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1. Introduction

The present chapter will be focused on different gene delivery systems used in gene therapy approaches with the purpose of inserting into individual cells and/or tissues to treat diseases. By correcting genetic defects via genome manipulation, gene therapy can truly revolutionize medical intervention for treating monogenetic inherited/acquired diseases or polygenetic conditions.

Gene therapy has undergone a remarkable development in the last 20 years. Particularly important advances have been made in the improvement of gene transfer and expression technology, with current efforts focusing on the design of safer and long-term gene expression vectors as well as systems possessing cell-type specificity for transgene delivery and regulatability of its expression by small molecules. The foreign genetic material can be administered in vivo, ex vivo or in vitro depending on the nature of a disease. A successful gene therapy system must perform several functions. In all cases, the therapeutic gene must first be delivered across the cell membrane, which is a significant barrier. Once delivered inside the cell, the therapeutic gene may exist episomally or be integrated into the host genome depending on the nature of the gene transfer vector. Moreover, an important issue is the replication and segregation of the therapeutic gene during cell division in order to maintain long-lasting gene expression. These specifications will be discussed for each gene delivery system along the chapter.

- Current viral vector systems will be detailed in this chapter: Adenovirus, Retrovirus, Lentivirus, Adeno-associated Virus, Sendai Virus and Herpes Simplex Virus.
- Non-viral vector systems will be also discussed: naked DNA (including plasmids, DNA transposons), DNA-lipid complexes and nanoparticles (including Qdots and the new technology of magnetic nano-particles) which have been actively developed as additional tools for crossing the cell membrane.
• Novel systems were the viral and non-viral methods merge will be also addressed.

2. Non viral gene delivery systems

2.1. Naked DNA

Naked DNA such as plasmids remain popular as vectors for gene therapy today for their low immunogenicity and low risk of causing insertional mutagenesis. However, their episomal feature resulting in transient gene expression makes them unsuitable as gene therapy vectors when long-term gene expression is needed for treatment. DNA transposons have the properties of naked DNA and plasmids as well as the ability to insert transgenes into host chromosomes for long-term expression. DNA transposons are natural genetic elements residing in the genome as repetitive sequences that move through a direct cut-and-paste mechanism.

A simple transposon is characterized by terminal inverted repeats flanking a gene encoding transposase, an enzyme required for its translocation (Meir et al., 2011). The cut-and-paste process, called transposition, makes DNA transposons particularly attractive as gene delivery tools. To turn DNA transposons into a gene delivery tool, a two-plasmid system, consisting of a helper plasmid expressing the transposase and a donor plasmid with the terminal repeat sequences flanking genes of interest, has been developed. Using this system, transposons have been utilized extensively as genetic tools in invertebrates and in plants for transgenesis and insertional mutagenesis (Spradling et al., 1982; Hayes et al., 2003).

Plasmid DNA is an attractive alternative due to its inherent simplicity and because it can easily be produced in bacteria and manipulated using standard recombinant DNA techniques. It shows very little dissemination and transfection at distant sites following delivery and can be re-administered multiple times into mammals without inducing an antibody response against itself (Jiao et al., 1992). Also, considerable long term foreign gene expression from naked plasmid DNA (pDNA) is possible even without chromosome integration if the target cell is post-mitotic or has low mitotic rate and if an immune reaction against the foreign protein is not generated (Herweijer et al., 2001; Wolff et al., 1992). The poor expression levels represent a major constraint in the use of these vectors for gene transfer/therapy. However, the low efficient expression by direct injection of naked plasmids was improved by ballistic technology, cationic lipids and neutral polymers (Prud’homme et al., 2001; Gao et al., 1995; Lemieux et al., 2000) and most efficiently by electroporation (Sandri et al, 2003), an established technology that transiently permeabilizes cell membrane by short voltage pulse, allowing the uptake of a wide spectrum of biological molecules.

The stability of DNA vectors with high-molecular-weight is a central point for the improvement of gene delivery. All high-molecular-weight DNA vectors are susceptible to damage. The self-compacting option (self-entangling) can be defined as the folding of single DNA molecules into a configuration with mutual restriction by the individual segments of bent DNA. A negatively charged phosphate backbone makes DNA self-repulsive, so it is reasonable to assume that a certain number of ‘sticky points’ dispersed within DNA could facilitate the
entangling. Tolmachov, proposes that the spontaneous entanglement of vector DNA can be enhanced by the interlacing of the DNA with sites capable of mutual transient attachment through the formation of non-B-DNA forms, such as interacting cruciform structures, inter-segment triplexes, slipped-strand DNA, left-handed duplexes (Z-forms) or G-quadruplexes. (Tolmachov, 2012).

2.2. Liposomes

The liposomes, lipids arranged in lamellar structures, are concentric bilayered vesicles surrounded by a phospholipid membrane. They are related to micelles which are generally composed of a monolayer of lipids. The amphiphilic nature of liposomes, their ease of surface modification, and a good biocompatibility profile make them an appealing solution for increasing the circulating half-life of peptides, proteins, cDNAs and siRNAs (Bhavsar et al., 2012). They may contain hydrophilic compounds, which remain encapsulated in the aqueous interior, or hydrophobic compounds, which may escape encapsulation through diffusion out of the phospholipid membrane (Figure 1).

