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Animal models for muscular dystrophy: valuable tools for the development of therapies

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Since the identification of dystrophin as the causative factor in Duchenne muscular dystrophy, an increasing amount of information on the molecular basis of muscular dystrophies has facilitated the division of these heterogeneous disorders into distinct groups. As more light is being shed on the genes and proteins involved in muscular dystrophy, diagnosis of patients has improved enormously. In addition to naturally occurring animal models, a number of genetically engineered murine models for muscular dystrophy have been generated. These animal models have provided valuable clues to the understanding of the pathogenesis of these disorders. Furthermore, as therapeutic approaches are being developed, mutant animals represent good models in which they can be tested. The present review focuses on the recent advancements of gene transfer-based strategies, with a special emphasis on animal models for Duchenne and limb-girdle muscular dystrophies.

INTRODUCTION

Muscular dystrophy (MD) refers to a number of clinically and genetically heterogeneous disorders whose molecular basis has been elucidated in the last decade or so. The identification of dystrophin as the defective protein in Duchenne muscular dystrophy (1,2) was soon followed by the isolation of a number of dystrophin-associated proteins in skeletal muscle. These proteins form a large oligomeric complex named the dystrophin–glycoprotein complex (DGC) (Fig. 1) (3–6) that bridges across the sarcolemma and connects the extracellular matrix and the actin cytoskeleton (7,8). To date, the core skeletal muscle DGC is composed of dystrophin, the sarcoglycans (α-, β-, γ- and δ-SG), dystroglycans (α- and β-DG), sarcospan (SSPN) (9) and the syntrophins. In addition, a number of extra- and intracellular proteins are less tightly associated with the DGC, such as nitric oxide synthase (nNOS) (10), dystrobrevin (11,12), caveolin 3 (13) and laminin-2 (14).

The finding that expression of DGC components was perturbed in dystrophic muscle subsequently led to the recognition of the involvement of this complex in various forms of MD (15–17). Mutations in genes encoding the sarcoglycans are responsible for autosomal recessive forms of limb-girdle muscular dystrophies (LGMD 2C–2F) (18–27). The laminin α2 chain, a basal lamina protein connected to the DGC, is responsible for about half of the 'occidental' or 'classical' forms of congenital muscular dystrophy (CMD) (reviewed in refs 28,29). The α7 integrin subunit, a transmembrane laminin receptor, is involved in human congenital myopathy (30). Interestingly, dystroglycan, sarcospan and syntrophins have not been associated with muscular dystrophies to date.

Despite the tremendous improvement in the understanding of the molecular basis of MD (31), no treatment is currently available. Thus, the development of therapies is the focus of numerous studies worldwide (32–34). The availability of animal models for these disorders (35) constitutes a critical asset, since it allows extensive pre-clinical studies on the safety and the functionality of various therapeutic approaches.

Three main avenues of research in the development of therapeutic approaches for MD have emerged in the past years and can be differentiated as follows: (i) *ex vivo* strategies where 'normal' or modified cultured cells (e.g. myoblasts, stem cells) are being transplanted into the skeletal muscle of a diseased recipient; (ii) *in vivo* strategies aiming at (a) introducing a 'normal' copy of the defective gene or a compensatory gene into the host myofibers by introduction of viral or non-viral vectors or (b) correcting the endogenous defective gene, using, for example, DNA–RNA chimeric oligonucleotides; and, more recently, (iii) pharmacological therapies. In the present review, we will focus mainly on viral gene transfer in animal models of DMD and LGMDs with sarcoglycan deficiency (Fig. 1).

MODELS FOR MUSCULAR DYSTROPHY

The currently available animal models for MD presented below are represented in schematically Figure 2.

