Anti-Cancer Drugs 2001, **12,** pp. 275–304

Review paper

Non-viral vectors in cancer gene therapy: principles and progress

AG Schatzlein[1]
[1]CRC Department of Medical Oncology, Beatson Laboratories, University of Glasgow, Switchback Road, Glasgow G61 1BD, UK.

This review focuses on the use of synthetic (non-viral) delivery systems for cancer gene therapy. Therapeutic strategies such as gene replacement/mutation correction, immune modulation and molecular therapy/'suicide' gene therapy type approaches potentially offer unique and novel ways of fighting cancer, some of which have already shown promise in early clinical trials. However, the specific and efficient delivery of the genetic material to remote tumors/metastases remains a challenge, which is being addressed using a variety of viral and non-viral systems. Each of these disparate systems has distinct advantages and disadvantages, which need to be taken into account when a specific therapeutic gene is being used. The review concentrates on particulate gene delivery systems, which are formed through non-covalent complexation of cationic carrier molecules (e.g. lipids or polymers) and the negatively charged plasmid DNA. Such systems tend to be comparatively less efficient than viral systems, but have the inherent advantage of flexibility and safety. The DNA–carrier complex acts as a protective package, and needs to be inert and stable while in circulation. Once the remote site has been reached the complex needs to efficiently transfect the targeted (tumor) cells. In order to improve overall transfection specificity and efficiency it is necessary to optimize intracellular trafficking of the DNA complex as well as the performance after systemic administration. Common principles and specific advantages or disadvantages of the individual synthetic gene delivery systems are discussed, and their interaction with tumor-specific and generic biological barriers are examined in order to identify potential strategies to overcome them. [© 2001 Lippincott Williams & Wilkins.]

Key words: Cancer, gene therapy, non-viral vector.

Introduction

Although genomic research has opened up many new avenues for therapeutic interventions based on genetic therapy, it will not be possible to realize the full potential of these new therapies until the issue of gene delivery has been resolved.[1] Cancer gene therapy promises efficacy and specificity superior to traditional approaches, and, furthermore, facilitates novel therapeutic strategies. The choice of vector and delivery strategy is, however, inseparable from the therapeutic approach, as the shortcomings of the former will inevitably affect the success of the latter. In this review recent developments are summarized, specifically with respect to the use of non-viral, DNA complexing gene delivery systems for cancer therapy, but also with a more general look at the biological barriers which such systems need to overcome.

Non-viral and viral vectors have both been used with some success in early clinical trials, demonstrating the principle feasibility of various cancer gene therapy approaches.[2] Improved genetic constructs have helped to redefine the minimum levels of transfection efficiency required for any given genetic strategy. Furthermore, the exploitation of bystander effects, 'multimode' strategies (e.g. combining 'suicide' with immune-mediated effects) and the combination of genetic therapy with traditional pharmacological intervention has helped to alleviate the gene delivery bottleneck to some degree.

Nevertheless, the high initial expectations in cancer gene therapy have been tempered—not least of all by the realization that the issue of suitable vector/delivery systems may ultimately jeopardize their clinical success. There is currently no practical method available that would allow safe *and* efficient gene delivery in most clinical situations. While viral vectors offer superior transfection efficiency they have been plagued by safety concerns,[3] highlighted recently by the death of a patient in clinical trials.[4,5] This review is concerned with 'non-viral' strategies as an important alternative. The term 'non-viral' describes, rather loosely, transfection strategies that range from the

Correspondence to AG Schatzlein, CRC Department of Medical Oncology, Beatson Laboratories, University of Glasgow, Switchback Road, Glasgow G61 1BD, UK.
Tel: (+44) 141 330 4354; Fax: (+44) 141 330 4127;
E-mail: A.Schatzlein@beatson.gla.ac.uk

injection of naked DNA to the use of sophisticated biolistic gene guns. (Alternative non-viral gene delivery systems have been devised using a wide array of techniques, many of which are based on physical devices such as gene guns,[6] jet injection,[7] electrical pulses[8] or even ultrasound.[9] In general their use is, however, limited to easily accessible or superficial sites. Bearing this in mind such systems can be quite efficient and could prove useful for the treatment of suitable tumors.[6] A more general overview including such systems can be found in a recent review.[10]

The focus of this review, however, will be on particulate gene delivery systems based on non-covalent complexes of the carrier molecules and the plasmid DNA. Such gene delivery systems are potentially useful in particular for the delivery of therapeutic genes to a solid tumor or metastasis, i.e. after systemic administration, but are currently fraught with a number of serious problems. In order to successfully transfect tumor cells at a remote site the system would need to be inert and stable while in circulation, yet, once the remote site has been reached, it should release its cargo and effect an efficient and specific transfection of all target cells. The development of a carrier system which could fulfil this task represents a formidable challenge, as a number of complex biological barriers need to be overcome. While some of these barriers are specific to gene therapy there are also other, tumor-specific challenges, such as the issue of genetic instability and heterogeneity of tumors, their tendency to acquire resistance, the formation of metastases or the induction of immune tolerance, which also may need to be addressed.

In the last decade the focus of development has been on non-viral gene delivery systems, which form complexes with DNA, such as cationic lipids (lipoplex) or polymers (polyplex).[11] These systems have since become invaluable research tools *in vitro* but their *in vivo* use has been dogged by a number of problems which hamper their broader therapeutic use.

Therapeutic strategies and clinical trials

Over the last decade a number of novel therapeutic strategies have been developed that are based on the therapeutic use of genes. Most therapeutic strategies for cancer gene therapy can be loosely grouped into three categories: molecular chemotherapy, mutation compensation/gene replacement and immune modulation approaches. The great initial expectation of potential clinical benefits from any of these approaches has been toned down somewhat because of the realization that the issue of delivery is currently holding back a wider application of these genetic strategies. In any case, in order to bring these novel approaches into the clinic it will be necessary to design strategies in which therapeutic gene and the capabilities of the chosen delivery system are matched closely, as both form part of an integrated therapeutic package.

For the synthetic gene delivery systems one of the challenges lies in their relatively low transfection efficiency, which makes it likely that their application will require a significant 'bystander effect'. This effect is based on the ability of certain genetic therapies to effect changes to, or destruction of, not only the relatively low proportion of transgene-expressing cells, but also in neighboring, untransfected cells. The bystander effects, which form an integral part of any therapeutic strategy and are potentially very important for the clinical outcome can be induced on three main levels:

- Local and direct, e.g. through toxin transfer to adjacent cells.[12,13]
- Regional, through the induction of downstream events, e.g. by destruction or growth inhibition of tumor vasculature.[14,15]
- Systemically, e.g. through the induction of an immune response.[16]

Different gene therapy strategies exploit such effects to a varying extent and sometimes the observed therapeutic effect may actually be the result of a combination of events on different levels.

While the possibility of harnessing a significant therapeutic effect is important in bypassing the shortcomings of current delivery systems we also need to consider other elements, e.g. specificity and safety, when selecting a therapeutic strategy because the genetic therapy and the delivery system need to be well matched.

Gene replacement/mutation compensation

The rationale behind this approach is the compensation of a genetic malfunction either by augmentation of a deficient tumor suppressor gene or by inhibition of a dominant oncogene.

In the case of overexpressed *oncogenes* the compensation can occur on the transcriptional, the translational or the protein level. Antisense strategies have commonly been used for the inhibition of oncogenes and some candidate drugs are currently being tested in clinical trials (e.g. transforming growth factor-β, erbB-2, k-*ras*, c-*myc*).[2,17] In addition, ribo-

zymes[18–20] and single-chain antibodies[21,22] have been developed to modulate oncogene products on the protein level. This strategy can also be used to modulate the expression of proteins that are involved in drug resistance.[23]

The reactivation of tumor *suppressor* genes or the replacement of mutated genes with their wild-type counterpart (e.g. *p53*,[24–26] retinoblastoma,[27] BRCA1[28]) aims to re-establish the balance between growth and apoptosis, activate cell cycle controls and DNA repair mechanisms so that the tumorigenic phenotype is reverted. Apart from p53 there are some other inducers of apoptosis such as Bax,[29] Bcl-X_s or the caspases, which have recently received more attention (for a recent review, see Favrot *et al.*[30]).

For this therapeutic strategy the combination with chemo- or radiotherapy may show synergistic or at least additive effects. The combination of radiotherapy and p53 delivered i.v. led to complete regression of established xenografts in nude mice.[26] Issues such as the involvement of multiple genes, tumor heterogeneity and the appearance of 'tumor suppressor resistance', i.e. the continued cancer growth despite successful re-expression of the suppressor genes, represent additional challenges for this approach.

To achieve complete ablation of the malignant phenotype a very high rate of transfection, probably close to 100%, would theoretically be required, as even a minute fraction of unmodified cells would suffice for re-growth of the tumor. Currently transfection rates of synthetic delivery systems are significantly lower than required and only a fraction of the target cells would express the transgene. On the other hand, there is also evidence for a tumor suppressor-based bystander effect, as the rate of tumor cell kill in some experiments exceeds the number of transfected cells significantly.[30] Thus transfection efficiencies significantly below 100% may be sufficient to be clinically beneficial.

Immune-modulation strategies

This approach is based on the harnessing of a tumor-specific host immune response to eliminate the tumor, thus employing the host's physiological immune response cascade to amplify therapeutic effects.[31–34] This amplification could prove critical in overcoming the current lack of efficient vectors. Modulation of the immune response can be achieved via stimulation and modification of immune effector cells, in order to enable them to recognize and reject cells that carry a tumor antigen. Alternatively, tumor cells themselves can be genetically modified to increase immunogenicity and trigger an immune response.

Harnessing the individual's immune response against the tumor is a very attractive therapeutic strategy as therapy of a local tumor could potentially also reach remote sites and (micro-)metastasis, thus alleviating the problems encountered on systemic administration of current synthetic vectors.

In the majority of cases viral vectors have been used to administer the transgene. Recent reports suggest that efficiency is probably less crucial for this approach: in immunocompetent mice the immune response seen after i.p. administration of a liposomal complex was similar to that resulting from the use of a retrovirus/liposome combination which transfected 100 times more efficiently.[35]

A potential advantage of immune-based approaches is a greater freedom of choice with regard to the administration modalities. Local administration can be a clinically important option, e.g. the intratumoral injection of allogeneic and xenogeneic MHC in cationic vesicles in a phase I/II trial resulted in some complete and partial local responses in the treatment of cutaneous metastasis.[36] Often a primary tumor, in contrast to the metastases, may be accessible to a locoregional administration. This could potentially also be useful for the treatment of remote sites because of the 'systemic bystander effect' afforded through the host's immune response. Consequently this approach would be interesting in avoiding the potential problems that exist for the systemic administration of synthetic gene delivery systems. In the future an improved understanding of tumors' escape of the immune system and novel insights into modulation of the tumor-directed immune response might help to create superior combinations of delivery systems and vector.

It should also be mentioned here that immune modulation effects can be induced through DNA alone, independent of any specific coding sequence. In particular, plasmids from microbial sources can be immunogenic due to differences in their methylation pattern, i.e. their lack of methylation of so-called CpG islands.[37] These bacterial sequences can induce high levels of cytokines, such as interferon-γ and tumor necrosis factor-α,[38] in particular in the lung.[39,40] The methylation of plasmid DNA and co-administration of dexamethasone significantly decreased the cytokine levels, and led to significantly higher levels of transgene expression on both first and second injection.[41] The immunogenicity of the plasmid alone has been shown to be of potential clinical importance as a mock plasmid inhibited tumor take in mice by 50%.[42] It is important to bear this in mind when designing experiments, as an immune response may contribute to the effects observed *in vivo*. Likewise

this should be considered for certain reporter genes and suicide vectors where immune-mediated effects may influence and extend the duration of gene expression.

Molecular chemotherapy

The two main approaches in this category are the modulation of drug resistance, i.e. chemo-/radio-sensitization or protection, and gene-directed enzyme prodrug therapies (GDEPT). Modulation of drug resistance can be used either to make cancer cells less resistant (chemo/radio-sensitization) or to make sensitive healthy cells more resistant (chemo/radio-protection).

Chemo/radio-*sensitization* can be achieved using similar strategies as with oncogenes to increase the sensitivity of drug-resistant cancer cells through down-regulation/inhibition of drug resistance proteins. Antisense oligonucleotides, ribozymes and single-chain antibodies have been shown to modulate the resistance phenotype of tumor cells.[23] Sensitization can also be achieved by modulation of the cellular response to cytotoxic drugs, e.g. downstream events in the apoptosis pathway—as has been shown for radio-sensitization through induction of expression of wild-type p53[26]—or by combination of an E1B-attenuated adenovirus with a double-suicide gene therapy approach.[43] Another approach to sensitization is based on concentrating radiation by expression of the sodium/iodide symporter gene (rNIS), which mimics the iodide transport of follicular cells of the thyroid thus increasing the uptake of radionucleotide,[44] or by using the noradrenaline transporter gene to induce uptake and accumulation of [^{131}I]meta-iodobenzylguanidine.[45]

Chemo/radio-*protection* gives protective resistance to sensitive cells through up-regulation/introduction of drug-resistance proteins. Enabling sensitive cells to tolerate higher doses of a cytotoxic drug would potentially make a higher proportion of cancer patients curable through reduction of dose-limiting toxicities. Among the potentially vulnerable organs, the bone marrow is frequently a site of manifestation of severe, dose-limiting side effects and strategies have been developed that would allow the incorporation of drug-resistance genes into marrow progenitor cells *ex vivo*.[46,47] Obviously one proviso for this type of approach is that a potent and efficient chemo- or radio-therapeutic strategy needs to be in place that will allow killing of the tumor cells.

In some cases these approaches will probably require a more permanent modification of the phenotype, i.e. for the duration of a course of radio- or chemotherapy. Consequently integrating vectors, i.e. retroviruses, have been used in most of these experiments. As the extent of potential bystander effects of, for example, using radionucleotides is unclear, it is currently difficult to define the boundary conditions for a suitable synthetic delivery system for this type of approach.

GDEPT/suicide gene therapy. The concept is based on the transfection of tumor cells with an enzyme, which can convert a relatively non-toxic prodrug into an active anti-cancer agent (*cf.* Figure 1). With this approach selectivity can be achieved on several levels in order to maximize the drug exposure of the tumor while minimizing side effects in healthy tissues: targeted delivery of the gene, tumor-specific activation of the transgene (e.g. tissue or disease specific promoter)[48,49] or the selection of an optimized enzyme–prodrug combination.[50,51]

One frequently used GDEPT combination is based on herpes simplex virus thymidine kinase (HSV*tk*) and gangciclovir (GCV). HSV*tk* phosphorylates the non-toxic nucleoside analog, which becomes a potent inhibitor of DNA polymerase and induces cell death. The system has been used in various tumor models but success was often limited to microscopic tumors. It has been suggested that the involvement of immune response may be part of the observed effects. There also have been reports of TK/GCV resistance developing.[52]

An important issue for this type of therapy is the extent and nature of the *bystander effect* (for a recent review, see Pope *et al.*[12]). There is clear evidence that gap junctional communication plays an important role in the bystander effect[12,13,53,54] and it has been suggested that this could be exploited by pharmacological manipulation.[55] When selecting enzyme–prodrug combinations the question of how permissive the tumor is likely to be to the spreading of a particular drug needs to be taken into consideration.[56] Bystander effects allow the successful therapy with only 10% of the cells expressing the transgene in mouse models.[55] The bystander effect can be improved through co-application of drugs such as lovastatin, apigenin and retinoic acid, which up-regulate gap junctional communication.[55,57] However, a significant proportion of human malignancies express only low levels of gap junctional proteins (i.e. connexin) and, consequently, are not amenable to this 'amplification'.[58]

An alternative, non-gap junction-based bystander effect was recently reported for the HSV transport protein VP22.[59] VP22 mediated transport of the prodrug-cleaving enzyme itself, which was sufficient

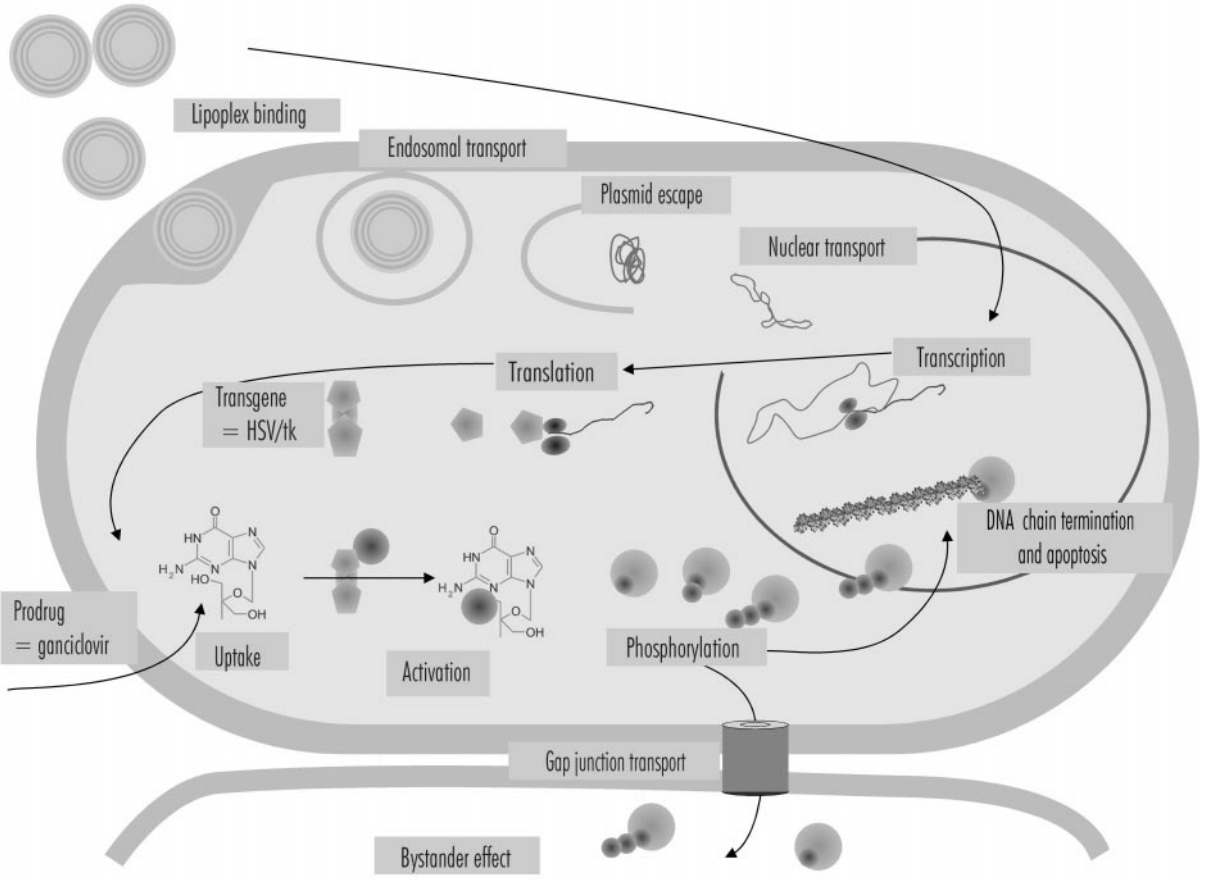

Figure 1. Suicide gene therapy (GDEPT) is one of the therapeutic strategies for cancer gene therapy. It is based on a two-step protocol requiring an initial transfection of the tumor cells and a subsequent treatment with a suitable prodrug. The plasmid codes for an enzyme, which is not normally expressed/active in the target cells and which specifically converts a non-toxic prodrug into a highly active anti-cancer agent. In this example HSV/*tk* activates the nucleotide analog GCV. The active analog interferes with DNA synthesis, which the leads to DNA chain termination and subsequent apoptosis. The activated drug can redistribute between adjacent cells connected by gap junctions and thus also act on neighboring cells that do not express the enzyme themselves, i.e. bystander effect.

to induce regression of tumors at a ratio or 50% wild-type cells in the absence of gap junctions.

