Modular Multifunctional Protein Vectors for Gene Therapy

Hugo Peluffo

Department of Histology and Embryology, Faculty of Medicine, University of the Republic (UDELAR) and Neurodegeneration Laboratory, Institut Pasteur de Montevideo Uruguay

1. Introduction

The introduction of genes into the organism or the regulation of the expression of endogenous genes has emerged in the last decade as a very potent strategy for correcting monogenic inherited diseases, treating acute disorders, and slowing down the progression of diseases without known cure. In addition it constitutes an important tool for research, which has been widely used and has contributed to show the mechanisms behind several physiological processes and pathologies.

Adequate carriers able to transfer DNA or RNA into target cells have been largely explored. However, this is an area under continuous expansion as there is no ideal vector suitable for all applications. In fact, no individual vector will meet all the characteristics for a perfect or ideal vector, as many of the needs are different and even contradictory. For example, immunogenicity is in most cases an undesirable side effect, while it is a valuable property when treating tumours as it contributes to their clearance. Another example of contradictory needs of one single vector would be the capacity of a vector to determine the overexpression of the transgenic protein for life. This would be an essential property for the treatment of inherited diseases produced by the lack of a particular protein, however for the treatment of acute injuries the lifelong expression of a therapeutic protein will probably be deleterious. Moreover, some vectors do not transduce post-mitotic cells like neurons or muscle fibres, which is a drawback for targeting these cell types but may be an advantage for the targeting of cancer cells. Thus, there is a need for diverse type of vectors for diverse therapeutic or experimental paradigms, and in particular versatile tuneable vectors would be very interesting. Moreover, several basic problems with the known vectors persist, like toxicity, oncogenicity, immunogenicity, low transfection efficiency, or poor bioavailability, which need further consideration and efforts.

Due to their natural efficiency, viruses have been modified to act as vectors, and they have shown a good degree of success. Non-viral vectors have also been developed by combining several properties necessary for transfection: nucleic acid attachment and condensation, cell attachment, cell entry, endosomal escape, intracellular trafficking, nuclear entry, and nucleic acid release. Some of these vectors are quite simple, as the ones formed by the combination of nucleic acids and lipid components or other carriers like polyethylene glycol (PEG). Others include the previous components but have in addition attached targeting molecules like antibodies, enabling these vectors to preferentially transfect a given tissue. In fact even

magnetic fields have been used to concentrate suitable engineered vectors to a given area (Corchero and Villaverde 2009).

An interesting type of non-viral vectors is the one based on multifunctional proteins (Aris and Villaverde 2004; Mastrobattista *et al.* 2006). The combination of functional domains in a single polypeptide is a simple yet powerful approach for the development of vectors suitable for gene therapy. In fact, this approach has generated the first prototypes of modular protein gene therapy vectors. Three general methods have been used for the engineering of these molecules: i) production of a recombinant protein by the direct fusion of the functional domains; ii) production of a recombinant protein by combining a known scaffold protein and several functional domains inserted into exposed regions of the scaffold protein; and iii) chemical conjugation of functional domains and proteins. Many of these vectors can be produced recombinantly, generating reproducible and stable stocks appropriate for the formulation of clinically usable drugs. Moreover, the modular nature of these versatile vectors enables the combination of different domains to fulfil the changing requirements of pathological end experimental situations.

2. Functional domains available

2.1 Nucleic acid attachment and condensation

Trans-membrane transport of DNA is an inefficient process, and thus the successful introduction of a transgene into a target cell must include two important steps regarding the plasmid or oligonucleotide DNA that is included in the vector. First, the extended DNA needs to be condensed into an ordered compact nano-particle, and second, once inside the nucleus, the DNA must be de-condensed and thus accessible to transcription. Basic peptides or polycations have been exploited for the interaction with the DNA backbone due to their electrostatic interactions (Bloomfield 1996; Saccardo *et al.* 2009)(see Table 1). When DNA is mixed with these condensing agents, smaller molecules of different shapes are formed mainly depending on DNA size (Vijayanathan *et al.* 2002). For instance, in the absence of DNA, the HNRK modular vector that uses poly-lysine for DNA condensation, self-organize as amorphous, polydisperse particulate entities ranging from a few nanometres up to around one micron. However, in the presence of DNA, protein-DNA complexes appear as tight and rather monodisperse spherical-like nanoparticles of around 80 nm in diameter (Domingo-Espín *et al.* 2011). The most widely used condensing agent is a poly-lysine chain (Saccardo *et al.* 2009). Poly-lysine polymers containing at least 6 lysines will efficiently condense DNA, however, additional 4 to 9 lysines are needed to fully condensate the DNA into smaller particles of 50-100nm, increasing in this way many folds the transfection capacity (Wadhwa *et al.* 1997). Other basic peptides used are the poly-arginine peptides, which not only induce DNA condensation, but also show membrane translocation potential (Futaki *et al.* 2001) and nuclear translocation capacity, determining in this way transgene expression (Kim *et al.* 2003; Vazquez *et al.*).

The condensed DNA by these peptides is partially protected from cellular acid nucleases of the lysosomal compartment (Krishnamoorthy *et al.* 2003; Ross *et al.* 1998; Wolfert and Seymour 1998) and serum nucleases, inducing an extended half-life in serum (Kumar *et al.* 2007) and in the circulation, making tissue targeting possible (Kawabata *et al.* 1995; Nishikawa *et al.* 2000a). For example, the addition of the acid nuclease inhibitor DMI-2 induced a 10-fold increase in receptor-mediated transfection in cultured cells exposed to a

Surfactant Protein A-poly-lysine modular vector or a transferrin-poly-lysine modular vector (Ross *et al.* 1998). Naturally DNA condensing proteins have also been used for the construction of modular vectors. For instance, Histones condense plasmid DNA and protects it from endonucleases, being the lysine-rich H1 Histone the most effective one (Pyhtila *et al.* 1976). Moreover, some nuclear localization signals like the NLS peptide from SV40 virus large T-antigen are lysine-rich peptides that when used as a tetramer can efficiently condense DNA without losing its nuclear localization properties (Ritter *et al.* 2003).

2.2 Cell attachment and cell targeting

When a viral or non-viral gene therapy vector is injected intravenously, most of the vectors will localize mainly in the liver but also in the kidneys, lungs and spleen. While this is normally a problem to circumvent for most gene therapy applications, it constitutes an advantage for the expression of molecules in the liver. There are many fetal metabolic diseases resulting from a defect or a deficiency of hepatocyte-derived proteins. Moreover, the liver can be considered as a platform to produce various proteins secreted into the blood. Therefore, many pioneer studies focused on the development of more efficient gene delivery systems for the introduction of therapeutic genes selectively into hepatocytes (Wu and Wu 1988). Intravenously injected plasmids are cleared from the circulation by the liver non-parenchymal cells by a scavenging receptor mediated mechanism (Kawabata *et al.* 1995). When Nishikawa and colleagues administered naked 32 PDNA into the tail vein of mice, about 40% and 10% of the radioactivity rapidly accumulated in the liver and kidneys, respectively (Nishikawa *et al.* 2000b). Again, the main cell-types targeted were the liver non-parenchymal cells: Kupffer cells and endothelial cells. When they injected a vector composed of 32 PDNA/polyornithine, little effect on the distribution of the DNA was observed. However, the injection of the 32 PDNA/Gal-pOrn galactose-mediated hepatocyte-targeting vector induced a 60% hepatic accumulation of radioactivity, but more interestingly, most of the targeted cells were now hepatocytes instead of Kupffer or endothelial cells. The same effect was observed at the level of luciferase transgene expression, indicating that the DNA/Gal-pOrn vector was not only able to adhere and enter preferentially into hepatocytes, but it could also transfect them.