Dystrophinopathy

The *mdx* mouse, a naturally occurring animal model for DMD, has been available for over a decade (36). Other mutations in the dystrophin gene have been found in mutant mice (*mdx*2-5^{cv}) that develop a dystrophic phenotype (37,38). Much controversy over the resemblance, or lack thereof, of the pathology between the *mdx* mouse and DMD patients has arisen. A double mutant lacking both dystrophin and utrophin (*mdx*/

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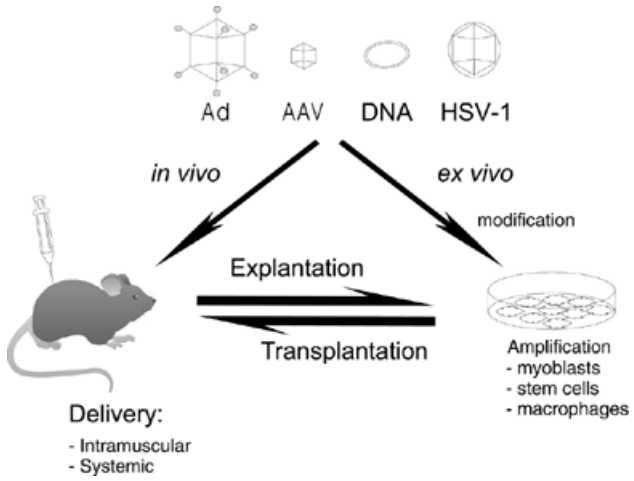

Figure 1. Schematic of vector-based therapeutic approaches for muscular dystrophy. Viral or non-viral vectors may be used for (i) *in vivo* gene therapy and may be delivered either directly into skeletal muscle or systemically; and (ii) *in vitro* gene therapy where they are used for infecting cultured cells that are then transplanted into the recipient animal. Ad, adenovirus; AAV, adeno-associated virus; DNA, naked plasmid DNA; HSV-1, herpes simplex virus.

utnr^{-}) has been generated and displays a phenotype closer to that of DMD patients, including a cardiomyopathy (39,40). These animals thus appear to be a more valid model for DMD. Early transgenic experiments, using full-length as well as mini-dystrophin constructs, have demonstrated that expression of ~20% of the dystrophin protein is sufficient to prevent the development of muscle pathology in *mdx* mice (41,42), thus generating great hope for a treatment for DMD, and by extension for the treatment of other types of MD. The dystrophic golden retriever dog (GRMD) represents a somewhat more attractive animal model for DMD than the murine models because of its larger size (43,44). Unfortunately, there is phenotypic variability between litters, and maintenance of a kennel of GRMDs is not straightforward.

Sarcoglycanopathy

The BIO 14.6 cardiomyopathic hamster, studied for several decades because of its cardiac phenotype, was recognized as a model for LGMD2F with δ-SG deficiency (45,46). Additionally, in the last 2 years, disruption of several sarcoglycans has been achieved in mice, thus providing models for all the sarcoglycanopathies known to date (47–52). All sarcoglycan-null animals display a progressive muscular dystrophy of variable severity. In addition, these models share the property of a significant secondary reduction in the expression of the other members of the sarcoglycan–sarcospan complex as well as some variable degree of disruption of other components of the DGC. Membrane integrity is disrupted in most of these animal models and can be assessed by the use of tracer dye markers (53). Importantly, unlike *Sgca*-null mice, *Sgcb*-, *Sgcg*- and *Sgcd*-null mouse models display a cardiac phenotype (48,50,52), and perfusion studies revealed abnormal vascular function in *Sgcb*- and *Sgcd*-null mice (50,52), thus providing new insights into the complexity of the pathological mechanisms of LGMD 2E and 2F. Surprisingly, although sarcospan expression is affected consistently by loss of the sarcoglycan subcomplex in sarcoglycan-deficient animal models, SSPN-null mice do not present with muscle pathology (54).

