ISSN 1990-7508, Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry, 2016, Vol. 10, No. 2, pp. 122–137. © Pleiades Publishing, Ltd., 2016.
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Transcriptomics and Proteomics in Studies of Induced Differentiation of Leukemia Cells

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Received August 10, 2015

Abstract—Induced differentiation of leukemia cells is in the focus of basic and applied biomedical studies for more than 30 years. During this period specific regulatory molecules involved in the maturation process have been identified by biochemical and molecular biological methods. Recent developments of high-throughput transcriptomic and proteomic techniques made it possible to analyze large sets of mRNA and proteins; this resulted in identification of functionally important signal transduction pathways and networks of molecular interactions, and thus extent existing knowledge on the molecular mechanisms of induced differentiation. Despite significant achievements in studies of induced differentiation, many problems associated with the molecular mechanism of cell maturation, a phenomenon of therapeutic resistance of leukemic cells still need better understanding and thus require further detailed study. Transcriptomics and proteomics methods provide a suitable methodological platform for the implementation of such studies. This review highlights the use of transcriptomic and proteomic methods in studies aimed at various aspects of the induced differentiation. Special attention is paid to the employment of the systems approach for investigation of various aspects of cell maturation. The use of the systems approach in studies of induced differentiation is an important step for transition from the formal data accumulation on expression of mRNA and proteins towards creating models of biological processes in silico.

Keywords: HL60 cell line, induced differentiation, transcriptomics, proteomics, systems biology

DOI: 10.1134/S1990750816020086

INTRODUCTION

Transcriptomics and proteomics are biological omics disciplines aimed at general analysis of all the transcripts and proteins of the cell in their structural and functional relationship. In contrast to classical biochemical methods, studies performed within the framework of systems biology employ an integrated approach in which all analyzed results are important. The advantages of high performance and sensitivity that provide transcriptomics and proteomics, are used in studies of biological processes in health and disease. In this context acute promyelocytic leukemia attracts much attention as a model for the study of cell differentiation.

Acute promyelocytic leukemia (APL) is a form of acute myeloid leukemia, which accounts for 5–15% of cases of leukemia in adults [1]. For a long time APL was characterized by a high degree of malignancy, due to the development of life-threatening bleeding caused by thrombocytopenia or disseminated intravascular coagulation [2]. In 1976, the antitumor effect of retinoic acid was recognized [3], and in 1980 it was found that all-*trans* retinoic acid (ATRA) was able to induce terminal differentiation of the HL60 promyelocytic leukemia cell line [4]. Introduction of ATRA-based drugs (Tretinoin, Vesanoid) to the clinical practice allowed to achieve remission in 90–95% of APL patients, with 5-year disease-free survival rate of 86% [5, 6]. Discovery of the differentiating effect of ATRA on tumor cells not only opened a new direction in the differentiating treatment of leukemia, but also made it possible to study the process of cell maturation [4]. Certain evidence exists that a chimeric receptor protein PML-RARα formed in the result of chromosomal translocation t(15;17)(q22;q21) is the etiological factor in the development of 95% of cases of promyelocytic leukemia [6, 7]. Despite detection of nuclear corepressors (N-CoR, SMRT, HDAC) and coactivators (P/CAF, p300/HAT) [6], involved in the cell response to the action of ATRA (Fig. 1), the mechanism of induced differentiation of leukemic cells still remains unclear.

Besides ATRA, the APL cell differentiation can be also induced by such compounds as dimethyl sulfoxide (DMSO), vitamin D_3, 12-O-tetradecanoylphorbol-13-acetate (TPA), arsenic trioxide (ATO); interestingly, tumor cell differentiation occurs in various directions of myeloid differentiation (granulocytic or monocyte-macrophage) in dependence of selected inductor [8].

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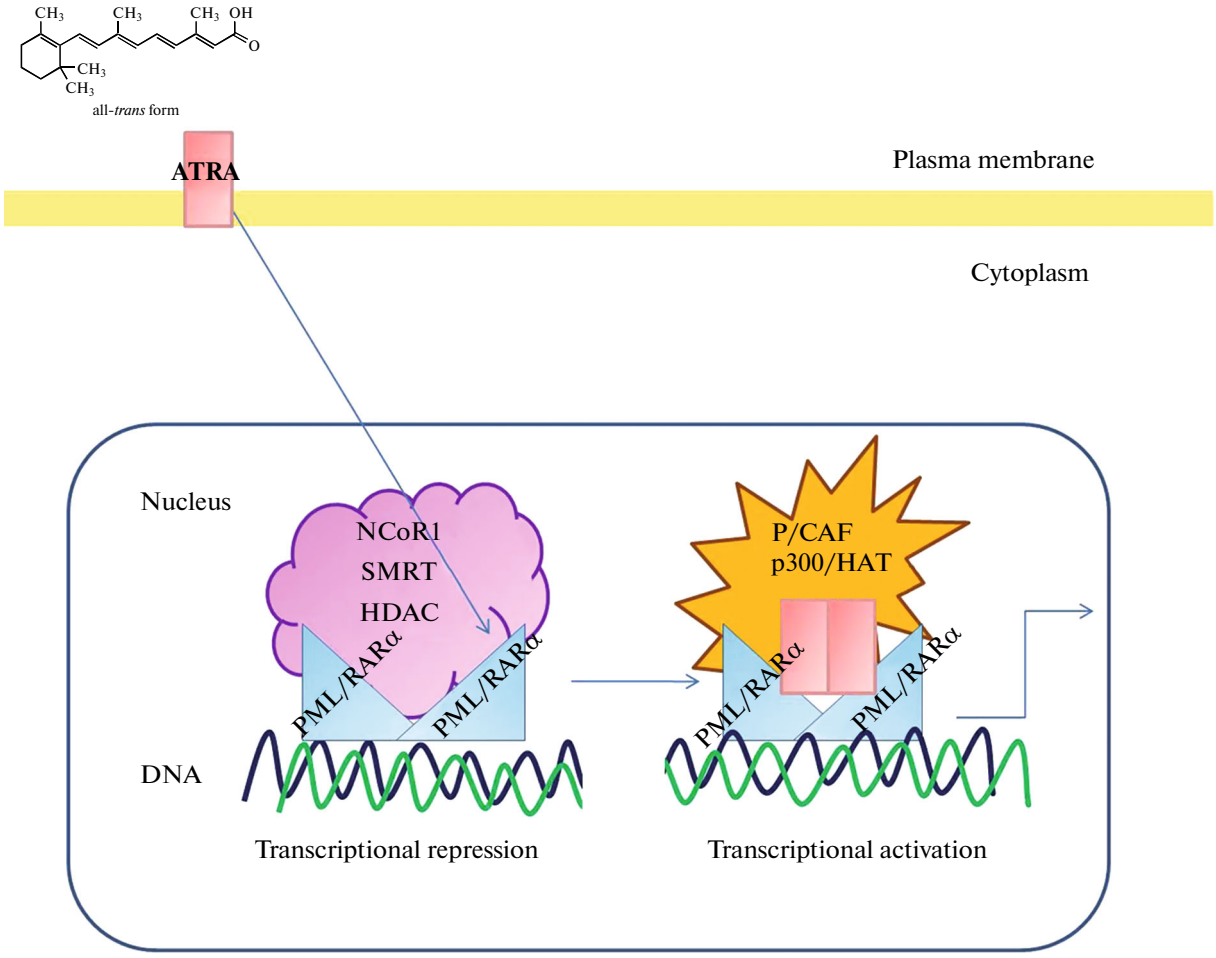

Fig. 1. The effect of ATRA on leukemia cells containing a chimeric receptor protein PML-RARα formed during translocation t(15;17)(q22;q21).

