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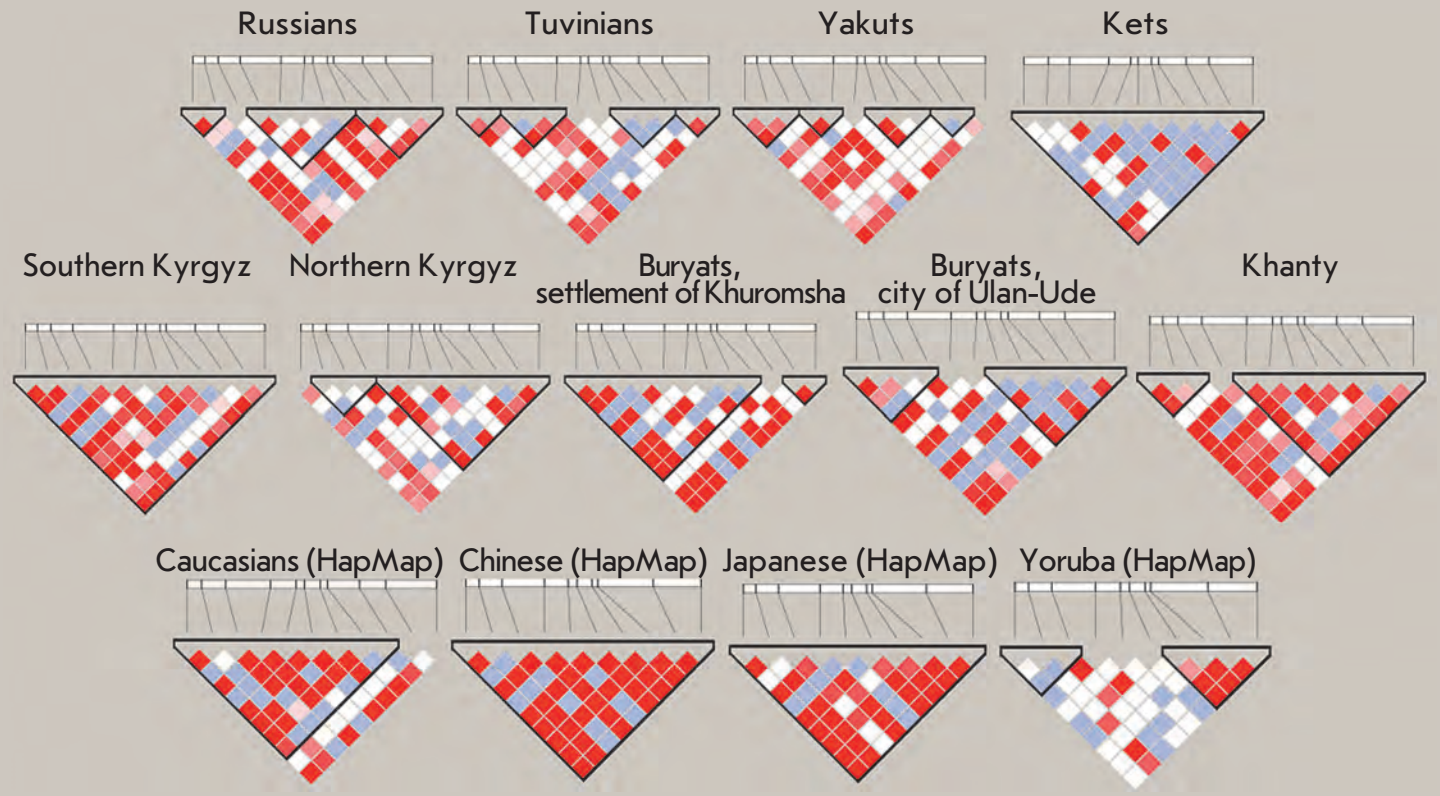
ActaNaturae

Regulation of Immunity via Multipotent Mesenchymal Stromal Cells



Letter from the Editors

Dear readers of Acta Naturae,

We are delighted to bring to your attention the 12th issue of our journal, the first for 2012. In spite of some skeptical voices, our journal is alive; the backlog of manuscripts is huge and we remain optimistic about the future. However, some problems do indeed exist; we are still yet to attain our deserved place in PubMed. Unfortunately, this process has dragged on, and for no fault of ours. We have had to overcome a large number of obstacles, first and foremost of which are the constantly changing requirements that we are subjected to. At this juncture, it looks as though our struggle has entered its final stage, and we are hopeful that this leap year will be one of fortune for the journal in this and other respects.

Let us return to our main topic, the current issue of the journal. The "Forum" section opens with an article devoted to the life and work of Academician Alexander Evseevich Braunstein, whose 110th anniversary we celebrate this year. Alexander Evseevich is remembered as a remarkable man and one of the 20th century's most revered scientists in the field of biochemistry. The article is written by Prof. T.V. Demidkina, a follower of Alexander Evseevich, who heads the Laboratory created by him at the Engelhardt Institute of Molecular Biology. Another material presented in the "Forum" section is an interview with V.A. Richter, in which he shares his thoughts concerning the promotion of innovative biomedical developments and the role of the government in the process. We hope that the material presented in the "Forum" section will be of interest to a wide audience.

The scientific section of the journal opens with two reviews devoted to topical issues in cell biology: the investigation of induced pluripotent stem cells (I.A. Muchkaeva et al.) and immunomodulation by multipotent mesenchymal stromal cells (Y.P. Rubtsov et al.). The authors of both reviews consider these problems from the standpoint of their medical application, thereby arousing special interest. As usual, the bulk of the papers presented in this issue are in the field of biomedicine (see the papers written by N.A. Orlova, L.Z. Velsher et al., and M.A. Volodina et al.)

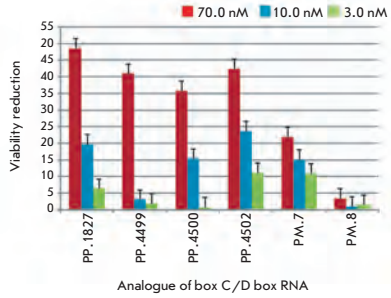
A series of reports are devoted to different aspects of molecular genetics, mostly related to medicine (see the works of S.A. Borinskaya et al., E.A. Trifonova et al., and G.A. Stepanov et al.). Finally, there are reports in which the technologies for obtaining and testing biomolecules and cells, including those with modified properties, are discussed (see the works of E.M. Smekalova et al., E.S. Kolotova et al., O.N. Solopova et al., and O.V. Bondar et al.).

The presented papers differ in terms of the themes and methods used. However, the high degree of modernity and professionalism with which they were completed are a common feature of all of them, hence our optimism about the future of our journal. ●

Analogues of Artificial Human Box C/D Small Nucleolar RNA As Regulators of Alternative Splicing of a pre-mRNA Target

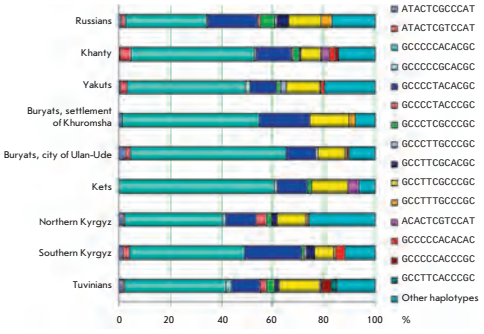
G. A. Stepanov, D. V. Semenov, E. V. Kuligina, O. A. Koval, I. V. Rabinov, Y. Y. Kit, V. A. Richter

Artificial analogues of U24 snoRNAs directed to nucleotides in 28S and 18S rRNAs, as well as pre-mRNAs and mature mRNAs of human heat shock cognate protein (hsc70), were designed and synthesized in this study. It was demonstrated that the transfection of cultured human cells with artificial box C/D snoRNA targeted to pre-mRNAs induce partial splicing impairments. It was found that transfection with artificial snoRNAs directed to 18S and 28S rRNA nucleotides, significant for ribosome functioning, induce a decrease in MCF-7 cell viability.



Influence of artificial box C/D RNAs on the viability of MCF-7 cells

The Genetic Diversity and Structure of Linkage Disequilibrium of the *MTHFR* Gene in Populations of Northern Eurasia



The distribution of haplotypes in the populations studied

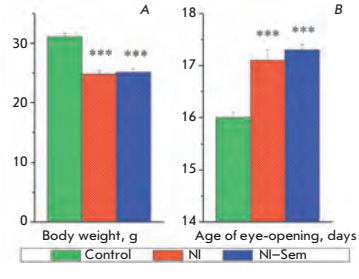
E.A. Trifonova, E.R. Eremina, F.D. Urnov, V.A. Stepanov

The structure of the haplotypes and linkage disequilibrium (LD) of the methylenetetrahydrofolate reductase gene (*MTHFR*) in 9 population groups from Northern Eurasia and populations of the international HapMap project was investigated in the present study. The data suggest that the architecture of LD in the human genome is largely determined by the evolutionary history of populations; however, the results of phylogenetic and haplotype analyses seems to suggest that in fact there may be a common "old" mechanism for the formation of certain patterns of LD.

Correction of Long-Lasting Negative Effects of Neonatal Isolation in White Rats Using Semax

M. A. Volodina, E. A. Sebentsova, N. Yu. Glazova, D. M. Manchenko, L. S. Inozemtseva, O. V. Dolotov, L. A. Andreeva, N. G. Levitskaya, A. A. Kamensky, N. F. Myasoedov

Adverse experience during the early postnatal period induces negative alterations in physiological and neurobiological functions, resulting in long-term disorder in animal behavior. The aim of the present work was to study the long-lasting effects of chronic neonatal stress in white rats and to estimate the possibility of their correction using Semax, an analogue of ACTH fragment (4–10). Early neonatal isolation was used as a model of early-life stress. It was shown that neonatal isolation leads to a delay in physical development, metabolic disturbances, and a decrease in the corticosterone stress response in white rats. These changes were observed during the first two months of life. Semax administration weakened the influence of neonatal isolation on the animals' body weight , reduced metabolic dysfunction, and led to an increase in stress-induced corticosterone release to the control values.



Neonatal isolation effects on the body weight of rats at the age of 15 days (A) and on the time of eyeopening (B)

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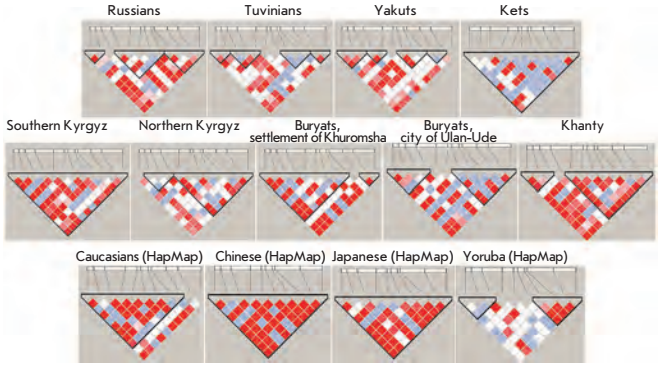

IMAGE ON THE COVER PAGE
The structure of linkage disequilibrium in the *MTHFR* gene in the populations studied (E.A. Trifonova *et al.*)

The 110th Anniversary of Academician Alexander Evseevich Braunstein

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Alexander Evseevich Braunstein, the well-known enzymologist, was born on May 26, 1902, into a medical family. His father was an ophthalmologist, a professor of Kharkov Medical Institute and the President of the Kharkov Medical Society.

In 1913, Alexander Braunstein enrolled in a gymnasium, directly entering the third grade, since by that time he had already received a thorough and all-round home education. Alexander Braunstein was fluent in German, English, and French, which, by his own admission, assisted him greatly in his scientific work and was an additional source of income. Alexander Braunstein began to display an interest in the sciences at an early age. In his childhood, he was keen on chemistry and had a small chemistry laboratory at home. Having graduated from the gymnasium in 1920, he entered Kharkov Medical Institute, being as it were at the time the only place in the Ukraine where one could receive higher education in the field of natural sciences.

A.E. Braunstein began his scientific research in 1925 as a postgraduate student of the very first postgraduate course in the USSR. His scientific adviser at the Institute of Biochemistry of the People's Commissariat for Health, in Moscow, was V.A. Engelhardt. In 1928, A.E. Braunstein defended his candidate dissertation devoted to the study of the interrelation between glycolysis and the phosphate metabolism in red blood cells. During the period from 1928 to 1936, Alexander Evseevich Braunstein immersed himself in the investigation of the processes of oxidative and respiratory phosphorylation and detoxification of aromatic compounds. In the aforementioned, as well as subsequent studies, Braunstein's excellent knowledge of chemistry and three foreign languages helped him to perform the research at a very high and contemporary biochemical level. In the XX century, biochemistry was making very rapid progress and shifting from the description of metabolic processes to the establishment of their molecular mechanisms.

In 1936, A.E. Braunstein took the helm at the Laboratory of Intermediate Nitrogen Metabolism at the Gorky All-Union Institute of Experimental Medicine. In 1937, working in cooperation with M.G. Kritsman, he discovered a new enzymatic reaction representing the transfer of an amino group from α-amino acids to keto acids; this reaction was named *transamination* [1]. This discovery, one of the greatest in biochemistry in the XX century, was of fundamental importance in understanding the pathways of assimilation and dissimilation of nitrogen. From then on, A.E. Braunstein's research was almost entirely focused on the study of the enzymatic transformations of amino acids and the establishment of the role of transamination reactions in metabolism. This work was interrupted by the Second World War and resumed in 1945 at the Institute of Biological and Medical Chemistry, USSR Academy of Medical Sciences.

Among the investigations performed by A.E. Braunstein in that period, the following should be noted: the experimental confirmation of his hypothesis put forth in 1939; according to the hypothesis, the concerted action of transaminase and glutamate dehydrogenase plays a key role in the metabolism of nitrogen and its conjugation with energy processes in the cell [2]. Following the discovery in 1944-1945 (in the USA and Great Britain) of the participation of pyridoxal-5'-phosphate (vitamin B6) in the enzymatic reactions of transamination and decarboxylation, intensive studies of the role of pyridoxal-5'-phosphate in nitrogen metabolism began at the laboratory headed by A.E. Braunstein. Previously unknown pyridoxal-5'phosphate-dependent reactions of the transformation of tryptophan, serine, cysteine, and their analogs were discovered. In 1949, A.E. Braunstein published a brilliant paper concerning the pathways of the transformation of *L*-tryptophan in animals [3]. Firstly, the metabolic reactions suggested in the work were later empirically confirmed via experimental studies performed soon af-

Alexander Evseevich Braunstein

terwards. Secondly, the hypothesis that the participation of pyridoxal-5'-phosphate in the by-then-known enzymatic transformations of amino acids could be explained by the electron properties of a coenzyme molecule was suggested; the hypothesis was a prerequisite for the general theory of pyridoxal catalysis. Thirdly, the mechanism of the breakdown of *L*-kynurenine, different from those given by non-Russian researchers, was suggested. In 1998, evidence confirming the validity of Braunstein's mechanism was obtained [4]. It should be noted that all the suggestions and conclusions E.A. Braunstein made owed a debt to his solid knowledge of organic chemistry.

The general theory of pyridoxal-5'-phosphate-dependent catalysis was formulated by A.E. Braunstein and M.M. Shemyakin in 1952–1953. This theory considered the electron peculiarities of pyridoxal-5'-phosphate, which is responsible for its ability to catalyze various chemical reactions, and postulated that the protein matrix of enzymes is one of the factors behind their reaction specificity [5]. A year later, American researchers (Snell E.E. et al.) proposed an analogous theory [6]. These works not only formed the basis for the explanation of the mechanism of the action of pyridoxal-5'-phosphate enzymes, but also spurred studies of the mechanisms in other coenzyme-dependent reactions.

In 1960, A.E. Braunstein was invited by V.A. Engelhardt to work for the Institute of Radiation and Chemical Biology, USSR Academy of Sciences (later renamed into the Institute of Molecular Biology, the USSR Academy of Sciences), at which he organized the Laboratory of Chemical Principles of Biocatalysis. In his autobiography (1981), A.E. Braunstein wrote: "*Since then, our group has focused primarily on the study of the molecular structure and detailed catalytic mechanism of the main types of pyridoxal-5'-phosphate-dependent enzymes by applying novel chemical and physical approaches*". In the recently established institute, V.A. Engelhardt brought together scientists specializing in various scientific fields and with various scientific interests, thereby enabling the performance of complex studies, which greatly promoted the development of molecular enzymology in Russia. At present, the followers of A.E. Braunstein, a founder of molecular enzymology in Russia, work in many different fields of physicochemical biology.

Among the most important works of A.E. Braunstein and his colleagues, the following should be noted: the dynamic molecular

model of enzymatic transamination developed in 1986 [7]; the determination of the amino acid sequence of aspartate aminotransferase from the cytosol of the pig's heart [8] (in collaboration with Yu.A. Ovchinnikov and researchers from the Institute of Bioorganic Chemistry); and the determination of the spatial structure of aspartate aminotransferase from the cytosol of the hen's heart (1977) (in collaboration with B.K.Vainstein and researchers from Shubnikov Institute of Crystallography) [9]. A.E. Braunstein summed up the main results of his versatile research in a book [10] published after his death on July 1, 1986.

In 1936, A.E. Braunstein married Sofia Vilgelmovna Kreiden. His wife played an important role in his scientific career, always supporting him; A.E. Braunstein wrote: "*her irresistible charm and devoted care maintain a unique atmosphere of harmony and happiness at our home*". Anyone who has had an occasion to work alongside, or indeed meet Alexander Evseevich, remembers not only his unique scientific knowledge, but also his unfailing kindness, friendliness, and their hospitality with Sofia Vilgelmovna.

A.E. Braunstein paid a great deal of attention to scientific and organizational work. He sat on the editorial boards of many Russian and non-Russian journals, academic boards, and was a member of various biochemical societies. His encyclopedic knowledge assisted in the development of the principles of a classification system for enzymes and a biochemical nomenclature.

A.E. Braunstein received wide international recognition for his work, commanding significant scientific authority in Russia and abroad. Many foreign scientists visited and worked at the Laboratory of Chemical Principles of Biocatalysis.

It is hard to overstate the influence of Braunstein's ideas on today's physicochemical biology and molecular enzymology. A.E. Braunstein was elected a full-fledged member of the Academy of Medical Sciences (1945) and the USSR Academy of Sciences (1964). He was awarded honorary doctorates from the Universities of Brussels, Greifswald, and Paris VII, and he held honorary membership in scientific societies and academies of sciences in a number of countries, including the Nation Academy of Sciences of the USA.

A.E. Braunstein won the State Prize (1941) and the Lenin Prize (1980) in the field of science and technology, and he was awarded the title of Hero of Socialist Labor (1972), and two Orders of the Red Banner of Labor. ●

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V. Richter: "The low toxicity and originality of the pharmaceutical agent are its major advantages"

There is no question that the government needs to provide vigorous financial support to Russian researchers looking to design new drugs. A different question is to what extent should the government participate in the financing and at what point should industrial investors join in?

Vladimir Richter

In the course of implementation of the initial stage of the strategy Development of Pharmaceutical and Medical Industries in the Russian Federation over the Period up to 2020 and Future Prospects, there are signs of the first active initiatives of the government focused on funding research-and-development in the field of new drugs. To be more exact, there are the results of competitions for government grants for a number of activities to be implemented within a particular Federal target programme (FTP). One of the goals consists in providing financial support in the development of products that could reach the market in the nearest future. This year, the Ministry of Education and Science of the Russian Federation announced that it was holding an open competition for the right to negotiate contracts for conducting preclinical studies for promising, cutting-edge drugs. We interviewed **Vladimir Richter**, the Deputy Director for Science at the Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, whose project, Preclinical Studies of a Pharmaceutical – Antitumour Agent Based on Human Milk-Derived Peptide Lactaptin, won the competition.

Vladimir Aleksandrovich, please tell us about this innovative pharmaceutical agent? Do you intend to carry out preclinical trials?

For many years, surgery, chemotherapy, and radiation therapy aimed at removing a primary tumor have been the major means for treating oncological diseases. Meanwhile, metastatic tumor growth has remained the main agent causing subsequent death of patients. Our novel oncotherapeutic agents are based on the principle of targeted therapy. Put in another way, agents suppressing the viability of tumor cells, without causing normal cell death, are being designed.

Several years ago we established that human milk contains the protein factor that induces apoptosis (i.e., programmed cell death) of cancer cells. We purified this factor to a homogeneous state and found out that it was a peptide, which is a fragment of kappa-casein present in milk. It consists of 74 amino acid residues and has a molecular weight of 8.6 kDa. The novel peptide became known as lactaptin (this name represents its peptide origin and apoptotic properties).

Then, we used genetic engineering techniques to obtain a number of producers of various analogues of this peptide in *E. coli* and compared the effect they have on cancer cells. The properties of the RL2 analogue were the most similar to those of the natural peptide. This analogue was used to test efficacy against various cell lines. Human breast adenocarcinoma cells MCF7 demonstrated the

highest levels of sensitivity to the agent. Therefore, all subsequent experiments aimed at elucidating the mechanism of *in vitro* apoptotic action of the RL2 analogue were carried out using this cell line.

Judging from the fact that you proceeded to preclinical trials, the agent showed good results on model animals. Is it true?

Yes, it is. We established that our peptide induces apoptosis of several murine oncotransformed cell lines. We attempted to develop a method to treat malignant neoformations in mice and obtained very encouraging results: after the intravenous introduction of RL2 to tumor-grafted mice, tumor growth was significantly held back. Moreover, RL2 therapy considerably increases the lifespan of mice with ascytic tumors. We compared the action of our peptide with that of the standard drug cyclophosphamide, which has been conventionally used for chemotherapy. The cytotoxic action of these agents is similar. Hence, we ascertained that our peptide specifically acts on a selected target and can thus be considered to be a potential antitumor agent.

What is the advantage of your pharmaceutical agent?

The low toxicity and originality of the pharmaceutical agent are its major advantages. The drug candidate is based on a nontoxic and non-immunogenic protein derived from human milk. The patent research has demonstrated that proteolytic fragments of kappa-casein (including lactaptin) had not previously been used for cancer therapy.

Speaking about the rivals: aren't you afraid that pharmaceutical giants may leave you behind, since they have much more resources?

For a start, let's just say that there have been few antitumor pharmaceuticals of peptide or protein nature on the market thus far. It is quite possible that some companies will do their best and leave us behind. However, such a course of events is not guaranteed. Even registered patents do not ensure 100% protection. We are positively aware of the fact that it will be very difficult for us to bring the new agent to registration as a drug even with preclinical trials being successful. At this stage, there is nothing we can do but work strenuously and hope that we will be able to strike a partnership with a large pharmaceutical company.

Are you going to conduct trials yourself or do you intend to share this work with partners?

Most of the work will be done by us. Since regulations for conducting preclinical studies suppose that certain work is done by authorized organizations, we are going to engage these organizations in conducting the individual stages of the trial.

Are off-budget funds required to conduct the preclinical trials under a Government Contract?

Our partner is a small commercial enterprise which has been participating in the research and investing funds into project implementation for a long time. Today, it is this small enterprise that equips the working area to produce pilot scale batches of the pharmaceutical agent.

Was it difficult to carry out the project expertise?

At the stage where the objective was formulated, we held long-lasting correspondence with the supervising expert. At first, we did not understand each other very well, since we knew nothing about the specific requirements imposed on medical products as we were used to dealing with fundamental science only. We gained valuable experience and are grateful for the patience of our expert during project preparation. ●

Interview by
Elena Novoselova

18-20 of June, 2012 M.V. Lomonosov Moscow State University

The sixth international conference "Progress and trends in bionanoscopy"

Invites to participate!

The conference is devoted to high resolution biological microscopy (scanning probe, electron, optical and fluorescent), to issues of data processing and computer modeling, as well as to innovation activities in these fields.

Nobel Prize winning physicist **Ivar Giaever** - speaker at 2011 "Progress and trends in bionanoscopy" conference

Conference:

The conference will include the talks of specialists in bionanoscopy: Russian and international research groups

- Plenary sessions
- Poster sessions
- Practical training
- Bioimage contest

For those who wish to participate:

Scientific results presented at the conference will be recommended to the expanded publication in the ActaNaturae journal.

Deadline for abstract submission
March 31

More information on the conference webpage:
www.nanoscopy.org/bionanoscopy/

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Molecular Mechanisms of Induced Pluripotency

I.A. Muchkaeva*, E.B. Dashinimaev, V.V. Terskikh, Y.V. Sukhanov, A.V. Vasiliev
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ABSTRACT **In this review the distinct aspects of somatic cell reprogramming are discussed. The molecular mechanisms of generation of induced pluripotent stem (iPS) cells from somatic cells via the introduction of transcription factors into adult somatic cells are considered. Particular attention is focused on the generation of iPS cells without genome modifications via the introduction of the mRNA of transcription factors or the use of small molecules. Furthermore, the strategy of direct reprogramming of somatic cells omitting the generation of iPS cells is considered. The data concerning the differences between ES and iPS cells and the problem of epigenetic memory are also discussed. In conclusion, the possibility of using iPS cells in regenerative medicine is considered.**
KEYWORDS **reprogramming; iPS cells; ES cells; differentiation; transformation; pluripotency.**
ABBREVIATIONS **ESCs – embryonic stem cells; iPSCs – induced pluripotent stem cells; Chd1 – chromodomain helicase DNA-binding protein; BAF – rg/Brahma-associated factors; miRNA, miR – microRNA; TERRA – telomeric-repeat-containing RNA; Cdkn – cyclin-dependent kinase inhibitor; VPA – valproic acid; siRNA – small interfering RNA; KMOS – Klf4, c-Myc, Oct4, Sox2; KOS – Klf4, Oct4, Sox2; LNOS – Lin28, Nanog, Oct4, Sox2; GSK-3 – glycogen synthase kinase 3; ROS – reactive oxygen species; HIF1 – hypoxia-inducible factor 1; VEGF – vascular endothelial growth factor.**

INTRODUCTION

Pluripotent stem cells are capable both of self-renewal and generation of all the cell types that constitute the three germ layers. Until recently, pluripotent stem cells were derived from cultures obtained from the internal cell mass of the blastocyst (embryonic stem cells – ESCs) [1, 2]. However, the procedure of obtaining ESCs was burdened with numerous practical and ethical issues that made it impossible to use ESCs in clinical practice. Because of this, the global scientific community pressed on with its active search for an appropriate method for obtaining cells with characteristics similar to those of ESCs. Certain progress was achieved in 1997, when Wilmut *et al.* reprogrammed breast somatic cells via the transfer of their nuclei into oocytes after the second meiotic division (somatic cell nuclear transfer, SCNT) [3–6]. In 2001, Tada *et al.* achieved the same result via the fusion of mouse thymocytes with ESCs [7]. However, all attempts aimed at eliminating the technical complexity and low reproducibility of these methods failed, as did the attempts aimed at using these techniques for primate cells.

In 2006, based on accumulated data, Takahashi and Yamanaka assumed that an unfertilized cell and ESCs contain pluripotency-determining factors [8]. The method for the introduction of genes playing a key role in early development using lentiviral constructs was described in their studies on mouse fibroblasts [8] and, subsequently, on human cells [9]. It was successfully demonstrated that the ectopic gene expression of only four transcriptions factors, Oct4, Sox2, Klf4, and c-Myc (subsequently referred to as the KMOS canonical gene set, or the "Yamanaka cocktail"), is sufficient for the reprogramming of fibroblasts into a pluripotent state. The cells obtained using this procedure were referred to as induced pluripotent stem cells (iPSCs); the phenomenon of reprogramming into a pluripotent state was referred to as induced pluripotency. Many characteristics of iPSCs are identical to those of ESCs (e.g., gene expression profiles, morphology, telomerase activity, the character of DNA methylation and histone modification). Furthermore, iPSCs are capable of *in vitro* generation of the tissue cells of the three germ layers; they form mature teratomas after they are injected into immunodeficient mice. Chimeric animals were successfully created; their descendants included the ones obtained from the reprogrammed cells [10, 11]. At the time of writing, a significant number of studies have been published reporting that human iPSCs have been obtained via various methods [12]. Cell reprogramming techniques characterized by higher efficiency and safety compared to the transfection of viral

vectors have been designed for potential clinical use [13]. iPSCs from patients with various inherited diseases have been obtained [13, 14]. There are two extensive research areas associated with cell reprogramming: namely, fundamental research of cell plasticity and the genetic mechanisms underlying the early development of the organism and neoplasias, and the technologies for reprogramming somatic cells in order to conduct substitution cell therapy [15]. The cell technologies using iPSCs are capable of providing patient-specific cell lines, including those obtained from the carriers of inherited diseases. These cell lines can be used for the simulation of various diseases and for the testing of new pharmaceutical agents.

MOLECULAR MECHANISMS UNDERLYING PLURIPOTENCY INDUCTION

Autoregulatory loop. The equilibrium between Klf4 and c-Myc. The impact of the Ink4/Arf locus

A trove of data has been published to support the hypothesis that pluripotency is regulated by three transcription factors, Oct4, Sox2, and Nanog [16]. It was demonstrated [17, 18] that the combination of Oct4, Sox2, and Nanog factors activates the promoters of both their own genes and the genes of each other, thus forming an autoregulatory loop. Data exists indicating that the autoregulatory loop enhances the stability of the pluripotency gene expression [19, 20]. The three factors under consideration are also capable of initiating the cascades of both active and inactive genes (involving up to several hundreds of them). The expression of the *Oct4, Sox2*, and *Nanog* genes serves as the basis for the transcriptional network, which ensures the pluripotency of ESCs by enhancing pluripotency gene transcription and simultaneously suppressing the activity of the genes associated with the differentiation and development [21–23].

In their pioneering studies, Takahashi and Yamanaka proceeded with the analysis of 24 genes and subsequently elucidated that four genes (*Oct4, Sox2, Klf4* and *c-Myc*) are sufficient for cell transfer into the pluripotent state. Whereas the first two genes are pluripotency master genes, the *Klf4* and *c-Myc* genes were selected for different reasons. The transcription factor c-Myc is known to increase the proliferation rate [24], which is an essential condition for successful reprogramming [25]. Moreover, the hyperexpression of this gene results in an increase in the p53 protein level. It was demonstrated in a number of studies that the expression of *Klf4* leads to an increase in the level of the p21 protein (a cyclin-dependent kinase inhibitor) [23] resulting in proliferation suppression, on one hand, and reduces the cellular level of p53, which has a positive effect on the reduction of the apoptosis risk [26], on the other hand. Thus, one can assume that *c-Myc* and *Klf4* are mutually complementary, their action being oppositely directed. Therefore, equilibrium between the expressions of these two genes is important for successful reprogramming [27].

The inhibition of the *Ink4/Arf* locus, which contains *Cdkn2a* and *Cdkn2b* encoding three powerful tumor suppressors, p16 (*Ink4a*), p19 (*Arf*), and p15 (*Ink4b*), is one of the key characteristics of pluripotent stem cells. It is the *Arf* gene that activates p53 and p21 in mouse cells, whereas the *Ink4a* gene mostly has these functions in human cells. It was demonstrated [28] that the *Ink4/Arf* locus is completely suppressed both in iPSCs and ESCs by epigenetic bivalent domain marks (the emergence of histone modifications repressing H3K27me3); however, the locus can be reactivated upon cell differentiation. Oct4, Sox2, and Klf4 jointly suppress this locus, increasing both the reprogramming kinetics and the number of iPSC colonies, thereby facilitating the enhanced generation of iPSCs. It should also be noted that some researchers directly attribute the activation of the *Ink4/Arf* locus to the overall aging of the organism. Therefore, it is more difficult to reprogram cells taken from an old donor in comparison to those taken from a young one. In this case, the suppression of the *Ink4/Arf* locus can considerably increase the efficiency and rate of reprogramming [25].

Epigenetic regulation of gene expression in pluripotent stem cells

ESCs can be distinguished from the differentiated cells due to certain epigenetic characteristics. Thus, the key pluripotency genes (*Oct4* and *Nanog*) are demethylated in ESCs and can be actively transcribed, whereas the differentiation results in the suppression of these genes via *de novo* DNA methylation. It is of interest that the methylation marks are removed during reprogramming. This enables the reactivation of the endogenous transcription of these genes [29].

In addition to DNA methylation, ESCs and the differentiated cells also have different histone modification patterns. Thus, the suppression of the genes responsible for the development and differentiation of ESCs is regulated via combinations of the activation (H3K4me3) and repression (H3K27me3) of histone modifications. Transcription regulation is mediated by Polycomb-group proteins, which suppress gene expression by means of their binding to the histones containing H3K27me3. This mechanism is considered to be a tool for the transcriptional flexibility of ESCs, which is conditioned by stable repression of the development-associated genes without the irreversible inactivation of these genes. Taking into account the fact that bivalent domains can

be found virtually only in ESCs and are an important characteristic of the pluripotent status, one can assume that the regeneration of "bivalent domains" is also a key stage in the reprogramming of somatic cells in iPSCs. It has been demonstrated in a number of studies that chromatin in the completely reprogrammed iPSCs contains bivalent histones that are identical to those in ESCs [10, 30].

At the time of writing, neither study completely represents the picture of the relationship between the transcription factors, chromatin modifications, and cascades of pluripotency genes during cell reprogramming. Nevertheless, not much data has been published relating to the analysis of the expression of transcription factors in human and mouse ESCs [31] and in iPSCs [32], which can be used as a basis to construct the reprogramming model. Based on the data available, one can assume that Oct4, Sox2, Nanog, and Klf4 are the key units of the reprogramming process, during which nucleosome recovery, assemblage of diacetylases, activation of Polycomb proteins, and chromatin remodeling occur. In 2010, Pereira *et al.* demonstrated in their studies devoted to the fusion of ESCs and lymphocytes that the components of the Polycomb complex play a significant functional role in epigenetic remodeling. ESCs deficient in Polycomb-group proteins lost their ability to remodel the somatic cell genome [33].

The mechanism underlying the suppression of the expression of differentiation genes in pluripotent cells comprises the binding of one or several pluripotency factors to the target gene promoters [34]. The binding between the reprogramming factors and their target genes can be facilitated by nucleosome remodeling complexes, such as Chd1 (chromodomain helicase DNA binding protein 1) [35] and BAF (rg/Brahma-associated factors – ATP-dependent chromatin-remodeling complex) [36]. These complexes improve the reprogramming efficiency and kinetics. Their regulatory role presumably consists in the reactivation and maintenance of the endogenous pluripotency gene expression in the absence of exogenous factors. This assumption is based on the fact that the endogenous pluripotency signals, as well as telomerase and the repressed X-chromosome in female cells, are reactivated by the end of the reprogramming process, whereas the activity of the retroviral genes is suppressed, although no clearly pronounced differentiation and relationship between these processes have been detected [37].

The role of microRNAs in pluripotency maintenance

A considerable improvement in reprogramming efficiency after the *Lin28* gene is added to the set of reprogramming factors has been observed in a number of studies devoted to the reprogramming of somatic cells to a pluripotent state [38]. The major contribution of the *Lin28* gene is thought to be its participation in microRNA (miRNA) processing. It was assumed that in ESCs, *Lin28* inhibits *let7* miRNA processing [39], a well-known tumor growth suppressor gene. This gene participates in the suppression of c-Myc activity. Furthermore, it was demonstrated that the key reprogramming factors Oct4, Sox2, and Nanog are capable of initiating the miR-90 family, whose members are expressed in ESCs under normal conditions and participate in the proliferation regulation, as well as the self-maintenance, of these cells. The activation of mir-290 during reprogramming may also be a result of chromatin remodeling by c-Myc [40]. The miRNA cluster (miR-203–367) promoter is one of the targets of the pluripotency transcription factors Oct4, Sox2, Nanog, and Rex1. Its products can indirectly induce TGF-β/Nodal/Activin signaling pathways (this signaling pathway plays a significant role in the maintenance of the pluripotency status of ESCs and in the suppression of their differentiation) by inhibiting some pathway regulators, which in turn has a positive effect on the maintenance of cells in an undifferentiated state.

The effect of cell aging and immortalization on reprogramming

Studies devoted to the interrelationship between reprogramming and the processes of cell aging and immortalization are of considerable interest. It was repeatedly reported in the early studies that telomerase activity increases in somatic cells and telomeric DNA regions last considerably longer in the course of reprogramming. Thus, the reprogrammed cells acquire immortality, typical of ESCs [8, 9, 41]. Yehezkel *et al.* [42] thoroughly studied the telomere length, the methylation in subtelomeric regions, and expression of the telomeric-repeat-containing RNA (referred to as TERRA) in iPSCs. In addition to supporting the previously ascertained data regarding telomerase activation and telomere elongation in iPSCs, it was demonstrated that subsequent differentiation resulted both in a considerable reduction in telomerase expression in the cells under study and in a strong shortening of their telomeric regions. The results obtained in that study provided evidence in support of the significant role of telomerase and the telomere state in the maintenance of the pluripotent status. The subtelomeric regions in iPSCs were hypermethylated compared with the initial cells, whereas the level of TERRA was increased relatively. It is assumed that regulation of the expression of telomeric-repeat-containing RNA can also participate in the reprogramming processes, along with regulation of the expression of the telomerase catalytic component (telomerase reverse transcriptase, TERT) [42].

The essential role of TERRA expression in the reprogramming processes was attested to by the results of study [43], in which the cells of patients with dyskeratosis congenital (a genetic disease associated with telomere dysfunction due to premature telomere shortening) were investigated. Contrary to expectations it turned out that the cells reprogrammed via *Oct4*, *Sox2*, *KLf4*, and *c-Myc* also elongate the telomeric regions and restore telomerase activity. The mechanism of restoration of telomerase activity was attributed to the restoration of TERRA expression [43].

Utikal *et al.* demonstrated that the acquisition of immortal status is an essential and limiting factor for the reprogramming of somatic cells into a pluripotent state, which may also attest to the fact that the reprogramming and transformation mechanisms are similar [25].

THE COMPLEXITY, MULTISTAGENESS, AND STOCHASTICITY OF PLURIPOTENCY INDUCTION

Low reprogramming efficiency is one of the problems of cell reprogramming through the addition of transcription factors. In the previous procedure of KMOS transfection, approximately 0.01–0.1% of the transfected cells were subjected to reprogramming; this index is considerably lower than that obtained when the cell fusion or nuclear transfer technique is used. Several hypotheses for explaining such a low yield of reprogrammed cells have been put forth:

1) iPSC formation requires specifically narrow ranges of the expression levels of transcription factors. Upon simultaneous transfection of several genes within a lentiviral construct, the distribution of gene expression over cells obeys the probability law (because of different viral copy counts per cell and random integration into the genome). This is presumably the reason why only a small fraction of the transfected cells acquire the "proper" set of expression levels of the reprogramming factors. The pluripotent status of ESCs is known to be quite sensitive to the expression levels of pluripotency genes; e.g., a 50% variation in the Oct4 expression level results in ESC differentiation [44].

2) The populations of reprogrammed somatic cells are heterogeneous and contain a certain amount of cells that are more susceptible to reprogramming than the other cells. For example, some cells (most likely, the dividing ones) during the transfection contain chromatin in a relatively decondensed state facilitating the reprogramming.

3) An unusually high expression level of the exogenous reprogramming factors activates the stress-associated genes that suppress proliferation. Thus, the expression of the *Cdkn1a* and *Cdkn2a* genes, the inhibitors of cyclin-dependent kinases that are involved in various differentiation pathways, increases in the transfected fibroblasts [45]. This fact is explained by the addition of transcription factors, since it is already known that the *Cdkn1a* expression is induced by the Klf4 factors, whereas *Cdkn2a* is activated due to the aberrant expression of *c-Myc* [46]. Thus, the internal self-preservation mechanisms are activated in the transfected cells; these mechanisms suppress uncontrollable proliferation, finally resulting in a low percentage content of cells with a chance of overcoming the barrier of proliferation suppression and achieving the pluripotent state.

4) Insufficient number of reprogramming factors. When using the techniques of reprogramming via cell fusion and nuclear transfer, a somatic cell or its nucleus is subjected to the action of all the components of the pluripotency transcriptional network. These components act at all levels, whereas only a limited number of factors act on the cells upon reprogramming via lentiviral transfection. These factors can activate the transcription cascades only at the very beginning, which makes the reprogramming process more vulnerable to and dependent on random variations.

Based on these hypotheses, one can conclude that the reprogramming induced by the transfection of transcription factors is characterized by a low efficiency and multistageness, as well as being strongly dependent upon stochastic processes. The significance of random variations upon iPSC formation is supported by data showing that the resulting reprogrammed cells are appreciably heterogeneous in terms of the general profile of pluripotency gene expression, epigenetic profile, and morphology. It was demonstrated that the iPSCs originating from the same parental cells reactivate the expression of endogenous *Oct4* at different time points during the entire reprogramming process, attesting to the multistageness of epigenetic rearrangements and the reprogramming in general [27]. However, when comparing the reprogramming method under consideration with the previously proposed techniques of nuclear transfer and cell fusion, one must allow for the fact that the method proposed by Takahashi and Yamanaka has a number of undeniable advantages, such as a relatively low cost and the simplicity of the reprogramming technique. The universality of the approach should also be noted, since it enables reprogramming of human cells, which had previously been impossible.

NEW METHODS FOR REPROGRAMMING SOMATIC CELLS TO A PLURIPOTENT STATE

The original technique for the transfection of reprogramming transcription factors using lentiviral vectors has a number of substantial drawbacks that impede its application in clinical practice. Virus integration into the host genome (up to 20 insertions per reprogram-

ming procedure) increases the risk of tumor formation, since virus incorporation into the target cell genome can accidentally activate or inactivate the host genes, thus increasing the mutagenesis risk. Continuous transgene overexpression is also problematic because of possible incomplete transgene suppression during the reprogramming and upon subsequent differentiation. Even the presence of several pluripotent stem cells in the transplanted tissue may result in tumor development [47].

One of the strategies to solve these problems is based on the reduction of the number of viral vectors introduced, which is achieved via the construction of polycistronic viral vectors carrying several target genes. Constructs encoding the four major reprogramming genes (KMOS) were designed in [47, 48]. The number of viral integrations into the genome was successfully reduced to 3–5 insertions per cell and the homogeneous expression of all four genes in one cell was provided via the use of a single lentiviral construction containing KMOS. Mouse fibroblasts were successfully reprogrammed via the integration of the polycistronic lentiviral vector encoding three genes, *Oct4, Sox2, Klf4* (KOS), followed by the removal of the vector from the genome. This approach enhances the attractiveness of the method due to the fact that the viral material is completely eliminated from the reprogrammed cells [49]. A similar procedure was applied in [50]: a specific piggiBag transposon containing KMOS was used; subsequently, it was eliminated to obtain iPSCs from mouse embryonic fibroblasts, which were free of transgenes and vector sequences. This method has also been used for human cells in some studies [51]. A considerable drawback of the aforementioned approach is that the elimination process of a number of transposons following the reprogramming is difficult to control; moreover, it does not guarantee a 100% result.

The technique for iPSC generation via plasmid transfection of the major pluripotency factors (KMOS or LNOS – *Lin28, Nanog, Oct4, Sox2*) based on temporary expression of the inserted genes was the next approach in the attempts to resolve the problem of viral integration into the genome. Successful application of this technique for iPSC generation from various cell cultures, including hepatocytes and HEK293 cells, has been reported in a number of studies [52–55]. In the course of improving the technique, the cells were successfully transfected with a single plasmid construct encoding the KMOS canonical gene set. This construct was eliminated from the cells following the reprogramming [54]. It should be noted that one of the drawbacks when using plasmid constructs (compared to the viral vector-based methods) is an extremely low reprogramming efficiency, since this method was mostly used to generate iPSCs from mouse embryonic cells and cell lines known for their lability. However, efficiency in reprogramming human fibroblasts was enhanced by 1% by using riP/EBNA1 episomal plasmid vectors that can encode six genes (*Oct4, Sox2, Klf4, c-Myc, Lin28*, and *Nanog*) at once [56].

Another unavoidable drawback of the techniques based on plasmid vectors is the possibility that residual DNA vectors may be present in the target cells after the reprogramming, and therefore the theoretical possibility of insertion mutagenesis [56]. Several methods were designed while searching for approaches that would eliminate the possibility of the incorporation of foreign DNA into the host DNA. Human iPSCs were generated using the Sendai transgenic virus with a reproductive cycle based only on RNAs, which contains neither the stage of DNA reverse transcription (as is the case in lentiviral vectors) nor the stage of integration into the host genome [57]. The advantage of this method is the relatively high gene introduction into various cells and tissues; the drawbacks include the complicated handling of the Sendai virus and the compulsory purification of the reprogrammed cell to remove the replicating virus [57].

Another reprogramming approach without the use of DNA vectors is based on the delivery of reprogramming factor proteins directly into the cells. A specific complex of recombinant proteins consisting of the polyarginine protein-transducing subunit bound to all four major reprogramming factors (KMOS) was designed to create proteins capable of penetrating through the plasma membrane of somatic cells [58]. This approach is relatively simple; the risk of changes in the target cells caused by exogenous genetic sequences decreases when using this technique [58]. However, in a later study in which human cells were used as an object [59], low efficiency was reported for the method. The efficiency of reprogramming using KMOS proteins conjugated to the cell-penetrating peptide (CPP), which contains a large percentage of basic amino acids and is capable of penetrating through the cell membrane, was equal to 0.001%, which is lower than that in the methods based on viral integration by two orders of magnitude.

Transfection of the *in vitro* synthesized mRNA of transcription factors is another promising method for reprogramming somatic cells without the use of DNA vectors. The researchers used mRNA of the LNOS genes [60] to successfully reprogram human neonatal fibroblasts to a pluripotent state. However, despite the fact that the result was achieved, a low reprogramming efficiency (0.0005%) was also observed. The authors attributed this problem to the high cytotoxicity of large mRNA doses [60]. However, the difficulties were overcome via the use of synthetic mRNA of the KMOS and *Lin28* genes, which was comprised of modified ribonu-

cleotides [61]. Combined with the use of the interferon inhibitor B18R and cell culturing under low oxygen content, this technique enabled the attainment of low cytotoxicity and transfection. Thanks to these modifications, the reprogramming efficiency increased by two orders of magnitude and reached 4.4% compared with the 0.04% that was obtained using viral transfection. A large-scale research project focused on the reprogramming of a wide range of somatic cells (including human cells) and analysis of the resulting iPSCs was subsequently carried out [61].

THE USE OF SMALL MOLECULES FOR REPROGRAMMING

The use of low-molecular-weight compounds, the so-called small reprogramming molecules, is one of the approaches to the reprogramming of human somatic cells. Combined with the earlier designed methods, these molecules are capable of either functional substitution of particular reprogramming factors or facilitating the increase in efficiency of the process. Thus, BIX-01294 (BIX), an inhibitor for the G9a histone methyltransferase, was used. The application of this agent in addition to the transfection using the *Klf4*, *c-Myc*, and *Sox2*, as well as the *Klf4* and *Oct4* sets within the lentiviral vectors, considerably enhanced (by a factor of 6–10) the yield of the reprogrammed cells [45]. This is attributed to the specific activity of BIX, which facilitates chromatin de-condensing and therefore can functionally substitute the c-Myc transcription factor [45]. 2-propylvaleric acid (valproic acid, VPA) is another compound capable of considerably increasing the reprogramming efficiency [62]. It can specifically inhibit DNA methyltransferases and histone deacetylase. According to [38], the use of this small molecule, in addition to the standard KMOS set, enhances the reprogramming efficiency by 1–2 orders of magnitude and allows one to dispense with the *c-Myc* oncogene. The positive effect of 5-azacytidine (5-azaC) on the yield of reprogrammed cells was demonstrated using the same strategy for DNA methyltransferase inhibition [29]. Reprogramming efficiency can also be increased via the introduction of a small interfering RNA (siRNA), which inhibits the transcripts of the commitment-associated genes [29]. The positive effect of CHIR99021, a specific inhibitor of glycogen synthase kinase 3 (GSK-3), on efficiency in the reprogramming of mouse embryonic fibroblasts has been described. The yield of iPSC colonies was considerably increased by using CHIR99021. The number of reprogramming factors was reduced to two, *Klf4* and *Oct4*, thanks to the use of this reagent in a number of experiments [63].

