

Dose-response and control of adeno-associated viral vectors based preclinical and clinical gene therapy

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ABSTRACT Human gene therapy needs to express exogenous DNA at the targeting cells, producing a practical and efficient therapeutic dosage at an appropriate time (quantitative pharmacology) with a safe manner. Recombinant adeno-associated virus (rAAV) vectors possess a number of properties and recent progress in rAAV production made it rapidly become the reagent of choice for therapeutic gene transfer. Over 60 clinical trials of gene therapy based on rAAV have been carried out. The dose response reaction between rAAV vectors and gene expression activity or clinical outcome is one of major aspects of these trials. Most studies showed that vector genomes (vg) and gene expression had a concentration-dependent relationship during a certain scope. However, gene expression can be affected by viral serotypes, tissue tropisms, cell targeting, drug regulation, injection route, age and sex, etc. Thus, these aspects should be carefully considered by scientists, pharmacologists and physicians during animal experiments or clinical trials.

KEY WORDS gene therapy; viral vector; dose-response; quantitative pharmacology; clinical therapy

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Gene therapy has been a prolific area of research over the past decade and continues to receive considerable attention. More than 1450 clinical trials have been carried out. However, the use of genetic material as therapeutic agents has produced many novel and challenging obstacles, including the formulation or packaging of the DNA, *in vivo* delivery, penetration of biological barriers, DNA elimination within the cell and from the tissue compartments of the whole body, control of product expression and overt toxicity. The current challenge is to resolve each of these obstacles to produce a practical and efficient therapeutic dosage at a right site and at an appropriate time with a safe manner. However, during early 90's, the relationship between the amount of transgene administered to a host and therapeutic response often received little attention in preclinical gene therapy studies. There are several possible reasons for this approach. Firstly, for many diseases under consideration for gene therapy, sensitive and reproductive animal models are not available. Secondly, a single dose therapeutic response in an animal model may be sufficient to warrant human studies. Thirdly, dose escalation studies aimed at determining the maximum response and toxicity of a transgene require amounts of material that are often difficult to produce^[1].

Recombinant adeno-associated virus (rAAV) vectors possess a number of properties that may make them suitable for clinical gene therapy. It has never been as-

sociated with any human tumors or other acute pathology. The size restriction of the small AAV genome has been partly overcome by developing trans-splicing vectors, which could efficiently package twice the size of the vector genome. The increases in efficiency gained with self-complementary vector (scAAV) made it a more promising vector for gene therapy. In additional, recent years, several novel AAV serotypes, including AAV7, AAV8, AAV9, AAV10, AAV11, AAV12 and over 120 AAV variants have been found in human or nonhuman primate tissues, which made possible for the exponential progress of rAAV-based vectors. More importantly, high-titer rAAV vectors has been successfully produced in the absence of helper adenovirus by Dr. Xiao Xiao and his colleagues^[2], and recent developments indicated that rAAV production in insect cells was compatible with current good manufacturing practice production on an industrial scale. Thus, obtaining $\times 10^{14}$ particles of rAAV per liter of cell culture in ex-scale is now possible. This yield not only supports development of rAAV therapeutics from tissue culture to small animal models, but also to large animal models, to toxicology studies, to phase I/II clinical trials and ever beyond. Based on preclinical studies in mice, rabbits, dogs and, nonhuman primates, over 60 clinical trials have now been approved, with most based on rAAV2 vectors.

1 DOSE RESPONSE

A number of preclinical studies had revealed interesting insights into the relationship between gene dose and activity. Human MSCs could indeed be transiently transduced *in vitro* by the rAAV2 vector with efficiencies of up to 65% with a 10,000 fold viral dose increase^[3]. When rAAV2 vectors at doses ranging from 4.0×10^8 to 1.1×10^{13} vector genomes (vg) per mouse, a 2-log-range linear dose-response curve of transgene expression was obtained from 3.7×10^9 to 3.0×10^{11} vg/mouse. Vector doses above 3.0×10^{11} vg/mouse resulted in disproportionately smaller increases in both the number of transduced hepatocytes and levels of transgene expression, followed by saturation at doses above 1.8×10^{12} vg/mouse^[4]. The relationship between vector dose and AADC enzymatic activity in tissue extracts was also linear when rAAV vectors carry-

ing human AADC cDNA was infused into the striatum of parkinsonian monkeys^[5]. The dose-response results in RPE65-mutant dogs indicated that the highest 1.5-log unit range of rAAV vector doses proved efficacious^[6]. But the relationship between gene expression level and the vector dose was not always positively linear. When the mice were infected with 5×10^{10} genome copies(gc) virus, high levels of hFIX in the plasma of five rAAV8-hFIX virus-infected mice were detected 2 weeks after injection. Much lower levels of hFIX were detected in mice infected with higher dose (2.5×10^{12}) of rAAV8 vector^[7]. From 10^8 to 10^{11} viral particles (VP), vAAV8 resulted in a concentration-dependent expression of GFP in liver, while GFP production in pancreas injected with rAAV8 saturated at 10^{10} VP^[8].

