The Role of Myostatin During Postnatal Myogenesis and Sarcopenia

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Abstract

Myostatin, a TGF-β superfamily member, is a key negative regulator of embryonic and postnatal muscle growth. In order to further elucidate the role of myostatin during postnatal growth, several lines of investigation were undertaken in mice. Analysis of myostatin downstream target genes identified several known and unknown genes. From these, the regulation of an androgen receptor binding co-factor, ARA70, was selected for further investigation. Reverse Northern analysis on the differentially expressed cDNA library indicated an increased expression of *ARA70* in *myostatin*-null muscles, which was later confirmed by Northern blot and semi-quantitative PCR analysis. In corroboration, treatment of myoblast cultures with exogenous myostatin resulted in the down-regulation of *ARA70,* confirming that myostatin is a negative regulator of *ARA70* gene expression. The role of myostatin during sarcopenia, a progressive age-related loss of skeletal muscle mass and strength, was also investigated. The atrophy associated with sarcopenia is frequently correlated with insufficient muscle regeneration, resulting from an impaired propensity of satellite cells to activate and a subsequent decline in myogenesis. Myostatin is a known inhibitor of postnatal satellite cell activation and muscle regeneration, thus muscle mass and regeneration, and satellite cell behaviour were examined in young and aged *myostatin*-null mice. *Myostatin*-null mice had increased individual muscle weights, as a consequence of massive fibre hypertrophy and hyperplasia, and an increased proportion of type IIB fibres. Aging induced oxidative fibre type changes and atrophy in the wild-type muscle while no fibre type switching was observed in the *myostatin*-null muscle and atrophy was minimal. No decrease in satellite cell numbers was observed with aging in both genotypes; though a gradual decline in the number of activated satellite cells was noted during aging. However, both young and aged *myostatin*-null mice displayed increased satellite cells and activation compared to wild-type mice, suggesting a greater myogenic potential in the *myostatin*-null satellite cells. Consistent with this, aged *myostatin*-null myoblasts proliferated faster and displayed a higher fusion index during differentiation than the aged wild-type myoblasts, confirming that the reduced sarcopenia in the *myostatin*-null mice was due to a preserved increase in the

myoblast myogenic activity. An increase in a Pax7-only myoblast population from *myostatin*-null muscle indicated an enhanced satellite cell self-renewal process, consistent with the increased satellite cell number observed on the *myostatin*-null muscle fibres. Additionally, muscle regeneration of aged *myostatin*-null muscle following notexin injury was accelerated, and fibre hypertrophy and type were recovered with regeneration, unlike the aged wild-type muscle. Testing the therapeutic value of a myostatin antagonist, Mstn-ant1, indicated that a short term blockade of myostatin by the antagonist significantly enhanced muscle regeneration in aged mice after injury and during sarcopenia. Antagonism of myostatin led to satellite cell activation, increased Pax7 and MyoD protein levels, and greater myoblast and macrophage cell migration culminating in enhanced muscle regeneration in the aged mice.

In conclusion, the hypertrophic phenotype associated with *myostatin*-null mice may in part result from increased androgen receptor (AR) activity due to the up-regulation of *ARA70*, given that increased expression of the *AR* leads to hypertrophy. Additionally, the increased muscle mass in *myostatin*-null mice is likely to result from an augmented myogenic potential and self-renewal process. Overall, a prolonged absence of myostatin reduced sarcopenia and the associated loss of muscle regenerative capacity. Furthermore, the antagonism of myostatin displayed significant therapeutic potential in the alleviation of sarcopenia, through the restoration of the myogenic and inflammatory responses in the aged environment. Thus, the research work clearly demonstrates the role of myostatin in sarcopenia, and documents for the first time a valid therapeutic for alleviating sarcopenia.

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"Therefore, since we are surrounded by such a great cloud of witnesses, let us throw off everything that hinders and the sin that so easily entangles, and let us run with perseverance the race marked out for us."
Hebrews 12:1

Table of Contents

List of Figures

List of Tables

List of Abbreviations

Abbreviated terms and products

3’	3 prime
5’	5 prime
α	alpha
A	adenine
Act RIIA/B	activin receptor type II A/B
ANOVA	analysis of variance
AR	androgen receptor
ARA70	androgen receptor associated protein-70
ARE	androgen response element
ARLBD	androgen receptor ligand binding domain
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
β	beta
BCIP	5-bromo-4-chloro-3-indoyl phosphate
BF	*m. biceps femoris*
bHLH	basic helix-loop-helix
BLAST	Basic Local Alignment Search Tool
BMP	bone morphogenetic protein
bp	base pair(s)
BrdU	5-bromo-2’-deoxy-uridine
BSA	bovine serum albumin
°C	degrees Celsius
CDK	cyclin-dependent kinase
cDNA	complimentary DNA
CEE	chicken embryo extract
Ci	Curie
CKI	cyclin-dependent kinase inhibitor
cλ	carrageen lambda
CLFS	chronic low frequency stimulation
CSF	colony stimulating factor

d	distilled
DAPI	4',6-diamidino-2-phenylindole
dCTP	2'-deoxycytidine 5'-triphosphate
DEPC	diethyl pyrocarbonate
DHT	5α-dihydrotestosterone
DIG	digoxigenin
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDL	*m. extensor digitorum longus*
EDTA	ethylenediaminetetraacetic acid
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
g	gravity
g	gram(s)
G	guanidine
G0, 1, 2	gap 0, 1 or 2 phase of the cell cycle
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gas	*m. gastrocnemius*
GFP	green fluorescent protein
H & E	haematoxylin and eosin
HIV	human immunodeficiency virus
HLH	helix-loop-helix
hr	hour(s)
HRP	horseradish peroxidase
HS	horse serum
ICC	immunocytochemistry
IgG	immunoglobin G
IGF	insulin-like growth factor
IPTG	isopropyl thio-β-galactoside
k	kilo (10^3)

kDa	kilo Dalton(s)
KO	*myostatin*-null
L	litres
LacZ	β-galactosidase reporter gene
LAP	latency associated protein
LB	Lennox L broth
LBD	ligand binding domain
LMP	low melting point
m	meter
m	milli (10^{-3})
m	*musculus*
M	molar, moles per litre
M	mitotic phase of the cell cycle
μ	micro (10^{-6})
μl	microlitre
μM	micromolar
mA	milliamp(s)
mATPase	myofibrillar adenosine triphosphatase
mdx	muscle dystrophy mouse phenotype
MHC	myosin heavy chain
min	minute(s)
MOPS	3-(N-morpholine)-propane-sulfonic acid
MQ	milli Q
MRF	myogenic regulatory factor
mRNA	messenger RNA
MSTN	myostatin
n	nano (10^{-9})
NBT	nitroblue tetrazolium chloride
NDS	normal donkey serum
NGS	normal goat serum
NIH	National Institutes of Health
nm	nanometre
nM	nanomolar (10^{-9})
NP-40	Nonidet P-40

NSS	normal sheep serum
OCT	optimal cutting temperature
OD	optical density
P	probability
PAGE	polyacrylamide gel electrophoresis
Pax	paired-box
PBS	phosphate buffer saline
PBS-T	phosphate buffer saline with Tween 20
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PEG	polyethylene glycol
PET	polyethylene terephthalate
pH	hydrogen ion concentration
PVP	polyvinylpyrrolidone
Quad	*m. quadriceps*
Rb	retinoblastoma susceptibility gene product
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
RSRR	arginine-serine-arginine-arginine
RT	reverse transcriptase
RT-PCR	reverse transcription-polymerase chain reaction
S	synthesis phase of the cell cycle
SDS	sodium dodecyl sulphate
s	second(s)
SEM	standard error of the mean
Smad	mammalian homolog to mothers against decapentaplegic
SSC	saline sodium citrate
SSH	suppressive subtraction hybridisation
TA	*m. tibialis anterior*
TAE	tris acetate EDTA
Taq	DNA polymerase from *Thermus aquaticus*
TBS	tris buffered saline
TBS-T	TBS with Tween 20

TE	tris-EDTA
TGF-β	transforming growth factor-β
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
tRNA	transfer RNA
U	units of enzyme
UTP	uridine triphosphate
UV	ultraviolet
VO2max	highest oxygen consumption rate during maximal exercise
WT	wild-type
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight
XAR	X-ray autoradiography
X-gal	X-5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YNB	yeast nitrogen base
YPD	yeast peptone dextrose
ZAMS	Zymosan A activated mouse serum

Abbreviations and locations of commercial suppliers

Amersham	Amersham plc, Buckinghamshire, UK
BD Biosciences	Becton Dickinson Biosciences, Franklin Lakes, NJ, USA
BDH	BDH, Merck KGaA, Darmstadt, Germany
BD PharMingen	BD PharMingen, San Diego, CA, USA
Biolab	Biolab Scientific, Auckland, New Zealand
Bio-Rad	Bio-Rad Laboratories, Hercules, CA, USA
Boehringer	Boehringer Mannheim, Mannheim, Germany
DSHB	Developmental Studies Hybridoma Bank, Iowa City, IA, USA
Bromley Park Hatcheries	Bromley Park Hatcheries, Tuakau, New Zealand
Clontech	Clontech Laboratories, Mountain View, CA, USA
Corning	Corning Inc., Life Sciences, Acton, MA, USA
Dage-MTI	Dage-MTI, Michigan City, IN, USA

Dako	DakoCytomation, Glostrup, Denmark
Diagnostic Instruments	Diagnostic Instruments, Sterling Heights, MI, USA
Esco	Esco Product, Oak Ridge, NJ, USA
Gibco	Gibco BRL, Invitrogen, Carlsbad, CA, USA
Hybaid	Hybaid, Franklin, MA, USA
Invitrogen	Invitrogen, Carlsbad, CA, USA
Kodak	Kodak, Rochester, NY, USA
Leica	Leica Microsystems GmbH, Wetzlar, Germany
Millipore	Millipore Corporation, Billerica, MA, USA
Molecular Devices	Molecular Devices, Sunnyvale, CA, USA
Molecular Probes	Molecular Probes, Invitrogen, Carlsbad, CA, USA
Muromachi	Muromachi, Tokyo, Japan
NanoDrop Technologies	NanoDrop Technologies Wilmington, DE, USA
Novagen	Novagen, Madison, WI, USA
Novex	Novex, San Diego, CA, USA
Nunc	Nunc A/S, Roskilde, Denmark
Olympus	Olympus Optical, Tokyo, Japan
OriGene	OriGene Technologies, Rockville, MD, USA
Oxoid	Oxoid Limited, Hampshire, UK
Owl Separation Systems	Owl Separation Systems, Portsmouth, NH, USA
PerkinElmer	PerkinElmer Wellesley, MA, USA
Promega	Promega Corporation, Madison, WI, USA
Qiagen	QIAGEN, Hilden, Germany
Research Diagnostics	Research Diagnostics, Flanders, NJ, USA
R&D Systems	R&D Systems, Minneapolis, MN, USA
Roche	Roche Diagnostics GmbH, Mannheim, Germany
Sakura	Sakura Finetek USA Inc, Torrance, CA, USA
Santa Cruz	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Sigma	Sigma, St Louis, MO, USA
Stratagene	Stratagene Cloning Systems, La Jolla, CA, USA
Thermo Scientific	Thermo Fisher Scientific Inc, Waltham, MA, USA
Venom Supplies	Venom Supplies Limited, Tanunda, Australia

Chapter One

Review of Literature

In this thesis, the role of myostatin during postnatal growth and sarcopenia was examined. Thus, to provide background for the work, this Chapter reviews the current literature on skeletal muscle structure, and prenatal and postnatal myogenesis. Sarcopenia is then discussed, focussing on the phenotype of sarcopenic muscle and the causes behind the condition. Later, the structure and function of myostatin is addressed with particular emphasis on the regulation of postnatal myogenesis and muscle wasting. Finally, the aims of this thesis are reviewed.

1.1 The structure of skeletal muscle

Vertebrate skeletal muscle is composed of numerous individual contractile cells (myocytes) commonly referred to as muscle fibres (Figure 1.1). Each muscle contains, or is surrounded by, connective tissue sheaths; endo-, peri- and epimysium (Borg and Caulfield 1980). While the whole muscle is surrounded by the epimysium, the perimysium groups tens to several hundred myofibres into fascicles. Each myofibre contains of a large number of contractile myofibrils, and possesses an extracellular basal lamina (Timpl and Dziadek 1986). It is also individually surrounded by the endomysium. Microscopic studies on myofibrils have identified alternating light (I-bands) and dark (A-bands) areas, as well as a band transversally bisecting the I-band (the Z-line). The pattern of bands between one Z-line and the next is referred to as a sarcomere. Myofibrils display this specific banding pattern due to the arrangement of two types of protein filaments; thick filaments, mainly consisting of myosin, and thin filaments, largely containing actin. The thin filament consists of two strings of actin units and a strand of nebulin arranged in a double helical formation and is associated with tropomyosin and troponin, both of which are involved in the regulation of muscle contraction (Ebashi *et al.,* 1969). Each myofibre is connected with a single motor neuron, which can innervate over one thousand myofibres, allowing them to respond to impulses conducted by motor neurons of the spinal cord or brain stem.

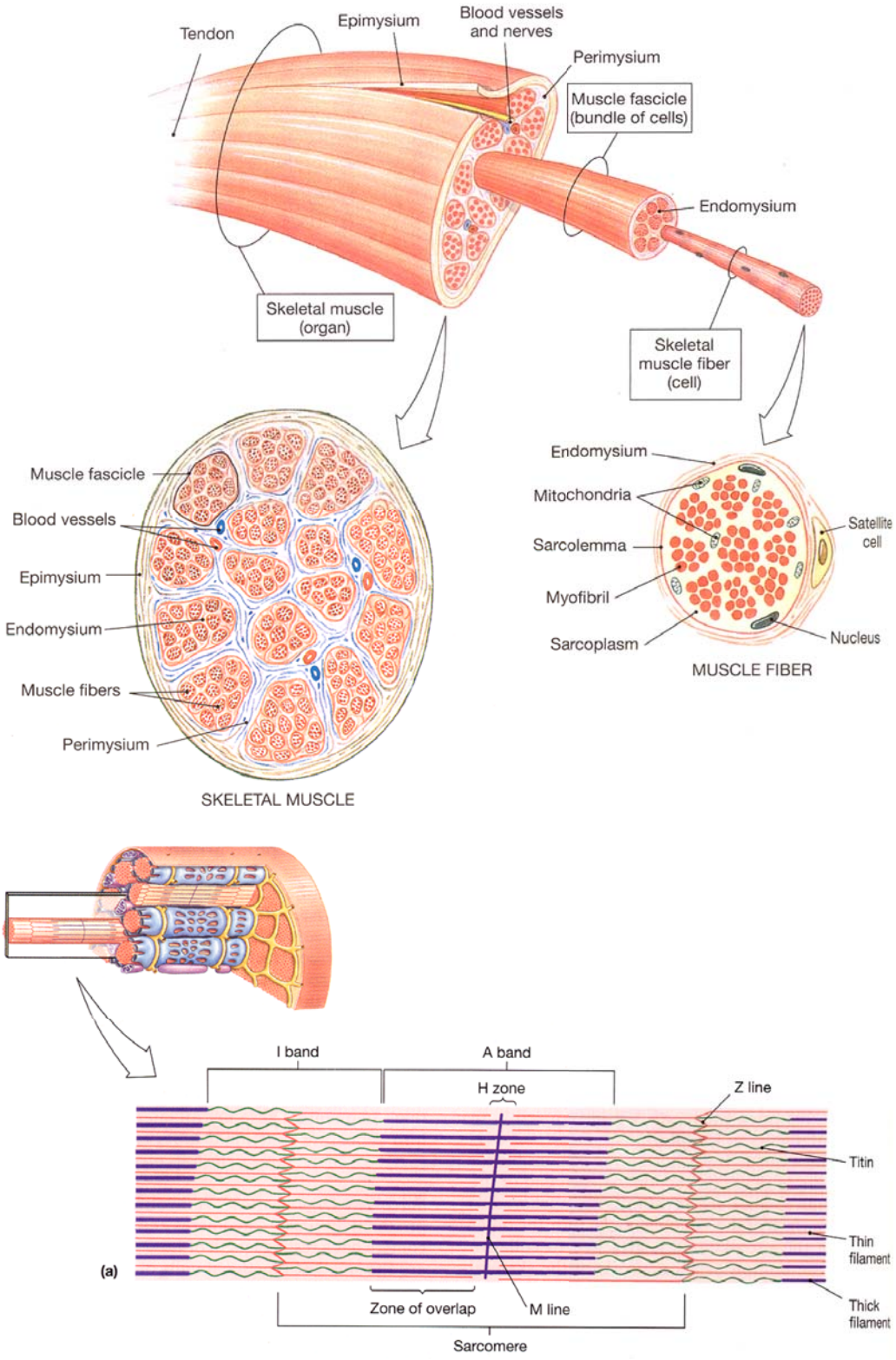

Figure 1.1 The structure of skeletal muscle

A skeletal muscle consists of fascicles enclosed by the epimysium. The bundles are separated by the perimysium, and within each fascicle, the muscle fibres are surrounded by the endomysium. Each myofibre contains numerous myofibrils, consisting of repeating sarcomeres along the myofibril length. Adapted from Martini (1998).

The junction between the muscle and the terminal branches of the axon is referred to as the motor end plate or neuromuscular junction, and is responsible for the transduction of neural signals to the muscle fibre. Myofibers also express specific proteins for contractile function and metabolic enzymes. Individual muscles are composed of a mixture of fibres types and thus have different physiological properties, depending on what proteins each myofiber expresses (discussed further in Section 1.4.1). As muscle fibres are the basic contractile units of skeletal muscles, the proportion of each fibre type within a muscle determines its overall contractile property.

1.2 Skeletal muscle function

During muscle contraction the sarcomere length decreases. However, only the I-band shortens, while the A-band length remains the same. A theory for these phenomena was first proposed in 1954, which described the shortening of the sarcomere as a result of the thick and thin filaments sliding past one another (Huxley and Niedergerke 1954; Huxley and Hanson 1954). Examination of rigor muscle provided the initial evidence of the thick and thin filaments being linked by projections (myosin heads), to form cross-bridges with the actin molecules (Huxley 1953; Huxley 1957). These cross-bridges were proposed to cause the relative motion of the filaments. The sliding filament theory later detailed the steps involved in muscle contraction, and includes the following. Prior to contraction, the myosin heads are tightly bound to the actin filament. The binding of an ATP molecule induces the release of the myosin head from actin, while ATP hydrolysis alters the myosin head angle. Calcium then binds to troponin, which shifts the tropomyosin, leading to the exposure of the myosin-binding sites on the actin filament. This allows the myosin heads to re-bind to the actin filament. The subsequent release of the products of ATP hydrolysis induces a conformational change in the myosin head back to its initial state, pulling the thin filaments inward, resulting in sarcomere shortening, and thus contraction. The initiation of this muscle contraction process is via nervous impulses, which alter the electrical state of the muscle fibre membrane. An interconnective system of transverse tubules (t-tubules) transfers this change into the fibre interior causing the release of calcium from the sarcoplasmic reticulum surrounding each myofibril (Huxley 1967). This change in calcium concentration produces an alteration in the thin

filament structure allowing the cross-bridges to form and the muscle to contract. Connective tissue within the muscle combines with the contractile myofibres creating a functional unit. Myofibre contraction is then transformed into movement via myotendinous junctions at each end of the myofibre, which attach the muscle to the skeleton via tendons.

1.3 Prenatal myogenesis

1.3.1 Prenatal muscle development

In vertebrates, skeletal muscle is derived from the somites, which are segmental blocks of paraxial mesoderm that develop on each side of the neural tube (reviewed by Christ and Ordahl (1995) and Cossu *et al*. (1996)). Somites differentiate dorsally into dorsal cells of the dermomyotome (Figure 1.2), and ventrally into the sclerotome. While the sclerotome gives rise to cells that form the ribs and vertebrae, emanating from the dermomyotome are the muscle precursor cells (myoblasts) as well as cells that contribute to other tissues. Dermomyotomal cells migrate dorsally to give rise to the epaxial myotome (source of trunk and back muscles) and ventrally to give rise to the hypaxial myotome (source of limb muscle). Three categories of myoblasts exist; embryonic, foetal and adult (or satellite cells). All of these cell types originate from the hypaxial region of the somite in the early embryo (White *et al.,* 1975; Feldman and Stockdale 1992; Smith and Miller 1992). Following migration to the limb bud, embryonic myoblasts begin to fuse into primary fibres. After a period of time, the primary myoblast population is replaced by foetal myoblasts, which are subsequently replaced by a population of myoblasts that have adult or satellite cell-like features (Miller and Stockdale 1986a; Miller and Stockdale 1986b). Satellite cells first emerge during late foetal development coinciding with the formation of the basal lamina, and are the last myoblast population to appear during muscle development (Ontell and Kozeka 1984; Feldman and Stockdale 1991; Feldman and Stockdale 1992; Hartley *et al.,* 1992). In contrast to this sequential appearance of the different classes of myoblasts, a human skeletal muscle study has demonstrated that all the human myoblast populations concurrently exist as soon as the first primary myotubes are formed (Edom-Vovard *et al.,* 1999). Furthermore, the authors suggested that it is the proportion

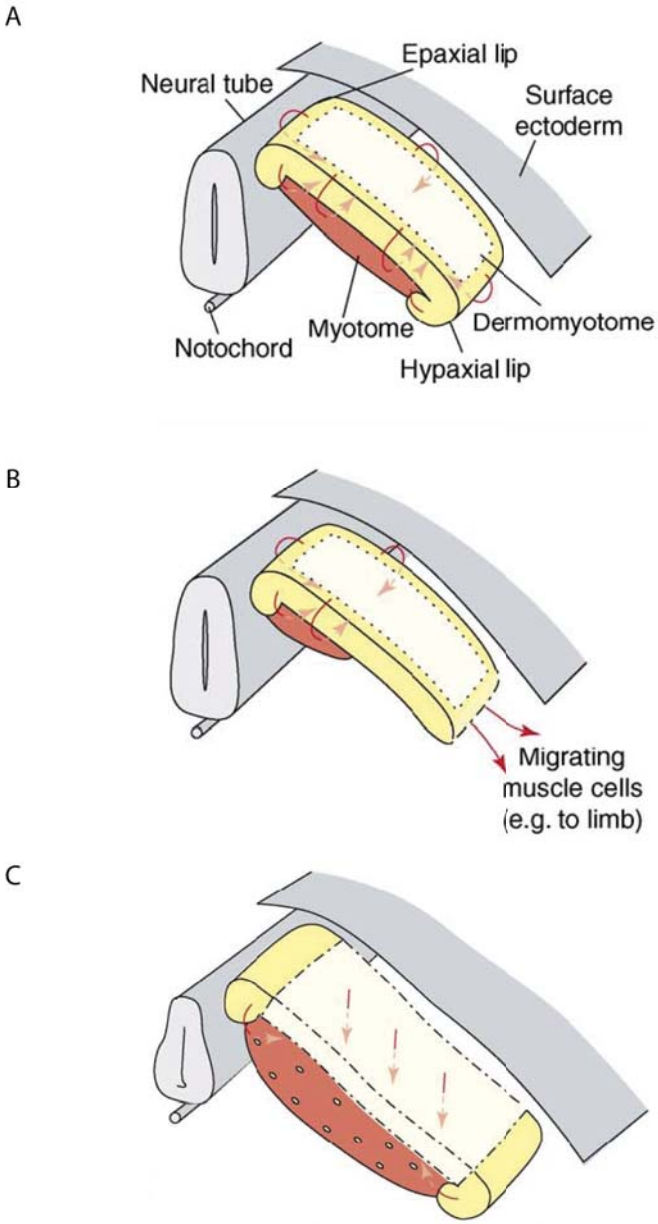

Figure 1.2 Prenatal myogenesis

(A) During the early stages of myogenesis, cells delaminate from the edges of the epithelial dermomyotome, initially mainly from the epaxial lip and then from the other edges. Cells from the hypaxial lip are a major source of myogenic cells. They express Myf5 and Mrf4, and form the skeletal muscle of the early myotome. (B) At certain axial levels, Pax-positive cells delaminate and migrate from the hypaxial dermomyotome to found muscle masses elsewhere. Some of these cells retain their Pax-positive progenitor cell status, contributing to later muscle growth. (C) At later stages, the central region of the dermomyotome loses its epithelial structure and Pax-positive cells enter into the muscle mass of the myotome. These provide progenitor cells for all subsequent muscle growth in the muscle masses of the trunk. Modified from Buckingham *et al*. (2006)

of the myoblast populations that differs during development, rather than their sequential appearance. Various studies support the hypothesis that muscle precursor cells, including the myogenic satellite cell population, emanate from the multipotential mesodermal cells of the somite (Schultz and McCormick 1994; Ordahl *et al.,* 2000). This concept has been challenged by studies suggesting that precursors of the satellite cell may be multipotential cells of nonsomitic origin (De Angelis *et al.,* 1999; Ordahl 1999). In one of these studies, cells isolated from the embryonic dorsal aorta were shown to possess a similar morphological appearance and gene expression profile to that of satellite cells (De Angelis *et al.,* 1999). However, agreement on the precise origin of muscle precursor cells remains unresolved.

1.3.2 Fibre formation

Muscle mass is increased by three mechanisms: muscle precursor cell proliferation; formation of muscle fibres (both prominent during embryonic development); and fibre hypertrophy, which begins during foetal development and dominates postnatally. Several waves of fibre formation occur during early development (reviewed by Stockdale (1997)). The primary wave occurs during embryonic development and produces primary fibres. These are formed from the first population of myoblasts and act as a scaffold for future fibres. This process involves myoblasts, from differing populations, fusing together to produce myofibres (Stockdale and Holtzer 1961). The second myogenic wave is initiated during late embryonic/early foetal development and forms the secondary fibres responsible for much of the muscle mass. This muscle growth is due to the rapid increase in fibre number, nucleation, and fibre diameter, occurring on the surface of the primary fibres (reviewed by Patel *et al*. (2002)).

1.3.3 Myogenic regulatory factor signalling

Basic helix-loop-helix (bHLH) proteins are a large family that function primarily as transcriptional activators. Two distinct domains characterise this group of proteins: the helix-loop-helix (HLH) domain (which facilitates dimerisation) and the basic region (containing positively charged amino acids that mediate binding to DNA). Myogenic regulatory factors (MRFs) are a subgroup of this family, as they share a region of homology with the two functionally

significant domains. It is generally accepted that MRFs play a vital regulatory role in the development of skeletal muscle (Buckingham 1992). This family of transcription factors is exclusively expressed in skeletal muscle, and includes MyoD (Tapscott *et al.,* 1988; Sassoon *et al.,* 1989; Weintraub 1993), myogenin (Braun *et al.,* 1989a; Edmondson and Olson 1989; Wright *et al.,* 1989), Myf5 (Braun *et al.,* 1989b) and MRF4, (also called herculin and Myf6) (Rhodes and Konieczny 1989; Miner and Wold 1990). All of the MRF family members are expressed in somites as well as developing muscle during embryogenesis, and function by forming heterodimeric DNA-binding complexes with other bHLH transcription factors to regulate gene expression (Weintraub *et al.,* 1989; Lassar *et al.,* 1991; Martin *et al.,* 1992). Over the years, the essential role of the MRF family during myogenesis has been established, particularly through the practice of gene disruption technologies (Braun *et al.,* 1992; Rudnicki *et al.,* 1992; Hasty *et al.,* 1993; Nabeshima *et al.,* 1993; Patapoutian *et al.,* 1995; Zhang *et al.,* 1995). For example, Kassar-Duchossoy *et al*. (2004) demonstrated, using triple-mutant mice, that MyoD, Myf5, and MRF4 are all essential for the specification of the skeletal muscle lineage. In addition, inactivation of myogenin has been shown to result in perturbed muscle differentiation and neonatal lethality (Hasty *et al.,* 1993; Nabeshima *et al.,* 1993). Various studies indicate that these transcription factors are activated sequentially (which appears to be species specific) during the skeletal muscle developmental program. For example, in mice, *Myf5* mRNA is first detected in the day 8 somite and is later reduced after day 14 (Ott *et al.,* 1991). On day 8.5, *myogenin* mRNA appears and is continually expressed throughout foetal development (Sassoon *et al.,* 1989). *MRF4* mRNA is transiently expressed on days 10 and 11, then again from day 16 and continues to be until after birth (Bober *et al.,* 1991), whereas *MyoD* mRNA is expressed throughout development after appearing around day 10.5 (Sassoon *et al.,* 1989).

1.3.4 Regulation of myogenesis by paired-box transcription factors

Other key regulators of myogenesis are the paired-box (Pax) transcription factors, which include Pax3 and Pax7. During the maturation of the somite, Pax3 and Pax7 have been used to identify cells of the dermomyotome and within the muscle mass of the myotome (Jostes *et al.,* 1990; Goulding *et al.,* 1991). Both *Pax3* and *Pax7* have been shown to be expressed in a population of muscle

precursor cells that are maintained throughout embryonic development in an uncommitted state (Kassar-Duchossoy *et al.,* 2005; Relaix *et al.,* 2005). *Pax3* expression marks the early stages of myogenic specification as well as playing a crucial role in the specification and/or migration of limb myogenic precursors (Bober *et al.,* 1994; Goulding *et al.,* 1994; Williams and Ordahl 1994). Differential modulation of Pax3 delineates the medial dermomyotome containing precursors of axial (back) muscle, and the lateral dermomyotome containing precursors of the limb muscle (appendicular muscle) (Williams and Ordahl 1994). In addition, *Pax3*-null mice (Splotch mice) have been shown to result in major disruptions in the early limb muscle development, suggesting Pax3 is necessary for the formation of limb muscles (Franz *et al.,* 1993; Goulding *et al.,* 1994). This is possibly through the generation of myogenic precursors or through the migration of cells to the limb buds. A number of studies indicate that muscle precursor cells from the lateral portion of the dermomyotome migrate into the limb bud where they differentiate and fuse to form the primary myotubes of the limb muscles (Chevallier *et al.,* 1977; Christ *et al.,* 1977). For this to proceed, *Pax3* is repressed as dermomyotome-derived cells activate the MRF family of transcriptional activators, which control a later step(s) in myogenic specification and the terminal differentiation of muscle (Williams and Ordahl 1994).

1.4 Postnatal myogenesis

1.4.1 Fibre types

Mammalian skeletal muscle is composed of various fibre types which differ in their contractile rate as well as their structural and metabolic properties. This allows the fibres, and thus the muscle as a whole, to perform various functional capabilities. Identification of the different fibre types has been performed using a number of histochemical staining techniques. A number of studies have utilised the pH lability of myofibrillar adenosine triphosphatase (mATPase) (Guth and Samaha 1969; Brooke and Kaiser 1970), as the myosin of slow muscle was found to be more alkali-labile than that of fast muscle (Sreter *et al.,* 1966). Other methods such as immunohistochemistry and electrophoretic analysis have also been performed to identify the various fibre types within a muscle. Perhaps more commonly, fibre types are often classified on the myosin

heavy chain (MHC) isoform(s) they express. Four major fibre types have been identified in the adult skeletal muscles of small mammals using the MHC classification system, though approximately eleven isoforms have been identified in total (Pette and Staron 2000). These four main fibre types are; slow type I, which are suggested to originate from the primary fibres (Crow and Stockdale 1984), and fast type IIA, B and D(X) (for reviews see Pette and Staron (2001) or Schiaffino and Reggiani (1996)). Functionally, these MHC isoforms have been correlated with shortening velocity. Studies indicate that type IIB fibres are the fastest contracting fibres, followed by IID, IIA and I being the slowest (Bottinelli *et al.,* 1991; Bottinelli *et al.,* 1994; Galler *et al.,* 1994).

Embryonic, foetal and adult cell populations contain myoblasts that have the capacity to form various fibre types. Clonal *in vitro* cultures of embryonic chick myoblasts have demonstrated the ability to form single parental myoblasts of three different fibre types (Rutz and Hauschka 1982; Miller and Stockdale 1986a). This suggests that myoblasts are committed to a specific fibre type and this commitment is retained in the progeny cells. However, in contrast, mouse or rat embryonic myoblast cultures reportedly only express a slow *MHC* isoform, while foetal myoblasts will only express fast *MHC* isoforms (Vivarelli *et al.,* 1988; Pin and Merrifield 1993). Thus, it is unclear whether myoblasts have a predetermined fibre type fate, or whether it is species-specific.

In addition to fibres expressing a single *MHC* isoform, fibres can also co-express two or more *MHC* isoforms, thus creating intermediate fibres (Pette and Staron 1990; Schiaffino and Reggiani 1996). These fibres are classified according to the isoforms they express. For example, co-expression of *MHCI* and *MHCIIA* is referred to as type I/IIA. This co-expression may be brought about by the functional pressures upon the muscle, or an indication of fibre type transitions initiated by various conditions. Along with transitions in the expression of *MHC* isoforms, skeletal muscle also demonstrates a high degree of adaptive potential in its metabolic properties, as specific exercise programs have shown to induce shifts from anaerobic to aerobic metabolism (via alterations in enzyme levels and activities) (Gollnick *et al.,* 1973; Holloszy and Booth 1976; Green *et al.,* 1983). Overall, muscle fibres demonstrate a significant degree of plasticity, and are capable of changing their phenotype in response to various conditions such as

neuromuscular or hormonal alterations, mechanical loading/unloading, as well as aging (discussed in detail in Section 1.5.1.2).

1.4.1.1 Neuromuscular innervation

Innervation has been shown to be a crucial factor in the establishment and maintenance of the muscle fibre properties (reviewed by Pette and Vrbová (1985)). Only during the earliest stage of embryonic muscle formation are there no influences from motor neurons (Christ and Ordahl 1995). However, during the later stages of muscle development, it has been identified that the site of innervation on the primary myotubes is crucial to the placement of new secondary myotubes, as secondary myotubes centre on these innervation sites (Duxson 1992). Through cross-innervation experiments, the role of innervation during the establishment of muscle types has been demonstrated (Buller *et al.,* 1960). With cross-innervation, slow muscles become fast when innervated with a fast nerve (and vice versa). This induced change is reportedly brought about through the neural impulse patterns, which are specific for each nerve type. Furthermore, the phenotype of a muscle can be altered with defined electrical simulation. Applying chronic low frequency stimulation (CLFS) to a fast muscle mimics the tonic low frequency impulse pattern typically delivered to a slow twitch muscle (Salmons and Vrbova 1969), thus inducing the expression of slow *MHC* isoforms. When CLFS has been performed in rabbit *m. tibialis anterior* (TA) muscles, MHC type IID isoforms are sequentially changed with MHC type IIA, which are later changed for MHC type Iβ (Leeuw and Pette 1993; Hamalainen and Pette 1997; Peuker *et al.,* 1998).

1.4.1.2 Hormones

Two types of hormones that can heavily influence the muscle fibre type composition are testosterone and thyroid hormones. While testosterone-induced changes have been observed in the sexually dimorphic temporalis muscle of the guinea pig (Lyons *et al.,* 1986), thyroid hormones generally have the greatest impact on fibre type compositions. Hypothyroidism is known to induce fast to slow fibre type transitions while the converse transitions are observed with hyperthyroidism (Nwoye and Mommaerts 1981; Caiozzo *et al.,* 1992; Larsson *et al.,* 1994; Canepari *et al.,* 1998). Furthermore, thyroid hormones are important

during muscle development and maturation, as low or high levels respectively result in the delay or acceleration of the transition from developmental to adult MHC isoforms (Gambke *et al.,* 1983; Butler-Browne *et al.,* 1987; Mahdavi *et al.,* 1987).

1.4.1.3 Mechanical loading/unloading

Increased neuromuscular activity produced through exercise has been shown to induce similar alterations in muscle as CLFS. However, the extent of change which occurs as a result of exercise is significantly reduced. Endurance exercise training is well known to increase enzymatic activities of aerobic-oxidative energy supply, and induce fast to slow MHC transitions (Holloszy and Booth 1976; Green *et al.,* 1983). Furthermore, mechanical loading also induces fast to slow MHC transitions. Several studies indicate that overload to a muscle in a stretched position can also cause the repression of fast-type genes, and induce the activation of slow-type genes (Loughna *et al.,* 1990; Goldspink *et al.,* 1991). Conversely, unloading through hind-limb and spaceflight has been shown to result in deceases in MHCI with a concomitant increase in MHCII.

1.4.2 Fibre number

It is generally believed that the number of fibres that form during foetal muscle development defines the fibre number in adult muscle, as little to no new fibre formation occurs after birth (Ontell and Kozeka 1984; Ross *et al.,* 1987; Ontell *et al.,* 1988). Thus, foetal muscle proliferation and fusion is a pivotal determining factor in regards to fibre number. One mechanism for the control of myoblast proliferation and thus fibre number is through innervation. Ross *et al*. (1987) demonstrated that innervation has been directly correlated with the regulation of secondary myotubes, as nerve terminals on the primary fibres regulate mitosis of myoblasts to promote secondary fibre formation. A second mechanism which may regulate myoblast numbers is through insulin-like growth factors (IGFs) and transforming growth factor-beta (TGF-β) family members (Olson *et al.,* 1986; Bischoff 1994; Cusella-De Angelis *et al.,* 1994; Thomas *et al.,* 2000) (discussed further in Section 1.6.4).

1.4.3 Fibre hypertrophy

The augmentation of muscle mass from the neonatal stage is largely due to increases occurring in the existing muscle fibres. Muscle hypertrophy is an increase in muscle size, which relates to the cross-sectional area of each muscle cell. Any change in fibre volume reflects alterations in the number of nuclei per unit fibre length and/or the volume of cytoplasm per nucleus. While one reflects nuclear turnover (nuclear addition or loss) the other reflects turnover of cell constituents (e.g. protein synthesis or degradation). Numerous studies indicate that muscle fibre hypertrophy is correlated with an increase in myonuclear number (Cabric and James 1983; Winchester and Gonyea 1992; Allen *et al.,* 1995; McCall *et al.,* 1998), and is reliant on the proliferation and contribution of satellite cells (Rosenblatt *et al.,* 1994; Adams and Haddad 1996).

Conversely, a stretch-overload study using avian muscle demonstrated that hypertrophy can also occur without satellite cell proliferation (Lowe and Alway 1999). In addition, Dupont-Versteegden *et al*. (1999) have suggested that increases in myofibre size can be obtained without addition of nuclei and that a considerable plasticity in the nuclear domain of myofibres exists.

1.4.4 Satellite cells

1.4.4.1 Satellite cell location and origin

Satellite cells were first described in 1961 through studies using frog muscle fibres (Katz 1961; Mauro 1961). Using electron microscopic studies, Mauro (1961) described the position of satellite cells on the muscle fibre as being wedged between the plasma membrane and the basement membrane (basal lamina). It is generally believed that satellite cells are derived from the somites (Armand *et al.,* 1983), and are responsible for the majority of postnatal myogenesis. However, recent studies have challenged this theory by suggesting that additional muscle progenitors may exist, including bone marrow-derived cells or cells of haematopoietic origin. Green fluorescent protein- (GFP) labelled bone marrow-derived cells have been shown to contribute to muscle myofibres and express satellite cell markers when transplanted into irradiation-induced damaged muscle (LaBarge and Blau 2002). In other studies, bone marrow-derived cells injected directly or intravenously into injured muscle have demonstrated that these

cells were capable of contributing to the myofibres (Ferrari *et al.,* 1998; Gussoni *et al.,* 1999; Ferrari *et al.,* 2001; Fukada *et al.,* 2002). However, Sherwood *et al*. (2004) recently compared the myogenic properties of endogenous satellite cells with those of bone marrow-derived, haematopoietic stem cell-derived, and circulating cells engrafting the skeletal muscle. The findings from this study indicated that there were significant differences in the myogenic properties within the different cell populations. Although each cell population was able to localise the same anatomic compartment as the myogenic satellite cells, they did not display any intrinsic myogenicity (Sherwood *et al.,* 2004).

Lastly, muscle-derived side population cells (basis on Hoechst dye exclusion) (Asakura *et al.,* 2002) injected intramuscularly (McKinney-Freeman *et al.,* 2002), or when co-cultured with myoblasts (Asakura *et al.,* 2002), have also been shown to contribute to myofibres. In accordance with these findings, muscle-resident $CD45^{+}Sca1^{+}$ cells (referred to as stem cells) have also been shown to generate myogenic cells in co-culture and *in vivo* after muscle injury or activation (Polesskaya *et al.,* 2003). These findings suggest a further muscle progenitor cell population.

1.4.4.2 Role of satellite cells

In addition to providing the location of satellite cells, Mauro (1961) also hypothesized that these satellite cells were remnants from embryonic development which remain dormant until repair of an existing fibre was required. Satellite cells are now defined as mitotically quiescent mononuclear cells that, in response to regenerative cues, are capable of proliferating to form myoblasts, which can terminally differentiate and fuse into multinucleated myotubes (Beauchamp *et al.,* 1999; Beauchamp *et al.,* 2000). During development, satellite cells proliferate and later become mitotically quiescent in the mature muscle (Schultz *et al.,* 1978). Rodent studies indicate that cessation of proliferation in postnatal muscle occurs within weeks after birth (Moss and Leblond 1971; Snow 1979). Furthermore, the percentage of satellite cells has been shown to decline from 30% of muscle nuclei in neonatal muscle to only 5% in adult mouse muscles (Bischoff 1994). In relation to the postnatal function of satellite cells, various studies indicate that they have a crucial role in muscle maintenance and repair (discussed in Sections 1.4.5.2 and 1.5.2). Techniques involving the isolation of single muscle fibres, along with their

resident satellite cells, via the enzymatic digestion of a muscle have provided significant insight into the mechanism for this maintenance and repair process. *In vitro* studies using isolated fibres have confirmed that satellite cells give rise to myoblasts and differentiating myotubes (Bischoff 1975; Konigsberg *et al.,* 1975; Yablonka-Reuveni and Rivera 1994; Rosenblatt *et al.,* 1995; Beauchamp *et al.,* 2000). Furthermore, it was recently shown through individually grafting myofibres into radiation-ablated muscles of dystrophic mdx-nude mice that satellite cells associated with the myofibre give rise to progeny competent to generate thousands of myonuclei and were capable of extensive self-renewal (Collins *et al.,* 2005).

Although satellite cells have traditionally been considered to be unipotent, more recently, studies have suggested that they are in fact multipotent cells. *In vitro* studies have demonstrated that satellite cells are also capable of differentiating into osteogenic and adipogenic lineages (Asakura *et al.,* 2001; Wada *et al.,* 2002; Shefer *et al.,* 2004). It remains unclear however, whether these alternative pathways are brought about through transdifferentiation of the satellite cells, are representations of differing cell types within the satellite cell niche, or simply a reflection of gene stimulation within the context of the myogenic progeny. Furthermore, it is also unknown if satellite cells possess the ability to alter their differentiation program *in vivo*.

1.4.4.3 Markers of satellite cells

The availability of molecular markers in recent times has allowed accurate identification of satellite cells at a light microscope level. An array of markers has been shown to detect satellite cells. These include Pax7 (Seale *et al.,* 2000), M-cadherin (Irintchev *et al.,* 1994), CD34 (Beauchamp *et al.,* 2000), c-Met (Cornelison and Wold 1997), lysenin (Nagata *et al.,* 2006), caveolin 1 (Galbiati *et al.,* 2001), along with others (see reviews (Hawke and Garry 2001; Charge and Rudnicki 2004)). Numerous studies utilising satellite cell markers have detailed the expression profile of satellite cells during various stages of myogenesis (Figure 1.3). For example, once quiescent satellite cells become activated, they begin to express MyoD while co-expressing Pax7 and Myf5 (Cooper *et al.,* 1999; Zammit *et al.,* 2004), as well as genes associated with cell cycling such as the proliferating cell nuclear antigen (PCNA) (Johnson and Allen 1993). With the

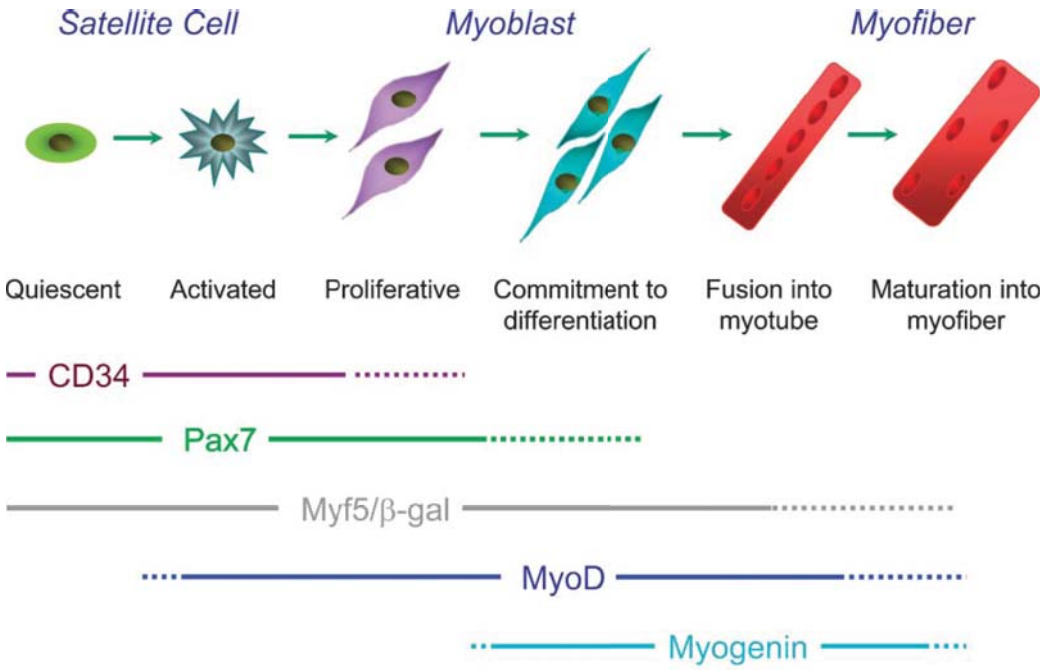

Figure 1.3 Schematic of satellite cell myogenesis and markers

Satellite cells are quiescent in normal adult muscle and can be activated upon stimulation. Once activated, satellite cells divide to produce satellite cell-derived myoblasts that further proliferate, before committing to differentiation and fusing to form myotubes, which then mature into myofibers (for clarity, satellite cell self-renewal is not included). CD34, Pax7, and Myf5/β-gal are expressed in quiescent satellite cells. Satellite cell activation is marked by the rapid onset of MyoD expression, whereas myogenin later marks the commitment to differentiation. Myf5/β-gal denotes the fusion protein product of the targeted allele of the Myf5nlacZ/1 mouse (Tajbakhsh *et al.,* 1997). Adapted from Miller *et al.* (Miller *et al.,* 1999) and Zammit *et al*. (2006)

onset of myogenic differentiation, myogenin is expressed coinciding with a decline in MyoD and PCNA (Yablonka-Reuveni and Rivera 1994).

1.4.4.4 Satellite cell self-renewal

A replenishment of the satellite cell pool is required, as it is estimated that a 1-2% myonuclear turnover occurs per week (Schmalbruch and Lewis 2000). In addition, it is thought that *in vivo*, satellite cells undergo only one or two divisions prior to differentiation (Grounds and McGeachie 1987). A mechanism for satellite cell self-renewal first proposed in 1971, suggested either a stochastic event, or an asymmetrical cell division resulting in one daughter cell being committed to myogenic differentiation while the second proliferated and returned to a quiescent-like state (Moss and Leblond 1971). *In vitro* studies using myoblast cultures, have demonstrated that upon serum withdrawal, a population of cells, referred to as "reserve cells", stop cycling and down-regulate *MyoD*, a characteristic mimicking quiescence cells (Kitzmann *et al.,* 1998; Yoshida *et al.,* 1998). After isolating these reserve cells and returning them to growth conditions, they again began to proliferate and express *MyoD* (Yoshida *et al.,* 1998). Using single fibre cultures, a similar behaviour has been observed in the associated satellite cells. As previously described, proliferating satellite cells can suppress *Pax7* expression while maintaining *MyoD* and differentiate, or alternately, down-regulate *MyoD*, maintain *Pax7*, and enter a quiescent-like state (Zammit *et al.,* 2004). This clearly suggests a mechanism for maintaining the satellite cell pool via a self-renewal process. However, it remains unconfirmed whether the satellite cell population is self-sufficient in restoring the satellite cell pool through self-renewal, or whether it relies on a non-satellite progenitor cell. These putative non-satellite progenitor cells are suggested to be multipotent stem cells residing outside of the satellite cell niche (Figure 1.4). Proposed candidates include interstitial cells (Tamaki *et al.,* 2002; Polesskaya *et al.,* 2003; Kuang *et al.,* 2006), endothelial-associated cells (De Angelis *et al.,* 1999) and side population cells (Asakura *et al.,* 2002). To date, it remains unclear how crucial these cell types are to the replenishment of the satellite cell pool.

In addition, it is currently unresolved whether Pax7 is critical for satellite cell self-renewal. Seale *et al*. (2000) first reported that a complete absence of satellite cells in *Pax7*-null skeletal muscle, suggesting that an induction of Pax7

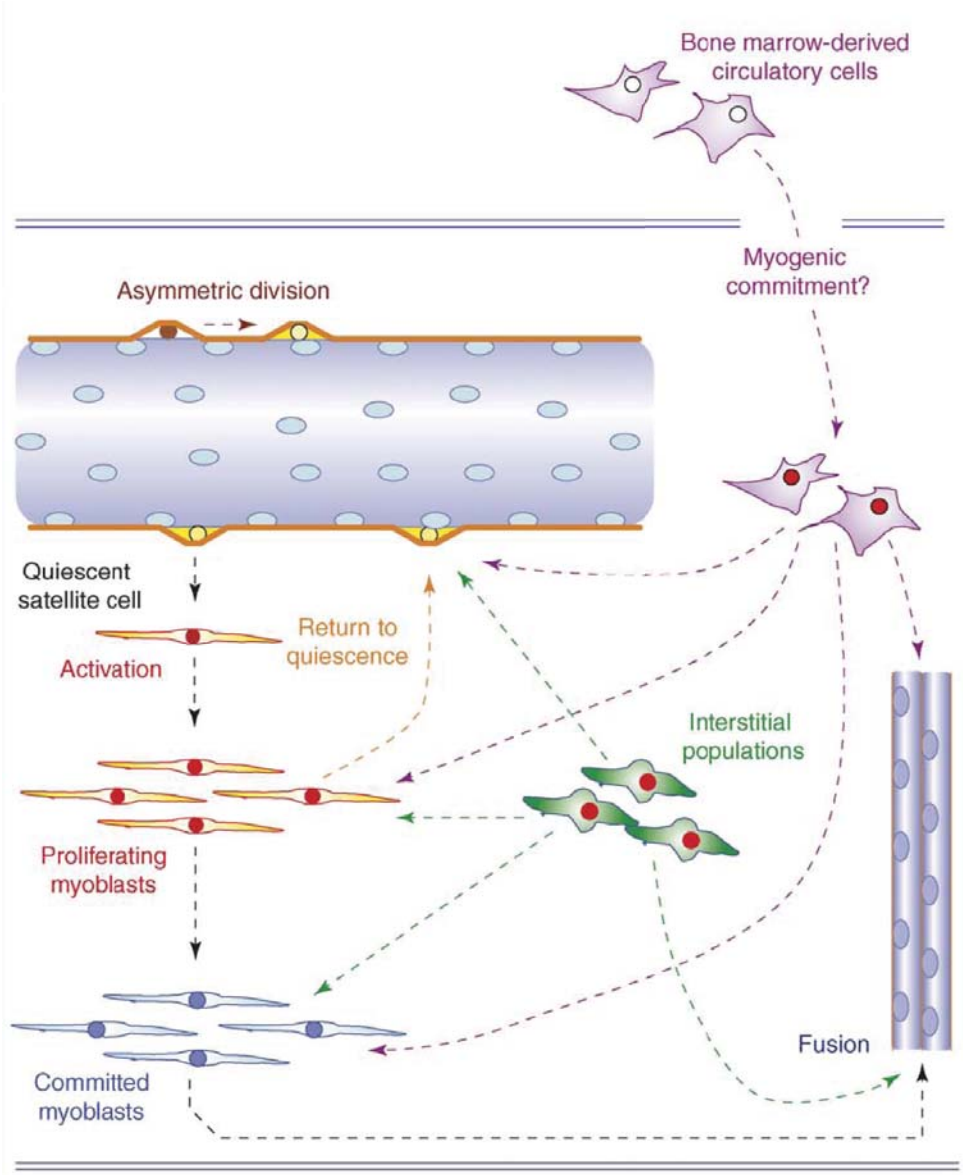

Figure 1.4 Proposed mechanisms for satellite cell self-renewal

Satellite cells generate proliferative myoblasts, which subsequently commit to differentiation, differentiate and fuse to repair or replace damaged myofibres. Satellite cell self-renewal could result from asymmetric division and/or by the cycling myoblasts returning to quiescence. Non-satellite cell phenotypes derived from the muscle interstitium and circulation may also give rise to small numbers of myonuclei and cells in the satellite cell position. Reproduced from Collins (2006)

induces satellite cell specification. However, other studies have demonstrated the presence of satellite cells in *Pax7*-null mice, though the mice display a rapid depletion of satellite cells during early postnatal growth (Seale *et al.,* 2000; Oustanina *et al.,* 2004). Asymmetrical division has also been correlated with divergent fates of daughter cells. Numb, a Notch antagonist has been found to be localised asymmetrically in dividing myogenic cells in *Drosophila* embryos (Ruiz Gomez and Bate 1997; Carmena *et al.,* 1998; Park *et al.,* 1998) and in murine cells (Conboy and Rando 2002). This asymmetric segregation of Numb appears to determine the fate of daughter myoblasts during terminal divisions ($Numb^+$ cells differentiate, while $Numb^-$ proliferate and possibly return to a quiescent state), thus providing another mechanism for satellite cell self-renewal.

