Characterisation of Mighty Expression during Skeletal Muscle Regeneration

by

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Abstract

Satellite cells are a distinct lineage of myogenic precursors that are responsible for the growth of muscle during post-natal life and for its repair after damage. During muscle growth and regeneration satellite cells are activated in response to growth signals from the environment, which induces the expression of one or both of the two MRFs, Myf-5 or MyoD. Activated satellite cells migrate to the site of injury and proliferate before these transcription factors go on to activate transcription of myogenic genes. The myoblasts can then adopt one of two fates. Some myoblasts initiate terminal differentiation and are able to either fuse into existing myofibres to repair them, or fuse with other myoblasts to form new fibres. Other myoblasts do not differentiate but instead return to quiescence and adopt a satellite cell position on repaired or newly formed fibres.

Mighty, a downstream target of myostatin that was discovered by the Functional Muscle Genomics Laboratory has recently been shown to induce cell hypertrophy in cell culture through enhanced differentiation and fusion of myoblasts. Myostatin-null mice have hypertrophic muscles and an improved muscle regeneration phenotype. These mice have also been shown to have higher basal levels of Mighty in skeletal muscle than wild-type mice. In this thesis the expression profile of Mighty during skeletal muscle regeneration was characterised in relation to MyoD.

During regeneration Mighty gene expression was induced at day five post-injury in both wild-type and myostatin-null mice. In the myostatin-null mice Mighty gene expression remained elevated at day seven post injury in contrast to the

levels in the wild-type, which had decreased at this time point. By day-14 and day-28 post-injury Mighty levels were decreased. The up-regulation of Mighty occurs at the time of peak myotube formation in regenerating skeletal muscle, consistent with a role for Mighty in enhancing differentiation and fusion of myoblasts. The extended up-regulation of Mighty in the myostatin-null muscle may be responsible for the enhanced regeneration phenotype of these mice.

Analysis of the myotube and reserve cell populations, which are an *in vitro* model of satellite cells, from both C_2C_{12} cells and Mighty over-expressing clones (Clone 7 and Clone 11) showed that Mighty expression down-regulates two satellite cell markers, CD34 and Sca-1. Both these molecules have been recently shown to be involved in myoblast fusion and reserve cell specification, although their exact role in these processes is not yet known. Expression of Sca-1 is associated with a slowly proliferating non-dividing state while CD34 is associated with the population of reserve cells that do not fuse when notch signalling is inhibited. The results of this thesis indicate that Mighty over-expression may cause the enhanced fusion phenotype by regulating these two molecules.

In conclusion the data in this thesis supports a role for Mighty in the myotube formation phase of regeneration and may be able to enhance regeneration by recruiting more myoblasts to terminal differentiation by altering CD34 and Sca-1 expression.

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List of Abbreviations

α	alpha
β	beta
μg	micrograms
μl	microlitres
μM	micromolar
μU	microunit
β-gal	beta galactosidase
BMP	bone morphogenic protein
bp	base pair(s)
BSA	bovine serum albumin
^{o}C	degrees celsius
c-λ	carageenen lambda
cDNA	complementary DNA
DAPI	4’,6-diamidino-2-phenylingole dihydrochloride
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco’s modified eagle medium
DNA	deoxyribonucleic acid
E	embryonic day
FGF	fibroblast growth factor
g	gram(s)
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDF-8	growth/differentiation factor-8
h	hour(s)
HGF	hepatocyte growth factor

HPRT	hydroxanthine guanine phosphoribosyl transferase
HRP	horse radish peroxidase
HS	horse serum
ICC	immunocytochemistry
IGF	insulin-like growth factor
LIF	leukaemia inhibitory factor
kDa	kilo Daltons
MARs	matrix attachment regions
MHC	myosin heavy chain
ml	millilitres
MNF	myocyte nuclear factor
mm	millimetre(s)
MRF	myogenic regulatory factor
NDS	normal donkey serum
NGS	normal goat serum
NO	nitric oxide
NOS	nitric oxide synthase
nm	nanometers
NSS	normal sheep serum
O/N	overnight
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline + tween-20
PCR	polymerase chain reaction
PEG	polyethylene glycol
pI	isoelectric point

PVP	polyvinylpyrrolidone
rcf	relative centrifugal force
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
s	second(s)
Sca-1	stem cell antigen-1
SP	side population
TA	*tibialis anterior*
TBS	Tris buffered saline
TBS-T	Tris buffered saline + Tween-20
TGF-β	transforming growth factor beta
TNF-α	tumour necrosis factor alpha
trunc	truncated
VEGF	vascular endothelial growth factor
x	times

Chapter One

Literature Review

In this literature review, skeletal muscle structure and function will be discussed briefly, followed by a short review of the myogenic processes occurring during embryonic myogenensis. This will be followed by a review of the satellite cell, and its role in regeneration of skeletal muscle following injury. Finally the current knowledge about Mighty will be put forward.

1.1 Muscle structure and formation

1.1.1 Skeletal muscle physiology

Skeletal muscle consists of three sheaths of connective tissue surrounding muscle cells, nerves and blood vessels. The epimysium surrounds the entire muscle, the perimysium divides the muscle up into bundles of muscle fibres called fascicles and the endomysium surrounds individual multinucleate muscle cells/fibres. Each muscle fibre is a single, multinucleate cell with a well defined internal structure. The internal structure of the myofibre is organised into bundles of myofilaments, which contain the proteins responsible for the contractile properties of the muscle organised into repeating units. These repeating units are called sarcomeres (Figure 1.1). Each sarcomere contains filaments of both thick and thin protein fibres. The major protein of the thick filaments is myosin, which is wrapped around a core of the protein titin. The major protein of the thin filament is actin, which is also associated with tropomyosin, troponin c and nebulin. The arrangement of these proteins within

the resting sarcomere allows three distinct regions to be described. The H zone is the central section of the sarcomere where only myosin filaments are present. The I band is the end zone where only actin filaments are present, and the A band is that area where the actin and myosin filaments overlap. The boundary of the sarcomere is defined by the Z lines, the part of the sarcomere where the thin filaments attach to connectins. The myosin filaments connect together at the M line, in the middle of the H zone. Contraction of the muscle involves action potentials from innervating neurons causing calcium release. Calcium floods into the myofibrils from the sarcoplasmic reticulum in response to the signal, and by binding to troponin C shifts the regulatory tropomyosin molecules away from the active site of the actin. The movement of the tropomyosin molecules allows the actin and myosin to interact, pulling the two Z lines at either end of the sarcomere towards each other, shortening the sarcomere length. Although each sarcomere only shortens a small amount, the combination of thousands of sarcomere shortenings results in a visible contraction of the muscle overall. Muscle contains three basic types of myofibres; fast, slow and intermediate fibres. Fast fibres, also known as Type II-B fibres can contract rapidly, and rely mainly on glycolysis for energy production. These fibres fatigue rapidly. Slow fibres, also known as Type I fibres, take three times as long to contract as fast fibres; however they can keep contracting for extended periods of time. As such muscle composed of slow fibres has more mitochondria and capillaries than muscle composed of fast fibres. Slow fibres also contain stores of oxygen bound to myoglobin, and can aerobically produce energy and thus contractions for long periods of time. The third kind of myofibres are intermediate fibres, also called Type II-A fibres. Intermediate fibres have characteristics in-between those of fast and slow fibres (Martini, 1998).