Some clinical assessment of gene dose-response had been reported, although mostly with respect to toxicity. Dr. Jan van Lunzen (University Medical Center, Hamburg Eppendorf, Germany), presented updated Phase I data from a study of tgAAC09 in healthy volunteers not infected with HIV in a poster presentation at the 14th Conference on Retroviruses and Opportunistic Infections (Los Angeles). The Phase I clinical trial was a double-blind, placebo-controlled, dose-escalation safety study that also monitored immune responses to HIV antigens. In this Phase I study, 80 healthy volunteers received a single intramuscular injection of tgAAC09 at different doses (10^9 – 10^{11} , Dnase Resistant Particles, DRP). Additionally, 21 of the 50 European volunteers received a booster vaccination of either tgAAC09 at the highest dose tested, or placebo. The results demonstrated that vaccination with tgAAC09 appeared to be safe and well tolerated and stimulated a modest immune response against gag, the principal HIV protein encoded by tgAAC09. In animal models, tgAAC09 elicited both T- and B-cell responses. In this trial, HIV-specific T-cell responses were observed in 20 percent of participants receiving the highest dose of tgAAC09 tested; however antibody responses were not observed. "Although the responses in this study are modest overall, it is very encouraging to see a 20% participant response at this dose threshold," said Dr. van Lunzen. "Given the dose-response relationship observed in this trial, it is our hope that higher doses may

enhance the vigor of the immune response elicited by tgAAC09". In phase II trial, 91 healthy HIV-negative volunteers at five clinical sites in Africa had received two intra-muscular injections of placebo or tgAAC09 at 3 different dosage levels either 6 or 12 months apart. The reported safety data reflected results following the first vaccination and re-vaccination in 91 and 82 volunteers, respectively. To date, the vaccine had been well tolerated, and no severe local or systemic reactions to vaccination had been reported. Further study of this and other AAV-based vaccines was warranted, as they provided a novel approach for using the power of the immune system to fight such a devastating infection (TARGETED GENETICS CORPORATION, <http://www.targen.com>). Another Phase I/II study carried out by the same corporation was designed to assess the safety and potential efficacy of different doses of tgAAC94 administered directly to affected joints of subjects with inflammatory arthritis. tgAAC94 is an investigational therapy that utilizes an rAAV vector to deliver the gene encoding a soluble form of the receptor for TNF-alpha (TNFR:Fc). In March 2006, the Company received approval from the FDA to amend its protocol for the tgAAC94 clinical trial to include a higher dose group and increase the number of patients. 127 adults had been randomized into three dose levels to receive a single intra-articular injection of either tgAAC94 or placebo into the knee, ankle, wrist, metacarpophalangeal or elbow, followed by an open-label injection of tgAAC94 after 12 to 30 weeks, depending on when arthritis symptoms in the target joint meet criteria for re-injection. The interim data reported by investigators at leading scientific meetings supported the safety and tolerability of single and repeat intra-articular injections of tgAAC94 to affected joints at doses up to 1×10^{13} (DRP/mL) of joint fluid in subjects with and without systemic TNF-alpha antagonists. This interim data also suggested that treatment with tgAAC94 might lead to improvements in signs and symptoms of arthritis in injected joints (TARGETED GENETICS CORPORATION, [HTTP://WWW.TARGEN.COM](http://www.targen.com)).

However, not all the results had dose relationship between vector amount and clinical outcome. In nervous system, twelve patients with moderate PD received gene therapy with the GAD gene via an rAAV vector.

Each received one of three doses of vector, differing from lowest to highest by a factor of $10(10^9 - 10^{11} \text{ vg/ml})^{[9]}$. In the off state, the total mean UPDRS motor scores at baseline, 1 month, 3 months, 6 months, and 12 months were 39.2, 33.9, 31.6, 28.3 and 29.8 respectively. Improvement from baseline to 12 months was 24.7% ($P = 0.0038$ for 12 months vs. baseline). This mean improvement reflected no improvement in 2 patients, 0–20% improvement in 4 patients, 20%–40% in 2 patients, and more than 40% in 4 patients. In the on state, the scores were 22.1, 18.8, 16.5, 16.3 and 16.1. Improvement from baseline to 12 months was 27.2% ($P = 0.0098$). In both off and on states, improvements in the limb contralateral to the treated side was greater than that in the limb unilateral. No change in ADL scores or dyskinesias was observed during the study. Blinded analysis of PET scans at baseline and 12 months indicated decreased glucose metabolism on the treated side, versus no change on the untreated side. There were no treatment-related adverse effects over the course of the year. High anti-AAV antibody titers were found at baseline in two patients; these did not change during the study. But the improvements seen were not dose-dependent. Several factors might have contributed to the absence of such an effect. First, the small sample size per dose group was not powered to detect differences in effectiveness by the clinical outcome measures reported here. Second, Parkinson's disease could be quite heterogeneous in presentation, and it was possible that this therapy might be most effective for some rather than all of the symptoms, because this study was not randomised, the different dose groups were not tightly matched for disease severity and symptom expression. Finally, more uniform and pronounced effects might be achieved if bilateral surgery had been done. Although the apparent laterality and time course of benefits lent support to a specific biological effect and this hypothesis was reinforced by the blinded regional hemispheric 18F-fluorodeoxyglucose PET metabolic changes in these patients, concerned regarding possible placebo effects cannot yet be completely excluded from this study.