1.4.5 Skeletal muscle regeneration

Skeletal muscle is a highly regenerative tissue that is second only to bone marrow with regards to its regenerative capacity. This extensive regenerative capacity has been demonstrated through repetitive injury studies (Sadeh *et al.,* 1985; Luz *et al.,* 2002). Various injury models such as crush, freeze, and chemical injuries have previously been performed to examine the regenerative mechanisms discussed here.

1.4.5.1 Muscle injury and the degenerative phase

Muscle regeneration is characterised by an initial degenerative phase, which is later integrated with a regenerative phase. The degenerative phase is generally initiated by necrosis of the muscle fibres elicited by a disruption of the myofibre sarcolemma. Any disruption of this kind would induce an increase in the permeability of the myofibre, resulting in a cascade of molecular and cellular events. It is likely that the increased calcium influx after membrane damage results in a calcium-dependent proteolysis, driving tissue degeneration via the action of calpains. These are calcium-activated proteases which cleave myofibrillar and cytoskeletal proteins (Bodensteiner and Engel 1978; Belcastro *et al.,* 1994; Spencer *et al.,* 1995; Williams *et al.,* 1999). An initial stage of muscle recovery is the influx of mononucleated cells, predominantly consisting of inflammatory cells. This process is believed to be initiated by factors released within the injured muscle, which activate resident inflammatory cells. Activation

of these cells causes them to release chemotactic signals attracting circulating inflammatory cells to the injury site (reviewed by Tidball (1995)). The first inflammatory cells to rapidly migrate to the site of injury are neutrophils, which release trophic factors to activate satellite cells within 2 hr of injury (Goetsch *et al.,* 2003). The infiltrating neutrophils may be phagocytic, however, they are also capable of releasing proteases that can participate in the degradation of cellular debris resulting from the muscle damage. The second inflammatory cell type to migrate into the damaged area is macrophages (Orimo *et al.,* 1991). Macrophages are antigen-presenting cells that play an important role in the regulation of cellular immune responses in injured muscle. Studies using rat skeletal muscle have demonstrated that macrophages which migrate to the injury site are phagocytic, and are at highest concentrations where the tissue is most damaged (Honda *et al.,* 1990; McLennan 1993). Various other roles macrophages may play during muscle repair and remodelling have also been proposed. For example, muscle regeneration using transplanted myogenic cells can be severely impaired when the host monocyte-macrophages are completely depleted (Lescaudron *et al.,* 1999). Interestingly, conditioned media from peritoneal macrophage cultures or macrophage cell lines have been shown to increase the proliferation rate and the number of *MyoD* expressing cells in myoblasts cultures (Cantini and Carraro 1995; Massimino *et al.,* 1997; Merly *et al.,* 1999). Furthermore, intensive physical exercise studies have demonstrated the stimulation of peritoneal macrophages (Fehr *et al.,* 1988; Lotzerich *et al.,* 1990). This suggests that a systemic factor is released following muscle damage, which can elicit an inflammatory response throughout the body. However, currently the identity of the macrophage-derived factor(s) that induce these changes is unknown.

Various techniques have been employed to measure muscle injury. For example, increased serum levels of muscle proteins, such as creatine kinase, have been measured to observe the extent of muscle damage due to muscle degenerative diseases, such as muscular dystrophies (Percy *et al.,* 1979). Uptake of low-molecular-weight dyes, such as Evans blue or procion orange, by the myofibre have also been shown to be reliable indicators of sarcolemmal damage (Matsuda *et al.,* 1995; Straub *et al.,* 1997).

1.4.5.2 Muscle regenerative phase

Following the degenerative and inflammatory responses to muscle injury is the process of muscle repair and regeneration. The ability of adult skeletal muscle to regenerate, even after total disruption (Studitsky 1964; Carlson 1986), has largely been attributed to satellite cells (Partridge 2002). Mammalian myonuclei within a muscle fibre are terminally differentiated, and therefore cannot replace themselves. After injury, regeneration occurs via the activation and proliferation of satellite cells (Figure 1.5A). These cells later differentiate and fuse to provide the myonuclei required to repair or replace the damaged myofibres (Moss and Leblond 1971; Snow 1978; Bischoff 1994). Numerous nuclear radiolabelling experiments have demonstrated the contribution of dividing myogenic cells (emanating from the resident satellite cells) to regenerating myofibres, thus providing clear evidence that satellite cells indeed function as myogenic precursors (Snow 1977b; Snow 1978; Trupin *et al.,* 1982; Schultz *et al.,* 1985; Darr and Schultz 1987). Furthermore, it has been demonstrated that following a chemical injury, the normally quiescent satellite cells become activated, re-enter the cell cycle and exhibit a significant proliferative capacity between 2 and 3 days following injury (Goetsch *et al.,* 2003). This proliferation is vital for muscle regeneration, as demonstrated by the impaired regenerative capacity after exposure to colchicine (an inhibitor of mitotic division) or after irradiation (prevents satellite cell proliferation) (Pietsch 1961; Quinlan *et al.,* 1995). Satellite cell proliferation is generally followed by a differentiation process, where myoblasts withdraw from the cell cycle and form small basophilic (reflecting high protein synthesis) centronucleated myotubes (Goetsch *et al.,* 2003) (Figure 1.5B). The initial phase of myogenic cell expansion is essential for this latter phase as muscle repair is reliant on the number of myoblasts available for fibre formation and repair. Newly formed myofibres often initially express embryonic or developmental forms of *MHC* (Whalen *et al.,* 1990), and contain centrally located myonuclei. These nuclei can be located in discrete portions or along the entire regenerating new fibre, suggesting cell fusion is focal to the site of injury (Blaveri *et al.,* 1999). After fusion of the myogenic cells has concluded, the myofibres further increase in size through cytoplasmic expansion, while the myonuclei move to the fibre periphery (Figure 1.5C). Thus the newly regenerated

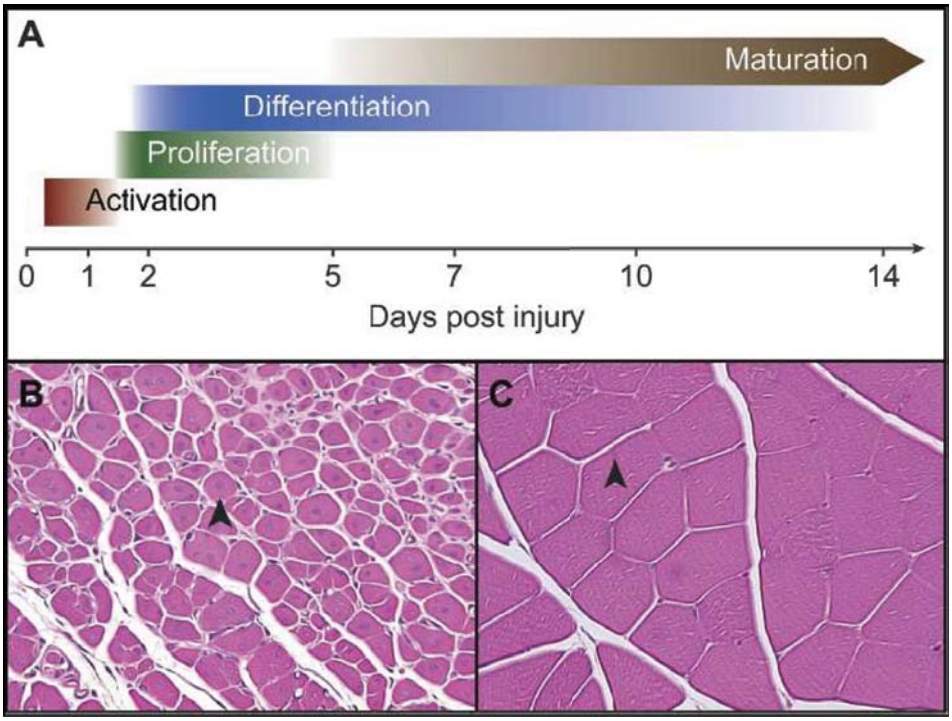

Figure 1.5 Muscle repair is dependent on myogenesis

(A) Illustration of the regenerative stages after injury including: satellite cell activation (within 2 hr of injury); satellite cell proliferative stage (peaking at days 2-3); differentiation (characterised by centrally located nuclei within the regenerating myofibers); and maturation. (B) Following cardiotoxin injury, regeneration results in the formation of small myofibres containing centrally located nuclei (indicated by arrowhead). (C) With further regeneration, these nuclei move to the myofibre periphery. Reproduced from Shi and Garry (2006).

fibres morphologically and functionally resemble the undamaged fibres within the muscle. During this regenerative period, numerous genes associated with myogenesis are systematically expressed. For example, studies indicate that *MyoD* is induced within 2-6 hr after injury correlating to the activation of satellite cells (Grounds *et al.,* 1992; Megeney *et al.,* 1996), while *Myf*5 and *myogenin* display a delayed expression peaking within 5 days of injury (Goetsch *et al.,* 2003). In addition, various components of the Notch/Numb pathway (which plays a role in the activation of satellite cells), as well as transcriptional regulators (including Pax3 and Pax7, Forkhead, and Sox family members) are up-regulated during the repair and regenerative period (Goetsch *et al.,* 2003; Turk *et al.,* 2005).

Collectively, the various studies described herein, indicate that the regenerative process after injury shares a high degree of similarity with prenatal and neonatal myogenesis.

1.4.5.3 Collagen formation

Myofibres and connective tissue have an imperative association essential for effective muscle function, normal transmission of force, and maintenance of the tensile strength of a muscle. Following an injury, the functional continuity of the tendon-myofibre-tendon unit is often disrupted (Grounds 1991; Hurme *et al.,* 1991). Therefore, successful repair of the damaged muscle is reliant on a balance between the regeneration of muscle fibres and connective tissue synthesis. These are two simultaneous processes which are supportive of, but also competitive with, each other.

Normally collagen forms a sheath around the fusing myoblasts during myotube formation (Allbrook 1973; Bailey *et al.,* 1979). However, excessive proliferation of fibroblasts, and thus synthesis of connective tissue, can lead to excessive scarring and fibrosis. This may lead to the formation of a dense mechanical barrier that can impede myofibre regeneration (McMinn 1967; Allbrook 1973; Burry 1975).

1.5 Sarcopenia

1.5.1 Phenotype of the aged muscle

A significant characteristic of the aging process in mammals is the profound loss of skeletal muscle mass and strength, commonly referred to as sarcopenia. Imaging techniques have confirmed an age-related loss of muscle mass within specific muscle groups (Coggan *et al.,* 1993; Frontera *et al.,* 2000). In humans, much of the observed muscle loss disproportionately occurs in the lower body (Janssen *et al.,* 2000). The rate of muscle loss after 50-years of age appears fairly constant at approximately 1-2% annually (Sehl and Yates 2001; Hughes *et al.,* 2002).

A number of studies have indicated that the functional decrease in muscle strength associated with aging is proportionally greater than the reduction in muscle mass (Overend *et al.,* 1992; Welle *et al.,* 1996b; Lynch *et al.,* 1999; Martin *et al.,* 2000). Contrary to this, others dispute that the decline in muscle mass is sufficient to account for the decreases in strength (Young *et al.,* 1984; Alway *et al.,* 1996; Frontera *et al.,* 2000). Irrespective of these inconsistencies, a decrease in muscle efficiency is largely responsible for the age-related decline in muscle strength and is most likely due to the loss of and/or alteration in the contractile properties of the muscle motor units (Faulkner *et al.,* 1995; Kadhiresan *et al.,* 1996). With advancing age, a decrease in muscle protein synthesis is known to occur (Welle *et al.,* 1993; Welle *et al.,* 1996a; Balagopal *et al.,* 1997). This loss is particularly prominent in the resident mitochondria, possibly due to the age-related mutations and deletions that occur in mitochondrial DNA (Barazzoni *et al.,* 2000). In addition, the decrease in mitochondrial protein synthesis has also been correlated with a decline in mitochondrial oxidative enzymes and VO2max (the highest rate of oxygen consumption attainable during maximal or exhaustive exercise) that occurs with age (Rooyackers *et al.,* 1996).

Various physiological characteristics associated with the age-related loss of muscle mass and strength include decreases in muscle fibre numbers or specific fibre types, atrophy of existing fibres, loss of motor innervation, defective muscle regeneration, altered vasculature, and increased fibrosis.

1.5.1.1 Muscle fibre loss

Whether a loss of fibres occurs with age is somewhat contentious, and is most likely species-specific (Caccia *et al.,* 1979; Hooper 1981; Sato *et al.,* 1984; Eddinger *et al.,* 1985; Larsson and Edstrom 1986; Alnaqeeb and Goldspink 1987; Brown 1987; Daw *et al.,* 1988; Lexell *et al.,* 1988; Timson and Dudenhoeffer 1990). Likewise, it is credible that muscle type is important for the analysis of fibre number, as the degree of age-related atrophy appears to be muscle-specific (as muscles vary in their fibre type composition). In saying this, human studies which have described a fibre loss, showed no discrimination between the fibre types (Sato *et al.,* 1984; Lexell *et al.,* 1988). The inconsistencies as to whether a loss of fibres occurs may also be due to the occurrence of fibre splitting, which complicates the analysis of the overall fibre number (Alnaqeeb and Goldspink 1987). Fibre splitting or branching has been associated with muscle regeneration and is possibly related to incomplete fusion of fibres regenerating within the same basal lamina (Bourke and Ontell 1984; Blaivas and Carlson 1991; Blaveri *et al.,* 1999). A number of studies report that fibre splitting is a common characteristic observed in aging mouse muscles, and has been suggested to correlate with its abnormal regenerative capacity (Bockhold *et al.,* 1998; Charge *et al.,* 2002). In the muscles of aged rats, the increased fibrosis observed within the regenerating muscle has provided an explanation for the observed myofibre branching (Sadeh 1988; Blaivas and Carlson 1991). One major cause of fibre loss that has been clearly identified is the process of denervation, which is known to occur with aging (refer to Section 1.5.1.4 for further discussion).

1.5.1.2 Muscle fibre type changes

Aging has been suggested to cause fast to slow fibre type transitions. Various studies indicate fibre type shifts within the MHCII isoforms or to an MHCI isoform (Kugelberg 1976; Alnaqeeb and Goldspink 1987; Larsson *et al.,* 1993; Musaro *et al.,* 1995). In addition, aging has been associated with a relative increase in muscle fibres co-expressing two or more *MHC* isoforms, signifying that there is less distinction between the slow and fast fibre types with age (Andersen *et al.,* 1999).

It has been difficult to determine however, the exact cause of these transitions, as aging is a multifactorial condition, and it is unknown whether fibre

type changes represent primary or secondary events. Two factors implicated in the age-related fibre type transitions are denervation via a selective loss of fast α-motor neurons (Hashizume *et al.,* 1988; Ansved and Larsson 1990) (discussed further in Section 1.5.1.4), and alterations in the level of thyroid hormones, which can modulate *MHC* gene expression and the protein composition of muscles (Sieck *et al.,* 1996).

1.5.1.3 Muscle fibre atrophy

As previously discussed, muscle fibre size is dependent on any alterations in the number of nuclei per unit fibre length, as well as the volume of cytoplasm. By using a number of muscle atrophy models, it has been shown that the fibre cross-sectional area and the myonuclear number are both consistently decreased with age (Allen *et al.,* 1995; Allen *et al.,* 1996; Kasper and Xun 1996b; Kasper and Xun 1996a; Allen *et al.,* 1997). However, it is unclear how the myonuclear domain (nucleo-cytoplasmic ratio) changes during age-related atrophy. For example, Vassilopoulos *et al*. (1977) demonstrated that the myonuclear domain remained unchanged with age, while Manta *et al*. (1987) showed a decrease. Interestingly, one study showed that human males who maintained physical activity into their eighties demonstrated compensatory hypertrophy of muscle fibres to compensate for a decrease in fibre number (Aniansson *et al.,* 1992). However, this observation appears to be uncommon among other aging studies.

The observed fibre atrophy is generally associated with type II fibres (Lexell *et al.,* 1988; Coggan *et al.,* 1992; Lexell 1995), which functionally would weaken the muscle due to the greater power that these fibres generate compared to type I fibres. Little is known about the control of fibre size in normal adult muscle fibres, and it is unclear why fibre size declines with age. One possibility is that a deficiency in satellite cell function results in a reduction in the number of nuclei within a muscle fibre. Alternatively, modifications intrinsic to the fibres could reduce the recruitment of satellite cells into the fibre during re-modelling, as well as reduce the production of muscle fibre-specific proteins. A number of studies have demonstrated that the synthesis of muscle protein declines by approximately 30% with aging (Welle *et al.,* 1993; Welle *et al.,* 1996a; Balagopal *et al.,* 1997). Of note, a selective reduction of muscle protein synthesis appears to occur, resulting in a decrease in the rate of MHC synthesis, while the synthesis of

sarcoplasmic protein is either maintained or increased with age (Balagopal *et al.,* 1997). More specifically, mRNA levels of both *MHC IIA* and *IID* isoforms are significantly reduced with age (Balagopal *et al.,* 2001). However, it is unclear whether this is related to transcriptional or pre-transcriptional defects, or mRNA instability. Since the ability of a muscle to synthesize MHC has been highly correlated with muscle strength (Balagopal *et al.,* 1997), this decline in MHC production is particularly relevant to the fibre atrophy observed during aging. And finally, extrinsic factors such as innervation or systemic factors are highly likely to be of major importance to fibre atrophy. There is no doubt that a loss of motor neurones occurs during aging (Ansved and Larsson 1990). This is highly pertinent to fibre size as denervation causes rapid atrophy of the fibres through loss of cytoplasmic volume and myonuclei (Viguie *et al.,* 1997), and can possibly result in the loss of the fibre.

1.5.1.4 Innervation

As previously described, innervation appears to be vital in fibre development, and also during maintenance of fibre size and type. In fact, following denervation, muscle mass can decline by over 50% within 1 month (Gutmann 1962). The denervation reported to occur with age is related to motor neuron cell death or neuromuscular junction remodelling (Pettigrew and Gardiner 1987; Hashizume *et al.,* 1988; Ansved and Larsson 1990), and has been directly linked with the progressive loss of muscle mass (Viguie *et al.,* 1997). In humans, a 50% loss of motor neurons can occur in individuals over 60-years of age (Tomlinson and Irving 1977). Numerous studies have shown not only a reduced number of motor units (consisting of a motor neuron and all of the fibres it innervates) with age (Campbell *et al.,* 1973; Doherty and Brown 1993; Luff 1998), but also an increase in the motor unit size (Sica *et al.,* 1976). This would indicate that if a fibre has lost contact with its motor neuron, it becomes reinnervated by another neuron by axonal branching. A selective loss of specific motor neuron types has been correlated with both fibre type grouping and fibre atrophy grouping, resulting in a reduction in the mosaic composition of a muscle (Grimby *et al.,* 1982; Larsson *et al.,* 1991). In addition, neuromuscular junction (Lexell 1997; Luff 1998) and axon myelination (Grover-Johnson and Spencer 1981; Kazui and Fujisawa 1988; Knox *et al.,* 1989) abnormalities, which would

impede stimulations for muscle contractions, have also been observed in aged rodents and humans. These observations are highly suggestive of neural degeneration occurring with age. Interestingly, innervation studies using rats have reported conflicting results as to whether aging is associated with an impaired ability to reinnervate muscle fibres (Kanda and Hashizume 1991; Carlson and Faulkner 1996; Carlson and Faulkner 1998), possibly indicating other influencing factors.

1.5.1.5 Regeneration

Various studies indicate that the efficacy of old muscle to regenerate is reduced due to a decreased rate of regeneration, as well as a reduced amount of muscle replaced (Carlson and Faulkner 1988; Sadeh 1988; Carlson and Faulkner 1989). Successful regeneration of the muscle is believed to be heavily reliant on an adequate satellite cell population and effective innervation (Grounds and Yablonka-Reuveni 1993). As previously discussed, satellite cells have a crucial role in muscle regeneration (Schultz and Jaryszak 1985), with their activation and proliferation being intimately linked to the regeneration capabilities of the muscle. However, it is generally accepted that this process is reduced or retarded with age (discussed further in Section 1.5.2). In addition, the participation of satellite cells during regeneration is significantly dependent on the inflammatory response. Of note, macrophage infiltration has been shown to be retarded after injury in old rats compared to young rats (Sadeh 1988). As macrophages are required for the removal of necrotic tissue and the promotion of satellite cell activation, any impediment in macrophage infiltration would result in delayed myofibre regeneration. This has been confirmed in a study demonstrating that an age-related decline in macrophage activity contributed to slower wound healing in aged mice (Danon *et al.,* 1989). Circulating leukocytes, hormones and cytokines (as well as the efficacy of cells responding to them) have also been suggested as potential candidates responsible for the altered macrophage activity (Grounds 1987; Cannon 1995; Rall *et al.,* 1996).

1.5.1.6 Vasculature

A number of studies have indicated changes in regards to vasculature within skeletal muscle. Reduced blood supply (McCully and Posner 1995),

decreased capillary density (Coggan *et al.,* 1992) and alterations in the vascular pathology (Cooper *et al.,* 1994) have all been reported to occur with age. Decreases in vascularity would have serious negative effects on muscle regeneration due to subsequent reductions in the inflammatory cell infiltration.

1.5.1.7 Fibrosis

With age, an increase in interstitial fibrotic connective tissue is frequently observed. In mice, endomysial collagen is reported to double between 3- and 26-weeks of age (Marshall *et al.,* 1989). Increased fibrosis is also reported to occur in regenerating muscles of aged animals (Carlson and Faulkner 1989; Ullman *et al.,* 1990). Furthermore, increases in the external lamina encircling the satellite cells has also been reported to occur with age (Snow 1977a), thus impacting their activation and proliferative capabilities. All of these fibrotic alterations would substantially reduce the myogenic capacity of a muscle.

1.5.2 Satellite cell activation

Aged muscle appears to have impaired satellite cell function. This would significantly contribute to the age-related loss and weakening of a muscle, as well as reduce the regenerative capacity. To date, it is unclear whether satellite cell numbers decline with age, although much of the confusion may arise through variations in muscle types and animal species (Gibson and Schultz 1983; Nnodim 2000; Conboy *et al.,* 2003; Schafer *et al.,* 2005; Shefer *et al.,* 2006). The ability of satellite cells to become activated as well as their proliferative capacity has also been shown to decrease with age (Schultz and Lipton 1982; Dodson and Allen 1987; Johnson and Allen 1993; Barani *et al.,* 2003; Conboy *et al.,* 2003; Machida and Booth 2004). Furthermore, satellite cells from aged muscle show an extended latent period between an activation stimulus and proliferation (Tatsumi *et al.,* 1998; Yablonka-Reuveni *et al.,* 1999). For example, an autoradiographic study performed on crush injured muscles has shown that myoblast proliferation is retarded in aged mice compared to young mice (McGeachie and Grounds 1995). In humans a decrease in the satellite cell pool and proliferative ability has been observed from early childhood years. Renault *et al*. (2000) showed that satellite cells from human neonatal and infant muscle were capable of 60 and 45 replications respectively. In comparison, 9-year-old and 60-year-old cells were

only capable of 20-30 replications. Additionally, with increasing age, satellite cells fused to form thinner, more fragile myotubes.

A mechanism for the impaired regenerative capacity associated with age-related myopathies such as sarcopenia has been suggested to result from both the macro- and microenvironment (Carlson and Faulkner 1989; Coggan *et al.,* 1992; Carlson and Faulkner 1996; Chakravarthy *et al.,* 2000). Surgical procedures have eloquently demonstrated that introduction of old muscle to a young environment rejuvenates the capacity of the satellite cells to contribute to the muscle regeneration (Carlson and Faulkner 1989; Conboy *et al.,* 2005). This clearly indicates that the aged environment is a major component in the decline of satellite cells participating during muscle regeneration. Interestingly, cross-transplantation of *m. extensor digitorum longus* (EDL) muscles between young and old rats demonstrated that the mature skeletal muscle was equally capable as young skeletal muscle in recovering from the transplantation (Carlson and Faulkner 1989). However, young nerve-muscle autografts functioned significantly better than old nerve-muscle autografts, suggesting that a deficiency in re-innervation with increasing age accounts for the poorer muscle regeneration in the aged muscle (Carlson and Faulkner 1996).

One molecular mechanism shown to be directly linked with the impaired proliferative capacity of aged satellite cells is the Notch signalling pathway, as induced expression of activated *Notch* restores the regeneration capacity of aged muscle (Conboy *et al.,* 2003; Luo *et al.,* 2005). Furthermore, parabiosis studies, whereby young and old mice were surgically joined to establish a shared circulatory system, have shown that as yet unknown circulating factors in the young mice restored both Notch signalling and the muscle regeneration capacity in the aged mice (Conboy *et al.,* 2005).

Various other factors have been suggested to affect the efficiency of skeletal muscle regeneration within the aged environment. These include thickening of the basal lamina (Snow 1977a), accumulation of collagen within the muscle (Marshall *et al.,* 1989), and reduced capillary density (Coggan *et al.,* 1992). In addition, aging has been shown to result in a decrease in inflammatory factors and macrophages essential for the normal satellite cell response to injury (Danon *et al.,* 1989). Reduced serum levels of growth factors such as IGF-I, which acts as a potent mitogen for satellite cells in culture (Allen and Boxhorn

1989; Doumit *et al.,* 1993), have also been reported in both aged rats and humans (Ullman *et al.,* 1990; Reeves *et al.,* 2000).

1.5.3 Hormonal and growth factor regulation

Several hormones and growth factors which have multifactorial roles, including the regulation of muscle fibres, satellite cells and motor neurons, are thought to alter with age. The age-related decline of both testosterone and IGF-I have been suggested to be the most significant of the endocrine changes associated with sarcopenia. Studies in human males have shown that free testosterone declines by approximately 40% between 25- and 75-years of age, correlating with a decrease in muscle strength (Nankin and Calkins 1986; Vermeulen *et al.,* 1996; van den Beld *et al.,* 2000). In addition, the decline in free testosterone, and to a lesser extent IGF-1, has been linked with a reduction in the rate of MHC synthesis (Balagopal *et al.,* 1997). Circulatory levels of IGF-I are also known to decline with age. For example, in a human study, a 25% decrease in the level of IGF-I was observed in aged muscles compared with adult muscles (Welle *et al.,* 2002). IGF-I normally exerts several anabolic effects in muscle including increased protein synthesis, myoblast proliferation and differentiation, as well as enhanced re-innervation of the muscle fibres (Bischoff 1994; Caroni *et al.,* 1994; Butterfield *et al.,* 1997). Thus it is likely that decreases in testosterone and IGF-I levels in both animals and humans play a significant role in the decline of skeletal muscle mass.

1.5.4 MRF expression

As the expression of MRFs relate to the level of myogenesis occurring within a muscle, their periodic detection can indicate the myogenic state of a muscle. For example, during the neonatal stage, *MyoD* and *myogenin* are expressed at relatively high levels, however, the expression of both MRFs decline in adult muscles to a barely detectable level (Eftimie *et al.,* 1991; Witzemann and Sakmann 1991; Musaro *et al.,* 1995). With further aging the expression of *MyoD* and *myogenin* mRNA again increases (Musaro *et al.,* 1995; Marsh *et al.,* 1997; Gomes and Booth 1998; Kostrominova *et al.,* 2000; Alway *et al.,* 2001). Various studies have indicated that these changes in *MyoD* and *myogenin* expression in both adult and senescent muscle may be elicited either by denervation (Eftimie *et*

al., 1991; Voytik *et al.,* 1993; Weis 1994; Kostrominova *et al.,* 2000) or by regeneration (Fuchtbauer and Westphal 1992; Grounds *et al.,* 1992; Kami *et al.,* 1995; Koishi *et al.,* 1995; Marsh *et al.,* 1997; Cooper *et al.,* 1999; Launay *et al.,* 2001). In a study which analysed MyoD and myogenin protein levels in skeletal muscles of senile rats, Dedkov *et al*. (2003) demonstrated that the higher level of MyoD and myogenin protein was associated with their accumulation in the nuclei of both muscle fibres and satellite cells. Furthermore, these authors speculated that the up-regulation of the MyoD and myogenin protein in the muscle fibre nuclei was associated with a loss of innervation, whereas in the satellite cell nuclei it was an indication of their participation in the regenerative process relating to the denervation and/or re-innervation of the fibres.

Overall these studies demonstrate that while aged muscle is able to respond to myogenic-inducing stimuli, it does not exhibit a high level of myogenic efficacy during this response, due to numerous factors.

1.5.5 Treatment

1.5.5.1 Testosterone replacement

An overview of the numerous studies of testosterone replacement in elderly humans (recently reviewed by Gruenewald and Matsumoto (2003)) has provided a highly variable conclusion. Some authors report a modest increase in lean mass, muscle strength and protein synthesis (Urban *et al.,* 1995; Sih *et al.,* 1997; Snyder *et al.,* 1999; Kenny *et al.,* 2001) or increased grip strength (Sih *et al.,* 1997), while others observed no change (Clague *et al.,* 1999; Snyder *et al.,* 1999). Furthermore, a number of studies have addressed whether testosterone replacement positively alters lower body strength (Sih *et al.,* 1997; Clague *et al.,* 1999; Snyder *et al.,* 1999; Kenny *et al.,* 2001; Brill *et al.,* 2002; Ferrando *et al.,* 2002), however, only a small number reported an increase. Although testosterone treatment appears to have minimal effectiveness in increasing in lean muscle mass, these studies do not necessarily indicate aged males are non-responsive (or have a low response) to testosterone, as aspects such as dosage could factor into the treatment response.

1.5.5.2 IGF-I

Studies involving the over-expression of *IGF-I* in transgenic mice have demonstrated increased satellite cell proliferation, mobilisation of non-muscle stem cells, and improved response to injury and regeneration in skeletal muscle of aged mice (Musaro *et al.,* 2001; Musaro *et al.,* 2004; Mourkioti and Rosenthal 2005). In addition, viral-mediated expression of *IGF-I* in mice has been shown to block age-related muscle atrophy (Barton-Davis *et al.,* 1998). Barton-Davis *et al*. (1998) hypothesized that this was achieved by enhancing muscle regeneration through resident satellite cell activation. Other studies adopting an IGF-I infusion procedure have demonstrated that IGF-I treatment results in muscle hypertrophy (Adams and McCue 1998), by increasing the proliferation potential of skeletal muscle satellite cells (Chakravarthy *et al.,* 2000). In addition, the hypertrophy observed following IGF-I treatment has also been suggested to be due to an enhancement of protein synthesis (Bark *et al.,* 1998) or increased nerve sprouting (Vergani *et al.,* 1997).

1.5.5.3 Exercise

Depending on the type of exercise training programme, increases in strength can be achieved. For example, resistance training, such as weightlifting, is a more powerful stimulus of muscle hypertrophy than endurance-type exercise regimes such as swimming (Klitgaard *et al.,* 1990). Another resistance training study reported significant muscle strength and size gains even in frail elderly people (Fiatarone *et al.,* 1990). Functionally, exercise programs have demonstrated increases in the size of both type I and type II muscle fibres, as well as muscle protein synthesis (Larsson 1982; Welle *et al.,* 1995; Yarasheski *et al.,* 1999; Hasten *et al.,* 2000; Balagopal *et al.,* 2001).

Although some clinicians have shown reluctance in regards to recommending high-intensity resistance training for elderly subjects, most studies have indicated that resistance training can be safely undertaken (Campbell *et al.,* 1994; Campbell *et al.,* 1999).

1.6 Myostatin

One group of growth factors which play a number of vital roles in the regulation of both prenatal and postnatal myogenesis is the TGF-β superfamily.

This superfamily consists of a large number of growth and differentiation factors. A prominent member of this superfamily is myostatin, also referred to as growth and differentiation factor 8 (GDF8). First identified in mice, *myostatin* has been shown to be expressed in both embryonic and adult skeletal muscle, where it elicited its effect through an autocrine fashion to inhibit muscle development (McPherron *et al.,* 1997).

1.6.1 Expression of myostatin

Myostatin can be detected in mice as early as 9.5 days postcoitum in approximately one third of the somites, and by 10.5 days it is expressed in almost every somite (McPherron *et al.,* 1997). With further development, *myostatin* can be detected in a large proportion of the developing muscles. Expression continues in the adult where it is almost exclusively expressed in the muscle tissue at varying amounts (McPherron *et al.,* 1997; Carlson *et al.,* 1999; Wehling *et al.,* 2000). This muscle-specific expression has been strongly associated with the fast fibre types (Carlson *et al.,* 1999). In addition, other studies using different animal species have also detected low levels of *myostatin* mRNA or protein in the porcine lactating mammary gland (Ji *et al.,* 1998), purkinje fibres and cardiomyocytes of the sheep heart (Sharma *et al.,* 1999), murine adipose tissue (McPherron *et al.,* 1997), serum in humans (Gonzalez-Cadavid *et al.,* 1998), several tissues of tilapia (Rodgers *et al.,* 2001), Atlantic salmon (Ostbye *et al.,* 2001), and rainbow trout (Rescan *et al.,* 2001), and in the brain and ovary of brook trout (Roberts and Goetz 2001).

1.6.2 Structure of myostatin

Myostatin was first discovered in 1997 through a degenerate polymerase chain reaction (PCR) on murine DNA, using oligonucleotides corresponding to highly conserved regions within the TGF-β family members (McPherron *et al.,* 1997). By screening a murine skeletal muscle library, a full length cDNA sequence containing a single open reading frame, encoding a 376 amino acid protein, was obtained. The predicted sequence contained distinct properties shared by TGF-β superfamily members; a secretion signal sequence, a proteolytic processing site, an amino-terminal propeptide domain and a carboxyl-terminal domain consisting of the active ligand (McPherron *et al.,* 1997) (Figure 1.6).

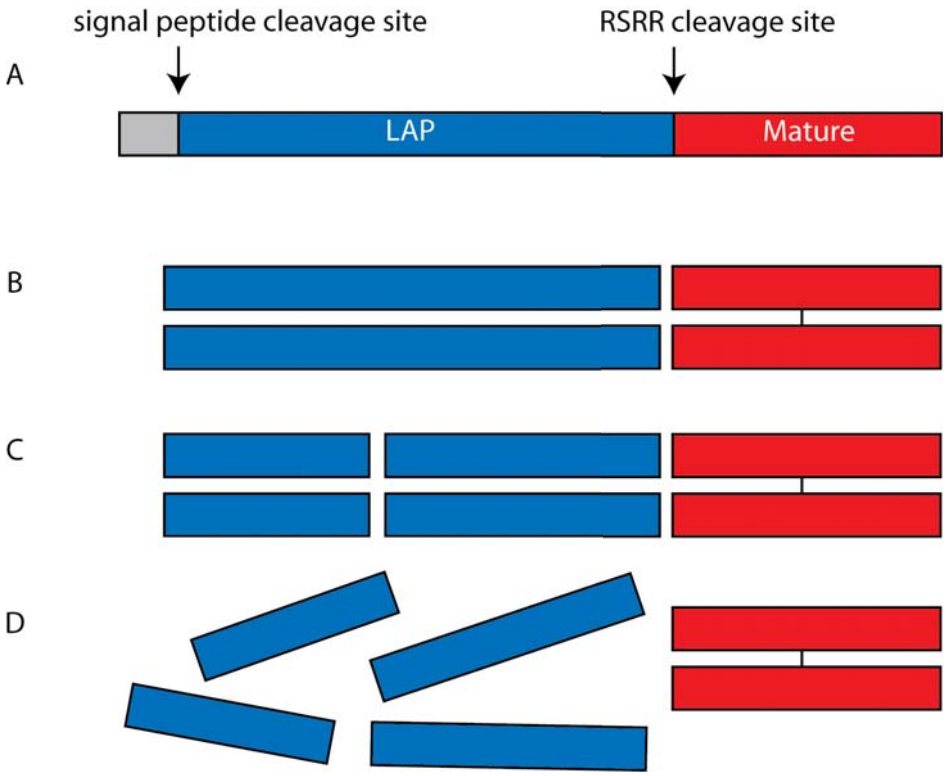

Figure 1.6 The structure and processing of myostatin protein

(A) The myostatin precursor molecule undergoes two proteolytic processing events: one which removes the amino-terminal signal sequence, and a second that generates the mature carboxyl-terminal fragment by cleaving at a RSRR site. (B) Following proteolytic processing, the LAP and the disulfide-linked mature dimer remain noncovalently bound in a latent complex. (C) Activation of latent myostatin occurs through proteolytic cleavage of the LAP. (D) This causes the dissociation of the latent complex, allowing receptor binding. Adapted from Lee (2004)

This carboxyl-terminal domain has been shown to contain the conserved pattern of nine cysteine residues, which allow the formation of the cysteine knot and homodimerisation essential for TGF-β activity (McPherron *et al.,* 1997). Myostatin undergoes post-translational modification whereby it is cleaved between the propeptide domain and the carboxyl-terminal domain, at an RSRR cleavage site, to form the amino-terminal latency associated protein (LAP) and the active carboxyl-terminal mature processed peptide (McPherron *et al.,* 1997).

1.6.3 Myostatin receptors

Like the other TGF-β family members, both the unprocessed and mature myostatin are capable of forming disulfide-linked dimers (Daopin *et al.,* 1992; Daopin *et al.,* 1993; McPherron *et al.,* 1997; Lee and McPherron 2001). Lee and McPherron (2001) have demonstrated that the carboxyl-terminal dimer is capable of binding to activin type II receptors, Act RIIB, and to a lesser extent, Act RIIA. Lee and McPherron also proposed that following proteolytic processing, the myostatin carboxyl-terminal dimer remains in a latent complex with its propeptide. Analogous to other TGF-β family members, the LAP molecule has been shown to bind mature myostatin and confer latency (Lee and McPherron 2001; Thies *et al.,* 2001). Follistatin, which can bind to Activins and bone morphogenetic proteins (BMPs) to inhibit their activity (de Winter *et al.,* 1996; Iemura *et al.,* 1998), has also been reported to bind myostatin and compete for receptor binding (Lee and McPherron 2001). Upon release from the latent complex, the carboxyl-terminal dimer signals through the activin type II receptor, possibly resulting in the activation of a type I receptor and the Smad proteins (Lee and McPherron 2001).

1.6.4 Function of myostatin

1.6.4.1 Identifying the effect of myostatin on muscle mass

Various experimental approaches have been adopted in order to establish the biological function of myostatin, primarily through knocking out the *myostatin* gene, or through its inhibition. McPherron *et al*. (1997) was the first to demonstrate that 3-6-month-old homozygous *myostatin*-null mice were significantly larger than their wild-type counterparts (Figure 1.7A).

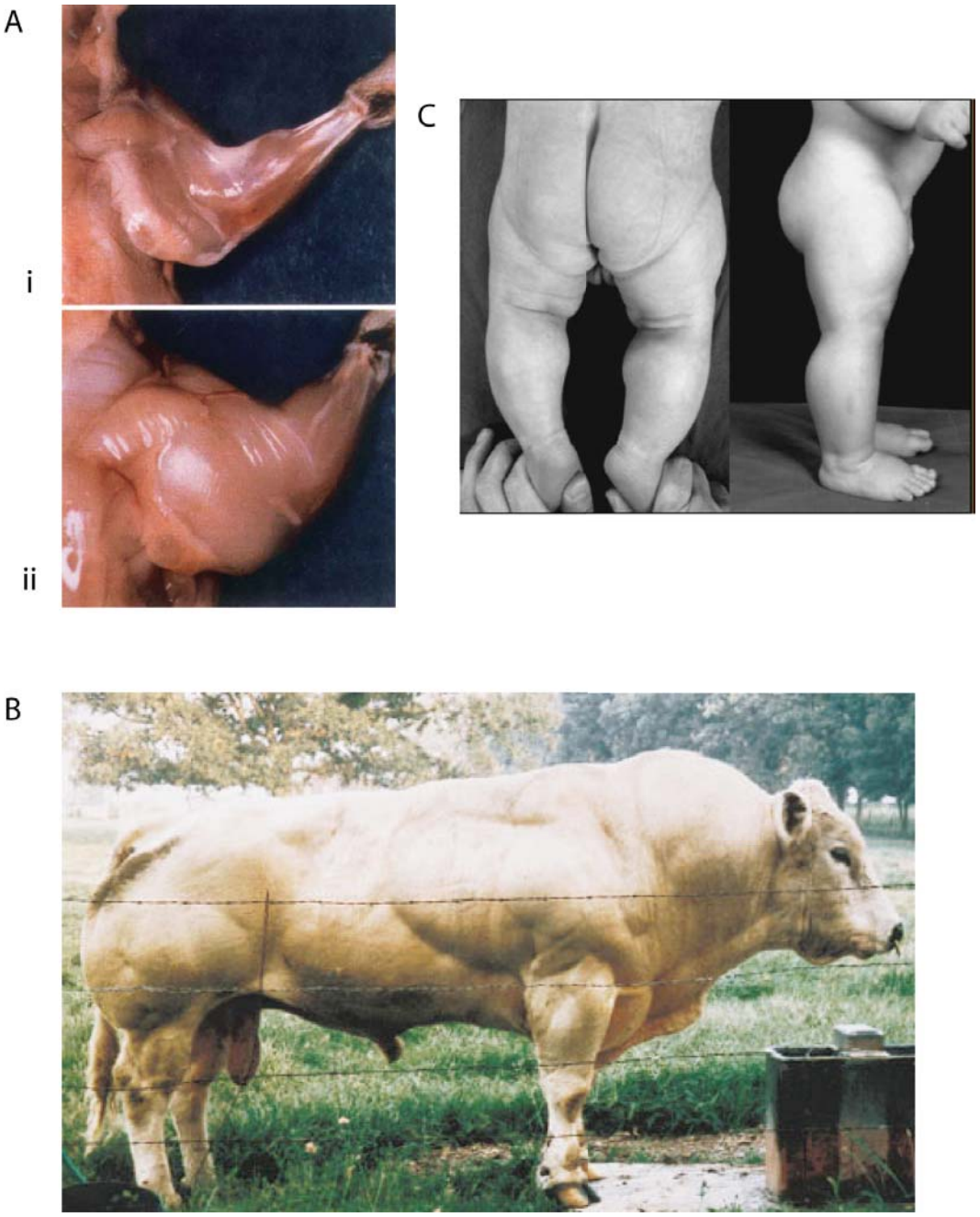

Figure 1.7 The* myostatin-*null phenotype is characterised by heavy muscling

(A) Increased skeletal muscle is observed in *myostatin*-null mice (ii) compared to wild-type littermates (i). Reproduced from McPherron *et al*. (1997). (B) A full blood Belgian Blue bull showing the double muscling phenotype. Adapted from McPherron and Lee (1997). (C) Heavy muscling can be observed in a human child at six-days-old (left) and at seven-months-old (right). The child possesses a myostatin mutation resulting in an inactive myostatin protein. Adapted from Schuelke *et al*. (2004).

This significant increase was reportedly due to a two to three-fold increase in muscle mass throughout the body, resulting from both muscle cell hyperplasia and hypertrophy (McPherron *et al.,* 1997). These results clearly indicated that myostatin was acting as a powerful negative regulator of muscle growth.

The heavy muscling observed in the *myostatin*-null mice is also known to occur in several bovine breeds, the most obvious being Belgian blue and Piedmontese, commonly referred to as double-muscled cattle (Figure 1.7B). Mapping of the bovine *myostatin* gene placed it at the same interval as the muscular hypertrophy locus that causes the double-muscle phenotype (Charlier *et al.,* 1995; Smith *et al.,* 1997). It was later discovered that the Belgian blue *myostatin* gene contained an 11 base pair (bp) deletion in the third exon, which resulted in a frame shift and subsequent early stop codon. This was found to produce an inactive truncated myostatin peptide (Grobet *et al.,* 1997; Kambadur *et al.,* 1997; McPherron and Lee 1997). Similarly, the Piedmontese *myostatin* gene was found to contain a mutation in exon three, leading to a cysteine to tyrosine substitution in the mature region of the protein, thus disrupting the formation of the cysteine knot (Kambadur *et al.,* 1997; Berry *et al.,* 2002).

Transgenic mice expressing a mutant form of *myostatin* lacking the normal cleavage site (thus preventing dimerisation and cleavage), have also been reported to have increased skeletal muscle mass resulting from muscle hypertrophy (Zhu *et al.,* 2000). In addition, Lee and McPherron (2001) investigated possible myostatin inhibitors which could promote muscle growth. These authors reported that by over-expressing *follistatin* or the *myostatin* propeptide though a skeletal muscle-specific promoter (the myosin light chain promoter MLC1), the binding of myostatin to Act RIIB could be inhibited, resulting in significant increases in muscle mass. Lee and McPherron (2001) further suggested that these or other molecules which act to block myostatin signalling may be potential therapeutic agents for enhancing muscle growth. More recently, the inhibition of myostatin through the administration of a myostatin inhibitory antibody to adult mice has shown significant increases in muscle size and grip strength, due to muscle fibre hypertrophy (Whittemore *et al.,* 2003). Interestingly, apart from the increase in fibre size, the muscle appeared histologically normal, no effect on organ size and histology was observed, and normal serum parameters were maintained, suggesting a high degree of specificity. More recently, a naturally occurring

myostatin mutation, consisting of a G to A bp transition in intron 1, has been identified in a human child (Schuelke *et al.,* 2004) (Figure 1.7C). The mutation has caused the production of a misspliced mRNA. This is predicted to give rise to a severely truncated protein, due to the 108 bp insertion adding a single lysine residue, followed by a premature termination codon. Phenotypic observations of the child appear very similar to those observed in *myostatin*-null mice, and consist of a large increase in skeletal muscle mass and decreased fat accumulation.

1.6.4.2 The mechanism of myostatin during cell proliferation

Recent studies have indicated that myostatin regulates early myogenesis by controlling myoblast proliferation and differentiation. Cell cycle analyses have revealed that myostatin causes myoblasts to accumulate in the G0/G1 and G2 phases of the cell cycle, resulting in a decrease of cells in the S phase (Thomas *et al.,* 2000; Joulia *et al.,* 2003). Progression of cells through the cell cycle phases is predominantly controlled by cyclin-dependent kinases (CDKs), their associated cyclins and CDK inhibitors (CKIs). Thomas *et al*. (2000) reported that myostatin arrests cell cycle progression through the up-regulation of *p21* (previously shown to be involved in cell cycle arrest in the G1 and G2/M phases (Dulic *et al.,* 1998)), whilst down-regulating *Cdk2*. This regulation effectively results in reduced Cdk2 activity which normally, along with its associated cyclin, cyclin-E, acts by phosphorylating the retinoblastoma-susceptible gene product, Rb. Rb normally acts by binding to and repressing the activity of specific transcription factors. Upon phosphorylation by CDKs, Rb releases the bound transcription factor, thus allowing transcription of specific S phase genes (La Thangue 1996). Thus, by myostatin up-regulating *p21* and down-regulating *Cdk2*, a reduction in the transcription of S phase-specific genes occurs. This in turn causes the cells to arrest at the G0/G1 phase and stop growing (Thomas *et al.,* 2000; Langley *et al.,* 2002; Joulia *et al.,* 2003). Conversely, inhibition of endogenous *myostatin* synthesis in C2C12 myoblasts, through the expression of an antisense *myostatin* mRNA, leads to decreased levels of *p21* gene expression and increased cell proliferation (Joulia *et al.,* 2003).

1.6.4.3 The mechanism of myostatin during cell differentiation

Numerous studies have also shown myostatin negatively regulating myoblast differentiation. Culturing C2C12 myoblasts in low serum media containing increasing concentrations of recombinant mature myostatin has been shown to reversibly block the differentiation of myoblasts (Langley *et al.,* 2002). Molecular analysis has indicated that this inhibition by myostatin results from the repression of *MyoD*, *Myf*5, *myogenin*, and *p21* expression, leading to the suppression of myogenic differentiation.

In corroboration, others have reported that *myostatin* over-expressing myoblasts have also been associated with the suppression of multinucleated myotube formation (Rios *et al.,* 2002; Joulia *et al.,* 2003). This suppression related to decreases in both MyoD and myogenin protein levels. A prerequisite for the initiation of myoblast differentiation is the arrest of the cell cycle in the G0/G1 phase (Gu *et al.,* 1993). This process involves the up-regulation of *p21*, which is enhanced by MyoD (Halevy *et al.,* 1995). Furthermore, MyoD may also induce cell cycle arrest by binding to Rb, thereby enhancing its expression (Martelli *et al.,* 1994) and inhibiting its phosphorylation (Gu *et al.,* 1993; Zhang *et al.,* 1999). This in turn would lead to a decline in S phase gene expression. Thus, myostatin-induced decreases in *myogenin* and *MyoD* expression, and subsequent decreases in p21, may account for the poor cell cycle exiting and the decline in myogenic differentiation observed in the myoblast cultures.

