is similar to that of erythrocytes, but not leukocytes.[11,12] With the development of high-throughput sequencing technology, RBCs were deeply sequenced, and 287 known and 72 putative novel miRNAs were identified in mature RBCs.[13] A recent study showed that RBCs contain a large number of Ago 2-miRNAs functional complexes. Encouragingly, several reports have revealed that RBCs can transfer miRNAs to recipient cells through vesicles (exosomes) in malaria-infected humans,[14–16] which provide clues to function of RBC miRNAs in humans. Previous studies have focused on the expression of RBC miRNAs in packed RBC units during preservation,[12,17] which cannot be equated to analysis of RBC miRNAs in vivo. Rivkin et al[18] generated miR-142 gene deletion mice and found that miR-142 regulates the erythrocyte cytoskeleton morphology and biomechanical properties and the capacity to cope with oxidative stress, thus affecting the survival of RBCs in vivo, which suggest that miRNAs in RBCs may exert function during the aging process. However, there is little research in this area, and further experimental studies are needed.

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In this study, we reported for the first time, that the global miRNA expression profiles of mature RBCs during the aging process by using a high-throughput miRNA sequencing analysis, and we correlated this information with two selected senescence variables for the first time. We tested the hypothesis that miR-22-3p regulates PKLR expression level, thus affecting the survival of RBCs in vivo. Overall, our studies demonstrate the possible roles of miRNAs during the RBC cellular aging process.

2. MATERIALS AND METHODS

2.1. Sample collection

RBCs from six blood donors (all males, 27.17 ± 0.69 years old, type O blood) were collected and stored in Ethylene Diamine Tetraacetic Acid (EDTA)-Na^+ solutions at the blood bank of the Department of Transfusion Medicine, the First Medical Center, Chinese PLA General Hospital. The study protocol was approved by the Ethics Committee of Chinese PLA General Hospital (S2024-428-01), in accordance with the Declaration of Helsinki.

2.2. Percoll density gradient fractionation of RBCs

Whole blood was centrifuged for 5 minutes at 2500 rpm. The plasma and buffy coat layers were aspirated for the initial removal of plasma and leukocytes, mature RBCs were then filtered for leucocyte removal and washed with PBS three times. Finally, purified mature RBCs were obtained. We routinely checked this material for residual platelet (PLT) and white blood cell (WBC) contamination in a hematology analyzer (BC-2800; MINDRAY, Shenzhen, China). Typical readings were nearly 0 WBCs/L and 0 PLTs/L.

Fractionation of the RBC population into subpopulations of different ages was conducted via density gradient centrifugation using a Percoll solution (Sigma, St. Louis, Missouri). We modified the original protocol from Bosch et al,[19] used Percoll for sample separation and detected the total number of different fractions. In detail, the preparation of the different Percoll density gradients was based on a Percoll stock solution with a density of 1.131 g/mL. The Percoll stock solution was diluted with NaCl buffer to the desired final densities of 1.096, 1.093, 1.088, and 1.085 g/mL. A total of 1 mL of each density fraction was carefully transferred to a 10 mL tube, starting with the densest solution. Next, 400 µL of the prepared blood suspension was carefully pipetted onto the pre-formed Percoll gradient and centrifuged at 3000 rcf for 20 minutes at 4°C.

The RBC layers consisting of different subpopulations were collected separately in clean tubes. The samples were washed 1:1 with 0.9% NaCl buffer and centrifuged (800 rcf, 10 minutes, 4°C) twice. After centrifugation, the supernatant was discarded. The hematocrit of the RBC pellets was adjusted to 40%, and the purified RBCs were subjected to the detection of PK, hematological indices and miRNA expression.

2.3. Detection of PK

We used a kit to detect PK activity (Nanjing Jiancheng Bio Company, Nanjing, China) according to the manufacturer's protocol. The formula was used to convert PK activity into RBC age.[4]

2.4. Detection of hematological indices

We used a BC-2800 analyzer (MINDRAY) for measuring the hematological indices, including RBC count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell volume distribution width (RDW-CV), WBC count, and PLT level.

2.5. Small RNA library construction and sequencing

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, California) following the manufacturer's protocol. The total RNA quantity and purity were analyzed by a Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, California). Approximately 1 µg of total RNA was used to prepare a small RNA library according to the TruSeq Small RNA Sample Prep Kit protocol (Illumina, San Diego, California). In addition, we then performed single-end sequencing (36 bp) on an Illumina Hiseq 2500 at LC-BIO (Hangzhou, China) following the vendor's recommended protocol.

2.6. Data processing and analysis of differentially expressed miRNAs

Briefly, the raw reads were subjected to the Illumina pipeline filter (Solexa 0.3), and the dataset was then further processed with an in-house program, ACGT101-miR (LC Sciences, Houston, Texas), to remove adapter dimers, junk, low complexity, common RNA families (rRNA, tRNA, snRNA, and snoRNA) and repeats. Subsequently, unique sequences with 18 to 26 nucleotides in length were mapped to specific species precursors in miRBase 20.0 by a BLAST search to identify known miRNAs. Differentially expressed miRNAs based on normalized deep-sequencing reads were analyzed by selectively using the Fisher exact test, Chi-squared 2X2 test, Chi-squared nXn test, Student *t* test, and ANOVA based on the different experimental designs. The significance threshold was set to 0.01 and 0.05 for each test. The analysis method of miRNA has been recorded in previous studies.[20]

2.7. Real-time polymerase chain reaction

Total RNA was extracted from cells by using Trizol reagent (TIANGEN, Beijing, China) according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) for miR-144-3p and miR-22-3p was performed using SYBR Green (TIANGEN), with U6 as an endogenous control. Real-time PCR analysis of PKLR mRNA was also performed using SYBR Green, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The relative amounts of miRNA or mRNA were quantified using the comparative threshold cycle (CT) method.

