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CELL TECHNOLOGIES IN BIOLOGY AND MEDICINE

Proteomic Profiling of HL-60 Cells during ATRA-Induced Differentiation

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Acute promyelocytic leukemia, a form of acute myeloid leukemia, is characterized by cell differentiation arrest at the promyelocyte stage. Current therapeutic options include administration of all trans-retinoic acid (ATRA), but this treatment produces many side effects. ATRA is known to induce differentiation of leukemic cells into granulocytes, but the mechanism of this process is poorly studied. We performed comparative proteomic profiling of HL-60 promyelocytic cells at different stages of ATRA-induced differentiation to identify differentially expressed proteins by high-resolution mass spectrometry and relative quantitative analysis without isotope labels. A total of 1162 proteins identified by at least two unique peptides were analyzed, among them 46 and 172 differentially expressed proteins were identified in the nuclear and cytosol fractions, respectively. These differentially expressed proteins can represent candidate targets for combination therapy of acute promyelocytic leukemia.

Key Words: *acute promyelocytic leukemia; trans-retinoic acid; targeted therapy*

At the time of the first description of the nosological entity in 1950s, acute promyelocytic leukemia (OPL) was characterized by high mortality rate, primary due to hemorrhagic complications. The median survival was less than 1 week. The disease poorly responded to treatment: the first available therapy with 6-mercaptopurine led to remission in only 5-14% patients; introduction of anthracyclines (including daunorubicin) made it possible to increase the probability of remission to 58% [4]. Discovery of differentiation-inducing effect of all trans-retinoic acid (ATRA) on leukemic cells and introduction of differentiating therapy into clinical practice made a revolution in the treatment of OPL and led to an increase in 5-year complete remission rate to 85% [23]. The main complications of ATRA monotherapy is the development of drug resistance and the risk of severe side effect, so-called differentiation syndrome or retinoic acid syndrome. In some cases, a good clinical result can be achieved due to combination of ATRA with other drugs, such as arsenic trioxide and idarubicin [8,10]. This combination therapy allows reducing the dose of ATRA and, consequently, the risk of complications and side effects associated with differential therapy. Thus, the search for new ATRA agonists is an urgent problem of modern oncology.

The search for new potential targets of tumor therapy is a laborious process and screening of potential regulators of biological processes such as differentiation and proliferation is a very important step in this process. Proteomic approaches, primarily mass spectrometry, are a suitable tool in detection of molecules involved in the realization of cell fate and having therapeutic potential [1,3,26]. Regulatory molecules,

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such as cell receptors (*e.g.* c-kit [12]) and molecules of signal pathways, *e.g.* mammalian target of rapamycin (mTOR)-mediated autophagy pathway, are generally considered among potential targets [11]. Many differentiation-related molecules, including transcription factors acting as biological signaling end-points, are located in the cell nucleus. In light of this, it seems interesting to study the dynamics of nucleus proteome of OPL cells in response to treatment with differentiation inducer ATRA.

Our aim was to identify nuclear proteins, whose content changes during ATRA-induced granulocyte differentiation of HL-60 cells, and that can serve as potential targets of combined therapy of promyelocytic leukemia. HL-60 promyelocytic leukemia cell line was chosen as the model because these cells differentiate into mature granulocytes under the action of ATRA [12,18].

MATERIALS AND METHODS

Cell cultures. Cell culture line HL-60 were obtained from the Cryobank of the V. N. Orekhovich Research Institute of Biomedical Chemistry. After defrosting, the cells were cultured in RPMI-1640 growth medium with 10% fetal calf serum, 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM L-glutamine (all reagents were from Gibco) in a CO_2 incubator under standard conditions (37°C, 5% CO_2, 80% humidity). After attaining cell concentration of 10^6 cells/ml culture, the cells were subcultured at a ratio of 1:3. The cells were counted in a Goryaev's chamber.

MTT test. The sensitivity of HL-60 cells to ATRA was evaluated by the effect of the agent on their proliferative activity. After centrifugation, the cells were resuspended in growth medium to a concentration of 10^6/ml and transferred to wells of 24-well flat-bottom plate (1 ml per well). In 2 h after cell seeding into the wells, 1 ml growth medium containing ATRA (Sigma) in concentrations of 200, 180, 160, 140, 120, 110, 100, 80, 60, 40, 20, and 2 μM was added so that the final concentration of ATRA was 100, 90, 80, 70, 60, 50, 40, 30, 20, 10, or 1 μM. In the control group, 1 ml growth medium was added.

The cells were incubated under standard conditions for 96 h, then, 200 μl of aqueous solution of MTT (Sigma) in a concentration of 5 mg/ml (final concentration 0.5 mg/ml) was added to each well and the plates were placed in the incubator for 3 h. After incubation, the content of the wells was carefully collected, transferred into Eppendorf test tubes, and centrifuged in a table centrifuge. Then, the supernatant was gently removed from the tubes, 100 μl DMSO (PanEco) was added, and the tubes were vortexed until the precipitate was fully dissolved. Optical density of the resultant solution was measured on an Infinite 200 PRO plate reader (Tecan) at λ=550 nm (reference measurements were made at λ=690 nm). Proliferative activity of cells was calculated by the formula D2/D1×100%, where D1 and D2 are optical densities corresponding to cell number before and after incubation with ATRA, respectively.

Flow cytofluorometry. Expression of surface markers on cells was analyzed by flow cytofluorometry. To this end, the cells were precipitated by centrifugation and resuspended in 1 ml PBS with 1% fetal calf serum (Gibco) and 0.1% NaN_3 (Sigma) to cell concentration of 10^6/ml; the washout procedure was repeated three times. Finally, the cells resuspended in 0.1 ml of the same solution were incubated at 4°C for 60 min with 10 μl monoclonal antibodies directly labeled with phycoerythrin (Becton Dickinson); phycoerythrin-labeled isotypic antibodies (Becton Dickinson) served as the negative control. After precipitation and washing, the cells were resuspended in 0.25 ml of the above buffer and fixed in 0.25 ml 4% paraformaldehyde over 4 min at room temperature. Then, the cell suspension was brought to 1 ml with buffer and filtered to remove cell aggregates (30 μ-pore filter).

Analysis was performed on FACSAria III cytofluorometer sorter (Becton Dickinson). The instrument was operated and the primary data were analyzed using FACSDiva software.

Marker expression was evaluated by the fluorescence intensity histogram of the label conjugated with specific monoclonal antibodies. Ten thousand events were recorded at minimum cell suspension flow rate. To exclude noise and objects less than cells, the registration threshold for direct light scattering was set at 20,000.

Further analysis of experimental data was performed using FlowJo software. To compare fluorescence intensity of the test sample and isotypical control, the results of experimental sample and control were superimposed in the histogram mode.

