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## Gene Delivery Systems: Tailoring Vectors to Reach Specific Tissues

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### 1. Introduction

The gene therapy concept was developed more than forty years ago. Edward Tatum in a historic lecture affirmed: *"We can anticipate that viruses will be used effectively for man's benefit, in theoretical studies concerning somatic cell genetics and possibly in genetic therapy... We even can be somewhat optimistic about the long-range possibility of therapy based on the isolation or design, synthesis, and introduction of new genes into defective cells of particular organs"*. Two years later Marshall Nirenberg predicted that: *"in 25 years it would be possible to program the cells with synthetic messages"*, but called to attention that *"it should be postponed until having the sufficient wisdom to use this knowledge for the benefit of mankind"*.

Initial efforts to construct vectors were achieved by Rogers and Pfudere in 1968. However, it was not until 1990 that the first gene therapy clinical trial was carried-out in a patient with adenosine deaminase (ADA) deficiency (Friedmann, 1992). Within the last several years, significant advances have been attained in therapies for cancer, AIDS, Parkinson's disease, X-linked severe combined immunodeficiency (X-SCID), hemophilias, cystic fibrosis, Leber's congenital amaurosi, and  $\beta$ -thalassemia, among others. Nonetheless, an adolescent patient suffering from a urea cycle defect died of an anaphylactic reaction a few hours after being injected with an adenoviral vector. Soon after, five children participating in a clinical trial to correct a X-SCID developed a leukemia-like condition due to the activation of an oncogene (Edelstein, 2007). Recently, the tragic death of a volunteer in an clinical trial for rheumatoid arthritis using an adenoassociated virus vector was initially associated to a side effect of the vector (Williams, 2007). However, further studies showed that this was caused by an infection with *Histoplasma capsulatum*, which was produced by an immunosuppression condition induced for a simultaneous systemic anti-TNF-alpha therapy in the form of the drug adalimumab (Williams, 2007). All these failures halted the progress of gene therapy and seriously questioned the efficacy of the procedure. It also prompted the need for more basic studies on the immunogenic aspects and how to make safer, and more efficient vectors.

During the last 35 years a long list of viral and non-viral vectors have been developed to design a vector that allows gene delivery to specific cell types, has a high gene transfer efficiency, produces therapeutic levels of gene expression during long-term periods, and minimizes the generation of side effects (Verma & Weitzman, 2005). Although the field has made great strides in producing an ideal vector, one of the main challenges remains to aim for a cell-specific vector. Most probably a vector left in the general circulation without a specific targeting signal, will be sequestered by the liver or may end-up in cells with no need of the

transfected gene product. In addition, to date gene therapy still encounters two main problems: (i) gene delivery to the central nervous system (CNS), as the gene or its product are naturally blocked by the blood brain barrier; and (ii) gene delivery into poorly circulated tissues such as bone, where up until now, it has only been achieved with low transfection efficiencies.

In this chapter, we will explore the modifications carried out in viral and non-viral vectors to enhance their natural tropism. Regardless of the vector, modification objectives can be summarized in: (i) tropism expansion to permit gene delivery to cells not easily transfected with non-modified vector, (ii) cell-type specific transfection reducing side effects, and (iii) stealth vector improvement to reduce protein interactions or cells that limit its therapeutic activity, e.g. immune system (Yu & Schaffer, 2005).

## 2. Retroviral vectors

Retroviruses are a family of enveloped, diploid, and positive-stranded RNA virus. This family is formed by alpharetrovirus, betaretrovirus, deltaretrovirus, epsilonretrovirus, gammaretrovirus, lentivirus, and spumavirus (foamy virus), from which only the last three have been used as gene therapy vectors (Verma & Weitzman, 2005). The genome basically consists of three genes: (i) *gag*, which encodes for viral matrix, capsid and nucleocapsid proteins, (ii) *pol*, which encodes for protease, reverse transcriptase and integrase, and (iii) *env*, which encodes a bipartite membrane-anchored surface (SF) protein (Escors & Breckpot, 2010). In addition, complex retroviruses (e.g. lentivirus and foamy virus) have accessory genes for the regulation of gene expression, assembly and replication (Escors & Breckpot, 2010). In the viral life cycle, glycoproteins (GPs) play an important role in viral tropism, receptor recognition and cell entry (Liang et al., 2009). The binding of the viral GP to the cellular surface protein induces a conformational change into the viral GP and leads to fusion and insertion of the capsid into the cytoplasm (Yu & Schaffer, 2005). Most of the advantages and limitations of retrovirus targeting are related with this cell entry mechanism.

Retroviral vectors used in gene therapy have properties that allow the transduction of a broad array of cells (Cronin et al., 2005; Frecha et al., 2008). However, the use of targeted retroviral vectors allowing the delivery of the transgene to cells which are not normally transduced, or limiting the delivery to cells which should not be transduced would be advantageous (Cronin et al., 2005; Frecha et al., 2008; Lavillette et al., 2001). In general, retroviral targeting can be achieved by: (i) engineering the GP to produce a vector that recognizes a specific cell surface protein (direct targeting), (ii) pseudotyping with GPs derived from other retrovirus and that recognize specific receptors, (iii) using ligands that block the cell entry of the vector to the cells expressing the receptor for the ligand, while allow the transduction of others cell types (indirect targeting), or (iv) using biospecific antibodies, chemically crosslinking ligands and grafting polymers (Fig. 1) (Lavillette et al., 2001; Verhoeven & Cosset, 2004; Yu & Schaffer, 2005).

### 2.1 Retroviral targeting by engineering the glycoproteins

Direct targeting consists of the insertion of ligands or antibody fragments into the viral GP (Fig. 1), which allows recognition of cell surface proteins different from those naturally used by the vector (Lavillette et al., 2001). Direct modification of a GP was first reported in 1990, with the successful fusion of CD4 with the GP of a gammaretroviral vector (Young et al., 1990). Later, a functional murine leukemia virus (MLV) vector bearing a GP protein fused with an antibody fragment against a hapten (4-hidroxy-5-iodo-3-nitrophenacetyl caproate) demonstrated binding of the modified retroviral vector to hapten, and the ability to package a transgene and transduce culture cells (Russell et al., 1993).

After these pioneer works revealed the feasibility of GP engineering, different authors studied the real potential of this targeting approach. The fusion of the erythropoietin polypeptide with the GP of a MLV vector showed for the first time that a chimeric GP was not only correctly incorporated into the virus envelope but also allowed the specific transduction of cells expressing the EPO receptor (Kasahara et al., 1994). A similar result was reported with a gammaretrovirus bearing a GP fused with a single-chain antigen-binding site against a cell surface expressed on human carcinoma cells (Kasahara et al., 1994).

