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Vectors and delivery systems in gene therapy

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Summary

Gene therapy provides modern medicine with new perspectives that were unthinkable two decades ago. Progress in molecular biology and, especially, molecular medicine is now changing the basics of clinical medicine. Reports of long-term side effects of the first successful human gene therapy study have slowed the penetration of DNA usage into clinical routine. However, the main safety problem lies in the secure and efficient delivery of genes into target cells and tissues. A number of older and more recently discovered techniques have been developed for therapeutic gene transfer. A variety of viral and non-viral possibilities are available for basic and clinical research. This review summarizes the delivery routes and methods for gene transfer used in gene therapy. The best known are viral vectors derived from adenoviruses and adeno-associated viruses. Specific properties of lentiviruses and retroviruses ensure their status in current gene therapy procedures. Recent advances in the preparation of lipoplexes and greater safety support the use of naked DNA transfer. The latest approach, using bacteria as vectors for gene therapy or as *in situ* producers of therapeutic proteins (alternative gene therapy), must undergo critical evaluation in further experiments.

key words: gene therapy • vectors • gene transfer • oligodeoxynucleotides • alternative gene therapy

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BACKGROUND

Gene technology has become one of the most intensively developing strategies for current clinical research. Gene therapy offers new treatment possibilities for many common acquired and inherited human diseases where conventional clinical procedures are less effective. These diseases include monogenic disorders, such as cystic fibrosis, but also more complex disorders, such as cardiovascular diseases, diseases of nervous system, autoimmune diseases, and cancer.

In the case of genetic diseases caused by a mutation in a specific gene or its product, gene therapy usually involves the delivery of a functional copy of this gene into a target cell or tissue to achieve a therapeutic benefit [1]. However, it can also be a tool for the treatment of non-genetic and polygenic disorders by delivering genes that stimulate immune response, suicidal genes inducing cell death, genes modifying cellular information or developmental program, or genes producing a therapeutic protein with specific functions [2].

The generic aim of all these strategies is to achieve a stable expression of transgenes in the target tissue for as long as required, in an appropriately regulated form, without side effects, for example interaction with host genome, toxicity, or cellular carcinogenic transformation. Two different approaches have been defined during the development of gene therapy: In so-called “*ex vivo*” gene therapy, specific cells isolated and purified from a patient are genetically modified and re-infused [3]. In contrast, “*in vivo*” gene therapy involves direct gene transfer into the tissue of patients using a vector [4].

Recent sobering reports on the long-term outcome of the first successful clinical gene therapy study have renewed the discussion about the safety of DNA use in the treatment of diseases [5]. Therefore, current studies are aimed at developing effective and safe techniques and vectors to deliver genes into tissue as well as to improve the regulation of transgene expression. This article summarizes the current situation in the field of gene delivery techniques.

GENE THERAPY VECTORS

The simplest way of gene delivery is injecting naked DNA encoding the therapeutic protein, but because of low efficiency there is a need to use special molecules and methods to improve gene delivery. A vector can be described as a system fulfilling several functions, including (a) enabling delivery of genes into the target cells and their nucleus, (b) providing protection from gene degradation, and (c) ensuring gene transcription in the cell. The administration of gene therapy vectors requires that they should not only be targeted and safe, but also protected from degradation, sequestration, or immune attack. The ideal DNA vehicle should also be suitable for clinical application. However, it has to be inexpensive and easy to produce and purify in large amounts and at high concentrations.

Two kinds of vectors have been employed as vehicles for gene transfer. Viral vectors for gene transduction, such as retroviral, adenoviral, and adeno-associated viral vectors, and non-viral vectors for gene transfection, such as plas-

mids and liposomes. However, each vector has its own advantages and disadvantages; none of these types of vectors has been found to be ideal for both safe and efficient gene transfer and stable and sufficient gene expression.

Viral delivery systems

Viral techniques use various classes of viruses as a tool for gene delivery. Viruses introduce their DNA into the cells with high efficiency. Therefore it is possible to take advantage of this by introducing a foreign gene into the virus and then using the properties of the virus to deliver this gene with high efficiency into the target cells [6]. Gene therapy vectors are being developed by genetic modification of retroviruses, adenoviruses, poxviruses, parvoviruses (adeno-associated viruses), herpesviruses, and others [7]. Unlike wild-type viruses, these vectors are used to transfer therapeutic genes into target cells and thus are engineered by deleting the essential genes which allow replication, assembling, or infection. These genes can be replaced by a therapeutic gene to make the genome of a gene therapy vector. Such vectors lose their ability to reproduce in target cells and can be replicated only in a cell line which supplies the deleted function. Replication deficiency ensures the safety of viral vectors, but on the other hand, vectors need to be produced in large amounts of virus particles. For this purpose, there are specialized cell lines called “packaging cell lines” (PCLs) engineered to replace a function of a deleted viral gene and for the production of recombinant viruses [8].

Removed virus genes (for example the gene for a capsid protein) are inserted into the genome of the packaging cells and can be expressed there. To produce an active vector, a defective vector with a deleted capsid protein gene is introduced into the PCL. Replicase enzyme encoded in the vector ensures the production of many copies of the defective vector genome. The capsid protein gene encoded in the packaging cell genome is also transcribed and its mRNA translated to produce capsid proteins. This enables the production of the active therapeutic viral vector. The process by which the function of the deleted viral gene is supplied by a protein encoded in the PCL genome is called “complementation” [9].

The interactions between a vector and a host-cell genome cannot be completely eliminated. In the process of recombination, the defective therapeutic vector can receive the deleted viral gene back. The recombinant vector genome is then no longer defective. These replication-competent vectors, called “reactivants”, can lose the therapeutic gene during the recombination and are the subject of infection control studies.