Preparation of samples for proteomic analysis. HL-60 cells in the growth medium were placed in 75-cm^2 culture flasks (cell concentration 10^6/ml, 10 ml medium), and 5 ml growth medium containing ATRA in a concentration of 150 μM (final concentration 50 μM) was added, and the flasks were incubated in a CO_2 incubator under standard conditions for 3, 24, or 96 h. After incubation, the cells were washed three times by centrifugation/resuspension in 10 ml PBS and the tubes with cell pellet were frozen in liquid nitrogen. The cells cultured without ATRA were used as the control corresponding to time point 0.

Lysis of cells and isolation of nuclear fraction. Nuclear fraction proteins were isolated by chemical extraction [16]. Tubes with HL-60 cell pellet were

thawed, 300 µl cold lysing buffer containing 10 mM HEPES-NaOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5% NP-40, 0.1 mM EDTA, and complete protease inhibitor cocktail (Roche) was added, the tubes were incubated for 15 min on ice and centrifuged for 10 min (6000 rpm) at 4°C. The supernatant containing the cytosolic fraction was collected and stored for further analysis. The obtained pellet containing nuclei was washed twice with lysing buffer without NP-40. For isolation of nuclear fraction, extraction buffer containing 20 mM HEPES-NaOH (pH 7.9), 25% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.1 mM EDTA, and protease inhibitor cocktail (Roche) was added, the tubes were incubated for 30 min on ice and centrifuged for 10 min (6000 rpm). The supernatant containing the nuclear fraction was collected and stored for further analysis.

Total protein concentration in samples of each fraction was measured by the colorimetric method with bicinchonic acid (BCA) using commercial Pierce BCA Protein Assay Kit (Pierce) according to manufacturer's recommendations.

Preparation of cytosolic and nuclear fraction proteins for mass spectrometric analysis. Hydrolysis of proteins was carried out according to FASP Protocol (Filter-Aided Sample Preparation) [24]. Protein mixture aliquots (100 µg) were placed in YM-10 Microcon device concentrating filters (Millipore). The samples were washed by adding 200 µl buffer containing 8 M urea with addition of 100 mM tris HCl (pH 8.5) followed by centrifugation at 11,000*g* for 15 min at 20°C. The washing procedure was repeated 3 times. The samples were then alkylated. To this end, the material was placed in concentrating filters,100 µl alkylating solution containing 50 mM iodacetamide was added, the mixture was incubated for 30 min at 25°C with shaking (600 rpm), and then precipitated for 15 min at 20°C. After alkylation, the samples were washed twice with 200 µl buffer followed by centrifugation at 11,000*g* for 40 min at 20°C. To samples in concentrating filters, 40 µl buffer for trypsinolysis containing 100 mM solution of tetraethylammonium bicarbonate (pH 8.5); then, trypsin solution was added to each sample (total weight of the enzyme/total mass protein weight 1:100 and incubated fovernight at 37°C.

After incubation with the enzyme, peptide samples were centrifuged at 11,000*g* and 20°C for 15 min and the filtrate was collected. The filters were then washed with 30% formic acid by centrifugation at 11,000*g* and 20°C for 15 min and the filtrates were also collected. The peptide mixtures obtained for the cytosol and nuclear fractions was lyophilized on a rotary concentrator and dissolved in 100 µl 0.1% formic acid. The resultant samples were analyzed by mass spectrometry.

Liquid chromatography mass spectrometry. Liquid chromatography mass spectrometry was performed for each experimental time point in 5 technical repeats. The peptide mixture was applied on concentrating Zorbax 300SB-C18 column (5 mm×0.3 mm; particle diameter 5 µ; Agilent Technologies) and mobile phase C for concentration column loading and washout (5% acetonitrile in 0.1 % formic acid and 0.05% trifluoroacetic acid) was supplied at a flow rate of 3 µl/min over 5 min. The peptides were separated on a Zorbax 300SB-C18 analytical column (3.5 µ, 150 mm×75 µ; Agilent Technologies) using mobile phase B gradient (80% acetonitrile in 0.1% formic acid) at a flow rate of 0.3 µl/min. The following parameters of acetonitrile gradient were used: the analytical column was washed with mobile 5% phase B for 5 min, after which the concentration of mobile phase B was linearly increased to 60% over 80 min and to 100% over the next 5 min; then, the analytical column was washed with 100% mobile phase B for 10 min, after which the concentration of mobile phase B was reduced to 5% over 5 min, and during the next 15 min, the analytical column was equilibrated with 5% mobile phase B.

Mass spectrometry was performed on an Orbitrap Velos hybrid mass spectrometer (Thermo Fisher Scientific) with orbitrap mass analyzer. The maximum time of accumulation of 10^6 ions for MC-scanning with resolution of 30,000 (for m/z=400) in the m/z range of 300-2000 in the positive ionization mode was 50 msec. Five most intense ions recorded in the MS-scan were selected for further fragmentation, if their absolute intensity exceeded 5000 rel. units. HCD fragmentation mode with normalized collision energy of 35% was used. Dynamic exclusion from tandem analysis was applied: the duration of exclusion was 90 sec after the ion was fragmented at least once and MS/MS-spectrum was acquired over 30 sec. The list of exclusion consisted of 500 ions. The maximum time of accumulation of 5×10^4 ions for MS/MS-scan acquisition with resolution of 7500 (for m/z=400) in the m/z range of 300-2000 in the positive ionization mode was 100 msec.

The recorded mass spectrometric data were processed using MaxQuant 1.5.5.0 (Max Planck Institute of Biochemistry).

Protein identification and relative quantitative analysis based on the area under the peak of the parent precursor ion. The proteins were identified using MaxQuant 1.5.5.0 software with Andromeda algorithm, FASTA file containing amino acid sequences of human proteins (29-03-2016), and FASTA file with inverted sequences to calculate the frequency of false positive identification (FDR). Carbamidomethylation of cysteine and oxidation of methionine were used as fixed and variable modifications, respectively. The tolerance for parent and daughter ions was 20 ppm.

For proteins and peptides, the threshold FDR was set at 0.01.

The quantitative analysis was carried out on the basis of area under the peak of the parent ion with calculation of LFQ (label-free quantification intensity) using built-in MaxQuant algorithm [5]. Statistical analysis was performed using Perseus 1.6.0.7 software (Max Planck Institute of Biochemistry). Mass spectrometry data for all experimental points (0, 3, 24, and 96 h) were compared. In addition, the data obtained at the experimental points 3, 24, and 96 h were pairwise compared with the control point 0 h. Heat maps reflecting changes in the expression of genes of proteins differentially expressed throughout the differentiation period were constructed.

The functional annotation was performed using GeneOntology Biological Process module and geneXplain platform software. Functional annotation groups containing at least 20 proteins (p<0.005) for the cytosol fraction and 7 proteins (p<0.005) for the nuclear fraction were considered.