In addition to the chromosomal translocation t(15;17)(q22;q21), detailed genomic analysis of myeloid leukemia cells revealed additional genetic mutations blocking cell maturation. For example, a extensive deletion in the p53 coding gene resulted in the loss of expression of this tumor suppressor [9], amplification of c-MYC and FMS-like tyrosine kinase receptor (FTL3) genes caused their overexpression and stimulated proliferation [10–12]. All these results suggest both heterogeneous nature of acute myeloid leukemia (AML), and indicate that the cell has a flexible regulatory system that allows to overcome the differentiation block through activation of various signaling pathways. Understanding the complex system of molecular bypasses crucial for maintenance of the balance in proliferation/differentiation may open the way for the development of new approaches for the AML treatment. In turn, identification of the components of the molecular mechanisms requires large-scale studies of quantitative and qualitative composition of molecules at different levels of cell organization.

The hypothesis on the involvement of specific mRNAs or proteins in the differentiation process is often verified by molecular biological approaches, such as transfection of expression constructs into tumor cells or, alternatively, gene knockdown. Such studies, demonstrating a clear effect of the test molecule, are characterized by low productivity; moreover observed decrease in the proliferation and development of apoptosis can also originate from non-specific inhibition of protein synthesis [13]. On the other hand, the differentiation process is accompanied by sequential changes in the content of the mRNA and proteins: some of them are necessary for formation of a mature phenotype, while others may serve as regulatory molecules, driving the cell towards proliferation or differentiation.

Independently, transcriptomics and proteomics, provide a powerful methodological platform for investigation of the molecular composition of cells at the level of mRNA and protein, but the most complete understanding of the mechanism of implementation

of the cell differentiation requires complex consideration and integration of transcriptomic and proteomic data. This problem may be solved by bioinformatics methods, which provide a systemic approach to the analysis of transcripts and proteins obtained in experiments. Studies performed in the framework of systems biology allow represent an important step for transition from the formal data accumulation on expression of mRNA and proteins towards creating models of biological processes in silico.

This review summarizes the results of studies of leukemic cell differentiation, primarily obtained by means of differential profiling; it contains some examples of the use of the systems biology for decoding mechanisms of induced differentiation.

1. TRANSCRIPTOMIC AND PROTEOMIC METHODS AS A METHODOLOGICAL PLATFORM FOR STUDIES OF INDUCED DIFFERENTIATION

Transcriptome analysis, i.e., analysis of all types of RNA of the biological object is used to accomplish many tasks, such as mRNA cataloging in the cell, determination of the transcription start sites, quantitative analysis of transcript level at different stages of cell development and under certain physiological or pathological conditions. Transcriptomics has a great potential in the genome-wide analysis on high-density RNA microarrays. Since concentration of certain proteins in the cell is very low so that proteomic methods of their analysis are basically inapplicable, the transcriptome analysis is the only approach allowing to investigate induced differentiation by detecting and determining the expression level of corresponding transcripts.

Using transcriptome analysis it is possible to register the behavior of immediate response genes immediately after addition of the inductor. Expression of such genes can significantly vary without detectable changes at the protein level, due to low rates of translation and therefore proteomic methods can not register these changes. Since transcriptomic profiling provides information about the expression of all genes in the cell, transcriptomic data are used in bioinformatics studies to search for signaling pathways and to generate interactome models [14]. The disadvantage of transcriptomic methods consists in the lack of information on the effectiveness of translation and post-translational modifications of corresponding protein products. The level of mRNA expression often poorly correlates with the levels of the corresponding protein content; this may be attributed to alternative splicing, and different rates of translation and degradation of mRNA and proteins [15, 16]. Finally, the phenotypic features of cells directly related to functions of these cells in the body, are mainly determined by qualitative composition and the levels of proteins, analyzed by proteomic techniques.

Proteomic analysis, i.e., analysis of all the proteins of the cell, tissue, or any other biological object, aims at identifying qualitative and quantitative composition in a particular mixture of proteins. Changes in the concentration and composition of proteins are of great importance for determination of the status of the biological system and signs of impairments of its normal physiological state induced by chemical or physical agents [17, 18].

Methods of proteomic analysis include mass spectrometry, which may be used independently for performance of high-throughput protein analysis or in combination with other proteomic techniques, primarily two-dimensional (2D) gel electrophoresis. In the context of systems biology, proteomics based on the use of mass spectrometry, provides various types of experimental data. Firstly, using mass spectrometric analysis it is possible to identify all the elements of the primary structure of proteins, including post-translational modifications. Secondly, using quantitative mass spectrometry proteomics can determine relative or absolute content of the protein of interest in a sample [19–21]. Finally, mass spectrometry based proteomics is well suited for mapping of protein-protein interactions using an affinity isolation of protein complexes and their subsequent quantitative mass spectrometric analysis [22].

In studies of induced differentiation, differential profiling (i.e., determation of relative protein content) often employs a platform that combines 2D-gel electrophoresis with identification of proteins by mass spectrometry, most frequently by using MALDI-TOF (Matrix Assisted Laser Desorption/Ionization Time-of-Flight) [23]. Relative quantitative analysis by 2D-gel electrophoresis can register changes in the protein content in the sample. The considerable advantage of this approach consists in the ability to study post-translational modifications.

Quantitative mass spectrometry analysis is widely used in studies of induced differentiation. Methodical features of quantitative mass spectrometry have been considered in a series of review articles [24–28].

Changes in protein content in the cell registered by 2D-gel electrophoresis or tandem mass spectrometry are often verified using Western blot analysis based on the use of antibodies, or the target mass spectrometry methods.

2. DIFFERENTIAL PROFILING IN STIDUES ON EXPRESSION OF SURFACE MARKERS, POST-TRANSLATIONAL MODIFICATIONS, DIFFERENT DIRECTIONS OF LEUKEMIA CELLS MATURATION

Use of data on expression patterns of proteins and transcripts for hypothesizing the mechanism, underlying the action of a certain inducer of differentiation, may be defined as the differential profiling for analysis of induced maturation of cells. Differential profiling

based on the use of methods of transcriptomics and proteomics, allows to perform simultaneous analysis of thousands of transcripts and proteins, and to obtain information on formal quantitative differences between the cells before and after exposure to an inducer.

2.1. Differential Profiling for Comparison of Physiological and Induced Differentiation

Using differential profiling it is possible to determine differences between leukemia cells matured after treatment with inducers of differentiation from normal blood cells. The Tagliafico's group performed transcriptomic profiling of HL60 cells treated with vitamin D_3 and normal peripheral blood monocytes [29]. They found decreased expression of genes of major histocompatibility complex (MHC) class II in peripheral blood monocytes as compared to monocytes obtained during the differentiation of HL60 cells [29]. On the one hand, this suggests differences in molecular mechanisms of physiological and induced differentiation; on the other hand, this fact indicates that vitamin D_3 influences the function of antigen presentation in myeloid cells.

High density RNA microarrays were used for analysis of HL60 cells treated with vitamin D_3 [29]; authors determined an increase in expression of genes, encoding protein products structurally related to cell surface receptors and functionally associated with the response to external stimuli [29]. The latter can be associated with the functions of the immune response and reflects the acquisition of the mature phenotype by the vitamin D_3-treated cells.

2.2. Differential Profiling for Comparison of Various Directions in Leukemia Cell Differentiation

Besides differences in normal hematopoiesis and induced hematopoiesis of leukemia cells, differential profiling can be used to study processes determining the phenotypic divergence of maturing leukemia cells derived from a single progenitor cell and treated by various inductors. For example, dimethyl sulfoxide (DMSO), and also ATRA and actinomycin D induce differentiation of HL60 promyelocytic cells into mature granulocytes, while vitamin D_3, phorbol esters, sodium butyrate and the compound NSC67657 induce monocyte-macrophage differentiation [30]. This may be attributed to the fact that these substances act through different molecular targets within the cell.

