Advances in Gene, Molecular and Cell Therapy ©2007 Global Science Books

Overcoming Barriers to Achieve Safe, Sustained and Efficient Non-Viral Gene Therapy

Dominic J. Glover[1] • Ljudmila Glouchkova[2] • Hans J. Lipps[2] • David A. Jans[1,3*]

[1] Nuclear Signalling Laboratory, Department of Biochemistry and Molecular Biology, Monash University, Victoria 3800, Australia
[2] Institute of Cell Biology, Witten/Herdecke University, 58448 Witten, Germany
[3] Australian Research Council Centre of Excellence for Biotechnology and Development, Australia

Corresponding author: * **David.Jans@med.monash.edu.au**

ABSTRACT

Adverse events in using viral-based vectors to deliver and integrate therapeutic DNA into patients in recent clinical trials have revitalized efforts to improve the efficacy of the alternative, the much safer non-viral vectors. Non-viral vectors are generally orders of magnitude less efficient than viral counterparts due their inability to overcome numerous biological barriers to DNA delivery, but deeper understanding of these barriers, and formulation of new approaches to transcend them, has enabled the generation of non-viral vectors for DNA delivery that comprise multiple functional modules mimicking the ability of viruses to traffic through the cell to the nucleus. These novel, multifunctional vectors may be combined with strategies for episomal replication and faithful expression of therapeutic DNA bereft of the potential dangers of integration into the host genome, with enormous potential for future clinical as well as research applications.

Keywords: gene therapy, non-viral gene delivery, stable expression, episomal replication, site-specific integration

CONTENTS

INTRODUCTION

Successful treatment of genetic diseases by gene therapy requires efficient delivery of therapeutic DNA into specific cells, followed by safe and long-term expression of the encoded gene product at physiologically relevant levels. In order for foreign genes (transgenes) to be expressed, they must reach the cell nucleus, but this journey requires numerous obstacles to be overcome. Limiting factors in therapeutic gene delivery can be extracellular, such as undesired interactions with blood plasma proteins in the circulatory system, with extracellular matrix proteins, and with cells that are not the intended delivery targets, or intracellular, such as entrapment in endosome/lysosome vesicles, degradation by nucleases, and slow movement through the dense cytoplasm, and through the nuclear envelope (NE) into the nucleus (**Fig. 1**). Once in the nucleus, the transgene must be replicated and passed onto daughter cells during each cell cycle, whilst evading transcriptional silencing e.g. by *de novo* cytosine methylation. Due to the difficulties in delivering transgenes and maintaining their stable expression, gene therapy has thus far been of limited medical benefit.

To circumvent the barriers to therapeutic DNA delivery, transgenes can be packaged into viruses, which have evolved mechanisms to exploit or circumvent cellular signalling and transport pathways to assist in overcoming cellular barriers during infection of mammalian cells. This ability to transfect cells actively, along with the propensity of some of them such as retroviruses to integrate transgenes into the host cell genome for persistent expression, has been exploited in viral-vector design, with recombinant vectors from diverse sources used in clinical trials with notable successes (Cavazzana-Calvo *et al*. 2000; Hacein-Bey-Abina *et al*. 2002; Gaspar *et al*. 2004). However, due to the invasive properties and tropisms of viruses, important biosafety issues have arisen that must be considered when using viral-based vectors in humans. Of most concern is the danger of insertional mutagenesis caused by ectopic chromosomal integration of viral DNA, which can disrupt the expression of a tumour-suppressor gene and/or activate a protooncogene

Received: 10 July, 2007. Accepted: 27 November, 2007.

Invited Review

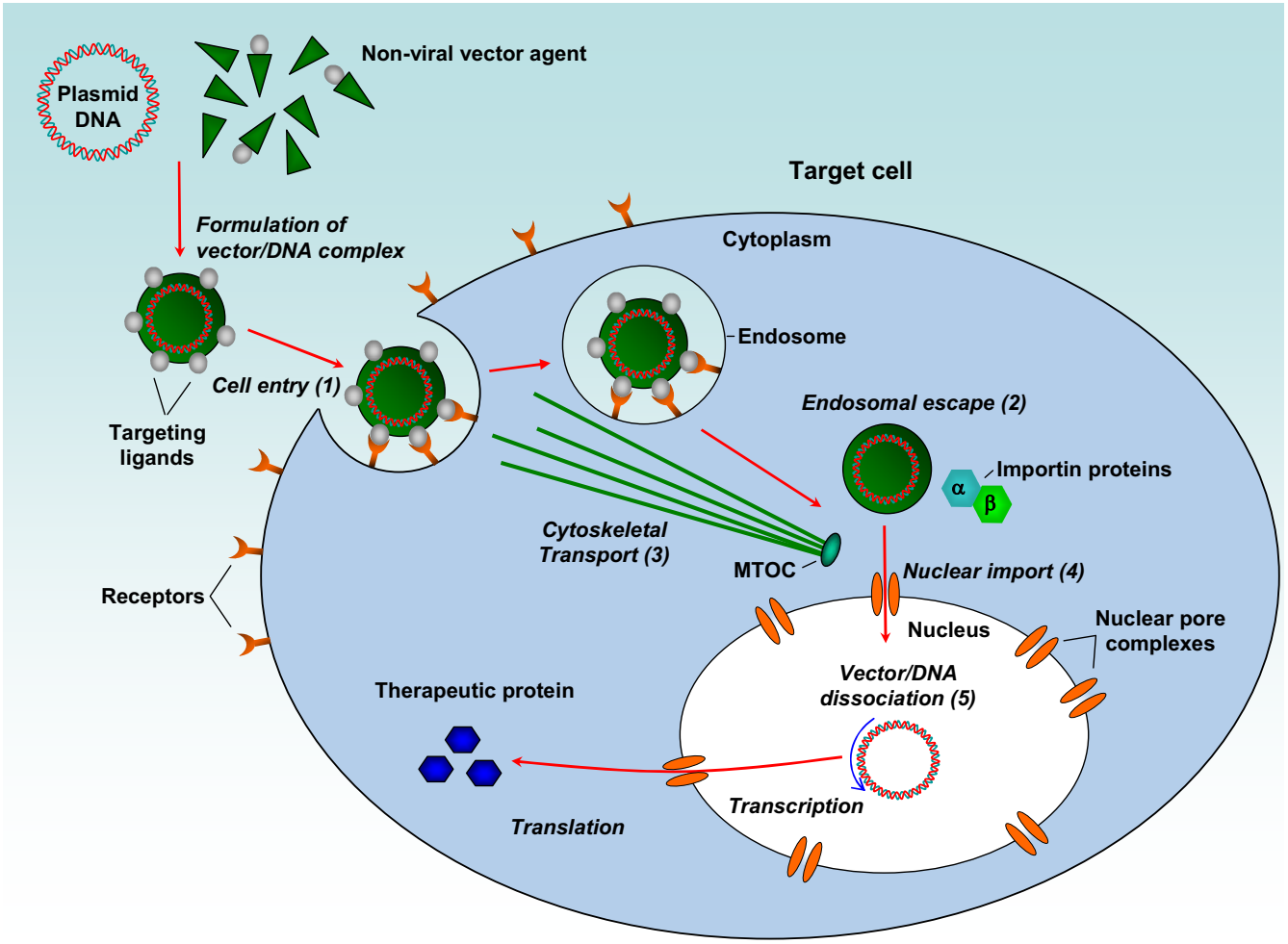

Fig. 1 The trafficking and intracellular barriers to non-viral gene delivery. A non-viral vector must reach the cell nucleus in order for its cargo, a therapeutic gene, to be expressed. Vectors must bind and traverse the cell membrane, generally through non-specific endocytosis, or if coupled with ligands, through internalisation by binding to specific receptors (1). Once inside the cell, the vector/DNA complex must escape from endosome vesicles (2), avoid degradation from cellular nucleases and proteases, and traffic through the structured cytoplasm (3), and enter the nucleus (4). The delivered gene must be unpackaged, transcribed and persistently maintained during cell replication (5).

(Woods *et al*. 2003). This problem was highlighted all too poignantly in a clinical trial to treat children with X-linked severe combined immunodeficiency (SCIDX1) caused by a lack of expression of the interleukin-2 receptor γ-chain gene (*IL2RG*) (Hacein-Bey-Abina *et al*. 2002). After treatment with a retroviral vector that effected expression of a complementing *IL2RG* transgene, three of 16 treated children developed a type of T-cell leukaemia, which has thus far resulted in the death of one patient (Hacein-Bey-Abina *et al*. 2003a, 2003b). It seems clear that the leukaemia was caused by insertional mutagenesis, whereby the retroviral vector used in this trial integrated close to, and exerted enhancer activity on the promoter of the distal growth-promoting LIM domain only 2 gene (*LMO2*) (McCormack *et al*. 2003; Woods *et al*. 2003; Nam and Rabbitts 2006; Shou *et al*. 2006). Activation of *LMO2*, a known protooncogene, is associated with T-cell tumorigenesis in childhood cancer (McCormack *et al*. 2003). In view of the other significant drawbacks of viral-based vectors, including their immunogenicity and cytotoxicity (Dewey *et al*. 1999; Hollon 2000), it seems clear that safer methods of gene transfer and sustained expression are required for human gene therapy.

Commonly used non-viral gene delivery agents include diverse lipid formulations and cationic polymers that bind to DNA and collapse its structure into nanosized particles to assist with cell binding and internalisation (**Fig. 2**). In contrast to viral-mediated gene delivery, non-viral vectors have a number of safety advantages, including reduced pathogenicity and diminished capacity for insertional mutagenesis. Furthermore, unlike live viruses, non-viral vectors are easier to manufacture, with less significant limitations in packaging large amounts of DNA. Unfortunately, the non-viral vectors currently in use are characterised by inefficient therapeutic DNA delivery, due to the fact that although particular non-viral vectors can condense DNA, confer cell internalisation, and in some cases promote endosomal escape, there are still many barriers to gene delivery that standard non-viral vectors are unable to overcome. In addition, the non-viral vector itself may encounter problems not normally encountered with viral vectors, such as instability and aggregation of the vector when complexed with DNA. The transgene expression obtained from non-viral vectors is usually transient, which can be sufficient for certain applications, such as the killing of cancer cells (Anderson *et al*. 2004), but for the treatment of inherited genetic diseases, the transgene needs to be expressed long-term. The latter is generally not achievable using non-viral vectors since the DNA delivered to the nucleus is not replicated and/or is lost during mitosis.

Considerable focus has been on improving the efficiency of non-viral vectors through structural modifications and inclusion of specific functional groups to either the carrier or the DNA molecule. These multifunctional vectors mimic key properties of viruses, overcoming the cellular barriers to DNA delivery through mechanisms analogous to those of viral vectors. This review examines the intracellular barriers to DNA delivery and transgene expression, and their impact on the design of novel non-viral vectors. Parallels will be drawn to virus-based gene delivery, with focus on the strategies employed by viruses and other

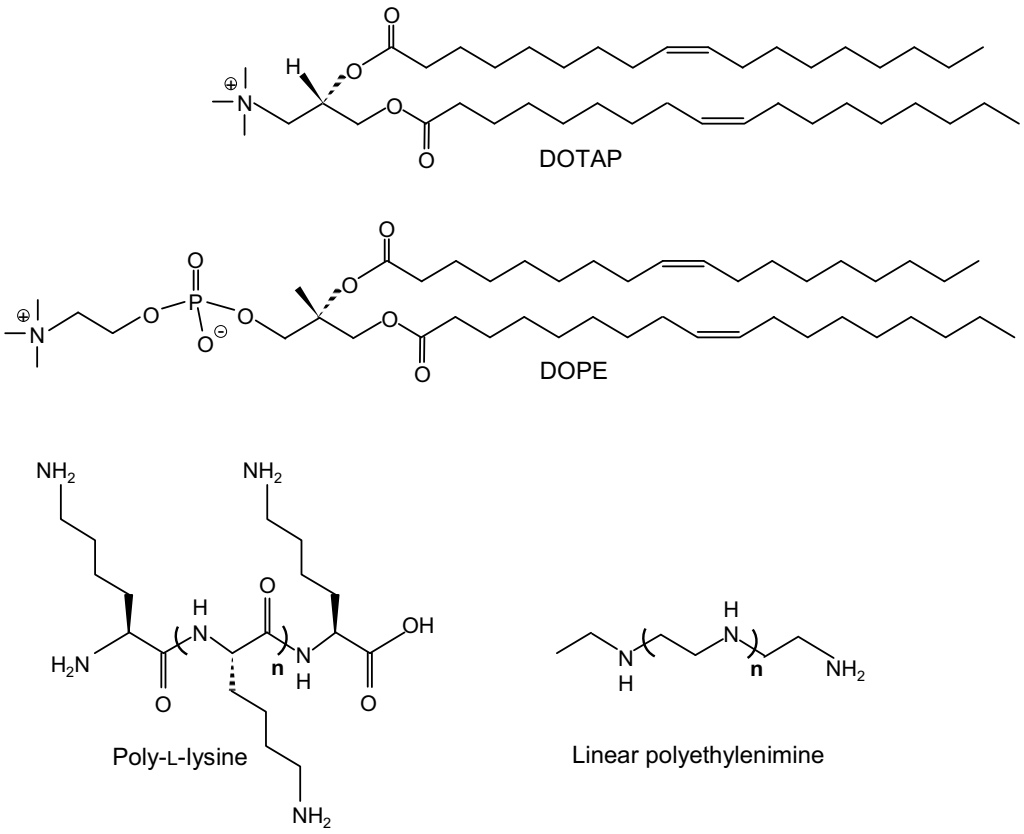

Fig. 2 Structure of commonly used cationic non-viral vectors for gene delivery. Liposome vectors are composed of cationic lipids such as DOTAP (1,2-dioleoyl-3-trimethylammonium propane), which are often mixed with neutral lipids such as DOPE (dioleoyl-phosphatidylethanolamine) to further enhance transfection efficiency. Polymers of cationic residues may be employed to condense and delivery DNA, including polylysine, and PEI (polyethylenimine).

agents such as bacterial toxins that interface with intracellular processes and actively traffic through cells. Importantly, since novel non-viral vectors can be "built from the ground up", they may be specificity engineered to be free of the safety problems that inherently hamper their viral counterparts. Furthermore, solutions compatible with non-viral vectors for obtaining safe and persistent transgene expression are discussed.