Many different domains of known proteins and sugars have been used for cell targeting of modular vectors, like galactose (Wu and Wu 1987), transferrin (Wagner *et al.* 1990), foot-and-mouth disease virus integrin interacting peptide (Aris *et al.* 2000; Aris and Villaverde 2003; Domingo-Espín *et al.* 2011), nerve growth factor (Ma *et al.* 2004; Zeng *et al.* 2004), surfactant protein A (Ross *et al.* 1995), rabies virus glycoprotein (Kumar *et al.* 2007), tetanus toxin fragment Hc (Box *et al.* 2003; Knight *et al.* 1999), cholera toxin b chain (Barrett *et al.* 2004), and neurotensin (Navarro-Quiroga *et al.* 2002). In an interesting study, Arango-Rodríguez and colleagues showed that they could target only substantia nigra neurotensin high affinity receptor positive neurons by means of a modular vector that displayed neurotensin, while no other neurons were transfected (Arango-Rodríguez *et al.* 2006). *In vivo*, many of these targeting systems have shown success (see Table 1). An additional interesting targeting strategy is the use of antibodies (Berhanu and Rush 2008; Buschle *et al.* 1995; Thurnher *et al.* 1994). For instance, the use of the 1E3 antibody against the Tn antigen expressed on many carcinomas coupled to polylysine induced an important increase in the transfection of a cancer cell line (Thurnher *et al.* 1994). Another vector, named fkAbp75-*ipr*, possess several

Name	Functional Domains	Production Method	Particle Size with nucleic acid (nm)	Gene	Target cells	Administration route	Time of transgene expression	Functional effect	Toxicity	Authors
ASOR-PL	Asialoosomucoid glycoprotein, Poly-Lys	Chemical conjugation	ND	Human serum albumin	Asialoglyco-protein receptor+ Hepatocytes	i.v.	At least 4 weeks	Partial correction of analbuminemia and hypercholesterolemia	ND	(Wilson et al. 1992; Wu et al. 1991; Wu and Wu 1987)
F105-P	α HIV-gp160 Fab fragment, protamine	Expressed in COS cells	ND	c-myc, MDM2, VEGF siRNA	HIV-gp160 overexpressing cancer cells	i.v.	ND	Partial inhibition of tumour growth	No interferon response detected	(Song et al. 2005)
Cal-PL	Galactose, Poly-Lys	Chemical conjugation	10-12	Human Factor IX	Asialoglyco-protein receptor+ Hepatocytes	i.v.	At least 140 days	No, but increased blood human Factor IX	ND	(Peraltes et al. 1994)
Man-PL	Mannose, Poly-Lys	Chemical conjugation	220	Chloramphenicol acetyltransferase	Mannose receptor+ hepatic Kupffer cells	i.v.	At least 2 days	ND	ND	(Nishikawa et al. 2000a)
PEI600/DNA/NL4-10K	PEI600, NCF loop4, Poly-Lys	Chemical synthesis and charge interaction assembly	180.4±5.5	Luciferase	DRG neurons	i.t.	At least 2 days	ND	No	(Zeng et al. 2007)
MC192-P-L-I	MAB MC192 α p75 ^{NTR} , Poly-Lys	Chemical conjugation	ND	GDNF	p75 ^{NTR} + cells	Cell foam at nerve transection site	At least 8 weeks	Neuroprotection of motor neurons after peripheral nerve transection	ND	(Barati et al. 2006)
fkA β p75- <i>ipr</i>	MAB MC192 α p75 ^{NTR} , HA2, Poly-Lys, SV40-NLS	Chemical conjugation	ND	TrkA siRNA	p75 ^{NTR} + cells	i.c.v. Osmotic pump	20 days	Impaired spatial memory after TrkA downregulation	ND	(Berhanu and Rush 2008)
249AL	β -Galactosidase scaffold, integrin binding FMDV RGD, Poly-Lys	Bacterial recombinant	20-40x100-200 filaments	GFP	α v β 3 and other α v integrin+ cells	i.c.	3 days	ND	No	(Aris et al. 2000; Peluffo et al. 2003)
NLS ζ	β -Galactosidase binding FMDV RGD, Poly-Lys, SV40-NLS	Bacterial recombinant	ND but probably similar to 249AL	Cu/Zn SOD	α v β 3 and other α v integrin+ cells	i.c.	3 days	Neuroprotection from excitotoxic brain lesion after Cu/Zn SOD overexpression	No, in fact neuroprotective per se	(Aris and Villaverde 2003; Peluffo et al. 2006)

Name	Functional Domains	Production Method	Particle Size with nucleic acid (nm)	Gene	Target cells	Administration route	Time of transgene expression	Functional effect	Toxicity	Authors
LDL-DNA	Several domains of the Apo B100 protein of LDL	Isolated from human and rat plasma	ND	GFP and luciferase	Apoprotein B100 receptor+ cells	i.v.	2 days	ND	ND	(Guevara et al.)
RVG+9R	Rabies virus glycoprotein (29aa), Poly-Arg	Chemical synthesis	ND	GFP, siRNA, Cu/ZnSOD siRNA, and FvEJ siRNA	Acetylcholine receptor+ cells	i.v.	2-3 days	Downregulation of genes, protection from mortal viral encephalitis	No inflammation nor specific antibodies produced	(Kumar et al. 2007)
Gal-pOrn-mHA2	Galactose, Poly-Orn, mHA2	Chemical synthesis	130.9 ± 22.6	Luciferase	Asialoglyco-protein receptor+ Hepatocytes	i.v.	2 days	ND	ND	(Nishikawa et al. 2000b)
Fusogenic-karyophilic-NT-polyplex	Neurotensin, HA2, Poly-Lys, SV40-NLS	Charge interaction assembly and chemical conjugation	<200	GFP, GDNF	High affinity Neurotensin receptor+ cells	i.c.	2 months	Neuroprotection and functional recovery in a model of Parkinson's Disease	ND	(Arango-Rodriguez et al. 2006; Gonzalez-Barrios et al. 2006; Navarro-Quiroga et al. 2002)
SPKRANL1-2/PEI600	SPKR, NGF loops 1 and 2, poly-His	Recombinant and charge interaction assembly	100-200	Luciferase	TkA and p75NTR+ cells	i.t.	3 days	ND	ND	(Ma et al. 2004)
DNA/THPEI/PEG	Transferrin, PEI, PEG	Charge interaction assembly and chemical conjugation	200-400	Luciferase	Transferrin receptor+ cells	i.v.	2 days	Preferential tumour targeting	ND	(Ogris et al. 1999)
Tat-PTD-DRBD	Tat-PTD, DRBD, poly-His	Recombinant	ND	Luciferase siRNA	Non-selective	i.n.	3-4 days	No, but downregulation of luciferase	ND	(Eguchi et al. 2009)
Tt-HSV-TK	Biotinylated-DNA, streptavidin, biotinylated-transferrin	Chemical conjugation	ND	herpes simplex virus thymidine kinase	Transferrin receptor+ cells	i.v.	At least 7 days	Decreased metastasis and increased survival	ND	(Sato et al. 2000)

Abbreviations: RGD: Arg-Gly-Asp; NGF: Nerve growth factor; i.v.: intravenous; i.c.: intracerebral; i.t.: intrathecal; i.n.: intranasal; i.c.v.: intracerebroventricular; i.n.: intranasal; LDL: low-density lipoprotein; HA2: peptide from influenza virus hemagglutinin subunit HA-2; i.t.: intrathecal; SPKR: DNA binding motif derived from Histone H1; FMDV: foot-and-mouth disease virus; PEG: poly (ethylene glycol); PEI: polyethyleneimine; Tat-PTD: Tat peptide transduction domain; DRBD: (ds)RNA-binding domain; GDNF: Glial derived neurotrophic factor; TK: thymidine kinase.