2 DOSE CONTROL

2.1 Elements of construct and strength of gene

expression In later 1990's, in order to obtain a suitable system in Canavan's disease clinical trial, we once conducted a study to compare a range of mammalian CNS expression cassettes in rAAV-2 vectors both *in vitro* and *in vivo* using strong endogenous promoter sequences, with or without a strong post-regulatory element (WPRE) and polyadenylation signal. Changes in these elements led to transgene expression varying by over three orders of magnitude. Individual promoter had distinct ability to express reporter or therapeutic genes in different cell types. In experiments conducted in primary cell culture and in 100 stereotactically injected rats, we observed highly efficient and stable (15 months) gene expression in neurons and limited expression in glia; the highest expression occurred with endogenous, nonviral promoters such as neuron-specific enolase (NSE) and β -actin^[10]. The post-regulatory element, WPRE, was inserted into in the preproenkephalin (PPE) promoter construct increased expression six-fold in both cortical and striatal cultures and three-fold in nigral cultures compared with the construct lacking WPRE. Higher gene expression was also found *in vivo*, with a 13-fold difference in striatum and 35-fold difference in hippocampus 2 weeks after injection^[11]. Similar phenomena also existed in the other constructs. When CAG promoter was constructed into rAAV1 vector, the highest EGFP expression was found in the inner hair cells and other cochlear cells^[11]. The CMV and NSE promoter drove the higher EGFP expression, but only a marginal activity was observed in EF-1 α promoter driven constructs. RSV promoter failed to drive the EGFP expression. Myo promoter driven EGFP was exclusively expressed in the inner hair cells of the cochlea. When driven by CAG promoter, reporter gene expression was detected in inner hair cells at a dose as low as 3×10^7 genome copies, and continued to increase in a dose-dependent manner. In the mouse lung, the RSV promoter performed significantly better than a human CMV promoter in the airway epithelium. However, CAG promote exhibited even higher expression than either of the strong viral promoters alone, showing a 38-fold increase in protein expression over the RSV promoter^[12]. Thus, tissue or cell specific promoters are capable of restricting gene expression in desirable cells and facilitating per-

sistent or regulated transgene expression.

2.2 Regulatable system for quantitative gene expression

The ability to regulate transgene expression over a long period would be critical for gene therapies that require intermittent production of the gene product. In addition, regulatable expression might be necessary for gene products that have small differences between effective and toxic doses (small therapeutic window). A number of laboratories had started to develop drug-sensitive promoter systems that allow for control of transgene expression and some of these systems had been tested *in vivo*. Most notable were the tetracycline-dependent vectors originally developed by Gossen and Bujard^[13]. Now, In the Tet-repressor system, elements of the bacterial tet operon were transferred into mammalian cells; the tet-repressor was fused to the VP16 transactivation domain (Fig 1). In the presence of tetracycline or its derivative doxycycline (Dox), this fusion protein would not bind a promoter which harbored a tet-repressor binding site. Later a mutant tet-VP16 fusion was developed which only binds to DNA in the presence of Dox. In many cases the leakiness of the regulated promoters had presented a problem. This could be avoided by the simultaneous expression of a tet-repressor fused to the KRAB protein (tTS), which kept the inducible gene inactive in the absence of Dox. This vector driven shRNA by TRE/U6 promoter had now been developed by Clontech. A number of investigators had constructed vectors suitable for gene therapy by incorporating a tetracycline-dependent promoter and had demonstrated regulated gene expression in cultured cells and *in vivo*. Dox induced a time- and dose-dependent release of GDNF *in vitro* in human glioma cells infected with rAAV-rfTAM2 serotype 2 virus^[14]. In a *in vivo* trial, 8 weeks after a single injection of the rAAV-rfTAM2-GDNF vector encapsidated into AAV serotype 1 capsids in the rat striatum, the GDNF protein level was 60 pg/mg tissue in Dox-treated animals whereas in untreated animals, it was undistinguishable from the endogenous level (approximately 4 pg/mg tissue)^[14]. Repeated Dox inductions of transgene expression in the nonhuman primate retina could be achieved using a Tet-inducible system via rAAV vector administration over a long period (2.5 years). Maximum erythropoietin (EPO) secretion in the anterior chamber depended

upon the rAAV serotype and the nature of the promoter driving rTA expression^[15]. Other inducible systems including rapamycin-inducible promoter^[16], hypoxia-response element^[17] and Cre/loxP system^[18] had also been successfully used to regulate rAAV vectors expressing genes. Together, these studies indicated the

potential of regulatable gene expression in whole animals. Gene regulation by a secondary drug might provide a quantal response (on/off switch) or a graded response (concentration-dependent effect) depending on the system.

