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Fibrotic pathways in the dystrophin deficient heart

A Dissertation submitted by

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ABSTRACT

Duchenne muscular dystrophy is the most prevalent and severe of the muscular dystrophies. A hallmark of dystrophic muscle is fibrosis which is a result of chronic cycles of degeneration and regeneration. Cardiac fibrosis interferes with contractility and rhythm of the heart and cardiomyopathy is a leading cause of mortality in DMD. While gene therapy may offer a cure in the future, there exists an immediate need to ameliorate the symptoms of DMD.

The *mdx* mouse is the most widely used animal model in DMD research. The initial study of this dissertation examined cardiac parameters of the *mdx* mouse at different ages (3, 6 and 12 months) and found a progressive cardiac involvement. Compromised systolic and diastolic function was evident from 6 months of age. An underlying morphological link between diastolic dysfunction is cardiac fibrosis and this was significantly increased in all ages examined.The role of proinflammatory cytokine TGF-β in fibrosis was investigated and mRNA levels were found to be significantly elevated at 3 months of age, which did not continue with the other two age groups examined. This suggests that TGF-β may be involved in the initiation of fibrotic pathways, but not in the progression of fibrosis. These results were supported by mRNA levels of procollagen and fibronectin which significantly decreased at 12 months of age. This implies a shift away from the initial active collagen synthesis toward a decreased collagen degradation maintaining the high levels of fibrosis.

Study 2 aimed to determine if insulin-like growth factor-1 could ameliorate the symptoms of the dystrophic heart by generating a novel transgenic mouse that overexpressed IGF-1 exclusively in the *mdx* heart. This study found IGF-1 overexpression to be beneficial to the dystrophic heart at 12 months of age. It mediated cardiac hypertrophy at 6 and 12 months of age which appeared to be a physiological adaptation as evidenced by the significantly improved cardiac function and rate of contractility at 12 months of age. IGF-1 mediated a dramatic reduction in ventricular fibrosis by 12 months of age to levels not significantly different than control mice. Thus IGF-1 appears to be capable of resolving the inflammatory response and thereby reducing fibrosis. This study supported the previous findings that at 6 and 12 months of age TGF-β mRNA levels do not correlate with the degree of ventricular fibrosis.

The final study of this dissertation further characterised the *mdx*:MyoD(-/-) mouse as a model of the cardiomyopathy associated with DMD. MyoD is required for efficient muscle regeneration and its removal from the germline of the *mdx* mouse resulted in significantly higher levels of ventricular fibrosis. This implies that the milder phenotype of the *mdx* mouse may be due in part to increased regeneration. As MyoD is only expressed in skeletal muscle, this also suggests that skeletal muscle pathology plays a role in dystrophin deficient cardiomyopathy.

One surprising finding from this study was an improvement in cardiac function in the face of increased ventricular fibrosis. This may be a compensatory mechanism due to the increased skeletal pathology placing an extra work load on the heart. Cardiac mRNA levels of TGF-β did not correlate with the increased levels of ventricular fibrosis observed in the *mdx*:MyoD(-/-) supporting the findings of the previous two studies. While procollagen gene expression also did not correlate with fibrosis, fibronectin mRNA levels did suggesting that fibronectin may be a better marker for active collagen synthesis than procollagen.

The experiments in this dissertation revealed for the first time the beneficial effects of IGF-1 on the dystrophic heart which was characterised by cardiac hypertrophy, improved function and decreased fibrosis. The detrimental effects of increased skeletal muscle pathology on the dystrophic heart were evidenced by increased ventricular fibrosis in *mdx* mice also lacking MyoD. All three studies of this dissertation highlighted the complex nature of the multifactorial molecular pathways involved in cardiac fibrosis.

CERTIFICATION OF DISSERTATION

I certify that the ideas, experimental work, results, analysis and conclusions reported in this dissertation are entirely my own effort, except where otherwise acknowledged. I also certify that the work is original and has not been previously submitted for any other award, except where otherwise acknowledged.

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Signature of Candidate	**Date**

ENDORSEMENT

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Signature of Supervisor/s	**Date**
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ABBREVIATIONS

AAV	Adeno-associated virus
ACE	Angiotensin Converting Enzyme
Ad-v	Adenovirus
AO	Antisense Oligonucleotide
bHLH	basic helix-loop-helix family
BDM	2,3-butanedione
BMD	Becker Muscular Dystrophy
BSA	bovine serum albumin
BW	body weight
cDNA	complimentary deoxyribonucleic acid
C57	C57BL10ScSn
CK	Creatine kinase
CSA	Cyclosporin A
DGC	Dystrophin Glycoprotein Complex
DMD	Duchenne Muscular Dystrophy
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DP	Developed Pressure
+dP/dt	Rate of Contraction over Time
-dP/dt	Rate of Relaxation over Time
E-C	Excitation coupling
ECG	Electrocardiography
ECM	extra cellular matrix
EDL	Extensor digitorum longus

F-actin	Filamentous actin
FXMD	feline muscular dystrophy cat
GATA-2	gata binding proteins
Gm	gram
GRMD	Golden Retriever Muscular Dystrophy dog
HDAC	histone deactylases
HW	heart weight
IGF-1	insulin-like growth factor-1
IGF-BP	insulin-like growth factor binding protein
IGF-1R	insulin-like growth factor receptor
IL	interleukin
LIMP	Large inhibitor of matrix metalloproteinases
MAPK	Mitogen-activated protein kinase pathway
Kb	Kilobases
mdx	muscular dystrophy mouse
mg	milligram
mm	millimeter
MMP	Matrix metalloproteinases
MRI	Magnetic Resonance Imaging
mRNA	messenger Ribonucleic Acid
MyoD	myogenic regulator
MEF2	myocyte enhancer factor 2
MHC	myosin heavy chain
nNOS	neuronal Nitric Oxide Synthase
NO	nitric oxide

NFATc1	nuclear factor activated T cells c1
PI3K	Phosphatidylinositol 3-kinase pathway
PSR	Picrosirius red
ROS	Reactive Oxygen Species
RT-PCR	Real time Polymerase Chain Reaction
sec	second
SAC	stretch activated channel
SEM	standard error of mean
SOC	store operated channel
TGF-β	transforming growth factor β
TL	tibial length
TIMP	tissue inhibitor of matrix metalloproteinases
TNF-α	tumor necrosis factor-α
TRPC1	transient receptor potential canonical 1
XLDC	x-linked dilated cardiomyopathy

CHAPTER 1 LITERATURE REVIEW AND SCOPE OF STUDY

1.1 DUCHENNE MUSCULAR DYSTROPHY

1.1.1 Brief description of Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD), an X-linked myopathy, is the most prevalent and severe of the muscular dystrophies, with an incidence of one in every 3500 live male births (Scott et al. 1988). The disease is due to the absence of the sub-sarcolemmal protein dystrophin. Lack of dystrophin disrupts the dystrophin-glycoprotein complex that spans the sarcolemma and severs the link between the extracellular matrix and the cytoplasm (Campbell & Kahl 1989). The ability to sustain eccentric contractions is greatly reduced in dystrophic muscle and leads to abortive cycles of regeneration and degeneration (Deconinck & Dan 2007). Regeneration fails to be maintained, with necrosis resulting in muscle loss and replacement with fibrous tissue. Boys with DMD exhibit muscle weakness and wasting and usually die before their third decade of life due to respiratory or cardiac complications (Eagle et al. 2002).

1.1.2 History of the disease

The most complete early description of DMD was presented in 1851 by Edward Meryon, and included case reports of eight affected boys from three different families (Emery, 1993). He noted that the disease appeared to be hereditary with a male predilection and was associated with muscle damage, while sparing the nervous system. Upon histological analysis, Meryon found that the sarcolemma in these patients was destroyed and was subsequently acknowledged that this is where the protein deficit occurs. The early history of DMD has been reviewed by Emery (Emery, 2003).

In 1861 the French neurologist, Guillaume Duchenne, described a further forty cases including a more detailed histological analysis of muscle biopsies and the disease was consequently named after him (Emery, 2003).

Sir William Gowers presented a detailed description of the clinical manifestations of DMD in a series of papers published in 1879 in the Lancet (Gowers 1879). In particular he described a common clinical manifestation of the disease, now termed the Gowers manoeuvre, upon where the patient pushes on his legs or thighs while attempting to rise from the floor.

In 1955 an allelic form of DMD with a milder phenotype was classified by Becker and termed Becker Muscular Dystrophy (BMD) (Becker & Kiener 1955). The clinical manifestations of BMD are variable ranging from some patients losing the ability to walk by their late teens, to others only experiencing cramps during exercise or mild proximal muscle weakness (Emery, 2003). Cardiac failure is the leading cause of death for BMD patients, with 50% of BMD deaths due to cardiomyopathy, compared to 20% for DMD patients (Finsterer & Stollberger 2003).

In 1987 the cDNA of the protein deficient in DMD was cloned and sequenced (Koenig et al. 1987). The protein termed dystrophin was identified (Hoffman, Brown & Kunkel 1987) and was found to be localised to the cytoplasmic surface of the sarcolemma (Watkins et al. 1988).

Studies on the function of dystrophin have been facilitated by the muscular dystrophy mouse (*mdx*), a murine model of DMD discovered in a colony of C57Bl/10ScSn (Bulfield et al. 1984). A dystrophin-like protein, utrophin, was identified in 1992 which is expressed in

foetal and regenerating muscle and has been suggested to play a similar role to dystrophin (Tinsley, et al. 1992).

1.1.3 Clinical signs of DMD

Early delayed gross motor development

DMD boys are normal at birth with the initial notable signs being a delay in the average age of walking and an abnormal gait. Clinical signs that usually present between ages 3-5 years include an awkward gait, inability to run, hypertrophy of the calf muscles, difficulty climbing stairs and difficulty rising from the floor resulting in the Gowers manoeuvre (Emery, 1993). An excess of muscular constituents, in particular creatine kinase (CK) are found in the serum of DMD patients indicating muscle degeneration (Moser 1984). Elevated CK levels occur early in DMD and later in BMD (Zatz et al. 1991). The rate of decline of the enzymes activity is significantly elevated in DMD, which correlates with the greater loss of muscle mass observed in DMD (Zatz et al. 1991).

The mean age for diagnosis of DMD using creatine kinase levels is 4.9 years, which has remained unchanged over the last 20 years (Ciafaloni et al. 2009). This delay in diagnosis impacts on genetic counselling for families and delays initiation of steroid treatment, the mainstay of therapy for DMD, which has been shown to be more beneficial the earlier it is commenced (Merlini et al. 2003).

Muscle hypertrophy

A common manifestation of DMD is hypertrophy of skeletal muscles. Calf hypertrophy is commonly observed (Kanamori 1988) (see Figure 1.1) as is hypertrophy of the infraspinatus muscle (Pradhan & Mittal 1995). The hypertrophied muscles display muscle weakness due to

the dystrophin deficiency rendering the sarcolemma fragile and more susceptible to inflammation and cell death (Yiu & Kornberg 2008).

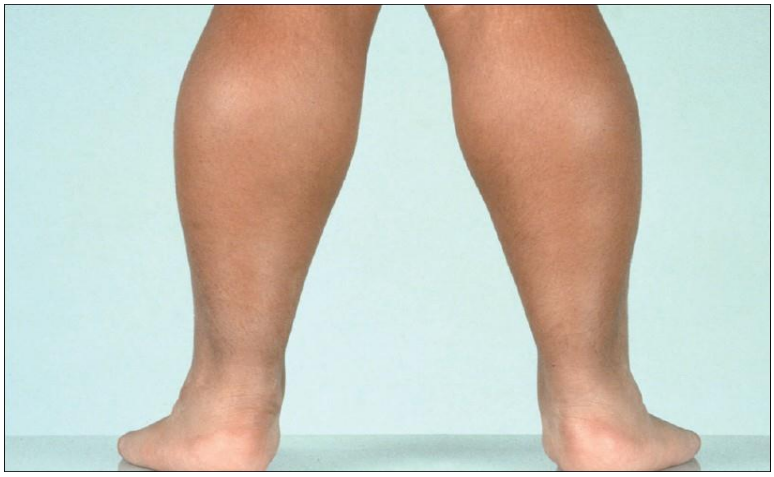


Figure 1.1 Calf hypertrophy in DMD patient (Yiu & Kornberg 2008)

Joint contractures

During late childhood joint contractures occur due to shortening of muscle fibres and tendon contractures which may result in toe walking. Painful joint contractures combined with muscle weakness leads to ambulatory difficulties with most boys being confined to a wheelchair by the age of 11years (Emery, 2003).

Progression of kyphoscoliosis

As the disease progresses the proximal pelvic muscles weaken and a compensatory kyphoscoliosis becomes evident. Adequate wheelchair support is required in an attempt to minimise the effects. However, if the curvature of the spine progresses beyond 20-30 degrees there will be a concomitant deterioration of cardiac and pulmonary function. A study of 51 Duchenne patients found that all exhibited scoliosis and once the curve was greater than 35

degrees, vital capacity was less than 40% of the predicted normal value (Smith, Koreska & Moseley 1989). Spinal fusion is often indicated to counteract scoliosis and retain vital capacity.

Intellectual impairment

Different isoforms of dystrophin are expressed in the brain and retina. In particular the carboxy-terminal truncated isoform of dystrophin contributes to normal brain function. One third of DMD patients exhibit mild to moderate intellectual impairment with a wide range of phenotypes in those affected (Muntoni, Torelli & Ferlini 2003).

DMD patients usually show normal visual acuity, however electroretinography has shown defects in several patients (Muntoni, Torelli & Ferlini 2003).

Respiratory dysfunction

Pulmonary function decreases progressively with age, resulting in hypercapnic respiratory failure. A predictor of mortality is often when the vital capacity falls below 1L, with a high likelihood of mortality occurring within the subsequent three years (Phillips et al. 2001). Nocturnal ventilation has reduced the number of deaths due to respiratory failure and has increased life expectancy from 14.4 years in the 1960's to 25.3 years for those ventilated since the 1990's (Eagle et al. 2002).

Cardiomyopathy

Although advances in respiratory care have improved life expectancy for DMD boys, cardiomyopathy significantly shortens their life expectancy (Eagle et al. 2002) with 20% of

DMD patients and 50 % of BMD patients dying due to cardiac complications (Finsterer & Stollberger 2003).

The clinical symptoms of cardiomyopathy are first evident at age 10, and are present in all patients over 18 years of age (Nigro et al. 1990). While most DMD patients remain asymptomatic until their early teens despite the progression of cardiac dysfunction, this may be due to either limited energy expenditure, muscle weakness or being wheelchair bound (Ogata, Ishikawa & Minami 2009).

Early detection of cardiac dysfunction is important to enable effective management and early treatment may prolong life expectancy (Ogata, Ishikawa & Minami 2009). Ultrasonic tissue characterisation may enable this as it has the capability to detect abnormalities in myocardial features prior to the onset of changes of systolic function and overt cardiomyopathy (Giglio et al. 2003). Electrocardiography (ECG) can also detect abnormalities from an early age. Typical changes include short PR interval, right ventricular hypertrophy and deep Q waves (Thrush et al. 2009). Further diagnostic techniques capable of detecting cardiac changes prior to clinical manifestations include the use of magnetic resonance imaging (MRI). MRI analysis of the T2 relaxation time in the myocardium of a group of DMD patients without symptoms of cardiac dysfunction were found to be decreased, indicating altered tissue composition (Mavrogeni et al. 2005). The progressive decrease of the T2 relaxation time with age furthers the notion that cardiac involvement occurs early in DMD and increases with age (Mavrogeni et al. 2005). In support of this, another MRI study found the ratio between cardiac phosphocreatine to adenosine in carriers with DMD and BMD patients to be reduced in the absence of left ventricular dysfunction, suggesting that cardiac metabolic dysfunction precedes clinical symptoms (Crilley et al. 2000).

As the disease progresses there is an increase in cardiac fibrosis, left ventricular dysfunction and the incidence of arrhythmias (de Kermadec et al. 1994; Melacini et al. 1996). Post mortem examination of hearts from DMD boys reveals severe fibrosis of the epimyocardial portion of the free wall of the left ventricle (Frankel & Rosser 1976). The progressive cardiac fibrosis impacts diastolic function more than systolic function. Echocardiographic measurements of DMD patients under 12 demonstrate normal systolic function, while diastolic myocardial velocities are reduced (Mertens et al. 2008). In the last phase of DMD systolic function also becomes impaired and may lead to heart failure and death (Finsterer & Stollberger 2003).

1.2 DYSTROPHIN

1.2.1 The Dystrophin gene

The dystrophin gene, located at Xp21, is the largest mammalian gene comprising 2300 kb of genomic DNA of which only 14 kb are transcribed into mRNA (Koenig et al. 1987).The enormous size of the dystrophin gene has been implicated in its high mutation rate of 1 in 10,000 (Koenig et al. 1987; Koenig, Monaco & Kunkel 1988).

1.2.2 Dystrophin isoforms

There are three full length isoforms, derived from three independent promoters which are expressed in brain, muscle and purkinje fibres which conduct an electrical stimulus enabling the heart to contract. Shorter transcripts are expressed in the retina, brain, and schwann cells. These are shown in Figure 1.2 (Muntoni, Torelli & Ferlini 2003).

1.2.3 Dystrophin gene mutations

Intragenic deletions represent the most common mutation, which account for 65 -72% of mutations (Aartsma-Rus et al. 2006; Emery, 2002). There are two deletion "hot spots"; Exons 45-55 and Exons 2-19 (Koenig & Kunkel 1990). The clusters of these two hot spots allow for multiplex polymerase chain reaction (qPCR) screening of only 19 of the 79 exons to enable identification of 90% of these deletions (Beggs & Kunkel 1990). Although this is a very effective technique it cannot identify the less common event of duplications or genotype female carriers which would require quantitative polymerase chain reaction (qPCR) (Abbs & Bobrow 1992).

The remaining mutations (approximately 30%) arise from duplications (5-15% of cases), point mutations resulting in nonsense, frame shift mutations, intronic deletions, or exonic

insertions of repetitive sequences (Muntoni, Torelli & Ferlini 2003).

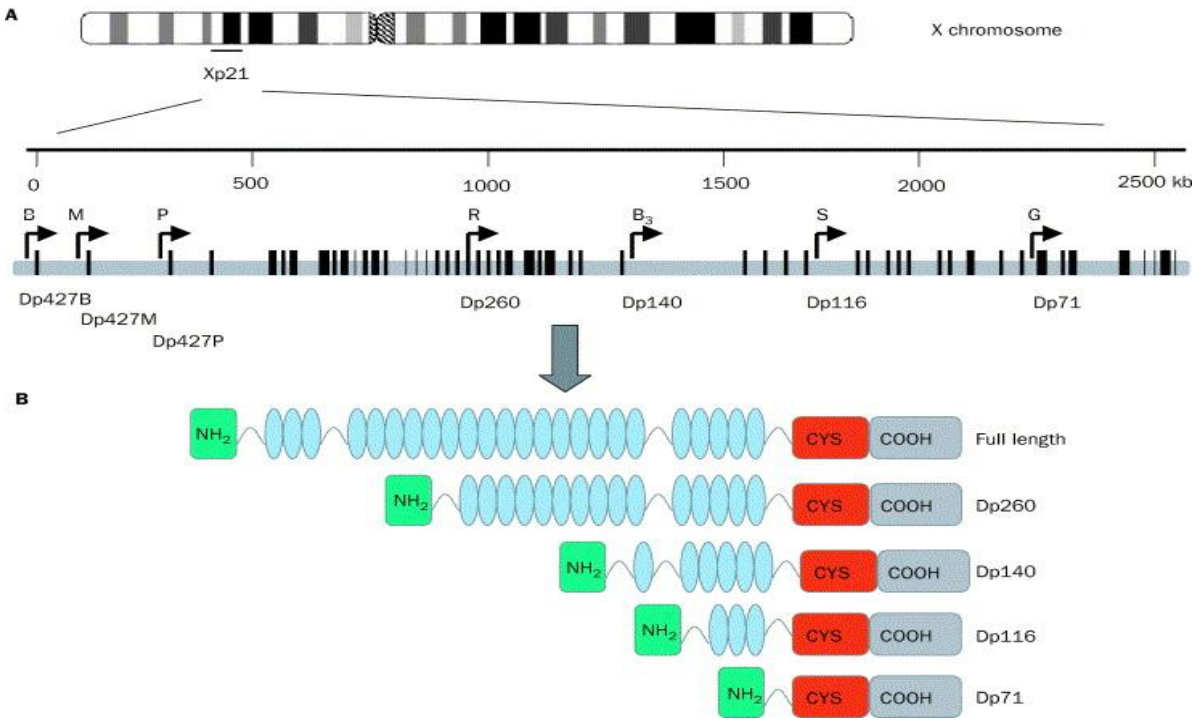


Figure 1.2 Genomic organisation of the dystrophin gene (Muntoni, Torelli & Ferlini 2003)

A. The X chromosome highlighting Xp21, the dystrophin region which is enlarged below. The black lines on the gene represent the 79 exons and arrows indicate promoters in the brain (B), muscle (M), and purkinje fibres (P), retina (R), brain 3 (B3), schwann cells (S) and general (G). General promoters stimulate expression of protein detected in most non-muscle tissues including brain, retina, kidney, liver, and lung and are present in cardiac but not skeletal muscle. Dp represents the dystrophin protein.

B. The domains of the different dystrophin proteins. The amino terminal is in green and is followed by the spectrin like domain in blue, the cysteine rich region in red and the carboxy-terminal in grey.

The reading frame hypothesis and genotype/phenotype correlation

The size of the deletion of the dystrophin gene does not correlate with the severity of the phenotype. Small deletions of exon 44 have been shown to result in DMD pathology, whereas large deletions involving 50% of the dystrophin gene have resulted in the milder phenotype of BMD (England et al. 1990; Koenig, Monaco & Kunkel 1988).

The severity of the DMD phenotype, however is dependent on whether the mutation disrupts the reading frame of the dystrophin gene as shown in figure 1.3. Mutations that maintain the reading frame result in abnormal but partly functional dystrophin and are associated with BMD. Mutations that disrupt the reading frame (frame shift) result in unstable mRNA that leads to the production of nearly undetectable concentrations of truncated dystrophin, resulting in DMD (Monaco et al. 1988). Over 4700 mutations have been reported in the Leiden DMD mutation database of which 91% are in agreement with this reading frame hypothesis (Aartsma-Rus et al. 2006).

Patients with identical exonic deletions may display different phenotypes. These patients may have different deletion breakpoints within introns as intronic sequences may contain motifs that affect gene splicing, and these different deletion breakpoints may affect exon skipping events (Gualandi et al. 2003).

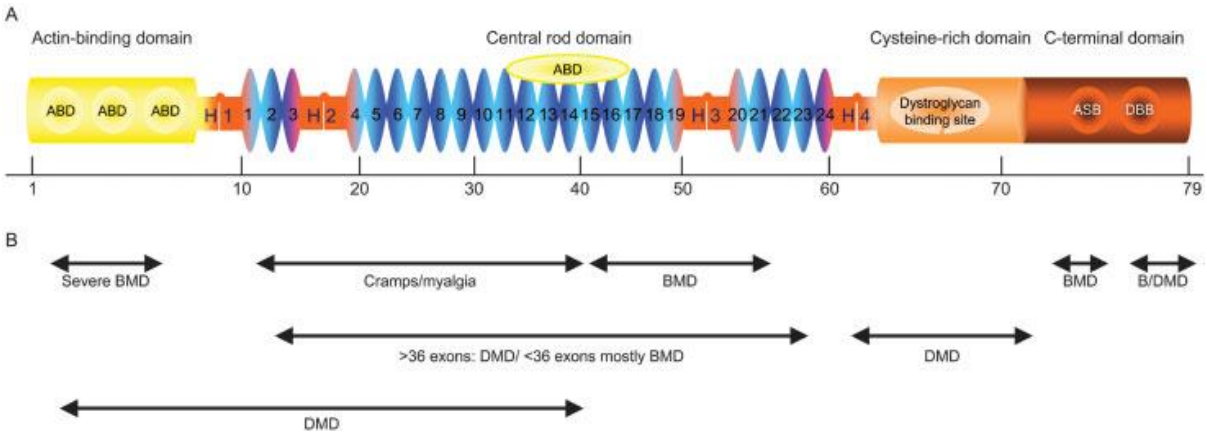


Figure 1.3 Diagrammatic representation of dystrophin protein and common exon deletions in DMD. Adapted from (Aartsma-Rus et al. 2006).

(A) Dystrophin protein showing domains. The N-terminal actin-binding domain (ABD yellow), central rod domain which contains 24 spectrin like repeats (1-24 blue) interrupted by four hinge regions (H1-4 orange), a cysteine rich domain (light orange) and the C-terminal domain (dark orange) containing syntrophin and dystrobrevin binding domains (ASD and DBD). An additional actin binding domain is present between repeat units 11 and 17. The location of exons is shown beneath the protein structure.

(B) Location of in-frame deletions and corresponding phenotypes. In-frame deletions involving only the central rod domain are usually associated with a mild phenotype unless the deletion is excessively large. A deletion of the N-terminal domain causes severe BMD, but a deletion of both the N-terminal and part of the central rod domain results in DMD.Mutations involving the cysteine rich domain usually results in DMD, whereas deletions of the syntrophin binding domains Exons 71-74 have been reported for some BMD patients. Mutations in Exon 74 or higher are found in both DMD and BMD patients.

1.2.4 Revertant fibres

The *mdx* mouse has a nonsense mutation of the dystrophin gene at Exon 23 and as such there should be no functional dystrophin expressed by muscle cells (Sicinski et al. 1989). However, although *mdx* muscle is negative for dystrophin detected by immunoblotting, a very small percentage of myofibres are dystrophin positive as observed by immunofluorescence microscopy (Hoffman et al. 1990). This is also observed in DMD patients (Fanin et al. 1995; Klein et al. 1992). It has been suggested that this is due to a naturally occurring spontaneous exon skipping, and these dystrophin expressing fibres are termed revertant fibres (Wilton et al. 1999).

1.3 THE DYSTROPHIN PROTEIN

1.3.1 Dystrophin structure

The dystrophin gene was cloned in 1985 (Kunkel et al. 1985) which was followed by the identification of the protein product, dystrophin (Hoffman, Brown & Kunkel 1987). Dystrophin is approximately 400kb in size and is localised to the sarcolemma of skeletal, smooth and cardiac muscle cells (Zubrzycka-Gaarn et al. 1988). It is not present in the muscle cells of DMD boys or in various dystrophic animal models including the *mdx* mouse, the golden retriever muscular dystrophy dog (GRMD) and the dystrophic cat.

As illustrated in figure 1.4, dystrophin is an integral component of the membrane associated proteins known collectively as the dystrophin-glycoprotein complex (DGC) (Campbell & Kahl 1989). The DGC is compromised of sarcoplasmic proteins α-dystrobrevin, syntrophins and neuronal nitric oxide synthase (nNOS)), transmembrane proteins (β-dystroglycan, the sarcoglycans, caveolin-3 and sarcospan) and extracellular proteins (α-dystroglycan and laminin) (Davies & Nowak 2006). Dystrophin binds to the DGC through its C terminus while the N terminus binds it to the cytoskeleton through filamentous (F) actin. The DGC therefore facilitates a mechanical link between the intracellular cytoskeleton and the extracellular matrix (ECM) (Davies & Nowak 2006). This mechanical link is imperative for membrane integrity (Campbell & Kahl 1989; Rybakova, Patel & Ervasti 2000).

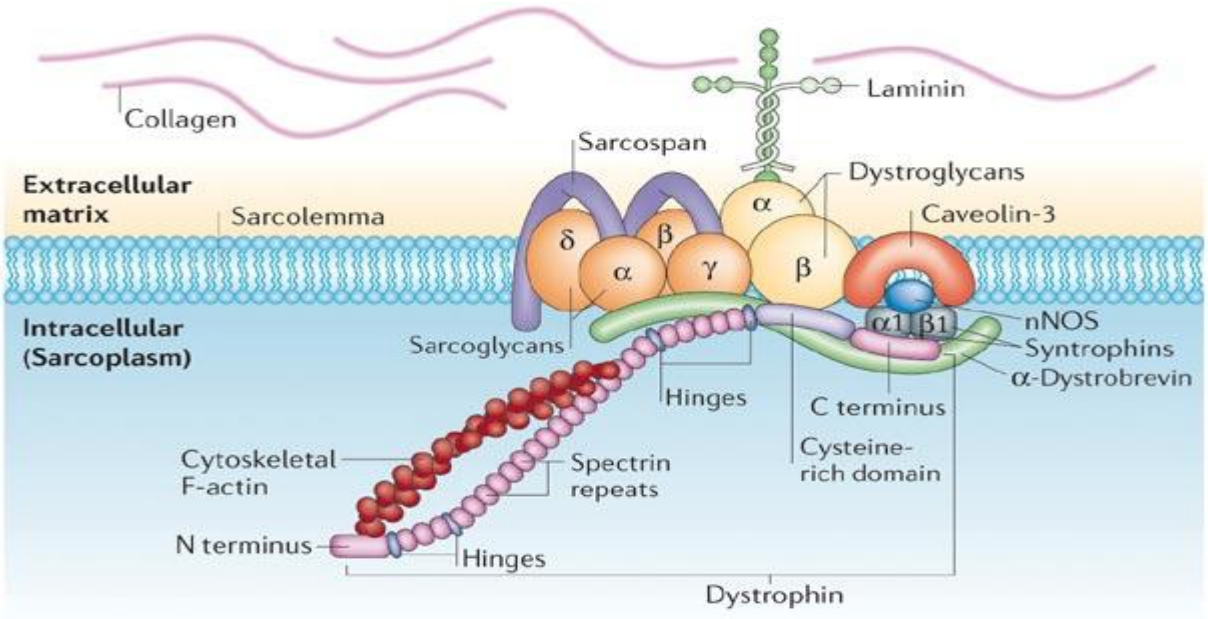


Figure 1.4 The structure of the human dystrophin-glycoprotein complex (DGC) (Davies & Nowak 2006). Dystrophin binds to the DGC through its C terminus. The DGC is comprised of sarcoplasmic proteins α-dystrobrevin, syntrophins and neuronal nitric oxide synthase (nNOS)), transmembrane proteins (β-dystroglycan, the sarcoglycans, caveolin-3 and sarcospan) and extracellular proteins (α-dystroglycan and laminin). The N terminus of dystrophin binds to the cytoskeleton through filamentous (F)-actin. Dystrophin has four structural domains. The amino terminus binds to F-actin (Way et al. 1992) and is followed by a large rod domain with 24 spectrin-like repeats and four hinge sections that confer flexibility and membrane resilience (Koenig & Kunkel 1990). Next is the WW domain which interacts with β-dystroglycan and is a motif found in proteins involved in signalling and regulatory functions (Chung & Campanelli 1999). The cysteine rich domain which follows is involved in calcium and calmodulin binding. Finally the c-terminal region which binds the c-terminus region of dystrobrevin and the alternatively spliced region binds syntrophin (Sadoulet-Puccio, Rajala & Kunkel 1997).

N terminal domain of dystrophin

The N terminal domain appears to be required for normal dystrophin function (Norwood et al. 2000). Transgenic *mdx* mice that expressed an isoform of dystrophin that contained the cysteine-rich and C-terminal domains of dystrophin, but lacked the N-terminal actin-binding and central spectrin-like repeat domains resulted in a severe pathology (Cox et al. 1994). Generally a deletion in the N-terminal results in severe BMD, whereas a deletion of the N-terminal and the part of the central rod domain results in DMD (Aartsma-Rus et al. 2006).

Cysteine-rich region of dystrophin

Transgenic mice that expressed an isoform of dystrophin that had deletions in the cysteine – rich region resulted in a disruption of the interaction between dystrophin and the DGC, leading to a severe dystrophic pathology (Rafael et al. 1996). Deletions in this region obliterate the β-dystroglycan binding site, which leads to a loss of β-dystroglycan and the sarcoglycan complex. This suggests that the cysteine rich region is imperative for a functional dystrophin and assembly of the DGC at the sarcolemma. Likewise mutations in the cysteine-rich area of the dystrophin gene results in a DMD phenotype (Aartsma-Rus et al. 2006).

Central rod domain of dystrophin

Transgenic mice with a truncated central rod domain displayed normal DGC formation and therefore no dystrophic pathology (Chamberlain et al. 1997).This suggests that the large central rod domain does not appear to be critical for a functional dystrophin.

Many BMD patients with mild pathologies have large in-frame deletions of this region and produce a shorter, but still functional dystrophin (England et al. 1990). Generally boys with

deletions in this region that are greater than 36 exons in size result in DMD, whereas deletions less than 36 exons display a milder BMD phenotype (Aartsma-Rus et al. 2006).

C-terminal end of dystrophin

Deletion of the C-terminal end of dystrophin in transgenic *mdx* mice appears to have no effect on the function of the protein suggesting that this region is not required for the assembly of the DGC (Rafael et al. 1996).

Inter- and intra-phenotypic variability is observed in BMD and DMD. For example deletions at the C-terminus of the dystrophin gene in boys generally results in the milder BMD phenotype, however one patient missing the C-terminus produced a truncated Becker-like dystrophin but displayed severe DMD phenotype (Hoffman et al. 1991).

Figure 1.3 illustrates the domains of the dystrophin protein, common exon deletions and the phenotype they most often result in.

1.3.2 Roles of dystrophin

Evidence of a mechanical role for dystrophin

Loss of dystrophin results in a loss of the DGC and the structural link between ECM and cytoskeleton.

Dystrophin anchors the DGC of the sarcolemma to the underlying cytoskeleton (Campbell & Kahl 1989) and is required for an intact DGC with a dramatic reduction in all of the DGC proteins at the sarcolemma observed in DMD patients and *mdx* mice (Matsumura et al. 1994). When dystrophin is expressed in revertant fibres the DGC is restored (Matsumura et al. 1994).

The DGC and other proteins form rib-like lattices known as costameres (Minetti et al. 1992). Costameres anchor the myofibrils to the sarcolemma and act as mechanical couplers between the contractile apparatus and the ECM. Loss of the DGC disrupts the costameric lattice which may lead to membrane fragility (Rybakova, Patel & Ervasti 2000).

The dystrophic membrane shows increased susceptibility to eccentric contraction induced damage.

Studies have shown that when mechanical stress is placed upon the sarcolemma during contraction it ruptures (Head, Williams & Stephenson 1994; Moens, Baatsen & Marechal 1993; Petrof et al. 1993). Loss of the DGC appears to render the sarcolemma susceptible to damage from muscle contraction thus supporting the hypothesis that the primary role of dystrophin is as a structural support.

The dystrophic membrane displays increased permeability.

The lack of this structural link renders the membrane fragile and increases its permeability. The increased permeability of the membrane leads to elevation of the cytoplasmic concentration of albumin and immunoglobins as reviewed by Deconinck and Dan (Deconinck, & Dan 2007).

Increased membrane permeability can be visualised by incorporation of the membrane impermeant dye Evans Blue (Hamer et al. 2002). Muscle enzymes such as pyruvate kinase and creatine kinase leak into the serum in DMD patients (Zatz et al. 1991) and *mdx* mice indicating muscle degeneration.

Calcium hypothesis

Dystrophin deficiency leads to an increase in intracellular calcium in myocytes of muscle biopsies from DMD patients (Imbert et al. 1995) and *mdx* mice (Allen 2004; Woolf et al. 2006). The process of calcium entry may occur via ionic channels or via membrane tears. Increased membrane permeability enables large proteins such as albumin and creatine kinase to enter and leave the cell and may also enable increased calcium influx (McNeil & Khakee 1992).

It has also been proposed that it is the calcium accumulation that brings about the observed increase in membrane permeability (Allen, Whitehead & Yeung 2005; Whitehead et al. 2006). Further studies suggest that the increased calcium levels are due to calcium entry via ionic channels. Calcium influx was found to be almost totally inhibited by Ni^{2+}, a non-specific cation channel inhibitor, suggesting that calcium entry is dependent upon permeable channels as opposed to membrane damage (Fraysse et al. 2004). Furthermore *mdx* mice are more likely to have open stretch-activated channels (SACS) and these channels seem to be more sensitive to the effects of contractions (Allen 2004). These channels are further opened by stretch which exacerbates ionic influx and results in a decrease in the force of contraction. Gadolinum and streptomyocin block SACs in muscle fibres and are capable of blocking increase in $[Na^+]$ in *mdx* mouse muscle (Allen 2004), improving force and decreasing membrane permeability in isolated muscle and in the whole mouse (Whitehead et al. 2006).

A recent study implicates transient receptor potential canonical 1 (TRPC1) as the calcium channel involved in the pathogenesis of DMD (Gervasio et al. 2008). TRPC1 are widely expressed calcium permeable channels in skeletal muscle (Venkatachalam & Montell 2007) and cardiac muscle (Williams & Allen 2007). It has been suggested that TRPC1 is a store

activated channels (SOC) or stretch activated channels (SAC) (Ducret et al. 2006). TRCP1 has been shown to form SACs in vertebrate cells (Maroto et al. 2005). Another contradictory study on TRPC1 knockout mouse displayed no apparent pathology, suggesting that TRPC1 is not an obligatory component of SAC or SOC ion channels in vascular smooth muscles (Dietrich et al. 2007). The function of TRPC in cardiomyocytes and their possible involvement in cardiomyopathy has been reviewed by Dietrich (Dietrich et al. 2007).

TRPC1 have been associated with DMD. SAC inhibitors stretomyocin and GsMTx4 reduced the resting calcium in isolated ventricular myocytes of old *mdx*, suggesting SACs are involved in the abnormal calcium handling seen in *mdx* mice. Furthermore an increased expression of TRPC1 was seen in old *mdx* mice (Williams & Allen 2007). In support of these results, suppression of TRPC1 decreases SOC and SAC occurrence and activity in *mdx* mice (Vandebrouck et al. 2002).

Regulatory proteins, caveolins, have been implicated in the regulation of TRPC1 (Gervasio et al. 2008) and higher levels of muscle specific caveolin-3 have been observed in *mdx* muscle (Vaghy et al. 1998). TRPC1 binds to caveolin-3 and is regulated by src kinase (Gervasio et al. 2008). Hydrogen peroxide, a reactive oxygen species (ROS), increased src kinase activity and enhanced calcium influx, but only in myoblasts expressing both TRPC1 and caveolin-3. In *mdx* muscle a ROS scavenger, tiron and a src inhibitor, PP2, decreased calcium entry and mediated force recovery (Gervasio et al. 2008). As ROS production is increased in *mdx* and DMD (Whitehead, Yeung & Allen 2006), these findings implicate a ROS-Src-TRPC1/caveolin-3 pathway in the pathogenesis of the disease (Gervasio et al. 2008). Chronic calcium overload results in activation of calcium dependant proteases, particularly calpains (MacLennan, McArdle & Edwards 1991). Proteases are a downstream calcium

activated target involved in protein degradation as evidenced in dystrophic skeletal muscle (Allen, Whitehead & Yeung 2005).

1.3.3 Dystrophin in the cardiomyocyte

In human and rat cardiac muscle, dystrophin is continuous with the cytoplasmic surface of the plasma membrane at costameric and non-costameric regions (Kaprielian et al. 2000; Kostin et al. 1998). It is absent at intercalated discs but, unlike skeletal muscle, is also present at transverse tubules. T-tubules are important in excitation contraction coupling and do not serve in the transmission of contractile force (Kaprielian et al. 2000). The presence of dystrophin in cardiomyocyte T-tubules suggests a diverse role for dystrophin in cardiomyocytes.

1.3.4 Utrophin

The dystrophin gene demonstrates over 80% homology with the autosomal gene utrophin which is located on chromosome 6 (Tinsley et al. 1992). Utrophin is found in all tissues and is expressed in high levels in DMD muscle.

Utrophin is present in developing skeletal muscle and is localised around the sarcolemma (Khurana et al. 1991), whereas in adult muscle it becomes restricted to myotendinous and neuromuscular junctions (Ohlendieck et al. 1991). This pattern of localisation suggests that utrophin may perform the function of dystrophin in foetal and developing muscle fibres. Furthermore utrophin appears to be able to functionally substitute for dystrophin (Perkins & Davies 2002).