Table 1. Modular protein vectors effective in vivo

functional domains (see Table 1), being one of them the monoclonal antibody MC192 against p75^{NTR}, the low affinity neurotrophin receptor (Berhanu and Rush 2008). By injecting it intracerebroventricularly coupled to siRNA against TrkA, one of the high affinity neurotrophin receptors, Berhanu and Rush were able to down-regulate TrkA expression in p75^{NTR} expressing cells and correlate this with functional alterations like impaired spatial memory.

2.3 Endosomal escape

A limiting step for receptor-mediated gene delivery is the escape from endosomes, as the vector needs to gain access to the cytosol to enter the nuclei. Fusogenic peptides are reported to strongly enhance *in vitro* gene transfer after being incorporated into carrier systems by chemical linkage (Box *et al.* 2003; Fisher and Wilson 1997; Navarro-Quiroga *et al.* 2002; Nishikawa *et al.* 2000b; Ogris *et al.* 2001; Wagner *et al.* 1992) or by ionic interaction (Gottschalk *et al.* 1996; Plank *et al.* 1994), but co-treatment of the cells with the vector and the fusogenic peptide may also be effective (Read *et al.* 2005). The most widely used method for endosome escape is based on the amino-terminal motif of influenza virus hemagglutinin subunit HA2 (Plank *et al.* 1994; Wagner *et al.* 1992). For example, Nishikawa and colleagues showed that when an acid-sensitive fusogenic peptide derived from HA2 was incubated with mouse erythrocytes at pH 5.0, it induced hemolysis while it did not show any significant hemolytic activity at pH 7.4 (Nishikawa *et al.* 2000b). Interestingly, the same study showed that the *in vivo* liver transgene expression obtained after intravenous injection of the vector DNA/Gal-pOrn-mHA2 was 300 fold higher than that obtained with the same vector lacking the HA2 domain (Nishikawa *et al.* 2000b).

Other domains used for DNA condensation or vector purification as polylysine or polyhistidine have shown endosome disrupting activities (Read *et al.* 2005; Zauner *et al.* 1997). The most effective ones were histidine rich polyplexes formed by the condensation of approximately 50 monomers of Cys-His₆-Lys₃-His₆-Cys and DNA (Read *et al.* 2005). In this study, the endosomolytic agent chloroquine, which normally enhance the transfection capacity of most non-viral vectors, did not enhance the transfection with the histidine rich polyplexes while it enhanced transfection with other non-viral vectors, suggesting that the poly-his domains are in fact endosomolytic. Though polycations like polylysine may be toxic to cells, especially if they have membrane-disrupting activity, this polyhistidine vector showed no toxicity. Histidine becomes positively charged when the pH decrease to less than 7 and thus becomes useful for the permeabilization of the endosomal membrane induced by acidification of endosomes, increasing cell transfection (Midoux *et al.* 1998). *In vivo*, many of these endosomal escape systems have shown success (see Table 1). For an extensive review on different strategies and domains used for endosomal escape please refer to Ferrer-Mirallès *et al.* (Ferrer-Mirallès *et al.* 2008).

For the introduction of siRNA and DNA into cells, several cationic peptide transduction domains or also called cell-penetrating domains have been used. TAT, 8xArg, Hph-1or Antp domains can deliver a wide variety of cargo into primary cells, to most tissues, and are in addition being evaluated in clinical trials (Gump and Dowdy 2007). For instance, when the Tat-domain was combined with a poly-His domain and the (ds)RNA-binding domain DRBD, the vector coupled to siRNA could successfully down-regulate Luciferase expression in the nasal and tracheal passages for 4 days after intranasal administration (Eguchi *et al.* 2009). An important characteristic of these systems using cell-penetrating peptides is that they are not cell-specific, and thus should be used for general non-selective transfection. A

careful evaluation of the toxicity of this cell penetrating domains in *in vivo* settings has to be performed as toxicity of Tat protein has been reported (Cardozo *et al.* 2007), specially for the CNS (Bonavia *et al.* 2001; Nath *et al.* 1996). In addition, toxicity of the Antp domain has also been reported for many cell types (Cardozo *et al.* 2007).

2.4 Nuclear translocation

The transgene expression levels obtained after plasmid DNA injection into the cytoplasm or the nucleus showed that de nuclear double membrane and its pores are important barriers for naked DNA (Liu *et al.* 2003; Pollard *et al.* 1998). The selection of macromolecules that will be actively imported into the nucleus occurs at the nuclear pore complex, which is composed of more than 50 different proteins. The pore complex will recognise importin proteins bound to short (normally 4-8 amino acids) nuclear localization signals which can be located almost anywhere in the amino acid sequence of the protein, and which are rich in the positively charged amino acids lysine and arginine and usually contains proline (Pouton 1998). This mechanism has been exploited for the design of modular protein vectors, introducing nuclear localization sequences like the SV40 NLS peptide from the T antigen (Aris and Villaverde 2003; Fritz *et al.* 1996). For instance, Aris and co-workers introduced this nuclear localization sequence into the 249AL modular vector (see Table 1) and they observed an enhanced transgene expression with the resulting vector termed NLSCt (Aris and Villaverde 2003). However, studies performed in cells in culture show that even in the presence of nuclear localizations sequences, complexes of more than 60nm seem to be excluded (Chan *et al.* 2000). This data are in contrast to the high transfection efficiency obtained, even *in vivo*, with different modular protein vectors that exceeds this size, reaching 200nm (see Table 1). One can speculate that in fact some molecules of up to 200nm can be imported into the nucleus by being flexible, or that during the interaction of the vector with the nuclear import machinery the vector is disassembled and only the DNA is imported.

Another important step for efficient transgene expression may be the release of the nucleic acid from the vector once in the nucleus. Several studies have addressed the possible enhancement of the release of the DNA by the cellular reducing conditions. For example, histidine rich polyplexes were able to release the complexed DNA when exposed to the reducing agent Dithiothreitol (DTT), suggesting that in cells a similar mechanism would occur (Read *et al.* 2005). In fact, the increase in the cellular antioxidant and reducing agent glutathione, induced an important 200 fold increase in the transfection observed with the histidine rich polyplexes, but only a 3fold increase was observed with the PEI/DNA vector, another non-viral vector with no reduction-dependent release of DNA. Though this is an interesting phenomenon, it is difficult to understand why the cytosol reducing conditions do not disassemble the vector too early, determining that the DNA is released into the cytosol instead of inside the nucleus, not favouring the transfection process.

2.5 Trophic vectors/functional vectors

An attractive possibility is the combination of the effects mediated by the overexpression of a transgene and the direct effects of the vector per se. In fact, as modular vectors normally take advantage of a cell attaching motif for receptor mediated endocytosis, they tend to display intrinsic activities. More importantly, the use of trophic factors or toxin domains for cell attachment and internalization is ideal, as their natural mechanism of action includes the attachment to high affinity cell surface receptors, the endocytosis to early endosomes,

and even being transported to the cell soma in the case of neurons (Lalli and Schiavo 2002). An interesting modular vector was produced combining a polylysine tail with the loop 4 of the nerve growth factor (NGF) (Zeng *et al.* 2004; Zeng and Wang 2005). This “trophic vector” maintained the trophic effects of NGF, was able to condensate DNA, and when combined with polyethylenimine (PEI600), transfected cells in culture that expressed NGF receptors but not cells without these receptors. Interestingly, the DNA-PEI600 showed a size of 445nm and an zeta potential of 6,2mV, but the addition of the NGF loop4 poly-lysine peptide to the complex induced the formation of smaller 180nm particles with a zeta potential of 23,2mV (Zeng *et al.* 2007). This shows that the addition of targeting peptides to non-specific DNA/condensing products complexes may in fact contribute to enhance not only the targeted delivery but also to decrease the particle size and charge of the resulting vector. A somehow more complex trophic vector including NGF loops was also produced. It combined the loops 1 and 2 of NGF and the SPKR4 domain derived from histone H1 DNA binding motif, linked together by a α -helical linker (Ma *et al.* 2004). Both NGF-loop derived vectors could even transfer a transgene *in vivo* preferentially to dorsal root ganglia neurons (which express NGF receptors) after intrathecal spinal cord injection (Ma *et al.* 2004; Zeng *et al.* 2007). Several toxins have been used as cell attachment motifs (Andreu *et al.* 2008; Box *et al.* 2003; Knight *et al.* 1999), and some motifs of these toxins can in fact display trophic effects (Chaib-Oukadour *et al.* 2004), and have thus been used to design trophic vectors.