Small molecules, such as arginine methyltransferase inhibitor AMI-5 and transforming growth factor β inhibitor A-83-01, facilitate the reprogramming process [64]. The induction of mouse fibroblasts by *Oct4* only and the addition of the two aforementioned small compounds resulted in the generation of iPSCs that expressed the typical pluripotency markers and could be differentiated into cells of three germ layers and produce viable chimeric mice. AMI-5 activity is comparable to the joint effect of three components (CHIR99021, Parnate and VPA). AMI-5 inhibits the activity of PRMT 1/3/4/6 and belongs to the family of proteins that catalyze mono- or dimethylated arginine residues. However, it remains to be determined how AMI enhances Oct-4-induced cell programming.

Interesting results were obtained when studying the effect of vitamin C on iPSC generation [65]. It turned out that the treatment of cells undergoing reprogramming with vitamin C in combination with the activation of the *Klf4*, *c-Myc*, and *Oct4* genes resulted in a considerable decrease in the p53 and p21 levels, as well as in the concentration of reactive oxygen species (ROS). It has been assumed that this factor enhances the reprogramming efficiency, since an increase in the ROS level is usually observed upon transfection with viral vectors. Sodium butyrate has a positive effect on the generation of iPSCs from adult and embryonic human fibroblasts [51]. It is suggested that sodium dutytate promotes the expression of DNA demethylase and H3 acetylation, which ultimately facilitates the expression of endogenous pluripotency factors, including *Oct4* and *Dppa2* (developmental pluripotency associated 2). The screening of various low-molecular-weight compounds was performed in one of the recent studies [66] focused on the role of small molecules in the processes of reprogramming and maintenance of the pluripotent status. A "cocktail" consisting of three molecules, PD98059 (mitogen-activated protein kinase inhibitor), CHIR99021 (glycogen synthase kinase inhibitor), and Y27632 (Rho kinase inhibitor), was selected based on the results of the study. The cocktail demonstrated a considerable effect on the ability of human ESCs to maintain their undifferentiated state under various culturing conditions.

DIRECT REPROGRAMMING OF SOMATIC CELLS

The so-called direct reprogramming can be attributed to areas that require special attention. This strategy presupposes the use of various methods for the transdifferentiation of a specialized cell type into another one, bypassing the stage of formation of pluripotent stem cells. If the method for direct reprogramming is designed, it would be possible to use cell technologies in clinical practice.

Study [67] can be mentioned among such research; in the study, mature exocrine cells from mouse pancreas

were *in vivo* reprogrammed into β-similar cells using adenoviral transcription of the genes of three transcription factors, *Ngn3 (Neurog3), Pdx1,* and *Mafa.* The morphology, ultrastructure, expression of the major markers, and key functions (insulin synthesis) of the induced β-cells were identical to those of the intact cells [67]. The data on the influence of the Oct4 transcription factor on the plasticity of mouse keratinocytes were published. Plasmid transfection of the *Oct4* gene was used to obtain a modified cell culture capable of differentiating into neural lineages under certain culturing conditions [68]. This field of research entered its next cycle of development in 2010, when it was demonstrated [69] that a short-term expression of the *Oct4* gene is sufficient in order to change the direction of differentiation of human keratinocytes, including the neural and mesenchymal lineage commitment.

The study where direct reprogramming of embryonic and neonatal mouse fibroblasts was induced *in vitro* [70] is also worthy of mention. A combination of 19 genes specific for the neural tissue and neurogenesis were used to successfully select three genes that perform cell transdifferentiation in the neuronal direction. Retroviruses carrying the *Ascl1, Brn2*, and *Myt1l* genes were used to infect fibroblast cultures and to observe the formation of functional neurons with a complex morphology. It also turned out that the formation of such characteristics of neural cells as the expression of certain neuron-specific voltage-dependent channel proteins, which are required to generate the action potential, can be carried out using a single *Ascl1* gene. However, joint expression of additional factors is required to make neuronal cell conversion easier and to provide for their complete maturation [70]. A similar result was reportedly obtained using human fibroblasts [71]: the cell phenotype was changed towards dopaminergic neurons after the *Lmx1a* and *FoxA2* genes were additionally inserted into the cells. It was proposed that astrocytes be used as an alternative source for the generation of cells with the characteristics of dopaminergic neurons [72].

Despite the apparent complexity related to the transdifferentiation of cells derived from one germ layer into cells derived from another germ layer, a number of studies have looked into the problem of cell plasticity. In these studies, evidence attesting to such a possibility was obtained both *in vitro* and *in vivo* [73–75].

Soda *et al.* successfully transdifferentiated glioblastoma cells into endothelial cells [73]. It was demonstrated that glioblastoma cells can be transdifferentiated into vascular endothelium and produce functional blood vessels that are insensitive to the inhibition of the VEGF receptor. The results of this study attest to the existence of a different mechanism of resistance of glioblastoma cells to anti-VEGF-therapy. The reprogramming of terminally differentiated hepatocytes to neuron-like cells has been reported [74].

Results of a successful direct reprogramming of mouse and human fibroblasts towards the neuronal differentiation direction have been published [75]. The reprogrammed cells, in which the expression of the *Ascl1* (*Mash1), Nurr1,* and *Lmx1a* genes was induced, were very similar to brain dopaminergic neurons in terms of the specific protein production, dopamine release, and pace-making activity. Researchers place their hopes on direct reprogramming of one type of cells to dopaminergic neurons that may be usefull in investigating and treating some neurodegenerative diseases, such as Parkinsons's disease.

THE DIFFERENCES BETWEEN iPSCs AND ESCs. EPIGENETIC "MEMORY" OF iPSCs

Despite the fact that many characteristics of iPSCs are rather similar to those of ESCs, there are also significant differences between these cell types. Among others, there are differences in the levels of control of pluripotency gene expression and in the formation of viable organisms after these cells are transplanted into a developing blastocyst to generate chimeric mice. Evidence has been obtained in support of the fact that the methylation levels of CpG islands in ESCs and iPSCs are similar [76]. A full genome analysis of the CpG islands localized in the functional regions comprising more than 14,000 genes revealed the difference in the methylation levels of 46 genes. The total CpG methylation of the promoter regions in pluripotent cells is higher in comparison to that found in somatic cells. Two ESC and iPSC lines derived from material that was genetically identical to ESCs were compared [77]. In animal chimera experiments, viable mice were successfully obtained from two ESC lines, whereas no animals were obtained from iPSCs. After thorough comparison of the RNA transcript profiles, it was ascertained that the transcription of the imprinted gene cluster *Dlk1-Dio3* in iPSCs is considerably lower than that in the ESC lines. It was detected that the region on chromosome 12 containing the key genes for fetal development was silenced in the iPSC line. Over 60 iPSC-like cell lines were also tested; a similar result was observed in most cases. It should be noted that this gene cluster was activated in a number of iPSC lines. Chimeric living mice were subsequently obtained from these cell lines. Thus, the state of this imprinted cluster allows one to introduce another characteristic for the adequacy of iPSC reprogramming [77]. iPSCs can be differentiated into definitive endoderm precursor cells to design approaches to the cell therapy of

damaged tissues of endodermal origin despite the fact that there are some differences between them at the molecular level [78].

It has been assumed that in addition to their potential application for the purpose of regenerative medicine, human ESCs and iPSCs can be used to simulate human inherited disorders. Meanwhile, before using these cells as a model for a particular disease, one needs to assess whether they contain any chromosomal rearrangements, which have put limits on the application of reprogrammed cells. Significant differences between the chromosomal characteristics of iPSCs and ESCs have been revealed [79]. iPSCs were obtained from the skin cells of three patients with a fragile X syndrome (FX) that is responsible for delayed mental development. Unlike ESCs obtained from patients with the FX syndrome, *FMR1* gene expression in certain types of differentiated cells from the same patients was reduced due to anomalous duplications of triplet repeats. It was demonstrated that iPSCs contain a mutated *FMR1* gene, which was not changed in the course of reprogramming, despite the pluripotent status [80]. The study made it apparent that iPSCs are not always suitable candidates for the simulation of diseases associated with epigenetic changes, including imprinting. In a similar study [81], DNA methylation patterns were analyzed in the genomes of 15 cell lines (four ESC lines, five human iPSC lines and the tissues from which these iPSCs were derived, and the differentiated cells obtained from the aforementioned two stem cell lines). Significant differences between iPSCs and ESCs were revealed; the methylation patterns near the chromosome ends and centres of iPSCs remained identical to those in the differentiated cells from which they were obtained. It is clear that reprogramming is a means for acquiring a pluripotent status other than obtaining cells from the embryos. Relying on these data, one can conclude that formation of certain cell types from reprogrammed cells may be restricted. The fact that reprogrammed stem cells have an epigenetic "memory" agrees with the recently published results of a comparison of iPSCs, ESCs, and pluripotent mouse cells obtained using the nuclear transfer procedure [82]. It was demonstrated that iPSCs contain residual epigenetic marks; however, these marks can be eliminated upon continuous culturing or by using specific agents that rearrange the chromatin structure. It was also ascertained that pluripotent stem cells obtained by nuclear transfer reprogram the epigenetic profile more efficiently in comparison with iPSCs.

In addition to the epigenetic "memory," the existence of gene duplications or deletions associated with genomic instability is the significant comparative characteristic of iPSCs. Genotyping of single nucleotide substitutions was used to compare 69 ESC lines and 37 iPSC lines between, as well as with linear and primary human cell cultures [83]. The results of this thorough study attest to the fact that pluripotent cells in general (and iPSCs to a larger extent) tend to accumulate duplications in the genomic regions containing the pluripotency genes and oncogenes, as well as to accumulate deletions in the region containing tumor growth suppressing genes.

Many researchers attribute the differences between iPSCs and ESCs to the reprogramming procedure and the existence of viral insertions into the genome. The transcription profiles of human ESCs and iPSCs were compared using methods without the use of viral constructs [84]. The transcription profiles of ESCs and iPSCs were shown to be largely similar; however, some differences were detected, which cannot be attributed to viral integration into the genome.

THE POTENTIAL OF USING iPSCs IN CLINICAL PRACTICE

Allogenic organ transplantation is associated with a number of problems, such as limited tissue engraftment and the necessity to use immunosuppressors. It is believed that these problems can be overcome by reprogramming the patient's own cells, because the cells grafted to the recipient will be genetically identical to his own cells. The method proposed is undoubtedly superior to the existing transplantation techniques because of the possibility of *in vitro* study and repair of the pathological mutations in the cells. For example, sicklemia has been successfully repaired using iPSCs on a mouse model [85]. The formation of normal erythrocytes from hematopoietic precursor cells obtained from completely reprogrammed skin cells was observed in [85].

Many diseases, such as diabetes mellitus type 1, the Parkinson's and Alzheimer's diseases, etc. are very difficult to study and cure both because the damaged organ is difficult to reach (hence the difficulties associated with the search for donor tissue) and because no methods have been designed for the continuous cultivation of the proper cell lines. When simulating these disorders, autological iPSCs can be obtained, followed by their differentiation in a culture into the required cell line to produce adequate test systems for the screening of pharmaceutical agents. These test systems can also be used to investigate the diseases accompanied by pathological motoneuronal death (e.g., in patients with amyotrophic lateral sclerosis or spinal muscular amyotrophy). The lack of cell materials originating from patients at late stages of the development of a disease is one of the main problems associated with the study of degenerative pathologies. Since iPSCs presumably have to undergo all the differentiation stages *in vitro,*

same as the recipient's cells before the disease develops *in vivo*, this technology can make it possible to study the early stages of a particular disease. Active research has been carried out; iPSCs have been already obtained in some laboratories from patients with Hungtington's disease, sicklemia, myodystrophy, the Down's syndrome, etc. [13, 14, 86, 87].

Considerable differences between the same cell types differentiated from ESCs and iPSCs have been revealed [88]. Study of teratoma formation in C57BL/6 and 129/SvJ mice has demonstrated that the disruption of gene expression in some cells differentiated from iPSCs may result in a T cell-dependent immune response in an isogenic recipient. Thus, the currently available reprogramming technologies are still a long way from clinical application. One of the primary tasks consists in the design of methods that would enable the epigenetic differences between iPSCs and ESCs to be minimized.

APPROACHES TO THE CLINICAL USE OF iPSCs

The use of oncogenes to obtain iPSCs is one of the major problems impeding the therapeutical use of these cells. The *c-Myc* oncogene is hyperexpressed in approximately 70% of human tumors; therefore, the hyperexpression of an inserted transgene makes the use of iPSCs dangerous [89]. In order to solve this problem, iPSCs obtained from humans and mice were subjected to study. No postnatal tumor development was observed in chimeric mice obtained from iPSCs without introduction of *c-Myc*, whereas oncological diseases developed in ~15% of the animals obtained from iPSCs with exogenous *c-Myc* [90]. *Oct4*, *Sox2*, and *Klf4* can also be associated with the emergence of different types of tumors; therefore, researchers increasingly try to avoid the transduction of these oncogenes [54, 56, 61, 91]. In order to achieve the necessary results, target cells are selected that would endogenously express the required factor at an adequate level, hence its introduction would be rendered unnecessary. Thus, the endogenous *Sox2* gene is strongly expressed in neutral stem cells; these cells were successfully reprogrammed in a number of experiments by inserting *Oct4* and *Klf4* only [45, 92] or even *Oct4* alone [92, 93]. Meningiocytes and keratinocytes can be regarded as promising cells for reprogramming because of their relatively high levels of *Sox2* [94], *c-Myc*, and *Klf4* [95, 96] expression. It has also been discovered that it is easier to derive iPSCs from amniotic fluid cells because of the fact that they are relatively weakly differentiated [97, 98]. The rate of iPSC formation from amniotic fluid cells is at least twice faster than that of iPSC formation from fibroblasts, whereas the reprogramming efficiency in the former case is higher by an order of magnitude. One of the approaches to reprogramming consists in the replacement of oncogenes for small molecules [38, 45]. Teratogenicity of iPSCs is a significant issue, since there may remain a certain amount of undifferentiated iPSCs that are dangerous for the recipient after these cells are differentiated into the specialized cells intended for transplantation [99]. The search for selection methods that would ensure the isolation of iPSCs from the differentiated cells continues. The karyotypic instability of pluripotent cell lines has been revealed via the study of the chromosome composition of ESCs and iPSCs [100], attesting to the necessity for a thorough cytogenetic analysis of iPSCs and initial cell lines.

The similarities and differences between ESCs and iPSCs are being actively investigated at the molecular and functional levels. The results of these studies may influence the therapeutic applicability of iPSCs. This field of research (as well as the development and optimization of differentiation protocols and the establishment of reliable criteria for the application of specialized cells generated from iPSCs) requires an analysis of the genomic and epigenomic statuses of human iPSCs. ●

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Regulation of Immunity via Multipotent Mesenchymal Stromal Cells

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ABSTRACT **Immune cells responsible for inflammation development are involved in tissue damage caused by wounding and various pathologies. Control of immune cell activation could be of significant benefit for regenerative medicine and the treatment of patients with autoimmune and degenerative diseases. It is a proven fact that MCSs (multipotent mesenchymal stromal cells) are capable of suppressing immune responses via the inhibition of dendritic cell maturation and via the restraining of the T, B, and NK cell function in the course of autoimmune diseases and various forms of inflammation. MSCs can be isolated easily from almost every type of tissue or organ and subsequently expanded *in vitro*. These cells are self-renewable and can be differentiated into various cell types of mesenchymal lineage. The current review contains a collection and critical analysis of data regarding the molecular mechanisms responsible for cross-talk between immune cells and MSCs. Some of these mechanisms can be used for the development of new practical approaches for the treatment of autoimmune diseases.**

KEYWORDS **immune system; multipotent mesenchymal stromal cells; inflammation; autoimmune disease; regeneration; immune suppression.**

ABBREVIATIONS **MSCs – multipotent mesenchymal stromal cells; CD – cluster of differentiation; SDF-1 – stem cell-derived factor-1; CXCR4 C-X-C chemokine receptor 4; VEGF – vascular endothelial growth factor; IGF-1 – insulin-like growth factor-1; BDNF – brain-derived neurotrophic factor; TGF-β – tumour growth factor; BMP– bone morphogenetic protein; IL-10 – interleukin-10; TNF-α – tumour necrosis factor; NK – natural killers; DC – dendritic cells; IFN-γ – interferon gamma; MHC – major histocompatibility complex; IDO – indoleamine-2,3-dioxygenase; PGE2 – prostaglandin E2; ICAM – intercellular adhesion molecule; VCAM – vascular cell adhesion protein; IL-1β – interleukin-1 beta; GVHD – graft versus host disease; EAE – experimental autoimmune encephalomyelitis; TLR – Toll-like receptor; HLA-G5 – non-classical molecule of histocompatibility complex class I antigen, G5 isoform.**

INTRODUCTION: GENERAL CHARACTERISTICS OF MSC

Multipotent mesenchymal stromal cells (MSCs) were originally characterized in the pioneering study of Friedenstein *et al.* in 1971 [1]. It was shown in that study that a heterogeneous fraction of cells bearing morphological resemblance to fibroblasts can adherently grow in a culture, tolerate numerous passages, and be isolated from bone marrow cells. MSCs express a set of markers on their surface (suggesting their mesenchymal origin) and are capable of differentiating into adipose, bone, and cartilage cells [1] and, to a lesser extent, into other cell types. The set of markers characteristic of MSCs includes CD105, CD166, CD54, CD90, CD55, CD13, CD73, Stro-1, and CD44; meanwhile, the surface of an MSC does not contain the hematopoietic markers CD14, CD45, CD34 and CD133 [2]. It was subsequently ascertained that cells with similar properties can be isolated not only from the bone marrow, but also from other sources (in particular, from adipose tissue) [3].

A detailed study of the properties of MSCs has demonstrated that self-sustaining clones can be derived from a fraction of single cells [4]. MSC populations from different sources can be passaged, as opposed to terminally differentiated cells; culture heterogeneity is strongly passage-dependent [5]. The rates of growth and division of MSCs in a culture gradually decrease due to telomere shortening at chromosome ends [6, 7].

The absence of any "reliable" surface markers renders the *in vivo* identification and study of MSCs extremely difficult; therefore, we have yet to determine whether MSCs are an artifact of *in vitro* isolation and cultivation of a complex cell mixture, or whether in-

deed this population exists in the organism. Opinions concerning the nature of MSC differ considerably. It has been clearly demonstrated in a number of studies that MSCs resemble fibroblasts (another stromal cell type) in terms of many characteristics [8]. The authors of a number of studies compare MSCs with the population of pericytes; i.e. vascular endothelium-associated cells that carry a set of markers on their surface, differing from that in MSCs to only a small extent [9, 10]. Nevertheless, the interest of researchers and medical investigators in MSCs is primarily a result of the unique properties of MSCs, which make these cells a promising object for cell and gene therapy; issues of their origin and philogeny ultimately fade into insignificance.

MSCS MIGRATE TO THE LESION LOCUS

When transplanted into animals with induced lesions or internal pathologies, MSCs are capable of migrating to the lesion site or to the inflammation focus. This discovery was confirmed by the results of experiments devoted to the systemic transplantation of variously labelled cells into recipients with the above-mentioned lesions (fluorescent protein-expressing cells were used, cells from male donors were transplanted into female recipients, human cells were used for heterologous transplantation into mice or rats) [11–15]. After a short period of time, the transplanted cells can be detected at the lesion site. MSC migration to the lesion (inflammation) site depends on chemokines, which is indirectly evidenced by the results of an analysis of chemokine receptor expression by MSCs. These cells express a wide range of chemokine receptors [16–18]. The contribution of most of them to the directed migration of MSCs has not yet been ascertained; however, it has been shown that SDF-1 and its receptor called C-X-C chemokine receptor type 4 (CXCR4) play the key role in this process. The CXCR4 level increases significantly in cells under stress conditions [16, 19, 20]. Disruption of signaling through this receptor using biochemical or genetic methods impairs MSC migration to the lesion/inflammation sites [19]. CXCR4 plays an essential role, since this receptor is also responsible for the retention of the hematopoietic stem cells in the bone marrow. Stem cells may leave the bone marrow as a result of systemic lesions due to the competition between MSCs and hematopoietic cells for the CXCR4 ligand – SDF-1 [21, 22]. For some time it was believed that MSC migration to the damaged tissue was indicative of active participation of these cells in tissue repair and regeneration. Additional studies of the behavior and migration of MSCs upon heterological transplantation clearly show that the proportion of MSCs that reach the lesion site post-transplant is very low. Moreover, the cells do not remain in the tissue and soon disappear. In this context, the initial assumption that the major role of MSCs was the direct replacement of the damaged-tissue cells through differentiation was dismissed[10]. Instead, the hypothesis that MSCs can facilitate the division and differentiation of stem and precursor cells, thus regulating their recruitment and survival upon stress conditions and injuries by secreting soluble factors, was proposed [23]. Therefore, it was suggested that MSCs serve as a mobile supplier of the factors necessary for tissue repair and regeneration.

SECRETORY POTENTIAL OF MSCS AND REGENERATIVE PROCESSES

MSCs possess a unique property, which is secretion of a wide range of biologically active molecules, such as growth factors, cytokines, hormones, and low molecular weight mediators, which regulate the key physiological processes [23]. Factor production and the ability to produce/destroy the cell matrix underlie the physiological effect that MSCs have on the damaged tissue [24–26]. It has been demonstrated that the production of soluble factors by MSCs can support tissue cells, in addition to resident stem and precursor cells under inflammatory conditions and hypoxia, which inevitably accompany wound and pathological lesions [27–29]. It has been proven that the secretion of proangiogenic factors, such as VEGF, IGF-1, etc., by MCSs accelerates vascular growth and maturation at the lesion site [30–32]; the secretion of neurotrophic factors (in particular BDNF) facilitates the recovery of damaged neurons [33–35]; and the secretion of morphogenic proteins of the TGF-β family facilitates bone and cartilage tissue repair after a fracture [36–38] (*Fig. 1*). It is very likely that immediate contact with the surrounding cells and structures (microenvironment) also plays a significant role in the regenerative function of MSCs; however, few experimental studies exist to support this idea.

IMMUNE CELLS IN TISSUE DAMAGE AND REGENERATION

When discussing the specific conditions accompanying tissue healing and repair processes, specific attention should be focused on the contribution of immune cells. It is a known fact that the immune system of mammals, including the human immune system, is a complex protective mechanism consisting of numerous types of cells that fight against infectious agents of different origin. The oldest immunity segment in terms of its evolution is represented by the cells responsible for recognizing foreign molecules and providing an immediate response to their presence [39]. These cells use molecular signalling to "pass the baton" to the adaptive immunity cells responsible for the development of the powerful immune response that is typically accompanied by the release of significant amounts of cytotoxic and proinflammatory

molecules [40, 41]. Unfortunately, it is not easy to control this powerful and complex mechanism and accurately measure the adequate strength and direction of the attacks. The immune response is accompanied by acute or chronic damage to tissues and organs [39].

There exists a quite definite order of immune system reactions that accompany any damage to internal organs, injury, or infection. Tissue-resident mast cells, dendritic cells (DC), and macrophages act as damage sensors [38]. They initiate the cascade of immune reactions via the release of proinflammatory cytokines, chemokines, and factors that facilitate the migration and stimulation of other cell types. Cytokines and adhesion molecules, which ensure rapid neutrophil accumulation at the lesion site, play the key role in this process [39]. In turn, cytokine and chemokine production by neutrophils causes macrophage migration and the release of additional proinflammatory cytokines, such as IFN-γ and TNF-α [40]. The secretion of larger amounts of inflammatory cytokines recruits T and B cells by accelerating their activation and maturation. These cells accumulate at the lesion site, thus enhancing inflammation due to the production of new doses of cytokines and proinflammatory factors, often resulting in an undesired lesion and subsequent cell death in the surrounding tissue [41–44]. In turn, the inflammatory response initiates the molecular mechanisms that suppress activation and division of immune system cells. These mechanisms include an increase in sensitivity of the activated cells to apoptosis, an upregulation of the expression of anti-inflammatory cytokine (IL-10 and TGF-β) receptors on the surface of immune cells, production of these cytokines by activated cells, the elevated production of negative coactivator molecules, the activation of regulatory cells and an increase in their number [45–47]. All these events result in the completion of the acute phase of the immune response, the death of injured and activated cells, and the phagocytosis of dead cells and their fragments by professional phagocytes [48]. Meanwhile, the production of such factors as TGF-β causes fibrotic changes in the tissue structure and facilitates the replacement of the original tissue with fibrin and connective tissue [49, 50]. A significant role belongs both to the cells of the surrounding tissue and to vascular endothelial cells, which by secretion and release of factors from the extracellular matrix direct the migration of particular effector cells into the lesion site [51]. To summarize, it should be emphasized that cells of the immune system are involved into all phases of the regenerative processes in tissues (*Fig. 1*). The participation of these cells actually defines the timeline and efficiency of the healing. Furthermore, the level of tissue inflammation and lesion considerably depends on the interaction between tissue cells and cells of the immune system.

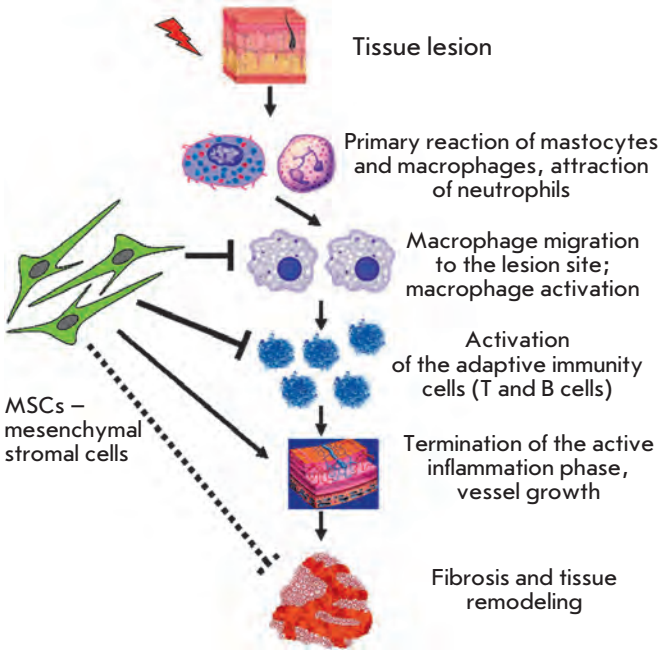

Fig. 1. Main events following damage to inflammatory / wound tissue and the involvement of immune cells. The effect of MSCs on particular steps is shown with arrows in the case of positive influence; and with blunt-end arrows, in the case of negative (inhibitory) influence.

MSC ANTIGEN PRESENTATION

Taking into account the secretory potential of MSCs and the effect on the microenvironment at the lesion site, the positive effect of MSCs in different models of tissue regeneration can (at least to some extent) be accounted for by their influence on cells of the immune system (*Fig. 1*). In this context, the immunological properties of MSCs have been studied rather thoroughly. Unfortunately, this does not apply to the molecular mechanisms being responsible for these properties. In immunological terms, MSCs strongly differ from body cells by their almost complete inability to be recognized by the immune system due to their phenotypic features [52, 53]. As a result of this property, MSCs are a promising object for application in transplantology, since it allows one to bypass the problem of immunological compatibility. In comparison with other cell lineages, MSCs express an extremely insignificant amount of MHC I and MHC II molecules and carry no costimulatory molecules CD40, CD80, or CD86, which are required for T cell activation [54]. Meanwhile, MHC expression recovers during the differentiation, resulting in the recognition and destruction of the MSC progeny by the recipient's immune system cells [55]. MSCs do not cause allogeneic mixed lymphocyte reaction in completely heterologous cultures [54]. MSC-mediated

expression of MHC may vary depending on culturing conditions. In particular, MSCs activate the expression of MHC genes in the presence of small IFN-γ concentrations, which results in their capability of antigen presentation (*in vitro*). High doses of IFN-γ do not have this effect [56].

It has recently been demonstrated that MSCs can suppress the immune response by inhibiting NK maturation, suppressing the functions of T and B lymphocytes and natural killer (NK) cells [57–60].

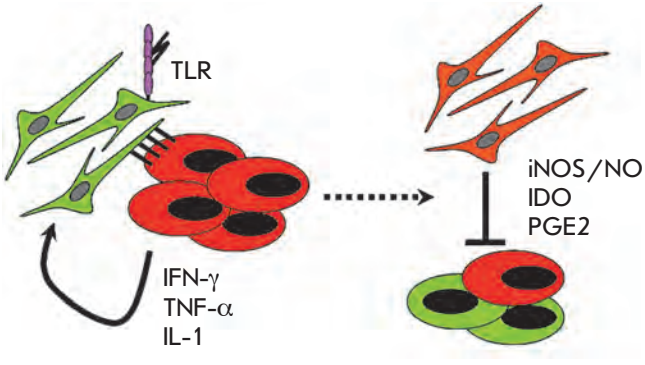

Fig. 2. Schematic representation of the key factors involved in immunosuppression stimulation by MSC (on the left) and soluble effector molecules mediating the inhibitory effect of MCS on T cell function (on the right).

MSC IMMUNOREGULATION *IN VITRO*

Most of the data on the immunological properties of MSCs has been obtained as a result of experiments on *in vitro* cocultivation or the joint incubation of MSCs and cells of the immune system. In these types of experiments, human blood leukocytes, or individual populations (e.g., T cells), were placed into the MSC-containing culture following activation. The effect of MSCs on the immune cells or, vice versa, the effect of immune cells on MSCs was then determined by measuring the cell division rate, the metabolic activity, the level of activation marker expression, the apoptosis level, and the secretion of cytokines and growth factors, etc. The following major regularities and mechanisms which have an impact on the results of the interaction between MSCs and cells of the immune system have been revealed [57–60] (*Fig. 2, 3*). It turns out that MSCs have different effects on different types of cells of the immune system. Naive (non-activated) T cells survive and divide in culture better in the presence of MSCs and MSC culture supernatants. Meanwhile, the activated T cells are susceptible to immunosuppression in the presence of MSCs. It has been ascertained that MSCs reduce the proliferative potential of T cells, the expression of activation markers and coactivatory molecules, and their ability to secrete proinflammatory cytokines, such as IFN-γ and TNF-α [58, 59, 61]. A similar effect was also observed for dendritic cells. After coculturing human or murine dendritic cells with MSCs, with DC maturation characterized by the expression of the molecules of the major histocompatibility complex on the cell surface, the capability of processing and representing protein antigen peptides to CD4 and CD8 T cells decreased in comparison to the control cocultures [60, 62, 63]. The effect also consisted in the reduction of the level of costimulatory molecules required for productive antigene presentation for T cells. Moreover, MSCs have a negative impact on the activation of immune cells of other types (in particular, NK [64, 65] and B cells [57, 66, 67]) in a culture. Inhibition of division and secretion of various immunoglobulins (IgA, IgM, IgG), as well as a decrease in chemokine receptor (CXCR4, CXCR5, CXCL12) expression manifesting itself in the suppression of cell chemotaxis, is observed for B cells [57, 64]. A set of factors secreted by MSCs have a negative impact on antigen production by plasma cells as a result of the activity of the CCL2 and CCL7 ligands that are formed as a result of the activity of matrix metalloproteinases being released from MSCs [65] (*Fig. 3*).

In early studies, the influence of MSCs on immune cells was determined in a blood mononuclear cell culture activated by preliminary incubation with antibodies against a T cell receptor or with nonspecific activators of the immune response (hemagglutinin, superantigens) [57–60]. For this assays T cells are the most convenient cell population, since it is the most abundant and the best characterized fraction of cells of the immune system. It is for this reason that the mechanism of the MSC effect on T cells has been studied appreciably well. It has been ascertained from the experiments on the MSC effect on activation and the effector function of T cells that only MSCs that were pre-incubated with activated T cells display immunosuppressive properties [68] (*Fig. 2*). Furthermore, incubation of MSCs with individual, purified proinflammatory cytokines (e.g., with IFN-γ) results in the emergence of these properties in MSCs (and MSC culture supernatants) [69–72]. This fact implies that cytokines stimulate MSCs, and this "activation" underlies the manifestation of immunosuppressive properties by MSCs (*Fig. 2*).

ACTIVATION OF IMMUNOSUPPRESSIVE PROPERTIES OF MSC REQUIRES PRELIMINARY STIMULATION OF MSC WITH PROINFLAMMATORY CYTOKINES

Which cytokines are critical for the manifestation of MSC's immunosuppressive properties? The answer to this question has been obtained using blocking antibod-

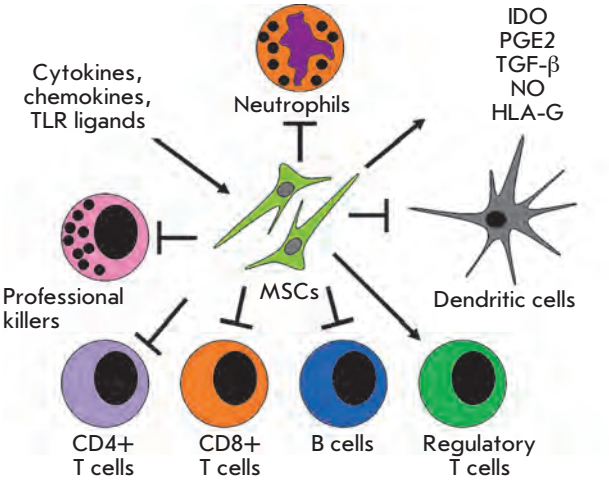

Fig. 3. Spectrum of MSC-mediated immunosuppression cellular targets. MSC immunosuppression inducers are presented in the frame on the left-hand side, the main molecules – mediators of suppression – on the right-hand side. MSCs induce neutrophil apoptosis, inhibit dendritic cell maturation and secretion of proinflammatory cytokines (IFN-γ, IL-12, TNF-α), slow down proliferation and B-cell differentiation towards plasma cells, decrease immunoglobulin secretion, limit division of NK, CD4 and CD8 T cells, and limit the secretion of proinflammatory cytokines and the maturation of cytotoxic T cells from CD8 T cells. At the same time, MSCs stimulate IL-10 production by dendritic and regulatory T cells and boost expansion of regulatory T cells. The arrows indicate the positive effect of MSCs on cell function, whereas the blunt-end arrows indicate the negative effect of MSCs.

ies against various proinflammatory cytokines in cocultures of MSCs and activated T cells [69–72]. The use of this approach has demonstrated that the neutralization of IFN-γ, and decrease in the level of the IFN-γ receptor by the over-expression of microRNAs in MSCs, which interfere with the mRNA of one of its subunits, and the use of MSCs from IFN-γ receptor knockout mice result in a considerable reduction in the ability of these modified MSCs to suppress T-cell activation in a culture [69]. An alternative pathway for MSC activation by proinflammatory cytokines requires simultaneous participation of several proteins, in particular IFN-γ, TNF-α, and IL-1β. The requirement in these cytokines has been confirmed in *in vitro* experiments with blocking antibodies to the corresponding cytokines. It is worth noting that the blockage of any one or two different cytokines (pairwise) allowed a negligible restriction of the immunosuppressive properties of MSCs in the culture [69]. Only the simultaneous blocking of all three factors resulted in a pronounced physiological effect.

MOLECULAR MECHANISMS OF MSC-MEDIATED IMMUNOREGULATION

It has been demonstrated, by a study of the molecular differences between "regular" and activated MSCs, that the expression of a number of genes, controlling suppression mechanisms, is triggered after treatment of MSCs by cytokines (*Fig. 2*). In particular, the level of indolamine-2,3-dioxygenase (IDO) in MSCs increases as a result of the action of proinflammatory cytokines [73]. It was revealed in earlier studies that IDO is a negative regulator of the T cell function. The secreted form of this enzyme is believed to diminish the level of free tryptophan (rapidly dividing activated T cells require large amounts of this amino acid) [74]. Moreover, tryptophan catabolite kynurenine, which is a product of IDO enzyme activity, also suppresses T-cell activation [74]. The experiments, in which a synthetic IDO inhibitor or MSCs from IDO-deficient mice was used, lend further credence to the significant role of this protein in MSC-mediated immunosuppression [69, 74, 75].

An alternative pathway of MSC activation based on simultaneous stimuli from IFN-γ, TNF-α, and IL-1β has also been ascertained at the molecular level and relies mainly on a considerable increase in the expression of the *iNOS* (inducible NO synthase) gene by MSCs. iNOS is an enzyme responsible for NO production by cells under stress conditions. The level of *iNOS* gene transcription under normal conditions is extremely low. The level of *iNOS* is known to significantly increase in many cells of the immune system under the action of cytokines and other stress factors [76]. An increase in the level of iNOS in MSC upon activation may attest to the fact that these cells enhance NO production. According to the existing data, the effect of NO on stimulated T cells consists in the suppression of cell division, cytokine secretion, and presumably, in an increase in the level of cell death. It has been shown by using inhibitors and iNOS-deficient MSCs that iNOS or NO activity is required for MSCs to be able to manifest their immunosuppressive properties [76].

It is interesting to note that recently obtained data appears to indicate that various immunosuppressive mechanisms may depend on the presence/absence of intercellular contacts. In the case of contact cocultivation of MSCs and activated T cells, a predominant increase in the level of TNF-α (but not IFN-γ) was observed in the system. Therefore, the immunosuppression was predominantly iNOS-dependent. On the other hand, the use of the contactless model resulted in the initiation of the alternative program that required IFN-γ production and, therefore, used NO production for immunosuppression [69].

ALTERNATIVE MECHANSIMS OF MSC-MEDIATED IMMUNOSUPPRESSION

The mechanisms responsible for the MSC-mediated neutralization of the activation of the cells of the immune system are not confined to only IDO and NO secretion. It has been shown that MSCs permanently express the inducible enzyme cyclooxygenase-2 (COX-2), which is responsible for the synthesis of prostaglandin E2 (PGE2) from arachidonic acid. PGE2 is a lipid that negatively affects T cell activation. Incubation of MSCs in the presence of blood lymphocytes results in a considerable increase in the PGE2 level in a culture [59, 75, 77, 78]. This may imply interaction between MSCs and T cells, leading to the enhanced synthesis of immunosuppressor molecules. Incubation of MSCs in the presence of IFN-γ and TNF-α causes a boost in the COX-2 expression level and PGE2 secretion, thus attesting to the fact that the production of this regulatory molecule can be controlled by the inflammation level [77]. The introduction of PGE2 inhibitors into a mixed culture consisting of T cells and MSCs resulted in a significant decrease in the immunosuppression level [77, 78].

It has been demonstrated that when incubated with lymphocytes or proinflammatory cytokines, MSCs secrete enhanced levels of IL-10 and TGF-β; anti-inflammatory cytokines that have a negative effect on the activation and division of T cells. The immunosuppressive effect that has been observed *in vitro* in the absence of antibodies can be partially eliminated by blocking antibodies against these cytokines [79]. It is believed that the secretion of IL-10 and TGF-β by activated MSCs accelerates the expansion of regulatory T cells, a minor population of CD4 lymphocytes, which are powerful negative immune response regulators, rather than just having a direct impact on T cells [80].

The nonclassical molecule of the histocompatibility complex class I antigen, G5 (HLA-G5), is another soluble factor that presumably participates in the MSC-mediated regulation of the immune response. Molecules of this type play a significant role in the establishment of immunological tolerance during pregnancy. The soluble HLA-G5 isoform is secreted by MSCs in the presence of contacts between MSCs and T cells in heterologous mixed cultures. HLA-G5 suppresses T cell proliferation and the cytotoxic properties of NK cells; simultaneously, it accelerates the division of regulatory T cells [65].

It has recently been established that MSCs express a set of Toll-like receptors (TLR), which are responsible for the recognition of the molecular patterns of various pathogens and innate immunity cell activation [81]. A MSC culture expresses a whole set of TLR (TLR1–TLR8) [82]. Stimulation of MSCs by incubating them with ligands of various TLR (such as LPS) results in the translocation of the NF-κB transcription factor to the nucleus and activation of the program, which simultaneously enhances the immunosuppressive properties of MSCs and increases IL-6 secretion in most cases [83, 84]. An increase in MSC activity upon TLR ligation can be easily accounted for by the fact that signal transduction pathways from the IFN-γ receptor and TLR intersect [83, 84]. Thus, the effect of TLR ligation may result (similarly to that for IFN-γ) in increased secretion of PGE2 and IDO [81].

The aforementioned mechanisms of MSC activation and immunosuppression are mediated by soluble factors. Meanwhile, mechanisms of MSC-mediated suppression of the immune response that depend on intercellular contacts have been described. One of the most well studied examples is the cell adhesion molecules ICAM-1 and VCAM-1 [85, 86], whose level on the surface of an MSC increases significantly in the presence of inflammation factors. These molecules are responsible for directed leukocyte migration and their penetration of the walls of blood vessels. It has been shown that an enhancement of the MSC-mediated expression of ICAM-1 and VCAM-1 is one of the possible immunosuppressive mechanisms, since the use of blocking antibodies against these molecules has reduced the level of MSC-mediated immunosuppression in a culture [85]. The results of experiments using cultures were supported by the data of *in vivo* experiments, in which MSCs with the ICAM-1 and VCAM-1 genes knocked out were used for immunosuppression [85]. Unfortunately, unambiguous interpretation cannot be made of the results of these experiments, since the nonspecific contribution of the genetic defect to cell mobility cannot be distinguished from the direct contribution of ICAM-1 and VCAM-1 to the suppression of the T cell function.

MSC-MEDIATED IMMUNOSUPPRESSION *IN VIVO*

The ability of MSC to suppress the immune response in the context of the entire organism *in vivo* was first detected during skin grafting experiments on monkeys. Transplanted MSCs decelerated the development of the immune response to the graft [68]. Moreover, it turned out that MSCs can be used in case of severe GVHD reaction (graft versus host disease). The transplantation of MSCs to mice, in which the lethal GVHD reaction after bone marrow transfer had been observed, enhanced their survival rate [87, 88]. At the time of writing, the mechanisms responsible for the improvement in clinical presentation have not been reliably determined; they have been only partially characterized in additional experiments using animals. Thus, it has been demonstrated that IFN-γ-deficient T cells are unsusceptible to MSC-mediated suppression in the GVHD model. In this

system, the pre-activation of MSC by IFN-γ resulted in a fivefold increase in the immunosuppressive properties of MSCs as compared with those of the control cells [87–90].

It is tempting to use the immunosuppressive effect of MSCs upon human autoimmune diseases, such as diabetes mellitus, arthritis, multiple sclerosis, and systemic lupus erythematosus. In the experimental autoimmune encephalitis (EAE) model, an analogue of multiple sclerosis in mice, systemic transplantation of MSCs to the affected mice prevented the development of inflammatory infiltrates (T and B cells, macrophages) and that of the demyelination process in the CNS; moreover, it reduced the response of T cells to MOG peptides which originate from myelin [91]. The medium in which MSCs had been cultured suppressed the activation of CD4+ T cells under EAE conditions by reducing STAT-3 protein phosphorylation [92]. Infiltration of CD4+ T cells into the spinal cord of MSC-transplanted mice and the level of proinflammatory TNF-α and IL-17 cytokines were reduced [91]. In another study, MSC transplantation from Balb/c mice to B57BL/6 recipients with pronounced EAE symptoms caused alleviation of symptoms, such as reduction in the infiltration of immune cells in the CNS and a decrease in the blood level of IFN-γ and IL-17 cytokines [93].

In the collagen-induced arthritis mouse model, systemic transplantation of MSCs from human adipose tissue considerably reduced the probability of disease progression and its severity. The levels of inflammation and Th1-type immune response significantly decreased. The injection of MSCs resulted in the suppression of the expansion of the antigen-specific cells synthesizing IFN-γ and IL-17 [94]. Moreover, the enhanced secretion of anti-inflammatory IL-10 cytokine in the draining lymph nodes adjacent to the inflamed joints, and an increased number of CD4+CD25+Foxp3+ regulatory T cells were observed [94]. MSCs responded to collagen by suppressing the *in vitro* activation and division of T cells obtained from patients with rheumatoid arthritis and enhancing IL-10 secretion by T cells [95]. Furthermore, MSCs stimulated the formation of regulatory T cells capable of suppressing the response of T cells to collagen and reducing the level of the enzymes that destroy the intercellular matrix in synovial cells [95]. However, the results of an independent study using an induced arthritis model demonstrated that transfer of an specific subpopulation of MSCs expressing the Flk-1 marker, on the contrary, results in enhanced arthritic manifestations due to increased IL-6 secretion and Th17-type differentiation [96].

In the case of acute renal failure, the introduction of MSCs led to a recovery of renal function through a reduction in the level of proinflammatory cytokines (IL-1β, TNF-α, IFN-γ) [97]. The participation of MSCs in the regulation of the progression of fibrosis has been studied in a case of acute renal failure in rats. Along with the decrease in the IL-6 and TNF-α levels, the introduction of MSCs resulted in a reduction in fibrotic changes and recovery of the renal function. Moreover, an enhancement of the level of anti-inflammatory cytokines was observed [98]. In an experimental model of pulmonary fibrosis, the level of lung inflammation was reduced by the introduction of MSCs, presumably due to the secretion of a IL-1 receptor antagonist [99]. Upon autoimmune diabetes mellitus type 1, disease progression in prediabetic NOD mice was checked through the allogenic transfer of MCSs, which enhanced the type II immune response [72, 99, 100]. The prevention of β-cell destruction, followed by the progression of diabetes, was achieved through a single intravenous injection of MSCs; this can be accounted for by the induction of regulatory T cells [99, 100]. When introduced to rats with streptozotocin-induced β-cell damage, culture-expanded bone marrow MSCs migrated to the pancreatic gland, increased the level of insulin secretion, and facilitated the normalization of the level of blood glucose [101]. Furthermore, an increase in the PDX-1 and insulin levels in the Langerhans islets was observed, which assumes β-cell activation in mice receiving MSCs [101].

CONCLUSIONS

In conclusion, it should be noted that reassuring data concerning the potential in using MSCs and drugs based on the factors secreted by them in the therapy of autoimmune diseases and regenerative medicine is already available. The data above provide convincing evidence that the immunosuppressive potential of MSCs can be enhanced by incubating the cells with inflammation factors and cytokines. Moreover, there is a possibility of obtaining genetically modified MSCs with improved immunosuppressive characteristics. However, it should be remembered that the infeasibility of strict control of the state of MSCs in a culture and the insufficiently proved genetic stability of these cells obstruct the implementation of MSC-based cell technologies. The accumulation of data on the ability of MSC to support and accelerate tumor growth by secreting factors that positively impact tissue regeneration is another major reason for concern [102]. ●

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Analogues of Artificial Human Box C/D Small Nucleolar RNA As Regulators of Alternative Splicing of a pre-mRNA Target

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ABSTRACT Small nucleolar RNAs (snoRNAs) play a key role in ribosomal RNA (rRNA) biogenesis. Box C/D snoRNAs guide the site-specific 2'-O-ribose methylation of nucleotides in rRNAs and small nuclear RNAs (snRNAs). A number of box C/D snoRNAs and their fragments have recently been reported to regulate post-transcriptional modifications and the alternative splicing of pre-mRNA. Artificial analogues of U24 snoRNAs directed to nucleotides in 28S and 18S rRNAs, as well as pre-mRNAs and mature mRNAs of human heat shock cognate protein (hsc70), were designed and synthesized in this study. It was found that after the transfection of MCF-7 human cells with artificial box C/D RNAs in complex with lipofectamine, snoRNA analogues penetrated into cells and accumulated in the cytoplasm and nucleus. It was demonstrated that the transfection of cultured human cells with artificial box C/D snoRNA targeted to pre-mRNAs induce partial splicing impairments. It was found that transfection with artificial snoRNAs directed to 18S and 28S rRNA nucleotides, significant for ribosome functioning, induce a decrease in MCF-7 cell viability.

KEYWORDS small nucleolar box C/D RNAs; post-transcriptional RNA modification; alternative splicing of pre-mRNA.

ABBREVIATIONS rRNAs – ribosomal RNAs; snoRNAs – small nucleolar RNAs; snRNAs – small nuclear RNAs; RT-PCR – reverse transcription polymerase chain reaction; RP-HPLC – reverse phase high-performance liquid chromatography; MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FAM – carboxyfluorescein.

