Chapter 17

Efficient AAV Vector Production System: Towards Gene Therapy For Duchenne Muscular Dystrophy

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Choice of vector

Successful gene therapy requires an adequate level of long-term transgene expression in the target tissues. While various viral vectors have been considered for the delivery of genes *in vivo*, an adeno-associated virus (AAV)-based vector is emerging as the gene transfer vehicle with the most potential for use in the neuromuscular gene therapies. The advantages of the AAV vector include the lack of disease associated with a wild-type virus, the ability to transduce non-dividing cells, and the long-term expression of the delivered transgenes.[1] Some serotypes of recombinant AAV (rAAV) exhibit a potent tropism for striated muscles.[2] Therefore, a supplementation of secretory protein can be achieved with this vector to use intramuscular injection.[3] Since a 5-kb genome is considered to be the upper limit for a single AAV virion, various truncated genes could be provided to meet size capacity, if nessessarry.[4]

Due to ingenious cloning and preparation techniques, adenovirus vectors are efficient delivery systems of episomal DNA into eukaryotic cell nuclei.[5] The utility of adenovirus vectors has been increased by capsid modifications that alter tropism, and by the generation of hybrid vectors that promote chromosomal insertion.[6] Also, gutted adenovirus vectors devoid of all adenoviral genes allow for the insertion of large transgenes, and trigger fewer cytotoxic and immunogenic effects than do those only deleted in the E1 regions of the adenovirus early genes.[7] Human artificial chromosomes (HACs) have the capacity to deliver genes in any size into host cells without integrating the gene into the host genome, thereby preventing the possibility of insertional mutagenesis and genomic instability.[8]

Long-term correction of genetic diseases requires permanent integration of therapeutic genes into chromosomes of the affected cells. However, retrovirus vector integration can trigger deregulated premalignant cell proliferation with unexpected frequency, most likely driven by retrovirus enhancer activity on the LMO2 gene promoter. [9] A goal in clinical gene therapy is to develop gene transfer vehicles that can integrate exogenous therapeutic genes at specific chromosomal loci as a safe harbor, so that insertional oncogenesis is prevented. AAV can insert its genome into a specific locus, designated AAVS1, on chromosome 19 of the human genome.[10] The AAV Rep78/68 proteins and the Rep78/68-binding sequences are the trans- and cis-acting elements needed for this reaction. A dual high-capacity adenovirus-AAV hybrid vector with full-length human dystrophin-coding sequences flanked by AAV integration-enhancing elements was tested for targeted integration.[11]

1.2. AAV biology

AAV is a small (20-26nm) non-enveloped dependent parvovirus with a single-stranded linear genome that contains two open reading frames (*rep* and *cap*).[12] The viral genome is characterized by the inverted terminal repeats (ITRs) to flank these open reading frames (Figure 1A). The genome encodes four replication proteins (Rep78, Rep68, Rep52, and Rep40) and three capsid proteins (Cap: VP1, VP2, and VP3). The large Rep (Rep78 and Rep68) proteins regulate AAV gene expression and hold nicking activity at the terminal resolution site as well as binding activity at Rep binding elements to process AAV replication (Figure 1B). The small Rep proteins (Rep52 and Rep40) are used for the accumulation of single-stranded viral genome followed by packaging within AAV capsids.

The minimum sets of regions in helper adenovirus that mediate AAV vector replication are the E1, E2A, E4, and VA.[13] A human embryonic kidney cell line 293 encodes the E1 region of the Ad5 genome.[14] The helper plasmid assembling E2A, E4, and VA regions (Ad-helper plasmid) is cotransfected into the 293 cells, along with plasmids encoding the AAV vector genome (vector plasmid) as well as *rep* and *cap* genes (AAV-helper plasmid). AAV vector is produced as efficiently as when adenovirus infection is employed as a helper virus. Furthermore, contamination of most adenovirus proteins can be avoided in AAV vector stock made by this helper virus-free method.

1.3. Vector application using various serotypes

The preparation of AAV vector for gene therapy study of neuromuscular diseases is greatly facilitated. Although AAV2 has been the serotype most extensively studied in preclinical and clinical trials, recently we have focused on the use of AAV vectors pseudotyped with capsid protein of alternative serotypes. A number of primate AAV serotypes have been characterized in the literature and are designated. There is divergence in homology and tropism for various AAV serotypes. For instance, the homology with capsid protein is only about 60% between AAV2 and AAV5[15], therefore the capsid structure could be responsible for the improved transduction efficiency.

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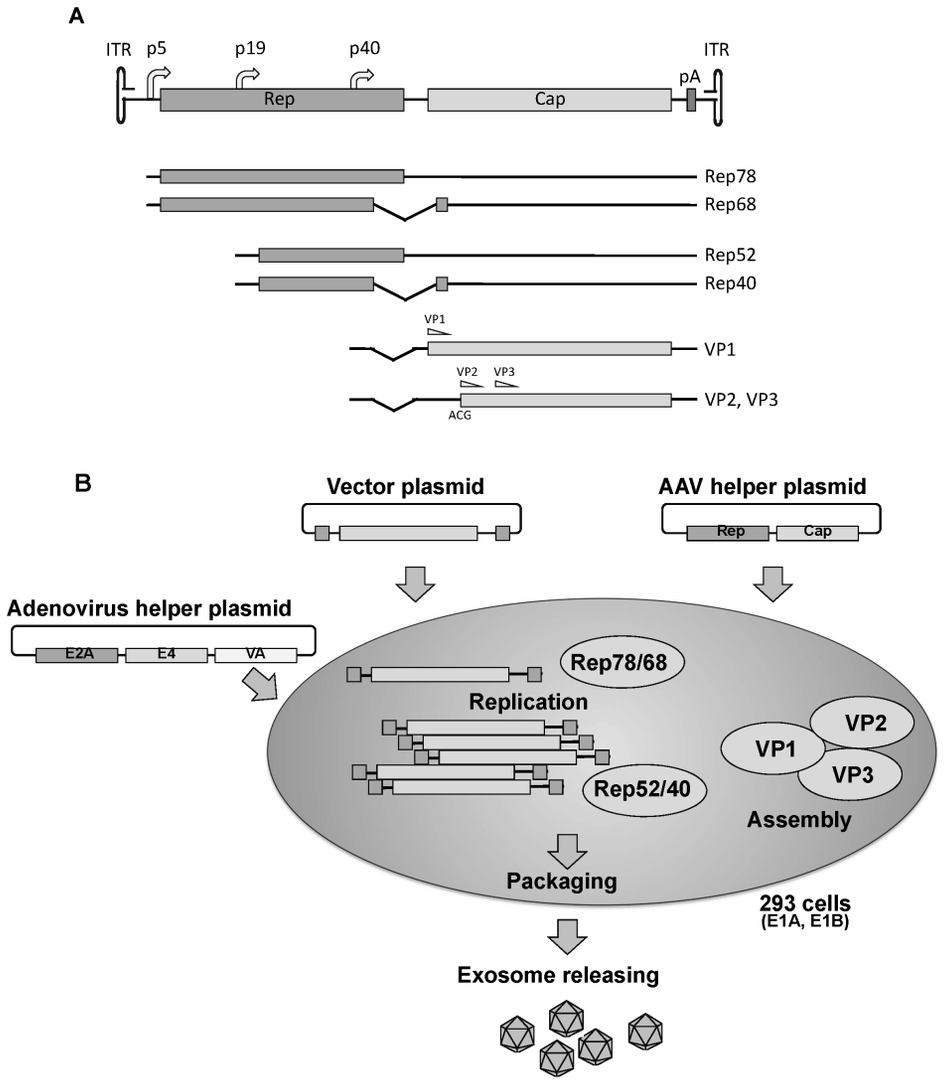

Figure 1. A) The *rep* and *cap* genes flanked by ITRs. The large Rep proteins (Rep78 and Rep68) are produced from transcripts using p5 promoter, while small Rep (Rep52 and Rep40) are produced from p19 promoter. (B) Recombinant AAV production. AAV has productive infection in the presence of adenovirus helper regions (E1, E2A, E4, and VA). This process is characterized by genome replication, assembly of the capsid proteins (VP1, VP2, and VP3), and packaging leading to virion production along with exosome releasing.