Bystander effects are not necessarily limited to neighboring cells but can also exploit downstream effects: targeting of tumor angiogenesis can potentiate the bystander effect as cessation of blood supply induces death of multiple dependent tumor cells.[15,60] If the anti-angiogenic factors are excreted efficiently the transfected producer site can be remote from the tumor.[14] Thus it is possible to circumvent the problems of systemic administration in favor of a local application.

Clinical trials

Current clinical trial protocols give a good indication of the status quo of genetic therapy in general and for cancer in particular. More than 3278 patients have so far participated in clinical trials in gene therapy.[2] Cancer gene therapy accounts for the majority of these studies with 252 out of 396 reported trials and 69.2% of all patients enrolled.[2] The predominance of cancer-related gene therapy also becomes obvious from the type of therapeutic gene used (*cf*. Figure 2).

Immune modulation approaches account for more than half of the patients, with cytokines and antigen-based approaches being of similar importance (24.1 and 27.2%, respectively). Suicide gene/GDEPT approaches account for another major proportion of the patients treated so far (16.6%). The fact that more than 80% of the agents were delivered in locally or directly into the tumor is not necessarily by choice, but rather highlights the challenges/barriers that the targeted *systemic* delivery of genes represents.[2] The vectors used in these trials have been predominantly of viral origin. Non-viral vectors, e.g. lipofection, are only

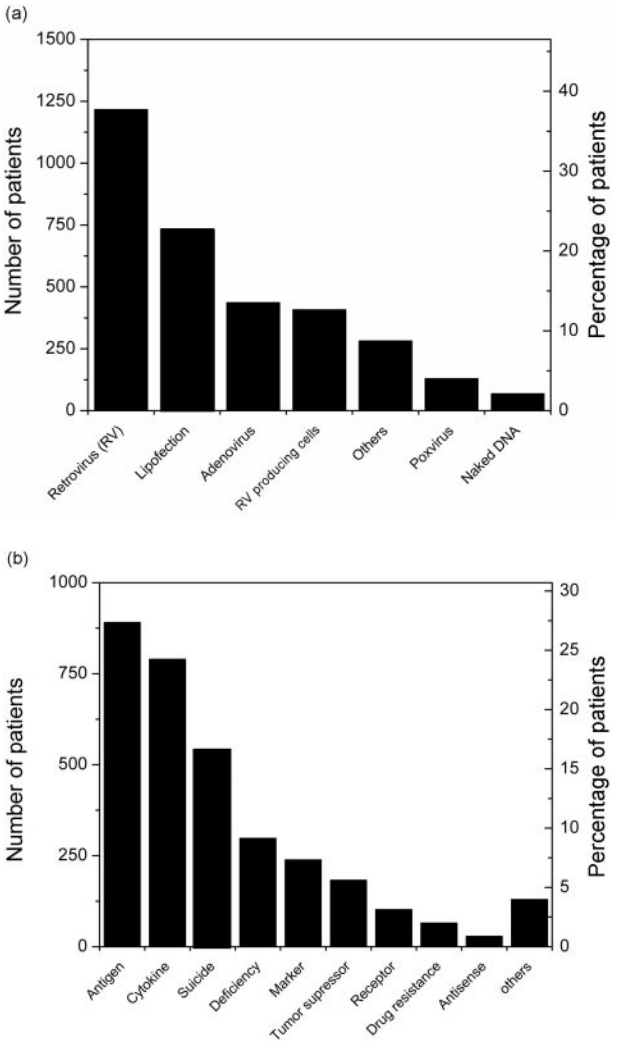

Figure 2. Gene therapy clinical trials. The number of patients and the respective percentage of the total number of patients that have been entered into various gene therapy trials by treatment category (a) and vector/gene delivery strategy (b).

being used in about 20% of the protocols (*cf*. Figure 2).[2] However, the balance of safety versus efficiency may have to be re-evaluated in the light of the recent death of a patient undergoing adenovirus mediated gene therapy.[61]

Gene delivery systems

Naked DNA

The use of the plasmid on its own (so-called naked DNA) represents the simplest possible delivery system, and direct injection of DNA has been used quite efficiently to transfect the skeletal muscle[62] and various organs, e.g. liver,[63] but has also been used successfully for direct intratumoral injections.[64] Many other reports have since demonstrated the use of naked DNA for local and regional administration, in particular the muscle, where it is currently the method of choice for DNA-based vaccines. The low levels of nuclease activity in muscle tissue compared to other organs and the serum is probably one of the main reasons for the relatively good success with intramuscular injection of naked DNA.[65] The systemic administration of naked DNA, i.e. by i.v. injection, is in general much less efficient because serum nucleases clear the various topoisoforms of plasmid DNA from the plasma within minutes.[66,67]

Regional vascular administration to the liver can be achieved by direct injection of naked DNA into afferent liver vessels combined with occlusion of the efferent vessels.[68] A similar effect can be achieved through the rapid injection of naked DNA in large volumes, the so-called hydrodynamic transfection, which allows efficient transfection of the liver by naked DNA after i.v. administration.[69,70] Using injection volumes equivalent to 8–12% of body mass appears to temporarily reverse the direction of venous flow between the heart/inferior vena cava and the liver, effectively 'backwashing' the plasmid into the organ.[69] A positive correlation between intra-parenchymal pressure and the levels of gene expression after direct injection into hepatic vessels exists, suggesting pressure in the capillary bed may temporarily dilate the fenestrae of the endothelial cells.[68,71] The high volume also causes effective exclusion of blood components, i.e. nucleases, from the bolus or at least a very significant dilution. The method poses the risk though that the high pressure may impair the sinusoidal sieving capability and damage the hepatocytes.[70,72] Using a similar principle it is also possible to transfect the hind limb muscles by direct injection into the afferent blood vessels and occlusion of efferent vessels.[73] These results highlight the fact that it imperative to protect DNA from degradation by nucleases, in particular in the blood.

In order to protect DNA on systemic application it is usually complexed through interaction with cationic lipids, polymers or peptides. The resulting complex not only protects the DNA from the attack of nucleases, but also acts as a package that changes the biological behavior of the complexed DNA: as long as the association persists the physicochemical properties of the delivery system (co-)determine the biological fate of the DNA. Consequently delivery systems can potentially improve transfection efficiency and specificity on multiple levels through their interaction with the various biological barriers.

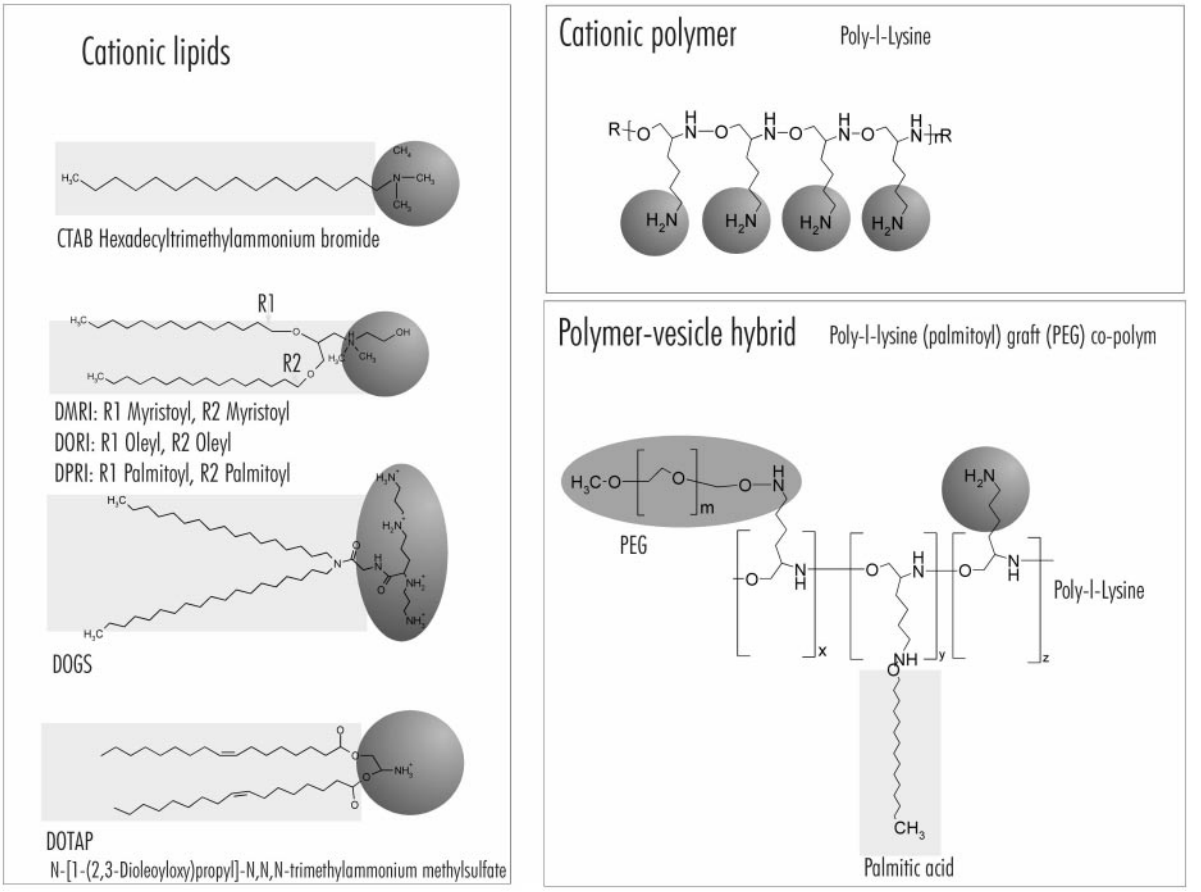

Figure 3. Structure of synthetic vector components—general structure and various examples of cationic lipids (a) and polymers (b) used as synthetic vectors for gene delivery. The common principles of cationic lipids are the cationic, hydrophilic head group which interacts with the DNA and the lipophilic 'tail' which is responsible for the self-assembly into vesicles and similar structures. Polymers such as PLL or PEI possess a high number of cationic groups per molecule, but usually lack the ability to self-assemble. Modified graft co-polymers such as PLP are hybrids which combine properties of both classes of synthetic vectors.

Synthetic gene delivery systems

Synthetic gene delivery systems encapsulate or complex the plasmid DNA to protect it and modulate its interaction with the biological system. The vast majority of these systems are based on the use of cationic lipids or polymers (or combinations thereof), which show a strong electrostatic interaction with the anionic DNA. Due to this interaction the DNA is condensed and becomes part of the carrier system.

Cationic lipids. Cationic lipids are amphiphiles that self-assemble into macromolecular aggregates such as vesicles (*cf.* Figure 3). Their interaction with each other, with helper lipids and the DNA needs to be regarded on a molecular as well as colloidal level to appreciate the complexity of these systems. On the molecular level the interaction of the cationic amphiphiles with the DNA and with the various cellular elements determines the intracellular fate of the complex, but morphology and colloidal properties, on the other hand, may be equally important, in particular *in vivo*.

The membrane of cationic vesicles carries positive charges, which are freely mobile within the respective membrane layers. On contact with the negatively charged DNA the attraction between the opposite charges sets in motion a highly dynamic complexation and aggregation process.[74]

Initially DNA adsorbs to the oppositely charged vesicle as helices with DNA strands running in parallel. The DNA-covered vesicle can now in turn adsorb to neighboring vesicle membranes, with subsequent aggregation and flattening of unilamellar vesicles.[74] At higher lipid concentrations large multilamellar complexes are formed, apparently by adsorption of a

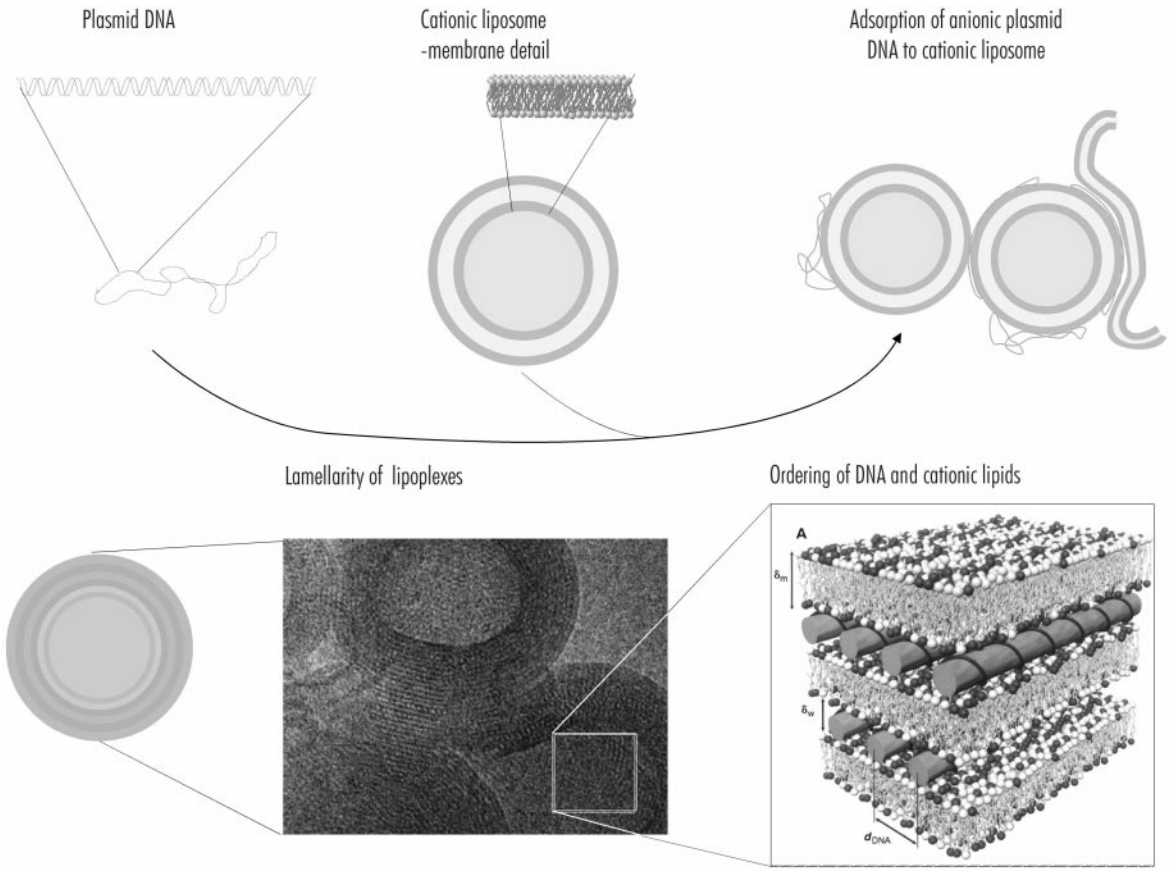

Figure 4. Lipoplexes—proposed structure (lower panel) and mechanism of assembly on multilamellar lipoplexes (upper panel). Negatively charged plasmid DNA and positively charged lipid head groups interact. Plasmid initially adsorbs/binds to the vesicle surface. This in turn allows adsorption of other cationic vesicles to the bound DNA. Increasingly tight binding of adsorbing vesicles leads to flattening and eventually membrane rupture. The ruptured membrane forms an additional layer of membranes deposited on the inner vesicle (as observed by electron microscopy, reprint with permission[263]). The plasmid DNA is tightly bound between those layers in a highly ordered lattice (reprint with permission;[76] © 1997 American Association for the Advancement of Science).

neighboring vesicle, followed by either its rupture and subsequent 'peeling over' or by fusion of both liposomes (*cf*. Figure 4).

The morphology of the specific aggregates is determined by kinetic parameters (e.g. speed of mixing and volumes) as well as stoichiometric constraints (lipid:DNA ratio). The process is not easily controlled, which may explain why nominally identical preparations may yield different experimental results. Lipoplexes tend to contain more than one plasmid molecule and the size of the complexes frequently increases compared to the original size of the vesicles. Lipoplexes can be formed over a relatively wide range of ratios, ranging from aggregates, which contain relatively more DNA, to those with a significant excess of the cationic lipid. Around the point of charge neutrality, i.e. an equal amount of phosphate ions and cations, complexes tend to aggregate very rapidly due to the lack of electrostatic repulsion. Only complexes formed at higher lipid:DNA ratios show reasonable colloidal stability over extended periods of time, thus making the positive charges of the complexes an important issue for the interaction with cells *in vitro* and also for the biodistribution of the complex *in vivo*. Complexation of cationic lipid and DNA also leads to a condensation of the DNA, whereby DNA strands and multiple layers of vesicle membranes arrange themselves in a three-dimensional lattice (*cf*. Figure 4); the closeness of neighboring DNA strands depends on the density of positive charges on the vesicle surface.[75–78]

Initial reports on the use of cationic lipid to improve transfection efficiencies *in vitro* sparked a new field of research.[79,80] Since then the research over the last decade has focused to a great extent on the identifica-

tion of 'better' lipids. The work has been based on empirical modification of cationic lipid structure and subsequent *in vitro* screening for reporter gene expression. A plethora of compounds has been screened and these systems have been reviewed by a number of authors.[81–85] In general it has been difficult to identify clear structure–activity relationships that would allow the rational design of the 'ideal' cationic lipid. There is also evidence that the importance of a particular parameter, e.g. lipid chain length and asymmetry, may differ from cell type to cell type.[86]

As hopeful candidates were subjected to *in vivo* experiments, however, it became clear that the correlation between *in vitro* and *in vivo* transfection was in general quite poor: lipoplexes that efficiently mediate transgene expression of tumor cells *in vitro* actually inhibited gene expression in a dose-dependent way when given as direct intratumor injection.[64] This observation highlights one of the defining challenges for the field: the *discrepancies between in vivo and in vitro* experiments not only challenge our understanding of the mechanism of non-viral transfection, but also pose the question whether or not *in vitro* experiments are valid and useful.