An important consideration regarding many acute injuries is that the therapeutic time window is short. In those cases, a direct trophic or functional effect of the vector per se could extend the therapeutic window, giving time for the transgene to be expressed and mediate its own effects. For example, the neuroprotection observed after an acute brain injury using the vector termed NLSCt was partially mediated by the transgene overexpressed, but also partially mediated by the RGD integrin-interacting motif of the vector itself (Peluffo *et al.* 2006; Peluffo *et al.* 2007). In this experimental setting the direct injection of the vector into the lesioned brain area was performed 4 hours after the lesion. The CNS is a tissue that tolerates injuries very badly due to its high dependence on blood flow and oxygen consumption, and its poor regeneration capacity. This determines that the therapeutic window for the treatment of acute injuries is very short. Interestingly, even in this experimental paradigm, the modular recombinant NLSCt vector overexpressing the anti-oxidant enzyme Cu/Zn superoxide dismutase (SOD) could mediate neuroprotection (Peluffo *et al.* 2006). These studies shows the wide possibilities of combining the vectors themselves with active protein domains like trophic factors, which will exert rapid direct effects, which in turn may increase the therapeutic window or the potency of the effect of the transgene used.

3. Immunogenicity and inflammation

The introduction of modular protein vectors into the organism may be accompanied by a humoral or cell-mediated immune response against the inserted motifs, which in many cases are derived from viral molecules. However, when injected intravenously, the Rabies virus glycoprotein (29aa)-Poly-Arg vector (RVG-9R) (see Table 1) did not induce an antibody response or an increase in several pro-inflammatory cytokines evaluated (Kumar *et al.* 2007). In another example, when the recombinant 249AL vector (see Table 1) was injected into the normal postnatal brain, no changes were observed in glial activation, demyelination, recruitment of cytotoxic CD8 lymphocytes, or expression of IL1 β . Interestingly, when a very similar vector termed NLSCt (see Table 1) was injected into the postnatal brain after an

excitotoxic injury, an increase in macrophage/microglia number and in the levels of IL1 β and Cox2 enzyme were observed in the lesion (Gonzalez *et al.* 2011). Most interesting, the same set of studies discovered that this vector, with or without accomplished control DNA, besides inducing an inflammatory response, also induced a decrease in the brain lesion volume and in the number of degenerating neurons (Peluffo *et al.* 2006; Peluffo *et al.* 2007), an effect that was mediated by the prototypic RGD-integrin interacting motif of the vector (Peluffo *et al.* 2007). These data may suggest that the modulation of the inflammation by the vector may be beneficial under some circumstances. Another vector termed Tat-PTD-DRBD (see Table 1) did not induce interferon (IFN)- α or tumour necrosis factor (TNF)- α responses when incubated with primary human peripheral blood mononuclear cells (Eguchi *et al.* 2009). Thus, the overall data suggests that these types of vectors are less immunogenic and pro-inflammatory than most viral and other non-viral vectors.

4. Administration routes and transgene expression

If these types of vectors are useful for gene therapy applications is still an open question, and adequate testing of these vectors in preclinical and actual clinical studies need to be performed. In fact, it has been well established that there is no ideal vector for all gene therapy applications, being the characteristics of each vector critical for each pathological paradigm. The use of modular protein vectors is limited to pathologies accepting an acute treatment, but would be ineffective for chronic ones as the transgene expression that they determine is normally short lived. The time of transgene expression varies from a few days to more than two months, depending on the doses and the method of administration. For instance, multifunctional recombinant vectors can induce the *in vivo* brain expression of a reporter gene after direct injection into the brain in a model of acute brain injury, lasting the transgenic protein in the brain for 3 days (Peluffo *et al.* 2003), but another vector was able to determine expression in normal brain for two months after intracerebral injection (Navarro-Quiroga *et al.* 2002). In the case of other administration routes and pathologies, as for example the intravenous administration of these vectors, the time for transgenic protein expression in the liver may range from a few days to more than 4 months (Perales *et al.* 1994). In another study, the liver-selective and transient overexpression of the therapeutic protein human coagulation factor IX could be achieved using a synthetic modular glycoprotein vector, and secreted factor IX into the serum could be detected for 30 days (Ferkol *et al.* 1993). This same paradigm could be used for vaccination, overexpressing transiently the desired immunogenic protein (Chen and Huang 2005). Even the use of modular vectors coupled to plasmids producing shRNA show potent downregulation of an endogenous gene during 20 days when infused with osmotic pumps into the nervous system (Berhanu and Rush 2008). In all this approaches, the transient expression of a protein by means of multifunctional vectors would be desirable when compared to viral vector inoculation, which present higher risks of oncogenic and inflammatory complications, may produce very high levels of transgenic protein, and will produce the transgenic protein for life or for extended periods.

5. Pharmacokinetics and biodistribution

Various approaches have been undertaken to overcome the interaction of vectors with blood components to avoid aggregation as well as embolisms. Moreover for most strategies, the phagocytic clearing system of the organism must be eluded. Pharmacokinetic analysis has

shown that physicochemical properties of the vectors such as molecular weight, electrical charge and immunogenicity (or pre-existing antibodies in the organism) are important determinants for the *in vivo* success of the treatment. In addition, the volume and shape of the final vector is also important as it determines if the complex will be internalized into the cell and the cell nucleus. P^{32} -plasmid DNA is rapidly eliminated from the circulation after intra-venous injection in mice (Kawabata *et al.* 1995), mainly by a scavenger receptor mechanism-mediated uptake by hepatic phagocytes (Kawabata *et al.* 1995; Takakura *et al.* 1999). Thus, the *in vivo* plasmid delivery needs to modify its physicochemical properties by condensing carriers. One interesting example is the Mannose-Poly-Lysine vector (Man-PL), which was designed to accommodate plasmid DNA for the mannose receptor-mediated transfection of liver endothelial cells and Kupffer phagocytes. After intravenous injection, the Man-PL- P^{32} DNA vector disappeared from the plasma with a half-life of 1 minute, being 80% of the radioactivity recovered from the liver at 10 minutes (mainly in the mannose receptor+ target cells) and less than 1% at lungs or kidneys at 1 hour after (Nishikawa *et al.* 2000a). This vector had a size of 220nm and a zeta potential of 12,1mV, while the DNA alone had a size of 200nm and a zeta potential of -36,4. This type of study clearly shows the importance of the condensation of plasmid DNA into small less charged particles. In accordance, it has been described that positively charged DNA complexes can activate the alternative complement pathway (Plank *et al.* 1996). The conjugation of vectors with hydrophilic polymers has been shown to decrease their interaction with plasmatic proteins and blood cells, increasing their half-life in circulation. One of the most used polymers is poly (ethylene glycol) (PEG). In an interesting study, Ogris and colleagues compared the blood stability of DNA/transferrin/PEI vectors with or without covalently linked PEG. The non-PEGylated vectors aggregated in plasma, bound several plasmatic proteins like IgM, fibrinogen, fibronectin, and complement C3, and also induced erythrocyte aggregation (Ogris *et al.* 1999). Interestingly, the PEGylated vector showed stable complex size, reduced surface charge, reduced binding of plasmatic proteins and erythrocyte aggregation, and most important, increased *in vivo* circulation half-life combined with enhanced transfection selectivity towards tumours.