In order to expand existing knowledge on the mechanisms determining directions of the induced differentiation, HL60 cell proteins were fractionated by 2D-gel electrophoresis and analyzed by MALDI-TOF before and after treatment of these cells with ATRA (granulocytic differentiation) and compound NSC67657 (monocytic differentiation) [31]. Differential expression of certain proteins and corresponding transcripts was verified by Western blot analysis and PCR in real-time (qRT-PCR), respectively. Proteomic analysis revealed changes in the expression of 25 proteins associated with both granulocytic and monocytic differentiation. At the same time, the contents of 10 proteins changed only during monocytic differentiation induced by NSC67657. Figure 2 shows altered expression of biologically significant proteins in the course of induced differentiation. One can see the increased expression of ICAT (beta-catenin-interacting protein, ICAT), involved in inhibition of the signaling pathway Wnt/β-catenin, and also tumor suppressor TRIT1 and kinase FYN, involved in cell differentiation; IKBKG protein, which is a regulatory subunit of the IKK complex, and BCL2L15 protein, involved in apoptosis. Proteins KIR and CD19 demonstrated decreased expression. Proteomic analysis of ATRA induced granulocytic differentiation revealed 15 differentially expressed proteins. There was increased expression of tumor suppressors CUTL1 and CLDN23, a negative regulator of MAP kinases BRAP, the retinoic acid receptor RXRC, transcription factor USF2. At the same time DEPDC6, a negative regulator of the signaling pathway mTORC1 and mTORC2, and PRDX1, a regulator of redox processes demonstrated decreased expression [31].

Interestingly, the signaling pathways mTORC1 and mTORC2 and Wnt/β-catenin, demonstrate functional links and their components demonstrate differential expression during granulocytic and monocytic differentiation. Activation mTORC through the signaling pathway PI3K-Akt leads to stimulation of the Wnt/β-catenin signaling pathway, which in turn exhibits an activating effect on the cell cycle regulators c-MYC and cyclin D1 [32].

2.3. Differential Profiling to Study the Phenomenon of Resistance of Leukemic Cells

Resistance to therapy is one of the leading causes of high mortality in APL. Using differential profiling it is possible to compare inductor-sensitive and resistant leukemia cells; such analysis is useful for elucidation of mechanisms underlying the phenomenon of resistance.

Based on the results indicating an important role of protein kinase C (PKC) in macrophage differentiation [33, 34], a clone of HL525 cells deficient in PKC protein β isoform was created [35]. Treatment of HL60 and HL525 cells with phorbol-12-myristate-13-acetate (PMA), a PKC activator, induced macrophage differentiation only in HL60 cells, while HL525 cells were insensitive to the PMA effect. Transcriptome analysis reveals differential expression of PRKX kinase mRNA in PMA-sensitive and resistant cells [35]. Specificity of PRKX expression was evaluated by analyzing expression of this gene in HL60 cells treated with DMSO (granulocytic differentiation),

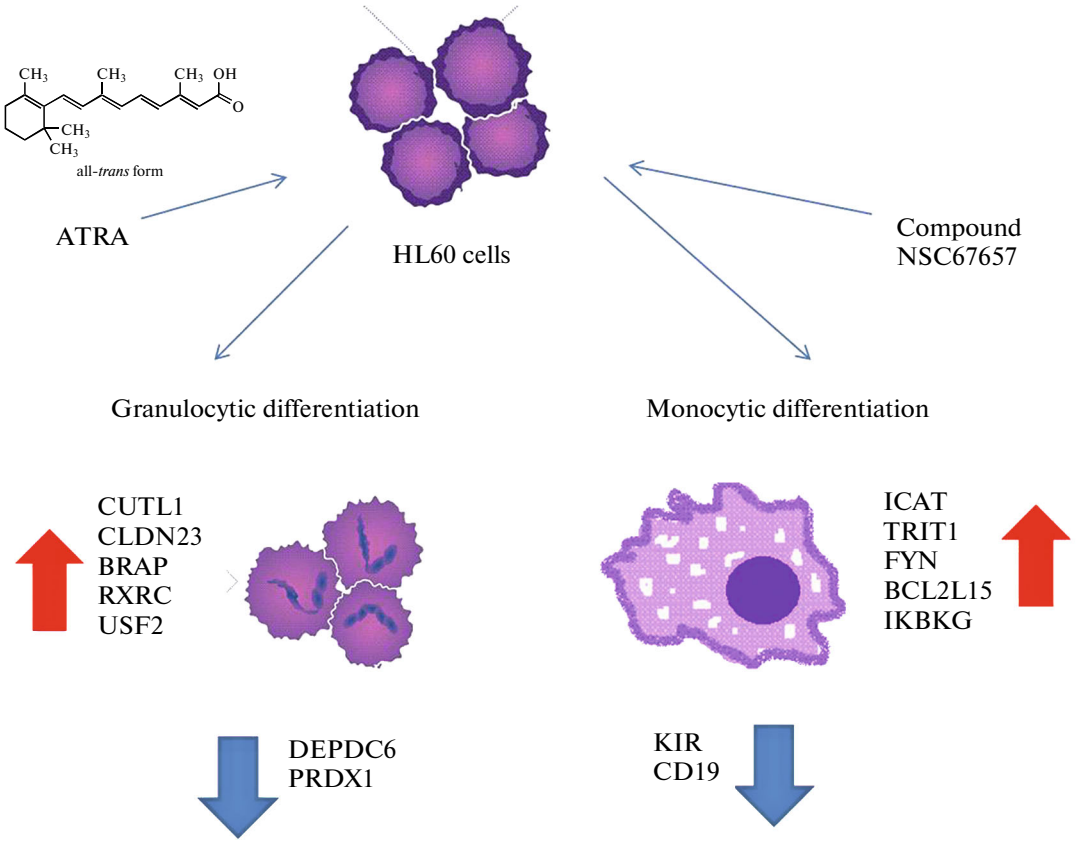

Fig. 2. Altered expression of biologically significant proteins during induced granulocytic and monocytic differentiation of HL60 cells. Asterisks show: ↑—increased expression; ↓—decreased expression.

or vitamin D_3 (monocytic differentiation), or PMA (macrophage differentiation). Experiments revealed that increased PRKX expression was observed in all directions of cell differentiation. PRKX expression was specific for blood cells as compared to other examined tissues (heart, lungs, brain, kidney, liver, and pancreas). Analysis of PRKX expression in lymphocytes and granulocytes from blood of healthy donors and also in the PMA-induced HL60 cells lines and erythroid cell line K562, revealed myeloid specificity of PRKX expression [35]. Inhibition of PRKX translation by antisense oligodeoxyribonucleotides resulted in resistance of leukemia cells proliferation to the effect of PMA; expression of the surface differentiation marker CD11b was also absent after treatment of these cells with PMA. Transfection of HL525 cells with the gene encoding PKCβ, and subsequent treatment of cells with PMA increased PRKX gene expression; this suggests a crucial regulatory role of kinase PKCβ for PRKX expression [35].

A few years later, Zheng et al. performed genome-wide transcriptome profiling of HL60 cells sensitive to a well-known inducer of macrophage differentiation TPA (12-O-tetradecanoylphorbol-13-acetate) and TPA-resistant HL525 cells [36]. In the case of TPA-sensitive HL60 cells increased expression of genes encoding cell growth inhibitor JunD, transcription factors TCF3 and KLF4, and also FosB, EGR1 and EGR2, was noted 15 min after TPA addition. In the case of TPA-resistant HL525 cells the authors found expression deficit of the myeloid-specific protein kinase PRKX and PKCβ [36]. This is consistent with previous results [35] and is probably the cause for the development of the resistance phenomenon.

Subsequent search for PRKX protein kinase targets by means of the yeast two-hybrid system and co-immunoprecipitation of HL60 cell proteins revealed interaction between PRKX kinase and the transcription factor Smad6 in differentiating myeloid cells [37]. It should be noted that PRKX kinase, involved in formation of leukemia cell resistance to the inductor, is still poorly investigated [38].