1.6.4.4 Effect on muscle fibres

Numerous studies have been undertaken to determine whether the increases in muscle mass associated with the interference of myostatin, results from muscle fibre hyperplasia and/or hypertrophy. It appears that the increases in muscle mass are largely dependent on the mode of treatment (i.e. transgenically mutated or through inhibition) and perhaps is also species-specific. Histological analysis of mouse *tibialis cranialis* muscle has indicated that the *myostatin*-null phenotype is associated with significant hyperplasia, as total cell number was 86% higher than their wild-type counterparts (McPherron *et al.,* 1997). In accordance with this, the amount of extracted DNA from pooled *myostatin*-null muscle was approximately 50% more than from wild-type muscle. This confirmed that hyperplasia accounted for a large proportion of the observed increased muscle

mass. Furthermore, analysis of individual cross-sectional fibre areas in the *m. gastrocnemius* (Gas) muscle showed an increase of up to 49% in the *myostatin*-null muscle. Consistent with this was an increase in the protein to DNA ratio (w/w) in the *myostatin*-null muscles compared to the wild-type muscles.

In agreement with these findings, over-expression of *myostatin* propeptide in mice has also been shown to cause both muscle fibre hyperplasia and hypertrophy (Lee and McPherron 2001). However, hyperplasia did not significantly contribute to the increased muscle mass observed in either the dominant negative mice, or in mice over-expressing the *myostatin* prodomain (Zhu *et al.,* 2000; Yang *et al.,* 2001). In these two cases, fibre hypertrophy, which occurred in both fast glycolytic fibres and fast oxidative glycolytic fibres, produced the observed muscle mass increases. Therefore, these studies suggest that total myostatin suppression is required to produce both muscle fibre hyperplasia and hypertrophy, while low or moderate inhibition of myostatin is more likely to result in fibre hypertrophy.

Surprisingly, the increase in muscle mass seen in double-muscled cattle reportedly results from muscle fibre hyperplasia only (Wegner *et al.,* 2000). Whether the differences between the two animal species relates to the inbreeding of double-muscled cattle, which inadvertently selects additional genes influencing muscle hypertrophy, remains unclear. Another explanation for this species discrepancy is a differential expression pattern of *myostatin* during embryogenesis. *Myostatin* is observed in bovine foetuses at a stage which can influence myoblast proliferation, however, unlike in murine muscle, its expression declines significantly during muscle differentiation (Kambadur *et al.,* 1997; Deveaux *et al.,* 2003). These observations are supported by *in vitro* evidence using bovine myoblasts, which indicates that *myostatin* expression peaks at the onset of fusion, decreases during terminal differentiation, and is later undetected in the myotubes (Deveaux *et al.,* 2003).

In addition to influencing muscle fibre size and number, myostatin can also affect the fibre type composition of a muscle. Wegner *et al*. (2000) reported that the number of type I fibres was unaffected in the Belgian Blue cattle, suggesting that the additional fibres were type IIB and IIA. Since *myostatin* is preferentially expressed in fast fibre types (Carlson *et al.,* 1999; Deveaux *et al.,*

2003), it is logical that fast fibre hyperplasia is associated with *myostatin* deficient animals.

In mice, myostatin appears to significantly influence the overall fibre type composition of the muscle. Comparison between *myostatin*-null and wild-type adult muscles has indicated that loss of myostatin results in a larger proportion of fast fibres with a concurrent reduction of slow fibres in *soleus* and EDL muscles (Girgenrath *et al.,* 2005). The effect of this alteration in fibre type composition would functionally result in an overall faster and more glycolytic muscle phenotype. Of note, Girgenrath *et al*. (2005) reported no change in the distribution of fibre types in EDL muscles from adult animals treated with a myostatin antibody. This would suggest that the fibre type alteration observed in *myostatin*-null mice is a consequence of the early developmental processes.

1.6.4.5 Myostatin and satellite cells

In addition to regulating muscle during embryogenesis, myostatin also appears to be a major factor during postnatal muscle growth and repair. While myostatin regulates early myogenesis by influencing proliferation and differentiation, it has been suggested that myostatin regulates postnatal myogenesis through satellite cell activation. Of note, *myostatin* expression has been detected in satellite cells (McCroskery *et al.,* 2003), indicating that myostatin may act by inhibiting satellite cell activation and proliferation. In support of this, not only do *myostatin*-null mice reportedly possess a greater number of satellite cells per muscle fibre, they also display an increased number of activated satellite cells in comparison to wild-type mice (McCroskery *et al.,* 2003). These results suggest a direct link between myostatin and the activity and recruitment of satellite cells during postnatal myogenesis, correlating with the muscle hyperplasia and hypertrophy observed in the *myostatin*-null phenotype. It is also interesting that type IIB muscle fibres express the highest amounts of *myostatin* (Carlson *et al.,* 1999) while containing the lowest numbers of satellite cells (Gibson and Schultz 1982). As previously discussed, satellite cells are a major component of muscle regeneration following injury or age-related muscle wasting. More recent studies have proposed a role for myostatin during the muscle repair process. A notexin-induced muscle regeneration study using young wild-type and *myostatin*-null mice has described accelerated migration and

enhanced accretion of myogenic cells and macrophages, as well as improved muscle regeneration and reduced fibrosis in the *myostatin*-null mice (McCroskery *et al.,* 2005). *Ex vivo* assays corroborated these findings, as reduced satellite cell activation and chemotactic movements of both myoblasts and macrophages were observed with the addition of recombinant myostatin (McCroskery *et al.,* 2005). Similarly, the muscles of aged *myostatin*-null mice reportedly regenerate more robustly after cardiotoxin injury, and attain larger diameter myofibres than their wild-type counterparts (Wagner *et al.,* 2005). Wagner *et al*. (2005) reported that the initial markers of regeneration were also enhanced in the absence of myostatin. Furthermore, satellite cells isolated from *myostatin*-null muscle also demonstrated a greater capacity for activation and faster proliferation and differentiation than wild-type cells, suggesting myostatin functions to inhibit these processes (McCroskery *et al.,* 2003; Wagner *et al.,* 2005). Cell cycle analysis of the *myostatin*-null and wild-type satellite cells indicated that myostatin was up-regulating *p21*, while decreasing the level and activity of *Cdk2* protein (McCroskery *et al.,* 2003). Thus myostatin was negatively regulating the G1 to S progression to maintain satellite cell quiescence.

Collectively, these results indicate that myostatin can negatively regulate muscle regeneration by controlling satellite cell activation, proliferation and differentiation, whilst also regulating myoblast and macrophage migration to the site of injury. Furthermore, inhibition of *myostatin* expression and signalling, through gene inactivation or myostatin inhibiting antibodies, have shown to result in significantly reduced amounts of fibrosis and fat infiltration following muscle regeneration (Bogdanovich *et al.,* 2002; Wagner *et al.,* 2002; McCroskery *et al.,* 2005; Wagner *et al.,* 2005)

1.6.5 Muscle wasting

The role of myostatin in muscle wasting has been investigated using a number of wasting models and human diseases. Immobilisation studies using rodents, have demonstrated significant increases in *myostatin* expression in association with the induced muscle atrophy (Carlson *et al.,* 1999; Wehling *et al.,* 2000). The observed increases may relate to myostatin inhibiting satellite cell proliferation, as the peak of *myostatin* expression reportedly occurs at the same time as satellite cell proliferation decreases during hind-limb unloading (Darr and

Schultz 1989; Schultz *et al.,* 1994). Interestingly, the increase in expression appears to vary according to the muscle observed and is strongly associated with the MHC IIB isoform (Carlson *et al.,* 1999). In humans, a clinical study has been performed to establish the relationship between myostatin levels and the muscle atrophy associated with human immunodeficiency virus (HIV) infection. Measurements of serum and intramuscular concentrations of myostatin protein were shown to increase in the HIV-infected patients with weight loss, compared to the healthy subjects (Gonzalez-Cadavid *et al.,* 1998). The increase in myostatin protein was also inversely correlated with the fat-free mass index. These results strongly suggest that increased myostatin levels contribute to HIV-associated muscle wasting, and raise the question whether myostatin is involved in the muscle loss associated with other pathologies.

A number of human and rodent studies have also been performed in order to determine if there is a correlation between myostatin and age-related muscle wastage. A study involving the comparison of serum myostatin-immunoreactive protein levels, fat-free mass and muscle mass in young, middle-aged, and physically frail old humans indicated that serum myostatin-immunoreactive protein levels increased with age (Yarasheski *et al.,* 2002). Furthermore, both fat-free mass and muscle mass were inversely correlated with serum myostatin-immunoreactive protein concentrations, suggesting that myostatin was acting as a negative regulator of muscle growth during aging. However, in a murine study, Kawada *et al*. (2001) reported that the content of myostatin protein in Gas and *plantaris* muscles was initially low and increased until their wet weight/body weight ratio reached a peak. The level of myostatin then remained unchanged with further aging, despite the occurrence of muscle atrophy. In contrast, a study examining the levels of *myostatin* mRNA and protein in rats during aging reported that while the level of *myostatin* mRNA in the Gas decreased with age, myostatin protein increased progressively (Baumann *et al.,* 2003). Although the results from these studies are somewhat conflicting, it appears that myostatin protein levels rather than mRNA may increase with age.

The phenotype observed in animals lacking functional myostatin (either through transgenic manipulation or myostatin inhibiting treatments), starkly contrasts the phenotype associated with *myostatin* over-expression. Transgenic mice over-expressing myostatin protein reportedly demonstrate an 18–24%

decrease in lower hind- and forelimb muscle mass (Reisz-Porszasz *et al.,* 2003). This reduced muscle mass was associated with an 18% decrease in fibre cross-sectional area and myonuclear number in the *m. quadriceps* (Quad) and Gas muscles. Furthermore, systemic over-expression of *myostatin* in adult mice has been shown to induce significant muscle and fat loss equivalent to that observed in human cachexia syndromes (Zimmers *et al.,* 2002). This again raises the question as to whether *myostatin* expression is involved in human cachetic-type conditions as well as other pathologies.

1.6.6 Adipogenesis

While the effect of myostatin on skeletal muscle is reasonably well characterised, its influence on adipogenesis and adipose tissue, remains reasonably unclear. A number of murine studies have reported significant decreases in adipose tissue in association with loss of myostatin function. For example, Lin *et al*. (2002) described decreases in fat pad weights and total lipid content by 12-weeks of age in *myostatin*-null mice in comparison to wild-type mice. In agreement, McPherron and Lee (2002) reported wild-type mice fat pads weighing approximately 2-4 times those of *myostatin*-null mice at 5- to 6-months of age. With advancing age, wild-type fat pad weights continued to increase in size while the *myostatin*-null did not. Moreover, mean total body fat was reduced by 70% in *myostatin*-null mice, and serum leptin levels were also significantly lower. Despite having a normal food intake, body temperature and a reduced metabolic rate, the *myostatin*-null gonadal fat pad had approximately 25% fewer cells, as well as a reduced fat cell size, compared to the wild-type fat pad. These results suggest that loss of functional myostatin not only affects muscle mass, but also adipose tissue mass.

In addition, *myostatin* prodomain transgenic mice have demonstrated a significant decrease in epididymal fat pad weight (Yang *et al.,* 2001). However, Whittemore *et al*. (2003) reported no change in the fat pad mass when myostatin was inhibited with a blocking antibody. In accordance with these studies, over-expression of *myostatin* in *myostatin*-transgenic male mice reportedly have significantly higher mean epididymal fat pad weights compared with wild-type controls (Reisz-Porszasz *et al.,* 2003).

Only a small number of *in vitro* studies have been performed to determine the role of myostatin during adipogenesis. Contradictory to *in vivo* data, Rebbapragada *et al*. (2003) first reported that myostatin blocks BMP7-induced adipogenesis in both pluripotent mesenchymal precursor cells (C3H 10T1/2 cells) and preadipocytes (3T3-L1 cells) by competing for binding of the ActRIIB receptor. However, a more recent study has demonstrated that recombinant myostatin treatment of C3H10T1/2 cells, which were first differentiated using azacytidine, induced early and late markers of adipogenesis, while myogenic markers were inhibited (Artaza *et al.,* 2005). Thus, this later study is in agreement with the observations of reduced fat accumulation associated with the *myostatin*-null phenotype.

1.6.7 The actions of androgens and their relationship to myostatin

Androgens are steroidal hormones generally believed to elicit positive growth effects in muscle, increasing both muscle size and strength. Belonging to this group of hormones is testosterone, which is secreted by testicular Leydig cells and is considered the primary circulating androgen (reviewed Mooradian *et al*. (1987)). While testosterone is converted to 5α-dihydrotestosterone (DHT) in reproductive tissues by the cytoplasmic enzyme 5α-reductase, in skeletal muscle, testosterone remains in its unconverted form due to the low levels of 5α -reductase (Bartsch *et al.,* 1980). Numerous clinical studies demonstrate that testosterone can augment muscle strength and increase muscle size (Brodsky *et al.,* 1996; Mauras *et al.,* 1998; Bhasin *et al.,* 2001; Sinha-Hikim *et al.,* 2002). Testosterone is a ligand of the androgen receptor (AR), a ligand-dependent transcription factor belonging to the steroid hormone receptor superfamily (Chang *et al.,* 1988b; Chang *et al.,* 1988a). Binding to the AR results in the formation of complexes, and forces conformational changes, which lead to dimerisation, nuclear localisation and DNA binding. This allows the AR to interact with androgen response elements (AREs) in various androgen target genes (reviewed by Chang *et al*. (1995)).

Investigations into the effects of concentric and eccentric loading on skeletal muscle in human subjects have indicated that *AR* mRNA concentration increases during both types of loading (Bamman *et al.,* 2001). Conversely, blockade of the AR during electrical stimulation in rats reportedly suppresses

muscle mass increases normally associated with the hypertrophy model (Inoue *et al.,* 1994). Clinical trials in humans have indicated that administration of testosterone can result in increased muscle mass in hypogonadal men (Bhasin *et al.,* 1997), HIV-infected men (Bhasin *et al.,* 2000) and healthy men (Bhasin *et al.,* 1996; Bhasin *et al.,* 2001).

As both myonuclei (Dorlochter *et al.,* 1994) and satellite cells (Doumit *et al.,* 1996) express *AR*, it is believed that they are direct targets of androgens. A number of studies have demonstrated that the administration of testosterone can increase satellite cell numbers. Joubert *et al*. (1994) reported that administration of testosterone to rats before puberty resulted in a transient increase in satellite cells. Furthermore, this observed increase in cell proliferation led to an increase in myonuclei number and the activation of quiescent satellite cells (Joubert and Tobin 1989; Joubert and Tobin 1995). A human study involving weight-lifters also reported muscle fibre hypertrophy as well as an increased number of fibres with centrally located nuclei in steroid-using athletes compared to nonsteroid-using athletes (Kadi *et al.,* 1999). The authors suggested that these observations were brought about through the process of satellite cell activation which had been enhanced by the steroid use. Indeed, increases in satellite cell number, and subsequent fusion with muscle fibres, have been shown following testosterone treatment (Sinha-Hikim *et al.,* 2003). However, *in vitro* studies have provided conflicting results in relation to satellite cell behaviour following testosterone treatment. While some report a modest effect of testosterone on primary myoblast proliferation (Powers and Florini 1975), others do not observe any change (Doumit *et al.,* 1996).

Since myostatin has been shown to negatively regulate myogenesis in a robust manor, the question of an interaction between myostatin and testosterone has been raised. Of note, the human *myostatin* promoter contains AREs in the 5'-regulatory region (Ma *et al.,* 2001), suggesting *myostatin* may be a direct target of the AR. However, a human study using healthy aged males failed to show any correlation between *myostatin* and *AR* mRNAs in muscle or with circulating testosterone levels (Marcell *et al.,* 2001). Furthermore, a strength training program conducted with young men indicated that suppression of endogenous testosterone, by the gonadotropin-releasing hormone analogue, goserelin, resulted in similar *MyoD*, *myogenin*, *myostatin*, and *AR* mRNA levels as observed in the placebo

group. Therefore, these studies suggest little interaction between testosterone and myostatin with respect to the androgen hormone suppressing *myostatin* expression or action. However, the effect of myostatin on testosterone and *AR* expression and/or activity has received minimal attention, and thus requires further study to investigate their potential relationship.

1.7 Aims of this thesis

A considerable number of studies have addressed the structure of skeletal muscle and the nature of myogenesis. With the aid of technological advancements in the molecular and microscopic fields, significant insights into muscle biology have been made possible. Many regulators of muscle growth, as well as myogenic-related genes and markers have now been identified. The discovery of myostatin, a negative regulator of muscle growth, has also increased the understanding of myogenesis and muscle development. Previous studies have provided insights into the structure and function of myostatin as well as its signalling and the mechanism through which it regulates myogenesis. However, despite the current level of understanding of myostatin and its regulation of myogenesis, many questions remain. In particular, how myostatin influences postnatal myogenesis, and whether it is involved in the muscle wasting commonly associated with aging. The degree and efficacy of postnatal myogenesis is of significant relevance to human health and quality of life. Currently, treatments for various myopathies are limited, and their possible side effects are frequently questioned. The overall aim of the work detailed in this thesis was to further elucidate the role of myostatin during postnatal myogenesis and sarcopenia. The following aims were formulated then addressed using *in vitro* and *in vivo* experiments.

1. Determine potential myostatin downstream targets to further elucidate how myostatin regulates postnatal myogenesis.

2. Examine the physiological effect of aging in wild-type and *myostatin*-null murine muscle, and investigate variations between the two genotypes to determine the role of myostatin during sarcopenia.

3. Determine how aging and a prolonged absence of myostatin affects the behaviour of satellite cells/primary myoblasts and the overall myogenic process.

4. Test the efficacy of a truncated myostatin protein to antagonise myostatin signalling and determine the effect of a short term blockade of myostatin during postnatal myogenesis.

These investigations enabled the rigorous testing of the hypothesis that myostatin profoundly regulates myogenesis during postnatal growth and sarcopenia, and that its inhibition will permit increased myogenesis and muscle regeneration.

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Chapter Two

Materials and Methods

2.1 Materials

Common laboratory chemicals and reagents were obtained from BDH, Roche, Sigma and Invitrogen. Cell culture medium components were from Invitrogen (Dulbecco's Modified Eagle's Medium (DMEM), horse serum (HS) and foetal bovine serum (FBS)) and Sigma (Phenol red, collagenase). Antibiotics (penicillin, streptomycin, ampicillin), and carrageen lambda (cλ), were obtained from Sigma. Radiolabelled nucleotides were obtained from Amersham. Specialised equipment and commercially available kits described in this thesis are referred to in the relevant Methods Sections.

2.1.1 Animals and ethical approval

Myostatin-null mice (C57BL/10 background) were obtained from S.-J. Lee (The Johns Hopkins University, Baltimore, MD). These mice have a disrupted myostatin gene by homologous targeting (McPherron *et al.,* 1997), involving the deletion of the mature carboxyl-terminal region, which was replaced with a neo cassette (resulting in a non-functional myostatin). Genotypic analysis allowed the selection of homozygous mutant pups. Periodically, mice were randomly chosen to perform genotypic analysis in order to maintain the integrity of the homozygous mutant mouse line.

The wild-type mouse strain C57BL/10 was bred at the Ruakura Small Animal Colony. All animals were handled in accordance with the guidelines of the Ruakura Animal Ethics Committee (AgResearch, Hamilton, New Zealand). Specific experimental procedures were approved by the University of Waikato and Ruakura Animal Ethics Committees. The approved ethics application numbers for work described in Chapters 3-6 are as follows.

Chapter 3 - ethics #630 (University of Waikato) and #10311 (Ruakura)

Chapter 4 - ethics #630 (University of Waikato) and #10157/10311 (Ruakura)

Chapter 5 - ethics #630 (University of Waikato) and #10311 (Ruakura)

Chapter 6 - ethics #643 (University of Waikato) and #10637 (Ruakura)

2.1.2 Common solutions

Common solutions were prepared as described in Sambrook *et al*. (1989) unless stated otherwise. The recipes for the common solutions are listed below:

Church and Gilbert hybridisation buffer
0.5 M $Na_2HPO_4.H_2O$
7% sodium dodecyl sulphate (SDS)
1 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0)
0.35% H_3PO_4
Made in diethyl pyrocarbonate (DEPC)-treated milli Q (MQ) H_2O

MOPS (3-(N-morpholine)-propane-sulfonic acid) (10X)
200 mM MOPS (pH 7.0)
100 mM NaOAc
10 mM EDTA (pH 8.0)
Made in DEPC-treated MQ H_2O

Phosphate buffer saline (PBS)
1 PBS tablet (Oxoid)
100 ml H_2O

PBS-T
100 ml PBS
0.2 ml Tween 20

Protein lysis buffer
0.5 ml 1 M 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris; pH 7.5)
0.5 ml 5 M NaCl
0.1 ml 0.5 M EDTA
10 µl NP-40
8.89 ml dH_2O
½ Protease inhibitor tablet (Complete; Roche)

Red blood cell lysis buffer

9 parts 0.83% NH_4Cl with 1 part 2.06% Tris (pH 7.65)

Adjust to pH 7.2 if required and filter with a 0.22 µm Millex-GP filter unit (Millipore)

Saline sodium citrate (SSC) (20X)

3 M NaCl

0.3 M sodium citrate (pH 7.0)

Scotts tap water

166 mM $MgSO_4$

36 mM $NaHCO_3$

1X SDS running buffer

50 ml 20X NuPAGE™ MES SDS Running Buffer (Invitrogen)

950 ml dH_2O

Tris acetate EDTA (TAE) solution

40 mM Tris-acetate

2 mM EDTA (pH 8.0)

Tris buffered saline (TBS)

50 mM Tris (pH 7.5)

150 mM NaCl

TBS-Tween (TBS-T)

50 mM Tris (pH 7.5)

150 mM NaCl

0.2% Tween 20

Tris-EDTA (TE)

10 mM Tris-Cl (at desired pH)

1 mM EDTA (pH 8.0)

Transfer buffer (pH 8)

25 mM Tris

190 mM glycine

20% methanol

10X Trypsin

2.5% trypsin in PBS

2.1.3 Loading dyes

DNA loading dye

15% Ficoll

0.25% bromophenol blue

0.25% xylene cyanol

1% SDS

1 mM EDTA (pH 8.0)

Protein loading dye

75 µl NuPAGE™ 4X LDS sample buffer (Invitrogen)

15 µl β-mercaptoethanol

RNA loading dye (2X)

10% MOPS (10X)

20% deionised formaldehyde

50% deionised formamide

0.02% bromophenol blue

5% glycerol

1 mM EDTA (pH 8.0)

40 µg/ml ethidium bromide

2.1.4 Staining solutions

Coomassie Blue Stain

2.5 g Coomassie Brilliant Blue R-250

45% methanol

10% acetic acid

Coomassie Blue Destain

45% methanol

10% acetic acid

Eosin 1% solution

1% eosin

0.01% acetic acid

Crystal of thymol

Gills haematoxylin

0.4% haematoxylin

2 mM $NaIO_3$

100 mM $Al_2(SO_4)_3$

25% ethylene glycol

4% glacial acetic acid

Ponceau S stain

0.1% (w/v) Ponceau S

0.1% (v/v) acetic acid

2.1.5 mATPase staining working reagents

Acidic pre-incubation media

3.90 g CH_3COONa

3.70 g KCl

dH_2O to 500 ml

Adjust to pH 4.6 with 100% glacial acetic acid

Basic pre-incubation media (pH 9.4)

0.95 g NaOH

1.90 g NaCl

1.98 g glycine

2.78 g $CaCl_2.2H_2O$

dH_2O to 500 ml

ATP Incubation media

0.255 g ATP

150 ml basic pre-incubation media (pH 9.4)

Adjust to pH 9.4 with 1N NaOH

Pre-incubation wash (pH 9.4)

Basic pre-incubation media

Ammonium sulphide

1 ml 20% $(NH_4)_2S$

dH_2O to 200 ml

1% $CaCl_2$

13.2 g $CaCl_2.2H_2O$

dH_2O to 1 L

2% $CoCl_2$

36.6 g $CoCl_2.6H_2O$

dH_2O to 1 L

2.1.6 Van Gieson solutions

Van Gieson solution

10 ml 1% aqueous acid fuchsin

90 ml saturated Picric acid

0.25 ml concentrated HCl

Solution A

1% haematoxylin in absolute alcohol

Solution B

4 ml 30% Ag ferric chloride

1 ml concentrated HCl

100 ml dH_2O

2.1.7 Bacterial growth media

LB media

Bacteria were grown in LB Broth (Lennox L Broth) which was prepared by adding 20 g LB Broth base to 1 L H_2O, then autoclaved and stored at 4°C.

LB plates

Bacterial colonies were grown on LB Agar (Lennox L Agar) plates. LB Agar plates were prepared by adding 32 g LB Agar to 1 L H_2O, followed by autoclaving. Molten LB Agar (20 ml) was poured into Petri dishes (10 cm; Nunc) and left to set before storing at 4°C.

2.1.8 Yeast two-hybrid media and buffers

Yeast growth media

Yeast peptone dextrose media (YPD; rich medium)

20 g peptone

10 g yeast extract

20 g glucose

0.1 g NaOH (if for plates)

20 g agarose (if for plates)

1 L H_2O

Yeast nitrogen base- (YNB) ura-his-leu-trp (selective medium)

1.7 g YNB (without amino acids)

0.6 g his-ura-trp-leu dropout mix

20 g glucose (or 20 g galactose/10 g raffinose for X-5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; if for plates)

20 g agar (if for plates)

1 L H_2O

Selective media

Combinations of the following amino acids were added to the YNB-ura-his-leu-trp media; 0.02 mg/ml ura, 0.02 mg/ml his, 0.06 mg/ml leu, and/or 0.04 mg/ml trp

Buffers

10X LiOAc

1 M lithium acetate

50% polyethylene glycol-3350 (PEG)

250 g polyethylene glycol-3350

500 ml H_2O

10X TE

0.1 M Tris (pH 7.5)

0.01 M EDTA

1X TE/LiOAc

1 part 10X TE, 1 part 10X LiOAc, and 8 parts H_2O

1X TE/LiOAc/PEG

1 part 10X TE, 1 part 10X LiOAc, and 8 parts 50% PEG

2.1.9 Cell culture media

DMEM

50 ml 2X DMEM

8 ml HCO_3^-

1 ml penicillin G (200 U/ml) and streptomycin (200 µg/ml)

41 ml H_2O

Satellite cell proliferation media

DMEM supplemented with 20% FBS (v/v), 10% HS (v/v), and 1% chick embryo extract (CEE) (v/v).

2.1.10 Fixatives

Ethanol Fixative

15 ml 50 mM glycine

35 ml absolute ethanol

Adjust pH to 2.0 with HCl

10% buffered formalin

32.5 g Na_2HPO_4

20 g $NaH_2PO_4.H_2O$

500 ml formalin

4.5 L H_2O

4% paraformaldehyde

2 g paraformaldehyde

50 ml PBS

Heat to ~50°C then add 2 M NaOH until almost clear. Remove from heat, and add concentrated HCl until pH 7.2 once cooled. Filter with a 0.22 µm Millex-GP filter unit (Millipore)

20:2:1 fixative

20 parts ethanol

2 parts formalin

1 part acetic acid

2.2 Methods

Standard molecular biology methods were performed as described by Sambrook *et al*. (1989) unless otherwise stated.

2.2.1 RNA extraction

Total RNA was isolated from tissues and cultured cells using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. For muscle tissue, approximately 50-100 mg of sample was homogenised in 1 ml TRIZOL. After 5 min incubation at room temperature, 200 µl chloroform per 1 ml TRIZOL was added and the tubes were shaken for 15 s before incubating at room temperature for a further 2-3 min. The samples were centrifuged at 12,000 x g for 15 min at 4°C, and the aqueous phase was transferred to a new tube. Total RNA was precipitated with 500 µl isopropanol per 1 ml TRIZOL, incubating for 10 min at room temperature. The samples were centrifuged at 14,000 x g for 10 min at 4°C and the pellet was washed in 75% ethanol and re-spun, before resuspending in 25-60 µl DEPC-treated MQ H_2O.

For cultured cells, media was removed and the cells were washed twice with PBS. The cells were then lysed by adding 2 ml TRIZOL per 10 cm dish (Nunc), and the lysate was transferred into 1.5 ml tubes. After 5 min incubation at room temperature, 400 µl chloroform was added before shaking the tubes for 15 s. Following a further 3 min incubation at room temperature, the samples were centrifuged at 12,000 x g for 15 min, and the aqueous phase was transferred to a new tube before total RNA was precipitated with 1 ml isopropanol. Samples were then centrifuged at 14,000 x g for 10 min before the pellet was washed in 75% ethanol and resuspended in 40 to 60 µl of DEPC-treated MQ H_2O.

Total RNA was later quantified by UV spectrophotometry measuring at 260/280 nm using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies).

2.2.2 First-strand synthesis

SuperScript II Reverse Transcriptase (Invitrogen) was utilised for all first-strand synthesis for subsequent PCRs. Reverse transcriptase (RT) reactions were performed according to the manufacturer's protocol. For each RT reaction, 0.5 µg

of Oligo(dT) was annealed to 5 µg of total RNA in a 10 µl reaction containing 1 mM dNTPs. This annealing reaction was incubated at 65°C for 5 min followed by a 1 min incubation on ice. A 9 µl reaction mixture containing 1X RT Buffer, 10 mM $MgCl_2$, 20 mM dithiothreitol (DTT) and 40 U RNaseOUT was then added and the reaction was incubated at 42°C for 2 min. SuperScript II RT (50 U) was added to the mixture before incubating at 42°C for 50 min and 70°C for 15 min. Rnase H (2 U) was then added and the mixture was incubated for a further 20 min at 37°C. All RT reactions were stored at -20°C until subsequent use in PCR amplifications.

2.2.3 PCR

All PCR amplifications were performed using *Taq* DNA Polymerase (Roche) according to the manufacturer's instructions. PCRs (50 µl) contained 0.2 mM dNTPs, 0.2 µM forward primer, 0.2 µM reverse primer, 1X PCR buffer and varying concentrations of RT reaction. A Hybaid MBS 0.5S PCR System (Hybaid) was used for all PCR thermocycling. PCR cycling temperatures and times are described in relevant sections where PCR was performed.

2.2.4 Electrophoresis of RNA

Total RNA was fractionated by electrophoresis using agarose gels containing 1% agarose, 1X MOPS and 0.66 M formaldehyde. A volume of 10 µg of total RNA was mixed with an appropriate volume of RNA loading dye before incubating at 65°C for 5 min. Following RNA loading, electrophoresis was performed at 40-80 V at 4°C until the desired separation was achieved. Resulting gels were photographed with a Bio-Rad Gel Doc 2000 system (Bio-Rad) to assess the integrity of the RNA using the 28S and 18S ribosomal bands.

2.2.5 Electrophoresis of DNA

Depending on the size of the DNA fragments and the required separation, agarose gels contained 0.8-2% agarose and 300 ng/ml ethidium bromide in 1X TAE buffer. Electrophoresis was performed using an Owl electrophoresis system (Owl Separation Systems) containing 1X TAE. DNA samples were mixed with DNA loading dye and then loaded before electrophoresis at 30-100 V until the

desired separation was achieved. Fractionated DNA was visualised and photographed using the Gel Doc system described above.

2.2.6 Recovery of DNA

Following DNA separation by low melting point (LMP) agarose gel electrophoresis, the section of gel containing the desired DNA fragment was excised under UV light and incubated at 70°C until melted. The recovery of DNA was performed using the Wizard DNA Purification System (Promega) as follows. A volume of 1 ml DNA purification resin was added before the resin/DNA was passed through a Wizard Minicolumn, followed by 2 ml 80% isopropanol. The Minicolumns were then centrifuged at 10,000 x g for 2 min before the DNA was eluted in 50 µl MQ H_2O with centrifugation at 10,000 x g for 20 s.

2.2.7 DNA ligations

The DNA and specific vectors were digested separately with the appropriate restriction endonucleases to generate complementary 3' or 5' extensions for cloning. Ligation reactions contained 25-50 ng vector, a 3- or 6-fold molar excess of insert over vector, 1X ligation buffer (Invitrogen) and 1 U T4 DNA ligase; ligations were performed at 16°C for 18 hr.

2.2.8 Transformation of competent cells

Transformation of DH5α and BL21 competent cells was performed using the following protocol. Plasmid DNA (1-20 ng) was added to 100 µl of the competent cells (Invitrogen) before incubating on ice for 30 min. The cells were heat shocked at 42°C for 45 s and then cooled on ice for 3 min before adding one volume of LB broth. The transformed cells were incubated at 37°C for 60 min without antibiotic before plating onto LB agar plates.

2.2.9 Growth of bacteria

Plated bacteria

Transformed bacterial cells (approximately 100 µg/ml) were streaked across LB agar plates containing the appropriate antibiotic(s). Plates were incubated at 37°C for 12-18 hr until colonies were approximately 1-2 mm in diameter.

Liquid cultures

Single colonies were picked using sterile tips and transferred to 3 ml of LB broth (for a miniprep culture) containing 100 µg/ml ampicillin. The cultures were then incubated at 37°C overnight with shaking (200 rpm).

2.2.10 Plasmid DNA extraction

Plasmid DNA from miniprep cultures of transformed bacteria was extracted and purified using the QIAprep Miniprep System (Qiagen) according to the manufacturer's instructions. Cultures were initially centrifuged at 4,000 x g for 4 min. The resulting bacterial pellets were resuspended in 250 µl Buffer P1, followed by 250 µl Buffer P2 and 350 µl Buffer P3, inverting the tubes 4-6 times between buffer additions to mix thoroughly. The samples were then centrifuged at 20,000 x g for 10 min before the supernatants were transferred to QIAprep spin columns. The columns were centrifuged at 20,000 x g for 1 min, washed with 0.75 ml Buffer PE and centrifuged again. Plasmid DNA was eluted in 50 µl MQ H_2O by centrifuging at 20,000 x g for 1 min.

2.2.11 Restriction endonuclease digestions

Plasmid DNA was digested with 5 U of the appropriate restriction endonuclease (specified in relevant sections) per µg DNA. Restriction digestions were performed in 1X restriction endonuclease buffer (as recommended by the supplier) in a volume such that the total enzyme concentration was less than 10%. Digestion reactions were performed 37°C (or temperature specified by supplier) for 1 hr. The resulting digest products were later used for agarose gel electrophoresis or purified for subsequent use.

2.2.12 Northern and Southern blotting

2.2.12.1 Northern blotting

Following electrophoresis and assessment of RNA integrity (described in Section 2.2.4), the RNA gels were soaked in 10X SSC for 10 to 20 min to remove the formaldehyde. RNA was transferred to Hybond N+ membrane (Amersham) by capillary transfer as described in Sambrook *et al*. (1989), using 10X SSC as the transfer buffer. After the transfer, the membranes were washed and cross-linked using a UV Stratalinker 1800 (Stratagene). The membranes were pre-hybridised

with Church and Gilbert hybridisation buffer in Hybaid bottles at 55°C for 1 hr prior to hybridisation. After denaturing the prepared ^{32}P-labelled cDNA probes with 4 M NaOH for 5 min, the radiolabelled probes were added to the membranes in fresh Church and Gilbert hybridisation buffer. Hybridisation was performed overnight in a Hybaid oven at 55°C. The following day the membranes were rinsed with 5X SSC, and washed at 55°C for 15 min with 2X SSC + 0.5% SDS and 1X SSC + 0.5% SDS. The membranes were then sealed in plastic before exposing to X-ray autoradiography (XAR) film (Kodak).

2.2.12.2 Southern blotting

Following DNA electrophoresis (as described in Section 2.2.5), the Northern blotting protocol (described above) was essentially performed, with the exception that after electrophoresis, the gels were soaked (and transferred) in 1.5 M NaCl and 0.5 M NaOH for 30 min.

2.2.13 Radiolabelling of probes

Androgen receptor associated protein-70 (ARA70) cDNA for a Northern and Southern blot radioactive probe was generated as described in Section 3.2.4 of Chapter 3. Similarly, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA for a Southern blot radioactive probe was generated as described in Section 3.2.7 of Chapter 3. The process of radiolabelling the cDNA was performed as follows. cDNAs were labelled using a Rediprime II random prime labelling system (Amersham) according to the manufacturer's instructions. Initially, cDNA was diluted to 25 ng in a final volume of 45 µl, and then denatured by heating to 100°C for 5 min before adding to a Rediprime random priming mix. A volume of 2.5 µl of [α-^{32}P]dCTP (25 µCi) was added and mixed before incubating at 37°C for 10 min.

2.2.14 Suppressive subtraction hybridisation methods

2.2.14.1 mRNA isolation

Poly $(A)^+$ RNA was purified from total RNA (TRIZOL) using a Fast Track 2.0 mRNA isolation kit (Invitrogen) according to the following protocol. Total RNA (2 mg) was dissolved in 15 ml DEPC-treated H_2O before the sodium chloride concentration was adjusted to 0.5 M with 950 µl of 5 M NaCl. The RNA

mix was passed through an 18-gauge needle and incubated at 42°C for 10 min. A tube of Oligo(dT) (75 mg) was added per sample before incubating for 2 min at room temperature. Samples were then gently rocked for 1 hr before centrifuging at 3,000 x g for 5 min. The Oligo(dT) was washed twice in binding buffer, followed by a wash in low salt wash buffer until the buffer was clear. After resuspending in 800 µl of low salt wash buffer, the Oligo(dT) was transferred to a spin column and centrifuged at 5,000 x g for 10 s. This was repeated until all cellulose was transferred to the column. Steps using low salt wash buffer were performed until the flow-through was less than 0.05 (OD260). The Oligo(dT) was later resuspended in two aliquots of 200 µl elution buffer, with 30 s centrifugation spins in between. After collecting the 400 µl of eluate, 60 µl 2 M sodium acetate, 1 ml 100% ethanol and 40 µg glycogen was added before storing at -80°C overnight. The following day, the solution was thawed and centrifuged at 15,000 x g for 25 min at 4°C. The pellet was then air dried and resuspended in elution buffer. The mRNA concentration was determined by UV spectrophotometry at 260 nm using a Helios γ spectrophotometer (Thermo Scientific).

2.2.14.2 Construction of subtracted libraries

To perform suppressive subtraction hybridisation (SSH), cDNAs were synthesised from 2 µg of poly $(A)^+$ RNA from *m. biceps femoris* (BF) of *myostatin*-null mice ('driver') and wild-type mice ('tester') using a PCR-Select cDNA subtraction kit (Clontech) as previously described by Diatchenko *et al*. (1996).

To select for double-muscle-directed transcripts, cDNAs derived from *myostatin*-null muscle were ligated to oligonucleotide linkers and hybridised with excess cDNAs derived from wild-type muscle tissue. First and second strand cDNA was subtracted as described in the manufacturer's protocol of the PCR-Select cDNA subtraction kit (Clontech). After hybridisation, differential transcripts were selectively amplified by suppression.

2.2.14.3 Cloning of cDNAs

Amplified cDNAs were shotgun cloned into the pGEM-T Easy vector (Promega) and transformed into DH5α competent cells (Invitrogen) (as described in Section 2.2.8). Colonies containing inserts were grown overnight in LB broth

with ampicillin, from which 1 µl was used to seed PCR amplification of inserts with Nested primer 1 and 2R. PCR conditions were; 94°C for 30 s, followed by 25 cycles of 95°C for 30 s and 68°C for 3 min.

Nested primer 1 5'-TCGAGCGGCCGCCCGGGCAGGT

Nested primer 2R 5'-AGCGTGGTCGCGGCCGAGGT

2.2.14.4 Differential library screening

PCR amplified transcripts were dot-blotted onto Hybond N+ membrane (Amersham) using a dot-blot apparatus (Bio-Rad) according to the manufacturer's instructions. For each PCR, 50 µl of the following combined mixture was dot-blotted onto duplicate membranes: 5 µl PCR product and 140 µl denaturing mix (0.3 M NaOH/15% Ficoll (Sigma) and 0.5% bromophenol blue). The membranes were autocross-linked in a UV Stratalinker 1800 (Stratagene) and pre-hybridised in Church and Gilbert hybridisation buffer at 60°C for 1 hr. Hybridisation with cDNA probes was performed overnight in a Hybaid oven at 55°. The following day, the membranes were washed with 2X SSC + 0.5% SDS for 15 min (x2) at 55°C, followed by 1X SSC + 0.5% SDS for a further 15 min. The membranes were then sealed in plastic and exposed to XAR film (Kodak).

2.2.14.5 cDNA probes

The wild-type and *myostatin*-null cDNA probes were synthesised in a 20 µl RT reaction from poly $(A)^+$ RNA using a superscript pre-amplification kit (Invitrogen). Each poly $(A)^+$ RNA (2 µg) was incubated at 70°C for 10 min with 1 µl Oligo(dT) (0.5 mg), and MQ H_2O to 7 µl. A further 12 µl of the following mix was then added: 2 µl 10X RT buffer, 2 µl 25 mM $MgCl_2$, 1 µl 10 mM dNTP mix (-dCTP), 2 µl 0.1 M DTT and 5 µl [α-^{32}P]dCTP, before incubating at 42°C for 5 min. A volume of 1 µl (200 U) Superscript II RT was added per tube, which were then incubated at 42°C for 40 min before termination at 70°C for 15 min. A volume of 1 µl RNase H was added, and tubes were incubated at 37°C for 20 min before 29 µl MQ H_2O was added to make a total of 50 µl. The cDNA probes were passed over a G-50 Sephadex column (Boehringer) to remove the unincorporated radioactive ^{32}P according to the manufacturer's instructions.

2.2.15 Yeast two-hybrid interactions

For yeast two-hybrid studies, the DupLEX-A™ Yeast Two-Hybrid System (OriGene) was used to identify protein-protein interactions between the androgen receptor ligand binding domain (ARLBD) and ARA70.

2.2.15.1 Cloning of ARLBD and ARA70

The cDNAs for ARA70 (both full length and truncated) and ARLBD were obtained by reverse transcription-PCR (RT-PCR) (as described in Section 2.2.3). First-strand cDNA was synthesised in a 20 µl RT reaction from 5 µg total RNA (from murine tissue) using SuperScript II pre-amplification kit (Invitrogen). PCR was performed with 2 µl of the RT reaction under the following conditions:
ARLBD; 94°C for 2 min, then 30 cycles of 94°C for 20 s, 55°C for 30 s, and 72°C for 1 min, followed by a final 75°C for 5 min step
Full length ARA70; 94°C for 2 min, then 25 cycles of 94°C for 20 s, 55°C for 30 s, and 72°C for 2 min, followed by a final 72°C for 5 min step
Truncated ARA70; 94°C for 2 min, then 25 cycles of 94°C for 15 s, 60°C for 30 s, and 68°C for 1.5 min, followed by a final 68°C for 7 min step.
The primers used for the amplification of ARA70 were:
Forward 5'-GCGAATTCATGAACACATCCCTG
Full length reverse 5'-GCGAATTCTCACATCTGTAGAGG
Truncated reverse 5'-GAATTCTCAAAGCCACTCTGACAAGGA
The primers used for ARLBD were:
Forward 5'-GGGATCCGTATGACTCTGGGAGCA
Reverse 5'-ACGGATCCCTCACTGTGTGTGGAA

The following molecular manipulations were performed as described in the relevant sections of this Chapter. All amplicons were electrophoresed in LMP agarose gels, excised, and Wizard purified before ligating into pGEM-T Easy (Promega). DH5α cells were transformed by the ligation mixture and incubated at 37°C before performing minipreps on each culture. The pEG202 bait plasmid and the pJG4-5 target plasmid were prepared by performing enzymatic digests using EcoR1 and BamH1 respectively. The ARA70 inserts were cloned into pEG202 as EcoR1 fragments, while the ARLBD was cloned into pJG4-5 as a BamH1 fragment. DH5α cells were then transformed by ligation mixtures and all

constructs were sequenced to confirm the absence of mutations. In preparation for yeast transformations, the cultures were incubated at 37°C and later minipreped.

2.2.15.2 Yeast transformation

Interactions between the encoded fusion proteins were assessed by co-transforming the bait and target plasmids together with the reporter plasmid, pJK103, into yeast strain EGY194 as follows. A 5 ml culture of yeast EGY194 was grown in YPD overnight at 30°C (yeast were grown with shaking unless stated otherwise), and used to inoculate 60 ml YPD to an absorbance of 0.1 at 600 nm. At an absorbance of 0.5-0.7, the cultures were centrifuged at 1,500 x g for 5 min, and resuspended in 0.3 ml 1X TE/LiOAc. The resuspended cells were aliquoted into 1.5 ml tubes and 100 ng of each plasmid DNA (bait, target and reporter) along with 50 µg denatured carrier DNA was added. A volume of 0.3 ml 1X TE/LiOAc/PEG was added and the tube was mixed by inversion. The cells were incubated at 30°C for 30 min, before the addition of 40 µl DMSO (dimethyl sulphoxide) and a further incubation at 42°C for 15 min (without shaking). The cells were then centrifuged at 12,000 x g for 10 s and resuspended in 0.5 ml H_2O. To assess protein interactions, the transformed yeast cells were grown on selection plates as described in Section 3.2.3 of Chapter 3.

2.2.16 *In situ* hybridisation

2.2.16.1 Overview of the in situ *hybridisation procedure*

In situ hybridisation experiments were performed to detect *ARA70* mRNA in muscle tissues. Initially, an approximately 500 bp *ARA70* fragment was cloned into a pGEM-T Easy vector (Promega) containing T7 and SP6 RNA polymerase promoters. After the vector was linearised by restriction enzymes, digoxigenin (DIG) -UTP-labelled, single-stranded RNA probes were generated using a DIG RNA labelling kit (Roche). RNA labelling with DIG-UTP by *in vitro* transcription with SP6 and T7 RNA polymerase permitted the generation of 'sense' and 'antisense' transcripts with DIG-UTP being incorporated every 20-25 nucleotides. The DIG-labelled probes were then introduced to the muscle sections where the 'antisense' probe (SP6 probe) could bind to any complementary ARA70 mRNA within the tissues. The probes were later immuno-detected with anti-DIG alkaline phosphatase fab fragments as a bound antibody conjugate, due to the enzyme

alkaline phosphatase catalysing a chemical reaction to produce a visible, dark indigo coloured, precipitate.

2.2.16.2 Generation of DIG-labelled RNA in situ *probes*

The sequences of the designed primers used for the generation of a 502 bp fragment were:

Forward 5'-GCGAATTCATGAACACATCCCTG

Reverse 5'-CCATCAGATGCTCAGGGATTTGA

PCR was performed using 2 µl of RT reaction (from murine kidney RNA) (as described in Section 2.2.3), under the following conditions: 94ºC for 2 min, then 35 cycles of 94ºC for 20 s, 55ºC for 30 s, and 72ºC for 2 min, followed by a termination step at 72ºC for 5 min. The amplicon was electrophoresed in a 1% LMP agarose gel before the fragment was excised and Wizard purified. The fragment was ligated into pGEM-T Easy (Promega), and transformed into DH5α cells. Endonuclear restriction digests were performed using EcoR1 and BamH1 to establish the presence and orientation of the insert respectively (refer to relevant sections within this Chapter for descriptions of these molecular manipulations). Additionally, the construct was sequenced to confirm the absence of mutations. A further restriction digest was performed using the restriction enzymes Sph1 and Sal1 to linearise the vector/ insert in preparation for the *in situ* probe making. The resulting digest products were mixed as follows: 57 µl linearised plasmid; 2 µl glycogen; 20 µl 3 M sodium acetate; 600 µl 100% ethanol; 143 µl H_2O. Samples were incubated at -20°C for 2 hr before centrifuging for 10 min at 14,000 x g. The supernatant was removed, and after the addition of 600 µl 70% ethanol, the samples were spun for 10 min at 14,000 x g. The resulting pellet was dried and then resuspended in 20 µl H_2O. The Roche DIG RNA labelling kit (SP6/T7) protocol was performed in order to generate DIG-labelled single-stranded RNA probes. For this, 2 µl of purified linearised plasmid was added to 11 µl DEPC-treated H_2O, before the following was added while incubating on ice.

Plasmid linearised by Sal1		Plasmid linearised by Sph1	
10X NTP labelling mix	2 µl	10X NTP labelling mix	2 µl
10X transcription buffer	2 µl	10X transcription buffer	2 µl
RNase inhibitor	1 µl	RNase inhibitor	1 µl
RNA polymerase T7	2 µl	RNA polymerase SP6	2 µl

Samples were mixed gently, centrifuged briefly, and incubated at 37ºC for 2 hr. The reaction was later terminated by the addition of 2 µl 0.2 M EDTA. The SP6 and T7 probes were then precipitated with 2.5 µl 4 M LiCl, 5 µl, 10 mg/ml yeast tRNA, and 75 µl 100% ethanol. Samples were incubated at -70ºC for 30 min then centrifuged at 13,000 x g for 15 min. The pellet was washed in 70% ethanol and re-centrifuged for a further 5 min. Once the supernatant was removed, the pellet was dried before being resuspended in 15 µl premix hybridisation buffer.

A dot-blot procedure was later performed to determine the relative concentrations of the SP6 and T7 probes. Both probes were diluted with TE buffer to the following dilutions: 1:100, 1:250, 1:500, 1:1,000, 1:2,000, and 1:3,000. A quantity of 1 µl of each probe dilution was spotted onto N+ nitrocellulose membrane (Amersham). The spots were dried and cross-linked before the membrane was submerged in 1X blocking buffer (diluted in TBS) for 45 min. Sheep anti-DIG alkaline phosphatase (Fab fragments) were then added to the blocking buffer at a dilution of 1:5,000 for 30 min. The membrane was washed in TBS-T for 10 min (x3), and incubated with a colour solution (1 ml 1 M Tris-HCl (pH 9.5), 0.2 ml 5 M NaCl, 0.5 ml 1 M $MgCl_2$, 8.3 ml DEPC-treated H_2O containing 45 µl nitroblue tetrazolium chloride (NBT) and 35 µl 5-bromo-4-chloro-3-indoyl phosphate (BCIP)) for 5-10 min depending on colour development.

2.2.16.3 De-waxing and pre-treatment

TA muscle tissues and sections were prepared as described in Section 2.2.18.2. Prepared muscle sections were washed in 2X SSC for 30 min, digested with Proteinase K (10 mg/ml in 100 mM Tris/HCl pH 8.0, 1 mM $CaCl_2$) for 15 min at 37°C, and washed with 2X SSC. Tissue sections were post-fixed with 0.25% glutaraldhyde diluted to 1:100 in DEPC-treated PBS for 5 min, and then washed twice in DEPC-treated PBS for 5 min. Sections were treated with 1% acetic anhydride in 0.1 M triethanolamine for 5 min, rinsed in 2X SSC with 0.5 ml of acetic anhydride for 5 min, and then washed in 2X SSC for 5 min.

2.2.16.4 Pre-hybridisation

Approximately 50 µl pre-hybridisation solution (50% formamide, 2X SSC, 150 mM NaCl, 4 µg/ml bovine serum albumin (BSA), 5% dextran sulfate,

0.5 mg/ml salmon sperm DNA, and 0.25 mg/ml yeast tRNA) was added to each section and incubated for 60 min at 55°C in a humidified chamber equilibrated with 50% formamide in 2X SSC.

2.2.16.5 Hybridisation

The pre-hybridisation solution was later removed, and approximately 50 µl of either sense or anti-sense RNA probe diluted in pre-hybridisation solution (at equal molar concentrations) was added to each tissue section. The slides were then incubated overnight at 55°C in the equilibrated chamber. The following day the probes were removed and the slides were immersed in 50% formamide in 2X SSC (preheated to 60°C) for 10 min (x2). Slides were further washed in 2X SSC for 15 min (x2), 1X SSC for 15 min (x2) and 0.2X SSC for 30 min (x2) at 37°C.

