UNIVERSITY OF SOUTHERN QUEENSLAND

MODIFICATION OF THE MYOSTATIN AND NITRIC OXIDE SIGNALLING PATHWAYS AS TREATMENTS FOR DUCHENNE MUSCULAR DYSTROPHY

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Abstract

Duchenne muscular dystrophy (DMD) is a fatal X-linked disease affecting 1 in 3500 live male births that arises from faulty production of the sub-sarcolemmal protein, dystrophin. Pathology associated with dystrophin deficiency is complex but primarily characterised by progressive muscular atrophy and fibrosis resulting from cycles of damage and repair. Four separate studies in this dissertation used murine models of DMD (*mdx* mice) to examine the effects of myostatin absence and modulation of the NO-cGMP pathway on muscle function and fibrosis.

Transgenic mice lacking the ability to produce the endogenous inhibitor of muscle growth, myostatin, were generated in both dystrophic (2KO) and non-dystrophin ($Mstn^{-/-}$) strains. Myostatin absence caused cardiac hypertrophy and rescued cardiac function in *mdx* mice but was associated with increased cardiac fibrosis.

Earlier studies suggested that dietary supplementation with the nitric oxide synthase (NOS) substrate, L-arginine, improved cardiac function but also increased muscle fibrosis at higher doses. This study aimed to optimise L-arginine dosing by treating *mdx* mice with 1%, 2% and 5% L-arginine in drinking water and assessment of cardiac function and fibrosis. Low-dose L-arginine had minimal to no effect on both cardiac function and cardiac fibrosis.

Patch-clamp, calcium imaging and cell shortening experiments showed *mdx* cardiomyocytes had smaller calcium transients accompanied by depressed calcium currents but retained relatively strong cell shortening. Overexpression of cardiac nNOS in *mdx* mice resulted in normalisation of L-type activation and inactivation as well as force-frequency relationships, increases in calcium transient height and a prolonged peak time interval. In addition, there was also evidence of an additional outward current observed in *mdx* cells.

Chronic administration of the PDE5 inhibitor tadalafil was used to examine whether cGMP-mediated pathways affected mRNA expression of the profibrotic genes TNF-α, TGF-β, fibronectin and PC1. Tadalafil did not significantly alter expression of any of the genes tested in twelve month old *mdx* but the co-precipitate in manufacture, HPMCP, may possess profibrotic properties. Neither treatment altered cardiac or skeletal muscle fibrosis.

Studies in this dissertation highlight potentially beneficial treatment options in addition to defining possible drawbacks. Although further research is required, controlled myostatin administration or manipulation of the NO-cGMP pathway targeted toward increasing sub-sarcolemmal nNOS show promise as palliative treatments for DMD.

Certification of Dissertation

I hereby certify that the work reported in this thesis is entirely my own effort, except where acknowledged. I also certify that the work is original and has not been previously submitted for assessment in any other course of study at this, or any other institution.

Signature of candidate	Date

Endorsement

Signature of supervisor/s	Date

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Table of Contents

Chapter 1: Introduction 1
1.1 Duchenne Muscular Dystrophy 1
1.1.1 History of DMD 1
1.1.2 Incidence 1
1.2 Dystrophin: gene and protein product 2
1.2.1 The dystrophin gene 2
1.2.2 Dystrophin protein 4
1.3 Clinical manifestations of DMD 6
1.3.1 Initial presentation 6
1.3.2 Diagnosis 7
1.3.3 Clinical progression 10
1.4 Pathology of dystrophic muscle 12
1.4.1 Mechanical damage hypothesis 13
1.4.2 Altered calcium homoeostasis 14
1.4.3 Downstream contributors to pathology 17
1.4.4 Fibrosis 18
1.5 Animal models of DMD 23
1.5.1 Dystrophin deficient mouse (*mdx*) 23
1.5.2 Other animal models 27
1.6 Current treatments 29
1.6.1 Pharmacological support 33
1.7 Future therapies 38
1.7.1 Gene modification 38
1.7.2 Cell therapy 44
1.7.3 Utrophin up-regulation 45
1.7.4 Nitric oxide manipulation 46
1.7.5 Myostatin inhibition 53
1.8 Aims and scope of the study 55
Chapter 2: Materials and Methods 62
2.1 Ethical considerations 62
2.2 Experimental animals 62
2.2.1 Breeding 62

2.2.2 Genotyping protocols 63
2.3 In vivo cardiac function - Millar catheter 68
2.4 Ex vivo cardiac function - Isolated Langendorff heart 69
2.5 Assessment of cardiac hypertrophy 71
2.6 Assessment of collagen content 71
2.7 Measurement of gene expression by qRT-PCR 73
2.7.1 RNA extraction 74
2.7.2 First strand cDNA synthesis 76
2.7.3 qRT-PCR evaluation of gene expression 77
2.8 Single cell methodologies 79
2.8.1 Cell isolation 79
2.8.2 Assessment of contractility 80
2.8.3 Calcium imaging 82
2.8.4 Voltage clamp 83
2.9 Statistical analysis 86
Chapter 3: Myostatin knock-out in the dystrophic heart 87
3.1 Introduction 87
3.3 Results 92
3.3.1 Genotyping 92
3.3.2 Morphometry 92
3.3.3 *Ex vivo* measurements - Langendorff 94
3.3.4 Histology 97
3.3.5 Gene expression 98
3.4 In vivo measurements – Millar catheter 100
3.4.1 Arterial measurements 100
3.4.2 Ventricular measurements 103
3.5 Discussion 106
Chapter 4: L-Arginine dose optimisation in *mdx* mice 113
4.1 Introduction 113
4.2 Materials and methods 117
4.3 Results 118
4.3.1 Dosing 118
4.3.2 Morphometry 119
4.3.3 In vivo measurements 122

4.3.4 *Ex vivo* measurements 128
4.3.5 Histology 130
4.4 Discussion 133
Chapter 5: Cardiac nNOS overexpression in dystrophic cardiomyopathy 140
5.1 Introduction 140
5.2 Materials and methods 142
5.3 Results 144
5.3.1 Genotyping 144
5.3.2 Voltage clamp measurements 144
5.3.3 L-type calcium channel rundown 147
5.3.4 Contractility and calcium handling measurements 148
5.3.4 Multi-variant analysis 151
5.4 Discussion 153
Chapter 6: Effect of tadalafil on profibrotic gene expression in *mdx* mice 157
6.1 Introduction 157
6.2 Materials and methods 161
6.3 Results 162
6.3.1 Gene expression 162
6.3.2 Histology 168
6.4 Discussion 170
Chapter 7: Conclusions and future directions 174
References 178

List of Figures

Figure 1.2.1 Representation of dystrophin protein synthesis. 3
Figure 1.2.2 Dystrophin and the DGC. 5
Figure 1.3.1 Gowers' sign 7
Figure 1.4.3 Summary of pathogenesis and disease progression in DMD. 22
Figure 1.6.1 Kaplan-Meier curve for loss of ambulation in corticosteroid treated patients. 34
Figure 1.7.4 Diagram showing the NO-cGMP pathway. 51
Figure 1.8.1 Summary of the four studies conducted and reported in this dissertation. 60
Figure 1.8.2 Summary of experimental techniques used in each of the four studies. 61
Figure 2.8.1 Inverted Olympus IX70 microscope 82
Figure 2.8.2 Inverted Olympus CK30 microscope 84
Figure 2.8.3 L-type calcium channel inactivation protocol 85
Figure 2.8.4 Calcium current–voltage relationship protocol 85
Figure 3.1 Summary of experimental animal numbers and techniques used in Chapter 3. 91
Figure 3.2 Myostatin genotyping gel. 92
Figure 3.3 Assessment of cardiac hypertrophy. 93
Figure 3.4 Coronary flow rate. 94
Figure 3.5 Developed pressure in the Langendorff isolated heart preparation. 95
Figure 3.6 Rates of left ventricular pressure change measured during Langendorff experiments. 96
Figure 3.7 Representative images of ventricular sections stained with picrosirius red. 97
Figure 3.8 Cardiac collagen content measured by digital quantification of picrosirius red staining. 98
Figure 3.9 TNF-α expression in the heart determined by qRT-PCR. 99
Figure 3.10 TGF-β expression in the heart determined by qRT-PCR. 99
Figure 3.11 Arterial pressure development. 101

Figure 3.12 Rates of arterial pressure change during Millar catheterisation. 102
Figure 3.13 Mean arterial blood pressure measured during Millar catheterisation. 103
Figure 3.15 Ventricular pressure development during Millar catheterisation. 104
Figure 3.16 Rates of ventricular pressure change measured during Millar catheterisation. 105
Figure 4.1 Localisation of nNOS to the DGC. 113
Figure 4.2 Chemical structure of L-arginine 114
Figure 4.3 Profibrotic pathway associated with excess L-arginine.... 115
Figure 4.4 Summary of techniques and mouse numbers used in Chapter 4. 117
Figure 4.5 Body and TA weights. 120
Figure 4.6 Normalised heart weight to body weight or tibial length. 121
Figure 4.7 Heart rate measured by Millar catheter. 122
Figure 4.8 EDP, ESP and pulse pressure measured at aortic arch by Millar catheter. 123
Figure 4.9 Rates of arterial blood pressure change measured by Millar catheter. 124
Figure 4.10 Mean arterial blood pressure (MABP). 125
Figure 4.11 Left ventricular EDP, ESP and developed pressure measured by Millar catheter. 126
Figure 4.12 Rates of ventricular pressure change measured by Millar catheter. 127
Figure 4.13 Left ventricular developed pressure (Langendorff)..... 128
Figure 4.14 Rates of left ventricular pressure change..... 129
Figure 4.15 Coronary flow measured during Langendorff experiments. 130
Figure 4.16 Representative images of picrosirius red-stained cardiac and skeletal muscle sections. 131
Figure 4.17 Cardiac collagen content assessed by digital quantification of picrosirius red staining. 132
Figure 4.18 TA collagen content assessed by digital quantification

of picrosirius red staining. 132
Figure 5.1 Summary of techniques and experimental numbers used in Chapter 5. 143
Figure 5.2 Detection of nNOS transgene in cardiac tissue by PCR. 144
Figure 5.3 Current-voltage (IV) relationship as determined by voltage clamp. 145
Figure 5.4 L-type calcium channel inactivation measured by voltage clamp. 146
Figure 5.5 L-type calcium channel rundown at different pulse rates. ... 147
Figure 5.6 Force-frequency relationships. 148
Figure 5.7 Cell shortening characteristics. 150
Figure 5.8 Calcium transient characteristics for each genotype. 151
Figure 5.9 Calcium transient-cell shortening relationship..... 152
Figure 6.1 Diagram representing the NO/cGMP pathway. 158
Figure 6.2 Summary of mouse numbers and techniques used in Chapter 6. 162
Figure 6.3 TNF-α gene expression in heart, diaphragm and tibialis anterior assessed by qRT-PCR. 163
Figure 6.4 TGF-β mRNA expressions in heart, diaphragm and tibialis anterior assessed by qRT-PCR. 164
Figure 6.5 Procollagen α1 mRNA expression in heart, diaphragm and tibialis anterior assessed by qRT-PCR. 165
Figure 6.6 Fibronectin gene expressions in heart, diaphragm and tibialis anterior assessed by qRT-PCR. 166
Figure 6.7 Representative images of cardiac and skeletal muscle sections stained with picrosirius red. 168
Figure 6.8 Collagen content in heart, diaphragm and tibialis anterior. . 169

List of Tables

Table 1.6.1 Multi-disciplinary team approach to the management of DMD. 31

Table 1.7.4 Potential roles of NO in the treatment of dystrophinopathy 52

Table 2.2.1 Digestion solution 64

Table 2.2.5 Primers for myostatin null genotyping PCR 65

Table 2.2.6 Reagents and amounts for myostatin null genotyping PCR. 65

Table 2.2.7 Cycling conditions for myostatin null genotyping PCR. ... 66

Table 2.2.2 Primers for nNOS cardiac overexpression strain genotyping PCR. 67

Table 2.2.3 Reagents and amounts for nNOS cardiac overexpressing genotyping PCR. 67

Table 2.2.4 Cycling conditions for nNOS cardiac overexpressing genotyping PCR. 68

Table 2.6.1 Tissues used for assessment of collagen content via picrosirius red staining. 71

Table 2.6.2 Picrosirius red staining protocol..... 73

Table 2.7.1 Reagents and ratios for gDNA check PCR. 75

Table 2.7.2 Cycling conditions for gDNA check PCR. 76

Table 2.7.3 SuperScript III First-Strand Synthesis SuperMix. 77

Table 2.7.4 SYBR GreenER qPCR SuperMix. 77

Table 2.7.5 Amplification cycling conditions for qRT-PCR evaluation of gene expression. 78

Table 2.7.6 Primers used for genes of interest. 78

Table 2.8.1 Solutions used in cell isolation..... 80

Table 4.1 Average water consumption and amount of L-arginine received per mouse. 118

Table 6.1 Summary of profibrotic gene expressions with tadalafil and HPMCP administration in selected *mdx* muscle. 167

List of Abbreviations

±dP/dt	rate of pressure change over time
2KO	*Mstn*$^{-/-}$/*mdx*
AAV	adeno-associated virus
ACE	angiotensin converting enzyme
AchR	acetylcholine receptors
ActRII	activin type-2 receptors
ADHD	attention-deficit hyperactivity disorder
Akt	protein kinase B (PKB)
ANP	atrial natriuretic peptide
AO	antisense oligonucleotide
ARB	angiotensin receptor blocker
ATP	adenosine triphosphate
BMD	Becker's muscular dystrophy
bp	base pairs
bpm	beats per minute
C57	C57BL/10ScSn
cdk	cyclin-dependant kinase
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
CICR	calcium-induced calcium release
CK	creatine kinase
CXMDJ	dystrophic beagle
DGC	dystrophin-glycoprotein complex
DMD	Duchenne muscular dystrophy
DNA	deoxyribonucleic acid
ECG	electrocardiogram
ECM	extracellular matrix
EDP	end diastolic pressure
EDTA	ethylenediaminetetraacetic acid
EMG	electromyography
eNOS	endothelial nitric oxide synthase
ESP	end systolic pressure
Galgt2	GalNAc transferase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	guanylate cyclase
GDF-8	growth and differentiation factor-8
gDNA	genomic DNA
Grb2	Growth Factor Receptor-bound Protein 2
GRC	growth factor-regulated channel
GRMD	golden retriever muscular dystrophy dog
GTP	Guanosine-5'-triphosphate

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HPMCP	hydroxypropyl methylcellulose phthalate
HR	heart rate
Hz	Hertz
ip	intraperitoneal injection
IP_3	inositol triphosphate
IQ	intelligence quotient
IV	current-voltage relationship
kb	kilo bases
kDa	kilo Dalton
kg	kilogram
KO	knock-out
LV	left ventricle
LVEDP	left ventricular end diastolic pressure
LVESP	left ventricular end systolic pressure
M	molar
MABP	mean arterial blood pressure
mdx	C57BL/10ScSn *mdx* (muscular dystrophy X-linked)
mg	milligram
min	minute
mL	millilitre
MLPA	multiplex ligation-dependent probe amplification
mM	millimolar
mmHg	millimetres of mercury
MMP	matrix metalloprotease
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
ms	millisecond
Mstn	myostatin
mV	millivolt
MΩ	mega ohm
NADPH	nicotinamide adenine dinucleotide phosphate
NF- κB	nuclear factor kappa-B
ng	nanogram
nM	nanomolar
nm	nanometre
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
^oC	degree centigrade
PC1	procollagen α1
PCR	polymerase chain reaction

PDE5	phosphodiesterase 5
pH	potential hydrogen
PKA	protein kinase A
PKG	protein kinase G
PLB	phospholamban
pmol	picomolar
PP	pulse pressure
qRT-PCR	quantitative real time PCR
Rb	retinblastoma protein
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RyR	ryanodine receptor
RyR2	cardiac ryanodine receptor
SAC	stretch-activated channel
sec	second
SEM	standard error of the mean
SERCA	sarcoplasmic reticulum calcium ATPase
snRNP	small nuclear ribonucleoproteins
SOC	store-operated channel
SR	sarcoplasmic reticulum
TA	tibialis anterior
TGF- β	transforming growth factor-β
TNF- α	tumour necrosis factor-α
tris	tris(hydroxymethyl)aminomethane
TRPC1	transient receptor potential, canonical
U	unit
VEGF	vascular endothelial growth factor
w/v	weight per volume
XO	xanthine oxidase
µL	microlitre
µM	micromolar
µm	micrometre

Chapter 1: Introduction

1.1 Duchenne Muscular Dystrophy

1.1.1 History of DMD

The earliest documented case of Duchenne muscular dystrophy (DMD) was reported by Charles Bell in 1830 when he described the awkward movements of an eighteen year old male (Emery 1993). The disease was subsequently named after Guillaume-Benjamin-Amand Duchenne who described it in 1861, and whose research, along with that of physicians Edward Meryon and William Gowers, now forms the basis of recognition and understanding of this complex and severe muscle disorder.

More than a century elapsed after initial clinical descriptions until more detailed insights into the nature of DMD were reported. Okinaka et al. (1959) discovered that serum levels of creatine kinase (CK), an indicator of tissue damage, are greatly elevated in DMD patients. Wilcox et al. (1985) determined that DMD was caused by a defective gene found in the short arm of the X-chromosome enabling Hoffman et al. (1987) to identify and characterise the protein dystrophin. Subsequently, Zubrzycka-Gaarn et al. (1988) demonstrated the important, protective role that dystrophin plays in the maintenance of myocyte integrity.

More recently, advances in the treatment of respiratory insufficiency such as nocturnal ventilation, cough assist devices and surgery to correct scoliosis, combined with corticosteroid administration, have increased both the quality of life and life span of DMD patients (Eagle et al. 2007). Refinements of molecular techniques have not only provided insight into the genetic origins of the disease but also the promise of novel treatments.

1.1.2 Incidence

DMD, the most common of the muscular dystrophies affecting one in 3500 live male births, is caused by a mutation in the dystrophin gene (Hoffman et

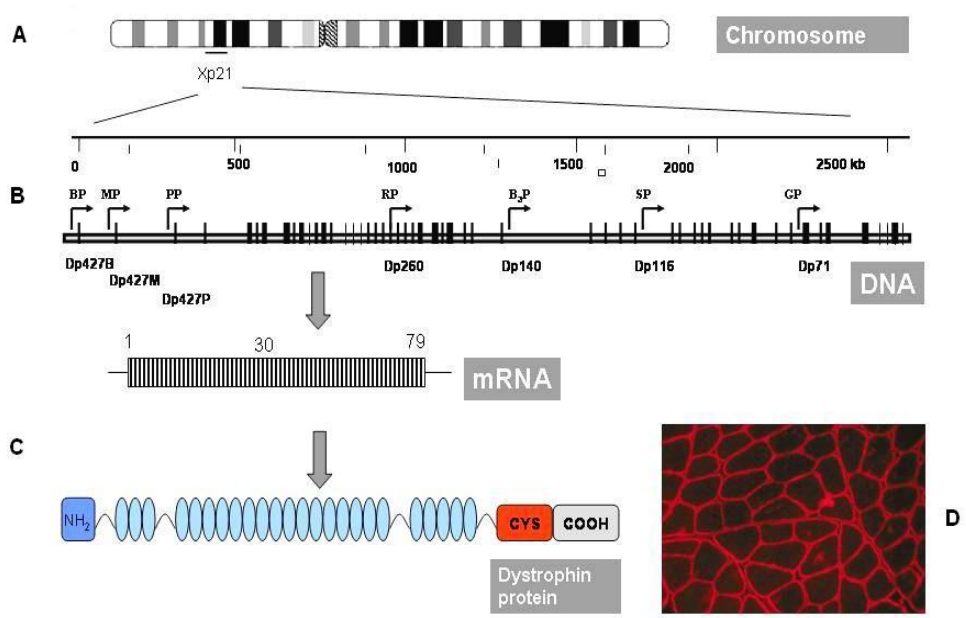
al. 1987; Emery 1991). It is estimated that up to one third of mutations are spontaneous, while remaining cases are inherited from a carrier mother in a typical X-linked recessive pattern (Emery 1991). While DMD is considered a predominantly male disease, 2-20% of female carriers also display muscle weakness (HoogerwaardBakker, et al. 1999). Subclinical cardiac disease is also evident in some female carriers including dilated cardiomyopathy and left ventricular dilatation (Hoogerwaardvan der Wouw, et al. 1999).

1.2 Dystrophin: gene and protein product

1.2.1 The dystrophin gene

The dystrophin gene is located on the short arm of the X-chromosome at position Xp21.1 (Hoffman et al. 1987). This is the largest human gene characterised, in excess of 2.4Mb and comprising 0.1% of the total human genome (Koenig et al. 1987; Roberts 2001). This complex gene has 79 exons and seven independent promoters making it potentially susceptible to extensive modification. The genomic locus is approximately 200 times greater in size than the final RNA transcript (14kb) with an average size of exons 200bp and average size of introns 35kb (Koenig et al. 1987). Figure 1.2.1 describes the position of the dystrophin gene, size and position of promoters as well as assembly and localisation of the dystrophin protein.

The most important promoters initiate the production of the different isoforms of the dystrophin protein. These have been named for the tissues in which they have been found – muscle, brain and Purkinje fibres (Blake et al. 2002). Products of the dystrophin gene are mostly found in skeletal, smooth and cardiac muscles as well as the brain; smaller isoforms of dystrophin are also found in retina, kidneys, liver, lungs and nervous system (Blake et al. 2002).



(Scheuerbrandt 2009)

Figure 1.2.1 Representation of dystrophin protein synthesis.

A: Location on the X-chromosome; **B:** Size, location of promoters, position of exons and relative size of introns; **C:** Assembly of mRNA and final protein; **D:** Immunofluorescence indicating localisation of dystrophin protein to sarcolemma.

Whole exon deletions account for approximately two thirds (64%) of genetic alterations that cause DMD. Deletions from one or more exons have a one in seven chance of causing a reading-frame shift resulting in an in-frame stop codon downstream (Prior et al. 1995; Aartsma-Rus et al. 2009). Other common forms of mutation are point mutations (27%) and duplications (8%) (Aartsma-Rus et al. 2009). Rare mutations such as missense, translocation, inversion and cryptic exon mutations account for approximately 1% of cases (Aartsma-Rus et al. 2009). While mutations occur throughout the gene, deletions are clustered within two mutation 'hot-spots' located approximately 500kb and 1200kb from the 5' end of the gene within exons 1-20 and 45-55 respectively (Koenig & Kunkel 1990). These mutations alter genetic coding, resulting in the production of truncated and ineffective versions of dystrophin (Prior et al. 1995).

Mutations that cause a reading frame shift will halt production of dystrophin and result in the severe pathology associated with DMD while those that

maintain the reading frame and allow production of a truncated though partially functional protein, result in Becker's muscular dystrophy (BMD) (Monaco et al. 1988). BMD has a milder phenotype and progresses slower than DMD (Becker & Kiener 1955).

1.2.2 Dystrophin protein

It is important that the structure and function of dystrophin is well understood as the absence of this protein is integral to the development of pathology in patients with DMD.

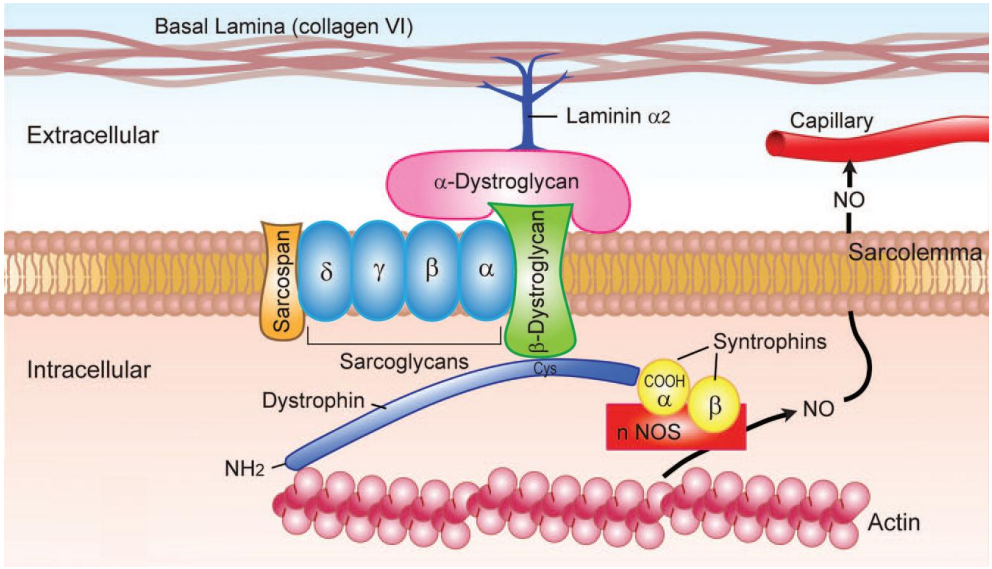
Protein Structure

The mRNA for full length dystrophin following translation is approximately 14kb long and produces a large protein of approximately 3685 amino acids (Blake et al. 2002). Although full-length functional protein weighs 427kDa, transcripts of dystrophin have been measured as low as 71kDa. The protein is comprised of four structural regions with a group of rod-bundles encompassing approximately 70% of the protein's length found near the amino-terminal. This terminal, along with regions of the rod domain, directly bind to actin forming a link between dystrophin and the cytoskeletal contractile machinery. There is also a cysteine-rich domain located after the rod domain. This is a complex region consisting of β-sheets, tightly turning α-helices and supporting zinc molecules (Roberts 2001). Finally, a carboxy-terminal domain completes the protein.

Role of Dystrophin

Dystrophin is associated with a group of proteins that interacts with the sarcolemma, termed the dystrophin-glycoprotein complex (DGC) (Figure 1.2.2). Dystrophin plays a vital role in the maintenance of myocyte integrity by forming a link between the contractile machinery and sarcolemma via the DGC (Carlson 1998; Emery 2002). Dystrophin is spread over the surface of the myocyte in an even, regular distribution, termed costameres that disperse mechanical forces generated during muscle contraction (Pardo et al. 1983; Petrof et al. 1993; Watkins et al. 1997). In addition, dystrophin and

associated proteins further stabilise the sarcolemma by anchoring the cytoskeleton to the extracellular matrix (Campbell 1995).



(Verhaert et al. 2011)

Figure 1.2.2 Dystrophin and the DGC.

This complex consists of dystrophin, dystroglycan, sarcoglycans and syntrophins. The amino-terminal of dystrophin binds F-actin, while the cysteine-rich region binds β-dystroglycan, which in turn binds sarcoglycans within the sarcolemma. Syntrophins are bound to the carboxy-terminal end of dystrophin and are also associated with nNOS.

Dystrophin and associated proteins may play a role in organisation of the cytoskeleton and extracellular matrix-mediated signal transduction (Brown & Lucy 1997; Rando 2001b). Rando (2001b) posited that the primary role of the DGC is as a transmembrane signalling complex, as proteins such as the dystrobrevins, sarcoglycans and syntrophins are involved in signalling pathways. The DGC is also associated with well-characterised signalling cascades involving calmodulin (Niebroj-Dobosz et al. 1989), neuronal nitric oxide synthase (nNOS) (Brenman et al. 1996) and the adaptor molecule Growth Factor Receptor-bound Protein 2 (Grb2) (Yang et al. 1995). Disruption in dystrophin synthesis results in reductions of associated proteins and disassembly of the DGC, suggesting that it may also act as a scaffold for the localisation of these proteins (Ohlendieck & Campbell 1991; Emery 1993).

Dystrophin and the DGC also play important roles in myocyte calcium homoeostasis. Abnormal calcium handling is a well-recognised feature of dystrophic pathology (Bodensteiner & Engel 1978; Bertorini et al. 1982) and leads to cell necrosis through activation of proteolytic and phospholipolytic pathways (Turner et al. 1991; Alderton & Steinhardt 2000a). In the *mdx* mouse, a murine model of DMD, dystrophic tissue has increased calcium channel permeability and abnormal regulation of stretch-activated calcium channels (Hopf et al. 1996; Tutdibi et al. 1999; Yeung et al. 2005; Williams & Allen 2007b). In addition, forced expression of either full-length or mini-dystrophin in DMD myotubes restored normal L-type calcium channel properties and calcium homoeostasis preventing calcium-induced damage (Friedrich et al. 2004; Marchand et al. 2004). Dystrophin may influence calcium homoeostasis indirectly as increased mitochondrial uptake of calcium and subsequent alterations of cellular metabolism have also been reported (Robert et al. 2001; Vandebrouck et al. 2006). Alterations in activity of the sodium/calcium exchanger have also been observed in dystrophic tissue (Deval et al. 2002).

1.3 Clinical manifestations of DMD

1.3.1 Initial presentation

DMD is caused by a genetic fault and is present from prenatal life. However it is usually not suspected until the child reaches the age of 3-5 years and delayed motor development becomes apparent (Miller & Hoffman 1994). This is demonstrated by delayed walking (18 months instead of 13 months), difficulty running, jumping or climbing and a tendency to stumble or fall (Driscol 2001). These signs are not pathognomic for DMD but are observed frequently and lead to further investigation. Talipes is also common, where weakness of the anterior tibial and peroneal muscles causes the child to walk on his toes (McCance & Huether 1998).

Other characteristic signs of DMD include hypertrophy of the gastrocnemius muscle and adoption of a broad-based, waddling gait as the child increases his stability by moving his centre of gravity to a position

behind his hips and in front of his knees (Emery 1993). Also, a manoeuvre known as Gowers' sign, where the child climbs his own legs to stand is indicative of DMD (Fig 1.3). Signs of intellectual disability, such as lowered IQ, delayed speech and increased incidence of attention-deficit hyperactivity disorder (ADHD) are also detected in approximately 30% of boys with DMD (Anderson et al. 2002; Hendriksen & Vles 2008). Symptoms of intellectual involvement are not linked with severity of physical weakness (Leibowitz & Dubowitz 1981).

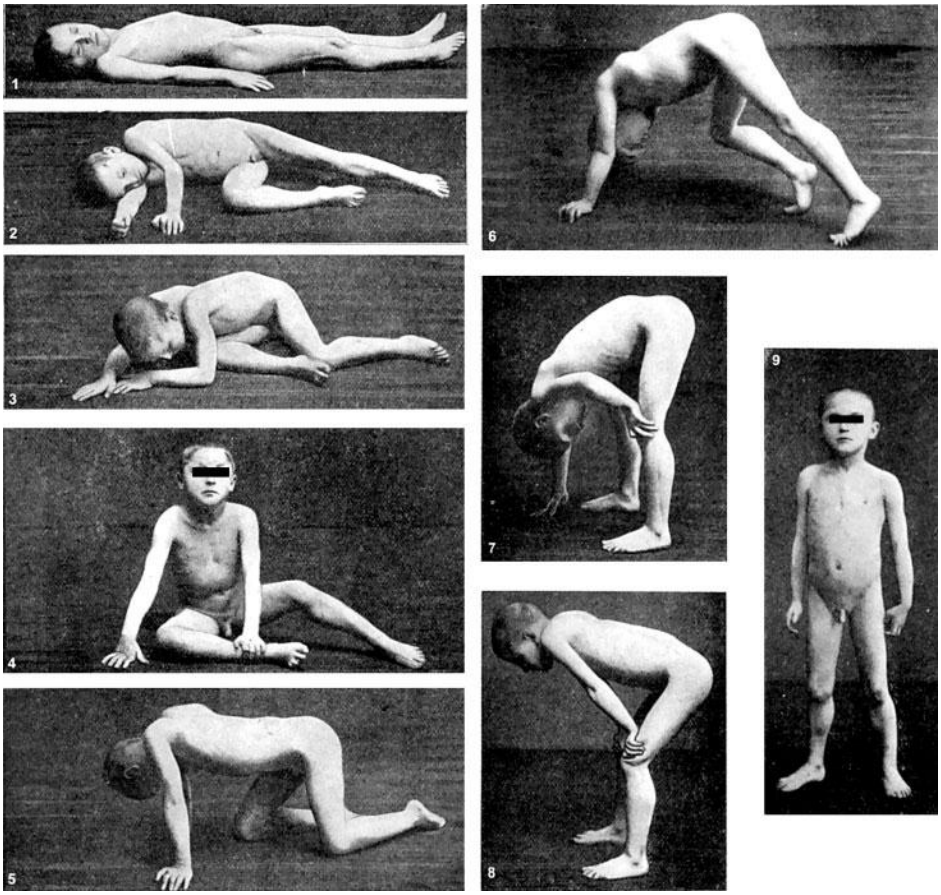


Figure 1.3.1 Gowers' sign represents the different positions taken by a DMD patient to rise from lying prone to standing (Dejerine (1914) cited in Tyler 2003).

1.3.2 Diagnosis

There are many techniques that can be employed to confirm a diagnosis of DMD including serum tests, histopathology and molecular tests. Advances in molecular techniques have allowed diagnosis of DMD and identification of the causative mutation. Electromyography and muscle magnetic

resonance imaging (MRI) have also been explored as means of characterising DMD but are not first-line diagnostic tests (Finsterer & Stollberger 2003).

Positive plasma tests for markers of tissue damage strengthen suspicion of DMD. Other muscle disorders may also elevate concentrations of these substances, however raised concentrations are indicative of DMD if present in conjunction with other clinical observations (Emery 2002). Plasma concentrations of CK are elevated from birth and can be as high as 50-200 times normal, with peak concentrations evident between the ages of 2-3 years (Rosalki 1989; Zatz et al. 1991). Conversely, a CK plasma concentration of less than ten times normal is counter-indicative and a differential diagnosis should be considered (Jones et al. 2003). While elevations of alanine transaminase, aspartate transaminase, aldolase and lactate dehydrogenase also occur in DMD, they are less useful as diagnostic indicators due their lack of muscle specificity (Tay et al. 2000; Korones et al. 2001).

Despite recent advances in molecular testing, muscle biopsy remains the 'gold standard' for diagnosis (Muntoni 2001). Histological analysis of dystrophic muscle reveals degenerating necrotic fibres, inflammation and macrophage infiltration, clusters of regenerating fibres with centrally located nuclei, high variability of fibre size and eventually, marked fibrosis and fat infiltration (Skinner 1995; Blake et al. 2002; Jones et al. 2003). Immunohistochemistry using antibodies against dystrophin or Western blot analysis may be employed to detect the presence and quantity of dystrophin and is useful in differentiating between DMD and the related, though milder, BMD. DMD biopsies may show less than 5% of normal levels of dystrophin, while BMD shows an intermediate level of dystrophin (Hoffman et al. 1988; Nicholson et al. 1993).

Molecular techniques identifying mutations within the dystrophin gene have become the first-line definitive diagnosis of DMD. Early multiplex polymerase chain reactions (PCR) pioneered by Chamberlain et al. (1988)

and Beggs et al. (1990) examined eighteen regions of the gene prone to deletions and were able to detect 90-98% of deletions within these hotspots. Unfortunately, these techniques were limited in that they did not examine the whole gene and were unable to identify duplication or point mutations. More recently, multiplex ligation-dependent probe amplification (MLPA) techniques have been developed that are more sensitive than previous PCR methods and are able to examine all coding areas of the gene for duplications as well as deletions (Schwartz & Duno 2004; Gatta et al. 2005). MLPA can also identity female carriers of a defective dystrophin gene, aiding genetic counselling. Hedge et al. (2008) described the application of targeted high-density oligonucleotide comparative genomic hybridisation microarray that permits high resolution analysis of the entire gene and is able to detect deletions, duplications and intronic mutations. If these techniques do not reveal a mutation despite a clinical presentation of DMD, specific identification and analysis of point mutations can be initiated (Schwartz & Duno 2004). Direct sequencing of the gene is not routinely used as a diagnostic tool as it is both costly and labour intensive (Flanigan et al. 2003).

Electromyography (EMG) and muscle MRI reveal abnormalities peculiar to DMD and may be employed to track the progress of the disease rather than as diagnostic tools. Alterations in signal conductivity are also evident in patients with DMD and can be detected by using electrocardiograms (ECG) and EMG. During a study into the nature of cardiac involvement in DMD conducted from 1976 to 1987, it was determined that patients with DMD exhibited tall R waves and wide Q waves in the ECG when viewed by the right precordial leads and left precordial leads, respectively (Nigro et al. 1990). Sanyal & Johnson (1982) found this pattern was evident in 70-80% of DMD cases. It is not uncommon for an EMG to show muscle fibrillations with sharp, positive waves and complex repetitive discharges (Desmedt & Borenstein 1975; Driscol 2001). Short duration, low amplitude polyphasic motor unit potentials have been observed in proximal muscles (Jones et al. 2003). There is also a tendency of muscles to become electrically silent as the disease progresses (Jones et al. 2003).

1.3.3 Clinical progression

DMD is characterised by progressive muscle weakness as repeated cycles of muscle necrosis and regeneration eventually render repair mechanisms of dystrophic tissue ineffective (Deconinck & Dan 2007). Activation of satellite cells causes them to multiply and differentiate into functional muscle tissue. Relentless cycles of damage and repair are repeated, eventually shortening muscle fibres and slowly replacing them with fat and fibrous tissue. The muscle groups most affected are those involved in the pelvic and pectoral girdles, quadriceps and gastrocnemius muscles (McCance & Huether 1998). The lower limbs are affected first, with many sufferers never being able to jump (Jones et al. 2003). Most boys with DMD lose their initial calf muscle hypertrophy by the age of ten, and are wheelchair-bound by the age of twelve years (Emery 2002; Jones et al. 2003). Limb contractures occur due to uneven muscle degradation, fibrosis and tendon shortening (Do 2002).

Osteoporosis is also seen in many patients as the disease progresses and they become progressively weaker. Lack of physical activity combined with long-term corticosteroid treatment results in loss of bone minerals and bone strength that is detectable even before wheelchair dependence, an effect that progresses with age (Aparicio et al. 2002; Soderpalm et al. 2007). Loss of mineral density combined with an increased tendency of patients to fall results in frequent fractures of the long bones and is a major contributing factor in loss of ambulation. It is estimated that 20-40% of DMD sufferers become wheelchair-bound as a direct result of fracture (McDonald et al. 2002).

Respiratory muscle weakness severely impacts on patients' quality and length of life, with respiratory failure remaining a major cause of death (Eagle et al. 2002). If left untreated, vital capacity decreases 8-12% per year from the age of ten years, with boys whose vital capacity is less than one litre having a life expectancy of only one to two years (Baydur et al. 1990; Phillips et al. 2001). Obstructive sleep apnoea is common and progressively

worsens until patients suffer hypercapnic chronic respiratory failure (Suresh et al. 2005; Toussaint et al. 2007). Life expectancy is less than one year once the patient is experiencing diurnal hypercapnia, although this may be extended through proper intervention.

90% of boys with DMD develop some form of scoliosis after they become wheelchair-dependent (Rodillo et al. 1988; Biggar et al. 2004). As spinal deformity worsens, respiratory function is further compromised and vital capacity declines (Smith et al. 1989). Surgical intervention to alleviate the effects of scoliosis is usually required by the age of 14-15 years (Kinali et al. 2007). The impact of these invasive surgical techniques is exacerbated as patients with DMD are more likely to develop malignant hyperthermia when under the influence of drugs such as halothane or succinylcholine (Kelfer et al. 1983; Hayes et al. 2008).

Remodelling occurs as muscle architecture becomes increasingly disrupted, leading to infiltration of fatty and connective tissue into muscle fibres. After damage, fibroblasts produce collagen in order to bind functioning muscle fibres together into cohesive units. In DMD, this action is repeated so often that extensive scar tissue is formed within the muscle that impairs function by impeding contraction and hampering blood flow (Bockhold et al. 1998).

It has been hypothesised that without the force-distributing effect of dystrophin, repeated cycles of muscle contraction damages myocytes, triggering calcium overload (Petrof et al. 1993). Excess calcium within the myocyte initiates numerous and uncontrolled mini-contractions that further damage or rupture the cell (Tay et al. 1992). Increased intracellular calcium concentration also activates cytotoxic proteases, such as calpains, whose actions promote apoptosis. The immune system is then activated and cytotoxic T-lymphocytes are attracted to the area to kill damaged myocytes and initiate neutrophil and macrophage infiltration. Dystrophin-deficient muscle is sensitive to free radicals released as the immune system attempts to remove any dead and damaged cells, resulting in further tissue damage (Rando et al. 1998).

Cardiac involvement in DMD is common. The dystrophic heart exhibits signs of valve dysfunction, changes in wall thickness, arrhythmias, tachycardia and heart murmurs (Emery 1994). The area of the heart most affected is the posterolateral wall of the left ventricle (Nigro et al. 1990). Abnormalities of heart function can be detected as early as ten years of age, although preclinical abnormalities, such as tachycardia, atrial or ventricular arrhythmias as well as complex or sustained ventricular ectopy can be observed in 25% of patients by the age of six years (Nigro et al. 1990; Chenard et al. 1993). By the age of eighteen years, it is expected that some form of cardiac abnormality will be detected in all cases of DMD (Nigro et al. 1990). Congestive heart failure is a commonly observed sequel due to ventricular weakness and volume overload (Nigro et al. 1990). Dilated cardiomyopathy and pulmonary congestion are caused by sustained increased pressures and, if left untreated, may lead to death.

Death is usually a result of either respiratory or cardiac failure (Boland et al. 1996). Until this decade, respiratory failure was the primary cause of death with some studies attributing it as the cause of up to 90% of DMD deaths (Ishikawa et al. 1995). Respiratory therapies in the form of nocturnal monitoring, mechanical cough assistance as well as spinal restraints have been employed to combat or prevent respiratory insufficiency (Eagle et al. 2002). To those DMD patients with access to these treatments, life expectancy has been extended to 25-30 years of age (Konagaya et al. 2005; Eagle et al. 2007). As the treatment of respiratory dysfunction improves, cardiomyopathies are becoming more prominent and are currently estimated to cause approximately 50% of DMD-related deaths (McCance & Huether 1998; Eagle et al. 2002).

1.4 Pathology of dystrophic muscle

The consequences of dystrophin loss are complex and encompass a wide range of structural and functional changes. Sarcolemmal fragility leads to repeated cycles of cell damage and repair, ultimately exhausting the ability of muscle tissue to regenerate and resulting in progressive muscle wasting (Deconinck & Dan 2007). Histological investigation of dystrophic muscle

reveals clusters of necrotic fibres infiltrated by macrophages and lymphocytes, as well as evidence of regeneration such as centrally nucleated fibres of varying size (Schmalbruch 1984; McDouall et al. 1990). Regenerative ability diminishes with age and eventually muscle fibres are replaced with connective and fatty tissues (Bockhold et al. 1998). In time, fibrosis and remodelling contribute to muscle dysfunction and joint contracture (Do 2002). Dystrophic muscle is also prone to inflammation, vascular dysfunction, metabolic abnormalities and increased fatigue resulting in significant skeletal, respiratory and cardiac myopathies (Tidball & Wehling-Henricks 2007). Synaptic dysfunction and intellectual disability are also observed in some patients (Shiao et al. 2004; Hendriksen & Vles 2008).

There have been a number of hypotheses put forward to explain the disease's natural history, although the precise mechanisms involved in progression of dystrophic pathology are not yet fully understood.

1.4.1 Mechanical damage hypothesis

Dystrophin plays a vital role in the stabilisation of sarcolemmal integrity. Loss of this protein leads to disassembly of the DGC and excessive fragility of muscle fibres as the DGC is no longer able to anchor the cytoskeleton to the extracellular matrix (Rybakova et al. 2000; Durbeej & Campbell 2002). Without the organising presence of dystrophin, other cytoplasmic proteins such as vinculin, desmin and spectrin also become dislocated and are no longer able to form costameres (Pardo et al. 1983). In healthy tissue, these lattice-like structures found on the cytoplasmic surface of the sarcolemma act to distribute forces generated during contraction to the basal lamina (Danowski et al. 1992). Dystrophin loss disorganises surrounding proteins, leading to disrupted force distribution by costameres. Subsequently, contractile forces create sarcolemmal lesions that eventually result in cell death (Williams & Bloch 1999). The presence of proteins such as albumin and immunoglobulins in fibres (Straub et al. 2000) as well as increased permeability of dystrophic muscle to dyes such as Evans blue and Procion orange (Matsuda et al. 1995) support this hypothesis.

1.4.2 Altered calcium homoeostasis

Robust regulation of calcium flux in myocytes is vital for proper muscle function. Early investigations of muscle biopsies taken from DMD patients revealed the presence of hypercontracted fibres and accumulation of calcium, suggesting that dysregulation of calcium kinetics was affecting muscle function and survival (Cullen & Fulthorpe 1975; Bodensteiner & Engel 1978). These findings prompted further investigations into calcium homoeostasis in dystrophin-deficient tissue.

The sarcolemmal loss of dystrophin and subsequent cytoskeletal disorganisation leads to abnormal calcium handling in favour of calcium influx. Consequently, high internal calcium concentrations cause aberrations in mitochondrial metabolism and activation of calcium-dependant proteases that hasten cell death (Alderton & Steinhardt 2000a; Robert et al. 2001). Calcium influx soon exceeds the ability of the cell to maintain homoeostasis by buffering mechanisms (Tinsley et al. 1998; Vandebrouck et al. 2006). This activates proteases such as calpains, that break down membrane components and induce further calcium influx, ultimately causing cell death (Carafoli & Molinari 1998; Raymackers et al. 2003). A similar vicious cycle has been suggested where mechanical stresses upon the sarcolemma produce microlesions that allow the passive influx of calcium, causing increasing concentrations that trigger calcium-induced proteolysis. Localised subsarcolemmal proteolysis activates calcium leak channels, inducing ongoing cycles of calcium leakage and damage ultimately resulting in cell death (Turner et al. 1993; Alderton & Steinhardt 2000a; Hopf et al. 2007).

It has been hypothesised that a number of calcium channels, particularly those associated with the transient receptor potential, canonical (TRPC1) gene, are variants of the same channel (Beech et al. 2004; Allen et al. 2005; Hopf et al. 2007). These channels include calcium leak, store-operated (SOC) and stretch-activated channels (SAC) (Vandebrouck et al. 2002; Maroto et al. 2005). Franco-Oregon and Lansman (2002) demonstrated that

SAC have an increased likelihood of being open in dystrophic muscle allowing intracellular concentrations of calcium to reach pathological levels. Increased calcium influx by these channels through dystrophin-deficient membranes has also been demonstrated in skeletal muscle by Vandebrouck et al. (2002) and in the heart by Williams and Allen (2007b). The growth factor-regulated channel (GRC) is another TRP family channel that is elevated in dystrophic tissue and activated by growth factors and stretch. These stimuli also cause the channel to translocate from the cytosol to the sarcolemma in a calcium-dependent manner that can be blocked by inhibiting sarcoplasmic reticulum (SR) calcium release via ryanodine receptors (RyR) (Iwata et al. 2003).

L-type calcium channels in dystrophic skeletal myotubes showed unaltered peak current with decreased total current and either unaltered or reduced voltage-dependent inactivation (Imbert et al. 2001; Friedrich et al. 2004). In the dystrophic heart, peak L-type calcium current is unchanged but channel inactivation is decreased (Sadeghi et al. 2002; Woolf et al. 2006). Decreased inactivation in both skeletal and cardiac muscle increases channel availability for opening and allows an increased calcium influx; however, this increased opening may be mitigated by decreased current density also observed in dystrophic myotubes (Imbert et al. 2001).

Calcium handling pathways within myocytes are also altered due to dystrophin loss. Inositol triphosphate (IP_3) receptors and concentrations of IP_3 were elevated in dystrophic myocytes (Liberona et al. 1998) and enhance calcium influx though activation of SOC and release of SR calcium stores (Kiselyov et al. 1998). Receptor inhibition by Bcl-2 lowered intracellular and mitochondrial calcium concentrations in myotubes derived from C57BL/10ScSn (C57) and C57BL/10ScSn *mdx* (*mdx*) mice (Basset et al. 2006). The loss of dystrophin is thought to remove a negative control on IP_3 receptors to enhance the slow release of calcium (Balghi et al. 2006). Altered RyR activity has also been implicated in dystrophinopathy, with phosphorylation of the receptor shown to increase, decrease or not change SR calcium release in the heart (Song et al. 2001; Viatchenko-Karpinski &

Gyorke 2001). β-adrenoceptor modulation of protein kinase A (PKA) action on RyR open probability also leads to depletion of SR calcium, that in turn activates SOC and increases intracellular calcium concentration (Valdivia et al. 1995; Vandebrouck et al. 2002). Increased calcium-induced calcium release (CICR) has also been seen in DMD myotubes (Deval et al. 2002). In addition, high cytosolic calcium concentrations enhance mitochondrial calcium uptake leading to mitochondrial overload and alteration of metabolism that accelerates cellular necrosis through enhanced production of reactive oxygen species (ROS) (Robert et al. 2001; Brookes et al. 2004).

Increased calcium influx may lead to high cytosolic concentrations of calcium, causing depletion of SR calcium through exaggerated CICR and SR leak. Store depletion triggers calcium influx through SOC further elevating intracellular calcium concentration and triggering mitochondrial overload and activation of proteases. High intracellular calcium concentrations increased production of ROS (Brookes et al. 2004) that activated nuclear factor kappa-B (NF-κB) (Kumar & Boriek 2003) and increased expression of TRPC1 proteins (Paria et al. 2003). Thus, the lack of dystrophin leads to enhanced expression of channels involved in increased calcium influx. Quinlan et al. (2004) demonstrated in the hearts of young *mdx* mice that, although the hearts exhibited abnormal calcium handling, they did not show signs of cardiac failure until older age, suggesting aberrant calcium handling is a primary feature of dystrophinopathy, not one secondary to cardiac failure.

Studies in *mdx* mice showed that high concentrations of calcium in muscle fibres were inhibited by the calcium and sodium channel blockers, nifedipine and amiloride (Hopf et al. 1996; Tutdibi et al. 1999). The calcium channel blocker, diltiazem, was investigated in humans by Pernice et al. (1988) but was found to have no clinical benefit, while overexpression of calpastatin, an endogenous calpain inhibitor, showed decreased necrosis in *mdx* mice (Spencer & Mellgren 2002). Transgenic introduction of a truncated form of dystrophin normalised L-type calcium channel properties in *mdx* mice (Friedrich et al. 2004).

1.4.3 Downstream contributors to pathology

Decreased availability of nitric oxide (NO), chronic inflammation and alterations in gene regulation also play roles in advancing pathology in DMD. It has been suggested that the full range of pathology is due to a complex sequence of events where effects of the genetic loss of dystrophin are compounded by downstream abnormalities in production and handling of free radicals (Rando 2001a). These abnormalities lead to changes in cell signalling, exacerbation of existing pathology and further disruption of free radical production and stoichiometry (Tidball & Wehling-Henricks 2007).

Dystrophic tissue is also in a state of chronic inflammation due to repeated cycles of muscle degeneration and regeneration (Deconinck & Dan 2007). This conclusion is supported by alterations in cytokine and chemokine signalling, leukocyte adhesion, presence of invasive cell markers and activation of the complement system (Haslett et al. 2002). Macrophage infiltration into inflamed areas acts through free radical-mediated mechanisms to initiate apoptosis (Nguyen & Tidball 2003a). While the exact mechanism of NO protection remains unknown, it has been shown to inhibit leukocyte adhesion (Liu et al. 1998) and free radical production (Clancy et al. 1992; Abu-Soud & Hazen 2000), induce leukocyte apoptosis (Albina et al. 1993) and scavenge cytotoxic free radicals (Miles et al. 1996). Similar levels of protection to cytotoxic effects are achieved by depletion of immune cells (Spencer et al. 2001) or overexpression of a neuronal nitric oxide synthase (nNOS) transgene (Wehling et al. 2001). Taken together, these results suggest that chronic inflammation exacerbates pathology in dystrophic tissue. Further, chronic inflammation may be ameliorated by regaining physiological NO concentrations.

Selective gene dysregulation is also an outcome of dystrophin loss. Goldspink et al. (1999) found that genes for a number of protein kinases, including p38 mitogen-activated protein kinase and c-Jun N-terminal kinases as well as genes responsible for calcineurin expression, were upregulated by mechanical stress in the hearts of *mdx* mice. Timmons

(2005) found that this pattern of gene upregulation was also present in those with DMD as well as healthy people during endurance training, suggesting that these alterations in gene regulation may represent a compensatory mechanism within the integrin signalling pathway (Deconinck & Dan 2007).

Decreased energy production in dystrophic muscle results in higher rates of fatigue observed in boys with DMD. Frascarelli et al. (1988) showed that eight to ten year old boys with DMD were able to sustain a 60-70% contraction of biceps brachii for 10 seconds while unaffected boys could sustain the same contraction for 45 seconds. Evidence of altered energy production include reduced concentrations of adenosine triphosphate (ATP) in isolated muscle fibres (Austin et al. 1992) and impaired glyceraldehyde 3-phosphate dehydrogenase (GAPDH) function (Souza & Radi 1998; Mohr et al. 1999). In addition, calcium overload of mitochondria may lead to altered metabolism and contribute to disordered calcium handling (Robert et al. 2001; Constantin et al. 2006) while modification of the cysteine domain within GAPDH leads to deactivation of the enzyme (Souza & Radi 1998; Mohr et al. 1999).

1.4.4 Fibrosis

Irrespective of the pathogenesis, the most prominent characteristic of dystrophic muscle is the progressive deposition of collagenous extracellular matrix resulting in severe decreases in skeletal and cardiac muscle function (Morrison et al. 2000). Cycles of damage and repair in dystrophic muscle disrupt the balance of collagen synthesis and degradation. Over time, the regenerative ability of the muscle becomes increasingly compromised, further unbalancing collagen regulation with affected muscles undergoing connective and fatty tissue infiltration (Bockhold et al. 1998).

Excessive fibrosis is linked with vascular impairment, conduction disruptions and progressive muscle weakening (Morrison et al. 2000; Deconinck & Dan 2007). The chaotic nature of dystrophinopathy leads to uneven muscle weakening about joints that results in joint contracture and

progressive scoliosis (Do 2002; Kinali et al. 2007). Joint contracture and scoliosis have significant outcomes on the quality and quantity of life for patients with DMD as they can lead to pain, disfigurement, compromised organ function and diminished respiratory function. Myocardial fibrosis is closely linked to altered cell signalling during inflammatory processes and affects systolic and diastolic function (Weber et al. 1989).

Molecular regulators of fibrosis

There are many factors that influence the development of fibrosis. The next section describes the effects of the cytokines, transforming growth factor-β (TGF-β) and tumour necrosis factor-α (TNF-α), as well as the expression of the proteins procollagen and fibronectin.

Transforming growth factor-β

TGF-β is a regulatory protein involved in inflammation, embryonic development, tissue repair and immunity that is activated by proteases, integrins, pH and reactive oxygen species (Passerini et al. 2002). Aberrations in TGF-β activation are linked with autoimmune disorders, fibrosis, oncogenesis and inflammation (Taipale et al. 1994; Yu & Stamenkovic 2000). Concentration and localisation of this pleiotropic cytokine alters the action of cells such as satellite cells and fibroblasts and exerts a strong influence on tissue repair and development of fibrosis (Murakami et al. 1999). TGF-β has a well-established connection to increased fibrosis in diseases such as liver cirrhosis and chronic pulmonary fibrosis (Khalil et al. 1989; Milani et al. 1991). Involvement of TGF-β in dystrophic fibrosis is also strong, with clear links to skeletal muscle fibrosis (Bernasconi et al. 1995). TGF-β concentrations in boys with DMD peak between the ages of two to six years and generally increase with the degree of fibrosis and age (Bernasconi et al. 1995). This progression may not be linear, as there is conflicting evidence that while TGF-β concentrations vary throughout the time-course of the disease, they may not reflect the level of fibrosis (Goldspink et al. 1994; Gosselin et al. 2004). For example, a study by Mezzano et al. (2007) found unaltered autocrine TGF-β signalling in *mdx* fibroblasts isolated from the diaphragm.

TGF-β acts though Smad proteins to alter transcription and increase collagen synthesis (Mauviel 2005). TGF-β also stabilises the collagen precursor procollagen α1 mRNA, while decreasing collagen degradation by matrix metalloproteases (MMP) (Eghbali et al. 1991). TGF-β exerts a strong influence on the extracellular matrix (ECM) as it is able to both induce expression and prevent degradation of ECM proteins (Siwik & Colucci 2004; Verrecchia & Mauviel 2007).

Tumour necrosis factor-α

TNF-α is recognised as a potent proinflammatory cytokine that also plays roles in induction of apoptosis, inhibition of tumour growth and viral disease and in postnatal growth and tissue regeneration (Li & Schwartz 2001; Locksley et al. 2001). Evaluating the precise role of this cytokine in fibrosis is complex as it acts in a tissue-selective manner. For example, it decreases fibrosis during liver injury repair (Hernandez et al. 2000) but acts as a mediator of inflammatory fibrosis in intestinal fibrosis associated with Crohn's disease (Theiss et al. 2005). TNF-α may also increase collagen degradation via increased MMP activity (Siwik & Colucci 2004).

Upregulation of TNF-α in DMD contributes to pathology through direct toxicity and augmentation of the inflammatory response (Porreca et al. 1999). Gene knock-out studies using *mdx* diaphragm have indicated that TNF-α plays a role in advancing deterioration in dystrophic muscle (Spencer et al. 2000; Gosselin et al. 2003). These studies have been supported by the pharmacological blockage of TNF-α by infliximab reducing skeletal muscle necrosis (Grounds & Torrisi 2004). Taken together, these findings indicate that although upregulation of TNF-α in dystrophic muscle is likely to be associated with increased pathology, other physiological roles of this cytokine must also be taken into account. Further study is required to predict whether manipulation of this cytokine will be beneficial in the treatment of DMD (Tidball & Wehling-Henricks 2004).

Procollagen α1

The procollagen α1 gene is responsible for the encoding of the α1 chain of collagen and is tightly regulated at the transcriptional level during tissue repair (Slack et al. 1993; Karsenty & Park 1995). Procollagen is the precursor protein to collagen, a major component of the ECM and connective tissue, and is synthesised by fibroblasts. Increased expression of this protein is seen during tissue remodelling and fibrotic changes as a result of stimuli such as mechanical strain or TGF-β signalling (Bishop & Lindahl 1999; Lindahl et al. 2002). Increased gene expression, as reflected by increased procollagen mRNA concentrations, has also been observed in *mdx* skeletal muscle (Goldspink et al. 1994). Interestingly, the same study also found that there was a matching age-related decrease in collagen concentrations in both *mdx* and control strains, suggesting that progressive fibrosis seen in dystrophic muscle is more likely related to impaired collagen degradation than enhanced gene expression (Goldspink et al. 1994).

Fibronectin

Cellular fibronectin is an insoluble glycoprotein that forms a major component of the ECM and binds integrins, collagen, fibrin and other glycoproteins such as proteoglycan (Pankov & Yamada 2002). It has roles in growth, cell adhesion, migration, and differentiation and is important in embryonic development (George et al. 1993), wound healing (Valenick et al. 2005) and maintenance of tissue integrity (White et al. 2008). Numerous tissue-specific splicing variants of fibronectin occur, with differing traits of cell adhesion, ligand binding and solubility allowing precise control of ECM composition (Pankov & Yamada 2002). While full understanding of the different roles of fibronectin isoforms is yet to be achieved, altered fibronectin regulation is associated with oncogenesis and fibrosis (Williams et al. 2008).

Fibronectin expression was increased in DMD myotubes (Meola et al. 1986). Fibroblasts isolated from *mdx* diaphragms showed increased synthesis of fibronectin while co-culture of dystrophic and control

fibroblasts increased fibronectin expression in control cells (Mezzano et al. 2007). In contrast, scanning electron microscopy of cells cultured from DMD biopsies revealed decreased surface fibronectin and disrupted organisation (Delaporte et al. 1990).

Pathogenesis in dystrophic muscle results from a complex interaction between the structural and functional consequences of dystrophin absence. Current knowledge suggests that primary contributors to pathology include sarcolemmal fragility, disordered calcium regulation and decreased production of NO. These are compounded by downstream contributors including alterations in free radical regulation, gene expression, cell signalling pathways and chronic inflammation. Figure 1.4.3 summarises pathways involved in dystrophic pathogenesis.

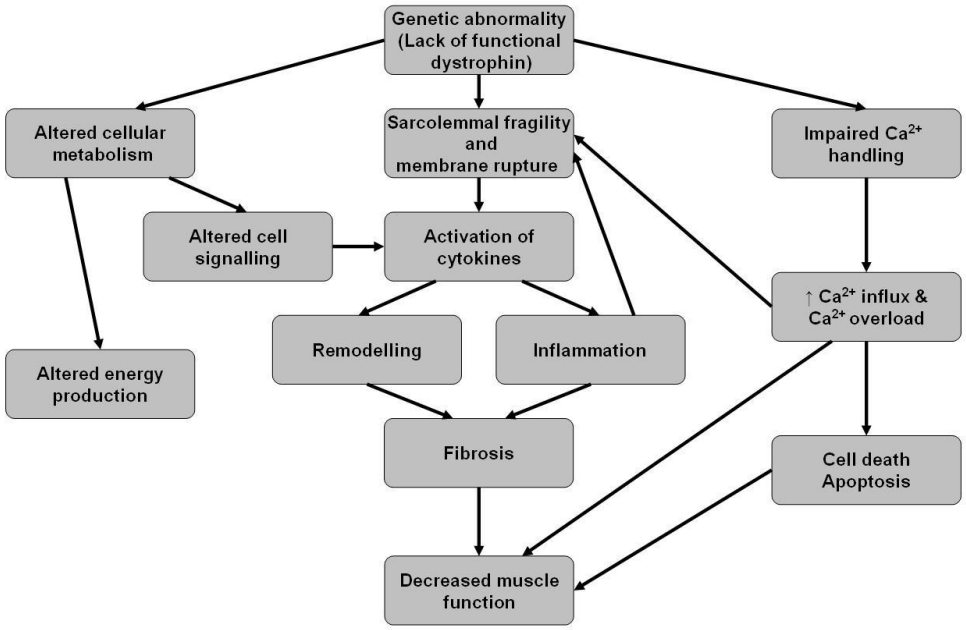


Figure 1.4.3 Summary of pathogenesis and disease progression in DMD.

Animal models of disease have long been used to further the understanding of processes and mechanisms that bring about disordered health. The following section discusses animal models important to the study of dystrophy.

1.5 Animal models of DMD

An animal model of human disease should mimic the development of the pathology of the human disease by having a similar genetic basis and possessing key pathologic hallmarks of the disease. For DMD, an animal model should display muscle weakness, respiratory insufficiency, cardiomyopathy and similar histological changes. The animal should also be readily available, easy to breed and handle and be extensively characterised with a phenotype that is reproducible over many generations (Willmann et al. 2009).

Much of the current level of knowledge of DMD has been gained from careful experimentation using various animal models of muscular dystrophy. It is apparent that as scientific knowledge of the pathophysiology of DMD expands, there are shortfalls in each commonly used animal model, with no single model fulfilling all criteria. As clinical application of definitive gene therapies remain distant, research into the development and application of alternative pharmacological treatment options is required. There are several animal models of DMD that may be employed to facilitate this research.

1.5.1 Dystrophin deficient mouse (*mdx*)

Sicinski et al. (1989) demonstrated that the *mdx* mouse, first described by Bulfield et al. (1984), has a point mutation that causes a shift in reading frame resulting in a premature stop codon located in exon 23 of the dystrophin gene (Sicinski et al. 1989). This naturally occurring model arose from a spontaneous mutation in a colony of C57BL/10ScSn (C57) mice.

Dystrophic pathology in this strain does not occur as a steady progression but advances though a number of stages. There is an acute necrotic phase at approximately 21 days of life that is marked by fibre necrosis and elevated serum CK resulting in skeletal muscle degeneration (McGreachie et al. 1993; Grounds & Torrisi 2004; Messina et al. 2006). Evidence of muscle regeneration such as a marked increase of centronucleated fibres of varying

size is seen from the age of two months (McGreachie et al. 1993; Gillis 1999). This widespread necrosis subsides to low level chronic pathology at approximately 60 days of age, with mice then entering a period of muscular hypertrophy between 5-12 months of age (Spencer & Tidball 1996). High rates of protein turnover are observed throughout life, as are signs of muscle remodelling such as hypertrophy, hyperplasia and proliferation of connective and fatty tissues (MacLennan & Edwards 1990; Lynch et al. 2001). From approximately one year of age, the *mdx* mouse starts to exhibit gradual muscle atrophy and fibrosis, progressing to generalised weakness in later life (Lefaucheur et al. 1995; Pastoret & Sebille 1995). A marked decrease in regenerative ability accompanied by respiratory pathology is evident after the age of sixteen months (Pastoret & Sebille 1995; Gayraud et al. 2007). Weakness in paraspinal and intercostal muscles permits the development of progressive thoracolumbar kyphosis observed in these mice between the ages of ten and seventeen months (Laws & Hoey 2004).

With the exceptions of the heart and diaphragm, *mdx* mice showed less myofibre degradation and fibrosis than humans with DMD (Stedman et al. 1991; Lefaucheur et al. 1995). The diaphragm showed comparable levels of muscle degeneration, fibrosis and functional defects as DMD patients (Stedman et al. 1991; Coirault et al. 1999) as well as contraction-induced damage (Anderson et al. 1998). Coirault et al. (1999) also found that muscle weakness in the diaphragm was due to alterations in myosin molecular motors. The severe pathology seen in the diaphragm makes it the most representative skeletal muscle for dystrophinopathy.

Mdx mice develop significant cardiomyopathy, with inflammation accompanying advanced necrosis and fibrosis (Lefaucheur et al. 1995; Quinlan et al. 2004), altered contractility and changes in relaxation and response to exogenous calcium (Sapp et al. 1996). Echocardiography and ECGs have also demonstrated tachycardia, decreased heart rate variability, alterations in left ventricular dimensions and conduction properties consistent with abnormal autonomic modulation of the heart (Bia et al. 1999; Chu et al. 2002; Quinlan et al. 2004). The *mdx* heart progresses from

normal function in young mice to dilated cardiomyopathy and significant fibrosis in older mice. By the age of one year, significant changes in fibrosis and function are evident. Patterns of fibrotic changes differ to those observed in humans with *mdx* exhibiting widespread, patchy fibrosis instead of the postero-lateral wall fibrosis seen in DMD (Quinlan et al. 2004).

Altered calcium homoeostasis is another feature of *mdx* pathology (Fraysse et al. 2004). Basal cytosolic concentrations of calcium in *mdx* mice were similar to wild-type mice (De Backer et al. 2002), but concentrations of the calcium buffer, parvalbumin, were elevated (Sano et al. 1990; Gailly et al. 1993). Alloatti et al. (1995) found substantially greater concentrations of calcium in ventricular myocytes and Robert et al. (2001) showed increased concentrations in the subsarcolemmal compartment and SR.

The *mdx* mouse has several advantages that make it useful in dystrophic research. While skeletal myopathy is not as pronounced as in DMD, cardiomyopathy closely resembles the human condition (Quinlan et al. 2004). Other advantages include a short life-span, short generation time, low cost and ready availability. Also, the nature of the mutation causing dystrophin deficiency in these mice means that they share close genetic homology to approximately one third of DMD cases (Partridge 1997; Aartsma-Rus et al. 2009).

Shortcomings of the *mdx* mouse include a relatively mild phenotype with skeletal muscle degeneration and fibrosis only becoming marked in later life, compounded by a lack of widespread fibrosis after necrosis (Stedman et al. 1991; Rolland et al. 2006). Compensation for the lack of dystrophin may in part be due to upregulation of utrophin, a structural analogue (Deconinck et al. 1997; Grady et al. 1997). The presence of fibres that spontaneously express dystrophin (revertant fibres) complicate interpretation of studies aimed at restoring dystrophin expression (Grounds et al. 2008). An additional shortcoming is that perinatal mortality in *mdx* litters is approximately 3.5 times greater than in wild-type mice (Torres & Duchen 1987).

Many transgenic models based on *mdx* mice have been developed to either test mechanistic theories or in an attempt to produce a murine model that resembles the human disease more closely. These include *mdx 52* mice, designed as a model with exon 52 of the dystrophin gene disrupted (Araki et al. 1997); *mdx:utrophin* knock-out mice, lacking both dystrophin and utrophin, developed to remove the compensatory effects of utrophin upregulation in *mdx* mice and to share many pathological hallmarks with DMD (Deconinck et al. 1997; Grady et al. 1997); and *mdx:myoD* knock-out mice designed to produce a model of muscular dystrophy with a diminished ability to regenerate muscle (Megeney et al. 1996).

Myostatin knock-out and myostatin knock-out/*mdx* mice

Myostatin knock-out mice developed by McPherron et al. (1997b) produced excessive skeletal muscle growth due to cellular hypertrophy and hyperplasia. These mice also exhibited decreased total body fat (Lin et al. 2002). Similar results were seen in *mdx* mice with myostatin inhibition or when crossed with myostatin null mice to create a strain lacking both myostatin and dystrophin. Removal of myostatin resulted in an amelioration of the dystrophic phenotype with benefits seen in skeletal muscle such as increased mass, improved function and decreased fibrosis (Bogdanovich et al. 2002; Wagner et al. 2002).

