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Cancer Gene Therapy: The New Targeting Challenge

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1. Introduction

Cancer is characterized by genetic alterations due, for instance, to mutations in genomic DNA caused by chemicals (mutagens such as pollutants or nitrosamines, and polycyclic aromatic hydrocarbons), radiations (e.g., prolonged exposure to ultraviolet radiation from the sun, which can lead to melanoma or other skin malignancies), and viral infections (e.g., papilloma virus; human T-cell leukemia viruses 1, 2, 3, and 4; and herpes simplex virus). Mutations in genes involved in cell proliferation, tumor suppressor genes, or proto-oncogenes may lead to uncontrolled cell proliferation into a tumor. Currently, the most widely used treatments for cancer are combinations of surgery, radiotherapy and chemotherapy. However, the effectiveness of these treatments is variable. Consequently, means of potentiating conventional treatments, as well as new strategies, need to be developed.

Gene therapy is generally perceived as a treatment for rare genetic diseases, in which replacing the deficient gene by its normal counterpart has proved successful, most notably in severe combined immunodeficiency (SCID) (Fischer et al., 2010), adrenoleukodystrophy (Cartier et al., 2009), and β -thalassemia (Cavazzana-Calvo et al., 2010). However, cancer is the main focus of basic and clinical research on gene therapy (<http://www.wiley.com//legacy/wileychi/genmed/clinical/>). Variable levels of success have been achieved using a broad range of genes encoding tumor suppressor proteins such as p53, antiangiogenic proteins such as anti-vascular endothelial growth factor (VEGF), inflammatory cytokines, and other proteins (Lane et al., 2010), (Candolfi et al., 2010), (Adachi et al., 2010).

One of the main hurdles in gene therapy is selective delivery of recombinant vectors to the target tissue. In cancer gene therapy, administration of the vector within the tumor may be of interest, but some tumors are not readily accessible and vector dissemination to healthy cells cannot be ruled out. Today, accurate tumor targeting is a major goal of cancer gene therapy.

In this chapter, we will focus on the methods developed to improve targeting in cancer gene therapy, most notably gene-directed enzyme prodrug therapy (GDEPT), which is a major focus of research at our laboratory.

2. Gene-directed enzyme prodrug therapy (GDEPT)

Cytotoxic chemotherapy is often associated with severe systemic toxicities. Gene-directed enzyme prodrug therapy (GDEPT) or suicide gene therapy consists in selective delivery to

the tumor of a gene encoding a drug-metabolizing enzyme that catalyzes the *in situ* conversion of a non-toxic prodrug to a toxic active drug (Figure 1). GDEPT can be used to increase the levels of an enzyme produced by the tumor or to introduce an enzyme that is not expressed endogenously. The local production of the cytotoxic drug within the tumor is expected to result in greater effectiveness and less toxicity, compared to systemic drug delivery.

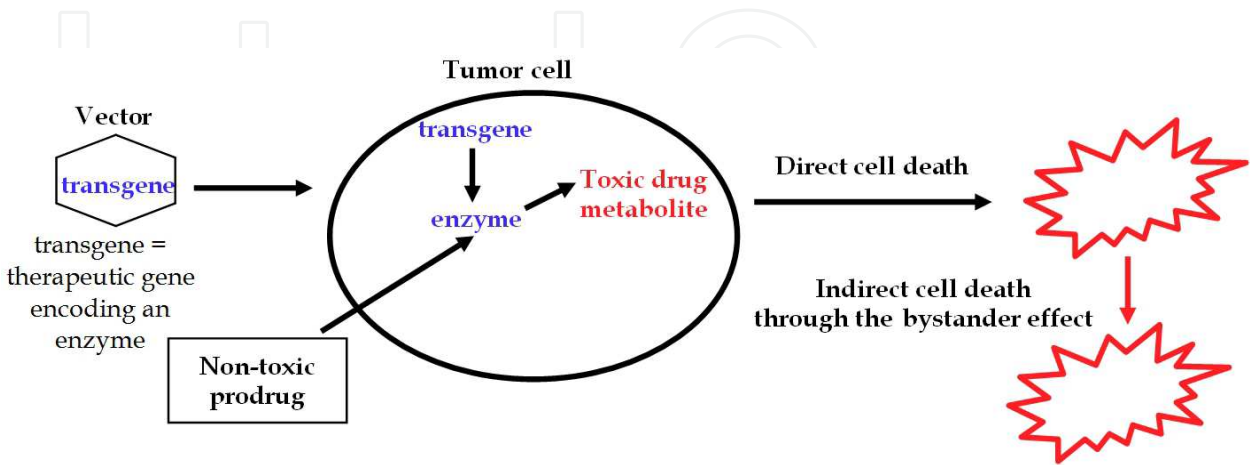


Fig. 1. Principle of gene-directed enzyme prodrug therapy (GDEPT)

Several studies have been performed with different enzyme and prodrug combinations. The most widely studied combinations are herpes simplex thymidine kinase/ganciclovir, cytosine deaminase/5-fluorouracile, and cytochrome P450 (CYP)/oxazaphosphorines (cyclophosphamide [CPA] and ifosphamide) (Altaner, 2008) (Table 1)

Enzymes	Source	Prodrug	Drug	Indication
Herpes simplex thymidine kinase	Herpes simplex virus	Ganciclovir	Ganciclovir triphosphate (GCV-TP)	Glioma, pancreatic cancer
Cytosine deaminase	<i>Escherichia coli</i>	5-Fluorocytosine (5-FC)	5-Fluorouracil (5-FU)	Glioblastoma, Colorectal cancer
Cytochrome P450	Rat/human/dog	Cyclophosphamide (CPA)	4-OH Cyclophosphamide (4-OH CPA)	Head and neck cancer, lung cancer, Burkitt's lymphoma
Nitroreductase	<i>Escherichia Coli</i>	CB1954	N-acetoxy derivatives	Cancer cells in general

Table 1. Enzyme/prodrug combinations used in GDEPT

2.1 Cytochrome P450 (CYP)/cyclophosphamide (CPA) combination

The chemotherapeutic prodrug CPA is widely used for the treatment of both solid tumors and hematological malignancies. Enzymatic bioactivation, chiefly via human CYP2B6 (Gervot et al., 1999), produces the metabolite 4'-OH-CPA, which undergoes spontaneous decomposition to acrolein and phosphoramidate mustard. Phosphoramidate mustard is an

electrophilic alkylating agent that causes the formation of intra- and interstrand DNA cross-links, which eventually lead to apoptotic cell death (Schwartz & Waxman, 2001). In patients treated with CPA, this prodrug is activated by CYP2B6 in the liver, and the active metabolites enter the bloodstream, which transports them not only to the tumor but also to healthy tissues where they may cause severe side effects including cardiotoxicity, renal toxicity, bone marrow suppression, and neurotoxicity (Fraiser et al., 1991) (Langford, 1997). To prevent these side effects, CYP2B-based gene-directed enzyme prodrug therapy was developed by D.J. Waxman and colleagues and, more recently, by our group (Waxman et al., 1999), (Jounaidi, 2002), (Jounaidi et al., 2006), (Tychopoulos et al., 2005). CYP2B expressed in tumor cells results in the *in situ* conversion of CPA to cytotoxic metabolites. Moreover, the diffusible 4'-OH-CPA metabolite can enter neighboring cells, where it is converted to phosphoramidate mustard, leading to the death of nontransfected tumor cells (Wei et al., 1995), (Tychopoulos et al., 2005). This bystander effect plays a major role in the CYP2B-based GDEPT strategy, and several studies of various suicide gene and prodrug combinations have shown that complete eradication of the tumor is possible even when the suicide gene product is expressed by less than 10% of the cells (Portsmouth et al., 2007).