INTRODUCTION

The class of small nucleolar RNAs (snoRNAs) consists of two major families: box C/D RNAs and box H/ACA RNAs. These RNAs act as a recognizing and targeting element in RNA–ribonucleoprotein complexes and participate in the modification of nucleotides in eukaryotic ribosomal RNAs. The RNAs belonging to the box C/D RNA family guide the 2'-O-methylation of rRNA nucleotides. Box C/D RNAs contain conserved structural elements, CUGA (box D) and RUGAUGA (box C), near the 5' and 3' termini, respectively. The box C/D RNAs possess a guide sequence region (a sequence complementary to the region of an RNA target). Certain RNAs contain two guide sequences and two box C/D pairs (C, D, C', and D') [1].

Cavaille J. et al. [2] demonstrated that if the RNA contains structural elements determining its membership in the family of box C/D RNAs, then it is sufficient to have the corresponding region of the box C/D snoRNA (complementary to the RNA target) in order to determine the methylation target. It was shown that 2'-O-methylation of RNA nucleotides without natural 2'-O-methyl groups can be guided by analogues of box C/D RNAs [2].

One of the key approaches to the study of the properties of box C/D RNAs is the design of DNA constructs that are expressed in a cell yielding either short non-natural snoRNAs or pre-mRNA fragments, the processing of which results in the formation of snoRNAs targeted to the pre-specified rRNA nucleotides [2]. This approach was used to design methods for the directed nucleotide modification in eukaryotic RNAs and for mapping functionally important rRNA sites, which are sensitive to

de novo 2'-O-methylation [3, 4]. The range of targets for artificial small nucleolar RNAs is not confined to rRNAs and snRNAs. The participation of snoRNAs in mRNA maturation is of increasing interest. It has already been revealed that box C/D RNAs can interact both with the products of transcription by RNA-polymerase I localized in the nucleolus and with RNA-polymerase II products [2]. Moreover, small nucleolar RNA HBII-52 (MBII-52) takes part in the processing of pre-mRNA of the serotonin receptor 5-HT_{2C}R [5, 6]. Thus, the structure of box C/D small nucleolar RNAs is a promising basis for the development of constructs for the directed regulation of gene expression in human cells.

The effect of synthetic analogues of natural box C/D RNAs on pre-mRNA-target splicing and processing of 18S and 28S rRNA in human cells was investigated in this study. The analogues of human U24 box C/D RNA directed to pre-mRNA of human heat shock cognate protein hsc70 and human rRNA were designed. The transfection of the MCF-7 cell line (human breast adenocarcinoma cells) with synthetic analogues was shown to result in a partial disruption of splicing (the elimination of an exon from the pre-mRNA-target). It was found that the transfection of MCF-7 cells with synthetic analogues of box C/D RNAs directed to rRNAs induces a decrease in cell viability.

EXPERIMENTAL

Artificial box C/D RNAs synthesis

Synthetic analogues of box C/D RNAs were obtained via the *in vitro* transcription of PCR-amplified DNA templates with T7 RNA polymerase (Fermentas, Lithuania).

Transfection of MCF-7 cells with synthetic RNAs. Isolation of total cellular RNA

MCF-7 cells (from the Russian cell culture collection of vertebrates, Institute of Cytology, Russian Academy of Sciences, St. Petersburg) were cultured in an IMDM medium with 10 mM L-glutamine and 40 μg/ml of gentamicin in the presence of 10% fetal bovine serum at 37°C. Synthetic analogues of box C/D RNAs were pre-incubated with the lipofectamine reagent (Invitrogen, United States) according to the manufacturer's protocol and were subsequently added to the culture medium. After the MCF-7 cells had been incubated for 18 h, total RNA was isolated using the Trizol reagent (Invitrogen, United States) according to the manufacturer's protocol.

Isolation of cytoplasmic and nuclear fractions of MCF-7 cell lysate

Upon completion of incubation, the MCF-7 cells were ice-cooled. The medium was collected. The cells were washed twice with phosphate buffered saline (PBS) and lysed on ice for 10 min (0.5% Triton X-100 in buffer A containing 150 mM NaCl, 50 mM Tris-HCl pH 7.5, 10 mM EDTA). The lysate was suspended and coated onto a 10% sucrose solution in buffer A, followed by centrifugation for 20 min at 600 *g*. The isolation of RNA from the supernatant (the cytoplasmic fraction of MCF-7) and nucleolar precipitate suspended in buffer A was carried out using the Trizol reagent. RNA concentrations in the samples were determined spectrophotometrically (λ = 260 nm), taking into account the extinction coefficient for RNA (ε_{260} = 25 l/mol cm).

Analysis of pre-mRNA *HSPA8* splicing variants by RT-PCR

The reverse transcription of pre-mRNA in the *HSPA8* and cDNA amplification was performed in the reaction mixture for RT-PCR "Real Best Master Mix RT" (Vektor-Best, Novosibirsk, Russia) using the primers hsp2.1 (5'-ACTGAACGGTTGATCGGTGA-3') and hsp8.2 (5'-AGATGAGCACGTTTCTTTCT-3'). The products were analyzed in a 4% polyacrylamide gel. The quantity of amplification products in the gel was ascertained using the Gel-Pro Analyzer 3.1 software. Sanger sequencing was performed using fluorescently labelled terminators of DNA-polymerase in the BigDye 3.1 mixture, followed by the separation of DNA on an ABI3100 analyzer (Applied Biosystems, Inter-Institute Sequencing Centre, Siberian Branch of the Russian Academy of Sciences).

Fluorescently labelled box C/D RNA synthesis and analysis of the accumulation of fluorescently labelled RNA in human cells

Fluorescently labelled RNA was obtained via *in vitro* transcription by T7 RNA polymerase (Fermentas, Lithuania) using Flu-12-UTP (Biosan, Novosibirsk, Russia). The RNA transcript was isolated via ion-pair RP-HPLC on a Milichrome A-02 liquid chromatograph using the ProntoSIL-120-5-C18 sorbent and a 2.0 × 7.5 mm column. The accumulation of fluorescently labelled RNA in MCF-7 cells was analyzed via fluorescence microscopy (The Centre for Collective Use of Microscopic Analysis of Biological Objects, Siberian Branch of the Russian Academy of Science). For this purpose, 3×10^4 MCF-7 cells were seeded onto a slide plate of the Culture Slide chamber (BD Falcon, United States), then they were transfected with fluorescently labelled RNA after 24 h, followed by incubation for 18 h. The medium was removed post-incubation. The cells were washed twice with PBS; the specimens were embedded into a drop of the DAPI/Antifade dye (Millipore, United States) and covered with a cover slip. The

specimens were analyzed on an Axioskop 2 Plus microscope (Carl Zeiss, Germany).

Analysis of 2'-O-methylation of G1702 of the 18S rRNA

2'-O-methyl groups of rRNA were detected via partial alkaline hydrolysis as previously described in [7]. The total RNA of the MCF-7 cells (2.5–5.0 μg) was incubated in a 50 mM Na_2CO_3 solution (pH 9.0) for 18 min at 90°C. The hydrolysis products were recovered by ethanol precipitation. Reverse transcription was carried out using primer 18.1702 (5'-GCCGATCCGAGGGCCTCACT-3'), complementary to the region 1731–1750 of the 18S rRNA, using M-MLV reverse transcriptase (Biosan, Novosibirsk, Russia). The rRNA region was sequenced via reverse transcription in the presence of ddNTP, according to [8].

Analysis of MCF-7 cell viability via the MTT assay

In order to analyze the effect of the analogues of box C/D RNAs on the viability of MCF-7 cells, the cells were cultured in a 96-well plate (3×10^4 cells per well). After 24 h, the RNA–lipofectamine complex was added to the culture mixture until concentrations of 3.0, 10.0, and 70.0 nM were obtained. MCF-7 cells were incubated with RNA for 3 days; the MTT solution in PBS was subsequently added to the medium until a final concentration of 0.5 mg/ml. The mixture was incubated at 37°C for 90 min. After the medium was removed, MTT-formazan crystals were dissolved in 100 μl of isopropanol. The absorbance of the solution was determined (λ = 570 nm, control at λ = 620 nm) on an Apollo 8 LB 912 multichannel spectrophotometer (Berthold Technologies, Germany). The data were presented as a decrease in viability (100% – MTT index) against the control (cells incubated under identical conditions with lipofectamine alone).

RESULTS

The effect of the synthetic analogues of box C/D RNAs on RNA processing in human cells was studied using the designed analogues of human natural U24 box C/D RNA. Human U24 RNA contains CUGA and AUGAUGU (GUGAUGA) sequences (for D and C (C') boxes, respectively) and two guide sequences directing the 2'-O-methylation of C2338 and C2352 in 28S rRNA (*Fig. 1A*) [9]. The resulting analogues contain conserved regions that are identical to U24 box C/D RNA and the regions complementary to those of the RNA-targets designed so that the target nucleotide in the RNA target was complementary to the fifth nucleotide upstream from box D (D') (CUGA) of the analogue of box C/D RNA [2]. All the resulting RNAs contained two sets of box C/D

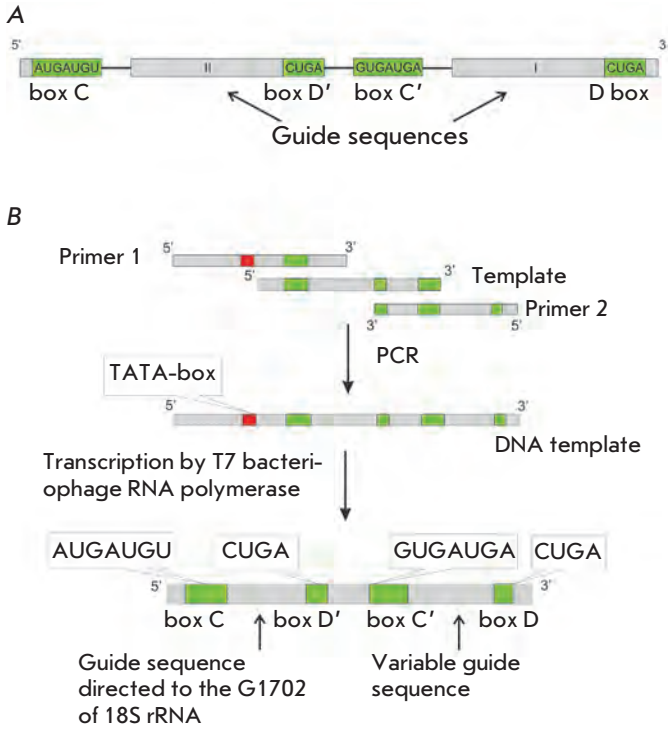

Fig. 1. (A) – Structure of U24 box C/D RNA; (B) – Scheme of synthesis of the artificial box C/D RNA.

RNA (C/D and C'/D', respectively) and thus two guide sequences (*Fig. 1B*).

One of the guide sequences (the D-box-dependent one) was directed to pre-mRNA nucleotides of the *HSPA8* gene encoding the human heat shock cognate protein (hsc70). Suppressing the expression of hsp-70-related proteins, including the hsc70 protein, results in the death of cultured cancer cells. The *HSPA8* gene is considered to be a promising target for gene-targeted cancer therapy [10]. Nucleotides, the modification of which may have a negative effect on the excision of the second intron upon pre-mRNA splicing, were selected for application as targets of the synthetic analogues: adenosine – the splicing branch point, splice donor and splice acceptor sites, the first and the last intron nucleotides. The second guide sequence (the D'-dependent sequence) was directed to G1702 in human 18S rRNA. The sequences of the analogues of box C/D RNAs are specified in *Table 1*.

It was ascertained via the transfection of MCF-7 cells with synthetic analogues of box C/D RNA and analysis of the variants of the alternative splicing of pre-mRNA of the *HSPA8* gene that both variants of alternative splicing of this pre-mRNA (the major and minor ones) can be identified in the control MCF-7 cells

Table 1. Synthetic analogues of U24 box C/D RNA directed to the key nucleotides involved in the splicing of pre-mRNA of the gene HSPA8

Notation	Nucleotide sequence*	Target nucleotide in the hsc70 pre-mRNA
PM.7	5′-UGCAG***AUGAUGU***AAAAUAGCGACGGG**C**GGUG***CUGA***GAG AUG***GUGAUGA***CAAAUGAAAACACUU**U**CAAU***CUGA***UGCA-3′	Adenosine – splicing branch point
PM.8	5′-UGCAG***AUGAUGU***AAAAUAGCGACGGG**C**GGUG***CUGA***GAG AUG***GUGAUGA***AAAUUAGGAACUCAC**C**AAAA***CUGA***UGCA-3′	Splice donor site
PM.9	5′-UGCAG***AUGAUGU***AAAAUAGCGACGGG**C**GGUG***CUGA***GAG AUG***GUGAUGA***AAAUUAGGAACUCA**C**CAAA***CUGA***UGCA-3′	First intron nucleotide
PM.10	5′-UGCAG***AUGAUGU***AAAAUAGCGACGGG**C**GGUG***CUGA***GAG AUG***GUGAUGA***ACAGAUGCCAAACGU**C**UGAU***CUGA***UGCA-3′	Splice acceptor site
PM.11	5′-UGCAG***AUGAUGU***AAAAUAGCGACGGG**C**GGUG***CUGA***GAG AUG***GUGAUGA***UACAGAUGCCAAACG**U**CUGA***CUGA***UGCA-3′	Last intron nucleotide

*__AUGAUGU__ – conserved elements of box C/D RNA; **A** – nucleotides that are complementary to the target nucleotide.

(*Fig. 2, lane K*). It was demonstrated via the sequencing of these forms that they differ by either the presence or absence of the second exon. The content of the minor form, i.e., the form without the second exon, increases in the cells transfected with synthetic box C/D RNAs directed to the nucleotides that are key ones for pre-mRNA splicing *(Fig. 2A, lanes 1–5)*. Thus, it was found that the analogues of box C/D RNAs directed to the selected pre-mRNA nucleotides of the *HSPA8* gene have an impact on the pre-mRNA target splicing and result in the excision of the second exon.

The transfection efficiency, distribution, and stability of the synthetic analogues of box C/D RNAs were assessed via RT-PCR of nuclear and cytoplasmic RNA of the MCF-7 cells transfected with PM.8 RNA. It is clear from the data shown in *Fig. 3* that, 3 h after the passive transfection of the cells with PM.8 RNA in the absence of lipofectamine, RNA could be detected neither in the nuclear nor in the cytoplasmic fraction of MCF-7 cells (*Fig. 3, lanes 1, 7*). After the cells were transfected with the analogue of box C/D RNA in the presence of lipofectamine, PM.8 RNA was found both in the cytoplasmic and nuclear fractions even 26 h post-transfection (*Fig. 3, lanes 5, 11*). All these facts lead one to the conclusion that synthetic box C/D RNAs in the presence of lipofectamine can efficiently penetrate into the cell cytoplasm and nucleus, where they can participate in the processing of the pre-mRNA-target.

The RT-PCR data are in close correlation with the results of the analysis of the distribution of FAM-labelled PM.8 RNA in MCF-7 cells via fluorescent micro-

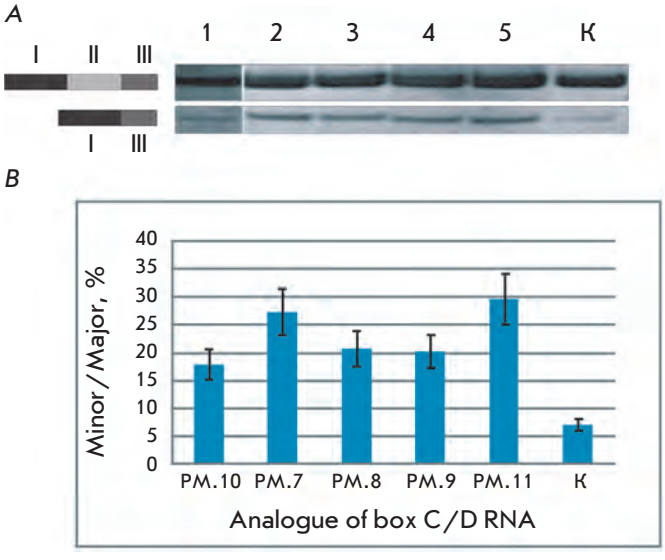

Fig. 2. Influence of U24 RNA analogues on pre-mRNA *HSPA8* splicing. (*A*) – RT-PCR products of pre-mRNA *HSPA8* splicing variants. The lanes correspond to the cDNA amplification products of 1 – 5 cells transfected with analogues PM.10, PM.7, PM.8, PM.9, PM.11, respectively; K – control MCF-7 cells incubated with lipofectamine. PCR products were analyzed on a 4% native polyacrylamide gel. Pre-mRNA *HSPA8* splicing variants are schematically represented. (*B*) – the ratio between the yields of the PCR products of the minor and major pre-mRNA hsc70 splicing variants. PM.7 – PM.11 – the cells were transfected with box C/D RNA analogues PM.7 – PM.11, respectively. K – control MCF-7 cells were incubated with lipofectamine alone.

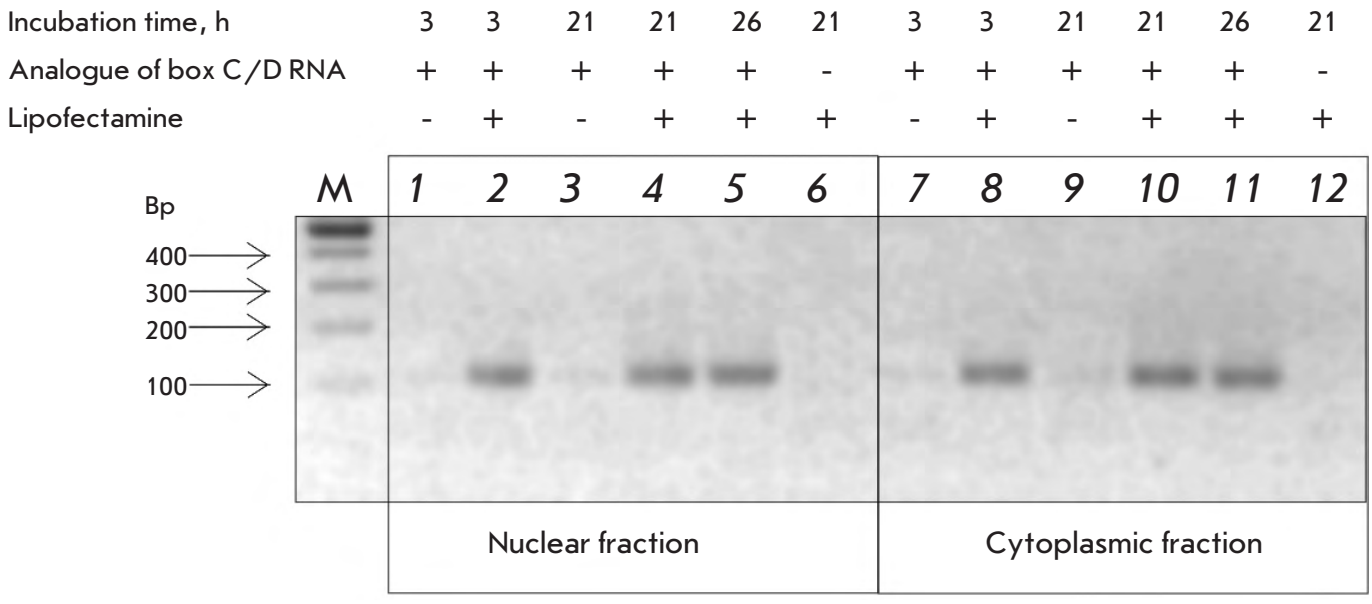

Fig. 3. Integrity of artificial box C/D RNA within MCF-7 cells. Human cells were transfected with the PM.8./lipofectamine complex (*2*, *4*, *5*, *8*, *10*, *11*), or PM.8 without lipofectamine (*1*, *3*, *7*, *9*) for the time indicated. Control cells were incubated with lipofectamine alone (*6*, *12*). RT-PCR products of RNA isolated from the nuclear (*1—6*) or cytoplasmic fraction (*7—12*). DNAs were analyzed on a 2% agarose gel. M – DNA molecular weight marker.

scopy. Thus, after the cells are incubated in a medium with FAM-labelled PM.8 box C/D RNA in complex with lipofectamine, RNA is captured, internalized, and then distributed both over the cytoplasm and the cell nucleus (*Fig. 4*).

It is known that rRNA nucleotides, which directly participate in the formation of ribosomal active sites or are located in close proximity to them, are more likely to undergo post-transcriptional modifications (pseudouridylation and 2'-O-methylation) [11]. These data permit the assumption that the post-transcriptional modifications have a substantial effect on the rRNA structure during the assembly process and eventually determine the ribosomal functionality [11–13].

The induction of 2'-O-methylation of the target of the second guide sequence (G1702 of the 18S rRNA) was analyzed in order to assess the ability of an analogue of box C/D RNA to direct rRNA modification. Since G1702 is one of the key nucleotides of the decoding site of human ribosomes, it was selected to be the target [14]. No additional 2'-O-methylated nucleotide at position G17012 of 18S rRNA was detected in the structure of rRNAs from the MCF-7 cells transfected with synthetic analogues via partial alkaline hydrolysis (*Fig. 5*). The presence of the 2'-O-methylated nucleotide within rRNA results in a decrease in the yield of the cDNA product (among the products

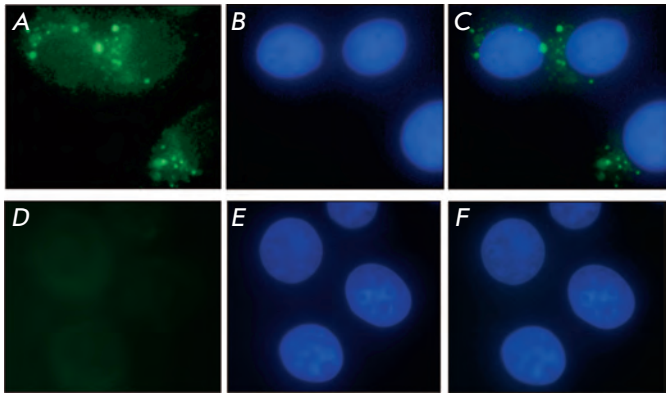

Fig. 4. Accumulation of box C/D RNA analogue PM.8 within MCF-7 cells. Fluorescence microscopy images of the transfected (*A—C*) and control cells (*D—F*): (*A*, *D*) – green filter (FAM-labelled box C/D RNA analogue); (*B*, *E*) – blue filter (staining with DAPI); (*C*, *F*) – merged images.

of reverse transcription of statistically hydrolyzed rRNA), the length of which is determined by the position occupied by a nucleotide that is downstream adjacent to the 2'-O-methylated nucleotide [8]. However, it is clear from *Fig. 5* that the yield of the cDNA transcript corresponding to the 2'-O-methylated G1702 of 18S rRNA does not decrease as a result of

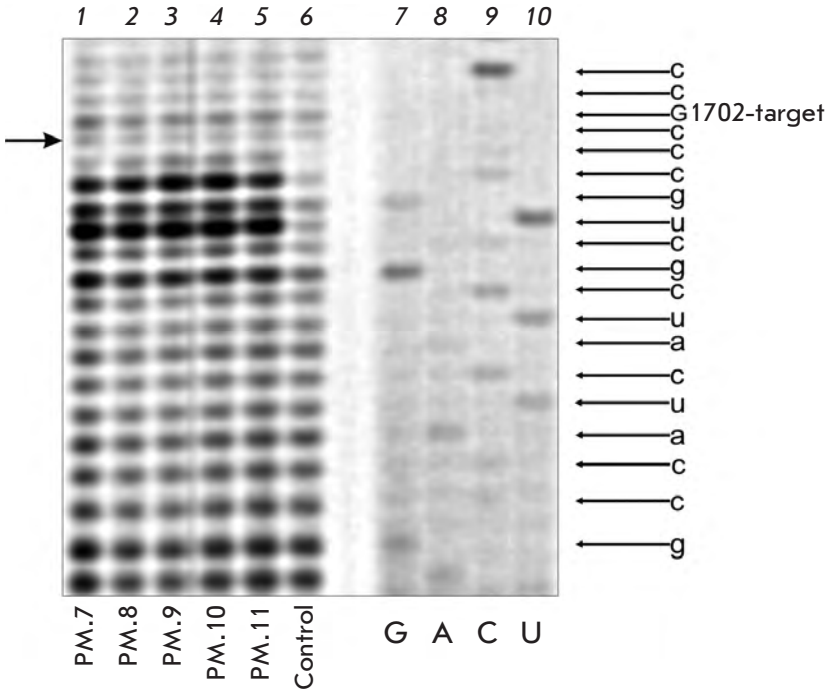

Fig. 5. Analysis of 2′-O-methylation of G1702 in 18S rRNA. RT products: (*1–5*) – rRNA isolated from MCF-7 cells transfected with box C/D RNA analogues PM.7–PM.11, respectively; (*6*) – rRNA isolated from control MCF-7 cells; (*7–10*) – sequencing of the corresponding region of human 18S rRNA. RT products were separated on a 12% denaturing polyacrylamide gel. The left arrow points to the position of the cDNA product, whose diminishing intensity could be an indicator of the 2′-O-methylation of G1702 in 18S rRNA.

Table 2. Synthetic analogues of U24 box C/D RNA directed to the nucleotides in human 18S and 28S rRNA

Notation	Nucleotide sequence*	Target nucleotide in human rRNA
PP.1827	5′-GGGUGCAG***AUGAUGU***AAAAUAGCGACGGGCGGUG***CUGA*** GAGAUG***GUGAUGA***CCUUGUUACG**A**CUUU***CUGA***UGCACCC-3′	U1827 18S
PP.4499	5′-GGGUGCAG***AUGAUGU***AAAAUAGCGACGGGCGGUG***CUGA*** GAGAUG***GUGAUGA***ACGGUCUAAA**C**CCAG***CUGA***UGCACCC-3′	G4499 28S
PP.4500	5′-GGGUGCAG***AUGAUGU***AAAAUAGCGACGGGCGGUG***CUGA*** GAGAUG***GUGAUGA***GACGGUCUAA**A**CCCA***CUGA***UGCACCC-3′	U4500 28S
PP.4502	5′-GGGUGCAG***AUGAUGU***AAAAUAGCGACGGGCGGUG***CUGA*** GAGAUG***GUGAUGA***ACGACGGUCU**A**AACC***CUGA***UGCACCC-3′	U4502 28S

* See note in *Table 1*.

the transfection of human cells with any analogue of box C/D RNAs.

The effect of synthetic analogues of box C/D RNAs on the post-transcriptional modification of rRNAs in human cells was analyzed using the designed and obtained analogues of U24 box C/D RNA, whose first guide sequence was directed to the nucleotides of human 18S and 28S rRNA. The nucleotide sequences of the analogues of box C/D RNAs are listed in *Table 2*. The targets selected are the key nucleotides of the ribosomal functional sites: U1827 of the 18S rRNA can be found in the ribosomal decoding centre; G4499, U4500, and U4502 of the 28S rRNA, in the peptidyl transferase centre [3, 4]. The second guide sequence of the analogues (the D'-box-dependent one) is directed to G1702 of the human 18S rRNA (*Table 2*).

A comparative analysis of the effect of the analogues of box C/D RNAs on MCF-7 cell viability was performed. The effect of the analogues of box C/D RNA was assessed after the cells were incubated in a medium with initial RNA concentrations of 3.0, 10.0, and 70.0 nM. It is clear from *Fig. 6* that the greatest reduc-

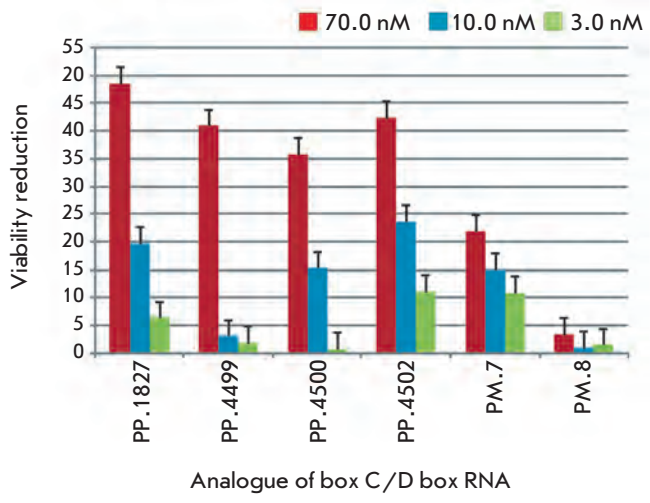

Fig. 6. Influence of artificial box C/D RNAs on the viability of MCF-7 cells. The MCF-7 cells were transfected with the RNA/lipofectamine complex and incubated for three days. The data represent the viability reduction (the average decrease in the MTT index and SD in 3 independent experiments) of cells treated with 3.0 nM (green); 10.0 nM (blue); and 70.0 nM (red) of RNA. The relative reduction in viability by 0% corresponds to the MTT index of the cells treated with lipofectamine alone.

tion in viability (> 35%) was induced by box C/D RNAs directed to U1827 of the 18S rRNA, G4499, U4500, and U4502 of the 28S rRNA. Meanwhile, the decrease in cell viability and proliferation caused by the analogues of U24 RNA, whose first guide sequence was directed to pre-mRNA of the hsc70 protein, was less than 22% (*Fig. 6, PM.7 and PM.8*).

MCF-7 cells were transfected with the analogues of box C/D RNA (*Table 2*) in order to analyze the induction of *de novo* 2'-O-methylation of target nucleotides; 2'-O-methylated rRNA nucleotides were detected by the partial alkaline hydrolysis method. It was ascertained that the contribution of rRNA forms containing the 2'-O-methylated target nucleotide in the total RNA pool of the transfected MCF-7 cells was lower than the sensitivity threshold of the detection technique.

The data relating to the changes in cell viability under the action of box C/D RNA, combined with the observed absence of the modification of the target nucleotides, allows one to assume that the effect of the analogues on human cells can be stipulated by the participation of box C/D RNAs not only in 2'-O methylation of rRNA nucleotides, but in other stages of post-transcriptional rRNA processing and ribosomal assembly, as well.

DISCUSSION

The conserved structural elements of box C/D RNA, namely, C/C' (RUGAUGA) and D/D' (CUGA) boxes, and the guide sequence ensure the ability of these RNAs to participate in the formation of a catalytic complex with proteins of the methyltransferase complex and direct 2'-O-methylation of a pre-specified nucleotide [1, 2, 15]. The guide sequence region of box C/D RNAs is a sequence consisting of 10–21 nucleotides, which is complementary to the RNA target; the nucleotide to be methylated is complementary to the fifth nucleotide of snoRNA upstream from the D box [7].

rRNAs and snRNAs are the major targets of box C/D RNAs in eukaryotic cells. Meanwhile, snoRNAs participating in pre-mRNA processing have been detected [6]. It was ascertained earlier that box C/D RNAs can interact with the transcripts synthesized by RNA polymerase II and guide 2'-O-methylation of a pre-specified nucleotide of the RNA target. The efficiency of the target nucleotide modification is substantially lower than that of the RNA targets synthesized in the nucleus by RNA polymerase I [2]. Moreover, it is a known fact that the chemical modification (in particular, methylation) of the 2'-OH groups of the oligonucleotides participating in pre-mRNA splicing significantly affects the efficiency of pre-mRNA maturation stages [16]. Therefore, the analogues of box C/D RNAs directing the 2'-O-methylation of pre-mRNA nucleotides are a promising model for the design of agents for splicing regulation.

The analogues of U24 small nucleolar box C/D RNA directed to pre-mRNA nucleotides of the *HSPA8* gene encoding heat shock cognate protein (hsc70) were designed and obtained in the present study (*Fig. 1*). It was ascertained that the transfection of human cells with synthetic analogues of box C/D RNA directed to the splice donor and splice acceptor sites of the second intron, adenosine the splicing branch point, the first and the second nucleotides of the first intron of pre-mRNA of the *HSPA8* (*Table 1*) results in an increase in the amount of the splicing product of pre-mRNA without the second exon (*Fig. 2*). Two key mechanisms can be proposed for the influence of the analogues of box C/D RNA on pre-mRNA target processing: 2'-O-methylation of the target nucleotide and complementary interaction between the antisense region of the box C/D RNA with the target RNA. Both pathways may theoretically result in the inhibition of certain splicing steps and, consequently, in a change in variants of alternative splicing of the pre-mRNA target.

The methods for detection of 2'-O-methylated nucleotides, which are currently in widespread application, do not allow to reveal these modifications in

mRNA and pre-mRNA with an adequate level of efficiency. Therefore, it cannot be unambiguously stated whether the methylation of the nucleotide target actually occurs, and whether or not the effect on splicing is caused by 2'-O-methylation of the pre-mRNA target. The key points of pre-mRNA splicing are sensitive to the modifications of the 2'-OH groups of ribose residues to different extents [16]. The resulting data do not permit one to claim considerable differences in the efficiency of splicing suppression by the analogues of box C/D RNA directed to various pre-mRNA nucleotides; therefore, the possibility cannot be excluded that the changes in the ratio between the forms of the alternative splicing of the pre-mRNA target being observed are induced by splicing inhibition by antisense RNA via the mechanism that was described for various oligonucleotide derivatives [17–19].

In order to participate in splicing, artificial RNA has to interact with the pre-mRNA target inside the nucleus. It was demonstrated that the analogues of box C/D RNA in the presence of lipofectamine are capable of efficient penetration into human cells (*Figs. 3, 4*). It is clear from *Fig. 3* that the analogue of box C/D RNA was reliably detected by RT-PCR 26 h following the single transfection in the presence of lipofectamine in the nuclear and cytoplasmic RNA fractions of MCF-7 cells. The resulting data attest to the fact that the artificial box C/D RNAs are potentially available for interaction with RNA targets located inside the nucleus. The accumulation of synthetic RNA in cells was confirmed via fluorescence microscopy (*Fig. 4*). Furthermore, it was ascertained that the synthetic analogues of box C/D RNA in the presence of lipofectamine can be efficiently conserved and detected via RT-PCR in human cells 72 h after the single transfection (the data are not illustrated). Meanwhile, in the case of transfection in the absence of lipofectamine, synthetic RNAs cannot be detected in human cells via RT-PCR as early as 3 h after having been introduced into the culture medium (*Fig. 3*).

For all the analogues of U24 snoRNAs obtained, one of the two guide sequences was designed so as to direct the 2'-O-methyaltion of G1702 in human 18S rRNA (*Table 1*). It was shown earlier that the transfection of human cells with synthetic analogues of box C/D RNAs directed to rRNA nucleotides (*Table 2*) induces the termination of reverse transcription on the target nucleotides [20, 21]. However, no additional 2'-O-methyl group at the specified position was detected from the analysis of 2'-O-methylation of G1702 in 18S rRNA of the cells transfected with the analogues of box C/D RNA via partial alkaline hydrolysis (*Fig. 5*). Neither of the *de novo* 2'-O-methylation of rRNA nucleotides – the targets of the analogues specified in *Table 2* (after cell transfection with the corresponding RNAs) – was revealed. Meanwhile, the positions of a number of known 2'-O-methylated nucleotides in human rRNA was successfully determined using this method.

The following data are to be given additional consideration in order to interpret the absence of a modification of the target nucleotides (with an exception for the known limitations of the analysis methods [8]). It is a known fact that the participation of box C/D RNA in the 2'-O-methylation of human cell rRNA is possible only provided that this RNA can be recognized by the proteins that are the subunits of the methyltransferase complex (namely, fibrillarin, NOP56p, NOP58p, and 15.5 kDa) and that the complex is formed with the participation of box C/D RNAs [22–24]. Therefore, the low yield of the targeted 2'-O-methylation observed can be explained by the low efficiency of the assembly of catalytically competent methyltransferase complexes with the analogues of box C/D RNA. Moreover, it was previously shown by *Liu B. et al.* [3, 4, 25] (who studied the transfection of yeast cells with DNA constructs encoding the analogues of box C/D RNA) that expression and maturation of artificial snoRNAs take place in the transfected cells. It also turned out that the expression of box C/D RNAs directed to a number of rRNA nucleotides resulted in a significant decrease in the proliferation rate and viability of the cells. The combination of the resulting data made it possible to arrive at a conclusion that it is 2'-O-methylation of rRNA nucleotides, guided by the analogues of box C/D RNA, that is the major reason for the influence of DNA constructs on the proliferation rate of the cells. However, for a number of box C/D RNAs [3, 4, 25], only a low level of 2'-O-methylation (or no modification at all) in the target nucleotides was revealed. On the other hand, the post-translational modifications (2'-O-methylation and pseudouridilation) are known to occur at the stage of maturation of the 47S rRNA precursor [7, 26–28]. The quality of the rRNA transcript is verified at the stage of the assembly of functional ribosomes; the incorrect nonfunctional RNA transcripts undergo degradation in exosomes [29]. The synthetic analogues of box C/D RNA are directed to the rRNA nucleotides immediately participating in the formation and functioning of the ribosomal active sites. Presumably, 2'-O-methylation of rRNA, guided by synthetic analogues of box C/D RNAs, has a significant effect on the rRNA structure and ribosomal functionality; this results in a rapid degradation of modified RNA and its low content in the transfected cells [25, 29].

Despite the fact that no modification of the target nucleotide was observed, artificial box C/D RNAs may

complementarily interact with the rRNA target, thus participating in the regulation of the maturation of rRNA transcripts and in ribosomal assembly and functioning. Hence, synthetic analogues of box C/D RNA can participate in the vital activity of the transfected cells and, therefore, affect the viability and proliferation of human cells.

The effect of the analogues of box C/D RNA on the viability of human MCF-7 cells was assessed via the MTT assay. The data obtained made it possible to arrive at the conclusion that cell transfection with synthetic analogues, whose first guide sequence is directed to the nucleotides of 18S and 28S rRNA (*Table 2*), results in a decrease in their viability by 36–48%; the initial concentration of synthetic RNA in the medium being 70.0 nM (*Fig. 6, PP.1827, PP.4499, PP.4500, PP.4502*). Meanwhile, synthetic analogues simultaneously directed to the pre-mRNA of heat shock cognate protein hsc70 and on G1702 in 18S rRNA (*Table 1*) reduced the cell's viability by only 20–25% within an initial concentration range of 3.0–70.0 nM (*Fig. 6, PM.7, PM.8*).

The rates of proliferation and monolayer formation decreased considerably, and cell morphology changed after the MCF-7 cells were transfected with synthetic analogues of box C/D RNA directed to U1827 in 18S rRNA, G4499, U4500, and U4502 in 28S rRNA. The rate of the decrease in viability varied for different analogues and depended on the target nucleotide and the initial RNA concentration in the culture medium (*Fig. 6*).

The difference in the influence of synthetic analogues of box C/D RNA on MCF-7 cell viability attests to the fact that these RNAs are involved in the regulation of vital processes in human cells during the transfection. The fact that this difference is a result of changes in the structure of the guide sequence allows one to assume that post-transcriptional processing of pre-rRNA is the major process modulated by the analogues of box C/D RNA. It should be noted that rRNA nucleotides comprising the ribosomal active sites (the decoding and peptidyl transferase ones) were selected as targets (*Table 2*). It was shown earlier by *Liu B. et al.* [3, 4, 25] that the expression of box C/D RNAs directed to these nucleotides in yeast cells induces the suppression of their growth and results in the partial degradation of rRNA.

The suppression of human cell viability induced by the analogues of box C/D RNA attests to the fact that they participate in the regulation of the viability process of the transfected cells. The fact that no target nucleotide modification occurred allows one to put forward a hypothesis that the analogues of box C/D RNA directed to rRNA are weakly involved into the 2'-O-methylation of rRNA nucleotides; however, there is a presumption that they participate in the other stages of post-transcriptional rRNA processing and in ribosome maturation.

CONCLUSIONS

It has been shown in this study that the transfection of MCF-7 cells (human breast adenocarcinoma cells) with synthetic analogues of box C/D RNA directed to pre-mRNA nucleotides of heat shock cognate protein hsc70 results in the disruption of splicing of the pre-mRNA target. The transfection of MCF-7 cells with analogues of box C/D RNA directed to the nucleotides in 18S and 28S rRNA that play the key role in ribosome functioning induces a decrease in cell viability. The data obtained attest to the high potential of designing constructs for the regulation of human gene expression and translation based on the snoRNA structure. ●

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Risk of HIV Infection and Lethality Are Decreased in *CCR5del32* Heterozygotes: Focus Nosocomial Infection Study and Meta-analysis

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ABSTRACT **_CCR5del32_ Homozygous deletion in the chemokine receptor R5 gene provides almost complete protection to individuals against HIV infection. However, data relating to the protective effect for _CCR5del32_ heterozygous individuals have been contradictory. The frequency of the _CCR5del32_ allele in population control cohorts was compared with that of a group of children (27 Kalmyks and 50 Russians) infected by G-subtype HIV-1 in a nosocomial outbreak. The frequency of the _CCR5del32_ allele was shown to be lower among the infected children in comparison with that of the control group; however, the difference was small and statistically insignificant. Similar results were obtained in a number of earlier studies. The insignificance of the small differences could be a result of one of two reasons. (i) The fact that there is no protective effect of the heterozygous state, and that the phenomenon depends only on the fluctuation of allele frequencies. In this case, there would be no differences even if the infected cohort is enlarged. (ii)The protective effect of the heterozygous state is real; however, the size of the studied cohort is insufficient to demonstrate it. In order to discern between these two reasons, a meta-analysis of data from 25 published articles (a total of 5,963 HIV-infected individuals and 5,048 individuals in the control group, including the authors' own data) was undertaken. A conclusion was drawn from the meta-analysis that the _CCR5del32_ allele protects individuals against the HIV infection even in a heterozygous state (OR=1.22, 95%CI=1.10–1.36). The risk of HIV infection for _CCR5 wt/del32_ heterozygotes was lower by at least 13% as compared to that for wild type _CCR5 wt/wt_ homozygotes. Prior to this study, no data of the type or any conclusions had been published for Caucasians. The mortality rate in the 15 years following the infection was found to be approximately 40% lower for _CCR5del32_ heterozygotes in comparison with that for the wild type homozygotes in the studied group. The size of the studied group was insufficient to claim difference validity (OR=2.0; p=0.705), even though the effect quantitatively matched the published data. The features of the meta-analysis influencing the threshold level and the statistical validity of the effects are being discussed. The level of the _CCR5del32_ protective effect on the chances to be infected with HIV and on the outcome of the HIV infection was assessed for various ethnic groups.**

KEYWORDS **HIV; nosocomial infection; lethality risk; infection risk; chemokine receptor gene; allele _CCR5del32_; meta-analysis.**

ABBREVIATIONS **HIV – human immunodeficiency virus; AIDS – acquired immune deficiency syndrome; PCR – polymerase chain reaction.**

INTRODUCTION

Having started with a single case detected in 1981, the AIDS epidemic is now one of the most important health care issues both in Russia and the rest of the world [1]. The evolution of the epidemic in Russia was characterized by the formation of clusters of nosocomial infection that took place in 1988–1989. The outbreak of the infection began with a HIV-infected child at a children hospital in Elista. Antiepidemic measures were not taken, which resulted in the spread of the epidemic throughout medical institutions in Kalmykia, the Rostov and Volgograd districts, as well as Stavropol Territory. A single focus (focus of infection) was responsible for infecting more than 260 children and their mothers [2, 3]. Many of them have long passed away (*Fig. 1*).

Some HIV-infected patients demonstrated rapid progression of the disease (2–3 years) and emergence of AIDS symptoms, whereas in other patients HIV symptoms took a considerable length of time to manifest. The differences in the rate of disease progression may be the result of a combination of external factors (infection conditions, concomitant diseases, ongoing treatment) and the individual genetic characteristics of a patient [4].

Among the human genes affecting the progression of the HIV infection, the *CCR5* gene that encodes the CC chemokine receptor 5, which mediates HIV binding to the cell membrane and penetration of certain viral strains into the cell, plays the most significant role [4]. A 32 bp deletion in the *CCR5* gene (*CCR5del32* (rs333)) results in the production of a nonfunctional protein. Individuals with homozygous deletion bear no functional CCR5 receptors, whereas the density of these receptors in individuals with heterozygous deletion is reduced. The CCR5del32 allele occurs predominantly in European populations. Its frequency is the highest in northern European countries (up to 15–18%). It is lower than 3–5% in most Asian populations. This allele is absent in almost all individuals of the native populations of America and Oceania [5–7].

CCR5del32 homozygous individuals (their proportion in European populations is equal to 1–2%) show high, although not absolute, resistance to infection. There are very few CCR5del32 homozygous individuals among HIV-infected individuals. Only 12 cases have been reported among the more than 20, 000 patients examined; for most of them, the virus was tropic for CXCR4, but not CCR5 [8–13]. The protective effect of *CCR5del32* homozygosity has been confirmed both in a number of epidemiological studies (an increased homozygote frequency among HIV-negative individuals who had a risk of infection) and via *in vitro* infecting of CD^+ cells derived from individuals of various genotypes [14].

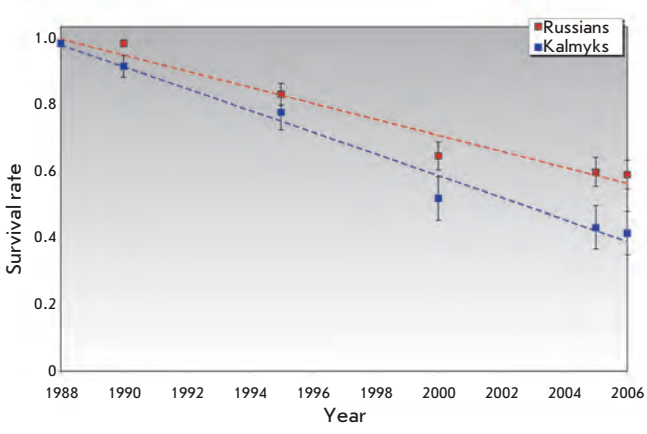

Fig. 1. Dynamics of the survival rate for individuals with nosocomial HIV infection: Russians (Rostov-on-Don – 107, Elista – 13 individuals) and Kalmyks (Elista – 57 individuals).

The protective effect of *CCR5del32* heterozygosity also manifested itself in the development of AIDS symptoms in HIV-infected individuals. It is not unlikely that the possibility of a symptomatic undiagnosed bearing of HIV by CCR5del32/+ heterozygotes may facilitate the spread of the infection. The viral load in HIV-infected *CCR5del32* heterozygous individuals is lower, the CD4+ T cell count decreases at a slower rate, and AIDS symptoms develop slower both in adults [8, 11, 13–17] and in children (most of whom were perinatally infected) [18]. The frequency of *CCR5del32* heterozygosity was considerably higher in the group of individuals who were infected in the 1980s and survived a period of 10 years post-infection [11].

However, data indicating that *CCR5del32* heterozygosity protects against HIV infection remain controversial. In a number of studies, the heterozygosity frequency among infected individuals has been found to be higher than that among the healthy ones who were at risk of infection, or than that of the total sample of the same population; points which may indicate the fact that individuals with the *CCR5wt/del32* genotype have partial HIV-1 resistance [10, 12]. This has not been observed in other studies; the difference between the frequencies of *CCR5wt/del32* heterozygotes and/or *del32* alleles between groups of HIV-positive and HIV-negative individuals have been either absent or statistically insignificant [8, 19–21]. In this study, the effect of *CCR5del32* heterozygosity on the survival rate of children with focus nosocomial HIV infection and the risk of infection upon transmission route through injection were analyzed. In addition, a meta-analysis of the

published data was performed in order to assess the potential decrease in the risk of infection in heterozygous individuals for the *CCR5del32* allele.