2.4. Differential Profiling for Studies of Post-Translational Modifications in Leukemia Cells

Differential proteomic profiling allows to study post-translational modifications (PTM), modulating functional activity of proteins without significant changes in their content in the cell. Phosphorylation is

one of the most common PTMs that regulate many cellular processes [39]. In order to determine which phosphorylated proteins are involved in the apoptotic process, HL60 cells have been treated with etoposide [40]. Proteomes of proliferating and apoptotic cells were compared by 2D-gel electrophoresis; then tyrosine-phosphorylated proteins were identified using Western blot analysis and MALDI-TOF mass spectrometry. Apoptotic cells differed from proliferating cells in the content of 18 tyrosine-phosphorylated proteins. Etoposide induced phosphorylation of 25K GTP-binding protein, causing activation of this protein for participation in signal transduction in the cell. Treatment of cells with etoposide caused phosphorylation of the transcription factor NFκB, regulating cell proliferation, migration, and apoptosis, and protein HP1, involved in heterochromatin formation and transcription inhibition. In addition etoposide also caused phosphorylation of following proteins: hnRNPH and hnRNPL proteins involved in regulation of alternative splicing; Hsp60 and GRP78 proteins involved in the response to stress; protein disulfide isomerase acting as a chaperone; proteasomal subunit beta type, belonging to the ubiquitin-proteasomal system; profilin involved in cytoskeleton rearrangement, and mitochondrial ATP synthase beta-chain [40]. These observations indicate that the effect of etoposide results in deep reorganization of the molecular apparatus of the cell, and affects all cell compartments.

2.5. Differential Profiling for Analysis of Surface Markers of Leukemia Cells

A particular problem in the study of differentiation processes consists in determining the level of expression of surface membrane markers that provide crucial information about the stage of cell development, potential interactions of cells of interest with other cells and the intercellular substance. Using the proteomic method, Hofmann et al. investigated surface proteins of HL60 and NB4 tumor cells induced by ATRA [41]. Using the mass spectrometry analysis of membrane-enriched fractions these authors identified 500 membrane proteins, including 137 proteins, which were annotated in CD markers databases. Both APL cell lines had similar changes in expression of surface CD markers reflecting transition of leukemia cells towards granulocytic differentiation. ATRA-treated cell lines HL60 and NB4 were characterized by increased in expression of CD11b, CD11c, CD35, CD36, CD38, CD54, CD55, CD66a, CD300a, CY24B and decreased expression of CD71. These proteins belong to the markers of granulocytic differentiation and proliferation arrest [41]. The surface markers of differentiating cells may be responsible for interaction with the microenvironment and perception of external signals. In addition, proteomic analysis of surface proteins may be used to monitor the cell response to various pharmacological agents and this has clear clinical importance as an alternative to immunophenotyping of surface markers by flow cytometry.

Using high-throughput transcriptomic and proteomic methods it is possible to analyze simultaneously a large number of molecules, including cell surface receptors or maturation markers (genes MHC class II, surface markers CD11b, CD11c, etc.). These methods have a great potential for the development of diagnostic panels and methods for evaluation of response to anticancer therapy. Transcriptomic and proteomic studies resulted in identification of components and/or regulators of signaling pathways, such as signaling pathways NFκB, Wnt/β-catenin, mTORC1 and mTORC2, as well as protein kinases PRKX, PKCβ, FYN and proteins involved in apoptosis. Transcriptomics and proteomics methods detected cell response to the inductor and revealed changes in metabolic systems, cell response to stress, and in the ubiquitin-proteasomal system. Differential profiling is the first step in identification of potential regulatory molecules involved in the differentiation of leukemia cells. The above considered examples demonstrate that annotation of differentially expressed transcripts and proteins determined in the experiments may be carried out by means of literature data or available bioinformatics databases. In the simplest case detection of changes in the expression level of transcripts or proteins observed before and after exposure to a differentiation inducer suggests a regulatory role for these molecules.

3. DIFFERENTIAL PROFILING IN STUDIES OF MECHANISM OF ACTION OF ANTITUMOR AGENTS ALTERNATIVE TO ATRA

Differential profiling is useful in studies of changes in expression of proteins and transcripts of leukemic cells treated with various potentially differentiating and antitumor agents: this helps to elucidate possible mechanisms of their action [42].

For a long time ATRA-based drugs (Tretinoin, Vesanoid), served as an impressive example of implementation of achievements in fundamental biological research to clinical practice. There is a wide range of drugs that exhibit differentiating and antitumor effect. The table summarizes known effects of chemical compounds on leukemia cells investigated by proteomics and transcriptomics methods.

The spectrum of pharmacological activities of the compounds listed in this table significantly differs. For example, 5-aza-2'-deoxycytidine (5-aza-dC) is an inhibitor of DNA methyl transferases involved in epigenetic suppression of tumor suppressor genes [43], adafostin (NSC 680410) is an active homologue of the compound AG957, ATP-independent noncompetitive inhibitor of Bcr/abl kinase [48–50]. The latter is a molecular marker for chronic myeloid leukemia

Results of studies of mechanism of differentiating and anti-tumor actions of chemical compounds alternative to ATRA by transriptomics and proteomics methods

Compound studied	Methods of analysis	Research object	Biologically important differentially expressed molecules		Proposed mechanism of action
			increased expression	decreased expression	
5-aza-dC	Transcriptomic profiling on high-density RNA chips	MV4-11 and HL60 cells	HOXA4, HOXD4, HOXD8, HOXD12, CD9 and RGS2	–	Reactivation of hypermethylated genes [43]
Adafostin (NSC 680410)	2D-gel electrophoresis followed by mass spectrometry	HL60 and K562 cells	Proteins involved in oxidative stress response: CALM, ERP29, GSTP1; apoptosis inducing proteins LAMA, FLNA, TPR, GDIS	Proteins involved in oxidative stress response: PDIA1	Oxidative stress [44]
Lovastatin	Quantitative mass spectrometry (SILAC)*	HL60 cells	–	HMG-CoA synthase and farnesyl diphosphate synthase (FDP)	Inhibition of endogenous cholesterol synthesis [45]
Genistein	2D-gel electrophoresis followed by mass spectrometry	HL60 cells	–	hnRNP A1, hnRNP C, stagmin-1	Inhibition of proliferation and such oncogenes as C-MYC [46]
Arsenic trioxide (ATO)	Quantitative mass spectrometry (SILAC)	HL60 cells	Neutrophil elastase and alpha-mannosidase	Fatty acid synthase (FAS) and phosphatase 1 alpha	Cytotoxic effect due to decreased content of fatty acid synthase (FAS) [47]

*SILAC—isotope labeling of amino acids in cell culture.

(CML); it also stimulates and expression of the C-KIT receptor, which enhances the mitogenic effect of the stem cells factor (CSF) [51]. Genistein, a tyrosine kinase inhibitor, influences expression of such important regulatory molecules as NF-κB, AKT, PTEN, p38MAPK [52–54]. Lovastatin is widely used for lowering blood cholesterol in patients with cardiovascular diseases. However, for all these compounds share one important feature: they exhibit a pronounced antitumor activity towards APL cells, but mechanisms of this antitumor activity still remains to be clarified.

3.1. 5-Aza-2'-Deoxycyitidine

The pyrimidine analog 5-aza-dC is involved in the inhibition of DNA methylation, which level is often elevated in carcinogenesis; this results in suppression of important genes, e.g., cell cycle inhibitor $p16^{INK4a}$, tumor suppressor $p14^{ARF}$, retinoic acid receptor RARβ2 and many others [43, 55]. During differential transcriptome profiling of myeloid leukemic cell line MV4-11 much attention was paid to genes demonstrating increased expression after treatment of cells with 5-aza-dC, compared to control. Such genes have been defined as reactivated [43]. Using microarray for such reactivated genes as *HOXA4, HOXD4, HOXD8, HOXD12, CD9*, and *RGS2* a significant increase in their expression was found; this increase was then confirmed by qRT-PCR on the MV4-11 cell line and three APL cell lines, including HL60 cells [43].