Nevertheless, different studies have exposed the limitations of this approach. The first evidence of this limitation was observed with vectors carrying the Ram-1 phosphate transporter or the epidermal growth factor (EFG) into the GP, which showed a reduced transduction efficiency although the chimeric GPs were correctly expressed, processed, and exposed in the viral envelope (Cosset et al., 1995). A similar observation was reported for a gammaretroviral vector with a GP bearing a peptide binding to the  $\alpha_v\beta_3$  integrin (Wu et al., 2000a), or with the stromal cell derived factor 1-alpha (Katane et al., 2002). These low transductions efficiencies were associated with loss or impairment of viral and cell surface proteins binding and subsequent membrane fusion and penetration of the viral core into the cytosol (Frecha et al., 2008; Lavillette et al., 2001; Yu & Schaffer, 2005). In addition, these results strongly suggest that only few set of peptides sequences can be inserted into the GPs without altering the mechanism of retrovirus cell entry (Verhoeven & Cosset, 2004). The insertion of spacer sequences between the inserted peptide and the GP has proved to overcome this issue, allowing a significant improvement in the transduction efficiency of the modified vectors (Kayman et al., 1999).

## 2.2 Retroviral targeting by pseudotyping

Pseudotyping involves the production of viral particles bearing GP derived from other enveloped viruses (Fig. 1). This strategy was developed due to the narrow cellular transduction profile observed in vectors with native GPs (Cronin et al., 2005; Yu & Schaffer,

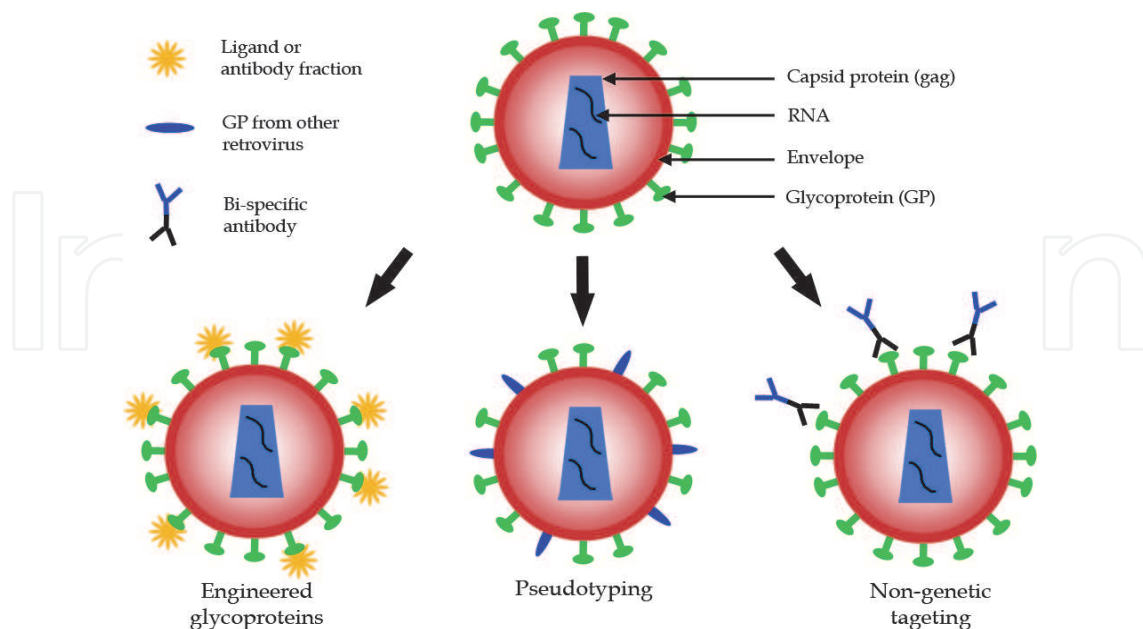


Fig. 1. Strategies for retroviral targeting. Retroviral vectors can be modified by ligand or antibody fraction insertion (engineered glycoproteins), use of glycoprotein (GP) from other retrovirus (pseudotyping), or the use of bi-specific antibodies (non-genetic targeting).

2005). Production of pseudotyped retroviral vectors is a well-established protocol (Bischof & Cornetta, 2010), which in most cases is used to allow virus purification, improve the cellular transduction, extend the range of transduced cells, and reduce cell toxicity (Cronin et al., 2005). However, it should be possible to employ natural tropism in GPs used for pseudotyping to target vectors bearing these proteins.

A large set of GPs has been evaluated for vector pseudotyping, among which vesicular stomatitis virus glycoprotein (VSV-G) is the most used [reviewed in (Bischof & Cornetta, 2010; Cronin et al., 2005)]. However, only a few GPs have allowed the transduction of different cell types compared to those observed VSV-G pseudotyped vectors. The use of a GP derived from rabies virus (RV) for pseudotyping an HIV-1 lentiviral vector allowed the transduction of neurons usually non-transduced with VSV-G vectors, since the RV glycoprotein induced a retrograde transport along axons (Mazarakis et al., 2001). The infusion of an animal model of familial amyotrophic lateral sclerosis (ALS) with a RV-HIV-1 vector carrying the gene of vascular endothelial growth factor (VEGF) delayed the onset of the disease and slowed progression in treated animals. Although treatment was initiated at the onset of paralysis CNS tropism of the vector was still significantly improved (Azzouz et al., 2004).

An interesting tropism was also observed for an HIV-1 vector pseudotyped with a GP from the lymphocytic choriomeningitis virus (LCMV) (Miletic et al., 2004). *In-vitro* and *in-vivo* evaluation of a vector pseudotyped with a LCMV GP showed that it transduced, almost exclusively, astrocytes; while VSV-G pseudotypes vectors infected neurons as well as astrocytes. In addition, LCMV-HIV-1 vector presented a specific transduction of infiltrating tumor cells, while VSV-G-HIV-1 vectors transduced mostly normal brain cells in infiltrating tumor areas. An HIV-1 vector pseudotyped with an Ebola Zaire virus-derived GP appeared useful in the treatment of airway diseases (e.g. cystic fibrosis). This vector allowed *in-vitro* and *in-vivo* transduction of airway epithelial cells, which was not observed with a VSV-G vector (Kobinger et al., 2001).

A recent modification of this approach used an HIV-1 vector pseudotyped with the GP and the fusion protein from a measles virus. The GP had an epidermal growth factor (EGF) or a single chain antibody against CD20 (Funke et al., 2008). The CD20-bearing vector was able to transduce primary and CD20-positive B cells both alone or within a cell mixture, while a VSV-G vector did not transduce these cell types.

### 2.3 Indirect retroviral targeting

This strategy is based on the finding that inclusion of certain peptides into the GP significantly impairs transduction of some cells. This prohibits gene delivering into cells that should not be transduced and extends viral tropism (Lavillette et al., 2001; Yu & Schaffer, 2005). First evidence of this approach was discovered while producing a MLV vector bearing a GP fused with epidermal growth factor (EGF). Although the chimeric GP was correctly expressed and processed, and the modified vector had the ability to bind to the EGF receptor, this manipulation completely inhibited vector transduction into cells expressing the EGF receptor (Cosset et al., 1995). This transduction inhibition was produced because of the modified vector was directed to the late endosome and destroyed by lysosomal enzymes.

A similar result was observed with a vector carrying the stem cell factor (SCF, a c-Kit receptor ligand), which selectively inhibited vector transduction on c-Kit-expressing cells (Fielding et al., 1998). When a mixture of EGF receptor-positive cancer cells and c-Kit-



positive hematopoietic cells were exposed to the above mentioned vectors, cancer cells were selectively transduced by the SCF-displaying vector, whereas hematopoietic cells were selectively transduced by the EGF-displaying vector. *In-vivo* proof-of-concept of this strategy was established after intravenous infusion of an EGF-displaying HIV-1 vector, showing preferable transduction of spleen with very low levels on EGF-receptor rich tissues (e.g. liver). Furthermore, the VSV-G vector transduced heart, skeletal muscle, lung, brain, kidney, ovaries and bone-marrow (Peng et al., 2001). Transduction inhibition of human and canine cells was also observed for a spleen necrosis virus bearing EFG, which was restored after cleavage via factor Xa at a site located between EFG and GP (Merten et al., 2003).