An interesting vector for improving pharmacokinetics could be the use of natural circulating molecules, like the low-density lipoprotein (LDL). In fact it has been shown that LDL can act as a vector when mixed with plasmid DNA and injected intravenously, reaching several organs including the brain, heart, kidneys and spleen (Guevara *et al.*). The LDL molecule is composed of a highly hydrophobic core, surrounded by a shell of phospholipids and unesterified cholesterol, as well as a single copy of Apo B100 protein (Segrest *et al.* 2001). The B100 protein contains several motifs that explain the vector profile of the LDL: i) a motif that enable nucleic acid binding, ii) a motif that mediate cellular uptake, and iii) a motif that is apparently involved in transferring DNA into the cell nucleus (Guevara *et al.*). Interestingly, low-density particles composed of lipid, Apo B100, RNA, and core protein of hepatitis C virus were reported in the plasmas of individuals infected with this virus (Andre *et al.* 2002). This and other studies suggested that virus might utilize the potential of the LDL particle to act as a vector as a mechanism for persistent chronic infection.

6. Preclinical studies

Many interesting preclinical studies have been performed with modular multifunctional protein vectors (see Table 1). The first studies showing *in vivo* functional effects using

modular protein vectors were made by Wu and colleagues. By injecting intravenously the asialoorosomucoid glycoprotein-polylysine vector (ASOR-PL, see Table 1), they targeted hepatocytes and were able to partially and temporary correct analbuminemia and hypercholesterolemia by overexpressing human serum albumin or functional LDL receptor respectively (Wilson *et al.* 1992; Wu *et al.* 1991; Wu and Wu 1987). In another study, neuroprotection from an acute brain injury was achieved after direct intracerebral injection of the NLSCt vector overexpressing the antioxidant enzyme Cu/ZnSOD (see Table 1). In this experimental setting the overexpression of the therapeutic protein could not only induce reduced infarct volume but also functional improvement of the animals (Peluffo *et al.* 2006). Here, the vector was injected directly into the lesioned brain by a tightly controlled microinjector, using a similar protocol reported for the injection of cells into the human Parkinsonian brain (Brundin *et al.*), or for the intracerebral injection of adeno-associated viral vectors for the treatment of infantile lysosomal storage disease (Worgall *et al.* 2008). Thus, this direct intracerebral injection approach could show some benefits in clinical cases of focal traumatic or ischemic injuries, where a delimited area is lesioned and where in some cases even a decompressing craniectomy is needed leaving a direct entrance to the brain parenchyma. Modular protein vectors have also been used for neuroprotection after acute peripheral nerve transection. For example, Barati and colleagues (Barati *et al.* 2006) delivered a polylysine-based polyplex targeting p75^{NTR} positive cells accomplishing the plasmid encoding for GDNF after a peripheral nerve transection (see Table 1). They showed an almost complete reversal in neuronal death caused by GDNF transgene expression. Though this is a very interesting study, the authors performed a subtle pre-lesion to the nerve one week before the nerve transection injury to upregulate p75^{NTR} receptor, and thus the same experiment should be repeated but under more clinically relevant conditions. In another preclinical setting, the intravenous injection (once a day during 3 consecutive days) of the RVG-9R vector accomplished to the antiviral siRNA siFvEJ (see Table 1) was able to induce 80% survival of animals 30 days after their inoculation with a fatal flavivirus (Kumar *et al.* 2007). Though many modular vectors have been shown to mediate over-expression or down-regulation of reporter genes, they need to be tested *in vivo* in clinically relevant models for the establishment of their real potential.

7. Conclusion

More complex modular protein vectors including all the important domains for efficient nucleic acid delivery need to be engineered. They should include domains for DNA attachment and condensation, cell attachment and endocytosis, endosomal escape, cytosol trafficking towards the nucleus, nuclear import, and DNA release. HNRK (Domingo-Espín *et al.* 2011), fkAbp75-ipr (Berhanu and Rush 2008), and the fusogenic-karyophilic-NT-polyplex (Navarro-Quiroga *et al.* 2002) (see Table 1) are three prototypes of this increasingly complex vectors, but additional domains have to be inserted. In addition, an interesting strategy could be the exploitation of several domains that have dual functions, like poly-his with DNA attachment and endosomal escape properties, like melittin with endosomal escape properties and nuclear import potential, or histones with DNA attachment and nuclear import potential. Considering the fact that for example several cellular nuclear proteins have several nuclear localization domains, the introduction of several domains with the same function in the same vector may further increase their efficiency. In fact, dual targeting of cancer cell lines using both transferrin and RGD domains showed synergistic effects (Nie *et al.*). Furthermore, the

combination of engineered modular protein vectors with engineered plasmids for long term-regulated expression *in vivo* will be essential. For instance, the pEPI DNA vector was the first prototype of episomal vector whose function relies exclusively on chromosomal elements, replicating autonomously in low copy numbers in all cells tested (Piechaczek *et al.* 1999). This vector was further engineered to show regulated expression and to be removed from transduced cells when transgene expression is no longer needed (Rupprecht *et al.*). Finally, vectors have to be tested *in vivo*, but evaluating biological effects and not only reporter gene expression, and the comparison of different vectors in a same *in vivo* experimental setting will also contribute to the selection of the best prototypes.

8. Acknowledgments

This work was supported by the Comisión Sectorial de Investigación Científica (CSIC) of the Universidad de la República (UDELAR) and by the Agencia Nacional de Investigación e Innovación (ANII) of the Ministerio de Cultura, Uruguay.

9. References

- Andre, P., F. Komurian-Pradel, S. Deforges, M. Perret, J.L. Berland, M. Sodoyer, S. Pol, C. Brechot, G. Paranhos-Baccala and V. Lotteau. 2002. Characterization of low- and very-low-density hepatitis c virus rna-containing particles. *J Virol* 76, no. 14: 6919-28.
- Andreu, A., N. Fairweather and A.D. Miller. 2008. Clostridium neurotoxin fragments as potential targeting moieties for liposomal gene delivery to the CNS. *ChemBiochem* 9, no. 2: 219-31.
- Arango-Rodriguez, M.L., I. Navarro-Quiroga, J.A. Gonzalez-Barrios, D.B. Martinez-Arguelles, M.J. Bannon, J. Kouri, P. Forgez, W. Rostene, R. Garcia-Villegas, I. Jimenez and D. Martinez-Fong. 2006. Biophysical characteristics of neurotensin polyplex for *in vitro* and *in vivo* gene transfection. *Biochim Biophys Acta* 1760, no. 7: 1009-20.
- Aris, A., J.X. Feliu, A. Knight, C. Coutelle and A. Villaverde. 2000. Exploiting viral cell-targeting abilities in a single polypeptide, non-infectious, recombinant vehicle for integrin-mediated DNA delivery and gene expression. *Biotechnol Bioeng* 68, no. 6: 689-96.
- Aris, A. and A. Villaverde. 2003. Engineering nuclear localization signals in modular protein vehicles for gene therapy. *Biochem Biophys Res Commun* 304, no. 4: 625-31.
- Aris, A. and A. Villaverde. 2004. Modular protein engineering for non-viral gene therapy. *Trends Biotechnol* 22, no. 7: 371-7.
- Barati, S., P.R. Hurtado, S.H. Zhang, R. Tinsley, I.A. Ferguson and R.A. Rush. 2006. Gdnf gene delivery via the p75(ntr) receptor rescues injured motor neurons. *Exp Neurol* 202, no. 1: 179-88.
- Barrett, L.B., M. Berry, W.B. Ying, M.N. Hodgkin, L.W. Seymour, A.M. Gonzalez, M.L. Read, A. Baird and A. Logan. 2004. Ctb targeted non-viral cDNA delivery enhances transgene expression in neurons. *J Gene Med* 6, no. 4: 429-38.
- Berhanu, D.A. and R.A. Rush. 2008. Targeted silencing of trkA expression in rat forebrain neurons via the p75 receptor. *Neuroscience* 153, no. 4: 1115-25.
- Bloomfield, V.A. 1996. DNA condensation. *Curr Opin Struct Biol* 6, no. 3: 334-41.