RESULTS

Effect of ATRA on cell proliferation. The effect of ATRA on proliferation of HL-60 cells was assessed using MTT test based on the ability of mitochondrial dehydrogenases in live, but not dead cells to convert water-soluble yellow MTT into insoluble purple-blue intracellular crystals of MTT-formazan. As optical density (D) at 540-570 nm of formazan dissolved in DMSO is proportional to the number of viable cells (n), MTT-test can be used to assess viability and proliferation of cell cultures *in situ* [7].

First, we studied the effect of ATRA on proliferative activity of HL-60 cells (Fig. 1). It was found that the presence of low concentrations of ATRA (1-30 mM) in the medium did not affect cell proliferation. After increasing ATRA concentration above 30 μM, the number of viable cells decreased and reached minimum at ATRA concentration of 70 μM. ATRA in concentrations of 70 μM produced a pronounced cytotoxic effect; no viable cells were found in these cases. It is known that HL-60 cells lose proliferation capacity after differentiation into granulocytes. Thus, our primary task was to determine the concentration of ATRA that inhibited cell proliferation, but did not produce pronounced cytotoxic effect. Our findings suggest that ATRA in our experimental model was most effective in a concentration of 50 μM, because the number of viable cells in this case remained almost unchanged over 96 h of the experiment.

Changes in the expression of CD11b and CD14 in HL-60 cells under the influence of ATRA. To confirm that ATRA in a concentration of 50 μM does induce differentiation of HL-60 cells, we studied changes in the expression of CD11b and CD14 marker molecules on their surface under the influence of ATRA (Fig. 2). Simultaneous expression of CD11b (complement receptor 3) and CD14 (a component of the receptor complex CD14/TLR4/MD2 recognizing LPS) is a characteristic feature of mature granulocytes. Unlike native cells, cells cultured in a medium with 50 μM ATRA demonstrated high expression of these markers, which indicated their differentiation towards granulocytes [17].

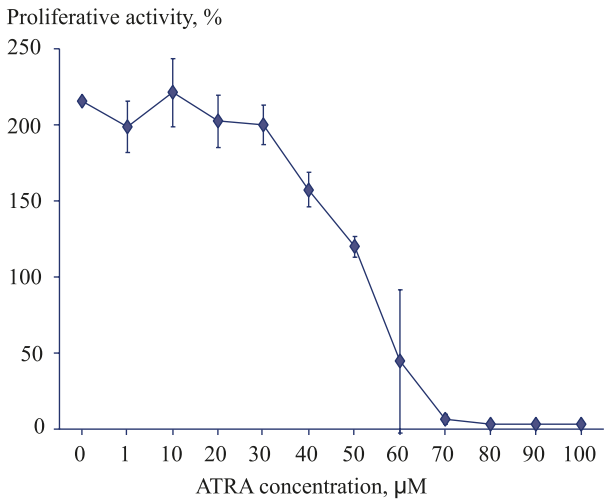

Fig. 1. Effect of different ATRA concentrations on proliferation of HL-60 cells.

Changes in proteome of HL-60 cells during ATRA-induced differentiation. In the proteomic analysis of HL-60 cells at different stages of granulocytic differentiation, nuclear proteins are particular interest, because this particular compartment functions as a regulatory center of eukaryotic cells. Nuclear proteins include proteins of the nuclear matrix, transport system of the nucleus, and transcription factors. The latter are molecules involved in the regulation of gene expression. They can play a role in the formation of various pathologies, including tumors [22]. In the context of promyelocytic leukemia, the role of nuclear proteins as the key regulators of cell fate suggests that studies of nuclear proteome is a promising field for the search of potential targets for new drugs [25].

We performed comparative liquid chromatography mass spectrometry of nuclear and cytosolic fractions of HL-60 cells at different stages of their ATRA-induced differentiation (0, 3, 24, and 96 h from the start of the experiment). A total of 1162 proteins (at least by two peptides) were identified. The analysis of mass spectrometry data revealed 172 differentially expressed proteins in the cytosolic fraction (FDR<10^{-6}) and 46 proteins FDR<10^{-6}) in the nuclear fraction (comparison in all time points). Heat maps reflecting

