Transfer and Expression of Small Interfering RNAs in Mammalian Cells Using Lentiviral Vectors

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ABSTRACT RNA interference is a convenient tool for modulating gene expression. The widespread application of RNA interference is made difficult because of the imperfections of the methods used for efficient target cell delivery of whatever genes are under study. One of the most convenient and efficient gene transfer and expression systems is based on the use of lentiviral vectors, which direct the synthesis of small hairpin RNAs (shRNAs), the precursors of siRNAs. The application of these systems enables one to achieve sustainable and long-term shRNA expression in cells. This review considers the adaptation of the processing of artificial shRNA to the mechanisms used by cellular microRNAs and simultaneous expression of several shRNAs as potential approaches for producing lentiviral vectors that direct shRNA synthesis. Approaches to using RNA interference for the treatment of cancer, as well as hereditary and viral diseases, are under active development today. The improvement made to the methods for constructing lentiviral vectors and the investigation into the mechanisms of processing of small interfering RNA allow one to now consider lentiviral vectors that direct shRNA synthesis as one of the most promising tools for delivering small interfering RNAs.

KEYWORDS lentiviral vectors; shRNA; RNA interference.

INTRODUCTION RNA interference is commonly used to inhibit gene expression. The advantages of this method include its simplicity, the possibility of quickly and significantly reducing the expression of any gene of interest, and the high specificity of the action. These properties render RNA interference a useful tool for investigating the role of specific genes in various cellular processes. For this purpose, entire libraries of siRNAs directed against a large number of genes have been created. Methods for applying RNA interference to the treatment of hereditary diseases, various neurodegenerative diseases, cancer, and as an antiviral therapy agent are currently under development. The search for new targets, the influence on which is efficient for treating a variety of diseases, is yet another application for RNA interference.

RNA INTERFERENCE RNA interference is a sequence-specific mechanism of suppressing gene expression, which is induced by the presence of exogenous or endogenous double-stranded RNA (dsRNA) in a cell [1]. This evolutionarily conserved mechanism functions in virtually all eukaryotic organisms. The sources of exogenous dsRNA include viruses or artificially introduced dsRNA. Endogenous dsRNA is formed as a result of the transcription of a cell’s own genes and often performs regulatory functions. The cleavage of long dsRNA by the Dicer protein, which belongs to the RNase type III family (Fig. 1), resulting in the formation of small 21- to 25-nucleotide-long siRNA duplexes is the shared stage of all types of RNA interference. The duplex contains a pair of unpaired nucleotides and a pair of hydroxyl groups at the 3'-ends and monophosphates at the 5'-ends (Fig. 1). This structure of RNA duplexes enables their normal processing by a protein belonging to the Ago family, which plays a key role in the formation of the RISC complex (RNA-induced silencing complex) [2]. The RNA fragments formed as a result of Dicer-mediated cleavage of dsRNA are included in the structure of a RLC complex (RISC-loading complex) containing Dicer and TRBP proteins. During the next phase, the formation of a
pre-RISC complex (complex preceding RISC) occurs. The structure of this complex includes the Ago-2 protein, which cleaves the RNA duplex, so that only the guide strand is retained in the complex [3]. This strand determines the specificity of expression suppression, while the other strand (known as the passenger strand) is removed from the complex [4]. The selection of the guide strand is independent of the prospective target; the strand whose 5’-end is characterized by a lower thermodynamic stability becomes the guide strand [5]. During the next phase, the guide strand forms a part of the RISC complex and binds to the site of the target mRNA according to the principle of complementarity (Fig. 1). The process of mRNA destruction involves two stages. First, a primary gap appears in the mRNA molecule, which is attributed to the endonuclease activity of the PIWI-domain of the Ago protein. This is followed by the destruction (degradation) of the target mRNA by cellular exonucleases [6]. If the complementarity of siRNA and mRNA is incomplete, the primary gap is not formed; hence, mRNA is not subjected to degradation. It is important to mention that even if the complementarity between the guide strand and mRNA is incomplete, suppression of gene expression can occur at the translation stage in a similar fashion to miRNA [7]. An alternative mechanism of siRNA action is associated with the formation of the RITS (RNA-induced transcriptional silencing) complex, which includes the Ago-1 protein. The target mRNA is recognized by the RITS complex due to its interaction with RNA polymerase II during transcription [8]. During the next phase, the histone methyltransferases that methylate histones can become a part of the RITS complex, resulting in chromatin compaction and inhibition of the expression at the epigenetic level.

