Recombinant adenoviruses and adenovirus penton vectors: from DNA transfer to direct protein delivery into cell

Review Article

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Summary

Recombinant adenovirus (rAd) is one of the most frequently used vectors with 171 human gene therapy trials involving 644 patients in 2002. Its success stems mainly from the capacity of rAd to efficiently penetrate various cell lines or tissues and from the ease of producing large amounts of this vector on industrial scale. Recently, in parallel to gene transfer by viral vectors, a new technology aiming to deliver proteins rather than genetic material in human cells has emerged, using small vehicle peptides often derived from viral proteins. In the first part of this review, I summarize the different types of rAd vectors used for gene transfer, their advantages and their limits. In the second part, I summarize the main vectors used for direct protein delivery into human cells and I show how an amazing nanoparticle called "adenovirus dodecahedron" can be used for this emerging therapeutic area.

I. DNA transfer

DNA transfer by adenovirus (Ad) can be achieved using two different strategies. The first consists in modifying the Ad genome in order to introduce a transgene encoding the therapeutic protein, which will then be delivered to target cells by virus penetration. Such vectors are called: recombinant adenoviruses (rAd). The second strategy using Ad capsid proteins as vectors allows non-encapsidated plasmid DNA to be introduced into cells.

A. Recombinant adenoviral vectors

Gene therapy is a promising approach for the treatment of either innate or acquired diseases. Among vectors that are used to transfer DNA into cells, Ads have several advantages. They can infect dividing as well as resting cells with high efficiency and can be easily produced, purified and concentrated to high titres. In addition, a sizeable part of the viral genome, non-essential for virus development in cell cultures used for the vector replication, can be removed, permitting the insertion of the majority of various cDNAs encoding the therapeutic gene.

Numerous gene therapy trials have been performed, in particular for cancer treatment, using different therapeutic genes (tumor suppressor, thymidine kinase, cytokines…). A considerable effort has also been deployed in the area of central nervous system diseases, as Ads infect non-dividing cells and can be transported by the retrograde route from the injection point to distant locations.

In the first generation of rAd vectors, the E1 region involved in the initiation of the virus replication cycle is deleted, in order to avoid formation of recombinant replicative particles (RCA). The major limiting factor of such vectors is immunogenicity, which triggers both inflammation and a cytotoxic response against the transfected cells. It has been observed that this phenomenon results in transgene extinction a few days post-application, due to cell death and to the negative viral promoter regulation by some cytokines (Qin et al, 1996). Surprisingly, viral proteins can be still expressed in cells infected with E1-defective rAd (Yang et al, 1994). Therefore, it has been proposed to delete from the adenovirus genome additional regions involved in the replication process. The E4 region is crucial for virus replication and E1-E4-defective rAds were generated in a transcomplementing cell lines (Yeh et al, 1996), where

vectors are produced by double recombination in E. coli (Crouzet et al, 1997; **Figure 1**). These rAds were tested for gene transfer in murine immuno-competent liver and showed a prolonged transgene expression due to lower viral protein expression as well as the absence of RCA during production (Gao et al, 1996, Dedieu et al, 1997, Wang et al, 1997). Interestingly, E1-E4-defective rAds have also been shown to be efficient for gene therapy of brain diseases. The local injection (with a stereotaxic pump) in rat striatum was associated with an attenuated immune response and prolonged transgene expression (Do Thi et al, 2004).

Recently, the elaboration of helper-dependent rAds called gutless vectors represent a very promising development area in gene therapy. Use of the helper Ad encoding all adenoviral proteins made possible the encapsidation of up to 30 000 base pairs transgenes in rAd containing only the two inverted terminal repeats (ITRs) and the encapsidation sequence ( ) of the original virus. In order to improve the yield of recombinant gutless vector production, the helper virus encapsidation sequence has been flanked by two loxP sequences permitting excision of this sequence in the presence of the Cre recombinase.

Serial passages of both gutless rAd and helper virus in 293 cells expressing the Cre recombinase resulted in an increased gutless rAd titre whereas the helper virus titre remained constantly low, limiting rAd stock contamination by this undesired particle (Parks et al, 1996). The resulting gutless vectors harbouring no viral coding regions elicit no cytotoxic immune response against Ad-infected cells, thus permitting a prolonged expression of the transgene as attested by -antitrypsin expression in baboons for over 50 weeks (Morral et al, 1999).

