Effects of Helper-Dependent Adenovirus Mediated Full-Length Utrophin on Dystrophic Muscle.

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Abstract

Helper-dependent adenovirus vector-mediated full-length dystrophin expression in muscle leads to significant mitigation of the dystrophic phenotype of the *mdx* mouse. However, dystrophin, as a neoantigen, elicits antibody formation. As an alternative approach, we evaluated gene transfer of full-length murine utrophin, a functional homologue of dystrophin that is normally present only at the neuromuscular junction. A single injection into the *mdx* mouse tibialis anterior muscle with a helper-dependent adenovirus vector encoding utrophin provided very good transduction, with 58% of fibers demonstrating sarcolemmal utrophin expression in the neonatally injected mice while 35% utrophin positive fibers were found in mice injected in adult age. The presence of utrophin prevented extensive necrosis in the neonates (improved centronucleation index), halted further necrosis in the adults and led to restoration of sarcolemmal expression of dystrophin associated proteins up to 1 year post-injection. Marked physiological improvement was observed in both neonates and adults. Neither increased humoral nor cellular immune responses were evident. However, there was a time-related decline of the initial high utrophin expression. Although viral DNA persisted in animals that were injected in the neonatal stage, viral DNA levels decreased in muscles of mice injected in the adult age. These results demonstrate that although utrophin gene transfer leads to amelioration of the dystrophic phenotype, the effects are not sustained upon loss of utrophin expression. As the level of utrophin expression may dictate the extent of recovery

from the dystrophic phenotype, we hypothesized that the delivery of a greater amount of utrophin would improve upon the initial results and further alleviate the dystrophic pathology. Through the use of higher titer adenovirus preparations, and pre-treatment of the *mdx* muscle with the histone deacetylase inhibitor, Trichostatin A, an increase in the initial and long-term transduction levels was achieved. This increased transduction had a significant effect on measured parameters such as serum creatine kinase levels, distribution of fiber diameter size and force generation. These results suggest that a critical determinant of the efficacy of utrophin gene transfer is the level of utrophin expression per fiber that is achieved during the initial transduction period. In conclusion, this study indicates that murine utrophin when expressed at adequate levels can successfully compensate for murine dystrophin in the *mdx* dystrophic model.

Résumé

L'expression de la dystrophine complète dans le muscle à l'aide d'un vecteur adénoviral dépendant d'un virus auxiliaire (adénovirus évidé ou « gutted ») mène à la réduction significative de la pathologie chez la souris *mdx*. Cependant, la dystrophine agit comme un néoantigène et provoque ainsi la formation d'anticorps. Comme approche alternative, nous avons évalué le transfert de l'ADNc complet de l'utrophine, une protéine ayant une fonction homologue à la dystrophine et qui est normalement présente seulement à la jonction neuromusculaire. Chez les souris *mdx* nouveau-nées, une seule injection de vecteurs adénoviraux contenant l'utrophine dans le muscle tibial antérieur a mené à une transduction élevée du muscle (58% des fibres montrant l'expression de l'utrophine à la membrane cellulaire). De plus, l'injection de ce même vecteur recombinant chez les souris adultes a permit l'expression de l'utrophine dans 35% des fibres. La présence de l'utrophine a empêché la nécrose massive chez les souris nouveau-nées (index amélioré des fibres à noyaux centraux), arrêté la progression de la nécrose chez les adultes et a mené à la restauration de l'expression sarcolemmale des protéines associées à la dystrophine jusqu'à un an après l'injection. Nous avons observé l'amélioration physiologique chez les nouveau-nés et les adultes. Aucune réaction immunitaire humorale ou cellulaire accrues n'était évidente. Cependant, il y avait un déclin de l'expression de l'utrophine dans le temps en fonction du niveau de son expression initiale. Bien que l'ADN viral ait persisté chez les animaux injectés à

l'âge néonatal, les niveaux d'ADN viraux ont diminué dans les muscles des souris injectées à l'âge adulte. Ces résultats démontrent que le transfert du gène de l'utrophine mène à l'amélioration du phénotype dystrophique mais les effets ne sont pas soutenus suite à la perte de l'expression de l'utrophine. Le niveau de l'expression initiale d'utrophine peut dicter l'ampleur du rétablissement du phénotype dystrophique. Nous avons présumé qu'un niveau initial élevé d'utrophine améliorerait les résultats préliminaires et allégerait davantage la pathologie dystrophique. Avec l'utilisation de préparations de vecteurs adénoviraux ayant des titres plus élevés ainsi qu'avec un prétraitement des souris *mdx* avec l'inhibiteur d'histone désacétylase, Trichostatin A, une augmentation de la transduction initiale et de longue durée a été réalisée. Cette transduction redoublée a eu un effet significatif sur les paramètres mesurés tels que les niveaux sériques de la créatine kinase, la distribution du diamètre des fibres et la génération de la force musculaire. Ces résultats suggèrent qu'une cause déterminante de l'efficacité du transfert du gène de l'utrophine est le niveau de l'expression d'utrophine par fibre au moment de la transduction initiale. En conclusion, cette étude indique que l'utrophine murine, une fois exprimée à des niveaux adéquats, peut compenser avec succès la dystrophine murine dans le modèle dystrophique *mdx*.

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Abbreviations

AAT	α_1-antitrypsin
AAV	adeno-associated-virus
AdV	adenovirus
ALT	alanine aminotransferase
ANOVA	analysis of variance
APC	antigen presenting cell
ApoE	apolipoprotein E
AST	aspartate aminotransferase
BMD	Becker muscular dystrophy
bp	base pair
CAR	coxsackie adenovirus receptor
CB	chicken β-actin promoter/CMV enhancer
cDNA	complementary DNA
CFTR	cystic fibrosis transmembrane conductance regulator
CK	creatine kinase
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
DAPs	dystrophin-associated proteins
dko	double knock-out, dystrophin/utrophin-deficient mouse
DMD	Duchenne muscular dystrophy
DNA	deoxyribonucleic acid

DPC	dystrophin associated protein complex
EBV	Epstein-Barr virus
EBNA 1	EBV nuclear antigen 1
EF-hand	motif that contains approximately 40 residues and is involved in binding intracellular calcium
ERK	extracellular signal related kinase
FVIII	coagulation factor VIII
GRMD	golden retriever muscular dystrophy
IL-6	interleukin 6
IL-12	interleukin 12
ITR	inverted terminal repeat
kb	kilobase
kDa	kilodalton
lacZ	β-galactosidase gene
LTR	long terminal repeat
MCK	muscle creatine kinase
MOI	multiplicity of infection
mRNA	messenger RNA
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming unit

RNA	ribonucleic acid
RSV	rous sarcoma virus
RT-PCR	reverse transcription-PCR
SDS	sodium dodecyl sulfate
TA	tibialis anterior
TSA	Trichostatin A
Utr+	utrophin positive
Utr-	utrophin negative
WW domain	domain that contains 2 conserved tryptophans (W) spaced by 20-22 aminoacids.
ZZ	zinc finger

CHAPTER 1 – Introduction

Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD), a muscle disorder, is characterized by the progressive weakness and wasting of voluntary muscles that control body movement. During the course of this disease, fatty and connective tissue replace muscle tissue (Engel et al., 1994). DMD occurs almost exclusively in boys with an incidence rate of 1 in 3500 newborn males (Emery, 1993).

DMD is an X-linked recessive disorder in which a mutation exists within the large dystrophin gene, located at position Xp21 (Hoffman et al., 1987). It is transmitted through carrier mothers and passed on from generation to generation. However, 1/3 of cases represent new mutations where the mother is not a carrier and only this one child is affected (Gardner-Medwin, 1970;Emery, 1980;Moser, 1984). The clinical onset is usually observed between the ages of two and five years old. By DNA analysis, the disease can also be detected or ruled out in babies where there is a positive family history. Diagnosis of DMD has improved a great deal. Female carriers can be positively identified and a healthy embryo can be selected for implantation with in-vitro fertilisation techniques (Fassati et al., 1994).

As mentioned previously, nearly all patients are male. Occasionally, the disease has been observed in females due to Turner (XO) or Turner mosaic (X/XX or X/XX/XXX) syndromes (Ferrier et al., 1965), a structurally abnormal X

chromosome (Berg and Conte, 1974), or an X–autosomal translocation (Lindenbaum et al., 1979). Infrequently, some heterozygote females suffer from the disease due to a failure of inactivation of the mutation-bearing X chromosome (Gomez et al., 1977).

Initial symptoms of DMD include difficulty rising from the floor, climbing stairs, falling easily, a waddling gait and pseudohypertrophy which is a characteristic prominence of the calf muscles (Duchenne de Boulogne, 1973). The progression of the disorder varies from child to child but it is steady with no remissions. A wheelchair is usually necessary by late childhood or early adolescence. Breathing is also affected in the later stages of DMD. The life span is shortened and victims usually die in their 20s from respiratory or cardiac failure.

A milder dystrophinopathy exists called Becker muscular dystrophy (BMD) (England et al., 1990). It is similar to DMD by the fact that it is X-linked, except that it progresses at a much slower rate. BMD occurs in approximately 1 out of 30 000 male births. Symptoms usually appear in boys at about age 12 or in some cases even later. These individuals stop walking at an average age of 25-30. Like DMD, BMD results from mutations in the dystrophin gene. However the genetic defects are different from DMD leading to a truncated or altered protein. The majority of patients (70%) have in-frame mutations which produce internal deletions or duplications within the protein. As a result, people with BMD have some dystrophin, but it's not enough. Having some dystrophin protects the

muscles of those with Becker from degenerating as badly or as quickly as those of people with Duchenne (Rowland, 1995).

Dystrophin

The dystrophin gene is among the largest known, with 2.4 megabases (Mb) of DNA consisting of 79 exons, representing 1.5% of the X chromosome and 0.1% of the total human genome. However, only 0.6% of the gene encodes mRNA for dystrophin. The entire gene and its 12 kb mRNA transcript have been cloned (Koenig et al., 1987). To date, seven different specific dystrophin transcripts have been identified: three full-length and four truncated isoforms. Muscle, cortical and Purkinje cell type dystrophins have a molecular weight of 427 kDa. Alternate promoters drive the expression of the truncated dystrophin transcripts which have molecular weights of 260, 140, 116 and 71 kDa (Ahn and Kunkel, 1993). They each have unique amino terminuses whereas the cysterine rich and carboxyl terminus are the lone constants amongst all dystrophin transcripts. For the duration of this thesis, the dystrophin in question will be muscle dystrophin.

The mutation in the dystrophin gene frequently leads to the absence of the dystrophin protein. The high frequency of mutations in the dystrophin gene is attributed to its large size (Coffey et al., 1992). Mutations affecting the dystrophin gene can either be large DNA rearrangements (deletions or duplications) or small mutations (single base changes). Large deletions account for 60-65% of DMD

cases. The large mutations are distributed unevenly, with hot spots occurring where the introns are particularly long (between the first 20 exons of the gene) and in the extremely large intron between exons 44 and 45 (Koenig et al., 1987;Koenig et al., 1989;Den Dunnen et al., 1989). Another 5% of mutations arise from the duplication of large segments and the remaining 30% are accounted for by point mutations (Den Dunnen et al., 1989). The mutations result in an unstable mRNA or premature termination of translation resulting in an incomplete and nonfunctional protein. It can be then prematurely degraded due to instability. Some inframe mutations do lead to a truncated dystrophin associated with the Becker phenotype (Koenig et al., 1987). A relationship has been found between the site of the deletion and the clinical syndrome. C-terminal deletions appear inconsequential while those within the cysteine rich region result in more severe types while Becker phenotypes result from deletions within the rod domain or the N-terminal region (Koenig et al., 1989;Scott et al., 2002).

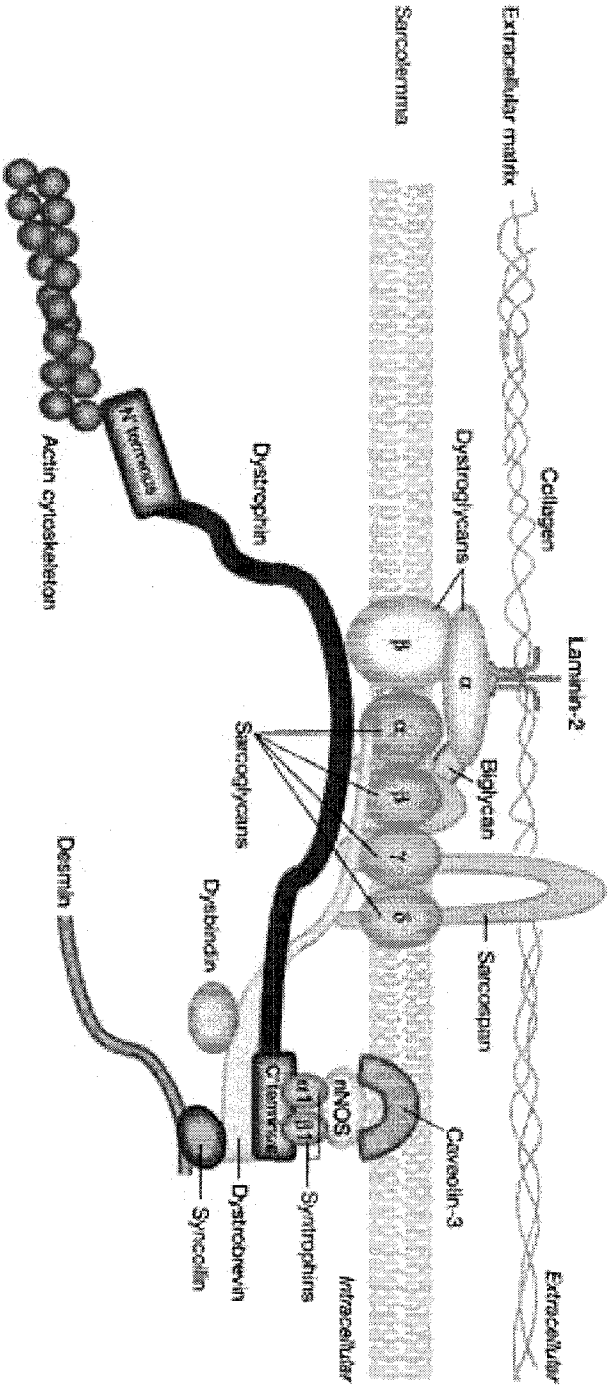
Dystrophin is a subsarcolemmal cytoskeletal protein with a molecular weight of 427 kDa. It has been found to have a conserved protein sequence between humans and rodents. Dystrophin was reported to constitute only 0.002% of total skeletal muscle protein, but it accounts for 2% of total sarcolemmal protein and 5% of subsarcolemmal cytoskeletal protein in muscle (Ervasti and Campbell, 1993). However, recent recalculations propose its amount in total normal muscle to be 13 fold higher at 0.026% (Rybakova et al., 2006;Ervasti, 2007). Dystrophin was predicted to be a rod-shaped cytoskeletal protein after an analysis of its amino acid sequence (Koenig et al., 1988). The

protein is composed of four structural domains. The amino-terminal end consists of 240 amino acids that form an actin binding domain in addition to having a high homology with several actin binding proteins such as α-actinin, β-spectrin and fimbrin (Hemmings et al., 1992). The second and largest section of the protein consists of 24 spectrin-like repeats of 109 amino acids in the from of a triple helix that are interspersed with four proline rich hinge regions that are thought to confer elasticity and flexibility (Koenig and Kunkel, 1990) (Matsumura et al., 1993). The second section also contains a second actin binding domain located at spectrin repeats 11 through 17 (Rybakova et al., 2002). The third domain is a cysteine rich domain containing 15 cysteines and a section of 142 amino acids that show homology to the carboxyl terminal of α-actinin (Koenig et al., 1988). It also encodes WW (tryptophan) and ZZ (zinc finger) domains encompassing two calcium binding EF (α-helices) hand-like motifs (Koenig et al., 1988;Bork and Sudol, 1994;Ponting et al., 1996). The final or C-terminal domain, containing 420 amino acids is highly conserved across species and has considerable homology with utrophin, a dystrophin related protein (Love et al., 1989).

Dystrophin is connected with a large oligomeric complex of sarcolemmal proteins including α and β-dystroglycan, α, β, γ, and δ-sarcoglycan and syntrophin (Figure 1). These proteins are collectively referred to as DAPs, dystrophin-associated proteins. Dystrophin interacts with many other proteins to act as linker between the actin cytoskeleton and the extracellular matrix. The amino terminus of dystrophin attaches to cytoskeletal actin while the cysteine rich region through its WW, EF and ZZ domains anchors dystophin to the

Figure 1. Schematic model of the sarcolemma depicting dystrophin and its associated proteins. Adapted from (Nowak and Davies, 2004).

Figure 1


Extracellular matrix
Collagen
Laminin-2
Dystroglycans
Sarcolemma
β
α
α
β
γ
δ
Biglycan
Sarcoglycans
Sarcospan
Caveolin-3
Extracellular
Intracellular
nNOS
α1
β1
Syntrophins
Dystrobrevin
Syncoilin
Desmin
Dystrophin
Dystrobrevin
N-terminus
Actin cytoskeleton

sarcolemma by binding to the transmembrane protein β-dystroglycan (Ishikawa-Sakurai et al., 2004). The latter then binds the extracellular protein α-dystroglycan which then joins to laminin thus completing the link and leading to the attachment of the entire complex to extracellular matrix. The C-terminus also interacts with components of the complex by binding to two syntrophin subunits (α and β1) and the cytoplasmic protein dystrobrevin.

Pathogenesis of DMD

Multiple factors are thought to contribute to muscle damage in a dystrophin-deficient environment. Despite intense muscle regeneration, dystrophic muscle is characterized by its inability over time to adequately repair itself whereby muscle undergoes necrosis and is eventually replaced by fat and connective tissue possibly due to regenerating fibers that do not always attain normal size (Carpenter and Karpati, 1979).

Dystrophin is thought to play a structural role by maintaining the structural integrity of the muscle fiber membrane during contractions and the resulting structural deformation, especially those involving stretch (Engel et al., 1994). The absence of dystrophin from the sarcolemma results in a marked reduction of overall levels of DAPs as well as loss of DAPs from the sarcolemma in skeletal muscle of DMD patients and animal models such as the *mdx* mouse (Ohlendieck and Campbell, 1991) (Matsumura and Campbell, 1994). DAPs mRNA levels are normal but the complex is not properly assembled and/or integrated into the

sarcolemma or are degraded in the absence of dystrophin (Ibraghimov-Beskrovnaya et al., 1992;Roberds et al., 1993). Due to the loss of the physical link between the cytoskeleton and the extracellular matrix, the sarcolemma becomes fragile and the dystrophin deficient fibers show an increased sensitivity to mechanical stress and are rendered susceptible to degeneration during repeated cycles of muscle contraction and relaxation (Petrof et al., 1993;Moens et al., 1993). Furthermore, abnormal muscle architecture such as fiber splitting and branching has been observed in older *mdx* muscle (Head et al., 1992). These combined factors are then thought to render dystrophic membranes vulnerable to stress, although fast-twitch skeletal muscles of dystrophic pups were found to be resistant to stress induced injury (Head et al., 1992;Petrof et al., 1993;Moens et al., 1993;Clarke et al., 1993;Grange et al., 2002). This is thought to lead to muscle cell necrosis and weakness of the overall muscle (Ohlendieck et al., 1993;Matsumura et al., 1993) resulting from sarcolemmal (physical) breaks during contraction (Ohlendieck et al., 1993). Despite no direct evidence to support the presence of membrane tears, the membrane stiffness of *mdx* myotubes was found to be fourfold lower than of normal myotubes (Pasternak et al., 1995). As well, an increase in membrane permeability is observed in *mdx* muscles due to the augmented plasma concentrations of muscle specific enzymes such as creatine kinase and the increased uptake of vital dyes by dystrophin deficient fibers (McArdle et al., 1994). Thus, in the absence of dystrophin, it is proposed that stretched contractions lead to transient membrane

tears (Petrof et al., 1993) which allow extracellular calcium to rapidly enter the fiber down its electrochemical gradient.

Another theory that leads to a rise in intracellular calcium concentration involves dystrophin having a role in ion channel accumulation in the membrane whereby the loss of dystrophin leads to a misdistribution and/or malfunctioning of these channels (Franco, Jr. and Lansman, 1990). Dystrophin may have a role in calcium homeostasis. As a result of the sarcolemma's increased fragility, the abnormal cell membrane experiences transient membrane disruptions leading to an increase in calcium inflow. In comparison to normal controls, intracellular calcium levels are elevated in DMD and *mdx* muscles (Fong et al., 1990;Turner et al., 1991). The intracellular calcium concentration was found to be threefold higher in the subsarcolemmal space in *mdx* muscle (Mallouk et al., 2000). An increased calcium leak channel activity has been observed in the sarcolemma of cultured dystrophic myotubes (Fong et al., 1990;Turner et al., 1991) (Vandebrouck et al., 2002a;Iwata et al., 2003). The activity of these leak channels was associated with contractile activity whereas an inhibitor of the protease calpain, leupeptin was found to inhibit the calcium influx from these channels in dystrophic muscle (Turner et al., 1993). Calcium activated proteases have been thought to play a role in protein degradation and necrosis in dystrophin deficient muscle (Turner et al., 1988). In particular calpain has been shown to degrade cytoskeletal and membrane proteins in skeletal muscle (Zaidi and Narahara, 1989;Goll et al., 2003) and possess greater activity in *mdx* muscle compared to wild-type muscle (Bonuccelli et al., 2003). As well, muscle

degeneration and necrosis was reduced when *mdx* muscles were injected with leupeptin, a calpain inhibitor, and in the context of transgenic mice overexpressing calpastatin, an endogenous calpain inhibitor (Badalamente and Stracher, 2000;Spencer and Mellgren, 2002). However, membrane permeability was unaffected in these mice. It was proposed that the localized influx of calcium through the membrane tears leads to the insertion of the leak channels in the membrane and the activation of proteases. The leak channels are then activated by the proteases which initiate the constant flow of calcium resulting in a self perpetuating cycle (Alderton and Steinhardt, 2000). Furthermore, young *mdx* diaphragm muscles, isolated before the onset of pathology, show an increase in calcium flow through calcium/stretch activated channels which appear to be activated due to the increased stress placed on the dystrophin deficient sarcolemma (Franco, Jr. and Lansman, 1990;Franco-Obregon, Jr. and Lansman, 1994;Kumar et al., 2004). In addition, these mechanosensitive channels were also found in greater quantity in *mdx* fibers (Squire et al., 2002;Vandebrouck et al., 2002b). Furthermore, the administration of stretch activated channel inhibitors (streptomycin, gadolinium and GsMTx4) prevented the increase of intracellular calcium following eccentric contractions and led to an improvement in the dystrophic pathology of *mdx* fibers (Yeung et al., 2003;Yeung et al., 2005;Whitehead et al., 2006). The data suggested stretch activated channels to be the primary source of calcium entry instead of membrane tears following stretched contractions leading to increased membrane permeability whereby the increased calcium concentration could then potentially activate the calcium leak

channels. The rise in intracellular calcium concentration leads to a loss of calcium homeostasis and the subsequent activation of diverse degradative pathways. The enhanced calcium influx results in the irregular hyperactivation of signaling cascades involved in the inflammatory response (Whitehead et al., 2006). Excess calcium is thought to overload mitochondria resulting in a malfunctioning oxidative phosporylation process in *mdx* mice leading to the increased production of reactive oxygen species (ROS) (Kuznetsov et al., 1998;Nethery et al., 2000;Brookes et al., 2004). ROS induced damage has also been shown to be more prevalent in *mdx* mice (Rando et al., 1998) whereas the use of anti-oxidants or the reduction of hydroxyl radicals in *mdx* lowered muscle damage (Bornman et al., 1998;Buetler et al., 2002). The involvement of ROS has also been proposed in the activation of transcription factor NF-κB which regulates the expression of TNF-alpha and IL-8, pro-inflammatory cytokines known to be augmented in *mdx* muscle before the onset of necrosis (Kumar and Boriek, 2003). This then creates a cycle due to the fact that TNF-alpha can stimulate the production of mitochondrial ROS (Reid and Li, 2001) which can then activate NF-κB. An anti-TNF-alpha antibody was found to lower necrosis and infiltrates in injected *mdx* mice (Grounds and Torrisi, 2004). ROS can also directly inflict damage since they can be produced by inflammatory cells such as neutrophils and macrphages. Levels of ROS can be reduced by nitric oxide which acts as a scavenger (Tidball, 2005). However, neuronal nitric oxide synthase (nNOS) is greatly reduced in dystrophic muscle (Brenman et al., 1995;Chang et al., 1996), leading to a decrease in nitric oxide production and allowing ROS levels to go

unchecked and rise gradually. This observation correlates with the increased number of inflammatory mediators and chemoattractants observed in dystrophin deficient muscle prior to the onset of disease symptoms (Spencer et al., 2001;Porter et al., 2002;Porter et al., 2003). Thus, the activation of inflammatory pathways due to unusual calcium homeostasis may contribute to muscle cell death.

Besides a structural role, dystrophin may contribute to signaling pathways. The localization of dystrophin and the entire DAP complex at the sarcolemma is ideal to act as a signaling scaffold and respond to extracellular stressors (Rando, 2001;Chakkalakal et al., 2005). The binding of alpha-dystroglycan to the extracellular matrix ligand laminin results in the recruitment of Rac1 and other signaling molecules known to be involved in actin remodeling (Oak et al., 2003). Besides Rac1, other signaling molecules such as calmodulin, nNOS, Grb2, extracellular signal related kinase (ERK) and diacylglycerol kinase are known to interact with components of the DAP complex (Brenman et al., 1995;Yang et al., 1995a;Abramovici et al., 2003;Kumar et al., 2004;Barton, 2006). Dystrophin and syntrophin both interact with calmodulin (Madhavan et al., 1992;Anderson et al., 1996b;Newbell et al., 1997;Iwata et al., 1998) and are phosphorylated by a calcium-calmodulin-dependent protein kinase, CaM kinase II (Walsh et al., 1995;Madhavan and Jarrett, 1999). Calmodulin mediated activities have also been shown to be decreased in dystrophic muscle (Niebroj-Dobosz et al., 1989). The interaction of CaM kinase II with the DAP complex (Madhavan and Jarrett, 1994) is interesting due to the protein kinase's involvement in cell survival

pathways, particularly those regulated by PI3K and Akt in addition to certain processes controlling apoptosis (Franklin and McCubrey, 2000). The Grb2 adaptor protein may also link the DAP complex to cell survival pathways. It participates in signaling pathways involving receptor tyrosines kinases and Ras signaling (Lowenstein et al., 1992;Chardin et al., 1995). Through its interaction with β-dystroglycan (Yang et al., 1995a), Grb2 could lead to the Ras/MAPK pathway via other adaptor proteins such as SOS (Chardin et al., 1995). Various membrane signaling complexes use this pathway for cellular survival including the integrins and caveolins (Wary et al., 1998;Bonni et al., 1999;Cary et al., 1999).

As already alluded to, the nNOS enzyme is thought to play a role in muscle damage. It associates with the DAP complex by interacting with syntrophins which bind to dystrophin and α-dystrobrevin (Brenman et al., 1996;Brenman and Bredt, 1996). Due to nNOS's decreased production of nitric oxide (NO) in dystrophin deficient muscle, many cellular defense mechanisms are jeopardized. NO is thought to upregulate cGMP signaling pathways through activation of guanylate cyclase (Denninger and Marletta, 1999) in addition to its actions as a cytoprotective free radical (Darley-Usmar et al., 1995); both of which contribute to cell survival. cGMP production controls processes such as contractile function, the recruitment of calcium and the metabolism of glucose (Grozdanovic and Baumgarten, 1999). Furthermore, NO acts as local vasodilator mediating muscle tone. In its absence, vasoconstriction goes unchecked during contraction which may lead to ischemic injury in dystrophic muscle (Thomas et

al., 1998;Sander et al., 2000). The localization of signaling components at the level of the DAP complex leads to the notion that an important function of dystrophin and the DAP complex may be to initiate appropriate downstream signaling cascades regulating cell survival and cellular defense in response to extracellular stimuli. The absence of dystrophin and the entire complex undoubtedly leads to the malfunction of signaling events which may contribute to disease progression.

Clinical Treatment

There is no specific treatment to stop or reverse the progression of DMD. Instead, attempts are made to slow the progression and alleviate some of the disease symptoms. A number of therapies are offered to DMD patients. Due to the formation of contractures (fixtures) in various joints which can restrict flexibility, physical therapy is used to maintain mobility. As well, orthopedic techniques involving braces are used to prevent khyposcoliosis (spine curvature) (Luque, 1982). However, life can be prolonged through tracheotomy assisted ventilation during the late stages of the disease.

Drug therapy to improve muscle strength involves the use of anti-inflammatory corticosteroids, in particular prednisone and its derivative deflazacort. They have been found to retard the progression of muscle deterioration but they tend to cause various side effects (DeSilva et al., 1987;Mendell et al., 1989;Fenichel et al., 1991;Griggs et al., 1993). Deflazacort is

the less harmful of the two with fewer side effects (Mesa et al., 1991;Bonifati et al., 2000). Besides the benefits to muscles, deflazacort has been found to help in respiration (Biggar et al., 2001), possibly due to its ability to improve the growth and repair capabilities as shown in the *mdx* diaphragm (Anderson et al., 1996a). The positive effects of deflazacort have been attributed to many reasons: its ability to promote laminin expression, myogenic repair and increase utrophin expression via the NO pathway and/or the calcineurin/NFAT signaling cascade (Anderson et al., 2000;Anderson and Vargas, 2003;St Pierre et al., 2004). Besides corticosteroids, anticonvulsants are used to control muscle seizures, immunosuppresants and antibiotics are given to delay some muscle damage and fight off recurring respiratory infections.