We found that choice of AAV serotypes and promoters could be quite useful for targeted transgene expression. For instance, the transgene expression of rAAV5 with the Rous sarcoma virus (RSV) promoter was preferentially found in the granular cells of the gerbil hippocampus, whereas transgene expression of rAAV2 with the RSV promoter was found in the pyramidal and granular cells.[16] Since AAV3 vector can specifically transduce cochlear inner hair cells with high efficiency *in vivo*, rAAV-mediated transduction might be promising for gene replacement strategies to correct recessive genetic hearing loss due to monogenic mutation.[17] Also, there is a significant difference in transgene expression by various AAV serotypes transduced into muscle. We observed that intramuscular injection of AAV5-IL-10 promoted a much higher serum level of secreted transgene product, as compared to AAV2-

mediated transfer.[18] We further demonstrated that AAV1 could more efficiently transduce the muscle than AAV5. Intramuscular single injection of modest doses of rAAV1 expressing IL-10 ($6x10^{10}$ g.c. per rat) introduced therapeutic levels of the transgene expression over the long-term to treat pulmonary arterial hypertension.[3] rAAV1-mediated sustained IL-10 expression also significantly ameliorated hypertensive organ damage to improve survival rate of Dahl salt-sensitive rats.[19] Furthermore, this protein supplementation therapy by rAAV1-mediated muscle transduction was quite effective to prevent vascular remodeling and end-organ damage in the stroke-prone spontaneously hypertensive rat.[20] Interestingly, alpha-sarcoglycan expression with single intramuscular injection of rAAV8 was widely distributed in the hind limb muscle as well as cardiac muscle, and persisted for 7 months with a reversal of the muscle pathology and improvement in the contractile force in the alpha-sarcoglycan-deficient mice.[21] Intravenous administration of rAAV8 into the hind limb in dogs resulted in improved transgene expression in the skeletal muscles lasting over a period of 8 weeks.[22] Moreover, rAAV9 would be administered systemically with excellent cardiac tropism.[23] Further strategies have been attempted to discover novel AAV capsid sequences from primate tissue, which can be used to develop newer-generation rAAVs with a greater diversity of tissue tropism for clinical gene therapy.

1.4. scAAV

Clinical gene therapy often requires rapid transduction with reasonable efficiency. In the case of AAV, second strand synthesis of the vector genome in the nucleus is the rate-limiting step for efficient transduction. Therefore, self-complementary AAV (scAAV) vector would be quite promising to promote efficient transduction regardless of DNA synthesis or annealing.[24] The scAAV vectors can bypass the inter-molecular annealing or second-strand synthesis by using intra-molecular annealing to immediately form transcriptionally active double-stranded DNA (Figure 2). Although immediate and efficient transduction could be observed with scAAV, the maximal insert size of the transgene cassette is reduced to 3.3 kb.[25]

2. Effective production strategies of rAAV

2.1. Principle of production

To gain acceptance as a medical treatment with a dose of over $1x10^{13}$ genome copies (g.c.)/kg body weight, therapeutic strategies with AAV vectors require a scalable and provident production method. However, the production and purification of recombinant virus stocks with conventional techniques entails cumbersome procedures not suited to the clinical setting. Therefore, development of effective large-scale culture and purification steps are required to meet end-product specifications.

A production protocol of AAV vectors in the absence of a helper virus[13] is widely employed for triple plasmid transduction of human embryonic kidney 293 cells.[1] The adenovirus regions that mediate AAV vector replication (namely, the VA, E2A and E4 regions) were assembled into a helper plasmid. When this helper plasmid is co-transfected into 293

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cells along with plasmids encoding the AAV vector genome and *rep-cap* genes, the AAV vector is produced as efficiently as when using adenovirus infection. Importantly, contamination of most adenovirus proteins can be avoided in AAV vector stock made by this helper virus-free method.

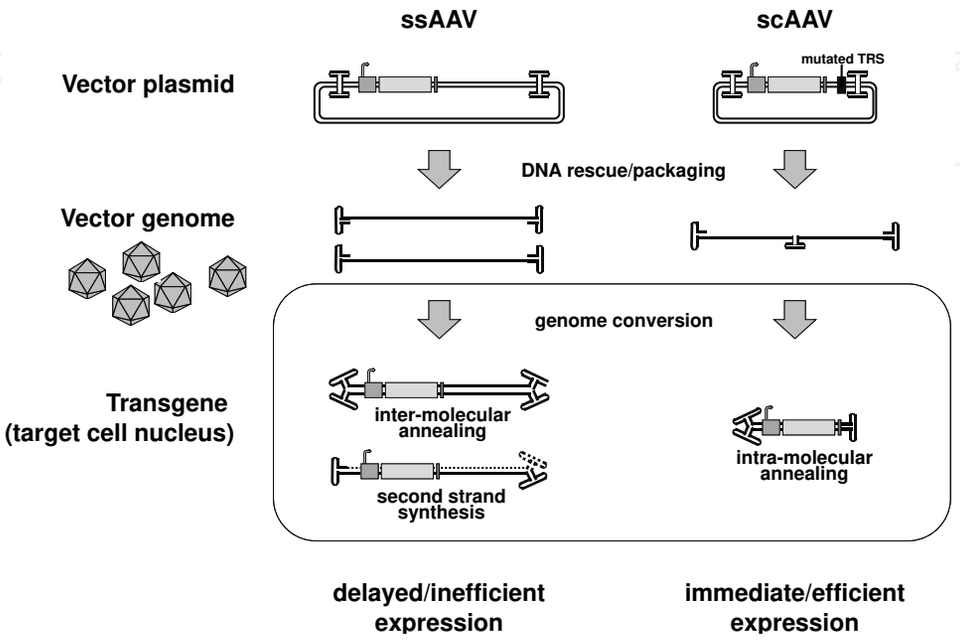

Figure 2. DNA rescue and transduction of a conventional single-stranded AAV (ssAAV) and a self-complementary AAV (scAAV) vector. Full-length ssAAV vector genome of both polarities are rescued from the vector plasmid and individually packaged into the AAV capsids. As a genome conversion in the transduced cell nucleus, the single-to-double stranded conversion of the DNA goes through the inter-molecular annealing or second strand synthesis. In contrast, a scAAV vector with half the size of the ssAAV genome has a mutation in the terminal resolution site (TRS) to form a vector genome with wild-type ITRs at the both ends and mutated ITR at the center of symmetry. After uncoating in the target cell nucleus, this DNA structure can readily fold into transcriptionally active double-stranded form through intra-molecular annealing.

Although various subtypes of the 293 cells harbor the E1 region of the adenovirus type 5 genome, to utilize a 293 cell stably expressing Bcl-xL (293B) has great advantage to support E1B19K function and protect cells from apoptosis.[26] Despite improvements in vector production, including the development of packaging cell lines expressing Rep/Cap or methods to regulate Rep/Cap,[27] maintaining such cell lines remains difficult, as the early expression of Rep proteins is toxic to cells.

We developed a large-scale transfection method of producing AAV vectors with an active gassing system that uses large culture vessels to process labor-effective transfection in a closed system.[28] This vector production system achieved reasonable production efficiency by improving gas exchange to prevent pH drop in the culture medium. Also, vector purification with the dual ion-exchange membrane adsorbers was effective and allowed higher levels of gene transfer *in vivo*.[29] Furthermore, the membrane adsorbers enabled the effective recovery of the AAV vector in the supernatant exosomes of the transduced cells culture.

This rapid and scalable viral purification protocol is particularly promising for considerable *in vivo* experimentation and clinical investigations (Figure 3).

