DISEASE TREATING METHOD

This invention belongs to the field of medicine, in particular, to the treatment methods involving administration of the preparations containing genetic material, and can be used for treatment of the impaired hematopoiesis connected with chromosomal defects and subsequent death of blood stem cells (BSCs) caused by the therapy with cytostatics or high radiation doses; for treatment of the diseases connected with the impairments of basic mechanisms involved in BSC differentiation towards specific hematopoietic elements without division, which develop in hemoblastoses caused by malignant transformations of hematopoietic cells making them unable to normally differentiate and leading to their uncontrolled differentiation; and for treatment of the diseases caused by chromosomal defects in stem cells (SCs), the treatment of which requires the appearance of genetically, phenotypically, and physiologically healthy SCs in the body.

A treatment method is known that is based on repair of point mutations in cells [U.S. patent no. 5,795,972, of August 18, 1998].

This method displays relatively low treatment efficiency and has a limited range of administration, because it requires that the mutations are precisely determined before commencement of the treatment.

Since a reliable proof that a particular mutation is the cause of a cancer is yet lacking, development of the method mentioned above requires a meticulous and long-term research into detection of particular mutations that require correction, thereby necessitating the development of the methods that would be applicable on the background of already available knowledge about the causes of cancer without emphasis on particular changes in the genome that induced malignization.

The methods involving a local administration of DNA fragments for treatment of precancerous states in the patient's skin are also known [US, no. 5,955,059, of September 21, 1999; US, no. 5,470,577, of November 28, 1995].

The applicability of these methods is limited, because neither particular DNA sequences nor DNA sources in the corresponding patents are specified. They propose using both the natural and synthetic DNA from "any appropriate source", for example,

salmon DNA with a length of 200 bp down to mononucleotides and nucleosides, including dimers, which are the most efficient according to the tests performed by the authors. However, the effect and consequences of administration of the foreign DNA on the human organism are yet to be studied in detail, which predetermines a certain danger in applying these methods.

The most close to the proposed use of fragmented DNA in its essence is the method based on administration of the preparation Polydan, which is a high purified standardized mixture of the sodium salts of polychlorohydrates of DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) derivatives produced from the sturgeon milt, administered as a slow (for 1.5–2 min) subcutaneous or intramuscular injections of the preparation previously heated in a vial in a hand to the temperature of patient's body [registration no. 95/292.8; registration no. 000552/01-2001].

The disadvantage of the closest design is a limited application field, as the active substance of the preparation is isolated from the tissues of a xenogeneic organism and contains both DNA and RNA molecules. Despite that the active substance of the preparation Polydan acts on the BSC genome in a manner similar to that of human DNA, namely, mimics the presence of chromosome breaks due to the presence of double-stranded breaks of the DNA fragments delivered to the nucleus, thereby activating BSCs to a terminal differentiation, and, due to a partial homology between the DNA of Polydan preparation and human chromosomal DNA, creates the conditions for the events of homologous recombination and preservation of the physical length and structure of chromosomes sufficient for a chain of successive mitoses, the substance of Polydan preparation introduces foreign sequences into the recipient genome.

This action of the preparation Polydan rescues the hematopoietic function of BSCs via the restoration and preservation of the functional size of BSC chromosomes and, presumably, does not influence the overall high-specialized properties of differentiated blood cells concurrently activating them to differentiation by its mimicking action; nonetheless, such rescued BSCs cannot be regarded as completely restored to their initial genetic state, as any other differentiation direction where the chromosomal loci affected by the recombination with foreign genetic material are involved will inevitably lead to defects in this BSC developmental pathway. The internal chromosome structure during such recombination becomes absolutely unpredictable, and the long-term

outcome of the leukocyte stimulation with the preparations of foreign DNA has not yet been studied.

The problem to be solved consists in the expansion of the application range of fragmented DNA to the treatment of the impaired hematopoiesis connected with chromosomal defects and subsequent BSC death, for example, in the therapy with cytostatics or high radiation doses; treatment of the diseases connected with the impairments of basic mechanisms involved in BSC differentiation towards specific hematopoietic elements without division, which develop in hemoblastoses caused by malignant transformations of hematopoietic cells making them unable to normally differentiate and leading to their uncontrolled proliferation; and for treatment of the diseases caused by chromosomal defects in stem cells (SCs), the treatment of which depends on the appearance of genetically, phenotypically, and physiologically healthy SCs in the body.

The necessary result is achieved by that the fragmented DNA is introduced into the patient's body; in addition, the fragmented DNA is allogeneic and consists of the fragments with a length of one to ten nucleosome units and constituting the complete genome of a patient in association with nuclear matrix proteins; the fragmented DNA is administered at an amount equal to or exceeding the amount of the DNA contained in the patient's blood plasma and tissue fluids but no more than 1500 ng/mL.

The effect of fragmented DNA is based on the ability of BSCs to respond to the action of fragmented DNA preparations. The DNA fragments delivered to the BSC nuclear space via the natural mechanism of homologous recombination substitute for the mutant regions in genes or restore the initial physical length of chromosomes, which were the reasons why BSCs lost their viability, thereby making unviable or mutant BSCs viable again. In this process, BSC chromosomes acquire the physical parameters that allow the BSCs to perform a series of successive mitoses and, in an optimal variant, the genome of a treated BSC becomes completely corrected and identical to the genome of an initial healthy BSC. Simultaneously, the fragments of extracellular DNA delivered to the nuclear space with their double-stranded ends mimic the appearance of functional breaks in BSC chromosomes, which are the particular inducers of initiating BSC terminal differentiation. This mimicking effect of extracellular DNA fragments present within the

BSC nucleus triggers the cell differentiation mechanisms and activates and maintains the BSC in a continuous active state of committed division.

The graphic sheet contains:

Fig. 1, Fig. 2, and Fig. 3: The effect of mouse and human DNA i.p. administered to mice before and after a lethal-dose irradiation on the mouse survival;

Fig. 5: Spleens from the mice exposed to a sub-lethal irradiation (a) treated with exogenous DNA and (b) untreated with exogenous DNA. Arrows indicate the spleen colonies appearing after treatment of irradiated mice with extracellular DNA from human placenta.

The proposed use of fragmented DNA for treating diseases is implemented as follows.

The fragmented DNA isolated from genetically and physiologically healthy donors is introduced into the patient's body (by intravenous, intramuscular, or intracutaneous injections; subcutaneously; intraperitoneally; by washing or application to mucosae and/or skin; by oral administration with or without preliminary neutralization of the acid stomach medium or using a special capsule insoluble in the stomach; intrarectally; intravaginally; intraocularly; intrarhinally; by intraocular injection; or by inhalation).

The amount of the administered preparation should be such that the concentration of fragmented DNA in the patient's blood and tissue fluids does not exceed the maximum permissible dose, amounting to 1500 ng/mL.

In the case BSCs are treated *ex vivo* for further autotransplantation, the BSCs should be treated with the fragmented DNA preparation at a concentration not exceeding 30 μg/mL.

When introduced into the patient's body, the fragmented DNA is delivered to the actively dividing cells of the body, including BSCs, via a natural delivery mechanism (the bloodstream and the specific receptors located on the surface of actively dividing cells).

Then the fragmented extracellular DNA delivered to the intracellular space is deposited with the interchromosomal compartment of the nucleus and undergoes an intranuclear processing.

In this case, the DNA fragments, being deposited in the interchromosomal compartment of the nucleus, substitute for the mutant regions of the chromosomes that have made BSCs unviable via the natural mechanism of homologous recombination. As a consequence of the mentioned events, a rescue, complete or partial, of BSC functions takes place as well as the activation of mitotic process and triggering of BSC differentiation. In this action of exogenous fragmented DNA on BSCs, of great importance is the ability of BSCs (and several other SC types, including embryonic SCs) to completely remodel their genome during several (one to three) first mitoses, bringing it to a committed state. In this process, numerous breaks occur in the BSC genome, which are an integral part of the genome remodeling. If the DNA fragments of an appropriate size in association with nuclear matrix proteins are present in the SC nucleus at this particular moment, they becomes accessible for integration into the genome via the homologous recombination mechanism; in addition, the activity of this process increases manifold owing to the numerous physiological functional breaks that appear in the chromatin. Moreover, the activating effect of DNA fragments on BSC differentiation is connected with their mimicking action. The fragments of extracellular DNA delivered to the nuclear space supplement the nucleus with numerous double-stranded breaks, which are recognized by the cell as its own functional chromosome breaks formed and rapidly repaired during BSC terminal differentiation. This mimicking effect of the extracellular DNA fragments localized within the BSC nucleus triggers the cell differentiation mechanisms as well as activates the committed BSC division and keeps it in a continuously active state.