2.8. Prediction of target genes of miRNAs

To predict the genes targeted by differentially expressed miRNAs, two computational target prediction algorithms (TargetScan 50 and miRanda 3.3a) were used to identify miRNA binding sites. Finally, the data predicted by both algorithms were combined and the overlaps were calculated. The Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of these differentially expressed miRNA targets were also annotated. Rich factor was calculated by prediction algorithms as reported in previous studies.[21]

2.9. Transfection of K562 cells with miRNA mimics or inhibitor

To validate the concept that differentially expressed miRNAs in the aging RBCs regulate cellular PK activity, we selected miR-22-3p for experiments. miR-22-3p mimics (inhibitor) and negative control (Genepharma Technologies, Suzhou, China)

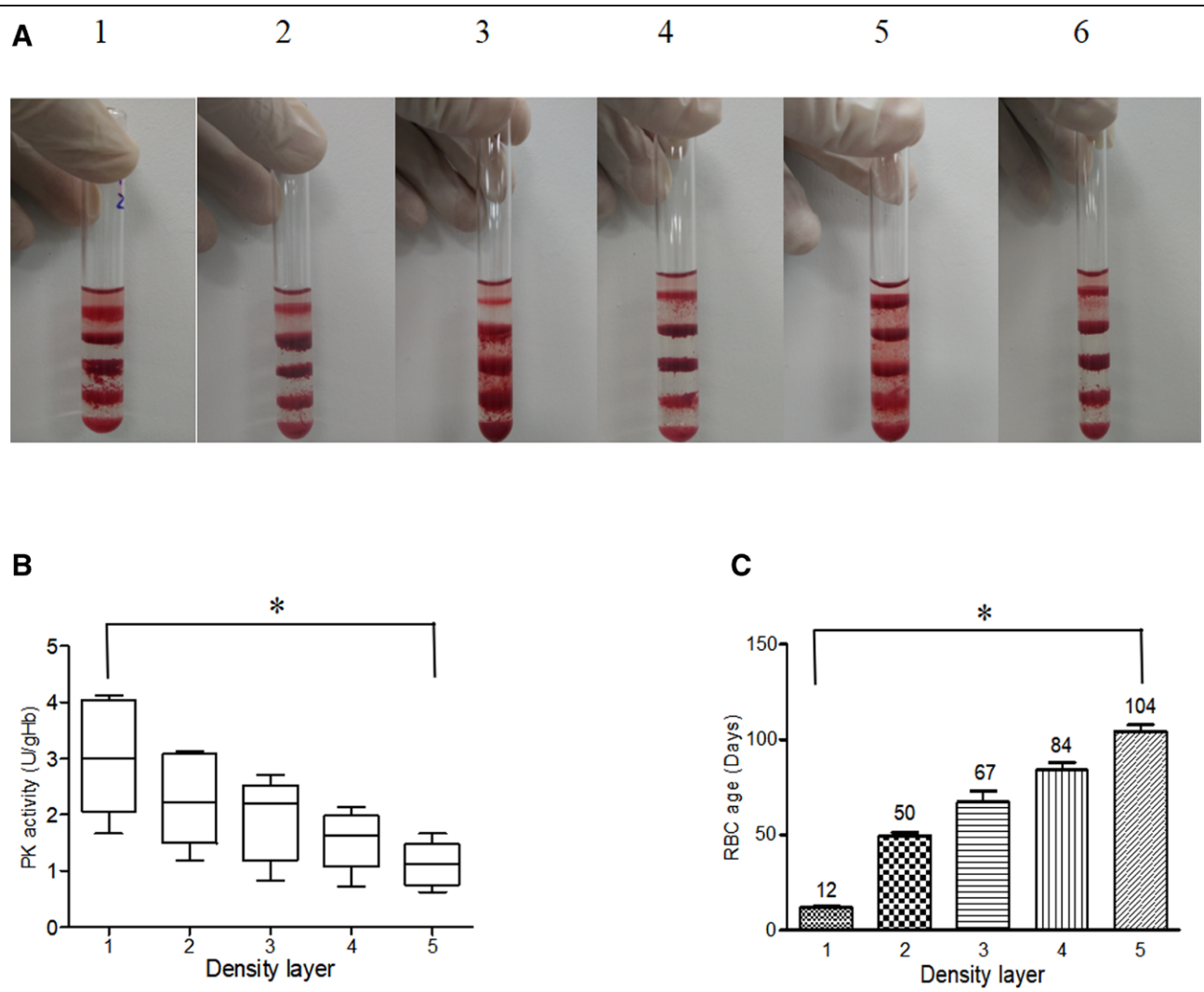

Figure 1. Ages of the RBCs from five layers. (A) RBCs were separated by Percoll density centrifugation with final densities of 1.096, 1.093, 1.088, and 1.085 g/mL (n = 6). (B) RBCs from the five layers were subjected to PK assay. (C) RBC ages of the five layers were determined by PK activity. *$P < .05$. PK = pyruvate kinase, RBC = red blood cell.

were transfected according to the manufacturer's protocol. The cells were cultured in replicates, and each cell culture was analyzed in triplicate for detection. First, Luciferase reporter assays were performed in the 293T cell. Cells were co-transfected with miR-22-3p mimics or miRNA NC (negative control), miR-22-3p inhibitor or miRNA inhibitor NC, using Lipofectamine 2000 (Invitrogen). Cell extracts were prepared for 24 hours before co-transfection, both the miRNA mimics (inhibitor) and the negative control were incubated with Lipo2000 for 20 minutes at room temperature. The cell suspensions ($2–3 \times 10^5$ cells/transfection) were then added to the transfection solutions, mixed, and cultured at 37°C for 72 hours. The cells were then harvested for analyses. Total RNA was isolated from an aliquot of the transfected cells and subjected to real-time PCR assays for miR-22-3p and PKLR; U6 and GAPDH were used as endogenous controls respectively. The transfection efficiency was confirmed, and PK protein levels were analyzed by western blotting.