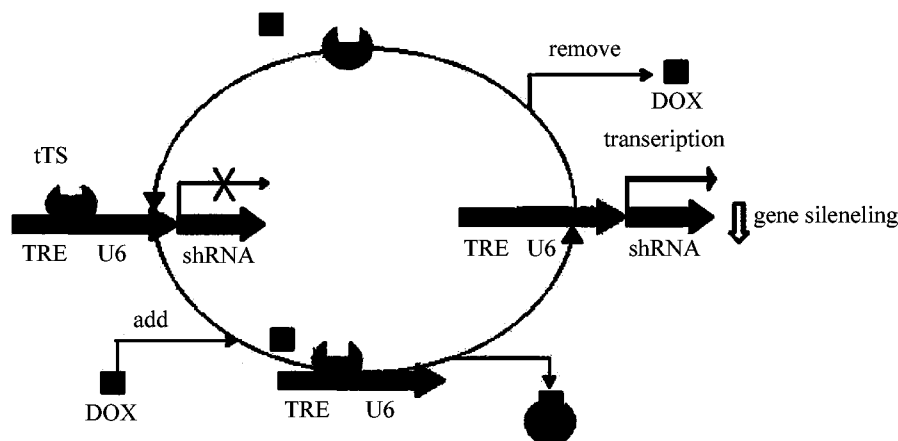


Fig 1 The Knockout Single Vector Inducible RNAi System uses a modified form of the tightly regulated tetracycline-controlled gene expression system. In the absence of Dox, tTS binds the tetO sequences within the TRE/U6 promoter and actively silences transcription of the shRNA. When Dox is added to the culture medium, tTS dissociates from the TRE, relieving transcriptional suppression and allowing high level transcription of the shRNA from the hybrid TRE/U6 promoter.

2.3 Viral serotypes, tissue tropisms, targeting and gene expression efficiency

Although novel AAV serotypes and variants made possible for the exponential progress of rAAV-based vectors^[19], the diverse tissue tropisms of AAV serotypes (Table 1) and their potential to evade preexisting neutralizing antibodies against the common human AAV2 made it difficult to fix quantification during preclinical and clinical studies. From certain perspectives, the promiscuity of viral vectors is more of a liability than a benefit, as the systemic delivery of vector generally leads to unwanted vector uptake by many different cell types in multiple organs.

Several researchers had already compared the transduction efficiencies of serotypes of rAAV vectors in different tissues. While results pertaining to AAV serotype tissue tropism were generally difficult to interpret due to inter-study variations in vector titers and doses, promoters, and transgen. A general hierarchy of transduction efficiency in major tissues had been established (Table 1). It is important to note that the final transduction efficiency as well as the kinetics of transgene expression varies significantly among different serotypes.^[32-33] In lung airway epithelia, we found that

rAAV5 was more efficient at mediating gene transfer than rAAV2, without adversely affecting lung development^[34]. Latter, it was found that rAAV9 could effectively transduce murine nasal and lung airway epithelia, at the peak of gene expression rAAV9-mediated human alpha-1-antitrypsin gene expression in serum was approximately 60-fold better than that of rAAV5^[35]. Most interestingly, rAAV9 could be readministered in the presence of high levels of serum-circulating neutralizing antibodies as early as 1 month after initial exposure, with minimal effect on overall reporter gene expression, rendering it a promising gene transfer vector candidate for use in humans^[20]. It can be further confirmed that consistent high-level transduction was found in the lung transduced with rAAV9. Abundant AP-positive cells were seen in alveolar cells and vasculature, but not in bronchioles^[21]. rAAV8 had been proved to be more efficient than rAAV1 or rAAV2 vectors for gene delivery to all of the structures analyzed, including the cerebral cortex, hippocampus, olfactory bulb, and cerebellum^[22]. In a detail study, rAAV type 2/1, 2/2, 2/5, 2/7, or 2/8 at high titers transduced comparable brain volumes in all targeted re-