1.4 PATHOPHYSIOLOGY OF DYSTROPHIN DEFICIENT MUSCLE

1.4.1 Initiation of necrosis

Loss of dystrophin at the sarcolemma severs the link between the contractile protein actin and the cell membrane resulting in a marked reduction of all the other dystrophin associated proteins (Matsumura et al. 1994). This loss of integrity of the cell membrane disrupts normal calcium homeostasis and allows an influx of calcium into the cytoplasm, activating calcium dependent proteases (MacLennan, McArdle & Edwards 1991). Affected myofibres are more susceptible to inflammation and cell death (Matsumura & Campbell 1993).

Although many of the processes that occur early in dystrophic muscle are stereotypical of necrosis, histological analysis points towards apoptosis preceding necrosis and characterising the onset of dystrophic pathology. This is followed by necrosis in the later stages of the disease (Tidball et al. 1995).

1.4.2 Myocyte regeneration and fibrosis

Inflammatory cytokines initiate the inflammatory process and cell death which leads to scarring and fibrosis. Tumor necrosis factor alpha (TNF-α), an early pro-inflammatory cytokine is upregulated in DMD patients (Porreca et al. 1999). However the exact role of this cytokine is contentious, with some studies finding TNF-α plays a role in the progressive deterioration of the *mdx* diaphragm (Gosselin & Martinez 2004), whereas others have found TNF-α to be associated with promoting muscle development, postnatal growth and regeneration (Li, & Schwartz 2001). The differences in the role TNF-α plays may be due to the cytokine influencing different types of muscles in different ways, and the stage of the disease.

Pleiotropic cytokine transforming growth factor-β (TGF-β) has also been suggested as a

mediator of fibrosis in DMD (Bernasconi et al. 1999), *mdx* mice (Andreetta et al. 2006) and the canine model of DMD (Passerini et al. 2002). Differences in the amount or localisation of this cytokine may influence the balance between muscle regeneration and the development of fibrosis (Murakami et al. 1999).

T lymphocytes have also been shown to play a role in fibrosis particularly in the diaphragm (Morrison et al. 2000). Furthermore, the expression of two myogenic regulatory factors, MyoD and myogenin have been shown to decrease as fibrosis progresses in *mdx* mice (Jin et al. 2000).

1.4.3 Oxidative stress

The abnormal membrane structure which leads to altered calcium homeostasis also results in excessive generation of reactive oxygen free radical species (ROS) (Whitehead, Yeung & Allen 2006). Dystrophin deficient muscle shows an increased production of ROS and decreased DGC signalling resulting in decreased expression of the enzyme neuronal nitric oxide synthase (nNOS). nNOS is responsible for the production of nitric oxide (NO) which mediates vasodilation and protects against ischemia (Rando 2001). Muscle biopsies from BMD patients show a correlation between disease severity and decreased nNOS at the sarcolemma (Chao et al. 1996). *Mdx* mice also have decreased nNOS (Bia et al. 1999). However, loss of nNOS alone does not explain subsequent cell death and degeneration as evidenced by an absence of muscular dystrophy in nNOS deficient mice (Chao, Silvagno & Bredt 1998). Rather it has been suggested that decreased nNOS at the sarcolemma results in a reduction in NO-mediated protection against ischemia, which combines with the increased susceptibility of dystrophic muscle to metabolic stress resulting in muscle degeneration (Rando 2001).

Oxidative stress is elevated in the failing myocardium and may be a regulator of the ECM through effects on matrix metalloproteinases (MMPs) and collagen synthesis (Siwik & Colucci 2004).

1.4.4 Muscle regeneration

Muscle satellite cells allow regeneration of new muscle fibres, but these are also dystrophin deficient and subsequently degenerate as shown in Figure 1.5. Furthermore DMD muscle precursor cells have been shown to replicate less than controls, which may be due to shortened telomere length resulting from previous replications (Decary et al. 2000). A decreased expression of myogenic regulator, MyoD (which is necessary in the regenerative process for the proliferation of satellite cells) is observed in *mdx* mice (Jin et al. 2000).

The continuous nature of cycles of regeneration and degeneration cycle results in increased fibrosis within the muscle with chronic accumulation of fibrosis being indicative of DMD and *mdx* muscle (Chamberlain et al. 2007).

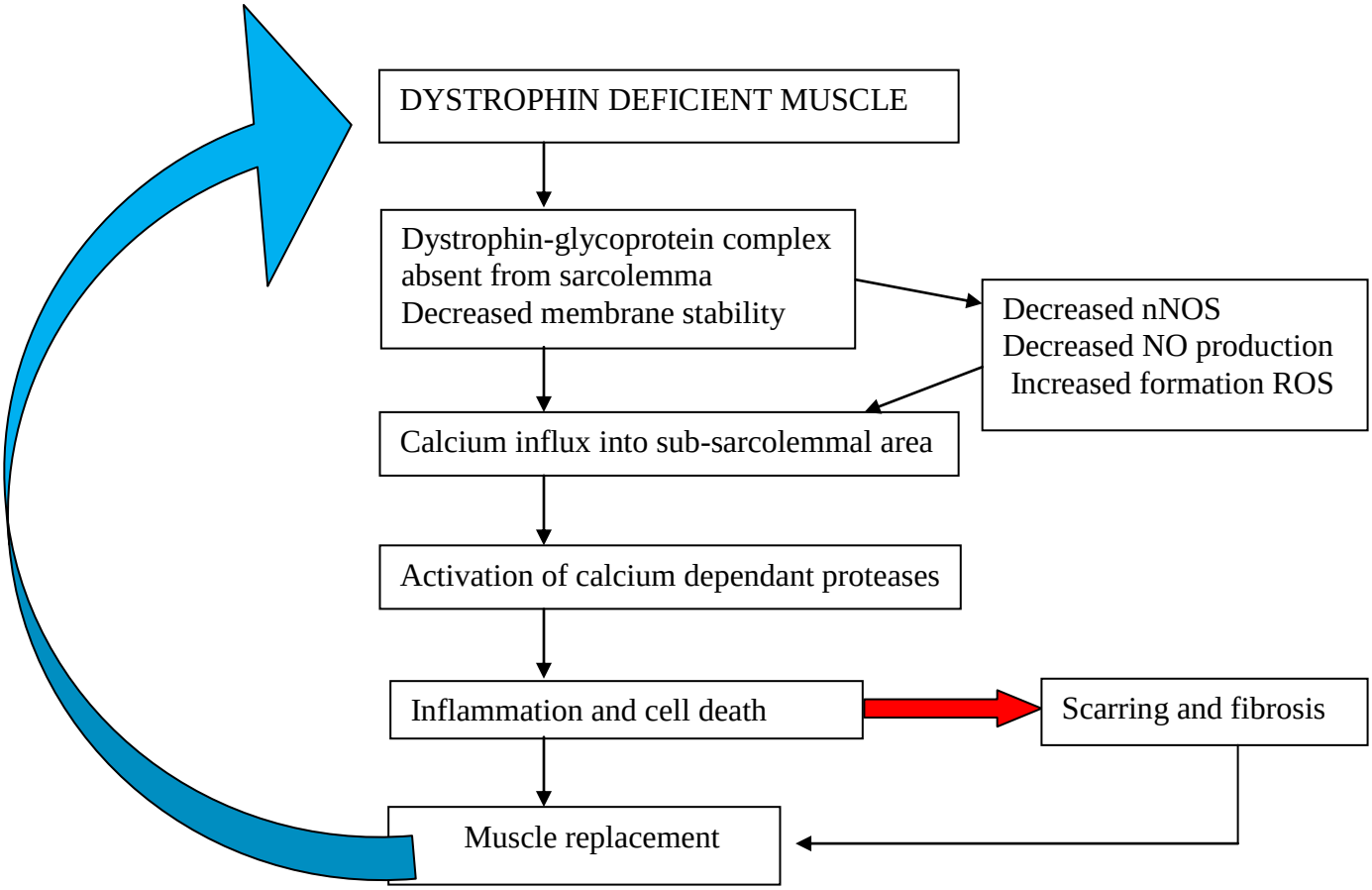


Figure 1.5 Pathophysiological processes occurring in dystrophin deficient muscle.

A lack of dystrophin results in a loss of the DGC at the sarcolemma, rendering the membrane fragile. Decreased nNOS and NO production results in increased ROS. Increased calcium influx into the sub-sarcolemmal area activates calcium dependant proteases. Subsequent initiation of the inflammatory response and cell death result in scarring and fibrosis. The cycle of degeneration and regeneration is ongoing as replaced muscle is also dystrophin deficient, and the extent of fibrosis is increased with every cycle.

1.5 ANIMAL MODELS OF DMD

1.5.1 Range of animal models used in DMD research

There is a wide range of naturally occurring and genetically manipulated animals used to study DMD. The most commonly used dystrophin deficient animal is the *mdx* mouse, which arose from a spontaneous mutation in a colony of C57B1/10ScSn mice (Bulfield et al. 1984). Other dystrophin deficient animals include the golden retriever muscular dystrophy dog (GRMD) (Kornegay et al. 1988), the dystrophic cat (Carpenter et al. 1989), the dystrophic chicken (Dawson 1966), cow (Poukka 1966) sheep (Paulson, Pope & Baumann 1966), zebrafish (Chambers et al. 2001) and the nematode Caenorrhabditis elegans (Mariol & Segalat 2001).

1.5.2 Hypertrophic feline muscular dystrophy cat

The feline muscular dystrophy cat (FXMD) lacks dystrophin and displays a true muscle hypertrophy, unlike the psuedohypertrophy seen in DMD and BMD. It exhibits a progressive histology characterised by necrosis and regeneration, but not fibrosis (Carpenter et al. 1989). For this reason it is seldom used as a model for DMD research.

1.5.3 Golden retriever muscular dystrophy dog

The golden retriever muscular dystrophy (GRMD) dog is the best representation of DMD. Like DMD, the GRMD displays muscle weakness, necrosis and regeneration, elevated serum creatine kinase levels, and endomysial and perimysial fibrosis resulting in a severe myopathy (Valentine et al. 1990). GRMD dogs present clinical symptoms within the first weeks of life involving limb and masticatory muscles. Impairment of the diaphragm and the intercostal muscle leads to respiratory failure (Ambrosio et al. 2008). Like DMD and BMD, GRMD dogs also develop dilated cardiomyopathy (Chetboul et al. 2004) with death occurring early

at 2 years of age due to respiratory or cardiac failure (Ambrosio et al. 2008).

The phenotype of the GRMD model is variable, which makes functional analysis difficult to interpret and compare (Banks & Chamberlain 2008). One recent case reported a GRMD dog lacking dystrophin, with high serum CK levels, but a very mild phenotype. The dog's two brothers carried the same dystrophin mutation, but displayed severe phenotypes, with one dying at just two weeks of age (Ambrosio et al. 2008). Clinical observations of musculoskeletal, morphological, gastrointestinal, respiratory, cardiovascular, and renal features have been used to identify three distinguishable phenotypes which may go some way to explain this issue. They range from mild (grade I), moderate (grade II) to severe (grade III) and perhaps reflect the inter- and intra- phenotypic variability observed in DMD and BMD patients (Ambrosio et al. 2009). Qualitative and quantitative measurements in the GRMD dog model have recently been reviewed (Willmann et al. 2009).

A limitation of the GRMD model is the high cost of maintenance and ethical issues involved in canine research. Although the GRMD has its limitations as an animal model for DMD, it is more useful for clinical trials than the *mdx* mouse which displays a much milder phenotype. Recent reports involving the GRMD dog include prednisone studies (Liu, J. M. et al. 2004), gene delivery using an adeno-associated virus (Yue et al. 2008), human adult stem cells transplantation (Kerkis et al. 2008), haematopoietic stem cell studies (Parker et al. 2008) and diaphragmatic transplantation (Krupnick et al. 2008).

1.5.4 *mdx* mouse

Genetic mutation in the *mdx*

The *mdx* mouse has a point mutation in Exon 23, which introduces a premature stop codon

leading to an absence of full length dystrophin (Sicinski et al. 1989) and attenuation of the DGC (Ohlendieck et al. 1991).

Life span

Compared to wild type mice, *mdx* have a shorter life span, which is reduced in females by 17% and in males by 19% (Chamberlain et al. 2007; Pastoret & Sebille 1995). They exhibit a progressive dystrophic muscle histopathology with old males being prone to spontaneous rhabdomyosarcomas (Chamberlain et al. 2007).

***Mdx* skeletal muscles undergo chronic cycles of degeneration and regeneration**

Degeneration and regeneration of muscle fibres is observed at 2-4 weeks of age as evidenced by an increased number of differentiating myofibres with centralised nuclei and increased heterogeneity of myofibre size (McGeachie et al. 1993). Necrosis has also been observed at this age (Grounds & Torrisi 2004). The muscle breakdown peaks at 28 days, then decreases and stabilises at around 12 weeks of age to a relatively low level of damage (McGeachie et al. 1993). In older *mdx* mice regeneration declines while the necrotic process continues (Pastoret & Sebille 1995). Further evidence for the chronic cycles of degeneration is a continual leak of cytosolic enzymes such creatine kinase and pyruvate kinase into the serum throughout the *mdx* lifespan (Bulfield et al. 1984).

Mdx skeletal muscles exhibit a heterogeneous response to dystrophin deficiency. Extraocular muscles do not exhibit the dystrophic phenotype (Porter et al. 1998), whereas hindlimb muscles undergo a progressive dystrophic process and the diaphragm displays a more severe pathology at all stages of the disease (Dupont-Versteegden & McCarter 1992). A gene expression profiling study suggested that inflammation-mediated repair may be lower in the

more severely affected muscles, and this may compromise regeneration resulting in a more severe pathology (Porter et al. 2004).

Body weight

Body weight increases at 8 weeks old (Connolly et al. 2001) with body and muscle weights declining dramatically after 52 weeks (Pastoret & Sebille 1995).

Hypertrophy

Generalised hypertrophy of the trunk and upper and lower limb muscles is evident in *mdx* at 6 months of age with the extensor digitorum longus (EDL) and soleus muscle displaying a dramatic increase in muscle mass of 28% and 42% respectively (Lynch & Hinkel 2001) and the tibialis anterior increasing by 58% (Pastoret et al. 1995).

Contradictory results regarding hypertrophy in old *mdx* mice exist, with the muscle mass of EDL and soleus muscles from *mdx* mice displaying a sustained hypertrophy at 28 months 17% and 22% respectively greater than controls (Lynch et al. 2001). Whereas another study found that the tibialis anterior muscle mass of *mdx* mice was reduced to 50% of control values by 24 months (Pastoret et al. 1995), suggesting not all skeletal muscles exhibit hypertrophy .

Force of contraction

Although *mdx* muscles are larger, they are weaker than control mice when the force they produce is normalised to muscle weight or cross sectional area. Throughout their lifespan, normalised power output of *mdx* muscles is on average 20 % lower than control mice (Lynch et al. 2001). A contradictory study suggested *mdx* muscles are bigger and stronger, however

force values were not normalised to weight or cross sectional area (Bobet, Mooney & Gordon 1998).

Calcium homeostasis in *mdx* skeletal muscle

Calcium homeostasis is altered in both *mdx* and DMD muscle. Studies report an increase in basal cytosolic calcium levels and sarcolemmal permeability in *mdx* muscle fibres (Fraysse et al. 2004). Abnormal calcium handling is exhibited in skeletal and cardiac myocytes (Lucas-Heron et al. 1987).

The increased resting cytosolic calcium and sarcolemmal permeability in *mdx* myofibres is exacerbated by exercise, leading to excessive calcium signalling which intensifies cellular damage (Fraysse et al. 2004). Exercise also reduces strength (Burdi et al. 2006; Pierno et al. 2007) and increases serum levels of creatine kinase (Rolland et al. 2006).

Respiratory Muscle Involvement

The diaphragm of the *mdx* mouse is the skeletal muscle that best reflects the progressive degeneration, fibrosis and functional deficit observed in limb muscles of DMD patients (Stedman et al. 1991). Fibrosis is up to seven times more than that of control diaphragm, and ten times that of the *mdx* hind-limb muscle (Stedman et al. 1991). However, this is not initially reflected in the respiratory response, with *mdx* mice at 5 months of age exhibiting no difference in ventilation during air breathing and in response to hypercapnia when compared to controls (Gayraud et al. 2007). By 15 months of age the accessory muscles of respiration exhibit similar but less severe histological changes (Stedman et al. 1991) and this is accompanied by an altered response to hypercapnia at 16 months of age (Gayraud et al. 2007).

Cardiac Involvement

An early indicator of cardiac involvement in *mdx* mice is cardiomyocyte membrane damage. Using Evans blue dye infiltration, membrane leakage has been observed in *mdx* heart at 3 months of age which persists up to 18 months (Van Erp et. al. submitted). Membrane leakage does not appear to affect cardiac function as this does not decrease until 15 months of age (Van Erp, Irwin & Hoey 2006).

Cardiac calcium homeostasis

Mdx cardiomyocytes have elevated levels of intracellular calcium, leading to decreased cardiomyocyte function and necrosis resulting from calcium dependant proteases (Alloatti et al. 1995). *Mdx* cardiomyocytes exhibit abnormal calcium signalling response to mechanical stress and this is accompanied with an increased production of ROS (Jung et al. 2008). The two mechanisms combine synergistically to modify calcium signalling (Jung et al. 2008).

Cardiac Fibrosis

Chronic cycles of degeneration in cardiomyocytes of *mdx* leads to cardiac fibrosis (Quinlan et al. 2004; Van Erp, Irwin & Hoey 2006). High levels of fibrosis have been observed in *mdx* hearts at 6 months of age (Van erp et. al. submitted) and 9 -10 months of age (Spurney et al. 2008). By 12 months of age fibrosis has been reported at 8% collagen which increased to 12% collagen by 24 months of age (Cohn et al. 2007).

It is difficult to compare results on the quantitative analysis of fibrosis between studies as results vary substantially. One study reported 17 month old *mdx* hearts displaying 7-9% collagen (Quinlan et al. 2004) whereas another study found younger 15 month old *mdx* hearts

exhibiting 13% collagen (Van erp et. al. submitted). Differences may be attributed to inconsistencies in methodology or inherent variations in different *mdx* mouse colonies worldwide.

Fibrosis in *mdx* hearts have been reported to affect all the regions of the left and right ventricle approximately equally (Quinlan et al. 2004). This differs from the hearts of DMD patients where fibrosis is most severe in the posterobasal portions of the left ventricle with a sparing of the right ventricle (Frankel & Rosser 1976). Differences in the distribution of cardiac fibrosis between DMD patients and the *mdx* mouse are also reflected in echocardiographic differences (Quinlan et al. 2004). High levels of fibrosis can eventually lead to decreased cardiac systolic and diastolic function (Van Erp, Irwin & Hoey 2006).

***Transforming Growth Factor* (TGF- β)**

TGF-β is a profibrotic cytokine known to play an important role in inflammation and wound repair with its expression related to fibrosis (Passerini et al. 2002). TGF-β increases collagen synthesis primarily by increased transcription (Mauviel 2005). It also increases collagen by stabilising procollagen mRNA and by decreasing matrix metalloproteinase (MMP) activity which leads to a decreased breakdown of collagen (Eghbali et al. 1991; Li, McTiernan & Feldman 2000).

Although levels of TGF-β expression have been shown to be increased early in life in the *mdx* diaphragm (at age 6 weeks) and early in the life of the GRMD (up to 60 days) its levels do not continue to increase with age and fibrosis (Gosselin et al. 2004). Data concerning TGF-β expression in the *mdx* heart is limited. One study reported no increase in TGF-β mRNA expression in *mdx* hearts at 15 months old even though there is a significant increase

in fibrosis at that age and while long term pirfenidone treatment lowered TGF-β expression, it did not reduce fibrosis (Van Erp, Irwin & Hoey 2006).

Unpublished data from our laboratory also confirms that although there is ongoing fibrosis in young *mdx* hearts it does not correlate with TGF-β mRNA expression (Van Erp et. al. 2009 submitted). These findings suggest that there is a lack of direct correlation between TGF-β expression and ventricular fibrosis. This inconsistency may be partly explained by a shift toward reduced degradation of collagen rather than active collagen deposition maintaining ventricular fibrosis in older *mdx* hearts (Van Erp, Irwin & Hoey 2006).

Procollagen

The cardiac collagen network consists of collagen type I and III, with type I being predominant (ratio of approx. 70:30) (Weber et al. 1989). Collagen type I is synthesised in fibroblasts as procollagen I. When procollagen I is secreted into the ECM proteinases remove the N-terminal and C-terminal propeptides to form collagen type I (Lopez et al. 2001). Serum levels of the carboxy terminal propeptide of procollagen type I have been used as a marker of extracellular collagen type I synthesis in hypertensive heart disease (Querejeta et al. 2000). To the authors knowledge cardiac mRNA levels of procollagen type I have not be determined in *mdx* mice. This research project examined for the first time, procollagen mRNA levels as a marker of collagen type I synthesis in *mdx* mice and two transgenic strains.

Fibronectin

Fibronectin plays a pivotal role in the ECM as it provides the structural network for the formation of the fibrotic network as it binds to integrin receptors and links cardiac myocytes

to the basal lamina (Chen, H. et al. 2004). In the adult heart fibronectin is expressed within fibroblasts and around the microvasculature (Farhadian et al. 1995). Fibronectin polymerisation into the ECM has been shown to be required for the deposition of collagen type I (Sottile & Hocking 2002). Fibronectin expression is increased during myocardial injury and inflammation (Farhadian et al. 1995). It acts as a chemoattractant for fibroblasts (Jane-Lise et al. 2000) and plays a role in wound repair and remodelling (Ulrich et al. 1997). Fibronectin has also been shown to stimulate several pathways involved in cardiomyocyte hypertrophy (Chen et al. 2004).

To date there is a lack of studies involving cardiac gene expression of fibronectin in the *mdx* mouse. As this gene plays an integral role in cardiac fibrosis this research project determined cardiac mRNA levels of fibronectin in the *mdx* mouse and two transgenic mouse models to further elucidate fibrotic pathways.

Cardiac function

Contractile properties are markedly altered in atria of *mdx* hearts. In particular half-relaxation time at 12 – 14 weeks of age is significantly prolonged in the *mdx* heart (Sapp, Bobet & Howlett 1996). Normal ventricular function has been observed in *mdx* mice hearts at 8 weeks with decreased function occurring at 9-10 months of age which was characterised by decreased fractional shortening and left ventricular end diastolic dimension increase, suggesting dilated cardiomyopathy (Quinlan et al. 2004).

Ex vivo experiments have highlighted the abnormal vulnerability of dystrophin deficient cardiomyocytes to mechanical stress which results in sarcolemmal damage and impaired contractile function (Danialou et al. 2001). Even in young *mdx* mice, the heart is unusually

susceptible to mechanical stress with 8 week old *mdx* mouse hearts displaying abnormal function under ex-vivo perfused conditions with both short term (isoproterenol induced heart failure) and chronic afterload increases (aortic constriction) (Danialou et al. 2001). These results suggest that dystrophin may protect cardiomyocytes from mechanical stress and workload-induced damage (Danialou et al. 2001).

It has been shown in young *mdx* mice that β_1-adrenoreceptor function is normal (Lu & Hoey 2000a). In old *mdx* β_1-adrenoreceptor function deteriorates with disease progression as evidenced by reduced potency of (-)-isoprenaline, and decreased cardiac contractility (Lu & Hoey 2000b). The aged *mdx* mouse exhibits cardiac dysfunction similar to DMD patients with *mdx* hearts at 15 months of age exhibiting a 26% decrease in left ventricular developed pressure, 29% decrease in contractility and 21% decrease in rate of relaxation compared to age matched controls (Van Erp, Irwin & Hoey 2006).

Electrocardiographic and heart rate variability changes

Electrocardiography (ECG) of *mdx* hearts also supports a progression from normal cardiac function in young *mdx* mice to a dilated cardiomyopathy in adult *mdx* mice (Quinlan et al. 2004). By 12-24 months of age *mdx* mice display autonomic dysfunction, impaired conduction, arrhythmias, deteriorated left ventricular function and dilated cardiomyopathy (Wehling-Henricks et al. 2005).

Mdx hearts display a significant tachycardia and decreased heart rate variability with a heart rate 15% faster than control mice (Chu et al. 2002). Heart rate variability is an indicator of autonomic function with depressed heart rate variability indicative of cardiac autonomic dysfunction (Gehrmann et al. 2000). It is suggested that the decreased heart rate variability

observed in *mdx* hearts reflects an imbalance in the autonomic nervous system modulation of heart rate, with decreased parasympathetic activity and increased sympathetic activity (Chu et al. 2002).

Evidence from different studies supports old *mdx* mice as a better model for DMD cardiac disorders than young *mdx* mice with the progression of cardiomyopathy observed in *mdx* mice at 42 weeks of age being comparable to the cardiac involvement of DMD patients (Quinlan et al. 2004).

Limitations of the *mdx* mouse model

The use of the *mdx* mouse as an animal model for DMD has been questioned due to its milder phenotype and lack of widespread fibrosis and fatty infiltration (Megeney et al. 1999). This mild phenotype may be a result of upregulation of utrophin (BostickYueLong et al. 2008) and utrophin/dystrophin double knockout mice have been suggested to resemble DMD pathology more closely than *mdx* mice (Deconinck et al. 1997). It has also been proposed that the reduced dystrophic features of the *mdx* mouse may be due to increased skeletal muscle regeneration. Removal of the myogenic regulator MyoD from the germline of *mdx* mice resulted in a more severe skeletal muscle pathology suggesting that increased regeneration does play a role in the milder phenotype of *mdx* mice (Megeney et al. 1999).

The *mdx* mouse displays distinct stages throughout its life span and different degrees of pathology depending on age and muscle type. For example evidence supports old *mdx* mice as a better model for DMD cardiac disorders and skeletal muscle pathology than young *mdx* mice (Chamberlain et al. 2007; Quinlan et al. 2004).

Experimental data on the *mdx* mouse is difficult to compare due to differences in methodologies and animal strains and housing. A recent review of quantitative and qualitative measurements of the *mdx* mouse including life span, body weights, force of contraction, biochemistry, histology and respiratory and cardiac physiology highlighted the need for standardised experimental protocols (Willmann et al. 2009).

Studies on cardiac involvement in the *mdx* mouse are limited and there exists a need for further characterisation of the model to enable targeting and comparison of potential therapies. Fibrosis is a predominant feature of the dystrophic heart, however the molecular pathways involved in cardiac fibrosis are unclear. Temporal gene expression profiling studies have been reported in *mdx* skeletal muscles, but not cardiac muscle (Porter et al. 2004; Porter et al. 2003). Research is required to evaluate genes involved in cardiac fibrosis over the life span of the *mdx* mouse to assess which genes are associated with the initiation of fibrosis and remodelling of the ECM.

1.6 THERAPEUTIC STRATEGIES

Currently there is no cure for DMD. While gene or cell therapy is promising, at present palliative care is aimed at ameliorating the symptoms of DMD. The only drug treatment proven to be able to preserve or maintain skeletal muscle function in DMD boys are steroids and they have been termed the "gold standard" to which other potential treatments are compared to. Steroids have been shown to improve muscle strength and function, prolong walking, improve respiratory and heart function and lower the risk of scoliosis (Bushby et al. 2004).

1.6.1 Steroids

Two randomised double-blind control trials on DMD boys found the steroid prednisone improved and stabilised muscle strength for up to three years, with the main side effect being excessive weight gain (Fenichel et al. 1991). Steroid use has been shown to be more beneficial to DMD patients than not being treated, with treated boys walking longer and having a decreased risk of scoliosis, however there is an increased risk of vertebral compression fractures and long bone fractures (King et al. 2007). The benefits of prednisone treatment may be optimised by commencing treatment before muscle function begins to decline as prednisone is effective in prolonging not recovering function (Merlini et al. 2003).

A comparative study of prednisone and deflazacort (an oxazolone derivative of prednisone) found deflazacort to be as efficient as prednisone in slowing down the decline of muscle strength in DMD boys (Reitter 1995). Deflazacort treatment prolongs mobility by 3-5 years, and both respiratory and cardiac function is improved (Biggar et al. 2006).While deflazacort treatment may have the advantage of resulting in less weight gain than prednisone, it leads to growth limitations and assymptomatic cataracts (Biggar et al. 2006).

Although prednisone is the most widely used drug to treat DMD, the exact mechanism by which it confers muscle improvement is unclear. The beneficial effects of steroids in DMD are thought to be associated with increased circulating insulin-like growth factor (IGF-1) levels (Rifai et al. 1995). Prednisone has been shown to reduce the number of anti-inflammatory cells in *mdx* suggesting an immunosuppressive role (Wehling-Henricks, Lee & Tidball 2004). It has also been reported that prednisone can reduce calcium influx and the size of calcium pools in *mdx* (Metzinger et al. 1995) and satelite cell myogenisis is enhanced by prednisone (Passaquin et al. 1993).

1.6.2 Exon skipping strategies

The concept of revertant fibres suggested that exon-skipping could be manipulated to produce shorter, but functional dystrophin (Wilton et al. 1999). If a deletion, duplication or point mutation of the dystrophin gene disrupts the reading frame resulting in DMD, the reading frame can potentially be restored by exon skipping with antisense oligonucleotides (AOs). If the region skipped is part of a non-essential region, like the rod domain, the result is a shorter but functional dystrophin protein.

Two kinds of chemically protected AOs are being used in animal and clinical trials, 2'O-methy-phosphorothioates (2'O-methyls) and morpholinos (www.treat-nmd.eu/soc/eng/dmd). Comparative studies of the two chemistries found that some exons are skipped more efficiently by 2'O methyls, others by morpholinos, whereas for some both chemistries appeared to be equally efficient (Heemskerk et al. 2009).

2'O-Methyl Antisense Oligonucleotides

Preclinical experiments in cell cultures, mice and dogs have shown successful exon-skipping

with 2'O methyl AOs (Gebski et al. 2003; McClorey et al. 2006). A small clinical trial of 4 DMD boys who were already confined to wheelchairs, with deletions of the dystrophin gene at exons 50, 52, 48-50 and 19-50 involved injections of 2'O methyl AO against Exon 51 (PRO051) into the tibialis anterior muscle (van Deutekom et al. 2007). After four weeks of treatment dystrophin expression was observed at the sarcolemma at 17-35% that of normal expression in 64-97% of muscle fibres (van Deutekom et al. 2007). While the study proved the principle of exon skipping in DMD boys, the expression of dystrophin was low and it highlighted the need to commence treatment while the muscles are still intact.

Morpholino Antisense Oligonucleotides

Preclinical experiments conducted on eight different morpholino AOs and tested in cell cultures of normal and dystrophic human muscle and non-dystrophic mice containing the human DMD gene found the morpholino AO H51A to be the most successful in exon skipping in *mdx* mice (Arechavala-Gomeza et al. 2007). One limitation of morpholinos is their inability to enter the heart. This has been addressed by a peptide-conjugated morpholino AO with systemic injections of this AO into *mdx* mice resulting in dystrophin expression in skeletal and cardiac muscle (Yin et al. 2008).

Another type of AOs, peptide nucleic acids (PNAs) are showing promising results at improving cell delivery. They have a peptide backbone, are water soluble and intramuscular injections into the tibialis anterior of 8 week old *mdx* mice restored dystrophin expression (Ivanova et al. 2008).

Another approach combines exon skipping with gene therapy, thus genetically instructing muscles to produce the AOs themselves (Goyenvalle et al. 2004). This technique would

negate the need for repeated injections. Modified U7-snRNAs (small nuclear RNA), containing the information for the construction of AOs, were placed into the DNA of type-2 adeno-associated viruses (AAV) and injected locally and systemically into *mdx* mice which resulted in a shorter, but functional dystrophin in 80% of fibres which persisted for more than one year (Goyenvalle et al. 2004).

Following on from that study, a "universal" U7snRNA vector has been developed that brings about exon skipping by universal proteins, heterogeneous nuclear ribonucleoproteins A1/A2 (hnRNP) (Goyenvalle et al. 2009). Advantages of this approach include shorter approval time compared to the many different AOs required for different exon skipping and the ability to perform multi-exon skipping by using two or more U7snRNAs (Goyenvalle et al. 2009).

75% of DMD patients could potentially benefit from exon skipping (Aartsma-Rus et al. 2004). It is important to recognise, however that this treatment is not a cure and results in the less severe BMD phenotype. Although BMD patients lead active lives into middle age and older, 50% of patients die due to cardiac failure (Finsterer & Stollberger 2003). Exon-skipping treatment needs to be repeated and it cannot repair lost muscle, therefore limiting its benefits for older patients. Careful genotyping is required for this type of treatment to enable targeting of deletions, and large or multiple deletions may not be amenable to this technique.

PTC124

High-throughput screens of about 800,000 low molecular weight compounds to identify compounds that promoted read through of premature stop codons, but not normal stop codons resulted in the development of the drug PTC124 (Welch et al. 2007). PTC124 has been shown to promote dystrophin production in muscle cells from humans, and in *mdx* mice

which expressed dystrophin nonsense alleles (Welch et al. 2007). Phase I clinical trials of PTC124 on 61 healthy adults showed no serious side effects which has been followed by the commencement of "proof of principle" studies on the extensor digitorum brevis muscle of 38 DMD boys (www.treat-nmd.eu/soc/eng/dmd). It is important to note that while PTC124 offers a promising therapeutic option, it will only be for the 13-15% of DMD boys with nonsense mutations.

1.6.3 Vectors for gene delivery

There are three main classes of vectors used for gene therapy in DMD, adenoviruses (Ad-v), adeno-associated viruses (AAV), and plasmids.

Adenoviruses

Ad-v are capable of delivering the mini-dystrophin gene to *mdx* mice, however they illicit a high immune response and have a limited capacity (Chamberlain 2002). In an attempt to overcome the size limitation, gutted Ad-v vectors have been developed that have the advantage of carrying full length dystrophin (Clemens et al. 1996).

Adeno-associated virus vector

AAV vectors are capable of transporting mini- or micro-dystrophin genes into skeletal and cardiac muscle, resulting in the expression of a shorter, but functional dystrophin (Chakkalakal et al. 2005). Preclinical experiments transferring a modified dystrophin gene using AAV serotype 6 into *mdx* mice utilising a vasculature permeabilising agent showed wide spread muscle specific expression of a functional mini dystrophin (Gregorevic et al. 2004) and an amelioration of cardiomyopathy in *mdx* was observed with injection of an AAV serotype 9 (Bostick Yue Lai et al. 2008).

Although AAV vectors exhibit a lower immune response than Ad-v vectors, studies on dogs treated with AAV serotype 2 have shown an immune response against the transgene product resulting in a loss of the transduced myofibres (Yuasa et al. 2007). These results highlight the difference between the immune response of murine models and larger animals, with dogs being more susceptible to immunogens than mice emphasising the need to characterise the immune response in larger animals before using AAV vectors for human trials. It has been demonstrated that a brief course of immunosuppressants can overcome the immune response in dogs and enable long-term expression of canine micro-dystrophin in the skeletal muscles of the canine x-linked dystrophic dog (Wang et al. 2007). Regional vascular delivery has been successfully demonstrated in *mdx* mice and non-dystrophic macaque monkeys at doses suitable for clinical trials (Rodino-Klapac et al. 2007).

Like AOs, AAV vector delivery of a mini-dystrophin gene will not cure DMD, rather change it to a milder BMD. However one advantage of AAV vector delivery over AOs is that it has the potential to benefit all DMD patients as it is not mutation specific.

Plasmids

Naked plasmid DNA can transfer genes to muscle cells with minimal immunogenicity and toxicity, however when administered by intramuscular injection they exhibit low transfection efficiency. Although electroporation may overcome this hurdle and has been shown to result in higher levels of transfection of full length dystrophin in skeletal muscles of *mdx* mice, it is also associated with muscle damage (Murakami et al. 2003). Pre-treatment of muscles with bovine hyaluronidase prior to electroporation further enhances transfection without increasing the muscle damage related to the electroporation process (McMahon et al. 2001).

The first clinical trial with plasmid DNA gene delivery commenced in 2005 with 9 DMD and BMD patients aged over 15 (Fardeau et al. 2005). The plasmid, which contained full length dystrophin was injected into a single muscle. Although the level of expression of dystrophin was low, no local or general adverse effects were seen (Fardeau et al. 2005). While this technology could potentially represent a treatment for all DMD patients the transfection efficiency is low and retention may be limited requiring repeated administration (Fardeau et al. 2005).

1.6.4 Myoblast transplantation

Myoblast transplantation aims to introduce muscle precursor cells containing the full length dystrophin gene into dystrophic muscle. One advantage of this technique would be that the dystrophin would be under the control of its normal control sequences. However, a limitation to the technique is the low amount of normal dystrophin expressed after transplantation (Skuk et al. 2006). Injections of normal myogenic cells (derived from relatives) into the tibialis anterior of 9 DMD patients, resulted in 8 patients having full length dystrophin in only 3.5 to 26% of muscle fibres that was restricted to the sites of injection (Skuk et al. 2006). Although the patients were immunosuppressed with tacrolimus, acute rejection may not have been efficiently controlled in all cases thus reducing the amount of new dystrophin observed (Skuk et al. 2006).

Myoblast transplantation has the potential to be combined with other treatments. For example blocking myostatin with the antagonist follistatin in combination with myoblast cell transfer improved myoblast transfer and muscle function in *mdx* mice (Benabdallah et al. 2008). A promising source for myoblasts involves using the myogenic regulator (MyoD). MyoD has

been shown to convert fibroblasts (non-muscle cells) into muscle cells (Weintraub et al. 1989).

1.6.5 Stem cells

Stem cells offer a possible therapy for DMD as they are a source of healthy muscle precursor cells that could repopulate dystrophic muscles cells, replacing defective dystrophin with a functional protein.

Embryonic stem cells

Embryonic stem cells have the potential of developing into muscle cells. A recent study found that differentiating embryonic stem cells need transcription factor Pax3 to form myogenic (muscle forming) cells, necessitating the introduction of this gene into the X-chromosome of the stem cells (Darabi et al. 2008). Injection of these embryonic stem cells locally and systemically into the *mdx* mouse resulted in improved muscle function, however new dystrophin was observed in only 11% to 16% of muscle fibres (Darabi et al. 2008).

Muscle stem cells

Two types of adult stem cells have been identified, mesioangioblasts which are associated with the walls of large blood vessels within muscle tissue, and pericytes which are associated with small blood vessels. Pericytes have the advantage of being able to cross the vasculature and fuse and form myotubes which enables them to be injected arterially (Dellavalle et al. 2007).

Mesioangioblasts delivered systemically to the GRMD dog restored dystrophin to the muscles and improved function and mobility (Sampaolesi et al. 2006). Six GRMD dogs

received mesioangioblasts from the skeletal muscle of healthy unrelated dogs (heterologous cells), while four dogs received mesioangioblasts from their own tissue (autologous cells) that had been transduced with a lentiviral vector expressing human dystrophin. The dogs receiving the heterologous treatment showed the most improvement, with expression of dystrophin in up to 70% of the muscle fibres. While dystrophin expression was high there was a lack of correlation between the amount of dystrophin detected and improvement in muscle function. Furthermore the study has been recently criticized for not taking into account the benefits that may have arisen from the immunosuppressive drug cyclosporin A (CSA) that was used (Grounds & Davies 2007). CSA has been shown to have both beneficial and detrimental effects on muscular dystrophy (De Luca et al. 2005; Stupka et al. 2004).

A double-blind phase I clinical trial on 8 DMD boys tested the safety of autologous transplantation of muscle derived CD133+ cells (Torrente et al. 2007). CD133 cells contain the marker protein CD133 in their membrane and have been shown to be able to repair muscle cells and form new ones in damaged muscle tissue (Torrente et al. 2004). The trial found that there were no local or systemic side effects associated with the transplantation in all treated patients (Torrente et al. 2007). This preliminary outcome leads the way for further clinical trials to test the efficacy of the treatment.