Congenital muscular dystrophy (CMD) with deficiency in laminin α2 chain

As many as five murine models for laminin α2-deficient CMD are now available, of which two knock-out strains were generated recently (dy^{3K} and dy^{W}) (55,56). The long-known strains *dy* (57) and dy^{2J} (58–60) present a muscle pathology and a dysmyelination of the peripheral nervous system (61), the latter being less severely affected since it expresses a truncated form of the protein. Recently, another spontaneous mutant strain (named dy^{PAS} mice) lacking the α2 chain of laminin was observed fortuitously (62). These mice, as well as the dy^{W} and dy^{3K}, present with a severe phenotype, close to that of the *dy* mouse, and, since their genetic defect is known, may become more widely used.

Interestingly, transgenic experiments have demonstrated that muscle-specific expression of the laminin α2 chain indeed restored the muscle phenotype in *dy* and dy^{W} mice but did not prevent the occurrence of the neuropathic phenotype in these mice (56), thus demonstrating the importance of a widespread expression of this protein.

Dysferlinopathy

Recently, a deletion in the dysferlin gene has been identified in SJL mice, a spontaneous strain used as a model for different human disorders for several decades (63). This mouse develops a progressive muscular dystrophy affecting primarily proximal muscle groups (63) and thus represents a novel model for LGMD 2B and Miyoshi myopathy.

Other models

Although dystroglycan has not yet been associated with a human disorder, it nevertheless constitutes an essential component of the DGC, and *in vitro* blockade of the α-dystroglycan interaction with laminin induced a dystrophic phenotype in myotubes (64). Furthermore, dystroglycan deficiency in mice leads to embryonic lethality (65) whereas chimeric mice develop a muscular dystrophy (66). Disruption of integrin α7 also leads to a dystrophic phenotype in mice (67). In addition, mice chimeric for the α5 integrin subunit also develop muscle pathology, detectable at a very early age (68), whereas α5 integrin-deficient mice die early in embryogenesis (69). Finally, deficiency of α-dystrobrevin, a cytoplasmic protein linked to dystrophin, leads to dystrophic changes in the skeletal muscle of *adbn*-null mice although the DGC appears preserved (11). As in *mdx* mice, the diaphragm was the most affected muscle, and myopathic changes were also detected in the heart of *adbn*-null mice (11). It is noteworthy that triple mutant animals lacking dystrophin, utrophin and α-dystrobrevin did not appear more severely affected than the *mdx*/*utrn*-null animals (11).