MicroRNAs differ from siRNAs by their mechanism of action and some features of their processing. Transcription of miRNAs is carried out by RNA polymerase II. The resulting RNAs undergo capping and polyadenylation [9]. Certain miRNAs are encoded by individual genes, while others are encoded by entire gene clusters. miRNAs can be transcribed together with mRNAs; the sequence encoding miRNA is located in the intron of a protein-coding gene [7]. As a result of the transcription, pri-miRNA (miRNA precursor) is formed. Its structure includes a sequence of the future miRNA, the terminating loop, and flanking sequences [10]. Processing of pri-miRNAs occurs in the nucleus with assistance from a complex consisting of two types of RNase III, Drosha and DGCR8 (in mammals). Approximately 65-bp-long hairpin-like miRNA precursors are formed as a result of the processing (Fig. 1) [11]. Transport of pre-miRNAs

**Fig. 1.** Cellular processing of small interfering RNAs. pri-miRNAs are transcribed by RNA polymerase II; the resulting transcripts undergo capping and polyadenylation. After these processes, pre-miRNAs are spliced out under the influence of Drosha (RNase type III). shRNA is transcribed by RNA polymerase III to form shRNA with a triphosphate at its 5’-end. Both hairpin structures (pre-miRNA and shRNA) are transported from the nucleus to the cytoplasm by the Exportin-5 protein. In the cytoplasm, the Dicer protein splices out the sequences of future miRNAs and siRNAs from the hairpin structures and exogenous double-stranded RNAs. As a result of the processing by the Dicer protein, 21- to 25-bp-long RNA duplexes having a pair of unpaired nucleotides at their 3’-ends, OH-groups at their 3’-ends, and monophosphates at their 5’-ends are formed. The guide strand is loaded into the RISC protein complex, which binds to the mRNA that is complementary to the sequence of the guide strand. Meanwhile, the passenger strand is removed from the complex.
to the cytoplasm is facilitated by the Exportin-5 protein (Fig. 1). In the cytoplasm, pre-miRNAs are cleaved by the Dicer protein, resulting in the formation of an approximately 22-bp-long duplex [12]. Unlike the siRNA duplex, the miRNA duplex typically contains unpaired nucleotides in the middle. The miRNA is subsequently included in the RISC complex in a similar fashion to siRNA [13].

In contrast to siRNA, miRNA is usually fully complementary only to a small fragment of the mRNA (several nucleotides long). The miRNA fragment, which is completely complementary to mRNA, most frequently comprises the nucleotides 2–8 from its 5’-end and is known as a “seed region.” The “seed region” determines specific miRNA targets [14]. miRNA usually binds to the mRNA site, which is located in the 3’-untranslated region and is represented by multiple copies of the same mRNA. Since the length of the region that must be fully complementary is rather small, several different mRNAs can act as targets for a single miRNA. It is presumed that full complementarity of miRNA and target mRNA can lead to mRNA degradation, while partial complementary binding of miRNA to mRNA can disrupt translation [7, 15].

The introduction of long dsRNAs to mammalian cells induces interferon response; hence, short chemically synthesized siRNAs are used. Their structure is similar to that of natural siRNAs [16]. However, the effect of synthetic siRNAs is short-term (only a few days), which is attributed to their degradation by cellular nucleases. Moreover, the concentration of these siRNAs decreases during cell division. These drawbacks can be avoided if one uses vectors that direct the synthesis of siRNA precursors: small hairpin RNAs (shRNAs). shRNAs contain the sequence of the siRNA guide strand (21–29 bp long), followed by a loop consisting of approximately 9 nucleotides, and a sequence that is complementary to the siRNA guide strand (Fig. 2A). The use of this structure enables to achieve long-term suppression of gene expression [17].