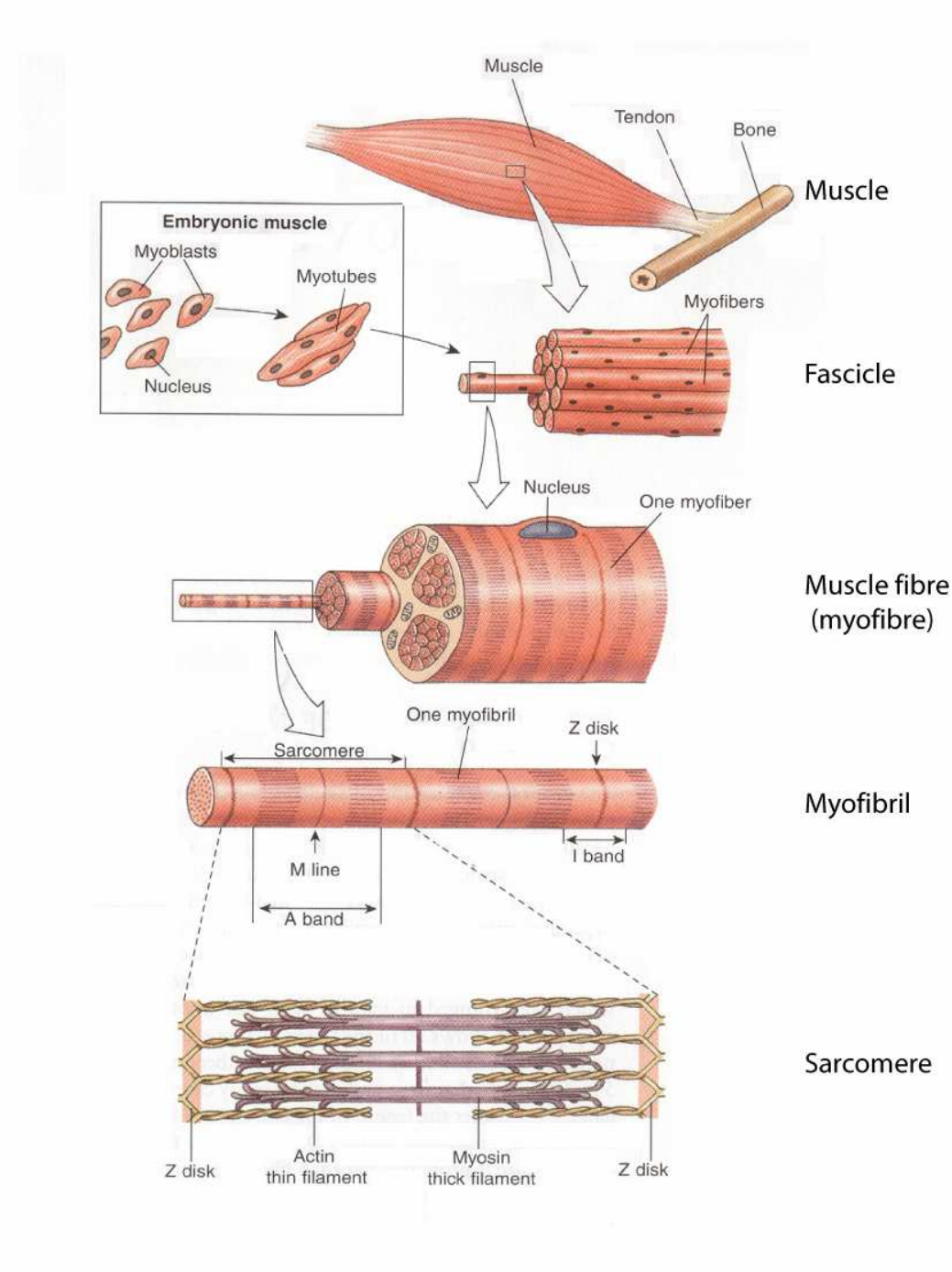

Figure 1.1: Skeletal muscle structure.
Muscle consists of bundles of cells called fascicles. Each multinucleate cell contains bundles of myofibrils which are composed of repeating units of sarcomeres. Each sarcomere is composed of highly ordered thick and thin filaments. Modified from Randall *et al*., 1997.

1.1.2 Myogenesis

1.1.2.1 Formation of somites and myogenic precursors

During embryogenesis the somites form from the paraxial mesoderm as balls of epithelial cells. Somites are paired on either side of the neural tube and notochord and form in a rostral-caudal direction. As embryogenesis continues the somites differentiate dorsally to form dermomyotome and ventrally to form sclerotome. The dermomyotome is divisible into two segments, the epaxial domain which gives rise to the muscles of the back, and the hypaxial domain which form the muscles of the body walls and limbs (Brand-Saberi and Christ, 1999). Signals from the tissues surrounding the somite influence the formation of the dermomyotome. The neural tube, notochord and surface ectoderm all secrete factors that influence this process (Cossu *et al*., 1996). Bone morphogenetic protein 4 (BMP-4) secreted from the dorsal neural tube up-regulates the expression of Wnt-1 and Wnt-3a in the neural tube. These Wnt's go on to activate expression of Wnt-11 in the medial lip of the dermomyotome, ultimately causing precursor cells to move to the myotome compartment of mature somites. Noggin, which inhibits BMP-4 signalling, is produced by the somite and prevents BMP-4 mediated inhibition of myotome formation. Sonic hedgehog (Shh) produced by the notochord inhibits Wnt-11 near the site of production, keeping Wnt-11 actions localised to the dorso-medial lip (Marcelle *et al*., 1997; Reshef *et al*., 1998).

1.1.2.2 Migration to the limb bud

The cells of the dermomyotome have been shown to be the precursors of the limb muscle. Cells from the somites migrate from the dermomyotome to the limb buds in a complicated and highly regulated process. Several factors must

be expressed correctly for migration to occur. Pax3, a paired domain transcription factor, has been shown to be essential for muscle precursor migration to the limb buds, as well as for the maintenance of the hypaxial dermomyotome (Relaix *et al*., 2004). In migratory cells Pax3 activates the expression of c-met (Epstein *et al*., 1996) which along with its ligand, scatter factor/HGF, is essential for precursor migration. In mice where either the c-met receptor or its ligand is knocked out, the development of the dermomyotome happens normally and migratory cells are formed but fail to delaminate and migrate away from the somites, resulting in loss of muscle masses formed by these cells (Dietrich *et al*, 1999). Lbx1 is a homeobox factor that has also been shown to influence migration of muscle precursors. In knock-out mice, cells delaminate and are able to migrate to form ventral muscle masses such as the diaphragm and tongue, but fail to respond to migration cues from the limb bud (Gross *et al*, 2000). Migration of muscle precursors also involves factors that are involved with cell adhesion and cytoskeletal organisation such as calpain (Dedieu *et al*, 2004) fibronectin (Brand-Saberi *et al*., 1993) and N-cadherin (Brand-Saberi *et al*., 1996). Cells that migrate to the limb bud are maintained in an undifferentiated state. Activation of Notch by Delta1 has been shown to inhibit differentiation in the chick limb, and may also act in this capacity during the migration phase (Delfini *et al*., 2000). BMP-4 has been shown to inhibit myogenesis during dermomyotome formation (Marcelle *et al*., 1997; Reshef *et al*., 1998) and is another candidate for inhibition of myogenesis during migration.

1.1.2.3 Muscle formation in the limb bud

After migration to the limb bud, cells undergo a period of proliferation to increase cell numbers. Proliferation is associated with cells that are both Myf-5 and Pax3 positive in the chick limb bud (Delfini *et al*., 2000). Several factors have been implicated in this proliferative stage, including *msx1*, which is a homeobox factor (Houzelstein *et al*., 1999), hepatocyte growth factor (Yamane *et al*., 2004) and fibroblast growth factor (Itoh *et al*., 1996). The formation of skeletal muscle requires the induction of the myogenic regulatory factors (MRFs), which are members of the basic helix-loop-helix transcription factor family. They are induced and specify cells to the muscle lineage. Members of this family have a conserved DNA binding domain that recognises Ephrussi-boxes (E-boxes) within the promoters of most myogenic genes. They also contain a heterodimerisation site that allows them to form dimers with other members of the E-protein family and bind to the consensus DNA binding site of CANNTG (Weintraub *et al*., 1991; Olson and Klein, 1994; Rudnicki and Jaenisch 1995). The first of these family members to appear in the mouse embryo limb bud is Myf-5, followed by myogenin and MyoD, then MRF4 (Hannon *et al*., 1992). MyoD and Myf-5 appear to be involved in the specification of myoblast precursors and have partially overlapping roles in myogenesis. Lack of either factor does not result in a loss of skeletal muscle although Myf-5-null mice die from rib defects (Braun *et al*., 1992; Rudnicki *et al*., 1992). Double MyoD/Myf-5 knock-out mice completely lack myoblasts and differentiated muscle fibres (Kablar *et al*., 2003), indicating that either factor is sufficient to specify cells to the myogenic lineage. Myogenin is required for terminal differentiation of myoblast cells. Mice that lack this factor form very little skeletal muscle yet still have myogenic cells in the limb buds (Nabeshima

et al., 1993). The expression of MRF4 has an early peak in the embryo before it tapers off, only to be expressed again after myogenin (Lin-Jones and Hauschka, 1996). MRF4 expression in skeletal muscle continues to remain high from this point and is expressed throughout adult life (Hinterberger *et al*., 1991). MRF4 plays a role similar to myogenin during myogenesis. In myogenin-null mice muscle fibre formation can be restored by constitutively expressed MRF4 (Sumariwalla and Klein, 2001). More recently, two MRFs have been shown to interact with chromatin remodelling enzymes in the process of initiating myogenic gene transcription. Myogenin acts by cooperating with Mef2D and Brg1 chromatin remodelling enzymes to alter the chromatin around the promoters of myogenic genes, making transcription of these genes more likely (Ohkawa *et al*., 2006). MyoD also targets Brg1 chromatin remodelling enzymes to the promoters of approximately one third of MyoD induced genes. It has been theorised that MyoD activates myogenin by binding to Pbx sites in conjunction with Pbx1/Meis proteins. After chromatin structure is altered, MyoD is then capable of binding to the E-box sites of the promoter and inducing transcription (de la Serna *et al*., 2005).

Once commitment to the myogenic lineage and terminal differentiation occurs, myoblasts fuse together in two waves to form primary and secondary fibres. Primary myotubes begin to form in the mouse *extensor digitorum longus* (EDL) by embryonic day-12 (E-12) (Ontell and Kozeka, 1984a), in the mouse soleus by E-14 (Ontell *et al*., 1988) and in the rat lumbrical muscle by E-15 (Ross *et al*., 1987). There is a lag time of two days after the formation of the primary myofibres before the formation of secondary fibres occurs. Secondary fibres initially form in close association with primary fibres and as they mature they

disassociate from the primary fibre. A single primary fibre may have secondary fibres of varying maturity associated with it (Ontell and Kozeka, 1984a; Ontell and Kozeka, 1984b; Ross *et al*., 1987; Ontell *et al*., 1988).

The association of myoblasts and their fusion to form myotubes requires several cell adhesion molecules. Neural cell adhesion molecule (NCAM) is up-regulated as myotubes begin to form and incubation of myoblasts with antibodies against this molecule prevents aggregation of fusion competent myoblasts. Both N-cadherin and M-cadherin are expressed in myoblasts and are up-regulated at the beginning of myoblast fusion. Other cell adhesion molecules, such as ADAM-12 (a disintegrin and metalloproteinase) and the integrin VLA-4 are expressed on myoblasts at the time of myotube formation (Abmayr *et al*., 2003). Although animals that have had NCAM and N-cadherin knocked-out have been generated, these animals are normal and fertile, indicating that other molecules involved in the fusion process are capable of compensating for their loss (Abmayr *et al*., 2003). More recently other molecules involved in the fusion process have been identified. Myoferlin and dysferlin are required for myoblast fusion. In the case of myoferlin-null animals, myotube formation is impaired, although muscle still forms, indicating that other members of this family, or other adhesion molecules compensate for its loss (Doherty *et al*., 2005). Melanoma cell adhesion molecule (M-CAM) has also been shown to be involved in the fusion process. However, it is the down-regulation of this molecule that allows fusion to occur, not the up-regulation as seen with other molecules (Cerletti *et al*., 2006).