The disagreement of *in vitro* and *in vivo* data is not something that is unique to lipoplexes, but also creates problems with other synthetic delivery systems. Initially this phenomenon cast some doubt on the validity of *in vitro* transfection experiments in general, but new insights into the interaction of DNA complexes with biological systems has facilitated the development of improved *in vitro* models, which take some of these limitations into account. Specifically, it has become clear that the biological interactions of colloidally stable complexes, which in general carry an excess of positive charge, are governed to large extent by non-specific electrostatic forces. In (over-)simplified *in vitro* experiments this does not pose a particular problem, as the environment is largely devoid of potential interaction partners—apart from the target cells. In fact in this situation the electrostatic interaction between complexes and cells surfaces is essential for efficient uptake. The *in vivo* situation is quite different as only a minute fraction of the potential interactions are with the target cells. The systems' promiscuity means that it is not the interaction with those target cells but the sum of all the other non-specific interactions, e.g. with components of the vascular compartment, that is observed. The discrepancy between *in vitro* and *in vivo* results will hence be greater for highly charged complexes but will be less significant for complexes in which the non-specific electrostatic interaction has been minimized and replaced by specific interactions.

Polymers

Poly-L-ysine (PLL). PLL has arguably been the most widely used poly-cationic polymer for gene delivery, particularly for *in vitro* applications. As the limitations of PLL emerged an array of modifications have been introduced in order to overcome them, which have frequently been adopted for other systems (*cf.* Figure 3).

PLL forms complexes with DNA at a broad range of DNA-to-polymer ratios with the sizes of the resulting particles ranging from 50 to 700 nm.[87,88] The intensity of binding between polymer and DNA determines the degree of condensation and the efficiency of intracellular release, and depends itself on the number of available ε-amino groups. There is probably an optimum for the strength of the coupling—strong enough to give stability in biological fluids but still able to dissociate within the cell. The binding requires more than 8–20 units to yield sufficiently stable transfection competent complexes,[87,89,90] but also has an upper limit for high molecular weight PLL.[91] Likewise, PLL of increasing molecular weight tends to improve DNA condensation but has also been found to be more toxic.[89,92] The toxicity of the PLL polyplexes can be reduced by an order of magnitude without loss of transfection efficiency through amphiphilic modification.[93]

The colloidal stability of poly-amino acid-based polyplexes in electrolyte solutions and biological fluids is often problematic.[88,94] Furthermore, the PLL polyplexes tend to be rapidly cleared from the systemic circulation with a plasma half-life of 5 min.[95] A popular strategy to alleviate these problems is based on the use of PEG chains to create a steric barrier to minimize blood clearance and increase plasma half-life.[93,96–101] Alternative strategies include the reaction of pendant groups of a hydrophilic HPMA polymer with PLL copolymer to create a hydrophilic coating.[102]

The attachment of *lipophilic* elements has been used in the past to modify properties of PLL.[103,104] This 'lipopoylysine' was based on a low molecular weight PLL (MW 3000) to which an average of two PE groups per molecule were conjugated. More recently Kim *et al.* coupled stearyl fatty acid chains to PLL and used this conjugate in conjunction with low-density lipoprotein (LDL) to form complexes of 100 nm, which were transfection competent.[105]

We have recently combined lipophilic and hydrophilic modifications of PLL through preparation of a palmitoyl-PEG–PLL amphiphilic graft copolymer (*cf.* Figure 3), which forms DNA complexing vesicles in the presence of cholesterol.[93] These complexes have a neutral ζ-potential and are one to two orders of

magnitude less cytotoxic than the parent polymers. They improve gene transfer to human tumor cell lines in comparison to the parent homopolymers despite the absence of receptor-specific ligands and chloroquine. Chloroquine has been found to improve transfection efficiency of unmodified PLL significantly[106] and has frequently been shown to actually be mandatory for transfection.[100,107,108]

In general, the PLL polyplex transfection efficiency is greatly improved through the use of endosomolytic peptides[109] or 'adenofection' (inclusion of inactivated adenovirus).[110,111] The combination of both chloroquine and membrane active peptides has been found to have a synergistic effect on endosomal lysis.[112] A novel strategy to improve endosomal escape of PLL is based on the covalent modification of PLL with histidyl residues, which then buffer the endosomal acidification and thus produce the 'proton sponge' effect.[113]

Another important strategy to improve PLL polyplexes is based on the use of *targeting* moieties to improve uptake and specificity of delivery. Among the earliest reports of targeting ligands coupled to PLL are asialoorosomucoid/asialoglycoprotein receptors to target the liver,[114,115] and the transferrin and its receptor, which is very active in rapidly growing cells.[116] These ligands have since been the most frequently used moieties but other modifications of PLL have included the coupling of targeting residues such as monoclonal antibodies,[106] various sugars,[117,118] peptides[119] and epidermal growth factor (EGF)[120,121] to the polymer chain to improve (specific) uptake of the complexes (see Zauner *et al.*[122] for a recent review). Interestingly the combination of both strategies, the PEG chains and the targeting moiety, may be necessary to maximize the beneficial effects, as the steric cushion of long hydrophilic chains often reduces non-specific binding to cells[100,123] which then needs to be replaced by an alternative uptake mechanism.[102]

The application of PLL-based gene delivery systems has been limited to *in vitro* use, by and large. Recently, however, reporter gene expression in the liver was reported after i.v. administration of galactosylated PLL polyplexes with slightly negative ζ-potential that were targeting hepatocytes via the asialoglycoprotein receptor.[124,125] A galactosyl-D-lysine/D-serin copolymer carrying a C-terminal PEG5000 apparently also targets the liver *in vivo* achieving expression levels of more then 10^6 RLU/mg with other organs expressing more than 3 orders of magnitude less transgene.[126]

Polyethylenimine (PEI). PEI polymers have recently emerged as a new and promising polymer for non-viral gene delivery.[127] Most PEIs are highly branched molecules where every third nitrogen is protonable, hence making this molecule an efficient '*proton sponge*' with excellent buffering capacity. The buffering capacity actually increases from 20 to 45% with a drop in pH from 7 to 5 (as it would occur during processing in the endosome).[128] PEI thus appears to act as an endosomolytic reagent in its own right. It has been proposed that PEI, due to its high buffering capacity, is buffering H^+ influx into the endosome. Hence this 'proton sponge' effect would then reduce acidification and DNA degradation, and ultimately cause osmotic lysis of the endosome.[127]

Packaging of additional low molecular weight PEI into polyplexes formed from high molecular weight PEI exploits the principle successfully as the improved transfection efficiency is apparently not due to increased (charge-mediated) uptake of the complexes, but their improved buffering capacity and subsequent endosomal escape.[129]

As with other synthetic vectors, toxicity of the polymer/complex is an important issue *in vitro* and *in vivo* as the therapeutic index is in general quite low. This is obviously related to the absolute amount of polymer the cells are exposed to. In a recent report, between 25 and 50% of cells treated lost their viability at DNA:PEI ratios of 1:1 (w/w) and 100% of the cells died at a ratio of 10:1 (w/w), respectively.[130]

Toxicity has also been observed *in vivo* after PEI doses greater than 40–100 μg per mouse[131–133] at N/P ratios (polymer amines to DNA phosphor) of 6:1 and greater. The acute nature of the reported symptoms suggested lung infarction as the cause of death, but the activation of complement by positively charged polyplexes may also have been involved.[134] The central role of colloidal stability of the complexes in the blood is also being highlighted by the fact that polyplexes formed in higher ionic strength buffer were dramatically more toxic (own observations and [132]).

PEI has been conjugated to a number of different groups in order to improve polyplex uptake and *targeting*. Cell-specific targeting has been shown with carbohydrate moieties,[135–137] integrin-binding peptides,[138] transferrin and antibodies.[133,139] The specific uptake as a result of receptor-mediated endocytosis is, however, sensitive to interference from non-specific interactions. For this reason lower ratios of polymer to DNA, i.e. polyplexes with less excess positive charge, are used with advantage for targeting. Another potential source for interference stems from the presence of other ligands, as can be seen through the loss of specificity reported when higher amounts of inactivated adenovirus are added to improve endosomal escape.[130]

Locoregional administration of PEI-based carriers to the brain,[127,140–142] kidney[143] and lung[144] demonstrated early on that PEI also has potential for gene delivery *in vivo*. For the local application *in vivo* complexes close to charge neutrality were most efficient, whereas highly positive complexes had a tendency to be toxic.[140,144] This is in contrast to most reports on systemic administration *in vivo* where a significant excess of PEI appears to be more efficient.

Most of the PEI formulations studied were prepared using branched PEI of varying molecular weight (0.6–800 kDa), but a linear PEI of 22 kDa has also been examined. While higher molecular weight polyplexes were found to be more efficient *in vitro*[127,145] the smaller linear 22 kDa PEI complexes were more efficient than either the branched 25 kDa PEI[141,146] or the higher molecular weight PEI (50–750 kDa)[133,140] on systemic administration *in vivo*.

As with other synthetic vectors, the *systemic administration* of the complexes *in vivo* remains a challenge: the *biodistribution* of reporter gene activity after tail vein injection closely resembles that of cationic lipoplexes and is dominated by the lung. The highest expression levels in the lung have been reported for the linear PEI (22 kDa) and levels in other organs are routinely 2 orders of magnitude lower.[131,133,146–148]

A combination of covalent and non-covalent modification of PEI complexes with non-ionic polyoxyethylene ethers showed some success in reducing the preference of transfection in the lung resulting in a rank order of expression of liver > spleen > lung.[149] On the other hand, transfection with PEI complexes can actually be targeted to the lung to some extent through the conjugation to anti-PECAM antibody.[133] The targeted polyplex showed a 20-fold higher expression than an untargeted PEI polyplex.

Peptide-based gene delivery systems

Peptides are the third group of compounds which have been explored as synthetic gene delivery systems.[150] These carriers take advantage of the versatility of amino acids to create peptides which carry one or more domains associated with specific functions, i.e. DNA binding/condensation, targeting/uptake, endosomal escape and possibly nuclear targeting. The blueprint for the different functions is frequently based on proven elements of other systems or adapted from viral vectors.

The binding/condensation is, as in most other synthetic systems, based on the electrostatic interaction between the anionic plasmid and cationic amino acid residues, frequently lysine. Thirteen lysine residues were reported to be required for the efficient complexation and condensation of plasmid DNA, yielding particles which were smaller and more transfection competent than those made from peptides with only eight lysine repeats or low molecular weight polylysine.[92] Similar numbers of residues have been reported from other peptide-based systems (12 amino acid peptide,[151] 16 lysine residues,[152] 12 aminoethyl side chains[153]). For lysine itself it has been suggested that a slightly higher number of residues (more than 8–20) is required for optimum activity,[87,89,90] but it is not clear whether the difference is significant. As for the other complexing systems, an excess of positive charges will stabilize the peptide-based particulate carriers, and is thus likely to contribute as a non-specific element to cell binding and uptake. Targeting elements, such as integrin targeting RGD peptides, have been included into such systems to improve specificity and increase cellular uptake.[90,154] Although some peptide-based complexes show remarkable stability to serum when incubated *in vitro*,[153,155,156] not much is yet known about their behavior *in vivo*.

Systemic and cellular barriers to gene delivery

The barriers to synthetic gene delivery systems exist on a systemic as well as on a cellular level. While on the cellular level the challenges lie in the efficient transport of the plasmid DNA to the nucleus, the systemic barriers hamper the specific transport of the DNA carrier to the tumor, in particular after systemic administration.

Systemic barriers and biological behavior

Systemic administration. Most therapeutic strategies for cancer gene therapy require efficient and specific delivery of the therapeutic cDNA to the primary tumor, but also tumors/metastases at remote sites. This represents a considerable challenge, as there are a number of biological barriers which seriously hamper the *in vivo* application of synthetic delivery systems, in particular after i.v. administration.

The blood compartment. Injection of naked DNA into the systemic circulation leads to its rapid clearance from the blood and degradation through serum nucleases.[66,67] Cationic lipid- and polymer-based formulations complex DNA through charge–charge interactions and thus protect it efficiently from degradation. To yield colloidally stable complexes the complexing agent (polymer or lipid) is usually used in

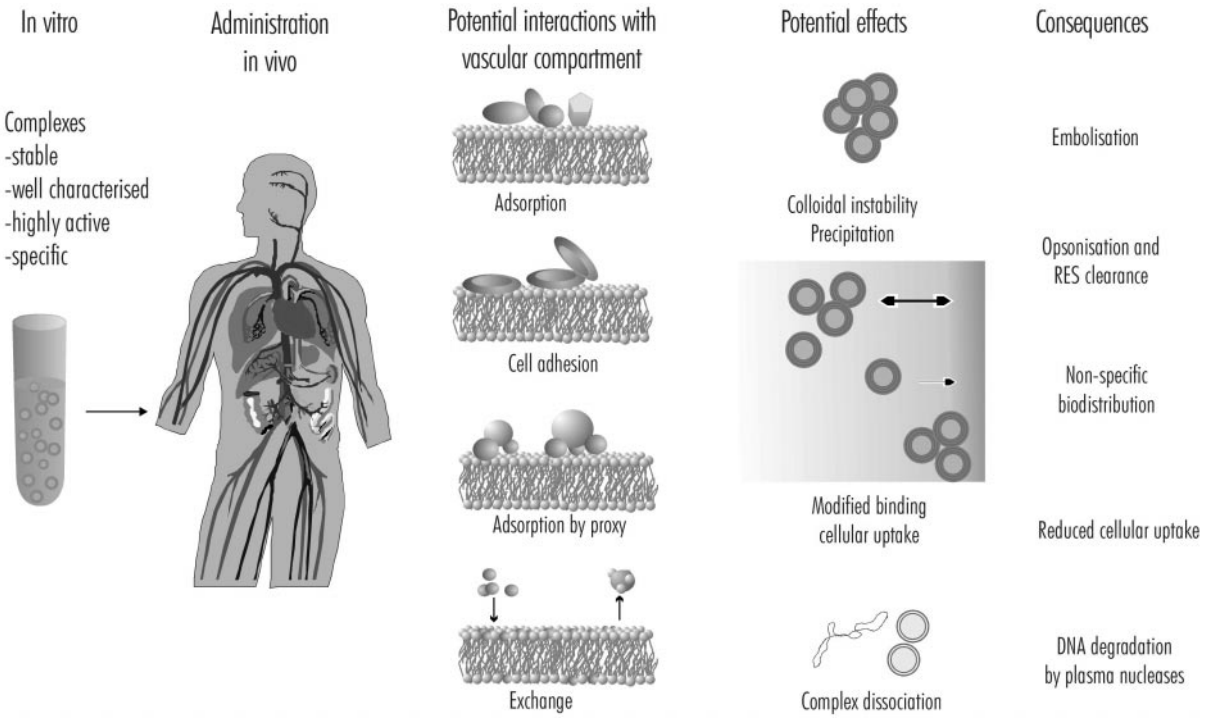

Figure 5. Systemic barriers encountered after i.v. administration of DNA complexes. *In vitro* complexes with an excess of positive charges repulse one another and are thus colloidally stable. After i.v. administration such complexes interact intensely with elements of the vascular compartment. This can lead to non-specific binding of various biological macromolecules and proteins (e.g. opsonins), but also cellular elements such as erythrocytes or platelets. A combination of negatively and positively charged elements can bind alternately ('by proxy') and thus modify the complex further. The binding events in combination with the exchange of cationic elements can potential cause complex destabilization, dissociation and, ultimately, degradation.

excess and the resulting complex carries excess positive charge. This charge facilitates complex endocytosis *in vitro*,[157] but also forms the basis for extensive non-specific interaction with cells, proteins or other molecules *in vivo*. As a result of such interactions complexes are subject to modifications, which can render them instable and frequently reduce their transfection capacity (*cf*. Figure 5).

Serum proteins tend to interact intensely with particulate drug carriers in general. In the case of the charged DNA complexes it is mainly the negatively charged proteins which adsorb to the DC-chol lipoplexes.[158] In some cases this adsorption can eventually reverses the surface charge.[159] The adsorption can be minimized through the removal or neutralization of these plasma proteins,[158] or, in the case of DOTAP lipoplexes, through the inclusion of cholesterol,[160] which has been shown to reduce plasma protein binding[161] and increase circulation half-life of vesicles.[162]

Among the proteins that interact strongly with DNA complexes are albumin, lipoproteins (HDL and LDL) and macroglobulin.[163] Albumin has been shown to release DNA from PLL polyplexes[95] (but not lipoplexes[164]) but also modulates complex binding and adsorption,[165] and nuclear uptake.[164] An indication of complexity of potential complex–plasma interaction comes from the observation of a transfection-enhancing effect, which compensates for the loss of uptake through increased intracellular efficiency.[166] A positive effect of serum on transfection efficiency was also observed with complexes formed from a novel cationic lipid DC-6-14, which were most efficient in the presence of serum.[167] The nature of these enhancing effects remains unclear.

Cellular elements such as erythrocytes and platelets also interact with DNA complexes. PEI polyplexes give rise to an extensive erythrocyte aggregation *in vitro* and, when formed in high ionic strength medium, can be highly toxic upon injection, presumably because of lung embolism after complex aggregation.[132] Platelets form part of the aggregates recovered shortly after i.v. injection of various lipoplexes, and the injection also causes a biphasic reduction of platelets (30% after 24 h) and leukocytes (73% after 24 h).[168]

In vitro studies can give some insight into the interaction of charged complexes with blood components; however, the *in vivo* situation is far more

complex. In addition to the blood components, the vasculature has the potential to influence the processing and biodistribution of the complex. In addition to a direct interaction with the endothelial cells, physical parameters such as complex size also determine the interaction with the *vasculature*.

Gene delivery systems used for systemic applications have a size distribution similar to other colloidal carrier systems (0.05–1.0 μm). Such particulate drug carriers leave the vascular bed only in specialized organs with increased fenestration of the vascular endothelium, e.g. the liver, or in tumors where the endothelial lining is highly irregular.[169,170] Consequently access of DNA complexes to the parenchyma of most organs is limited and endothelial cells take up most of the complex material.[168,171–173] These barriers are not necessarily absolute as other studies were able to demonstrate gene expression not only in the endothelium but also in the parenchyma of many organs.[174]

In the tumor *extravasation* is potentially facilitated by the irregularities and mosaic nature of the endothelium.[170] Particulate drug carriers can extravasate the 'leaky' tumor vasculature and accumulate in perivascular clusters within the tumor. This so-called 'enhanced penetration and retention' (EPR) effect[175] has in fact been employed successfully to target vesicles and polymers to tumors[176–178] and may also allow some limited tumor targeting with suitable synthetic DNA vector systems. The endothelial size exclusion barrier can potentially be lowered using special administration modes[69,73] or pharmacological intervention, such as low-dose vascular endothelial growth factor treatment which increased average and maximum size (from 400 to 800 nm) of endothellial pores in human colon carcinoma xenografts.[179] In addition to this mechanism, positively charged lipoplexes or cationic vesicles seem to accumulate in regions with a high angiogenic activity such as the tumor neovasculature probably because of their charge.[180] It is currently unclear whether a similar mechanism exists for polymer-based complexes.