2.10. Statistical analyses

All analyses were performed by GraphPad Prism (version 5) or R (version 2.10.1) software. Inter-group comparisons were performed using one-way ANOVA or Student *t* test. Significance was determined by a *P* value of less than .05. Error bars indicate the standard deviation (SD). The correlations of miRNA profiles and cell indices between two groups were calculated by Pearson correlation analysis.

3. RESULTS

3.1. Determination of RBC age by PK activity

Blood samples were collected from six individuals (male, type O, 27.17 ± 0.69 years old), and five RBC layers were produced by Percoll density centrifugation (**Fig. 1A**). Calculation of the proportion of each RBC subpopulation to the total number of RBCs revealed that middle-aged RBCs with densities of 1.088 to 1.093 g/mL constituted 34% of the total RBC number, the portion of young RBCs was calculated to be 8%, and the old fraction that included RBCs with densities greater than 1.096 g/mL represented 15%. We detected the PK activity of RBCs from five layers and determined their ages (**Fig. 1B**). We found that the greater the density was, the older the RBC age was. According to the density of the RBCs, from smallest to largest, the ages were calculated as described in a previous study,[4] which were 12, 50, 67, 84, and 104 days (D), respectively.

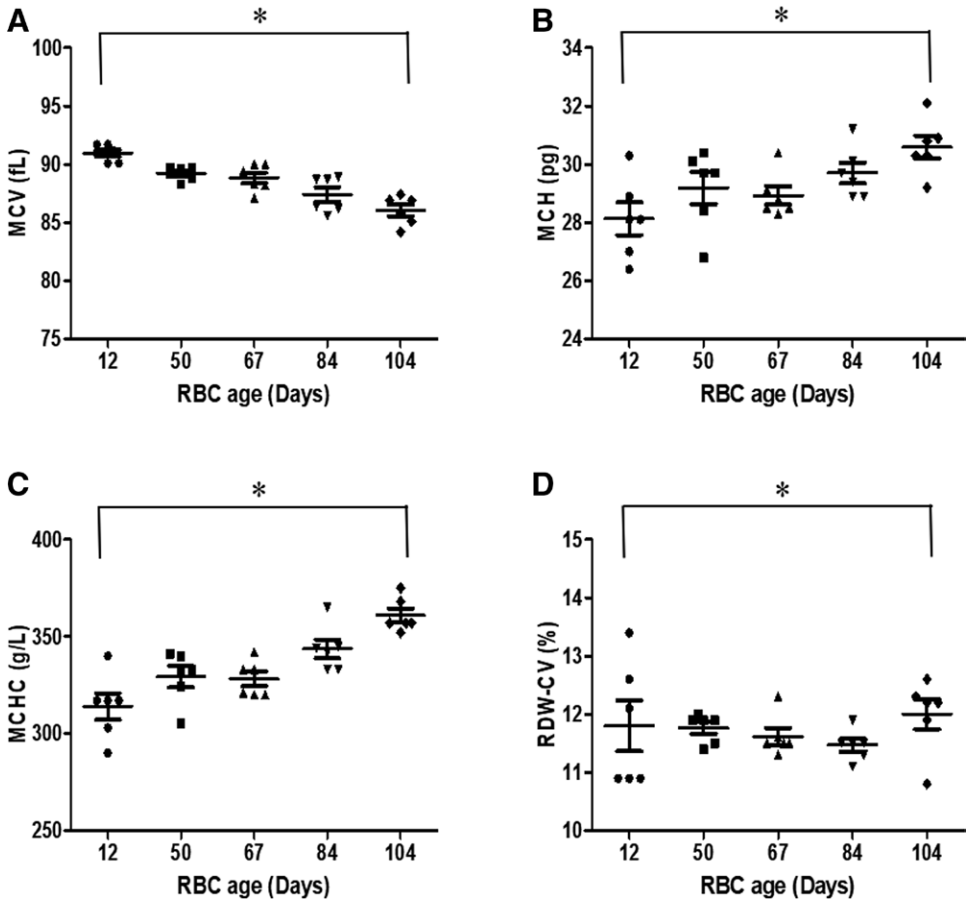

Figure 2. Changes in the RBC indices during the aging process. RBCs from the five layers separated by Percoll were analyzed by an automated hematology analyzer. MCV (A), MCH (B), MCHC (C), and RDW (D) values of all samples from each density layer were calculated. *$P < .05$. MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, MCV = mean corpuscular volume, RBC = red blood cell, RDW = red blood cell distribution width.

3.2. RBC indices significantly changed during the RBC aging process

The RBC indices from the five layers were analyzed using an automated hematology analyzer in order to understand the physiological changes of RBC during the aging process. We observed that the MCV levels of RBCs steadily decreased during the aging process (**Fig. 2A**). Unlike the significantly increased MCH and MCHC levels in RBCs (**Fig. 2B and C**), RDW-CV levels showed no obvious change trend related to RBC aging (**Fig. 2D**).

3.3. Overall status analysis of miRNAs derived from RBCs

We mixed each of the five layers of RBCs from six people into one piece, and then performed miRNA high-throughput sequencing on the five pieces of RBCs. As to examine whether the amounts of miRNAs derived from RBCs changed along with the aging process, we measured a total of 336 miRNAs in RBCs and found that the type and number of RBC miRNAs changed with RBC aging (miRNAs with more than 1 read in the sequencing data were considered expressed miRNAs). Figure 3A exhibits the top 10 miRNA levels with the highest expression levels in mature RBCs, we found that miR-451a accounted for 77% of total expression amounts of all miRNAs. In addition, we conducted KEGG (**Fig. 3B**) and GO (**Fig. 3C**) enrichment analyses of miRNAs derived from erythrocytes. The main pathways in which miRNAs derived from RBCs participated were "cancer" and "heart development."