Figure 1. Non-viral gene delivery using lipoplexes. DNA is complexed with cationic liposomes and is internalized through receptor mediated endocytosis. After their internalization large amounts of complexes are degraded in the endolysosomal compartments. Only a small fraction enters into the nucleus and elicits desired gene expression. (From: Pankajakshan Divya and Devendra K. Agrawal, 2013).
Liposomes can be designed to adhere to cellular membranes to deliver drugs or cDNAs after endocytosis (Bangham et al., 1995). The first formulation was prepared in 1986 by the Christian Dior laboratories in collaboration with the Pasteur Institute (Bangham et al., 1995). Liposomes have been used as delivery systems due to their versatility, charge and surface functionalities that improve their effectiveness in vivo. Presumably, the lack of widespread medical impact is due to their limited biological stability. Hydrophobic nanoparticles such as unmodified liposomes are rapidly cleared via the reticuloendothelial system. Liposome formulations with prolonged circulation time have been obtained upon functionalization with PEG (polyethylene-glycol, a biologically inert polymer). The longer circulation half-life of these PEG-coated liposomes may allow a better control of therapeutic drug delivery (Gabizon A et al, 1988).

There are additional unique mesophase structures of lipids formed as a result of lipid structure polymorphisms, which include cubic-, hexagonal- or sponge-phases. These structures have been utilized for gene, vaccine and drug delivery and provide the advantages of stability and production feasibility compared with liposomes (Shanmugam et al., 2011).

2.3. Nanoparticles and nanotechnology

Nanotechnology is a rapidly expanding field, encompassing the development of man-made materials in the 5–200 nanometer size range. This dimension vastly exceeds that of standard organic molecules, but its lower range approaches that of many proteins and biological macromolecules. The origin of nanotechnology can be traced back to 1959 when physicist Richard Feynman (1960) recognized the potential of manipulating individual atoms and molecules at the nanometer scale and suggested that materials at this scale possess unique physical properties. The nanotechnology field encompasses concepts and approaches deeply rooted in physics, polymer and colloidal chemistry, pharmaceutics, biomaterials, as well as cell and molecular biology and biophysics. The main theme of nanotechnology is the development and use of nanometer-scale materials that display unique functional properties not shown by bulk materials. Nanomaterials can interact with biological systems at a molecular and supra-molecular level, they can be coated to respond to specific cell environments and even to induce desired physiological responses in cells, while minimizing unwanted side effects. The first practical applications of nanotechnology can be traced to advances in communications, engineering, physics, chemistry, biology, robotics, and medicine. Nanotechnology has been utilized in medicine for therapeutic drug delivery and the development of treatments for a variety of diseases and disorders. An intracellular nanoparticle, consequently, may act as a drug depot within the cell and provide an intracellular sanctuary to protect therapeutic compounds from efflux or degradation.

Several synthetic strategies exist to prepare ferromagnetic iron oxide nanoparticles (Gobe et al., 1983). For maghemite (\(\text{c-Fe}_2\text{O}_3\)) and magnetite (\(\text{Fe}_3\text{O}_4\)) nanoparticles, this precipitation technique requires alkalization of a solution of metal salt with subsequent hydrolysis in microemulsions. Additionally, biosynthetic routes exist utilizing “magnetic bacteria”; the resulting nanoparticles typically range from 50 to 100 nm in diameter (Matsunaga et al., 1998). The synthesis of iron oxide nanoparticles has also been achieved by sonochemical decomposition of iron pentacarbonyl (Faraji et al., 2009), thermal decomposition of other iron
complexes (Rockenberger et al., 1999), and by thermal decomposition of iron pentacarbonyl followed by oxidation. When optimized, these methods may afford monodisperse nanoparticles with sizes ranging from 3 to 20 nm for magnetite and 4 to 16 nm for maghemite (Sun et al., 2002). The circulation times of these particles can be greatly increased simply by hydrophilic surface modification with PEG. Furthermore, iron oxide nanoparticles also display fairly easy surface modification capabilities that presents an attractive prospect for direct drug or biomolecule payload attachment (Longmuir et al., 2006).

Nanoparticle-based delivery (NBD) has emerged as a promising approach to improve the efficacy and the development of new therapies (Probst et al., 2012).

2.3.1. Quantum dots (Qdots)

Quantum dots are luminescent nanoparticles typically used for imaging in biological systems. Their primary components — core (cadmium with selenium or tellurium), shell, and coating — give the photochemical properties. Qdots have small size and versatile surface chemistry and offer superb optical properties for real-time monitoring as transport vehicles at both cellular and systemic levels. Qdots offer great potential providing mechanisms for monitoring intracellular and systemic nanocarrier distribution, degradation, drug release, and clearance. They can be manufactured with diameters from a few nanometers to micrometers and a narrow size distribution using techniques requiring high annealing temperatures (Yum et al., 2009). Capping of quantum dots with ZnS has been shown to augment stability and enhance luminescence (Park et al., 2009). However, ZnS capping alone is not sufficient to fully stabilize the core, especially in biological systems. PEGylation plays a dual role in increasing biocompatibility and improving the core stability in biological systems (He et al., 2010).

Though the direct use of QDots for drug delivery remains questionable due to their potential long-term toxicity, the QDot core can be easily replaced with other organic drug carriers or more biocompatible inorganic contrast agents (such as gold and magnetic nanoparticles) based on their similar size and surface properties, facilitating translation of well characterized NBD vehicles to the clinic, maintaining NBD imaging capabilities, and potentially providing additional therapeutic functionalities such as photothermal therapy and magneto-transfection.