Exon-skipped stem cells

Isolated CD133+ stem cells obtained from the muscles of DMD boys, have been subjected to exon-skipping to repair the dystrophin gene and then transferred into *mdx* mice with a lentivirus (Benchaouir et al. 2007). This resulted in muscle regeneration, with dystrophin in regenerating fibres and restored muscle function (Benchaouir et al. 2007). However future

research needs to examine the effect of integration of the lentivirus on the control of housekeeping genes or tumor suppressing genes.

1.6.6 Alternative drug therapies for dystrophic muscle

Utrophin upregulation

Utrophin has been suggested as a possible pharmacological intervention for the absence of dystrophin. Old *mdx* mice have been shown to upregulate utrophin in cardiac myocytes, perhaps as a compensatory mechanism (Bostick et al. 2008). Although it is insufficient to prevent the dilated cardiomyopathy observed in old *mdx* mice, it may in part explain the milder phenotype of the *mdx* mouse.

Using gene therapy, expression of full length utrophin in transgenic *mdx* mice have resulted in improvements in function and prevention of dystrophic manifestations (Tinsley et al. 1998) and likewise overexpression of utrophin in the GRMD dog ameliorated it's dystrophic pathology (Cerletti et al. 2003).

While *in utero* overexpression of utrophin in *mdx* mice has been shown to prevent the increase of mechano-sensitive calcium channel activity, this did not prevent increased CK levels, centronucleation of muscle fibres and susceptibility to mechanical stress with the effects of utrophin being dose dependant (Squire et al. 2002).

Utrophin based drug therapy has the advantage that it could be administered systemically as utrophin expression in tissue other than muscle does not appear to be toxic (Fisher et al. 2001). Utrophin levels can be stimulated by treatment with heregulin (Krag et al. 2004), activation of calcineurin signalling and increased nitric oxide levels. However these potential

drug-based therapies may induce significant side-effects as highlighted in a review by Miura (Miura & Jasmin 2006).

Inhibition of myostatin

Myostatin is a negative regulator of skeletal muscle growth and inactivation of the myostatin gene results in increased musculature which may reverse muscle wasting in DMD (Bogdanovich et al. 2005). Myostatin inhibitors include myostatin antibodies, myostatin propeptide, follistatin and follistatin-related protein. A myostatin-specific inhibitor derived from follistatin has been developed and transgenic mice expressing this have shown increased skeletal muscle mass and strength (Tsuchida 2008). *Mdx* mice crossed with these transgenic mice also resulted in increased muscle mass and function (Tsuchida 2008). Follistatin delivered with AAV-type-1 vectors into single muscles has been tested on *mdx* mice at three weeks old and seven months old and resulted in increased muscle strength and reduced inflammation and fibrosis (Haidet et al. 2008). Therefore, while inhibiting myostatin does not address the protein deficiency in DMD, this treatment may have the potential to limit the symptoms.

Inhibition of transforming growth factor (TGF-β)

TGF-β is a pleiotropic cytokine which plays a role in inflammation, embryonic development, cell growth and wound repair. GRMD dogs and *mdx* mice have increased amounts of TGF-β (Passerini et al. 2002). Further to this TGF-β levels are high in the skeletal muscles of DMD boys, with expression peaking at 2-6 years of age (Bernasconi et al. 1995).

The correlation between ventricular fibrosis and TGF-β is unclear as highlighted by anti-fibrotic drug, pirfenidone reducing levels of TGF-β in old *mdx* hearts, but not reducing

fibrosis (Van Erp, Irwin & Hoey 2006). Experiments administering pirfenidone to younger mice during the acute inflammatory stage would be valuable to determine if early administration could inhibit upregulation of inflammatory or profibrotic cytokines, like TGF-β, to prevent fibrosis forming.

L-arginine and neuronal nitric oxide synthase (nNOS)

A lack of dystrophin results in a decrease in the concentration of neuronal nitric oxide (nNOS) (a component of the DGC) and its mislocalisation from the sarcolemma to the cytosol (Crosbie 2001). As nNOS produces nitric oxide (NO) from L-arginine, lower levels of nNOS results in decreased levels of NO. NO plays important roles in fibrosis and can reduce muscle inflammation and membrane lysis (Wehling, Spencer & Tidball 2001). Administration of NO precursor, L-arginine showed a reduction of cardiac fibrosis and improved coronary blood flow and heart function (Van erp submitted for publication).

Upregulation of Insulin-like growth factor -1

Insulin-like growth factor-1 (IGF-1) is of interest as possible therapy for DMD as it has been shown to increase muscle mass and strength in skeletal muscles of *mdx* mice while limiting fibrosis (Barton et al. 2002). IGF-1 will be discussed further in section 1.7.

1.6.7 Therapies to alleviate symptoms of cardiomyopathy

Current treatment for DMD cardiomyopathy involves angiotensin-converting enzyme (ACE) inhibitors and beta-blockers. ACE inhibitors and β-blockers have been shown to reverse symptoms of cardiomyopathy in DMD and BMD and prolong survival (Ishikawa, Bach & Minami 1999). A combinatorial treatment has been demonstrated to have a beneficial effect on long term survival and to be more effective when commenced on asymptomatic patients with left ventricular dysfunction as opposed to symptomatic patients (Ogata, Ishikawa & Minami 2009).

Angiotensin II is thought to play a role in cardiac fibrosis in various cardiomyopathies by influencing both collagen synthesis and degradation (Gonzalez et al. 2002). ACE inhibitors impart an antihypertensive effect and may inhibit angiotensin II effects on fibroblast proliferation and their ability to synthesise collagen I and III resulting in an amelioration of fibrosis. Spontaneous hypertensive rats treated with ACE inhibitor quinapril displayed a marked reduction in left ventricular fibrosis as a result of decreased procollagen type I C-terminal peptide (as a marker of collagen synthesis) and increased collagen type I pyrindinoline cross-linked C-terminal telopeptide (as a marker of collagen type I degradation) (Lopez et al. 2001). This reduction in ventricular fibrosis was also observed in a human trial of patients with hypertensive heart disease who were treated with ACE inhibitor lisinopril which was accompanied by improved left ventricular diastolic function (Brilla, Funck & Rupp 2000). While angiotensin II may mediate the synthesis of collagen type I, and thus ACE inhibitors reduce collagen type I, another study involving hypertensive rats treated with ACE inhibitors found myocardial fibrosis still present suggesting other profibrotic factors (such as aldosterone) are involved (Querejeta et al. 2004).

1.6.8 Gene therapy for DMD cardiomyopathy

While there has been extensive research into skeletal muscle gene therapy for DMD, studies on the heart are limited. Issues that need to be clarified include which gene to use as full-length dystrophin exceeds the packaging of most viral vectors. AAV mediated expression of microdystrophin (lacking the C-terminal domain and a deletion from repeat 4 to 23 in the rod domain) in the heart was found to restore the DGC and protect the heart after mechanical challenge by β- isoproterenol (Yue et al. 2003). One limitation to the use of this microgene is the unknown consequence of C-terminal deletion (Duan 2006).

To determine the level of transduction of dystrophin required to protect the heart one study challenged the hearts of heterozygous female *mdx* mice with β-isoproterenol (Yue et al. 2004). As a result of random X-chromosome inactivation these mice express dystrophin in approximately 50% of heart cells. The study found that 50% dystrophin expression was sufficient to attenuate stress-induced cardiomyopathy (Yue et al. 2004).

The pathogenesis of cardiomyopathy associated with DMD has not been clearly defined. It has been suggested that skeletal muscle pathology may play a role in cardiac pathology (Megeney et al. 1999). Further research needs to clarify this as treating the heart alone may not be sufficient to ameliorate DMD cardiomyopathy.

1.7 INSULIN-LIKE GROWTH FACTOR-1

1.7.1 Introduction

DMD muscle is characterised by chronic cycles of regeneration and degeneration associated with the activation of satellite cells to replace muscle cells. As the disease progresses the regenerative capacity of dystrophic muscle is exhausted leading to fibrosis and necrosis. Upregulation of IGF-1 warrants investigation as a potential treatment for DMD as it exhibits proliferative and differentiating effects on muscle growth, increasing muscle mass and strength. Studies on skeletal muscles of *mdx* mice have also shown that IGF-1 overexpression reduces fibrosis, a hallmark of dystrophic muscle (Barton et al. 2002).

While gene therapy, stem cells or myoblast treatment are many years away from providing a cure for DMD, there is a potential for IGF-1 treatment alone or as an adjunct therapy to ameliorate dystrophic symptoms in the interim.

Insulin-like growth factor-1

IGF-1 is a 70 amino acid single chain polypeptide with three stabilising bridges, thus it has a similar shape to insulin and their genes shows a high degree of sequence homolgy. The gene for human IGF-1was first isolated in 1985 and mapped to q22-q24 chromosome 12 (Bell et al. 1985). The gene consists of 6 exons and 5 introns and spans more than 80kb of cDNA (Bell et al. 1985).

IGF-1 is an endogenous hormone with 75% being synthesised in the liver under the control of circulating growth hormone (GH) (Schwander et al. 1983). The remaining 25% of IGF-1 is produced by a number of different cell types, including muscle and acts locally in an autocrine or paracrine manner.

1.7.2 IGF-1 Isoforms

The IGF-1 gene encodes at least four different IGF-1 isoforms. Differential promoter use, alternative splicing and post translational modification of the IGF-1 gene gives rise to six human isoforms and four rat isoforms (Barton 2006a). The isoforms differ in structure and function.

The rodent gene has 6 exons which give rise to classes 1 and 2, derived from Exon 1 or 2 respectively. Transcripts which exclude Exon 5 are termed transcripts A and those which exclude Exon 6 are termed transcript B.

The human gene also has 6 exons, and its alternative splicing is classified the same as the rodent gene except for an additional C transcript which is produced by an internal splice site within Exon 5 resulting in a frameshift and premature termination in Exon 6 (Barton 2006a).

The isoforms that initiate at Exon 1 (class 1) are widespread in all tissue and act in an autocrine or paracrine manner (Shavlakadze et al. 2005). Enhanced autocrine/paracrine action of IGF-1 has been shown to mediate muscle growth (Eppler et al. 2007).

Isoforms initiating at Exon 2 (class 2) are more abundant in the liver, are highly responsive to GH and work in an endocrine manner (Shavlakadze et al. 2005). High levels of circulating IGF-1 has been implicated in an increased risk for several common cancers (Pollak 2004).

Alternative splicing does not affect the mature IGF-1 protein, but does produce different E peptide extensions and it has been suggested that the isoform from which IGF-1 is produced can affect its potency as a mediator of the hypertrophic response (Barton 2006a).

Isoforms with the E peptide extension IGF-1a and IGF-1b promote muscle hypertrophy, with conjecture as to the duration and efficiency of the isoforms. It has been suggested that rat IGF1-b (also termed mechano growth factor) is more efficient than IGF-1a at producing hypertrophy (Goldspink 2005). Other studies have found both isoforms to be equally efficient in producing hypertrophy in young mice with only IGF-1a being effective in older mice and rat IGF-1b potency diminishing with age (Barton 2006b). These studies implicate rat IGF-1b in activating proliferation and IGF-1a appears to preferentially enhance differentiation. In support of this, exercise induces mRNA expression of rat IGF-1b prior to IGF-1a mRNA in rat muscles, which is consistent with IGF-1b activating proliferation (Haddad & Adams 2002). Another study found that rat tibialis anterior muscle post injury displayed an early increase in IGF-1b which activated satellite cells and this was followed by IGF-1a expression which sustained protein synthesis in order to finish the repair process (Hill, Wernig & Goldspink 2003).

1.7.3 The IGF-1/GH axis

The IGF-1/GH axis consists of:

- two ligands (IGF-1 and IGF-2)
- two cell surface receptors (IGF-1R and IGF-2R)
- seven binding proteins (IGF-1BP 1 to 7) and associated degrading enzymes.

IGF-1 receptor

IGF-1 acts exclusively by binding to the tyrosine kinase IGF-1 receptor, which is a transmembrane protein. It consists of two extracellular alpha subunits with IGF-1 binding sites and two beta subunits with three tyrosine residues which undergo phosphorylation and

therefore activation upon binding to IGF-1 (Dupont & LeRoith 2001). IGF-1 and its receptor are imperative for normal growth with knockout mice lacking IGF-1 showing severe growth retardation and mice lacking IGF-1 receptors dying at birth (Liu et al. 1993).

Binding proteins

IGF-1 is bound to IGF-BPs in the circulation and throughout the extracellular matrix (Sitar et al. 2006). The binding proteins release IGF-1 slowly from the blood to the tissues, which increases IGF-1 half life to approximately 20 hours and greatly prolongs the growth effects of GH secretion, which has a half life of less than 20 minutes. The binding proteins directly control the interaction between IGF-1 and its receptor, transporting IGF-1 in the plasma and providing tissue and cell localisation (Jones & Clemmons 1995). IGF-BP3 may play an important role in cardiac hypertrophy (Ito et al. 1993).

1.7.4 IGF-1 signalling pathways

IGF-1 stimulates both proliferation and differentiation of myoblasts (Coolican et al. 1997). Once IGF-1 is released from its binding protein it binds to the IGF-1 receptor located at the cell surface. This can involve activation of three pathways - the mitogen-activated protein kinase pathway (MAPK), the phosphatidylinositol 3-kinase pathway (PI3K) and the calcium-calmodulin dependant protein kinase pathway as illustrated in Fig 1.6 (Mourkioti & Rosenthal 2005).

The MAPK pathway mediates cellular proliferation (Engert, Berglund & Rosenthal 1996). It induces DNA synthesis and mitogenesis, activating cell cycle progression markers such as cyclin D, cdk4, c-fos and c-jun (Mourkioti & Rosenthal 2005). (See Figure 1.6)

The PI3K pathway, which is activated during differentiation, initiates protein synthesis and increases intracellular calcium. It prevents cell death by inhibiting pro-apoptotic proteins and inducing expression of anti-apoptotic proteins. Activation of the PI3K pathway also suppresses protein breakdown and expression of atrophy genes (Sacheck et al. 2004). (See Figure 1.6).

IGF-1 signalling of the calcium-calmodulin dependant protein kinase pathway induces histone deacetylases (HDACs) and myocyte enhancer factor (MEF2) and also expression of GATA-binding protein 2 (GATA-2) and nuclear factor of activated T-cells c1 (NFATc1) implicated in the regulation of skeletal muscle hypertrophy as illustrated in Fig 1.6 (Musaro et al. 1999).

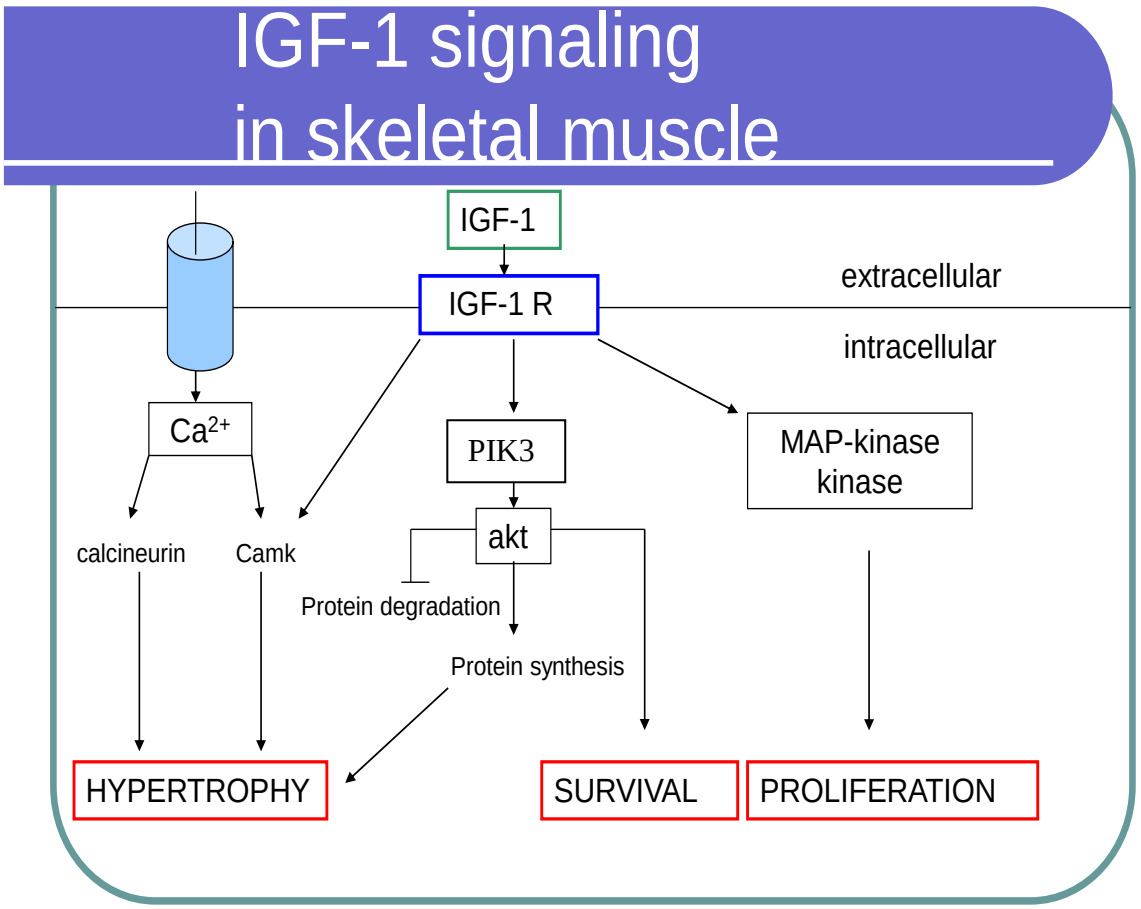


Figure 1.6 IGF-1 signaling pathways in skeletal muscle adapted from Mourkioti (Mourkioti & Rosenthal 2005). The binding of IGF-1 to the IGF-1 receptor located at the cell surface induces phosphorylation of the receptor which can activate three pathways.

1. MAP kinase pathway – activating cell cycle progression markers. 2. PI3K pathway – activating expression of markers for terminal muscle differentiation, cell survival, protein synthesis and protein degradation. 3. Calcium-calmodulin dependant protein kinase pathway which is implicated in the regulation of skeletal muscle hypertrophy.

1.7.5 IGF-1 and skeletal muscle

Comparison of studies on the effects of IGF-1 on skeletal muscle is complicated by the many different modes of delivery and the IGF-1 isoform or receptor that is used. Methods of delivery include transgenic overexpression, viral mediated delivery, plasmid injection, systemic administration and bioengineered tissue as summarised in Tables 1.1 and 1.2.

Table 1.1 Summary of studies involving transgenic overexpression of IGF-1 gene in skeletal muscle of mice.

Citation	Comments on study
Coleman et al., 1995	Avian skeletal alpha actin promoter, hIGF-1. Uniform hypertrophy observed.
Criswell et al., 1998	Hypertrophy in fast and slow muscle.
Chakravarthy et al., 2000	IGF-1 overexpression extends satellite cell regenerative capacity.
Musaro et al., 2001, Musaro et al., 2004	IGF-1 overexpression mediated hypertrophy and increased force generation particularily in fast fibres.
Barton et al., 2002	IGF-1 overexpression in *mdx* showed hypertrophy and hyperplasia and reduction of fibrosis of diaphragm.
Fiorotto et al. 2003	IGF-1 overexpression induced muscle hypertrophy
Shavlakadze et al. 2004	IGF-1 overexpression reduced necrosis in *mdx* mouse.
Shavlakadze et al. 2005	IGF-1 overexpression reduced skeletal muscle atrophy resulting from denervation
Song et al. 2005	IGF-1 overexpression blocks angiotensin II-induced skeletal muscle wasting.
Pelosi et al., 2007	IGF-1 overexpression accelerated the regenerative process of injured skeletal muscle, modulating the inflammatory response and limiting fibrosis.

Table 1.2 Summary of different modes of IGF-1 delivery to skeletal muscle of mice

MODE OF DELIVERY	EXAMPLES OF STUDIES
Viral mediated delivery	• IGF-1 stimulated muscle cell differentiation and myofiber hypertrophy (Coleman et al. 1995). • IGF-1 stimulated muscle regeneration via the activation of satellite cells, blocking the age-related loss of skeletal muscle function (Barton-Davis et al. 1998). • IGF-1 induced local skeletal muscle hypertrophy and attenuated age-related skeletal muscle atrophy, restoring and improving muscle mass and strength (Musaro et al. 1999). • Improvement of dystrophic muscle by rAAV/microdystrophin vector is augmented by IGF-1 co-delivery (Abmayr et al. 2005). • Comparison of IGF-1A and IGF-1B found both equally effective in increasing muscle mass with IGF-IB t only effective in growing animals (Barton 2006b).
Systemic administration	• IGF-I treatment ameliorates muscle wasting and improves the functional properties of skeletal muscles of dystrophic mice (Lynch et al. 2001). • Administration of IGF-1 improves fatigue resistance of skeletal muscles from dystrophic *mdx* mice (Gregorevic et al. 2004). • IGF-I enhanced oxidative status and reduced contraction-induced damage in skeletal muscles of *mdx* dystrophic mice (Schertzer et al. 2006).
Bioengineered tissue	• Paracrine release of IGF-1 from a bioengineered tissue stimulates skeletal muscle growth *in vitro* (Shansky et al. 2006)

1.7.6 Transgenic overexpression of IGF-1

Two main mouse strains have been developed using different transgenic constructs that overexpress IGF-1 specifically in skeletal muscle and they each exhibit a different phenotype (Shavlakadze et al. 2005).

The two different promoters used in the transgenic constructs are:

1. skeletal α-actin which is the major actin isoform in adult skeletal muscle and is only active in differentiated skeletal and cardiac muscle. Although there is a greater expression of the transgene with this promoter in skeletal muscle, it is also expressed in the heart and circulation (Delaughter et al., 1999, Shavlakadze et al., 2005).

2. myosin light chain 1/3 which is activated by differentiated muscle cells. It has the advantage of not being expressed in cardiac muscle so that IGF-1 overexpression effects can be observed in skeletal muscle alone.

It is also important to take in to consideration the isoform expressed, with most studies using either rat or human IGF-1Ea, as this isoform is expressed in skeletal and cardiac muscle and does not enter the circulation, but remains in the muscle bed (Musaro et al. 2001).

1.7.7 Effects of IGF-1 on skeletal muscle

It has been well documented that overexpression of IGF-1 in the skeletal muscles of transgenic mice induces hypertrophy (Barton-Davis et al. 1998; Barton-Davis, Shoturma & Sweeney 1999; Barton 2006b; Barton et al. 2002; Coleman et al. 1995; Criswell et al. 1998; Fiorotto, Schwartz & Delaughter 2003; Musaro et al. 2001; Sacheck et al. 2004; Shavlakadze

et al. 2004). The hypertrophy is accompanied with increased muscle strength and extended muscle mass and strength in aging mice (Musaro et al. 2001).

IGF-1 transgenic overexpression in skeletal muscles of normal mice has been shown to modulate the inflammatory response and limit fibrosis after cardiotoxin induced muscle injury as illustrated in Fig 1.7 (Pelosi et al. 2007). IGF-1 is a powerful enhancer of the regeneration response inducing proliferation and differentiation of satellite cells and extending their replicative life span (Chakravarthy et al. 2000). IGF-1 has also been shown to increase recruitment and production of proliferating bone marrow cells (Musaro et al. 2004).

These attributes of IGF-1 overexpression suggest it could be beneficial for dystrophic muscle. Treatment of skeletal muscles of *mdx* mice with recombinant human IGF-1 reduced fibrosis, necrosis and improved fatigue resistance (Gregorevic, Plant & Lynch 2004). Transgenic mice overexpressing IGF-1 in skeletal muscles have been crossed with *mdx* mice (Barton et al. 2002). These IGF-1+/*mdx* mice showed an amelioration of the histopathological changes normally seen in the *mdx* mouse. In particular IGF-1+/*mdx* mice displayed hypertrophy which was accompanied with increased strength and a reduction of fibrosis and necrosis (Barton et al. 2002). In 21 day old IGF-1/*mdx* mice (the age of acute onset of necrosis) myofibre damage was reduced without hypertrophy, thus suggesting a protective role for IGF-1 (Shavlakadze et al. 2004). Further evidence of this protective role, is the potential of overexpression of IGF-1 in *mdx* mice to reduce the susceptibility of dystrophic muscle to exercise induced damage (Ridgley et al. 2009).

IGF-1 may reduce muscle damage by attenuating excitation-contraction coupling (E-C) coupling failure. Dystrophic skeletal muscles display abnormal E-C (De Luca et al. 2001) and

overexpression of IGF-1 in the dystrophic EDL muscle attenuates this failure (Schertzer et al. 2008).

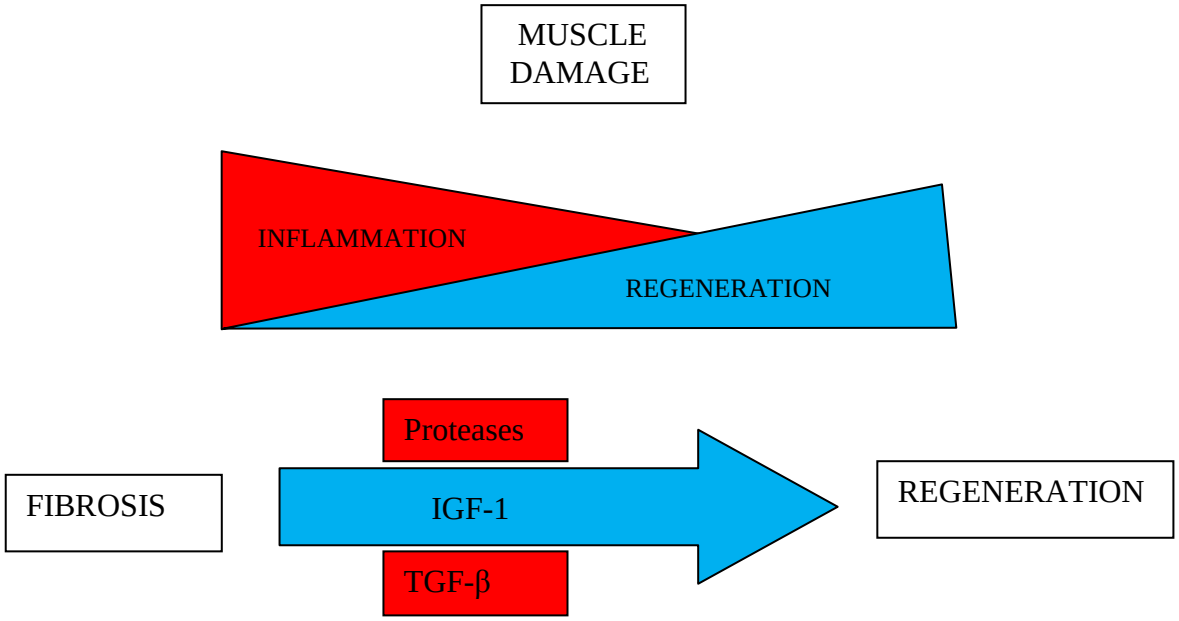


Figure 1.7 Diagrammatic representation of the role of IGF-1 in the coordination between inflammation and regeneration in skeletal muscle. Adpated from (Mourkioti & Rosenthal 2005)**.** Muscle injury breaks capillaries and initiates the inflammation process. Regeneration of skeletal muscle activates TGF-β initiating fibrosis which replaces the space within myofibres with connective tissue. Fibrotic tissue initially aids muscle repair, however continued accumulation of fibrotic tissue fills the space new muscle cells would normally occupy. This results in the muscle not being able to regain normal contractile properties which leads to decreased strength. Effective muscle regeneration depends on an exact balance between pro- and anti- inflammatory factors. IGF-1 reduces the chronic inflammatory response and moves the balance toward muscle regeneration.

1.7.8 IGF-1 and the heart

IGF-1 has been shown to promote cardiac growth, improve cardiac contractility, cardiac output, stroke volume and ejection fraction as reviewed by Ren et al.(1999). IGF-1 is an endogenous regulator of myocardial function and associated with its general anabolic effects, it improves cardiac function through a direct inotropic effect on normal and various disease models hearts (Ren, Samson & Sowers 1999).

Physiological hypertrophy as evidenced by improved cardiac function

An increased level of IGF-1 has been shown to result in cardiac hypertrophy. Several studies have found this to be due to increased cardiomyocyte size (Duerr et al. 1995; Ito et al. 1993; Santini et al. 2007). While other studies have reported increased number of cardiomyocytes with IGF-1 overexpression, a different isoform of IGF-1(IGF-1B) was used (Reiss et al. 1996; Welch et al. 2002).

The cardiac hypertrophy mediated by IGF-1 appears to be a physiological adaptation as evidenced by an improvement in function (Cittadini et al. 1998; Duerr et al. 1995; Santini et al. 2007; Serose et al. 2005). One study found the cardiac hypertrophy to be initially a physiological adaptation which progressed to a pathological condition (Delaughter et al. 1999). This may be due to the cDNA for the IGF-1 being driven by a skeletal α-actin promoter which resulted in high levels of systemic IGF-1.

The inotropic effect of IGF-1 overexpression may be due to IGF-1 mediating an increase in the sensitivity of cardiomyocytes to calcium (Ren 2002). Rat hearts immersed in a bathing solution of IGF-1 resulted in cardiomyocytes being more sensitive to calcium through a wortmannin-sensitive pathway (Cittadini et al. 1998).

Evidence suggests that IGF-1 plays a protective role against myocardial injury. It has been shown to inhibit cardiac necrosis and apoptosis (Buerke et al. 1995; Davani et al. 2003; Li, Q. et al. 1997; Santini et al. 2007). Further supporting this cardioprotective role, IGF-1 has been shown to stimulate the gene expression of detoxifying proteins and antioxidant enzymes produced in pathological conditions (Santini et al. 2007). In the diabetic rat IGF-1 overexpression protects the heart against diabetic cardiomyoapthy by reducing angiotensin synthesis (Kajstura et al. 2001). Further to this, angiotensin is thought to play a role in cardiac fibrosis (Gonzalez et al. 2002) and attenuation of the renin-angiotensin system by ACE inhibitors has been shown to be beneficial to the dystrophic heart (Ishikawa, Bach & Minami 1999).

IGF-1 plays an important role in the repair of injured hearts, restoring function and limiting fibrosis. Transgenic mice, overexpressing IGF-1 driven by the α-myosin heavy chain promoter were subjected to two different modes of injury, ligation of the coronary artery and direct cardio toxin injection. As α-myosin heavy chain is only expressed in cardiac muscle, the use of this promoter restricts the overexpression of IGF-1 to the heart excluding effects on other tissues. IGF-1 mediated a restoration of cardiac function in these transgenic mice by blocking scar formation and enabling myocardial reconstruction (Santini et al. 2007). In support of these findings, administration of IGF-1 (systemically and by direct myocardial injection) to the hearts of δ-sarcoglycan deficient hamsters conferred improved cardiac function accompanied by a reduction in interstitial fibrosis (Serose et al. 2005).

IGF-1 is thought to restore stucture and function to damaged cardiac tissue by resolving inflammation at the site of injury, preventing scar formation and enabling tissue replacement

as illustrated in Figure 1.8. A study highlighting this observed anti-inflammatory cytokines interleukin-4 (IL-4) and interleukin-10 (IL-10) to be significantly upregulated in transgenic IGF-1 overexpression mouse hearts after cardio toxin injection (Santini et al. 2007). Furthermore, proinflammatory cytokine interleukin-6 (IL-6) was down regulated (Santini et al. 2007). Other pro-inflammatory cytokines (in particular TGF-β) were not affected (Santini et al. 2007).

1.7.9 Cardiac signalling pathways initiated by IGF-1

There is evidence for differing signalling pathways initiated by IGF-1 in skeletal and cardiac muscle (Rommel et al. 2001; Santini et al. 2007). In skeletal muscle IGF-1 is thought to mediate cellular growth by initiating the P13K phosphorylation pathway via Akt and mTOR intermediates (Rommel et al. 2001). In cardiomyocytes, PI3K signalling is thought to be through PDK1/SGK1signalling intermediates, independent of Akt (Santini et al. 2007). This pathway is illustrated in Figure 1.8.

With regard to muscle hypertrophy, skeletal and cardiac muscle appear to share a similar signalling system which is regulated by calcineurin controlling associations between NFAT and GATA transcription factors (Molkentin et al. 1998; Mourkioti & Rosenthal 2005). Hypertrophic stimuli acting on the cell membrane leads to an elevation of intracellular Ca^{2+} which in turn activates calcineurin. Calcineurin dephosphorylates NF-AT3, resulting in its translocation to the nucleus, where it interacts with GATA4 to synergistically activate transcription of hypertrophic genes (Molkentin et al. 1998).

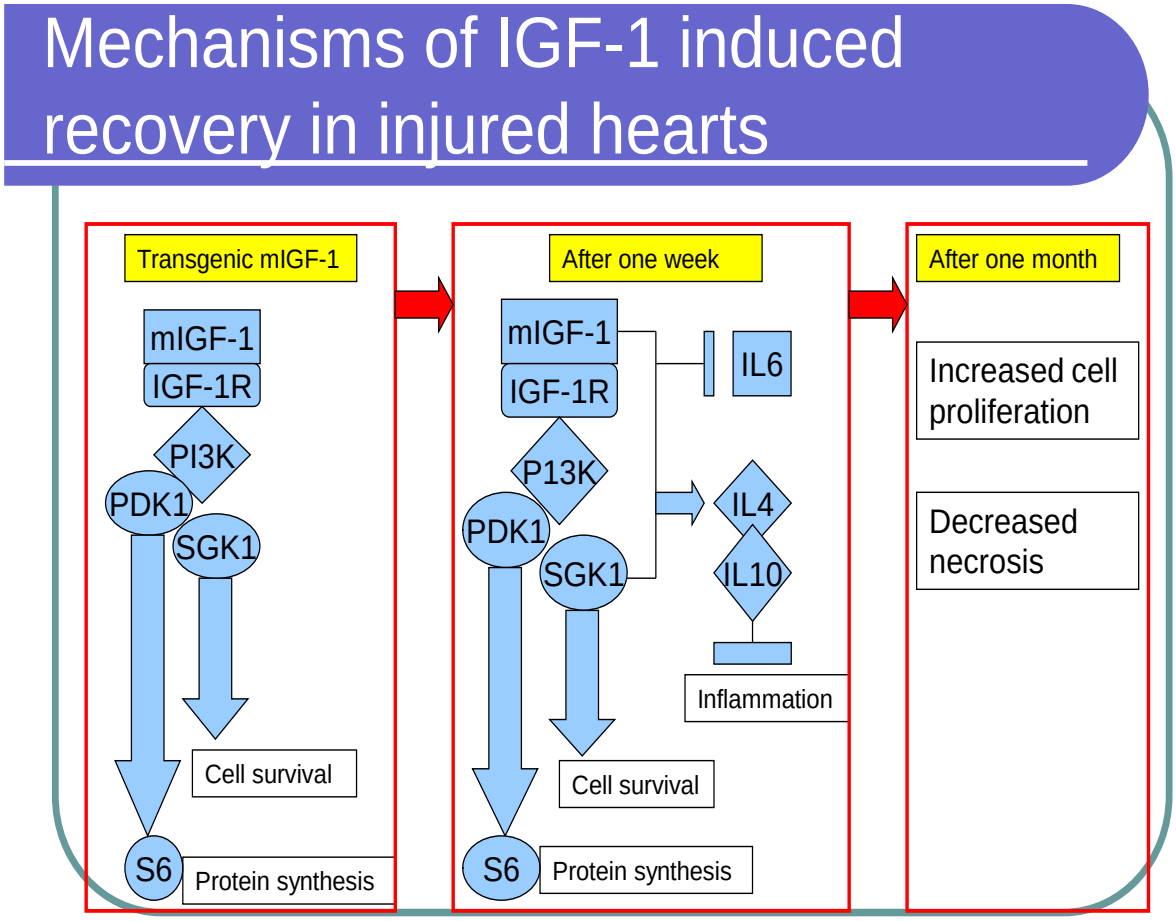


Figure 1.8 Mechanisms of IGF-1 to induce cardiac repair. Adapted from Santini (Santini et al. 2007)

A. IGF-1 overexpressed exclusively in the hearts of transgenic mice induces protein synthesis and cell survival pathways in the myocardium through a PDK1/SGK1 phosphorylation cascade.

B. Following myocardial infarction (MI), pathways mediated by IGF-1 result in a rapid decrease in proinflammatory cytokine IL6 that promotes fibrosis and cardiac dysfunction, while activating cytokines such as IL4 and IL10 that resolve inflammation.

C. This compliant tissue environment is amenable to cardiac wall replacement, as demonstrated by increased proliferation of cells at 4 weeks post injury (Beltrami et al.2003).

Data regarding transgenic mice that overexpress IGF-1 exclusively in the heart enable the evaluation of the effects of IGF-1 on the myocardium. Researchers have used this to observe the effects of IGF-1 on various cardiac disease states and after cardiac injury. IGF-1 has been shown to have a cardioprotective effect, and to mediate repair of the injured heart restoring function, while limiting fibrosis by resolving inflammation (Santini et al. 2007). It is important to assess whether the beneficial actions of IGF-1 seen in conditions such as myocardial infarction could be translated to the severe dystrophic changes observed in DMD. Rather than an acute necrotic episode and inflammatory response of myocardial infarction, dystrophic muscle is characterised by relentless cycles of degeneration and fibrosis. Transgenic *mdx* mice that overexpress IGF-1 in skeletal muscle have shown that IGF-1 plays a protective role (Shavlakadze et al. 2004), increases muscle mass and strength with a reduction of fibrosis and necrosis (Barton et al. 2002). Clearly there exists a need to investigate the effects of IGF-1 overexpression on the dystrophic heart.

1.8 MYOGENIC REGULATOR MyoD

1.8.1 Introduction

The myogenic regulator, MyoD is vital for muscle differentiation and is expressed in skeletal muscle but not in the heart. It is a member of the basic helix-loop-helix family (bHLH).The mouse MyoD1 gene was isolated in 1991 (Zingg, Alva & Jost 1991). The MyoD gene family includes MyoD, Myf-5, myogenin, and MRF4 (Chen & Goldhamer 1999).

1.8.2 MyoD and satellite cell function

Satellite cells in adult skeletal muscle are situated underneath the basement membrane and are mitotically quiescent (Yablonka-Reuveni & Rivera 1994) and do not express any of the myogenic regulatory family (Cornelison & Wold 1997). Once activated, satellite cells isolated from skeletal muscles of mice expressed either MyoD or myf5 first among the MRFs which was followed by expression of proliferating cell nuclear antigen (a marker for cell proliferation) (Cornelison & Wold 1997). Following proliferation, myogenin and MRF-4 expression marks the entry of myoblasts into differentiation, which is rapidly followed by terminal withdrawal from the cell cycle and expression of muscle specific structural proteins as illustrated by Fig 1.9 (Yablonka-Reuveni & Anderson 2006).

In the *mdx* mouse, satellite cells are thought to be active, participating in the ongoing regeneration that is characteristic of dystrophic muscle (Anderson et al. 1998). Cell cultures from *mdx* mice showed an earlier decline in MyoD expression than controls and an earlier increase in myogenin expression (Yablonka-Reuveni & Anderson 2006). This suggests that dystrophic muscle exhibits a more rapid differentiation than normal muscle (Yablonka-Reuveni & Anderson 2006).