Animal Models

During the course of this study, the animal model for dystrophin deficiency used was the *mdx* mouse (Bulfield et al., 1984). The dystrophic phenotype arises due to a mutation on the X chromosome in C57BL/10ScSn inbred mice. The molecular basis of the mutation is a C to T nucleotide change at position 3185 that converts a CAA glutamine codon to TAA stop codon. This results in premature termination of translation at 27% of the length of the dystrophin polypeptide (Sicinski et al., 1989). This truncated and unstable dystrophin is unable to attach to the sarcolemma. A marked secondary DAP deficiency is a consequence of the absence of sarcolemmal dystrophin (Ohlendieck and

Campbell, 1991). *Mdx* mice and DMD patients have similar biochemical and histological defects. However, unlike in humans, affected mice show little if any disability in addition to having a normal life span. Furthermore, muscle regeneration does not seem to be compromised which may explain the relative lack of muscle weakness. At birth, few muscle fibers are affected but necrosis increases suddenly at day 20. However, the intensity of the fiber necrosis decreases after day 60 and keeps occurring at low frequencies during the life of the animal (Tanabe et al., 1986). There is excessive atrophy with loss of normal muscle fibers. Instead of connective tissue, regenerating fibers continuously replace the necrotic fibers. During regeneration, debris is removed by macrophages and increased formation of myofibers with the formation and fusion of myoblasts proceeds. As an indicator of previous damage, the new fibers contain central nuclei. Both of these traits are in contrast to the observations made in humans. Unlike other muscles, the diaphragm of the *mdx* does correctly reflect DMD pathology. It demonstrates progressive fibrosis and a loss of function (Stedman et al., 1991) possibly due to its constant active state (Muller et al., 2001). The mild consequences of the dystrophin deficiency in *mdx* mice are still not understood. Possible reasons include a greater replicative potential of murine satellite cells (Grounds and McGeachie, 1992) or that the small-caliber muscle fibers of the mouse uphold less mechanical stress than larger fibers in humans. This has led to criticism of the *mdx* mouse as a reasonable model for DMD. It does however provide a good model for the early presymptomatic phase during

which there is excessive damage to muscle and maximum benefit could be reaped from treatment (Wells, 2006).

Other dystrophin mutant mice have been discovered or generated (*mdx*$^{2\text{-}5cv}$) (Chapman et al., 1989). In general, these mice are similar to the conventional *mdx* aside from certain differences. For example, the *mdx*^{4cv} and *mdx*^{5cv} have ten fold lower numbers of revertant fibers than *mdx* presumably due to the different locations of the point mutation. Another related mouse model that more closely resembles the DMD phenotype is the dystrophin and utrophin double knock out (mdx/utrn–/–, *dko*) mouse (Grady et al., 1997;Deconinck et al., 1997a). It displays a more severe disease progression with a lifespan of approximately 20 weeks. By comparison to the mdx, *dko* mice exhibit growth retardation, weight loss, spinal curvature (khyposcoliosis) and joint contractures, early diaphragm pathology and premature death.

It should also be mentioned that there are canine and feline animal models for muscular dystrophy as well (Valentine et al., 1986;Carpenter et al., 1989;Valentine et al., 1990). The GRMD canine model of DMD resulted from a splice mutation in a litter of golden retrievers that leads to a loss of exon 7 and subsequent loss of the open reading frame (Cooper et al., 1988). The GRMD dog shares many similarities with DMD but some pups tend to die at birth due to excessive myopathy. Their genetic outbred background along with the difficulty to get reasonable n values makes them complicated to work with. These models were also not used in this study due the increased costs attributed to the acquisition and maintenance of these animals.

Experimental Treatment

Cell based therapy for DMD involves the introduction of normal cells into a dystrophic milieu in the hope that the normal cells will fuse with the diseased muscle. One of the first procedures involved the transfer of myoblasts in DMD patients (Karpati et al., 1993a). In this experiment, bicep muscles of DMD patients were injected with purified normal myoblasts derived from biopsy specimens of the fathers. The transfer efficiency was concluded to be poor based on, amongst others, the dystrophin content of the muscle and on the lack of donor derived dystrophin DNA and messenger RNA in the injected muscle. A recent clinical trial yielded 11% dystrophin positive fibers after 30 million cells had been injected by 25 parallel injections in a 1 cm^3 area (Skuk et al., 2004). A denser pattern of injections resulted in only a slight increase in percentage of positive fibers (up to 26%) (Skuk et al., 2006). Another study tested the intramuscular *ex vivo* transfer of self cells from *mdx* mice that had first been retrovirally transduced with recombinant versions of dystrophin (Dunckley et al., 1993;Fassati et al., 1997;Kobinger et al., 2003;Li et al., 2005). However, the use of retroviruses does pose a problem considering their threat for insertional mutagenesis. Thus, myoblast therapy currently seems unviable as an effective treatment for DMD.

Another cell based approach involves the transfer of stem cells (aka side population cells, myogenic progenitors) which have been shown to confer some

dystrophin positivity in *mdx* mice and GRMD dogs that underwent intravenous injection from a dystrophin positive donor (Gussoni et al., 1999;Dell'Agnola et al., 2004). As well, some engraftment in skeletal muscle was observed in a DMD patient that had received bone marrow transplantation from a dystophin positive donor for the treatment of X-linked severe combined immune deficiency (Gussoni et al., 2002). Despite the initial potential of this approach, all these procedures displayed low efficiencies in terms of level of dystrophin expression and incorporation of donor nuclei. However, the recent use of lentivirus-treated stem cells with microdystrophin for intra-arterial delivery into mdx^{5cv} mice provided modest results (5-8% engraftment) (Bachrach et al., 2006). Yet significant improvements still need to be made in order for this method to be a viable therapy for DMD.

Besides cell based therapy, therapies have evolved to target the various aspects of DMD pathogenesis. Briefly, some of the goals of these approaches are: an increase in cellular adhesion and muscle stability (through overexpression of α7β1-integrin (Burkin et al., 2001), a reduction in inflammation and necrosis (by blocking tumor necrosis factor alpha (Grounds and Torrisi, 2004) and the maintenance of the regenerative capacity (by overexpressing insulin-like growth factor-1 (Barton-Davis et al., 1998;Musaro et al., 2001;Barton et al., 2002;Abmayr et al., 2005) or by blocking the myostatin protein (Bogdanovich et al., 2002;Wagner et al., 2002). Furthermore, *mdx* mice treated with proteasome inhibitors demonstrated the restoration of members of the DAP complex in addition to a truncated dystrophin. The sarcolemma had less damage

and displayed better integrity (Bonuccelli et al., 2003;Assereto et al., 2006). All of the above mentioned approaches were successful in improving to some extent the pathology in *mdx* cultured myotubes and/or in dystrophic mice (Nowak and Davies, 2004) (Odom et al., 2007). An overview of many current therapies employed to treat DMD is shown in Figure 2. However, all of these approaches fail to address the fundamental problem in DMD, which is the underlying genetic defect that causes the absence of dystrophin.

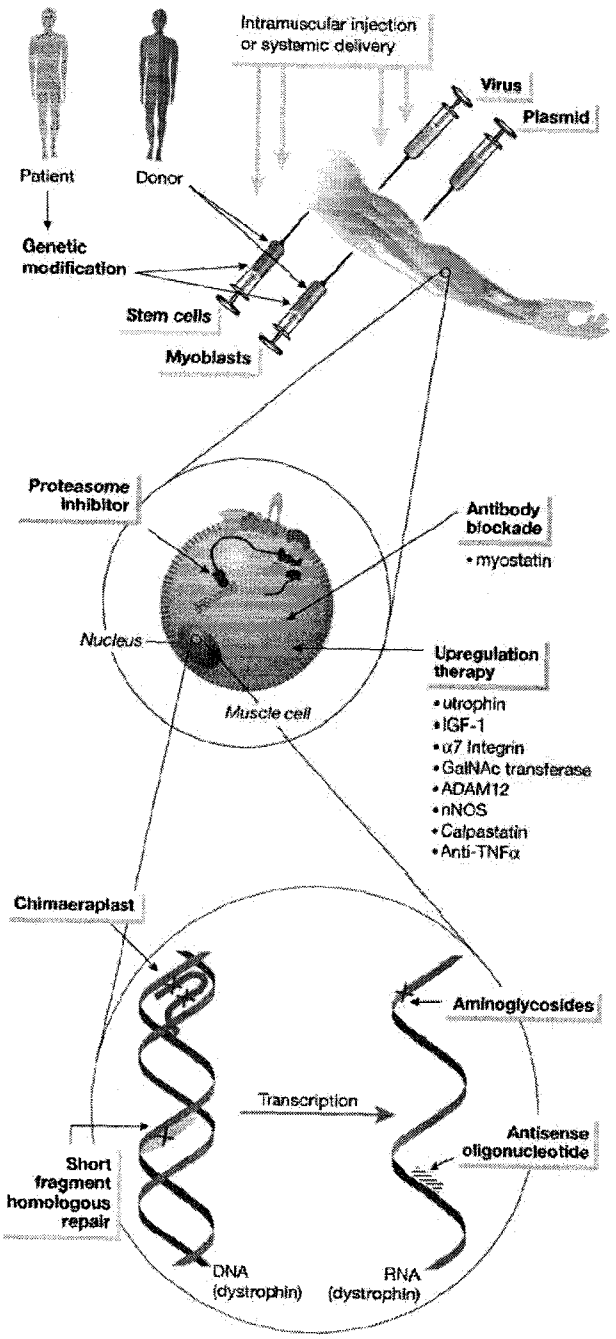
Dystrophin Based Therapy

To assess whether the restoration of dystrophin in a dystrophic context would improve the disease phenotype, transgenic *mdx* mice were constructed expressing a mini or full length dystrophin protein. A reversal of the dystrophic symptoms was observed in these mice even in transgenic lines where dystrophin expression was at 20% of wild-type levels (although higher levels ~70% were required in the quadriceps) (Phelps et al., 1995). Some recovery was also noticed in mice expressing low levels of dystrophin indicating that high expression levels are not required for some disease correction to occur (Wells et al., 1992;Cox et al., 1993;Lee et al., 1993;Phelps et al., 1995;Wells et al., 1995;Sakamoto et al., 2002). Therefore, dystrophin based therapies appeared to be the most viable options to treat the dystrophic pathology.

In an attempt to reintroduce dystrophin, the precise correction of the mutation has been attempted by two ways. The first method was by short

Figure 2. Summary of the wide range of approaches used to treat Duchenne muscular dystrophy. Adapted from (Nowak and Davies, 2004).

Figure 2


Intramuscular injection
or systemic delivery
Virus
Plasmid
Patient
Donor
Genetic modification
Stem cells
Myoblasts
Proteasome inhibitor
Antibody blockade
• myostatin
Nucleus
Upregulation therapy
• utrophin
• IGF-1
• α7 integrin
• GalNAc transferase
• ADAM12
• nNOS
• Calpastatin
• Anti-TNFα
Muscle cell
Chimaeraplast
Aminoglycosides
Transcription
Antisense oligonucleotide
Short fragment homologous repair
DNA (dystrophin)
RNA (dystrophin)

fragment homologous replacement of a nonsense mutation in *mdx* myoblasts *in vitro* which was shown to induce gene repair. However, efficiencies were deemed very low due to the lack of expression of full-length dystrophin transcript and the adverse effects experienced by the transfected cells due to the lipofection protocol (Kapsa et al., 2001). Chimaeric oligonucleotide directed mismatch repair was also found to correct the *mdx and* GRMD mutation. Unfortunately, this approach was also inefficient due the localized and minimal amount of dystophin expression after intramuscular chimeraplast injection (Rando et al., 2000;Bertoni and Rando, 2002;Bertoni et al., 2003).

Since some DMD cases arise from a loss of the open reading frame, DMD patients can be converted to a BMD phenotype by the removal of one or more exons around the mutation site. This method involves the modification of the dystrophin transcript by induction of exon skipping. Using antisense oligonucleotides, dystrophin expression was restored *in vitro* in *mdx* muscle cultures and DMD myoblasts (Dunckley et al., 1998;Wilton et al., 1999;van Deutekom et al., 2001;Aartsma-Rus et al., 2003;Aartsma-Rus et al., 2004). *In vivo* experiments in *mdx* mice using direct intramuscular injection of 2-O-methyl phosporothioate antisense oligonucleotides also restored dystrophin expression (Mann et al., 2001;Lu et al., 2003;Wells et al., 2003;Bremmer-Bout et al., 2004). Intravenous delivery to multiple muscles (Lu et al., 2005) and long term expression using adeno-associated viruses (AAVs) carrying antisense sequences have also been demonstrated in *mdx* (Goyenvalle et al., 2004;Denti et al., 2006). Clinical trials using antisense oligonucleotides targeting dystrophin

restoration are currently underway in two locations. There is however a small possibility that the newly formed dystrophin may elicit an immune response to the novel epitope at the newly formed junction that restores the open reading frame. Furthermore, each treatment using antisense oligonucleotides requires optimization for almost every mutation in the gene.

Dystrophin deficiency also arises from point mutations that result in a premature stop codon. Dystrophin restoration was demonstrated by treatment of *mdx* mice with gentamicin, an aminoglycoside antibiotic resulting in read through of the premature stop codon in exon 23 of the dystrophic mouse (Barton-Davis et al., 1999). However, a repeat of this experiment did not yield the same results (Dunant et al., 2003) and clinical trials failed to give a definitive answer (Wagner et al., 2001;Politano et al., 2003). Recently, a small molecule drug termed PTC 124, which also induces read through and thought to be more effective than gentamicin has shown favourable preliminary results (Welch et al., 2007) and is progressing to clinical trials.

Dystrophin Gene Therapy

As mentioned, necrosis and fiber loss in dystrophic muscle fibers can be avoided by establishing dystrophin expression in affected fibers (Phelps et al., 1995;Acsadi et al., 1996;Deconinck et al., 1996). As well, the presence of dystrophin can benefit neighboring fibers that did not receive the gene by reducing the overall mechanical stress (Deconinck et al., 1996). Dystrophin gene

therapy is a promising mode of action to deliver a functional dystrophin in the treatment of DMD. In gene therapy, a normal gene is delivered to target cells to provide a functional protein in order to overturn the harmful effects of a genetic mutation. Due to the large size of the dystrophin gene, a cDNA based version is used for gene therapy purposes. In DMD, the most severely affected tissue is skeletal muscle, making skeletal muscle fibers the target cells (Karpati and Acsadi, 1994). A large and elongated cell, a muscle fiber is composed of hundreds of myonuclei, each of which in turn contains a full genome (Grounds, 1991). Due to the fact that the dystrophin protein remains in close proximity to each myonucleus, a majority of myonuclei must possess a normal allele in order for the entire muscle fiber to collectively contain dystrophin (Karpati and Acsadi, 1993). Therefore, it is possible for only a portion of a fiber to become treated (Carpenter and Karpati, 1979). Furthermore, muscle fibers are post-mitotic cells in addition to being surrounded by an extracellular matrix (basal lamina) that could pose difficulties for gene therapy (Karpati and Acsadi, 1994).

Previously, many approaches have been taken to transfer genes into muscle fibers. Plasmid expression vectors (Wolff et al., 1990;Acsadi et al., 1991), microprojectiles (Yang et al., 1990) and cationic liposomes containing the therapeutic gene (Wagner et al., 1991;Curiel et al., 1991) are examples of methods which were initially unsuccessful. However, some progress has been made in plasmid gene transfer. In certain studies, systemic delivery resulted in 40% dystrophin expression in fibers, restoration of some DAP proteins, and a decrease in central nucleation (Liu et al., 2001;Zhang et al., 2004;Liang et al.,

2004). In one study, dystrophin expression lasted 6 months post injection, despite the detection of anti-dystrophin antibodies (Zhang et al., 2004). It is assumed that injection of naked dystrophin DNA into dystrophic muscle did not induce an immune response in previous studies because the plasmid injection failed to produce a high level of expression necessary to evoke an immune response (Acsadi et al., 1991;Acsadi et al., 1996). These advancements led to the first gene therapy trials involving plasmid gene transfer in DMD patients. Dystrophin expression was found up to 3 weeks post injection in radial muscle fibers in six of nine patients but the levels were very low (Romero et al., 2002;Romero et al., 2004). Despite the general inefficiency linked with plasmid injection, this method does avoid the anti-vector immunogenicity usually involved with viral vectors. Groups have also increased transfection efficiency by applying an electrical field across the muscle following local intramuscular injection (Aihara and Miyazaki, 1998;Mir et al., 1999;Mathiesen, 1999) but this procedure was accompanied by muscle damage. In vivo electroporation of dystrophin has also been demonstrated with some success (Vilquin et al., 2001;Murakami et al., 2003;Gollins et al., 2003;Molnar et al., 2004) but this procedure appears highly unlikely to be attempted with patients. Unlike direct intramuscular injection, multiple muscles have been transduced with plasmid vectors using a rapid limb perfusion system. Vectors within a large volume of solution were administered into the circulation of an isolated limb via a major artery that had been pre-treated with vasoactive drugs (Budker et al., 1998;Zhang et al., 2004). This procedure was improved upon when the same rapid perfusion was done via a peripheral

vein without the need of vasoactive agents and the invasive cannulation of a deep artery (Hagstrom et al., 2004). These methods yielded improved efficiencies compared to direct intramuscular injections, yet were still lower than those obtained with viral vectors.

Viral vectors are another option in gene therapy. The herpes virus has a large insert capacity (152 kb), but its tropism for muscle cells is poor (Suhr and Gage, 1993). Most retroviruses require replicating target cells for efficient transduction except for lentiviruses, a sub-group of retroviruses, which can permanently transduce muscle cells and their precursors for stable transgene expression (Kafri et al., 1997;MacKenzie et al., 2002). However, their insert capacity of 7 kb is too small for full-length dystrophin (Temin, 1989) in addition to their risk of germ-line insertion. In the treatment of DMD, adenoviruses and AAV are currently the most promising vectors for gene transfer into muscle fibers.

AAV are single stranded DNA viruses first isolated as contaminants from adenovirus preparations. They are characterized by their reduced inflammatory and immunological responses (Jooss et al., 1998). However, antibodies against the dystrophin transgene were detected in dystrophin-deficient skeletal muscle (Yuasa et al., 2002). The insert capacity of AAV (4.7 kb) poses the same problem as retroviruses. Despite this, promising results have been obtained with AAV vectors expressing a greatly truncated dystrophin micro-gene necessitated by the size constraints for AAV packaging (Wang et al., 2000;Watchko et al., 2002;Fabb et al., 2002;Harper et al., 2002;Gregorevic et al., 2004;Yoshimura et al., 2004;Gregorevic et al., 2006). The major hurdle of restricted, localized

expression at the site of injection (O'Hara et al., 2001) associated with any intramuscular vector mediated gene delivery was overcome with AAV. Extensive transduction of the skeletal and cardiac musculature along with improved pathology and muscle force was observed in *mdx* mice after a single systemic injection (Gregorevic et al., 2004). However, vascular endothelial growth factor (VEGF) was used to permeablize the microvasculature in conjunction with low AAV titers. VEGF was not needed with high vector titers. Similar results were obtained in *mdx/utr⁻ dko* mice where a single injection of the same AAV significantly rescued the mice from most dystrophic pathologies and prolonged their lifespan beyond one year (Gregorevic et al., 2006). The systemic delivery was a significant breakthrough for it allows the envisioning of DMD patients being treated with AAV compared to intramuscular treatment which would require a multitude of injections in every muscle throughout the body. However, in DMD patients, AAV-mediated micro-dystrophin expression would presumably produce at best the equivalent of a Becker muscular dystrophy phenotype (England et al., 1990;Love et al., 1990). As a result, two strategies have evolved in order to confer at least a minidystophin expression with AAV infection. The first method involves trans-splicing whereby the transgene is split at splice sites and inserted into two different vectors and expression of the large protein occurs in co-infected cells. RNA processing is crucial in this method as well as the environment and location of the splice sites which can influence transcription and splicing efficiency (Cartegni et al., 2002;Liu et al., 2005;Ibrahim et al., 2005;Lai et al., 2005). Another system uses recombination in which two AAV vectors carry two

ends of a transgene containing a large overlapping sequence. The large gene is obtained when cells are co-infected and homologous recombination occurs between the overlapping sequences. The serotype of AAV used, the arrangement of the overlapping sequence and the tissue of choice all play a role in determining the efficiency of this method (Duan et al., 2001;Halbert et al., 2002;Ghosh et al., 2006;Ghosh et al., 2007). Regardless of method employed, it is reasonable to assume that it would be a considerable scale-up challenge to translate these AAV studies to human trials considering the difficult nature of AAV production. Experiments involving systemic delivery to mice require 10^{12}-10^{13} genomes which convert into 5 x 10^{13}-10^{14} genomes/kg. It would then take an astonishing 10^{15}-10^{16} AAV genomes to treat a 20kg child (Wells, 2006). As gene replacement therapy with full-length dystrophin is the optimal choice for complete recovery from the dystrophic pathology, using fully deleted adenoviral vectors (AdV) appear ideal for the eventual treatment of DMD due to their decreased immunogenicity and their capacity to carry the full-length dystrophin cDNA (14 kb).

Adenoviruses are non-enveloped icosohedral viruses containing linear double stranded DNA 35 kb in length. Adenoviruses infect a variety of post-mitotic cells, such as skeletal muscle, lung, brain and heart. Adenoviruses are very attractive vectors for the expression and delivery of therapeutic genes due to their ability to deliver their genome to the nucleus and to replicate very efficiently. Adenoviruses are popular as gene vectors for a variety of reasons: they're easy to manipulate, they can be grown to high titers, they are capable of

infecting many different cell types and as already mentioned, they transduce both replicating and nonreplicating cells such as muscle fibers and neurons (Leppard, 1997).

An adenoviral gene vector can incorporate only 2 kb of foreign DNA into its genome without affecting its stability or infectivity. The addition of longer sequences requires the deletion of additional viral genes, and early generations were deleted of various early transcriptional units (adenoviruses have four early regions termed E1-E4) in an attempt to increase insert capacity. The first generation of adenoviral vectors was rendered replication deficient by deleting the E1 region. These vectors were also deleted for their E3 region.

First generation adenoviruses (E1/E3 deleted) with a capacity of ~8.3 kb were capable of incorporating the minidystrophin cDNA (6.5 kb) which was based on the protein discovered in BMD patients (England et al., 1990). The cDNA used was deleted for most of the rod domain (between exons 17 and 48) and had a molecular weight of 200 kd. Intramuscular injection of this virus revealed dystrophin expression in 50% of the myofibers for a duration of up to 3 months (Ragot et al., 1993). In mouse models, dystrophin expression increased in muscle fibers transduced with adenoviruses but the transgene expression was only transient due to an immune response against the vector and transgene (Yang et al., 1998;Yuasa et al., 1998) due in part to residual viral gene expression which led to the elimination of transduced muscle fibers (Acsadi et al., 1996) . A prolonged dystrophin expression of up to 2 months was observed in diseased muscles subjected to adenoviral-mediated dystrophin transfer followed

by a thirty day treatment of FK506 (Lochmuller et al., 1996). FK506 is an inhibitor in the signaling pathway from the T cell receptor to the nucleus, therefore halting cell proliferation, differentiation and cytokine production (Sawada et al., 1987;Schreiber and Crabtree, 1992). FK506 was also responsible for suppressing the host humoral response against the vector following the first viral administration (Lochmuller et al., 1996). The potency of the immune response has also been shown in the canine model system. Increased dystrophin expression was observed in dogs that had been treated with cyclosporin, an immunosuppressive drug, after viral injection (Howell et al., 1998).

In addition to their immunogenicity, another disadvantage for adenoviruses was that they were found to infect myoblasts, myotubes and regenerating fibers but were not highly infective for mature muscle fibers (Acsadi et al., 1994a;Acsadi et al., 1994b) possibly due to a lack of viral receptors (Acsadi et al., 1994b;Huard et al., 1995;Feero et al., 1997). The gene uptake efficiency was two fold lower in adult mice (Acsadi et al., 1994b;Nalbantoglu et al., 1999;Nalbantoglu et al., 2001) which translated into a reduced transgene expression in adult mice compared to a more elevated expression in neonates (Ragot et al., 1993;Vincent et al., 1993;Acsadi et al., 1996).

To overcome some of the drawbacks of first-generation vectors, these results prompted the creation of the most recent generation of adenoviruses. The third generation of adenoviral vectors was fully deleted (also called "gutted" viruses) due to the removal of their entire genome. Termed helper-dependent adenovirus (Kumar-Singh and Chamberlain, 1996;Hardy et al., 1997;Morsy et al.,

1998;Amalfitano et al., 1998;Lieber et al., 1999;Steinwaerder et al., 1999), these viruses retained only the inverted terminal repeats (ITRs) at either end of the virus which are essential for replication along with a sequence necessary for proper packaging (packaging signal). They required the aid of a helper virus in order to be propagated, with all the remaining viral proteins are provided for in *trans* (Parks et al., 1996).

Due to their increased insert capacity, these viruses are able to accommodate the full length version of the dystrophin cDNA. In terms of transduction in dystrophic muscle, they have also been able to confer a more sustained level of dystrophin expression, restoration of DAP proteins, attenuation of dystrophin pathology and an improvement in muscle function in addition to inducing reduced cellular immune responses following intramuscular injection (Kochanek et al., 1996;Clemens et al., 1996;Haecker et al., 1996;Chen et al., 1997;Chen et al., 1999;Gilbert et al., 2001;Gilbert et al., 2002;Gilchrist et al., 2002;DelloRusso et al., 2002;Gilbert et al., 2003;Dudley et al., 2004;Matecki et al., 2004). However, the appearance of inflammatory infiltrates due to cellular immunity and the presence of anti-dystrophin antibodies were still observed.

Despite the advancements with helper dependent adenoviruses, researchers still face challenges. It appears the episomal adenoviral genomes are gradually lost from transduced fibers and even with the lowered immune response, adaptive responses to the vector and humoral immunity to the vector and the dystrophin transgene in dystrophin deficient muscle can occur (Muruve et al., 1999;Gilbert et al., 2003;Dudley et al., 2004;Muruve et al., 2004).

Antibodies are generated against dystrophin because many dystrophin mutations arise from large deletions which prevent the expression of many epitiopes. Therefore, an exogenous full length dystrophin may be perceived as a neoantigen in a dystrophin deficient environment (Ohtsuka et al., 1998). Incidentally, similar to previous work with first generation adenoviruses (Kay et al., 1995), some researchers are using immunomodulatory molecules in conjunction with helper dependent adenoviruses. Known to block costimulatory signals beween T cells and antigen presenting cells, CTL4AIg was delivered to *mdx* mice along with a dystrophin expressing helper dependent adenovirus in an attempt to prolong transgene expressing (Jiang et al., 2004a;Jiang et al., 2004b). An alternative option to circumventing some of the immune responses would be to use a transgene capable of substituting for dystrophin without being considered a neoantigen such as utrophin.

Utrophin

Utrophin is a functional homologue of dystrophin. The name is a contraction of ubiquitous dystrophin. It was first identified by screening a human fetal muscle library using a 3' dystrophin probe (Love et al., 1989). The gene is ~1MB in length and is localized to human chromosome 6q24 while the murine isoform is located on chromosome 10 (Tinsley et al., 1992).

Utrophin is ubiquitously expressed (Nguyen et al., 1991;Khurana et al., 1991;Nguyen et al., 1992;Khurana et al., 1992;Blake et al., 1996). In a muscle

myofiber, utrophin is found restricted to the acetylcholine receptor rich crests located at neuromuscular junctions where it binds to components of the dystrophin associated complex much like dystrophin does at the sarcolemma (Khurana et al., 1991;Nguyen et al., 1992;Matsumura et al., 1992;Campanelli et al., 1994). Besides the myofiber, utrophin is also present in intramuscular nerves and blood vessels in muscle. Pre-natally, utrophin is also found at the level of the sarcolemma in fetal skeletal muscle. It then gradually disappears and is eventually replaced by dystrophin soon after birth, leading to both proteins having a distinct localization pattern in adult muscle. Because of this phenomenon, utrophin is proposed by some to be the autosomal fetal/embryonic/neonatal form of dystrophin (Takemitsu et al., 1991;Clerk et al., 1993).

Like dystrophin, utrophin also exists as different isoforms. Two full-length utrophin proteins termed A and B (Dennis et al., 1996;Burton et al., 1999) along with three short C-terminal isofoms are known to exist (Wilson et al., 1999). The utrophin A protein is the abundant isoform in skeletal muscle while B is predominantly found in the vascular endothelium (Weir et al., 2002).

The utrophin sequence has many similarities with that of dystrophin. The N-terminal domain of utrophin binds F-actin with similar affinity as dystrophin however the modes in which the bindings occur and their respective binding sites on actin are different (Rybakova et al., 2002;Rybakova and Ervasti, 2005;Rybakova et al., 2006). Utrophin has one actin binding domain while dystrophin has two; unlike dystrophin, utrophin lacks the basic, actin binding spectrin repeats in the rod domain. Its sole actin binding domain stretches from

the amino terminus through the first ten spectrin repeats. The cysteine rich and C-terminal regions have an 80% homology between the two proteins and thus have similar C-terminal binding partners. However, dystrophin and utrophin do exhibit different modes of contact with β-dystroglycan (Ishikawa-Sakurai et al., 2004). The rod domain is the least conserved sequence with a 35% homology.

Utrophin Based Therapy

Due to the fact the utrophin is upregulated and re-localized to the sarcolemma in certain dystrophin deficient situations (such as DMD) and to the many similarities in sequence and domains between utrophin and dystrophin, it was proposed that utrophin may be a viable substitute for dystophin in a dystrophic environment (Khurana et al., 1991;Clerk et al., 1993;Karpati et al., 1993b). There is currently ample evidence demonstrating that the substantial increase of extrasynaptic utrophin in skeletal muscle of dystrophin-deficient mouse and dog models will greatly mitigate the dystrophic phenotype. Initial evidence supporting this theory came when *mdx* transgenics (transgenic mice engineered onto an *mdx* background) expressing a truncated utrophin were found to have a striking improvement in their dystrophic phenotype (Tinsley et al., 1996). These animals were found to have a lower amount of fibrosis/necrosis, central nucleation, serum creatine kinase levels in addition to restoring components of the dystrophin complex back to the sarcolemma. In addition, physiological parameters including mean normalized tetanic force and force drop

after sarcolemmal disruption were also improved (Deconinck et al., 1997b). These results were substantiated in studies involving *mdx* transgenics expressing full-length utrophin (Tinsley et al., 1998). An explanation for the greater effectiveness of full-length utrophin may be the presence of the complete actin binding domain, unlike the truncated version which has an incomplete domain (Ervasti, 2007). In these studies, it became evident that the level of utrophin expression dictated the extent of recovery from the dystrophic phenotype. The highest expressing transgenic lines were found to attain almost a complete reversal of pathology. Taking into account the abundance of utrophin in these lines and the amount of dystrophin in normal muscle, it was calculated that the amount of utrophin required to rescue most of the dystrophic phenotype would be approximately half the amount of dystophin found in normal muscle or ~11 times the amount of utrophin in a *mdx* muscle (Ervasti, 2007).

