The AAPS Journal 2006; 8 (4) Article 83 (http://www.aapsj.org).

Themed Issue: Drug Delivery Systems for Targeted Drug Delivery
Guest Editors - Victor C. Yang, Joseph P. Balthasar, Yoon Jeong Park and Jun Feng Liang

Optimizing Targeted Gene Delivery: Chemical Modification of Viral Vectors and Synthesis of Artificial Virus Vector Systems

Submitted: April 10, 2006; Accepted: August 23, 2006; Published: December 8, 2006

Sabine Boeckle[1] and Ernst Wagner[1]

[1]Pharmaceutical Biology - Biotechnology, Department of Pharmacy, Ludwig-Maximilians- Universität München

ABSTRACT

In comparison to classical medicines, gene therapy has the potential to mediate the highest possible level of therapeutic specificity. Every normal or diseased cell can switch on or off a gene expression cassette in a tissue-, disease-, and time-dependent fashion, by use of specific transcription factors that are active only in a given unique situation. In practice, we face the problem in realizing the concept: the delivery of nucleic acids into target cells is very ineffective and presents a formidable challenge. Key issues for future developments include improved targeting, enhanced intracellular uptake, and reduced toxicity of gene vectors. The currently used classes of vectors have complementary characteristics, such as high intracellular efficiency of viral vectors on the one hand and low immunogenicity and greater flexibility of nonviral vectors on the other hand. The merge of viral and nonviral vector technologies is highlighted as an encouraging strategy for the future; concepts include chemically modified viral vectors ("chemo-viruses") and synthesis of virus-like systems ("synthetic viruses"). Examples for the development of vectors toward artificial synthetic viruses are presented.

KEYWORDS: Gene transfer, nonviral vector, receptor targeting, synthetic virus, viral vector

INTRODUCTION

In gene therapy, different types of therapeutic nucleic acids may be applied to achieve various effects at the molecular genetic level (Table 1). The nucleic acids are thereby mostly used to turn on or restore a gene function in a specific fashion (gain of gene function) using targeting strategies as explained in the following 2 paragraphs. A relatively new field in gene therapy applies nucleic acids to yield loss of specific gene functions (loss of gene function), by turning off genes with antisense oligonucleotides or double-stranded small interfering (si)RNA. Both gain-of-function and loss-of-function approaches might revolutionize modern molecular medicine, provided that appropriate nucleic acid delivery and targeting technologies (vector systems) are available.

Specificity for the target tissue may be achieved by 2 major strategies: (1) the therapeutic vector may be preferentially transported toward the target tissue, taken up by the target cells, and delivered into the nucleus of target cells (targeted delivery), or (2) the vector may be delivered into several cell types, but the carried nucleic acid is under the control of specific promoter/enhancer elements that allow gene transcription in the target cells only (targeted transcription).

The concept of targeted transcription was used, for example, to achieve highly specific gene expression in colon carcinoma cells. Specificity was realized using synthetic promoters[1] that are activated after binding of β-catenin/T-cell factor, 2 factors that accumulate in the nucleus of colon cancer cells due to a deregulated Wnt signaling pathway. Brunori et al[2] applied this tumor-specific transcriptional regulation to control the production of attenuated adenoviruses. Since adenoviral replication was driven by the β-catenin/T-cell factor, viruses were produced in colon carcinoma cells only.

Initial efforts to realize the concept of targeted delivery were made about 20 years ago using nonviral vectors.[3] Since then, this concept has been applied for most vector types and, in many encouraging studies, effective and specific targeted delivery was demonstrated. The development of a targeted delivery system is not only essential for specific gene expression in the target tissue but also such delivery systems will allow the administered therapeutic dose to be reduced, thereby lessening toxic side effects and costs for the treatment. In addition, targeted delivery may be combined with other targeting strategies such as targeted transcription. Therefore, targeted delivery will present the main focus of this review. Before introducing the concept in detail, the characteristics and limitations of current viral and nonviral vector systems will be reviewed, outlining why improved targeted delivery remains the key issue.

Corresponding Author: Ernst Wagner, Pharmaceutical Biology - Biotechnology, Department of Pharmacy, Ludwig-Maximilians-Universität München, Butenandtstr 5-13, D-81377 Munich, Germany. Tel: +49 89 2180 77840; Fax: +49 89 2180 77791; E-mail: ernst.wagner@cup.uni-muenchen.de

Table 1. Strategies Toward Specific Gene Therapy*

Therapeutic Nucleic Acids:
DNA (oligonucleotides, plasmid, viruses, artificial chromosomes, bacteria)
RNA (oligonucleotides, ribozymes, siRNA, mRNA, viruses)
Effects of Therapeutic NAs at the Molecular Genetic Level:
Gain of gene function (gene substitution/replacement; trans-splicing)
Loss of gene function (antisense, RNA interference)
Strategies to Achieve Specificity for the Target Tissue:
Targeted delivery of NAs
Targeted transcription of expression cassettes

*NAs indicates nucleic acids.

Characteristics of Current Viral Vectors

Several viral vector systems have been used for nucleic acid delivery; their main advantages and limitations are listed in Table 2. Since it is the nature of viruses to deliver their genes into host cells, they present good candidates for the development of effective gene delivery. Natural evolution, however, optimized them for infecting and replicating their genome in the host cells, but not necessarily for survival of the transduced host cell and maintenance of the expressed genes. Nevertheless, replication-defective viruses, where viral genes were partly replaced by therapeutic genes, represent the first generation of viral vectors applied in gene therapy.

Retroviral vectors and adenoviral vectors have been most commonly used in clinical trials (www.wiley.co.uk/genetherapy/clinical/). Other viruses that were used to develop viral vectors include adeno-associated virus (AAV), herpes virus, pox virus, and more recently lentivirus. Viruses in general are strong in cellular uptake and intracellular efficiency, therefore few viral particles are sufficient for the transduction of cells. Most frequently, replication-defective viral vectors are applied that can replicate only in special producer cells but not in the host body cells. For some applications, however, viral replication after the application of viral vectors can be used to multiply the therapeutic effect. In tumor therapy, for example, attenuated replicative viruses[2,4] were used that can only replicate within tumor cells. The virus replication process directly kills the infected tumor cells (oncolytic viruses) and/or strongly amplifies the copy number of the therapeutic gene carried by the viral vector.

Gene transfer activity of viral vectors is dependent on the type of virus used for transduction. Murine retroviral vectors, in contrast to lentiviral or adenoviral vectors, can transduce dividing cells only. This specificity has been applied in clinical trials for the treatment of glioblastoma.[5] Retrovirus-producing cells were locally injected into glioblastoma, and the retroviral vectors transduced tumor cells but not nondividing normal brain cells. The transduced tumor cells expressed the therapeutic gene encoding for thymidine kinase. The produced enzyme converted the administered prodrug ganciclovir, into a cytotoxic agent locally. This concept gave encouraging results in preclinical

Table 2. Strengths and Weaknesses of Currently Used Vectors

Vector	Greatest Advantages	Greatest Disadvantages
Retrovirus	Ex vivo stable transduction of blood cells	Low efficiency in vivo; risk of insertional mutagenesis
Adenovirus	High short-term expression in vivo (in liver)	Immunogenicity and inflammatory responses
Adeno-associated virus	Long-term expression in vivo	Small genome
Viral Vectors in General	High efficiency on particle basis	No repeated administration because of immunogenicity
Naked DNA	Simple (eg, for vaccination)	Low efficiency
Physically enhanced delivery of DNA (electroporation; gene gun, hydrodynamic delivery)	High expression in vivo	Limited localized area, device required
Complex-based gene transfer (lipoplexes, polyplexes)	High expression ex vivo; expression in vivo (localized and systemic)	Short-term expression; toxicity of cationic carriers
Nonviral Vectors in General	High flexibility; lower immunogenicity	Low efficiency on particle basis

and initial clinical evaluation. Clinical phase III trials, however, could not demonstrate a patient benefit.[5] Similar to other clinical gene therapy trials, this was mainly because of limited gene transfer efficiency.

