

Research Paper

## Gene expression analysis of human red blood cells

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### Abstract

Understanding of molecular mechanisms governing the enucleating phenomena of human erythrocytes is of major importance in both fundamental and applied studies. Total RNA (n=7) from human RBCs (purity of erythrocyte preparation >99,99%) was tested using 2100 Bioanalyzer (*Agilent*, USA), and transcribed to cDNA. Microarray analysis was performed with the Human Genome Focus GeneChip (*Affymetrix*, USA), containing 8500 transcripts corresponding to 8400 human genes. Here we report that human RBCs contain typical eukaryotic RNA with 28S- and 18S-rRNA standard bands. Microarray studies revealed the presence of transcripts of 1019 different genes in erythrocytic RNA. Gene Ontology analysis recognized 859 genes involved in general biological processes: 529 genes for cellular metabolism, 228 genes for signal transduction, 104 genes for development, 107 genes for immune response, 62 genes for protein localization, 53 genes for programmed cell death, and 5 genes for autophagy. A number of genes responsible for transcription, translation, RNA-stabilisation as well as for apoptosis and anti-apoptosis have been identified for the first time in circulating human RBCs. The presented data shed new light on the genetic determination of erythropoiesis, apoptosis and may have implications on the pathophysiology and diagnosis of various diseases involving red blood cells.

Key words: red blood cell, gene expression analysis

### Introduction

Human erythrocytes discard their nucleus during maturation, and are thought not to be able to synthesise proteins. Research in this field can be divided into the following: (I) gene expression analysis of erythropoietic progenitor cells; (II) biochemical characterization of nucleotide and protein synthesis during the life cycle of nucleated erythrocytes in vertebrates; (III) molecular aspects of malaria pathogenesis during RBC development; (IV) and genomic and proteomic analysis of gene expression in normal adult human erythrocytes.

Thus, current array data showed that most genes

expressed in haematopoietic stem cells are developmentally regulated and associated with cell self-renewal <sup>1</sup> as well as survival, differentiation and/or migration/adhesion <sup>2</sup>. Genes enriched in committed progenitors were mostly associated with haematopoietic differentiation, immune regulation, and metabolism <sup>1</sup>. It was shown, that both the rate and extent of transcription in mature erythrocyte nuclei from chicken <sup>3</sup> and newt <sup>4</sup> were much reduced as compared to reticulocytes from this species. Previous studies of malaria pathogenesis did not shed light on gene regulation mechanisms in respect to erythrocyte

development, though some regulatory elements have been proposed<sup>5</sup>.

A part of human erythrocytic proteins were identified<sup>6, 7, 8, 9</sup>. The number of genomic studies of RBCs is at present limited and represented either by micronucleus assay as a marker of classification of RNA- positive reticulocytes and erythrocytes<sup>10, 11, 12</sup> or by attempts to describe of human erythroid gene activity, planned to be finished in 5-10 years<sup>13</sup>. At present no information regarding gene expression in human RBCs is available.

In contrast, according to recent data, there is a strong evidence that anucleate platelets contain a functional spliceosome<sup>14</sup>, mRNAs<sup>15</sup>, rRNA, rough endoplasmic reticulum and polyribosomes, as well as numerous translation factors including 3'-UTR RNA- and poly(A)-binding protein<sup>16</sup>. It is therefore believed that platelets maintain functionally intact protein translational capabilities accompanied by posttranslational modifications<sup>17</sup>. Recently we were able to detect RNA in washed human RBCs<sup>18</sup>. In this study we used microarray technique to identify genes possibly present and translated in human RBCs.

## Study design

### Cell isolation

Whole blood was taken from healthy human volunteers (n=7) and collected in tubes containing Natrium heparin. RBCs were isolated as described earlier<sup>18</sup>. In short, an open syringe without piston was closed at the tip, filled with whole blood and kept upright, and then centrifugated at 800g for 20 min at 4°C. The resulting plasma was discarded. After opening the syringe at the tip, about 2/3 of the sedimented RBCs were allowed to carefully drop out of the syringe. Special attention was paid to not disturb the WBC layer above the RBCs. This simple method to purify RBCs was superior to methods using density gradients or magnetic beads.