Thus, the preparation of the fragmented allogeneic DNA isolated from physiologically and genetically healthy donors introduced into the body is adsorbed by BSCs during their division via a natural delivery mechanism typical of the actively dividing cells (a receptor-mediated pinocytosis). The fragmented DNA delivered to the nuclear compartment, the interchromosomal space, is deposited there and involved into homologous exchange with the corresponding chromosome loci when the BSC genome is remodeled and, correspondingly, numerous chromosome breaks are formed in the

chromatin. Due to the natural mechanism of homologous recombination, the DNA fragments deposited in the interchromosomal space substitute for the mutant chromatin regions, the mutations in which have led to BSC inviability, and can substitute for any mutant loci in SC genes, making them nonmutant. Correspondingly, the BSC genome becomes partially or completely corrected, thereby restoring the BSC mitotic potential or even completely restoring the impaired BSC genome. Concurrently, the fragments of extracellular DNA delivered to the nuclear space supplement the nucleus with numerous double-stranded ends, which the cell recognizes as its own functional chromosome breaks, which are formed and rapidly repaired during the BSC terminal differentiation. This mimicking action of the extracellular DNA fragments located within the BSC nucleus triggers the cell differentiation mechanisms as well as activates BSC division and maintains them in an active committed state.

The effect achieved by using the proposed invention can be substantiated by the following theoretical data.

According to the modern classification, the stem cells include the totipotent morula cells, pluripotent blastocyst cells (forming all body tissues), and multipotent regional germinative cells [Orkin SH, Morrison SJ. Stem-sell competition. Nature. 2002. V. 418. no. 6893. P. 25-27]. The regional or tissue-specific SCs are preserved over the entire life of an organism, are self-sustained, and provide a constant replacement of the lost cell populations. The tissue-specific SCs contribute to regeneration and maintenance of numerous if not all mammalian tissues, including the blood, liver, intestines, skeletal muscles, and central nervous system. Until recently, it has been considered that the tissue-specific SCs are determined into certain cell types and distributed in the tissues wherefrom they originate. Nonetheless, numerous papers that have recently appeared demonstrate a more pronounced plasticity of regional SCs [Bedada FB, Technau A, Ebelt Y, Schulze M, Beaun T. Activation of myogenic differentiation pathways in adult bone marrow-derived stem cells. Moll. Cell. Biology. 2005. V. 25. no. 21. P. 9509-9519; Kues WA, Petersen B, Mysegades W, Carnwath JW, Niemann H. Isolation of murine and porcine fetal stem cells from somatic tissue. Biology of reproduction. 2005. V. 72. P. 1020-1028]. For example, it has been confirmed that the neuronal SCs isolated from the mouse brain differentiate into the blood, skeletal muscle, and endothelial cells; the SCs of human adipose tissue, into bone, muscle, and cartilage cells; and mammalian dermal

SCs, into skeletal muscle, neuronal, glial, and adipose cells. It has been demonstrated that some rare cell populations, the so-called multipotent progenitor cells, are able to differentiate into derivatives of all three germinal layers. The ability to differentiate into the cells of unrelated tissues has been also demonstrated for SCs of the bone marrow, initially involved in formation and maintenance of the blood cell population.

The discovery of SC plasticity and their ability to differentiate into the cells of ectodermal, mesodermal, and endodermal origins, as well as the ability of SCs to migrate from the bone marrow and to supplement the SC pools of various tissues made it possible to formulate the concept of a common mechanism of tissue regeneration with SCs as a main player. This formed the background and induced an active development of a new direction, regenerative medicine, which is based on using SCs for restoration of the cell composition of organs and tissues impaired due to diseases, exposure to chemical agents, traumas, or age-related degenerative changes.

Among the main and most frequent negative actions on the regenerative processes in the body are X-ray and chemotherapies used for treating cancer diseases. Administration of hard radiation or cytostatics of various natures cause the death not only of cancer cells, but also of all actively proliferating cells, including SCs. In particular, a consequence of such exposures is a partial or total disruption of hematopoiesis, which leads to a negative prognosis. As is known, the postembryonic hematopoiesis is a physiological blood regeneration process compensating for physiological degeneration of differentiated cells.

High-dose chemotherapy causes an especial decrease in the blood regeneration potential; without a compensatory treatment, this leads to a high rate of toxic mortality. The method combining high-dose chemotherapy and grafting of autologous hematopoietic precursor cells is now widespread. The only source for restoration of hematopoiesis is hematopoietic SCs of the bone marrow. The SCs ($CD34^+$) for autografting are isolated from the bone marrow or peripheral blood, frozen in the presence of cryostabilizer (DMSO), and used for restoration of the hematopoietic function of the bone marrow impaired by radiation or high doses of cytostatics [Blume KG, Thomas ED. A review of autologous hematopoietic cell transplantation. American Society for Blood and Marrow Transplantation. 2000. P. 1-12].

The proposed use of fragmented DNA for treating diseases is fundamentally different from the method based on the action of mediator colony-stimulating molecules that are the growth factors stimulating SC activity. The BSC activity is regarded as an increase in the BSC proliferative potential and retention of the ability to differentiate and develop into particular cell types; the growth factors control these particular BSC properties. The growth factors include colony-stimulating factors (CSFs), interleukins, and inhibitory factors. All these substances are glycoproteins with a molecular weight of about 20 kDa and act as both the circulating hormones and local mediators controlling hematopoiesis and differentiation of specific cell types. The effect of growth factors expands on the BSCs, colony-forming units (CFUs), committed cells, and mature cells. In particular, a decrease in erythrocyte counts and, correspondingly, a decrease in oxygen partial pressure (PO_2) are the signals for erythropoietin production. Erythropoietin acts on the sensitive cells, CFU-E, thereby stimulating their proliferation and differentiation. Interleukins act on the polypotent SCs, the majority of CFUs, and sometimes on terminally differentiating cells. The inhibitory factors give an opposite effect, they slow down the hematopoiesis. A deficiency in these factors can be a cause of leukemia, characteristic of which is a considerable increase in the blood leukocyte counts. A factor inhibiting leukemia was isolated; this factor slows down the proliferation and differentiation of monocytes, macrophages. All these actions are regulatory events and are determined by the mediator molecules of growth factors, which act on various cellular mechanisms triggering or controlling the processes involved in chromatin replication and transcription of various proteins that mediate the metabolism of a proliferating and differentiating BSC. In these processes, growth factors are used for the restoration of blood hematopoietic function.

When using fragmented DNA for treatment, the impaired SC chromatin is repaired, thereby making the SC able to perform a normal mitosis and respond to the action of growth factors. In brief, if there is no object to be stimulated, no growth factor will help. On the other hand, the fragments of extracellular DNA delivered to the nuclear space mimic by their double-stranded ends the appearance of functional breaks in BSC chromosomes, which are inducers of BSC differentiation. This mimicking action of the extracellular DNA fragments localized within the BSC nucleus triggers the cell differentiation mechanisms as well as activates and maintains the committed BSC division.

As we see it, the mechanism involved in emergence and maintenance of the mutations in SC induced by mutagens or related to age-dependent processes is as follows.

The genomic DNA of any organism is subject to mutagenic action of environmental factors, such as ionizing radiation and chemical mutagens, and various internal factors, including the errors of the cell repair systems. The known repair mechanism corrects DNA damages or incorrect nucleotide substitutions and covalently links single-stranded (SSBs) and double-stranded (DSBs) breaks after mutational events [Hoeijmakers JH. Genome maintenance mechanisms for preventing cancer. Nature. 2001. V. 411. P. 366-374]. When the damages involve both DNA strands and have a considerable length, repair of such regions present a certain problem, and the corresponding damages frequently lead to mutations. DSBs represent an example of the damages that are almost uncorrectable by the repair mechanism, as they affect both the chromosome integrity and a direct integrity of DNA molecule. A rapid mechanism of DSB repair is known; it involves the proteins KU70/KU-86 and nuclear DNA-dependent protein kinase, which join the DNA ends and restore the genome integrity [Pfeiffer P. The mutagenic potential of DNA double-strand break repair. Toxicol. Lett. 1998. V. 96-97. P. 119-129]. It is impossible to restore the nucleotides of short DNA fragments that were lost because of simultaneous multiple breaks in DNA molecule. As a consequence of such losses, chromosome aberrations appear after the restoration of chromosome integrity; in this process, DSBs are the source of mutations.