Despite their low in vivo efficiency, murine retroviral vectors are useful for ex vivo gene transfer because of their ability to efficiently integrate into the host cell genome. In particular, they have been optimized for gene transfer into hematopoietic stem cells. Gene therapy of human severe combined immunodeficiency syndrome (SCID)-X1 can be regarded as the first success story in gene therapy.[6-8] Fifteen out of 16 patients with X-SCID have benefited from gene therapy. Correction of the immunodeficiency has been maintained now for 7 years in the first patients thus treated. The therapy, however, was associated with the incidence of leukemia in 3 treated children.[7,8] Two of the 3 children were cured by standard chemotherapy, 1 died. Insertional oncogenesis by the retroviral vector was identified as the cause of leukemia, highlighting a major drawback of randomly integrating vector systems.

Adenoviral vectors do not integrate into the host genome, therefore gene expression is only short term. Adenoviral vectors can efficiently transfer genes into both dividing and nondividing cells. Both natural immunity against adenovirus (the virus that causes human respiratory diseases) and acute inflammatory and immunological responses limit clinical application to few areas such as localized cancer gene therapy. Systemic administration of a large amount of adenovirus (eg, into the liver) may cause serious side effects and have even caused the death of one patient as described by Marshall[9] and Raper et al.[10] To improve viral vector safety, a strategy was to reduce the number of remaining adenoviral genes—from "first generation" adenoviruses (only one early gene segment was removed) to so-called high-capacity "gutless" adenoviruses, where all viral genes have been replaced.[11] Unfortunately, even high-capacity "gutless" adenoviruses triggered host responses.[12,13] Such host responses are believed to be independent of the type of virus and of viral gene expression, but they are likely related to the viral infection process and to viral proteins of the vector particle. Inflammatory, apoptotic, and immunological host responses to viruses, therefore, in general exclude repeated in vivo administration of viral vectors.

Characteristics of Current Synthetic Vectors

Nonviral vectors[14,15] are more flexible in terms of type and size of the delivered nucleic acid; a broad range from small siRNA, for interfering with gene expression, up to large artificial chromosomes can be used. The great advantage of nonviral vectors (Table 2) is their lower immunogenicity in comparison to viral vectors, since they present no or less immunogenic proteins or peptides. Vectors are usually based on chemically defined lipids or polycations and can be generated protein free or using nonimmunogenic human or humanized proteins and peptides only.

The real weakness of nonviral vectors is their low efficiency in intracellular delivery, which currently is partly compensated by administration of large amounts of vector. Direct injection of milligram-size quantities of plasmid DNA is usually well tolerated but still only yields very low levels of transfection. Nevertheless direct DNA application led to protein expression that was sufficient for triggering immune responses and was used for human DNA vaccination trials.[16] To enhance the delivery of naked DNA, a series of strategies uses physical forces, such as electric pulses, mechanical forces, or hydrodynamic pressure. Electroporation enhanced transgene expression by 2 or 3 orders of magnitude,[17] sufficient for stimulating strong immune responses in DNA vaccine applications. Specificity of gene expression is obtained by the localized action of the physical field applied for electroporation. It is important to note that the efficiency of some physical methods is already very high. Hydrodynamic delivery to rodent livers was indeed so successful[18,19] that a similar pressure-mediated method was recently adapted to larger species.[20]

In complex-based gene transfer vectors, plasmid DNA is condensed with cationic lipids (lipoplexes) or polycations (polyplexes). Although the most potent lipoplexes or polyplexes have reached efficiencies of viral vectors at least in some in vivo applications, far more (100- to 1000-fold) gene copies per cell are required for successful transfection. Therefore, rather high systemically applied doses of plasmid DNA are used in preclinical studies (10-100 μg/mouse, which would equal 100-mg quantities for humans). Since plasmid DNA is not inert and contains immunological active sequences such as nonmethylated CpG sequences, the application of high DNA doses can negatively affect the long-term expression.[21,22] In addition, the cationic reagents used in lipoplexes and polyplexes can exert toxic side effects thereby limiting the doses that can be administered.

Key Steps for Improved Gene Delivery

As described in the previous sections, viral and nonviral vectors have partly complementary characteristics, such as high intracellular delivery of viral vectors but lower immunogenicity and greater flexibility of nonviral vectors. A combination of those complementary positive characteristics into a novel improved vector type would be desirable. Novel vector types should also provide capacities to overcome further limitations common to both viral and nonviral

vectors, such as nonspecific interaction with blood components and nontarget cells,[23] clearance by the reticuloendothelial system, induction of significant toxicity, including apoptotic and inflammatory responses, and low vector specificity for the target tissue.

Key steps where gene transfer vectors require optimization are listed in Table 3. They refer to (1) improved extracellular delivery and targeting, (2) enhanced intracellular delivery and persistence of expression, and (3) reduced toxicity and host cell response. The following sections will review solution strategies that have been established to overcome both general and vector-specific limitations to improve gene delivery.

Extracellular Delivery and Targeting

Because naked DNA is degraded by serum nucleases, condensation or encapsulation of DNA within vectors using viral capsids, cationic polymers, or lipids has greatly improved the stability of the delivered DNA. However both viral and nonviral vectors suffer from weak to nonexisting specificity to the target tissue (see previous section). To increase specificity, the concept of targeted delivery was developed, which is currently the most attractive concept to achieve specificity and, in principle, this strategy is applicable for all current vectors.

To provide a vector with the ability to distinguish between target and nontarget tissue, cell binding ligands have to be incorporated that recognize target-specific cellular receptors. In addition, vector domains with undesired binding potential to blood or nontarget cells (eg, natural receptor binding proteins in viral vectors, positive surface charge in nonviral vectors) have to be shielded or removed.

During the past few years, a series of viral vectors have been engineered to avoid gene transfer through their native receptors and to redirect them to a variety of specific receptors.[24] Retargeting of viral vectors was performed genetically, for example, by sequence integration of retargeting ligands into the genome of the vector.