### RNA isolation and probe synthesis

Total RNA from RBCs was purified using reagents provided in the PAXgene Blood RNA Kit (Qiagen, Germany) and tested with RNA LabChip Kit by 2100 Bioanalyzer (Agilent, USA). cDNA was synthesised from 5µg total RNA using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen, USA) and purified according to the manufactures' instruction (GeneChip Sample Cleanup Module, Affymetrix, USA). Biotin-labeled cRNA was synthesized using the BioArray HighYield RNA Transcript Labeling Kit (Enzo Life Sciences, USA) and purified using GeneChip Sample Cleanup Module (Affymetrix, USA). Yield and size distribution of the labeled transcripts

were determined with NanoDrop (Kisker, Germany) and 2100 Bioanalyzer (Agilent, USA). Fragmentation was carried out using the fragmentation buffer from GeneChip Sample Cleanup Module (Affymetrix, USA).

### Microarray and gene ontology analysis

10 µg of fragmented cRNA were hybridised to the Human Genome Focus Array (Affymetrix, USA). After hybridisation, GeneChips were automatically stained with streptavidin-phycoerythrin by using a fluidic station (Affymetrix, USA). Microarrays were scanned by GeneChip Scanner (Affymetrix, USA). The resulting images were processed by the accompanying software (Microarray Suite 5.0; Affymetrix, USA). A global scaling approach was used to normalize signal intensities (TGT value = 500). Genes that were present in all 7 arrays are reported and subjected to further analysis. For classification of the resulting genes the gene ontology browser (Netaffx, Affymetrix, USA) was used.

## Results and discussion

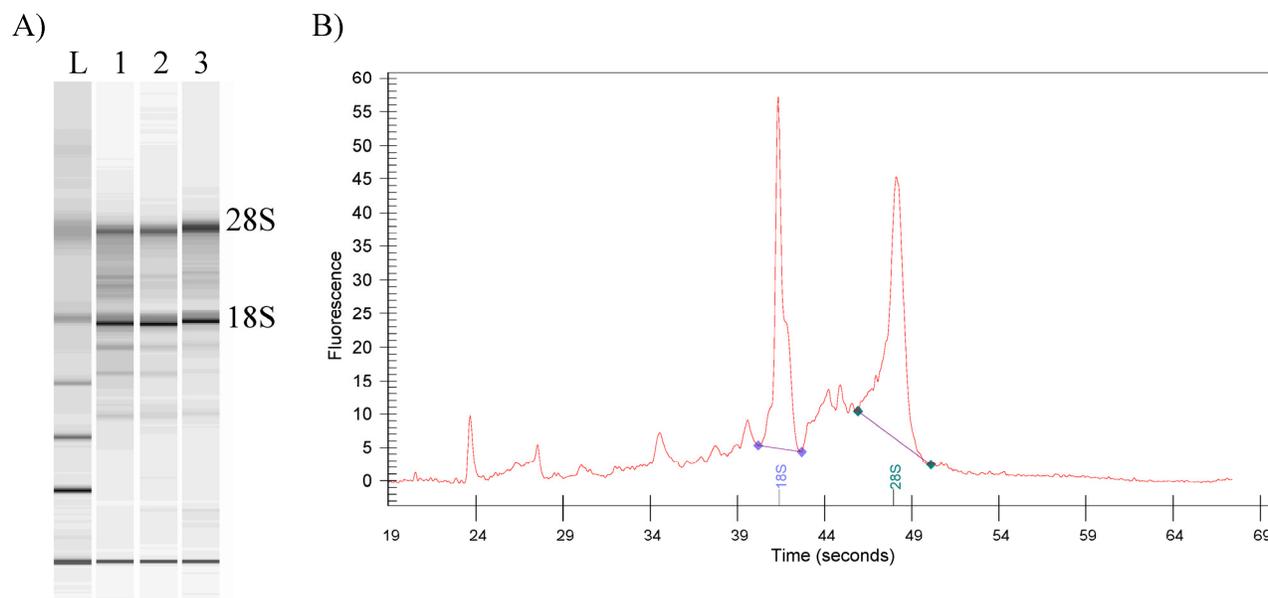
### Characterization of total RNA of human RBCs

The purity of erythrocyte fraction achieved 99,99997% and was confirmed by Pappenheim staining of blood slides, flow cytometry (MÖLAB, Germany) and FACS analysis (Cytomics FC 500 CXP, Beckman Coulter, Germany) using labeling with human leucocyte- (CD45) or platelet specific (CD42) antibodies (Table 1).