A variant of this strategy takes advantage of protease-activatable gene delivery vehicles. Matrix metalloproteinases (MMPs) are commonly overexpressed in angiogenesis, inflammation, and cancer invasion. The inclusion of a MMPs-cleavable peptide within an EGF-retroviral vector permitted that in the presence of exogenous MMPs, the infectivity of the MMP-EGF vector but not of the EGF-vector, could be restored. This MMPs-sensitive vector could efficiently discriminate between two different cell types, infecting only MMP-positive cells (Peng et al., 1997).

#### 2.4 Non-genetic retroviral targeting

This strategy involves the use of antibodies or adapter molecules bound to the GP (Fig. 1) (Lavillette et al., 2001; Yu & Schaffer, 2005). This was the first approach used for cell targeting of a gammaretrovirus by using two biotinylated antibodies bridged by streptavidin: one against a GP and the other one against a specific cell membrane marker (Roux et al., 1989). This strategy allowed the specific transduction of cells expressing class I or II major histocompatibility complex (MHC) molecules by using monoclonal antibodies against these MHC molecules and against GP from the retroviral vector (Roux et al., 1989).

For targeting of a retroviral vector to folate receptor-expressing epidermoid carcinoma cells, a myeloproliferative sarcoma retrovirus was modified with folic acid (Reddy et al., 2001). Although receptor binding was observed, this vector was not able to induce gene expression. Similar results were observed for a MLV vector bearing a single chain anti-folate receptor antibody to produce vectors targeting ovarian cancer cells (Pizzato et al., 2001), manifesting the limitations of folate receptor targeting. A modification of this approach combines genetic modification of the GP by inclusion of an IgG-binding domain and the conjugation with an antibody that reacts with specific cell surface molecules expressing the antigen (Ohno et al., 1997). By changing the monoclonal antibody it was possible to transduce efficiently and specifically a variety of cell lines. The potential of this approach was confirmed by *in-vivo* evaluation of modified vectors conjugated with antibodies against surface proteins expressed in cancer cells, indicating that after intravenous infusion the vector preferentially transduced these cells (Liang et al., 2009). This strategy has been used both with gammaretroviral and lentiviral vectors. It represents a powerful tool that can be easily modified to obtain a site-specific gene expression for the treatment of cancers, genetic, infectious and immune diseases.

### 3. Adenoviral vectors

An adenovirus (AdV) is a non-enveloped virus formed by an icosahedral protein capsid surrounding a lineal double-stranded DNA of 36 kb (Douglas, 2007). The AdV capsid is characterized by the presence of 252 different capsomers and long fibers protruding from

each of the twelve vertices. Seven polypeptides form this complex capsid: hexon (II), penton base (III), fiber (IV), IIIa, VI, VIII, and IX (Campos & Barry, 2007; Sharma et al., 2009). The cell receptor depends on the virus subgroup: A, E and F subgroups use the cell surface coxsackievirus B and adenovirus receptor (CAR); B1 and B2 subgroups use CD46, CD80/86, receptor X, or heparan sulfate proteoglycan (HSPG); C subgroup uses CAR, HSPG, MHC-I, vascular cell adhesion molecule-I (VCAM-I), or integrins; and D subgroup uses CAR, sialic acid, or CD46 (Arnberg, 2009; Campos & Barry, 2007; Sharma et al., 2009).

Adenoviral vectors have been used as tools for gene therapy since the late 80s (Friedmann, 1992), and the first clinical trial was started in 1993 (Douglas, 2007). Since then, over 392 clinical trials using AdV vectors have been carried out. Currently AdV are the most used vectors for gene therapy and represent 24% of clinical trials total (Edelstein, 2007). In addition, the first commercially approved gene therapy product, Gendicine, is based on an AdV vector engineered to express p53 for treatment of patients with head and neck squamous cell carcinoma (Peng, 2005).

AdV vectors are mainly derived from serotypes 2 (AdV2) and 5 (AdV5). They have the advantage of inducing high levels of gene expression, are able to infect both dividing and non-dividing cells, and can be purified to high titers. Furthermore, AdV2 and AdV5 have high bloodstream stability with a reduced risk of insertional mutagenesis, since their genome remains extrachromosomal (Douglas, 2007; Edelstein, 2007; Volpers & Kochanek, 2004). Nonetheless, several drawbacks have been identified during *in-vivo* evaluation of these vectors: (i) presence of pre-existing antibodies that rapidly neutralize the vector, (ii) after intravenous administration the vector is mainly taken up by liver cells, limiting the vector to reach its target tissue in adequate concentrations, and (iii) use of high doses in an effort to overcome these problems has not proven to be an adequate and safe approach (Arnberg, 2009). Three strategies can be used to solve these issues: (i) use of vectors from other subgroups different from AdV2 and AdV5 (subgroup C), (ii) use of non-human serotypes, and (iii) retargeting of the vector to cells or tissues of interest (Arnberg, 2009). In this section we will explore different strategies for AdV retargeting that include genetic modification of the capsid, use of molecular adaptors and chemical modification of the capsid (Fig. 2) (Campos & Barry, 2007).

### 3.1 Genetic modification of the AdV capsid

Genetic modification of the viral capsid involves incorporation of foreign peptides into exposed regions of the capsid. In this manner the gene encoding for the peptide is inserted into the vector's genome (Fig. 2) (Campos & Barry, 2007). The possibility of altering the AdV vector capsid was first evaluated by the insertion of octapeptides into the hexon (Crompton et al., 1994) or the fiber knob (Krasnykh et al., 1998). This established the possibility of producing viable vectors with foreign proteins present on the vector capsid surface. Further experiments revealed the possibility of expanding the vector tropism by inserting a heparin-binding domain (Wickham et al., 1996), or an Arg-Gly-Asp (RGD)-containing peptide into the fiber knob (Dmitriev et al., 1998).