B. Adenovirus pentons and dodecahedra

The use of recombinant adenovirus for gene therapy has been tempered by strong immune responses that develop to the virus and virus-infected cells. In addition, notwithstanding that the rAd are replication-defective, they introduce into the recipient cell together with the gene of interest viral genes that might lead to fortuitous recombination if the recipient is infected by wild-type adenovirus. The use of an Ad structural complex called the penton has been proposed as an alternative vector for
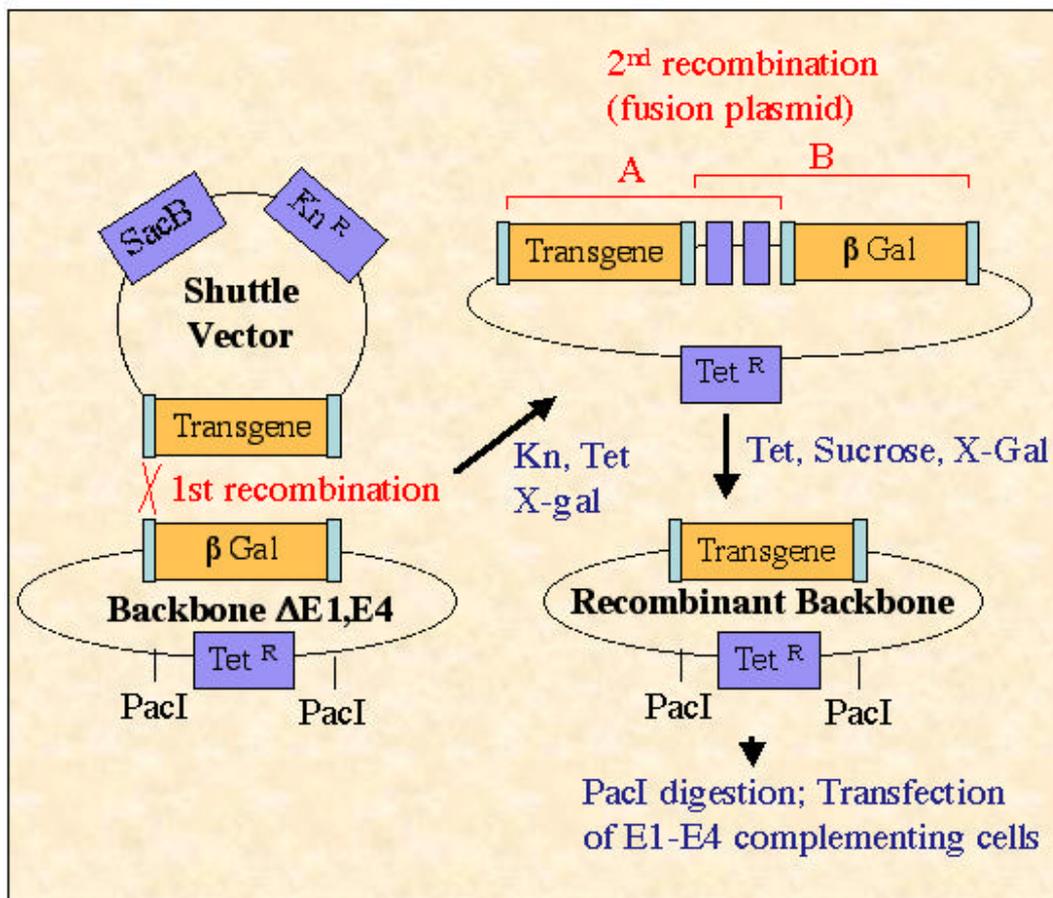

Figure 1: Principle of recombinant adenovirus production**.** Shuttle vector with the transgene cloned between the left ITR and the pIX sequences of adenovirus is transfected in recombination competent E. coli containing the E1-/E4- adenovirus backbone. Selection with Kanamycin (Kn) and Tetracyclin (Tet) results in the survival of a ß-galactosidase positive plasmid fusion. A second selection in presence of Tet and sucrose (that confere sensitivity to SacB positive bacteria) lead to the selection of either -Gal positive backbone (case A) or the desired recombinant backbone (case B). After PacI digestion, the recombinant backbone is transfected in a E1, E4 complementing cell line.