Lentiviral vectors are optimal tools for the delivery of shRNAs into cells. An important feature of the life cycle of lentiviruses consists in their ability to integrate their genomes (in combination with proviral DNA) into cellular DNA. In addition, lentiviruses, as opposed to simple retroviruses, are capable of infecting nondividing cells. Despite the fact that such lentiviruses as the equine infectious anemia virus, feline immunodeficien-

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**Fig. 2.** Schematic representations of the vectors that direct shRNA synthesis. A – The expression cassette is inserted between two LTR-sequences of the lentiviral vector. The expression of shRNA is directed by the H1 promoter. Transcription is initiated from the sequence of future siRNA (sense), followed by the “loops,” the inverted sequence, which is complementary to siRNA (α-sense), and termination sequence (thymines). The puromycin-resistance (puro) gene is also present in the vector; it enables the selection to be carried out [47]. B – The expression cassette is cloned into the 3’-LTR sequence. The cassette is doubled during reverse transcription; two copies of the expression cassette are formed following the integration of the provirus. A marker gene (e.g., green fluorescent protein (GFP) gene) under the control of the CMV promoter can also be cloned using this vector [45]. C – The expression cassette encoding shRNA fused with tRNA (S4tRNALys3-shRNA). After the transcription, the chimeric RNA undergoes processing in a similar fashion to normal tRNA, resulting in shRNA release [52].
cy virus and the bovine immunodeficiency virus are used as templates for lentiviral vectors, the human immunodeficiency virus type 1 (HIV-1) remains the most commonly used virus for vector production. This is associated with the fact that the life cycle of this virus is better understood than those of the other viruses [18, 19].

LENTIVIRAL VECTORS

Replication-incompetent systems based on lentiviruses are used for gene transfer and expression [20]. These systems enable the integration of the encoding target gene into the genome of the target cell’s DNA (transgene). A typical lentiviral vector contains cis-elements of the viral genome, which are required for the assembly and integration of the viral particle and the sequence encoding the target gene. All trans-elements of the viral genome are removed from the vector. Cotransfection of a vector and the plasmids encoding viral proteins is the main approach used for obtaining lentiviral vectors [19]. In order to reduce the risk of occurrence of replication-competent particles due to recombination, the components of the viral genome required for the assembly of lentiviral vectors are typically divided into three or four plasmids: one or two packaging plasmids, the vector plasmid, and the plasmid encoding the viral envelope protein. Constructs with all cis-elements (except for RRE and the splice donor site that is required for post-transcriptional processing of mRNA) removed are used today in third generation packaging systems. A heterologous promoter (usually CMV) and the polyadenylation signal of the SV40 virus are used instead of a long terminal repeat (LTR). The rev and gag/pol genes are integrated into the cells using various expression cassettes. Humanization of the gag/pol genes is also employed, which enables their expression independently of rev. This also renders RRE removal from the packaging system possible [21]. It is important that such significant modifications of the packaging system do not affect the efficiency of the transduction by the lentiviral vector and significantly reduce the risk of occurrence of replication-competent particles due to homologous recombination. In order to reduce the risk of a nonhomologous recombination, a trans-lentiviral packaging system has been developed where the coding region of the gag/pol is divided into two parts and is incorporated into the structure of two different expression plasmids [22].

Pseudotyping of lentiviral vectors

In order to increase the tropism of lentiviral particles, the HIV-1 envelope protein is frequently replaced with the G protein of the vesicular stomatitis virus (VSV). These pseudotyped lentiviral particles enable the transduction of virtually all cell types. This modification not only expands the tropism of viral particles, but also increases their stability. Another important property of VSV-G is its ability to facilitate the penetration of the vector into the cell via endocytosis, thus reducing the need for auxiliary membrane proteins [23]. The main drawback of VSV-G pseudotyped lentiviral particles consists in their rapid elimination by the components of the immune system from the circulatory system [24].

One of the major problems encountered during the use of small interfering RNAs is the insufficient specificity of their delivery into the target cells. In addition to VSV-G, heterologous glycoproteins of lyssaviruses, the lymphohyptic choriomeningitis virus, alphavirus and baculoviruses can also be used to carry out pseudotyping [25]. The transduction efficiency of liver cells is increased with the use of the hepatitis C virus or baculovirus-envelope proteins [26]. Pseudotyping of lentiviral particles by the envelope proteins of the Rabies virus enables the lentiviruses to infect the cells of the central nervous system in vivo [27]. The envelope proteins of other viruses are frequently used to ensure more efficient tissue-specific transduction.

