During the experiment of neural induction, the order of treatment was just reversed for the control experiment (Fig.1). Again, the surprising phenomenon of muscle differentiation, small number of slender cells containing two to three nuclei, could be recognized in the culture dish. Considering the advantages of MSCs, this phenomenon was expected to develop the large-scale induction system of skeletal muscle cells from patient's own MSCs. Thus, the induction experiment was repeated, and finally a new method to systematically and efficiently induce skeletal muscle lineage cells with high purity from large population of MSCs was established [23].

## II. Induction systems of skeletal muscle cells from MSC

Human and rat MSCs were passaged at least for three times, and then plated on plastic dishes at 1,700~1,900 cells/cm$^2$. They were first treated with the trophic factors bFGF, FSK, platelet-derived growth factor (PDGF) and neuregulin for three days. After this treatment (C-MSCs), Pax7 expression could be recognized in MSCs (Fig.2). They were then transfected with a plasmid expression vector containing constitutive active form of Notch gene (The mouse Notch1 intracellular domain (NICD) cDNA was subcloned into

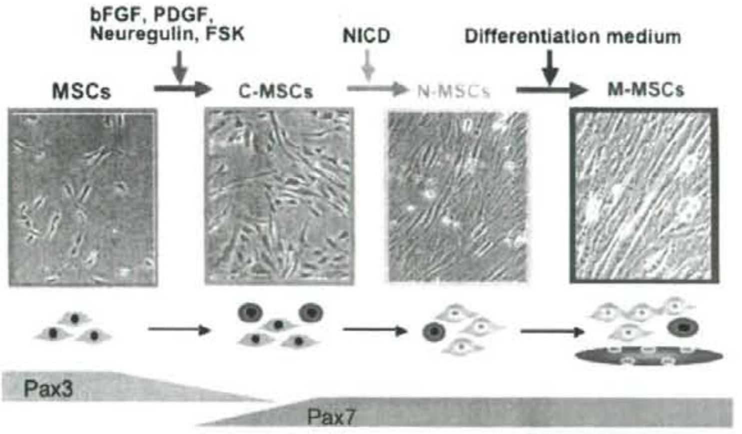

**Figure 2.** Induction of skeletal muscle cells from MSCs (human). MSCs originally express Pax3 become positive to Pax7 after trophic factor stimulation (C-MSCs). NICD transfection induced MyoD- and myogenin expression in N-MSCs. These N-MSCs fuse to form multinucleated myotubes by differentiation medium, expressing the marker of maturity, such as myosin heavy chain (MHC).

pCI-neo, a cytomegalovirus promoter-containing mammalian expression vector) by lipofection followed by G418 selection, and allowed to recover to 100% confluency. At this stage (N-MSCs), a large majority of MSCs developed into mononucleated myogenic cells expressing MyoD and myogenin, while a small population of Pax7 (+) satellite-like cells also existed (Fig.2). Cells were then supplied with a differentiation medium of either 2% horse serum, Insulin-Transferrin-Selenite (ITS)-serum free medium or the supernatant of the original untreated MSCs [23], and the final muscle lineage population (M-MSCs) was acquired (Fig.2). M-MSCs contained three kinds of muscle-lineage cells. The first population included post-mitotic multinucleated myotubes, which expressed myogenin, Myf6/MRF4 (a marker for mature skeletal muscle) and contractile proteins of skeletal myosin, myosin heavy chain, and troponin, all related to skeletal muscle characteristics. In fact, some multinucleated cells exhibited spontaneous contraction *in vitro*. They are also positive for p21, a marker for post-mitotic muscle lineage cells. The second group was mononucleated myoblasts which expressed MyoD and myogenin. The third group was composed of satellite-like cells and were immunopositive for Pax7 and c-Met, both markers for muscle satellite cells [23].

However, it is critical to determine if these MSC-derived skeletal muscle cells integrate into the host tissue and are genuine muscle cells. In the following sections, the effectiveness of these induced cells is verified by a transplantation experiment using animal models of muscle degeneration and dystrophy.