human gene therapy. The penton is a complex of two oligomeric proteins, a penton base and fiber. This complex located on the 12 vertices of the Ad capsid **(Figure 2)** is responsible for cell attachment, internalisation, and virus escape into the cytoplasm. The penton retains many of the advantages of adenovirus for gene transfer such as efficiency of entry and wide a range of cell and tissue targets. Because it consists of two proteins instead of at least 11 contained in an Ad virion, it is potentially a safer alternative to rAd. Interestingly, for some Ad serotypes such as human adenovirus of serotype 3 (Ad3), twelve pentons are capable of interacting together in a symmetric manner thus forming a sub-viral nanoparticle called dodecahedron (Dd) (Norrby et al, 1966). Expression of the Ad3 penton base alone or its co-expression with fiber protein using the baculovirus system leads respectively to the formation of base-dodecahedron (Bs-Dd) and penton-dodecahedron (Pt-Dd) **(Figure 2)**. The Dds structures solved by cryo-microscopy revealed that these particles contain an internal cavity with a volume close to 100 nm^3 (Schoehn et al, 1996).

Adenoviruses use primary receptors for high affinity interaction with invaded cell. We have recently shown that Ad3 dodecahedra recognize two types of receptors. The first is the Ad3 receptor (Sirena et al, *in press*) and, in addition, they have a capacity to strongly interact with cell surface glycoaminoglycans (Vives et al, Virology *in press*). This explains the remarkable penetration efficiency of Dds observed for a wide spectrum of cells. It has been proposed to use these particles as a gene transfer vector. In theory, the Dds internal cavity can only accommodate

small oligonucleotides of about 100-mers. To overcome this limitation and attach DNA externally to the particle, a bi-functional peptide capable of interacting with both Dds and plasmid DNA (thanks to a polylysine stretch) has been designed, resulting in efficient gene expression in cells transfected by this system (Fender et al, 1997). Under certain conditions, this system permitted a gene expression comparable to that obtained with RAds *in vitro* and superior to cells transfected with cationic lipids. Following the same idea it has been reported that other penton base serotypes like Ad7 can transfect cells *in vitro* in an integrin-dependent manner. (Bal et al, 2000)

A modified version of this vector has been examined using the non-dodecameric Ad5 penton base fused to heregulin and a stretch of 10 lysines (HerPBK10). The rationale for this idea was to conserve the endocytotic properties of the Ad penton base (internalisation and endosomolytic activity) together with the targeting of HER-2/3 or HER2/4 heterodimeric receptors which are over-expressed in certain aggressive breast cancers (Medina-Kauwe et al, 2001).

II. Protein delivery

To date, direct protein delivery in the cell is mainly based on the use of small protein or peptide vectors. However, adenovirus capsid protein complexes can also be engineered to become a novel protein transduction vector.
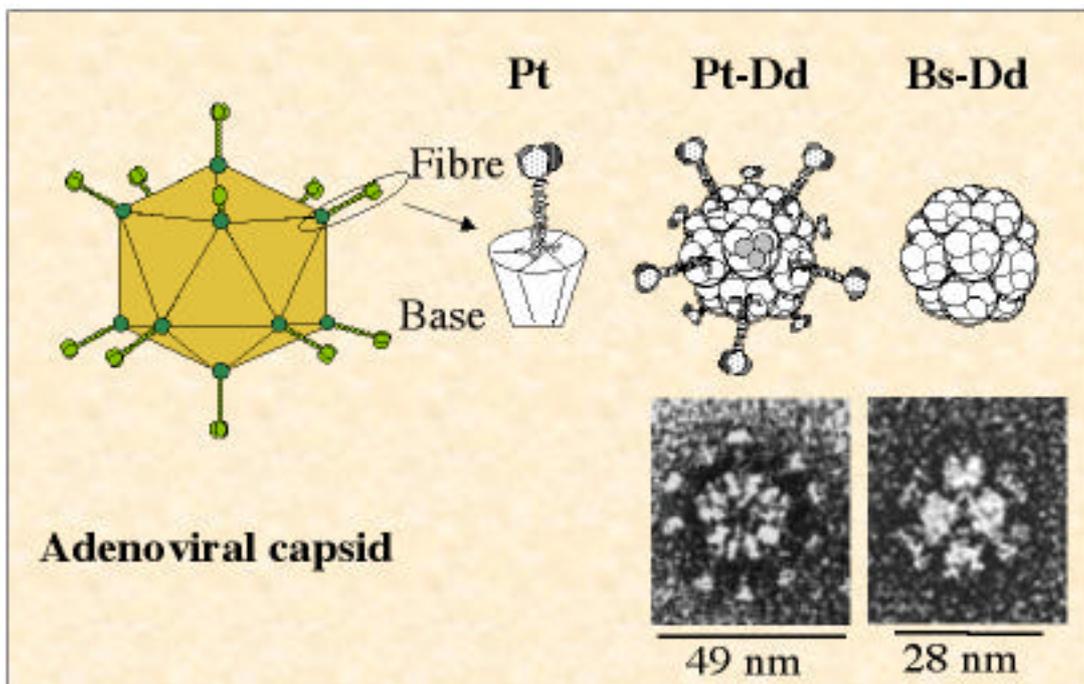