2.3.2. Magnetic nanoparticles (MNPs)

Magnetofection is a methodology developed in the early 2000’s (Scherer et al., 2002). It is based on the association of MNPs with non-viral or viral vectors in order to optimize gene delivery in the presence of a magnetic field, and to concentrate therapeutic complexes in target areas. The association of viral vector-based gene delivery with nanotechnology now offers the possibility to develop more efficient and less invasive gene therapy strategies for a number of major pathologies and diseases (See details in Section 3.5 of this chapter).
3. Viral gene delivery systems

3.1. General overview of viruses as vectors for gene delivery

For centuries the health sciences have invested sweat and tears in order to fight against viral infections affecting humans, animals and plants. It is reasonable to imagine that the idea of viruses as therapeutic agents was quite a shock when first presented. The enormous advances in Molecular Biology, Biochemistry, Genomics and Human Medicine, among others, have provided to the Virology field the necessary tools for manipulating them on their behalf.

These recombinant vectors are viruses where the genome has been altered in a controlled way by experimental manipulation. For any procedure to generate a recombinant virus the starting point is to clone and manipulate its genome. Thus DNA virus genomes may be cloned directly while RNA virus genomes may be cloned as cDNA. These molecules can then be modified by site-specific alteration, or more drastically, segments may be removed and replaced with foreign DNA sequences. Then the process must be completed by recreating infectious virus particles. This requires specific techniques and is not yet possible for all virus types (Dimmock et al., 2007).

Regarding the biological value of these viral vectors, there are constantly novel potential applications such as vaccines, carriers of nucleic acid sequences for regulating gene expression and agents for gene therapy. In order to become a therapeutic agent the DNA has to be carried into the cell and ultimately reach the nucleus; therefore it is mandatory to be provided of a strategy for membrane cross and lysosomal scape. This is something that naked DNA is very poorly equipped to achieve. By contrast, the nucleic acid that is inside an infectious virus particle can avoid these issues. First, viruses have evolved specific interactions with cell surface molecules that lead to their efficient entry and, second, if that entry involves arrival in the cytoplasm within an endocytic vesicle, then viruses have mechanisms to allow efficient escape. This process of virus-mediated gene delivery into a cell is known as transduction (Dimmock et al., 2007).

It is clear then that, to be potentially useful as a gene delivery vector, a virus should have a number of specific features (Figure 2).

However, there is no virus that can meet all the criteria for an ideal gene delivery vector and there are some significant drawbacks that will be addressed in the following sections. Thus, each application is likely to need its own vector, chosen and then tailored to fulfill the precise requirements.

3.2. Current viral vectors systems

3.2.1. Adenoviral vectors (Ad)

The discovery and initial description of Adenoviruses (AdV) took place in the early 1950s. They were first isolated from human adenoid tissue cultures (Rowe et al., 1953). Since then several different serotypes of human, avian, reptilian, amphibian and other mammalian
Adenoviruses have been isolated and characterized giving birth to the *Adenoviridae* family of over 50 members.

The focus on AdV for gene transfer was based on basic research. The establishing of the biology of AdV and their capacity to efficiently deliver the viral genome to the target cells became relevant then. More importantly, since AdV was not oncogenic in humans and the genomes of common AdV were completely defined and easy to modify, the production of recombinant AdV (RAdV) was achieved. In the context of gene delivery, serotypes 5 and 2 of the subgroup C have been used the most because their structure and biology is well described and there are convenient biologic reagents available to produce recombinant subgroup C gene transfer vectors in large quantities. Regarding safety, AdVs of subgroup C can cause minor to mild respiratory infections sometimes associated with conjunctival compromise (Ginsberg et al., 1994).

AdV virions consist of a ~36 kb linear double-stranded DNA genome encased within a non-enveloped icosahedral particle (Figure 3).
The experimental manipulation of its genome has allowed the production of a diversity of recombinant viral particles where most of the replication genes are removed. The deletion of the E1 and E3 regions in first-generation AdV allows ~7 kb of foreign DNA to be inserted into the vector genome (Volpers and Kochanek, 2004). Another feature of these vectors is that they can be grown to extremely high titers in the HEK 293 cell line, with burst sizes typically between $10^3$-$10^4$ viral particles (VP) per cell and final concentrations reaching $10^{13}$ VP/ml, after CsCl density gradient centrifugation. Whereas AdV vectors can achieve high expression in many target organs when used in vivo, expression of the transgene is limited to days or weeks, mainly because innate and adaptive immune host defenses against the virus. For applications where persistent expression is required to achieve a therapeutic goal, the modern, third-generation, high capacity AdV vectors have become the most efficient alternative (Hackett NR and Crystal RG, 2009). Other important obstacle in the use of RAd for gene transfer is the process of cell attachment and internalization used by the viral particles. The target cell must express the cell membrane receptor CAR (Coxsackie-Adenovirus Receptor) in order to be susceptible to the adenoviral infection (Figure 4).

For those transduction-refractory tissues, modern virology has developed modified-tropism RAds with modifications in fiber/high affinity receptor or the penton-integrin of the capsid. In an extensive survey of the tropism of AdV5-derived vectors but with fibers derived from different serotypes, the fiber genes of AdV16 were found to be better at targeting fibroblasts and chondrocytes, AdV35 at targeting dendritic cells and melanocytes and AdV50 better at targeting myoblasts and hematopoietic stem cells (Havenga et al., 2002). The addition of an oligolysine motif to the C-terminus of the fiber protein, giving the virus an affinity for polyanions such as heparin sulphate, profoundly affects the range of cell types that can be...
infected in vitro allowing cells lacking CAR to be transduced by RAdVs (Bouri et al., 1999). For a more detailed description of Adenoviral vectors see section 3.3 of the present chapter.