Methods that enable the presentation of various cellular receptors and their corresponding antibodies on the surface of viral particles are becoming more common [28–30]. The general principle in this approach is to create a fusion protein which can be successfully integrated into the envelope of the vector particles to ensure a relative stability of these particles, on the one hand. On the other hand, this protein carries a fragment of the ligand required for binding to the receptor. Most frequently, this chimeric protein is based on a glycoprotein of the amphotropic murine leukemia virus (A-MLV) and the hemagglutinins of the influenza and measles viruses. These viral envelope proteins are modified in such a way that they can no longer recognize their natural receptors, thus avoiding nonspecific infection. Lentiviral vectors containing the epidermal growth factor (EGF) or an anti-CD20 single-chain variable antibody fragment (scFv) on their surface, which are fused with the hemagglutinin of the measles virus and intended for infecting B cells, have been produced based on this scheme [31]. Another approach consists in producing lentiviral particles containing the glycoprotein A-MLV fused with anti-CD3 scFv or with interleukin-7 (IL-7), presented on their surface [32, 33]. This system enables the infection of T cells. Two ligands can be simultaneously used for pseudotyping: the stem cell factor (SCF) fused with the A-MLV glycoprotein and thrombopoietin (TPO) conjugated to the hemagglutinin of the influenza virus. Transduction of CD34+ cells with lentiviral particles carrying either thrombopoietin, or SCF, or both ligands on their surface, has proved sig-
nificantly more efficient than the use of VSV-G as an envelope protein [34].

Utilization of viral surface envelope proteins is not the only way of presenting cell receptor ligands on the surface of viral particles. In this case, the utilized protein must contain a transmembrane domain; the surface of the viral vector must contain an envelope protein that can facilitate the fusion of the virus with the cell. Modified envelope proteins of the Sindbis virus or VSV-G, which have lost the ability to bind to their “native” receptor, are used for this purpose. The Sindbis virus has two surface envelope proteins, E1 and E2. The E1 protein is responsible for fusion with the cell, and E2 is responsible for binding to the receptor. The E1 protein functions independently of E2. A lentiviral vector containing the transmembrane form of SCF and a modified envelope protein of the Sindbis virus was produced according to this principle [35]. In the absence of the transmembrane domain, which is necessary for localization on the surface of lentiviral particles, the protein is attached to the transmembrane domain of VSV-G or human leukocyte antigen (HLA) [36]. For pseudotyping of lentiviral particles with antibodies, the packaging system must contain not only genes encoding light and heavy chains of antibodies, but also genes encoding Igα and Igβ proteins which are required for antibody exposure on lentiviral particle surface.

This scheme was used to obtain lentiviral particles with surfaces containing anti-surface protein (CD20, DS-SIGN and CD3) antibodies [37–39].

Sindbis virus envelope proteins are also used for the pseudotyping of lentiviral particles by antibodies. For this purpose, the E2 protein is modified by incorporating the Fc-binding domain of protein A (ZZ-domain), which binds to immunoglobulin IgG, into its structure. Transduction using these lentiviral particles is only possible in the presence of monoclonal antibodies. The selection of antibodies determines the tropism of the lentivirus, enabling one to design viral particles that are specific with respect to cells of various origins without modifying the packaging system [40]. The disadvantages of Sindbis viral envelope proteins include the dependence of the protein E1 activity on pH (the pH value must lie within the range of 4.5–5.0). The reduced stability of these chimeric proteins during the pseudotyping of lentiviral particles can also be regarded as a drawback. The reduced efficiency of target cell infection using these lentiviral particles (which, however, can be compensated for by high specificity) should also be mentioned here.

Another approach that can provide specific infection of cells is the use of proteins as a component of the viral envelope, whose binding to a specific surface receptor results in a significant reduction in the efficiency of the transduction of those cells, the introduction of a transgene into which is undesirable [29]. This contamination-preventing protein can be bound to a viral glycoprotein using an amino acid sequence that is sensitive to certain proteases. The infection in this case involves two stages: first, the ligand on the viral surface binds to the cell receptor, and then cleavage of the peptide insertion occurs under the influence of certain proteases. After the insertion is cleaved, the glycoprotein can bind to its specific receptor on the cell surface. This approach enables to infect cells in the presence of specific proteases.

The use of tissue-specific promoters

Non-specific cellular transduction, and therefore, transgene expression in these cells can cause a variety of adverse effects. In particular, transgene expression in antigen-presenting cells (APC) can result in the development of an immune response and T cell activation [41]. Tissue-specific promoters are used to reduce the effect of the non-specific infection. Pseudotyping and the use of tissue-specific promoters enable to achieve transgene expression exclusively in the desired cells. However, tissue-specific promoters can be quite weak, and the level of expression of the target gene may be insufficient. The enhancers of stronger promoters can be used to strengthen these promoters. The enhancer of the CMV promoter used in combination with a variety of tissue-specific promoters provides a multifold increase in the expression of the target gene without decreasing the promoter specificity [42]. The site of transgene incorporation into the genome of the target cell is determined randomly; however, the incorporation takes place preferentially in the transcriptionally active regions. It is important to make an allowance for the fact that the incorporated transgene can accidentally come under the control of a strong promoter. In this case, its expression will be independent of the tissue specificity of the promoter. Insulators that block the effects of the neighboring enhancers are used to avoid this effect [28].