Figure 2: Adenovirus pentons and dodecahedron. Diagram of the icosahedric adenovirus capsid (left). A non-covalent complex of the fiber and the penton base protein called the penton (Pt) is found at each of the twelve virion vertices. The trimeric fibre is responsible for primary receptor recognition and enables the interaction of the pentameric penton base with cellular integrins. Human Ad3 pentons are capable of interacting together through the penton base and form a highly symmetric complex made of 12 units. The fibre is not required for this process as attested by the formation of both penton dodecahedron (Pt-Dd) and base dodecahedron (Bs-Dd).

A. Transduction peptides

A number of obstacles currently limit the effectiveness of gene therapy. One of the most formidable is the delivery of the desired genes to a sufficient number of target cells to elicit a therapeutic response. Recently, a series of virus-encoded and some regulatory proteins were found to be able to cross biological membranes. For example, peptides derived from the *Drosophila* Antennapedia homeodomain are internalised by cells in culture (Derossi et al, 1994) and transferred to cell nuclei where they can directly and specifically interfere with transcription (Le Roux et al, 1995, Derossi et al, 1996). The HIV-1 Tat protein was reported to be rapidly taken up by the cell *in vitro* where it can specifically transactivate the HIV-LTR (Frankel and Pabo 1988, Green and Loewenstein 1988). The Tat protein is composed of 86 amino acids and contains a highly basic region and a cysteine-rich region. It was found that Tat-derived peptides as short as 11 amino acids are sufficient for transduction of proteins (Fawell et al, 1994, Nagahara et al, 1998). The precise mechanism by which the 11-amino acid transduction domain crosses lipid bilayers was for a long time poorly understood but a recent study demonstrated that Tat or Antenapedia peptide endocytosis was promoted by interaction with cellular glycosaminoglycans (Console et al, 2003). A Tat-galactosidase fusion protein that was delivered efficiently into brain tissue and skeletal muscle *in vivo* has been generated recently (Schwarze et al, 1999). These findings suggest that protein therapies may be successfully developed provided that problems caused by immune response and toxicity that might be associated with long-term expression of novel proteins *in vivo* can be solved.

The herpes simplex virus type 1 tegument protein VP22 was also reported to exhibit a unique property of spreading between neighbouring cells. VP22 is a basic, 38-kDa phosphorylated protein encoded by the viral UL49 gene (Knopf and Kaerner 1980, Elliot and Meredith 1992). The transport of VP22 occurs *via* a mechanism potentially involving actin microfilaments. VP22 is exported from the cytoplasm of expressing cells and imported into neighbouring cells where it accumulates in the nucleus (Elliot et al, 1997). These properties aroused interest in VP22 as a delivery vehicle for therapeutic proteins (Dilber et al, 1999). VP22-directed delivery of proteins could be achieved either by transfection of genes encoding VP22 with a fused target protein gene, or by exogenous application of a protein extract containing VP22-fusion (Elliot et al, 1997).

However, the initial enthusiasm for results obtained with these peptides is tempered by a recent controversy whereby the nuclear homing of such fusion proteins was shown to be an artefact occurring during cell permeabilisation step (Lundberg and Johasson 2001).

B. Protein delivery using Ad dodecahedron

We are convinced that the high endocytotic capacity of adenoviruses can be used for direct protein delivery.