Retargeting was first tested with retroviral vectors.[25] The challenge was to incorporate ligands without strongly reducing vector production and cell transduction efficiency. This was, for example, achieved using a ligand-modified envelope protein from another virus that was less sensitive to ligand incorporation.[26] Another approach was to genetically insert the ligand-modified proteins, without replacing the natural envelope proteins.[27]

Targeted AAV vectors have been designed by genetic incorporation of an antibody binding protein A domain, which upon attachment of the appropriate targeting antibody can redirect AAV to the desired receptors.[28] Specific transduction of distinct human hematopoietic cell lines was possible

Table 3. Main Barriers for Gene Delivery and How to Overcome Them*

Extracellular Delivery and Targeting	
Degradation of DNA by serum nucleases	DNA condensation or encapsulation with cationic polymers or lipids
Nonspecific interactions with blood components	Surface shielding with hydrophilic polymers
Targeting of DNA to diseased tissue	Specific targeting ligands in combination with surface shielding; exploit vascular abnormalities (eg, passive targeting to tumors)
Intracellular Delivery and Persistence of Gene Expression	
DNA uptake by cells	Specific targeting ligands to enhance receptor-mediated endocytosis
Endosomal escape	Membrane active peptides (eg, melittin analogs) Polycationic "proton sponges" (eg, PEI)
Transport from the cytosol into the nucleus	Small particles to enhance cytosolic migration Nuclear localization signal peptides
Gene silencing	Use mini-circles (devoid of bacterial sequences)
Loss of transgene	Mini-chromosomes with episomal elements for maintenance Expression cassettes for transgene integration
Host Response and Toxicity	
Acute toxicity	Surface shielding to avoid aggregation in blood Removal of contaminating polycations, bacterial LPS
Cytotoxicity and long-term toxicity,	Biodegradable cationic gene carriers
Immune cell responses	Transient immunosuppression

*PEI, indicates polyethylenimine; and LPS, lipopolysaccharide.

applying retargeted AAV vectors with antibodies against CD29, c-kit receptor, and CXCR4.[28]

Adenoviral vectors have been retargeted genetically by incorporation of cell binding peptides into viral coat proteins, as reviewed in Wickham.[24] Alternatively, 2-component systems have been applied, where a bifunctional recombinant bridging molecule binds to the vector and to a target cell receptor. This method confers a new targeting specificity, as successfully demonstrated in vitro for more than 20 receptor-ligand pairs[24] (eg, the epidermal growth factor [EGF] receptor,[29] or the CD105 protein).[30]

However, up to now all approaches to retarget adenoviral vectors with or without replacing cellular entry proteins could not prevent the rapid uptake of viruses by the liver. Also, with retargeted retroviral and AAV vectors no satisfactory in vivo targeting results were obtained for various reasons. These problems highlight the need for further strategies to reduce unspecific interactions that trigger viral clearance.

For the targeting of nonviral lipoplexes and polyplexes, various cell-targeting ligands were covalently attached to a lipid anchor (in lipoplexes) or a DNA-binding cationic polymer (in polyplexes). Targeting ligands that have been evaluated for this purpose include small chemical compounds,[31] vitamins,[32] carbohydrates,[33] peptide ligands,[34] growth factors, and proteins[35-37] or antibodies.[38-40] More details can be found in reviews by Schatzlein[41] and Wagner et al.[42] Successful in vitro targeting has been described by several groups. Transfection of target cells with targeted complexes led to significantly (10- to 1000-fold) enhanced gene expression in comparison to ligand-free complexes. In addition, gene expression was strongly reduced in competition experiments with targeted complexes in the presence of free ligands. This apparent target specificity can be misleading since interaction to other (nontarget) cells was not tested. In fact, the in vivo situation is different. Apart from a few cases where systemic targeting indeed was successful (for example, as described by Wu et al[43]) it was found that irrespective of the presence of a targeting ligand, systemic administration of different lipoplexes and polyplexes via the tail vein of mice resulted in highest gene expression in the lung tissue; for example, see Kircheis et al.[44] In addition, gene transfer at effective DNA doses was associated with acute toxicity. Ex vivo experiments revealed that positively charged polyplexes induced aggregation of erythrocytes. It was proposed that both unspecific lung expression and toxicity are attributed to the aggregation of initially small polyplexes with blood components and their subsequent entrapment in the lung capillary bed.[45]

Obviously the positive surface charge of many nonviral complexes prohibits specific gene transfer in vivo, and shielding agents have to be attached to nonviral vector particles to prevent unspecific interactions. The hydrophilic polymer polyethylene glycol (PEG) has been used to shield lipoplexes[32,46] and polyplexes.[36,44,47] PEG-shielding reduced gene transfer efficiency of complexes, but the efficiency was at least partly restored by incorporation of targeting ligands. Another approach used the serum protein transferrin both for surface shielding and targeting.[37,48] Applying such strategies, systemic targeting of tumors was demonstrated using the folic acid receptor,[32] transferrin receptor,[37,44,49] or epidermal growth factor (EGF) receptor[36] as target, providing first proof-of-concept that systemic targeting is possible, at least with nonviral shielded vectors. Applying such polyplexes systemically, tumor-specific gene expression of tumor necrosis factor (TNF)-α as transgene and therapeutic effects were demonstrated in several mouse models.[50,51] Targeting efficiencies, however, are still not perfect and transfection efficiencies were very poor. Apparently, particle shielding is a double-edged sword: the shielding improves specificity for the target tissue but impairs intracellular delivery and efficiency of vector particles.

Intracellular Delivery and Persistence of Gene Expression

Once the vectors have reached the target cells, vector particles are internalized via cell fusion in the case of some enveloped viruses or via receptor-mediated endocytosis, macropinocytosis, phagocytosis, or related processes[52-55] in the case of lipid-free viruses and most lipoplexes and polyplexes. For successful transgene expression, several intracellular barriers then have to be overcome (Table 3): vector particles need to survive in and escape from the endosome, traffic through the cytoplasm toward the nucleus, enter the nucleus, and expose the DNA to the cell's transcription and translation machinery. Especially for first-generation nonviral vectors, some of these intracellular barriers present formidable hurdles. The first step, intracellular uptake, can be taken rather easily by nonviral vectors, as appropriate receptor binding ligands may enhance binding and intracellular uptake of particles into endosomal vesicles; however, many formulations do not mediate subsequent release of particles to the cytosol. Although the "proton sponge" effect of polyethylenimine (PEI, see section "Optimizing Synthetic Viruses") is believed to contribute to endosomal release,[56] this effect is far from being sufficient. Nuclear entry of nonviral vectors is another big hurdle, which is currently only easily overcome in rapid-dividing cells; for example, transfection of nondividing cells with lipoplexes or PEI polyplexes was several log units less effective compared with transfection of mitotic cells where the nuclear envelope had broken down.[57,58]

In contrast, viral vectors are astonishingly effective in most steps of intracellular delivery. Adenovirus, for example, enters the cell via active endocytosis. The viral endosomal disruption function induces endosomal release. For intracellular trafficking, the virus migrates along the cytosolic cytoskeleton toward the nucleus. Finally the virus uses active nuclear transfer of its viral genome through the nuclear pores. This process requires less than 1 hour, and less than 10 viral particles per cell are sufficient for gene expression in both dividing and nondividing cells. From this perspective, viruses such as adenoviruses present ideal natural templates for the optimization of nonviral vectors.