3.4. MiRNA expression patterns at different RBC ages

First, we compared miRNA profiles from the D50 RBC group to that from the D12 RBC group, and miRNA profiles in the D104 RBC group to that in the D84 RBC group, and found that, of the miRNAs tested, 15 RBC miRNAs were differentially expressed as follows: 10 RBC miRNAs were markedly downregulated in the D50 RBC group compared with that in the D12 RBC group (fold-change > 2, the top 10 highest expression levels, Table 1), and 5 RBC miRNAs with high expression levels were significantly upregulated in the D104 RBC group compared with the D84 RBC group (fold-change >2, Table 2). Second, in order to verify the trends of PK activity and RBC indices during RBC aging process, we performed Pearson correlation analysis and found that any two values among the PK activity and; MCV, MCH, and MCHC levels were significantly correlated (Table 3). Third, to correlate the miRNAs that exhibited expression profile changes with the aging variables that were tested, a normalized miRNA sequencing data set was uploaded into a statistics program, and we selected PK activity, MCV, MCH, and MCHC level as the template for the correlation analysis. The analysis revealed that a total of 51, 45, 54, and 54 miRNAs exhibited expression levels that correlated with reduced PK activity and, changed MCV, MCH, and MCHC levels, respectively. Interestingly, as many as 32 miRNAs overlapped among the four groups related with different types of RBC aging variables, and the results are shown in the Venn diagram (**Fig. 4A**) and heat map (**Fig. 4B**). Of all these miRNAs, miR-144-3p exhibited the highest expression level and miR-22-3p were the most significantly correlated with PK activity.

Figure 3. Overall status of miRNAs derived from RBCs. A miRNA sequencing analysis was conducted using the mature RBCs from five layers, miRNAs with the top 10 highest expression level were shown (A). We conducted KEGG (B) and GO (C)-enrichment analyses of the miRNAs derived from erythrocytes. GO = Gene Ontology, HTLV = human T-cell lymphotropic virus, KEGG = Kyoto Encyclopedia of Genes and Genomes, MAPK = mitogen-activated protein kinase, miRNA = microRNA, PI3K-Akt = phosphatidylinositol-3-kinase-protein kinase B, RBC = red blood cell.

3.5. Confirmation of selected miRNA expression in RBCs

Expression levels of the selected miRNAs (miR-144-3p and miR-22-3p) associated with RBC aging values were confirmed by real-time PCR analyses as shown in Figure 5A and B. The results of real-time PCR are in agreement with the sequencing results.

Table 1

Markedly altered miRNAs in the D50 RBC group compared with the D12 RBC group.

miRNA	D12	D50	D67	D84	D104	Fold-change (D12/D50)
Downregulated (D50 vs D12)						
miR-223-3p	4562.38	272.82	276.67	334.00	337.56	16.72*
miR-27a-3p	1697.09	807.19	831.57	1218.79	962.82	2.10*
let-7c-5p	937.36	28.60	32.44	19.21	15.03	32.77*
miR-23a-3p	894.44	397.51	297.65	270.00	177.35	2.25*
miR-143-3p	446.82	120.77	102.59	54.19	186.42	3.70*
miR-27b-3p	428.13	145.97	143.78	179.32	185.91	2.93*
miR-125b-5p	251.84	38.23	41.18	34.48	59.95	6.58*
miR-99b-5p	232.34	5.21	0	4.92	8.06	44.56*
let-7d-3p	209.60	94.71	106.47	111.34	53.41	2.21*
miR-23b-3p	200.66	89.93	62.17	43.35	28.21	2.23*

miRNA = microRNAs, RBC = red blood cell.

*P value <.001, calculated by Fisher exact test.

Table 2

Markedly altered miRNAs in the D104 RBC group compared with the D84 RBC group.

miRNA	D12	D50	D67	D84	D104	Fold-change (D104/D84)
Upregulated (D104 vs D84)						
miR-142-3p	3858.86	4841.41	5182.13	5818.07	12892.06	2.22*
miR-144-5p	2914.86	2992.43	3292.84	2424.77	5235.83	2.15*
miR-19b-3p	1737.70	2404.78	2397.94	2804.59	6018.45	2.15*
miR-19a-3p	687.28	1119.69	1095.80	1409.93	3411.62	2.41*
miR-96-5p	253.46	251.11	266.56	339.92	702.34	2.07*

miRNA = microRNAs, RBC = red blood cell.

*P value <.001.

Table 3

Correlations between any two values of PK, MCV, MCH, and MCHC levels by Pearson's correlation analysis.

Relevance	PK	MCV	MCH	MCHC
PK				
Pearson correlation coefficient	1	0.978**	−0.949*	−0.953*
P value		.004	.014	.012
N	5	5	5	5
MCV				
Pearson correlation coefficient	0.978**	1	−0.982**	−0.991**
P value	.004		.003	.001
N	5	5	5	5
MCH				
Pearson correlation coefficient	−0.949*	−0.982**	1	0.994**
P value	.014	.003		.001
N	5	5	5	5
MCHC				
Pearson correlation coefficient	−0.953*	−0.991**	0.994**	1
P value	.012	.001	.001	
N	5	5	5	5

MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, MCV = mean corpuscular volume, PK = pyruvate kinase.