Sub-viral particles, dodecahedra, which retain the endocytotic features of Ad and are devoid of genetic material are ideal candidates for this purpose. The proof of the principle of using Dds for protein delivery has been made with a large multimeric protein (Fender et al, 2003). In this study, a monoclonal antibody against the penton base was shown to be efficiently delivered to cells by Dds. On average about $5,7 \cdot 10^5$ Mabs was found per cell using Pt-Dd vector while the efficiency of Bs-Dd in such transfer was lower. In order to attach other proteins to Dd, we are currently developing a system based on WW domains. Indeed, WW domains were found to interact with Ad penton base protein, which is a building block of Dd (Galinier et al, 2003). These domains, containing a linear sequence of about 35 amino-acids including two conserved tryptohanes (W), are known to interact with PPxY motifs in partner proteins (Sudol et al, 1995) and two such motifs are conserved in penton base sequences. In a novel study, we have shown that three WW domains fused in tandem to any protein enable fusion protein attachment to Dds and its subsequent delivery into human cells (**Figure 3**, manuscript in preparation). The use of WW domains as an adaptor to attach the protein of interest to our particles makes of Dds a versatile vector for proteins delivery. The efficiency of this new system is surprisingly high with an average of 10^7 WW-fused proteins imported per cell. Moreover, contrary to the results obtained for Mabs delivery with Dd, a comparable amount of protein is delivered by both Pt-Dd and Bs-Dd showing that the WW domain does not neutralize our vectors.

III. Conclusion

Its remarkable penetration efficacy has made adenovirus one of the most used gene therapy vectors. Several different types of rAds vectors have been designed, helped by the available knowledge of Ad genetics. The major problem with rAd vectors, their immunogenicity, has been at least partially overcome with the appearance of gutless rAd devoid of viral coding sequences. The alternative approach that benefits from virus endocytotic efficacy without the viral genome is to use the adenovirus capsid protein responsible for viral internalisation. Several studies have reported that the Ad penton base alone or penton base-derived dodecameric particle can be modified to attach plasmid DNA thus leading to transgene expression *in vitro*.

Very recently, another therapeutic approach based on direct protein delivery has appeared. Obviously, this kind of approach cannot be used for applications, like hereditary diseases, that require a sustained protein activity. However, it can be used in a particular therapeutic window where a temporary but massive protein delivery is sufficient. Among the possible applications of protein transduction, cancer therapy with toxins or apoptotic proteins would be one of the major challenges. However, the most promising uses for this new technology are *ex vivo* applications. In this vein, the delivery of antigens to prime dendritic cells for cancer vaccination or the delivery of pro-proliferating proteins to expand primary cells *in*
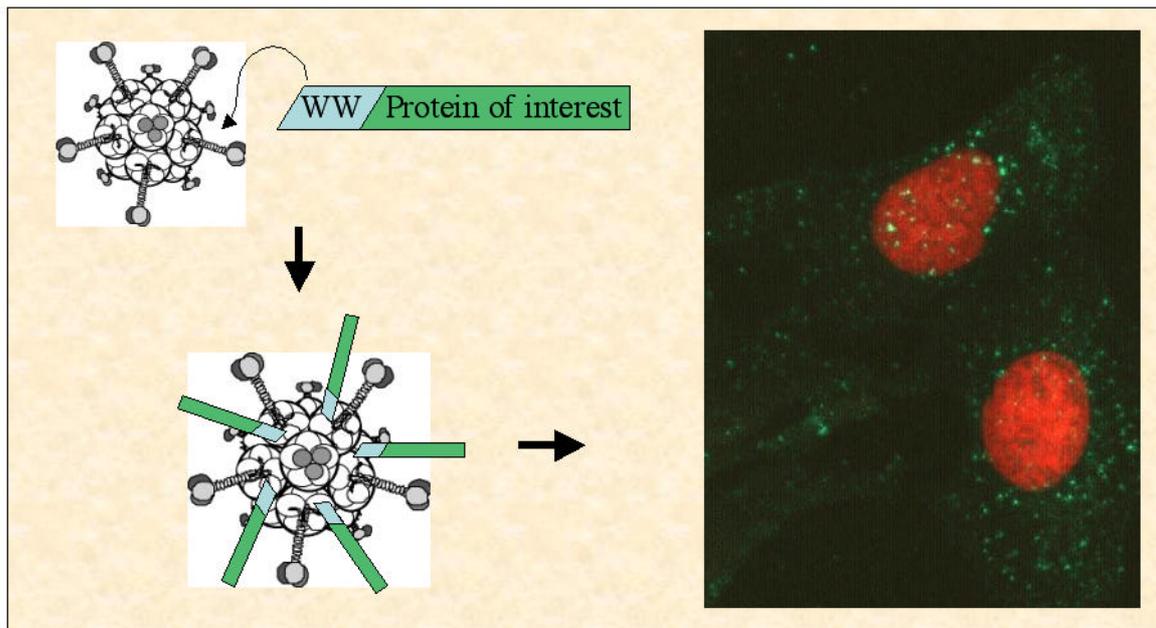
Figure 3: WW motifs can be use as a universal adaptor. The protein to be delivered to the cell is fused with WW domains to permit its attachment to the dodecahedron vector. The resulting complex is efficiently delivered into the cell as reflected by the green immuno-fluorecence detection of the protein of interest in HeLa cells (nuclei are counterstained in red)