Viruses currently developed as transfer vectors fall into two classes, following their different strategies for replication and survival. Non-lytic viruses, including retroviruses and lentiviruses, produce virions from the cellular membrane of an infected cell, leaving the host cell relatively intact. Lytic viruses, including human adenovirus and herpes simplex virus families, destroy the infected cell after replication and virion production. This native nature of the original viruses determines the use of each recombinant replication-defective viral vector in clinical applications. Despite some limitations on the use of viral vectors regarding safety and

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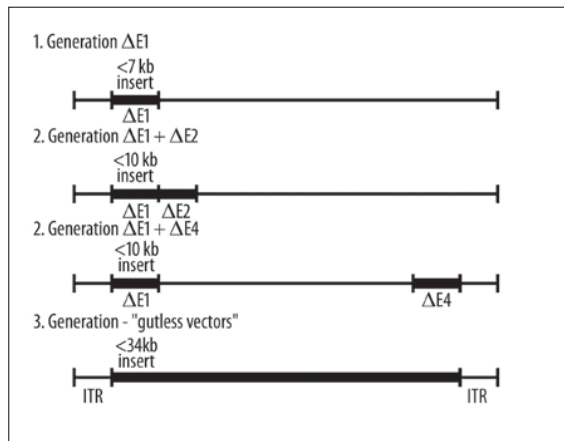


Figure 1. Three generations of adenoviral vectors have been developed.

1. Generation has a deletion in E1 region that ensures viral replication and DNA synthesis. However, these E1 deleted viruses can be complemented by co-infection of another DNA virus and also the function of E1 region may be supplied by cytokines. Still notable immunogenicity and low-level replication of first generation led to the preparation of second generation, that was constructed by the deletion of other viral regions, either E2 or E4 region. Second generation has significantly reduced cytopathic effects and immune responses. Third generation called "gutless vectors" was developed by deleting all viral genes except inverted terminal repeats and packaging signal and is characterized by highly reduced immunogenicity and capacity up to 34 kb.

reproducibility, they are still the most used gene transfer vehicles [10].

Adenoviruses

Adenoviruses are non-enveloped, double stranded DNA viruses with a diameter of 70 nm, an icosahedral conformation, and a genome size of about 36 kb encoding 50 viral polypeptides. They are the best described and most used systems for gene transfer, originally prepared to overcome the limitations of retroviral vectors. More than 50 different serotypes of adenoviruses are known, and the most frequently utilized for gene therapy are type 2 and type 5. Due to their facility to infect tissue, the group of adenoviruses associated with many human diseases (typically diseases of the respiratory system) is suitable for use as gene transfer vectors. Recombinant adenoviruses can be obtained *in vitro* at very high concentrations (10^{10} – 10^{11} viral particles/ml) and are easy to manipulate. The capacity for foreign DNA is up to 8.5 kb, which suffices for most therapeutic genes. Transduction into recipient cells and internalization is mediated through interaction of a 182-kDa fiber protein in the viral coat with the cell-surface coxsackie and adenovirus receptor, and interaction of the arginine-glycine-aspartic acid motives in the penton base with cellular integrins. Targeted gene transfer (recognition of cell-specific receptors) can be achieved by modification of capsid proteins, fiber proteins, or penton bases [11].

Three generations of adenoviral vectors have been investigated in the past in order to decrease cytopathic effects

and immune responses (Figure 1) [12]. The first generation contains a deletion in the E1 region encoding products which control the activation of DNA synthesis and late viral expression. E1-deleted adenoviruses can be complemented by co-infection with another virus (papillomavirus, cytomegalovirus). This first generation is defective in the production of infection particles in the target cells and has notable immunogenicity. Therefore, for further attenuation of the virus, other parts of the viral genome were deleted. The second generation also has a deletion in the E2 or E4 locus in addition to that in the E1. Long-term expression without cytopathic effects has, however, not been achieved yet. The third generation, called "gutless vectors", contains only cis-acting inverted terminal repeats and a packaging signal. These vectors are characterized by highly reduced immunogenicity, but are not able to replicate unless complemented [13]. To achieve the required quantities of adenoviral vectors, the PCL HEK 293 was used. Recently introduced PCLs overcome the problem of recombination and the production of replication-competent adenoviruses in HEK 293 [14].

Adenoviruses can infect dividing as well as quiescent cells with equally high efficiency [15]. Thus, gene delivery into non-dividing target cells with a high level of differentiation has become easier. Among other advantages, adenoviral vectors are characterized by their complexity, stability, and broad cell specificity. Integration of the virus into the host genome does not occur and the viral sequences are absent in the progeny of transduced cells. The consequence of the non-integrative nature of adenoviral vectors is a transient expression of foreign delivered genes [11].

It is evident that the recipient's immune system plays a pivotal role in adenovirus-mediated gene transfer [16]. The majority of the human population has been exposed to adenoviral infection and thus has antibodies against different serotypes. Consequently, almost 90% of injected adenoviral vectors are degraded within 24 hours, often even before reaching the target cells. Acute-phase responses after injection (rapid release of proinflammatory cytokines) can be caused by the capsid protein [17]. The main limitation of adenoviral vectors is the B- and T cell-mediated inflammatory response resulting from an early activation of immune cells. This influences the efficiency of gene delivery as well as the health of the patient [18]. Other disadvantages of adenoviruses are their short-term expression, particularly in dividing cells, difficult or limited transduction of the cells with reduced or no expression of attachment and internalization receptors, transient expression of foreign genes, and immunogenicity [19].

Present studies are focused on inhibiting inflammatory responses against adenoviral vectors and circumventing the degradation of adenoviral particles. A promising approach in this area is the use of vectors with capsid proteins derived from different adenoviral serotypes [20]. On the other hand, immunogenicity can be useful, for example in cancer therapy, as it stimulates the patient's immune system against tumor cells [21].

Retroviruses

These small RNA viruses, replicating through a DNA intermediate, were among the first constructed human gene thera-

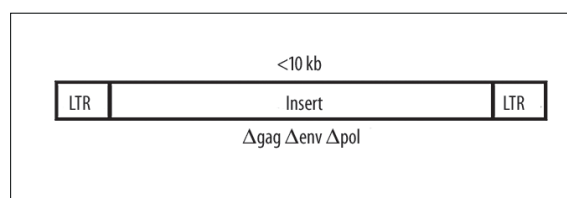


Figure 2. Construction of retroviral vectors is based on removal of genes required for virus replication and insertion of therapeutic gene in their place. This deletion of *gag*, *env* or *pol* genes makes the vector replication defective, unless complemented in packaging cell line. Long terminal repeats (LTR) are signal sequences, which control the gene expression.