2.2.16.6 Immunological detection

The slides were washed with TBS for 10 min (x2), after which, blocking buffer (TBS containing 0.1% Triton X-100 and 2% normal sheep serum (NSS)) was added for 30 min. The sections were then covered with TBS containing 0.1% Triton X-100, 1% NSS, and sheep anti-DIG alkaline phosphatase (Fab fragments) at 1:500 for 2 hr in a humid chamber. The slides were again washed with TBS for 10 min (x2) before incubating for 10 min with a pre-colour solution (100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 50 mM $MgCl_2$). A colour solution containing 1 ml pre-colour solution, 4.5 µl NBT (75 mg/ml), 3.5 µl/ml BCIP (50 mg/ml), and 1 mM levamisole was then added to each section overnight. The following day the reaction was stopped by incubating in a stop buffer (10 mM Tris-HCl (pH 8.1), 1 mM EDTA) for 5 min, and dipping in dH_2O. The slides were washed in PBS for 5 min before counterstaining with 4',6-diamidino-2-phenylindole (DAPI; 1:1,000 in PBS; Molecular Probes) for 1 min. The sections were re-washed and mounted with aqueous mounting medium (Dako). Images were obtained using an Olympus BX50 microscope (Olympus) fitted with a Dage-MTI DC-330 colour camera (Dage-MTI) and UV filter.

2.2.17 Generation and purification of a myostatin truncation

The truncated myostatin protein used in the antagonist experiments described in this thesis was generated and purified by Carole Berry and Dr Gina

Nicholas (AgResearch, Hamilton, New Zealand), according to a previously published protocol (Sharma *et al.,* 1999). A pET protein expression system (Novagen) was used to express the truncated myostatin protein, referred to as Mstn-ant1. A portion of bovine myostatin cDNA spanning amino acids 267-350 was PCR amplified as a BamHI fragment to produce a truncated portion of the processed region, and subsequently cloned into a pET 16-B vector. The myostatin coding sequence was placed in frame with the 10 histidine residues. PCR was performed at 94°C for 30 s, 50°C for 1 min, and 72°C for 1 min for 35 cycles, followed by a single 72°C extension step for 5 min. The primers used for the amplification of the truncated myostatin sequence were:

Forward 5'-GAGGATCCGGATTTTGGGCTTGAT

Reverse 5'-CGGATCCTCCATATTAATTGGACACAT

The amplicon was excised from a LMP gel and Wizard purified before ligating into pGEM-T Easy (Promega). DH5α cells were transformed with the ligation mixture and incubated at 37°C before minipreping and sending for sequencing to confirm absence of mutations. The insert was cloned into a pET 16-B vector as a BamH1 fragment, and then transformed into DH5α cells, which were incubated at 37°C and later minipreped. Plasmid DNA was transformed into BL21 *E. coli* (Invitrogen) competent cells (refer to relevant sections within this chapter for descriptions of these molecular manipulations). An overnight culture of cells transformed with the recombinant myostatin expression vector was diluted (1:50) and incubated at 37°C to an OD of 0.8 (595 nm) in 1 L of LB broth containing 50 mg/L ampicillin. The fusion protein was induced by 0.5 mM isopropyl thio-β-galactoside (IPTG) for 2 hr. The bacteria were collected by centrifugation (4,500 x g for 30 min) and resuspended in 40 ml of lysis buffer (50 mM Tris (pH8.0), 200 mM NaCl, 10% glycerol). The bacteria were then sonicated, and the resulting lysate was centrifuged at 10,000 x g for 30 min. The truncated protein was purified from the supernatant by Ni-Agarose (Qiagen) affinity chromatography. For this, the supernatant was bound to the Ni-Agarose for 2 hr at 4°C, then packed into a column and washed with lysis buffer containing 50 mM imidazole. Column fractions were eluted with lysis buffer containing 200 mM imidazole, and pooled and dialysed against two changes of 100 mM Tris-HCl (pH 8.0) containing 500 mM NaCl for 2 hr. In order to test the purity of the truncated protein, 3 µg was

separated on a NuPAGE™ 4-12% Bis-Tris gel (Invitrogen), and stained with Coomassie Blue stain.

2.2.18 Tissue preparation

2.2.18.1 Freezing muscles for sectioning

Dissected muscles were coated in Tissue-Tek OCT compound (Sakura) before dipping in liquid nitrogen-cooled isopentane for approximately 10 s. Tissues were then stored at -80°C until further use. Muscle sections were cut at 10 µm using a Reich-Jung Cryocut 1800 cryostat (Leica), then air dried and stored at -20°C. Before staining, the slides were thawed in an airtight container at room temperature.

2.2.18.2 Formalin fixing and paraffin embedding of tissues

Excised fat pads and muscles were fixed in 10% formalin at room temperature for 48 hr and then washed in 70% ethanol until processing. A Leica TP 1050 tissue processor (Leica) was used to embed the fat pads with paraffin wax. This process involved an automated sequence described in Table 2.1.

Table 2.1 Paraffin fixation of tissues

Solution	*Temperature*	*Time*
70% ethanol (x2)	room temperature	30 min
85% ethanol	room temperature	60 min
95% ethanol	room temperature	60 min
100% ethanol (x2)	room temperature	60 min
xylene (x3)	room temperature	60 min
paraffin (x3)	60°C	60 min

A MQ H_2O bath was preheated to 42°C before the sections were cut to a thickness of 7-10 µm using a Leitz Wetzlar microtome (Leica). Cut sections were floated in the water bath and allowed to flatten before transferring onto polysine-coated slides (Esco). The slides were dried overnight on a warming tray before storing at room temperature. In preparation of haematoxylin and eosin (H & E) staining or *in situ* hyridisation, sections were first de-waxed by immersing slides

into clean xylene twice for 10 min each, and then re-hydrated through a descending ethanol series. The slides were then stained as described in Sections 2.2.16 and 2.2.19.

2.2.19 H & E staining

H & E staining enabled the visualisation of the nuclei (haematoxylin, purple colouration) and cytoplasm (eosin, pink colouration) in both tissues and cells. The staining procedure for tissues and cells are described in Tables 2.2 and 2.3 respectively.

Table 2.2 H & E staining protocol for tissue sections

Procedure	*Time*
Stain with Gills haematoxylin	5 min
Rinse with tap water	until clear
Blue with Scott's tapwater	2 min
Rinse with tapwater	2 min
Stain with 1% eosin Y	2 min
Rinse in tapwater	until clear, plus 2 min
50% ethanol	3 dips
70% ethanol	3 dips
95% ethanol	2 min
100% ethanol	2 min x2
xylene	5 min x2

Table 2.3 H & E staining protocol for myoblasts/myotubes

Procedure	*Time*
Remove media and rinse cells with PBS	
Stain with Gill's haematoxylin	5 min
Wash with dH_2O	2 min x2
Blue with Scott's tapwater	2 min
Rinse with dH_2O	
Stain with 1% eosin Y	2 min
100% ethanol	5 min x3
xylene	5 min x2

Once the slides dried, DPX mounting solution was applied before placing a coverslip over the specimens.

2.2.20 Van Gieson staining

Van Gieson staining enabled the visualisation of nuclei (purple colouration) cytoplasm (brown colouration) and collagen (pink colouration) within a muscle section. The staining procedure is described in Table 2.4. The Weigert's iron haematoxylin was prepared by mixing equal parts of solution A and solution B (see Section 2.1.6).

Table 2.4 Van Gieson staining protocol for muscle sections

Procedure	Time
Stain in Weigert's iron haematoxylin	10 min
Rinse with H_2O	until clear, plus 2 min
Stain in Van Gieson solution	5 min
Rinse with H_2O	dip x3
90% ethanol with 6 drops saturated picric acid	1 min
100% ethanol with 6 drops saturated picric acid	1 min
xylene	5 min (x2)

Once the slides dried, DPX mounting solution was applied before placing a coverslip over the tissue sections.

2.2.21 Fibre typing through mATPase

For fibre type analysis, muscle sections were stained for mATPase activity using a modified method of Brooke and Kaiser (1970). The staining procedure is described in Table 2.5. Fresh tissue sections were required for the staining procedure. In addition, the ATP incubation medium and the ammonium sulphide solution (which was maintained in a dark environment) needed to be made fresh just prior to their use.

Table 2.5 Fibre typing staining protocol for muscle sections

Procedure	*Time*
Acidic pre-incubation media (pH 4.6) (at 25°C)	6 min
Pre-incubation wash	30 s x2
ATP incubation medium (at 37°C, with shaking)	30 min
1% $CaCl_2$	1 min
1% $CaCl_2$	2 min
1% $CaCl_2$	3 min
2% $CoCl_2$	2 min x3
Rinse in dH_2O	x15-25
Rinse in ammonium sulphide	1 min
Rinse in dH_2O	x25

Once the slides dried, a 10% glycerol mounting solution was applied before placing a coverslip over the tissue sections.

2.2.22 Immunocytochemistry

2.2.22.1 Mac1 immunocytochemistry

The analysis of macrophage infiltration into injured muscle was performed on muscle sections using a previously published immunocytochemistry (ICC) protocol (McCroskery *et al.,* 2005). Muscle sections were fixed in 2% paraformaldehyde for 10 min at room temperature, rinsed in PBS (x3), then permeablised in 0.3% Triton X-100 in PBS for 30 min. The sections were blocked with 10% normal donkey serum (NDS) in TBS for 1 hr at room temperature before incubating overnight at 4˚C with a goat anti-Mac1 antibody (1:400; Integrin α M (m-19); Santa Cruz) in 5% NDS in TBS. The sections were washed for 4 min in PBS (x3) and incubated with a biotinylated donkey anti-goat secondary antibody (1:400; Amersham) in 5% NDS in TBS for 30 min at room temperature. The washes were repeated before adding streptavidin-conjugated Alexa Fluor 488 (1:400; Molecular Probes) in 5% NDS in TBS for 30 min at room temperature. The sections were washed with PBS, and later counterstained with DAPI (Molecular Probes) at 1:1,000 in PBS for 5 min. A final rinse in PBS was performed before mounting with fluorescent mounting medium (Dako).

2.2.22.2 PCNA ICC

In order to detect proliferating satellite cells in isolated muscle fibre cultures, the following protocol was performed. After the required culturing time, the muscle fibres were fixed with 100% methanol and washed in PBS for 5 min (x3). The fibres were then permeablised in 0.5% TritonX-100 in PBS for 10 min and blocked with 10% normal goat serum (NGS) and 0.35% cλ in PBS for 1 hr at room temperature. Mouse anti-PCNA antibody (1:100; Dako) in 5% NGS and 0.35% cλ in PBS was added overnight at 4°C. Fibres were re-washed before the primary antibody was detected using Alexa Fluor 546 goat anti-mouse IgG conjugated secondary (1:300; Molecular Probes) in 5% NGS and 0.35% cλ in PBS for 1 hr at room temperature. After re-washing, the fibres were counterstained with DAPI (1:1,000 in PBS; Molecular Probes) before mounting with fluorescent mounting media (Dako).

2.2.22.3 CD34 ICC

Satellite cells were detected with CD34 antibodies according to an adapted method of Beauchamp *et al*. (2000). After the required culturing time, fibres were fixed with 4% paraformaldehyde and rinsed with PBS (x3). The fibres were permeablised with 0.5% TritonX-100 in PBS for 10 min and blocked with 10% NGS in PBS for 30 min at room temperature. Fibres were then incubated overnight at 4°C with rat anti-mouse CD34 monoclonal antibody (1:100; clone RAM34, BD PharMingen) in 0.35% cλ in PBS. After washing in TBS-T for 5 min (x3), the primary antibody was detected using a biotinylated goat anti-rat secondary antibody (1:300; Amersham) in 0.35% cλ in PBS for 2 hr at room temperature. The washes were repeated before incubating with streptavidin-conjugated Alexa Fluor 488 (1:400; Molecular Probes) in 0.35% cλ in PBS for 1 hr at room temperature. Following a further washing, the fibres were counterstained with DAPI (Molecular Probes) at 1:1,000 in PBS for 5 min before mounting with fluorescent mounting media (Dako).

2.2.22.4 Desmin and MyoD/Pax7 ICC

For the detection of desmin or MyoD and Pax7 in primary myoblast cultures, the following protocol was performed. After the required culturing time,

the actively growing primary myoblasts were fixed with 20:2:1, rinsed with PBS (x3) and permeablised with 0.1% triton X-100 in PBS.

For the detection of desmin, the myoblasts were blocked with 10% NSS and 0.35% cλ in PBS for 1 hr at room temperature before incubating overnight at 4˚C with mouse anti-desmin (1:200; Sigma) in 5% NSS and 0.35% cλ in PBS. After washing in TBS-T for 5 min (x3), the fibres were incubated with biotinylated sheep anti-mouse secondary antibody (1:300; Amersham) in 5% NSS and 0.35% cλ in PBS for 1 hr at room temperature. The washes were repeated before incubating with streptavidin-conjugated Alexa Fluor 488 (1:400; Molecular Probes) in 5% NSS and 0.35% cλ in PBS for 1 hr at room temperature. The fibres were re-washed, then counterstained with DAPI (Molecular Probes) at 1:1,000 in PBS for 5 min before mounting with fluorescent mounting media (Dako).

For the detection of MyoD/Pax7, the myoblasts were blocked in a blocker solution (5% NSS, 5% NGS and 0.35% cλ in PBS) for 1 hr at room temperature before incubating overnight at 4˚C with rabbit anti-MyoD (1:100; Santa Cruz) and mouse anti-Pax7 (1:100; ascites fluid obtained from the Developmental Studies Hybridoma Bank; DSHB) in blocker solution. After washing in TBS-T for 5 min (x3), the cells were incubated with biotinylated donkey anti-rabbit secondary antibody (1:300; Amersham) in blocker solution for 1 hr at room temperature. The washes were repeated before incubating with streptavidin-conjugated Alexa Fluor 488 and Alexa Fluor 546 goat anti-mouse IgG conjugated secondary (1:300; Molecular Probes) in blocking media for 1 hr at room temperature. Following a further wash, the cells were counterstained with DAPI (Molecular Probes) at 1:1,000 in PBS for 5 min and mounted with fluorescent mounting media (Dako).

2.2.22.5 ARA70 ICC

ARA70 was detected in primary myoblast cultures as follows. After the required culturing time, actively growing primary myoblasts were fixed with 4% paraformaldehyde for 5 min at 37ºC, rinsed with PBS (x3), and permeablised with 0.1% Triton-X 100 in PBS for 10 min at room temperature. Cells were then blocked with 0.35% cλ in PBS for 1 hr at room temperature, before incubating with goat anti-ARA70 polyclonal antibody (1:25; Santa Cruz) in blocking buffer. Cells were washed in TBS-T for 5 min (x3), and incubated with biotinylated donkey anti-goat secondary antibody (1:300; Amersham) for 1 hr. The washes

were repeated before incubating with streptavidin-conjugated to fluorescein isothiocyanate (FITC; 1:300; Molecular Probes) for 1 hr at room temperature. After re-washing in TBS-T, the cells were counterstained with DAPI (Molecular Probes) at 1:1,000 in PBS, and rinsed in PBS before mounting with fluorescent mounting media (Dako).

2.2.23 Isolation of cells and myofibres

2.2.23.1 Satellite cell isolation by tissue dissociation and adherence to Matrigel-coated plates

The isolation of satellite cells described here was adapted from previously published protocols (Allen *et al.,* 1997; Partridge 1997; Yablonka-Reuveni *et al.,* 1999). Hind-limb muscle from mice was dissected out and minced in PBS before being enzymatically digested in DMEM (without serum) containing 0.2% collagenase type 1A (w/v) (>125 CDU/mg). The minced muscle was incubated at 37°C, with constant shaking for 90 min (70 rpm). The slurry was centrifuged at 1,400 x g for 10 min, after which, 10 ml of PBS was added to the pellet before triturating the solution for 5 min. The slurry was then passed through a 100 μm cell strainer (BD Biosciences) before spinning at 1,000 x g for 10 min. The cell pellet was suspended in 8 ml warm satellite cell proliferation media and pre-plated on uncoated 10 cm plates (Nunc) for 2 hr at 37°C and 5% CO_2. The cell suspension was later transferred to plates coated with 10% Matrigel (BD Biosciences), and incubated for 24 or 48 hr at 37°C and 5% CO_2. Cells were later washed with PBS before trypsinising off for further usage.

2.2.23.2 Satellite cell isolation by tissue dissociation and density centrifugation

The isolation of satellite cells described here was adapted from a previously published protocol (Yablonka-Reuveni and Nameroff 1987). Satellite cells were isolated as described in Section 2.2.23.1; however, after passing the slurry through a 100 μm cell strainer (BD Biosciences), the filtering was repeated using a 70 μm cell strainer (BD Biosciences). The cell suspension was then spun at 1,000 x g for 10 min and resuspended in PBS. A 90% Percoll solution was prepared using 9 parts Percoll and 1 part 10X PBS. Both 70% and 40% gradient solutions were prepared by diluting the 90% Percoll solution with the required volume of PBS. The gradient was made by overlaying the 40% solution with the

70% solution. The cell suspension was placed on top of the gradient before spinning at 2,000 x g for 20 min at 4°C with the brake off. The layer of satellite cells was then transferred to a tube with PBS. Cells were again spun at 2,000 x g for 10 min, and later used for 5-bromo-2'-deoxy-uridine- (BrdU) labelling.

2.2.23.3 Isolation of single myofibres

The isolation of single myofibres described here was adapted from a previously published protocol (Rosenblatt *et al.,* 1995). TA muscles were dissected out and digested in 0.2% (w/v) type 1A collagenase (>125 CDU/mg) in DMEM (without serum) for 60 min at 37ºC with rocking (70 rpm). The digested muscles were then transferred to DMEM containing 10% HS and 0.5% CEE before the fibres were separated by gentle trituration using a glass Pasteur pipette. Isolated fibres were transferred to 4- or 8-well chamber slides (BD Biosciences) coated with 10% Matrigel (BD Biosciences) and either cultured in fresh media for 24 to 72 hr at 37°C and 5% CO_2, or fixed immediately with relevant fixative.

2.2.23.4 Isolation of peritoneal macrophages

Macrophages were isolated by a peritoneal lavage technique. This involved freshly killed mice being injected intraperitoneally with 5 ml ice cold sterile PBS with a 23-gauge needle. The abdomen was massaged for 2 min before the fluid, containing macrophages, was withdrawn from the abdomen cavity. This cell solution was stored on ice while the process was repeated. Macrophages were kept on ice until subsequent use.

2.2.23.5 Isolation of bone marrow-derived macrophages

After killing the mice, hind-limb muscle was cut away from the bone before removing the bone by cutting the tibia just above the ankle, and the femur close to the hip socket. The bones were placed in DMEM media while using a 10 ml syringe and 26-gauge needle to flush out the bone marrow by forcing the media through the bone cavity. Once all of the marrow was harvested, the cell solution was filtered through a 70 µm cell strainer (BD Biosciences) before spinning at 300 x g for 7 min at room temperature. The cell pellet was resuspended in medium (approximately 1 ml per mouse), and 10 µl of the cell suspension was mixed with 90 µl of red blood cell lysis buffer in order to count

the cells on a hemacytometer. The cells were then plated at 5 x 10^6 cells/plate in DMEM with 10% FBS and 10% L929 conditioned medium (containing colony stimulating factor-1 (CSF-1)), for 5 days to induce macrophage differentiation. For macrophage activation, approximately 1 x 10^7 cells were seeded onto 10 cm plates (Nunc) and left overnight to adhere to the plate surface.

2.2.24 Culture of C2C12 myoblasts

C2C12 myoblasts were grown according to standard techniques (Thomas *et al.*, 2000). C2C12 myoblasts were cultured in DMEM containing 10% FBS, 7.22 nM Phenol red, 1 x 10^5 U/L penicillin, and 100 mg/L streptomycin. The medium was buffered with 41.9 mM $NaHCO_3$ and gaseous CO_2. Myoblasts were cultured at 37°C and 5% CO_2 in a ThermoForma Series II water jacketed incubator 3250 (Thermo Scientific). The culture dishes, seeding densities of cells, and treatments for specific experiments are described in the Materials and Methods of Chapters 3 and 6.

2.2.25 Production of CEE

The following protocol was adapted from the Gibco product sheet and Yu-Li Wang's Laboratory, Lab Protocols (http://ylwang.umassmed.edu/index.htm). Fertilised Cobb eggs (Bromley Park Hatcheries) were incubated at 37.5°C and 65-70% humidity for 9 days. The eggs shells were wiped clean with sterile water, followed by 70% ethanol (x2). With sterile scissors, the wide end of the shell was cut open and the embryo was removed with a sterile surgical spoon. The embryos were weighed and 1 ml 1X Earles Balanced Salt solution (Invitrogen) per gram of embryo weight was added. The solution was blended on a high speed for 15 s, 3 times, with 15 s rests on ice between blending. The solution was then centrifuged at 3,000 x g for 15 min at 4°C. The supernatant was later aliquoted out and stored at -80°C. Sterility of the CEE was checked by preparing DMEM containing 10% FBS and 1% CEE media and incubating for 48 hr at 37°C. Following the incubation period, the media was observed for any bacterial or fungal growth.

2.2.26 Trypsinisation of cells

The harvesting of all cell types was performed as follows. The culturing medium was removed before rinsing the cells with PBS (x3). Trypsin (1X) was

then added to the cells for 5-10 min at 37°C. Once the cells had lifted off the culturing dish, fresh media was added to inactivate the trypsin. The cell density was established using a haemacytometer before re-plating for further culturing, or alternatively, the cells were used for RNA or protein extraction.

2.2.27 Fixation of single fibres and myoblasts

4% paraformaldehyde fixing

Approximately 0.5 ml 4% paraformaldehyde was added to the bottom of each chamber well before removing the majority of the top media layer. The slides were then incubated at 37ºC for 5-10 min. The fixative was removed before the fibres were rinsed repeatedly with PBS.

100% methanol fixing

The media was first removed before adding ice cold 100% methanol. The slides were incubated at 4ºC for 10 min and then air dried for a further 10 min. The muscle fibres were then rinsed repeatedly with PBS.

Ethanol fixing

Once the media was removed, the muscle fibres were left for approximately 30 s before adding 0.5 ml of ethanol fixative (Section 2.1.10). The slides were then incubated at -20ºC for 20 min. The fixative was later removed and the fibres were rinsed repeatedly with washing buffer (BrdU labeling and detection kit; Roche).

20:2:1 fixing

Once the media was removed, the cells were rinsed with PBS (x3). 20:2:1 fixative was added for 30 s, and then removed before re-rinsing the cells.

2.2.28 BrdU-labelling

2.2.28.1 Labelling of single fibres

After incubating the isolated fibres for 48 hr in DMEM containing 10% HS, 0.5% CEE and 10 μM BrdU (Roche) at 37°C and 5% CO_2, the fibres were fixed with ethanol fixative (refer to Section 2.1.10). A BrdU ICC was then performed according to the BrdU labeling and detection kit (Roche) protocol as follows. Fibres were incubated with mouse anti-BrdU antibody in incubation

buffer (1:10) for 30 min at 37ºC. Following 3 washes with the washing buffer solution, the fibres were incubated with anti-mouse-Ig-fluorescein in PBS (1:10) for 40 min at 37ºC. The fibres were then washed twice for 5 min with washing buffer before counterstaining with DAPI (Molecular Probes) at 1:1,000 in PBS for 5 min at room temperature. A further two 5 min washes were performed, and the slides were then mounted with Dako fluorescent medium.

2.2.28.2 Labelling of satellite cells

Satellite cell proliferation was investigated by *in vivo* BrdU-labelling. Mice were intraperitoneally injected with BrdU (30 mg/kg; Roche) as a single pulse 2 hr before euthanizing. Satellite cells were isolated following the protocol described in Section 2.2.23.2. Cells were fixed in 70% ethanol for 30 min on ice, and then centrifuged at 1,200 x g for 10 min at 10ºC before treating with 2 M HCl and 0.5% TritonX-100 for 30 min at room temperature. After re-centrifuging, the acid treatment was neutralised with 0.1 M disodium tetraborate buffer (pH 8.5). The cells were again centrifuged before incubating in 70% ethanol at -20ºC overnight. The following day the cells were re-centrifuged and permeablised in 0.5% Tween-20 in PBS at 4ºC for 10 min. After a further centrifugation, the cells were incubated for 45 min at 37ºC with a monoclonal anti-BrdU-FLUOS antibody (1:25, Roche) in incubation buffer (Roche). PBS was added before a final centrifuge. Cells were resuspended in PBS and analysed using a FACScan flow cytometer (BD Biosciences).

2.2.29 Proliferation assay

For all proliferation assays, the previously published photometric endpoint assay was performed (Oliver *et al.,* 1989). After the required culturing time, the cells were fixed in 100 µl of 10% formaldehyde in 0.9% saline. The fixative was removed and 100 µl of methylene blue stain (1% methylene blue and 0.01 M borate buffer, pH 8.5) was added to each well for 30 min at room temperature. After the removal of the staining solution, the cells were washed four times with 200 µl borate buffer, followed by the addition of 200 µl 0.1 M HCl:70% ethanol (1:1). The absorbance of the cells was read on a VersaMax microplate reader (Molecular Devices) at 655 nm, a measure directly proportional to cell number.

2.2.30 Chemotaxis assays

Chemotaxis assays were performed using an adapted method previously described by McCroskery *et al*. (2005). For the analysis of myoblast and macrophage migration, the two cell types were isolated as described in Section 2.2.23. Chemotaxis assays were performed using cell culture inserts (BD Biosciences), containing polyethylene terephthalate (PET) 0.8 μm membranes, in 24-well plates (Corning). The insert membranes were treated with 1% Matrigel (BD Biosciences) in DMEM and then dried overnight at 37°C. The relevant test or control media (specified in Section 6.2.7 of Chapter 6) was added to the bottom wells, while 75,000 cells were added to the top wells (inserts). The plates were then incubated at 37ºC and 5% CO_2 for 4 hr for macrophages or 7 hr for myoblasts. After the required incubation period, the top surfaces of the membranes were washed with pre-wet swabs to remove cells that did not migrate. The membranes were then fixed, stained in Gill's haematoxylin and wet-mounted onto slides. Migrated cells were counted on four representative fields per membrane and the average number plotted. All assays were performed in duplicate.

2.2.31 Protein isolation

2.2.31.1 Cell protein

Protein isolated from cell cultures was performed as follows. Cells harvested by typsinisation were spun down at 1,000 x g for 5 min and resuspended in approximately 200 μl PLB before freezing at -80ºC. Alternatively, cells were scraped off the culturing plate surface with 200 μl PLB and transferred to 1.5 ml tubes before freezing at -80ºC. Cell lysates were later thawed and repeatedly passed through a 20-gauge needle before centrifuging at 14,000 x g for 5 min to pellet the cell debris. The supernatant (protein extract) was collected and stored at -80°C until further use.

2.2.31.2 Muscle protein

Required muscles were dissected out, frozen in liquid nitrogen, and stored at -80°C. To isolate the muscle protein, 50 mg of each muscle was homogenised

in 1 ml PLB. The muscle samples were spun at 14,000 x g for 5 min before collecting the supernatant and storing at -80°C until further use.

2.2.32 Estimation of protein concentrations

Total protein concentrations were estimated using the Bradford Assay (Bradford 1976). Total protein from each sample (1-2 µl) was added to dH_2O to make up a final volume of 100 µl. The Protein Assay Dye Reagent (Bio-Rad) was diluted one in five before 1.2 ml was mixed with each protein sample. The absorbencies were then determined at 595 nm using a Heλios γ UV spectrophotometer (Thermo Scientific). The absorbances of BSA standards (0 to 15 µg) were also measured to generate a standard curve with which the unknown samples were compared.

2.2.33 SDS polyacrylamide gel electrophoresis and membrane transfer

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed using precast NuPAGE™ 4-12% Bis-Tris gels (Invitrogen). The required amount of total protein (10 to 20 µg) was mixed with an appropriate volume of protein loading dye and boiled for 5 min prior to loading. Electrophoresis of the proteins was performed using 1X SDS Running Buffer (Invitrogen) at 40 mA until the desired separation was attained. After electrophoresis, the protein was transferred to nitrocellulose membrane (Bio-Rad) by electroblotting using a XCell II Blot Module (Invitrogen) which was run at 30 V for approximately 1 hr. Following transfer, the membranes were stained in Ponceau S to assess protein loading. Alternatively, gels were stained with Coomassie Blue stain to visualise the protein.

Ponceau S staining

Membranes were stained for 1 min and then rinsed in TBS-T until bands were clearly defined.

Coomassie Blue staining

Gels were stained for 30 min in Coomassie Blue stain, and then destained in Coomassie Blue destaining solution until bands were clearly defined.

2.2.34 Western blotting

For the analysis of MyoD, membranes were blocked in BSA blocker (0.3% BSA, 1% PEG, and 1% poly vinyl perrolidone (PVP) in TBS-T) for 1 hr at room temperature, and then incubated with mouse anti-MyoD (BD PharMingen) at 1:10,000 overnight at 4°C. Alternatively, membranes were blocked in 5% milk in TBS-T overnight at 4°C, and then incubated with polyclonal rabbit anti-MyoD antibody (Santa Cruz) at 1:200 for 3 hr at room temperature.

For the analysis of Pax7, myostatin and GAPDH, the membranes were blocked overnight at 4°C in 5% milk in TBS-T, and then incubated with one of the following: mouse anti-Pax7 antibody (ascites fluid obtained from the Developmental Studies Hybridoma Bank) at 1:500 for 3 hr; purified myostatin antibody (0.5 mg/ml; produced in house) at 1:2,000 for 3 hr; or mouse anti-GAPDH (1:10,000; Research Diagnostics) for 1 hr at room temperature.

All membranes were washed in TBS-T for 5 min (x5), then incubated with either goat anti-mouse IgG horseradish peroxidise (HRP) conjugate (Dako) (1:5,000, MyoD; 1:2,000, Pax7 and myostatin; or 1:10,000, GAPDH), or goat anti-rabbit IgG HRP conjugate (1:2,000; Dako) in their respective blocking solutions for 1 hr at room temperature. The washes were repeated before HRP activity was detected using Western Lightning™ Chemiluminescence Reagent Plus (PerkinElmer). XAR film (Kodak) was exposed to the membranes and the developed films were scanned and analysed using a GS-800 densitometer and Quantity One imaging software (Bio-Rad).

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Chapter Three

Myostatin negatively regulates the expression of the steroid receptor co-factor ARA70

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Myostatin Negatively Regulates the Expression of the Steroid Receptor Co-Factor ARA70

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3.1 Introduction

The maintenance of adult skeletal muscle mass is the net result of the balance between muscle synthesis and degradation. When the equilibrium of molecular, physiological, and pathological determinants favours one mechanism over the other, the consequence is hypertrophy, or alternatively, atrophy of muscle. An extreme example of widespread skeletal muscle hypertrophy is dramatically illustrated in *myostatin*-deficient animals. The individual muscles of *myostatin*-null mice exhibit a 2-3 fold increase in size over wild-type, due to a combination of hypertrophy and hyperplasia (McPherron *et al.,* 1997). Myostatin is a secreted member of the TGF-β family of signalling molecules (McPherron *et al.,* 1997; McPherron and Lee 1997) that acts to restrict muscle growth via several mechanisms during embryonic, foetal and postnatal growth. *Myostatin* expression is predominately seen in skeletal muscle. Although *myostatin* mRNA levels do not differ between males and females, myostatin protein levels have been found to be sexually dimorphic. This was reported by McMahon *et al*. (2003) who showed that mature myostatin was 40-60% lower in males when compared to females. Furthermore, in an independent study, Reisz-Porszasz *et al*. (2003) indicated that over-expression of *myostatin* led to sex-specific (males only) atrophy in mice.

During growth, myoblasts enter the cell cycle and proliferate until a cascade of signals, initiated by activatation of MyoD, causes myoblasts to permanently withdraw from the cell cycle (Crescenzi *et al.,* 1990; Sorrentino *et al.,* 1990), differentiate and fuse into multinucleated myotubes. However, in response to myostatin signalling there is an increase in *p21* expression and a decrease in Cdk2 protein activity that results in an accumulation of hypophoshorylated Rb leading to the arrest of myoblasts in the G1 and G2 phases of the cell cycle (Thomas *et al.,* 2000). When functional myostatin is absent, deregulated cell cycle control of myoblasts results in an increased number of myoblasts and, consequently, muscle fibres (hyperplasia) (Thomas *et al.,* 2000). In addition to proliferation, myostatin also appears to regulate the differentiation step of myogenesis by regulating the activity and the expression levels of the MRF *MyoD* (Langley *et al.,* 2002).

Satellite cells are myogenic precursor cells that are mitotically quiescent in mature muscle (Schultz *et al.,* 1978), but in response to injury are activated, re-

enter the cell cycle, and are responsible for postnatal growth of muscle (Snow 1977a; Snow 1977b; Bischoff 1989). Myostatin has been shown to maintain satellite cell quiescence by inhibiting the G1 to S progression and, moreover, negatively regulate satellite cell renewal (McCroskery *et al.,* 2003). Increased satellite cell activation in *myostatin*-deficient animals leads to an increase in myonuclei, and thus myofibre size (hypertrophy) (McCroskery *et al.,* 2003).

A second well known example of hypertrophy is observed in individuals that have been administered testosterone. The effects of testosterone on skeletal muscle have been well documented and include increased type I and II muscle fibre volume, myonuclear number, protein synthesis and satellite cell number, decreased protein degradation, and increased myogenesis and decreased adipogenesis of pluripotent stem cells (review by Herbst and Bhasin, (2004)). The action of testosterone is mediated through the AR, a ligand-dependent transcription factor that is a member of the steroid hormone receptor superfamily (Chang *et al.,* 1988b; Chang *et al.,* 1988a). When testosterone binds, the AR undergoes a series of conformational changes that allows the AR to interact with AREs in various androgen target genes (Chang *et al.,* 1995). ARA70, also known as nuclear receptor coactivator 4, RFG and ELE1, has been identified as a type II coregulator that stabilises the ligand-bound AR enhancing the transactivational potential of the AR (Yeh and Chang 1996; Yeh *et al.,* 1998). AR coactivators, including ARA70, are recruited by the AR dimers on the ARE of DNA to facilitate further recruitment of histone acetylases and regulate chromatin structure and transcription (Spencer *et al.,* 1997).

In this study we demonstrate that murine *ARA70* is expressed in myoblasts during myogenesis. We further demonstrate that myostatin is a potent negative regulator of *ARA70* gene expression. Given that increased *AR* expression and reduced myostatin levels lead to hypertrophy, we propose a model where we hypothesize that the absence of myostatin, by increasing *ARA70* expression, increases the activity of AR leading to the hypertrophy phenotype which is associated with male growth and driven by testosterone.

3.2 Materials and Methods

3.2.1 Animals

Myostatin-null mice (C57BL/10 background) were obtained from S.-J. Lee (The Johns Hopkins University, Baltimore, MD). Wild-type mice (C57BL/10) were bred at the Ruakura Small Animal Colony. Animal manipulations described in this paper were approved by the Animal Ethics Committee (AgResearch Ruakura) and the University of Waikato Animal Ethics Committee (University of Waikato, Hamilton, New Zealand). Male mice only were utilised in the experiments reported herein.

3.2.1 SSH

Total RNA from BF muscle of both wild-type and *myostatin*-null mice was prepared using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. Poly $(A)^+$ RNA was isolated from 2 mg of total RNA by Fast Track 2.0 mRNA kit (Invitrogen) according to the manufacturer's recommendations. To perform subtraction hybridisation, complementary DNAs were synthesised from 2 µg of poly $(A)^+$ RNA. SSH was then performed between *myostatin*-null mice ('driver') and wild-type mice ('tester') using the PCR-Select cDNA Subtraction Kit (Clontech) according to the manufacturer's recommendations. Differentially expressed cDNAs were amplified and sub-cloned into the pGEM-T Easy vector (Promega) and transformed into DH5α competent cells (Invitrogen). Colonies containing inserts were grown overnight in LB broth (ampicillin), from which 1 µl was used for PCR amplification of inserts with Nested primer1 and 2R supplied with the kit (Clontech). PCR conditions were as stated in the manufacturer's instructions.

3.2.2 Differential library screening

The PCR amplified differential transcripts were denatured and dot-blotted onto Hybond N+ membrane (Amersham), using dot-blot apparatus (Bio-Rad) according to the manufacturer's instructions. For each PCR, 50 µl of the following combined mixture was dot-blotted onto duplicate membranes: 5 µl of PCR product and 140 µl of denaturing mix containing 0.3 M NaOH, 15% Ficoll (Sigma), and 0.5% bromophenol blue. The dot-blotted membranes were

autocross-linked by UV irradiation in a UV Stratalinker 1800 (Stratagene) then pre-hybridised in Church and Gilbert hybridisation buffer (0.5 M Na_2HPO_4, pH 7.2, 7% SDS, 1 mM EDTA) at 60°C for approximately 1 hr prior to hybridisation. To compare relative levels of isolated differential transcripts between *myostatin*-null and wild-type tissues, ^{32}P-labelled cDNA probes were synthesised in a 20 µl RT reaction from 2 µg poly $(A)^+$ RNA, using superscript pre-amplification kit (Invitrogen) according to the manufacturer's instructions. The cDNA probes were passed over a G-50 Sephadex column (Boehringher) to remove unincorporated ^{32}P according to the manufacturer's instructions. One of the duplicate membranes was then hybridised with the *myostatin*-null cDNA probe, while the other was hybridised with the wild-type cDNA probe, overnight at 55°C. After hybridisation, membranes were washed twice with 2X SSC + 0.5% SDS for 15 min at 55°C, then in 1X SSC + 0.5% SDS for a further 15 min at 55°C and exposed to XAR film (Kodak) and dot-blot duplicate intensities compared. Differentially expressed transcripts were identified via dot-blot intensity differences (the autoradiogram was scanned in a GS-800 Calibrated Densitometer (Bio-Rad) and the relative OD of the dots was measured using Quantity One 4.2.2 software (Bio-Rad)), then sequenced by dye terminator sequencing. The partial sequences obtained were then compared with entries in the GenBank database using the BLAST homology search program.

3.2.3 Yeast two-hybrid interactions

The DupLEX™ Yeast Two-Hybrid System (OriGene) was used to detect protein-protein interactions. The open reading frame of mouse *ARA70* (bp 35-1912; amino acid residues 1-625; GenBank accession number NM_019744) encoding the full length *ARA70* was cloned into the pEG202 bait plasmid (carrying the *HIS3* gene) containing the Lex-A DNA binding domain using *Eco*R1 restriction sites (LexA-FL-ARA70). A truncated form of *ARA70* (bp 35-1564; amino acid residues 1-510) encoding amino-terminal region was also cloned in the pEG202 bait plasmid using *Eco*R1 restriction sites. A portion of the *AR* (bp 1842-2732 amino acid residues 604-899; GenBank accession number NM_013476) containing the LBD was cloned into the pJG4-5 target plasmid (carrying the *TRP1* gene) containing the B42 DNA binding domain using *Bam*H1 restriction sites. Interaction between the encoded fusion proteins was assessed by

co-transforming the bait and target plasmids together with a reporter plasmid, pJK103, carrying the *lacZ* reporter gene under the control of LexA operators and the *URA3* gene into yeast strain EGY194 (MATa *trp1 his 3 ura3 leu2*:4 LexAop-LEU2). Transformed yeast cells were plated onto medium lacking histidine, uracil and tryptophan and grown at 30°C for 3 days to select for the presence of the three plasmids. Three independent colonies were then transferred to medium lacking histidine, uracil, tryptophan and leucine for 3 days at 30ºC to select for positive interactions. Positive clones were then tested for expression of the second reporter gene, *lacZ*, by growth on medium containing X-gal and lacking in histidine, uracil, and tryptophan. Expression of the target-B42 activation domain fusion protein, B42-ARLBD, is galactose-inducible in this system and therefore galactose growth-dependence was also tested.

3.2.4 Northern blot analysis

RNA was isolated from various mouse tissues using TRIZOL reagent according to the manufacturer's instructions. Northern analysis was performed essentially as described by Sambrook *et al*. (1989). Total RNA (10 µg) was fractionated by 0.66 M formaldehyde, 1% agarose gel electrophoresis. RNA was transferred to Hybond N+ membrane (Amersham) by capillary transfer using 10X SSC. The membrane was pre-hybridised in Church and Gilbert hybridisation buffer at 55°C for 1 hr, followed by hybridisation with ^{32}P-labelled cDNA probes in fresh Church and Gilbert hybridisation buffer at 55°C overnight. The membrane was washed at 55°C with 2X SSC + 0.5% SDS, and then 1X SSC + 0.5% SDS. *ARA70* was PCR amplified using an RT-PCR kit (Invitrogen). Five micrograms of total RNA from kidney was used in a RT reaction and PCR was performed with 2 µl of the RT reaction at 94°C for 20 s, 55°C for 20 s, and 72°C for 1 min for 35 cycles. This was followed by a single 72 °C extension step for 5 min. The primers for *ARA70* were:

Forward 5'- CCATCAGATGCTCAGGGATTTGA

Reverse 5'- GCGAATTCATGAACACATCCCTG

Probe cDNAs were radioactively labelled using [α^{32}P]dCTP (Amersham) and Rediprime II labelling kit (Amersham), according to the manufacturer's instructions.

3.2.5 Isolation of mouse primary myoblasts and ICC

Primary myoblasts from the entire hind-limb muscles were isolated and cultured according to the protocols of Allen *et al*. (1997) and Partridge (1997) with slight modifications. To detect ARA70 protein, isolated primary myoblasts were grown and then fixed on chamber slides (Nunc) coated with 10% Matrigel (BD Biosciences), and incubated with goat anti-ARA70 polyclonal antibody (1:25; Santa Cruz) for 1 hr. The primary antibody was detected using biotinylated anti-goat secondary antibody (1:300; Amersham) followed by streptavidin-conjugated to FITC (1:300; Molecular Probes). All nuclei were counterstained with DAPI (Molecular Probes) and slides visualised under fluorescence illumination.

3.2.6 *In situ* hybridisation

Mouse TA muscle tissue was fixed in 4% paraformaldehyde in PBS for 16 hr at room temperature, washed, dehydrated through an ascending ethanol series, and embedded in paraffin wax. Sections were then cut at 7 μm and mounted on microscope slides (Esco). For the generation of DIG probes, a 502 bp fragment of *ARA70* cDNA (bp 46-597; GenBank accession no. NM_019744) was cloned into the pGEM-T Easy vector (Promega) and transcribed according to the manufacturer's recommendation (DIG RNA Labeling Kit; SP6/T7; Boehringer). Paraffin-embedded sections were dewaxed, and *in situ* hybridisations were performed according to the method of Greiwe *et al*. (2001) with slight modifications. All nuclei were counterstained with DAPI (Molecular Probes).

3.2.7 Semi-quantitative RT-PCR and Southern blotting

Total RNA was isolated from wild-type or *myostatin*-null mouse muscle tissues or C2C12 myoblast cells using TRIZOL reagent according to the manufacturer's instructions, then used as template in a RT reaction using Oligo(dT) primers and SuperScript II First-Strand Synthesis System for RT-PCR (Invitrogen), according to the manufacturer's instructions. The resulting cDNA was used as template in semi-quantitative PCR conditions for *ARA70* and *GAPDH* were 94°C for 20 s, 55°C for 30 s, and 72°C for 2 min for 20 cycles and a final extension at 72°C for 5 min. The primers used for the PCRs were:

ARA70

Forward 5’ - GCGAATTCATGAACACATCCCTG

Reverse 5’ - GCGAATTCTCACATCTGTAGAGG

GAPDH (GenBank accession number NM_008084)

Forward 5’-GTGGCAAAGTGGAGATTGTTGCC

Reverse 5’-GATGATGACCCGTTTGGCTCC

Thirty microlitres of the semi-quantitative PCR products were fractionated on a 0.8% or 1.2% agarose gel, transferred to a Hybond N+ membrane (Amersham) in SB solution (1.5 M NaCl, 0.5 M NaOH) and hybridised to an *ARA70* or *GAPDH* [α^{32}P]dCTP -labelled probe (prepared as above) in Church and Gilbert buffer at 55ºC. After washes in 2X SSC + 0.5% SDS and 1X SSC + 0.5% SDS at 55°C, the membranes were exposed to XAR film (Kodak). The autoradiogram was scanned and the relative OD of bands was measured as above.

3.2.8 C2C12 myoblast culture

C2C12 myoblasts were grown prior to assay in DMEM buffered with 41.9 mM $NaHCO_3$ and 5% gaseous CO_2. Phenol red (7.22 nM) was used as a pH indicator, and 1X 105 IU/l penicillin, 100 mg/l streptomycin, and 10% FBS were added to media. For assaying, C2C12 myoblasts were seeded on a 10 cm plate (Nunc) at a density of 25,000 cells/cm^2. Following a 16 hr attachment period, differentiation media consisting of DMEM containing 2% HS and 0 or 15 μg/ml recombinant myostatin (prepared as described by Thomas *et al*. (2000) were added. Cells were collected for RNA extraction after 0, 12, 24, 48 and 72 hr and processed for semi-quantitative RT-PCR as above. Cells cultured without myostatin were also collected for protein extraction after 0, 12, 24, 48 and 72 hr.

3.2.9 Western blot analysis

Cell extracts were prepared and total protein estimated exactly as described by Thomas *et al*. (2000). Fifteen micrograms of total protein was resolved by 4-12% SDS-PAGE (Invitrogen) then transferred to nitrocellulose membrane (Bio-Rad) by electroblotting. Equal protein loadings were confirmed with Ponceau S staining. Western blots were performed using standard procedures. Polyclonal rabbit anti-MyoD (Santa Cruz) was used at a 1:200 dilution. Detection of the primary antibody was carried out with a HRP

conjugated anti-rabbit IgG (Dako) at a 1:2,000 dilution. Peroxidase activity was detected using Western Lightning™ Chemiluminescence Reagent Plus (PerkinElmer). The autoradiogram was scanned and the relative OD of bands was measured as above.

3.3 Results

3.3.1 ARA70 is a downstream target of myostatin

Myostatin is a powerful inhibitor of muscle growth with both hypertrophy and hyperplasia of muscle observed in the absence of myostatin (Kambadur *et al.,* 1997; McPherron *et al.,* 1997). At embryonic and foetal myogenic stages, myostatin appears to function by regulating cell cycle progression (Thomas *et al.,* 2000). Key transcriptional regulators of muscle development, such as Pax3, MyoD, and Myf5, are known to be down-regulated in response to myostatin signalling in myogenic cells resulting in inhibition of both proliferation and differentiation of muscle (Amthor *et al.,* 2002). To further elucidate the molecular mechanisms by which myostatin affects myogenesis, SSH was performed to investigate differential gene expression patterns postnatally. For this analysis total RNA from BF muscle was isolated from wild-type and *myostatin*-null mice and a library of cDNA representative of differentially expressed mRNAs was generated. Around 400 cDNA clones were dot-blotted and analysed using reverse Northern blotting techniques. Genes that were differentially expressed were selected for sequencing and the derived sequences were used to search GenBank. Results revealed several known and well characterised genes that were regulated by myostatin in addition to various genes of unknown function (Table 3.1). Here we describe a murine cDNA clone that contained sequence homology to a human gene known as nuclear receptor coactivator 4, or ARA70. This gene was significantly up-regulated in the *myostatin*-null muscle (Figure 3.1).

Table 3.1. Genes up-regulated in* myostatin-*null mice.

Differentially expressed genes in wild-type and *myostatin*-null mice were identified by SSH and a selection is tabulated below.

Description	Function	Homology	Accession No.
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	Ketogenic pathway	89%	NM_017268
Actinin alpha 3	Myofibril protein	99%	NM_013456
Adenine nucleotide translocase-1	Translocates ADP/ATP	95%	U27315
Anaphase-promoting complex subunit 5	Ubiquitin ligase	84%	NM_021505
Apolipoprotein B editing complex 2	C to U editing enzyme	95%	NM_009694
ARA70	Coactivator	99%	AK016355
ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1	Mitochrondrial ATP synthase subunit	99%	NM_007505
Carbonyl reductase 1	Reducing enzyme	98%	NM_007620
Core binding factor beta	Transcription factor subunit	98%	BC006763
Cullin 1, containing frame shift errors	Ubiquitin pathway	95%	BC004836
DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 13	Putative RNA helicase	95%	NM_020494
Desmin gene	Intermediate filament	92%	Z18892
Diphosphoinositol polyphosphate phosphohydolase type II	Metabolism of diphosphoinositol polyphosphates	90%	AF253473
Dual specificity phosphatase TS-DSP2	Affects MAP kinase	95%	AF237619
Fibroblast growth factor inducible 14	Transmembrane receptor	95%	NM_008015
MAP Kinase Kinase	Activates MAP kinase member	98%	X97052
Mitogen activated protein kinase kinase kinase 4	Apoptosis	89%	NM_011948
Muscle creatine kinase	Energy metabolism	98%	NM_007710
Muscle glycogen phosphorylase	Glycogen pathway	95%	NM_011224
Nebulin	Actin binding protein	98%	Y16350
Nucleoside-diphosphate kinase 2	Enzyme (NTP/NDP)	97%	NM_008705
Ornithine aminotransferase	Glutamine metabolism	98%	BC008119
Phosphorylase kinase, gamma subunit	Glycogen metabolism	97%	J03293
SG2NA beta isoform	Intracellular protein	82%	AF307777
Tropomyosin 1, alpha	Myofibrillar protein	97%	NM_024427
GLUT4 vesicle protein	Unknown	95%	AF099138
Hypothetical RING finger containing protein	Unknown	84%	AK004898
Mitochondrion DNA sequence	Unknown	95%	J01420
Novel gene	Unknown	92%	HS209A6
Nuclear protein UKp68	Unknown	93%	AB032932
Prion protein	Unknown	96%	BC006703
Similar to hypothetical protein FLJ10342	Unknown	99%	BC003291
Small muscle protein, X-linked	Unknown	77%	NM_025357
Unknown EST	Unknown	100%	AK017368
Unnamed protein product	Unknown	96%	AK007809
Unnamed protein product	Unknown	90%	AK007596

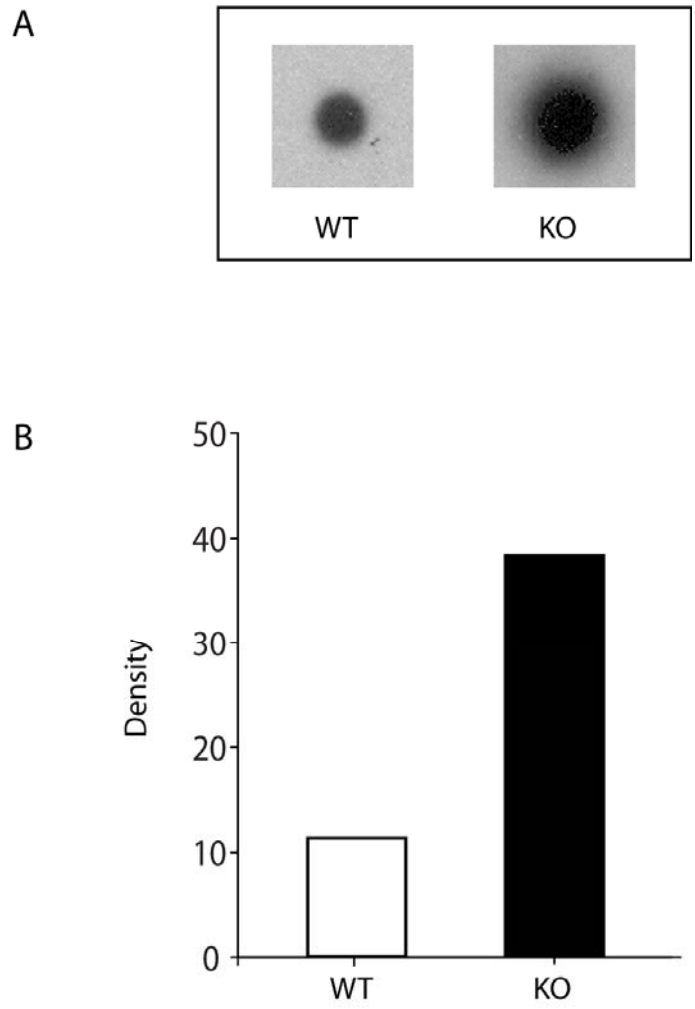

Figure 3.1 ARA70 is revealed as a downstream target of myostatin by SSH

Total RNA from BF muscle was isolated from wild-type (WT) and *myostatin*-null (KO) mice and a library of cDNA representative of expressed mRNAs was generated. Individual PCR-amplified products were gridded on nitrocellulose membranes as dot-blot arrays after two SSHs were performed. Membranes were hybridised against ^{32}P-labelled cDNA probes from wild-type and *myostatin*-null poly $(A)^+$ RNA. (A) The reverse Northern dot-blot for the cDNA clone subsequently identified by DNA sequencing and BLAST analysis as *ARA70* is shown. (B) Autoradiogram of the dot-blot shown in panel A was analysed by densitometry.