gions except for rAAV 2, which transduced much smaller brain volumes^[32]. Pseudotyped vectors derived from rAAV7 and rAAV8 have increased transduction efficiency in the murine CNS, with the rank order rAAV 7 > rAAV8 > rAAV5 > rAAV2 = rAAV6. Efficiency of gene transfer to the orthotopic tumor (4C8 glioblastoma tumor) was increased when using rAAV6, -7, and -8 capsid proteins in comparison with serotype 2, with the order rAAV 8 = rAAV7 > rAAV6 > rAAV2 > rAAV5^[23]. AAV 7, 8, 9, and Rh10 vectors expressing cDNA for a lysosomal enzyme transduced neurons, but not astrocytes or oligodendrocytes, in the cortex, striatum, hippocampus, and thalamus^[39]. Another report showed that both rAAV8 and rAAV9 were able to transduce myocardium at 20- and 200-fold (respectively) higher levels than rAAV2^[25]. The mechanism(s) underlying such distinct transduction profiles is currently unclear, it is likely that differences between serotypes arise due to differences in cellular uptake and intracellular trafficking mechanisms of AAV serotype vectors in each tissue type. These diverse infectious pathways are in turn thought to arise from structural differences between AAV serotypes at the capsid level. Efforts to understand cell surface receptor usage and intracellular trafficking pathways exploited by AAV continue to provide significant insight into the biology of rAAV vectors^[19].

Table 1 Transduction efficiency in major tissues of rAAV serotype vectors

Tissue	Optimal serotype(s)	Reference
lung	AAV9	(20, 21)
CNS	AAV7, AAV8	(22, 23)
Heart	AAV8, AAV9	(24, 25)
Photoreceptor cells	AAV7, AAV8	(26)
Kidney	AAV2	(27)
Pancreas	AAV8	(28, 29)
Skeletal muscle	AAV1, AAV7	(30, 31)

2.4 Kinetics of cellular uptake and intracellular trafficking Ledley and Ledley in 1994 reported the kinetics of gene therapy at a cellular level^[37], which outlined the likely events that might affect transgene expression and proposed a 6 compartment linear model to describe the uptake, intracellular trafficking and expression of exogenous genes (Fig. 2A). Their classical

pharmacological compartmental approach, although theoretical in nature, provided a basis for developing strategies to enhance or modulate transgene expression at a cellular level.

DNA viral vector uptake through the cellular membrane, with escape from the endocytic/lysosomal compartment and transport into the nucleus, which is regulated by capsid and DNA interactions with the host cell; and effective expression of transgenes by the promoter and regulatory elements, which includes production of the transcript and its interaction with cellular factors. For instance, there are two separate issues affecting the targeting and expression of AAV2 in the brain. With respect to the first issue, AAV has a neurotropism, largely attributable to its protein capsid, which makes it ideal for the study or manipulation of neurons. This tendency to infect neurons can be accentuated through the use of a neuron-specific promoter such as NSE, which we found confers 99% neuronal specificity^[40]. In a previous kinetic study by Bartlett et al^[38], wildtype AAV2 capsids labeled with a fluorophore were taken up into the nuclei of neuronal cells *in vivo* within minutes, and by 24 h AAV2 particles were also found in scattered microglia. These experiments suggested that viral binding and uptake primarily determined the neurotropism of AAV2.

The first cellular receptor to be identified for AAV was heparan sulfate proteoglycan (HSPG), other coreceptors had been proposed, such as the FGF-1 receptor^[39] and v5 integrin^[40] (Fig2 B). Yet all cells in a tissue are not equally permissive to AAV infection, for example, we found that irrespective of the hepatic stellate cells (HSC) were readily transduced whereas other liver cells such as hepatic cells were transduced poorly if at all, suggesting that both extra- and intra-cellular factors are responsible for differences in expression among liver cells with AAV2^[41]. Distribution of receptors and coreceptors also accounts for differences in susceptibility to AAV transduction between cell types. Endocytosis and intracellular trafficking mechanism were different with altering of AAV serotypes or hybrid vectors^[38, 42-45] and the intracellular events underlying disassembly (uncoating) and nuclear translocation of AAV virions remain largely unknown^[33, 46]. Both AAV and rAAV deliver a single-stranded DNA genome.

which must be converted into double-stranded DNA (dsDNA) by the host cell. It is the rate-limiting step. scAAV vector have been developed to facilitate robust transgene expression at a minimal dose with rapid transgene expression within 3 days^[47], but at the same dose, single strand rAAV vector required 14 days for the attainment of expression levels comparable to those observed using scAAV at day 3. For most of the vector DNA, when the dose is high enough to deliver multiple genomes to the nucleus, they can join end to end to

form concatemers, which also can circularize (Fig 2B). More recently, the molecular fate of both wild-type (wt) AAV in humans and rAAV in animals had been demonstrated to be persistent as episomal circles. It is not clear that the linear rAAV genome is inherently subjected to loss of gene expression through nucleolytic degradation or by other mechanisms. Rather, the linear form of the genome represents a transient episomal phase in normal cells due to the recombinogenic activity of the free DNA ends^[48].