py vectors [22]. The retroviral genome consists of few genes, including those essential for replication, and long terminal repeats (LTRs). By constructing a transfer vector, genes such as *gag* (capsid), *pol* (reverse transcriptase), or *env* (envelope glycoprotein) are deleted and replaced by the therapeutic gene (of a possible size of up to 10 kb) (Figure 2). These deletions make the virus unable to replicate and, thus, the vector can produce viral particles only if transfected into a PCL [23]. The minimal vector containing the therapeutic gene can transfect these cells during the production of defective recombinant retrovirus, which is able to deliver the transgene, but not initiate a productive infection. The PCL carries the genes needed in *trans* for replication of viral elements, while the vector contains the sequences necessary in *cis* for viral RNA replication, packaging, and integration as well as the therapeutic gene. This division of *trans* components into the PCL and *cis* components into the vector played an important role in the development of all viral vector systems. The main idea behind this system is that recombinant viral vectors cannot replicate and produce viral particles after transduction of target cells (because they do not encode the *trans* genes required for replication). The potential risk of recombination between retroviruses and PCLs, generating replication-competent retroviruses, has been eliminated in the last years. The *trans* components are divided into different chromosomal locations within the PCL genome. This makes full recombination virtually impossible [24].

Targeted retrovirus-mediated gene transfer with high tissue specificity can be achieved by fusing part of the *env* gene to a coding sequence for the ligand to a tissue-specific receptor. Most vectors derived from retrovirus are based on Moloney murine leukemia virus (MoMLV). Because of their integrative nature, retroviruses maintain the viral sequences also during cell division [25]. This can present an advantage in the treatment of inherited and chronic diseases, but also a risk of inserting mutagenesis and toxicity based on over-expression of the therapeutic gene.

There are many important limitations of retroviral vectors: (a) low vector titer (10^7 infection particles/ml), (b) low transfection efficiency, demonstrated in *in vitro* experiments, (c) particle instability and difficulty to concentrate, and (d) inability to transduce non-dividing post-mitotic cells, particles infecting only proliferating cells. Therefore, the retroviral vectors are suitable for *ex vivo* gene therapy, for example for transducing CD34+ bone marrow hematopoietic stem

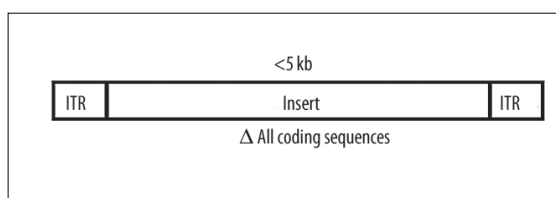


Figure 3. Vectors derived from adeno-associated viruses can be constructed by deleting all coding sequences of the virus (including *rep* and *cap* genes) except inverted terminal repeats (ITRs) and inserting the therapeutic gene. Production of recombinant AAV vectors is achieved by co-infection with helper virus or plasmid carrying the *rep* and *cap* genes necessary for replication and encapsidation. Expression of foreign gene is driven by ITRs.

cells or peripheral blood lymphocytes [26]. Despite all the disadvantages, retroviral gene delivery systems have been already used in a number of clinical trials [27].

To overcome the limitations of retroviruses, vectors derived from lentiviruses have been constructed. Lentiviruses (e.g. human immunodeficiency virus type 1: HIV-1) are a special group of retroviruses with the ability to infect both proliferating and quiescent cells. To expand the spectrum of target cells, it is possible to replace the genes for surface glycoproteins by genes from another viral genome (ebola virus, vesicular stomatitis virus) in the PCL of the vector. The HIV-1 vectors have 6 of all 9 genes deleted, keeping only *gag*, *pol*, and *rev* genes. Further modification was conducted by deleting the terminal signal sequences. Lentiviral transfer systems ensure long-term expression and efficient transfer without inflammatory responses [28]. An efficient transduction was achieved by infecting the cells of the lympho-hematopoietic and central nervous systems, whereas in liver and muscle cells the level of transgene expression was poor [29]. The great potential of these vectors should be further studied in future preclinical and clinical experiments.

Adeno-associated virus

Adeno-associated virus (AAV) belongs to the parvoviruses. It is a single-stranded, non-enveloped DNA virus of 4.5 kb size which causes a latent infection of human cells. Parvoviruses represent an alternative to malignancy-related retroviruses. Besides the low risk of insertional mutagenesis, parvoviruses also infect a wide spectrum of mammalian cells and can thus be used as vectors for gene therapy in a number of experimental animal species [30].

Most AAV-based vectors are derived from adeno-associated virus serotype 2, but also from other serotypes (AAV 1, AAV 3A, AAV 4, AAV 5, AAV 6). Wild-type AAV 2 is non-pathogenic and integrates site-specifically into the long arm of chromosome 19 after infection of human cells [31]. Recombinant AAV 2 vectors are generated by insertion of a therapeutic gene between two inverted terminal repeats (ITRs), replacing all coding sequences, including *rep* and *cap* genes, except ITRs (Figure 3) [32]. This recombinant AAV plasmid is co-transfected in HEK 293 cells with a "helper" plasmid containing *rep* and *cap* AAV genes and adenoviral E2A, E4, and VA genes needed for the appropriate expression of the AAV genes, but lacking the AAV inverted terminal repeats.

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So the productive infection is dependent on the presence of a helper virus (adenovirus, herpes simplex virus, vaccinia virus). *Rep* and *cap* genes are necessary for replication and encapsidation, as well as for site-specific integration (*Rep* 78 and *Rep* 68 proteins). Consequently, because of the removal of all internal coding sequences, the recombinant AAV vectors lacking these genes are replication-defective unless co-transfected with a helper virus bearing these functions [33]. The AAV-based vectors contain only 145 bp of the original AAV genome and their packaging capacity for a DNA insert is 4–5 kb.

As the infection of human cells is latent (long-term persistence of a proviral genome), AAV vectors can ensure the long-lasting expression of a therapeutic gene. Beside this, the main advantages of AAV vector systems are: high stability, safety and efficiency, heat-inactivation resistance, wide host range, and broad tissue tropism. Unlike retroviruses, which are of murine origin, the adeno-associated viral transfer systems are derived from human viruses and therefore may be more feasible for human gene therapy. Recent studies indicate that the main limitation of small packaging capacity will also be overcome [34].