In our laboratory, we are developing a GDEPT strategy based on human CYP2B6, the human CYP isoform that preferentially metabolizes CPA (Gervot et al., 1999). One of the main difficulties is the relatively low affinity of CYP2B6 for CPA. Modifications aimed at increasing the efficiency of CYP2B6 (V_{\max}/K_m) in catalyzing the 4-hydroxylation of CPA have therefore been evaluated. We used site-directed mutagenesis of the active site of CYP2B6 to produce a double mutant (I114V/V477W) characterized by a 4-fold increase in CPA-4-hydroxylation efficiency compared to the wild-type CYP2B6 (CYP2B6wt), ascribable chiefly to an increase in enzyme affinity (Nguyen et al., 2008). Recently, we obtained a triple CYP2B6 mutant (CYP2B6TM) that is 8 times more efficient than CYP2B6wt (unpublished results from our laboratory).

Another means of improving the efficiency of CYP2B6-mediated GDEPT is co-expression in the tumor cells of NADPH-cytochrome P450 reductase (RED). RED is a FAD- and FMN-containing enzyme that catalyzes the transfer from NADPH of electrons required for CYP-dependent enzyme reactions. Within tumors, where RED expression is heterogeneous (Fitzsimmons et al., 1996; L. J. Yu et al., 2001), CYP-GDEPT results in high levels of CYP expression, and RED availability can limit the rate of CYP-catalyzed enzyme reactions and, therefore, of prodrug bioactivation. To ensure the production of both CYP2B6 and RED by the same cancer cell, a CYP2B6wt-RED fusion protein having both 4-hydroxylase activity and reductase activity was built. This fusion protein proved more efficient than CYP2B6wt alone for metabolizing CPA in several pulmonary cell lines (Tychopoulos et al., 2005). Recently, we produced a CYP2B6TM-RED fusion protein that is 10 times more efficient than CYP2B6wt-RED in activating CPA (unpublished results from our laboratory).

These studies show that improving the efficiency of CYP2B6 is feasible. This method may allow the use of lower CPA dosages with no loss of cytotoxic effectiveness within the tumor but with less activation by hepatic CYP2B6 and, therefore, a possible decrease in cytotoxic effects on non-tumor tissue. Preliminary results in various human pulmonary and head-and-neck cancer cell lines show that expression of the CYP2B6TM-RED protein sensitized the cancer cells to lower doses of CPA compared to expression of CYP2B6wt-RED (unpublished results from our laboratory).

3. Gene therapy vectors

The most important step in any gene therapy protocol is the development of efficient vectors for delivering the transgene to its target. The ideal vector should be administered by a non-invasive route, penetrate only into the targeted cells in order to limit adverse side effects, and express the transgene in amounts sufficient to produce strong therapeutic effects. A wide range of vectors have been developed including viral vectors, polymers, liposomes, nanoparticles, and bare DNA.

Today, about 70% of clinical gene therapy trials worldwide use viral vectors such as retroviruses, adenoviruses, and adeno-associated viruses (AAV) or lentiviruses (Table 2) to transfer transgenes and 64.5% of these trials are conducted in patients with cancer (<http://www.wiley.com//legacy/wileychi/genmed/clinical/>).

However, retroviral vectors used to treat SCID have been responsible for leukemia caused by transgene insertion into proto-oncogene regions (Hacein-Bey-Abina et al., 2003). This side effect has severely slowed the development of gene therapy. However, we now have safer vectors such as the lentivirus used for gene therapy of adrenoleukodystrophy (Cartier et al., 2009) and β -thalassemia (Cavazzana-Calvo et al., 2010). Transgenes from recombinant lentivirus may be integrated mainly within intragenic or intronic regions (S. H. Yang et al., 2008).

Here, we will focus on three viruses that are presently widely used in gene therapy, namely, adenoviruses, AAVs, and lentiviruses.

3.1 Adenoviruses

Adenoviruses cause mild upper airway diseases. They are non-enveloped icosahedral viruses composed of a nucleocapsid and double-stranded linear DNA genome of about 35 kb with inverted terminal repeat (ITR) sequences at each end. There are 51 classified human adenovirus serotypes; serotypes 2 and 5 are those used most widely in *ex vivo* and *in vivo* gene therapy. They are very convenient vectors, because they can accommodate relatively large segments of DNA, up to 8 kb. Moreover, their transduction efficiency is high. To avoid a strong immune response after vector delivery, non-replicative recombinant adenoviruses lacking some of the early genes involved in the immune response are used. Deletion of the E1 sequence renders the virus unable to produce infectious viral particles in infected cells, and the E3 region is not necessary for viral production since it encodes proteins involved in evading host immunity. Thus, deletion of E1 and E3 is used to decrease the host immune response to the viral proteins (Alba et al., 2005).

Adenoviral vectors allow episomal and, therefore, transient transgene expression by infected cells (no integration of the foreign DNA into the genome of the host cell) (Russell, 2009) (Alemany & Curiel, 2001).

To infect cells, adenoviruses use the coxsackie-adenovirus receptor (CAR) and integrins as primary cell surface attachment components (Figure 2). The adenovirus (Ad) fiber knob binds with high-affinity to the CAR receptor and the viral penton base interacts with integrins (Bergelson, 1999). CAR plays a significant role in liver transduction and, consequently, most of the adenoviral particles administered intravenously are sequestered in the liver (Vrancken Peeters et al., 1996). However, the mechanism of adenoviral infection *in vivo* is controversial, especially as the introduction of mutations that abrogate CAR binding does not significantly impact the infectivity of adenoviral vectors.