3.2. Adafostin

The study of mechanism of adafostin action was stimulated by detection of its antitumor activity against AML and gliomas that do not express Bcr/abl [56, 57]. To identify the mechanism of adafostin action a comparative study of HL60 (Bcr/abl negative

cell line) and K562 (Bcr/abl positive cell line) cell proteomes was carried out at 6 h, 12 h and 24 h after cell treatment with adafostin [44]. On the basis of results on cell survival, HL60 cell line (Bcr/abl negative cell line) was 5–10 times more sensitive to adafostin compared with line K562. Proteomic analysis of adafostin treated cells resulted in identification of 49 differentially expressed proteins in HL60 and K562 cells lines compared to the control; the content of 19 proteins differed between Bcr/abl positive and negative cell lines. K562 cells (Bcr/abl positive cell line) showed increased content of 8 proteins, including GSTP1, ERP29 and PCNA, and decreased SNP29 content (in HL60 cells these proteins were either not detected, or their content remained unchanged). HL60 cells demonstrated decreased expression of 10 proteins, including LAM2, KRYM, PCBP1. Annotation of differentially expressed proteins by means of the PANTHER database revealed that these proteins are mainly involved in oxidative stress response (CALM, ERP29, GSTP1, PDIA1) or induction of apoptosis (LAMA, FLNA, TPR, GDIS). Subsequent cell transfection with a gene encoding the antioxidant enzyme glutathione-S-transferase (GSTP1) (its content increased in K562 cells), resulted in increased resistance of these cells to adafostin. Moreover, treatment of K562 and HL60 cells with adafostin in combination with the antioxidant L-NAC attenuated the adafostin effect on both tumor cell lines. To test the hypothesis on involvement of reactive oxygen species (ROS) in the adafostin effect, K562 and HL60 cells were incubated with hydroquinone and hydrogen peroxide, which are known as ROS sources. This treatment resulted in changes of the proteomic profile similar to that observed after cell treatment with adafostin [44]. These observations suggest the peroxide nature of the adafostin action.

3.3. Lovastatin

The hypolipidemic drug lovastatin inhibited cell proliferation and induced apoptosis in cell models, including AML cells [58]. The effect of lovastatin on HL60 cell line was investigated using quantitative mass spectrometry analysis by the SILAC method [45], which is the preferred method for comparative analysis of protein expression in cells before and after treatment with pharmacological agents [59]. Quantitative analysis of 3228 proteins revealed changes in the content of 122 proteins. This included decreased content of enzymes HMG-CoA-synthase and farnesyl diphosphate synthase (FDP), involved in cholesterol biosynthesis. Authors suggested that reduction of cell proliferation is accompanied by inhibition of endogenous cholesterol synthesis [60, 61]. Endogenous synthesis of cholesterol includes formation of geranyl pyrophosphate and farnesyl pyrophosphate intermediates involved in prenylation of C-terminal cysteine residues of proteins. Attachment of hydrophobic groups changes physico-chemical properties of proteins and influences protein-protein interactions as well as protein interactions with membranes. Prenylation of Ras proteins is involved in the development of various types of malignant tumors, including colorectal carcinoma, melanoma, prostate carcinoma and hepatocellular carcinoma [62, 63]. Probably protein prenylation is involved in the formation of the leukemic phenotype.

3.4. Genistein

Genistein is a tyrosine kinase inhibitor [52–54]. Treatment of HL60 cells with genistein resulted in altered staining intensity of 40 protein spots on 2D-gel electrophoresis [46]. Fourteen of the 40 protein spots were identified using MALDI-TOF/TOF mass spectrometry. Among them hnRNP A1 and hnRNP C proteins involved in transport and processing of RNA demonstrated decreased content. Earlier, for the *hnrnp a1* gene the antiapoptotic effect on tumor cells was demonstrated [64]. For another differentially expressed protein, hnRNPC, previous study demonstrated its ability to enhance translation of proto-oncogene c-MYC, and to stimulate translation of antiapoptotic proteins [65]. The study of the genistein action on HL60 cells also revealed a decrease in the content of p53-regulated protein stagmin-1 [46]. Inhibition of stagmin-1 in cancer cells may prevent cell cycle progression in proliferating cells [66]. Thus, using proteomic methods of analysis, the authors were able to link the inducing effect of genistein with changes in the content of proteins involved in the regulation of cell proliferation and cell life cycle.

3.5. Arsenic Trioxide (ATO)

ATO-based pharmacological agents (Assadin, Trisenox) are currently used along with ATRA in clinical practice for the APL treatment [67]. Using the SILAC method followed by mass spectrometry analysis, Xiong and Wang found that cultivation of HL60 cells in the presence of ATO resulted in the altered expression of 56 proteins [47]. They found increased content of nuclear histones, neutrophil elastase, alpha-mannosidase, and decreased content of fatty acid synthase (FAS), and phosphatase-1alpha. Levels of differentially expressed proteins were confirmed by Western blot analysis. Since increased content of FAS was already determined in cells of various human carcinomas [68], Xiong and Wang hypothesized that decreased content of FAS may underlie the cytotoxic effect of arsenite on tumor cells. To confirm this hypothesis, these authors treated ATO-induced tumor cells with palmitate, the end product of fatty acids synthesis, and in accordance of the proposed hypothesis they observed attenuation of the cytotoxic effect [47]. Increased expression of neutrophil elastase and alpha-mannosidase indicates transition of leukemia cells

towards granulocytic differentiation. The mechanism by which FAS supports cell proliferation still requires better understanding; it is suggested that intensive synthesis of fatty acids is necessary for growth of rapidly dividing cells as well as for maintenance of a high level of glycolysis (the Warburg effect), characteristic for tumor cells [69].

Transcriptomic and proteomic studies of the action of anticancer drugs alternative to ATRA on APL cells revealed changes associated with regulation of redox processes, biosynthesis of cholesterol and fatty acids, and glucose metabolism. At the same time, transcriptomic and proteomic studies allowed to detect importance of epigenetic modifications of chromatin in APL cells, as well as emphasize involvement of the proto-oncogene c-MYC and p53 tumor suppressor in regulation of cell proliferation/differentiation.

4. DIFFERENTIAL PROFILING IN STUDIES OF SIGNALING PATHWAYS INVOLVED IN THE PROCESS OF INDUCED DIFFERENTIATION OF LEUKEMIA CELLS

Differential profiling is successfully used in studies of functional activity of biological signaling pathways. This functional approach is often combined with traditional molecular biological methods based on the introduction of expression constructs into the cell that model situations of overexpression of a protein product, thus confirming the functional significance of putative regulatory molecules [70, 71].

4.1. Transcription Factor HOXA9

HOXA9 protein is a transcription factor with poorly known particular targets. Its importance was demonstrated in mice with knockdown of the HOXA9 gene: such animals demonstrated various developmental defects of myeloid and lymphoid systems [72]. HOXA9 overexpression in human bone marrow cells resulted in activation of proliferation of blood stem cells [73]. Genes of the HOX family became reactivated after the treatment of myeloid leukemia cell line MV4-11 with 5-aza-dC (see Section 3.1).