MATERIALS AND METHODS

Blood samples from the collection of the Biotechnology Laboratory (Ivanovsky Institute of Virology, Russian Academy of Medical Sciences) were used in this study. The samples were obtained as a result of planned medical examinations of individuals with nosocomial HIV infection, during the period spanning 1991–2007. Consent letters were obtained from the parents of each of the examined children giving permission to use some of the samples obtained for research purposes. This sample of HIV-infected patients is unique, since there is no variability of infection development associated with differences in viral strains. All of the patients were infected with the same viral strain (HIV-1 subtype G) originating from the original child that had been infected (focus nosocomial infection) [22, 23]. Furthermore, most patients belonged to two ethnic groups (Russians and Kalmyks), thereby reducing the possible influence of genetic heterogeneity in each cohort. Anonymous data on patients' birth dates and death dates in cases of fatal outcomes were obtained for 107 HIV-infected patients in the Rostov district (all Russians) and 60 HIV-infected patients from Elista (47 Kalmyks and 13 Russians). Blood samples of HIV-infected children (50 Russians and 27 Kalmyks, age varied from less than 1 year to 16 years; median age 2.7 years) were used for the study. Blood samples taken from healthy volunteers were used as control samples. The first control group consisted of students of the Rostov State Medical University (the majority of whom were born in 1986–1990). According to the results of the survey, they were second-generation Russians and were born in the Rostov district. The second control group consisted of Kalmyks living in Elista (ethnicity was established based on the survey results). Blood samples were collected in full compliance with the informed-consent procedure. The genetic study project obtained approval from the Ethics Committee of the Institute of the Institute of General Genetics, Russian Academy of Sciences.

Genomic DNA was extracted from venous blood samples (up to 50 μl) using a commercial kit DNAPrep (IsoGene, Russia) at the Biotechnology Laboratory of the Institute of Virology, Russian Academy of Medical Sciences (equipped for handling infected samples), according to the manufacturer's procedure.

Genotyping was performed using PCR amplification of DNA samples. The primers and amplification conditions were described in [24]. PCR products underwent 2% agarose gel electrophoresis in order to determine the length of DNA fragments.

Estimated value of the protective effect of CCR5del32 allele in the heterozygous state

The effects observed for all samples were uniformly characterized using the odds ratio measure (*OR*), which was calculated as the ratio between the chance of bearing the *wt/wt* genotype in HIV-positive and HIV-negative individuals:

$$OR = \frac{P(wt/wt|HIV+)}{1-P(wt/wt|HIV+)} / \frac{P(wt/wt|HIV-)}{1-P(wt/wt|HIV-)} = \\ = \frac{P(wt/wt|HIV+)\,P(wt/del|HIV-)}{P(wt/del|HIV+)\,P(wt/wt|HIV-)},$$

where $P(*|\,\text{HIV+})$ and $P(*|\,\text{HIV−})$ are the genotype frequencies in the samples of infected and healthy individuals, respectively. The risk ratio (*RR*), which is determined as the ratio between morbidities for various genotypes, was assessed using the following formula:

$$RR = \frac{P(HIV+|wt/wt)}{P(HIV+|wt/del)} = \frac{Se}{1-Se} \cdot \frac{1-P(wt/wt)}{P(wt/wt)},$$

where *Se* is the test sensitivity for disposition, i.e., the frequency of the risk *wt/ wt* genotype in patients, and *P*(*wt / wt*) is the population frequency of the risk genotype.

The statistical significance of frequency differences was assessed using the two-tailed Fisher's exact test.

The meta-analysis was conducted using a freeware package of statistical programs for epidemiologists WinPepi v.10 (2010) [25]. The package allows one to estimate the median *OR* value based on the fixed effects model (Mantel–Haenszel test) and the random effects model (Der Simonian–Laird method). The choice between the two models was made based on a dataset analysis (Cochran's Q test).

RESULTS

Allele and genotype frequencies in HIV-infected patients and in the control groups

CCR5 genotyping was carried out for each child from the sample of children with nosocomial HIV infection and the control group individuals; *CCR5del32* allele bearers were revealed (*Table 1*). Genotype distribution in all groups did not differ significantly from the Hardy–Weinberg equilibrium. The *CCR5del32* allele frequency was first ascertained in the Kalmyk population and was equal to 0.021 ± 0.012. The low allele frequency in the Kalmyk population correlates with its frequency in the neighboring population of the Caucasus (3–5%) and the low frequency in the populations of Central Asia of close origin with the Kalmyks (e.g., 1.1% for the Mongolians in China [26]). No bearers of this allele were detected in the sample consisting of 27 HIV-infected

Table 1. Distribution of the genotype and allele frequencies over the CCR5 gene in HIV-infected children and in the control samples

Group	N	Number of individuals (genotype frequencies, %)			Allele frequency and statistical error (±SE)		Comparison of the HIV+ and control groups
		wt/wt	wt/del	del/del	wt	del	
HIV, Kalmyk children	27	27	0	0	1	0	OR = 2.85 p = 0.558
Control, Kalmyks in Elista	70	67 (95.71)	3 (4.28)	0	0.979 ± 0.012	0.021 ± 0.012	
HIV, Russian children	50	39 (78.0)	11 (22.0)	0	0.890 ± 0.031	0.110 ± 0.031	OR = 1.21 p = 0.690
Control, Russians in the Rostov district	99	73 (73.7)	25 (25.3)	1 (1.0)	0.864 ± 0.024	0.136 ± 0.024	

Table 2. The frequencies of the CCR5del32 (rs333) allele in groups of Russians and Kalmyks

Population	N	CCR5del32 allele frequency	$CI_{95\%}$	Reference
Russians: Leningrad district	33	0.166	0.083–0.300	[27]
Kostroma	54	0.157	0.091–0.252	[28]
St. Petersburg	50	0.130	0.069–0.223	[29]
Moscow	83	0.139	0.088–0.208	[30]
Moscow	176	0.122	0.088–0.164	[31]
Ryazan	78	0.12	0.072–0.188	[32]
Lipetsk	48	0.104	0.045–0.192	M.M. Garaev's own data
Novosibirsk	53	0.104	0.051–0.187	[33]
Lys'va	186	0.100	0.070–0.138	[34]
Moscow, ethnicity not specified	171	0.091	0.062–0.129	[35]
Rostov-on-Don	99	0.136	0.089–0.198	This study
Russian children, HIV	50	0.110	0.054–0.198	This study
Kalmyks	70	0.021	0.004–0.063	This study
Kalmyk children, HIV	27	0	0–0.073	This study

Kalmyk children (the differences from the frequency in the control group were insignificant: $p = 0.558$, based on the exact Fischer's test).

According to the published data, the *CCR5del32* allele frequency in Russians varies from 0.104 to 0.157 (*Table 2*) (see review in [7]).The control group was formed of volunteers (the students of the Rostov State Medical University) since most infected Russian children within the sample under study were undergoing treatment in Rostov-on-Don hospitals; whereas the *CCR5del32* allele frequency in Russians of the Rostov district was unknown. According to the survey results, they were second-generation Russians and had been born in the Rostov district. The *CCR5del32* allele frequency in this group was equal to 0.136 = 0.024, which lies within the frequency variability range in various geographic groups of Russians. The *CCR5del32* allele frequency in HIV-infected Russian children turned out to be slightly lower (0.110 ± 0.031); however, the differences were of no significant degree ($OR = 1.21$, $p = 0.69$).

For such a small sample size, the lower *CCR5del32* allele frequency in HIV-infected individuals as compared with that in the control group may be a random effect or result from the protective action of this allele.

In a number of studies, data on the lower *CCR5del32* allele frequency and/or lower *wt/del32* zygote frequency among HIV-infected individuals as compared

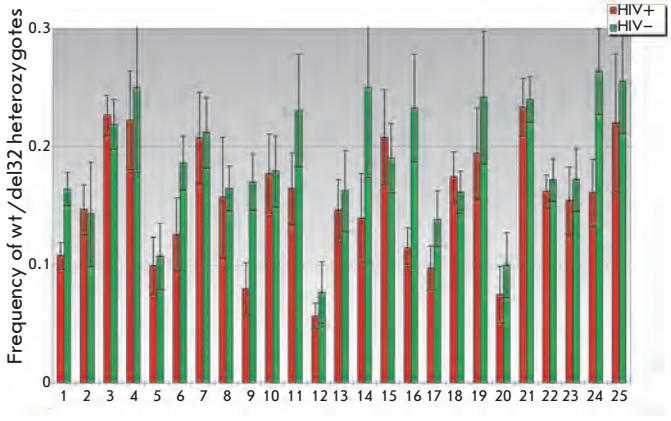

Fig. 2. Frequencies of heterozygous bearers of the CCR5del32 allele among HIV-infected (HIV+) and healthy (HIV–) individuals. 1 – Belgians and French [10]; 2 – Swiss (HIV-infected [36], control group [37]); 3 – Euro-Americans [38]; 4 – Danes [39]; 5 – Italians, Milan [40]; 6 – Australians [41]; 7 – Finns [42]; 8 – Slovenians [43]; 9 – Spaniards, Asturias [44]; 10 – Moscow residents [19]; 11 – Russians (HIV-infected – Muscovites, control – Russians, Ryazan) [20]; 12 – Spaniards, southern Spain [45]; 13 – Spaniards [46]; 14 – Hungarians [47]; 15 – Russians, Perm district [34]; 16 – Euro-American females [48]; 17 – Germans, Munich [49]; 18 – Euro-Americans, Seattle [50]; 19 – Poles [51]; 20 – Italians [52]; 21 – Estonians [21]; 22 – Germans [13]; 23 – Slovaks [53]; 24 – Poles, Szczecin [54]; 25 – Russians, Rostov district (the present study).

Table 3. Mortality rate (by 2006) in the samples studied depending on genotype

Genotype	Russians	Kalmyks
wt/wt	12 of 39 (30.8%)	11 of 27 (40.7%)
wt/del32	2 of 11 (18.2%)	–
Total	14 of 50 (28.0%)	11 of 27 (40.7%)

with the population control group have been obtained. In many cases, these differences do not reach any significant level, whereas an opposite ratio between the frequencies has been revealed in some studies (*Fig. 2* and the database of allele frequencies (Genome Analysis Laboratory, Institute of General Genetics, Russian Academy of Sciences), http://vigg.ru/institute/podrazdelenija/otdel-genomiki-i-genetiki-cheloveka/laboratorijaanaliza-genoma/allefdb/ccr5-hiv/). The meta-analysis of the published data was performed in the groups of HIV-infected patients and control groups in order to assess the eventuality of a protective effect of heterozygosity for *CCR5del32*.

Meta-analysis: does heterozygosity for *CCR5del32* reduce the risk of infection?

For the meta-analysis, articles comparing the allele and genotype frequencies in the samples of HIV-infected patients and the corresponding control samples of uninfected individuals were selected from over 360 articles found in the PubMed database upon inquiry "CCR5 AND deletion AND HIV" (September 2011). The publications studying Asian, African, and Latin American populations with a*CCR5del32* allele frequency of 1–3% or lower were eliminated from the analysis.

As a result of the differences in the *CCR5del32* allele frequencies in populations of European origin (from 5–8% in southern Europe to 15–18% in northern Europe) [7], the ethnicity of the control group individuals (and in some cases, their membership in subgroups within an ethnic group) has to closely match the ethnicity of the group of infected individuals. Therefore, the publications in which the ethnicity of the groups was not indicated or the samples were not ethnically homogeneous were also eliminated. A total of 25 Caucasian groups, including our sample, were selected for the meta-analysis: 5,967 HIV-infected individuals and 5,410 control group individuals (*Table 3*).

The frequency of homozygous deletion bearers was 4 of 5,967 HIV-infected individuals against 63 of 5,410 individuals in the control group. This ratio corresponds to $OR = 17.6$ at $p = 4.4 \times 10^{-16}$. In this case, the relative risk value is approximately equal to the *OR* value; i.e., the infection probability of deletion homozygous individuals was lower than that of the bearers of the other genotypes by a factor of 17.6. Close estimations of the protective effect of homozygocity for deletion were obtained in separate studies of Euro-Americans, where the groups of seronegative individuals were compared with groups of seropositive individuals and the population control groups [9, 38], and in some of the other studies [10, 11]. Therefore, *CCR5del32/ CCR5del32* homozygous individuals were eliminated from the subsequent analysis. The ratio between heterozygous bearers of the *CCR5del32* allele and the individuals without this allele (i.e., the ratio between the *wt/CCR5del32* and *wt/wt* genotypes in the groups of HIV-infected individuals and the population control group) were considered to assess the risk of infection.

It was demonstrated via a comparison of the genotype frequencies that the frequency of *wt/CCR5del32* heterozygotes in HIV-infected individuals was higher

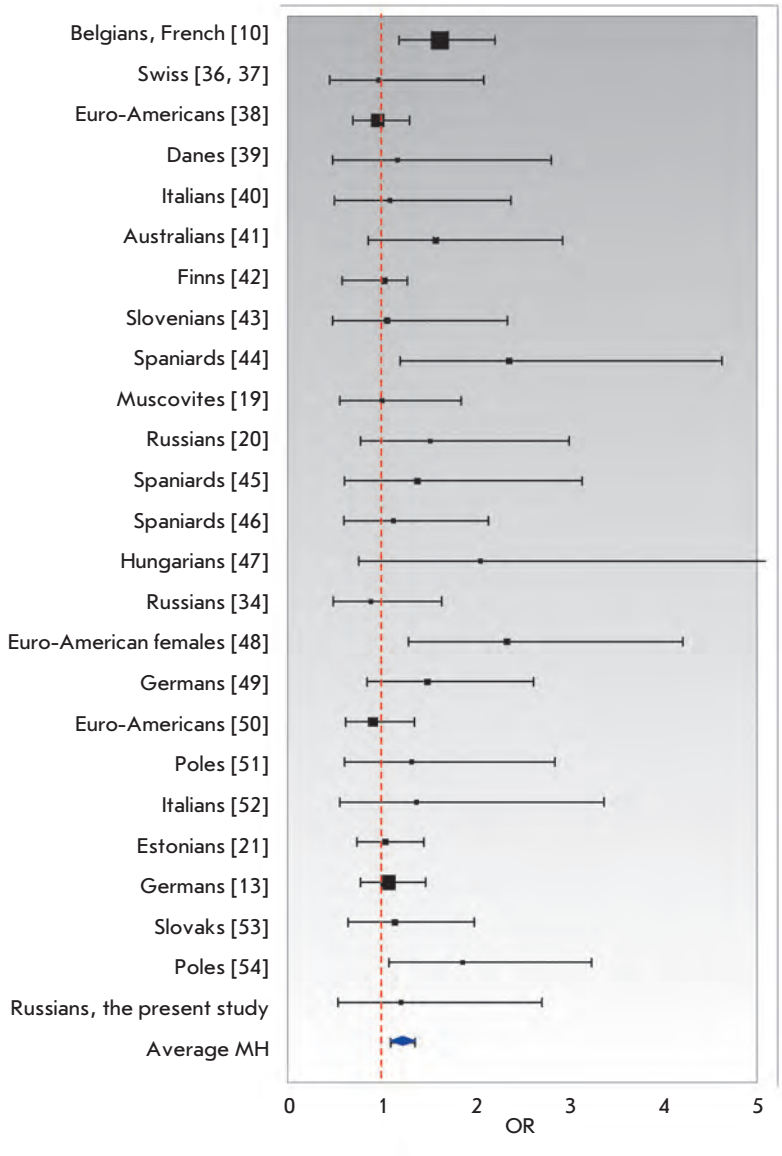

Fig. 3. The estimated odds ratios (OR) and the corresponding 95% confidence intervals for 25 samples of Caucasians (specified in the legend to Fig. 2). The vertical dashed line corresponds to OR = 1 (no effects). The points on the right-hand side of this line indicate the protective effect of the wt/CCR5del32 genotype. The size of the square markers is conventionally proportional to sample size. The lower diamond-shaped marker corresponds to the Mantel–Haenszel (MH) estimate of the averaged OR.

than that in healthy individuals in only 4 out of 25 studies (*Fig. 2*). Based on the assumption that the effect was random, the probability of the event "the heterozygote frequency in affected individuals is higher than that in healthy ones in no more than 4 studies out of 25" was equal to 4.7×10^{-7} (similar to the probability of getting no more than four heads after a coin is tossed 25 times).

The odds ratio (*OR*) was determined for each sample; the *OR* values were then averaged with allowance made for the population size and degree of homogeneity of the effects. The results are presented in graphical form (*Fig. 3*).

It was found via a meta-analysis carried out on the basis of the results of 25 studies that the *wt/CCR5del32* heterozygote frequency against that of *wt/wt* homozygotes in the samples of HIV-infected individuals was reliably lower than that in the control ($p = 0.0002$ with the two-tailed Fischer's exact test and $p = 0.00018$ with the χ^2 test). With the Cochran's Q test, heterogeneity of the data was insignificant: $\chi^2 = 25.29$,($p = 0.39$).The variability fraction caused by the heterogeneity of *OR* values $I^2 = 5.1\%$ ($CI_{95\%} = 0-36.4\%$). This value is considerably lower than the critical value (50%), which allows one to use the "fixed effects model," employing the Mantel–Haenszel test (MH average) for averaging the *OR* values. The total value of the effect is appreciably low: $OR = 1.22$, $CI_{95\%} = 1.10-1.36$. However, these estimations have a large stability margin: 28 studies, in which the genotype frequencies would be the same for the samples of HIV-infected individuals and in the control group ($OR = 1$), need to be added to reduce the to-

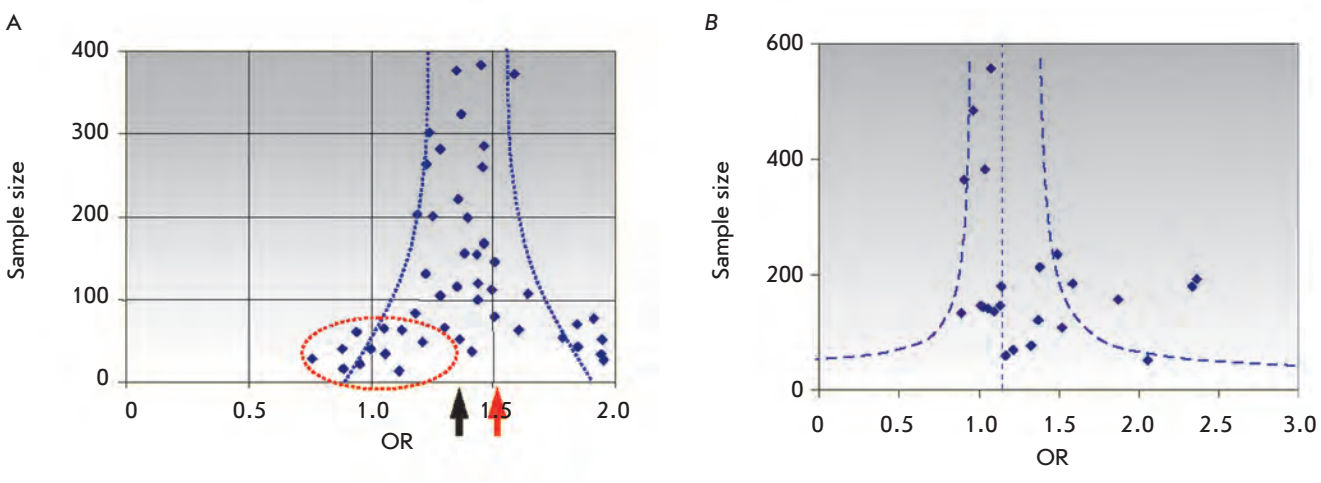

Fig. 4. Dependence of the effect intensity (OR) on the composition of the samples selected for the meta-analysis (funnel plot). The strong asymmetry of this plot may indicate publication bias. (A) – The hypothetic distribution of the studies over the size of the samples studied and effect intensity. For the elimination of less willingly published results obtained using small samples with weak effects (dots in a dashed oval), the OR value turns out to be overestimated (red arrow) with respect to the real OR (black arrow). (B) – Distribution of the studies over sample size and effect intensity for 25 publications selected for the meta-analysis in this study (insignificant asymmetry).

tal value of the effect to an insignificant level $OR = 1$.

The possibility of publication bias is usually taken into account when performing a meta-analysis. The publication bias is a result of the fact that the researchers and journal editorial boards are more willing to publish positive rather than negative or "zero" results. Moreover, studies with significant effects obtained using small samples are often published. All of these facts may result in the overestimation of the averaged effect value in a meta-analysis (*Fig. 4A*). Constructing the dependence of the effect value (*OR*) on sample size (a funnel plot) is the standard method for checking for data symmetry. A strongly asymmetric plot may attest to selective presentation of the data in publications. In our case, asymmetry is insignificant (*Fig. 4B):* the Kendall rank correlation between *OR* and the sample size is equal to 0.21 at $p = 0.187$; asymmetry based on the regression test [55] is unreliable ($p = 0.148$).

Thus, a statistically significant, although weak, protective effect of heterozygocity for the *CCR5del32* allele with respect to HIV-infected individuals (OR = 1.22 at $p = 2 \times 10^{-4}$) was established by the meta-analysis, which included our own experimental data. An appreciably low *OR* value explains the reason why no significant differences in heterozygote frequencies between the HIV-infected individuals and those from the control groups have been found in the majority of articles. The data demonstrate that at a deletion allele frequency of 10% and $OR = 1.22$, the significant effect ($p = 1.22$ at 80% strength) can be detected only when the total sample size is 4,500 (2,250 affected and 2,250 healthy individuals).

The estimated value $OR = 1.22$ does not mean that the risk of infection in the bearers of the *wt/wt* homozygous genotype is higher than that in the bearers of the *CCR5del32* allele by 22%. *OR* is the ratio between the chances, but not the risks of infection. The risk ratio parameter (*RR*), which is determined as the ratio between morbidities for various genotypes, cannot be directly estimated in context-dependent case-control studies. One may only propose various indirect estimations of RR based on *OR* and population allele frequency values or morbidity data [56]. Moreover, the inequality $OR \geq RR$ is always valid. According to the results of this meta-analysis, the corresponding estimations are as follows: $SE = 0.851$ and $P(wt/wt) = 0.835$; therefore, RR = 0.13. Thus, the infection probability for *wt/wt* homozygous individuals is higher than that for the bearers of the *CCR5del32* allele by at least 13%.

Our estimation is based on a comparison of the ratios between the *wt/CCR5del32* and *wt/wt* genotype frequencies in HIV-infected individuals and in the population control group. It is evident that the HIV-infected individuals had contacted the virus and had been infected; whereas the population control individuals had not contacted the virus (the fraction of the individuals who contacted the virus and/or HIV-infected ones is assumed to be negligibly small in the populations of European origin under study). The protective effect of

heterozygosity can be assessed more precisely by using the control group consisting of individuals who had contacted the virus but remained HIV-negative. However, the fraction of individuals in the existing high-risk groups (haemophiliacs; sexual partners of HIV-infected individuals; injection drug users; men practicing receptive penile–anal contacts with men (MSM); prostitutes) who had contacted the virus differs largely and sometimes cannot be determined. The random-effects model can be used when carrying out a meta-analysis of the results of these studies. However, because of the sample heterogeneity, *OR* estimations to a significant extent show the probability of contacting the virus in various risk groups rather than showing the direct protective effect of the allele in the group of individuals who contacted the virus.

This phenomenon can be illustrated by comparing the frequencies of homozygous genotypes for the deletion allele among uninfected individuals in two risk groups: haemophiliacs [57] and MSM individuals [38]. Among the uninfected individuals in the MSM group, the *CCR5del32/del32* homozygote frequency was equal to 4.5% (5 of 11 individuals); whereas the frequency among the haemophiliacs was equal to 16.3% (7 of 43 individuals), given the fact that the population frequency of these homozygotes in the Caucasian populations is less than 1–2%. The differences in the frequencies of individuals with homozygous deletion are statistically significant in the two risk groups ($p = 0.038$, two-tailed Fischer's test). A higher *CCR5del32/del32 homozygote* frequency among haemophiliacs is accounted for by a higher risk of infection; for the individuals receiving intense therapy with blood-based products in 1978–1985, it was equal to 94% [57]. Since almost 100% of patients who were administered blood-based samples contacted the virus, it can be assumed that the higher frequency of homozygotes for the deletion allele (i.e., a more pronounced protective effect) cannot be achieved because of the genetic heterogeneity of the indicator of resistance to infection with macrophagotropic HIV strains [58], similar to how the selection of protective alleles of different genes ensuring resistance to infection (e.g., malaria) is carried out in the same population under natural conditions.

Effect of heterozygosity for the CCR5del32 allele on the survival rate of HIV-infected individuals

The variability in the progression of the HIV infection into AIDS and the mortality rate were observed previously for both samples (for Russians and Kalmyks). The variability is caused by nongenetic factors (infection age, which varies from several months to 14 years in these samples; the median age is 2.5 years), intensity of parenteral interventions, and concomitant diseases [3].

By 2006, the mortality rate in the sample studied was equal to 32.5% (25 of 77 individuals). The mortality rate was 28.0% (14 of 50) among the infected Russian children and 40.7% (11 of 27), among Kalmyk children. By 2006, the rate of mortality in Russians in the studied group was lower than that for the Kalmyks by 31.2%; however, these differences were insignificant for the given sample sizes ($p = 0.311$). Nevertheless, the regressions describing the general mortality dynamics differ to a significant degree: the slope angle of the regression for Russian children is equal to -0.016 ± 0.004, against -0.025 ± 0.002 for Kalmyk children ($p = 0.02$, two-tailed Z test).

The effect of the carriage of the *CCR5del32* allele on the survival rate of infected individuals was tested, as well as whether the differences in longevity post-infection could be linked to the differences in the *CCR5del32* frequency in the two ethnic groups.

In the sample subjected to study, no deletion allele was detected in HIV-infected Kalmyk children; all of them had the *wt/wt* genotype. Among the infected Russian children with the *wt/wt* genotype, the rate of mortality was 30.8% (12 of 39%), and 18.2% (2 of 11) among the bearers of the CCR5del32 allele (*Table 3*). Thus, the mortality rate in Russian children with the *wt/CCR5del32* genotype 15 years after the infection was lower by 40.9% compared with that of the children without the deletion allele, although these differences are statistically insignificant ($OR = 2.0$; $p = 0.705$). The limited sample size makes it impossible to accept or refute the hypothesis that the differences in longevity in the two ethnic groups are caused by the differences in the *CCR5del32* allele frequencies. Nevertheless, it should be noted that our insignificant estimations literally line up with previously published data. According to the results of the meta-analysis of 19 cohorts of HIV-infected individuals (a total of 1,635 Caucasians), the protective effect of heterozygous bearing of the *CCR5del32* allele manifested itself in a 39% decrease in the risk of death [17]. In order to attain statistical significance at this level of the effect ($OR = 2$), the sample size for the infected individuals has to be at least 550 individuals at a mortality rate of 30% and 400 people at the instant when mortality is as high as 60%.

Thus, in the study that focused on 507 HIV-infected Poles who were observed over a period of 15 years prior to the introduction of antiretroviral therapy, the differences in the rate of mortality between the bearers of the *wt/del32* and *wt/wt* genotypes were equal to 49% (the overall mortality rate being 19%). These differences were statistically significant ($p = 0.026$), whereas the differences were insignificant ($p = 0.23$) for individuals receiving treatment (442 individuals) [59].

DISCUSSION

The effect of the heterozygous bearing of the *CCR5del32* deletion allele on the risk of HIV infection in populations of European origin (without allowance for the route of infection transmission, viral serotype, and differences in antiretroviral therapy) was first assessed based on the meta-analysis of published data. The protective effect observed was rather small (OR = 1.22) but was statistically significant and corresponded to a decrease of at least 13% in the risk of infection in the individuals with the *CCR5wt/del32* genotype, according to the calculations. The small OR value explains why the differences between the frequencies of genotypes and/or CCR5del32 allele between the groups of HIV-infected individuals and the control group detected in most articles are statistically insignificant.

The size of the samples that are to be studied in order to demonstrate the reliability of this phenomenon reversely depends on the allelic abundance in the population. In particular, in Chinese populations, where the abundance of the *CCR5del32* allele is lower than that in Europeans, no significant protective effect of heterozygous bearing of *wt/del32* has been detected via a meta-analysis: OR = 1.156 ($CI_{95\%}$ = 0.808–1.654) [60].

The *CCR5del32* allele is predominantly present in populations of European origin; its abundance in southern European population groups (Spaniards, Italians, and Greeks) is equal to 5–8%, being as high as 15–18% in northern European population groups (Finns, Estonians, Mordvinians, Tatars, etc.) [7]. In Russians, the frequency of the *CCR5del32* allele is appreciably high (10–17% in different regions), whereas the frequency of this allele is equal to 2% in the other group that was studied (Kalmyks). Can the differences in the frequency of the protective *CCR5del32* allele play a substantial role in the prevention of the HIV infection at the population level or can they be accounted for by the differences in the mortality rate of HIV-infected individuals?

Hypothetically, the population effects caused by the presence of the deletion allele can be assessed as follows. Let us assume q to be the frequency of the deletion allele, and S_{ww}, S_{wd}, and S_{dd} – the survival rates of the infected individuals with *wt/wt*, *wt/CCR5del32*, or *CCR5del32/CCR5del32* genotypes, respectively. Then, the population average survival rate S_{pop} is higher than the survival rate of the individuals with the *wt/wt* genotype by the following figure:

$$\Delta S = S_{pop} - S_{ww} = (1-q)^2 S_{ww} + 2q(1-q)S_{wd} + q^2 S_{dd} - S_{ww} \approx 2(S_{wd} - S_{ww})q.$$

The terms of q^2 order were neglected in the latter equality. Thus, the protective effect of heterozygous bearing of the *CCR5del32* allele (a 40% decrease in the mortality rate for the infected individuals) for a 10% frequency of this allele provides an 8% decrease in the general death rate for the HIV-infected individuals against the group without bearers of this allele. A decrease in the risk of infection due to the presence of the *CCR5del32* allele in the population is calculated identically. If the probability of infection of heterozygous individuals is reduced by 13%, the general infection frequency for the population is reduced by 3.3%. At a 15% allele frequency, the decrease in the infection rate would be 5.6%; whereas the decrease in the mortality rate of the HIV-infected individuals would be 12%.

To summarize, protection against the HIV infection and the reduction in mortality rates in HIV-infected individuals at the population level is rather small even in groups with a high frequency of the *CCR5del32* allele (15%). In addition to *CCR5del32*, there are other genes which affect both the susceptibility to HIV infection and the course of progression of the HIV infection [61] and have the ability to contribute to interpopulation differences. Thus, Russians and Kalmyks differ in terms of the frequencies of the protective *C/C* genotype at polymorphism in the regulatory site of the interleukine 10 gene *IL*10–592 A/C (49%in Russians in the Rostov district and 33% in Kalmyks in Elista) and in terms of the frequencies of the protective CCR2-64I allele (12% in Russians of the Rostov district and 23% in Kalmyks in Elista) (the data obtained by the authors have yet to be published). However, the possible contribution of these genes to interpopulation differences in the progression of the HIV infection requires further studies. The authors express the hope that samples with nosocomial infections will no longer be available. ●

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The Genetic Diversity and Structure of Linkage Disequilibrium of the *MTHFR* Gene in Populations of Northern Eurasia

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ABSTRACT **The structure of the haplotypes and linkage disequilibrium (LD) of the methylenetetrahydrofolate reductase gene (*MTHFR*) in 9 population groups from Northern Eurasia and populations of the international HapMap project was investigated in the present study. The data suggest that the architecture of LD in the human genome is largely determined by the evolutionary history of populations; however, the results of phylogenetic and haplotype analyses seems to suggest that in fact there may be a common "old" mechanism for the formation of certain patterns of LD. Variability in the structure of LD and the level of diversity of *MTHFR* haplotypes cause a certain set of tagSNPs with an established prognostic significance for each population. In our opinion, the results obtained in the present study are of considerable interest for understanding multiple genetic phenomena: namely, the association of interpopulation differences in the patterns of LD with structures possessing a genetic susceptibility to complex diseases, and the functional significance of the pleiotropic *MTHFR* gene effect. Summarizing the results of this study, a conclusion can be made that the genetic variability analysis with emphasis on the structure of LD in human populations is a powerful tool that can make a significant contribution to such areas of biomedical science as human evolutionary biology, functional genomics, genetics of complex diseases, and pharmacogenomics.**
KEYWORDS **genome; linkage disequilibrium; populations of Northern Eurasia; methylenetetrahydrofolate reductase; haplotype.**
ABBREVIATIONS **CD – complex diseases; LD – linkage disequilibrium; MTHFR – methylenetetrahydrofolate reductase; SNP – single nucleotide polymorphism; HC – homocysteine.**

INTRODUCTION

Genetic variability underlies the human phenotypic variation and plays a significant role in explaining the differences between individuals in their susceptibility to complex diseases (CD) and in determining the metabolic pathways involved in the development of pathological processes. Single nucleotide polymorphisms (SNPs) represent the most common type of genome variability. Thanks to the efforts of the International SNP Consortium, ~ 10 million SNPs with an approximate density of 1 polymorphism per 300 bp have currently been identified [1]. Each new allele of a polymorphic variant emerges from an already existing haplotype, the ancestral variant of a particular marker originally being associated with its alleles. New haplotypes are formed via the accumulation of new mutations and recombinations. The coinheritance of alleles in a haplotype manifests itself at the population level as the linkage disequilibrium (LD).

At the time of writing, the architecture of LD in the human genome is the subject of active discussions and research [2–7]. It has been shown in a number of studies that blocks of associated sites demonstrating no signs of substantial recombination in the evolutionary history of our species can be distinguished in the genome. These blocks are delimited by regions with a higher rate of recombination, the so-called "hotspots" [8, 9]. The patterns of linkage disequilibrium in modern human populations are the result of complex evolutionary processes, including the demographic population history (alteration of the effective population size, structure of population stratification, and migrations), as well as gene-specific factors, such as the mutation and recombination rates and selection pressure. Analysis of the structure of LD enables to reconstruct the demographic history of modern populations and plays a key role in the mapping of the CD genes [10].

Along with the whole-genome patterns of LD that have been investigated in modern genome-wide studies [11–15], the structure of LD in separate, functionally significant genomic sites (in particular, in the genetic loci associated with common human diseases) is of considerable interest. The importance of the analysis of the haplotype structure of these genomic regions is rooted in the necessity of revealing the functionally significant variants of these genes which make possible their participation in their common component of the inherited susceptibility to CD, on the one hand, and to the significance of the assessment of the evolutionary genetic mechanisms of the generation of genetic variability in these genomic loci, on the other hand. These mechanisms were presumably formed with allowance for such factors as natural selection, genetic drift and migration, as well as via comparison of the genetic-demographic scenarios obtained by the analysis of the fine structure of the candidate genes of CD with those based on the data of conventionally neutral genetic systems and the results of whole-genome studies.

In this study, the methylenetetrahydrofolate reductase (*MTHFR*) gene was selected for use as a locus to investigate the LD structure in populations of various ethnic origins. According to the results of numerous studies, the polymorphic variants of this gene are associated with the development of a number of complex conditions, such as cardiovascular and oncological diseases, neural tube defects, abnormal pregnancy, as well as other pathological processes. The enzyme methylenetetrahydrofolate reductase catalyzes the only intracellular reaction of formation of 5-methyltetrahydrofolate, which is required for homocysteine (HC) conversion into methionine. A decrease in the activity of this enzyme is frequently caused by mutations in the *MTHFR* gene and results in the accumulation of HC and the development of moderate hyperhomocysteinemia.

A significant number of studies have been devoted to the role of genetic variability in the *MTHFR* locus. The results of these studies attest to the small contribution of individual SNPs of the *MTHFR* gene to the structure of inherited susceptibility to CD; moreover, the data for many ethnic groups are often contradictory. Since analysis of the LD structure in the candidate genes and identification of the haplotypes associated with the disease and their tagSNPs is considered to be one of the strategies for identifying the genetic variants underlying CD susceptibility with the highest potential [3, 16, 17], the assessment of the genetic variability of the candidate genes at the level of SNPs and haplotypes in different population samples is a rather topical task.

EXPERIMENTAL

Populations

The data presented in this study were obtained via a molecular genetic DNA analysis of individuals from nine population groups inhabiting different regions of Northern Eurasia and belonging to seven ethnic groups (*Table 1*). The total sample included 837 individuals. The populations studied represent two Eurasian race types, Caucasian and Mongoloid, and speak languages of four

Table 1. Linguistic and anthropological characteristics of the populations

Ethnic group	Population, community	N	Locality	Linguistic classification, family/group	Race and anthropological type
Tuvinians	Settlement of Bai-Taiga	134	Tuva Republic	Altaic/ Turkic	Mongoloid (Central Asian)
Buryats	City of Ulan-Ude, Settlement of Khuromsha	60 60	Buryat Republic	Altaic/ Mongolic	Mongoloid (Central Asian)
Yakuts	Settlement of Dyupsya	81	Sakha Republic	Altaic/ Turkic	Mongoloid (Central Asian)
Kyrgyz	North (Settlements of Kegety, Taldy-Su), South (city of Osh)	85 111	Republic of Kyrgyzstan	Altaic/ Turkic	Mongoloid (South Siberian)
Kets	Settlement of Kellog	38	Turukhanskii district, Krasnoyarsk krai	Paleo-Asian/ Ket	Mongoloid (North Asian)
Khanty	Settlement of Russinskii	142	Surgut district, Khanty-Mansi Autonomous Okrug	Uralic/ Finno-Ugric	Uralic (transitory)
Russians	City of Tomsk	126	Tomsk oblast	Indo-European/ Slavic	Caucasian (East Caucasian)

Table 2. Characteristics of the *MTHFR* gene SNPs studied

№	SNP	Position on a chromosome (according to the NCBI database)	dbSNP alleles	Ancestral allele	Mutation type	Localization in the *MTHFR* gene (according to data from the NCBI database)
1	rs3753588	11863904	A/G	G		Intron 1
2	rs2066470	11863057	C/T	C	Synonymous (39 Pro/Pro)	Exon 2
3	rs17037397	11862163	A/C	C		Intron 2
4	rs7533315	11860683	C/T	C		Intron 3
5	rs4846052	11857951	C/T	T		Intron 4
6	rs1801133	11856378	C/T	C	Non-synonymous (222 Val/Ala)	Exon 5
7	rs6541003	11855867	A/G	G		Intron 5
8	rs2066462	11854896	C/T	C	Synonymous (352 Ser/Ser)	Exon 7
9	rs1801131	11854476	A/C	A	Non-synonymous (429 Ala/Glu)	Exon 8
10	rs17375901	11852516	C/T	C		Intron 9
11	rs2274976	11850927	A/G	G	Non-synonymous (594 Gln/Arg)	Exon 12
12	rs1537516	11847861	C/T	C		3'-untranslatable region

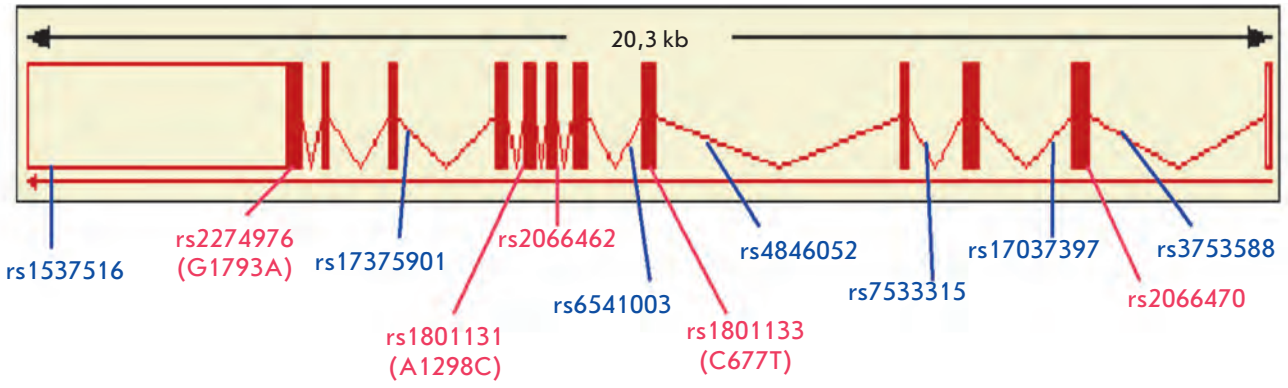


Fig. 1. Localization of the SNPs studied in the *MTHFR* gene. The blue color indicates the polymorphisms located in introns and 3'UTR; red, in exons.

linguistic families (Altaic, Paleo-Asiatic, Indo-European, and Uralic). Only individuals, nonmetisized in at least three generations, participated in the study. Ethnicity, genealogy, and membership of the individuals in sub-ethnic groups (in some cases) were ascertained on the basis of questionnaires. The sample of Tuvinians was collected in the Tuva Republic (settlement of Bai-Taiga). Two Buryat populations were examined in the Buryat Republic (the city of Ulan-Ude and settlement of Khuromsha). The Yakut sample was collected in the settlement of Dyupsya located in the eastern Ust-Aldan ulus (district) of Sakha Republic (Yakutia). The Central Asian populations are represented by Kyrgyz. Two Kyrgyz samples were made up of northern (the settlements of Kegety and Taldy-Su) and southern (the city of Osh) populations of the Republic of Kyrgyzstan; they belong to different sub-ethnic groups. The Russian sample was represented by residents of Tomsk.

The Ket population was collected in the settlement of Kellog, Turukhanskii district, Krasnoyarsk krai. The Khanty population was collected in the settlement of Russkinskii, Khanty-Mansi Autonomous Okrug.

The analysis also included data on the Caucasian (residents of the state of Utah, USA), Chinese (residents of Beijing, China), Japanese (residents of Tokyo, Japan), and Yoruba (residents of Ibadan, Nigeria) populations presented in the HapMap database [11].

Polymorphisms

The following 12 SNPs of the *MTHFR* gene were selected for use as markers to study LD patterns: rs3753588, rs2066470, rs17037397, rs7533315, rs4846052, rs1801133 (C677T), rs6541003, rs2066462, rs1801131 (A1298C), rs17375901, rs2274976 (G1793A), and rs1537516 (*Fig. 1*). *Table 2* briefly characterizes the studied loci of the *MTHFR* gene. Ten of 12 SNPs resulted from the transitions (3 A→G and 7 C→T), two SNPs resulted from the transversions (A→C). The selected polymorphic variants are distributed in a relatively uniform manner over the gene sites (exons, introns, and 3'-untranslated regions); the minor allele frequency in most loci is at least 5% (according to the data from the NCBI database). Genotyping was carried out in accordance with the previously described protocols [18–20].

Methods for the statistical processing of the results

Statistical analysis was performed using conventional software packages: Statistica 6.0, ARLEQUIN, and Haploview 4.0. The distribution pattern of the resulting data was determined using the Kolmogorov–Smirnov test; haplotype frequencies were determined using the EM algorithm. The LD between SNP pairs was assessed using the Levontin's D' coefficient and Pearson's correlation coefficient r^2. The block structure was determined using the Solid Spine of the LD algorithm [21] provided by the Haploview 4.1 software, with the specified D' threshold ≥ 0.8. The levels of genetic diversity and interpopulation differentiation were calculated via an analysis of the molecular variation (AMOVA). The selective neutrality of polymorphisms was studied using the Ewens–Watterson test [22]. The role of selection pressure in the formation of LD patterns and the level of genetic diversity in the populations was assessed using the conventional Tajima's and Fu's statistic tests of neutrality [23, 24].

RESULTS AND DISCUSSION

Genetic diversity and haplotype structure at the *MTHFR* locus in populations

The gene pool of modern human populations was formed as a result of sequential evolutionary demographic processes: continuous evolution of genetic diversity in Africa and population variance as modern humans migrated, with partial isolation and reduction of the gene flow in inverse proportion to the migration distance. In different geographic areas, the populations have both a common and unique evolutionary history; the "fingerprints" of these histories can be observed in the modern human genome as LD patterns [3, 25–27].

The distribution of genotypes and the allele frequency, the observed heterozygosity, and the significance of goodness-of-fit of the *MTHFR* gene SNPs to Hardy–Weinberg proportions are presented in *Table 3*. All 12 loci appeared to be polymorphic in almost all the populations analyzed (with the exception of rs2066470 in the Ket population). The minor allele frequency varied from 0 to 39%; seven SNPs (rs3753588, rs7533315, rs4846052, rs1801133, rs6541003, rs1801131, and rs1537516) were identified in all the populations with a frequency higher than 5%. The resulting data lie within the range of variations of allele frequencies and genotypes of *MTHFR* polymorphisms which had been previously published and listed in the databases of the Caucasian and Mongoloid populations. In all the samples, the distribution of the genotype frequencies of almost all markers fitted into the Hardy–Weinberg proportions (with the exception of loci rs17375901, rs2066470, rs3753588, rs2274976, and rs1537516 in the Buryat subpopulation from the settlement of Khuromsha). Low and medium heterozygosity values were observed in the majority of cases, which was consistent with the world data. The highest heterozygosity for the loci combination was detected in the Yakut population (0.28); the lowest heterozygosity value was recorded in the residents of the settlement of Khuromsha (0.18). It is obvious that these values do not represent the SNP heterozygosity level of the populations examined, since the number of the loci taken into account was too small. These values are of some interest as they provide information pertaining to the degree of polymorphism in the *MTHFR* gene. As for the deviation from the Hardy–Weinberg proportions observed in this study, it could be a result of the shift in the estimated frequency values due to the small size of the sample. On the other hand, the cases of a reliable deviation of the distribution from the expected one may represent the specificity of population-genetic processes in the population, which can be associated both with the parameters of the genetic-demographic structure of the population and with the linkage with a functionally significant locus. We consider the latter reason to be more plausible.

The C677T (rs1801133) polymorphic variant is one of 12 SNPs in the *MTHFR* gene which has been best studied. The missense mutation in C677T (substitution of cytosine by thymine at position 677) results in alanine

Table 3. Distribution of genotypes and minor alleles of the polymorphic variants of the *MTHFR* gene in the samples under study

№	SNPs studied	Genotype, allele	Frequency, %								
			Tuvinians (*N*=134)	Southern Kyrgyzes (*N*=111)	Northern Kyrgyzes (*N*=85)	Kets (*N*=38)	Buryats, city of Ulan-Ude (*N*=60)	Buryats, settlement of Khuromsha (*N*=60)	Yakuts (*N*=81)	Khanty (*N*=142)	Russians (*N*=126)
1	2	3	4	5	6	7	8	9	10	11	12
1	rs3753588	AA	2	1	1	0	0	3	4	2	1
		AG	13	14	22	11	17	5	20	28	18
		GG	85	85	77	89	83	92	76	70	81
		A	8	8	12	5	8	6	14	16	10
		H_e	0.16	0.15	0.23	0.10	0.17	0.13	0.24	0.28	0.18
		p	0.21	0.48	1.00	1.00	1.00	0.008	0.15	1.00	1.00
2	rs2066470	CC	90	85	78	100	83	92	76	79	83
		CT	10	14	18	0	17	5	19	19	16
		TT	0	1	1	0	0	3	5	2	1
		T	5	8	10	0	8	6	14	11	9
		H_e	0.13	0.16	0.20	0	0.17	0.13	0.25	0.20	0.16
		p	1.00	0.53	1.00	0	1.00	0.007	0.05	0.67	1.00
3	rs17037397	AA	0	0	0	0	0	0	3	0	0
		AC	12	11	8	16	20	5	17	26	11
		CC	88	89	92	84	80	95	80	74	89
		A	6	5	4	8	10	3	11	13	6
		H_e	0.12	0.11	0.09	0.17	0.20	0.07	0.20	0.23	0.11
		p	1.00	1.00	1.00	1.00	1.00	1.00	0.24	0.13	1.00
4	rs7533315	CC	55	69	60	68	78	63	63	75	53
		CT	38	29	39	29	19	35	37	23	42
		TT	7	2	1	3	3	2	0	2	5
		T	26	16	21	17	13	19	18	13	26
		H_e	0.38	0.28	0.34	0.31	0.24	0.32	0.31	0.23	0.38
		p	1.00	0.74	0.18	1.00	0.21	0.67	0.06	0.72	0.36
5	rs4846052	CC	46	55	40	55	58	58	40	51	30
		CT	41	42	52	39	36	35	53	39	53
		TT	13	3	8	6	6	7	7	10	17
		T	34	24	34	25	23	24	34	29	43
		H_e	0.45	0.37	0.46	0.38	0.35	0.38	0.45	0.41	0.50
		p	0.33	0.12	0.23	1.00	1.00	0.73	0.13	0.68	0.47
6	rs1801133 (C677T)	CC	67	53	53	79	72	55	61	67	50
		CT	28	37	44	18	25	42	33	29	37
		TT	5	10	3	3	3	3	6	4	13
		T	19	28	24	12	16	24	23	18	31
		H_e	0.32	0.41	0.38	0.24	0.27	0.37	0.36	0.29	0.44
		p	0.26	0.36	0.14	0.41	0.62	0.48	0.75	0.57	0.10
7	rs6541003	AA	43	54	38	53	60	58	37	49	29
		AG	46	43	52	42	35	35	52	40	56
		GG	11	3	10	5	5	7	11	11	15
		G	34	24	36	26	23	24	32	31	43
		H_e	0.45	0.38	0.47	0.39	0.35	0.38	0.47	0.43	0.50
		p	0.85	0.12	0.34	1.00	1.00	0.73	0.47	0.44	0.28

Table 3 (Contd.)