3.2.2. Adeno-associated virus Vectors (AAV)

AAV is a human parvovirus within the genus Dependovirus. It was originally observed as a contaminant of laboratory preparations of adenovirus (Carter et al., 2009). Viral particles are small (20–30 nm) and non-enveloped, containing single-stranded DNA molecules with plus
and minus strands packaged with equal efficiency (Daly, 2003). No human disease has been associated with AAV infection which is an important feature when thinking in AAV as gene vectors. Six serotypes of AAV have been described with AAV2 being the most widely used for gene-transfer studies (Hermonat et al., 1984). AAV2 cell entry is mediated by binding to heparin sulfate proteoglycans and αvβ5 integrin; fibroblast growth factor receptor-1 (FGFR-1) may also be involved. The distribution of these molecules on many different cell types can explain the prolonged in vivo expression following AAV treatment seen in the liver, brain, skeletal muscle, lung, and hematopoietic stem cells of animal models (Daly, 2003). AAV vectors contain no viral genes that could elicit undesirable immune or inflammatory responses. The primary host reaction that might have an unwanted impact is the production of neutralizing antibodies against the viral particles.

One major concern when developing AAV vectors for gene delivery is that DNA constructs larger than the wild-type 4.7 kb sequence do not package well and vector titers decrease sharply thus constituting an insertional limitation for the cDNAs. Another important issue is the frequently seen integration of AAV genome in the host cell chromosomes. However, the available evidence indicates that integration of wild type AAV in vivo does not reflect the experimental in vitro observations, but appears to be a rather rare event and AAV genomes mostly persist as episomes, as has also been demonstrated for AAV vectors (Carter et al., 2009).

3.2.3. Retroviral vectors (RV)

Retroviruses are lipid-enveloped viruses; with nucleocapsids containing two copies of a linear, positive-stranded 7–11 kb RNA genome. The family Retroviridae contains various viruses that have shown potential utility for gene therapy, such as the gammaretrovirus (or simple retrovirus), spumaviruses and lentiviruses (or complex retrovirus). Following attachment and receptor-mediated entry into host cells, viral reverse transcriptase and integrase enzymes mediate reverse transcription and integration of the virus genome into the host-cell chromatin. Retroviral vectors have the ability for stable integration and allow long-term expression so that theoretically a single administration could have a sustained, potentially even, lifelong curative effect (Schambach et al., 2009). As for any viral vector, replication-deficiency is a condition. To achieve this goal, the retroviral coding sequences have to be removed, which creates at least 6 kb space for the transgene of interest. Since neither structural proteins nor replication enzymes are encoded by the target cell, the generation of replication-competent virus is prevented. The gammaretroviruses cannot infect quiescent, non-dividing cells, which is a handicap of the vectors derived from these retroviruses. However, this can be overcome by the use of lentiviral vectors.

For most RV, taking advantage of the insertional mechanism, the simplest application is in the production of cell lines that express a transgene introduced on a retroviral vector. For modified, transgenic animals, the lentivirus group must be used because gammaretrovectors are silenced during embryonic development. RV vectors can also be used in the delivery of toxic genes to cancer cells, which are actively dividing. Another area of application is gene discovery. The integration of the viral genome can reveal function by insertional inactivation of a gene in the host cell chromosome (Somia, 2003).
3.2.4. Herpes Simplex Virus Vectors (HSV)

HSV-1 is a double-stranded DNA virus, with a capsid surrounded by a dense layer of proteins - the tegument - enveloped in a lipid bilayer with surface proteins. It has evolved to persist in a lifelong nonintegrated latent state without causing disease in the immune-competent host. Among the herpes family Herpes Simplex Virus type 1 (HSV-1) is an attractive vehicle because in natural infection, the virus establishes latency in neurons, a state in which viral genomes may persist for the life of the host as intranuclear episomal elements. Although the wild-type virus may be reactivated from latency under the influence of a variety of stresses, completely replication-defective and non-lytic viruses can be designed (Goins et al., 2003). HSV-1 has a broad host range and does not require cell division for infection and gene expression. Accordingly, HSV may be generally useful for gene transfer to a variety of normal and disease tissues. The overall size of the HSV-1 genome (152 kb) represents an attractive feature for employing the vector for the transfer of large amounts of exogenous genetic sequences. Approximately one-half of the HSV-1 coding sequences are nonessential for virus replication in cell culture. At least 44 kb of HSV sequence can potentially be removed in order to accommodate a transgene (Wolfe et al., 2009).

The obstacles that need to be addressed in order to take advantage of the full potential of these vectors include elimination of residual vector toxicity, design of promoter cassettes that provide sufficient level and duration of transgene expression, and targeting of transgene expression to specific cell populations through the use of tissue-specific promoters, or by altering the virus host range through modifying receptor utilization for attachment and entry (Wolfe et al., 2009).