Chemical cytostatics and hard γ-radiation are widely used in treating cancer; these agents interfere with the integrity of double-stranded DNA due to an alkylating effect in the former case and direct action of X rays and the free radicals generated by ionization in the latter case. Multiple DSBs formed in DNA in the case of such exposures lead to arrest of the synthetic processes in the nucleus and impairment of the cell cycle. Eventually, the self-destruction program is triggered, and either the cell enters the apoptosis or acquires profound genetic changes. These particular genetic changes, which are generated in all cells of the body (especially, in actively proliferating cells), lead either to cell death or appearance of mutant progeny.

Unfortunately, such treatments cause death not only of cancer cells, but also of other actively dividing cells of the body, such as epithelial cells, leukocytes, the SCs of

white and red blood lineages, hair follicle cells, and others. The most dangerous side effect is destroying of the blood cells providing the active immune response. Several researchers postulated the existence of a natural mechanism that can influence the genetic component of the cell in a multicellular organism utilizing the extracellular genomic DNA from biological fluids as an external genome standard [Yakubov LA, Petrova NA, Popova KA, Semenov DV, Nikolin VP, Oskina IN. Role of extracellular DNA in maintaining of stability and variability of cellular genomes. Doklady Biochemistry and Biophysics. 2002. V. 382. P. 31-34; Yakubov LA, Popova NA, Nikolin VP, Semenov DV, Bogachev SS, Os'kina IN. Extracellular genomic DNA protects mice against radiation and chemical mutagens. Genom Biol. 2003. V. 5. P. 3]. According to the proposed mechanism, the DNA-binding receptors located on the cell surface deliver the fragments of genomic DNA from the ambient medium to the nucleus, where these fragments substitute for the corresponding homologous fragments in the cell genomic DNA via the mechanism of homologous recombination.

Then we will consider how the mutated DNA fragments from the cells exposed to mutagenic effects are involved in progressive development of mutational changes in the body.

The elements of DNA turnover in the body, such as the extracellular DNA of the bloodstream, DNA transport to the nucleus, and somatic homologous recombination, can be the essence of the mechanisms capable of removing any fixed or newly formed mutations from the genome. The efficiency of this process significantly depends on the quality and properties of the external DNA standard. On the one hand, this mechanism can remove mutations by substituting the exogenous nonmutant sequences for them and, on the other, this mechanism can introduce mutations into the cell, by substituting mutant exogenous homologous fragments for healthy sequence of the cell. The latter variant is especially applicable to the situation occurring in the body immediately after exposure to a strong mutagenic agent. It is known that chemical mutagens as well as hard irradiation induces the apoptosis; consequently, the DNA fragments carrying a large number of manifold defects including lethal mutations are released into the blood [Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res. 1977. V. 37. P. 646-650; Nikolin VP, Kaledin VI, Matienko NA, Gruntenko EV. Cis-Diamminedichloroplatinum (II) as a potential

chemotherapeutic agent against neoplastic lesions in the liver. J. Clinical Hemat. Oncol. 1979. V. 9. P. 198-207].

The mechanism of DNA transport delivers all these fragments to cells and homologous recombination incorporates these fragments into the cell genome. Then the cells that received the DNA fragments carrying lethal mutations die and successively release their genetic DNA carrying mutant sequences into environment. The cells that received the DNA molecules lacking lethal mutations survive and then can form the start population for selection, for example, of a cancer clone. The pool of apoptotic DNA fragments can contain the fragments formed by repair without taking into account the homology. Such fragment will contain the linked DNA fragments from different genomic regions from the same or different chromosomes. Such chimeric fragments will be integrated into to the genome of a recipient cell taking into account the homology of the terminal regions of the fragment. As these ends can originate from different chromosome regions or different chromosomes, the recombination events can lead to various chromosome aberrations. It is assumed that the mutant DNA fragments from the cells being exposed to a strong mutagen will be repeatedly delivered from dead cells to living cells. These fragments will induce the same mutations and chromosome aberrations in living cells; correspondingly, these fragments during many rounds of cell death will spread the defect that was caused by the primary exposure to mutagen.

According to the existing theoretical and experimental data, underlying our reasoning, if the bloodstream of an organism exposed to a strong mutagen or a high radiation dose contains healthy DNA fragments constituting together the whole genome homologous to the recipient genome, they will compete in the system of DNA turnover with the DNA fragments released into the blood as a result of the above described events after exposure to a mutagen. The healthy DNA fragments will be a substrate for the repair homologous recombination occurring in the nucleus, and their involvement in repair processes will lead to correction of mutant loci and, as a consequence, to generation of a healthy population of the corresponding cell group. The preparation of fragmented DNA contains the pool of healthy fragments of specially treated human DNA, which take part in the direct action on the damaged SC genome eventually leading to the restoration of chromosome integrity and the BSC ability to perform a series of successive mitoses with subsequent differentiation.

The proposed use of fragmented DNA for the treatment involving the action on BSCs can be applied in the case of any genetic abnormality by correcting it in the pool of BSCs, which are then grafted into the same organism.

The effect achieved when using the proposed invention can be grounded with the following theoretical data.

Each cell of the body contains genomic DNA, coding for the information about both all the proteins of all cells of the body and the spatial arrangement of the genes in the nucleus, necessary for a correct spatial and temporal expression pattern of these proteins. Thus, the genomic DNA is the template of the life of an organism that comprises the information about both the overall organism and its development. Initially, the genomic DNA is identical in all the cells of the organism. However, the genomic DNA of each cell with the growth of the organism is exposed to mutational impacts caused by both environmental factors and the errors occurring during cell replication and repair. It is known that the repair mechanisms change incorrect or defective DNA bases to join again the ends of DNA molecule after SSBs and DSBs in the strands immediately after mutational events. For this purpose, the repair mechanisms can utilize the second DNA strand. In the cases when the impaired regions are very long and involve both DNA strands, the repair becomes problematic and a mutation can emerge. Thus, environmental mutagenic factors and mistakes of the cell repair and replication are the sources of somatic mutations in the cell. The pluripotent SCs are most important for the restoration potential of the body. The defects in genomic structure of these cells always entail the development of a defect tissue and a pathology. The only pathogenetically justified approach to treatment of the diseases connected with genetic abnormalities, including the abnormalities in SC genome, is the change of the genome of these cells in an individual by any method that would restore the genetic homeostasis characteristic of the healthy cell.

Numerous methods of gene therapy (gene targeting) have been developed in the modern experimental medicine; however, all these methods have serious shortcomings and are rather inefficient for a complex eukaryotic organism.

The classical gene targeting methods are directed to correction of chromosome mutations using the viral constructs capable of efficiently introducing the working gene