CELL BINDING AND INTERNALISATION

Complexes formed between commonly used non-viral vectors (**Fig. 2**) and DNA are highly charged foreign particles that interact strongly with blood plasma proteins, and the innate and acquired immune system of the patient. This results in rapid opsonisation and removal of non-viral vectors from the blood circulation after intravenous administration, thereby limiting the opportunity for cellular internalisation. To prevent non-specific interactions and extend circulation half-life, the vector can be shielded from the extracellular environment by attachment of hydrophilic molecules such as polyethylene glycol (PEG) (Lee *et al*. 2002; Moffatt *et al*. 2006), biodegradable agents such as polyhydroxyethyl L-asparagine/L-glutamine (Metselaar *et al*. 2003), or by encapsulating the vector either in a lipid vesicle (Mastrobattista *et al*. 2001), or in amphiphilic "block" copolymers, which have a linear architecture comprised of a hydrophilic segment covalently jointed to a hydrophobic segment, and which in aqueous solution can encapsulate DNA into a cell-membrane-like vesicle that is thicker, less permeable, and more stable than a lipid vesicle (Richard *et al*. 2005; Chang *et al*. 2007). Generally, unshielded highly positive vector/DNA complexes enter cells through non-specific endocytic mechanisms including macropinocytosis, phagocytosis, and possibly through simple charge interactions with cellular networks of polyanions (Zuhorn *et al*. 2002; Kueltzo and Middaugh 2003). Therefore, an added benefit of shielding charged complexes is the reduction in uptake by undesired cells. Furthermore, ligands or antibodies can be coupled with the hydrophilic surface modifications of the complex to promote robust cell-specific attachment and receptor-mediated uptake.

A wide variety of targeting ligands, including full-length proteins (Ogris *et al*. 2003; Kakudo *et al*. 2004), vitamins (Hattori and Maitani 2005), carbohydrates (Tang *et al*. 2007), and hormones (Rosenkranz *et al*. 1992, 2003), have been used for targeted DNA and drug delivery (see **Table 1**). Some ligands themselves may assist in reducing surface charge of complexes thereby reducing non-specific uptake (Kakudo *et al*. 2004). Monoclonal antibodies have also been used to effect cell-specific DNA delivery when conjugated to vectors (Chiu *et al*. 2004; Ikegami *et al*. 2006) or through inclusion in multidomain protein vectors in the form of a recombinant fusion of a single chain variable fragment (scFv) of the variable regions of the heavy and light chains of an immunoglobulin (Uherek *et al*. 1998; Li *et al*. 2001). Two or more different ligands or antibodies may be attached to a single vector to create heterovalent-ligand vector constructs capable of binding to multiple receptors (see Handl *et al*. (2004) for review). Heterovalent constructs appear to bind with increased affinity compared to their corresponding monomers, and can exhibit specificity for target cells that highly express a combination of two receptor types when untargeted cells at most express only one of the two receptors (Caplan and Rosca 2005). Such highly specific targeting may be particularly useful for cancer therapy in delivering cytotoxic drugs/genes. Heterovalent ligands are useful for targeting surface antigens that are attractive targets, but poor in terms of being internalised (Zhang *et al*. 2004). In this case, one ligand/antibody would target against a highly specific receptor, whilst a second ligand/antibody binds an internalizable receptor. Tan *et al*. (2003), for example linked liposomes to an antibody specific to E-selectin to confer endothelial-tissue selectivity, and a second antibody specific for the transferrin receptor to offer rapid internalisation of the liposome. As an alternative to delivering molecules to single cells, physical methods of delivery can be applied at the tissue level, with the aim being to create transient permeabilization of cell membranes through which vector/DNA complexes may enter cells. Such techniques include particle bombardment (Walther *et al*. 2004), hydrodynamic pressure (Tsoulfas *et al*.

Table 1 Desired properties and components of non-viral vectors to enhance cell-specific gene delivery.

Component	Function	Examples
Nucleic acid carrier	Binding and condensation of nucleic acid into defined and ordered structures. Protection of DNA from nuclease.	Poly-L-lysine (Chan and Jans 1999; Chan *et al*. 2000; Lee *et al*. 2002) Cationic lipids (Farhood *et al*. 1995) Polyethylenimine (Sonawane *et al*. 2003) Protamine (Li *et al*. 2001; Masuda *et al*. 2005) Histones (Balicki *et al*. 2002; Wagstaff *et al*. 2007) Transcription factors (Binding only) (Paul *et al*. 1997; Chan *et al*. 1998; Uherek *et al*. 1998; Chan and Jans 2001)
Surface modification	Extracellular stability and inactivity	PEG (Lee *et al*. 2002) PHEA/PHEG (Metselaar *et al*. 2003) Lipid bilayer (Mastrobattista *et al*. 2001)
Ligand / antibody	Cell-specific binding and internalization	Full-length proteins e.g. transferrin (Ogris *et al*. 2003; Kakudo *et al*. 2004) Vitamins e.g. folate (Hattori and Maitani 2005) Carbohydrates e.g. mannose (Tang *et al*. 2007) Hormones e.g. insulin (Rosenkranz *et al*. 1992; Rosenkranz *et al*. 2003) Monoclonal antibodies e.g. Herceptin (Chiu *et al*. 2004; Ikegami *et al*. 2006) Recombinant ScFv antibodies e.g. anti-ErbB2 scFv (Uherek *et al*. 1998; Li *et al*. 2001)
Endosomolytic group	Disruption of endosome to allow escape of vector/DNA into cytoplasm	Polyethylenimine (Sonawane *et al*. 2003) Helper lipids (e.g. DOPE) (Farhood *et al*. 1995; Simberg *et al*. 2001) Fusiogenic proteins e.g. Influenza HA-2 (Uherek *et al*. 1998; Gaur *et al*. 2002; Lee *et al*. 2002; Navarro-Quiroga *et al*. 2002; Rittner *et al*. 2002; Futaki *et al*. 2005) Protonatable peptides (Midoux and Monsigny 1999; Kichler *et al*. 2003)
Microtubule-association sequence	Cytoplasmic trafficking towards the cell nucleus	NFκB p50 protein (Mesika *et al*. 2005) Possible DNA sequences e.g. transcription factor binding sites (Vaughan and Dean 2006)
Nuclear localization sequence (NLS)	Conferring recognition by the cellular nuclear import machinery for active transport into the nucleus	T-ag NLS ($PKKKRKV^{132}$) (Zanta *et al*. 1999) opT-NLS ($SSDDEATADAQHAA^{124}PPKKKRKVEDP^{135}$) (Chan and Jans 1999; Zanta *et al*. 1999; Chan *et al*. 2000) M9 NLS (GNQSSNFGPMKGCNFGGRSSGPYGGGGQYFAKPRNQGGY) (Subramanian *et al*. 1999; Byrnes *et al*. 2002; Harmon *et al*. 2003; Bremner *et al*. 2004) Histones (Balicki *et al*. 2002; Wagstaff *et al*. 2007) Protamine (RSQSRSRYYRQRQRSRRRRRR) (Masuda *et al*. 2005)

2006), ultrasound (Rodriquez-Porcel *et al*. 2005), and *in vivo* electroporation using electrodes specially designed to deliver electric pulses that open 'pores' in membranes (Matsuda *et al*. 2007).

ENDOSOMAL ESCAPE

After entry into the cell by endocytosis, the vector/DNA complex must exit from the endosome to escape degradation in the lysosomal pathway. A number of strategies have been successfully employed to enhance release of DNA from endosomes. One of the first methods used was chloroquine, an endosomotropic agent that accumulates in endosomes, buffering the pH and promoting the release of vectors such as polylysine (Cheng *et al*. 2006). However, chloroquine and similar lysosomotropic reagents would be required at levels toxic to humans, and are therefore not amenable to *in vivo* human gene therapy. Viruses that enter cells by endocytosis have evolved sophisticated mechanisms that use pH-sensitive fusogenic proteins for endosome escape. For example, the hemagglutinin subunit HA-2 from the influenza virus undergoes a conformational change in the low pH endosome milieu to interact with and perturb the endosomal membrane (Han *et al*. 2001). Coupling of fusogenic peptides such as the HA-2 subunit to polycation vectors augments gene transfer, and thereby provides an alternative to endosomotropic agents (Navarro-Quiroga *et al*. 2002). Other fusogenic sequences that have been exploited to elicit endosome escape of non-viral vectors include the translocation domains of the diphtheria (Uherek *et al*. 1998) and anthrax toxins (Gaur *et al*. 2002), or from designed amphipathic sequences (Rittner *et al*. 2002) such as GALA (Futaki *et al*. 2005) or KALA (Lee *et al*. 2002) (see **Table 1**).

Liposomes are often formulated to include fusogenic lipids such as DOPE (**Fig. 2**) which fuse to and destabilize the lipid bilayer of the endosomal membrane (Farhood *et al*. 1995; Simberg *et al*. 2001). An alternative to the inclusion of fusogenic lipids into liposomes, is the entrapment of fusogenic proteins within the liposome, which can function to disrupt the endosomal vesicle and possibly the liposome itself (Mastrobattista *et al*. 2002; Kakudo *et al*. 2004). Endosomes may be disrupted by non-viral vectors in an indirect manner, as exemplified by PEI polymers that contain many titratable amine groups which become protonated in the low pH of endosomes, creating a passive chloride influx, in turn causing osmotic swelling and rupture of the endosome (Sonawane *et al*. 2003). The proton sponge activity of PEI can be mimicked using peptides with histidine residues either positioned in the core of the peptide, or substituted onto polylysine, with protonation of imidazole groups of the histidine residues occurring in the slightly acidic endosomal milieu (Midoux and Monsigny 1999; Kichler *et al*. 2003). A novel approach has recently been reported where a cationic peptide is used in conjunction with a light-inducible anionic phthalocyanine dendrimer, that upon 689-nm irradiation produces photochemical destabilization of endosome membranes; this results in >100-fold enhancement of transgene expression (Nishiyama *et al*. 2005).

TRAFFICKING THROUGH THE CYTOPLASM

Diffusion of large molecules is limited in the crowded and highly structured milieu of the cytoplasm, thereby potentially preventing therapeutic DNA in the cytoplasm from reaching the nucleus. The diffusional mobility of DNA microinjected into the cytoplasm is size dependent, with small DNA fragments <250 bp able to diffuse widely in the cytoplasm, whilst plasmid DNA >2 kb is unable to diffuse freely from the site of injection (Lukacs *et al*. 2000; Shimizu *et al*. 2005). This size-dependent reduction of cytoplasmic diffusion is not solely due to macromolecular crowding (Dauty and Verkman 2004; Sanabria *et al*. 2007); instead, the presence of the highly dense cytoskeleton causes sterical hindrances in the mobility of large molecules, as shown by the greatly enhanced diffusion of large molecules including plasmid DNA after disruption or reorganization of the cytoskeleton (Dauty and Verkman 2005; Shimizu *et al*.

2005; Geiger *et al*. 2006).

In the context of normal intracellular trafficking, endogenous proteins, organelles, and vesicles are transported along the cytoskeletal network, in an analogous fashion to a rail system. For example, the nuclear-acting cancer suppressor proteins p53 (Giannakakou *et al*. 2000), retinoblastoma protein $p110^{Rb}$ (Roth *et al*. 2007), and parathyroid hormone related protein (PTHrP) (Lam *et al*. 2002) are transported along microtubule (MT) components of the cytoskeleton towards the nucleus. MTs are long, hollow cylinders made of tubulin that extend from the vicinity of the plasma membrane to the MT organizing centre (MTOC), a structure that is typically in close proximity to the nucleus (**Fig. 1**) (Caviston and Holzbaur 2006). Most MT transport towards the MTOC is catalyzed by the molecular motor dynein, whereas molecules may be moved away from the MTOC along MTs by the motor kinesin.

Non-viral vectors can also be trafficked in MT-dependent fashion, although the mechanism(s) are poorly understood. The primary mode of transport appears to be mediated by the natural endocytic mechanism of the cell (Soldati and Schliwa 2006). Whilst still within endosomes, both liposome and PEI vector/DNA complexes appear to be rapidly transported through the cell along MTs dependent on the action of dynein (Hasegawa *et al*. 2001; Suh *et al*. 2003; Kulkarni *et al*. 2005). This endosomal trafficking of vector/DNA complexes is dependent on MTs, since MT depolymerization through nocodazole treatment abolishes movement of the complexes (Suh *et al*. 2003; Kulkarni *et al*. 2006). Although it has been reported that the endosomal trafficking of PEI/DNA complexes is directed towards the perinuclear region (Suh *et al*. 2003), over a longer time course, the movement appears to be a back-and-forth motion about their starting positions (Kulkarni *et al*. 2005, 2006), most likely as a result of switching between dynein- or kinesin directed transport (Kural *et al*. 2005); this ultimately ends when the complex exits the endosome. The endocytic transport of PEI/DNA is comparable to viral exploitation of the endocytic machinery during infection; the influenza virus, for example, is rapidly moved in endosomes by dynein to the perinuclear region, prior to an intermittent back and forth movement in the perinuclear region (Lakadamyali *et al*. 2003). After acidification of the endosome in the perinuclear region, the influenza virus envelope fuses with the endosomal membrane to release viral genes into the cytoplasm adjacent to the nucleus (Lakadamyali *et al*. 2003).

In a similar fashion, non-viral vectors that are trafficked in endosomes towards the MTOC and released after endosome acidification may end up in the cytoplasm close to the nucleus, thereby increasing the propensity of the non-viral vector to enter the nucleus. It should be noted, however, that endocytic transport of viral and non-viral vectors towards the MTOC most likely coincides with trafficking to lysosomes (Hasegawa *et al*. 2001) or possibly aggresomes (Garcia-Mata *et al*. 2002), resulting in degradation of the vector and DNA. In this regard, it has been suggested that the bidirectional MT-dependent transport of endosome-entrapped viruses might be advantageous in avoiding degradation whilst trafficking towards the nucleus (Dohner *et al*. 2005); bidirectional MT-dependent transport may confer a similar benefit to non-viral vectors. Alternatively, it has recently been reported that naked plasmid DNA in the cytoplasm may be trafficked along MTs through the action of adaptor proteins that bind both the DNA and dynein (Vaughan and Dean 2006). At this stage, the identity of the adaptor proteins and mechanism whereby DNA is linked to dynein is unknown, as is whether this MT trafficking is specific to certain DNA sequences. It is known that viruses use specific protein sequences for interaction with dynein to promote transport to the nucleus (Dohner *et al*. 2005; Radtke *et al*. 2006). An intriguing possibility is therefore to attach dynein-association sequences directly to non-viral vectors or DNA, to enhance cytoplasmic transport to the perinuclear region.

TRANSPORT INTO THE NUCLEUS

The NE that encapsulates the nucleus is an effective barrier to gene delivery (Capecchi 1980; Graessmann *et al*. 1989; Pollard *et al*. 1998). To enter the nucleus, molecules must pass through nuclear pore complexes (NPCs), which are multimeric structures that straddle the inner and outer membranes of the NE, with a central channel of 9 nm (Goldberg and Allen 1992) that prevents molecules >45 kDa from passively diffusing into the nucleus. In the case of naked DNA, fragments <300 bp can passively diffuse into the nucleus (Ludtke *et al*. 1999; Lukacs *et al*. 2000); since this is larger than the upper molecular weight limit for passive nuclear entry, it is thought that these linear DNA fragments "thread" their way through the NPC (Salman *et al*. 2001). Molecules of DNA >300 bp, even when condensed by a non-viral vector, are too large to passively diffuse through the NPC and are excluded from the nucleus, except when cells are undergoing mitosis (Ludtke *et al*. 1999, 2000). During cell division, the NE is temporarily disassembled and DNA-vector complexes can be sequestered within the daughter cell nuclei (Fasbender *et al*. 1997; Tseng *et al*. 1999; Grosse *et al*. 2006). This is the basis of the ease with which conventional non-viral vectors transfect rapidly dividing immortalised cell lines, but show only poor transfection rates in non-dividing cells (Fasbender *et al*. 1997). Since efficient transfection of slow dividing or terminally differentiated cells is required for *in vivo* human gene therapy of genetic diseases, there is considerable interest in improving the nuclear import efficiency of non-viral vectors.