Important progress has been achieved in the field of target infection by capsid modification. Utilization of the notable tissue selectivity of another parvovirus (B19) resulted in the development of recombinant parvovirus B19 vectors in which the AAV genome is encapsidated into B19 capsid structures. Parvovirus B19 uses erythrocyte P antigen as a cellular receptor for selective infection of human hematopoietic cells [35,36]. The generation of such vectors has been achieved by transfecting HEK 293 cells containing recombinant AAV plasmid with a helper plasmid containing AAV *rep* and B19 *cap* genes. During the ensuing adenoviral infection the recombinant AAV genome was encapsidated into the B19 structures. Thus, the produced vectors are characterized by a high efficiency of cell-specific transgene delivery and therefore may be potentially used in the gene therapy of human hemoglobinopathies [37]. Despite the pathogenicity of parvovirus B19, the recombinant B19 vectors are highly safe because they do not contain any B19 virulence gene.

Adeno-associated viral gene delivery systems have been already successfully used in the phase I clinical trials for the gene therapy of cystic fibrosis and hemophilia B, and further clinical studies are considered for hemophilia A, α antitrypsin deficiency, and Leber congenital amaurosis [38]. Preclinical application of AAV vectors showed a promising development in the treatment of human diseases such as myocardial infarction, liver cirrhosis, or colon and pancreatic tumors [39]. Considering this as well as the fact that host cellular immune responses have not yet been observed, AAV vectors seem to be a promising and effective system for human gene therapy.

Herpes simplex virus

Herpes simplex virus is an enveloped, double-stranded DNA virus 150 kb in size. It is a natural human pathogen, replicating in epithelial cells, but able to stay in a latent state in non-dividing cells (neurons). Gene transfer vectors are based on Herpes simplex virus type-1 (HSV 1) and can be

prepared by (a) cloning the therapeutic gene into a plasmid containing the HSV origin and a packaging signal followed by transfecting the cells and infecting with helper HSV virus, or (b) cloning the gene into a plasmid surrounded by specific HSV sequences and this plasmid is co-transfected to cells with HSV virus [40]. The subsequent recombination between the homologous sequences in the plasmid and the virus results in the introduction of the therapeutic gene into HSV. A reporter gene, such as the green fluorescent protein, helps to identify the recombinant viruses. Because of the pathogenicity of herpes simplex virus, the key role in the construction of vectors is inactivating the pathogenic genes of the virus [41]. This can be reached either by deleting the genes needed for replication and encapsidation, such as immediate early genes, or by removing the genes essential for growth in quiescent cells. This determines the safety and efficiency of the transduction of non-dividing cells, which offers a potential for clinical neurology [42].

The main advantage of HSV-based vectors is their large cloning capacity for foreign genes (30–40 kb). The genome of HSV is one of the biggest among viral vectors. A number of genes can be deleted and this enables the introduction of a very large gene or even of several smaller genes. On the other hand, after entering a latent state, the HSV turns off the expression of all genes, including the gene of interest [43]. Only one small region in the HSV genome stays active during latency. This LAT region is under the control of two promoters, LATP1 and LATP2, and produces the latency-associated transcripts. This limitation can be overcome by inserting the foreign gene into the LAT region [44].

Poxviruses and Alphaviruses

Poxviruses derived from the vaccinia virus are engineered by introducing the therapeutic gene directly into the vaccinia genome without any inactivation or disabling [45]. The cloning capacity is large and the vector is not integrated into the host genome, but the ability of replication can cause side effects such as immune response.

Alphaviruses are members of the *Togaviridae* family and are enveloped, single-stranded RNA viruses of icosahedral conformation. There are three different viruses with similar properties useful in gene therapy: Semliki forest virus (SFV), Sindbis virus (SIN), and Venezuelan equine encephalitis virus (VEE) [46]. Most alphavirus vectors used as gene vehicles are replication incompetent. They encode only the non-structural genes, while the structural genes necessary for replication are located in the helper plasmid genome. To eliminate or at least reduce the possibility of generating infectious replication-competent particles, structural genes can be localized to two different helper plasmids. However, the replication-competency of alphaviral vectors can also be advantageous when used in intratumoral gene delivery [47,48]. Replication-competent vectors contain all the genes of the wild-type genome, including the therapeutic gene, and are also able to transduce neighboring and surrounding tumor cells.

Because of their extensive host-cell toxicity and broad host range, alphaviruses are suitable for cancer gene therapy of various tumors. Many clinical trials have showed that intratumoral injection of recombinant alphaviruses induced ap-

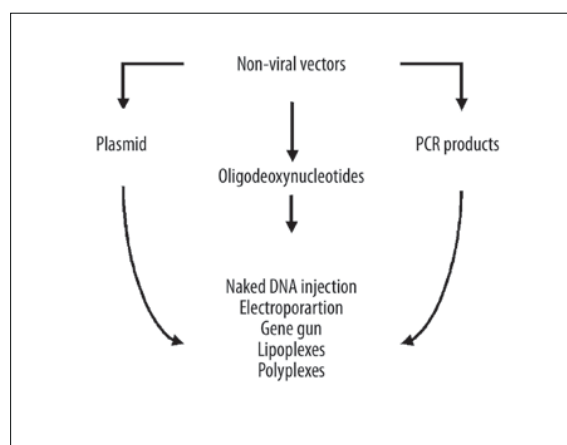


Figure 4. In comparison to virus-derived vectors, non-viral vectors have several advantageous qualities, mainly the safety of administration without immunogenicity, but also almost unlimited transgene size and the possibility of repeated administration. Therapeutic gene can be introduced into the target cell either as an insert in plasmid with regulation sequences, what enables the regulation control of expression (inducible promoter) or as a PCR product. The simplest way of gene introduction is an injection of naked DNA into target cells. However, this approach does not reach the transfection effectivity of viral vectors. Thus, many techniques have been developed to improve the introduction of therapeutic gene. Physical methods like electroporation (short electric pulses induce the membrane potential and produce membrane pores) and gene gun (DNA is coated onto small gold particles) lighten the entry of transgene into target cells. In addition, lipoplexes (DNA-liposomes complexes) and polyplexes (DNA-polymers complexes) improve the stability of DNA and also facilitate the entry into the cell. Synthetic oligodeoxynucleotides do not encode any therapeutic gene, but allow the expression inactivation of target genomic gene.

optosis of tumor cells [48]. The great potential of alphaviruses is also given by their immunogenicity and ability to induce cytotoxic T-cell responses. They are suitable for vaccine production and immunization against infectious agents, but also tumors [49,50]. In conclusion, despite some safety problems related to their use as vectors, alphaviruses provide a promising tool in immunization and protection against pathogenic viruses and tumors, cancer gene therapy, target gene delivery, and expression of foreign gene.