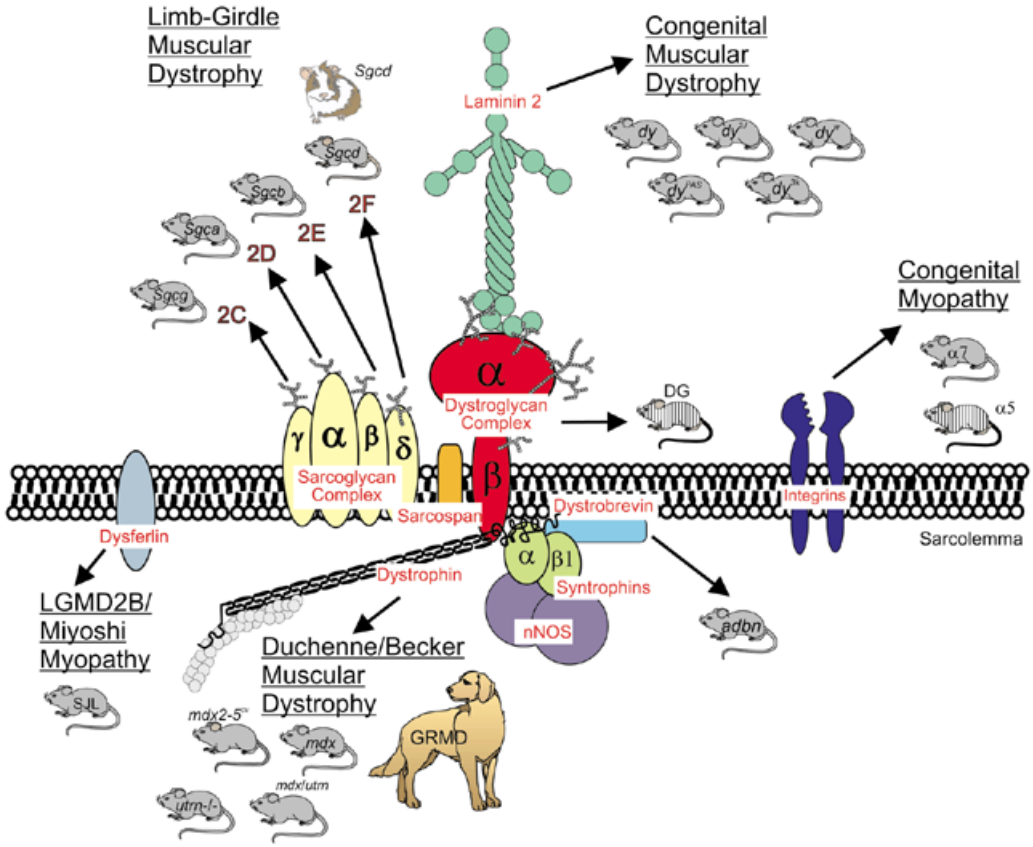

Figure 2. Animal models of muscular dystrophies. Chimeric animals for dystroglycan and integrin α5 are striped. See text for references. Not drawn to scale.

EX VIVO APPROACHES

Myoblast transfer

Myoblast therapy initially generated great hope, but early clinical trials showed little success (70). Importantly, recent studies demonstrated that persistence of donor myoblasts did not necessarily lead to restored expression of dystrophin at the sarcolemma of DMD recipient patients (71). Nevertheless, this avenue of research is still being pursued, with revived interest since some functional benefit was obtained in immunosuppressed *mdx* mice (72,73). Functional benefit is obtained despite the observation that myoblast transplantation is hindered greatly by the poor survival of injected myoblasts. Poor survival is due not only to inflammatory reactions to the transplanted myoblasts and to the therapeutic gene product (74) but also to the intrinsic characteristics of the muscle-derived cells that are transplanted (75,76). Nevertheless, the surviving ~1% of the transplanted cells are then responsible for new muscle formation (76). Most relevant to the potential for treating DMD is the finding that dystrophin itself appears to induce rejection of transplanted wild-type myoblasts in the *mdx* mouse (77). Other proteins may also contribute to rejection, as pointed out in a recent report that demonstrated the importance of using donor myoblasts that match the host muscle for myosin heavy chain expression (78).

Myoblast transplantation has also been investigated in animal models of CMD with deficiency in the α2 chain in laminin. Moderate success at restoring laminin α2 expression was obtained in skeletal muscle of *dy*/*dy* mice by human and murine myoblast transplantation (79,80).

Stem cells

A new avenue of research for the treatment of muscular dystrophies is now being explored, namely the use of stem cells (81). Two recent reports (82,83) provide *in vitro* and *in vivo* evidence that bone marrow transplantation allowed recruitment of stem cells into muscle of *mdx* mice. Furthermore, expression of dystrophin was demonstrated, although at levels that would not be likely to provide functional benefit (83). Nevertheless, as transplantation techniques are optimized, this approach constitutes an attractive means for systemic targeting of muscle groups.

A recent report suggests that blood-borne macrophages may play an essential role in triggering *de novo* muscle regeneration and should thus be taken into account for developing satellite cell transplantation (84).

IN VIVO APPROACHES

One of the major hurdles to vector-based therapies in MD patients is the large volume and wide distribution of the target

tissue. Skeletal muscle may constitute >40% of the human body, and some muscles, such as the diaphragm and intercostal muscles, are not easily accessible to a route of administration such as intramuscular injections. In addition, the heart is affected in DMD and in a subset of LGMD patients. Thus, as detailed below, systemic delivery appears a necessity. Unfortunately, only focal transduction has been obtained from systemic delivery, even with the use of permeabilizing agents.