# III. Mechanism of induction

To examine the induction events leading from MSCs to M-MSCs, we investigated the expression of genes related to myogenesis in these cells by RT-PCR [23]. Before trophic factor treatment, MSCs expressed Pax3, Six1 and Six4 while Pax7, MyoD and myogenin were not. After treatment with trophic factors bFGF, FSK, PDGF and neuregulin (C-MSCs), Pax3 was down-regulated instead Pax7 expression was recognized which persisted after NICD introduction (N-MSCs) and final population of M-MSCs. Expression of MyoD and myogenin was firstly detectable in N-MSCs and persisted in the M-MSCs. These results were also confirmed by Western analyses. Myf6/MRF4, a marker for mature skeletal muscle, was detectable only in the final MSC-M population. While expression of Six1 and Six4 persisted for the entire period, another myogenic factor, myf5 was not detected in any induction step. In this way, the induction process mimicked some aspects of conventional skeletal muscle development since Pax3, Pax7, MyoD, Myogenin and Myf6/MRF4, all of which are related to muscle development [30-33], could be detected in a sequential manner. However, as MSCs used in this induction system possess different characteristics from the conventional myogenic progenitor cells, it is possible that some of mechanisms should differ, especially in the initial step converting MSCs to MyoD-positive N-MSC population. For this initial step, cytokine pre-treatment and the subsequent NICD transfection are critical and required for MSC-derived cells to acquire competence for myogenic induction. In fact, when we reversed the order of cytokine treatment and NICD transfection, muscle-lineage markers were not detected nor were multinucleated cells observed.

It is well established that Notch signaling inhibits myogenic differentiation; Deltal/Jagged1 inhibits MyoD expression, blocks the differentiation of myoblasts, and prevents the formation of myotubes [34, 35]. Hes 1/5, downstream effectors of Notch, are reported unrelated to the inhibition of the myogenic pathway in C2C12 myoblasts, while others report that Hes1 up-regulation results in the prevention of myogenesis [36, 37].

We examined the expression of Hes family members to judge whether conventional Notch pathway was activated in our induction process [38-40]. The expression of Hes 1/5 was not significantly upregulated by NICD transfection (N-MSCs). The forced expression of Hes 1/5 in place of NICD failed to induce skeletal muscle lineage cells, suggesting that Hes 1/5 signaling is not involved in the muscle induction event in MSCs. Hes 6, another Hes family member known to induce the myogenic differentiation program, was slightly up-regulated, while muscle induction by the forced expression of Hes6 in place of NICD could barely elicit muscle lineage cells.

In our induction system, NICD transfection up-regulated MyoD while it has been shown to inhibit myogenic differentiation in cultured muscle cells and in the embryo [34, 35]. We re-expressed NICD in rat N-MSCs and analyzed MyoD expression. N-MSCs were transfected with pCI-neo-NICD by lipofection, followed by G418 selection, and were brought to RT-PCR. Interestingly, the down-regulation of MyoD was recognized after re-expression of NICD in N-MSCs as well as in C2C12 cells. Furthermore, after the re-expression of NICD, cells were subjected to differentiation medium containing 2% horse serum to analyze myotube formation. The differentiation into multinucliated myotubes was significantly suppressed by re-expression of NICD in N-MSCs as well as C2C12 cells. These results collectively suggest that cellular response to NICD in MSCs is different from that of conventional myogenic progenitor cells, but once they differentiate into myogenic lineage cells by this induction system, they behave like real myogenic cells such as C2C12 cells [34, 35].

Our results showing that NICD introduction accelerates the induction of skeletal muscle cells from MSCs are surprising from the viewpoint of conventional Notch signaling in myogenesis. We consider our results do not refute the known role of Notch-Hes signals in myogenesis, but rather reflecting the distinct cellular responses of MSCS to Notch signals; for example, the repertoire of proteins, second messengers and other active factors may well be quite different between conventional myogenic progenitor cells and MSCs. Notably, as described above, we observed the induction of neuronal cells from MSCs by NICD introduction. A yet unknown signaling pathway downstream of Notch may be involved in these events. Further studies are nevertheless needed to identify the factor involved in this phenomenon.

Bone marrow (mostly hematopoietic cells) contains a small population of myogenic stem cells known to express c-Kit, CD45 and CD34 [1-3, 7, 41, 42]. Hematopoietic cells are generally non-adherent and cells we used were adherent MSCs. However, even though we used adherent MSCs, several percent of cells are positive to above markers. To exclude the possibility that the production of muscle-lineage cells was due to the vast proliferation of myogenic stem cells contained in MSCs, human MSCs negative for c-Kit, CD45 and CD34 were isolated by FACS and subjected to the induction process [43]. We confirmed that isolated cells could also be driven to be muscle-lineage cells as efficiently as the unsorted MSCs. Therefore, in our system, it appears that it is not a small fraction of bone-marrow-derived myogenic stem cells, but rather the major population of MSCs contribute to the production of muscle lineage cells.