1	2	3		5	6	7	8	9	10	11	12
8	rs2066462	CC	94	88	93	89	97	97	89	74	94
		CT	6	12	7	11	3	3	11	26	6
		T	3	6	3	5	2	2	6	13	3
		H_e	0.06	0.12	0.07	0.10	0.05	0.05	0.11	0.23	0.06
		p	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.13	1.00
9	rs1801131 (A1298C)	AA	44	62	34	58	60	58	46	51	40
		AC	35	35	54	37	36	35	48	38	48
		CC	21	3	12	5	4	7	6	11	12
		C	38	20	39	24	21	24	30	30	36
		H_e	0.38	0.33	0.49	0.37	0.33	0.38	0.43	0.42	0.47
		p	0.79	0.56	0.25	1.00	1.00	0.73	0.29	0.32	0.69
10	rs17375901	CC	98	96	89	97	98	94	94	94	91
		CT	2	4	11	3	2	3	6	6	9
		TT	0	0	0	0	0	3	0	0	0
		T	1	2	5	1	1	5	3	3	4
		H_e	0.02	0.04	0.11	0.03	0.03	0.11	0.07	0.06	0.09
		p	1.00	1.00	1.00	1.00	1.00	0.003	1.00	1.00	1.00
11	rs2274976 (G1793A)	AA	0	0	0	0	0	3	3	1	0
		AG	10	30	11	8	17	4	17	23	13
		GG	90	70	89	92	83	93	80	76	87
		A	5	15	5	4	8	5	13	13	6
		H_e	0.11	0.26	0.11	0.10	0.17	0.11	0.20	0.23	0.12
		p	1.00	0.12	1.00	1.00	1.00	0.003	0.24	1.00	1.00
12	rs1537516	CC	84	85	80	89	83	92	80	69	82
		CT	16	14	20	11	17	5	17	29	17
		TT	0	1	0	0	0	3	3	2	1
		T	8	8	10	5	8	6	13	16	10
		H_e	0.15	0.16	0.18	0.13	0.17	0.013	0.20	0.27	0.18
		p	1.00	0.53	1.00	1.00	1.00	0.007	0.24	0.53	0.34
Average H_o across 12 loci			0.21	0.24	0.27	0.19	0.21	0.18	0.28	0.27	0.27
Average H_e across 12 loci			0.23	0.23	0.26	0.19	0.21	0.20	0.27	0.27	0.23

Note: N – the number of individuals per sample; H_e – expected heterozygosity; H_o – observed heterozygosity; p – significance of goodness-of-fit to Hardy–Weinberg proportions. The statistically significant differences are indicated in bold.

replacement for valine in the enzyme catalytic domain. In individuals homozygous and heterozygous for the polymorphic allele, the *in vitro* activity of the enzyme is reduced by 70% and 35%, respectively. The 677T mutant allele frequency in world populations varies from total absence in the Dendi tribe to 55% in Spanish populations [28–30]. In Russia, the frequency of the 677T allele is 29% in residents of the Moscow region and 32% in residents of Siberia [31, 32]. In the examined populations, the frequency of this allele varies from 12% in the Ket population to 31% in the Russian sample.

The A1298C transition (rs1801131) resulting in the replacement of a glutamic acid residue by alanine in the enzyme regulatory domain is the second common polymorphism in the *MTHFR* gene. The enzymatic activity is reduced in individuals carrying the 1298C allele, although this reduction is not as significant as that in the ones carrying the 677T allele. According to some studies, MTHFR activity falls by 40–50% and a biochemical profile similar to that in homozygous carriers of the 677T allele is observed in compound heterozygous individuals [33]. The lowest frequency of the 1298C allele was detected in residents of Senegal (4%), whereas the highest frequency was detected in the Israeli and New Guinean populations (41%) [34, 35]. In Russians, the frequency of this allele varies from 24 to 38% [31]. In the populations under study, allele C occurs with a frequency ranging from 20% (in the southern

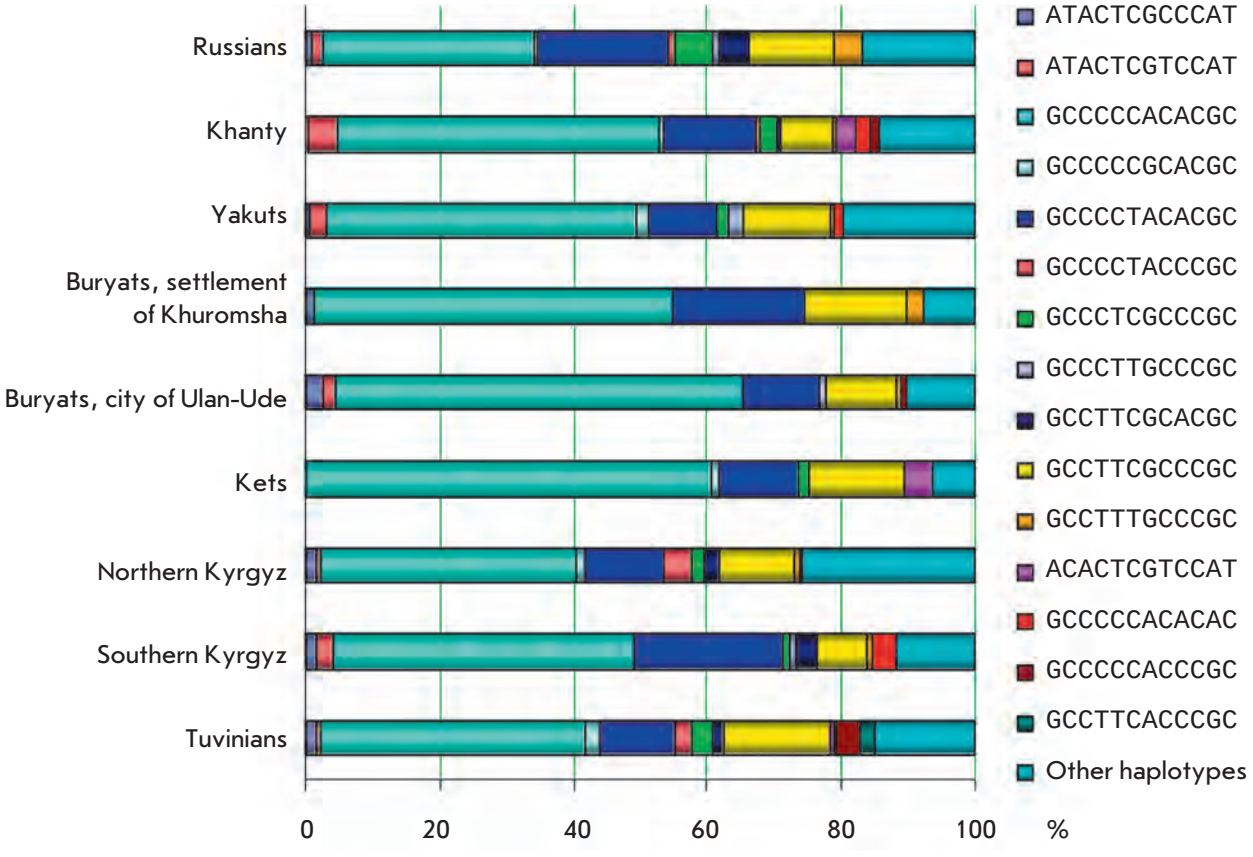


Fig. 2. The distribution of haplotypes in the populations studied.

Kyrgyzes) to 39% (in the northern Kyrgyzes).

It has recently been ascertained that another *MTHFR* gene SNP, rs2274976 (G1793A), impacts the HC level. This locus has been subjected to less investigation in comparison with C677T and A1298C. It is a known fact that the frequency of missense mutation G1793A varies from 1.3% in the Ashkenazi Jewish population to 26.6% in the Indonesian populations of Java [36]. It has been demonstrated that G1793A homozygosity results in an increase in the blood HC level by 40% [37, 38]. In the populations under analysis, the minimum frequency of allele A was observed in the Ket population sample, whereas the maximum frequency of the allele (15%) was observed in the southern Kyrgyz population.

A total of 160 haplotypes were detected in our samples; the theoretically possible number was 4,096. The maximum number of haplotypes was revealed in the Tuvinian population (47); the minimum number was revealed in the Ket population (11). A high level of haplotype diversity was observed in most samples, bar the Ket, Buryat, and southern Kyrgyz populations. If each mutation that resulted in the formation of a fixed polymorphic variant is assumed to be a unique event, and the mutation rate is assumed to be negligibly low, it should be postulated that only 12 haplotypes could be formed as a result of the mutation process. In this case, a considerable part of the haplotype diversity even in such a physically small genome region as an approximately 20-thousand-bp long *MTHFR* locus should have been formed due to the recombination events (see below).

The distribution of haplotypes occurring in the populations studied with a frequency of over 2% is presented in *Fig. 2*. The haplotypes with a frequency of more than 5% are referred to as the major ones. Three major haplotypes, GCCCCCACACGC, GCCCCTACACGC, and GCCTTCGCCCGC, were detected in all the populations studied, bar the Russian sample; the sum of their frequencies is more than 83% of the chromosomes observed in the Ket and Buryat populations, and more than 61% in the other samples. The degrees of haplotype diversity detected in the populations studied were different; nevertheless, all the samples contained identical major haplotypes, attesting to the fact that there can indeed be a common mechanism for the formation of the LD patterns.

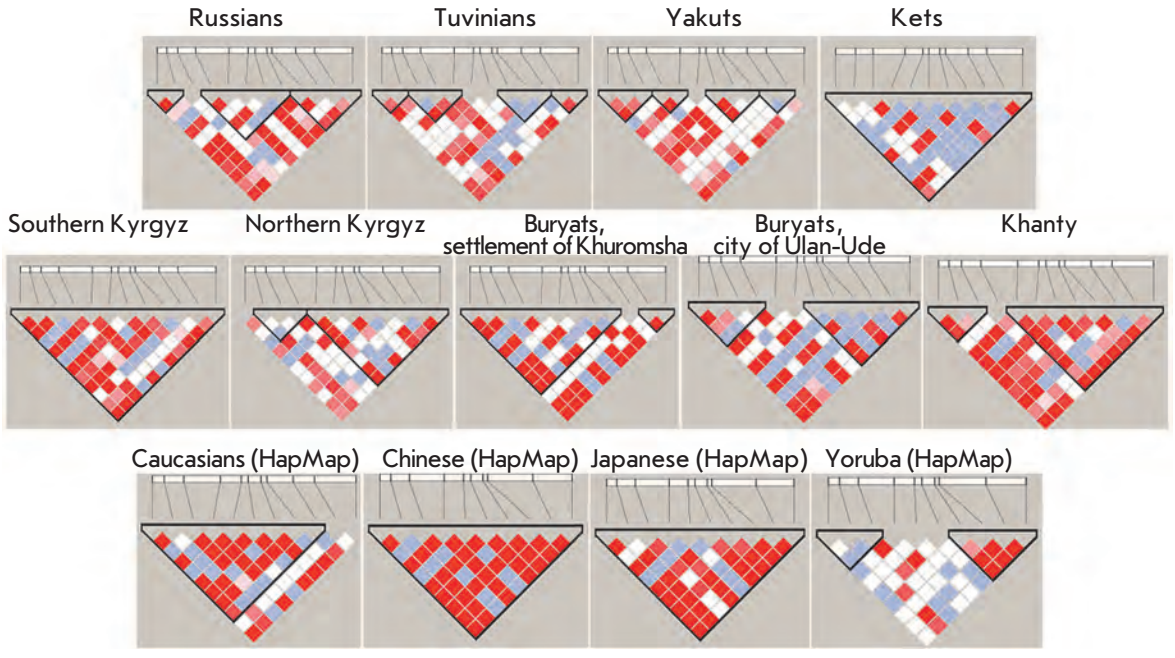


Fig. 3. The structure of linkage disequilibrium in the *MTHFR* gene in the populations studied. The color scheme shows the strength of adhesion between SNPs: bright red – a strong link (D'=1, LOD>2), red and pink – a significant link (D'<1, LOD>2), white – poor link (D'<1, LOD<2). The lilac cell denotes the impossibility to calculate the linkage disequilibrium due to the low frequency of the minor allele polymorphism (D'=1, LOD<2). Localization of SNPs for each population is as follows (from left to right): 1 – rs3753588, 2 – rs2066470, 3 – rs17037397, 4 – rs7533315, 5 – rs4846052, 6 – rs1801133, 7 – rs6541003, 8 – rs2066462, 9 – rs1801131, 10 – rs17375901, 11 – rs2274976, 12 – rs1537516 (there is no polymorphism rs3753588 in the populations from the HapMap project). In the Yoruba population, marker rs17375901 was excluded from the analysis because of its monomorphicity.

Architecture of linkage disequilibrium for the *MTHFR* gene in certain Eurasian populations

The structure of LD between the investigated loci of the *MTHFR* gene in 13 population samples is shown in *Fig. 3*. The maximum linkage between the SNPs studied was demonstrated for the southern Kyrgyz, Ket, Chinese, and Japanese populations. In these samples, all the allelic variants of the *MTHFR* gene under analysis belong to the same haplotype block. A single block was also observed in the Caucasian individuals from the HapMap project; however, it did not contain the rs2274976 and rs1537516 markers. Two blocks were detected in the northern Kyrgyz population: the first one comprising three SNPs (rs2066470, rs17037397, and rs7533315), the second one encompassing a 10-thousand-bp-long region and containing eight SNPs. Two blocks were also revealed in the Khanty population: the first one containing rs3753588, rs2066470, and rs17037397; the second one, identical to block № 2 in the northern Kyrgyz population. Strong linkage between the first nine SNPs belonging to the first block (9-thousand-bp long), as well as that between rs2274976 and rs1537516 forming the second small block were revealed in the Buryat population of the settlement of Khuromsha. A significant linkage between many polymorphic variants was also detected in the Buryat population of the city of Ulan-Ude; however, two different blocks (3- and 8-thousand-bp long) were represented in the LD structure of this population. Three blocks were observed for the Russian population: the first block was made up of two closely located SNPs (rs3753588 and rs2066470), the second block comprised five polymorphisms (rs7533315, rs4846052, rs1801133, rs6541003, and rs2066462), whereas the third block was made up of four SNPs (rs1801131, rs17375901, rs2274976, and rs1537516).

Four small haplotype blocks consisting of two or three neighbouring SNPs can be detected in the Tuvinian and Yakut populations. Two blocks were observed in the Yoruba populations: the first one consisted of 2 thousand bp and contained rs2066470, rs17037397, and rs7533315; the second block was appreciably long (7 thousand bp) and comprised four loci (rs2066462, rs1801131, rs2274976, and rs1537516). Thus, the haplotype blocks (with lengths varying from 847 bp to 16 thousand bp) were represented by several (3–6) major

haplotypes, the sum of which provided more than 90% of the chromosomes observed. The composition and size of the blocks varied depending on the population structure. It is particularly remarkable that the functionally significant C677T and A1298C polymorphisms were not linked in all the populations, bar the southern Kyrgyz and Ket samples, as well as populations from the HapMap project. It should also be noted that close linkage between the SNPs that form the second block in the Yoruba population was observed in many populations.

At the time of writing, the degree of haplotype diversity and length of linkage blocks in various genome regions have been identified in many human populations [39, 40]. A considerable variability of the haplotype structure was revealed; it was considerably different when passing from one genome region to another and frequently alternated with the regions with a low LD level. The length of haplotype blocks varied from 1 to 100 (and more) thousand bp [9]. Some researchers have reported on the consistent spatial distribution of the haplotype blocks in several human genome regions, referring to the common mechanism of formation of these blocks in various populations as the possible reason for this phenomenon [3, 41]. An African versus non-African dichotomy was also described in several segments of the human genome [42, 43], the haplotype blocks with the greatest length in non-African populations (> 44 thousand bp) in comparison with those in African populations (> 22 thousand bp) [9]. Patterns of meiotic recombination were revealed, attesting to the fact that the haplotype blocks are confined by the regions with a low LD level corresponding to those with high recombination indices [10, 44]. The relationship between the recombination frequency, chromatin structure, and the various structural and functional components of the nucleus were also assessed within the framework of intense research relating to the recombination in the human genome. It was demonstrated via this analysis that various potential factors, both local (DNA nucleotide sequence, chromatin structure) and the ones unrelated to the structure of the recombination site directly, may affect the recombination indices of a specific chromosome segment. An assumption was made that recombination is the major causal factor accounting for the formation of the linkage blocks resulting from the disintegration of long haplotypes in chromosome regions with a comparatively high recombination level. This hypothesis was confirmed by the fact that several genome regions in various populations possess an identical LD structure [45–47]. Meanwhile, data have been obtained attesting to a significant interpopulation variation in the degree and pattern of LD within the same genome region [10, 48–50]. These results attest to the fact that the pattern of LD revealed for a specific population or sample presumably cannot be automatically extrapolated onto other populations, at least in particular genome regions. It is unlikely that one common map of linkage disequilibrium in the genome will appear to be useful for the selection of genetic markers for performing association studies in a number of populations, since the interaction between various population-specific factors and genome-specific mechanisms upon the formation of the LD structure cannot be neglected.

The population-specific nature of the formation of LD patterns was confirmed in this study. The strongest linkage (all SNPs belong to the same block) between the loci under study was detected in the Ket, southern Kyrgyz, Chinese, and Japanese populations. Close linkage was also observed in the Buryat populations. Two explanations to the observed retention of block structure and length can be proposed: either common ancestors or positive selection; the latter frequently resulting in an increase in the length of the block containing a useful allele [51]. Since the minimum number of haplotypes among all the populations investigated was revealed in the Ket and Buryat samples, there is a probability that the ancestral effect could have taken place in this specific case. However, a number of other factors also affect the increase in the LD structure [49]; namely, genetic isolation, population subdivision or mixing, balancing selection, the bottleneck effect, small population size, and other reasons. The influence of the aforementioned factors on certain populations cannot be ignored.

It has been demonstrated that the length of LD patterns in the human genome determines the potential and design of association studies that use SNPs for the mapping of the genes underlying complex indices. According to the current estimations, the number of markers required for a LD-based genome scan of different populations varies from 120, 000 to several millions and is attestable to the following facts: the cost of genotyping is tremendous, and there can be problems with the validity of the statistical conclusion. An assumption was made that the number of markers required for CD mapping will be considerably lower in populations with a high degree of LD [52].

Selection of tagSNPs in the *MTHFR* gene

According to the estimations made by different researchers, the human genome contains more than 7.5 million common SNPs with a minor allele frequency (MAF) of at least 5% [21, 53], which are partly responsible for the inherited risk of developing many CDs. Today, tagSNP selection aimed at broadening the genetic coverage is one of the most efficient strategies for designing a genetic marker panel for ana-

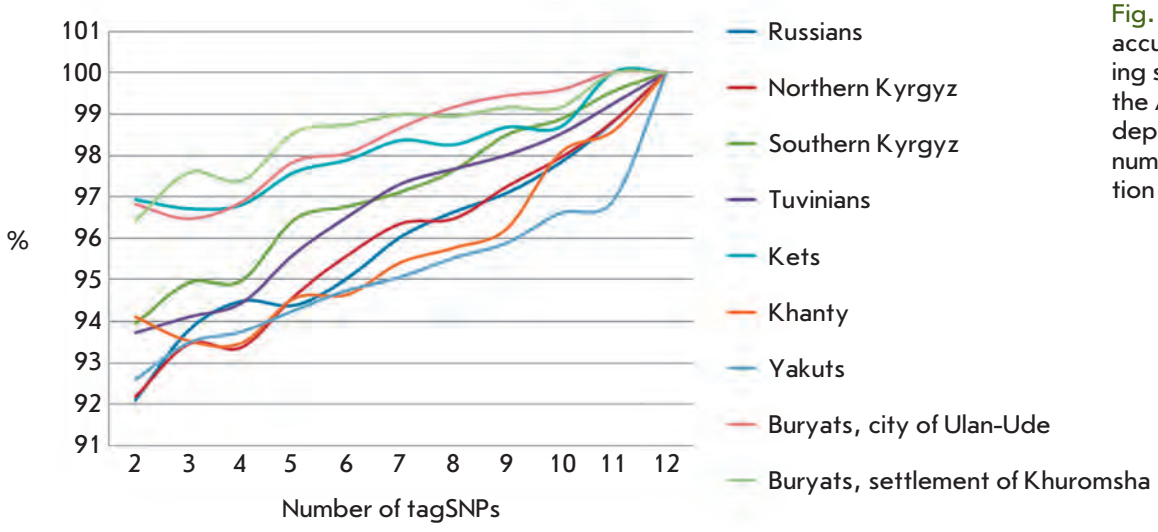


Fig. 4. Variability in accuracy in predicting set tagSNPs for the *MTHFR* gene, depending on their number and population sample.

lyzing the association with CD [54]. In this case, the gene coverage is defined as the percentage of the set of all common SNPs with MAF of 5% that demonstrate threshold correlation with at least one SNP from the specified polymorphism array [55]. The tagSNP approach has advantages due to knowledge of the LD block structure of the human genome. Moreover, this strategy considerably reduces effort and cost in genotyping [53, 56]. Because of the undeniable potential benefit of tagSNP selection for association studies, a proposition was made that tagSNPs be efficiently identified using various algorithms.

Two methods (namely, STAMPA and Tagger) were used in this study for tagSNP identification. The genotype data are used in the STAMPA method, whereas no data on the haplotypes and block architecture of the genome region under study are required [57]. This algorithm is based on the hypothesis that the correlation between SNPs tends to decrease as the physical distance between them increases; the allelic SNP variant can be determined based on knowledge of the allelic variants of the nearest tagSNPs from each side. A prognostic significance of more than 90% is achieved in all populations already when two tagSNPs of the *MTHFR* gene are selected (*Fig. 4*). This is presumably because the gene is small and the polymorphic sites are located physically close to each other. However, to achieve a prognostic significance of 99–100%, the number of tagSNPs needs to be considerably high. In our opinion, this fact is a significant drawback of this algorithm.

The tagSNPs in the *MTHFR* gene were also identified in this study using the Tagger algorithm provided by the HaploView software [21]. An aggressive tagging algorithm based on tagSNP identification in two- and three-marker haplotypes, within which all the polymorphic variants should be closely linked with one another (LOD > 3), was used in this method [26]. The tagSNPs detected using the aforementioned algorithm are listed in *Table 4*.

The prognostic significance of this set of tags with respect to the entire polymorphism array under study is equal to 100%. According to the results obtained, unequal tagSNP sets were observed in different populations at the specified threshold of prognostic significance, which was presumably caused by the variation of the LD structure and the haplotype diversity of the *MTHFR* gene within the samples. This fact was confirmed by the statistically significant correlation between the haplotype diversity and the variability of the number of tagSNPs ($r^2 = 0.85$; $p < 0.01$). The problem of the possible transfer of tagSNPs from one population to another is rather significant due to the considerable topicality of association analysis using tagSNPs identified on the basis of the HapMap project data. An appreciably high prognostic significance of tagSNP sets in several genome regions selected for CEU, CHB, and JPT with regard to the Caucasian and Mongoloid populations has been recently shown in a series of experiments [58–60]. Nevertheless, it has been determined that the extrapolation level of tagSNP decreases when the set of tagSNPs established for CEU populations is used in association studies in the African and several isolated Caucasian populations [26, 61, 62]. At the same time, it has been demonstrated that the most universal tagSNPs providing maximum genetic coverage in the other populations are found in the Yoruba population because of the minimum strength of LD in this sample [53].

A comparative analysis of the efficiency of STAMPA and Tagger algorithms depending on the level of

Table 4. *MTHFR* gene tagSNPs identified using the Tagger algorithm

Population	Number of tagSNPs	***MTHFR* gene SNPs**											
Russians	9	1	2	3	4	5	6	7	8	9	10	11	12
Northern Kyrgyz	11	1	2	3	4	5	6	7	8	9	10	11	12
Southern Kyrgyz	8	1	2	3	4	5	6	7	8	9	10	11	12
Tuvinians	11	1	2	3	4	5	6	7	8	9	10	11	12
Kets	8	1	2	3	4	5	6	7	8	9	10	11	12
Khanty	9	1	2	3	4	5	6	7	8	9	10	11	12
Yakuts	10	1	2	3	4	5	6	7	8	9	10	11	12
Buryats, city of Ulan-Ude	8	1	2	3	4	5	6	7	8	9	10	11	12
Buryats, settlement of Khuromsha	7	1	2	3	4	5	6	7	8	9	10	11	12

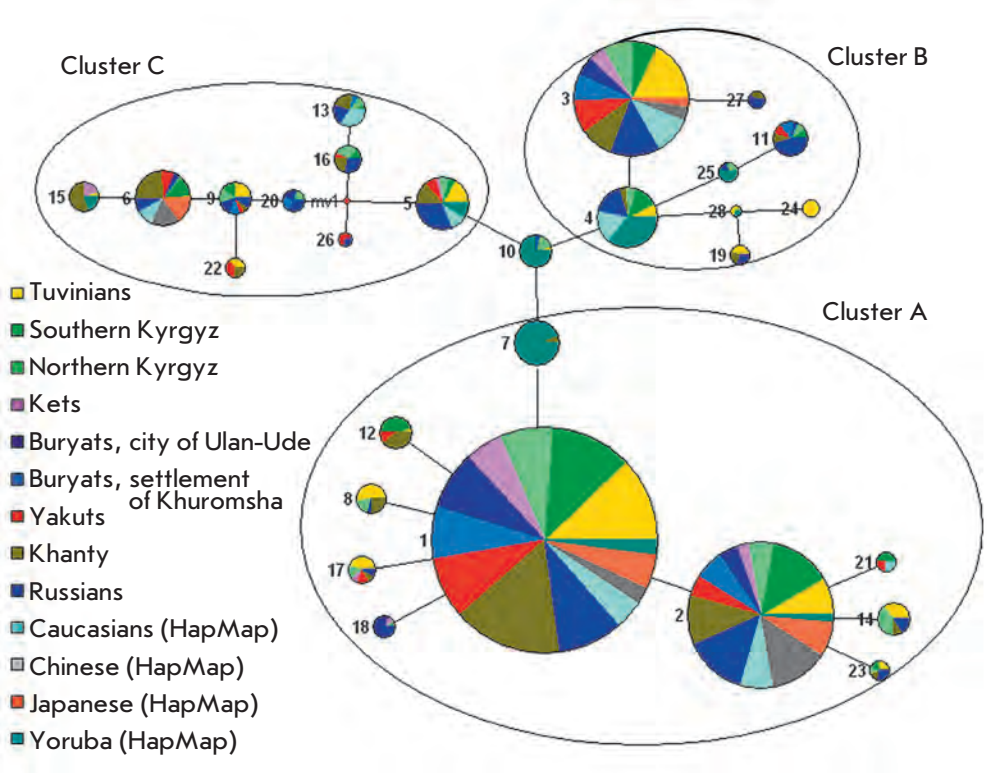
Note: The following SNP numeration is used: 1 – rs3753588, 2 – rs2066470, 3 – rs17037397, 4 – rs7533315, 5 – rs4846052, 6 – rs1801133, 7 – rs6541003, 8 – rs2066462, 9 – rs1801131, 10 – rs17375901, 11 – rs2274976, 12 – rs1537516. The *MTHFR* gene tag SNPs are shown in bold type at grey background.

Table 5. Comparative characteristics of STAMPA and Aggressive tagging algorithms for tagSNP determination

Population	90% prognosis accuracy		95% prognosis accuracy		98% prognosis accuracy		100% prognosis accuracy		Haplotype diversity	Number of blocks
	STAMPA	Tagger	STAMPA	Tagger	STAMPA	Tagger	STAMPA	Tagger		
	Number of *MTHFR* gene tagSNPs									
Russians	2	8	6	9	12	9	10	9	0.69	3
Northern Kyrgyz	2	10	6	11	12	11	10	11	0.77	2
Southern Kyrgyz	2	7	4	8	12	8	9	8	0.62	1
Tuvinians	2	10	5	11	12	11	9	11	0.82	4
Kets	2	7	2	8	12	8	10	8	0.49	1
Khanty	2	8	7	9	12	9	10	9	0.78	3
Yakuts	2	9	7	10	12	10	12	10	0.72	4
Buryats, city of Ulan-Ude	2	7	2	8	12	8	5	8	0.59	3
Buryats, settlement of Khuromsha	2	6	2	7	12	7	5	7	0.55	2

prognostic significance was carried out in this study. It is clear from *Table 5* that the minimum number of tagSNPs at a prognostic significance of 90–95% is determined using the STAMPA algorithm, whereas the Tagger method is more efficient at a prognostic significance of 98–100%.

TagSNPs are widely used in various genetic studies as a tool that efficiently represents genetic diversity. Nevertheless, the quality of the selected tagSNPs depends on the original array in which they were characterized. If the original marker density was low, the selected tagSNP will "capture" less information than is required for the analysis. The required marker density in the initial data array varies within different genome regions depending on a number of factors, such as the recombination level, LD structure, SNP frequencies, mutation character, and the demographic history of a population [17].



	Haplotype
№	1 2 3 4 5 6 7 8 9 10 11
1	C C C **C** C **A** C A C G C
2	C C C **C T A** C A C G C
3	C C **T** T C G C **C** C G C
4	C C **T** T C G C A C G C
5	C C C T C G C **C** C G C
6	**T A** C T C G **T C C A T**
7	C C C T C **A** C A C G C
8	C C C **C** C **A** C **C** C G C
9	**T A** C T C G C **C C A T**
10	C C C T C G C A C G C
11	C C **T** T **T** G C **C** C G C
12	C C C **C** C **A** C A C **A** C
13	**T** C C T C G **T C T** G **T**
14	C C C **C T A** C **C** C G C
15	C **A** C T C G **T C C A T**
16	**T** C C T C G C **C T** G **T**
17	C C C **C** C G C A C G C
18	C **A** C **C** C **A** C A C G C
19	C C **T C** C **A** C A C G C
20	**T** C C T C G C **C C A T**
21	C C C **C T A** C A C **A** C
22	C **A** C T C G C **C C A T**
23	C C C **C T** G C A C G C
24	C C **T** T C **A** C **C** C G C
25	C C **T** T **T** G C A C G C
26	**T A** C T C G C **C** C G **T**
27	C **A T** C G C **C** C G C
28	C C **T** T C **A** C A C G C

Fig. 5. The median tree of the haplotypes occurring with a frequency of more than 0.1% in the total sample. Mutant alleles are shown in bold letters; ancestral haplotype is denoted as №10. Node diameter represents haplotype frequency in the total sample. Numeration of SNPs in haplotypes is as follows: 1 – rs2066470, 2 – rs17037397, 3 – rs7533315, 4 – rs4846052, 5 – rs1801133, 6 – rs6541003, 7 – rs2066462, 8 – rs1801131, 9 – rs17375901, 10 – rs2274976, 11 – rs1537516.

Phylogenetic analysis of the relationships between haplotypes at the *MTHFR* locus and the assessment of the selective neutrality of the polymorphisms under study

The phylogenetic analysis of the relationships between the haplotypes, which are determined on the basis of diallelic markers and characterized by a frequency of occurrence in the total sample of more than 0.1%, were carried out by constructing phylogenetic trees (networks) of haplotypes using the median network algorithm implemented in the Network software. The haplotype consisting of ancestral alleles was used as the ancestral haplotype (the data was taken from the NCBI database). The results obtained attest to the fact that all the haplotypes observed in the human populations under analysis originated from a common ancestral variant, which occurs in the Yoruba population (with a frequency of approximately 12%) and in the Russian and southern Kyrgyz populations (with a frequency of 1%) (haplotype 10 in *Fig. 5*). This fact supports the hypothesis of the Recent African Origin of modern humans. The fact that haplotypes № 4 and 7, which are the closest ones to the ancestral variant, occur in the Yoruba population with a significant frequency provides further potency to this hypothesis.

All the haplotypes observed lie within six mutation steps from their common ancestor and can be subdivided into three major clusters, A, B, and C, which are formed from the CC**T**TCGCACGC, CCCTCGC**C**CGC, and CCCTC**A**CACGC haplotypes (№ 4, 5, and 7, respectively; *Fig. 3*). Cluster A is represented by 10 haplotypes, two of which (№ 1, 2) are the most widespread (their total frequency being more than 50% of the frequency of all haplotypes in the total sample) and were revealed in all the populations studied. It should be noted that the tree structure in this cluster is of a strongly pronounced star-shaped character, which obviously indicates a sudden population expansion in the demographic history. Cluster B contains eight haplotypes, including haplotype № 3, which is the third most frequently occurring haplotype and is represented in all the populations, bar the Yoruba sample. It is worthy of note that haplotypes № 8, 12, 17, 18, 21, 14, 23, 27, 11, 24, 19 of clusters A and B located at the branch tips

of the phylogenetic tree occur only in northern Eurasian populations and presumably emerged relatively recently. Haplotypes belonging to cluster C contain a large number of mutant alleles; they are likely to have been formed as a result of recombination events.

Since the SNP mutation rate and their diversity observed in the modern populations can be assessed, it is possible to calculate the time of origin of this haplotype lineage. It has been known that diversity assessments based on the phylogeny of DNA nucleotide sequences are independent of demographic processes [63] and suitable for determining the evolutionary age of genetic lineages. The term "age" is understood as the coalescence time (coalescence to the common ancestor); i.e., as the generation time of the diversity observed. With allowance for these facts, the variation of the alleles of the same SNP in the same locus was considered to be a mutation step in order to assess the haplotype coalescence time. An identical mutation rate ($1*10^{-8}$ mutations per locus per generation) was set for all the polymorphic variants [64]. The generation time was assumed to be equivalent to 20 years. The haplotype coalescence time was assessed using the Network software. In general, the age of diversity generation determined on the basis of 12 SNPs in the *MTHFR* gene was equal to 314,000±135,000 years. The median haplotype trees obtained by dividing the *MTHFR* gene into two blocks (the first one comprising rs3753588, rs2066470, rs17037397, rs7533315, rs4846052, rs1801133, rs6541003; and the second one comprising rs2066462, rs1801131, rs17375901, rs2274976, rs1537516) were analyzed in order to assess the accuracy of the results. This analysis was performed because of the fact that the specified regions of the *MTHFR* gene occur in different LD blocks in most populations, including the Yoruba sample. Therefore, an independent phylogenetic analysis of two blocks of the *MTHFR* gene may be less prone to the possible errors added because of recombination. Finally, the coalescence time for the first block turned out to be equal to 350,000±188,000 years; the age of the ancestral haplotype of the second block was assessed as 306,000±188,000 years.

Although the phylogenetic analysis that was performed is an appreciably powerful and efficient tool to characterize the evolutionary relationships between the haplotypes, it should be noted that particular care needs to be taken when interpreting the absolute assessments of the coalescence time (i.e., time expressed in years), since the key parameters underlying these assessments include the mutation rate and the absence of recombination within the genome region under analysis.

According to the results of a number of studies [65, 66], a relatively recent and rapid expansion of human populations from Africa left a considerable footprint on our genome by forming a structure of genetic variations in human populations, which is of biomedical significance, among other factors. It should be noted that the genomic variability causing the phenotypic difference between two individuals is only 0.1%. In fact, most of these variations in DNA should be evolutionary neutral; however, a great number of polymorphisms affecting the phenotype have been revealed, which can serve as selection objects or can be subjected to subsequent selection [65].

When analyzing the selective neutrality of *MTHFR* gene polymorphisms using the Ewens–Watterson test, neutrality deviation was detected only at rs4846052 and rs6541003 in the Russian samples (the F criterion observed for the Ewens–Watterson test was equal to 0.99 ($p = 0.039$) and 0.84 ($p = 0.041$), respectively) and the Caucasian populations from the HapMap project ($F = 0.79$ ($p = 0.021$) and $F = 0.98$ ($p = 0.030$), respectively). All three functionally significant SNPs causing an increase in the HC blood level turned out to be selectively neutral. This can presumably be attributed to the fact that even a certain variation in phenotype can be selectively neutral, provided that it does not affect reproductive efficiency [67]. Nevertheless, data concerning the selection of the 677T allele in the Spanish population have been reported. They were based on the study of the variation of the distribution of the frequencies of genotypes of alleles of the C677T polymorphism during the XX century. An increase in the number of individuals with the 677TT genotype has been noted in the population. This increase was caused by the fact that many women took folic acid during the second and third trimesters of pregnancy, resulting in an increase in the viability of the carriers of the 677T allele during early stages of embryogenesis [68]. Furthermore, the selective significance of the T allele was supported by the results of the analysis of the distribution of the frequencies of alleles, genotypes, and haplotypes of the C677T and A1298C polymorphisms in the *MTHFR* gene in the Israeli, Japanese, and African populations. According to these data, the 677T allele is found in the haplotypes with a selective advantage [69].

It is a known fact that the 677T allele frequency in world populations is very heterogeneous, varying from complete absence in the representatives of African tribes to 55% in Spanish populations [28–30]. Moreover, the allele frequency gradient is observed in Europe in the north–southward direction [70]. It has been demonstrated that the frequency of 677TT homozygous individuals in North America increases in the direction from Western Canada (Alberta) to the Southeastern United States (Atlanta), reaching a peak in Mexico [71]. The mechanisms of gradient

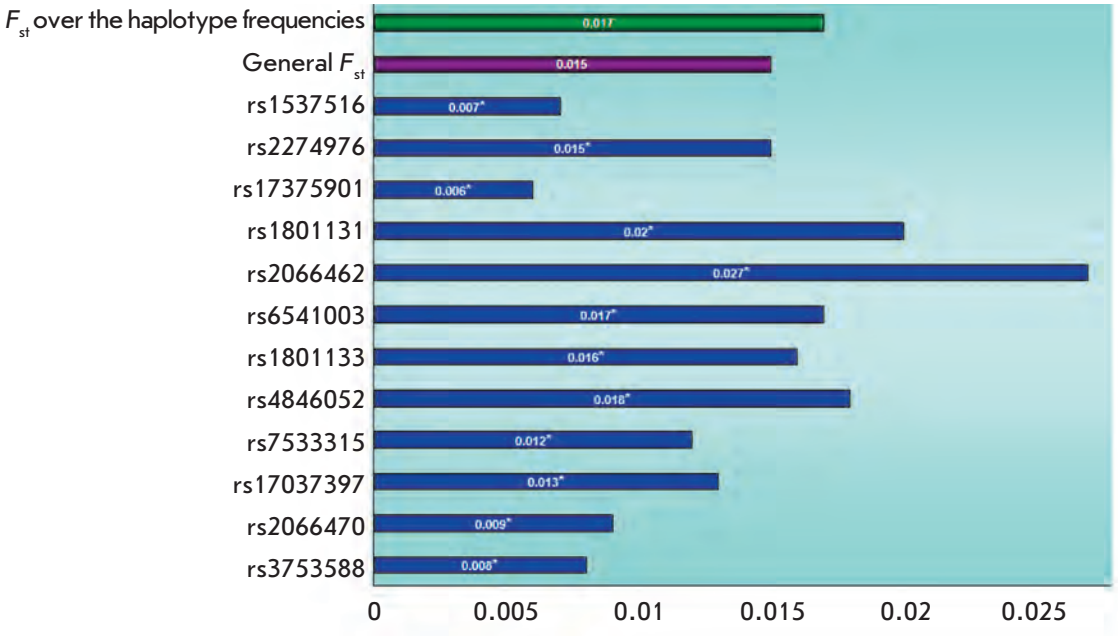


Fig. 6. The overall genetic differentiation of the total sample studied over the polymorphisms of the *MTHFR* gene. Note: * indicates a statistically significant difference ($p<0.05$).

generation have not as yet been reliably ascertained; however, there are at least three hypotheses, which are based on the assumption that the high 677T allele frequency is caused by the action of natural selection. The first hypothesis postulates that a decrease in MTHFR activity during the famine reduces homocysteine remethylation, thus saving monocarbon radicals in the tetrahydrofolate metabolism for essential DNA and RNA synthesis. According to the second hypothesis, the carriers of the mutant gene are less likely to develop colon cancer; therefore, the mutation frequency in the population may gradually increase [18]. The third hypothesis considers the gene–medium interactions between MTHFR and the folic acid content as the major factor for the accumulation of 677TT homozygous individuals in the population. Evidence supporting the latter theory was obtained in a number of experimental studies [34, 70, 71].

The role of selection pressure in the formation of LD patterns and the level of genetic diversity in populations was assessed using the standard statistical tests of neutrality proposed by Tajima and Fu [23, 24]. The value of Tajima's D test turned out to be negative in all the populations studied; however, it was statistically insignificant. The value of the Fu's *F*s test was negative and statistically significant in the populations of Tuvinians ($Fs = -11.28$, $p < 0.01$), northern Kyrgyz ($Fs = -24.15$; $p < 0.00001$), Yakuts ($Fs = -19.76$, $p < 0.00001$), and Khanty ($Fs = -10.31$, $p < 0.01$), attesting to either a possible effect of negative selection on a specific genome region in these populations or population expansion. However, the mixing of these populations with neighboring ones could have also resulted in an increase in DNA diversity and the *F*s test.

In general, the data obtained attest to the fact that stabilizing selection has an impact on the rs4846052 and rs6541003 loci in the Caucasian populations from the HapMap project and that the negative selection possibly affects specific haplotypes of the *MTHFR* gene in the populations of Tuvinians, northern Kyrgyz, Yakuts, and Khanty. It should be noted that these four populations are characterized by a higher level of haplotype diversity (more than 70%) and low LD level among all the groups under analysis.

Genetic differentiation and relationships between the populations under study

The data relating to the degree of gene differentiation (F_{st}) in the total sample with respect to each of the markers selected are presented in *Fig. 6*. It should be noted that all the polymorphic variants studied demonstrated a reliable differentiation. It has been shown that the differences in allele frequencies at the rs4846052, rs1801133, rs6541003, rs2066462, rs1801131, and rs2274976 loci contribute most to interpopulation diversity. The lowest degree of interpopulation diversity is typical of rs17375901. The level of genetic differentiation of the populations under study with respect to the allele frequencies of the 12 *MTHFR* gene SNPs under investigation was equal to 0.015, and equal to 0.017 with respect to haplotype frequencies. The assessment was carried out using the F_{st} coefficient.

The phylogenetic analysis of the genetic relationship between the populations was carried out based on

a data array for 13 populations. The resulting dendrogram showing the genetic relationships between the populations is presented in *Fig*. 7. Two major clusters can be isolated in the phylogenetic tree. The first cluster comprises the Yoruba, HapMap Caucasians, northern Kyrgyzes, Tuvinians, and Yakuts; the second cluster consists of the Khanty, Ket, Buryat, southern Kyrgyz, Japanese, and Chinese populations. This approach reveals the considerable differences between the gene pools of the Caucasian (Russians, HapMap Caucasians) and Mongoloid (Japanese, Chinese, Buryats, southern Kyrgyzes) populations, as well as the close genetic relationship between the Yoruba and HapMap Caucasian populations, and the Chinese and Japanese populations. It is of interest that the northern and southern Kyrgyz are located in different clusters at a significant genetic distance. The resolution capacity of the tree constructed based on only 11 loci is insufficient to make any definite conclusions relating to the details of the genetic relationships between the populations under study and only attests to the degree of genetic variations between them with respect to the *MTHFR* locus.

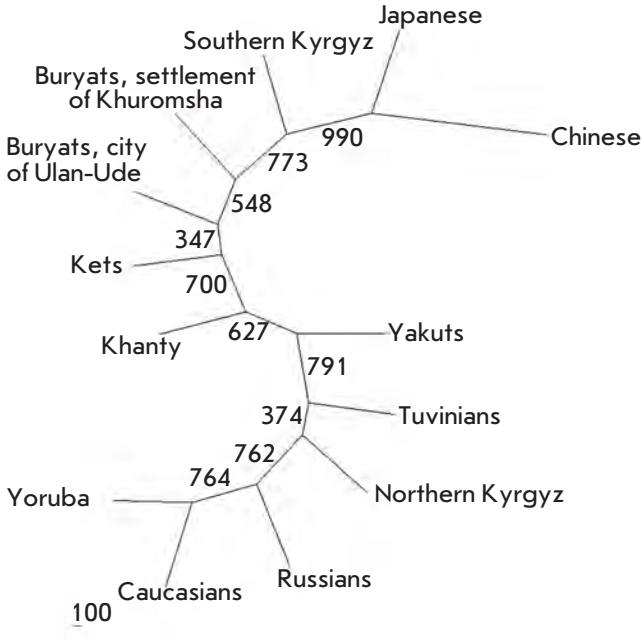


Fig. 7. Dendrogram of the genetic relationships between the populations studied. The length of the branches of the phylogenetic tree corresponds to genetic distances.

CONCLUSIONS

This investigation of the architecture of linkage disequilibrium of the *MTHFR* locus in nine populations inhabiting northern Eurasia was based on a conception assuming that the human genome has a block structure. The data relating to the Caucasian, Chinese, Japanese, and Yoruba populations obtained in the HapMap project were used as a basis for population comparisons.

A population-specificity of the LD structure of the *MTHFR* gene in various ethnoterritorial groups inhabiting northern Eurasia was revealed in this study. In addition, similarity in LD architecture was detected for certain populations, attesting to the role of evolutionary history in the organization of the block structure of LD.

Different degrees of haplotype diversity were established for the populations under study; nevertheless, identical major haplotypes were identified in all the samples with the exception of the Yoruba population, attesting to the fact that there may have been a common mechanism of formation of LD patterns in the *MTHFR* gene. The phylogenetic analysis of haplotypes showed that all the haplotypes observed in the populations under study originated from a common ancestral variant, thus attesting to the significant role of recombination in the generation of genetic diversity in the *MTHFR* locus and the possibility of a sudden population expansion. The age of generation of the diversity with respect to 12 SNPs of the *MTHFR* gene was 314,000±135,000 years.

Data supporting the impact of stabilizing selection on the rs4846052 and rs6541003 loci in HapMap Caucasian populations and that of negative selection on certain haplotypes of the *MTHFR* gene in populations of Tuvinians, northern Kyrgyz, Yakuts, and Khanty, which are characterized by the highest level of haplotype diversity (over 70%) and a low level of LD among all the groups under study, were also obtained in this work. All the investigated SNPs of the *MTHFR* gene showed reliable differentiation. Data on separate loci demonstrated that the variations with respect to allele frequencies at loci rs4846052, rs1801133, rs6541003, rs2066462, rs1801131, and rs2274976 contribute most to the interpopulation diversity. The lowest degree of interpopulation diversity is typical of the rs17375901 marker, which is monomorphic in the Yoruba population.