copies into the cell genome. Nonetheless, providing a random insertion of a gene and the regulatory regions, these approaches are not free from several problems, including the dependence of gene expression on the integration site, silencing of the integrated virus genome, and insertion mutagenesis associated with carcinogenesis [Khon DB, Sadelain M, Glorioso JC. Occurrence of leukaemia following gene therapy of X-linked SCID. Nature Rev. Cancer. 2003. P. 477-488; Persons DA, Nienhuis AW. Gene therapy of hemoglobin disorders. Hematol. Rep. 2003. V. 2, P. 348-355]. An attractive alternative to these methods could be the correction or replacement of a mutant gene within the chromosome via the natural homologous recombination with a DNA template lacking the corresponding mutation delivered to the cell; however, the efficiency of this method is very low, as demonstrated by early attempts to use it [Yanez RJ, Porter AC. Therapeutic gene targeting. Gene therapy. 1998. V. 5, P. 149-159; Rothstein RJ. One-step gene disruption in yeast. Methods Enzymol. 1983. V. 101. P. 202-211]. Historically, the strategy of gene replacement developed in yeasts and later, in mammalian cells, to overcome the problems of insertion mutagenesis consisted in the use of linear recombinant DNA with its ends homologous to the regions flanking the target gene and the gene itself replaced with the selected marker [Thomas KR, Capecchi MR. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell. 1987. V. 51. no. 3. P. 503-512]. This type of gene targeting is named “ends-out”, as the ends of the construct correspond to two chromosome DNA sequences located outside of the target gene. The ends of an ends-out gene targeting construct are recombinogenic and enhance replacement of the gene sequence confined between the terminal homologies. It has been recently demonstrated that when replacing a whole yeast gene with a heterologous sequence or when substituting a single base pair by this method, the process in initiated by two independent strand invasions, which implies the search for homology and the recombination events performed by the two terminal segments of a therapeutic construct [Langston LD, Symington LS. Gene targeting in yeast is initiated by two independent strand invasions. Proc Natl Acad Sci USA. 2004. V. 101. no. 43. P. 15392-15397]. Earlier, a similar mechanism was proposed for explaining the ends-out gene targeting in mammalian cells [Li J, Read LR, Baker MD. The mechanism of mammalian gene replacement is consistent with the formation of long regions of heteroduplex DNA associated with two crossing-over events. Mol Cell Biol. 2001. V. 21. no. 2. P. 501-510]. As the somatic homologous recombination (HR) has been preserved in the evolution for

billions years from the protists to mammals, it is evident that this mechanism must perform an absolutely necessary and universal function in all the kingdoms of the living world and must perform it efficiently. The somatic HR in unicellular yeasts is the main mechanism of precision DSB repair; therefore, its efficiency must be very high to provide the rescue of an individual. Initial experiments with microinjections of plasmids into the cell nucleus led to discovery that the plasmids always integrated into the cell genome as a single concatemer comprising all (frequently, more than 100) injection plasmids linked into one molecule in a tail-to-head manner and the mechanism worked in a very efficient manner [Capecchi M. Generating mice with targeted mutations. Nature Medicine 2001. V. 7. P. 1086-1090]. However, a low efficiency of the classical gene targeting utilizing HR in mammalian cells is explainable by the discrepancy between the problem specified for HR and the HR properties and possibilities selected by the evolution for performance of its own yet still unknown function. The data described in the literature make it possible to identify some factors that are critical for the operation of HR mechanism.

The linear size of the DNA fragments delivered to the site of recombination complex is important for an efficient HR. For example, a 100-fold exponential increase in gene targeting efficiency was observed with the increase in homology length of the rescue construct from 2 to 14.5 kbp; however, the achieved efficiency, 10^{-5} events of gene rescue per cell, was still insufficient for using this method without selection [Deng C, Capecchi MK. Reexamination of gene targeting frequency as a function of the extent of homology between the targeted vector and the target locus. Mol. Cell Biol. 1992. V. 12. P. 3365-3371]. A drastic contrast to these data is the results of using small fragment homologous replacement (SFHR) for correcting a mutant gene; the reported efficiency was 1–20% [Gruenert D, Bruscia E, Novelli G, Colosimo A, Dallapiccola B, Sangiuolo F, Goncz K. Sequence-specific modification of genomic DNA by small DNA fragments. Clin. Invest. 2003. V. 112, P. 637-641]. The gene targeting efficiency obtained in these experiments suggests that HR either prefers to operate with short fragments or ligates these short fragments with ligase IV in a head-to-tail manner into the multimers, which are most efficient in recombination events.

The necessary condition for obtaining the recombinant product in mammalian cells is the appropriate length of the terminal homology, which amounts to about 200 bp

[Lin Y, Lukacsovich T, Waldman AS. Multiple pathways for repair of DNA double-strand breaks in mammalian chromosomes. Mol. Cell Biol. 1999. V. 19. P. 8353-8360; Nassif N, Engels W. DNA homology requirements for mitotic gap repair in Drosophila. Proc Natl Acad Sci USA. 1993. V. 90. no. 4. P. 1262-1266].

The principal condition for HR is a linear from of the delivered therapeutic DNA as a source double-stranded ends. Circular forms of therapeutic plasmids are almost not involved into the homologous exchange. Generation of DSBs and formation of double-stranded ends of DNA molecule locally activates and boosts the chromosomal and extrachromosomal recombination and gene targeting [Kucherlapati RS, Eves EM, Song KY, Morse BS, Smithies O. Homologous recombination between plasmids in mammalian cells can be enhanced by treatment of input DNA. Proc Natl Acad Sci USA. 1984. V. 81, P. 3153-3157]. A recent publication in the *Nature* [Urnov FD, Miller JC, Lee YL, Beausejour CM, Rock JM, Augustus S, Jamieson AC, Proteus MH, Gregory PD, Holmes MC. Highly efficient endogenous human gene correction using designed zinc-finger nucleases. Nature. 2005. V. 435. P. 646-651] reports using of this principle for induction of the correction of the IL2R gene where a point mutation in exon 5 causes a lethal hereditary disease, severe combined immunodeficiency (SCID), linked to the X chromosome. The authors demonstrated a high (up to 20%) level of gene correction by transfecting the cells with genetic constructs expressing synthetic zinc-finger nucleases, capable of specifically cleaving the sequence in the vicinity of the mutation in IL2R gene in genomic DNA, and a plasmid carrying the nonmutant fragment of the same gene covering the region of SCID mutation.

Presumably, a principle ability of HR to very efficiently perform gene correction is connected with the initiation of the processes by emergence of free double-stranded DNA ends in the nuclear space independently of the origin of these ends—whether they are the ends of a broken chromosome formed as a result of DSBs or the double-stranded ends of DNA fragments delivered to the nucleus and representing typical ends-out structures. Any unmodified fragment of genomic DNA with its two ends homologous to two sequences of chromosomal DNA and the central part capable of substituting for the corresponding genomic fragment and correcting a mutation is a typical ends-out construct. This means that the ends of the fragment will initiate the search for homology and the HR with genomic DNA, which will lead to replacement of the genomic DNA

fragment with the central part of the fragment delivered from the outward space to the cell. When using genetically correct exogenous DNA, these events will lead to correction of a genetic defect [Yakubov L, Popova N, Nikolin V, Semenov D, Bogachev S, Oskina I. Extracellular genomic DNA protects mice against radiation and chemical mutagens. Genome Biology. 2003. V. 5. P. 3].

Another important moment likely to influence the HR efficiency and rescue defective chromosomal loci is the phenomenon of genome opening, observed in SCs and other differentiating cells when they enter the differentiation pathway; this phenomenon was discovered by several research teams yet left without the deserved attention [Farzaneh F, Zalin R, Brill D, Shall S. DNA strand breaks and ADP-ribosyl tranferase activation during cell differentiation. Nature. V. 300. P. 362-366; Johnstone AP, Williams GT. Role of DNA breaks and ADP-ribosyl transferase activity in eukaryotic differentiation demonstrated in human lymphocytes. Nature. V. 300. P. 368-370; Vatolin AY, Okhapkina EV, Matveeva NM, Shilov AG, Baiborodin SI, Philimonenko VV, Zhdanova NS, Serov OL. Scheduled perturbation in DNA during in vivo differentiation of mouse embryo-derived cells. Mol. Reprod. Develop. 1997. V. 47. P. 1-10].

When studying the ribosyltransferase activity, generation of numerous SSBs in the chromatin of differentiating cells was observed, suggesting the involvement of genetic material in the remodeling, which is an obligatory and integral part of differentiation and subsequent changes in gene expression. Different organization of genetic material after chromatin remodeling as result of generated SSBs or DSBs is observed in different tissues of the same individual. The number of breaks per haploid genome of differentiating cells is estimated in various papers as several hundreds (100–300 breaks per haploid genome). It has been also demonstrated that this phenomenon is not a defect in the repair system; it is rather a necessary and obligatory step in the cell terminal differentiation. For example, when about 600 additional breaks were induced in the cell by hard radiation at the moment when the cell had the maximal number of immanent breaks, the rate of repair of these 600 breaks was the same as that in the nondifferentiating cells. However, the number of breaks reduced to the initial level. It is important for the essence of this invention that the number of rearrangements in the genome significantly increases at the moment of terminal differentiation and generation of chromatin breaks. In particular, it has been demonstrated that the rate of sister

chromatid breaks is five–sixfold higher during the preimplantation period, i.e., during the time when the chromatin remodeling and functional chromatin breaks are observed. In the first three mitoses, when the dramatic genome remodeling is observed, other recombination events also take place, in particular, shuffling of minisatellites and extension of simple repeat chain. The putative mechanism of such exchanges in the genome is homologous recombination. Nonetheless, the first step in all these events is the generation of functional breaks in chromatin DNA.