Previous findings have also shown utrophin, when expressed via a first generation AdV (deleted in the E1+E3 regions) to be sufficient in replacing dystrophin at the sarcolemma and in alleviating some symptoms of dystrophy (Gilbert et al., 1998;Gilbert et al., 1999;Ebihara et al., 2000;Wakefield et al., 2000;Cerletti et al., 2003). In a direct comparison to dystrophin, utrophin was found to be more efficient in stopping pathology in addition to having a greater and more sustained expression than dystrophin in adult *mdx*. However, the expression was found to decrease gradually, especially in adult mice, presumably due to the immunogenicity of the viral vector (Gilbert et al., 1999;Ebihara et al., 2000).

Another attractive feature of utrophin, as alluded to earlier, involves increasing the expression of a protein that is already present in dystrophic muscle which may prevent any transgene immunogenicity concerns that could be associated with dystrophin replacement therapy. The overexpression of utrophin, similar to dystrophin, even in non-muscle tissues was found to be non-toxic (Cox et al., 1993;Fisher et al., 2001b).

Instead of exogenous utrophin delivery, another method is the upregulation of endogenous utrophin by pharmacological intervention. One study tested the small peptide domain of the neurally secreted factor heregulin. When injected intraperitoneally into *mdx* mice (Krag et al., 2004), heregulin was found to increase utrophin expression and improved the disease phenotype; however the results were not as striking as those obtained with adenoviral gene transfer. In another study, transgenic mice expressing calcineurin were found to have an increased amount of utrophin mRNA (Chakkalakal et al., 2003). This increase was attributed to the signaling of an effector of calcineurin, NFAT. Utrophin upregulation has also been observed after administration of nitric oxide and nitric oxide donors such as L-arginine and hydroxyurea (Chaubourt et al., 1999;Chaubourt et al., 2002). Recently, it has been reported that the Ets-2 repressor factor (ERF) downregulates extra-synaptic utrophin A in skeletal muscle, thus providing another target for utrophin upregulation (Perkins et al., 2007).

Rationale and Objective

Our group had previously shown that human and murine dystrophin expression via a helper-dependent adenovirus in *mdx* mice was attainable yet it was subject to eventual loss of expression, especially in adult mice, presumably due to the immunogenicity caused by dystrophin's neoantigen state (Gilbert et al., 2003;Dudley et al., 2004). Previous work, in the context of truncated transgenes expressed by first generation adenoviruses, had also shown utrophin to be preferable to dystrophin (Gilbert et al., 1998;Gilbert et al., 1999;Ebihara et al., 2000). Thus, it was assumed that the ideal vector in treating the dystrophic pathology in *mdx* would be a helper-dependent adenovirus expressing the full-length isogenic form of utrophin.

In terms of therapeutic solutions for DMD, the ideal vector would combine low toxicity with strong and stable transgene expression. Thus, the aim of this project was to create and amplify to a high titer a helper-dependent (fully deleted) adenovirus encoding two copies of full-length murine utrophin cDNA capable of high transgene expression. The purified recombinant adenovirus was tested in vitro by western blot and subsequently injected into neonate and adult mdx mice for in vivo evaluations of dystrophic indices at various time points (10, 30, 60, 90, 180, 240 and 365 days post-injection). The injected muscles were examined for utrophin expression, the restoration of the dystrophin-associated protein complex, and the reversal of the dystrophic phenotype. In addition mice underwent physiological tests to assess muscle force generation parameters

CHAPTER 2 - Materials and Methods

Cell culture. Cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Burlington, Canada) supplemented with 30 µg/ml gentamicin, 20 mmol/l L-glutamine, and 10% fetal bovine serum (BioWhittaker, Walkersville, MD) at 37 °C in an atmosphere of 5% CO_2.

Construction of HdAdV-mUTR. To construct HdAdV-mUTR, pCMVUtrFl44 and pUC19 (MBI Fermentas, Burlington, Canada) were digested with *Bam*HI and *Xba*I, and the larger fragments of both digestions were ligated together to generate pUCFlagUtr-*Xba*I. This plasmid was then digested with *Xba*I, treated with Klenow and *Asc*I linker, and self-ligated to generate pUCFlagUtr/*Asc*I. The Flag tag was removed from the 5' end of pUCFlagUtr/*Asc*I by digestion with *Bam*HI and *Sac*II and replaced with a PCR-generated 5' end of murine utrophin flanked by *Bam*HI and *Sac*II sites and containing an *Asc*I site to generate pUCmUtrFL. The 5' utrophin PCR product was generated using the following primers: 5'-tacaggatccggcgcgccaccatggccaagtatggggac-3' and 5'-gtcatctgttcctggatctc-3'. This plasmid was digested with *Asc*I to release murine utrophin, which was then ligated with pCAGGS45 that had been pre-treated with *Xho*I and *Asc*I linker to generate pCBmUTR. The construction of HdAdV-mUTR also required pCBZ-ITR containing a dystrophin sequence, part of which served as a stuffer. This plasmid was generated from pAdDysFl and pCBLacZ.46 pAdDysFl was constructed by inserting the full-length cDNA of human dystrophin into pMCKG. This was done by first digesting pRSVDys47 with *Sal*I and *Not*I to

remove the full-length dystrophin cDNA. The ends were blunted by treatment with Klenow, ligated with *Spe*I linkers, and digested with *Spe*I. Plasmid pMCKG was digested with *Spe*I and dephosphorylated by treatment with calf intestinal phosphatase. The purified dystrophin DNA fragment and the linearized pMCKG were then ligated with each other. pCBLacZ was treated with *Hind*III followed by T4 DNA polymerase. *Asc*I linkers were added and the plasmid was digested with *Sal*I and *Asc*I to generate a 5.7-kilobase fragment. This fragment was inserted into a 13.8-kilobase fragment generated from pAdDysFI, which had been treated with *Sph*I followed by addition of *Mlu*I linkers. The 13.8-kilobase fragment was generated by digestion with *Sal*I and *Mlu*I followed by treatment with shrimp alkaline phosphatase. The two fragments from pCBLacZ and pAdDysFI were ligated together to form pCBZ-ITR. pCBZ-ITR was digested with *Bam*HI and *Sal*I along with pCBmUTR and both fragments were ligated together to form pAdV-mUTR. This plasmid was linearized with *Not*I and treated with shrimp alkaline phosphatase before digestion with *Sal*I to lose the plasmid backbone. Then two identical fragments were ligated together head to head and transfected into 293Cre4 cells (Parks et al., 1996) with LipofectAMINE (Life Technologies, Burlington, Canada) according to the manufacturer's recommendations. The virus was amplified, purified, and titered as described in detail previously (Gilbert et al., 2003). In brief, the titer (virus particles per milliliter) was determined by measuring the optical density at 260 nm and the level of helper virus contamination, which was determined by measuring the cytopathic effect after serial dilution on 293A cells was found to be 0.01%.

Western blot analysis of muscle tissues. Muscle sections were treated in sample buffer consisting of 62 mmol/l Tris–HCl (pH 6.8), 15% sodium dodecyl sulfate, 10% glycerol, 0.02% bromophenol blue, 10 µmol/l phenylmethylsulfonyl fluoride, 2.5 U aprotinin/ml, 0.5 µg leupeptin/ml. The protein concentration was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL) and the samples were treated with 5% β-mercaptoethanol before Western blot analysis. The blots were incubated with a monoclonal antibody specific for the amino terminus of utrophin (NCL-DRP2; Novocastra, Newcastle-upon-Tyne, UK). Protein expression (utrophin, dystrophin, β-dystroglycan) from *mdx* and *scid* muscles was analyzed by Western blotting using 20 µg of muscle protein (Gilbert et al., 2003). The blots were also stained with a monoclonal antibody against vinculin (V2638; Sigma-Aldrich, St. Louis, MO) as a loading control (Gilbert et al., 2003).

Intramuscular injection and histochemistry. Experiments performed on animals were carried out according to the guidelines of the Canadian Council on Animal Care. As described previously (Acsadi et al., 1996), 10 or 30 µl of AdVs, at a titer of 1.45×10^{12} virus particles/ml, were injected into the right TA of neonatal (2–4-day-old) or adult (6–8-week-old) *mdx* mice (C57BL/10ScSn-*mdx*/J; Jackson Laboratory, Bar Harbor, ME). Contralateral TA muscles which served as control were injected with sucrose-based virus storage buffer. Before the injection, the adult *mdx* mice were anesthetized by intraperitoneal injection of 2.5% avertin. Injections in neonatal (2–4-day-old) *scid* mice (C3SnSmn.CB17-*Prkdc*^{scid}/J; Jackson Laboratory, Bar Harbor, ME) were conducted in the same

manner. The animals were killed at 10, 30, 60, 90, 180, and 365 days after injection, and the TA muscles were removed and snap-frozen in liquid nitrogen–cooled isopentane. Cryostat sections were immunostained for utrophin as described previously (Gilbert et al., 1999) using a monoclonal antibody specific for the amino terminus of utrophin. The staining was visualized using either horseradish peroxidase- or Cy-3-conjugated streptavidin (Jackson Immuno Research Laboratories, West Grove, PA). For each injected muscle, the total number of transduced fibers was determined by counting the number of utr^+ fibers on a single cryostat section, which spans the entire TA cross-section. Some sections were also stained using an antibody against β-dystroglycan (NCL-43DAG; Novocastra, Newcastle-upon-Tyne, UK), and α-sarcoglycan (NCL-50DAG; Novocastra, Newcastle-upon-Tyne, UK) (Gilbert et al., 2003). Utrophin-positive muscle fibers with centrally located nuclei were viewed by double-staining TA cross-sections with a utrophin antibody (NCL-DRP2; Novocastra, Newcastle-upon-Tyne, UK) and Hoechst. With regards to the trichostatin A (TSA, Sigma) injections, nine week old *mdx* mice were treated intraperitoneally with TSA for a duration of 3 months at a dose of 0.6mg/kg/day. A subset of these mice were injected in their TA muscles with HdAdV-mUTR at a dose of 4.5×10^{10} viral particles on the third day following the commencement of their TSA treatment. At ~3 months post-injection, the TAs of these mice were subjected to physiological experiments to determine force generating capacity. Mice were subsequently sacrificed and utrophin expression in the TAs was analyzed as described above.

Cellular and humoral response. We measured the humoral response against murine utrophin by transfecting dishes of 293A cells with 1μg of a plasmid containing murine utrophin (pCBmUTR) using Tranfectin (Bio-Rad Laboratories, Hercules, CA) according to the recommended protocol. The next day, the cells were lysed and 50 μg of protein was separated on a 5% sodium dodecyl sulfate gel and processed for Western blot analysis as described previously (Gilbert et al., 2001). The sera of *mdx* mice injected with HDAdV-mUTR were used as primary antibody. The presence of utrophin antibodies in the tested serum was implied by the detection of a utrophin band on the blot (Gilbert et al., 2003). The presence of cellular infiltrates caused by HDAdV-mUTR was verified by staining consecutive TA sections for macrophages or $CD8^+$/$CD4^+$ T lymphocytes using rat monoclonal antibodies Mac1, anti-CD8a (Ly 2; Cedarlane, Hornby, Canada), and anti-CD4 (L3T4, BD Biosciences Pharmingen, Mississauga, Canada), respectively. Mac1 was prepared from supernatant of rat hybridoma (M1/70.15.11.5HL) obtained from the American Type Culture Collection (Rockville, MD). A rat anti-mouse biotinylated antibody (Jackson Immuno Research Laboratories, West Grove, PA) followed by horseradish peroxidase–conjugated streptavidin (Jackson Immuno Research Laboratories, Grove, PA) was used to visualize the signal (Gilbert et al., 2003). Each section was analyzed for infiltrates in predominantly utr^+ areas (greater than 50% transduction).

Real-time PCR for analysis of adenoviral DNA. Real-time PCR using TA muscle sections has been described in detail previously (Dudley et al., 2004). In brief, 70 cryostat sections from each muscle were treated with lysis buffer (100

mmol/l Tris–HCl pH 8.5, 5 mmol/l EDTA, 0.2% sodium dodecyl sulfate, 200 mmol/l NaCl containing 20 mg/ml proteinase K) and left overnight in 0.5 ml at 50 ℃. The samples were then treated with a phenol/chloroform extraction followed by isopropanol precipitation. DNA pellets were re-suspended in TE buffer and read on a spectrophotometer. DNA (200 ng) along with 2.5 µl of 10x PCR buffer, minus magnesium (Invitrogen, Burlington, Canada), 0.75 µl of 50 mmol/l magnesium chloride, 2.5 µl of 2.5 mmol/l deoxyribonucleotide triphosphate, 7.5 pmol of probe, and 10 pmol of each primer in 25-µl volumes was used to run the PCR in a Smart Cylcer (Cepheid, Sunnyvale, CA). The protocol consisted of 95 ℃ for 10 minutes followed by 45 cycles of 94 ℃ for 1 minute, 58 ℃ for 1 minute, and 72 ℃ for 1 minute. PCR products were generated using primers and probe for a portion of the promoter (5'-cccacttggcagtacatcaa-3', 5'-atggggagagtgaagcagaa-3', 5'-FAM-cgtcaatgacggtaaatggcccgcct-TAMRA-3'; Operon Biotechnologies, Huntsville, AL). Values were then normalized against amounts obtained using primers and probe for the murine adipsin gene (5'-acgtgagacccctacccttg-3', 5'-gactaccccgtcatggtacg-3', 5'-TET-tcacccatgcaggacgcaggcctgatgt-TAMRA-3'; Operon Biotechnologies, Huntsville, AL). In regards to TSA experiments, 293A cells were infected with HdAdV-mUTR at an MOI of 300 viral particles. Five hours later, half of these samples were treated with 25nM TSA. RNA was isolated from these cells 24 hours later using TRIZOL (Invitrogen, Burlington, Canada) and samples then underwent reverse transcription using MMLV-RT (Invitrogen, Burlington, Canada) according to the manufacturer's protocols. Equal amounts of DNA along with 2.5 µl of 10x PCR

buffer, minus magnesium (Invitrogen, Burlington, Canada), 0.75 µl of 50 mmol/l magnesium chloride, 2.5 µl of 2.5 mmol/l deoxyribonucleotide triphosphate, 12.5 pmol of each primer and 2.5 µl SYBR Green I (Roche Diagnostics, Laval, Canada) in 25-µl volumes was used to run the PCR in a Smart Cycler (Cepheid, Sunnyvale, CA). The protocol consisted of 95 °C for 10 minutes followed by 40 cycles of 95 °C for 30 seconds, 52 °C for 30 seconds, and 72 °C for 30 seconds. PCR products were generated using primers for a portion of the utrophin cDNA (5'-aaacagggaggcacattgtc-3', 5'-gcccaggtcattgtagagga-3'; Operon Biotechnologies, Huntsville, AL). Values were then normalized against amounts obtained using primers for the murine GAPDH gene (5'-gtgttcctacccccaatgtg-3', 5'-aggagacaacctggtcctca-3'; Operon Biotechnologies, Huntsville, AL).

In vivo *measurement of force generation.* This protocol had been described in great length previously (Gilbert et al., 2003;Dudley et al., 2004). Neonatal and adult *mdx* mice muscles, treated and contralateral untreated, were analyzed for force generation capabilities and their ability to withstand stressful conditions. In brief, the distal tendons of TAs were isolated and tied to a lever arm of a force transducer/length servomotor system (model 305B dual mode; Cambridge Technology, Watertown, MA). The TA was placed at optimal muscle length (L_0, the length at which maximal twitch force is achieved), after which it was subjected to two twitch stimulations and the mean value was considered as maximal isometric twitch force. The muscle was then stimulated at 120 Hz for 300 ms to measure maximal isometric tetanic force. A series of high-stress eccentric (lengthening) contractions was performed next to determine the

muscle's ability to withstand such pressures. Each contraction involved supramaximal stimulation at 120 Hz for a total of 300 ms; the muscle was held at L_0 during the initial 100 ms (isometric component) and then lengthened through a distance of 25% of L_0 during the last 200 ms (eccentric component). Once stimulation had stopped, peak muscle length was held for an additional 100 ms, followed by a return to L_0 during the next 100 ms. This was repeated five times with a 2-minute interval between each contraction. To determine the final level of isometric force production, a 120-Hz stimulation was performed at L_0. To take into account the magnitude of mechanical stress placed upon the muscle, the isometric force deficit induced by each eccentric contraction was normalized by dividing the percentage force drop by the level peak stress (N/cm^2) attained during the preceding eccentric contraction.

Effects of Interferon-gamma (IFN-γ) on promoter shutdown. C2C12 cells were mock infected or infected with AdCMVLacZ or HdCBLacZ at an MOI of 500 infectious particles. 20 hours later, half of the cells in each group were treated with IFN-γ (50U/mL). The cells were harvested 24 hours later and β-galactosidase activity was measured by luminometry using the Galacto-star System (Applied Biosystems, Bedford, Mass.).

Creatine kinase assay. The sera of experimental *mdx* mice was used to measure for levels of creatine kinase enzyme. The assay was conducted using a Creatine Kinase (NAC) Assay kit (DCL, Charlottetown, Canada) according to the manufacturer's protocol. The levels were subsequently read and analyzed using the Softmax Pro software (Molecular Devices Corp., Sunnyvale, CA).

Measurement of muscle fiber diameters. Using ImageJ (NIH, Bethesda, Maryland) photos of TA cross sections were used to determine the fiber diameters of experimental mice. Depending on the subset measured, every visible fiber was measured at its smallest width where the connecting line can pass through the midpoint at the center of the fiber.

Effects of Trichostatin A on promoter expression. C2C12 cells were infected with HdCBLacZ at an MOI of 120 infectious particles. 5 hours post-infection, cells were treated with three different concentrations of TSA (25nM, 250nM and 1μM). Cells were harvested 24 hours later and assayed for total protein using the BCA Protein Assay Kit (Pierce, Rockford, IL) and for β-galactosidase expression using the Galacto-Star System (Applied Biosystems, Bedford, Mass.).

Statistical analysis. Unless stated otherwise the data are expressed as the mean ±SEM. The data were analyzed using an unpaired two-tailed *t*-test or with an analysis of variance. Comparisons of the response to eccentric contractions were analyzed by two-way analysis of variance. Statistical significance was set at $P< 0.05$.

RESULTS

CHAPTER 3 - *In vitro* characterization of HdAdV-mUTR

Verification of utrophin expression cassette. Before proceeding with the construction of the adenovirus, the utrophin expression cassette needed to be validated. Thus, a western blot analysis was done on 293A cells transfected with the pCBmUtrFL plasmid encoding the full-length utrophin expression cassette which would then be used for the viral production (Figure 3). A plasmid encoding a flag-tagged utrophin transgene used previously (Guo et al., 1996) was chosen as a positive control. Non-transfected 293A cells were used as negative control. The transfection of pCBmUtrFL was done in duplicate and robust expression was seen in both samples at levels equivalent to the positive control.

Generation of a helper-dependent adenovirus expressing murine utrophin. The helper dependent adenovirus expressing utrophin (HdAdV-mUTR) was modeled after the previously described vector that was used for gene transfer of the full-length dystrophin cDNA (Gilbert et al., 2003;Dudley et al., 2004). Briefly, it consists of two copies of the full-length murine utrophin cDNA driven by the hybrid chicken β-actin promoter/CMV enhancer (CB), arranged in head-to-head fashion. A genomic fragment from the human dystrophin locus (nucleotides 9910 to 12101, Genbank accession number NM 004010) was included as stuffer sequence to attain optimal viral packaging size (Figure 4). In addition, the fully deleted adenovirus vector contained a packaging signal and was flanked at both

ends by inverted terminal repeats (ITR) which are essential for DNA replication. Analysis of HdAdV-mUTR viral DNA was done by restriction analysis and Southern blotting (data not shown). The infectivity of the virus was verified *in vitro* by western blot of infected 293A cells (Figure 5).

Figure 3. Western blot analysis of protein expression obtained from a plasmid encoding utrophin cDNA. Cell lysates from 293A transfected cells were electrophoresed on a SDS-PAGE. The first lane contained the negative control, a mock transfected 293A sample. Lanes 2 and 3 contain lysates of cells transfected with the plasmid of interest, pCBmUtrFl while the last lane contains lysates obtained with the positive control, pFLAG-Utr. Full-length utrophin gives a band at 395 kDa. However, lower bands represent degradation products.

Figure 3

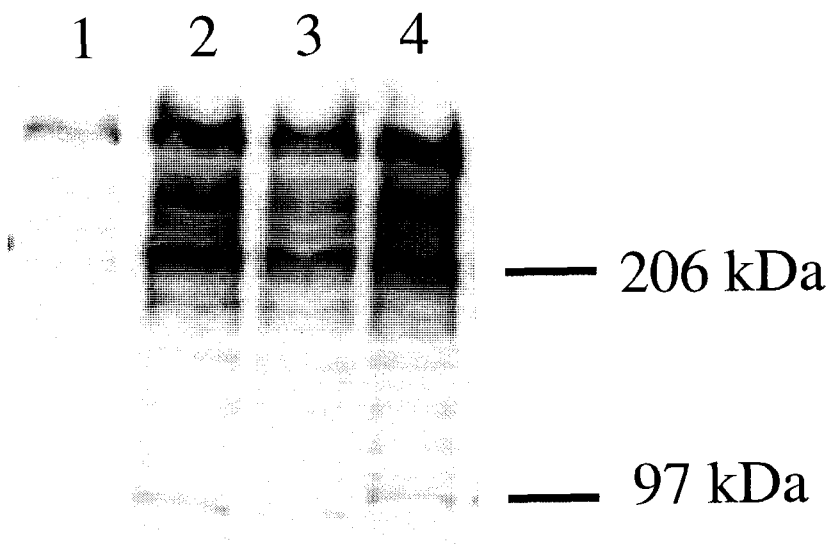


Figure 4. Schematic map of HdAdV-mUTR. Fully deleted "gutted" adenovirus incorporating a double murine utrophin cDNA expression cassette controlled by the hybrid CB promoter, consisting of the chicken β-actin promoter and CMV enhancer. Note the inclusion of human dystrophin genomic sequences as stuffer regions necessary to attain optimal viral packaging size. The 3' end of dystrophin was included in order to optimize any advantages the the 3' untranslated region may have on transgene expression and persistence. The adenovirus vector is flanked at both ends by AdV-specific inverted terminal repeats (ITR) which are essential for DNA replication. The total size of the recombinant AdV is approximately 31.4 kb.

Figure 4

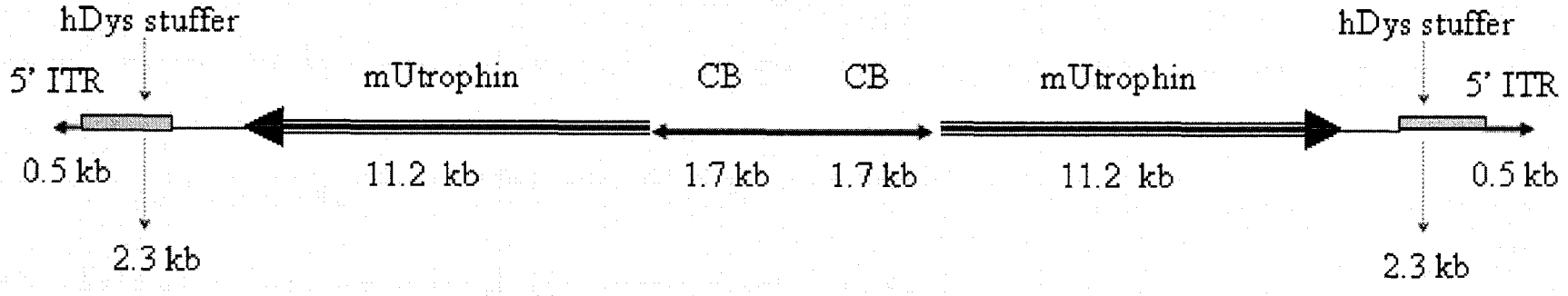
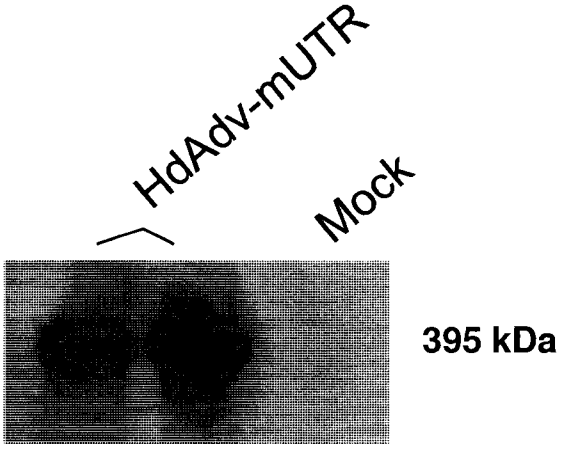

hDys stuffer
5' ITR
mUtrophin
CB
CB
mUtrophin
hDys stuffer
5' ITR
0.5 kb
11.2 kb
1.7 kb
1.7 kb
11.2 kb
0.5 kb
2.3 kb
2.3 kb

Figure 5. Western blot analysis of HdAdV-mUTR utrophin expression. Cell lysates obtained from infected 293A cells at an MOI of 300 viral particles (as opposed to infectious particles; with a ratio of virus to infectious particles of ~ 100:1) were electrophoresed on a SDS-PAGE. The first two lanes are samples from cells infected with HdAdV-mUTR while the last lane represents a negative control, a mock infected 293A sample.

Figure 5



CHAPTER 4 – Analysis of *mdx* neonates following HdAdV-mUTR injection

Strong initial widespread transduction in *mdx* neonates persists at early timepoints before gradually decreasing. The extent of gene transfer obtained after a single intramuscular injection of the purified HdAdV-mUTR (1.45×10^{10} virus particles) was first evaluated in neonate *mdx* mice. Tibialis anterior (TA) muscle sections were immunostained for utrophin at 10, 60 and 240 days post-injection (Figure 6). In the first group of neonates (n=5) at 10 days post-injection, the mean number of sarcolemmal utr-positive (utr+) fibers in injected vs control-injected TA was 1596 +/- 297 vs 114 +/- 76, which corresponds to a ~58 % transduction level. Utrophin did not display a mosiac pattern, indicative of fibre type specificity but rather a uniform distribution. Utrophin expression was stable at the two early time points (at least up to 60 days). However, at the 240 day time point, despite significant expression in some mice, the total number of utr+ fibers dropped to an average of 188 +/- 193 vs 18 +/- 12 , but the difference between the injected and uninjected sides remained significant (two-tailed paired p value=0.0115) (Figure 7).

Western blot analysis of utrophin levels in neonate muscle sections mimics *in vivo* results. Western blot analyses were performed on injected (+) and control (-) muscle sections. Vinculin, a cytoskeletal protein, was used as a loading control. Western analysis on sections from mice 10 days post-injection was not possible due to the small size of the sections at that age. At 60 days

post-injection, there was strong utrophin expression seen in injected muscles compared to control samples (Figure 8). However by 240 days post-injection, as utrophin expression decreased *in vivo*, the difference in utrophin expression between injected and control samples by western analysis was not as evident throughout all the samples (data not shown).

Restoration of dystrophin associated proteins after HdAdV-mUTR administration in neonate *mdx*. The functional consequences of utrophin expression were also assessed in these cohorts of animals. Lack of dystrophin in DMD patients and *mdx* mice leads to the loss of the dystrophin associated protein complex (DPC) from the sarcolemmal membrane. The localization of two members of the DPC was analyzed at all time points. The immunostaining of both α -sarcoglycan and β –dystroglycan was detected on the sarcolemmal membrane in utr+ fibers at all time points (10 and 240 days are shown), indicating that utrophin expression could indeed restore the DPC (Figure 9).

Efficient utrophin transducion in *mdx* neonates prevents muscle degeneration. The *mdx* TA muscle is characterized by cycles of necrosis and regeneration. A hallmark of muscle regeneration, the presence of central nucleation of muscle fibers was verified in neonate *mdx* mice injected with HdAdV-mUTR (Figure 10). A consistent and significant difference was observed in the percentage of central nucleation between utr+ and utrophin negative (utr-)

fibers, even at the 240 day time point. These data suggest that the initial utrophin expression protected the muscle fibers from undergoing necrosis.

Persistent utrophin expression improves muscle physiological indices in neonate *mdx* mice. Muscle physiological analyses were carried out at 60 and 240 days post-injection on neonate *mdx* mice treated with HDAdV-mUTR (Figure 11). At both time points, AdV injected muscles showed increased resistance to eccentric contraction-induced stress compared to contralateral control muscles. At 60 days post-injection (Figure 11A), force generation was increased as compared to contralateral uninjected muscle, and at the latter time point, there was at the very least a tendency towards improved force generation in the AdV injected muscles. Furthermore, up to 240 days post-injection (Figure 11B), the injected muscles were significantly less hypertrophic, as shown by lower individual muscle weights and lower muscle to body weight ratios, as compared to contralateral control muscles. Taken together, these results show that a single intramuscular injection of HdAdV-mUTR in the neonate period was sufficient to have a long-term, significant impact on the pathological progression that is usually observed in *mdx* mice.

No increase in immune response in *mdx* neonates due to increased utrophin. Cellular immune factors did not seem to be involved in the loss of utr+ fibers since no increase in cellular infiltrates (either CD4+, CD8+ cells, or macrophages) were ever detected in abundance in the vicinity of utr+ fibers at

any time point compared to contralateral control muscles (Figure 12B). As well, the sera of injected mice were checked for the presence of anti-utrophin antibodies by western blot analysis at all timepoints. Sera from mice showing high and low levels of transduction were tested at different concentrations of sera. No antibodies were ever detected in the tested sera (Figure 12A).