## IV. Application of M-MSCs to muscle degenerative disease model

As induced multinucleated myotubes in M-MSCs are already post-mitotic, single cells of MyoD-positive myoblasts and Pax7-positive satellite cells were subjected to clonal culture (clonal M-MSCs) to exclude non-muscle cells and transplanted into muscle degenerative disease models [43]. To estimate how workable these clonally-cultured M-MSCs are in the repair of degenerated muscles, human cells were transplanted into immunosuppressed rats whose gastrocnemius muscles were damaged with cardiotoxin pretreatment [43]. Cells were labeled by means of a GFP-encoding retrovirus and then transplanted by local injection (L.I.) into muscles or by intravenous injection (I.V.). Two weeks after transplantation, GFP-labeled cells incorporated into newly formed immature myofibers, exhibited centrally located nuclei in both L.I. and I.V. treated animals. The ratio (%) of GFP (+) fibers in total fibers (1500 fibers with centrally located nuclei were counted for each sample) was 37.1±9.9 % in L.I. and 22.6±7.9 % in I.V. Four weeks after transplantation, GFP-positive myofibers exhibited mature characteristics with peripheral nuclei just beneath the plasma membrane. Functional differentiation of grafted human cells was also confirmed by the detection of human dystrophin in GFP-labeled myofibers. These findings indicate that clonal-M-MSCs are able to incorporate into damaged muscles and contribute to regenerating myofiber formation, regardless of the transplantation method [43].

Clonal M-MSCs contained Pax7-positive satellite cells which integrated into the satellite cell position after transplantation, namely the plasma membrane and the basal lamina inbetween [43]. The ratio of Pax7/GFP (+) cells in total Pax7-positive cells at 2 weeks was 17.2±4.2 % in L.I. and 5.9±2.8 % in I.V. In general, muscle satellite cells are known to contribute to the regeneration of myofiber formation upon muscle damage [44]. To confirm the contribution of transplanted satellite cells to muscle regeneration as *in vivo* satellite cells, the following experiment was performed. Four weeks after the initial transplantation of human clonal-M-MSCs intraveneously, cardiotoxin was re-administered into the same muscles without additional transplantation. Two weeks after the second cardiotoxin treatment (6 weeks after initial transplantation), many regenerating GFP-positive myofibers with centrally-located nuclei were observed. This implies that, upon transplantation of clonal-M-MSCs to the muscles of patients, those retained as satellite cells should be able to contribute to future muscle regeneration [23].

Transplantation of muscle lineage cells is a potential therapeutic approach for muscle degenerative disorders such as Duchenne muscular dystrophy (DMD), a severe progressive muscle wasting disease that results from a mutation in the dystrophin gene. The *mdx*-mouse, an animal model for DMD, was used for this experiment. The *mdx*-mouse is characterized by the absence of the muscle membrane associated protein, dystrophin. We locally injected GFP-labeled human clonal-M-MSCs into cardiotoxin-pretreated muscles of *mdx*-nude mice. Immunohistochemistry revealed the incorporation of transplanted cells into newly formed myofibers which expressed human dystrophin after transplantation as same as in case of above rat experiment [23].

## V. Perspective

Cell transplantation therapy also offers hope for the treatment of intractable muscle degenerative disorders. Indeed, ES cells, stem cells derived from adult and prenatal muscle tissues, and myogenic stem cells from bone marrow are powerful candidates for transplantation therapy [1-5, 41]. Compared to these sources, the MSC system offers several important advantages. Firstly, our induction system does not depend on a rare stem cell population, but can utilize the general population of adherent MSCs, which can be easily isolated and expanded. MSCs provide hopeful possibilities for clinical application, since they can efficiently expand *in vitro* and a therapeutic scale of induced cells are available. Thus functional skeletal muscle cells can be obtained within a reasonable time course on a therapeutic scale. Secondary, transplantation of MSC-derived cells should pose fewer ethical problems than ES cells and other kinds of stem cells, since bone marrow transplantation has already been widely performed. Hopefully, this MSC differentiation system may contribute substantially to eventual cell-based therapies for muscle disease.

Transplantation of untreated MSCs is reported to be effective to various kinds of degenerative models. In these reports, MSCs or cells derived from bone marrow are sometimes observed to penetrate into host tissue and thereby differentiate as mature neurons and skeletal muscle cells and so on [45, 46]. However, the ratio of so called "spontaneous differentiation" or "transdifferentiation" is extremely low and thus cannot be expected to the clinical application. Rather, transplantation of MSCs may contribute to the functional recovery in degeneration models by trophic supply, since they are known to produce various kinds of cytokines and trophic factors [47]. Needless to say, substantial supply of lost cells is crucial to the cell based therapy in degenerative diseases such as muscle dystrophy. Therefore, it is desirable to develop a systematic induction system to obtain large amounts of purposeful cells those confirmed to be morphologically and physiologically functional. Moreover, the practical application to human degenerative diseases depends on the ability to control their differentiation into functional cells with high efficiency and purity. As mentioned, $10^7$ MSCs can be harvested from 20-100 ml of bone marrow aspirate within two to three weeks. If an induction procedure takes the shortest and most perfect course, $10^7$ MSCs give rise to nearly $10^7$ skeletal muscle cells within 5-7 weeks when taking into account the term necessary for NICD introduction, G418 selection and trophic factor administration. Therefore these induction systems may be useful since large amounts of purposeful cells can be obtained from the bone marrow for transplantation therapy within a reasonable time course.