Although different studies indicated the feasibility to target vectors to specific cell types, genetic modification often resulted in failure to rescue viable viruses, or in an impaired virus packing, peptide exposure, and vector transduction (Leissner et al., 2001; Wu et al., 2005). Because the insertion of a pre-selected peptide into a fiber knob often fails to generate an adenovirus vector, use of random peptide libraries displayed directly on the AdV capsid allows isolation of viable vectors with high affinity for specific tissues or cells (Miura et al.,

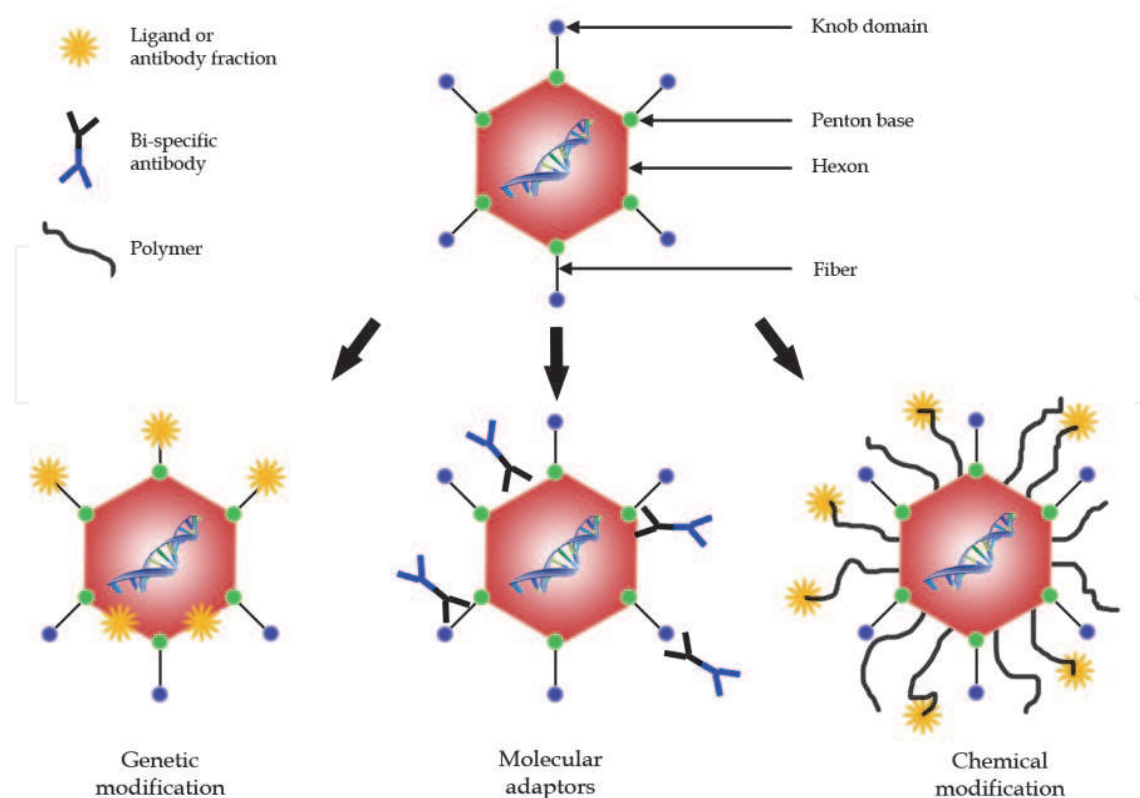


Fig. 2. Strategies for Adenoviral vectors targeting. Adv vectors can be modified by ligand or antibody fraction insertion (genetic modification), use of bi-specific antibodies (molecular adaptors), or conjugation with polymers bearing or not a ligand (chemical modification).

2007). A similar approach involves the pre-selection of peptides by using a phage display peptide library, from which peptides with high affinity for a certain cell type are selected and then inserted into the Adv vector capsid. This has allowed the targeting of Adv vectors to epithelium cells (Nicklin et al., 2001b), tumor cells (Rittner et al., 2007), and neurons (Schmidt et al., 2007).

Most efforts to modify the Adv vector capsid have been on vector targeting to tumor cells (Bachtarzi et al., 2008). Some examples of this approach include: (i) production of an Adv vector bearing a CAR protein fused with EGF producing an enhanced gene transfer efficiency in pancreatic carcinoma cells (Wesseling et al., 2001); (ii) a vector with a polylysine motif in the fiber for a CAR-independent binding to HSPG for specific transduction of different breast cancer cells (Ranki et al., 2007); and (iii) vectors with high affinity for prostate adenocarcinoma (Wu et al., 2010), and human colon carcinoma cells (Rittner et al., 2007).

In addition to cancer cell, genetically modified Adv vectors have been used as tools for gene delivery to other cell types. The insertion of an RGD-modified Adv allowed the production of a vector that mediated cell entry via RGD binding to integrins. Administration into the synovial lining improved the outcome of gene therapy for arthritis (Bakker et al., 2001). Targeting to endothelial cells, an important target in vascular gene therapy, was possible after insertion of the SIGYPLP peptide into the fiber protein in combination with fiber mutations that block natural CAR binding (detargeting) (Nicklin et al., 2001b). For specific kidney gene delivery, the intravenous administration of HTTHREP- and HITSLLS-bearing Adv vectors resulted in selective renal targeting, specifically of tubular epithelium and glomeruli (Denby et al., 2007).



Although genetically modified AdV vectors have been widely studied, most of these modifications have been carried out only into fiber or hexo. Recently it was determined that protein IX in the capsid is a viable platform for the insertion of single-chain variable-fragment antibodies (scFv) or single-domain antibodies (sdAb) for AdV vector retargeting. Even so, only sbAb enhanced virus infection of cells expressing the targeted receptor. Thus, proving that the nature of the ligand can significantly affect vector targeting as already had been observed with fiber and hexo (Poulin et al., 2010).

### 3.2 Molecular adaptors for AdV targeting

Although genetic modification is the most direct form of vector targeting it has the disadvantage of not all peptides can be inserted into the vector capsid. An approach to overcome this difficulty is to use molecular adaptors consisting of a capsid-binding domain fused with a cell-binding domain (Fig. 2) (Campos & Barry, 2007). This strategy has the advantages of not requiring a correct processing of the cell-binding peptide, since it is not translated with the viral capsid proteins; and that virtually any cell-binding peptide can be fused with the capsid.

The primary adaptor molecules are bi-specific antibodies against a capsid protein and a cellular receptor. The use of a single-chain bi-specific antibody directed against human EGFR and fiber knob significantly increased gene transfer into primary glioma cells and organotypic glioma spheroids, while reducing natural tropism dramatically (Grill et al., 2001). The human epithelial cell adhesion molecule (EpCAM) is highly expressed in malignant lesions of the stomach and esophagus. By using bi-specific antibodies against adenovirus fiber knob protein and EpCAM a specific transduction of gastric and esophageal cancer cell lines was observed with reduced transduction in normal cells (Heideman et al., 2001). For other epithelial tumors, e.g. colon, lung and breast, it was possible to target vectors by using a bi-specific adapter protein, which fused the ectodomain of CAR with a single-chain anti-carcinoembryonic antigen (CEA) antibody. This adaptor molecule allowed the targeting of AdV vectors to CEA-positive epithelial tumor cells in cell culture, in subcutaneous tumor grafts, and in hepatic tumor grafts (Li et al., 2007). A similar strategy was used for vector targeting to pancreatic carcinoma cells by using a vector bearing an EGF peptide fused with CAR (Wesseling et al., 2001). The combination of this adaptor molecule with the insertion of an RGD-containing peptide into the fiber knob resulted in a significant enhancement of gene transfer.

### 3.3 Chemical modification of the AdV capsid

The third strategy for AdV targeting is the chemical modification of the viral capsid by using primary-amine reactive groups (Fig. 2) (Campos & Barry, 2007). The capsid modification with polyethylene glycol (PEG - PEGylation) is a promising strategy to diminish vector toxicity and immune response. Although other polymers have also been used PEGylation can be employed to modify tropism. Polymer-coated vectors can be targeted to specific cells by incorporation onto the polymer molecules that bind to specific cell-surface proteins (Campos & Barry, 2007; Volpers & Kochanek, 2004).