Presumably, in the presence of therapeutic fragmented DNA in the nuclear space during the genome remodeling in the first three SC mitoses, the HR takes place between the extracellular exogenous fragments of therapeutic DNA and the SC chromatin regions that became accessible, and this HR leads to the rescue of defective loci. This is what rescues the SC from death. In this process, the initial abilities of the SC to undergo a series of successive mitoses and to determine the direction of terminal differentiation are restored. On the other hand, the fragments of extracellular DNA delivered to the nuclear space with their DSBs mimic the generation of functional breaks in BSC chromosomes, which are the inducers of BSC terminal differentiation. This mimicking action of the extracellular DNA fragments localized within the BSC cells triggers the mechanism of cell differentiation as well as activates and maintains the committed BSC division.

The stated technical problem is solved, as it is based on the developed theoretical concept of the extracellular DNA turnover in the body, which has been confirmed by numerous experiments. Below we formulate the main postulates of the concept of the extracellular DNA turnover in the eukaryotic organism.

Extracellular fragmented genomic DNA containing mutant sequences at the frequencies matching to the frequencies of the corresponding mutations in the cells is formed as a result of the programmed cell death, or apoptosis, and is always present in the intercellular space and blood plasma [Anker P, Mulcahy H, Chen XQ, Stroun M. Detection of circulating turnover DNA in the blood (plasma/serum) of cancer patients. Cancer Metastasis Rev. 1999. V. 18. P. 65-73; Giacona MB, Ruben GC, Iczkowski K, Roots T, Porter D, Sorenson G. Cell-free DNA in human blood plasma: length measurements in patients with pancreatic cancer and healthy controls. Pancreas. 1998. V. 17. P. 89-97]. Cells are capable of engulfing the formed DNA or chromatin fragments via a receptor-mediated mechanism and transporting these fragments to the cell nuclei

[Yakubov LA, Deeva EA, Zarytova VF, Ivanova EM, Ryte AS, Yurchenko LY, Vlassov VV. Mechanisms of oligonucleotide uptake by cells: Involvement of specific receptors? Proc Natl Acad Sci USA. 1989. V. 86, P. 6454-6458; Shestova OE, Andreeva AY, Vlasov VV, Yakubov LA. Transport of complexes of oligonucleotides with cell-surface proteins to the cell nucleus. Dokl Akad Nauk. 1999. V. 368. P. 264-267. In Russian]. The delivered fragments recombine with the genomic DNA in the nuclei via the mechanism of HR and are able to initiate this process themselves. Presumably, the combination of these three processes composes the mechanism capable of controlling the genetics of somatic cells at the level of the overall organism by correcting mutations or inducing genetic changes in the cells with involvement of the extracellular DNA of tissue fluids and blood plasma as an external genomic standard. This mechanism can form the background for the genome integrity of the organism's cells. This mechanism can eliminate the emerged and fixed mutations as well as insert mutations into cells with the help of mutant sequences of the extracellular DNA standard. If this mechanism could use the DNA standard almost free of harmful mutations, its only function will be elimination of mutations from cells of the body. Since the allelic genes are usually highly homologous and frequently differ in relatively small regions of changed sequences, the other aspect of the function of this putative mechanism could be the replacement of sequences of the allelic genes in chromosomes with alternative sequences, similar to substitution of nonmutant sequences for mutant variants.

The stated technical problem is also solved by that the complete genome of an individual is introduced into the organism as a set of polynucleotide molecules that are fragments of the genomic DNA, where it circulates, is engulfed by the SC, and reaches the nucleus, where it is deposited and involved in the HR with the cell genomic DNA during the BSC genome remodeling, which is accompanied by generation of physiological SSBs. Thus, the possibility arises to correct the genetic defects present in the SC by introducing into the organism a complete genome as a set of polynucleotide molecules that are fragments of the genomic DNA free of genetic defects.

The proposed method implies using of the fragments of genomic DNA comprising the complete genome of a physiologically and genetically healthy individual associated with the proteins of nuclear matrix. The size of exogenous fragments fits the size of the fragments circulating in the blood plasma of the treated organism, which

appeared due to apoptotic nucleolysis (1–30 nucleosome units). In the prototype methods, a xenogeneic DNA in combination with RNA molecules is used. This DNA is not closely related, is not associated with the proteins of nuclear matrix, and does not fit the size of the natural apoptotic DNA of the organism, amounting to 1–30 nucleosome units.

These distinctive characteristic features of the use of fragmented DNA for treating diseases based on the ability of BSCs to respond to the preparation of fragmented DNA were studied in the experiments with laboratory mouse strain (*in vivo*). The proposed use of fragmented DNA for treating diseases based on the ability of BSCs to respond to the preparation of fragmented DNA was studied in the experiments with isolated human SCs (*ex vivo*).

The restoration of hematopoiesis, BSC rescue, and, as a consequence, saving of mice from death after a lethal exposure to hard radiation was studied in the experiments with mice (in an *in vivo* system). We conducted a set of experiments to study the protective effect of fragmented DNA preparation at various irradiation doses. There are early classical experiments in which the lethally irradiated mice (which lost their own hematopoietic cells) received suspension of the bone marrow or the fraction enriched with BSCs. After this injection, the cell colonies formed by the donor BSC progenies developed in the spleen. Each BSC in the spleen forms one colony and is named the spleen CFU. The proliferative activity is modulated by colony-stimulating factors and interleukins. Colony counting makes it possible to assess the number of CSs delivered by infusion to the bloodstream. Thus, it has been found that 50 SCs is present for 10^5 cells of the bone marrow; 3.5 SCs, per 10^5 cells of the spleen; and 1.4 SCs, per 10^5 blood leukocytes. Study of the purified BSCs with electron microscope demonstrates that the BSC in their ultrastructure are similar to small dark lymphocytes.

Study of the cell composition of spleen colonies detected two differentiation lineages of the studied SCs. One lineage gives rise to the multipotent cell that is the ancestor of granulocyte, erythrocyte, monocytes, and megakaryocyte hematopoietic lineages (CFI-GEMM). The second lineage gives rise to the multipotent cell that is the ancestor of lymphopoiesis (CFU-L).

According to the theoretical concept proposed here, the results of these experiments could also stem from the effect of extracellular DNA present in the introduced BSC specimen as well as from the integration of live SCs into lymphoid organs and colony formation in the spleen. This DNA genetically corresponded to the genome of healthy BSCs, and its action on the damaged immanent mouse SCs could lead to the restoration of SC viability via the HR mechanism described in this invention. We verified this hypothesis under analogous conditions using either DNA isolated from mouse (allogeneic) or the preparation of fragmented DNA isolated from human placenta as a therapeutic agent.

Materials and Methods

Three-months-old CBA/Lac female mice kept and bred at the animal breeding facility of the Institute of Cytology and Genetics were used in the experiments; 9–10 mice were kept in separate plastic cages. Water and feed were freely available. The mice were fed standard granulated PK120-1 (Laboratorsnab, Moscow, Russia) and irradiated with an IGUR-1 (Russia, 137Cs) γ-radiation unit. Four experiments were performed.

In the first three experiments, DNA at different doses and of different origins were tested. The DNA from CBA mouse organs or human placental DNA was i.p. administered to mice before and after irradiation.

The lifespan of mice in each group were recorded.

In experiment 4, the effect of human placental DNA on BSCs was assessed in the mice exposed to a sub-lethal radiation dose. These mice received i.p. the human placental DNA before irradiation. Mice were euthanized on day 10 to count the number of spleen hematopoietic colonies. Bone marrow is a radiosensitive tissue and, consequently, the BSCs exposed to γ-radiation died. It was assumed that the exogenous DNA allowed BSCs to survive by having gained access to the cells and becoming involved in repair. The rescued BSCs migrated to colonize the lymphoid organs, such as lymph nodes, spleen, and thymus. This promoted further hematopoiesis recovery and mouse survival.

Experiment 1

The mice were irradiated at a dose of 9.6 GR (1.4 GR/min). Ten mice (with label) received i.p. 1 mg of the DNA isolated from organs of CBA mice 30 min before irradiation (group 2). The remaining mice after irradiation were randomly separated in three groups.