In an attempt to identify downstream molecular targets of the HOXA9 protein, the Dorsam's group transfected the HOXA9 gene into two myeloid tumor cell lines U937 and K562, and also in the lymphoid tumor cell line Jurkat, and performed subsequent cDNA analysis of each cell line on high density microarrays [74]. Altered expression levels of some transcripts were then validated by qRT-PCR. Using a cDNA microarray analysis authors identified 220 genes characterized by at least 2-fold change in their expression in response to overexpression of the transfected HOXA9 gene. Analysis of genes expression profiles in populations of myeloid and lymphoid cells showed that, depending on the cell type, the transcription factor HOXA9 functions as a repressor or an activator of transcription. Gene profiles demonstrating the same behavior in myeloid cell included the following genes *CD36, ID2, LYN, JUNB*, which showed decreased expression, while expression of *EPS8, ALDH1, TCN1, MYB* increased. In the lymphoid cell lines expression of these genes remained unchanged. Genes corresponding to potential HOXA9 targets, were found among the records of the Human Stem Cell Transcriptome Database, containing information on transcripts expressed in the bone marrow stem cells; at the same time the study of induced differentiation revealed increased expression of *ID2* (see Section 5) and the surface marker CD36 (see section 2.5). It is possible that the transcription factor HOXA9 is involved in maintenance of immature phenotype of myeloid progenitor cells.

4.2. Insulin/Insulin-Like Growth Factor 1 (IGF1) Signaling Pathway

This signaling pathway plays different roles in the proliferation and differentiation of hematopoietic cells. On the one hand, the insulin/IGF1 signaling pathway stimulates cell growth via the activation of AKT kinase [75]. On the other hand, the insulin/IGF1 signaling pathway transmits differentiation signals mediated by SHC protein [76]. In order to search new effector molecules of the of insulin/IGF1 signaling pathway, HL60 cells were treated with insulin and then a comparative study of proteomes of experimental and control cell samples was performed using the 2D-gel electrophoresis/MALDI-TOF platform [77]. Using this approach, it was found that the content of CLIC1 and SRp20 proteins changed after treatment of HL60 cells with insulin; it should be mentioned that earlier these proteins were not considered in the context of the insulin/IGF1 signaling pathway. Being located in the plasma membrane of cells, CLIC1 protein functions as an ion channel [78]. In human hematopoietic cells CLIC1 protein is located in the nucleus [79]; this suggests a regulatory role of CLIC1, which is also a structural protein homologue of the superfamily of glutathione-S-transferases (GST), involved in redox reactions [80]. Besides determination of differential expression of CLIC1 protein in response to activation of the insulin/IGF1 signaling pathway, Saeki et al. registered CLIC1 translocation from nuclear matrix in the nucleolus [77].

Quantitative analysis by 2D-gel electrophoresis revealed an insulin-mediated decrease of SRp20 protein content in HL60 cells [77]. SRp20 protein is a factor involved in regulation of alternative splicing of certain RNA precursors, including *srp20* RNA [81]. Treatment of HL60 cells with proteasome inhibitor MG-132 after activation of the insulin/IGF1 signaling pathway attenuated the decrease in the SRp20 protein content [77]. Thus, the proteomic analysis revealed proteins with significantly altered levels in response to

activation of the insulin/IGF1 signaling pathway and these never considered as its molecular targets.

4.3. MAPK Signaling Pathway

Triggering processes of sequential phosphorylation, mitogen-activated protein kinases (MAPKs) are involved into signal transduction in the cells that mediate proliferation, differentiation, cell response to stress, and apoptosis [82, 83]. MAPK signaling pathways include the following key kinases: extracellular signals regulated kinases 1 and 2 (ERKs), and also JNK kinase, p38 and ERK5. ERKs kinases are important for the development of the hematopoietic system [84]. Activation of MKK/ERK signaling induced by phorbol esters and cytokines resulted in megakaryocytic differentiation of erythroleukemia and megakaryoblastic tumor cells [85–87]. Changes in the level of ERKs kinases during cells differentiation were studied by proteomic methods [88]. In order to identify protein targets responsive to stimulation of the MKK/ERK signaling pathway during induced differentiation, erythroleukemia K562 cells were treated either by PMA alone, or PMA in combination with a compound U0126, an MKK1/2 inhibitor. Constitutively active genes of MKK1 and MKK2 kinases were also co-transfected into intact K562 cells (without PMA treatment) [88]. Treatment of cells with PMA resulted in quantitative changes in the levels of 41 proteins; 25 of these proteins coincided with differentially expressed proteins after co-transfection of mutant forms of MKK1/2 (activation of the MKK/ERK signaling pathway), and after exposure to U0126. Besides known targets of the MKK/ERK signaling pathway (MKK2, stagmin, cytokeratin 8, eIF-4E, MNK1) these included proteins regulators of apoptosis Bcl2 and MCL1, which showed increased expression. For many types of cancer, particularly for breast cancer, expression of cytokeratin 8 correlated with an increased risk of metastasis [89]. At the same time, it was shown that treatment with retinoids resulted in a decrease of tumor cell proliferation, while expression of cytokeratin 8 increased [90]. Interestingly, activation of the MKK/ERK signaling pathway resulted in an increase in the content of retinal aldehyde dehydrogenase 2, which catalyzes the final step of the biosynthesis of retinoic acid, the inducer of differentiation in vivo and in vitro [88]. These results may indicate a link between ERKs and retinoic acid signaling pathways.

5. SYSTEMS BIOLOGY OF INDUCED DIFFERENTIATION OF LEUKEMIA CELLS

Regulation and realization of the differentiation process involves different levels of organization of the cell (DNA, mRNA, proteins), and full understanding of the mechanism of differentiation requires integration and annotation of results obtained during systemic measurements of biological molecules. This is the task of the systems approach to the study of differentiation. Although the systems approach is infrequently employed in studies of induced differentiation of leukemia cells, it has a great potential.

In order to study the synergistic effect of ATO and ATRA on NB4 tumor cells, Zheng et al. employed transcriptome analysis, proteomic analysis, and computational biology techniques. ATRA-induced NB4 cells were characterized by altered expression of 1113 genes and by 793 differentially expressed proteins.

The transcriptome analysis, performed at 6 h after the onset of induction, revealed increased expression of genes encoding factors and cofactors, belonging to the families C/EBP (C/EBPα, C/EBPβ, C/EBPε), and HLH (ID1 and ID2), and also genes encoding IRF1, SMARCD2, and TADA3L proteins, and a decrease in expression of the *HHEX* gene. At later time points from the onset of differentiation, altered expression of genes encoding proteins involved in regulation of the metabolism and transport of calcium into the cell (ITPR2, NUCB2), as well as increased expression of IFR-responsive genes were registered. At later stages of differentiation, increased levels of expression of genes (*BCL2A1, PDCD6IP, CASP1, CASP7, MADD*) responsible for cell survival were also detected. The differentially expressed proteins also included proteins involved in regulation of calcium metabolism and transport, cytoskeletal organization, and cell cycle control [91].

Transcriptome and proteome analyses of NB4 cells treated with ATO, revealed 487 and 982 molecules with altered expression level, respectively. The level of expression of 316 of 487 (65%) of transcripts was also influenced by ATRA. Among the differentially expressed genes there were genes encoding differentiation markers (PECAM1 and SELPLG), regulators of apoptosis (BAK1, BCL2, MADD), and also genes *CDC7L1, PLK3*, regulating cell cycle and growth. In contrast to ATRA-induced differentiation, ATO influenced gene expression of the ubiquitin-proteasomal system and oxidative stress response. At the protein level recorded differential expression of molecules involved in metabolism regulation, cytoskeleton organization, protein synthesis was registered [91].