3.2.5. Sendai Virus Vectors (SeV)

Since its isolation in 1953 in Japan, Sendai virus (SeV) has been widely used as a research tool in cell biology and in the industry, but the application of SeV as a recombinant viral vector has been investigated only recently. Sendai virus (SeV) is a nonsegmented negative-strand RNA virus belonging to the Paramyxoviridae family. As SeV can infect various animal cells with an exceptionally broad host range and is not pathogenic to humans, various applications have been explored for SeV as a recombinant viral vector capable of transient but strong gene expression (Nakanishi and Otsu, 2012). Its RNA nature is advantageous for applications in which chromosomal integration of exogenous genes can be undesirable. These viral vectors are currently being tested in regenerative medicine to reprogram cell genomes to a pluripotent state with a surprisingly high efficiency (Nishimura et al., 2011; MacArthur et al., 2012) and as recombinant viral vaccines for influenza prevention (Le et al., 2011).

3.3. Recombinant adenoviral vectors

Although the pathologies associated with wild-type Adenovirus (AdV) infections are generally mild, there is a potential risk of using fully replication-competent AdV for gene transfer because the inflammatory host responses may alter organ function. There is also the possibility of overwhelming infection if AdV replication is allowed to progress when there are deficiencies
in the host defense system. These situations became then a major reason to develop recombi-
nant, non-pathogenic viral particles.

Regardless experimentation with different viral gene delivery systems, adenoviral vectors 
continue to be widely used for gene transfer strategies (Kovesdi et al., 1997; De Gruijl et al, 
2012; Youngjoo et al, 2013; Fishbein I et al, 2013). They were also the first viral vector system 
to be developed. However this was more by chance than intentionally while working in the 
production of live adenoviral vaccines propagated in monkey cell lines. Infection of tissue 
culture cells with AdV vaccine accidentally contaminated with simian virus 40 (SV40) resulted 
in the production of the SV40 T antigen, even after removal of the SV40 virions from the AdV 
stocks by immunodepletion. Analysis of this adenovirus revealed that the T-antigen gene from 
SV40 had recombined into the E3 region of the Ad genome. This demonstrated the possibility 
that AdV could carry foreign genes and express them as well as demonstrating the dispensa-
bility of the E3 genes for \textit{in vitro} replication (Roy-Chowdhury and Horwitz, 2002; Campos et 
al., 2007).

The first wild type adenoviruses subjected to “vectoring” process were AdVs derived from 
the human serotypes 5 (Ad5) and 2 (Ad2). First-generation replication-deficient Ad5 vectors 
were developed by deleting the E1 genes, necessary for expression of E2 and late genes 
required for AdV DNA synthesis, capsid protein expression, and viral replication. Further 
deletions included the E3 genes which are involved in the evasion of host immune defenses 
but dispensable for replication of the virus \textit{in vitro}. Therefore, because of this experimental 
manipulation leading to viral replication impairment, there was the need to develop a 
biological system capable of providing the genes required for the virus propagation. This led 
to the creation of the HEK 293 cell line, which was transfected with sheared adenovirus-type 
5 (Ad5) genomic DNA and stably expresses the E1 genes (Graham et al., 1977).

Although the first-generation AdV vectors are generally considered replication defective, there 
is some low level expression of viral antigens that limits the duration of transgene expression 
\textit{in vivo}, due to elimination of transduced cells by the cellular immune system (McConnell and 
Imperiale, 2004). To avoid this response and allow long-term episomal expression a new 
recombinant vector was developed. They are referred as “gutless” or high-capacity Adenovi-
rus (HC-AdV) lack all viral coding sequences except the \textit{cis}-elements required for the genome 
replication and encapsidation. Therefore, they need to be assisted by Helper Adenoviruses 
that provide all necessary replication genes in \textit{trans}; therefore, they are also called Helper-
Dependent adeno vectors (HD-AdV) (Amalfitano, 1999). In addition, these vectors have a 
much higher packaging capacity of \~{}35 kb of foreign DNA, enabling the expression of large 
transgenes or the inclusion of human genomic regulatory elements (Palmer and Ng, 2005). See 
Table 1 for comparison of the profile of different vector generations.

A variety of applications can be found for these vectors. The most widely explored are Gene 
Therapy for prostate, colon, cervix, ovary and CNS tumors, for genetic diseases such as 
Hemophilia, Duchene Muscular Dystrophy, Familial Hypercholesterolemia, Autoimmune 
Diabetes; viral vaccines; supplementary therapy for degenerative conditions as Parkinson, 
Alzheimer and Rheumatic Arthritis and production of recombinant proteins among others.
One of the most spectacular results in gene therapy using HD-AdV was published by Kim et al., 2001. They used a gutless AdV harboring the apoE gene to treat apoE-deficient C57BL/6 mice which display spontaneous hypercholesterolemia. These mice received a single intravenous (i.v) injection of HD-AdV-ApoE at the 12th week of age. This isolated intervention managed to normalize plasma apoE concentrations and therefore diminish the plasma cholesterol level to values found in wild-type mice. However, the most outstanding finding involves the duration of the protective effect of this gene therapy, being of at least 2.5 years, the lifespan of these mice.

Many of these AdV vectors produced in accordance with regulated quality standards, are now being used for human clinical trials. For detailed information the reader is referred to the following reviews: Russell, 2000; Józkowicz and Dulak, 2005; Campos, 2007.

### 3.4. Adenoviral vectors in tissue-specific gene transfer: The skeletal muscle

The skeletal muscle gene transfer approach using Adenoviral Vectors has created controversy. There are several studies with heterogeneous efficiency rates and, in some cases, divergent outcomes. On this section we will intend to present and discuss these results as well as to introduce a novel technology that might overcome the difficulties experienced in the transduction of this tissue.