Considering the advantages of MSCs, we can expect the possibility of establishing "auto-cell transplantation system" in muscle dystrophy (Fig.3). Nevertheless, the major matter is that how to replace the mutated gene in patient's MSCs. Probably, genetic manipulation is possible after the isolation and expansion of MSCs. Without resolution of this matter, our system will not lead to the fundamental "auto-cell transplantation therapy" in such hereditary disease. Another way is to utilize MSCs with the same HLA subtype from a healthy donor, namely allo-cell transplantation. This method may minimize the risks of rejection and be more realistic way for the clinical application. Needless to say, the bone marrow should at least be 'normal and healthy' for transplantation (Fig.3).

There are several problems that need to be solved in the future. First, while there have been few reports of tumor formation after transplantation of untreated MSCs, further studies are needed to ensure the safety and efficacy of manipulated MSCs over a long period using primates and nude-mice/rats. In fact, recent reports have raised the possibility of transformation in the long term cultivation of MSCs [48, 49]. Furthermore, yet we introduced NICD by plasmid but not by retrovirus or lentivirus vectors, the safety of induced cells should carefully be estimated. Although the expression of introduced NICD

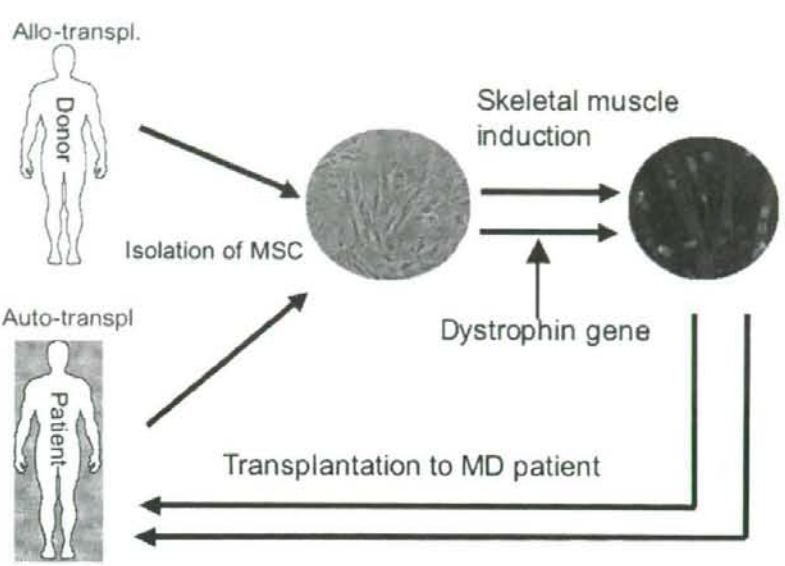

**Figure 3.** Schematic diagram of Allo- and Auto-transplantation therapy based on MSC-derived skeletal muscle cells. MSCs are isolated either from healthy donor with same HLA subtype or from patient and are subjected to the muscle induction. Those cells are transplanted back to the muscle dystrophy patient. "Auto-transplantation" system escapes not only from ethical problem but also from immuno-rejection. However, the replacement of the mutated gene is necessary in this case.

was very faint by RT-PCR in clonal-M-MSCs probably due to the diluting out of the transfected NICD plasmid, it would be more desirable to establish alternative system using protein introduction or signal activation. Second, as the potential differentiation may differ with age, individual, race, and sex, each of these characteristics must be examined in the future. Finally, MSCs have been shown to be heterogeneous in terms of growth kinetics, morphology, phenotype and plasticity. With the development of specific markers and detailed characterization of heterogeneous general adherent MSCs, their properties and plasticity can be studied and defined with more accuracy. Finally, the efficiency and safety of this system need to be examined using primate and higher mammal models such as dystrophy dog.

From the point view of basic research, the role of NICD in myogenic differentiation of MSCs needs to be clarified. As this induction was also suggested to be independent of Hes1/5 actions and the conventional Notch signaling pathway, it will be reasonable to consider that distinct cellular responses to Notch signals; for example, the repertoire of second messengers and active factors in MSC may well be different from conventional myogenic precursor cells, or the susceptibility of MSCs to the Notch signal is probably different from that of known myogenic precursor cells. Thus further studies are needed to identify the factor involved in this phenomenon.