Non-viral delivery systems

Non-viral techniques of gene transfer represent a simple and, more importantly, safer alternative to viral vectors. Thanks to their relatively simple quantitative production and their low host immunogenicity, non-viral vectors are attractive tools in gene therapy [51]. In the past, the low levels of transfection and expression of a gene in host cells were among the viral vectors' greatest disadvantages. However, ongoing studies and the development of new vectors that show transfection efficiency just like that of viral vectors point towards their great potential. A non-viral, precisely defined molecule of DNA with the required gene, whose expression is dependent on the cell transcription and translation apparatus, cre-

ates the basis of such vectors. (Figure 4). The main advantage is a virtually unlimited clone capacity, low toxicity and immunogenicity of non-viral vectors, and the ability of repeated application [52].

The simplest technique of non-viral gene transfer is the use of so-called naked DNA. Such a naked plasmid DNA was used in several pre-clinical and clinical trials [53]. Intramuscular injection of naked DNA secures a relatively low level of expression, but sufficient for use in DNA vaccination. DNA vaccination encoding viral antigens caused in several animal experiments an immune response which was protective against various infections [54].

Another successful use of naked DNA with the gene for vascular endothelial growth factor (VEGF) was recorded in the field of therapeutic angiogenesis in critical limb and myocardial ischemia [55,56]. Despite naked DNA application to various tissues, either intravascular or intratracheal, the best result for therapeutic purpose seems to be achieved after intramuscular injection. Moreover, some positive results were gained in cancer therapy by intratumoral injection of tumor suppressor genes or cytokines [57]. An alternative form of the naked plasmid method is the application of PCR products that encode the therapeutic gene instead of plasmid DNA. The experiments showed the same or even higher transfer efficiency. Low transfection efficiency and short-term expression still remain the main disadvantages of naked DNA gene transfer compared with viral vectors [58].

Since *in vitro* transfection by non-viral DNA is more effective than *in vivo* (which is a more complex system), several methods and systems were developed that increase transfection efficiency and expression level. One of the methods that improve DNA penetration of the cell is electroporation [59]. This is a process of transmembrane potential induction by short electric impulses which produce small pores in the membrane. This makes it easier for DNA to get into the cell. *In vivo* use of electroporation is done by injecting naked DNA followed by electric pulses from electrodes that are located *in situ* in the target tissues. Successful use of electroporation was observed in transfecting muscles, brain, skin, liver, and tumors.

Since every tissue is specific and has its own characteristics, there are no generally accepted optimal conditions of electroporation that are suitable for effective transfection. These are dependent both on the amplitude and duration of the electric pulses and on the amount and concentration of DNA. Under *in vitro* conditions, a voltage of several kilovolts and a pulse duration of several microseconds appeared to be optimal. In contrast, under *in vivo* conditions several tens or hundreds of kilovolts and duration of milliseconds are needed [60].

Similarly to electroporation, another method, called the "gene-gun", does not require the presence of complicated and potentially toxic delivery systems [61]. The gene material transfer is mediated by miniature particles of gold on which the DNA is bound. These particles are then shot into the cell under great pressure and speed (with the help of compressed helium) and so pass the membrane barrier. At first, the gene-gun was developed for gene transfer into plant cells; however, its use has expanded to gene transfer

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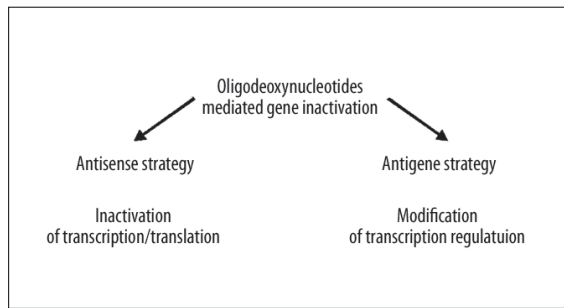


Figure 5. Synthetic oligodeoxynucleotides (ODNs) represent an alternative tool for down-regulation of gene expression (gene inactivation). There are two different strategies known for gene-specific expression interruption. Antisense strategy means introduction of gene-specific oligodeoxynucleotides and their complementary binding to target gene or mRNA. This process inhibits the transcription or translation of target gene by antisense inactivating without affecting other genes. The aim of antisense strategy is to avoid the interaction between transcription factor (TF) and its target gene by introduction of specific oligodeoxynucleotide, which acts as a decoy for transcription factor. In this process, the transcription activation is stopped as a consequence of decoy-TF interaction.

into the mammalian cells [62]. Effective exploitation of the gene-gun was also achieved in the realm of DNA vaccination. The latest clinical experiments focus on cancer vaccines against various human tumors [63]. Both methods (electroporation and the gene-gun) have been shown to be much more efficient in DNA transfer into the cell than the transfer of naked DNA only.

Though non-viral vectors are much safer than viral, there is still a need to improve their safety. One of the possible approaches is to remove all sequences that do not encode the therapeutic gene. For example, so-called “minicircle DNA” systems are double-stranded superhelix DNA molecules that contain, except for transgene and its expression regulation components, only lambda phage attachment sequences [64].

Oligodeoxynucleotides

Gene therapy using synthetic oligodeoxynucleotides is based on inactivating the genes related to disease processes [65]. The main aim is to inhibit the expression of target genes *in vivo*. For this purpose, two different approaches using oligodeoxynucleotides (ODN) are known (Figure 5). The antisense strategy is based on disrupting the transcription of the desired gene with the help of gene-specific antisense ODNs without influencing other genes. The alternative is the use of ribozymes, specific RNA molecules with catalytic activity, to interrupt the translation process by cleavage of unique sequences in the specific mRNA. In general, the antisense strategy affects the transcriptional or translational level and the result is the suppression of expression by ODNs complementary to disease-related genes [66,67].