1.2: The Satellite Cell

Post-natal Muscle growth and regeneration

1.2.1 Satellite cell physiology

The satellite cell is a myogenic precursor cell that is responsible for the growth and repair of skeletal muscle during post-natal life. Satellite cells were first identified by Katz (1961) in frog muscle and have since been shown to exist in all vertebrate muscle. Quiescent satellite cells are mono-nuclear and are located at the outside edge of mature myofibres, between the fibre and the basal lamina (Figure 1.2). Other distinguishing anatomical features that identify the satellite cell are a high nuclear-to-cytoplasmic ratio, an increase in nuclear heterochromatin and a smaller nuclear size compared to myonuclei. As satellite cells activate they appear as swellings on the myofibre with cytoplasmic processes extending from the poles. They undergo an increase in the nuclear-to-cytoplasm ratio and have more intracellular organelles (Schultz and McCormick, 1994). Satellite cells that are associated with muscle fibres appear at embryonic day-19 in the mouse (Cardasis and Cooper, 1975). The dorsal dermomyotome, which during embryogenesis provides the precursor cells for the limb musculature, also provides the cells that populate the satellite cell niche (Gros *et al*., 2005).

Satellite cells can not be identified using phase contrast light microscopy. Immunocytochemistry allows for identification of these cells by the expression of several gene products. Two recently characterised markers for satellite cells are Sca-1 and CD34. Stem cell antigen-1 (Sca-1, also known as Ly-6A) was first identified in the haematopoietic system as an antigen that allowed for

purification of stem cells from bone marrow (Spangrude *et al*., 1988). Sca-1 expression is also found within the spleen, thymus, kidney and within the vasculature of the brain, heart and liver (van de Rijn *et al*., 1989). More recently Sca-1 has been identified on cells from mouse carcinomas, sarcomas and melanomas (Lollini *et al*., 1992), on murine oesteoblasts (Horowitz *et al*., 1994) and liver sinusoidal endothelial cells (Luna *et al*., 2004). The gene family to which Sca-1 belongs (the Ly-6 family) is located on chromosome 15 (LeClair *et al*., 1987). Sca-1 is a membrane protein attached to the membrane via a glycosylphospha-idilinositol moiety. This attachment has been shown to be essential for Sca-1 to activate T-cells (Su *et al*., 1991).

Sca-1 has also been identified in muscle. Jankowski *et al*. (2001) showed that Sca-1 is expressed on the surface of some muscle derived stem cells. Shen *et al*. (2003) identified Sca-1 as being up-regulated 19.5-fold at the beginning of C_2C_{12} differentiation. Sca-1 was expressed by a sub-population of differentiating C_2C_{12} cells at the time of cell cycle withdrawal. Blocking Sca-1 expression with antibodies resulted in cells that remained proliferative and did not form myotubes (Epting *et al*., 2004). Mitchell *et al*. (2005) also described two myoblast populations, one that was Sca-1 negative and proliferated rapidly and underwent differentiation, and one that was Sca-1 positive and proliferated slowly and did not readily differentiate. They hypothesised that Sca-1 allows a subset of cells to remain in an undifferentiated state much like that of the reserve cell population described by Yoshida *et al*. (1998).

CD34 is a cell surface glycophosphoprotein member of the sialomucin family of surface molecules. It was discovered during the development of antibodies that

recognise subsets of marrow cells but not mature blood or lymphoid cells. CD34 exists as a splice variant. The full length form is made from exons one through eight of the gene, and has a 73 amino acid intracellular domain. The truncated form is made when exon X is inserted between exons seven and eight, introducing a premature stop codon. The resulting truncated protein has only a 16 amino acid intracellular domain. Characterisation of the function of CD34 is not complete, but several roles have been theorised based on the homology CD34 has with other proteins. CD34 is thought to be involved in cell-cell adhesions and in haematopoietic cell differentiation. CD34 is expressed by early lymphohematopoietic stem and progenitor cells, small vessel endothelial cells and embryonic fibroblasts (Krause *et al*., 1996).

More recently, CD34-positive cells have been found within adult tissues not of haematopoietic origin. CD34 positive cells have been identified as fibroblasts within human breast tissue (Yamazaki and Eyden, 1995) and as fibroblasts associated with the interstitial cells of Cajal in the human intestine (Vanderwinden *et al*., 1999). CD34 is used as a marker of muscle-derived stem cells. These cells are capable of participating in skeletal muscle repair, as shown by the expression of dystrophin in dystrophin-deficient (mdx) mice (Lee *et al*., 2000; Torrente *et al*., 2001). CD34-positive cells, isolated from mouse muscle by adherence characteristics, have a decreased fusion index compared to CD34-negative cells yet are capable of restoring dystrophin expression in mdx muscle to a greater degree (Jankowski *et al*., 2002). CD34 has also been used as a marker of quiescent satellite cells. Beauchamp *et al*. (2000) showed that CD34 was expressed by quiescent satellite cells. These cells also expressed Myf-5, indicating that they were committed to the myogenic lineage, and m-cadherin, a

known satellite cell marker. Thus CD34 is both a marker of satellite cells and of a population of muscle-derived stem cells.

Other markers of satellite cells have been identified by several different groups. Cornelison and Wold (1997) showed that c-met receptor tyrosine kinase was expressed by both the quiescent and activated cells seen in fibre culture and in mononucleated muscle-derived cells. Syndecan 3 and 4 from the syndecan family of heparan sulphate proteoglycans are also expressed specifically in satellite cells (Cornelison *et al*., 2001). Satellite cells can be marked by the levels of lipid components in their plasma membranes. Nagata *et al*. (2006) showed that quiescent satellite cells bound lysenin to their plasma membrane, while activated satellite cells and myotubes did not. Garry *et al*. (1997) demonstrated that myocyte nuclear factor (MNF) was expressed persistently only in quiescent satellite cells and that as cells are activated, the expression declines. Sox8 has persistent expression in satellite cells and proliferating muscle precursor cells, before down-regulation during differentiation (Schmidt *et al*., 2003). Caveolin-1 has recently been identified on quiescent satellite cells, and its expression is down-regulated as they are activated (Volonte *et al*., 2005). M-cadherin, a member of the calcium dependent cell adhesion molecule family, has also been identified as a marker of satellite cells. Beauchamp *et al*. (2000) showed that 80% of quiescent satellite cells express M-cadherin, while Wernig *et al*. (2004) have demonstrated that up to 94% of cells occupying the satellite cell niche were positive for M-cadherin. Myf-5 has also been shown to be expressed by at least 80% of quiescent satellite cells (Beauchamp *et al*., 2000). In situ hybridisations have revealed that Pax7 localises to satellite cells and

Western blot analysis indicates that this protein is also expressed in proliferating muscle precursor cells (Seale *et al*., 2000).

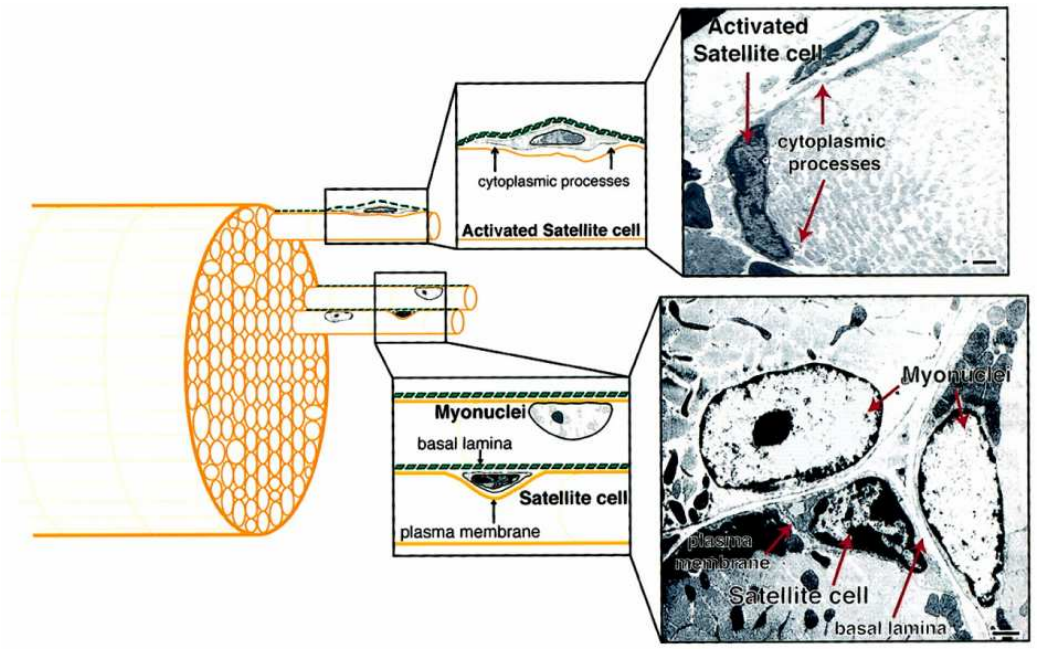

Figure 1.2: Satellite cell position on myofibres
Satellite cells reside between the basal lamina and the myofibre membrane (sarcolemma). Hawke and Garry, 2001.