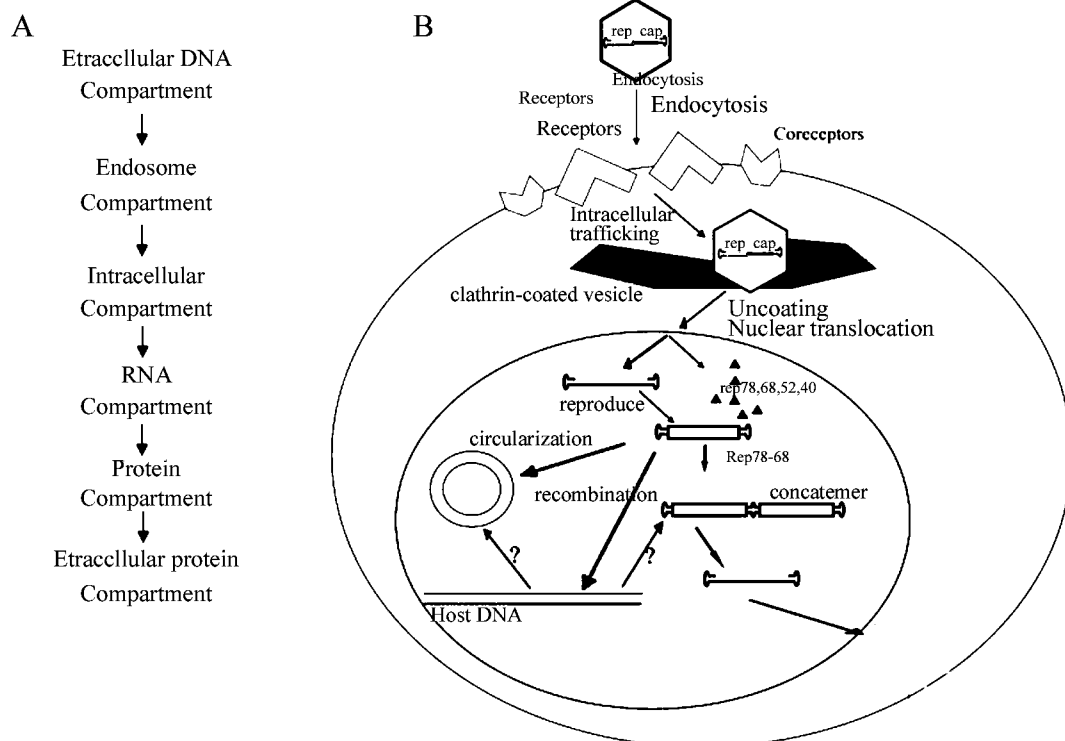


Fig 2 A. Compartmental model describing the intracellular disposition of transgenes and their products (37); B. Cellular uptake intracellular trafficking nuclear translocation and fate in nuclear of AAV.

2.5 Drug enhanced gene therapy Often the success of multi-drug treatments is due to additive or synergistic responses and is best when the individual drugs act via different mechanisms to produce similar responses. This can lead to much lower, and therefore safer, drug doses required to achieve the same effect. The possibility that conventional drug treatments may complement rAAV-gene-based treatments is important for optimizing clinically relevant gene therapies. Already, several interesting drug-gene combinations have been reported, mostly for cancer-directed gene therapies. Combination therapy in which rAAV-hIFN-beta was used together with low-dose cyclophosphamide re-

sulted in complete regression of both established retroperitoneal and disseminated disease^[49]. In HepG2 cells transduced with rAAV vectors encoding LFv2IRE, AP20187 induces LFv2IRE homodimerization and transphosphorylation minutes after drug administration, resulting in the phosphorylation of a canonical substrate of the insulin receptor tyrosine kinase, IRS-1. AP20187 activation of LFv2IRE is dependent on the dose of drug and the amount of chimeric receptor expressed in rAAV-transduced cells. Finally, AP20187-dependent activation of LFv2IRE results in insulin-like effects, such as induction of glycogen synthase activity and cellular proliferation^[50]. The uptake via the endo-

somal system could also be enhanced pharmacologically with drugs that inhibited endosomal acidification or by agents that enhanced the rate of DNA release from the endosomes^[48]. Antiproteasome treatments with MG-132 led to a 50-fold enhancement in transduction efficiency. Not only regulating expression and enhancing therapeutic effects, conventional medicines were also increasingly being used to overcome some of the obstacles faced with gene therapy, such as reducing unwanted immune responses to transgenes. However, multi-agent therapy introduces further pharmacological considerations such as potential interactions between the drug(s) and the transgene product, dosing regimes and toxicity of the combined treatment. When conventional drugs are used to supplement the effects of gene therapies, additive or synergistic responses also may need to be considered.