Transgene expression can be regulated at the post-transcriptional level. The mechanism underlying this regulation is based on RNA interference. Over 200 miRNAs exhibiting tissue-specific expression have been identified thus far. It has been demonstrated that the introduction of four sites recognized by miR-142 miRNA, which is expressed mainly in hematopoietic cells, into the gene encoding the green fluorescent protein (GFP) reduces the level of fluorescence exclusively in these cells [28, 43]. Taking into account the fact that new miRNA expression patterns are continuously identified in various cells, it can be assumed that this method is of significant interest for precise control of the expression of the introduced genes.
Small hairpin RNAs

shRNAs are siRNA precursors. They are typically expressed using U6 or H1 RNA polymerase III promoters (mouse or human) [43]. These promoters are small in size (about 400 bp long); transcription is initiated at the +1 position, and in the case of the U6 promoter it is desirable for the transcription to be initiated with guanine [44]. A sequence of 5–6 thymine residues acts as a transcription termination signal, resulting in the formation of double-stranded shRNA containing an unpaired 3’-end, which is essential for further processing by the Dicer protein. U6 and H1 promoters provide a stable and a relatively high level of shRNA expression in all cell types. shRNAs obtained as a result of RNA polymerase III-mediated transcription have neither 5’-caps nor 3’-poly (A) sequences; they are not processed by the Drosha protein. Their transport to the cytoplasm is carried out by the Exportin-5 protein [12]. The use of the RNA polymerase III promoter during the production of lentiviral vectors that direct the synthesis of shRNA allows one to attain a high level of shRNA expression in virtually all cell types. There are approaches in which the cassettes expressing shRNA are cloned into the 3’-LTR region of a lentiviral vector [45]. During the synthesis of a provirus, 3’-LTR is used as a template for 5’-LTR. As a result, two copies of the expression cassette are incorporated into the proviral insertion (Fig. 2B).

The lentiviral vector frequently includes marker genes. Genes encoding fluorescent proteins or antibiotic resistance genes are typically employed. The presence of marker genes in a vector enables the selection of transduced cells and evaluation of the transduction efficiency. Lentiviral vectors that direct the synthesis of shRNA were used in the production of cell lines characterized by a stable suppression of the expression of the activated oncogenes detected in acute myeloid leukemias. The puromycin resistance gene was introduced into the vector as a marker gene (Fig. 2A) [46, 47].

When constructing the vectors that direct the shRNA synthesis, it is important to take into account the fact that the increased level of shRNA expression in cells may have adverse consequences. It was demonstrated that transduction of mouse hepatocytes using an adeno-associated virus-based vector, the shRNA transcription in which is controlled by the U6 promoter, results in liver lesions in 50% of cases [48]. A total of 49 different vectors, each encoding a unique shRNA, were used in the study [48]. The toxic effect of these vectors is associated with the competition between shRNAs and cellular miRNAs for interaction with the Dicer and Exportin-5 proteins involved in the processing of both types of small RNAs. It is of significance that the resulting shRNA contains triphosphate at its 5’-end, which can cause an interferon response and stop the translation of cellular proteins. The presence of two unpaired nucleotides at the 3’-end of the shRNA stem is essential for efficient operation of the processing proteins (Exportin-5 and Dicer). An increase in the number of unpaired nucleotides significantly reduces the functional activity of these shRNAs [49–51]. The formation of triphosphate at the 5’-end can be avoided using an approach characterized by simultaneous transcription of shRNA and tRNA [52]. The chimeric RNA processed by cellular endonucleases is synthesized, resulting in the formation of shRNA containing monophosphate at its 5’-end (Fig. 2C). The use of tRNA promoters allows one to prevent the emergence of nonspecific responses; the expression level of shRNAs is considerably lower than when Polymerase III promoters are used.