Recent developments also suggest that AAV vector production in insect cells would be compatible with current good manufacturing practice production on an industrial scale.[30]

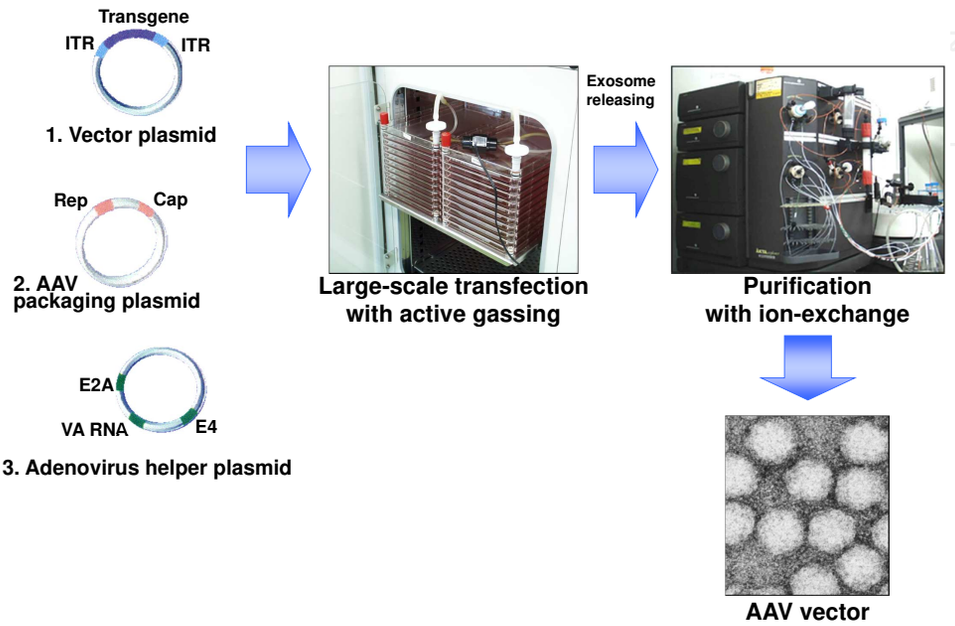

Figure 3. A scalable triple plasmid transfection using active gassing. When (1) a vector plasmid encoding the transgene cassette flanked by ITRs is co-transfected into human embryonic kidney 293 cells with (2) an AAV packaging plasmid harboring *rep-cap* genes and (3) an adenovirus helper plasmid, the AAV vector is produced as efficiently as when using adenovirus infection. A large-scale transduction method to produce AAV vectors with an active gassing system makes use of large culture vessels for labor- and cost-effective vector production in a closed system. Samples containing vector particles are further purified with a quick two-tier CsCl gradient centrifugation and an ion-exchange chromatography to obtain highly purified vector stocks.

2.2. Large-scale production with active gassing

Our protocol utilizes the transfection of 293B cells in one 10-Tray flask (CF10; Nalge Nunc International, Rochester, NY) with a surface area of 6320 cm^2 by using an active gassing at 500 ml/min. Typical transduction procedure is conducted with one or two CF10 to meet downstream purification protocol. Although previous protocols for recombinant virus production in a large culture vessel had the problem of insufficient transduction efficiency because of inadequate gas exchange, this method to use active gassing significantly improves productivity of the vectors and is linearly scalable from the small 225-cm^2 flask.[3]

The 293B cells are cultured in Dulbecco's modified Eagle's medium and Nutrient Mixture F-12 (D-MEM/F-12, Invitrogen, Grand Island, NY) with 10% fetal bovine serum (SIGMA-ALDRICH, St Louis, MO), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 incubator. Cells are initially plated at 8 x 10^7 cells per CF10 to achieve a monolayer of 20 to 40% confluency when cells attached to surface of the flask. The volume of medium

utilized per flask is 1120 ml. Subsequently, cells are grown for 48-72 h until reaching 70-90% confluence and are consequently transfected with appropriate triple plasmids. An aquarium pump (Nisso, Tokyo, Japan) should be used to circulate the gas through the CF10 with 5% CO_2 and humidity in an incubator.

Half of the medium in the CF10 tissue culture flask are exchanged with fresh D-MEM/F-12 containing 10% FBS, 1 h before transfection of the 293 cells. Subsequently, the cells are co-transfected with 650 μg of each plasmid: a proviral vector plasmid, an AAV helper plasmid, as well as an adenoviral helper plasmid, using calcium phosphate co-precipitation. Each plasmid was added to 112 ml of 300 mM $CaCl_2$. This solution was gently added to the same volume of 2 x HBS (290 mM NaCl, 50 mM HEPES buffer, 1.5 mM Na_2HPO_4, pH 7.0) and gently inverted 3 times to form a uniform solution. This solution was immediately mixed with fresh D-MEM/F-12 containing 10% FBS to produce a homogeneous plasmid solution mixture. Subsequently, the medium in the culture flask was replaced with this plasmid solution mixture. At the end of a 6-12 h incubation, the plasmid solution mixture in the culture flask was replaced with pre-warmed fresh D-MEM/F-12 containing 2% FBS.

2.3. Purification phase

The culture supernatant sample for the ion-exchange procedure is processed by centrifugation and filtration. The culture supernatant fluid 72-96 h after the transduction is sampled and then clarified with an appropriate amount of the activated charcoal (Wako Pure Chemical Industries, Osaka, Japan). Insoluble debris is removed by a centrifugation at 3,000 *g* for 15 min and filtration. The elucidated culture supernatant is enriched with a hollow fiber cross flow membrane (100,000 NMWC, GE Healthcare, Pittsburgh, PA). For the material obtained from a CF10, 5 mM $MgCl_2$ (final concentration) with 2,500-5,000 units of Benzonase nuclease is added to incubate for 30 min at 37 ^oC. Sequentially, 5 mM EDTA (final concentration) is added to terminate the reaction. Place 38 ml of the sample solution in a semi-sterile ultracentrifuge tube (Ultrabottle #3430-3870; Nalge Nunc, Rochester, NY) and remove the cell debris by centrifugation at 10,000*g* for 15 minutes at 4 ^oC to achieve cleared lysates. The sample is quickly concentrated by the brief two-tier CsCl (1.25 and 1.60 g/cm^3) step gradient centrifugation for 3 h and then the vector fraction is dialyzed in the MHA buffer (3.3 mM MES 3.3 mM HEPES [pH 8.0], 3.3 mM NaOAc).

Chromatography can be performed using an appropriate FPLC system, such as AKTA explorer 10S (Amersham Biosciences, Piscataway, NJ, USA) equipped with a 50 ml Superloop. The sample which passed through the Mustang™ S membrane (optional treatment, PALL corporation, NY) is dialyzed against MHA buffer and further loaded onto an anion-exchange membrane (acrodisc unit with Mustang™ Q membrane, PALL corporation, equilibrated with MHA buffer) at a rate of 3 ml/min. The membrane is then washed with 10 column volumes of MHA buffer. Bound virus on the Mustang™ Q membrane is eluted over a 50 column volume span with a 0-2 M linear NaCl gradient in MHA buffer and 0.5-1 ml fractions are collected. Recombinant rAAV particle number is determined by quantitative PCR of DNase I-treated stocks with plasmid standards. The final titer of the purified vectors

from a CF10 usually ranges around 5 x 10^{13} genome copies (g.c.), although it depends on the vector constructs and transgene.

3. AAV-mediated therapeutic approach to neuromuscular disease

3.1. DMD gene replacement therapy

Duchenne muscular dystrophy (DMD) is the most common form of childhood muscular dystrophy and is an X-linked recessive disorder with an incidence of one in 3500 live male births.[31] DMD causes progressive degeneration and regeneration of skeletal and cardiac muscles due to mutations in the *dystrophin* gene, which encodes a 427-kDa subsarcolemmal cytoskeletal protein.[32] DMD is associated with severe, progressive muscle weakness and typically leads to death between the ages of 20 and 35 years. Due to recent advances in respiratory care, much attention is now focused on treating the cardiac conditions suffered by DMD patients. The approximately 2.5-megabase *dystrophin* gene is the largest gene identified to date, and because of its size, it is susceptible to a high sporadic mutation rate. Absence of dystrophin and the dystrophin-glycoprotein complex (DGC) from the sarcolemma leads to severe muscle wasting. Whereas DMD is characterized by the absence of functional protein, Becker muscular dystrophy, which is commonly caused by in-frame deletions of the *dystrophin* gene, results in the synthesis of an incompletely functional protein.