- Bonavia, R., A. Bajetto, S. Barbero, A. Albin, D.M. Noonan and G. Schettini. 2001. Hiv-1 tat causes apoptotic death and calcium homeostasis alterations in rat neurons. *Biochem Biophys Res Commun* 288, no. 2: 301-8.
- Box, M., D.A. Parks, A. Knight, C. Hale, P.S. Fishman and N.F. Fairweather. 2003. A multi-domain protein system based on the hc fragment of tetanus toxin for targeting DNA to neuronal cells. *J Drug Target* 11, no. 6: 333-43.
- Brundin, P., R.A. Barker and M. Parmar. Neural grafting in parkinson's disease problems and possibilities. *Prog Brain Res* 184: 265-94.
- Buschle, M., M. Cotten, H. Kirlappos, K. Mechtler, G. Schaffner, W. Zauner, M.L. Birnstiel and E. Wagner. 1995. Receptor-mediated gene transfer into human t lymphocytes via binding of DNA/cd3 antibody particles to the cd3 t cell receptor complex. *Hum Gene Ther* 6, no. 6: 753-61.
- Cardozo, A.K., V. Buchillier, M. Mathieu, J. Chen, F. Ortis, L. Ladriere, N. Allaman-Pillet, O. Poirot, S. Kellenberger, J.S. Beckmann, D.L. Eizirik, C. Bonny and F. Maurer. 2007. Cell-permeable peptides induce dose- and length-dependent cytotoxic effects. *Biochim Biophys Acta* 1768, no. 9: 2222-34.
- Chaib-Oukadour, I., C. Gil and J. Aguilera. 2004. The c-terminal domain of the heavy chain of tetanus toxin rescues cerebellar granule neurones from apoptotic death: Involvement of phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathways. *J Neurochem* 90, no. 5: 1227-36.
- Chan, C.K., T. Senden and D.A. Jans. 2000. Supramolecular structure and nuclear targeting efficiency determine the enhancement of transfection by modified polylysines. *Gene Ther* 7, no. 19: 1690-7.
- Chen, W.C. and L. Huang. 2005. Non-viral vector as vaccine carrier. *Adv Genet* 54: 315-37.
- Corchero, J.L. and A. Villaverde. 2009. Biomedical applications of distally controlled magnetic nanoparticles. *Trends Biotechnol* 27, no. 8: 468-76.
- Domingo-Espín, J., E. Vazquez, J. Ganz, O. Conchillo, E. García-Fruitós, J. Cedano, U. Unzueta, V. Petegnief, N. Gonzalez-Montalbán, A.M. Planas, X. Daura, H. Peluffo, N. Ferrer-Miralles and A. Villaverde. 2011. The nanoparticulate architecture of protein-based artificial viruses is supported by protein-DNA interactions. *Nanomedicine* In Press.
- Eguchi, A., B.R. Meade, Y.C. Chang, C.T. Fredrickson, K. Willert, N. Puri and S.F. Dowdy. 2009. Efficient sirna delivery into primary cells by a peptide transduction domain-dsRNA binding domain fusion protein. *Nat Biotechnol* 27, no. 6: 567-71.
- Ferkol, T., G.L. Lindberg, J. Chen, J.C. Perales, D.R. Crawford, O.D. Ratnoff and R.W. Hanson. 1993. Regulation of the phosphoenolpyruvate carboxykinase/human factor ix gene introduced into the livers of adult rats by receptor-mediated gene transfer. *Faseb J* 7, no. 11: 1081-91.
- Ferrer-Miralles, N., E. Vazquez and A. Villaverde. 2008. Membrane-active peptides for non-viral gene therapy: Making the safest easier. *Trends Biotechnol* 26, no. 5: 267-75.
- Fisher, K.J. and J.M. Wilson. 1997. The transmembrane domain of diphtheria toxin improves molecular conjugate gene transfer. *Biochem J* 321 (Pt 1): 49-58.
- Fritz, J.D., H. Herweijer, G. Zhang and J.A. Wolff. 1996. Gene transfer into mammalian cells using histone-condensed plasmid DNA. *Hum Gene Ther* 7, no. 12: 1395-404.
- Futaki, S., T. Suzuki, W. Ohashi, T. Yagami, S. Tanaka, K. Ueda and Y. Sugiura. 2001. Arginine-rich peptides. An abundant source of membrane-permeable peptides

- having potential as carriers for intracellular protein delivery. *J Biol Chem* 276, no. 8: 5836-40.
- Gonzalez, P., H. Peluffo, L. Acarin, A. Villaverde, B. Gonzalez and B. Castellano. 2011. Il-10 overexpression does not synergize with the neuroprotective action of rgd-containing vectors after postnatal brain excitotoxicity, but modulates the main inflammatory cell responses. . Under second revision in the *Journal of Neuroscience Research*.
- Gonzalez-Barrios, J.A., M. Lindahl, M.J. Bannon, V. Anaya-Martinez, G. Flores, I. Navarro-Quiroga, L.E. Trudeau, J. Aceves, D.B. Martinez-Arguelles, R. Garcia-Villegas, I. Jimenez, J. Segovia and D. Martinez-Fong. 2006. Neurotensin polyplex as an efficient carrier for delivering the human gdnf gene into nigral dopamine neurons of hemiparkinsonian rats. *Mol Ther* 14, no. 6: 857-65.
- Gottschalk, S., J.T. Sparrow, J. Hauer, M.P. Mims, F.E. Leland, S.L. Woo and L.C. Smith. 1996. A novel DNA-peptide complex for efficient gene transfer and expression in mammalian cells. *Gene Ther* 3, no. 5: 448-57.
- Guevara, J., Jr., N. Prashad, B. Ermolinsky, J.W. Gaubatz, D. Kang, A.E. Schwarzbach, D.S. Loose and N.V. Guevara. 2010. Apo b100 similarities to viral proteins suggest basis for ldl-DNA binding and transfection capacity. *J Lipid Res* 51, no. 7: 1704-18.
- Gump, J.M. and S.F. Dowdy. 2007. Tat transduction: The molecular mechanism and therapeutic prospects. *Trends Mol Med* 13, no. 10: 443-8.
- Kawabata, K., Y. Takakura and M. Hashida. 1995. The fate of plasmid DNA after intravenous injection in mice: Involvement of scavenger receptors in its hepatic uptake. *Pharm Res* 12, no. 6: 825-30.
- Kim, H.H., W.S. Lee, J.M. Yang and S. Shin. 2003. Basic peptide system for efficient delivery of foreign genes. *Biochim Biophys Acta* 1640, no. 2-3: 129-36.
- Knight, A., J. Carvajal, H. Schneider, C. Coutelle, S. Chamberlain and N. Fairweather. 1999. Non-viral neuronal gene delivery mediated by the hc fragment of tetanus toxin. *Eur J Biochem* 259, no. 3: 762-9.
- Krishnamoorthy, G., B. Roques, J.L. Darlix and Y. Mely. 2003. DNA condensation by the nucleocapsid protein of hiv-1: A mechanism ensuring DNA protection. *Nucleic Acids Res* 31, no. 18: 5425-32.
- Kumar, P., H. Wu, J.L. McBride, K.E. Jung, M.H. Kim, B.L. Davidson, S.K. Lee, P. Shankar and N. Manjunath. 2007. Transvascular delivery of small interfering rna to the central nervous system. *Nature* 448, no. 7149: 39-43.
- Lalli, G. and G. Schiavo. 2002. Analysis of retrograde transport in motor neurons reveals common endocytic carriers for tetanus toxin and neurotrophin receptor p75^{ntr}. *J Cell Biol* 156, no. 2: 233-9.
- Liu, G., D. Li, M.K. Pasumathy, T.H. Kowalczyk, C.R. Gedeon, S.L. Hyatt, J.M. Payne, T.J. Miller, P. Brunovskis, T.L. Fink, O. Muhammad, R.C. Moen, R.W. Hanson and M.J. Cooper. 2003. Nanoparticles of compacted DNA transfect postmitotic cells. *J Biol Chem* 278, no. 35: 32578-86.
- Ma, N., S.S. Wu, Y.X. Ma, X. Wang, J. Zeng, G. Tong, Y. Huang and S. Wang. 2004. Nerve growth factor receptor-mediated gene transfer. *Mol Ther* 9, no. 2: 270-81.
- Mastrobattista, E., M.A. Van Der Aa, W.E. Hennink and D.J. Crommelin. 2006. Artificial viruses: A nanotechnological approach to gene delivery. *Nat Rev Drug Discov* 5, no. 2: 115-21.