Biodistribution. While i.v. administered naked plasmid DNA tends to be found mainly in the lung, liver and blood,[172] complexation of the DNA to cationic lipids tends to increase accumulation in lung and liver.[66,172] In most *in vivo* studies with lipo- and polyplexes the lung has been shown to be the predominant organ of accumulation over the first few hours,[181–183] with a possible later redistribution phase, e.g. of the lipids to the liver.[184] Unsurprisingly the large extent of non-specific interaction of complexes with the vascular compartment also determines their biodistribution: within a few minutes after the i.v. injection the charged particles coat the luminal surface of the small blood vessels of most organs (with the highest levels found in the lung, lymph nodes, Peyer's patches, ovary and the adrenal medulla). In the liver it is mainly the phagocytotic Kupfer cells, and not the hepatocytes, which take up the complexes. However, with the exception of the spleen the lipoplexes are retained by the vasculature.[168,180]

The *transgene expression* coincides by and large with the plasmid biodistribution, predominantly in the lung. A direct comparison of *in vivo* efficiency of the different non-viral systems is difficult because of numerous differences in experimental design. However, taking the highest expression levels for luciferase reporter systems driven by a cytomegalovirus (CMV) promoter, expression levels are quite similar for lipoplexes and polyplexes with peak expression of about 10^7 RLU/mg protein (± 1 order of magnitude).[131,146,147,182,183] The fact that in the lung expression levels are often found to be at least 2 orders of magnitude higher than in other transfected organs with a similar DNA uptake (such as lymph nodes and ovaries)[168] suggests that this organ may have a natural susceptibility for transfection/transgene expression.

Cationic vesicles that accumulate in the lung in the absence of DNA also promote (dose-dependent) plasmid uptake and retention in the lung within the first hour(s) after injection.[168,182,183,185,186] This could be due to combination of different mechanisms (*cf*. Figure 5).

The coating of complexes with plasma proteins could lead to shielding of the surface charges, thus abolishing the electrostatic inter-complex repulsion. In addition, adsorbed cellular material could act as glue linking neighboring complexes. These effects would facilitate the formation of aggregates, which in turn could accumulate in the lung through a size exclusion/*sieve effect*. Alternatively it has been suggested that (partially) unshielded complexes could adsorb to the endothelial cells of the first major capillary bed that they encounter, i.e. the lung, through non-specific *electrostatic interaction*. It is also conceivable that the complexes 'piggy back' into cells retargeted by a more specific mechanism of receptor-mediated uptake conferred by some of the adsorbed proteins.

There is, however, some evidence which suggests that a different mechanism is involved in the *binding and uptake of lipoplexes and polyplexes*. When two doses of a DOTMA-based luciferase lipoplex were administered within 15 min the expression increased by 1–2 orders of magnitude compared with the expression of a single dose.[146] A mock plasmid in the first lipoplex was equally efficient, suggesting that

the cationic lipid component of the complex may have acted as a scavenger for serum proteins, in a similar fashion as has been shown for liposomes,[162] or that the additional cationic lipid may have improved endothelial binding and uptake of the second dose. The sequential injection of a mock polyplex, and a luciferase polyplex on the other hand, did not increase levels of luciferase expression with the second dose. Only when two doses of the luciferase polyplex were given within 15 min did the transgene expression increased by 1 order of magnitude.[146]

The observations could potentially be explained by an initial non-specific electrostatic interaction that promotes adsorption of cationic complexes to the cell and a subsequent step in which interaction with the cell membrane and/or various receptors leads to endocytosis of the complex. The non-specific component would be common to all positively charged complexes, while the actual uptake may be mediated by a different mechanism.

There are indications that polymers may be able to utilize an active mechanism for transport across the endothelium. Linear PEI complexes that reach the lung seem to be able to cross the lung endothelial cell efficiently within a short period of time.[148] This results in transgene expression in the lung parenchyma within 2 h after injection, while expression in endothelial cells was rarely observed. The authors suggest that a non-destructive process such as transcytosis might be responsible for the rapid transport across the endothelium. The lack of apoptosis or inflammation is taken as evidence that the extravasation is not caused by a hydrodynamic effect related to the relatively large injection volume.[69,70] The luciferase expression reached peak levels of nearly 10^7 RLU/mg of protein after 24 h but drops quite rapidly at 48 h. The observed transport mechanism may well be related to the tendency of lung alveolar cells and endothelial cells to actively accumulate natural polyamines.[187–190] More research is needed to be able to judge the potential applicability of this phenomenon to turn the lung 'tropism' of cationic carriers to an advantage for the delivery of genetic material to the lung parenchyma. The observations are also in contrast to reports of transgene expression in the lung after the use of lipoplexes, which reportedly tend to transfect mainly the endothelial cells.[168]

Understanding the mechanism of accumulation in the lung is important in order to either exploit this accumulation so that a systemic delivery of genetic material to the lung can be optimized or, alternatively, to minimize these effects if gene delivery to remote sites is required.

Strategies for the minimization of non-specific interactions have been based on the experiences gained with other particulate drug delivery systems. One promising approach has been based on the creation of a steric barrier through incorporation of hydrophilic polymers into a particulate drug carrier, which minimizes protein adsorption.[191] In the case of charged carriers this coating of the carrier surface also masks electrostatic charges of polyplexes,[98,100] lipoplexes[123] and hybrid systems.[93] The coating of complexes with this and similar hydrophilic polymers has also been shown to reduce non-specific interaction of PEI polyplexes *in vivo*, thus reducing accumulation in the lung[149] and increasing transgene expression in the tumor in a mouse xenograft model.[132]

The reduction of non-specific electrostatic interactions between DNA complexes and components of the vascular compartment is a prerequisite for targeted delivery of the genetic material to remote sites. Reduction of non-specific interactions also decreases overall complex uptake. Therefore, it is necessary to *replace the non-specific* uptake with a more specific mechanism. Both lipoplexes and polyplexes have frequently been modified by the addition of receptor-specific ligands to improve complex uptake. Only a minority of these targeted complexes have, however, been administered systemically. One reason for this discrepancy may be the fact that many of these targeted complexes still possess a considerable potential for non-specific interaction. When cells that express the targeted receptor are being transfected *in vitro* non-specific interactions frequently improve efficiency, but invariably also reduce targeting specificity.[120] On administration *in vivo* the complexes are exposed to an environment of dramatically increased interaction potential and the non-specific interactions prevail.

Transferrin (Tf) is one of the most frequently used targeting ligands *in vitro*. The Tf receptor is expressed in high numbers in rapidly growing cells and potentially interesting for tumor targeting. Tf-modified PEI polyplex (Tf–PEI) when stabilized through PEG chains showed significantly reduced uptake in the lung and was able to transfect a s.c. xenograft after i.v. injection in mice,[132,192] but there is also evidence for an increased non-specific, non-receptor-mediated endocytotic uptake of Tf complexes.[193,194]

Glycosylation (galactose, mannose) of carrier systems to target hepatocytes has mainly been used in conjunction with polylysine-based systems.[117,125,126] This strategy may also benefit from the default redistribution of complexes towards the liver, which has previously been observed. The use of a galactosyl-D-lysine/D-serine copolymer with C-terminal PEG5000

targets the liver *in vivo* with expression of more then 10^6 RLU/mg of protein. Expression levels in other organs, including the lung, were more than 3 orders of magnitude lower.[126] The targeting of specially formulated DOTAP:Chol complexes to hepatic asialoglycoprotein receptor also increased liver expression 7-fold; the expression in the lung was nevertheless by far the highest.[195]

In general, a higher *dose* of transfection agent will give rise to higher levels of transfection, but the considerable toxicity of cationic lipids and polymers limits the total amount can be administered safely. Frequently the onset of toxic symptoms is quite rapid, suggesting the observed toxicity may be related to the physicochemistry of the complexes, i.e. their lack of colloidal stability upon i.v. injection, which may result in aggregation and subsequent blockage of small capillaries, most likely in the lung.[131,132] There is, however, also some evidence that links toxicity to the total amount of either cationic lipid (1–2 mg/mouse)[159,172,182,195] or polymer (40–100 μg/mouse).[131,133] Other signs of toxicity have been linked to inflammation induced by the unmethylated CpG islands.[40–42]

Locoregional administration. One way to circumvent the problems of systemic administration is by direct injection either via the afferent vasculature or by direct injection into the parenchyma of the affected organs or the tumor itself. This strategy is particularly promising if a strong systemic bystander effect, such as an immune response, is induced, which may then act at remote sites, e.g. micro-metastases.

Locoregional administration can be divided into vascular administration, the direct injection into the parenchyma and injection into fluid filled cavities within the organ. The delivery of plasmid DNA to the lung, i.e. by inhalation or instillation, represents a special case of local delivery to a body cavity, which we will not cover in detail (for recent reviews, see Eastman and Scheule[196] and Marshall *et al.*[197]).

Regional vascular administration, i.e. via afferent blood vessels, reduces non-specific uptake in other organs, i.e. the lung, and minimizes exposure to other components of the vascular compartment. A recent example of this administration modality is the intracoronary infusion of lipoplexes based on viral envelope liposomes in a rat model, which led to expression of β-galactosidase in 50% of the heart myofibers.[198] Complex extravasation and access to the parenchyma remain potential problems, depending on the fenestration of the endothelium. In the case of tumors, the irregular and leaky vasculature may lead to an accumulation of the carrier due to enhanced penetration and retention.[175]

Injection into *cavities* within an organ, e.g. the brain ventricles, completely avoids potential destabilization of the DNA complex by the blood. Depending on the specific cavity, however, the respective biological fluid (e.g. cerebrospinal fluid) may nevertheless contain destabilizing components. In addition the complexes also need to be able to get across the epithelial lining of those cavities to gain access to the parenchyma itself. Examples are the intraventricular administration of lipoplexes[199] and PEI polyplexes[142] to the brain or the intra-renal-pelvic injection of a lipoplex formulation. In all cases the authors report some expression in the parenchyma. A potentially important application of this route of administration is the i.p. injection, e.g. for the treatment of ovarian cancers. Recent examples in animal models demonstrated regression of i.p. tumors and induction of an extensive immune response after i.p. administration of a lipoplex that expresses mycobacterial heat shock protein (hsp65).[200] In a similar experiment the i.p. injection of lipoplex with a tumor necrosis factor-α-expressing plasmid improved median survival of the treated group in an animal model of disseminated i.p. cancer.[201] In a recent report different lipid formulations were compared after i.p. administration in a peritoneal tumor model.[167] The efficiencies of transfection of tumor cells were reported as 1% for a novel cationic lipid DC-6-14l and 0.23–0.62% for the commercially available lipoplexes.

For direct *injection into the tumor* the specific injection modalities can be quite important: hydrodynamic factors, i.e. total injection volume, number of injection sites, speed of injection[147] and complex size[141] need to be taken into consideration as they will influence distribution and efficiency of transfection. When naked DNA and PEI polyplexes were injected directly into tumor xenografts at 'normal' speed the luciferase expression from polyplexes was almost 10-fold lower than that measured from naked DNA.[147] When a slow injection regimen (5 min) was employed, however, the situation was reversed and linear PEI polyplexes gave the highest expression. The efficiency of complexes administered by direct injection can be improved further by using targeting, e.g. Tf-targeted PEI polyplex gave rise to expression levels 10–100 times higher than from naked DNA after direct injection into s.c. growing xenografts.[192] Thus injection of a formulation consisting of small particles given in a high volume, administered on multiple injection sites with a slow rate of injection would probably be the best regimen for direct intratumor administration of DNA complexes.

The problem of spread within the tumor is potentially less critical when the therapeutic strategy is based on immune modulation: direct injection of DNA in a lipoplex was reported to be significantly more efficient than naked DNA in induction of interferon-α2 expression in a model of human basal cell carcinoma (BCC) in immune-deficient mice.[202] Likewise, a high percentage of tumor rejection was observed on the direct intratumor injection of IL-12 coding plasmid complexed to a non-condensing polymer [polyvinyl pyrrolidon (PVP)] in a mouse xenograft model.[203] In a direct comparison of intratumor injection of reporter plasmids formulated with a proprietary polymer, a lipoplex and a recombinant adenoviral vector in a intracranial glioma xenograft model the polyplex formulation was considered to be superior to the lipoplex and of equal efficiency as the viral vector.[204] The direct tumor injection of an HLA/H-2K-based immunotherapeutic lipoplex has also been successfully tested in phase I/II clinical trials, where many of the patients showed at least a local response in a cutaneous nodule.[36]

The three routes for local administration have been compared directly for administration of a lipoplex formulation, which was injected into mice intra-renal-pelvic, intra-renal-arterial and intra-renal-parenchymal.[205] Interestingly the intra-renal-parenchymal administration, theoretically the simplest route, was found to be less efficient than both other routes.

It is difficult to generalize such results or to predict the suitability of a formulation even for local administration from *in vitro* experiments, especially as the formulation of DNA as a complex does not in all cases improve the efficiency of transgene expression.[64]

One of the expectations in the use of direct injection is that the therapeutic genes would show their effect in the immediate vicinity. This may in many cases be a serious limitation but can also be seen to increase safety and specificity of delivery. However, after direct intramuscular injection of 2 μm microspheres into mice significant expression was reported in the contralateral limb and several internal organs.[206] These carriers gave rise to prolonged expression in the injected muscle (more than 21 days) and also distributed expression throughout the body, e.g. in the brain and fetal tissues.[206] Thus it would appear that local injection does not necessarily guarantee specific expression in the target cells only, a fact that could have potentially important safety implications.

Cellular barriers and intracellular trafficking

While the specific delivery of DNA complex to the tumor has proven to be a challenge it has also become obvious that it will be necessary to optimize the transfection process on a cellular level. Intracellular trafficking of the DNA complex can be organized into a series of steps that involve the initial binding, internalization through endosomal uptake, trafficking in the endosome/lysosome compartment, escape from the endosome/lysosome compartment and transport to the nucleus (*cf.* Figure 6). It is important to understand the specific interaction of a synthetic gene delivery system with these potential barriers in order to optimize its intracellular processing.

There is some evidence that suggests that the delivery of plasmid DNA across the cell membrane is not a major limitation for the overall transfection efficiency of non-viral gene delivery systems.[207–209] Routinely plasmid DNA is taken up in relatively large quantities by cells in *in vitro* experiments. However, only when cells take up more than 10^6 plasmids molecules does their transgene expression approach 100%.[209] This is probably equivalent to 10–100 plasmid molecules having reached the nucleus,[39,210] suggesting that there is significant potential for optimization.

One limitation for the binding and uptake of plasmid DNA lies in the targeting of specific cells or receptors. For the majority of complexes studied to date the nature of the initial binding to the cell is thought to be ionic, based on the excess positive charge of complexes and the negative charge of the cell surface, i.e. the membrane-associated proteoglycans.[157,208,211] In fact a positive correlation has been shown to exist between the ζ-potential of complexes and their cellular uptake.[212] The non-specific electrostatic interactions of charged complexes tend to over-ride specific interaction, i.e. ligand–receptor binding, and thus interfere with specific delivery not only *in vivo* but also *in vitro*.[120] Once the complexes have been taken up by the cell the intracellular processing of DNA appears to follow a common route irrespective of the mode of uptake, as for $CaPO_4$ precipitation, lipofection or electroporation, the trafficking through the endosomal and lysosomal compartment seems to be mandatory.[207]

Endosome. The process of endosomal escape is one of the main intracellular barriers encountered in non-viral gene delivery. The escape of the plasmid DNA from the endosome into the cytoplasm before lysosomal degradation occurs is a prerequisite for the subsequent transport to the nucleus. Here some intriguing differences between polyplexes and lipoplexes become apparent: not only are different mechanisms involved in the endosomal escape, but also the subsequent processing shows differences.

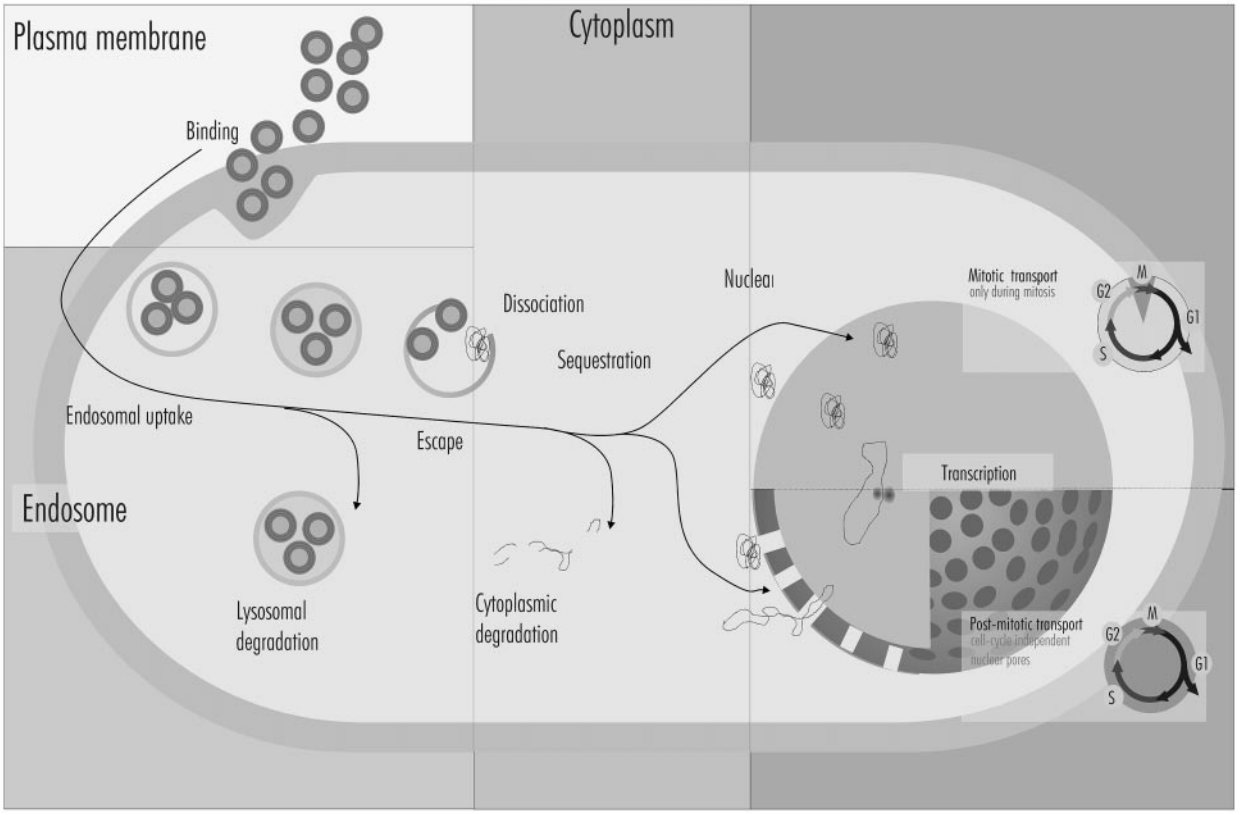

Figure 6. Intracellular barriers to synthetic gene delivery systems. Trafficking of the plasmid DNA to the nucleus requires transport across at least four cellular compartments: the plasma membrane, the endosome, the cytoplasm and, finally, the nucleus. Cellular uptake depends on either non-specific or receptor-mediated endocytosis. Endosomal escape of the complex or the dissociated plasmid DNA needs to occur before lysosomal degradation sets in. In the cytoplasm the DNA can be sequestered or degraded if it is unprotected or not transferred to the nucleus rapidly. Access to the nucleus and the transcription machinery depends on transport through the nuclear pores (post mitotic) or break down of the nuclear membrane during mitosis.