Sharma et al. (1999) reported the presence of myostatin in foetal and mature hearts as well as upregulation of the protein in tissue surrounding the site of myocardial infarction, suggesting roles in cardiac development and remodelling. Studies into the role of myostatin in the dystrophic heart have had varying results with one study reporting no change in heart mass or fibrosis (CohnLiang, et al. 2007) while another reported an increase in cell proliferation that resulted in an increase in cardiac mass (Artaza et al. 2007).

nNOS cardiac overexpression/*mdx* mice

Research by Wehling-Hendricks et al. (2005) with transgenic nNOS knock-out mice showed that these mice exhibited the features of cardiomyopathy similar to DMD. They had modifications to calcium cycling caused by

abnormal calcium channel activity that led to increased intracellular calcium concentrations (Sears et al. 2003). Alterations in calcium handling led to abnormalities in myocyte contraction in response to varying rates of stimulation. The removal of NO was accompanied by an increase in production of xanthine oxidase (XO), linked to decreased myofibre calcium sensitivity, decreased myocyte contractility and increased production of free radicals (Khan et al. 2004). Reduced concentrations of NO also led to abnormalities in vascular function that further reduced the function of the heart. Many of these symptoms were also observed in *mdx* mice. Bia et al. (1999) showed that dystrophin deficiency in the *mdx* mouse resulted in decreased cardiac nNOS that was associated with abnormal ECG readings. Elevated production of cardiac NO by introduction of a nNOS transgene in *mdx* mice led to improvements in ECG abnormalities, heart rate variability, inflammation and fibrosis (Wehling-Henricks et al. 2005). Taken together, these findings suggest that increasing NO production in dystrophic myocytes may have important beneficial effects that should be investigated as a possible treatment for DMD-associated cardiomyopathy.

1.5.2 Other animal models

The range of models includes cows, sheep, dogs, cats, chickens, mice, zebra fish and nematodes, but only the canine and murine models are widely used. As many of these models have significant differences in phenotype and genetic origin to DMD, only the two main canine models will be discussed.

Golden retriever muscular dystrophy dog (GRMD)

This animal has been recognised as an excellent model for DMD and is the best characterised canine model with several breeding colonies established in the United States, Japan, France and Italy. Disruption of dystrophin production is due to a base pair change in the splice site of intron 6 resulting in exon 7 skipping and a premature stop codon in exon 8 arising from this loss of reading frame (Sharp et al. 1992). Dystrophin absence produces a rapidly progressive and fatal disease that shares many pathological features with DMD. There is however a large phenotypic variation even between pups of the same litter, with some pups surviving for only days while others

remain ambulatory for years (Ambrosio et al. 2008). The predicted length of dystrophin in the GRMD model is ~5% of unaffected dystrophin, however, Schatzberg et al. (1998) showed low levels of nearly full-length dystrophin by Western blot analysis in some dogs. It was hypothesised that some animals may be able to overcome their frameshift mutation and synthesise dystrophin by an as yet unknown, alternate mechanism.

GRMDs exhibited elevated serum CK, muscle necrosis and faulty muscle regeneration resulting in atrophy, weakness, significant fibrosis and joint contracture (Kornegay et al. 1988; Valentine et al. 1988). Consequently, delayed growth, gait abnormalities and development of kyphosis were common (Valentine et al. 1988; Kornegay et al. 2003). Selective muscle degeneration, particularly involving the tongue, masticatory and trunk muscles progresses until reduced respiratory capacity was observed as a result of significant pharyngeal and oesophageal dysfunction (Kornegay et al. 2003). Dilated cardiomyopathy was pronounced with echocardiography showing decreased fractional shortening and decreased left ejection fraction as well as marked left ventricular free wall dysfunction (Chetboul et al. 2004). By three months of age, systolic dysfunction and left ventricular dilation were evident and by six to seven months, systolic and diastolic indices were severely impaired (Chetboul et al. 2004). Histological changes in the heart were observed from the age of approximately three months and share many characteristics with DMD such as cellular hypertrophy, interstitial fibrosis and macrophage infiltration (Moise et al. 1991).

Shortcomings of the model include the degree of symptom variability between littermates that necessitate careful selection of experimental and control animals. High rates of perinatal mortality in severely affected animals are also seen. The high expenses of obtaining, breeding and housing animals as well as the need for a veterinary facility are also disadvantages (Willmann et al. 2009).

Dystrophic Beagle (CXMDJ)

In an attempt to create a canine model of DMD that was smaller and easier to maintain, healthy beagles were mated to an affected GRMD (Shimatsu et al. 2003). The beagle was chosen due to the breed's smaller size and prior use in toxicology studies during drug development (Willmann et al. 2009). The phenotype is similar, though milder than the GRMD, but with increased rates of pup survival (Shimatsu et al. 2003). Involvement of the diaphragm was more pronounced and was affected shortly after birth while skeletal muscle pathology was not usually evident until approximately two months of age (Shimatsu et al. 2003). Cardiac pathology was similar to GRMD although it progressed more slowly and was less severe (Yugeta et al. 2006). Although the CXMDJ is not yet fully characterised, increased survival rate and smaller size make it an attractive alternative to the GRMD model.

Recognition and understanding of the altered processes that result from dystrophin absence allows intelligent treatment strategies to be developed. Section 1.6 discusses treatments that are currently being employed to manage DMD.

1.6 Current treatments

The wide-reaching effects of dystrophinopathy dictate that a multidisciplinary and individualised approach to the management of patients be undertaken. Table 1.6.1 outlines the various treatment specialists that may be required to manage complications of dystrophin loss. Anticipation and early treatment of complications limit their severity and maintain quality of life for sufferers of DMD. Improvements in management of dystrophinopathy have modified the disease's natural history and extended patients' life expectancy into adulthood (Manzur et al. 2008b). Diet, psychosocial support, genetic counselling for family members, maintenance of ambulation and management of respiratory and cardiac consequences are all important in the approach to DMD.

Careful management of diet is an important, ongoing aspect of DMD treatment as malnutrition, failure to thrive and obesity have impacts upon

other aspects of the disease (Willig et al. 1995). Nutritional advice is recommended for all patients, especially those receiving long-term corticosteroid administration as this medication impairs carbohydrate tolerance that should be monitored by measurement of blood and urine glucose levels (Angelini 2007). Advice usually includes limiting simple sugar and sodium intake while increasing dietary proportion of complex carbohydrates (Angelini 2007). Dietary supplementation of vitamin D and calcium to prevent osteoporosis remains controversial as increasing dietary intake and exposure to sunlight produce longer-lasting effects (Dawson-Hughes 1991; Bachrach 2005). A high-fibre diet, adequate bowel regime and use of laxatives where necessary have also been suggested as ways to prevent constipation (Yiu & Kornberg 2008).

Psychosocial support for both the family and sufferer of DMD also has an immense effect on mental health and quality of life. Behavioural problems, especially those associated with depression, become significant at pathological epochs of the disease such as loss of ambulation (Hinton et al. 2006). Treatment from a psychologist or psychiatrist may be required. Intellectual involvement is expressed as impairments in language, visual-spatial skills, fine motor skill, attention and memory (Cyrulnik et al. 2008). Patients with DMD should be encouraged to attend mainstream education for as long as possible, however severity of mental or physical symptoms may necessitate a more specialised educational setting (Polakoff et al. 1998). Screening and determination of female carriers allows genetic counselling, antenatal diagnosis and ongoing surveillance for cardiomyopathy (Politano et al. 1996; Manzur et al. 2008b).

Table 1.6.1 Multi-disciplinary team approach to the management of DMD.

	Core team	Complication treatment
Doctors	Paediatrician Neurologist General practitioner	Geneticist Muscle histopathologist Orthopaedic surgeon Pulmonologist/intensivist Cardiologist General surgeon
Therapists	Physiotherapist	Orthotist Dietitian Speech and language therapist Occupation therapist Wheel chair specialist Psychologist
Associated professionals	Hospital/community nurse	Family care officer Social services worker

(Adapted from Manzur et al. 2008b)

Maintenance of ambulation is a critical factor in delaying the onset of joint contracture, development of scoliosis and preservation of respiratory function (Deconinck & Dan 2007; Kinali et al. 2007). Ambulation also has an important psychological effect on DMD patients (Hinton et al. 2006). Maintenance of bone mineral density in the face of muscle weakness, inactive lifestyle and corticosteroid treatment can be bolstered with improved dietary intake or supplementation of vitamin D and calcium (Bachrach 2005; Quinlivan et al. 2005). Physical therapy in the form of mild activities such as swimming, standing or use of a vibrating platform assists in maintaining muscle strength and range of joint movement (Caulton et al. 2004). The use of nocturnal splints and stretching of tendons about joints, especially those about the Achilles tendon, hip flexors and iliotibial bands, delays onset of contracture and scoliosis (Rodillo et al. 1988). Despite measures to halt their progression, relentless fibrotic processes advance the development of contracture and scoliosis to the point where corrective surgery is required. The criteria for scoliosis surgery is a spinal curvature of greater than 25 degrees and vital capacity <30% predicted (Kinali et al. 2007). The mean age of DMD patients for surgery to alleviate scoliosis is

14-15 years (Kinali et al. 2007). While advantages of scoliosis surgery include increased patient comfort, deformity prevention and improved respiratory function, there is no evidence that it increases life-span (Cheuk et al. 2007; Kinali et al. 2007).

Advances in the treatment of respiratory insufficiency have improved the lifespan of patients with DMD, with life expectancies increasing to 25-30 years (Konagaya et al. 2005; Eagle et al. 2007). Monitoring should be commenced before wheelchair dependence and include spirometric measures of forced vital capacity and peak flow, pulse oximetry, determination of carbon dioxide tension, and regular polysomnography (Bach et al. 1997; Phillips et al. 2001). Determination and monitoring of other respiratory conditions, such as obstructive sleep apnoea, oropharyngeal aspiration or asthma should also be conducted (Finder et al. 2004). It is also prudent for patients to ensure prophylactic vaccinations for seasonal influenza and pneumococcal infections with swift treatment of infection by early administration of antibiotics, chest physiotherapy and respiratory support (Finder et al. 2004). Patients should be taught methods for manual and mechanical assisted airway clearance as effective airway clearance prevents the development of respiratory complications such as atelectasis or pneumonia and delays respiratory failure (Bach et al. 1997). Manually assisted coughing by glossopharyngeal breathing and breath stacking in conjunction with upper abdominal compression aids airway clearance (Kang & Bach 2000). Use of a mechanical insufflator-exsufflator that assists coughing by providing a positive pressure breath followed by negative pressure has been shown to produce greater peak cough values to manual techniques (Bach 1993). Garstang et al. (2000) demonstrated mechanical insufflator-exsufflator use produced superior clearance and less trauma to mucosal surfaces when compared to tracheal suction. Treatment of hypercapnia by nocturnal non-invasive intermittent positive pressure ventilation results in delayed onset of daytime hypercapnia and improvements in quality of life and life expectancy (Konagaya et al. 2005; Eagle et al. 2007). Constant ventilation via nasal, mouthpiece or

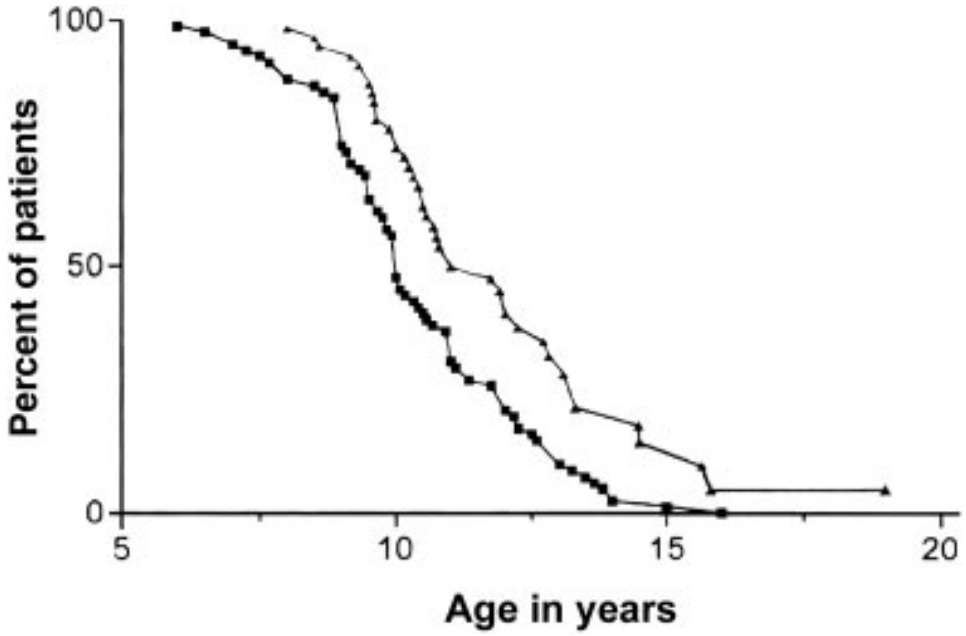
tracheotomy may be required as the disease progresses (Toussaint et al. 2006).

1.6.1 Pharmacological support

The main advantages of pharmacological treatment compared to genetic strategies are centred on increased feasibility and include relative ease of administration, systemic delivery and potential benefit to all DMD patients regardless of their individual mutation. Currently the only proven effective pharmacological treatments are corticosteroids and drugs used to treat heart failure, including angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARB), β-adrenoceptor antagonists and diuretics (Griggs et al. 1991; Biggar et al. 2004; McNally & Towbin 2004).

Corticosteroids

To date, the only effective pharmacological treatment to prevent muscle weakening in DMD has been the corticosteroids such as prednisolone/prednisone or the oxazoline analogue of prednisone, deflazacort (Griggs et al. 1991; Biggar et al. 2004). Daily doses of prednisone or deflazacort produce long-term benefits including improvement in muscle function (Mendell et al. 1989; Fenichel et al. 1991; Griggs et al. 1991), preservation of respiratory and cardiac function (Silversides et al. 2003) and prolongation of ambulation (Angelini et al. 1994). These positive effects are thought to stem from stabilisation of muscle strength (Bonifati et al. 2000). King et al. (2007) also demonstrated delays of onset and progression of scoliosis associated with long-term steroid treatment, illustrated in Figure 1.6.1 comparing steroid-treated and non-treated DMD patients' age at loss of ambulation.



(Angelini 2007)

Figure 1.6.1 Kaplan-Meier curve for loss of ambulation in corticosteroid treated patients.

Steroid-treated (filled triangles) DMD patients show a delay of ambulation loss compared to steroid-naïve patients (filled squares).

Early commencement of steroid treatment in ambulatory patients, before significant loss of muscle function, allows greater stabilisation of muscle strength and delays wheelchair dependence (Dubowitz et al. 2002; Merlini et al. 2003). While there is no official consensus on preferred steroid or dosing regimen, it is generally accepted that daily doses of 0.75mg/kg for prednisone and 0.9mg/kg for deflazacort are the most effective (Mendell et al. 1989; Griggs et al. 1991; Manzur et al. 2008a). Many clinicians halt steroid treatment as the patient becomes wheelchair-dependent, although benefits to respiratory and cardiac function suggest continuation past loss of ambulation may still be obtained (TREAT-NMD 2009).

Precise mechanisms of action of these drugs are yet to be fully elucidated, however potential mechanisms have been suggested to explain their benefits in DMD. These include inhibition of muscle proteolysis (Elia et al. 1981; Rifai et al. 1995), stimulation of myoblast differentiation and enhancement of muscle repair (Bal & Sanwall 1980; Anderson et al. 2000), sarcolemmal

stabilisation (Jacobs et al. 1996), anti-inflammatory and immunosuppressive ability (Kissel et al. 1991), modulation of cytostolic calcium (Metzinger et al. 1995; Vandebrouck et al. 1999), utrophin upregulation (Pasquini et al. 1995) and modulation of muscle fibre genes (Muntoni et al. 2002).

The major aim of steroid treatment is prolongation of ambulation, although this parameter is difficult to test. Instead, a number of functional tests have been devised to ascertain effectiveness of treatment. Steroid treatment improved the ability to lift weights (Mendell et al. 1989; Griggs et al. 1991) and shortened times taken to rise from prone (Gowers' sign), walk nine metres and climb four stairs (Mendell et al. 1989; Griggs et al. 1991; Rahman et al. 2001; Beenakker et al. 2005). Improvements were also seen in leg function, average muscle scores and pulmonary function assessed by forced vital capacity (Mendell et al. 1989; Griggs et al. 1991; Rahman et al. 2001; Beenakker et al. 2005). Silversides et al. (2003) demonstrated improvement in left ventricular function with deflazacort treatment.

There are significant adverse effects associated with steroid treatment. Excessive weight gain was reported by Mendell et al. (1989) and Griggs et al. (1991) with prednisone treatment, and a trend to increased weight gain with deflazacort treatment reported by Angelini et al. (1994). Increased appetite has been reported with prednisone administration (Griggs et al. 1991) but not with deflazacort treatment (Angelini et al. 1994). A significant increase in the risk of developing a Cushingoid appearance with administration of prednisone at doses $\geq$0.75mg/kg/day was also reported (Mendell et al. 1989; Griggs et al. 1991), while Angelini et al. (1994) reported that two out of eleven patients treated with deflazacort developed this adverse effect. Dermatological changes include increased hair growth and acne at higher doses of prednisone (Mendell et al. 1989; Griggs et al. 1991). An increased risk of behavioural changes such as hyperactivity, irritability or euphoria, has been reported (Mendell et al. 1989; Griggs et al. 1991; Angelini et al. 1994; Beenakker et al. 2005). Deflazacort treatment increased the incidence of asymptomatic cataracts (Reitter 1995). Other

possible side effects associated with corticosteroid treatment include hypertension, hyperglycaemia, glycosuria, hypokalaemia and osteoporosis.

Osteoporosis is seen early in the natural history of patients with DMD, with lowered bone mineral density observed even at diagnosis (Angelini et al. 1994; Bianchi et al. 2003). Long-term steroid treatment exacerbated osteoporosis and contributed to growth inhibition and bone fracture (Kissel et al. 1991; Hardiman et al. 1993). Vertebral fractures, uncommon in steroid-naive DMD patients, were increased with steroid administration and contributed to decreasing stature and development of scoliosis (Talim et al. 2002; Bothwell et al. 2003). It has been proposed that shorter limbs and delayed growth may be beneficial to DMD patients as there is evidence that rapid growth may exacerbate muscle deterioration (Biggar et al. 2004).

Bisphosphonate treatment has been suggested as one intervention to combat the decreased bone mineral density observed with dystrophinopathy as these drugs inhibit bone reabsorption by decreasing the number and activity of osteoclasts (Quinlivan et al. 2005). Use of bisphosphonates is standard practice in adults with osteoporosis but relatively uncommon in children, although studies have shown increased bone density, normalisation in linear growth and decreased fracture rates in treated children (Brumsen et al. 1997; Glorieux et al. 1998). Side effects associated with this class of drug include hypocalcaemia, exacerbation of vitamin D insufficiency, formation of sclerotic bands and delayed mineralisation (Quinlivan et al. 2005).

Cardiac therapy

Cardiac disorders have increased in importance as improvements in the treatment of respiratory insufficiency have extended life expectancy (Finder et al. 2004). Cardiac repercussions of dystrophinopathy are evident early in life with MRI revealing sub-clinical signs in patients before the age of six years (Nigro et al. 1990; Silva et al. 2007) while clinical signs are usually detected between the ages of ten and fifteen years (Bahler et al. 2005; Giatrakos et al. 2006). Regular monitoring should be commenced before loss of ambulation and include ECG and echocardiography (or MRI for

patients whose acoustic windows are too restricted due to fibrosis or thoracic deformity) with the addition of Holter monitoring once rhythm irregularities have been detected (Surgery 2005). Treatment is aimed at reducing cardiac afterload to prevent dilated cardiomyopathy. This may be achieved by administration of ACE inhibitors, blockade of angiotensin receptors and/or β-adrenoceptors, control of fluid overload by diuretics such as spironolactone and aggressive management of arrhythmias (McNally & Towbin 2004).

Steroid treatment also benefits the heart. A long-term cohort study conducted by Markham et al. (2008) analysed cardiac dysfunction in steroid-treated and steroid-naive DMD patients, showing that steroid treatment commenced before clinical evidence of heart failure retarded development of ventricular dysfunction. Kaplan-Meier freedom from ventricular dysfunction at 1500 days was 93% in the treated group compared to 53% in the untreated group (Markham et al. 2008).

β-Adrenoceptor antagonists and ACE inhibitors promoted favourable cardiac remodelling, prevented left ventricular dysfunction and reduced progression of cardiomyopathy (Duboc et al. 2005; Shaddy et al. 2007). A retrospective study analysing survival rates of DMD patients who were administered combination therapy of β-adrenoceptor antagonists and ACE inhibitors revealed that this treatment improved long-term survival (Ogata et al. 2009). They also found that this benefit was greater if treatment was commenced before symptoms of cardiac failure became clinically evident. Five year and seven year survival rates for patients who were started treatment after they were symptomatic were 81% and 71% respectively while those who were treated before the onset of cardiac symptoms had five year and seven year survival rates of 97% and 84% respectively (Ogata et al. 2009).

ARBs may be an attractive alternative to ACE inhibitors as they are already used successfully in the treatment of other cardiomyopathies (McNally 2008). Cohn et al. (2007) demonstrated ARBs' applicability to skeletal

dystrophinopathy when they showed a decrease in fibrosis in *mdx* mice treated with this class of drug; however cardiac benefits have yet to be demonstrated. Unpublished data from our laboratory showed no improvement in cardiac function with administration of the ARB, losartan, in *mdx* mice. ACE inhibitors may be more beneficial as a DMD treatment as this class of drug also inhibits kininase II, leading to a downstream increase in bradykinin concentrations. Bradykinin, acting through generation of prostaglandins and NO, is a known vasodilator. ARBs act through alternate pathways that do not result in the accumulation of bradykinin (Cockcroft et al. 1993).

1.7 Future therapies

Potential treatments currently being explored include genetic modification methods such as introduction of shortened versions of dystrophin into muscle cells by viral vectors, antisense oligonucleotide mediated exon skipping and strategies to promote read-though of premature stop codons. Implantation of myogenic stem cells and pharmacological upregulation of the dystrophin analogue utrophin, manipulation of pathways involving NO and inhibition of myostatin are also under investigation.

1.7.1 Gene modification

Viral vectors

After early attempts to deliver naked DNA for encoding dystrophin proved ineffective, attention shifted to adenovirus as vectors for delivery of the dystrophin gene (Liang et al. 2004). Regions of viral DNA responsible for replication and regulation were removed in an attempt to create an unviable virus and diminish immune responses in order to deliver sustained transgene expression (Liang et al. 2004). Researchers then ‘gutted’ the virus by removing all of the viral genes to allow a larger transgene to be delivered (Scott et al. 2002). Initial enthusiasm was marred as researchers observed production and replication of competent viruses followed by the sudden death of a patient being treated with this therapy, leading to widespread mistrust of adenovirus safety (Verma 2000; Hartigan-O'Connor et al. 2002).

Attention turned to adeno-associated viruses (AAV) as they are not associated with human disease and have numerous serotypes and genomic variants, eleven of which have been identified as being useful in gene delivery (Xiao et al. 1999; Rodino-Klapac et al. 2007). Due to a limited ability of AAVs to carry large transgenes, mini- and micro-dystrophins have been developed. Design of these transgenes was based on studies of Becker's muscular dystrophy and *mdx* genotypes that differentiated between sections of the dystrophin gene that are functionally critical and those that may be deleted. Modified dystrophin transgenes have had their 5' and 3' untranslated regions and C-terminals removed while mini-dystrophin has had 5 spectrin repeats removed and micro-dystrophin has had 4 spectrin repeats removed (England et al. 1990; Crawford et al. 2000; Wang et al. 2000; Harper et al. 2002).

Current hurdles include generation of circulating antibodies that intercept vectors, preventing them from reaching their target (Jiang et al. 2006), transgene expression limited by cell-mediated cytotoxic T-cell response (Manno et al. 2006), and the need to develop a systemic mode of delivery ensuring transgene distribution to multiple muscle groups. Production of large quantities of vector also remains a challenge (Rodino-Klapac et al. 2007). Circulating antibodies may be overcome by pre-treatment screening of patients or plasmapheresis (Hodges et al. 2005) while short-term immunosuppression may mediate T-cell responses (Manno et al. 2006). Vascular delivery offers a possible solution to systemic distribution though use of AAV6 and AAV8 serotypes that cross vascular endothelium and transduce muscle (Rodino-Klapac et al. 2007).

Mdx mice treated with mini- and micro-dystrophin genes demonstrated decreased Evans blue dye permeability and centronucleation but without protection against eccentric contraction-induced damage, highlighting the need to improve this form of treatment (Watchko et al. 2002; Liu et al. 2005). The modulation of 'booster' genes, such as overexpression of insulin growth factor-1, may provide potential for amelioration of contraction-induced damage (Abmayr et al. 2005). Other potential strategies include

inhibition of myostatin allowing maintenance of muscle mass (Lee 2004); or overexpression of CT GalNAc transferase (Galgt2) to strengthen linkage of α-dystroglycan to extracellular matrix improving membrane stability (Nguyen et al. 2002).

Antisense oligonucleotide exon skipping

The aims of antisense oligonucleotide (AO) therapy in the treatment of dystrophin deficiency are to conceal coding regions needed for inclusion of specific faulty exons from the splicing machinery. In this way, the induction of 'exon skipping' and restoration of reading frame allow a shortened but partially functional dystrophin to be produced. It is hoped that the severe dystrophinopathy seen in DMD can be converted into a milder BMD-like phenotype. This method has been verified in both *mdx* mice (Mann et al. 2001; Alter et al. 2006) and humans with DMD (van Deutekom et al. 2007; Kinali et al. 2009) and has progressed to clinical trials.

The first clinical trial was conducted by van Deutekom et al. (2007) and was aimed at inducing exon 51 skipping in four boys with DMD aged between 10-13 years as proof of concept in humans. The trial employed a local intramuscular injection of 2'O-methyl AO into the tibialis anterior muscle. mRNA composition and dystrophin expression were assessed from muscle biopsies taken 28 days after treatment and showed 64-97% dystrophin gene expression in fibres, while there was 3-12% dystrophin protein compared to healthy tissue (van Deutekom et al. 2007). A phase I-IIa systemic trial conducted by Prosensa Theraputics BV to induce exon 51 skipping in DMD boys aged between 5-15 years recently concluded. The aim of this trial was to induce systemic exon skipping by delivering treatment by a series of subcutaneous abdominal injections ranging from 0.5-10mg/kg/injection. The drug was well tolerated with no serious side effects reported and there was a modest improvement in walking (Goemans et al. 2011).

Kinali et al. (2009) described local intramuscular injections of morpholino AO against exon 51. The phase I trial assessed safety and biochemical efficacy of morpholino AOs in humans. Two doses were tested; 0.09mg and

0.9mg, both delivered in 0.9mL saline over nine injections of 0.01mL solution into extensor digitorum brevis muscle, with the contralateral muscle used as a control. Biopsies were taken 3-4 weeks after treatment to assess dystrophin expression that was increased with the higher dose. The 0.9mg dosing regimen showed 17% greater dystrophin expression compared with contralateral controls, with dystrophin-positive fibres expressing 42% dystrophin protein compared to normal tissue (Kinali et al. 2009). A systemic phase II trial of exon 51 skipping conducted by AVI Biopharma using morpholino AOs delivered by weekly intravenous injection has also recently concluded. This to assessed changes in muscle function as well as safety and tolerance. Nineteen ambulant DMD boys, divided into four groups, were treated in a dose escalation trail. Doses ranged from 0.5mg/kg/week to 4.0mg/kg/week and were delivered over a period of twelve weeks (Cirak et al. 2011). This study reported that the drug was well tolerated and that there was a dose-dependent increase in dystrophin positive fibres. There was also a reduction in inflammation as well as a return of α-sarcoglycan and nNOS to the sarcolemma leading the researchers to conclude that there is strong potential for this therapy to be disease-altering (Cirak et al. 2011).

There are difficulties associated with AO-mediated exon skipping. Application to duplication mutations is proving problematic in that the AOs are too efficient and skip both the original and the duplication, often resulting in an out-of-frame mutation (Aartsma-Rus & van Ommen 2007). This method is not suitable for large deletions of greater than 36 exons or with mutations involving the cysteine-rich domain or actin-binding sites as these are essential for protein function (Aartsma-Rus et al. 2009). The order of intron removal may be important and warrants further research to improve low success rates with multi-exon skipping (Aartsma-Rus et al. 2006).

Ideal delivery methods remain problematic. Systemic administration in *mdx* mice by intravenous or subcutaneous injection has restored body-wide skeletal muscle dystrophin accompanied by improvement in muscle

function (Alter et al. 2006; Fletcher et al. 2007). Methods need to be refined as the majority of the AOs do not reach target tissues, instead accumulating in the liver and kidneys. Due to AO clearance and muscle turnover, AO treatment effects are transitory, necessitating life-long administration for lasting effectiveness and the impact of long-term treatment remains unknown (van Ommen et al. 2008). Experiments into long-term expression have included integration of AO into small nuclear ribonucleoproteins (snRNP) genes, to be delivered via AAV vector (Goyenvalle et al. 2004; Denti et al. 2006), or the use of lentiviral vectors to incorporate the AO into patient-derived myogenic stem cells with subsequent transplantation of these modified cells (Benchaouir et al. 2007).

Ideally, treatment should be commenced early in the disease process when there is relatively little muscle replacement as this form of treatment is dependent on muscle transcripts of dystrophin (van Deutekom et al. 2007). AO treatment is customised, necessitating analysis and determination of each patient's mutation and to ensure that it is expressed in the majority of dystrophin transcripts (Aartsma-Rus et al. 2009). MLPA to screen all 79 exons should be used to identify the type of mutation and determine whether any unexpected or unusual splicing occurs (Aartsma-Rus et al. 2009). Production of individualised AOs is difficult and expensive for these reasons. Under current regulations, each new AO is considered a new drug and is required to undergo expensive and lengthy medical trials (Hoffman 2007; van Ommen et al. 2008). This process may prove difficult as it may not be possible to get enough trial patients for rare mutations; this has led some researchers to suggest that regulatory processes may have to be amended to accommodate the treatment of these children (Goyenvalle & Davies 2011).

Read-though of premature stop codons

High doses of aminoglycoside antibiotics relax the stringency of codon recognition permitting read-through of nonsense point mutations and is therefore applicable in 10-15% of DMD cases (Barton-Davis et al. 1999; Wells 2006; Aartsma-Rus et al. 2009). Gentamicin administration was

effective in *mdx* mice causing transcription and sarcolemmal expression of dystrophin that was accompanied by a decrease in serum CK (Barton-Davis et al. 1999). Human trials with gentamicin had mixed results. Politano et al. (2003) administered four ambulant or recently confined to wheelchair DMD patients who had nonsense point mutations with two cycles of six day treatments with an interval of seven weeks. Three of the patients with UGA stop codons showed increases in dystrophin expression while the fourth with a UAA codon did not respond to treatment (Politano et al. 2003). A trial by Wagner et al. (2001) involving four DMD boys treated daily over a period of two weeks was unable to detect dystrophin in either pre- or post-treatment muscle biopsies; however there was a decline in serum CK concentrations. More recently, Malik et al. (2010) conducted clinical trials aimed at determining the long-term feasibility of gentamicin-induced readthrough of stop codons. They found increased levels of dystrophin and decreased serum CK accompanied by stabilisation of strength and increased vital capacity with six months of treatment. These researchers also reported a need for caution as the development of an immunogenic epitope was also detected in at least one patient.

There was a need to develop a drug that has read-through capabilities but without the side-effect profile of gentamicin as high doses cause gastrointestinal disturbances, ototoxicity and nephrotoxicity in humans (Wells 2006). PTC124, marketed as Ataluren, allows ribosomal read-though of premature stop codons, permitting continued gene translation and production of functional protein. Efficacy has been demonstrated in *mdx* mice where it restored dystrophin protein and lowered plasma CK concentrations (Hamed 2006; Welch et al. 2007). A phase I trial conducted by Hirawat et al. (2007) in healthy volunteers demonstrated the drug was absorbed from the oral route with good bioavailability and was well tolerated. A phase IIb trial involving 165 DMD boys with nonsense point mutations also showed little toxicity but was unable to demonstrate efficacy in protecting patients with DMD (Welch et al. 2007; Nelson et al. 2009).

1.7.2 Cell therapy

Myoblast transfer was first demonstrated in *mdx* muscle fibres by Partridge et al. (1989) who transferred healthy myoblasts from C57 to *mdx* mice resulting in restored dystrophin expression. This initial success was muted as early myoblast transplantation in humans provoked an immune response and rejection of transplanted cells (Tremblay et al. 1993; Gussoni et al. 1999). Problems encountered with cell therapies that lead to low treatment efficiency included very limited diffusion of transplanted cells from the injection site, extensive die-off of transplanted cells within the first few days of administration and the need for adequate immunosuppression to prevent rejection (Peault et al. 2007). Rapid cell death has been addressed by injecting large numbers of cells (Skuk et al. 1999) while low diffusion rates have been increased by modulation of MyoD (Smythe & Grounds 2001) and use of matrix metalloproteinase (El Fahime et al. 2000). Initial immune suppression employed the anti-tumour drug cyclophosphamide. However, this compound not only killed rapidly proliferating cells but also killed the newly transplanted cells (Karpati et al. 1993). Immune suppression by FK506 has allowed increased rates of transplantation but has been linked to nephrotoxicity, diabetes and increased risk of oncogenesis (Kinoshita et al. 1994; Peault et al. 2007).

Alternatives include autologous muscle-derived stem cells that were modified to correct any genetic fault by short fragment homologous replacement or chimeraplasty (Kapsa et al. 2001; Bertoni & Rando 2002). Short fragment homologous replacement has successfully converted *mdx* to wild-type nucleotide in 15-20% of cultured loci and up to 0.1% of tibialis anterior muscle, a correction that persisted for more than three weeks. Adverse effects on cell viability indicate this form of therapy still requires further optimisation (Kapsa et al. 2001). Bertoni and Rando (2002) reported successful gene repair using RNA-DNA chimeric oligonucleotides (chimeraplasts) to express full-length dystrophin in 2-15% of wild-type cells. Bone marrow-derived stem cells differentiated into myogenic cells and assisted in muscle regeneration; however, the low efficiency of this

technique means it remains clinically unviable (Ferrari et al. 1998; Gussoni et al. 1999). Deasy et al. (2009) demonstrated that muscle-derived stem cells found in blood vessel walls were potentially useful in the treatment of dystrophic muscle. These pericytes, which were transduced to overexpress vascular endothelial growth factor (VEGF), increased angiogenesis and skeletal muscle repair and reduced fibrosis in *mdx* mice, but they did not increase the number of dystrophin-positive fibres (Deasy et al. 2009).

A problem with many of these options is that it remains difficult to obtain enough suitably viable cells for an effective therapy. Therefore, the challenge is developing a technique to rapidly isolate and harvest stem cells with myogenic potential.

1.7.3 Utrophin up-regulation

Utrophin shares 85% homology with dystrophin and is similar in function (Perkins & Davies 2002). This protein is highly expressed in foetal development (Clerk et al. 1993) but usually restricted to myotendonous and neuromuscular junctions in adult life (Khurana et al. 1991; Ohlendieck et al. 1991). The aim of utrophin upregulation is to express utrophin along the length of myofibres and replace dystrophin by forming utrophin-glycoprotein complexes. This potential therapy is still in the early stages of development, but upregulation of utrophin, by either germline or somatic gene transfer, restored sarcolemmal expression of DGC proteins and improved dystrophic phenotype in *mdx* mice (Tinsley et al. 1996).

Administration of heregulin peptide, a small peptide region of ectodomain in *mdx* mice, led to a three-fold increase in utrophin expression and an amelioration of dystrophic features such as muscle degradation, inflammation and reduced susceptibility to damage caused by eccentric contraction (Krag et al. 2004) although assessment of toxicity and tissue specificity are yet to be conducted (Miura & Jasmin 2006). Administration of transgene containing an activated form of calcineurin in *mdx* mice resulted in reductions in variability of muscle fibre size, degree of centronucleation and inflammation as well as improvement in sarcolemmal

stability (Chakkalakal et al. 2004). Activation of this pathway may contribute to cardiac hypertrophy that may worsen an already compromised dystrophic heart (Molkentin et al. 1998). Alternatively, expression of utrophin along entire myofibres and accompanying stabilisation of membrane integrity has been demonstrated with the overexpression of Galgt2 (Nguyen et al. 2002). Finally, supplementation of NO either with the precursor L-arginine or molsidomine increased utrophin expression in *mdx* mice (Barton et al. 2005; Voisin et al. 2005), possibly acting though modulation of utrophin breakdown via calpain-mediated processes (Waheed et al. 2005). These treatments decreased necrosis and contraction-induced damage (Barton et al. 2005; Voisin et al. 2005).

These therapies show potential for improving the genotype and phenotype of patients with DMD but need further development to be clinically viable. Safe, systemic delivery without compromising immune response is yet to be achieved. In addition, these potential therapies do not have the capacity to produce full-length dystrophin in the long-term. Taken together, these facts highlight the need for continuing research into concurrent treatments to reduce complications of aberrant dystrophin expression such as dysregulation of calcium homoeostasis, inflammation and fibrosis.

1.7.4 Nitric oxide manipulation

NO plays important roles in the processes of inflammation, cell signalling and vascular control. In muscle, NO is primarily produced by nNOS which is normally anchored to the sarcolemma by the DGC (Figure 1.2.2). Other isoforms of NOS exist within the myocyte but are not expressed as significantly as nNOS (Nakane et al. 1993). The loss of dystrophin and subsequent disruption of the DGC also results in the translocation and reduced gene expression of nNOS, causing NO concentrations to be greatly decreased (Brenman et al. 1995; Chang et al. 1996; Bia et al. 1999).

Increased antioxidant concentrations at the sarcolemma (Touboul et al. 2005), elevated enzymes linked to oxidative stress (Austin et al. 1992) and by-products of oxidative damage are found in muscles of DMD patients

(Kar & Pearson 1979; Jackson et al. 1984) and in their exhaled air (Grinio et al. 1984). These findings indicate that dystrophic muscle is exposed to increased oxidative stress. Rando et al. (1998) demonstrated that not only is dystrophic tissue prone to alterations in metabolism but it has an increased susceptibility to oxidative damage compared to healthy tissue. The hypothesis that NO loss plays an important role in dystrophinopathy was further strengthened by Wehling et al. (2001) when they demonstrated an amelioration of pathology associated with oxidative stress with over-expression of a muscle-specific transgene for nNOS in *mdx* mice.

Altered vascular control may also contribute to DMD disease progression. It has been postulated that without NO acting through cyclic guanosine monophosphate (cGMP)-mediated pathways to produce vasodilatation, affected muscle is damaged by repeated periods of ischaemia during times of increased oxygen requirements (Thomas et al. 1998; Crosbie 2001). Interestingly, nNOS knock-out mice on a C57 background do not develop dystrophic-like pathology such as sarcolemmal fragility, while nNOS knock-out mice on an *mdx* background are phenotypically indistinct to *mdx* (Crosbie et al. 1998). These results indicate that not only are dystrophic muscles already nNOS deficient but that perturbations in NO alone do not contribute directly to dystrophinopathy. However, impaired NO signalling may play strong secondary roles in DMD.

NO deficiency also leads to defects in neuromuscular junction architecture (Shiao et al. 2004). Abnormal distribution and decreased concentration of acetylcholine receptors (AChR) has been observed in *mdx* mice and has been associated with impaired synaptic transmission (Rafael et al. 2000; Carlson & Roshek 2001). Pharmacological donation of NO or activation of protein kinase G (PKG), as well as genetic overexpression of guanylate cyclase (GC) or PKG normalised AChR levels at the synapse, indicating disorganisation of these receptors may be a sequel to dystrophin loss (Jones & Werle 2000; Godfrey & Schwarte 2003; Schwarte & Godfrey 2004).

nNOS co-precipitated with cardiac RyR (RyR2) suggests that it forms an interactive complex with these receptors and acts to modulate cardiac function (Damy et al. 2004). In addition, nNOS was activated by calcium (Schmidt et al. 1992) causing NO production to follow CICR and the cardiac cycle (Pinsky et al. 1997). In nNOS knockout mice, NO derived from this source had negative or neutral effects on left ventricular inotropy and a positive effect on cardiac lusitropy (Ashley et al. 2002; Sears et al. 2003; Khan et al. 2004). Conversely, NO derived from endothelial NOS (eNOS) increased open probability of RyR2, increasing calcium transient amplitude and force of contraction (Petroff et al. 2001). Thus it can be seen that the actions of NO in muscle are complex and closely linked to the site of production.

Consequences of cardiac fibrosis include muscle weakening and conduction defects, with the most affected areas being the posterolateral and lateral free walls (Finsterer & Stollberger 2003). These areas are the foci for ventricular arrhythmias and contribute to abnormal systolic function (Miyoshi 1991). If fibrosis is a result of oxidative stress, normalisation of NO may reduce superoxide production through inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase or other profibrotic free radicals (Clancy et al. 1992; Cave et al. 2005; Tidball & Wehling-Henricks 2007; Williams & Allen 2007a). Wehling-Henricks et al. (2005) demonstrated that decreased fibrosis, amelioration of ventricular pathology and improved cardiac function in *mdx* mice were achieved though NO normalisation. However, fibrosis may be secondary to inflammation, in which case the anti-inflammatory role of NO may also protect the myocardium (Wehling-Henricks et al. 2005).

Watkins and Cullen (1988) found that satellite cell counts in DMD patients were increased by 200-300% in dystrophic tissue compared to unaffected tissue. However, overuse of repair mechanisms over time results in satellite cell depletion, ultimately rendering dystrophic muscle unable to keep up with the relentlessly destructive processes of dystrophinopathy. Impaired NO production may contribute to pathology as muscle repair is slowed after

crush injury in mice with administration of NOS inhibitors (Anderson 2000). The action of NO in muscle repair may be through stimulation of hepatocyte growth factor that accelerates the cell cycle and enhances muscle growth and repair (Tatsumi et al. 2002) or through promotion of myoblast fusion to form myotubes through GC and cGMP-mediated pathways (Lee et al. 1994). NO upregulation of follistatin also influences muscle growth and repair as follistatin increased myogenesis through regulation of myoblast fusion (Pisconti et al. 2006) and inhibition of myostatin (Lee & McPherron 2001; Zimmers et al. 2002).

It has therefore been suggested that reductions in NO would have an adverse effect on muscle regeneration (Anderson 2000). In contrast, Wehling et al. (2001) demonstrated that normalisation of NO concentration led to a decrease in centronucleated fibres, indicating that this treatment led to an improvement of dystrophic pathology and not to an increase in tissue regeneration. This study also showed that, although NO is capable of modifying blood vessel development (Montrucchio et al. 1997; Powell et al. 2000), increased levels of NOS did not change *mdx* blood vessel density (Wehling et al. 2001). This suggests positive results gained though NO restoration were not due to angiogenesis.

These actions show clear benefits of NO in dystrophic muscle and further investigation into modulating NO pathways is warranted. This could be achieved by two strategies: transgenic replacement of the enzyme responsible for the synthesis of NO or through pharmacological enhancement of NO production or action. As transgenic manipulation of NOS is not yet a viable option for the treatment of DMD, pharmacological interventions should be investigated.

L-arginine is one such candidate for pharmacological treatment. L-arginine is the substrate of NOS used to synthesise NO and is a semi-essential amino acid required for growth. As such, elevated concentrations of L-arginine may cause hypotension, increased heart rate and release of hormones such as insulin, glucagon, prolactin and growth hormone (Goumas et al. 2001;

Todorovic et al. 2001). L-arginine may be metabolised to support glucose synthesis or catabolised to produce energy. The L-arginine-NO pathway is also involved in inflammation, tissue repair and fibrosis (Mane et al. 2001). It should be noted that L-arginine is also the substrate for eNOS that has been linked to the stretch-dependent stimulation of NO production involved in modulation of RyR open probability in cardiomyocytes (Petroff et al. 2001). Therefore, increasing L-arginine concentrations may increase NO production leading to enhanced satellite cell activation and muscle fibre production as well as reduction of fibrosis and ultimately increasing muscle function.

The actions of NO may be either through direct modification of target proteins, such as is involved in modulation of RyR2 open probability or indirectly through increased activity of PKG by enhanced cGMP synthesis (Petroff et al. 2001; Gonzalez et al. 2008). In the heart, regulation of cGMP is by PKG activation of phosphodiesterase 5 (PDE5) which inactivates cGMP by hydrolysis to 5'-GMP (Rybalkin et al. 2002). Figure 1.7.4 illustrates the NO-cGMP-PDE5 pathway. Previous strategies to increase cGMP concentrations through enhanced NO production have been complicated by PDE5 feedback inhibition, therefore a more efficient strategy to test cGMP-dependent effects is by PDE5 inhibition (Kim et al. 2001; Forfia et al. 2007).

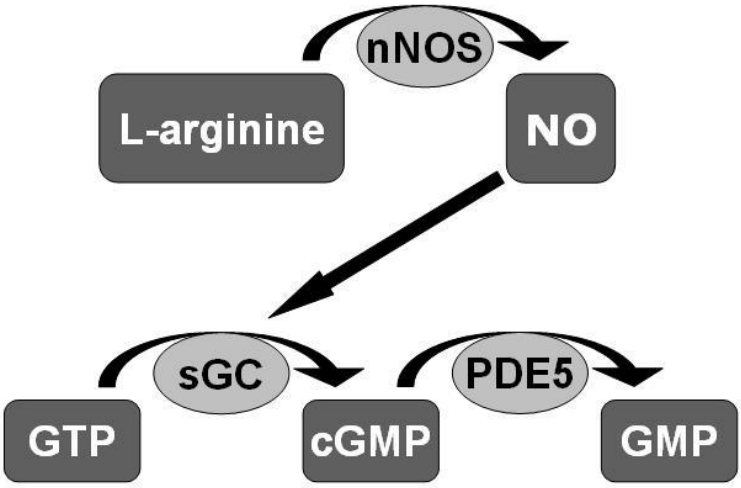


Figure 1.7.4 Diagram showing the NO-cGMP pathway.

nNOS synthesises NO from the substrate L-arginine. NO acts through soluble GC to convert GTP into cGMP. Deactivation of cGMP occurs as it is hydrolysed by PDE5 into GMP.

A study by Nagayama et al. (2009) investigating PDE5 inhibition in advanced cardiac hypertrophy found decreased hypertrophy, chamber remodelling and fibrosis, improvement in basal and β-adrenergic stimulated contractility and relaxation, as well as enhanced calcium handling. Clinical trials of PDE5 inhibition in heart failure described improvement in endothelial function that resulted in improved exercise capacity and decreased pulmonary and vascular resistances (Guazzi et al. 2007; Lewis et al. 2007). Benefits of PDE5 inhibition have also been reported in the *mdx* heart as enhanced cGMP signalling resulting in decreased sarcolemmal fragility and improvements in cardiac function and mitochondrial metabolism (Khairallah et al. 2008).

Mechanisms involved in PDE inhibition include improved endothelial responses mediated by cGMP signalling (Guazzi et al. 2007; Lewis et al. 2007), enhanced calcium handling (Layland et al. 2002; MacLennan & Kranias 2003; Yang et al. 2007) and altered mitochondrial function (Costa et al. 2005). Enhanced endothelial responses related to increased cGMP concentrations allowed vasodilatation in chronic heart failure and coronary artery disease, improved exercise capacity and was protective against

workload-induced ischaemia (Katz et al. 2000; Halcox et al. 2002). PDE inhibition acted on calcium regulation by decreasing L-type current through a PKG-mediated pathway (Yang et al. 2007), decreasing myofilament calcium sensitivity through downstream phosphorylation of troponin1 (Layland et al. 2002) and affecting SR calcium dynamics by increased phosphorylation of phospholamban (PLB) (MacLennan & Kranias 2003).

NO influences many diverse aspects of myocyte function, from mediating inflammation to cardiac regulation and vasodilation. NO also influences learning and memory and synaptic formation as well as playing roles in the cell cycle and proteolysis. A fuller understanding of the manipulation of NO or downstream effectors such as cGMP may yield important options for the treatment of dystrophinopathy. Table 1.7.4 summarises potential links between defective NO production or regulation and dystrophic pathology.

Table 1.7.4 Potential roles of NO in the treatment of dystrophinopathy

Dystrophin deficiency	NO function
Muscle inflammation	Mediates inflammation
Muscle wasting	Regulates proteolysis
Muscle necrosis/apoptosis	Influences cell death
Intellectual defects	Influences learning and memory
Cardiomyopathy	Cardiac regulation
Metabolic defects	Influences mitochondrial metabolism
Synaptic dysfunction	Regulates synapse function and formation
Vascular defects	Vasodilatation
Increased intracellular calcium	Alters calcium handling

Adapted from (Tidball & Wehling-Henricks 2007)

1.7.5 Myostatin inhibition

Myostatin, a member of the TGF-β superfamily and also known as growth and differentiation factor-8 (GDF-8), is recognised as an endogenous negative regulator of muscle growth (McPherron et al. 1997b; Zimmers et al. 2002). The protein is well-conserved and has been observed in cattle, pigs, chicken, mice and man (Grobet et al. 1997; McPherron et al. 1997b; Jiang et al. 2002; Schuelke et al. 2004; Zhiliang et al. 2004). Inhibition of myostatin produces excessive muscle growth and decreased total body fat, while overexpression or administration of active protein causes cachexia (Lee & McPherron 2001; Zimmers et al. 2002). Myostatin upregulation has also been observed in other muscle-wasting conditions such as Human Immunodeficiency Virus (HIV) infection, space flight and muscle disuse (Gonzalez-Cadavid et al. 1998; Lalani et al. 2000; Reardon et al. 2001). Myostatin is thought to play roles in cardiac development and remodelling as it is expressed in both foetal and adult hearts and is elevated in tissue bordering myocardial infarcts (Sharma et al. 1999). This supports the view presented by McPherron et al. (1997b) that the primary role of myostatin is regulation of muscle fibre growth during embryonic development as well as during post-natal growth and repair.

Myostatin synthesis and signalling occur in a similar fashion to other TGF-β family members. It is initially synthesised as a 375 amino acid protein that undergoes two proteolytic events that first cleave the N-terminal then expose the C-terminal and actiatves the protein (McPherron et al. 1997b). Synthesis is predominantly in skeletal muscle and the protein circulates within the blood as an inactive propeptide until activated at the target site (McPherron et al. 1997b). Signalling occurs through activin type-2 receptors (ActRIIA/B) that bind the C-terminal with high affinity. A cascade of events follows where these receptors recruit activin-like kinase-4 or -5 that are phosphorylated and activate a serine/threoine kinase domain and cause the phosphorylation of Smad 2 and 3. These interact with Smad 4 causing it to move into the nucleus, bind directly with DNA and regulate gene expression (Lee & McPherron 2001; Rebbapragada et al. 2003).

The primary action of myostatin is the suppression of proliferation and differentiation of myogenic cells. However, myostatin also increased cell survival, inhibited protein synthesis and decreased total body fat (Taylor et al. 2001; McPherron & Lee 2002; Joulia et al. 2003). Myostatin inhibited satellite cell proliferation though upregulation of the cyclin-dependent kinase (cdk) inhibitor p21 and decreasing cdk-2 as well as decreasing phosphorylated retinblastoma protein (Rb), with a net result of preventing satellite cells from entering the cell cycle (Thomas et al. 2000; Rios et al. 2002). p21 increased myocyte survival by lowering rates of apoptosis in differentiating myoblasts (Wang & Walsh 1996). Differentiation of myoblasts and satellite cells was inhibited by myostatin through decreased expression of the myogenic regulatory factors MyoD, Myf-5 and myogenin, resulting in decreased formation of multinucleated myotubes (Langley et al. 2002; Joulia et al. 2003). It is thought that myostatin holds myogenic cells in a quiescent state until favourable conditions for muscle growth or repair cause suppression of myostatin and release of these cells into the cell cycle.

Myostatin inhibition in *mdx* mice resulted in phenotypic improvements such as decreased fibrosis and increased skeletal muscle hyperplasia and hypertrophy that also corresponded to improvements in strength and function (Bogdanovich et al. 2002; Wagner et al. 2002). Despite these benefits, histological analysis of skeletal muscle from myostatin-inhibited *mdx* mice revealed that many hallmarks of dystrophinopathy were still present. Increased heterogeneity of fibre size, fibre splitting, centralised nuclei, patchy necrosis and inflammation as well as persistently elevated serum CK concentrations indicated primary disease processes were still in effect (Bogdanovich et al. 2002; Wagner et al. 2002). The effects of myostatin in the heart have been less consistent. Cohn et al. (2007) reported no increase in cardiac mass or improvement in cardiac fibrosis with myostatin inhibition and concluded that myostatin plays significantly different roles in cardiac and skeletal muscle. Other studies have found myostatin expression decreased cardiomyocyte proliferation and differentiation as well as modulation of cardiac excitation-contraction coupling through enhanced β-adrenoceptor responsiveness and increased

calcium release from the SR (Artaza et al. 2007; Rodgers et al. 2009b). Myostatin inhibition may be useful in combination with other treatments regimes despite not correcting the underlying genetic and molecular faults associated with DMD. There is a paucity of definitive studies into this growth factor, which needs to be addressed to elucidate the potential for modulation of myostatin as a therapy for muscular dystrophy.

1.8 Aims and scope of the study

DMD is a fatal X-linked disease caused by a genetic fault that prevents the production of the sarcolemmal protein dystrophin, and affects one in 3500 live male births (Emery 2002). It is characterised by progressive weakening and atrophy of muscles that leads to death due to respiratory or cardiac failure. Improvements in respiratory care have extended the life expectancy of DMD patients from their early teens into their late twenties but have also highlighted the need for improved cardiac care. Some form of cardiomyopathy is observable in patients as young as six years of age, with clinical symptoms usually becoming evident as patients enter their early teens (Nigro et al. 1990; Chenard et al. 1993). Death due to dilated cardiomyopathy accounts for 30% of DMD deaths (Finsterer & Stollberger 2003).

The absence of dystrophin causes a complex pattern of pathology that, despite extensive research, still requires further understanding in order to provide effective treatment for boys with DMD. Pathology associated with DMD includes sarcolemmal fragility leading to necrosis, inflammation and fibrosis, abnormal calcium handling and cell signalling disturbances causing protease activation and altered metabolism as well as the dislocation of nNOS from the sarcolemma resulting in aberration of NO-mediated pathways and mishandling of ROS (Rando 2001a; Durbeej & Campbell 2002; Hopf et al. 2007).

Much of our current knowledge of dystrophinopathy has been gathered through careful experimentation using animal models of DMD. Of these, the most widely used are the *mdx* mouse and GRMD dog. These models have

their individual strengths and weaknesses that should be considered during project design, but together have been invaluable in furthering understanding of the disease. Transgenic modification to the *mdx* mouse has also allowed investigation of specific aspects of DMD pathogenesis. To this end, nNOS cardiac overexpressing *mdx* and myostatin knock-out *mdx* were generated and studied through the course of this dissertation to explore the actions of NO and myostatin on the dystrophic heart.

Up to this time, the therapy of DMD has centred on corticosteroid treatment. While this offers some anti-inflammatory benefit and prolongation of ambulation (Angelini et al. 1994), there is still a need for more specific antifibrotic therapy. Cyclic GMP and NO-mediated pathways are an area of interest, not only in DMD research but in a wide spectra of disorders such as acute heart failure (Shin et al. 2007), claudication (Wang et al. 2001), depression and psychosis (Bobon et al. 1988; Kanes et al. 2007), pulmonary conditions (Ghofrani et al. 2006; Calverley et al. 2007) and erectile dysfunction (Corbin 2004). The potential of NO signalling in dystrophic pathology further highlights the importance of new research in this field, with emphasis on possible cardiac benefits as well as skeletal muscle effects.

A number of promising new gene-based therapies are on the horizon, but unfortunately most of these will not fully address the causative genetic faults associated with DMD. Therapies such as viral vectors, AO-mediated exon skipping and read-through of nonsense mutations still need to overcome significant obstacles before they become clinically viable treatment options. Hurdles including prohibitive costs, individualised drug development, difficulties in systemic delivery and lack of knowledge of long-term consequences are yet to be resolved. In addition, these therapies will likely produce a truncated form of dystrophin and will therefore not yield a total cure. Ancillary treatments aimed at improving the quality and quantity of life for those affected by DMD, particularly including drugs or modulators to attenuate fibrotic pathways, are still required.

Research questions that are addressed by this dissertation focus on NO-cGMP pathways or the effect of myostatin absence in alleviating cardiomyopathy in DMD. These studies are listed below individually, with their results presented in the subsequent chapters of this dissertation.

Study 1: Myostatin knock-out in the dystrophic heart

Myostatin is an endogenous inhibitor of muscle growth, thus inactivation or absence of this protein leads to increased muscle mass. Experimentation using myostatin knock-out mice bred onto a *mdx* background aimed to identify whether absence of myostatin had a direct effect on the dystrophic heart and whether subsequent increases in skeletal muscle mass had beneficial or detrimental cardiac effects. The postulation was that cardiac function would improve following myostatin disruption, as cellular hypertrophy (McPherron et al. 1997b) would be beneficial in maintaining systolic function in dystrophic hearts. However, there was a possibility that additional cardiac output and metabolic demands of a markedly increased skeletal muscle mass would negate any positive benefits of cardiac hypertrophy. This series of experiments also aimed to address these questions.

Myostatin knock-out mice were crossed with *mdx* mice to produce a double knock-out strain lacking both myostatin and dystrophin. Cardiac function was assessed in C57, *mdx* and double knockout strains using the isolated Langendorff heart technique. Collagen content of hearts and diaphragms were also assessed by picrosirius red staining. Determination of TGF-β, TNF-α, procollagen 1α and fibronectin gene expression was conducted by qRT-PCR.

Study 2: L-arginine optimisation

Based partially on previous research showing some benefits of treatment with the NO donor L-arginine (Barton et al. 2005; Voisin et al. 2005), it was hypothesised that increasing this substrate would prove beneficial to the dystrophic heart, but that dose optimisation is clearly needed. There is a need to assess dose-related effects of L-arginine on ventricular function,

arterial pressure development and cardiac and skeletal muscle fibrosis as this information will indicate the usefulness and safety of this supplement in boys with DMD.

Optimisation trials were conducted to determine the best dosing regimen for L-arginine administration. Doses of 1%, 2% and 5% L-arginine in drinking water were administered *ad libitum* to age and sex-matched C57 and *mdx* mice over a period of three months. Heart function was assessed using the isolated Langendorff heart technique, with arterial pressure development measured by a Millar catheter positioned within the aorta. Cardiac hypertrophy was investigated. Hearts were preserved for later sectioning and assessment of cardiac collagen content by picrosirius red staining. Collagen content of the tibialis anterior muscle was also assessed by picrosirius red staining.

Study 3: Cardiac nNOS overexpression in dystrophic cardiomyopathy

Little is understood about the impact on calcium handling in the hearts from nNOS overexpression *mdx* mice, although there is increased cardiac NO production resulting in improved heart rate variability, ameliorated ECG abnormalities and reduced cardiac fibrosis in these mice (Wehling-Henricks et al. 2005).

In order to ascertain whether the normalisation of NO production in the dystrophic heart was beneficial, transgenic mice that overexpress cardiac nNOS were crossed with *mdx* mice to generate a dystrophin-deficient model with NO concentrations closer to that of wild-type mice. It was hypothesised that increasing cardiac NO concentrations in *mdx* mice would directly improve calcium handling through S-nitrolysation of L-type calcium channels (Paolocci et al. 2000; Sun et al. 2006) and RyR2 function (Vandebrouck et al. 2002; Damy et al. 2004). Benefits may also be seen as a result of cGMP-mediated actions such as improved vasodilatation and mitochondrial function (Crosbie 2001; Khairallah et al. 2008). Cardiomyocytes were isolated and L-type calcium channel function was

assessed by voltage clamping, while calcium handling and contractility were assessed using Fura-2 dye and cell shortening techniques.

Study 4: Tadalafil administration in *mdx* mice

It was hypothesised that PDE5 inhibition would be beneficial to dystrophic hearts, based on mounting evidence of the role of cGMP in many chronic inflammatory diseases. Inhibition of these pathways was expected to result in reduced fibrosis, improved function and reduced expression of profibrotic and proinflammatory genes such as TGF-β, TNF-α, procollagen 1α and fibronectin.

Inhibition was achieved by administration of oral tadalafil mixed in normal mouse chow. This drug, although similar to sildenafil, possesses a longer half-life (17.5 hours), requiring less frequent dosing. The drug is prescribed for the treatment of erectile dysfunction and approved for treatment of pulmonary arterial hypertension. Asai et al. (2007) showed decreased muscle damage following tadalafil treatment in dystrophic skeletal muscle. To date, this is the only study that has tested the efficacy of tadalafil treatment in dystrophin deficiency, but has only examined the vasodilatory effect of PDE5 inhibition in skeletal muscle. There remains a need for further understanding the cardiac effects of PDE5 inhibition and their potential in the treatment of DMD.

Age and gender-matched C57 and *mdx* mice were treated for a period of 12 weeks by *ad libitum* feeding. Cardiac function was assessed by the isolated Langendorff heart technique, after which the heart was dissected transversely. One half of the heart and the diaphragm were prepared for collagen assessment by picrosirius red staining while the remainder was used in determination of TGF-β, TNF-α, procollagen 1α and fibronectin gene expression.

Study 1 aimed to examine the cardiac consequences of myostatin inhibition and whether these responses are mediated through direct modulation of the myocardium or indirectly by increased muscle mass. Studies 2, 3, and 4

were designed to examine stages of the NO-cGMP pathway in order to assess their applicability to the treatment of DMD as well as to isolate the direct effects of NO from those mediated through cGMP. Studies 1-4 are summarised in Figure 1.8.1. Figure 1.8.2 outlines the various techniques used in each study.

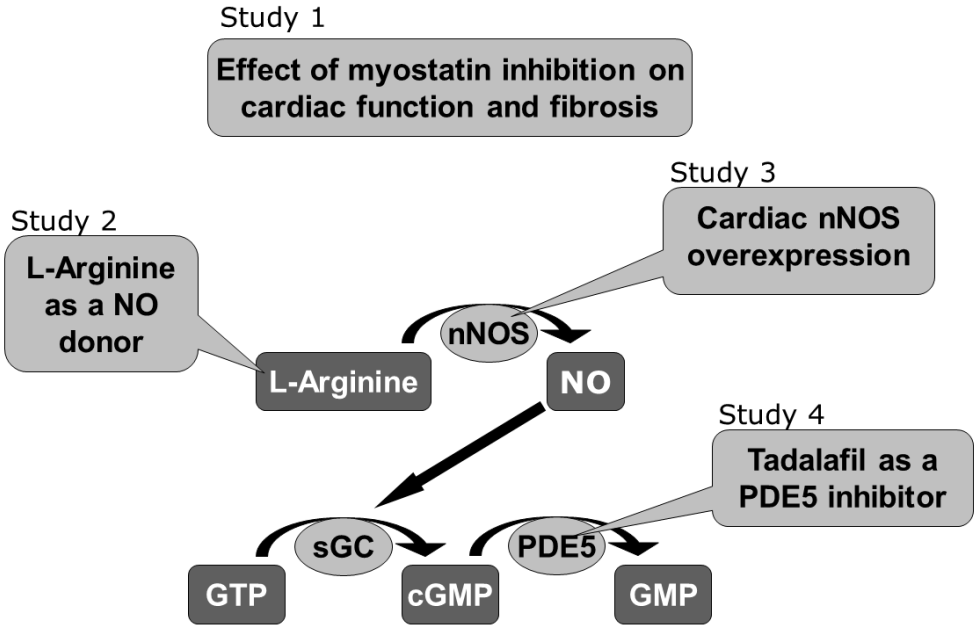


Figure 1.8.1 Summary of the four studies conducted and reported in this dissertation.

Study 1 was designed to examine the consequences of the genetic absence of myostatin on dystrophic hearts, while Studies 2, 3 and 4 are linked, each involving steps in the NO-cGMP pathway.

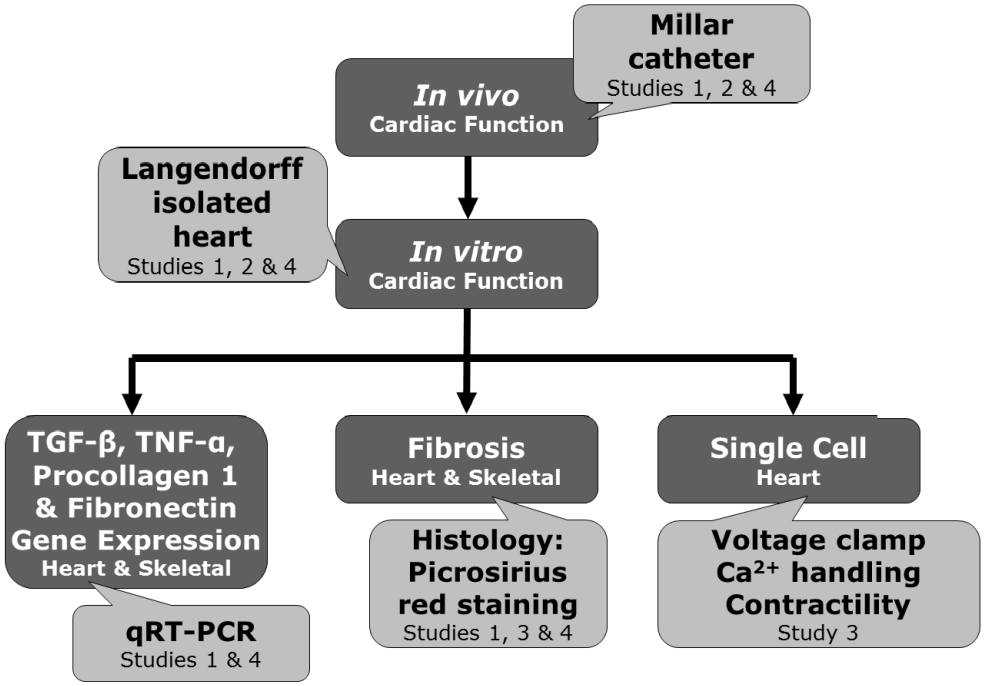


Figure 1.8.2 Summary of experimental techniques used in each of the four studies.

Chapter 2: Materials and Methods

2.1 Ethical considerations

Procedures for animal care and experimental protocols were approved by the University of Southern Queensland (USQ) Animal Ethics Committee. These protocols are based on the National Health and Medical Research Committee Australian Code of Practice.

2.2 Experimental animals

Several transgenic mouse strains were bred and used for these studies, in addition to C57 BL/10ScSn (C57) and C57 BL/10ScSn *mdx* mice, which served as controls. C57 mice were used to represent a healthy control, while data obtained from *mdx* represented the dystrophic strain. All animals were housed at the USQ Animal House in a controlled environment that consisted of a 12 hour light/dark cycle and air-conditioned to 22^{o}C. Unless administered treated diets, mice were maintained on a standard diet of water and Barastock rat and mouse pellets *ad libitum*. *Mdx* and C57 mice were bred from existing colonies housed at the USQ Animal House while transgenic mice were imported under contract by the Animal Resources Centre, Murdoch, Western Australia and re-derived in compliance with quarantine requirements. C57 BL/10ScSn myostatin knock-out (*Mstn*$^{-/-}$) mice were generously provided by Professor Se-Jin Lee from the Johns Hopkins School of Medicine, Baltimore. Disruption of the myostatin gene in these mice was achieved by gene targeting. C57 BL/10ScSn *mdx* nNOS cardiac over-expressing mice (nNOS) mice were generously provided by Professor James Tidball from the University of California, Los Angles. Transgenic mice were bred with existing C57 and *mdx* colonies to obtain appropriate experimental animals.

2.2.1 Breeding

Myostatin KO mouse breeding regime

Generation of these transgenic mice and establishment of this breeding colony was conducted as previously described by Wagner et al. (2002). Female *mdx* were crossed with male C57 BL/10ScSn mice that were

heterozygous for the myostatin null mutation (*Mstn*$^{+/-}$) to obtain males that were heterozygous for the myostatin null and hemizygous for the dystrophin null conditions. These males were back-crossed to *mdx* females to generate *Mstn*$^{+/-}$/*mdx* mice. Matching pairs of *Mstn*$^{+/-}$/*mdx* mice were used to create a colony that produced *Mstn*$^{-/-}$/*mdx* (2KO) and *Mstn*$^{+/+}$/*mdx* mice. Heterozygous pups were used to maintain this colony.

nNOS cardiac overexpression mouse breeding regime

Generation of these transgenic mice has been described by Wehling et al. (2001). Establishment of this breeding colony was also conducted as described by Wehling et al. (2001). Female *mdx* mice were crossed with male nNOS transgenic mice to obtain males that were heterozygous for the nNOS transgene and hemizygous for the dystrophin null condition. These males were back-crossed to *mdx* females to generate nNOS$^{+/-}$/*mdx* mice that were matched with similar pairs to create a colony that produced nNOS$^{-/-}$/*mdx* and nNOS$^{+/+}$/*mdx* mice. Heterozygous pups were used to maintain this colony.

2.2.2 Genotyping protocols

DNA was isolated by salt extraction and final genotyping of pups was conducted by polymerase chain reaction (PCR) to determine suitability for experimentation. Heterozygous pups were kept to maintain breeding colonies while pups that were homozygous for the genes of interest were selected for experimentation. All PCRs were conducted in sterile conditions with both positive and negative controls to ensure freedom from contamination. All primers were purchased from Invitrogen Australia (Mulgrave, Australia).

DNA extraction

DNA was extracted from tail tips taken from pups between the ages of 10-14 days or from ear punch tissue if DNA was required from older mice. Volumes described below are for tail tip tissue, with volumes halved for ear punch tissue. All tubes and solutions were autoclaved to ensure freedom from contamination. Tissues were placed immediately in 700µL of digestion

solution (Table 2.2.1) containing 30µL of Proteinase K (Merck) and digested at 55^{o}C overnight. Samples were chilled on ice for ten minutes and 250µL of 6M saturated salt solution was added. Tubes were mixed by inversion and chilled on ice for a further five minutes to precipitate out unwanted material. Samples were centrifuged at 13,000rpm for five minutes before 700µL of the supernatant was carefully transferred to a fresh tube. An equal volume of isopropanol was added, and the solution was gently mixed to precipitate the DNA. Samples were centrifuged as above and the supernatant was discarded. The pellet was washed with 500µL of 70% ethanol and centrifuged again. The supernatant was carefully removed and the pellet allowed to dry for twenty to thirty minutes. The DNA was resuspended in 50µL of autoclaved TE solution (10mM Tris-Cl pH 7.6, 1mM EDTA) and allowed to dissolve overnight at room temperature. Genomic DNA (gDNA) was stored at 4^{o}C with 1:20 dilutions used in genotyping protocols within days of extraction.