Naked DNA transfer

Renewed enthusiasm for plasmid DNA as a non-viral gene transfer vector (85), and thus a safer alternative to viral vectors, seems to have arisen with the development of more efficient delivery strategies. Intravascular injection of plasmid DNA under high hydrostatic pressure has been shown to lead to high efficiency of reporter gene product expression in several muscle groups of rat hindlimbs (86). In addition, high-level and long-lasting gene expression of reporter gene products has been obtained by optimized electroporation conditions (87).

Further studies with therapeutic transgenes nevertheless are warranted in animal models of MD to provide data more directly relevant to these diseases and to support the encouraging results obtained with reporter genes in wild-type animals. This may prove important since the mechanisms for uptake of naked plasmid DNA remain unclear (88) and uptake mechanisms may differ in dystrophic muscle.

Virally mediated gene transfer

The initial hopes generated by adenoviral vectors have been dampened by the identification of major drawbacks such as (i) the transient expression of the transgene resulting from both humoral and cellular immune responses against viral antigens and transgene products (89,90); and (ii) the inability to transduce mature myofibers efficiently (91–93). Improved adenoviral vectors, such as the so-called gutted adenovirus, recently have emerged and combine the advantages of being less immunogenic and of being able to accommodate larger therapeutic genes such as dystrophin and laminin α2 chain along with appropriate regulatory sequences (94–98). Further modification of adenoviral vectors is of interest in order to enhance muscle cell transduction (99) or to promote genomic integration (100).

In the last few years, adeno-associated vectors (AAVs) were developed and hold great promise because of their low immunogenicity and their potential for integration. In addition, transduction of mature myofibers is achieved effectively with AAVs (101). Unfortunately, AAV gene transfer is only possible for a restricted number of MDs since they can only accommodate up to 5 kb of exogenous DNA, thus excluding their use for gene transfer of the dystrophin, utrophin or laminin α2 chain genes. Nevertheless, recent reports on the modification of AAV and the use of dual viruses to accommodate larger inserts (102,103) are opening up some new opportunities for AAV-mediated gene transfer of large therapeutic genes. In addition, other types of virus are being investigated as alternatives to adenoviruses. Interesting results have been obtained with the herpes simplex virus type 1 (HSV-1)-(104,105) as well as Epstein–Barr virus (EBV)-based minichromosome vectors (106).

Since the size of the dystrophin cDNA (14 kb) precludes its insertion into conventional viral vectors, with the exception of gutted adenoviruses, mini-dystrophin genes have been engineered and tested for their ability to rescue dystrophic muscle. Several versions of dystrophin minigenes have proven successful at improving the muscle phenotype in *mdx* mice (107–109) and are expected to convert a DMD phenotype into a milder BMD phenotype.

An alternative to delivering dystrophin to dystrophic muscle is to introduce utrophin, a dystrophin homolog (110–112), as this should alleviate any immune response elicited by dystrophin itself. Studies using transgenic animals initially demonstrated that either full-length or truncated utrophin could indeed functionally replace dystrophin in skeletal muscle of *mdx* mice (113–116). Substantial efforts have since been made to deliver the utrophin gene via adenoviral vectors and have indeed led to improvement of the muscle pathology in *mdx* mice (117,118). In addition, a recent report demonstrated that adenovirus-mediated gene transfer of a utrophin minigene in the skeletal muscle of double mutant mice leads to protection against the dystrophic process (119).