3.3.2 Murine ARA70 is highly homologous to human ARA70

GenBank sequence analysis revealed that there are two identical entries for putative murine ARA70 (NM_019744 and AF159461), which correspond to the gene we identified in the SSH analysis, and another extremely homologous sequence that differs by only 1 amino acid substitution (AK129020). These 3 entries we have designated as the α-form of ARA70. A further entry, (BC031528), is also highly homologous excepting 6 amino acid substitutions and a 27 bp inframe deletion resulting in the loss of 9 amino acids in the carboxyl-terminal portion of the protein. This we have designated the β-form of ARA70 (Figure 3.2). Two additional entries show significant sequence homology to the above sequences, one has an amino-terminal 66 amino acid truncation (AK032951) and the other, 1 internal amino acid substitution and a carboxyl-terminal 242 amino acid truncation (AK014479). Murine ARA70α is 82% homologous to human ARA70 at DNA level. When protein sequences were compared, it was revealed that murine ARA70α shared approximately 79% sequence homology to human ARA70 (Figure 3.2). The form of ARA70 used for experiments reported herein is the α-form, identical to the NM_019744 and AF159461 entries.

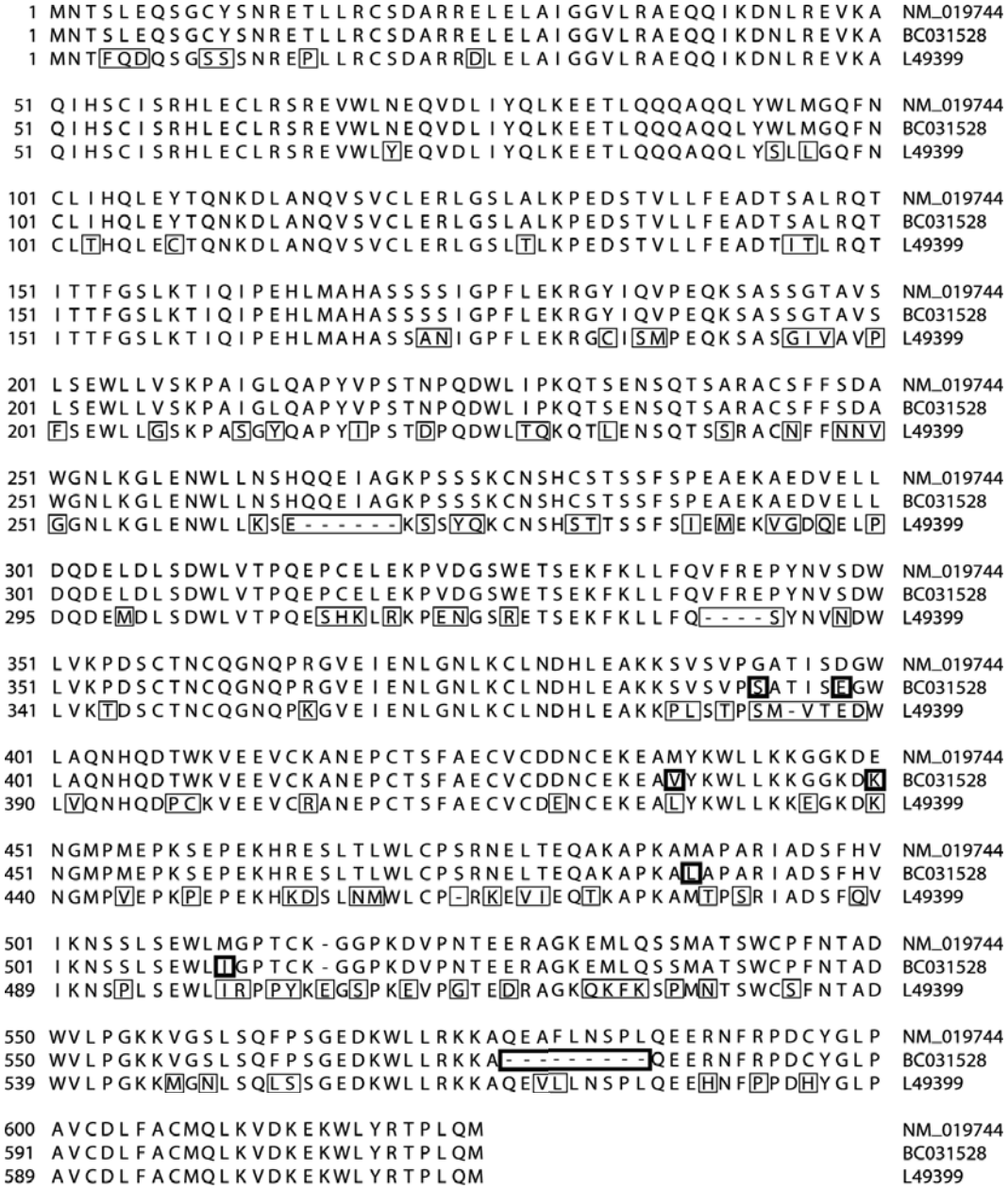

Figure 3.2 Murine ARA70 shows high homology to human ARA70

Amino acid sequence comparison between murine ARA70α (NM_019744), murine ARA70β (BC01528), and human ARA70 (L49399) are depicted. Amino acid residues differing between the murine α- and β-forms are highlighted in bold boxes and differences between the human and murine α-form are boxed.

3.3.3 Murine ARA70 interacts directly with the ARLBD

To confirm and characterise the putative murine ARA70 as an AR coactivator, we used the yeast two-hybrid system to determine if mouse ARA70 binds to the LBD of mouse AR. Previously, the binding between human ARA70 and ARLBD has been characterised using the yeast two-hybrid system where it was shown that ARA70 amino acid residues 321-441 bound to ARLBD with highest binding affinity (Zhou *et al.,* 2002). Furthermore, the carboxyl-terminal region of ARA70 is thought to contain a repression domain that influences protein folding and decreases the binding affinity to ARLBD. Deletion of this region (amino acid residues 499-614) increased binding to ARLBD 4-fold (Zhou *et al.,* 2002). We therefore expressed both of these forms of ARA70 as a bait fusion protein to the LexA activation domain: the full length ARA70 (LexA-FL-ARA70) and the truncated form with the carboxyl-terminal region deleted (LexA-1-510-ARA70). These fusion proteins were tested for interaction in the yeast two-hybrid system with the LBD (amino acids residues 604-899) of the AR expressed as a target fusion protein to the B42 activation domain (B42-ARLBD) (Figure 3.3). In yeast co-transformed with LexA-FL-ARA70 and B42-ARLBD there was no transactivation of either *lacZ* or *LEU2* reporters and hence no growth of yeast was observed on leucine deficient agar plates. These data indicate that full length ARA70 did not bind to ARLBD. In contrast, when yeast were co-transformed with the truncated form of ARA70, LexA-1-510-ARA70, and B42-ARLBD, transactivation of the *lacZ* reporter occurred when yeast were grown on agar containing galactose but not when the carbohydrate source was glucose. This result is consistent with the galactose dependency of the *GAL1* promoter used to drive the expression of the B42-ARLBD fusion protein. Similarly, transactivation of *LEU2* in yeast co-transformed with LexA-1-510-ARA70 and B42-ARLBD expression plasmids grown on medium deficient in leucine occurred only in the presence of galactose. These data, therefore, clearly indicate that 1-510-ARA70 and ARLBD can interact directly in the yeast cells.

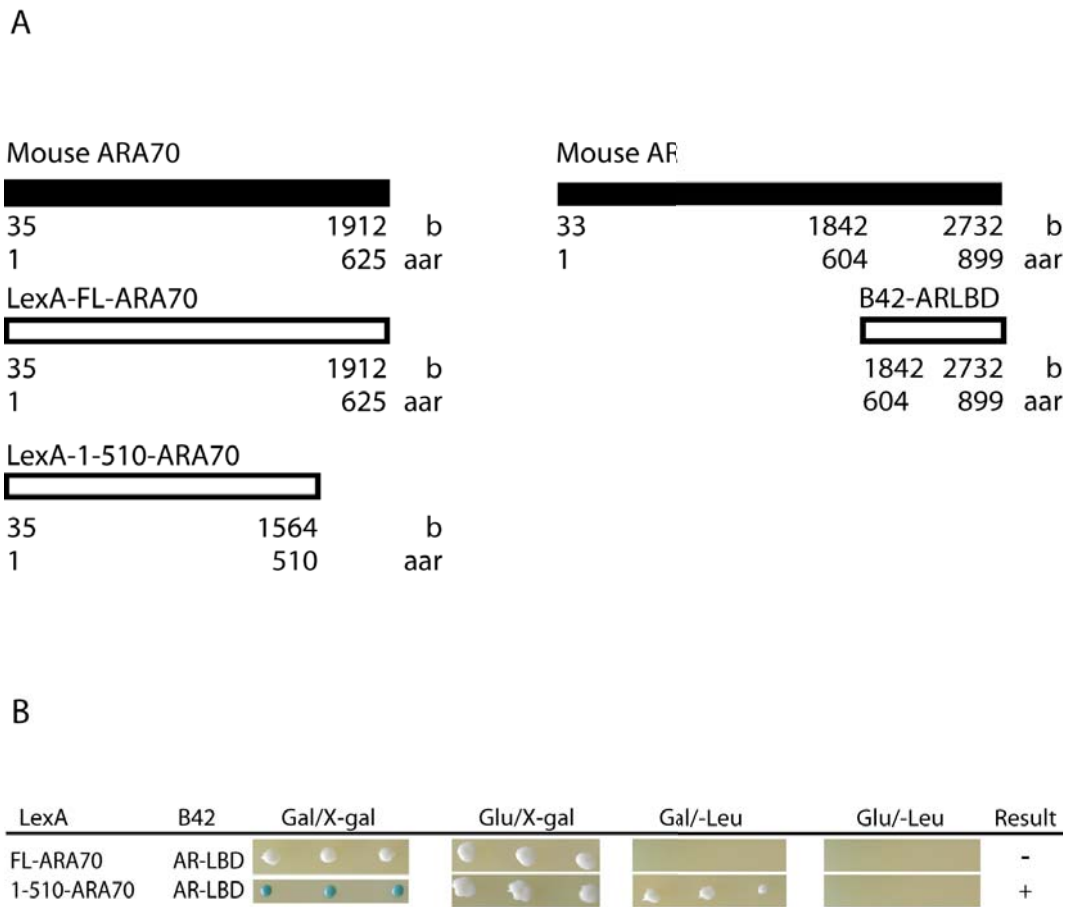

Figure 3.3 ARA70 interacts directly with ARLBD

Interactions between pairs of fusion proteins containing either an amino-terminal LexA DNA-binding domain or B42 transactivation domain were determined by yeast two-hybrid assay using *lacZ* and *LEU2* reporter genes under the control of *LexA* operators. (A) Numbers below the schematic layout of ARA70 indicate the nucleotide numbers correctly corresponding to the GenBank mouse entry NM_019744 (b) and residue numbers of the amino acid sequence (aar). Similarly for ARLBD GenBank mouse entry NM_013476. Bars indicate the positions of the cDNAs used for the bait plasmids LexA-FL-ARA70 and LexA-1-510-ARA70 and the target plasmid B42-ARLBD. (B) Three independent transformants were tested for their ability to transactivate the *lacZ* locus as visualised by X-gal staining on plates containing either galactose (Gal) or glucose (Glu), to either induce or repress, respectively, expression of the B42 fusion protein. Similarly, transactivation of the *LEU2* locus, which enables yeast to grow in the absence of leucine, was tested on plates containing either galactose (Gal) or glucose (Glu).

3.3.4 *ARA70* is expressed in various tissues

To analyse the expression profile of the *ARA70* gene we performed Northern blot analysis. Northern analyses in mouse indicated that *ARA70* is expressed as an mRNA of approximately 3.3 kilobases in many tissues, including brain, heart, intestine, kidney, liver, lung, testis and skeletal muscle (Figure 3.4A). To assess if ARA70 protein was present in primary myoblasts we performed immunofluorescent staining on primary myoblast cell cultures. Immunofluorescence revealed ARA70 protein was localised throughout the myoblast cytoplasm (Figure 3.4Bi). Counterstaining with DAPI to identify cell nuclei showed ARA70 protein was consistently present in all cells (Figure 3.4Bii). Immunofluorescence was not detected in the absence of primary antibody (Figure 3.4Biii). To assess the distribution and level of *ARA70* expression in muscle cells we performed *in situ* hybridisation on paraffin-embedded transverse sections of TA muscle (Figure 3.4C). A fragment from the *ARA70* open reading frame sequence was used to generate sense and anti-sense DIG-labelled RNA *in situ* probes. Expression of *ARA70* mRNA was clearly detectable in muscle tissue (Figure 3.4Ci). Control sections hybridised with the sense probe displayed only background signal verifying specificity of the antisense probe (Figure 3.4Cii). *ARA70* expression did not appear to be fibre type specific as the staining intensity was uniform throughout the muscle section. Furthermore, the pattern of staining within the individual fibres indicated that *ARA70* mRNA was localised throughout the cytoplasm. These data are in agreement with previous studies showing ARA70 protein localised in the cytoplasm of human breast cancer cells (Kollara *et al.*, 2001).

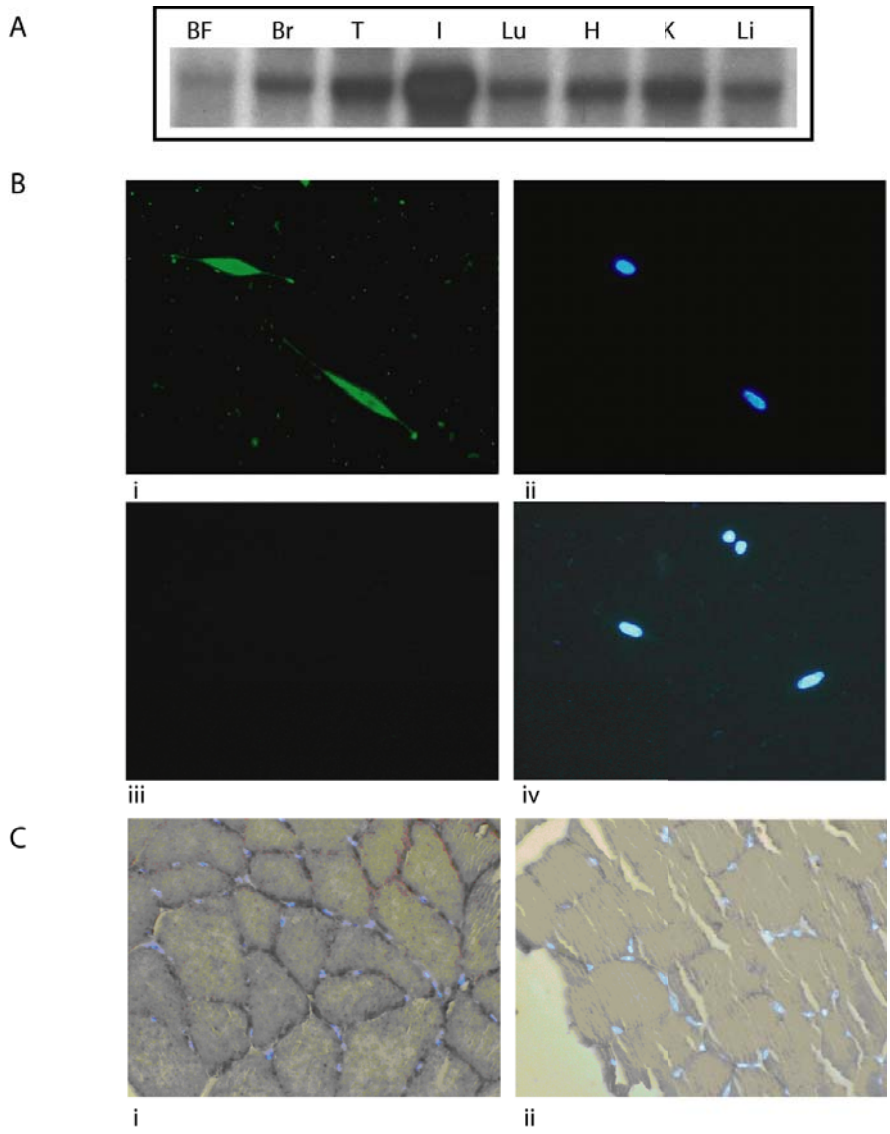

Figure 3.4 ARA70 is expressed in various mouse tissues

(A) Northern analysis of the tissue distribution of *ARA70*. Total RNA (15 μg) extracted from mouse BF muscle; Br, brain; T, testes; I, intestine; Lu, lung; H, heart; K, kidney; L, liver; was fractionated by gel electrophoresis and transferred to Hybond N+ membrane and probed with an *ARA70* ^{32}P-labelled cDNA probe. Ethidium bromide-stained 18 S and 28 S rRNA bands from the gel were assessed to have equal loading and showed RNA integrity (data not shown). (B) Immunofluorescent staining of ARA70 in primary myoblasts. (i) Primary myoblasts isolated from hind-limb muscle of 4-week-old mice were cultured and immunostained for ARA70. (ii) DAPI staining of myonuclei of the corresponding field is shown. (iii) Background immunofluorescence when anti-mouse secondary antibody was used in the absence of primary antibody is shown. (iv) DAPI staining of negative control. (C) Expression of *ARA70* in TA muscle. (i) *In situ* hybridisation of transverse TA muscle sections was performed using a DIG-labelled *ARA70* antisense probe. (ii) Negative control using a DIG-labelled *ARA70* sense probe. Slides were counterstained with DAPI.

3.3.5 *ARA70* expression is negatively regulated by myostatin

SSH results revealed differential expression of *ARA70* between wild-type and *myostatin*-null mice. In the absence of myostatin, increased expression of *ARA70* was observed. To further analyse the effect of myostatin on *ARA70*, we used two independent gene expression analyses. Northern analysis was performed on the total RNA from BF muscle of wild-type and *myostatin*-null mice (Figure 3.5A). The results indicate that wild-type BF muscle exhibited a lower level of *ARA70* expression than *myostatin*-null muscle. Similarly, semi-quantitative RT-PCR analysis also confirmed that there is increased expression of *ARA70* in *myostatin*-null as compared to wild-type BF muscle (Figure 3.5B-C). Thus the Northern and semi-quantitative RT-PCR analyses together corroborated the original observation in SSH that in BF, in the absence of myostatin, *ARA70* expression was up-regulated.

3.3.6 Myostatin inhibits the expression of *ARA70* in C2C12 cells

Three independent techniques, SSH, Northern, and semi-quantitative RT-PCR, of wild-type and *myostatin*-null mice strongly indicated a down-regulation of *ARA70* expression in muscle in the presence of myostatin. To further investigate this phenomenon we used an *in vitro* muscle model. C2C12 myoblasts were cultured under differentiating conditions for up to 72 hr in the presence or absence of exogenous myostatin. Semi-quantitative RT-PCR showed *ARA70* expression was initiated between 12 and 24 hr under differentiating conditions in myoblasts that were cultured without the addition of exogenous myostatin (Figure 3.6A-B). *ARA70* expression was maximal after 72 hr under differentiating conditions in the control myoblasts. Expression of MyoD protein was also measured in control myoblasts and was significantly increased during early differentiation, by 12 h, (Figure 3.6C-D) as previously shown (Langley *et al.*, 2002) and was maximal at 24 hr. These data clearly demonstrate that initiation of *ARA70* expression was delayed by approximately 12 hr relative to increases in MyoD protein. *ARA70* expression was not detected by semi-quantitative RT-PCR in C2C12 myoblasts cultured under differentiating conditions in the presence of exogenous myostatin at any of the time points investigated. These *in vitro* data confirm *in vivo* tissue results, that myostatin down-regulates expression of *ARA70*.

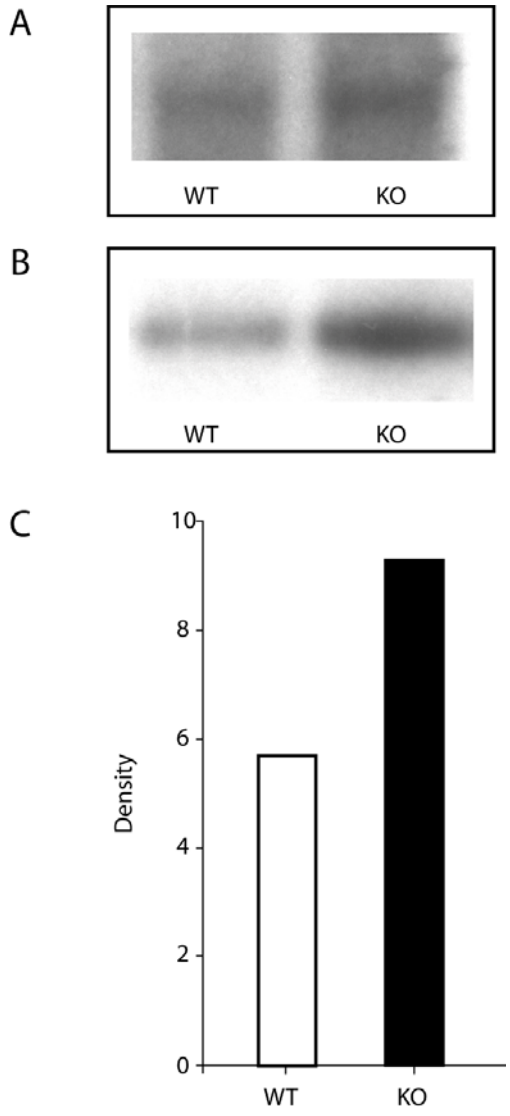

Figure 3.5* ARA70 *mRNA expression is increased in* myostatin-*null muscle

(A) Northern analysis of *ARA70* in wild-type (WT) and *myostatin*-null (KO) mice. Total RNA (15 μg) extracted from BF muscle was fractionated by gel electrophoresis and transferred to Hybond N+ membrane and probed with an *ARA70* ^{32}P-labelled cDNA probe. Ethidium bromide-stained 18 S and 28 S rRNA bands from the gel were assessed to have equal loading and showed RNA integrity (data not shown). (B) Semi-quantitative RT-PCR analysis of *ARA70* from BF muscles from WT and KO mice. Primers specific for full length *ARA70* amplify the expected 1878 bp product in a sequential RT then PCR amplification. Amplicons were then transferred to nitrocellulose membrane and probed with an *ARA70* ^{32}P-labelled cDNA probe. (C) Autoradiogram of the Southern blot shown in panel B was analysed by densitometry and normalised to *GAPDH*.

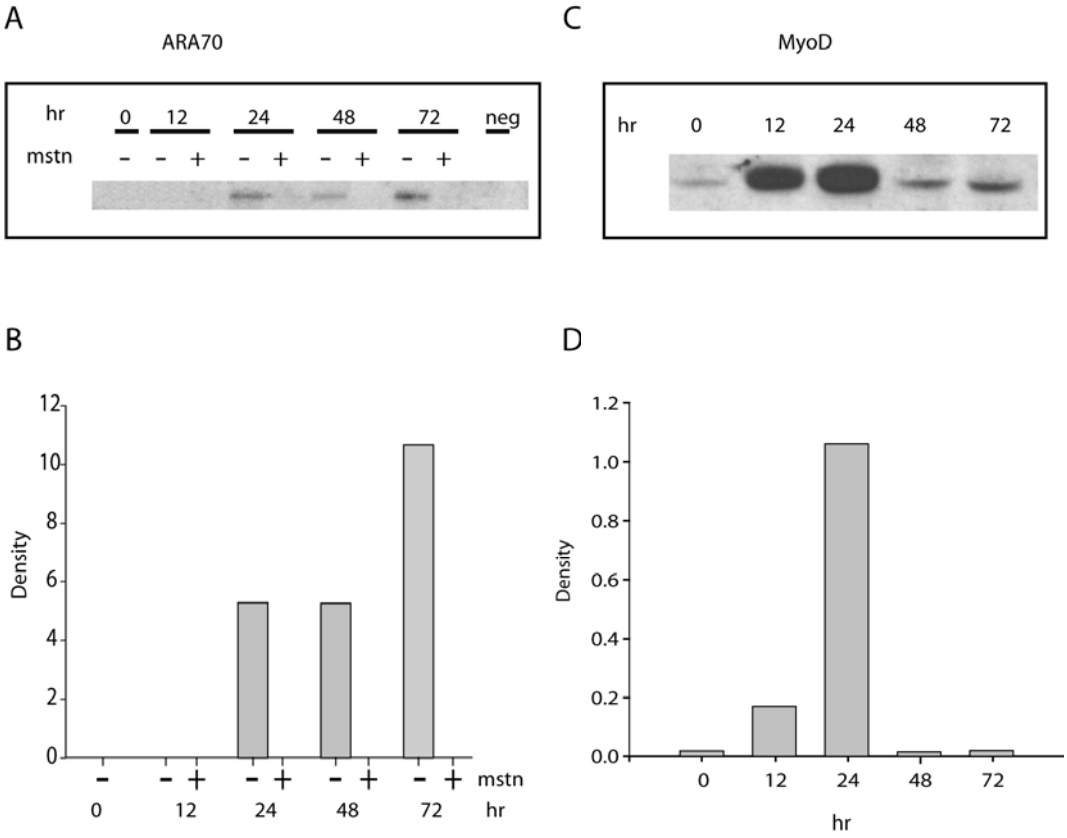

Figure 3.6 Myostatin down-regulates* ARA70 *in differentiating C2C12s

(A) Semi-quantitative RT-PCR analysis of *ARA70* derived from myoblasts cultured in differentiating medium with (+) or without (-) exogenous myostatin for 0, 12, 24, 48 or 72 hr. Primers specific for full length *ARA70* amplify the expected 1878 bp product in a sequential RT then PCR amplification. Amplicons were then transferred to nitrocellulose membrane and probed with an *ARA70* ^{32}P-labelled cDNA probe. Amplicons were not detected in the absence of template (negative control). (B) Autoradiogram of the Southern blot shown in panel A was analysed by densitometry and normalised to *GAPDH*. (C) Western blot analysis of MyoD. Total protein (15 µg) derived from myoblasts cultured in differentiating medium without exogenous myostatin for 0, 12, 24 or 72 hr were resolved by 4-12% SDS-PAGE, transferred to nitrocellulose membrane, and probed with polyclonal rabbit anti-MyoD, goat anti-rabbit IgG HRP and detected using chemiluminescence. (D) Autoradiogram of the Western blot shown in panel C was analysed by densitometry.

3.4 Discussion

Myostatin is a potent negative regulator of myogenesis and it was recently demonstrated that myostatin functions by regulating both the proliferation and differentiation steps of myogenesis by different mechanisms. Myostatin has been shown to regulate myoblast cell cycle progression through Retinoblastoma-dependent (Thomas *et al.,* 2000) and independent (Langley *et al.,* 2004) mechanisms. However, during differentiation, myostatin has been shown to function by regulating the activity and expression of MyoD (Langley *et al.,* 2002). To further analyse the molecular mechanism by which myostatin functions, we performed a molecular genetic screen and describe here that myostatin could potentially regulate muscle growth by controlling the expression of a steroid receptor coactivator, *ARA70*.

To isolate downstream target genes of myostatin, we performed SSH on total RNA from 4- to 6-week-old wild-type and *myostatin*-null mice, by which stage we observe generalised heavy muscling in the *myostatin*-null mice. We have recovered several novel, as well as some known, genes in this subtraction; however, here we chose to characterise *ARA70* as a downstream target gene of myostatin. Sequence analysis revealed that in addition to the expected canonical ARA70, there is an internally deleted variant (Figure 3.2). When compared to the canonical isoform, the β-form displays 6 amino acid substitutions and a 9 amino acid deletion in the carboxyl-region of the peptide. At this stage it is not known if both proteins are encoded by two non-allelic genes with different expression patterns or if these differences are due to RNA processing. The homology search revealed that murine ARA70 is highly homologous to human ARA70. We tested the ability of the murine ARA70 α-isoform to interact with ARLBD in the yeast two-hybrid system. The result indicated that full length α-isoform of ARA70 did not interact with the ARLBD (Figure 3.3). This result is consistent with the previous observation that the interaction between the full length human AR and human ARA70 is ligand-dependent in yeast (Yeh and Chang 1996). Thus the lack of interaction seen here between full length ARA70 and ARLBD could be due to lack of availability of the ligand or may also be due to incorrect folding of murine ARA70 in yeast. Mapping experiments with human ARA70 have clearly indicated that the carboxyl-terminal region of ARA70 has an inhibitory effect on

ARA70 binding to AR in yeast (Zhou *et al.,* 2002). Thus we conducted further yeast two-hybrid studies with ARA70 in which the carboxyl-terminal portion was deleted. The results indicated that the truncated ARA70 did specifically interact with ARLBD (Figure 3.3), confirming that the murine ARA70 behaves much like human ARA70 with respect to its interactions with AR.

The expression analysis indicates that *ARA70* is expressed ubiquitously (Figure 3.4A). Since *ARA70* is highly expressed in muscle, we further monitored the expression of *ARA70* in myoblasts during myogenic differentiation of C2C12 myoblasts (Figure 3.6). The peak expression of *ARA70* was detected much later during differentiation (72 hr), subsequent to peak expression of MyoD (24 hr). This result suggests that ARA70 could be involved in regulating the late differentiation events of myogenesis, such as increased protein synthesis, in combination with AR.

The major finding of this study is the identification of *ARA70* as a downstream target gene of the potent negative regulator of muscle growth, myostatin. Several independent lines of evidence suggest *ARA70* expression is negatively regulated by myostatin; reverse Northerns, Northern blot analysis and semi-quantitative RT-PCR analysis using RNA from wild-type and *myostatin*-null mice indicate that in the absence of myostatin there are increased levels of expression of *ARA70*. In corroboration of these data, when myoblasts were treated with exogenous myostatin there was reduced expression of *ARA70*, confirming that the *ARA70* gene is either directly or indirectly repressed by myostatin. What might be the physiological consequences of myostatin regulating the *ARA70* gene expression? Studies have shown that both AR (Inoue *et al.,* 1993; Inoue *et al.,* 1994) and testosterone (Ferrando *et al.,* 1998) induce skeletal muscle hypertrophy by inducing protein synthesis. In contrast, lack of myostatin also leads to generalised hypertrophy of muscles. Since increased levels of *ARA70* are observed in the *myostatin*-null muscles, it is quite possible that the up-regulated ARA70 protein could increase the activity of AR, therefore leading to increased protein synthesis.

Thus, we propose that the hypertrophy seen in animals that lack myostatin could not only be due to increased satellite cell activation (McCroskery *et al.,* 2003) but also a result of increased protein synthesis due to enhanced AR activity owing to increased expression of *ARA70*. Currently efforts are underway to

generate an *ARA70* over-expressing transgenic mice line to assess the hypertrophy phenotype since transient over-expression of *ARA70* in C2C12 cells has failed to induce hypertrophy in the myoblasts.

3.5 Acknowledgements

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Chapter Four

Prolonged absence of myostatin reduces sarcopenia

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Prolonged Absence of Myostatin Reduces Sarcopenia

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4.1 Introduction

Myostatin, a TGF-β superfamily member, is a negative regulator of muscle growth (McPherron *et al.,* 1997). It is expressed in both embryonic and adult skeletal muscle, suggesting that myostatin acts as a regulator of both prenatal and postnatal myogenesis. *Myostatin*-null mice are significantly larger than their wild-type counterparts due to a two- to three-fold increase in muscle mass resulting from both muscle cell hyperplasia and hypertrophy (McPherron *et al.,* 1997). Studies indicate myostatin regulates cell cycle progression and MRF levels, thereby controlling myoblast proliferation and differentiation during developmental myogenesis (Thomas *et al.,* 2000; Langley *et al.,* 2002). In addition, myostatin also influences postnatal muscle growth. Recently it was shown that myostatin regulates myogenesis through the inhibition of satellite cell activation in mice (McCroskery *et al.,* 2003). Furthermore, myostatin levels increased with muscle atrophy due to unloading in mice (Carlson *et al.,* 1999), and with severe muscle wasting in HIV patients (Gonzalez-Cadavid *et al.,* 1998). Cachexia, induced by systemic administration of myostatin, has also been shown to cause muscle wasting in mice, suggesting that myostatin may be directly involved in cachetic-type conditions in humans (Zimmers *et al.,* 2002). Together, these studies suggest that increased levels of myostatin lead to muscle wasting. Possible mechanisms of wasting through myostatin include the inhibition of satellite cell activation and proliferation, and inhibition of differentiation or myoblast fusion.

Studies measuring myostatin levels during aging (Kawada *et al.,* 2001; Baumann *et al.,* 2003) have yielded conflicting results regarding the role of myostatin in age-related muscle wastage, or sarcopenia. Sarcopenia is the profound loss of skeletal muscle mass and strength (Brooks and Faulkner 1994) associated with normal aging in mammals. This atrophy has previously been attributed to various factors including a decrease in muscle fibre number, an alteration in the fibre type, atrophy of existing fibres (Alnaqeeb and Goldspink 1987), loss of motor innervation (Carry *et al.,* 1993), and defective muscle regeneration (Grounds 1998) which involves muscle satellite cells (Schultz and Jaryszak 1985; Schultz and McCormick 1994). Aged muscle appears restricted in its ability to promote satellite cell activation, proliferation and differentiation,

therefore impairing its ability to regenerate (Welle 2002). It has been suggested that this reduced satellite cell activation is a function of the environment of aged muscle (Carlson and Faulkner 1989). Furthermore, using parabiotic pairing experiments, Conboy *et al*. (2005) recently showed that systemic factors from young mice promote the proliferation and regenerative capacity of satellite cells in aged mice. In addition, we believe that negative regulators, such as myostatin, are essential in the regulation of satellite cell-mediated muscle regeneration. Therefore, to observe the effects of prolonged absence of myostatin and muscle wasting during the aging process we compared young and old wild-type and *myostatin*-null muscle and its regeneration.

Here we show that, irrespective of age, a lack of functional myostatin resulted in muscle fibre hyperplasia and hypertrophy, and alterations in the fibre types. *Myostatin*-null muscle fibres had an increased number of associated satellite cells which demonstrated a greater propensity to undergo activation. In addition, muscle regeneration following notexin damage was accelerated in *myostatin*-null mice.

4.2 Materials and Methods

4.2.1 Animals

Myostatin-null mice (C57BL/10 background) were obtained from Se-Jin Lee (Johns Hopkins University, Baltimore, MD). Wild-type C57BL/10 mice were bred at the Ruakura Small Animal Colony. All animals were handled in accordance with the guidelines of the Ruakura Animal Ethics Committee (AgResearch, Hamilton, New Zealand) and the University of Waikato Animal Ethics Committee (University of Waikato, Hamilton, New Zealand).

4.2.2 Tissue processing

Mice were euthanized by CO_2 inhalation followed by cervical dislocation. TA and BF muscles were dissected out and frozen in isopentane cooled in liquid nitrogen. Frozen 10 μm transverse sections were cut from the mid-belly region of the muscles and used for histochemical analysis. For total fibre number, muscle sections were stained with H & E while for fibre type analysis, muscle sections were either immunostained using monoclonal antibodies specific for MHC types or stained for mATPase activity using a modified method of Brooke and Kaiser (1970). For immunostaining, muscle sections were blocked in 0.2% TritonX-100, 0.2% BSA and 10% NSS in PBS for 1 hr at room temperature followed by an overnight incubation at 4˚C with antibodies against type I (BA-D5, 1:100), type IIA (SC-71, 1:50) or type IIB (BF-F3, 1:100) MHC (Schiaffino *et al.,* 1989) (American Type Culture Collection) in 0.2% BSA and 5% NSS in PBS-T. The sections were then washed with PBS and fixed in 10% buffered formalin for 5 min before incubating with a biotinylated sheep anti-mouse immunoglobulin secondary antibody (1:300, Amersham) in 0.2% BSA and 5% NSS in PBS-T for 1 hr at room temperature. The sections were then washed and incubated with streptavidin-conjugated Alexa Fluor 488 (1:400, Molecular Probes) in 0.2% BSA in PBS-T for 1 hr. After washing, the sections were counterstained with DAPI at 1:1,000 (Molecular Probes) in PBS before mounting with fluorescent mounting medium (Dako) and viewing under fluorescent illumination using an Olympus BX50 microscope (Olympus) and SPOT RT camera and software (Diagnostic Instruments).

For mATPase staining, sections were incubated in an acid pre-incubation medium (pH 4.6) (95 mM CH_3COONa, 99 mM KCl) followed by an ATP incubation medium (pH 9.4) (2.8 mM ATP, 47.5 mM NaOH, 65 mM NaCl, 52.75 mM glycine, 37.8 mM $CaCl_2$). Fibre types were distinguished by the intensity of staining (type I > type IIX > type IIB > type IIA).

4.2.3 *In vivo* BrdU-labelling of satellite cells

Satellite cell proliferation was investigated by *in vivo* BrdU-labelling. Wild-type and *myostatin*-null mice were intraperitoneally injected with BrdU (30 mg/kg; Roche) as a single pulse 2 hr before euthanizing. Satellite cells were isolated following an adapted protocol of Yablonka-Reuveni and Nameroff (1987). Briefy, 1- and 6-month-old wild-type and *myostatin*-null mice (n = 10 per group) were killed by CO_2 gas followed by cervical dislocation. Hind-limb muscles were dissected out, minced and digested in 0.2% (w/v) type 1A collagenase (>260 CDU/mg) in DMEM for 90 min at 37ºC. The muscle slurry was triturated then passed through a 70 μm cell strainer (BD Biosciences) before loading onto 70% and 40% Percoll gradients (Sigma) and centrifuged at 2,000 x g for 20 min at 25ºC. The interface between the two gradient solutions was recovered and cells were resuspended in PBS. In order to detect BrdU-incorporation, an *In Situ* Cell Proliferation Kit, FLUOS (Roche) was used. Cells were fixed for 30 min in 70% ethanol on ice and treated with 2N HCL and 0.5 % TritonX-100 for 30 min at room temperature before neutralising in 0.1 M disodium tetraborate buffer (pH 8.5). Cells were permeablised in 0.5% Tween-20 in PBS and incubated for 45 min at 37ºC with a monoclonal anti-BrdU-FLUOS antibody (1:25, Roche) in incubation buffer (Roche). Cells were analysed by a FACScan (BD Biosciences) flow cytometer. The Percoll isolated cells purified by this method were >95% myogenic (McCroskery *et al.,* 2003).

4.2.4 Single myofibre isolation and culture

Single fibres were isolated as previously described by Rosenblatt *et al*. (1995). Briefly, 1- and 24-month-old wild-type and *myostatin*-null mice were euthanized by CO_2 gas followed by cervical dislocation. TA muscles were dissected out and digested in 0.2% (w/v) type 1A collagenase (>260 CDU/mg) in DMEM for 60 min at 37ºC. Muscles were transferred to DMEM containing 10%

HS and 0.5% CEE and fibres were separated by gentle trituration. Isolated fibres were transferred to 4-well chamber slides (BD Biosciences) coated with 10% Matrigel (BD Biosciences) and either fixed immediately after isolation at 37ºC for 10 min in 4% paraformaldehyde in PBS (for the detection of CD34) or cultured in DMEM containing 10% HS, 0.5% CEE and 10 μM BrdU (Roche) for 48 hr at 37ºC in 5% CO_2 (for the detection of BrdU-incorporation).

Satellite cells were detected with CD34 antibodies according to an adapted method of Beauchamp *et al*. (2000). Briefly, fibres were fixed with 4% paraformaldehyde, washed in PBS, permeablised in 0.5% TritonX-100 in PBS for 10 min and blocked in 10% NGS in PBS for 30 min at room temperature. Rat anti-mouse CD34 monoclonal antibody (clone RAM34; BD PharMingen) at 1:100 in 0.35% cλ in PBS was incubated overnight. Primary antibody was detected using biotinylated goat anti-rat IgG polyclonal antibody (Amersham) at 1:300 in 0.35% cλ in PBS for 2 hr at room temperature followed by streptavidin-conjugated Alexa Fluor 488 (Molecular Probes) at 1:400 in 0.35% cλ in PBS for 1 hr at room temperature. Fibres were counterstained with DAPI (Molecular Probes) at 1:1,000 in PBS for 5 min before mounting.

To detect BrdU-incorporated cells, the BrdU labeling and detection kit (Roche) protocol was followed. Fibres were counterstained with DAPI (Molecular Probes) at 1:1,000 in PBS for 5 min before mounting.

CD34- or BrdU-positive nuclei were counted as a percentage of total myonuclei in order to normalise the data and exclude any discrepancies in the lengths of the fibres analysed.

4.2.5 Muscle regeneration following notexin injury

Twenty four-month-old male mice were anaesthetised with Ketamine/Rompun (10% ketamine hydrochloride at 100 mg/ml, 5% Rompun 2% at 20 mg/ml) at 0.1 ml/7 g body weight. A small incision over the right TA muscle was made before 10 μl of Notexin (10 μg/ml) was injected into the muscle. The incision was then closed using a Michelle clip. Mice were euthanized on days 1, 7, 10 or 28 following the notexin administration. Both right and left (regenerating and control respectively) TA muscles were removed, weighed, and then frozen in isopentane cooled in liquid nitrogen. Frozen 10 μm transverse sections were cut from the mid-belly region of the TA and stained with H & E or immunostained

using monoclonal antibodies specific for type I, IIA and IIB MHCs as described previously.

4.2.6 Statistical analysis

All data are presented as means and SEM. Angular transformed data (percentages of type IIA fibres and BrdU-positive cells) and untransformed data (fibre numbers and areas and percentages of CD34-positive cells) were analysed by analysis of variance (ANOVA) to assess the effects of age and the *myostatin*-null genotype. The pooled residual standard deviation from these analyses was used to assess particular comparisons of interest, the wild-type versus *myostatin*-null difference at each age and changes between ages.

4.3 Results

4.3.1 Lack of myostatin alters fibre morphology

In order to assess the effect of myostatin and aging on muscle, three different parameters of muscle fibre physiology (fibre number, fibre type and fibre size) were determined in young and aged wild-type and *myostatin*-null muscle.

Aging muscle generally undergoes varying degrees of muscle fibre atrophy. Hence, the extent of muscle atrophy was assessed by measuring cross-sectional areas of various fibre types of 6- and 24-month-old wild-type and *myostatin*-null TA muscle. The TA muscle was chosen because a number of previous studies concerning age-related muscle wasting suggest that TA muscle is consistently affected by aging (Rowe 1969; Hooper 1981; Alnaqeeb and Goldspink 1987; Marsh *et al.,* 1997a). Furthermore, lack of myostatin is known to affect the physiology of TA muscle (McPherron and Lee 2002). Histochemical staining of type I, IIA, B and X fibres in TA and BF muscles, indicated that typically, type IIB was the largest fibre type, type IIX being an intermediate, while type IIA and type I were generally the smallest fibres (Figure 4.1A). Analysis of 6- and 24-month-old wild-type TA type IIB/X cross-sectional areas clearly suggested a significant decrease in the fibre areas due to atrophy resulting from increasing age (Figure 4.1B) ($P < 0.001$). In contrast, measurements of *myostatin*-null type IIB/X fibres indicated significant fibre hypertrophy in both 6- and 24-month-old TA muscle suggesting the hypertrophy observed was not only maintained in the aged muscle, but also little fibre atrophy had occurred during the aging process (Figure 4.1B).

During aging, murine muscle undergoes a switch in fibre type from fast glycolytic to fast oxidative fibre type. To investigate fibre type changes, immunostaining using antibodies against type I, IIA, and IIB MHCs were performed. Our results indicate that between 6- and 24-months, an increase of over 54% in type IIA fibres occurred in the wild-type TA muscle (Figure 4.1C) ($P < 0.05$). In contrast, *myostatin*-null TA indicated no significant increase in type IIA fibres with age. Moreover, type IIA fibres were minimally represented in 1-, 6- and 24-month-old *myostatin*-null TA in comparison to wild-type muscle. For example, at 24-months of age, type IIA fibres occupied close to 9% of the wild-

type TA, while just over 1% in *myostatin*-null TA (Figure 4.1C) ($P < 0.001$). Although a previous report suggested a very low number of type I fibres in mouse TA (Wang and Kernell 2001), we found no fibres of this type in any of the TA muscles tested. However type I fibres were found to be abundant in BF muscles, and therefore provided a positive control for both immuno- and histochemical staining procedures.

Fibre number was also measured during aging in both genotypes. One-month-old *myostatin*-null TA displayed a high degree of hyperplasia with a 57% increase in total fibre number compared to 1-month-old wild-type TA (Figure 4.1D) ($P < 0.001$). Six-month-old *myostatin*-null TA muscle contained fewer fibres than 1-month-old *myostatin*-null muscle but 40% more fibres than 6-month-old wild-type muscle ($P < 0.001$). Within genotypes, no statistically significant change in fibre number was observed between 6- and 24-months of age.

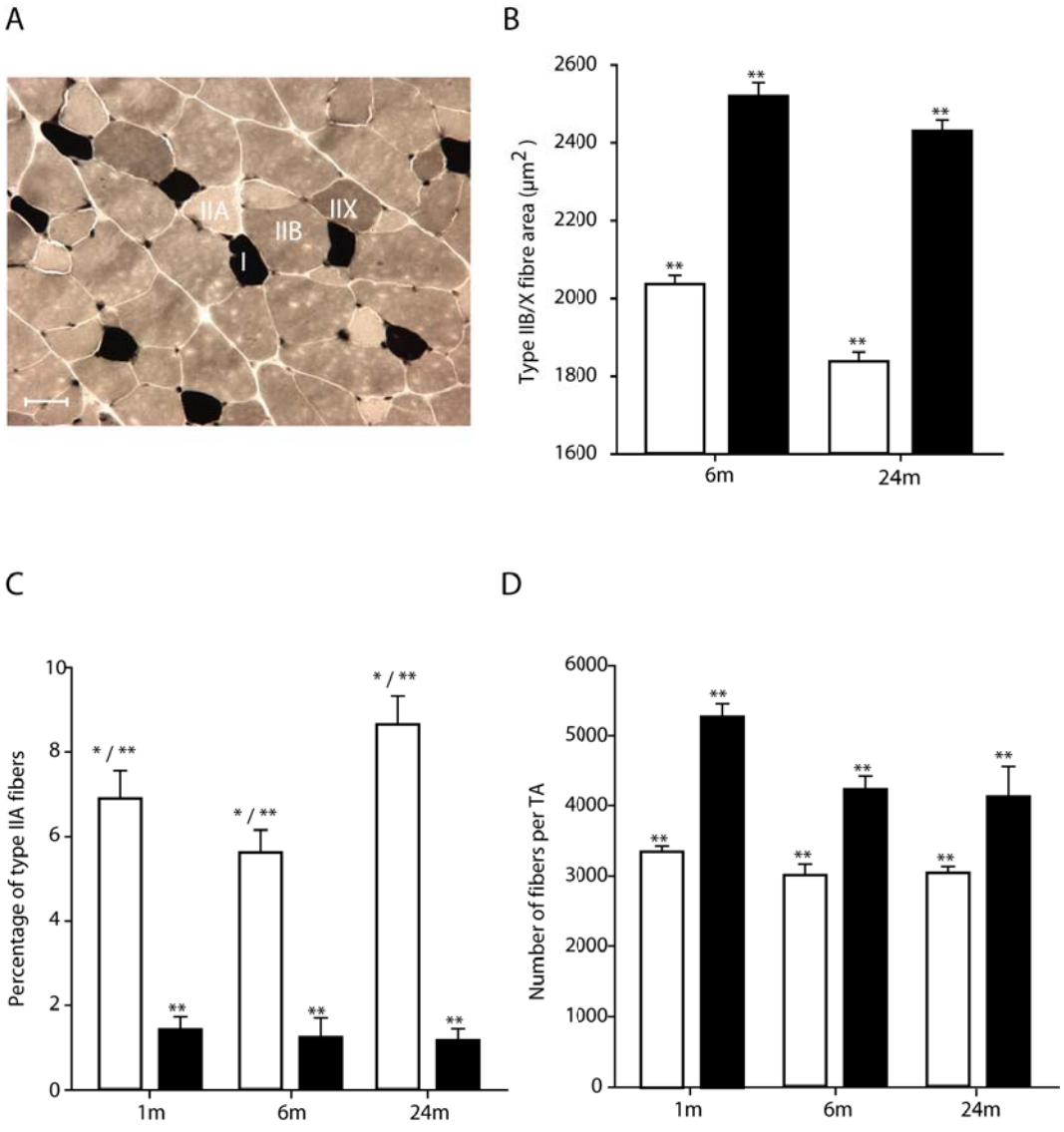

Figure 4.1 Long term absence of myostatin alters muscle fibre morphology

(A) Example of mATPase stained BF muscle cross-section. Fibre types were determined by staining intensity, type I > type IIX > type IIB > type IIA (from darkest to lightest). Scale bar, 20µm. (B) Averages of type IIB/X fibre areas in TA cross-sections from 6- and 24-month-old wild-type (WT) (empty bars) and *myostatin*-null (KO) (solid bars) muscle (n = 5 per group). ** = $P < 0.001$ when WT is compared to KO and when WT 6m is compared to WT 24m. (C) Percent of type IIA fibres in TA cross-sections from 1-, 6- and 24-month WT and KO mice (n = 5 per group). ** = $P < 0.001$ when WT groups are compared to KO groups, * = $P < 0.05$ when WT 1m and WT 6m is compared to WT 24m. (D) Number of fibres in TA cross-sections from 1-, 6-, and 24-month-old WT and KO muscles (n = 5 per group). ** = $P < 0.01$ when WT is compared to KO, and when KO 1m is compared to KO 6m. All data are expressed as mean ± SEM.

4.3.2 Myostatin decreases satellite cell number and activation

Satellite cell numbers and or their activation are believed to be adversely affected by progressive aging leading to a reduction in regeneration capabilities (Carlson and Faulkner 1989; Bockhold *et al.,* 1998; Conboy and Rando 2002). *Myostatin* is expressed in satellite cells and lack of *myostatin* in young mice leads to a greater number of satellite cells per unit fibre length as well as an increase in their propensity to become activated during *in vitro* studies (McCroskery *et al.,* 2003). To elucidate the effects of myostatin and aging on satellite cell behaviour, the total number of satellite cells and their ability to become activated was quantified from 1- and 24-month-old wild-type and *myostatin*-null mice.