Table 2.2.1 Digestion solution used in DNA extraction.

Reagent	To make up 80mL (final concentration)
1M Tris-Cl pH 8*	4ml (50mM)
0.25M EDTA pH 8*	6.4ml (20mM)
25% SDS^	6.4ml (2%)

*sterilised by autoclaving; ^ filter sterilised

Myostatin null genotyping

The original myostatin null genotyping protocol was supplied by Professor Se-Jin Lee from the Johns Hopkins School of Medicine, Baltimore, but was optimised for use in our laboratory. Primers used for determining the presence or absence of myostatin are presented in Table 2.2.5 and PCR reagent ratios summarised in Table 2.2.6. Amplification cycling conditions are listed in Table 2.2.7. A ThermoHybaid thermocycler (HBPX220 Integrated Sciences) was used in PCR amplification of target sequences.

Table 2.2.5 Primers for myostatin null genotyping PCR.

Name	Sequence	Band size (bp)
Wild type		220
TIP#13	5'-AGA AGT CAA GGT GAC AGA CAC AC-3'	
TIP#168	5'-GGT GCA CAA GAT GAG TAT GCG G-3'	
***Mstn* KO**		332
TIP#169	5'-GGA TCG GCC ATT GAA CAA GAT G-3'	
TIP#170	5'-GAG CAA GGT GAG ATG ACA GGA G-3'	

Table 2.2.6 Reagents and amounts for myostatin null genotyping PCR.

Reagent	Final Concentration	Volume per reaction (µL)
gDNA		0.5
TIP#13 (20 µM)	10 pmol	0.5
TIP#168 (20 µM)	10 pmol	0.5
TIP#169 (20 µM)	10 pmol	0.5
TIP#170 (20 µM)	10 pmol	0.5
10X Qiagen Buffer*	1x	2.5
dNTP (10mM each)	0.2 mM	0.5
HotStarTaq Plus	1 U	0.2
Sterile water		19.3
Total		25

*contains 15mM $MgCl_2$. In this reaction, final concentration is 1.5mM.

Table 2.2.7 Cycling conditions for myostatin null genotyping PCR.

Temperature	Time	Number of cycles
95°C	5 min	1
94°C	30 sec	35-40
55°C	30 sec	
72°C	1 min	
72°C	15 min	1

This PCR used QIAGEN buffer and HotStarTaq (Cat #203603). A 10mM mix of dNTP was used (Fisher Biotech Cat# DN-10M). The PCR product was run on a 1.8% agarose gel to distinguish bands.

Interpretation of results

A single band at 220bp indicated the animal was wildtype ($Mstn^{+/+}$), while a single band at 332bp indicated the animal was homozygous for the deleted myostatin gene ($Mstn^{-/-}$). If two bands at 220bp and 332bp were present, the animal was heterozygous.

nNOS genotyping

Genotyping for nNOS cardiac overexpressing mice was conducted by PCR using primers and amplification times supplied by Professor James Tidball from the University of California, Los Angles. The reaction mix was optimised for use in our laboratory. Primers are presented in Table 2.2.2. PCR reagent ratios have been summarised in Table 2.2.3 and amplification cycling conditions in Table 2.2.4. A ThermoHybaid thermocycler (HBPX220 Integrated Sciences) was used in PCR amplification of target sequences.

Table 2.2.2 PCR primers for detection of the nNOS cardiac overexpression transgene.

Name	Sequence	Band size (bp)
nNOS		420
h761HSAfwd	5'-CCC GAG CCG AGA GTA GCA GT-3'	
VP1downstreamII	5'-CCC TTC CCT GTT GGC TAC T-3'	

Table 2.2.3 Reagents and quantities for nNOS cardiac overexpressing genotyping PCR.

Reagent	Final Concentration	Volume per reaction (µL)
5x Buffer	1x	5.0
$MgCl_2$ (25mM)	4mM	4.0
DMSO (100%)	4%	1.0
h761HSAfwd (100µM)	0.5µM	0.13
VP1downstreamII (100 µM)	0.5µM	0.13
dNTP (10mM each)	0.2µM	0.5
GoTaq (5U/µL)	2.5U	0.5
gDNA		1.0
Sterile water		12.74
Total		25

Table 2.2.4 PCR Cycling conditions for detection of the nNOS cardiac overexpression transgene.

Temperature	Time	Number of cycles
95°C	3 min	30
95°C	1 min	
95°C	30 sec	
59°C	1 min	
72°C	1 min	
72°C	10 min	1

This PCR protocol used GoTaq 5x buffer and Taq DNA polymerase (Promega). A 10mM mix of each dNTP was used (Fisher Biotech Cat# DN-10M). The PCR product was run on a 1.8% agarose gel to distinguish bands.

Interpretation of results

This PCR was designed to detect the presence or absence of the nNOS transgene. A single band at 420 bp indicated the presence of the transgene (nNOS positive).

2.3 In vivo cardiac function - Millar catheter

This procedure was conducted to measure *in vivo* cardiac function. Parameters recorded included heart rate (HR), end systolic pressure (ESP), end diastolic pressure (EDP) and rates of pressure change over time (+ and - dP/dt). An electrocardiogram (ECG) and temperature probe was also instrumented.

Mice were anaesthetised with ketamine 50 mg/kg and xylazine 20 mg/kg (Lyppard Australia, Brisbane) by intraperitoneal injection (ip) and placed in a supine position. Lead II ECG leads and a rectal temperature probe were positioned. An incision was made from the mandible to the upper thorax to expose the ventral cervical region. The trachea, right carotid artery and right vagus nerve were identified and a tracheotomy was conducted to ensure adequate air flow during the procedure. The right carotid was carefully separated from the vagus nerve before a silk ligature was secured at the

superior end of the carotid. A second ligature was positioned (but not tightened) at the inferior end of the vessel. The inferior end of the carotid was occluded using a micro-aneurysm clip to halt blood flow. A small incision was made in the carotid between the clip and the superior ligature and the head of the Millar catheter (Model number: SPR-671; Catalogue number: 840-6719, Millar Instruments Inc., Houston, Texas) was inserted into the vessel and advanced to the clip. The head of the Millar catheter occluded blood flow allowing the clip to be removed and the catheter to be advanced further toward the heart. The inferior ligature was tightened once the head of the catheter was moved into the thoracic cavity to ensure the vessel was sealed. The Millar catheter was positioned in the aortic arch and measurements of arterial pressure development were conducted. The catheter head was then gently advanced into the left ventricle through the aortic valve and measurements of ventricular pressure development taken. The head was then retreated to the aortic arch and a second set of arterial measurements was taken. At completion, the catheter head was withdrawn to a point just superior to the inferior ligature and the ligature tightened to fully occlude the vessel and prevent exsanguination. The animal was then transported to the Langendorff area for further experimentation.

Data was acquired using a PowerLab recording system (ADInstruments, Castle Hill, Australia) and analysed using Chart5 software (ADInstruments, Castle Hill, Australia).

2.4 Ex vivo cardiac function - isolated Langendorff heart

Unless otherwise stated, all chemicals used during the isolated Langendorff heart technique were purchased from Sigma-Aldrich, Sydney. This procedure was conducted to measure *in vitro* cardiac function. Parameters recorded included left ventricular end systolic pressure (LVESP), left ventricular end diastolic pressure (LVEDP), rates of pressure change over time (+ and -dP/dt), and coronary flow rate. The heart was paced to 420 beats per minute (bpm).

When Millar catheter experiments were conducted, mice were brought immediately to the Langendorff area at completion of *in vivo* assessment of arterial blood pressure. If mice were showing signs of recovery from the initial dose of anaesthetic, an incremental dose of ketamine/xylazine was administered to insure full surgical anaesthesia. Otherwise, mice were anaesthetised with 50mg/kg sodium pentobarbitone by IP injection. Hearts were excised via thoracotomy and placed into modified Krebs-Henseleit solution (in mM: NaCl 119; $NaHCO_3$ 1.2; KCl 4.7; KH_2PO_4 1.2; $CaCl_2$ 2.5; $MgSO_4$ 1.2; glucose 11; Na pyruvate 1; ethylene-diamine tetraacetic acid (EDTA) 0.5; 2,3-butanedione monoxime (BDM) 10; and bubbled with carbogen (95% O_2, 5% CO_2) to maintain a pH of 7.4. The aorta was cannulated and the heart was perfused retrogradely with Krebs-Henseleit solution without BDM (in mM: NaCl 119; $NaHCO_3$ 1.2; KCl 4.7; KH_2PO_4 1.2; $CaCl_2$ 2.5; $MgSO_4$ 1.2; glucose 11; Na pyruvate 1; ethylene-diamine tetraacetic acid (EDTA) 0.5) at a pressure of 80mmHg. The left atrium was removed and a small polyethylene drain was inserted into the cardiac apex to prevent volume overload in the left ventricle. Both the heart and perfusion solution were maintained at a temperature of $37\pm0.5^{o}C$ and the preparation was allowed to equilibrate. A small, fluid-filled, polyvinyl chloride plastic balloon attached to a pressure transducer (MLT844 Physiological Pressure Transducer, ADInstruments) and microsyringe was inserted into left ventricular chamber through the mitral valve. The microsyringe was used to control the volume of the balloon that was inflated to produce a left ventricular end diastolic pressure of 10mmHg.

The heart was paced at 420bpm by a Grass SD9 stimulator (West Warwick, Rhode Island) attached to silver wire that was embedded into the left ventricular apex and earthed to the aortic cannula. Developed cardiac pressures and coronary perfusion pressure were measured by MLT844 physiological pressure transducers (ADInstruments). Coronary flow was monitored by a TS410 transit-time tubing flowmeter (Transonic Systems Inc, Ithaca, USA). Data was acquired using a PowerLab recording system (ADInstruments) and analysed using Chart5 software (ADInstruments). Data was excluded if coronary flow exceeded 5mL/min.

Hearts and left atria were weighed before the heart was separated into superior and inferior sections. The superior section was fixed in preparation for picrosirius red staining (see Section 2.6) while the inferior section was frozen in liquid nitrogen-cooled isopentane to prevent tissue fracture. Frozen tissue was stored at -80^{o}C until RNA extraction (see Section 2.7).

2.5 Assessment of cardiac hypertrophy

Tissues were dissected and trimmed of fat and connective tissue before being blotted dry and weighed. Heart weights were normalised to tibial length (studies 1, 2 and 3) or to whole body weight (studies 1 and 4) to determine their proportional size.

2.6 Assessment of collagen content

Unless otherwise stated, all chemicals used during the assessment of collagen content were purchased from Sigma-Aldrich, Sydney. Collagen content was assessed as an indicator of the extent of fibrosis. All muscles were processed and assessed by the method below. This technique was used in studies 1, 3 and 4. Table 2.6.1 shows the tissues that were used in these studies.

Table 2.6.1 Tissues used for assessment of collagen content by picrosirius red staining.

Study	Heart	Diaphragm	Tibialis anterior muscle
1	✓		✓
3	✓	✓	✓
4	✓	✓	

Muscles were fixed sequentially in Telly's fixative (70% ethanol, 37% formaldehyde, glacial acetic acid) for 72 hours; modified Bouin's solution (saturated picric acid, 37% formaldehyde, glacial acetic acid) for 24 hours; and washed daily in 70% ethanol for a further 3 days. Muscles were stored in 70% ethanol until being embedded in paraffin wax and sectioned at 7μm

on a Reichert-Jung Biocut 2035 microtome. Sections were taken from the mid-section of the heart and tibialis anterior (TA) muscle, while the diaphragm was cut into strips and pinned to cork to be processed, with sections taken transversely through the muscle. Sections were placed on Menzel-Glaser polysine coated slides and stained using the selective collagen dye, picrosirius red solution (1% w/v Sirius Red F3B, Chroma Dyes, in saturated picric acid). Table 2.6.2 details the staining procedure used.

Slides were cover-slipped using Depex and examined on a Nikon Eclipse E600 light microscope. Codes were assigned randomly to each animal and slides were viewed blinded to genotype or treatment group. Images were captured with a QImaging MicroPublisher 5.0 RTV digital camera (Burnaby, Canada). Five images of each section were captured at 400×magnification, analysed using analySIS software (version 5, Soft Imaging System, Münster, Germany) and averaged to determine collagen percentage. In the case of the heart, two images were taken from the right and left ventricular walls and one from the septum. Images from the TA and diaphragm were acquired from over the entire muscle section.

Table 2.6.2 Picrosirius red staining protocol.

Stage	Solution	Time
De-waxing	Xylene	5 min
	Xylene	5 min
	Xylene	5 min
Rehydration	Ethanol (100%)	3 min
	Ethanol (100%)	3 min
	Ethanol (90%)	3 min
	Ethanol (70%)	3 min
	Lithium carbonate	1 min
	Water	2 min
	Distilled water	1 min
Picrosirius red staining	Phosphomolybdic acid	5 min
	Water	Brief rinse
	Picrosirius red (0.1%)	90 min
	Hydrochloric acid (0.01M)	30 sec
	Ethanol (70%)	30 sec
	Ethanol (90%)	30 sec
	Ethanol (100%)	30 sec
	Ethanol (100%)	30 sec
	Ethanol (100%)	30 sec
	Ethanol (100%)	30 sec
Preparation for mounting	Xylene	5 min
	Xylene	5 min
	Xylene	5 min

2.7 Measurement of gene expression by qRT-PCR

Quantitative real time PCR (qRT-PCR) was used to measure gene expression in hearts and diaphragms of mice in studies 3 and 4. Genes of interest were TGF-β, TNF-α, procollagen 1 and fibronectin. β-actin was

used as the housekeeper gene. Cardiac tissue (inferior portion) was taken after completion of Langendorff experiments and frozen immediately in liquid nitrogen-cooled isopentane (2-methylbutane CHROMASOLV) to prevent tissue fracture. Diaphragms were dissected immediately following removal of heart (during Langendorff equilibration period), rolled and frozen in liquid nitrogen-cooled isopentane. Tissues were stored at -80^{o}C until RNA extraction.

2.7.1 RNA extraction

Unless otherwise stated, products used in RNA extraction were purchased from Ambion Australia. RNA was extracted from frozen tissue shaved on a Microm HM550 cryostat (Zeizz, Australia) by Trizol extraction and used to derive cDNA for qRT-PCR evaluation of gene expression. All surfaces were cleaned with RNAse zap wipes and sterile distilled water before the procedure to ensure non-contamination. Samples were kept on dry ice whenever possible to maximise RNA yield.

800µL of Trizol was added to samples and homogenised for two minutes, after which it was passed vigorously through a 26 gauge needle approximately ten times to shear genomic DNA (gDNA). Homogenised samples were then incubated for five minutes at room temperature before 160µL of chloroform was added. The sample-chloroform solution was mixed vigorously by hand for fifteen seconds and incubated for three minutes. Samples were centrifuged at 14,000rpm for ten minutes in a refrigerated centrifuge set to 4^{o}C that separated the solution into phases. The aqueous (upper) phase was carefully transferred to a fresh tube and the lower phase discarded. 400µL of chilled (-20^{o}C) isopropanol was added to precipitate RNA from solution. 1.3µL of GlycoBlue was added to enable easy visualisation of aqueous phase and the solution was mixed by inversion before a ten minute incubation at 4^{o}C. Samples were centrifuged for ten minutes at 4^{o}C to form pelleted RNA. Supernatants were carefully removed using a pipette and the pellet washed vigorously with 800µL of RNAse free 75% ethanol. Samples were centrifuged as above for five minutes to reform the RNA pellet and remove supernatant (ethanol). RNA was dissolved in

30µL of RNAse free water and incubated at 62°C for ten minutes before the additions of 0.5µL of SUPERaseIn RNAse inhibitor, 0.6µL of TURBO-DNA free DNAse enzyme and 3µL of TURBO DNA-free DNase buffer. Samples were mixed and briefly centrifuged before being incubated at 37°C for thirty minutes to allow inactivation of RNAses and degradation of gDNA. After incubation, 3.4µL of TURBO-DNA free DNase inactivation reagent was added to halt the reaction. Samples were vortexed and incubated at room temperature for one minute before being centrifuged at 13,000rpm for ninety seconds to form the inactivation resin into a pellet. The supernatant (containing the RNA) was carefully transferred to a fresh tube and yield quantified using a GeneQuant (GE Healthcare Australia Pty. Ltd, Rydalmere, Australia). 50ng/µL stocks were made up for each sample and stored at -80°C. A gDNA check PCR using primers for β-actin was conducted to ensure there was no contamination that would invalidate gene expression analysis. Table 2.7.1 summarises reagents and ratios for this PCR. Amplification cycling times are given in Table 2.7.2. A ThermoHybaid thermocycler (HBPX220 Integrated Sciences) was used in PCR amplification of target sequences.

Table 2.7.1 PCR reagents and ratios for gDNA check.

Reagent	Volume per reaction (µL)
RNA (50ng)	1 (stock RNA)
Primer: β-actin fwd (10µM)	2
Primer: β-actin rev (10µM)	2
10x NEB buffer	2.56
dNTP (10mM each)	0.25
$MgCl_2$ (25mM)	1.5
NEB Taq polymerase	0.15
RNAse/DNAse free water	15.54
Total	25

Table 2.7.2 PCR cycling conditions for gDNA check.

Temperature	Time	Number of cycles
94°C	2 min	1
94°C	30 sec	30
56°C	1 min	
72°C	2 min	
11°C	hold	

This PCR utilised New England Biolabs buffer and Taq polymerase and was run with appropriate positive and no-template controls. A 10mM mix of each dNTP was used (Fisher Biotech Cat# DN-10M). The PCR product was run on a 2% agarose gel to distinguish bands.

Interpretation of results

This PCR was designed to detect the presence of contaminating gDNA in RNA samples. The presence of a band at 127bp indicated that the sample still contained gDNA and needed to undergo further DNAse treatment. Absence of a band indicated that the sample was free of gDNA contamination and was ready for use in synthesis of complementary DNA (cDNA).

2.7.2 First strand cDNA synthesis

A SYBR GreenER two-Step qRT-PCR kit (Invitrogen) was used to synthesise cDNA and run qRT-PCR evaluation of gene expression. SuperScript III First-Strand Synthesis SuperMix (Invitrogen) reagents were combined as summarised in Table 2.7.3. Samples were mixed gently and incubated at 25°C for ten minutes. They were then incubated for a further thirty minutes at 50°C before the reaction was terminated by incubation at 85°C for five minutes and samples placed on ice. 1µL of *E. coli* RNAse H was added and samples were incubated at 37°C for twenty minutes to ensure residual RNA was degraded. Purified cDNA was stored at -20°C until required for evaluation of gene expression.

Table 2.7.3 SuperScript III First-Strand Synthesis SuperMix.

Reagent	Volume per reaction (µL)
2x RT Reaction mix (includes SuperScript III & RNAseOUT)	10
RT Enzyme mix (includes 2.5µM oligo$(dT)_{20}$, 2.5ng/µL random hexamers, 10mM $MgCl_2$ & dNTPs)	2
RNA (100ng)	2
DEPC-treated water	6
Total	20

2.7.3 qRT-PCR evaluation of gene expression

cDNA was used in qRT-PCR evaluation of gene expression within days of synthesis. 80µL of sterile water was added to each sample to dilute 1:5; the diluted sample was combined with components from the SYBR GreenER qPCR SuperMix Universal module as summarised in Table 2.7.4. Amplification cycling conditions (given in Table 2.7.5) were performed using a Rotor-Gene 3000 apparatus and analysed using Roto-Gene software (version 6) (Corbett Life Science). Primers for the genes of interest are presented in Table 2.7.6. Whole mouse RNA (Invitrogen) was used as a calibrator. Primer efficiencies were determined for each primer set before evaluation of gene expression.

Table 2.7.4 SYBR GreenER qPCR SuperMix.

Reagent	Volume per reaction (µL)
SYBR GreenER qPCR SuperMix Universal	12.5
Forward primer (300nM)	3.75
Reverse primer (300nM)	3.75
cDNA template (1:5 dilution)	5
Total	25

Table 2.7.5 Amplification cycling conditions for qRT-PCR evaluation of gene expression.

Temperature	Time	Number of cycles
95°C	10 min	1
95°C	15 sec	40
58°C	60 sec	
72°C	20 sec	
Melt 72-95°C (increasing at 0.1°C increments)	5sec pauses between increments	1

Table 2.7.6 Primers used for genes of interest.

Name	Sequence
TGF-β	
Forward	5'-CCG CAA CAA CGC CAT CTA TG-3'
Reverse	5'-CTC TGC ACG GGA CAG CAA T-3'
TNF-α	
Forward	5'-CCC TCA CAC TCA GAT CAT CTT CT-3'
Reverse	5'-GCT ACG ACG TGG GCT ACA G-3'
Procollagen 1	
Forward	5'-GCT CCT CTT AGG GGC CAC T-3'
Reverse	5'-CCA CGT CTC ACC ATT GGG G-3'
Fibronectin	
Forward	5'-ATG TGG ACC CCT CCT GAT AGT-3'
Reverse	5'-GCC CAG TGA TTT GAC CAA AGG-3'
β-Actin	
Forward	5'-CCC AGA TCA TGT TTG AGA CCT-3'
Reverse	5'-CCA TCA CAA TGC CTG TGG TA-3'

2.8 Single cell methodologies

Cardiomyocytes from C57, *mdx* and nNOS cardiac overexpressing mice in Study 3 were isolated and used in the assessment of contractility and calcium handling. Isolated cells were also used to assess L-type calcium channel function by the voltage clamp technique. All single cell experiments were conducted at 37 ± 1^{o}C maintained by a custom-made, in-line heat exchanger. All solutions were filtered (0.2μm filter), equilibrated with carbogen (95%O_2; 5% CO_2) for at least 20min and adjusted to pH 7.4 at room temperature before use.

2.8.1 Cell isolation

Unless otherwise stated, all chemicals used during the cell isolation were purchased from Sigma-Aldrich, Sydney (solutions are described in Table 2.8.1). Experimental animals were administered an ip injection of 0.5mL heparin diluted in phosphate-buffered saline (PBS) to 100U/mL to inhibit blood coagulation. After a period of twenty minutes, mice were anaesthetised using 50mg/kg sodium pentobarbitone ip. A thoracotomy was performed, the heart dissected and placed into isolation solution (in mM: NaCl 130; KCl 5.4; $MgCl_2$ 3.5; glucose 10; HEPES 5; $NaHPO_4$ 0.6; taurine 20) where the thymus was removed to expose the aorta. The heart was cannulated via the aorta and perfused retrogradely on a Langendorff apparatus with isolation solution warmed to 37^{o}C for three minutes. It was then perfused with enzyme solution 1 (Table 2.8.1) for five minutes before the ventricles were dissected free and placed into a small volume of enzyme solution 2 (Table 2.8.1). Ventricles were dissociated coarsely using sterile scissors before being transferred to a tube containing 5mL of enzyme solution 2 and placed into a 37^{o}C shaking water bath for five minutes.

The cell solution was made up to 10mL with wash solution (Table 2.8.1) and centrifuged at 600rpm for three minutes, after which the pellet was removed, transferred to a fresh tube and resuspended in approximately 5mL of solution 2. This tube was placed back into the water bath for a further five minute digestion. The supernatant was set to one side and allowed to

settle; this constituted the first yield of isolated cardiomyocytes. These steps were repeated in order to produce three yields of isolated cells harvested at digestion times of five, ten and fifteen minutes. Cells were inspected for yield size and quality before being allowed to settle. The supernatant was carefully removed and cells suspended in approximately 3mL of storage solution (in mM: NaCl 120; KCl 5.6; $MgSO_4$ 5; $CaCl_2$ 0.2; sodium pyruvate 5; glucose 20; taurine 20; HEPES 10) until use. Isolated cells were harvested in the morning and used for cell shortening, calcium imaging and patch clamp experiments in the afternoon of the same day. All chemicals used in preparation of cell isolation solutions were purchased from Sigma-Aldrich with the exception of collagenase type II being purchased from Worthington.

Table 2.8.1 Solutions used in cell isolation.

Solution	Reagent	Volume or weight
BSA solution	Isolation solution	40mL
	Bovine serum albumin	400mg
Enzyme solution 1 collagenase + protease	Isolation solution	25mL
	BSA solution	5mL
	Collagenase II	30mg
	Protease	5mg
	$CaCl_2$ (1M)	1.5uL
Enzyme solution 2 collagenase	Isolation solution	16.67mL
	BSA solution	3.3mL
	Collagenase II	20mg
	$CaCl_2$ solution(1M)	1uL
Wash solution	BSA solution	33.35mL*
	$CaCl_2$ solution (0.1M)	333.5µL

* Portion remaining after preparation of enzyme solutions 1 and 2.

2.8.2 Assessment of contractility

Unless otherwise stated, all chemicals used during the assessment of contractility were purchased from Sigma-Aldrich, Sydney. Isolated

cardiomycytes were placed in a 0.5mL chamber situated on the stage of an inverted Olympus IX70 microscope (Figure 2.8.1) and allowed to settle for five minutes. Cells were superfused with perfusion solution (in mM: NaCl 140; KCl 5.4; MgCl 1.2; HEPES 5; glucose 10; CaCl 1.8) that was maintained by a Minipuls 3 peristaltic pump (Gilson Incorporated, Middleton, USA) to produce a flow rate of 6mL/min and allowed to equilibrate for five minutes. Perfusion solution was warmed to 37^{o}C by a custom made, in-line heat exchanger. Cells were field stimulated at 0.5, 1, 2, 3 and 6Hz by pulses delivered by a pair of platinum electrodes within the chamber. Stimulation was controlled by an S48 Stimulator (Grass Technologies, West Warwick, USA) attached to an Ebony amplifier (Audio Assemblers, Melbourne, Australia) and was set to a 5ms square pulse with voltage set above threshold to maximise capture. Myocyte edge movement was acquired using a video edge-detector system (IonOptix, Milton, USA) coupled to a Myocam (IonOptix, Milton, USA) mounted to the microscope. Contractility data was acquired and analysed using IonWizard and SoftEdge Myocyte Cell Length Acquisition software (IonOptix, Milton, USA). Force-frequency experiments were conducted on unloaded cells to prevent calcium buffering by FURA-2 AM.

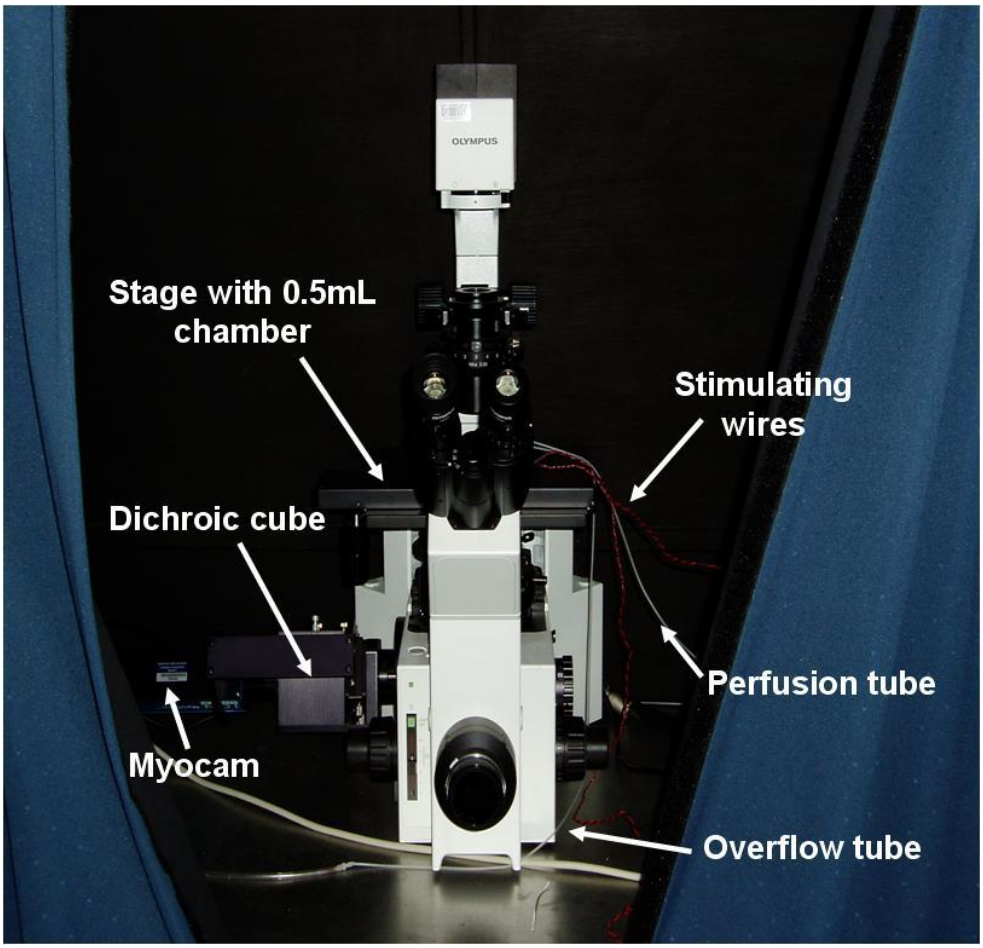


Figure 2.8.1 Inverted Olympus IX70 microscope and apparatus used in contractility and calcium imaging experiments.

2.8.3 Calcium imaging

Isolated cardiomyocytes were removed from storage solution and suspended in approximately 1mL of perfusion solution (as described above but with 250μM [Ca^{2+}]) and loaded with the calcium-sensitive dye FURA-2 AM by adding 5μL of 1mM FURA-2 AM (diluted in 100% DMSO) and 2μL of 20% pluronic acid. The cells were incubated in the dark for fifteen minutes at room temperature. The supernatant was carefully removed and cells resuspended in an equal volume of perfusion solution (as described above but with 500μM [Ca^{2+}]). Again, 5μL of 1mM FURA-2 AM and 2μL of 20% pluronic acid was added and cells incubated for a further fifteen minutes in the dark at room temperature. The resultant supernatant was removed carefully and cells resuspended in perfusion solution made up to 500μM calcium concentration and incubated in the dark for a final ten minutes. Cells were then considered loaded and calcium-tolerant. Experiments were

conducted using an inverted Olympus IX70 microscope (pictured in Figure 2.8.1) attached to a Lambda DG4 (Sutter Instrument Company, SDR Clinical Technology, Sydney, Australia) light source within a darkened environment to prevent bleaching of the FURA-2 AM dye.

Isolated cells were placed in a 0.5mL chamber situated on the stage of the microscope and allowed to settle for five minutes. Cells were superfused with perfusion solution for five minutes before being field-stimulated. Calcium transient and shortening data were collected from cell contractions induced by single pulses. Changes in cell length and calcium transients were recorded simultaneously by splitting the microscope light with a dichroic cube situated between the microscope and the myocam. The video edge detector received red light, with the remaining light diverted to the photomultiplier tube for measurement of fluorescence. Assessment of intracellular calcium was conducted by whole cell photometry. The emission ratio at 510nm was measured during alternate excitation at 340nm and 380nm and was used to determine changes in intracellular calcium concentrations. Contractility measurements were conducted as described in section 2.8.3. Data acquisition and analysis were performed using IonWizard and SoftEdge Myocyte Cell Length Acquisition software (IonOptix, Milton, USA). There was contamination of the outward current meaning that transient time constants (tau) could not be calculated.

2.8.4 Voltage clamp

Isolated cardiomyocytes were placed in a 0.5mL chamber situated on the stage of an inverted Olympus CK30 microscope (pictured in Figure 2.8.2) and allowed to settle for five minutes. The cells were then superfused with carbogenated perfusion solution (in mM: NaCl 140; KCl 5.4; $MgCl_2$ 1.2; HEPES 5; glucose 10; $CaCl_2$ 1.8) that was maintained by a Minipuls 3 peristaltic pump (Gilson Incorporated, Middleton, USA) to produce a flow rate of 3mL/min and allowed to equilibrate for five minutes. Perfusion solution was warmed to 37^{o}C by a custom made, in-line heat exchanger. Cells were patched using electrodes (1-2 MΩ resistance) pulled from filamented borosilicate glass (World Precision Instruments, Sarasota, USA)

on a Flaming/Brown micropipette puller (Sutter Instrument Company, Novato, USA) and filled with an electrode solution containing (in mM: CsF 135; NaCl 10; HEPES 10; EGTA 10; glucose 7.7; MgATP 2; pH 7.2). Data was acquired using a HEKA EPC-9 and analysed using Pulse (v8.4) software. The system was earthed and situated within a Faraday cage to prevent 50Hz electrical interference.

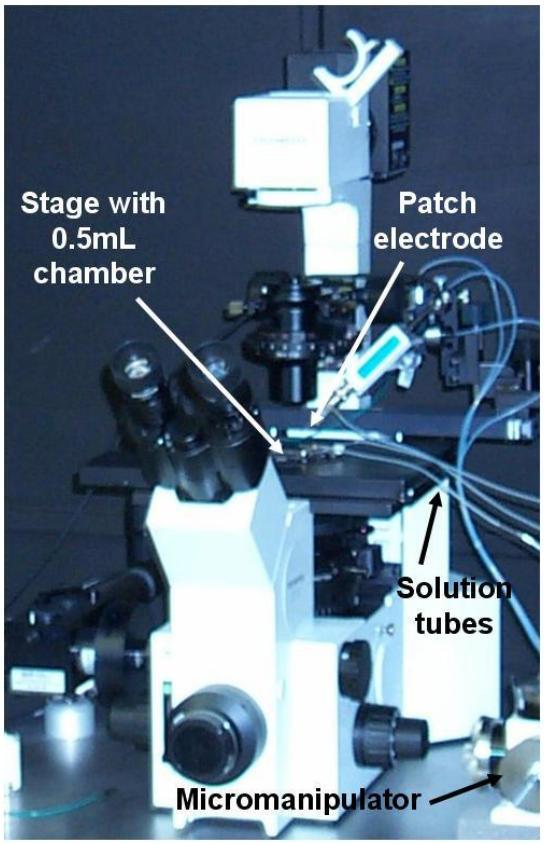


Figure 2.8.2 Inverted Olympus CK30 microscope used in voltage clamp experiments.

L-type calcium channel inactivation

The voltage step protocol for the L-type calcium channel inactivation experiments is represented in Figure 2.8.3. Briefly, cells were stepped from a holding potential of -70 up to -40mV for 65ms to inactivate the sodium current. Membrane potential was then stepped up to 0mV and the calcium current amplitude (*a*) was recorded before the membrane potential was returned to -40mV. Test potentials stepped from -40 up to +50mV in 10mV increments and a second measurement of calcium current amplitude (*b*) was taken at 0mV. *b* was expressed as a percentage of *a* at all test potentials.

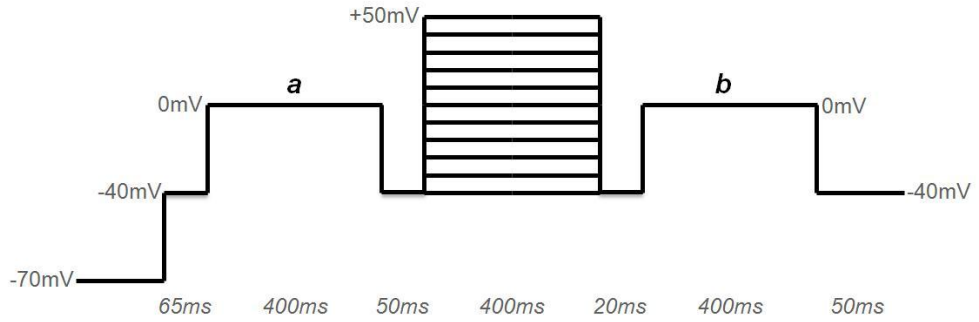


Figure 2.8.3 L-type calcium channel inactivation protocol, showing voltage steps used.

Calcium current–voltage relationship

The voltage step protocol for the calcium current–voltage relationship experiments is represented in Figure 2.8.4. Briefly, patched cells were stepped from a holding potential of -70 to -40mV for 65ms to inactivate the sodium current, then stepped through a series of test potentials ranging from -40mV up to +60mV in 10mV increments before membrane potential was returned to -40mV. Peak calcium current was measured at each test potential and plotted as a current-voltage curve representing mean data for each group.

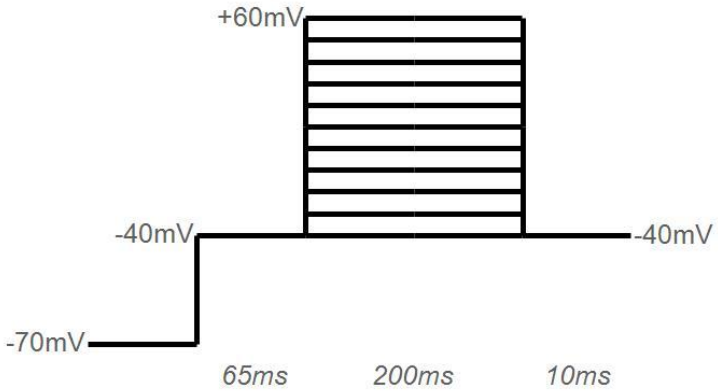


Figure 2.8.4 Calcium current–voltage relationship protocol, with voltage increments.

Calcium current rundown

Sodium currents were inactivated by stepping the holding potential of -70mV up to -40mv for 65ms. Ten test potentials to 0mV were then pulsed at 1, 2, 4 and 8Hz and resulting peak calcium current amplitudes were

recorded. Test amplitudes were expressed as a percentage of the first response current.

2.9 Statistical analysis

Data is presented as mean ± standard error of the mean (SEM). Data was compared by unpaired Student's *t*-test, with $P<0.05$ considered statistically significant. The only exception to this was during study 2, where correlation of percent cell shortening to calcium transient height was conducted by Hotelling's *t*-test, with $P<0.05$ considered statistically significant.

Chapter 3: Myostatin knock-out in the dystrophic heart

3.1 Introduction

Myostatin is a well-recognised negative regulator of muscle growth. Since its discovery, the relevance of myostatin (or GDF-8) to the treatment of muscle wasting diseases such as muscular dystrophy has been investigated (McPherron et al. 1997a; Bogdanovich et al. 2002; Qiao et al. 2008). Structurally, myostatin is a member of the TGF-β superfamily and is similarly activated by enzymatic cleavage of an inhibitory propeptide (McPherron et al. 1997a; Zimmers et al. 2002). In skeletal muscle, inhibition of myostatin resulted in excessive muscle growth accompanied by decreased adiposity, while overexpression or administration of the active protein caused cachexia (Lee & McPherron 2001; Zimmers et al. 2002). The action of myostatin is not restricted to skeletal muscle; myocardial development and repair are also affected as myostatin is expressed in both foetal and adult cardiac tissue as well as being upregulated in tissue bordering myocardial infarcts (Sharma et al. 1999). It is also upregulated in Akt overexpression and volume-overload hypertrophic hearts (Cook et al. 2002; Shyu et al. 2006).

The principal action of myostatin is the suppression of myogenic cell proliferation and differentiation, although there is evidence that it also increases cell survival, inhibits protein synthesis and decreases adiposity (Taylor et al. 2001; McPherron & Lee 2002; Joulia et al. 2003). Myostatin upregulated p21, an inhibitor of cyclin-dependent kinase (cdk), resulting in decreased levels of cdk-2, phosphorylated retinoblastoma protein and prevention of satellite cells from entering the cell cycle (Thomas et al. 2000; Rios et al. 2002). Increased p21 has also been linked to decreased myoblast apoptosis (Wang & Walsh 1996). Myostatin decreased expression of a number of myogenic regulatory factors including MyoD, Myf-5 and myogenin, thus slowing differentiation of myoblasts and satellite cells by preventing myotube formation (Langley et al. 2002; Joulia et al. 2003). Myostatin may hold myogenic cells in a quiescent state until muscle growth

or repair suppresses the protein's activity and allows these cells into the cell cycle.

Myostatin inhibition in dystrophin-deficient muscle taken from *mdx* mice has yielded encouraging results. Drug therapies, such as administration of blocking antibodies (Bogdanovich et al. 2002) and the inhibitory myostatin propeptide (Bogdanovich et al. 2005; Qiao et al. 2008), have demonstrated effective myostatin inhibition with corresponding functional improvement in dystrophic skeletal muscle. Myostatin inhibition has produced both hyperplasia and hypertrophy that translated to improved muscle strength and function (Bogdanovich et al. 2002; Wagner et al. 2002). Histological analysis revealed that, while there was improvement in fibrosis, classic hallmarks of dystrophinopathy such as heterogeneity of size, fibre splitting, centralised nuclei, patchy necrosis, inflammation and elevated serum CK remained (Bogdanovich et al. 2002; Wagner et al. 2002).

Initial research suggested that myostatin may play different roles in different types of muscle. Cohn et al. (2007) found that, unlike in skeletal muscle, myostatin inhibition in the heart produced no change in cardiac morphometry, fibrosis or function. However, further studies showed that cardiomyocyte proliferation and differentiation were affected by the level of myostatin expression, and together with enhanced β-adrenoceptor responsiveness and SR calcium release, translated to improved cardiac excitation-contraction coupling (Artaza et al. 2007; Rodgers et al. 2009a). Moreover, myostatin has myocardial modulatory functions such as negatively regulating myocyte growth in response to stress (Bish et al. 2010). Myostatin is also upregulated in the hearts of heart failure patients, where it may impact upon hypertrophic growth (George et al. 2010). Thus, it is emerging that myostatin may indeed play a modulatory function in the myocardium.

This evidence of both skeletal and cardiac regulation makes myostatin inhibition an attractive treatment option for DMD, especially if applied in conjunction with already validated therapies such as corticosteroids in

skeletal muscle, or β-adrenoceptor antagonists and ACE inhibitors in the heart. Induction of hyperplasia in dystrophic muscle may counteract the atrophy observed in the later stages of the disease, preserving strength and improving quality of life for boys with DMD. For example, myostatin inhibition in *mdx* mice has resulted in phenotypic improvements such as decreased fibrosis and increased skeletal muscle hyperplasia and hypertrophy that also corresponded to improvements in strength and function (Bogdanovich et al. 2002; Wagner et al. 2002). In contrast, relatively few studies have focused on the ramifications of myostatin inhibition in the dystrophic heart. To this end, this study has been designed to investigate whether myostatin absence affected cardiac morphology and function, and to assess the possibility that the increased demands of an increased skeletal muscle mass outweighed benefits gained from cardiac hypertrophy.

3.2 Materials and methods

Myostatin knock-out mice (*Mstn*$^{-/-}$) were crossed with *mdx* mice to produce a double knock-out strain lacking both myostatin and dystrophin (2KO). Significant obstacles were encountered in the breeding and genotyping of myostatin knock-out strains (both the 2KO and *Mstn*$^{-/-}$) that had detrimental outcomes on experimental animal numbers and time taken to complete this study. Myostatin-null mice were very poor breeders, with low fertility and few pups born. Subsequently, both male and female *Mstn*$^{-/-}$ and 2KO mice were used in experiments to increase group numbers and improve statistical validity.

There were unexpected responses to anaesthesia during *in vivo* assessment of cardiac function by Millar catheter in mice with an *mdx* background (*mdx* and 2KO). These mice experienced severe orthostatic hypotension and rapidly developing Cheyne-Stokes-like breathing patterns after being placed in a supine position, so reliable Millar catheter results could not be obtained. Therefore, the Langendorff isolated heart preparation was used to measure cardiac function *ex vivo*, with *mdx* mice excluded from *in vivo* evaluation of heart function.

Assessment of cardiac hypertrophy was conducted by normalising whole heart weight to body weight. Cardiac function and arterial pressure development was assessed in C57 and *Mstn*$^{-/-}$ strains using Millar catheter and the Langendorff isolated heart techniques, while *mdx* based strains (*mdx* and 2KO) were assessed using Langendorff only. Cardiac fibrosis was assessed histologically by collagen staining using picrosirius red. Gene expression levels of TGF-β and TNF-α were investigated using qRT-PCR. These procedures have been detailed in Chapter 2 and their use in this study summarised in Figure 3.1. Error bars represent the standard error of the mean (SEM).

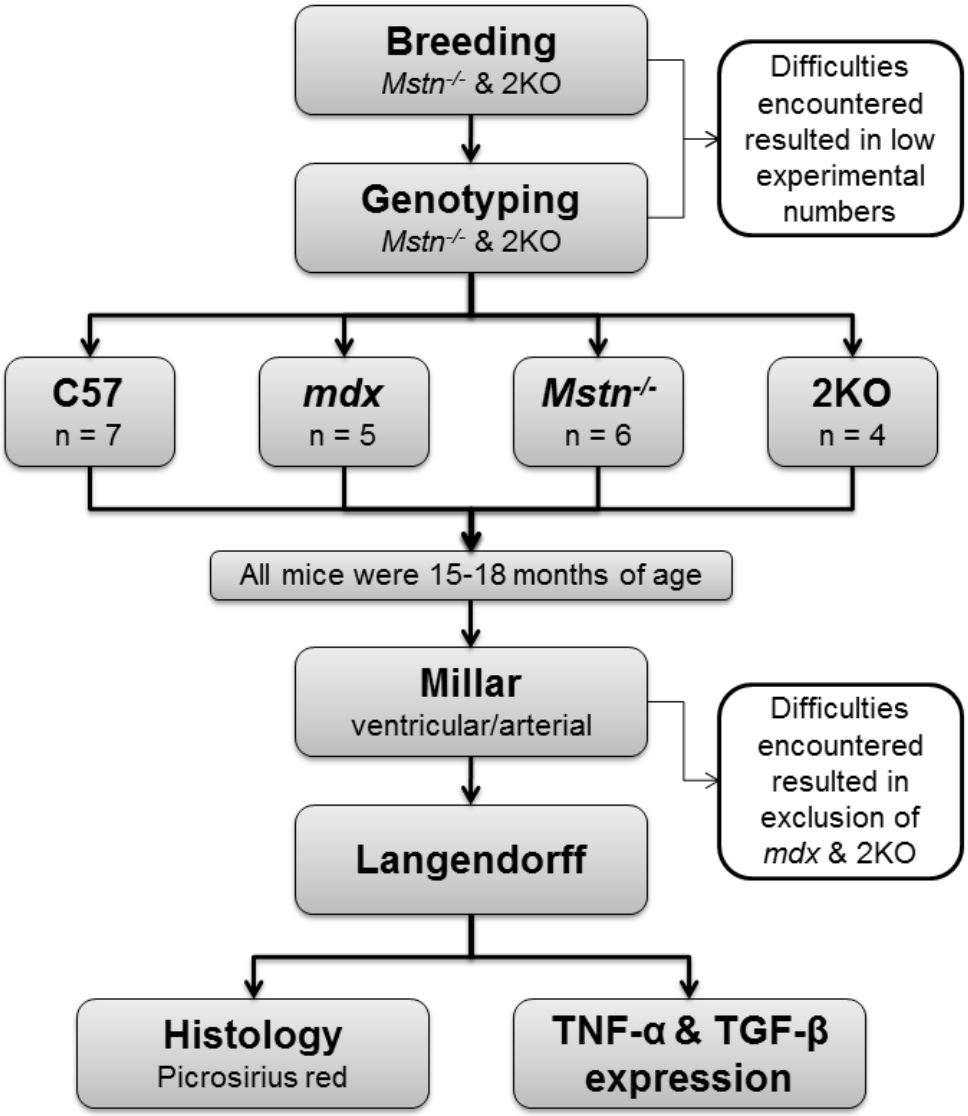


Figure 3.1 Summary of experimental animal numbers and techniques used in Chapter 3.

3.3 Results

3.3.1 Genotyping

A single band at 220bp indicated the animal was wildtype (*Mstn*$^{+/+}$), while a single band at 332bp indicated the animal was homozygous for the deleted myostatin gene (*Mstn*$^{-/-}$). If two bands at 220bp and 332bp were present, the animal was heterozygous. Figure 3.2 shows a genotyping gel with wildtype, myostatin null and heterozygous results.

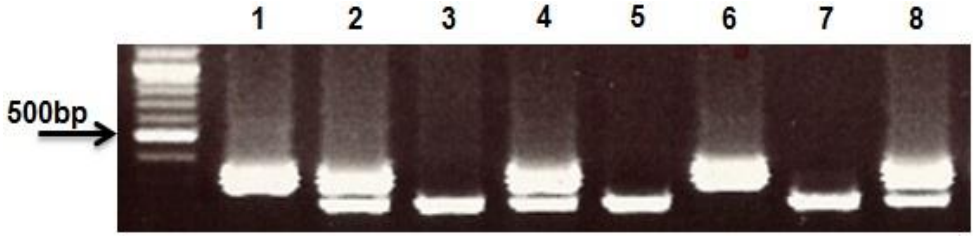


Figure 3.2 Myostatin genotyping gel.

Samples from lanes 1 and 6 show a single band at 332bp, indicating myostatin null mice. Samples from lanes 2, 4 and 8 show both bands at 220bp and 332bp, indicating heterozygous mice. Samples 3, 5 and 7 show a single band at 220bp indicating wildtype mice. The DNA size marker was a commercial 100bp ladder purchased from New England Biolabs (NEB).

3.3.2 Morphometry

Measurements of body weight and heart weight were used to determine whether the genetic disruption of myostatin affected cardiac mass in a similar fashion to skeletal muscle. The removal of myostatin resulted in increases in both body mass and whole heart mass (Figures 3.3A & B). Cardiac hypertrophy was observed in myostatin null mice with a significantly increased heart weight normalised to body weight indicating their increased body mass was accompanied by a proportionally greater increase in cardiac mass (Figure 3.3C).

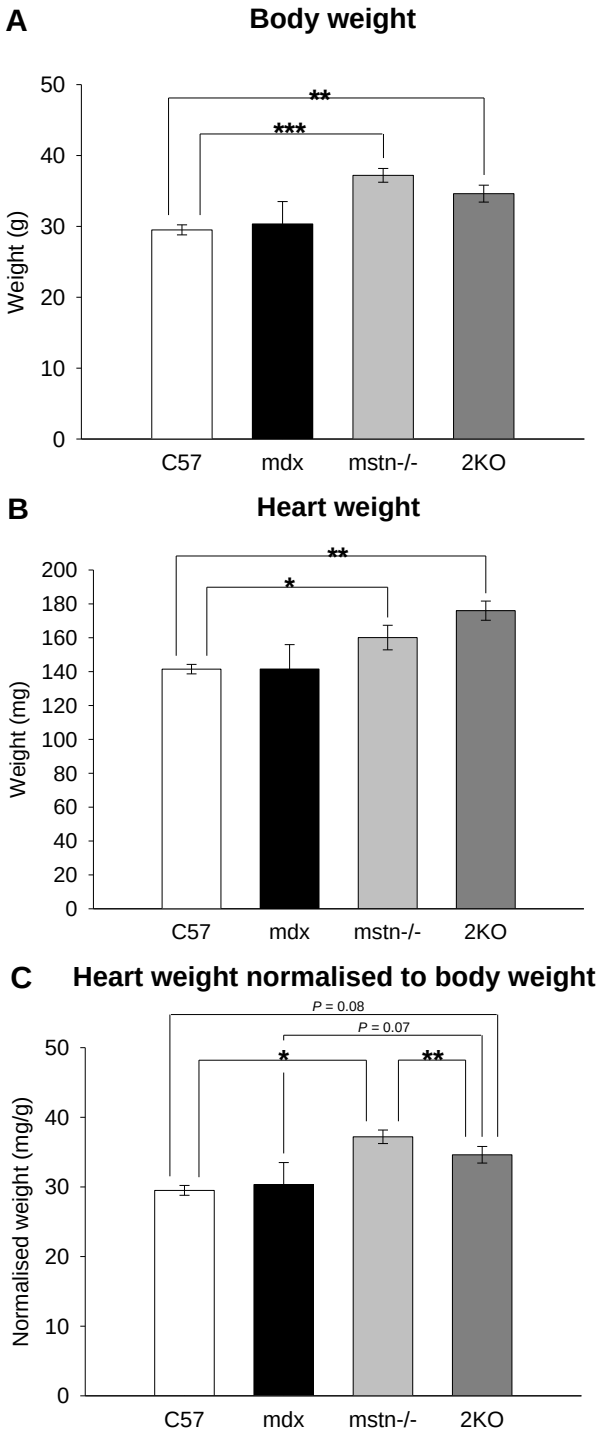


Figure 3.3 Assessment of cardiac hypertrophy.

A: Myostatin KO groups (*Mstn*$^{-/-}$ and 2KO) had significantly greater body mass compared to C57 but were not heavier than *mdx*. C57 and *mdx* did not differ in body weight. **B:** Heart weights of myostatin KO groups were significantly greater than C57 but not *mdx*. **C:** Normalised heart weights of myostatin KO strains were greater than their C57 and *mdx* controls.
(**P*<0.05; ***P*<0.01; ****P*<0.001)

3.3.3 *Ex vivo* measurements - Langendorff

Ex vivo measurements of cardiac parameters were conducted using the Langendorff isolated heart preparation to examine heart function in the absence of non-cardiac endocrine or nervous influence. Hearts were electrically paced at 420bpm in all groups to facilitate comparison of rate-dependent parameters such as LVDP and dP/dt and to allow investigation of heart function at an organ level. Rate of coronary flow was similar for all groups (Figure 3.4). Raw ESP and EDP data have not been presented as EDP was set to a physiological level of 10mmHg and instead developed pressure values are shown. Developed pressure was significantly decreased in *mdx* compared to C57 ($P<0.05$) (Figure 3.5). C57, *Mstn*$^{-/-}$ and 2KO developed pressures did not differ. Of note, 2KO developed pressure was equivalent to C57 values and significantly greater than *mdx* indicating a rescue of dystrophic heart function was achieved in the absence of myostatin in *mdx* hearts. Rates of ventricular contraction and relaxation (as indicated by ±dP/dt) were not significantly different (Figure 3.6).

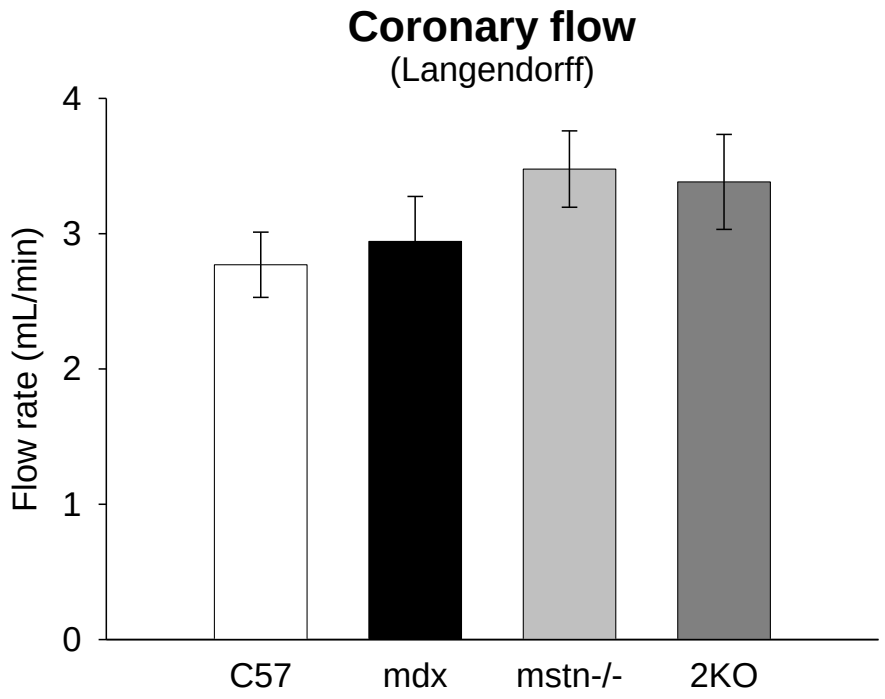


Figure 3.4 Coronary flow rate.

There were no significant differences in coronary flow between groups.

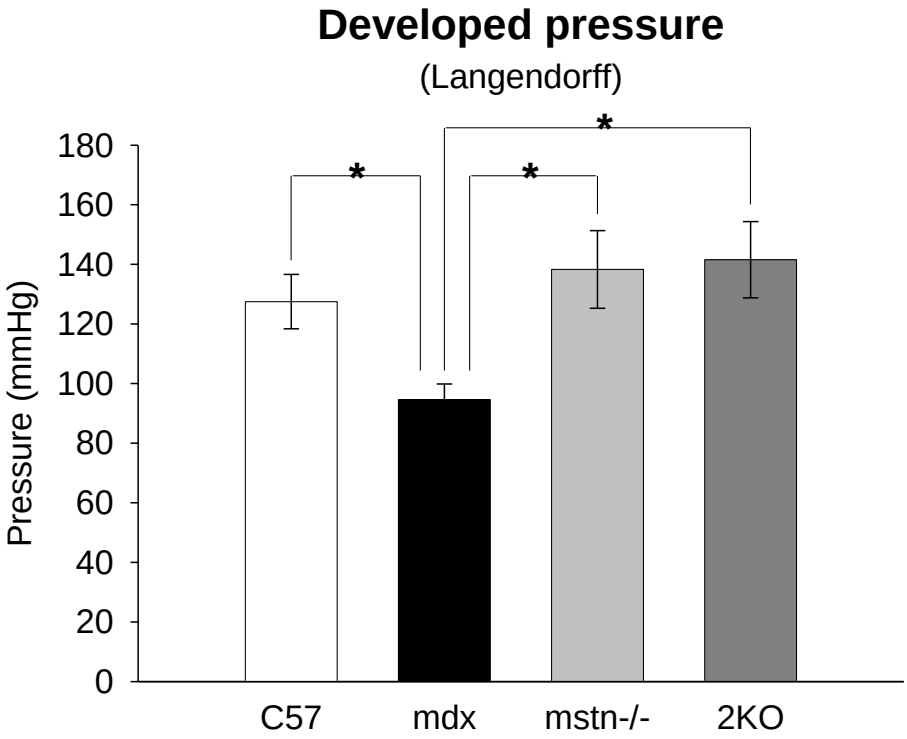


Figure 3.5 Developed pressure in the Langendorff isolated heart preparation.

EDP was set to a physiological level (10mmHg) by adjustment of Langendorff balloon size. Developed pressure therefore reflects changes in ESP. *Mdx* developed pressure was significantly lower than that of C57. Myostatin absence alone (*Mstn*$^{-/-}$) did not alter developed pressure, however this parameter was normalised in the 2KO group. (*P<0.05)

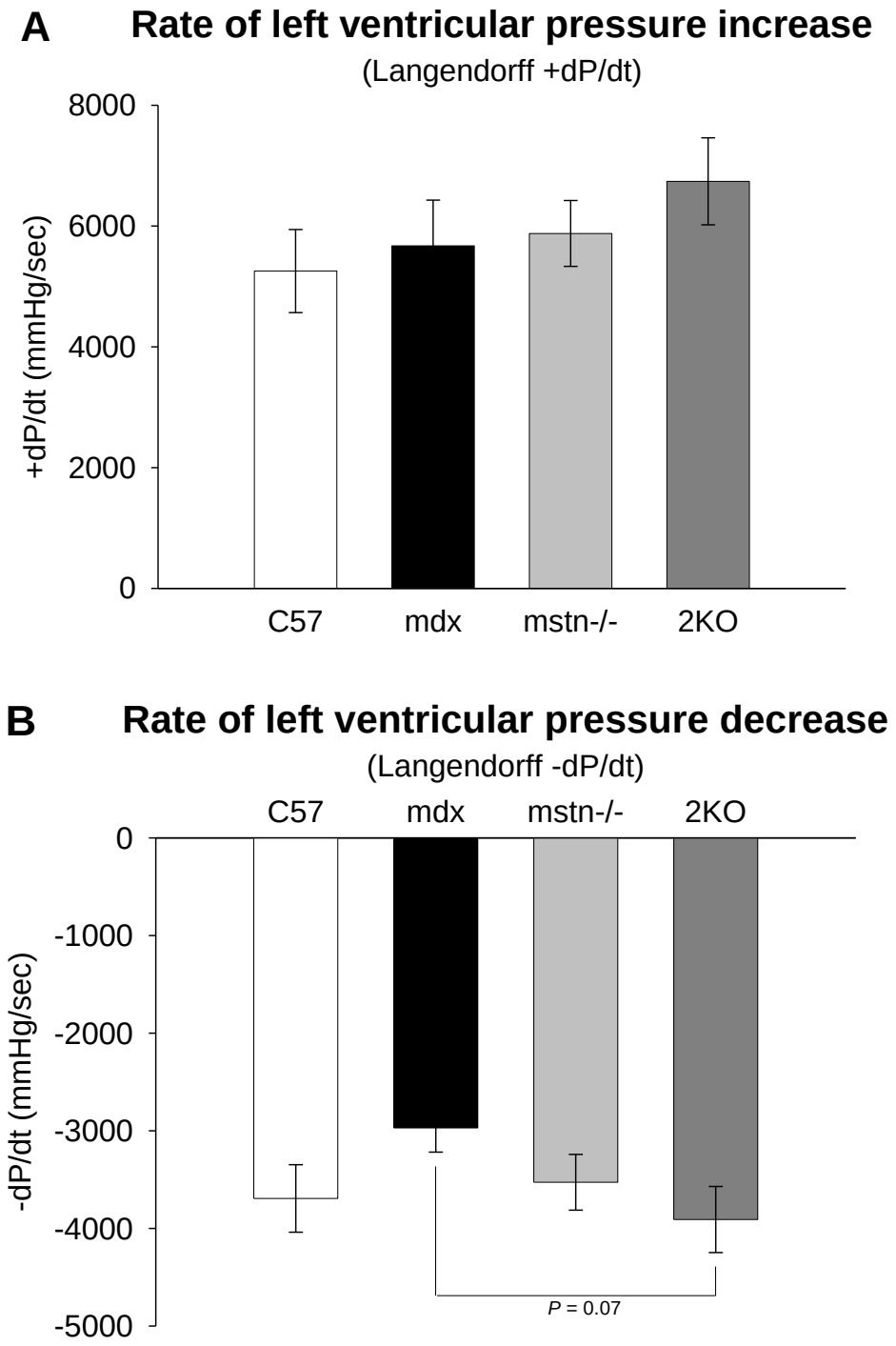


Figure 3.6 Rates of left ventricular pressure change measured during Langendorff experiments.

A: Rate of ventricular contraction as measured by +dP/dt. **B:** Rate of ventricular relaxation as measured by -dP/dt. Rates of left ventricular contraction and relaxation were not altered by myostatin absence.

3.3.4 Histology

Measurement of cardiac fibrosis was conducted using picrosirius red staining of ventricular collagen and then digital assessment of collagen content. Representative images of collagen stained ventricular tissue are presented in Figure 3.7. Groups on a dystrophic background showed marked increases in collagen content (*mdx* $P<0.001$; 2KO $P<0.05$) compared to those on a C57 background (C57 and *Mstn*$^{-/-}$ respectively) (Figure 3.8). Likewise, myostatin KO groups had significantly greater collagen content (*Mstn*$^{-/-}$ $P<0.05$; 2KO $P<0.05$) compared to myostatin-expressing control groups (C57 and *mdx* respectively). Interestingly, the absence of myostatin combined with a dystrophic background (2KO) appeared to potentiate the development of cardiac fibrosis (Figure 3.8).

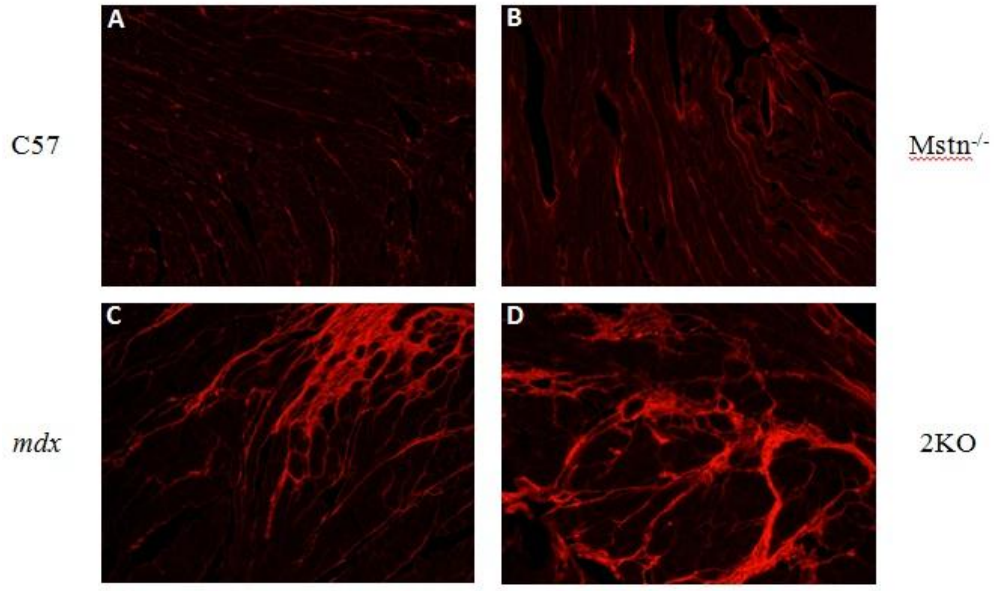


Figure 3.7 Representative images of ventricular sections stained with picrosirius red.

Images A (C57) and B (*Mstn*$^{-/-}$) are ventricles from a C57 (wild type) background, while images C (*mdx*) and D (2KO) are from a *mdx* (dystrophic) background. Images A and B have significantly less collagen than C and D.

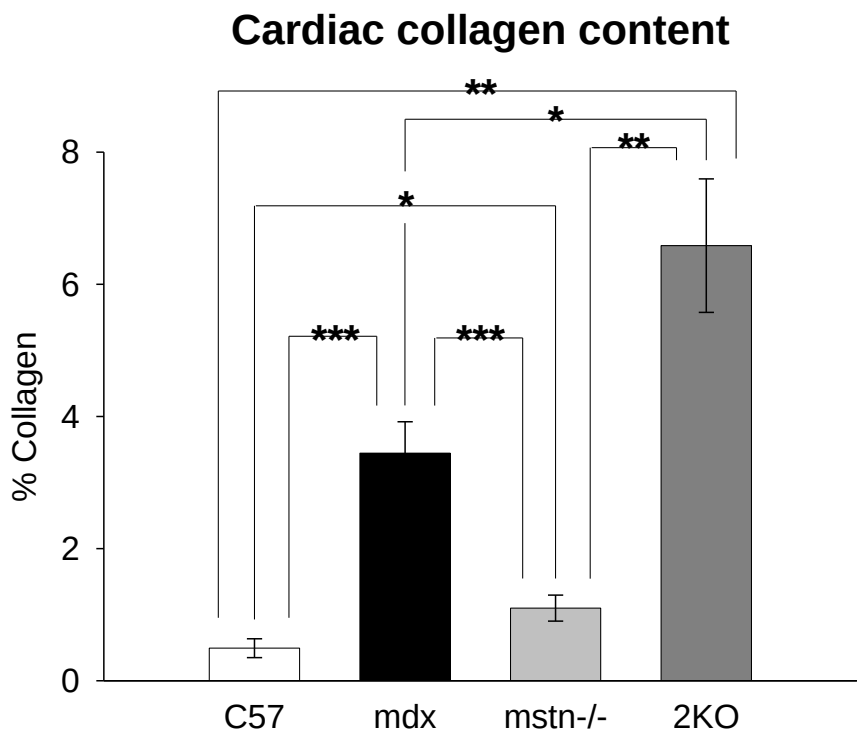


Figure 3.8 Cardiac collagen content measured by digital quantification of picrosirius red staining.

Mdx groups (*mdx* and 2KO) had significantly greater cardiac collagen compared to C57 groups (C57 and *Mstn*$^{-/-}$). Myostatin KO groups (*Mstn*$^{-/-}$ and 2KO) had significantly greater cardiac collagen compared to their myostatin control groups (C57 and *mdx* respectively). (**P*<0.05; ***P*<0.01; ****P*<0.001)

3.3.5 Gene expression

Possible mechanisms for the increased collagen deposition during histological assessment of cardiac fibrosis were tested by measurement of mRNA for the profibrotic cytokines TNF-α and TGF-β by qRT-PCR. TNF-α gene expression was not significantly different between groups. Double-knockout animals (2KO) showed a great variability in gene expression for this cytokine (Figure 3.9). *Mdx* mice had a significantly greater level of TGF-β gene expression compared to C57 and *Mstn*$^{-/-}$ groups (*P*<0.05) but not 2KO (Figure 3.10).

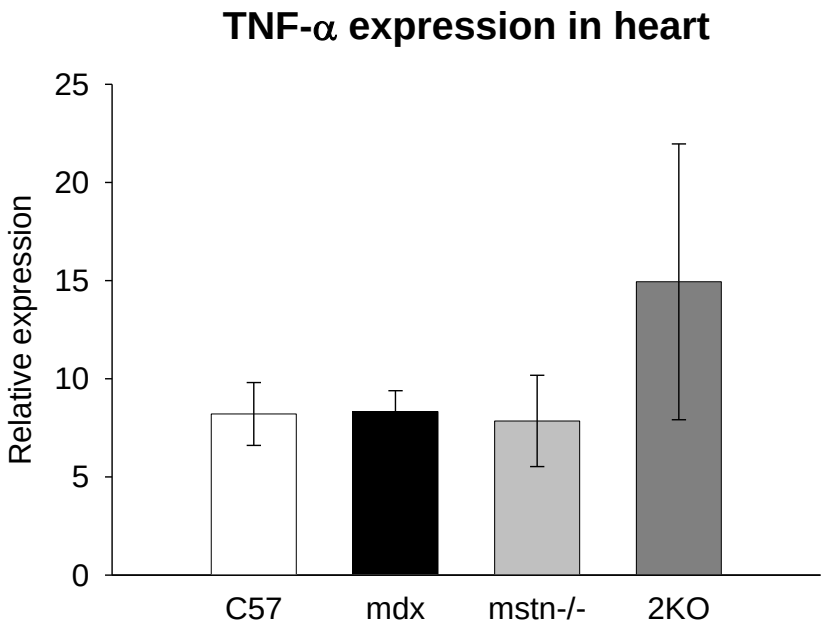


Figure 3.9 TNF-α expression in the heart determined by qRT-PCR.