PEG-modified AdV vectors were conjugated with an anti-HER2/neu monoclonal antibody leading to an enhanced and specific transduction of HER2/neu over-expressed breast cancer cells. Furthermore, a significant reduction of the innate immune response against the vector was accomplished (Jung et al., 2007; Kim et al., 2011). Conjugation of PEG-AdV vectors with

an RGD peptide led to a specific  $\alpha_v\beta_3$  integrin vector cell entry. This resulted in a significant improvement in transduction and specificity of gene delivery into endothelial cells. These events have implications on the treatment of rheumatoid arthritis, inflammatory bowel disease and epithelial tumors (Eto et al., 2005; Ogawara et al., 2006). Transduction of bone marrow derived human mesenchymal stem cells (MSC) can be significantly improved by using a PEGylated AdV conjugated with a blocked poly-L-lysine. However, modification on vector tropism was not clearly reported in this study (Park et al., 2010).

Although a great part of systemically administrated vector particles is scavenged by Kupffer cells in the liver, the use of a PEG- or a dextran-coated vectors allowed the specific transduction of hepatocytes independent of the presence of Kupffer cells, emphasizing the potential for therapeutic liver-directed gene transfer (Prill et al., 2010). Similar result can be observed by using a multivalent hydrophilic polymer based on poly-[N-(2-hydroxypropyl)methacrylamide] conjugated with EGF or VCAM, producing a CAR-independent binding and uptake into EGF- or VCAM-positive target cells selectively in mixed culture and also in xenografts *in-vivo* (Fisher et al., 2001).

#### 4. Adeno-associated viral vectors

Adeno-associated virus (AAV) are non-enveloped virus belonging to the *Parvoviridae* family that need a helper virus, such as AdV or herpes simplex, for efficient infection and replication (Flotte, 2004). AAV are formed by a single-stranded DNA genome of 4.7 kb that contains two open reading frames (ORFs): (i) *rep*, which encodes for proteins Rep78, Rep68, Rep52 and Rep40, involved in virus genome replication, packing and integration; and (ii) *cap*, which encodes for the capsid proteins VP1, VP2 and VP3 (Wu et al., 2006). These ORFs are flanked by two Inverted Terminal Repeats (ITRs), involved in complementary DNA synthesis, Rep binding proteins, and site-specific genome integration in human chromosome 19 (Wu et al., 2006).

AAV capsid is formed by 60 subunits of VP1, VP2 and VP3 in a 1:1:10 ratio (Michelfelder & Trepel, 2009). These proteins share the C-terminal sequence, while the N-terminal sequences differ according to the start codon, being VP1>VP2>VP3 (Wu et al., 2006). Capsid structure has been elucidated for AAV2, AAV3, AAV4, AAV8 and AAV9 serotypes (Govindasamy et al., 2006; Lerch et al., 2010; Mitchell et al., 2009; Nam et al., 2007; Xie et al., 2002). The difference within capsid proteins allows for each serotype to use a specific receptor: (i) AAV2 and AAV3 use the HSPG, (ii) AAV1, 4 and 5 use glycans with sialic acid ends, and (iii) AAV8 uses the 37/67 kDa laminin receptor (Nam et al., 2007; Summerford & Samulski, 1998; Wu et al., 2006). AAV can infect a wide number of tissues including liver, lung, central nervous system, muscle, and heart; although as a result of the differences in receptor used, each serotype has a characteristic tropism (Flotte, 2004; Verma & Weitzman, 2005).

From the first clinical trial for cystic fibrosis patients with AAV vectors in 1995, over 75 clinical trials have been conducted. Pathologies such as  $\alpha_1$ -antitrypsin deficiency, Alzheimer's disease, Canavan's disease, hemophilia B, Leber congenital amaurosis, Parkinson's disease and muscular dystrophy; have attained promising results and no direct side effects have been associated with the vector (Warrington & Herzog, 2006). Although AAV vectors have shown to be promising tools for therapeutic gene delivery, they cannot transduce all cell types and could be useful to restrict its transduction to specific cell types. Strategies to modify the natural tropism of AAV vectors include: (i) the insertion of ligands into the viral capsid, (ii) use of chimeric or mosaic capsids, and (iii) conjugation with ligands

through non-genetic modifications (Fig. 3) (Choi et al., 2005; Kwon & Schaffer, 2008; Michelfelder & Trepel, 2009).

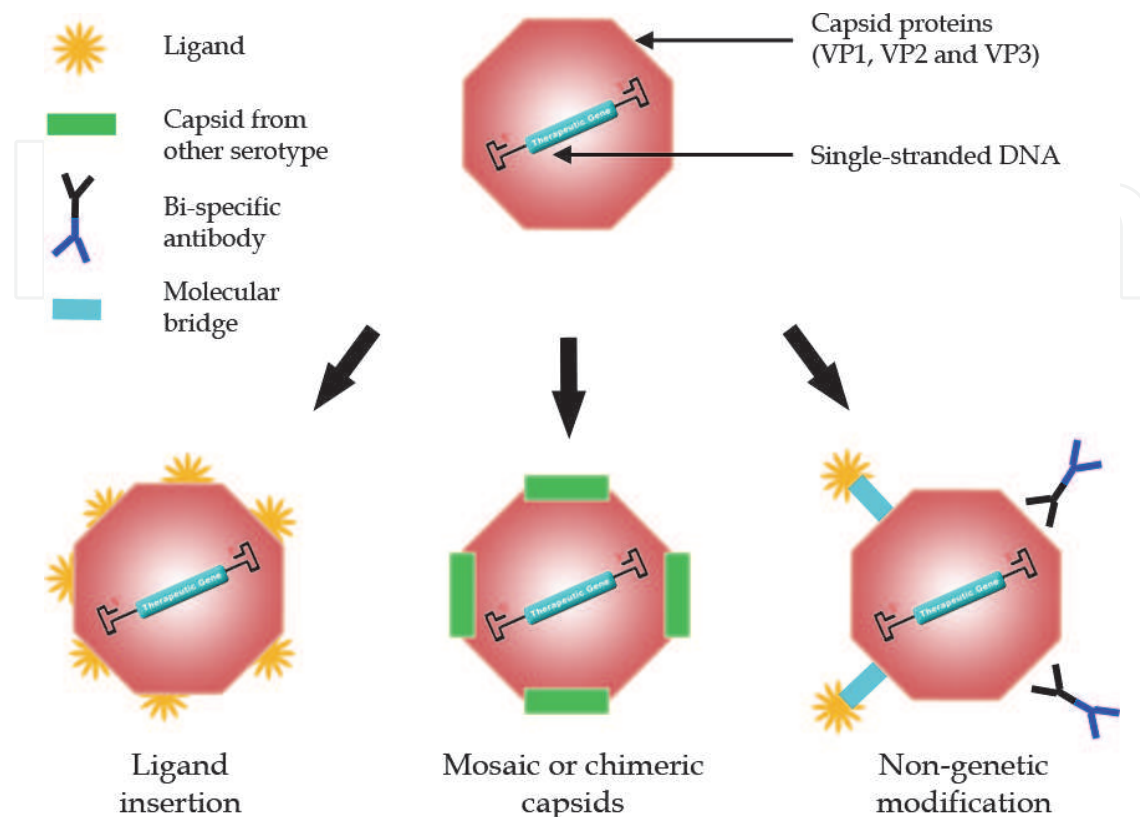


Fig. 3. Strategies for AAV vectors retargeting. AAV vectors can be modified by insertion of ligands into the capsid's proteins (ligand insertion), mixing capsid proteins from different serotypes (mosaic or chimeric capsids), or absorption of molecules onto the capsid using bi-specific antibodies or ligands fused with molecular bridges (non-genetic modification).