Classically, proteins that normally localize to the nucleus possess a specific targeting signal called a nuclear localization sequence (NLS) (Jans *et al*. 2000; Quimby and Corbett 2001; Pemberton and Paschal 2005), typically a short cluster of basic amino acids recognised by members of the importin (Imp) superfamily of proteins, either directly by Imp β or one of the many homologs thereof, or indirectly through the Imp α component of the Imp α/β heterodimer (Jans *et al*. 2000). Imp β confers docking to the NPC for energy-dependent transport of the protein into the nucleus, and mediates release within the nucleus of the NLS-containing cargo upon interaction with the monomeric guanine nucleotide binding protein Ran, in activated GTP-bound form. In order to transfect non-dividing cells, viruses have evolved to make use of the above nuclear import pathway through the inclusion of NLSs on viral proteins bound to the viral genome. For example, the immunodeficiency virus type 1 (HIV-1) enzyme integrase (IN) has recently been shown to bind and transport plasmid DNA into the nucleus of mechanically perforated cells (Hearps and Jans 2006). This efficient import of DNA is conferred by an NLS located within IN, which confers recognition by the Imp α/β heterodimer and subsequent transport through the NPC.

Numerous studies have attempted to enhance nuclear import of non-viral vectors through the addition of an NLS to the non-viral vector. For much of this work, the NLS of choice has been the minimal NLS ($PKKKRKV^{132}$) of the simian virus SV40 large tumour antigen (T-ag). The minimal T-ag NLS has been shown to enhance reporter gene expression when conjugated or complexed directly to the DNA molecule or the agent used to compact DNA. In one study, a T-ag NLS peptide (T-ag residues 126-135) conjugated to the end of a linear DNA fragment condensed with a cationic lipid or PEI, was shown to increase expression 1000-fold of a reporter gene, with enhancement abolished when the NLS was replaced with a non-functional mutant form (Zanta *et al*. 1999). A similar approach linking the minimal T-ag NLS (amino acids 126-132) to plasmid DNA increased nuclear accumulation of the conjugated DNA in permeabilized cells but did not enhance transgene expression when microinjected into the nucleus (Sebestyen *et al*. 1998). This abolishment of transgene expression was most likely due to the >100 NLS peptides conjugated to the DNA molecule directly interfering with transcriptional activity of the transfected DNA (Ciolina *et al*. 1999).

The minimal T-ag NLS, however, is a relatively inefficient NLS. There is clear evidence that the inclusion of protein kinase CK2 phosphorylation sites N-terminal to the T-ag minimal NLS enhance recognition by Imp αβ by up to 100-fold (Rihs *et al*. 1991; Jans and Jans 1994; Hubner *et al*. 1997; Xiao *et al*. 1997, 1998), as long as the cyclin-dependent protein kinase site (T^{124}) flanking the NLS is rendered non-phosphorylatable by alanine substitution (Jans *et al*. 1991). The "optimized" T-ag NLS ("opT-NLS") constitutes T-ag amino acids 111-135, with CK2 sites present and functional, and cdk site non-functional (Akhlynina *et al*. 1997, 1999). When attached to a DNA compaction agent such as polylysine or histones, the opT-NLS is recognised with very high affinity by the nuclear import machinery, leading to a significant enhancement in both the nuclear delivery and expression of DNA (Chan and Jans 1999; Chan *et al*. 2000; Chan and Jans 2001; Wagstaff *et al*. 2007).

Positively charged NLSs can associate with DNA (Ritter *et al*. 2003), and since simple cations themselves can enhance transfection, appropriate methods must be used to demonstrate NLS function in enhancing gene delivery in a given experimental system. A useful method, for example, is quantitation of the direct binding of Imps to DNA-protein complexes to demonstrate functionality of NLS/mutated NLS sequences, thereby enabling the dependence of transfection enhancement on NLS function to be demonstrated (Chan and Jans 1999; Chan *et al*. 2000).

The M9 NLS sequence derived from the heterogeneous nuclear ribonucleoprotein A1 differs from the T-ag NLS in that M9 is recognised by importin β2 (transportin) rather than importin α/β. As an alternative to the T-ag NLS, M9 has been examined for use in nuclear DNA delivery (Subramanian *et al*. 1999; Byrnes *et al*. 2002a, 2002b; Bremner *et al*. 2004), whereby the M9 peptide, when bound to plasmid DNA, was able to increase reporter gene expression 63-fold in confluent non-dividing cells (Subramanian *et al*. 1999). Addition of the lectin wheat germ agglutinin, an inhibitor of NPC function, abolished nuclear import.

Nuclear-acting DNA-binding proteins such as chromatin components and transcription factors that contain intrinsic NLSs have been employed as vectors to both condense DNA and/or enhance its delivery to the nucleus in a "piggy back" mechanism. The GAL4 transcription factor in fusion with targeting ligands has been used to deliver plasmid DNA, containing the requisite GAL4 DNA-recognition motif, to cells expressing the appropriate cell surface receptors (Paul *et al*. 1997; Uherek *et al*. 1998), albeit with low efficiency. It was subsequently shown that for transcription factors such as GAL4, the NLS and DNA-binding domain overlap, leading to mutually exclusive binding between DNA and Imp proteins, and suboptimal nuclear import (Chan *et al*. 1998; Chan and Jans 2001). To overcome this, the nuclear import pathway of transcription factors may be "switched" by including an additional, heterologous NLS, such as the opT-NLS (Chan and Jans 2001).

Intriguingly, endogenous cellular transcription factors may facilitate the nuclear import of exogenous plasmids. Dean (1997, 1999) demonstrated that the SV40 enhancer, a DNA sequence rich in binding sites for transcription factors, could facilitate the nuclear import of plasmids. Most likely, a number of general cellular transcription factors bind their respective specific binding motifs (DBSs or DNA binding sites) within the SV40 enhancer, to result in protein-DNA complexes that can be recognised by Imps in order to be transported into the nucleus. The SV40 enhancer in this context can be seen as a sequence able to facilitate nuclear targeting of the DNA incorporating it. Other DNA sequences functioning analogously to the SV40 enhancer in this sense have been reported (Langle-Rouault *et al*. 1998; Mesika *et al*. 2001, 2005), some of which function in a cell-specific manner by the presence of promoter elements that are bound by transcription factors expressed exclusively in unique cell types. One such cell specific sequence for facilitating nuclear targeting of DNA is the promoter of the smooth muscle γ-actin (SMGA), whose ability to localize DNA to the nucleus is limited to smooth muscle cells (Vacik *et al*. 1999).

The natural capacity of histones to bind via simple charge interactions to DNA, combined with their predominant Imp β mediated nuclear import (Johnson-Saliba *et al*. 2000; Baake *et al*. 2001) has led to their application as non-viral vectors. The core histones H2A and H2B, as monomers or H2A/H2B dimers, are capable of nuclear import of DNA bound to them (Balicki *et al*. 2002; Wagstaff *et al*. 2007). Nuclear delivery may be further improved though the addition of the op-T- NLS to H2B, again through "switching" of the nuclear import pathway from Imp β to Imp α/β (Wagstaff *et al*. 2007). Interestingly, both H2A and H2B appear to have protein transduction ability, with transfection occurring through a non-endocytic and temperature independent route (Rosenbluh *et al*. 2005; Wagstaff *et al*. 2007). Thus, histones such as H2B engineered to have optimised nuclear targeting are excellent prospects for non-viral gene delivery approaches, in that they seem to possess, in addition to DNA condensing and efficient nuclear targeting properties, the ability to mediate entry into cells (Wagstaff *et al*. 2007), without the need for other agents such as liposomes, or dependent on cell surface receptor expression on the part of the target cells.

Highly basic polymers such as polylysine and protamine have been used as potential agents to enhance nuclear targeting when complexed with DNA due to the fact that they are highly basic, resembling typical NLS sequences (Vitiello *et al*. 1996; Sorgi *et al*. 1997; Masuda *et al*. 2005). Importantly, it has been shown that Imp recognition and nuclear import of polylysine is inhibited when the latter is complexed with DNA, indicating that polylysine does not function as a conventional NLS (Chan and Jans 1999). Protamine has been reported to improve nuclear delivery of bound DNA (Masuda *et al*. 2005); although we have observed that reduction of Imp binding to protamine occurs when the latter is bound to DNA (unpublished observation).

The need for an NLS may be bypassed through coupling of plasmids to the Imp β binding protein (IBB) derived from Imp α (Carrière *et al*. 2003). Noncovalently plasmid-bound IBB was able to mediate binding to Imp β, and increase transfection efficiency 20-fold of cationic lipid/DNA complexes. However, inclusion of an NLS onto non-viral vectors may have additional benefits besides nuclear gene import, as intriguingly, the NLS may also facilitate trafficking along MTs towards the nucleus (Mesika *et al*. 2005; Salman *et al*. 2005). Indeed, Imps have been shown to facilitate MT association of NLS-containing proteins, leading to active transport on the MT network towards the nucleus (Lam *et al*. 2002).

RELEASE OF DNA FROM NON-VIRAL VECTORS

Once inside the nucleus, the non-viral vector itself may present a barrier to expression, as the agent used to condense DNA could potentially interfere with access of the cellular transcription machinery to the promoter and thereby reduce or prevent transgene expression, as has been observed for DNA-bound cationic polymers and lipids *in vitro* (Schaffer *et al*. 2000; Prasad *et al*. 2003). However, premature release of the DNA from the vector may expose the DNA to enzymatic degradation before expression can occur (Lechardeur *et al*. 1999), so that an often overlooked requirement in non-viral gene delivery is the controlled dissociation or removal of the compaction agent. Intracellular separation of the non-viral vector and passenger DNA is thought to be as a result of competing electrostatic charge interaction between the DNA and compaction agent in the presence of various intracellular cations, anions, and DNA-binding proteins. In the case of liposomal-based vectors, DNA displacement from the vector is linked to endosomal escape, driven by the negatively charged phosphatidylserine lipids of the endosomal membrane which neutralize the charge of the cationic lipids in the liposomal formulation (Xu and Szoka 1996; Zelphati and Szoka 1996; Harvie *et al*. 1998;

Mui *et al*. 2000). The helper lipid DOPE (**Fig. 2**) in various liposomal formulations in particular appears to facilitate DNA release by partially neutralizing the positive charge of the cationic lipid through a salt bridge between the charged amine group of the cationic lipid and the negatively charged phosphate group of DOPE (Harvie *et al*. 1998; Hirsch-Lerner *et al*. 2005). That lipid dissociates from the DNA before import into the nucleus is in agreement with the observation from microinjection experiments that reporter gene expression is inhibited when bound to liposomes (Zabner *et al*. 1995; Pollard *et al*. 1998; Hama *et al*. 2006).

In contrast, polycation/DNA complexes appear to release from each other in the nucleus through exchange of the polycations in the complexes with the protein components of the surrounding chromatin (Cereghini and Yaniv 1984; Jeong and Stein 1994; Masuda *et al*. 2005), and perhaps by competitive interactions with cations such as spermine and spermidine (Pollard *et al*. 1998; Schaffer *et al*. 2000). The rate of polycation dissociation from DNA is dependent upon the degree of polymeration (i.e. the number of charged residues in the polycation), with shorter polycation chains dissociating faster than longer chains both *in vitro* and within the nucleus (Ziady *et al*. 1999; Balhorn *et al*. 2000; Schaffer *et al*. 2000; Brewer *et al*. 2002). The degree of polymerisation potentially influences the extent of the persistence of gene expression, with the use of longer chain polylysine molecules reported to result in more sustained expression of reporter transgenes than the use of shorter polylysines (Ziady *et al*. 1999), due to the slower release of DNA. The chemistry of the cationic polymer also appears to be able to influence DNA release, although the affinity for DNA of the binding interaction does not appear to be critical; arginine-rich polycations like protamine bind more tightly to DNA than lysine-rich polycations (Mascotti and Lohman 1997; Plank *et al*. 1999), but appear to dissociate more efficiently from DNA and consequently interfere less with transcription than polylysine vectors, as shown in nuclear microinjection experiments (Masuda *et al*. 2005). This suggests additional mechanisms other than competitive charge interactions may be involved in the removal of physiologically "natural" polycations such as protamine from plasmid DNA. In developing sperm, protamine functions to not only condense DNA, but also to repress gene expression completely (Wykes and Krawetz 2003; Vilfan *et al*. 2004), so that rapid removal of protamine after fertilization is required for gene expression, through the action of chromatin remodelling proteins that replace protamine on the paternal chromatin with histones (McLay and Clarke 2003). Although further work is required to establish the mechanism (Masuda *et al*. 2005), remodelling of introduced protamine/DNA vector complexes appears to occur in analogous fashion within the nucleus of somatic cells. Ultimately, deeper understanding of chromatin remodelling mechanisms should enable their considered application to non-viral vector design, and may prove central to achieving sustained transgene expression from introduced therapeutic DNAs.