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## CHAPTER XVI

# GENE THERAPY FOR DUCHENNE MUSCULAR DYSTROPHY

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Duchenne muscular dystrophy (DMD) is caused by various distinct mutations, ranging from point mutations to large deletions, in the *dystrophin* gene. These mutations have led to a variety of therapeutic modalities for muscular dystrophy, including gene replacement, gene correction, and modification of the gene product. Gene replacement therapy provides an impersonal approach for treating DMD. Adeno-associated virus (AAV) vector-mediated truncated *micro-dystrophin* gene delivery has been successful in some animal models of DMD. However, recent evidence of immune-mediated loss of vector persistence in dogs and humans suggests that immune modulation might be necessary to achieve successful long-term transgene expression in these species. In this chapter, we focus on the methods that have been developed for gene replacement therapy using vectors based on the AAV.

## 1. Introduction

### 1.1. *Background of Duchenne Muscular Dystrophy*

Duchenne muscular dystrophy (DMD) is the most common form of childhood muscular dystrophy. DMD is an X-linked recessive disorder with an incidence of one in 3500 live male births.[1] 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.[2] 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.

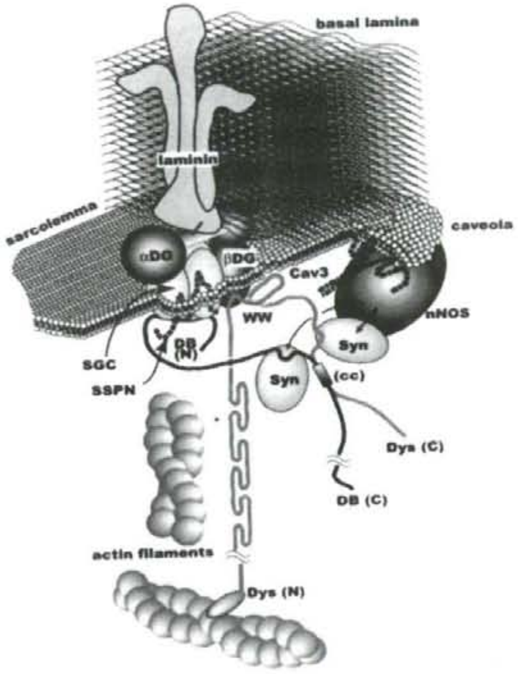

Fig. 1. Dystrophin-glycoprotein complex. Molecular structure of the dystrophin-glycoprotein complex and related proteins superimposed on the sarcolemma and subsarcolemmal actin network (redrawn from Yoshida *et al.*,[3] with modifications). cc, coiled-coil motif on dystrophin (Dys) and dystrobrevin (DB); SGC, sarcoglycan complex; SSPN, sarcospan; Syn, syntrophin; Cav3, caveolin-3; N and C, the N and C termini, respectively; G, G-domain of laminin; asterisk indicates the actin-binding site on the dystrophin rod domain; WW, WW domain.

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 (Figure 1). 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 a partially functional protein.

## 2. Gene-replacement Strategies using Virus Vectors

### 2.1. *Choice of Vector*

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 adeno-associated virus (AAV)-based vector is emerging as the gene transfer vehicle with the most potential for use in DMD 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.[4] Serotypes 1, 6, 8 and 9 of recombinant AAV (rAAV) exhibit a potent tropism for striated muscles.[5] Since a 5-kb genome is considered to be the upper limit for a single AAV virion, a series of rod-truncated micro-dystrophin genes is used in this treatment.[6]

Due to ingenious cloning and preparation techniques, adenovirus vectors are efficient delivery systems of episomal DNA into eukaryotic cell nuclei.[7] 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.[8] 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 (from bases 343 to 2270).[9] Human artificial chromosomes (HACs) have the capacity to deliver a large gene (roughly 6-10 megabases) into host cells without integrating the gene into the host genome, thereby preventing the possibility of insertional mutagenesis and genomic instability.[10]

A goal in clinical gene therapy is to develop gene transfer vehicles that can integrate exogenous therapeutic genes at specific chromosomal loci, so that insertional oncogenesis is prevented. AAV can insert its genome into a specific locus, designated AAVS1, on chromosome 19 of the human genome.[11] 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.[12]

Gene correction is a process whereby sequence alterations in genes can be corrected by homologous recombination-mediated gene conversion between the recipient target locus and a donor construct encoding the correct sequence.[13] The introduction of a corrective sequence together with a site-specific nuclease to induce a double-stranded break (DSB) at sites responsible for monogenic disorders would activate gene correction. Pairs of designated zinc-finger protein with tandem DNA binding sites fused to the cleavage domain of the Fok1 protein were introduced into model systems or cell lines and produced corrections in 10–30% of cases tested.[14]

### 2.2. *Modification of the dystrophin Gene and Promoter*

Due to the large deletion in its genome, the gutted adenovirus vector can package 14-kb of full-length *dystrophin* cDNA. 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.[15] However, further improvements are needed to regulate the virus-associated host immune response before clinical trials can be performed.