TNF-α expression did not significantly differ between groups however, there was an increase in TNF-α expression observed in 2KO. This did not achieve statistical significance due to a large SEM.

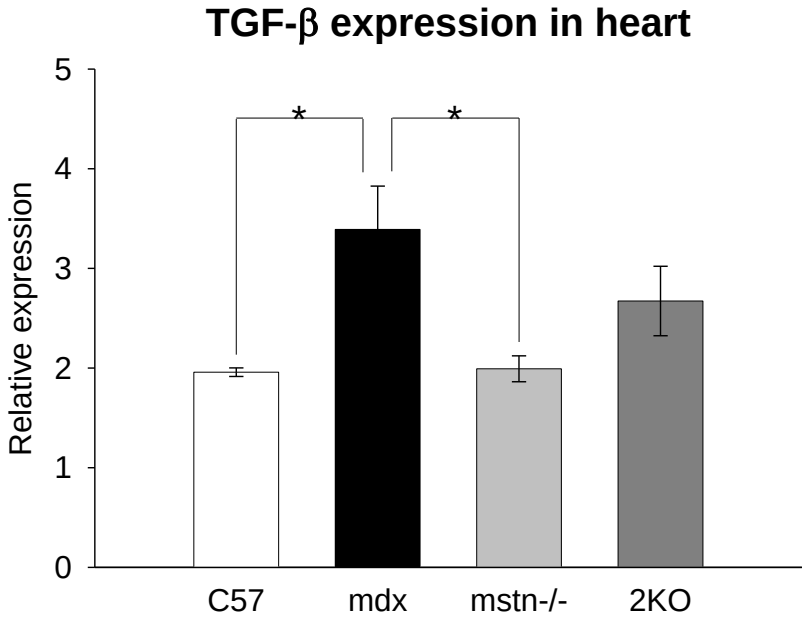


Figure 3.10 TGF-β expression in the heart determined by qRT-PCR.

Mdx had significantly greater TGF-β expression compared to C57 and *Mstn*$^{-/-}$ but not 2KO. (*P<0.05)

3.4 In vivo measurements – Millar catheter

Mdx and 2KO mice of this age group did not tolerate Millar experiments due to orthostatic hypotension, so that the experiments comparing mdx, 2KO, C57 and *Mstn*$^{-/-}$ were unable to be performed. The *mdx* and 2KO mice were reserved for Langendorff experimentation (*ex vivo* experiments as described above) as it was felt that these experiments would yield a greater amount of information. The following section describes *in vivo* measurements of heart function by Millar catheter for *Mstn*$^{-/-}$ and C57. Therefore these experiments aim to characterise the effect of myostatin absence in otherwise healthy mice.

3.4.1 Arterial measurements

Measurements of heart rate during Millar catheterisation showed no significant difference (C57: 357.5±82.9 bpm; *Mstn*$^{-/-}$: 345.0±82.7 bpm). Measurements of arterial developed pressures were conducted to ascertain whether the removal of myostatin had an effect on vascular smooth muscle, and to determine whether these effects were similar to those observed in the myocardium. Arterial Millar catheter data showed there was a decrease in EDP and ESP with myostatin removal (Figures 3.11A & B). This did not translate to an altered pulse pressure as both EDP and ESP were reduced in parallel (Figure 3.11C). MABP (Figure 3.13) was significantly decreased in *Mstn*$^{-/-}$ possibly indicating a degree of orthostatic hypotension. Rates of ventricular contraction and relaxation (as indicated by positive and negative changes of pressure over time (±dP/dt)) indicated myostatin removal significantly decreased the rate of cardiac contraction but not relaxation (Figure 3.12).

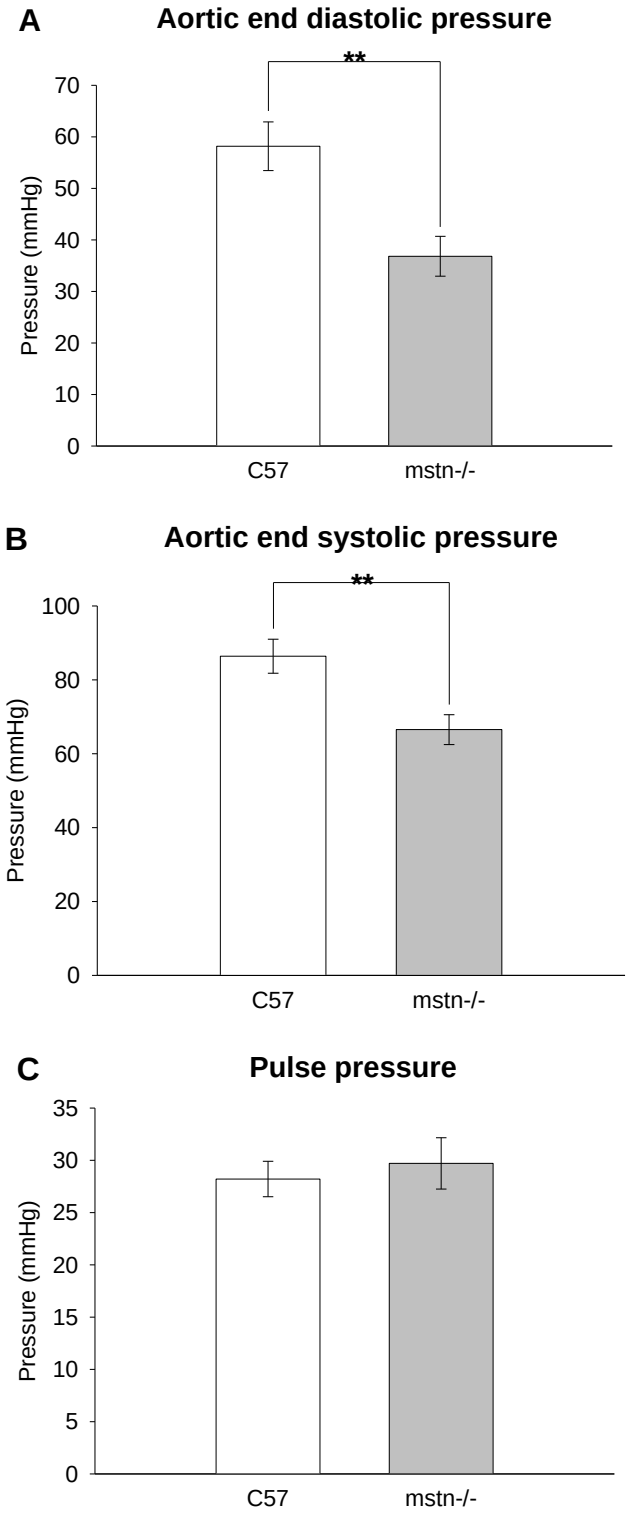


Figure 3.11 Arterial pressure development.

EDP (A) and ESP (B) were significantly decreased in the aorta with myostatin absence, however this did not translate to an altered pulse pressure (C). (**P<0.01)

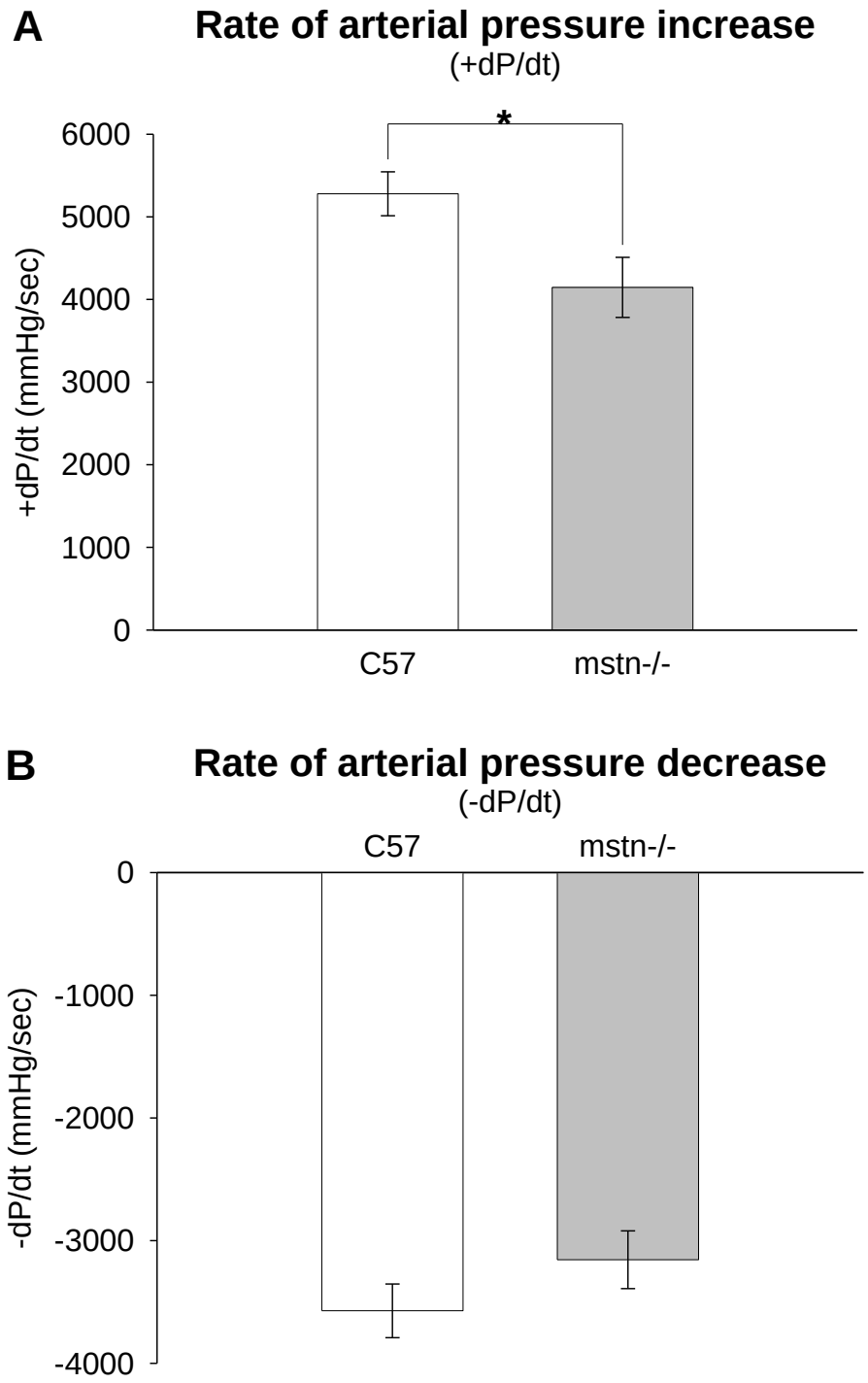


Figure 3.12 Rates of arterial pressure change during Millar catheterisation.

A: Rate of arterial contraction, as indicated by +dP/dt, was decreased by myostatin removal. **B:** Rate of arterial relaxation, as indicated by –dP/dt, did not differ between groups. (*P<0.05)

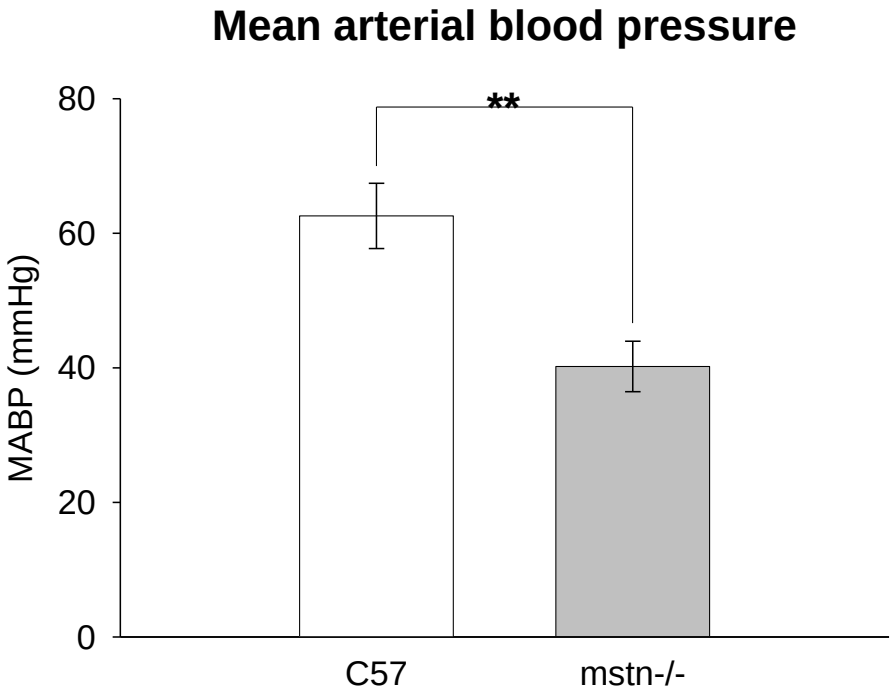


Figure 3.13 Mean arterial blood pressure measured during Millar catheterisation.

MABP was significantly decreased by approximately 20mmHg in myostatin-null mice compared to controls. (**P<0.01)

3.4.2 Ventricular measurements

Ventricular measurements of developed pressures obtained by Millar catheter were conducted to determine the effect of the absence of myostatin from the myocardium. The only significant difference between C57 and *Mstn*$^{-/-}$ groups was a decreased EDP observed in the *Mstn*$^{-/-}$ group. Unlike arterial data, this was not accompanied by a decrease in ESP (Figures 3.14A & B). Developed pressure was unaltered by myostatin absence (Figure 3.14C). Rates of ventricular pressure change were not affected by the absence of myostatin (Figure 3.15).

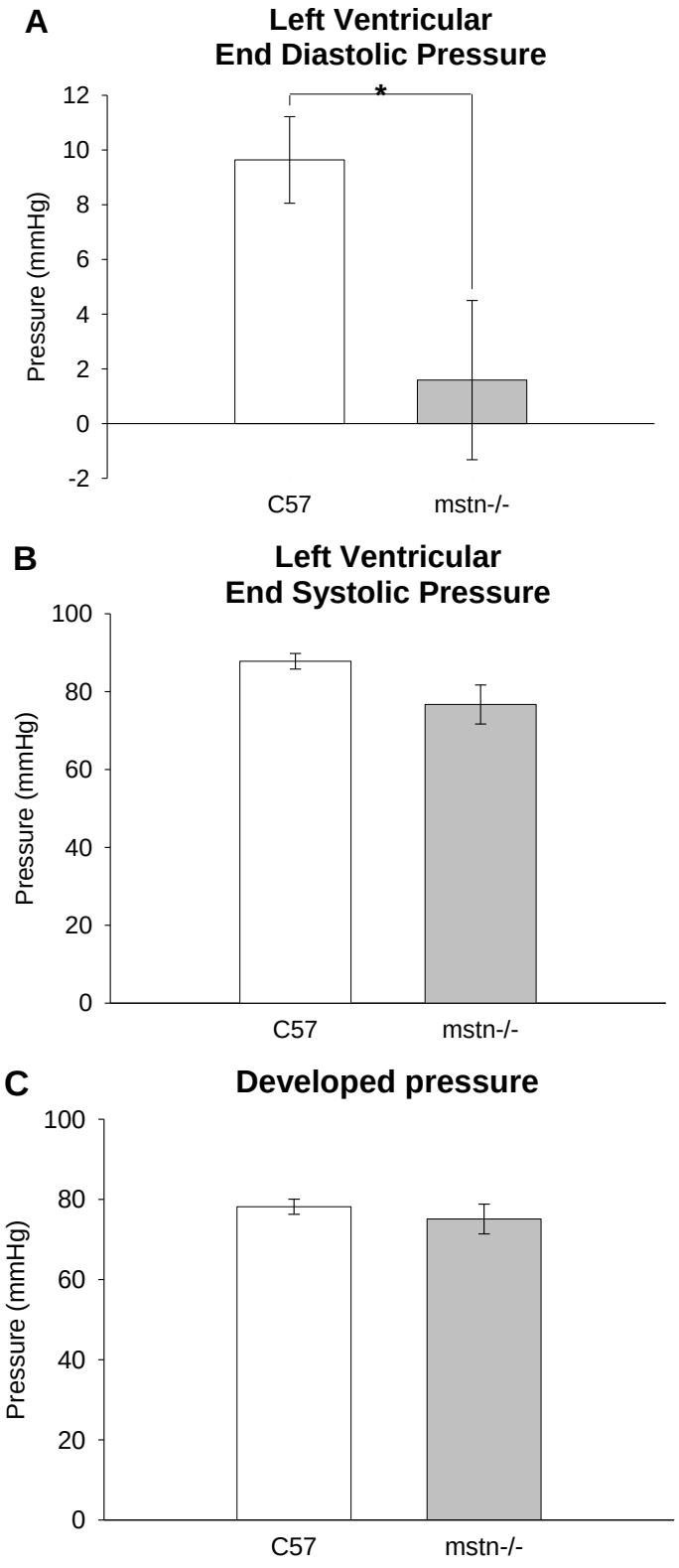


Figure 3.15 Ventricular pressure development during Millar catheterisation.

Mstn$^{-/-}$ had a significantly lower ventricular EDP (**A**) but similar ESP (**B**). **C:** The observed change in EDP did not translate to a significantly altered developed pressure. (*P<0.05)

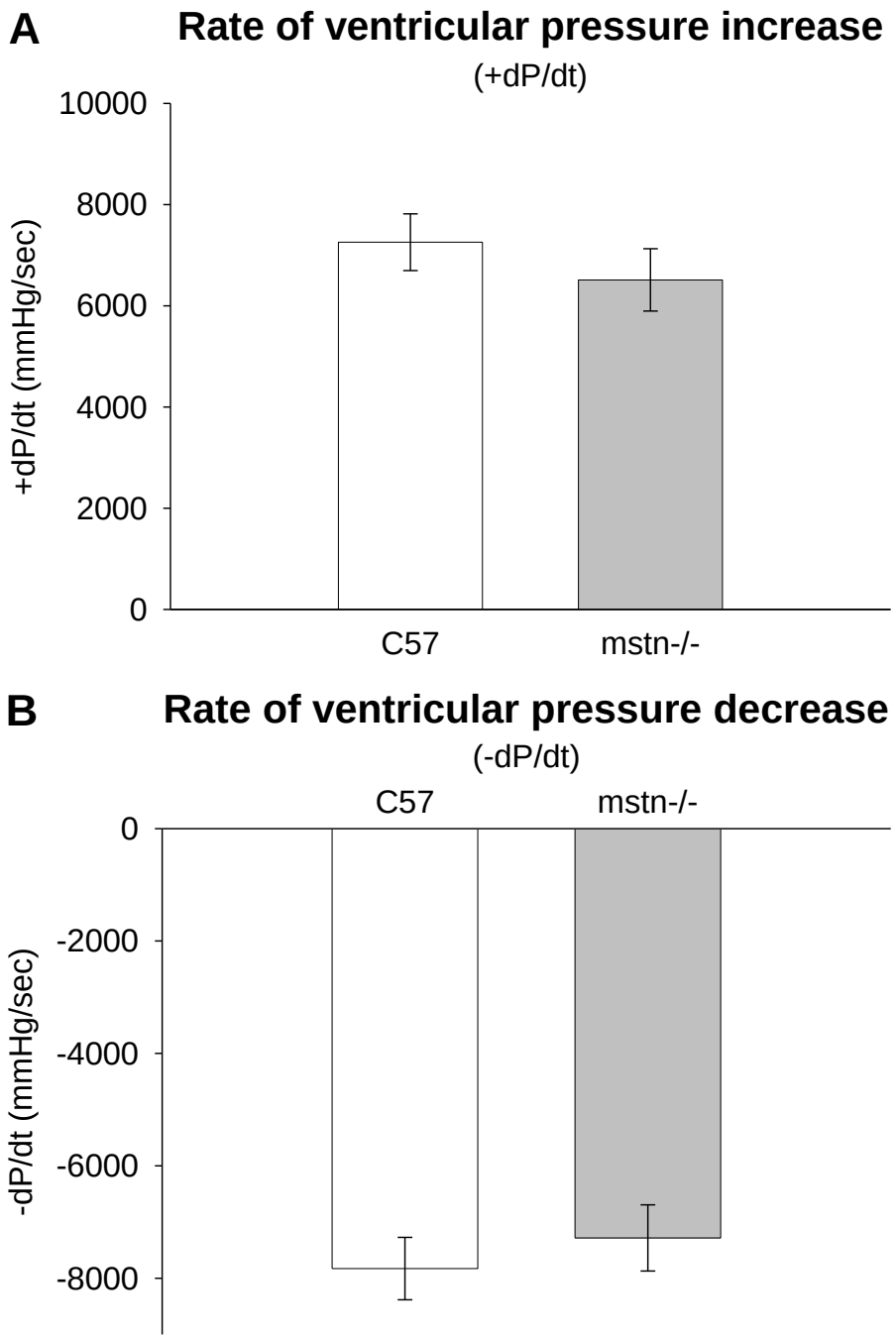


Figure 3.16 Rates of ventricular pressure change measured during Millar catheterisation.

Rates of contraction (A: +dP/dt) and relaxation (B: -dP/dt) were not altered by myostatin absence.

3.5 Discussion

Myostatin has been studied extensively as a negative regulator of skeletal muscle growth but there has been little research into the role of this protein in the heart, particularly in the setting of muscular dystrophy. The current study used 15-18 month old murine models of DMD (*mdx*) and wild-type (C57) animals that were bred with myostatin KO animals to produce *mdx*/myostatin-null (2KO) and C57/myostatin-null ($Mstn^{-/-}$) models. Investigations determined whether the absence of myostatin affected cardiac morphometry, function and fibrosis in both dystrophic and non-dystrophic animals.

Cardiac hypertrophy

The absence of myostatin was associated with increases in cardiac muscle mass and body weight. Myostatin KO genotypes ($Mstn^{-/-}$ and 2KO) had significantly greater heart masses (normalised to body weight) compared to C57 and *mdx* controls, indicating that absence of myostatin resulted in cardiac hypertrophy (Figure 3.3).

In contrast to this finding, an early study into the cardiac effects of myostatin absence in dystrophic hearts concluded that myostatin played little or no role in the regulation of cardiomyofibre growth or function (CohnLiang, et al. 2007). They found no change in left ventricular (LV) mass, cardiomyocyte cross-sectional area or parameters of cardiac function such as systolic and diastolic LV dimensions, intra-ventricular septum dimension or ejection fraction (CohnLiang, et al. 2007). However, more recent studies have found that myostatin plays a significant role in the regulation of heart growth and repair. *In vitro* work by Morissette et al. (2006) on non-dystrophic cardiac myocytes found that inhibition of myostatin increased cell size and protein synthesis. These findings have been supported by Rodgers et al. (2009a) who reported myostatin KO mice exhibited physiological, eccentric cardiac hypertrophy accompanied by improved cardiac performance. These results correlate with those of the current study.

Full understanding of the pathways involved in myostatin signalling is yet to be achieved. Initial theories explaining why the inhibition of myostatin results in muscular hypertrophy suggested that myostatin held muscle progenitor cells, such as satellite cells, in a quiescent state and that removal of the protein allowed activation of these cells and entry into the cell cycle (McCroskery et al. 2003; Wagner et al. 2005). In contrast, recent work by Amthor et al. (2009) reported that muscular hypertrophy associated with myostatin inhibition may not require increased precursor cell activity. They found that muscle fibres did not have an increased number of myonuclei or satellite cells and that myostatin did not have a significant effect on satellite cell proliferation *in vitro*. In addition, they found that expression of myostatin receptors in postnatal satellite cells dropped below detectable limits (Amthor et al. 2009). These findings suggest that regulation of muscle fibre growth by myostatin is more complex than first suspected.

Myostatin inhibited activation of cardiac growth factors such as p38 mitogen-activated protein (MAP) kinase, Akt, glycogen synthase kinase (GSK3β) and insulin-like growth factor-1 (IGF-1) and inhibition or absence of myostatin allowed greater activation of these factors and consequent cardiac hypertrophy (Morissette et al. 2006; Morissette et al. 2009; Rodgers et al. 2009a). The action of myostatin is likely to lie upstream of p38 and Akt as expression of MKK, a preferential activator of p38, rescued Akt activation in myostatin-expressing cells (Morissette et al. 2006). These findings demonstrate that not only is myostatin an important mediator of cardiac growth, but that the influence of this protein is dynamic.

Cardiac function

Cardiac function was assessed by two techniques; a Millar catheter was used in control strains (C57 and *Mstn*$^{-/-}$) to measure *in vivo* pressure development in the aortic arch and left ventricle, and the Langendorff isolated heart technique was used in all strains to measure *ex vivo* left ventricular pressure development.

Ex vivo assessment of cardiac function

Ex vivo assessment of cardiac function was conducted using the Langendorff isolated heart technique. These experiments used both dystrophic (*mdx* and 2KO) and non-dystrophic (C57 and *Mstn*$^{-/-}$) murine models. The absence of myostatin did not alter coronary flow (Figure 3.4) suggesting that while myostatin-null hearts were larger, the heart's blood flow requirements were unaltered. The slight rise in coronary flow at a fixed perfusion pressure in the myostatin KO hearts may also argue against a significant impact of myostatin on vascularity or capillary density, countering suggestions that myostatin modifies aortic pressure through shifts in the growth of vascular beds. It is important to note that there was no significant increase in the rate of LV contraction (Figure 3.6B) observed in 2KO, nor was there an alteration in the rate of LV relaxation when compared to *mdx* (Figure 3.6A). Also, the absence of myostatin did not alter developed pressure in non-dystrophic genotypes but rescued this parameter in the dystrophic genotype (2KO) (Figure 3.5). Taken together, these results indicate that the genetic absence of myostatin produced a functional rescue of the dystrophic heart that may have been, at least in part, due to physiological cardiac hypertrophy.

The literature is currently divided on the role of myostatin in the heart as some researchers have reported myostatin had no effect on cardiac function (CohnLiang, et al. 2007) while others have demonstrated improved function in myostatin-null cardiac cells (Rodgers et al. 2009a). Functional measurements by Cohn et al. (2007) found the genetic absence of myostatin was not associated with changes in cardiac function, however this paper also reported no functional differences between dystrophic and wild-type hearts. Quinlan et al. (2004) found that *mdx* mice had developed dilated cardiomyopathy by the age of forty-two weeks and exhibited decreased fractional shortening and heart rate and increased LV EDP, LV ESP and LV mass. Using cultured H9C2 cells, Rodgers et al. (2009a) showed that myostatin-null cells had significantly larger calcium transients and enhanced cell shortening. They concluded that myostatin decreased excitation-contraction coupling and that myostatin absence resulted in cardiac

hypertrophy, increased calcium release from the sarcoplasmic reticulum and ultimately larger calcium transients and stronger contractions.

Cardiac fibrosis

There was an approximate two-fold increase in cardiac fibrosis associated with the genetic disruption of myostatin (Figure 3.8). In line with previous findings of Quinlan et al. (2004), there was a marked increase in cardiac fibrosis observed in dystrophic genotypes compared to non-dystrophic genotypes. However, the increase in fibrosis associated with myostatin absence was surprising, especially considering the functional rescue observed in 2KO mice. This observation suggests the possibility that the impaired function observed in dystrophic hearts may not be as influenced by fibrosis as previously theorised and may be more associated with processes involved in volume overload and dilated cardiomyopathy. This is supported by a similar finding of improved cardiac function despite increased cardiac fibrosis in pirfenidone-treated *mdx* mice (Van Erp et al. 2006).

The functional role of myostatin in fibrosis is still being explored. One study using prolonged cold ischaemia to induce myofibrosis in rat abdominal aorta found that increases in fibrosis were associated with a down-regulation of myostatin (Gao et al. 2007). Another study found that systemic gene delivery by adeno-associated virus serotype 8 of the myostatin propeptide, known to inhibit the actions of myostatin, decreased fibrosis in skeletal muscle (Qiao et al. 2008). Conversely, myostatin has also been shown to be profibrotic in a rat model of Peyronie's disease where it induced fibrotic plaque formation in both smooth and skeletal muscles (Cantini et al. 2008). In this study, myostatin had a three-fold overexpression in myofibroblasts, increasing their number and collagen synthesis.

Expression levels of TNF-α and TGF-β, two cytokines involved in fibrosis, were also investigated. No difference in TNF-α expression was found in heart muscle of either dystrophic or non-dystrophic mice (Figure 3.9). TGF-β expression was increased in *mdx* compared to non-dystrophic strains but not 2KO (Figure 3.10). This study failed to correlate any relationship of

these cytokines, the absence of myostatin and the development of cardiac fibrosis. The role of TGF is complex and other studies have shown there is a non-linear relationship between this cytokine and the progression of fibrosis (Gosselin et al. 2004; Van Erp et al. 2006). Given that fibrosis is a net result of collagen deposition and breakdown, TGF mRNA expression may be more significant in earlier stages of active collagen synthesis. In the *mdx* diaphragm, TGF-β mRNA levels initially peaked and then declined (Gosselin et al. 2004), and in DMD boys, skeletal muscle levels of TGF-β were not directly proportional to the degree of fibrosis.

In vivo assessment of cardiac function

Mice on a dystrophic genetic background (*mdx* and 2KO) were not able to tolerate Millar catheter experiments. It was suspected that they rapidly developed severe orthostatic hypotension and exhibited a Cheyne-Stokes like breathing pattern that made measurement of thoracic pressures impossible. Reasons for orthostatic hypotension are unlikely to be related to changes in muscle mass and associated peripheral resistance changes but more likely due to an age-related reduction in autonomic function in these old mice. In addition, there is evidence that DMD patients also have an elevated risk of having abnormal responses to general anaesthesia (Larsen et al. 1989). Cases of malignant hyperthermia-like reactions to general anaesthetics in muscular dystrophy patients were first reviewed by Cobham and Davis (1964). Since then, research has identified that not only is the dystrophic plasma membrane vulnerable to anaesthetics but that hallmarks of dystrophinopathy such as deranged calcium homoeostasis, diminished NO availability and an increased susceptibility to ROS are major contributors to these reactions (Endo et al. 1983; Farrell 1994; Haycock et al. 1998). These mice were therefore reserved for Langendorff experiments. As a result, data gathered from non-dystrophic genotypes (C57 and *Mstn*$^{-/-}$) are presented and represent the response to myostatin absence in otherwise healthy mice.

The genetic absence of myostatin was associated with decreased mean arterial blood pressure (MABP) (Figure 3.13), verified by decreased arterial

EDP and ESP (Figure 3.11). Ventricular measurements mirrored these findings with decreases in EDP and ESP observed (Figure 3.15). Heart rate, pulse pressure and rate of arterial pressure decrease were unaltered, as were ventricular measurements of developed pressure and rates of ventricular contraction and relaxation (as indicated by ±dP/dt).

Very little research has been conducted on the role of myostatin in vascular smooth muscle. Possible mechanisms for the observed lowering of MABP may include myostatin inhibition of atrial natriuretic peptide (ANP), alterations in capillary density (Rehfeldt et al. 2005) and changes in vessel wall myofibrosis (Gao et al. 2007). ANP influences MABP as it acts as a vasodilator and has a strong influence on fluid homoeostasis. Therefore the genetic absence of myostatin may decrease MABP by removal of ANP inhibition allowing increased loss of fluid via the renal system and increased vasodilatation. Peripheral resistance, and therefore MABP, is also influenced by vessel length. The genetic removal of myostatin has been associated with decreased capillary density suggesting that, although there is an increased muscle mass linked with myostatin inhibition (Rehfeldt et al. 2005), this may not result in a significantly increased vascular bed. Arterial measurements also indicated that the rate of blood pressure increase (arterial +dP/dt) was lower in *Mstn*$^{-/-}$ mice (Figure 3.12A). One possible mechanism is increased fibrosis of the arterial wall (Gao et al. 2007) having a negative impact on vessel compliance that resulted in prolonged vasodilation. Thus the surge in blood flow during systole into an already dilated vessel is not translated into a sharp rise in blood pressure.

While the cause of intolerance to anaesthesia in 2KO and *mdx* could not be determined with certainty, it is likely to be linked to lowered blood pressure, which was also observed in the non-dystrophic mice lacking myostatin. Furthermore, the advanced age of these mice may also have contributed as their baroreflex may have been blunted. Placing these mice in dorsal recumbency and surgical stimulation of their great vessels may have elicited a state of haemodynamic crisis and agonal breathing. *Ex vivo* experiments showed normal coronary flow in both *mdx* and 2KO mice and improved left

ventricular function in 2KO mice, suggesting that cardiac insufficiency was not implicated in these deaths.

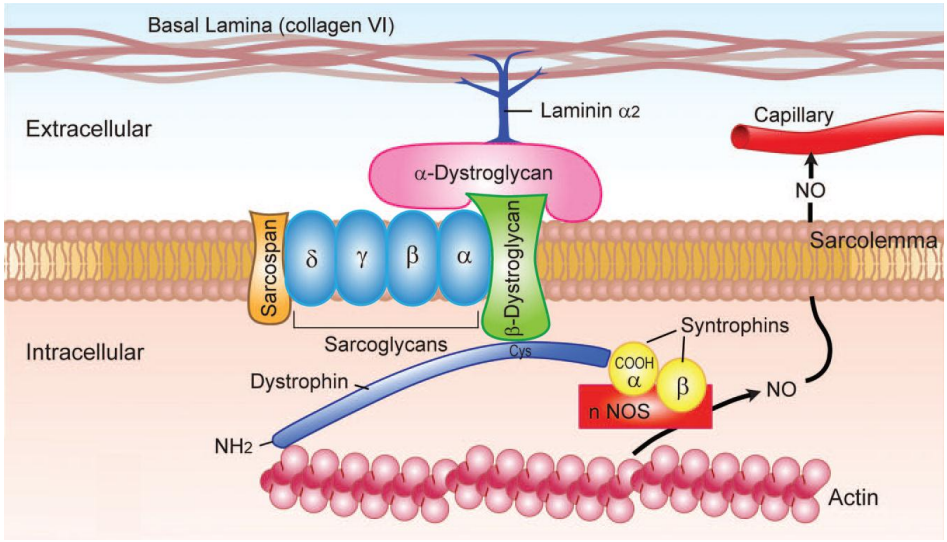
This study was limited by a number of factors. Experimental numbers were low due to poor breeding characteristics of myostatin KO animals. Increasing experimental numbers would allow clarification of some statistically marginal data; for example, greater statistical power may determine whether 1% L-arginine supplementation produces fibrosis-limiting results in the heart (Figure 4.17). Low animal numbers also necessitated the use of both male and female mice, a factor that may also confound interpretation of data. Also, further optimisation of DNA extraction and PCR genotyping protocols may remove and non-specific amplification in wildtype samples.

In summary, the genetic absence of myostatin was associated with cardiac hypertrophy, decreased MABP, increased cardiac fibrosis and improved cardiac function. Improvements in cardiac function seem to be linked to physiological cardiac hypertrophy and possibly augmented myocardial calcium handling, while decreased MABP may have been influenced by the removal of ANP inhibition, changes in capillary density and increased arterial wall myofibrosis. This study demonstrates the functional rescue of LVDP in isolated dystrophic hearts by myostatin inhibition suggesting this avenue is worth further investigation as it may provide a potential treatment option for DMD-related cardiomyopathy. The evidence showing improved cardiac function is tempered by an increase in fibrosis in double knockout mice and some observed anaesthetic difficulties. These issues need to be further evaluated in the future, particularly as cardiac fibrosis contributes greatly to the extent and clinical manifestation of cardiomyopathy in boys with DMD.

Chapter 4: L-Arginine dose optimisation in *mdx* mice

4.1 Introduction

Reduced availability of NO due to disruption of the DGC at the sarcolemma is a significant consequence of dystrophin loss observed in DMD (Brenman et al. 1995; Chang et al. 1996; Bia et al. 1999). NO is synthesised by three isoforms of nitric oxide synthase (NOS). Neuronal NOS (nNOS) is the primary source of NO in muscle tissue, although NO derived from endothelial NOS (eNOS) also plays important roles in influencing myocellular calcium dynamics by altering the open-probability of cardiac ryanodine receptors (RyR2) and sarcoplasmic reticulum calcium ATPase (SERCA) (Schmidt et al. 1992; Nishida & Ortiz de Montellano 1998; Petroff et al. 2001). nNOS is normally localised to the sarcolemma through association to DGC members α-dystrobrevin, α1-syntrophin and dystrophin (Brenman et al. 1996; Grady et al. 1999; Rando 2001a) (Figure 4.1). Destabilisation of this complex results in translocation and reduced gene expression of nNOS, consequently reducing nNOS to levels insufficient to meet cellular demands for NO (Chang et al. 1996; Crosbie 2001).



(Verhaert et al. 2011)

Figure 4.1 Localisation of nNOS to the DGC.

Diagram describing the sarcolemmal localisation of nNOS with the DGC components α1-syntrophin and dystrophin.

NO is produced in a reaction catalysed by isoforms of NOS using L-arginine (2-amino-5-guanidino-pentanoic acid) as the substrate. Oxidisation of the guanidino group (Figure 4.2) consumes five electrons and forms NO and L-citrulline (Ignarro 1990).

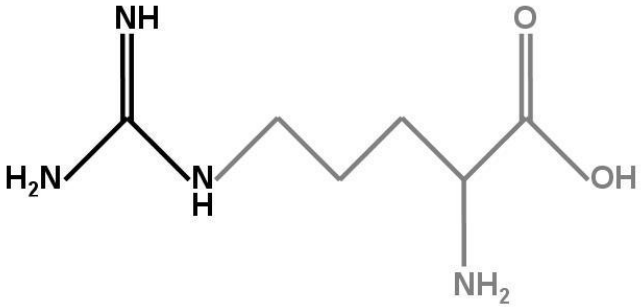
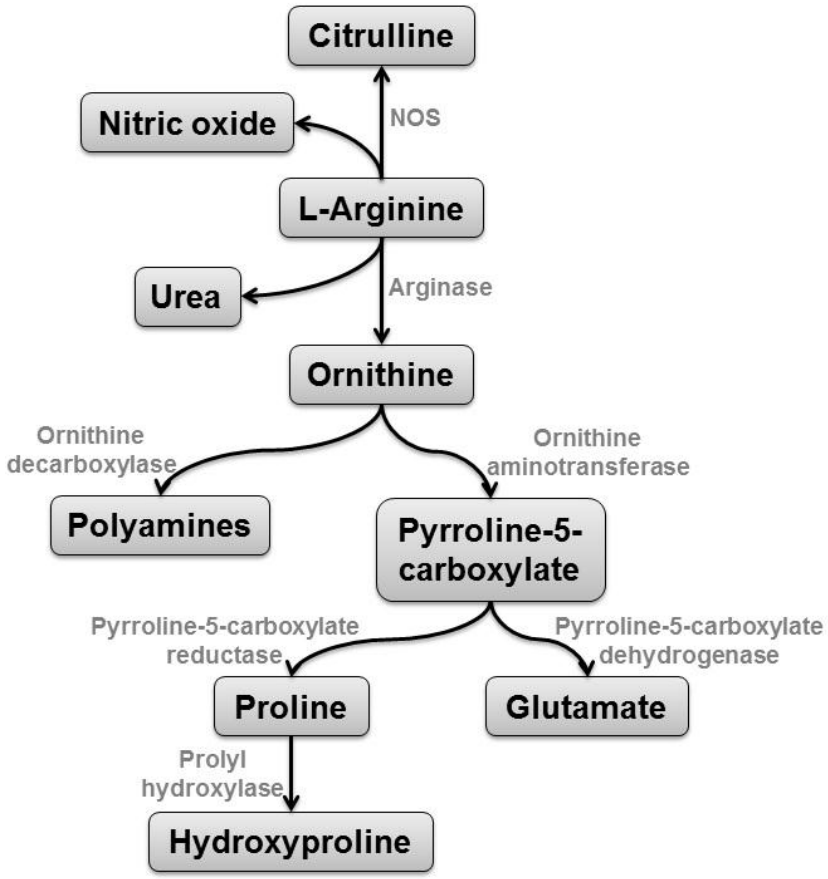


Figure 4.2 Chemical structure of L-arginine, highlighting the guanidine group (shown in black).

L-Arginine is a conditionally essential amino acid that is required for growth. Depending on energy requirements, it may be either metabolised to support glucose synthesis or catabolised to produce energy. L-Arginine is also involved in the synthesis of L-ornithine, L-glutamate and polyamines as well as protein degradation through the ubiquitin-proteosome pathway (Wu & Morris 1998). The L-arginine-NO pathway plays significant roles in inflammation, tissue repair and fibrosis (Mane et al. 2001; Wehling et al. 2001). It has been postulated that supplementation of L-arginine and subsequent enhancement of NO production may be beneficial in dystrophin-deficient muscle (Chaubourt et al. 1999; Barton et al. 2005; Voisin et al. 2005; Hnia et al. 2008). However, there is a need for accurate dose optimisation as elevated concentrations of L-arginine may cause hypotension, tachycardia and increased release of insulin, glucagon, prolactin and growth hormone (Goumas et al. 2001; Todorovic et al. 2001). Excessive concentrations of L-arginine have also been linked to increased fibrosis through downstream production of proline through the pathway illustrated in Figure 4.3 (Mori & Gotoh 2004).



(adapted from Mori & Gotoh 2004)

Figure 4.3 Profibrotic pathway associated with excess L-arginine.

Arginase acts upon L-arginine to produce ornithine, which is later converted to proline. Proline is converted to hydroxyproline, a major component of collagen.

Studies investigating the usefulness of dietary supplementation of L-arginine in *mdx* mice have reported encouraging results in skeletal muscle, including decreased contraction-induced muscle damage, decreased serum creatine kinase (CK) levels and inflammation, enhanced muscle regeneration, translocation of utrophin (a structural analogue of dystrophin) to the sarcolemma and decreased Evans blue dye uptake (Chaubourt et al. 1999; Barton et al. 2005; Voisin et al. 2005; Hnia et al. 2008). In addition, Archer et al. (2006) reported improved skeletal muscle function (by improved distance running) and reduced contraction-induced damage in *mdx* mice with combined L-arginine and deflazacort therapy despite an increase in Evans blue penetration immediately post-exercise.

Until recently, little work has been conducted exploring the cardiac consequences of L-arginine supplementation in muscular dystrophy. Marques et al. (2010) used long-term (15 months) L-arginine treatment in *mdx* mice and found that, while there was a decrease in inflammatory cell density, there was no alteration in cardiac fibrosis. Another study showed that six months of arginine butyrate treatment (250mg/kg/day delivered by intraperitoneal injection) improved skeletal muscle parameters but did not result in benefits to cardiac function or fibrosis (Guerron et al. 2010). A further study by Wehling-Henricks et al. (2010) found long term L-arginine treatment (5mg/mL in drinking water from three weeks to 18 months of age) did not increase cardiac sarcolemmal utrophin or cardiac function, and, in fact exacerbated both skeletal and cardiac fibrosis.

It was hypothesised that increasing the availability of the NOS substrate, L-arginine, would result in increased concentrations of NO allowing the normalisation of NO-dependent processes and decreased cardiac pathology. However, Van Erp (2005) demonstrated that while 10% w/v L-arginine administered in drinking water improved cardiac contractility, it also significantly increased cardiac fibrosis. This evidence, coupled with the involvement of L-arginine in profibrotic pathways, warranted and highlighted the need to assess dose-related effects of L-arginine supplementation on ventricular function, arterial pressure development and cardiac muscle fibrosis.

This study tested this hypothesis in the dystrophic heart by treating *mdx* mice for a period of three months with doses of 1%, 2% and 5% w/v L-arginine in drinking water. Cardiac effects were examined using a Millar catheter to measure *in vivo* cardiac function, the Langendorff isolated heart technique to measure *ex vivo* cardiac function and picrosirius red staining to assess cardiac fibrosis.

4.2 Materials and methods

Doses of 1%, 2% and 5% L-arginine in drinking water were administered *ad libitum* to *mdx* mice over a period of three months and compared to control data obtained from age and sex-matched *mdx* and C57 mice. Mice were anaesthetised using 50/20 mg/kg ip ketamine/xylazine and *in vivo* cardiac function was assessed using a Millar catheter positioned within the left ventricle, with arterial pressure development measured by altering the position of the Millar catheter within the aorta. Mice were then euthanised and their hearts suspended on a Langendorff apparatus where *ex vivo* assessment of cardiac function was conducted. Hearts and tibialis anterior (TA) muscles were fixed in Telly's fixative and modified Bouin's solution. Samples were preserved in 70% ethanol for later sectioning and histological assessment of cardiac collagen content by picrosirius red staining. Changes in cardiac wet weight were also determined. These techniques have been detailed in Chapter 2 and their use in the current study along with experimental numbers, summarised in Figure 4.4.

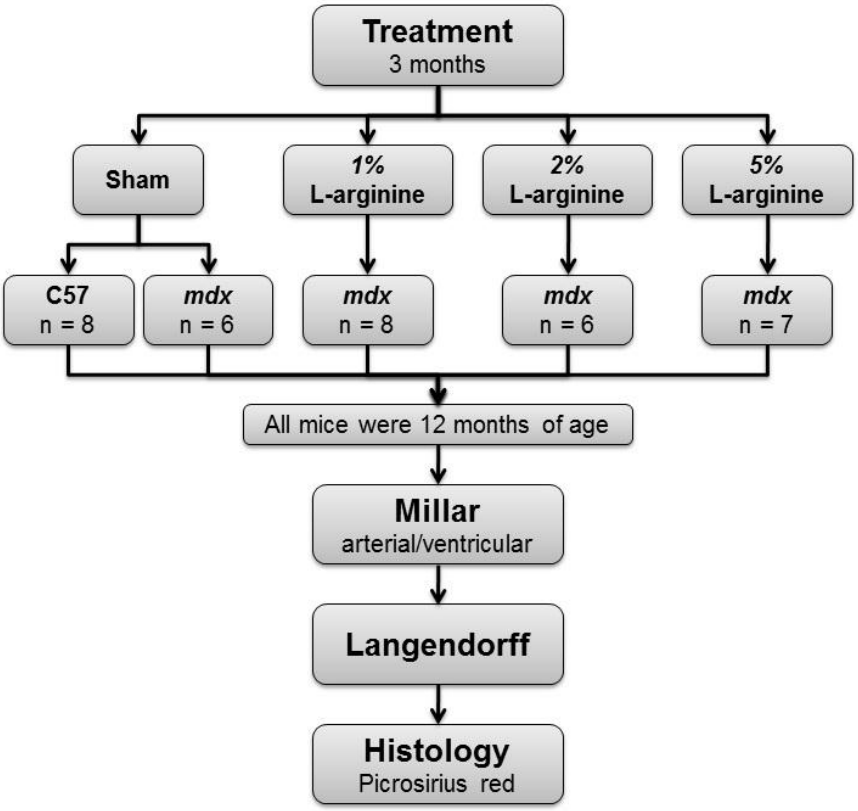


Figure 4.4 Summary of techniques and mouse numbers used in Chapter 4.

4.3 Results

4.3.1 Dosing

Water consumption was monitored for the final fifty-three days of treatment to record the relative amounts of L-arginine each group was receiving. Groups were separated into different cages containing ten mice and the drug was administered in drinking water. Data has been represented in Table 4.1 as water consumed relative to each control and average values of mL water/day/mouse and mg L-arginine/day/mouse.

Table 4.1 Average water consumption and amount of L-arginine received per mouse.

Group	Total volume consumed (mL)	Relative volume consumed to C57	Relative volume consumed to *mdx*	mL of water /mouse/day	g of L-arginine /mouse/day
C57	2097	1	0.88	3.96	0
mdx	2378	1.13	1	4.49	0
***mdx* 1% L-arginine**	3306	1.58	1.39	6.24	0.06
***mdx* 2% L-arginine**	2828	1.35	1.19	5.34	0.11
***mdx* 5% L-arginine**	2699	1.29	1.13	5.09	0.25

Table 4.1 shows water consumption and consequent dose of L-arginine received for each of the treated groups. All *mdx* groups had higher rates of water consumption compared to C57. Calculation of average amounts of drug per mouse showed that experimental groups received the correct dosing.

4.3.2 Morphometry

All *mdx* groups except the sham *mdx* group were significantly heavier than C57 (Figure 4.5A). Sham *mdx* and C57 did not differ in either heart or body weight (Figure 4.5B). All *mdx* groups had significantly greater TA weights compared to C57; however L-arginine treatment in *mdx* mice did not alter TA weight at any of the doses (Figure 4.5C). These data indicate that *mdx* mice underwent skeletal muscle hypertrophy but not cardiac hypertrophy and that L-arginine treatment at these doses did not significantly alter muscle mass. Normalised heart weights also reflected this finding. Figure 4.6A shows heart weight normalised to body weight and showed *mdx* sham and *mdx* 5% L-arginine mice had degree of cardiac wasting proportional to their mass when compared to C57 mice. However, when heart weight was normalised to tibial length, no differences were detected (Figure 4.6B). This discrepancy is likely due to skeletal muscle hypertrophy, common in *mdx* mice at this age.

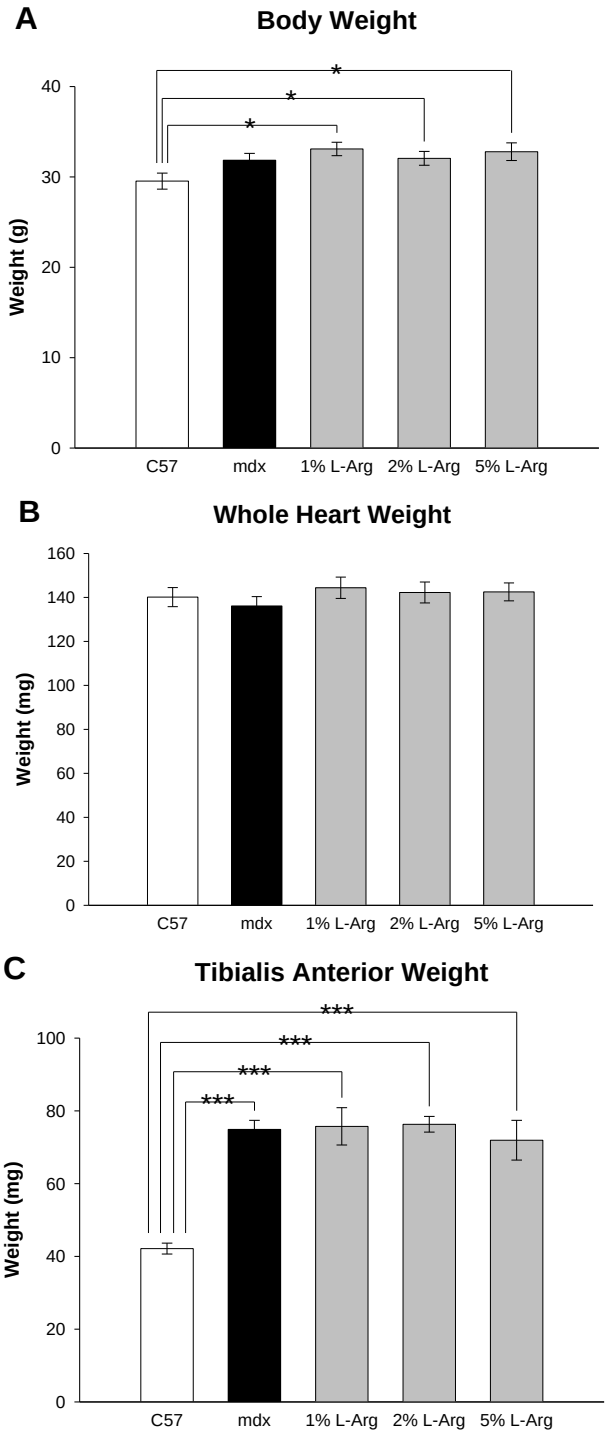


Figure 4.5 Body and TA weights.

A: There was no difference in body weight between *mdx* and C57 or treated groups, although treated *mdx* groups were significantly heavier than the C57 control group. **B:** There was no significant difference in whole heart weight between any of the groups. **C:** TA weight was increased in *mdx* and treated groups compared to C57 controls but was not altered by L-arginine supplementation in *mdx*. (*P<0.05; ***P<0.001)

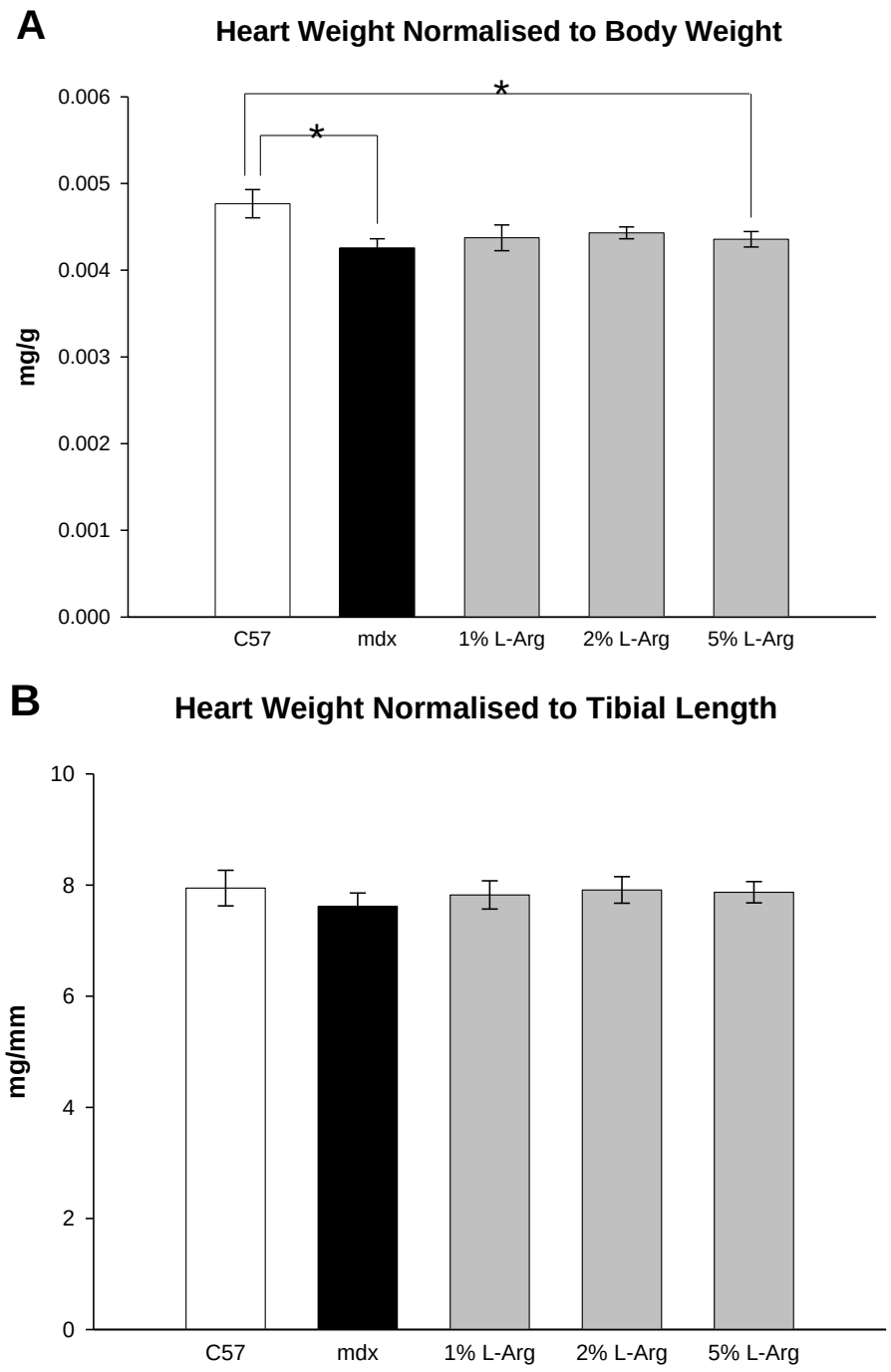


Figure 4.6 Normalised heart weight to body weight or tibial length.

Tibial length did not differ between strains or treatment groups (data not shown). **A:** Heart weight normalised to body weight indicated that C57 had greater heart weights as a proportion of body weight compared to *mdx*. **B:** Heart weight normalised to tibial length indicated that *mdx* heart weight as a proportion of body size did not differ from C57 values and that L-arginine treatment did not alter this parameter. (*P<0.05)

4.3.3 In vivo measurements

In vivo measurements of cardiac parameters were conducted using a Millar catheter positioned within either the aorta or the left ventricle. With the exception of heart rate, data from these experiments have been presented as arterial and ventricular data sets. *In vivo* heart rates reflect neurohumoral and pharmacological influences such as sympathetic nervous system activation and depth of anaesthesia. Heart rates were not different in sham *mdx* or C57 mice, nor in 1% or 5% L-arginine treated groups; however 2% L-arginine-treated *mdx* mice showed a significantly higher heart rate compared to *mdx* sham and C57 (Figure 4.7).

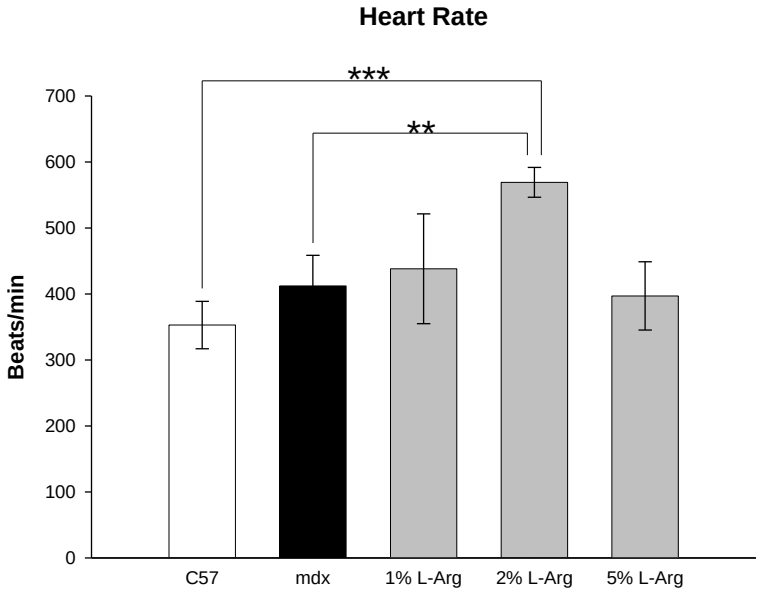


Figure 4.7 Heart rate measured by Millar catheter.

2% L-arginine treatment produced a significantly higher heart rate in *mdx* mice compared to either C57 or untreated *mdx*. 1% and 5% L-arginine treated groups did not differ from either of the control groups. (**P<0.01; ***P<0.001)

Arterial measurements

End diastolic pressure (EDP), end systolic pressure (ESP) and pulse pressure (PP) of C57 and *mdx* controls did not differ during Millar catheter arterial measurements, nor did L-arginine treatment alter these parameters from *mdx* control values under the current dosing regimen (Figure 4.8). 1% L-arginine treatment produced increased EDP and ESP but not PP compared to C57 (Figures 4.8A & B). 5% L-arginine treatment produced increased

ESP compared to C57 (Figure 4.8B). PP was increased in 2% and 5% L-arginine groups compared to C57 (Figure 4.8C).

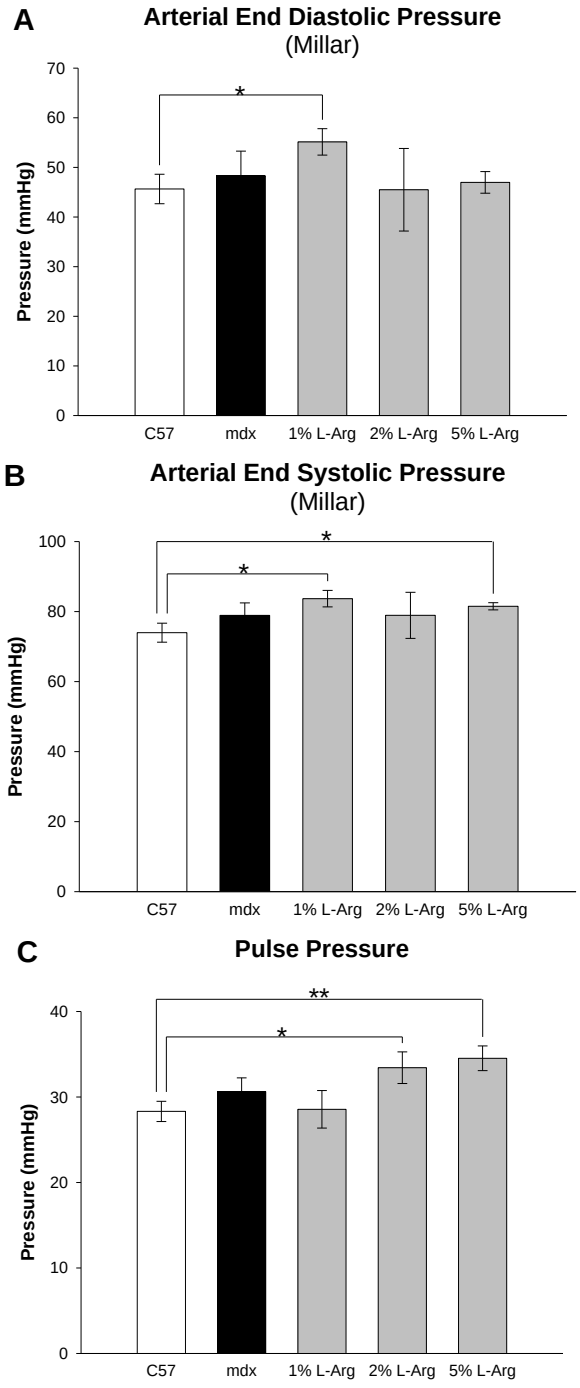


Figure 4.8 EDP, ESP and pulse pressure measured at aortic arch by Millar catheter.

A: 1% L-arginine treatment increased arterial EDP compared to C57. **B:** 1% and 5% treatment groups had increased arterial ESP compared to C57. **C:** Slight elevations in ESP for 2% and 5% L-arginine groups resulted in an increased pulse pressure compared to C57. L-arginine supplementation did not alter ESP, EDP or pulse pressure in *mdx* mice. (**P*<0.05; ***P*<0.01)

Rates of pressure change (±dP/dt) of L-arginine treatment groups were not significantly different to their *mdx* controls (Figure 4.9). The *mdx*, 2% and 5% L-arginine groups exhibited greater rates of relaxation compared to C57.

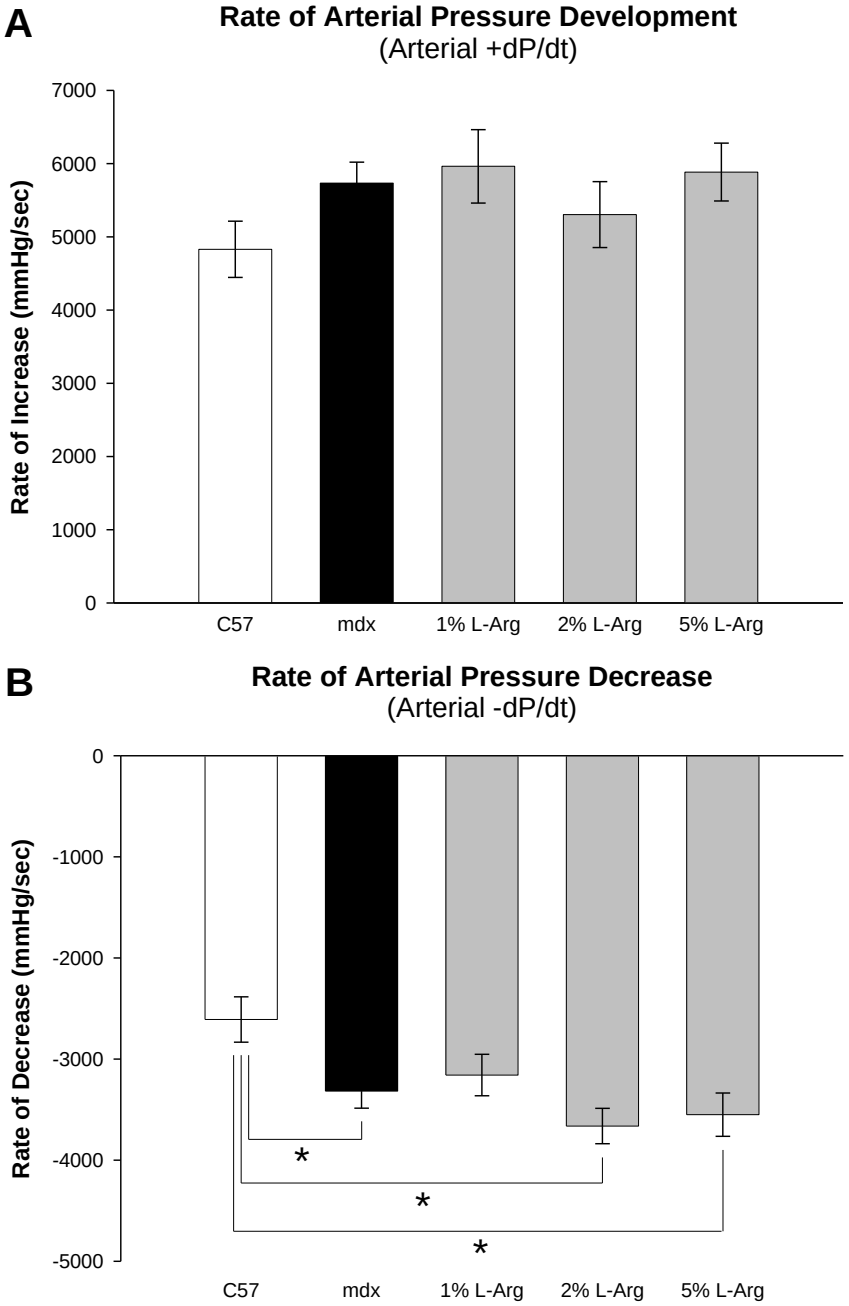


Figure 4.9 Rates of arterial blood pressure change measured by Millar catheter.

A: Rates of arterial pressure increase did not differ between any of the tested groups. **B:** With the exception of the 1% L-arginine group, *mdx* groups had greater rates of arterial pressure decline compared to C57 but not *mdx*. (*P<0.05)

Mean arterial blood pressure did not differ between C57 and *mdx* controls, nor did L-arginine treatment cause this parameter to change (Figure 4.10).

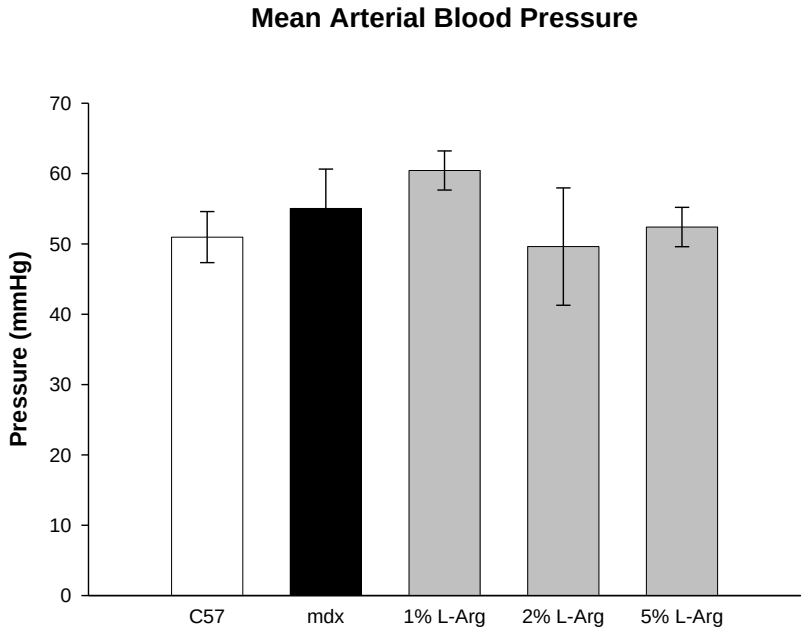


Figure 4.10 Mean arterial blood pressure (MABP).

There were no differences observed in MABP between any of the groups.

Ventricular measurements

L-arginine treatment groups did not increase mean values for EDP (Figure 4.11A). The 1% L-arginine group had significantly higher ESP compared to C57 (Figure 4.11B). Developed pressure was uniform across all groups (Figure 4.11C). EDP, ESP and developed pressure of L-arginine-treated groups did not differ from their *mdx* control. *In vivo* rates of pressure change within the left ventricle showed that 1% L-arginine treatment produced increased rates of ventricular contraction and relaxation compared to C57 but not *mdx* (Figure 4.12). L-Arginine treatment did not alter these parameters (Figure 4.12).