Figure 6. Utrophin expression following injection of neonatal *mdx* mice with HdAdV-mUTR. The TAs of four day old animals were injected once with purified HdAdV-mUTR. Cryostat sections were immunostained for utrophin at the indicated timepoints post-injection. Scale bar = 190 µm for top two panels and 450 µm for bottom panel.

Figure 6

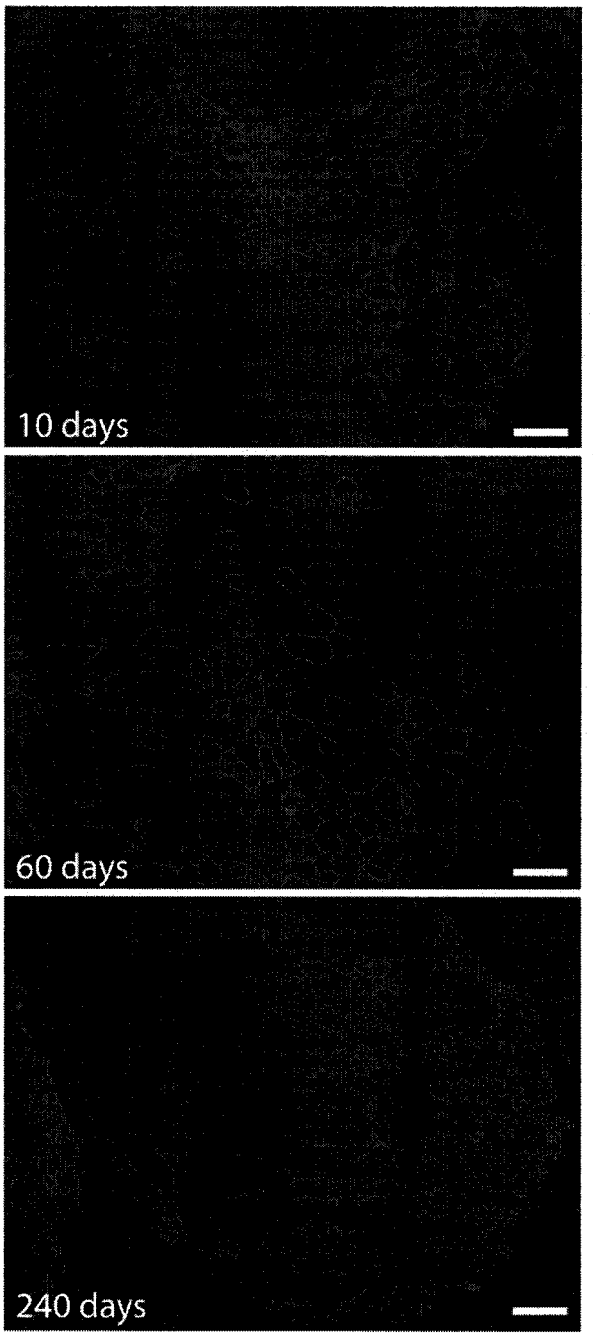


Figure 7. Utrophin expression in *mdx* neonates throughout the course of this study. A scatterplot representing the quantification of utrophin positive fibers for each animal. The lines represent the means of each timepoint. The values for the contralateral control sides were 114+/-76, 74+/-57, and 18 +/- 12 at 10, 60 and 240 days respectively.

Figure 7

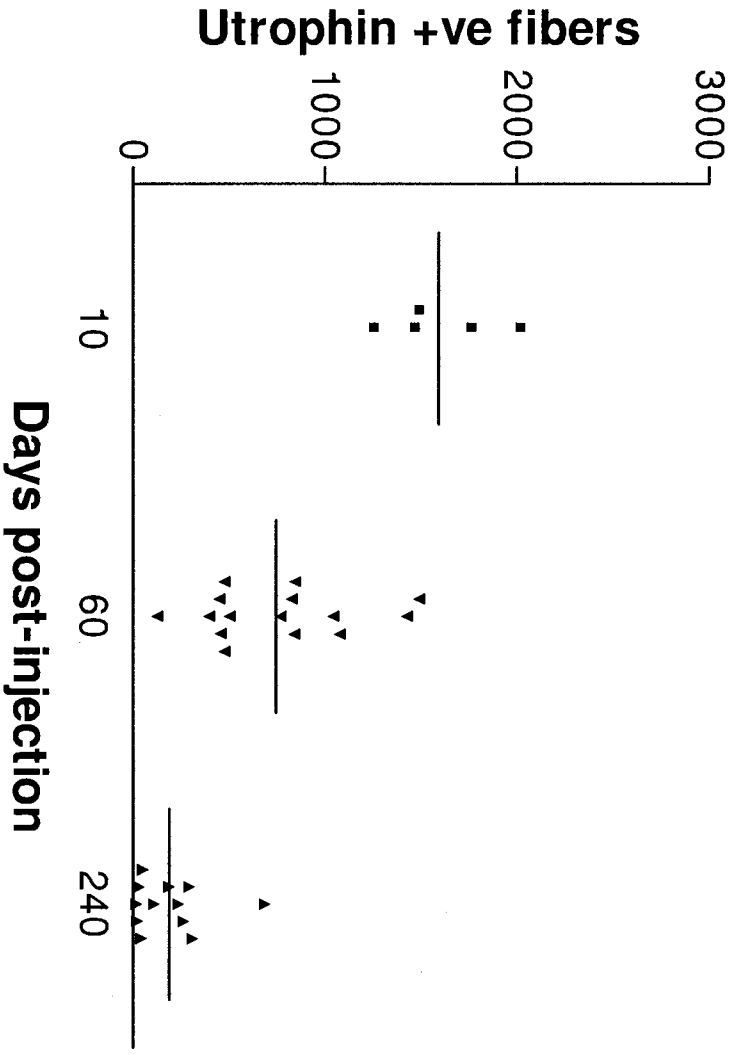

Utrophin +ve fibers
3000
2000
1000
0
10
60
240
Days post-injection

Figure 8. Evidence of exogenous utrophin expression in neonatal *mdx* mice by western blot analysis. Twenty micrograms of each muscle extract was analyzed using an antibody against utrophin or vinculin which served as a loading control. Injected (+), uninjected (-).

Figure 8

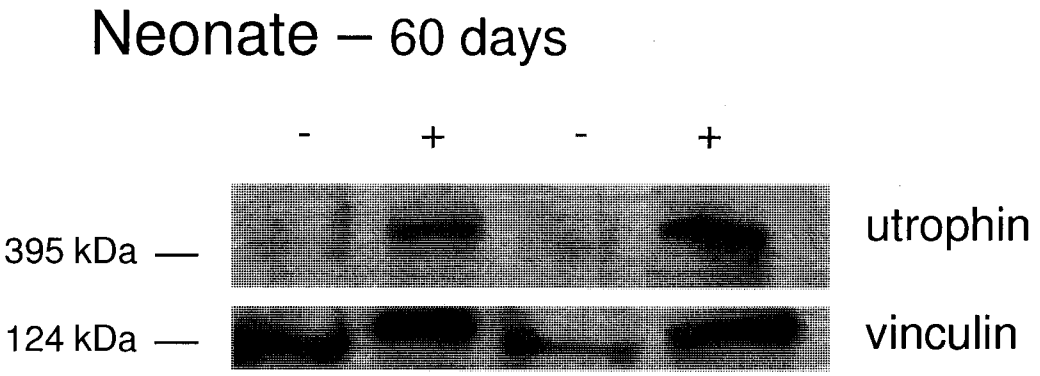
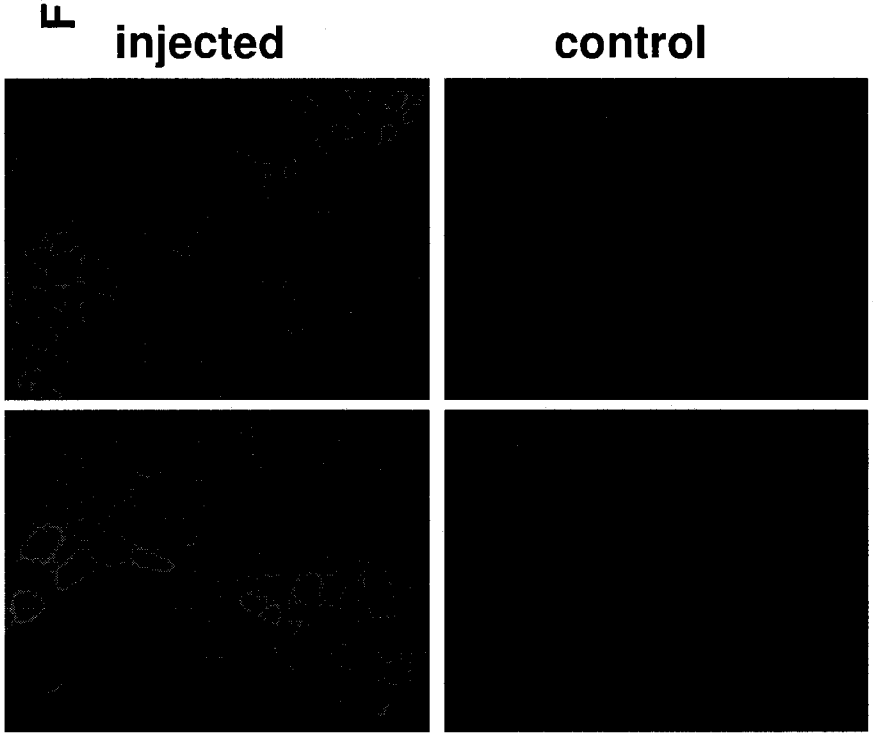
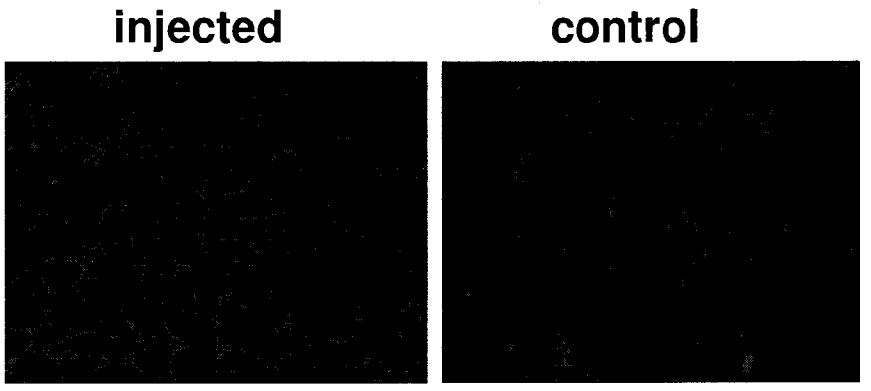


Figure 9. Restoration of the dystrophin protein complex in neonatal *mdx*. TA muscle cross sections of neonate *mdx* mice stained for utrophin, α -sarcoglycan and β -dystroglycan 10 (A) and 240 (B) days post-injection. A. Virus injected samples are shown on the left while contralateral control samples are shown on the right. For utrophin, images at magnifications at both 100x (top) and 200x (bottom) are shown. B. Serial staining for utrophin and dystrophin associated proteins. Asterisk marks same fiber in each section. Scale bar = 190 μm.

Figure 9A


injected
control
utrophin


injected
control
α-sarcoglycan

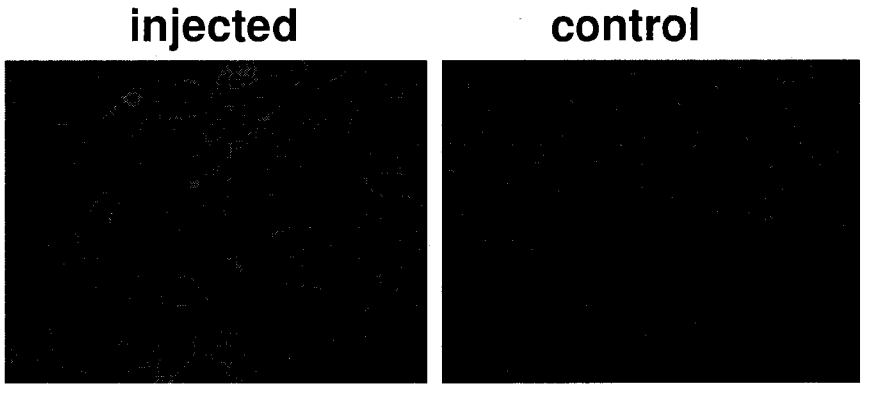

injected
control
β-dystroglycan

Figure 9B

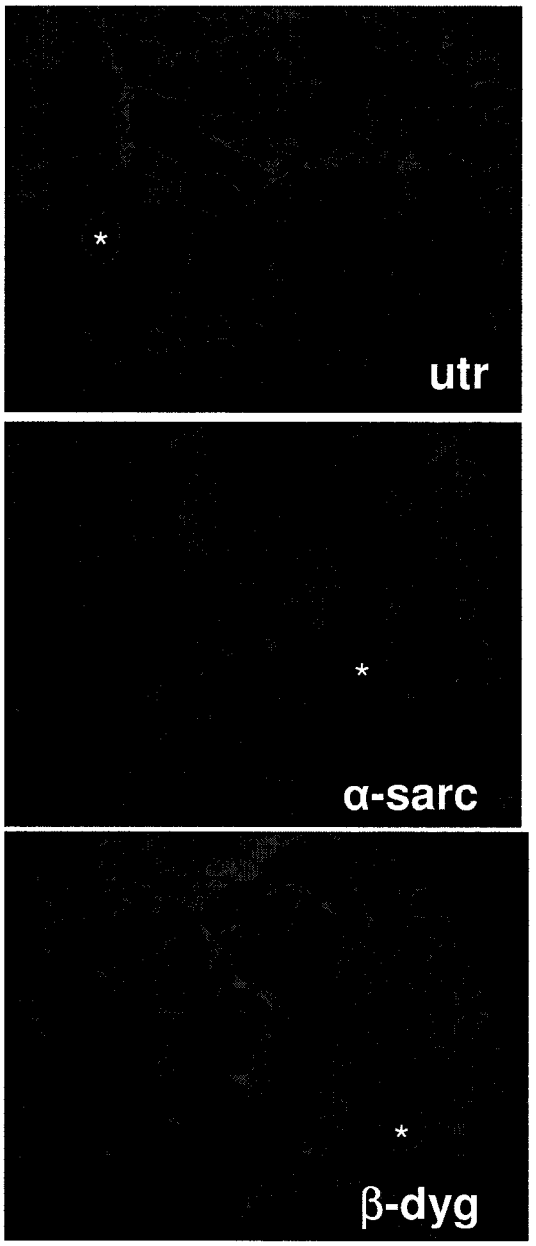


Figure 10. Prevention of muscle degeneration due to utrophin expression. At the indicated timepoints, the percentage of muscle fibers with central myonuclei was determined in utrophin-positive (total + fibers counted = 7067, 2203, and 2020 for the three timepoints) compared to utrophin-negative (total - fibers counted = 6134, 2113 and 1750 for the three timepoints) fibers in neonatal *mdx*. The error bars represent SEM. N=5, 15, 12 for 10, 60 and 240 days respectively. Statistical significance was determined by a one-way ANOVA, $p < 0.0001$.

Figure 10

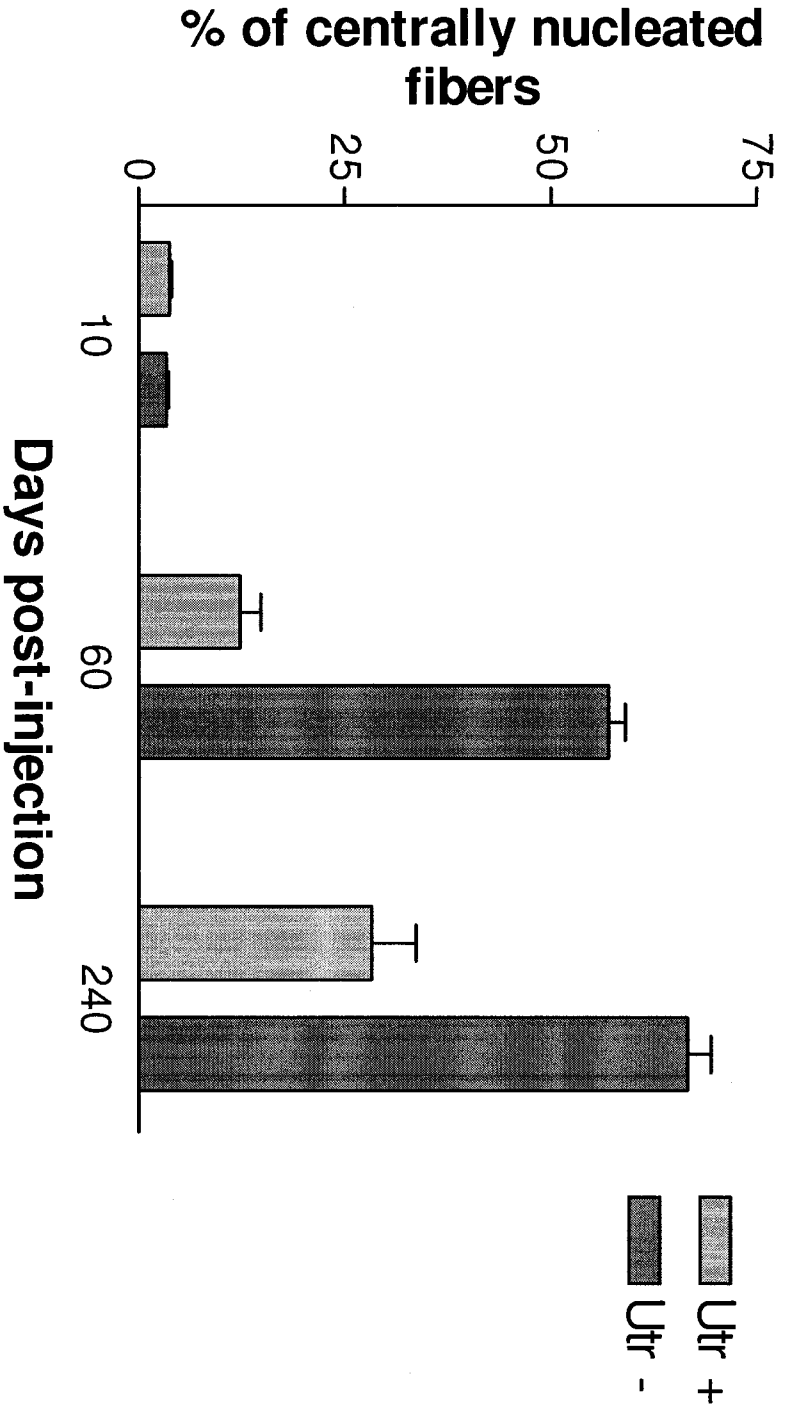

% of centrally nucleated fibers
0
25
50
75
10
60
240
Days post-injection
Utr +
Utr -

Figure 11. Improvement of force generation in muscles after injection of HdAdV-mUTR in the neonatal period. The right TA of neonatal *mdx* mice was injected with HdAdV-mUTR and the contralateral TA with buffer. Muscles were analyzed at 60 (A) and 240 (B) days post-injection. Each individual muscle was tested for force generating capacity, resistance to stress induced by lengthening contractions, weight and ratio of muscle weight to body weight. All error bars represent SEM.

Figure 11

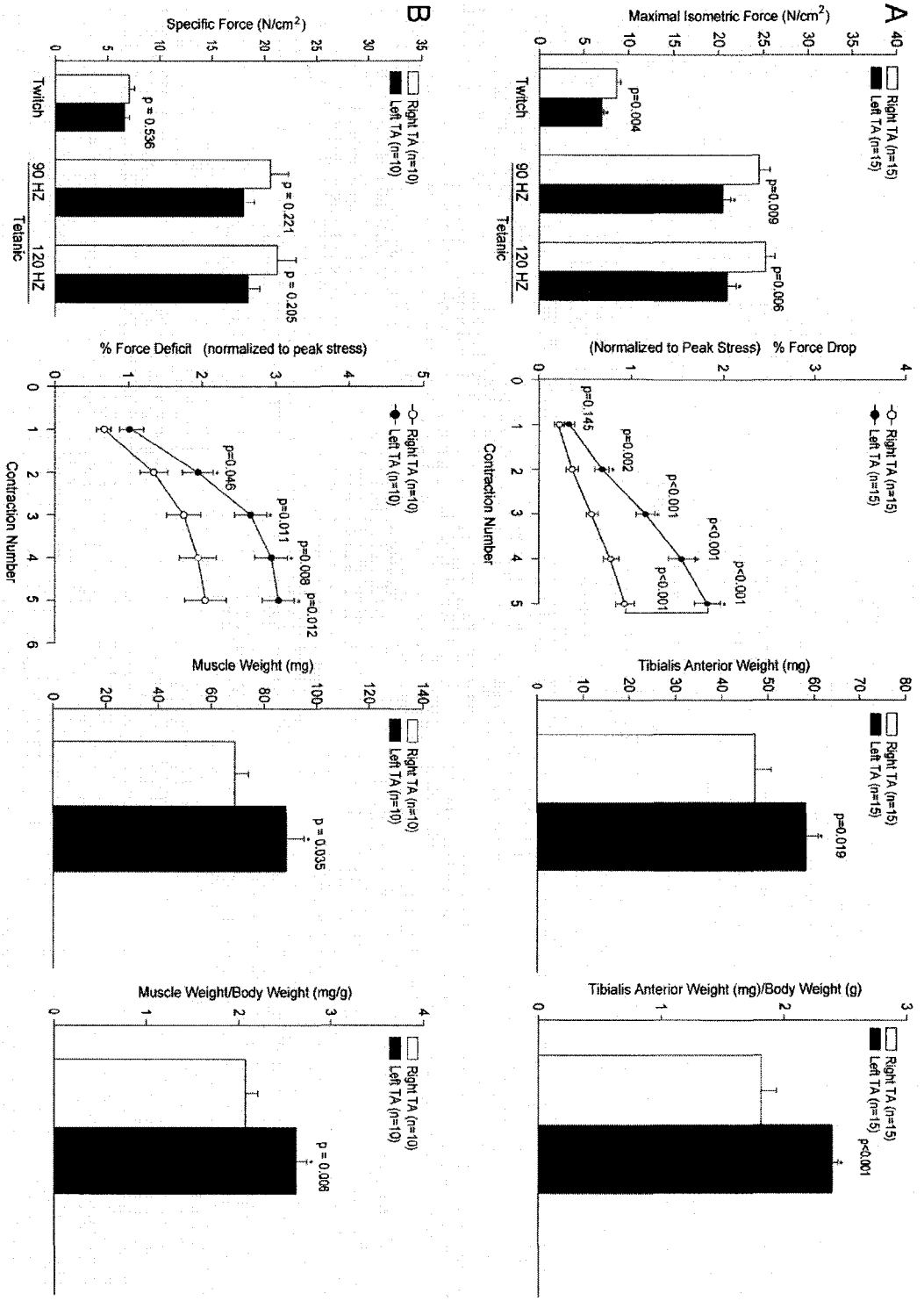
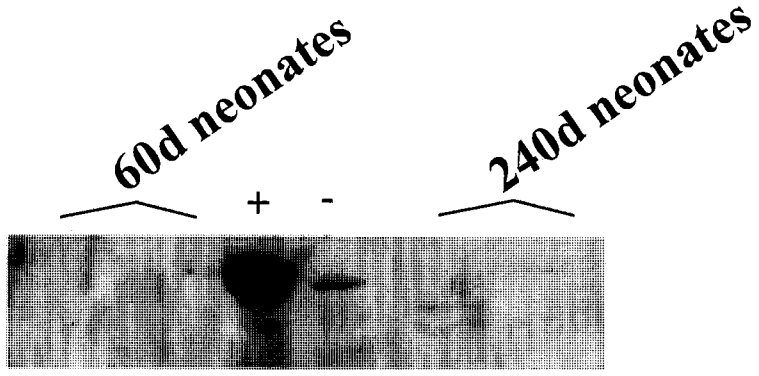

A
Maximal Isometric Force (N/cm²)
Right TA (n=15)
Left TA (n=15)
p=0.004
p=0.009
p=0.056
Twitch
90 HZ
120 HZ
Tetanic
(Normalized to Peak Stress) % Force Drop
p=0.145
p=0.002
p<0.001
p<0.001
p<0.001
Contraction Number
Tibialis Anterior Weight (mg)
p=0.019
Tibialis Anterior Weight (mg)/Body Weight (g)
p<0.001
B
Specific Force (N/cm²)
Right TA (n=10)
Left TA (n=10)
p = 0.536
p = 0.221
p = 0.205
% Force Deficit (normalized to peak stress)
p=0.046
p=0.011
p=0.008
p=0.012
Muscle Weight (mg)
p = 0.035
Muscle Weight/Body Weight (mg/g)
p = 0.008

Figure 12. Treatment of *mdx* muscle with HdAdV-mUTR results in a very mild immune response. (A) Antibodies against utrophin were not detected in the sera of *mdx* mice injected with HdAdV-mUTR at all timepoints. The tested sera, used at two different concentrations, were from mice injected during the neonatal stage, and that displayed low or high utrophin transduction. Mock transfected 293A cells (-) or cells transfected with a plasmid encoding murine utrophin (+) served as controls. (B) Low level of cellular immunity in TAs of *mdx* mice exposed to HdAdV-mUTR. The first panel of cryostat sections depict utrophin immunostaining while the second shows immunostaining for macrophages. The third column is a merged picture of the co-staining. The panels originate from an *mdx* mouse injected as a neonate and euthanized at 50 days post-injection. Scale bar = 95 µm.

Figure 12

A



B

CHAPTER 5 – Analysis of *mdx* adults following HdAdV-mUTR injection

Initial high transduction in adult *mdx* after HDAdV-mUTR injection is not maintained long term. Six to eight week old *mdx* mice were injected in the TA at a single site with the same preparation of HdAdV-mUTR that was used in the neonates, but at the higher dose of 4.35 x 10^{10} virus particles. Subsequently, TA muscle sections of adult *mdx* mice were analyzed for utrophin expression at 10, 30, 60, 90, 180 and 365 days post-injection (Figure 13). At the 10 day time point, the mean number of utrophin positive fibers in the adults (n=7) was 685 +/- 505 and 112 +/- 80 for AdV and control injected TAs respectively, which corresponds to ~23% transduction level. (Overall, the number of utr+ fibers corresponds to an approximate 14 and 6 fold increase in utr expression over control levels in neonates and adults respectively). High levels of transduction were also obtained at the 30 day time point, with the mean number of utrophin positive fibers (n=7) being 770 +/- 223 which corresponds to a ~35% transduction level. Despite these very high initial levels, utrophin expression dropped significantly at 60 days and beyond. The decrease was observable both on immunostaining of TA cross-sections (Figure 13) and on immunoblots (data not shown). A scattergraph depicting the total number of utr+ fibers per TA shows the sudden and dramatic drop of utrophin (Figure 14). Remarkably, despite this average drop there were still some mice which continued to express utrophin at relatively high overall amounts and high concentrations on a per fiber basis even at 180 days post-injection.

Western blot analysis of utrophin levels in adult muscle sections mimics *in vivo* results. Western blot analyses were done on injected (+) and control (-) muscle sections. At 10 and 30 days post-injection, there was strong utrophin expression seen in injected muscles compared to control samples (Figure 15). However by 60 days post-injection, as utrophin expression decreased *in vivo*, the difference in utrophin expression between injected and control samples by western analysis was not as evident throughout all the samples (data not shown). Vinculin was used as a loading control.

Restoration of dystrophin associated proteins after HdAdV-mUTR administration in adult *mdx*. The functional consequences of utrophin expression were also assessed in these cohorts of animals. The localization of two members of the DPC was analyzed at all time points (Figure 16). The immunostaining of both α -sarcoglycan and β –dystroglycan was detected on the sarcolemmal membrane in utr+ fibers at all time points (10 and 180 days are shown), indicating that utrophin expression could indeed restore the DPC. Arrows indicate the normal localization of utrophin at the neuromuscular junction in *mdx* muscle.

Efficient utrophin transducion in *mdx* adults halts further muscle degeneration. In order to compare the effect of HdAdV-mUTR between *mdx* mice injected in the neonate and adult stage, these *mdx* mice were subjected to

the same analyses as those performed in the neonatally injected mice. Adult *mdx* already demonstrate underlying pathology at the age at which they are injected, having undergone at least one cycle of necrosis/regeneration, and thus have a substantial number of centrally located nuclei. Nevertheless, there still were significant differences in the percentage of central nucleation between utr+ and utr- fibers (Figure 17). Even 365 days post-injection, the utrophin expressing fibers showed decreased central nucleation, indicating that the AdV injection provided some long-term protection.

Adult muscle physiological indices appear to be a function of utrophin expression. Adult *mdx* mice treated with HdAdV-mUTR were also evaluated for muscle physiological studies at 60 and 180 days post-injection (Figure 18). At 60 days post-injection, AdV injected muscles showed increased resistance to eccentric contraction-induced stress compared to contralateral control muscles (Figure 18A). However, no difference was seen in this parameter at 180 days post-injection, indicating that there was neither improvement nor deleterious consequence from HdAdV-mUTR (Figure 18B). Unlike the effect observed in neonates, no difference was seen between AdV injected muscles and contralateral control muscles in the magnitude of force generation or in the state of hypertrophy as assessed by muscle weight at any of the time points. These results indicate that adenovirus-mediated utrophin expression can prevent progression of the dystrophic pathology in the adults for a certain period of time

but that the effects are not sustained due to progressive decline of gene expression.

Very mild immune response in *mdx* adults due to increased utrophin. Immune factors did not seem to be involved in the loss of utr+ fibers since no increase in cellular infiltrates (either CD4+, CD8+ cells, or macrophages) were ever detected in abundance in the vicinity of utr+ fibers at any time point compared to contralateral control muscles (as detected by immunofluorescent or H&E staining) (Figure 19B,C). As well, the sera of injected mice were checked for the presence of anti-utrophin antibodies by western blot analysis at all timepoints (Figure 19A). Sera from mice showing high and low levels of transduction were tested at different concentrations of sera. No antibodies were ever detected in the tested sera.

HDAdV-mUTR mediated utrophin expression in *scid* mice follows *mdx* pattern of expression. In order to determine whether there was any involvement of the immune system in the loss of utrophin expression, injections of HdAdV-mUTR were performed in the immune deficient *scid* mouse. Utrophin expression was determined at 10, 30, 90 and 180 days post-injection (Figure 20). Similar to the observations in the *mdx*, the transgene expression was quite high at the two earlier timepoints but decreased dramatically by 90 days post-injection (Figure 21).