The mice of group 1 i.p. received physiological saline solution (0.2 mL) 30 min after irradiation and in the next 2 days.

The mice of group 3 received the DNA of CBA mice (1 mg in 0.2 mL of physiological saline solution) 30 min after irradiation and additionally 0.5 mg of the DNA each next 2 days.

The mice of group 4 received the DNA isolated from human placenta according to the same scheme.

Figure 1 shows the data on the effect of mouse and human DNA preparations on the survival rate of the mice after exposure to a lethal dose of γ-radiation.

Experiment 2

The mice were irradiated in a γ-unit (^{137}Cs) at a dose of 9.5 GR (1.3 GR/min). One group of mice (n = 10) i.p. received the DNA isolated from organs of CBA mice: 1–2 mg 20 min before the exposure and ½ of the initial dose 30 min, 24 h, and 48 h after the exposure. The control group i.p. received physiological saline solution at a dose of 0.2 mL according to the scheme of DNA administration.

Figure 2 shows the characteristics of the radioprotective action of allogeneic DNA i.p. administered to the mice before and after the exposure to a lethal γ-radiation dose.

Experiment 3

The mice were irradiated in a γ-unit (^{137}Cs) at a dose of 9.5 GR (1.3 GR/min). The control mice (n = 10) i.p. received 0.2 mL of physiological saline solution 10 min before and 30 min, 24 h, and 48 h after the exposure. The preparation of human placental

DNA was administered 20 min before the irradiation (1 mg) and 30 min, 24 h, and 48 h after the irradiation (0.5 mg). The DNA isolated from mice was administered according to the analogous scheme.

Figure 3 shows the radioprotective action of allogeneic DNA i.p. administered to mice before and after the lethal γ-irradiation.

Experiment 4

CBA mice (n = 20) were exposed to γ-radiation at a dose of 800 R (8.21 Gy). The control mice (n = 10) i.p. received 1 mg of human placental DNA before the irradiation and additionally 0.5 mg of the DNA 30 min after the exposure, 0.25 mg on day 2, and 0.17 mg on day 3 after the exposure. The control mice (n = 10) received physiological saline solution (0.2 mL) according to the same scheme. On day 10, the animals were euthanized to determine the number of spleen hematopoietic colonies (Table 1).

Table 1. The number of spleen colonies formed in mice after the exposure to sub-lethal radiation dose combined with exogenous DNA therapy as compared with the untreated controls

Control mice	0	0	0	0	0	1	1	1	1	2	**0.6** ± 0.22
Treated mice	0	0	1	3	4	5	9	24	30	33	**10.9** ± 4.09

Despite a small sample size and a wide variation in the number of spleen colonies in the treated group, the differences between the samples were significant at $p > 0.95$. The average number of spleen colonies increased 18-fold after i.p. DNA administration as compared with the control. The effect of administered DNA was specific in that it either occurred or not in a particular individual. It is encouraging that 50% of the treated mice positively responded to the treatment (Fig. 4).

Thus, administration of allogeneic DNA and xenogeneic human placental DNA to mice allows the animals to survive after irradiation with a lethal dose; i.e., the specially treated fragmented DNA in combination with nuclear matrix proteins displays a pronounced radioprotective action. The observed survival of experimental animals is connected with the preservation of BSC viability provided by the action of the DNA preparations and the subsequent endogenous colony formation in the mouse spleen. The variation of individual sensitivity in the experiments on both survivability and counting

of CFU-S is most likely connected with whether or not the therapeutic DNA was delivered to the BSCs at the moment when the functional breaks manifesting the CS transition to the terminal differentiation were generated.

Utilization of the BSC ability to respond to the fragmented DNA preparation, described in the proposed method for using DNA for treatment of the mentioned diseases, was studied in experimentally isolated human SCs (*ex vivo*).

The bone marrow SCs of hemoblastosis patients, which are damaged as a result of leukemization of the bone marrow and exposure to high-dose programmed chemotherapy with cytostatics, are very convenient objects to study the therapeutic and regenerative properties of fragmented DNA preparation. This is determined by that, on the one hand, SCs are able to give rise to all hematopoietic lineages and, on the other, to maintain the constancy of their quantitative and qualitative composition without any external inflow. In addition, the mechanisms of SC self-reproduction and differentiation are concurrently the mechanism controlling the kinetic equilibrium in the overall pool of hematopoietic cells. In hemoblastosis, the fundamental mechanisms of SC homeostasis are damaged, and the underlying causes are particularly determined by the presence of both the primary genetic defects that cause the development of malignant hemoblastoses and the secondary damages of their genetic machinery connected with the used treatment by high doses of cytostatics.

Based on the information briefed above, the goal of this study was to determine the modulatory effect of fragmented human DNA preparation on the proliferative activity of mononuclear cells and differentiation of stem hematopoietic cells of the bone marrow in hemoblastosis patients after programmed chemotherapy.

The main goal of this stage of the work was to assess *ex vivo* the effect of human exogenous DNA on various functional parameters of the bone marrow cells of hemoblastosis patients treated with programmed polychemotherapy. The program of this research stage comprised the study of the action of fragmented DNA preparation on the proliferative activity of bone marrow mononuclear cells and the generation of hematopoietic colonies by hematopoietic SCs.

Results

The object of the study was the mononuclear cells (MNCs) of the bone marrow. The MNC fraction was obtained from the bone marrow aspirate by separation using Ficoll–Verografin density gradient (ρ = 1.078 g/cm^3). The experimental group was formed of 10 hemoblastosis patients after the second-line programmed chemotherapy.

The bone marrow was sampled by trepanation biopsy, performed during planned diagnostic examination of the patients after the programmed high-dose therapy with cytostatics. The trepanation biopsy of bone marrow was performed at the specialized Hematology and Transplantation Department with the Immunopathology Clinics of the Institute of Clinical Immunology, Siberian Branch of the Russian Academy of Medical Sciences (Table 2).

Table 2. Characterization of the bone marrow MNCs

Patients	**Yield of MNCs (10^6/mL)**	**CD34+ (%)**
V-kin	2.4	3.16
G-k	2.8	3.24
L-v	5.1	4.35
P-na	2.8	4.26
Sh-ra	4.8	2.60
D-va	2.04	5.63
K-n	2.16	3.44
K-ch	8.0	4.94
K-va	3.8	1.71
Kol-va	1.7	3.87
Donors		
M-va	9.75	2.52
S-va	2.66	2.47
P-va	8.9	2.58
Ch-va	4.66	2.09
Sh-ko	20.0	5.99
Z-i	15.0	3.47
S-v	16.8	3.02

After the biopsy, the bone marrow samples were suspended in the RPMI-1640 medium containing 5% fetal bovine serum (FBS). To obtain the MNC fraction, this suspension was layered onto the Ficoll–Verografin density gradient and centrifuged at 400×g for 30 min. The interface band, containing MNCs, was collected and washed three times with the RPMI-1640 medium containing 5% FBS by centrifugation at 200×g for 10 min. After the third centrifugation, the supernatant was replaced with the RPMI-1640

medium containing 20% FBS. The final MNC concentration was adjusted to 1×10^5 viable cells per 1 mL nutrient medium.

The bone marrow MNCs isolated from part of the patients were frozen using a programmed cell cooling to –80ºC in the presence of a cryostabilizer (dimethyl sulfoxide). The frozen cells were stored at –80ºC until used but no longer than 2 months. Before assaying, the cells were thawed and washed three times with the RPMI-1640 medium containing 5% FBS. After resuspending the pellet, the concentration of viable MNCs, as in the case of native bone marrow cells, was adjusted to 1×10^5 cells per 1 mL of complete nutrient medium.

The effect of the fragmented human exogenous DNA preparation on the proliferative activity of bone marrow MNC was estimated.

The model for assessing the effect of exogenous DNA on the MNC mitotic activity was the intensity of their proliferation. The proliferative activity of bone marrow MNCs was assessed in the tests for spontaneous and induced proliferative activities using standard techniques. The anti-CD3 monoclonal antibodies were used for inducing the proliferation of bone marrow MNCs. The MNCs incubated with the fragmented DNA (experiment) and not incubated (control) were assayed in triplicate. The proliferative activity was tested on day 3 of cultivation in 96-well immunological plates. The intensity of cell proliferation was assessed according to the incorporation of ^{3}H-thymidine into the DNA of proliferating cells. The activity of proliferation was expressed as the number of counts per minute (cpm).