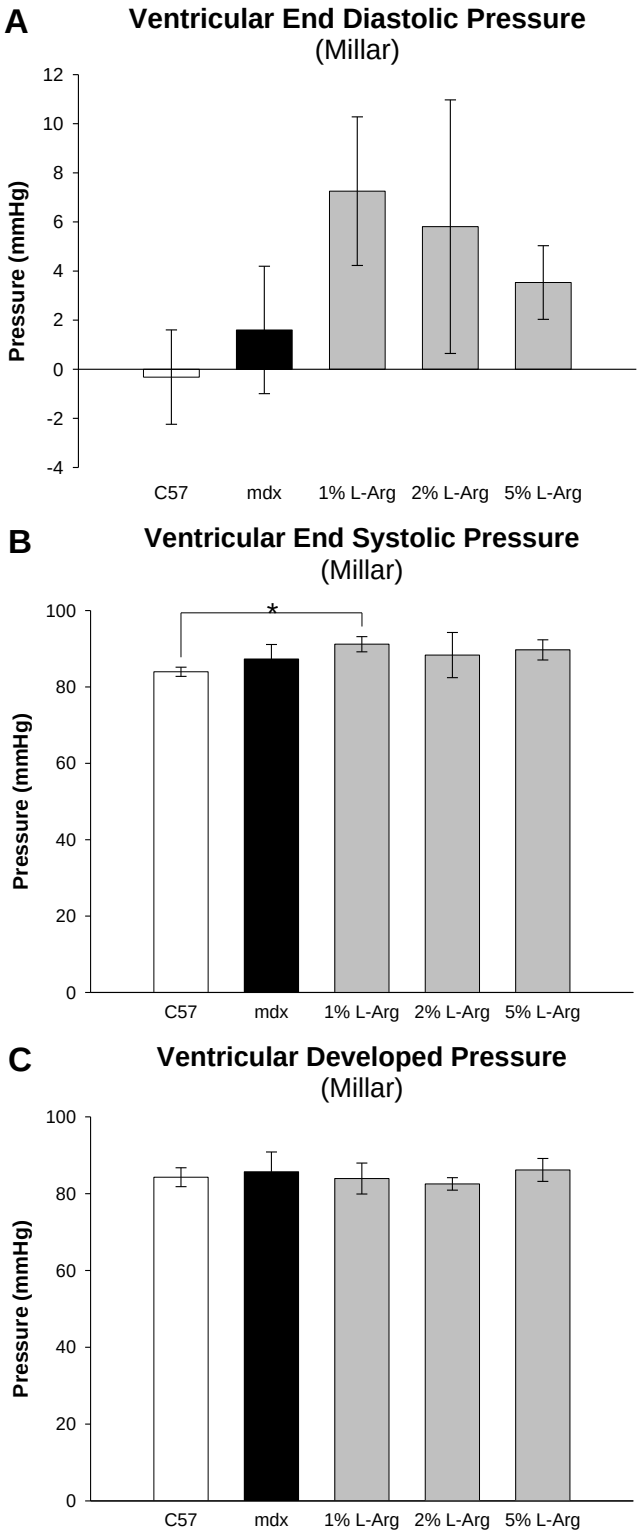


Figure 4.11 Left ventricular EDP, ESP and developed pressure measured by Millar catheter.

A: There were no differences in EDP between groups. **B:** The 1% L-arginine group had significantly greater ESP compared to C57 but not *mdx*. **C:** Developed pressure was not altered by L-arginine supplementation at these doses. (*P<0.05)

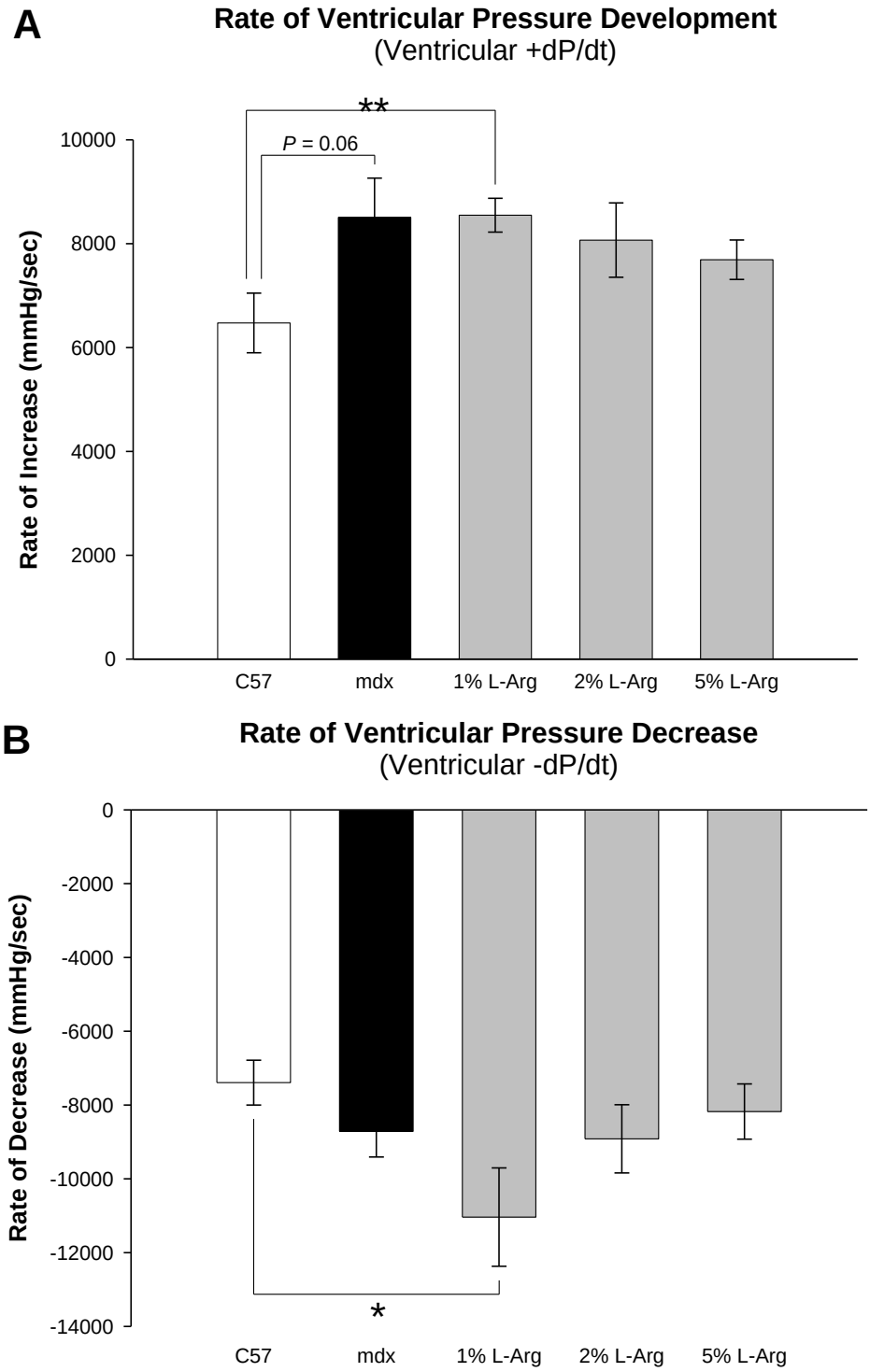


Figure 4.12 Rates of ventricular pressure change measured by Millar catheter.

A: L-arginine treated groups did not differ from the *mdx* control group. **B:** A similar trend was observed in relaxation rates with the 1% L-arginine treatment group exhibiting a greater rate compared to C57 but not *mdx* controls. 2% and 5% groups did not differ from either control. (**P*<0.05; ***P*<0.01)

4.3.4 *Ex vivo* measurements

Ex vivo measurement of cardiac function were conducted using the Langendorff isolated heart preparation. Raw ESP and EDP data have not been presented as EDP was set to a physiological level of 10mmHg and developed pressure was then used as the measure of systolic function. L-arginine treatment did not alter developed pressure compared to *mdx* (Figure 4.13). The 5% L-arginine treated group had greater developed pressure compared to C57 ($P<0.05$). Positive and negative rates of pressure change over time (±dP/dt) were not altered by L-arginine treatment (Figure 4.14). Coronary flow rates of L-arginine treated groups were unchanged (Figure 4.15).

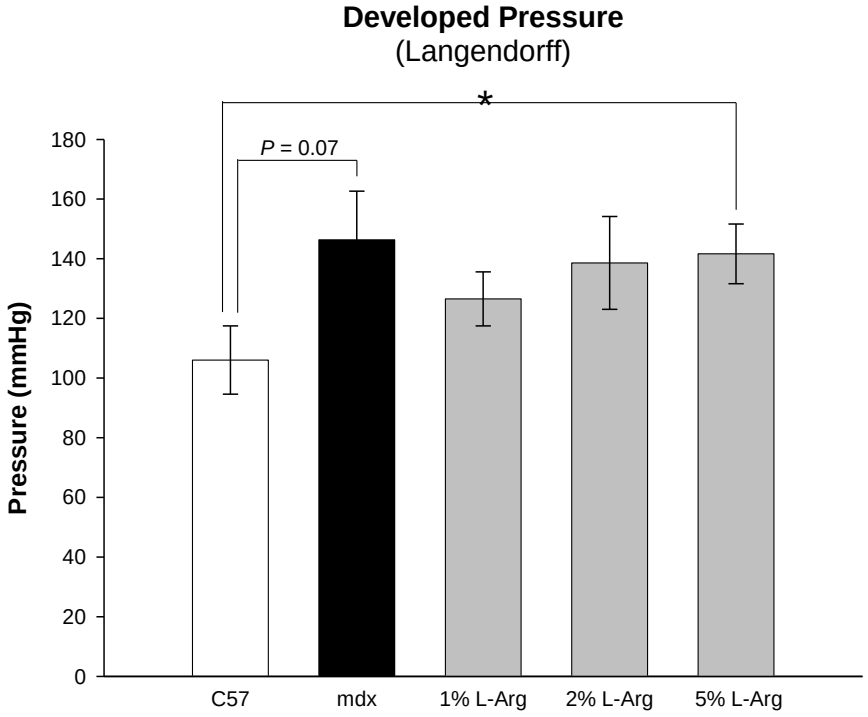


Figure 4.13 Left ventricular developed pressure (Langendorff).

End diastolic pressure was set to a physiological level (10mmHg) by adjustment of Langendorff balloon size. 5% L-arginine treatment produced significantly higher developed pressure compared to C57 but did not differ from *mdx*. Treated *mdx* groups did not differ from *mdx* control. (*$P<0.05$)

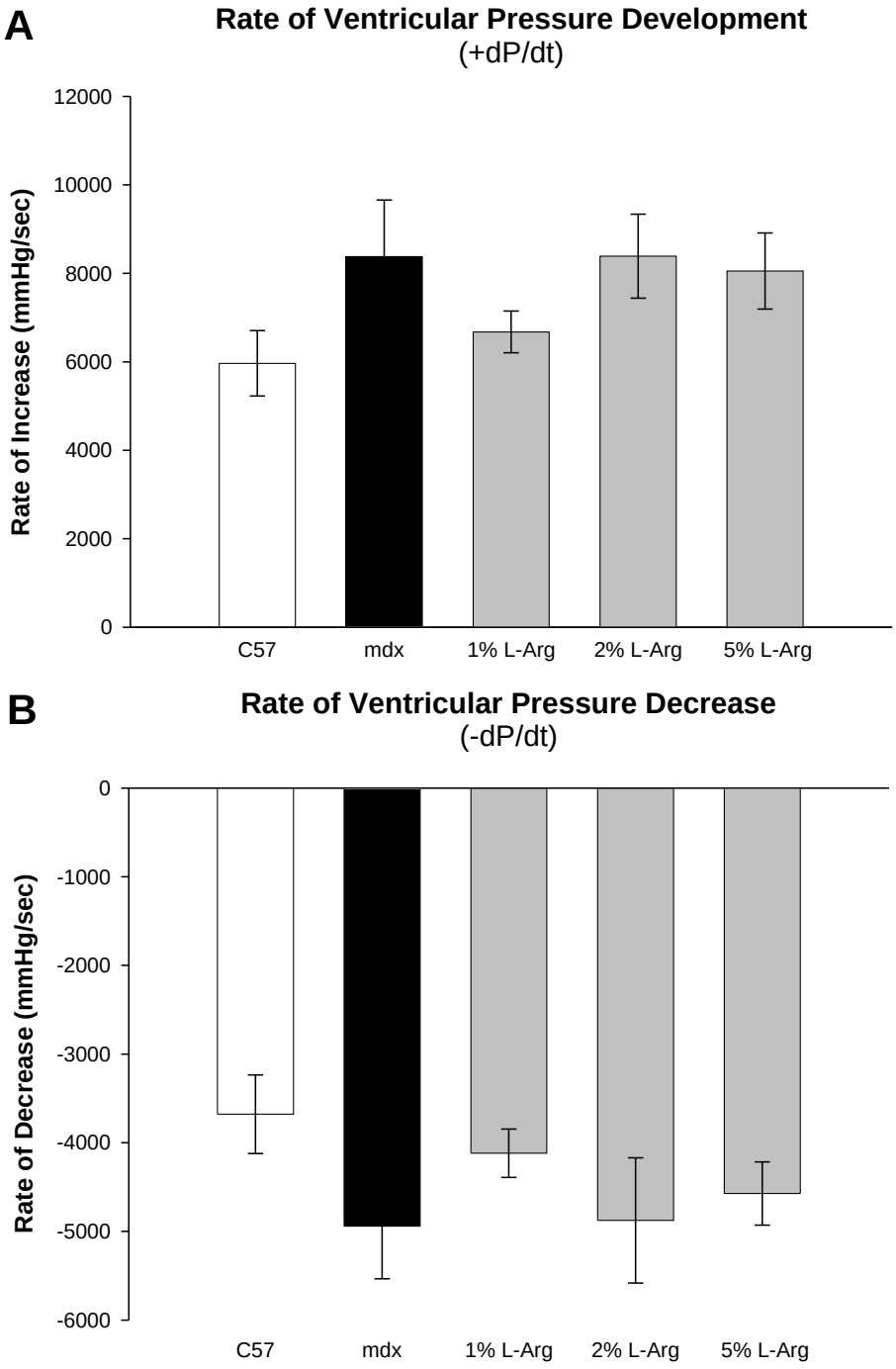


Figure 4.14 Rates of left ventricular pressure change.

Rates of cardiac contraction and relaxation, as indicated by rates of pressure increase and decrease, were not statistically different between any of the groups. L-Arginine treatment at these doses did not alter this parameter in *mdx*.

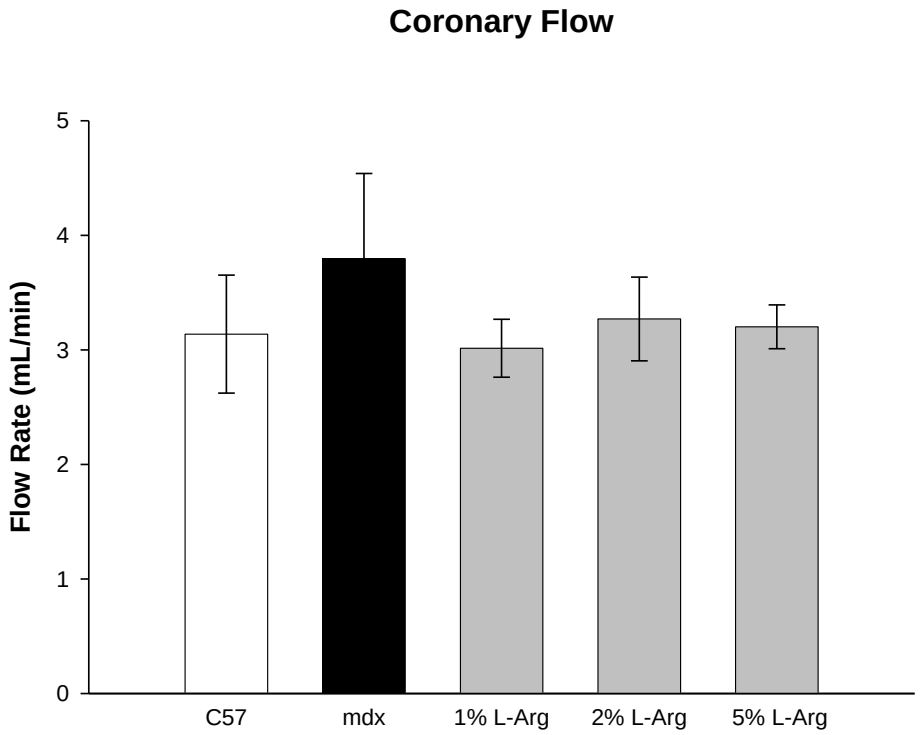


Figure 4.15 Coronary flow measured during Langendorff experiments.

There was no difference in coronary flow between groups.

4.3.5 Histology

Histological measurement of cardiac and skeletal fibrosis was conducted using digital quantification of picrosirius red staining of collagen in these tissues. There were no significant changes in cardiac collagen content with any L-arginine treatment level (Figure 4.17). All *mdx* groups had significantly greater levels of cardiac fibrosis compared to C57 (Figure 4.16). Decreased skeletal muscle fibrosis was seen in 2% and 5% L-arginine treated groups (Figure 4.18). All *mdx* groups exhibited significantly greater levels of skeletal muscle fibrosis compared to C57 (Figure 4.17). Representative images of picrosirius red-stained skeletal and cardiac muscles are shown in Figure 4.16.

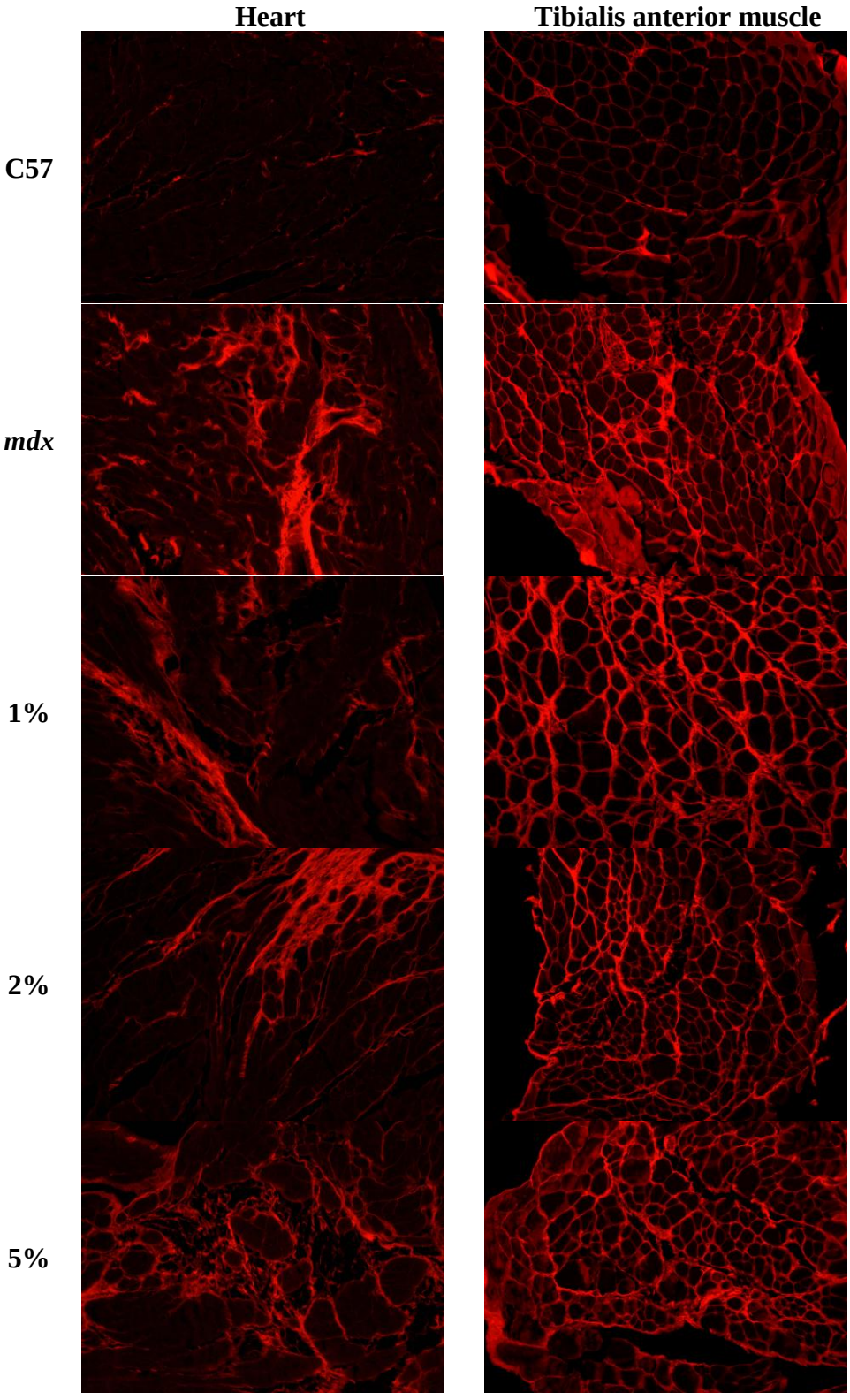


Figure 4.16 Representative images of picrosirius red-stained cardiac and skeletal muscle sections.

All *mdx* sections show increased collagen staining compared to C57 sections. 2% and 5% L-arginine-treated groups had significantly less collagen compared to *mdx* controls in skeletal but not cardiac muscle.

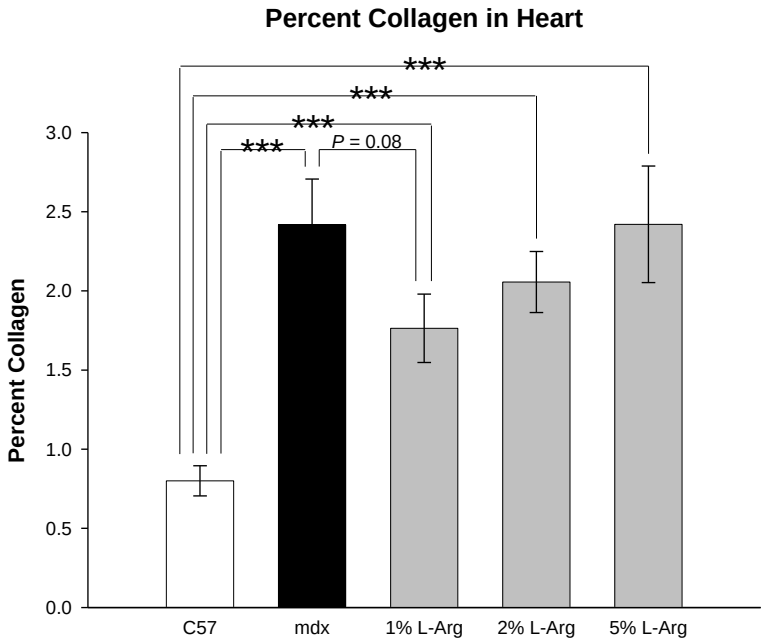


Figure 4.17 Cardiac collagen content assessed by digital quantification of picrosirius red staining.

Mdx groups had significantly greater cardiac collagen than C57. Although statistical significance was not achieved (*P*=0.08), mean values for cardiac collagen were reduced with low-dose L-arginine. (****P*<0.001)

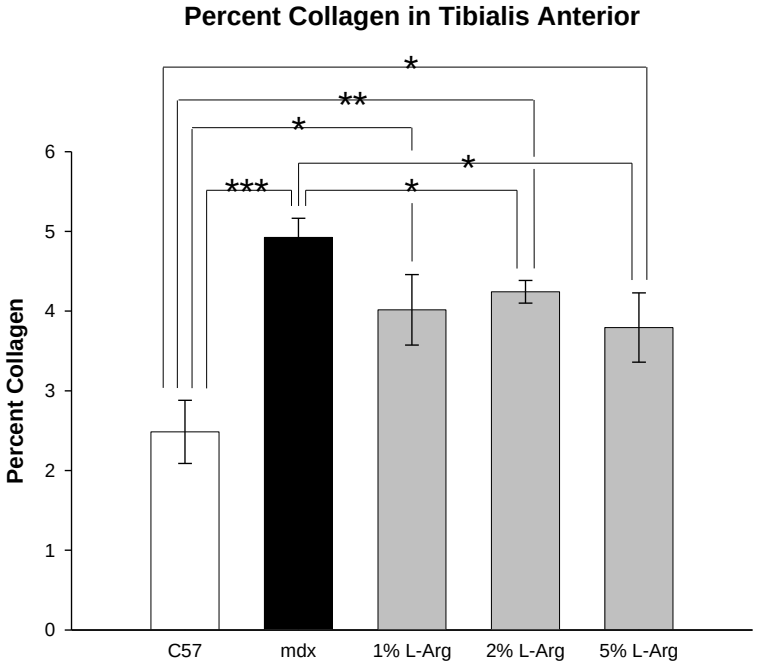


Figure 4.18 TA collagen content assessed by digital quantification of picrosirius red staining.

There was increased in skeletal muscle collagen in *mdx* groups compared to C57. 2% and 5% L-arginine treatment groups had significantly lower values then the *mdx* control group. (**P*<0.05; ***P*<0.01; ****P*<0.001)

4.4 Discussion

Previous work from our laboratory (Van Erp 2005) demonstrated that 10% w/v L-arginine in drinking water improved cardiac contractility and coronary flow in *mdx* mice but increased cardiac fibrosis and cardiac mass. As a result, this study determined whether a lower dose of dietary L-arginine afforded cardiac benefits while limiting detrimental morphological changes.

Morphology

L-arginine treatment did not alter body weight or skeletal muscle hypertrophy. All *mdx* mice (treated and untreated) exhibited increased skeletal muscle size, validating findings that show this parameter to be a major contributor to increased body mass (Shavlakadze et al., 2010). In support, tibial length did not differ between groups (data not shown), further suggesting that muscle bulk, rather than increased skeletal size was responsible for body weight increases. No differences in gross cardiac mass were observed. When normalised to body weight, the cardiac mass of *mdx* control and 5% L-arginine groups were significantly lower than C57 values reflecting previous data generated from our laboratory that indicated higher doses of L-arginine decreases cardiac mass (Van Erp 2005). 1% and 2% L-arginine treatment groups did not statistically differ from C57 values, but were also not statistically different from untreated *mdx*. Interpretation of this data needs to be taken with caution as *mdx* mice experience skeletal muscle hypertrophy and therefore heart weights were subsequently normalised to tibial length to better reflect cardiac mass as a proportion of body size. This normalisation showed that not only were heart weights similar across all groups, but that L-arginine treatment did not alter cardiac mass.

Cardiac function

Cardiac function was assessed using both Millar catheter and Langendorff isolated heart techniques to observe differences between *in vivo* and *ex vivo* pressure development. It was expected that *in vivo* measurements would reflect influences such as hormonal and neural inputs, particularly in relation to autonomic parameters that are altered in dystrophic hearts (Nigro

et al. 1990; Lu & Hoey 2000; Chu et al. 2002; Wehling-Henricks et al. 2005). Use of a Millar catheter allowed measurement of both ventricular and aortic pressure development. This is the first study to measure *in vivo* aortic pressure development in *mdx* mice. *In vivo* readings are complicated by choice of anaesthetic regime and level of anaesthesia, also evident as abnormal responses to general anaesthetics in DMD patients (Takagi 2000; Takagi & Nakase 2008). The well-established technique of the Langendorff isolated heart offered the advantage of pressure conductance under paced and regulated conditions, thus minimising the impact of varying heart rates and diastolic pressures on force development.

In vivo assessment of cardiac function

The heart rate of the 2% L-arginine group during Millar experiments was increased compared to either of the control groups. Given that there was no decrease in MABP, this result was unexpected; if a decrease in MABP due to the vasodilatory effect of NO on smooth muscle had been evident, it is expected that the heart would compensate by increasing HR. However in the absence of this phenomenon, the net effect of increased NO on HR was expected to be inhibitory. NO derived from eNOS attenuated sympathetic stimulation, most likely through PKG-dependent phosphorylation of troponin I and calcium desensitisation, while also potentiating parasympathetic effects (Seddon et al. 2007; Hofmann et al. 2009). NO derived from nNOS inhibits L-type calcium channel influx while also stimulating SR calcium uptake leading to a net negative inotropic effect (Seddon et al. 2009). Therefore in the absence of a change in MABP, the effects of NO are more likely to cause a decrease in HR. Taken together, the observed rise in HR in the 2% treated group was more likely an effect of anaesthesia and the 2% L-arginine treatment and also unrelated to the absence or presence of dystrophin. Despite the differences in HR observed in the 2% treatment group, no other associated changes to *in vivo* and *ex vivo* cardiac function suggest an interaction between 2% L-arginine treatment and non-myocardial factors. For example, Guo et al. (2009) demonstrated modulation of HR by increased NO through medullary cardiac reflexes.

In general, *in vivo* pressure development showed small changes in arterial measurements but little observable alteration in ventricular measurements. Ventricular developed pressure was not altered by L-arginine treatment. While NO, particularly that derived from eNOS, alters inotropic responses to stretch and mitochondrial respiration (Trochu et al. 2000; Balligand et al. 2009), changes in pressure development observed in aortic Millar measurements were most likely due to non-significant alterations in vascular tone. Mechanisms such as the Anrep effect or altered mitochondrial respiration are unlikely to be responsible for these changes as they would also have been evident in ventricular Millar and Langendorff derived data. Additional evidence eliminating the Anrep effect is also seen in the unchanged MABP, indicating no alteration in cardiac afterload.

Rates of pressure change (±dP/dt) within the artery represent changes of blood pressure and, to a large extent, indicate the quality of vessel compliance. With the exception of the 2% L-arginine treated group, +dP/dt was generally higher in *mdx* compared C57 groups regardless of treatment. This suggests, in conjunction with increased -dP/dt observed in most of the *mdx* groups, that vessel compliance is impaired with dystrophin deficiency. This is most likely due to ongoing fibrotic changes within the vascular smooth muscle resulting in wall stiffening and reduced compliance. Evidence of vascular changes linked to the absence of dystrophin has been shown in altered vasodilation response to sheer stress that may lead to long-term alterations in arterial structure (Loufrani et al. 2004). It should be noted that -dP/dt in those hearts treated with 1% L-arginine were no longer different to healthy control (C57) hearts and represents a return to normal values for this parameter. While quantification of arterial smooth muscle fibrosis was not conducted in this study, measurement of both cardiac and skeletal muscle fibrosis showed reductions in collagen content with L-arginine supplementation (especially evident in hearts of the 1% L-arginine-treated group), implying there may also be reduced smooth muscle fibrosis in this group.

Interpretation of ventricular rates of pressure change differ from that of arterial ±dP/dt as ventricular values are indicators of cardiac contractility and not vascular compliance. Ventricular pressure increase (+dP/dt) is a measure of cardiac contraction and the rate of pressure decline (-dP/dt) is a measure of cardiac relaxation. All *mdx* groups (regardless of treatment) exhibited greater rates of ventricular pressure increase compared to C57 controls that was most likely due to increased cardiac fibrosis resulting in myocardial stiffening. There was no difference between *mdx* control and treated groups indicating that L-arginine supplementation did not affect cardiac contraction. The only observable difference in cardiac relaxation was seen in the 1% L-arginine-treated group. This is an important finding as improved lusitropy may enhance ventricular filling thus improving cardiac function, allowing better tissue perfusion, decreased functional ischaemia and in turn lessening necrotic changes (Amrani et al. 1992; Susic et al. 1999; Chazalette et al. 2005).

MABP was unaltered by L-arginine treatment at any of the doses used in this study. It was expected that increasing the NOS substrate would lead to vasodilatation through cGMP and PKG-mediated pathways. Although few studies have been directed toward understanding dystrophic smooth muscle, dystrophin loss is known to cause abnormalities in nNOS intracellular localisation in skeletal and cardiac muscles that leads to significant attenuation of NO function (Feron et al. 1996; Bia et al. 1999). It may be possible that blood vessels affected by dystrophin loss are less responsive to minor alterations in NO concentrations, thus doses of L-arginine used in this study may have been too low to elicit a vasodilatory response.

Ex vivo assessment of cardiac function

It was surprising that there were no differences in developed pressure detected between C57 and *mdx* groups, however Van Erp et al. (2010) has also described that in similar aged mice there is no difference in this parameter. The current study extended these findings to show that there was no difference in cardiac function as measured by ±dP/dt or coronary flow. Furthermore, L-arginine treatment had no effect on cardiac function.

Other studies have shown marked differences between *mdx* and C57 in terms of cardiac function. Quinlan et al. (2004) used echocardiography to establish that, by 42 weeks of age, *mdx* mice had developed dilated cardiomyopathy and diminished cardiac function. However, this study also reported that there was no detectable cardiac dysfunction using cardiac catheterisation (Millar catheter) in 44 week old *mdx* mice compared to healthy controls. Also using echocardiography, Wehling-Henricks et al. (2010) found decreased fractional shortening and left ventricular ejection fraction in older *mdx* mice (18 months of age). It remains unclear why the developed pressures of *mdx* mice exceeded those of their C57 controls in this study. However, these elevated values may provide evidence that the *mdx* heart was in a compensatory state at 12 months of age (the time of data collection). Given that *in vivo* measurements showed no differences in either HR or MABP but that *ex vivo* measurement of developed pressure showed *mdx* hearts to have elevated function suggests that these hearts have had to work harder in order to achieve normal arterial blood pressure parameters – a phenomenon only observable once the heart has been removed from the diseased system for which it was compensating. This study clearly demonstrates the importance of an understanding of *ex vivo* function in the context of an *in vivo* setting.

Fibrosis

All *mdx* groups had increased ventricular collagen deposition compared to the C57 control group (Figure 4.16). Any potential benefit of L-arginine to cardiac fibrosis was lost with higher doses. This observation suggests that the absence or mislocation of nNOS in dystrophic muscle may have potentiated profibrotic pathways through a lack of competition for a common substrate (L-arginine) (Mori & Gotoh 2004; Wehling-Henricks et al. 2010).

In contrast, L-arginine treatment was beneficial in regards to skeletal muscle fibrosis. 2% and 5% L-arginine treatment groups had lower values then the *mdx* control group, indicating that these doses may be sufficient to decrease skeletal muscle collagen deposition. There are a number of possible

mechanisms where increased levels of NO may have limited the development of fibrosis in skeletal muscle: (1) upregulation and transposition of utrophin, a structural analogue of dystrophin, into the sarcolemma may have compensated for the lack of dystrophin and decreased muscle pathology (Voisin et al. 2005); (2) NO is known to act by cGMP-activated pathways to induce vasodilatation allowing increased tissue perfusion and decreased necrosis (Amrani et al. 1992; Susic et al. 1999; Chazalette et al. 2005); (3) increased NO may also result in decreased myofilament calcium sensitivity and altered calcium handling in both skeletal and cardiac muscle, ultimately resulting in a negative inotropic effect; this attenuation of contraction may have acted to limit contraction-induced damage (Seddon et al. 2007; Hofmann et al. 2009; Seddon et al. 2009); (4) NO is an important signalling molecule in initiation of muscle repair systems (Anderson 2000) and finally, (5) superoxide scavenging and anti-inflammatory properties of NO have been well documented (Nguyen & Tidball 2003b).

A limitation of this study was that greater statistical power may have clarified interpretation of a number of figures. For example, Figure 4.12 shows *mdx* hearts may have greater +dP/dt compared to C57 hearts (P=0.06). If this were the case, this data would indicate that not only did sham *mdx* and 1% L-arginine-treated *mdx* hearts have greater rates of ventricular contraction compared to C57 hearts, but that 2% and 5% L-arginine supplementation decreased contraction time to that of C57 control data. This figure may then indicate dietary supplementation of L-arginine at 2-5% in drinking water resulted in less violent ventricular contractions that may in turn act to minimise contraction-induced damage in *mdx* hearts. Increasing the size of the data sets may also clarify Figures 4.13, perhaps indicating 1% and 2% treatment to be beneficial to *mdx* developed pressure, and 4.17, which suggests 1% treatment may possess fibrosis-limiting properties.

It should also be noted that dietary supplementation represents a systemic dosing regimen. As such, increased availability of L-arginine would be

expected to enhance the actions of both nNOS and eNOS. Activation of both enzymes may complicate interpretation of results. For example, in addition to its effects on vascular tone through cGMP-mediated pathways, eNOS has been associated with cardiac calcium handling through modulation of RyR2 activity (Petroff et al. 2001; Lim et al. 2008). Stretch-dependent stimulation of NO production by eNOS increases the open probability of RyR2, in turn increasing calcium transient amplitude causing a subsequent increase in cardiac inotropy (Petroff et al. 2001). However, nNOS may also modulate RyR2 function as it has been shown to co-precipitate with RyR2 in both human (Damy et al. 2004) and mouse (Barouch et al. 2002) studies suggesting they form an interactive complex.

This study demonstrated that cardiac function in 12 month old dystrophic mice is unchanged and that low dose L-arginine has minimal to no effect. This is surprising given that cardiac fibrosis is significantly elevated in *mdx* mice. Furthermore L-arginine treatment did not reduce cardiac fibrosis. Evidence from this study indicates that low dose L-arginine is of no benefit while higher doses are potentially detrimental (Van Erp 2005; Wehling-Henricks et al. 2010). Given the large increase in fibrosis, there are likely to be adaptive and compensatory measures occurring in order to maintain cardiac function. These were not measured in the current study but may be due to changes in cardiac myocyte function with respect to calcium handling. Studies involving single cardiac myocytes may be able to elucidate these mechanisms as they would be isolated from the surrounding fibrotic pathology. Williams and Allen (2007b) have shown that *mdx* cardiomyocytes have altered calcium transients, increased basal intracellular calcium, increased RyR expression, decreased phospholamban phosphorylation and altered Na^{+}/Ca^{2+} exchanger function. These results suggest that there are adaptive changes occurring at the single cardiac myocyte level that may not be detected at the organ level. Chapter 5 of this thesis investigates L-type calcium channel characteristics in conjunction with single myocyte properties including contractility, force-frequency relationships and calcium transient analysis in isolated ventricular myocytes.

Chapter 5: Cardiac nNOS overexpression in dystrophic cardiomyopathy

5.1 Introduction

DMD was thought to be predominantly a skeletal muscle disease, however cardiac complications are now seen as major contributing factors to morbidity and mortality (Nigro et al. 1990; Eagle et al. 2002). Alterations in cardiomyocyte calcium handling associated with dystrophin deficiency have been observed in both human (Bodensteiner & Engel 1978; Jackson et al. 1985) and murine (*mdx* mice) forms of the disease (Williams & Allen 2007b). Calcium mishandling in dystrophic muscle is of particular importance as it promotes premature cell death through activation of intracellular proteases (Alderton & Steinhardt 2000b). Further, sarcolemmal micro-ruptures and activation of stretch-induced calcium influx increase the susceptibility of mitochondria in dystrophic cardiomyocytes to irreversible loss of membrane potential and initiation of cell death (Jung et al. 2008). Cardiac ryanodine receptor (RyR2) sensitivity is also increased after oxidisation (Pessah et al. 2002) by increased concentrations of reactive oxygen species present in these cells and contributes to irregular calcium signal amplification, impaired intracellular calcium regulation and altered excitation-contraction coupling (Williams & Allen 2007a; Jung et al. 2008; Ullrich et al. 2009). In young *mdx*, cardiomyocyte calcium mishandling is present before signs of heart failure are evident, suggesting abnormal regulation of calcium plays an significant role in compromising the dystrophic heart (Quinlan et al. 2004). Thus, further understanding of calcium dynamics within the dystrophic heart is required to develop alternate treatment options in the future.

NO is involved in varied and sometimes contradictory actions that are determined by subcellular compartmentalisation and regulation of NOS isoforms (Barouch et al. 2002). Of importance to this study, NO exerts modulatory actions on cardiac cells including inhibiting L-type calcium channels (Mery et al. 1993), stimulation of sarcoplasmic reticular calcium release (Xu et al. 1998; Petroff et al. 2001) and having direct effects on L-

type channels and RyR2 (by S-nitrosylation). NO also exerts downstream effects on muscle contractility and the open-probability of the mitochondrial transition pore through cGMP. Defective NO/cGMP signalling has also been linked to mitochondrial dysfunction and premature cell death (Burelle et al. 2010). A major source of NO in cardiomyocytes is nNOS, which in normal tissue is directly associated with the DGC near the sarcolemma. Significantly decreased levels of nNOS protein expression are seen in both humans with DMD and *mdx* mice (Brenman et al. 1995; Bia et al. 1999). Absence of dystrophin and subsequent DGC disruption also results in the remaining nNOS being absent from the sarcolemma (Brenman et al. 1995); further, relocalisation of nNOS to the sarcolemma has been recognised as a marker for restoration of the DGC (Wells et al. 2003).

Chao et al. (1998) showed that transgenic mice lacking both dystrophin and nNOS showed no further exacerbation of skeletal muscle pathology than *mdx*, supporting the finding that nNOS was severely disrupted with dystrophin loss. This coupled with the finding by Bia et al. (1999) that cardiac nNOS was reduced by approximately 80% in *mdx*, argues that this disruption is mirrored in the heart. Although nNOS over-expression in *mdx* mice has demonstrated increased cardiac NO production resulting in improved heart rate variability, ameliorated ECG abnormalities and reduced cardiac fibrosis, little is understood about the impact on calcium handling in these hearts (Wehling-Henricks et al. 2005). Therefore the presence or absence of cardiac nNOS appears to play a role in ameliorating or exacerbating the dystrophic process. As a result, there is a strong need to further understand the calcium dynamics of dystrophic cardiomyocytes with special regard to the roles of nNOS derived NO.

The major aim of this study was to determine whether overexpressing cardiac nNOS would benefit dystrophic cardiomyocytes at the level of the single cell. To this end, a transgenic murine model was bred that overexpressed cardiac nNOS on a background of dystrophin deficiency (+nNOS/*mdx*). +nNOS/*mdx* mice have previously been shown to have a two-fold increase in expression of nNOS in cardiac tissue leading to 18%

increase in nNOS activity (Wehling-Henricks et al. 2005). Cardiac myocytes from these mice, along with those from C57 and *mdx* mice (controls), were isolated and used to assess L-type calcium channel function, calcium handling and contractility.

5.2 Materials and methods

nNOS cardiac overexpression mice were crossed with *mdx* to produce a line that possessed increased production of cardiac NO on a background of dystrophic pathology. Generation of the transgene and breeding strategy used are described in Wehling et al. (2001). Briefly, rat brain nNOS was cloned downstream of the human skeletal actin promotor and the vp1 intron. nNOS cDNA was followed by the SV-40 large T poly-A site, with ClaI-DraI-digested DNA injected into the pronucleus of parent mice (Wehling et al. 2001). Experimental mice were males aged 5-6 months, with genotypes verified by PCR genotyping. Euthanasia was performed using 50mg/kg ip sodium pentobarbitone, after which cardiac myocytes were isolated from C57, *mdx* and +nNOS/*mdx* (nNOS) mice and used to assess L-type calcium channel function, contractility and calcium handling properties. While there was no difference in the appearance of isolated cells from the different lines used, it was noted that C57 cells were more tolerant to isolation compared to cells from *mdx* or nNOS hearts. There was no difference to isolation tolerance between the two *mdx* lines (*mdx* and nNOS) used in the study.

L-type calcium channel function was assessed using the voltage-clamp technique. Current-voltage relationship, L-type calcium channel inactivation and L-type calcium channel rundown were measured. Contractility experiments were conducted both in the absence and presence of the fluorescent calcium binding dye FURA-2 AM to test whether this dye acted as a calcium buffer and thus influencing calcium dynamics. Subsequently force-frequency experiments were conducted in the absence of FURA-2 AM to reflect cell shortening data. Additional cell shortening and calcium handling experiments were conducted using isolated cardiomyocytes that had been loaded with FURA-2 AM in order to measure the characteristics of calcium transients in conjunction with those of their resultant contractions.

Techniques are detailed in Chapter 2 and experimental animal numbers summarised in Figure 5.1.

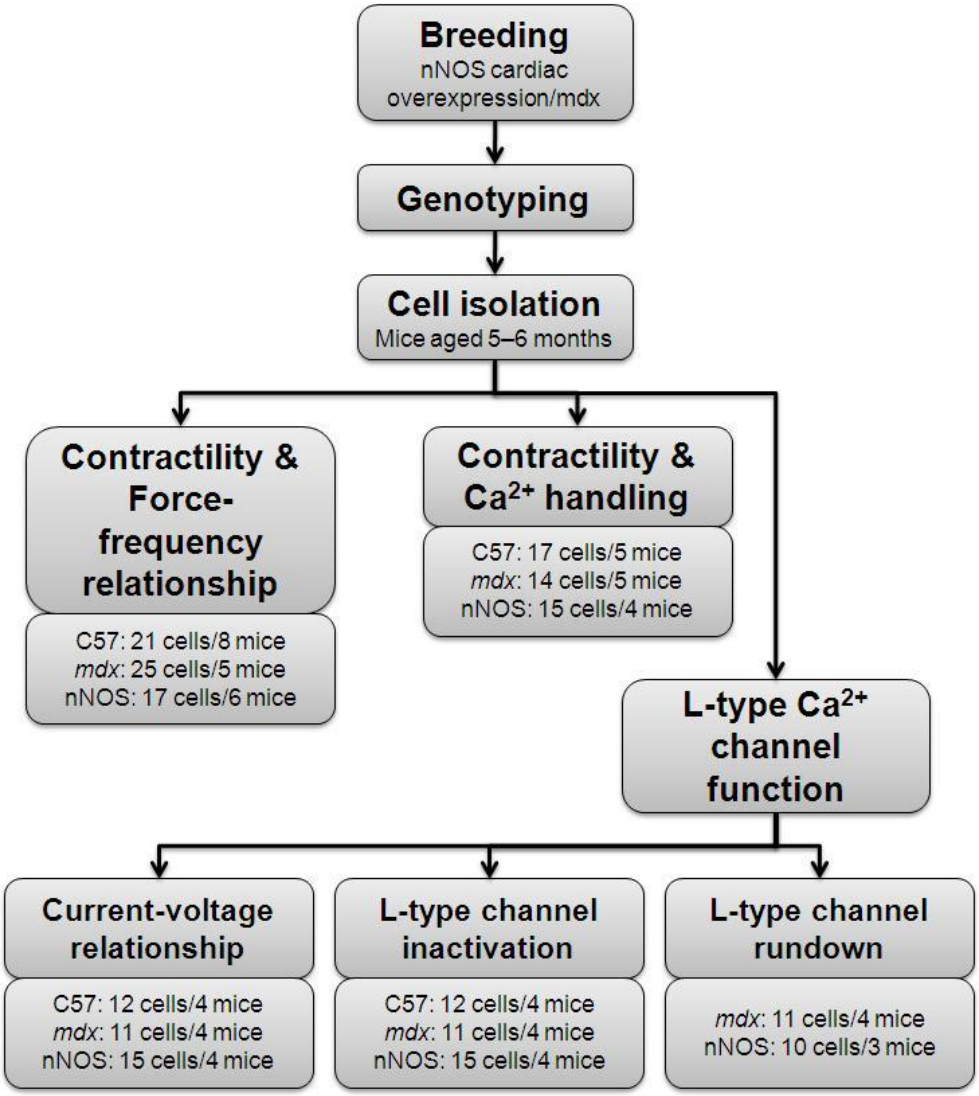


Figure 5.1 Summary of techniques and experimental numbers used in Chapter 5.

5.3 Results

5.3.1 Genotyping

Cardiac nNOS overexpressing mice supplied by Professor James Tidball (University of California) were bred with an existing colony of *mdx* mice to produce a strain of *mdx* with this characteristic (nNOS Tg+/*mdx*). Confirmation of transgene expression was accomplished using PCR. Figure 5.2 shows a genotyping gel that shows both positive and negative results.

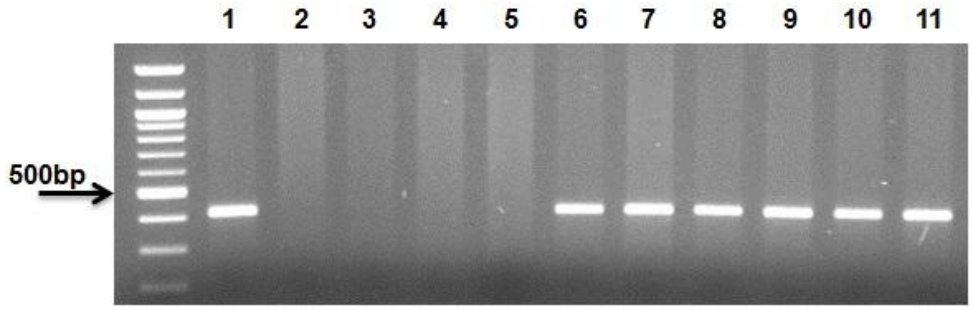


Figure 5.2 Detection of nNOS transgene in cardiac tissue by PCR.

This PCR was designed to detect the presence or absence of the nNOS cardiac overexpression transgene. As can be seen in columns 1 and 6-11, a single band at 420bp indicated the presence of the transgene (nNOS positive). A 100bp NEB ladder was used to identify band size.

5.3.2 Voltage clamp measurements

The current-voltage (IV) relationship (Figure 5.3) shows that *mdx* myocytes have depressed L-type calcium currents at potentials from -40 to 0mV when compared to C57 myocytes. The IV relationship was 'normalised' in *mdx* cardiomyocytes that overexpressed nNOS such that data from this group closely paralleled the IV relationship obtained from C57 cardiac myocytes.

Experiments were performed to determine if the kinetics of inactivation were altered and if this was ameliorated by nNOS overexpression. Woolf et al. (2006) have previously demonstrated that *mdx* myocytes have a delayed calcium channel inactivation. Briefly, membrane potential was stepped up to 0mV and the calcium current amplitude (*a*) was recorded. Test potentials were stepped from -40 up to +50mV in 10mV increments and a second measurement of calcium current amplitude (*b*) was taken (Figure 5.4B); *b*

was expressed as a percentage of *a* at all test potentials (Figure 5.4A). This protocol has been previously used in studies of L-type calcium currents to examine the voltage dependence of L-type calcium channel decay (Findlay 2002). These experiments show that, in C57 myocytes, 100% of the L-type calcium currents were inactivated at 0mV, whereas in untreated *mdx* cardiac myocytes, 100% inactivation occurred at approximately -15mV. The overexpression of the nNOS gene in *mdx* cardiac myocytes returned the voltage dependence of inactivation to that of C57 values (Figure 5.4A). Of note, there was an additional outward current observed in *mdx* myocytes at potentials where 100% of the calcium current was inactivated.

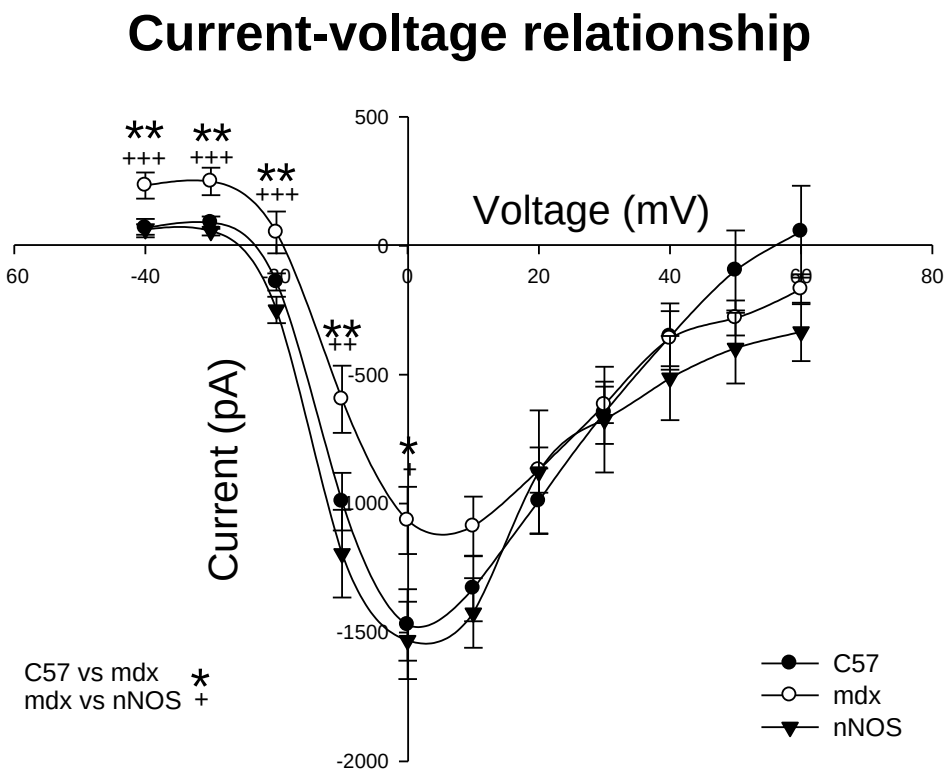


Figure 5.3 Current-voltage (IV) relationship as determined by voltage clamp.

Mdx cells had a significantly reduced peak current curve compared to C57 or nNOS cells for voltages lower than 0mV. The nNOS cardiac overexpression IV curve closely followed C57. Both the threshold of activation and reversal potential were similar between groups.
(Significance symbols: * C57 vs mdx; + mdx vs nNOS)
(* $P<0.05$; ** $P<0.01$; *** $P<0.001$)

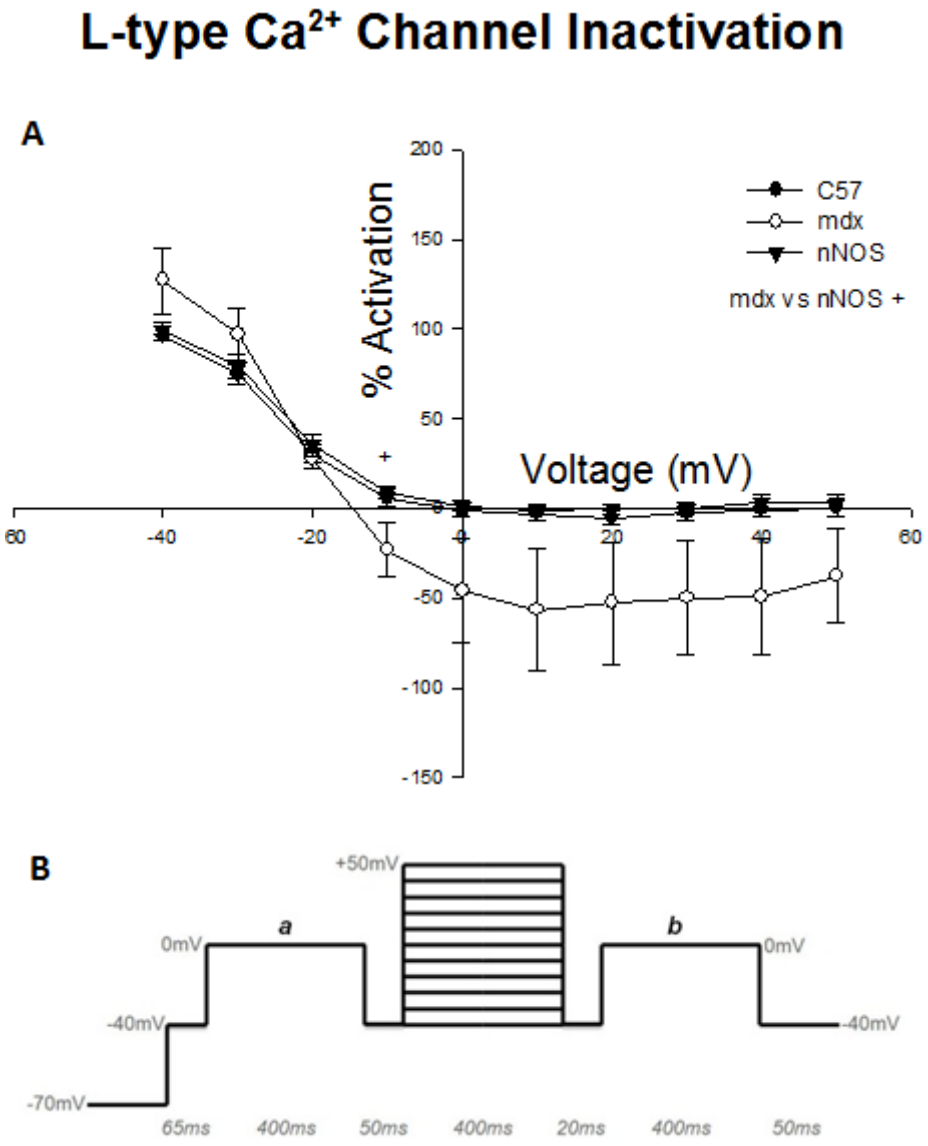


Figure 5.4 L-type calcium channel inactivation measured by voltage clamp.

A: Shows voltage dependence of decay of L-type calcium current. Both C57 and nNOS cardiac overexpression groups had 100% channel inactivation at 0mV, whereas *mdx* 100% inactivation occurred at -15mV. nNOS cardiac overexpression data did not differ from C57 values. **B:** Voltage stepping protocol for L-type channel inactivation experiments. (+ $P<0.05$)

5.3.3 L-type calcium channel rundown

These experiments were conducted to examine the kinetics of L-type calcium channel inactivation with respect to dependence on time. Briefly, L-type calcium currents were elicited at test potentials of 0mV at 1, 2 4 and 8Hz and expressed as a percentage of the initial test current. The level of rundown was significantly different at the first pulse regardless of stimulation frequency (Figure 5.5).

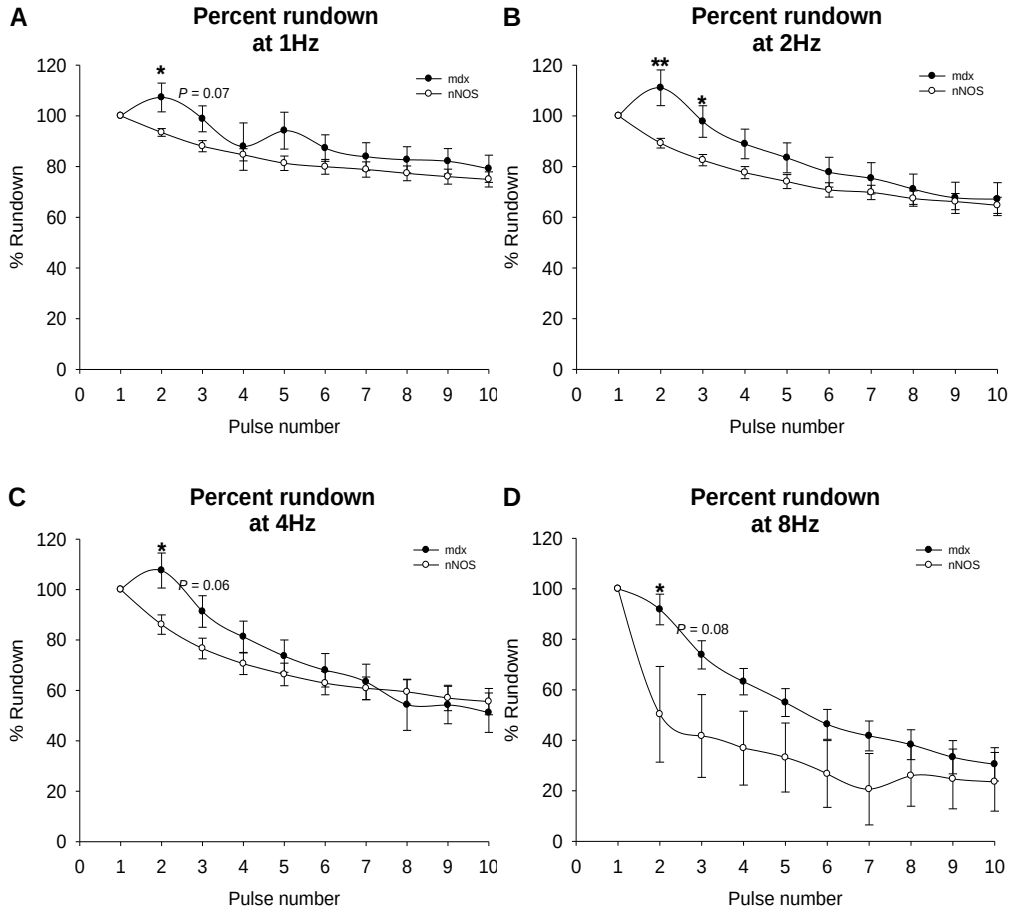


Figure 5.5 L-type calcium channel rundown at different pulse rates.

Although nNOS data was consistently lower than *mdx*, especially at 8Hz pulse rate, nNOS L-type calcium channel rundown was not significantly different to *mdx*. (* $P<0.05$; ** $P< 0.01$)

5.3.4 Contractility and calcium handling measurements

Force-frequency measurements

Contractility and calcium handling experiments consisted of determining a force-frequency relationship and measurement of intracellular calcium using FURA-2 AM-loaded isolated cardiomyocytes. Force-frequency experiments were conducted on FURA2-naive cells to obtain shortening responses that were free of any potential calcium binding that may have occurred when using the dye. These experiments revealed *mdx* cardiomyocytes had significantly greater cell shortening at all pulse frequencies tested compared to C57. Overexpression of cardiac nNOS in *mdx* cardiomyocytes returned the force-frequency relationship to that seen in C57 cells (Figure 5.6). C57 and nNOS cardiac overexpression groups were not significantly different at any of the tested pulse frequencies (Figure 5.6).

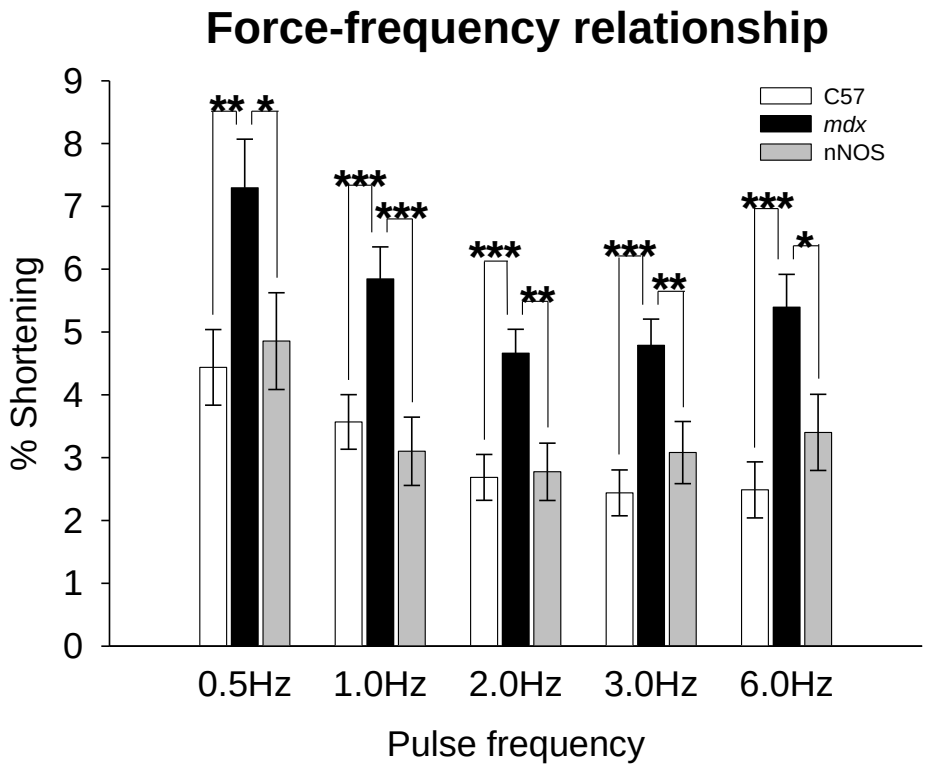


Figure 5.6 Force-frequency relationships.

Mdx displayed significantly greater cell shortening at all frequencies tested. C57 and nNOS cardiac overexpression groups did not differ.
(* $P<0.05$; ** $P<0.01$; *** $P<0.001$)

Cell shortening measurements

Data for cell shortening was collected from single pulse FURA-2 AM-loaded cells and were conducted in conjunction with measurement of calcium transients. Representative traces of shortening events from single pulses have been included for comparison between genotypes (Figure 5.7A). These are not composite traces but actual events that were chosen to represent each genotype as they most closely resemble mean values for percent shortening, time to peak shortening and decay constant (tau). As described above, C57 and nNOS cardiac overexpression groups did not differ in percent shortening but were significantly less than *mdx* values for this parameter (Figure 5.7B). Time to peak shortening did not differ between groups. The cell shortening decay constant (tau) was greater in *mdx* compared to C57 (Figure 5.7D). In summary, *mdx* myocytes have a significantly greater cell shortening, unchanged time to peak and a slower relaxation velocity (tau). Overexpression of the nNOS only reduced the cell shortening parameter to C57 values leaving time to peak and decay constant unchanged.

Calcium transient measurements

Representative traces of calcium transients have also been included for comparison between genotypes as traces of single pulse transients (Figure 5.8A). C57 and *mdx* transient heights were not statistically different. nNOS cardiac overexpression mice had significantly greater transient height compared to C57 and *mdx* groups (Figure 5.8B). There was no difference in time to transient peak in C57 and *mdx* groups. nNOS cardiac overexpression mice had a significantly greater time to transient peak compared to C57 but not *mdx* (Figure 5.8C). Transient decay constant did not show significant differences between genotypes (Figure 5.8D).

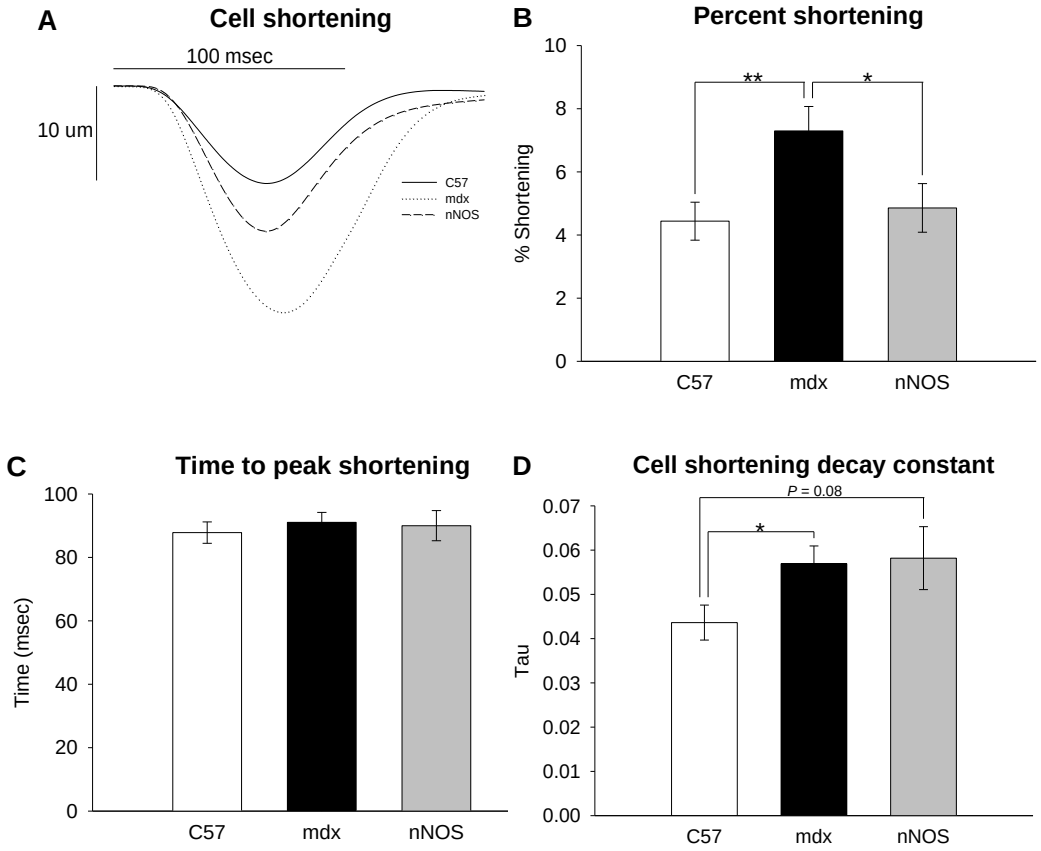


Figure 5.7 Cell shortening characteristics.

A: Representative traces of cell shortening. **B:** *Mdx* had significantly greater percent-shortening compared to C57 and nNOS cardiac overexpression mice. C57 and nNOS cardiac overexpression groups did not differ. **C:** No difference in time taken to peak shortening was detected. **D:** *Mdx* and nNOS cardiac overexpression groups did not differ in decay constant (tau) but had greater values compared to C57. (* $P<0.05$; ** $P<0.01$)

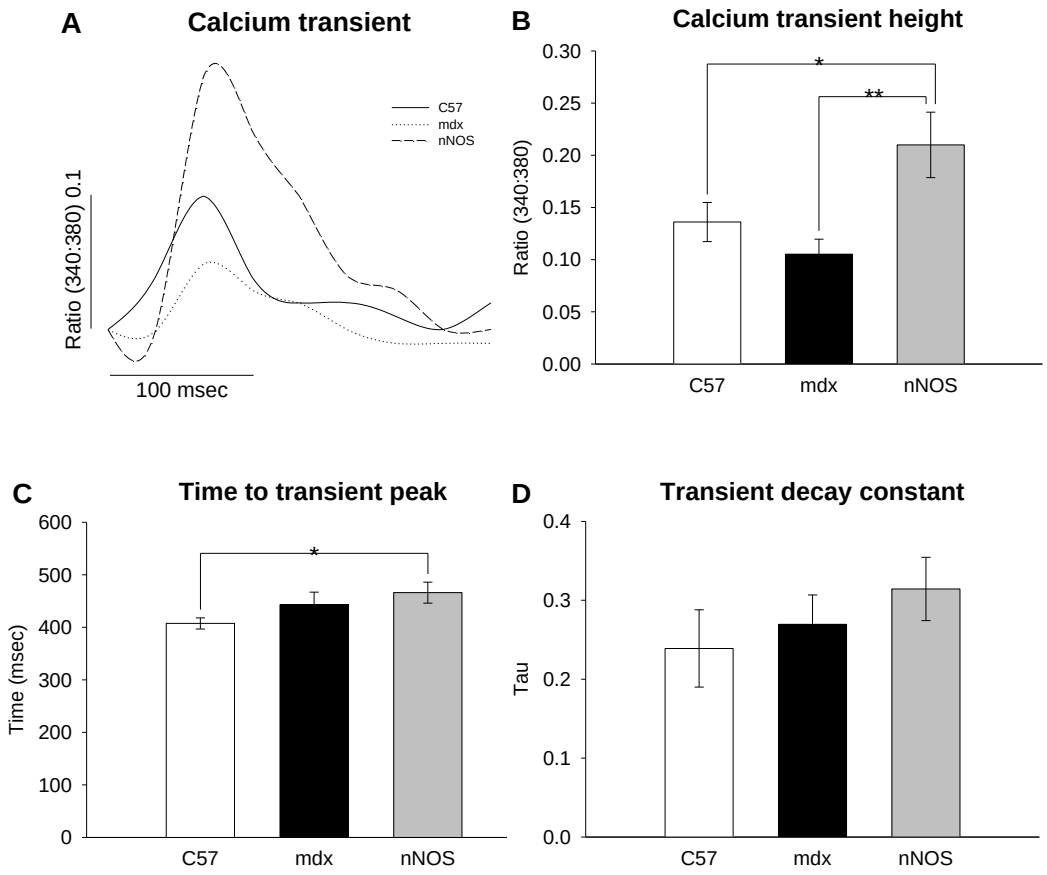


Figure 5.8 Calcium transient characteristics for each genotype.