Viral DNA content in skeletal muscle. To determine how the kinetics of fiber loss correlated with the amount of viral DNA present at any time point in neonate and adult *mdx*, as well as the *scid* mice, Real-Time PCR was used to quantitate viral DNA present in the TA at the different time points (Figure 22). In the adult *mdx*, similar to utrophin expression, the amount of viral DNA also decreased dramatically but slightly earlier, between 10 and 30 days post-injection. On the other hand, minimal loss of viral DNA occurred either in the neonate *mdx* or in the *scid*s. These data suggest that both loss of the episomal DNA and decreased gene expression from the AdV, possibly due to promoter shutdown, are major contributing factors to the observed loss of utr+ fibers.

Shutdown of CB promoter in the presence of murine interferon gamma. To determine whether cytokines such as interferon gamma might be responsible for the observed loss of transgene expression, an *in vitro* study was conducted in which C2C12 cells were infected with HdCBLacZ or AdCMVLacZ. Half of these samples were subsequently treated with murine interferon gamma to investigate whether the CB promoter (which includes the CMV enhancer) can be downregulated similar to the CMV promoter/enhancer (Harms and Splitter, 1995;Qin et al., 1997;Sung et al., 2001). Based on these previous experiments involving interferon gamma and viral vectors, samples were treated with interferon gamma once at a dose of 50 units/mL 20 hours post-infection. Cells were then harvested 24 hours post-treatment and beta-galactosidase expression was quantitated by lumunometry (Figure 23). There was a small (18%) but

significant decrease ($p=0.04$) in CB promoter expression in the presence of interferon gamma. The CMV promoter activity was more affected (27% decrease) by interferon gamma. Therefore, the inclusion of the CMV enhancer in the CB promoter may play a role in promoter shutdown especially in an *in vivo* context where interferon gamma may be present in higher doses and for longer durations.

Figure 13. Utrophin expression following injection of mature *mdx* mice with HdAdV-mUTR. Six to eight week old *mdx* mice were injected once in the TA with HdAdV-mUTR. TA cryostat sections of mature *mdx* mice immunostained for utrophin were analyzed for utrophin expression at 10, 30, 60, 90, 180 and 365 days post-injection. Scale bar = 190 µm for top row and 450 µm for bottom two rows.

Figure 13

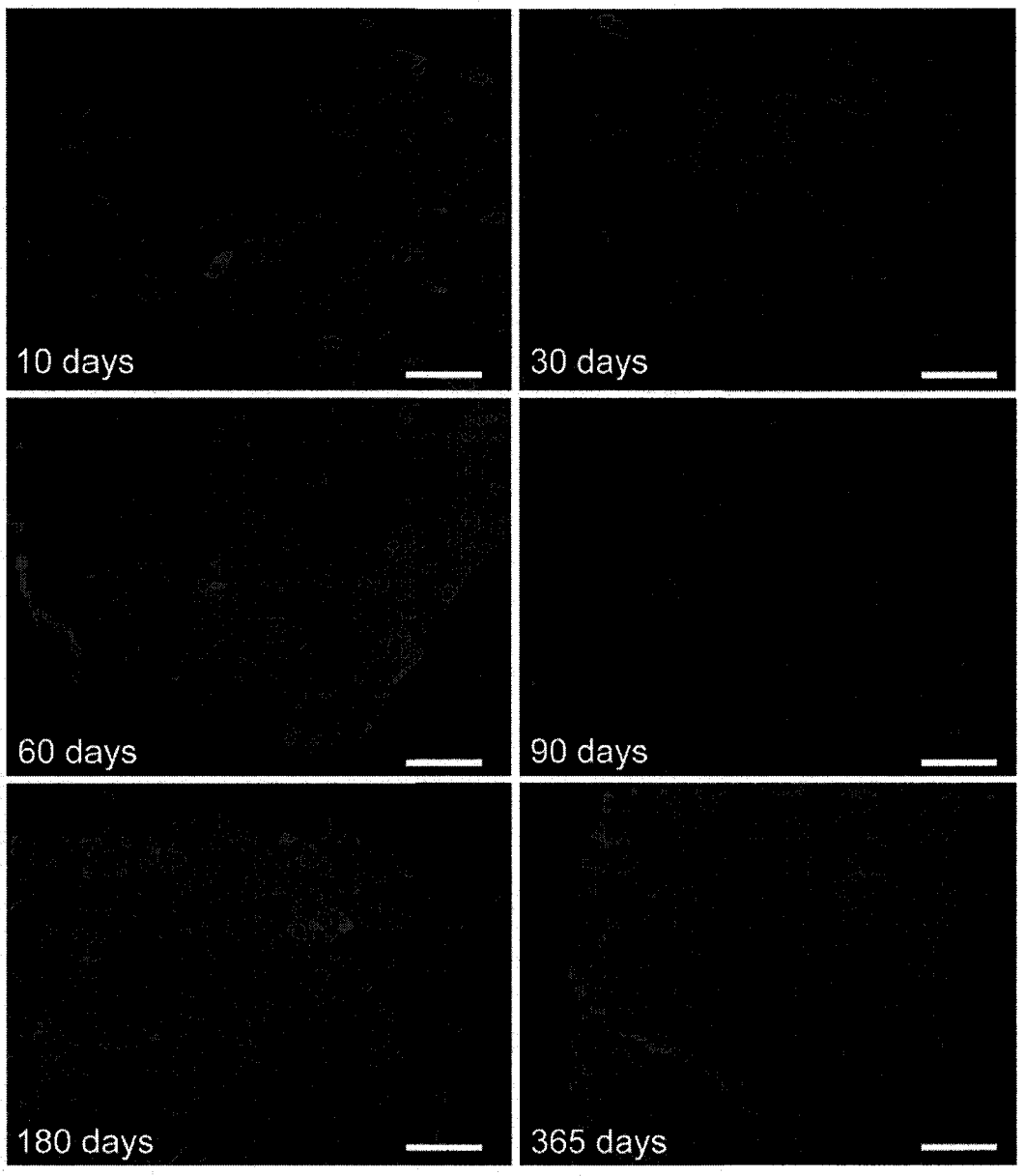


Figure 14. Utrophin expression in *mdx* adults throughout the course of this study. A scatterplot representing the quantification of utrophin positive fibers per animal. The lines represent the means of each timepoint. The values for the contralateral control sides were 112+/-80, 267+/-60, 21+/-12, 13+/-8, 21+/-21 and 30+/-27 at 10, 30, 60, 90, 180 and 365 days respectively.

Figure 14

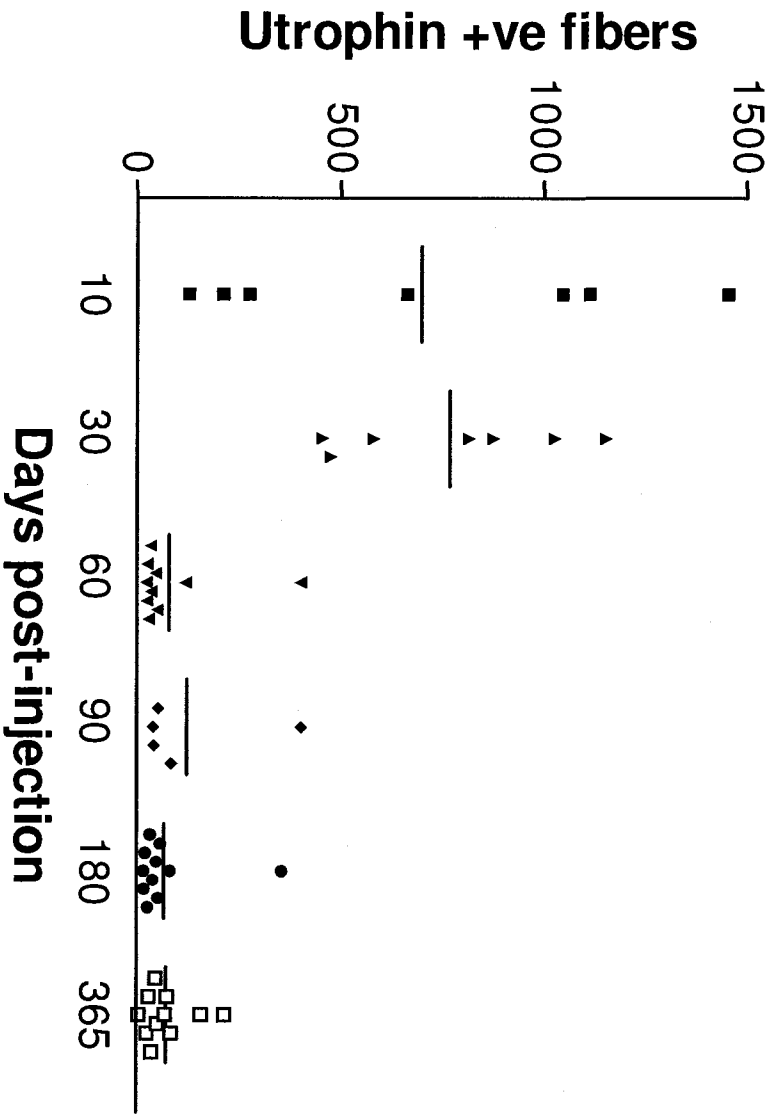

Utrophin +ve fibers
0
500
1000
1500
10
30
60
90
180
365
Days post-injection

Figure 15. Evidence of exogenous utrophin expression in mature *mdx* mice by western blot analysis. Twenty micrograms of each muscle extract was analyzed using an antibody against utrophin or vinculin which served as a loading control. Injected (+), uninjected (-).

Figure 15

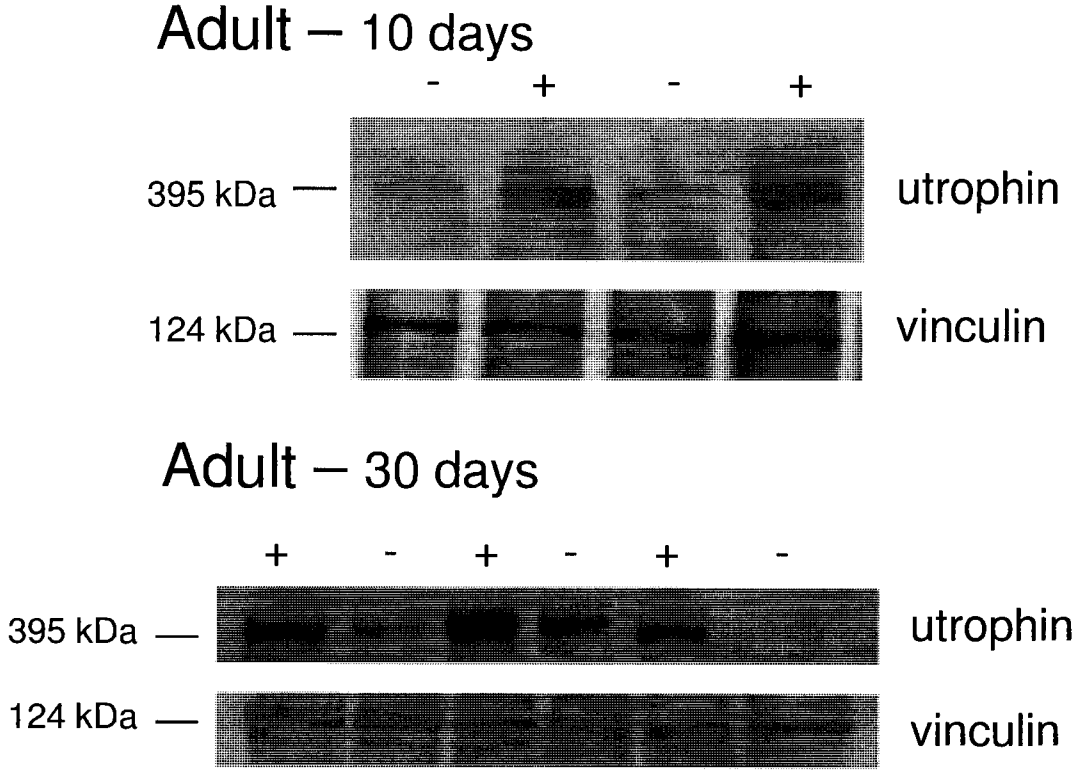

Adult – 10 days
- + - +
395 kDa
utrophin
124 kDa
vinculin
Adult – 30 days
+ - + - + -
395 kDa
utrophin
124 kDa
vinculin

Figure 16. Restoration of the dystrophin protein complex in adult *mdx*. Anterior tibialis muscle cross sections of adult *mdx* mice injected with HdAdV-mUTR were stained for utrophin, α-sarcoglycan and β-dystroglycan at 10 (A) and 180 (B) days post-injection. A. Virus injected samples are shown in the top row while contralateral control samples are shown at the bottom. Arrows indicate the normal localization of utrophin at the neuromuscular junction in *mdx* muscle. B. Inset box highlights utrophin transduced area along with serial staining for dystrophin associated proteins, α-sarcoglycan and β-dystroglycan at 180 days post-injection. Scale bar = 190 μm

Figure 16A

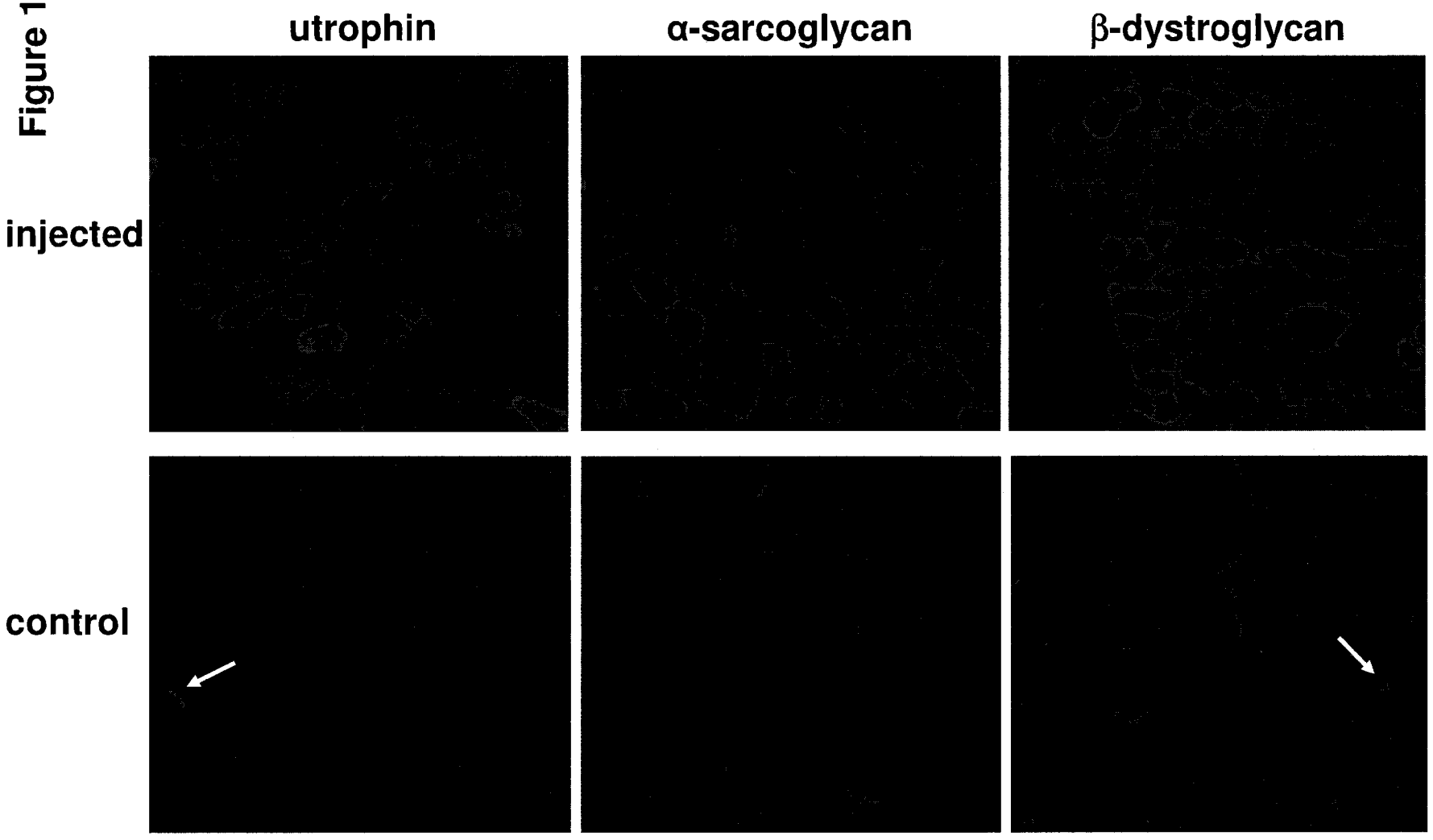


Figure 16B

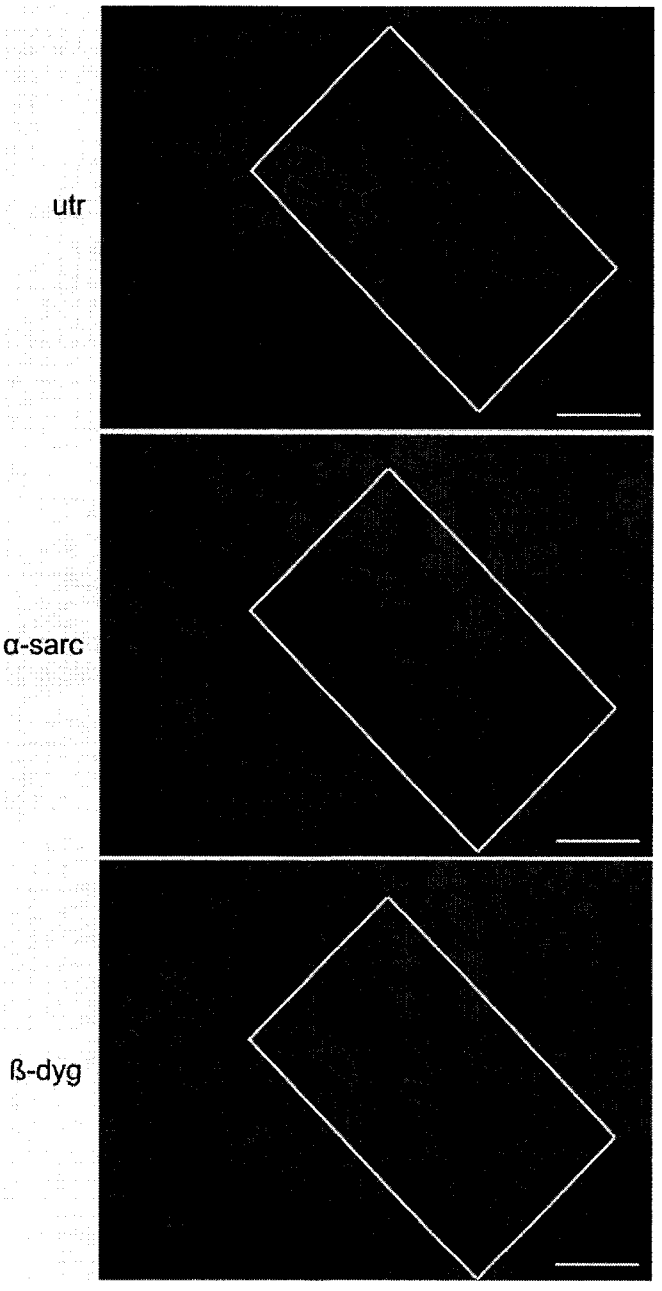


Figure 17. Muscle degeneration halted due to utrophin expression. At the indicated timepoints, the percentage of muscle fibers with central nuclei was determined in utrophin-positive (total + fibers counted = 4610, 5230, 973, 534, 1217 and 1617 for the five timepoints) compared to utrophin-negative (total - fibers counted = 16993, 9610, 3233, 900, 1800 and 1650 for the five timepoints) fibers in adult *mdx*. The error bars represent SEM. N=7, 7, 10, 6, 9, 11 at 10, 30, 60, 90, 180 and 365 days respectively. Statistical significance was determined by a one-way ANOVA, $p < 0.0001$.

Figure 17

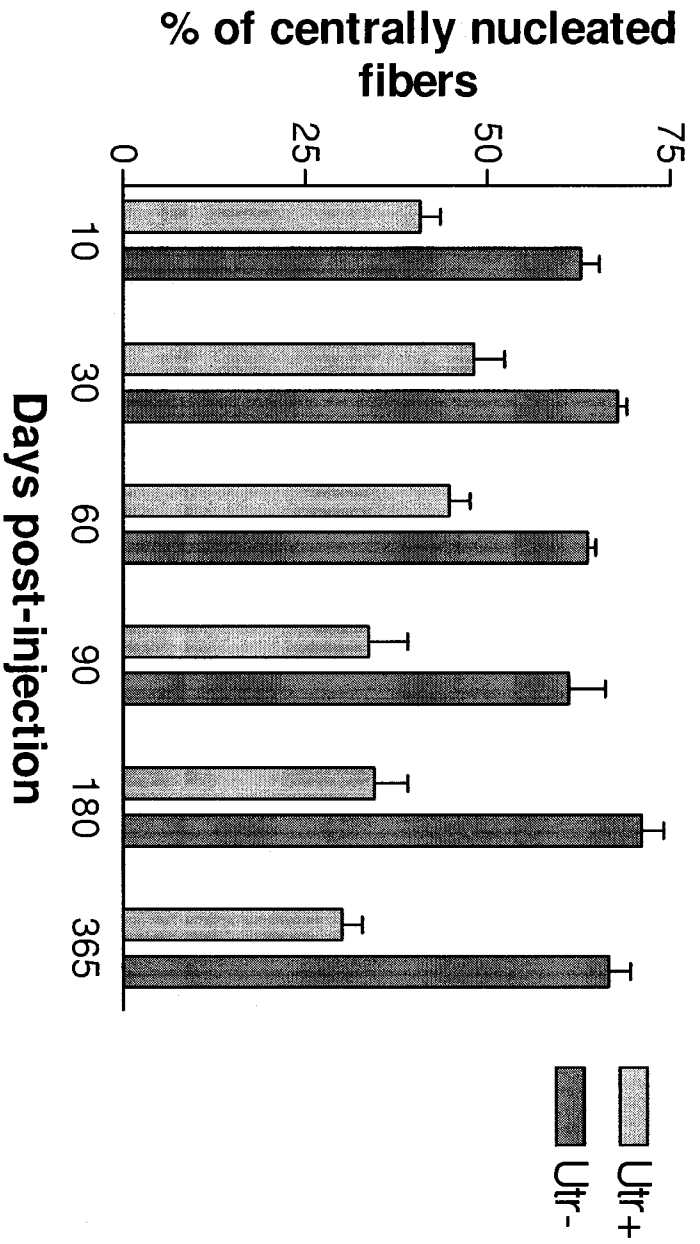

% of centrally nucleated fibers
0
25
50
75
10
30
60
90
180
365
Days post-injection
Utr+
Utr-

Figure 18. Physiological analyses of *mdx* injected with HdAdV-mUTR in the adult stage. The right TA of mature *mdx* mice was injected with HdAdV-mUTR and the contralateral TA with buffer. Muscles were analyzed at 60 (A) and 180 (B) days post-injection. Each individual muscle was tested for force generating capacity and resistance to stress induced by lengthening contractions. All error bars represent SEM. * $p<0.04$

Figure 18

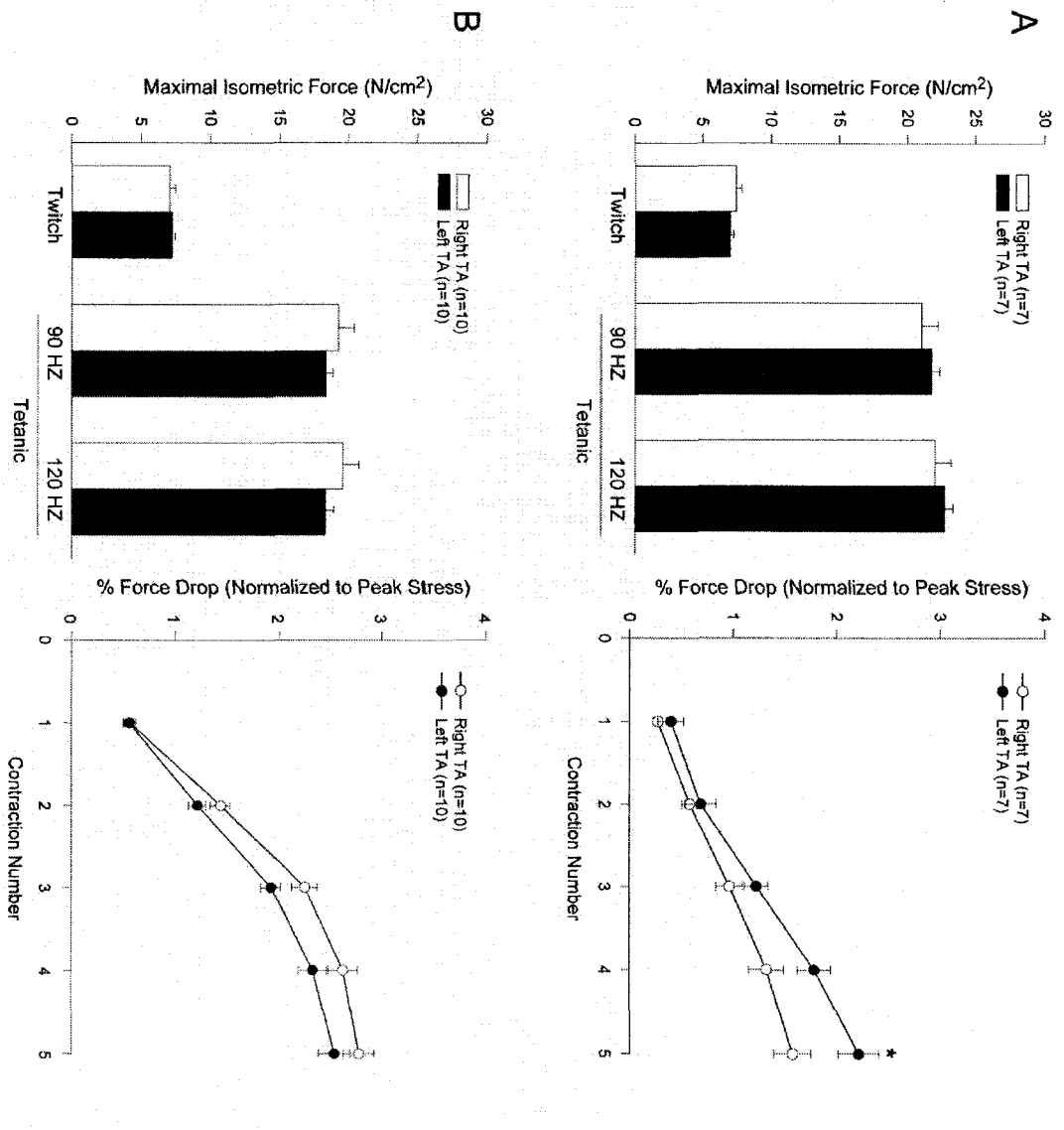

A
Maximal Isometric Force (N/cm2)
Right TA (n=7)
Left TA (n=7)
Twitch
90 HZ
120 HZ
Tetanic
% Force Drop (Normalized to Peak Stress)
Contraction Number
*
B
Maximal Isometric Force (N/cm2)
Right TA (n=10)
Left TA (n=10)
Twitch
90 HZ
120 HZ
Tetanic
% Force Drop (Normalized to Peak Stress)
Contraction Number

Figure 19. Treatment of adult *mdx* muscle with HdAdV-mUTR results in a very mild immune response. (A) Antibodies against utrophin were not detected in the sera of adult *mdx* mice injected with HdAdV-mUTR at all timepoints. The tested sera, used at two different concentrations, were from mice that displayed low or high utrophin transduction. Mock transfected 293A cells (-) or cells transfected with a plasmid encoding murine utrophin (+) served as controls. (B) Low level of cellular immunity in TAs of *mdx* mice exposed to HdAdV-mUTR. The first column of cryostat sections depict utrophin immunostaining while the second shows immunostaining for specific immune components (top: macrophages, bottom: CD8). The third column is a merged picture of the co-staining. The panels in both rows originate from *mdx* mice injected in the adult stage and euthanized at 10 and 30 days post-injection respectively. Scale bar = 95 μm. (C) Decreased presence of cellular infiltrates in TAs injected with HdAdV-mUTR in the adult stage (30 days post-injection). Cryostat sections were stained with hematoxylin and eosin. Top two panels originate from injected muscle shown at low and high magnification. Bottom panel is of contralateral control side.