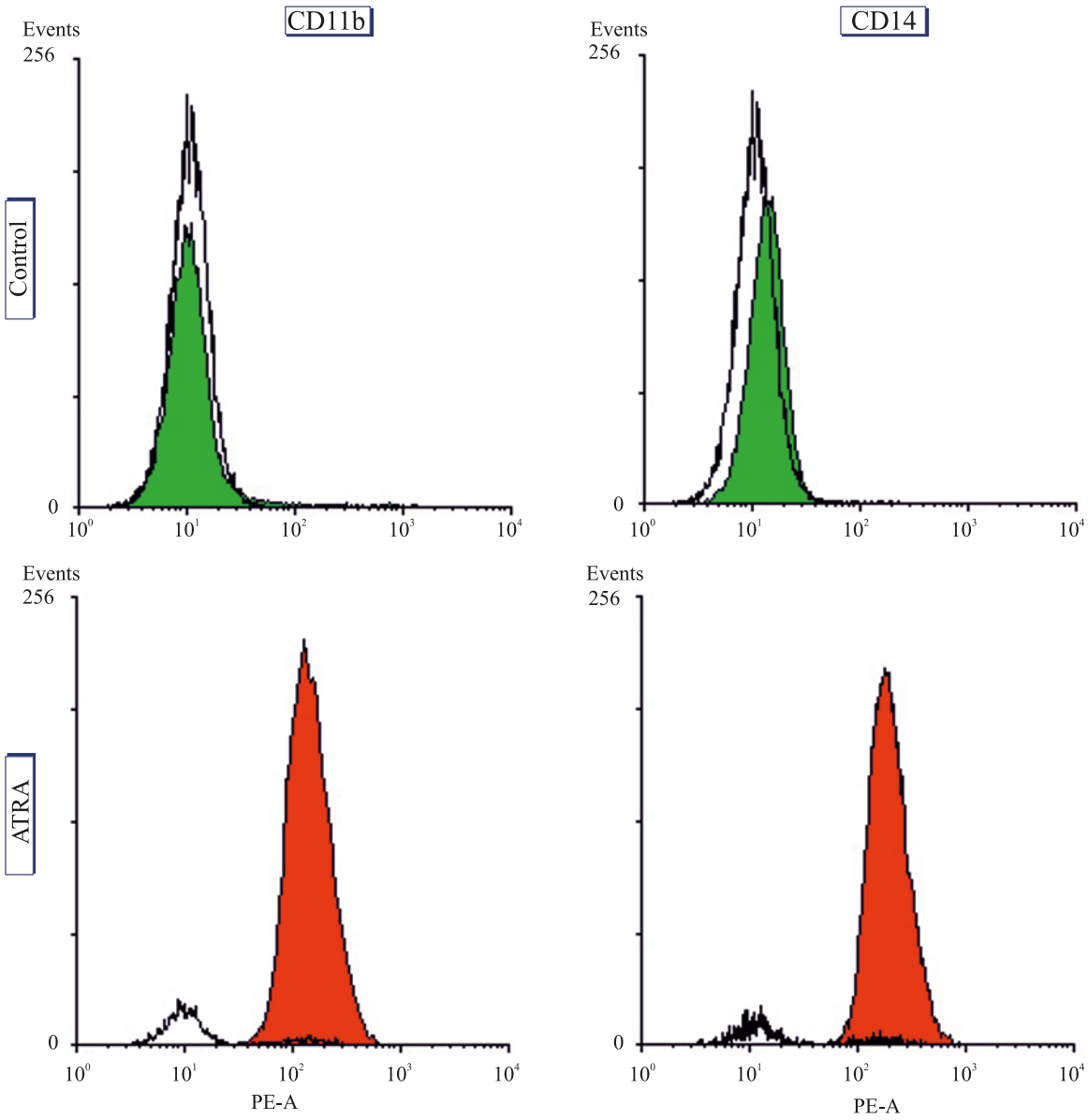

Fig. 2. Changes in the expression of CD11b and CD14 in HL-60 cells after 96-h culturing in the presence of 50 μM ATRA. Abscissa: fluorescence intensity reflecting the expression of surface marker; ordinate: number of events (cells). Isotypical control is shown by black line.

changes in the gene expression are presented in Figures 3 and 4.

Differentially expressed proteins of nuclear fraction. In the analysis of protein expression in nuclear fractions two main clusters can be isolated in all time points: by 96 h of cell differentiation, expression of 31 proteins increased under the action of ATRA and expression of 5 proteins decreased (Table 1).

Among the proteins whose content increased, special attention deserved SRSF1 (serine/arginine-rich splicing factor 1), SARS protein (serine tRNA-ligase) that interacts with VEGFA promoter in the nucleus preventing its binding to the proto-oncogene c-ICC [14], protein CLIC1 (chloride intracellular channel protein 1) involved in the regulation of the cell cycle [21], and ALOX5AP protein involved in the synthesis of leukotrienes [6]. According to the Uniprot database annotation, these proteins are located in the nucleus or its membrane.

Among proteins whose content, on the contrary, decreased, protymosin alpha (PTMA) involved in the functioning of the immune system, SND1 protein (Staphylococcal nuclease domain-containing protein 1), a phase transcription factor that regulates STAT6, MYB, and EBNA2 [19], and DNA- and RNA-binding protein NONO (Non-POU domain-containing octamer-binding protein) capable of regulating transcription [15] are worthy of note. According to the

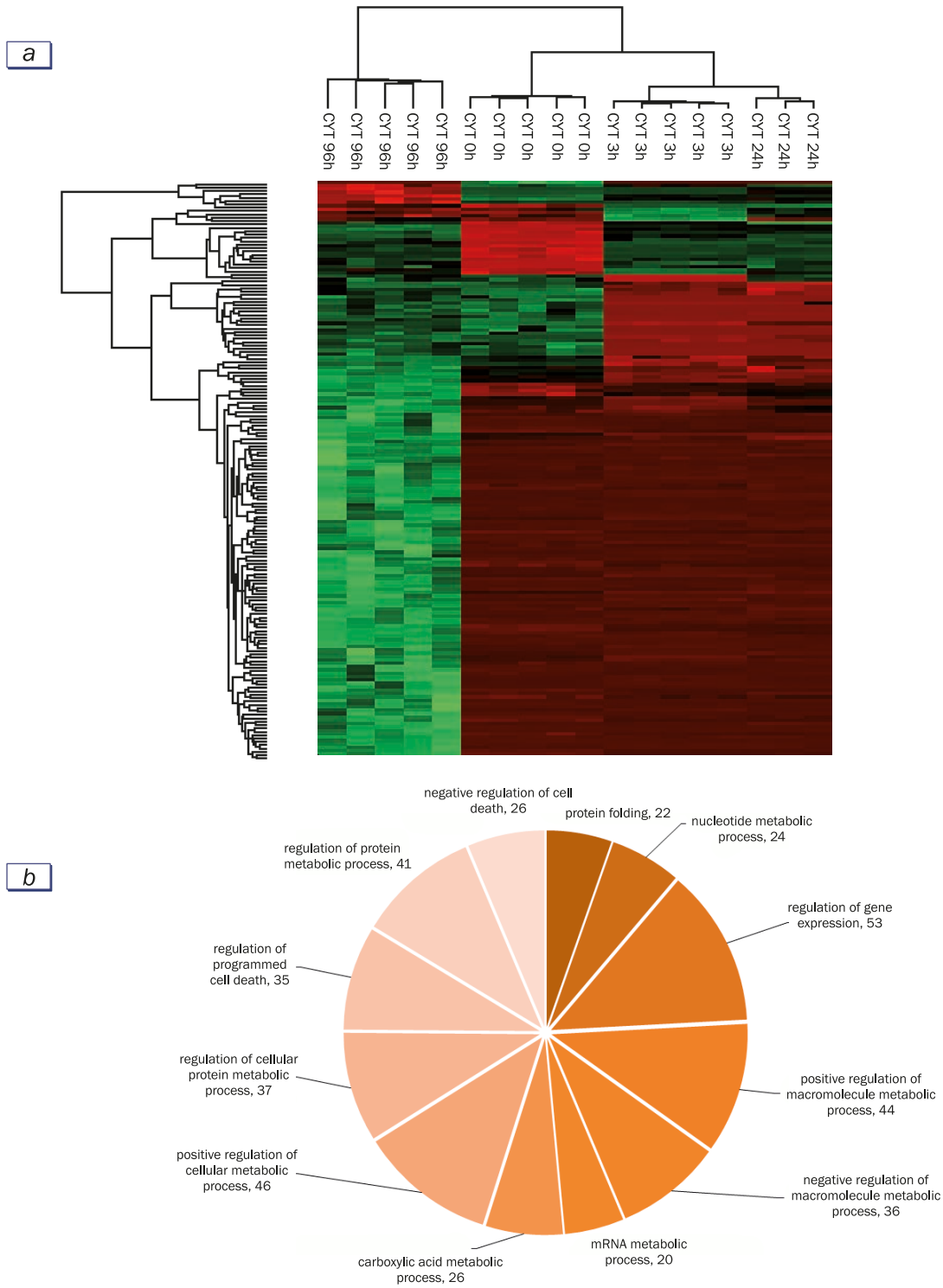

Fig. 3. Differentially expressed proteins of cytosolic fraction: 172 proteins (FC>3, multiple-sample tests ANOVA, FDR=0.0002) (*a*), functional classification of differentially expressed proteins of cytosol fraction according to GeneOntology database (Biological processes, minimal hits per group=20, p=0.005) (*b*).

Uniprot database, these proteins are located in the nucleus.