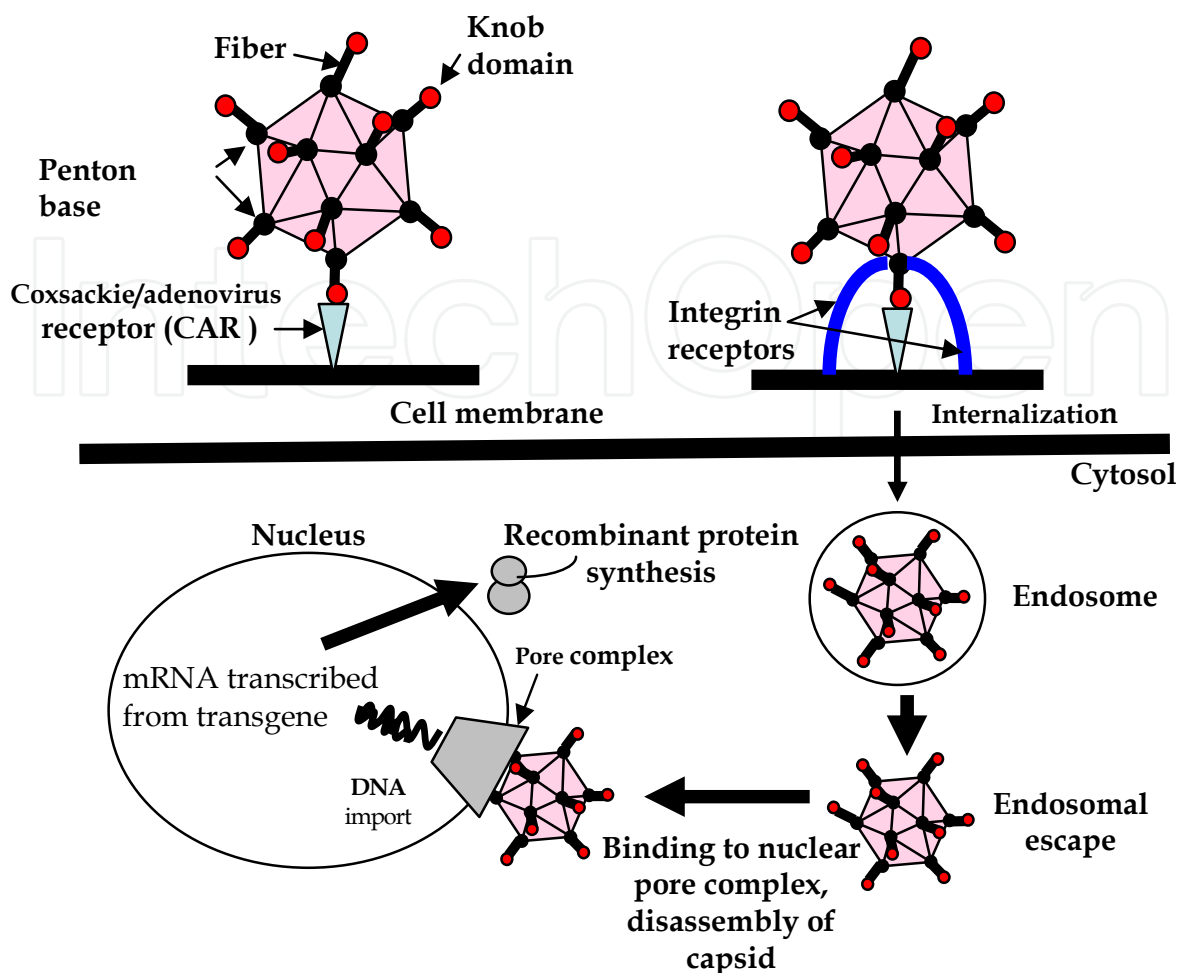


Fig. 2. Schematic representation of adenoviral attachment and internalization

Although immune responses have been limited, they have sometimes restricted the efficiency of adenoviral vectors in clinical trials. Increased immunogenicity has been reported, and many patients have pre-existing immunity to the adenoviral serotypes used in gene therapy. Cell-mediated recognition of the viral capsid components or nucleic acids has received considerable attention and is thought to be chiefly regulated by toll-like receptors (TLRs). Innate immune responses to viruses are initiated by the infected cells, which activate the interferon response to block viral replication, while simultaneously releasing chemokines that attract neutrophils, mononuclear cells, and natural killer cells. In 2010, adenoviruses were still the most widely used vectors for gene therapy. Nevertheless, the use of adenoviral vectors relative to other vectors decreases year on year.

3.2 Adeno-associated viruses (AAV)

Adeno-associated viruses (AAV) are small non-enveloped DNA viruses belonging to the parvovirus family. The single-strand DNA genome of about 4.8 kb comprises two open reading frames (*rep* and *cap*) flanked by inverted terminal repeats (ITRs). Twelve serotypes have been isolated from primate or human tissues (Schmidt et al., 2008). Advantages of AAVs include an apparent lack of pathogenicity, an ability to infect both non-dividing and dividing cells, and stable integration into the host genome at a specific site of the human

chromosome 19 when the vector includes the *rep* gene. In the absence of the *rep* gene, chromosomal integration occurs infrequently and at random sites (Huser et al., 2010). The AAV infection cycle is initiated by the binding of the viral capsid to cell surface receptors. One of the main receptors involved is heparan sulfate proteoglycan (HSPG); moreover, several co-receptors contribute to transduction (Asokan et al., 2006). Receptor binding mediates endocytosis, endosomal escape and, finally, transport to the nucleus. AAV vectors are constructed by replacing the viral DNA with an expression cassette encoding the gene of interest under transcriptional control of a suitable promoter. Vector production is achieved by transfection of a cell line with three plasmids: one contains the expression cassette flanked by the ITRs; another contains *rep cap* helper sequences, and the third is an adenoviral helper plasmid encoding the adenoviral E2a, E4, and VA helper genes (Grimm & Kleinschmidt, 1999). AAVs have become very popular as gene therapy vectors because of both their ability to mediate stable and efficient gene expression and their good safety profile. The major drawbacks of AAVs are the small amount of DNA that the virus can carry, which results in low capacity; and the difficulty of producing the vector in high titers (Michelfelder & Trepel, 2009). AAVs have been used in at least 80 clinical trials (as of 2011), in strategies based on the delivery of cytotoxic genes, tumor suppressor genes, and other types of genes.

3.3 Lentiviruses

Lentiviruses are retroviruses that include the human immunodeficiency virus 1 (HIV-1). They have a lipid envelope and two identical single-stranded genomic RNA molecules that require a reverse transcriptase for conversion to DNA. The HIV genome is composed of two

	Adenovirus	Adenovirus-associated virus	Retrovirus	Lentivirus
Genome integration	Rarely	No (in absence of <i>rep</i> gene) Yes (in presence of <i>rep</i> gene)	Yes	Yes
Transgene expression	Transient	Stable	Stable	Stable
Immune response	Marked	According to conditions (animal, transgene, injection conditions,...)	Absent to moderate	Absent to moderate
Target cells	Quiescent or dividing	Quiescent or dividing	Dividing	Quiescent or dividing
Transgene size	up to 8 kb	limited	8-9 kb	8-9 kb
Main use in gene therapy	<i>in vivo</i>	<i>in vivo</i>	<i>ex vivo</i> – <i>in vivo</i>	<i>ex vivo</i>
Titer	>10 ¹¹	>10 ¹¹	>10 ⁸	>10 ⁸
Genotoxicity	No	No	Mutagenesis-related risks	No

Table 2. Characteristics of four viral vectors: adenovirus, adenovirus-associated virus, retrovirus, lentivirus.

regulatory genes, *tat* and *rev*, which are necessary for viral replication; and four accessory genes, *vif*, *vpr*, *vpu*, and *nef*, which are not required for *in vitro* replication or growth but are crucial for *in vivo* replication. The *tat* and *rev* proteins are involved in regulating HIV gene expression at the transcriptional and post-transcriptional levels, respectively (Pauwels et al., 2009).

Lentiviral particle production involves co-transfection by calcium phosphate precipitation of gag-pol, env, and vector plasmids into HEK 293T cells. Viral particles are then recovered from the cell medium, concentrated, and filtered. Finally, the viral titer is determined (Dull et al., 1998) (Kutner et al., 2009). The transgene present in recombinant lentiviruses is integrated into the host genome via an integrase and is therefore expressed in a stable manner over time. Among retroviruses, lentiviruses efficiently infect both dividing and non-dividing cells (Naldini et al., 1996) without inducing genotoxicity with insertional mutagenesis (Montini et al., 2009), since they are integrated mainly within intragenic or intronic regions. Lentiviruses (e.g., the HIV) use cell receptors such as CD4 and the co-receptors CCR5 or CXCR4 to penetrate the cells. Lentiviral vectors express various types of proteins that are recognized by cell receptors and co-receptors, leading to a very broad tropism.