If the first 2–8 nucleotides of the siRNA guide strand are complementary to the “seed region” of a particular miRNA molecule, then this siRNA molecule can function as a miRNA. This can trigger a nonspecific action from siRNA. The ability of siRNA to act as miRNA can be used to suppress the expression of certain genes (e.g., the CCR5 gene) [53]. siRNA, which is specific with respect to the CCR5 gene, is complementary to the “seed region” located in the 3’-UTR of mRNA. This siRNA caused the degradation of mRNA and resulted in disruption of translation in a fashion similar to the action of miRNA. When selecting shRNA sequences, one should bear in mind that the 5’-end of the guide strand of the duplex formed as a result of processing must be characterized by a lower thermodynamic stability. Inconsistency with these rules can result in the following: the passenger strand will become part of the RISC complex, instead of the guide strand, leading to a reduced specificity of the shRNA action. It is assumed that the H1 promoter is better suited for in vivo application as compared to the U6 promoter, since the H1 promoter is less toxic despite its lower efficiency [54]. Successful application of lentiviral vectors guiding the shRNA synthesis was demonstrated using animal models of various diseases [55–57]. In particular, the expression of shRNA persisted for 9 months following the injection of lentiviral particles, and suppression of the reporter gene expression in mouse brain cells was maintained [58].

The adverse effects associated with the use of shRNAs (interferon response, competition with cellular miRNAs, nonspecific action) can be avoided by employing various approaches. Several of them are based on the adaptation of the artificial shRNA processing to the mechanisms used by cellular miRNAs [59]. To achieve this objective, the sequence of the guide strand of the future miRNA can be replaced with an artificial sequence, while conserving the structure of miRNA pre-
cursors. miRNAs are transcribed by Polymerase II; thus, it is preferable to use the promoters of this enzyme when constructing the vector. It was demonstrated that expression of shRNA under the control of the U6 promoter in an adeno-associated virus-based vector is 10 times more efficient than the expression of miR-30 under the control of the same promoter. However, the suppression level of the reporter gene was approximately the same, while the toxic effect of the construct containing miR-30 was much lower [60, 61]. The fact that the Dicer protein can select both strands of the miRNA duplex is a drawback of miRNA-based systems. Cell transduction with lentiviral vectors carrying the gene for the nerve growth factor receptor (NGFR), whose first intron contains an integrated sequence encoding pri-miR-223 (200 bp) under the control of the integrated EF1α promoter, results in stable expression of the NGFR gene and miRNA (Fig. 3A). The sequence of the guide strand in the “stem” of miRNA can be replaced by other guide strand sequences from other miRNAs or siRNAs [62]. An approach enabling one to achieve stable expression of the mouse BIC gene and its miR-155 product characterized by an altered sequence of the guide strand has also been developed (Fig. 3B) [63]. The vector containing a fragment of the BIC gene (including the sequence of miR-155) directed the successful expression of both the source miRNA and miRNA with an altered sequence of the guide strand (Fig. 3B) [63]. General rules for constructing artificial miRNAs have yet to be developed, which is primarily due to insufficient knowledge with regard to their processing.

Simultaneous synthesis of several small interfering RNAs

In some cases, the simultaneous expression of multiple siRNAs is preferable (e.g., during antiviral therapy). This is attributed to the fact that some viruses mutate at a high rate, and the probability of developing resistance to specific siRNA among them is high. The use of multiplex constructs enabling the synthesis of several siRNAs significantly reduces the probability of emergence of resistant forms of the virus.

Therefore, a lentiviral vector that directs the synthesis of long hairpin RNAs (lhrRNAs) containing the “loop-stem” structure was constructed. Processing of these lhrRNAs occurs with assistance from the Dicer protein; several siRNAs are formed under the influence of the latter. The lhrRNA (the precursor of siRNA) nucleotide sequence is selected according to the same principle as per shRNA selection process. Suppression of HIV-1 replication was achieved with assistance from the 50- to 80-bp-long lhrRNA, which acts as a precursor for 2–3 siRNAs against various parts of the general region of tat/rev genes, (Fig. 4A) [64]. A similar approach was used to suppress the replication of the hepatitis B and C viruses [65, 66]. The efficiency of lhrRNA processing by the Dicer protein decreases as the siRNA sequence approaches the “loop,” resulting in the formation of various amounts of siRNA and a nonuniform suppression of target gene expression.