On their identification, the sarcoglycans were soon recognized as interesting candidates for viral gene transfer because their cDNAs are <1.5 kb and can thus be accommodated easily by adenovirus as well as AAVs. Our laboratory initially demonstrated that adenoviral gene transfer could deliver δ-SG successfully to skeletal muscle of the BIO 14.6 hamster (120) and restore the DGC at the sarcolemma of transduced fibers, thus protecting the myofibers against sarcolemmal damage and the dystrophic process. Since this study, we and others have investigated gene transfer approaches further in various animal models of sarcoglycanopathy. Adenoviruses have now proven successful at restoring the DGC in β-, γ- and α-SG deficient mice, animal models for LGMD 2E, 2C and 2D, respectively (52,121,122), and at preventing the development of muscular dystrophy (121,122). We recently demonstrated that >80% of myofibers were transduced efficiently by an adenoviral vector expressing the human α-SG and that the expression persisted for at least 7 months after a single intramuscular injection in the quadriceps muscle of newborn *Sgca*-null mice (Fig. 3) (121). Nevertheless, transduction was restricted to the injected muscle because adenoviral particles cannot cross the fascia between muscle groups (Fig. 3). Importantly, we ascertained maintenance of sarcolemal integrity in injected mice by contrast agent-enhanced magnetic resonance imaging (MRI) (Fig. 4), a technique that should prove most useful in patients to assess skeletal muscle damage in the course of muscular dystrophy and following therapeutic approaches (121,123).

AAV-mediated gene transfer of δ-SG in the BIO 14.6 hamster was shown to correct the dystrophic phenotype (124–126). Recently, AAV-mediated rescue of skeletal muscle of γ-SG deficient mice was also demonstrated (122). Nevertheless, the transduction efficiency obtained with AAVs consistently appears lower than that of adenovirus (<50% for AAVs compared with >80% for adenovirus) (121,122).

Considering the relatively small size of AAV particles, systemic delivery should conceptually be achieved more easily than with adenoviral vectors. A recent report demonstrated, albeit on a regional scale, transduction of several muscle groups of the cardiomyopathic hamster hindlimb following perfusion of AAV particles using histamine and papaverine to

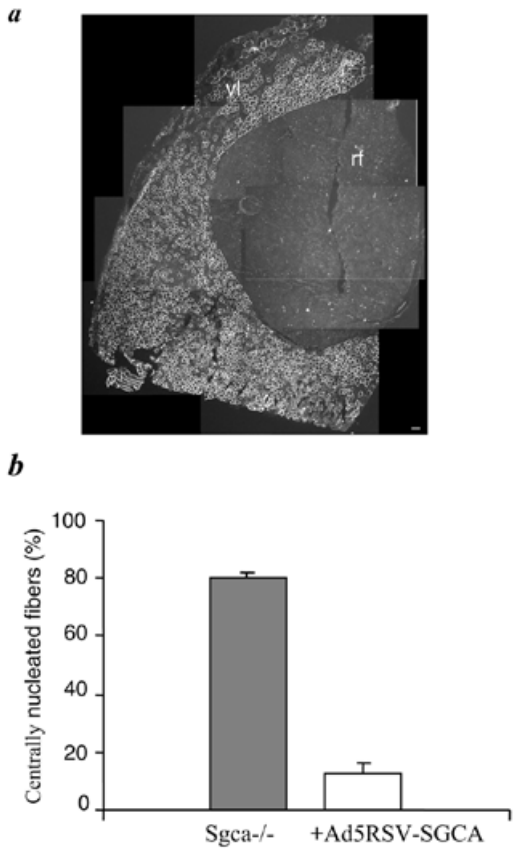

Figure 3. Adenoviral injection confers sustained expression of human α-SG and prevents the degeneration–regeneration process. (**a**) Mice were sacrificed 15 weeks following injection of Ad5RSV-SGCA in the quadriceps femoris of neonate α-SG-deficient mice. Quadriceps femoris muscles were harvested and analyzed by immunofluorescence using a rabbit polyclonal antibody against α-SG. Composites represent images taken at a magnification of 5×. Vl, vastus lateralis; rf, rectus femoris. Bar, 100 µm. (**b**) Percentage of fibers containing centrally located nuclei in either non-injected (*Sgca*$^{-/-}$) or injected (+Ad5RSV-SGCA) vastus lateralis muscles 15 weeks after intramuscular injection of Ad5RSV-SGCA. A total of 553 and 2112 fibers were counted from the non-injected and injected muscles, respectively.

enhance diffusion (124). These preliminary results are indeed encouraging and may open up the way to studies aimed at improving perfusion techniques.