The second approach, the antigene (decoy) strategy, is based on modifying transcriptional regulation and uses the *cis*-ele-

ment double-stranded oligodeoxynucleotides as a “decoy” for specific transcriptional factors (TFs). Every TF activates the transcription of specific genes. Therefore the binding of decoy ODNs to TF inhibits the interaction between this TF and the target gene regulation sequence and, consequently, the transcription process is stopped [68]. Indeed, the exact molecular structure and sequence of each transcriptional factor has to be known. Thus the aim is to prevent the TF from interacting with the target gene and to avoid activation of the expression of genes encoding cell cycle regulatory proteins, cytokines, etc. The antigene decoy strategy is being used in TF-driven diseases, particularly in the cardiovascular gene therapy of diseases such as restenosis after angioplasty (target TFs: E2F, NFκB), hypertension (angiotensin), inflammation (NFκB), graft rejection (E2F, NFκB), and other.

The main advantage of the decoy approach is the simplicity of ODN synthesis and manipulation and also the tissue specificity of the target TF. On the other hand, there are some limitations in the use of decoys, the high rate of degradation by endocytosis or nucleases as well as a short lifetime being among the most important. These can be overcome by the use of proper delivery systems that increase the stability of ODNs.

An alternative antigene strategy is the technology called “RNA interference”, in which the introduction of double-stranded RNA leads to inhibition of the expression of the specific gene [69]. The keystone is the effect of small interfering RNAs which recognize and degrade the target mRNA. Small interfering RNAs are short oligonucleotides of about 19–23 nucleotides characterized by a high specificity of gene targeting, high efficiency of expression reduction (up to 90%), and a long-lasting effect. Therefore RNA interference also provides a strong tool for the regulation of gene function [70]. Both oligodeoxynucleotide-based gene therapy methods are used in the treatment of pathological processes, where the inactivation of a specific gene or inhibition of an undesired physiological function caused by dominantly acting mutant alleles is needed [71].

A different approach in the treatment of diseases caused by dysfunctions or mutations in specific genes is the repair of these genes. This attractive strategy can be achieved by gene targeting using RNA/DNA oligonucleotides that encode the functional (“healthy”) alternative of the gene. The possibility of homologous recombination between the functional and dysfunctional copy of the gene (similar sequences) is the basis of this method [72]. However, it must be noted that reviewing the results of this technique would be truly preliminary.

Lipoplexes and polyplexes

In order to facilitate the effective transfer of non-viral DNA into the cells, synthetic vectors improving the admission of DNA into the cell and protecting it from undesirable degradation were created. The most used are derived from lipids or synthetic polymers.

Plasmid DNA can be covered by lipids into organized structures such as liposomes or micelles. This complex of DNA with lipids is called a lipoplex [73]. Lipoplexes can be divided into two types:

Anionic and neutral liposomes. At first, anionic and neutral lipids were used for the construction of synthetic vectors. Even though they were characterized by safety, compatibility with body fluids, and the possibility of tissue-specific gene transfer, the level of transduced cell expression was relatively low. In addition, the construction of these lipoplexes was very demanding, and so attention turned to cationic lipids. At present, however, new neutral and anionic liposomes suitable for *in vivo* gene therapy are being constructed [74].

Cationic liposomes. In contrast to neutral and anionic liposomes, which need DNA implementation into the vehicle, cationic liposomes naturally create complexes with negatively charged DNA. Their positive charge, moreover, allows interactions with the negatively charged cell membrane and thus penetration into the cell is permitted [75]. One of the advantages is endocytosis of such a lipoplex followed by the release of the DNA into the cytoplasm. Cationic liposomes also ensure effective protection against the degradation of the foreign DNA by the cell. The specificity and structural demands of transfection are determined by the differences in the structures of various cationic liposomes, especially by the hydrophobic anchor.

The interactions of liposomes with DNA and the subsequent lipoplex formation are dependent on several physical conditions (pH, charge) as well as structural characteristics of the liposomes. The most frequent use of DNA-liposome complexes is in gene transfer into cancer cells, where the applied genes stimulate anti-tumor immune processes or genes decreasing the activity of oncogenes [76]. Recent studies revealed the ability of lipoplex gene transfer into the epithelial cells of the respiratory tract, which supports their usage in the therapy of respiratory diseases and cystic fibrosis [74]. Their expression in all main organs, mainly in lungs, was observed after intravenous administration of lipoplexes. Targeted transfection can be gained, to some extent, by the addition of tissue-specific target ligand.

The *in vivo* process of lipoplex transfection consists of several parts and it is suggested that the transfection is based on endocytosis of the host cell. Nevertheless, the molecules on the cell surface, which play a crucial role during the interaction with the liposome/DNA complex, have not been identified yet. Some studies point to the key role of proteoglycans in this process. The endosome produced by endocytosis is degraded in the cell and the DNA is released into the cytoplasm. This process is mediated by so-called “helper lipids”, which are present in the liposome itself. So the important factor for successful transfection is the presence of an endosome disruption mechanism.

Another important step is the unpacking of the DNA, since DNA is inactive when coated in a liposome. One of the hypotheses is the interaction between cationic lipoplexes and anionic cell lipids that leads to the neutralization of these complexes (formation of neutral ion pairs) and to release of DNA from the liposome/DNA complex. Such released DNA is ready for expression.

Transport of DNA into the cell nucleus mediated by lipofectins is considered by most of the studies to be a key step in gene transfer [77]. This process is dependent on the size of the DNA as well as on its conformation (linear DNA is

transported more effectively to the nucleus) and is secured by the nuclear pore complex (NPC), which is present in the nuclear membrane [78]. NPC binds the sequence called the “nuclear localization signal” (NLS).

Vectors based on a complex of polymers with DNA are called polyplexes. Most of them consist of cationic polymers and their production is regulated by ionic interactions. In contrast to lipoplexes, some polyplexes (polylysins) are not able to release intracellular DNA into the cytoplasm [79]. For this purpose, co-transfection with endosome-lytic agents (inactivated adenovirus) is needed. On the other hand, polymers such as polyethylenimine have a mechanism of endosome disruption and there is thus no need for transfection with endosome-lytic agents. Polyethylenimine is used as a vector in aerosol inhalation gene therapy. It is a non-invasive and relatively effective gene transfer, especially into the respiratory tract, with permanent gene expression in this target region without undesirable expression in other tissues [80].

The size of the polymer determines the transfection efficiency and is specific for each individual gene transfer. In general, the longer the polymer chain, the better the condensation of DNA that is so protected against degradation. For DNA to be active it must be released from the complex, and that is why the polymer cannot be too big. It is the size of the aerosol particles that determines the place of action, and thus the specificity of inhalation gene therapy. The preference of respiratory tissues as a target area suggests the potential of this method in apoptotic gene transfer into lung tumors [81].