Since these vectors were first introduced, they have been modified in several ways with the goal of improving their safety profile. Now, these viral vectors are being increasingly used. However, their lack of tissue specificity may limit their use, and several methods have been developed to improve their ability to target the desired site.

4. Current strategies for viral vector targeting

Today, the major goal in cancer gene therapy is to improve tumor targeting, thus preventing transgene expression by normal cells and therefore diminishing the risk of toxic side effects. Initially, the vector was injected directly into the tumor. However, vectors are now available that target the tumor after being administered systemically.

Efforts to improve viral vector targeting can modify the binding of the virus to the cell and entry of the virus into the cell (entry targeting/transductional regulation) or the events that occur once the virus is in the cell (post-entry targeting/transcriptional regulation). Several approaches have been devised such as envelope or capsid modifications, the use of various adapters, placement of transgene expression under specific promoter control, and modifications of the transgene sequence.

4.1 Pseudotyping: Envelope or capsid modification

Viral vectors infect their natural host-cell populations preferentially and with the greatest efficiency. Viral infection occurs when host-cell receptors recognize the viral envelope proteins. Pseudotyping consists in changing the plasmid encoding the expression of envelope proteins. The result is a shift in the range of host cells and, consequently, in the tissue tropism of the viral vector. The vector surface is modified via the incorporation of foreign envelope glycoproteins that have a restricted natural population of host-cell receptors (Frecha et al., 2008). This technique was the first to be used for modifying viral tropism, particularly in retroviruses such as lentiviruses, which have an envelope. Adenoviral vectors have no envelope, and the viral attachment protein must therefore be incorporated into a protein capsid instead of a lipid bilayer.

Lentiviral vector pseudotyping is usually achieved using the vesicular stomatitis virus G (VSV-G) protein, which exhibits a broad tropism for various cell types. Additional advantages of VSV-G-pseudotyped lentivirus are the higher viral titers compared to those obtained with other envelope proteins and the improved vector particle purification due to increased stability of the virus. However, when used in high concentrations, lentiviral vectors bearing VSV-G may exert cytotoxic effects (Chen et al., 1996). Fortunately, this drawback can be overcome either by improving purification of the lentiviral particles using gradient centrifugation to eliminate unincorporated transgene particles (Ricks et al., 2008) or by using other proteins for pseudotyping. VSV-G-pseudotyped particles are convenient to use *ex vivo* to express a transgene in a broad spectrum of cell lines. However, VSV-G-pseudotyped viruses can be inactivated by human serum (DePolo et al., 2000). In clinical trials of cancer gene therapy, the objective is to limit the tropism of the vector to the cancer cells.

Miletic et al., worked on a gene therapy strategy for malignant gliomas, which are the most common primary brain tumors and carry a poor prognosis due to their infiltrative growth (Miletic et al., 2004). Miletic and co-workers compared the expression of various pseudotyped lentiviruses in normal brain cells and malignant glioma cells. VSV-G pseudotyped lentiviruses infected the neurons and astrocytes, whereas the tropism of lymphocytic choriomeningitis virus glycoprotein (LCMV-GP) pseudotypes was virtually confined to the astrocytes. LCMV-G-pseudotyped lentivirus was specifically and efficiently transduced in rat gliomas, whereas VSV-G-pseudotyped lentivirus was considerably less efficient in transducing glioma cells.

Another protein often used to target cancer cells is the modified sindbis virus envelope. Pariente et al., (2007) used it successfully to target prostate cancer cells.

Transduction efficiency is low after tumor cell infection with adenoviruses. One reason is the limited expression of the coxsackievirus-adenovirus receptor (CAR) in tumor cells. To overcome this obstacle, the adenovirus fiber can be modified by removing interactions with both CAR and integrins, the main components involved in adenovirus transduction (Einfeld et al., 2001). This modification diminishes the native tropism and enhances the efficacy of specific targeting ligands in redirecting the adenovirus to the target tissues.

Malignant gliomas are refractory to adenovirus-mediated gene therapy, chiefly because CAR is not expressed by the tumor cells. Zheng et al. identified several receptors that were over-expressed in tumor cells, and they created a series of pseudotyped adenoviral vectors. Some of these vectors enhance gene transfer to tumors and warrant further development for glioma gene therapy. (Zheng et al., 2007)

Yu et al., (L. Yu et al., 2005) reported increased infection of esophageal and oral carcinoma cells with adenoviruses whose Ad5 fiber was substituted with fibers from Ad11 or Ad35, compared to unmodified adenoviruses. Similarly, attaching the Ad3 fiber to the Ad5 backbone was particularly effective for targeting ovarian cancer and squamous cell carcinoma of the head and neck.

The efficacy of pseudotyping may be limited by the lack of tissue specificity and ubiquitous expression of some of the receptors. Furthermore, the viral envelope modifications may diminish viral stability and limit viral production, leading to low titers.

4.2 Use of adapters: Antibody/ligand

Another technique consists in fusing special adapters or proteins to the envelope proteins. These adapters determine the affinity of the vector for the target.

4.2.1 Antibody

A protein can be specifically targeted by the use of specific antibodies, antibody fragments, or single-chain antibodies fused to the viral membrane. There are two main methods for using antibodies to improve targeting by vectors.

- The entire antibody or an antibody fragment directed against both a viral envelope protein and a tumor cell membrane receptor can be used as a bridge to attach the virus to specific cells.
- An antibody fragment (usually the fragment crystallizable region Fc) can be expressed at the viral envelope and the rest of the antibody can be directed against a specific antigen of the target cells.

For prostate cancer gene therapy, Kraaij et al. developed a targeted method based on bi-specific antibodies constructed as conjugates between an anti-adenovirus fiber knob Fab' fragment and an anti-prostate specific membrane antigen (PSMA) (Kraaij et al., 2005). These bi-functional antibodies, used as a bridge between capsid proteins and cell surface receptors, were selective for the prostate cancer cell lines. They may hold promise for gene therapy of prostate cancer.

Another strategy, developed by Zhang et al., consists in binding trastuzumab (or Herceptin®, a monoclonal antibody directed against the human epidermal growth factor receptor (HER-2)) to the lentivirus envelope. Thus, the vector targets cells that overexpress HER-2, such as prostate cancer cells, to which it delivers the transgene. Zhang et al. engineered these lentiviruses to express thymidine kinase and showed that prostate cancer cell lines infected by these lentiviruses became vulnerable to ganciclovir. (Zhang et al., 2009) Poulin et al. worked on a new adenoviral vector and investigated the usefulness of capsid protein IX (a minor protein of the adenoviral capsid) as a platform for presenting single-chain variable-fragment antibodies and single-domain antibodies for virus targeting. Given the ability of this protein to fuse to large polypeptides, Poulin et al. decided to test large targeting ligands such as antibodies. Presence in the vector of single-chain variable-fragment antibodies was not sufficient to ensure accurate targeting, contrary to the presence of single-domain antibodies (Poulin et al., 2010).