Successful therapy for DMD requires the restoration of dystrophin protein in skeletal and cardiac muscles. While various viral vectors have been considered for the delivery of genes to muscle fibers, the AAV-based vector is emerging as an appropriate gene transfer vehicle with the most potential for use in DMD gene therapies. As for another candidate vehicle, the gutted adenovirus vector can package 14-kb of full-length *dystrophin* cDNA due to the large deletion in virus genome. Multiple proximal muscles of seven-day-old utrophin/dystrophin double knockout mice (*dko* mice), which typically show symptoms similar to human DMD, were effectively transduced with the gutted adenovirus bearing full-length murine *dystrophin* cDNA.[33] However, further improvements are needed to regulate the virus-associated host immune response before clinical trials can be performed.

A series of truncated *dystrophin* cDNAs containing rod repeats with hinge 1, 2, and 4 were constructed (Figure 4A).[4] Although AAV vectors are too small to package the full-length *dystrophin* cDNA, AAV vector-mediated gene therapy using a rod-truncated *dystrophin* gene provides a promising appraoch.[34] The structure and, particularly, the length of the rod are crucial for the function of micro-dystrophin.[35] An AAV type 2 vector expressing micro-dystrophin (DeltaCS1) under the control of a muscle-specific MCK promoter was injected into the tibialis anterior (TA) muscles of dystrophin-deficient *mdx* mice,[36] and resulted in extensive and long-term expression of micro-dystrophin that exhibited improved force generation. Likewise, AAV6 vector-mediated systemic *micro-dystrophin* gene transfer was effective in treating *dko* mice.[37] The potential for ameliorating the pathology of advanced-stage muscular dystrophy by systemic administration of AAV6 vectors encoding a micro-dystrophin expression construct was also demonstrated.[38] Furthermore, AAV9 vector-mediated

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micro-dystrophin transduction of *mdx* mice accomplished prevention of cardiac fibrosis as well as heart failure.[23] The transduction efficiency achieved with rAAV9 was nearly complete, with persistent expression for 74 weeks after transduction (Figure 4BC). Both the strong affinity of the rAAV9 for cardiac tissue and the therapeutic effect of the expressed micro-dystrophin might be involved in the prevention of the degeneration of the cardiomyocytes and cardiac fibrosis.

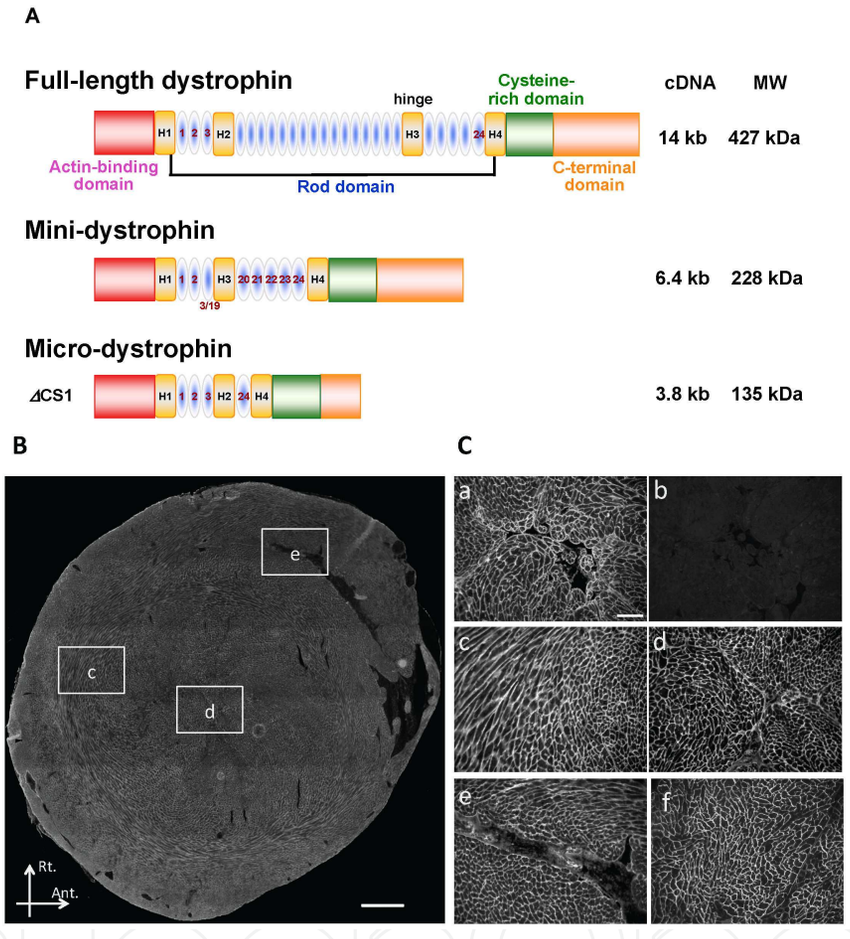

Figure 4. AAV9 vector-mediated *cardiac* transduction. (A) Structures of full-length and truncated dystrophin. Helper-dependent adenovirus vector can package 14-kb of full-length dystrophin cDNA because of the large-sized deletion in its genome. A mini-dystrophin is cloned from a patient with Becker muscular dystrophy, which is caused by in-frame deletions resulting in the synthesis of partially functional protein. A truncated micro-dystrophin cDNAs harboring only four rod repeats with hinge 1, 2, and 4 and a deleted C-terminal domain (delta CS1) is constructed to be packaged in the AAV vector. (B) Transverse section of *mdx* mouse heart at mid-ventricular level 24 weeks after transduction of *micro-dystrophin*, stained with anti-dystrophin antibody NCL-DysB. Scale bar, 500 μm. (C) Expression of dystrophin in C57BL10 hearts at the sarcolemma (a), while it is absent in *mdx* hearts (b). Magnified views of sections from the center of the left ventricle at 28 weeks (c-e) show micro-dystrophin expression in the areas indicated in B (scale bar, 100 μm). At 74 weeks after transduction, *mdx* mice still retain extensive expression of micro-dystrophin (f).

The impact of codon usage optimization on micro-dystrophin expression and function in the *mdx* mouse was demonstrated to compare the function of two different configurations of codon-optimized *micro-dystrophin* genes under the control of a muscle-restrictive promoter

(Spc5-12).[39] Codon optimization of micro-dystrophin significantly increased micro-dystrophin mRNA and protein levels after intramuscular and systemic administration of plasmid DNA or rAAV8. By randomly assembling myogenic regulatory elements into synthetic promoter recombinant libraries, several artificial promoters were isolated whose transcriptional potencies greatly exceed those of natural myogenic and viral gene promoters.[40]

3.2. Intravascular vector administration by limb perfusion

Although recent studies suggest that vectors based on AAV are capable of body-wide transduction in rodents,[21] translating the characteristics into large animals with advanced immune system remains a lot of challenges. Intravascular delivery can be performed as a form of limb perfusion, which might bypass the immune activation of DCs in the injected muscle.[41] We performed limb perfusion-assisted intravenous administration of rAAV8-lacZ into the hind limb of Beagle dogs (Figure 5A).[42] Administration of rAAV8 by limb perfusion demonstrated extensive transgene expression in the distal limb muscles of canine X-linked muscular dystrophy in Japan ($CXMD_J$) dogs without obvious immune responses for the duration of the experiment over four weeks after injection.