An alternative to polyplexes can also be the use of polymer nanoparticles [82]. Two types of such complexes are characterized: gelatin-DNA and chitosan-DNA. *In vitro* transfection does not reach the level of lipoplexes or polyplexes, but in comparison with naked DNA, transfection using nanoparticles shows increased expression *in vivo* when administered intratracheally or intramuscularly.

Hybrid vectors

Because every type of vector has its advantages and limitations, a promising approach to overcome the limitations and exploit the advantages of the individual types of vectors is their combination: the construction of hybrid vectors (Figure 6). A hybrid vector combines the main advantages of the systems from which it is derived [83]. Several types of hybrid vectors are known:

- a. Virosomes are produced by the fusion of lipoplexes (liposomes with DNA) with inactivated HVJ virus (hemagglutinating virus of Japan) or influenza virus [84]. Their potential lies in gene transfer into the respiratory tract, because it was shown that the efficiency of transduction is higher than in cationic liposomes or viral vectors. In addition, they are very well tolerated from the immunological view, so even repeated injection does not influence the efficiency and safety of transfer;
- b. The second type is represented by hybrids that were created by simply mixing cationic liposomes or polymers with adenoviral vector. These are effective mainly in cells which do not have viral receptors. Besides, it was proved that an inactivated adenovirus attachment improves the

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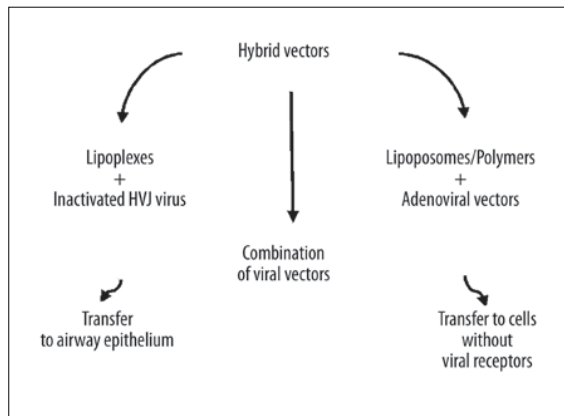


Figure 6. Every type of gene vectors has its own pros and cons. Combination of two or many vectors (hybrid) enables to overcome individual disadvantages and unite the advantages of each vector (see in text).

efficiency of the transfer mediated by cationic liposomes or polymers [85];

- c. Hybrid viruses can be constructed by a combination of various types of viral vectors, and they represent a system which employs the main advantages of both maternal viruses [86]. An example is a hybrid of adenovirus with Epstein-Barr virus (EBV), which is characterized by high titer (an attribute of adenoviruses) and long-term presence in cell in the form of a stable episome.

Regulation and gene expression in non-viral vectors

As the main limitation of non-viral vector systems is the absence of stable and long-lasting expression, many recent studies focus on researching a system capable of long-lasting expression.

The reason for short-term transgene expression is its inactivation, which can be caused by various processes. One of them is so-called "gene dilution". This process takes place mainly in dividing cells with a plasmid vector that is not integrated and in which the number of copies of extrachromosomal DNA reduces with each cell cycle. Repression of this barrier is possible when viral replicons, such as EBV and SV40, are added into a non-viral vector [87]. Sequences from these viruses ensure extrachromosomal replication, the presence of the vector as a stable episome, and also a high efficiency of transfer. Plasmids that are based on the EBV replicon consist of the EBNA1 gene (nuclear antigen 1) together with the origin of replication oriP. EBNA1 secures nuclear episome transfer with the oriP sequence and binding to the nuclear matrix and chromosome. Episome replication is thus synchronized with the chromosome and thus stable expression is facilitated in dividing cells.

Another possibility of expression inactivation is condensation of the chromatin and histone deacetylation caused by the influence of the histone-deacetylase complex, whose mechanism of action is still not fully known. Nevertheless, it is known that the expression reactivation of such inactivated genes can be gained by inhibitors of histone-deacetylase (e.g. trichostatin A). The main problem, however, is the non-specific effect of inhibitors and thus their potential toxicity [88].

It is apparent that the ultimate step in non-viral gene transfer is DNA transport into the nucleus, because the RNA-polymerase necessary for transcription is present in the nucleus. Classic plasmids remain in the cytoplasm after penetration into the cell and are gradually inactivated or degraded. One of the possible solutions is the above-mentioned EBV system (EBNA1 + oriP). An alternative to EBV is the use of the cytoplasm T7 expression system [89]. Since T7 RNA-polymerase does not have a nuclear localization signal, there is no need for transport to the nucleus. This T7 RNA-polymerase is supplied to cells with a plasmid and is highly specific to its own T7 promoter, which is present in the vector and which regulates the expression of the therapeutic gene.

One of the claims for gene therapy is also the ability to direct a therapeutic gene into specific cells, and this is called "tissue specificity of vectors". The reason is undesirable expression in other tissues (e.g. after VEGF gene transfer, angiogenesis appeared in non-target cells). The principal strategy for tissue specificity is the exploitation of cell-specific promoters as, for example, LCR (locus control region). LCR activates inactive chromatin and so creates a transcription-active domain. This process is tissue specific.

Vectors based on chromosomal sequences, artificial operational and structural units, which do not interfere with the genome and secure expression from an arbitrary place in the genome, have great potential. Particular attention is directed towards the construction and use of artificial chromosomes [90]. They represent an independent structural and operational unit whose replication is stable and autonomous, dependent on the cell cycle. The capacity is virtually limitless, although all the necessary regulation sequences are included. Artificial chromosomes have not been used as vectors in gene therapy; however, in the future they represent an attractive alternative for gene transfer.

An important aspect of vector applications in gene therapy is the possibility of regulating the expression of a therapeutic gene. In practice, the most used are the so-called "inducible systems". A substance activating the promoter of the therapeutic gene is administered after vector transfer into the target tissue, thus ensuring its expression [91]. This inducer should be highly specific to the promoter activation which regulates the transcription of the target gene and, similarly, the promoter should be highly specific to activation by this inducer. Among the basic requirements are also the repeatability and reversibility of induction as well as the safety and availability of the inducer. Application of the inducer is possible either *per os* or intravascularly. Possible undesirable over-expression can be stopped by inducer omission. Currently used systems employ substances such as tetracycline, ecdysone (insect hormone), or rapamycin as inducers [92]. Their limitation was a slight toxicity and the development of immune reaction. A possible solution to eliminate immune reaction could be the use of a regulation system with an activator derived only from human transcription factors. Such a potential inducer is the endogenous human transcription factor PPAR γ 2 that was used in several studies; it shows safety and ensures long-lasting expression. Its effects, however, have to be proved in further studies. Promoters induced by hypoxia or radiation and cell systems based on exploitation of bacteria as vectors for gene transfer also have extensive regulation capability for targeted expression [93].