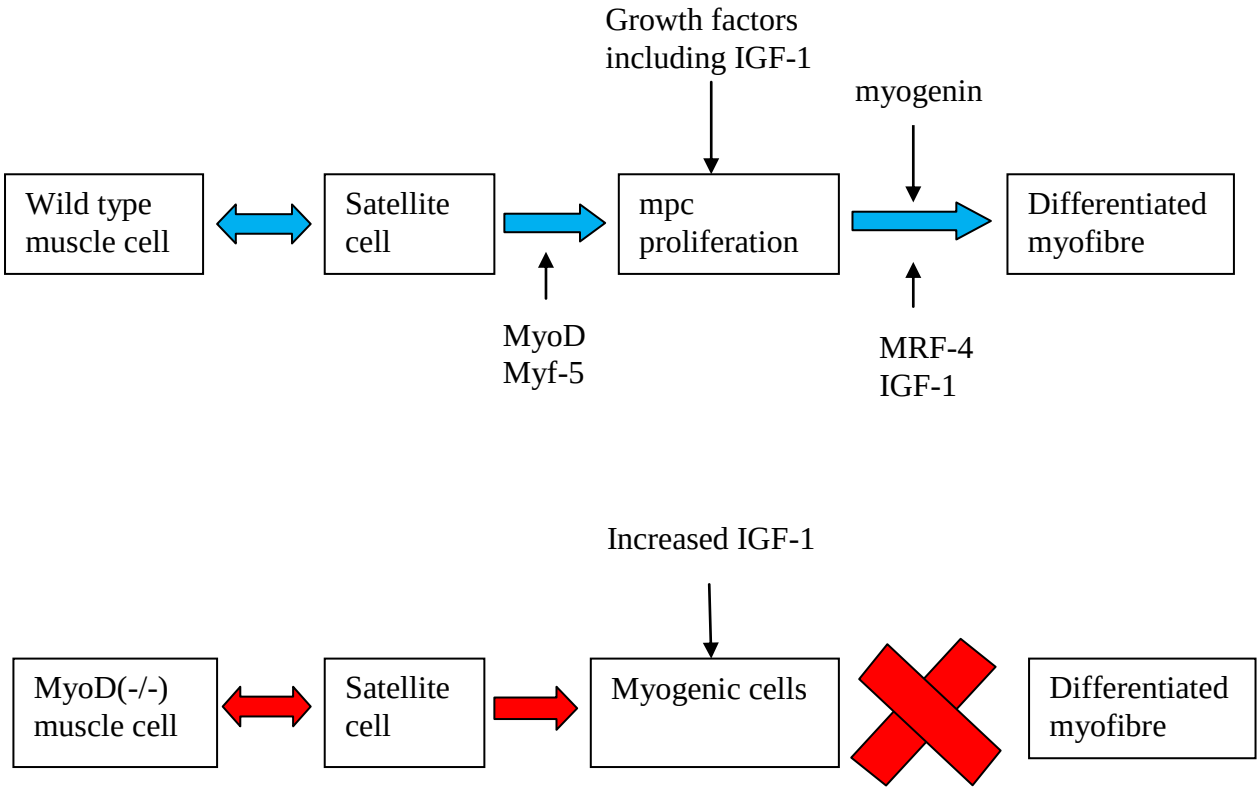


Figure 1.9 Role for MyoD in satellite cell function adapted from Megeney and Seale (Megeney et al. 1996; Seale & Rudnicki 2000).

Upregulation of MyoD appears to be required for entry into myogenic precursor cells proliferative phase before terminal differentiation. MyoD is upregulated with entry into cell cycle, Myf-5 and MRF4 are expressed intermediate, and myogenin is expressed last (Yablonka-Reuveni & Rivera 1994). Lack of MyoD leads to increased numbers of myogenic cells due to stem cell self renewal, as opposed to progression through the development program (Megeney et al. 1996). IGF-1 and other growth factors promote proliferation of myogenic precursor cells (mpcs). Following proliferation mpcs undergo terminal differentiation in the formation of myocytes, which requires the expression of myogenin and MRF4 before fusing with exisiting or new fibres.

1.8.3 MyoD null mice

To elucidate the role of MyoD in myogenesis, mice with a null mutation of the MyoD gene were generated (Rudnicki et al. 1992). Unexpectedly these mice were viable and fertile however mRNA levels of Myf-5 were increased (Rudnicki et al. 1992). Myf-5 is normally reduced during gestation when MyoD mRNA first appears. This suggests that Myf5 expression can be repressed by MyoD and that Myf5 may be capable of functionally replacing MyoD (Rudnicki et al. 1992). However either MyoD or Myf5 is required for the formation of skeletal muscle with mice deficient for both MyoD and Myf5 displaying a complete lack of skeletal myoblasts and muscle (Rudnicki et al. 1993).

1.8.4 MyoD and dystrophin double knockout mice

To further examine the role of MyoD in muscle regeneration, mice lacking MyoD were then interbred with *mdx* mice which are characterised by ongoing cycles of muscle degeneration and regeneration (Megeney et al. 1996). The milder phenotype of the *mdx* mouse compared to DMD patients is thought to be due, in part, to increased muscle regeneration (Anderson, Bressler & Ovalle 1988). Transgenic null MyoD dystrophic mice displayed more severe dystrophic changes in skeletal muscle than *mdx* mice, highlighting the importance of MyoD in muscle regeneration as highlighted in Figure 1.10 (Megeney et al. 1996). These changes include curvature of the spine, an abnormal waddling gait characterised by weight bearing on the tarsal joints, and reduced muscle mass (Megeney et al. 1996). While DMD patients do not have a defiency in MyoD, the phenotype of the *mdx*/MyoD(-/-) model correlates to that observed in DMD patients.

Mdx mice are characterised by skeletal muscle hypertrophy (Anderson, Ovalle & Bressler 1987), and this was reduced or absent in *mdx*/MyoD(-/-) mice (Megeney et al. 1996). The increased skeletal myopathy suggests a dramatic decrease in their regenerative capacity,

however several centrally located nuclei were observed, indicating that some regenerative potential did still did exist in these mice (Megeney et al. 1996). This study also highlighted a unique role for MyoD in proliferation during regeneration, which Myf5 cannot substitute for as shown in figure 1.9 (Megeney et al. 1996).

Apart from the diaphragm, the *mdx* mouse does not exhibit the muscle fibre loss and widespread fibrosis observed in DMD boys (Stedman et al. 1991). Further to this, unlike *mdx* skeletal muscle, DMD muscle has reduced satellite cell populations and proliferative potential of myogenic precursor cells (Webster & Blau 1990). Therefore the *mdx*/MyoD(-/-) mouse with its reduced proliferative potential of myogenic cell and increased skeletal myopathic phenotype, offers a transgenic model that more closely resembles DMD.

As MyoD is not expressed in cardiac muscle, *mdx*/MyoD(-/-) double knockout mice also provide an insight into the effect of skeletal muscle damage on cardiomyopathy. It has been suggested that the cardiomyopathy in boys with DMD may not be wholly due to the lack of dystrophin in the cardiomyocytes, but may also be due to dystrophic changes in skeletal muscle leading to postural changes which place extra work load on the heart (Megeney et al. 1999). In support of this, BMD patients have greater mobility and exhibit a more severe cardiomyopathy than DMD, suggesting that increased mobility places a heavier work load on the dystrophic heart resulting in a more severe cardiomyopathy (Finsterer & Stollberger 2003).

The *mdx* mouse exhibits a progressive cardiomyopathy (Quinlan et al. 2004) that appears to be exacerbated by removal of the MyoD gene (Megeney et al. 1999). At 5 months of age the *Mdx*/MyoD(-/-) double knockout mice demonstrates a more severe cardiomyopathy

highlighted by increased levels of fibrosis compared to *mdx* mouse, suggesting this model may represent DMD cardiomyopathy more closely than the *mdx* mouse (Megeney et al. 1999).

To date the only study regarding cardiac involvement in these transgenic mice, examined heart weight, fibrosis and activation of stress-activated protein kinases (Megeney et al. 1999). This transgenic model warrants further characterisation to validate its use as a model for dystrophin deficient cardiomyopathy which may resemble DMD more closely than the *mdx* mouse.

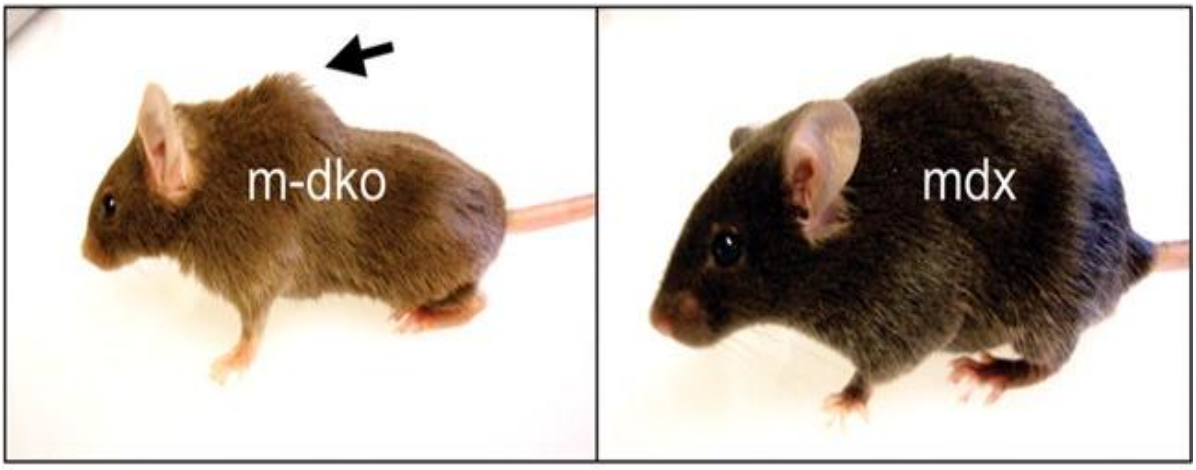


Figure 1.10 Photos of *mdx*:MyoD(-/-) (left) and *mdx* mice (right) at 5 months of age. Adapted from Duan (Duan 2006).

The increased skeletal muscle pathology of the *mdx*:MyoD(-/-) mouse is characterised by a decreased muscle mass and increased kyphosis as indicated by the arrow.

1.9 AIMS AND SCOPE OF STUDY

1.9.1 Age study

The aim of the initial study in this research project was to further characterise the *mdx* mouse as a model of cardiomyopathy associated with DMD. Functional studies on the *mdx* heart are few and this study aimed to provide baseline measurements of cardiac function. *Mdx* mice appear to display a progressive cardiomyopathy, so it is imperative to examine changes in cardiac function of the animal with age. This study used three age groups, 3, 6, 12 months in an attempt to observe early changes in cardiac function.

Cardiac fibrosis is a predominant feature of cardiac muscle in DMD and *mdx* mice. Despite this it has been poorly reported and the complex molecular pathways involved have not been fully elucidated. Any future therapies aimed at alleviating fibrosis would need clarification of the mechanisms involved. To this end, the study examined the degree of cardiac fibrosis at different age groups and mRNA levels of genes involved in the inflammatory process (TGF-β) and genes involved in the remodelling of the ECM (procollagen and fibronectin). Using three age groups enabled aspects of the molecular pathway involved in the early stages of fibrosis to be compared to those later in the life of the animal. Therefore this study aimed to test the hypothesis that the *mdx* mouse is a valid model of dystrophin deficient cardiomyopathy, and Figure 1.11 provides an overview of this.

1.9.2 IGF-1 study

The second study addressed the effects of overexpression of IGF-1 on the dystrophic heart. The growth enhancing effects of IGF-1 have been well documented, and it has been reported to reduce fibrosis in dystrophic skeletal muscle and improve cardiac function in other animal models of cardiac dysfunction. This study aimed to assess the effect of IGF-1 on the

dystrophic heart by generating a novel mouse model that overexpressed IGF-1 exclusively in the *mdx* mouse heart. Morphological and *in vivo* cardiac functional analyses were performed at two ages, 6 and 12 months, to enable a comparison of the effects of IGF-1 on a younger and older animal.

This study also aimed to examine cardiac fibrosis and the pathways involved. In particular the gene expression levels of TGF-β, procollagen and fibronectin were evaluated. Once again the two age groups provided an opportunity to determine any differences in the fibrotic pathways of the younger animal compared to the older one.

This study tested the hypothesis that overexpression of IGF-1 exclusively in the dystrophic heart, has the ability to ameliorate the symptoms of ventricular fibrosis and improve cardiac function. Figure 1.11 provides an overview of this IGF-1 study.

1.9.3 MyoD study

The main aim of the third study was to further characterise the *mdx*:MyoD(-/-) mouse as a model that may resemble the cardiomyopathy associated with DMD more closely than the *mdx* mouse. Further to this, as the MyoD gene is only present in skeletal muscle, its removal provides a model that enables assessment of the impact of exacerbated skeletal muscle pathology on the myocardium.

The study involved histological analysis to determine the level of cardiac fibrosis and *in vivo* functional experiments were performed on this mouse model for the first time. Young, 3 month old mice were used in an attempt to assess early cardiac changes. Cardiac gene

expression of TGF-β, procollagen and fibronectin provided further valuable data on the molecular events involved in fibrosis.

This final study tested the hypothesis that the *mdx*:MyoD mouse is a better model for the cardiomyopathy associated with DMD than the *mdx* mouse. Figure 1.11 provides an overview of the MyoD study.

The experimental design for all three studies aimed to examine cardiac involvement at

- the organ level with the Langendorff technique utilising the *ex vivo* isolated heart,
- the tissue level with histological analysis of ventricular fibrosis and
- the molecular level by qRT-PCR analysis of cardiac genes involved in fibrosis.

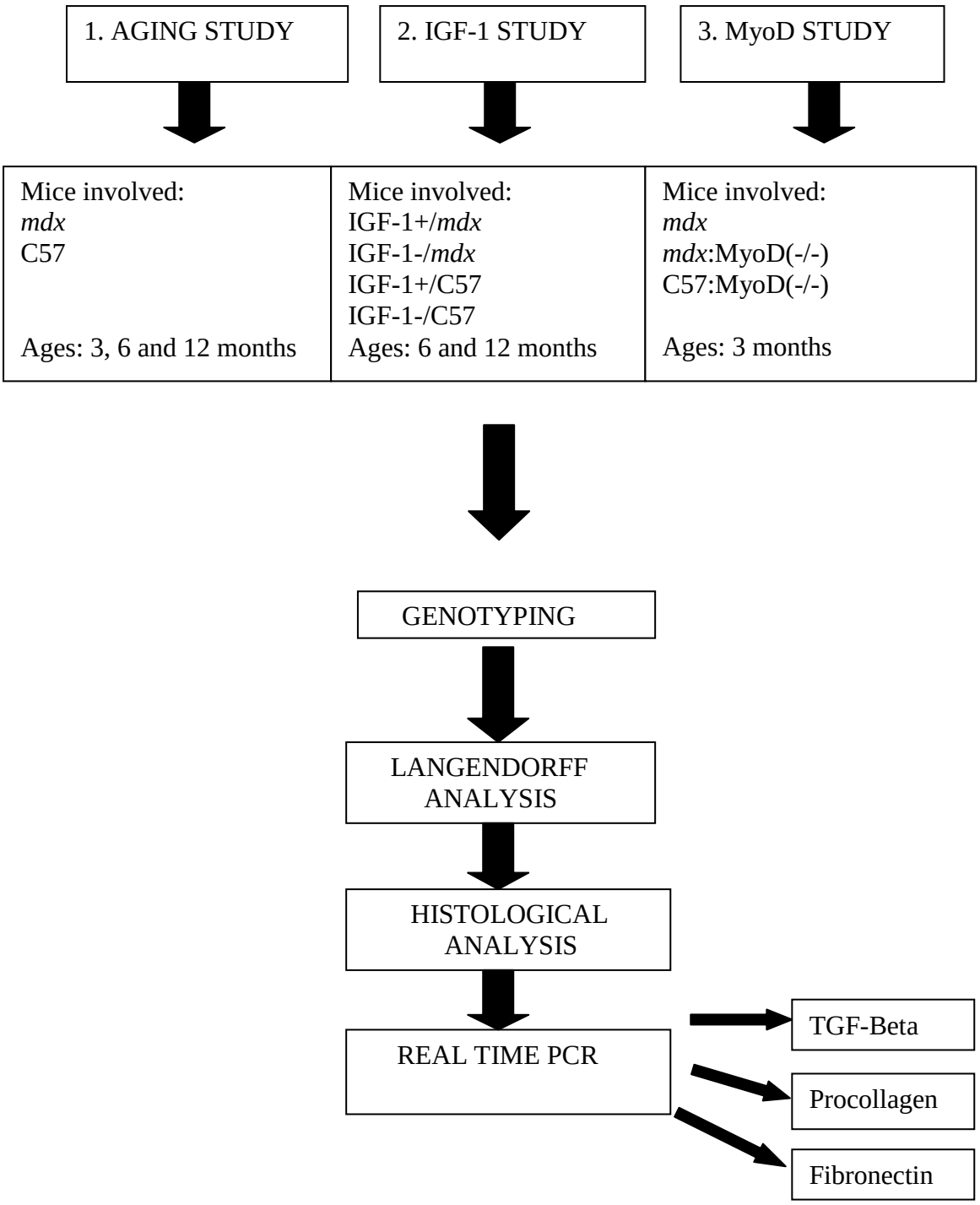


Figure 1.11 Overview of research project. Three studies involved are 1. Age study, 2. IGF-1 study and 3. MyoD study.

CHAPTER 2. MATERIALS AND METHODS

2.1 ANIMALS

Mdx mice (C57BL/10scsn *mdx*) and C57 (C57BL/10ScSn) control mice were bred and housed at the University of Southern Queensland animal house in accordance with the National Health and Medical Research council code of practice. Mice were subjected to a 12 hour light/dark cycle at 20-25^{0}C with free access to food and water. All experiments were approved by the University of Southern Queensland animal ethics committee.

2.1.1 *mdx* and C57 mice

Mdx and C57 mice used in the age study were sourced from and bred at the University of Southern Queensland animal house. Experimental mice were genotyped and used at 3, 6 and 12 months of age. Only males were used in experiments to reduce variability due to sex.

2.1.2 IGF-1 mice

This project utilised foundation mice that overexpressed muscle specific IGF-1 (mIGF-1) under a myosin heavy chain (MHC) promoter which restricted overexpression to the heart. These mice were provided generously by Professor Miranda Grounds from the University of Western Australia.

The experimental mice were generated by crossing the MHC/mIGF-1 mice, which were on an FVB background, with *mdx* females. The resultant F1 generation were genotyped using a PCR (described in 2.2.3) for IGF-1 using interleukin used as a control gene. Male mice that were positive for the IGF-1 transgene were backcrossed with *mdx* females. The resultant F2

generation were genotyped and consisted of *mdx* positive for the mIGF-1 transgene (IGF-1+/*mdx*) and *mdx* negative for the IGF-1 transgene (IGF-1-/*mdx*).

Likewise the control strain, C57 were generated in the same way and the F2 generation resulted in C57 positive for mIGF-1 (IGF-1+/C57) and C57 negative for the mIGF-1 (IGF-1-/C57).

These F2 generation mice were allowed to grow out to either 6 months or 12 months of age. Only males were used for experiments to reduce variability due to sex.

2.1.3 MyoD mice

This project utilised transgenic MyoD mice that had a disruption of the MyoD gene, caused by insertion of a vector which resulted in MyoD not being expressed in skeletal muscles. These transgenic mice were provided generously by Professor Miranda Grounds from the University of Western Australia.

The experimental mice were generated by crossing MyoD deficient mice with *mdx* females. The resultant F1 generation were genotyped for absence of the MyoD gene using PCR (described in 2.2.4). The male mice lacking MyoD were backcrossed with *mdx* females. The resultant F2 generation were genotyped and consisted of *mdx* positive for MyoD and *mdx* negative for the MyoD gene.

Likewise, C57 mice were crossed with MyoD deficient mice which resulted in C57 positive for MyoD and C57 negative for the MyoD. Male mice without the MyoD gene were backcrossed with C57 females. The resultant F2 generation provided C57 mice lacking the

MyoD gene and they were allowed to grow out to 3 months. Only males were used for experiments to reduce variability due to sex.

2.2 GENOTYPING

2.2.1 DNA extraction

DNA for genotyping was obtained by removing approximately 2mm of the tip of the mouse tail which was digested in 50mM of Tris-Cl (pH 8), 20mM of EDTA (pH 8), 2% SDS and 10mg/ml of Proteinase K (Merck) and incubated at 55°C for at least 6 hours. The digest was then chilled on ice for 10 minutes and 250µL of a saturated salt solution added. After 5 minutes centrifugation at 13,000rpm, isopropanol was then added to the supernatant to precipitate the DNA. The DNA pellet was washed with 70% ethanol and resuspended in 50µL of 10mM Tris-Cl 1mM EDTA.

2.2.2 *mdx* PCR

All *mdx* and C57 mice were genotyped using a high resolution melt assay to ensure integrity of the strains (Trebbin & Hoey 2009).

2.2.3 IGF-1 PCR

In order to test for the presence of the IGF-1 transgene the following PCR reaction was performed. IGF-1 primers (Invitrogen) at a concentration of 10 µL were used in the reaction with the following oligo sequences (5' to 3'):

IGF-1 upstream TTC CTG ACA GTG TCT GTG

IGF-1 downstream GAG CTG ACT TTG TAG GCT TCA

Interleukin primers (Invitrogen) at a concentration of 10µM were used as a control gene to ensure that a false negative result did not occur. The oligo sequences for these primers were as follows:

IL-2A CTA GGC CAC AGA ATT GAA AGA TCT

IL-2B GTA GCT GGA AAT TCT AGC ATC C

The reagents used in the reaction were 10xThermopol buffer (NEB), 10mM dNTP, 25mM $MgCl_2$, and Taq polymerase (NEB). The cycling conditions were 94°C for 2 minutes, 94°C for 1 minute, 55° C for 2 minutes, 72°C for 3 minutes, then going back to the second step and repeating 31 times. The final step being 72°C for 10 minutes.

2.2.4 MyoD PCR

To detect the absence of the MyoD gene a PCR reaction was performed using 20µg/mL stock MyoD primers (Invitrogen) with the following oligo sequences (5' to 3'):

MyoDF1: 5'- CTT CTA TGA TGA TCC GTG TTT CGA C-3'

MyoDR1: 5'- CTG CAG ACC TTC GAT GTA GCG-3'

Neo and SJT primers (Invitrogen), with a stock concentration of 20µg/mL were used as a control gene to ensure that a false negative mouse did not occur. The oligo sequences for these primers were as follows:

Neo 1F 5'- TCG TGC TTT ACG GTA TCG CCG CTC CCG ATT -3'

SJT 61 5'- CCC AAG CTC CGC CCT ACT ACA CTC CTA TTG GCT TGA GGC G-3'

The reagents used in the reaction were; 10x magic buffer (1x consisting of 16.6mM ammonium sulphate, 67 mM TRIS pH8.8, 6.7mM $MgCl_2$,5mM beta-mercaptoethanol,

6.7μM EDTA), 10mM dNTP, 10mg/ml bovine serum albumin (BSA), dimethyl sulphoxide (DMSO) and Taq polymerase (NEB).

The cycling conditions were 93°C for 2 minutes, and then 30 times 93°C for 30 seconds, 57°C for 30 seconds, 65°C for 2 minutes. The final step consisted of 65°C for 10 minutes.

2.3 FUNCTIONAL EXPERIMENTS

Functional experiments performed in all studies involved the Langendorff perfused heart system as described below.

2.3.1 Mouse numbers for functional experiments age study

Functional experiments were conducted on *mdx* and C57 mice at 3, 6 and 12 months of age and the numbers are shown in Table 2.1.

Table 2.1 Mouse numbers for functional experiments age study

	3 months of age	**6 months of age**	**12 months of age**
mdx	n=5	n=7	n=5
C57	n=5	n=8	n=11

2.3.2 Mouse numbers and groups for functional experiments IGF-1 study

Functional experiments were conducted 6 and 12 month old mice. The resultant groups and mouse numbers are shown in Table 2.2.

Table 2.2 Mouse numbers and groups for functional experiments IGF-1 study

	6 months of age	**12 months of age**
IGF-1-/*mdx*	n=7	n=5
IGF-1+/*mdx*	n=7	n=12
IGF-1-/C57	n=8	n=11
IGF-1+/C57	n=5	n=5

2.3.3 Mouse numbers and groups for functional experiments MyoD study

All experiments were conducted on 3 month old mice. The resultant groups and mouse numbers used for functional experiments are shown in table 2.3.

Table 2.3 Mouse numbers and groups for Langendoff experiments MyoD

mdx	***mdx*:MyoD(-/-)**	**C57:MyoD(-/-)**
n=5	n=5	n=6

2.3.4 Langendorff Experiment

Mice were weighed and then anaethetised by intraperitoneal injection of sodium pentobarbitone solution (Boehringer Ingelheim) at a concentration of 100mg/kg. A

thoracotomy was performed and the excised heart was rinsed in Krebs physiological solution containing (mM): NaCl, 119; $NaHCO_3$, 22; KCl, 4.7; KH_2PO_4, 1.2; $CaCl_2.2H_20$, 2.5; $MgCl_2$,1.2; glucose, 11; sodium pyruvate, 10; EDTA, 0.5 with 10mM of 2,3-butanedione monoxime (BDM).

The aorta was isolated and immediately cannulated using dysilk veterinary suture on a 23 gauge needle attached to Langendorff apparatus. Lungs and the left atria were removed. An apical drain (made from polyethlylene tubing) was inserted into the left ventricle to prevent accumulation of fluid in the heart by thesbian veins. The heart was perfused retrogradely at a pressure of 80mmHg with Krebs at 37^oC and bubbled with carbogen ($95\%O_2$, $5\%CO_2$) to oxygenate and maintain a pH of 7.4. Data from hearts with a coronary flow greater than 5 mL/min were excluded from experimental analysis.

A polyvinyl chloride balloon attached to a physiological pressure transducer (MLT844 AD instruments, Castle Hill NSW, Australia) was inserted into the left ventricle. The balloon was connected to a water filled microsyringe and the volume of water was adjusted to achieve an end diastolic pressure of 10mmHg.

The heart was stimulated via a Grass SD9 stimulator set at 7Hz, 5ms pulse width, to achieve a heart paced at 424bpm.The heart was equilibrated at this pressure for 10 minutes and then function was recorded as mmHg, ensuring an end diastolic pressure of 10mmHg.

At completion of the experiment the heart was removed and weighed and tibial length was measured. The superior section of the ventricles were snap frozen in liquid nitrogen cooled

isopentane and stored at -80^{o}C until required for qRT-PCR experiments. The inferior sections of the ventricles were placed in Telly/Bouins for histological analysis.

2.4 HISTOLOGY

Histological analysis in all studies involved picrosirius red staining of ventricular sections of the heart.

2.4.1 Mouse numbers for histology

Histological analysis was performed on tissue from 5 mice per group for all studies.

2.4.2 Histological analysis

The inferior sections of Langendorff perfused hearts were placed in Tellys fixative (100mL 5% ethanol, 5mL glacial acetic acid and 10mL of 37% formaldehyde) for three days and in a modified Bouins solution (85mL saturated picric acid, 5mL glacial acetic acid and 10mL 37% formaldehyde) for one day. The heart sections were washed once a day in 70% ethanol for three days, and then stored in 70% ethanol.

The heart sections were then embedded in paraffin wax and 7µm sections cut on a Reichert-Jung Biocut 2035 microtome and floated onto microscope slides (Menzel-Glaser polysine coated slides). The slides were stained with the collagen selective stain, 0.1% w/v picrosirius red (Sirius red F3B, Chroma dyes, in saturated picric acid) and visualised with a Nikon eclipse E600 microscope. The images were captured with a cooled CCD digital camera (micropublisher 5.0, OImaging, Canada), with a magnification of x40.

Percentage collagen was quantified using analySIS software (Soft imaging system,

GmbH Munster, Germany). Five regions of the section, involving left and right ventricles, were viewed and the average percentage collagen determined by colour separation density analysis.

2.5 RNA ANALYSIS

RNA analysis of TGF-β, procollagen and fibronectin gene expression were performed in each study.

2.5.1 Mouse numbers and groups for RNA analysis

RNA analyses were performed on tissue from 5 mice per group for all studies.

2.5.2 RNA Extractions

The superior ventricular sections, stored at -80 ^{o}C after Langendorff experiments, were sectioned with a cryostat at 6μm thickness. The cryostat-sectioned tissue samples were lysed in 150μL of TRIzol reagent, homogenised and volume made up to 800μL with TRIzol. After passing the sample through a 26 gauge needle, 160μL of chloroform was added and the samples centrifuged at 12,000g for 10 minutes at 4°C. The aqueous layer containing the RNA was decanted and RNA was precipitated by adding 400 μL of isopropyl alcohol. The RNA pellet was washed with RNase free ethanol, dried and then resuspended and treated with RNA aqueous kit (Ambion, Austin, Texas).

The RNA was quantified using Pharmacia Biotech Gene Quant II and a 50ng/μL stock prepared. To ensure that the RNA was not contaminated with DNA, a genomic PCR was run. β-actin F561 and β-actin R688 primers (Invitrogen) were used at a concentration of 10μm each, 10x NEB buffer, 10mM dNTP, 25mM $MgCl_2$, and Taq polymerase (NEB). The

following cycling conditions were used: 94°C for 2 mins x1; 94°C for 30 secs, 56°C for 1min and 72°C for 2 mins x 30; and finally held at 11°C.

2.5.3 Preparation of cDNA

cDNA was prepared from the extracted RNA using Super Script III First Strand Synthesis SuperMix and incubated at 25°C for 10 mins, 50°C for 30 mins and 85°C for 5 mins.

2.5.4 mRNA RT-PCR Reaction

The primers (Genebank) shown in Table 2.4 were used at a concentration of 200nM with β-actin serving as a housekeeping gene.

Table 2.4 Primers used in qRT-PCR

Gene	**Forward Primer**	**Reverse Primer**
TGF-β	CCG CAA CAA CGC CAT CTA TG	CTC GCA GGG ACA GCA AT
TNF-α	CCC TCA CAC TCA GAT CAT CTT CT	GCT ACG ACG TGG GCT ACA G
β-actin	CCC AGA TCA TGT TTG AGA CCT	CCA TCA CAA TGC CTG TGG TA
Fn1	TGT GAC AAC TGC CGT AGA CC	GAC CAA CTG TCA CCA TTG AGG
Col α1	GCA AAG AGT CTA CAT GTC TAG	CCT ACA TCT TCT GAG TTT GG

The reaction included SYBER GreenER^{TM}qPCR SuperMix Universal mix, and a 1 in 5 dilution of cDNA. The Rotor-Gene real time PCR thermocycler was used with the following cycling conditions: 50°C for 2 mins, 95°C for 10 mins, and 40 cycles of 95°C for 15 secs and 60°C. Rotor-Gene software analysed results using the following formula: Pfaffl formula $R=E_{target}^{\Delta CP\ target} / E_{reference}^{\Delta CP\ reference}$

2.6 Statistical analysis

All data is presented as mean ± SEM, comparisons made using the unpaired students t-test and ANOVA. $P<0.05$ was considered statistically significant.

CHAPTER 3: EFFECT OF AGE ON CARDIAC FUNCTION IN *mdx* MICE

3.1 INTRODUCTION

DMD boys usually die before their third decade of life due to respiratory or cardiac complications (Eagle et al. 2002). Although improved respiratory care has increased the life expectancy of DMD patients, cardiomyopathy still results with 20% of DMD and 50% of BMD patients dying from cardiac complications (Finsterer & Stollberger 2003). DMD associated cardiomyopathy is progressive with clinical symptoms first evident in patients at 10 years of age, and present in all patients over 18 years (Nigro et al. 1990).

The *mdx* mouse is the best characterised mouse model for DMD. The effects of dystrophin deficiency on the skeletal muscles of the *mdx* mouse have been well characterised, however there are fewer studies documenting cardiac involvement.

The *mdx* mouse has been shown to exhibit abnormal adrenoreceptor responses (Lu & Hoey 2000b), calcium channel dysfunction (Williams & Allen 2007) and increased fibrosis (Cohn et al. 2007; Quinlan et al. 2004; Van Erp, Irwin & Hoey 2006). Electrocardiographic differences observed in the *mdx* mouse include tachycardia, decreased heart rate variability and increased heart rate at 10 -12 weeks of age (Chu et al. 2002), and older *mdx* mice (42 weeks) exhibit dilated cardiomyopathy (Quinlan et al. 2004).

Due to the prominent role cardiomyopathy plays in the morbidity and mortality of DMD patients, cardiac involvement in the *mdx* mouse needs to be further characterised. As cardiomyopathy in DMD patients is progressive, a temporal study on cardiac involvement in the *mdx* mouse is required to serve as a preliminary study for establishing a baseline for future studies utilising transgenic models.

In order to evaluate the progression of cardiac involvement in the *mdx* mouse and to optimise the targeting of potential DMD therapies, this initial study concentrated on the time frame of pathology. The study aimed to evaluate age related changes in cardiac morphology, function, fibrosis, and gene expression of TGF-β, procollagen and fibronectin in *mdx* compared to C57 control mice. Age groups of 3, 6 and 12 months were chosen to enable observation of cardiac involvement and progression with age. These studies also provided quantitative data regarding *mdx* and C57 mice for later comparisons with novel transgenic strains such as IGF-1 cardiac overexpression dystrophic mice and MyoD deficient *mdx* mice (*mdx*:MyoD(-/-)). Quantitative data was established for the first time for mRNA levels of TGF-β, procollagen, fibronectin in the *mdx* heart. In addition these preliminary studies enabled expertise to be gained in *ex vivo* experiments measuring cardiac function, molecular techniques and histological assessment of fibrosis.

3.2 RESULTS

3.2.1 Morphology

At 3 and 12 months of age *mdx* mice exhibited a higher heart weight normalised to tibial length (mg/mm) than age matched C57 mice ($P< 0.05$). Normalised heart weight was highest at 3 months for *mdx* mice, compared to 6 and 12 months of age ($P<0.05$) (see Table 3.1 and Figure 3.1) Tibial length was not significantly different between *mdx* and C57 mice at 3 months of age, however heart weights were significantly higher (Table 3.1) resulting in a higher heart weight/tibial length ratio in *mdx* mice.

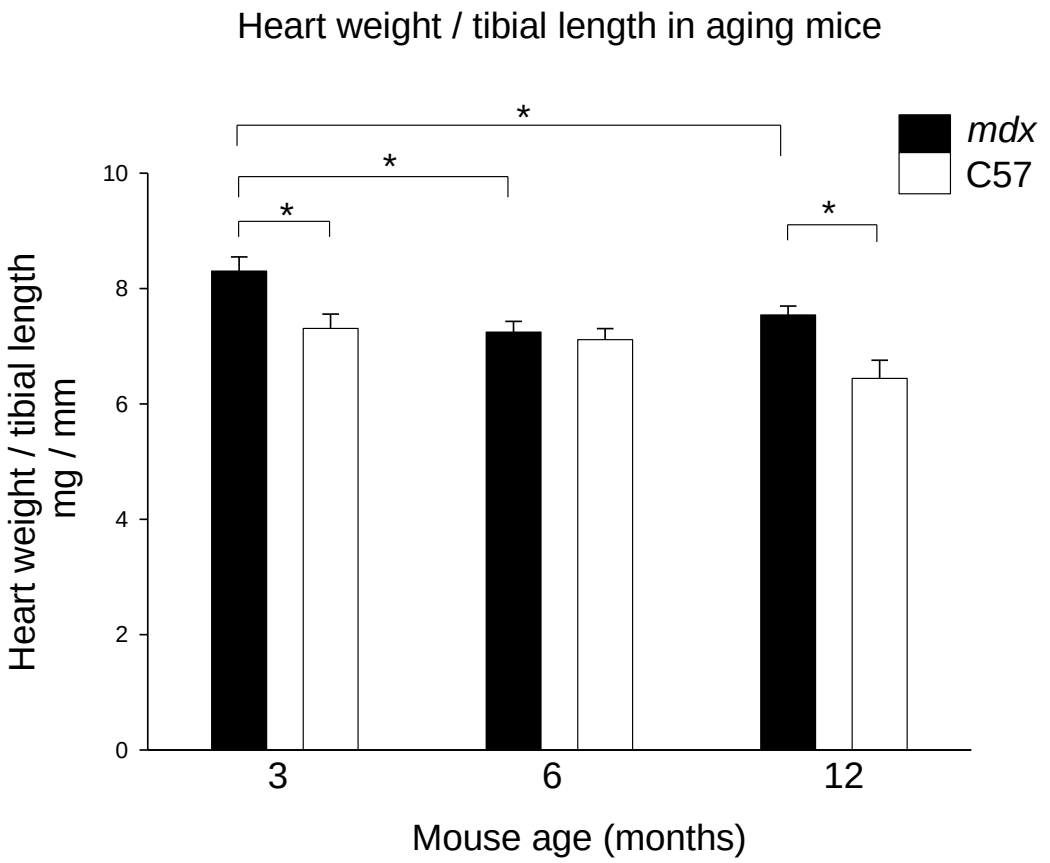


Figure 3.1 Morphometry data from *mdx* and C57 mice at 3, 6 and 12 months of age. Heart weight (gm) normalized to tibial length (mm). Heart weight normalised to tibial length was significantly greater for *mdx* mice at 3 months compared to 6 and 12 month old *mdx* mice, and compared to age matched controls. In addition, normalised heart weight of *mdx* mice at 12 months of age was significantly greater than age matched controls. (* P<0.05, **P<0.01, ***P<0.001)

3.2.2 Cardiac function

Ex vivo cardiac function, measured via Langendorff mounted hearts as left ventricular developed pressure in mmHg, was not significantly different between *mdx* mice hearts and C57 mice at 3, 6 and 12 months of age (see fig 3.2).

Rate of contraction, measured as a positive change in pressure (+dp/dt in mmHg/second) decreased in *mdx* mice by 36% at 6 months of age compared to 3 month old *mdx* mice ($P<0.001$) (see fig 3.3A).

Rate of relaxation, measured as a negative change in pressure (–dp/dt in mmHg/second) decreased in 6 month old *mdx* mice compared to 3 month old *mdx* mice ($P<0.001$). It also decreased in 12 month old *mdx* mice compared to 3 month old *mdx* mice ($P<0.05$).
C57 mice also showed a decrease in rate of relaxation at 6 and 12 months of age compared to 3 months ($P<0.01$). These results are shown in Figure 3.3B.