Proteins whose content uniquely varies at different stages of differentiation (Fig. 5). The results of pairwise comparison of the data of mass spectrometric analysis of samples obtained at each time point with the control are presented in Table 2. In 3 h after the addition of ATRA, the content of 16 proteins in the nuclear fraction, including regulators of alternative splicing, DNA repair and apoptosis, changed by more

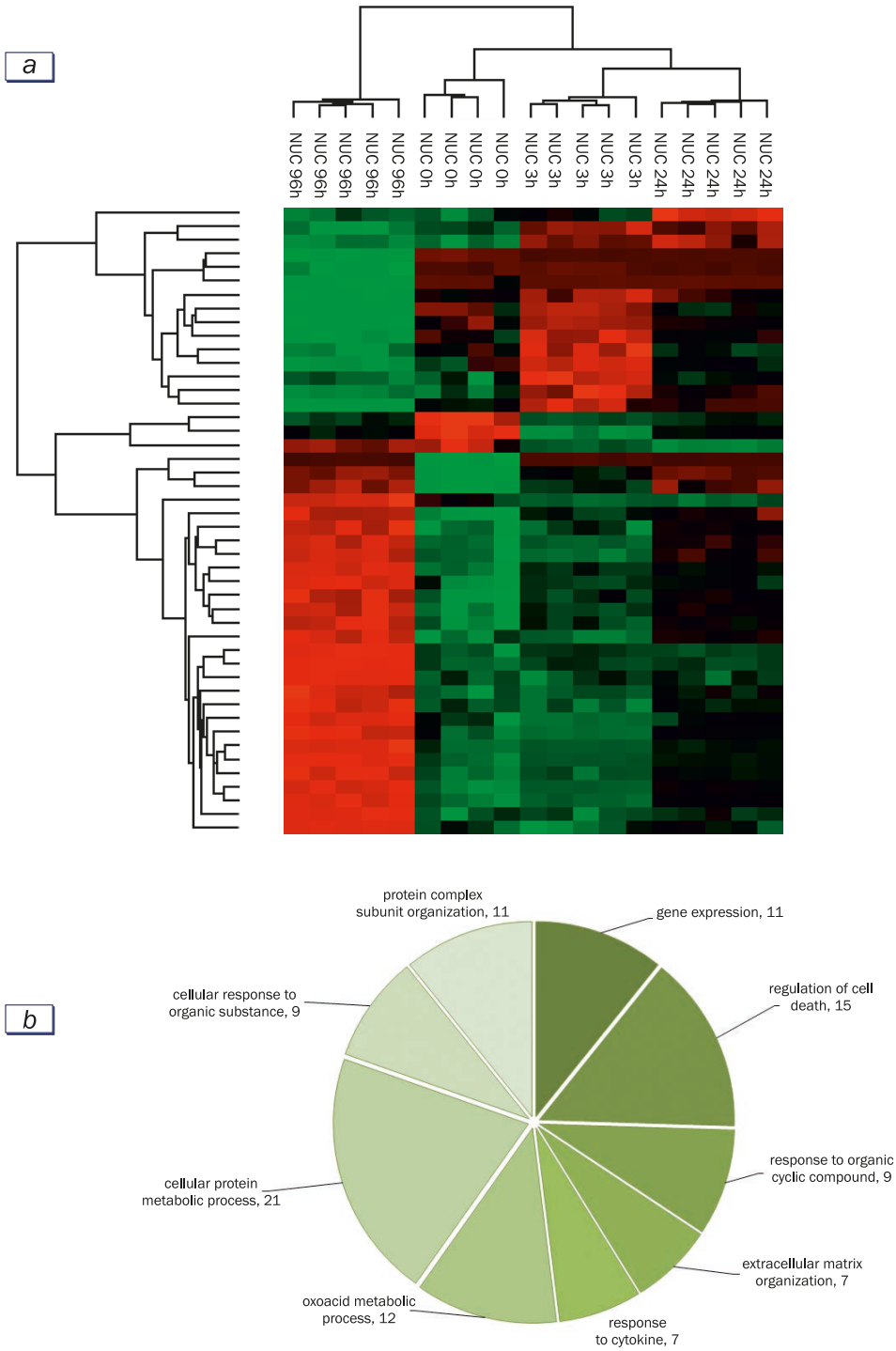

Fig. 4. Differentially expressed proteins of the nuclear fraction: 46 proteins (FC>3, multiple-sample tests ANOVA, FDR=0.0002) (*a*), functional classification of differentially expressed proteins of the nuclear fraction according to GeneOntology database (Biological processes, minimal hits per group=20, p=0.005) (*b*).

than 3 times in comparison with the control. According to the Uniprot 13 database annotation, these proteins were located in the nucleus. Of particular interest is protein UBE2V1 (CROC1) mediating activation of C-Fos proto-oncogene transcription [13]. It is involved in DNA reparation and affects cell cycle and differentiation. This protein is also involved in activation of NF-κB mediated by IL-1β, TNF, TRAF6, and TRAF2.

In 24 h after the start of differentiation, 9 unique differentially expressed proteins were revealed in the nucleus, including nucleotide exchange factor (BAG2), deubiquitinylation regulator (SHMT2), and

TABLE 1. Nuclear Fraction Proteins whose Content Differed from the Control at All Time Points of ATRA-Induced Differentiation of HL-60 Cells