When long-term maintenance of gene expression is desired, further measures will have to be taken to prevent early loss of the transgene. Transgene integration into the host cell genome allows persistence of gene expression but bears the risk of insertional oncogenesis since current viral vectors are randomly integrating. Efforts to mediate integration of nonviral vectors and to control the integration site include the use of new expression cassettes such as the Sleeping Beauty transposon/transposase system[59] and site-specific bacteriophage recombinases.[60] Transgene maintenance can also be achieved using mini-chromosomes[61,62] or mini-circles.[63] Mini-circles are completely free of bacterial plasmid backbone sequences, which were found to induce immunostimulation and gene silencing.[21,22] In first promising results, these mini-circles have been shown to prolong the in vivo gene expression.[63]

Reduced Toxicity and Reduced Host Cell Response

All efforts that are made to increase the in vivo gene delivery efficiency of vectors will allow substantial reduction of the therapeutic vector dose. This obviously will strongly reduce the acute and also the long-term toxicity of vector application. Shielding the positive surface charge of lipoplexes and polyplexes with PEG greatly reduced acute toxicity of nonviral vectors.[44] To further improve the safety profile of nonviral vectors, excess cationic reagents that are not incorporated in the nonviral vector should be removed (see section "Generate Purified Vector Particles"), since these reagents exert significant cellular toxicity. In addition, preferentially biodegradable cationic DNA carriers[64-69] should be applied to reduce any long-term toxicity hazards.

The infection or transfection process of many viral and nonviral vectors can trigger host cell responses, such as inflammatory and immune responses. Although the immunogenicity of nonviral vectors can be reduced to a minimum, up to now no solution was found to prohibit host cell response after viral vector application. Strategies of immune-suppressive cotreatment, resulting in transient immune suppression during and shortly after the infection process, may help to avoid immune responses and early loss of expressing cells. In addition, chemical modification of the virus such as PEGylation may contribute to reduce immunogenicity of viral vectors.

Chemically Modified Viral Vectors and Synthetic Virus-Like Systems

As outlined in Table 2, the currently used classes of viral and nonviral vectors have complementary strengths, such as high intracellular efficiency of viral vectors, and high systemic potential with low immunogenicity of shielded nonviral vectors. The merge of viral and nonviral vector research efforts could be an encouraging strategy for the future optimization of both vector classes. Viral vectors may greatly benefit from chemical modifications to develop chemoviruses; nonviral vectors may benefit from modifications mimicking viral intracellular delivery functions to generate synthetic viruses. In addition, hybrid strategies (including both nonviral and viral gene vector parts) may also provide interesting solutions (see Figure 1).

The combination of chemical and genetic modification of recombinant viruses may be considered as a top-down approach. The aim is to delete all undesired viral functions from the viral vector (such as illustrated by the development of high-capacity gutless adenoviral vectors), and to mask the original surface of the virus with retargeting ligands and shielding shell/domains using chemical or recombinant procedures (Figure 1, left).

Coating of adenoviral vectors with hydrophilic polymers such as PEG[70-72] or poly-N-2-hydroxypropyl-methacrylamide

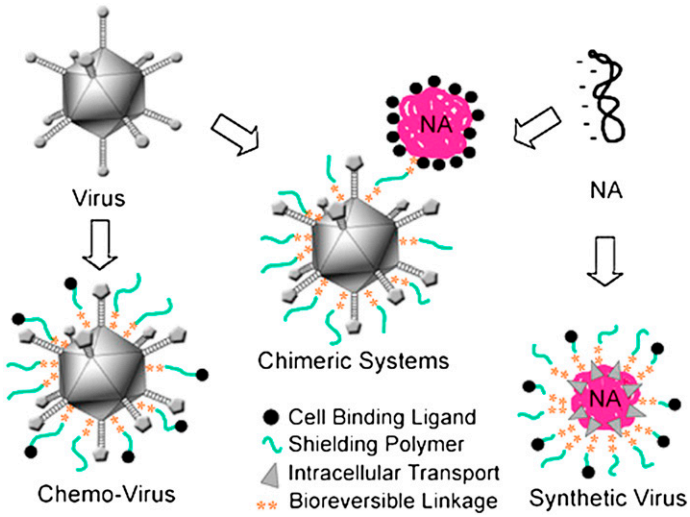

Figure 1. Chemically modified viral vectors (lower left); synthetic virus-like systems (lower right); and hybrid systems containing both viral and nonviral nucleic acid (center). NA, nucleic acid; chemo-virus, chemically-modified virus.

(pHPMA)[73] shielded the virus from interaction with its native receptor or neutralizing antibodies and the incorporated ligands enabled retargeting. A very encouraging example of systemic adenovirus retargeting was recently described.[74] PEGylated adenoviruses were retargeted using anti-E-selectin antibodies. These viruses showed longer persistence in the blood circulation compared with unmodified viruses and selectively targeted inflamed skin in mice resulting in local gene expression.

For combination of genetic and chemical adenoviral vector particle modifications, a novel cystein-based vector platform was developed by Kreppel and colleagues.[75] They genetically introduced cysteines at solvent-exposed positions of the adenovirus fibers. The corresponding thiol groups (36 per adenovirus) were highly reactive in controlled chemical coupling to protein ligands such as transferrin. Such particles were efficiently targeted to the transferrin receptor pathway. This targeting concept via cysteine modifications could also be successfully combined with PEGylation of adenovirus amino groups, providing shielding (detargeting) against nontarget interactions.

Another strategy is to generate hybrid vectors combining viral and nonviral elements. For example, a virus particle (eg, adenovirus) can be covalently linked to a separate nonviral formulation (eg, expression plasmid) (Figure 1, center). The viral vector genome (within the virus) and the separate nonviral gene expression cassette (outside the virus) may synergize in some applications; for example, a helper plasmid attached to the surface of the virus may activate virus replication or regulate the viral gene expression cassette. In addition, the nonviral part may be modified with targeting ligands to allow retargeting of the hybrid vector, whereas the virus part may confer greatly improved intracellular efficiency.[76,77]

Another type of hybrid strategy incorporates a whole viral genome into a nonviral formulation. A plasmid, encoding for the conditionally replicating oncolytic adenovirus dl1520, was integrated into liposomes.[78] Like the parent virus, this plasmid generates infectious particles following transfection of p53-defective tumor cells, but in contrast to the virus, the particles can also transfect coxsackievirus and adenovirus receptor (CAR)-negative tumor cells. The antitumor efficacy of this infectious plasmid was demonstrated with human tumor xenografts in mice after both local and intravenous administration. In contrast to oncolytic viruses, systemic activity was retained, even in the presence of neutralizing antibodies.[78]

A complementary concept (Figure 1, right) to the *top-down approach* is to rebuild a new, synthetic virus based on the *bottom-up approach.*[15] Key questions of this concept are: (1) How many synthetic components are required to generate efficient artificial viruses? (2) How do we achieve coordinate assembly of all components into one defined, supramolecular complex?

One of the first prototypes of synthetic virus-like particles was generated by Plank et al.[33] The particles contained a targeting ligand and a membrane-active peptide to induce endosomal release. More specifically, the system contained a synthetic tetra-antennary carbohydrate ligand for hepatic asialoglycoprotein receptor binding and a synthetic acidic peptide analog derived from the N-terminal HA-2 subunit of influenza virus hemagglutinin. Both components were covalently attached to polylysine, a polycationic carrier for DNA binding. The polyplex components were found to compact DNA to small particles with virus-like dimension of ~100 nm. Application of these particles to hepatocytes resulted in efficient, ligand-specific gene expression, which was highest in the presence of the endosome-destabilizing peptide. This early approach, however, displayed also clear limitations: compared with viral vector particles the system was still very inefficient; the complex formulation was a crude heterogeneous composition that did not contain any measures to shield the particle surface, and therefore particles aggregated with time. For the generation of artificial viruses, the assembly, purification, and stability will be critical and challenging. Further aspects and examples relevant for development toward artificial viruses will be discussed in the next section.