However, this method is still complicated to use, as it requires the production of monoclonal antibodies, which is both time-consuming and costly. In addition, a specific tumor cell antigen must be obtained, which may be difficult. Finally, the titer of vectors that express the antibody in their envelope is sometimes low.

4.2.2 Ligand

The first attempts at inserting a ligand into the viral membrane used various types of ligand such as growth factors, hormones, and peptides, which were inserted at various sites of the viral surface.

Morizono et al., (Morizono et al., 2009) used a strategy based on a lentiviral vector bearing the biotin-adapter-peptide. In earlier studies of adenoviral or AAV vectors, peptides that were biotinylation substrates were inserted and associated with biotinylated sites, bound avidin, neutravidin, or streptavidin. (Parrott et al., 2003; Pereboeva et al., 2007; Stachler et al., 2008)

Similarly, Liu and colleagues (Liu et al., 2011) used a serotype 5 adenoviral vector (Ad5) whose fiber knob was deleted and replaced by a biotin-acceptor peptide. The advantage of this new adenoviral vector is that no CAR-dependent cell uptake and transduction occurs; moreover when the vector is biotinylated, biotinylated antibodies can be used to achieve targeting. AAV vectors can also be biotinylated.

A hybrid approach using an antibody and a protein ligand has been described in two papers by a group working at the University of California, Los Angeles. (Joo & Wang, 2008), (L. Yang et al., 2006). This group of researchers engineered a lentiviral vector whose surface bears two distinct molecules, an antibody conferring target specificity to the engineered vector and a pH-dependent fusogenic protein that allows the engineered vector to penetrate the target cells. Evaluation by image processing showed highly specific incorporation of this lentivirus into the cells.

Hajitou et al. (Hajitou et al., 2006) developed an AAV vector combined with a double cyclic peptide (RGD-4C) of an fd-tet phage. Their aim was to target αV integrins, a cell surface receptor that is overexpressed in tumors and interacts with the RGD peptide. The native tropism of AAV for mammalian cells is eliminated, since there is no AAV capsid formation and the ligand peptides allow homing to tissue specific receptors. To obtain chimeric viruses, Hajitou et al. inserted an eukaryotic gene cassette from the AAV into an intergenomic region of the RGD-4C phage. The vector was functional and efficiently targeted human Kaposi sarcoma (KS 1767 cells) grafted in nude mice *in vivo*. Using a ganciclovir cytotoxicity strategy, Hajitou et al. obtained a decrease in tumor volume in mice receiving this vector compared with those given a non-specific vector. Using the same strategy, Bauerschmitz et al. (Bauerschmitz et al., 2002) used an adenovirus modified with a RGD domain to target ovarian cancer cells. As seen with the other approaches involving transductional targeting, limited viral production and stability may occur when the viral envelope is modified.

4.3 Tissue-specific promoter

A promoter is a DNA region that is located upstream of the gene and plays a key role in regulating gene expression. The insertion of a cell-specific regulated promoter upstream from the transgene may limit the expression of the promoter to the targeted cells. Several cancer-specific promoters have been found effective in cancer gene therapy, including prostate stem cell antigen (PSCA) promoter in prostate cancer (Petrigliano et al., 2009), carcinoembryonic antigen (CEA) promoter in gastric cancer (Tanaka et al., 2006), and alpha-fetoprotein (AFP) enhancer and albumin promoter in hepatocellular carcinoma (He et al., 2000).

These promoters are tissue-dependent, however. A universal tumor-specific promoter targeting tumor cells of any origin would be of considerable interest. For instance, given that hypoxia is a common physiological feature of tumor tissue, an optimized hypoxia-responsive promoter (OBHRE) may be effective in increasing the therapeutic window of cytotoxic cancer gene therapy (Binley et al., 2003). In a range of cell types, this promoter expresses high levels of transgene in hypoxic tissue but has minimal activity in normoxia. Moreover, the OBHRE promoter in a recombinant adenovirus allowed high-level expression of the transgene in tumor cells but was not expressed in normal tissues such as the liver, spleen, lung, and kidney. Binley et al. developed a GDEPT strategy using CYP2B6 or thymidine kinase as the transgene in combination with CPA and ganciclovir, respectively. Direct administration of the gene therapy vector containing OBHRE into established tumor models was effective, and this method limited the toxic effects due to hepatic sequestration of the adenovirus.

A characteristic promoter of cancer cells is the prostate stem cell antigen (PSCA) promoter. Petrigliano et al. (Petrigliano et al., 2009) used the PSCA promoter to develop a lentiviral

vector targeting prostate cells. PSCA is consistently expressed by high-grade prostate intraepithelial neoplasias and invasive prostate cancers (Watabe et al., 2002). The lentiviral vector carried a cytotoxic thymidine kinase gene and was combined with ganciclovir treatment. Lentiviral gene therapy vector driven by a short PSCA promoter induced prostate-specific cellular toxicity *in vivo* and *in vitro*. This strategy could be used to treat local and advanced metastatic prostate cancer.

However, one of the main problems with the specific promoter strategy is that faithful reconstitution of a complete gene sequence promoter can be difficult. Moreover, transcriptional targeting cannot prevent the sequestration of therapeutic viruses in normal tissues, which may result in toxicity and loss of efficacy.

5. A new strategy for viral vector targeting: micro RNAs (miRNA)

In addition to the above-mentioned methods, microRNAs (miRNAs) may hold potential for improving viral vector targeting, as they are involved in the post-transcriptional regulation of gene expression.

5.1 microRNAs (miRNAs)

The small non-coding RNAs (~20-25 nucleotides) known as miRNAs regulate gene expression at the post-transcriptional level. They are involved in a variety of biological processes including development, differentiation, apoptosis, and cell proliferation. They repress gene expression by binding to their complementary target sites in mRNAs, thereby increasing the degradation or preventing the translation of the transcripts. Thus, cells that express an miRNA complementary to an mRNA do not express the protein coded by this mRNA: miRNAs are endogenous negative gene regulators. (Figure 3).

In 1993, miRNAs were identified for the first time, in the nematode *Caenorhabditis elegans*, in which they were encoded by the *lin-4* and were complementary to mRNA for the *lin-14* gene (R. C. Lee et al., 1993). The *lin-4* gene product is a small RNA of 22 nucleotides (i.e., a miRNA) that is specific of the 3'UTR of the *lin-14* gene and therefore inhibits the production of the *lin-14* protein, thus preventing the transition from larval stage L1 to stage L2. Since the discovery of miRNAs, their mechanisms of action and biogenesis have been studied in detail, and they have been shown to play a major role in physiological processes, development, and disease.

Briefly, miRNA biogenesis involves four stages: transcription of pri-miRNA; cleavage by Drosha to release a precursor pre-miRNA; export of the precursor to the cytoplasm; and cleavage of the pre-miRNA precursor by Dicer. All miRNAs are processed from precursor molecules called pri-miRNAs (Y. Lee et al., 2002), which are transcribed from independent miRNA genes or are portions of introns of protein-coding RNA polymerase II transcripts. Typically, a single pri-miRNA often contains sequences of several different miRNAs.