3.3. Systemic transduction and immunological issues

In comparison with fully dystrophin-deficient animals, targeted transgenic repair of skeletal muscle, but not cardiac muscle, paradoxically elicits a five-fold increase in cardiac injury and dilated cardiomyopathy.[43] Because the dystrophin-deficient heart is highly sensitive to increased stress, increased activity by the repaired skeletal muscle provides the stimulus for heightened cardiac injury and heart remodeling. In contrast, a single intravenous injection of AAV9 vector expressing micro-dystrophin efficiently transduces the entire heart in neonatal *mdx* mice, thereby ameliorating cardiomyopathy.[44]

Since a number of muscular dystrophy patients can be identified through newborn screening in future, neonatal transduction may lead to an effective early intervention in DMD patients. After a single intravenous injection, robust skeletal muscle transduction with AAV9 vector throughout the body was observed in neonatal dogs.[45] Systemic transduction was achieved in the absence of pharmacological intervention or immune suppression and lasted for at least six months, whereas rAAV9 was barely transduced into the cardiac muscle of dogs. Likewise, *in utero* gene delivery of full-length murine *dystrophin* to *mdx* mice using a high-capacity adenoviral vector resulted in effective protection from cycles of degeneration and regeneration.[46]

Neo-antigens introduced by AAV vectors evoke significant immune reactions in DMD muscle, since increased permeability of the DMD muscle allows leakage of the transgene products from the dystrophin-deficient sarcolemma of muscle fibers.[47] rAAV2 transfer into skeletal muscles of normal dogs resulted in low levels of transient expression, together with intense cellular infiltration, and the marked activation of cellular and humoral immune responses.[48] Furthermore, an *in vitro* interferon-gamma release assay showed that canine splenocytes respond to immunogens or mitogens more strongly than do murine splenocytes. Therefore, co-administration of immunosuppressants, cyclosporine (CSP) and myco-

phenolate mofetil (MMF) was attempted to improve rAAV2-mediated transduction. The AAV2 capsids can induce a cellular immune response via MHC class I antigen presentation with a cross-presentation pathway,[49] and rAAV2 could also stimulate human dendritic cells (DCs).[50] Whereas the non-immunogenic nature of AAV6 in murine studies, rAAV6 also elicited robust cellular immune responses in dogs.[51] In contrast, other serotypes, such as rAAV8, induce T-cell activation to a lesser degree.[42] The rAAV8-injected muscles showed lowed rates of infiltration of $CD4^+$ and $CD8^+$ T lymphocytes in the endomysium than the rAAV2-injected muscles.[42]

Resident antigen-presenting cells, such as DCs, myoblasts, myotubes and regenerating immature myofibers, should play a substantial role in the immune response against rAAV. Our study also showed that MyD88 and co-stimulating factors, such as CD80, CD86 and type I interferon, are up-regulated in both rAAV2- and rAAV8-transduced dog DCs (Figure 5B).[42]

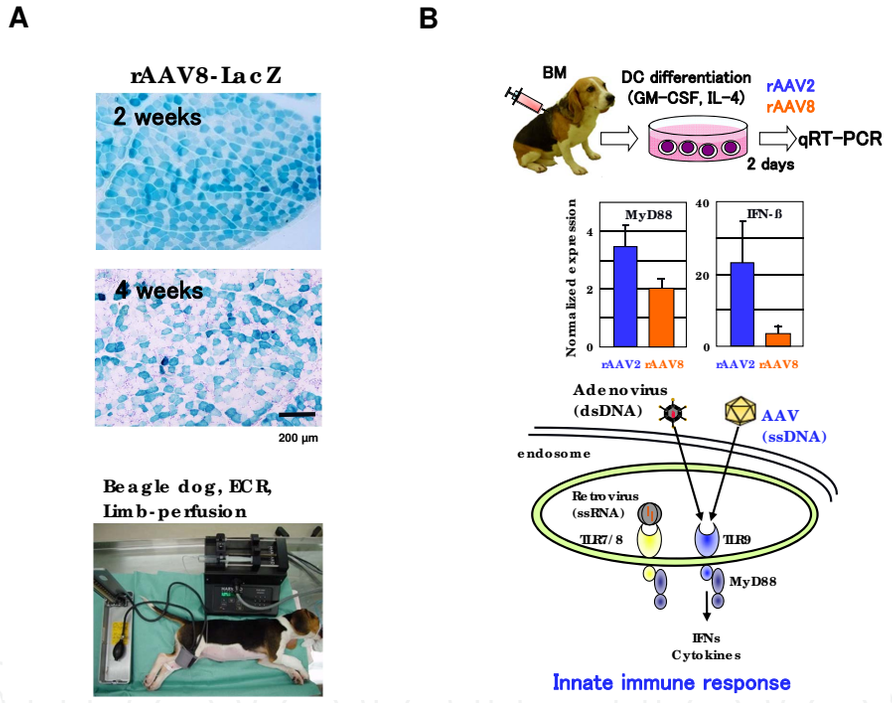

Figure 5. rAAV-mediated transduction of dog. (A) Intravascular vector administration by limb perfusion. A blood pressure cuff is applied just above the knee of an anesthetized $CXMD_J$ dog. A 24-gauge intravenous catheter is inserted into the lateral saphenous vein, connected to a three-way stopcock, and flushed with saline. With a blood pressure cuff inflated to over 300 mmHg, saline (2.6 ml/kg) containing papaverine (0.44 mg/kg, Sigma-Aldrich, St. Louis, MO) and heparin (16 U/kg) is injected by hand over a 10 second period. The three-way stopcock is connected to a syringe containing rAAV8 (1 x 10^{14} vg/kg, 3.8 ml/kg). The syringe is placed in a PHD 2000 syringe pump (Harvard Apparatus, Edenbridge, UK). Five minutes after the papaverine/heparin injection, rAAV8-LacZ is injected at a rate of 0.6 ml/sec. Two minutes after the rAAV injection, the blood pressure cuff is released and the catheter is removed. Four weeks after the transduction, the expression slightly fell off. (B) AAV-mediated stimulation of innate immune response via TLR9/MyD88 pathway. Bone marrow (BM)-derived dendritic cells (DCs) were obtained from humerus bones and cultured in RPMI (10% FCS, p/s) for 7 days with canine GM-CSF and IL-4. DCs were transduced with rAAV2- or rAAV8-*lacZ* (1x10^6 vg/cell for 4 hours, and mRNA levels of MyD88 and IFN-ß were analyzed. Untransduced cells were used as a normalization standard to demonstrate relative value of expression. Results are representative of two independent experiments. Error bars represent s.e.m., n = 3.

4. Safety and potential impact of clinical trials

4.1. Clinical trials for muscle transduction

While low immunogenicity was considered a major strength supporting the use of rAAV in clinical trials, a number of observations have recently provided a more balanced view of this procedure.[52] An obvious barrier to AAV transduction is the presence of circulating neutralizing antibodies that prevent the virion from binding to its cellular receptor.[53] This potential threat can be reduced by prescreening patients for AAV serotype-specific neutralizing antibodies or by performing therapeutic procedures such as plasmapheresis before gene transfer. Another challenge recently revealed is the development of a cell-mediated cytotoxic T-cell (CTL) response to AAV capsid peptides. In the human factor IX gene therapy trial in which rAAV was delivered to the liver, only short-term transgene expression was achieved and levels of therapeutic protein declined to baseline levels 10 weeks after vector infusion.[52] This was accompanied by elevation of serum transaminase levels and a CTL response toward specific AAV capsid peptides. To overcome this response, transient immunosuppression may be required until AAV capsids are completely cleared. Additional findings suggest that T-cell activation requires AAV2 capsid binding to the heparan sulfate proteoglycan (HSPG) receptor, which would permit virion shuttling into a DC pathway, as cross-presentation.[54] Exposure to vectors from other AAV clades, such as AAV8, did not activate capsid-specific T-cells.

The initial clinical studies lay the foundation for future studies, providing important information about vector dose, viral serotype selection, and immunogenicity in humans. The first virus-mediated gene transfer for muscle disease was carried out for limb-girdle muscular dystrophy type 2D using rAAV1. The study, consisting of intramuscular injection of virus into a single muscle, was limited in scope and the main conclusion was to establish the safety of this procedure in phase I clinical trials. The first clinical gene therapy trial for DMD began in March 2006.[55] This was a Phase I/IIa study in which an AAV vector was used to deliver micro-dystrophin to the biceps of boys with DMD. The study was conducted on six boys with DMD, each of whom received an injection of mini-dystrophin-expressing rAAV2.5 in a muscle of one arm and a placebo in the other arm. Dystrophin-specific T cells were detected after treatment, providing evidence of transgene expression even when the functional protein was not visualized in skeletal muscle.[56] The potential for T-cell immunity to self and non-self dystrophin epitopes should be considered in designing and monitoring experimental therapies for this disease. Basically, this issue is in common with the treatment of genetic diseases. Although concerns regarding risk of an immune response to the transgene product limited the ability to achieve therapeutic efficacy, rAAV2-mediated gene transfer to human skeletal muscle can persist for up to a decade.[57]

4.2. Gene therapy medicine

After more than two decades of expectations, the field of gene therapy appears close to reaching a regulatory approval by proposing rAAV-mediated muscle transduction. European medicine agency eventually recommends first gene therapy medicine for approval.