MIMICKING VIRUSES TO ENHANCE NON-VIRAL VECTORS

Thus far, this review has focussed on functional modules and modifications that can be applied to non-viral vectors to improve their efficiency of transgene delivery (**Table 1**). Ultimately, all of the required functional modules would ideally be incorporated into a single vector, thereby allowing the vector to overcome sequentially all of the various cellular barriers encountered on its journey through the cell. Two approaches have been used to create multifunctional vectors, either by the assembly of the coding sequences of diverse protein domains in the same reading frame to give a single chimeric protein with modular functions, or through linking the various functional groups via different coupling chemistries (**Fig. 3**). Since in principle the domains of multifunctional vectors are modular, they may be tailored to specific applications such as the delivery/expression in distinct tissue and/or cell types, and perhaps permit easier transition between animal models and potential application in the clinic. Whilst the creation of such vectors requires considerable work, recent *in vivo* animal experiments provide

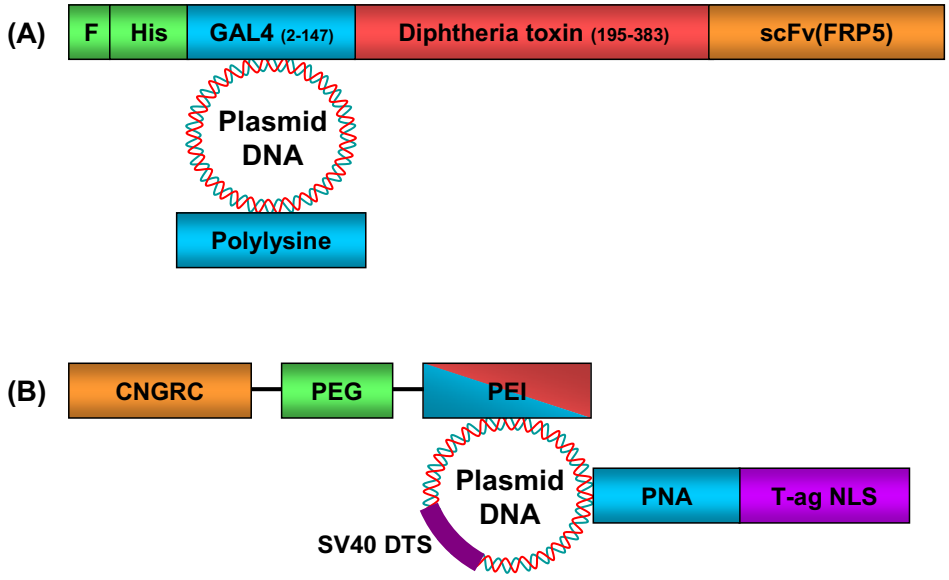

Fig. 3 Strategies for the construction of multifunctional non-viral vectors for gene delivery. (**A**) Modular protein vectors are constructed by combining functional domains from diverse organisms to be expressed as a single linear fusion protein. An example of such a modular vector is 'GD5' (Uherek *et al*. 1998), which consists of a DNA-binding domain from the yeast GAL4 transcription factor; a single-chain scFv antibody specific for the tumour-associated ErbB2/HER2 antigen; and the diphtheria toxin translocation domain for endosomal disruption in acidic pH. Bacterially expressed GD5 protein is bound to DNA that is then condensed with polylysine. Inclusion of additional domains including a polyhistidine (His) and a FLAG epitope (F) are useful for the purification and detection of the protein. (**B**) Alternatively, multifunctional vectors may be created by the coupling of synthetic molecules and peptides via different chemistries. In one such example, the CNGRC peptide specific for CD13-expressing tumour cells, and polyethylenimine (PEI) for DNA binding and endosome disruption, were linked together via a phenyl(di)boronic acid-salicylhydroxamic acid coupling strategy that included a polyethylene glycol (PEG) linker (Moffatt *et al*. 2006). To enhance nuclear import of the PEI/DNA complex, a peptide nucleic acid (PNA) containing the SV40 large T antigen nuclear localization sequence (T-ag NLS) was coupled to the DNA; in addition, the SV40 DNA nuclear targeting sequence (DTS) was cloned into the plasmid DNA. The individual domains are not depicted to scale; domains with similar function are identically coloured.

an optimistic outlook for their future in non-viral gene delivery (Moffatt *et al*. 2006). These novel multidomain/multifunctional vectors essentially mimic the ability of viruses to interact with and actively traffic though different compartments within cells. Importantly, they have clear safety advantages over viral-counterparts, and can allow the incorporation of additional synthetic moieties to expand the ability of the vector to transport diverse molecules/drugs to the nucleus beyond those compatible with viruses (Rosenkranz *et al*. 2003; Nishiyama *et al*. 2005).

Whilst transient transgene expression may be acceptable in some applications, such as targeted cancer gene therapy, the treatment of genetic defects requires the transgene to be expressed long-term at physiologically relevant levels. The simplest strategy to achieve persistent expression is to ensure stable retention of the desired transgene, and transfer thereof to daughter cells of that receiving the DNA in the first place, with mechanisms to prevent the silencing of transgene expression. Viruses exploit the host cell DNA replication mechanisms to either integrate their genome into that of the host cell (e.g. HIV-1) or are maintained in an episomal state (e.g. SV40, Herpes Simplex Virus - HSV – see below). Problems with respect to safety when transgenes are integrated into the host cell DNA (see above), and reproducibility in terms of the maintenance of stable transgene expression demonstrate the need for alternative strategies, highlighting the need for approaches that combine the safety and delivery advantages of multifunctional non-viral vectors, with the ability to maintain delivered DNA in a active form within the nucleus. In theory, non-viral, episomally-replicating plasmid DNA appears to be able to overcome most of the drawbacks of viral vector-based replication and integration.

REPLICATION OF DNA IN EUKARYOTIC CELLS

Replication of mammalian genomes is tightly regulated to ensure precision and minimize damage that would lead to cell death or genome instability (Gilbert 2002, 2004). Extrachromosomal vectors for use in gene therapy must be replicated with the same level of precision to obtain sustained gene expression in proliferating cells. Many of the basic biochemical mechanisms of DNA replication were first studied in prokaryotic cells such as *Escherichia coli*, where replication is generally initiated at a single site in the circular chromosome – the origin of replication (or replicator). A protein complex – the initiator – binds to specific sequences in the replicator and determines the timing and specificity of initiation. In contrast to prokaryotes, DNA replication in eukaryotes occupies only part of the cell cycle, the S phase, with each chromosome duplicated only once per cell cycle, producing two daughter chromosomes. To ensure the timely and precise replication of large eukaryote genomes, DNA synthesis in eukaryotic cells initiates at multiple origins distributed over the chromosome (*ca*. 60000 in human cells; (Hubermann and Riggs 1966). Establishment of replication origins and their subsequent selection for activation are the key steps that ensure efficient replication.

The first step, binding of the origin recognition complex (ORC) to chromosomal DNA occurs in G1 phase of the cell cycle. The ORC, consisting of six subunits (Orc1-6) that are conserved in all eukaryotes, recruits the additional initiation factors Cdc6p and Cdt1. Subsequently in late G1-phase, the ORC-Cdc6p-Cdt1 complex "loads" MCM(2-7) proteins onto chromatin, the resulting assembly being the pre-replication complex (pre-RC) that renders the region of the chromosome competent for replication. At the onset of S phase, selected replication origins are licensed for activation (Blow and Dutta 2005), whereby helicase activation and DNA replication itself are initiated by protein kinases such as cyclin-CDK and Cdc7-Dbf4 (DDK), which phosphorylate Cdc6p and the MCM proteins. This results in release of Cdc6p from the DNA to enable the single strand DNA-binding protein (RPA), together with Cdc45p, to bind to the origin of replication. This complex in turn is believed to activate helicase activity of MCM(2-7), followed by recruitment of the DNA polymerase and commencement of DNA synethesis. As S-phase proceeds, different regions of the genome are replicated at different times, according to a temporal and spatial program that is dictated by chromosome structure (Jackson and Pombo 1998; Gilbert 2002; Sadoni *et al*. 2004), and epigenetic factors such as chromatin structure, transcriptional status and localization within the nucleus (Jackson and Pombo 1998; Sadoni *et al*. 2004).

Considerable effort has been invested into creating non-integrating episomally replicating vectors, of which four types of systems have been described for mammalian cells. In order to mimic yeast chromosome replication, putative mammalian origins of replication (ori) have been inserted into plasmids. More complex methods to create episomal vectors include the construction of human artificial chromosomes (HACs) by applying a "bottom-up" approach in which the functional elements of the chromosome (telomeres, centromere and an origin of replication) are assembled, or the "top-down" approach, in which natural chromosomes are fragmented into minichromosomes. Another class of episomally replicating vectors mimics viruses such as SV40, Epstein-Barr virus (EBV), and HSV, which encode the gene products necessary to ensure episomal replication in eukaryotic cells. Functionality in this respect of the latest generation of episomally replicating plasmid DNAs ("small circular episomes") relies on the inclusion of chromosomal scaffold/matrix attachment region (S/MAR) sequences into the plasmid encoding the transgene.

PLASMID VECTORS CONTAINING ORIGIN OF REPLICATION

The best characterized chromosomal origins of replication are those of the simple eukaryote *Saccharomyces cerevisiae* (Newlon and Theis 1993), where origin function is performed by ~125 bp, AT-rich DNA elements with an 11 bp core sequence. These replicators are referred to as autonomous replicating sequences (ARS) represent the binding site for the ORC, and hence are able to support episomal replication in yeast.

Attempts to construct analogous vectors in mammalian cells by inserting putative mammalian replication origins into plasmids, have failed in most cases due to defective replication, resulting in either the loss of these constructs from the cell, or integration into the genome (see Lipps *et al*. (2003) for review). In one attempt, various short mammalian origins were cloned into a yeast artificial chromosome (YAC) vector. Most of these constructs integrated into the host genome, but some were maintained as an episome after long-term culture in selective medium (Nielsen *et al*. 2000). The same authors identified a 36 bp human consensus sequence that supports episomal replication in mammalian cells (Price *et al*. 2003). Recently, the human homolog of monkey replication origin orc8 (hors8) was identified, which when cloned into plasmid DNA was capable of conferring sustained episomal replication in HeLa cells (Callejo *et al*. 2006); association of hors8 with DNA replication initiator proteins such as Orc2 and Cdc6 was confirmed in quantitative chromatin immunoprecipitation (ChIP) assays.

Comparison of various mapped mammalian origins of replication showed no sequence homologies, but rather a number of structural features such as AT-rich regions and CpG islands (Calos 1998; Gilbert 2004). Therefore it is now generally assumed that activation of a mammalian origin of replication is strongly influenced by epigenetic factors, such as chromatin structure, ongoing transcription or global nuclear architecture (Gilbert 2002, 2004), all of which need to be considered when constructing episomally replicating vectors for stable transgene expression in mammalian cells.

ARTIFICIAL CHROMOSOMES

The first YAC was constructed in 1983, but it took a further 14 years for the construction of a mammalian counterpart

(Murray and Szostak 1983; Harrington *et al*. 1997). In theory, the advantages of human artificial chromosomes (HACs) over other vector systems are several-fold. First, the mitotic stability and maintenance as non-integrating DNA ensures that HACs do not disturb the stability of host genome and its gene activities. Second, since HACs have a basically unlimited cloning capacity for therapeutic genes in their natural genomic context, regulatory elements that are physically quite far apart in the genome can be included to ensure faithful expression of a therapeutic transgene.

Two different approaches have been applied for the design of a mammalian artificial chromosome. The first human artificial chromosome (HAC) was generated by the "bottom-up" approach, where long synthetic arrays of α-satellite DNA (a major component of natural human centromeres), PCR-generated telomeric DNA, and ~100 kb random genomic DNA fragments were introduced into HT1080 cells, resulting in linear 6-10 Mbp HACs (Harrington *et al*. 1997). Unfortunately, these HACs were structurally unstable and generally integrated into the host genome (see Lipps *et al*. (2003); Conese *et al*. (2004); Basu and Willard (2005); Glover *et al*. (2005) for review), although there was an exception of a circular telomere-absent HAC that demonstrated stability without integration into the host genome (Ebersole *et al*. 2000).

The alternative "top down" approach has involved truncating human chromosomes in living cells to a minimal length by irradiation or telomere fragmentation (Farr *et al*. 1992). Such mini-chromosomes are generally linear, can be as small as 0.5 to 1 Mbp, and contain telomeres at their termini and centromeric regions with long arrays of the centromeric α-satellite DNA. It has to be assumed they also carry genomic origins of replication, but control and initiation of DNA replication has not been studied (see Lipps and Bode 2001; Lipps *et al*. 2003; Conese *et al*. 2004; Basu and Willard 2005; Glover *et al*. 2005). The mitotic stability of "bottom-up" and "top down"-created HACs is improved by including α-satellite sequences, but it has become apparent in recent years, that α-satellite DNA is neither sufficient nor essential for the formation of fully functional human centromeres, whilst stable neocentromeric mini-chromosomes, which lack α-satellite sequences, have been described (Willard 2001; Wong *et al*. 2002, 2005). Both types of mini-chromosomes seem to be able to be maintained stably after propagation in cell lines and even in whole animals (Tomizuka *et al*. 1997; Shen *et al*. 2000; Voet *et al*. 2001).

A number of studies have used HACs or mini-chromosomes to achieve long-term transgene expression. Generally, insertion of a transgene into these large constructs involves the use of targeted site-specific recombination between two minichromosome vectors, using either the Cre-loxP (Kuroiwa *et al*. 2000; Moralli *et al*. 2001; Kuroiwa *et al*. 2002) or the FLP-FRT systems (O'Gorman *et al*. 1991). In one study, a HAC was constructed in bacterial cells by linking *c*. 70 kb of alphoid DNA to a 156 kb bacterial artificial chromosome carrying the human HPRT hypoxanthine-guanine phosphoribosyltransferase gene locus (Kotzamanis *et al*. 2005). A selectable marker and eGFP as reporter gene were then inserted by Cre-loxP recombination using the arabinose inducible *cre* gene within the bacterial host. Isolation of the construct from the bacterial host and transfection into HPRT-deficient human HT1080 cells resulted in the generation of minichromosomes that stably express HPRT, the selection marker, and eGFP. Similar 'top down' approaches have been used to create alphoid sequence-rich circular HACs that stably express HPRT long-term in the absence of selection, with complementation of the HPRT deficiency (Grimes *et al*. 2001; Mejia *et al*. 2001).

Telomere-directed chromosome breakage was used to generate a minichromosome from human chromosome 21 (Katoh *et al*. 2004). This 21ΔpqHAC vector was mitotically stable in human cells and able to express an introduced eGFP gene stably. It was subsequently used to transfect human mesenchymal stem cells (hiMSCs), with the eGFP reporter, under the control of the osteopontin gene promoter whose activity is upregulated during differentiation, specifically expressed in hiMCSs that differentiated into the osteocytes (Ren *et al*. 2005). A satellite DNA-based artificial chromosome that was created in mouse cells was shown to stably express reporter genes in $CD34^+$ cord cells (Vanderbyl *et al*. 2005), while a HAC encoding the human erythropoietin gene was maintained stably and produced human erythropoietin for at least 12 weeks in human primary fibroblasts (Kakeda *et al*. 2005). Truncated mini-chromosomes have also been used to achieve the expression of genes such as the human cystic fibrosis transmembrane conductance regulator (CFTR): insertion of the CFTR locus and its upstream sequences were incorporated into a mini-chromosme, with the presence of the CFTR transcript and protein demonstrated, and a resultant chloride secretory response to cAMP detectable in transfected cells (Auriche *et al*. 2002).

In a number of *in vivo* studies, artificial chromosomes, mini-chromosomes and neocentromere-based chromosomes have been introduced into mice either by microcell-mediated chromosome transfer or pronuclear injection (see Kuroiwa *et al*. (2002); Lipps *et al*. (2003); Basu and Willard (2005) for review) and shown to be able to be transmitted through the germ line. Recently, a circular HAC containing the guanosine triphosphate cyclohydrolase I (GCH1-HAC) and a linear HAC containing the human globin gene cluster (globin-HAC) from HT1080n that were transferred into mouse embryonic stem (ES) cells were stably maintained for 3 months (Suzuki *et al*. 2006). Finally, chimeric mice created using genetically modified ES cells showed that a circular GCH1-HAC could be retained in mouse tissue and transmitted to progeny. Although mammalian artificial chromosomes allow the faithful expression of transgenes and are mitotically stable in the absence of selection, they are difficult to create and deliver into desired cells due to their extremely large size (Glover *et al*. 2005), and hence not ideal for general application in human gene therapy.