Optimizing Synthetic Viruses

A synthetic virus-like vector, in its ideal form, should be available in defined, purified, and stable form. After the controlled assembly to a defined multiplex system, particles should be purified from all components used for vector preparation. In comparison to a standard nonviral vector, the artificial virus should respond in a more dynamic manner to alterations in their biological micro-environment such as changes in pH or disulfide-reducing conditions, and the vector should undergo programmed structural changes compatible with the different gene delivery steps. This would result in improved shielding and targeting characteristics during systemic circulation and outside the target cell, and in enhanced intracellular trafficking triggered by the intracellular environment. Thus, virus-like entry functions have to be activated and presented in a bioresponsive fashion. The following selected examples illustrate how to tackle upcoming challenges.

Generate Purified Vector Particles

Nonviral vector formulations usually contain an excess of cationic transfection reagent that is not complexed to DNA. In previous studies, not much attention was paid to the presence of these "contaminating" reagents and their effect on the transfection process or on the overall toxicity. Boeckle et al[79]

developed an efficient method for the purification of PEI/DNA complexes using size exclusion chromatography that allowed complete removal of excess PEI (see Figure 2). PEI polyplexes were chosen because of their established high efficiency in vitro and in vivo.[80] Notably, purified PEI polyplexes demonstrated low cellular and systemic toxicity. At lower DNA concentrations, gene transfer with purified particles, however, was less efficient than with polyplexes containing free PEI (Figure 2). Mechanistic studies showed that free PEI was essential for intracellular gene delivery steps. It is therefore likely that free PEI contributes to endosomal release, in agreement with recent data,[56] confirming that both free PEI and PEI polyplexes contribute to the proton sponge mechanism of PEI, in that both lead to buffering of vesicular pH and accumulation of chloride in vesicles, which triggers endosome rupture. Therefore, the desired endosomal release activity currently mediated by free PEI might be replaced by alternative agents such as membrane-destabilizing peptides that are stably integrated into the vector.

Improved Endosomal Release Peptides

Endosomal release was found to be a major bottleneck for the majority of nonviral vectors.[33,77] Amongst various membrane-active peptides tested to overcome this barrier, melittin has displayed a particular strong membrane-destabilizing activity and, when incorporated into PEI polyplexes, successfully enhanced gene transfer.[81] Of interest, the covalent attachment of melittin to PEI strongly influenced the peptide's membrane activity. Melittin-PEI conjugates have been synthesized with PEI covalently attached to the C or the N terminus of melittin (C-Mel-PEI or N-Mel-PEI). Although free melittin is not known to show pH-specific membrane activity, N-Mel-PEI was significantly less lytic than C-Mel-PEI at pH 7. In contrast, at endosomal pH 5, N-Mel-PEI was more lytic than C-Mel-PEI. The differences in lytic activity depending on the microenvironment were found to have a strong effect on the biological activity of the corresponding DNA polyplexes. N-Mel-PEI polyplexes were less toxic in transfection and displayed significantly higher gene transfer efficiency than C-Mel-PEI polyplexes.[82]

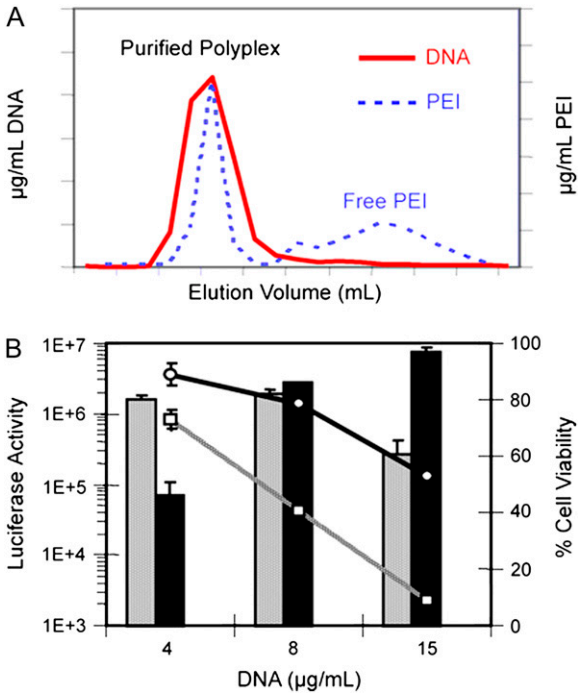

Figure 2. Purification of polyplexes. (A) Size exclusion chromatography (SEC) of PEI polyplexes was performed as described.[79] (B) CT 26 cells were transfected with increasing concentrations of nonpurified and purified PEI22kDa polyplexes. Gene expression activity (luciferase expression) in bars and cellular viability (as indicator for cytotoxicity) is presented as lines: light bars and line, nonpurified polyplexes; dark bars and line, purified polyplexes.

The concept of targeted and endosomolytic polyplexes was extended into therapeutic studies.[83] Synthetic RNA viruses were generated containing synthetic RNA (poly IC), PEI as a condensing agent, EGF linked via a PEG spacer as targeting ligand, and covalently attached melittin (N-mel) as endosomal release agent. These polyplexes induced rapid cell killing in EGF receptor overexpressing glioblastoma cells, whereas tumor cells not overexpressing the EGF receptor were not affected. Nude mice carrying orthotopically implanted human glioblastoma tumors were treated with the synthetic RNA virus by implanting Alzet osmotic micropumps (DURECT Corp, Cupertino, CA) into the tumors. Whereas control animals died within 30 days after tumor implantation, all treated animals survived for >1 year and were completely cured. Similar results were also achieved with other EGF receptor overexpressing tumors.[83]

Obviously for the endosomal release capacity of polyplexes, lytic activity at pH 5 is important, whereas lytic activity at pH 7 mediates cellular toxicity. Consistently, an acidic-modified melittin analog of C-mel (CMA3) was designed to have even higher membrane activity at endosomal pH. This peptide mediated the best gene transfer activity upon incorporation into PEI polyplexes.[84] The next challenging step toward artificial viruses had to prove whether such peptide-conjugates would enhance endosomal release of purified polyplexes. For targeted delivery, PEG-shielded and EGF-receptor-targeted PEI polyplexes with or without CMA3-PEI conjugate were used.[84] After purification, polyplexes with the melittin analog indeed displayed significantly higher gene expression than polyplexes without melittin (Figure 3).