1.2.2 Satellite cell function

Satellite cells are a distinct lineage of myogenic precursors that are responsible for the growth of muscle during post-natal life and for its repair after damage. During muscle growth and regeneration satellite cells are activated in response to growth signals from the environment, which induce the expression of one or both of the two MRFs, Myf-5 or MyoD. Proliferating satellite cells will all express MyoD and some maintain Myf-5 expression (Cooper *et al*., 1999). These transcription factors go on to activate transcription of myogenic genes. Activated satellite cells can migrate from different areas of muscle to proliferate and participate in repair. The myoblasts can then adopt one of two fates. Some myoblasts initiate terminal differentiation and are able to either fuse into existing myofibres to repair them, or fuse with other myoblasts to form new fibres. Other myoblasts do not differentiate but instead return to quiescence and adopt a satellite cell position on repaired or newly formed fibres (Hawke and Garry 2001). Figure 1.3 illustrates this process. The mechanisms controlling satellite cell activation, proliferation, differentiation and self-renewal are discussed in the following sections.

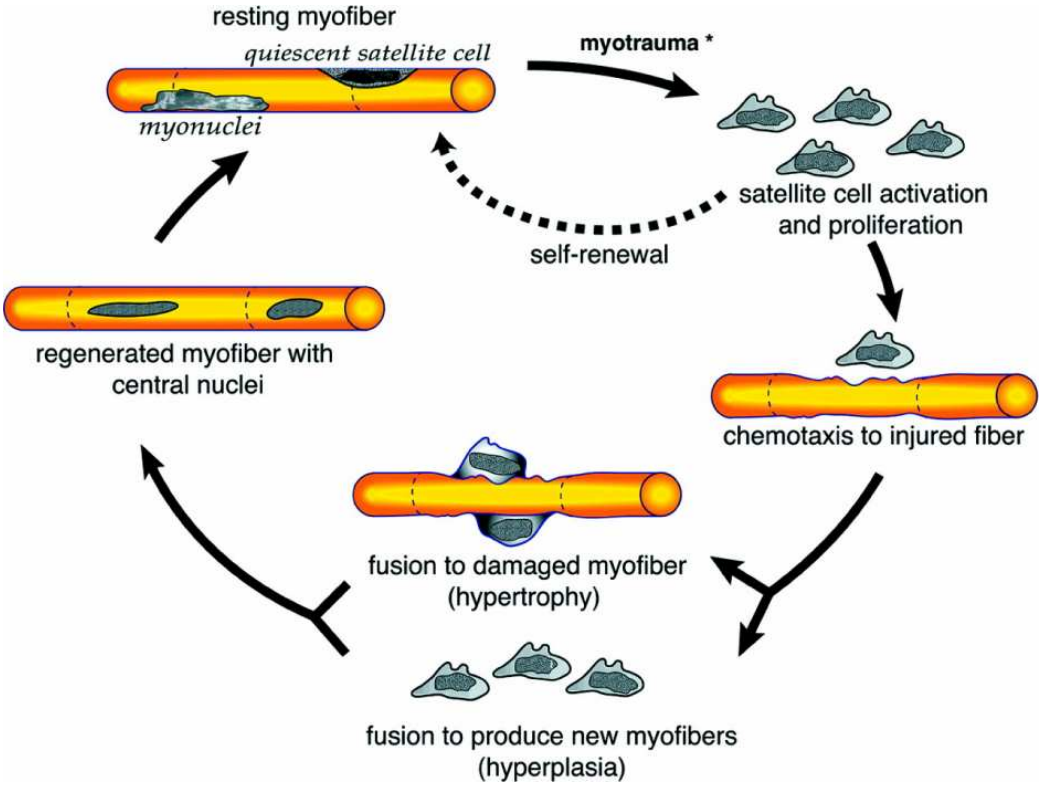

Figure 1.3: Satellite cell function
In response to activating signals, such as myotrauma, satellite cells activate, proliferate and differentiate to repair muscle. Hawke and Garry, 2001.

1.2.3 Regulation of satellite cell activation, proliferation and differentiation

For satellite cells to play a role in the post-natal growth and repair of muscle they must be activated, migrate to the site of growth or injury, and proliferate before entering terminal differentiation. Various growth factors play roles in these processes, both as positive and negative regulators. Injured fibres produce many of the factors involved.

Hepatocyte growth factor (HGF), the ligand for the c-met receptor, is expressed during muscle injury. The expression of HGF is proportional to the extent of muscle damage and exogenous injection of HGF into undamaged muscle of rats activates satellite cells (Tatsumi *et al*., 1998). Addition of exogenous HGF to regenerating muscle increases the number of satellite cells present, but depending on the time of injection can delay the regeneration process by inhibition of differentiation (Miller *et al*., 2000). HGF-mediated increases in satellite cell proliferation are dose dependent (Gal-Levi *et al*., 1998). Caveolin-1 has recently been identified as a marker of quiescent satellite cells and is a downstream target of HGF signalling. HGF signalling down-regulates caveolin-1 as a part of normal satellite cell activation (Volonte *et al*., 2005).

Insulin-like growth factors (IGF) I and II both play a role in the activation of satellite cells. IGF-I is involved in activation of satellite cell proliferation via the IGF-I receptor (Yang and Goldspink, 2002). IGF-I stimulates proliferation and differentiation of myoblasts and can activate satellite cells, causing hypertrophy (Florini *et al*., 1996; Gal-Levi *et al*., 1998). More recently the IGF-I transcript has been shown to be alternately spliced into two different isoforms. One form, called mechano-growth factor, peaks one day after mechanical injury to muscle

and activates satellite cells and enhances their proliferation. IGF-IEa, the canonical form of IGF-I, peaks seven days after injury and enhances both proliferation and differentiation of the activated satellite cells (Yang and Goldspink 2002; Hill and Goldspink, 2003). IGF-II has been shown to stimulate myoblast differentiation at low concentrations and inhibit differentiation at high concentrations (Florini *et al*., 1996). IGF-II also influences regeneration of skeletal muscle after injury. The first stages of regeneration after injury are delayed by IGF-II treatment. However, late regeneration, when fibre enlargement occurs, is enhanced by IGF-II treatment (Kirk *et al*., 2003).

Fibroblast growth factor (FGF) isoforms 1, 2, 4, 6, and 9 all act to stimulate myoblast proliferation (Sheehan and Allen, 1999), while reducing satellite cell differentiation (Spizz *et al*., 1986). FGF-2, like HGF, is released during muscle injury in proportion to the extent of injury (Clarke *et al*., 1993). When FGF-2 is neutralised with antibodies, the number of regenerating fibres in damaged muscle is decreased (Lefaucher and Sebille, 1995).

Nitric oxide (NO) may be a mediator of satellite cell activation. Inhibition of nitric oxide synthase (NOS) is capable of inhibiting injury induced satellite cell activation. NOS activity is reduced in dystrophic muscle (Anderson, 2000) and addition of an NO donor to dystrophic muscle that has been damaged with bupivacaine significantly improves the regeneration of the muscle (Marques *et al*., 2005).

Vascular endothelial growth factor (VEGF) receptors Flk-1 and Flk-2 are predominantly expressed on quiescent satellite cells of normal muscle. After

induction of muscle injury via ischemia they are present on activated satellite cells as well as newly formed myofibres, but very rarely on mature fibres. VEGF is a chemo-attractant for myoblasts and increases the differentiation of C_2C_{12} cells in culture. VEGF is capable of reducing the number of apoptotic cells during differentiation, indicating that it acts as a survival factor for myoblasts (Germani *et al*., 2003).

Leukaemia inhibitory factor (LIF) is a cytokine that belongs to the interleukin 6 family. It is capable of inhibiting myoblast differentiation *in vitro* (Jo *et al*., 2005) and administration to undamaged muscle is capable of increasing muscle fibre size (Gregorevic *et al*., 2002). LIF appears to act as a myoblast survival factor (White *et al*., 2001a). Addition of LIF to dystrophic mice that have received donor myoblasts results in an increase in donor derived dystrophin expression and improves the regeneration of the muscle (White *et al*., 2001b). LIF has also been shown to enhance regeneration in normal mice. Barnard *et al*. (1994) showed that administration of LIF to the site of a crush injury resulted in an increased regeneration rate and hypertrophy.

Notch signalling plays a role in the regulation of satellite cell activation, proliferation and differentiation during muscle growth and repair. The repair of aged muscle can be restored to the levels seen in young mice by forced activation of the Notch receptor by antibodies against it (Conboy *et al*., 2003). Notch signalling inhibits MyoD expression and results in a population of cells that proliferate (Kopan *et al*., 1994). Inhibition of Notch signalling, by antagonists such as Numb, allows the myogenic program to be activated and commitment of the muscle precursors to occur (Nofziger *et al*., 1999; Conboy

and Rando, 2002). Inhibition of Notch signalling influences the self-renewal of satellite cells, and is capable of inducing myotube hypertrophy in cell culture by recruiting a sub-population of reserve cells into committed myogenensis (Kitzmann *et al*., 2006).

Macrophages play an important role in muscle regeneration after injury. After injury muscle releases chemo-attractant cytokines and growth factors for macrophage and polymorphonuclear leukocyte migration and proliferation (Robertson *et al*., 1993). One such chemo-attractant is TNF-α, which has been shown by Chen *et al.* (2005) to be required for muscle regeneration. Although in some circumstances expression of TNF-α can cause cachexia (Tracey *et al*., 1990), the expression of TNF-α in muscle increases due to myofibre injury (Zador *et al*., 2001). Blockade of TNF-α signalling by a double knock-out of the TNF-α receptors resulted in regeneration defects due to a lack of p38-mediated signalling events.

Both neutrophils (Kuipers *et al*., 1983) and macrophages expressing the ED1+ and ED2+ antigens are involved with phagocytosis of damaged myofibres and cellular debris (Honda *et al*., 1990; Massimino *et al*., 1997). Macrophages secrete several factors, including LIF and FGF, to attract satellite cells to the site of injury and enhance their proliferation and differentiation (Robertson *et al*., 1993; Massimino *et al*., 1997). Depletion of the macrophage population by irradiation prior to muscle transplants abolishes regeneration in the host (Lescaudron *et al*., 1999). Macrophages have also been proposed to provide structural support for muscle precursor cells which can aid their growth and

prevent susceptibility to apoptosis through secretion of soluble mitogenic factors and cell contact-mediated survival signals (Chazaud *et al*, 2003).