In order to analyse satellite cell numbers per unit fibre length, satellite cells attached to single fibres isolated from 1- and 24-month-old wild-type and *myostatin*-null TA muscle were counted using the cell surface marker CD34. The mean percentage of fibre nuclei that were satellite cells in 1-month-old wild-type fibres was 5% compared to 11% in 1-month-old *myostatin*-null fibres (Figure 4.2A) ($P < 0.001$). Aging appeared to have little effect on satellite cell number as no significant change in the satellite cell number was observed between 1- and 24-month-old wild-type and *myostatin*-null fibres.

Since not only the number of satellite cells but also the activity of satellite cells is relevant to the ability of a muscle to regenerate, satellite cell activation was investigated using *in vitro* and *in vivo* BrdU-labelling. *In vitro* BrdU-labelled satellite cells attached to isolated fibres indicated the average percentage of activated satellite cells per fibre in 1-month-old wild-type TA was 6.5% as opposed to 10% in 1-month-old *myostatin*-null TA muscle (Figure 4.3A) ($P < 0.001$). However, during aging, satellite cell activation was reduced in both the wild-type and *myostatin*-null 24-month-old mice ($P < 0.001$). It is noteworthy that at 24-months, there was still twice the number of activated satellite cells per fibre in *myostatin*-null muscle fibres as compared to wild-type fibres ($P < 0.05$). As an independent measure, the number of fibres with one or more activated satellite cell attached was also counted. On average, 80 to 90% of 1-month-old wild-type and *myostatin*-null fibres in explant cultures contained activated satellite cells (Figure 4.3B). In contrast, only 16% of 24-month-old wild-type fibres and 31% of *myostatin*-null contained BrdU-positive nuclei ($P < 0.001$).

Finally, the propensity of satellite cells to become activated was also measured using *in vivo* BrdU-incorporation. FACS analysis of BrdU-labelled satellite cells indicated similar trends to the *in vitro* labelled satellite cells. The percentage of activated satellite cells from 1-month-old wild-type muscle was 8.5% as opposed to 14.8% in 1-month-old *myostatin*-null muscle (Figure 4.3D) ($P < 0.001$). With increasing age the percentage of activated satellite cells in both wild-type and *myostatin*-null 6-month-old muscle dropped significantly to 2% and 5% respectively ($P < 0.001$).

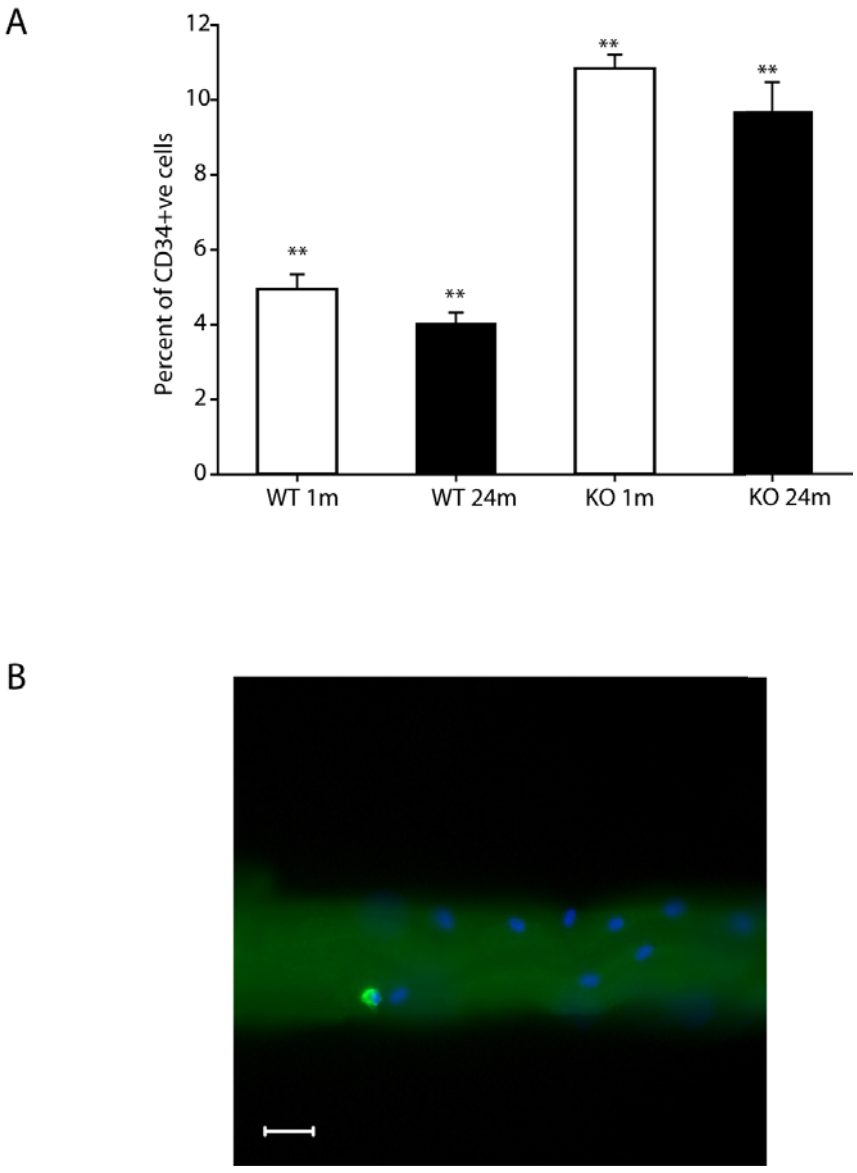

Figure 4.2 Satellite cell number is increased in* myostatin*-null mice

(A) Percent of satellite cells per 100 myonuclei on fibres isolated from 1- and 24-month-old wild-type (WT) and *myostatin*-null (KO) TA muscle. Satellite cells were visualised by immunostaining for CD34 and total nuclei by DAPI counterstaining. Fibres were isolated from 3 animals per group and in excess of 1,000 nuclei per group were counted. ** = $P < 0.001$ when WT is compared to KO. Data are expressed as mean ± SEM. (B) Example of a CD34-positive satellite cell attached to an isolated muscle fibre. Scale bar, 20 µm.

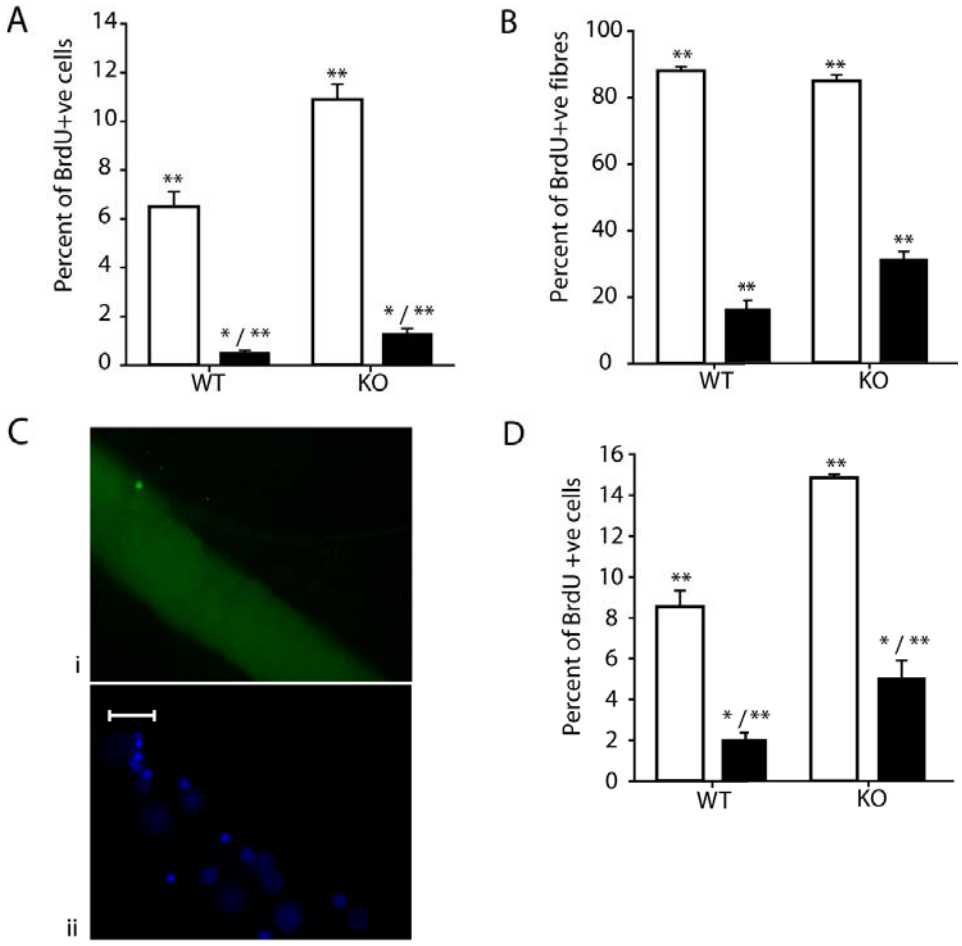

Figure 4.3 Increased cell activation is retained in the* myostatin-*null fibres

(A) Percent of activated satellite cells per 100 myonuclei on fibres isolated from 1- (empty bars) and 24- (solid bars) month-old wild-type (WT) and *myostatin*-null (KO) TA muscle. Activated satellite cells were represented by *in vitro* BrdU-incorporation and total nuclei by DAPI counterstaining. Fibres were isolated from 3 animals per group and over 1,000 nuclei per group were counted. ** = $P < 0.001$ when WT 1m is compared to KO 1m or WT 24m and when KO 1m is compared to KO 24m, * = $P < 0.01$ when WT 24m is compared to KO 24m. (B) Percent of fibres with activated satellite cells attached. Fibres were isolated from 1- and 24-month-old WT and KO TA muscle and in excess of 100 fibres per group were assessed. ** = $P < 0.001$ when WT 1m is compared to WT 24m, when KO 1m is compared to KO 24m, and when WT 24m is compared to KO 24m. (C) Example of a BrdU-labelled satellite cell attached to an isolated TA fibre (i). The fibre was counterstained with DAPI to visualise all myonuclei (ii). Scale bar, 20 μm. (D) Percent of BrdU-positive cells determined through flow cytometry. Satellite cells were BrdU-labelled *in vivo* and isolated from 1- and 6-month-old WT and KO hind-limb muscle using Percoll gradients. A minimum of 10,000 cells per sample group were analysed in triplicate. ** = $P < 0.001$ when WT 1m is compared to WT 6m or KO 1m and when KO 1m is compared to KO 6m, * = $P < 0.05$ when WT 6m is compared to KO 6m. All data are expressed as mean ± SEM.

4.3.3 Lack of myostatin enhances muscle regeneration

Notexin, a potent myotoxin, causes muscle necrosis, which induces regeneration. Compared to young muscle, aged muscle has a reduced capacity to regenerate. Recent work from our laboratory has shown that young *myostatin*-null mice have a greater capacity to regenerate than their wild-type counterparts (McCroskery *et al.,* 2005). It is unknown however, whether aged *myostatin*-null mice retain this greater ability to regenerate. Therefore, muscle regeneration in 24-month-old wild-type and *myostatin*-null mice were compared.

Histochemically stained day-1 wild-type and *myostatin*-null TA muscle sections displayed substantially damaged muscles indicated by necrotic fibres interspersed with surviving fibres. At this stage, an increased number of infiltrating cells were observed in *myostatin*-null TA compared to wild-type TA. In contrast, by day 7, greater amounts of infiltrating cells were present in wild-type TA relative to the *myostatin*-null muscle. However, unlike the wild-type muscle, the *myostatin*-null TA contained a large number of nascent myotubes and centrally located nuclei, indicating earlier regeneration than in the wild-type muscle. These nascent myotubes and centrally located nuclei were not largely observed until day 10 in the wild-type muscle (Figure 4.4Ai). By day 10, remaining non-regenerated areas were larger and the nascent myotubes within these areas were less advanced in wild-type TA relative to *myostatin*-null TA (Figure 4.4Ai-ii). At days 7 and 10, a greater number of original fibres appeared to survive the notexin damage in *myostatin*-null TA as compared to the wild-type muscle. In both wild-type and *myostatin*-null muscles, smaller fibres within less extensively damaged areas had centrally located nuclei at days 7 and 10 whereas larger fibres did not. Assessment of fibre areas indicated original and nascent myotubes were substantially larger in *myostatin*-null TA compared to those observed in wild-type TA. Early collagen formation was observed by day 7 in wild-type TA and remained high in day-10 and -28 muscles (Figure 4.4Ai, iii). In contrast, little collagen was observed in *myostatin*-null TA at each time point (Figure 4.4Aii, iv). By day 28, wild-type and *myostatin*-null TA appeared largely regenerated (Figure 4.4Aiii-iv). Immunohistochemical analysis of wild-type day-28 TA indicated a significant increase of type IIA fibres in regenerated TA (148%) compared to non-injured TA (Figure 4.4B) ($P < 0.001$). In contrast, a

slight (statistically insignificant) increase was observed between day-28 regenerated and non-injured *myostatin*-null muscles. In addition, a low percentage of type I fibres (0.15%; $P < 0.05$, data not shown) were detected in day-28 regenerated wild-type muscle. No type I fibres were observed in the *myostatin*-null muscle.

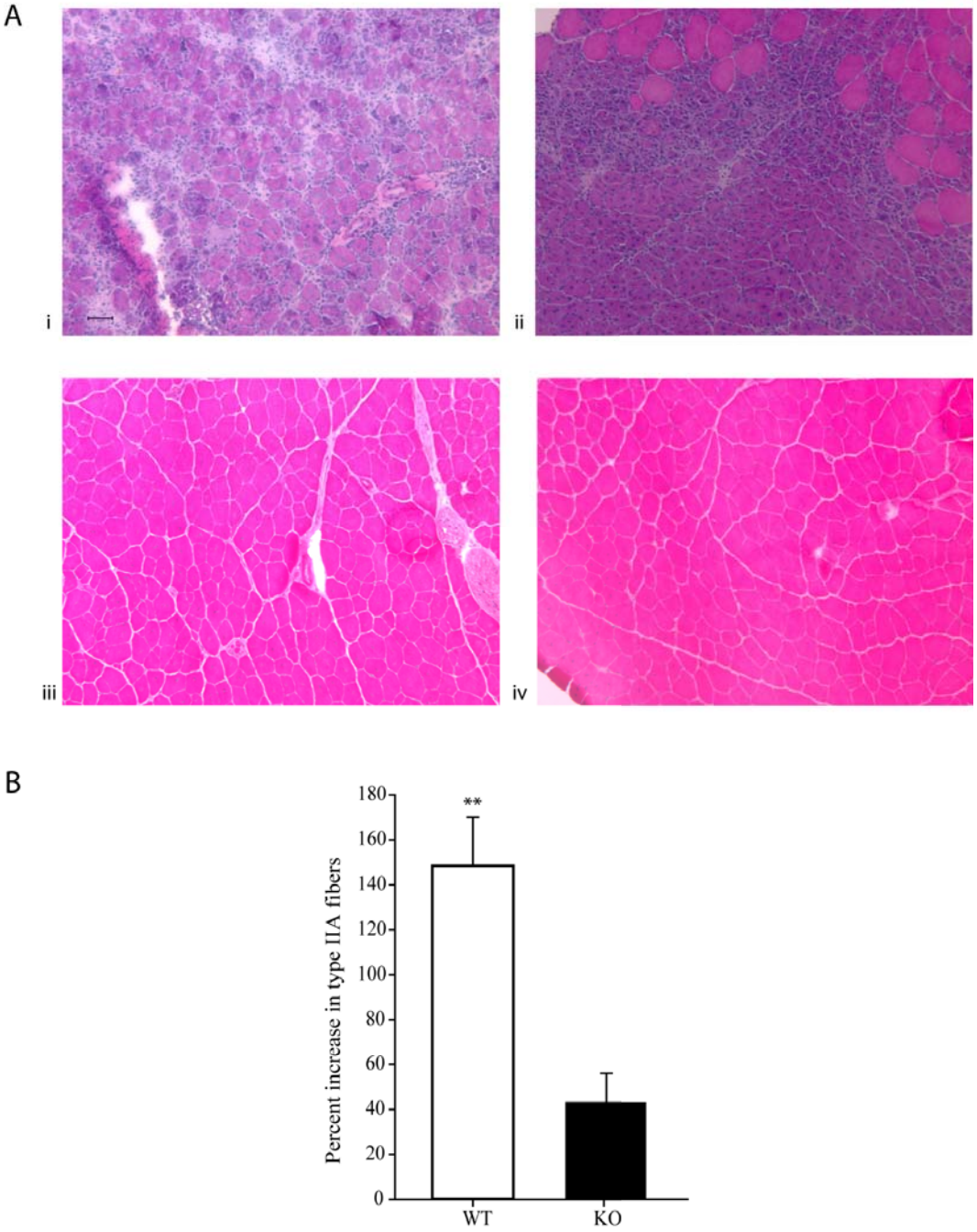

Figure 4.4 Muscle regeneration is enhanced in the* myostatin-*null muscle

(A) H & E stained cross-sections of day-10 wild-type (i) and *myostatin*-null (ii), and day-28 wild-type (iii) and *myostatin*-null (iv) TA muscles following notexin administration. Scale bar, 50 µm. (B) Percentage increase of type IIA fibres in muscle cross-sections from day-28 wild-type (WT) and *myostatin*-null (KO) notexin injured TA muscles (n = 3 per group). Data are expressed as mean ± SEM. ** = $P < 0.001$.

4.4 Discussion

One of the most striking effects of aging in muscle is the associated loss in muscle mass resulting in loss of strength and endurance. Furthermore, aging muscle has a marked reduction in its regenerative capabilities after muscle damage. It has been difficult to establish a primary cause and to formulate a unified theory explaining the molecular basis behind the aging muscle phenotype. Although the roles of several positive regulators have been extensively studied (Mezzogiorno *et al.,* 1993; Allen *et al.,* 1995; Marsh *et al.,* 1997b; Barton-Davis *et al.,* 1998; Yablonka-Reuveni *et al.,* 1999), the role of negative regulators during age-related muscle wasting is not known. In the current study we explored the involvement of myostatin, a known negative regulator of muscle growth, during the aging process.

Well established effects of aging on muscle are atrophy of the muscle and its individual fibres, a shift towards oxidative fibres, and impairment of satellite cell activation and subsequent muscle regeneration. In this current study, we show that the prolonged absence of myostatin in mice reduces fibre atrophy associated with aging (Figure 4.1B).

Currently, satellite cells are believed to be largely responsible for muscle growth and maintenance throughout life (see Hawke and Garry (2001) for review). Previously it has been suggested that satellite cell numbers decline during aging (Gibson and Schultz 1983; Shefer *et al.,* 2006) while others report no change (Nnodim 2000; Conboy *et al.,* 2003). Here we report that satellite cell activation rather than number is adversely affected during the aging process.

We have previously shown in young mice that myostatin is involved in the maintenance of satellite cell quiescence (McCroskery *et al.,* 2003) and that a lack of myostatin results in increased activation of satellite cells. Myostatin acts by inhibiting cell cycle progression from G0 to S phase. In its absence, cell cycle progression can proceed resulting in an increase in satellite cell activation and proliferation as observed in the *myostatin*-null mice. This increased cell number and activation would provide a mechanism for greater myoblast recruitment and subsequent fibre formation and enlargement leading to the fibre hypertrophy observed in the young *myostatin*-null mice. We now show that the prolonged absence of myostatin maintains the increased satellite cell number and activation

even in aged muscle (Figure 4.2A, 4.3A). The increased cell number and activation would provide an essential resource during aging, when a significant pressure on the maintenance of the fibres would be present in response to the aging process. Therefore we propose that lack of myostatin, from prenatal through to old age, leads to increased self-renewal of satellite cells and efficient replacement of lost muscle fibres, leading to increased muscle growth and reduced muscle wasting. However, at this time, it is not known whether a prolonged lack of myostatin provides increased resistance to the loss of muscle mass also.

With aging, murine muscle undergoes specific fibre type switches, with functional and metabolic consequences. Specifically, numerous reports suggest a shift from glycolytic fibres to oxidative fibres with increasing age (Grimby *et al.,* 1982; Alnaqeeb and Goldspink 1987; Larsson *et al.,* 1993). Myostatin also influences fibre type composition, as an increase in type II fibres, possibly at the expense of type I, can be observed in *myostatin*-null muscle (Holmes and Ashmore 1972; Girgenrath *et al.,* 2005). This in itself would lead to muscle hypertrophy due to the larger size of the type II fibres. In agreement with previous findings of Alnaqueeb and Goldspink (1987) and Larsson *et al*. (1993), the number of type IIA fibres in this current study increased with age within the wild-type muscle (Figure 4.1C). Assessment of the total fibre number indicated that they remained constant between 6- and 24-month-old muscles. Similar studies are somewhat conflicting as to whether a loss of fibres occurs during aging in rodents (Hooper 1981; Alnaqeeb and Goldspink 1987; Brown 1987; Timson and Dudenhoeffer 1990) and it has been suggested that the occurrence of fibre splitting complicates the analysis of fibre number (Alnaqeeb and Goldspink 1987). However, as the fibre number did not change with aging, it was concluded that the observed increase in type IIA fibres was at the expense of either type IIB or X fibres through fibre type shifts in the wild-type muscle. In contrast, all *myostatin*-null muscles displayed minimal type IIA fibres. This indicates an alteration in the fibre type composition with the loss of myostatin, as well as a resistance to an increase of type IIA fibres, which was associated with aging in the wild-type mice (Figure 4.1C). The role played by myostatin in the determination of fibre types is still unclear, although Girgenrath *et al*. (2005) have suggested that myostatin may influence the fibre type during prenatal myogenesis and postnatal terminal differentiation, therefore playing a role in the determination of the adult

fibre type. One mechanism Girgenrath *et al*. (2005) proposed is that an absence of myostatin inhibits the proliferation or differentiation of primary myoblasts leading to a decline in the number of slow fibres, or conversely, increases the proliferation or differentiation of secondary myoblasts, resulting in a higher number of fast fibres. Regardless of the mechanism, increased type IIB fibres would cause the muscle to remain predominantly glycolytic during aging.

Aging is also thought to negatively influence satellite cell behaviour. These cells are heavily involved in the regenerative process after muscle injury. Aging has a significant effect on the muscle regenerative capacity, since the proliferative potential of satellite cells in skeletal muscles of aged rodents is decreased as compared with young adults (Schultz and Lipton 1982). Furthermore, some reports also suggest that the poor regenerative capacity of skeletal muscle is also due to a decrease in the number of satellite cells (Snow 1977). Since we observed that lack of myostatin maintains the increased number of satellite cells and activation propensity in 24-month-old *myostatin*-null mice compared to 24-month-old wild-type mice (Figure 4.2A, 4.3A), it was questioned whether aged *myostatin*-null muscle would demonstrate an increased propensity to regenerate following notexin damage. Overall, findings suggested a lack of myostatin did result in a more efficient regeneration, even in the aged mice. Nascent fibres formed faster, muscle and fibre hypertrophy and fibre type composition were preserved, and the formation of scar tissue was greatly reduced (Figure 4.4A). Interestingly, senescent *myostatin*-null mice were virtually able to recapitulate the enhanced regeneration seen in young adult *myostatin*-null mice. This is in agreement with a recent study by Wagner *et al*. (2005), who have shown that regeneration after injury in senescent *myostatin*-null muscle was more robust than the wild-type controls. In common with the prevention of fibre atrophy during the aging process, the subsequent muscle regeneration following notexin damage would be heavily reliant on satellite cell availability and activation. Undoubtedly, an increased number of satellite cells and activation propensity, as observed in the *myostatin*-null mice, would be advantageous during this regenerative process.

Recently, Conboy *et al*. (2005), using a parabiosis model, have suggested that systemic factors contribute to the aging process. Indeed the results presented

here clearly supports that, in addition to the positive acting factors, negative regulators of myogenesis also play a role in the aging of skeletal muscle.

4.5 Acknowledgements

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Chapter Five

Enhanced myogenic potential of myoblasts is maintained in aged myostatin-null mice

This study will be communicated as a manuscript to the Journal of Cellular Physiology.

Enhanced myogenic potential is maintained with a prolonged absence of myostatin

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5.1 Introduction

The progressive loss of skeletal muscle mass and strength associated with aging is referred to as sarcopenia. Sarcopenia can have a profound effect on a person's quality of life due to reduced mobility and independence and increased risk of falls (Grimby and Saltin 1983; Harris 1997; Roubenoff and Hughes 2000; Roubenoff 2001). A substantial factor related to this loss is a decreased regenerative capacity reported to occur in aged muscle (Carlson and Faulkner 1989; Grounds 1998). Essential to muscle regeneration is the involvement of quiescent satellite cells associated with the muscle fibres (Zammit *et al.,* 2002). In response to various stimuli, satellite cells become activated, leading to myoblasts fusing to existing fibres or the formation of new fibres (Schultz and McCormick 1994). With increasing age, the activation potential of satellite cells is thought to decrease, thus contributing to the impaired muscle regeneration (Schultz and Lipton 1982; Conboy *et al.,* 2005). It has been suggested that this reduced satellite

cell activation is a function of the environment of aged muscle (Carlson and Faulkner 1989; Conboy *et al.,* 2005) indicating that various factors within the environment may positively or negatively regulate myogenesis and muscle regeneration.

A potent inhibitor of muscle growth is myostatin, a TGF-β superfamily member (McPherron *et al.,* 1997). Postnatally, *myostatin* has been shown to be expressed in adult skeletal muscle (McPherron *et al.,* 1997) and satellite cells (McCroskery *et al.,* 2003). In addition, myostatin is known to inhibit myoblast proliferation and differentiation (Thomas *et al.,* 2000; Taylor *et al.,* 2001), as well as inhibit satellite cell activation, thus maintaining the quiescent status of satellite cells (McCroskery *et al.,* 2003). Increased myostatin protein levels have frequently been correlated to the loss of muscle associated with aging (Schulte and Yarasheski 2001; Yarasheski *et al.,* 2002; Baumann *et al.,* 2003) suggesting myostatin has a significant role in the reduced muscle regeneration observed during sarcopenia. In accordance with these findings, recently we (Chapter 4), and others, have shown that a prolonged absence of myostatin reduced sarcopenia in mice (Wagner *et al.,* 2005).

In addition, myostatin also influences adipogenesis as animals lacking functional myostatin have significantly reduced fat accumulation (Arthur 1995; Lin *et al.,* 2002; McPherron and Lee 2002; Short *et al.,* 2002; Wiener *et al.,* 2002). Generally in animals, fat accumulates with increasing age until old age when a decline occurs. The accumulation of adipose tissue is through the formation of new adipocytes from precursor cells, which occurs throughout life (Bertrand *et al.,* 1978; Miller *et al.,* 1984), and adipocyte hypertrophy.

Although the role of myostatin in aging has been researched, to date the biology of *myostatin*-null myoblasts derived from aged satellite cells has not been investigated. Here we describe how a prolonged absence of functional myostatin affects the aging process in regards to the myogenic nature of satellite cells as well as adipose tissue. We found that the muscle hypertrophy, increased myoblast proliferation and differentiation, and decreased fat accumulation observed in young *myostatin*-null mice, was retained in aged *myostatin*-null mice.

5.2 Materials and Methods

5.2.1 Animals and tissue processing

Myostatin-null mice (C57BL/10 background) were obtained from S.-J. Lee (The Johns Hopkins University, Baltimore, MD). The wild-type mouse strain C57BL/10 was bred at the Ruakura Small Animal Colony. All animals were handled in accordance with the guidelines of the Ruakura Animal Ethics Committee (AgResearch, Hamilton, New Zealand) and the University of Waikato Animal Ethics Committee (University of Waikato, Hamilton, New Zealand). Male wild-type and *myostatin*-null mice aged 1-, 6- and 24-months, were killed by CO_2 gas followed by cervical dislocation. Individual muscles: BF; TA; Gas; and the Quad muscle group were dissected out and weighed. Gas was then frozen in liquid nitrogen for protein isolation. Epididymal fat pads were dissected and weighed before fixing in formalin and embedding in paraffin. Ten micrometer sections of each fat pad were cut and stained with H & E.

5.2.2 Primary myoblast assays

Satellite cells were isolated from the hind-limb muscles from 1-, 6- and 24-month-old wild-type and *myostatin*-null mice according to published protocols (Allen *et al.,* 1997; Partridge 1997; Yablonka-Reuveni *et al.,* 1999; McCroskery *et al.,* 2005). After 48 hr culturing, the cells were trypsinised off and utilised in a proliferation assay as previously described (Oliver *et al.,* 1989; McCroskery *et al.,* 2003), plated on 8-well chamber slides (BD Biosciences) for ICC analysis (described below) or plated on 6-well plates (Nunc) for differentiation assays. After a 3 hr attachment period, the culturing media was replaced with DMEM with 2% HS. The differentiating myoblast cultures were used for protein isolation or stained with H & E for histological analysis after culturing for 0, 6, 12, 24, 48 or 72 hr.

5.2.3 ICC analysis

Actively growing primary myoblasts were fixed with 20 parts 70% ethanol/2 parts 37% formaldehyde/1 part glacial acetic acid for 30 s and permeablised in 0.1% triton X-100 in PBS for ICC analysis of desmin or MyoD and Pax7. For desmin, the myoblasts were blocked with 10% NSS and 0.35% cλ

in PBS for 1 hr at room temperature before incubating overnight at 4˚C with mouse anti-desmin (1:200; Sigma) in 5% NSS and 0.35% cλ in PBS. Biotinylated sheep anti-mouse secondary antibody (1:300; Amersham) and streptavidin-conjugated Alexa Fluor 488 (1:400; Molecular Probes) were used as secondary and tertiary antibodies respectively. For MyoD/Pax7 staining, the myoblasts were blocked with 5% NSS, 5% NGS and 0.35% cλ in PBS (blocking solution) for 1 hr at room temperature before incubating overnight at 4˚C with rabbit anti-MyoD (1:100; Santa Cruz) and mouse anti-Pax7 (1:100; ascites fluid obtained from the Developmental Studies Hybridoma Bank) in blocking solution. The cells were incubated with biotinylated donkey anti-rabbit secondary antibody (1:300; Amersham) for 1 hr before incubating for a further hour with streptavidin-conjugated Alexa Fluor 488 (1:400) and Alexa Fluor 546 goat anti-mouse IgG conjugated secondary (1:300) (Molecular Probes). All cells were stained with DAPI (Molecular Probes) for 5 min and mounted with fluorescent media (Dako) before viewing under fluorescent illumination.

5.2.4 Protein analysis of Gas muscle and primary myoblasts

Gas muscles were homogenised in 1 ml PLB (0.05 M Tris pH 7.5, 0.25 M NaCl, 5 mM EDTA, Complete™ protease inhibitor tablet (Roche) and 0.1% NP40). Protein was collected from differentiating primary myoblasts isolated from 6- and 24-month-old wild-type and *myostatin*-null mice as previously published (McCroskery *et al.,* 2003). Bradford's reagent (Bio-Rad) was used to estimate total protein. Total protein (10 μg for muscle, 15 μg for myoblasts) was separated on a NuPAGE™ 4-12% Bis-Tris gel (Invitrogen) and transferred to nitrocellulose membranes (Bio-Rad). The membranes were stained in Ponceau S to visually assess equal protein loading. A Western blot for myostatin was performed as previously published (Sharma *et al.,* 1999). For MyoD, BSA blocker (0.3% BSA, 1% PEG, and 1% PVP in TBS-T) was added for 1 hr at room temperature before incubating with mouse anti-MyoD (1:10,000; BD PharMingen) overnight at 4°C. For GAPDH, 5% milk in TBS-T was added overnight at 4°C before incubating with mouse anti-GAPDH (1:10,000; Research Diagnostics) for 1 hr at room temperature. Both membranes were incubated with goat anti-mouse IgG HRP conjugate (1:5,000 for MyoD, 1:10,000 for GAPDH;

Dako) for a further hour at room temperature. HRP activity was detected using Western Lightning™ Chemiluminescence Reagent Plus (PerkinElmer).

5.2.5 Image and statistical analysis

Images of fat pads and primary myoblasts and myotubes were obtained using an Olympus BX50 microscope (Olympus) and a SPOT-RT 4.01 camera and software (Diagnostic Instruments). The images were then analysed using ImageJ software (National Institutes of Health; NIH). All data are presented as means ± SEM. To determine the significance ($P < 0.05$) of differences between groups, comparisons were made using Student's t test.

5.3 Results

5.3.1 Lack of functional myostatin increases muscle weights

Lack of functional myostatin has been shown to result in muscle hypertrophy, as observed in both Belgian blue cattle and *myostatin*-null mice (Kambadur *et al.,* 1997; McPherron *et al.,* 1997; McPherron and Lee 1997). Conversely, aging can cause loss of muscle mass in both animals and humans (Alnaqeeb and Goldspink 1987; Lexell *et al.,* 1988; Holloszy *et al.,* 1991; Gallegly *et al.,* 2004). In order to determine any correlation between a lack of myostatin and muscle mass during aging, wet muscle weights of BF, Gas, TA and Quad from 1-, 6- and 24-month-old wild-type and *myostatin*-null mice were measured (Figure 5.1A). From the outset, 1-month-old *myostatin*-null muscle weights were significantly increased from that of 1-month-old wild-type muscle. For example, 1-month-old *myostatin*-null BF and Quad muscles were 50 and 72% heavier than their respective wild-type muscles ($P < 0.001$). Both wild-type and *myostatin*-null muscle weights increased significantly from 1- to 6-months of age. However, the *myostatin*-null muscles increased significantly more than the wild-type. For example, *myostatin*-null Quad and TA increased 300% and 170% respectively compared to a 250% and 130% increase observed in the wild-type muscles ($P < 0.001$). With further aging, a significant decrease in wet weight was only observed in the wild-type and *myostatin*-null Quad muscles (16% and 24% loss respectively) ($P < 0.05$). However, all *myostatin*-null muscle weights were significantly larger than their wild-type counterparts irrespective of age.

5.3.2 Age alters MyoD levels in *myostatin*-null & wild-type muscles

MyoD expression has been shown to indicate the level of myogenesis occurring within a muscle (Grounds *et al.,* 1992). To investigate MyoD protein levels in 1-, 6- and 24-month-old wild-type and *myostatin*-null muscles, Western blot analyses were performed (Figure 5.1B). The results indicated that in the wild-type muscle there was a gradual increase in the expression levels of MyoD during postnatal growth. In the *myostatin*-null muscle, no significant up-regulation of MyoD levels was seen in the muscles of the mice between 1- and 6-months of age.

When relative comparisons were made between the two genotypes, it was discovered that MyoD levels were significantly higher in 1-month-old *myostatin*-null muscle compared to 1-month-old wild-type muscle (Figure 5.1C) ($P < 0.05$). However, at 6-months of age, MyoD was significantly higher in the wild-type muscle compared to the *myostatin*-null muscle ($P < 0.05$) of the same age. The level of MyoD increased significantly between 6- and 24-months of age ($P < 0.05$). Of note, no significant difference was observed between the two genotypes at 24-months of age.

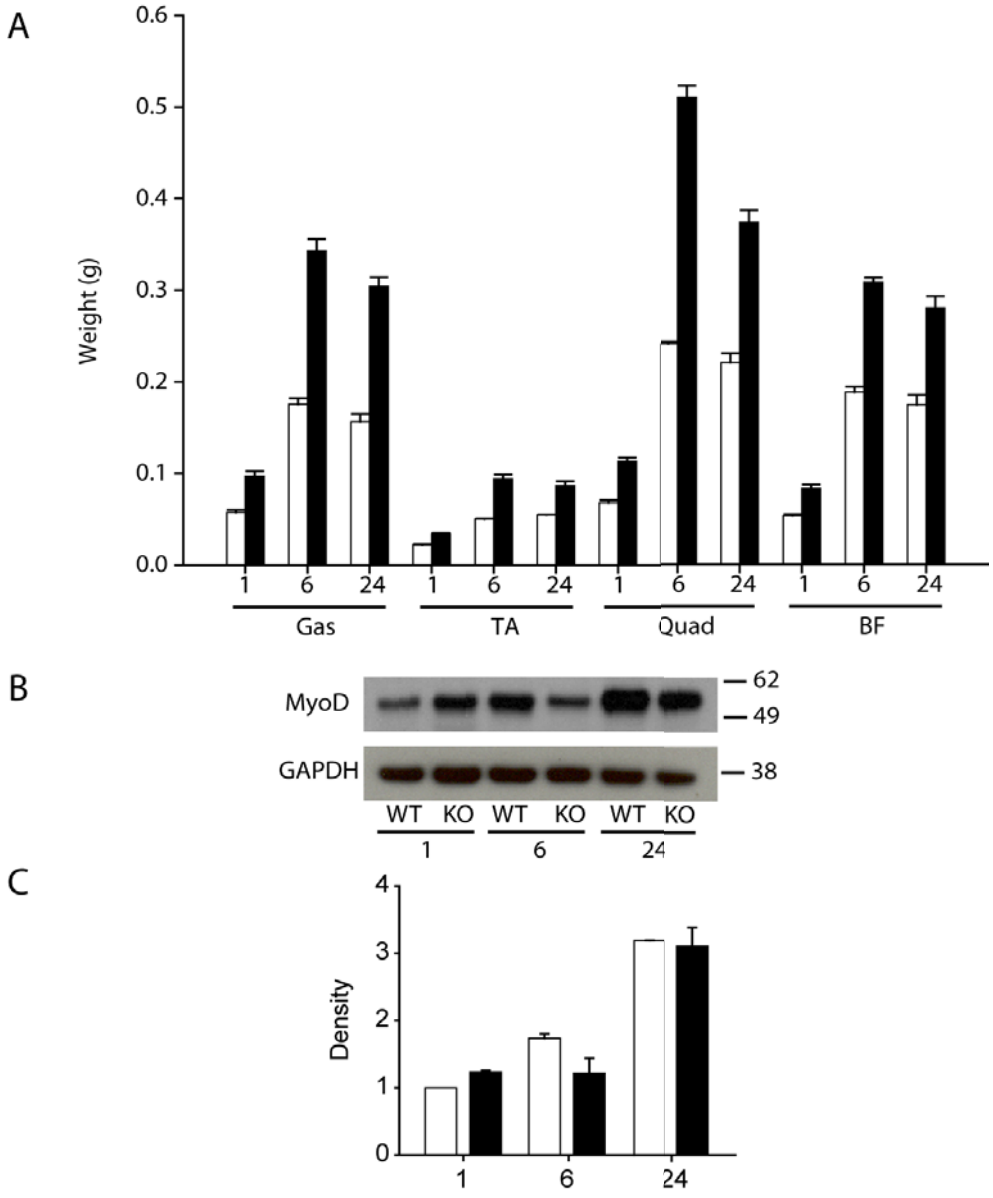

Figure 5.1 Lack of myostatin alters muscle weights and MyoD levels

(A) *Gastrocnemius* (Gas), *tibialis anterior* (TA), *quadriceps* (Quad) and *biceps femoris* (BF) muscle weights were measured in 1-, 6-, and 24-month-old wild-type (empty bars) and *myostatin*-null (solid bars) mice (n = 10). $P < 0.001$ when wild-type is compared to *myostatin*-null and when 1-month is compared to 6-month, $P < 0.05$ when 6-month Quad is compared to 24-month Quad. (B) Western blot analysis was performed on 1-, 6-, and 24-month-old wild-type (WT) and *myostatin*-null (KO) mice muscles to determine MyoD protein levels. The Western blot was analysed by densitometry and normalised to GAPDH protein levels. Normalised data were used to generate the MyoD (C) graph shown (empty bars = wild-type, solid bars = *myostatin*-null) (n = 5). $P < 0.001$ when 1-month WT is compared to 6-month WT, $P < 0.05$ when 1- and 6-month WT is compared to 1- and 6-month KO respectively, and when 6-month is compared to 24-month. All data are expressed as mean ± SEM.

5.3.3 Aged *myostatin*-null myoblasts display an increased proliferation

Previously, it has been shown that exogenous myostatin inhibits the proliferation of C2C12 myoblasts (Thomas *et al.,* 2000) and that *myostatin*-null myoblasts from young mice proliferate significantly faster than their wild-type counterparts (McCroskery *et al.,* 2003). To determine if this greater proliferative capacity of the *myostatin*-null myoblasts is maintained with age, myogenic cells were isolated from 24-month-old wild-type and *myostatin*-null mice, and a proliferation assay was later performed. The myogenic purity of the 1- and 24-month-old wild-type and *myostatin*-null primary myoblast cultures were first assessed through ICC using an antibody for desmin, a marker expressed specifically on muscle precursor cells (Kaufman and Foster 1988). Analysis of the immunostaining indicated that each culture was 90-94% myogenic as determined by the percentage of desmin-positive cells (Figure 5.2A) ($P < 0.001$). The proliferation assay indicated that indeed, the *myostatin*-null myoblasts possessed greater proliferation rates compared to the wild-type cultures (Figure 5.2B) ($P < 0.05$).

5.3.4 Differentiation and fusion is increased in *myostatin*-null cultures

Myostatin has been shown to strongly inhibit myoblast differentiation (Langley *et al.,* 2002; Rios *et al.,* 2002; Joulia *et al.,* 2003). In support of these reports, myotube hypertrophy was observed in the differentiating *myostatin*-null cultures in relation to the wild-type cultures irrespective of age (Figure 5.3A). In addition, aging has been correlated with a decrease in the differentiation potential of myoblasts (Charge *et al.,* 2002; Lees *et al.,* 2006). Analysis of cell fusion within each culture indicated that fusion was significantly greater in the *myostatin*-null cultures (Figure 5.3B). For example, the fusion index for 6-month *myostatin*-null cultures was 71% as opposed to 56% in the wild-type cultures ($P < 0.001$). Aging had a minimal effect on the fusion index as any observed decreases were not statistically significant.

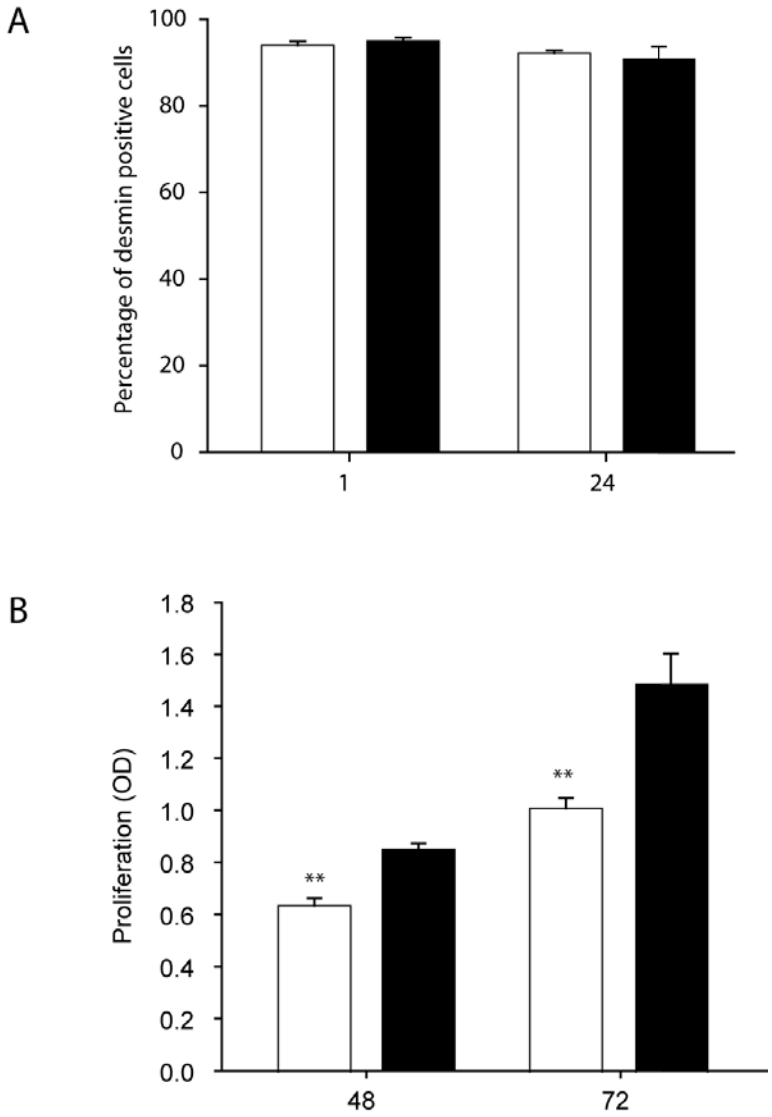

Figure 5.2 Aged* myostatin-*null myoblasts display increased proliferation

(A) The number of desmin-positive cells in 1- and 24-month wild-type (empty bars) and *myostatin*-null (solid bars) myoblast cultures were determined to assess myogenic purity. Myoblasts were isolated from 3 mice of each age and genotype, cells were plated in duplicate, and a minimum of 1,000 cells per culture were counted. (B) A proliferation assay was performed with primary myoblasts isolated from 24-month-old wild-type (empty bars) and *myostatin*-null (solid bars) mice. Cell proliferation was determined using a methylene blue photometric endpoint assay. Proliferation after 48 and 72 hr is graphically shown. Assays were performed in triplicate using cells isolated from 6 animals. ** = $P < 0.05$ when wild-type is compared to *myostatin*-null. All data are expressed as mean ± SEM.

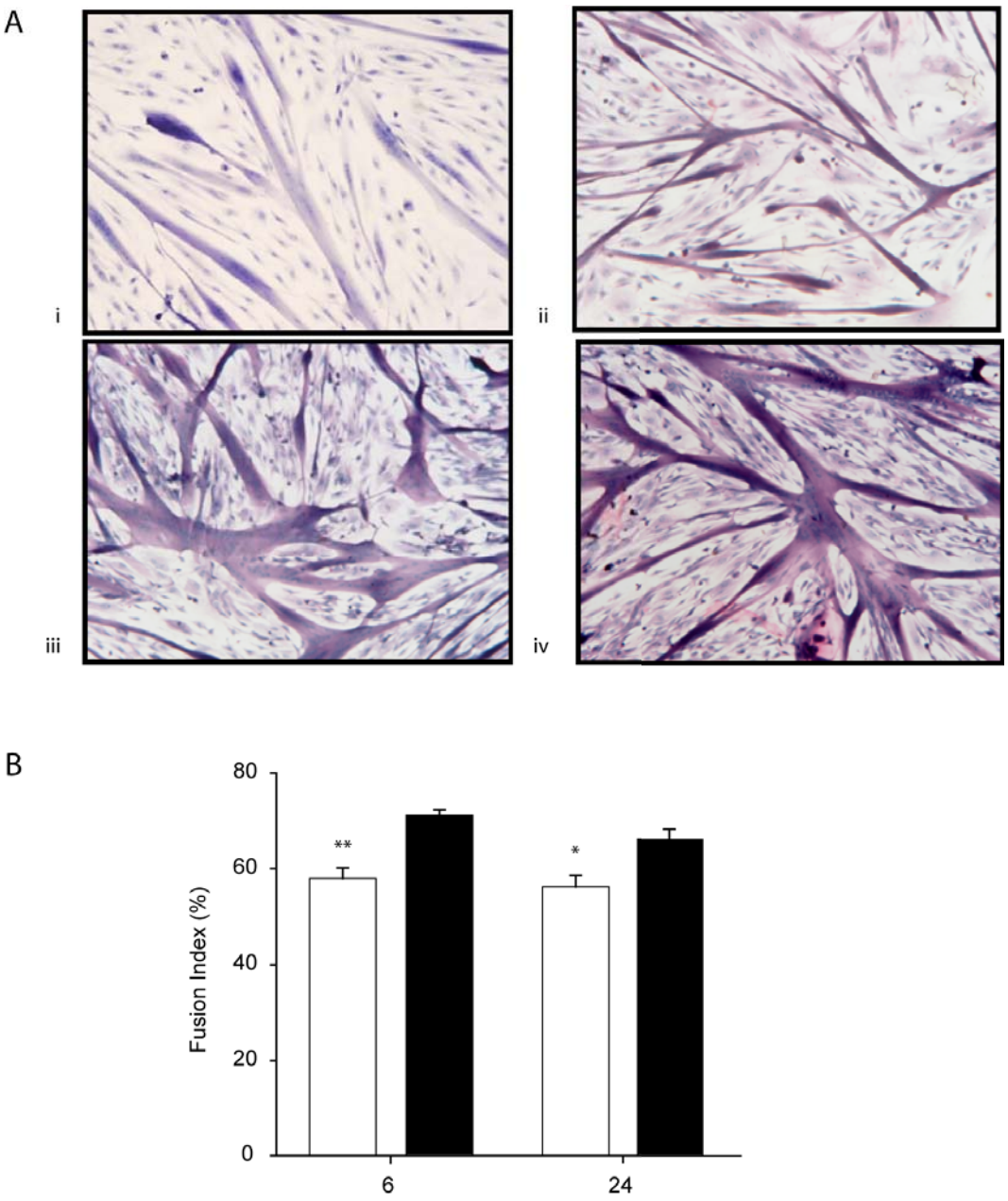

Figure 5.3 Increased differentiation is retained in* myostatin-*null myoblasts

(A) Examples of wild-type 6- (i) and 24- (ii) month, and *myostatin*-null 6- (iii) and 24- (iv) month H & E stained differentiating myotubes after 96 hr culturing. (B) The fusion indexes (number of myotube nuclei/total nuclei) of wild-type (empty bars) and *myostatin*-null (solid bars) differentiating myotubes was determined after 96 hr culturing. Myoblasts isolated from 6 mice per age group and genotype were plated in triplicate and 10 images per plate were obtained for analysis. ** = $P < 0.001$ when 6-month wild-type is compared to 6-month *myostatin*-null, * = $P < 0.05$ when 24-month wild-type is compared to 24-month *myostatin*-null. All data are expressed as mean ± SEM.

5.3.5 Myostatin levels are altered with age during differentiation

To determine whether aging would influence myostatin protein levels, Western analysis of differentiating myoblasts isolated from 6- and 24-month-old wild-type mice was performed over 72 hr of incubation (Figure 5.4A). The 6-month-old differentiating myoblasts displayed a consistent level of processed myostatin until 48 hr of differentiation, after which, the level decreased (Figure 5.4B). In contrast, the 24-month-old differentiating myoblasts displayed a low level of processed myostatin at 0 hr, relative to the 6-month-old myoblasts, and slowly increased until 48 hr.

5.3.6 MyoD & Pax7 are differentially expressed in myoblasts cultures

Activated satellite cells have been shown to co-express Pax7 with MyoD (Zammit *et al.,* 2004; Nagata *et al.,* 2006). However, later the cells may down-regulate Pax7 or MyoD depending on the myogenic fate of the cells. To assess the ratio of MyoD-only, Pax7-only, and MyoD/Pax7-positive cells within the primary myoblast cultures isolated from 1- and 24-month-old wild-type and *myostatin*-null mice, MyoD and Pax7 co-immunostaining was performed (Figure 5.4C). Although the percentage of MyoD/Pax7-positive cells was similar in the 1-month-old cultures, a greater percentage of MyoD-only cells were observed in the wild-type cultures (~16%) compared to the *myostatin*-null cultures (~6%) ($P < 0.001$). This corresponded to a greater percentage of Pax7-only cells observed in the *myostatin*-null cultures (~16%) compared to the wild-type cultures (~3%) ($P < 0.001$). With age, a significant decrease in MyoD-only cells (~13%) ($P < 0.05$) and a corresponding increase in Pax7-only cells (~19%) ($P < 0.001$) was observed in the 24-month-old wild-type cultures compared to the 1-month-old wild-type cultures. In contrast, the percentage of MyoD/Pax7 cells decreased (~18%) ($P < 0.05$), while the percentage of Pax7-only cells increased (~16%) ($P < 0.001$), in the 24-month-old *myostatin*-null cultures compared to the 1-month-old *myostatin*-null cultures.