Since the promoters of RNA polymerase III are relatively small (200–400 bp), a single vector can incorporate several siRNA sequences, each controlled by its own promoter. Different RNA polymerase III promoters (U6, H1 and 7SK) are used in this case, since the utilization of identical promoters may induce recombination between their sequences and deletion of one or several expression cassettes in 80% of the cases [67]. A vector ensuring the synthesis of four shRNAs under the control of the mouse U6, H1 and human U6, 7SK promoters has been constructed. In this case, the mouse H1 and human U6 promoters were fused into a single
bidirectional promoter (Fig. 4B). Suppression of the expression of four different genes was achieved using this vector [68].

Clusters encoding polycistronic miRNAs, which form several pre-miRNAs, can be used for simultaneous expression of multiple siRNAs. Transcription of the miR-17-92 gene cluster gives rise to double-stranded pri-miRNAs approximately 1 kbp in length. The latter are precursors of six different pre-miRNAs. The mir-17-92 gene cluster was used to create lentiviral vectors that direct the synthesis of four HIV1-specific miRNAs. Sequences encoding pre-miRNAs and containing 40 nucleotides on each side of the “loop-stem” structure were obtained from the gene cluster and were incorporated into the vector. Sequences of the guide strands of the future miRNAs were replaced with segments specific with respect to HIV-1 (Fig. 4C) [69]. Due consideration was given to such features of the original structure of miRNAs as mismatches and thermodynamic stability during the replacement of the sequences of the guide strand.

The use of small interfering RNA

Approaches for the clinical application of small interfering RNAs are currently being developed. Dozens of siRNA-based medicinal agents designed to treat different kinds of diseases are currently undergoing clinical trials. Only one drug, which is based on the lentiviral delivery of shRNA, has been tested thus far. The use of lentiviral vectors directing the synthesis of shRNAs is constrained by the fact that they are relatively unsafe. This is attributed to possible nonspecific responses, which can be caused by shRNA expression in cells and the probable insertional mutagenesis. However, the use of lentiviral vectors for siRNA delivery has a number of significant advantages. They can be used to achieve stable and prolonged shRNA synthesis in dividing and nondividing cells, making their application rather...
promising for the treatment of chronic diseases.

The mechanism of RNA interference is a component of the antiviral defense system of the organism; therefore, the use of RNA interference in chronic viral infections [59, 70, 71] (including the diseases caused by the hepatitis B and C and HIV-1 viruses) is of considerable interest. However, the use of RNA interference may result in the emergence of resistant forms of the virus, which limits the application of this method [72]. Contemporary methods enable the creation of lentiviral vectors that can simultaneously encode three or four shRNAs which are specific with respect to various viral genes. This can significantly reduce the probability of emergence of resistant forms of the virus. Existing methods of siRNA delivery to T cells and macrophages (HIV-1 targets) are inefficient. The use of lentiviral vectors can be an efficient approach to introducing siRNAs into cells targeted by HIV-1. However, in the case of lentiviral vectors directing the synthesis of shRNAs, which are specific with respect to viral genes, reduction in the efficiency of lentiviral particles and their titer is possible [73]. Thus, point mutations that do not affect the synthesis of the proteins required for the assembly of viral particles are introduced into the genes used in the packaging system. Selection of these mutations complicates the process of vector construction, especially if shRNAs are selected for the conserved HIV-1 sites. After the infection with HIV-1, the viral envelope protein binds to the CD4+ receptor exposed on the surface of the target cells; the virus uses the CCR5 cell receptor as a co-receptor. It has been demonstrated that homozygous deletion of the human CCR5 gene renders cells resistant to the HIV-1 infection, and the mutation apparently has almost no effect on the normal functioning of the cells [74]. It was demonstrated that shRNA-mediated suppression of the CCR5 receptor expression also renders cells resistant to infection by the virus in vitro and in vivo [75–78]. Several proteins whose functions are not essential to T cells or macrophages and which play an important role in the life cycle of HIV-1 have been identified [79].

The optimal approach is to obtain HIV-1-resistant T cells and macrophages from their common progenitors. To achieve this objective, transduction of early hematopoietic precursor cells was carried out using lentiviral vectors that direct the synthesis of shRNAs that are specific with respect to the CCR5 or CXCR-4 gene. The descendants of these cells (T cells and macrophages) acquired resistance to the virus [80–82]. There is an approach that enables the expression of shRNA, along with the other genes. The lentiviral vector that was successfully used to provide the synthesis of a false target for the viral TAT protein in addition to the expression of shRNA specific with respect to the general region of the tat/rev genes is an example of the latter concept. This false target impedes the action of the TAT protein and synthesis of ribozyme, which is specific with respect to the CCR5 receptor [83, 84]. The efficiency of this vector was tested on humanized mice; stable inhibition of HIV-1 at different stages of the life cycle was achieved using this vector [85]. Clinical trials demonstrated the safety of using this vector in autologous bone marrow transplants in patients with HIV-1 and lymphoma. The patients with lymphoma at the remission stage, which resulted from a conventional treatment regimen, exhibited no side effects associated with the introduction of shRNAs. A detectable level of shRNA expression persisted in patients for 24 months.