Cytosolic or nuclear microinjection of plasmid DNA still complexed to the cationic lipids prevents transgene expression[213] and it seems to be essential that the DNA is released from a lipoplex *before* it reaches the cytosol.

This step has significant potential for improvement of the overall transfection efficiency but the underlying mechanism is poorly understood. Anionic liposomes with a composition similar to that of endosomal membranes can—in contrast to most soluble ionic molecules found in the cell—mediate complex dissociation.[214] A similar mechanism has been proposed to destabilize the endosomal membrane and thus induces flip-flop of anionic lipids from the cytoplasmatic side of the endosomal membrane with subsequent release of the DNA.[214] The situation is different for polyplexes where the release from the complex does not seem to be a precondition for gene expression.[213]

In any case it is likely that there is only a limited window of opportunity for DNA or complexed DNA to escape from the endosome and maintain its activity: in the lysosomal environment degradation would probably occur quite rapidly. While some of the DNA-complexing agents have a built-in escape mechanism it remains unclear for most how the escape occurs in detail. The escape mechanisms probably all ultimately lead to the disruption of the endosomal membrane in one form or another. In the case of DOPE-containing lipoplexes the de-complexation process and the ability to escape from the endosome can both be linked to the fusogenic properties of the DOPE helper lipid.[77,214,215] It is less clear how the destabilization of the endosomal membrane is induced in the case of other lipoplexes, but their transfection efficiency frequently correlates with their ability to escape the endosome.[216]

In contrast polyplexes formed from PEI-based polymers have a built-in mechanism for the induction of endosomal escape based on the broad buffering capacity of the polymer, the so-called 'proton sponge' effect, which induces osmotic lysis of the endosome.[128,217] The effect can also be potentially in operation with other polymers with inherent buffering capacity[218] or harnessed through partial substitution of a polymer such as PLL with buffering groups, e.g. histidyl groups, to thus confer the buffering capacity at low pH.[113]

Virus-enhanced transfection. Viruses possess the ultimate machinery to overcome the cellular barriers, and, consequently, various groups have tried to exploit these properties using non-viral systems in order to optimize intracellular trafficking and in particular endosomal escape.

In one approach the whole virus, either replication deficient or inactivated, with all its proteins is combined with the non-viral delivery system. Variations of such systems, mainly based on adenovirus ('adenofection'), have been tested over the years with polyplex and lipoplex systems. In general such combinations have been found to be able to improve transfection efficiencies by 1–2 orders of magnitude.[110,219,220] Although the improvements have frequently been linked to the increased endosomal escape additional effects related to other viral proteins may contribute to the improved gene expression further downstream of this event.[221] Use of the whole virus, be it as a replication-deficient recombinant virus or in an inactivated form, also means that the safety concerns and issues of immunogenicity need to be addressed in almost the same way as with viral delivery systems. A strategy that attempts to avoid this problem is based on the use of only the essential viral components, which are required to trigger efficient endosomal escape. A mixture of such proteins can be incorporated into a vesicle-based carrier system to mix properties of virus with non-viral carriers.[222,223] A further reduction of viral elements is achieved through the use of the pH-sensitive virus-derived endosomolytic peptides alone.[224]

Well-characterized are the influenza virus-derived peptides based on the N-terminus of the hemagglutinin subunit 2 (HA2). Hemagglutinin undergoes a pH-dependent conformational change into a membrane-active helical peptide, which mediates a membrane fusion reaction and has also been used as part of non-viral carriers.[224,225] Through sequence optimization the induction of conformational changes and subsequent membrane lysis can be made pH sensitive.[226] The effects of such peptides (e.g. the *H. influenza*/INF7) are, however, not necessarily limited to endosomolytic activities, as some have also been shown to increase gene expression when bound to a polyplex and injected into the cytoplasm or nucleus.[112] This observation of multiple functions combined in a relatively small peptide also demonstrates just how well optimized viruses are for 'transfection'.

Other substances with endosomolytic activity such as chloroquine have frequently been used to enhance gene expression in combination with less potent transfection agents. PLL-based polyplexes normally depend to some extent on such agents for efficient transfection. The chloroquine-mediated enhancement of polyplex transfection is significantly less efficient than the endosomolytic peptides, typically less than 10-fold. Interestingly the endosomolytic peptides and chloroquine have synergistic effects for the PLL polyplex-mediated transfection, but actually inhibit transfection with DOTAP lipoplexes.[112] The reasons for this difference are currently unclear, but serve to demonstrate that distinct mechanisms maybe involved in the intracellular trafficking of the various types of gene delivery systems.

Cytoplasm. The endosome has long been seen as the critical compartment in terms of plasmid stability, with the cytoplasmatic environment representing a more or less passive transition compartment *en route* to the nucleus. However, recent reports have challenged this view: depending on the time required for the transport to the nucleus the plasmid stability in the cytoplasm could in fact be a crucial factor in determining transfection efficiencies, as it may limit the amount of plasmid DNA that can actually be transported into the nucleus. This becomes even more significant if the plasmids' access to the nucleus is linked to mitosis, i.e. if there is a delay between the endosomal escape and the entry to the nucleus during the next cell division.

Naked plasmid DNA, single and double stranded, when microinjected directly into the cytosol has an apparent half-life of 50–90 min, probably due to nuclease-mediated degradation.[227] In contrast the plasmid DNA was found to be mostly intact 48 h after transfection with a lipoplex.[39] Lipoplexes tend to be more stable than polyplexes and offer excellent protection for nucleases, be it extracellular, endosomal or cytoplasmatic. The use of specific nuclease inhibitors that inhibit degradation of plasmid DNA in the endosomes/lysosomes, for example, can improve transfection efficiency of PLL polyplexes significantly more than that of lipoplexes.[227,228]

Although plasmid DNA seems to be protected quite effectively from nucleases, this will probably not be

useful in improving cytoplasmatic stability of plasmid DNA, as transgene expression from lipoplexes involves an obligatory de-complexation step: while the lipoplex appears still intact in intracellular structures, the cationic lipids were not observed together with DNA in nucleus[39] and, furthermore, lipoplexes when injected directly into the cytoplasm or the nucleus do not give rise to gene expression.[208,213]

The situation is quite different for polyplexes (PLL or PEI based), which apparently allow transgene expression after microinjection into the nucleus[213] and enhance gene expression when injected into the cytoplasm.[112,113,229] Here the dissociation of polymer and DNA does not necessarily occur in the endosome, and PLL polyplex has been found intact in the cytoplasm.[230]

The improvement of gene expression after cytoplasmatic injection of polyplexes has been reported to be better for higher molecular weight PLL[112] and PEI was found to be superior to PLL in this respect.[213] It has been proposed that the complexation of the DNA to the polymer may offer protection from cytosolic nucleases, thus increasing the amount of intact DNA that can reach the nucleus. A less likely explanation is that the polymers may act as a nuclear homing sequence.[231]

Nucleus. To be efficiently expressed the exogenous DNA has to gain access to the nuclear transcription machinery. The efficiency of intracellular trafficking into the nucleus is apparently quite low with probably only one in 10^4–10^5 plasmids that have been taken up by the cell eventually being expressed.[39,209,210]

A threshold value of 10^6 plasmid molecules/cell has been reported for efficient expression of a transgene after transfection with a lipoplex *in vitro*.[209] A subpopulation of 30% of these cells, which showed relatively rapid intracellular transport of the vector, was found to express the transgene with an efficiency of almost 100%.[209] However, the maximal plasmid dose is limited: the direct injection of plasmid into muscle fibers showed a plateau for the transfection efficiency (30%), which did not increase appreciably even when a 200-fold higher dose was used.[232] The toxicity of the complexes will also prevent the use of higher doses as strategy to increase transgene expression. Thus the transport of the plasmid DNA into the nucleus can in fact be a major barrier to efficient transgene expression. There are two alternative routes that would allow transport of cytoplasmatic DNA to the nucleus: (i) the breakdown of the nuclear membrane during mitosis or (ii) transport through the nuclear pores.

Mitosis. The question whether or not mitosis is a precondition for efficient transport of plasmid DNA to the nucleus for all non-viral vectors has not been answered conclusively to date. Microinjection into the cytoplasm of post-mitotic myotubes suggests that naked DNA does not depend on mitosis to reach the nucleus.[233] While earlier reports suggested that lipoplex access to the nucleus is independent of cell cycle,[234] a number of recent studies demonstrate that mitosis is probably required for efficient transfection with lipoplexes and other non-viral systems.[39,151,229,235,236]

When comparing the transfection efficiencies of lipoplex and polyplex preparations between growing and confluent cell populations, the PLL polyplexes showed less dependency on cell cycle.[229] However, when subpopulations of cells with known cell cycle status were transfected with non-viral vectors (TF–PLL, Tf–PEI, Lipofectamine and an AV enhanced PLL polyplex) and recombinant AV, both lipoplexes and polyplexes were, in contrast to the viral system, highly dependent on the phase of the cell cycle.[237] Cells in G_1 phase were significantly more refractive to transfection and also had expression levels 1–2 orders of magnitude lower than cells closer to division, i.e. late S/G_2. This differential was found to be quite similar for both lipoplexes and polyplexes, with the PLL polyplex showing the highest differential. The observation was not due to a difference in the uptake of complexes, which was in fact slightly lower in G_2. Even when G_1 cells had been cultured long enough so that they would go through a cell division again, expression did not improve, indicating that DNA stability was not sufficient over this prolonged time period (45 h).

These findings would suggest that mitosis is the most important factor in determining access to the nucleus. On the other hand, an inverse relationship of plasmid size and transfection efficiency has been observed using lipoplexes.[238,239] As the nuclear pores act as a size-exclusion barrier (see below) this would suggest that plasmid access to the nucleus is not exclusively dependent on mitosis, but may also occur through interaction with the nuclear pore complex.

Transport through nuclear pores. This mechanism of DNA entry into the nucleus exhibits a size dependency. Small DNA fragments (ON/plasmids) enter the nucleus by passive diffusion, whereas larger fragments are transported through the nuclear pore complex in an energy-dependent manner. The cut-off of the nuclear pores (9–10 nm diameter) is in the order of 50 kDa for proteins[240] and 300 bp for nucleotides.[241] Depending on cell type and metabolic activity the nuclear pore density varies between 3 and

80 μm^{-1} with a distance of 0.1 to 0.6 μm between pores.[242] Transport of larger molecules, and even 25 nm colloidal gold, is an active process, however.[243]

The transport efficiency of larger DNA molecules is inversely proportional to the plasmid size. A significant drop in the rate of nuclear transport has been reported for linear DNA fragments bigger than 1–1.5 kb.[241] A similar effect was observed with plasmids of various sizes delivered as lipoplex.[239] Depending on the nature of the transfected cells, the smallest construct (2.9 kb) was between 6 and 77 times more efficient than the largest construct 52.6 kb). Furthermore, it has been reported that a 50% size reduction of a plasmid, i.e. removal of bacterial origin of replication and antibiotic resistance gene through recombination, was able to increase expression 2- to 10-fold or higher.[238,244]

On the other hand, plasmid vectors consisting of concatemers of copies of a reporter gene with a size in excess of 12 kb were able to increase expression levels 14 times compared to the same copy number of the smaller plasmids that contained only one gene copy per plasmid.[245]

The observations are not necessarily contradictory but in fact suggest that two access routes exist and may work in parallel: the access during mitosis which is independent of plasmid size and the access trough nuclear pore complex with its intrinsic size restrictions. The importance of each route will depend on the boundary conditions of the experiment (e.g. time, dose, plasmid size, etc.), but will probably also depend on biological variables, e.g. cell line. The mitotic nuclear access could potentially be useful to improve preferential transfection of rapidly dividing cells, i.e. tumor cells. It may also be possible to optimize transfection of target cells through modulation of their cell cycle.

Two strategies have been proposed for the optimization of post-mitotic nuclear transport:

(i) Direct—through the attachment of nuclear localization sequences (NLS) onto the plasmid DNA so that such peptides/proteins tags may then redirect intracellular protein transport to the nucleus.
(ii) Indirect—through binding or inclusion of nucleotide sequences into the plasmid with affinity to cellular proteins which then mediate the actual nuclear transport.

Although numerous NLS have been identified,[246] many of the tested peptide sequences have been derived from SV40 NLS. After non-covalent inclusion of such NLS peptides into a lipoplex, a moderate 3-fold increase of expression levels was observed.[247] Likewise the non-covalent attachment of NLS to the plasmid (using the non-covalent sequences specific binding of peptide nucleic acid tags) was able to increase expression after PEI polyplex-mediated transfection 8-fold.[248] The covalent coupling of the NLS peptides to PLL also increased the affinity for the nuclear transport protein importin and resulted in 50% improved transfection efficiency.[231] However, SV40-derived NLS peptides covalently linked to plasmid DNA induced nuclear plasmid accumulation only in digitonin-permeabilized cells, but not after injection into the cytoplasm.[249] A corresponding observation using two different techniques for covalent attachment[250] showed specific interaction with the nuclear pore complex, but lack of nuclear accumulation and progressive plasmid degradation in the cytoplasm.

The attachment of a single NLS peptide resulted in a 10- to 1000-fold enhancement of transfection irrespective of the cationic vector or the cell type used.[251] The authors suggest that multiple peptides per DNA could lead to a threading of a individual DNA strand through more then one of the nuclear pores, thus effectively preventing the transfer to the nucleus that a single NLS molecule could mediate.

PLL and PEI have been reported to promote nuclear transport,[213] but for PLL this is not due to a specific interaction with the nuclear pore complex.[231] The temporary stabilization of plasmid DNA in the cytoplasm may instead be responsible for the effect by increasing the amount of intact plasmid available at the time of the next mitosis. On the other hand, it has been shown that PEI itself accumulates in the nucleus[252] and improved nuclear transport of associated DNA has been reported specifically for PEI.[213,231]

Recently it has been demonstrated that the plasmid DNA itself can gain post-mitotic access through the nuclear pore complex.[253] This process is sequence specific and requires the SV40 enhancer region, which is known to bind to a number of general transcription factors.[254,255] The import through the nuclear pore complex is a multi-step process, which can be disrupted through transcription inhibitors. Transcription factors that are translated in the cytoplasm use the cell's nuclear import machinery for their import into the nucleus. Plasmid DNA with sequences which promote binding of such transcription factors can then virtually 'piggy back' across the nuclear membrane as part of an importin complex.[255] Pre-incubation of plasmid DNA with transcription factors/nuclear proteins also gave rise to higher transfection levels.[256]

Transgene expression. As final step in the optimization of the intracellular processing of a gene delivery system the transgene expression needs to be considered. In general strong, non-specific promoters, such as the cytomegalovirus immediate–early promoter, are used to drive the gene expression from reporter gene constructs. Such promotors will be useful to gauge transfection efficiency when comparing different synthetic delivery systems or administration modalities, but promoter specificity and regulation of expression need to be selected with the therapeutic strategy in mind. Disease- and tissue-specific promoters may also be useful to add extra levels of selectivity to synthetic gene delivery systems.[257,258]

The duration and time course of gene expression reported *in vivo* vary from a few days in most cases to over 19 months.[259] Frequently the expression maximum after i.v. administration is reached within the first 48 h and expression levels fall to background levels within a week, e.g. 10% of maximum gene expression after 72 h,[172] 0.1% of maximum after 96 h.[183]

Although it is currently not clear which factors limit the duration of gene expression there is evidence that processes are involved on a molecular and histological level: for direct injection into the muscle the duration of transgene expression depends largely on the absence of a specific immune response, as myofibers which express immune stimulatory proteins such as hepatitis B virus surface antigen (HsBAg) are eliminated within 10 days.[260] Green fluorescent protein (GFP) expression in murine muscle fibers, on the other hand, was stable for more than 60 days,[232] but when produced by transplantable murine tumors provoked an immune response that limited tumor growth.[261] The issue is complicated further by the fact that DNA by itself can induce an immune response to specific methylated sequences, so called CpG islands.[40,41]

While the elimination of cells that express the transgene occurs on a histological level there are also indications that difference exists between cells in terms of their capability to eliminate foreign DNA. Apparently nuclear plasmid DNA is excluded/degraded much faster in primary cells (fibroblasts) than in transformed cells (HeLa).[207] Similar data were reported for C2C12 myoblasts and myotubes derived from primary cells.[262] The degradation of plasmid in the nucleus is thus one of the factors limiting the duration of gene expression. The underlying mechanisms are currently unknown, but it is conceivable that in transformed cells the DNA machinery may, as a result of multiple mutations, be less efficient than in primary cells.

Conclusions

The feasibility of genetic therapy using synthetic gene delivery systems has been established in animal experiments and clinical trials.

The main problem for the non-viral systems has so far been the comparatively low efficiency of these systems, especially *in vivo*. For successful use in cancer therapy destruction, or at least reversal of the transformed phenotype, of all the affected cells is necessary. Hence the specific transfection of 100% of the tumor cells would be desirable. This is, however, unlikely to be possible with any gene delivery system. To put the transfection rates of current systems into perspective one needs to address the question of how efficient the transfection process actually needs to be. This will depend on many parameters but most importantly on the chosen therapeutic strategy. Many of the strategies for the genetic therapy of tumors provide a biological amplification that will influence not only the transfected cells but also, to a varying degree through a bystander effect, the neighboring tissue.

The specific advantages and limitations need to be taken into account when a specific therapeutic strategy is being devised, as the delivery strategy forms an integral part of any approach to genetic therapy. Suitable combinations of vector and therapeutic strategy will need to take into account efficiency and specificity of the delivery as well as the direct and indirect (bystander) therapeutic effects of the therapeutic gene. Currently immune modulation strategies and GDEPT approaches would appear to be particularly suited because of their potential to induce a considerable bystander effect. However, there is evidence from other strategies for significant indirect therapeutic effects and more research will be required before the feasibility of specific genetic approaches to cancer therapy can be assessed.