Significantly, animal models of LGMD 2E (β-SG deficiency) (52) and LGMD 2F (δ-SG deficiency) (50,127) display cardiomyopathy, as do human patients affected with these diseases (128–130). Gene transfer to the heart is thus an issue that needs to be taken into account, and promising results of transduction of the myocardium were obtained in the hamster by intrapericardial injection of adenoviral particles containing a reporter gene (131).

RNA–DNA oligonucleotides

The use of RNA–DNA chimeric oligonucleotides, or chimeraplasts, recently has been developed in order to correct point mutations directly in a gene of interest by taking advantage of the endogenous DNA repair machinery (132). This approach should therefore allow long-term correction. Two recent reports demonstrated the rescue of dystrophin expression in *mdx* and GRMD skeletal muscle following intramuscular injections of chimeraplasts (133,134). Expression of dystrophin nevertheless was restricted to myofibers directly surrounding the injection site (133,134) and it thus appears that other delivery methods may need to be investigated, in particular systemic delivery, which is theoretically feasible.

PHARMACOLOGICAL THERAPY

Up-regulation of compensatory proteins

As compensation of dystrophin by utrophin in dystrophic skeletal muscle appears to be efficient in mice, much effort has been made in investigating means of up-regulating endogenous utrophin along the sarcolemma of dystrophin-deficient myofibers. In that respect, reports that heregulin had the potential to induce utrophin expression in skeletal muscle held great promise (135,136). Recently, a novel promoter that potentially could serve as a target for up-regulation of utrophin was identified and may widen the possibilities for induction of this protein (137). A large-scale search for other small molecules that may up-regulate utrophin currently is under way. The results will be most interesting and will no doubt generate a quantity of potential candidates to be tested *in vitro* and *in vivo*.

An intriguing study using adenovirus vectors expressing β-gal or green fluorescent protein in *mdx* mice recently pointed out a potential mechanism for endogenous utrophin up-regulation involving cytokines released during the immune response (138). A better understanding of this mechanism may therefore provide valuable information for designing strategies to up-regulate utrophin expression.

Aminoglycoside antibiotics

Possibly the most encouraging therapeutic approach to DMD has emerged from investigations aimed at treating cystic fibrosis by suppressing nonsense mutations resulting in premature stop codons in the cystic fibrosis transmembrane conductance regulator gene (*CFTR*) (139,140). Encouraging preliminary results showed that restoration of dystrophin levels to 10–20% of normal was detected in skeletal muscle of *mdx* mice after subcutaneous injections of gentamicin (141). Importantly, such levels of dystrophin expression supported functional benefits to treated muscles. This report constitutes the first *in vivo* use of an aminoglycoside antibiotic to overcome a nonsense mutation. Indeed, more thorough investigations need to be performed, in particular to assess the secondary effects due to aminoglycoside antibiotic treatment, mainly nephrotoxicity and ototoxicity (142). Nevertheless, this class of antibiotics holds promise for pharmacological treatment of ~5–15% of DMD patients with premature stop mutations. It is worth mentioning that due to the extended half-life of dystrophin, such treatment is expected to be long lasting and gentamicin administration may not need to be repeated too often.