<table>
<thead>
<tr>
<th>Ad vector generation</th>
<th>AdV genome deletions</th>
<th>foreign DNA insertion capacity</th>
<th>propagation in cell lines</th>
<th>type of transgene expression</th>
<th>host immune response</th>
<th>risks</th>
</tr>
</thead>
<tbody>
<tr>
<td>first</td>
<td>E1 and/or E3</td>
<td>6.5 kb</td>
<td>HEK293 (express E1)</td>
<td>transient</td>
<td>significant</td>
<td>Possibility of the production of replication-competent adenovirus (RCA)</td>
</tr>
<tr>
<td>second</td>
<td>E2 (complete or partial)</td>
<td>8kb</td>
<td>293-C2 (express E2)</td>
<td>AE1-2a</td>
<td>transient</td>
<td>important; especially after repeated administration No production of RCA</td>
</tr>
<tr>
<td>third</td>
<td>most of the genome. ITR and packaging seq. retained (&quot;gutless&quot;, HC or HD-Ad)</td>
<td>35-37 kb</td>
<td>293 cells expressing Cre; require helper virus</td>
<td>long-lasting</td>
<td>low or none</td>
<td>No production of RCA</td>
</tr>
</tbody>
</table>

Table 1. Comparison of adenoviral vectors generations (Russell, 2000).
Direct gene transfer into skeletal muscle cells \textit{in vitro} and \textit{in vivo} using either plasmid DNA or recombinant viruses has medical applications in vaccination and gene therapy and also has been widely used in studies of developmental and physiological regulation of muscle gene expression (Hallauer et al., 2000). Many factors regarding muscle tissue barriers, immune response, systemic dissemination, potential toxicity and specific properties of each viral system need to be taken into account when selecting the proper approach for skeletal muscle. It is well known that skeletal muscle is a highly developed and organized tissue in which the constituent myofibers become post-mitotic in fetal life. The mononucleated myogenic precursor cells (satellite cells) that are located between the extracellular matrix and the plasma membrane of myofibers are known to be capable of fusing together or with preexisting myofibers in response to various types of stimuli, mainly to injury (Chargé et al., 2004). These satellite cells are relatively easy to isolate and cultivate \textit{in vitro} and can also be efficiently transduced using virtually any viral vector in contrast with the mature multinucleated myofibers. Thus, is believed that viral transduction during skeletal muscle maturation might require mitotically active myoblasts (van Deutekom et al., 1998). However, because some of the viral vectors have been shown to transduce post-mitotic, immature myofibers \textit{in vitro} and \textit{in vivo} other factors are also likely to be involved in the poor level of viral transduction of mature myofibers (Wolff et al., 1990; Acsadi et al., 1994 and Huard et al., 1996).