Current synthetic gene delivery systems are based on three main groups of materials, i.e. cationic lipids, polymers and peptides, which form complexes with the therapeutic plasmid DNA. The formation of these complexes with the cationic carriers is in general based on the electrostatic interaction with the plasmid DNA. The morphology and physicochemistry of the complexes are difficult to control as they depend not only on the stoichiometry of DNA and complexing agent but also on kinetic parameters. Stable complexes, which are able to protect the DNA from nucleases and condense it efficiently, are usually generated with excess of the cationic carrier compound. The resulting particles are positively charged and colloidally stable but have a high potential for non-

specific interaction. The non-specific interaction results in a tendency for promiscuous binding to biological surfaces and molecules. *In vitro* this is advantageous as it increases cellular uptake and subsequent expression of the complex. In the more complex *in vivo* environment these interactions compromise targetability and stability of the complexes. This is one of the causes for the discrepancies between results obtained in experiments *in vitro* and *in vivo*.

While some of these problems can be reduced through the use of locoregional administration, this route is not suitable for the direct therapy of remote sites or disseminated disease. This phenomenon is part of the 'systemic barrier' to gene delivery, which makes the targeted delivery of genetic material to a tumor a difficult challenge. Thus, in order to create gene delivery systems that can target remote tumors or metastases after systemic administration it is essential to find means to overcome this systemic barrier.

Current systems when injected systemically will accumulate predominantly in the lung and expression levels in other organs are 2–3 orders of magnitude lower. To create delivery systems which give a more even biodistribution it is first of all important to minimize the non-specific electrostatic interactions. Furthermore, to compensate for a lack of non-specific binding and to allow targeted uptake, specific interactions such as ligand–receptor binding will be required. Various groups have introduced strategies which have been able to alleviate some of the problems, but more research is needed to find generally applicable approaches.

While the transfection efficiencies of the different systems vary over a broad range, depending on the experimental conditions and exact formulation used, the highest levels that have been achieved for either of these synthetic gene delivery systems are quite comparable. Although total transgene expression is a valid measure of the transfection efficiency, it is important to characterize the interaction of complexes with the different biological barriers in more detail in order to properly understand and optimize their intracellular processing. This detailed characterization is also important in order to be able to correlate the physicochemistry of such complexes to their biological behavior, in particular to their interaction with the cellular barriers to gene delivery. These barriers limit transgene expression of gene delivery systems once they have reached the target cells. The major hurdles for the complexes are the escape from endosome/lysosome into the cytoplasm and the efficient transport into the nucleus. Various strategies to enhance the efficiency of these steps have been discussed. The basic trafficking of the different classes of gene delivery appears to be the same; however, specifically for intracellular stability, complex dissociation, endosomal escape and transport to the nucleus, significant differences in their intracellular processing are emerging. We may be able to exploit such differences, and use current gene delivery systems as tools to gain a better understanding of the cellular and molecular processes involved in the transfection process. Furthermore, it may possible to exploit their complementary properties to overcome current challenges and create synthetic gene delivery systems, which have been optimized for a particular therapeutic strategy to maximize the clinical benefits of cancer gene therapy. Given the complexity of the evolutionary optimized viral systems it seems likely that future non-viral systems might also be based on a number of optimized modules rather than monomolecular systems.

References

1. Anderson WF. Human gene therapy. *Nature* 1998; **392**: 25–30.
2. Gene therapy clinical trials. *J Gene Med* 2000; URL: http://www.wiley.co.uk/genetherapy/clinical
3. Verma IM, Somia N. Gene therapy—promises, problems and prospects. *Nature* 1997; **389**: 239–42.
4. Lehrman S. Virus treatment questioned after gene therapy death. *Nature* 1999; 517–8.
5. Fox JL. Gene therapy safety issues come to fore. *Nat Biotechnol* 1999; **17**: 1153.
6. Hui KM, Chia TF. Eradication of tumor growth via biolistic transformation with allogeneic MHC genes. *Gene Ther* 1997; **4**: 762–7.
7. Sawamura D, Ina S, Itai K, *et al*. *In vivo* gene introduction into keratinocytes using jet injection. *Gene Ther* 1999; **6**: 1785–7.
8. Oshima Y, Sakamoto T, Yamanaka I, Nishi T, Ishibashi T, Inomata H. Targeted gene transfer to corneal endothelium *in vivo* by electric pulse. *Gene Ther* 1998; **5**: 1347–54.
9. Lauer U, Burgelt E, Squire Z, *et al*. Shock wave permeabilization as a new gene transfer method. *Gene Ther* 1997; **4**: 710–5.
10. Luo D, Saltzman WM. Synthetic DNA delivery systems. *Nat Biotechnol* 2000; **18**: 33–7.
11. Felgner PL, Barenholz Y, Behr JP, *et al*. Nomenclature for synthetic gene delivery systems. *Hum Gene Ther* 1997; **8**: 511–2.
12. Pope IM, Poston GJ, Kinsella AR. The role of the bystander effect in suicide gene therapy. *Eur J Cancer* 1997; **33**: 1005–16.
13. Touraine RL, Ishii-Morita H, Ramsey WJ, Blaese RM. The bystander effect in the HSV*tk*/ganciclovir system and its relationship to gap junctional communication. *Gene Ther* 1998; **5**: 1705–11.

14. Takayama K, Ueno H, Nakanishi Y, *et al*. Suppression of tumor angiogenesis and growth by gene transfer of a soluble form of vascular endothelial growth factor receptor into a remote organ. *Cancer Res* 2000; **60**: 2169–77.
15. Chen QR, Kumar D, Stass SA, Mixson AJ. Liposomes complexed to plasmids encoding angiostatin and endostatin inhibit breast cancer in nude mice. *Cancer Res* 1999; **59**: 3308–12.
16. Vile RG, Castleden S, Marshall J, Camplejohn R, Upton C, Chong H. Generation of an anti-tumour immune response in a non-immunogenic tumour: HSVtk killing *in vivo* stimulates a mononuclear cell infiltrate and a T_h1-like profile of intratumoural cytokine expression. *Int J Cancer* 1997; **71**: 267–74.
17. Marcusson EG, Yacyshyn BR, Shanahan Jr WR, Dean NM. Preclinical and clinical pharmacology of antisense oligonucleotides. *Mol Biotechnol* 1999; **12**: 1–11.
18. Turner PC. Ribozymes. Their design and use in cancer. *Adv Exp Med Biol* 2000; **465**: 303–18.
19. Suzuki T, Anderegg B, Ohkawa T, *et al*. Adenovirus-mediated ribozyme targeting of Her-2/neu inhibits *in vivo* growth of breast cancer cells. *Gene Ther* 2000; **7**: 241–8.
20. Cobaleda C, Sanchez-Garcia I. *In vivo* inhibition by a site-specific catalytic RNA subunit of RNAse P designed against the *bcr–abl* oncogenic products: a novel approach for cancer treatment. *Blood* 2000; **95**: 731–7.
21. Kasono K, Heike Y, Xiang J, *et al*. Tetracycline-induced expression of an anti-c-myb single-chain antibody and its inhibitory effect on proliferation of the human leukemia cell line k562. *Cancer Gene Ther* 2000; **7**: 151–9.
22. Cochet O, Kenigsberg M, Delumeau I, *et al*. Intracellular expression of an antibody fragment-neutralizing p21 *ras* promotes tumor regression. *Cancer Res* 1998; **58**: 1170–6.
23. Piche A, Rancourt C. A role for intracellular immunization in chemosensitization of tumor cells? *Gene Ther* 1999; **6**: 1202–9.
24. Nielsen LL, Maneval DC. P53 tumor suppressor gene therapy for cancer. *Cancer Gene Ther* 1998; **5**: 52–63.
25. Baselga J. New horizons: gene therapy for cancer. *Anti-Cancer Drugs* 1999; **10**(suppl 1): S39–42.
26. 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**: 2941–52.
27. Claudio PP, Howard CM, Pacilio C, *et al*. Mutations in the retinoblastoma-related gene Rb2/p130 in lung tumors and suppression of tumor growth *in vivo* by retrovirus-mediated gene transfer. *Cancer Res* 2000; **60**: 372–82.
28. Tait DL, Obermiller PS, Hatmaker AR, Redlin-Frazier S, Holt JT. Ovarian cancer BRCA1 gene therapy: Phase I and II trial differences in immune response and vector stability. *Clin Cancer Res* 1999; **5**: 1708–14.
29. Tai YT, Strobel T, Kufe D, Cannistra SA. *In vivo* cytotoxicity of ovarian cancer cells through tumor-selective expression of the *bax* gene. *Cancer Res* 1999; **59**: 2121–6.
30. Favrot M, Coll JL, Louis N, Negoescu A. Cell death and cancer: replacement of apoptotic genes and inactivation of death suppressor genes in therapy. *Gene Ther* 1998; **5**: 728–39.
31. Tuting T, Storkus WJ, Lotze MT. Gene-based strategies for the immunotherapy of cancer. *J Mol Med* 1997; **75**: 478–91.
32. Tuting T, Wilson CC, Martin DM, *et al*. DNA vaccines targeting dendritic cells for the immunotherapy of cancer. *Adv Exp Med Biol* 1998; **451**: 295–304.
33. Gomez-Navarro J, Curiel DT, Douglas JT. Gene therapy for cancer. *Eur J Cancer* 1999; **35**: 867–85.
34. Kouraklis G. Progress in cancer gene therapy. *Acta Oncol* 1999; **38**: 675–83.
35. Lukacs KV, Porter CD, Pardo OE, *et al*. *In vivo* transfer of bacterial marker genes results in differing levels of gene expression and tumor progression in immunocompetent and immunodeficient mice. *Hum Gene Ther* 1999; **10**: 2373–9.
36. Hui KM, Ang PT, Huang L, Tay SK. Phase I study of immunotherapy of cutaneous metastases of human carcinoma using allogeneic and xenogeneic MHC DNA–liposome complexes. *Gene Ther* 1997; **4**: 783–90.
37. Pasquini S, Deng H, Reddy S, Giles-Davis W, Ertl H. The effect of CpG sequences on the B cell response to a viral glycoprotein encoded by a plasmid vector. *Gene Ther* 1999; **6**: 1448–55.
38. Li S, Wu SP, Whitmore M, *et al*. Effect of immune response on gene transfer to the lung via systemic administration of cationic lipidic vectors. *Am J Physiol* 1999; **276**: L796–804.
39. Zelphati O, Liang X, Hobart P, Felgner PL. Gene chemistry: Functionally and conformationally intact fluorescent plasmid DNA. *Hum Gene Ther* 1999; **10**: 15–24.
40. Yew NS, Wang KX, Przybylska M, *et al*. Contribution of plasmid DNA to inflammation in the lung after administration of cationic lipid:PDNA complexes. *Hum Gene Ther* 1999; **10**: 223–34.
41. Tan Y, Li S, Pitt BR, Huang L. The inhibitory role of CpG immunostimulatory motifs in cationic lipid vector-mediated transgene expression *in vivo*. *Hum Gene Ther* 1999; **10**: 2153–61.
42. Whitmore M, Li S, Huang L. LPD lipopolyplex initiates a potent cytokine response and inhibits tumor growth. *Gene Ther* 1999; **6**: 1867–75.
43. Freytag SO, Rogulski KR, Paielli DL, Gilbert JD, Kim JH. A novel three-pronged approach to kill cancer cells selectively: concomitant viral, double suicide gene, and radiotherapy. *Hum Gene Ther* 1998; **9**: 1323–33.
44. Mandell RB, Mandell LZ, Link Jr CJ. Radioisotope concentrator gene therapy using the sodium/iodide symporter gene. *Cancer Res* 1999; **59**: 661–8.
45. Boyd M, Cunningham SH, Brown MM, Mairs RJ, Wheldon TE. Noradrenaline transporter gene transfer for radiation cell kill by ^{131}I meta-iodobenzylguanidine. *Gene Ther* 1999; **6**: 1147–52.

46. Longhurst SJ, Rafferty JA, Arrand JR, *et al*. Recombinant adeno-associated virus-mediated expression of O^6-alkylguanine-DNA-alkyltransferase protects human epithelial and hematopoietic cells against chloroethylating agent toxicity. *Hum Gene Ther* 1999; **10**: 301–10.
47. Jelinek J, Rafferty JA, Cmejla R, *et al*. A novel dual function retrovirus expressing multidrug resistance 1 and O^6-alkylguanine-DNA-alkyltransferase for engineering resistance of haemopoietic progenitor cells to multiple chemotherapeutic agents. *Gene Ther* 1999; **6**: 1489–93.
48. Zhao JQ, Hoare SF, McFarlane R, *et al*. Cloning and characterization of human and mouse telomerase RNA gene promoter sequences. *Oncogene* 1998; **16**: 1345–50.
49. Dachs GU, Dougherty GJ, Stratford IJ, Chaplin DJ. Targeting gene therapy to cancer: a review. *Oncol Res* 1997; **9**: 313–25.
50. Connors TA. The choice of prodrugs for gene directed enzyme prodrug therapy of cancer. *Gene Ther* 1995; **2**: 702–9.
51. Niculescu-Duvaz I, Springer CJ. Gene-directed enzyme prodrug therapy: a review of enzyme/prodrug combinations. *Expert Opin Invest Drugs* 1997; **6**: 685–703.
52. Benedetti S, Dimeco F, Pollo B, *et al*. Limited efficacy of the HSV-tk/GCV system for gene therapy of malignant gliomas and perspectives for the combined transduction of the interleukin-4 gene. *Hum Gene Ther* 1997; **8**: 1345–53.
53. Elshami AA, Saavedra A, Zhang H, *et al*. Gap junctions play a role in the 'bystander effect' of the herpes simplex virus thymidine kinase/ganciclovir system *in vitro*. *Gene Ther* 1996; **3**: 85–92.
54. Ishii-Morita H, Agbaria R, Mullen CA, *et al*. Mechanism of 'bystander effect' killing in the herpes simplex thymidine kinase gene therapy model of cancer treatment. *Gene Ther* 1997; **4**: 244–51.
55. Touraine RL, Vahanian N, Ramsey WJ, Blaese RM. Enhancement of the herpes simplex virus thymidine kinase/ganciclovir bystander effect and its antitumor efficacy *in vivo* by pharmacologic manipulation of gap junctions. *Hum Gene Ther* 1998; **9**: 2385–91.
56. Bridgewater JA, Knox RJ, Pitts JD, Collins MK, Springer CJ. The bystander effect of the nitroreductase/CB 1954 enzyme/prodrug system is due to a cell-permeable metabolite. *Hum Gene Ther* 1997; **8**: 709–17.
57. Park JY, Elshami AA, Amin K, Rizk N, Kaiser LR, Albelda SM. Retinoids augment the bystander effect *in vitro* and *in vivo* in herpes simplex virus thymidine kinase/ganciclovir-mediated gene therapy. *Gene Ther* 1997; **4**: 909–17.
58. Ruch RJ. The role of gap junctional intercellular communication in neoplasia. *Ann Clin Lab Sci* 1994; **24**: 216–31.
59. Dilber MS, Phelan A, Aints A, *et al*. Intercellular delivery of thymidine kinase prodrug activating enzyme by the herpes simplex virus protein, VP22. *Gene Ther* 1999; **6**: 12–21.
60. Feldman AL, Restifo NP, Alexander HR, *et al*. Antiangiogenic gene therapy of cancer utilizing a recombinant adenovirus to elevate systemic endostatin levels in mice. *Cancer Res* 2000; **60**: 1503–6.
61. Wadman M. NIH under fire over gene-therapy trials. *Nature* 2000; **403**: 237.
62. Wolff JA, Malone RW, Williams P, *et al*. Direct gene transfer into mouse muscle *in vivo*. *Science* 1990; **247**: 1465–8.
63. Hickman MA, Malone RW, Lehmann-Bruinsma K, *et al*. Gene expression following direct injection of DNA into liver. *Hum Gene Ther* 1994; **5**: 1477–83.
64. Yang JP, Huang L. Direct gene transfer to mouse melanoma by intratumor injection of free DNA. *Gene Ther* 1996; **3**: 542–8.
65. Barry ME, Pinto-Gonzalez D, Orson FM, McKenzie GJ, Petry GR, Barry MA. Role of endogenous endonucleases and tissue site in transfection and CpG-mediated immune activation after naked DNA injection. *Hum Gene Ther* 1999; **10**: 2461–80.
66. Niven R, Pearlman R, Wedeking T, *et al*. Biodistribution of radiolabeled lipid–DNA complexes and DNA in mice. *J Pharm Sci* 1998; **87**: 1292–9.
67. Houk BE, Hochhaus G, Hughes JA. Kinetic modeling of plasmid DNA degradation in rat plasma. *PharmSci* 1999; **1**: http://mc.pharmsci.org/manuscripts/Houk003/frame_composed.html.
68. Zhang G, Vargo D, Budker V, Armstrong N, Knechtle S, Wolff JA. Expression of naked plasmid DNA injected into the afferent and efferent vessels of rodent and dog livers. *Hum Gene Ther* 1997; **8**: 1763–72.
69. Liu F, Song Y, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther* 1999; **6**: 1258–66.
70. 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**: 1735–7.
71. Hirano T, Fujimoto J, Ueki T, *et al*. Persistent gene expression in rat liver *in vivo* by repetitive transfections using hvj-liposome. *Gene Ther* 1998; **5**: 459–64.
72. Fraser R, Bowler LM, Day WA, Dobbs B, Johnson HD, Lee D. High perfusion pressure damages the sieving ability of sinusoidal endothelium in rat livers. *Br J Exp Pathol* 1980; **61**: 222–8.
73. Budker V, Zhang G, Danko I, Williams P, Wolff J. The efficient expression of intravascularly delivered DNA in rat muscle. *Gene Ther* 1998; **5**: 272–6.
74. Huebner S, Battersby BJ, Grimm R, Cevc G. Lipid–DNA complex formation: reorganization and rupture of lipid vesicles in the presence of DNA as observed by cryoelectron microscopy. *Biophys J* 1999; **76**: 3158–66.
75. Salditt T, Koltover I, Radler JO, Safinya CR. Two-dimensional smectic ordering of linear DNA chains in self-assembled DNA–cationic liposome mixtures. *Phys Rev Lett* 1997; **79**: 2582–5.
76. Radler JO, Koltover I, Salditt T, Safinya CR. Structure of DNA–cationic liposome complexes: DNA intercalation in multilamellar membranes in distinct interhelical packing regimes. *Science* 1997; **275**: 810–4.
77. Koltover I, Salditt T, Radler JO, Safinya CR. An inverted hexagonal phase of cationic liposome–DNA complexes related to DNA release and delivery. *Science* 1998; **281**: 78–81.
78. Koltover I, Salditt T, Safinya CR. Phase diagram, stability, and overcharging of lamellar cationic lipid–DNA self-assembled complexes. *Biophys J* 1999; **77**: 915–24.
79. Felgner PL, Gadek TR, Holm M, *et al*. Lipofection—a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci USA* **1987; 84**: 7413–7.