vitro before a safe re-injection to the patient could be considered. Once again, Ad-derived vectors like the Ad3 dodecahedron could play an important role in this restricted but valuable area.

Acknowledgments

References

Bal HP, Chroboczek J, Schoehn G, Ruigrok RW, Dewhurst S. **(2000)** Adenovirus type 7 penton purification of soluble pentamers from Escherichia coli and development of an integrin-dependent gene delivery system. **Eur J Biochem** 267, 6074-81

Console S, Marty C, Garcia-Echeverria C, Schwendener R, Ballmer-Hofer K. **(2003)** Antennapedia and HIV transactivator of transcription (TAT) "protein transduction domains" promote endocytosis of high molecular weight cargo upon binding to cell surface glycosaminoglycans. **J Biol Chem** 278, 35109-14.

Crouzet J, Naudin, L., Orsini C, Vigne E, Ferrero L, Le Roux A, Benoit P, Latta M, Torrent C, Branellec D, Denefle P, Mayaux JF, Perricaudet M, Yeh P. **(1997)** Recombitional construction in E.coli of infectious adenoviral genomes. **Proc Natl Acad Sci** 94, 1414-1419.

Dedieu JF, Vigne E., Dedieu JF, Vigne E, Torrent C, Jullien C, Mahfouz I, Caillaud JM, Aubailly N, Orsini C, Guillaume JM, Opolon P, Delaere P, Perricaudet M, Yeh P. **(1997)** Long term delivery into the livers of immunocompetent mice with E1 /E4 defective adenoviruses. **J.Virol** 71, 4626-4637.

Derossi, D., Calvet, S., Trembleau, A., Brunissen, A., Chassaing, G, Prochiantz, A. **(1996)** Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent **J. Biol. Chem** 271, 18188-18193

Derossi, D., Joliot, A. H., Chassaing, G. **(1994)** The third helix of the Antennapedia homeodomain translocates through biological membranes **J. Biol. Chem**. 269, 10444-10450.

Dilber, M. S., Phelan, A., Aints, A., Mohamed, A. J., Elliott, G., Edvard Smith, S. C. I., O'Hare, P. **(1999)** Intercellular delivery of thymidine kinase prodrug activating enzyme by the herpes simplex virus protein, VP22. **Gene Ther**. 6, 12-21

Do Thi NA, Saillour P, Ferrero L, Dedieu JF, Mallet J, Paunio T. **(2004)** Delivery of GDNF by an E1,E3/E4 deleted adenoviral vector and driven by a GFAP promoter prevents dopaminergic neuron degeneration in a rat model of Parkinson's disease. **Gene Ther,** in press.

Elliott, G. D., Meredith, D. M. **(1992)** The herpes simplex virus type 1 tegument protein VP22 is encoded by gene UL49. **J. Gen. Virol.** 73, 723-726

Elliott, G., O'Hare, P. **(1997)** Intercellular trafficking and protein delivery by a herpesvirus structural protein. **Cell** 88, 223-233

Fawell, S., Seery, J., Daikh, Y., Moore, C., Chen, L. L., Pepinsky, B., Barsoum, J. **(1994)** Tat-mediated delivery of heterologous proteins into cells. **Proc. Natl. Acad. Sci** 91, 664-668

Fender P, Ruigrok RW, Gout E, Buffet S, Chroboczek J. **(1997)** Adenovirus dodecahedron, a new vector for human gene transfer. **Nat Biotechnol** 15, 52-6

Fender P, Schoehn G, Foucaud-Gamen J, Gout E, Garcel A, Drouet E, Chroboczek J. **(2003)** Adenovirus dodecahedron allows large multimeric protein transduction in human cells. **J Virol**. 77, 4960-4.