A: Representative calcium transient traces for each genotype. **B:** Transient heights of C57 and *mdx* groups did not show significant differences. nNOS cardiac overexpression mice had significantly greater transient height compared to C57 and *mdx*. **C:** Time to transient peak was not different between C57 and *mdx* groups. nNOS cardiac overexpression mice had a significantly greater time to transient peak compared to C57 but not *mdx* groups. **D:** Transient decay constant (tau) did not differ significantly between genotypes. (* $P<0.05$; ** $P<0.01$)

5.3.4 Multi-variant analysis

Results from the preceding sections have shown differences in both cell shortening and calcium transients. Multi-variant analysis was performed to understand the relationship between calcium transient height and percent cell shortening. Data has been presented as the centroids of the data sets for each genotype with accompanying standard errors for each variable. Figure 5.9 shows *mdx* myocytes have a comparatively small transient size for a larger cell shortening when compared to C57. The nNOS cardiac overexpression group showed a significantly larger transient size was

required to elicit a similar sized contraction (Figure 5.9). Comparisons between the other groups did not achieve statistical significance. Taken together, overexpression of cardiac nNOS causes a larger transient for a similar sized cell shortening, although nNOS overexpression caused a reduction in cell shortening when examined in isolation from other variables (Fig 5.7B).

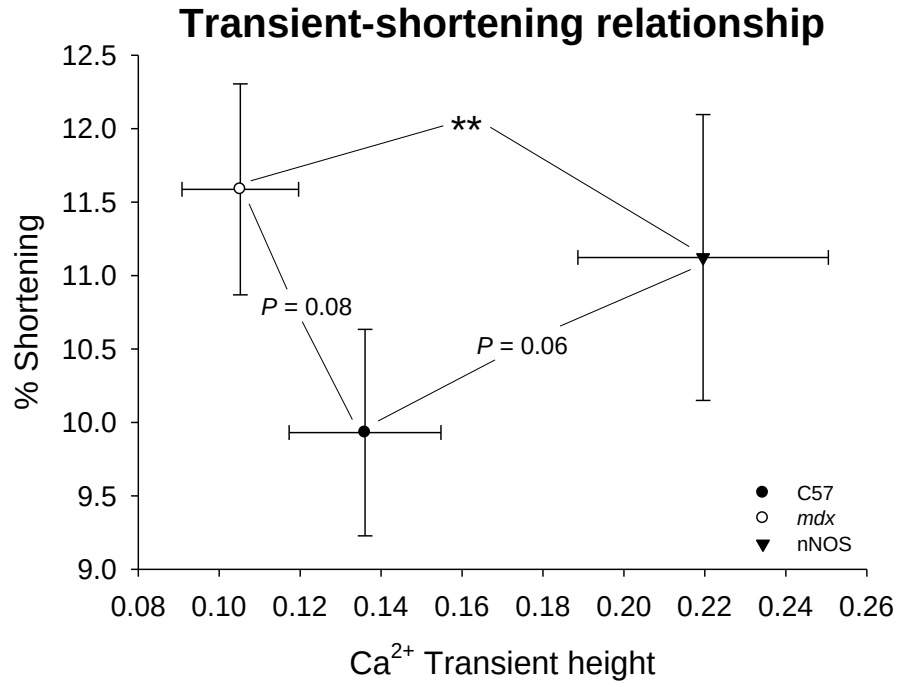


Figure 5.9 Calcium transient-cell shortening relationship.

Mdx was significantly different to the nNOS cardiac overexpression group but not C57. nNOS required a greater transient to achieve a similar contraction. C57 did not differ from either of the other genotypes.
(** $P<0.01$)

5.4 Discussion

Altered calcium handling has been a well-recognised, if not well understood, characteristic of dystrophic cardiac muscle. It has not been until recent years that the mechanisms involved in the abnormal calcium dynamics of this disease have been explored. Published research using transgenic *mdx* mice that overexpress cardiac nNOS showed positive functional outcomes (Wehling-Henricks et al. 2005). Therefore, this study aimed to explore the effect of increased NO availability on L-type calcium channel function in dystrophic cardiomyocytes.

The results of this study have shown that the overexpression of cardiac nNOS normalises a number of L-type channel properties. While the threshold of activation and reversal potential were similar for all groups, there was reduced peak amplitude evident in *mdx* cells. Interestingly, there was also evidence of an additional outward current observed in *mdx* cells that, to some degree, may account for the reduced L-type current peak also observed. The exact nature of the additional current could not be determined during these experiments. However, there is evidence that the function of other channels (e.g. sodium channels) and Na^{+}/Ca^{2+} exchange are abnormal with dystrophin loss (Fanchaouy et al. 2009; Koenig et al. 2011). The reduction in peak amplitude and additional current observed in *mdx* cells were abolished in the nNOS overexpression group indicating a normalisation of L-type channel activation.

Likewise, altered L-type voltage-dependant channel inactivation was also normalised with the overexpression of nNOS in *mdx* cardiomyocytes. L-type calcium channels in *mdx* cells were found to be completely inactivated at more negative potentials compared to C57 or nNOS overexpressing cells. Conversely, Woolf et al. (2006) found *mdx* cardiomyocytes showed prolonged inactivation of these channels. The difference between the two studies may be explained by age as the results of the earlier study were obtained using young (12-14 week old) mice whereas the results of the current study were from 12 month old animals.

Channel rundown can be defined as spontaneously decreasing channel activity over time and in non-dystrophic cells has previously been attributed to a loss of unidentified cytoplasmic factors, reversal of protein kinase A phosphorylation and calcium-dependant proteolysis (McDonald et al. 1994; Zhen et al. 2006), although protein kinase A phosphorylation and proteolysis are unlikely to be critical processes (Byerly & Yazejian 1986; Belles et al. 1988; McDonald et al. 1994; Kameyama et al. 1997). However, these hitherto less significant processes may have increased importance when considering dystrophic pathology as both NO availability and calcium-dependant proteolysis are adversely affected by dystrophin absence (Bia et al. 1999; Williams & Allen 2007b). Rundown experiments from the current study show that L-type channel activity of the nNOS overexpressing group was only significantly altered at the commencement of the pulse run. RyR2 function and SR load are also likely to be involved in rundown process. However, actions of NO on SR load are still being defined as nNOS-derived NO enhanced (Stoyanovsky et al. 1997), inhibited (Zahradnikova et al. 1997) or did not alter (Sears et al. 2003) SR calcium load.

Mdx ventricular myocytes are nNOS deficient (Bia et al. 1999), therefore it is reasonable to conclude these cells are relatively free of calcium-desensitising effects of NO such as decreased L-type function and decreased SR calcium load (Williams & Allen 2007b; Wang et al. 2008). Both of these responses would be expected to result in an increased inotropic response. Consistent with this hypothesis, force-frequency experiments from this current study demonstrated that isolated *mdx* cardiomyocytes had a greater percent shortening at frequencies up to 6Hz when compared to C57 and that the overexpression of nNOS in these cells normalised this parameter. These results were also reflected in single-pulse FURA2 loaded cell shortening experiments where overexpression of cardiac nNOS did not affect rates of contraction (time to peak shortening) or relaxation (shortening decay constant) but did return percent shortening to C57 values. These findings are supported by Sadeghi et al. (2002) who found *mdx* cardiac L-type channels

had an altered steady-state activation that was shifted towards more positive potentials.

In the present study, nNOS increased calcium transient height and prolonged transient peak time interval compared to C57 but not *mdx* (although the latter may have simply been a product of increased peak height). Multi-variant analysis of percent shortening and peak transient height revealed data that is consistent with NO decreasing calcium sensitivity, showing that the overexpression of nNOS caused *mdx* cells to require greater transient amplitudes to elicit similar sized contractions. Conversely, Williams and Allen (2007b) found *mdx* had increased transient amplitude. Their results were unexpected as standard models of heart failure show diminished transient heights (O'Rourke et al. 1999). Although not a finding of this study, an increased L-type calcium transient decay constant has also been observed in other studies on human heart failure and *mdx* hearts, which may be linked to actions downstream of NO such as reduced SERCA2 function as a result of phospholamban dephosphorylation (Schwinger et al. 1995; Williams & Allen 2007b).

Studies have shown *mdx* hearts are nNOS deficient, resulting in a relatively increased sensitivity to calcium. As the disease progresses, calcium sensitivity is further increased in *mdx* due to the action of increased ROS production on RyR2 (Williams & Allen 2007a; Jung et al. 2008; Ullrich et al. 2009). Results from this study supporting this hypothesis include relatively small calcium transients and depressed calcium current in *mdx* causing relatively strong percent shortening. Tempering the finding that relative loss of nNOS in *mdx* myocytes increased calcium sensitivity is that their elevated resting intracellular calcium concentrations (Alloatti et al. 1995) may also decrease calcium sensitivity though protease activation (e.g. calpains) and degradation of troponin I (Carrozza et al. 1992; Gao et al. 1997; Papp et al. 2000).

Although outside the scope of this study, it may be hypothesised that increasing concentrations of cardiac NO in *mdx* mice may directly improve

calcium handling, possibly through S-nitrosylation of L-type calcium channels (Paolocci et al. 2000; Sun et al. 2006) and correction of RyR2 function (Vandebrouck et al. 2002; Damy et al. 2004). One possible method to examine SR calcium pump activity may be to compare calcium transients from cardiomyocytes isolated from the mice used in this study that have been paced at different frequencies. Examination of transient characteristics from these cells may clarify the effect that increasing NO availability has on RyR2 function and SR calcium pump activity in dystrophic hearts.

This study did not differentiate between direct and indirect actions of increased NO availability. In non-dystrophic isolated cardiac myocytes, endogenous nNOS-derived NO was shown to also have indirect effects on calcium dynamics through actions on the Na^{+}/K^{+}-ATPase pump and downstream changes at the Na^{+}/Ca^{2+} exchanger (Zhou et al. 2002; Xu et al. 2003). Downstream benefits may also result from cGMP-mediated actions such as improved vasodilatation, improved mitochondrial function and changes in protein kinase A phosphorylation (Crosbie 2001; Khairallah et al. 2008). Thus, it may be important to develop studies that differentiate between direct and indirect actions of NO in order to develop intelligent treatments for DMD.

Chapter 6: Effect of tadalafil on profibrotic gene expression in *mdx* mice

6.1 Introduction

In DMD, the absence of dystrophin results in complex muscle cell pathologies that range from sarcolemmal fragility to altered calcium handling (Durbeej & Campbell 2002; Williams & Allen 2007b). The detailed knowledge of the disease's intricacies needed to develop effective therapies can only be gained through careful examination of each of these pathological features. Bia et al. (1999) showed that the absence of dystrophin resulted in a marked decrease of sarcolemmal nNOS, leading not only to decreased levels of available NO but to alterations in the downstream effects of NO (Crosbie 2001). Various studies have shown beneficial effects of increasing NO availability to dystrophic pathology but few have differentiated between direct and indirect effects of NO (Crosbie 2001; Wehling et al. 2001; Van Erp 2005; Wehling-Henricks et al. 2005; Hnia et al. 2008). Differentiating between direct and indirect actions of NO will allow more focussed approaches to the treatment of DMD. Figure 6.1 describes the NO/cGMP pathway. nNOS uses L-arginine as the substrate to produce NO that acts upon sGC to produce cGMP from GTP. Inactivation of cGMP occurs by PDE5-mediated hydrolysis of cGMP to GMP.

NO is directly involved in mitigating oxidative stress and attenuating fibrosis (Wehling et al. 2001). This is particularly important in DMD as not only are increased concentrations of by-products of oxidative damage present (Kar & Pearson 1979; Jackson et al. 1984), but dystrophic tissues are particularly prone to oxidative stress (Rando et al. 1998). Reduced NO availability contributes to this pathology as NO's antioxidant and anti-inflammatory actions are diminished (Cave et al. 2005; Wehling-Henricks et al. 2005; Tidball & Wehling-Henricks 2007). Muscle growth and repair are also enhanced by the stimulation of hepatocyte growth factor by NO (Tatsumi et al. 2002). In addition, NO has a strong connection to cardiac CICR, having direct modulatory effects on L-type calcium channels, where it enhances cardiac lusitropy (Ashley et al. 2002; Sears et al. 2003; Khan et

al. 2004), and on RyR2 where it causes increased force of contraction through augmented SR calcium release (Petroff et al. 2001).

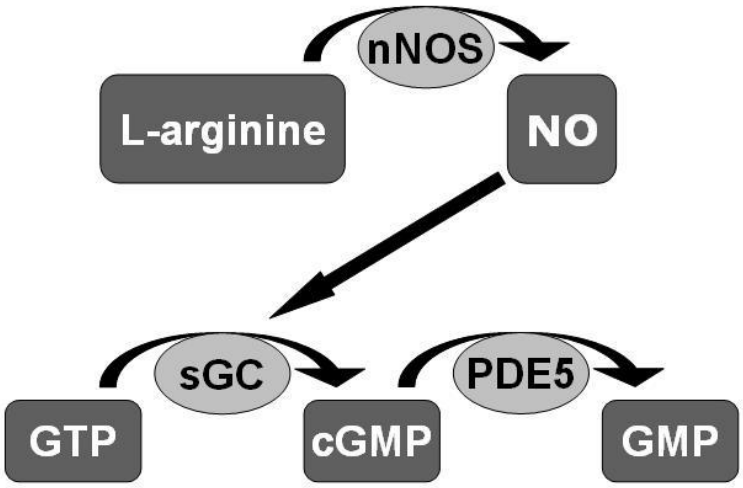


Figure 6.1 Diagram representing the NO/cGMP pathway.

nNOS synthesises NO from the substrate L-arginine. NO acts through soluble GC to convert GTP into cGMP. Deactivation of cGMP occurs as it is hydrolysed by PDE5 into GMP.

Processes modulated by cGMP are also relevant to dystrophic pathology. Functional ischaemia, when the blood flow of working muscles is restricted to the extent that metabolic demands are unable to be met, is also well documented in DMD (Mendell et al. 1971; Thomas et al. 1998; Sander et al. 2000). Increased concentrations of cGMP have been linked with improved endothelial responses in both skeletal (Thomas et al. 1998; Crosbie 2001) and cardiac muscles (Guazzi et al. 2007; Lewis et al. 2007). In support, studies by Halcox et al. (2002) and Katz et al. (2000) showed that acute PDE5 inhibition using sildenafil in the setting of chronic heart failure and coronary heart disease was protective against work-load induced ischaemia and improved exercise capacity. In addition, calcium dynamics are also modulated by cGMP-mediated pathways. L-type calcium current is inhibited through a PKG-mediated pathway, resulting in phosphorylation of troponin1 and decreased calcium sensitivity (Layland et al. 2002; Yang et al. 2007). Furthermore, neuromuscular junction architecture may be affected by cGMP as defects in *mdx* acetylcholine receptor distribution were

normalised by genetic overexpression of GC or PKG production (Jones & Werle 2000; Rafael et al. 2000; Carlson & Roshek 2001; Godfrey & Schwarte 2003; Schwarte & Godfrey 2004; Shiao et al. 2004). Finally, muscle repair is aided through cGMP-mediated pathways by the formation of myotubes through promotion of myoblast fusion (Lee et al. 1994).

The effectiveness of PDE5 inhibition in dystrophic muscle has not been extensively studied, although these compounds have shown functional improvements in both cardiac and skeletal muscles (Rao & Xi 2009). Benefits to the dystrophic heart were reported with both transgenic overexpression of cardio-specific GC and acute PDE5 inhibition using sildenafil (Khairallah et al. 2008). These included decreased sarcolemmal fragility, improved cardiac function and enhanced mitochondrial metabolism. Asai et al. (2007) found tadalafil administration improved blood flow, prevented contraction-induced damage and decreased centronucleation in *mdx* skeletal muscle. They also demonstrated that these benefits were abolished by using the vasoconstrictive dose of angiotensin II, thus concluding PDE5 inhibition protected dystrophic myofibres through prevention of functional ischaemia.

This study used the selective PDE5 inhibitor tadalafil, marketed as Cialis for the treatment of erectile dysfunction and as Adcirca for pulmonary arterial hypertension. The drug is orally available in mice and has a half-life of 17.5 hours (Asai et al. 2007). Like related drugs, sildenafil (Viagra) and vardenafil (Levitra), tadalafil maintains cGMP concentrations by inhibiting hydrolysis of cGMP to 5'-GMP.

The genes of interest to this study are TNF-α, TGF-β, procollagen α1 and fibronectin. TNF-α is a much-studied cytokine that is involved in tissue growth and repair, inflammation, apoptosis, inhibition of tumour growth and viral disease (Li & Schwartz 2001; Locksley et al. 2001). Upregulation of TNF-α in DMD contributes to pathology through direct toxicity and augmentation of the inflammatory response (Porreca et al. 1999). Aberrant TGF-β regulation has been linked to profibrotic processes in liver cirrhosis

and chronic pulmonary fibrosis as well as being associated with skeletal muscle fibrosis in DMD (Khalil et al. 1989; Milani et al. 1991; Bernasconi et al. 1995). TGF-β and mechanical strain also increase the expression of procollagen α1 that encodes the α1 collagen chain. Increased expression of this gene has been observed in *mdx* skeletal muscle (Goldspink et al. 1994; Lindahl et al. 2002). Finally, fibronectin influences growth, wound healing, cell differentiation and tissue integrity and was found to be increased in *mdx* diaphragms but decreased in cultured myogenic cells derived from DMD patient muscle biopsies (Delaporte et al. 1990; Valenick et al. 2005; Mezzano et al. 2007; White et al. 2008).

Previous chapters of this dissertation have examined modulation of NO availability through increasing the available NOS substrate (L-arginine) or by increasing production of NO by overexpression of cardiac nNOS. However, these studies did not examine whether these effects were direct or downstream of increased NO availability. The current study examined outcomes of tadalafil administration and the co-precipitate during manufacture, the enteric coating hydroxypropyl methylcellulose phthalate (HPMCP), on the expression levels of a number of profibrotic genes as well as direct measurements of fibrosis in the diaphragm, TA and cardiac muscles of *mdx* mice.

6.2 Materials and methods

Male nine month old *mdx* mice were treated with 60 mg/kg/day tadalafil or the co-precipitate, 60mg/kg/day HPMCP, for a period of three months. Drugs were administered in a mixture of standard mouse pellets and 5% dextrose solution with animals allowed to eat *ad libitum*. Age and gender-matched C57 and untreated *mdx* were used as controls.

The hypothesis was that chronic treatment with tadalafil would improve cardiac function, as measured by Millar catheter and Langendorff isolated heart techniques. However, these measurements could not be completed. Tadalafil-treated mice experienced severe orthostatic hypotension and rapidly developed a Cheyne-Stokes breathing pattern that precluded measurement of arterial and ventricular pressure development. The *in vivo* experiments were then discontinued. Data collected during Langendorff experiments yielded supra-physiological values that were unable to be corrected. Consequently, this *ex vivo* data did not accurately reflect cardiac function and was also excluded. These experiments were to be followed by assessment of skeletal and cardiac muscle fibrosis using digital quantification of picrosirius red staining of tissue collagen and measurement of profibrotic gene expression by qRT-PCR; these experiments form the remainder of this chapter.

Data presented includes quantification of profibrotic gene expression in both skeletal and cardiac muscles as well as direct histological measurement of muscle fibrosis in the same tissues. Skeletal muscles used were the diaphragm and the tibialis anterior (TA) muscle. Genes of interest were TNF-α, TGF-β, procollagen α1 (PC1) and fibronectin. Techniques used in this study are detailed in Chapter 2. A summary of mouse numbers, techniques and difficulties encountered is provided in Figure 6.2.

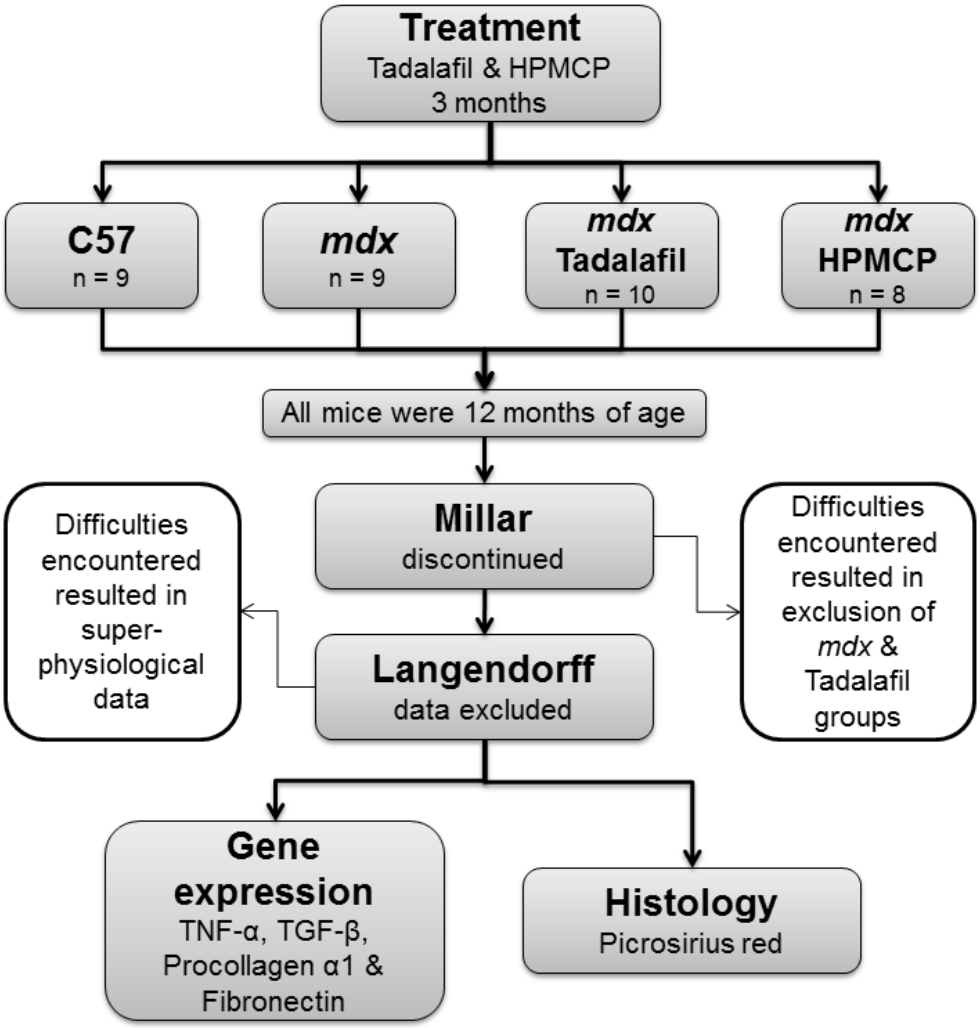


Figure 6.2 Summary of mouse numbers and techniques used in Chapter 6.

6.3 Results

6.3.1 Gene expression

Gene quantification studies were conducted to determine whether treatment with tadalafil or HPMCP altered fibrosis through modulation of profibrotic gene expression. Quantification of profibrotic gene mRNA was conducted using qRT-PCR, with β-actin used as the housekeeper gene. Genes of interest were TNF-α, TGF-β, PC1 and fibronectin. The expression of these genes was measured in heart, diaphragm and TA muscles and is summarised in Table 6.1.

TNF-α expression

TNF-α expression was elevated in the hearts of HPMCP mice (P<0.05) but remained unaltered in both skeletal muscles (Figure 6.3B & C). Tadalafil administration did not alter TNF-α expression in any of the tested muscles (Figure 6.3).

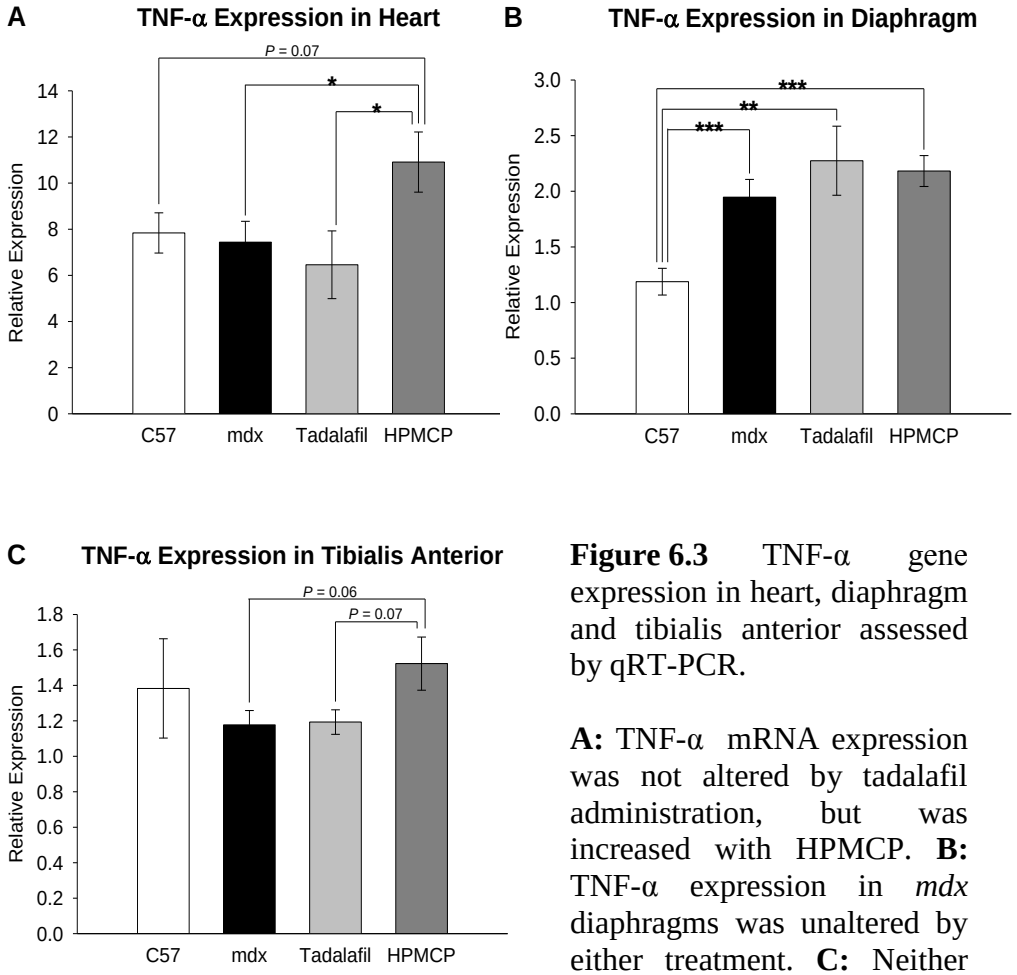


Figure 6.3 TNF-α gene expression in heart, diaphragm and tibialis anterior assessed by qRT-PCR.

A: TNF-α mRNA expression was not altered by tadalafil administration, but was increased with HPMCP. **B:** TNF-α expression in *mdx* diaphragms was unaltered by either treatment. **C:** Neither tadalafil nor HPMCP administration caused changes in TNF-α expression in *mdx* TA muscle. (* P<0.05; ** P<0.01; *** P<0.001)

TGF-β expression

TGF-β expression was generally elevated in *mdx* groups (*mdx*, tadalafil and HPMCP) in the heart and TA muscle but not diaphragm when compared to C57 (heart $P<0.001$ for all groups; diaphragm $P<0.05$ for HPMCP; TA $P<0.001$ for all groups) (Figure 6.4). TGF-β expression was not altered by either tadalafil or HPMCP treatment compared to *mdx* control data suggesting that cGMP did not play a role in regulating this cytokine (Figure 6.4). HPMCP administration caused a significant increase in TGF-β mRNA expression in the diaphragms of mdx mice compared to healthy controls (C57) but this increase was not statistically different to *mdx* control data.

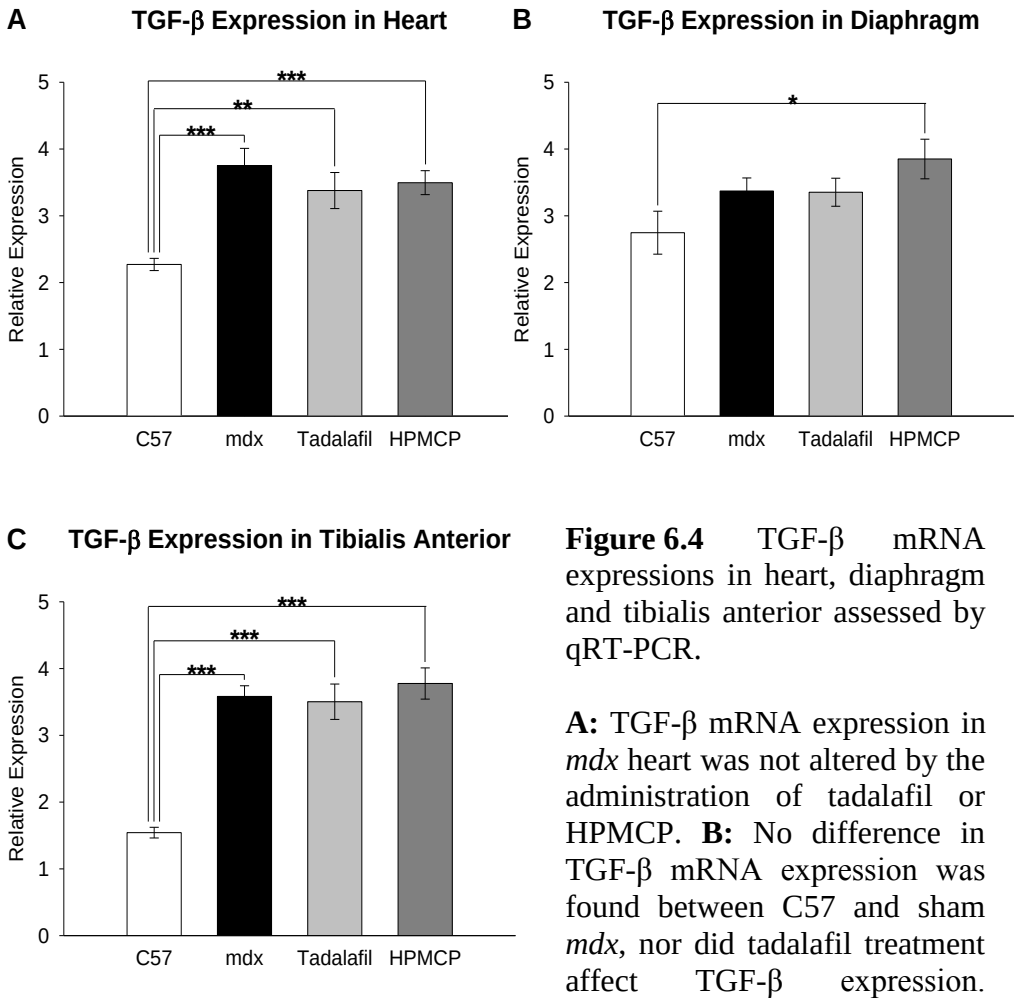


Figure 6.4 TGF-β mRNA expressions in heart, diaphragm and tibialis anterior assessed by qRT-PCR.

A: TGF-β mRNA expression in *mdx* heart was not altered by the administration of tadalafil or HPMCP. **B:** No difference in TGF-β mRNA expression was found between C57 and sham *mdx*, nor did tadalafil treatment affect TGF-β expression. HPMCP treatment caused TGF-β expression to increase in *mdx* diaphragms compared to C57 but not compared to *mdx* controls. **C:** Neither treatment caused alterations in TGF-β expression in *mdx* TA muscle.

* $P<0.05$; ** $P<0.01$; *** $P<0.001$)

Procollagen α1 expression

PC1 expression was increased in the hearts, diaphragms and TA muscles of *mdx* mice compared to C57. Treatment with tadalafil did not change PC1 mRNA expression in either cardiac or skeletal muscles. HPMCP administration increased PC1 expression in heart (P<0.01) and diaphragm (P<0.01) of *mdx* mice but not their TA muscles (Figure 6.5).

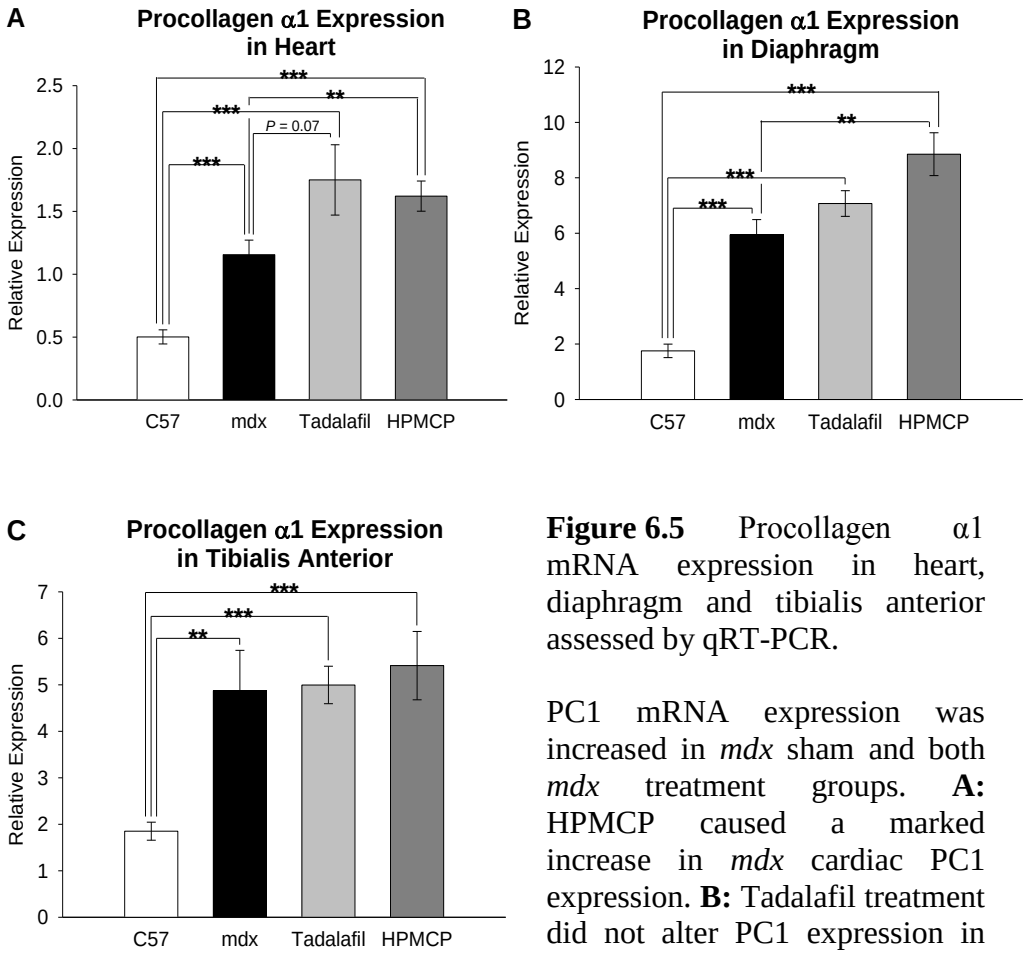


Figure 6.5 Procollagen α1 mRNA expression in heart, diaphragm and tibialis anterior assessed by qRT-PCR.

PC1 mRNA expression was increased in *mdx* sham and both *mdx* treatment groups. **A:** HPMCP caused a marked increase in *mdx* cardiac PC1 expression. **B:** Tadalafil treatment did not alter PC1 expression in *mdx* diaphragm; however HPMCP treatment caused an increase in this tissue. **C:** Neither treatment caused alterations in PC1 expression in *mdx* TA muscle. (** P<0.01; *** P<0.001)

Fibronectin expression

Fibronectin expression in the heart was increased by HPMCP administration (P<0.01) but not tadalafil administration (Figure 6.6A). Tadalafil administration caused a decrease in fibronectin expression in *mdx* diaphragm (P<0.05) (Figures 6.6B). *Mdx* groups had greater levels of fibronectin expression in heart (P<0.001) and TA muscles (P<0.01) but not diaphragm compared to C57.

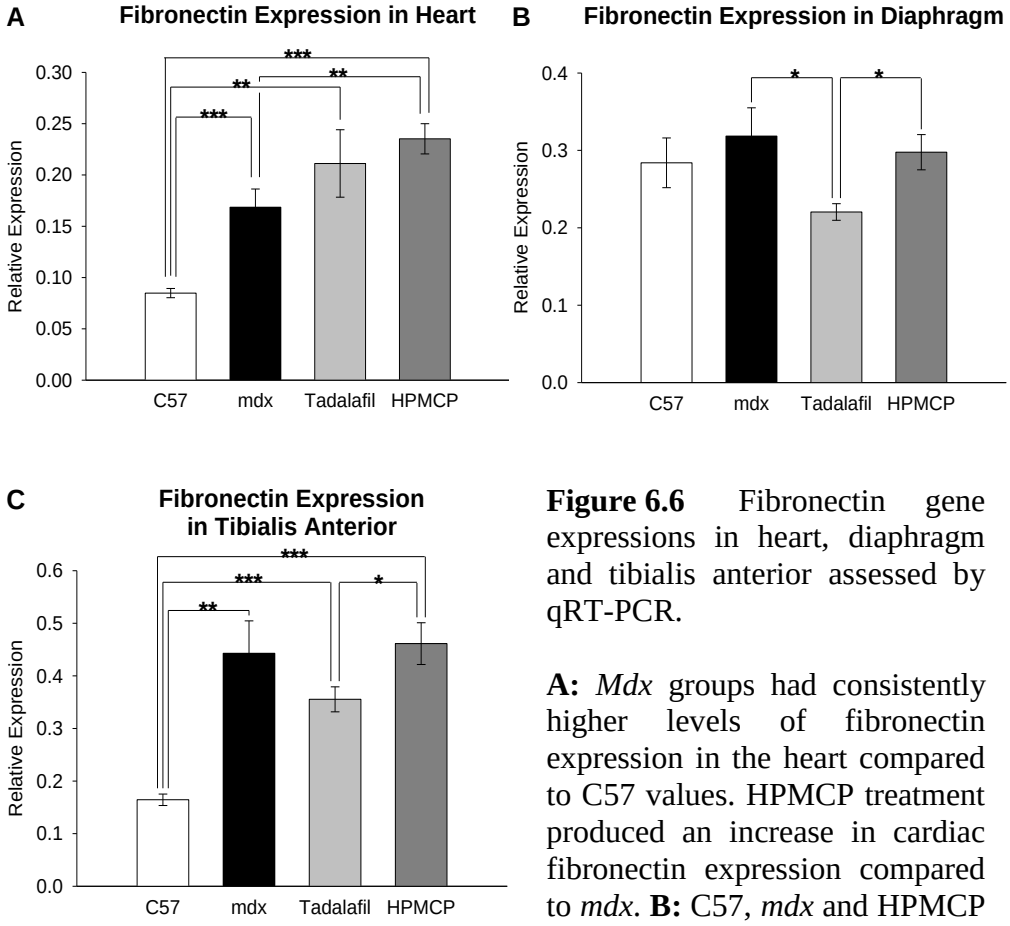


Figure 6.6 Fibronectin gene expressions in heart, diaphragm and tibialis anterior assessed by qRT-PCR.

A: *Mdx* groups had consistently higher levels of fibronectin expression in the heart compared to C57 values. HPMCP treatment produced an increase in cardiac fibronectin expression compared to *mdx*. **B:** C57, *mdx* and HPMCP fibronectin expression did not differ in the diaphragm. Tadalafil treatment produced decreased fibronectin mRNA expression in this muscle. **C:** *Mdx* groups had higher fibronectin expression in the TA muscle. Tadalafil treatment caused a reduction in fibronectin expression. (* P<0.05; ** P<0.01; *** P<0.001)

Table 6.1 Summary of profibrotic gene expression with tadalafil and HPMCP administration in selected *mdx* muscle.

	TNF-α		TGF-β		PC1		Fibronectin	
	Tad	HPMCP	Tad	HPMCP	Tad	HPMCP	Tad	HPMCP
Heart	-	↑*	-	-	-	↑**	-	↑**
Dia	-	-	-	↑* vs C57 not *mdx*	-	↑**	↓*	-
TA	-	-	-	-	-	-	-	-

Table 6.1 shows tadalafil treatment decreased mRNA expression of fibronectin in *mdx* diaphragms while HPMCP administration upregulated TNF-α, PC1 and fibronectin in *mdx* hearts as well as increasing expression of TGF-β and PC1 in *mdx* diaphragms. (* $P<0.05$; ** $P<0.01$)

6.3.2 Histology

Histological measurement of cardiac and skeletal fibrosis was conducted using digital quantification of picrosirius red staining of collagen. Tissues tested included the heart, diaphragm and TA muscle. Representative images of stained sections from these muscles are presented in Figure 6.7. Reflecting the underlying disease process, *mdx* muscle had greater collagen content ($P<0.001$ for all tissues examined) (Figure 6.8). Tadalafil and HPMCP-treated groups did not differ from their *mdx* control indicating that cGMP did not play a role in preventing fibrosis.

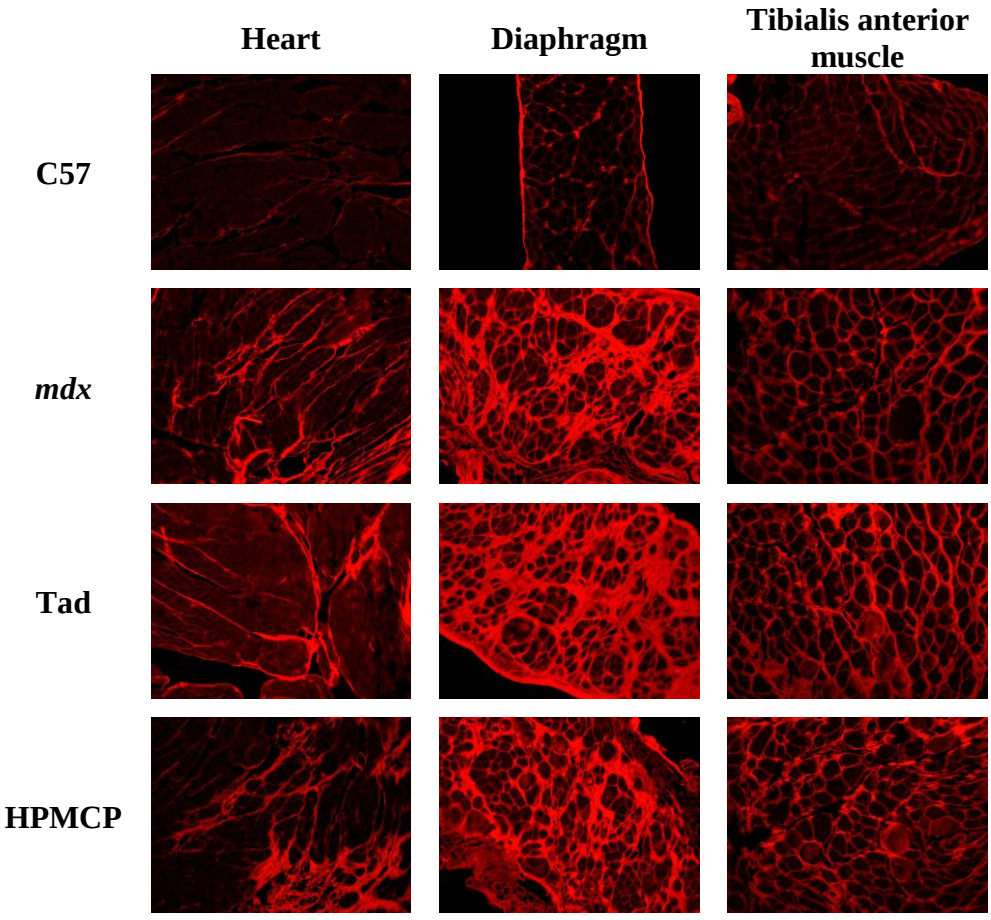


Figure 6.7 Representative images of cardiac and skeletal muscle sections stained with picrosirius red.

Neither tadalafil nor HPMCP treatment affected collagen levels in either cardiac or skeletal muscle.

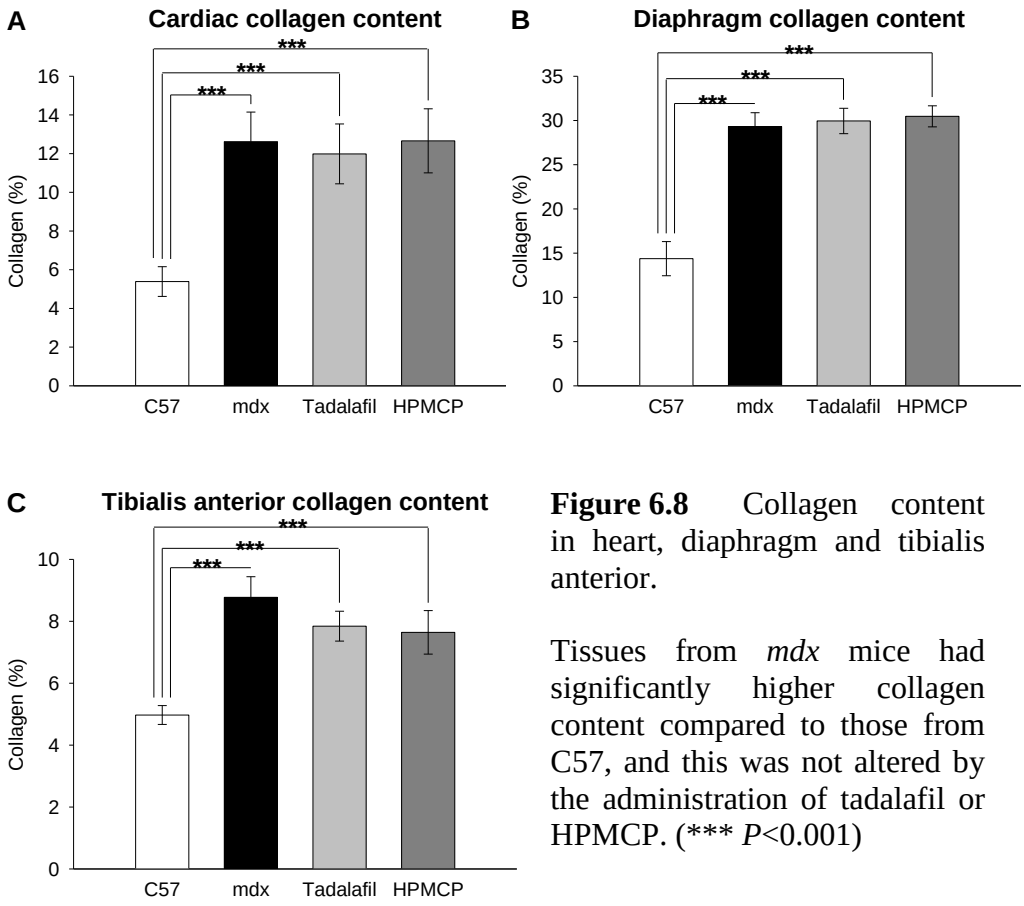


Figure 6.8 Collagen content in heart, diaphragm and tibialis anterior.

Tissues from *mdx* mice had significantly higher collagen content compared to those from C57, and this was not altered by the administration of tadalafil or HPMCP. (*** $P<0.001$)

6.4 Discussion

Muscle fibrosis is perhaps the most prominent characteristic of dystrophic pathology. It produces important pathological changes including progressive muscle weakness, molecular and electrical signal disturbances and joint contracture (Bockhold et al. 1998; Morrison et al. 2000; Deconinck & Dan 2007; Kinali et al. 2007). Therefore, it is important to understand the processes involved in the development of fibrotic changes in order to develop more directed treatment strategies. In this study, PDE5 inhibition was investigated to determine whether preventing the breakdown of cGMP decreased the development of muscle fibrosis in a dystrophic setting. Tadalafil was chosen as a PDE5 inhibitor as the drug's long half-life allowed for easier administration. HPMCP, commonly used as an enteric coating since the early 1970's, was also tested as it is a co-precipitate of tadalafil during drug manufacture. Tadalafil treatment did not alter fibrosis in twelve month old *mdx* mice; there were no changes in profibrotic gene expression or direct measurement of muscle collagen in either cardiac or skeletal muscles. HPMCP administration caused increases in the expression of each of the profibrotic genes in selected tissues but did not alter tissue collagen. While cGMP may have functional benefits for dystrophic cardiac (Khairallah et al. 2008) and skeletal muscles (Asai et al. 2007), results from this study suggest that cGMP has minimal if any role in reducing established dystrophic fibrosis.

However, PDE5 inhibition may produce improvements in dystrophic muscle. Aberrant calcium handling has been long recognised as an important trigger of dystrophic pathology (Bodensteiner & Engel 1978; Allen et al. 2005). In addition to its important role in vasodilatation, the cGMP-PKG pathway also mediates calcium dynamics. cGMP acts upon the SR though a second messenger pathway to change the phosphorylation state of the RyR2 (Takasago et al. 1991) and decrease myofilament calcium sensitivity by troponin 1 phosphorylation (Shah et al. 1994; Layland et al. 2002). More specific to dystrophic pathology, Ascah et al. (2011) found PDE5 inhibition using sildenafil reduced cellular calcium loading and

mitochondrial calcium uptake in *mdx* hearts. However, further work into defining the roles of cGMP-mediated pathways in dystrophin deficiency is needed as cGMP also affects other important signalling molecules. For example, it may activate or inhibit PDEs responsible for the inactivation of PKA (Fischmeister et al. 2005).

PDE5 inhibition has produced functional benefits that protect both cardiac and skeletal dystrophin-deficient myocytes. Sildenafil-treated *mdx* mice showed enhanced cardiac cGMP signalling and subsequent improvements in contractile performance, metabolic status and sarcolemmal integrity (Khairallah et al. 2008). In *mdx* skeletal muscle, administration of clenbuterol, 8-CPT cGMP and tadalafil increased cGMP availability with benefits observed in decreased contraction-induced muscle damage and reduced muscle turnover (Asai et al. 2007). It was theorised that functional improvements may result in improved cell survival that would have been reflected as a reduction in fibrotic replacement of dead cells in the heart wall.

While not borne out in this study, evidence indicates that PDE5 inhibition may play a role in attenuating fibrosis in other conditions. In pulmonary fibrosis, beneficial effects of PDE5 inhibition were derived from prevention of lipid peroxidation, cytokine production and/or release and neutrophil accumulation, while in hepatic fibrosis a decline in PC1 mRNA expression was seen in bile duct-ligated rats treated with udenafil (Choi et al. 2009; Yildirim et al. 2010). In muscle tissues, sildenafil administration led to decreased expression of profibrotic factors in smooth muscle and, in addition to reducing cardiac fibrosis, PDE5 inhibition reversed eccentric remodelling, maladaptive molecular signalling and reduced function associated with sustained pressure overload in a murine model (Zhang et al. 2010; Sirad et al. 2011).

With one exception, the results of this study show PDE5 inhibition using tadalafil did not alter the mRNA expressions of TNF-α, TNF-β, PC1 or fibronectin in *mdx* heart or skeletal muscle. The exception was a decreased

fibronectin expression in *mdx* diaphragms to a level that was no longer significantly different to healthy controls. While the exact reason for this down-regulation remains unclear, fibronectin upregulates cGMP (Huntley et al. 2006; Chamorro-Jorganes et al. 2011). It may have been that increased concentrations of cGMP as a result of PDE5 inhibition suppressed or removed this stimulus. This situation, coupled with the absence of other upregulating factors such as during wound repair, allowed fibronectin expression to remain low. However, this explanation does not account for the unchanged levels of fibronectin expression found in the other muscles. In addition, the decrease in diaphragm fibronectin expression was not reflected as decreased collagen in this tissue.

HPMCP administration caused increases in various genes in different tissues. It increased expression of TNF-α, PC1 and fibronectin in *mdx* hearts while also increasing expression of TGF-β and PC1 in *mdx* diaphragms. It should be noted that these increases did not result in corresponding increases in tissue collagen. HPMCP is a cellulose-based enteric coating that has been used since the early 1970's and has been deemed safe by the U.S. National Formulary, European Pharmacopeia, and Japanese Pharmacopeia. In commercial uses, it may have additives such as pigments or plasticisers added, however the HPMCP used in this study was a co-precipitate with tadalafil during manufacture and as such was pure. Thus, the upregulation of profibrotic genes in this setting can only be attributed to HPMCP. To the best knowledge of the author, there have been no previous studies investigating the effects of HPMCP on gene expression. Early toxicology reports conducted in 1973 using rats and dogs reported diarrhoea with high doses and slight increases in the weights of livers, lungs, adrenals and ovaries in Wistar rats (reported in Brown 2007). Given the findings of this study, it may be prudent to evaluate effect of HPMCP on pro-fibrotic gene expression in wild type mice using more modern techniques and procedures.

Finally, the age of the mice is also of importance. The mice used in this study commenced treatment at the age of nine months and were treated for three months, in contrast to the young mice used in other studies that found

PDE5 inhibition to be functionally beneficial (Asai et al. 2007; Khairallah et al. 2008). Given that *mdx* mice undergo a 'crisis' period at approximately 21 days of age (McGreachie et al. 1993; Grounds & Torrisi 2004), the mice used in this study would have already developed marked fibrotic changes. This study has shown PDE5 inhibition does not ameliorate the established muscle fibrosis characteristic of adult/aged dystrophin deficiency. Therefore, in order to gain maximum benefit, future studies investigating antifibrotic actions of enhanced cGMP signalling in dystrophin deficiency should be targeted at very young mice as a preventive protocol.

Chapter 7: Conclusions and future directions

DMD is the result of mutations in the dystrophin gene that lead to either the non-production of dystrophin or the production of a non-functional dystrophin protein. The dystrophin gene is the largest in the human genome leaving it susceptible to mutation; one third of DMD cases arise from spontaneous mutation with remaining cases being inherited from a carrier mother. This disease affects one in 3500 live male births and is not bound by social or economic boundaries.

The lack of dystrophin results in a complex range of serious pathologies that are always fatal. Most notably, the loss of this single protein leads to progressive muscle wasting characterised by the continuing replacement of muscle with fatty and fibrotic tissue, leading to weakness and eventual joint and spinal contractures. Dystrophin is important because it provides mechanical support to muscle cells linking contractile machinery to the cytoskeleton thus protecting both from contraction-induced injury. Further, dystrophin is an integral part of a complex of proteins at the sarcolemma acting as an anchor for the localisation of other molecules important in cell signalling pathways.

The full ramifications of dystrophin deficiency are still being investigated although DMD has been extensively studied and treatments have been developed that have prolonged mobility and life expectancy. It is only through a full and complete understanding of these complex interactions that directed treatments for those suffering DMD can be developed. Developing effective treatments for DMD is not only important to current patients and their families but implications for developing techniques that lead to a treatment for a genetic disease apply to a broad range of ailments. The study of DMD is not simply the study of a single disease, as understanding the genetic aetiology and complex pathology also opens avenues to the treatment of other diseases. While a number of promising gene-based therapies are being explored, there remains a need for new

therapies that are targeted at improving the quality and quantity of life for those affected by DMD.

The aim of this work was to explore the effects of myostatin absence and modulation of the NO-cGMP pathway in the dystrophic setting focussing on cardiac function and muscle fibrosis. The rationale behind these studies was that modifications of these pathways are potentially novel ways to treat this disease. Modulation was achieved through pharmacological manipulation of the NO-cGMP pathway and generation of myostatin knock-out *mdx* and nNOS cardiac overexpression *mdx* strains of mice.

Chapter 3 describes the effects of myostatin absence in the dystrophic heart. Absence of this endogenous inhibitor of muscle growth in *mdx* hearts resulted in decreased MABP, cardiac hypertrophy accompanied by increased cardiac function and increased cardiac fibrosis. This study showed a functional rescue of the dystrophic heart can be achieved through the absence of myostatin but this benefit was tempered by a significant increase in cardiac fibrosis. Difficulties with anaesthetic usage were also noted. These drawbacks have particular impact on boys with DMD as they are characteristic of the human disease. While results from this study are encouraging in terms of functional benefits, future work examining the potential of myostatin modulation should focus on pharmacological inhibition as well as attempting to define the paradox of increased cardiac function together with increased fibrosis.

An early target for manipulation of the NO-cGMP pathways is altering the availability of the NOS substrate, L-arginine. Previous studies investigating this treatment option have shown that while functional benefits are possible, increases in muscle fibrosis, accompanied by potential exacerbation of contractures, bring the long-term use of this supplement into question. The focus of chapter 4 was to determine whether there was a safe long-term dose for dietary L-arginine supplementation. Results from this study indicated that low-dose L-arginine showed no functional benefit and did not reduce cardiac fibrosis.

Detailed in chapter 5, overexpression of cardiac nNOS in *mdx* mice resulted in normalisation of a number of single cell parameters in isolated cardiomyocytes. *Mdx* cardiomyocytes showed diminished calcium transients and depressed calcium currents but also had relatively strong cellular contractions. nNOS overexpression in *mdx* cardiomyocytes increased calcium transient height and prolonged the peak time interval. In addition, L-type calcium channel activation and inactivation properties were also normalised. *Mdx* cardiomyocyte calcium sensitivity was decreased with overexpression of nNOS, also bringing this parameter closer to normal values. Finally, the force-frequency relationship of *mdx* nNOS-overexpressing cells was not different to healthy controls. These results lend credence to NO playing an important, if secondary, role in dystrophic pathology. Of interest, the presence of an additional outward current in *mdx* cardiomyocytes, possibly revealed by the L-type current inactivation, was observed in these cells. Future study should investigate the applicability of normalising nNOS concentrations to the sarcolemma as well as identifying additional aberrations in the movement of other important ions in dystrophic cells.

Chapter 6 showed chronic PDE5 inhibition using tadalafil did not alter nRNA expressions of TNF-α, TGF-β, fibronectin or PC1 in either cardiac or skeletal muscles of *mdx* mice. However, there was evidence that HPMCP may elevate expression of these profibrotic cytokines in a dystrophic setting. Direct measurements of cardiac and skeletal muscle fibrosis also showed no benefit from chronic PDE5 inhibition. Future research should be directed to confirming whether HPMCP has profibrotic properties, with a particular focus on using more up-to-date techniques to gather information currently unavailable. If proven, there are important implications for not only boys with DMD but for sufferers of other fibrotic conditions such as cystic fibrosis or pulmonary fibrosis as HPMCP has been commonly used in pharmaceutical manufacture as an enteric coating since the early 1970's. Future study should also focus on early commencement of chronic PDE5 inhibition in DMD given current evidence indicating benefits from augmenting cGMP signalling with respect to ameliorating functional

ischaemia in dystrophic muscle. Caution should be taken as cGMP has many actions and chronic inhibition may have unforeseen outcomes.

The studies in this dissertation aimed to evaluate the effects of myostatin absence and modulation of the NO-cGMP pathways in an established stage of the dystrophic pathology. To this end, mice used were either old (twelve months of age) or very old (fifteen to eighteen months of age). Although not achieving statistical significance, many of the results reported here have shown strong trends toward functional improvement on the background of either maintained or increased fibrosis. Earlier intervention using these treatments may produce these benefits before the development of significant pathology and bring about measureable functional improvements.

Clinical trials investigating the potential and safety of molecular therapies such as AO-mediated exon skipping offer hope for mitigating dystrophic pathology, but there are still hurdles to be overcome before these treatments become widely available. Until methods for early diagnosis and treatments that provide persistent functional relief for sufferers of DMD are readily available, there remains need for better palliative treatments to be developed. The work detailed in this dissertation shows that if administered before the onset of significant fibrosis, directed myostatin inhibition and NO modulation have real potential to improve cardiac function in those with DMD.

References

Aartsma-Rus, A & van Ommen, GJ 2007, 'Antisense-mediated exon skipping: a versatile tool with therapeutic and research applications', *RNA*, vol. 13, no. 10, pp. 1609-24.

Aartsma-Rus, A, Van Deutekom, JC, Fokkema, IF, Van Ommen, GJ & Den Dunnen, JT 2006, 'Entries in the Leiden Duchenne muscular dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule', *Muscle Nerve*, vol. 34, no. 2, pp. 135-44.

Aartsma-Rus, A, Fokkema, I, Verschuuren, J, Ginjaar, I, van Deutekom, J, van Ommen, GJ & den Dunnen, JT 2009, 'Theoretic applicability of antisense-mediated exon skipping for Duchenne muscular dystrophy mutations', *Hum Mutat*, vol. 30, no. 3, pp. 293-9.

Abmayr, S, Gregorevic, P, Allen, JM & Chamberlain, JS 2005, 'Phenotypic improvement of dystrophic muscles by rAAV/microdystrophin vectors is augmented by Igf1 codelivery', *Mol Ther*, vol. 12, no. 3, pp. 441-50.

Abu-Soud, HM & Hazen, SL 2000, 'Nitric oxide modulates the catalytic activity of myeloperoxidase', *J Biol Chem*, vol. 275, no. 8, pp. 5425-30.

Albina, JE, Cui, S, Mateo, RB & Reichner, JS 1993, 'Nitric oxide-mediated apoptosis in murine peritoneal macrophages', *J Immunol*, vol. 150, no. 11, pp. 5080-5.

Alderton, JM & Steinhardt, RA 2000a, 'Calcium influx through calcium leak channels is responsible for the elevated levels of calcium-dependent proteolysis in dystrophic myotubes', *J Biol Chem*, vol. 275, no. 13, pp. 9452-60.

Alderton, JM & Steinhardt, RA 2000b, 'How calcium influx through calcium leak channels is responsible for the elevated levels of calcium-dependent proteolysis in dystrophic myotubes', *Trends Cardiovasc Med*, vol. 10, no. 6, pp. 268-72.

Allen, DG, Whitehead, NP & Yeung, EW 2005, 'Mechanisms of stretch-induced muscle damage in normal and dystrophic muscle: role of ionic changes', *J Physiol*, vol. 567, no. Pt 3, pp. 723-35.

Alloatti, G, Gallo, MP, Penna, C & Levi, RC 1995, 'Properties of cardiac cells from dystrophic mouse', *J Mol Cell Cardiol*, vol. 27, no. 8, pp. 1775-9.

Alter, J, Lou, F, Rabinowitz, A, Yin, H, Rosenfeld, J, Wilton, SD, Partridge, TA & Lu, QL 2006, 'Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology', *Nat Med*, vol. 12, no. 2, pp. 175-7.

Ambrosio, CE, Valadares, MC, Zucconi, E, Cabral, R, Pearson, PL, Gaiad, TP, Canovas, M, Vainzof, M, Miglino, MA & Zatz, M 2008, 'Ringo, a Golden Retriever Muscular Dystrophy (GRMD) dog with absent dystrophin but normal strength', *Neuromuscul Disord*, vol. 18, no. 11, pp. 892-3.

Amrani, M, O'Shea, J, Allen, NJ, Harding, SE, Jayakumar, J, Pepper, JR, Moncada, S & Yacoub, MH 1992, 'Role of basal release of nitric oxide on coronary flow and mechanical performance of the isolated rat heart', *J Physiol*, vol. 456, pp. 681-7.

Amthor, H, Otto, A, Vulin, A, Rochat, A, Dumonceaux, J, Garcia, L, Mouisel, E, Hourde, C, Macharia, R, Friedrichs, M, Relaix, F, Zammit, PS, Matsakas, A, Patel, K & Partridge, T 2009, 'Muscle hypertrophy driven by myostatin blockade does not require stem/precursor-cell activity', *Proc Natl Acad Sci U S A*, vol. 106, no. 18, pp. 7479-84.

Anderson, JE 2000, 'A Role for Nitric Oxide in Muscle Repair: Nitric Oxide-mediated Activation of Muscle Satellite Cells', *Mol. Biol. Cell*, vol. 11, no. 5, pp. 1859-2449.

Anderson, JE, Weber, M & Vargas, C 2000, 'Deflazacort increases laminin expression and myogenic repair, and induces early persistent functional gain in mdx mouse muscular dystrophy', *Cell Transplant*, vol. 9, no. 4, pp. 551-64.

Anderson, JE, Garrett, K, Moor, A, McIntosh, L & Penner, K 1998, 'Dystrophy and myogenesis in mdx diaphragm muscle', *Muscle Nerve*, vol. 21, no. 9, pp. 1153-65.

Anderson, JL, Head, SI, Rae, C & Morley, JW 2002, 'Brain function in Duchenne muscular dystrophy', *Brain*, vol. 125, no. Pt 1, pp. 4-13.

Angelini, C 2007, 'The role of corticosteroids in muscular dystrophy: a critical appraisal', *Muscle Nerve*, vol. 36, no. 4, pp. 424-35.

Angelini, C, Pegoraro, E, Turella, E, Intino, MT, Pini, A & Costa, C 1994, 'Deflazacort in Duchenne dystrophy: study of long-term effect', *Muscle Nerve*, vol. 17, no. 4, pp. 386-91.

Aparicio, LF, Jurkovic, M & DeLullo, J 2002, 'Decreased bone density in ambulatory patients with duchenne muscular dystrophy', *J Pediatr Orthop*, vol. 22, no. 2, pp. 179-81.

Araki, E, Nakamura, K, Nakao, K, Kameya, S, Kobayashi, O, Nonaka, I, Kobayashi, T & Katsuki, M 1997, 'Targeted disruption of exon 52 in the mouse dystrophin gene induced muscle degeneration similar to that observed in Duchenne muscular dystrophy', *Biochem Biophys Res Commun*, vol. 238, no. 2, pp. 492-7.

Archer, JD, Vargas, CC & Anderson, JE 2006, 'Persistent and improved functional gain in mdx dystrophic mice after treatment with L-arginine and deflazacort', *Faseb J*, vol. 20, no. 6, pp. 738-40.

Artaza, JN, Reisz-Porszasz, S, Dow, JS, Kloner, RA, Tsao, J, Bhasin, S & Gonzalez-Cadavid, NF 2007, 'Alterations in myostatin expression are associated with changes in cardiac left ventricular mass but not ejection fraction in the mouse', *J Endocrinol*, vol. 194, no. 1, pp. 63-76.

Asai, A, Sahani, N, Kaneki, M, Ouchi, Y, Martyn, JA & Yasuhara, SE 2007, 'Primary role of functional ischemia, quantitative evidence for the two-hit mechanism, and phosphodiesterase-5 inhibitor therapy in mouse muscular dystrophy', *PLoS One*, vol. 2, no. 8, p. e806.

Ascah, A, Khairallah, M, Daussin, F, Bourcier-Lucas, C, Godin, R, Allen, BG, Petrof, BJ, Des Rosiers, C & Burelle, Y 2011, 'Stress-induced opening of the permeability transition pore in the dystrophin-deficient heart is attenuated by acute treatment with sildenafil', *Am J Physiol Heart Circ Physiol*, vol. 300, no. 1, pp. H144-53.

Ashley, EA, Sears, CE, Bryant, SM, Watkins, HC & Casadei, B 2002, 'Cardiac nitric oxide synthase 1 regulates basal and beta-adrenergic contractility in murine ventricular myocytes', *Circulation*, vol. 105, no. 25, pp. 3011-6.

Austin, L, de Niese, M, McGregor, A, Arthur, H, Gurusinghe, A & Gould, MK 1992, 'Potential oxyradical damage and energy status in individual muscle fibres from degenerating muscle diseases', *Neuromuscul Disord*, vol. 2, no. 1, pp. 27-33.

Bach, JR 1993, 'Mechanical insufflation-exsufflation. Comparison of peak expiratory flows with manually assisted and unassisted coughing techniques', *Chest*, vol. 104, no. 5, pp. 1553-62.

Bach, JR, Ishikawa, Y & Kim, H 1997, 'Prevention of pulmonary morbidity for patients with Duchenne muscular dystrophy', *Chest*, vol. 112, no. 4, pp. 1024-8.

Bachrach, LK 2005, 'Taking steps towards reducing osteoporosis in Duchenne muscular dystrophy', *Neuromuscul Disord*, vol. 15, no. 1, pp. 86-7.

Bahler, RC, Mohyuddin, T, Finkelhor, RS & Jacobs, IB 2005, 'Contribution of Doppler tissue imaging and myocardial performance index to assessment of left ventricular function in patients with Duchenne's muscular dystrophy', *J Am Soc Echocardiogr*, vol. 18, no. 6, pp. 666-73.

Bal, E & Sanwall, W 1980, 'A synergistic effect of glucocorticosteriods and insulin on the differentiation of myoblasts', *Journal of Cell Physiology*, vol. 102, pp. 27-36.

Balghi, H, Sebille, S, Constantin, B, Patri, S, Thoreau, V, Mondin, L, Mok, E, Kitzis, A, Raymond, G & Cognard, C 2006, 'Mini-dystrophin expression down-regulates overactivation of G protein-mediated IP3 signaling pathway in dystrophin-deficient muscle cells', *J Gen Physiol*, vol. 127, no. 2, pp. 171-82.

Balligand, JL, Feron, O & Dessy, C 2009, 'eNOS activation by physical forces: from short-term regulation of contraction to chronic remodeling of cardiovascular tissues', *Physiol Rev*, vol. 89, no. 2, pp. 481-534.