These pri-miRNAs of about 100 nucleotides are folded into hairpin structures and characterized by imperfectly base-paired stems. These molecules are then processed by a multiprotein complex including the RNase III type endonuclease Drosha and DGCR8 (DiGeorge syndrome critical region gene 8). The hairpin structures are recognized in the nucleus by DGCR8, a double-stranded RNA-binding protein (dsRBP). DGCR8 and the Drosha complex process the pri-miRNAs to pre-miRNA hairpins composed of about 70 nucleotides. Pre-miRNAs are then transported from the nucleus to the cytoplasm by exportin 5. In the cytoplasm, they undergo a final maturation step consisting in cleavage by

Dicer, which is complexed with TAR RNA binding protein (TRBP). This cleavage step releases an miRNA duplex of about 20 nucleotides. Mature miRNAs are integrated into a ribonucleoprotein complex called RNA induced silencing complex (RISC) or miRNA-induced silencing complex (miRISC). The components of miRISC complexes are mature miRNAs, Dicer and TRBP proteins, and proteins of the Argonaute family (AGO).

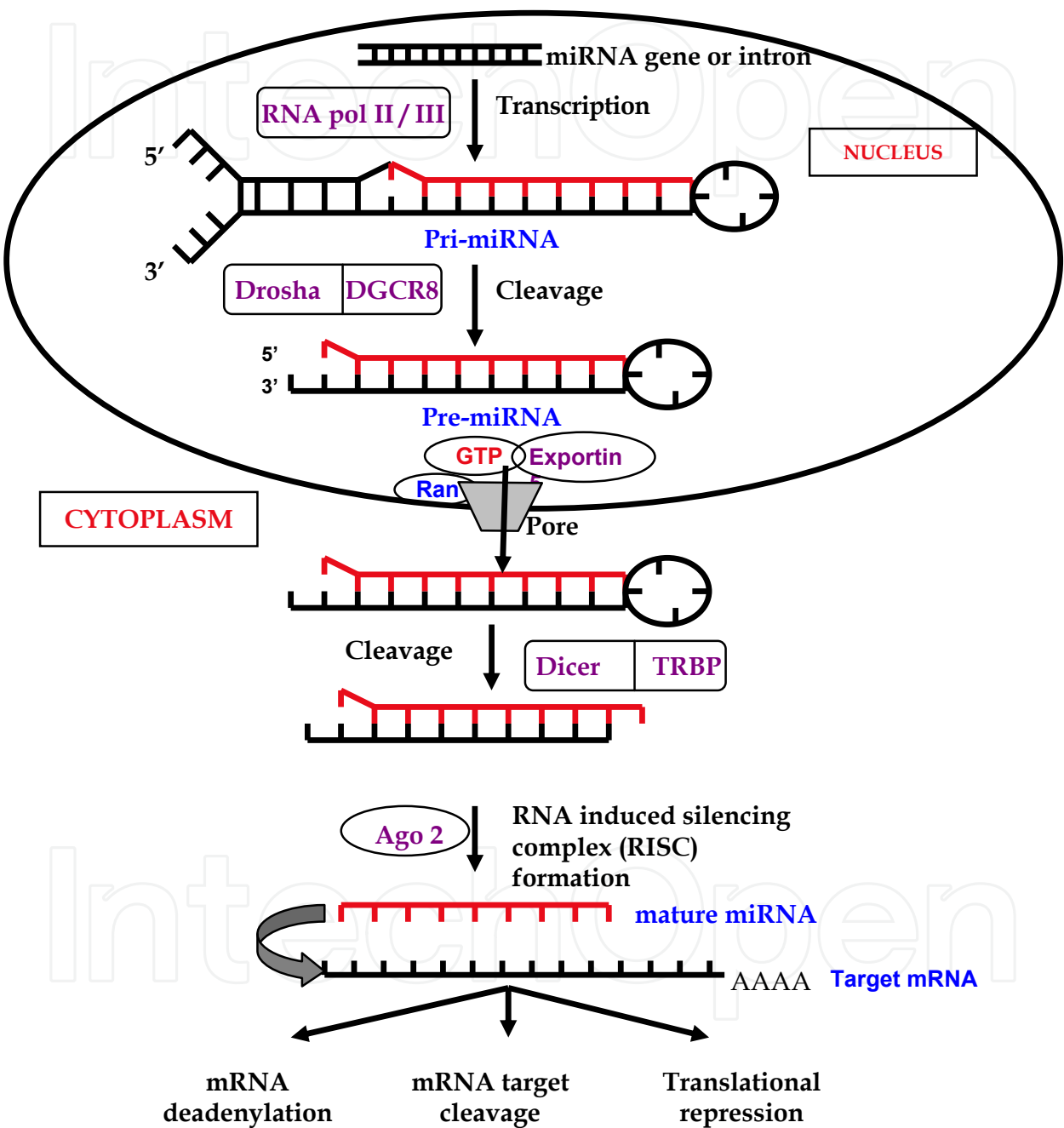


Fig. 3. Principle of miRNA biogenesis

AGO proteins represent the key components of miRISCs; in mammals, four AGO proteins (AGOs 1, 2, 3, and 4) have been identified. They are involved in the miRNA repression function via protein synthesis repression, whereas only AGO2 contributes to the RNA interference (RNAi) function. (Jaskiewicz & Filipowicz, 2008).

Binding of miRNAs to complementary target sites on mRNAs prevents the translation of the transcript or accelerates its decay. The regulation of miRNAs depends on the binding of the first 2 – 8 bases of their mature sequence to the 3'UTR of target genes. To date, 1048 human miRNA precursor sequences have been deposited in the miRBase (<http://www.mirbase.org>) (Kozomara & Griffiths-Jones)

There is now sound evidence that miRNAs are involved in the pathogenesis of conditions such as cancer and inflammatory responses. It has been shown that miRNA expression is deregulated in cancer cells. The differences in miRNA expression between normal and malignant cells may be related to the location of miRNA genes in cancer-associated regions, to epigenetic mechanisms, and to alterations in the miRNA processing machinery (Calin & Croce, 2006). Several studies suggest that miRNAs may contribute to oncogenesis by acting either as tumor suppressors (excessive regulation) or as oncogenes (insufficient regulation).

5.2 Targeting strategy using miRNA

Recently, researchers have started to evaluate endogenous miRNA-mediated regulation as a means of targeting the expression of exogenous genes. Naldini and co-workers demonstrated that endogenous miRNAs could be broadly exploited to regulate transgene expression in various cell lines. This very elegant approach to the control of protein expression relies on the potent regulatory properties of miRNAs. Several studies demonstrated that miRNA expression in cancer cells is deregulated compared to normal cells. The idea is to use this deregulation to modulate the expression of the transgene (B. D. Brown et al., 2007a) (Figure 4). Naldini and colleagues first developed a vector characterized by suppression of transgene expression in hematopoietic cells. The vector contains target sequences for the hematopoietic cell-specific miRNA miR 142-3p; thus, transgene expression is specifically suppressed in all hematopoietic cell lines but is not affected in other cell types. (B. D. Brown et al., 2007a)