80. Felgner PL, Ringold GM. Cationic liposome-mediated transfection. *Nature* 1989; **337**: 387–8.
81. Zabner J. Cationic lipids used in gene transfer. *Adv Drug Del Rev* 1997; **27**: 17–28.
82. Mahato RI, Rolland A, Tomlinson E. Cationic lipid-based gene delivery systems: pharmaceutical perspectives. *Pharm Res* 1997; **14**: 853–9.
83. Li S, Huang L. Nonviral gene therapy: promises and challenges. *Gene Ther* 2000; **7**: 31–4.
84. Lasic DD, Templeton NS. Liposomes in gene-therapy. *Adv Drug Del Rev* 1996; **20**: 221–66.
85. Gao X, Huang L. Cationic liposome-mediated gene transfer. *Gene Ther* 1995; **2**: 710–22.
86. Balasubramaniam RP, Bennett MJ, Aberle AM, Malone JG, Nantz MH, Malone RW. Structural and functional-analysis of cationic transfection lipids—the hydrophobic domain. *Gene Ther* 1996; **3**: 163–72.
87. Kwoh DY, Coffin CC, Lollo CP, *et al*. Stabilization of poly-L-lysine/DNA polyplexes for *in vivo* gene delivery to the liver. *Biochim Biophys Acta* 1999; **1444**: 171–90.
88. Lucas P, Milroy DA, Thomas BJ, Moss SH, Pouton CW. Pharmaceutical and biological properties of poly(amino acid)/DNA polyplexes. *J Drug Target* 1999; **7**: 143–56.
89. Plank C, Tang MX, Wolfe AR, Szoka Jr FC. Branched cationic peptides for gene delivery: role of type and number of cationic residues in formation and *in vitro* activity of DNA polyplexes. *Hum Gene Ther* 1999; **10**: 319–32.
90. Harbottle RP, Cooper RG, Hart SL, *et al*. An RGD-oligolysine peptide: a prototype construct for integrin-mediated gene delivery. *Hum Gene Ther* 1998; **9**: 1037–47.
91. Erbacher P, Roche AC, Monsigny M, Midoux P. The reduction of the positive charges of polylysine by partial gluconoylation increases the transfection efficiency of polylysine/DNA complexes. *Biochim Biophys Acta* 1997; **1324**: 27–36.
92. Wadhwa MS, Collard WT, Adami RC, McKenzie DL, Rice KG. Peptide-mediated gene delivery: influence of peptide structure on gene expression. *Bioconjug Chem* 1997; **8**: 81–8.
93. Brown MD, Schätzlein AG, Brownlie A, *et al*. Preliminary characterisation of novel amino acid based polymeric vesicles as gene delivery agents. *Bioconjug Chem* 2000; **11**: 880–91.
94. Tang MX, Szoka FC. The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes. *Gene Ther* 1997; **4**: 823–32.
95. Dash PR, Read ML, Barrett LB, Wolfert MA, Seymour LW. Factors affecting blood clearance and *in vivo* distribution of polyelectrolyte complexes for gene delivery. *Gene Ther* 1999; **6**: 643–50.
96. Wolfert MA, Schacht EH, Toncheva V, Ulbrich K, Nazarova O, Seymour LW. Characterization of vectors for gene therapy formed by self-assembly of DNA with synthetic block co-polymers. *Hum Gene Ther* 1996; **7**: 2123–33.
97. Katayose S, Kataoka K. Water-soluble polyion complex associates of DNA and poly(ethylene glycol)–poly(L-lysine) block copolymer. *Bioconjug Chem* 1997; **8**: 702–7.
98. Katayose S, Kataoka K. Remarkable increase in nuclease resistance of plasmid DNA through supramolecular assembly with poly(ethylene glycol)–poly(L-lysine) block copolymer. *J Pharm Sci* 1998; **87**: 160–3.
99. Toncheva V, Wolfert MA, Dash PR, *et al*. Novel vectors for gene delivery formed by self-assembly of DNA with poly(L-lysine) grafted with hydrophilic polymers. *Biochim Biophys Acta* 1998; **1380**: 354–68.
100. Robinson P, Hawtrey A, Ariatti M, Govender P, Jagganath D. Effect of polyethylene glycol conjugated to DNA-transfecting complexes targeted at the transferrin receptor of hela cells. *Drug Del* 1997; **4**: 115–9.
101. Choi YH, Liu F, Kim JS, Choi YK, Park JS, Kim SW. Polyethylene glycol-grafted poly-L-lysine as polymeric gene carrier. *J Controll Rel* 1998; **54**: 39–48.
102. Dash PR, Read ML, Fisher KD, *et al*. Decreased binding to proteins and cells of polymeric gene delivery vectors surface modified with a multivalent hydrophilic polymer and retargeting through attachment of transferrin. *J Biol Chem* 2000; **275**: 3793–802.
103. Zhou XH, Klibanov AL, Huang L. Lipophilic polylysines mediate efficient DNA transfection in mammalian-cells. *Biochim Biophys Acta* 1991; **1065**: 814.
104. Zhou XH, Huang L. DNA transfection mediated by cationic liposomes containing lipopolylysine—characterization and mechanism of action. *Biochim Biophys Acta* 1994; **1189**: 195–203.
105. Kim JS, Kim BI, Maruyama A, Akaike T, Kim SW. A new non-viral DNA delivery vector: the terplex system. *J Control Rel* 1998; **53**: 175–82.
106. Coll JL, Combaret V, Metchler K, *et al*. *In vitro* targeting and specific transfection of human neuroblastoma cells by CHCE7 antibody-mediated gene transfer. *Gene Ther* 1997; **4**: 156–61.
107. Shewring L, Collins L, Lightman SL, Hart S, Gustafsson K, Fabre JW. A nonviral vector system for efficient gene transfer to corneal endothelial cells via membrane integrin. *Transplantation* 1997; **64**: 763–9.
108. Pouton CW, Lucas P, Thomas BJ, Uduehi AN, Milroy DA, Moss SH. Polycation–DNA complexes for gene delivery: a comparison of the biopharmaceutical properties of cationic polypeptides and cationic lipids. *J Control Rel* 1998; **53**: 289–99.
109. Sosnowski BA, Gonzalez AM, Chandler LA, Buechler YJ, Pierce GF, Baird A. Targeting DNA to cells with basic fibroblast growth factor (FGF2). *J Biol Chem* 1996; **271**: 33647–53.
110. Curiel DT, Agarwal S, Wagner E, Cotten M. Adenovirus enhancement of transferrin polylysine-mediated gene delivery. *Proc Natl Acad Sci USA* 1991; **88**: 8850–4.
111. 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**: 6099–103.
112. Wolfert MA, Seymour LW. Chloroquine and amphipathic peptide helices show synergistic transfection *in vitro*. *Gene Ther* 1998; **5**: 409–14.
113. Midoux P, Monsigny M. Efficient gene transfer by histidylated polylysine/pDNA complexes. *Bioconjug Chem* 1999; **10**: 406–11.

114. Wu GY, Wu CH. Receptor-mediated *in vitro* gene transformation by a soluble DNA carrier system. *J Biol Chem* 1987; **262**: 4429–32.
115. Wu CH, Wilson JM, Wu GY. Targeting genes: delivery and persistent expression of a foreign gene driven by mammalian regulatory elements *in vivo*. *J Biol Chem* 1989; **264**: 169857.
116. Cotten M, Langle-Rouault F, Kirlappos H, *et al*. Transferrin-polycation-mediated introduction of DNA into human leukemic cells: stimulation by agents that affect the survival of transfected DNA or modulate transferrin receptor levels. *Proc Natl Acad Sci USA* 1990; **87**: 4033–7.
117. Mahato RI, Takemura S, Akamatsu K, Nishikawa M, Takakura Y, Hashida M. Physicochemical and disposition characteristics of antisense oligonucleotides complexed with glycosylated poly(L-lysine). *Biochem Pharmacol* 1997; **53**: 887–95.
118. Fajac I, Briand P, Monsigny M, Midoux P. Sugar-mediated uptake of glycosylated polylysines and gene transfer into normal and cystic fibrosis airway epithelial cells. *Hum Gene Ther* 1999; **10**: 395–406.
119. Ziady AG, Ferkol T, Gerken T, Dawson DV, Perlmutter DH, Davis PB. Ligand substitution of receptor targeted DNA complexes affects gene transfer into hepatoma cells. *Gene Ther* 1998; **5**: 1685–97.
120. Schaffer DV, Lauffenburger DA. Optimization of cell surface binding enhances efficiency and specificity of molecular conjugate gene delivery. *J Biol Chem* 1998; **273**: 28004–9.
121. Xu B, Wiehle S, Roth JA, Cristiano RJ. The contribution of poly-L-lysine, epidermal growth factor and streptavidin to EGF/pll/DNA polyplex formation. *Gene Ther* 1998; **5**: 1235–43.
122. Zauner W, Ogris M, Wagner E. Polylysine-based transfection systems utilizing receptor-mediated delivery. *Adv Drug Del Rev* 1998; **30**: 97–113.
123. Hong K, Zheng W, Baker A, Papahadjopoulos D. Stabilization of cationic liposome–plasmid DNA complexes by polyamines and poly(ethylene glycol)–phospholipid conjugates for efficient *in vivo* gene delivery. *FEBS Letters* 1997; **400**: 233–7.
124. Nishikawa M, Takemura S, Takakura Y, Hashida M. Targeted delivery of plasmid DNA to hepatocytes *in vivo*: optimization of the pharmacokinetics of plasmid DNA/galactosylated poly(L-lysine) complexes by controlling their physicochemical properties. *J Pharmacol Exp Ther* 1998; **287**: 408–15.
125. Hashida M, Takemura S, Nishikawa M, Takakura Y. Targeted delivery of plasmid DNA complexed with galactosylated poly(L-lysine). *J Control Rel* 1998; **53**: 301–10.
126. Hisayasu S, Miyauchi M, Akiyama K, Gotoh T, Satoh S, Shimada T. *In vivo* targeted gene transfer into liver cells mediated by a novel galactosyl-D-lysine/D-serine copolymer. *Gene Ther* 1999; **6**: 689–93.
127. Boussif O, Lezoualc'h F, Zanta MA, *et al*. A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. *Proc Natl Acad Sci USA* 1995; **92**: 7297–301.
128. Suh J, Paik HJ, Hwang BK. Ionization of poly(ethylenimine) and poly(allylamine) at various pHs. *Bioorg Chem* 1994; **22**: 318–27.
129. Godbey W, Wu K, Hirasaki G, Mikos A. Improved packing of poly(ethylenimine)/DNA complexes increases transfection efficiency. *Gene Ther* 1999; **6**: 1380–8.
130. Diebold SS, Kursa M, Wagner E, Cotten M, Zenke M. Mannose polyethylenimine conjugates for targeted DNA delivery into dendritic cells. *J Biol Chem* 1999; **274**: 19087–94.
131. Goula D, Benoist C, Mantero S, Merlo G, Levi G, Demeneix BA. Polyethylenimine-based intravenous delivery of transgenes to mouse lung. *Gene Ther* 1998; **5**: 1291–5.
132. 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.
133. Li S, Tan Y, Viroonchatapan E, Pitt BR, Huang L. Targeted gene delivery to pulmonary endothelium by anti-PECAM antibody. *Am J Physiol* 2000; **278**: L504–11.
134. Plank C, Mechtler K, Szoka Jr FC, Wagner E. Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. *Hum Gene Ther* 1996; **7**: 1437–46.
135. Zanta MA, Boussif O, Adib A, Behr JP. *In vitro* gene delivery to hepatocytes with galactosylated polyethylenimine. *Bioconjug Chem* 1997; **8**: 839–44.
136. Bettinger T, Remy JS, Erbacher P. Size reduction of galactosylated PEI/DNA complexes improves lectin-mediated gene transfer into hepatocytes. *Bioconjug Chem* 1999; **10**: 558–61.
137. Diebold SS, Lehrmann H, Kursa M, Wagner E, Cotten M, Zenke M. Efficient gene delivery into human dendritic cells by adenovirus polyethylenimine and mannose polyethylenimine transfection. *Hum Gene Ther* 1999; **10**: 775–86.
138. Erbacher P, Remy JS, Behr JP. Gene transfer with synthetic virus-like particles via the integrin-mediated endocytosis pathway. *Gene Ther* 1999; **6**: 138–45.
139. Kircheis R, Kichler A, Wallner G, *et al*. Coupling of cell-binding ligands to polyethylenimine for targeted gene delivery. *Gene Ther* 1997; **4**: 409–18.
140. Abdallah B, Hassan A, Benoist C, Goula D, Behr JP, Demeneix BA. A powerful nonviral vector for *in vivo* gene transfer into the adult mammalian brain: polyethylenimine. *Hum Gene Ther* 1996; **7**: 1947–54.
141. Goula D, Remy JS, Erbacher P, *et al*. Size, diffusibility and transfection performance of linear PEI/DNA complexes in the mouse central nervous system. *Gene Ther* 1998; **5**: 712–7.
142. Lemkine GF, Goula D, Becker N, Paleari L, Levi G, Demeneix BA. Optimisation of polyethylenimine-based gene delivery to mouse brain. *J Drug Target* 1999; **7**: 305–12.
143. Boletta A, Benigni A, Lutz J, Remuzzi G, Soria MR, Monaco L. Nonviral gene delivery to the rat kidney with polyethylenimine. *Hum Gene Ther* 1997; **8**: 1243–51.
144. Ferrari S, Moro E, Pettenazzo A, Behr JP, Zacchello F, Scarpa M. Exgen 500 is an efficient vector for gene delivery to lung epithelial cells *in vitro* and *in vivo*. *Gene Ther* 1997; **4**: 1100–6.

145. Godbey WT, Wu KK, Mikos AG. Size matters: molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle. *J Biomed Mater Res* 1999; **45**: 268–75.
146. Bragonzi A, Boletta A, Biffi A, *et al*. Comparison between cationic polymers and lipids in mediating systemic gene delivery to the lungs. *Gene Ther* 1999; **6**: 1995–2004.
147. Coll JL, Chollet P, Brambilla E, Desplanques D, Behr JP, Favrot M. *In vivo* delivery to tumors of DNA complexed with linear polyethylenimine. *Hum Gene Ther* 1999; **10**: 1659–66.
148. Goula D, Becker N, Lemkine GF, *et al*. Rapid crossing of the pulmonary endothelial barrier by polyethylenimine/DNA complexes. *Gene Ther* 2000; **7**: 499–504.
149. Nguyen HK, Lemieux P, Vinogradov SV, *et al*. Evaluation of polyether–polyethyleneimine graft copolymers as gene transfer agents. *Gene Ther* 2000; **7**: 126–38.
150. Mahato RI. Non-viral peptide-based approaches to gene delivery. *J Drug Target* 1999; **7**: 249–68.
151. Wilke M, Fortunati E, Van DenBroek M, Hoogeveen AT, Scholte BJ. Efficacy of a peptide-based gene delivery system depends on mitotic activity. *Gene Ther* 1996; **3**: 1133–42.
152. Colin M, Harbottle RP, Knight A, *et al*. Liposomes enhance delivery and expression of an RGD-oligolysine gene transfer vector in human tracheal cells. *Gene Ther* 1998; **5**: 1488–98.
153. Murphy JE, Uno T, Hamer JD, Cohen FE, Dwarki V, Zuckermann RN. A combinatorial approach to the discovery of efficient cationic peptoid reagents for gene delivery. *Proc Natl Acad Sci USA* 1998; **95**: 1517–22.
154. Hart S, Collins L, Gustafsson K, Fabre J. Integrin-mediated transfection with peptides containing arginine–glycine–aspartic acid domains. *Gene Ther* 1997; **4**: 1225–30.
155. Huang CY, Uno T, Murphy JE, *et al*. Lipitoids–novel cationic lipids for cellular delivery of plasmid DNA *in vitro*. *Chem Biol* 1998; **5**: 345–54.
156. Schwartz B, Ivanov MA, Pitard B, *et al*. Synthetic DNA-compacting peptides derived from human sequence enhance cationic lipid-mediated gene transfer *in vitro* and *in vivo*. *Gene Ther* 1999; **6**: 282–92.
157. Mislick KA, Baldeschwieler JD. Evidence for the role of proteoglycans in cation-mediated gene transfer. *Proc Natl Acad Sci USA* 1996; **93**: 12349–54.
158. Yang JP, Huang L. Overcoming the inhibitory effect of serum on lipofection by increasing the charge ratio of cationic liposome to DNA. *Gene Ther* 1997; **4**: 950–60.
159. Li S, Rizzo MA, Bhattacharya S, Huang L. Characterization of cationic lipid–protamine–DNA (LPD) complexes for intravenous gene delivery. *Gene Ther* 1998; **5**: 930–7.
160. Crook K, Stevenson BJ, Dubouchet M, Porteous DJ. Inclusion of cholesterol in DOTAP transfection complexes increases the delivery of DNA to cells *in vitro* in the presence of serum. *Gene Ther* 1998; **5**: 137–43.
161. Semple SC, Chonn A, Cullis PR. Influence of cholesterol on the association of plasma proteins with liposomes. *Biochemistry* 1996; **35**: 2521–5.
162. Oja CD, Semple SC, Chonn A, Cullis PR. Influence of dose on liposome clearance: critical role of blood proteins. *Biochim Biophys Acta* 1996; **1281**: 31–7.
163. Zelphati O, Uyechi LS, Barron LG, Szoka Jr FC. Effect of serum components on the physico-chemical properties of cationic lipid/oligonucleotide complexes and on their interactions with cells. *Biochim Biophys Acta* 1998; **1390**: 119–33.
164. Wang J, Guo X, Xu Y, Barron L, Szoka Jr FC. Synthesis and characterization of long chain alkyl acyl carnitine esters. Potentially biodegradable cationic lipids for use in gene delivery. *J Med Chem* 1998; **41**: 2207–15.
165. Oupicky D, Konak C, Dash PR, Seymour LW, Ulbrich K. Effect of albumin and polyanion on the structure of DNA complexes with polycation containing hydrophilic nonionic block. *Bioconjug Chem* 1999; **10**: 764–72.
166. Yang JP, Huang L. Time-dependent maturation of cationic liposome–DNA complex for serum resistance. *Gene Ther* 1998; **5**: 380–7.
167. Kikuchi A, Aoki Y, Sugaya S, *et al*. Development of novel cationic liposomes for efficient gene transfer into peritoneal disseminated tumor. *Hum Gene Ther* 1999; **10**: 947–55.
168. McLean JW, Fox EA, Baluk P, *et al*. Organ-specific endothelial cell uptake of cationic liposome–DNA complexes in mice. *Am J Physiol Heart Circ Physiol* 1997; **42**: H387–404.
169. Jain RK. Delivery of molecular and cellular medicine to solid tumors. *Adv Drug Del Rev* 1997; **26**: 71–90.
170. Hashizume H, Baluk P, Morikawa S, *et al*. Openings between defective endothelial cells explain tumor vessel leakiness. *Am J Pathol* 2000; **156**: 1363–80.
171. Griesenbach U, Chonn A, Cassady R, *et al*. Comparison between intratracheal and intravenous administration of liposome–DNA complexes for cystic fibrosis lung gene therapy. *Gene Ther* 1998; **5**: 181–8.
172. Liu F, Qi H, Huang L, Liu D. Factors controlling the efficiency of cationic lipidmediated transfection *in vivo* via intravenous administration. *Gene Ther* 1997; **4**: 517–23.
173. Hofland HE, Nagy D, Liu JJ, *et al*. *In vivo* gene transfer by intravenous administration of stable cationic lipid/DNA complex. *Pharm Res* 1997; **14**: 742–9.
174. Zhu N, Liggitt D, Liu Y, Debs R. Systemic gene expression after intravenous DNA delivery into adult mice. *Science* 1993; **261**: 209–11.
175. Maeda H. The tumor blood vessel as an ideal target for macromolecular anticancer agents. *J Control Rel* 1992; **19**: 315–24.
176. Yuan F, Leunig M, Huang SK, Berk DA, Papahadjopoulos D, Jain RK. Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft. *Cancer Res* 1994; **54**: 3352–6.
177. Gabizon A, Isacson R, Libson E, *et al*. Clinical-studies of liposome-encapsulated doxorubicin. *Acta Oncol* 1994; **33**: 779–786.
178. Vasey PA, Kaye SB, Morrison R, *et al*. Phase I clinical and pharmacokinetic study of PK I [*n*-(2-hydroxypropyl)-methacrylamide copolymer doxorubicin]: first member of a new class of chemotherapeutic agents–drug–polymer conjugates. Cancer Research Campaign Phase I/II Committee. *Clin Cancer Res* 1999; **5**: 83–94.