Syndecan-3 and syndecan-4, which are co-receptors for tyrosine kinases, have been shown to be expressed by satellite cells. Syndecan-3-null mice display a dystrophic phenotype, and isolated satellite cells show defects in MyoD nuclear localisation and thus differentiation. Syndecan-4-null mice do not display an overt dystrophic phenotype but their satellite cells have an impaired ability to activate, proliferate, fuse and differentiate, and like the syndecan-3-null cells they have MyoD localisation defects. Thus signalling through these receptors is necessary for correct satellite cell behaviour (Cornelison *et al*., 2004).

Transforming growth factor-beta (TGF-β), a member of the TGF-β superfamily, is a regulator of myogenensis. Several groups have reported that TGF-β is an inhibitor of myoblast differentiation in cell culture (Florini *et al*., 1986; Massague *et al*., 1986; Olson *et al*., 1986). TGF-β is also capable of inhibiting satellite cell proliferation and satellite cell differentiation (Allen and Boxhorn, 1987). Allen and Boxhorn (1989) showed that TGF-β cannot reduce proliferation in the presence of mitogens such as IGF-I and FGF, although inhibition of differentiation was maintained. Thus the role of TGF-β in post-natal myogenesis and regeneration is to enhance satellite cell accumulation by inhibition of premature differentiation. Bone morphogenic protein (BMP), another member of the TGF-β super-family, also inhibits terminal differentiation of myoblasts, by decreasing the activity of MyoD and myogenin (Katagiri *et al*., 1997).

Myostatin, also known as growth/differentiation factor-8 (GDF-8), is also a member of the TGF-β superfamily. Myostatin shares many characteristics with the TGF-β family members. It has a hydrophobic amino acid core that acts as a secretion signal, conserved cysteine residues at the c-terminus, and a proteolytic processing site (Arnold *et al*., 2001; Sharma *et al*., 2001). The Belgian Blue and Peidmontese cattle breeds are characterised by heavy muscling and both have natural mutations in the myostatin gene (Kambadur *et al*., 1997; McPherron *et al*., 1997). Myostatin is synthesised as a precursor protein (52 kDa) and proteolytically processed to form a 26 kDa mature peptide in myoblasts. The mature myostatin protein is then secreted from the myoblasts (Thomas *et al.*, 2000). *In vitro* myostatin negatively regulates both proliferation and differentiation of myoblasts through two mechanisms. Proliferation is inhibited by up-regulation of p21, a cyclin-CDK inhibitor, causing some of the myoblasts to arrest in the G1-phase of the cell cycle (Thomas *et al.*, 2000). Inhibition of differentiation in myoblasts occurs through a Smad3 dependent down-regulation of MyoD (Langley *et al.*, 2002).

Myostatin is a negative regulator of satellite cells at many levels. Skeletal muscle of myostatin-null mice contains more satellite cells per fibre than wild-type mice. These satellite cells also have a greater propensity to activation and proliferation than cells isolated from wild-type mice (McCroskery *et al*., 2003). More recently McCroskery *et al*. (2005) have demonstrated in an *ex vivo* system that the presence of myostatin inhibits the activation of satellite cells on isolated muscle fibres, maintaining the quiescent state. Myostatin also prevents chemotaxis of satellite cells and macrophages, inhibiting their ability to participate in regeneration events after muscle damage. After muscle injury,

myostatin-null mice have twice the number of myogenic cells, identified by MyoD at the site of injury than wild-type mice. Healing is more complete with less fibrosis and scar tissue when myostatin is absent (McCroskery *et al*., 2005).

1.2.4 Satellite cell heterogeneity

Studies of satellite cells on isolated fibres have shown that they can be divided into two populations based on the time taken to complete the cell cycle. The majority (80%) take 32 hours to complete the cell cycle, while the remaining cells divide much more slowly (Schultz, 1996). Satellite cells also display heterogeneity with respect to fusion ability. Highly proliferative satellite cells predominantly fuse with existing myofibres, while slowly proliferating satellite cells predominantly fuse together (Rouger *et al*., 2004). Distinct sub-populations of cells can be derived from satellite cell culture. When C_2C_{12} cells are induced to differentiate a proportion of the cells commit to terminal differentiation, while a different population withdraw from the cell cycle and do not differentiate. Isolation and culture of undifferentiated cells results again in the formation of two populations of cells, one differentiated, one undifferentiated (Yoshida *et al*., 1998). Satellite cells also exhibit this heterogeneity with respect to proliferation (Barani *et al*., 2003) and fusion (Baroffio *et al*., 1996), indicating that the C_2C_{12} culture is a good *in vitro* model of satellite cells. In addition to the heterogeneity seen with respect to cell cycle time, fusion and ability to terminally differentiate, satellite cells differentially express satellite cell markers such as CD34 and Myf-5, as described in Section 1.2.1.

1.2.5 Satellite cell self-renewal

To maintain the capability of muscle to repair during post-natal life the pool of cells responsible for this repair, the satellite cells, must be maintained. Satellite cells are persistent in degenerative myopathies such as muscular dystrophy, indicating an ability to self-renew (Bischoff, 1994). Pathways involved for self-renewal of satellite cells are complex, as the cell must either withdraw from a proliferative state to G0 or undergo cell cycle arrest at the G1/S transition.

Evidence for self-renewal has come from cell labelling studies. When cells expressing a nuclear localising LacZ gene are injected into dystrophic muscle, nuclei that stain positive for β-Gal staining are derived from donor nuclei. Nuclei staining positive are found not only within existing myofibres, but within the satellite cell niche. Isolation and culture of these cells gives rise to further myoblasts, indicating that they are functional satellite cells (Blaveri *et al*., 1999; Heslop *et al*., 2001). Evidence for a role of the MRF's in self-renewal has come from studies of MyoD-null mice. MyoD-null cells have higher levels of Myf-5, display differentiation defects, and have an increased tendency towards self-renewal than wild-type cells. One theory to account for this is that Myf-5 is involved in specification of the cells towards self-renewal (Megeney *et al*., 1996; Sabourin *et al*., 1999). Pax7 may also be involved in satellite cell self-renewal or muscle-stem-cell specification. Pax7-null mice appear to completely lack satellite cells yet they still have a population of cells called side population cells (SP). SP cells from normal mice, when cultured under certain conditions, will give rise to cells from both the haematopoietic and the myogenic lineages. SP cells from the Pax7-null mice have an increased tendency towards forming haematopoietic colonies, indicating that Pax7 is involved in satellite cell

specification (Asakura *et al*., 2002). Dedifferentiation of terminally differentiated cells may be another way in which the satellite cell niche is repopulated. Forced expression of *msx1,* a homeobox transcription factor, in C_2C_{12} myoblast lines is capable of causing myotubes to cease expression of MRF's and give rise to proliferating mononuclear cells (Odelberg *et al*., 2000).

Asymmetrical cell division, which results in one daughter cell adopting a differentiation committed state and the other returning to quiescence is proposed to be a mechanism allowing myoblasts to self renew. Zammit *et al*. (2004) have shown that the majority of satellite cells co-express Pax7 and MyoD after activation. Some of these cells will down-regulate Pax7 and differentiate, while others maintain Pax7, down-regulate MyoD and withdraw from differentiation, presumably to repopulate the quiescent satellite cell niche. Notch signalling is also coming to be understood as a regulator of satellite cell quiescence. Proliferative progeny of satellite cells localise Numb, an inhibitor of Notch signalling, asymmetrically during division, with one daughter cell becoming Numb+ and one becoming Numb-. Numb+ cells go on to express markers of myogenic commitment, such as Myf-5 and desmin, while Numb- cells retain Pax3 expression and may re-enter the quiescent state (Conboy and Rando, 2002). Self-renewal of satellite cells is negatively regulated by myostatin. Myostatin-null mice have a higher proportion of CD34-positive cells per unit fibre length than their wild-type counterparts, indicating that activated satellite cells have self-renewed to a greater extent than seen in wild-type mice (McCroskery *et al*., 2003).

1.2.6 Other sources of myogenic precursors

Other possible sources of myogenic precursors that participate in muscle regeneration have been identified. Mouse neural tube (Tajbakhsh *et al*., 1994), the periventricular region of the mouse brain (Galli *et al*., 2000) and human adipose tissue (Zuk *et al*., 2001) all contain cells that are capable of expressing myogenic markers. The haematopoietic system is another possible source of myogenic cells. Circulating AC133+ cells isolated from blood can express the myogenic markers Myf-5, M-cadherin and Pax7, and will differentiate into muscle when co-cultured with committed myogenic cells (Torrente *et al*., 2004). Ferrari *et al*. (1998) reported that donor bone-marrow-derived nuclei that stained positive for β-Gal were present within muscle fibres of regenerating TA of immune deficient mice, although at a lower frequency than that seen in control muscles injected with donor satellite cells. Recently, however, some doubt has been present as to the extent that bone-marrow derived cells can participate in functional restoration of muscle. Lapidos *et al*. (2004) demonstrated that bone marrow derived cells could fuse with existing myofibres, but their ability to produce muscle specific genes to alleviate a muscular dystrophy phenotype was impaired.