Protein	AN Uniprot	Gene	ANOVA *p*-value
Increase in protein content by 96 h			
Staphylococcal nuclease domain-containing protein 1	Q7KZF4	*SND1*	1.21×10^{-8}
Heat shock protein HSP 90-beta	P08238	*HSP90AB1*	1.27×10^{-8}
Prothymosin alpha	P06454	*PTMA*	2.56×10^{-9}
60S ribosomal protein L3	P39023	*RPL3*	1.10×10^{-13}
Eukaryotic translation initiation factor 3 subunit I	Q13347	*EIF3I*	3.29×10^{-9}
Eukaryotic translation initiation factor 4 gamma 1	Q04637	*EIF4G1*	2.59×10^{-10}
Non-POU domain-containing octamer-binding protein	Q15233	*NONO*	5.27×10^{-9}
S-adenosylmethionine synthase isoform type-2	P31153	*MAT2A*	2.82×10^{-8}
Elongation factor 2	P13639	*EEF2*	6.04×10^{-10}
Multifunctional protein ADE2	P22234	*PAICS*	6.39×10^{-9}
L-lactate dehydrogenase A chain	P00338	*LDHA*	2.29×10^{-8}
Polypyrimidine tract-binding protein 1	P26599	*PTBP1*	4.10×10^{-9}
Glyceraldehyde-3-phosphate dehydrogenase	P04406	*GAPDH*	1.04×10^{-8}
Elongation factor 1-beta	P24534	*EEF1B2*	3.92×10^{-8}
Deoxyuridine 5-triphosphate nucleotidohydrolase, mitochondrial	P33316	*DUT*	5.66×10^{-9}
Decrease in protein content by 96 h			
40S ribosomal protein S8	P62241	*RPS8*	1.39×10^{-9}
Serine/arginine-rich splicing factor 1	Q07955	*SRSF1*	3.21×10^{-12}
Histone H4	P62805	*HIST1H4A*	2.22×10^{-8}
Serine — tRNA ligase, cytoplasmic	P49591	*SARS*	9.42×10^{-13}
Moesin	P26038	*MSN*	6.50×10^{-11}
Tyrosine — tRNA ligase	P54577	*YARS*	8.70×10^{-10}
Glutamate dehydrogenase 1	P00367	*GLUD1*	9.19×10^{-10}
Endoplasmin	P14625	*HSP90B1*	4.68×10^{-9}
Cofilin-1	P23528	*CFL1*	6.93×10^{-9}
Peptidyl-prolyl cis-trans isomerase B	P23284	*PPIB*	9.29×10^{-10}
Galectin-1	P09382	*LGALS1*	3.47×10^{-10}
Protein disulfide-isomerase	P07237	*P4HB*	6.63×10^{-10}
Annexin A1	P04083	*ANXA1*	1.94×10^{-9}
Protein disulfide-isomerase A4	P13667	*PDIA4*	2.13×10^{-8}
Plastin-2	P13796	*LCP1*	8.18×10^{-10}
Chloride intracellular channel protein 1	O00299	*CLIC1*	1.26×10^{-8}
Coactosin-like protein	Q14019	*COTL1*	7.51×10^{-11}
Malate dehydrogenase, mitochondrial	P40926	*MDH2*	5.64×10^{-13}
NAD-dependent malic enzyme, mitochondrial	P23368	*ME2*	2.16×10^{-14}
Intercellular adhesion molecule 1	P05362	*ICAM1*	4.50×10^{-10}
Synaptic vesicle membrane protein VAT-1 homolog	Q99536	*VAT1*	4.50×10^{-9}
Phosphoserine aminotransferase	Q9Y617	*PSAT1*	2.98×10^{-11}
10 kDa heat shock protein, mitochondrial	P61604	*HSPE1*	2.39×10^{-9}
Annexin A6	P08133	*ANXA6*	4.42×10^{-11}
6-phosphogluconate dehydrogenase, decarboxylating	P52209	*PGD*	2.08×10^{-12}
78 kDa glucose-regulated protein	P11021	*HSPA5*	1.33×10^{-14}
Annexin A5	P08758	*ANXA5*	2.25×10^{-12}
Protein disulfide-isomerase A3	P30101	*PDIA3*	4.15×10^{-14}
Cathepsin D	P07339	*CTSD*	2.67×10^{-14}
Metalloproteinase inhibitor 1	P01033	*TIMP1*	5.75×10^{-10}
Arachidonate 5-lipoxygenase-activating protein	P20292	*ALOX5AP*	8.32×10^{-9}

TABLE 2. Nuclear Fraction Proteins Whose Content Significantly Differed from the Control Only in One Time Point: 3, 24, or 96 h (Proteins Identified by at Least Two Proteotypic Peptides) (p<0.01)

Protein	AN Uniprot	Gene	FC	Intracellular localization, Uniprot database annotation
3 h vs. 0 h				
Probable 28S rRNA (cytosine(4447)-C(5))-methyltransferase	P46087	*NOP2*	0.2	Nucleus
40S ribosomal protein S10	P46783	*RPS10*	0.3	Nucleus
Eukaryotic translation initiation factor 3 subunit E	P60228	*EIF3E*	3.3	Nucleus (PML bodies), cytoplasm
Acylamino-acid-releasing enzyme	P13798	*APEH*	3.3	Cytoplasm
Methylosome subunit pICln	P54105	*CLNS1A*	3.9	Nucleus (PML bodies)
N-alpha-acetyltransferase 50	Q9GZZ1	*NAA50*	3.9	Nucleus
Small nuclear ribonucleoprotein G;	P62308	*SNRPG*	4.0	Nucleus, cytoplasm
Ubiquitin-conjugating enzyme E2 variant 1	Q13404	*UBE2V1*	4.4	Nucleus
Protein arginine N-methyltransferase 1	Q99873	*PRMT1*	5.3	Nucleus, cytoplasm
Myeloblastin	P24158	*PRTN3*	5.7	Secretory protein
Proteasome subunit beta type-1	P20618	*PSMB1*	7.3	Nucleus, cytoplasm
Heterogeneous nuclear ribonucleoprotein K	P61978	*HNRNPK*	7.3	Nucleus, nucleoplasm
Eukaryotic translation initiation factor 3 subunit B	P55884	*EIF3B*	7.8	Cytoplasm
Superoxide dismutase [Cu-Zn]	P00441	*SOD1*	8.7	Nucleus, mitochondria, cytoplasm
RNA-binding protein 8A	Q9Y5S9	*RBM8A*	10.7	Nucleus
Translin	Q15631	*TSN*	13.7	Nucleus, cytoplasm
24 h vs. 0 h				
BAG family molecular chaperone regulator 2	O95816	*BAG2*	0.5	Cytoplasm
Serine hydroxymethyltransferase	P34897	*SHMT2*	2.2	Nucleus, mitochondria, cytoplasm
Cytosolic non-specific dipeptidase	Q96KP4	*CNDP2*	2.2	Cytoplasm
GTP-binding protein SAR1a	Q9NR31	*SAR1A*	2.6	Endoplasmic reticulum, Golgi complex
Eukaryotic translation initiation factor 3 subunit H	O15372	*EIF3H*	3.4	Cytoplasm
Farnesyl pyrophosphate synthase	P14324	*FDPS*	3.5	Cytoplasm
Eukaryotic translation initiation factor 1	P41567	*EIF1*	3.5	Nucleus, cytoplasm
Lymphocyte cytosolic protein 2	Q13094	*LCP2*	4.6	Cytoplasm
UTP — glucose-1-phosphate uridylyltransferase	Q16851	*UGP2*	5.2	Cytoplasm
96 h vs. 0 h				
60S ribosomal protein L11	P62913	*RPL11*	0.04	Nucleus
DNA replication licensing factor MCM3	P25205	*MCM3*	0.05	Nucleus
DNA replication licensing factor MCM4	P33991	*MCM4*	0.07	Nucleus
DNA replication licensing factor MCM7	P33993	*MCM7*	0.07	Nucleus
DNA replication licensing factor MCM5	P33992	*MCM5*	0.08	Nucleus, cytosol
ATP-dependent RNA helicase DDX18	Q9NVP1	*DDX18*	0.09	Nucleus, chromosomes
DNA replication licensing factor MCM6	Q14566	*MCM6*	0.09	Nucleus

TABLE 2.