(http://www.ema.europa.eu/ema) The European Medicines Agency's Committee for Medicinal Products for Human Use has recommended the authorization of Glybera (rAAV1-expressing LPL S447X variant) for marketing in the European Union. It is intended to treat lipoprotein lipase deficiency in patients with severe or multiple pancreatitis attacks, despite dietary fat restrictions.

5. Challenges and future perspectives

5.1. Immunomodulation to augment clinical benefits

To regulate host immune response against vectors and transgene products, treatments involving immunosuppressants and other strategies have been attempted in the animal models. A brief course of immunosuppression with a combination of anti-thymocyte globulin (ATG), CSP and MMF was effective in permitting AAV6-mediated, long-term and robust expression of a canine micro-dystrophin in the skeletal muscle of a dog DMD model.[58] To establish the feasibility of multiple AAV1 injections for extending the treatment to whole body muscles, the dystrophic *mdx* mouse was repeatedly transduced with AAV1 vector, and the immune response was characterized.[59] By blocking the T-B crosstalk with anti-CD40 Abs and CTLA4/Fc fusion protein, a five-day-long immunomodulation treatment was found to be sufficient for totally abrogating the formation of anti-AAV1 antibodies.

There have been numerous reports to develop the therapeutic potential of mesenchymal stem cells (or mesenchymal multipotent stromal cells MSCs).[60] Because of their immunomodulatory properties, increasing experimental and early clinical observations indicate that allogeneic, and even xenogeneic, MSCs may be useful for tissue transplantation.[61] In fact, the immune tolerance with MSCs is well investigated in various animal studies. Infusion of syngeneic MSCs into a sensitized mouse model of kidney transplantation resulted in the expansion of donor-specific T- regulatory cells into lymphoid organs, prolonged allograft survival and promoted the development of tolerance.[62]

5.2. Pharmacological intervention

The use of a histone deacetylase (HDAC) inhibitor depsipeptide effectively enhances the utility of rAAV-mediated gene therapy.[63] In contrast to adenovirus-mediated transduction, the improved transduction with rAAV induced by the depsipeptide is due to enhanced transgene expression rather than to increased viral entry. The enhanced transduction is related to the histone-associated chromatin form of the rAAV concatemer in the transduced cells. Since various HDAC inhibitors are approved in clinical usage for many diseases to achieve therapeutic benefits, the application of such inhibitors to the rAAV-mediated gene therapy is theoretically and practically reasonable.

5.3. In situ gene therapy

Transplantation of genetically modified vector-producing cells is a possible future treatment for genetic diseases as an *in situ* gene therapy. MSCs are known to accumulate at the site of inflammation or tumors, and therefore can be utilized as a platform for the targeted delivery of therapeutic agents.[64] The MSCs-based targeted gene therapy should enhance the therapeutic efficacy, since MSCs would deliver therapeutic molecules in a concentrated fashion. This targeted therapy can also reduce systemic adverse side effects, because the reagents act locally without elevating their systemic concentrations. We developed the genetically-modified MSCs that produce viral vectors to augment therapeutic efficacy of systemic gene therapy.[65] MSCs isolated from the SD rats bone marrow were transfected with retroviral vector components by nucleofection. As a result, the injection of luciferase-expressing vector-producing MSCs caused significantly stronger signal of bioluminescence at the site of subcutaneous tumors in mice compared with luciferease-expressing non-vector-producing MSCs.[66] Furthermore, tumor-bearing nude mice were treated with the vector-producing MSCs combined with HSV-*tk*/GCV system to demonstrate improved anti-tumor effects. This study suggests the effectiveness of vector-producing MSCs in systemic gene therapy. The therapeutic benefit of this strategy should be further examined by using rAAV-producing MSCs in the various animal models of inflammatory diseases including neuromuscular disorders.

5.4. Capsid modification

A DNA shuffling-based approach for developing cell type-specific vectors is an intriguing possibility to achieve altered tropism. Capsid genomes of AAV serotypes 1-9 were randomly reassembled using PCR to generate a chimeric capsid library.[67] A single infectious clone (chimeric-1829) containing genome fragments from AAV1, 2, 8, and 9 was isolated from an integrin minus hamster melanoma cell line previously shown to have low permissiveness to AAV. Molecular modeling studies suggest that AAV2 contributes to surface loops at the icosahedral threefold axis of symmetry, while AAV1 and 9 contribute to two-fold and five-fold symmetry interactions, respectively.

A versatile rAAV targeting system to redirect rAAV-mediated transduction to specific cell surface receptors would be useful. Insertion of an IgG binding domain of protein A into the AAV2 capsid at amino acid position 587 could permit antibody-mediated vector retargeting, although producing mosaic particles is required to avoid low particle yields.[68] Alternatively, a targeting system using the genetic fusion of short biotin acceptor peptide along with the metabolic biotinylation via a biotin ligase was developed for the purification and targeting of multiple AAV serotypes.[69]

6. Conclusions and outlook

Although an increasing number of scalable methods for purification of rAAV have been described, in order to generate sufficient clinical-grade vector to support clinical trials we need to further improve a large-scale GMP-compatible system for production and purification. To

translate gene transduction technologies into clinical practice, development of an effective delivery system with improved vector constructs as well as efficient immunological modulation must be established. A novel protocol that considers all of these issues would help improve the therapeutic benefits of clinical gene therapy.

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References

[1] Okada, T., K. Shimazaki, T. Nomoto, T. Matsushita, H. Mizukami, M. Urabe, et al. (2002). "Adeno-associated viral vector-mediated gene therapy of ischemia-induced neuronal death." Methods Enzymol 346: 378-393.

[2] Inagaki, K., S. Fuess, T. A. Storm, G. A. Gibson, C. F. McTiernan, M. A. Kay, et al. (2006). "Robust systemic transduction with AAV9 vectors in mice: efficient global cardiac gene transfer superior to that of AAV8." Mol Ther 14: 45-53.

[3] Ito, T., T. Okada, H. Miyashita, T. Nomoto, M. Nonaka-Sarukawa, R. Uchibori, et al. (2007). "Interleukin-10 expression mediated by an adeno-associated virus vector prevents monocrotaline-induced pulmonary arterial hypertension in rats." Circ Res 101: 734-741.

[4] Yuasa, K., Y. Miyagoe, K. Yamamoto, Y. Nabeshima, G. Dickson and S. Takeda (1998). "Effective restoration of dystrophin-associated proteins in vivo by adenovirus-mediated transfer of truncated dystrophin cDNAs." FEBS Lett 425: 329-336.

[5] Okada, T., J. Ramsey, J. Munir, O. Wildner and M. Blaese (1998). "Efficient directional cloning of recombinant adenovirus vectors using DNA-protein complex." Nucleic Acids Res. 26: 1947-1950.

[6] Okada, T., N. J. Caplen, W. J. Ramsey, M. Onodera, K. Shimazaki, T. Nomoto, et al. (2004). "In situ generation of pseudotyped retroviral progeny by adenovirus-mediated transduction of tumor cells enhances the killing effect of HSV-tk suicide gene therapy in vitro and in vivo." J Gene Med 6: 288-299.

[7] Hammerschmidt, D. E. (1999). "Development of a gutless vector." J Lab Clin Med 134: C3.

[8] Hoshiya, H., Y. Kazuki, S. Abe, M. Takiguchi, N. Kajitani, Y. Watanabe, et al. (2008). "A highly Stable and Nonintegrated Human Artificial Chromosome (HAC) Containing the 2.4 Mb Entire Human Dystrophin Gene." Mol Ther.

[9] Hacein-Bey-Abina, S., A. Garrigue, G. P. Wang, J. Soulier, A. Lim, E. Morillon, et al. (2008). "Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1." J Clin Invest 118: 3132-3142.