2.6 Route of viral vector gene delivery and outcome of therapy

The route of viral vector administration has been shown to influence the extent of transgene expression. For DNA given by non-intravenous methods, bioavailability of the construct and the gene product may vary considerably according to how and where the vector was administered to the patient. Moreover, immunological responses *in vivo* to transgenes appear to be quite different depending on the route of administration. Successful delivery of rAAV vector into mammalian model organisms has been carried out using various methods. It is difficult to make generalizations about which delivery method leads to the most effective silencing, however, because different viral types and tissues have different requirements for effective delivery, especially for animals of different sizes. For example, intraperitoneal and intravenous delivery of rAAV8 effectively transduced exocrine acinar cells as well as endocrine beta-cells, while local pancreatic intraductal delivery of rAAV6 showed the best efficiency in the beta-cells among all AAV serotypes tested in this study^[51].

2.6.1 Direct administration

The direct injection of an AAV vector into the target tissue leads to the infection of distant non-target tissues with the vector via blood circulation. The rAAV2 vector, when administered by instillation to the bronchial epithelium of rhesus, was distributed to the heart, liver, jejunum, ki-

dey, lymph nodes spleen, pancreas, and brain^[52]. The rAAV2 vector, when injected into the liver of rhesus fetuses, was distributed to the lymph node, liver, skin, spleen, lung, and esophagus of human infants^[53]. The rAAV2 vector, when injected into the muscle, was distributed to the liver and lymph nodes^[54]. Thus, detailed evaluation of vector biodistribution to various tissues is a necessary part of the assessment of the safety of the vector in context of administering a gene therapy strategy with *in vivo* administration. Direct administration may be ideal for some tissues by avoiding both rapid clearance from the central compartment and the endothelial cell/basement membrane barrier. Although there were still many problems exist in direct administration, some positive results had been got by different groups. Intrathecal administration of rAAV-IDUA delivers vector to brain cells, and was both minimally invasive and effective^[55]. An intramuscular injection of AAV-PGIS prevented monocrotaline-pulmonary arterial hypertension in rats and provided a new therapeutic alternative for preventing pulmonary arterial hypertension in human^[56]. A single VTA injection of rAAV9 compared with injections into the striatum, resulted in higher enzyme levels in more regions of the brain. Widespread distribution of the vector genome after rAAV9 VTA injection resulted in even further distribution of the enzyme product, by secretion and uptake by surrounding cells, and complete correction of the storage lesions throughout the entire brain^[57]. Clinical trial done by Doring and his colleague for PD with rAAV based gene (GAD) therapy was achieved by delivering unilaterally to the subthalamic nucleus contralateral to the most affected side of the body^[9].

2.6.2 Systemic administration

Intravenous injection is currently the most popular mode of delivery of rAAV vectors in ongoing clinical trials. Intravenous administration is attractive pharmacologically as it is simple and because it may provide access to tissues where direct injection of genetic material is not practical. However, transgenes administered intravenously must cross the endothelial cell barrier and basement membrane before reaching target cells in the parenchyma^[58]. The transcytosis across barrier epithelial and endothelial cells appears to be serotype and cell-type specific^[59]. This can be a very slow process com-

pared to general tissue distribution, metabolism, and excretion, hindering efficient gene delivery. It has been demonstrated that distribution pattern with intravenously administered rAAV2 varied from monkey to monkey^[60]. Intravenous delivery of rAAV1 into 1-day-old neonatal mice resulted in broad distribution of expression but successful disease correction in a mouse model for Pompe disease^[61]. It was observed that intravenous injections of rAAV5-based vectors encoding the human coagulation factor IX (hFIX) gene was more efficiency than with intraperitoneal (i. p.) injections in mice^[8]. However, the rAAV vector containing the murine Pah-cDNA pseudotyped with capsids from AAV serotype 8 delivered into the liver of PKU mice via single intraportal or tail vein injections have no distinct difference^[62]. Moreover, combination systemic and direct (brain) injections of rAAV vectors encoding hASM could have better global reversal of pathology translated to normal weight gain and superior recovery of motor and cognitive functions compared to animals treated by either brain or systemic injection alone. All of the animals treated by combination therapy survived in good health to an investigator-selected 54 weeks, whereas the median lifespans of the systemic-alone, brain-alone, or untreated ASM knockout groups were 47, 48, and 34 weeks, respectively^[61].