Barouch, LA, Harrison, RW, Skaf, MW, Rosas, GO, Cappola, TP, Kobeissi, ZA, Hobai, IA, Lemmon, CA, Burnett, AL, O'Rourke, B, Rodriguez, ER, Huang, PL, Lima, JA, Berkowitz, DE & Hare, JM 2002, 'Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms', *Nature*, vol. 416, no. 6878, pp. 337-9.

Barton-Davis, ER, Cordier, L, Shoturma, DI, Leland, SE & Sweeney, HL 1999, 'Aminoglycoside antibiotics restore dystrophin function to skeletal muscles of mdx mice', *J Clin Invest*, vol. 104, no. 4, pp. 375-81.

Barton, ER, Morris, L, Kawana, M, Bish, LT & Toursel, T 2005, 'Systemic administration of L-arginine benefits mdx skeletal muscle function', *Muscle Nerve*, vol. 32, no. 6, pp. 751-60.

Basset, O, Boittin, FX, Cognard, C, Constantin, B & Ruegg, UT 2006, 'Bcl-2 overexpression prevents calcium overload and subsequent apoptosis in dystrophic myotubes', *Biochem J*, vol. 395, no. 2, pp. 267-76.

Baydur, A, Gilgoff, I, Prentice, W, Carlson, M & Fischer, DA 1990, 'Decline in respiratory function and experience with long-term assisted ventilation in advanced Duchenne's muscular dystrophy', *Chest*, vol. 97, no. 4, pp. 884-9.

Becker, PE & Kiener, F 1955, 'A new x-chromosomal muscular dystrophy.', *Arch Psychiatr Nervenkr Z Gesamte Neurol Psychiatr*, vol. 193, no. 4, pp. 427-48.

Beech, DJ, Muraki, K & Flemming, R 2004, 'Non-selective cationic channels of smooth muscle and the mammalian homologues of Drosophila TRP', *J Physiol*, vol. 559, no. Pt 3, pp. 685-706.

Beenakker, EA, Fock, JM, Van Tol, MJ, Maurits, NM, Koopman, HM, Brouwer, OF & Van der Hoeven, JH 2005, 'Intermittent prednisone therapy in Duchenne muscular dystrophy: a randomized controlled trial', *Arch Neurol*, vol. 62, no. 1, pp. 128-32.

Beggs, AH, Koenig, M, Boyce, FM & Kunkel, LM 1990, 'Detection of 98% of DMD/BMD gene deletions by polymerase chain reaction', *Hum Genet*, vol. 86, no. 1, pp. 45-8.

Belles, B, Hescheler, J, Trautwein, W, Blomgren, K & Karlsson, JO 1988, 'A possible physiological role of the Ca-dependent protease calpain and its inhibitor calpastatin on the Ca current in guinea pig myocytes', *Pflugers Arch*, vol. 412, no. 5, pp. 554-6.

Benchaouir, R, Meregalli, M, Farini, A, D'Antona, G, Belicchi, M, Goyenvalle, A, Battistelli, M, Bresolin, N, Bottinelli, R, Garcia, L & Torrente, Y 2007, 'Restoration of human dystrophin following transplantation of exon-skipping-engineered DMD patient stem cells into dystrophic mice', *Cell Stem Cell*, vol. 1, no. 6, pp. 646-57.

Bernasconi, P, Torchiana, E, Confalonieri, P, Brugnoni, R, Barresi, R, Mora, M, Cornelio, F, Morandi, L & Mantegazza, R 1995, 'Expression of transforming growth factor-beta 1 in dystrophic patient muscles correlates with fibrosis. Pathogenetic role of a fibrogenic cytokine', *J Clin Invest*, vol. 96, no. 2, pp. 1137-44.

Bertoni, C & Rando, TA 2002, 'Dystrophin gene repair in mdx muscle precursor cells in vitro and in vivo mediated by RNA-DNA chimeric oligonucleotides', *Hum Gene Ther*, vol. 13, no. 6, pp. 707-18.

Bertorini, TE, Bhattacharya, SK, Palmieri, GM, Chesney, CM, Pifer, D & Baker, B 1982, 'Muscle calcium and magnesium content in Duchenne muscular dystrophy', *Neurology*, vol. 32, no. 10, pp. 1088-92.

Bia, BL, Cassidy, PJ, Young, ME, Rafael, JA, Leighton, B, Davies, KE, Radda, GK & Clarke, K 1999, 'Decreased myocardial nNOS, increased iNOS and abnormal ECGs in mouse models of Duchenne muscular dystrophy', *J Mol Cell Cardiol*, vol. 31, no. 10, pp. 1857-62.

Bianchi, ML, Mazzanti, A, Galbiati, E, Saraifoger, S, Dubini, A, Cornelio, F & Morandi, L 2003, 'Bone mineral density and bone metabolism in Duchenne muscular dystrophy', *Osteoporos Int*, vol. 14, no. 9, pp. 761-7.

Biggar, W, Politano, L, Harris, V, Passamano, L, Vajsar, J, Alman, B, Palladino, A, Comi, L & Nigro, G 2004, 'Deflazacort in Duchenne mucsular dystrophy: a comparison of two different protocols', *Neuromuscular Disorders*, vol. 14, pp. 476-82.

Bish, LT, Morine, KJ, Sleeper, MM & Sweeney, HL 2010, 'Myostatin is upregulated following stress in an Erk-dependent manner and negatively regulates cardiomyocyte growth in culture and in a mouse model', *PLoS One*, vol. 5, no. 4, p. e10230.

Bishop, JE & Lindahl, G 1999, 'Regulation of cardiovascular collagen synthesis by mechanical load', *Cardiovasc Res*, vol. 42, no. 1, pp. 27-44.

Blake, DJ, Weir, A, Newey, SE & Davies, KE 2002, 'Function and genetics of dystrophin and dystrophin-related proteins in muscle', *Physiol Rev*, vol. 82, no. 2, pp. 291-329.

Bobon, D, Breulet, M, Gerard-Vandenhove, MA, Guiot-Goffioul, F, Plomteux, G, Sastre-y-Hernandez, M, Schratzer, M, Troisfontaines, B, von Frenckell, R & Wachtel, H 1988, 'Is phosphodiesterase inhibition a new mechanism of antidepressant action? A double blind double-dummy study between rolipram and desipramine in hospitalized major and/or endogenous depressives', *Eur Arch Psychiatry Neurol Sci*, vol. 238, no. 1, pp. 2-6.

Bockhold, KJ, Rosenblatt, JD & Partridge, TA 1998, 'Aging normal and dystrophic mouse muscle: analysis of myogenicity in cultures of living single fibers', *Muscle Nerve*, vol. 21, no. 2, pp. 173-83.

Bodensteiner, JB & Engel, AG 1978, 'Intracellular calcium accumulation in Duchenne dystrophy and other myopathies: a study of 567,000 muscle fibers in 114 biopsies', *Neurology*, vol. 28, no. 5, pp. 439-46.

Bogdanovich, S, Perkins, KJ, Krag, TO, Whittemore, LA & Khurana, TS 2005, 'Myostatin propeptide-mediated amelioration of dystrophic pathophysiology', *FASEB J*, vol. 19, no. 6, pp. 543-9.

Bogdanovich, S, Krag, TO, Barton, ER, Morris, LD, Whittemore, LA, Ahima, RS & Khurana, TS 2002, 'Functional improvement of dystrophic muscle by myostatin blockade', *Nature*, vol. 420, no. 6914, pp. 418-21.

Boland, BJ, Silbert, PL, Groover, RV, Wollan, PC & Silverstein, MD 1996, 'Skeletal, cardiac, and smooth muscle failure in Duchenne muscular dystrophy', *Pediatr Neurol*, vol. 14, no. 1, pp. 7-12.

Bonifati, MD, Ruzza, G, Bonometto, P, Berardinelli, A, Gorni, K, Orcesi, S, Lanzi, G & Angelini, C 2000, 'A multicenter, double-blind, randomized trial of deflazacort versus prednisone in Duchenne muscular dystrophy', *Muscle Nerve*, vol. 23, no. 9, pp. 1344-7.

Bothwell, JE, Gordon, KE, Dooley, JM, MacSween, J, Cummings, EA & Salisbury, S 2003, 'Vertebral fractures in boys with Duchenne muscular dystrophy', *Clin Pediatr (Phila)*, vol. 42, no. 4, pp. 353-6.

Brenman, JE, Chao, DS, Xia, H, Aldape, K & Bredt, DS 1995, 'Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy', *Cell*, vol. 82, no. 5, pp. 743-52.

Brenman, JE, Chao, DS, Gee, SH, McGee, AW, Craven, SE, Santillano, DR, Wu, Z, Huang, F, Xia, H, Peters, MF, Froehner, SC & Bredt, DS 1996, 'Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains', *Cell*, vol. 84, no. 5, pp. 757-67.

Brookes, PS, Yoon, Y, Robotham, JL, Anders, MW & Sheu, SS 2004, 'Calcium, ATP, and ROS: a mitochondrial love-hate triangle', *Am J Physiol Cell Physiol*, vol. 287, no. 4, pp. C817-33.

Brown, P 2007, *Pharmacology/Toxicology Review and Evaluation NDA Number 22,210*, viewed 16/7/2012, <http://www.accessdata.fda.gov/drugsatfda_docs/nda/2009/022210s000pharmr.pdf>.

Brown, S & Lucy, J 1997, 'Functions of dystrophin', in S Brown & J Lucy (eds), *Dystrophin: Gene, Protein and Cell Biology*, Cambridge Universtiy Press, Cambridge, pp. 184-92.

Brumsen, C, Hamdy, NA & Papapoulos, SE 1997, 'Long-term effects of bisphosphonates on the growing skeleton. Studies of young patients with severe osteoporosis', *Medicine (Baltimore)*, vol. 76, no. 4, pp. 266-83.

Bulfield, G, Siller, WG, Wight, PA & Moore, KJ 1984, 'X chromosome-linked muscular dystrophy (mdx) in the mouse', *Proc. Natl. Acad. Sci. USA*, vol. 81, no. 4, pp. 1189-92.

Burelle, Y, Khairallah, M, Ascah, A, Allen, BG, Deschepper, CF, Petrof, BJ & Des Rosiers, C 2010, 'Alterations in mitochondrial function as a harbinger of cardiomyopathy: lessons from the dystrophic heart', *J Mol Cell Cardiol*, vol. 48, no. 2, pp. 310-21.

Byerly, L & Yazejian, B 1986, 'Intracellular factors for the maintenance of calcium currents in perfused neurones from the snail, Lymnaea stagnalis', *J Physiol*, vol. 370, pp. 631-50.

Calverley, PM, Sanchez-Toril, F, McIvor, A, Teichmann, P, Bredenbroeker, D & Fabbri, LM 2007, 'Effect of 1-year treatment with roflumilast in severe chronic obstructive pulmonary disease', *Am J Respir Crit Care Med*, vol. 176, no. 2, pp. 154-61.

Campbell, KP 1995, 'Three muscular dystrophies: loss of cytoskeleton-extracellular matrix linkage', *Cell*, vol. 80, no. 5, pp. 675-9.

Cantini, LP, Ferrini, MG, Vernet, D, Magee, TR, Qian, A, Gelfand, RA, Rajfer, J & Gonzalez-Cadavid, NF 2008, 'Profibrotic role of myostatin in Peyronie's disease', *J Sex Med*, vol. 5, no. 7, pp. 1607-22.

Carafoli, E & Molinari, M 1998, 'Calpain: a protease in search of a function?', *Biochem Biophys Res Commun*, vol. 247, no. 2, pp. 193-203.

Carlson, CG 1998, 'The dystrophinopathies: an alternative to the structural hypothesis', *Neurobiol Dis*, vol. 5, no. 1, pp. 3-15.

Carlson, CG & Roshek, DM 2001, 'Adult dystrophic (mdx) endplates exhibit reduced quantal size and enhanced quantal variation', *Pflugers Arch*, vol. 442, no. 3, pp. 369-75.

Carrozza, JP, Jr., Bentivegna, LA, Williams, CP, Kuntz, RE, Grossman, W & Morgan, JP 1992, 'Decreased myofilament responsiveness in myocardial stunning follows transient calcium overload during ischemia and reperfusion', *Circ Res*, vol. 71, no. 6, pp. 1334-40.

Caulton, JM, Ward, KA, Alsop, CW, Dunn, G, Adams, JE & Mughal, MZ 2004, 'A randomised controlled trial of standing programme on bone mineral density in non-ambulant children with cerebral palsy', *Arch Dis Child*, vol. 89, no. 2, pp. 131-5.

Cave, A, Grieve, D, Johar, S, Zhang, M & Shah, AM 2005, 'NADPH oxidase-derived reactive oxygen species in cardiac pathophysiology', *Philos Trans R Soc Lond B Biol Sci*, vol. 360, no. 1464, pp. 2327-34.

Chakkalakal, JV, Harrison, MA, Carbonetto, S, Chin, E, Michel, RN & Jasmin, BJ 2004, 'Stimulation of calcineurin signaling attenuates the dystrophic pathology in mdx mice', *Hum Mol Genet*, vol. 13, no. 4, pp. 379-88.

Chamberlain, JS, Gibbs, RA, Ranier, JE, Nguyen, PN & Caskey, CT 1988, 'Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification', *Nucleic Acids Res*, vol. 16, no. 23, pp. 11141-56.

Chamorro-Jorganes, A, Calleros, L, Griera, M, Saura, M, Luengo, A, Rodriguez-Puyol, D & Rodriguez-Puyol, M 2011, 'Fibronectin upregulates cGMP-dependent protein kinase type Ibeta through C/EBP transcription factor activation in contractile cells', *Am J Physiol Cell Physiol*, vol. 300, no. 3, pp. C683-91.

Chang, WJ, Iannaccone, ST, Lau, KS, Masters, BS, McCabe, TJ, McMillan, K, Padre, RC, Spencer, MJ, Tidball, JG & Stull, JT 1996, 'Neuronal nitric oxide synthase and dystrophin-deficient muscular dystrophy', *Proc Natl Acad Sci U S A*, vol. 93, no. 17, pp. 9142-7.

Chao, DS, Silvagno, F & Bredt, DS 1998, 'Muscular dystrophy in mdx mice despite lack of neuronal nitric oxide synthase', *J Neurochem*, vol. 71, no. 2, pp. 784-9.

Chaubourt, E, Fossier, P, Baux, G, Leprince, C, Israel, M & De La Porte, S 1999, 'Nitric oxide and l-arginine cause an accumulation of utrophin at the sarcolemma: a possible compensation for dystrophin loss in Duchenne muscular dystrophy', *Neurobiol Dis*, vol. 6, no. 6, pp. 499-507.

Chazalette, D, Hnia, K, Rivier, F, Hugon, G & Mornet, D 2005, 'alpha7B integrin changes in mdx mouse muscles after L-arginine administration', *FEBS Lett*, vol. 579, no. 5, pp. 1079-84.

Chenard, AA, Becane, HM, Tertrain, F, de Kermadec, JM & Weiss, YA 1993, 'Ventricular arrhythmia in Duchenne muscular dystrophy: prevalence, significance and prognosis', *Neuromuscul Disord*, vol. 3, no. 3, pp. 201-6.

Chetboul, V, Carlos, C, Blot, S, Thibaud, JL, Escriou, C, Tissier, R, Retortillo, JL & Pouchelon, JL 2004, 'Tissue Doppler assessment of diastolic and systolic alterations of radial and longitudinal left ventricular motions in Golden Retrievers during the preclinical phase of cardiomyopathy associated with muscular dystrophy', *Am J Vet Res*, vol. 65, no. 10, pp. 1335-41.

Cheuk, DK, Wong, V, Wraige, E, Baxter, P, Cole, A, N'Diaye, T & Mayowe, V 2007, 'Surgery for scoliosis in Duchenne muscular dystrophy', *Cochrane Database Syst Rev*, no. 1, p. CD005375.

Choi, SM, Shin, JH, Kim, JM, Lee, CH, Kang, KK, Ahn, BO & Yoo, M 2009, 'Effect of udenafil on portal venous pressure and hepatic fibrosis in rats. A novel therapeutic option for portal hypertension', *Arzneimittelforschung*, vol. 59, no. 12, pp. 641-6.

Chu, V, Otero, JM, Lopez, O, Sullivan, MF, Morgan, JP, Amende, I & Hampton, TG 2002, 'Electrocardiographic findings in mdx mice: a cardiac phenotype of Duchenne muscular dystrophy', *Muscle Nerve*, vol. 26, no. 4, pp. 513-9.

Cirak, S, Arechavala-Gomeza, V, Guglieri, M, Feng, L, Torelli, S, Anthony, K, Abbs, S, Garralda, ME, Bourke, J, Wells, DJ, Dickson, G, Wood, MJ, Wilton, SD, Straub, V, Kole, R, Shrewsbury, SB, Sewry, C, Morgan, JE, Bushby, K & Muntoni, F 2011, 'Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: an open-label, phase 2, dose-escalation study', *Lancet*, vol. 378, no. 9791, pp. 595-605.

Clancy, RM, Leszczynska-Piziak, J & Abramson, SB 1992, 'Nitric oxide, an endothelial cell relaxation factor, inhibits neutrophil superoxide anion production via a direct action on the NADPH oxidase', *J Clin Invest*, vol. 90, no. 3, pp. 1116-21.

Clerk, A, Morris, GE, Dubowitz, V, Davies, KE & Sewry, CA 1993, 'Dystrophin-related protein, utrophin, in normal and dystrophic human fetal skeletal muscle', *Histochem J*, vol. 25, no. 8, pp. 554-61.

Cobham, IG & Davis, HS 1964, 'Anesthesia for Muscle Dystrophy Patients', *Anesth Analg*, vol. 43, pp. 22-9.

Cockcroft, JR, Sciberras, DG, Goldberg, MR & Ritter, JM 1993, 'Comparison of angiotensin-converting enzyme inhibition with angiotensin II receptor antagonism in the human forearm', *J Cardiovasc Pharmacol*, vol. 22, no. 4, pp. 579-84.

Cohn, RD, Liang, HY, Shetty, R, Abraham, T & Wagner, KR 2007, 'Myostatin does not regulate cardiac hypertrophy or fibrosis', *Neuromuscul Disord*, vol. 17, no. 4, pp. 290-6.

Cohn, RD, van Erp, C, Habashi, JP, Soleimani, AA, Klein, EC, Lisi, MT, Gamradt, M, ap Rhys, CM, Holm, TM, Loeys, BL, Ramirez, F, Judge, DP, Ward, CW & Dietz, HC 2007, 'Angiotensin II type 1 receptor blockade attenuates TGF-beta-induced failure of muscle regeneration in multiple myopathic states', *Nat Med*, vol. 13, no. 2, pp. 204-10.

Coirault, C, Lambert, F, Marchand-Adam, S, Attal, P, Chemla, D & Lecarpentier, Y 1999, 'Myosin molecular motor dysfunction in dystrophic mouse diaphragm', *Am J Physiol*, vol. 277, no. 6 Pt 1, pp. C1170-6.

Constantin, B, Sebille, S & Cognard, C 2006, 'New insights in the regulation of calcium transfers by muscle dystrophin-based cytoskeleton: implications in DMD', *J Muscle Res Cell Motil*, vol. 27, no. 5-7, pp. 375-86.

Cook, SA, Matsui, T, Li, L & Rosenzweig, A 2002, 'Transcriptional effects of chronic Akt activation in the heart', *J Biol Chem*, vol. 277, no. 25, pp. 22528-33.

Corbin, JD 2004, 'Mechanisms of action of PDE5 inhibition in erectile dysfunction', *Int J Impot Res*, vol. 16 Suppl 1, pp. S4-7.

Costa, AD, Garlid, KD, West, IC, Lincoln, TM, Downey, JM, Cohen, MV & Critz, SD 2005, 'Protein kinase G transmits the cardioprotective signal from cytosol to mitochondria', *Circ Res*, vol. 97, no. 4, pp. 329-36.

Crawford, GE, Faulkner, JA, Crosbie, RH, Campbell, KP, Froehner, SC & Chamberlain, JS 2000, 'Assembly of the dystrophin-associated protein complex does not require the dystrophin COOH-terminal domain', *J Cell Biol*, vol. 150, no. 6, pp. 1399-410.

Crosbie, RH 2001, 'NO vascular control in Duchenne muscular dystrophy', *Nat Med*, vol. 7, no. 1, pp. 27-9.

Crosbie, RH, Straub, V, Yun, HY, Lee, JC, Rafael, JA, Chamberlain, JS, Dawson, VL, Dawson, TM & Campbell, KP 1998, 'mdx muscle pathology is independent of nNOS perturbation', *Hum Mol Genet*, vol. 7, no. 5, pp. 823-9.

Cullen, MJ & Fulthorpe, JJ 1975, 'Stages in fibre breakdown in Duchenne muscular dystrophy. An electron-microscopic study', *J Neurol Sci*, vol. 24, no. 2, pp. 179-200.

Cyrulnik, SE, Fee, RJ, Batchelder, A, Kiefel, J, Goldstein, E & Hinton, VJ 2008, 'Cognitive and adaptive deficits in young children with Duchenne

muscular dystrophy (DMD)', *J Int Neuropsychol Soc*, vol. 14, no. 5, pp. 853-61.

Damy, T, Ratajczak, P, Shah, AM, Camors, E, Marty, I, Hasenfuss, G, Marotte, F, Samuel, JL & Heymes, C 2004, 'Increased neuronal nitric oxide synthase-derived NO production in the failing human heart', *Lancet*, vol. 363, no. 9418, pp. 1365-7.

Danowski, BA, Imanaka-Yoshida, K, Sanger, JM & Sanger, JW 1992, 'Costameres are sites of force transmission to the substratum in adult rat cardiomyocytes', *J Cell Biol*, vol. 118, no. 6, pp. 1411-20.

Dawson-Hughes, B 1991, 'Calcium supplementation and bone loss: a review of controlled clinical trials', *Am J Clin Nutr*, vol. 54, no. 1 Suppl, pp. 274S-80S.

De Backer, F, Vandebrouck, C, Gailly, P & Gillis, JM 2002, 'Long-term study of Ca(2+) homeostasis and of survival in collagenase-isolated muscle fibres from normal and mdx mice', *J Physiol*, vol. 542, no. Pt 3, pp. 855-65.

Deasy, BM, Feduska, JM, Payne, TR, Li, Y, Ambrosio, F & Huard, J 2009, 'Effect of VEGF on the Regenerative Capacity of Muscle Stem Cells in Dystrophic Skeletal Muscle', *Mol Ther*.

Deconinck, AE, Rafael, JA, Skinner, JA, Brown, SC, Potter, AC, Metzinger, L, Watt, DJ, Dickson, JG, Tinsley, JM & Davies, KE 1997, 'Utrophin-dystrophin-deficient mice as a model for Duchenne muscular dystrophy', *Cell*, vol. 90, no. 4, pp. 717-27.

Deconinck, N & Dan, B 2007, 'Pathophysiology of duchenne muscular dystrophy: current hypotheses', *Pediatr Neurol*, vol. 36, no. 1, pp. 1-7.

Delaporte, C, Dautreaux, B, Rouche, A & Fardeau, M 1990, 'Changes in surface morphology and basal lamina of cultured muscle cells from Duchenne muscular dystrophy patients', *J Neurol Sci*, vol. 95, no. 1, pp. 77-88.

Denti, MA, Rosa, A, D'Antona, G, Sthandier, O, De Angelis, FG, Nicoletti, C, Allocca, M, Pansarasa, O, Parente, V, Musaro, A, Auricchio, A, Bottinelli, R & Bozzoni, I 2006, 'Body-wide gene therapy of Duchenne muscular dystrophy in the mdx mouse model', *Proc Natl Acad Sci U S A*, vol. 103, no. 10, pp. 3758-63.

Desmedt, JE & Borenstein, S 1975, 'Relationship of spontaneous fibrillation potentials to muscle fibre segmentation in human muscular dystrophy', *Nature*, vol. 258, no. 5535, pp. 531-4.

Deval, E, Levitsky, DO, Marchand, E, Cantereau, A, Raymond, G & Cognard, C 2002, 'Na(+)/Ca(2+) exchange in human myotubes: intracellular

calcium rises in response to external sodium depletion are enhanced in DMD', *Neuromuscul Disord*, vol. 12, no. 7-8, pp. 665-73.

Do, T 2002, 'Orthopedic management of the muscular dystrophies', *Curr Opin Pediatr*, vol. 14, no. 1, pp. 50-3.

Driscol, D 2001, 'Duchenne and Becker Muscular Dystrophies', *Contempory Ob/GYN*, vol. 46, pp. 97-101.

Duboc, D, Meune, C, Lerebours, G, Devaux, JY, Vaksmann, G & Becane, HM 2005, 'Effect of perindopril on the onset and progression of left ventricular dysfunction in Duchenne muscular dystrophy', *J Am Coll Cardiol*, vol. 45, no. 6, pp. 855-7.

Dubowitz, V, Kinali, M, Main, M, Mercuri, E & Muntoni, F 2002, 'Remission of clinical signs in early duchenne muscular dystrophy on intermittent low-dosage prednisolone therapy', *Eur J Paediatr Neurol*, vol. 6, no. 3, pp. 153-9.

Durbeej, M & Campbell, KP 2002, 'Muscular dystrophies involving the dystrophin-glycoprotein complex: an overview of current mouse models', *Curr Opin Genet Dev*, vol. 12, no. 3, pp. 349-61.

Eagle, M, Baudouin, S, Chandler, C, Giddings, D, Bullock, R & Bushby, K 2002, 'Survival in Duchenne Muscular Dystrophy: improvements in life expectancy since 1967 and the impact of home nocturnal ventilation.', *Neuromuscul Disord*, vol. 12, no. 10, pp. 926-9.

Eagle, M, Bourke, J, Bullock, R, Gibson, M, Mehta, J, Giddings, D, Straub, V & Bushby, K 2007, 'Managing Duchenne muscular dystrophy--the additive effect of spinal surgery and home nocturnal ventilation in improving survival', *Neuromuscul Disord*, vol. 17, no. 6, pp. 470-5.

Eghbali, M, Tomek, R, Sukhatme, VP, Woods, C & Bhambi, B 1991, 'Differential effects of transforming growth factor-beta 1 and phorbol myristate acetate on cardiac fibroblasts. Regulation of fibrillar collagen mRNAs and expression of early transcription factors', *Circ Res*, vol. 69, no. 2, pp. 483-90.

El Fahime, E, Torrente, Y, Caron, NJ, Bresolin, MD & Tremblay, JP 2000, 'In vivo migration of transplanted myoblasts requires matrix metalloproteinase activity', *Exp Cell Res*, vol. 258, no. 2, pp. 279-87.

Elia, M, Carter, A, Bacon, S, Winearls, CG & Smith, R 1981, 'Clinical usefulness of urinary 3-methylhistidine excretion in indicating muscle protein breakdown', *Br Med J (Clin Res Ed)*, vol. 282, no. 6261, pp. 351-4.

Emery, A 1994, *Muscular Dystrophy: The Facts*, Oxford University Press, Oxford.

Emery, A 2002, 'The muscular dystrophies', *Lancet*, vol. 359, no. 9307, pp. 687-95.

Emery, AE 1991, 'Population frequencies of inherited neuromuscular diseases--a world survey', *Neuromuscul Disord*, vol. 1, no. 1, pp. 19-29.

Emery, AE 1993, *Duchenne Muscular Dystrophy*, 2nd edn, Oxford University Press, Oxford.

Endo, M, Yagi, S & Ishizuka, T 1983, 'Changes in the Ca-induced Ca release mechanism in the sarcoplasmic reticulum of the muscle from a patient with malignant hyperthermia.', *Biomed Res*, vol. 4, pp. 83-92.

England, SB, Nicholson, LV, Johnson, MA, Forrest, SM, Love, DR, Zubrzycka-Gaarn, EE, Bulman, DE, Harris, JB & Davies, KE 1990, 'Very mild muscular dystrophy associated with the deletion of 46% of dystrophin', *Nature*, vol. 343, no. 6254, pp. 180-2.

Fanchaouy, M, Polakova, E, Jung, C, Ogrodnik, J, Shirokova, N & Niggli, E 2009, 'Pathways of abnormal stress-induced Ca2+ influx into dystrophic mdx cardiomyocytes', *Cell Calcium*, vol. 46, no. 2, pp. 114-21.

Farrell, PT 1994, 'Anaesthesia-induced rhabdomyolysis causing cardiac arrest: case report and review of anaesthesia and the dystrophinopathies', *Anaesth Intensive Care*, vol. 22, no. 5, pp. 597-601.

Fenichel, GM, Florence, JM, Pestronk, A, Mendell, JR, Moxley, RT, 3rd, Griggs, RC, Brooke, MH, Miller, JP, Robison, J, King, W & et al. 1991, 'Long-term benefit from prednisone therapy in Duchenne muscular dystrophy', *Neurology*, vol. 41, no. 12, pp. 1874-7.

Feron, O, Belhassen, L, Kobzik, L, Smith, TW, Kelly, RA & Michel, T 1996, 'Endothelial nitric oxide synthase targeting to caveolae. Specific interactions with caveolin isoforms in cardiac myocytes and endothelial cells', *J Biol Chem*, vol. 271, no. 37, pp. 22810-4.

Ferrari, G, Cusella-De Angelis, G, Coletta, M, Paolucci, E, Stornaiuolo, A, Cossu, G & Mavilio, F 1998, 'Muscle regeneration by bone marrow-derived myogenic progenitors', *Science*, vol. 279, no. 5356, pp. 1528-30.

Finder, JD, Birnkrant, D, Carl, J, Farber, HJ, Gozal, D, Iannaccone, ST, Kovesi, T, Kravitz, RM, Panitch, H, Schramm, C, Schroth, M, Sharma, G, Sievers, L, Silvestri, JM & Sterni, L 2004, 'Respiratory care of the patient with Duchenne muscular dystrophy: ATS consensus statement', *Am J Respir Crit Care Med*, vol. 170, no. 4, pp. 456-65.

Findlay, I 2002, 'Voltage-dependent inactivation of L-type Ca2+ currents in guinea-pig ventricular myocytes', *J Physiol*, vol. 545, no. Pt 2, pp. 389-97.

Finsterer, J & Stollberger, C 2003, 'The heart in human dystrophinopathies', *Cardiology*, vol. 99, no. 1, pp. 1-19.

Fischmeister, R, Castro, L, Abi-Gerges, A, Rochais, F & Vandecasteele, G 2005, 'Species- and tissue-dependent effects of NO and cyclic GMP on cardiac ion channels', *Comp Biochem Physiol A Mol Integr Physiol*, vol. 142, no. 2, pp. 136-43.

Flanigan, KM, von Niederhausern, A, Dunn, DM, Alder, J, Mendell, JR & Weiss, RB 2003, 'Rapid direct sequence analysis of the dystrophin gene', *Am J Hum Genet*, vol. 72, no. 4, pp. 931-9.

Fletcher, S, Honeyman, K, Fall, AM, Harding, PL, Johnsen, RD, Steinhaus, JP, Moulton, HM, Iversen, PL & Wilton, SD 2007, 'Morpholino oligomer-mediated exon skipping averts the onset of dystrophic pathology in the mdx mouse', *Mol Ther*, vol. 15, no. 9, pp. 1587-92.

Forfia, PR, Lee, M, Tunin, RS, Mahmud, M, Champion, HC & Kass, DA 2007, 'Acute phosphodiesterase 5 inhibition mimics hemodynamic effects of B-type natriuretic peptide and potentiates B-type natriuretic peptide effects in failing but not normal canine heart', *J Am Coll Cardiol*, vol. 49, no. 10, pp. 1079-88.

Franco-Obregon, A & Lansman, JB 2002, 'Changes in mechanosensitive channel gating following mechanical stimulation in skeletal muscle myotubes from the mdx mouse', *J Physiol*, vol. 539, no. Pt 2, pp. 391-407.

Frascarelli, M, Rocchi, L & Feola, I 1988, 'EMG computerized analysis of localized fatigue in Duchenne muscular dystrophy', *Muscle Nerve*, vol. 11, no. 7, pp. 757-61.

Fraysse, B, Liantonio, A, Cetrone, M, Burdi, R, Pierno, S, Frigeri, A, Pisoni, M, Camerino, C & De Luca, A 2004, 'The alteration of calcium homeostasis in adult dystrophic mdx muscle fibers is worsened by a chronic exercise in vivo', *Neurobiol Dis*, vol. 17, no. 2, pp. 144-54.

Friedrich, O, Both, M, Gillis, JM, Chamberlain, JS & Fink, RH 2004, 'Mini-dystrophin restores L-type calcium currents in skeletal muscle of transgenic mdx mice', *J Physiol*, vol. 555, no. Pt 1, pp. 251-65.

Gailly, P, Hermans, E, Octave, JN & Gillis, JM 1993, 'Specific increase of genetic expression of parvalbumin in fast skeletal muscles of mdx mice', *FEBS Lett*, vol. 326, no. 1-3, pp. 272-4.

Gao, C, Xiong, W & Li, Y 2007, 'The myofibrosis mechanism of differentiation growth factor-8 on rat abdominal aorta grafts', *Transplant Proc*, vol. 39, no. 10, pp. 3420-3.

Gao, WD, Atar, D, Liu, Y, Perez, NG, Murphy, AM & Marban, E 1997, 'Role of troponin I proteolysis in the pathogenesis of stunned myocardium', *Circ Res*, vol. 80, no. 3, pp. 393-9.

Garstang, SV, Kirshblum, SC & Wood, KE 2000, 'Patient preference for in-exsufflation for secretion management with spinal cord injury', *J Spinal Cord Med*, vol. 23, no. 2, pp. 80-5.

Gatta, V, Scarciolla, O, Gaspari, AR, Palka, C, De Angelis, MV, Di Muzio, A, Guanciali-Franchi, P, Calabrese, G, Uncini, A & Stuppia, L 2005, 'Identification of deletions and duplications of the DMD gene in affected males and carrier females by multiple ligation probe amplification (MLPA)', *Hum Genet*, vol. 117, no. 1, pp. 92-8.

Gayraud, J, Matecki, S, Hnia, K, Mornet, D, Prefaut, C, Mercier, J, Michel, A & Ramonatxo, M 2007, 'Ventilation during air breathing and in response to hypercapnia in 5 and 16 month-old mdx and C57 mice', *J Muscle Res Cell Motil*, vol. 28, no. 1, pp. 29-37.

George, EL, Georges-Labouesse, EN, Patel-King, RS, Rayburn, H & Hynes, RO 1993, 'Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin', *Development*, vol. 119, no. 4, pp. 1079-91.

George, I, Bish, LT, Kamalakkannan, G, Petrilli, CM, Oz, MC, Naka, Y, Sweeney, HL & Maybaum, S 2010, 'Myostatin activation in patients with advanced heart failure and after mechanical unloading', *Eur J Heart Fail*, vol. 12, no. 5, pp. 444-53.

Ghofrani, HA, Osterloh, IH & Grimminger, F 2006, 'Sildenafil: from angina to erectile dysfunction to pulmonary hypertension and beyond', *Nat Rev Drug Discov*, vol. 5, no. 8, pp. 689-702.

Giatrakos, N, Kinali, M, Stephens, D, Dawson, D, Muntoni, F & Nihoyannopoulos, P 2006, 'Cardiac tissue velocities and strain rate in the early detection of myocardial dysfunction of asymptomatic boys with Duchenne's muscular dystrophy: relationship to clinical outcome', *Heart*, vol. 92, no. 6, pp. 840-2.

Gillis, JM 1999, 'Understanding dystrophinopathies: an inventory of the structural and functional consequences of the absence of dystrophin in muscles of the mdx mouse', *J Muscle Res Cell Motil*, vol. 20, no. 7, pp. 605-25.

Glorieux, FH, Bishop, NJ, Plotkin, H, Chabot, G, Lanoue, G & Travers, R 1998, 'Cyclic administration of pamidronate in children with severe osteogenesis imperfecta', *N Engl J Med*, vol. 339, no. 14, pp. 947-52.

Godfrey, EW & Schwarte, RC 2003, 'The role of nitric oxide signaling in the formation of the neuromuscular junction', *J Neurocytol*, vol. 32, no. 5-8, pp. 591-602.

Goemans, NM, Tulinius, M, van den Akker, JT, Burm, BE, Ekhart, PF, Heuvelmans, N, Holling, T, Janson, AA, Platenburg, GJ, Sipkens, JA, Sitsen, JM, Aartsma-Rus, A, van Ommen, GJ, Buyse, G, Darin, N, Verschuuren, JJ, Campion, GV, de Kimpe, SJ & van Deutekom, JC 2011, 'Systemic administration of PRO051 in Duchenne's muscular dystrophy', *N Engl J Med*, vol. 364, no. 16, pp. 1513-22.

Goldspink, G 1999, 'Changes in muscle mass and phenotype and the expression of autocrine and systemic growth factors by muscle in response to stretch and overload', *J Anat*, vol. 194 (Pt 3), pp. 323-34.

Goldspink, G, Fernandes, K, Williams, PE & Wells, DJ 1994, 'Age-related changes in collagen gene expression in the muscles of mdx dystrophic and normal mice', *Neuromuscul Disord*, vol. 4, no. 3, pp. 183-91.

Gonzalez-Cadavid, NF, Taylor, WE, Yarasheski, K, Sinha-Hikim, I, Ma, K, Ezzat, S, Shen, R, Lalani, R, Asa, S, Mamita, M, Nair, G, Arver, S & Bhasin, S 1998, 'Organization of the human myostatin gene and expression in healthy men and HIV-infected men with muscle wasting', *Proc Natl Acad Sci U S A*, vol. 95, no. 25, pp. 14938-43.

Gonzalez, DR, Fernandez, IC, Ordenes, PP, Treuer, AV, Eller, G & Boric, MP 2008, 'Differential role of S-nitrosylation and the NO-cGMP-PKG pathway in cardiac contractility', *Nitric Oxide*, vol. 18, no. 3, pp. 157-67.

Gosselin, LE, Barkley, JE, Spencer, MJ, McCormick, KM & Farkas, GA 2003, 'Ventilatory dysfunction in mdx mice: impact of tumor necrosis factor-alpha deletion', *Muscle Nerve*, vol. 28, no. 3, pp. 336-43.

Gosselin, LE, Williams, JE, Deering, M, Brazeau, D, Koury, S & Martinez, DA 2004, 'Localization and early time course of TGF-beta 1 mRNA expression in dystrophic muscle', *Muscle Nerve*, vol. 30, no. 5, pp. 645-53.

Goumas, G, Tentolouris, C, Tousoulis, D, Stefanadis, C & Toutouzas, P 2001, 'Therapeutic modification of the L-arginine-eNOS pathway in cardiovascular diseases', *Atherosclerosis*, vol. 154, no. 2, pp. 255-67.

Goyenvalle, A & Davies, KE 2011, 'Challenges to oligonucleotides-based therapeutics for Duchenne muscular dystrophy', *Skelet Muscle*, vol. 1, no. 1, p. 8.

Goyenvalle, A, Vulin, A, Fougerousse, F, Leturcq, F, Kaplan, JC, Garcia, L & Danos, O 2004, 'Rescue of dystrophic muscle through U7 snRNA-mediated exon skipping', *Science*, vol. 306, no. 5702, pp. 1796-9.

Grady, RM, Teng, H, Nichol, MC, Cunningham, JC, Wilkinson, RS & Sanes, JR 1997, 'Skeletal and cardiac myopathies in mice lacking utrophin and dystrophin: a model for Duchenne muscular dystrophy', *Cell*, vol. 90, no. 4, pp. 729-38.

Grady, RM, Grange, RW, Lau, KS, Maimone, MM, Nichol, MC, Stull, JT & Sanes, JR 1999, 'Role for alpha-dystrobrevin in the pathogenesis of dystrophin-dependent muscular dystrophies', *Nat Cell Biol*, vol. 1, no. 4, pp. 215-20.

Griggs, RC, Moxley, RT, 3rd, Mendell, JR, Fenichel, GM, Brooke, MH, Pestronk, A & Miller, JP 1991, 'Prednisone in Duchenne dystrophy. A randomized, controlled trial defining the time course and dose response. Clinical Investigation of Duchenne Dystrophy Group', *Arch Neurol*, vol. 48, no. 4, pp. 383-8.

Grinio, LP, Orlov, ON, Prilipko, LL & Kagan, VE 1984, 'Lipid peroxidation in children with Duchenne's hereditary myopathy', *Biull Eksp Biol Med*, vol. 98, no. 10, pp. 423-5.

Grobet, L, Martin, LJ, Poncelet, D, Pirottin, D, Brouwers, B, Riquet, J, Schoeberlein, A, Dunner, S, Menissier, F, Massabanda, J, Fries, R, Hanset, R & Georges, M 1997, 'A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle', *Nat Genet*, vol. 17, no. 1, pp. 71-4.

Grounds, MD & Torrisi, J 2004, 'Anti-TNFalpha (Remicade) therapy protects dystrophic skeletal muscle from necrosis', *FASEB J*, vol. 18, no. 6, pp. 676-82.

Grounds, MD, Radley, HG, Lynch, GS, Nagaraju, K & De Luca, A 2008, 'Towards developing standard operating procedures for pre-clinical testing in the mdx mouse model of Duchenne muscular dystrophy', *Neurobiol Dis*, vol. 31, no. 1, pp. 1-19.

Guazzi, M, Samaja, M, Arena, R, Vicenzi, M & Guazzi, MD 2007, 'Long-term use of sildenafil in the therapeutic management of heart failure', *J Am Coll Cardiol*, vol. 50, no. 22, pp. 2136-44.

Guerron, AD, Rawat, R, Sali, A, Spurney, CF, Pistilli, E, Cha, HJ, Pandey, GS, Gernapudi, R, Francia, D, Farajian, V, Escolar, DM, Bossi, L, Becker, M, Zerr, P, de la Porte, S, Gordish-Dressman, H, Partridge, T, Hoffman, EP & Nagaraju, K 2010, 'Functional and molecular effects of arginine butyrate and prednisone on muscle and heart in the mdx mouse model of Duchenne Muscular Dystrophy', *PLoS One*, vol. 5, no. 6, p. e11220.

Guo, ZL, Tjen, ALSC, Fu, LW & Longhurst, JC 2009, 'Nitric oxide in rostral ventrolateral medulla regulates cardiac-sympathetic reflexes: role of synthase isoforms', *Am J Physiol Heart Circ Physiol*, vol. 297, no. 4, pp. H1478-86.

Gussoni, E, Soneoka, Y, Strickland, CD, Buzney, EA, Khan, MK, Flint, AF, Kunkel, LM & Mulligan, RC 1999, 'Dystrophin expression in the mdx mouse restored by stem cell transplantation', *Nature*, vol. 401, no. 6751, pp. 390-4.

Halcox, JP, Nour, KR, Zalos, G, Mincemoyer, RA, Waclawiw, M, Rivera, CE, Willie, G, Ellahham, S & Quyyumi, AA 2002, 'The effect of sildenafil on human vascular function, platelet activation, and myocardial ischemia', *J Am Coll Cardiol*, vol. 40, no. 7, pp. 1232-40.

Hamed, SA 2006, 'Drug evaluation: PTC-124--a potential treatment of cystic fibrosis and Duchenne muscular dystrophy', *IDrugs*, vol. 9, no. 11, pp. 783-9.

Hardiman, O, Sklar, RM & Brown, RH, Jr. 1993, 'Methylprednisolone selectively affects dystrophin expression in human muscle cultures', *Neurology*, vol. 43, no. 2, pp. 342-5.

Harper, SQ, Hauser, MA, DelloRusso, C, Duan, D, Crawford, RW, Phelps, SF, Harper, HA, Robinson, AS, Engelhardt, JF, Brooks, SV & Chamberlain, JS 2002, 'Modular flexibility of dystrophin: implications for gene therapy of Duchenne muscular dystrophy', *Nat Med*, vol. 8, no. 3, pp. 253-61.

Hartigan-O'Connor, D, Barjot, C, Crawford, R & Chamberlain, JS 2002, 'Efficient rescue of gutted adenovirus genomes allows rapid production of concentrated stocks without negative selection', *Hum Gene Ther*, vol. 13, no. 4, pp. 519-31.

Haslett, JN, Sanoudou, D, Kho, AT, Bennett, RR, Greenberg, SA, Kohane, IS, Beggs, AH & Kunkel, LM 2002, 'Gene expression comparison of biopsies from Duchenne muscular dystrophy (DMD) and normal skeletal muscle', *Proc Natl Acad Sci U S A*, vol. 99, no. 23, pp. 15000-5.

Haycock, JW, Mac Neil, S & Mantle, D 1998, 'Differential protein oxidation in Duchenne and Becker muscular dystrophy', *Neuroreport*, vol. 9, no. 10, pp. 2201-7.

Hayes, J, Veyckemans, F & Bissonnette, B 2008, 'Duchenne muscular dystrophy: an old anesthesia problem revisited', *Paediatr Anaesth*, vol. 18, no. 2, pp. 100-6.

Hegde, MR, Chin, EL, Mulle, JG, Okou, DT, Warren, ST & Zwick, ME 2008, 'Microarray-based mutation detection in the dystrophin gene', *Hum Mutat*, vol. 29, no. 9, pp. 1091-9.

Hendriksen, JG & Vles, JS 2008, 'Neuropsychiatric disorders in males with duchenne muscular dystrophy: frequency rate of attention-deficit hyperactivity disorder (ADHD), autism spectrum disorder, and obsessive--compulsive disorder', *J Child Neurol*, vol. 23, no. 5, pp. 477-81.

Hernandez, I, de la Torre, P, Rey-Campos, J, Garcia, I, Sanchez, JA, Munoz, R, Rippe, RA, Munoz-Yague, T & Solis-Herruzo, JA 2000, 'Collagen alpha1(I) gene contains an element responsive to tumor necrosis factor-alpha located in the 5' untranslated region of its first exon', *DNA Cell Biol*, vol. 19, no. 6, pp. 341-52.

Hinton, VJ, Nereo, NE, Fee, RJ & Cyrulnik, SE 2006, 'Social behavior problems in boys with Duchenne muscular dystrophy', *J Dev Behav Pediatr*, vol. 27, no. 6, pp. 470-6.

Hirawat, S, Welch, EM, Elfring, GL, Northcutt, VJ, Paushkin, S, Hwang, S, Leonard, EM, Almstead, NG, Ju, W, Peltz, SW & Miller, LL 2007, 'Safety, tolerability, and pharmacokinetics of PTC124, a nonaminoglycoside nonsense mutation suppressor, following single- and multiple-dose administration to healthy male and female adult volunteers', *J Clin Pharmacol*, vol. 47, no. 4, pp. 430-44.

Hnia, K, Gayraud, J, Hugon, G, Ramonatxo, M, De La Porte, S, Matecki, S & Mornet, D 2008, 'L-arginine decreases inflammation and modulates the nuclear factor-kappaB/matrix metalloproteinase cascade in mdx muscle fibers', *Am J Pathol*, vol. 172, no. 6, pp. 1509-19.

Hodges, BL, Taylor, KM, Chu, Q, Scull, SE, Serriello, RG, Anderson, SC, Wang, F & Scheule, RK 2005, 'Local delivery of a viral vector mitigates neutralization by antiviral antibodies and results in efficient transduction of rabbit liver', *Mol Ther*, vol. 12, no. 6, pp. 1043-51.

Hoffman, EP 2007, 'Skipping toward personalized molecular medicine', *N Engl J Med*, vol. 357, no. 26, pp. 2719-22.

Hoffman, EP, Brown, RH, Jr. & Kunkel, LM 1987, 'Dystrophin: the protein product of the Duchenne muscular dystrophy locus', *Cell*, vol. 51, no. 6, pp. 919-28.

Hoffman, EP, Fischbeck, KH, Brown, RH, Johnson, M, Medori, R, Loike, JD, Harris, JB, Waterston, R, Brooke, M, Specht, L & et al. 1988, 'Characterization of dystrophin in muscle-biopsy specimens from patients with Duchenne's or Becker's muscular dystrophy', *N Engl J Med*, vol. 318, no. 21, pp. 1363-8.

Hofmann, F, Bernhard, D, Lukowski, R & Weinmeister, P 2009, 'cGMP regulated protein kinases (cGK)', *Handb Exp Pharmacol*, no. 191, pp. 137-62.

Hoogerwaard, EM, van der Wouw, PA, Wilde, AA, Bakker, E, Ippel, PF, Oosterwijk, JC, Majoor-Krakauer, DF, van Essen, AJ, Leschot, NJ & de Visser, M 1999, 'Cardiac involvement in carriers of Duchenne and Becker muscular dystrophy', *Neuromuscul Disord*, vol. 9, no. 5, pp. 347-51.

Hoogerwaard, EM, Bakker, E, Ippel, PF, Oosterwijk, JC, Majoor-Krakauer, DF, Leschot, NJ, Van Essen, AJ, Brunner, HG, van der Wouw, PA, Wilde, AA & de Visser, M 1999, 'Signs and symptoms of Duchenne muscular dystrophy and Becker muscular dystrophy among carriers in The Netherlands: a cohort study', *Lancet*, vol. 353, no. 9170, pp. 2116-9.

Hopf, FW, Turner, PR & Steinhardt, RA 2007, 'Calcium misregulation and the pathogenesis of muscular dystrophy', *Subcell Biochem*, vol. 45, pp. 429-64.

Hopf, FW, Turner, PR, Denetclaw, WF, Jr., Reddy, P & Steinhardt, RA 1996, 'A critical evaluation of resting intracellular free calcium regulation in dystrophic mdx muscle', *Am J Physiol*, vol. 271, no. 4 Pt 1, pp. C1325-39.

Huntley, BK, Sandberg, SM, Noser, JA, Cataliotti, A, Redfield, MM, Matsuda, Y & Burnett, JC, Jr. 2006, 'BNP-induced activation of cGMP in human cardiac fibroblasts: interactions with fibronectin and natriuretic peptide receptors', *J Cell Physiol*, vol. 209, no. 3, pp. 943-9.

Ignarro, LJ 1990, 'Nitric oxide. A novel signal transduction mechanism for transcellular communication', *Hypertension*, vol. 16, no. 5, pp. 477-83.

Imbert, N, Vandebrouck, C, Duport, G, Raymond, G, Hassoni, AA, Constantin, B, Cullen, MJ & Cognard, C 2001, 'Calcium currents and transients in co-cultured contracting normal and Duchenne muscular dystrophy human myotubes', *J Physiol*, vol. 534, no. Pt. 2, pp. 343-55.

Ishikawa, Y, Bach, JR & Minami, R 1995, 'A management trial for Duchenne cardiomyopathy', *Am J Phys Med Rehabil*, vol. 74, no. 5, pp. 345-50.

Iwata, Y, Katanosaka, Y, Arai, Y, Komamura, K, Miyatake, K & Shigekawa, M 2003, 'A novel mechanism of myocyte degeneration involving the Ca2+-permeable growth factor-regulated channel', *J Cell Biol*, vol. 161, no. 5, pp. 957-67.

Jackson, MJ, Jones, DA & Edwards, RH 1984, 'Techniques for studying free radical damage in muscular dystrophy', *Med Biol*, vol. 62, no. 2, pp. 135-8.

Jackson, MJ, Jones, DA & Edwards, RH 1985, 'Measurements of calcium and other elements in muscle biopsy samples from patients with Duchenne muscular dystrophy', *Clin Chim Acta*, vol. 147, no. 3, pp. 215-21.

Jacobs, SC, Bootsma, AL, Willems, PW, Bar, PR & Wokke, JH 1996, 'Prednisone can protect against exercise-induced muscle damage', *J Neurol*, vol. 243, no. 5, pp. 410-6.

Jiang, H, Couto, LB, Patarroyo-White, S, Liu, T, Nagy, D, Vargas, JA, Zhou, S, Scallan, CD, Sommer, J, Vijay, S, Mingozzi, F, High, KA &

Pierce, GF 2006, 'Effects of transient immunosuppression on adenoassociated, virus-mediated, liver-directed gene transfer in rhesus macaques and implications for human gene therapy', *Blood*, vol. 108, no. 10, pp. 3321-8.

Jiang, YL, Li, N, Plastow, G, Liu, ZL, Hu, XX & Wu, CX 2002, 'Identification of three SNPs in the porcine myostatin gene (MSTN)', *Anim Biotechnol*, vol. 13, no. 1, pp. 173-8.

Jones, H, De Vivo, DC & Darras, BT 2003, *Neuromuscular disorders of infancy, childhood and adolescence. A clinician's approach.*, Butterworth-Heinemann, Oxford.

Jones, MA & Werle, MJ 2000, 'Nitric oxide is a downstream mediator of agrin-induced acetylcholine receptor aggregation', *Mol Cell Neurosci*, vol. 16, no. 5, pp. 649-60.

Joulia, D, Bernardi, H, Garandel, V, Rabenoelina, F, Vernus, B & Cabello, G 2003, 'Mechanisms involved in the inhibition of myoblast proliferation and differentiation by myostatin', *Exp Cell Res*, vol. 286, no. 2, pp. 263-75.

Jung, C, Martins, AS, Niggli, E & Shirokova, N 2008, 'Dystrophic cardiomyopathy: amplification of cellular damage by Ca2+ signalling and reactive oxygen species-generating pathways', *Cardiovasc Res*, vol. 77, no. 4, pp. 766-73.

Kameyama, A, Yazawa, K, Kaibara, M, Ozono, K & Kameyama, M 1997, 'Run-down of the cardiac Ca2+ channel: characterization and restoration of channel activity by cytoplasmic factors', *Pflugers Arch*, vol. 433, no. 5, pp. 547-56.

Kanes, SJ, Tokarczyk, J, Siegel, SJ, Bilker, W, Abel, T & Kelly, MP 2007, 'Rolipram: a specific phosphodiesterase 4 inhibitor with potential antipsychotic activity', *Neuroscience*, vol. 144, no. 1, pp. 239-46.

Kang, SW & Bach, JR 2000, 'Maximum insufflation capacity', *Chest*, vol. 118, no. 1, pp. 61-5.

Kapsa, R, Quigley, A, Lynch, GS, Steeper, K, Kornberg, AJ, Gregorevic, P, Austin, L & Byrne, E 2001, 'In vivo and in vitro correction of the mdx dystrophin gene nonsense mutation by short-fragment homologous replacement', *Hum Gene Ther*, vol. 12, no. 6, pp. 629-42.

Kar, NC & Pearson, CM 1979, 'Catalase, superoxide dismutase, glutathione reductase and thiobarbituric acid-reactive products in normal and dystrophic human muscle', *Clin Chim Acta*, vol. 94, no. 3, pp. 277-80.

Karpati, G, Ajdukovic, D, Arnold, D, Gledhill, RB, Guttmann, R, Holland, P, Koch, PA, Shoubridge, E, Spence, D, Vanasse, M & et al. 1993,

'Myoblast transfer in Duchenne muscular dystrophy', *Ann Neurol*, vol. 34, no. 1, pp. 8-17.

Karsenty, G & Park, RW 1995, 'Regulation of type I collagen genes expression', *Int Rev Immunol*, vol. 12, no. 2-4, pp. 177-85.

Katz, SD, Balidemaj, K, Homma, S, Wu, H, Wang, J & Maybaum, S 2000, 'Acute type 5 phosphodiesterase inhibition with sildenafil enhances flow-mediated vasodilation in patients with chronic heart failure', *J Am Coll Cardiol*, vol. 36, no. 3, pp. 845-51.

Kelfer, HM, Singer, WD & Reynolds, RN 1983, 'Malignant hyperthermia in a child with Duchenne muscular dystrophy', *Pediatrics*, vol. 71, no. 1, pp. 118-9.

Khairallah, M, Khairallah, RJ, Young, ME, Allen, BG, Gillis, MA, Danialou, G, Deschepper, CF, Petrof, BJ & Des Rosiers, C 2008, 'Sildenafil and cardiomyocyte-specific cGMP signaling prevent cardiomyopathic changes associated with dystrophin deficiency', *Proc Natl Acad Sci U S A*, vol. 105, no. 19, pp. 7028-33.

Khalil, N, Bereznay, O, Sporn, M & Greenberg, AH 1989, 'Macrophage production of transforming growth factor beta and fibroblast collagen synthesis in chronic pulmonary inflammation', *J Exp Med*, vol. 170, no. 3, pp. 727-37.

Khan, SA, Lee, K, Minhas, KM, Gonzalez, DR, Raju, SV, Tejani, AD, Li, D, Berkowitz, DE & Hare, JM 2004, 'Neuronal nitric oxide synthase negatively regulates xanthine oxidoreductase inhibition of cardiac excitation-contraction coupling', *Proc Natl Acad Sci U S A*, vol. 101, no. 45, pp. 15944-8.

Khurana, TS, Watkins, SC, Chafey, P, Chelly, J, Tome, FM, Fardeau, M, Kaplan, JC & Kunkel, LM 1991, 'Immunolocalization and developmental expression of dystrophin related protein in skeletal muscle', *Neuromuscul Disord*, vol. 1, no. 3, pp. 185-94.

Kim, D, Rybalkin, SD, Pi, X, Wang, Y, Zhang, C, Munzel, T, Beavo, JA, Berk, BC & Yan, C 2001, 'Upregulation of phosphodiesterase 1A1 expression is associated with the development of nitrate tolerance', *Circulation*, vol. 104, no. 19, pp. 2338-43.

Kinali, M, Main, M, Eliahoo, J, Messina, S, Knight, RK, Lehovsky, J, Edge, G, Mercuri, E, Manzur, AY & Muntoni, F 2007, 'Predictive factors for the development of scoliosis in Duchenne muscular dystrophy', *Eur J Paediatr Neurol*, vol. 11, no. 3, pp. 160-6.

Kinali, M, Arechavala-Gomeza, V, Feng, L, Cirak, S, Hunt, D, Adkin, C, Guglieri, M, Ashton, E, Abbs, S, Nihoyannopoulos, P, Garralda, ME, Rutherford, M, McCulley, C, Popplewell, L, Graham, IR, Dickson, G,

Wood, MJ, Wells, DJ, Wilton, SD, Kole, R, Straub, V, Bushby, K, Sewry, C, Morgan, JE & Muntoni, F 2009, 'Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Duchenne muscular dystrophy: a single-blind, placebo-controlled, dose-escalation, proof-of-concept study', *Lancet Neurol*.

King, WM, Ruttencutter, R, Nagaraja, HN, Matkovic, V, Landoll, J, Hoyle, C, Mendell, JR & Kissel, JT 2007, 'Orthopedic outcomes of long-term daily corticosteroid treatment in Duchenne muscular dystrophy', *Neurology*, vol. 68, no. 19, pp. 1607-13.

Kinoshita, I, Vilquin, JT, Guerette, B, Asselin, I, Roy, R & Tremblay, JP 1994, 'Very efficient myoblast allotransplantation in mice under FK506 immunosuppression', *Muscle Nerve*, vol. 17, no. 12, pp. 1407-15.

Kiselyov, K, Xu, X, Mozhayeva, G, Kuo, T, Pessah, I, Mignery, G, Zhu, X, Birnbaumer, L & Muallem, S 1998, 'Functional interaction between InsP3 receptors and store-operated Htrp3 channels', *Nature*, vol. 396, no. 6710, pp. 478-82.

Kissel, JT, Burrow, KL, Rammohan, KW & Mendell, JR 1991, 'Mononuclear cell analysis of muscle biopsies in prednisone-treated and untreated Duchenne muscular dystrophy. CIDD Study Group', *Neurology*, vol. 41, no. 5, pp. 667-72.

Koenig, M & Kunkel, LM 1990, 'Detailed analysis of the repeat domain of dystrophin reveals four potential hinge segments that may confer flexibility', *J Biol Chem*, vol. 265, no. 8, pp. 4560-6.

Koenig, M, Hoffman, EP, Bertelson, CJ, Monaco, AP, Feener, C & Kunkel, LM 1987, 'Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals', *Cell*, vol. 50, no. 3, pp. 509-17.

Koenig, X, Dysek, S, Kimbacher, S, Mike, AK, Cervenka, R, Lukacs, P, Nagl, K, Dang, XB, Todt, H, Bittner, RE & Hilber, K 2011, 'Voltage-gated ion channel dysfunction precedes cardiomyopathy development in the dystrophic heart', *PLoS One*, vol. 6, no. 5, p. e20300.

Konagaya, M, Sakai, M, Wakayama, T, Kimura, S, Kuru, S & Yasuma, F 2005, 'Effect of intermittent positive pressure ventilation on life-span and causes of death in Duchenne muscular dystrophy', *Rinsho Shinkeigaku*, vol. 45, no. 9, pp. 643-6.

Kornegay, JN, Tuler, SM, Miller, DM & Levesque, DC 1988, 'Muscular dystrophy in a litter of golden retriever dogs', *Muscle Nerve*, vol. 11, no. 10, pp. 1056-64.

Kornegay, JN, Cundiff, DD, Bogan, DJ, Bogan, JR & Okamura, CS 2003, 'The cranial sartorius muscle undergoes true hypertrophy in dogs with

golden retriever muscular dystrophy', *Neuromuscul Disord*, vol. 13, no. 6, pp. 493-500.

Korones, DN, Brown, MR & Palis, J 2001, '"Liver function tests" are not always tests of liver function', *Am J Hematol*, vol. 66, no. 1, pp. 46-8.

Krag, TO, Bogdanovich, S, Jensen, CJ, Fischer, MD, Hansen-Schwartz, J, Javazon, EH, Flake, AW, Edvinsson, L & Khurana, TS 2004, 'Heregulin ameliorates the dystrophic phenotype in mdx mice', *Proc Natl Acad Sci U S A*, vol. 101, no. 38, pp. 13856-60.

Kumar, A & Boriek, AM 2003, 'Mechanical stress activates the nuclear factor-kappaB pathway in skeletal muscle fibers: a possible role in Duchenne muscular dystrophy', *Faseb J*, vol. 17, no. 3, pp. 386-96.

Lalani, R, Bhasin, S, Byhower, F, Tarnuzzer, R, Grant, M, Shen, R, Asa, S, Ezzat, S & Gonzalez-Cadavid, NF 2000, 'Myostatin and insulin-like growth factor-I and -II expression in the muscle of rats exposed to the microgravity environment of the NeuroLab space shuttle flight', *J Endocrinol*, vol. 167, no. 3, pp. 417-28.

Langley, B, Thomas, M, Bishop, A, Sharma, M, Gilmour, S & Kambadur, R 2002, 'Myostatin inhibits myoblast differentiation by down-regulating MyoD expression', *J Biol Chem*, vol. 277, no. 51, pp. 49831-40.

Larsen, UT, Juhl, B, Hein-Sorensen, O & de Fine Olivarius, B 1989, 'Complications during anaesthesia in patients with Duchenne's muscular dystrophy (a retrospective study)', *Can J Anaesth*, vol. 36, no. 4, pp. 418-22.

Laws, N & Hoey, A 2004, 'Progression of kyphosis in mdx mice', *J Appl Physiol*, vol. 97, no. 5, pp. 1970-7.

Layland, J, Li, JM & Shah, AM 2002, 'Role of cyclic GMP-dependent protein kinase in the contractile response to exogenous nitric oxide in rat cardiac myocytes', *J Physiol*, vol. 540, no. Pt 2, pp. 457-67.

Lee, KH, Baek, MY, Moon, KY, Song, WK, Chung, CH, Ha, DB & Kang, MS 1994, 'Nitric oxide as a messenger molecule for myoblast fusion', *J Biol Chem*, vol. 269, no. 20, pp. 14371-4.

Lee, SJ 2004, 'Regulation of muscle mass by myostatin', *Annu Rev Cell Dev Biol*, vol. 20, pp. 61-86.

Lee, SJ & McPherron, AC 2001, 'Regulation of myostatin activity and muscle growth', *Proc Natl Acad Sci U S A*, vol. 98, no. 16, pp. 9306-11.

Lefaucheur, JP, Pastoret, C & Sebille, A 1995, 'Phenotype of dystrophinopathy in old mdx mice', *Anat Rec*, vol. 242, no. 1, pp. 70-6.

Leibowitz, D & Dubowitz, V 1981, 'Intellect and behaviour in Duchenne muscular dystrophy', *Dev Med Child Neurol*, vol. 23, no. 5, pp. 577-90.

Lewis, GD, Lachmann, J, Camuso, J, Lepore, JJ, Shin, J, Martinovic, ME, Systrom, DM, Bloch, KD & Semigran, MJ 2007, 'Sildenafil improves exercise hemodynamics and oxygen uptake in patients with systolic heart failure', *Circulation*, vol. 115, no. 1, pp. 59-66.

Li, YP & Schwartz, RJ 2001, 'TNF-alpha regulates early differentiation of C2C12 myoblasts in an autocrine fashion', *FASEB J*, vol. 15, no. 8, pp. 1413-5.

Liang, KW, Nishikawa, M, Liu, F, Sun, B, Ye, Q & Huang, L 2004, 'Restoration of dystrophin expression in mdx mice by intravascular injection of naked DNA containing full-length dystrophin cDNA', *Gene Ther*, vol. 11, no. 11, pp. 901-8.

Liberona, JL, Powell, JA, Shenoi, S, Petherbridge, L, Caviedes, R & Jaimovich, E 1998, 'Differences in both inositol 1,4,5-trisphosphate mass and inositol 1,4,5-trisphosphate receptors between normal and dystrophic skeletal muscle cell lines', *Muscle Nerve*, vol. 21, no. 7, pp. 902-9.

Lim, G, Venetucci, L, Eisner, DA & Casadei, B 2008, 'Does nitric oxide modulate cardiac ryanodine receptor function? Implications for excitation-contraction coupling', *Cardiovasc Res*, vol. 77, no. 2, pp. 256-64.

Lin, J, Arnold, HB, Della-Fera, MA, Azain, MJ, Hartzell, DL & Baile, CA 2002, 'Myostatin knockout in mice increases myogenesis and decreases adipogenesis', *Biochem Biophys Res Commun*, vol. 291, no. 3, pp. 701-6.

Lindahl, GE, Chambers, RC, Papakrivopoulou, J, Dawson, SJ, Jacobsen, MC, Bishop, JE & Laurent, GJ 2002, 'Activation of fibroblast procollagen alpha 1(I) transcription by mechanical strain is transforming growth factor-beta-dependent and involves increased binding of CCAAT-binding factor (CBF/NF-Y) at the proximal promoter', *J Biol Chem*, vol. 277, no. 8, pp. 6153-61.

Liu, M, Yue, Y, Harper, SQ, Grange, RW, Chamberlain, JS & Duan, D 2005, 'Adeno-associated virus-mediated microdystrophin expression protects young mdx muscle from contraction-induced injury', *Mol Ther*, vol. 11, no. 2, pp. 245-56.

Liu, P, Xu, B, Hock, CE, Nagele, R, Sun, FF & Wong, PY 1998, 'NO modulates P-selectin and ICAM-1 mRNA expression and hemodynamic alterations in hepatic I/R', *Am J Physiol*, vol. 275, no. 6 Pt 2, pp. H2191-8.

Locksley, RM, Killeen, N & Lenardo, MJ 2001, 'The TNF and TNF receptor superfamilies: integrating mammalian biology', *Cell*, vol. 104, no. 4, pp. 487-501.

Loufrani, L, Dubroca, C, You, D, Li, Z, Levy, B, Paulin, D & Henrion, D 2004, 'Absence of dystrophin in mice reduces NO-dependent vascular function and vascular density: total recovery after a treatment with the aminoglycoside gentamicin', *Arterioscler Thromb Vasc Biol*, vol. 24, no. 4, pp. 671-6.

Lu, S & Hoey, A 2000, 'Changes in function of cardiac receptors mediating the effects of the autonomic nervous system in the muscular dystrophy (MDX) mouse', *J Mol Cell Cardiol*, vol. 32, no. 1, pp. 143-52.

Lynch, GS, Hinkle, RT, Chamberlain, JS, Brooks, SV & Faulkner, JA 2001, 'Force and power output of fast and slow skeletal muscles from mdx mice 6-28 months old', *J Physiol*, vol. 535, no. Pt 2, pp. 591-600.

MacLennan, DH & Kranias, EG 2003, 'Phospholamban: a crucial regulator of cardiac contractility', *Nat Rev Mol Cell Biol*, vol. 4, no. 7, pp. 566-77.

MacLennan, PA & Edwards, RH 1990, 'Protein turnover is elevated in muscle of mdx mice in vivo', *Biochem J*, vol. 268, no. 3, pp. 795-7.

Malik, V, Rodino-Klapac, LR, Viollet, L, Wall, C, King, W, Al-Dahhak, R, Lewis, S, Shilling, CJ, Kota, J, Serrano-Munuera, C, Hayes, J, Mahan, JD, Campbell, KJ, Banwell, B, Dasouki, M, Watts, V, Sivakumar, K, Bien-Willner, R, Flanigan, KM, Sahenk, Z, Barohn, RJ, Walker, CM & Mendell, JR 2010, 'Gentamicin-induced readthrough of stop codons in Duchenne muscular dystrophy', *Ann Neurol*, vol. 67, no. 6, pp. 771-80.

Mane, J, Fernandez-Banares, F, Ojanguren, I, Castella, E, Bertran, X, Bartoli, R, Alvarez, M & Gassull, MA 2001, 'Effect of L-arginine on the course of experimental colitis', *Clin Nutr*, vol. 20, no. 5, pp. 415-22.

Mann, CJ, Honeyman, K, Cheng, AJ, Ly, T, Lloyd, F, Fletcher, S, Morgan, JE, Partridge, TA & Wilton, SD 2001, 'Antisense-induced exon skipping and synthesis of dystrophin in the mdx mouse', *Proc Natl Acad Sci U S A*, vol. 98, no. 1, pp. 42-7.

Manno, CS, Pierce, GF, Arruda, VR, Glader, B, Ragni, M, Rasko, JJ, Ozelo, MC, Hoots, K, Blatt, P, Konkle, B, Dake, M, Kaye, R, Razavi, M, Zajko, A, Zehnder, J, Rustagi, PK, Nakai, H, Chew, A, Leonard, D, Wright, JF, Lessard, RR, Sommer, JM, Tigges, M, Sabatino, D, Luk, A, Jiang, H, Mingozzi, F, Couto, L, Ertl, HC, High, KA & Kay, MA 2006, 'Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response', *Nat Med*, vol. 12, no. 3, pp. 342-7.