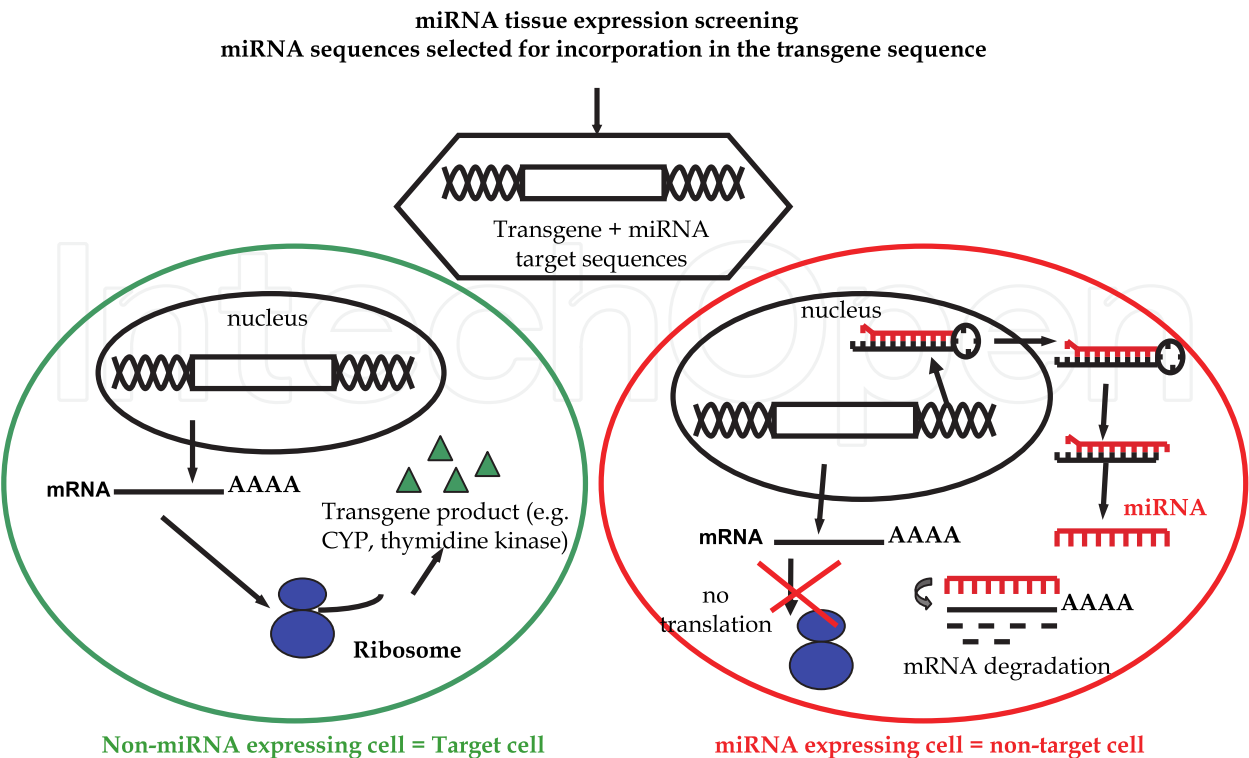


Fig. 4. Principle of miRNA targeting strategy

During the development of this technique, one issue was determination of the amount of endogenous miRNA needed to obtain effective target mRNA suppression. Brown et al., (B. D. Brown et al., 2007b) investigated this issue and concluded that target suppression depended on a threshold miRNA concentration.

Suzuki et al., (Suzuki et al., 2008) worked on a suicide gene therapy strategy based on the herpes simplex virus thymidine kinase (HSVtk) gene and ganciclovir (GCV), with adenoviral vectors. Based on the literature and their experiments, they showed that intratumorally injected adenoviral vectors were disseminated into the systemic circulation and transduced in the liver, resulting in hepatotoxicity. They therefore decided to produce a vector capable of preventing the hepatotoxicity of adenoviruses without altering the antitumor effects of suicide gene therapy. They hypothesized that insertion of sequences complementary to miR122a (which is highly expressed in the liver) into the 3'-UTR of a transgene expression cassette in adenoviral vectors would reduce hepatic transduction without affecting transgene expression in the tumor.

They constructed several vectors; among them, one had four tandem copies of sequences with perfect complementarities to miR122a. The copy number of miRNA target sequences is expected to play an important role in the regulation of transgene expression. An increase in the number of miRNA sequences leads to greater suppression of transgene expression (Doench et al., 2003); thus, four copies are better than two (B. D. Brown et al., 2007b). However, considerable work remains needed to determine the best number of copies and the best spacing elements between tandem copies of miRNA.

Simultaneously, Ylosmaki et al. have developed an adenoviral vector containing sequences complementary to miR 122. They tested the expression of a protein encoded by the vector in Huh7 cells. Huh7 cells resemble normal hepatocytes in that they have a high level of miR 122 expression. As mentioned previously, this strategy prevented transgene expression in the liver, thus avoiding adenovirus-induced hepatotoxicity.

An increasing number of studies combine tissue promoter regulation with miRNA regulation. For instance, Wu C et al. (Wu et al., 2009) developed a baculoviral vector, a strategy that could be extended to other viral vectors. To target glioblastoma cells, they used thymidine kinase/ganciclovir, and a glial fibrillary acidic protein (GFAP) gene promoter. Expression of the herpes simplex virus thymidine kinase gene was controlled by adding the repeated target sequences of three miRNAs that are enriched in astrocytes but downregulated in glioblastoma cells. To determine which miRNA sequences should be used, they reviewed the literature on miRNA expression in gliomas and normal brain tissues.

Downregulated miRNAs are miR 128, 137, 299, 31, 107, 132, 133a, 133b, 154, 323, 330, 127, 134, 181a, and 181b (Ciafre et al., 2005) (Silber et al., 2008); there is only one upregulated miRNA, namely, miR 10b. Wu and colleagues used these results to construct targeting vectors. Suicide gene expression controlled by specific miRNA sequences exerted selective cellular effects *in vitro* and *in vivo*. Glioma cells were specifically targeted, and ganciclovir was toxic in these cells. Wu et al. concluded that incorporating miRNA regulation into a transcriptional targeting vector provided a high level of control over transgene expression. The crucial steps in developing an efficient system include selection of a relevant tissue-specific promoter and determination of relative miRNA expressions in tumor cells and their normal counterparts. The next step is selection of miRNAs that are downregulated in tumor cells and expressed at high levels in normal cells.

This approach has also been studied in another cancer treatment strategy based on oncolytic viruses. Thus, Leja et al., (Leja et al., 2010) worked on an oncolytic adenovirus. Their aim was to abolish the hepatic tropism of the adenovirus, and therefore the occurrence of hepatotoxicity, without altering the antitumoral effects in neuroendocrine cells. They used not only a specific promoter but also miR 122 sequences. Similar to Suzuki et al. (Suzuki et al., 2008) and Ylosmaki et al. (Ylosmaki et al., 2008), Leja et al. found that hepatic tropism and expression were abolished.

Edge et al. (Edge et al., 2008) used another oncolytic virus, the vesicular stomatitis virus (VSV). They incorporated let-7 miRNA complementary sequences within the VSV to eliminate toxicity for normal cells without preventing expression in cancer cells *in vitro* and *in vivo*.

This approach has also been found effective in diseases other than cancer. Thus, an miR 142-3p regulated lentiviral vector has been used in hemophilia B (B. D. Brown et al., 2007a); miR 122 regulated transgene expression improved targeting to the heart (Geisler et al.); and a lentiviral vector containing miR 142 sequences regulated UGT1A1 expression in the liver (Schmitt et al., 2010)).