[10] Kotin, R. M., R. M. Linden and K. I. Berns (1992). "Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by non-homologous recombination." Embo J 11: 5071-5078.

[11] Goncalves, M. A., G. P. van Nierop, M. R. Tijssen, P. Lefesvre, S. Knaan-Shanzer, I. van der Velde, et al. (2005). "Transfer of the full-length dystrophin-coding sequence into muscle cells by a dual high-capacity hybrid viral vector with site-specific integration ability." J Virol 79: 3146-3162.

[12] Srivastava, A., E. W. Lusby and K. I. Berns (1983). "Nucleotide sequence and organization of the adeno-associated virus 2 genome." J Virol 45: 555-564.

[13] Matsushita, T., S. Elliger, C. Elliger, G. Podsakoff, L. Villarreal, G. J. Kurtzman, et al. (1998). "Adeno-associated virus vectors can be efficiently produced without helper virus." Gene Ther 5: 938-945.

[14] Graham, F. L., J. Smiley, W. C. Russell and R. Nairn (1977). "Characteristics of a human cell line transformed by DNA from human adenovirus type 5." J Gen Virol 36: 59-74.

[15] Chiorini, J. A., F. Kim, L. Yang and R. M. Kotin (1999). "Cloning and characterization of adeno-associated virus type 5." J Virol 73: 1309-1319.

[16] Nomoto, T., T. Okada, K. Shimazaki, H. Mizukami, T. Matsushita, Y. Hanazono, et al. (2003). "Distinct patterns of gene transfer to gerbil hippocampus with recombinant adeno-associated virus type 2 and 5." Neuroscience Letters 340: 153-157.

[17] Liu, M., Y. Yue, S. Q. Harper, R. W. Grange, J. S. Chamberlain and D. Duan (2005). "Adeno-associated virus-mediated microdystrophin expression protects young mdx muscle from contraction-induced injury." Mol Ther 11: 245-256.

[18] Yoshioka, T., T. Okada, Y. Maeda, U. Ikeda, M. Shimpo, T. Nomoto, et al. (2004). "Adeno-associated virus vector-mediated interleukin-10 gene transfer inhibits atherosclerosis in apolipoprotein E-deficient mice." Gene Ther 11: 1772-1779.

[19] Nonaka-Sarukawa, M., T. Okada, T. Ito, K. Yamamoto, T. Yoshioka, T. Nomoto, et al. (2008). "Adeno-associated virus vector-mediated systemic interleukin-10 expression ameliorates hypertensive organ damage in Dahl salt-sensitive rats." J Gene Med 10: 368-374.

[20] Nomoto, T., T. Okada, K. Shimazaki, T. Yoshioka, M. Nonaka-Sarukawa, T. Ito, et al. (2009). "Systemic delivery of IL-10 by an AAV vector prevents vascular remodeling and end-organ damage in stroke-prone spontaneously hypertensive rat." Gene Ther 16: 383-391.

[21] Nishiyama, A., B. N. Ampong, S. Ohshima, J. H. Shin, H. Nakai, M. Imamura, et al. (2008). "Recombinant adeno-associated virus type 8-mediated extensive therapeutic gene delivery into skeletal muscle of alpha-sarcoglycan-deficient mice." Hum Gene Ther 19: 719-730.

[22] Ohshima, S., J. H. Shin, K. Yuasa, A. Nishiyama, J. Kira, T. Okada, et al. (2009). "Transduction Efficiency and Immune Response Associated With the Administration of AAV8 Vector Into Dog Skeletal Muscle." Mol Ther 17: 73-91.

[23] Shin, J. H., Y. Nitahara-Kasahara, H. Hayashita-Kinoh, S. Ohshima-Hosoyama, K. Kinoshita, T. Chiyo, et al. (2011). "Improvement of cardiac fibrosis in dystrophic mice by rAAV9-mediated microdystrophin transduction" Gene Ther. 18: 910-919.

[24] McCarty, D. M., P. E. Monahan and R. J. Samulski (2001). "Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis." Gene Ther 8: 1248-1254.

[25] Wu, J., W. Zhao, L. Zhong, Z. Han, B. Li, W. Ma, et al. (2007). "Self-complementary recombinant adeno-associated viral vectors: packaging capacity and the role of rep proteins in vector purity." Hum Gene Ther 18: 171-182.

[26] Yamaguchi, T., T. Okada, K. Takeuchi, T. Tonda, M. Ohtaki, S. Shinoda, et al. (2003). "Enhancement of thymidine kinase-mediated killing of malignant glioma by BimS, a BH3-only cell death activator." Gene Ther 10: 375-385.

[27] Okada, T., H. Mizukami, M. Urabe, T. Nomoto, T. Matsushita, Y. Hanazono, et al. (2001). "Development and characterization of an antisense-mediated prepackaging cell line for adeno-associated virus vector production." Biochem Biophys Res Commun 288: 62-68.

[28] Okada, T., T. Nomoto, T. Yoshioka, M. Nonaka-Sarukawa, T. Ito, T. Ogura, et al. (2005). "Large-scale production of recombinant viruses by use of a large culture vessel with active gassing." Hum Gene Ther 16: 1212-1218.

[29] Okada, T., M. Nonaka-Sarukawa, R. Uchibori, K. Kinoshita, H. Hayashita-Kinoh, Y. Nitahara-Kasahara, et al. (2009). "Scalable purification of adeno-associated virus sero-

type 1 (AAV1) and AAV8 vectors, using dual ion-exchange adsorptive membranes." Hum Gene Ther 20: 1013-1021.

[30] Cecchini, S., A. Negrete and R. M. Kotin (2008). "Toward exascale production of recombinant adeno-associated virus for gene transfer applications." Gene Ther 15: 823-830.

[31] Emery, A. E. (1991). "Population frequencies of inherited neuromuscular diseases--a world survey." Neuromuscul Disord 1: 19-29.

[32] Hoffman, E. P., R. H. Brown, Jr. and L. M. Kunkel (1987). "Dystrophin: the protein product of the Duchenne muscular dystrophy locus." Cell 51: 919-928.

[33] Kawano, R., M. Ishizaki, Y. Maeda, Y. Uchida, E. Kimura and M. Uchino (2008). "Transduction of full-length dystrophin to multiple skeletal muscles improves motor performance and life span in utrophin/dystrophin double knockout mice." Mol Ther 16: 825-831.

[34] Wang, B., J. Li and X. Xiao (2000). "Adeno-associated virus vector carrying human minidystrophin genes effectively ameliorates muscular dystrophy in mdx mouse model." Proc Natl Acad Sci U S A 97: 13714-13719.

[35] Sakamoto, M., K. Yuasa, M. Yoshimura, T. Yokota, T. Ikemoto, M. Suzuki, et al. (2002). "Micro-dystrophin cDNA ameliorates dystrophic phenotypes when introduced into mdx mice as a transgene." Biochem Biophys Res Commun 293: 1265-1272.

[36] Yoshimura, M., M. Sakamoto, M. Ikemoto, Y. Mochizuki, K. Yuasa, Y. Miyagoe-Suzuki, et al. (2004). "AAV vector-mediated microdystrophin expression in a relatively small percentage of mdx myofibers improved the mdx phenotype." Mol Ther 10: 821-828.

[37] Gregorevic, P., J. M. Allen, E. Minami, M. J. Blankinship, M. Haraguchi, L. Meuse, et al. (2006). "rAAV6-microdystrophin preserves muscle function and extends lifespan in severely dystrophic mice." Nat Med 12: 787-789.

[38] Gregorevic, P., M. J. Blankinship, J. M. Allen and J. S. Chamberlain (2008). "Systemic microdystrophin gene delivery improves skeletal muscle structure and function in old dystrophic mdx mice." Mol Ther 16: 657-664.

[39] Foster, H., P. S. Sharp, T. Athanasopoulos, C. Trollet, I. R. Graham, K. Foster, et al. (2008). "Codon and mRNA sequence optimization of microdystrophin transgenes improves expression and physiological outcome in dystrophic mdx mice following AAV2/8 gene transfer." Mol Ther 16: 1825-1832.