179. Monsky WL, Fukumura D, Gohongi T, *et al*. Augmentation of transvascular transport of macromolecules and nanoparticles in tumors using vascular endothelial growth factor. *Cancer Res* 1999; **59**: 4129–35.
180. Thurston G, McLean JW, Rizen M, *et al*. Cationic liposomes target angiogenic endothelial cells in tumors and chronic inflammation in mice. *J Clin Invest* 1998; **101**: 1401–13.
181. Osaka G, Carey K, Cuthbertson A, *et al*. Pharmacokinetics, tissue distribution, and expression efficiency of plasmid [^{33}P]DNA following intravenous administration of DNA/cationic lipid complexes in mice: use of a novel radionuclide approach. *J Pharm Sci* 1996; **85**: 612–8.
182. Song YK, Liu DX. Free liposomes enhance the transfection activity of DNA/lipid complexes *in vivo* by intravenous administration. *Biochim Biophys Acta* 1998; **1372**: 141–50.
183. Song YK, Liu F, Liu D. Enhanced gene expression in mouse lung by prolonging the retention time of intravenously injected plasmid DNA. *Gene Ther* 1998; **5**: 1531–7.
184. Barron LG, Meyer KB, Szoka FC. Effects of complement depletion on the pharmacokinetics and gene delivery mediated by cationic lipid DNA complexes. *Hum Gene Ther* 1998; **9**: 315–23.
185. Hillery E, Cheng S, Geddes D, Alton E. Effects of altering dosing on cationic liposome-mediated gene transfer to the respiratory epithelium. *Gene Ther* 1999; **6**: 1313–6.
186. Barron LG, Gagne L, Szoka Jr FC. Lipoplex-mediated gene delivery to the lung occurs within 60 minutes of intravenous administration. *Hum Gene Ther* 1999; **10**: 1683–94.
187. Hoet PH, Nemery B. Polyamines in the lung: polyamine uptake and polyamine-linked pathological or toxicological conditions. *Am J Physiol* 2000; **278**: L417–33.
188. Morgan DM. Uptake of polyamines by human endothelial cells. Characterization and lack of effect of agonists of endothelial function. *Biochem J* 1992; **286**: 413–7.
189. Hoet PH, Dinsdale D, Lewis CP, Verbeken EK, Lauweryns JM, Nemery B. Kinetics and cellular localisation of putrescine uptake in human lung tissue. *Thorax* 1993; **48**: 1235–41.
190. Sokol PP, Longenecker KL, Kachel DL, Martin WJd. Mechanism of putrescine transport in human pulmonary artery endothelial cells. *J Pharmacol Exp Ther* 1993; **265**: 60–6.
191. Blume G, Cevc G. Liposomes for the sustained drug release *in vivo*. *Biochim Biophys Acta* 1990; **1029**: 91–7.
192. Kircheis R, Schuller S, Brunner S, *et al*. Polycation-based DNA complexes for tumor-targeted gene delivery *in vivo*. *J Gene Med* 1999; **1**: 111–20.
193. Simoes S, Slepushkin V, Gaspar R, de Lima MC, Duzgunes N. Gene delivery by negatively charged ternary complexes of DNA, cationic liposomes and transferrin or fusigenic peptides. *Gene Ther* 1998; **5**: 955–64.
194. Simoes S, Slepushkin V, Pires P, Gaspar R, de Lima MP, Duzgunes N. Mechanisms of gene transfer mediated by lipoplexes associated with targeting ligands or pH-sensitive peptides. *Gene Ther* 1999; **6**: 1798–807.
195. Templeton NS, Lasic DD, Frederik PM, Strey HH, Roberts DD, Pavlakis GN. Improved DNA: liposome complexes for increased systemic delivery and gene expression. *Nat Biotechnol* 1997; **15**: 647–52.
196. Eastman JS, Scheule RK. Cationic lipid:DNA complexes for the treatment of cystic fibrosis. *Curr Opin Mol Ther* 1999; **1**: 186–96.
197. Marshall J, Yew NS, Eastman JS, Jiang C, Scheule RK, Cheng SH, Cationic lipid-mediated gene delivery to the airways. In: Hung M-c, Huang L, Wagner E, eds. *Nonviral vectors for gene therapy*. San Diego, CA: Academic Press 1999.
198. Sawa Y, Kaneda Y, Bai HZ, *et al*. Efficient transfer of oligonucleotides and plasmid DNA into the whole heart through the coronary artery. *Gene Ther* 1998; **5**: 1472–80.
199. Zou L, Huang L, Hayes R, *et al*. Liposome-mediated NGF gene transfection following neuronal injury: Potential therapeutic applications. *Gene Ther* 1999; **6**: 994–1005.
200. Lukacs KV, Nakakes A, Atkins CJ, Lowrie DB, Colston MJ. *In vivo* gene therapy of malignant tumours with heat shock protein-65 gene. *Gene Ther* 1997; **4**: 346–50.
201. Namiki Y, Takahashi T, Ohno T. Gene transduction for disseminated intraperitoneal tumor using cationic liposomes containing non-histone chromatin proteins: cationic liposomal gene therapy of carcinomatosa. *Gene Ther* 1998; **5**: 240–6.
202. Hottiger MO, Dam TN, Nickoloff BJ, Johnson TM, Nabel GJ. Liposome-mediated gene transfer into human basal cell carcinoma. *Gene Ther* 1999; **6**: 1929–35.
203. Mendiratta SK, Quezada A, Matar M, *et al*. Intratumoral delivery of IL-12 gene by polyvinyl polymeric vector system to murine renal and colon carcinoma results in potent antitumor immunity. *Gene Ther* 1999; **6**: 833–9.
204. Goldman CK, Soroceanu L, Smith N, *et al*. *In vitro* and *in vivo* gene delivery mediated by a synthetic polycationic amino polymer. *Nat Biotechnol* 1997; **15**: 462–6.
205. Lai LW, Moeckel GW, Lien YH. Kidney-targeted liposome-mediated gene transfer in mice. *Gene Ther* 1997; **4**: 426–31.
206. Baranov A, Glazkov P, Kiselev A, *et al*. Local and distant transfection of MDX muscle fibers with dystrophin and *lacZ* genes delivered *in vivo* by synthetic microspheres. *Gene Ther* 1999; **6**: 1406–14.
207. Coonrod A, Li FQ, Horwitz M. On the mechanism of DNA transfection: efficient gene transfer without viruses. *Gene Ther* 1997; **4**: 1313–21.
208. 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**: 18997–9007.
209. Tseng WC, Haselton FR, Giorgio TD. Transfection by cationic liposomes using simultaneous single cell measurements of plasmid delivery and transgene expression. *J Biol Chem* 1997; **272**: 25641–7.
210. Capecchi MR. High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. *Cell* 1980; **22**: 479–88.

211. Pires P, Simoes S, Nir S, Gaspar R, Duzgunes N, Pedroso de Lima MC. Interaction of cationic liposomes and their DNA complexes with monocytic leukemia cells. *Biochim Biophys Acta* 1999; **1418**: 71–84.
212. Takeuchi K, Ishihara M, Kawaura C, Noji M, Furuno T, Nakanishi M. Effect of zeta potential of cationic liposomes containing cationic cholesterol derivatives on gene transfection. *FEBS Lett* 1996; **397**: 207–9.
213. Pollard H, Remy JS, Loussouarn G, Demolombe S, Behr JP, Escande D. Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. *J Biol Chem* 1998; **273**: 7507–11.
214. Xu Y, Szoka Jr FC. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry* 1996; **35**: 5616–23.
215. Wrobel I, Collins D. Fusion of cationic liposomes with mammalian cells occurs after endocytosis. *Biochim Biophys Acta* 1995; **1235**: 296–304.
216. El Ouahabi A, Thiry M, Pector V, Fuks R, Ruysschaert JM, Vandenbranden M. The role of endosome destabilizing activity in the gene transfer process mediated by cationic lipids. *FEBS Lett* 1997; **414**: 187–92.
217. Boussif O, Zanta MA, Behr JP. Optimized galenics improve *in-vitro* gene-transfer with cationic molecules up to 1000-fold. *Gene Ther* 1996; **3**: 1074–80.
218. van de Wetering P, Moret EE, Schuurmans-Nieuwenbroek NM, van Steenbergen MJ, Hennink WE. Structure–activity relationships of water-soluble cationic methacrylate/methacrylamide polymers for nonviral gene delivery. *Bioconjug Chem* 1999; **10**: 589–97.
219. Meunier-Durmort C, Picart R, Ragot T, Perricaudet M, Hainque B, Forest C. Mechanism of adenovirus improvement of cationic liposome-mediated gene transfer. *Biochim Biophys Acta* 1997; **1330**: 8–16.
220. Meunier-Durmort C, Grimal H, Sachs LM, Demeneix BA, Forest C. Adenovirus enhancement of polyethylenimine-mediated transfer of regulated genes in differentiated cells. *Gene Ther* 1997; **4**: 808–14.
221. Dunphy EJ, Redman RA, Herweijer H, Cripe TP. Reciprocal enhancement of gene transfer by combinatorial adenovirus transduction and plasmid DNA transfection *in vitro* and *in vivo*. *Hum Gene Ther* 1999; **10**: 2407–17.
222. Kaneda Y, Uchida T, Kim J, Ishiura M, Okada Y. The improved efficient method for introducing macromolecules into cells using HVJ (sendai virus) liposomes with gangliosides. *Exp Cell Res* 1987; **173**: 56–69.
223. Ponimaskin E, Bareesel KK, Markgraf K, *et al*. Sendai virosomes revisited: reconstitution with exogenous lipids leads to potent vehicles for gene transfer. *Virology* 2000; **269**: 391–403.
224. Wagner E, Plank C, Zatloukal K, Cotten M, Birnstiel ML. Influenza-virus hemagglutinin-HA-2 N-terminal fusogenic peptides augment gene-transfer by transferrin polylysine DNA complexes—toward a synthetic virus-like gene-transfer vehicle. *Proc Natl Acad Sci USA* 1992; **89**: 7934–7938.
225. Schoen P, Chonn A, Cullis PR, Wilschut J, Scherrer P. Gene transfer mediated by fusion protein hemagglutinin reconstituted in cationic lipid vesicles. *Gene Ther* 1999; **6**: 823–32.
226. Mechtler K, Wagner E. Gene transfer mediated by influenza virus peptides: the role of peptide sequences. *New J Chem* 1997; **21**: 105–11.
227. Lechardeur D, Sohn KJ, Haardt M, *et al*. Metabolic instability of plasmid DNA in the cytosol: A potential barrier to gene transfer. *Gene Ther* 1999; **6**: 482–97.
228. Ross GF, Bruno MD, Uyeda M, *et al*. Enhanced reporter gene expression in cells transfected in the presence of dmi-2, an acid nuclease inhibitor. *Gene Ther* 1998; **5**: 1244–50.
229. Zauner W, Brunner S, Buschle M, Ogris M, Wagner E. Differential behaviour of lipid based and polycation based gene transfer systems in transfecting primary human fibroblasts: a potential role of polylysine in nuclear transport. *Biochim Biophys Acta* 1999; **1428**: 57–67.
230. Vitiello L, Chonn A, Wasserman JD, Duff C, Worton RG. Condensation of plasmid DNA with polylysine improves liposome-mediated gene transfer into established and primary muscle cells. *Gene Ther* 1996; **3**: 396–404.
231. Chan CK, Jans DA. Enhancement of polylysine-mediated transferrinfection by nuclear localization sequences: polylysine does not function as a nuclear localization sequence. *Hum Gene Ther* 1999; **10**: 1695–702.
232. Utvik JK, Nja A, Gundersen K. DNA injection into single cells of intact mice. *Hum Gene Ther* 1999; **10**: 291–300.
233. Dowty ME, Williams P, Zhang G, Hagstrom JE, Wolff JA. Plasmid DNA entry into postmitotic nuclei of primary rat myotubes. *Proc Natl Acad Sci USA* 1995; **92**: 4572–6.
234. Tseng W, Purvis NB, Haselton FR, Giorgio TD. Cationic liposomal delivery of plasmid to endothelial cells measured by quantitative flow cytometry. *Biotechnol Bioeng* 1996; **50**: 548–54.
235. Fasbender A, Zabner J, Zeiher BG, Welsh MJ. A low rate of cell proliferation and reduced DNA uptake limit cationic lipid-mediated gene transfer to primary cultures of ciliated human airway epithelia. *Gene Ther* 1997; **4**: 1173–80.
236. Mortimer I, Tam P, MacLachlan I, Graham RW, Saravolac EG, Joshi PB. Cationic lipid-mediated transfection of cells in culture requires mitotic activity. *Gene Ther* 1999; **6**: 403–11.
237. 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**: 401–7.
238. Darquet AM, Rangara R, Kreiss P, *et al*. Minicircle: an improved DNA molecule for *in vitro* and *in vivo* gene transfer. *Gene Ther* 1999; **6**: 209–18.
239. Kreiss P, Cameron B, Rangara R, *et al*. Plasmid DNA size does not affect the physicochemical properties of lipoplexes but modulates gene transfer efficiency. *Nucleic Acids Res* 1999; **27**: 3792–8.
240. Pante N, Aebi U. Molecular dissection of the nuclear pore complex. *Crit Rev Biochem Mol Biol* 1996; **31**: 153–99.
241. Ludtke JJ, Zhang G, Sebestyen MG, Wolff JA. A nuclear localization signal can enhance both the nuclear transport and expression of 1 kb DNA. *J Cell Sci* 1999; **112**: 2033–41.
242. Stewart M. Nuclear pore structure and function. *Semin Cell Biol* 1992; **3**: 267–77.
243. Feldherr CM, Akin D. EM visualization of nucleocytoplasmic transport processes. *Electron Microsc Rev* 1990; **3**: 73–86.

244. Darquet AM, Cameron B, Wils P, Scherman D, Crouzet J. A new DNA vehicle for nonviral gene delivery: supercoiled minicircle. *Gene Ther* 1997; **4**: 1341–9.
245. Leahy P, Carmichael GG, Rossomando EF. Transcription from plasmid expression vectors is increased up to 14-fold when plasmids are transfected as concatemers. *Nucleic Acids Res* 1997; **25**: 449–50.
246. Boulikas T. Putative nuclear-localization signals (NLS) in protein transcription factors. *J Cell Biochem* 1994; **55**: 32–58.
247. Aronsohn AI, Hughes JA. Nuclear localization signal peptides enhance cationic liposome-mediated gene therapy. *J Drug Target* 1998; **5**: 163–9.
248. Branden LJ, Mohamed AJ, Smith CI. A peptide nucleic acid–nuclear localization signal fusion that mediates nuclear transport of DNA. *Nat Biotechnol* 1999; **17**: 784–7.
249. Sebestyen MG, Ludtke JJ, Bassik MC, *et al*. DNA vector chemistry: The covalent attachment of signal peptides to plasmid DNA. *Nat Biotechnol* 1998; **16**: 80–5.
250. Neves C, Escriou V, Byk G, Scherman D, Wils P. Intracellular fate and nuclear targeting of plasmid DNA. *Cell Biol Toxicol* 1999; **15**: 193–202.
251. Zanta MA, Belguise-Valladier P, Behr JP. Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. *Proc Natl Acad Sci USA* 1999; **96**: 91–6.
252. Godbey WT, Wu KK, Mikos AG. Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. *Proc Natl Acad Sci USA* 1999; **96**: 5177–81.
253. Dean DA. Import of plasmid DNA into the nucleus is sequence specific. *Exp Cell Res* 1997; **230**: 293–302.
254. Dean DA, Dean BS, Muller S, Smith LC. Sequence requirements for plasmid nuclear import. *Exp Cell Res* 1999; **253**: 713–22.
255. Vacik J, Dean B, Zimmer W, Dean D. Cell-specific nuclear import of plasmid DNA. *Gene Ther* 1999; **6**: 1006–14.
256. Collas P, Husebye H, Alestrom P. The nuclear localization sequence of the SV40 T antigen promotes transgene uptake and expression in Zebrafish embryo nuclei. *Transgenic Res* 1996; **5**: 451–8.
257. Dachs GU, Patterson AV, Firth JD, *et al*. Targeting gene expression to hypoxic tumor cells. *Nat Med* 1997; **3**: 515–20.
258. Sarvesvaran J, Going JJ, Milroy R, Kaye SB, Keith WN. Is small cell lung cancer the perfect target for anti-telomerase treatment? *Carcinogenesis* 1999; **20**: 1649–51.
259. Wolff JA, Ludtke JJ, Acsadi G, Williams P, Jani A. Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. *Hum Mol Genet* 1992; **1**: 363–9.
260. Davis HL, Millan CL, Watkins SC. Immune-mediated destruction of transfected muscle fibers after direct gene transfer with antigen-expressing plasmid DNA. *Gene Ther* 1997; **4**: 181–8.
261. Stripecke R, Carmen Villacres M, Skelton D, Satake N, Halene S, Kohn D. Immune response to green fluorescent protein: implications for gene therapy. *Gene Ther* 1999; **6**: 1305–12.
262. Vitiello L, Bockhold K, Joshi PB, Worton RG. Transfection of cultured myoblasts in high serum concentration with DODAC:DOPE liposomes. *Gene Ther* 1998; **5**: 1306–13.
263. Battersby BJ, Grimm R, Huebner S, Cevc G. Evidence for three-dimensional interlayer correlations in cationic lipid–DNA complexes as observed by cryo-electron microscopy. *Biochim Biophys Acta* 1998; **1372**: 379–83.

(Received 13 January 2001; revised form accepted 20 January 2001)