- Midoux, P., A. Kichler, V. Boutin, J.C. Maurizot and M. Monsigny. 1998. Membrane permeabilization and efficient gene transfer by a peptide containing several histidines. *Bioconjug Chem* 9, no. 2: 260-7.
- Nath, A., K. Psooy, C. Martin, B. Knudsen, D.S. Magnuson, N. Haughey and J.D. Geiger. 1996. Identification of a human immunodeficiency virus type 1 tat epitope that is neuroexcitatory and neurotoxic. *J Virol* 70, no. 3: 1475-80.
- Navarro-Quiroga, I., J. Antonio Gonzalez-Barrios, F. Barron-Moreno, V. Gonzalez-Bernal, D.B. Martinez-Arguelles and D. Martinez-Fong. 2002. Improved neurotensin-vector-mediated gene transfer by the coupling of hemagglutinin ha2 fusogenic peptide and vp1 sv40 nuclear localization signal. *Brain Res Mol Brain Res* 105, no. 1-2: 86-97.
- Nie, Y., D. Schaffert, W. Rodl, M. Ogris, E. Wagner and M. Gunther. Dual-targeted polyplexes: One step towards a synthetic virus for cancer gene therapy. *J Control Release*.
- Nishikawa, M., S. Takemura, F. Yamashita, Y. Takakura, D.K. Meijer, M. Hashida and P.J. Swart. 2000a. Pharmacokinetics and in vivo gene transfer of plasmid DNA complexed with mannosylated poly(l-lysine) in mice. *J Drug Target* 8, no. 1: 29-38.
- Nishikawa, M., M. Yamauchi, K. Morimoto, E. Ishida, Y. Takakura and M. Hashida. 2000b. Hepatocyte-targeted in vivo gene expression by intravenous injection of plasmid DNA complexed with synthetic multi-functional gene delivery system. *Gene Ther* 7, no. 7: 548-55.
- Ogris, M., S. Brunner, S. Schuller, R. Kircheis and E. Wagner. 1999. Pegylated DNA/transferrin-pei complexes: Reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther* 6, no. 4: 595-605.
- Ogris, M., R.C. Carlisle, T. Bettinger and L.W. Seymour. 2001. Melittin enables efficient vesicular escape and enhanced nuclear access of nonviral gene delivery vectors. *J Biol Chem* 276, no. 50: 47550-5.
- Peluffo, H., L. Acarin, A. Aris, P. Gonzalez, A. Villaverde, B. Castellano and B. Gonzalez. 2006. Neuroprotection from nmda excitotoxic lesion by cu/zn superoxide dismutase gene delivery to the postnatal rat brain by a modular protein vector. *BMC Neurosci* 7: 35.
- Peluffo, H., A. Aris, L. Acarin, B. Gonzalez, A. Villaverde and B. Castellano. 2003. Nonviral gene delivery to the central nervous system based on a novel integrin-targeting multifunctional protein. *Hum Gene Ther* 14, no. 13: 1215-23.
- Peluffo, H., P. González, A. Aris, L. Acarin, A. Villaverde, B. Castellano and B. González. 2007. Rgd domains neuroprotect the immature brain by a glial dependent mechanism. *Ann Neurol* 62: 251-56.
- Perales, J.C., T. Ferkol, H. Beegen, O.D. Ratnoff and R.W. Hanson. 1994. Gene transfer *in vivo*: Sustained expression and regulation of genes introduced into the liver by receptor-targeted uptake. *Proc Natl Acad Sci U S A* 91, no. 9: 4086-90.
- Piechaczek, C., C. Fetzer, A. Baiker, J. Bode and H.J. Lipps. 1999. A vector based on the sv40 origin of replication and chromosomal s/mars replicates episomally in cho cells. *Nucleic Acids Res* 27, no. 2: 426-8.

- Plank, C., K. Mechtler, F.C. Szoka, Jr. and E. Wagner. 1996. Activation of the complement system by synthetic DNA complexes: A potential barrier for intravenous gene delivery. *Hum Gene Ther* 7, no. 12: 1437-46.
- Plank, C., B. Oberhauser, K. Mechtler, C. Koch and E. Wagner. 1994. The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems. *J Biol Chem* 269, no. 17: 12918-24.
- Pollard, H., J.S. Remy, G. Loussouarn, S. Demolombe, J.P. Behr and D. Escande. 1998. Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. *J Biol Chem* 273, no. 13: 7507-11.
- Pouton, C.W. 1998. Nuclear import of polypeptides, polynucleotides and supramolecular complexes. *Adv Drug Deliv Rev* 34, no. 1: 51-64.
- Pyhtila, M.J., I. Miettola, T. Kurkela and A. Hoyhtya. 1976. Protection of deoxyribonucleic acid from nuclease action by histones. *Acta Chem Scand B* 30, no. 8: 797-8.
- Read, M.L., S. Singh, Z. Ahmed, M. Stevenson, S.S. Briggs, D. Oupicky, L.B. Barrett, R. Spice, M. Kendall, M. Berry, J.A. Preece, A. Logan and L.W. Seymour. 2005. A versatile reducible polycation-based system for efficient delivery of a broad range of nucleic acids. *Nucleic Acids Res* 33, no. 9: e86.
- Ritter, W., C. Plank, J. Lausier, C. Rudolph, D. Zink, D. Reinhardt and J. Rosenecker. 2003. A novel transfecting peptide comprising a tetrameric nuclear localization sequence. *J Mol Med* 81, no. 11: 708-17.
- Ross, G.F., M.D. Bruno, M. Uyeda, K. Suzuki, K. Nagao, J.A. Whitsett and T.R. Korfhagen. 1998. Enhanced reporter gene expression in cells transfected in the presence of dmi-2, an acid nuclease inhibitor. *Gene Ther* 5, no. 9: 1244-50.
- Ross, G.F., R.E. Morris, G. Ciraolo, K. Huelsman, M. Bruno, J.A. Whitsett, J.E. Baatz and T.R. Korfhagen. 1995. Surfactant protein a-polylysine conjugates for delivery of DNA to airway cells in culture. *Hum Gene Ther* 6, no. 1: 31-40.
- Rupprecht, S., C. Hagedorn, D. Seruggia, T. Magnusson, E. Wagner, M. Ogris and H.J. Lipps. Controlled removal of a nonviral episomal vector from transfected cells. *Gene* 466, no. 1-2: 36-42.
- Saccardo, P., A. Villaverde and N. Gonzalez-Montalban. 2009. Peptide-mediated DNA condensation for non-viral gene therapy. *Biotechnol Adv* 27, no. 4: 432-8.
- Sato, Y., N. Yamauchi, M. Takahashi, K. Sasaki, J. Fukaura, H. Neda, S. Fujii, M. Hirayama, Y. Itoh, Y. Koshita, K. Kogawa, J. Kato, S. Sakamaki and Y. Niitsu. 2000. *In vivo* gene delivery to tumor cells by transferrin-streptavidin-DNA conjugate. *Faseb J* 14, no. 13: 2108-18.
- Segrest, J.P., M.K. Jones, H. De Loof and N. Dashti. 2001. Structure of apolipoprotein b-100 in low density lipoproteins. *J Lipid Res* 42, no. 9: 1346-67.
- Song, E., P. Zhu, S.K. Lee, D. Chowdhury, S. Kussman, D.M. Dykxhoorn, Y. Feng, D. Palliser, D.B. Weiner, P. Shankar, W.A. Marasco and J. Lieberman. 2005. Antibody mediated *in vivo* delivery of small interfering rnas via cell-surface receptors. *Nat Biotechnol* 23, no. 6: 709-17.
- Takakura, Y., T. Takagi, M. Hashiguchi, M. Nishikawa, F. Yamashita, T. Doi, T. Imanishi, H. Suzuki, T. Kodama and M. Hashida. 1999. Characterization of plasmid DNA binding and uptake by peritoneal macrophages from class a scavenger receptor knockout mice. *Pharm Res* 16, no. 4: 503-8.