Manzur, AY, Kinali, M & Muntoni, F 2008b, 'Update on the management of Duchenne muscular dystrophy', *Arch Dis Child*, vol. 93, no. 11, pp. 986-90.

Manzur, AY, Kuntzer, T, Pike, M & Swan, A 2008a, 'Glucocorticoid corticosteroids for Duchenne muscular dystrophy', *Cochrane Database Syst Rev*, no. 1, p. CD003725.

Marchand, E, Constantin, B, Balghi, H, Claudepierre, MC, Cantereau, A, Magaud, C, Mouzou, A, Raymond, G, Braun, S & Cognard, C 2004, 'Improvement of calcium handling and changes in calcium-release properties after mini- or full-length dystrophin forced expression in cultured skeletal myotubes', *Exp Cell Res*, vol. 297, no. 2, pp. 363-79.

Markham, LW, Kinnett, K, Wong, BL, Woodrow Benson, D & Cripe, LH 2008, 'Corticosteroid treatment retards development of ventricular dysfunction in Duchenne muscular dystrophy', *Neuromuscul Disord*, vol. 18, no. 5, pp. 365-70.

Maroto, R, Raso, A, Wood, TG, Kurosky, A, Martinac, B & Hamill, OP 2005, 'TRPC1 forms the stretch-activated cation channel in vertebrate cells', *Nat Cell Biol*, vol. 7, no. 2, pp. 179-85.

Marques, MJ, Barbin, IC, Taniguti, AP, Oggian, DS, Ferretti, R & Santo Neto, H 2010, 'Myocardial fibrosis is unaltered by long-term administration of L-arginine in dystrophin deficient mdx mice: a histomorphometric analysis', *Acta Biol Hung*, vol. 61, no. 2, pp. 168-74.

Matsuda, R, Nishikawa, A & Tanaka, H 1995, 'Visualization of dystrophic muscle fibers in mdx mouse by vital staining with Evans blue: evidence of apoptosis in dystrophin-deficient muscle', *J Biochem*, vol. 118, no. 5, pp. 959-64.

Mauviel, A 2005, 'Transforming growth factor-beta: a key mediator of fibrosis', *Methods Mol Med*, vol. 117, pp. 69-80.

McCance, K & Huether, S 1998, *Pathophysiology: The Biological Basis for Disease in Adults and Children*, 3 edn, Missouri.

McCroskery, S, Thomas, M, Maxwell, L, Sharma, M & Kambadur, R 2003, 'Myostatin negatively regulates satellite cell activation and self-renewal', *J Cell Biol*, vol. 162, no. 6, pp. 1135-47.

McDonald, DG, Kinali, M, Gallagher, AC, Mercuri, E, Muntoni, F, Roper, H, Jardine, P, Jones, DH & Pike, MG 2002, 'Fracture prevalence in Duchenne muscular dystrophy', *Dev Med Child Neurol*, vol. 44, no. 10, pp. 695-8.

McDonald, TF, Pelzer, S, Trautwein, W & Pelzer, DJ 1994, 'Regulation and modulation of calcium channels in cardiac, skeletal, and smooth muscle cells', *Physiol Rev*, vol. 74, no. 2, pp. 365-507.

McDouall, RM, Dunn, MJ & Dubowitz, V 1990, 'Nature of the mononuclear infiltrate and the mechanism of muscle damage in juvenile dermatomyositis and Duchenne muscular dystrophy', *J Neurol Sci*, vol. 99, no. 2-3, pp. 199-217.

McGreachie, JK, Grounds, MD, Partridge, AJ & Morgan, JE 1993, 'Age-related changes in replication of myogenic cells in mdx mice: quantitive autoradiographic studies', *J Neurol Sci*, vol. 119, no. 2, pp. 169-79.

McNally, EM 2008, 'Duchenne muscular dystrophy: how bad is the heart?', *Heart*, vol. 94, no. 8, pp. 976-7.

McNally, EM & Towbin, JA 2004, 'Cardiomyopathy in muscular dystrophy workshop. 28-30 September 2003, Tucson, Arizona', *Neuromuscul Disord*, vol. 14, no. 7, pp. 442-8.

McPherron, AC & Lee, SJ 2002, 'Suppression of body fat accumulation in myostatin-deficient mice', *J Clin Invest*, vol. 109, no. 5, pp. 595-601.

McPherron, AC, Lawler, AM & Lee, SJ 1997a, 'Regulation of Skeletal Muscle Mass in Mice by a New TGF-β Superfamily Member', *Nature*, vol. 387, no. 1 May, pp. 83-90.

McPherron, AC, Lawler, AM & Lee, SJ 1997b, 'Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member', *Nature*, vol. 387, no. 6628, pp. 83-90.

Megeney, LA, Kablar, B, Garrett, K, Anderson, JE & Rudnicki, MA 1996, 'MyoD is required for myogenic stem cell function in adult skeletal muscle', *Genes Dev*, vol. 10, no. 10, pp. 1173-83.

Mendell, JR, Engel, WK & Derrer, EC 1971, 'Duchenne muscular dystrophy: functional ischemia reproduces its characteristic lesions', *Science*, vol. 172, no. 3988, pp. 1143-5.

Mendell, JR, Moxley, RT, Griggs, RC, Brooke, MH, Fenichel, GM, Miller, JP, King, W, Signore, L, Pandya, S, Florence, J & et al. 1989, 'Randomized, double-blind six-month trial of prednisone in Duchenne's muscular dystrophy', *N Engl J Med*, vol. 320, no. 24, pp. 1592-7.

Meola, G, Scarpini, E, Velicogna, M, Mottura, A, Baron, PL, Beretta, S & Scarlato, G 1986, 'Analysis of fibronectin expression during human muscle differentiation', *Basic Appl Histochem*, vol. 30, no. 2, pp. 153-63.

Merlini, L, Cicognani, A, Malaspina, E, Gennari, M, Gnudi, S, Talim, B & Franzoni, E 2003, 'Early prednisone treatment in Duchenne muscular dystrophy', *Muscle Nerve*, vol. 27, no. 2, pp. 222-7.

Mery, PF, Pavoine, C, Belhassen, L, Pecker, F & Fischmeister, R 1993, 'Nitric oxide regulates cardiac Ca2+ current. Involvement of cGMP-inhibited and cGMP-stimulated phosphodiesterases through guanylyl cyclase activation', *J Biol Chem*, vol. 268, no. 35, pp. 26286-95.

Messina, S, Bitto, A, Aguennouz, M, Minutoli, L, Monici, MC, Altavilla, D, Squadrito, F & Vita, G 2006, 'Nuclear factor kappa-B blockade reduces

skeletal muscle degeneration and enhances muscle function in Mdx mice.', *Exp Neurol*, vol. 198(1), pp. 234-41.

Metzinger, L, Passaquin, AC, Leijendekker, WJ, Poindron, P & Ruegg, UT 1995, 'Modulation by prednisolone of calcium handling in skeletal muscle cells', *Br J Pharmacol*, vol. 116, no. 7, pp. 2811-6.

Mezzano, V, Cabrera, D, Vial, C & Brandan, E 2007, 'Constitutively activated dystrophic muscle fibroblasts show a paradoxical response to TGF-beta and CTGF/CCN2', *J Cell Commun Signal*, vol. 1, no. 3-4, pp. 205-17.

Milani, S, Herbst, H, Schuppan, D, Stein, H & Surrenti, C 1991, 'Transforming growth factors beta 1 and beta 2 are differentially expressed in fibrotic liver disease', *Am J Pathol*, vol. 139, no. 6, pp. 1221-9.

Miles, AM, Bohle, DS, Glassbrenner, PA, Hansert, B, Wink, DA & Grisham, MB 1996, 'Modulation of superoxide-dependent oxidation and hydroxylation reactions by nitric oxide', *J Biol Chem*, vol. 271, no. 1, pp. 40-7.

Miller, RG & Hoffman, EP 1994, 'Molecular diagnosis and modern management of Duchenne muscular dystrophy', *Neurol Clin*, vol. 12, no. 4, pp. 699-725.

Miura, P & Jasmin, BJ 2006, 'Utrophin upregulation for treating Duchenne or Becker muscular dystrophy: how close are we?', *Trends Mol Med*, vol. 12, no. 3, pp. 122-9.

Miyoshi, K 1991, 'Echocardiographic evaluation of fibrous replacement in the myocardium of patients with Duchenne muscular dystrophy', *Br Heart J*, vol. 66, no. 6, pp. 452-5.

Mohr, S, Hallak, H, de Boitte, A, Lapetina, EG & Brune, B 1999, 'Nitric oxide-induced S-glutathionylation and inactivation of glyceraldehyde-3-phosphate dehydrogenase', *J Biol Chem*, vol. 274, no. 14, pp. 9427-30.

Moise, NS, Valentine, BA, Brown, CA, Erb, HN, Beck, KA, Cooper, BJ & Gilmour, RF 1991, 'Duchenne's cardiomyopathy in a canine model: electrocardiographic and echocardiographic studies', *J Am Coll Cardiol*, vol. 17, no. 3, pp. 812-20.

Molkentin, JD, Lu, JR, Antos, CL, Markham, B, Richardson, J, Robbins, J, Grant, SR & Olson, EN 1998, 'A calcineurin-dependent transcriptional pathway for cardiac hypertrophy', *Cell*, vol. 93, no. 2, pp. 215-28.

Monaco, AP, Bertelson, CJ, Liechti-Gallati, S, Moser, H & Kunkel, LM 1988, 'An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus', *Genomics*, vol. 2, no. 1, pp. 90-5.

Montrucchio, G, Lupia, E, de Martino, A, Battaglia, E, Arese, M, Tizzani, A, Bussolino, F & Camussi, G 1997, 'Nitric oxide mediates angiogenesis induced in vivo by platelet-activating factor and tumor necrosis factor-alpha', *Am J Pathol*, vol. 151, no. 2, pp. 557-63.

Mori, M & Gotoh, T 2004, 'Arginine metabolic enzymes, nitric oxide and infection', *J Nutr*, vol. 134, no. 10 Suppl, pp. 2820S-5S; discussion 53S.

Morissette, MR, Cook, SA, Buranasombati, C, Rosenberg, MA & Rosenzweig, A 2009, 'Myostatin inhibits IGF-I-induced myotube hypertrophy through Akt', *Am J Physiol Cell Physiol*, vol. 297, no. 5, pp. C1124-32.

Morissette, MR, Cook, SA, Foo, S, McKoy, G, Ashida, N, Novikov, M, Scherrer-Crosbie, M, Li, L, Matsui, T, Brooks, G & Rosenzweig, A 2006, 'Myostatin regulates cardiomyocyte growth through modulation of Akt signaling', *Circ Res*, vol. 99, no. 1, pp. 15-24.

Morrison, J, Lu, QL, Pastoret, C, Partridge, T & Bou-Gharios, G 2000, 'T-cell-dependent fibrosis in the mdx dystrophic mouse', *Lab Invest*, vol. 80, no. 6, pp. 881-91.

Muntoni, F 2001, 'Is a muscle biopsy in Duchenne dystrophy really necessary?', *Neurology*, vol. 57, no. 4, pp. 574-5.

Muntoni, F, Fisher, I, Morgan, JE & Abraham, D 2002, 'Steroids in Duchenne muscular dystrophy: from clinical trials to genomic research', *Neuromuscul Disord*, vol. 12 Suppl 1, pp. S162-5.

Murakami, N, McLennan, IS, Nonaka, I, Koishi, K, Baker, C & Hammond-Tooke, G 1999, 'Transforming growth factor-beta2 is elevated in skeletal muscle disorders', *Muscle Nerve*, vol. 22, no. 7, pp. 889-98.

Nagayama, T, Hsu, S, Zhang, M, Koitabashi, N, Bedja, D, Gabrielson, KL, Takimoto, E & Kass, DA 2009, 'Sildenafil stops progressive chamber, cellular, and molecular remodeling and improves calcium handling and function in hearts with pre-existing advanced hypertrophy caused by pressure overload', *J Am Coll Cardiol*, vol. 53, no. 2, pp. 207-15.

Nakane, M, Schmidt, HH, Pollock, JS, Forstermann, U & Murad, F 1993, 'Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle', *FEBS Lett*, vol. 316, no. 2, pp. 175-80.

Nelson, SF, Crosbie, RH, Miceli, MC & Spencer, MJ 2009, 'Emerging genetic therapies to treat Duchenne muscular dystrophy', *Curr Opin Neurol*, vol. 22, no. 5, pp. 532-8.

Nguyen, HH, Jayasinha, V, Xia, B, Hoyte, K & Martin, PT 2002, 'Overexpression of the cytotoxic T cell GalNAc transferase in skeletal

muscle inhibits muscular dystrophy in mdx mice', *Proc Natl Acad Sci U S A*, vol. 99, no. 8, pp. 5616-21.

Nguyen, HX & Tidball, JG 2003a, 'Interactions between neutrophils and macrophages promote macrophage killing of rat muscle cells in vitro', *J Physiol*, vol. 547, no. Pt 1, pp. 125-32.

Nguyen, HX & Tidball, JG 2003b, 'Expression of a muscle-specific, nitric oxide synthase transgene prevents muscle membrane injury and reduces muscle inflammation during modified muscle use in mice', *J Physiol*, vol. 550, no. Pt 2, pp. 347-56.

Nicholson, LV, Johnson, MA, Bushby, KM, Gardner-Medwin, D, Curtis, A, Ginjaar, IB, den Dunnen, JT, Welch, JL, Butler, TJ, Bakker, E & et al. 1993, 'Integrated study of 100 patients with Xp21 linked muscular dystrophy using clinical, genetic, immunochemical, and histopathological data. Part 1. Trends across the clinical groups', *J Med Genet*, vol. 30, no. 9, pp. 728-36.

Niebroj-Dobosz, I, Kornguth, S, Schutta, HS & Siegel, FL 1989, 'Elevated calmodulin levels and reduced calmodulin-stimulated calcium-ATPase in Duchenne progressive muscular dystrophy', *Neurology*, vol. 39, no. 12, pp. 1610-4.

Nigro, G, Comi, LI, Politano, L & Bain, RJ 1990, 'The incidence and evolution of cardiomyopathy in Duchenne muscular dystrophy', *Int J Cardiol*, vol. 26, no. 3, pp. 271-7.

Nishida, CR & Ortiz de Montellano, PR 1998, 'Electron transfer and catalytic activity of nitric oxide synthases. Chimeric constructs of the neuronal, inducible, and endothelial isoforms', *J Biol Chem*, vol. 273, no. 10, pp. 5566-71.

O'Rourke, B, Kass, DA, Tomaselli, GF, Kaab, S, Tunin, R & Marban, E 1999, 'Mechanisms of altered excitation-contraction coupling in canine tachycardia-induced heart failure, I: experimental studies', *Circ Res*, vol. 84, no. 5, pp. 562-70.

Ogata, H, Ishikawa, Y & Minami, R 2009, 'Beneficial effects of beta-blockers and angiotensin-converting enzyme inhibitors in Duchenne muscular dystrophy', *J Cardiol*, vol. 53, no. 1, pp. 72-8.

Ohlendieck, K & Campbell, KP 1991, 'Dystrophin-associated proteins are greatly reduced in skeletal muscle from mdx mice', *J Cell Biol*, vol. 115, no. 6, pp. 1685-94.

Ohlendieck, K, Ervasti, JM, Matsumura, K, Kahl, SD, Leveille, CJ & Campbell, KP 1991, 'Dystrophin-related protein is localized to neuromuscular junctions of adult skeletal muscle', *Neuron*, vol. 7, no. 3, pp. 499-508.

Okinaka, S, Sugita, H, Momoi, H, Toyokura, Y, Kumagai, H, Ebashi, S & Fujie, Y 1959, 'Serum creatine phosphokinase and aldolase activity in neuromuscular disorders', *Trans Am Neurol Assoc*, vol. 84, pp. 62-4.

Pankov, R & Yamada, KM 2002, 'Fibronectin at a glance', *J Cell Sci*, vol. 115, no. Pt 20, pp. 3861-3.

Paolocci, N, Ekelund, UE, Isoda, T, Ozaki, M, Vandegaer, K, Georgakopoulos, D, Harrison, RW, Kass, DA & Hare, JM 2000, 'cGMP-independent inotropic effects of nitric oxide and peroxynitrite donors: potential role for nitrosylation', *Am J Physiol Heart Circ Physiol*, vol. 279, no. 4, pp. H1982-8.

Papp, Z, van der Velden, J & Stienen, GJ 2000, 'Calpain-I induced alterations in the cytoskeletal structure and impaired mechanical properties of single myocytes of rat heart', *Cardiovasc Res*, vol. 45, no. 4, pp. 981-93.

Pardo, JV, Siliciano, JD & Craig, SW 1983, 'A vinculin-containing cortical lattice in skeletal muscle: transverse lattice elements ("costameres") mark sites of attachment between myofibrils and sarcolemma', *Proc Natl Acad Sci U S A*, vol. 80, no. 4, pp. 1008-12.

Paria, BC, Malik, AB, Kwiatek, AM, Rahman, A, May, MJ, Ghosh, S & Tiruppathi, C 2003, 'Tumor necrosis factor-alpha induces nuclear factor-kappaB-dependent TRPC1 expression in endothelial cells', *J Biol Chem*, vol. 278, no. 39, pp. 37195-203.

Partridge, TA 1997, 'Models of dystrophinopathy, pathological mechanisms and assessment of therapies', in SC Brown & JA Lucy (eds), *Dystrophin: Gene, Protein and Cell Biology*, Cambridge University Press, Cambridge, pp. 310-4.

Partridge, TA, Morgan, JE, Coulton, GR, Hoffman, EP & Kunkel, LM 1989, 'Conversion of mdx myofibres from dystrophin-negative to -positive by injection of normal myoblasts', *Nature*, vol. 337, no. 6203, pp. 176-9.

Pasquini, F, Guerin, C, Blake, D, Davies, K, Karpati, G & Holland, P 1995, 'The effect of glucocorticoids on the accumulation of utrophin by cultured normal and dystrophic human skeletal muscle satellite cells', *Neuromuscul Disord*, vol. 5, no. 2, pp. 105-14.

Passerini, L, Bernasconi, P, Baggi, F, Confalonieri, P, Cozzi, F, Cornelio, F & Mantegazza, R 2002, 'Fibrogenic cytokines and extent of fibrosis in muscle of dogs with X-linked golden retriever muscular dystrophy', *Neuromuscul Disord*, vol. 12, no. 9, pp. 828-35.

Pastoret, C & Sebille, A 1995, 'mdx mice show progressive weakness and muscle deterioration with age', *J Neurol Sci*, vol. 129, no. 2, pp. 97-105.

Peault, B, Rudnicki, M, Torrente, Y, Cossu, G, Tremblay, JP, Partridge, T, Gussoni, E, Kunkel, LM & Huard, J 2007, 'Stem and progenitor cells in skeletal muscle development, maintenance, and therapy', *Mol Ther*, vol. 15, no. 5, pp. 867-77.

Perkins, KJ & Davies, KE 2002, 'The role of utrophin in the potential therapy of Duchenne muscular dystrophy', *Neuromuscul Disord*, vol. 12 Suppl 1, pp. S78-89.

Pernice, W, Beckmann, R, Ketelsen, UP, Frey, M, Schmidt-Redemann, B, Haap, KP, Roehren, R & Sauer, M 1988, 'A double-blind placebo controlled trial of diltiazem in Duchenne dystrophy', *Klin Wochenschr*, vol. 66, no. 13, pp. 565-70.

Pessah, IN, Kim, KH & Feng, W 2002, 'Redox sensing properties of the ryanodine receptor complex', *Front Biosci*, vol. 7, pp. a72-9.

Petrof, BJ, Shrager, JB, Stedman, HH, Kelly, AM & Sweeney, HL 1993, 'Dystrophin protects the sarcolemma from stresses developed during muscle contraction', *Proc Natl Acad Sci U S A*, vol. 90, no. 8, pp. 3710-4.

Petroff, MG, Kim, SH, Pepe, S, Dessy, C, Marban, E, Balligand, JL & Sollott, SJ 2001, 'Endogenous nitric oxide mechanisms mediate the stretch dependence of Ca2+ release in cardiomyocytes', *Nat Cell Biol*, vol. 3, no. 10, pp. 867-73.

Phillips, MF, Quinlivan, RC, Edwards, RH & Calverley, PM 2001, 'Changes in spirometry over time as a prognostic marker in patients with Duchenne muscular dystrophy', *Am J Respir Crit Care Med*, vol. 164, no. 12, pp. 2191-4.

Pinsky, DJ, Patton, S, Mesaros, S, Brovkovych, V, Kubaszewski, E, Grunfeld, S & Malinski, T 1997, 'Mechanical transduction of nitric oxide synthesis in the beating heart', *Circ Res*, vol. 81, no. 3, pp. 372-9.

Pisconti, A, Brunelli, S, Di Padova, M, De Palma, C, Deponti, D, Baesso, S, Sartorelli, V, Cossu, G & Clementi, E 2006, 'Follistatin induction by nitric oxide through cyclic GMP: a tightly regulated signaling pathway that controls myoblast fusion', *J Cell Biol*, vol. 172, no. 2, pp. 233-44.

Polakoff, RJ, Morton, AA, Koch, KD & Rios, CM 1998, 'The psychosocial and cognitive impact of Duchenne's muscular dystrophy', *Semin Pediatr Neurol*, vol. 5, no. 2, pp. 116-23.

Politano, L, Nigro, G, Nigro, V, Piluso, G, Papparella, S, Paciello, O & Comi, LI 2003, 'Gentamicin administration in Duchenne patients with premature stop codon. Preliminary results', *Acta Myol*, vol. 22, no. 1, pp. 15-21.

Politano, L, Nigro, V, Nigro, G, Petretta, VR, Passamano, L, Papparella, S, Di Somma, S & Comi, LI 1996, 'Development of cardiomyopathy in female carriers of Duchenne and Becker muscular dystrophies', *JAMA*, vol. 275, no. 17, pp. 1335-8.

Porreca, E, Guglielmi, MD, Uncini, A, Di Gregorio, P, Angelini, A, Di Febbo, C, Pierdomenico, SD, Baccante, G & Cuccurullo, F 1999, 'Haemostatic abnormalities, cardiac involvement and serum tumor necrosis factor levels in X-linked dystrophic patients', *Thromb Haemost*, vol. 81, no. 4, pp. 543-6.

Powell, JA, Mohamed, SN, Kerr, JS & Mousa, SA 2000, 'Antiangiogenesis efficacy of nitric oxide donors', *J Cell Biochem*, vol. 80, no. 1, pp. 104-14.

Prior, TW, Bartolo, C, Pearl, DK, Papp, AC, Snyder, PJ, Sedra, MS, Burghes, AHM & Mendell, JR 1995, 'Spectrum of Small Mutations in the Dystrophin Coding Region', *American Journal of Human Genetics*, vol. 57, no. 1, pp. 22-33.

Qiao, C, Li, J, Jiang, J, Zhu, X, Wang, B & Xiao, X 2008, 'Myostatin propeptide gene delivery by adeno-associated virus serotype 8 vectors enhances muscle growth and ameliorates dystrophic phenotypes in mdx mice', *Hum Gene Ther*, vol. 19, no. 3, pp. 241-54.

Quinlan, JG, Hahn, HS, Wong, BL, Lorenz, JN, Wenisch, AS & Levin, LS 2004, 'Evolution of the mdx mouse cardiomyopathy: physiological and morphological findings', *Neuromuscul Disord*, vol. 14, no. 8-9, pp. 491-6.

Quinlivan, R, Roper, H, Davie, M, Shaw, NJ, McDonagh, J & Bushby, K 2005, 'Report of a Muscular Dystrophy Campaign funded workshop Birmingham, UK, January 16th 2004. Osteoporosis in Duchenne muscular dystrophy; its prevalence, treatment and prevention', *Neuromuscul Disord*, vol. 15, no. 1, pp. 72-9.

Rafael, JA, Townsend, ER, Squire, SE, Potter, AC, Chamberlain, JS & Davies, KE 2000, 'Dystrophin and utrophin influence fiber type composition and post-synaptic membrane structure', *Hum Mol Genet*, vol. 9, no. 9, pp. 1357-67.

Rahman, MM, Hannan, MA, Mondol, BA, Bhoumick, NB & Haque, A 2001, 'Prednisolone in Duchenne muscular dystrophy', *Bangladesh Med Res Counc Bull*, vol. 27, no. 1, pp. 38-42.

Rando, TA 2001a, 'Role of nitric oxide in the pathogenesis of muscular dystrophies: a "two hit" hypothesis of the cause of muscle necrosis', *Microsc Res Tech*, vol. 55, no. 4, pp. 223-35.

Rando, TA 2001b, 'The dystrophin-glycoprotein complex, cellular signaling, and the regulation of cell survival in the muscular dystrophies', *Muscle Nerve*, vol. 24, no. 12, pp. 1575-94.

Rando, TA, Disatnik, MH, Yu, Y & Franco, A 1998, 'Muscle cells from mdx mice have an increased susceptibility to oxidative stress', *Neuromuscul Disord*, vol. 8, no. 1, pp. 14-21.

Rao, YJ & Xi, L 2009, 'Pivotal effects of phosphodiesterase inhibitors on myocyte contractility and viability in normal and ischemic hearts', *Acta Pharmacol Sin*, vol. 30, no. 1, pp. 1-24.

Raymackers, JM, Debaix, H, Colson-Van Schoor, M, De Backer, F, Tajeddine, N, Schwaller, B, Gailly, P & Gillis, JM 2003, 'Consequence of parvalbumin deficiency in the mdx mouse: histological, biochemical and mechanical phenotype of a new double mutant', *Neuromuscul Disord*, vol. 13, no. 5, pp. 376-87.

Reardon, KA, Davis, J, Kapsa, RM, Choong, P & Byrne, E 2001, 'Myostatin, insulin-like growth factor-1, and leukemia inhibitory factor mRNAs are upregulated in chronic human disuse muscle atrophy', *Muscle Nerve*, vol. 24, no. 7, pp. 893-9.

Rebbapragada, A, Benchabane, H, Wrana, JL, Celeste, AJ & Attisano, L 2003, 'Myostatin signals through a transforming growth factor beta-like signaling pathway to block adipogenesis', *Mol Cell Biol*, vol. 23, no. 20, pp. 7230-42.

Rehfeldt, C, Ott, G, Gerrard, DE, Varga, L, Schlote, W, Williams, JL, Renne, U & Bunger, L 2005, 'Effects of the compact mutant myostatin allele Mstn (Cmpt-dl1Abc) introgressed into a high growth mouse line on skeletal muscle cellularity', *J Muscle Res Cell Motil*, vol. 26, no. 2-3, pp. 103-12.

Reitter, B 1995, 'Deflazacort vs. prednisone in Duchenne muscular dystrophy: trends of an ongoing study', *Brain Dev*, vol. 17 Suppl, pp. 39-43.

Rifai, Z, Welle, S, Moxley, RT, 3rd, Lorenson, M & Griggs, RC 1995, 'Effect of prednisone on protein metabolism in Duchenne dystrophy', *Am J Physiol*, vol. 268, no. 1 Pt 1, pp. E67-74.

Rios, R, Carneiro, I, Arce, VM & Devesa, J 2002, 'Myostatin is an inhibitor of myogenic differentiation', *Am J Physiol Cell Physiol*, vol. 282, no. 5, pp. C993-9.

Robert, V, Massimino, ML, Tosello, V, Marsault, R, Cantini, M, Sorrentino, V & Pozzan, T 2001, 'Alteration in calcium handling at the subcellular level in mdx myotubes', *J Biol Chem*, vol. 276, no. 7, pp. 4647-51.

Roberts, RG 2001, 'Dystrophins and dystrobrevins', *Genome Biol*, vol. 2, no. 4, p. REVIEWS3006.

Rodgers, BD, Interlichia, JP, Garikipati, DK, Mamidi, R, Chandra, M, Nelson, OL, Murry, CE & Santana, LF 2009a, 'Myostatin represses

physiological hypertrophy of the heart and excitation-contraction coupling', *J Physiol*, vol. 587, no. Pt 20, pp. 4873-86.

Rodgers, BD, Interlichia, JP, Garikipati, DK, Mamidi, R, Chandra, M, Nelson, OL, Murry, CE & Santana, LF 2009b, 'Myostatin Represses Physiological Hypertrophy of the Heart and Excitation-Contraction Couping', *J Physiol*.

Rodillo, EB, Fernandez-Bermejo, E, Heckmatt, JZ & Dubowitz, V 1988, 'Prevention of rapidly progressive scoliosis in Duchenne muscular dystrophy by prolongation of walking with orthoses', *J Child Neurol*, vol. 3, no. 4, pp. 269-74.

Rodino-Klapac, LR, Chicoine, LG, Kaspar, BK & Mendell, JR 2007, 'Gene therapy for duchenne muscular dystrophy: expectations and challenges', *Arch Neurol*, vol. 64, no. 9, pp. 1236-41.

Rolland, JF, De Luca, A, Burdi, R, Andreetta, F, Confalonieri, P & Conte Camerino, D 2006, 'Overactivity of exercise-sensitive cation channels and their impaired modulation by IGF-1 in mdx native muscle fibers: beneficial effect of pentoxifylline', *Neurobiol Dis*, vol. 24, no. 3, pp. 466-74.

Rosalki, SB 1989, 'Serum enzymes in disease of skeletal muscle', *Clin Lab Med*, vol. 9, no. 4, pp. 767-81.

Rybakova, IN, Patel, JR & Ervasti, JM 2000, 'The dystrophin complex forms a mechanically strong link between the sarcolemma and costameric actin', *J Cell Biol*, vol. 150, no. 5, pp. 1209-14.

Rybalkin, SD, Rybalkina, IG, Feil, R, Hofmann, F & Beavo, JA 2002, 'Regulation of cGMP-specific phosphodiesterase (PDE5) phosphorylation in smooth muscle cells', *J Biol Chem*, vol. 277, no. 5, pp. 3310-7.

Sadeghi, A, Doyle, AD & Johnson, BD 2002, 'Regulation of the cardiac L-type Ca2+ channel by the actin-binding proteins alpha-actinin and dystrophin', *Am J Physiol Cell Physiol*, vol. 282, no. 6, pp. C1502-11.

Sander, M, Chavoshan, B, Harris, SA, Iannaccone, ST, Stull, JT, Thomas, GD & Victor, RG 2000, 'Functional muscle ischemia in neuronal nitric oxide synthase-deficient skeletal muscle of children with Duchenne muscular dystrophy', *Proc Natl Acad Sci U S A*, vol. 97, no. 25, pp. 13818-23.

Sano, M, Yokota, T, Endo, T & Tsukagoshi, H 1990, 'A developmental change in the content of parvalbumin in normal and dystrophic mouse (mdx) muscle', *J Neurol Sci*, vol. 97, no. 2-3, pp. 261-72.

Sanyal, SK & Johnson, WW 1982, 'Cardiac conduction abnormalities in children with Duchenne's progressive muscular dystrophy:

electrocardiographic features and morphologic correlates', *Circulation*, vol. 66, no. 4, pp. 853-63.

Sapp, JL, Bobet, J & Howlett, SE 1996, 'Contractile properties of myocardium are altered in dystrophin-deficient mdx mice', *J Neurol Sci*, vol. 142, no. 1-2, pp. 17-24.

Schatzberg, SJ, Anderson, LV, Wilton, SD, Kornegay, JN, Mann, CJ, Solomon, GG & Sharp, NJ 1998, 'Alternative dystrophin gene transcripts in golden retriever muscular dystrophy', *Muscle Nerve*, vol. 21, no. 8, pp. 991-8.

Scheuerbrandt, G 2009, *Research approaches for a Therapy of Duchenne Nuscular Dystrophy. Part 1: Exon Skipping*, viewed 08/09/09, <<http://www.treat-nmd.eu/userfiles/file/general/Exon%20Skipping%20Report%20April%202020 09.pdf>>.

Schmalbruch, H 1984, 'Regenerated muscle fibers in Duchenne muscular dystrophy: a serial section study', *Neurology*, vol. 34, no. 1, pp. 60-5.

Schmidt, HH, Pollock, JS, Nakane, M, Forstermann, U & Murad, F 1992, 'Ca2+/calmodulin-regulated nitric oxide synthases', *Cell Calcium*, vol. 13, no. 6-7, pp. 427-34.

Schuelke, M, Wagner, KR, Stolz, LE, Hubner, C, Riebel, T, Komen, W, Braun, T, Tobin, JF & Lee, SJ 2004, 'Myostatin mutation associated with gross muscle hypertrophy in a child', *N Engl J Med*, vol. 350, no. 26, pp. 2682-8.

Schwarte, RC & Godfrey, EW 2004, 'Nitric oxide synthase activity is required for postsynaptic differentiation of the embryonic neuromuscular junction', *Dev Biol*, vol. 273, no. 2, pp. 276-84.

Schwartz, M & Duno, M 2004, 'Improved molecular diagnosis of dystrophin gene mutations using the multiplex ligation-dependent probe amplification method', *Genet Test*, vol. 8, no. 4, pp. 361-7.

Schwinger, RH, Bohm, M, Schmidt, U, Karczewski, P, Bavendiek, U, Flesch, M, Krause, EG & Erdmann, E 1995, 'Unchanged protein levels of SERCA II and phospholamban but reduced Ca2+ uptake and Ca(2+)-ATPase activity of cardiac sarcoplasmic reticulum from dilated cardiomyopathy patients compared with patients with nonfailing hearts', *Circulation*, vol. 92, no. 11, pp. 3220-8.

Scott, JM, Li, S, Harper, SQ, Welikson, R, Bourque, D, DelloRusso, C, Hauschka, SD & Chamberlain, JS 2002, 'Viral vectors for gene transfer of micro-, mini-, or full-length dystrophin', *Neuromuscul Disord*, vol. 12 Suppl 1, pp. S23-9.

Sears, CE, Bryant, SM, Ashley, EA, Lygate, CA, Rakovic, S, Wallis, HL, Neubauer, S, Terrar, DA & Casadei, B 2003, 'Cardiac neuronal nitric oxide synthase isoform regulates myocardial contraction and calcium handling', *Circ Res*, vol. 92, no. 5, pp. e52-9.

Seddon, M, Shah, AM & Casadei, B 2007, 'Cardiomyocytes as effectors of nitric oxide signalling', *Cardiovasc Res*, vol. 75, no. 2, pp. 315-26.

Seddon, M, Melikian, N, Dworakowski, R, Shabeeh, H, Jiang, B, Byrne, J, Casadei, B, Chowienczyk, P & Shah, AM 2009, 'Effects of neuronal nitric oxide synthase on human coronary artery diameter and blood flow in vivo', *Circulation*, vol. 119, no. 20, pp. 2656-62.

Shaddy, RE, Boucek, MM, Hsu, DT, Boucek, RJ, Canter, CE, Mahony, L, Ross, RD, Pahl, E, Blume, ED, Dodd, DA, Rosenthal, DN, Burr, J, LaSalle, B, Holubkov, R, Lukas, MA & Tani, LY 2007, 'Carvedilol for children and adolescents with heart failure: a randomized controlled trial', *JAMA*, vol. 298, no. 10, pp. 1171-9.

Shah, AM, Spurgeon, HA, Sollott, SJ, Talo, A & Lakatta, EG 1994, '8-bromo-cGMP reduces the myofilament response to Ca2+ in intact cardiac myocytes', *Circ Res*, vol. 74, no. 5, pp. 970-8.

Sharma, M, Kambadur, R, Matthews, KG, Somers, WG, Devlin, GP, Conaglen, JV, Fowke, PJ & Bass, JJ 1999, 'Myostatin, a transforming growth factor-beta superfamily member, is expressed in heart muscle and is upregulated in cardiomyocytes after infarct', *J Cell Physiol*, vol. 180, no. 1, pp. 1-9.

Sharp, NJ, Kornegay, JN, Van Camp, SD, Herbstreith, MH, Secore, SL, Kettle, S, Hung, WY, Constantinou, CD, Dykstra, MJ, Roses, AD & et al. 1992, 'An error in dystrophin mRNA processing in golden retriever muscular dystrophy, an animal homologue of Duchenne muscular dystrophy', *Genomics*, vol. 13, no. 1, pp. 115-21.

Shavlakadze, T, Chai, J, Maley, K, Cozens, G, Grounds, G, Winn, N, Rosenthal, N & Grounds, MD 'A growth stimulus is needed for IGF-1 to induce skeletal muscle hypertrophy in vivo', *J Cell Sci*, vol. 123, no. Pt 6, pp. 960-71.

Shavlakadze, T, Chai, J, Maley, K, Cozens, G, Grounds, G, Winn, N, Rosenthal, N & Grounds, MD 2010, 'A growth stimulus is needed for IGF-1 to induce skeletal muscle hypertrophy in vivo', *J Cell Sci*, vol. 123, no. Pt 6, pp. 960-71.

Shiao, T, Fond, A, Deng, B, Wehling-Henricks, M, Adams, ME, Froehner, SC & Tidball, JG 2004, 'Defects in neuromuscular junction structure in dystrophic muscle are corrected by expression of a NOS transgene in dystrophin-deficient muscles, but not in muscles lacking alpha- and beta1-syntrophins', *Hum Mol Genet*, vol. 13, no. 17, pp. 1873-84.

Shimatsu, Y, Katagiri, K, Furuta, T, Nakura, M, Tanioka, Y, Yuasa, K, Tomohiro, M, Kornegay, JN, Nonaka, I & Takeda, S 2003, 'Canine X-linked muscular dystrophy in Japan (CXMDJ)', *Exp Anim*, vol. 52, no. 2, pp. 93-7.

Shin, DD, Brandimarte, F, De Luca, L, Sabbah, HN, Fonarow, GC, Filippatos, G, Komajda, M & Gheorghiade, M 2007, 'Review of current and investigational pharmacologic agents for acute heart failure syndromes', *Am J Cardiol*, vol. 99, no. 2A, pp. 4A-23A.

Shyu, KG, Lu, MJ, Wang, BW, Sun, HY & Chang, H 2006, 'Myostatin expression in ventricular myocardium in a rat model of volume-overload heart failure', *Eur J Clin Invest*, vol. 36, no. 10, pp. 713-9.

Sicinski, P, Geng, Y, Ryder-Cook, AS, Barnard, EA, Darlison, MG & Barnard, PJ 1989, 'The molecular basis of muscular dystrophy in the mdx mouse: a point mutation', *Science*, vol. 244, no. 4912, pp. 1578-80.

Silva, MC, Meira, ZM, Gurgel Giannetti, J, da Silva, MM, Campos, AF, Barbosa Mde, M, Starling Filho, GM, Ferreira Rde, A, Zatz, M & Rochitte, CE 2007, 'Myocardial delayed enhancement by magnetic resonance imaging in patients with muscular dystrophy', *J Am Coll Cardiol*, vol. 49, no. 18, pp. 1874-9.

Silversides, CK, Webb, GD, Harris, VA & Biggar, DW 2003, 'Effects of deflazacort on left ventricular function in patients with Duchenne muscular dystrophy', *Am J Cardiol*, vol. 91, no. 6, pp. 769-72.

Sirad, F, Hlaing, S, Kovanecz, I, Artaza, JN, Garcia, LA, Rajfer, J & Ferrini, MG 2011, 'Sildenafil promotes smooth muscle preservation and ameliorates fibrosis through modulation of extracellular matrix and tissue growth factor gene expression after bilateral cavernosal nerve resection in the rat', *J Sex Med*, vol. 8, no. 4, pp. 1048-60.

Siwik, DA & Colucci, WS 2004, 'Regulation of matrix metalloproteinases by cytokines and reactive oxygen/nitrogen species in the myocardium', *Heart Fail Rev*, vol. 9, no. 1, pp. 43-51.

Skinner, HB 1995, *Current Diagnosis and Treatment in Orthopedics*, Appleton & Lange, Connecticut.

Skuk, D, Roy, B, Goulet, M & Tremblay, JP 1999, 'Successful myoblast transplantation in primates depends on appropriate cell delivery and induction of regeneration in the host muscle', *Exp Neurol*, vol. 155, no. 1, pp. 22-30.

Slack, JL, Liska, DJ & Bornstein, P 1993, 'Regulation of expression of the type I collagen genes', *Am J Med Genet*, vol. 45, no. 2, pp. 140-51.

Smith, AD, Koreska, J & Moseley, CF 1989, 'Progression of scoliosis in Duchenne muscular dystrophy', *J Bone Joint Surg Am*, vol. 71, no. 7, pp. 1066-74.

Smythe, GM & Grounds, MD 2001, 'Absence of MyoD increases donor myoblast migration into host muscle', *Exp Cell Res*, vol. 267, no. 2, pp. 267-74.

Soderpalm, AC, Magnusson, P, Ahlander, AC, Karlsson, J, Kroksmark, AK, Tulinius, M & Swolin-Eide, D 2007, 'Low bone mineral density and decreased bone turnover in Duchenne muscular dystrophy', *Neuromuscul Disord*, vol. 17, no. 11-12, pp. 919-28.

Song, LS, Wang, SQ, Xiao, RP, Spurgeon, H, Lakatta, EG & Cheng, H 2001, 'beta-Adrenergic stimulation synchronizes intracellular Ca(2+) release during excitation-contraction coupling in cardiac myocytes', *Circ Res*, vol. 88, no. 8, pp. 794-801.

Souza, JM & Radi, R 1998, 'Glyceraldehyde-3-phosphate dehydrogenase inactivation by peroxynitrite', *Arch Biochem Biophys*, vol. 360, no. 2, pp. 187-94.

Spencer, MJ & Tidball, JG 1996, 'Calpain translocation during muscle fiber necrosis and regeneration in dystrophin-deficient mice', *Exp Cell Res*, vol. 226, no. 2, pp. 264-72.

Spencer, MJ & Mellgren, RL 2002, 'Overexpression of a calpastatin transgene in mdx muscle reduces dystrophic pathology', *Hum Mol Genet*, vol. 11, no. 21, pp. 2645-55.

Spencer, MJ, Marino, MW & Winckler, WM 2000, 'Altered pathological progression of diaphragm and quadriceps muscle in TNF-deficient, dystrophin-deficient mice', *Neuromuscul Disord*, vol. 10, no. 8, pp. 612-9.

Spencer, MJ, Montecino-Rodriguez, E, Dorshkind, K & Tidball, JG 2001, 'Helper (CD4(+)) and cytotoxic (CD8(+)) T cells promote the pathology of dystrophin-deficient muscle', *Clin Immunol*, vol. 98, no. 2, pp. 235-43.

Stedman, HH, Sweeney, HL, Shrager, JB, Maguire, HC, Panettieri, RA, Petrof, B, Narusawa, M, Leferovich, JM, Sladky, JT & Kelly, AM 1991, 'The mdx mouse diaphragm reproduces the degenerative changes of Duchenne muscular dystrophy', *Nature*, vol. 352, no. 6335, pp. 536-9.

Stoyanovsky, D, Murphy, T, Anno, PR, Kim, YM & Salama, G 1997, 'Nitric oxide activates skeletal and cardiac ryanodine receptors', *Cell Calcium*, vol. 21, no. 1, pp. 19-29.

Straub, V, Donahue, KM, Allamand, V, Davisson, RL, Kim, YR & Campbell, KP 2000, 'Contrast agent-enhanced magnetic resonance imaging

of skeletal muscle damage in animal models of muscular dystrophy', *Magn Reson Med*, vol. 44, no. 4, pp. 655-9.

Sun, J, Picht, E, Ginsburg, KS, Bers, DM, Steenbergen, C & Murphy, E 2006, 'Hypercontractile female hearts exhibit increased S-nitrosylation of the L-type Ca2+ channel alpha1 subunit and reduced ischemia/reperfusion injury', *Circ Res*, vol. 98, no. 3, pp. 403-11.

Suresh, S, Wales, P, Dakin, C, Harris, MA & Cooper, DG 2005, 'Sleep-related breathing disorder in Duchenne muscular dystrophy: disease spectrum in the paediatric population', *J Paediatr Child Health*, vol. 41, no. 9-10, pp. 500-3.

Surgery, AAoPSoCaC 2005, 'Cardiovascular health supervision for individuals affected by Duchenne or Becker muscular dystrophy', *Pediatrics*, vol. 116, no. 6, pp. 1569-73.

Susic, D, Francischetti, A & Frohlich, ED 1999, 'Prolonged L-arginine on cardiovascular mass and myocardial hemodynamics and collagen in aged spontaneously hypertensive rats and normal rats', *Hypertension*, vol. 33, no. 1 Pt 2, pp. 451-5.

Taipale, J, Miyazono, K, Heldin, CH & Keski-Oja, J 1994, 'Latent transforming growth factor-beta 1 associates to fibroblast extracellular matrix via latent TGF-beta binding protein', *J Cell Biol*, vol. 124, no. 1-2, pp. 171-81.

Takagi, A 2000, '[Malignant hyperthermia of Duchenne muscular dystrophy: application of clinical grading scale and caffeine contracture of skinned muscle fibers]', *Rinsho Shinkeigaku*, vol. 40, no. 5, pp. 423-7.

Takagi, A & Nakase, H 2008, 'Malignant hyperthermia-like reactions in Duchenne or Becker muscular dystrophy: review and hypothesis', *Rinsho Shinkeigaku*, vol. 48, no. 2, pp. 101-5.

Takasago, T, Imagawa, T, Furukawa, K, Ogurusu, T & Shigekawa, M 1991, 'Regulation of the cardiac ryanodine receptor by protein kinase-dependent phosphorylation', *J Biochem*, vol. 109, no. 1, pp. 163-70.

Talim, B, Malaguti, C, Gnudi, S, Politano, L & Merlini, L 2002, 'Vertebral compression in Duchenne muscular dystrophy following deflazacort', *Neuromuscul Disord*, vol. 12, no. 3, pp. 294-5.

Tatsumi, R, Hattori, A, Ikeuchi, Y, Anderson, JE & Allen, RE 2002, 'Release of hepatocyte growth factor from mechanically stretched skeletal muscle satellite cells and role of pH and nitric oxide', *Mol Biol Cell*, vol. 13, no. 8, pp. 2909-18.

Tay, JS, Lai, PS, Low, PS, Lee, WL & Gan, GC 1992, 'Pathogenesis of Duchenne muscular dystrophy: the calcium hypothesis revisited', *J Paediatr Child Health*, vol. 28, no. 4, pp. 291-3.

Tay, SK, Ong, HT & Low, PS 2000, 'Transaminitis in Duchenne's muscular dystrophy', *Ann Acad Med Singapore*, vol. 29, no. 6, pp. 719-22.

Taylor, WE, Bhasin, S, Artaza, J, Byhower, F, Azam, M, Willard, DH, Jr., Kull, FC, Jr. & Gonzalez-Cadavid, N 2001, 'Myostatin inhibits cell proliferation and protein synthesis in C2C12 muscle cells', *Am J Physiol Endocrinol Metab*, vol. 280, no. 2, pp. E221-8.

Theiss, AL, Simmons, JG, Jobin, C & Lund, PK 2005, 'Tumor necrosis factor (TNF) alpha increases collagen accumulation and proliferation in intestinal myofibroblasts via TNF receptor 2', *J Biol Chem*, vol. 280, no. 43, pp. 36099-109.

Thomas, GD, Sander, M, Lau, KS, Huang, PL, Stull, JT & Victor, RG 1998, 'Impaired metabolic modulation of alpha-adrenergic vasoconstriction in dystrophin-deficient skeletal muscle', *Proc Natl Acad Sci U S A*, vol. 95, no. 25, pp. 15090-5.

Thomas, M, Langley, B, Berry, C, Sharma, M, Kirk, S, Bass, J & Kambadur, R 2000, 'Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation', *J Biol Chem*, vol. 275, no. 51, pp. 40235-43.

Tidball, JG & Wehling-Henricks, M 2004, 'Evolving therapeutic strategies for Duchenne muscular dystrophy: targeting downstream events', *Pediatr Res*, vol. 56, no. 6, pp. 831-41.

Tidball, JG & Wehling-Henricks, M 2007, 'The role of free radicals in the pathophysiology of muscular dystrophy', *J Appl Physiol*, vol. 102, no. 4, pp. 1677-86.

Timmons, JA, Larsson, O, Jansson, E, Fischer, H, Gustafsson, T, Greenhaff, PL, Ridden, J, Rachman, J, Peyrard-Janvid, M, Wahlestedt, C & Sundberg, CJ 2005, 'Human muscle gene expression responses to endurance training provide a novel perspective on Duchenne muscular dystrophy', *FASEB J*, vol. 19, no. 7, pp. 750-60.

Tinsley, J, Deconinck, N, Fisher, R, Kahn, D, Phelps, S, Gillis, JM & Davies, K 1998, 'Expression of full-length utrophin prevents muscular dystrophy in mdx mice', *Nat Med*, vol. 4, no. 12, pp. 1441-4.

Tinsley, JM, Potter, AC, Phelps, SR, Fisher, R, Trickett, JI & Davies, KE 1996, 'Amelioration of the dystrophic phenotype of mdx mice using a truncated utrophin transgene', *Nature*, vol. 384, no. 6607, pp. 349-53.

Todorovic, Z, Prostran, M & Vuckovic, S 2001, 'The influence of L-arginine on heart rate and tissue oxygen extraction in haemorrhaged rabbits', *Pharmacol Res*, vol. 43, no. 4, pp. 321-7.

Torres, LF & Duchen, LW 1987, 'The mutant mdx: inherited myopathy in the mouse. Morphological studies of nerves, muscles and end-plates', *Brain*, vol. 110 (Pt 2), pp. 269-99.

Touboul, D, Brunelle, A, Halgand, F, De La Porte, S & Laprevote, O 2005, 'Lipid imaging by gold cluster time-of-flight secondary ion mass spectrometry: application to Duchenne muscular dystrophy', *J Lipid Res*, vol. 46, no. 7, pp. 1388-95.

Toussaint, M, Chatwin, M & Soudon, P 2007, 'Mechanical ventilation in Duchenne patients with chronic respiratory insufficiency: clinical implications of 20 years published experience', *Chron Respir Dis*, vol. 4, no. 3, pp. 167-77.

Toussaint, M, Steens, M, Wasteels, G & Soudon, P 2006, 'Diurnal ventilation via mouthpiece: survival in end-stage Duchenne patients', *Eur Respir J*, vol. 28, no. 3, pp. 549-55.

TREAT-NMD 2009, *Standards of care for Duchenne muscular dystrophy. Brief TREAT-NMD recommendations*, TREAT-NMD, viewed 12/09/09, <http://www.treat-nmd.eu/userfiles/file/general/TREAT-NMD_DMD_interim_recommendations.pdf>.

Tremblay, JP, Malouin, F, Roy, R, Huard, J, Bouchard, JP, Satoh, A & Richards, CL 1993, 'Results of a triple blind clinical study of myoblast transplantations without immunosuppressive treatment in young boys with Duchenne muscular dystrophy', *Cell Transplant*, vol. 2, no. 2, pp. 99-112.

Trochu, JN, Bouhour, JB, Kaley, G & Hintze, TH 2000, 'Role of endothelium-derived nitric oxide in the regulation of cardiac oxygen metabolism: implications in health and disease', *Circ Res*, vol. 87, no. 12, pp. 1108-17.

Turner, PR, Fong, PY, Denetclaw, WF & Steinhardt, RA 1991, 'Increased calcium influx in dystrophic muscle', *J Cell Biol*, vol. 115, no. 6, pp. 1701-12.

Turner, PR, Schultz, R, Ganguly, B & Steinhardt, RA 1993, 'Proteolysis results in altered leak channel kinetics and elevated free calcium in mdx muscle', *J Membr Biol*, vol. 133, no. 3, pp. 243-51.

Tutdibi, O, Brinkmeier, H, Rudel, R & Fohr, KJ 1999, 'Increased calcium entry into dystrophin-deficient muscle fibres of MDX and ADR-MDX mice is reduced by ion channel blockers', *J Physiol*, vol. 515 (Pt 3), pp. 859-68.

Tyler, KL 2003, 'Origins and early descriptions of "Duchenne muscular dystrophy"', *Muscle Nerve*, vol. 28, no. 4, pp. 402-22.

Ullrich, ND, Fanchaouy, M, Gusev, K, Shirokova, N & Niggli, E 2009, 'Hypersensitivity of excitation-contraction coupling in dystrophic cardiomyocytes', *Am J Physiol Heart Circ Physiol*, vol. 297, no. 6, pp. H1992-2003.

Valdivia, HH, Kaplan, JH, Ellis-Davies, GC & Lederer, WJ 1995, 'Rapid adaptation of cardiac ryanodine receptors: modulation by Mg2+ and phosphorylation', *Science*, vol. 267, no. 5206, pp. 1997-2000.

Valenick, LV, Hsia, HC & Schwarzbauer, JE 2005, 'Fibronectin fragmentation promotes alpha4beta1 integrin-mediated contraction of a fibrin-fibronectin provisional matrix', *Exp Cell Res*, vol. 309, no. 1, pp. 48-55.

Valentine, BA, Cooper, BJ, de Lahunta, A, O'Quinn, R & Blue, JT 1988, 'Canine X-linked muscular dystrophy. An animal model of Duchenne muscular dystrophy: clinical studies', *J Neurol Sci*, vol. 88, no. 1-3, pp. 69-81.

van Deutekom, JC, Janson, AA, Ginjaar, IB, Frankhuizen, WS, Aartsma-Rus, A, Bremmer-Bout, M, den Dunnen, JT, Koop, K, van der Kooi, AJ, Goemans, NM, de Kimpe, SJ, Ekhart, PF, Venneker, EH, Platenburg, GJ, Verschuuren, JJ & van Ommen, GJ 2007, 'Local dystrophin restoration with antisense oligonucleotide PRO051', *N Engl J Med*, vol. 357, no. 26, pp. 2677-86.

Van Erp, C 2005, *Modifying function and fibrosis of cardiac and skeletal muscle from mdx mice*, PhD, <http://eprints.usq.edu.au/1521/>.

Van Erp, C, Irwin, NG & Hoey, AJ 2006, 'Long-term administration of pirfenidone improves cardiac function in mdx mice', *Muscle Nerve*, vol. 34, no. 3, pp. 327-34.

Van Erp, C, Loch, D, Laws, N, Trebbin, A & Hoey, AJ 2010, 'Timeline of cardiac dystrophy in 3-18-month-old MDX mice', *Muscle Nerve*, vol. 42, no. 4, pp. 504-13.

van Ommen, GJ, van Deutekom, J & Aartsma-Rus, A 2008, 'The therapeutic potential of antisense-mediated exon skipping', *Curr Opin Mol Ther*, vol. 10, no. 2, pp. 140-9.

Vandebrouck, A, Ducret, T, Basset, O, Sebille, S, Raymond, G, Ruegg, U, Gailly, P, Cognard, C & Constantin, B 2006, 'Regulation of store-operated calcium entries and mitochondrial uptake by minidystrophin expression in cultured myotubes', *Faseb J*, vol. 20, no. 1, pp. 136-8.

Vandebrouck, C, Imbert, N, Duport, G, Cognard, C & Raymond, G 1999, 'The effect of methylprednisolone on intracellular calcium of normal and dystrophic human skeletal muscle cells', *Neurosci Lett*, vol. 269, no. 2, pp. 110-4.

Vandebrouck, C, Martin, D, Colson-Van Schoor, M, Debaix, H & Gailly, P 2002, 'Involvement of TRPC in the abnormal calcium influx observed in dystrophic (mdx) mouse skeletal muscle fibers', *J Cell Biol*, vol. 158, no. 6, pp. 1089-96.

Verhaert, D, Richards, K, Rafael-Fortney, JA & Raman, SV 2011, 'Cardiac involvement in patients with muscular dystrophies: magnetic resonance imaging phenotype and genotypic considerations', *Circ Cardiovasc Imaging*, vol. 4, no. 1, pp. 67-76.

Verma, IM 2000, 'A tumultuous year for gene therapy', *Mol Ther*, vol. 2, no. 5, pp. 415-6.

Verrecchia, F & Mauviel, A 2007, 'Transforming growth factor-beta and fibrosis', *World J Gastroenterol*, vol. 13, no. 22, pp. 3056-62.

Viatchenko-Karpinski, S & Gyorke, S 2001, 'Modulation of the Ca(2+)-induced Ca(2+) release cascade by beta-adrenergic stimulation in rat ventricular myocytes', *J Physiol*, vol. 533, no. Pt 3, pp. 837-48.

Voisin, V, Sebrie, C, Matecki, S, Yu, H, Gillet, B, Ramonatxo, M, Israel, M & De la Porte, S 2005, 'L-arginine improves dystrophic phenotype in mdx mice', *Neurobiol Dis*, vol. 20, no. 1, pp. 123-30.

Wagner, KR, McPherron, AC, Winik, N & Lee, SJ 2002, 'Loss of myostatin attenuates severity of muscular dystrophy in mdx mice', *Ann Neurol*, vol. 52, no. 6, pp. 832-6.

Wagner, KR, Liu, X, Chang, X & Allen, RE 2005, 'Muscle regeneration in the prolonged absence of myostatin', *Proc Natl Acad Sci U S A*, vol. 102, no. 7, pp. 2519-24.

Wagner, KR, Hamed, S, Hadley, DW, Gropman, AL, Burstein, AH, Escolar, DM, Hoffman, EP & Fischbeck, KH 2001, 'Gentamicin treatment of Duchenne and Becker muscular dystrophy due to nonsense mutations', *Ann Neurol*, vol. 49, no. 6, pp. 706-11.

Waheed, I, Gilbert, R, Nalbantoglu, J, Guibinga, GH, Petrof, BJ & Karpati, G 2005, 'Factors associated with induced chronic inflammation in mdx skeletal muscle cause posttranslational stabilization and augmentation of extrasynaptic sarcolemmal utrophin', *Hum Gene Ther*, vol. 16, no. 4, pp. 489-501.

Wang, B, Li, J & Xiao, X 2000, 'Adeno-associated virus vector carrying human minidystrophin genes effectively ameliorates muscular dystrophy in mdx mouse model', *Proc Natl Acad Sci U S A*, vol. 97, no. 25, pp. 13714-9.

Wang, H, Kohr, MJ, Traynham, CJ, Wheeler, DG, Janssen, PM & Ziolo, MT 2008, 'Neuronal nitric oxide synthase signaling within cardiac myocytes targets phospholamban', *Am J Physiol Cell Physiol*, vol. 294, no. 6, pp. C1566-75.

Wang, J & Walsh, K 1996, 'Resistance to apoptosis conferred by Cdk inhibitors during myocyte differentiation', *Science*, vol. 273, no. 5273, pp. 359-61.

Wang, S, Cone, J, Fong, M, Yoshitake, M, Kambayashi, J & Liu, Y 2001, 'Interplay between inhibition of adenosine uptake and phosphodiesterase type 3 on cardiac function by cilostazol, an agent to treat intermittent claudication', *J Cardiovasc Pharmacol*, vol. 38, no. 5, pp. 775-83.

Watchko, J, O'Day, T, Wang, B, Zhou, L, Tang, Y, Li, J & Xiao, X 2002, 'Adeno-associated virus vector-mediated minidystrophin gene therapy improves dystrophic muscle contractile function in mdx mice', *Hum Gene Ther*, vol. 13, no. 12, pp. 1451-60.

Watkins, S, Swartz, D & Byers, T 1997, 'Localization of dystrophin in skeletal, cardiac and smooth muscle', in C Brown & J Lucy (eds), *Dystrophin: Gene, Protein and Cell Biology*, Cambridge University Press, Cambridge, pp. 81-92.

Watkins, SC & Cullen, MJ 1988, 'A quantitative study of myonuclear and satellite cell nuclear size in Duchenne's muscular dystrophy, polymyositis and normal human skeletal muscle', *Anat Rec*, vol. 222, no. 1, pp. 6-11.

Weber, KT, Pick, R, Jalil, JE, Janicki, JS & Carroll, EP 1989, 'Patterns of myocardial fibrosis', *J Mol Cell Cardiol*, vol. 21 Suppl 5, pp. 121-31.

Wehling-Henricks, M, Jordan, MC, Roos, KP, Deng, B & Tidball, JG 2005, 'Cardiomyopathy in dystrophin-deficient hearts is prevented by expression of a neuronal nitric oxide synthase transgene in the myocardium', *Hum Mol Genet*, vol. 14, no. 14, pp. 1921-33.

Wehling-Henricks, M, Jordan, MC, Gotoh, T, Grody, WW, Roos, KP & Tidball, JG 2010, 'Arginine metabolism by macrophages promotes cardiac and muscle fibrosis in mdx muscular dystrophy', *PLoS One*, vol. 5, no. 5, p. e10763.

Wehling, M, Spencer, MJ & Tidball, JG 2001, 'A nitric oxide synthase transgene ameliorates muscular dystrophy in mdx mice', *J Cell Biol*, vol. 155, no. 1, pp. 123-31.

Welch, EM, Barton, ER, Zhuo, J, Tomizawa, Y, Friesen, WJ, Trifillis, P, Paushkin, S, Patel, M, Trotta, CR, Hwang, S, Wilde, RG, Karp, G, Takasugi, J, Chen, G, Jones, S, Ren, H, Moon, YC, Corson, D, Turpoff, AA, Campbell, JA, Conn, MM, Khan, A, Almstead, NG, Hedrick, J, Mollin, A, Risher, N, Weetall, M, Yeh, S, Branstrom, AA, Colacino, JM, Babiak, J, Ju, WD, Hirawat, S, Northcutt, VJ, Miller, LL, Spatrick, P, He, F, Kawana, M, Feng, H, Jacobson, A, Peltz, SW & Sweeney, HL 2007, 'PTC124 targets genetic disorders caused by nonsense mutations', *Nature*, vol. 447, no. 7140, pp. 87-91.

Wells, DJ 2006, 'Therapeutic restoration of dystrophin expression in Duchenne muscular dystrophy', *J Muscle Res Cell Motil*, vol. 27, no. 5-7, pp. 387-98.

Wells, KE, Torelli, S, Lu, Q, Brown, SC, Partridge, T, Muntoni, F & Wells, DJ 2003, 'Relocalization of neuronal nitric oxide synthase (nNOS) as a marker for complete restoration of the dystrophin associated protein complex in skeletal muscle', *Neuromuscul Disord*, vol. 13, no. 1, pp. 21-31.

White, ES, Baralle, FE & Muro, AF 2008, 'New insights into form and function of fibronectin splice variants', *J Pathol*, vol. 216, no. 1, pp. 1-14.

Wilcox, DE, Affara, NA, Yates, JR, Ferguson-Smith, MA & Pearson, PL 1985, 'Multipoint linkage analysis of the short arm of the human X chromosome in families with X-linked muscular dystrophy', *Hum Genet*, vol. 70, no. 4, pp. 365-75.

Williams, CM, Engler, AJ, Slone, RD, Galante, LL & Schwarzbauer, JE 2008, 'Fibronectin expression modulates mammary epithelial cell proliferation during acinar differentiation', *Cancer Res*, vol. 68, no. 9, pp. 3185-92.

Williams, IA & Allen, DG 2007a, 'The role of reactive oxygen species in the hearts of dystrophin-deficient mdx mice', *Am J Physiol Heart Circ Physiol*, vol. 293, no. 3, pp. H1969-77.

Williams, IA & Allen, DG 2007b, 'Intracellular calcium handling in ventricular myocytes from mdx mice', *Am J Physiol Heart Circ Physiol*, vol. 292, no. 2, pp. H846-55.

Williams, MW & Bloch, RJ 1999, 'Differential distribution of dystrophin and beta-spectrin at the sarcolemma of fast twitch skeletal muscle fibers', *J Muscle Res Cell Motil*, vol. 20, no. 4, pp. 383-93.

Willig, TN, Bach, JR, Venance, V & Navarro, J 1995, 'Nutritional rehabilitation in neuromuscular disorders', *Semin Neurol*, vol. 15, no. 1, pp. 18-23.

Willmann, R, Possekel, S, Dubach-Powell, J, Meier, T & Ruegg, MA 2009, 'Mammalian animal models for Duchenne muscular dystrophy', *Neuromuscul Disord*, vol. 19, no. 4, pp. 241-9.

Woolf, PJ, Lu, S, Cornford-Nairn, R, Watson, M, Xiao, XH, Holroyd, SM, Brown, L & Hoey, AJ 2006, 'Alterations in dihydropyridine receptors in dystrophin-deficient cardiac muscle', *Am J Physiol Heart Circ Physiol*, vol. 290, no. 6, pp. H2439-45.

Wu, G & Morris, SM, Jr. 1998, 'Arginine metabolism: nitric oxide and beyond', *Biochem J*, vol. 336 (Pt 1), pp. 1-17.

Xiao, W, Chirmule, N, Berta, SC, McCullough, B, Gao, G & Wilson, JM 1999, 'Gene therapy vectors based on adeno-associated virus type 1', *J Virol*, vol. 73, no. 5, pp. 3994-4003.

Xu, KY, Kuppusamy, SP, Wang, JQ, Li, H, Cui, H, Dawson, TM, Huang, PL, Burnett, AL, Kuppusamy, P & Becker, LC 2003, 'Nitric oxide protects cardiac sarcolemmal membrane enzyme function and ion active transport against ischemia-induced inactivation', *J Biol Chem*, vol. 278, no. 43, pp. 41798-803.

Xu, L, Eu, JP, Meissner, G & Stamler, JS 1998, 'Activation of the cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation', *Science*, vol. 279, no. 5348, pp. 234-7.

Yang, B, Jung, D, Motto, D, Meyer, J, Koretzky, G & Campbell, KP 1995, 'SH3 domain-mediated interaction of dystroglycan and Grb2', *J Biol Chem*, vol. 270, no. 20, pp. 11711-4.

Yang, L, Liu, G, Zakharov, SI, Bellinger, AM, Mongillo, M & Marx, SO 2007, 'Protein kinase G phosphorylates Cav1.2 alpha1c and beta2 subunits', *Circ Res*, vol. 101, no. 5, pp. 465-74.

Yeung, EW, Whitehead, NP, Suchyna, TM, Gottlieb, PA, Sachs, F & Allen, DG 2005, 'Effects of stretch-activated channel blockers on [Ca2+]i and muscle damage in the mdx mouse', *J Physiol*, vol. 562, no. Pt 2, pp. 367-80.

Yildirim, A, Ersoy, Y, Ercan, F, Atukeren, P, Gumustas, K, Uslu, U & Alican, I 2010, 'Phosphodiesterase-5 inhibition by sildenafil citrate in a rat model of bleomycin-induced lung fibrosis', *Pulm Pharmacol Ther*, vol. 23, no. 3, pp. 215-21.

Yiu, EM & Kornberg, AJ 2008, 'Duchenne muscular dystrophy', *Neurol India*, vol. 56, no. 3, pp. 236-47.

Yu, Q & Stamenkovic, I 2000, 'Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis', *Genes Dev*, vol. 14, no. 2, pp. 163-76.

Yugeta, N, Urasawa, N, Fujii, Y, Yoshimura, M, Yuasa, K, Wada, MR, Nakura, M, Shimatsu, Y, Tomohiro, M, Takahashi, A, Machida, N, Wakao, Y, Nakamura, A & Takeda, S 2006, 'Cardiac involvement in Beagle-based canine X-linked muscular dystrophy in Japan (CXMDJ): electrocardiographic, echocardiographic, and morphologic studies', *BMC Cardiovasc Disord*, vol. 6, p. 47.

Zahradnikova, A, Minarovic, I, Venema, RC & Meszaros, LG 1997, 'Inactivation of the cardiac ryanodine receptor calcium release channel by nitric oxide', *Cell Calcium*, vol. 22, no. 6, pp. 447-54.

Zatz, M, Rapaport, D, Vainzof, M, Passos-Bueno, MR, Bortolini, ER, Pavanello Rde, C & Peres, CA 1991, 'Serum creatine-kinase (CK) and pyruvate-kinase (PK) activities in Duchenne (DMD) as compared with Becker (BMD) muscular dystrophy', *J Neurol Sci*, vol. 102, no. 2, pp. 190-6.

Zhang, M, Takimoto, E, Hsu, S, Lee, DI, Nagayama, T, Danner, T, Koitabashi, N, Barth, AS, Bedja, D, Gabrielson, KL, Wang, Y & Kass, DA 2010, 'Myocardial remodeling is controlled by myocyte-targeted gene regulation of phosphodiesterase type 5', *J Am Coll Cardiol*, vol. 56, no. 24, pp. 2021-30.

Zhen, XG, Xie, C, Yamada, Y, Zhang, Y, Doyle, C & Yang, J 2006, 'A single amino acid mutation attenuates rundown of voltage-gated calcium channels', *FEBS Lett*, vol. 580, no. 24, pp. 5733-8.

Zhiliang, G, Dahai, Z, Ning, L, Hui, L, Xuemei, D & Changxin, W 2004, 'The single nucleotide polymorphisms of the chicken myostatin gene are associated with skeletal muscle and adipose growth', *Sci China C Life Sci*, vol. 47, no. 1, pp. 25-30.

Zhou, L, Burnett, AL, Huang, PL, Becker, LC, Kuppusamy, P, Kass, DA, Kevin Donahue, J, Proud, D, Sham, JS, Dawson, TM & Xu, KY 2002, 'Lack of nitric oxide synthase depresses ion transporting enzyme function in cardiac muscle', *Biochem Biophys Res Commun*, vol. 294, no. 5, pp. 1030-5.

Zimmers, TA, Davies, MV, Koniaris, LG, Haynes, P, Esquela, AF, Tomkinson, KN, McPherron, AC, Wolfman, NM & Lee, SJ 2002, 'Induction of cachexia in mice by systemically administered myostatin', *Science*, vol. 296, no. 5572, pp. 1486-8.

Zubrzycka-Gaarn, EE, Bulman, DE, Karpati, G, Burghes, AH, Belfall, B, Klamut, HJ, Talbot, J, Hodges, RS, Ray, PN & Worton, RG 1988, 'The Duchenne muscular dystrophy gene product is localized in sarcolemma of human skeletal muscle', *Nature*, vol. 333, no. 6172, pp. 466-9.