Bioresponsive Elements

As mentioned earlier, the introduction of shielding agents such as PEG is a double-edged sword: particles with a

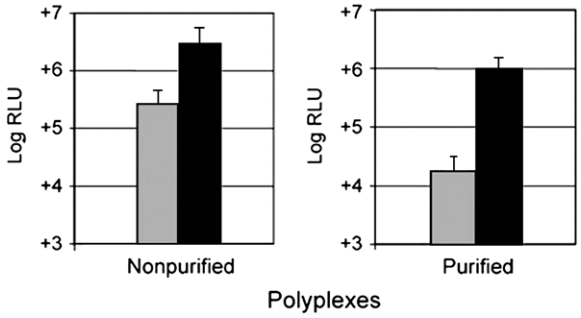

Figure 3. Membrane-active melittin analogs for enhanced endosomal escape. An acidic melittin analog CMA3 covalently attached to PEI25kDa (CMA3-PEI25kDa) enhances luciferase gene transfer of EGF-targeted, PEG-shielded polyplexes to EGFR-Renca cells. Results with nonpurified polyplexes (left graph) and SEC-purified polyplexes (right graph) are shown. The polyplex formulation consists of pCMVL luciferase plasmid complexed with 10% EGF-PEG-PEI25kDa, 10% PEG20kDa-PEI25kDa, and 80% of either PEI25kDa (light bars) or CMA3-PEI25kDa (dark bars). Luciferase expression is presented in relative light units (RLU) on a logarithmic scale. PEI indicates polyethylenimine; EGF, epidermal growth factor; PEG, polyethylene glycol; and EGFR, epidermal growth factor receptor.

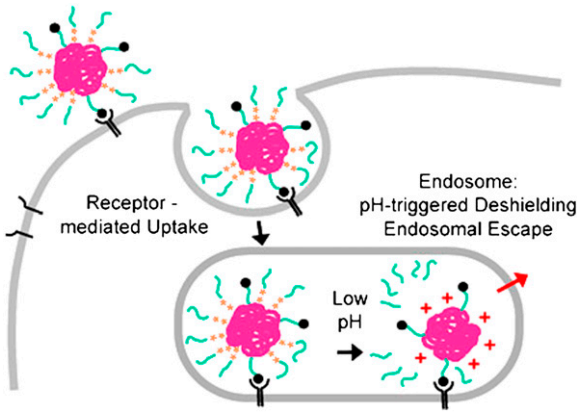

Figure 4. Toward synthetic viruses. Receptor-mediated uptake delivers ligand-targeted polyplexes into endosomal vesicles. Bioresponsive PEG conjugates are applied, which are cleavable within the endosomal pH environment, facilitating subsequent escape of polyplexes from the endosome. In comparison with the use of stable PEG conjugates, this results in strongly enhanced gene transfer in cell culture and in an in vivo tumor model.[85] PEG indicates polyethylene glycol.

higher degree of PEG shield show longer circulation time and better accumulation at the target site, but lower gene expression activity in the target cells. Apparently, the shield hampers intracellular uptake processes. Therefore an optimal nonviral system for systemic application must have a more dynamic character: its surface charge must be neutralized during circulation; but after entering the target cell, the membrane-interacting cationic surface should be re-exposed for efficient gene transfer. For this purpose, Walker et al[85] introduced bioresponsive PEG-polycation conjugates with a pH-labile linkage. Pyridyl hydrazones showed the appropriate acid-dependent hydrolysis; at pH 5 and 37°C, 90% PEG conjugates hydrolyzed within 10 minutes, while at pH 7.4 the half-life was 1.5 hours. DNA particles shielded with these bioreversible PEG conjugates remain stable for at least 4 hours at neutral pH and 37°C but are expected to lose their PEG shield upon reaching endosomal pH (Figure 4). Particles with such labile PEG shield displayed up to 100-fold higher gene transfer activity in cell culture models compared with polyplexes with the analogous stable PEG shield, and 10-fold higher systemic in vivo gene transfer efficiency in a mouse tumor model.[85] Engineering of nanoparticle structures with such dynamic virus-like domains is an encouraging direction in vector design.

Conclusions

Specificity of gene vectors in recognizing the target cells only and exploiting the proper intracellular trafficking routes are the key issues in vector optimization. These dominate many other issues; for example, any improvement in specificity will improve efficiency, enabling the administration of lower vector doses, which will also reduce toxicity and adverse host responses. To reach this goal, viral and nonviral vector technologies will have to be combined. First concepts already prove that this direction can be very encouraging; for example, the in vivo targeting potential of standard viral and nonviral vectors was very limited but has been greatly improved with the design of chemically modified viruses[74,75] and synthetic virus-like systems.[15,85]

Acknowledgments

We are very thankful to Manfred Ogris for inspiring discussions on synthetic viruses. This work was supported by the Deutsche Forschungsgemeinschaft, SFB 486 “Nanoman” and the European Community FP6 network project GIANT. It has been an exceptional pleasure to work together with an excellent research team. The skillful assistance of Olga Brück in preparing the manuscript is greatly appreciated.

References

1. Lipinski KS, Djeha HA, Gawn J, et al. Optimization of a synthetic beta-catenin-dependent promoter for tumor-specific cancer gene therapy. *Mol Ther*. 2004;10:150-161.

2. Brunori M, Malerba M, Kashiwazaki H, Iggo R. Replicating adenoviruses that target tumors with constitutive activation of the wnt signaling pathway. *J Virol*. 2001;75:2857-2865.

3. Wu GY, Wu CH. Receptor-mediated in vitro gene transformation by a soluble DNA carrier system. *J Biol Chem*. 1987;262:4429-4432.

4. McCormick F. Cancer gene therapy: fringe or cutting edge? *Nat Rev Cancer*. 2001;1:130-141.

5. Rainov NG. A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme. *Hum Gene Ther*. 2000;11:2389-2401.

6. Hacein-Bey-Abina S, Le Deist F, Carlier F, et al. Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. *N Engl J Med*. 2002;346:1185-1193.

7. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science*. 2003;302:415-419.

8. Fischer A, Hacein-Bey-Abina S, Lagresle C, Garrigue A, Cavazana-Calvo M. Gene therapy of severe combined immunodeficiency disease: proof of principle of efficiency and safety issues. Gene therapy, primary immunodeficiencies, retrovirus, lentivirus, genome. *Bull Acad Natl Med*. 2005;189:779-785.

9. Marshall E. Gene therapy death prompts review of adenovirus vector. *Science*. 1999;286:2244-2245.

10. Raper SE, Chirmule N, Lee FS, et al. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol Genet Metab*. 2003;80:148-158.

11. Kreppel F, Kochanek S. Long-term transgene expression in proliferating cells mediated by episomally maintained high-capacity adenovirus vectors. *J Virol*. 2004;78:9-22.

12. Schiedner G, Bloch W, Hertel S, et al. A hemodynamic response to intravenous adenovirus vector particles is caused by systemic Kupffer cell-mediated activation of endothelial cells. *Hum Gene Ther*. 2003;14:1631-1641.

13. Schiedner G, Hertel S, Johnston M, Dries V, van Rooijen N, Kochanek S. Selective depletion or blockade of Kupffer cells leads to enhanced and prolonged hepatic transgene expression using high-capacity adenoviral vectors. *Mol Ther*. 2003;7:35-43.

14. Niidome T, Huang L. Gene therapy progress and prospects: nonviral vectors. *Gene Ther*. 2002;9:1647-1652.

15. Wagner E. Strategies to improve DNA polyplexes for in vivo gene transfer: will "artificial viruses" be the answer? *Pharm Res*. 2004;21:8-14.

16. Wang R, Epstein J, Charoenvit Y, et al. Induction in humans of CD8+ and CD4+ T cell and antibody responses by sequential immunization with malaria DNA and recombinant protein. *J Immunol*. 2004;172:5561-5569.