Protein	AN Uniprot	Gene	FC	Intracellular localization, Uniprot database annotation
60S ribosomal protein L10	P27635	*RPL10*	0.10	Nucleus, cytosol, endoplasmic reticulum
60S acidic ribosomal protein P1	P05386	*RPLP1*	0.10	Cytosol, exosomes
RNA-binding protein FUS	P35637	*FUS*	0.10	Nucleus
Nucleolar and coiled-body phosphoprotein 1	Q14978	*NOLC1*	0.10	Nucleus
14-3-3 protein eta	Q04917	*YWHAH*	0.10	Cytosol, exosomes, mitochondria, plasma membrane
Peroxisomal multifunctional enzyme type 2	P51659	*HSD17B4*	0.11	Peroxisomes
Cytochrome c	P99999	*CYCS*	0.11	Mitochondria
Nucleolar RNA helicase 2	Q9NR30	*DDX21*	0.12	Nucleus
DNA replication licensing factor MCM2	P49736	*MCM2*	0.13	Nucleus
Eukaryotic translation initiation factor 3 subunit I	Q13347	*EIF3I*	0.14	Cytoplasm
Hsc70-interacting protein	P50502	*ST13*	0.14	Cytoplasm
FACT complex subunit SSRP1	Q08945	*SSRP1*	0.14	Nucleus, chromosomes
DAZ-associated protein 1	Q96EP5	*DAZAP1*	0.14	Nucleus
Splicing factor 3A subunit 3	Q12874	*SF3A3*	0.15	Nucleus (nuclear speckles)
Splicing factor, proline- and glutamine-rich	P23246	*SFPQ*	0.16	Nucleus, nuclear matrix
60S ribosomal protein L14	P50914	*RPL14*	0.17	Cytosol, exosomes, plasma membrane
40S ribosomal protein S28	P62857	*RPS28*	0.17	Cytosol
40S ribosomal protein S27	P42677	*RPS27*	0.17	Nucleus, cytosol
60S ribosomal protein L19	P84098	*RPL19*	0.17	Cytosol
Serine/threonine-protein kinase VRK1	Q99986	*VRK1*	0.18	Nucleus
DNA-dependent protein kinase catalytic subunit	P78527	*PRKDC*	0.19	Nucleus
Poly [ADP-ribose] polymerase 1	P09874	*PARP1*	0.19	Nucleus
40S ribosomal protein S17	P08708	*RPS17*	0.20	Cytosol, extracellular matrix, nucleoplasm
DNA topoisomerase 2-beta	Q02880	*TOP2B*	0.20	Nucleus
Unconventional myosin-Ig	B0I1T2	*MYO1G*	0.20	Plasma membrane
Nucleolar transcription factor 1	P17480	*UBTF*	0.21	Nucleus
Transformer-2 protein homolog beta	P62995	*TRA2B*	0.21	Nucleus
Heterogeneous nuclear ribonucleoprotein L	P14866	*HNRNPL*	0.21	Nucleus
RNA-binding protein EWS	Q01844	*EWSR1*	0.21	Nucleus, cytoplasm, plasma membrane
Beta-adrenergic receptor kinase 1	P25098	*ADRBK1*	0.22	Cytoplasm, plasma membrane
Eukaryotic translation initiation factor 4B	P23588	*EIF4B*	0.22	Cytosol
Paraspeckle component 1	Q8WXF1	*PSPC1*	0.22	Nucleus
Receptor of activated protein C kinase 1	P63244	*GNB2L1*	0.22	Nucleus, cytoplasm, plasma membrane
Septin-6	Q14141	*SEPT6*	0.23	Cytoplasm
Mitotic checkpoint protein BUB3	O43684	*BUB3*	0.24	Nucleus

TABLE 2.

Protein	AN Uniprot	Gene	FC	Intracellular localization, Uniprot database annotation
Trifunctional purine biosynthetic protein adenosine-3	P22102	*GART*	0.25	Cytosol, exosomes
Endothelial differentiation-related factor 1	O60869	*EDF1*	0.25	Nucleus
Eukaryotic translation initiation factor 2 subunit 2	P20042	*EIF2S2*	0.25	Cytoplasm
General transcription factor II-I	P78347	*GTF2I*	0.25	Nucleus
UPF0568 protein C14orf166	Q9Y224	*C14orf166*	0.26	Nucleus, cytoplasm
RNA-binding motif protein, X chromosome	P38159	*RBMX*	0.26	Nucleus
Replication protein A 70 kDa DNA-binding subunit	P27694	*RPA1*	0.27	Nucleus
Spectrin beta chain, non-erythrocytic 1	Q01082	*SPTBN1*	0.28	Cytoskeleton
Nucleoplasmin-3	O75607	*NPM3*	0.29	Nucleus
40S ribosomal protein S3	P23396	*RPS3*	0.29	Nucleus, cytoplasm, mitochondria
Eukaryotic translation initiation factor 4 gamma 1	Q04637	*EIF4G1*	0.29	Nucleus, cytoplasm
Heterogeneous nuclear ribonucleoprotein A1	P09651	*HNRNPA1*	0.29	Nucleus, cytoplasm
116 kDa U5 small nuclear ribonucleoprotein component	Q15029	*EFTUD2*	0.29	Nucleus
40S ribosomal protein S12	P25398	*RPS12*	0.29	Cytoplasm
Polyadenylate-binding protein 1	P11940	*PABPC1*	0.31	Nucleus, cytoplasm
S-adenosylmethionine synthase isoform type-2	P31153	*MAT2A*	0.31	Cytoplasm
Structural maintenance of chromosomes flexible hinge domain-containing protein 1	A6NHR9	*SMCHD1*	0.32	Nucleus
Aspartate--tRNA ligase, mitochondrial	Q6PI48	*DARS2*	0.32	Mitochondria
Pre-mRNA-processing-splicing factor 8	Q6P2Q9	*PRPF8*	0.33	Nucleus
40S ribosomal protein S5	P46782	*RPS5*	0.34	Nucleoplasm, cytoplasm, exosomes
10 kDa heat shock protein, mitochondrial	P61604	*HSPE1*	3.01	Mitochondria
Asparagine—tRNA ligase, cytoplasmic	O43776	*NARS*	3.03	Cytoplasm
Histone H1.5	P16401	*HIST1H1B*	3.03	Nucleus
Transaldolase	P37837	*TALDO1*	3.05	Cytoplasm
Peroxiredoxin-5, mitochondrial	P30044	*PRDX5*	3.07	Mitochondria, peroxisomes
Malate dehydrogenase, mitochondrial; Malate dehydrogenase	P40926	*MDH2*	3.16	Mitochondria
Talin-1	Q9Y490	*TLN1*	3.16	Cytoplasm
Intercellular adhesion molecule 1	P05362	*ICAM1*	3.21	Plasma membrane
Single-stranded DNA-binding protein, mitochondrial	Q04837	*SSBP1*	3.38	Mitochondria
NAD-dependent malic enzyme, mitochondrial	P23368	*ME2*	3.41	Mitochondria
Protein FAM49B	Q9NUQ9	*FAM49B*	3.42	Plasma membrane
Annexin A11	P50995	*ANXA11*	3.54	Nucleus, cytoplasm
Protein canopy homolog 2	Q9Y2B0	*CNPY2*	3.97	Nucleus, cytoplasm
Protein canopy homolog 2	Q9Y2B0	*CNPY2*	3.97	Nucleus, cytoplasm

TABLE 2.