- Thurnher, M., E. Wagner, H. Clausen, K. Mechtler, S. Rusconi, A. Dinter, M.L. Birnstiel, E.G. Berger and M. Cotten. 1994. Carbohydrate receptor-mediated gene transfer to human t leukaemic cells. *Glycobiology* 4, no. 4: 429-35.
- Vazquez, E., M. Roldan, C. Diez-Gil, U. Unzueta, J. Domingo-Espin, J. Cedano, O. Conchillo, I. Ratera, J. Veciana, X. Daura, N. Ferrer-Miralles and A. Villaverde. Protein nanodisk assembling and intracellular trafficking powered by an arginine-rich (r9) peptide. *Nanomedicine (Lond)* 5, no. 2: 259-68.
- Vijayanathan, V., T. Thomas and T.J. Thomas. 2002. DNA nanoparticles and development of DNA delivery vehicles for gene therapy. *Biochemistry* 41, no. 48: 14085-94.
- Wadhwa, M.S., W.T. Collard, R.C. Adami, D.L. Mckenzie and K.G. Rice. 1997. Peptide-mediated gene delivery: Influence of peptide structure on gene expression. *Bioconjug Chem* 8, no. 1: 81-8.
- Wagner, E., C. Plank, K. Zatloukal, M. Cotten and M.L. Birnstiel. 1992. Influenza virus hemagglutinin ha-2 n-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: Toward a synthetic virus-like gene-transfer vehicle. *Proc Natl Acad Sci U S A* 89, no. 17: 7934-8.
- Wagner, E., M. Zenke, M. Cotten, H. Beug and M.L. Birnstiel. 1990. Transferrin-polycation conjugates as carriers for DNA uptake into cells. *Proc Natl Acad Sci U S A* 87, no. 9: 3410-4.
- Wilson, J.M., M. Grossman, C.H. Wu, N.R. Chowdhury, G.Y. Wu and J.R. Chowdhury. 1992. Hepatocyte-directed gene transfer *in vivo* leads to transient improvement of hypercholesterolemia in low density lipoprotein receptor-deficient rabbits. *J Biol Chem* 267, no. 2: 963-7.
- Wolfert, M.A. and L.W. Seymour. 1998. Chloroquine and amphipathic peptide helices show synergistic transfection *in vitro*. *Gene Ther* 5, no. 3: 409-14.
- Worgall, S., D. Sondhi, N.R. Hackett, B. Kosofsky, M.V. Kekatpure, N. Neyzi, J.P. Dyke, D. Ballon, L. Heier, B.M. Greenwald, P. Christos, M. Mazumdar, M.M. Souweidane, M.G. Kaplitt and R.G. Crystal. 2008. Treatment of late infantile neuronal ceroid lipofuscinosis by cns administration of a serotype 2 adeno-associated virus expressing cln2 cDNA. *Hum Gene Ther* 19, no. 5: 463-74.
- Wu, G.Y., J.M. Wilson, F. Shalaby, M. Grossman, D.A. Shafritz and C.H. Wu. 1991. Receptor-mediated gene delivery *in vivo*. Partial correction of genetic analbuminemia in nagase rats. *J Biol Chem* 266, no. 22: 14338-42.
- Wu, G.Y. and C.H. Wu. 1987. Receptor-mediated *in vitro* gene transformation by a soluble DNA carrier system. *J Biol Chem* 262, no. 10: 4429-32.
- Wu, G.Y. and C.H. Wu. 1988. Receptor-mediated gene delivery and expression *in vivo*. *J Biol Chem* 263, no. 29: 14621-4.
- Zauner, W., A. Kichler, W. Schmidt, K. Mechtler and E. Wagner. 1997. Glycerol and polylysine synergize in their ability to rupture vesicular membranes: A mechanism for increased transferrin-polylysine-mediated gene transfer. *Exp Cell Res* 232, no. 1: 137-45.
- Zeng, J., H.P. Too, Y. Ma, E.S. Luo and S. Wang. 2004. A synthetic peptide containing loop 4 of nerve growth factor for targeted gene delivery. *J Gene Med* 6, no. 11: 1247-56.
- Zeng, J. and S. Wang. 2005. Enhanced gene delivery to pc12 cells by a cationic polypeptide. *Biomaterials* 26, no. 6: 679-86.

- Zeng, J., X. Wang and S. Wang. 2007. Self-assembled ternary complexes of plasmid DNA, low molecular weight polyethylenimine and targeting peptide for nonviral gene delivery into neurons. *Biomaterials* 28, no. 7: 1443-51.

Non-Viral Gene Therapy

Edited by Prof. Xubo Yuan

ISBN 978-953-307-538-9

Hard cover, 696 pages

Publisher InTech

Published online 07, November, 2011

Published in print edition November, 2011

This book focuses on recent advancement of gene delivery systems research. With the multidisciplinary contribution in gene delivery, the book covers several aspects in the gene therapy development: various gene delivery systems, methods to enhance delivery, materials with modification and multifunction for the tumor or tissue targeting. This book will help molecular biologists gain a basic knowledge of gene delivery vehicles, while drug delivery scientist will better understand DNA, molecular biology, and DNA manipulation.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Hugo Peluffo (2011). Modular Multifunctional Protein Vectors for Gene Therapy, Non-Viral Gene Therapy, Prof. Xubo Yuan (Ed.), ISBN: 978-953-307-538-9, InTech, Available from:
<http://www.intechopen.com/books/non-viral-gene-therapy/modular-multifunctional-protein-vectors-for-gene-therapy>

INTech
open science | open minds

InTech Europe

University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

© 2011 The Author(s). Licensee IntechOpen. This is an open access article distributed under the terms of the [Creative Commons Attribution 3.0 License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.