\textit{Retroviral vectors (RV)} can infect dividing myoblasts with a high efficiency although they remain incapable of infecting post mitotic myotubes or myofibers (Miller et al., 1990 and Salvatori et al., 1993). In addition, the ability to become stably integrated into the host cell genome, which can provide long-term, stable expression of the delivered gene, may also represent a risk for insertional mutagenesis. As mentioned in Section 3.2 of the present chapter, other limitations to the use of retroviruses are the gene insert capacity (less than 7 kb) and the relatively low production titers \((10^8–10^9\text{ plaque-forming units per milliliter (pfu/mL)})\). The recombinant vectors obtained from the \textit{Herpes Simplex Virus type 1 (HSV-1)} can persist in the host cell in a nonintegrated state and be prepared at adequately high titers \((10^7–10^9\text{ pfu / ml})\). They are capable of transducing muscle cells in most maturation stages while carrying large DNA fragments. However, are still unable to highly penetrate and transduce mature myofibers (Huard et al., 1997). \textit{Adeno-associated derived viral vectors (AAV)} have also been used to approach muscle cells. Although a long-term gene expression (up to 18 months) and a high efficiency of mature myofibers transduction have been observed in mouse skeletal muscle, the application of adeno-associated viral vectors for gene therapy may be limited by their restrictive gene insert capacity (Pruchnic et al., 2000). In the last few years a novel gene transfer viral vector has been used into skeletal muscle, the \textit{recombinant Sendai virus (SeV) vectors}. As explained before, the wild SeV is a non-segmented negative-strand RNA virus belonging to the Para-myxoviridae family that can infect various animal cells with an exceptionally broad host range. Shiotani et al., 2001 accomplished a significant overexpression of hIGF-1 in the adult rat Tibialis Anterior muscle when injected the tissue with a recombinant SeV vector (hIGF-1/SeV). They indicate a favorable gene delivery to mitotic myoblasts, post-mitotic immature and mature myofibers.
Despite this wide range of viral delivery systems, Adenoviral (AdV) vectors are probably the most prominent ones in this matter and have been extensible use to deliver genes into skeletal muscle. However, several obstacles have been identified in the application of adenovirus as gene delivery vehicles to skeletal muscle (Acsadi et al., 1994 and van Deutekom et al., 1998). The major limitations facing first generation adenoviral gene transfer to skeletal muscle are (1) the lack of transgene persistence due to the immune rejection of transduced myofibers; (2) the relatively low insert carrying capacity; (3) the reduced viral transducibility during muscle maturation; and (4) repeated administration associated with the production of neutralizing antibodies is limited to the viral capsid (Cao et al., 2001). During experiments of AdV gene transfer in animals of different ages it became clear that the transduction efficiency was related with the maturation state of the muscle. While the skeletal muscle of newborn mice achieved high levels of AdV infection, the mature muscle from adult animals was significantly less susceptible to infection under the same conditions (Huard et al., 1995). Here the high adenoviral transduction of newborn myofibers could be explained due partly to transduction of myoblasts and partly to the higher levels of CAR in these myofibers (Nalbantoglu et al., 1999). Several studies have shown that in developing human, mouse and rat muscle, expression of the primary AdV membrane receptor CAR is severely downregulated even at early ages with CAR mRNA being barely detectable in adult myofibers (Nalbantoglu et al., 1999). Furthermore, it has been demonstrated that forced expression of CAR in myotubes by different approaches, such as RAdV encoding hCAR or transgenic mice overexpressing the receptor, overcomes the poor AdV mediated transducibility of these cells (Nalbantoglu et al., 2001 and Kimura et al., 2001). On the other hand, basal lamina and glycocalyx surrounding mature skeletal muscle cells appear to be an anatomical barrier that may limit the access and distribution of exogenously introduced virus. (van Deutekom et al., 1998 and Cao et al., 2001). It has been reported that the extracellular matrix of mature myofibers may form a physical barrier and prevent the passage of some viral particles that are too big to pass through its pores, which are estimated at 40 nm in size. Adenoviral particles are about 70 nm and 100 nm in diameter and appear to be too large to penetrate the pores of the basal lamina (Cao et al., 2001). Regardless these difficulties some authors have published high rates of skeletal muscle transduction using AdV. In 2002, Sapru et al., was able to achieve nearly 100% of transduced fibers in the adult rat Soleus and more than 80% in the Tibialis Anterior muscle when infected with an adenoviral vector harbouring the cDNA of the GFP under the control of the CMV promoter (AdVCMV-GFP). These authors claim that the viral titer used was an important factor since they could increase the number of transduced fiber when the viral dose was doubled. Other major factor regarding skeletal muscle infection with AdV and AAV seems to be the fiber composition of the muscle, with suggested preferential transduction of slow fibers (Pruchnic et al., 2000 and Sapru et al., 2002). New AdV vectors lacking all viral genes, the Helper-Dependent AdV vectors, show a markedly decreased immunogenicity and hence, an improved persistence of transgene expression in muscle in vivo (Billbbo et al., 2005). These observations suggest that the limitations regarding the immunogenicity with the use of adenoviral vectors are being overcome. However, the inability of adenoviral vectors to efficiently transduce mature myofibers remains a major hurdle facing the widespread application of adenoviral gene transfer to skeletal muscle (van Deutekom et al., 1998).
On the next section we will introduce the novel combination of Magnetic Nanoparticles and Recombinant Adenoviral Vectors as an efficient alternative for gene delivery in transduction-resistant differentiated skeletal muscle cells.

3.5. Use of magnetic nanoparticles and magnetic fields to enhance viral vector-based gene delivery

Nowadays, the novel association of non-viral or viral vector-based gene delivery with nanotechnology offers the possibility to develop more efficient gene transfer strategies for a number of applications. In 2002 the concept of Magnetofection was first published by Scherer et al. Here, the Magnetic Drug Targeting (MDT) approach (Widder et al., 1978) classically used to concentrate magnetically responsive therapeutic complexes in target areas of the body by means of external gradient magnetic fields was applied for gene delivery. Therefore, Magnetofection is based on the association of Magnetic Nanoparticles (MNPs) with non-viral or viral vectors in order to optimize gene delivery when exposed to a magnetic field (Scherer et al., 2002).

There are currently several synthetic formulations of MNPs commercially available for biomedical applications such as cell separation, drug/gene delivery, magnetic resonance imaging (MRI) and hyperthermia (Gupta et al., 2005). Despite the differences, they all need to comprise some basic functionality to allow them to be associated with a gene delivery vector. Furthermore, the magnetic properties of these particles have to be sufficient to concentrate the vector at the target cells under a magnetic force and the formulation has to be biocompatible enough for application in living cells or organisms (Plank et al., 2011). Their general structure is based on a magnetic core of magnetite (Fe3O4) or maghemite (g-Fe2O3) coated with synthetic polymers that provides both protection and biological functionality. Occasionally specific organic linkers are added to this structure to generate new attachment sites for drugs or gene vectors (Yallapu et al., 2010). (Figure 5).

![General Structure of a Magnetic Nanoparticle](image)

For association with nucleic acids and/or viral particles, coatings comprised of cationic polymers such as polyethylenimine (PEI) are particularly useful (Mykhaylyk et al., 2007). The negatively charged phosphate backbone of nucleic acids as well as the negative electrokinetic
or zeta-) potential of all types of viral particles in aqueous media allow their assembling with cationic species and particles due to electrostatically induced aggregation (Scherer et al., 2002). The resulting magnetic-viral vectors complexes are then forced into sedimentation over the cell monolayer when a magnetic field is applied.

As detailed in Section 2.3, one of the most remarkable aspects of magnetofection enhancing viral gene transfer is the lack of need for virus interaction with cellular receptors. This has a particular significance for adenoviral vectors. It is known that for all the Wild-Type (except Subgroup B) and Recombinant Adenovirus cell entry takes place through receptor-mediated endocytosis via the Coxsackie--Adenovirus Receptor (CAR) (Meier and Greber, 2003). Various cell types differ widely in their level of CAR expression, and this may be a limiting factor for the transduction efficiency achievable with Adenovirus (Chorny et al., 2006). When AdVs are complex with MNPs all the viral surface proteins become hidden and unreachable for the CAR receptor. The cell entry in then achieved by unspecific clathrin-mediated endocytosis (Plank et al., 2011).