17. Mathiesen I. Electropermeabilization of skeletal muscle enhances gene transfer in vivo. *Gene Ther*. 1999;6:508-514.

18. Liu F, Song Y, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther*. 1999;6:1258-1266.

19. Zhang G, Budker V, Wolff JA. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum Gene Ther*. 1999;10:1735-1737.

20. Hagstrom JE, Hegge J, Zhang G, et al. A facile nonviral method for delivering genes and siRNAs to skeletal muscle of mammalian limbs. *Mol Ther*. 2004;10:386-398.

21. Chen ZY, He CY, Meuse L, Kay MA. Silencing of episomal transgene expression by plasmid bacterial DNA elements in vivo. *Gene Ther*. 2004;11:856-864.

22. Hodges BL, Taylor KM, Joseph MF, Bourgeois SA, Scheule RK. Long-term transgene expression from plasmid DNA gene therapy vectors is negatively affected by CpG dinucleotides. *Mol Ther*. 2004;10:269-278.

23. Plank C, Jr, Mechtler K, Jr, Szoka FC, Jr, Wagner E. Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. *Hum Gene Ther*. 1996;7:1437-1446.

24. Wickham TJ. Ligand-directed targeting of genes to the site of disease. *Nat Med*. 2003;9:135-139.

25. Russell SJ, Hawkins RE, Winter G. Retroviral vectors displaying functional antibody fragments. *Nucleic Acids Res*. 1993;21:1081-1085.

26. Hatziioannou T, Delahaye E, Martin F, Russell SJ, Cosset FL. Retroviral display of functional binding domains fused to the amino terminus of influenza hemagglutinin. *Hum Gene Ther*. 1999;10:1533-1544.

27. Gordon EM, Chen ZH, Liu L, et al. Systemic administration of a matrix-targeted retroviral vector is efficacious for cancer gene therapy in mice. *Hum Gene Ther*. 2001;12:193-204.

28. Ried MU, Girod A, Leike K, Buning H, Hallek M. Adeno-associated virus capsids displaying immunoglobulin-binding domains permit antibody-mediated vector retargeting to specific cell surface receptors. *J Virol*. 2002;76:4559-4566.

29. Dmitriev I, Kashentseva E, Rogers BE, Krasnykh V, Curiel DT. Ectodomain of coxsackievirus and adenovirus receptor genetically fused to epidermal growth factor mediates adenovirus targeting to epidermal growth factor receptor-positive cells. *J Virol*. 2000;74:6875-6884.

30. Nettelbeck DM, Miller DW, Jerome V, et al. Targeting of adenovirus to endothelial cells by a bispecific single-chain diabody directed against the adenovirus fiber knob domain and human endoglin (CD105). *Mol Ther*. 2001;3:882-891.

31. Hood JD, Bednarski M, Frausto R, et al. Tumor regression by targeted gene delivery to the neovasculature. *Science*. 2002;296:2404-2407.

32. Hofland HE, Masson C, Iginla S, et al. Folate-targeted gene transfer in vivo. *Mol Ther*. 2002;5:739-744.

33. Plank C, Zatloukal K, Cotten M, Mechtler K, Wagner E. Gene transfer into hepatocytes using asialoglycoprotein receptor-mediated endocytosis of DNA complexed with an artificial tetra-antennary galactose ligand. *Bioconjug Chem*. 1992;3:533-539.

34. Ziady AG, Ferkol T, Dawson DV, Perlmutter DH, Davis PB. Chain length of the polylysine in receptor-targeted gene transfer complexes affects duration of reporter gene expression both in vitro and in vivo. *J Biol Chem*. 1999;274:4908-4916.

35. Kim TG, Kang SY, Kang JH, et al. Gene transfer into human hepatoma cells by receptor-associated protein/polylysine conjugates. *Bioconjug Chem*. 2004;15:326-332.

36. Wolschek MF, Thallinger C, Kursa M, et al. Specific systemic nonviral gene delivery to human hepatocellular carcinoma xenografts in SCID mice. *Hepatology*. 2002;36:1106-1114.

37. Xu L, Pirollo KF, Tang WH, Rait A, Chang EH. Transferrin-liposome-mediated systemic p53 gene therapy in combination with radiation results in regression of human head and neck cancer xenografts. *Hum Gene Ther*. 1999;10:2941-2952.

38. Buschle M, Cotten M, Kirlappos H, et al. Receptor-mediated gene transfer into human T lymphocytes via binding of DNA/CD3 antibody particles to the CD3 T cell receptor complex. *Hum Gene Ther*. 1995;6:753-761.

39. Chiu SJ, Ueno NT, Lee RJ. Tumor-targeted gene delivery via anti-HER2 antibody (trastuzumab, Herceptin) conjugated polyethylenimine. *J Control Release*. 2004;97:357-369.

40. Zhang Y, Zhang YF, Bryant J, Charles A, Boado RJ, Pardridge WM. Intravenous RNA interference gene therapy targeting the human

epidermal growth factor receptor prolongs survival in intracranial brain cancer. *Clin Cancer Res*. 2004;10:3667-3677.

41. Schatzlein AG. Targeting of synthetic gene delivery systems. *J Biomed Biotechnol.* 2003;2003:149-158.

42. Wagner E, Culmsee C, Boeckle S. Targeting of polyplexes: toward synthetic virus vector systems. *Adv Genet.* 2005;53: 333-354.

43. Wu GY, Wilson JM, Shalaby F, Grossman M, Shafritz DA, Wu CH. Receptor-mediated gene delivery in vivo: partial correction of genetic analbuminemia in Nagase rats. *J Biol Chem*. 1991;266:14338-14342.

44. Kircheis R, Schuller S, Brunner S, et al. Polycation-based DNA complexes for tumor-targeted gene delivery in vivo. *J Gene Med*. 1999;1:111-120.

45. Chollet P, Favrot MC, Hurbin A, Coll JL. Side-effects of a systemic injection of linear polyethylenimine-DNA complexes. *J Gene Med*. 2002;4:84-91.

46. Monck MA, Mori A, Lee D, et al. Stabilized plasmid-lipid particles: pharmacokinetics and plasmid delivery to distal tumors following intravenous injection. *J Drug Target*. 2000;7:439-452.

47. Ogris M, Brunner S, Schuller S, Kircheis R, Wagner E. PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther*. 1999;6:595-605.

48. Kircheis R, Wightman L, Schreiber A, et al. Polyethylenimine/DNA complexes shielded by transferrin target gene expression to tumors after systemic application. *Gene Ther*. 2001;8:28-40.

49. Hildebrandt IJ, Iyer M, Wagner E, Gambhir SS. Optical imaging of transferrin-targeted PEI/DNA complexes in living subjects. *Gene Ther*. 2003;10:758-764.

50. Kircheis R, Ostermann E, Wolschek MF, et al. Tumor-targeted gene delivery of tumor necrosis factor-alpha induces tumor necrosis and tumor regression without systemic toxicity. *Cancer Gene Ther*. 2002;9:673-680.

51. Ogris M, Walker G, Blessing T, Kircheis R, Wolschek M, Wagner E. Tumor-targeted gene therapy: strategies for the preparation of ligand-polyethylene glycol-polyethylenimine/DNA complexes. *J Control Release*. 2003;91:173-181.