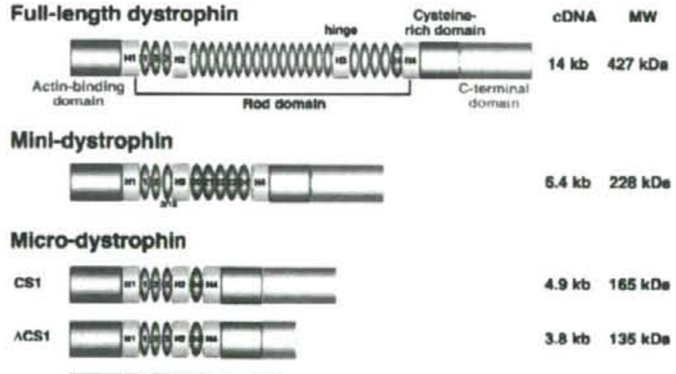

Fig. 2. Structures of full-length and truncated dystrophin. Helper-dependent adenovirus vector can package 14-kb of full-length dystrophin cDNA because of the large-sized deletion in its genome. A mini-dystrophin is cloned from a patient with Becker muscular dystrophy, which is caused by in-frame deletions resulting in the synthesis of partially functional protein. A series of truncated micro-dystrophin cDNAs harboring only four rod repeats with hinge 1, 2, and 4 (CS1); the same components, except that the C-terminal domain is deleted (delta CS1); or one rod repeat with hinge 1 and 4 (M3), are constructed to be packaged in the AAV vector.

A series of truncated *dystrophin* cDNAs containing rod repeats with hinge 1, 2, and 4 were constructed (Figure 2).[6] 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 approach.[16] The structure and, particularly, the length of the rod are crucial for the function of micro-dystrophin.[17] 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,[18] and resulted in extensive and long-term expression of micro-dystrophin that exhibited improved force generation.

The impact of codon usage optimization on micro-dystrophin expression and function in the *mdx* mouse was assessed to compare the function of two different configurations of codon-optimized *micro-dystrophin* genes under the control of a muscle-restrictive promoter (Spc5-12).[19] 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.[20]

### **2.3.** ***Use of Surrogate Genes***

An approach using a surrogate gene would bypass the potential immune responses associated with the delivery of exogenous dystrophin. Methods to increase expression of utrophin, a dystrophin paralog, show promise as a treatment for DMD. rAAV6 harboring a murine codon-optimized micro-utrophin transgene was intravenously administered into adult *dko* mice to alleviate the pathophysiological abnormalities.[21] The paralogous gene efficiently acted as a surrogate for *dystrophin*. Myostatin is extensively documented as being a negative regulator of muscle growth. Systemic gene delivery of myostatin propeptide, a natural inhibitor of myostatin, enhanced body-wide skeletal muscle growth in both normal and *mdx* mice.[22] The delivery of various growth factors, such as insulin-like growth factor-I (IGF-I), has been successful in promoting skeletal muscle regeneration after injury.[23]

Matrix metalloproteinases (MMPs) are key regulatory molecules in the formation, remodeling and degradation of all extracellular matrix

(ECM) components in pathological processes. MMP-9 is involved predominantly in the inflammatory process during muscle degeneration.[24] In contrast, MMP-2 is associated with ECM remodeling during muscle regeneration and fiber growth.