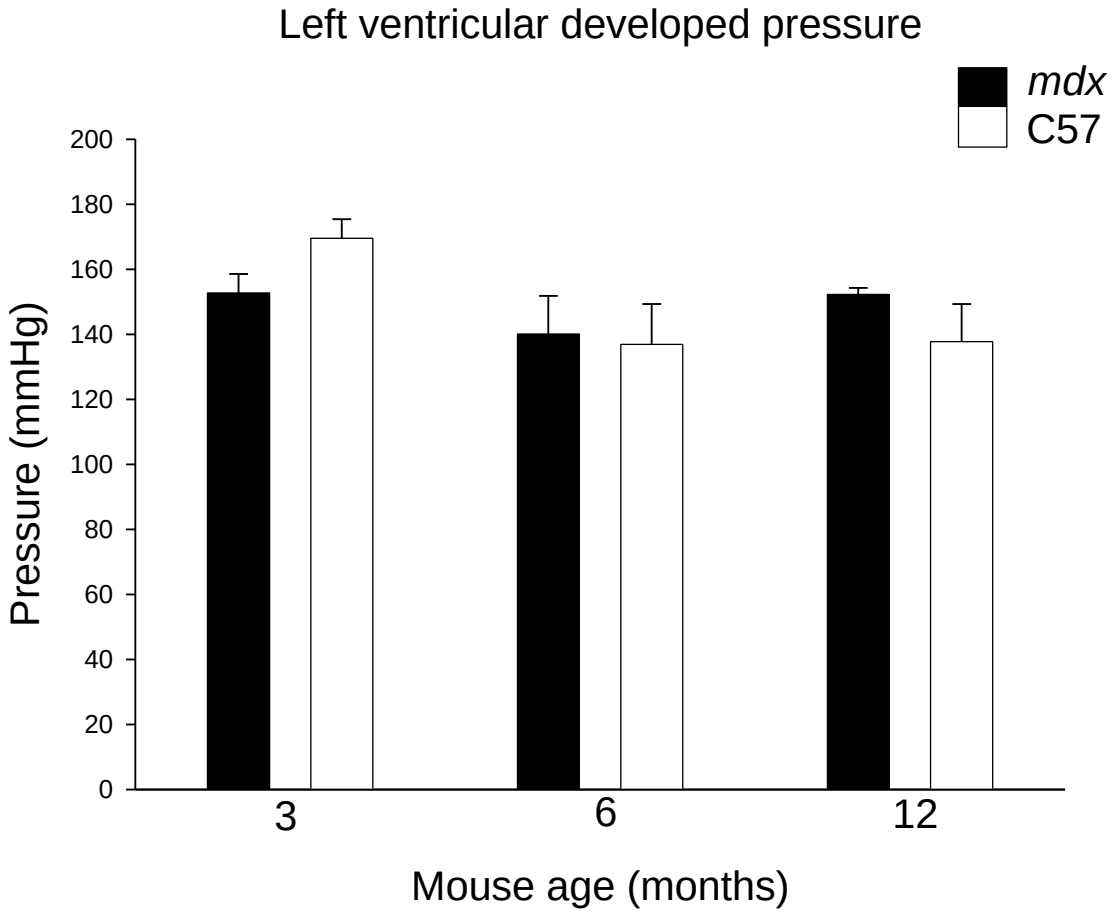
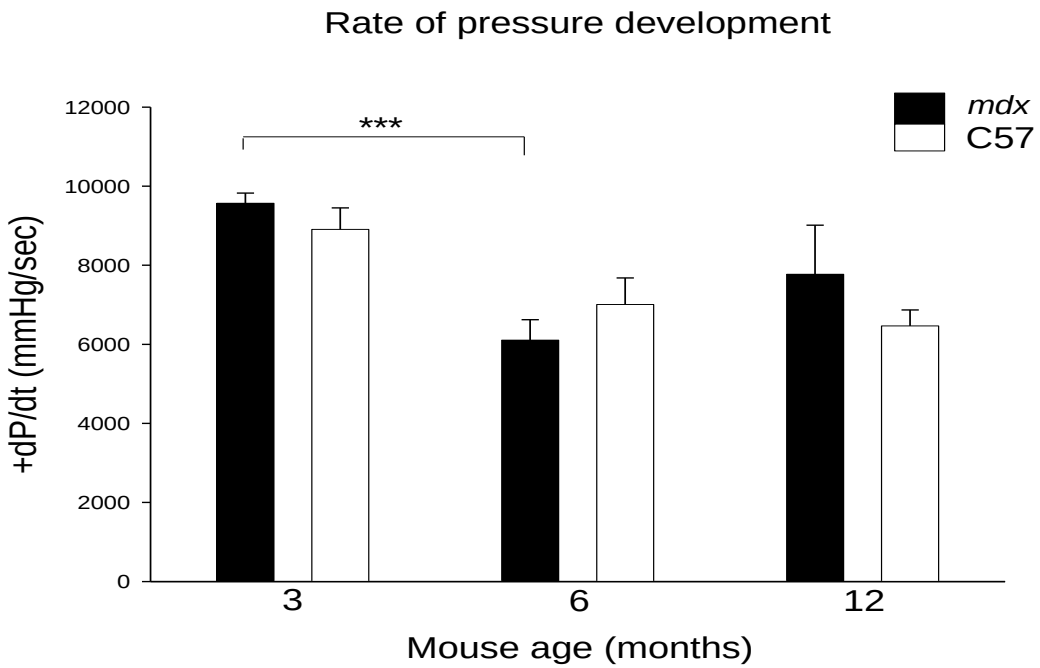


Figure 3.2 Cardiac function of *mdx* and C57 mice at 3, 6 and 12 months of age

Left ventricular developed pressure is not significantly different between *mdx* and C57 mice at 3, 6 and 12 months of age.

A.



B.

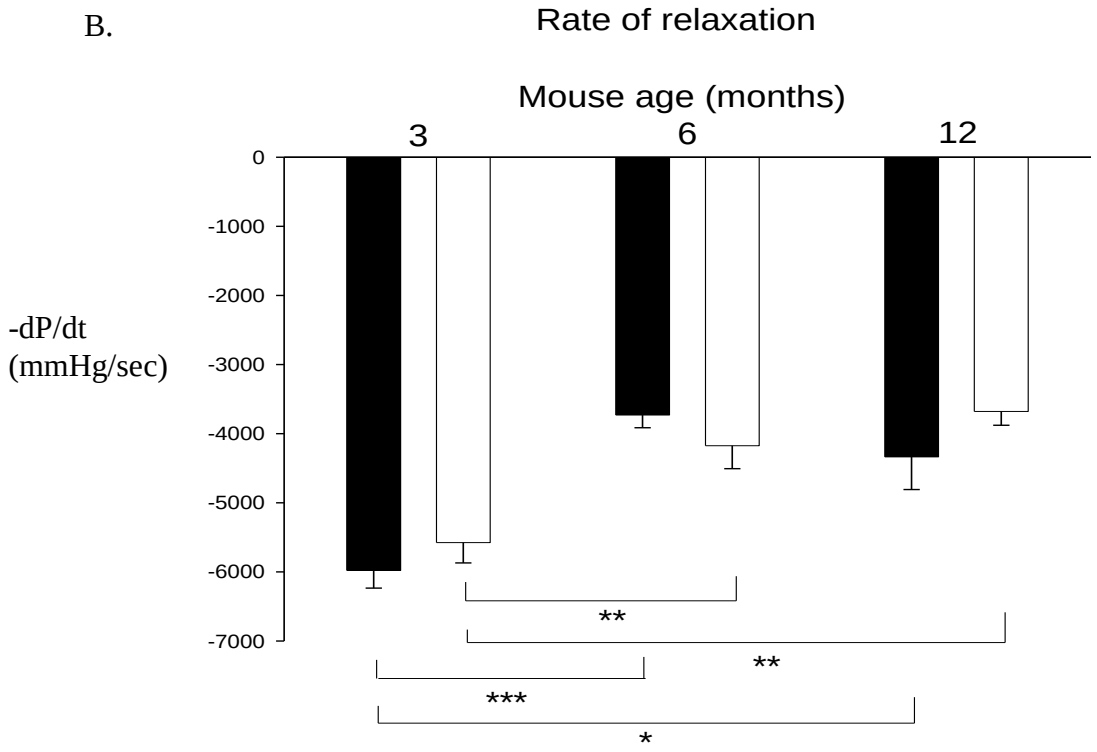


Figure 3.3 Rate of pressure development and rate of relaxation in *mdx* and C57 hearts at 3, 6 and 12 months of age. Contraction measured as rate of pressure development (+dP/dt) mmHg/sec (A), and rate of relaxation (-dP/dt) mmHg/sec (B). The rate of contraction was significantly greater at 3 months of age for *mdx* mice than at 6 months. Rates of relaxation were also greater for 3 month old *mdx* and C57 mice compared to older mice. (* P<0.05, **P<0.01, ***P<0.001)

3.2.3 Ventricular fibrosis

Mdx mouse hearts display 72-90% greater ventricular fibrosis at all ages compared to age matched C57 mice ($P<0.01$ and $P<0.001$ respectively) as shown by picrosirius red staining. There was no significant difference in the amount of cardiac fibrosis seen between *mdx* mice at 3, 6 and 12 months of age (see Figure 3.4 and representative photomicrographs Figure 3.5).

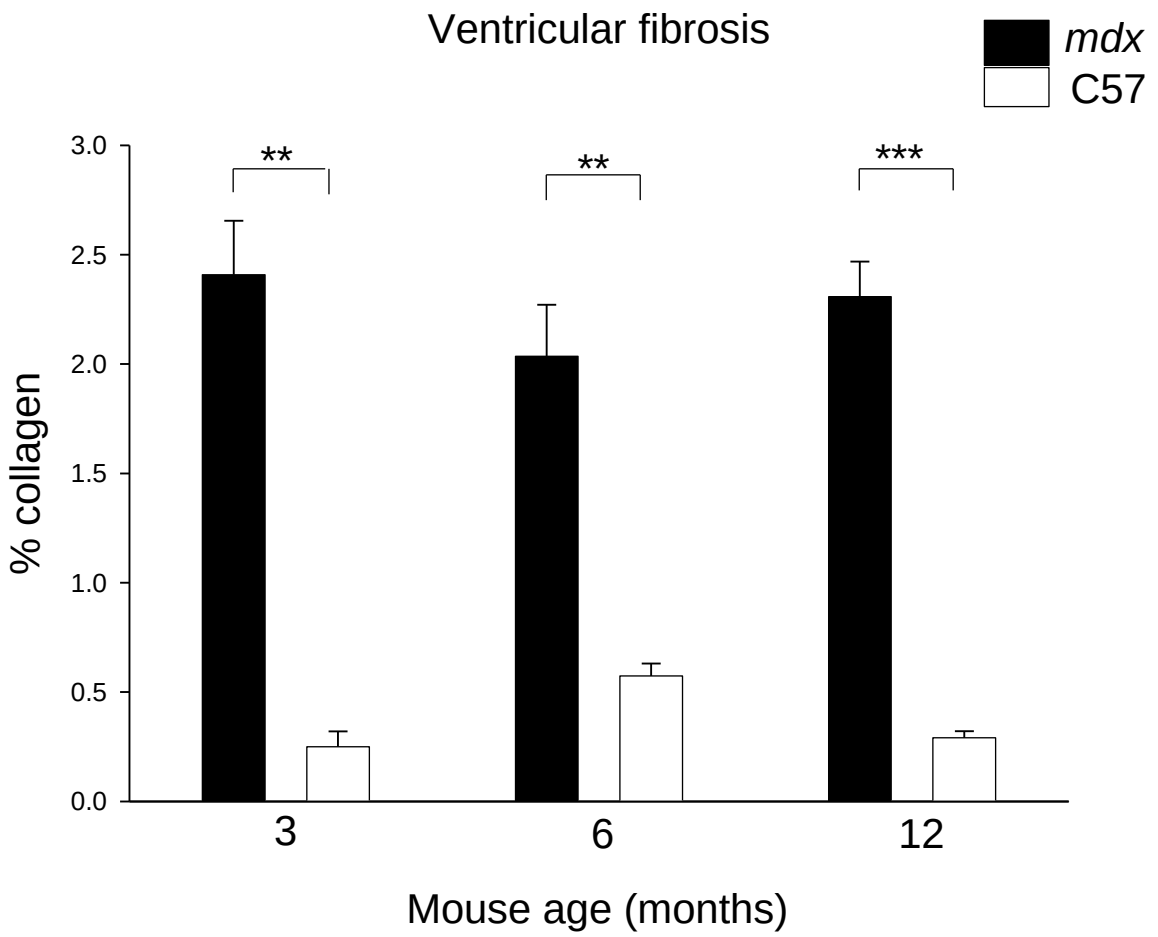


Figure 3.4 Ventricular fibrosis (expressed as % collagen) of *mdx* and C57 mice at 3, 6 and 12 months of age. At each of the ages, *mdx* mice exhibited significantly increased ventricular fibrosis compared to age matched controls, however *mdx* ventricular fibrosis did not increase significantly in the ages examined. (* P<0.05, **P<0.01, ***P<0.001)

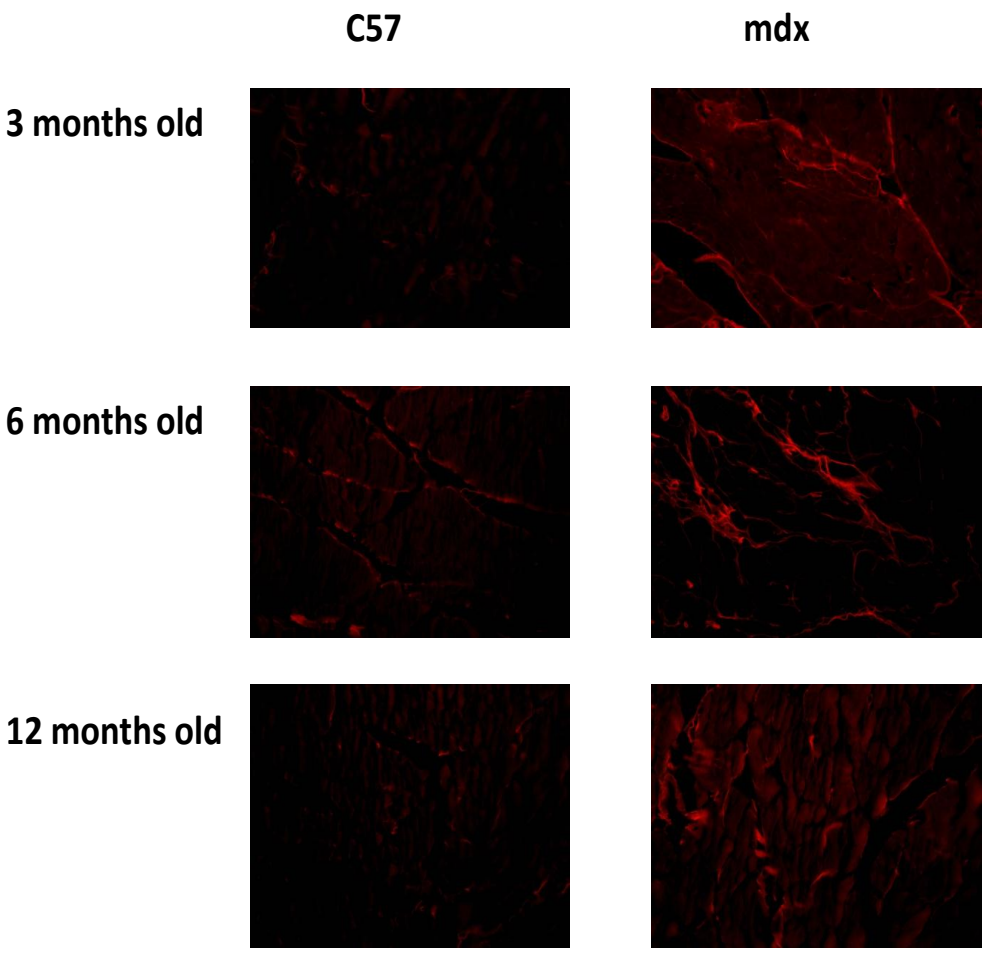


Figure 3.5 Representative photomicrographs of ventricular sections of C57 and *mdx* hearts at 3, 6 and 12 months of age (picrosirius red stained). Magnification x40. *Mdx* hearts exhibit significantly more fibrosis than C57 at all ages examined.

3.2.5 Gene expression of TGF-β, procollagen and fibronectin

Cardiac gene expression of TGF-β, relative to β-actin, decreased in *mdx* mouse at 12 months of age compared to 3 months ($P<0.001$). TGF-β expression of *mdx* mouse hearts compared to age matched C57 mice was not significantly different at 3 and 6 months of age, but by 12 months of age *mdx* mice showed less expression than 12 month old C57 mice ($P<0.05$) (Figure 3.6).

Cardiac gene expression of procollagen, also relative to β-actin, significantly increased in *mdx* mouse hearts at 6 months of age compared to 3 months ($P<0.001$) and then decreased at 12 months of age compared to 6 months ($P<0.01$). Procollagen gene expression was significantly increased in *mdx* mice at 6 months of age compared to C57 mice at that age ($P<0.01$), but at 12 months of age there was no significant difference between both strains (Figure 3.7A).

Cardiac gene expression of fibronectin, also relative to β-actin, was greater in *mdx* mouse hearts at 6 months of age compared to *mdx* mice at both 3 months of age ($P<0.001$) and 12 months of age ($P<0.005$). *Mdx* fibronectin mRNA at 6 months was significantly higher than age matched C57 mice, but at 12 months of age there was no significant difference in expression of fibronectin between *mdx* and C57 mice (Figure 3.7B).

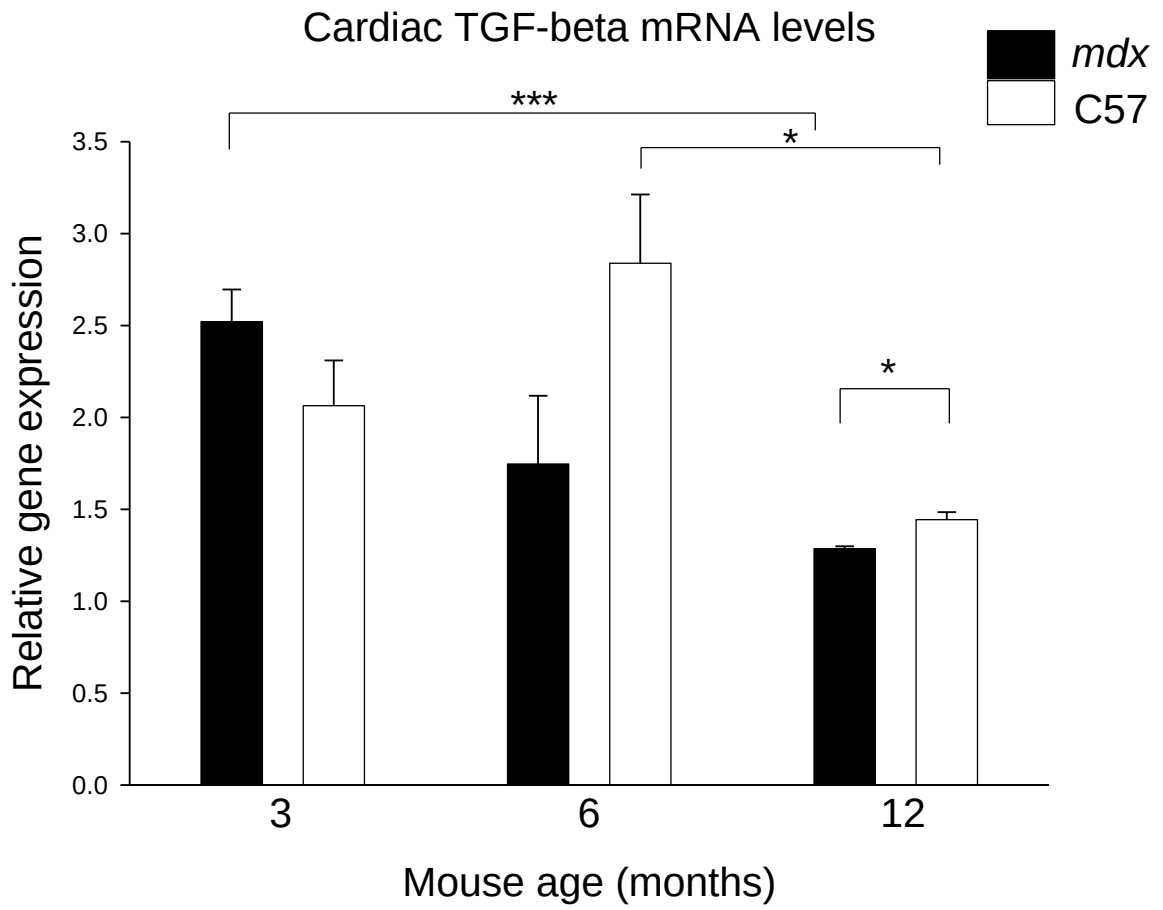


Figure 3.6 Cardiac gene expression levels of TGF-β mRNA as determined by quantitative real time PCR relative to β-actin for *mdx* and C57 mice at 3, 6 and 12 months of age. mRNA levels of TGF-β were significantly higher for 3 month old *mdx* mice compared to 12 month old *mdx* mice. 12 month old *mdx* mice also displayed significantly less TGF-β than age matched control. (* P<0.05, **P<0.01, ***P<0.001)

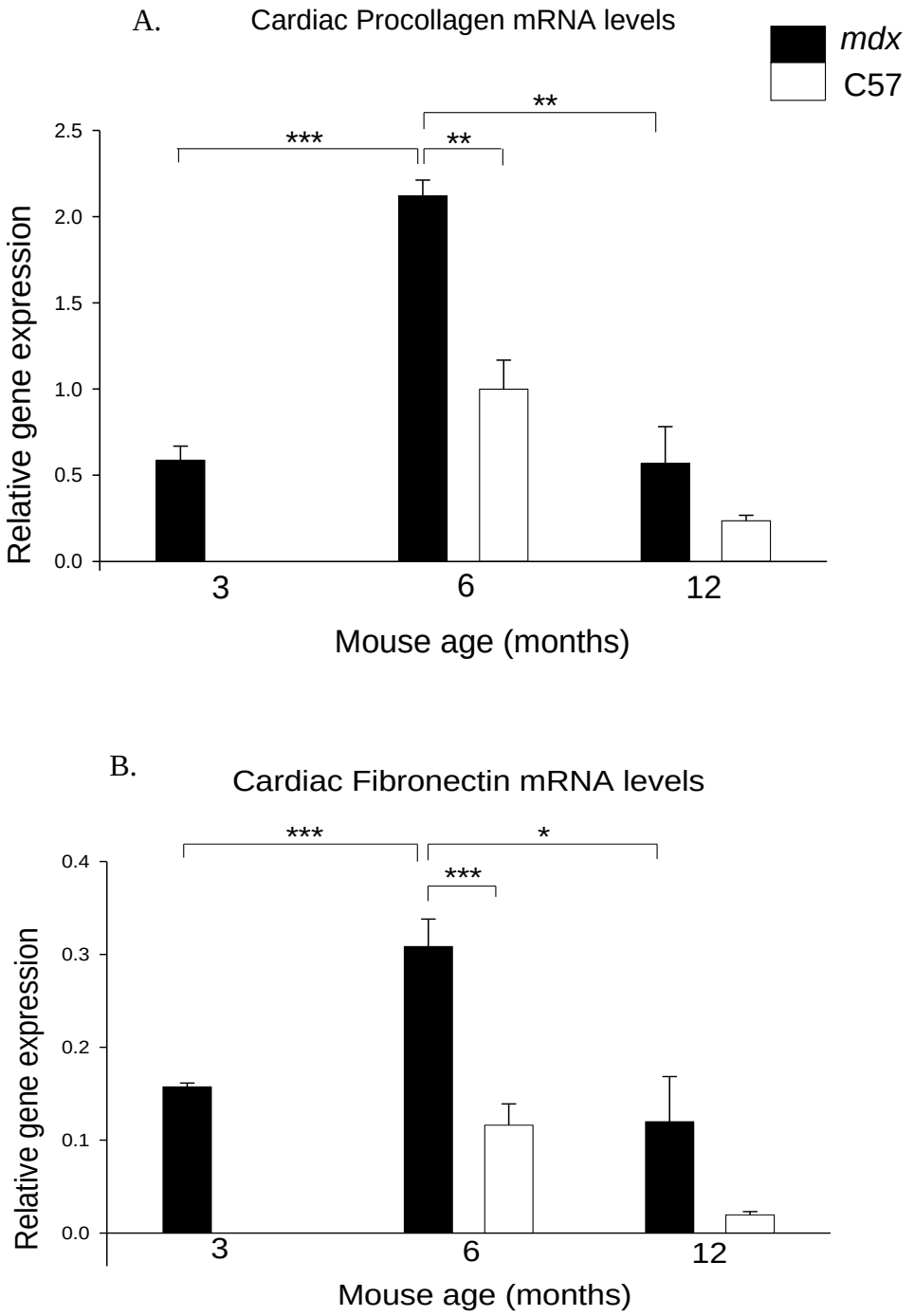


Figure 3.7 Cardiac gene expression of procollagen and fibronectin determined by quantitative real time PCR relative to β-actin of *mdx* at 3, 6 and 12 months of age and C57 at 6 and 12 months. Data for procollagen and fibronectin was not available for control mice at 3 months of age due to time constraints. mRNA levels of procollagen and fibronectin were significantly higher for 6 month old *mdx* hearts compared to 3 and 12 month old *mdx*, and age matched control. (* P<0.05, **P<0.01, ***P<0.001)

3.3 DISCUSSION

Previous studies have demonstrated that the skeletal muscles of *mdx* mice exhibit an abrupt onset of necrosis at 21 days of age, peaking at 28 days (McGeachie et al. 1993). Sarcolemmal damage is evident at 22 days of age accompanied with high amounts of necrosis (Grounds & Torrisi 2004; McGeachie et al. 1993). In contrast, cardiac muscle has intact sarcolemma at 24 and 28 day old, with data from our laboratory showing that membrane damage did not occur until 3 months of age (Cornford-Nairn et al 2009 submitted for publication).

This early sarcolemmal damage appears to be crucial in the progression of the dystrophic pathology and is exacerbated by inflammatory cell and cytokine infiltration leading to necrosis and later fibrosis (Grounds & Torrisi 2004; Hodgetts et al. 2006). Indeed by 3 months of age the present study found cardiac fibrosis to be significantly increased in *mdx* mice compared to controls, an effect that was maintained through all ages. The higher heart weight to tibial length ratio observed at 3 months of age is due to increased heart weight (see table 1) and may reflect a compensatory hypertrophy of the heart as the *mdx* mouse at this age has come out of a period of widespread skeletal muscle necrosis. In support of this study's findings, an increase in left ventricular mass at 2 months of age in *mdx* mice has been reported by others (Quinlan et al. 2004).

By 6 months of age normalised heart weights between *mdx* mice and age matched C57 were not significantly different and this has been observed by others (Danialou et al. 2001; Yue et al. 2004). This may reflect a stabilisation of growth after the peak phase of degeneration and regeneration (McGeachie et al. 1993).

At 12 months of age, *mdx* mice had a higher heart weight/tibial length ratio, due to an increased heart weight, compared to control mice. Indeed another study has shown an increased left ventricular mass of in *mdx* mice at 42 weeks (Quinlan et al. 2004). This cardiac hypertrophy does not appear to be a physiological adaptation but rather a pathological one, as functional data from this study showed no improvement in function or contraction, and a decrease in rate of relaxation. This pathological hypertrophy may possibly be either a compensatory mechanism due to the extra work load placed on the *mdx* mouse heart by the deterioration of skeletal muscles or due to the lack of dystrophin in the cardiomyocytes, or both.

Cardiac function has been shown to be normal in young *mdx* mice, progressing to a dilated cardiomyopathy by 42 weeks of age, with decreases in fractional shortening and heart rate, increases in left ventricular end diastolic and systolic diameter and calculated left ventricular mass (Quinlan et al. 2004). Cardiac dysfunction is not evident in *mdx* mice until 15 months of age when left ventricular pressure was seen to decrease by 26% (Van Erp, Irwin & Hoey 2006). The present study partly supports these findings with *ex vivo* measures of cardiac dysfunction in the *mdx* mouse heart not evident at the three age groups examined. At 6 month of age, however, *mdx* hearts displayed a decreased rate of relaxation and contractility, and at 12 months of age a decreased rate of relaxation with a trend to decreased contractility was also observed. These results suggest that at 3 months of age fibrosis and hypertrophy of the heart doesn't impact on cardiac function, however by 6 months of age the fibrosis is associated with diastolic dysfunction.

The compromised diastolic function is thought to be due to cardiac fibrosis which impairs the re-lengthening of cardiomyocytes during the diastolic phase of the cardiac cycle (Vasan &

Benjamin 2001; Weber et al. 1989). In addition to this, diastolic dysfunction has been associated with an increased activity of matrix metalloproteinases (MMP-2 and MMP-9), which are involved in matrix remodelling (Li et al. 2000). MMPs have been found to be upregulated in DMD (Pescatori et al. 2007; von Moers et al. 2005) and *mdx* mice (Porter et al. 2003).

Fibrosis was increased dramatically in *mdx* mouse hearts by between 72-90% at each age group compared to control mice. This finding is consistent with previous *mdx* mouse studies (Buyse et al. 2009; Quinlan et al. 2004; Van Erp, Irwin & Hoey 2006). A progression in cardiac fibrosis with age was not observed in this current study, with values between the age groups showing no significant difference. Although a progression in fibrosis has been shown to occur in other studies, it occurred after 12 months of age (Cohn et al. 2007).

The correlation between cardiac fibrosis and dysfunction is unclear. Cardiac expression of a mini dystrophin gene was capable of restoring the histology of *mdx* mouse hearts but not the cardiac function (Bostick et al. 2009).

Fibrosis may affect the electrical conduction between myocytes, and some ECG abnormalities found in *mdx* mice hearts (such as altered PR and QRS intervals and R waves) are thought to be due to increased fibrosis (Bostick Yue Long et al. 2008). In DMD patients ECG abnormalities have been attributed to fibrosis of the conduction system and/or autonomic nervous system (Finsterer & Stollberger 2003).

The mechanism leading to cardiac fibrosis is thought to be partially mediated by the inflammatory cytokine TGF-β (Khan & Sheppard 2006). TGF-β is a potent stimulator of

collagen-producing cardiac fibroblasts, and it was expected that its levels would increase in *mdx* hearts, reflecting increased fibrosis. While it is acknowledged that gene expression does not necessarily reflect protein transcription and translation, the increased fibrosis displayed in *mdx* mouse hearts compared to C57 control mice at all ages, was not reflected in TGF-β mRNA values. There was no significant difference in levels of this cytokine between *mdx* mice compared to age matched controls except at 12 months of age where C57 TGF- β mRNA levels were significantly higher than *mdx*. This lack of correlation in ventricular fibrosis and TGF- β mRNA levels is supported by previous work from our laboratory in 15 month old *mdx* mouse hearts showing no difference in levels of TGF-β mRNA compared to control mice despite increased levels of ventricular fibrosis (Van Erp, Irwin & Hoey 2006).

In this current study, however *mdx* hearts from younger ages (3 months) exhibited higher levels of TGF-β mRNA than 12 months old *mdx* mice with these 12 month old *mdx* mice also exhibiting a decreased expression compared to age matched controls. These results suggest that TGF-β may be involved in the initiation of profibrotic pathways, but it does not appear to be the main driver in the ongoing progression of fibrosis. In support of our findings, another study on the *mdx* diaphragm found that TGF-β mRNA levels peaked at 6 weeks of age, but not at 9 or 12 weeks of age (Gosselin et al. 2004). This study also suggested that TGF-β may be involved in initiation of the fibrotic pathway. The age of the *mdx* mouse where TGF-β mRNA peaked in the diaphragm was a considerably younger age than the 3 months used in this study, however previous studies from our laboratory have found no significant heart pathology at 21, 24 or 28 days (as measured by EBD leakage, macrophage infiltration or immunostaining), possibly indicating minimal involvement of cytokines in the heart at those ages. As sarcolemmal fragility and EBD leakage is first evident at 3 months of age in the *mdx* heart, this would be followed by the inflammatory response and therefore the increased TGF-

β mRNA levels seen in this study at 3 months of age may reflect the early involvement of TGF-β in initiating cardiac profibrotic pathways. It appears from this current cardiac study and previous skeletal muscle studies that there is a temporal difference between skeletal and cardiac TGF-β mediated pathways.

TGF-β levels in DMD boys shows an early increase up to 6 years of age, then declining to very low levels despite fibrosis continuing to increase (Bernasconi et al. 1995). The link between TGF-β mRNA levels and increased fibrosis is contentious. Another study, involving the anti-fibrotic drug pirfenidone, found that although it reduced TGF-β levels in the *mdx* mouse heart, improving cardiac function, it did not reduce fibrosis (Van Erp, Irwin & Hoey 2006). The mRNA expression of TGF-β also did not correlate with muscle fibrosis in *mdx* quadriceps and diaphragm (Zhou et al. 2006). Contradictory to these studies, TGF-β protein content has been shown to correlate with collagen deposition in the human myocardium (Li, McTiernan & Feldman 2000). This current study found that although fibrosis continued at 12 months of age in *mdx* mouse hearts, TGF-β levels decreased (compared to age matched controls and 3 month old *mdx* mice).

There are several possible explanations to account for the lack of correlation between TGF-β mRNA levels and fibrosis observed in this and other studies. It has been suggested that once profibrotic pathways have been established other mediators or autocrine processes take over (Bernasconi et al. 1995). As the sum balance of collagen deposition is a function of both synthesis and degradation, increased fibrosis observed in older *mdx* mice and DMD patients may not be due solely to TGF-β involvement in the synthesis of collagen, but rather a decreased rate of its degradation. TGF-β has been shown to inhibit matrix degrading proteases such as matrix metalloproteinases (MMPs) that are involved in the breakdown of

the ECM (von Moers et al. 2005). In particular MMP1 is involved in the breakdown of fibrillar proteins and MMP2 degrades type IV collagens. Increased levels of MMP2 and tissue inhibitors of matrix metalloproteinase 2 (TIMP2) leads to the formation of the large inhibitor of matrix metalloproteinases (LIMP) which in turn inhibits MMP1 (Kolkenbrock et al. 1991).

Gene expression profiling in the early stages of DMD show an increase in MMP2, TIMP1 and TIMP2 levels in DMD (Pescatori et al. 2007). DMD patients from 2 to 13 years of age also showed increased mRNA levels of MMP-2, TIMP-1 and TIMP-2 (von Moers et al. 2005). In the skeletal muscles of *mdx* mice several MMPs, and TIMP 1 and 3 show increased gene expression (observed at a later stage from 23 – 112 days of age) (Porter et al. 2003).

Two consequences of these increases are: (i). the metabolic effects of increased MMP-2 on collagen breakdown would be negated by increased transcription of its inhibitor, TIMP-2 and (ii). increased MMP-2 and TIMP-2 would result in increased generation of LIMP. The inhibition of MMP-1 by LIMP would be compounded with increased TIMP-1, resulting in reduced degradation of fibrillar collagens. In addition the inhibition and complex formation of MMP-2 would prevent it from degrading denatured collagens (von Moers et al. 2005).
A disturbance in the balance between MMPs and TIMPs has been shown to contribute to liver fibrosis (Iredale 1997) and myocardial fibrosis (Li, McTiernan & Feldman 2000). The activity of MMPs has been shown to be increased, and TIMPs decreased in the failing human heart (Li, McTiernan & Feldman 1999; Thomas et al. 1998). Increased MMP2 and MMP9 activity has also been shown to result in cardiac dilation and diastolic dysfunction in mice with cardiac restricted overexpression of TNFα (Li, et al. 2000).

Signalling pathways responsible for regulating MMP expression have not been clearly defined, however they are regulated at pre- and post-transcriptional levels by growth factors, cytokines (for example TNF-α, TGF-β) and matrikines (degraded products of matrix proteins) (Li, McTiernan & Feldman 2000). While TGF-β suppresses proteolytic activity through reduced proteinase synthesis and increased TIMP expression, it also increases MMP2 expression (Wahl et al. 1993).

Temporal gene expression analysis of skeletal muscle of the *mdx* mouse showed that the timing of induction of the molecular pathology differed from DMD (Porter et al. 2004). DMD patients exhibit altered expression of genes involved in the inflammatory response, ECM remodelling, muscle regeneration and energy metabolism conserved from presymptomatic patients (age <6 months) to older symptomatic patients (4-5 years of age) (Chen, et al. 2000; Pescatori et al. 2007). Disease progression does not appear to introduce changes in muscle gene expression profile. *Mdx* mice, however were characterised by minimal transcription alterations in the pre-necrotic phase (Porter et al. 2004). In the skeletal muscles of *mdx* mice it is not until the post necrotic phases that parallel changes in gene expression were observed, with approximately 67 % of the inflammatory genes induced in the *mdx* mouse displaying a similar pattern to DMD patients (Pescatori et al. 2007).

There is also a difference in transcription levels of the muscle genes that are expressed in different *mdx* skeletal muscles. For example collagen transcription is increased in the diaphragm at 112 days of age, whereas in the hindlimb it is decreased at that age (Porter et al. 2004). For this reason, gene profiling of a specific *mdx* skeletal muscle would not necessarily reflect cardiac muscle changes occurring at the same point.

In the hindlimb and diaphragm of *mdx* mice, levels of transcripts for the genes involved in the inflammatory response and ECM remodelling do not begin to increase until 23 days of age. Levels peaked at 50 days of age and then decreased again (Porter et al. 2004). Although observed changes in skeletal muscle do not necessarily reflect changes in cardiac muscle, the reported increased cardiac TGF-β levels at 3 months of age is occurring 40 days after the peak observed in the previous skeletal muscle study (Porter et al. 2004) thus further highlighting the temporal difference between skeletal and cardiac muscle in TGF-β mediated pathways. This skeletal muscle gene profiling study also supports the hypothesis that TGF-β is involved in initiating fibrotic pathways as inflammatory gene expression increased at just 23 days of age, peaking by 50 days and then decreasing (Porter et al. 2004).

Further elucidation of the complex interplay between collagen deposition and breakdown may be found by examining expression of other genes such as procollagen and fibronectin. Procollagen plays an important role in fibrosis as the precursor to collagen and fibronectin provides structural support for the formation of the fibrotic network. By 6 months of age *mdx* mice exhibited increased procollagen and fibronectin, compared to age matched controls and compared to 3 and 12 months old *mdx* mice. This may reflect active collagen deposition at 6 months compared to the other ages. The decreased levels at 12 months of age in the face of high fibrotic levels may reflect a shift in emphasis from active collagen synthesis to reduced collagen deposition to maintain the fibrosis.

Gene expression of procollagen and fibronectin has been shown to be upregulated in presymptomatic and symptomatic DMD patients (Chen et al. 2000; Pescatori et al. 2007). In skeletal muscles of *mdx* mice procollagen and fibronectin begin to increase at 23 days, peaking at 50 days, and then declining (Porter et al. 2003). The results of this study showed a

peak much later in life, reflecting again the temporal difference in levels of gene expression of fibronectin and procollagen between cardiac and skeletal muscle in the *mdx* mouse and the lack of correlation in the expression of these genes and the level of fibrosis.

Figure 3.8 summarises changes in skeletal and cardiac muscle of *mdx* mice across their lifespan, with particular emphasis on fibrosis, function and inflammatory gene response.

3.4 SHORTCOMINGS OF THE STUDY

One shortcoming of this study was the lack of procollagen and fibronectin data for C57 mice at 3 months of age. This deficit was due to time constraints placed on this research project and lack of available mice at that age.

Future research into the dystrophic changes in cardiac muscle in the *mdx* mouse would benefit from utilising younger age groups than used in this study and further *in vivo* experiments such as echocardiography and millar catheter. Ages under 3 months would enable the evaluation of early changes in cardiomyocytes and initiation of fibrotic pathways.

Due to the relentless nature of cardiac fibrosis associated with DMD it is imperative to further clarify the molecular pathways involved. In particular future research needs to address cardiac expression levels of MMPs and TIMPs as these proteases appear to be pivotal in collagen metabolism.

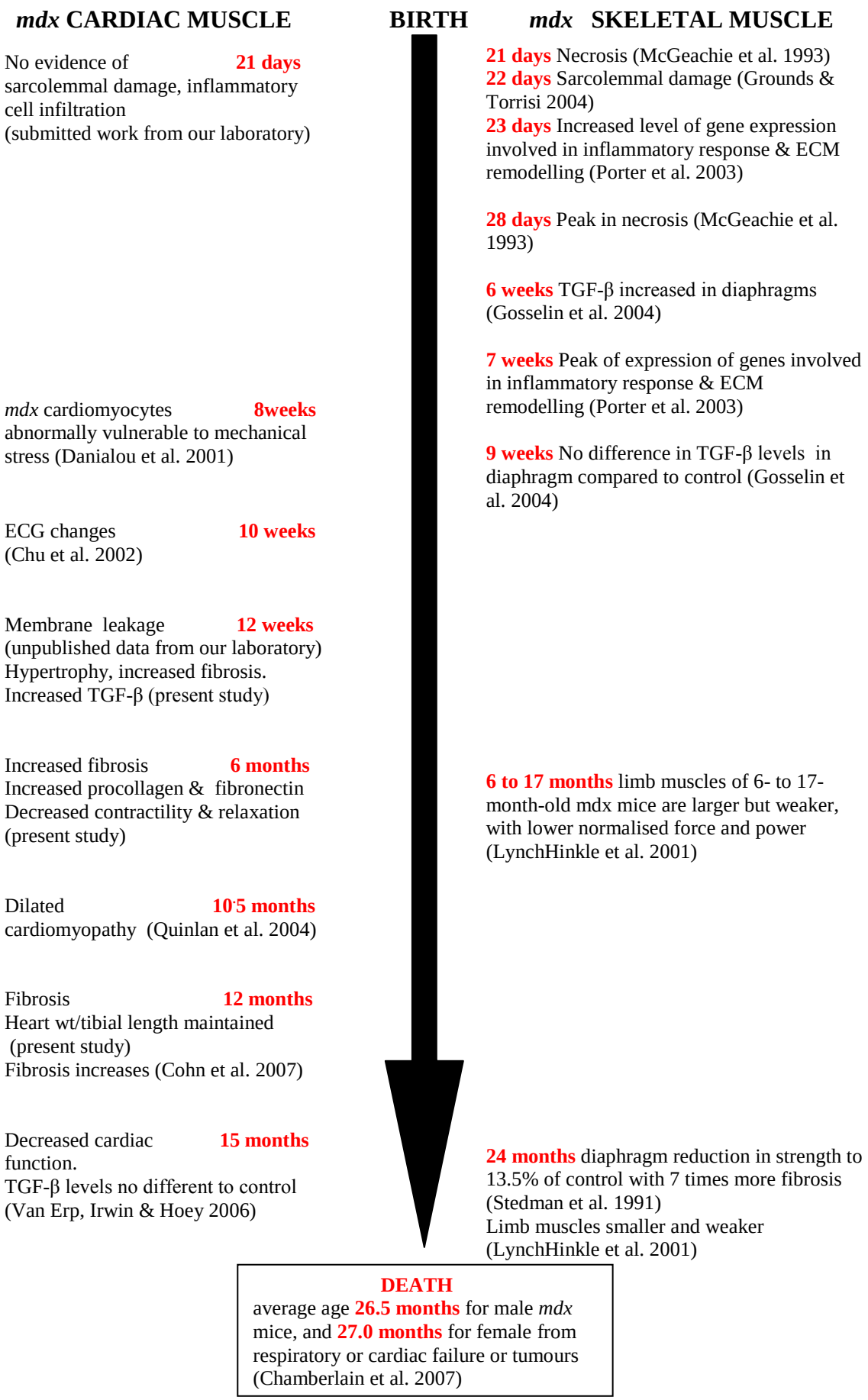


Figure 3.8 Timeline of dystrophic changes in skeletal and cardiac muscle in the *mdx* mouse.