A different source comes from a lineage of CD45+ cells that reside within the muscle. These cells, called side population (SP) cells for their ability to exclude Hoechst dye, are found in all muscle including that from Pax7-null mice which lack satellite cells. SP cells from normal mice will form haematopoietic colonies in cell culture when cultured alone, and differentiate into myotubes when co-cultured with committed myogenic precursors (Asakura *et al*., 2002). SP cells are also able to undergo myogenic commitment during regeneration in response

to Wnt signalling (Polesskaya *et al*., 2003). Induced muscle damage increases the number of CD45+ cells present, and a large number of these can acquire myogenic potential (Seale *et al*., 2003).

1.3 Mighty

1.3.1 Discovery and characterisation of Mighty

Novel downstream targets of myostatin have been identified by the Functional Muscle Genomics (FMG) Laboratory, AgResearch, Ruakura, in order to elucidate the mechanisms by which myostatin is able to negatively regulate muscle mass. One of the target genes, identified as being up-regulated 58% in myostatin-null mice by suppressive subtraction hybridisation, is a cDNA that has been called Mighty. At the time of discovery the protein encoded by this cDNA had no known function. Characterisation of the Mighty gene by homology searches, calculations of the theoretical size and pI of the protein, secondary structure predictions, protein domain homology searches, prediction of post-translational modifications and subcellular localisation predictions have all been carried out.

Mighty has been shown by Northern analysis to be up-regulated specifically in mouse skeletal muscle, from a 48% increase in the *m. gastrocnemius* (the lowest increase seen) to a 106% increase in the *m. masseter* in mice. The mouse Mighty cDNA shares 96% and 89% homology and the protein shares a 98% and 93% homology with the rat and the human respectively. Conservation of the Mighty sequence is seen to some extent throughout the animal kingdom, implying that the function of Mighty is biologically important. Mouse Mighty has been located to chromosome four and contains five exons and four introns. The predicted protein size is 22 kDa and the protein should have a pI of 8.91. There is a high probability that the protein is localised to the nucleus, however, any DNA binding domains are yet to be characterised. Several possible

phosphorylation sites exist within the protein, along with two possible myristoylation sites.

The Mighty protein is predicted to have a secondary structure that consists of three α-helices. Tertiary structure is difficult to predict as the sequence has little homology with proteins of known function. A possible forkhead domain is predicted for Mighty, but as it shares little homology with known proteins of this kind it is likely to be either a distantly related or a novel member of this family (Bishop, 2005).

1.3.2 Mighty cellular localisation

Results (unpublished) from the Functional Muscle Genomics Laboratory show that endogenous Mighty protein localises to speckles in the nucleus, to perinuclear regions and to the endoplasmic reticulum. Mighty has been shown to be co-localised with lamin, a protein of the nuclear lamina in the nuclear speckles. This localisation of Mighty with lamins, which have been shown to bind DNA at matrix attachment regions (MARs) (Luderus *et al*., 1992, 1994) of regulatory DNA sequences (Boulikas, 1994, 1995), may be a possible mechanism for Mighty to regulate gene expression.

1.3.3 Mighty over-expression

Over-expression of Mighty in the C_2C_{12} cell line causes myoblast hypertrophy but does not affect the rate of cell proliferation as measured by methylene blue assay. Mighty over-expressing clones show an enhanced fusion phenotype and the resulting myotubes are hypertrophied. Regulatory molecules of differentiation, such as MyoD and Myogenin, are up-regulated earlier in the

Mighty over-expressers. The differentiation marker myosin heavy chain (MHC) is seen earlier than in control cells. Conditioned media taken from Mighty clone cultures has the ability to alter the phenotype of normal C_2C_{12} cells to one like that of the over-expressers (Bishop, 2005).

1.3.4 Mighty promoter regulation

The Mighty promoter region has been shown to extend -1963 to +129 bp around the transcription start site. Promoter activity analysis indicates that the minimal promoter required extends from -158 to +129. Activity of the promoter decreases as more upstream elements are added, indicating that repressive as well as enhancer elements are found in these regions. Prediction of transcription factor binding sites suggest that the promoter contains TATA sequences, Sp1 binding sites, AP-1 binding sites, E-box motifs, two of which are MyoD specific, GATA binding motifs, an E2F binding site, a NFκB binding site, an E4BP binding site, an E47 binding site, MZF1 binding sites and AML-1a sites.

As expected, myostatin treatment is able to down-regulate the Mighty promoter in a dose-dependent fashion. Treatment of truncated fragments of the promoter with myostatin indicated that very little (80 bp upstream of transcription initiation) is required for myostatin-regulated inhibition. Further studies have shown that one pathway through which myostatin down-regulates the Mighty promoter is by binding to the ActIIB and ALK5 receptors which causes formation of inhibitory Smad 2/3 complexes. Myostatin is also able to negatively regulate the Mighty promoter via the MEK and pI3K pathways (Bishop, 2005).

1.4: Aims and Objectives

Satellite cells play an important role in post-natal growth and regeneration of muscle. Myofibre nuclei are post-mitotic and as such are unable to proliferate to meet an increase in muscle demand during growth and exercise-induced hypertrophy. Satellite cells are also activated after muscle injury, in response to various stimuli, to participate in the repair of damaged muscle fibres. Several factors regulate satellite cell activation. An important negative regulator of muscle satellite cells is myostatin. Lack of myostatin in myostatin-null mice results in larger muscles than their wild-type counterparts, with a larger pool of activated satellite cells that remain in the cell cycle longer before withdrawing for terminal differentiation. A genetic screen performed on myostatin-null and wild-type mouse muscle identified a novel gene 'Mighty' which is up-regulated in the knock-out muscle.

Mighty is a putative transcription factor that is up-regulated between 48% and 106% in the skeletal muscles of myostatin-null mice. Mighty is a putative transcription factor that has been shown to be involved in muscle hypertrophy. Over-expression of Mighty in C_2C_{12} cells results in an enhanced fusion and hypertrophic phenotype (Bishop, 2005). Thus our hypothesis is that Mighty is a transcription factor involved in the activation of satellite cells for growth and regeneration, like MyoD and Myf-5. The aims of this thesis were to firstly characterise the expression of Mighty in both quiescent and activated satellite cells from wild-type and myostatin-null mice, secondly to characterise Mighty expression during muscle regeneration, and thirdly to look at the effect of

Mighty over-expression on the reserve cell population of C_2C_{12} cells as an in vitro model of satellite cells.

Chapter Two

Materials and Methods

2.1 Materials

2.1.1 Oligonucleotide primers for PCR amplification

Oligonucleotide primers were designed for use in Polymerase Chain Reaction (PCR) amplifications to generate the respective gene products, and were obtained from Invitrogen. All oligonucleotides were re-suspended in 100 µl of sterile water and stored at -20^{o}C. Primers were diluted to 10 µM with sterile water for use in PCR reaction. All diluted primer aliquots were stored at -20^{o}C. The primers used are described in Table 1.

Table 1: Oligonucleotide Primers

Gene	Primer Sequence (5` to 3`)	Product size (bp)	Primer source
Mighty	Fwd: TGAAGCGGCCCATGGAGTTC Rev: GGTGGGCTGGTCCTTCTTCA	350	Designed in house
CD34	Fwd: AGCACAGAACTTCCCAGCAA Rev: CCTCCACCATTCTCCGTGTA	Trunc:416 Full: 250	Ref 12
Sca-1	Fwd: CGAGGGAGGGAGCTGTGAGGTT Rev: GAGGGCAGATGGGTAAGCAAAGAT	285	Ref 103
MyoD	Fwd: CGGCGGCAGAATGGCTACGA Rev: TGCAGTCGATCTCTCAAAGC	350	Ref 98
α-Tubulin	Fwd: GCTTCTTGGTTTTCCACAGC Rev: CATGGTAGGCTTTCTCAGCA	450	Designed in house

2.1.2 Mammalian cell lines

C_2C_{12} myoblasts (Yaffe and Saxel, 1977) were used for myotube and reserve cell enrichments and were sourced from the American Type Culture Collection (ATCC). C_2C_{12} cell lines over-expressing a Mighty gene construct were made in house. All cells were grown in Dulbecco's modified eagle medium (DMEM) +

10% fetal bovine serum + 20% horse serum for proliferation and in DMEM + 2% horse serum for differentiation, at 37^{o}C/5% CO_2.

2.1.3 Animals

Female and male wild type mice, strain C57BL/10, used in these experiments were bred at the Ruakura Small Animal Colony. Male and female myostatin-null mice, originally sourced from Se-Jin Lee (Johns Hopkins University, Baltimore, USA), were also bred in the Ruakura Small Animal Colony. All mice used in the study were 4-6 weeks of age. Animals were kept at constant temperature (20-22^{o}C) with a natural day/night cycle and food and water *ad libitum*. All animal manipulations were carried out according to the standard operating procedures approved by the Ruakura Animal Ethics Committee.

2.1.4 Antibodies

Antibodies used for immunocytochemisty (ICC) or Western blot in this thesis are described in Table 2.

Table 2: Antibodies

Antibody	Source	Use	Dilution
Mighty	QED Biosciences	Western	1:5000
Mighty	QED Biosciences	Cell ICC Tissue ICC	1:200 1:100
MyoD	Santa Cruz Biotechnology	Cell ICC	1:100
Desmin	Sigma Aldrich	Cell ICC	1:200

2.1.5 Solutions

Common solutions used were made according to Ausubel *et al*. (1987), Sambrook *et al*. (1989) and/or Lillie (1965) and are listed in the Appendix.

2.2 Methods

2.2.1 Isolation of satellite cells using Percoll gradients

Quiescent satellite cells were isolated from hind-limb muscle of mice using a protocol adapted from Yablonka-Reuveni and Nameroff (1987).