Figure 19

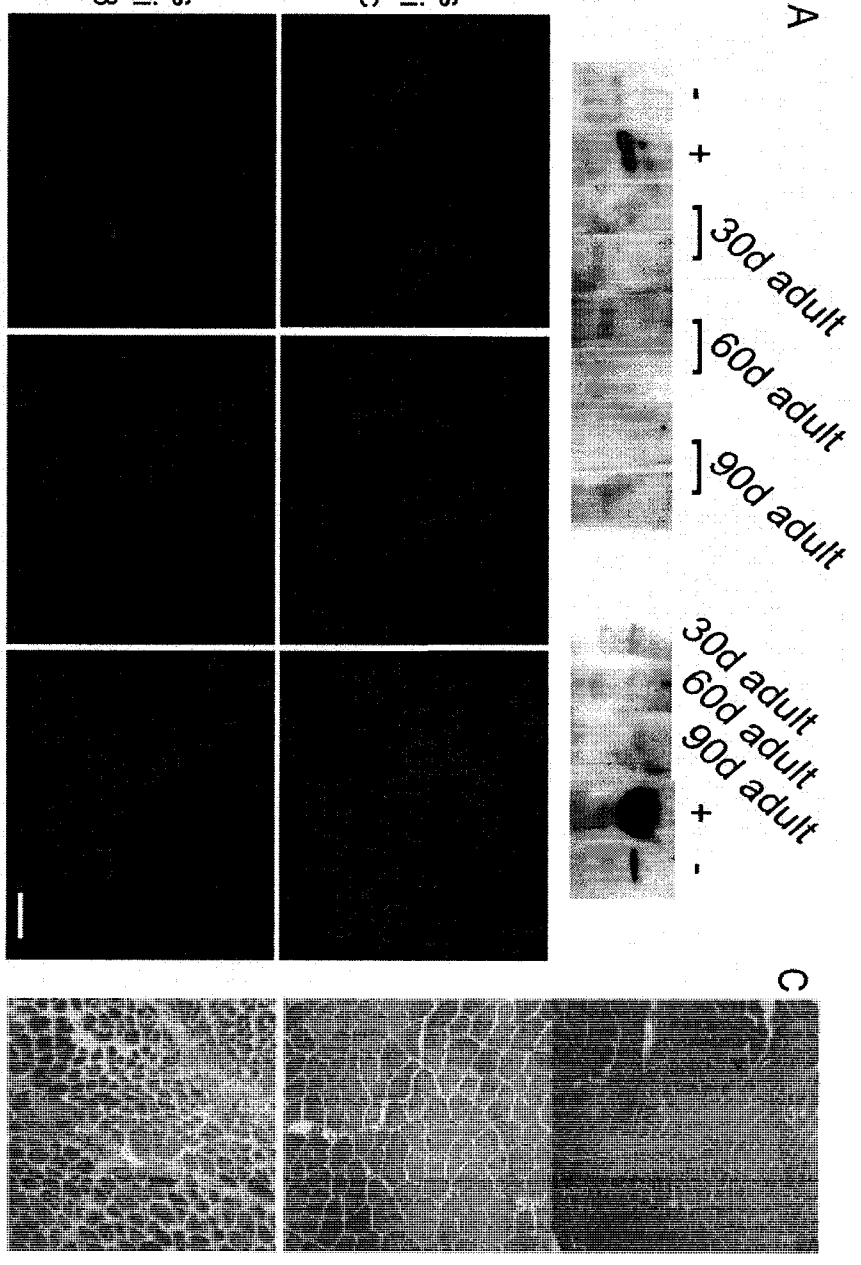

A
-
+
30d adult
60d adult
90d adult
30d adult
60d adult
90d adult
+
-
B
Adults
10d pi
Utr / mac
Adults
30d pi
Utr / CD8
C

Figure 20. Utrophin expression in muscles of *scid* mice injected in the neonatal period. Two to four day old *scid* mice were injected bilaterally in the TA with HdAdV-mUTR at a dose of 1.5 x 1010 virus particles. As with the *mdx* mice, *scid*s were euthanized at the indicated timepoints post-injection and utrophin expression was assessed on TA muscle sections.

Figure 20

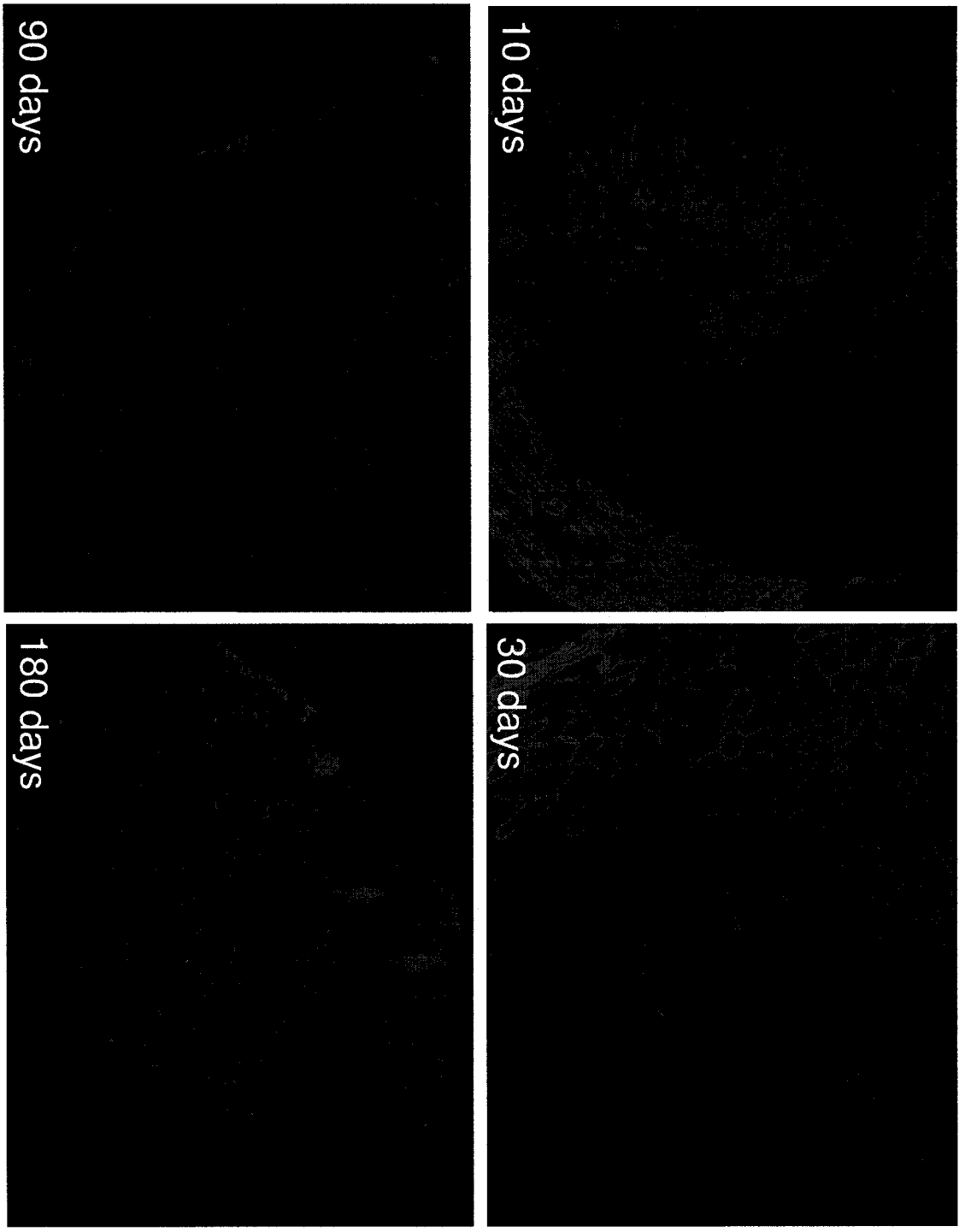


Figure 21. Utrophin expression throughout the course of the *scid* study. A scatterplot representing the quantification of utrophin positive fibers per animal. The lines represent the means of each timepoint.

Figure 21

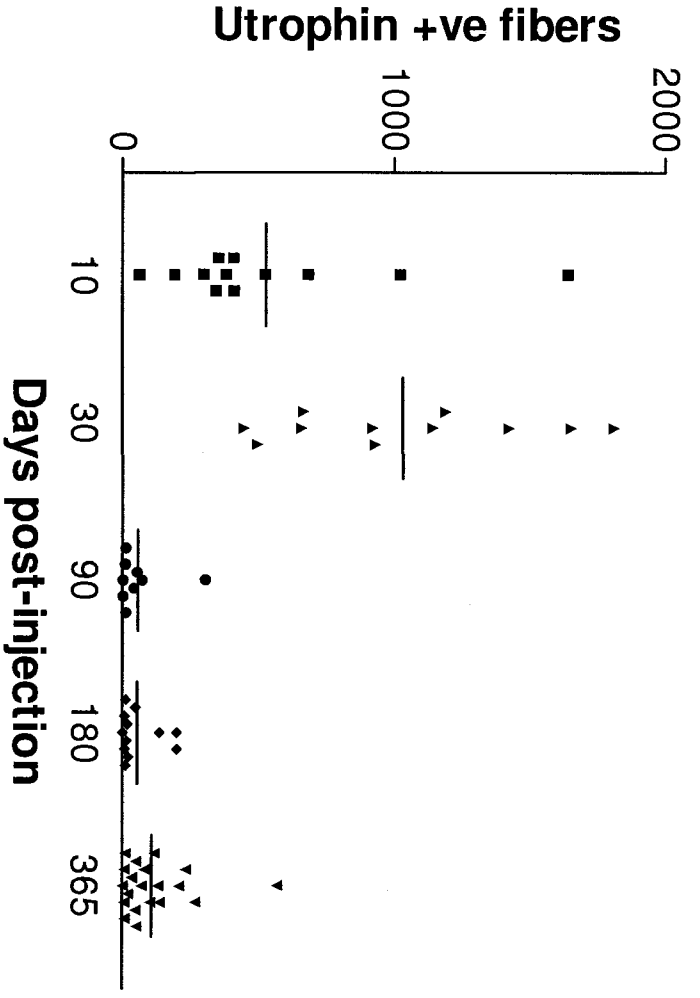
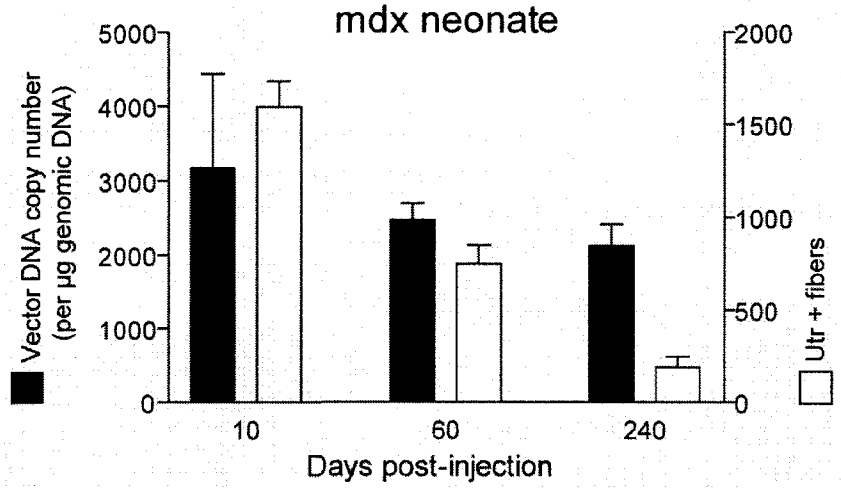
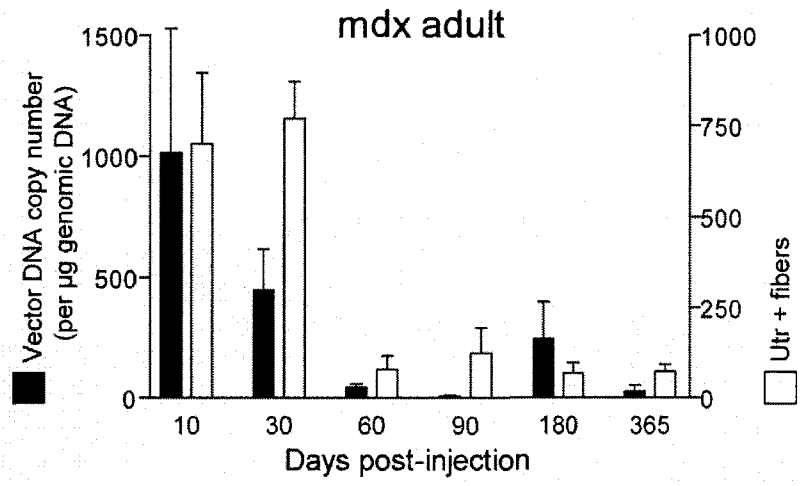

Utrophin +ve fibers
0
1000
2000
Days post-injection
10
30
90
180
365

Figure 22. Quantitation of viral vector DNA loss in TA muscle. Viral vector DNA was determined in three groups of animals at various timepoints by real-time PCR. The corresponding number of utrophin positive fibers at each timepoint is also shown for comparison. All error bars represent SEM. N=3,3,6 for the SCID timepoints, n=4,3,3 for *mdx* neonates and n=4,3,3,3,3,3 for the *mdx* adult timepoints. Statistical significance was determined by a Kruskal-Wallis test. (P = 0.03 for *mdx* adult mice and non-significant for *mdx* neonates and *scid* mice).

Figure 22


mdx neonate
Vector DNA copy number (per µg genomic DNA)
5000
4000
3000
2000
1000
0
2000
1500
1000
500
0
Utr + fibers
10
60
240
Days post-injection


mdx adult
Vector DNA copy number (per µg genomic DNA)
1500
1000
500
0
1000
750
500
250
0
Utr + fibers
10
30
60
90
180
365
Days post-injection

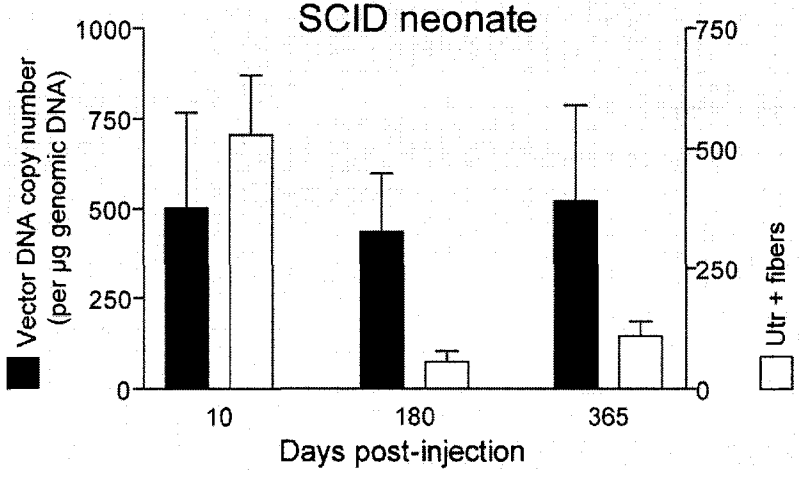
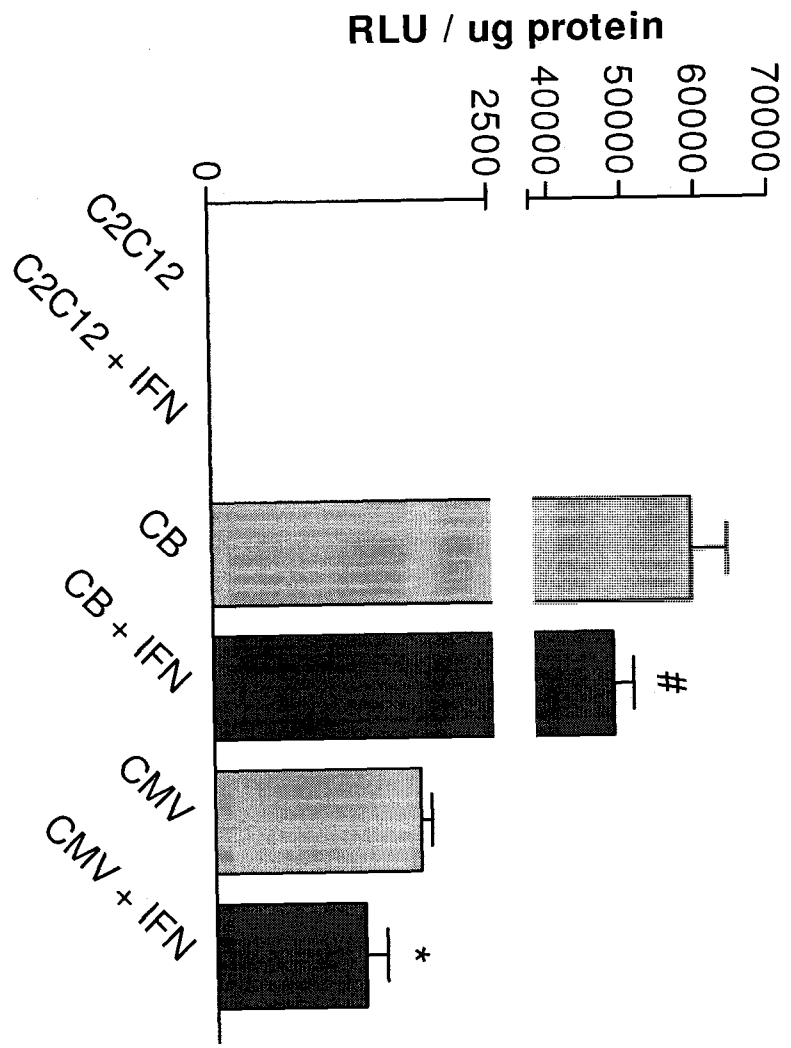

SCID neonate
Vector DNA copy number (per µg genomic DNA)
1000
750
500
250
0
750
500
250
0
Utr + fibers
10
180
365
Days post-injection

Figure 23. Effects of Interferon gamma on CB promoter activity. C2C12 cells were infected with a helper dependent adenovirus encoding LacZ under the control of the CB promoter (HdCBLacZ). Half of the HdCBLacZ samples were then treated with IFN-gamma once (50U/mL) and collected 24 hours later for assessment of beta-galactosidase activity. Samples were similarly infected with the first generation adenovirus AdCMVLacZ and treated with IFN-gamma for use as a positive control. Statistical significance was determined by a one-tailed unpaired t-test. #p=0.0422, *p=0.0203.

Figure 23


RLU / ug protein
0
2500
40000
50000
60000
70000
C2C12
C2C12 + IFN
CB
CB + IFN
CMV
CMV + IFN
#
*

CHAPTER 6 – Effects of increasing amounts of exogenous utrophin on dystrophic pathology.

HdAdV-mUTR of similar titers yield comparable levels of utrophin expression in neonate *mdx*. To determine the reproducibility of the results, two different batches of HdAdV-mUTR at similar titers (1.1 and 1.4 x 10^{12} virus particles/mL) were injected into neonate *mdx* mice which were then euthanized at 240 and 365 days respectively. The average number of utr+ fibers counted in both groups was similar: 168 +/- 35 versus 231 +/- 29 which was not significantly different (Figure 24A). As well, the distribution of utr+ fibers observed in every animal was also similar in both groups. There was a wide range of utrophin expression in both groups. The amount of central nucleation was also verified in both groups by counting the percentage of centrally nucleated fibers in utr+ positive fibers versus the percentage in utr- fibers in the virus injected muscles (Figure 24B). The percentage of central nucleation in utr+ against utr- fibers was found to be very similar in both groups. In the group sacrificed at 240 days, the amounts recorded were 17 +/- 8 and 55 +/- 4 compared to 19 +/- 2 and 64 +/- 1 in the 365 day group. Creatine kinase (CK) values, which serve as an indicator of muscle necrosis, were measured in both groups compared to twenty week old *mdx* mice which served as control mice (Figure 24C). CK values in both HdAdV-mUTR injected groups were not significantly different. Dystrophic muscle is composed of fibers of varying sizes due to the ongoing degeneration/regeration process. Therefore, diameters of the muscle fibers in each group were measured

and divided into three classes: generally utrophin positive area (+), largely utrophin negative area (-) and only utrophin positive fibers (utr). No evident shift in distribution of diameter sizes was observed with a similar pattern in sizes detected in both groups of HdAdV-mUTR (Figure 24D).

HdAdV-mUTR of a higher titer ameliorates various dystrophic parameters. To evaluate the influence of viral titers on the outcome measures, two different batches of HdAdV-mUTR at different titers (1.4 and 2.6 x 10^{12} virus particles/mL) were injected into adult *mdx* mice which were then euthanized at 180, 365 (batch 1: 1.4 x 10^{12} virus particles/mL) and 210 days (batch 2: 2.6 x 10^{12} virus particles/mL) respectively. The average number of utr+ fibers counted in the high titer (HT) group was found to be greater as compared to the other two timepoints: 116 +/- 37 compared to 68 +/- 29 and 73 +/- 18 respectively (Figure 25A). Although, this was not a significant difference, the distribution of utr+ fibers observed in every animal appeared to be better in the HT group - more animals in this group were near or above the mean average as compared to the 180 day group where one animal was responsible for the increase in mean for the entire group while the rest were located below the average. Surprisingly and inexplicably, CK values were found to be significantly lower (~50% lower) in the HT group at 210 days compared to the original group at 180 days post injection and the twenty week old control *mdx* (Figure 25B). Furthermore, the HT group demonstrated an improved distribution of fiber diameter size. As described previously, fiber diameters in each group were measured and divided into three

classes: generally utrophin positive area (+), largely utrophin negative area (-) and only utrophin positive fibers (utr). In comparing both groups, there was a slight but obvious shift in distribution of diameter sizes in the HT 210 utr group compared to other 210 groups and to its counterpart in the 180 group (Figure 25C). The HT 210 group clearly demonstrates a greater percentage of fibers at the larger fiber diameter range (50-100μm). Finally, an impressively high and widespread initial transduction was achieved in neonate *mdx* mice 10 days post-injection with a HdAdV-mUTR production of even higher titer (6.3×10^{12} virus particles/mL), ~4x higher than the production used in chapters 4 and 5 (Figure 25D).

Combination of HdAdV-mUTR and Trichostatin A improves muscle physiological indices. A recent study demonstrated an improvement in dystrophic parameters in adult *mdx* mice following treatment with a histone deacetylase inhibitor TSA (Minetti et al., 2006). Our group attempted to verify those findings by administering nine week old *mdx* mice with TSA daily intraperitoneally for a duration of three months. At the third day of treament, one group of mice were injected in the TA muscle with HdAdV-mUTR at a dose of 4.5×10^{10} viral particles (same dose as in chapter 5). At ~3 months post injection, the TA muscles of these mice were tested for muscle physiological parameters of force generating capacity and the ability to withstand stress. Three separate groups were tested: *mdx* untreated, *mdx* treated with TSA alone and *mdx* treated with TSA and injected with HdAdV-mUTR. Specific force was measured at three

different frequencies: 20, 90 and 120 Hz (Figure 26A). No difference amongst the three tested groups was observed at the lowest frequency. At 90 Hz, the combination of virus and TSA did yield a significant difference compared to the untreated mice while TSA by itself did not. However at 120 Hz, both groups of treated mice manifested significant differences in comparison to the untreated *mdx*, yet the mice treated with the combination of HDAdV-mUTR + TSA showed greater significance than mice with TSA alone ($p<0.01$ versus $p<0.05$). In terms of stress resistance to eccentric contractions, no significant difference was observed between the *mdx* untreated and the *mdx* treated with TSA alone (Figure 26B). However, commencing at the second contraction, mice treated with HdAdV-mUTR + TSA yielded significant differences in comparison to both other groups, an approximate 0.5-0.75 % difference in force drop demonstrating that the presence of a cytoskeletal protein at the level of the sarcolemma is essential to convey resistance to contraction induced stress. Furthermore, the combination of utrophin and TSA was found to bestow an increased specific force and a decreased force deficit compared to utrophin treatment by itself (as seen in Figure 18).

Combination of HdAdV-mUTR and Trichostatin A increases number of utrophin postive fibers through increased transcription driven by CB promoter. The number of utr+ fibers were counted in the injected TA muscles of adult *mdx* mice treated with TSA and injected with HdAdV-mUTR at 90 days post-injection (Figure 27A) . These results were then compared to data obtained

previously with adult *mdx* mice injected solely with HdAdV-mUTR (Figure 14). The combination of HdAdV-mUTR + TSA significantly increased the average number of utr+ fibers per TA compared to HdAdV-mUTR by itself, more than doubling the number of transduced fibers, 252 +/- 38 vs 120 +/- 33 respectively. To determine whether this increase could be (partially) attributed to the effect of TSA on the CB promoter, an *in vitro* study was conducted whereby a helper-dependent adenovirus encoding the LacZ transgene driven by the CB promoter (HdCBLacZ) was used to assess the effects of TSA on transgene expression in the infected C2C12 murine myoblast cell line (Figure 27B). The cells were incubated with three different concentrations of TSA: 25nM (which represents the normal plasma levels found in treated mice (Sanderson et al., 2004), 250nM and 1μM. All three concentrations resulted in increased beta-galactosidase expression as detected by chemiluminescence. Notably, the 25nM samples had an 11 fold induction compared to levels obtained by virus alone.

Combination of HdAdV-mUTR and Trichostatin A shown to increase utrophin levels *in vitro* by quantitative real-time RT-PCR analysis. To determine whether TSA could also increase CB-driven transcription of the utrophin transgene, 293A cells were infected *in vitro* with HdAdV-mUTR and one group was subsequently treated with Trichostatin A at a concentration of 25 nM. The cells were then harvested and RNA was isolated. Reverse transcription was carried out on all groups followed by real-time PCR (Figure 28). Once normalized to GAPDH transcript, the HdAdV-mUTR infected cells treated with TSA were

found to have a ~75% increase ($p=0.0269$) in utrophin transcript compared to TSA untreated cells. Thus incubation with TSA at levels found in plasma results in upregulation of utrophin transcription, presumably leading to higher levels of utrophin protein as observed in the TSA treated HdAdV-mUTR-injected mice.

Figure 24. Different batches of HdAdV-mUTR with similar titers yield comparable results. A. Scatterplot depicting the number of utrophin positive fibers counted in both groups with the horizontal bars representing the mean. B. The percentage of central nucleation in utrophin positive fibers (Utr+) and utrophin negative fibers (Utr-) in virus-injected muscles was verified in both groups. Error bars represent SEM. C. Creatine kinase (CK) values as indicators of muscle damage were measured in both groups. Twenty week old *mdx* mice served as control. Error bars represent SEM. D. Fiber diameter sizes were measured in both groups and categorized as follows: generally utrophin positive area (+), largely utrophin negative area (-) and exclusively utrophin positive fibers (utr).

Figure 24

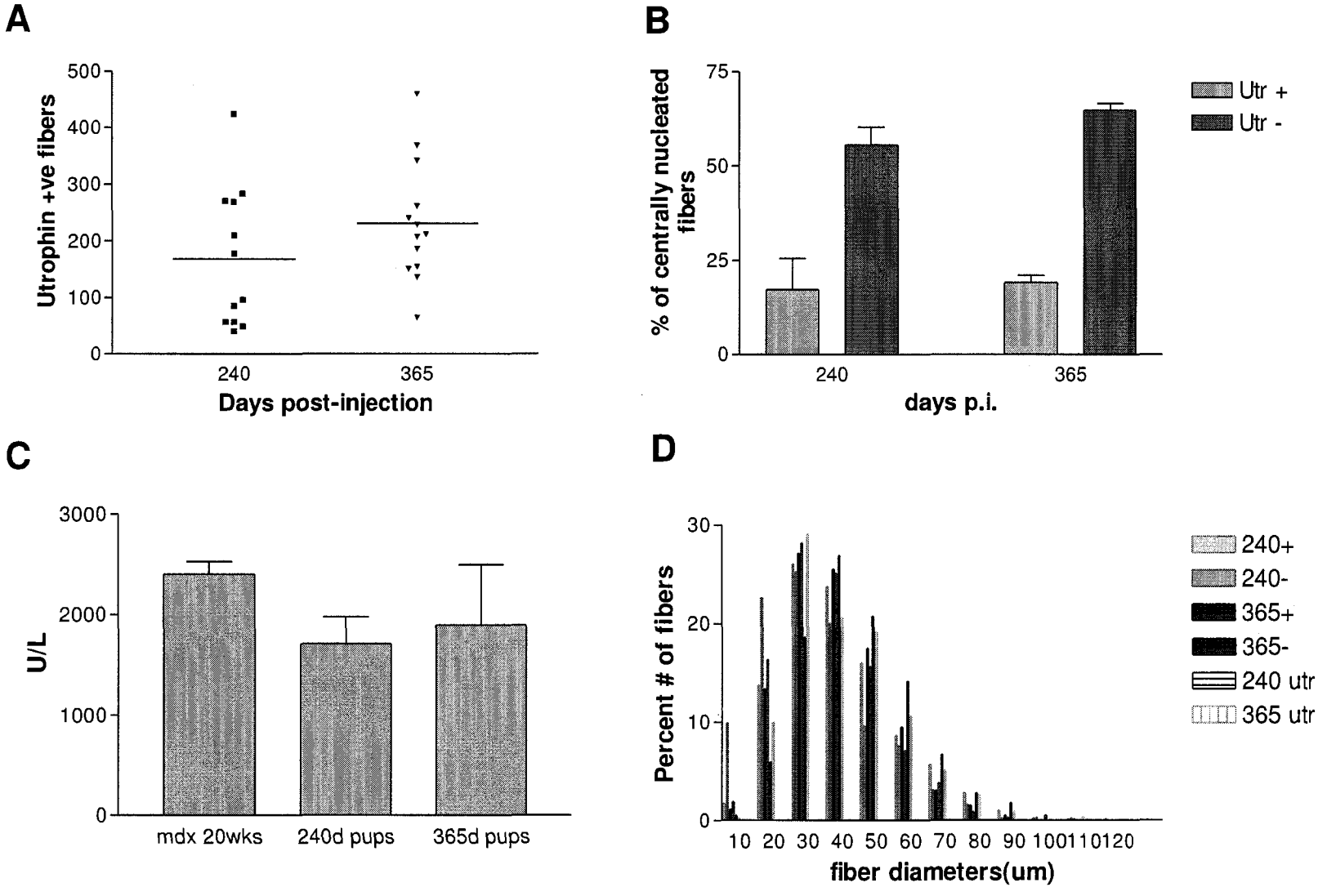

A
Utrophin +ve fibers
500
400
300
200
100
0
240
365
Days post-injection
B
% of centrally nucleated fibers
75
50
25
0
240
365
days p.i.
Utr +
Utr -
C
U/L
3000
2000
1000
0
mdx 20wks
240d pups
365d pups
D
Percent # of fibers
30
20
10
0
10 20 30 40 50 60 70 80 90 100 110 120
fiber diameters(um)
240+
240-
365+
365-
240 utr
365 utr

Figure 25. Mice injected with batches of HdAdV-mUTR with higher titers show improvement in dystrophic parameters. A. Scatterplot depicting the number of utrophin positive fibers counted in both groups with the horizontal bars representing the mean. B. Creatine kinase (CK) values as an indicator of muscle damage were measured in both groups. Twenty week old *mdx* mice served as control. Error bars represent SEM. *p=0.0032 compared to control and p=0.0061 compared to 180 day group. Significance was determined by a two-tailed unpaired t-test. C. Fiber diameter sizes were measured in both groups and categorized as follows: generally utrophin positive area (+), largely utrophin negative area (-) and exclusively utrophin positive fibers (utr). D. TA cryostat sections of neonatal mdx immunostained for utrophin at 10 days post-injection with a HdAdV-mUTR batch at a titer of 6.3 x 1012 virus particles/mL. Inset photo lower left is at lower magnification enabling a complete view of muscle section.