Thus, the architecture of LD in the human genome, and in the *MTHFR* locus in particular, is of a population-specific character and is to a significant extent determined by the evolutionary history of the population. It is obvious that the ethno-specific variability of haplotype blocks should be taken into consideration when analyzing the structure of LD and selecting tagSNPs during genetic mapping of common diseases both at the whole-genome level and for association studies, when a disease-associated variant is detected on the basis of its linkage with the nearest sites in a relatively narrow ge-

nome region. Subsequent investigation of the character of the genetic diversity and linkage disequilibrium in the genome of specific geographical, ethnic, or population groups will enable to reconstruct the genetic history of populations and detect the footprints of the natural selection associated with the adaptive variability. ●

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Hansenula Polymorpha TERT: A Telomerase Catalytic Subunit Isolated in Recombinant Form with Limited Reverse Transcriptase Activity

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ABSTRACT **Telomerase is a ribonucleoprotein, the main function of which is to synthesize telomeres, i.e. repetitive sequences which are localized at the ends of eukaryotic chromosomes. Telomerase maintains the stability of the genome in eukaryotic cells by replicating chromosomal ends. The structural and functional investigation of the telomerase complex is significantly restricted due to difficulties connected with the isolation of its main catalytic subunit in recombinant form. Herein, we describe a method developed for the isolation of the recombinant telomerase reverse transcriptase from thermotolerant yeast *Hansenula polymorpha*. A functional test performed for the isolated protein and the RNA/DNA duplex, simulating the interaction of telomerase RNA and telomere, reveals that the isolated catalytic subunit of telomerase possesses limited reverse transcriptase activity.**
KEYWORDS: telomerase reverse transcriptase; recombinant proteins; thermotolerant yeast *Hansenula polymorpha*.

INTRODUCTION

Telomerase is a ribonucleoprotein, the main function of which is to synthesize telomeres, i.e. repetitive sequences that are localized at the ends of eukaryotic chromosomes, unable to replicate in accordance with the classical replication mechanism. Telomerase exhibits its activity in cells capable of infinite division, such as germinal and stem cells, as well as in the majority of malignant tumors (85%). It is believed that the inhibition of the telomerase catalytic function halts the maintenance of the telomere length, thereby eliminating the infinite replication potential of tumor cells. Altogether, it allows telomerase to be considered as a universal target for various antitumor drugs [1]. The main components of telomerase are the protein, the telomerase reverse transcriptase (TERT), and the telomerase RNA, the matrix of which serves as a template for the synthesis of the telomere sequence [2]. One of the main difficulties in studying telomerase is the low stability of its catalytic subunit isolated in recombinant form [3]. The absence of data on the structure of telomerase prevents the docking of known substances with a view to searching for the potential effectors of this enzyme, as well as the difficulties attached to isolating the full-length functional telomerase reverse transcriptase, which thus impede the testing of the interactions between pharmacological agents and the target. At the time of writing, the TERT isolated from *Tribolium castaneum* is the only full-length telomerase reverse transcriptase that has been isolated [4]. The distinctive feature of this protein is the absence of the N-terminal domain typical for other telomerase reverse transcriptases. Data on the structure of the N-terminal domain in the telomerase catalytic subunit of *Tetrahymena thermophile* and its RNA-binding domain have been obtained [2, 5].

The use of thermophilic organisms is promising for structural and functional studies of proteins, since these organisms possess a more compact spatial organization, which facilitates its stabilization in a solution. Earlier, we identified the telomerase reverse transcriptase of thermotolerant yeast *Hansenula polymorpha* (hpTERT) and have shown for the first time that hpTERT can be expressed in the cells of *Escherichia coli* and that the recombinant protein can be isolated [6]. We have developed a method for the effective isolation of hpTERT expressed in *E. coli*. Specific expression constructions were used, enabling the production of the telomerase catalytic subunit with various affinity tags at either the C- or N-terminus of the protein. It was shown that the optimal vector for the expression and isolation of hpTERT is pET30aTEV, in which the open reading frame encodes hpTERT with 6His- and S-tags at the N-terminus. A test was performed that served to confirm the exhibition of reverse transcriptase activity by this

protein, thus proving that it is suitable for functional and structural studies. We believe that this report will be useful not only for researchers who study telomerase, but also for those who face problems in obtaining recombinant proteins that are unstable in their soluble form.

EXPERIMENTAL

Cloning of the *hpTERT* Gene into Various Expression Systems

The *hpTERT* gene was cloned using the following primers: 1) BamH1a/E2 (5'-aaggatccaaggtttgatcagtatgttgatga-3') and E2/Pst1/Rev (5'-tttctgcagttagaatgctttaagaagcga-3') for obtaining the pCDF plasmid, in which hpTERT is fused with a 6His tag at the N-terminus; 2) Nco1E2Fwd (5'-aaaaaccatgggaaggtttgatcagtatgttgat-3') and E2Sal1Rev (5'-tttttgtcgac gaatgctttaagaagcgaac-3') for obtaining pET33b+, in which hpTERT is merged with a 6His tag at the C-terminus; 3) HpET30F (5'-gacggagctcgaattttattagaatgctttaagaagcgaac-3') and HpET30S (5'-gtattttcagggcgccatgaggtttgatcagtatgttgat-3') for obtaining the pET30aTEV, in which hpTERT is merged with 6His- and S tags at the N-terminus. The pET30aTEV was kindly provided by Daniela Rhodes (MRS LMB, Cambridge, United Kingdom). DNA sequencing was carried out using a set of ABI PRISM® BigDye™ Terminator v. 3.1 reagents, followed by an analysis of reaction products by means of an Applied Biosystems 3730 DNA Analyzer.

Isolation and Purification of Recombinant hpTERT

E. coli BL21DE3 cells transformed with either a pCDF_hpTERT plasmid or pET33b_hpTERT or pET30_hpTERT were cultivated at 37°C until an optical density of 0.1–0.3 (OD_{600}); following this, the expression of the protein was induced by 0.1 mM isopropyl-thio-β-D-galactoside (IPTG) and the mixture was incubated upon stirring for 12–16 h at 16°C. The cells were collected via centrifugation at 5000 rev/min and cooled by liquid nitrogen; they were then disintegrated using a dismembrator (2000 rev/min, twice, for 30 sec each); the latter provided less denaturation of the protein during the isolation process. Disintegrated cells were re-suspended in buffer A: 50 mM NaH_2PO_4 (pH 7), 200 mM NaCl, 10% glycerol, 10 mM β-mercaptoethanol, and 0.05% Tween-20. Cell debris were separated by centrifugation at 15000 rev/min for 20 minutes. A cell lysate was then incubated with Ni-NTA-agarose for 30 min at 4°C; an affinity sorbent was separated from the unbound protein fraction by centrifugation at 3000 rev/min, followed by decantation of the supernatant. Ni-NTA-agarose was washed three times with buffer A containing 50 mM imidazole. The hpTERT protein bound to the affinity sorbent was eluted with buffer A containing 300 mM imidazole.

During additional purification by ion-exchange chromatography on SP-sepharose, the sample obtained in the previous stage was diluted to a total concentration of salts of 150 mM, and SP-sepharose pre-equilibrated in buffer B (50 mM NaH_2PO_4 (pH 7), 100 mM NaCl, 10% glycerol, 10 mM β-mercaptoethanol, and 0.05% Tween-20) was added. The bound protein fraction was washed away using a gradient of NaCl concentration (0.1–1 M) in an analogous buffer. Glycerol (up to 30%) was added to the sample; the latter was subsequently frozen under liquid nitrogen and stored at –80°C.

Testing of the Functionality of Purified hpTERT *in vitro*

The functionality of hpTERT was tested in a system containing 50 mM Tris-HCl, 1 mM DTT (dithiothreitol), 1 mM spermidine, 50 μM dCTP, 5 μM substrate (5'-cgccaccccgccaccc-3' RNA oligonucleotide and 5'-cgccaccccgccaccc-3', 5'-ggcgggcggggtg-3' DNA oligonucleotides were used), 3.75 μM [α-^{32}P] dGTP (800 Cu/mmol), and 5 μM hpTERT. Duplexes (DNA-DNA or DNA-RNA) were formed by hybridization of corresponding oligonucleotides. The reaction was performed at a temperature of 37°C for 30 min; the mixture was then treated with protein kinase K (0.3 mg/ml) and re-precipitated in alcohol. The products of the reaction were separated by gel electrophoresis in a 15% denaturing polyacrylamide gel (PAAG). Radioactive signals were detected by means of the Phosphorimager system.

RESULTS AND DISCUSSION

The gene of the hpTERT protein was cloned under the control of the T7 promotor into the following three expression systems, with the purpose of further isolation of the protein from *E. coli* cells: 1) pCDF with a 6His tag at the N-terminus of the hpTERT protein; 2) pET33b+ with a 6His tag at the C-terminus of the hpTERT protein; and 3) pET30aTEV with 6His- and S tags at the N-terminus of the hpTERT protein. Such location of tags, *i.e.* from different sides of the protein, relates to the ability of the amino acid sequence termini to fold inside a protein globule; the latter is most probably one of the reasons behind the decrease in the effectiveness of affinity chromatography. The S tag is a short sequence (4 kDa) which can be used for the stabilization of proteins in a solution. The expression of proteins was induced by IPTG; the proteins were purified by metal-chelate chromatography on Ni-NTA-aragose. The results on the isolation of the proteins expressed by using various constructions are shown in *Fig. 1*. The hpTERT protein is detected in all the samples eluted with

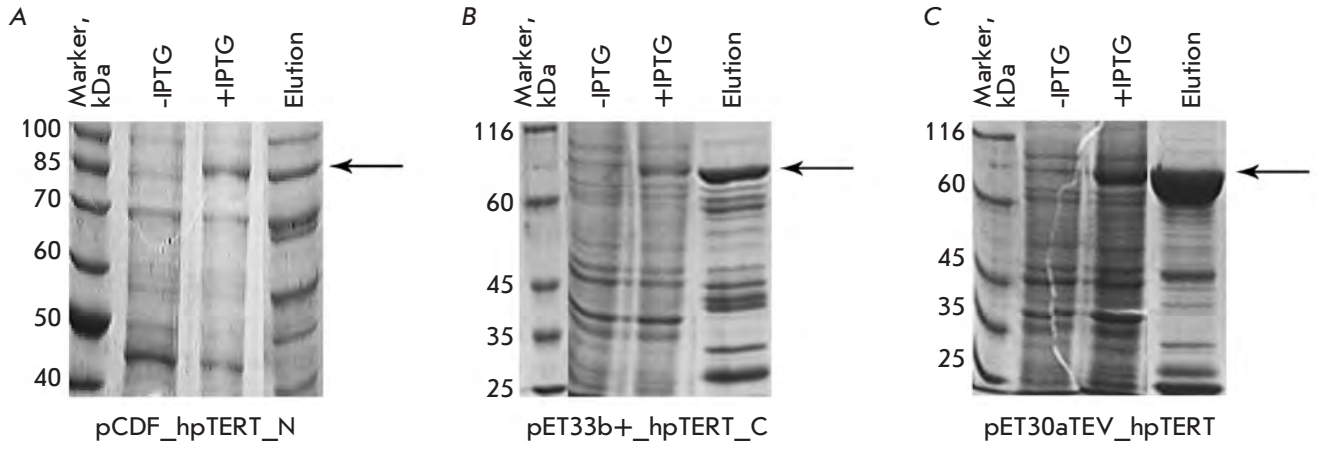

Fig. 1. Results of the expression and affinity isolation of recombinant hpTERT from *E. coli* cells transformed with different plasmids: *A* – the hpTERT is cloned into the pCDF vector with a 6His tag at its N-terminus; *B* – hpTERT is cloned into the pET33b+ vector with a 6His tag at its C-terminus; *C* – hpTERT is cloned into the pET30aTEV vector with 6His- and S tags at its N-terminus. The samples of *E. coli* cells before and after IPTG induction of expression and the sample of elution of hpTERT from Ni-NTA agarose were analyzed by denaturing PAGE electrophoresis. The zone corresponding to the mobility of the hpTERT protein in the gel is indicated by an arrow.

Ni-NTA-agarose. The latter indicates that the selection of thermotolerant yeast as a source for the production of the telomerase catalytic subunit was successful. However, in the cases occurring when the pCDF and pET33b+ vectors are used and a tag is located at the N-terminus or C-terminus or at both (*Figs. 1A, 1B*), a significant amount of impurities are detected on the resin, along with a target protein. It should be noted that the amount of impurities relative to the amount of the target protein is lower when the pET33b+ with a 6His tag at the C-terminus of the protein is used; in all likelihood, this reflects the close orientation of the N-terminus in hpTERT. The isolation of the protein with the help of the S tag (the pET30aTEV construction) gives a much better result (*Fig. 1C*). Apparently, a well-structured short N-terminal S tag significantly enhances the stability of the protein soluble form. The protein was isolated and additionally purified by ion-exchange chromatography on SP-sepharose (*Fig. 2*). The final characteristics of the protein sample obtained are the following: the concentration is 5 mg/ml, the yield is 5 mg/L of the *E. coli* cell culture, and the content of impurities is not more than 1%.

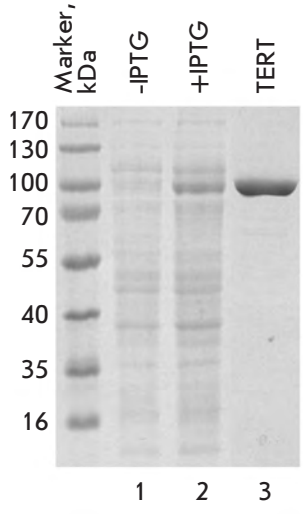

Fig. 2. The expression, isolation, and purification of hpTERT. Gel electrophoresis of the proteins from *E. coli* cells transformed with plasmid pET30aTEV_hpTERT without IPTG induction (-IPTG) and after IPTG induction of the hpTERT expression (+IPTG). The hpTERT sample corresponds to the protein obtained by affinity purification of cell lysate, followed by ion-exchange chromatography.

The functionality of the obtained protein was confirmed with the help of a constructed *in vitro* system. *H. polymorpha* telomeres consist of 18–23 repeats (5'-GGGTGGCG-3') [7]. On the basis of these data, a composition of the telomerase RNA fragment can be suggested and the DNA oligonucleotide representing a telomere can be modeled. Thus, the system contained the purified recombinant telomerase catalytic subunit, the substrate that is a hybrid RNA/DNA duplex with a free 3'-terminus (*Fig. 3A*), the mixture of nucleotides in which [α-^{32}P]dGTP was used for the visualization of oligonucleotide elongation. Either the analogous DNA/DNA duplex or the single-stranded telomerase DNA was used as a control. Since the telomerase catalytic subunit is a reverse transcriptase, such substrates cannot be used by hpTERT for elongation. Moreover, each reaction was performed in the presence and the absence of hpTERT (*Fig. 3B*). In lane *1* (*Fig. 3B*), a specific signal corresponding to the bind-

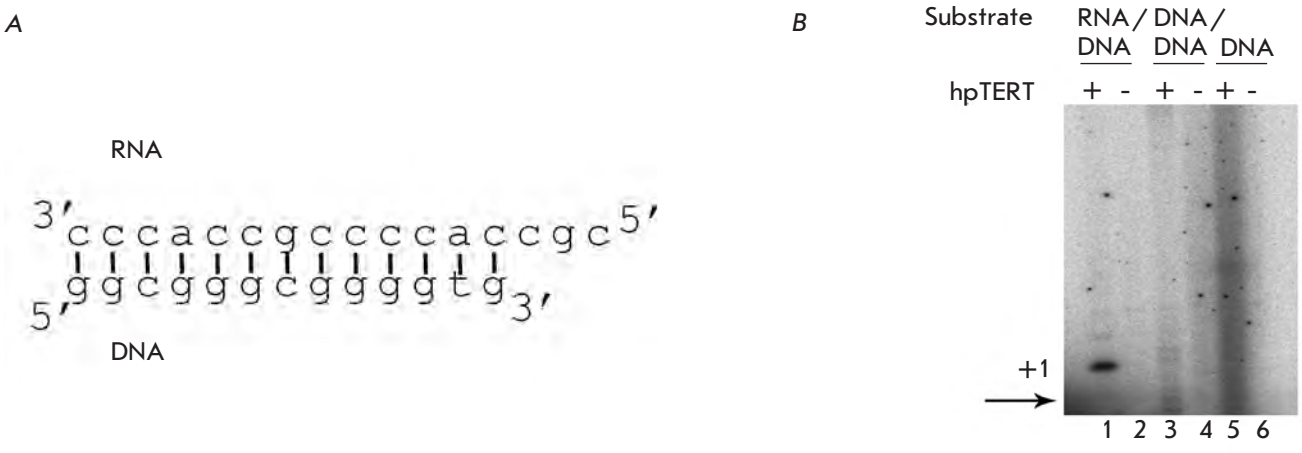

Fig. 3. Recombinant hpTERT isolated from *E. coli* cells transformed with the pET30aTEV vector acts as a reverse transcriptase. *A* — Scheme of the RNA/DNA duplex, which is used as a substrate for hpTERT; *B* — Products of the reaction of hpTERT (the presence of the protein is marked by +) and different substrates (the type of the used substrate is indicated above the picture). Products of the reaction are visualized via the introduction of radioactively labeled dGTP into the reaction mixture. The zone which corresponds to the mobility of the initial DNA oligonucleotide is marked by an arrow. The primer extension is present only in the first lane; this proves that recombinant hpTERT acts as a reverse transcriptase in this model system.

ing of dGTP to the DNA oligonucleotide in the RNA/DNA duplex can be observed. This zone is absent in systems with other substrates and in the absence of the protein, thereby eliminating the participation of the *E. coli* polymerases in this reaction. The RNA/DNA duplex used in this reaction was constructed so that the binding of three nucleotides was possible in the system. The signals corresponding to the binding of the second and third nucleotides can be seen in lane *1* (*Fig. 3B*), despite the fact that their intensity is much lower. In all likelihood, this is associated with the absence of the full-length telomerase RNA in the system, which is required for the reconstruction of telomerase activity *in vitro*. Nevertheless, the binding of even one nucleotide indicates that the protein has a specific reverse transcriptase activity, and that its functional structure is preserved.

Thus, the pET30aTEV construction with hpTERT, in which the 6His- and S-tags are located at the N-terminus of the protein, can be used for the production of the recombinant functional catalytic *H. polymorpha* telomerase subunit. This opens up new opportunities for the determination of the structure of the telomerase reverse transcriptase and the study of its functional mechanism. ●

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Conformational Differences between Active Angiotensins and Their Inactive Precursors

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ABSTRACT **The peptide conformation in the context of a protein polypeptide chain is influenced by proximal amino acid residues. However, the mechanisms of this interference remain poorly understood. We studied the conformation of angiotensins 1, 2 and 3, which are produced naturally in a sequential fashion from a precursor protein angiotensinogen and contain an identical peptide core structure. Using the example of angiotensins 1, 2 and 3, it was shown that similar amino acid sequences may have significant conformational differences in various molecules. In order to assess the conformational changes, we developed a panel of high-affinity mouse monoclonal antibodies against angiotensins 1, 2 and 3 and studied their cross-reactivity in indirect and competitive ELISAs. It was found that the conformations of inactive angiotensin1 and the corresponding fragment of angiotensinogen are similar; the same is true for the conformations of active angiotensins 2 and 3, whereas the conformations of homologous fragments in the active and inactive angiotensins differ significantly.**
KEYWORDS **peptides; conformation; angiotensins; angiotensinogen; monoclonal antibodies.**
ABBREVIATIONS **Ang1 – human angiotensin 1; Ang2 – human angiotensin 2; Ang3 – human angiotensin 3; ELISA – enzyme-linked immunosorbent assay; PAG – polyacrylamide gel; Hsp70 – heat shock protein with a molecular mass of 70 kDa; K_d – dissociation constant.**

INTRODUCTION

Since the appearance of the hybridoma technology in 1975 [1], a large number of monoclonal antibodies to various substances have been obtained. Despite this, new antibodies are still highly sought; namely, antibodies with particular properties, antibodies to particular epitopes and to newly discovered proteins and other organic and inorganic compounds. Normally, it is possible to obtain new proteins only in limited amounts, and frequently it is very difficult or even impossible to isolate them in their pure form with the natural conformation preserved. These kinds of proteins cannot be used for immunization to obtain antibodies; hence, in the majority of cases, the only viable option is immunization with synthetic peptides corresponding to particular fragments of the desired protein. Along with the apparent advantages, this approach also has a number of shortcomings: *i.e.*, peptides, when found in proteins, have a significantly lower degree of freedom than when they are in a free state. As a result, the antibodies against peptides are not always capable of binding to full-size proteins [2].

The oft-cited example of the structural differences between peptides found in their protein precursor and peptides in a free state is human angiotensinogen and its metabolites; *i.e.*, angiotensins 1, 2 and 3. Angiotensin 1 (Ang1) is a prohormone that consists of 10 amino acid residues and is produced from angiotensinogen as a result of the cleavage of the N-terminal peptide [3]. The Ang1 exhibits no physiological activity and plays the role of a substrate in the formation of angiotensins 2 and 3. Angiotensin 2 (Ang2) differs from angiotensin 1 by the absence of two C-terminal amino acid residues. Angiotensin 3 (Ang3) is shorter than angiotensin 2 by one N-terminal residue (*Figure*). Ang1 contains the same amino acids as Ang2; however, Ang1 is incapable of binding to the receptors of Ang2 and thereby cannot initiate effector functions [4]. The most likely causal factor behind this phenomenon is the conformational differences between angiotensins 1 and 2. To confirm this hypothesis, we obtained monoclonal antibodies against angiotensins 1, 2 and 3 and studied their cross-reactivity to various angiotensins and angiotensinogen.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Angiotensinogen	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu	Leu	Val	Tyr	Ser
Angiotensin 1	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu				
Angiotensin 2	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe						
Angiotensin 3		Arg	Val	Tyr	Ile	His	Pro	Phe						

Amino acid sequences of the precursors of angiotensin 2 and its metabolites.

EXPERIMENTAL

In this work human recombinant angiotensinogen ("Sigma", USA), angiotensins 1, 2 and 3 ("American Peptide", USA), recombinant Hsp70 from *Mycobacterium tuberculosis* obtained in our laboratory [5], BALB/c mice, and the mouse myeloma cell line Sp2/0 were used.

The Production of Monoclonal Antibodies against Angiotensins 1, 2, and 3

Mice were immunized in their hind paws with angiotensins conjugated with Hsp70: an adjuvant protein from *M. tuberculosis*, as described in [6]. The procedure was carried out twice with an interval of two weeks with a dosage of 100 μg of the conjugate per one immunization. The first immunization was performed using Freund's complete adjuvant and the second through Freund's incomplete adjuvant. On the third day following the second immunization, popliteal lymph node cells were hybridized with sp2/0 myeloma cells in accordance with the standard procedure [1]. The supernatants of the hybrids were tested by means of indirect [7] and competitive enzyme-linked immunosorbent assays (ELISAs) [8]; positive clones were cloned 2–4 times, monoclonal antibodies were produced in ascitic fluids of mice and were isolated by affine chromatography on protein G-sepharose [9]. The purity of the antibodies was controlled by electrophoresis in 12% polyacrylamide gel as described in [10].

Characterization of Monoclonal Antibodies Produced

The specificity of the obtained antibodies was determined by means of indirect and competitive ELISAs [7, 8]. The affinity of the antibodies against each target was assessed via the measurement of the dissociation constant (K_d) as described in [11] by Klotz, with modifications made by Friguet [12].

RESULTS AND DISCUSSION

The human and mouse angiotensins 1, as well as angiotensins 2 and 3, have identical amino acid sequences [13]. In addition, angiotensins 2 and 3 exhibit physiological activity and their introduction into the body at doses needed for immunization (10–50 μg/mouse) leads to a fatal outcome even in the case of intramuscular and subcutaneous injections. Altogether, it makes angiotensins extremely inconvenient immunogens; however, their conjugation with Hsp70, an adjuvant protein from *M. tuberculosis*, allowed to overcome the immunological tolerance and to eliminate the toxicity. As a result, we obtained monoclonal antibodies against each angiotensin.

The specificity of each antibody produced was determined by enzyme-linked immunosorbent assay (*Table 1*). Indirect ELISA revealed interaction between the antibodies and the sorbed targets. In the aforementioned system, part of the structural units of angiotensinogen and peptides is found inaccessible to antibodies and the other part is distorted. Through the competitive ELISA, we established the interaction of the antibodies with the protein and peptides in the single-phase system, *i.e.*, a solution; in turn, the determination of the dissociation constants allowed us to quantitatively estimate the interaction force (*Table 2*).

The most affine antibody obtained against angiotensin 1, AngC11 ($K_d = 1.3 \times 10^{-10}$), almost does not bind to sorbed Ang1 and also interacts with angiotensins 2 and 3 in neither indirect nor competitive ELISA. At the same time, AngC11 recognizes angiotensinogen both in the sorbed form and in solution. All these factors indicate that either the epitope of this antibody contains amino acid residues that detach when angiotensins 2 and 3 are formed, or that the structures of this fragment in Ang1, Ang2, and Ang3 differ to the extent that the antibody is only capable of binding to Ang1.

Table 1. Interaction of antibodies with angiotensins 1, 2, and 3 and with angiotensinogen in indirect and competitive ELISAs

Immunogen	Antibody	Indirect ELISA				Competitive ELISA			
		A-gen	A1	A2	A3	A-gen	A1	A2	A3
Ang 1	AngE9	-	+	-	-	-	+	-	-
	AngC9	+	+	-	-	+	+	±	±
	AngC11	+	±	-	-	+	+	-	-
Ang 2	AngIIE7	-	-	+	±	-	-	+	+
Ang 3	AngIII B7	-	-	+	±	n.d.	n.d.	n.d.	n.d.
	AngIII F7	-	-	+	±	n.d.	n.d.	n.d.	n.d.

In contrast, the antibodies obtained as a result of the immunization with angiotensins 2 and 3 recognize only angiotensins 2 and 3, without making any difference between them in competitive analysis and preferring Ang2 in indirect ELISA, regardless of which angiotensin immunization (second or third) is performed. The fact that angiotensin 2 was better recognized in indirect ELISA can be explained simply by its superior ability to sorb Ang2 than the shorter, less hydrophilic Ang3. None of the antibodies against Ang2 and Ang3 recognizes angiotensin 1 and the angiotensinogen in any mode of enzyme-linked immunosorbent assay. Despite this, Ang1 and angiotensinogen containing amino acid sequences are also found in the structures of angiotensins 2 and 3.

Table 2. Dissociation constants (K_d) of monoclonal antibodies against angiotensins 1 and 2 with different targets

Antibody	Kd, M			
	A-gen	Ang 1	Ang 2	Ang 3
AngE9	$>10^{-5}$	$4.7x10^{-7}$	$>10^{-5}$	$>10^{-5}$
AngC9	$4.0x10^{-8}$	$7.7x10^{-9}$	$3.0x10^{-5}$	$3.0x10^{-5}$
AngC11	$1.25x10^{-8}$	$1.3x10^{-10}$	$5.5x10^{-6}$	$2.3x10^{-5}$
AngIIE7	$>10^{-5}$	$>10^{-5}$	$6.0x10^{-7}$	$2.0x10^{-6}$

CONCLUSIONS

Summarizing the results, we can draw the following conclusions:

1. Angiotensin 1 in free state and in angiotensinogen has the same conformation;
2. The detachment of two amino acid residues from Ang1 changes the conformational structure of the entire peptide; the produced angiotensin 2 has a different conformation than the conformation of the identical fragments in angiotensin 1 and angiotensinogen;
3. The detachment of one amino acid residue from angiotensin 2 does not significantly alter the conformational structure of the peptide; the conformation of angiotensin 3 produced as a result is similar to the conformation of angiotensin 2 and differs completely from the conformation of the corresponding fragments in Ang1 and angiotensinogen;
4. When short peptides are used for producing monoclonal antibodies against proteins, the probability of a complete transformation of the protein antigenic determinants synthesized in the form of peptides should be taken into account; peptides sorbed in solid phase can also have significant conformational differences from dissolved peptides with the same amino acid sequence.

The transformation of inactive angiotensin 1 and angiotensinogen into their active forms, Ang2 and Ang3, is accompanied by a significant conformational rearrangement of the corresponding fragments in peptide and protein molecules. ●

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Monitoring of the Zeta Potential of Human Cells upon Reduction in Their Viability and Interaction with Polymers

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ABSTRACT **The dynamic light scattering (DLS) technique was applied in order to assess the zeta potential of the plasma membrane of human cells. At pH 7.4, the cell zeta potential for different types of cells showed variations over a wide range and was equal to −19.4 ± 0.8 mV for HeLa cells and −31.8 ± 1.1 mV for erythrocytes. The difference could presumably be attributed to the differences in the biochemical composition of the cell plasma membrane. As a result of the heating of HeLa cells, the zeta potential shifted towards more negative voltages by 4.2 mV. An increase in the zeta potential correlated with an increase in the content of phosphatidylserine on the cell surface, which is considered to be an early marker of apoptosis. The DLS technique was also used to study the interactions between the cells and membranotropic polymers, such as polycations and nonionogenic Pluronic L121.**
KEYWORDS **dynamic light scattering; zeta potential; HeLa cells, MCF-7, mononuclear leukocytes, erythrocytes; apoptosis; phosphatidylserine; membranotropic polymers.**
ABBREVIATIONS **DLS – dynamic light scattering; PBS – phosphate-buffered saline.**

INTRODUCTION

Determination of the morphological and biochemical features of human cells is necessary in order to perform an unbiased assessment of the functioning of various organs and systems of the organism [1], design new pharmaceuticals [2], and conduct fundamental research.

The parameters of living cells under normal and pathological conditions were studied using direct spectroscopy methods; i.e., the Raman, dielectric, and NMR spectroscopy techniques [3]. The cell-surface charge is the key biophysical parameter that depends on the composition of the cytoplasmic membrane and the physiological condition of cells. The cell-surface charge is assessed by measuring their electrokinetic potential (zeta potential), which characterizes the electrical double-layer potential on the cell surface. Microelectrophoresis and capillary electrophoresis have been conventionally used to record the zeta potential of animal cells [4]; however, these techniques are labour-consuming and yield poorly reproducible results. The electrophoretic light scattering technique based on dynamic light scattering (DLS), in which the shift in the frequency or in the oscillation phase of the laser beam depends on the mobility of particles/cells in an alternating electric field [5], is a promising alternative to these methods.

The DLS technique was previously applied primarily in order to study microorganism cells [5]. We used a Zetasizer Nano ZS analyzer (Malvern Instruments) to analyze the zeta potential of animal cells.

This work was aimed at assessing the analytical capabilities of the DLS technique for determining the zeta potential of normal human cells, as well as that of human cells upon apoptosis induction, and following treatment with membranotropic polymers.

The zeta potential of human blood cells (mononuclear cells, erythrocytes) and cell lines (HeLa, MCF-7) were compared; the influence of heat-induced apoptosis and adsorption of polycations and amphiphilic nonionogenic Pluronic L121 on the zeta potential of HeLa cells was assessed.

EXPERIMENTAL

Cell culture reagents were purchased from PanEco (Russia). Adenocarcinoma cells of uterine cervix HeLa and breast adenocarcinoma cells MCF-7 were cultured in a DMEM medium supplemented with 10% fetal bovine serum, 2 mM *L*-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin. The cells were grown in polystyrene vials until cell monolayers were obtained. The cells were subsequently suspended using a 0.05% trypsin solution in 0.53 mM EDTA. The concentration of the suspended cells in a buffered phosphate saline

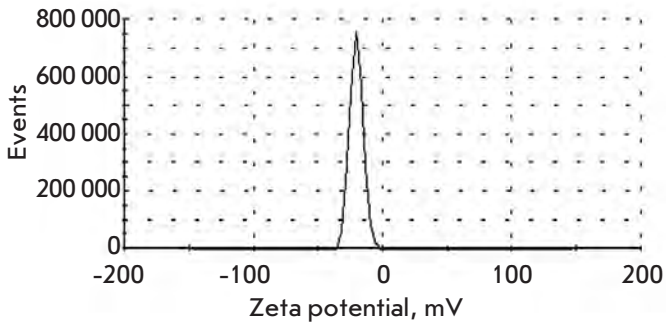

Fig. 1. Distribution of the zeta potential in HeLa cells (0.5×10^6 cells/ml) recorded using the dynamic light scattering technique.

(PBS) (1.7 mM KH_2PO_4, 5.2 mM Na_2HPO_4, 150 mM NaCl) was determined on a haemocytometer. Cell death was induced via heat shock (the cell suspension was heated for 30 min at 45°C).

Erythrocytes and mononuclear cells were isolated from the peripheral blood of conventionally healthy donors; 0.27% EDTA was used as an anticoagulant agent. Blood was decanted in a vial for 60 min in order to allow the erythrocytes to precipitate. The leukocyte-containing plasma was subjected to Ficoll-Paque density gradient separation (1.077 g/ml) at 400*g* for 40 min. The layer containing mononuclear cells was collected, washed by centrifugation, and subsequently suspended in PBS.

The zeta potential of the intact cells and those subjected to thermal shock or treatment with polymers was recorded in a suspension (0.5×10^6 cells/ml) using the electrophoretic light scattering technique on a Zetasizer Nano ZS analyzer (Malvern Instruments, Great Britain). The measurements were performed in a U-shaped cell with gold-plated electrodes at 25°C and pH 7.4 in a phosphate buffered solution containing no chlorine ions. The results were processed using the Dispersion Technology Software 6.2 (Malvern Instruments).

Various concentrations (10, 20, 40, 50, 80 μg/ml) of polyethyleneimine (60 kDa), poly(*L*-lysine) (~20 kDa) or ethylene oxide–propylene oxide block copolymer, Pluronic L121 (Sigma-Aldrich, USA) were added to the cell suspension (0.5×10^6 cells/ml) with the purpose of studying the interaction between the polymers and the cells. The mixture was incubated for 10 min; the cell zeta potential was subsequently determined.

In order to carry out flow cytometry, the cells were treated with a binding buffer containing FITC Annexin V and propidium iodide according to the manufacturer's protocol (BD Biosciences, USA). The analysis was performed on a BD FACSCalibur flow cytometry apparatus (BD Biosciences); the event count was > 20,000.

RESULTS AND DISCUSSION

The suspensions of human blood cells, as well as HeLa and MCF-7 cell lines, were used in this study. It was ascertained for HeLa cells that the zeta potential distribution curve has a maximum at −19.4 mV at pH 7.4 (*Fig. 1*). The zeta potential being recorded characterizes the electrical double layer potential on the cell surface [5]; its value should be dependent on the biochemical composition of plasma, provided that the solvent composition is constant.

The zeta potential values of the other cell types under study (MCF-7, mononuclear cells) were similar to that of HeLa cells, whereas the zeta potential of erythrocytes was equal to −31.9 mV (*Table*), which can be explained by the presence of sialic acid residues on the erythrocyte surface [6]. The negative values of the zeta potential of cell membranes at physiological pH values are presumed to be caused by the presence of nonionogenic groups within phospholipids, proteins, and their polysaccaride conjugates. The zeta potential value of phosphatidylcholine liposomes (phosphatidylcholine is the predominant lipid in animal cell membranes) is provided in the *Table* for comparative purposes. Under identical conditions, it is approximately equal to −62 mV. This fact indicates that lipids significantly contribute to the total negative charge of the cell membrane.

The changes in the zeta potential of HeLa cells subjected to heat shock were then subjected to analysis. The cell viability was assessed on a flow cytometry apparatus using a mixture of dyes, FITC Annexin V (possessing affinity to phosphatidylserine) and propidium iodide (PI), which stains necrotic cells. According

Zeta potential (ζ) of human cells and phosphatidylcholine liposomes, pH 7.4

Cells	ζ, mV*
HeLa	−19.4 ± 0.8
MCF-7	−20.9 ± 0.4
Mononuclear cells	−21.9 ± 0.2
Erythrocytes	−31.8 ± 1.1
Lyposomes (phosphatidylcholine)	−62.3 ± 1.5

*Zeta potential of cells was detected in independent triplicates. Right column shows the mean values ± standard deviation.

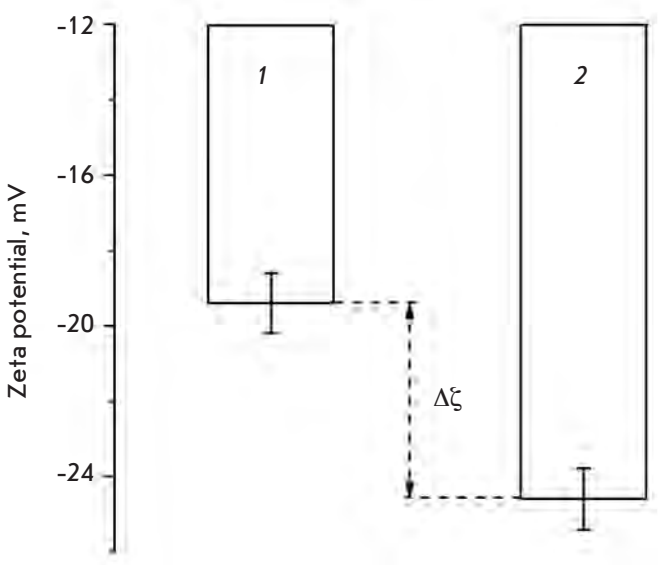

Fig. 2. The zeta potential of HeLa cells before and after cell heating (45°C, 30 min): 1 – intact cells, 2 – heated cells, Δζ – shift of the zeta potential of the cells after the treatment.

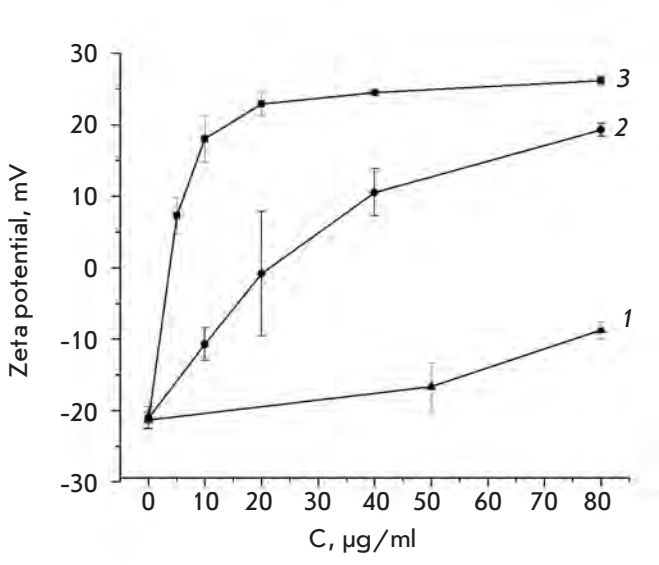

Fig. 3. Alteration of the zeta potential in HeLa cells treated with membranotropic polymers: 1 – Pluronic L121, 2 – poly(L-lysine), 3 – polyethyleneimine.

to the data of flow cytometry, cell incubation at 45°C for 30 min results in the emergence of 68% of FITC-positive cells and 62% of PI-positive cells, attesting to the fact that apoptosis and cell necrosis were induced.

According to the results obtained using the DLS technique, the average zeta potential of the heated cells shifted towards negative values by almost 4.2 mV, compared to the intact cells (*Fig. 2*). It is presumably a result of the redistribution of phosphatidylserine bearing a negatively charged carboxyl group from the inner to the outer lipid layer of the plasmalemma. The emergence of phosphatidylserine in the outer lipid monolayer of the cell membrane is one of the earliest markers of apoptosis and reduction in cell viability [6].

The results obtained attest to the fact that the DLS technique can be used to determine the changes in the biochemical composition of human cell membranes and, in particular, to detect phosphatidylserine in the outer lipid cell layer upon apoptosis induction. This approach is simple and does not require the use of expensive dyes.

It was of special interest to use the DLS technique to assess the effect of membranotropic polymers (used in cell technologies and for drug delivery) on the cell zeta potential. The interaction between HeLa cells and the model polycations, polyethyleneimine and poly(*L*-lysine), which have been widely used for DNA condensation and delivery to cells and for producing bioadhesive coatings for cell culturing [7], were studied. Another polymer, Pluronic L121, is a nonionogenic amphiphilic ethylene oxide–propylene oxide block copolymer. Block copolymers of this type are capable of irreversible interaction with cell membranes and of changing the activity of membrane transport agents, which is used to boost the efficacy of drug delivery to cells [8].

The addition of 20 μg/ml polylysine to the cells resulted in the neutralization of the cell zeta potential, presumably due to the electrostatic adsorption of the polycation on the surface of the negatively charged membrane (*Fig. 3*). At higher polylysine concentrations (above 20 μg/ml), the cell zeta potential becomes positive, reaching its maximum value in the presence of 80 μg/ml polylysine. Polyethyleneimine changes the charge sign of cells at a considerably lower concentration compared to polylysine (approximately 5 μg/ml), whereas the cell zeta potential is shifted to 26 mV at the saturating concentration of polyethyleneimine (*Fig. 3*). The effect of polyethyleneimine on the cell zeta potential is more pronounced due to the fact that this polycation is characterized by a higher density of the positive charge compared to polylysine.

The effect of Pluronic L121 on the cell surface charge was additionally assessed. Pluronic L121 is characterized by a low ratio between the hydrophilic and lipophilic parts of the molecule; its polypropylene oxide block exhibits affinity for the lipid bilayer [9]. Pluronic L121 forms nano-sized micelles in a buffered solution; according to our data, these micelles are characterized by a weakly negative zeta potential (approximately −6.7 mV).

It was ascertained that the treatment of HeLa cells with Pluronic L121 was accompanied by a noticeable shift in the cell surface charge into the positive region in proportion to the Pluronic concentration (*Fig. 3*). The changes observed can be attributed to the adsorption of nonionogenic Pluronic on the cell surface and the incorporation of its hydrophobic block into the membrane [10], which results in the alteration of the electrical double layer potential on the cell surface. The treatment of cells with Pluronic L121 within the analyzed concentration range did not lead to the complete neutralization of the cell charge, as was observed for polycations. Similar changes in the cell zeta potential in the presence of polymers were observed in the other cell types (MCF-7, mononuclear blood cells), which attests to the fact that the interaction between the polymers used and human cells is of a nonspecific character.

CONCLUSIONS

The zeta potential values of blood cells (erythrocytes, mononuclear cells), as well as HeLa and MCF-7 cell lines, were determined via the DLS technique. It was shown that phosphatidylserine, the early marker of apoptosis and reduction in cell viability, can be detected based on the increase in the total negative charge of the cells. The introduction of polycations and amphiphilic Pluronic L121 results in the neutralization of the negative cell zeta potential in direct proportion to the polymer concentration.

The results demonstrate that the dynamic light scattering technique can be used to study the properties of animal cell membranes, the changes in their biochemical composition, and their interaction with membranotropic polymers under various conditions. The DLS technique can be used in cell biology to analyze the condition of the membranes of various cells, in order to assess the effect of pharmaceutical agents and membranotropic substances. ●

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Targeted Therapy: A New Approach for the Treatment of Locally Advanced Oropharyngeal Cancer

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ABSTRACT **Presented herein is a clinical study comprising 48 patients (42 men and 6 women) of working age (40–70 years), all of whom are suffering from locally advanced oropharyngeal cancer. A modern approach is applied to treat these patients, *i.e.*, neoadjuvant targeted therapy, taking into account the biological profile of the tumor. The use of gefitinib causes an antitumor effect in 90.5% of cases as opposed to 56.5% when no drug is applied.**
KEYWORDS **oropharyngeal cancers; targeted therapy; quality of life; gefitinib.**
ABBREVIATIONS **SCCHN – squamous-cell carcinoma of the head and neck; EGFR – epidermal growth factor receptor.**

INTRODUCTION

Malignant tumors of the head and neck account for 20% of overall incidences of cancer. Squamous-cell carcinoma of the head and neck (SCCHN) is one of the most frequently observed types among other malignant tumors, the number of cases exceeding 600,000 annually [1]. In Russia, more than 80,000 patients with the pathology are registered every year; 3.5% of them are patients suffering from cancer of the mouth and throat. In the territory of the Russian Federation, the incidence of oropharyngeal cancers increased from 24.7 to 29.6 per 100,000 for the period from 1997 to 2007. In more than 70% of the cases, patients seek medical care when the disease is advanced (stages III, IV); in these stages, radical surgery is either impossible or severely restricted [1, 2]. The percentage of advanced oropharyngeal cancer cases has risen from 26.4 to 31.2%; the mortality rate from this is 15.6% [3]. The severity and urgency of this problem is beyond question, since the problem affects people of working age.

Using surgical intervention for patients of this group involves performing extended and combined surgeries, which have a mutilating effect, thereby significantly impairing the quality of life. Radiation therapy, either in combination with surgical treatment or alone at high doses of radiation, causes the development of severe complications (xerostomia, dysphagia, mucositis, etc.), which significantly limit its application, and impair psychosocial adaptation and rehabilitation of the patients.

In addition, during combined therapy, local recurrence occurs in 10–30% of patients with SCCHN, including those with histologically normal resection margins; the latter indicates a probable subclinical systemic extension of the tumor, occurring even prior to the stage of generalization. In this context, the complex approach plays an increasingly important role not only in surgery and radiation therapy, but in medicamental treatment as well; in other words, it is a systemic action on tumor cells.

For a significant period of time, drug therapy against squamous-cell oropharyngeal cancer has been applied for palliative purposes in the inoperable cases of advanced, frequently recurrent cancers that are characterized by the presence of distant metastases.

All current regimens of polychemotherapy, in which platinum-based or 5-fluorouracil (5 FU) drugs are used, provide an objective response rate of 57–80%; while the use of taxanes provides up to 36–40%. The overall life expectancy of patients does not increase in both the aforementioned cases [4–9]. The low response of tumors forces researchers to seek new approaches for systemic treatment.

The recent period in the history of anticancer therapy began in the mid-1990s, although its foundations were laid as a result of the achievements in fundamental biology in the last two decades [10]. In those studies, the molecular mechanisms of regulation of cellular proliferation and differentiation were revealed; the lat-

ter enabled the development of drugs acting in a completely novel way. [11].

In contrast to classic cytostatic drugs, whose action is based on disturbing the cell cycle, the drugs for targeted therapy affect only particular molecular targets, thereby blocking the earlier stages of carcinogenesis. There are drugs of the above-mentioned type that can be applied for the treatment of squamous-cell oropharyngeal cancer [2, 10, 12–14].

The main target in the case of SCCHN is the epidermal growth factor receptor (EGFR). The hyperexpression of epidermal growth factor receptors is noted in approximately 90–100% of cases of squamous-cell oropharyngeal cancer and is associated with the worst prognosis of the disease, a low differentiation of the tumor, and a decrease in the total and recurrence-free survival rates [15–17].

In 2004, three research groups published data indicating that mutations in the EGFR tyrosine kinase domain increase the response of tumors to the following EGFR tyrosine kinase inhibitors: gefitinib and erlotinib [12, 16, 18]. The majority of mutations found in the *EGFR* gene are either deletions in exon 19 (29 from 56.52% of cases), which lead to the loss of four amino-acid residues (leucine, arginine, glutamic acid, and alanine) in a protein molecule, or point mutations in exon 21 causing the replacement of leucine in position 858 by arginine (20 from 56.36% of cases) [12]. The presence of mutations in the *EGFR* gene is an important predictor of the probability of a more favorable outcome in gefitinib therapy. The high effectiveness of gefitinib was confirmed for the case of non-small cell lung cancer with a mutation in the *EGFR* gene: the objective response rate was 84.6% [19–21]. Since hyperexpression of EGFRs is observed in more than 80% of malignant tumors of the head and neck [22], we began studying the effectiveness of combined cisplatin, 5-fluorouracile and gefitinib (Iressa) in patients suffering from advanced (stages III, IV) squamous-cell oropharyngeal cancer with a mutation in *EGFR*.

Gefitinib (Iressa) was one of the first tyrosine kinase inhibitors introduced into clinical practice. According to its chemical structure, this drug is a derivative of anilinoquinazoline. Gifitinib selectively and reversibly binds to the ATP-binding site of the EGFR tyrosine kinase domain, thereby blocking its tyrosine kinase activity, *i.e.*, its ability to phosphorylate the signal proteins found after this site; the latter leads to the inhibition of proliferative signals [23, 24]. Gefitinib induces an increase in the level of the cyclin-dependent kinase p27 inhibitor in the cell, in turn causing a delay of the cell cycle in G1. Active studies of gefitinib are being performed within international clinical trials. In the Phase II clinical trial, application of gefitinib in 52 patients with recurrent/metastatic SCCHN allowed to achieve an objective response in 10.6% of them and to attain a level of disease control in 53%. Half of the patient cohort received gefitinib as plan B therapy. Thus, the median progression-free survival and overall survival were 3.4 and 8.1 months, respectively. The only clinically significant side effect observed was diarrhea [25].