CONCLUSIONS

Despite the advent of and astounding pace of progress in molecular medicine, the challenges faced in developing therapies for muscular dystrophies that may be applied to human patients are still daunting, and many more pre-clinical experi-

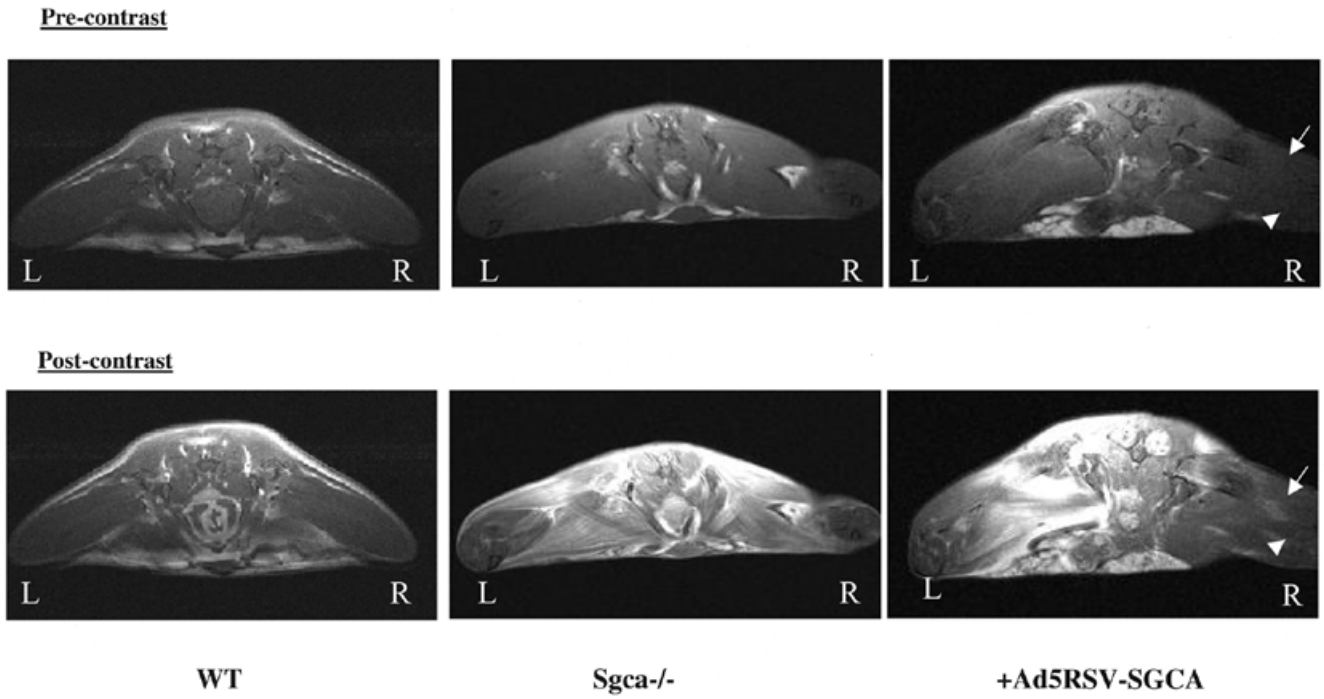

Figure 4. Adenoviral-mediated gene transfer of α-SG protects against sarcolemmal damage. Contrast agent-enhanced MRI of muscle groups from the pelvic girdle and proximal hindlimb muscles of wild-type (WT), non-injected (*Sgca*$^{-/-}$) and injected (+Ad5RSV-SGCA) α-SG-deficient mice. Neonate mice received intramuscular injections of Ad5RSV-SGCA in their right quadriceps femoris (arrows) and hamstring (arrowheads) muscles. Images were taken prior to (pre-contrast) and 15 min following (post-contrast) systemic injection of AngioMark (MS-325).

ments are warranted. In this respect, experiments on animal models will continue to provide crucial pieces of information in regard to issues such as the appropriate timing for intervention (the earlier the better seems to be a consensus), the risk–benefit ratio of current vectors and transgenes, and the assessment of functional benefit. It is worth mentioning that the accumulation of data on sarcoglycan gene transfer in animal models of sarcoglycanopathies has provided grounds for a phase I clinical trial for sarcoglycan-deficient LGMDs (143). Finally, although more data still need to be obtained, animal model studies have certainly demonstrated that gene therapy holds promise for muscular dystrophy and other diseases for which no other treatments currently are available.

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