Four to five week old mice were euthanised by CO_2 aspyhxiation and cervical dislocation. The entire hind limb muscle was dissected out and weighed. Approximately 2 g of muscle was used per Percoll (Sigma Aldrich) column as described below. The muscle was thoroughly minced using a scalpel in PBS then transferred to a 50 ml tube and centrifuged at 600 rcf at room temperature for five minutes before being digested in Dulbecco's modified eagle medium (DMEM) without serum and containing 0.2% collagenase Type 1A at 37^{o}C and with constant shaking (70 rpm). After 90 minutes of digestion, 10 ml PBS was added to each digest followed by centrifugation at 1400 rcf. The supernatant, containing the collagenase, was removed and replaced with 10 ml PBS, and then the slurry was triturated for five minutes before being passed through a 70 µm filter. The filtered cells were centrifuged at 1400 rcf for ten minutes before re-suspension in 3 ml PBS. During centrifugation, a 90% Percoll solution was made from 9 parts Percoll to 1 part 10x PBS. Percoll solutions of 40% and 70% were made from the 90% Percoll using sterile 1x PBS, then used to create a gradient by underlying 3 ml of 40% Percoll with 3 ml of 70% Percoll in a 15 ml tube. The cell suspension was laid on top of the gradient, and then the gradient was centrifuged in a Sorval RC-3B refrigerated centrifuge in a H2000B rotor at 1450 rcf for 20 minutes at 25^{o}C without a brake. The top layer of the gradient containing fibroblasts and debris was discarded, and then the satellite cells from

the 70%/40% interface were removed to a clean 15 ml tube. The cells were washed with PBS then centrifuged at 1700 rcf for ten minutes. This wash step was repeated two to three times. Cells were re-suspended either in protein lysis buffer for Western analysis or in Trizol (Invitrogen) for RNA extraction.

2.2.2 Isolation of satellite cells by adherence to Matrigel coated plates

Activated satellite cells (primary myoblasts) were isolated from hind limb muscle of mice using a protocol adapted from Allen *et al*. (1997) and Partridge (1997).

Entire hind limb muscle was dissected out and thoroughly minced with a scalpel in phosphate buffered saline (PBS). The minced muscle was transferred to a 50 ml tube and centrifuged at 600 rcf at room temperature for five minutes before being digested in DMEM without serum and containing 0.2% collagenase Type 1A at 37^{o}C and with constant shaking (70 rpm). After 90 minutes of digestion, the sample was centrifuged at 1400 rcf for 10 minutes. The supernatant, containing the collagenase, was removed and replaced with 10 ml PBS. The slurry was triturated for 5 minutes then passed through a 100 µm filter, then a 70 µm filter. The volume of the sample was made up to 35 ml with sterile PBS then centrifuged at 600 rcf for ten minutes. After centrifugation the supernatant was removed and the cells were re-suspended in 8 ml satellite cell proliferation media. The cells were pre-plated on uncoated 10 cm Nunc cell culture Petri dishes for two hours at 37^{o}C/5% CO_2 to allow fibroblasts to attach. After pre-plating, the cell suspension was plated onto Matrigel-coated Nunc plates and incubated at 37^{o}C/5% CO_2 for 48 hours. After 48 hours the cells were washed with sterile PBS then trypsinised and re-suspended in Trizol (Invitrogen) for

RNA extraction and cDNA synthesis or protein lysis buffer for Western analysis.

2.2.3 Notexin injection to induce muscle regeneration

Both wild-type and myostatin-null mice were used in this trial. Mice were anaesthetised by intraperitoneal injection of ketamine/rompun (ketamine hydrochloride 100mg/ml, xylazine hydrochloride 20 mg/ml) at 0.1 ml per 6 g body weight. Once anaesthetised, a small incision was made in the skin above the right *tibialis anterior* (TA) and 0.1 µg of notexin (*Notechis scutatus*, Venom Supplies Pty. Ltd., Tanunda, South Australia) was injected into the muscle. At time points of day 0, 1, 2, 3, 5, 7, 14 and 28, five mice were euthanised by CO_2 asphyxiation and cervical dislocation and had both the treated and control (contra-lateral) TA removed. The muscles from three mice were frozen in liquid nitrogen for RNA extraction. The muscles from the remaining two mice were fixed for tissue sectioning. Tissues were coated in Tissue Tek O.C.T compound (Sakura) then frozen in liquid nitrogen-cooled iso-pentane (BDH) for ten seconds. Tissues were then moved to liquid nitrogen. All tissues, for both RNA and tissue sectioning, were stored at -80^{o}C until use.

2.2.4 Isolation of Reserve cell populations

Reserve cells were isolated from cultures of both normal C_2C_{12} cells and from stably-transfected cell lines expressing Mighty, according to the method of Kitzmann *et al*. 1998. Cell cultures were plated on 10 cm diameter Nunc culture dishes at a density of 25,000 cells/cm^2 and allowed to attach for 16 hours. Differentiation media (DMEM + 2% horse serum) was added and the cultures were incubated at 37^{o}C/5% CO_2 for 72 to 96 hours. Following this a short

trypsinisation was carried out (five minutes, 0.15 % trypsin) and the myotubes were removed with two PBS washes. The reserve cells, which remain attached during this procedure, were then removed by a ten minute incubation with 0.25% trypsin. Isolated myotube or reserve cell populations were stored at -80^{o}C in Trizol (Invitrogen).

2.2.5 RNA isolation

From whole tissue:

Total RNA was isolated from tissue samples using Trizol (Invitrogen) according to the manufacturer's protocol.

TA muscles isolated from notexin-injected mice were homogenised with 1 ml Trizol per 50 mg of tissue. An initial spin at 12,000 rcf for ten minutes at 4^{o}C was carried out to pellet cellular debris. 200 µl of chloroform per 1 ml Trizol was added and incubated for two to three minutes before centrifugation at 12,000 rcf for 15 minutes at 4^{o}C. The upper aqueous phase was removed to a clean Eppendorf tube and had an equal volume of chloroform added. The samples were centrifuged at 12,000 rcf for 15 minutes at 4^{o}C, and then the upper aqueous layer was removed to a clean tube. Total RNA was precipitated by adding 500 µl of isopropanol per 1 ml of Trizol to the aqueous phase. Samples were incubated at room temperature for ten minutes then centrifuged at 12,000 rcf for ten minutes at 4^{o}C. The RNA pellet was washed with 1 ml 75% ethanol made with DEPC water, then centrifuged at 7,500 rcf for five minutes at 4^{o}C. The RNA pellets were dried for five to ten minutes then re-suspended in DEPC water. Incubation at 55-60^{o}C for ten minutes completed the re-suspension. RNA was stored at -80^{o}C.

From cultured cells:

Total RNA was isolated from cultured cells using Trizol (Invitrogen) according to the protocol mentioned above but with the changes outlined below.

Media was removed from the plates and the cells were washed twice with PBS. 1 ml Trizol was added to each plate to lyse the cells. All cells were removed from the plates by pipetting the Trizol over the surface several times. The Trizol was removed to Eppendorf tubes and had 200 µl chloroform added. From this point on the protocol was essentially as described above.

All RNA was quantified using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies). 1 µl of a 1:10 dilution of the RNA was pipetted onto the detector and the absorbance at 230, 260 and 280 nm was read and used to determine the quantity of RNA in the sample.

2.2.6 First Strand cDNA Synthesis

cDNA for use as a template in the PCR was generated using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) following the manufacturer's protocol.

1-5 µg total RNA was added to 1 µl 10 mM dNTPs and 1 µl $Oligo(dT)_{12-18}$ primer, then made up to 10 µl with DEPC-treated water. The RNA was incubated at 65°C for five minutes, then on ice for at least one minute. 9 µl of a reaction mix containing RT buffer, $MgCl_2$, DTT and RNaseOUT, was added to the RNA and incubated at 42°C for two minutes. 1 µl of Superscript II reverse transcriptase was added to each reaction. The reactions were incubated at 42°C

for 50 minutes then at 70°C for 15 minutes. 1 µl RNaseH was added to each tube then incubated at 37°C for 20 minutes. All cDNA was stored at -20°C.

2.2.7 Polymerase Chain Reaction (PCR)

PCR amplifications were carried out using Taq DNA Polymerase (Roche). PCR reactions (50 µl) contained 1 x PCR buffer, varying concentrations of template, both sense and antisense primers at 0.2 µM, and dNTPs at 0.2 mM. A Hybaid MBS 0.5S PCR System (Hybaid) was used for the thermocycling of the PCR reactions. PCR amplifications for Mighty contained 1 x Q solution in addition to the reagents listed above.

PCR amplifications were standardised on cDNA templates derived from either wild type mouse TA muscle or C_2C_{12} cells differentiated for 72 hours. PCR primers are described in Table 1 and cycle conditions are described in Table 3.

Table 3: PCR cycle conditions.

Wt TA cDNA

Product	Denaturation	Annealing	Extension
Mighty	94°C, 20 s	60°C, 45 s	72°C, 1 min
MyoD	94°C, 15 s	60°C, 40 s	72°C, 1 min

72 hr differentiated C_2C_{12}

Mighty	94°C, 20 s	60°C, 45 s	72°C, 1 min
Sca1	94°C, 20 s	62°C, 45 s	72°C, 45 sec
CD34	94°C, 20 s	58°C, 45 s	72°C, 45 sec
Tubulin	94°C, 20 s	55°C, 30 s	72°C, 1 min

To determine the linear increase during the amplification PCRs were carried out on the templates mentioned above. Cycle numbers of 20-30 were well

standardised to determine the linear increase in product. The PCR products were then run on a 1% agarose gel and stained with ethidium bromide.

2.2.8 RNA and DNA analysis

1.2% denaturing RNA gels were made according to the protocol in the Appendix. 0.5 to 1 µg of total RNA was mixed with an equal volume of 2 x RNA loading dye and incubated at 65^{o}C for five minutes before loading. Gel visualisation was carried out on a BioRad Gel Doc 2000.