3.5 CONCLUSION

This study determined the level of cardiac involvement of the *mdx* heart at 3, 6 and 12 months of age. At 3 months of age cardiac hypertrophy was observed which may reflect a compensatory mechanism as skeletal muscle comes out of a period of widespread necrosis. By 12 months of age the increased heart weight was accompanied by decreased diastolic function, suggesting a pathological hypertrophy.

Fibrosis was increased in the *mdx* heart at all the ages studied. While it did not affect LV developed pressure, by 6 months of age impaired rate of contraction and relaxation became evident. Decreased diastolic function was also observed at 12 months of age. As *mdx* mice at 15 months of age have been previously shown to exhibit impaired cardiac function (Van Erp, Irwin & Hoey 2006), these present results suggest a progression toward cardiac dysfunction commencing at 6 months of age.

Dystrophic fibrotic pathways are complex and have not been fully elucidated. This study highlights an involvement of TGF-β in the initiation of profibrotic pathways, a phenomenon which does not persist. This indicates that there may be a shift away from the initial increase in collagen synthesis, to a reduction in collagen degradation with age. Literature supports a role for MMPs and their inhibitors TIMPs in the decreased breakdown of the ECM.

There is a lack of cardiac data regarding genes involved in the remodelling of the ECM and this study provides novel data on fibronectin and procollagen mRNA levels in the *mdx* mouse heart. Both of these genes were found to be upregulated at 6 months of age in *mdx* hearts indicative of active collagen synthesis at this age. This increase correlates with high levels of ventricular fibrosis and the initial impairment in systolic and diastolic function observed. By

12 months of age the expression of these genes decreases even though levels of ventricular fibrosis are still significantly elevated. These results lend further support to the hypothesis of reduced rate of collagen degradation at older ages in the mdx mouse sustaining the high levels of fibrosis.

This study provides a useful baseline for the following experiments using novel transgenic mouse models. It also provided essential development of expertise in *ex vivo* cardiac function techniques, histological analysis of fibrosis and molecular techniques.

CHAPTER 4 OVEREXPRESSION OF IGF-1 PARTIALLY RESTORES FUNCTION IN THE *mdx* HEART

4.1 INTRODUCTION

DMD is a characterised by repetitive cycles of muscle damage and repair resulting in muscle wasting as the degenerative process overwhelms the capability of the muscle for regeneration (Moser 1984). After injury, muscle repair occurs in different phases: necrosis/degeneration and inflammation, regeneration and fibrosis. These appear to be interrelated (Huard, Li & Fu 2002).

Contraction induced damage results in necrosis, which is mediated by intrinsic proteases (Huard, Li & Fu 2002). Neutrophils invade the injured muscle and release free radicals and oxidants which cause lysing of the sarcolemma (Tidball 2005). The necrotic area is also infiltrated by small blood vessels, activated macrophages, mononuclear cells and T-lymphocytes (Huard, Li & Fu 2002). Activated lymphocytes secrete cytokines and growth factors. Cytokines, macrophages and other cellular components activate and attract fibroblasts into the area of injury (Mourkioti & Rosenthal 2005). Fibroblasts overproduce TGF-β and are thought to produce the majority of collagen. Collagen remodels the ECM, reduces vascularity and eventually replaces connective tissue with scar tissue (fibrosis) as reviewed by Prisk (Prisk & Huard 2003). Although fibrotic tissue provides an early support network for broken myofibres to reconnect, if it continues to increase it restricts the regenerative potential of the new myofibres by filling up space where new muscle fibres would normally be (Mourkioti & Rosenthal 2005).

IGF-1 has been shown to increase the regenerative capacity of dystrophic skeletal muscle by reducing the replacement of muscle fibres with fibrosis through modulation of the inflammatory response (Barton et al. 2002). Further, overexpression of IGF-1 in hepatic

stellate cells of cirrhotic rats showed attenuated fibrosis which was partly mediated by down regulation of TGF-β (Sanz et al. 2005). A recent study found IGF-1 gene transfer to cirrhotic livers improved liver function and reduced fibrosis which was associated with up regulation of MMPs and down regulation of TIMP-1 (Sobrevals et al. 2010).

Muscle cell regeneration involves the activation of satellite cells. Following muscle injury satellite cells proliferate, fuse with damaged myotubes, then differentiate and mature. IGF-1 is involved in the proliferation and differentiation of satellite cells and has been shown to extend their replicative life span (Chakravarthy et al. 2000). IGF-1 has also been shown to enhance bone marrow derived side population stem cells (Musaro et al. 2004). IGF-1 appears to be a crucial factor in mediating skeletal muscle growth (Florini, Ewton & Coolican 1996). It has an anabolic effect on skeletal muscle promoting muscle hypertrophy (Zdanowicz et al. 1995), and blocking age related loss of muscle mass and function (Barton-Davis et al. 1998).

Cardiac and skeletal muscles are markedly different in their postnatal capacity for cell replication during growth and regeneration (Grounds et al. 2002). Cardiac cells were previously thought to be incapable of replication after birth and there was thought to be no replacement with new cardiomyocytes after injury. Human studies, however, have shown that after myocardial infarction, there is a subpopulation of myocytes that are not terminally differentiated and do proliferate (Beltrami et al. 2001). The level of cardiomyocyte proliferation is low in the normal heart and increases with heart disease (Kajstura et al. 1998). It is unclear whether these proliferating cells are derived from resident cardiomyocytes or from circulating stem cells. One potential strategy for cardiomyocyte replacement after injury would therefore be to stimulate proliferation of these undifferentiated myocytes.

The subpopulation of undifferentiated myocytes in adult rat hearts exhibit all the properties expected for cardiac stem cells ie. self-renewing, clonogenic, and multipotent, and giving rise to a minimum of three differentiated cell types: myocytes, smooth muscle, and endothelial vascular cells (Beltrami et al. 2003). IGF-1 injected into a mouse heart after myocardial infarction promotes the survival and proliferation of cardiac stem cells (Beltrami et al. 2003).

Evidence suggests that IGF-1 plays an important role in cardiac regeneration and IGF-1 has been shown to increase cardiomyocyte replication (Reiss et al. 1996; Welch, S. et al. 2002). IGF-1 has also been demonstrated to play a protective role in the heart by preventing cardiomyocyte death (Li et al. 1997).

In the normal heart, hypertrophic growth (ie. enlargement of individual cardiomyocytes) is sufficient to maintain adequate cardiac function (Grounds et al. 2002). IGF-1 plays an important role in the hypertrophic response of the myocardium (Wang, P. H. et al. 1998). IGF-1 overexpressed or administered to the hearts of murine species induces cardiac hypertrophy (Duerr et al. 1995; Ito et al. 1993; Santini et al. 2007; Welch, S. et al. 2002). The hypertrophy mediated by IGF-1 appears to be a physiological adaptation as evidenced by an improved cardiac function as reviewed by Ren (Ren, Samson & Sowers 1999).

IGF-1 has been demonstrated to play a protective role in the myocardium by attenuating the renin angiotensin system. Overexpression of IGF-1 in the heart of the streptozotocin-induced diabetic rat reduced angiotensin II and apoptosis (Kajstura et al. 2001). Angiotensin has been implicated in collagen synthesis and degradation in various cardiomyopathies (Gonzalez et al. 2002).

While the inflammatory response is an integral component of muscle regeneration, it must be resolved to allow muscle repair (Pelosi et al. 2007). The ability of IGF-1 to resolve the inflammatory response and reduce fibrosis makes it a promising therapy for the dystrophic heart with its characteristic chronic inflammatory response and progressive fibrosis.

The first studies on IGF-1 overexpression in the skeletal muscle of *mdx* mice demonstrated that IGF-1 increased muscle mass and strength, decreased myonecrosis, and reduced fibrosis (Barton et al. 2002). The beneficial effects of IGF-1 on the heart has been assessed in normal rats (Cittadini et al. 1996), rats after myocardial infarction (Duerr et al. 1995), the δ-sarcoglycan deficient hamster (Serose et al. 2005), the diabetic rat (Kajstura et al. 2001), the tropomodulin-overexpression mouse (Welch, S. et al. 2002), patients with chronic heart failure (Donath et al. 1998), and healthy people (Donath et al. 1996). To date, however the effects of IGF-1 on the dystrophic heart have not been determined. This current study addressed this by generating transgenic mice with cardiac restricted overexpression of IGF-1 that have been crossed with *mdx* mice providing a novel animal model. These IGF-1/*mdx* mice offer a chronic model of injury, as opposed to the acute model of injury in the study by Santini and co-workers (Santini et al. 2007).

The aims of this current study were to ascertain whether cardiac overexpression of IGF-1 could improve function and ameliorate fibrosis in the dystrophic heart. Transgenic control mice that overexpressed cardiac restricted IGF-1 were also generated to compare any differences between the responses of normal cardiac muscle to dystrophic cardiac muscle. Levels of gene expression of TGF-β, procollagen and fibronectin were measured in an attempt to further elucidate profibrotic molecular pathways. Two age groups of 6 months and

12 months old were examined to determine the effect of IGF-1 overexpression with age of the animal.

4.2 RESULTS

4.2.1 Method for the generation of transgenic *mdx* and C57 mice overexpressing IGF-1 exclusively in the heart

Male IGF-1 mice were used which overexpressed the mIGF-1 isoform, comprising a Class 1 signal peptide and a C-terminal Ea extension peptide using an α myosin heavy chain (α-MHC) promoter to restrict overexpression exclusively to the heart. These animals were generously provided by Professor Miranda Grounds University of Western Australia. Figure 4.1 illustrates the rodent IGF-1 gene with the α-MHC promoter.

These transgenic mice were crossed with *mdx* and C57 female mice from the University of Southern Queensland Animal house. This F1 generation was genotyped for the presence of the IGF-1 gene as outlined in Section 2.2.3. Male mice positive for IGF-1 were then back crossed with *mdx* or C57 female mice. These mice were genotyped for IGF-1 to determine the four experimental groups, IGF-1+/*mdx*, IGF-1-/*mdx* and IGF-1+/C57, IGF-1-/C57. Subsequent experiments in this study were conducted on male mice to reduce variability due to sex of the mouse. Tables 4.1 and 4.2 outline breeding numbers and resultant positive and negative genotyped offspring from these matings.

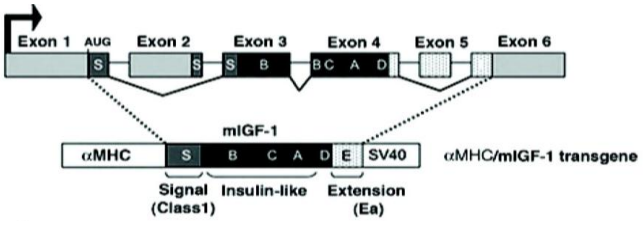


Figure 4.1 Schematic representation of the αMHC/mIGF-1 transgene construct. Adapted from Santini (Santini et al. 2007)

Table 4.1 Generation of IGF-1 + / *mdx* mice and IGF-1 -/ *mdx* mice

	POSITIVE **IGF-1 genotype**	**NEGATIVE** **IGF-1 genotype**
F1 ♂ IGF-1 x ♀ *mdx* 22 crosses	28 ♂ IGF-1 + /*mdx*	49 ♂ IGF-1 - / *mdx*
F2 ♂ IGF-1 +/*mdx* x ♀ *mdx* 20 crosses	22 ♂ IGF-1+ / *mdx*	44 ♂ IGF-1 -/ *mdx*

Table 4.2 Generation of IGF-1+/C57 mice and IGF-1-/C57

	POSITIVE **IGF-1 genotype**	**NEGATIVE** **IGF-1 genotype**
F1 ♂ IGF-1 x ♀ C57 17 crosses	9 ♂ IGF-1+/C57	14 ♂ IGF-1-/C57
F2 ♂ IGF-1 / C57 x ♀ C57 18 crosses	15 ♂ IGF-1+/C57	39 ♂ IGF-1-/C57

4.2.2 Morphology

Mdx mice that overexpressed IGF-1 in the heart (IGF-1+/*mdx*) displayed a significantly greater body weight at 12 months of age compared to IGF-1-/*mdx* mice at the same age ($P<0.05$). C57 mice that overexpressed IGF-1 in the heart (IGF-1+/C57) also displayed a significantly greater body weight at 12 months of age compared to age matched IGF-1-/C57 ($P<0.05$) (see Figure 4.2A).

Heart weights were increased in IGF-1+/*mdx* mice at 6 months of age by 20% ($P<0.01$), and by 33% at 12 months ($P<0.001$) compared to age matched IGF-1-/*mdx* mice. Similarly IGF-1+/C57 mice showed a 17% increase in heart weight at 6 months ($P<0.05$), and a 21% increase at 12 months ($P<0.001$) compared to age matched IGF-1-/C57 (see Figure 4.2B).

IGF-1+/*mdx* mice demonstrated a greater heart weight/body weight (HW/BW) ratio at 6 months of age ($P<0.05$) and 12 months of age ($P<0.01$) when compared to age matched IGF-1-/*mdx* mice. In comparison IGF-1+/C57 mice showed no significant difference in HW/BW at 6 and 12 months of age when compared to age matched IGF-1-/C57 control mice (see Figure 4.3A).

When heart weight was normalised to tibial length (HW/TL) IGF-1+/ *mdx* mice demonstrated a 21% increase at 6 months of age ($P< 0.01$) and a 26% increase at 12 months of age ($P< 0.001$) compared to age matched IGF-1+/ *mdx*. This trend was also evident in IGF-1+/C57 mice with an increase of 17% at 6 months of age ($P<0.01$) and 21% at 12 months of age ($P<0.01$) compared to age matched IGF-1-/C57 (see Figure 4.3B).

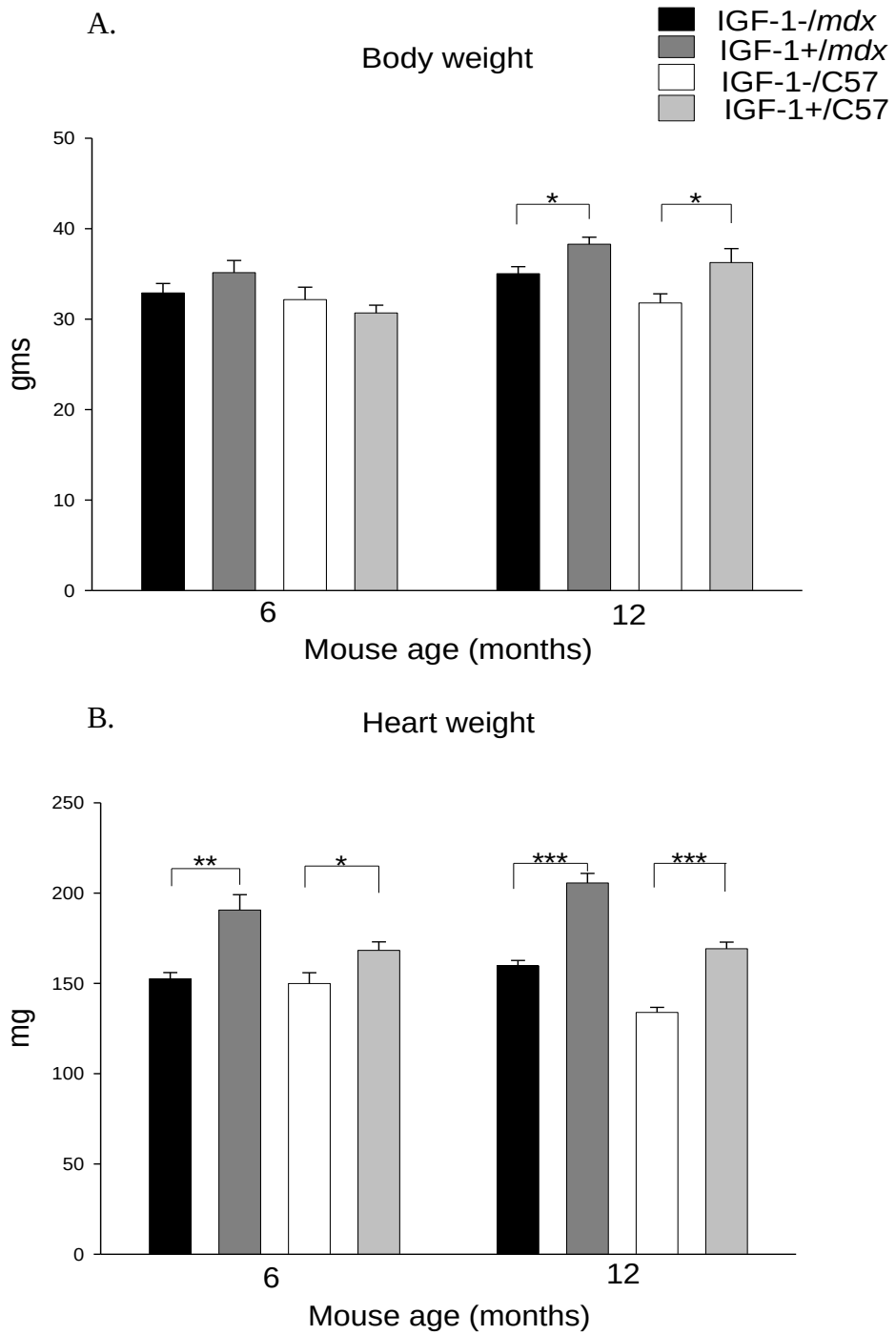


Figure 4.2 Body weight and heart weight of *mdx* and C57 mice negative and positive for the IGF-1 transgene at 6 and 12 months of age. Body weight (gms) (A); heart weight (mg). Body weight is significantly increased in both IGF-1+/*mdx* and IGF-1+/C57 mice at 12 months of ge. Heart weight is significantly increased in IGF-1+/*mdx* and IGF-1+/C57 mice at both ages. (* P<0.05, **P<0.01, ***P<0.001)

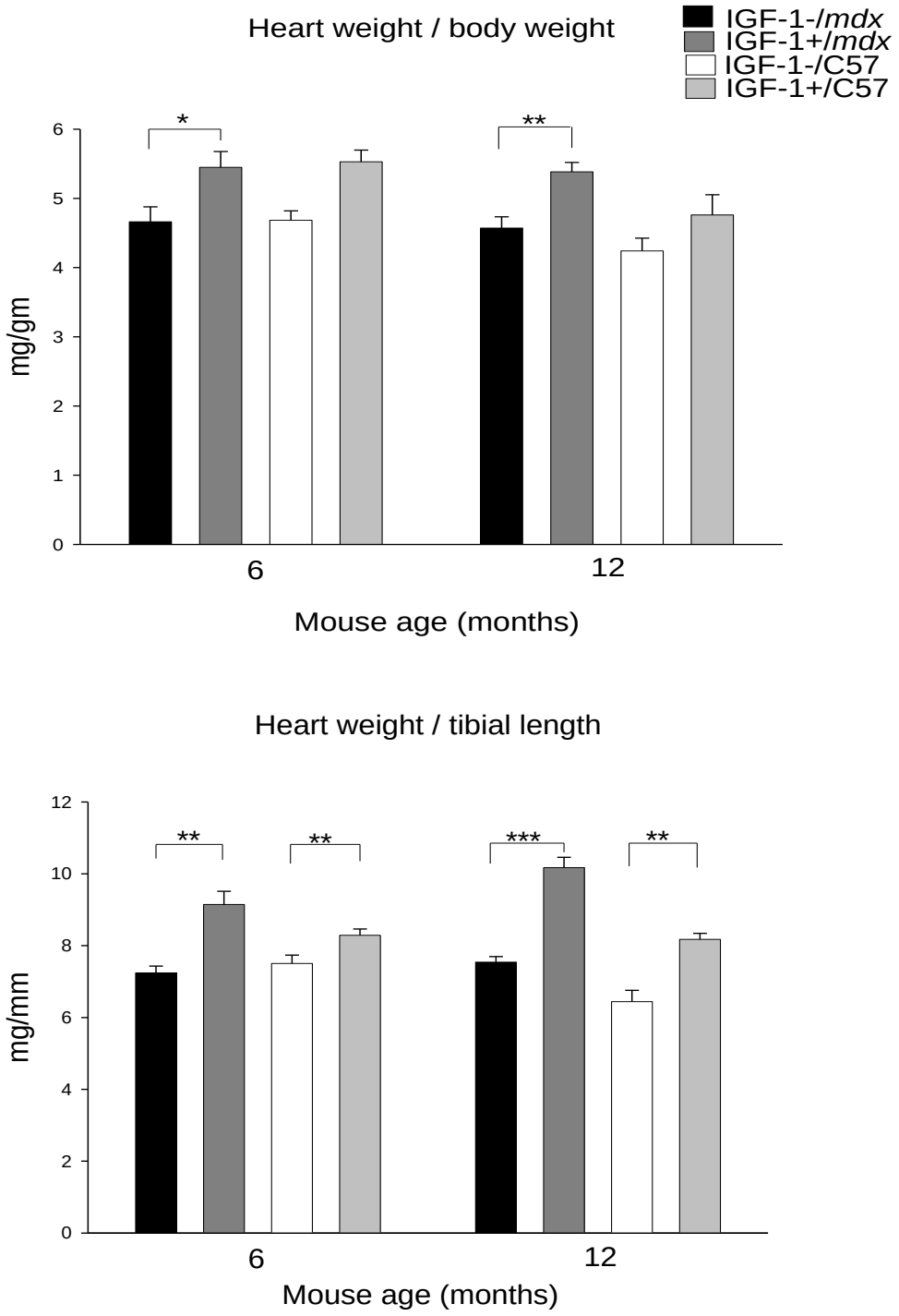


Figure 4.3 Morphometric data from *mdx* and C57 mice negative and positive for the IGF-1 transgene at 6 and 12 months of age. Heart weight normalised to body weight (mg/gm) (A), and heart weight normalised to tibial length (mg/mm) (B). Heart weight/body weight was increased only in IGF-1+/*mdx* mice at both age groups, however heart weight/tibial length was increased in both *mdx* and IGF-1+/C57 at both age groups. (* P<0.05, **P<0.01, ***P<0.001)

4.2.3 Cardiac function

Ex vivo cardiac experiments demonstrated a significant improvement in systolic function for IGF-1+/*mdx* at 12 months of age compared to 6 months ($P<0.05$), and an equivalent improvement compared to age matched IGF-1+/C57 mice ($P<0.05$) (see figure 4.4).
The rate of contraction (+dP/dt) also increased significantly in 12 month old IGF-1+/*mdx* mice compared to 6 months ($P<0.05$) (see figure 4.5A).

There were no significant difference in values between all groups of mice for the rate of relaxation (-dP/dt) (see figure 4.5B).

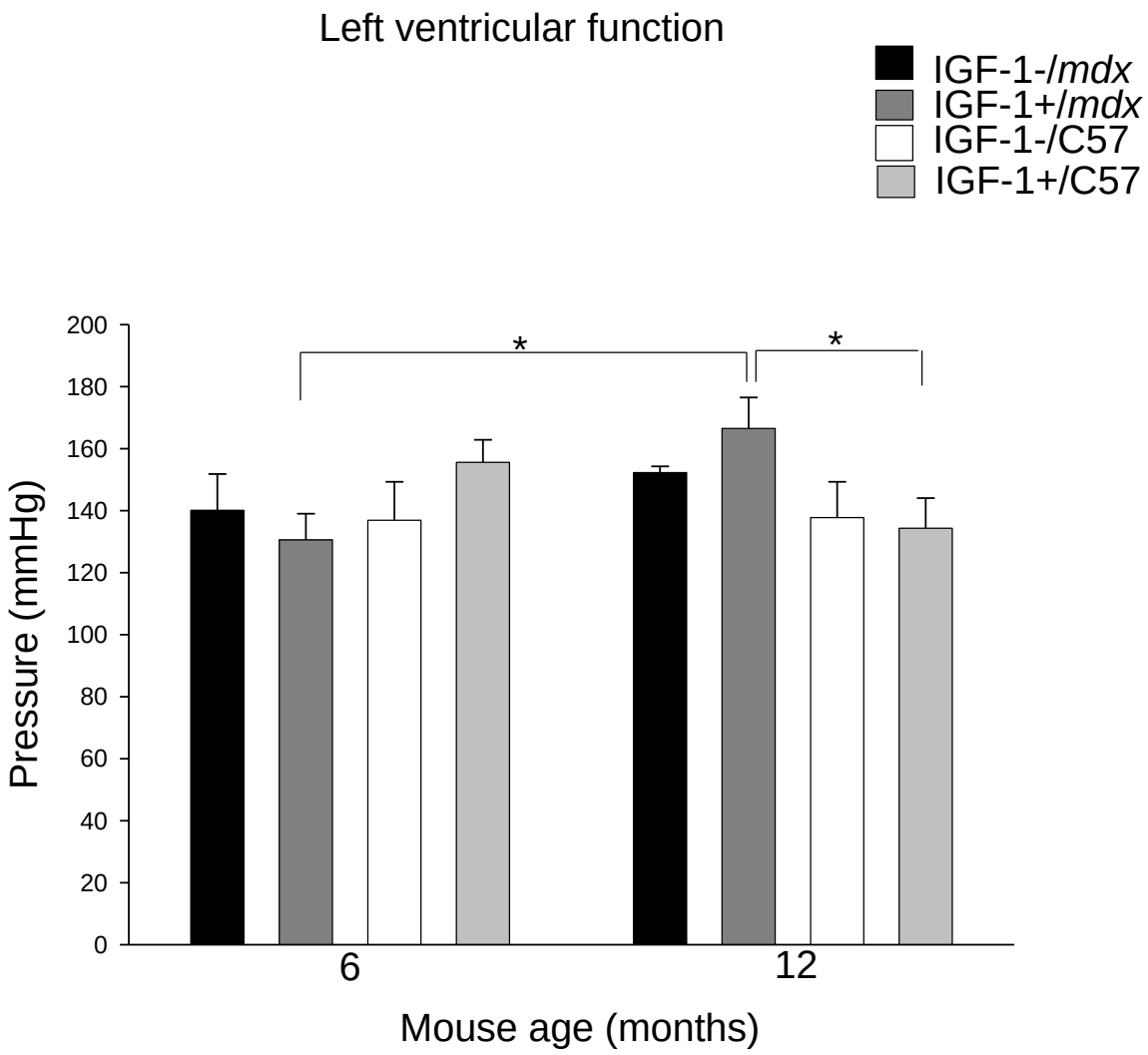


Figure 4.4 Cardiac function of *mdx* and C57 mice negative and positive for the IGF-1 transgene at 6 and 12 months of age. Left ventricular developed pressure (mmHg) from isolated hearts Langendorff perfused hearts. Cardiac function improved significantly in IGF-1+/*mdx* mice at 12 months of age compared to IGF-1+/*mdx* mice at 6 months and compared to age matched control IGF-1+/C57 mice. (* P<0.05, **P<0.01, ***P<0.001)

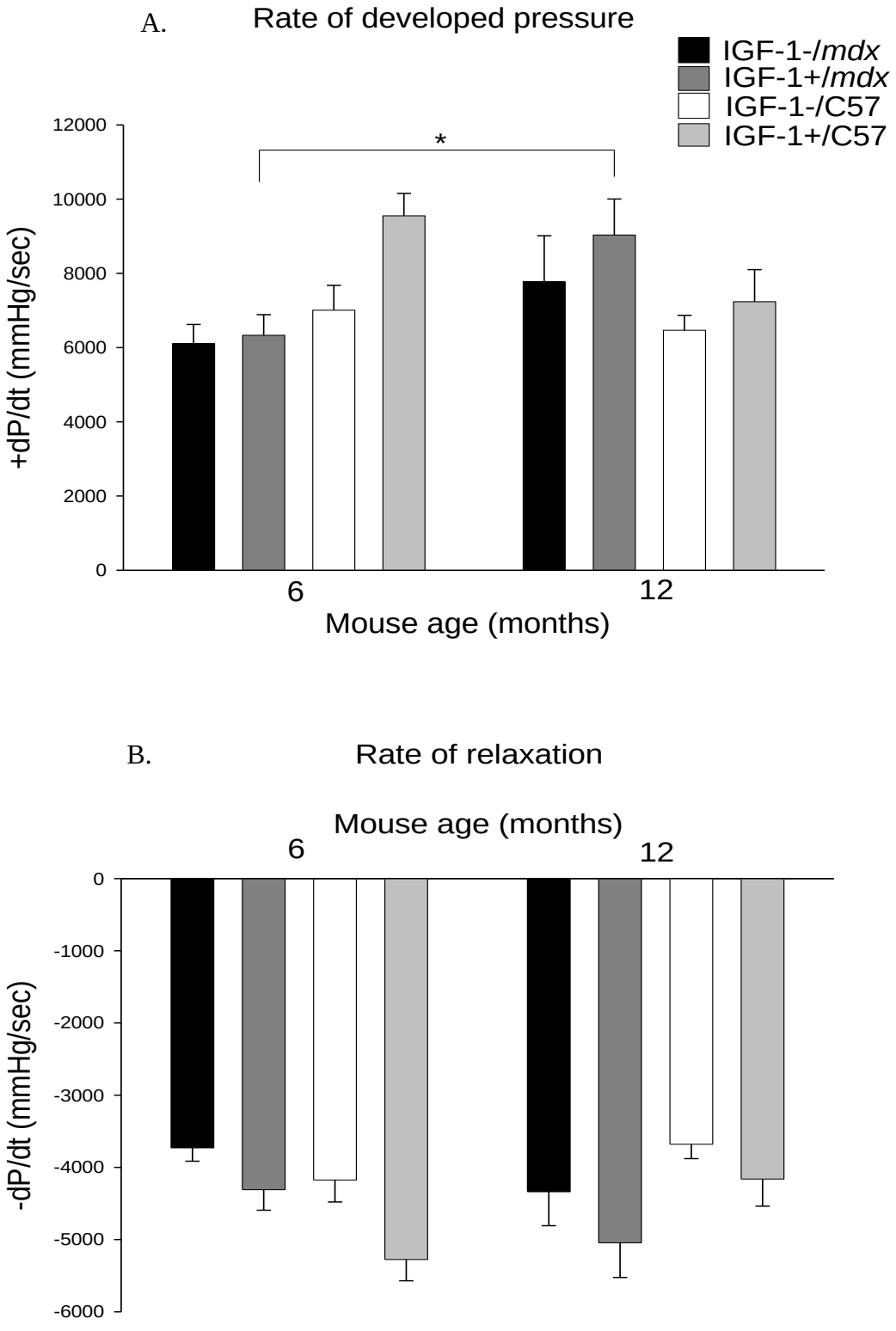


Figure 4.5 Rate of developed pressure and rate of relaxation for *mdx* and C57 hearts negative and positive for the IGF-1 transgene at 6 and 12 months of age. Contraction measured as rate of pressure development (+dP/dt) mmHg/sec (A), and rate of relaxation (-dP/dt) mmHg/sec (B). The rate of contraction increased significantly in IGF-1+/*mdx* mice at 12 months of age compared to IGF-1+/*mdx* at 6 months of age. (* P<0.05, **P<0.01, ***P<0.001)

4.2.4 Ventricular fibrosis

Ventricular fibrosis, measured by picrosirius red staining and expressed as % collagen, was significantly higher for *mdx* mice compared to C57 mice at 6 and 12 months of age ($P<0.01$ and $P<0.001$ respectively) (see Figure 4.6 and representative photomicrographs shown in Figure 4.7).

At 6 months of age IGF-1+/*mdx* mice displayed levels of fibrosis comparable to age matched *mdx* mice, however by 12 months of age IGF-1+/*mdx* mice displayed levels of fibrosis that was significantly less than age matched control *mdx* ($P<0.001$) and 6 month old IGF-1+/ *mdx* ($P<0.001$). The percentage collagen exhibited by 12 month old IGF-1+/*mdx* decreased to values not significantly different to age matched IGF-1+/C57 (see Figure 4.6 and representative photomicrographs shown in Figure 4.7).

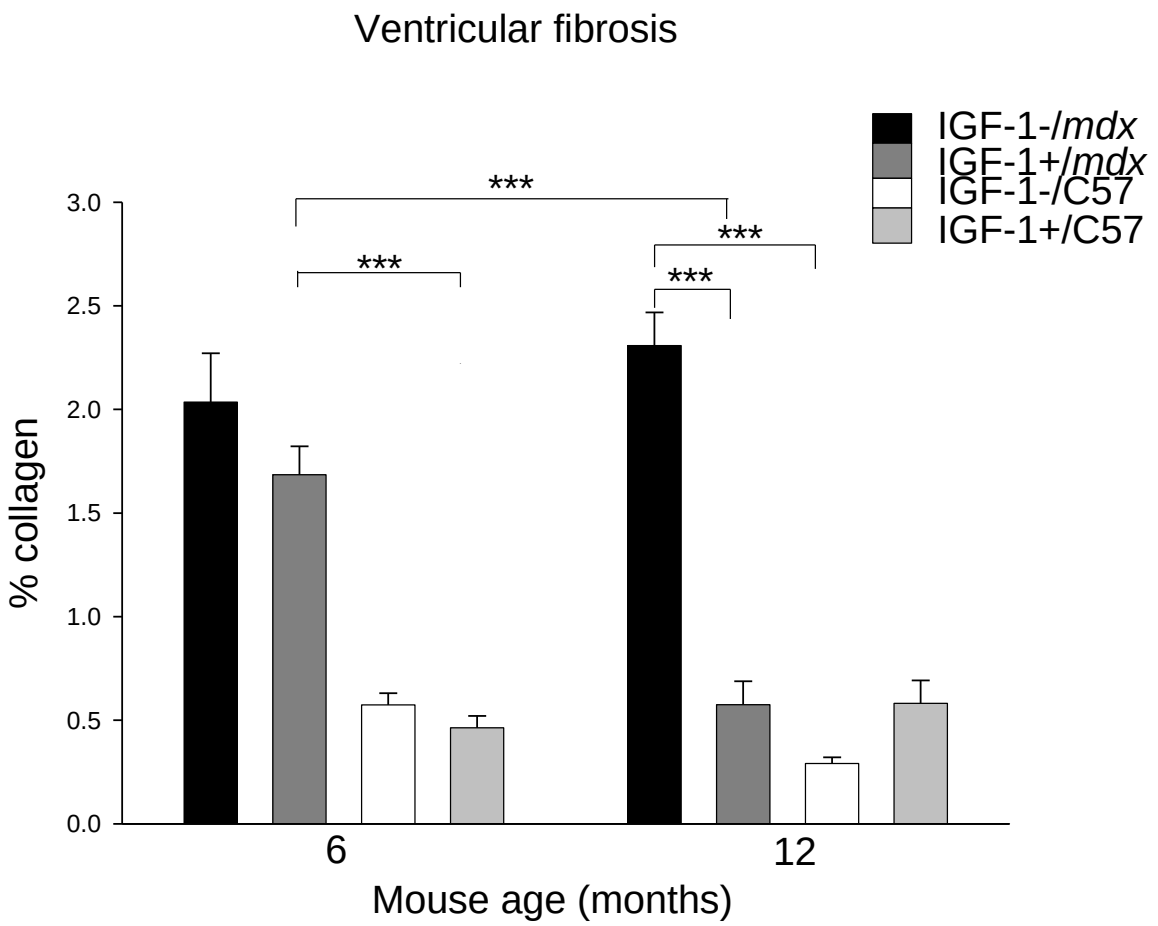


Figure 4.6 Ventricular fibrosis expressed as % collagen of *mdx* and C57 mice negative and positive for the IGF-1 transgene at 6 and 12 months of age.

Percentage collagen was significant higher for IGF-1+/*mdx* at 6 months, but decreased in IGF-1+/*mdx* at 12 months to levels not significantly different to age matched IGF-1+/C57.

(* P<0.05, **P<0.01, ***P<0.001)

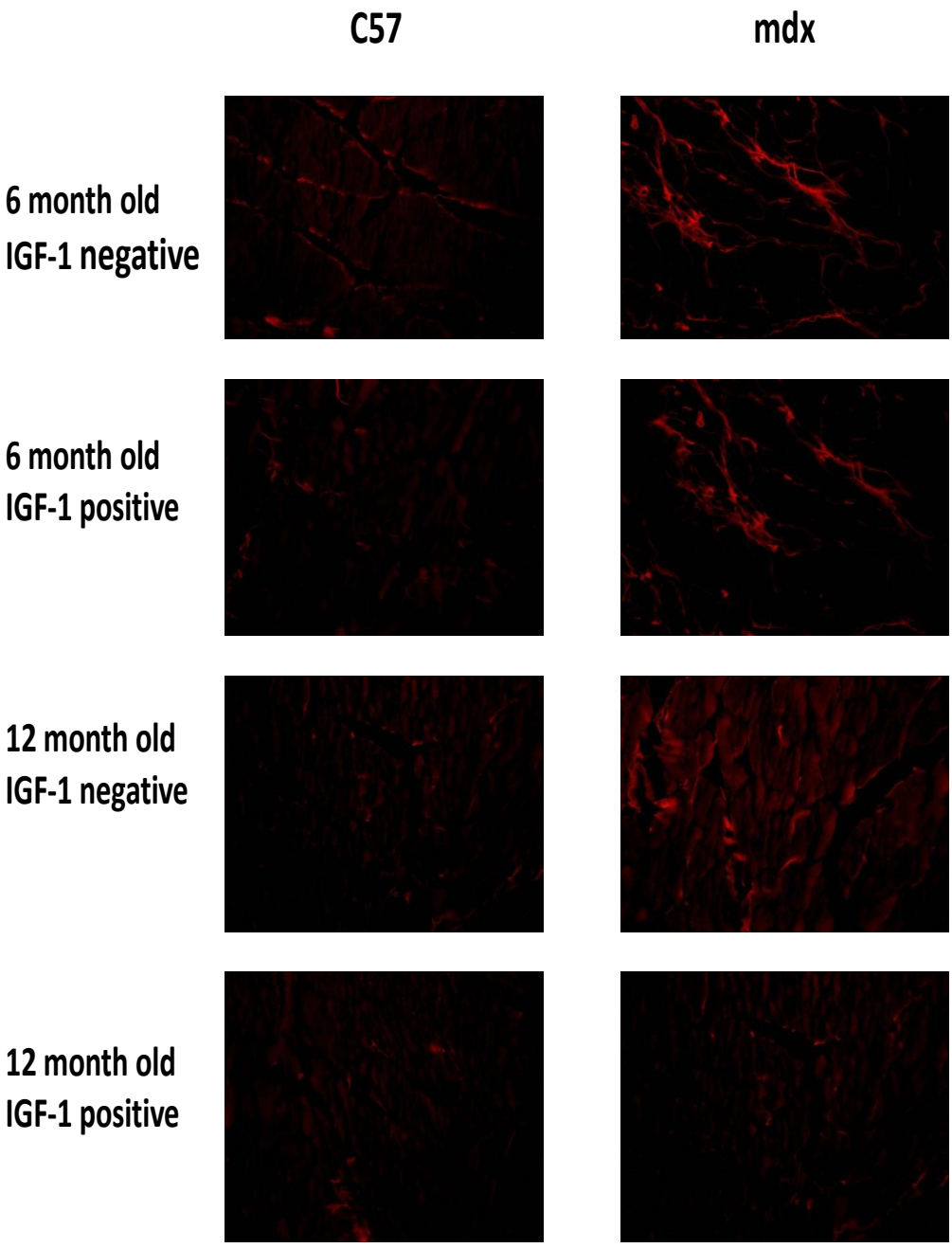


Figure 4.7 Representative photomicrographs of ventricular sections of IGF-1-/*mdx*, IGF-1+/*mdx*, IGF-1-/C57 and IGF-1+/C57 at 6 and 12 months of age (picrosirius red stained). Magnification x40. IGF-1 -/*mdx* hearts display significantly greater fibrosis than IGF-1-/C57 hearts at both age groups. By 12 months of age IGF-1+/*mdx* hearts display significantly less fibrosis than IGF-1-/*mdx* and the level of fibrosis is reduced to be not significantly different than age matched IGF-1+/C57.