EXPERIMENTAL

For the period from March 2009 to April 2011, 48 patients (42 men, 6 women) aged 40–75 years, a mean age of 57 years, were treated.

The diagram presented in *Fig. 1* clearly demonstrates that the bulk of the patients are men of working age. Oropharyngeal cancer is seven times more common in men than in women.

Within the patients, the tumor was distributed as follows (*Fig. 2*): the mouth floor in 8 (17%); the oropharynx in 18 (37%); the laryngopharynx in 12 (25%); the mobile part of the tongue in 8 (17%); and the retromolar area in 2 (4%). In the diagram, it can be clearly seen that oropharyngeal and laryngopharyngeal cancers prevail, while cancers of the mobile part of the tongue and of the retromolar area are less common.

The area of tumor involvement before the beginning of therapy was assessed by clinical examination of the lesion area, along with computed tomography and ultrasonic examination of regional lymph nodes.

Prior to therapy, the biological profile of the tumor, *i.e.* the expression of epidermal growth factor receptors and the presence of mutations in the *EGFR* gene, was determined in all patients. Mutations in the *EGFR* gene were revealed by polymerase chain reaction (PCR), and the expression of EGFRs was ascertained via the immunohistochemical method. Allele-specific PCR with primers specific to the L858R mutation in the *EGFR* gene was carried out on the DNA from paraffin blocks

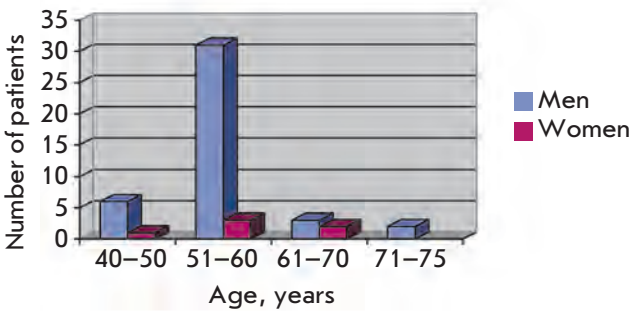

Fig. 1. Distribution of patients with oropharyngeal cancer by age.

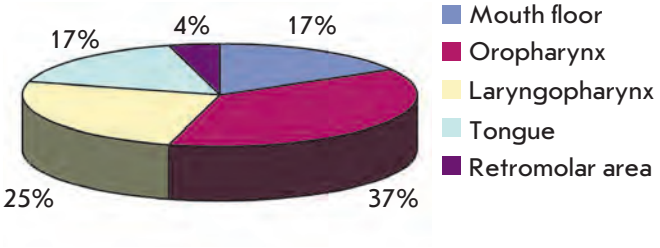

Fig. 2. Distribution of patients with oropharyngeal cancer by the localization of the primary tumor (%).

with an established tumor. The wild-type *EGFR* gene undergoes amplification, accompanied by an increase in Ct by 7–10 cycles under the same conditions, thus enabling the above-mentioned gene to be distinguished from the mutant.

Naive patients suffering from locally advanced squamous-cell oropharyngeal cancer (stages III, IV) were randomly divided into two groups:

The first group (studied) received cisplatin (100 mg/m^2, via intravenous administration, on the first day), 5-fluorouracil (500 mg/m^2, via intravenous administration, from the first to the fifth day) (four cycles with intervals of 21 days); and gefitinib (Iressa) (250 mg per os, daily, for 16 weeks).

The second group (control) received cisplatin (100 mg/m^2, via intravenous administration, on the first day) and 5-fluorouracil (500 mg/m^2, via intravenous administration, from the first to the fifth day) (four cycles with intervals of 21 days).

After four cycles, the tumor response was assessed clinically and in accordance with the RECIST criteria.

In the second stage of complex therapy, the patients in whom complete resorption of the tumor was achieved were treated with radiation therapy in accordance with the radical program: the primary tumor was irradiated at a total dose of 60–70 Gy, and the regional lymph nodes – at a total dose of 30–40 Gy. The patients with partial regression and stabilization of the tumor process underwent preoperational radiation therapy at a total dose of 30–40 Gy, followed by surgery.

RESULTS AND DISCUSSION

The effect of the therapy was assessed in 44 patients who went through a complete course of treatment. For four patients (16.7%), it was necessary to interrupt the treatment due to the appearance of toxic effects: in two (8.3%) of them, nephrotoxicity (stage III-IV) appeared; in the other two (8.3%), hematologic toxicity (stage IV) was observed. Hyperexpression of EGFRs was revealed in all 48 patients (100%). However, mutations in the *EGFR* were found in only three (6.8%).

A comparative analysis of the results of the therapies in both groups (*Table*) revealed that a complete clinical regression of the tumor was achieved in seven (33.3%) people from the group of 21 examined patients, and a mutation in the *EGFR* gene was found in two (4.5%) of those seven; in nine (42.9%) cases, a partial response (regression of the tumor of up to 85%) was observed; in three (14.3%) patients, stabilization of the tumor process (a decrease in the tumor by 18–20%) was detected; and in two (9.5%) patients, tumor progression was noted. In the control group of patients receiving only standard chemotherapy, a partial response (regression of the tumor of up to 57%) was observed in 13 (56.3%) of the 23 patients, while in 10 (43.5%) patients, the tumor continued to grow.

CONCLUSIONS

A significant increase was recorded in the effectiveness of the therapy in people suffering from oropharyngeal cancer by applying gefitinib, a drug used in targeted therapy. The effect of gefitinib is most pronounced when there are mutations in the *EGFR* gene.

Comparative analysis of the effectiveness of therapy in patients suffering from squamous-cell carcinoma of the head and neck, with and without the application of gefitinib

Group	Number of patients	Objective effect				Mutated *EGFR*	
		Complete response	Partial response	Stabilization	Progression	Yes	No
First (studied)	21	7 (33.3%)	9 (42.9%)	3 (14.3%)	2 (9.5%)	2 (4.5%)	20 (45.5%)
Second (control)	23	_	13 (56.5%)	_	10 (43.5%)	1 (2.3%)	21 (47.7%)
In total	100%					3 (6.8%)	41 (93.2%)

When therapy including gefitinib was used, the clinical tumor response was achieved in 90.5% of patients, which was twice higher than in the case of chemotherapy alone, 56.5%; and in 33.3% of cases, the result was achieved without surgery. The combination of targeted therapy with standard chemotherapy allows to increase the effectiveness of the therapy and to improve the prognosis for the disease. The results obtained in this work show the significant potential held by the application of this therapy scheme in conservative stages of treatment (chemoradiation therapy) during the early stages of the tumor; owing to this, organ-preserving complex treatment of patients suffering from squamous-cell oropharyngeal cancer may become possible. Clinical studies of the effectiveness of targeted drugs (erlotinib, gefitinib, cetuximab), applied in combination with chemoradiation therapy against squamous-cell carcinoma of the head and neck with a mutation in the *EGFR* gene, continue around the world. Altogether, the results of these studies will open up new opportunities for the treatment of the types of patients detailed above, improving their quality of life and enabling the performance of organ-preserving operations. ●

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Correction of Long-Lasting Negative Effects of Neonatal Isolation in White Rats Using Semax

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ABSTRACT **Adverse experience during the early postnatal period induces negative alterations in physiological and neurobiological functions, resulting in long-term disorder in animal behavior. The aim of the present work was to study the long-lasting effects of chronic neonatal stress in white rats and to estimate the possibility of their correction using Semax, an analogue of ACTH fragment (4–10). Early neonatal isolation was used as a model of early-life stress. Rat pups were separated from their mothers and littermates for 5 h daily during postnatal days 1–14. The pups of the control group were left undisturbed with the dams. Half of the rats subjected to neonatal isolation received an intranasal injection of Semax at a dose of 50 μg/kg daily, from postnatal day 15 until day 28. The other animals received intranasal vehicle injections daily at the same time points. It was shown that neonatal isolation leads to a delay in physical development, metabolic disturbances, and a decrease in the corticosterone stress response in white rats. These changes were observed during the first two months of life. Semax administration weakened the influence of neonatal isolation on the animals, body weight , reduced metabolic dysfunction, and led to an increase in stress-induced corticosterone release to the control values. So the chronic intranasal administration of Semax after termination of the neonatal isolation procedure diminishes the negative effects of neonatal stress.**
KEYWORDS **chronic stress; neonatal isolation; Semax; body weight; corticosterone; rat.**
ABBREVIATIONS **ACTH adrenocorticotropic hormone; MD – maternal deprivation; NI – neonatal isolation.**

INTRODUCTION

It is well known that the neonatal period of life is very important in the neurophysiological mechanisms development and the subsequent formation of mental functions. Aversive experience during the early postnatal period of human life (such as parental loss, abuse or parental neglect) results in an increase of psychopathology development probability in adulthood [1]. Children who had severe diseases in the neonatal period are exposed to painful and stressful influences, resulting in acute changes and permanent alterations in the structure and functions of the central nervous system [2]. Although the correlation between neonatal stress and behavioral disorders in adults has been demonstrated by a number of researchers, more study of the question are required. Animal experiments using various aversive actions enable to determine the relationship between the delayed changes in behavior, duration, and the type of actions, as well as to facilitate the search for methods for correcting the effects of neonatal stress. It has been demonstrated in numerous clinical studies that a disturbance of the socio-emotional mother-infant relationship during the first year of life is a significant stressor, which subsequently increases the risk of a number of mental disorders development [1, 3]. Long-term maternal isolation in the early postnatal period (neonatal maternal deprivation) also influences the behavior and physical development of various animal species.

There are a lot of studies devoted to the investigation of the long-lasting effects of neonatal maternal deprivation (MD). It has been demonstrated that the delayed effects of chronic MD depend on the duration of the daily deprivation of pups. Short-term chronic deprivation (15 min per day during the first 1–2 weeks of life) has a positive effect on the subsequent development of the animals. The rats that underwent such experience showed reduced anxiety and increased exploratory activity, as well as learning ability improvement [4–6]. Long-term separation of the rat pups from their moth-

er (for 3–6 h per day during the first few weeks of life) also causes long-lasting delayed changes in animal behavior and is considered to be the neonatal stress model. Two models of maternal deprivation are used in the experiments. In the first case, the pups of the same litter stay together during MD. In the second case, the pups are subjected to neonatal isolation (NI): the pups are placed into individual boxes, where they are separated both from their dams and littermates. An increase in the anxiety level and reduced exploratory activity were observed in most experiments in the animals subjected to MD [7–9]. Nevertheless, MD occasionally resulted in an increase in the animals exploratory activity [10]. The effect of long-term MD on the animals, learning ability is also controversial. Different researchers have detected both disturbance [11, 12] and improvement of the spatial learning ability in maternally deprived animals [13]. In some studies, no effect of MD on the spatial learning ability of rats was observed [14]. The influence of MD and NI on the functioning of the hypothalamic-pituitary-adrenal axis was demonstrated. However, the results were appreciably controversial, similar to those in the case of the animal behavior alteration. Thus, whereas some authors reported an increase in stress-induced corticosterone release in mice that were subjected to maternal deprivation [15, 16], others reported a decrease in this index in animals that were subjected to NI [17–19] or MD [20]. In a number of studies, no changes in the hormonal stress-response in animals that had experienced neonatal stress were revealed [7]. The inconsistency of the results could be due to the differences in the experimental protocols and in the age of the tested animals [18]. Thus development of an adequate neonatal stress model in animals and further study of the delayed effects of chronic long-term maternal deprivation is quite essential.

The heptapeptide Semax (MEHFPGP) is an ACTH fragment (4–10) analogue that has prolonged neurotropic activity [21]. This peptide possesses neuroprotective and neurotrophic effects [22, 23]; it also has antihypoxic and antihaemorrhagic action [21, 24]. Semax is used in medicine as a nootropic and neuroprotective agent [25]. It was demonstrated earlier that chronic neonatal administration of Semax results in enhancement of the exploratory behavior and a decrease in anxiety in rats. Moreover, the animals that received Semax in the neonatal period showed better learning ability in subsequent years. These alterations had a delayed long-lasting character [26]. Semax neonatal administration effects were opposite to the effects of neonatal stress: that let us to assume that Semax administration can correct the negative effects of neonatal stress. It was ascertained in our previous studies that the daily neonatal isolation of rat pups for 5 h during 1–2 weeks of postnatal development causes long-lasting alteration in the animal's behavior. An increase in anxiety and reduction in exploratory activity at the age of 1–2 months was observed in rats subjected to NI during the first weeks of postnatal development. Chronic intranasal administration of Semax during 15–28 days of life resulted in a considerable normalization of the emotional state of the animals exposed to NI [27].

The aim of the present work is to study the effects of neonatal isolation on physical development in rats and the hormonal stress-response, as well as to find out whether Semax administration during 15-28 postnatal days can correct the NI effects.

EXPERIMENTAL

Noninbreded white rat pups of both sexes were used. The animals were housed in a vivarium under the standard conditions with free access to food and water and were maintained on a 12 h light–dark cycle. The heptapeptide Semax (MEHFPGP) was synthesized in the Institute of Molecular Genetics, Russian Academy of Sciences.

The day of birth of the pups was considered as day 0 of life. Each litter was divided into three groups: the control group, the NI group (the animals were subjected to neonatal isolation), and the NI–Semax group (the animals were subjected to NI and subsequently received Semax). The pups from the control group were left undisturbed in their nest for the first two weeks of life. The pups from the NI and NI–Semax groups were daily placed into individual boxes for 5 h (days 1–14 of life). During the isolation, the pups were left in silent conditions at a temperature of 25 ± 2°C, and they were illuminated with moderate light. The rats from the NI–Semax group intranasally received a 0.05 mg/kg dose of an aqueous Semax solution during the period from day 15 to day 28 of life. The pups from the control and NI groups received an equivalent volume of the solvent during the same time period. During the experiment, the age of eye-opening and body weight were recorded for each animal (daily during the period from day 15 to day 28 of life; then, once a week). Blood glucose levels were measured at days 15, 30, and 48 of life. Blood samples were obtained from the tip of the tail in order to determine the glucose level; the glucose content was measured using a glucometer (Accu-Chek Performa Nano).

The level of food motivation in animals was assessed on day 42 of life. Prior to the experiment, the animals were deprived of food for 20 h. The blood glucose level was measured in hungry animals; after 30 min had elapsed, the rat was placed into an empty cage. After 5-min adaptation to the new conditions, a weighed portion of food was placed into the cage. Then, the follow-

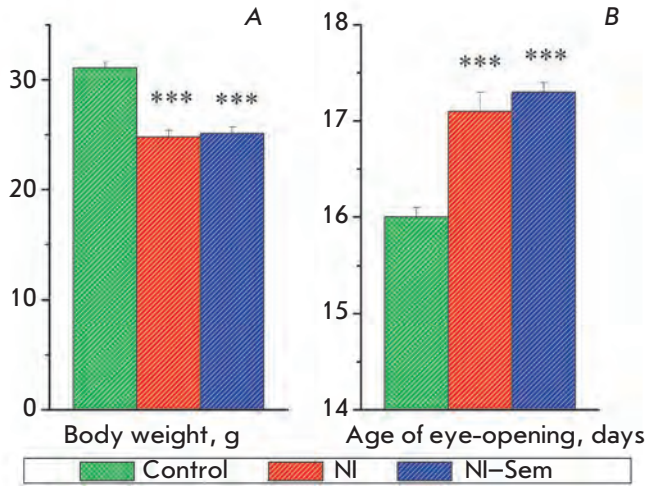

Fig. 1. Neonatal isolation effects on the body weight of rats at the age of 15 days (*A*) and on the time of eye-opening (*B*). The number of animals in groups: 81/74/77. *** ($p < 0.001$) – significant difference from the control.

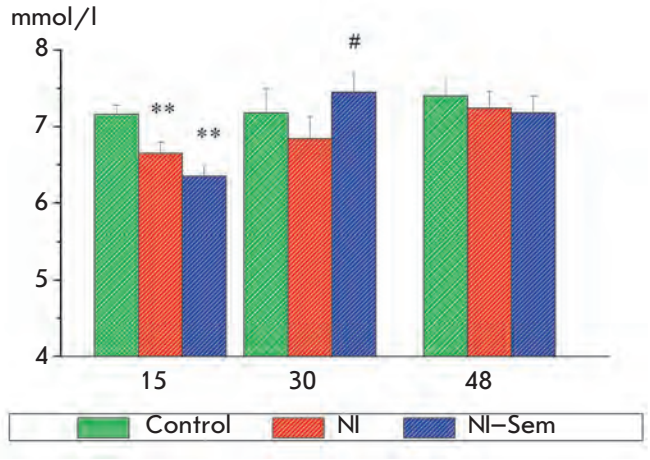

Fig. 2. Blood glucose level in rats. The x-axis – the age of animals (days); the y-axis – glucose concentration (mmol/l). The number of animals in groups: rats at the age of 15 days – 38/35/38; rats at the age of 30 and 48 days – 12/11/11. **($p < 0.01$) – significant difference from the control, #($p < 0.05$) – significant difference from the NI group.

ing parameters were recorded during 10 min: the latency to food intake, feeding duration, and the amount of food consumed. Next, the rat was placed in a cage with unlimited access to food. The glucose level was measured again after 30 min.

The alteration in corticosterone in rat blood as a response to an acute stressor was assessed on day 65 of life. In the beginning of the experiment, a rat was placed into an immobilizing device; a blood sample (200 μl) was obtained from the tail after cutting off the tip. The animal was subjected to forced swim stress at 24°C for 10 min. Ten min after the termination of the stressor exposure, the rat was repeatedly placed into the immobilizing device. The second blood sample was obtained; the animal was then placed back into its home cage. Sixty min after the termination of the forced swim stress, the rats were decapitated to obtain a blood sample. The sample was kept at 37°C for 20 min, and at 4°C for 60 min. The samples were then centrifuged (10 min, 5,000 rpm), and the serum was collected. The corticosterone level was subsequently assessed in the serum samples using the Corticosterone EIA Kit designed to determine corticosterone in biological fluids (Catalog № ADI-900-097, Enzo).

RESULTS

Animals of both sexes were used in the experiments. The factor Sex had a considerable effect only on the body weight alteration of the rats. Other parameters did not differ significantly in either males or females. It was shown via use of the two-way ANOVA method (factor 1 –Group; factor 2 – Sex) for analyzing the alteration in the body weight of the rats during the first two months of life that the Group ($F_{2,48} = 3.49$, $p < 0.04$) and Sex factors ($F_{1,48} = 34.91$, $p < 0.000001$) had a significant effect. However, no significant interaction between these factors was observed ($F_{2,48} = 0.33$, $p = 0.72$). No statistically significant differences in the influence of NI and Semax on animals of different sexes were revealed by comparison with the results obtained for the male and female groups. This fact allows us to present the results obtained for the entire group of rats.

It was demonstrated that daily isolation during the first two weeks of life results in body weight decrease in 15-day-old rat pups ($F_{2,229} = 39.60$, $p < 0.0001$; *Fig. 1A*) and delay in eye-opening ($F_{2,136} = 25.83$, $p < 0.0001$; *Fig. 1B*) versus animals of the control group. In addition, a significant decrease in the blood glucose level was detected 1 day after the last NI in rats that had experienced neonatal stress in comparison with those in the control group ($F_{2,107} = 9.53$, $p < 0.0001$; *Fig. 2*). On day 15 of life, the pups subjected to NI were randomly divided into the NI and NI–Semax groups. The animals from these two groups had identical body weight, glucose level at day 15 of life, and the age of eye-opening (*Figs. 1, 2*).

Half of the pups exposed to NI received a daily intranasal Semax injection during the period from postnatal day 15 to day 28 (the NI–Semax group). The remaining animals (the NI group) and the animals from the control

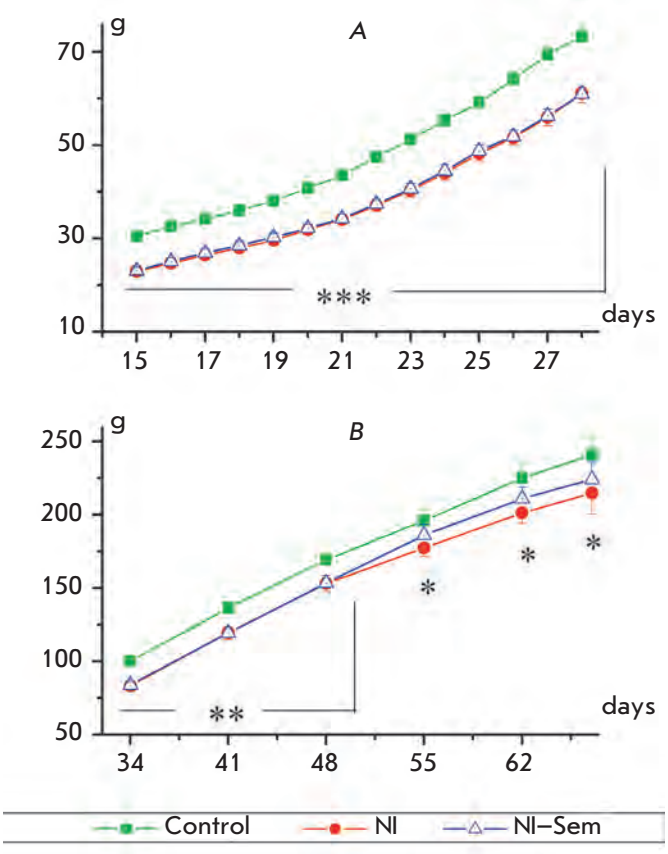

Fig. 3. The growth rate of rats during the first (A) and the second (B) months of life. The x-axis – the age of animals (days), the y-axis – the body weight (g). The number of animals in groups: 32/29/28. *($p < 0.05$), **($p < 0.01$), and ***($p < 0.001$) significant difference from the control.

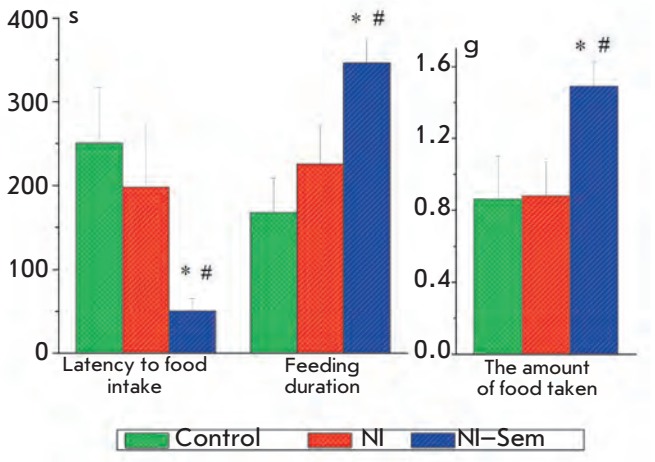

Fig. 4. The results of food motivation test for the rats at the age of 42 days. The rats were food-deprived for 20 h before testing. The number of animals in groups: 12/11/11. *($p < 0.05$) – significant difference from the control, #($p < 0.05$) – significant difference from the NI group.

group received distilled water. The lower body weight in rats from the NI and NI–Semax groups was observed during this period as compared to those in the control group ($F_{2,84} = 27.75$, $p < 0.0001$; *Fig. 3A*). No significant differences between the NI and NI–Semax groups were detected ($F_{1,53} = 0.03$, $p > 0.85$). The animals from the NI group had a lower body weight in comparison with that of the rats in the control group up to day 65 of life ($F_{1,25} = 4.63$, $p < 0.04$). Until day 48, the body weight of the rats in the NI–Semax group remained significantly lower than that in the control group. No significant differences were subsequently detected ($F_{1,26} = 2.87$, $p > 0.10$; *Fig. 3B*).

No significant differences in the blood glucose level in 30-day-old rats were revealed between the groups ($F_{2,32} = 1.09$, $p > 0.25$), although the blood glucose content in the pups from the NI group was lower as compared to the control values. Subsequent analysis demonstrated that the value of this parameter in the NI–Semax group was significantly higher than that in the NI group ($p < 0.02$ using the χ^2 test). No differences in the glucose level were observed for the groups of 48-day-old rats ($F_{2,31} = 0.74$, $p > 0.50$) (*Fig. 2*).

The food motivation level of the animals was assessed on day 42. In the rats from the NI group, the parameters characterizing the food motivation level were identical to those in the control group. The group of animals receiving Semax injections demonstrated a reduction of the latency to food intake, an increase in feeding duration and the amount of food consumed during the experiment, as compared to these parameters in the control and NI groups ($F_{2,31} > 3.3$, $p < 0.05$) (*Fig. 4*). The aforementioned changes attest to increased food motivation in the NI–Semax animals. Hence, the neonatal isolation experience did not affect the food motivation level of rats; Semax administration to the rat pups exposed to NI resulted in an increase in food motivation.

It was demonstrated that the blood glucose level in NI rats after 24-h food deprivation was significantly lower as compared with that in the control and NI–Semax groups ($F_{2,31} = 3.32$, $p < 0.05$). The glucose level after food deprivation in NI–Semax animals was identical to that in the control group. The repeated measurements (after the food intake) showed no significant differences in this parameter between the groups ($F_{2,31} = 0.46$, $p > 0.60$) (*Fig. 5*). Thus, neonatal isolation resulted in the reduction of the blood glucose level under conditions of food deprivation. Semax ad-

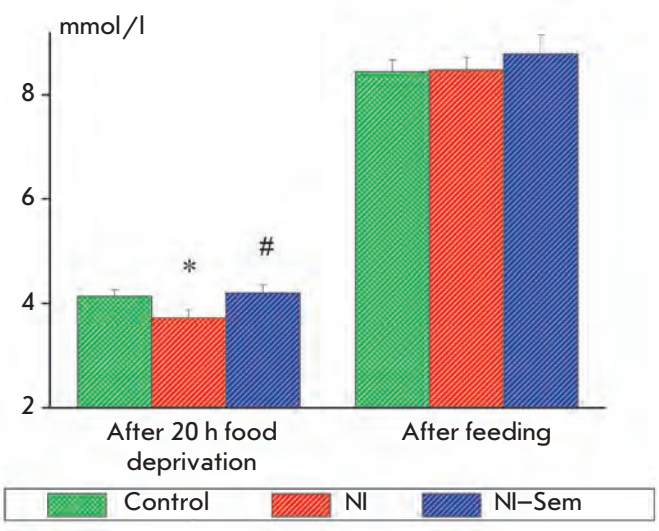

Fig. 5. Blood glucose level in rats subjected to 20-hour food deprivation (before and after food consumption). The y-axis – glucose concentration (mmol/l). The number of animals in groups: 12/11/11. *($p < 0.05$) – significant difference from the control, #($p < 0.05$) – significant difference from the NI group.

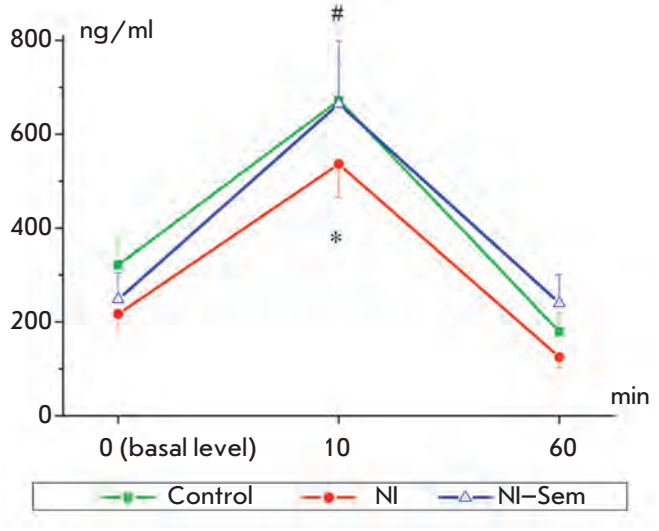

Fig. 6. Stress-induced changes in corticosterone level: basal (0), 10 and 60 min after exposure to stress. The y-axis – serum corticosterone concentration (ng/ml). The number of animals in groups: 13/12/11. *($p < 0.05$) – significant difference from the control, #($p < 0.05$) – significant difference from the NI group.

ministration eliminated the NI effect in case of this parameter.

The changes in the blood corticosterone level of the rats in response to a acute stressor were assessed at day 65 of life. The corticosterone basal level in NI rats was lower than that in the control group; however, this difference did not attain the level of statistical significance ($p > 0.05$). Ten minutes following stress termination, the blood corticosterone level in NI rats was significantly lower than that in the control and NI–Semax groups ($p < 0.05$ using the χ^2 test). No significant differences between the groups with respect to this parameter were obtained 1 h after stress termination (*Fig. 6*). Thus, neonatal isolation had resulted in a reduction in the stress-induced corticosterone release; whereas Semax administration had eliminated the isolation effect, bringing the corticosterone level to the control value.

DISCUSSION

The rat pups were isolated from their mothers and littermates for 5 h daily during the period from day 1 to day 14 of life. It was established earlier that rodents exhibit a weak response of the hypothalamic-pituitary-adrenal system to moderate stress during the period of early neonatal development (the period known as period of stress hyporeactivity). Ensured by specific maternal behavior, the suppression of the stress response plays a significant role in the normal development of the nervous system. It was demonstrated that the isolation of the pups from their mothers weakens the blockage of the hypothalamic-pituitary-adrenal system [28, 29]. NI procedure in the present work included daily food deprivation for 5 h, cold stress, and the absence of contact with the mother; so a combination of physical and emotional stress of high intensity was used. Furthermore, as mentioned above, the separation of the rat pups from their mothers resulted in a weakening of the neonatal stress-hyporeactivity. Thus, the actions used were stressors of high intensity. The model used can be considered to be a neonatal stress model.

It was demonstrated that chronic NI of rat pups during the period from day 1 to day 14 of life resulted in delayed eye-opening, which attests to the delayed physical development. The observed effects of NI are similar to those of MD [30, 31]. Changes in body weight is another parameter characterizing the physical development of the animals. In the model used, NI resulted in delayed somatic growth of the rat pups. The differences between the body weight of the NI rats and the control rats remained during the entire experiment time; i.e., at least up to the age of 2 months. There is a lack of consistency in the published data relating to the effect of MD on the body weight of the animals. In most of the studies, no changes in this parameter were detected [32, 33]. However, it was men-

tioned in a number of studies that the pups that had undergone MD had a reduced body weight [19, 34, 35]. The difference in the effects can presumably be attributed to the different conditions under which the pups were kept during the deprivation. Thus, it was demonstrated that NI at a temperature of 30°C did not affect the body weight of the animals, whereas NI at 22°C resulted in a decrease in body weight and growth deceleration [36]. In our experiments, during the NI procedure the pups were kept at 24–26°C. Presumably, a decrease in body temperature caused by the isolation of the pups from their mother and littermates plays a significant role in the development of the NI effects on their physical development. In addition, it was demonstrated that maternal deprivation causes suppression of the cell response to three major trophic hormones (growth hormone, prolactin, and insulin). These changes may result in somatic growth deceleration [37].

Thus, the daily isolation during the first two weeks of life resulted in the deceleration of the physical development of rat pups, which remained up to day 65 of life. Intranasal Semax injections to the 3- to 4-week-old rat pups weakened the effect of NI on the body weight of the animals; thus, these parameters approached the control level at days 55–65. This compensatory action of peptides on rat body weight is presumably based on the increased food motivation of the stressed animals that received Semax. In our case, the increased food motivation can be considered to be the adaptive response of the organism to the reduction in body weight caused as a result of neonatal isolation.

A significant decrease in the blood glucose level was recorded in rats that had experienced neonatal isolation at the age of 15 days. The glucose level was measured 24 h after the last isolation procedure; i.e., by the time the blood sample was collected, the rat pups had been in contact with their mothers for 1 day. Therefore, the decrease in the blood glucose level that was observed cannot be explained by food deprivation. The glucose level was not significantly different from the control values in the NI animals with unlimited access to food at the age of 30 and 48 days. However, under conditions of food deprivation, decreased glucose content was observed in NI rats compared to that in the control group. The resulting data attest to the fact that neonatal isolation causes long-lasting disturbances in metabolic processes in the rat organism. Semax administration to the animals exposed to NI resulted in an increase in the blood glucose level under conditions of both unlimited access to food and food deprivation, attesting to the fact that the peptide has a normalizing effect. It is well-known that it is necessary to maintain a physiological blood glucose level for the normal development of the brain in mammals. During the period when the nervous system is under development, hypoglycaemia may cause disorders both in cognitive functions and in the emotional status. These disorders do not disappear after the glucose level is normalized; they can manifest themselves in adulthood [38]. It was previously shown that NI during the first 1–2 weeks of life causes long-lasting behavioral changes: 1- to 2-month-old rats that had experienced NI demonstrated increased levels of anxiety and reduced exploratory activity. Subsequent Semax administration normalized the emotional state of the NI animals [27]. The abatement of NI-induced metabolic disturbances may have been one of the mechanisms of the positive effect of Semax on the emotional status of the animals that had experienced neonatal stress.

Study of the neonatal stress effect on the corticosterone level in blood demonstrated that NI had no effect on the basal level of this hormone; however, it resulted in a decrease in corticosterone release as a response to acute stress exposure. No effect of MD on the basal level of corticosterone had been observed in most of the previous studies [33, 39]. There has been no consistency in the published data relating to the effect of MD on stress-induced corticosterone release. It should be noted that most studies have been devoted to the investigation of the effects of maternal deprivation rather than neonatal isolation; this could account for the inconsistency in the results. Rees *et al*. [18] compared the effects of MD and NI and demonstrated that whereas MD did not affect the basal and stress-induced corticosterone release, NI resulted in a decrease in the stress-induced corticosterone release. The reduced hormonal response to stress in animals that had experienced neonatal isolation may be caused by the exposure to repeated stress episodes, which may have led to the reiterated release of corticosterone. The repeated activation of the hypothalamic-pituitary-adrenal system during the early neonatal period may result either in depletion of this system or in an increase in the efficiency of negative feedback [17, 35]. Semax administration to the rats subjected to NI boosted the level of stress-induced corticosterone release to the control level. Therefore, the subsequent administration of the peptide normalized the hormonal response to stress exposure, which had been disturbed by neonatal isolation.

It was previously demonstrated that daily neonatal isolation of white rat pups for 5 h during the period from day 1 to day 14 of life results in long-lasting changes in animal behavior [27]. It has been shown in this study that the neonatal exposure to stress also results in delay of physical development in the animals, disturbance of metabolic processes, and weakening of the hormonal response to acute stress. These changes were observed during the first 1–2 months of life; i.e., they were of delayed and long-term character. The

negative effects of the neonatal stress were reduced by the chronic intranasal administration of Semax after the termination of the procedure of neonatal isolation. The results obtained can be used to broaden the range of clinical applications of Semax; in particular, for the treatment of pathologies in children during the early postnatal period. ●

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Stable Expression of Recombinant Factor VIII in CHO Cells Using Methotrexate-Driven Transgene Amplification

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ABSTRACT **Prophylaxis and treatment of inherited clotting disorder hemophilia A requires regular administration of factor VIII. Recombinant factor VIII, which is produced in CHO or BHK cells, is equivalent to the plasma-derived one and is prevalent in current clinical practice in developed countries. Development of a biosimilar recombinant FVIII requires the creation of a highly productive clonal cell line and generation of monoclonal antibodies suitable for affinity purification of the product. Methotrexate-driven transgene amplification of genetic cassettes that code full-length and truncated variants of FVIII under the control of the CMV promoter was studied. It was shown that the expression level of the truncated variant of FVIII is 6.5 times higher than that of the full-length molecule. The transgene amplification procedure was sufficient for a twofold increase of the expression level in the transfected cells pool and subsequent selection of the clonal line, stably producing truncated FVIII at the level of 0.52 IU/ml during cultivation in a chemically defined protein-free culture medium. Four generated mouse monoclonal antibodies toward the heavy chain of FVIII were found suitable for binding the truncated variant of FVIII directly from the conditioned medium and elution of the FVIII with a more than 85% yield and normal pro-coagulant activity. The producer cell line and monoclonal antibodies obtained are sufficient for the development of upstream and downstream processes of biosimilar FVIII production. Generation of more productive cell lines by the use of stronger, nonviral promoters and shorter cDNA of FVIII will be the subject of further studies.**
KEYWORDS **coagulation factor VIII; B-domain deleted factor VIII; hemophilia A; heterologous protein expression systems.**
ABBREVIATIONS **FVIII – blood clotting factor VIII; rhFVIII – recombinant human FVIII, BDD-FVIII – FVIII with deleted B-domain, MTX - methotrexate, EG - ethylene glycol, EMCV - Encephalomyocarditis virus, IRES - internal ribosome entry site, DHFR - dihydrofolate reductase (EC 1.5.1.3); IU – international unit (1 IU of FVIII corresponds to it s content in 1 ml of pooled donor plasms), ORF – open reading frame, PBS – phosphate buffered solution.**

INTRODUCTION

Blood clotting factor VIII (FVIII) is a nonenzymatic cofactor for factor IXa forming a complex that binds factor X and activates it, realizing a major amplification loop of the blood coagulation cascade. Defects in the gene of FVIII result in hemophilia A, a recessive X-linked coagulation disorder with a prevalence of 1 case per 5,000 males.

Protein substitution therapy, performed on a regular basis, is the only effective treatment for hemophilia A. The traditional source of FVIII is donated blood plasma, which is in short supply and poses a significant risk of virus [1, 2] and prions [3] transmission, even after rigorous plasma batches screening and multiple viral inactivation procedures. Recombinant human FVIII (rhFVIII) for hemophilia A treatment may be obtained from cultured mammalian cells purified to clinical grade by affinity chromatography and three or four rounds of conventional chromatography and virus-inactivated by solvent-detergent treatment and nanofiltration or heating. Marketed variants of rhFVIII are expressed in Chinese hamster ovary (CHO) or baby hamster kidney (BHK) cells and are fully equivalent to the plasma-derived FVIII in replacement therapy.

Table 1. Primers used for FVIII-SQ BDD mutant construction. Restriction sites are underlined

O1KpnIfor	5'GCTGGTACCTCACAGAGAATATACA3'
O1HindIIIrev	5'GGAGAAGCTTCTTGGTTCAATG3'
O2HindIIIfor	5'CCAAGCTTCTCCCAAAACCCACCA GTCTTGAAAC3'
O2BlpIrev	5'CTGCCCATGCTGAGCAGATAC3'
Odelf	5'GCCACAACTCAGACTTTCG3'
8sq4f	5'TGTATTTGATGAGAACCGAAGC3'
8sq5r	5'GCCACTCTGAGCCCTGTT3'
CMVfor	5'CGCAAATGGGCGGTAGGCGTG3'
8sq15r	5'GAGTTCTTTGTTTCTGAGTGCC3'

Table 2. Primers used for sequence analysis of FVIII ORF

8sq1f	TGATCAGACCAGTCAAAGGGA
8sq2f	GATTGGATGCCACAGGA
8sq3f	GCCCTCAGCGGATTGGT
8sq4f	TGTATTTGATGAGAACCGAAGC
8sq5f	TGCCATTGAACCAAGAAGC
8sq6f	GAGAAACTGGGGACAACTGC
8sq7f	AGAAAGACTCACATTGATGGCC
8sq8f	ACAAAGTGGTAGTAGGAAAGGGTG
8sq9f	TGAAACAATTCAGACTCCCACT
8sq10f	GACAAGTGCCACAAATTCAG
8sq11f	TTTGTCCCTGAACGCTTGT
8sq12f	CAGCCCTTATACCGTGGAG
8sq13f	CAGATGGAAGATCCCACTTT
8sq14f	GGATCAATCAATGCCTGGAG
8sq15f	AGGAGTAATGCCTGGAGACC
8sq1re	GCAAGCCAGGGAGGGAC
8sq2re	TGGCAAACATATTGGTAAAGTA
8sq3re	AGGGGAGTCTGACACTTATTGC
8sq4re	GAGCAAATTCCTGTACTGTCACTT
8sq5re	GCCACTCTGAGCCCTGTT
8sq6re	CTTGGGATTTCCACTCTTCTTT
8sq7re	CTGCTGGAAGATGAGAAGAGTT
8sq8re	TGCTGGCTTGTATTAGGAGA
8sq9re	GCCTTGCCCAGAGTTCAG
8sq10re	AGTCAACAAAGCAGGTCCAT
8sq11re	ACTGTCTATTGCTCCAGGTGA
8sq12re	CTGAGAATGGGAATAGGGTGA
8sq13re	GGGTCAGGCACCGAGGA
8sq14re	GGATGCTTCTTGGCAACTGA
8sq15re	GAGTTCTTTGTTTCTGAGTGCC
IRESArev	AGGTTTCCGGGCCCTCACATTG

The major drawback of rhFVIII production techniques is the low expression level, which is caused by the unusual size and structural complexity of the target protein. Natural human FVIII is a 170- to 280-kDa glycoprotein, mainly present in circulation in the form of a noncovalent complex with the chaperone - von Willebrand factor (vWF) in a concentration of approximately 400 ng/ml. FVIII is expressed in the liver as a single-chain polypeptide containing the A1-A2-B-A3-C1-C2 domains. The mature, secreted protein is cleaved in the region between the B and A3 domains and forms a heterodimer of 90-200 kDa, heavy chain (A1-A2-B domains), and 80 kDa, light chain (A3-C1-C2 domains) [4]. A significant part of the FVIII molecule, the B-domain, may be deleted without compromising the clotting activity and plasma half-life of the truncated FVIII [5]. Replacement of the B-domain by the short linker peptide called SQ results in a significant increase in the rhFVIII expression level in CHO cells and the complete processing of the precursor protein to its mature form [6]. The B-domain-deleted FVIII (BDD-FVIII), which is marketed under the trade name ReFacto, has shown comparable efficiency with full-length FVIII variants and is as safe [7].

The aim of the present study was to generate a clonal cell line secreting rhFVIII at a significant level and to develop monoclonal antibodies for affinity purification of rhFVIII. Since the ability of cell lines bearing a single-copy genomic insert to produce enough rhFVIII has not been confirmed in published studies, FVIII or BDD-FVIII coding vectors suitable for insertion cassette amplification were evaluated.

EXPERIMENTAL PART

Construction of expression plasmids:

For pOptivec/F8 construction, pOptivec plasmid derived from a re-ligated linear pOptivec-TOPO vector (Invitrogen, USA) was restricted by *NotI* and ligated by T4-ligase with a *NotI-NotI* fragment of pCMV6-XL4/NM_000132 containing the full factor VIII gene (Origene, USA). The enzymes used were acquired from Fermentas, Lithuania, or Sibenzyme, RF.

For BDD-FVIII generation, the PCR fragments F1 (479 b.p.) and F2 (933 b.p.) that flank the deleted region were obtained using the primers O1KpnIfor, O1Hindrev and O2Hindfor, and O2Blprev, respectively (Supplementary *Table 1*). Oligonucleotides were synthesized by Evrogen JSC, RF. PCR was performed by a Tersus polymerase mix (Evrogen JSC, RF) on the PTC-100

Thermal Cycler (MJ Reseach, USA); purified PCR products were cloned to the pAL-TA vector (Evrogen JSC, RF) and fully sequenced using the BigDye Terminator v. 3.1 cycle sequencing kit (Applied Biosystems, USA), a ABI PRISM 3730 genetic analyzer (Applied Biosystems, USA), and the Chromas 1.45 program (Technelysium Pty Ltd, Australia) for data analysis.

The N-terminal FVIII gene fragment F3 was obtained by pCMV6-XL4/NM_000132 restriction with the *NotI* and *KpnI* enzymes. Assembly of the fragments F1-3 was performed in the PAL-TA vector by corresponding restriction enzymes resulting in pALTA/F123. PCR for clone analysis was performed with the Odelf specific primer and the vector-specific M13for and M13rev primers.

The *BlpI-BlpI* fragment of the pOptivec/F8 plasmid was exchanged for the *BlpI-BlpI* restriction fragment of pALTA/F123, resulting in the pOptivec/F8BDD plasmid. PCR for clone analysis was performed with two specific primer pairs: 8sq4f, 8sq5r and CMVfor, and 8sq15r. The ORFs of full-length FVIII and BDD-FVIII and expression vector functional elements (promoter, IRES, terminator) were sequenced using the specific primers listed in Supplementary Table 2.

Preparation of the assembled plasmids for transfection was done by transformation to a Stbl4 *E. coli* strain (#11635018 Invitrogen, USA), cultivation in a 0.5 L TB broth for 18 h, and purification by the EndoFree Plasmid MaxiKit (Qiagen, USA). For stable cell lines generation, plasmids were linearized by *PvuI* restriction, followed by ethanol precipitation. The precipitates were dissolved in PBS and filter-sterilized using 0.22 μm filters (Millipore, USA).

Cell culture: A DHFR-negative Chinese Hamster Ovary CHO DG-44 cell line (Invitrogen, USA) maintained in a chemically defined suspension medium was used. The cells were cultivated in a suspension culture as 30 mL batches in Erlenmeyer flasks (VWR, USA), with a CD DG-44 medium (Invitrogen, USA) supplemented with 8 mM L-glutamine (Invitrogen, USA) and 0.18% Pluronic F-68 (BASF Inc., USA). The culture flasks were maintained in a humidified incubator, 37˚C/8% CO_2 on a shaker, at a constant rotation rate of 130 rpm. Viability by trypan blue exclusion assay was assessed and cell count performed at each cell passage. Cells were passaged every 2-3 d and maximum cell concentration was set at $1.2x10^6$ viable cells in 1 ml: split ratio 1:4.

Transfection and selection of stably transfected cells: transfection was performed by the animal origin free reagent Lipofectamine 2000 (Invitrogen, USA) using 18 μg of linearized plasmid DNA per $1,5x10^7$ cells in 30 mL of the culture medium. Cells were cultivated 48 h post-transfection without medium change, then they were transferred to the selection nucleoside-free medium CD OptiCHO (Invitrogen) supplemented with 8 mM L-glutamine (Invitrogen, USA) and cultivated until cell viability reached 90% (10-20 d). During cultivation in the selection medium, the cells were passaged every 3 d or at a concentration of $3x10^5$ cells/mL. The levels of FVIII secretion were determined 48 h after transfection and at the end of cultivation in the selection medium. Three independent transfections were performed in the same conditions for each plasmid. The highest producing pool was selected for the methotrexate (MTX) induced amplification of *dhfr* and the FVIII genes.

Clonal cell lines generation: the selected pool of stably transfected cells was subjected to growth in the presence of increasing concentrations of methotrexate in a CD OptiCHO medium supplemented with 8 mM L-glutamine. At every subsequent step of the MTX-driven target gene amplification, the concentration of MTX was increased twofold. On each step, the cells were cultivated for at least 10 days, then 4 to 15 more days until cell viability reached 90%. The levels of secreted FVIII were measured by ELISA at the end of each step. The highest producing amplified pool was used for obtaining clonal cell lines by limiting dilution at 0.5 cells/well. Cloning was performed in the adherent culture, utilizing a medium CD CHO-A (Invitrogen, USA) (200 μl/well) containing 8 mM GlutaMAX (Invitrogen, USA) at 37˚C/ 5% CO_2 for 21 days. MTX was excluded from the cloning medium and was not used in further cultivation.

The growth of single colonies in wells was monitored and documented on days 10 and 14. The colonies were transferred to 48-well plates, and the conditioned medium from the wells with actively grown colonies was assayed by ELISA. The best secreting cell clones were further propagated in the adherent conditions and re-adapted to the suspension culture in 3 consecutive passages in 24-, 12-, and 6-well plates, utilizing a CD OptiCHO medium with 8 mM L-glutamine. The conditioned medium from 6-well plates was screened by ELISA; one clonal line was selected and expanded further by subsequent passages in 3, 15, 100, and 200 mL of CD OptiCHO.