Malignant tumors develop as a result of mutations leading to an abnormal expression of the genes that stimulate cell proliferation and impairing apoptosis. RNA interference is a useful tool for modulating gene expression. It is considered that methods based on the principle of RNA interference can be of considerable interest in the treatment of tumors. The classical approaches to the therapy of malignant diseases are characterized by a number of significant deficiencies associated with the nonspecificity of their action. The use of RNA interference enables to exert a specific effect on oncogenes at a relatively low cost. A total of 10 siRNA-based drugs are currently undergoing clinical trials. The main obstacle associated with the use of RNA interference in treating malignant diseases is the imperfections of the methods for siRNA delivery to tumor cells. One of the most convenient and efficient gene delivery systems is lentiviral vectors. The optimal approach for obtaining HIV-1-resistant T cells and macrophages is to introduce the synthesis of shRNAs into early hematopoietic cells using lentiviral vectors, which will be then transduced into T cells and macrophages. This approach is expected to provide stable inhibition of HIV-1 and malignant tumors in the long term.
transfer systems is based on the use of lentiviral vectors. These systems enable a highly specific integration of sequences encoding shRNA into the target cell’s genome. Methods for pseudotyping lentiviral particles are being developed and tissue-specific promoters are used in order to achieve this objective. Systems with multiplex shRNA expression show promise as well. Multiplex shRNA expression enables specific inhibition of multiple genes involved in tumor development. The use of multiple shRNAs specific to different regions of the same activated oncogene makes it possible to improve the efficiency of these systems [86].

Many human miRNAs are capable of inhibiting the growth of malignant tumors [87, 88]. Thereby, some of the research teams are working on the use of miRNAs for treating malignant tumors whose cells are characterized by a lower expression of oncosuppressor miRNAs. It was demonstrated that restoration of the expression of oncosuppressor miRNA decreases cell growth in patients suffering from non-small-cell lung carcinoma, breast cancer, liver cancer, and chronic lymphocytic leukemia [89–92]. However, the inefficiency of in vivo transduction still remains the major problem when using lentiviral vectors as the main form of therapy. Leukemia therapy is considered to be the most suitable area for the use of lentiviral vectors that direct shRNA synthesis (Fig. 5). Autologous transplantation of hematopoietic cells transduced with a lentiviral vector which is specific with respect to one or several activated oncogenes can also be promising. The safety of this approach was demonstrated using lentiviral vectors that direct the synthesis of shRNAs capable of inhibiting HIV-1 [93].

The search for new target genes that are involved in tumor development is also regarded as a promising application for shRNAs. Nowadays, gene expression profiles in cancer cells are being actively studied. This has already enabled the discovery of several genes whose increased expression is associated with specific types of tumors. Vast libraries of lentiviral shRNA-based vectors enabling the search for the genes that are considered promising for the development of novel chemotherapeutic agents have been created [94, 95]. The shRNA-induced inhibition of oncogene expression allows one to assess the contribution of these genes to the maintenance of the malignant status of tumor cells. A similar approach was used to transduce cells derived from a patient with acute myeloid leukemia using lentiviral vectors that direct shRNA synthesis, which are specific for c-kit and AML1-ETO oncogenes. The system was successfully used to investigate the inhibitory action of binase on the tyrosine kinase KIT receptor [96].

It is thought that shRNA can be successfully used in the gene therapy of neurodegenerative diseases, such as the Alzheimer’s, Parkinson’s and Huntington’s diseases. Their application is considered to be extremely promising, since lentiviruses are capable of overcoming the blood-brain barrier and infecting cells of the central nervous system (CNS). Lentiviral vectors enable to achieve a stable shRNA synthesis, which can be extremely important in the treatment of these chronic diseases. Pseudotyping of lentiviral vectors using the rabies virus envelope protein can increase efficiency in the infection of CNS cells.

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