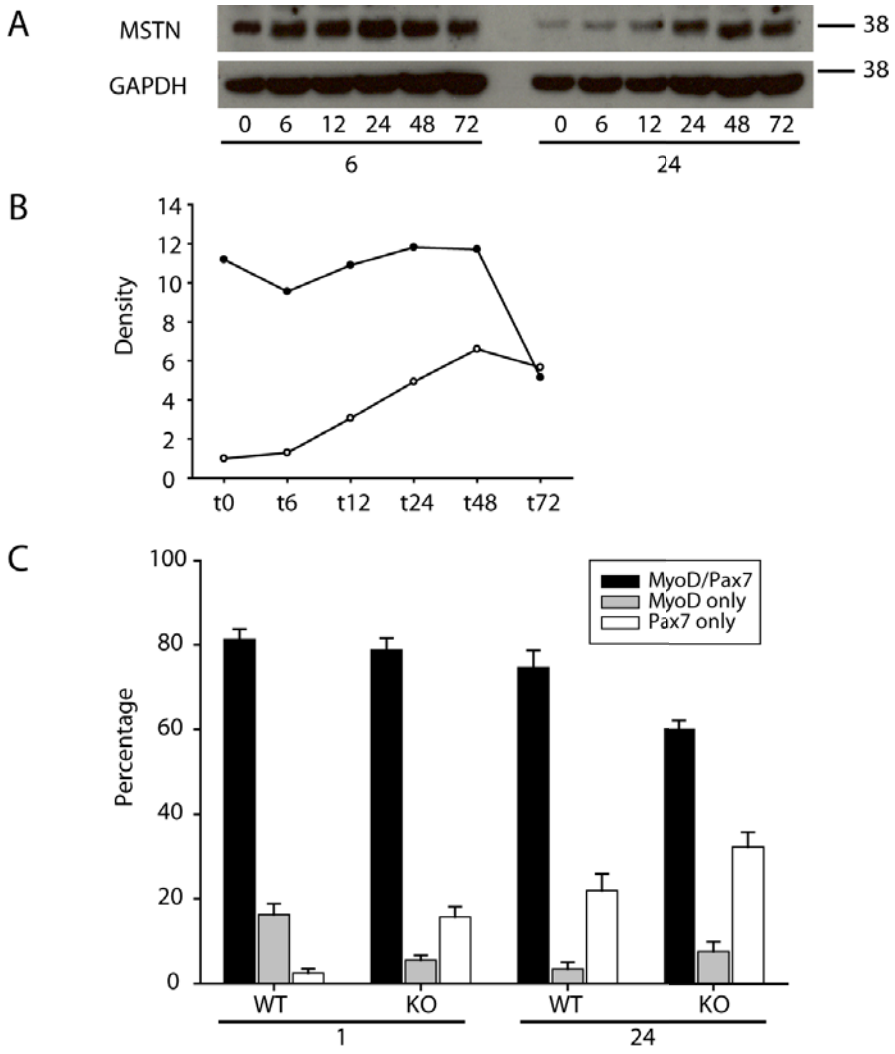

Figure 5.4 Myostatin levels and MyoD/Pax7 expression is altered with age

(A) Western blot analysis was performed on differentiating myoblasts isolated from 6- and 24-month-old wild-type mice to determine myostatin (MSTN) protein levels. The Western blot was analysed by densitometry and normalised to GAPDH protein levels. Normalised data were used to generate the graph shown (B) (solid dots = 6-month, empty dots = 24-month) (n = 6). (C) The percentage of myoblasts positive for MyoD/Pax7 (black bars), MyoD-only (grey bars), or Pax7-only (white bars) within 1- and 24-month-old wild-type and *myostatin*-null cultures was determined by MyoD and Pax7 co-immunostaining. Myoblasts were isolated from 3 mice of each age and genotype, cells were plated in triplicate, and a minimum of 1,000 cells per culture were counted. For MyoD/Pax7, $P < 0.001$ when 1-month compared to 24-month KO, $P < 0.05$ when 24-month WT compared to 24-month KO. For MyoD-only, $P < 0.001$ when 1-month WT is compared to 1-month KO, $P < 0.05$ when 1-month WT compared to 24-month WT. For Pax7-only, $P < 0.001$ when 1-month WT is compared to 1-month KO or 24-month WT, and when 1-month KO compared to 24-month KO. All data are expressed as mean ± SEM.

5.3.7 Epididymal fat is decreased in the *myostatin*-null mice

In addition to influencing myogenesis, myostatin is also known to alter adipogenesis, as decreased body fat is observed in *myostatin*-null mice (McPherron and Lee 2002). To investigate the effect of the presence or absence of myostatin on body fat in aged mice, epididymal fat pads from 1-, 6- and 24-month-old wild-type and *myostatin*-null mice were weighed (Figure 5.5A). Both genotypes indicated an increase of adipose tissue during development from 1- to 6-months of age. However, the increase observed in the wild-type mice was significantly greater than in the *myostatin*-null mice (1900% and 500% respectively, $P < 0.001$). The effect of further aging on the fat pad weights appeared minimal as no significant weight gain or loss was observed in either genotype. Visual assessment of H & E stained fat pad sections revealed a significant variation between the two genotypes in regards to the fat cell sizes (Figure 5.5B). Of note, the 1-month-old wild-type fat pad contained cells which were very irregular in size with the majority being very small. This differed considerably from the 6-month-old wild-type fat pad which contained large similar sized cells throughout the fat pad. In contrast to the 1-month-old wild-type fat pad, fat cells within the 1-month-old *myostatin*-null fat pads appeared very similar to that of the adult *myostatin*-null fat pads, though smaller in size. The average fat cell size from both wild-type and *myostatin*-null fat pads increased significantly from 1- to 6-months of age though the increase was substantially larger in the wild-type mice (1,000% and 100% respectively, $P < 0.001$) (Figure 5.5C). Although no significant change in cell size was observed in the *myostatin*-null mice between 6- and 24-months of age, the wild-type mice showed a small significant decrease (10%, $P < 0.05$).

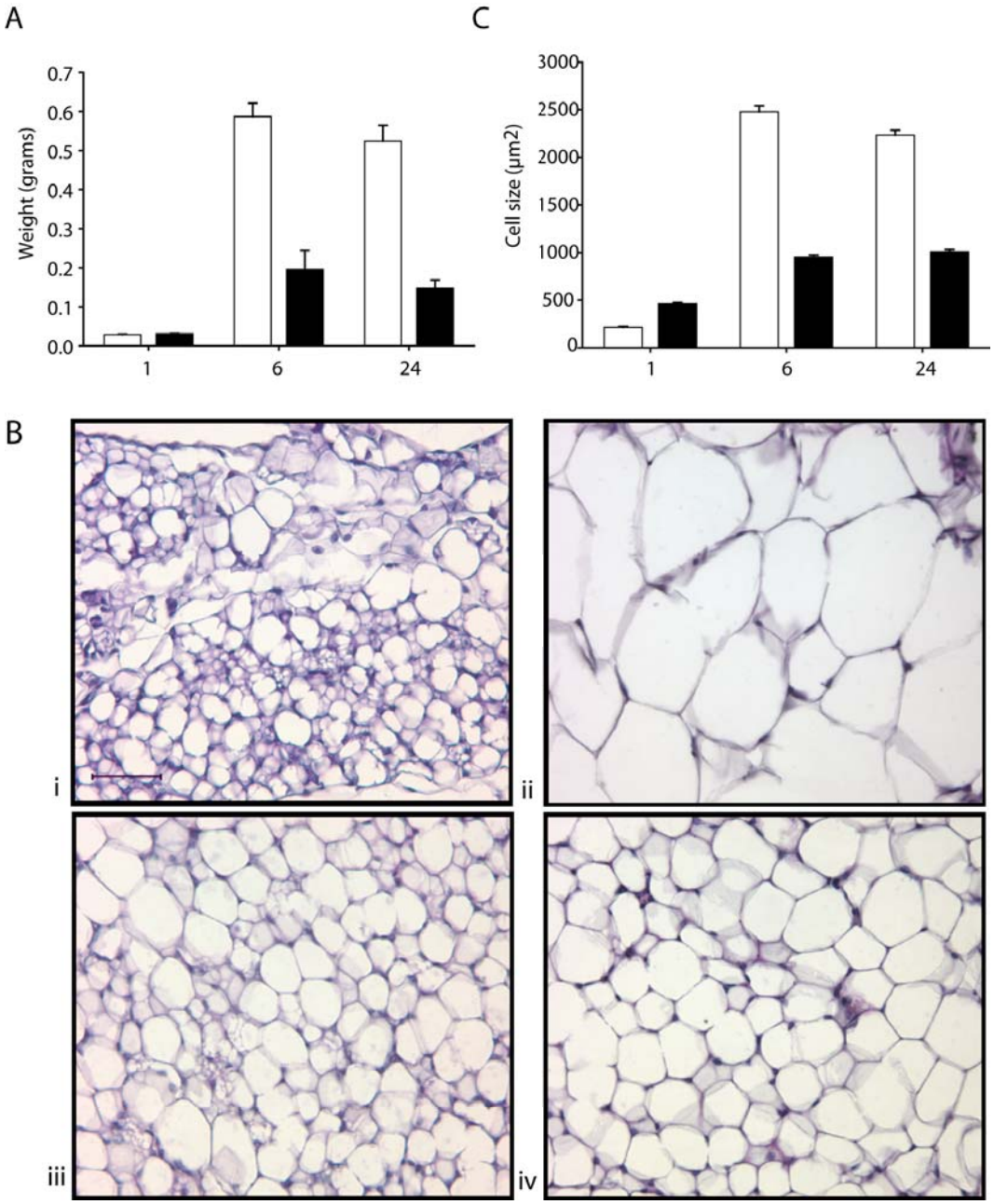

Figure 5.5* Myostatin-*null mice display a decreased fat accumulation

(A) Epididymal fat pads were measured in 1-, 6-, and 24-month-old wild-type (empty bars) and *myostatin*-null (solid bars) mice (n = 10). $P < 0.001$ when 1-month is compared to 6-month, and when 6- and 24-month wild-type is compared to 6- and 24-month *myostatin*-null. $P < 0.05$ when 6-month wild-type is compared to 24-month wild-type. (B) Examples of wild-type 1- (i) and 6- (ii) month, and *myostatin*-null 1- (iii) and 6- (iv) month H & E stained epididymal fat pads. (C) Average fat cell sizes within 1- and 24-month wild-type (empty bars) and *myostatin*-null (solid bars) fat pads are graphically shown (n =5). Approximately 2,000 cells were measured per age group and genotype. $P < 0.001$ when WT is compared to KO or when 1-month is compared to 6- and 24-month, $P < 0.05$ when 6-month WT is compared to 24-month WT. All data are expressed as mean ± SEM.

5.4 Discussion

Sarcopenia in elderly humans is known to contribute to frailty and falls, however the exact mechanism for the associated loss of muscle mass remains undetermined. Alternatively, it is well established that animals lacking functional myostatin have greater muscle mass (Grobet *et al.,* 1997; McPherron *et al.,* 1997; Zhu *et al.,* 2000). To address how a prolonged absence of myostatin would affect the normal aging process, the myogenic nature of aged wild-type and *myostatin*-null muscle was examined in mice. In agreement with previous studies, *myostatin*-null mice displayed significantly larger muscles compared to wild-type mice (Figure 5.1A), a difference retained in the aged mice. The greater muscle weights in the 1-month *myostatin*-null muscles corresponded to increased MyoD protein levels (Figure 5.1C), however higher MyoD levels were observed in the 6-month wild-type muscle compared to the age-matched *myostatin*-null muscle. This may suggest a lengthier period of myogenesis in the wild-type muscle due to a slower rate of myogenesis relative to the *myostatin*-null muscle. It is possibly that in young *myostatin*-null mice, the greater MyoD protein level reported here, and the increased satellite cell number and activation previously described (McCroskery *et al.,* 2003) leads to the establishment of the upper muscle mass limit earlier than the wild-type muscle. Conversely, an increased level of MyoD in the wild-type muscle at 6-months may indicate an earlier onset of age-related muscle wasting and associated myogenesis. Of note, a similar level of MyoD was observed in the 24-month wild-type and *myostatin*-null muscle, however, the level was significantly greater than at 1-month. As aging is known to impair satellite cell activation (Conboy *et al.,* 2003; Gallegly *et al.,* 2004), it is possible that an increased level of MyoD occurs in response to the poor satellite cell activation potential in the aged animals. Regardless, the similar MyoD level in the 24-month wild-type and *myostatin*-null muscles suggests a greater level of MyoD is not required to maintain the increased mass muscle observed in the *myostatin*-null muscle. It is possible that the increased muscle mass established in the young mice could be of more significance. Furthermore, the greater proliferation rate (Figure 5.2B) and differentiation fusion index (Figure 5.3B) observed in the *myostatin*-null myoblast cultures would theoretically equate to more efficient myogenesis in the aged *myostatin*-null mice.

Quiescent satellite cells have been shown to express Pax7 (Seale *et al.*, 2000), however, when they become activated, they co-express Pax7 with MyoD (Zammit *et al.,* 2004; Nagata *et al.,* 2006). Cells may down-regulate Pax7 and progress along the myogenic pathway, or maintain Pax7 and down-regulate MyoD, thereby withdrawing from the cell cycle and returning to a quiescence-like state (Zammit *et al.,* 2004; Nagata *et al.,* 2006). MyoD and Pax7 co-stained wild-type and *myostatin*-null myoblast cultures indicated a higher percentage of Pax7-only cells in the 1-month *myostatin*-null cultures compared to the 1-month wild-type cultures (Figure 5.4C). This would suggest a higher proportion of *myostatin*-null cells returning to a quiescence-like state, thus resulting in a larger satellite cell pool via increased self-renewal. The greater proportion of Pax7-only cells in the *myostatin*-null cultures appears to be at the expense of MyoD-only cells suggesting a decreased number of cells progress along the myogenic pathway. However, theoretically this would be compensated by the increased proliferation rate reported to occur in the *myostatin*-null cultures (McCroskery *et al.,* 2003), thus resulting in an increased number of myoblasts destined to terminally differentiate. Interestingly, the proportion of Pax7-only cells increased in both the wild-type and *myostatin*-null 24-month myoblast cultures. This may suggest that a higher proportion of aged myoblasts return to a quiescence-like state. Alternatively, it may be due to the decreased activation potential observed in aged satellite cells, which would result in a lower percentage of MyoD-positive cells. Interestingly, 24-month-old wild-type differentiating myoblasts displayed significantly reduced levels of processed myostatin. It is possible that due to aged myoblasts becoming refractory to proliferation and differentiation, a reduced level of myostatin is processed as a compensatory mechanism.

An alteration in adipogenesis is another characteristic of the aging process. Fat deposits are thought to increase until middle to early old age followed by a decline with increasing age. Fat cells are continually formed (Bertrand *et al.,* 1978; Miller *et al.,* 1984), though the overall number of cells may change throughout the lifespan depending on the rate of gain and loss of fat cells (Kirkland and Dax 1984; Silver *et al.,* 1993; Kirkland and Dobson 1997). Myostatin also influences adipogenesis as *myostatin*-null mice have demonstrated a significant decrease in fat accumulation compared to wild-type mice (Lin *et al.,* 2002; McPherron and Lee 2002). In agreement with this finding, results reported

here indicated that the increase in fat pad weight from 1- to 6-months of age was significantly greater in wild-type mice compared to *myostatin*-null mice (Figure 5.5A). Further aging appeared to have a minimal effect on wild-type and *myostatin*-null fat pad weights. Interestingly, fat cells within the 1-month-old *myostatin*-null fat pad were significantly larger than wild-type cells of the same age (Figure 5.5C), suggesting greater fat accumulation at this age. The average fat cell size increased significantly with age, however, the increase was substantially smaller in the *myostatin*-null mice indicating a reduced fat accumulation with increasing age. Further aging appeared to have little effect on the *myostatin*-null fat pad in regards to fat cell size, though an expected reduction was observed in the wild-type mice. Although the exact mechanism by which myostatin regulates fat metabolism is yet to be elucidated (and whether it is a direct or indirect effect of myostatin), it is intriguing how *myostatin*-null mice display a consistently low level of fat accumulation even in the aged mice.

Collectively our findings show that a prolonged absence of myostatin results in the retention of muscle hypertrophy even in aged animals. *In vitro* results indicate this is brought about through increased myoblast proliferation and differentiation.

5.5 Acknowledgements

I would like to acknowledge the contributions from the following co-authors. Alex Hennebry for his assistance with the myoblast isolations, Carole Berry for performing the Western blots, and Dr Ravi Kambadur and Dr Mridula Sharma for their supervision during experimental procedures and the preparation of the resulting manuscript.

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Chapter Six

Antagonism of myostatin enhances muscle regeneration during sarcopenia

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Antagonism of Myostatin Enhances Muscle Regeneration during Sarcopenia

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6.1 Introduction

Mammalian skeletal muscle mass is dependent on numerous factors including muscle fibre number, type and size. All three aspects are believed to be influenced by a variety of conditions, including aging, which can lead to atrophy of the individual muscle fibres, loss of fibre numbers and fibre type switches (Gutmann and Hanzlikova 1966; Rowe 1969; Alnaqeeb and Goldspink 1987; Lexell *et al.,* 1988; Holloszy *et al.,* 1991). A prominent influence on muscle fibre size is the involvement of satellite cells, a population of myogenic precursor cells associated with the muscle fibres. Satellite cells are located between the basal lamina and sarcolemma (Mauro 1961) and exist predominantly in a mitotically quiescent state (Schultz *et al.,* 1978). These cells are believed to be largely responsible for muscle regeneration (Partridge 2002) by giving rise to myoblasts, thereby enabling additional nuclei to fuse to existing fibres or form new myofibres (Bischoff 1994; Schultz and McCormick 1994). For the progression of this process, satellite cells must enter the cell cycle from the quiescent state and

undergo myogenesis. Clearly, the number of satellite cells as well as their proficiency to undergo this process would impact the degree to which muscle regeneration can occur. One factor thought to severely impact satellite cell behavior is aging. Satellite cells isolated from aged animals have demonstrated a significant lag when entering the cell cycle (Johnson and Allen 1995), a decreased proliferation and differentiation potential (Schultz and Lipton 1982; Lees *et al.,* 2006), and an increased susceptibility to apoptosis (Jejurikar *et al.,* 2006; Krajnak *et al.,* 2006). Additionally, various studies indicate that aged muscle is restricted in its ability to promote satellite cell activation (Decary *et al.,* 1997; Bockhold *et al.,* 1998; Conboy *et al.,* 2003). Using cross-age muscle transplantation it has been demonstrated that the poor regeneration associated with old animals is a function of the aged environment (Carlson and Faulkner 1989). In addition, it has also been suggested that insufficient up-regulation of Notch signaling is directly responsible for the impaired activation propensity of the aged satellite cells (Conboy *et al.,* 2003). More recently, parabiotic pairing experiments have indicated that systemic factors from young mice restored the activation of Notch signaling as well as promoted the proliferation and regenerative capacity of satellite cells in aged mice (Conboy *et al.,* 2005). Alternatively, negative regulators, such as myostatin, may inhibit satellite cell activity in the aged environment.

Myostatin is a TGF-β superfamily member functioning as a potent inhibitor of muscle growth (McPherron *et al.,* 1997). It is expressed both prenatally in the developing myotome and post-natally in adult skeletal muscle (McPherron *et al.,* 1997) suggesting myostatin plays a continual role in myogenesis. Indeed, *myostatin*-null animals display significantly greater muscle mass resulting from muscle fibre hyperplasia and hypertrophy (McPherron *et al.,* 1997). Studies indicate that myostatin influences myogenesis in C2C12 myoblast cultures through the regulation of cell cycle progression and MRFs (Thomas *et al.,* 2000; Langley *et al.,* 2002). Moreover, myostatin has been shown to inhibit satellite cell activation in mice (McCroskery *et al.,* 2003), which would have significant consequences during age-related muscle wasting and regeneration. Interestingly, increased myostatin has been correlated with muscle atrophy during unloading in mice (Carlson *et al.,* 1999), muscle wasting in HIV patients (Gonzalez-Cadavid *et al.,* 1998) and cachexia induced by systemic administration of myostatin (Zimmers *et al.,* 2002). Although the exact role of myostatin during

age-related muscle wasting remains unclear, a prolonged absence from prenatal stage as seen in *myostatin*-null mice, has demonstrated an overall reduction in sarcopenia (Wagner *et al.,* 2005). This being the case, the antagonism of myostatin has serious therapeutic potential in the alleviation of sarcopenia and impaired muscle regeneration observed in aged animals. In fact, we report here that a short term blockade of myostatin function through the administration of a truncated protein was able to significantly enhance muscle regeneration in aged mice. This regeneration occurred via a restoration of the myogenic and inflammatory responses in the aged mice, leading to increased satellite cell activity and enhanced macrophage and myoblast migration.

6.2 Materials and Methods

6.2.1 Generation of Mstn-ant1 and testing during C2C12 proliferation

An *E. coli* expression system was used to produce the truncated myostatin protein. Amplified cDNA, from the carboxyl-terminal region of myostatin, was purified and subsequently inserted into the cloning vector pET 16-B (Novagen) as previously described (Sharma *et al.,* 1999). For the generation of Mstn-ant1, the cDNA was truncated at the amino acid 350 to produce a truncated portion of the processed region. The protein was purified utilising a Ni-NTA agarose (Qiagen) affinity column (Sharma *et al.,* 1999). In order to test the purity of the antagonist, 3 µg was separated on a NuPAGE™ 4–12% Bis-Tris gel (Invitrogen), stained with Coomassie blue then destained. C2C12 myoblasts were grown (Thomas *et al.,* 2000) in uncoated 96-well microtitre plates (Nunc) at 1000 cells/well. Following a 16 hr attachment period, test media containing Mstn-ant1 was added to the actively growing cells before incubating for a further 48 or 72 hr. Cell proliferation was later assessed using a methylene blue photometric endpoint assay as previously described (Oliver *et al.,* 1989).

6.2.2 Animals

The wild-type mouse strain C57BL/10 was bred at the Ruakura Small Animal Colony. All animals were handled in accordance with the guidelines of the Ruakura Animal Ethics Committee (AgResearch, Hamilton, New Zealand) and the University of Waikato Animal Ethics Committee (University of Waikato, Hamilton, New Zealand).

6.2.3 Administration of Mstn-ant1 to notexin injured and aged mice

On day 0, 1-year-old mice were anaesthetised with 10% ketamine hydrochloride (100 mg/ml)/5% Rompun (20 mg/ml) at 0.1 ml/7 g body weight. A small incision was made over the left TA before 10 µl of notexin (10 µg/ml; Venom Supplies) was injected into the muscle. On days 1, 3 5 and 7, the antagonist treatment group received Mstn-ant1 at 6 µg/g body weight subcutaneously, while the control group received the equivalent volume of saline. Mice were euthanized on days 1, 2, 3, 7, 10 and 28 (4-6 mice per group per day) for tissue collection.

Additionally, mice aged 13- to 16-months were injected subcutaneously 3 times a week with Mstn-ant1 at 6 μg/g body weight, or the equivalent volume of saline, for 6 weeks (10 per group). TA and Gas muscles were collected for single fibre isolations and muscle protein respectively, while the remaining hind-limb muscle was collected for myoblast isolation. Bone marrow was also collected for macrophage cultures used in the chemotaxis assays.

6.2.4 Assessment of muscle regeneration and strength

TA muscles were dissected from each notexin injured mouse, weighed and frozen for protein isolation or tissue sectioning. Transverse sections (10 μm) were cut from the mid-belly region of each muscle. Muscle sections were stained with H and E to visualise and measure unregenerated and regenerated areas. To visualise areas of collagen deposited 10 and 28 days after injury, muscle sections were stained with Van Gieson. The amount of collagen was then measured within each section as a percentage of the total section area. Day-2, -3, -7 and -10 muscles sections were immunostained for Mac1, an antibody specific for infiltrating peripheral macrophages, as previously described (McCroskery *et al.*, 2005). Muscle strength was measured in a double-blind fashion in the aged mice injected with saline or Mstn-ant1 for 6 weeks at the commencement and completion of the trial using a grip strength apparatus measuring fore- and hind-limb strength) (MK-380S, Muromachi). The mice were pulled backward by the tail while the maximal force exerted by the mouse was digitally recorded. The mean of three grip tests for each animal was calculated and the mean maximal force of 10 animals per group was determined and expressed in Newtons as mean ± SEM.

6.2.5 Single fibre isolation and analysis of satellite cell activation

TA muscles collected from the Mstn-ant1 or saline injected mice were used to isolate single fibres following an adapted protocol of Rosenblatt *et al.* (1995) previously described (refer to Section 2.2.23.3). Isolated fibres were then cultured for 24 or 48 hr at 37ºC in 5% CO_2. In addition, single fibres were isolated from non-treated 1- and 24-month-old mice. These fibres were cultured with or without Mstn-ant1 at 5 μg/ml for 24, 48, or 72 hr at 37ºC in 5% CO_2. After the required culturing time, all fibres were fixed with 100% methanol, washed in

PBS, permeablised in 0.5% TritonX-100 in PBS for 10 min and blocked in 10% NGS and 0.35% cλ in PBS for 1 hr at room temperature. Mouse anti-PCNA antibody (Dako) at 1:100 in 5% NGS and 0.35% cλ in PBS was then added overnight. The primary antibody was detected using Alexa Fluor 546 goat anti-mouse IgG conjugated secondary (Molecular Probes) at 1:300 in 5% NGS and 0.35% cλ in PBS for 1 hr at room temperature. Fibres were counterstained with DAPI (Molecular Probes) before mounting with fluorescent mounting media (Dako) and viewing under fluorescent illumination. PCNA-positive nuclei were counted as a percentage of total myonuclei in order to normalise the data and exclude any discrepancies in the lengths of the fibres analysed.

6.2.6 Isolation of primary myoblasts and macrophages

Satellite cells were isolated from the hind-limb muscles from the Mstn-ant1 or saline injected mice according to published protocols (Partridge 1997; Yablonka-Reuveni *et al.,* 1999; McCroskery *et al.,* 2005). After 48 hr of culturing, the pooled cells were collected for protein isolation. In addition, primary myoblasts were isolated from non-treated 1- and 24-month-old mice for proliferation and chemotaxis assays. For a proliferation assay, the myoblasts were cultured with or without Mstn-ant1 at 10 μg/ml for 96 hr. Bone marrow derived macrophages were obtained by plating bone marrow cells at $5x10^6$ cells/plate in DMEM with 10% FBS and 10% L929 conditioned medium (containing CSF-1) for 5 days to induce macrophage differentiation (Suresh and Sodhi 1991). Peritoneal macrophages were obtained by lavage of the peritoneal cavity of 1- and 24-month-old non-treated mice with cold PBS.

6.2.7 Myoblast and macrophage chemotaxis assays

Chemotaxis assays were performed using cell culture inserts, containing PET 0.8 μm membranes (BD Biosciences) as previously described (McCroskery *et al.,* 2005). For macrophage chemotaxis, DMEM with Zymosan A (Sigma) activated mouse serum (ZAMS) was used as a chemoattractant at 33, 22 and 11%. For myoblast chemotaxis, DMEM with 2% HS and 5% CEE (optimal), DMEM with 2% HS (suboptimal) or DMEM with 5% CEE (suboptimal) were used as chemoattractants. Rescue experiments were performed with either 2.5 or 5 μg/ml of recombinant myostatin in the presence or absence of Mstn-ant1 at 5x the

myostatin concentration. The cells were incubated for 4 or 7 hr for macrophages and myoblasts respectively. Migrated cells were counted on four representative fields per membrane and the average number plotted. All assays were performed in duplicate.

6.2.8 Protein analysis

TA muscles from notexin injured mice were homogenised in 1 ml PLB (0.05 M Tris pH 7.5, 0.25 M NaCl, 5 mM EDTA, Complete™ protease inhibitor tablet (Roche) and 0.1% NP40). Bradford's reagent (Bio-Rad) was used to estimate total protein. Total protein (10 µg) was separated on NuPAGE™ 4-12% Bis-Tris gels (Invitrogen) and transferred to a nitrocellulose membrane (Bio-Rad). Membranes were stained in Ponceau S to visually assess equal protein loading. For analysis of MyoD, membranes were blocked in BSA blocker (0.3% BSA, 1% PEG, and 1% PVP in TBS-T) for 1 hr at room temperature then incubated with mouse anti-MyoD (BD PharMingen) at 1:10,000 overnight at 4°C. For analysis of Pax7, membranes were blocked in 5% milk in TBS-T overnight at 4°C, and then incubated with mouse anti-Pax7 antibody (ascites fluid obtained from the Developmental Studies Hybridoma Bank) at 1:500 for 3 hr at room temperature. The membranes were then incubated with goat anti-mouse IgG HRP conjugate (Dako) at 1:5,000 (MyoD) or 1:2,000 (Pax7) for 1 hr at room temperature. HRP activity was detected using Western Lightning™ Chemiluminescence Reagent Plus (PerkinElmer).

In addition, protein was collected from the primary myoblasts (McCroskery *et al.,* 2003) and Gas muscle isolated from the aged mice injected with saline or Mstn-ant1 for 6 weeks. Total protein (15 µg) was used for Western blot analyses of MyoD and Pax7.

6.2.9 Image and statistical analysis

Unregenerated/regenerated areas and collagen deposition was analysed using an Olympus SZ-PT stereomicroscope (Olympus), a 3 CCD camera (Dage-MTI) and Scion Image software (Scion). The obtained images were then measured using ImageJ software (NIH). Positive Mac1 staining was viewed under fluorescent illumination using an Olympus BX50 microscope (Olympus), SPOT-RT 4.01 camera and software (Diagnostic Instruments Inc.), and images of each

section were obtained for analysis. The number of Mac1-positive cells was counted per unit area from four images per muscle section. All data are presented as means ± SEM. ANOVA with Tukey test was used to determine the significance ($P < 0.05$) of data between groups.

6.3 Results

6.3.1 Antagonist production and biological activity

Previously we have shown that a prolonged absence of myostatin reduced sarcopenia in *myostatin*-null mice (refer to Chapter 4). We elected to test whether a specific molecule, a truncated version of myostatin, could antagonise myostatin and potentially offer a therapeutic option for treating sarcopenia. Myostatin protein is proteolytically processed at amino acid 266, thereby giving rise to the biologically active mature myostatin (McPherron *et al.,* 1997) which spans from amino acid 266-375. This 110 amino acid protein binds to Activin type IIB receptor as a dimer to transduce signalling (Lee and McPherron 2001). To antagonise myostatin, we produced a carboxyl-terminal truncation, spanning from amino acid 266 to 350, as a dominant negative protein. This carboxyl-terminal truncation, named Mstn-ant1, along with its amino acid length, is illustrated in Figure 6.1A. The Mstn-ant1 protein was expressed in an *E. coli* expression system and purified to homogeneity (Figure 6.1B). The biological activity of the antagonist Mstn-ant1 was determined by a C2C12 myoblast proliferation assay. The results indicated that Mstn-ant1 was able to significantly increase the proliferation rate of the treated myoblasts above the control myoblast level after 48 (Figure 6.1C) and 72 (data not shown) hr in culture ($P < 0.05$).

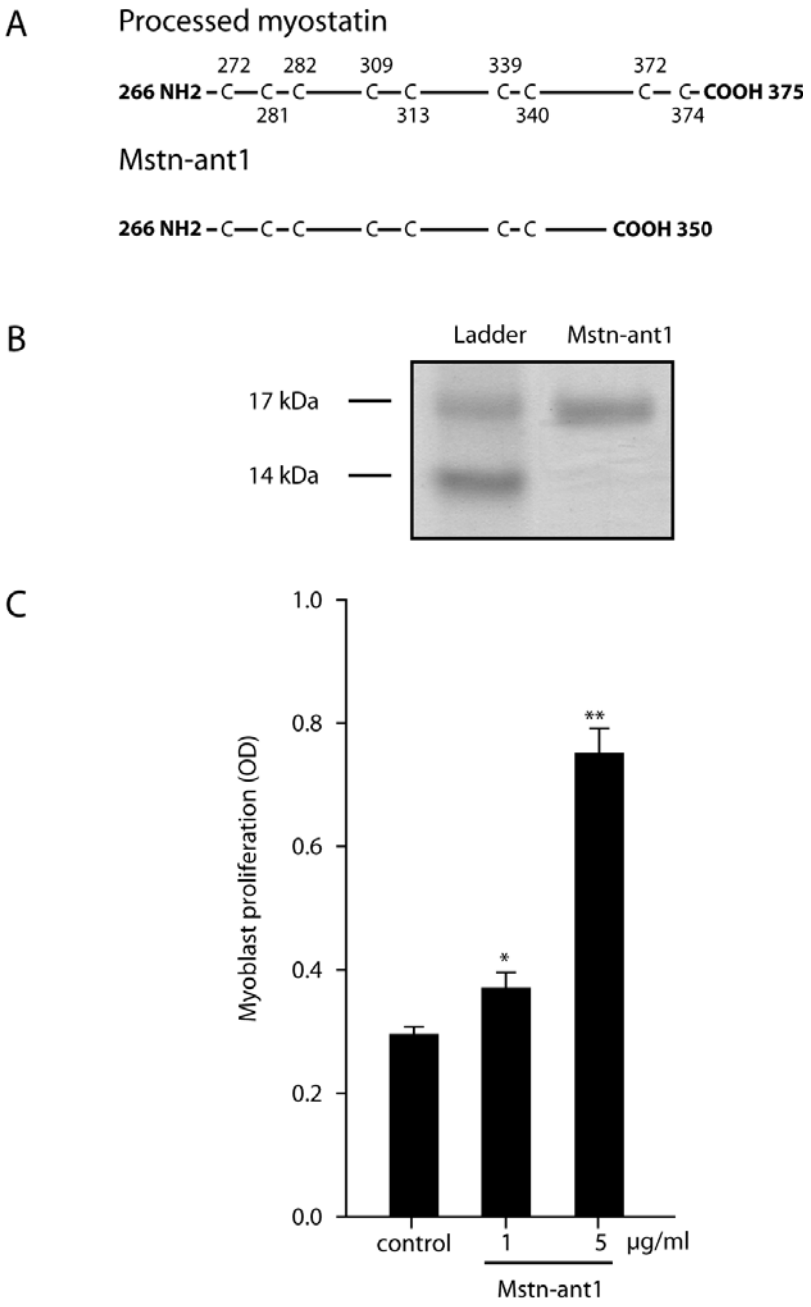

Figure 6.1 Structural representation and biological activity of Mstn-ant1

(A) Mstn-ant1 and processed myostatin are depicted, illustrating the length of the truncated protein (C = cysteine, numbers = amino acid number). (B) The Mstn-ant1 protein was separated on a SDS gel showing as a 17 kDa molecule, and later screened for its biological activity in a proliferating C2C12 cell culture. (C) C2C12 myoblasts were cultured with or without 1 µg/ml (1) or 5 µg/ml (5) of Mstn-ant1 for 48 hr. ** = $P < 0.001$ and * = $P < 0.05$ when control compared to antagonist treated myoblasts. Data are expressed as mean ± SEM.

6.3.2 Mstn-ant-1 enhances muscle regeneration following injury

Next we tested the utility of Mstn-ant1 in a regenerating model in mice. Typically after a notexin type injury, the injured muscle initially increases in weight due to a resulting oedema, followed by a decrease due to necrosis of the damaged muscle fibres which are cleared from the site of injury. The muscle weight then begins to increase again as regeneration of the fibres occur. The results indicated a trend in which the loss in muscle weight was less pronounced in the Mstn-ant1 treated muscles compared to the saline treated muscles at days 7 and 10 (Figure 6.2A). It is possible that the observed trend was due to a decreased muscle loss during the necrosis period, or conversely, due to advancement in new fibre formation resulting from the *in vivo* antagonism of myostatin by the Mstn-ant1 treatment. In support of this, analysis of the TA muscle sections indicated earlier nascent muscle fibre formation (regenerated areas) and an associated reduction in necrotic areas (unregenerated areas) in the muscles treated with Mstn-ant1 compared to saline treated muscles at days 7 and 10 (Figure 6.2B-C) ($P < 0.05$).

Since a measure of fibre areas can indicate the progression of regeneration occurring within a recovering muscle, individual fibre areas were measured to assess fibre regeneration 28 days after injury. Results indicated that the regenerated fibres from Mstn-ant1 treated mice were significantly larger than the saline treated muscles (Figure 6.3B) ($P < 0.05$).

Furthermore, following notexin injury, collagen deposits can be observed within the regenerated muscle (McCroskery *et al.,* 2005). Van Gieson staining of the saline and Mstn-ant1 treated muscles (Figure 6.3A) indicated reduced levels of collagen in the Mstn-ant1 treated muscles as compared to saline treated muscles at days 10 and 28 (Figure 6.3C) ($P < 0.05$).

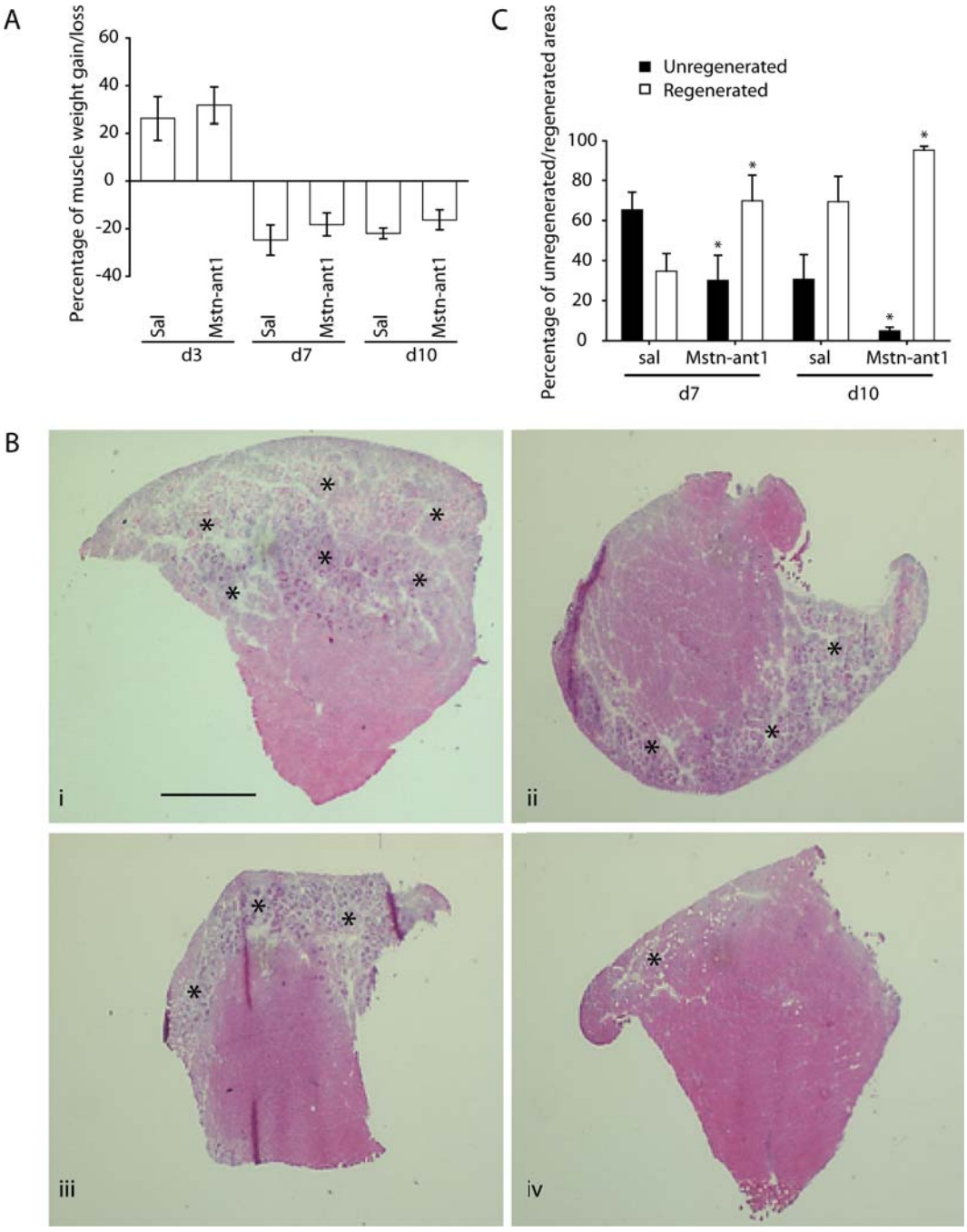

Figure 6.2 Antagonism of myostatin enhances muscle regeneration

(A) After notexin injury and treatment with Mstn-ant1, the percentage of muscle weight gain or loss from saline (Sal) and Mstn-ant1 treated muscles was analysed on days 3, 7 & 10 (d3, 7 & 10) (n = 4). (B) Sections were cut from each TA muscle and stained with H & E to visually determine the extent of muscle injury and regeneration in day-7 saline (i) and Mstn-ant1 (ii) and day-10 saline (iii) and Mstn-ant1 (iv) muscles (n = 4, * denotes unregenerated areas) (scale bar, 1 mm). (C) Using the H & E stained sections, the percentage of unregenerated and regenerated areas within each muscle were measured (n = 4). * = $P < 0.05$ when Sal compared to Mstn-ant1 treated. All data are expressed as mean ± SEM.

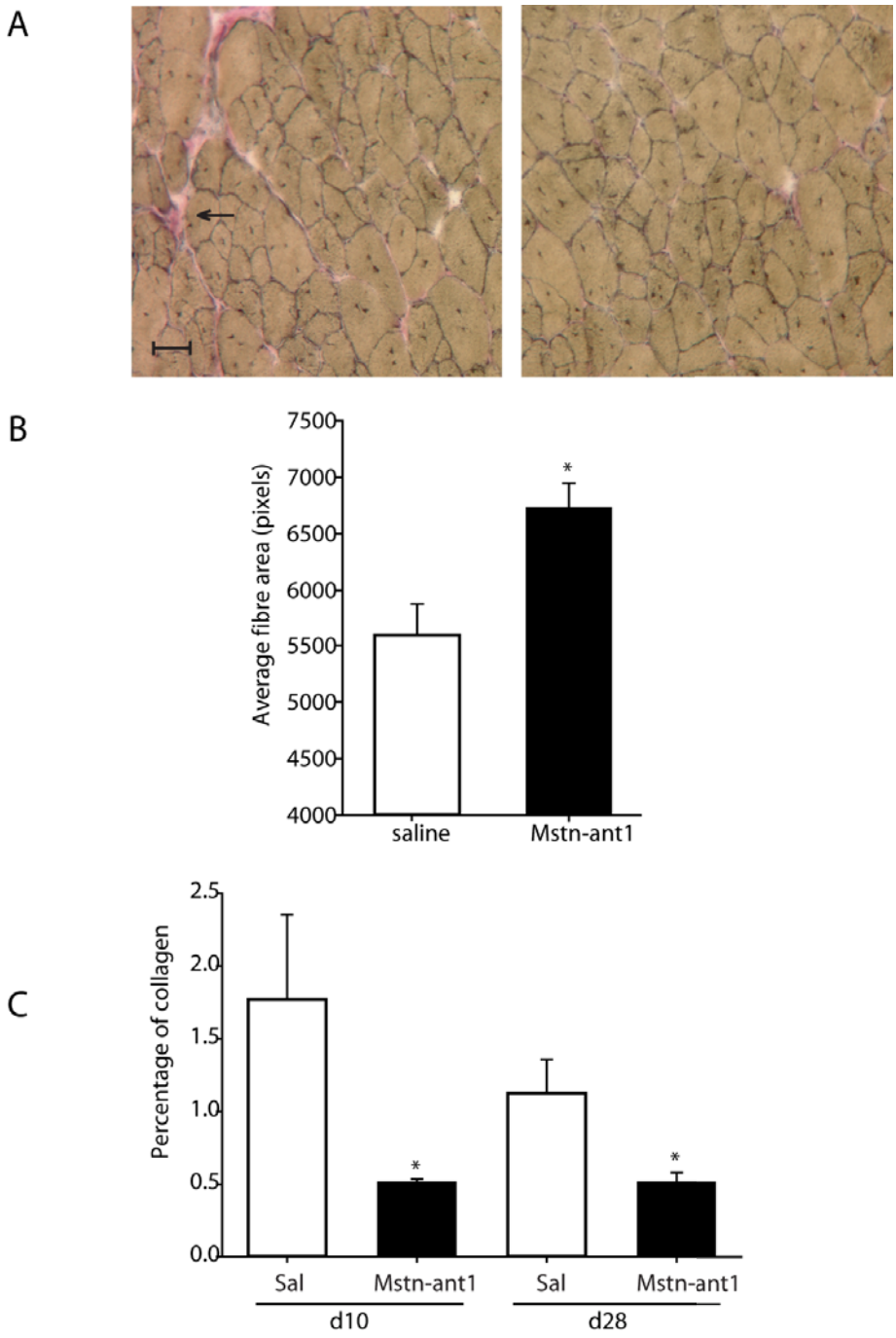

Figure 6.3 Mstn-ant1 increases fibre area and decreases collagen deposition

(A) Representative images of Van Gieson stained saline (i) and Mstn-ant1 (ii) treated muscle sections are shown (scale bar = 20 μm). Collagen is identified by pink staining (indicated by arrow). Individual regenerated fibre areas (B) as well as the percentage of collagen within each section (C) was measured in the saline (Sal) and Mstn-ant1 treated day-28 muscles using the Van Gieson stained sections (n = 4). *P < 0.05 when Sal is compared to Mstn-ant1 treated. All data are expressed as mean ± SEM.

6.3.3 MyoD and Pax7 levels are altered by Mstn-ant1 treatment

Both Pax7 and MyoD have been established as potent markers of myogenesis. While Pax7 levels can signify the satellite cell pool as well as satellite cell self-renewal (Seale *et al.,* 2000; Oustanina *et al.,* 2004), MyoD can signify the level of myogenesis occurring within a muscle (Grounds *et al.,* 1992). To investigate Pax7 and MyoD protein levels in the notexin injured muscles, Western blot analyses were performed. Analysis of the saline and Mstn-ant1 treated muscles indicated that Pax7 protein levels were higher with Mstn-ant1 treatment at days 3, 7, 10, and 28 (Figure 6.4A-B) ($P < 0.05$). Similarly, MyoD levels were also higher with Mstn-ant1 treatment compared to the saline treated muscles at days 3, 7, and 10 (Figure 6.4A,C) ($P < 0.05$). Comparable levels of MyoD were seen between the treatment groups at day 28, in contrast to the greater level of Pax7 observed at the same time point with the administration of Mstn-ant1.

Similar to a notexin injury, though to a lesser extent, aged muscle also displays a level of ongoing myogenesis due to muscle maintenance and repair in association with the aging process. Therefore, Pax7 and MyoD were also analysed in the muscles isolated from the aged mice treated for 6 weeks with saline, or Mstn-ant1. Protein isolated from Gas muscle at the completion of the trial indicated significantly higher Pax7 and MyoD protein levels in the Mstn-ant1 treated mice compared to the saline treated mice (Figure 6.5A,C,E) ($P < 0.05$). In addition, protein isolated from actively growing primary myoblasts also displayed increased levels of both Pax7 and MyoD (Figure 6.5B,D,F) with Mstn-ant1 treatment.

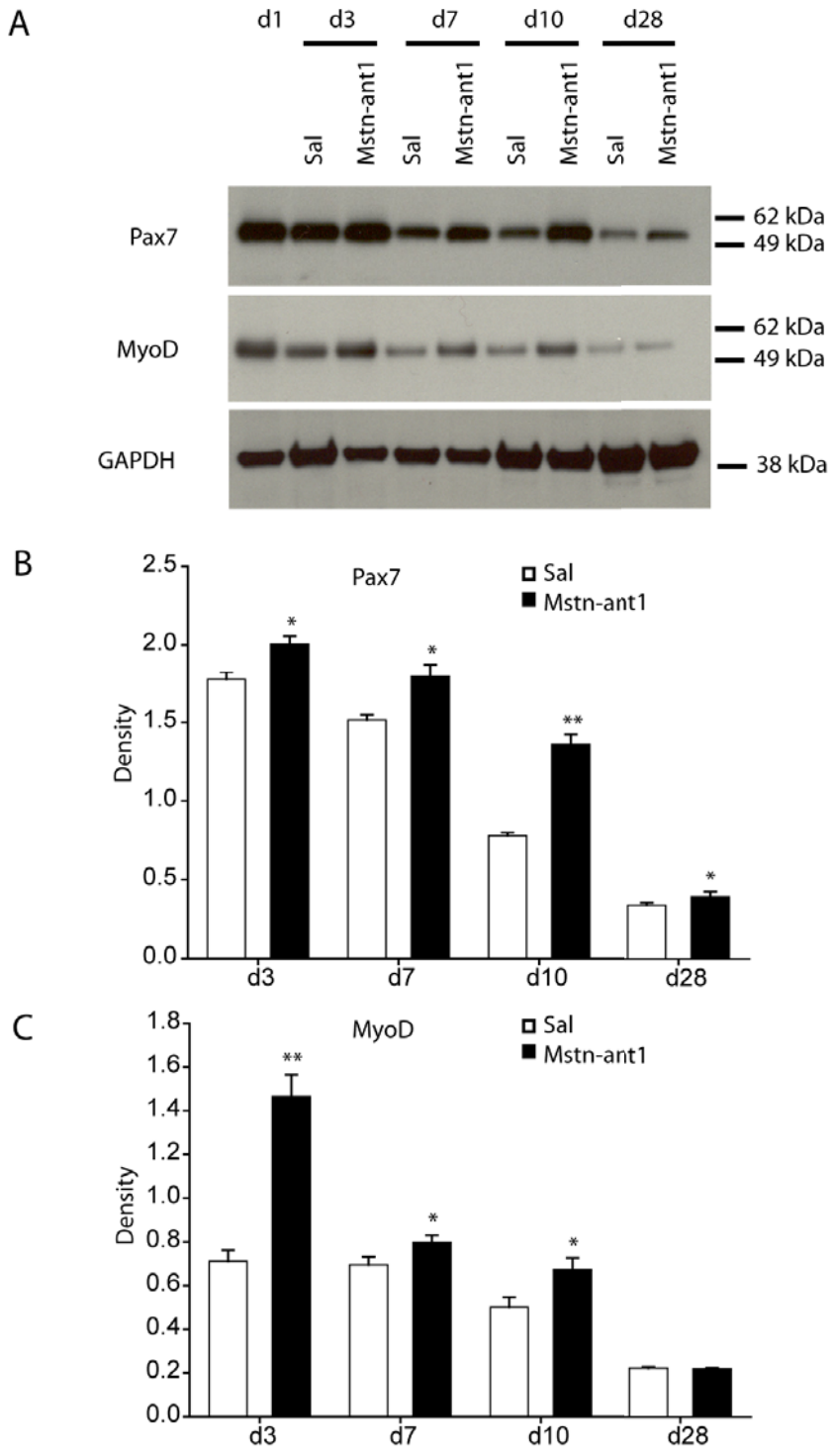

Figure 6.4 MyoD & Pax7 are increased by Mstn-ant1 treatment after injury

(A) Western blot analysis was performed on days 1, 3, 7, 10 & 28 saline (Sal) and Mstn-ant1 treated notexin injured muscles to determine Pax7 and MyoD protein levels. Each blot was analysed by densitometry and normalised to GAPDH protein levels. Normalised data were used to generate the Pax7 (B) and MyoD (C) graphs depicted (n = 4). ** = $P < 0.001$, * = $P < 0.05$ when Sal compared to Mstn-ant1. All data are expressed as mean ± SEM.