## 3. AAV-mediated transduction of animal models

### 3.1. *Vector Production*

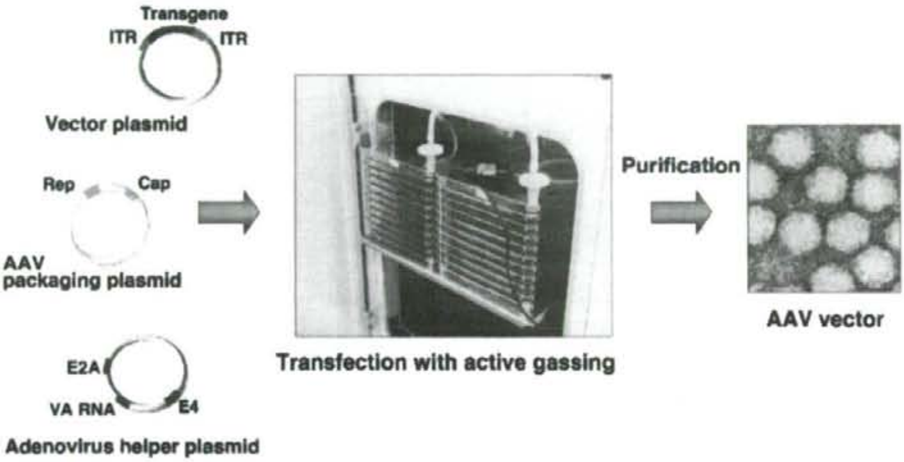

Fig. 3. A scalable triple plasmid transfection system using active gassing. When adenovirus helper plasmid is co-transfected into human embryonic kidney 293 cells along with a vector plasmid encoding the AAV vector and an AAV packaging plasmid harboring *rep-cap* genes, the AAV vector is produced as 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 transfection in a closed system. Samples containing vector particles are further purified with a two-tier CsCl gradient or dual ion-exchange chromatography to obtain highly purified vector particles.

To gain acceptance as a medical treatment with a dose of over $1\times10^{13}$ genome copies (g.c.)/kg body weight, AAV vectors require a scalable and economical production method. A production protocol of AAV vectors in the absence of a helper virus[25] is widely employed for triple plasmid transduction of human embryonic kidney 293 cells.[4] 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 human embryonic kidney 293 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 (Figure 3). Importantly, contamination of most adenovirus proteins can be avoided in AAV vector stock made by this helper virus-free method. Samples containing vector particles are further purified with a two-tier CsCl gradient or dual ion-exchange chromatography to obtain highly purified vector particles.[4]

Despite improvements in vector production, including the development of packaging cell lines expressing Rep/Cap, and of methods that induce the expression and regulation of Rep/Cap,[26] maintaining such cell lines remains difficult, as the early expression of Rep proteins is toxic to cells. A scalable method, using active gassing and large culture vessels, was developed to transfect rAAV in a closed system, in a labor- and cost-effective manner.[27] This vector production system achieved a yield of more than $5x10^{13}$ g.c./flask by improving gas exchange to maintain the physiological pH in the culture medium. Recent developments also suggest that AAV vector production in insect cells would be compatible with current good manufacturing practice production on an industrial scale.[28]

### 3.2. *Animal Models for the Gene Transduction Study*

Dystrophin-deficient canine X-linked muscular dystrophy was found in a golden retriever with a 3' splice-site point mutation in intron 6.[29] The clinical and pathological characteristics of dystrophic dogs are more similar to those of DMD patients than are those of *mdx* mice. A beagle-based model of canine X-linked muscular dystrophy, which is smaller and easier to handle than the golden retriever-based muscular dystrophy dog (GRMD) model, has been established in Japan, and is referred to as $CXMD_J$.[30] The limb and temporal muscles of $CXMD_J$ are affected by two-months-old, which is the age corresponding to the second peak of serum creatine kinase.

Interestingly, we found extensive lymphocyte-mediated immune responses to rAAV2-*lacZ* after direct intramuscular injection into $CXMD_J$ dogs, despite successful delivery of the same viral construct into mouse skeletal muscle.[31] In contrast to rAAV2, rAAV8-mediated transduction of canine skeletal muscles produced significantly higher transgene expression with less lymphocyte proliferation than rAAV2.[32]

It is increasingly important to develop strategies to treat DMD that consider the effect on cardiac muscle. The pathology of the conduction system in $CXMD_J$ was analyzed to establish the therapeutic target for DMD.[33] Although dystrophic changes of the ventricular myocardium were not evident at the age of 1 to 13 months, Purkinje fibers showed remarkable vacuolar degeneration when dogs were as young as four-months-old. Furthermore, degeneration of Purkinje fibers was coincident with overexpression of Dp71 at the sarcolemma. The degeneration of Purkinje fibers could be associated with the distinct deep Q waves present in ECGs and the fatal arrhythmias seen in cases of dystrophin deficiency.[33]