Protein	AN Uniprot	Gene	FC	Intracellular localization, Uniprot database annotation
ERO1-like protein alpha	Q96HE7	*ERO1L*	4.05	Endoplasmic reticulum
Protein S100-A9	P06702	*S100A9*	4.11	Nucleus, cytoplasm, microvillus membrane
Annexin A4; Annexin	P09525	*ANXA4*	4.11	Nucleus, exosomes
V-type proton ATPase subunit B, brain isoform	P21281	*ATP6V1B2*	4.47	Endosomes
Hexokinase-1	P19367	*HK1*	4.56	Mitochondria
Pyruvate kinase PKM	P14618	*PKM*	4.85	Nucleus (translocation in response to apoptotic stimulus), cytoplasm
Fructose-1,6-bisphosphatase 1	P09467	*FBP1*	5.24	Mitochondria
F-actin-capping protein subunit alpha-2	P47755	*CAPZA2*	5.39	Cytoskeleton, cytoplasm, exosomes
Protein-tyrosine-phosphatase; receptor-type tyrosine-protein phosphatase C	P08575	*PTPRC*	5.45	Plasma membrane
Leukocyte elastase inhibitor	P30740	*SERPINB1*	5.54	Cytoplasm
Protein-glutamine gamma-glutamyltransferase 2	P21980	*TGM2*	5.58	Cytoplasm, mitochondria
Cytochrome b-c1 complex subunit 1, mitochondrial	P31930	*UQCRC1*	5.62	Mitochondria
Tropomyosin alpha-4 chain	P67936	*TPM4*	5.64	Cytoskeleton
Proteasome subunit beta type-8	P28062	*PSMB8*	5.72	Nucleus
Core histone macro-H2A.1	O75367	*H2AFY*	6.01	Nucleus
Nicotinamide phosphoribosyltransferase	P43490	*NAMPT*	6.05	Nucleus
Pleckstrin	P08567	*PLEK*	6.10	Cytoplasm
Lactoylglutathione lyase	Q04760	*GLO1*	6.15	Nucleus, cytoplasm
Prostaglandin E synthase 3	Q15185	*PTGES3*	6.35	Cytoplasm
Dipeptidyl peptidase 3	Q9NY33	*DPP3*	6.44	Cytoplasm
Interleukin-8	P10145	*CXCL8*	6.46	Secretory protein
Protein S100-P	P25815	*S100P*	6.85	Nucleus, cytoplasm, microvillus membrane
Glutathione S-transferase omega-1	P78417	*GSTO1*	6.92	Cytoplasm
V-type proton ATPase subunit G 1	O75348	*ATP6V1G1*	7.10	Plasma membrane, exosomes
BTB/POZ domain-containing protein KCTD12	Q96CX2	*KCTD12*	7.27	Plasma membrane
Glucose-6-phosphate 1-dehydrogenase	P11413	*G6PD*	8.85	Cytoplasm, exosomes
Protein S100-A8	P05109	*S100A8*	8.89	Plasma membrane, secretory protein, cytoskeleton
Glutathione reductase, mitochondrial	P00390	*GSR*	9.09	Mitochondria
Arachidonate 5-lipoxygenase-activating protein	P20292	ALOX5AP	9.29	Mitochondria
Histone H1x	Q92522	*H1FX*	9.90	Nucleus
Tryptophan—tRNA ligase, cytoplasmic; T1-TrpRS;T2-TrpRS	P23381	*WARS*	9.90	Cytoplasm
Plasminogen activator inhibitor 2	P05120	*SERPINB2*	9.99	Secretory protein
C-C motif chemokine 2	P13500	*CCL2*	10.36	Secretory protein
Metalloproteinase inhibitor 1	P01033	*TIMP1*	20.12	Secretory protein

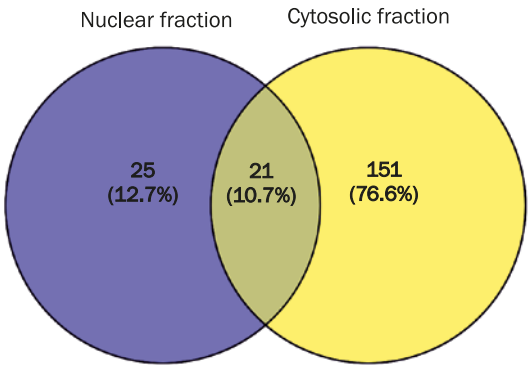

Fig. 5. Venn's diagram for differentially expressed proteins of nuclear and cytosolic fractions of HL-60 cells. The diagram shows the number of unique and common proteins for both fractions. The intersection between the fractions of differentially expressed proteins includes 21 proteins

MAPK signal pathway activator (CNDP2). The latter was shown to participate in the development of gastric cancer [27].

In 96 h, 110 proteins were uniquely differentially expressed in the nuclear fraction; 17 of them are involved in the functioning of the immune system: S100P, S100A8, SFPQ, S100A9, PKM1, CAPZA2, SERPINB1, CXCL8, ICAM1, CCL2, ANXA1, PRKDC, PSMB8, MYO1G, RPS17, PSPC1, and PTPRC (GO annotation, categories Biological process; Uniprot database). Thus, the content of CXCL8, a factor involved in neutrophil activation [20], was elevated by more than 6 times (FC=6.46). Proteins SFPQ and PSPC1 form a heterodimer that is a component of the nuclear paraspeckles [29]. We observed parallel reduction in the content of both components of the heterodimer (FC SFPQ=0.16 and FC PSPC1=0.22). Interestingly, the content of proteins involved in DNA replication and repair (MCM3, MCM4, MCM7, MCM5, DDX18, MCM6, FUS, NOLC1, DDX21, MCM2, SSRP1, PRKDC, PARP1, TOP2B, BUB3, and RPA1) was also reduced by more than 5 times. By the end of differentiation, proteins exhibiting activity of transcription factors were detected in the nuclei: UBTF (FC=0.21), EDF1 (FC=0.25), GTF2I (FC=0.25), RBMX (FC=0.25), and FUS (FC=0.1).

Our results confirm the general hypothesis that ATRA-induced differentiation of HL-60 cells is accompanied by significant changes in the content of various proteins involved in the regulation of gene expression in the nucleoplasm of these cells. The use of high-resolution comparative chromatography-mass spectrometry allowed us to identify some proteins that potentially can modulate development of tumors, including OPL. We referred to protein descriptions in Uniprot database, one of the most relevant protein databases. Deciphering of the mechanisms of action of the detected functionally active protein molecules as well as profound study of proteins, whose functions are currently unknown were beyond the scope of our study. Nevertheless, our results are of utmost interest, because they help to narrow the search for potential molecular targets for targeted anti-leukemia therapy.

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