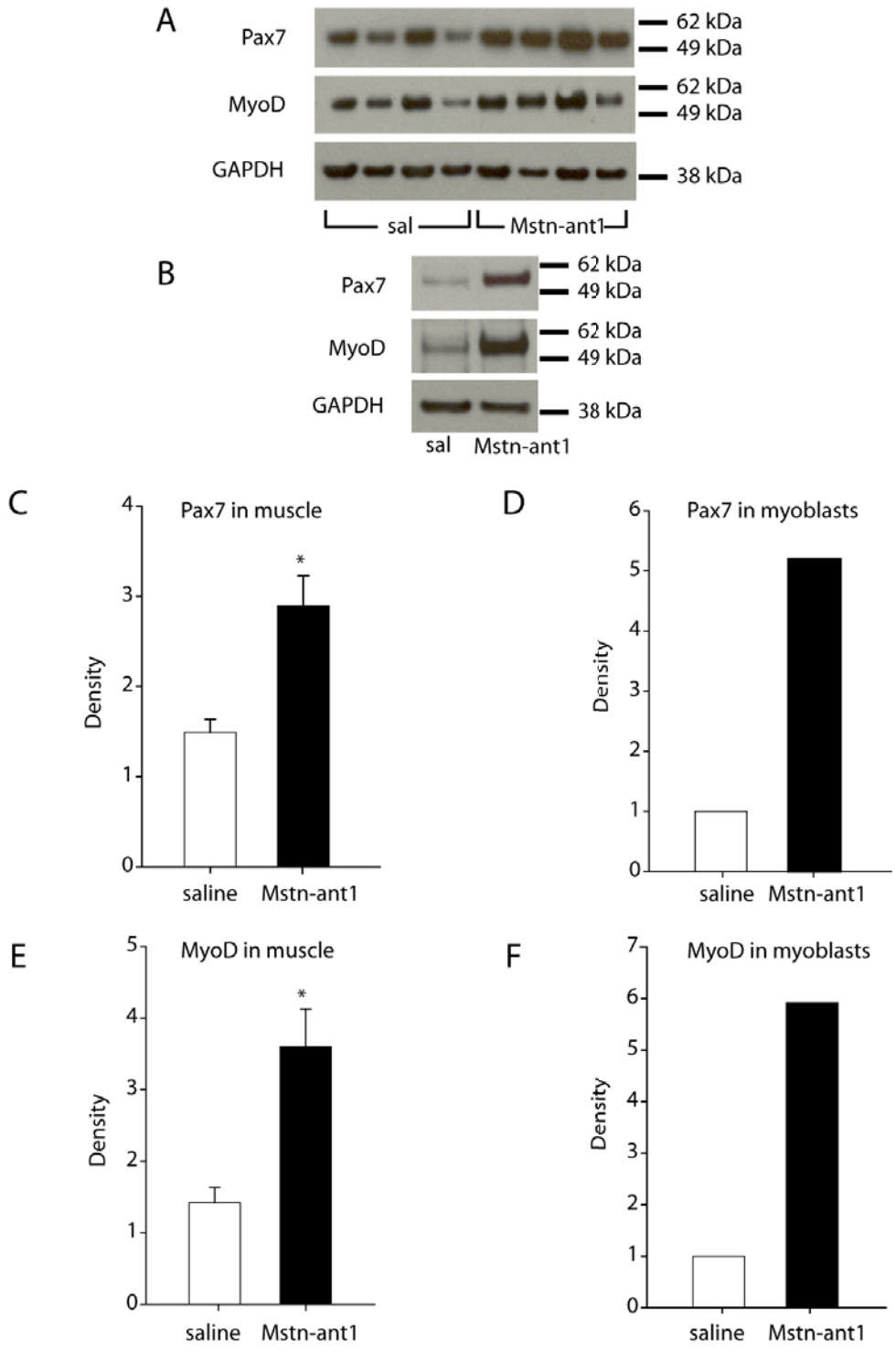

Figure 6.5 MyoD & Pax7 levels in aged muscle is increased by Mstn-ant1

Western analysis was performed on muscles (A) and pooled primary myoblasts (B) isolated from aged mice treated for 6 weeks with saline (Sal) or Mstn-ant1 to determine Pax7 and MyoD protein levels during sarcopenia-related muscle regeneration. Each blot was analysed by densitometry and normalised to GAPDH protein. Normalised data were used to generate the Pax7 (C-D) and MyoD (E-F) graphs shown (n = 10). * = $P < 0.05$ when Sal compared to Mstn-ant1. All data are expressed as mean ± SEM.

6.3.4 Satellite cell activation and proliferation and muscle strength is increased in response to Mstn-ant1 treatment

To investigate how Mstn-ant1 would affect satellite cell activation, isolated single fibres from 1- and 24-month-old mice were cultured with or without Mstn-ant1. Satellite cell activation was determined through ICC using an antibody for PCNA (Figure 6.6A), a marker for DNA replication. The fibres cultured with Mstn-ant1 consistently displayed a higher percentage of activated satellite cells per fibre than fibres cultured in media alone (Figure 6.6B-C) ($P < 0.05$).

Myostatin is also known to affect cell proliferation, thus to investigate the efficacy of the antagonist to increase myoblast proliferation, primary myoblasts from 1-month-old mice were cultured with or without Mstn-ant1. The result indicated that culturing with Mstn-ant1 increased myoblast proliferation by 15% (Figure 6.6D) ($P < 0.001$).

In addition to culturing isolated fibres with Mstn-ant1, satellite cell activation was also investigated in the fibres isolated from the aged mice injected with saline or Mstn-ant1 for 6 weeks. The fibres isolated from the Mstn-ant1 treated mice displayed a significantly increased percentage of activated satellite cells per fibre when compared to the saline treated mice (Figure 6.7A) ($P < 0.05$).

Furthermore, to test whether the 6 week treatment could increase muscle strength, grip tests were performed at the commencement and completion of the treatment period. The results indicated that the administration of Mstn-ant1 significantly increased the grip strength of the aged mice by 12% ($P < 0.05$) (Figure 6.7B). In contrast, no significant change was observed in the saline treated mice.

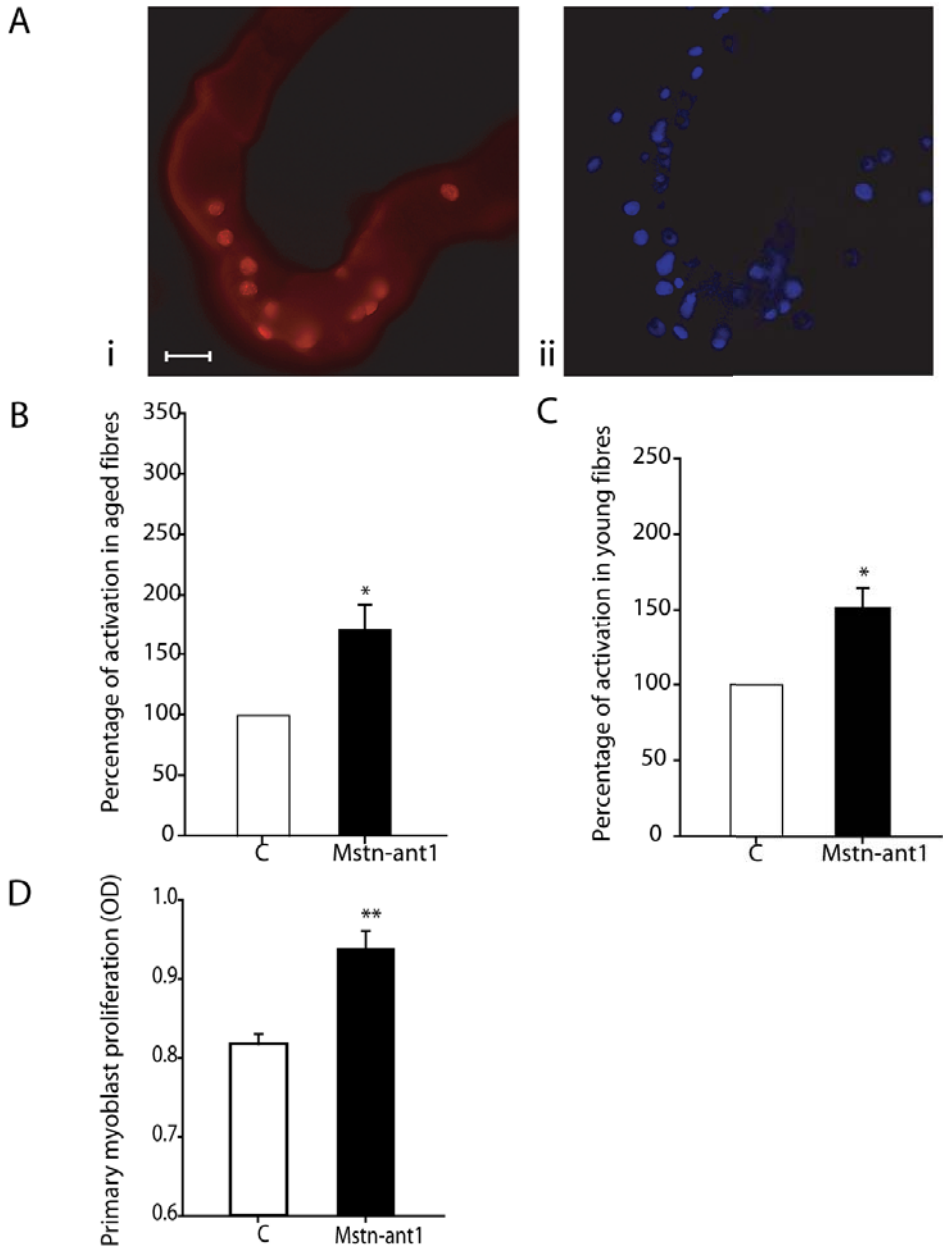

Figure 6.6 Mstn-ant1 increases satellite cell activation and proliferation

(A) Activated satellite cells on isolated single fibres were visualised by immunostaining for PCNA (i) and total nuclei by DAPI (ii) (scale bar = 20 μm). The number of satellite cells per 100 myonuclei was determined in fibres isolated from 24-month-old (B) and 1-month-old (C) mice after culturing for 48 or 24 hr respectively with or without (c = control) Mstn-ant1. Fibres were isolated from 12 animals and in excess of 1,000 nuclei per group were counted. * = $P < 0.05$ when Mstn-ant1 compared to control. (D) A proliferation assay was performed with primary myoblasts isolated from 1-month-old mice. The myoblasts were cultured with or without Mstn-ant1 and cell proliferation was determined using a methylene blue photometric endpoint assay. Proliferation after 96 hr is graphically shown. Assays were performed in triplicate using cells isolated from 4 animals. ** = $P < 0.001$. All data are expressed as mean ± SEM.

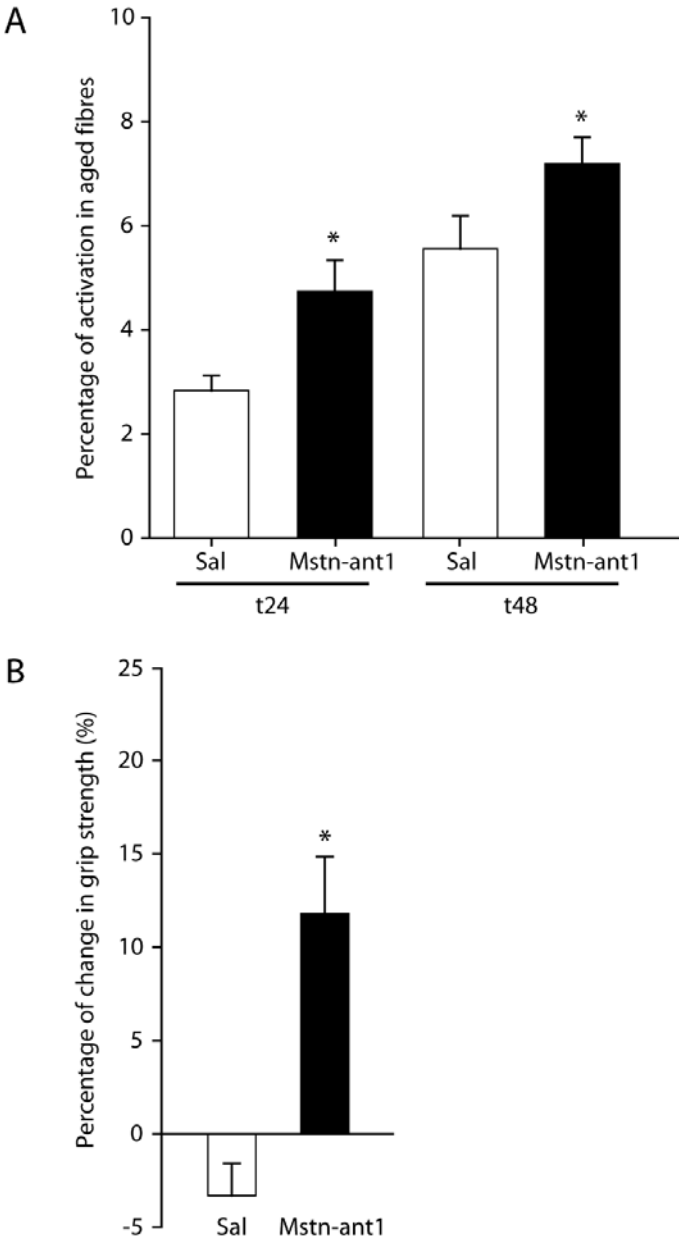

Figure 6.7 Cell activation and grip strength are increased by Mstn-ant1

(A) Satellite cell activation was assessed in single fibres isolated from aged mice treated for 6 weeks with saline (Sal) or Mstn-ant1. Single fibres were cultured for 24 and 48 hr (t24, t48). Activated satellite cells were visualised by immunostaining for PCNA and total nuclei by DAPI. The number of satellite cells per 100 myonuclei was then determined. Fibres were isolated from 10 animals in each treatment group and in excess of 1,000 nuclei per group were counted. * = $P < 0.05$ when Sal is compared to Mstn-ant1 treated. (B) Grip strength was assessed in aged mice treated for 6 weeks with Sal or Mstn-ant1. Grip strength was assessed on each animal at the trial commencement and completion (n = 10 per treatment group) giving a measurement in Newtons. The percentage of change between the commencement and completion grip strengths was then determined. * = $P < 0.05$. All data are expressed as mean ± SEM.

6.3.5 Antagonist treatment alters macrophage migration

Myostatin is believed to inhibit macrophage migration (McCroskery *et al.*, 2005). Therefore, the presence of macrophages was examined in the notexin injured muscles treated with saline or Mstn-ant1 using anti-Mac1 antibodies to determine the efficacy of the antagonist to enhance macrophage migration (Figure 6.8A). Indeed, a greater percentage of infiltrated nuclei were Mac1-positive in day-2 injured muscles which had been treated with Mstn-ant1, compared to saline treated muscles (Figure 6.8B) ($P < 0.05$). By day 3, the percentage had dropped in the Mstn-ant1 treated muscles below that of the saline treated day 3 muscles, and continued to be lower in day 7 and 10 muscles. This is indicative of a decreased macrophage requirement in the Mstn-ant1 treated muscles due to the earlier and increased number of macrophages observed at day 2.

6.3.6 Cell migration is altered with age and Mstn-ant1 treatment

Both myoblasts and macrophages are known to be influenced by chemotactic signals which affect their movement (Bischoff 1997; Jones 2000). To elucidate the effect of myostatin and aging on both myoblast and macrophage migration, a series of chemotaxis assays were performed. Results suggested that migration was significantly retarded in the myoblasts isolated from 24-month-old mice as compared to 1-month-old mice when in the presence of 5% CEE or 2% HS (Figure 6.9A) ($P < 0.05$). Similarly, migration of macrophages isolated from 24-month-old mice was also reduced compared to 1-month-old mice, irrespective of the chemoattractant concentration (Figure 6.9B) ($P < 0.05$). The efficacy of the Mstn-ant1 to rescue cell migration in response to myostatin treatment was then examined using primary myoblasts isolated from 24-month-old mice. With the addition of Mstn-ant1, cell migration was able to be rescued by hindering the chemo-inhibitory effect of myostatin (Figure 6.9C) ($P < 0.05$). To test the efficacy of Mstn-ant1 to enhance cell migration after prolonged *in vivo* treatment, bone marrow derived macrophages were isolated from mice injected for 6 weeks with saline or Mstn-ant1. Indeed, the macrophages from Mstn-ant1 treated mice were able to migrate more efficiently than macrophages from saline treated mice (Figure 6.9D) ($P < 0.05$).

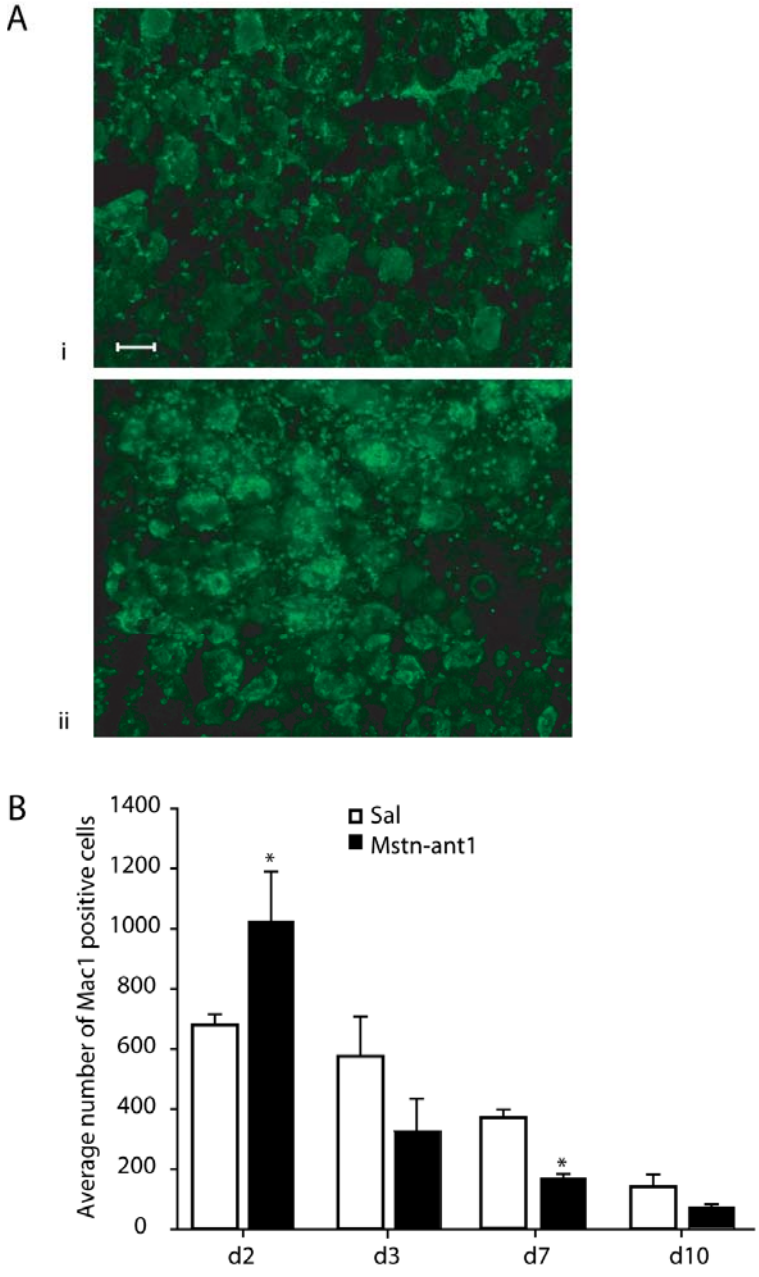

Figure 6.8 Antagonism of myostatin enhances macrophage infiltration

Representative images of Mac1 immunostained day-2 saline (A) and Mstn-ant1 (B) treated muscle sections after notexin injury are shown (scale bar = 50 μm). (C) The number of macrophages per unit area in regenerating muscle after notexin injury in saline (Sal) and Mstn-ant1 treated muscle sections was determined at day 2, 3, 7, & 10 (n = 4 per day and treatment group). * = $P < 0.05$ when Mstn-ant1 treatment is compared to Sal treatment.

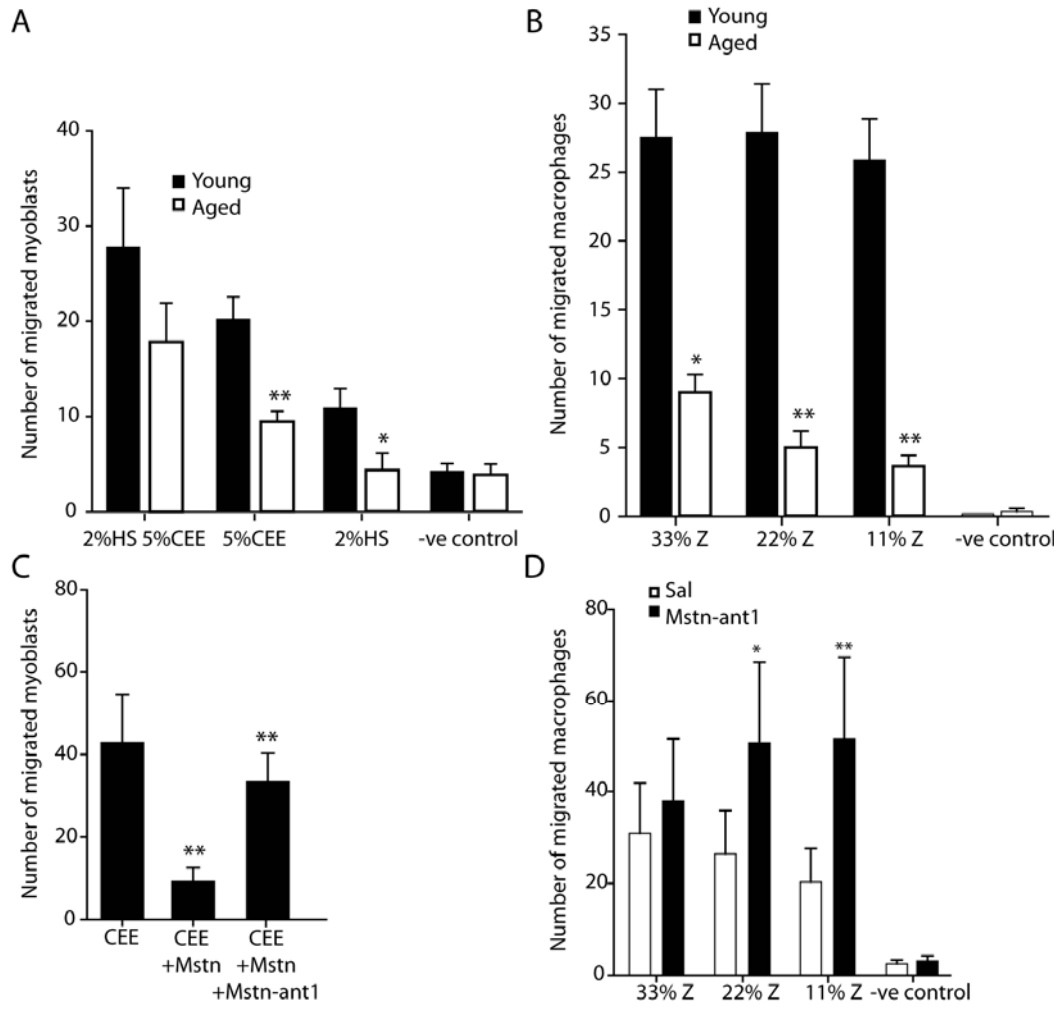

Figure 6.9 Aging decreases while Mstn-ant1 enhances cell migration

(A) Primary myoblasts isolated from 1- and 24-month-old mice were utilised in a migration assay using three concentrations of chemoattractant, 2% HS + 5% CEE (optimum concentration), 5% CEE, or 2% HS (suboptimal), while DMEM alone was a negative control (-ve control). ** = $P < 0.001$, * = $P < 0.05$ when 1-month-old is compared to 24-month-old. (B) Peritoneal macrophages isolated from 1- and 24-month-old mice were utilised in a migration assay using three concentrations of the chemoattractant ZAMS (Z), 33% (optimum concentration), 22% and 11% (suboptimal) and DMEM alone as a negative control. ** = $P < 0.001$, * = $P < 0.05$ when 1-month-old is compared to 24-month-old. (C) Primary myoblasts isolated from 24-month-old mice were used in a chemotaxis assay. CEE medium was a positive control, while CEE + myostatin (Mstn; 2.5 μg/ml) was a negative control. Mstn-ant1 was added to wells containing CEE medium + Mstn to rescue the chemo-inhibitory effect of Mstn. ** = $P < 0.001$ when CEE compared to CEE + Mstn, or CEE + Mstn compared to CEE + Mstn + Mstn-ant1. (D) Bone marrow derived macrophages from the saline (Sal) and Mstn-ant1 treated mice were used to assay macrophage migration after treatment. ** = $P < 0.001$, * = $P < 0.05$ when Mstn-ant1 treatment compared to Sal treatment. For all migration assays, migrated cells were counted on four representative fields per membrane and the average number was plotted. All data are expressed as mean ± SEM.

6.4 Discussion

Numerous studies indicate that aging considerably influences myogenesis through the muscle environment or systemic factors (Carlson and Faulkner 1989; Conboy *et al.,* 2005), expression of MRFs (Welle *et al.,* 2000) and satellite cell behaviour (Bockhold *et al.,* 1998; Conboy *et al.,* 2003). Recently, it has been shown that a prolonged absence of myostatin reduces sarcopenia in *myostatin*-null mice (Wagner *et al.,* 2005). However, as these mice lack functional myostatin from a prenatal stage, it was necessary to determine the effects of short term inhibition of myostatin during old age. A previous study has indicated that an increase in skeletal muscle mass and strength occurs following treatment with a myostatin inhibitory antibody (Whittemore *et al.,* 2003). In this study, we show that a short term administration of a myostatin antagonist restores the regenerative and myogenic capacity of aged muscle.

In order to examine the effect of a short term antagonism of myostatin, Mstn-ant1 was screened for its ability to neutralise myostatin function. Cultured myoblasts express and secrete myostatin, which regulates the proliferation rate of myoblasts (Thomas *et al.,* 2000; McFarlane *et al.,* 2005). Thus, antagonism of myostatin by Mstn-ant1 would result in an increase in the myoblast proliferation rate. Indeed, a C2C12 myoblast proliferation assay indicated that Mstn-ant1 effectively increased the proliferation of the myoblasts above that of the control (Figure 6.1C), thus confirming its biological activity.

In agreement with past studies showing that a lack of myostatin significantly enhances muscle regeneration and reduces fibrosis (McCroskery et al., 2005; Wagner et al., 2005), results reported here suggest that a blockade of myostatin through the administration Mstn-ant1 immediately after notexin injury can replicate this effect. Muscle weights, histology, greater regenerated areas at days 7 and 10, as well as significantly larger individual fibre areas at day 28 all indicated advancement of regeneration with Mstn-ant1 treatment (Figure 6.2, 6.3B). In addition, Mstn-ant1 treated muscles also displayed reduced levels of collagen (Figure 6.3C) suggesting myostatin is involved in fibrosis and collagen formation. In support of this, during fibroblast migration assays, myostatin can behave as a fibroblast chemoattractant (data not shown). Collectively, these results indicate that a prolonged absence of myostatin, as observed in the

myostatin-null mice, is not required to obtain enhanced muscle regeneration and reduced fibrosis. Rather, a short term blockade of myostatin during the regeneration period is sufficient to enhance the regeneration process.

During muscle regeneration, MyoD is expressed earlier and at higher levels in *myostatin*-null muscle as compared with wild-type muscle (McCroskery et al., 2005). Similarly, Western blot analysis performed on the regenerating muscle from mice treated with Mstn-ant1 showed increased levels of MyoD during regeneration, suggesting increased myogenesis directly resulting from a myostatin blockade by Mstn-ant1 (Figure 6.4C). In addition, Pax7, which is expressed in quiescent and proliferating cells (Seale et al., 2000), was higher with Mstn-ant1 treatment throughout the trial period suggesting an increase in satellite cell number, activation and/or self renewal compared to saline treated mice (Figure 6.4B). These higher Pax7 and MyoD levels could be due to increased numbers of satellite cells and the subsequent myogenesis, and increased satellite cell self renewal. Of note, while similar levels of MyoD were observed in the Mstn-ant1 and saline treated day 28 regenerated muscles, higher levels of Pax7 were seen in the Mstn-ant1 treated muscles compared to the saline treated muscle. Since Pax7 is a marker for satellite cell self-renewal (Oustanina et al., 2004; Zammit et al., 2004), the higher level of Pax7 indicates that Mstn-ant1 enhanced this self-renewal process. This is in accordance with an earlier finding that indicates myostatin inhibits satellite cell self-renewal (McCroskery et al., 2003). Similarly, an extended treatment with Mstn-ant1 to aged mice for 6 weeks also resulted in increased MyoD and Pax7 protein levels in both Gas muscle and primary myoblasts (Figure 6.5C-F). Aged muscle can undergo wasting due to a loss of fibres and atrophy of existing fibres (Alnaqeeb and Goldspink 1987), therefore a level of continued myogenesis is required to maintain and repair the muscle. Correspondingly, MyoD expression has been shown to be high in young animals, significantly decreasing in the adult, and later increasing again as the animal continues to age (Dedkov *et al.,* 2003). However, although the level of MyoD, as well as other MRFs, increases in the aged muscle, this does not restore the muscle's myogenic ability to that seen in a young muscle (Grounds 1998; Welle 2002; Conboy *et al.,* 2005). As myostatin is known to reduce the expression of MyoD (Langley *et al.,* 2002), it would be expected that myostatin would negatively regulate this age-related myogenesis. Indeed, by antagonising

myostatin, we were able to increase the level of myogenesis, as reflected by the increase in both MyoD and Pax7 protein levels. In support of these results, increased satellite cell activation was observed in 1- and 24-month-old single fibres when cultured in the presence of Mstn-ant1 (Figure 6.6B-C). Furthermore, *in vivo* administration of Mstn-ant1 to aged mice was able to increase the percentage of activated satellite cells per fibre (Figure 6.7A). This would suggest that antagonism of myostatin through subcutaneous injections of Mstn-ant1 can provide a means for increased myogenesis possibly by increasing satellite cell number or by priming the satellite cells to continue along the myogenic pathway. If indeed this is the case, then potentially, antagonism of myostatin in aged animals could positively contribute to the reversal of the decline in satellite cells and/or their activation potential that has been reported to occur in the aged muscle environment (Conboy *et al.,* 2003; Shefer *et al.,* 2006). Increasing satellite cell activation and number would be expected to result in an augmentation in force strength. As Figure 6.7B indicates, after a 6 week treatment of Mstn-ant1, grip strength was increased in the treated animals ($P < 0.05$).

As previously stated, a major component of the regeneration process is the inflammatory response. After injury, both macrophages and myoblasts migrate to the site of injury in response to signalling from inflammatory cytokines and various growth factors. In corroboration with a study using *myostatin*-null mice (McCroskery *et al.,* 2005), an accelerated migration and enhanced accretion of macrophages was observed with Mstn-ant1 treatment (day 2, Figure 6.8B), suggesting Mstn-ant1 rapidly and effectively antagonised the inhibitory effect of myostatin on macrophage migration. These findings were also recapitulated in chemotaxis assays whereby Mstn-ant1 effectively blocked the inhibitory effect of myostatin, thus rescuing the cell migration (Figure 6.9C). Aging also displayed a significantly negative effect on both primary myoblast and macrophage migration (Figure 6.9A-B). This could be due to a reduced propensity of the 24-month-old myoblasts and macrophages to respond to their respective chemoattractants or a reduced expression of receptors. Interestingly, after 6 weeks of Mstn-ant1 treatment, the migratory ability of macrophages was restored (to an unknown extent) in the aged mice (Figure 6.9D).

Collectively, the results presented here suggest that short term blockade of myostatin and its function through antagonist treatment can effectively enhance

muscle regeneration in aged mice after injury and during age-related muscle wasting. The ramifications of antagonist treatment for human health are potentially extensive. In addition, the efficacy of an antagonist treatment has been demonstrated here, as only four doses of an antagonist during the critical period of regeneration after injury was sufficient to significantly improve muscle recovery. Therefore, we propose that antagonism of myostatin is a viable option for treatment of deficient muscle regeneration and sarcopenia in humans, through a restoration of myogenic and inflammatory responses and decreased fibrosis.

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Chapter Seven

Perspectives and future directions

Since the discovery of myostatin, a significant amount of research has been undertaken to understand the biological mechanism and function of myostatin during prenatal and postnatal myogenesis. Initially much of this work focused on the phenotype of *myostatin*-null animals, followed by *in vitro* studies with myogenic cell lines to determine the mechanism by which it regulates the cell cycle. More recently, studies have concentrated on the action of myostatin on satellite cell regulation and self-renewal, and muscle regeneration after injury. In addition, correlations between myostatin levels and human conditions and diseases such as aging and HIV have been determined. Although *myostatin*-null animals enabled significant insights into the biological effects of myostatin, the effects of myostatin during prenatal and postnatal myogenesis were unable to be discriminated. To clarify this, a number of studies have attempted to block the action of myostatin postnatally. Conversely, myostatin over-expression studies have provided confirmation of the action of myostatin and its associated null phenotype. Additionally, myostatin over-expression studies have corroborated the putative role of myostatin during muscle wasting conditions such as cachexia and aging. While previous studies have provided invaluable insights into the structure, expression, and function of myostatin, a number of areas concerning postnatal myogenesis remain undetermined. Therefore, the primary aim of this thesis was to address a number of these areas to provide a greater understanding of myostatin. More specifically, myostatin downstream targets were investigated, along with the effect of both prolonged and short term blockade of myostatin function during *in vitro* and *in vivo* myogenesis.

Previous *in vitro* studies have indicated that myostatin regulates cell cycle progression during prenatal myogenesis via Rb-dependent (Thomas *et al.,* 2000) and independent (Langley *et al.,* 2004) pathways. A proposed mechanism for this regulation is through the down-regulation of cell cycle-related genes. However, to date, a systematic analysis of the global gene expression changes initiated by either the presence or lack of myostatin has not been described. Thus, in order to identify further myostatin downstream target genes, a SSH experiment was

performed and described here. The study revealed *ARA70*, a coregulator of AR, as a negatively regulated downstream target gene of myostatin. The administration of testosterone is known to elicit various effects, which are mediated through the AR (Chang *et al.,* 1988b; Chang *et al.,* 1988a), including increased muscle fibre volume, myonuclear number, protein synthesis and satellite cell number, decreased protein degradation, and increased myogenesis and decreased adipogenesis of pluripotent stem cells (reviewed by Herbst and Bhasin (2004)). Therefore, the down-regulation of *ARA70* was of particular interest, and required further analysis. Thus, Northern and Southern blot analyses using wild-type and *myostatin*-null muscle were performed, verifying that a down-regulation of *ARA70* by myostatin had occurred in the murine muscle. Furthermore, addition of exogenous myostatin to C2C12 cells resulted in the down-regulation of *ARA70* expression, confirming that the *ARA70* gene is either directly or indirectly repressed by Myostatin. Thus a novel finding of this study was the identification of *ARA70* as a downstream target gene of the potent negative regulator of muscle growth, myostatin. From these findings, it was proposed that since lack of myostatin and increased expression of *AR* leads to muscle hypertrophy, an absence of myostatin, in part, may induce the *myostatin*-null hypertrophy phenotype, by enhancing AR activity, through the up-regulation of *ARA70*. In order to prove this hypothesis, future work is necessary to delineate the normal function of ARA70 in myogenesis, as well as the role of ARA70 in signalling hypertrophy. This would necessitate the generation of an *ARA70* over-expressing transgenic mouse line, to assess the possibility of a resulting muscle hypertrophy phenotype.

In addition to ARA70, numerous other genes of unknown function were also shown to be down-regulated by myostatin. Therefore to fully investigate potential downstream targets of myostatin, a comprehensive analysis of these genes could be undertaken. This would require similar molecular techniques employed in this thesis.

A well known condition associated with aging is sarcopenia, defined as a progressive reduction in muscle mass and strength. In humans, this condition can have a profound effect on a person's quality of life, as it is frequently associated with reduced mobility and independence, as well as increased risk of falls (Grimby and Saltin 1983; Harris 1997; Roubenoff and Hughes 2000; Roubenoff

2001). However, to date there is no viable therapeutic intervention available for either the prevention or alleviation of sarcopenia. The muscle atrophy observed with sarcopenia has previously been attributed to alterations in the muscle fibres (Alnaqeeb and Goldspink 1987), which have been frequently correlated with insufficient or inadequate levels of muscle regeneration (Grounds 1998). It is believed that this results from impaired satellite cell involvement and myogenesis due to the aged environment (Conboy *et al.,* 2003). Parabiotic experiments have indicated that circulating factors positively influence the proliferation and regenerative capacity of satellite cells (Conboy *et al.,* 2005). In addition, negative regulators of muscle mass, such as myostatin, are most likely involved in the balancing of the signals that influence satellite cell activation and muscle regeneration. Currently it is unclear whether the impaired satellite cell behaviour results from a decline in positive factors and/or from an increase in negative regulators.

To investigate the role of myostatin during sarcopenia, in order to design myostatin-based therapies, the muscle phenotype and regenerative capacity were examined in young and old wild-type and *myostatin*-null mice. In agreement with other studies (McPherron *et al.,* 1997; Lee and McPherron 2001; Girgenrath *et al.,* 2005), the findings reported here show that young *myostatin*-null muscle displayed fibre hypertrophy and hyperplasia and an increased number of type IIB fibres, resulting in a more glycolytic and powerful muscle type. Additionally, this study shows that with aging, wild-type muscle became increasingly oxidative and displayed prominent fibre atrophy, whereas no fibre type switching and minimal atrophy was observed in the aged *myostatin*-null muscle. These findings are highly noteworthy as they indicate that myostatin has a substantial role during sarcopenia. As the *myostatin*-null muscle did not undergo significant fibre type shifts or atrophy associated with sarcopenia, the inhibition of myostatin, could in theory, ameliorate sarcopenia in humans, thus improving the quality of life in the elderly. Indeed, the aged animal trial using the myostatin antagonist did highlight the therapeutic potential of myostatin antagonism in sarcopenia.

It is generally accepted that mammalian muscle becomes increasingly oxidative with age, due to oxidative fibre type shifts (Kugelberg 1976; Alnaqeeb and Goldspink 1987; Larsson *et al.,* 1993; Musaro *et al.,* 1995), though the exact cause for these transitions has been difficult to determine. However, earlier

research has indicated that denervation via a selective loss of fast α-motor neurons (Hashizume *et al.,* 1988; Ansved and Larsson 1990), and alterations in hormone levels (Sieck *et al.,* 1996) may significantly contribute to this condition. It remains unclear why, and how, *myostatin*-null muscle is resistant to the oxidative changes. In a study involving the administration of a neutralising myostatin antibody to adult mice, no change in the distribution of fibre types or in the expression of MHC isoforms was observed (Girgenrath *et al.,* 2005). Thus, the authors speculated that the alteration in fibre type composition observed in *myostatin*-null mice was a consequence of the absence of myostatin during prenatal development and postnatal terminal differentiation. In order to confirm this, further work is necessary to understand prenatal and neonatal fibre development in the absence of myostatin. This would require an analysis of the primary and secondary myogenic waves using histological and molecular techniques, including *in situ* hybridisation and ICC for MHC isoforms, Pax7 and relevant MRFs. In addition, innervation and hormone levels would need to be assessed to determine if alterations are present in the *myostatin*-null muscle. Furthermore, attempting to forcibly alter the fibre types in the *myostatin*-null muscle through the application of CLFS, which has been shown to cause oxidative fibre type changes, may lead to a greater understanding of fibre type fate.

Further findings reported here demonstrated that *myostatin*-null muscle had increased satellite cell numbers and activation compared to wild-type muscle, suggesting a greater propensity to undergo myogenesis. However, satellite cell activation declined significantly with age in both wild-type and *myostatin*-null muscles. Although the *myostatin*-null fibres retained a higher percentage of activated satellite cells, it was surprising that the percentage declined to such an extent, given that the fibre hypertrophy was maintained in the aged *myostatin*-null mice. Whether this result alludes to a greater myogenic potential that *myostatin*-null satellite cells possess is unknown. However, other findings reported in this thesis suggest that the greater myogenic potential observed in *myostatin*-null satellite cells would significantly factor into the overall myogenic capacity of the aged muscle. Alternatively, it may indicate that aged *myostatin*-null muscle experiences decreased catabolic signalling via the ubiquitin-proteasome pathway. As this remains unknown, further work is required to clarify whether the two genotypes differ in the level of catabolic signalling. For this, a molecular analysis

of MuRF-1 and Atrogin-1, two muscle-specific ubiquitin E3 ligases associated with a number of atrophic conditions (Bodine *et al.,* 2001; Gomes *et al.,* 2001), could be performed. This would help to elucidate why and how reduced atrophy, or maintained hypertrophy, is observed in the *myostatin*-null muscle.

This study also describes accelerated regeneration (following notexin injury) in *myostatin*-null muscle compared to wild-type muscle. This finding is in agreement with an earlier study conducted in our laboratory, which reports an accelerated regeneration in young *myostatin*-null mice (McCroskery *et al.,* 2005). Although these regeneration experiments conducted in young and aged mice eloquently demonstrated the mechanism of myostatin regulation on regeneration, it did not allow myostatin signalling to be separated from other effects of prolonged absence of myostatin. This is relevant as the increased satellite cell number and activation associated with the *myostatin*-null phenotype would factor significantly during muscle regeneration. The latter use of a myostatin antagonist enabled the separation of the long term effects from a short term blockade of myostatin signalling during muscle regeneration.

Additionally, the current study reported here shows that fibre hypertrophy and type were restored after regeneration in the *myostatin*-null muscle, unlike the wild-type muscle, which displayed smaller and more oxidative fibres. These novel findings clearly indicate the significant impact of myostatin during muscle regeneration. It is unclear whether the smaller fibres observed in the wild-type muscle were due to incomplete fibre regeneration. Alternatively, and perhaps more likely, the smaller fibres were representative of the fibre atrophy associated with sarcopenia. In contrast, the observed oxidative fibre type shift is highly reflective of the aging process. Notexin injury is known to be highly damaging to the muscle fibres, and undoubtedly complete regeneration would require the re-establishment of muscle innervation. Therefore, the observed fibre type shifts may indicate an alteration in the re-innervation process. If this is the case, it is unknown why the *myostatin*-null muscle is unaffected in the way that the wild-type muscle appears to be. Further work is therefore required to understand this process, and to determine how myostatin has a significant influence on fibre type determination. This would entail a histological analysis of the regenerated muscle and associated motor neurons to determine the types of neurons innervating the muscle and the size of the motor unit. An analysis of this type would provide an

indication of whether myostatin is influencing the type of neuron a fibre is innervated by, or simply regulating the expression of the various MHC isoforms.

As previously discussed, muscle atrophy is commonly associated with aging. Furthermore, reduced satellite cell potential has been correlated with this age-related atrophy, as satellite cell activation potential and amount of proliferation are both thought to decrease with age (Schultz and Lipton 1982; Dodson and Allen 1987; Johnson and Allen 1993; Barani *et al.,* 2003; Conboy *et al.,* 2003; Machida and Booth 2004). Additionally, satellite cells from aged muscle display an extended latent period between an activation stimulus and proliferation (Tatsumi *et al.,* 1998; Yablonka-Reuveni *et al.,* 1999). Consequently, myoblast proliferation is retarded after injury (McGeachie and Grounds 1995). In humans, the proliferative ability of satellite cells starts to decline from early childhood years, and with increasing age, satellite cells form thinner, more fragile myotubes (Renault *et al.,* 2000). A mechanism for the impaired regenerative capacity associated with aging has been suggested to result from the aged environment (Carlson and Faulkner 1989; Coggan *et al.,* 1992; Carlson and Faulkner 1996; Chakravarthy *et al.,* 2000), however, this does not entirely explain these previous *in vitro* findings.

Myostatin has been shown to regulate satellite cell activation and myoblast proliferation and differentiation (Thomas *et al.,* 2000; Langley *et al.,* 2002; McCroskery *et al.,* 2003). Thus, it was investigated, and subsequently reported here, how a prolonged absence of myostatin affected the normal aging process, with regards to muscle atrophy and the myogenic nature of the satellite cells.

Irrespective of age, *myostatin*-null muscles were found to be consistently larger than the wild-type muscles. Young *myostatin*-null mice displayed increased MyoD protein levels compared to wild-type mice, however, at 6-months of age, MyoD levels were higher in the wild-type muscle compared to the age matched *myostatin*-null muscle. Thus it was proposed that the wild-type muscle had either: a lengthier period of myogenesis due to a lethargic rate of myogenesis; or conversely, experienced an earlier onset of age-related muscle wasting and associated myogenesis. Therefore, future work could be performed to investigate whether the wild-type muscle receives earlier atrophic signalling, or is simply more sensitive to it. As previously suggested, a molecular analysis of the

ubiquitin-proteasome pathway could be undertaken to establish any variation between the two genotypes.

Also reported in this thesis is an *in vitro* analysis of aged wild-type and *myostatin*-null satellite cells, which indicated that the *myostatin*-null myoblasts had a greater proliferation rate and differentiation fusion index. This suggests that an increased myogenic potential may account for the muscle hypertrophy and reduced sarcopenia observed in the aged *myostatin*-null mice. A molecular mechanism proposed to be directly linked with the impaired proliferative capacity of aged satellite cells is the Notch signalling pathway. Induced expression of activated Notch has been shown to restore the regenerative capacity of aged muscle (Conboy *et al.,* 2003; Luo *et al.,* 2005). Furthermore, parabiotic studies have demonstrated a restoration of the Notch signalling as well as the muscle regeneration capacity of aged mice (Conboy *et al.,* 2005). Interactions between myostatin and Notch signalling are yet to be understood. Since a lack of myostatin and an activation of Notch signalling both result in increased satellite cell activation and subsequent myoblast proliferation, it is tempting to speculate on a potential interaction between these two regulators of myogenesis. Thus an analysis of Notch signalling in wild-type and *myostatin*-null muscle could provide further answers as to how myostatin regulates satellite cell activation and proliferation. This would require a molecular study into the expression profile of the Notch signalling components during myogenesis, and investigations into any direct interactions using *in vitro* cell culture systems.

Recent studies have demonstrated heterogeneity in the myoblasts derived from satellite cells. Myoblasts expressing Pax7-only appear to be fusion incompetent and thus contribute to the self-renewed population, whereas myoblasts expressing MyoD-only, which are fusion competent, are destined to become new fibre myonuclei (Seale *et al.,* 2000; Zammit *et al.,* 2004; Nagata *et al.,* 2006). The findings presented here indicate that in the absence of myostatin there are increased Pax7-only myoblasts during aging. This suggests an enhanced self-renewal process in the *myostatin*-null mice, which is consistent with an earlier observation indicating that lack of myostatin increases the satellite cell number due to enhanced self-renewal (McCroskery *et al.,* 2003).

Increased fat accumulation is a well known characteristic of aging, however, *myostatin*-null mice reportedly demonstrate significantly decreased fat

accumulation compared to wild-type mice (Lin *et al.,* 2002; McPherron and Lee 2002). In agreement, analysis of the fat pads in this current study indicated that the size of both fat cell and pad were significantly decreased in the *myostatin*-null mice compared to the wild-type mice. Presently it is unknown whether the decreased fat accumulation in *myostatin*-null mice is a primary effect elicited by myostatin, or a secondary consequence due to the increased muscle mass, as a similar effect is seen in other muscle hypertrophy genetic models (Sutrave *et al.,* 1990; Musaro *et al.,* 2001). Interestingly, McPherron and Lee (2002) found that the *myostatin*-null mice gonadal fat pad contained approximately 25% fewer fat cells than the wild-type fat pad, while Whittemore *et al*. (2003) reported no change in the fat pad mass when myostatin was inhibited with a blocking antibody. Due to the low (and inconsistent) number of *in vitro* reports regarding the role of myostatin during adipogenesis, it is difficult to understand how a prolonged lack of myostatin results in a consistently low level of fat accumulation even in the aged mice. Therefore, future work could be performed to elucidate the mechanism by which myostatin regulates fat accumulation. One way of addressing this issue would be to study the adipogenic nature of preadipocytes isolated from *myostatin*-null and wild-type animals. An analysis of their proliferative capacity as well as the expression profile of relevant adipogenic early and late differentiation genes would allow a comparison between the two genotypes. Alternatively, myostatin or myostatin antagonist treatment of preadipocytes would possibly provide evidence for any regulation by myostatin. Furthermore, investigations into possible correlations between myostatin and known inducers of adipogenesis may help to clarify any regulation by myostatin.

A number of recent studies investigating the role of myostatin have adopted the use of antibodies which inhibit myostatin function (Bogdanovich *et al.,* 2002; Whittemore *et al.,* 2003; Bogdanovich *et al.,* 2005; Girgenrath *et al.,* 2005). The inhibition of myostatin appeared successful, however, most of the findings concentrated on the augmentation of muscle mass and strength, and fibre type analysis only. Therefore, to further elucidate the molecular mechanism of myostatin, especially during sarcopenia, a small molecule, designed to antagonise myostatin and its biological function, was employed in a series of *in vitro* and *in vivo* experiments. Overall, the short term blockade of myostatin reported here was found to significantly enhance muscle regeneration in aged mice after injury and

during age-related muscle wasting. This was achieved through enhanced macrophage migration, satellite cell activation, and myogenic-related gene expression. Thus the findings supported the hypothesis that myostatin signalling plays a major role in both muscle regeneration and age-related muscle wasting. From these results, it was proposed that antagonism of myostatin could lead to a restoration of the myogenic and inflammatory responses in the aged environment. The consequences of restoration are highly noteworthy both scientifically and medically, as this is the first demonstration of how the antagonism of myostatin has significant therapeutic potential in the alleviation of sarcopenia as well as other muscle wasting conditions observed in humans. Although a short term blockade of myostatin has revealed significant insights into the effects of myostatin signalling, a number of questions still remain unanswered. For example, how is the antagonist molecule blocking myostatin signalling? Is it due to competitive binding of the Act RIIB receptor, or through dimerisation of the active mature myostatin protein with the antagonist, thus rendering it inactivated? A series of receptor binding or competitive binding assays using Act RIIB receptor, the myostatin antagonist, and the active mature myostatin protein could be performed to address these questions. Furthermore, could the antagonist be utilised as a prophylactic molecule, to prevent sarcopenia? This would require an additional *in vivo* trial using young mice, which would regularly receive the antagonist until the age when sarcopenia is observed in the control mice. Extensive clinical trials would also be required to assess the safety of the antagonist as a treatment for muscle wasting in humans.

In conclusion, a prolonged absence of myostatin appeared to reduce age-related sarcopenia. Antagonism of myostatin was also found to effectively enhance myogenesis and the muscle regenerative capacity through a number of mechanisms. Therefore, the use of a myostatin antagonist appears to be a viable treatment option for deficient muscle regeneration and sarcopenia in humans. Additionally, it may also alleviative other debilitating human conditions such as cancer and HIV. Thus, myostatin antagonist treatment for human health is potentially extensive.

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