**Table 1** The purity of human RBCs fraction tested by independent methods.

blood cell count (n=5)	RBCs (cells/µL)	white blood cells (cells/µL)	platelets (cells/µL)
cytometer	6600000±2000	n.d.*	n.d.*
FACS	6300000±1520	n.d.*	n.d.*
blood slide	6400000±120	0±0	2±1
*according to the resolution options of the cytometer n.d. correspond to < 100 cells/µL			

Human erythrocyte lack a nucleus and are thought to be void of protein synthesis. In contrast, we have found that total RNA from human RBCs resembles typical eukaryotic RNA with 5S-80S sedimentation distributions, and contains standard 28S- and 18S-rRNA bands (Fig. 1). Total RNA from nucleated avian erythrocytes was discovered to have from 5 to 60 S sedimentation rates<sup>3</sup>. Identification of each unique RNA-class within the RNA pool as well as genetic mechanisms from both nucleated and anucleate erythrocytes awaits future studies.



**Figure 1:** Analysis of total human RNA of RBCs (Bioanalyzer 2100 Agilent, USA): A) L- RNA 6000 ladder (Ambion, USA); 1-3 - total RNA of human RBCs from different donors; B) typical electropherogram of total RNA of human RBCs

### Microarray analysis of RNA from human RBCs

Recent proteomic studies of RBCs based on 1D/2D-electrophoresis<sup>6, 7</sup> or mass spectrometry assay<sup>8, 9</sup>, allowed to recognize 272 proteins. Our data generated from microarray studies (n=7) evidence the presence of transcripts for 1019 genes in RNA of human RBCs including the above mentioned 272 proteins. The complete array dataset with genes reported has been deposited in the Gene Expression Omnibus database (accession number - GSE3674).

It was found 529 genes for cellular metabolism (among them 96 genes for protein biosynthesis), 228 genes for signal transduction (among them 112 genes for intracellular signalling cascade), 104 genes for development, 107 genes for immune response, 62 genes for protein localization, and only 53 genes for programmed cell death as well as 5 genes for autophagy. The function of remainder (160 genes) is yet unknown.

In our work the percentage of genes sorted according to key developmental functions corresponds to results presented by Kakhniashvili and Tyang<sup>8, 9</sup>. Interestingly, human RBCs contain 40-50% of genes encoding cell cycle processes (including 3-5% of genes for transcription/translation) as compared to only 10-20% of genes responsible for self-destruction processes.

For the first time we report about the presence of

genes in human RBCs encoding initiation, activation and regulation of transcription and translation (for instance RNA polymerises I,II,III, zinc/PHD finger-DNA-binding proteins, cysteinyl, lysyl-tRNA synthetase), important RNA-stabilising factor - poly(A) binding protein, anti-apoptotic proteins (for instance beclin 1, reticulon 4, BCL2, IAP) together with genes for RNA degradation (for example ribonuclease T2) as well as genes encoding typical apoptotic proteins such as cyclooxygenase, apoptotic protease activating factor, caspase 8. Other authors were able to show a protein synthesis in human platelets by megakaryocyte-derived mRNAs<sup>19</sup>. The finding of RNA in anucleate cells like erythrocytes support the idea of nucleus independent protein synthesis and supports data<sup>20</sup> about possible mechanism of globin m-RNA stability in human RBCs.

Further experiments are needed to understand the mechanisms and the biological meaning of these findings. But gene expression profiling of human erythrocyte could be an important key for understanding the machinery of anucleate protein synthesis and its meaning in the pathophysiology of diseases.

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### Conflict of Interest

The authors have declared that no conflict of interest exists.

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