6. Conclusion

Cancer gene therapy and, in particular, suicide gene therapy holds considerable promise as a substitute for conventional chemotherapy. However, several aspects of gene therapy remain to be improved. In particular, there is a need for developing enzymes such as mutant forms of human enzymes that are more efficient than the wild-type enzyme regarding specificity and kinetics for the prodrugs, as exemplified by our CYP2B6TM-RED and CPA combination.

The viral vectors used to achieve gene transfer may have a broad tropism and may therefore infect healthy tissue. An insufficient ability of vectors to target tumors has contributed to slow the development of cancer gene therapy. Researchers have therefore expended considerable effort to improve viral vector targeting, as discussed in this chapter. Moreover, the accumulation of knowledge about miRNAs has opened up a new field of gene regulation. Using miRNA properties to regulate transgene expression, and therefore targeting, in cancer gene therapy is both extremely elegant and quite simple. Future strategies should combine several targeting methods (Figure 5). Several groups have already constructed vectors characterized by a double targeting system consisting of specific promoters and miRNA. Today, the development of vectors characterized by both transductional and transcriptional targeting is within reach. It is reasonable to hope that safe vectors capable of specifically targeting cancer cells will be available soon and will open up new horizons for cancer gene therapy.

Last, new prodrugs with greater effectiveness are needed. Given that hypoxia is a common environmental feature in solid tumors, prodrugs specifically activated by hypoxia should be designed. For example, our previously described fusion gene expresses both CYP2B6 and RED catalytic activities, and we plan to use CPA treatment in combination with additional prodrugs known to be activated to cytotoxic metabolites under hypoxic conditions, such as AQ4N by CYP 2B6 or mitomycin C and tirapazamine by RED (J. M. Brown & Wang, 1998; Cavazzana-Calvo et al., 2010; Friery et al., 2000; McErlane et al., 2005).

Recent clinical trials confirmed the usefulness of cancer gene therapy and its potential for application in the clinical setting, as a substitute for conventional chemotherapy or, if the

result is only a decrease in tumor size, in combination with surgery and radiotherapy. We hope that the expected improvements in cancer gene therapy outlined above will further facilitate the use of this strategy for treating solid tumors.

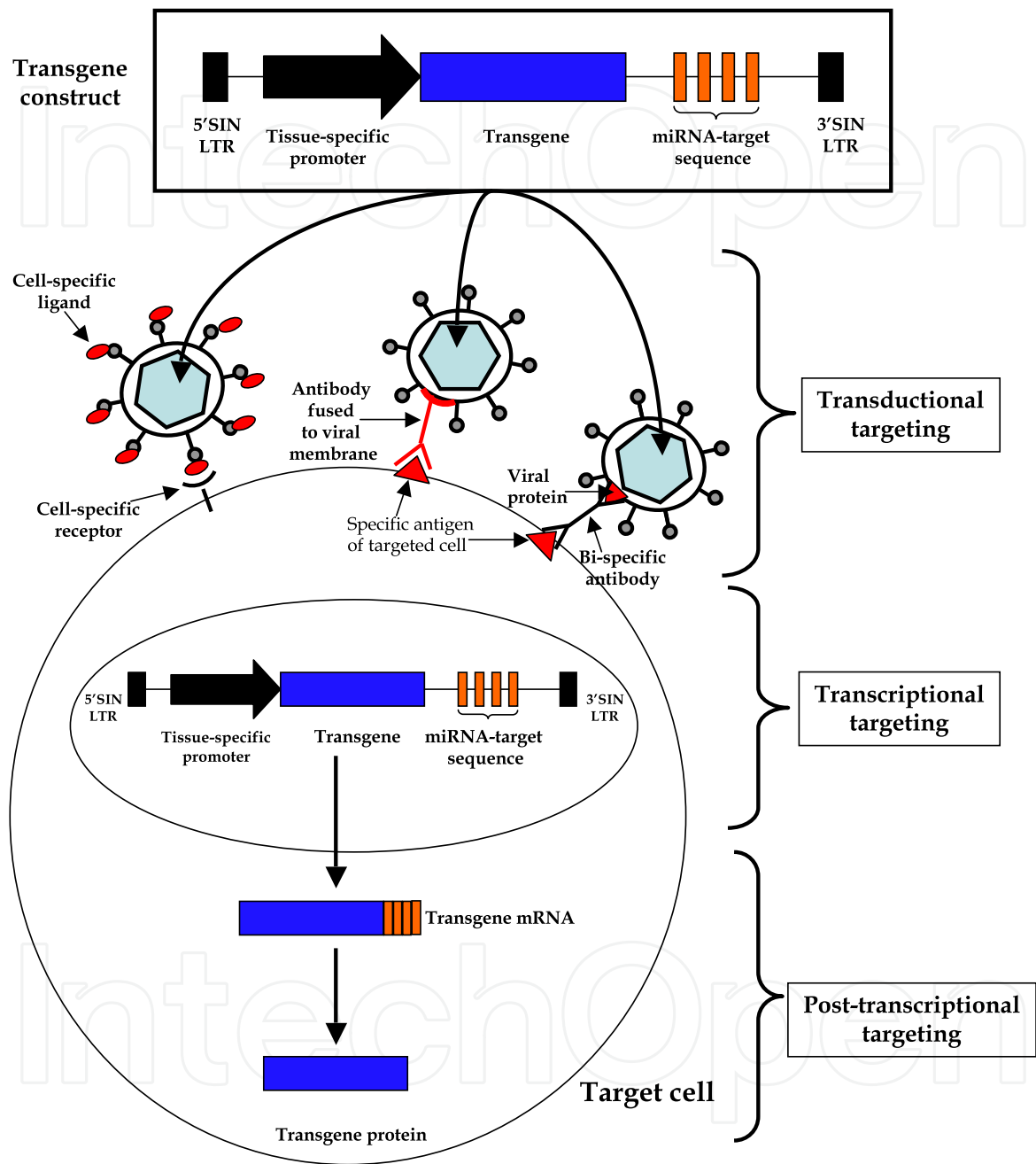


Fig. 5. Summary of various strategies for targeting lentiviral expression to cancer cells

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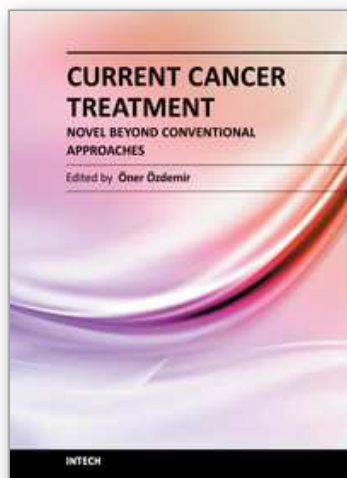
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Currently there have been many armamentaria to be used in cancer treatment. This indeed indicates that the final treatment has not yet been found. It seems this will take a long period of time to achieve. Thus, cancer treatment in general still seems to need new and more effective approaches. The book "Current Cancer Treatment - Novel Beyond Conventional Approaches", consisting of 33 chapters, will help get us physicians as well as patients enlightened with new research and developments in this area. This book is a valuable contribution to this area mentioning various modalities in cancer treatment such as some rare classic treatment approaches: treatment of metastatic liver disease of colorectal origin, radiation treatment of skull and spine chordoma, changing the face of adjuvant therapy for early breast cancer; new therapeutic approaches of old techniques: laser-driven radiation therapy, laser photo-chemotherapy, new approaches targeting androgen receptor and many more emerging techniques.

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