Small-scale production culture was done in shaking flasks at a 200-mL scale. Cells were seeded at $2,5x10^5$ cells/mL, cultured without medium change to a density of $3x10^6$ cells/mL (4-5 days), and then cultured for 3 more days with a daily addition of 4 mM glutamine and 3 mM glucose. Cell mass and debris were removed by centrifugation at 500 g for 5 min and subsequent filtration of supernatant by 0, 22 μm PES capsule filter (Millipore, USA). The clarified medium was stored frozen and thawed immediately before use.

ELISA: ELISA was performed as described in [8]. Antibody capture ELISA was used for the testing of anti FVIII mAbs, and a concentrate of plasma-derived FVIII (a generous gift from Dr. A.L. Berkovsky) in PBS at 200 ng/well was used for plate coating. Sandwich ELISA was utilized for secreted FVIII in the culture medium, polyclonal anti-FVIII antibodies (LifeSpan BioSciences, USA) at 50 ng/well were used for plate coating, and in-house developed anti-FVIII murine mAb A2 was used for detection. Frozen pooled normal human plasma serially diluted in 1% BSA-PBS was used as a quantity calibrator. All the samples tested were applied to plates undiluted or diluted immediately before testing by 1% BSA-PBS.

Western blotting: Whole-cell lysates were prepared with a modified RIPA buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) containing a protease inhibitor cocktail (Sigma, USA). Samples of the conditioned medium were clarified by centrifugation and concentrated 30-fold by trichloroacetic acid precipitation. The samples were normalized by total protein concentration, applied at 10 μg of total protein per lane, and resolved on 7.5% SDS-PAGE gels. Protein transfer, blocking, hybridization, and color development were done according to [8] using a Hybond C Extra membrane (GE Healthcare, USA) and a 3,3',5,5'-tetramethylbenzidine substrate (Sigma, USA).

Generation of mAbs: Immunization, fusion, and cloning of hybridomas were performed according to [8]. Female Balb/c mice (Harlan Labs, UK) were immunized subcutaneously with 100 ng of recombinant full-length FVIII (Kogenate FS) in 0.25 mL of 0.85% NaCl and 0.25 mL of complete Freund's adjuvant. (Pierce Biotechnology, USA). Two and four weeks after the initial injection, the animals were boosted with 100 ng of the same antigen in IFA. One week after the last injection, the mice were tail-bled and the serum antibody level was monitored by ELISA. One mouse with the highest titer of IgG was sacrificed for cell fusion. Splenocytes from the chosen mouse were fused with SP2/0 myeloma cells using polyethylene glycol. The fused cells were propagated in a selective medium, plated in 96-well plates, and then screened for anti-fVIII IgG titer. The cells from positive wells were expanded to 24-well plates and screened for titer and sensitivity to elution by 50% ethylene glycol in PBS. The wells with the highest titers and highest sensitivity to ethylene glycol elution were used for hybridoma cloning by limiting dilution (0.5 cells/w). Expanded hybridoma clones were re-screened by the same procedure and cloned again. The expanded clones were used for generation of the conditioned medium (10-100 mL) and ascites production in pristane-primed Balb/c mice. The ascitic fluid collected was stored frozen for further use.

Purification of monoclonal antibodies from the ascitic fluid and conditioned medium was performed by the same protocol – precipitation by ammonium sulphate, Protein G affinity chromatography using a HiTrap Protein G HP (GE Healthcare, USA) 1 ml column, concentration of eluted IgG by ultrafiltration, and polishing/desalting by size exclusion chromatography utilizing a Superdex 75 10/300 column (GE Healthcare, USA) and PBS as the mobile phase.

NHS-activated Sepharose 4 Fast Flow (GE HealthCare, USA) was used for mAb's coupling according to the resin manufacturer`s instruction. Antigen capture was performed in batch format, and 1 ml aliquots of the conditioned medium was mixed in microcentrifuge test tubes with 0.1 ml aliquots of affinity sorbents for 1 h at room temperature. Sorbents were settled by brief centrifugation, and the supernatants of the depleted medium were collected for further analysis. Sorbents were washed by 3 1 ml portions of PBS; bound proteins were eluted by the addition of 0.15 ml of 50% ethylene glycol in the PBS solution and 5 min incubation.

Coagulation assay: The clotting assay for the fVIII level was performed on a ThromboScreen 400c (Pacific Hemostasis A Fisher Scientific Company) optical coagulometer using the reagents kit "Factor VIII-test" (NPO Renam, RF) according to the kit manufacturer's protocol with some modifications. Culture media samples were diluted ten times by imidazole solution prior to the analysis, and eluates from the affinity columns were diluted 10-50 times by a imidazole solution supplemented with 1% BSA. For testing the conditioned media samples, calibration plasma samples were supplemented by 10% of the conditioned medium from non-transfected CHO DG-44 cells. In case of affinity column eluates, the calibration samples were supplemented by 2-10% of the elution solution.

RESULTS AND DISCUSSION

An expression construct of the full-length factor VIII gene pOptivec/F8 was created on the base of the pOptivec-TOPO vector. The SQ B-domain deletion mutant cloning strategy included minimization of the PCR fragments length to bypass PCR-mediated mutations. Two short PCR fragments, F1 and F2, flanking the deleted region were separately cloned and then assembled with F3 - a restriction fragment of pCMV6-XL4/NM_000132 corresponding to the N-terminal part of the FVIII protein. To obtain the expression plasmid, the pOptivec/F8BDD *BlpI-BlpI* fragment of the pOptivec/F8 expression plasmid was exchanged for the *BlpI-BlpI* fragment of the obtained F123 assembly (*Fig. 1*).

The resulting expression plasmid contained a strong CMV-promoter, a natural FVIII Kozak sequence, F8-

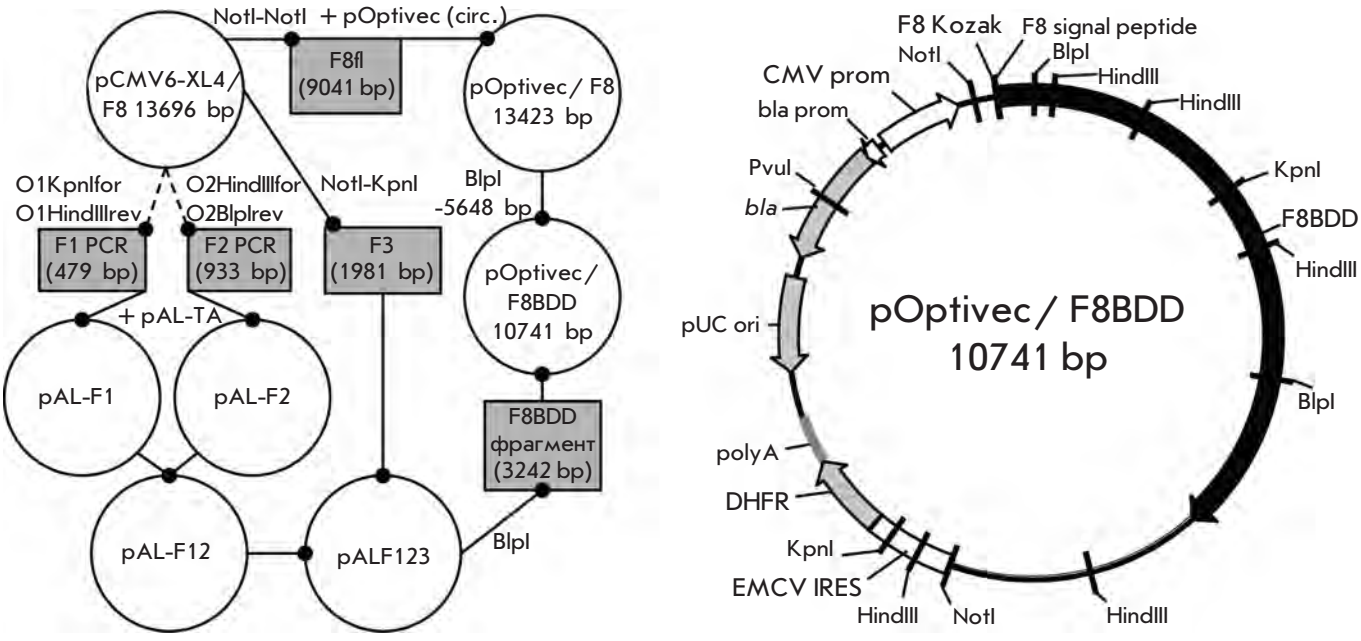

Fig. 1. General molecular cloning scheme and map of the pOptivec/F8BDD expression plasmid. Linear fragments shown as rectangles, plasmids – as circles. PCR steps are shown as dotted lines, restriction-ligation steps – as full lines. CMV-prom - cytomegalovirus promoter, F8 Kozak - natural Kozak sequence of the FVIII gene, F8 signal peptide – natural FVIII signal peptide sequence, F8BDD – ORF of the SQ B-domain deleted factor VIII, EMCV IRES - encephalomyocarditis virus internal ribosome entry site, dhfr - ORF of the dihydrofolate reductase, polyA – bovine growth hormone polyadenylation signal, pUC ori – bacterial replication origin, bla – ampicillin resistance gene, bla prom - ampicillin resistance gene promoter. Directions of genes transcription are shown by arrows. Restriction sites used for cloning procedures are in italics.

BDD ORF, followed by a encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) that allows 5' cap-independent translation initiation: the dhfr gene which allows clonal selection of transfectants and further MTX-driven amplification of the fVIII-IRES-dhfr cassette in *dhfr-* cells. For stable cell lines generation plasmids were linearized for destruction of the irrelevant ampicillin resistance gene.

Generation of the producer cell line

Transfection of DHFR-negative CHO DG-44 cells by linearized expression cassettes was performed in a serum-free medium utilizing an animal-origin free transfection reagent. Transfection efficiency was estimated by transfection of the control plasmid coding fluorescent eGFP protein; 10 to 20% of the cells expressed eGFP at 48 h post-transfection, and cell viability was above 85%. Three rounds of transfection were performed for each FVIII-coding plasmid, and stable pools were obtained by cultivation of the transfected cells in a selective medium for 15-20 days. The levels of secreted FVIII were determined by ELISA; in the case of full-length FVIII, less than 10 IU/l of secreted FVIII was found in all cell pools, and in the case of BDD-FVIII, a stable transfectant pool with a 71±10 IU/ml secretion level was found and used for transgene amplification.

The pool of stably transfected cells was treated by MTX, starting at the 25 nM level. After stabilization of the culture, determined as improvement in cell viability to more than 85%, the concentration of MTX was increased twofold and amplification continued (*Fig. 2*). It was found that a steady increase in the secreted BDD-FVIII level does not take place; the maximum BDD-FVIII level in the conditioned medium was attained after 5 subsequent amplification steps (0,5 μM of MTX). Further increase of MTX concentration resulted in an immediate tenfold drop in the product secretion rate and subsequent increase to the values of the initial culture (74±6 IU/l at 16 μM MTX). It was suggested that non-producing cells containing altered bicistronic mRNA [9] or amplified non-relevant genes, for example the gene of multiple-drug resistance protein 1 [10], take over the producing population.

The best producing cell pool, obtained at the level of 0.5 μM MTX, was used for the generation of clonal cell

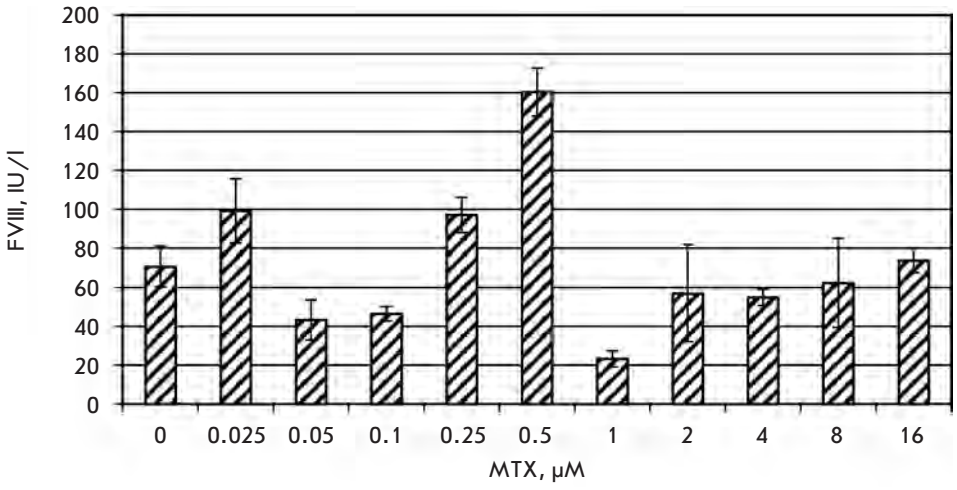

Fig. 2. Secretion level of BDD-FVIII for confluent cell pools by ELISA. Samples in duplicates, error bars represent a 95% confidence interval.

Table 3. Productivity of the 10 highest secreting clonal cell lines. Product concentration was measured for adherent cultures at the attainment of confluence

Clone number	18	22	17	9	1	2	15	3	4	16
Secreted FVIII , IU/l	502	475	434	416	410	399	395	379	378	375

lines by limiting dilution. Twenty-two clones derived from the wells with single cell colonies and secreting the target protein according to ELISA were grown to more than 10^6 cells in the adherent culture conditions and analyzed for the concentration of secreted FVIII (*Table 1*). Four clones with the highest production rate were further cultivated in the suspension culture and tested again for the concentration of FVIII after three and ten consequent passages (10 and 30 days of continuous culturing). Two clones, #18 and #22, showed no significant drop in FVIII production (data not shown), and the more productive clone #18 was chosen for subsequent use.

The conditioned medium from the chosen clonal cell line, designated DG-BDDFVIII-18, was used for the characterization of the secreted FVIII. According to Western blotting data (*Fig. 3*), the bulk of the secreted FVIII, as well as the intracellular precursor, was processed into the form of a two-chain protein. Low levels of the single-chain form were detectable only by the antibody toward the heavy chain, and no degradation products were detected by both antibodies, which is an indication of the proper short-term stability of FVIII in the culture medium used.

The pro-coagulant activity of FVIII in the conditioned medium was assessed based on its ability to decrease the clot time of the substrate plasma obtained from hemophilia A patients in the aPTT test. The activity of FVIII in the conditioned medium tested was 0.47 IU/ml, and the FVIII antigen level in the same sample was 0.52 IU/ml according to ELISA data; hereby, the recombinant FVIII secreted by the clonal cell line DG-BDDFVIII-18 has full specific activity.

The known industrial purification process for the BDD-FVIII protein consists of 4 stages of conventional chromatography and one round of affinity purification [11]. Therefore, the key element in the process is the monoclonal antibody, which is capable of capturing BDD-FVIII from the culture medium or from the intermediate concentrate and of subsequently releasing the product under mild elution conditions. The typical solution suitable for the elution of FVIII from immunoaffinity columns is PBS with 50% ethylene glycol [12].

A monoclonal antibody suitable for the affinity purification of BDD-FVIII was obtained by screening anti-FVIII hybridoma clones by ELISA, in which the wells were washed three times with a 50% ethylene glycol solution after the incubation of hybridoma culture supernatants and the control wells were washed with a PBS-Tween solution. The target clone is expected to show a significant signal decrease upon ethylene glycol wash. Out of the 34 individual clones derived from one cell fusion, four clones with a high mAb titer and the highest sensitivity to ethylene glycol wash were selected (*Table 3*).

All of the four mAbs selected recognized the heavy chain of BDD-FVIII on western blotting; i.e., their epitopes do not belong to the B-domain of FVIII. Pu-

Table 4. Properties of ethylene glycol-sensitive mAbs

Clone name	mAb titer in the ascitic fluid	Decrease in ELISA signal at ethylene glycol wash, %	Resin binding capacity, IU/ml	FVIII elution degree, %
A2	1:123 000	40%	2.6	89%
E3	1:68 500	39%	2.8	89%
A4	1:27 500	15%	1.6	>90%
B6	1:123 000	35%	3.4	86%

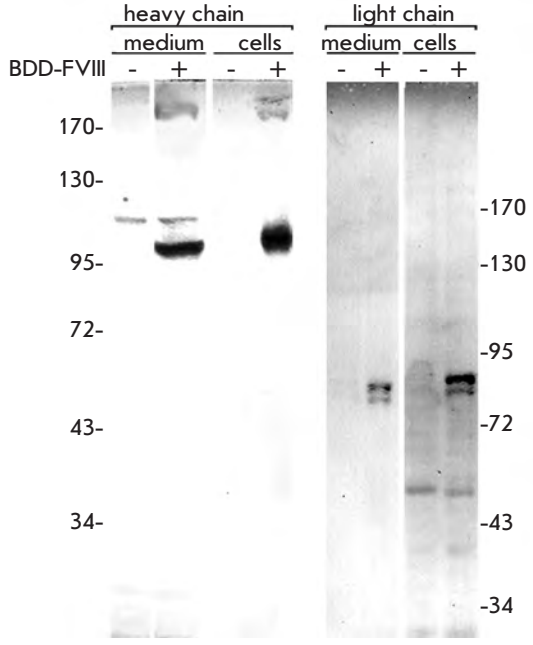

Fig. 3. Western blotting of secreted and intracellular BDD-FVIII. "Heavy chain" and "light chain" – probing by antibodies toward heavy and light chain, "BDD-FVIII -" and "BDD-FVIII +" – samples from nontransfected CHO DG-44 cells and cell line DG-BDDFVIII-18, respectively. SDS-PAGE in reducing conditions, molecular weights are shown in kDa.

rified mAbs were obtained from the ascitic fluid and immobilized on NHS-activated Sepharose at 1 mg of mAb per 1 ml of the settled resin ratio. Immunosorbents were used to capture BDD-FVIII directly from the conditioned culture medium with 1 h batch adsorption. Adsorption of BDD-FVIII in these conditions was incomplete (ca. 20-30%), but nearly the entire bound product was retained notwithstanding PBS wash and eluted with the 50% ethylene glycol solution. The levels of FVIII in the nonbound fractions and eluates were measured by plasma clot assays. The presence of biologically active FVIII in the eluates indicates that the elution conditions used did not significantly degrade the product. Thus, several mAbs suitable for large-scale affinity purification of recombinant FVIII were obtained.

CONCLUSIONS

The purpose of this study was to develop a recombinant FVIII producing stable cell lines and monoclonal antibodies for affinity purification of the secreted protein.

Expression constructs bearing completely sequence-verified cDNA's of full-length human FVIII and a B-domain deletion variant of human FVIII were obtained. Stably transfected cell pools were obtained; the BDD-FVIII variant showed a vastly increased level of secretion and was used for methotrexate-driven transgene amplification and subsequent cell cloning. A clonal cell line DG-BDDFVIII-18, capable of stable secretion of BDD-FVIII at the 500 IU/l level, was established. The target protein in the conditioned medium was found to be biologically active and almost entirely processed into its two-chain mature form. The cell line was obtained without the use of animal-origin substances and stably grows in a chemically defined medium as a suspension culture. Monoclonal antibodies toward the heavy chain of BDD-FVIII, suitable for affinity purification of the target protein in native form, were obtained.

Thus, both of the major components of the industrial FVIII production process were created – the animal-origin free clonal cell line and monoclonal antibodies for the affinity purification step. Generation of more productive clonal cell lines and overall FVIII production process development will be studied subsequently. ●

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Cytotoxic and Immunochemical Properties of Viscumin Encapsulated in Polylactide Microparticles

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ABSTRACT **Biodegradable polylactide microparticles with encapsulated cytotoxic protein viscumin were obtained via the ultrasound-assisted supercritical fluid technique. The size of the microparticles was 10–50 μM, as shown by electron microscopy. The time course of viscumin release from microparticles was studied using an immunoenzyme test system with anti-viscumin monoclonal antibodies. It was found that 99.91% of the cytotoxic protein was incorporated into polymer microparticles. Only 0.08% of the initially encapsulated viscumin was released from the microparticles following incubation for 120 h in a phosphate-buffered saline at neutral pH. Importantly, the method of ultrasonic dry supercritical fluid encapsulation failed to alter both the cytotoxic potency and the immunochemical properties of the encapsulated viscumin. Thus, this procedure can be used to generate biodegradable polylactide microparticles with encapsulated bioactive substances.**

KEYWORDS **biodegradable microparticles; viscumin; polylactide.**

ABBREVIATIONS **MLI – viscumin; RIP – ribosome inactivating protein; SCF – supercritical fluid; sc-CO_2 – supercritical carbon dioxide; PBS – phosphate buffered saline; TMB – tetramethylbenzidine; MSG – magnetostriction generator; SEM – scanning electron microscopy; BSA – bovine serum albumin; MTT – 3-(4,5-dimethylthiozolyl-2-yl)-2,5-diphenyltetrazolium bromide; LD_{50} – lethal dose for 50% of the cells.**

INTRODUCTION

Viscumin, a ribosome-inactivating lectin, occurs in leaf extracts from the parasitic plant Common Mistletoe (*Viscum album*). Viscumin has a molecular weight of 60 kDa and consists of two subunits, A and B, which are linked via a disulfide bond [1, 2]. This protein has found widespread application in anti-tumor therapy [3–6]. Its efficacy can be enhanced by encapsulating viscumin into biodegradable polymer microparticles, thus ensuring its chemical and spatial stabilization, as well as a prolonged release of the protein into the surrounding tissues. Hence, the toxin will have a prolonged effect on tumor cells.

Polylactides are a class of biodegradable polymers that belong to the homologous series of aliphatic polyesters that are finding increased application in biomedicine and pharmaceutics [7]. Polylactide is a polymer of lactic acid that contains asymmetric carbon atoms (*Fig. 1)* and can easily form optically active cyclic dimers (lactides), which are capable of polymerizing via catalytic opening of 1,4-dioxane rings, similar to the polymerization of glycolides.

Polylactide contains methyl groups, and thus it is a more hydrophobic compound as compared to polyglycolide. Polyactide dissolves more easily in organic solvents. Since a monomer of lactic acid exists in two stereometric forms, four morphologically different polylactides can be synthesized: two stereo-regular polymers, poly(*D*-lactide) and poly(*L*-lactide); a polymerized blend of the *D*- and *L*-lactic acids – poly(*D*,*L*-lactide); and poly(meso-lactide) – *D*- and *L*-lactide blends. The polymers synthesized from the optically active *D*- or *L*-lactic acid only are polycrystalline, whereas the optically inactive poly(*D*,*L*-lactides) possess an amorphous structure. This fact is significant in their practical application, since the hydrolysis rate of these compounds (which determines their biodegradation kinetics in a living organism) is inversely proportional to their degree of crystallinity. Glycolide is a simpler compound that exists only in single form. The identity

of the catalytic reaction of ring opening in glycolides and lactides enables copolymerization, yielding high-molecular-weight copolymers (polylactoglycolides); this fact considerably broadens the range of biodegradable synthetic materials that possess various biochemical and mechanical properties.

The ability of aliphatic polyesters to gradually biodegrade in the organism is used both to provide temporary protection to active molecules or drugs against rapid degradation by various enzymes and peptides and for the targeted delivery of these compounds to specific cells, tissues, and organs, as well as to control the release rate of these molecules from the polymer matrix, thus ensuring a more prolonged therapeutic effect [7]. For this purpose, a drug is encapsulated into a polymer carrier and is injected or introduced perorally into the organism [8, 9]. The method of dry supercritical fluid (SCF) encapsulation can be successfully used to incorporate various biologically active compounds (enzymes, peptides, proteins, and drugs) into polymer microparticles, the physicochemical and biological properties of which are almost completely retained [10–12]. This fact serves to differentiate SCF encapsulation from other methods, whose application assumes the use of high temperatures (up to 100°C and even higher) and toxic organic solvents; it is rather problematic to remove these solvents from the final product [10]. Supercritical carbon dioxide (sc-CO_2) allows one to perform the encapsulation of bioactive components into various amorphous polymers without using liquid solvents at temperatures close to room temperature and under moderate pressure (the critical parameters for CO_2: T_{cr} = 31°C, P_{cr} = 7.4 МПа). Sc-CO_2 can be easily and almost completely removed from the polymer by reducing pressure below the critical value [13].

The first model experiments devoted to the SCF encapsulation of viscumin into polylactide micromatrices were performed earlier [11]. It was revealed that the liberation time of viscumin can be controlled by varying the conditions of SCF encapsulation. This procedure actually enables the design of prolonged-action drugs with the desired release kinetics of the active substance from the polymer carrier.

Herein, the immunochemical and cytotoxic properties of viscumin, following its liberation from microparticles produced via the same procedure but with the use of ultrasound, were studied. Denser, fine particles (10–50 μm) were obtained using ultrasound.

EXPERIMENTAL

Viscumin was kindly provided by Professor U. Pfüller (Institute of Phytochemistry, University of Witten/Herdecke, Germany). *D,L*-polylactide PURASORB PDL 02 (PURAC Biochem bv, Netherlands) with a molecular mass M_w ~ 20000 was used as an initial biodegradable polymer. Carbon dioxide of special purity grade (99.99%, Balashikha Oxygen Plant, Moscow oblast, Russia) was used without any additional purification. Dry phosphate buffered saline (PBS, Flow Laboratories, Great Britain), two-component reagent kit for the substrate mixture based on tetramethylbenzidine (TMB) for ELISA and the streptavidin–peroxidase conjugate (IMTEK, Russia); and polystyrene plates (Costar, USA) were also used. Monoclonal antibodies MNA4 and biotinylated MNA9 against various epitopes of the viscumin A-subunit were obtained earlier [14, 15]. The remaining reagents were purchased from Sigma-Aldrich Corporation (USA).

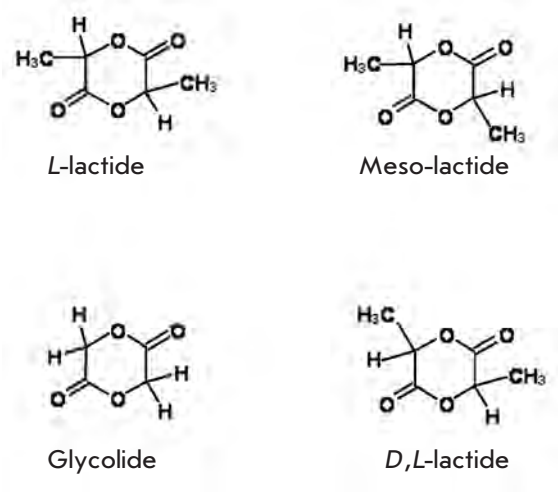

Fig. 1. Cyclic dimers for the synthesis of aliphatic polyesters.

Encapsulation of viscumin into polylactide microparticles

The encapsulation of viscumin into the polylactide carrier was performed on an experimental setup similar to that described in [16]. The major difference from the previously used equipment [11] was the powerful ultrasonic action (18 ± 0.2 kHz, up to 1 kW that the polymer/viscumin system in sc-CO_2 atmosphere was subjected to). This approach was implemented using a magnetostriction generator (MSG) with an acoustic concentrator and titanium inducer introduced into the high-pressure reaction chamber.

The formation of bioactive microparticles is attained via the following steps: The powder-like polylactide with a characteristic particle size of 100–200 μm (0.1 g) produced from preliminarily grinded initial polymer grains with a diameter of ~ 3÷4 mm

and lyophilized viscumin powder (1 mg) were loaded into the high-pressure reaction chamber. The chamber was pressurized; CO_2 at room temperature was fed until the pressure reached 5 MPa. The chamber heaters and nozzles were subsequently turned on. The temperature in the chamber was typically at 40°C; the nozzle temperature varied from 40 to 80°C. The pressure in the chamber increased with each rise in temperature. After the desired temperature was attained, the pressure in the chamber was brought to a pre-selected value of 10 ÷ 20 MPa. The MSG power control unit was then turned on; its power was varied within a range of 0.1–1.0 kW. The system was kept under these conditions for ~ 30 min to form the regime of SCF plasticization of the viscumin–polymer mixture in the reactor. A pulsed discharge of the plasticized mixture and carbon dioxide into the inlet chamber was subsequently performed using a pulsed valve, via a 0.5 mm diameter nozzle.

After the resulting product was stored in the inlet chamber under atmospheric conditions for 3 h (the time required for the complete removal of CO_2 from polymer particles and the final hardening of the particles), the microparticles being collected were placed into 1.5 ml glass vials and were stored at a temperature of +4°C prior to the subsequent analysis.

Scanning electron microscopy

The morphology of the surface of the polymer microparticles with viscumin encapsulated via the sc-CO_2 technique was studied using scanning electron microscopy (SEM) on a LEO 1450 microscope (Carl Zeiss, Germany). A small amount of the powder under study was applied on a conducting (carbon) adhesive tape with a thin (~ 0.05–0.1 μm) gold film, which was coated onto it via plasma spraying and ensured the required conductivity.

Investigation of the time course of viscumin liberation from polylactide microparticles

A dry powder of viscumin-containing polylactide microparticles (16.5 mg) was suspended in 2 ml of PBS; the suspension was centrifuged for 10 min at 12,100 *g*; and the supernatant was then sampled. The remaining polylactide particles were again suspended in 2 ml of PBS and then stirred on a rocker at 22°C. The supernatant was sampled, and the next PBS portion was added after 10, 30, 60, 120, 360, 1440, 2880, and 7200 min, respectively. The resulting samples were stored at +4°C.

Analysis of the amount of viscumin liberated upon degradation of polylactide microparticles

The amount of viscumin in the samples (i.e., in the supernatant sampled at different time points) was determined using the earlier described modified test system [14, 15]. Anti-viscumin monoclonal antibodies MNA4 at a concentration of 10 μg/ml in PBS were adsorbed onto a 96-well plate (100 μl per well). The antibodies were incubated for 24 h at +4°C and subsequently washed with a solution containing 20 mmol/l lactose and 0.05% Tween 20 in PBS. 100 μl of the buffer solution containing 0.1% of bovine serum albumin (BSA), 20 mmol/l lactose and 0.05% of Tween 20 in PBS were introduced into each well to block the vacant binding sites of the polystyrene surface. Following incubation for 1 h at 37°C, the antibodies were washed three times and 100 μl of the viscumin-containing samples under study in various dilutions were added to each well. Viscumin at different concentrations was used as a control sample. The incubation was performed for 1 h at 37°C. Biotin-labelled anti-viscumin monoclonal antibodies MNA9 at a concentration of 2 μg/μl were then applied. The following procedure included incubation for 1 h at 37°C, threefold washing, and incubation (1 h, 37°C) with the streptavidin–peroxidase conjugate and fivefold washing. The development was carried out for 20 min at 37°C using a TMB substrate buffer. The reaction was stopped by adding 50 μl of 10% sulfuric acid to each well. Colorimetric measurements were carried out on a Multiskan® PLUS-314 spectrophotometer at 450 nm.

The amount of viscumin incorporated into the microparticles was determined via solid-phase ELISA according to the above-described scheme. For this purpose, the sample of polylactide microparticles was completely hydrolyzed at 42°C for 48 h (5 mg of the sample in 10 ml PBS).

Evaluation of the cytotoxic properties of viscumin following its release from polylactide microparticles

The cytotoxic activity of viscumin liberated from biopolymer microparticles was determined using the MTT assay according to the earlier described procedure [17, 18]. The lethal dose of toxin (viscumin) for 50% of the cells (LD_{50}) was determined to assess the survival rate of the cells. Viscumin not subjected to encapsulation was used as the control. When calculating LD_{50}, the staining intensity of the cells cultured in the absence of a cytotoxic agent was assumed to be 100%. The results obtained in one of three typical experiments are presented (LD_{50} ± standard deviation).

RESULTS AND DISCUSSION

The typical SEM microimages of the experimental samples after their removal from the inlet chamber of the SCF setup are shown in *Fig. 2*. It is clear that the samples consist both of individual microparticles (10–50 μm) and particle agglomerates (up to 200 μm).

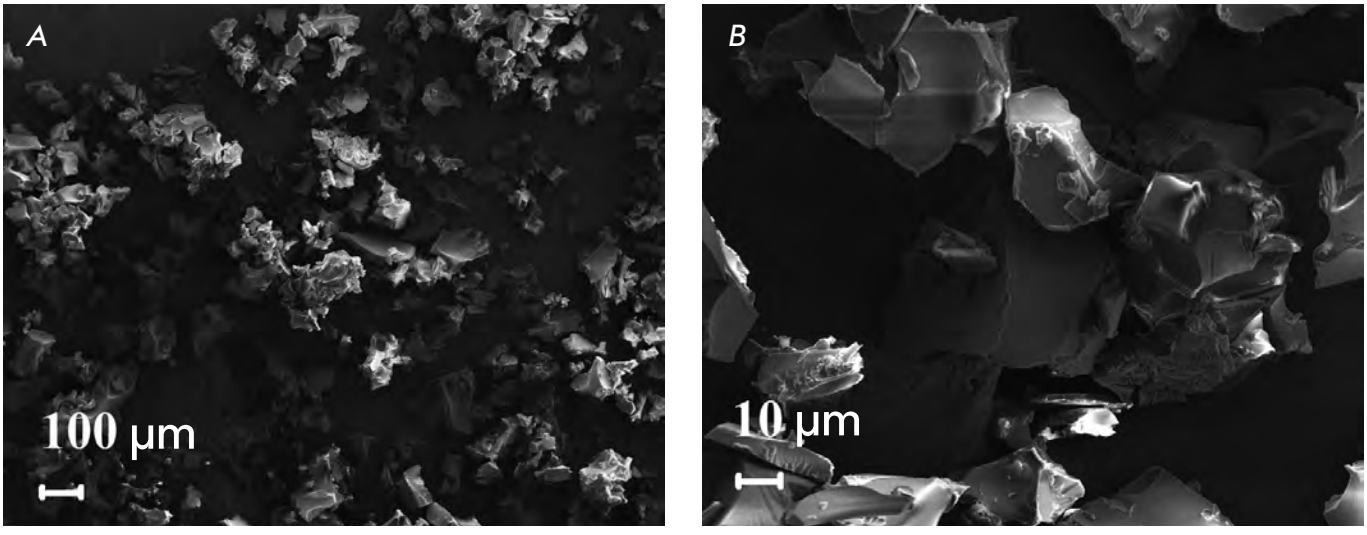

Fig. 2. Electronic microimages of polylactide microparticles with incorporated viscumin. (A) – overview, (B) – detailed structure.

Polylactide microparticles are dense bulk particles of irregular shape with an appreciably smooth surface.

The use of a powerful ultrasonic action made it possible not only to achieve more homogeneous stirring of the initial components (viscumin and polylactide), but to considerably reduce the viscosity of the polymer plasticized in sc-CO_2 due to the introduction of additional acoustic energy to the system, as well. The combination of these factors resulted in a cardinal change in the regime of subsequent dispersion of the resulting mixture into the inlet chamber under atmospheric pressure. In turn, the morphology of the bioactive polymer structures being formed drastically changed.

Thus, polylactide fibrous matrices consisting of porous microparticles of irregular shape (50–200 μm) formed when the magnetic stirrer was used alone under the same conditions [11]. Meanwhile, the use of intensive acoustic action resulted in the formation of dense individual microparticles with the characteristic size varying from 10 to 50 μm.

The previously described test system based on the monoclonal anti-viscumin antibodies MNA4 and biotinylated MNA-9 [14, 15] was used to determine the amount of viscumin in the samples. The system enables the specific determination of viscumin; its sensitivity threshold is approximately 0.8 ng/ml. The total amount of viscumin in a 5 mg sample consisting of polylactide particles was assessed using this test system. The total amount of viscumin appeared to be equal to 50 μg, which corresponds to 1 wt % and represents the amount of viscumin subjected to encapsulation. Presumably, this attests to the fact that the protein antigenic structure after the encapsulation remained unchanged.

It is reasonable to assume that some nonencapsulated viscumin could remain on the microparticle surface. In order to remove the unbound viscumin, 16.5 mg of the microparticles was washed twice in PBS (time points 0 and 10 min). The amount of viscumin in these samples was 0.145 μg (i.e., 0.09% of the total amount of the toxin subjected to encapsulation). Thus, the amount of encapsulated viscumin was equal to 99.91%. *Figures 3A* and *3B* show the amount of viscumin liberated from polylactide microparticles during a period from 0 to 120 h.

It is clear from *Fig. 3B* that the toxin amount in supernatants decreased with incubation time. This fact indicates the slow degradation of polymer microparticles with the gradual release of viscumin. An increase in the amount of viscumin in supernatants at points 6 and 24 h can be explained by longer incubation, when a greater amount of toxin is liberated from the degraded polymer. In addition, starting at point 48 h, viscumin was released more slowly. This type of kinetics of toxin release may be linked with the structure of polylactide microparticles; i.e., inside a microparticle it is denser than outside. A total of 0.134 μg of viscumin was released from the polylactide matrix after 120 h (except for the amount of viscumin detected in the samples after they were washed twice), which is equal to 0.08% of the initially encapsulated protein.

Viscumin belongs to type II ribosome-inactivating proteins; it can be used to remove eukaryotic target

cells [19–21]. It was demonstrated using the MTT assay that viscumin retains its cytotoxic activity after it is released from polylactide microparticles. The cytotoxic activity of viscumin in supernatants remained virtually the same as that of the untreated toxin: the concentration of the native viscumin causing the death of 50% of 3T3 cells (LD_{50}) after 48 h was equal to $7 \times 10^{-12} \pm 3 \times 10^{-12}$ M; LD_{50} of viscumin in the samples under analysis was equal to $7 \times 10^{-12} \pm 2 \times 10^{-12}$ M.

It should be mentioned that the morphology of the surface and internal structure of the polymer microparticles being formed can be varied over an appreciably wide range (from highly porous to virtually monolithic) by changing the regimes of SCF encapsulation and sputtering of the plasticized polymer blend; this will have a determining effect on the kinetics of release of bioactive components from these particles. The results obtained clearly demonstrated this fact. Thus, the viscumin-containing polylactide structures in [11] were represented by agglomerates of porous particles (with a characteristic porosity coefficient of 20–25%) of irregular shape, as opposed to the dense microparticles with smooth surfaces, obtained in the present work (*Figs. 3A* and *3B*). Correspondingly, the kinetics of viscumin release from these structures under identical conditions (suspension in PBS, slow stirring on a rocker at 22°C; samples were collected after two washings and after 30, 60, 120, 360, 1440, 2880, and 7220 min) was cardinally different from that shown in *Fig. 3A*.

Viscumin can be used in anti-tumor therapy [22]. The application of biocompatible biodegradable systems containing slowly releasing viscumin has considerable potential. Drug introduction via injection into the area of tumor growth becomes less traumatic due to the reduction in the size of the biodegradable microparticles containing viscumin and/or another specific anti-tumor cytotoxin.

The ultrasound-assisted technique of dry supercritical fluid encapsulation does not affect the cytotoxic and immunochemical properties of the viscumin incorporated into polylactide microparticles. The designed encapsulation technique ensures the gradual release of the encapsulated toxin from microparticles, which can provide a more prolonged therapeutic effect. ●

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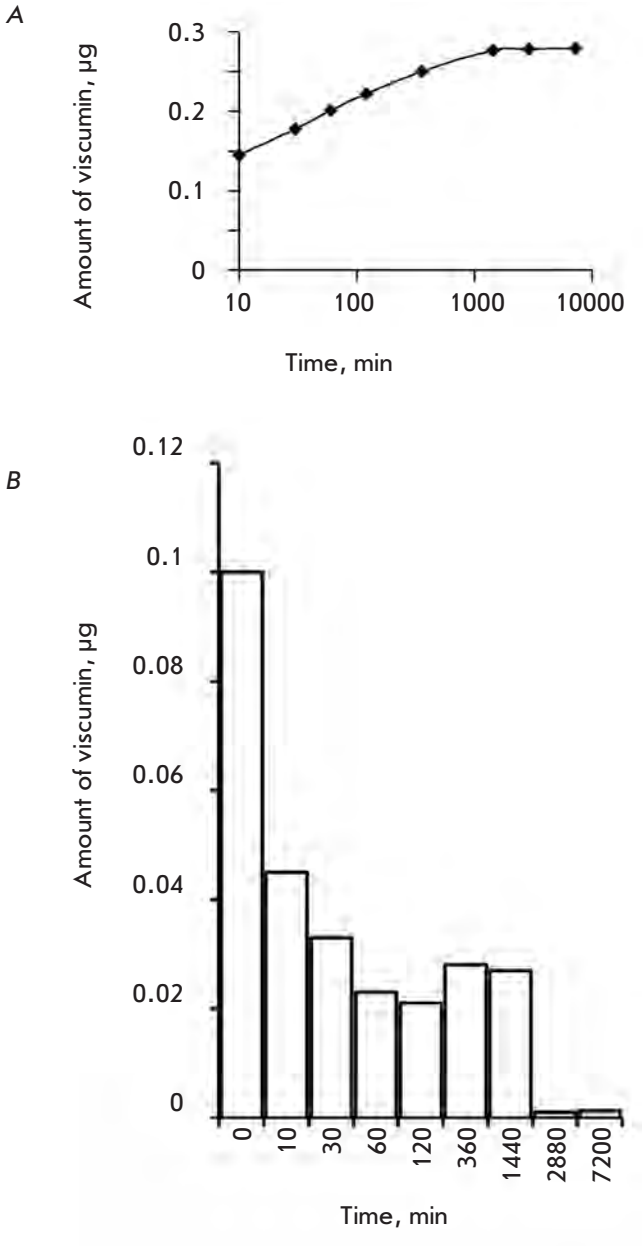

Fig. 3. Kinetics of the release of viscumin from polylactide microparticles. (A) – the overall amount of viscumin in the supernatants under analysis. (B) – the amount of viscumin released from microparticles as a function of incubation time.

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- Article title. Bold font. The title should not be too long or too short and must be informative. The title should not exceed 100 characters. It should reflect the major result, the essence, and uniqueness of the work, names and initials of the authors.
- The corresponding author, who will also be working with the proofs, should be marked with a footnote *.
- Full name of the scientific organization and its departmental affiliation. If there are two or more scientific organizations involved, they should be linked by digital superscripts with the authors' names. Abstract. The structure of the abstract should be very clear and must reflect the following: it should introduce the reader to the main issue and describe the experimental approach, the possibility of practical use, and the possibility of further research in the field. The average length of an abstract is 20 lines (1,500 characters).
- Keywords (3 – 6). These should include the field of research, methods, experimental subject, and the specifics of the work. List of abbreviations.
- INTRODUCTION
- EXPERIMENTAL PROCEDURES
- RESULTS AND DISCUSSION
- CONCLUSION

  The organizations that funded the work should be listed at the end of this section with grant numbers in parenthesis.
- REFERENCES

  The in-text references should be in brackets, such as [1].

RECOMMENDATIONS ON THE TYPING AND FORMATTING OF THE TEXT

- We recommend the use of Microsoft Word 2003 for Windows text editing software.
- The Times New Roman font should be used. Standard font size is 12.
- The space between the lines is 1.5.
- Using more than one whole space between words is not recommended.
- We do not accept articles with automatic referencing; automatic word hyphenation; or automatic prohibition of hyphenation, listing, automatic indentation, etc.
- We recommend that tables be created using Word software options (Table → Insert Table) or MS Excel. Tables that were created manually (using lots of spaces without boxes) cannot be accepted.
- Initials and last names should always be separated by a whole space; for example, A. A. Ivanov.
- Throughout the text, all dates should appear in the "day.month.year" format, for example 02.05.1991, 26.12.1874, etc.
- There should be no periods after the title of the article, the authors' names, headings and subheadings, figure captions, units (s – second, g – gram, min – minute, h – hour, d – day, deg – degree).
- Periods should be used after footnotes (including those in tables), table comments, abstracts, and abbreviations (mon. – months, y. – years, m. temp. – melting temperature); however, they should not be used in subscripted indexes (T_m – melting temperature; $T_{p.t.}$ – temperature of phase transition). One exception is mln – million, which should be used without a period.
- Decimal numbers should always contain a period and not a comma (0.25 and not 0,25).
- The hyphen ("-") is surrounded by two whole spaces, while the "minus," "interval," or "chemical bond" symbols do not require a space.
- The only symbol used for multiplication is "×"; the "×" symbol can only be used if it has a number to its right. The "·" symbol is used for denoting complex compounds in chemical formulas and also noncovalent complexes (such as DNA·RNA, etc.).
- Formulas must use the letter of the Latin and Greek alphabets.

- Latin genera and species' names should be in italics, while the taxa of higher orders should be in regular font.
- Gene names (except for yeast genes) should be italicized, while names of proteins should be in regular font.
- Names of nucleotides (A, T, G, C, U), amino acids (Arg, Ile, Val, etc.), and phosphonucleotides (ATP, AMP, etc.) should be written with Latin letters in regular font.
- Numeration of bases in nucleic acids and amino acid residues should not be hyphenated (T34, Ala89).
- When choosing units of measurement, SI units are to be used.
- Molecular mass should be in Daltons (Da, KDa, MDa).
- The number of nucleotide pairs should be abbreviated (bp, kbp).
- The number of amino acids should be abbreviated to aa.
- Biochemical terms, such as the names of enzymes, should conform to IUPAC standards.
- The number of term and name abbreviations in the text should be kept to a minimum.
- Repeating the same data in the text, tables, and graphs is not allowed.

GUIDENESS FOR ILLUSTRATIONS

- Figures should be supplied in separate files. Only TIFF is accepted.
- Figures should have a resolution of no less than 300 dpi for color and half-tone images and no less than 500 dpi.
- Files should not have any additional layers.

REVIEW AND PREPARATION OF THE MANUSCRIPT FOR PRINT AND PUBLICATION

Articles are published on a first-come, first-served basis. The publication order is established by the date of acceptance of the article. The members of the editorial board have the right to recommend the expedited publishing of articles which are deemed to be a priority and have received good reviews.

Articles which have been received by the editorial board are assessed by the board members and then sent for external review, if needed. The choice of reviewers is up to the editorial board. The manuscript is sent on to reviewers who are experts in this field of research, and the editorial board makes its decisions based on the reviews of these experts. The article may be accepted as is, sent back for improvements, or rejected.

The editorial board can decide to reject an article if it does not conform to the guidelines set above.

A manuscript which has been sent back to the authors for improvements requested by the editors and/or reviewers is reviewed again, after which the editorial board makes another decision on whether the article can be accepted for publication. The published article has the submission and publication acceptance dates set at the beginning.

The return of an article to the authors for improvement does not mean that the article has been accepted for publication. After the revised text has been received, a decision is made by the editorial board. The author must return the improved text, together with the original text and responses to all comments. The date of acceptance is the day on which the final version of the article was received by the publisher.

A revised manuscript must be sent back to the publisher a week after the authors have received the comments; if not, the article is considered a resubmission.

E-mail is used at all the stages of communication between the author, editors, publishers, and reviewers, so it is of vital importance that the authors monitor the address that they list in the article and inform the publisher of any changes in due time.

After the layout for the relevant issue of the journal is ready, the publisher sends out PDF files to the authors for a final review.

Changes other than simple corrections in the text, figures, or tables are not allowed at the final review stage. If this is necessary, the issue is resolved by the editorial board.

FORMAT OF REFERENCES

The journal uses a numeric reference system, which means that references are denoted as numbers in the text (in brackets) which refer to the number in the reference list.

For books: the last name and initials of the author, full title of the book, location of publisher, publisher, year in which the work was published, and the volume or issue and the number of pages in the book.

For periodicals: the last name and initials of the author, title of the journal, year in which the work was published, volume, issue, first and last page of the article. Must specify the name of the first 10 authors. Ross M.T., Grafham D.V., Coffey A.J., Scherer S., McLay K., Muzny D., Platzer M., Howell G.R., Burrows C., Bird C.P., et al. // Nature. 2005. V. 434. № 7031. P. 325–337.

References to books which have Russian translations should be accompanied with references to the original material listing the required data.

References to doctoral thesis abstracts must include the last name and initials of the author, the title of the thesis, the location in which the work was performed, and the year of completion.

References to patents must include the last names and initials of the authors, the type of the patent document (the author's rights or patent), the patent number, the name of the country that issued the document, the international invention classification index, and the year of patent issue.

The list of references should be on a separate page. The tables should be on a separate page, and figure captions should also be on a separate page.

The following e-mail addresses can be used to contact the editorial staff: vera.knorre@gmail.com, actanaturae@gmail.com, tel.: (495) 727-38-60, (495) 930-80-05