*P value <.05

**P value <.01

3.6. Bioinformatics prediction of the potential miRNA targets revealed a strong association with aging-related cellular functions

Potential target genes for the validated differentially expressed miRNAs (miR-144-3p and miR-22-3p) were identified by using two computational target prediction algorithms (TargetScan 50 and miRanda 3.3a), and the GO terms and KEGG pathways of these differentially expressed miRNA targets were annotated. The GO terms for miR-144-3p and miR-22-3p are shown in Figure 6A and B, respectively. We found that the overlapping biological pathways associated with the potential targets of miR-144-3p and miR-22-3p were "protein binding" and "cytoplasm." The potential targets of miR-22-3p were also predicted to be associated with "ATP binding" and "protein kinase" activity pathways, including PK, liver and red blood cells (PKLR). These findings suggested that miR-22-3p may be included in the PK metabolic pathway. Therefore, we selected miR-22-3p for the functional analysis related with PK metabolism.

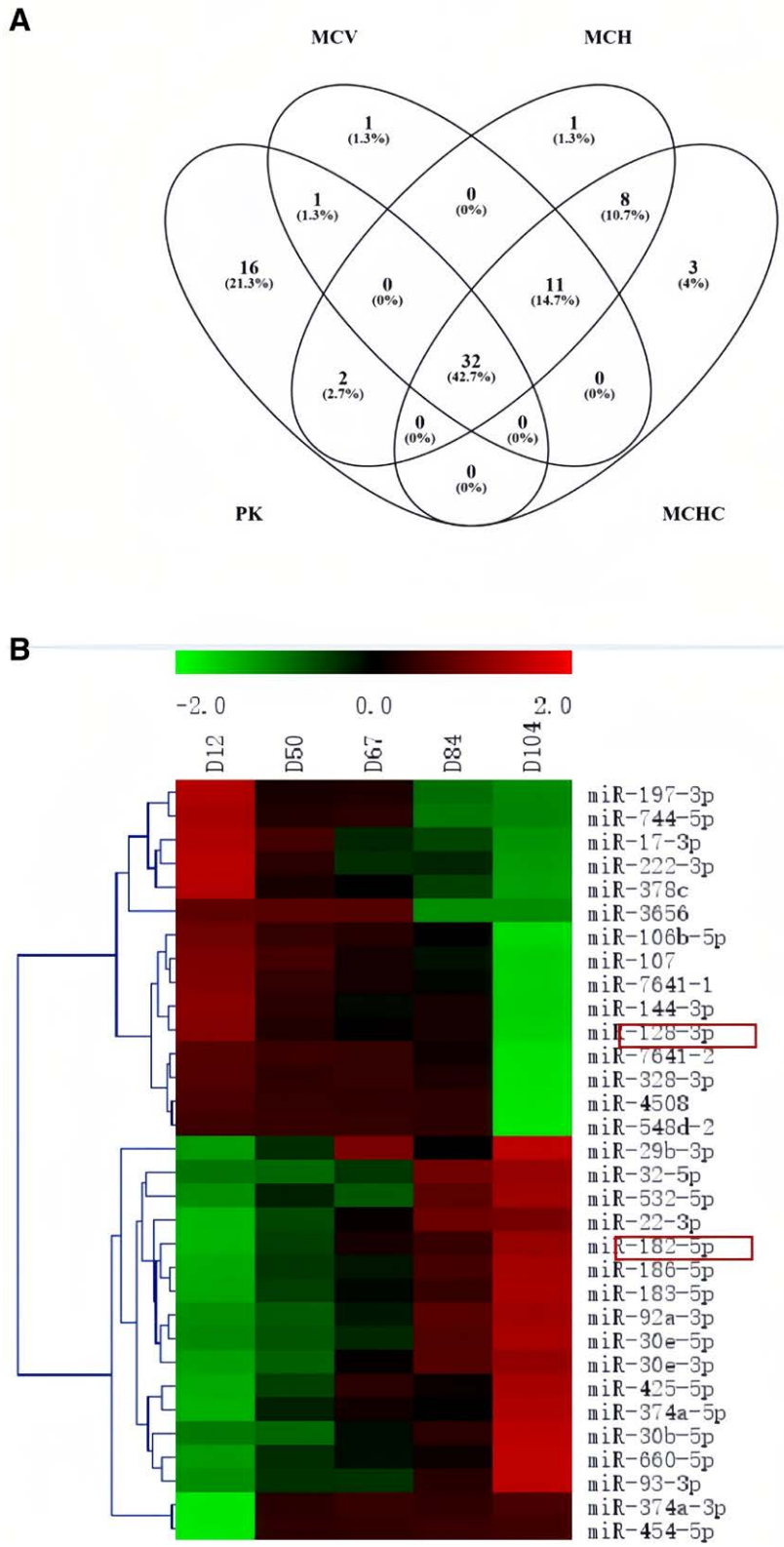

Figure 4. Expression levels of miRNAs are correlated with PK activity and, MCV, MCH, and MCHC levels. Pearson's correlation analyses were performed to identify miRNAs whose expression levels were significantly correlated with PK activity and, MCV, MCH, and MCHC levels ($P < .05$). (A) The Venn diagram displays the counts of miRNAs which overlap among any two groups with different indices. (B) Thirty-two miRNAs whose levels were correlated with all four parameters, were shown in the heat map; each column of the heat matrix is a time point, and each row indicates a miRNA. Red indicates upregulation and green indicates downregulation. MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, MCV = mean corpuscular volume, miRNA = microRNA, PK = pyruvate kinase.