The results demonstrating the effect of fragmented exogenous DNA on the spontaneous and induced (with anti-CD3 monoclonal antibodies) proliferations are consolidated in Tables 3 and 4. As is evident, the mitotic activity of bone marrow MNCs considerably decreased after 1-h preincubation with the DNA ($p < 0.05$). Note that this phenomenon was recorded not only in the case of additional proliferation stimulation with anti-CD3 monoclonal antibodies, but also for the spontaneous mitotic activity of the MNCs enriched with hematopoietic precursors. Based on the calculated index of influence (II), which characterizes the change in the intensity of cell proliferation in the experiment relative to the control, the level of mitotic activity of the bone marrow MNCs

after the treatment with exogenous DNA decreased approximately fourfold, amounting to no more than 30% (14–35%) of its initial level in the hemoblastosis patients.

Table 3. The effect of fragmented exogenous DNA on the spontaneous proliferation of bone marrow MNCs

Patient	**Diagnosis**	**Cells**	**Control, cpm**	**Experiment, cpm**	**II**
D-va	MM	FI	6833	1690	0.25
K-n	Lymphoma	TF	629	174	0.28
K-va	MM	FI	2646	644	0.24

Notes: MM, multiple myeloma; FI, freshly isolated cells; TF, thawed frozen cells; and II, index of influence of DNA.

Table 4. The effect of fragmented exogenous DNA on the stimulated proliferation of bone marrow MNCs

Patient	**Diagnosis**	**Cells**	**Control, cpm**	**Experiment, cpm**	**II**
D-va	MM	FI	16510	4038	0.24
K-n	Lymphoma	TF	6054	825	0.14
K-va	MM	FI	19122	6789	0.35

Notes: MM, multiple myeloma; FI, freshly isolated cells; TF, thawed frozen cells; and II, index of influence of DNA.

Thus, the obtained results demonstrate an active distinct influence of exogenous DNA on the proliferative status of the bone marrow MNCs, including stem hematopoietic cells.

Turning to the discussion of the obtained results, we proceed from the well-known mechanism of self-maintenance of the SC pool, which induces a certain number of quiescent SCs per unit time to differentiate without division into specific hematopoietic elements—the committed precursors of the erythroid, granulocyte–macrophage, and other hematopoietic lineages. In turn, differentiation causes an "excitation" in the same number (per unit time) of the quiescent cells. The "excited" cells proceed from the G_0 phase to the mitotic cycle and are unable to differentiate until

completion of division. After division, the daughter cell again return to the G_0 phase, supplementing the group of cells potentially capable of both differentiating and returning to the mitotic cycle. These basic mechanisms are disrupted during hemoblastoses; consequently, an uncontrolled proliferation of malignantly transformed hematopoietic cells, unable to differentiate normally, is observed.

On the other hand, the amount of labeled thymidine incorporated into the cells characterizes the fraction of dividing cells at a given time moment, i.e., actually it reflects the number of active, "excited", SCs that are in the state of mitosis and unable to differentiate. Consequently, a considerable decrease in the bone marrow MNC mitotic activity after their treatment with exogenous DNA suggests that the fraction of SCs in a quiescent state (G_0 phase) changes (increases); the quiescent SCs become able to differentiate to form committed specific hematopoietic elements. This inactivation of SCs is caused by the effect of the preparation Panagen on the chromosomal genetic loci in the cells having undergone a malignant transformation. In this process, as was demonstrated in another our invention [Russian patent no. 2313349 of January 16, 2006], the underlying mechanism can be connected with the substitution of nonmutant chromosomal loci for the mutant ones; correspondingly, the malignantly transformed cells partially or completely lose their malignant status.

This hypothesis can be tested by assessing the changes in differentiation of the stem hematopoietic cells contained in the mononuclear fraction of bone marrow.

Find below the assessment of the effect of human fragmented exogenous DNA on the differentiation of bone marrow stem hematopoietic cells.

The method of erythroid and granulocyte-macrophage colony formation on a semiliquid medium was the model for studying the differentiation of hematopoietic SCs [Pluzniik DH, Sachs L. The cloning of normal "mast" cell in tissue culture. J. cell. Comp. Physiol. 1965. V. 66. P. 319; Bradley TR, Metcalf D. The growth of mouse bone marrow cell in vitro. Aust J Exp Biol Med Sci. 1966. V. 44. P. 287-299]. It has been demonstrated using chromosome markers that the cells of one colony develop from a single progenitor cell. In addition, the possibility of obtaining secondary colonies of the same type by transferring a primary erythroid or granulocyte-macrophage colony onto new medium demonstrate that the source of colony growth *in vitro* is committed

hematopoietic stem cells [Metcalf D, Foester R. Behavior on transfer of serum stimulated bone marrow colonies. Proc Soc Exp Biol. 1967. V. 126. P. 758].

Thus, the number of growing colonies in a semiliquid medium characterizes, on the one hand, the total number of the SCs capable of committing to specific hematopoietic elements and, on the other, the direction of this differentiation, as can be estimated by the ratio of hematopoietic colonies of different types.

The change in mitotic activity induced by the preparation of fragmented DNA can influence in different manners the ability of SCs to commit to erythroid and granulocyte-macrophage hematopoietic lineages. In particular, this is determined by that the erythropoietic committed precursors in their natural mitotic activity significantly differ from other committed precursors. In the overall precursor pools, only 10% of the SCs synthesize DNA versus the committed erythropoietin-sensitive elements, 75% of which synthesize DNA. As the main part of the erythropoietin-sensitive cells are in the mitotic cycle, this suggests that a change in the proliferative activity of the bone marrow MNCs caused by exogenous DNA can first and foremost change the number of the SCs committed to erythropoiesis [Lajtha LG, Schofield R. Proliferative capacity of hemopoietic stem cells. In: Sympos. on Normal and Malignant Cell Growth: New York. 1969. P. 10].

Below is the estimation of the effect of human fragmented exogenous DNA on the formation of erythroid colonies.

The semiliquid media with methylcellulose supplemented with erythropoietin, recombinant interleukin-3, and several other inducers of erythroid cell proliferation and differentiation [Gol'dberg ED, Dygai AM, Shakhov VM. Cell culture methods in hematology. Tomsk University Press, Tomsk, 1992. 272 pp. In Russian] were used for cultivation and counting of the functionally active hematopoietic SCs committed to erythropoiesis.

At this stage of the study, we used only frozen bone marrow MNCs. The MNCs were thawed, washed as described above, and divided into two aliquots. In the experimental series, the bone marrow MNCs were preincubated for 1 h at 37ºC with exogenous DNA, which was added to the incubation mixture as a dose of 100 μg/mL. In

the control, the cell incubation medium was added at the same amount. On completion of the preincubation, the MNCs without washing at a dose of 1×10^5 viable CD34+ cells were transferred to the semiliquid cultivation media and poured into 24-well plates at a dose of 0.25 mL/well (0.25×10^5 cells/mL). The cells incubated with fragmented DNA (experiment) and not incubated with fragmented DNA (control) were assayed in four replicates (four wells), totally containing 1×10^5 bone marrow MNCs. The plates were cultivated in a CO_2-incubator at 37ºC and 100% humidity for 8–14 days. The number of erythroid colonies was estimated in dynamics and recorded on days 8 and 14. The cell aggregates formed during cultivation and containing over 100 erythroid elements at various degrees of maturation or groups (up to 50 cells) of small neighboring cells with the morphological traits characteristic of erythroid elements were regarded as the erythroid colonies (erythroid burst-forming units, BFU-E).

The counting results for erythroid colonies on days 8 and 14 as the range of colony numbers grown in the four wells for each studied patient are listed in Tables 5 and 6.

Table 5. The effect of human fragmented exogenous DNA on the number of erythroid colonies on day 8 of cultivation of bone marrow MNCs

Patient	**Diagnosis**	**Number of colonies (per 0.25×10^5 cells), control**	**Number of colonies (per 0.25×10^5 cells), experiment**
V-kin	Lymphoma	20–40	80–100
G-ik	Lymphoma	25–45	90–110
L-shev	LGM*	Absent	30–50
P-ina	LGM*	20–35	65–90
Sh-era	LGM*	Absent	32–54

*LGM, lymphogranulomatosis.