52. Goncalves C, Mennesson E, Fuchs R, Gorvel JP, Midoux P, Pichon C. Macropinocytosis of polyplexes and recycling of plasmid via the clathrin-dependent pathway impair the transfection efficiency of human hepatocarcinoma cells. *Mol Ther*. 2004;10:373-385.

53. Rejman J, Bragonzi A, Conese M. Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. *Mol Ther*. 2005;12:468-474.

54. Kopatz I, Remy JS, Behr JP. A model for non-viral gene delivery: through syndecan adhesion molecules and powered by actin. *J Gene Med*. 2004;6:769-776.

55. Bausinger R, von Gersdorff K, Ogris M, et al. The transport of nanosized gene carriers unraveled by live-cell imaging. *Angew Chem Weinheim Bergstr Ger*. 2006;118:1598-1602.

56. Sonawane ND, Jr, Szoka FC, Jr, Verkman AS. Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. *J Biol Chem*. 2003;278:44826-44831.

57. Brunner S, Sauer T, Carotta S, Cotten M, Saltik M, Wagner E. Cell cycle dependence of gene transfer by lipoplex, polyplex and recombinant adenovirus. *Gene Ther*. 2000;7:401-407.

58. Zabner J, Fasbender AJ, Moninger T, Poellinger KA, Welsh MJ. Cellular and molecular barriers to gene transfer by a cationic lipid. *J Biol Chem*. 1995;270:18997-19007.

59. Liu G, Aronovich EL, Cui Z, Whitley CB, Hackett PB. Excision of Sleeping Beauty transposons: parameters and applications to gene therapy. *J Gene Med*. 2004;6:574-583.

60. Portlock JL, Calos MP. Site-specific genomic strategies for gene therapy. *Curr Opin Mol Ther*. 2003;5:376-382.

61. Lipps HJ, Jenke AC, Nehlsen K, Scinteie MF, Stehle IM, Bode J. Chromosome-based vectors for gene therapy. *Gene*. 2003;304:23-33.

62. Magin-Lachmann C, Kotzamanis G, D'Aiuto L, Wagner E, Huxley C. Retrofitting BACs with G418 resistance, luciferase, and oriP and EBNA-1: new vectors for in vitro and in vivo delivery. *BMC Biotechnol*. 2003;3:2.

63. Chen ZY, He CY, Ehrhardt A, Kay MA. Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression in vivo. *Mol Ther*. 2003;8:495-500.

64. Lim YB, Kim SM, Suh H, Park JS. Biodegradable, endosome disruptive, and cationic network-type polymer as a highly efficient and nontoxic gene delivery carrier. *Bioconjug Chem*. 2002;13:952-957.

65. Kim YH, Park JH, Lee M, Kim YH, Park TG, Kim SW. Polyethylenimine with acid-labile linkages as a biodegradable gene carrier. *J Control Release*. 2005;103:209-219.

66. Wang J, Gao SJ, Zhang PC, Wang S, Mao HQ, Leong KW. Polyphosphoramidate gene carriers: effect of charge group on gene transfer efficiency. *Gene Ther*. 2004;11:1001-1010.

67. Anderson DG, Akinc A, Hossain N, Langer R. Structure/property studies of polymeric gene delivery using a library of poly(beta-amino esters). *Mol Ther*. 2005;11:426-434.

68. Zhong Z, Song Y, Engbersen JF, Lok MC, Hennink WE, Feijen J. A versatile family of degradable non-viral gene carriers based on hyperbranched poly(ester amine)s. *J Control Release*. 2005;109:317-329.

69. Kloeckner J, Boeckle S, Persson D, et al. DNA polyplexes based on degradable oligoethylenimine-derivatives: combination with EGF receptor targeting and endosomal release functions. *J Control Release*. 2006;[Epub ahead of print].

70. Croyle MA, Chirmule N, Zhang Y, Wilson JM. "Stealth" adenoviruses blunt cell-mediated and humoral immune responses against the virus and allow for significant gene expression upon readministration in the lung. *J Virol*. 2001;75:4792-4801.

71. Lanciotti J, Song A, Doukas J, et al. Targeting adenoviral vectors using heterofunctional polyethylene glycol FGF2 conjugates. *Mol Ther*. 2003;8:99-107.

72. O'Riordan CR, Lachapelle A, Delgado C, et al. PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody in vitro and in vivo. *Hum Gene Ther*. 1999;10:1349-1358.

73. Fisher KD, Stallwood Y, Green NK, Ulbrich K, Mautner V, Seymour LW. Polymer-coated adenovirus permits efficient retargeting and evades neutralizing antibodies. *Gene Ther*. 2001;8:341-348.

74. Ogawara K, Rots MG, Kok RJ, et al. A novel strategy to modify adenovirus tropism and enhance transgene delivery to activated vascular endothelial cells in vitro and in vivo. *Hum Gene Ther*. 2004;15:433-443.

75. Kreppel F, Gackowski J, Schmidt E, Kochanek S. Combined genetic and chemical capsid modifications enable flexible and efficient de- and retargeting of adenovirus vectors. *Mol Ther*. 2005;12:107-117.

76. Curiel DT, Wagner E, Cotten M, et al. High-efficiency gene transfer mediated by adenovirus coupled to DNA-polylysine complexes. *Hum Gene Ther*. 1992;3:147-154.

77. Wagner E, Zatloukal K, Cotten M, et al. Coupling of adenovirus to transferrin-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes. *Proc Natl Acad Sci USA*. 1992;89:6099-6103.

78. Yotnda P, Davis AR, Hicks MJ, Templeton NS, Brenner MK. Liposomal enhancement of the antitumor activity of conditionally replication-competent adenoviral plasmids. *Mol Ther*. 2004;9:489-495.

79. Boeckle S, von Gersdorff K, van der Piepen S, Culmsee C, Wagner E, Ogris M. Purification of polyethylenimine polyplexes highlights the role of free polycations in gene transfer. *J Gene Med*. 2004;6:1102-1111.

80. Zou SM, Erbacher P, Remy JS, Behr JP. Systemic linear polyethylenimine (L-PEI)-mediated gene delivery in the mouse. *J Gene Med*. 2000;2:128-134.

81. Ogris M, Carlisle RC, Bettinger T, Seymour LW. Melittin enables efficient vesicular escape and enhanced nuclear access of nonviral gene delivery vectors. *J Biol Chem*. 2001;276:47550-47555.

82. Boeckle S, Wagner E, Ogris M. C- versus N-terminally linked melittin-polyethylenimine conjugates: the site of linkage strongly influences activity of DNA polyplexes. *J Gene Med*. 2005;7:1335-1347.

83. Shir A, Ogris M, Wagner E, Levitzki A. EGF receptor-targeted synthetic double-stranded RNA eliminates glioblastoma, breast cancer, and adenocarcinoma tumors in mice. *PLoS Med*. 2006;3:e6.

84. Boeckle S, Fahrmeir J, Roedl W, Ogris M, Wagner E. Melittin analogs with high lytic activity at endosomal pH enhance transfection with purified targeted PEI polyplexes. *J Control Release*. 2006;112:240-248.

85. Walker GF, Fella C, Pelisek J, et al. Toward synthetic viruses: endosomal pH-triggered deshielding of targeted polyplexes greatly enhances gene transfer in vitro and in vivo. *Mol Ther*. 2005;11:418-425.