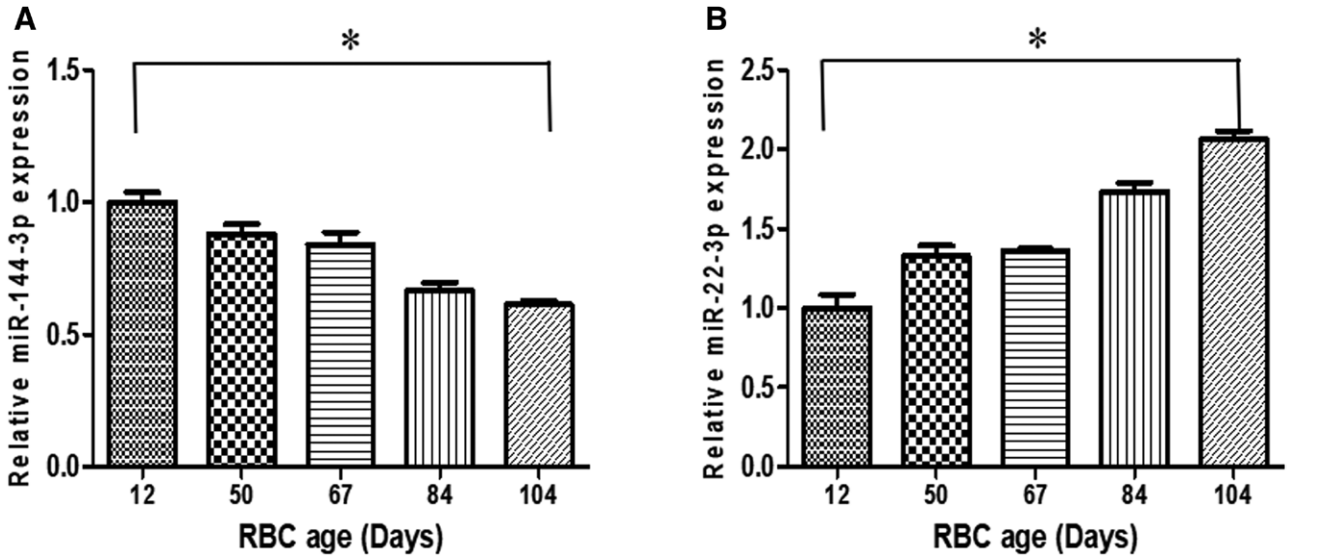

Figure 5. Confirmation of selected miRNA expression levels by real-time PCR in RBCs. Expression levels of miR-144-3p (A) and miR-22-3p (B) were detected by real-time PCR; U6 was used as the internal control. *P < .05. miRNA = microRNA, PCR = polymerase chain reaction, RBC = red blood cell.

3.7. Functional analysis of miR-22-3p

To confirm and demonstrate that miRNAs are involved in the biological mechanisms associated with reduced PK activity during the RBC aging process, miR-22-3p was selected for functional analysis. As shown in Figure 7A and B, the human erythroblast cell line K562 was employed for the miRNA functional analysis, due to the fact that RBCs cannot be transfected with nucleic acids and cultured in vitro. So, we transfected K562 cells with miR-22-3p mimics, miR-22-3p inhibitor, and negative controls, and found a significant increase in miR-22-3p expression level in the miR-22-3p-mimic group (**Fig. 7A**) and, a significant decrease of miR-22-3p amounts in the miR-22-3p-inhibitor group compared with the control group (**Fig. 7B**). Real-time PCR and western blotting were conducted to determine whether the expression of miR-22-3p had a negative effect on the levels of PKLR. The results showed that overexpression of miR-22-3p decreased the mRNA level of PKLR (**Fig. 7C**) and protein level of PK (**Fig. 7E**), whereas down-expression of miR-22-3p showed the opposite results, compared with the negative control (**Fig. 7D and F**). Therefore, we concluded that miR-22-3p might reduce PK protein levels by downregulating PKLR mRNA levels.

4. DISCUSSION

Many molecules and signaling pathways are involved in RBC aging and eryptosis, which are tightly regulated. Studies have shown that erythrocyte-derived miRNAs constitute the majority of miRNAs in the whole blood; however, the function of RBC miRNAs is not clear, especially in the field of RBC aging. Therefore, it is important to study the role of RBC miRNAs in the aging process.

In this study, we separated RBCs into five layers by percoll density gradient centrifugation. It has been reported that the age of RBCs will increase as the density increases.[3,4] Therefore, we used the percoll separation method to obtain RBCs of different ages. We observed significant changes in three important RBC indices (ie, the MCV, MCH, and MCHC levels) and found that the MCV values were significantly decreased in old RBCs compared with young RBCs. However, MCH and MCHC values increased along with RBC senescence. Changes in the levels of many key factors in energy metabolism, such as PK levels, have been reported to be associated with RBC aging events. Here, we have shown that PK levels decrease along with the increases in RBC density, which represent the degree of RBC aging and our results are consistent with the previous research.[19]

We performed a global miRNA expression profiling in parallel with aging values analyses from mature RBCs isolated by Percoll density separation. The results showed that there were 336 miRNAs expressed in RBCs, and the variety and number of miRNAs in RBCs changed during the aging process. The main pathways in which these miRNAs participate are "cancer" and "heart development," other key pathways included "regulation of cell development" and "cell differentiation." These findings show that miRNAs from RBCs may play important roles in regulating the cell life cycle. Multiple erythrocyte miRNAs are related to RBC physiological functions. For example, miR-451a and miR-144-3p are closely related to erythropoiesis, miR-30b-5p is related to oxygen and NO-related environment, and miR-96, miR-150, miR-196a, and miR-197 are associated with RBC storage in vitro.[12,13,22] Interestingly, we found that 10 miRNAs with the highest expression levels were significantly downregulated at the early stage of the RBC aging, and five miRNAs were upregulated at the terminal stage, and the expression levels of 32 miRNAs were correlated with RBC aging. As we know, the regulation of miRNAs maturation in the nucleated cell is accomplished by an RNA-induced silencing complex (RISC) that is initiated by Dicer 1[23,24]; however, mature RBCs lack Dicer 1,[15] so the changes in miRNAs derived from mature RBCs are probably due to degradation or the transfer of miRNAs through the RBC-derived extracellular vesicles. A complete understanding of this mechanism, however, requires further experimental evidence.