PLASMIDS ENCODING VIRAL PROTEINS

Mammalian viruses whose genomes are maintained episomally such as SV40, papillomaviruses (BPV, HPV) or EBV were among the very first replicons to be analyzed in higher eukaryotes (Chow and Broker 1994; Aiyar *et al*. 1998; Stillman 2005). For example, a 64 bp sequence, containing a binding site for SV40 large tumor-antigen (T-ag), is sufficient to initiate replication of the SV40 genome. T-ag is the only viral factor required *in trans* for replication, as the host cell provides all other replication proteins. T-ag functions as a replicative helicase, which permits SV40 replication to be uncoupled from the genomic replication machinery, thereby allowing SV40 to replicate multiple times during the cell cycle (DePamphilis 2003; Ohta *et al*. 2003; Blow and Dutta 2005). Replication of BPV and HPV occur in a similar manner, through the action of the viral E1 protein, a functional homolog of SV40 T-ag (Chow and Broker 1994; Li *et al*. 2003).

In contrast, replication of EBV from *oriP*, a 1.7 kb region of the EBV chromosome is dependent on host ORC and MCM(2-7) complexes and occurs only once per cell cycle in synchrony with the host cell chromosome (Dhar *et al*. 2001; Wang and Sugden 2005). The EBV *oriP* is composed of the dyad symmetry (DS) and family of repeats (FR) regions, both of which are *cis*-acting elements necessary for efficient replication, with the FR region also possessing transcriptional enhancer activity. The second critical component required for the synthesis of EBV vectors is the *trans*-acting protein EBNA-1 (EBV-encoded nuclear antigen 1), which binds to repeats in each *oriP*. Vectors with deleted DS region in the *oriP* fail to replicate efficiently, but can still provide long term gene expression, due to the supposed ability of EBNA-1 and the FR to provide nuclear retention of linked DNA.

A number of chromosomal DNA sequences were isolated that are able to support episomal replication when inserted into a vector system encoding the EBNA-1 protein

(Krysan *et al*. 1989; Calos 1996, 1998; Stoll and Calos 2002). In one approach, mammalian DNA fragments were randomly cloned into a replication-defective EBV *oriP* plasmid, with the expectation that if replicator sequences were present, they would ensure replication in human cells (Krysan *et al*. 1993). Indeed, plasmids bearing some of the inserted human sequences were able to replicate in mammalian cells over several months, albeit under selection. This implies that such vectors are not actively retained in the nucleus but are randomly distributed during mitotic division.

In another study, five previously unknown sequences capable of acting as origins of replication were identified using a novel two-step origin-trapping assay (Gerhardt *et al*. 2006). In this approach, human DNA sequences bound to ORC were enriched by ChIP (chromatin immunoprecipitation) using antibodies directed against human Orc2 protein and then inserted into an EBV-based plasmid with defective *oriP* to confirm their potential to serve as a replicator. While studies of this type are useful to identify replication-promoting sequences in EBNA-based vectors, there is concern about safety problems related to EBNA-1, which in lymphocytes can cause malignant transformation. The extent of these problems remains a matter of debate (e.g. see Kang *et al*. 2005).

Gene expression for up to six months in the absence of selection pressure has been reported for EBV-based vectors (Wade-Martins *et al*. 2000; Stoll *et al*. 2001; Sclimenti *et al*. 2003), though the episomal status of EBV-based vectors does not necessarily prevent epigenetic silencing through chromatin remoldelling (Hsieh 1999; Hong *et al*. 2001). To overcome this problem the transcriptional control elements with a potent chromatin remodeling capacity such as locus control region (LCR), can be included into the vectors. LCRs are tissue-specific transcriptional regulatory regions that are able to overcome chromatin position effects, and have transcriptional activating abilities with respect to *cis*-linked genes (Fraser and Grosveld 1998). For example, an EBV-based vector containing a human β-globin minigene construct that included the β-globin LCR was shown to be able to maintain physiological levels of β-globin expression in the absence of selection over two months of continuous culture (Chow *et al*. 2002); the DNaseI-hypersensitive (HS) sites that constitute the β-globin LCR were also found to confer cell-specific gene expression. HS sites 2, 3 and 4 were shown to act synergistically and gave close to a 100-fold increase in gene expression compared to the human β-globin alone in erythroid cells. More importantly, this efficient LCR also ensured long-term expression for at least 30 days, whereas a dramatic fall in gene expression was seen when minimal LCRs with only one HS element were used, suggesting that multiple LCR elements are required to maintain a transcriptionally competent open chromatin configuration. Preservation of expression by the complex LCR element was also shown to correlate with the preservation of active chromatin as judged by its sensitivity to nuclease digestion. In similar fashion to the smaller episome described above, this ~40 kb mini-gene was maintained without selection at 5-10 copies per cell, but a gradual and constant loss of the episomes was seen, associated with reduced levels of gene expression in individual clones. Expression from the globin mini-gene was up to ~20% of that from the natural chromosomal locus. Though this level of efficiency emphasizes the importance of the natural chromosomal locus in supporting physiological levels of expression, it is also obvious that this system, with fewer than 10 copies of the episome per cell, is able to achieve sufficiently high levels of expression of β-globin to be of value in treating haemoglobinpathies.

EBV-based vectors have been used for ectopic transgene expression in many cell types. Efficient extrachromosomal replication has been observed in cultured, proliferating mammalian cells of an EBV-based vector containing the human *SERPINA1* gene which encodes α1-antitrypsin (AAT). Importantly, a single injection of this plasmid into mice resulted in physiological levels of AAT in the blood serum of the animal for up to 9 months (Stoll *et al*. 2001). The same group demonstrated that injection of an EBV-based vector carrying the human gene encoding human factor IX (hFIX) into mice resulted in prolonged, up to 3 months, transgene expression in the liver. Furthermore, the presence of the EBNA-1 gene and its family of repeats binding sites in this vector ensured a 10 to 100-fold increase in hFIX serum levels compared to expression levels from vectors carrying the hFIX minigene without EBV components (Sclimenti *et al*. 2003).

A common limitation of episomal replicons such as EBV-based vectors, as well as for vectors reliant on S/MARs for episomal maintenance (see below), is the low efficiency with which transfected cells become established clones that stably express the transgene. In the case of the EBNA-1-based vectors, clones arise from 1-10% of transfected cells (Leight and Sugden 2001; Jenke *et al*. 2005). This low percentage is also observed when plasmids are isolated from an established cell clone and reintroduced into cells, implying that establishment occurs through a stochastic event that occurs infrequently and does not rely on DNA modification or a particular chromatin make-up.

S/MAR-BASED VECTORS

For mammalian cells it is now generally assumed that replication origins are selected and programmed by epigenetic features, such as gene expression, nuclear organization and chromosome structure (Gilbert 2002). Because of this and the observation that replication origins bind to the nuclear matrix prior to the onset S-phase (Cook 1999), S/MAR (scaffold matrix attachment region)-containing vectors have been constructed for use in proliferating cells. The nuclear matrix or scaffold is believed to play important roles in different aspects of chromatin function (Bode *et al*. 2000). S/MARs are the generally AT-rich sequences that confer attachment to this structure, and are critical for the organization of chromatin loops that define the boundaries of independent chromatin domains (Bode *et al*. 2000).

In one approach, three putative mammalian origins were cloned into an S/MAR-containing YAC vector. Using this YAC, two cell clones were shown to retain extra-chromosomal vectors when under selection, with one cell showing binding of the YAC to the nuclear matrix (Cossons *et al*. 1997; Nielsen *et al*. 2000). S/MAR-based vectors have also been developed using the strong human S/MAR from the γ-interferon gene cluster (Piechaczek *et al*. 1999). The prototype vector, pEPI-1, replicates in various mammalian cell lines, including primary human cells (Piechaczek *et al*. 1999; Schaarschmidt *et al*. 2004; Papapetrou *et al*. 2006). Nuclear fractionation procedures and FISH analyses revealed that it is bound to the nuclear matrix by interaction with the prominent matrix protein SAF-A, and associates with chromosomes during mitosis (Baiker *et al*. 2000; Jenke *et al*. 2002).

Importantly, this vector was retained for at least 100 cell generations in the absence of selection, while maintaining a copy number of <10 per cell. This is only possible if both replication and segregation to daughter cells during mitosis are highly efficient. It was shown that this vector replicates once per cell cycle during early S-phase and that the origin of replication can assemble at various regions on the vector, which implies that initiation of DNA replication occurs at almost random sites within this construct.

Detailed analysis of the prototype pEPI-1 vector showed that long-term propagation in proliferating cells (without selection pressure) and sustained reporter gene expression depended exclusively on the presence of a transription unit upstream of the S/MAR (Stehle *et al*. 2003); note that in this configuration, transcription actually runs into the S/MAR element. Using this arrangement as a deign parameter, second generation vectors were constructed using a transcription unit linked to oligomers of a synthetic S/MAR module (Jenke *et al*. 2004b), to give almost com-

letely synthetic vectors. This study showed that four copies of a 180 bp synthetic S/MAR module resulted in a vector with sustained gene expression at about 6 copies per cell. Vectors that contained only two synthetic S/MAR repeats, mostly integrated into the host chromosomes. This implies that an S/MAR element of at least 700 bp is needed for efficient extra-chromosomal maintenance.

As with all purportedly extra-chromosomal vectors, it is necessary to determine the extent to which they randomly integrate into the host genome. FISH analysis of stable cell clones with extra-chromosomal vectors enables potential integation events to be detected, as once a vector becomes integated it will be duplicated during S-phase and give identical fluorescent signals in the daughter chromatids of metaphase cells (Baiker *et al*. 2000; Jenke *et al*. 2004b). FISH analysis has been shown that integration of this vector type depends exclusively on the quality of the vector preparation, i.e. any nicked or linearized vector may become integrated.

A series of pEPI-1 variants have also been prepared with loxP sites (Kolot *et al*. 1999; Voziyanov *et al*. 1999; Thyagarajan *et al*. 2000) that lack all bacterial sequences, comprising essentially a 4 kb "minicircle" with only one active transcription unit and the S/MAR. These minicircles have an improved long-term and physically stability. Moreover, they can be transfected into dividing cells and established in the absence of any selection pressure, meeting a major requirement for gene therapeutic applications (Nehlsen *et al*. 2006). This class of vector provides a route to generating extra-chromosomal gene delivery systems that contain therapeutic genes with defined regulatory elements and little or no 'contaminating' bacterial or viral DNA.

In pEPI-1 the γ-interferon S/MAR and a strong promoter appear to be sufficient to maintain long-term expression in the absence of selection in Chinese Hamster Ovary and K562 erythroleukemia cells (Jenke *et al*. 2004a, 2005). Intriguingly, it was observed that the CMV promoter in this vector was not silenced by cytosine methylation, whereas silencing occurred within less than three weeks in cells where the episome integrated. It has also proved possible to introduce an S/MAR-based vector containing the γ-globin activator into K562 cells, and document reporter gene expression for four months of continuous culture together with a constantly elevated level of γ-globin mRNA compared to untransfected cells (Eleana F. Stavrou, pers. comm.). However, silencing of transgene expression has been observed for the same vector in the murine erythroleukemic cell line MEL (Papapetrou *et al*. 2006), implying that neither extra-chromosomal status nor the presence of a S/MAR alone is sufficient to prevent transgene silencing in all types of cells.

The S/MAR-based vector pEPI-1 has recently been used successfully to generate genetically modified pigs. The vector was introduced into the egg cell by sperm-mediated gene transfer (SMGT) (Manzini *et al*. 2006), and subsequently demonstrated to be present in 12 out of 18 fetuses in various different tissues in episomal state. In positive animals, about 79% of cells in all tissues that were tested expressed the eGFP reporter gene. Germ-line transmission of pEPI vector was not investigated in this study, so that the possibility that pEPI may be properly transmitted to the next generation (i.e. through meiotic as well as mitotic cell divisions) has not been examined. Nevertheless, successful transfer of an episomally replicating vector by SMGT, with the subsequent development of normal embryos containing the vector in all tissues in episomal state, clearly provides the basis for the future development of germ-line therapies using this approach.

All vector types discussed above - EBV-derived vectors either containing the EBV oriP and EBNA-1 or EBNA-1 together with chromosomal origins of replication, S/MAR-based vectors, artificial chromosomes and mini-chromosomes – exhibit a high mitotic stability in the absence of selection although they are not as stable as natural chromosomes. In the case of artificial chromosomes, mini-chromosomes or neocentromere-based chromosomes, mitotic stability is achieved by the presence of a functional centromere, demonstrable by detection of centromere specific protein CENP-A (see Basu and Willard 2005). In contrast, mitotic stability in the case of EBV- or S/MAR-based vectors does not appear to require a functional centromere. It is believed that nuclear retention and mitotic stability of these constructs, which varies between 96% (i.e. in 4% of the cells the vector is lost in one generation) in the case of EBV-based vectors to over 98% in the case of S/MAR-based vectors (Piechaczek *et al*. 1999; Leight and Sugden 2001; Schaarschmidt *et al*. 2004), is provided by the presence of EBNA-1 or the S/MAR. The mechanism of retention appears to be through co-segregation with mitotic chromosomes via an ill-characterized "piggyback" mechanism (Baiker *et al*. 2000; Wu *et al*. 2000).

Thus, taken together, although S/MAR-containing episomal vectors that passively, and importantly safely, exploit the cellular genomic DNA retention and segregation mechanisms, are promising prospects to achieve long-term transgene expression in conjunction with non-viral delivery. Optimiziation of these vectors, particularly with respect to their ability to become established reliably in eukaryotic cells upon delivery, appears to be the major stumbling block to their application in a whole range of areas, including in the clinic.

CONCLUDING REMARKS

In essence, gene therapy aims to engineer host cells to express a desired gene product, in many ways precisely emulating what happens during viral infection, but on a single gene scale. Indeed, many important lessons can be garnered from viruses in the design and engineering of non-viral vectors, including approaches to avoid the safety problems that dramatically limit the use of virus-based vectors in humans. Furthermore, whereas viruses depend on host mechanisms for their creation, non-viral vectors may be combined with synthetic molecules that allow for transfection and expression strategies unavailable to viruses. A 'perfect' non-viral vector may never be created, but the constant refinement of existing approaches, through the inclusion of novel functional modules within both the carrier and DNA molecule, will produce a range of non-viral vectors tailored for specific applications, including the safe and long-term expression of therapeutic genes in humans.

ACKNOWLEDGEMENTS

We acknowledge the support of the National Health and Medical Research Council (fellowship #334013/384109 and project grants #143710 and 284205) (DAJ), as well as the DFG (Deutsche Forschungsgesellschaft) and European Union (HJL).