#### 4.1 Targeting by ligand insertion

Mutagenesis analysis have permitted the identification of positions within the viral capsid that allow peptide insertion with little or no effect on DNA packaging and virus trafficking. For AAV2-derived vectors, peptides inserted in positions 1, 34, 138, 161, 459, 584, 587 and 588 (relative to VP1 start codon) are displayed on the vector surface, and allow production of vectors with similar viral titers to those observed for unmodified AAV2 vectors (Shi et al., 2001; Wu et al., 2000b). Most of the studies have used positions 138 (VP2 N-terminal), 587 and 588 (HSPG binding domain) to insert peptides ranging from 5 to 272 amino acids (Loiler et al., 2003; Michelfelder et al., 2009; Nicklin et al., 2001a; Perabo et al., 2006; Shi et al., 2001; White et al., 2007; White et al., 2004; Wu et al., 2000b; Yu et al., 2009). Capsid protein modifications have improved gene delivery to lung (Kwon & Schaffer, 2008), endothelial cells (Nicklin et al., 2001a), pancreatic islets (Loiler et al., 2003), vascular tissue (White et al., 2004), atherosclerotic lesions (White et al., 2007), muscle (Yu et al., 2009), myocardium (Yang et al., 2009), and cancer cells (Michelfelder et al., 2009).

Although the therapeutic effect of these vectors still remains to be seen, there is a long list of reports showing different advantages of peptide insertion within different positions of the viral capsid. Insertion after position 588 provided a complete AAV2 retargeting with inhibition

of binding to HSPG (Büning et al., 2003; Perabo et al., 2006). AAV vectors designed for vascular tissue targeting were constructed by insertion after position 587 of peptides MSLTTPPAVARP and MTPFPPTSNEANL, which showed increased transduction and specificity for venous endothelial cells and reduction of hepatocytes transduction (White et al., 2004). *In-vivo* experiments with these modified AAV vectors demonstrated a reduction in liver and kidney transduction, while a significantly higher and specific targeting towards vena cava cells was observed (White et al., 2004). Recently, two new peptides (CAPGPSKSC and CNHRYMQMC) were evaluated for AAV retargeting to atherosclerotic lesions (White et al., 2007). The modified vectors showed higher levels of *in vitro* transduction than those observed for the untagged vector in human, murine, and rat endothelial cells. *In vivo* experiments showed that substantial higher levels of both peptide-modified AAV2 vectors were detected in the brachiocephalic artery (the site of advanced atherosclerotic plaques) and aorta, whereas reduced levels of modified vector were detected in all other organs examined (White et al., 2007). Serpin receptor ligand inserted after position 138 increased by 15-fold the transduction of vector on a lung epithelial cell line expressing the serpin receptor (Wu et al., 2000b). Insertion of a peptide that binds to the luteinizing hormone receptor after position 139 allowed production of a modified vector able to specifically transduce ovarian cancer cells (a luteinizing hormone receptor-bearing cell line) in an HSPG-independent manner (Shi et al., 2001). Insertion after position 138 of the 28-amino acid ApoE-derived ligand led to a 90-fold increase in pancreatic islet cells *in-vitro* transduction. Additionally, a four-fold increase of human antitrypsin expression was observed compared to unmodified AAV vector transduction (Loiler et al., 2003). Viral dose can be reduced 100-fold with similar transduction results as a consequence of ApoE-derived ligand insertion with a critical impact on side effect reduction. Functional AAV-modified vectors with long peptides up to 30 kDa have been reported, yet a laborious producing protocol is necessary to increase yield (Warrington et al., 2004).

Although these studies have demonstrated the feasibility of AAV retargeting, there is only one study evaluating the therapeutic effect of a modified AAV vector. In the study, AAV vectors were designed for brain-endothelial targeting, using peptides identified by phage display (Chen et al., 2009). After intravenous administration of modified vectors in MPS VII mice, there was extensive expression of enzyme in brain, leading to correction of lysosomal storage and reversal of established behavioral deficits (Chen et al., 2009).

During the last years we have worked in the development of a gene therapy strategy for the Mucopolysaccharidosis IV A (Morquio A disease) by using AAV vectors (Alméciga-Díaz et al., 2010; Alméciga-Díaz et al., 2009; Gutierrez et al., 2008). Morquio A disease, as well as other mucopolysaccharidosis, has a marked bone involvement, and an effective therapy should focus on the treatment in these manifestations. Recently, we designed a bone-targeting AAV vector bearing an aspartic acid octapeptide inserted immediately after the N-terminal of the VP2 capsid protein. The vector was designed to interact with hydroxyapatite (HA), a main component of bone (Tomatsu et al., 2010). We observed that the unmodified AAV vector had low bone affinity, while the bone-targeting vector had significantly higher HA affinity with up to 36.6-fold higher vector genome copies in cortical bone compared with the unmodified vector matrix (Alméciga-Díaz CJ, Montaña A, Barrera L, Tomatsu S., unpublished data).

#### 4.2 Chimeric or mosaic capsids

A mosaic capsid AAV is a virion that is composed of a mixture of viral capsid proteins from different serotypes, which are mixed during viral assembly. On the other hand, a chimeric



capsid AAV is a vector produced by the insertion of a sequence from another wild-type AAV into the ORF of the capsid gene (Fig. 3) (Choi et al., 2005; Michelfelder & Trepel, 2009). AAV vectors produced by the mixture of capsid proteins of AAV serotypes 1 to 5 led to high-titer viral particles with mixtures of serotype 1, 2, or 3; whereas intermediate titers were observed from AAV5 mixtures (Rabinowitz et al., 2004). Transduction levels varied depending on capsid mixture, producing a synergistic effect in transduction when AAV1 capsids were combined with AAV2 or AAV3 (Rabinowitz et al., 2004). Similar results were observed for a vector produced by the mixture of AAV1 and AAV2 capsid proteins, which exhibited similar titers to those observed for native vectors. The biodistribution profile combined transduction characteristics of both parent vectors (i.e. muscle and liver transduction) (Hauck et al., 2003).

A variant of this strategy uses capsid proteins bearing foreign peptides mixed with wild-type capsids. An AAV2 vector containing wild-type and immunoglobulin-binding Z34C fragment of protein A-bearing capsid proteins; made possible the production of a vector with high virus particle and transduction titers, and the capacity to transduce selectively and efficiently MO7e (human megakaryoblastic leukemia cell line) and Jurkat cells (T lymphocyte cells line) (Gigout et al., 2005). A mosaic vector produced by the mixture of wild-type and integrin-targeted capsid proteins resulted in a vector with a 50 - 100-fold enhancement in endothelial cell gene transfer. This is higher compared to the vector produced only with integrin-targeted capsid (Stachler & Bartlett, 2006).