Figure 25

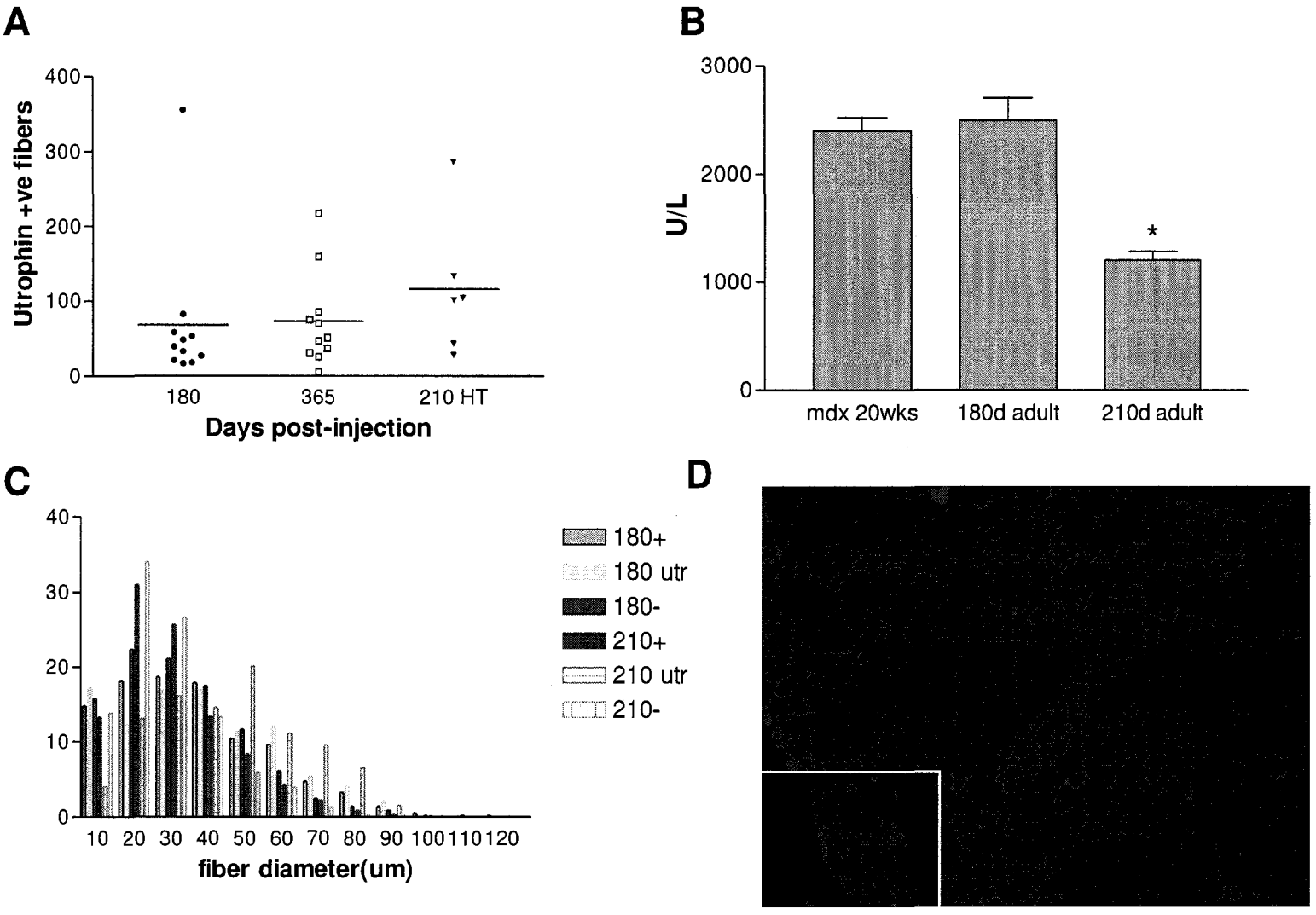

A
Utrophin +ve fibers
400
300
200
100
0
180
365
210 HT
Days post-injection
B
U/L
3000
2000
1000
0
*
mdx 20wks
180d adult
210d adult
C
40
30
20
10
0
10 20 30 40 50 60 70 80 90 100 110 120
fiber diameter(um)
180+
180 utr
180-
210+
210 utr
210-
D

Figure 26. Physiological analyses of TSA treated adult *mdx* injected with HdAdV-mUTR. Nine-week old *mdx* mice were treated daily intraperitoneally with TSA for ~90 days. In one group of treated mice, the right TA was injected with HdAdV-mUTR and muscles were analyzed at 90 days post-injection. Each individual muscle was tested for force generating capacity (A) and resistance to stress (B) induced by lengthening contractions. A. #$p<0.05$ and *$p<0.01$. B. $p<0.05$ between control *mdx* and HdAdV-mUTR+TSA following the 2nd contraction. $P<0.01$ and $p<0.001$ at the 2nd and following the 3rd contraction respectively between the TSA treated mice and HdAdV-mUTR+TSA *mdx* mice. All error bars represent SEM.

Figure 26

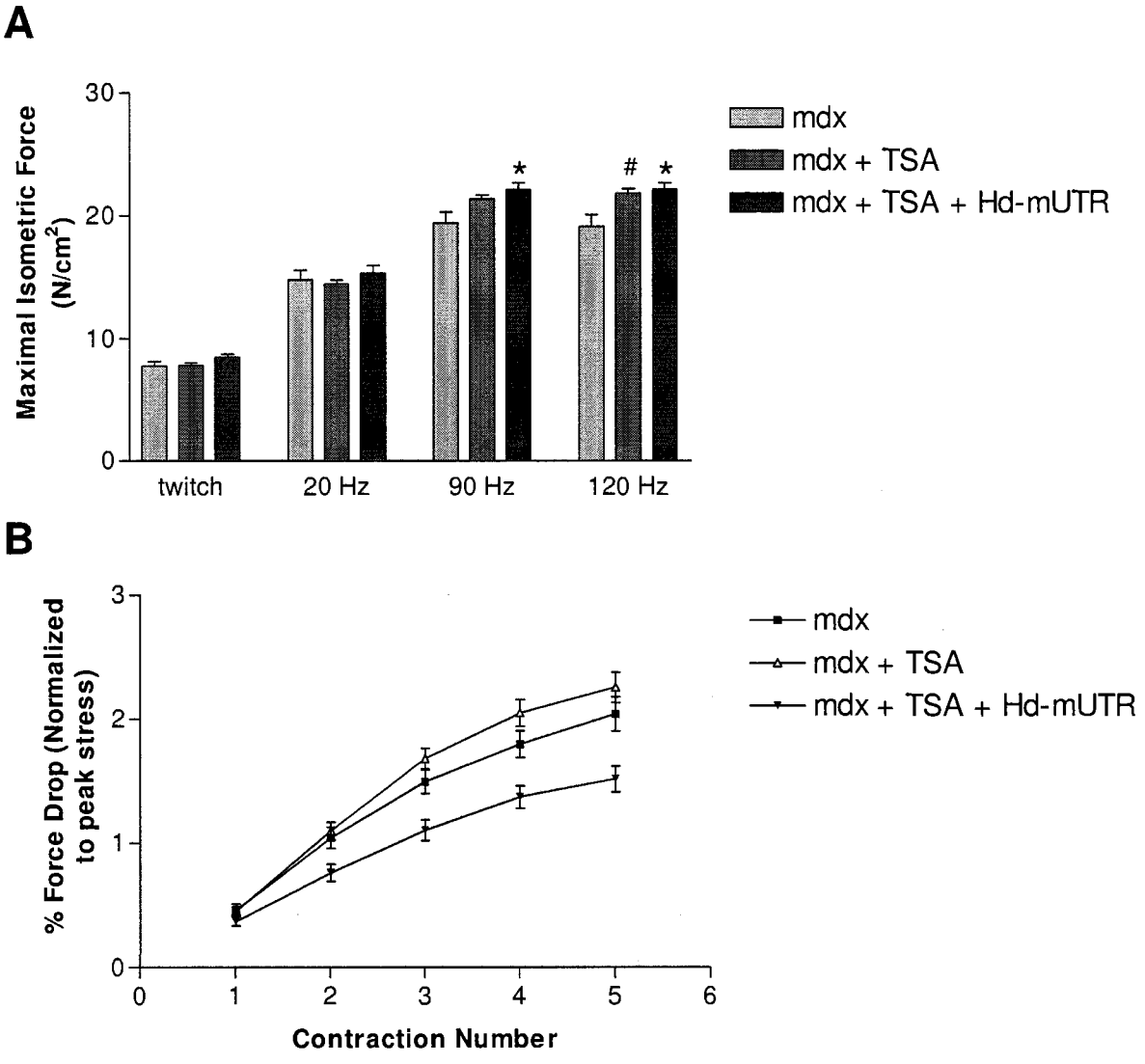

A
Maximal Isometric Force (N/cm²)
30
20
10
0
twitch
20 Hz
90 Hz
120 Hz
*
#
*
mdx
mdx + TSA
mdx + TSA + Hd-mUTR
B
% Force Drop (Normalized to peak stress)
3
2
1
0
0
1
2
3
4
5
6
Contraction Number
mdx
mdx + TSA
mdx + TSA + Hd-mUTR

Figure 27. Combination of HdAdV-mUTR and TSA increase utrophin levels through increased transcription. A. Scatterplot comparing the number of utrophin positive fibers at 90 days post-injection between mice injected with HdAdV-mUTR alone and those treated with both TSA and injected with HdAdV-mUTR. The horizontal bars represent the mean. P=0.0196 following a two-tailed unpaired t-test. B. An *in vitro* chemiluminescent study using C2C12 cells infected with the helper-dependent HdCBLacZ and treated with different amounts of TSA was done to determine the effects of TSA on the CB promoter. Samples were treated with TSA six hours post-infection. Cells were then harvested 48 hours post-treatment and beta-galactosidase expression from the sample lysates was measured on a luminometer. *p<0.0001 following a two-tailed unpaired t-test between the HdCBLacZ and HdCBLacZ + TSA 25nM samples.

Figure 27

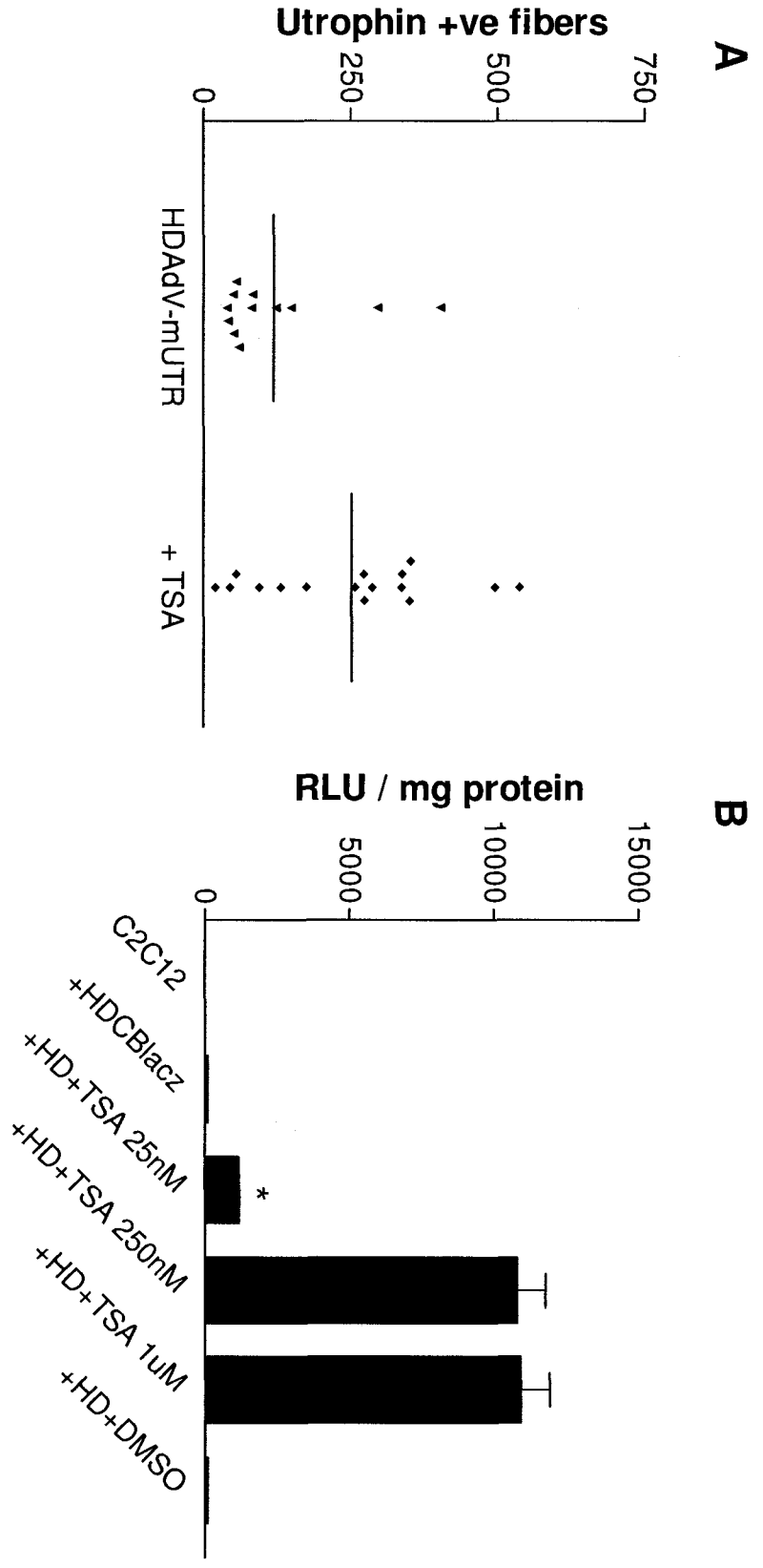
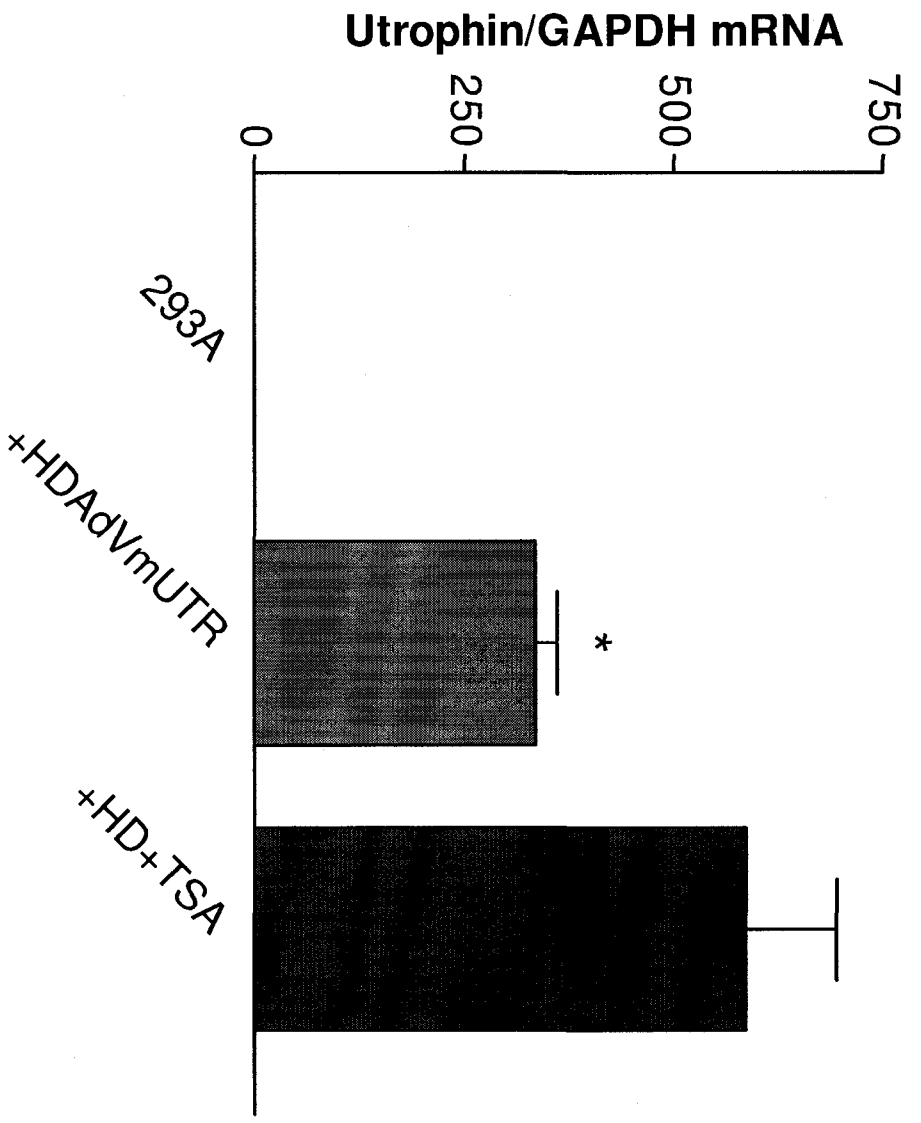

A
Utrophin +ve fibers
0
250
500
750
HDAdV-mUTR
+ TSA
B
RLU / mg protein
0
5000
10000
15000
C2C12
+HDCBlacz
+HD+TSA 25nM
+HD+TSA 250nM
+HD+TSA 1uM
+HD+DMSO
*

Figure 28. Increased utrophin RNA through the combination of HdAdV-mUTR and TSA. Bar graph showing the effects of TSA on relative utrophin transcript levels following HDAdVmUTR infection of 293A cells treated with and without TSA. Cells were treated with TSA six hours post-infection and harvested 48 hours post-treatment. Reverse transcription and real-time PCR was then performed on all samples. *Statistical significance was determined by a two-tailed unpaired t-test between the HdAdV-mUTR and HdAdV-mUTR + TSA samples; p=0.0269.

Figure 28



CHAPTER 7 - Discussion

Evolution of Adenoviral Vectors

Although initial studies involving first generation adenoviruses *in vitro* proved to be promising, these vectors were associated with a transient transgene expression and cellular toxicity. A disadvantage of using adenoviruses is the interference of humoral and cellular immune responses with transduction efficiency, in which CD8+ T cells play an important role (Yang et al., 1994a;Yang et al., 1994b;Dai et al., 1995;Yang et al., 1995b;Acsadi et al., 1996). Repeated injections of first generation vectors were also found to be inefficient because of rapidly induced antibodies against the expressed transgene and low grade synthesis of adenoviral gene products such as viral capsid antigens (Yang et al., 1994a;Yang et al., 1994b;Tripathy et al., 1996;Yang et al., 1996;Michou et al., 1997). Furthermore, the transgene expression can occur in many cells, including antigen presenting cells such as macrophages and dendritic cells if the promoter is not tissue specific (Gorman et al., 1982;Acsadi et al., 1996).

To overcome some of the drawbacks of first-generation vectors such as limited capacity for foreign DNA and immunogenicity, the next generation of vectors was deleted in the E2A and E4 region in addition to E1 and E3 (Lusky et al., 1998;Moorhead et al., 1999). These adenoviruses demonstrated decreased toxicity and inflammation (Gao et al., 1996;Dedieu et al., 1997;Wang et al., 1997). However, the transgene expression dramatically decreased as well (Armentano et al., 1997;Kaplan et al., 1997) due possibly to the absence of E4

open reading frames (ORF) 3 and 6 which increase late viral protein production by stabilizing the late viral mRNA (Ohman et al., 1993). Overall, much variation in results was observed with adenoviruses deleted in multiple early regions. In some instances, there was a reduced toxicity and longer transgene expression while in others, these characteristics were not observed. By comparison to first generation vectors, E2A deleted vectors did not confer much improvement (Lusky et al., 1998;O'Neal et al., 1998;Christ et al., 2000) while results involving vectors with E4 deletions were somewhat ambiguous (Gao et al., 1996;Wang et al., 1997;Brough et al., 1997;Lusky et al., 1998). This may be attributed to a variety of reasons including the type of target tissue, transgene immunogenicity, host strain genotypes, routes of administration, the expression of the viral genes, the influence of these genes on each other and heterologous promoters. In fact, the E4 region has been shown to exert some influence in *cis* and in *trans* on the CMV and RSV promoters resulting in prolonged adenovirus-mediated expression (Brough et al., 1997;Grave et al., 2000;Gilbert et al., 2001)with E4 ORF3 playing a crucial role in this promoter activation (Lusky et al., 1999;Armentano et al., 1999;Christ et al., 2000).

In general, stability of transgene expression was not obtained with second generation vectors despite the lowered toxicity observed with some constructs. Furthermore, any adenovirus vector still encoding some viral genes can in theory be complemented by a wild-type adenovirus infection in human patients (Rademaker et al., 2002). Thus, it is widely perceived that removal of all viral genes confers increased safety and provides the best alternative to overcoming

the cytotoxic T lymphocyte (CTL) responses initiated by residual viral expression (Goncalves and de Vries, 2006). This eventually lead to the creation of the helper dependent, fully deleted, high capacity adenovirus vector. These vectors have yielded successful gene therapy results in many tissues. For instance, stable long term expression involving significant rescue from disease phenotypes has been shown in animal models using helper-dependent adenoviruses encoding genes for α_1-antitrypsin (AAT), apolipoprotein E (apoE), coagulation factor VIII (FVIII), cystic fibrosis transmembrane conductance regulatory protein and uridine diphospho-glucuronosyl transferase 1A1 (Morral et al., 1999;Kim et al., 2001;Reddy et al., 2002;Koehler et al., 2003;Toietta et al., 2005). In some cases, a single administration of helper-dependent adenovirus resulted in the lifelong correction (2-3 years) in rodent models for familial hypercholesterolemia and Crigler-Najjar syndrome (Kim et al., 2001) (Toietta et al., 2005).

In particular, head to head comparisons of early generation to helper-dependent vectors demonstrated the superiority of the latter in terms of persistence of transgene expression and safety (Schiedner et al., 1998;Morral et al., 1999;Chen et al., 1999;Kim et al., 2001;Reddy et al., 2002). Increased durability of transgene expression from helper-dependent was demonstrated regardless of the transgene (human AAT, apoE or FVIII) or type of animal model (mice, rats, baboons). In the case of AAT, expression lasted for 24 months with only a slight decrease from peak levels. In comparison, AAT expression from first generation vectors was observed for only 3 to 5 months and its decrease was attributed to the removal of transduced hepatocytes by cellular immune

components (Morral et al., 1999). Similar results were observed with apoE and FVIII helper-dependent adenovirus-mediated expression which was maintained for 2.5 years and 40 weeks respectively, with minimal decrease in expression, by comparison to 28 days and 12 weeks respectively with early generation vectors (Kim et al., 2001;Reddy et al., 2002). These latter vectors were also associated with increased hepatotoxicity, with a 10-20 fold increase in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels being detected in both studies. However, in the FVIII study, unlike the apoE experiments, a rise in liver enzymes was also noted in animals treated with helper-dependent adenovirus vectors. This initial rise associated with both type of vectors was detected one day post administration and levels returned to baseline by day three. Enzyme levels rose again 10 fold at day 7 and returned to baseline levels by day 28 but this phenomenon was only observed in animals treated with the early generation vector (Reddy et al., 2002). These results suggested that the initial increase in AST and ALT was due to the presence of viral capsid proteins and the second spike in enzyme levels was attributed to residual viral expression from the vector backbone. A direct comparison carried out in skeletal muscle showed that β-galactosidase expression from a helper-dependent adenovirus persisted at a high level for 20 weeks whereas expression levels dropped at 6 weeks for the first generation vector (Chen et al., 1999). Cellular infiltrates were also detected in a small percentage of mice injected with the first generation vector as opposed to the absence of infiltration in the second experimental group. After an initial drop in the level of viral DNA in

both groups, the levels remained stable beyond the 2 week timepoint. The observed drop in expression from the first generation vector, despite the persistence of DNA, was attributed to promoter shutdown amongst other possibilities. However, one cannot rule out that differences in vector design between the two vectors may have influenced the outcome.

Another advantage provided by helper-dependent vectors is that the activities of tissue-specific promoters appear to be more stringently regulated (more "tissue specific") in the context of helper-dependent versus first generation vectors (Shi et al., 2002). This may be due to the presence of viral gene products in the latter vector modulating the activity of the promoter. Therefore, early generation adenoviruses are now mainly being used for gene transfer in cancer and infectious diseases where a local and/or transient expression is warranted. To tackle chronic disorders where an enduring expression is required, helper-dependent adenoviruses appear to be better suited (Goncalves and de Vries, 2006). Thus, in this study, the utrophin cDNA was delivered to skeletal muscle using the least immunogenic AdV, one that is fully deleted of all viral genes and in which the incorporated stuffer sequences were also of mammalian origin, to minimize immunogenicity further.

Vector Immunogenicity

As already alluded to, adenoviruses, regardless of generation type are known to elicit some type of immune response. Regarding first-generation adenoviruses, it was assumed that removal of the E1 region would prevent

expression of the remaining adenoviral regions. However, residual expression was still observed possibly due to E1A-like activities from cellular factors especially after high multiplicities of infection were employed (Imperiale et al., 1984;Kovesdi et al., 1986). This expression leads to the processing of viral peptides by antigen presenting cells (APCs) via MHC I molecules which can then trigger the activation of CD8 T cells (CTL). This in turn leads to vector transduced cells being eliminated by the growing number of CD8 T cells. As well, transduced APCs can present viral antigen via MHC class II which can lead to the stimulation of CD4 TH1 cells which can further enhance the activity of the CD8 T cells (Jooss et al., 1998;Wivel et al., 1999;Roth et al., 2002). Furthermore, MHC class II molecules on the surface of B cells also expressing viral antigens can be recognized by CD4 Th2 cells which in turn release various cytokines prompting the differentiation of the B cells into plasma cells producing anti-adenovirus antibodies capable of blocking viral infection, especially in the context of vector readministration.

Besides the expression of viral genes and the transgene triggering the adaptive immune response, factors associated with innate immunity also play a role during adenoviral administration due possibly to the presence of adenoviral capsid proteins. This response is not dependent on viral gene expression. It has been observed soon after administration and before initiation of viral gene expression (Muruve et al., 1999;Schnell et al., 2001;Zhang et al., 2001). It was also induced in the presence of transcriptionally inactive adenoviral vectors in both mice and non-human primates. Thus, all adenoviral vectors, including

helper-dependent adenoviruses are capable of provoking this response because the viral capsid is virutally the same across all the vector generations (Stilwell et al., 2003;Brunetti-Pierri et al., 2004;Muruve et al., 2004;Stilwell and Samulski, 2004). The activation of the immune response appears to be dose-dependent. In one study, two baboons were administered systemically two identical helper-dependent adenoviruses encoding LacZ with a difference in dose being the only variant. The animal receiving the lower dosage (5.6 X 10^{12} VP/kg) suffered a transient and mild toxic response (through elevated IL-6 levels) which returned to normal by 24 hours post-injection. However, the baboon receiving the higher dosage (1.1 X 10^{13} VP/kg) became moribound after its IL-6 levels increased 100 fold in comparison to its counterpart (Brunetti-Pierri et al., 2004). The nonlinear dose response associated with systemic administration of adenovirus has been attributed to its sequestration by the Kupffer cells located in the liver. However their precise role and the mechanism by which adenovirus-mediated innate responses occur still remain to be fully elucidated (Palmer and Ng, 2005).

Another study also tested the immune responses in mice after intravenous administration of first generation and helper-dependent adenoviruses. In this case, both types of vectors elicited expression of inflammatory cytokines and chemokines in the liver including TNF-α, macrophage inflammatory protein 2 and interferon-inducible protein 10. CD11b leukocytes trafficked to the liver in response to both types of vectors but only first generation infected animals had an increase in CD3 lymphocytes and inflammatory gene expression 7 days post infection while no increase in inflammatory cells or gene expression was

detected beyond the 24 hour mark in mice treated with the helper-dependent andenovirus. This led the authors to speculate that fully deleted adenoviruses elicit an intact innate but attenuated adaptive immune responses *in vivo* (Muruve et al., 2004).

A considerable role in eliminating adenoviral infections *in vivo* is played by the components of the innate immune response such as macrophages, complement and natural killer cells (Worgall et al., 1997). This innate antiviral response appears to be regulated, in part, by the transcription factor NF-κB due to its ability to activate the transcription and production of proinflammatory cytokines in addition to the control of signaling pathways (Ferreira et al., 1999). Activation of NF-κB occurs at early stages of infection, particularly at high multiplicities of infection (Clesham et al., 1998;Lieber et al., 1998). Components of signaling pathways such as p38 MAPK are also known to contribute to the innate response by leading to the expression of proinflammatory cytokines and chemokines which then attract granulocytes, natural killer cells and monocytes/macrophages to perform cytolytic activities, thus producing more cytokines/chemokines. Through this production and the expression of viral antigens by infiltrates such as macrophages, the adaptive branch is triggered resulting in an amplification of the entire immune response (Guidotti and Chisari, 2001;Liu and Muruve, 2003;Goncalves and de Vries, 2006). Thus, the innate immune response remains a major hurdle in the efficient use of helper-dependent adenoviruses.

Evasion Strategies for Immune Responses

Attempts have been made to avoid the host immune response by administering immunosuppressive agents such as IL-12, CTLA4Ig, cyclopsorin, and FK506 (Fang et al., 1995;Kay et al., 1995;Lochmuller et al., 1995;Vilquin et al., 1995;Yang et al., 1995c). A daily treatment of cyclosporin A, an inhibitor of activated T lymphocyte proliferation, was ineffective in increasing long term transgene expression after adenoviral injection into mouse diaphragm (Petrof et al., 1995). No prolonged expression was observed after IL-12 administration due to only selective impeding of certain humoral responses (Yang et al., 1995c). In the case of CTLA 4Ig, efficient secondary transduction was prevented due to low-level neutralizing antibodies despite administration of the immunosuppressive drug (Kay et al., 1995). In order to deal with the severity of the innate immune response, researchers have tried to "mask" the viral capsid through the use of a couple of retargeting strategies such as PEGylated adenoviruses or vectors encapsidated within bilamellar cationic liposomes (Yotnda et al., 2002;Mok et al., 2005). In both studies, there was no difference in hepatic transduction efficiencies yet serum IL-6 levels decreased 50-80% in comparison to control vectors. Despite these encouraging results, the mechanism by which these modifications attenuate innate immunity is still not clear and further studies are warranted.