In order to find the optimal magnetic vector formulations for plasmid, siRNA and viral vector delivery, Plank and co-workers have aimed at maximal association with the magnetic component but avoiding an excess of magnetic particles (Plank et al., 2011). They have published that an excess of magnetic nanoparticles can inhibit transfection/transduction efficiency and cause toxicity (Tresilwised et al., 2010 and Sanchez-Antequera et al., 2011). Therefore, finding the optimal MNPs-to-nucleic acid ratios (about 0.5–1 units of iron weight per unit of the nucleic acid weight for triplexes with an enhancer) as well as MNPs-to-virus ratios (2.5–10 fg iron per virus particle further referred to as fg Fe/VP) have turned out useful for a variety of magnetic nanoparticle types (Sanchez-Antequera et al., 2011). The complexes formulated in this way were efficient and hardly toxic in delivery of DNA and siRNA as well in delivery of adenoviral and lentiviral vectors in vitro and ex vivo. Particularly to this regard there are several studies that have demonstrated the higher efficiency of this method when compare with traditional viral transduction using Adenovirus, Adeno-associated Virus, Baculovirus, Lentivirus and Retrovirus (Chan et al., 2005; Chorny et al., 2009; Hughes et al., 2001; Kaikkonen et al., 2008; Mah et al., 2000 and 2002; Morizono et al., 2009; Raty et al., 2004; Tresilwised et al., 2010 and 2012).

Regarding our experimental field, development of reliable techniques for manipulation of gene expression in mature skeletal muscle fibers is critical for understanding molecular mechanisms involved in both physiology and physiopathology. As explained before, differentiated skeletal muscle myotubes and myofibers are refractory to most standard protocols for gene transfer in vitro and in vivo and the use of adenoviral vectors offers relatively low efficiency. It is believed that a maturation-dependent loss of the CAR receptor together with structural and biochemical changes are responsible for these decreased transduction efficiencies (Nalbantoglu et al., 1999). It has been proposed that these limitations can be overcome by achieving adenoviral cellular uptake via a CAR independent pathway using genetic modifications of the capsid proteins or chemical modifications of the virus. However, these strategies are not sufficient for rapid infection of the cells at the target site, as the delivery process itself is diffusion-limited (Haim et al., 2005 and Schillinger et al., 2005). Here, magnetofection provided us a powerful,
accessible and efficient tool for transducing differentiated myotubes of the C2C12 cell line (Pereyra A et al., 2011-Posters Sessions). A first generation (E1/E3-deleted), serotype 5, Recombinant Adenoviral vector harboring de cDNA of the Green Fluorescent Protein (RAdV-GFP) under the control of the CMV promoter was constructed in our laboratory. This vector was incubated with Atto550PEI-Mag2, a magnetic nanoparticle conjugated with a red fluorescent dye that allows particle tracking during the cellular uptake and internalization. Then the [RAdV-GFP-Atto550PEI-Mag2] complexes were incorporated to the supernatant of the mature myotubes cultures. The magnetic field required for sedimentation was provided by a commercial plate (Oz Biosciences®, Marseille, France) composed of cylindrical-permanent-Nd-Fe-B magnets. The same protocol was tested in undifferentiated C2C12 myoblast cultures and conventional RAdV transduction experiments using the same viral multiplicity of infection (MOI) were also performed for efficiency comparisons. (Figure 6)

As showed in Figure 7, the poor RadV-GFP transduction of mature myotubes was overcome by magnetofection. In myoblasts, were the conventional transduction protocols show an acceptable efficiency, the magnetofection method displayed an enhancer effect. The intracellular localization of the magnetic nanoparticles can be seen in Figure 8.

**Figure 6.** Magnetofection in cell culture. This basic scheme shows the process of magnetofection in cultured cells. The pre-incubated RAdV-MNPs complexes are introduced to the cell culture. Then the culture plate is exposed to a magnetic field created by the magnetic plate placed under the cells.
Figure 7. Magnetofection in mature C2C12 myotubes and myoblasts. The conventional RAdV transduction system was compared against magnetofection. The images were obtained 48 hs after incubation. The green fluorescence corresponds to the expression of the GFP protein encoded by the viral genome. Magnification 40X (Pereyra A et al., 2011-Posters Sessions).

Figure 8. This picture was taken with a Double Band filter in order to appreciate the cellular co-localization of the green and red (Atto550-PEI-Mag2) fluorescence. Magnification 40X (Pereyra A et al., 2011-Posters Sessions).
4. Final remarks

There is no doubt that genetic manipulation of cells, tissues and whole individuals has become a fundamental tool for both basic and clinical research. The information that can be retrieved from experimentation using gene transfer techniques is highly significant and the therapeutic interventions that can be made by gene therapy are positioned as a promissory future for medicine. In this chapter we intended to describe the pros and cons of the most commonly used viral and non-viral gene delivery systems as well as to introduce the novelties in this field such as magnetic nanoparticles and magnetofection technology. The current applications for all of these systems seems endless; from the traditional recombinant protein production to the cutting edge cell reprograming. It is certain then that is of major importance to continue working in the pursuit of the ideal gene delivery system with high efficiency, selective tissue-tropism, non-toxic, and long-lasting expression.

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