Table 6. The effect of human fragmented exogenous DNA on the number of erythroid colonies on day 14 of cultivation of bone marrow MNCs

Patient	**Diagnosis**	**Number of colonies (per 0.25×10^5 cells), control**	**Number of colonies (per 0.25×10^5 cells), experiment**
V-kin	Lymphoma	60–85	>150
G-ik	Lymphoma	65–80	>150
L-shev	LGM*	Absent	80–100
P-ina	LGM*	52–60	>150
Sh-era	LGM*	2–5	62–64

*LGM, lymphogranulomatosis.

As is evident from the quantitative characteristics listed in Tables 5 and 6, the number of generated erythroid colonies increased after the MNC preincubation with exogenous DNA in a statistically significant manner ($p < 0.001$). In addition, the increase in the number of erythroid colonies induced by the treatment with fragmented DNA preparation is most illustrative at the early time point, on day 8 of cultivation, when the increase in the number of formed erythroid colonies in experiment exceeded their number in the control three–fourfold and more.

Thus, these data are a certain confirmation of the postulated stimulatory effect of exogenous DNA on the ability of BSCs to be committed to erythroid differentiation. On the other hand, taking into account that a drastic increase in the number of erythroid colonies in the patients after high-dose chemotherapy occurs at the early time point of cultivation, we can assume that the action of exogenous DNA is not confined to the influence on the mitotic cycle but also involved other mechanisms.

One of the postulated mechanisms is the involvement of exogenous DNA in the repair of CS genetic machinery damaged by cytostatics. According to the results of the study, the involvement of exogenous DNA in the repair of SC genomes can be assumed in at least two of the five examined specimens of bone marrow MNCs (40%), when the restoration of SC ability to differentiate into erythroid precursors after the preincubation with fragmented DNA was observed on the background of a completely unobservable development of erythroid colonies in the control.

Thus, the action of human fragmented DNA preparation on the damaged hematopoietic precursors in the model of erythroid colony formation in 40% of the cases

is of qualitative rather than quantitative character, which is most likely mediated by the integration of exogenous DNA into the CS genetic machinery.

Other indirect data suggesting the integration of exogenous DNA into the CS genome are the specific morphophysiological features of the erythroid colonies formed after the preincubation with human fragmented DNA. In particular, it was noted that immature, BFU-E, colonies were predominant (70–90%) in the experimental wells as compared with the control wells. Formation of BFU-E is characteristic of young highly active SCs untypical of the initial stem precursors isolated from the hemoblastosis patients after programmed cytostatic chemotherapy (<5–10%).

A shortcoming of the model of erythroid colony formation is the inability to analyze the intricate competitive interactions between different hematopoietic lineages, where the bone marrow SCs can not only significantly change the pattern of their differentiation, but also the degree of response to various regulatory and restoration factors. Taking this into account, at the next stage of studying the effects of fragmented DNA preparation, we used a more complex model to assess the functional state of SCs, namely, the model of cocultivation of erythroid and granulocyte–macrophage colonies.

Below is the estimation of the effect of human fragmented exogenous DNA on the concurrent formation of erythroid and granulocyte-macrophage colonies.

To concurrently study the differentiation of erythroid and granulocyte–macrophage committed stem cells, we used a complete MethoCult® H 4434 (Stem Cell Technology, Canada, cat. no. 04434) methylcellulose medium. The complete methylcellulose medium H 4434 contains a wide set of growth factors. In addition to erythropoietin and interleukin-3, the complete medium contains stem cell growth factor (SCF), granulocyte-macrophage growth factor (GM-CSF), and other biologically active components maintaining proliferation and differentiation of the committed stem precursors.

The study was performed in four sets of experiments with the MNCs isolated from the bone marrow of the hemoblastosis patients after programmed cytostatic therapy. The protocol of experiments and the concentration of bone marrow MNCs in the experiment, i.e., after 1-h preincubation of MNCs with the human fragmented DNA

preparation, and in the control were identical to those used when studying the erythroid differentiation of committed precursors (see the pervious section). The only difference was that not only frozen, but also freshly isolated MNCs were used for studying the formation of erythroid and granulocyte-macrophage colonies.

The granulocyte-macrophage (CFU-GM), erythroid (BFU-E), and mixed colonies, i.e., the colonies containing both types of cell elements (CFU-GEMM) , were identified and counted using a microscope after completion of 14-day incubation at a temperature of 37ºC in a humid atmosphere containing 5% CO_2.

The results of counting the committed SCs forming the colonies of various types are consolidated in Table 7. The significant deviations in the number of formed hematopoietic colonies after the treatment of MNCs with exogenous DNA are marked with (+) and (–), respectively, indicating the increase and decrease of the characteristics in the experiment relative to the control.

Table 7. The effect of human fragmented exogenous DNA on the bone marrow MNC differentiation into erythroid and granulocyte–macrophage colonies

Diagnosis*	**Cells***	**Series**	**Number of colonies (per 1.0×10^5 cells)**			
			Total number	**BFU-E**	**CFU-GM**	**CFU -GEMM**
Lymphoma	TF	DNA─ DNA+	33 78^+	11 38^+	21 40^+	0 0
MM	FI	DNA─ DNA+	54 54	30 12^-	24 42^+	0 0
ALL	FI	DNA─ DNA+	220 200	116 82^-	96 88	8 30^+
MM	FI	DNA─ DNA+	82 64	32 30	50 28^-	0 6^+

Note: MM, multiple myeloma; ALL, acute lympholeukemia; FI, freshly isolated cells; and TF, thawed frozen cells.

These results demonstrate that a statistically significant two—threefold increase in the total number of the colonies both of erythroid and granulocyte–macrophage types formed in the presence of exogenous DNA was observed only when using the thawed frozen bone marrow cells, displaying an initially low number of colony-forming cells.

This confirms the earlier hypothesis on a possible involvement of exogenous DNA in restoration of the defects caused not only by programmed chemotherapy, but also by the freezing of bone marrow stem precursors. The latter fact, taking into account a wide application of cryoconserved stem cells in treatment of not only hematological, but also many other diseases, is not only of purely scientific interest, but of also of considerable applied value. From this standpoint, the human fragmented exogenous DNA in the complex with nuclear matrix proteins can be regarded as a new promising preparation for increasing the number of functionally active SCs after their cryoconservation, with an evident patenting potential.

The effects of human fragmented DNA preparation on the freshly isolated SCs are more intricate for interpretation. However, as is evident from the results of our studies, the studied exogenous DNA undoubtedly influences the SC differentiation. This appears in a selective competitive increase/decrease in the precursors committed for developing erythroid (BFU-E) or granulocyte-macrophage (CFU-GM) colonies as well as the increase in the number of immature stem precursors forming the mixed hematopoietic colonies (CFU-GEMM).

Any cell differentiation is a profound remodeling of the entire cell genome when a pluripotent cell selects its particular specific developmental pathway.

The discovered phenomena can be regarded as an indirect evidence of an active involvement of the extracellular exogenous DNA in the genesis of BSCs.

To implement the proposed use of fragmented DNA, we give an example of calculation of the amount of DNA preparation necessary for creating in the human blood plasma and interstitial fluids the DNA concentration range equal to or exceeding the own blood plasma DNA concentration but no more than 1500 ng/mL.

It is known the main amount of freely circulating body fluid is constituted by the blood volume, 5–6 L (1/10–1/12 of the human body weight) and the lymph volume, 1–2 L. Consequently, the total amount of freely circulating fluid in the adult human body is 6–8 L. The maximal amount of fragmented DNA preparation that could be administered to a patient without an evident and a hidden harm to his/her organism is about 9 mg; note that the minimal therapeutic amount of DNA preparation is 0.06 mg.

Claims

The use of fragmented DNA for treating diseases is based on introduction of fragmented DNA into patient's body **characterized in that** the fragmented DNA is allogeneic, obtained from genetically and physiologically healthy donors, and consists of the fragments with a length corresponding to one–ten nucleosome units that constitute the entire patient's genome in association with nuclear matrix proteins and administered in the amount providing that the fragmented DNA concentration in the patient's blood plasma is equal to or exceeds the concentration of own blood plasma DNA but no more than 1500 mg/mL.