Transgene Immunogenicity

Besides immune responses to the vector capsid and gene products, transgene immunogenicity is also thought to contribute to a decline in expression levels (Yang et al., 1996). To thoroughly test for acceptance of a transgene, injection into adult mice is required. Testing in neonates is not the ideal scenario due to their immature immune system and the opportunity for tolerization of a foreign antigen. Furthermore, no difference in the duration of transgene expression has been shown in immunodeficient and immunocompetent newborn mice (Acsadi et al., 1996). Our results in adult *mdx* show that the utrophin transgene did not elicit a response since we could not detect the production of any anti-utrophin antibodies. As we had hypothesized, the mice appeared to tolerate the exogenous utrophin. However, the loss of episomal DNA and the innate immune system contributed to the observed decrease in transgene expression.

Hybrid Adenoviral Vectors

Loss of episomal adenoviral DNA, especially in cells with high turnover, has been shown to occur both in immunocompetent and immunodeficient mice (Ehrhardt et al., 2003) and is considered one of the major drawbacks of adenoviral vectors. In an attempt to attain long-term persistence of adenovirus-mediated transgene expression, researchers have opted to use hybrid versions of helper-dependent adenoviruses combined with other viral systems. The purpose of these hybrid vectors is to utilize and combine the favourable aspects

of each individual vector system. Epstein-Barr virus (EBV), retrovirus and AAV are all examples of systems with which adenoviruses have been modified to build a chimeric vector (Fisher et al., 1996;Feng et al., 1997;Tan et al., 1999;Reynolds et al., 1999;Recchia et al., 1999;Leblois et al., 2000;Murphy et al., 2002;Ehrhardt et al., 2007). Epstein-Barr viruses (EBV) are interesting because of the ability of EBV episomes to persist in proliferating cells due to EBV nuclear antigen 1 (EBNA-1)-mediated replication and segregation. A dual adenovirus-EBV hybrid system was generated using two helper-dependent adenoviruses, one which encoded the transgene and the EBNA-1 cassettes flanked by recombinase recognition sites while the other vector encoded the recombinase. Following co-infection, the EBV episomes circularized. This method had a ~40% infection efficiency rate *in vitro,* with up to 98% of episomal viral DNA replicating and persisting in proliferating cell lines resulting in transgene expression for over 20 weeks (Dorigo et al., 2004;Kreppel and Kochanek, 2004).

Integrating Adenoviral Vectors

Another possible solution may involve the use of integrating adenoviruses. Adenoviruses are known to randomly integrate but at low frequencies (0.001% to 1%) *in vitro* (Harui et al., 1999;Mitani and Kubo, 2002), a process dependent on cell type, expression cassette and MOI used (Doerfler, 1970;Van Doren et al., 1984;Lucher, 1995;Hillgenberg et al., 2001). *In vivo* integration has also been attempted but it appears to occur too infrequently to lead to any substantial gene expression. In the few studies that *in vivo* integration may have occurred, it was

not verified and frequencies of chromosomal integration were not determined (Overturf et al., 1997;Brown et al., 1997).

Thus to increase the integration frequency, the efficient transduction capabilities of adenoviruses have been combined with the integrating abilities of transposons, retroviruses and AAVs. Indeed, a helper-dependent adenovirus-transposon hybrid vector has been created using a eukaryotic transposase (Sleeping Beauty) to integrate transgene sequences in mouse liver. Systemic *in vivo* delivery resulted in duration of Factor IX expression for more than six months in proliferating mouse liver (Yant et al., 2002). Stable integration of transgene has also been achieved following adenoviral delivery of the L1 element from L1 retrotransposon into cultured human cells (Soifer et al., 2001;Soifer and Kasahara, 2004). Retrotransposition frequency in HeLa cells was dose-dependent and found to be 0.35% at an MOI of 100 infectious particles / cell.

An alternate method uses retroviruses alongside adenoviruses in which all retroviral genes are carried on two adenoviral vectors. The packaging functions (gag/pol/env) are delivered separately from the LTR-flanked trangene and retroviral sequences. Upon co-infection, the transduced cells begin producing retroviral particles which are then released, and have the capability of infecting and integrating the therapeutic gene into the genome of neighbouring cells (Feng et al., 1997;Reynolds et al., 1999;Volpers and Kochanek, 2004). This approach has the added advantage of being able to circumvent or at least reduce the effect of the complement pathway which tends to inactivate retroviral vectors by particle

lysis (Welsh, Jr. et al., 1975;Mitani and Kubo, 2002). In one study, three hybrid vectors carrying gag/pol, env and retroviral vector were transduced *in vivo* into xenografts of a human cervical cancer cell line implanted in mice. The transduction efficiencies were determined to be 10-15% at 16 days post-adenoviral infection (Feng et al., 1997). Yet integration frequencies were not shown. Another study involving the *in vivo* transduction of four hybrid vectors into human melanoma xenografts in nude mice revealed that the percentage of selected colonies from tumors transduced with all four adenoviral-retroviral vectors increased over time upto 7.2% after four weeks post-administration (Caplen et al., 1999). Similar spreading of transgene expression and integration occurs through the use of helper-dependent adenoviruses which enable the incorporation of the entire retroviral genome, including the retroviral structural genes and the transfer vector, into one adenovirus (Soifer et al., 2002). However, some drawbacks involving retroviral-adenoviral vectors include the inability of retroviruses to re-infect hybrid-infected cells due the presence of retroviral envelope protein at the cell surface receptor site. These same hybrid infected retroviral producer cells can also become targets of the host CTL response. Furthermore, retroviruses require neighbouring cells to be undergoing division in order for infection to occur *in vivo*. This obstacle can be overcome with the use of lentiviral vectors which can infect cells regardless of their proliferation state (Mitani and Kubo, 2002).

Ideally, the integrated DNA should not harbour any outward promoter and enhancer activity such as those in the retroviral long terminal repeats and be site

specific within the genome to avoid oncogene activation and/or insertional mutagenesis (Goncalves and de Vries, 2006). Thus, AAV proteins are being used, in particular, the Rep68/78 proteins along with AAV ITRs with adenovirus to obtain stable transgene integration (Fisher et al., 1996;Recchia et al., 1999). Some adenovirus/AAV hybrid vectors only contained adenoviral packaging signals and ITRs along with the AAV ITR flanked transgene (Lieber et al., 1999). Although stable transduction of cultured cells comparable to recombinant AAV (and less than 1st generation AdV) was achieved, integration was found to occur randomly. However, the use of both the Rep proteins (in *trans*) and AAV-ITRs (in *cis*) allow for AAV site specific integration on human chromosome 19 (AAVS1 locus). Once again, two adenoviruses are used carrying the Rep protein (Rep78) and the ITR flanked transgene respectively. Despite low vector rescue, 35% targeted integration at AAVS1 was demonstrated in hepatoma cells. This latter hybrid method, as some others mentioned above, takes advantage of the helper-dependent adenovirus' ability to transduce efficiently, its large insert capacity and high titer but has the added advantage of integrating at a specific location (Recchia et al., 1999). However, in the case of this vector, integration only occurs in the presence of Rep proteins which are provided in a separate vector. The magnitude of Rep expression must be monitored for it can also inhibit adenovirus replication and elicit immune responses (Weitzman et al., 1996;Recchia et al., 1999).

Hybrid Adenoviral Vectors in Dystrophic Muscle

Hybrid vector methods have also been employed to treat DMD. In fact, C2C12 mouse myoblasts and myotubes have been used to produce retroviral vectors. Co-infection of first generation adenoviruses encoding gag/pol, amphotropic (10A1) env and retroviral vector respectivley resulted in retroviral vector production of 1.4 – 1.8 x 10^5 cfu/ml (Roberts et al., 2001). Retrovirus production seems to preferentially occur in post-mitotic cells as production increased 80% following addition of aphidicolin, a cell cycle inhibitor, to the myotubes. A follow-up study involving adenoviral-retroviral hybrid vectors also consisted of the two-step *in situ* retroviral production method described earlier. Dystrophic muscle was considered an attractive target tissue for this particular therapy because it is under a continuous state of degeneration and regeneration. Necrotic fibers are replaced by satellite cells which migrate, differentiate into myoblasts and fuse with existing myotubes. It has been speculated that mature myofibers, once infected with the hybrid vectors, would provide a platform from which functional retroviruses would be released into muscle where the vectors would be in close proximity to proliferating (dividing) and maturing muscle cells. *Mdx* TA muscle cells were shown to be efficient retroviral producer cells following adenoviral-retroviral vector infection. The hybrid vectors were based on first generation adenoviruses and thus encoded a 3.7kb microdystrophin gene. Administration of this hybrid into neonatal *mdx* mice along with vectors encoding gag, pol and env resulted in maintenance of a high transgene expression (~90% of TA fibers) upto 3 months post-injection. In the absence of gag, pol and env,

expression levels dropped beyond the four week timepoint. The expression of the microdystrophin was correlated with the restoration of two members of the DAP complex and in a slight decrease in the centronucleation index. A portion of the muscles demonstrated retroviral integration. However, frequency of chromosomal insertion was not determined (Roberts et al., 2002).

Another study found an adenovirus and AAV hybrid vector carrying the full-length dystrophin open reading frame was capable of REP-dependent site specific integration in HeLa cells. However, integration in AAV does not necessarily occur at a specific nucleotide but instead within a specfic stretch of DNA. This results in the formation of AAVS1-vector DNA junctions of various lengths. Furthermore, this hybrid vector was also shown to transduce neonatal rat cardiomycytes *in vitro* and gastrocnemius muscles of adult *mdx* mice *in vivo* (Goncalves et al., 2005).

Alternative Integration Strategies in Muscle

Besides hybrid vectors, other methods have also been employed to correct dystrophin deficient cells via DNA integration. *Mdx* mice were transplanted with dystrophin deficient mouse myoblasts that had been treated *ex vivo* with herpes simplex virus amplicons encoding a full length dystrophin sequence resulting in a non targeted integration (Bujold et al., 2002). In another study, muscle progenitor cells isolated from murine muscle were treated *ex vivo* with a lentiviral vector encoding human microdystrophin and redelivered to dystrophic murine muscle. Stable transduction of these side population cells

resulted in non specific integration of the foreign DNA (Bachrach et al., 2004). In a similar study involving lentiviral delivery of the minidystrophin gene,(Li et al., 2005).transgene expression in *mdx* muscles *in vivo* prevented subsequent fiber degeneration for over six months but transduction efficiency of the lentiviral vector was very low. However, dystrophin positive myofibers were observed following transplantation of lentiviral treated myoblasts (*ex vivo*) into *mdx* skeletal muscles (Li et al., 2005). Attempts have also been made with electroporating plasmids incorporating a full length dystrophin-bacteriophage phiC31 fusion protein into myogenic cells (Quenneville et al., 2004). This resulted in site specific integration but the transducion efficiency was too low along with a high cell mortality. Besides the loss of episomal DNA, a decrease in transgene expression may also be governed by the promoter used to regulate the transgene. A possible solution to the decreased utrophin expression we observed may involve a change in promoter to a tissue specific promoter.

Promoter Selection

For driving expression of therapeutic genes in skeletal muscle fibers, an ideal promoter and/or enhancer should be highly active in target cells and show specific activity in muscle cells to minimize toxicity and immunogenicity in non-target cells (Karpati and Acsadi, 1994). An obvious selection would be the natural muscle promoter for dystrophin (Klamut et al., 1990;Ahn and Kunkel, 1993). However, this promoter was found to be very weak or even inactive (Klamut et al., 1990).

The constitutive viral promoters (Rous sarcoma virus late promoter, RSV-LTR, or the cytomegalovirus promoter/enhancer, CMV) were found to be very active in muscle cells (Wolff et al., 1991). However, their activity is not restricted to the muscle (Gorman et al., 1982). Both promoters, when placed in first-generation vectors encoding dystrophin and injected into *mdx* muscle produced high expression levels after ten days in neonates (Acsadi et al., 1996). Use of CMV, in particular, resulted in increased sarcolemmal and cytoplasmic dystrophin staining in muscle fibers. Notably, lower levels were observed in young adult and old mice. As well, levels expressed by both promoters dropped considerably by day sixty in neonates (Acsadi et al., 1996).

The muscle-specific muscle creatine kinase (MCK) promoter has also been extensively used in DMD therapy. In first-generation viruses, MCK was found to increase dystrophin expression at the sarcolemma only, thus avoiding the overexpression experienced with CMV (Larochelle et al., 2001). MCK has also been placed along with dystrophin in encapsidated adenovirus minichromosomes yielding a transient transgene expression for 3-4 weeks (Kumar-Singh and Chamberlain, 1996). Although transgene expression is high, MCK is limited to differentiated skeletal and cardiac muscles (Urdal et al., 1983). In the case of fully deleted viruses encoding MCK and dystrophin, dystrophin expression was noted in a decent percentage of *mdx* muscle fibers (Kochanek et al., 1996;Clemens et al., 1996;Haecker et al., 1996;Chen et al., 1997;DelloRusso et al., 2002). However, the extent and strength of dystrophin expression was based on histological observation and not any quantitative measurements such

as the number of dystrophin positive fibers. Based on our group's analysis, the hybrid chicken β-actin promoter/CMV enhancer outperformed MCK and all of the afore-mentioned promoters (Gilbert et al., 2003;Dudley et al., 2004).

Levels of expression vary depending on the fragment of MCK promoter used. In our group, a 1.35 kb MCK promoter, shown to be efficient in first-generation vectors (Larochelle et al., 1997), was not able to generate a high level of expression in any *mdx* age group at any time point when inserted in fully deleted viruses (unpublished data). Despite these results, the MCK promoter may need to be revisited once again, especially since it too has been shown to be upregulated by TSA (unpublished data); thus any activity that it may be lacking in comparison to the CB promoter can be overcome with TSA co-treatment. Another possibility may be to use another derivative of the MCK, the CK6 promoter generated through *in vitro* mutagenesis which has been shown to be very powerful in dystrophic muscle within the context of AAV vectors (Hauser et al., 2000;Gregorevic et al., 2004;Gregorevic et al., 2006). However when dealing with muscle specific promoters for DMD treatment, promoters should demonstrate activity throughout the entire tissue without preferential expression for a specific fiber type (fast or slow) (Corin et al., 1995;Jerkovic et al., 1997;Swoap, 1998;Lupa-Kimball and Esser, 1998).

Adenoviral Retargeting Strategies

A majority of the breakthroughs involving helper-dependent adenoviral gene therapy have occurred in diseases involving the liver because hepatocytes

are readily infected by adenovirus. Despite improvements in muscle gene transfer, adenoviral transduction is more significant in neonates compared to adult mice. This could be attributed to a lack of sufficient CAR (coxsackie and adenoviral receptor) on the surface of muscle. Indeed, CAR has been found to be developmentally down-regulated on muscle cells thus affecting the ability of adenovirus to efficiently bind and infect mature muscle (Nalbantoglu et al., 1999).

In order to overcome this problem, several groups have pursued retargeting of the adenovirus. Some methods employed involve the modification of the capsid or the use of adaptor molecules, such as polyethylene glycol (PEG), which redirect the virus to alternate cell surface receptors (Wickham et al., 1996;Watkins et al., 1997;Smith et al., 1999;Romanczuk et al., 1999;Haisma et al., 2000;Dmitriev et al., 2000;Fisher et al., 2001a;Volpers et al., 2003). Another strategy involves "fiber swapping" where the fiber from a different serotype is genetically incorporated to allow the targeting of previously refractory cells (Zabner et al., 1999;Shayakhmetov et al., 2000;Havenga et al., 2001). For example, a hybrid AAV and adenovirus integrating vector was further modified by altering the adenoviral fibre. The fibre from the adenoviral serotype 5 was switched to the serotype 50 fibre in order to achieve a CAR independent transduction of DMD myoblasts and myotubes. The primary receptor for all group B adenoviruses (including serotype 50), CD46, was found readily on the surface of DMD myoblasts in comparison to CAR which was barely detected. Retargeted vectors were found to have a superior transduction efficiency of DMD muscle cells compared to the conventional vectors. Nearly 30 fold higher titers of

serotype 5 fibre vectors were necessary to achieve similar frequencies of transgene expressing cells in relation to vectors with serotype 50 fibres. The latter vector also transduced multiple fold higher precentages of dystrophin expressing DMD myoblasts (Goncalves et al., 2006). Specific cellular receptors besides CAR, such as integrins and heparan sulfate proteoglycans can also be selected by incorporating specific peptide ligands such as the arginine-glycine-aspartate (RGD) peptide (for integrins) and poly-lysine (for heparan sulfate) into specific regions within the fiber, hexon or penton base proteins located on the exterior of the adenovirus (Wickham et al., 1995;Wickham et al., 1996;Dmitriev et al., 1998;Vigne et al., 1999;Biermann et al., 2001;Dmitriev et al., 2002). One study involved the incorporation of the RGD or polylysine sequences into the HI loop of fiber knobs in helper dependent adenoviruses. Vectors with the polylysine modified knobs demonstrated 12 and 21 fold higher transgene activity (β-galactosidase) in murine C2C12 myoblasts and myotubes respectively compared to control vectors. However, an improvement in transduction was not observed when the same modified vectors were used to infect mature murine skeletal muscle (Bramson et al., 2004). Conversely, to render the vector more tissue-pecific, attempts have been made to reduce the background CAR-reactivity in non-target cells. This can accomplished by ablating the vector's ability to interact with CAR by removing the knob portion of the adenoviral fiber involved in interaction or by inserting point mutations within the same knob domain thus disturbing the contact between the fiber and CAR (Kirby et al., 1999;Magnusson et al., 2001).

Interpretation of HdAdV-mUTR Results

Our study represents an in-depth analysis of full-length, isogenic utrophin gene transfer using a fully deleted AdV for the treatment of dystrophin deficiency in the *mdx* mouse. As already shown by several groups, including our own, utrophin can replace dystrophin in many contexts, including in transgenic *mdx* mice or when administered by first-generation adenoviruses (Tinsley et al., 1996;Deconinck et al., 1997a;Gilbert et al., 1998;Rafael et al., 1998;Tinsley et al., 1998;Gilbert et al., 1999;Wakefield et al., 2000;Squire et al., 2002;Cerletti et al., 2003). First generation adenovirus expressing a truncated utrophin was found to maintain expression at 600 fibers (34% of the total fibers) up to 60 days in *mdx* neonates, restore proteins of the DAP complex, protect neonatal *mdx* muscle from necrosis as inferred by a reduction in central nucleation (15% vs 56% at 60 days), reduce permeability of neonatal *mdx* muscle to the Evans blue vital dye and improve force-generating capacity of neonatal *mdx* muscle after high stress contraction. However, utrophin expression peaked at 10 days post-injection in *mdx* adults at ~500 fibers (30% of total) and gradually decreased by 30 days post-injection. Furthermore, anti-utrophin antibodies were detected in the sera of *mdx* neonates and adults in addition to an increased presence of inflammatory infiltrates in highly transduced areas (Gilbert et al., 1999). In a direct comparison conducted by our group using truncated versions of dystrophin and utrophin encoded by first generation adenoviruses, utrophin transduced muscles demonstrated a higher initial and more sustained transduction (Lochmuller et al., 1996;Yang et al., 1996;Guibinga et al., 1998;Ebihara et al., 2000), less

inflammation, a greater ability to mitigate necrosis and performed better in muscle physiological tests in adult *mdx* mice (Ebihara et al., 2000). These results highlighted and reinforced the attractiveness of utrophin as a therapeutic candidate for DMD gene therapy.

In the present study, a single injection of HdAdV-mUTR in the TA provided very good transduction, resulting in up to 1,500–2,000 utr^+ fibers (~75% of total) in some neonates (both *mdx* and *scid*). Adult TAs were also relatively well transduced, with 35% of fibers being utr^+ 30 days after injection. The presence of utrophin prevented much of the necrosis in the neonatally injected mice as determined by the central nucleation index (Figure 10), and in the adults, further necrosis (after gene transfer) was markedly reduced in the utr^+ fibers even at late time points after HdAdV-mUTR administration (Figure 17). Dystrophin-associated proteins (α-sarcoglycan and β-dystroglycan) were also found to be present in the sarcolemma in the presence of utrophin up to 1 year after injection (Figure 16). Marked physiological improvement was observed in both neonates and adults (Figures 11 and 18). The improved physiological indices were similar to those ascertained following dystrophin gene replacement (Gilbert et al., 2003;Dudley et al., 2004) (Tables 1 and 2). However, unlike in our group's previous study, in which HdAdV-mDYS-injected adult *mdx* were immunosuppressed with tacrolimus (FK506) for 7 days and injected twice before the physiology experiments, the animal cohorts in the present study remained untreated (thus immunocompetent) before their single injection of HdAdV-mUTR (Table 2).

Table 1. Comparison of results between groups of neonatal *mdx* mice injected with helper-dependent adenoviral vector encoding murine dystrophin or murine utrophin.

Table 1

mDystrophin & mUtrophin - neonates	mDystrophin	mUtrophin
+ve fibers	10 days - 712 60 days - 590 365 days - 1381	10 days - 1596 60 days - 886 240 days - 189
Central Nucleation	10 days - <7.5 % 60 days - <15 % 365 days - ~10% ~45% diff	10 days - <4 % 60 days -12% 240 days -17% ~40% diff
Force Generation	Increased force generation compared to control at early timepoint.	
Stress Resistance	Similar Results (0.7% vs 1.5% drop)	
Reduced hypertrophy	Similar results at 240 days.	
Infiltrates	No significant difference at 60 days. 4x less at 365d	No observable difference to control.
Transgene Antibody	Detected at every timepoint.	Undetected up to 240 days.

Table 2. Comparison of results between groups of adult *mdx* mice injected with helper-dependent adenoviral vector encoding murine dystrophin or murine utrophin.

Table 2

mDystrophin & mUtrophin - adults	**mDystrophin**	**mUtrophin**
+ve fibers (%)	10 days - 276 (16) 30 days - 415 (24) 90 days - 88 (5) 180 days - 204 (11)	10 days - 693 (22) 30 days - 770 (35) 90 days - 124 (5) 180 days - 68 (3)
Central Nucleation	~ 20% difference between +ve and –ve fibers up to 180 days.	~ 25-30% difference between +ve and –ve fibers up to 365 days.
Force Generation	Similar results. No improvement was seen.	
Stress Resistance	Similar % of force drop (1 vs 2%) at 60 days.	
	Injected twice, 25% Higher titer, FK506+	Injected once, no treatment.
Reduced hypertrophy	Similar results. No improvement was seen	
Infiltrates	CD4, CD8 ↑ at 60 days.	No observable difference to control.
Transgene Antibody	Detected.	Not detected.

Aside from some differences, murine utrophin when delivered by a helper-dependent adenovirus gave comparable results to murine dystrophin in terms of the central nucleation index, muscle physiology, restoration of dystrophin-associated proteins and the level of cellular infiltrates (only in neonates). However, differences were observed regarding the greater initial transduction of utrophin followed by the larger drop in expression from the same transgene (Tables 1 and 2). One explanation for the differences in duration of transgene expression between utrophin and dystrophin transduced muscles may involve the inherent stability of each protein. Unlike utrophin expression in adult *mdx* mice, which drops between days 30 and 60 after injection, the amount of viral DNA decreased as dramatically but slightly earlier, between 10 and 30 days after injection. Although similar decreases in viral DNA were observed after dystrophin gene transfer (Dudley et al., 2004), dystrophin expression was maintained for much longer, supporting the notion that dystrophin has a longer half-life than utrophin (Ahmad et al., 2000;Squire et al., 2002) and highlighting the difference in kinetics and overall stability between these two homologous proteins. Furthermore, treatment with HdAdV-mUTR did not appear to induce any additional immune responses in the *mdx* muscle (both cellular and humoral) other than what is normally associated with the dystrophic phenotype (Figures 12 and 19). This is in contrast to the *mdx* mice injected with the dystrophin-expressing adenovirus, which developed antibodies against the transgene in both neonatal and adult mice and demonstrated increased cellular infiltrates in transduced areas of older mice (Tables 1 and 2) (Gilbert et al., 2003;Dudley et

al., 2004). Our group's previous results were comparable to another study of helper-dependent adenoviral delivery of full length murine dystrophin which similarly demonstrated high levels of transduction (~25% of the TA up to 1 month post-injection), a greater resistance to contraction-induced injury, no improvement in force generation and a slight increase in cellular infiltrates (DelloRusso et al., 2002). Taken together, the data show that, at early time points, gene transfer of full-length utrophin had a significant mitigating effect on the dystrophic phenotype in immunocompetent animals, and the magnitude of the effect was comparable to that demonstrated previously with murine dystrophin gene transfer (Gilbert et al., 2003;Dudley et al., 2004).

Utrophin expression did decline at later time points after HdAdV-mUTR administration, both in the neonates and in the adults. Loss of expression in the neonatal *mdx* and *scid* mice occurred despite the persistence of viral vector DNA (Figure 22). The decreased expression may be attributable to promoter shut-down as the cytomegalovirus enhancer (contained within the hybrid promoter) is susceptible to cytokine-induced down-regulation (Harms and Splitter, 1995). The CMV promoter along with other viral promoters has been shown to be susceptible to cytokines such as IFN-γ and TNF-β (Harms and Splitter, 1995;Qin et al., 1997;Sung et al., 2001). In contrast to other promoters such as RSV which was activated in the presence of IFN-γ, CMV activity was downregulated. The loss of gene expression in skeletal muscle due to promoter shutdown has also been alluded to in other gene therapy studies involving helper dependent adenoviruses (Chen et al., 1999). Thus, in the case of the hybrid CB promoter

which contains the CMV enhancer, IFN-γ produced by innate immune mediators such as NK cells may play a role in promoter shutdown leading to a decreased utrophin expression emanating from HdAdV-mUTR (Figure 23). Although there was no overt immune reaction, components of the innate immune response may have contributed to the observed effects. In contrast to the neonates, in the adult *mdx* mice, there was clearly a decrease in vector copy number over time. Thus loss of expression in the adults may be compounded through initial promoter down-regulation, followed by loss of episomal DNA owing to the consequences of the ensuing dystrophic pathology and not due solely to the immune system.

Levels of Utrophin Determine Extent of Correction

Despite the overall decrease in utr$^+$ fibers among all adult mice, there were a few animals in which high levels of utrophin persisted throughout the experiment (Figure 14). This suggests that a higher initial transduction rate may be needed to prevent the natural progression of the dystrophic phenotype and that, compared with dystrophin, extrasynaptic utrophin expression may be required above a certain threshold to compensate functionally for dystrophin deficiency. This has already been suggested by earlier studies in *mdx* utrophin transgenic mice as physiological improvement paralleled the utrophin expression of the various transgenic lines (Tinsley et al., 1998). This was also addressed by our experiments in which a higher utrophin transduction was aimed for through the use of higher titer viruses or by the co-treatment with TSA. By these methods, a higher and robust initial transduction was achieved. This increase in utrophin expression may have mitigated the dystrophic phenotype through the

appearance of a healthier and stronger muscle. This was confirmed by the sustained larger number of utr^+ fibers, a greater number of muscle fibers with larger diameters and lower CK values in the sera of treated *mdx* mice (Figure 25). Muscle physiological indices involving adult *mdx* muscles co-treated with HdAdV-mUTR and TSA also provided positive results. An improved stress resistance was attained through the combination therapy which was significantly better than either therapy yielded by itself. The most noteworthy result observed in these physiological experiments was the improvement in muscle force generation, a feat that our group had not been able to produce previously in mature *mdx* muscle following adenoviral gene transfer (Figure 26).

It is unclear how much extrasynaptic utrophin can be deemed sufficient for full compensation of dystrophin deficiency. Initial studies estimated this ratio of utrophin to dystrophin to be approximately 2:1 for certain *mdx* muscles, although recent data based on total protein content suggest that utrophin at half the amount of dystrophin found in a normal mouse, or 10 times the amount of utrophin in an *mdx* mouse, would be sufficient to rescue most of the known dystrophic phenotypes (Ervasti, 2006;Rybakova et al., 2006).

Future Directions: Multi Modal Approach

In conclusion, the physiological consequences of utrophin gene transfer to *mdx* muscle compare very favorably to what has previously been achieved by dystrophin gene transfer with a helper-dependent AdV. In the long term, both loss of episomal DNA and decreased gene expression from the AdV are major

contributing factors to the observed late decline of the utr$^+$ fiber numbers. In this regard, modification of the viral backbone by introducing a matrix attachment region (Baiker et al., 2000) or by inducing chromosomal integration (Lieber et al., 1999;Recchia et al., 1999;Goncalves et al., 2002;Murphy et al., 2002;Goncalves et al., 2005;Ehrhardt et al., 2007) may overcome loss of episomal DNA. Use of a muscle-specific promoter (Larochelle et al., 1997;Hauser et al., 2000) and insertion of sequences that allow for consistent transcriptional activity may also prevent loss of expression (Wang et al., 2005). However, when expressed in sufficient amounts (high levels), either at the early timepoints following HdAdV-mUTR injection, through the use of a higher titer or alongside TSA treatment, murine utrophin compares favourably with murine dystrophin. Eventually, for optimal long-term therapeutic effects, utrophin gene transfer may have to be combined with up-regulation of the endogenous utrophin A gene expression (Hirst et al., 2005) to attain sufficient overall levels of extrasynaptic utrophin on the sarcolemma. One possibility may involve the use of zinc-finger based transcription factors. Two such proteins, Jazz and Bagly bind to and activate transcription of utrophin promoter A *in vitro* (Corbi et al., 2000;Onori et al., 2007). Similar upregulation of endogenous utrophin has been observed in Jazz transgenic mice (Mattei et al., 2007). If this strategy were achievable, it would be competitive and complementary to others currently being evaluated for overcoming dystrophin deficiency (Chakkalakal et al., 2005).

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Appendix