Frankel A. D, Pabo, C. O. **(1988)** Cellular uptake of the tat protein from human immunodeficiency virus **Cell** 55, 1189-1193

Galinier R, Gout E, Lortat-Jacob H, Wood J, Chroboczek J. **(2002)** Adenovirus protein involved in virus internalization recruits ubiquitin-protein ligases. **Biochemistry** 41, 14299-305

Gao GP, Yang Y, Wilson JM. **(1996)** Biology of adenovirus vectors with E1 and E4 deletions for liver-directed gene therapy. **J. Virol** 70, 8934-43

Green, M, Loewenstein, P. M. **(1988)** Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein **Cell** 55, 1179-1188

Knopf, K. W., Kaerner, H. C. **(1980)** A herpes simplex virus type 1 mutant with a deletion in the polypeptide-coding sequences of the ICP4 gene **J. Gen. Virol**. 46, 405-414

Le Roux, I., Duharcourt, S., Volovitch, M., Prochiantz, A. and Ronchi, E. **(1995)** Promoter-specific regulation of gene expression by an exogenously added homedomain that promotes neurite growth **FEBS Lett.** 368, 311

Lundberg M, Johansson M.. **(2001)** Is VP22 nuclear homing an artefact? **Nat Biot** 19, 713-314

Medina-Kauwe LK, Kasahara N, Kedes L. **(2001)** 3PO, a novel nonviral gene delivery system using engineered Ad5 penton proteins. **Gene Ther** 8, 795-803.

Medina-Kauwe LK, Maguire M, Kasahara N, Kedes L. **(2001)** Nonviral gene delivery to human breast cancer cells by targeted Ad5 penton proteins. **Gene Ther** 8, 1753-61

Morral N, O'Neal W, Rice K, Leland M, Kaplan J, Piedra PA, Zhou H, Parks RJ, Velji R, Aguilar-Cordova E, Wadsworth S, Graham FL, Kochanek S, Carey KD. **(1999)** Administration of helper-dependent adenoviral vectors and sequential delivery of different vector serotype for long-term liver-directed gene transfer in baboons. **Proc Natl Acad Sci**, 12816-21

Nagahara, H., Vocero-Akbani, M. A., Snyder, E., Ho, A., Latham, G. D., Lissy, A. N., Becker-Hapak, M., Ezhevsky, A. S., Dowdy, F. S. **(1998)** Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration **Nat. Med.** 4, 1449-1452

Norrby E **(1966)** The relationship between the soluble antigens and the virion of adenovirus type 3. II. Identification and characterization of an incomplete hemagglutinin. **Virology** 4, 608-17.

Parks RJ, Chen L, Anton M, Sankar U, Rudnicki MA, Graham FL. **(1996)** A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. **Proc Natl Acad Sci** 93, 13565-70

Pomeranz, L, Blaho, J. **(1999)** Modified VP22 localizes to the cell nucleus during synchronized herpes simplex virus type 1 infection **J. Virol.** 73, 6769-6781

Qin L, Ding Y, Pahud DR, Robson ND, Shaked A, Bromberg JS. **(1997)**. Adenovirus-mediated gene transfer of viral interleukin-10 inhibits the immune response to both alloantigen and adenoviral antigen. **Hum Gene Ther** 8, 1365-74.

Schoehn G, Fender P, Chroboczek J, Hewat EA. **(1996)** Adenovirus 3 penton dodecahedron exhibits structural changes of the base on fibre binding. **EMBO J** 15, 6841-6

Schwarze, R. S., Ho, A., Vocero-Akbani, A., Dowdy, F. S. **(1999)** In vivo protein transduction: delivery of a biologically active protein into the mouse **Science** 385, 1569-1572.

Sudol M, Chen HI, Bougeret C, Einbond A, Bork P. **(1995)** Characterization of a novel protein-binding module--the WW domain. **FEBS Lett**. 369, 67-71. Review.

Wang Q, Greenburg G, Bunch D, Farson D, Finer MH. **(1997)** Persistent transgene expression in mouse liver following in vivo gene transfer with a delta E1/delta E4 adenovirus vector. **Gene Ther** 4, 393-400

Yang Y, Nunes FA, Berencsi K, Furth EE, Gonczol E, Wilson JM. **(1994).** Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. **Proc Natl Acad Sci** 91, 4407-4411.

Yeh P, Dedieu JF, Orsini C, Vigne E, Denefle P, Perricaudet M **(1996)**. Efficient dual transcomplementation of adenovirus E1 and E4 regions from a 293-derived cell line expressing a minimal E4 functional unit. **J. Virol** 70, 559-65.

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