Among the 10 miRNAs changed at the early stage of the RBC aging process, the expression level of miR-223-3p was significantly downregulated, because its level in the D12 RBC group was 3.4 times higher than that in the D50 RBC group. The literature[25] reports that miR-223 can affect the differentiation of erythroid progenitor cells through regulating its target gene LMO2 (LMO2 is an important positive regulator of hematopoietic stem cell [HSC] development and erythropoiesis). There exist possibility that miR-223-3p can be transferred to erythroid-oriented progenitor cells through the exosomes derived from RBCs and then inhibit erythropoiesis by downregulating LMO2

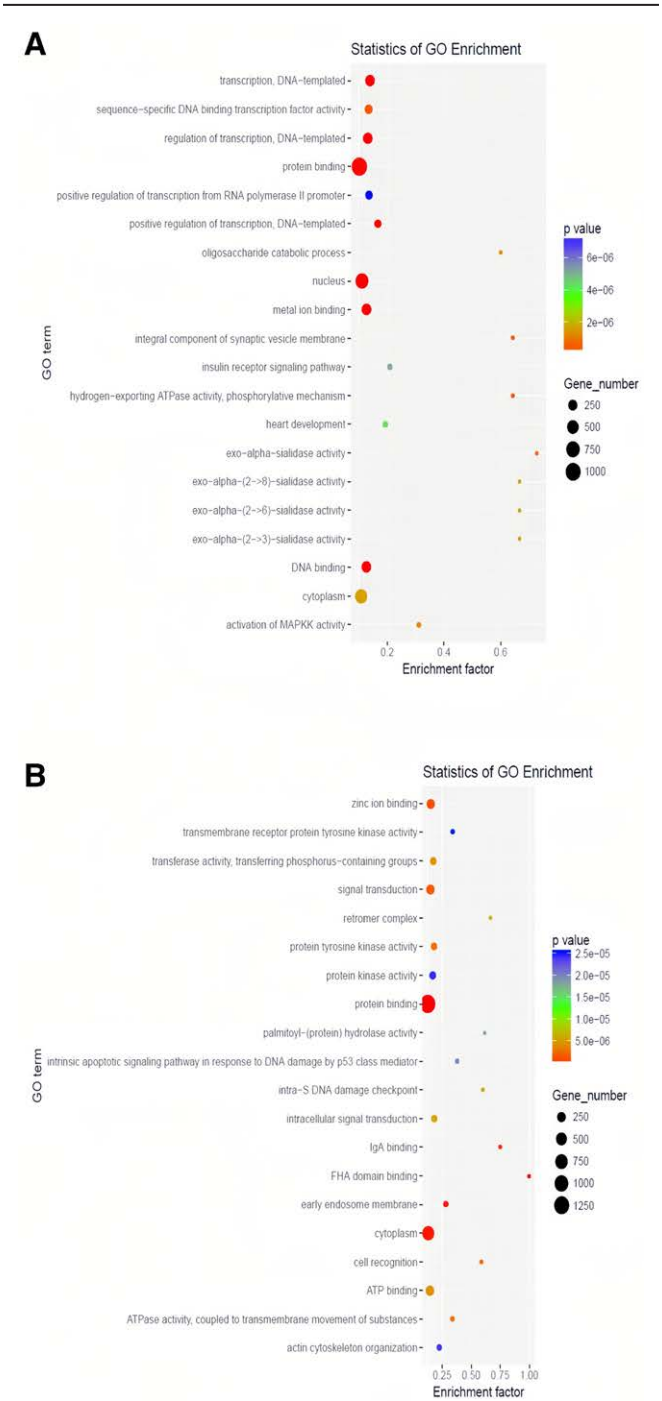

Figure 6. Prediction of the potential miR-144-3p and miR-22-3p targets. GO term annotations for miR-144-3p (A) and miR-22-3p (B) were analyzed by Target Scan 50 and miRanda 3.3a prediction algorithms. GO = Gene Ontology.

expression.[15,16] Therefore, the reduced amount of miR-223-3p in older RBCs may lead to the weakening of the inhibition of erythropoiesis during RBC aging. However, this conclusion still needs a lot of data to prove. Among the five miRNAs upregulated at the terminal stage of the RBC aging process, miR-142-3p was significantly higher in the D104 RBC group than in the D84 RBC group, which indicates that miR-142 is a key regulator of erythrocyte senescence as reported in a previous study[18] and likely a signal that appears at the terminal stage, followed by the rapid removal of senescent RBCs. In addition, other miRNAs with significant changes in Tables 1 and 2 also deserved attention and further study.

Our strategy has enabled the integration of data from different aspects of biology and identified miRNAs whose changes in their expression levels are positively or negatively correlated with changes in RBC indices and PK levels. Among those miRNAs, the expression levels of 51, 45, 54, and 54 miRNAs were significantly correlated with changing PK activity and, MCV, MCH, and MCHC levels, respectively. Interestingly, as many as 32 miRNAs were associated with four types of RBC aging variables. Among them, two miRNAs (ie, miR-144-3p and miR-22-3p) were confirmed to be significantly differentially expressed in RBCs during the aging process by real-time PCR.