### 3.3. *Immunological Issues of rAAV*

Neo-antigens introduced by AAV vectors evoke significant immune reactions in DMD muscle, since increased permeability of sarcolemma allows leakage of the transgene products from the dystrophin-deficient muscle fibers.[34] rAAV2 transfer into skeletal muscles of normal dogs resulted in low and transient expression, together with intense cellular infiltration, and the marked activation of cellular and humoral immune responses.[31] Furthermore, an *in vitro* interferon-gamma release assay showed that canine splenocytes respond to immunogens or mitogens more strongly than do murine splenocytes. In fact, co-administration of immunosuppressants, cyclosporine (CSP) and mycophenolate mofetil (MMF) improved rAAV2 transduction. The AAV2 capsids can induce a cellular immune response via MHC class I antigen presentation with a cross-presentation pathway, and rAAV2 has also been proposed to have an effect on human dendritic cells (DCs). In contrast, other serotypes, such as rAAV8, induced T-cell activation to a lesser degree.[32] Immunohistochemical analysis revealed that the rAAV2-injected muscles showed higher rates of infiltration of $CD4^+$ and $CD8^+$ T lymphocytes in the endomysium than the rAAV8-injected muscles.[32]

Resident antigen-presenting cells, such as DCs, myoblasts, myotubes and regenerating immature myofibers, might play a role in the immune response. A recent study also showed that mRNA levels of MyD88 and co-stimulating factors, such as CD80, CD86 and type I interferon, are elevated in both rAAV2- and rAAV8-transduced dog DCs *in vitro*.[32] 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.[35]

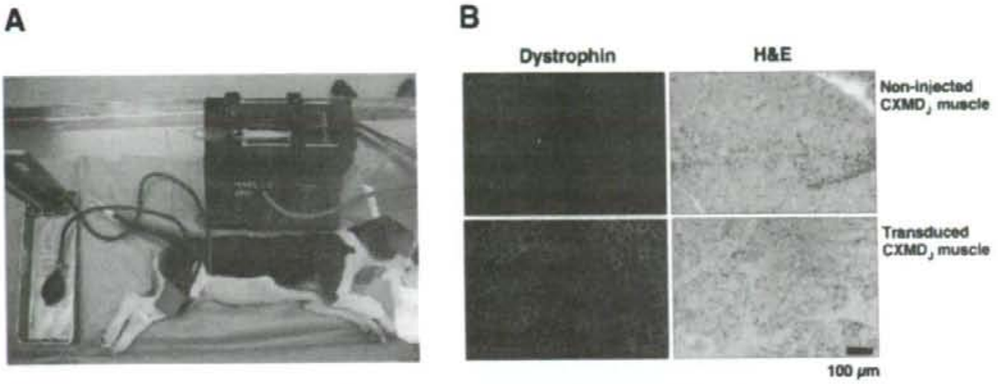

Fig. 4. Intravascular vector administration by limb perfusion. (A) A blood pressure cuff is applied just above the knee of an anesthetized $\text{CXMD}_J$ dog. A 24-gauge intravenous catheter is inserted into the lateral saphenous vein, connected to a three-way stopcock, and flushed with saline. With 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 expressing micro-dystrophin ($1 \times 10^{14}$ 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 is injected at a rate of 0.6 ml/sec. (B) Administration of rAAV8-micro-dystrophin by limb perfusion produces extensive transgene expression in the distal limb muscles of $\text{CXMD}_J$ dogs without obvious immune responses at four weeks after injection.

### 3.4. *Intravascular Vector Administration by Limb Perfusion*

Although recent studies suggest that vectors based on AAV are capable of body-wide transduction in rodents, translating this finding into large animals remains a challenge. Intravascular delivery can be performed as a form of limb perfusion, which might bypass the immune activation of DCs in the injected muscle.[32] We performed limb perfusion-assisted intravenous administration of rAAV8-lacZ into the hind limb of normal dogs and rAAV8-micro-dystrophin into the hind limb of $\text{CXMD}_J$ dogs (Figure 4).[32] Administration of rAAV8-micro-dystrophin by limb perfusion produced extensive transgene expression in the distal limb muscles of $\text{CXMD}_J$ dogs without obvious immune responses for as long as eight weeks after injection.

### 3.5. *Global Muscle Therapies*

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.[36] 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.[37]

Since a number of muscular dystrophy patients can be identified through newborn screening, 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.[38] Systemic transduction was achieved in the absence of pharmacological intervention or immune suppression and lasted for at least six months, whereas cardiac muscle was barely transduced in the dogs.

## 4. Safety and Potential Impact of Clinical Trials

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 discharged to establish the safety of this procedure in phase I clinical trials.[39] The first clinical gene therapy trial for DMD began in March 2006.[39] 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 was transduced with mini-dystrophin genes in a muscle of one arm in the absence of serious adverse events.

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.[40] An obvious barrier to AAV transduction is the presence of circulating neutralizing antibodies that prevent the virion from binding to its cellular receptor.[41] This potential threat can be reduced by prescreening patients for AAV serotype-specific neutralizing antibodies or by performing 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