Treatment of NB4 cells with combination of ATO together with ATRA were basically similar to those registered after cell treatment with ATRA only. At the same time, there was a group of genes differentially expressed only in response to the combined effect of ATO and ATRA as compared to the response of tumor cells to effect of each inducer separately. These included components of the ubiquitin-proteasomal system UBE2L6, PSMC2, PSMD13. It is possible that activation of the ubiquitin-proteasomal system involved in the degradation of the fused protein PML-RARα represents a basis of the synergistic effect of ATO and ATRA. In addition, the synergistic effect of ATRA and ATO also caused a decrease in expression of genes encoding proteins ARHGAP26, SH3GL1,

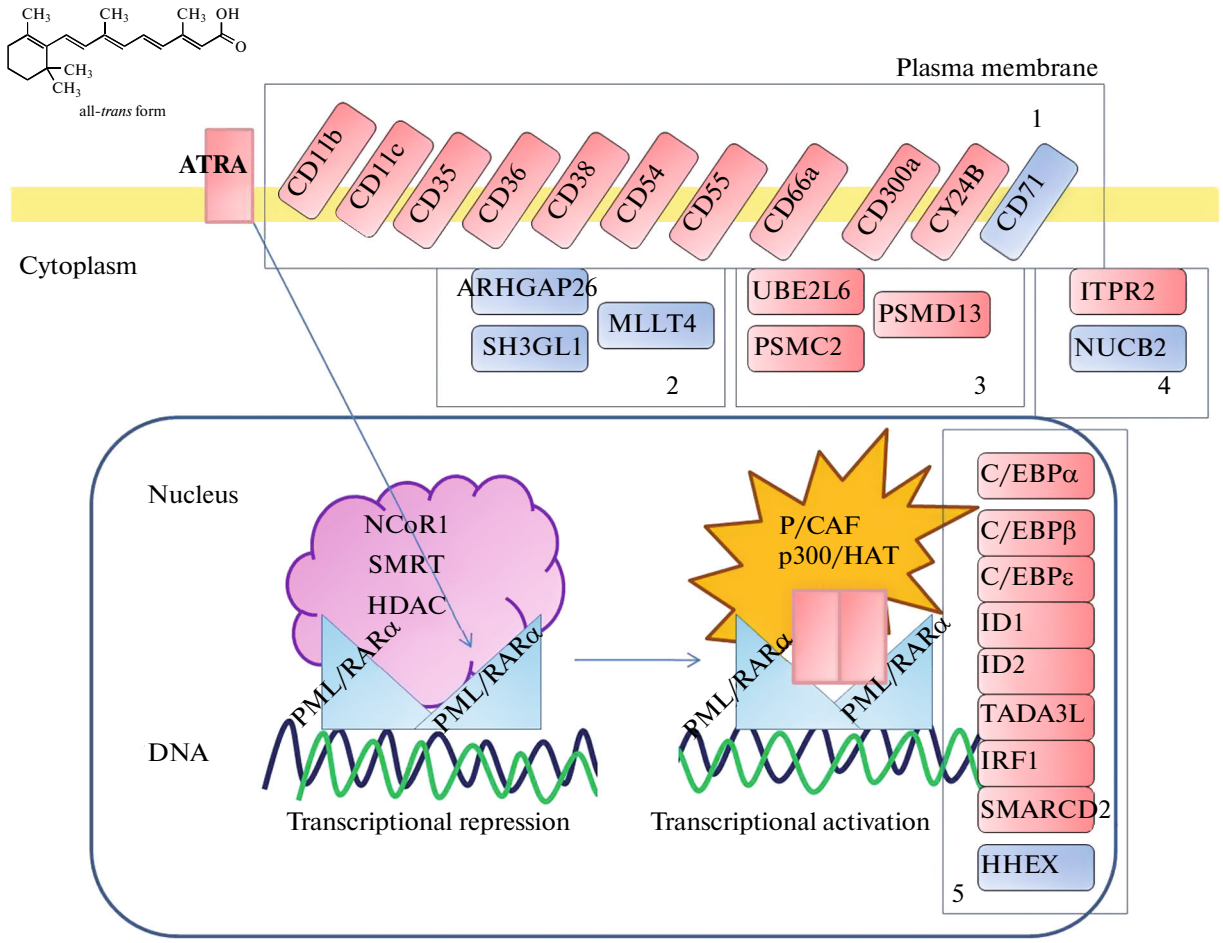

Fig. 3. Molecules of transcripts and proteins involved in ATRA-induced differentiation of NB4 cells containing chimeric receptor protein PML/RARα. (1) Surface CD-markers and membrane proteins; (2) genes encoding these proteins are involved in chromosomal translocations; (3) proteins of the ubiquitin-proteasome system; (4) proteins regulating calcium transport and metabolism; (5) transcription factors.

MLLT4, involved in formation of chromosomal translocations appeared in certain types of leukemias (translocation partners of the *MLL* gene). At the proteomic level 414 differentially expressed proteins were registered after combined treatment of cells with ATO and ATRA; these included proteins involved in translation, DNA repair and ubiquitin-dependent proteolysis [91].

The systems study was carried out on the NB4 cell line, carrying the translocation directly affecting ATRA receptor, and recognized in 95% of cases of APL [6]. Results of the study, along with the cited study by Hofmann et al. [41] (see Section 2.5) can significantly enrich the set of transcripts and proteins involved in the ATRA induced differentiation of leukemia cells (Fig. 3).

At the same time, the cell HL60 line is more frequently used as a research object in studies of induced differentiation; it carries a wild-type RARα receptor, but is characterized by an extensive deletion of the p53 gene and amplification of the C-MYC gene. Genetic defects affecting some of genes very important for the regulation of cell proliferation/differentiation, as well as responsiveness of HL60 cells to a large repertoire of inductors, suggest existence of a special mechanism of ATRA-induced differentiation, in which retinoic acid receptors play an important role. However, other mechanisms also exist. We have recently performed a systems study of ATRA-induced differentiation of HL60 cells by means of the use of high-resolution mass spectrometry, genome-wide transcriptome analysis and the GeneXplain software (Tikhonova et al., in preparation). Figure 4 shows a scheme demonstrating a possible sequence of molecular events during induced differentiation.