#### 4.3 Adsorption of receptor ligands

This strategy involves the binding of molecules onto the viral capsid to alter natural tropism of AAV vectors (Fig. 3). Initial approaches used bi-specific antibodies consisting of an anti-AAV antibody cross-linked with another antibody that binds specifically to a cellular receptor (Choi et al., 2005). In the first approach, an anti-AAV antibody was chemically cross-linked to Fab arms of the  $\alpha_{IIb}\beta_3$  integrin binding monoclonal AP-2 antibody, allowing the specific transduction of megakaryocyte cells expressing the integrin (Ponnazhagan et al., 1996).

In a second approach, a high-affinity biotin-avidin interaction was used as a molecular bridge for the adsorption of streptavidin-fused ligands (Ponnazhagan et al., 2002). This strategy did not affect virus production and allowed the vector to be specifically targeted to EGFR- or fibroblast growth factor receptor (FGFR)-positive cells after conjugation of EGF or FGF onto the biotinylated capsid. Recently the exogenous glycation of the viral capsid was presented as a potential alternative to redirect vectors from liver to skeletal and cardiac muscle after systemic administration in mice (Horowitz et al., 2011).

### 5. Non-viral vectors

Non-viral vectors for gene delivery offer several advantages over viral vectors, since these include lack of an immune response, simple synthesis, and low scale-up production cost (Niidome & Huang, 2002). Non-viral vectors may include lipoplexes (DNA-liposome complex), polyplexes (DNA-polymer complex), lipopolyplexes (DNA-liposome-polymer complex) and peptide-based complexes (Douglas, 2008); which can be mixed with physical methods (e.g. electroporation, ultrasound, gene gun or hydrodynamic infusion) to improve the gene delivery (Niidome & Huang, 2002). In addition, most used non-viral vectors do not integrate into the host genome reducing the risk of mutagenic events in the transfected cells

(Kim et al., 2010). However, transduction efficiencies are significantly lower than that of viral vectors (Douglas, 2008). The reader is referred to other chapters in this book edition for more thorough treatises of non-viral vectors.

Although some physical methods allow gene delivery to a specific cell type, we will only consider some strategies developed to modify vector tropism. It is important to note that although most of these studies have been evaluated exclusively *in-vitro*, they constitute proof-of-concept.

### 5.1 Lipoplexes

Lipoplexes are the result of a complex formation between cationic liposomes (artificial closed vesicles of lipid bilayer membranes) and genetic material. They can be considered a synthetic means of encapsulating genetic material until it reaches its cellular target (Tros et al., 2010). The main strategies for targeting of lipoplexes involve use of chelator lipids that bind metal-bearing ligands, or the modification with polymers bearing a ligand (Fig. 4).

The insertion of the chelator lipid 3 (nitrilotriacetic acid)-ditetradecylamine (NTA(3)-DTDA) in a lipoplex preparation allowed the engraftment of a His-tagged vascular epidermal growth factor (VEGF) ligand and specific transfection of cells expressing the VEGF receptor (Fig. 4) (Herringson et al., 2009). A similar approach was used to target small iRNAs to B lymphocytes by using a lipoplex bearing the NTA(3)-DTDA chelator lipid engrafted with a His-tagged CD4 or to cells expressing the receptor for tumor necrosis factor alpha (TNF $\alpha$ ) by engrafting with a His-tagged TNF $\alpha$  ligand (Herringson & Altin, 2009).

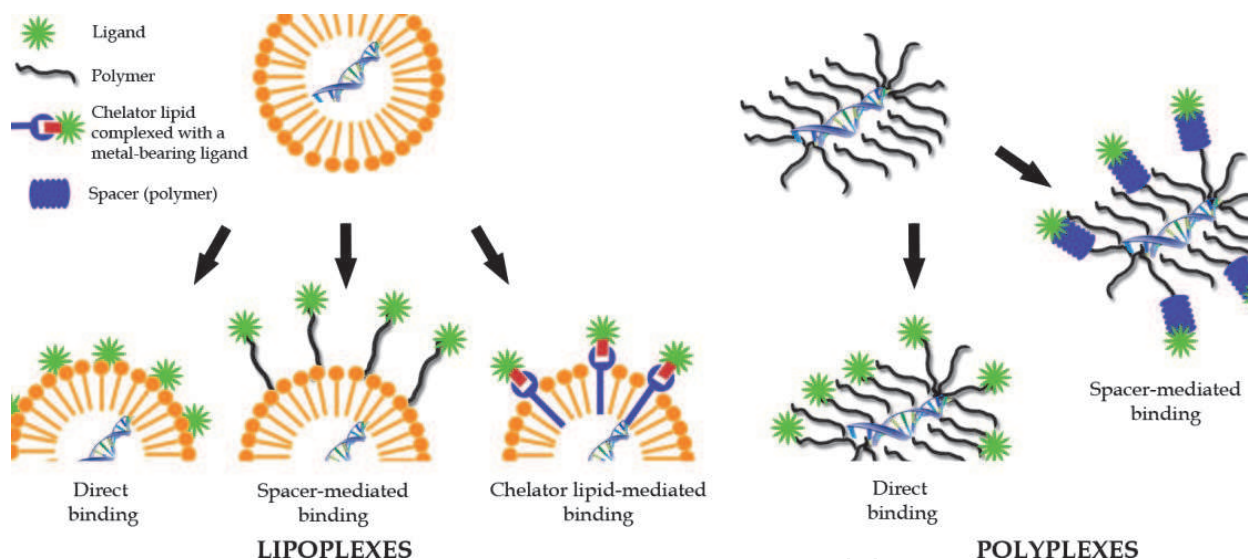


Fig. 4. Modification of lipoplexes and polyplexes. Ligands can be bound to non-viral vectors through direct binding, spacer (polymer)-mediated binding, or chelator lipid-mediated binding (ligand should be fused with a metal).

To improve hepatic specific delivery of a lipoplex after systemic administration, PEG molecules were added to the complex and galactose molecules were covalently affixed at the distal end of the PEG chain to allow an active targeting to the asialoglycoprotein-receptor present on hepatocytes. This resulted in an 18-fold increase in hepatic gene expression (Morille et al., 2009). Inclusion of a PEG conjugated with a nuclear localization signal (NLS) and added to a lipoplex preparation permitted transfection of endothelial cells in the

presence of serum. This method was used as a model for transfection of blood-brain barrier cells considered difficult targets for non-modified viral and non-viral vectors (Zhang et al., 2009). A similar strategy was used for targeting of a lipoplex to tumor cells expressing the tumor-associated glycoprotein (TAG)-72 (Kim et al., 2008). In this work, a Fab' fragment from an anti-TAG-72 monoclonal antibody was conjugated with PEG and included within the lipoplex, providing specific gene delivery to TAG-expressing tumor cells both *in-vitro* and *in-vivo*, while non-targeted lipoplexes did not produce the transfection of those cells.