REFERENCES

Aiyar A, Tyree C, Sugden B (1998) The plasmid replicon of EBV consists of multiple cis-acting elements that facilitate DNA synthesis by the cell and a viral maintenance element. *The EMBO Journal* **17**, 6394-6403

Akhlynina TV, Jans DA, Rosenkranz AA, Statsyuk NV, Balashova IY, Toth G, Pavo I, Rubin AB, Sobolev AS (1997) Nuclear targeting of chlorin e6 enhances its photosensitizing activity. *The Journal of Biological Chemistry* **272**, 20328-20331

Akhlynina TV, Jans DA, Statsyuk NV, Balashova IY, Toth G, Pavo I, Rosenkranz AA, Naroditsky BS, Sobolev AS (1999) Adenoviruses synergize with nuclear localization signals to enhance nuclear delivery and photodynamic action of internalizable conjugates containing chlorin e6. *International Journal of Cancer* **81**, 734-740

Anderson DG, Peng W, Akinc A, Hossain N, Kohn A, Padera R, Langer R, Sawicki JA (2004) A polymer library approach to suicide gene therapy for cancer. *Proceedings of the National Academy of Sciences of the USA* **101**, 16028-16033

Auriche C, Carpani D, Conese M, Caci E, Zegarra-Moran O, Donini P, Ascenzioni F (2002) Functional human CFTR produced by a stable minichromosome. *EMBO Reports* **3**, 862-868

Baake M, Doenecke D, Albig W (2001) Characterisation of nuclear localisation signals of the four human core histones. *Journal of Cellular Biochemistry* **81**, 333-346

Baiker A, Maercker C, Piechaczek C, Schmidt SB, Bode J, Benham C, Lipps HJ (2000) Mitotic stability of an episomal vector containing a human scaffold/matrix-attached region is provided by association with nuclear matrix. *Nature Cell Biology* **2**, 182-184

Balhorn R, Brewer L, Corzett M (2000) DNA condensation by protamine and arginine-rich peptides: analysis of toroid stability using single DNA molecules. *Molecular Reproduction and Development* **56**, 230-234

Balicki D, Putnam CD, Scaria PV, Beutler E (2002) Structure and function correlation in histone H2A peptide-mediated gene transfer. *Proceedings of the National Academy of Sciences of the USA* **99**, 7467-7471

Basu J, Willard HF (2005) Artificial and engineered chromosomes: non-integrating vectors for gene therapy. *Trends in Molecular Medicine* **11**, 251-258

Blow JJ, Dutta A (2005) Preventing re-replication of chromosomal DNA. *Nature Reviews Molecular Cell Biology* **6**, 476-486

Bode J, Benham C, Knopp A, Mielke C (2000) Transcriptional augmentation: modulation of gene expression by scaffold/matrix-attached regions (S/MAR elements). *Critical Reviews in Eukaryotic Gene Expression* **10**, 73-90

Bremner KH, Seymour LW, Logan A, Read ML (2004) Factors influencing the ability of nuclear localization sequence peptides to enhance nonviral gene delivery. *Bioconjugate Chemistry* **15**, 152-161

Brewer L, Corzett M, Balhorn R (2002) Condensation of DNA by spermatid basic nuclear proteins. *The Journal of Biological Chemistry* **277**, 38895-38900

Byrnes CK, Nass PH, Duncan MD, Harmon JW (2002a) A nuclear targeting peptide, M9, improves transfection efficiency in fibroblasts. *The Journal of Surgical Research* **108**, 85-90

Byrnes CK, Nass PH, Shim J, Duncan MD, Lacy B, Harmon JW (2002b) Novel nuclear shuttle peptide to increase transfection efficiency in esophageal mucosal cells. *Journal of Gastrointestinal Surgery* **6**, 37-42

Callejo M, Sibani S, Di Paola D, Price GG, Zannis-Hadjopoulos M (2006) Identification and functional analysis of a human homologue of the monkey replication origin ors8. *Journal of Cellular Biochemistry* **99**, 1606-1615

Calos MP (1998) Stability without a centromere. *Proceedings of the National Academy of Sciences of the USA* **95**, 4084-4085

Calos MP (1996) The potential of extrachromosomal replicating vectors for gene therapy. *Trends in Genetics* **12**, 463-466

Capecchi MR (1980) High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. *Cell* **22**, 479-88

Caplan MR, Rosca EV (2005) Targeting drugs to combinations of receptors: a modeling analysis of potential specificity. *Annals of Biomedical Engineering* **33**, 1113-1124

Carrière M, Escriou V, Savarin A, Scherman D (2003) Coupling of importin beta binding peptide on plasmid DNA: transfection efficiency is increased by modification of lipoplex's physico-chemical properties. *BMC Biotechnology* **3**, 14

Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, Gross F, Yvon E, Nusbaum P, Selz F, Hue C, Certain S, Casanova JL, Bousso P, Deist FL, Fischer A (2000) Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* **288**, 669-672

Caviston JP, Holzbaur EL (2006) Microtubule motors at the intersection of trafficking and transport. *Trends in Cell Biology* **16**, 530-537

Cereghini S, Yaniv M (1984) Assembly of transfected DNA into chromatin: structural changes in the origin-promoter-enhancer region upon replication. *The EMBO Journal* **3**, 1243-1253

Chan CK, Hubner S, Hu W, Jans DA (1998) Mutual exclusivity of DNA binding and nuclear localization signal recognition by the yeast transcription factor GAL4: implications for nonviral DNA delivery. *Gene Therapy* **5**, 1204-1212

Chan CK, Jans DA (1999) Enhancement of polylysine-mediated transferinfection by nuclear localization sequences: polylysine does not function as a nuclear localization sequence. *Human Gene Therapy* **10**, 1695-1702

Chan CK, Jans DA (2001) Enhancement of MSH receptor- and GAL4-mediated gene transfer by switching the nuclear import pathway. *Gene Therapy* **8**, 166-171

Chan CK, Senden T, Jans DA (2000) Supramolecular structure and nuclear targeting efficiency determine the enhancement of transfection by modified polylysines. *Gene Therapy* **7**, 1690-1697

Chang CW, Choi D, Kim WJ, Yockman JW, Christensen LV, Kim YH, Kim SW (2007) Non-ionic amphiphilic biodegradable PEG-PLGA-PEG copolymers enhances gene delivery efficiency in rat skeletal muscle. *Journal of Controlled Release* **118**, 245-253

Cheng J, Zeidan R, Mishra S, Liu A, Pun SH, Kulkarni RP, Jensen GS, Bellocq NC, Davis ME (2006) Structure-function correlation of chloroquine and analogues as transgene expression enhancers in nonviral gene delivery. *Journal of Medicinal Chemistry* **49**, 6522-6531

Chiu SJ, Ueno NT, Lee RJ (2004) Tumor-targeted gene delivery via anti-HER2 antibody (trastuzumab, Herceptin) conjugated polyethylenimine. *Journal of Controlled Release* **97**, 357-369

Chow CM, Athanassiadou A, Raguz S, Psiouri L, Harland L, Malik M, Aitken MA, Grosveld F, Antoniou M (2002) LCR-mediated, long-term tissue-specific gene expression within replicating episomal plasmid and cosmid vectors. *Gene Therapy* **9**, 327-336

Chow LT, Broker TR (1994) Papillomavirus DNA replication. *Intervirology* **37**, 150-158

Ciolina C, Byk G, Blanche F, Thuillier V, Scherman D, Wils P (1999) Coupling of nuclear localization signals to plasmid DNA and specific interaction of the conjugates with importin alpha. *Bioconjugate Chemistry* **10**, 49-55

Conese M, Auriche C, Ascenzioni F (2004) Gene therapy progress and prospects: episomally maintained self-replicating systems. *Gene Therapy* **11**, 1735-1741

Cook PR (1999) The organization of replication and transcription. *Science* **284**, 1790-1795

Cossons N, Nielsen TO, Dini C, Tomilin N, Young DB, Riabowol KT, Rattner JB, Johnston RN, Zannis-Hadjopoulos M, Price GB (1997) Circular YAC vectors containing a small mammalian origin sequence can associate with the nuclear matrix. *Journal of Cellular Biochemistry* **67**, 439-450

Dauty E, Verkman AS (2004) Molecular crowding reduces to a similar extent the diffusion of small solutes and macromolecules: measurement by fluorescence correlation spectroscopy. *Journal of Molecular Recognition* **17**, 441-417

Dauty E, Verkman AS (2005) Actin cytoskeleton as the principal determinant of size-dependent DNA mobility in cytoplasm: a new barrier for non-viral gene delivery. *The Journal of Biological Chemistry* **280**, 7823-7828

Dean DA (1997) Import of plasmid DNA into the nucleus is sequence specific. *Experimental Cell Research* **230**, 293-302

Dean DA, Dean BS, Muller S, Smith LC (1999) Sequence requirements for plasmid nuclear import. *Experimental Cell Research* **253**, 713-722

DePamphilis ML (2003) The 'ORC cycle': a novel pathway for regulating eukaryotic DNA replication. *Gene* **310**, 1-15

Dewey RA, Morrissey G, Cowsill CM, Stone D, Bolognani F, Dodd NJ, Southgate TD, Klatzmann D, Lassmann H, Castro MG, Lowenstein PR (1999) Chronic brain inflammation and persistent herpes simplex virus 1 thymidine kinase expression in survivors of syngeneic glioma treated by adenovirus-mediated gene therapy: implications for clinical trials. *Nature Medicine* **5**, 1256-1263

Dhar SK, Yoshida K, Machida Y, Khaira P, Chaudhuri B, Wohlschlegel JA, Leffak M, Yates J, Dutta A (2001) Replication from oriP of Epstein-Barr virus requires human ORC and is inhibited by geminin. *Cell* **106**, 287-296

Dohner K, Nagel CH, Sodeik B (2005) Viral stop-and-go along microtubules: taking a ride with dynein and kinesins. *Trends in Microbiology* **13**, 320-327

Ebersole TA, Ross A, Clark E, McGill N, Schindelhauer D, Cooke H, Grimes B (2000) Mammalian artificial chromosome formation from circular alphoid input DNA does not require telomere repeats. *Human Molecular Genetics* **9**, 1623-1631

Farhood H, Serbina N, Huang L (1995) The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochimica et Biophysica Acta* **1235**, 289-295

Farr CJ, Stevanovic M, Thomson EJ, Goodfellow PN, Cooke HJ (1992) Telomere-associated chromosome fragmentation: Applications in genome manipulation and analysis. *Nature Genetics* **2**, 275-282

Fasbender A, Zabner J, Zeiher BG, Welsh MJ (1997) 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 Therapy* **4**, 1173-1180

Fraser P, Grosveld F (1998) Locus control regions, chromatin activation and transcription. *Current Opinion in Cell Biology* **10**, 361-365

Futaki S, Masui Y, Nakase I, Sugiura Y, Nakamura T, Kogure K, Harashima H (2005) Unique features of a pH-sensitive fusogenic peptide that improves the transfection efficiency of cationic liposomes. *The Journal of Gene Medicine* **7**, 1450-1458

Garcia-Mata R, Gao YS, Sztul E (2002) Hassles with taking out the garbage: Aggravating aggresomes. *Traffic* **3**, 388-396

Gaspar HB, Parsley KL, Howe S, King D, Gilmour KC, Sinclair J, Brouns G, Schmidt M, Von Kalle C, Barington T, Jakobsen MA, Christensen HO, Al Ghonaium A, White HN, Smith JL, Levinsky RJ, Ali RR, Kinnon C, Thrasher AJ (2004) Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. *Lancet* **364**, 2181-2187

Gaur R, Gupta PK, Goyal A, Wels W, Singh Y (2002) Delivery of nucleic acid into mammalian cells by anthrax toxin. *Biochemical and Biophysical Research Communications* **297**, 1121-1127

Geiger RC, Taylor W, Glucksberg MR, Dean DA (2006) Cyclic stretch-induced reorganization of the cytoskeleton and its role in enhanced gene transfer. *Gene Therapy* **13**, 725-731

Gerhardt J, Jafar S, Spindler MP, Ott E, Schepers A (2006) Identification of new human origins of DNA replication by an origin-trapping assay. *Molecular and Cellular Biology* **26**, 7731-7746

Giannakakou P, Sackett DL, Ward Y, Webster KR, Blagosklonny MV, Fojo T (2000) p53 is associated with cellular microtubules and is transported to the nucleus by dynein. *Nature Cell Biology* **2**, 709-717

Gilbert DM (2002) Replication timing and transcriptional control: beyond cause and effect. *Current Opinion in Cell Biology* **14**, 377-383

Gilbert DM (2004) In search of the holy replicator. *Nature Reviews Molecular Cell Biology* **5**, 848-855

Glover DJ, Lipps HJ, Jans DA (2005) Towards safe, non-viral therapeutic gene expression in humans. *Nature Reviews Genetics* **6**, 299-310

Goldberg MW, Allen TD (1992) High resolution scanning electron microscopy of the nuclear envelope: demonstration of a new, regular, fibrous lattice attached to the baskets of the nucleoplasmic face of the nuclear pores. *The Journal of Cell Biology* **119**, 1429-1440

Graessmann M, Menne J, Liebler M, Graeber I, Graessmann A (1989) Helper activity for gene expression, a novel function of the SV40 enhancer. *Nucleic Acids Research* **17**, 6603-6612

Grimes BR, Schindelhauer D, McGill NI, Ross A, Ebersole TA, Cooke HJ (2001) Stable gene expression from a mammalian artificial chromosome. *EMBO Reports* **2**, 910-914

Hacein-Bey-Abina S, Le Deist F, Carlier F, Bouneaud C, Hue C, De Villartay JP, Thrasher AJ, Wulffraat N, Sorensen R, Dupuis-Girod S, Fischer A, Davies EG, Kuis W, Leiva L, Cavazzana-Calvo M (2002) Sustained correction of X-linked severe combined immunodeficiency by *ex vivo* gene therapy. *The New England Journal of Medicine* **346**, 1185-1193

Hacein-Bey-Abina S, von Kalle C, Schmidt M, Le Deist F, Wulffraat N, McIntyre E, Radford I, Villeval JL, Fraser CC, Cavazzana-Calvo M, Fischer A (2003a) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *The New England Journal of Medicine* **348**, 255-256

Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, Lim A, Osborne CS, Pawliuk R, Morillon E, Sorensen R, Forster A, Fraser P, Cohen JI, de Saint Basile G, Alexander I, Wintergerst U, Frebourg T, Aurias A, Stoppa-Lyonnet D, Romana S, Radford-Weiss I, Gross F, Valensi F, Delabesse E, Macintyre E, Sigaux F, Soulier J, Leiva LE, Wissler M, Prinz C, Rabbitts TH, Le Deist F, Fischer A, Cavazzana-Calvo M (2003b) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**, 415-419

Hama S, Akita H, Ito R, Mizuguchi H, Hayakawa T, Harashima H (2006) Quantitative comparison of intracellular trafficking and nuclear transcription between adenoviral and lipoplex systems. *Molecular Therapy* **13**, 786-794

Han X, Bushweller JH, Cafiso DS, Tamm LK (2001) Membrane structure and fusion-triggering conformational change of the fusion domain from influenza hemagglutinin. *Nature Structural Biology* **8**, 715-720

Handl HL, Vagner J, Han H, Mash E, Hruby VJ, Gillies RJ (2004) Hitting multiple targets with multimeric ligands. *Expert Opinion on Therapeutic Targets* **8**, 565-586

Harrington JJ, Van Bokkelen G, Mays RW, Gustashaw K, Willard HF (1997) Formation of de novo centromeres and construction of first-generation human artificial microchromosomes. *Nature Genetics* **15**, 345-355

Hasegawa S, Hirashima N, Nakanishi M (2001) Microtubule involvement in the intracellular dynamics for gene transfection mediated by cationic liposomes. *Gene Therapy* **8**, 1669-1673

Hattori Y, Maitani Y (2005) Folate-linked lipid-based nanoparticle for targeted gene delivery. *Current Drug Delivery* **2**, 243-252