2.6.3 Oral gene delivery and expression Oral vaccines were scavenged by intestinal M cells, rapidly taken up by the antigen-presenting cells (APCs) in Peyer's patches and the lamina propria, and could induce strong humoral immune responses. The hardness of AAV made it particularly suitable as an orally-delivered vector. It was resistant to temperature and pH extremes and solvents. We first demonstrated the feasibility of administering an rAAV vector orally to obtain long-term gene expression in the gastrointestinal system^[64]. In 2000, we used rAAV vaccine to generate autoantibodies that targeted a specific brain protein, the NR1 subunit of the *N*-methyl-*D*-aspartate (NMDA) receptor. After peroral administration of the rAAV vaccine, transgene expression persisted for at least 5 months and was associated with a robust humoral response in the absence of a significant cell-mediated response. This single-dose vaccine was associated with strong anti-epileptic and neuroprotective activity in rats

for both a kainate-induced seizure model and also a middle cerebral artery occlusion stroke model at 1 to 5 months following vaccination^[65]. In addition, we also provide evidence that oral administration of rAAV-TRAIL might be an important alternative route with practical significance for cancer gene therapy^[66]. More recently, a mucosal immunotherapy for Alzheimer's disease via oral vaccine with rAAV vector expressing Al 1-43 IL-10 induced the long-term expression of all in the epithelial cells and presented the Ap antigen to the mucosal immune system^[67]. Oral administration of rAAV A β reduced the accumulation of A β and attenuated cognitive and histological abnormalities in Tg2576 mice without lymphocytic infiltration or microhemorrhage in the brain^[68].

2.6.4 Cross/repeat administration Increased expression levels may be obtained by using alternative serotypes in combination with repeated administrations. Repeated administration demonstrated that increased gene transfer level was achieved with a second injection of rAAV1 following the first administration of rAAV2 or rAAV5. However rAAV1, rAAV2 or rAAV5 were inhibited when the animals were previously exposed to the same serotype^[30]. This phenomenon could be further confirmed by Sumner-Jones^[69], who reported that transduction efficiency as judged by reporter gene expression was markedly reduced on a second rAAV5/5 administration and effectively abolished on a third. In contrast, no significant change in gene expression from the second vector was observed in cross-administration. A humoral immune response was elicited against the viral capsid for all three serotypes following the initial exposure. Neutralizing antibody (NAB) levels were correlated with the vector dose injected. No significant cross-reactivity of NAB from a given serotype toward another was observed *in vitro*^[30].

2.7 Age and sex All transgenes administered by i. p. injection were more efficiently expressed in neonates than in adults^[8]. The expression was confined to the peritoneal tissue. Interestingly, a sex-related difference was observed in transgene expression in adults, whereas this difference was not apparent in neonates. But in another study, it was found the blood Phe concentrations decreased to normal levels (≤ 100 micromol or 1.7 mg/dL) 2 weeks after vector application, inde-

pendent of the sex of the PKU animals^[62]. When delivered alkaline phosphatase (AP) reporter gene via rAAV9 vector either in newborn or adult C57Bl/10 mice, it was observed that efficient transduction in multiple skeletal muscles and the heart, irrespective of the age or delivery route. Besides striated muscle, it was also found consistent high-level transduction in the lung. Abundant AP-positive cells were seen in alveolar cells and vasculature, but not in bronchioles. Interestingly, several organs demonstrated an age-dependent profile. In particular, the aorta, liver and kidney were preferentially transduced in adult mice while the inner layer of retina was strongly transduced only following the neonatal administration^[21].

3 CONCLUSION

In summary, the increasing discovery of new elements and construct of viral vectors, viral serotypes and variants continued provide us to develop suited gene therapy vectors for their diverse tissue tropisms and potential to quantitatively control gene expression and to evade preexisting neutralizing antibodies against immune response. Efforts to understand cell surface receptor usage and intracellular trafficking pathways exploited by viral vectors rAAV in particular, continue to provide significant insight into improving the outcome of clinical gene therapy. In this review, the elements of constructs, regulatable system, drug interaction, targeting, delivery route, age and sex etc have been well documented. In future, more attention should be paid to fine no/off switch of gene expression and to the immunity, insertion mutation and safety of the viral vectors.

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基因治疗用腺相关病毒载体的剂量效应与剂量控制

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摘要 人类基因治疗需要在靶细胞适时、适量、安全的产生具有疗效的药物, 这些正好是定量药理学研究范畴。重组腺相关病毒载体(rAAV)具有的各种特性以及近来在规模化制备方面取得的进展使其逐渐成为基因治疗的理想载体。超过60项以AAV为载体的基因治疗已经完成或正在展开。其中, rAAV载体的剂量与基因表达效率以及临床疗效之间的关系是研究者关注的焦点之一。多数研究认为, 在一定的剂量范围内, 基因表达水平具有剂量依赖性。然而, 基因表达水平同

时还受病毒载体构型、组织趋向性、细胞靶向性、注射途径, 甚至患者的年龄、性别等条件影响, 基因表达水平还可受到其他药物的调控。因此, 基因治疗实验过程中应结合定量药理学深入展开, 解决基因治疗中的定量与药效之间的关系。

关键词 基因治疗; 病毒载体; 剂量效应; 定量药理学; 临床治疗

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