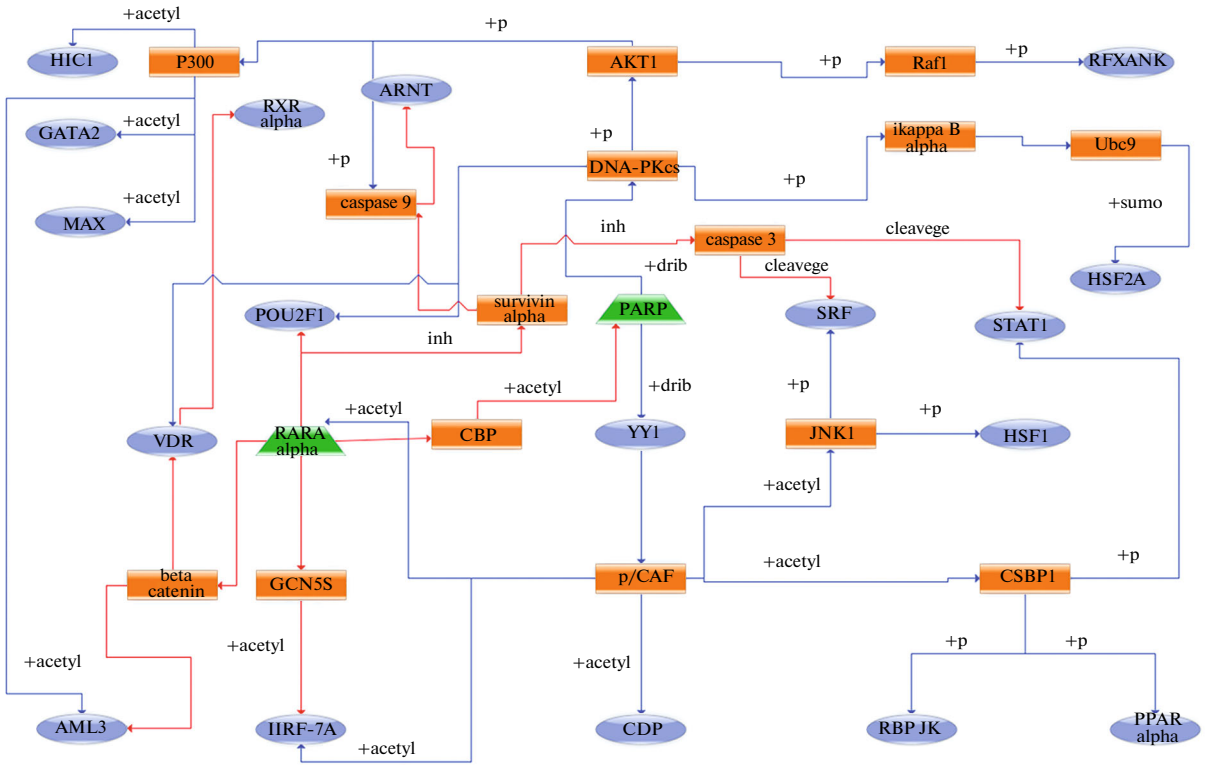

Fig. 4. Possible sequence of molecular events occurring in the process of ATRA-induced differentiation of HL60 cells. Designations: (1) trapezoid—a key regulatory molecule; oval—a transcription factor; rectangle—messenger molecules; (2) +p—phosphorylation; +acetyl—acetylation; +drib—poly-ADP-ribosylation; +sumo—sumoylation; inh—inhibition; (3); asterisk—registration for the proteomic level.

In general, transcriptomic and proteomic studies of induced differentiation of HL60 cells significantly extended modern understanding of the molecules involved in the granulocytic maturation of APL cells carrying the wild-type receptor RARα (Fig. 5).

Recent examples demonstrate that consolidation of the results obtained by using transcriptomic and proteomic techniques, as well as employment of bioinformatics tools for classification, interpretation of experimental data and generation of hypotheses, can serve as a basis for creation of a platform, which can be used for investigation of differentiation of tumor cells.

CONCLUSIONS

High-throughput transcriptomic and proteomic techniques opened the possibility of a large scale analysis of expression of mRNA and proteins during the process of induced differentiation of leukemia cells. Despite the fact that separate transcriptomic or proteomic profiling of leukemia cells during their maturation may be successfully used for identification of new regulatory molecules, the problem of combined interpretation of experimental (transcriptomic/proteomic) data still remains unsolved. One reason consists in weak correlation between quantitative data obtained at transcripts and protein levels. In addition, genome-wide transcriptome profiling data for a long time corresponded to a limited number of measurements obtained by proteomic methods. Recent developments of mass spectrometry and also development of new methods of quantitative analysis of mass spectrometry data allowed to quantify the major part of the cell proteome.

Using quantitative mass spectrometry methods it is possible to analyze mainly master canonical form of proteins; nevertheless potential applicability of mass spectrometry for the study of PTM and various splice variants of proteins opens new directions in research of induced differentiation of leukemia cells.

Finally, bioinformatics techniques are an important tool for the study of induced differentiation of leukemia cells, for the interpretation and integration of data obtained by proteomic and transcriptomic methods, thus providing the basis for a systems approach to the study of the biological process and this is essential for approximation of in silico modeling to real conditions in a living cell.

On the one hand, applicability of transcriptomics and proteomics opens perspectives for their use in

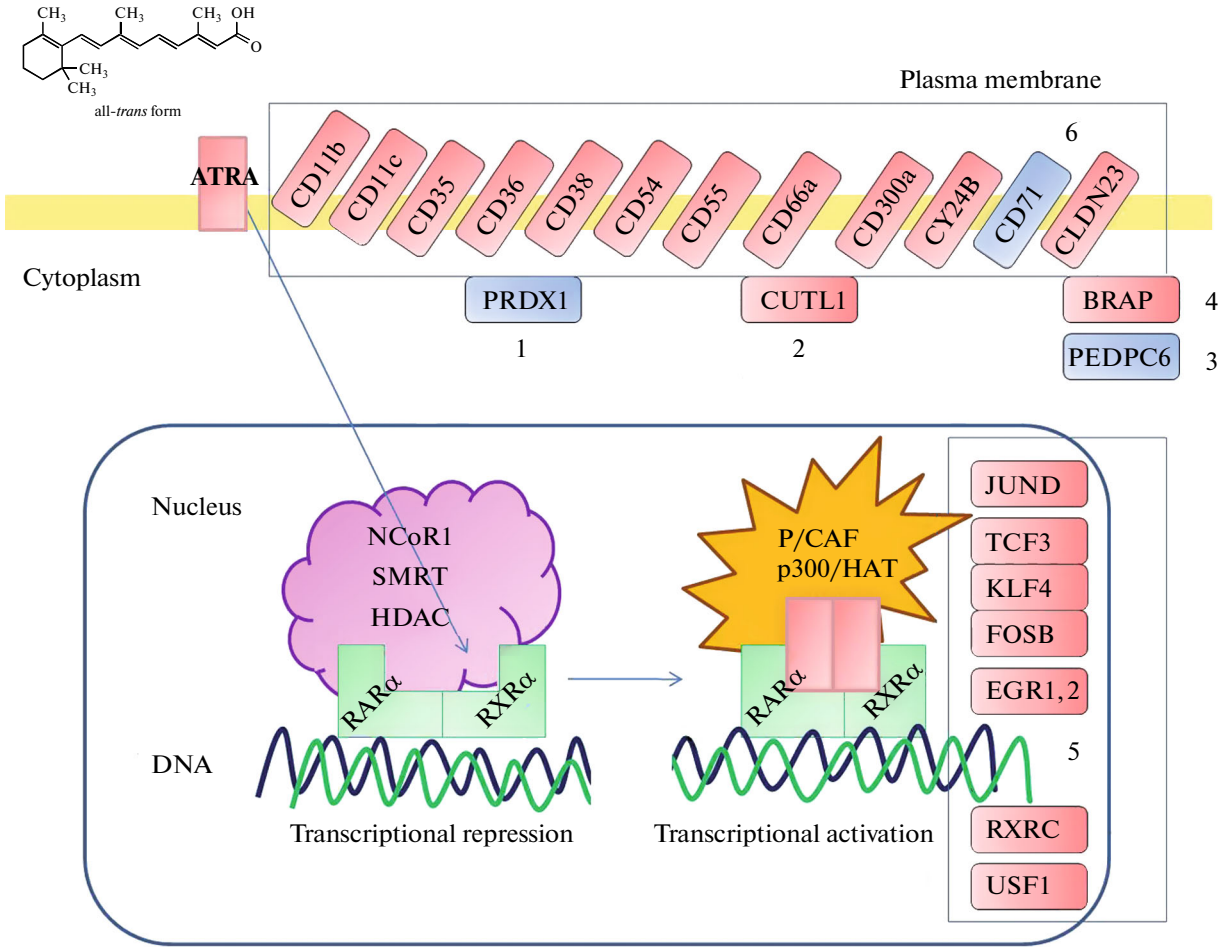

Fig. 5. Molecules of transcripts and proteins involved in ATRA-induced differentiation of HL60 cells the wild type receptor protein RARα. (1) Protein involved in redox; (2) tumor suppressor, (3) negative regulator of the proteins of the mTORC1 and mTORC2 signaling pathway; (4) negative regulator of MAP kinase; (5) transcription factors; (6) surface markers and membrane proteins.

clinical practice, for example, in analysis of surface markers; on the other hand, application these methods significantly extends our knowledge on mechanisms underlying the action of inducers of differentiation, particularly ATRA, thus highlighting elements of crucial signaling pathways (mTORC1 and mTORC2, Wnt/β-catenin, NFκB, apoptosis) and cell systems (ubiquitin-proteasome, redox). Acting on these systems it is possible to direct cell along the way of differentiation. Results of proteome and transcriptome analysis, treated by bioinformatics techniques provide a powerful screening platform that can generate theories of functional significance of transcripts and proteins in health and disease.

ACKNOWLEDGMENTS

This work was supported by the Russian Science Foundation (project no. 14-25-00132).

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Translated by A. Medvedev