Hearps AC, Jans DA (2006) HIV-1 integrase is capable of targeting DNA to the nucleus via an importin alpha/beta-dependent mechanism. *Biochemical Journal* **398**, 475-484

Hollon T (2000) Researchers and regulators reflect on first gene therapy death. *Nature Medicine* **6**, 6

Hong K, Sherley J, Lauffenburger DA (2001) Methylation of episomal plasmids as a barrier to transient gene expression via a synthetic delivery vector. *Biomolecular Engineering* **18**, 185-192

Hsieh CL (1999) *In vivo* activity of murine de novo methyltransferases, Dnmt3a and Dnmt3b. *Molecular and Cellular Biology* **19**, 8211-8218

Hubermann JA, Riggs AD (1966) Autoradiography of chromosomal DNA fibers from Chinese hamster cells. *Proceedings of the National Academy of Sciences of the USA* **55**, 599

Hubner S, Xiao CY, Jans DA (1997) The protein kinase CK2 site (Ser111/112) enhances recognition of the simian virus 40 large T-antigen nuclear localization sequence by importin. *The Journal of Biological Chemistry* **272**, 17191-17195

Ikegami S, Yamakami K, Ono T, Sato M, Suzuki S, Yoshimura I, Asano T, Hayakawa M, Tadakuma T (2006) Targeting gene therapy for prostate cancer cells by liposomes complexed with anti-prostate-specific membrane antigen monoclonal antibody. *Human Gene Therapy* **17**, 997-1005

Jackson DA, Pombo A (1998) Replicon clusters are stable units of chromosome structure: evidence that nuclear organization contributes to the efficient activation and propagation of S phase in human cells. *The Journal of Cell Biology* **140**, 1285-1295

Jans DA, Ackermann MJ, Bischoff JR, Beach DH, Peters R (1991) p34cdc2-mediated phosphorylation at T124 inhibits nuclear import of SV-40 T antigen proteins. *The Journal of Cell Biology* **115**, 1203-1212

Jans DA, Jans P (1994) Negative charge at the casein kinase II site flanking the nuclear localization signal of the SV40 large T-antigen is mechanistically important for enhanced nuclear import. *Oncogene* **9**, 2961-2968

Jans DA, Xiao CY, Lam MH (2000) Nuclear targeting signal recognition: a key control point in nuclear transport? *Bioessays* **22**, 532-544

Jenke AC, Eisenberger T, Baiker A, Stehle IM, Wirth S, Lipps HJ (2005) The nonviral episomal replicating vector pEPI-1 allows long-term inhibition of bcr-abl expression by shRNA. *Human Gene Therapy* **16**, 533-539

Jenke AC, Scinteie MF, Stehle IM, Lipps HJ (2004a) Expression of a transgene encoded on a non-viral episomal vector is not subject to epigenetic silencing by cytosine methylation. *Molecular Biology Reports* **31**, 85-90

Jenke AC, Stehle IM, Herrmann F, Eisenberger T, Baiker A, Bode J, Fackelmayer FO, Lipps HJ (2004b) Nuclear scaffold/matrix attached region modules linked to a transcription unit are sufficient for replication and maintenance of a mammalian episome. *Proceedings of the National Academy of Sciences of the USA* **101**, 11322-11327

Jenke BH, Fetzer CP, Stehle IM, Jonsson F, Fackelmayer FO, Conradt H, Bode J, Lipps HJ (2002) An episomally replicating vector binds to the nuclear matrix protein SAF-A *in vivo*. *EMBO Reports* **3**, 349-354

Jeong S, Stein A (1994) Micrococcal nuclease digestion of nuclei reveals extended nucleosome ladders having anomalous DNA lengths for chromatin assembled on non-replicating plasmids in transfected cells. *Nucleic Acids Research* **22**, 370-375

Johnson-Saliba M, Siddon NA, Clarkson MJ, Tremethick DJ, Jans DA (2000) Distinct importin recognition properties of histones and chromatin assembly factors. *FEBS Letters* **467**, 169-174

Kakeda M, Hiratsuka M, Nagata K, Kuroiwa Y, Kakitani M, Katoh M, Oshimura M, Tomizuka K (2005) Human artificial chromosome (HAC) vector provides long-term therapeutic transgene expression in normal human primary fibroblasts. *Gene Therapy* **12**, 852-856

Kakudo T, Chaki S, Futaki S, Nakase I, Akaji K, Kawakami T, Maruyama K, Kamiya H, Harashima H (2004) Transferrin-modified liposomes equipped with a pH-sensitive fusogenic peptide: an artificial viral-like delivery system. *Biochemistry* **43**, 5618-5628

Kang MS, Lu H, Yasui T, Sharpe A, Warren H, Cahir-McFarland E, Bronson R, Hung SC, Kieff E (2005) Epstein-Barr virus nuclear antigen 1 does not induce lymphoma in transgenic FVB mice. *Proceedings of the National Academy of Sciences of the USA* **102**, 820-825

Katoh M, Ayabe F, Norikane S, Okada T, Masumoto H, Horike S, Shirayoshi Y, Oshimura M (2004) Construction of a novel human artificial chromosome vector for gene delivery. *Biochemical and Biophysical Research Communications* **321**, 280-290

Kichler A, Leborgne C, Marz J, Danos O, Bechinger B (2003) Histidine-rich amphipathic peptide antibiotics promote efficient delivery of DNA into mammalian cells. *Proceedings of the National Academy of Sciences of the USA* **100**, 1564-1568

Kolot M, Silberstein N, Yagil E (1999) Site-specific recombination in mammalian cells expressing the Int recombinase of bacteriophage HK022. *Molecular Biology Reports* **26**, 207-213

Kotzamanis G, Cheung W, Abdulrazzak H, Perez-Luz S, Howe S, Cooke H, Huxley C (2005) Construction of human artificial chromosome vectors by recombineering. *Gene* **351**, 29-38

Krysan PJ, Haase SB, Calos MP (1989) Isolation of human sequences that replicate autonomously in human cells. *Molecular and Cellular Biology* **9**, 1026-1033

Krysan PJ, Smith JG, Calos MP (1993) Autonomous replication in human cells of multimers of specific human and bacterial DNA sequences. *Molecular and Cellular Biology* **13**, 2688-2696

Kueltzo LA, Middaugh CR (2003) Nonclassical transport proteins and peptides: an alternative to classical macromolecule delivery systems. *Journal of Pharmaceutical Sciences* **92**, 1754-1772

Kulkarni RP, Castelino K, Majumdar A, Fraser SE (2006) Intracellular transport dynamics of endosomes containing DNA polyplexes along the microtubule network. *Biophysical Journal* **90**, L42-4

Kulkarni RP, Wu DD, Davis ME, Fraser SE (2005) Quantitating intracellular transport of polyplexes by spatio-temporal image correlation spectroscopy. *Proceedings of the National Academy of Sciences of the USA* **102**, 7523-7528

Kural C, Kim H, Syed S, Goshima G, Gelfand VI, Selvin PR (2005) Kinesin and dynein move a peroxisome in vivo: a tug-of-war or coordinated movement? *Science* **308**, 1469-1472

Kuroiwa Y, Tomizuka K, Shinohara T, Kazuki Y, Yoshida H, Ohguma A, Yamamoto T, Tanaka S, Oshimura M, Ishida I (2000) Manipulation of human minichromosomes to carry greater than megabase-sized chromosome inserts. *Nature Biotechnology* **18**, 1086-1090

Kuroiwa Y, Yoshida H, Ohshima T, Shinohara T, Ohguma A, Kazuki Y, Oshimura M, Ishida I, Tomizuka K (2002) The use of chromosome-based vectors for animal transgenesis. *Gene Therapy* **9**, 708-712

Lakadamyali M, Rust MJ, Babcock HP, Zhuang X (2003) Visualizing infection of individual influenza viruses. *Proceedings of the National Academy of Sciences of the USA* **100**, 9280-9285

Lam MH, Thomas RJ, Loveland KL, Schilders S, Gu M, Martin TJ, Gillespie MT, Jans DA (2002) Nuclear transport of parathyroid hormone (PTH)-related protein is dependent on microtubules. *Molecular Endocrinology* **16**, 390-401

Langle-Rouault F, Patzel V, Benavente A, Taillez M, Silvestre N, Bompard A, Sczakiel G, Jacobs E, Rittner K (1998) Up to 100-fold increase of apparent gene expression in the presence of Epstein-Barr virus oriP sequences and EBNA1: implications of the nuclear import of plasmids. *The Journal of Virology* **72**, 6181-6185

Lechardeur D, Sohn KJ, Haardt M, Joshi PB, Monck M, Graham RW, Beatty B, Squire J, O'Brodovich H, Lukacs GL (1999) Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer. *Gene Therapy* **6**, 482-497

Lee H, Jeong JH, Park TG (2002) PEG grafted polylysine with fusogenic peptide for gene delivery: High transfection efficiency with low cytotoxicity. *Journal of Controlled Release* **79**, 283-291

Leight ER, Sugden B (2001) Establishment of an oriP replicon is dependent upon an infrequent, epigenetic event. *Molecular and Cellular Biology* **21**, 4149-4161

Li D, Zhao R, Lilyestrom W, Gai D, Zhang R, DeCaprio JA, Fanning E, Jochimiak A, Szakonyi G, Chen XS (2003) Structure of the replicative helicase of the oncoprotein SV40 large tumour antigen. *Nature* **423**, 512-518

Li X, Stuckert P, Bosch I, Marks JD, Marasco WA (2001) Single-chain antibody-mediated gene delivery into ErbB2-positive human breast cancer cells. *Cancer Gene Therapy* **8**, 555-565

Lipps HJ, Bode J (2001) Exploiting chromosomal and viral strategies: the design of safe and efficient non-viral gene transfer systems. *Current Opinion in Molecular Therapeutics* **3**, 133-141

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

Ludtke JJ, Zhang G, Sebestyen MG, Wolff JA (1999) A nuclear localization signal can enhance both the nuclear transport and expression of 1 kb DNA. *Journal of Cell Science* **112**, 2033-2041

Lukacs GL, Haggie P, Seksek O, Lechardeur D, Freedman N, Verkman AS (2000) Size-dependent DNA mobility in cytoplasm and nucleus. *The Journal of Biological Chemistry* **275**, 1625-1629

Manzini S, Vargiolu A, Stehle IM, Bacci ML, Cerrito MG, Giovannoni R, Zannoni A, Bianco MR, Forni M, Donini P, Papa M, Lipps HJ, Lavitrano M (2006) Genetically modified pigs produced with a nonviral episomal vector. *Proceedings of the National Academy of Sciences of the USA* **103**, 17672-17677

Mastrobattista E, Kapel RH, Eggenhuisen MH, Roholl PJ, Crommelin DJ, Hennink WE, Storm G (2001) Lipid-coated polyplexes for targeted gene delivery to ovarian carcinoma cells. *Cancer Gene Therapy* **8**, 405-413

Mastrobattista E, Koning GA, van Bloois L, Filipe AC, Jiskoot W, Storm G (2002) Functional characterization of an endosome-disruptive peptide and its application in cytosolic delivery of immunoliposome-entrapped proteins. *The Journal of Biological Chemistry* **277**, 27135-27143

Masuda T, Akita H, Harashima H (2005) Evaluation of nuclear transfer and transcription of plasmid DNA condensed with protamine by microinjection: the use of a nuclear transfer score. *FEBS Letters* **579**, 2143-2148

Matsuda T, Cepko CL (2007) Controlled expression of transgenes introduced by *in vivo* electroporation. *Proceedings of the National Academy of Sciences of the USA* **104**, 1027-1032

McCormack MP, Forster A, Drynan L, Pannell R, Rabbitts TH (2003) The LMO2 T-cell oncogene is activated via chromosomal translocations or retroviral insertion during gene therapy but has no mandatory role in normal T-cell development. *Molecular and Cellular Biology* **23**, 9003-9013

McLay DW, Clarke HJ (2003) Remodelling the paternal chromatin at fertilization in mammals. *Reproduction* **125**, 625-633

Mejia JE, Willmott A, Levy E, Earnshaw WC, Larin Z (2001) Functional complementation of a genetic deficiency with human artificial chromosomes. *The American Journal of Human Genetics* **69**, 315-326

Mesika A, Grigoreva I, Zohar M, Reich Z (2001) A regulated, NFkappaB-assisted import of plasmid DNA into mammalian cell nuclei. *Molecular Therapy* **3**, 653-657

Mesika A, Kiss V, Brumfeld V, Ghosh G, Reich Z (2005) Enhanced intracellular mobility and nuclear accumulation of DNA plasmids associated with a karyophilic protein. *Human Gene Therapy* **16**, 200-208

Metselaar JM, Bruin P, de Boer LW, de Vringer T, Snel C, Oussoren C, Wauben MH, Crommelin DJ, Storm G, Hennink WE (2003) A novel family of L-amino acid-based biodegradable polymer-lipid conjugates for the development of long-circulating liposomes with effective drug-targeting capacity. *Bioconjugate Chemistry* **14**, 1156-1164

Midoux P, Monsigny M (1999) Efficient gene transfer by histidylated polylysine/pDNA complexes. *Bioconjugate Chemistry* **10**, 406-411

Moffatt S, Wiehle S, Cristiano RJ (2006) A multifunctional PEI-based cationic polyplex for enhanced systemic p53-mediated gene therapy. *Gene Therapy* **13**, 1512-1523

Moralli D, Vagnarelli P, Bensi M, De Carli L, Raimondi E (2001) Insertion of a loxP site in a size-reduced human accessory chromosome. *Cytogenetics and Cell Genetics* **94**, 113-120

Murray AW, Szostak JW (1983) Construction of artificial chromosomes in yeast. *Nature* **305**, 189-193

Nam CH, Rabbitts TH (2006) The role of LMO2 in development and in T cell leukemia after chromosomal translocation or retroviral insertion. *Molecular Therapy* **13**, 15-25

Navarro-Quiroga I, Antonio Gonzalez-Barrios J, Barron-Moreno F, Gonzalez-Bernal V, Martinez-Arguelles DB, Martinez-Fong D (2002) Improved neurotensin-vector-mediated gene transfer by the coupling of hemagglutinin HA2 fusogenic peptide and Vp1 SV40 nuclear localization signal. *Brain Research: Molecular Brain Research* **105**, 86-97

Nehlsen K, Broll S, Bode J (2006) Replicating minicircles: Generation of nonviral episomes for the efficient modification of dividing cells. *Gene Therapy and Molecular Biology* **10**, 233-244

Newlon CS, Theis JF (1993) The structure and function of yeast ARS elements. *Current Opinion in Genetics and Development* **3**, 752-758

Nielsen TO, Cossons NH, Zannis-Hadjopoulos M, Price GB (2000) Circular YAC vectors containing short mammalian origin sequences are maintained under selection as HeLa episomes. *Journal of Cellular Biochemistry* **76**, 674-685

Nishiyama N, Iriyama A, Jang WD, Miyata K, Itaka K, Inoue Y, Takahashi H, Yanagi Y, Tamaki Y, Koyama H, Kataoka K (2005) Light-induced gene transfer from packaged DNA enveloped in a dendrimeric photosensitizer. *Nature Materials* **4**, 934-941