4.2.5 Gene expression of TGF-β, procollagen, and fibronectin

TGF-β

Gene expression of TGF-β, as calculated by RT-PCR and expressed relative to β-actin, increased in IGF-1+/*mdx* mice compared to *mdx* mice at 12 months ($P<0.001$). There was no significant difference in TGF-β mRNA levels when comparing IGF-1+/*mdx* mice and IGF-1+/C57 mice, or between IGF-1-/*mdx* mice and IGF-1-/C57 at 6 and 12 months of age (see figure 4.8).

Procollagen

Levels of procollagen mRNA, as calculated by RT-PCR and expressed relative to β-actin, were higher in *mdx* and IGF-1+/*mdx* mice at 6 months compared to age matched control ($P<0.01$ and $P<0.05$ respectively). This trend was also apparent when comparing IGF-1-/*mdx* to IGF-1+/*mdx* mice at 12 months of age ($P<0.01$ and $P<0.001$ respectively) (see figure 4.9A). By 12 months of age, however there was no significant difference in the level of procollagen gene expression between IGF-1-/*mdx* and IGF-1+/*mdx* mice relative to age matched controls (see figure 4.9A).

Fibronectin

Gene expression of fibronectin, as calculated by RT-PCR and expressed relative to β-actin, was greater in IGF-1-/*mdx* mice at 6 months of age compared to IGF-1+/*mdx* and IGF-1-/C57 mice at the same age (both $P<0.05$). Fibronectin mRNA levels decreased in IGF-1+/*mdx* mice at 12 months compared to IGF-1+/*mdx* mice at 6 months ($P<0.01$) to levels not significantly different to that of age matched IGF-1+/C57 mice (see figure 4.9B). There was a trend towards decreased fibronectin gene expression in IGF-1-/*mdx* at 12 months also. Larger error bars prevented this difference from reaching significance (see figure 4.9B).

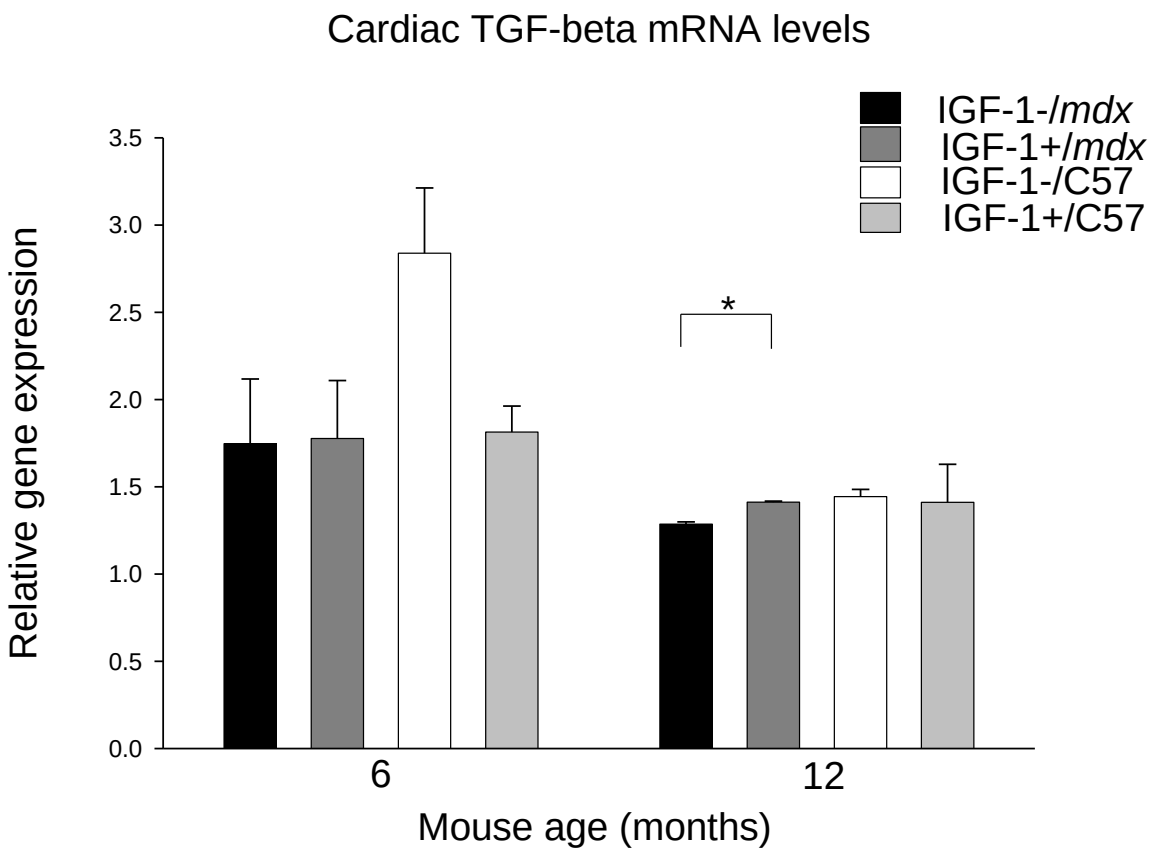


Figure 4.8 Cardiac gene expression of TGF-β mRNA as determined by quantitative real time PCR relative to β-actin for *mdx* and C57 mice positive and negative for the IGF-1 transgene at 6 and 12 months of age. TGF-β mRNA levels were significantly higher for IGF-1+/*mdx* mice at 12 months of age compared to age matched IGF-1-/*mdx* mice.

(* P<0.05, **P<0.01, ***P<0.001)

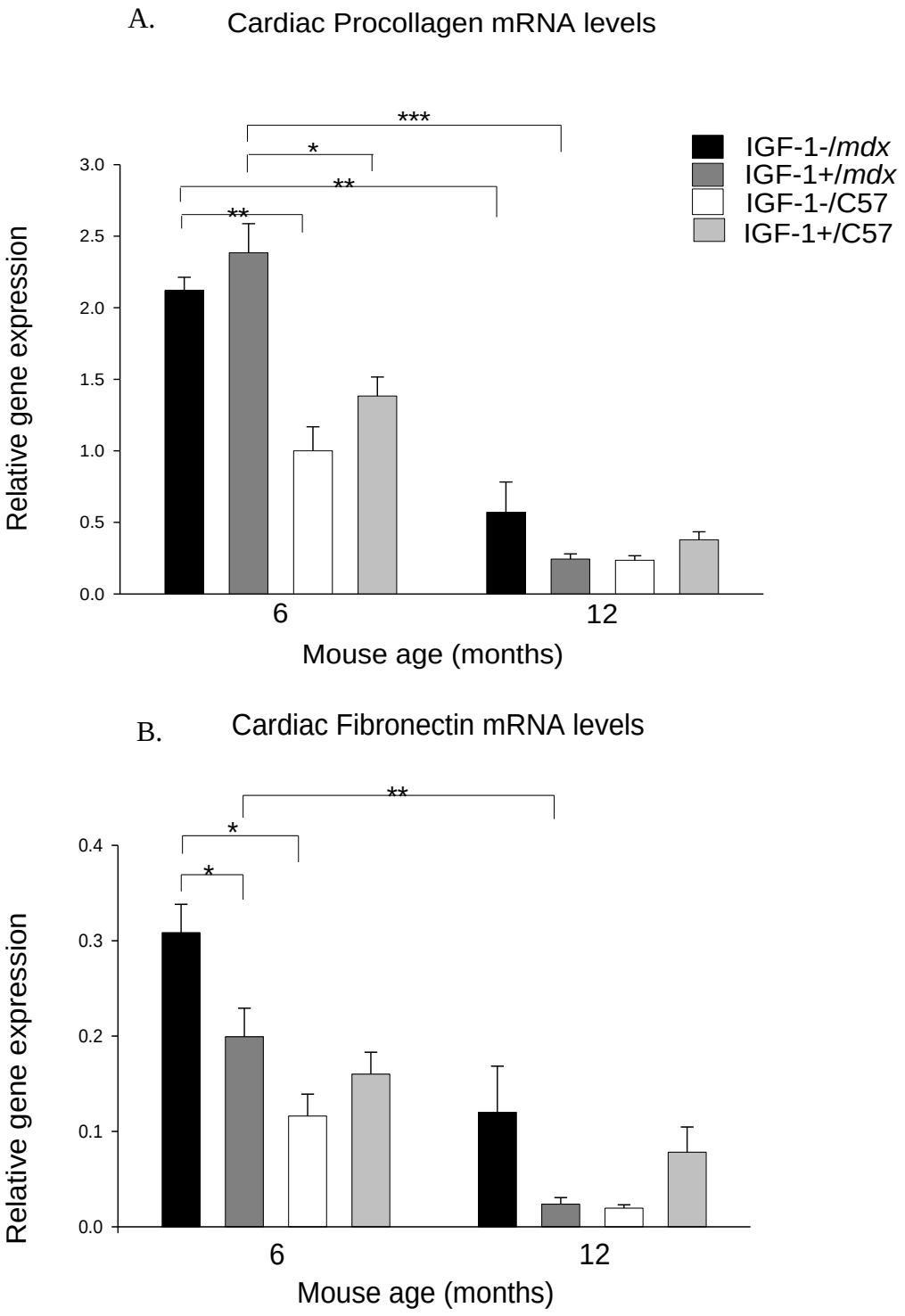


Figure 4.9 Cardiac gene expression for procollagen and fibronectin determined by quantitative real time PCR relative to β-actin for *mdx* and C57 mice positive and negative for the IGF-1 transgene at 6 and 12 months of age. mRNA levels of procollagen and fibronectin declined in an age dependant manner in the *mdx* transgenic mice. mRNA levels of procollagen were significantly higher in IGF-1+/*mdx* mice and IGF-1-/*mdx* mice at 6 months of age compared to age matched IGF-1+/C57 and IGF-1-/C57respectively and mRNA levels of fibronectin were significantly higher in IGF-1-/*mdx* mice at 6 months compared to age matched IGF-1+/*mdx* and IGF-1-/C57. (* P<0.05, **P<0.01, ***P<0.001)

4.3 DISCUSSION

4.3.1 Cardiac hypertrophy

IGF-1 has been shown to mediate cardiac hypertrophy in murine species (Duerr et al. 1995; Ito et al. 1993; Santini et al. 2007; Welch, S. et al. 2002). Transgenic IGF-1 overexpression increased heart weight in both *mdx* mice and C57, however the effect was more pronounced in the dystrophic heart. This suggests an enhanced growth promoting effect of IGF-1 in dystrophic muscle compared to normal muscle, reflected also in heart weight normalised to body weight with only IGF-1/*mdx* mice exhibiting a significant increase. This correlates to studies looking at overexpression of IGF-1 in the skeletal muscles of *mdx* and normal mice which found an increased growth promoting effect of IGF-1 on dystrophic muscle (Barton et al. 2002; Shavlakadze et al. 2004).

IGF-1 appears to mediate a more dramatic effect on heart weight in the older animal, with ventricular weights greater at 12 months in both transgenic strains compared to 6 months of age. This effect is also observed in the diaphragms of transgenic *mdx* mice overexpressing IGF-1 in skeletal muscle (Barton et al. 2002) and in the hearts of transgenic mice overexpressing IGF-1B in cardiac muscle (Reiss et al. 1996).

In order to further assess cardiac hypertrophy, heart weight was normalised to tibial length. In murine models, tibial length changes minimally after maturity and correlates better than body weight so it is useful for assessing changes in cardiomyocyte size with age (Yin et al. 1982). Cardiac hypertrophy was observed in *mdx* and C57 overexpressing IGF-1 at both ages examined, however the greatest increase was seen in the dystrophic heart at 12 months of age.

The growth promoting effects of IGF-1 are also dependent upon the mode of administration and the dose. Short term administration of IGF-1 has been shown not to increase heart weight (Ren 2002). IGF-1 administered by osmotic pump to normal rats resulted in a greater left ventricle weight normalised to tibial length with increased dose (2.2mg/kg/day and 3mg/kg/day) (Duerr et al. 1995). In comparison, the transgenic model used in this current study provides chronic increased levels of IGF-1. This provides a more consistent model than intermittently administered exogenous sources and enables a valid comparison of the effects of IGF-1 with increasing age.

Cardiac growth may be due to an increase in cardiomyocyte size and/or cellular proliferation. Studies have indicated that IGF-1 mediated increases in heart weight are due to increased cardiomyocyte size (hypertrophy), rather than number (hyperplasia) (Duerr et al. 1995; Ito et al. 1993; Santini et al. 2007). Although other studies have reported increased cardiomyocyte number in transgenic mice overexpressing IGF-1, a different isoform of IGF-1 was used (IGF-1B) (Reiss et al. 1996; Welch, S. et al. 2002). The degree to which hypertrophy occurs as opposed to hyperplasia may be dependent upon the age of the animal and the specific muscle. IGF-1 overexpressed in skeletal muscles of *mdx* mice exhibited more hyperplasia in the extensor digitorum longus at 3 months of age mice than at 14 months where the increased muscle size was due to hypertrophy. This was in contradiction to the diaphragm which displayed hyperplasia and hypertrophy at 3 months, followed by only hyperplasia at 14 months of age (Barton et al. 2002).

Cardiac hypertrophy observed in transgenic mice that overexpressed IGF-1 exclusively in the heart has been shown to be related to higher levels of atrial natriuetic peptide (ANP) at early ages, while other cardiac hypertrophy markers such as brain natriuretic peptide (BNP), α-

skeletal actin, β-myosin heavy chain and glutamate transporter were not affected (Santini et al. 2007).

4.3.2 Cardiac Function

In order to determine if the observed cardiac hypertrophy conferred a physiological change as opposed to a pathological one, functional experiments were performed on isolated mouse hearts. This showed that IGF-1 overexpression improved cardiac function in 12 month old *mdx* by 20% compared to aged matched controls. This increase was 22% greater than IGF-1/*mdx* hearts at 6 months of age, and was accompanied by a 30% increased rate of contraction. The improvement in cardiac function suggests the observed hypertrophy is a physiologic change. While IGF-1 has been shown in other models to improve cardiac function (Cittadini et al. 1998; Duerr et al. 1995; Santini et al. 2007; Serose et al. 2005), this current study is the first to report improvement in cardiac function with IGF-1 overexpression in the dystrophic heart.

The improved function at 12 months of age correlated with a dramatic reduction in levels of ventricular fibrosis. Even though IGF-I has been shown to stimulate fibroblasts (Petley et al. 1999) it has been shown to reduce fibrosis in skeletal muscles of *mdx* mice (Barton et al. 2002), in the injured heart (Santini et al. 2007) and the delta-sarcoglycan deficient heart (Serose et al. 2005).

IGF-1 appears to be capable of resolving the inflammatory response and thereby attenuating fibrosis. After myocardial infarction, transgenic overexpression of IGF-1 has been shown to decrease levels of proinflammatory cytokine IL-6 that promotes fibrosis while levels of anti-inflammatory cytokines IL-4 and IL-10 were increased (Santini et al. 2007).

An age related impact of IGF-1 is demonstrated by 12 month transgenic IGF-1+/ *mdx* mice with reduced ventricular fibrosis and improved cardiac function as compared to younger mice. One possible explanation is that IGF-1 overexpression in the *mdx* mouse heart may not be able to resolve fibrosis at 6 months of age due to increased circulating levels of TNF-α. TNFα is an early and potent proinflammatory cytokine that stimulates the inflammatory response. Elevated TNF-α increases catabolism within skeletal muscle and can interfere with IGF-1 signalling (Grounds & Torrisi 2004). TNF-α strongly inhibits IGF-1 gene and protein expression of muscle cells (Fernandez-Celemin et al. 2002; Frost, Lang & Gelato 1997). As TNF-α levels have been shown to be upregulated in the active stage of DMD (Porreca et al. 1999) and *mdx* mice (Porter et al. 2003) perhaps at 6 months of age TNF-α levels in the heart are high enough to inhibit IGF-1 signalling and thereby block its ability to resolve the inflammatory process. Future therapeutic strategies involving increasing levels of IGF-1 may prove more effective if combined with reducing TNF-α activity (Shavlakadze et al. 2004).

The mechanisms involved in the increased contractile performance mediated by IGF-1 are unclear and beyond the scope of this current study. Concomitant with its anabolic and antiapoptotic effects, IGF-1 enhanced calcium responsiveness of cardiomyocytes and intracellular Ca^{2+} replenishing capacity (Ren 2002), and this mechanism may be involved with improved systolic function.

4.3.3 TGF-β

Despite fibrosis decreasing at 12 months of age, this was not accompanied by a decrease in TGF-β levels. Values were not different between strains of mice, with only a slight increase in IGF-1/*mdx* at 12 months compared to *mdx* at the same age. Another study found that increased TGF-β levels were not part of the cardiac inflammatory response after cardiotoxin injury and myocardial infarction, but rather increased levels of interleukins 6 and 1β (Santini

et al. 2007). This highlights a selective involvement of certain cytokines in the cardiac inflammatory response (Santini et al. 2007).

4.3.4 Procollagen and fibronectin

Collagen and fibronectin are secreted by fibroblasts which are key mediators of the fibrotic response as reviewed by Tomasek (Tomasek et al. 2002). This current data showed decreased procollagen and fibronectin mRNA levels in IGF-1+/*mdx* mice at 12 months of age which was accompanied with decreased fibrosis. *Mdx* fibroblasts have been shown to synthesize more fibronectin than normal fibroblasts and incubating *mdx* fibroblasts in IGF-1 decreased fibronectin levels (Mezzano et al. 2007). From current results it is possible that this same effect exists at an organ level, not just in cell culture.

Fibrosis is a function of both synthesis and degradation of the ECM. The lack of clear temporal correlation observed in this study, between TGF-β, procollagen and fibronectin with fibrosis highlights the complex pathways involved and it is likely that other signalling molecules are involved. MMPs may participate in fibrosis and remodelling of the ECM via direct digestion of the ECM, regulation of matrikines and/or release of growth factors such as IGF-1 from the ECM (Taipale & Keski-Oja 1997). Elevated myocardial IGF-1 mRNA has been shown to have a positive correlation with MMPs 11 and 14 and TIMPS 1 to 4 therefore suggesting a link between IGF-1 expression in transgenic animals and alterations in ECM metabolism (Barton et al. 2005).

4.4 SHORTCOMINGS OF THE STUDY

The next phase of research involving this IGF-1+/*mdx* mouse model would be to further examine the molecular pathways involved in cardiac fibrosis. In particular, analysis of TNF-α would be valuable to ascertain if it prevented IGF-1 mediated reduction in fibrosis at 6 months of age. Other cytokines of interest would be proinflammatory IL-6 and anti-inflammatory IL-4 and 10 as these have been implicated in the resolution of the inflammatory response of the heart (Santini et al. 2007). Also of importance would be cardiac levels of MMPs and TIMPs to determine their involvement in the balance between collagen deposition and degradation in the dystrophic heart.

Only two age groups were examined in this current study. More frequent sampling is required to elucidate a signaling profile of the cytokines involved in fibrosis. Future research could also examine younger and older age groups. It would be valuable to study this model at younger ages to observe the effects on IGF-1 on initiation of fibrotic pathways and at 15 months of age where marked reduction in cardiac function has been observed (Van Erp, Irwin & Hoey 2006).

Future studies to further elucidate the mechanism behind improved cardiac function mediated by IGF-1 would benefit from single cell experiments such as cell shortening, rate of cell shortening and intracellular calcium levels, to determine changes at a cellular level.

Transgenic *mdx* mice overexpressing IGF-1 in skeletal muscle have been generated and histological and functional experiments have found improvements in skeletal muscle pathology (Barton et al. 2002). To date the cardiac involvement of these mice has not been

assessed to see if improvements in skeletal pathology results in an improvement of the cardiac phenotype.

4.5 CONCLUSION

The major findings of this study indicate that IGF-1 mediates cardiac growth, and by 12 months of age imparts a positive inotropic effect on the dystrophic heart which is accompanied with a marked reduction in ventricular fibrosis.

The observed cardiac growth appeared different from both normal growth of the immature heart, in which no changes in left ventricular function are usually observed, and from pathological growth which is associated with decreased contractile performance (Scheuer & Buttrick 1987).

Efficient repair of the IGF-1/*mdx* heart induced by IGF-1 may prevent the formation of an environment into which fibroblasts migrate thereby reducing fibrosis as observed in this study and in *mdx* skeletal muscle (Barton et al. 2002).

This study provides further evidence that TGF-β is not the principle driver in the progression of fibrosis in dystrophic cardiomyopathy as also suggested by others (Gosselin et al. 2004; Van Erp, Irwin & Hoey 2006). Indeed inflammation after myocardial infarction and cardiotoxin injury found levels of TGF-β not to be affected (Santini et al. 2007). The evidence suggests that the progressive fibrosis characteristic of dystrophic muscle may be a result of reduced degradation of collagen as opposed to active synthesis and this is further supported by the decreased mRNA levels of fibronectin and procollagen observed in this study. Indeed IGF-1 has been linked to ECM remodelling through its positive correlation

with MMPs 11 and 14 and TIMPs 1 and 2 which are involved in ECM metabolism (Barton et al. 2005).

The ability of IGF-1 overexpression to improve function and attenuate fibrosis in the dystrophic heart warrant further investigation of this growth hormone as a potential therapy to ameliorate the dystrophic phenotype. While IGF-1 therapy would not address the primary deficit associated with DMD, it may be beneficial in protecting the heart from the secondary symptoms of dystrophin deficiency. It is also acknowledged that it is difficult to translate the transgenic model to the clinical setting and delivery of IGF-1 to the patient's heart is an issue. AAV vectors offer a possible technique to deliver IGF-1 to a specific muscle and avoid side effects on other tissues. IGF-1Ea has been successfully injected into the skeletal muscles of *mdx* mice using AAV vectors, on its own (Barton 2006b) and in conjunction with microdystrophin (Abmayr et al. 2005).

CHAPTER 5 EFFECT OF MYOD AND DYSTROPHIN KNOCKOUT ON CARDIAC STRUCTURE AND FUNCTION

5.1 INTRODUCTION

The *mdx* mouse exhibits a milder phenotype than DMD. This may be due to upregulation of utrophin (Deconinck et al. 1997), or increased muscle regeneration (Anderson, Bressler & Ovalle 1988). Other mouse models that have been suggested as alternatives to the *mdx* mouse for studies into dystrophin deficient cardiomyopathy include the dystrophin/utrophin double knockout mouse (Deconinck et al. 1997), and the MyoD and dystrophin double knockout mouse (*mdx*:MyoD(-/-)) (Megeney et al. 1999).

Dystrophin /utrophin knockout mice exhibit a more severe progressive muscular dystrophy than *mdx* mice that results in premature death, however utrophin /*mdx* mice failed to develop cardiomyopathy (Deconinck et al. 1997). This could be due to the shorter life span of only 20 weeks of these mice which does not allow time for cardiomyopathy to develop.

It has been argued that the *mdx*:MyoD(-/-) mouse displays a phenotype more closely aligned to that observed in DMD, and in particular a more severe cardiomyopathy (Duan 2006). *mdx*:MyoD(-/-) mice are characterised by a marked kyphosis, a waddling gait characterised by weight bearing on the tarsal joints, and an increased skeletal myopathy leading to premature death (Megeney et al. 1996). The marked skeletal myopathic phenotype appears to be due to a reduction in skeletal muscle regeneration as a result of reduced activity of satellite cells (Megeney et al. 1996).

Myogenic transcription factor, MyoD is directly involved in satellite cell activation (Rudnicki et al. 1992). Knocking out the MyoD gene in the *mdx* mouse would determine if enhanced

muscle regeneration contributed to the milder phenotype of the *mdx* mouse. Indeed *mdx*:MyoD(-/-) mice have been shown to have reduced muscle hypertrophy and significantly reduced regeneration in skeletal muscles, however some regenerative potential did persist (Megeney et al. 1996). MyoD therefore appears to be required for efficient regeneration at the level of satellite cell formation, survival or function. MyoD may also be indirectly required by myofibres for the expression of growth factors essential for activating satellite cells (Megeney et al. 1996).

Controversy exists as to whether cardiomyopathy associated with DMD is a secondary phenomenon due to skeletal muscle pathology or is a result of dystrophin deficiency alone. A study of 9 DMD patients found that the severity of skeletal muscle pathology was associated with the extent of cardiac dysfunction (Hunsaker et al. 1982). X-linked dilated cardiomyopathy (XLDC) challenges this idea. Dystrophin is present in skeletal muscle, but absent from the heart of these patients. Lack of cardiac dystrophin alone is sufficient to cause severe cardiomyopathy (Muntoni et al. 1995). In support of this, a study involving γ-sarcoglycan mice inserted with a transgene to rescue γ-sarcoglycan only in skeletal muscle, found no beneficial effects on the heart (Zhu et al. 2002).

The *mdx*: MyoD(-/-) mouse model has the potential to distinguish if dystrophic cardiomyopathy is primarily due to dystrophin deficiency, or a secondary phenomenon associated with skeletal muscle pathology. As MyoD is expressed only in skeletal muscle, not in the heart, changes in cardiac involvement in the *mdx*:MyoD(-/-) mouse compared to the *mdx* mouse would be due to the increased level of skeletal muscle damage.

Mdx:MyoD(-/-) mice exhibit cardiac hypertrophy by 5 months of age predominantly in the left ventricle (Megeney et al. 1999). Ventricular fibrosis increased with age compared to *mdx* hearts, and was also localised to the left ventricle (Megeney et al. 1999). This escalation in the cardiac involvement of the model compared to age matched *mdx* mice is due to increased skeletal myopathy suggesting that dystrophin deficiency in cardiomyocytes is not the sole cause behind DMD cardiomyopathy. Further to this, the *mdx*:MyoD(-/-) mouse heart may reflect the progressive dilated cardiomyopathy observed in DMD more closely than the *mdx* mouse (Duan 2006).

To date cardiac functional data has not been assessed for the *mdx*:MyoD(-/-) mouse. If the cardiomyopathy of *mdx*:MyoD(-/-) mice more closely resembles DMD patients it would be an extremely useful model to assess potential therapies for dystrophic cardiomyopathy. This current study aims to examine *ex vivo* cardiac function of the *mdx*:MyoD(-/-) mouse to determine any changes in systolic and diastolic function. This will add to the body of knowledge along with the observed morphological changes and activation of protein kinases observed in other studies (Megeney et al. 1999).

Additional objectives of the current study were to determine levels of fibrosis and gene expression of TGF-β, procollagen and fibronectin which are implicated in profibrotic pathways in this transgenic model. Increased fibrosis is a feature of both *mdx* and DMD cardiomyopathy, however post mortem studies of DMD suggest that it is more extensive in human hearts and localised to the left ventricle (Frankel & Rosser 1976). A previous study showed that 5 month old *mdx*:MyoD(-/-) mice exhibit increased fibrosis compared to *mdx* which was localised to the left ventricle (Megeney et al. 1999). This current study examined the level of cardiac fibrosis of the *mdx*:MyoD(-/-) model at 3 months of age and determined

the levels of gene expression of TGF-β, procollagen, and fibronectin in an attempt to elucidate fibrotic molecular pathways, with particular reference to this transcription factor. This age group was chosen as the study aimed to examine levels of cardiac hypertrophy similar to those observed in the IGF-1 transgenic *mdx* mice experiments.

5.2 RESULTS

5.2.1 Generation of *mdx*:MyoD(-/-) mice

Male mice with the MyoD promoter, exon 1 and half of intron 1 deleted and replaced by a *VGKneo* cassette, were crossed with female *mdx* and C57 mice. Genotyping, as outlined in Chapter 2, resulted in three groups of mice: *mdx*:MyoD(-/-), C57:MyoD(-/-) and *mdx*.

5.2.2 Morphology

There was no significant difference in body weight, heart weight, heart weight normalised to body weight (HW/BW) or heart weight normalised to tibial length (HW/TL) between *mdx*, *mdx*:MyoD(-/-), C57:MyoD(-/-) mice (Figures 5.1 and 5.2).

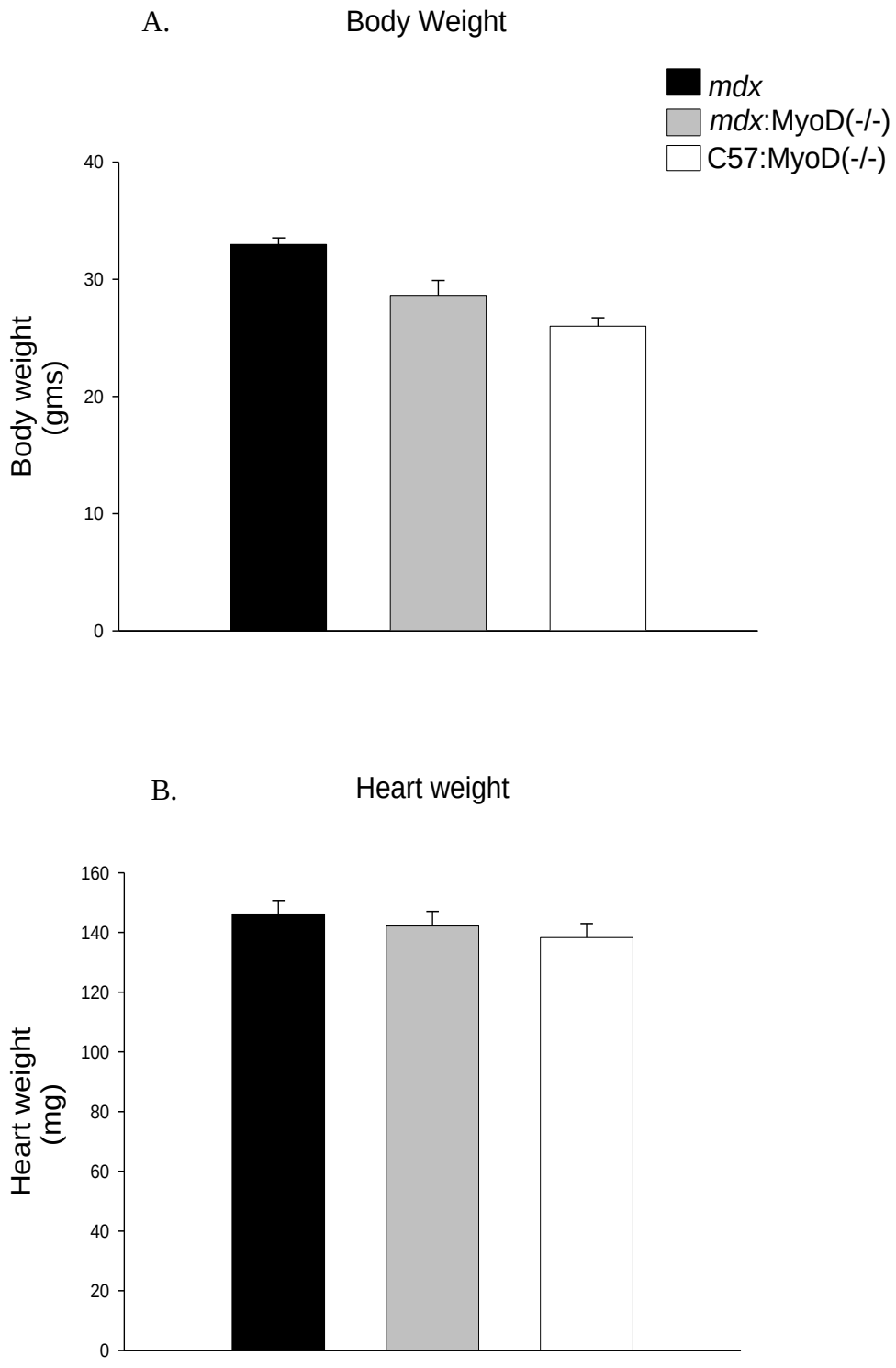


Figure 5.1 Body weight and heart weight from *mdx*, *mdx*:MyoD(-/-) and C57:MyoD(-/-) mice at 3 months of age. Body weight (gms) (A.), and heart weight (mg) (B.). There was no significant difference in body weight or heart weight between the different strains of mice. (* P<0.05, **P<0.01, ***P<0.001)

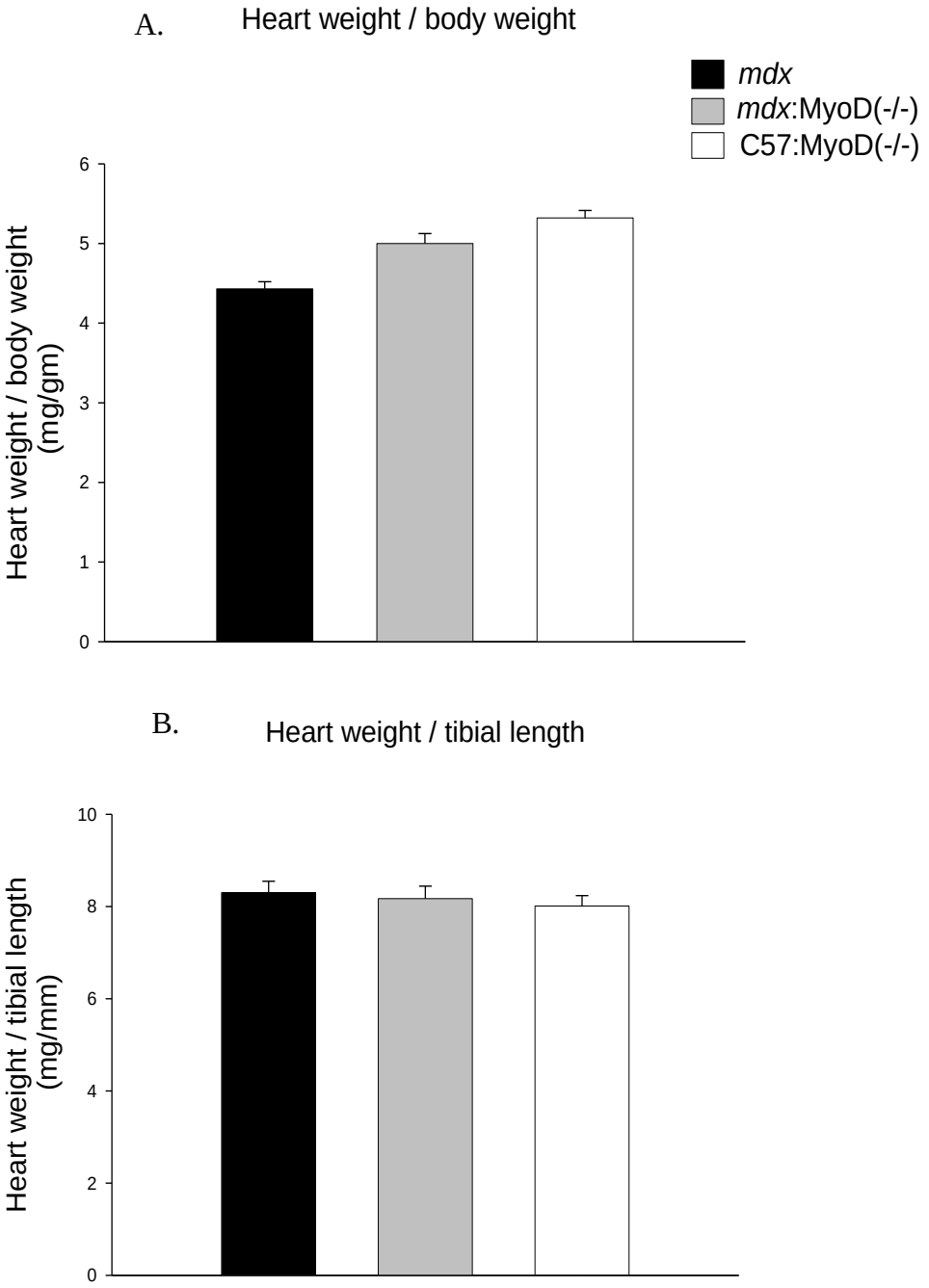


Figure 5.2 Morphological data from *mdx*, *mdx*:MyoD(-/-) and C57:MyoD(-/-) mice at 3 months of age. Heart weight / body weight (mg/gm) (A.), heart weight / tibial length (mg/mm) (B.). There was no significant difference in heart weight normalised to body weight or tibial length between the different strains of mice. (* P<0.05, **P<0.01, ***P<0.001)

5.2.3 Cardiac function

An improvement in cardiac function (mmHg), rate of contractility (mmHg/second), rate of relaxation (mmHg/second), was observed in *mdx*:MyoD(-/-), mice compared to *mdx* mice ($P<0.05$, $P<0.001$, $P<0.05$ respectively) as shown Figure 5.3 and 5.4. There was no significant difference in functional data between *mdx*:MyoD(-/-), and C57:MyoD(-/-) (Figure 5.3 and 5.4).