According to a previous report,[26] miR-144 is included in the list of mature miRNAs common in Ago2 IP RBCs (Agos in RBCs which are immunoprecipitated) as a functional protein complex and miR-144-3p is reported to show high expression level in erythrocyte,[27] further experiments are needed to demonstrate whether it exert function in regulating RBC aging processes. More interestingly, in accordance with our bioinformatics prediction analysis, one of the miR-22-3p target genes is PKLR gene encoding PK isozymes in humans, which is a key glycolytic enzyme associated with RBC aging and tumor occurrence. Our biological experiments showed that overexpression of miR-22-3p in the erythroblast cell line K562 cells had negative effects on the expression levels of PK, these findings are consistent with the former bioinformatics analyses. Moreover, we found that during RBC aging, miR-22-3p may be correlated with other factors, such as ATPase activity, actin cytoskeleton organization, and apoptotic signaling by analyzing the potential target genes, which mean that miR-22-3p may affect RBC senescence through many pathways. Previous studies have shown that the research direction of miR-22-3p and PKLR is related to the occurrence and development of cancer. For example, it has been reported that the expression level of miR-22-3p is closely related to the occurrence of various human cancers, such as multiple myeloma, liver cancer, pancreatic cancer,[28–30] in addition, PKLR is regulated by miR-338-3p, and controlled hepatocellular carcinoma by glycolysis.[2] Our studies show that the expression level of miR-22-3p has the potential to be associated with RBC senescence. However, further experimental verification is needed to establish the roles of miR-22-3p during RBC aging. In addition, CD34+ hematopoietic progenitor cells, as another type of RBC model cell line, can be used to verify the miR-22-3p functional experiments in the future.[31,32]

In general, by analyzing both aging data and miRNA expression profiles, we have identified significantly altered miRNAs, and performed functional analyses of miR-22-3p in erythroblast cells. The differential expression of miRNAs detected in the RBCs reflects systemic changes influencing the aging processes, especially changes in PK activity; and our functional analyses confirm the mechanism of PKLR regulated by miR-22-3p during RBC aging. However, the increasing density of the part from RBCs is not completely consistent with the RBC aging process, so more methods are needed to identify the aging degree of RBCs. Also, more samples of different ages and genders are needed to be included so as to conduct a more comprehensive analysis of RBC miRNAs.

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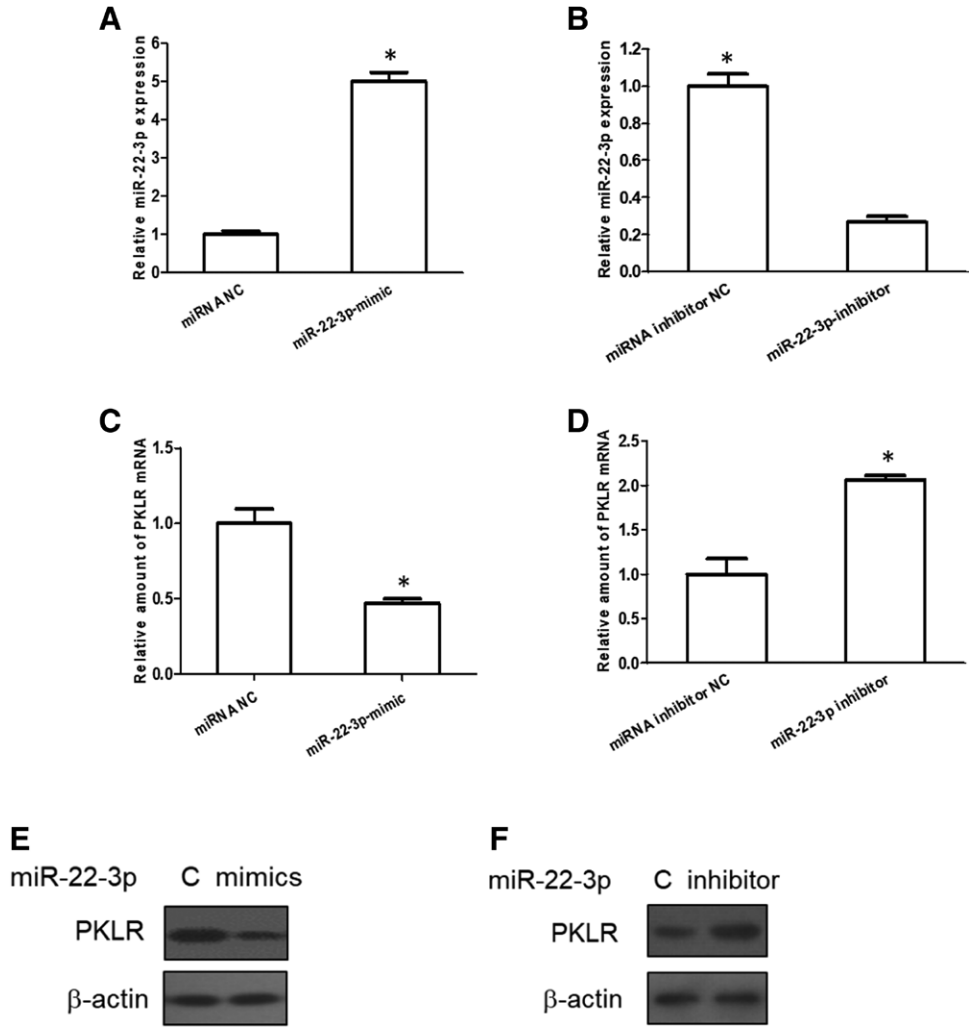

Figure 7. Expression of miR-22-3p affects expression levels of PK. To confirm that the miRNA mimics and inhibitor transfections were successful, total RNA was isolated from the K562 cells transfected with the miR-22-3p mimics, inhibitor, or negative control. miRNA real-time PCR analyses were conducted to measure the expression levels of miR-22-3p (A and B). The upregulation of miR-22-3p decreased the mRNA levels of PKLR (C) and protein levels of PK (E); the downregulation of miR-22-3p increased the mRNA levels of PKLR (D) and protein levels of PK (F). **P* value <.05; error bars indicate the SD. miRNA = microRNA, PCR = polymerase chain reaction, PK = pyruvate kinase, PKLR = PK, liver and red blood cells, RBC = red blood cell, SD = standard deviation.

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