O'Gorman S, Fox DT, Wahl GM (1991) Recombinase-mediated gene activation and site-specific integration in mammalian cells. *Science* **251**, 1351-1355

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

Ohta S, Tatsumi Y, Fujita M, Tsurimoto T, Obuse C (2003) The ORC1 cycle in human cells: II. Dynamic changes in the human ORC complex during the cell cycle. *The Journal of Biological Chemistry* **278**, 41535-41540

Papapetrou EP, Ziros PG, Micheva ID, Zoumbos NC, Athanassiadou A (2006) Gene transfer into human hematopoietic progenitor cells with an episomal vector carrying an S/MAR element. *Gene Therapy* **13**, 40-51

Paul RW, Weisser KE, Loomis A, Sloane DL, LaFoe D, Atkinson EM, Overell RW (1997) Gene transfer using a novel fusion protein, GAL4/invasin. *Human Gene Therapy* **8**, 1253-1262

Pemberton LF, Paschal BM (2005) Mechanisms of receptor-mediated nuclear import and nuclear export. *Traffic* **6**, 187-198

Piechaczek C, Fetzer C, Baiker A, Bode J, Lipps HJ (1999) A vector based on the SV40 origin of replication and chromosomal S/MARs replicates episomally in CHO cells. *Nucleic Acids Research* **27**, 426-428

Pollard H, Remy JS, Loussouarn G, Demolombe S, Behr JP, Escande D (1998) Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. *The Journal of Biological Chemistry* **273**, 7507-7511

Price GB, Allarakhia M, Cossons N, Nielsen T, Diaz-Perez M, Friedlander P, Tao L, Zannis-Hadjopoulos M (2003) Identification of a *cis*-element that determines autonomous DNA replication in eukaryotic cells. *The Journal of Biological Chemistry* **278**, 19649-19659

Quimby BB, Corbett AH (2001) Nuclear transport mechanisms. *Cellular and Molecular Life Sciences* **58**, 1766-1773

Radtke K, Dohner K, Sodeik B (2006) Viral interactions with the cytoskeleton: A hitchhiker's guide to the cell. *Cellular Microbiology* **8**, 387-400

Ren X, Katoh M, Hoshiya H, Kurimasa A, Inoue T, Ayabe F, Shibata K, Toguchida J, Oshimura M (2005) A novel human artificial chromosome vector provides effective cell lineage-specific transgene expression in human mesenchymal stem cells. *Stem Cells* **23**, 1608-1616

Richard P, Bossard F, Desigaux L, Lanctin C, Bello-Roufai M, Pitard B (2005) Amphiphilic block copolymers promote gene delivery *in vivo* to pathological skeletal muscles. *Human Gene Therapy* **16**, 1318-1324

Rihs HP, Jans DA, Fan H, Peters R (1991) The rate of nuclear cytoplasmic protein transport is determined by the casein kinase II site flanking the nuclear localization sequence of the SV40 T-antigen. *The EMBO Journal* **10**, 633-639

Ritter W, Plank C, Lausier J, Rudolph C, Zink D, Reinhardt D, Rosenecker J (2003) A novel transfecting peptide comprising a tetrameric nuclear localization sequence. *Journal of Molecular Medicine* **81**, 708-717

Rittner K, Benavente A, Bompard-Sorlet A, Heitz F, Divita G, Brasseur R, Jacobs E (2002) New basic membrane-destabilizing peptides for plasmid-based gene delivery in vitro and in vivo. *Molecular Therapy* **5**, 104-114

Rodriquez-Porcel M, Gheysens O, Chen IY, Wu JC, Gambhir SS (2005) Image-guided cardiac cell delivery using high-resolution small-animal ultrasound. *Molecular Therapy* **12**, 1142-1147

Rosenkranz AA, Lunin VG, Gulak PV, Sergienko OV, Shumiantseva MA, Voronina OL, Gilyazova DG, John AP, Kofner AA, Mironov AF, Jans DA, Sobolev AS (2003) Recombinant modular transporters for cell-specific nuclear delivery of locally acting drugs enhance photosensitizer activity. *The FASEB Journal* **17**, 1121-1123

Rosenkranz AA, Yachmenev SV, Jans DA, Serebryakova NV, Murav'ev VI, Peters R, Sobolev AS (1992) Receptor-mediated endocytosis and nuclear transport of a transfecting DNA construct. *Experimental Cell Research* **199**, 323-329

Roth DM, Moseley GW, Glover D, Pouton CW, Jans DA (2007) A microtubule-facilitated nuclear import pathway for cancer regulatory proteins. *Traffic* **8**, 673-686

Sadoni N, Cardoso MC, Stelzer EH, Leonhardt H, Zink D (2004) Stable chromosomal units determine the spatial and temporal organization of DNA replication. *Journal of Cell Science* **117**, 5353-5365

Salman H, Zbaida D, Rabin Y, Chatenay D, Elbaum M (2001) Kinetics and mechanism of DNA uptake into the cell nucleus. *Proceedings of the National Academy of Sciences of the USA* **98**, 7247-7252

Sanabria H, Kubota Y, Waxham MN (2007) Multiple diffusion mechanisms due to nanostructuring in crowded environments. *Biophysical Journal* **92**, 313-22

Schaarschmidt D, Baltin J, Stehle IM, Lipps HJ, Knippers R (2004) An epi-

somal mammalian replicon: Sequence-independent binding of the origin recognition complex. *The EMBO Journal* **23**, 191-201

Schaffer DV, Fidelman NA, Dan N, Lauffenburger DA (2000) Vector unpacking as a potential barrier for receptor-mediated polyplex gene delivery. *Biotechnology and Bioengineering* **67**, 598-606

Sclimenti CR, Neviaser AS, Baba EJ, Meuse L, Kay MA, Calos MP (2003) Epstein-Barr virus vectors provide prolonged robust factor IX expression in mice. *Biotechnology Progress* **19**, 144-151

Sebestyen MG, Ludtke JJ, Bassik MC, Zhang G, Budker V, Lukhtanov EA, Hagstrom JE, Wolff JA (1998) DNA vector chemistry: The covalent attachment of signal peptides to plasmid DNA. *Nature Biotechnology* **16**, 80-85

Shen MH, Mee PJ, Nichols J, Yang J, Brook F, Gardner RL, Smith AG, Brown WR (2000) A structurally defined mini-chromosome vector for the mouse germ line. *Current Biology* **10**, 31-34

Shimizu N, Kamezaki F, Shigematsu S (2005) Tracking of microinjected DNA in live cells reveals the intracellular behavior and elimination of extrachromosomal genetic material. *Nucleic Acids Research* **33**, 6296-6307

Shou Y, Ma Z, Lu T, Sorrentino BP (2006) Unique risk factors for insertional mutagenesis in a mouse model of XSCID gene therapy. *Proceedings of the National Academy of Sciences of the USA* **103**, 11730-11735

Simberg D, Danino D, Talmon Y, Minsky A, Ferrari ME, Wheeler CJ, Barenholz Y (2001) Phase behavior, DNA ordering, and size instability of cationic lipoplexes. Relevance to optimal transfection activity. *Journal of Biological Chemistry* **276**, 47453-47459

Soldati T, Schliwa M (2006) Powering membrane traffic in endocytosis and recycling. *Nature Reviews Molecular Cell Biology* **7**, 897-908

Sonawane ND, Szoka FC, Verkman AS (2003) Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. *The Journal of Biological Chemistry* **278**, 44826-44831

Sorgi FL, Bhattacharya S, Huang L (1997) Protamine sulfate enhances lipid-mediated gene transfer. *Gene Therapy* **4**, 961-968

Stehle IM, Scinteie MF, Baiker A, Jenke AC, Lipps HJ (2003) Exploiting a minimal system to study the epigenetic control of DNA replication: the interplay between transcription and replication. *Chromosome Research* **11**, 413-421

Stillman B (2005) Origin recognition and the chromosome cycle. *FEBS Letters* **579**, 877-884

Stoll SM, Sclimenti CR, Baba EJ, Meuse L, Kay MA, Calos MP (2001) Epstein-Barr virus/human vector provides high-level, long-term expression of alpha1-antitrypsin in mice. *Molecular Therapy* **4**, 122-129

Subramanian A, Ranganathan P, Diamond SL (1999) Nuclear targeting peptide scaffolds for lipofection of nondividing mammalian cells. *Nature Biotechnology* **17**, 873-877

Suh J, Wirtz D, Hanes J (2003) Efficient active transport of gene nanocarriers to the cell nucleus. *Proceedings of the National Academy of Sciences of the USA* **100**, 3878-3882

Suzuki N, Nishii K, Okazaki T, Ikeno M (2006) Human artificial chromosomes constructed using the bottom-up strategy are stably maintained in mitosis and efficiently transmissible to progeny mice. *The Journal of Biological Chemistry* **281**, 26615-26623

Tan PH, Manunta M, Ardjomand N, Xue SA, Larkin DF, Haskard DO, Taylor KM, George AJ (2003) Antibody targeted gene transfer to endothelium. *The Journal of Gene Medicine* **5**, 311-323

Tang CK, Lodding J, Minigo G, Pouniotis DS, Plebanski M, Scholzen A, McKenzie IF, Pietersz GA, Apostolopoulos V (2007) Mannan-mediated gene delivery for cancer immunotherapy. *Immunology* **120**, 325-335

Thyagarajan B, Guimaraes MJ, Groth AC, Calos MP (2000) Mammalian genomes contain active recombinase recognition sites. *Gene* **244**, 47-54

Tomizuka K, Yoshida H, Uejima H, Kugoh H, Sato K, Ohguma A, Hayasaka M, Hanaoka K, Oshimura M, Ishida I (1997) Functional expression and germline transmission of a human chromosome fragment in chimaeric mice. *Nature Genetics* **16**, 133-143

Tsoulfas G, Takahashi Y, Liu D, Yagnik G, Wu T, Murase N, Geller DA (2006) Hydrodynamic plasmid DNA gene therapy model in liver transplantation. *The Journal of Surgical Research* **135**, 242-249

Uherek C, Fominaya J, Wels W (1998) A modular DNA carrier protein based on the structure of diphtheria toxin mediates target cell-specific gene delivery. *The Journal of Biological Chemistry* **273**, 8835-8841

Vacik J, Dean BS, Zimmer WE, Dean DA (1999) Cell-specific nuclear import of plasmid DNA. *Gene Therapy* **6**, 1006-1014

Vanderbyl SL, Sullenbarger B, White N, Perez CF, MacDonald GN, Stodola T, Bunnell BA, Ledebur HC, Lasky LC (2005) Transgene expression after stable transfer of a mammalian artificial chromosome into human hematopoietic cells. *Experimental Hematology* **33**, 1470-1476

Vaughan EE, Dean DA (2006) Intracellular trafficking of plasmids during transfection is mediated by microtubules. *Molecular Therapy* **13**, 422-428

Vitiello L, Chonn A, Wasserman JD, Duff C, Worton RG (1996) Condensation of plasmid DNA with polylysine improves liposome-mediated gene transfer into established and primary muscle cells. *Gene Therapy* **3**, 396-404

Voet T, Vermeesch J, Carens A, Durr J, Labaere C, Duhamel H, David G, Marynen P (2001) Efficient male and female germline transmission of a human chromosomal vector in mice. *Genome Research* **11**, 124-136

Voziyanov Y, Pathania S, Jayaram M (1999) A general model for site-specific recombination by the integrase family recombinases. *Nucleic Acids Research* **27**, 930-941

Wade-Martins R, White RE, Kimura H, Cook PR, James MR (2000) Stable correction of a genetic deficiency in human cells by an episome carrying a 115 kb genomic transgene. *Nature Biotechnology* **18**, 1311-1314

Wagstaff KM, Glover DJ, Tremethick DJ, Jans DA (2007) Histone-mediated transduction as an efficient means for gene delivery. *Molecular Therapy* **15**, 721-731

Walther W, Stein U, Fichtner I, Schlag PM (2004) Low-volume jet injection for efficient nonviral *in vivo* gene transfer. *Molecular Biotechnology* **28**, 121-128

Wang J, Sugden B (2005) Origins of bidirectional replication of Epstein-Barr virus: Models for understanding mammalian origins of DNA synthesis. *Journal of Cellular Biochemistry* **94**, 247-256

Willard HF (2001) Neocentromeres and human artificial chromosomes: An unnatural act. *Proceedings of the National Academy of Sciences of the USA* **98**, 5374-5376

Wong LH, Saffery R, Anderson MA, Earle E, Quach JM, Stafford AJ, Fowler KJ, Choo KH (2005) Analysis of mitotic and expression properties of human neocentromere-based transchromosomes in mice. *The Journal of Biological Chemistry* **280**, 3954-3962

Wong LH, Saffery R, Choo KH (2002) Construction of neocentromere-based human minichromosomes for gene delivery and centromere studies. *Gene Therapy* **9**, 724-726

Woods NB, Muessig A, Schmidt M, Flygare J, Olsson K, Salmon P, Trono D, von Kalle C, Karlsson S (2003) Lentiviral vector transduction of NOD/SCID repopulating cells results in multiple vector integrations per transduced cell: Risk of insertional mutagenesis. *Blood* **101**, 1284-1289

Wu H, Ceccarelli DF, Frappier L (2000) The DNA segregation mechanism of Epstein-Barr virus nuclear antigen 1. *EMBO Reports* **1**, 140-144

Xiao CY, Hubner S, Jans DA (1997) SV40 large tumor antigen nuclear import is regulated by the double-stranded DNA-dependent protein kinase site (serine 120) flanking the nuclear localization sequence. *The Journal of Biological Chemistry* **272**, 22191-22198

Xiao CY, Jans P, Jans DA (1998) Negative charge at the protein kinase CK2 site enhances recognition of the SV40 large T-antigen NLS by importin: effect of conformation. *FEBS Letters* **440**, 297-301

Xu Y, Szoka FC (1996) Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry* **35**, 5616-5623

Zabner J, Fasbender AJ, Moninger T, Poellinger KA, Welsh MJ (1995) Cellular and molecular barriers to gene transfer by a cationic lipid. *The Journal of Biological Chemistry* **270**, 18997-19007

Zanta MA, Belguise-Valladier P, Behr JP (1999) Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. *Proceedings of the National Academy of Sciences of the USA* **96**, 91-96

Zhang Y, Zhang YF, Bryant J, Charles A, Boado RJ, Pardridge WM (2004) Intravenous RNA interference gene therapy targeting the human epidermal growth factor receptor prolongs survival in intracranial brain cancer. *Clinical Cancer Research* **10**, 3667-3677

Ziady AG, Ferkol T, Dawson DV, Perlmutter DH, Davis PB (1999) Chain length of the polylysine in receptor-targeted gene transfer complexes affects duration of reporter gene expression both *in vitro* and *in vivo*. *The Journal of Biological Chemistry* **274**, 4908-4916

Zuhorn IS, Kalicharan R, Hoekstra D (2002) Lipoplex-mediated transfection of mammalian cells occurs through the cholesterol-dependent clathrin-mediated pathway of endocytosis. *The Journal of Biological Chemistry* **277**, 18021-18028