[40] Li, X., E. M. Eastman, R. J. Schwartz and R. Draghia-Akli (1999). "Synthetic muscle promoters: activities exceeding naturally occurring regulatory sequences." Nat Biotechnol 17: 241-245.

[41] Hagstrom, J. E., J. Hegge, G. Zhang, M. Noble, V. Budker, D. L. Lewis, et al. (2004). "A facile nonviral method for delivering genes and siRNAs to skeletal muscle of mammalian limbs." Mol Ther 10: 386-398.

[42] Ohshima, S., J. H. Shin, K. Yuasa, A. Nishiyama, J. Kira, T. Okada, et al. (2008). "Transduction Efficiency and Immune Response Associated With the Administration of AAV8 Vector Into Dog Skeletal Muscle." Mol Ther.

[43] Townsend, D., S. Yasuda, S. Li, J. S. Chamberlain and J. M. Metzger (2008). "Emergent dilated cardiomyopathy caused by targeted repair of dystrophic skeletal muscle." Mol Ther 16: 832-835.

[44] Bostick, B., Y. Yue, Y. Lai, C. Long, D. Li and D. Duan (2008). "Adeno-associated virus serotype-9 microdystrophin gene therapy ameliorates electrocardiographic abnormalities in mdx mice." Hum Gene Ther 19: 851-856.

[45] Yue, Y., A. Ghosh, C. Long, B. Bostick, B. F. Smith, J. N. Kornegay, et al. (2008). "A single intravenous injection of adeno-associated virus serotype-9 leads to whole body skeletal muscle transduction in dogs." Mol Ther 16: 1944-1952.

[46] Reay, D. P., R. Bilbao, B. M. Koppanati, L. Cai, T. L. O'Day, Z. Jiang, et al. (2008). "Full-length dystrophin gene transfer to the mdx mouse in utero." Gene Ther 15: 531-536.

[47] Yuasa, K., M. Sakamoto, Y. Miyagoe-Suzuki, A. Tanouchi, H. Yamamoto, J. Li, et al. (2002). "Adeno-associated virus vector-mediated gene transfer into dystrophin-deficient skeletal muscles evokes enhanced immune response against the transgene product." Gene Ther 9: 1576-1588.

[48] Yuasa, K., M. Yoshimura, N. Urasawa, S. Ohshima, J. M. Howell, A. Nakamura, et al. (2007). "Injection of a recombinant AAV serotype 2 into canine skeletal muscles evokes strong immune responses against transgene products." Gene Ther.

[49] Li, C., M. Hirsch, A. Asokan, B. Zeithaml, H. Ma, T. Kafri, et al. (2007). "Adeno-associated virus type 2 (AAV2) capsid-specific cytotoxic T lymphocytes eliminate only vector-transduced cells coexpressing the AAV2 capsid in vivo." J Virol 81: 7540-7547.

[50] Zhang, Y., N. Chirmule, G. Gao and J. Wilson (2000). "CD40 ligand-dependent activation of cytotoxic T lymphocytes by adeno-associated virus vectors in vivo: role of immature dendritic cells." J Virol 74: 8003-8010.

[51] Wang, Z., J. M. Allen, S. R. Riddell, P. Gregorevic, R. Storb, S. J. Tapscott, et al. (2007). "Immunity to adeno-associated virus-mediated gene transfer in a random-bred canine model of Duchenne muscular dystrophy." Hum Gene Ther 18: 18-26.

[52] Manno, C. S., G. F. Pierce, V. R. Arruda, B. Glader, M. Ragni, J. J. Rasko, et al. (2006). "Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response." Nat Med 12: 342-347.

[53] Scallan, C. D., H. Jiang, T. Liu, S. Patarroyo-White, J. M. Sommer, S. Zhou, et al. (2006). "Human immunoglobulin inhibits liver transduction by AAV vectors at low AAV2 neutralizing titers in SCID mice." Blood 107: 1810-1817.

[54] Vandenberghe, L. H., L. Wang, S. Somanathan, Y. Zhi, J. Figueredo, R. Calcedo, et al. (2006). "Heparin binding directs activation of T cells against adeno-associated virus serotype 2 capsid." Nat Med 12: 967-971.

[55] Rodino-Klapac, L. R., L. G. Chicoine, B. K. Kaspar and J. R. Mendell (2007). "Gene therapy for duchenne muscular dystrophy: expectations and challenges." Arch Neurol 64: 1236-1241.

[56] Mendell, J. R., K. Campbell, L. Rodino-Klapac, Z. Sahenk, C. Shilling, S. Lewis, et al. (2010). "Dystrophin immunity in Duchenne's muscular dystrophy." N Engl J Med 363: 1429-1437.

[57] Buchlis, G., G. M. Podsakoff, A. Radu, S. M. Hawk, A. W. Flake, F. Mingozzi, et al. (2012). "Factor IX expression in skeletal muscle of a severe hemophilia B patient 10 years after AAV-mediated gene transfer." Blood 119: 3038-3041.

[58] Wang, Z., C. S. Kuhr, J. M. Allen, M. Blankinship, P. Gregorevic, J. S. Chamberlain, et al. (2007). "Sustained AAV-mediated Dystrophin Expression in a Canine Model of Duchenne Muscular Dystrophy with a Brief Course of Immunosuppression." Mol Ther 15: 1160-1166.

[59] Lorain, S., D. A. Gross, A. Goyenvalle, O. Danos, J. Davoust and L. Garcia (2008). "Transient immunomodulation allows repeated injections of AAV1 and correction of muscular dystrophy in multiple muscles." Mol Ther 16: 541-547.

[60] Nitahara-Kasahara, Y., H. Hayashita-Kinoh, S. Ohshima-Hosoyama, H. Okada, M. Wada-Maeda, A. Nakamura, et al. (2012). "Long-term engraftment of multipotent mesenchymal stromal cells that differentiate to form myogenic cells in dogs with Duchenne muscular dystrophy." Mol Ther 20: 168-177.

[61] Chiu, R. C. (2008). "MSC immune tolerance in cellular cardiomyoplasty." Semin Thorac Cardiovasc Surg 20: 115-118.

[62] Casiraghi, F., N. Azzollini, M. Todeschini, R. A. Cavinato, P. Cassis, S. Solini, et al. (2012). "Localization of Mesenchymal Stromal Cells Dictates Their Immune or Proinflammatory Effects in Kidney Transplantation." Am J Transplant. (in press)

[63] Okada, T., R. Uchibori, M. Iwata-Okada, M. Takahashi, T. Nomoto, M. Nonaka-Sarukawa, et al. (2006). "A histone deacetylase inhibitor enhances recombinant adeno-associated virus-mediated gene expression in tumor cells." Mol Ther 13: 738-746.

[64] Studeny, M., F. C. Marini, J. L. Dembinski, C. Zompetta, M. Cabreira-Hansen, B. N. Bekele, et al. (2004). "Mesenchymal stem cells: potential precursors for tumor stroma and targeted-delivery vehicles for anticancer agents." J Natl Cancer Inst 96: 1593-1603.

[65] Okada, T. and K. Ozawa (2008). "Vector-producing tumor-tracking multipotent mesenchymal stromal cells for suicide cancer gene therapy." Front Biosci 13: 1887-1891.

[66] Uchibori, R., T. Okada, T. Ito, M. Urabe, H. Mizukami, A. Kume, et al. (2009). "Retroviral vector-producing mesenchymal stem cells for targeted suicide cancer gene therapy." J Gene Med 11: 373-381.

[67] Li, W., A. Asokan, Z. Wu, T. Van Dyke, N. DiPrimio, J. S. Johnson, et al. (2008). "Engineering and selection of shuffled AAV genomes: a new strategy for producing targeted biological nanoparticles." Mol Ther 16: 1252-1260.

[68] Muzyczka, N. and K. H. Warrington, Jr. (2005). "Custom adeno-associated virus capsids: the next generation of recombinant vectors with novel tropism." Hum Gene Ther 16: 408-416.

[69] Arnold, G. S., A. K. Sasser, M. D. Stachler and J. S. Bartlett (2006). "Metabolic biotinylation provides a unique platform for the purification and targeting of multiple AAV vector serotypes." Mol Ther 14: 97-106.

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