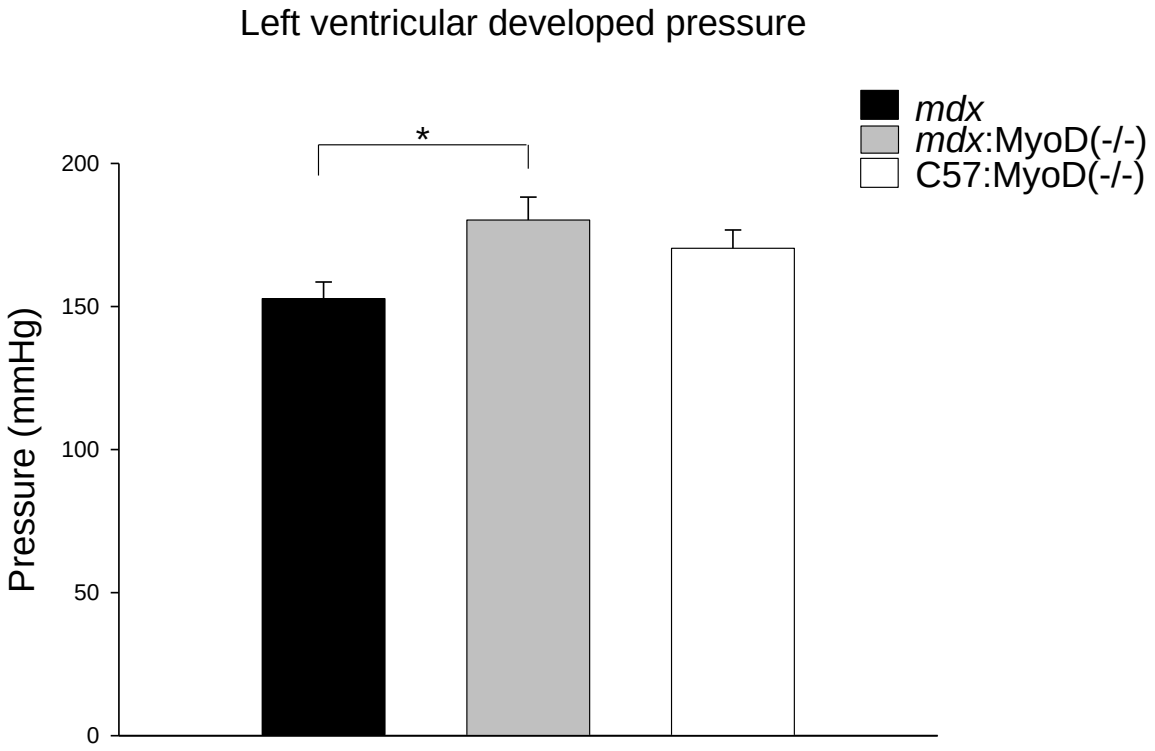


Figure 5.3 Cardiac function of *mdx*, *mdx*:MyoD(-/-) and C57:MyoD(-/-) mice at 3 months of age. Left ventricular developed pressure (mmHg) from isolated hearts Langendorff perfused hearts. There was a significant increase in left ventricular developed pressure in *mdx*:MyoD(-/-) hearts compared to *mdx*. (* $P<0.05$, **$P<0.01$, ***$P<0.001$)

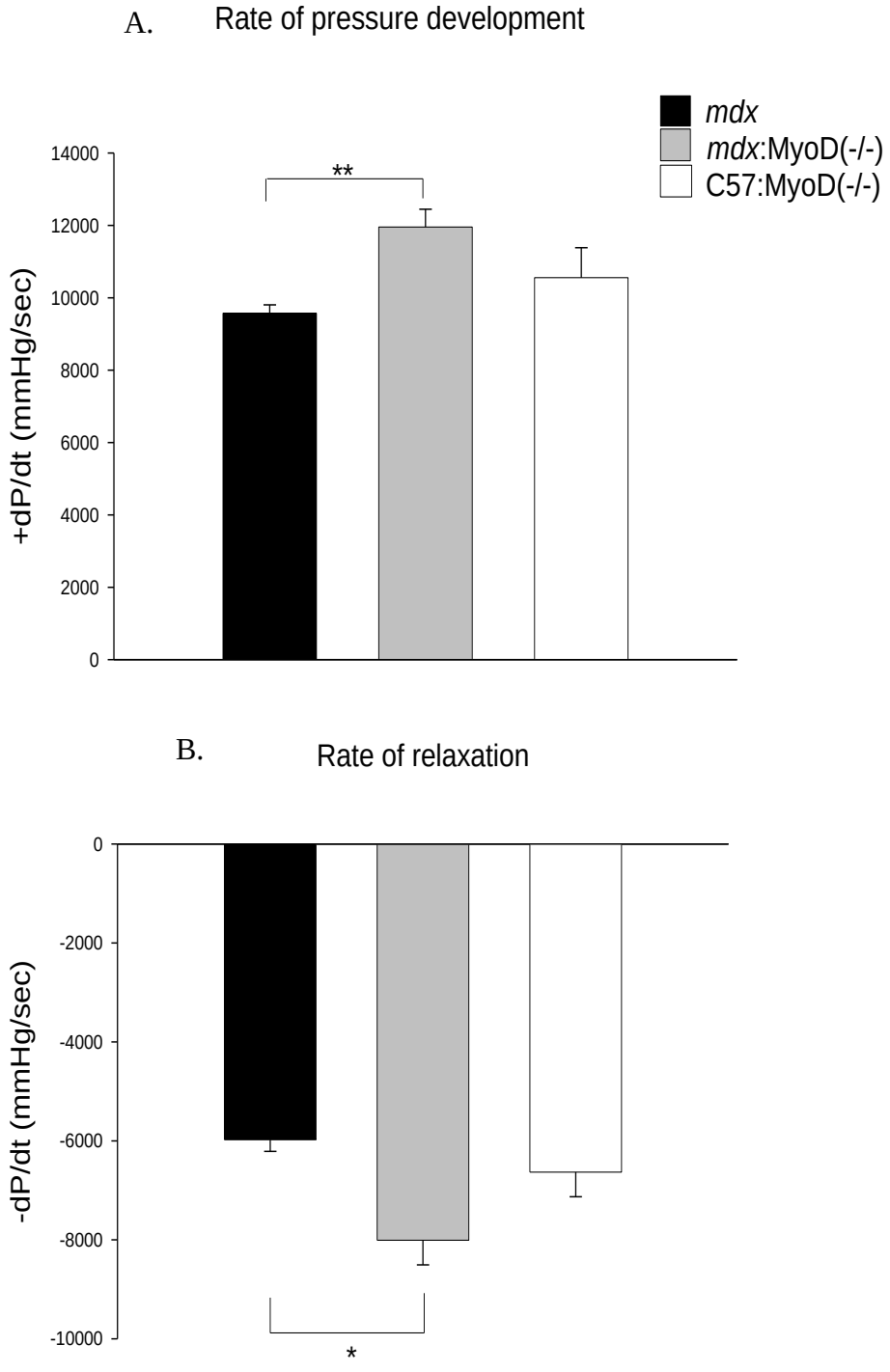


Figure 5.4 Rate of developed pressure and rate of relaxation of *mdx*, *mdx*:MyoD(-/-) and C57:MyoD(-/-) hearts at 3 months of age. Contraction measured as rate of pressure development (+dP/dt) mmHg/sec (A), and rate of relaxation (-dP/dt) mmHg/sec (B). Both the rate of contraction and rate of relaxation significantly increased in *mdx*:MyoD(-/-) hearts compared to *mdx*. (* P<0.05, **P<0.01, ***P<0.001)

5.2.4 Ventricular Fibrosis

Mdx:MyoD(-/-) mice exhibited a significant increase in ventricular fibrosis (expressed as % collagen) compared to *mdx* and C57:MyoD(-/-) mice ($P<0.05$ and $P<0.001$). *Mdx* mice also displayed more fibrosis than C57:MyoD(-/-) mice ($P<0.001$). (see Figure 5.5 and representative photomicrographs Figure 5.6)

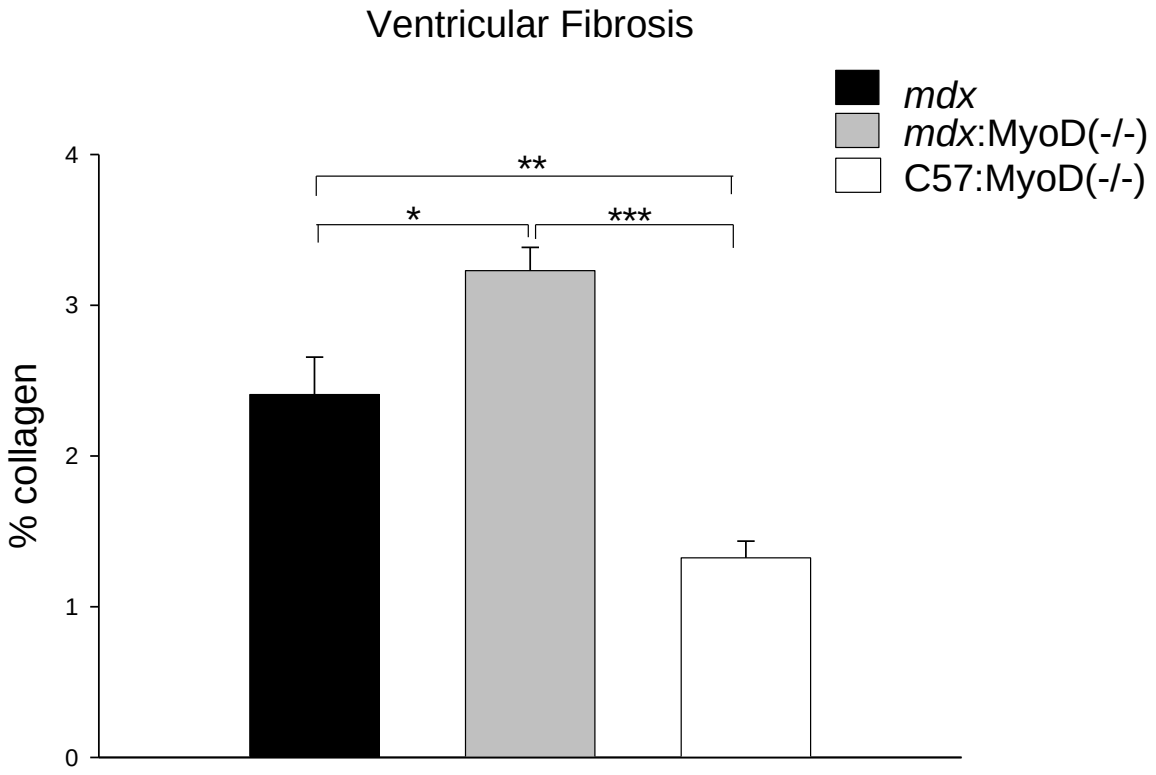


Figure 5.5 Ventricular fibrosis expressed as % collagen of *mdx*, *mdx*:MyoD(-/-) and C57:MyoD(-/-) mice at 3 months of age. Percentage collagen was significantly higher for *mdx*:MyoD(-/-) hearts compared to *mdx* and C57:MyoD(-/-) and *mdx* hearts displayed significantly more fibrosis than C57:MyoD(-/-). (* P<0.05, **P<0.01, ***P<0.001)

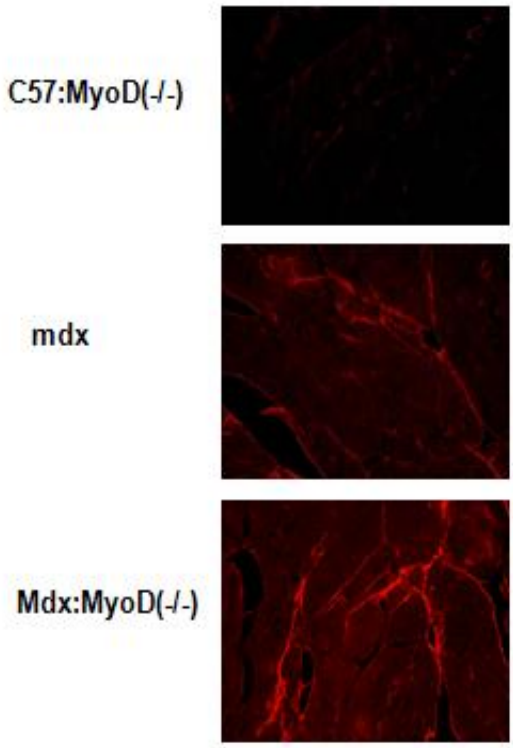


Figure 5.6 Representative photomicrographs of ventricular sections of C57:MyoD(-/-), *mdx* and *mdx*:MyoD(-/-) at 3 months of age (picrosirius red staining). Magnification x40. Removal of the MyoD gene from *mdx* mice results in significantly increased cardiac fibrosis compared to *mdx* and C57:MyoD(-/-) hearts.

5.2.5 Cardiac gene expression of TGF-β, procollagen and fibronectin

There was no difference in gene expression of TGF-β and procollagen between *mdx*, *mdx*:MyoD(-/-) and C57:MyoD(-/-) mice as determined by RT-PCR analysis relative to β-actin (see Figure 5.7 and 5.8A).

A significant increase in mRNA levels of fibronectin in *mdx*:MyoD(-/-) compared to C57:MyoD(-/-) mice ($P<0.05$) and in *mdx* compared to C57:MyoD(-/-) ($P<0.05$) was observed, however there was no significant difference between *mdx* and *mdx*:MyoD(-/-) (see Figure 5.8B). This paralleled the change in fibrosis (see fig 5.5)

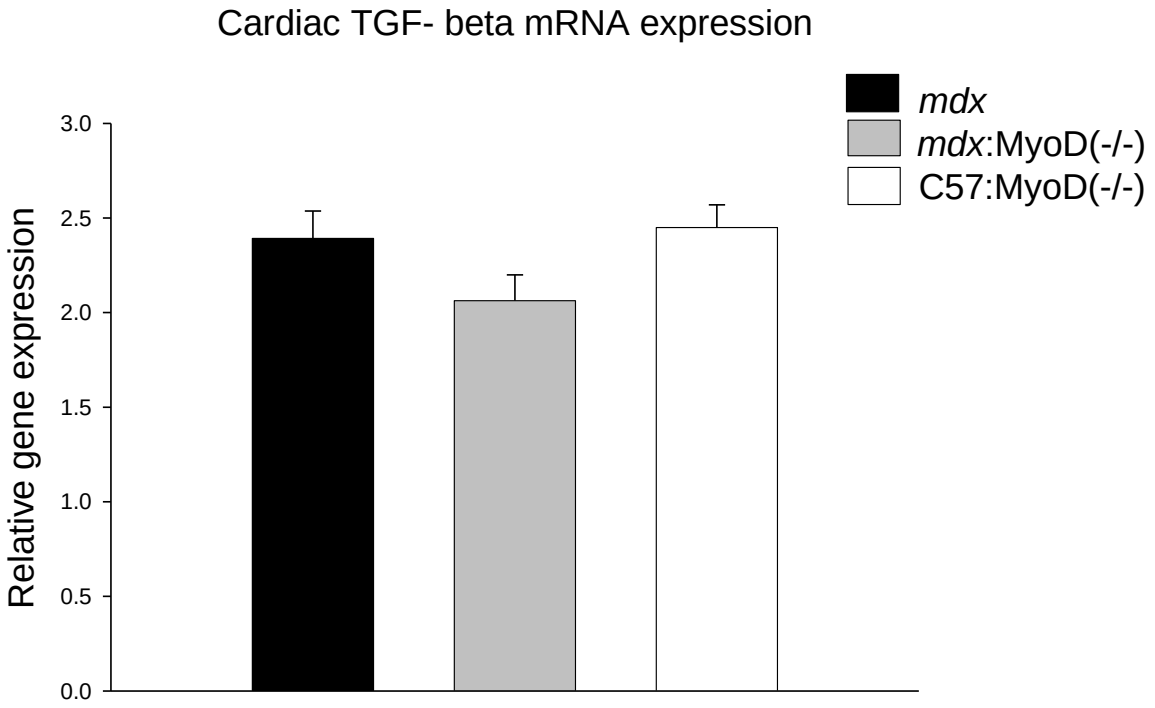


Figure 5.7 Cardiac gene expression levels of TGF-β mRNA as determined by quantitative real time PCR relative to β-actin for *mdx*, *mdx*:MyoD(-/-) and C57:MyoD(-/-) mice at 3 months of age. There was no significant difference in levels of TGF-β mRNA between all strains of mice. (* $P<0.05$, ** $P<0.01$, *** $P<0.001$)

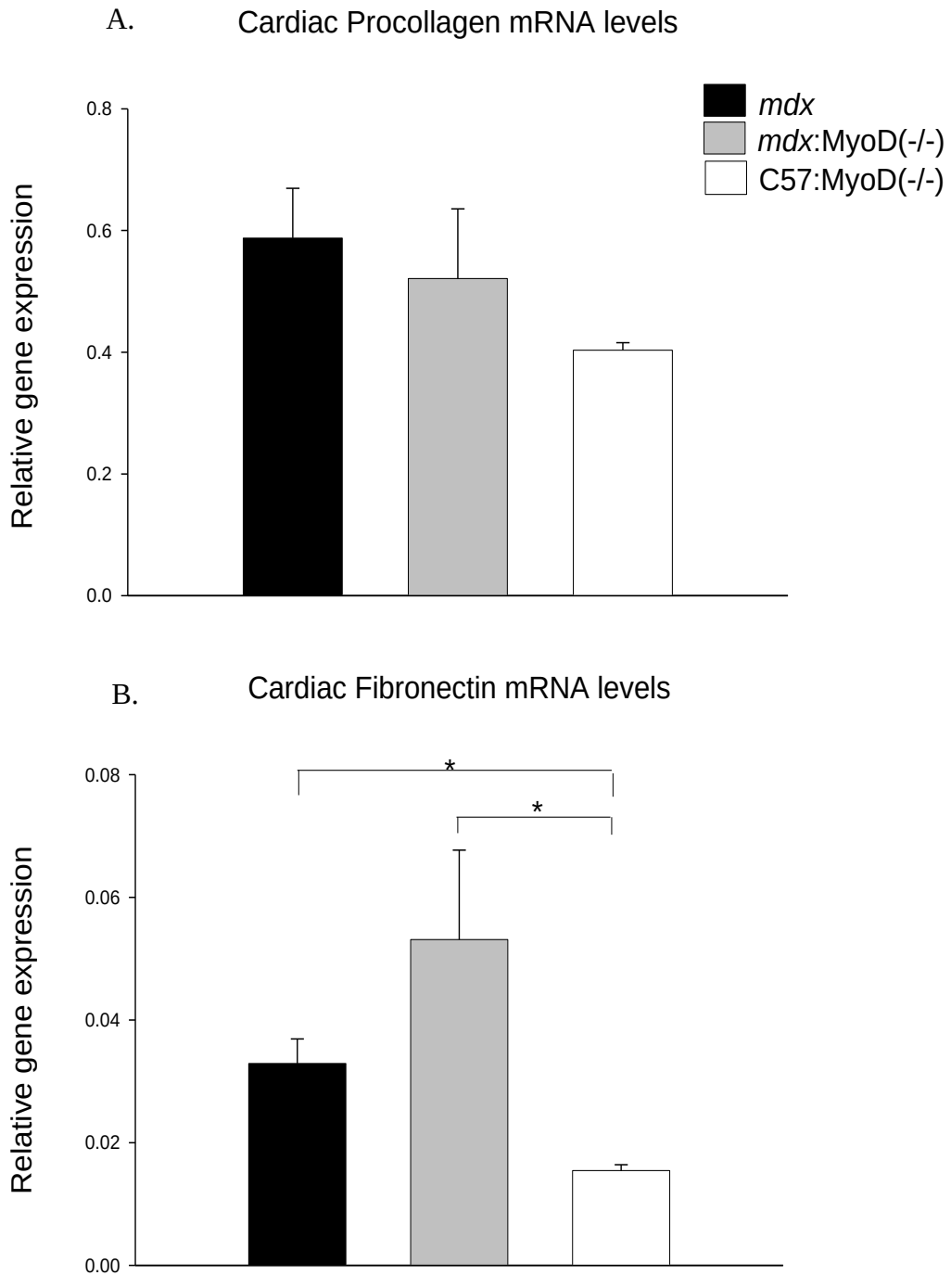


Figure 5.8 Cardiac gene expression of procollagen (A.) and fibronectin (B.) as determined by quantitative real time PCR relative to β-actin of *mdx*, *mdx*:MyoD(-/-) and C57:MyoD(-/-) mice at 3 months of age. There was no significant difference in procollagen mRNA levels, however levels of fibronectin mRNA was increased for both *mdx* and *mdx*:MyoD(-/-) hearts compared to C57:MyoD(-/-).(* P<0.05, **P<0.01, ***P<0.001)

5.3 DISCUSSION

At 3 months of age *mdx*:MyoD(-/-) mice do not exhibit cardiac hypertrophy as was expected, however it has been reported at 5 months of age with areas, predominantly in the left ventricle, showing an increase in cardiomyocyte size (Megeney et al. 1999). This may reflect progressive changes from a normal heart weight at 3 months to sporadic cellular hypertrophy by 5 months (Megeney et al. 1999).

Unexpectedly an increase in cardiac function, systolic and diastolic function was observed in *mdx*:MyoD(-/-) mice at 3 months of age compared to *mdx* mice. This surprising result warrants further investigation as previous data at 5 months of age, suggest that the *mdx*:MyoD(-/-) model displays a more severe cardiomyopathy than the *mdx* mouse.
The increased cardiac function occurred in the face of a significant increase in ventricular fibrosis in *mdx*:MyoD(-/-) mice compared to *mdx* mice and C57:MyoD$^{-/-}$ mice. This paradox may be an early compensatory mechanism of the heart as it is working harder due to the increased skeletal pathology placing an extra work load on the heart. Further, it has been shown in other studies that the changes in cardiac function are not always contingent upon levels of fibrosis (Cohn et al. 2007; Van Erp, Irwin & Hoey 2006). Cardiac hypertrophy did not accompany this increased cardiac function, however other studies have reported hypertrophy at 5 months of age (Megeney et al. 1999).

Ventricular fibrosis was determined by sampling two sections from the left ventricle, two sections from the right ventricle and one from the interventricular septum. Although it was not quantified, fibrosis appeared to be predominant in the left ventricle (data not shown). This was also a feature of *mdx*:MyoD(-/-) mice at 5 months of age, suggesting an accelerated left ventricle involvement in the development of cardiomyopathy in these mice (Megeney et al.

1999). On the other hand, *mdx* mice exhibit fibrosis in both left and right ventricles (Quinlan et al. 2004). The pattern of fibrosis exhibited by *mdx*:MyoD(-/-) mice more closely resembles DMD patients where autopsy studies found fibrosis to be more severe in the left ventricle with a sparing of the right ventricle (Frankel & Rosser 1976). Further to this, echocardiography studies of DMD patients showed myocardial degeneration localised initially to the left ventricular posterior wall, lateral wall and apex, and eventually the ventricular septum and left ventricular anterior wall (Sasaki et al. 1998). In contrast, ultrasound studies on the *mdx* mouse showed diffuse, rather than regional cardiac dyskinesia (Quinlan et al. 2004). The extent and localisation of cardiac fibrosis in the *mdx*:MyoD(-/-) model warrants further investigation, particularly in older animals.

The observed increased fibrosis did not correlate with gene expression levels of TGF-β in*mdx*:MyoD(-/-) mice which were not significantly different to *mdx* or C57:MyoD(-/-) mice. This finding is in accordance with results from the previous age study, in which higher levels of fibrosis observed in *mdx* mice at 6 and 12 months of age did not correlate to increased TGF-β levels. Levels of TGF-β may perhaps increase before 3 months initiating pro-fibrotic pathways. In support of this hypothesis, TGF-β has been shown to be increased early in the *mdx* diaphragm at 6 and 9 weeks, but not at 12 weeks (Gosselin et al. 2004).

Procollagen mRNA levels also did not correlate to fibrosis, with no significant difference in expression observed for *mdx*:MyoD(-/-) mice compared to *mdx* and C57:MyoD(-/-) mice. Fibronectin gene expression, however, was significantly increased in *mdx*:MyoD(-/-) and *mdx* mice compared to C57:MyoD(-/-) mice which paralleled with the observed increase in collagen deposition. This highlights the dynamic nature of the ECM and suggests that fibronectin may be a better marker for active collagen synthesis than procollagen.

The main findings of this study are that *mdx*:MyoD(-/-) mice display an increased cardiac pathology with higher levels of fibrosis than *mdx* at 3 months of age. As MyoD is only expressed in skeletal muscle, the increased cardiac involvement of *mdx*:MyoD(-/-) mice may reflect cardiomyopathy secondary to increased skeletal muscle damage. These results have important implications for potential cardiac therapies for DMD patients as they imply that perhaps treating just the heart will not be sufficient to alleviate the cardiac phenotype.

The observed increased cardiac fibrosis of *mdx*:MyoD mice compared to *mdx* and C57:MyoD(-/-) also supports the hypothesis that increased regeneration may play a role in the milder phenotype of the *mdx* mouse compared to DMD patients.

This study and others provide further support for the lack of correlation between TGF-β and progression of fibrosis with other signalling molecules thought to be involved (Gosselin et al. 2004; Van Erp, Irwin & Hoey 2006). Cardiac fibronectin mRNA levels paralleled increases in ventricular fibrosis implying that this gene may be a useful indicator of collagen synthesis.

5.4 SHORTCOMINGS OF THE STUDY

Due to time constraints on this study, only one age group was examined. Future research of the *mdx*:MyoD(-/-) model needs to address different ages as *mdx* mice exhibit a progressive cardiomyopathy. This study was the first to assess cardiac function of the model. One unexpected finding of the study was the increase in cardiac function in the *mdx*:MyoD(-/-) compared to the *mdx* mouse. Future research would benefit from older age groups to determine if this was a compensatory mechanism. Echocardiography would also provide valuable information regarding the cardiac hypertrophy observed at 5 months.

This study focused on three genes involved in collagen synthesis associated with fibrosis. As

the rate of collagen degradation also plays a pivotal role in collagen accumulation, future research would benefit from an examination of genes involved in collagen degradation such as MMPs and TIMPs.

In conclusion, the findings of increased cardiac involvement of the *mdx*/MyoD(-/-) transgenic mouse observed in this study and others, warrant its further characterisation as a model for DMD cardiomyopathy (Megeney et al. 1996; Megeney et al. 1999).

CHAPTER 6 CONCLUSIONS AND FUTURE DIRECTIONS

Duchenne muscular dystrophy is a debilitating neuromuscular disease that affects 1 in 3500 newborn boys. The disease is characterised by a progressive muscular weakness leading to most boys being wheelchair bound by the age of 11. Most patients die in early adulthood due to respiratory and cardiac failure. The last decade has seen improvements in ventilatory assistance which has prolonged the life span significantly. This in turn has resulted in an increased presentation of cardiac manifestations highlighting cardiomyopathy as a major contributor to mortality.

There is currently no treatment to cure DMD. While gene therapy offers a potential cure, this technology is still years away. An urgent need exists for therapies to alleviate the devastating consequences of the disease including cardiac failure, inflammation and fibrosis.

6.1 STUDY 1 Aging and inflammatory gene expression

The *mdx* mouse is the most commonly used animal model in DMD research. The degree of cardiac involvement in the model appears to be progressive. Cardiac hypertrophy and increased levels of fibrosis were observed at the youngest age examined, 3 months and by 6 months of age decreased systolic and diastolic function is evident supporting the initial hypothesis that the mdx mouse reflects dystrophin deficient cardiomyopathy.

Increased cardiac fibrosis is characteristic of the dystrophic heart and does not necessarily correlate with cardiac function. The fibrotic pathways involved are complex and multifactorial. While increased levels of TGF-β mRNA were observed at 3 months of age, they did not persist with age. This suggests that TGF-β may be involved in the initiation of profibrotic pathways activating collagen synthesis, but perhaps the fibrosis observed later in

the *mdx* mouse life span is a result of decreased collagen degradation. This hypothesis is supported by the literature which demonstrates a role for altered ECM metabolism mediated in part by MMPs and their inhibitors TIMPs (Li, McTiernan & Feldman 2000).

Little data exists regarding cardiac levels of genes involved in remodelling the ECM in the *mdx* mouse, and this study is the first to our knowledge to provide data on fibronectin and procollagen mRNA levels in this model. Expression levels of both of these genes decreased by 12 months of age which may further reflect a shift away from active collagen synthesis toward a decline in its degradation sustaining high levels of fibrosis as shown diagrammatically in figure 6.2.

6.2 STUDY 2 IGF-1 ameliorates fibrosis and improves function in *mdx* hearts at 12 months of age

Evidence from previously reported studies supports the hypothesis that IGF-1 overexpression is capable of ameliorating the fibrosis characteristic of skeletal dystrophic muscle (Barton et al. 2002) and improving cardiac function in various animal models of cardiac disease (Cittadini et al. 1996; Duerr et al. 1995; Kajstura et al. 2001; Santini et al. 2007; Serose et al. 2005). To examine the effects of IGF-1 on the dystrophic heart, this current study involved the generation of a novel transgenic mouse model that overexpressed cardiac restricted IGF-1 isoform Ea in the *mdx* mouse. Two age groups, 6 and 12 months, were utilised to observe any differences in the effect of IGF-1 overexpression with age of the animal.

IGF-1 induced cardiac growth at all ages examined. This hypertrophy was accompanied by improved left ventricular systolic function and increased rate of contraction in the dystrophic heart at 12 months. This demonstrates the involvement of IGF-1 in cardiac hypertrophy, with

functional benefits to *mdx* mice midway through their lifespan and therefore supports the initial hypothesis the study was based on.

IGF-1 overexpression ameliorated fibrosis and improved cardiac function at 12 months of age in the *mdx* mouse as illustrated in figure 6.1. The marked reduction in fibrosis at 12 months of age did not correlate with elevated TGF-β levels, once again supporting the hypothesis that this cytokine is not the main driver in the progression of fibrosis (Fig 6.2). This agrees with evidence from the literature suggesting TGF-β does not play a role in the cardiac inflammatory response after cardio toxin injury and myocardial infarction (Santini et al. 2007).

IGF-1 decreases fibronectin in cell culture (Mezzano et al. 2007), and in the whole heart by 12 months of age as shown in this current study. The reduction of fibronectin mRNA expression correlated with a reduction in procollagen mRNA and reduced fibrosis (Fig 6.2).

6.3 STUDY 3 Cardiac involvement in the *mdx*/MyoD(-/-) mouse model

Skeletal muscle regeneration involves activation of satellite cells. Myogenic regulator, MyoD plays an important role in satellite cell function as it is required for myogenic precursor cells to enter the proliferative phase prior to muscle differentiation (Megeney et al. 1996). It is expressed only in skeletal muscle.

Cardiac pathology in *mdx* mice at 3 months of age was exacerbated by removal of MyoD from the germline. The *mdx*/MyoD(-/-) double knock out mouse exhibited increased cardiac fibrosis which appeared secondary to the increased skeletal muscle myopathy observed by

others. This suggests that skeletal muscle damage may play a role in the degree of cardiomyopathy observed with dystrophin deficiency.

To further examine the effects of skeletal muscle pathology on the heart, it would be valuable to perform histological and functional experiments on the hearts of *mdx* mice that overexpress IGF-1 in skeletal muscle only, excluding the heart. These mice display reduced fibrosis and increased force generation in skeletal muscles (Barton et al. 2002). To date cardiac functional experiments have not been performed on this mouse model. Any cardiac improvements in this model would be attributable to decreased skeletal muscle pathology.

Surprisingly, cardiac function of the *mdx*:MyoD(-/-) mouse was found to increase and this could be regarded as an early compensatory mechanism resulting from the extra work load placed on the heart by the increased skeletal muscle pathology. Future research addressing cardiac function in older age groups is required to determine the duration of this phenomenon.

Mdx mice display a milder phenotype than DMD and this may be due in part to increased regeneration. The increased cardiac pathology exhibited by the *mdx*:MyoD(-/-) mice compared to *mdx* and C57:MyoD(-/-) lends support to this hypothesis (Fig 6.1).

The *mdx*:MyoD(-/-) model further highlighted the lack of correlation between levels of ventricular fibrosis and TGF-β mRNA levels, further supporting the notion of the lack of involvement of TGF-β in the progression of fibrosis in dystrophic hearts. Procollagen mRNA levels also did not correlate with higher levels of fibrosis exhibited by *mdx*:MyoD(-/-), however fibronectin levels did. The IGF-1 study also found a correlation between fibronectin

levels and fibrosis, with decreased fibrosis of IGF-1+/*mdx* hearts at 12 months of age accompanied with decreased fibronectin mRNA levels. This suggests that fibronectin may be a better marker of active collagen synthesis than procollagen (Fig 6.2).

The increased cardiac fibrosis of the *mdx*:MyoD(-/-) compared to the *mdx* mouse supports the hypothesis that it may reflect DMD cardiomyopathy more closely, however the improvement in cardiac function calls for further characterisation of the model.

All three studies in this dissertation highlighted the complex pathways involved in the initiation and progression of fibrotic pathways in the dystrophic heart. Future research into the molecular pathways of fibrosis needs to address cardiac levels of MMPs and TIMPs due to the important roles they play in the balance between ECM deposition and degradation. Expression of other cytokines such as proinflammatory IL- 6 and anti-inflammatory IL-4 and 10, are also of interest as these have been implicated in the inflammatory response of the heart.

While an attempt has been made in each study to examine suitable age groups, there exists a need to include younger and older age groups. Younger ages could provide a more detailed view of the initiation of fibrotic pathways, while the older age groups enable experiments to be performed on symptomatic cardiomyopathy.

Further research in each of the three studies would also benefit from additional cardiac *in vivo* analysis using echocardiography to provide data on cardiac output and ejection fraction and Millar catheter experiments to provide blood pressure and volume.

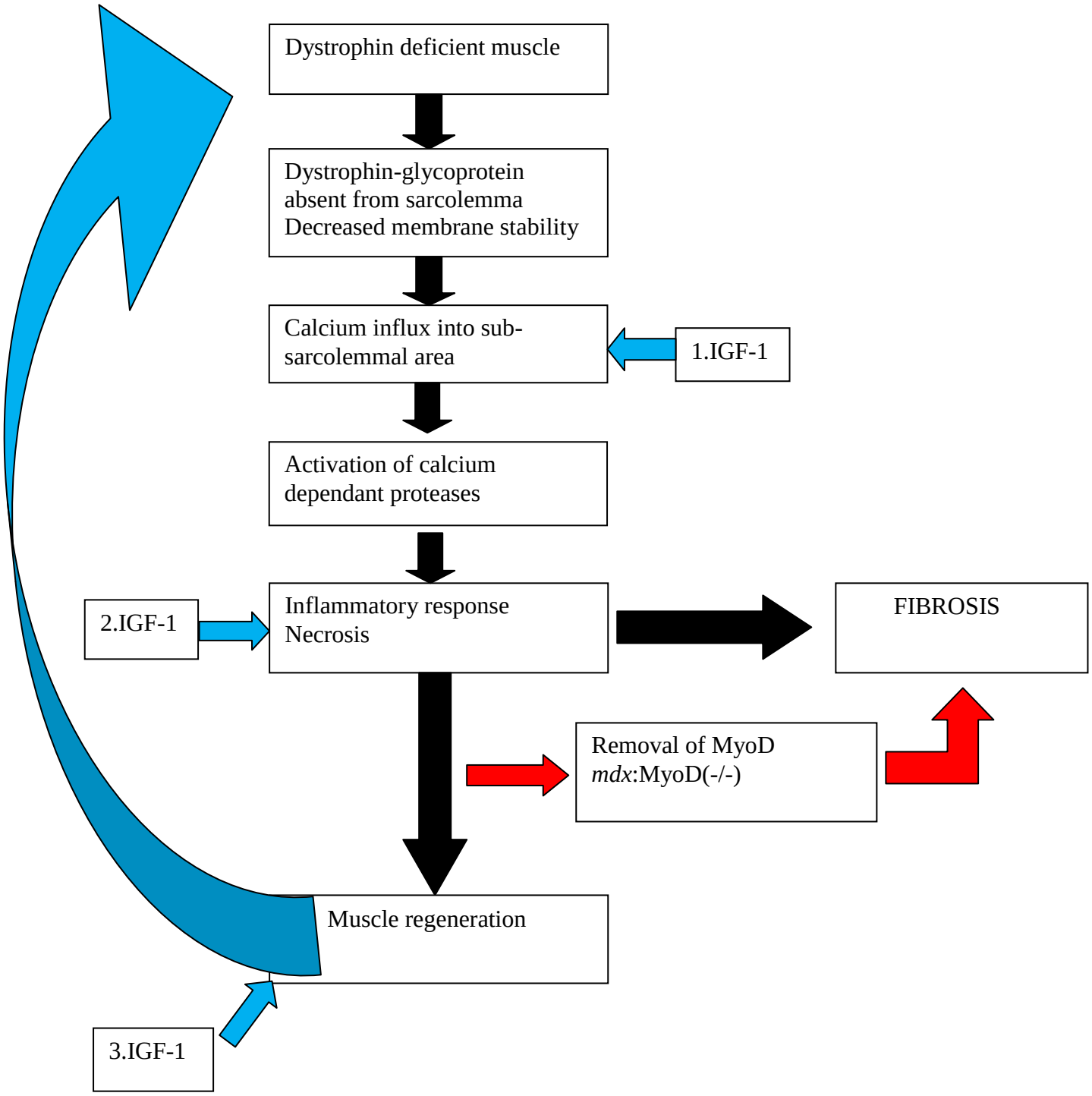
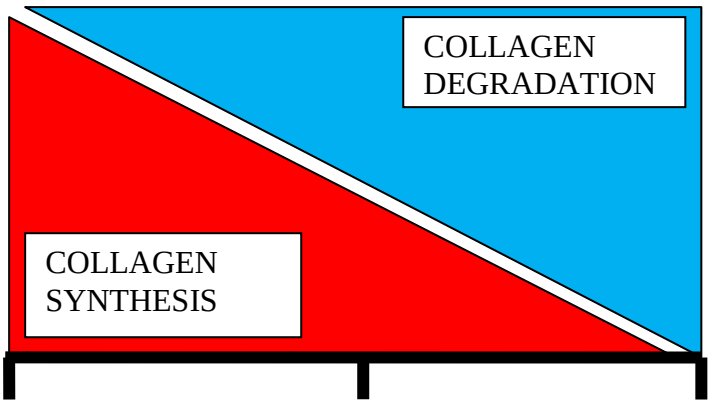


Figure 6.1 Flow chart combining IGF-1 and MyoD contributions to pathophysiology of dystrophic deficient muscle. Dystrophin deficiency in cardiomyocytes results in a loss of the DGC at the sarcolemma, increasing its permeability. This leads to an influx of calcium, activating calcium proteases.
1.IGF-1 may increase cardiac contractility by increasing cardiomyocyte calcium sensitivity. The inflammatory response is activated resulting in necrosis.
2.IGF-1 may limit fibrosis by inhibiting necrosis, resolving the inflammatory response and/or attenuating the renin-angiotensin system.
3.IGF-1 induces proliferation and differentiation of satellite cells enhancing regeneration. Removal of myogenic regulator MyoD from the germline of *mdx* mice reduces the efficiency of regeneration, increasing fibrosis.



Increased cardiac mRNA levels of TGF-β, FN, procollagen		
3 months of age	6 months of age	12 months of age
↑TGF-β *mdx* ↑FN *mdx*:MyoD	↑FN ↑Procollagen IGF-1+/*mdx* ↑FN ↑Procollagen *mdx*	No increase *mdx*

Figure 6.2 Diagrammatical representation of the balance between collagen synthesis and collagen degradation in the hearts of *mdx*, IGF-1+/*mdx* and *mdx*:MyoD(-/-) mice at 3, 6 and 12 months of age.

At 3 months of age increased mRNA levels of TGF-β in *mdx* mice and elevated mRNA levels of fibronectin (FN) in *mdx*:MyoD(-/-) suggesting active collagen synthesis contributing to high levels of fibrosis observed in both strains.

At 6 months of age *mdx* and IGF-1+/*mdx* mice both exhibit increased fibrosis that correlates with increased mRNA levels of FN and procollagen.

By 12 months of age ventricular fibrosis is elevated in the *mdx* mouse, but this no longer correlates with cardiac mRNA levels of these TGF-β, FN or procollagen suggesting a shift toward decreased collagen degradation maintaining high levels of fibrosis.

6.4 CONCLUSION

While gene therapy theoretically offers the best chance of a cure for DMD patients its implementation is still years away. In the interim, treatment strategies are aimed at alleviating the symptoms of DMD and focus on promoting muscle regeneration, hypertrophy or relieving fibrosis.

This current study provides evidence for the first time that IGF-1 overexpression can mediate cardiac hypertrophy in the dystrophic heart. Importantly this overexpression was associated with improved cardiac function and rates of contraction. One striking finding of this study was the ability of IGF-1 to significantly reduce ventricular fibrosis by 12 months of age. Cardiac fibrosis severely impacts on the life expectancy of DMD patients causing left ventricular dysfunction and arrhythmias, and eventually systolic dysfunction may lead to heart failure and sudden death (Finsterer & Stollberger 2003). While the difficulties of transferring findings from this transgenic model to the clinical situation are acknowledged, the results of this study implicate IGF-1 as a potential therapy that may enhance muscle mass, reduce ventricular fibrosis and improve function in the dystrophic heart. The complex interplay of factors involved in fibrotic pathways was further highlighted by this series of experiments and more research is required to further elucidate the exact molecular pathways.

Finally, there exists a need for animal models that more accurately reflects the cardiomyopathy associated with DMD. The *mdx*:MyoD(-/-) mouse characterised in this study offers potential as a model of cardiac fibrosis. However the unexpected findings of functional improvement in the face of fibrosis means that further research is required including *in vivo* studies (echocardiography or Millar catheter experiments) and the use of mice of different ages.

In summary the findings from this study may contribute to the knowledge of IGF-1 as a potential cardiac therapy, the prospect of an alternative mouse model for DMD cardiomyopathy, and further elucidation of cytokines, protein and growth factor involvement in fibrotic pathways in the dystrophic heart.

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