Alternative therapeutic systems

Bacterial delivery systems and cancer gene therapy

The therapy of cancer diseases is a rapidly developing area of recent gene therapy. The targeted therapy of only cancer cells without impact on other tissues still remains one of the main necessities of clinical practice. The specificity of cancer gene therapy remains imperfect and connected with healthy cell degeneration. To repress these limitations, new strategies are being developed. One is the exploitation of the unique microenvironment of cancer. It is characterized by several specific attributes, such as hypoxia or the presence of necrotic areas. Hypoxia can be elegantly used as a cancer indicator which can restrict therapeutic effects to these regions in several ways. The application of hypoxia response elements into vector promoters limits the expressions of therapeutic genes only to the tumor areas [94]. In such a way the expressions of genes for erythropoietin and VEGF have been successfully regulated. Another possibility is the induction of tumor-specific toxicity by administration of toxin precursors, which are activated under hypoxic conditions. Such a substance can be tirapazamine or AQ4N [95]. The latest approach in targeting the gene therapy is the use of bacterial systems as vectors for transfer and gene expression in tumor areas (Figure 7). The principle lies in the ability of some anaerobic bacteria to selectively colonize hypoxic areas (tumors) and replicate there. So it is possible to achieve selective expression of therapeutic genes specifically in tumors. As therapeutic genes, sequences coding for toxins, cytokines (TNF α , tumor necrosis factor α , which is directly cytotoxic for tumor cells), proteins stimulating the immune system (TNF α stimulates T-cell immunity), or genes coding enzymes that transform non-toxic precursors into toxic substance (cytosine deaminase converts 5-fluorocytosine to 5-fluorouracil, which negatively influences the synthesis of DNA and RNA) are used [96].

Currently known and tested bacterial vectors can be divided into two groups. Strictly anaerobic bacteria (the species *Clostridium* and *Bifidobacterium*) are used in *in vivo* experiments. *Clostridium* is the most important bacterial species for use as a vector (*C. beijerinckii*, producing nitroreductase, *C. sporogenes*, and *C. acetobutylicum*, producing cytosine-deaminase and TNF α) [97]. *Bifidobacterium*, since it is not pathogenic, is naturally present in the human gastrointestinal tract and provides higher safety. The level of colonization of tumor tissues by *B. longum*, however, did not reach the level of colonization by *Clostridia*, although the results are positive and promising in *in vivo* systems [98].

The second group consists of attenuated auxotrophic strains of *Salmonella typhimurium*, that require the presence of tumor-specific nutrition factors for selective replication. They use these factors for their own metabolism, thus prohibiting the tumor cells from utilizing them and growing [99]. In *in vivo* experiments, high levels of *Salmonella* (10^9 bacteria/g tissue) were obtained. A similar tumor inhibitory effect was shown by application of *Salmonella* producing thymidin-kinase.

Even though the above bacteria are characterized by high selectivity in tissue colonization, it is necessary to ensure the maximum level of specificity and therapeutic efficiency of bacterial vectors. For this purpose, promoters induc-

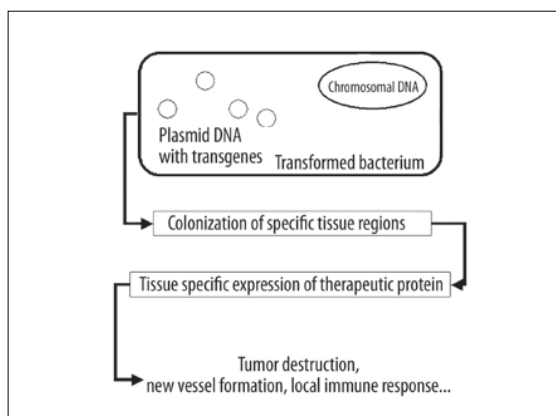


Figure 7. Bacteria provide an alternative tool for therapeutic gene application. Some bacterial species selectively colonize specific regions and tissues in the organism and thus can here express the therapeutic gene (present on a plasmid) or metabolize some substances (tumour-specific nutrients). The transgene expression can be induced by an inducible promoter (radiation-inducible promoter) and thereby the local and tissue specific gene expression is achieved. This approach has been proved as hopeful mainly in cancer gene therapy with the utilization of anaerobic bacteria (*Clostridium*, *Bifidobacterium*) colonizing the hypoxia regions.

ible by radiation were constructed. The transcription of such promoters is conditioned by irradiation with visible light. Bacteria are known for their resistance to irradiation (having a relatively small genome and effective DNA repair mechanisms). Therefore the localization of a therapeutic gene under the control of a radiation-inducible promoter ensures that cytotoxic proteins are expressed only in bacteria colonizing currently irradiated tissues [93]. This strategy allows eliminating the possibility of expression in non-tumor and hypoxic tissues (e.g. infarctions). Some experiments have proved that positioning the repressor-binding site in the promoter area of some genes (TNF α) can change this promoter into a hypoxia-inducible promoter and thus increase the expression of the gene after irradiation. These facts are supported by the assumption that positioning a repressor-binding site into an arbitrary promoter changes this promoter into a radiation-inducible promoter, though this has to be proved in further studies. The latest approach using prokaryotes in gene therapy is a system of transformed bacteria producing a therapeutic protein *in situ* under exogenous induction regulation [100].

CONCLUSIONS

A number of methods have been and are being invented for the efficient and safe delivery of therapeutic DNA. The increasing variety, however, also indicates that to now there is no clear ideal delivery system. The perspectives and hopes that are associated with gene therapy support research in this field of molecular biomedicine [101]. Although clinical trials have already started, there are still numerous limitations that must be solved before routine clinical use. Nevertheless, it can be expected that future research will bring tissue- and disease-specific delivery strategies and that this hurdle will be overcome at last.

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