Ligands can be also directly conjugated with liposome molecules (Fig. 4). A hyaluronic acid-liposome conjugate resulted in an increased gene delivery to breast cancer cells with high CD44 levels. Conversely, cells with low CD44 levels were not transfected (Surace et al., 2009). Conjugation of the peptide 4-fluorobenzoyl-RR-(L-3-(2-naphthyl)alanine)-CYEK-(L-citrulline)-PYR-(L-citrulline)-CR with a liposome permitted targeting of a lipoplex to CXCR4-expressing cells, with transfection efficiency depending upon CXCR4 expression levels (Driessen et al., 2008). Conjugation of a liposome with transferrin presented a significant increment in iRNA delivery to hepatocarcinoma cells, with reduced toxicity and enhanced specific gene silencing compared to non-modified lipoplexes (Cardoso et al., 2007). Transferrin-conjugated lipoplexes were also used to evaluate specific gene delivery to a metastatic mammary carcinoma cell line, although the results did not demonstrate a significant increase in cytotoxicity with modified lipoplexes in comparison with non-modified lipoplex (Lopez-Barcons et al., 2005).

Another strategy based on *ex-vivo* gene therapy assessed the challenge of nuclear entry by using plasmids transfected with a cationic lipid vector in MSC (Hoare et al., 2010). To enhance gene expression in MSC, they used NLS peptides to direct the plasmid to the nucleus. A significant increase was observed in reporter gene delivery compared to non-modified plasmid. They also noticed the lipid had a protective impact on the plasmid-NLS complex. Differentiation potential was not affected by NLS peptides.

## 5.2 Polyplexes

Polyplexes are non-viral vectors formed by genetic material coupled with cationic polymers including polylysine (PLL), polyarginine, PEG, polyethylenimine (PEI), polyamidoamine (PAMAM), dendrimers, and chitosan. The mechanism involves interaction between negatively charged DNA or iRNA molecules with positively charged molecules within the polymer (Tros et al., 2010). To target polyplexes to specific cells, they can be modified by incorporation of ligands as small molecules, vitamins, carbohydrates, peptides, or proteins (Wagner et al., 2005).

PEG-derived vectors are widely used as gene therapy tools as a result of their well-known capacity to enhance gene delivery and induce a longer DNA half-life time (Midoux et al., 2008). Due to the possibility to bind ligands in the terminal ends of the polymer (Fig. 4), PEG-derived vectors are commonly used for cell specific gene delivery using non-viral vectors (Hughes & Rao, 2005). PEG polyplexes have been conjugated with a transferrin peptide to target vectors to receptor-expressing cells in two prostate cancer cell lines (Nie et al., 2011). In addition they have been conjugated with EGFR ligand or lactose for specific transfection of human hepatocarcinoma cell lines (Klutz et al., 2011; Oishi et al., 2007).

PEG has also been used as a spacer to bind ligands to polyplexes (Fig. 4), although it has been associated in some cases with reduced transfection efficiencies. A complex of DNA and the HIV-derived TAT protein (which has important translocation abilities due to its strong

cell surface adherence) was conjugated with an ICAM-1 ligand by using a PEG spacer allowing an efficient and specific transfection of ICAM-1 expressing cells (Khondee et al., 2011). Similarly, a PEI-derived polyplex was engineered for enhanced and specific delivery of VEGF iRNA in prostate cancer cells. The conjugation with prostate cancer-binding peptide via a PEG spacer, showed a significantly higher VEGF inhibition than with the unmodified PEI polyplex in human prostate carcinoma cells (Kim et al., 2009). Anionic liposomes and cationic polymers do not display an efficient targeting to liver or tumors cells. Thus, a lipopolyplex was constructed with a liposome. A PEI polymer and a PEG polymer bearing a monoclonal antibody permitted an *in-vitro* and *in-vivo* transfection of tumor cells (Hu et al., 2010). A similar strategy was used to target an iRNA to tumor cells expressing the EGFR by using this lipopolyplex bearing the EGFR ligand (Hu et al., 2011).

Direct binding of ligands in other polyplexes has also been evaluated. Conjugation of folic acid with the aminomethacrylate-phosphoryl-choline based copolymer, allowed transfection of cells overexpressing the receptor for folic acid in some human tumors cells (Lam et al., 2009). A PEI-derived polyplex conjugated with an Arg-Gly-Asp peptide, which binds to the  $\alpha_v\beta_3$  integrin receptor, resulted in efficient transfection of HeLa cells with low and high densities of  $\alpha_v\beta_3$  integrin (Ng et al., 2009). As observed in viral vectors and lipoplexes, antibodies can be also conjugated with polyplexes to target genetic material to specific cell types. A successful overexpression of the human epidermal growth factor receptor-2 (HER2) by transfection with a polyplex conjugated with an anti-HER2 monoclonal antibody has been reported for a breast cancer cell line (Chiu et al., 2004). Another approach in adult MSCs used a modification of a PLL polymer with a palmitic acid substitution to target bone marrow stromal cells. A transfection efficiency similar to that of adenoviral vectors was obtained, opening the possibly to efficiently transfecting these cells without the side-effects and cell toxicity observed with viral vectors (Incani et al., 2007).

*In-vivo* evaluations of these modified polyplexes include use of a PEI-derived complex bearing an FGFR ligand for transfection of tumor cell lines. It demonstrated *in-vitro* and *in-vivo* specific transduction of tumor cells overexpressing the FGFR (Li & Huang, 2006), and the use of a poly[N-(2-hydroxypropyl)methacrylamide]-derived polyplex modified with an anti-P-selectin antibody. *In-vivo* specific and enhanced gene delivery to inflamed cremasteric venules was observed (Newman et al., 2009). A significant retargeting to tumor cells was also observed with a PEI polyplex carrying an antibody against a tumor-specific protein via a PEG spacer, with low expression in non-tumor cells (Duan et al., 2010).

## 6. Conclusions

Modification of gene therapy vectors for specific cell type gene delivery can have a significant impact on vector tropism limitations, side effects, and consolidation of gene therapy as a viable treatment. Vector modifications can be grouped in three basic strategies regardless of vector type: genetic modification (ligand insertion within the capsid), chemical modification (polymers or ligand cross-linking), or the use of adaptor molecules (bi-specific antibodies). Most studies have focused on vector design and proof-of-concept of specific gene delivery, while only few of them have evaluated the therapeutic impact of these modifications. In that sense, future studies should focus on assessing the potential of these modified vectors to improve gene therapy trials in an effort to advance from the bench science to the clinic trials. Special attention should be placed in the development of modified vectors for gene delivery to CNS and bone. Since, under normal conditions they show a



limited transfection profile with the currently available vectors. Finally, parallel research should be carried out in tissue-specific promoters to design safer integrating vectors, reduce immune response, and improve the processes of vector production.

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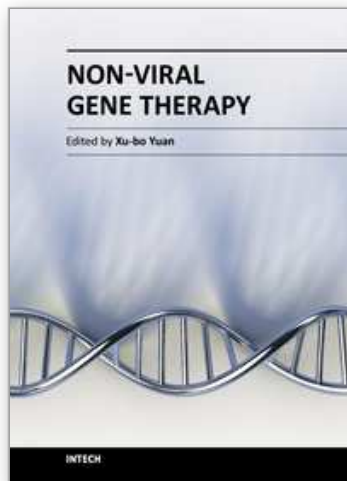
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This book focuses on recent advancement of gene delivery systems research. With the multidisciplinary contribution in gene delivery, the book covers several aspects in the gene therapy development: various gene delivery systems, methods to enhance 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.

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