DNA gels were made according to the protocol in the Appendix. To ensure the gels were consistent, the volume of agarose, the volume of ethidium bromide per gel and the same gel running conditions were used. To visualise the products of the PCR reactions, 5 µl of PCR product was combined with 1 µl of 10 x DNA loading dye and run on the gels. Agarose gels were exposed to UV light by a BioRad Gel Doc 2000, with the same exposure settings used each time. Pictures of the gels were saved for analysis using BioRad Quantity One 4.4.1 software.

2.2.9 Immunocytochemistry for Mighty on tissue sections

The tissues recovered from the regeneration trial described in Section 2.2.3 were sectioned using a Leica Cryocut 1800. Sections cut were 10 µm thick and stored unfixed on Esco POLYSINE microscope slides (Biolab Scientific) at -20^{o}C until analysis. For Mighty detection, the sections were thawed in a humid chamber, then a circle was drawn around the edge of the tissue with a PAP pen (Zymed Laboratories Inc.). Sections were blocked in PBS-T + 0.2% BSA + 10% normal donkey serum (NDS) for 30 minutes at room temperature. Primary rabbit anti-Mighty antibody was added at 1:100 in PBS-T + 0.2% BSA + 5% NDS

overnight at 4^{o}C in a humid box. As a negative control, primary antibody was not added to one section. The next day the slides were washed three times in PBS for four minutes each wash. Sections were fixed in 10% formalin for five minutes at room temperature then washed three times in PBS for four minutes each wash. Secondary antibody was added at 1:300 in PBS-T + 0.2% BSA + 5% NDS for one hour at room temperature. The slides were then washed three times in PBS-T 0.2% for four minutes per wash. Tertiary antibody was added at 1:400 in PBS-T + 0.2% BSA in a dark humid box for one hour at room temperature. Slides were washed twice in PBS for four minutes per wash, and then had DAPI added at 1:1000 in PBS and were incubated for five minutes. Slides were again washed twice in PBS for four minutes each wash, then were dried and had cover slips mounted with DakoCytomation fluorescent mounting medium (Med-bio Ltd). Slides were stored wrapped in tinfoil and at 4^{o}C. Slides were viewed using an Olympus BX50 Microscope (Olympus Optical Co., Germany) with an attached camera. Images were taken using Spot Basic software (Spotsoftware, Diagnostic Instruments Inc.). Comparisons of Mighty levels and of infiltrating nuclei numbers between the wild-type and myostatin-null tissues was done by simple visual estimation.

2.2.10 Immunocytochemistry on satellite and C_2C_{12} cells

Immunocytochemisty (ICC) was carried out for MyoD on Percoll-isolated satellite cells, for Desmin on Matrigel-isolated satellite cells and for Mighty on reserve cells derived from C_2C_{12} cells and Mighty over-expression clones.

Satellite cells isolated by the Percoll method (Section 2.2.1) were aliquoted onto Lab-Tek 8-well chamber slides that had been pre-coated with poly-L-lysine

(Sigma, 0.01% solution). After a 30 minute incubation to allow cells to attach to the slide, the excess suspension was removed and the cells were fixed, blocked and incubated with antibodies against MyoD as described in Table 4 and Table 5.

Matrigel-isolated satellite cells (Section 2.2.2) were allowed to proliferate for 24 hours on a 10 cm Matrigel-coated plate. The cells were then trypsinised and plated onto a Matrigel-coated Lab-Tek 8-well chamber slide at a cell density of 15,000 cells/cm^2 and allowed to attach and proliferate for a further 24 hours. Excess media was removed and the cells were fixed, blocked and incubated with antibodies against Desmin as described in Table 4 and Table 5.

C_2C_{12} cells and Mighty over-expressing clones were plated on Permanox slides (Invitrogen) and allowed to proliferate for 24 hours before being switched to differentiation media (DMEM + 2% HS). Cells were differentiated for 72 to 96 hours then subjected to a limited trypsinisation, as outlined in Section 2.2.4. The remaining reserve cells were then fixed, blocked and incubated with antibodies against Mighty as described in Table 4 and Table 5.

Table 4: Immunocytochemisty conditions.

ICC	Slide type	Fixation	Permeablisation	Blocking
MyoD	8-well, poly-l lysine coated	30 s of 20:2:1 fixative per well	0.5% Triton X-100 in PBS, 10 min at RT	0.35% C-λ in PBS + 10% NDS, 1 h RT
Desmin	8 well, Matrigel coated	30 s of 20:2:1 fixative per well	0.5% Triton X-100 in PBS, 10 min at RT	0.35% C-λ in PBS + 10% NSS, 1 h RT
Mighty	Permanox	30 s of 20:2:1 fixative per well	0.5% Triton X-100 in PBS, 10 min at RT	5% BSA in PBS + 5% NGS, 4 h RT

Table 5: Immunocytochemistry conditions continued

ICC	Primary	Secondary	Tertiary
MyoD	1:100 in 0.35% C-λ in PBS + 5% NDS, O/N at 4^{o}C	1:300 in 0.35% C-λ in PBS + 5% NDS, 1 h at RT	1:400 in 0.35% C-λ in PBS + 5% NDS, 1 h at RT in dark
Desmin	1:200 in 0.35% C-λ in PBS + 5% NSS, O/N at 4^{o}C	1:300 in 0.35% C-λ in PBS + 5% NSS, 1 h at RT	1:400 in 0.35% C-λ in PBS + 5% NSS, 1 h at RT in dark.
Mighty	1:200 in 2.5% BSA in PBS + 2.5% NGS, O/N at 4^{o}C	AF488 conjugated secondary at 1:300 in 2.5% BSA in PBS + 2.5% NGS, 1 h at RT	Not applicable

In between each step the slides were washed in PBS-T (satellite cells) or TBS-T (C_2C_{12}) for three rounds of five minutes. After the tertiary antibody incubation, all slides were stained with DAPI (1mg/ml) at 1:1000 in PBS for five minutes. All slides had cover slips mounted with DakoCytomation fluorescent mounting medium (Med-bio Ltd). Slides were stored wrapped in tinfoil and at 4^{o}C. Slides were viewed using an Olympus BX50 Microscope (Olympus Optical Co., Germany) with an attached camera. Images were taken using Spot Basic software (Spotsoftware, Diagnostic Instruments Inc.).

To determine the percentage of myogenic cells from the Percoll and Matrigel isolations, random images were taken from both the positive and negative wells.

All nuclei per image, as stained blue by DAPI, were counted, and then the number of cells that stained for MyoD or Desmin (green) were counted.

2.2.11 Haematoxylin and eosin staining

Sections on slides were submerged in Gill's haematoxylin stain for four minutes, then washed in tap water until the water ran clear. The slides were then submerged in Scott's tap water for three minutes then rinsed in tap water for two minutes. Slides were stained in eosin for two minutes then washed in tap water until the water ran clear then for a further two minutes. The sections were then dehydrated by 30 seconds in 50% ethanol, one minute in 70% ethanol, one minute in 95% ethanol, two minutes times two in 100% ethanol then five minutes times two in xylene. Cover slips were mounted using xylene-based DPX mounting for microscopy (BDH). Slides were viewed using an Olympus BX50 Microscope (Olympus Optical Co., Germany) with an attached camera. Images were taken using Spot Basic software (Spotsoftware, Diagnostic Instruments Inc.).

2.2.12 Western Blotting

Protein extraction from Percoll isolated satellite cells

Total protein was extracted from satellite cells for analysis by Western Blotting. Harvesting of the cells was performed as described in Sections 2.2.1 and 2.2.2. The cell lysate was passed through a 0.45 mm gauge syringe needle ten times. The cell lysate was then centrifuged at 12000 rcf at room temperature for ten min. The supernatant was removed and the protein concentration was determined by mixing 1 µl of protein with 99 µl PBS and 1.2 ml of a 1:5 dilution of Bio-Rad protein assay Dye Reagent Concentrate, then reading the absorbance at 595 nm. The

concentration was calculated from a standard curve of 0, 5, 10, 15 and 20 µg BSA standard prepared from a 5 µg /µl stock of BSA solution.

SDS polyacrylamide gel electrophoresis

Separation of protein for Western blotting was achieved using NuPage 4-12% gradient Bis-Tris pre-cast polyacrylamide gels (Invitrogen) in a XCELL II Mini gel apparatus (Novex) with 1 x Nupage MES SDS running buffer (Invitrogen). Samples were mixed with 4 x NuPage Sample Buffer (Invitrogen) containing β-mercaptoethanol before boiling for five minutes. The SeeBlue Plus2 Pre-Stained Standard (Invitrogen) was used as a molecular weight marker.

Western Blotting

Following electrophoresis, the acrylamide gels were removed from their pre-cast casing and washed in Western Blot Transfer Buffer. The protein was transferred to Trans-Blot (Bio-Rad) nitrocellulose membrane by electroblotting using the XCell II Blot Module (Invitrogen). The membranes were then stained with Ponceau S. solution for five minutes. Excess stain was washed off with MQ water and the blot scanned to show effective transfer of protein. After washing the membranes for five minutes in TBS-T, the membranes were blocked in BSA solution (0.3% BSA, 1% PVP, and 1% PEG in TBS-T) for three hours at room-temperature to block non-specific antibody binding. Primary antibody incubation used rabbit anti-Mighty antibody 1:5000 dilution in BSA solution at 4°C overnight, with gentle shaking. Following the incubation, the membranes were washed (five times five minutes) with TBS-T. The membranes were then incubated with goat anti-rabbit conjugated to horseradish peroxidase (HRP) (Amersham) 1:2000 dilution in BSA solution for

one hour. HRP activity was detected with ECL reagent (Western Lightning Chemiluminescense Reagent Plus).

2.2.13 Statistical analyses

Regeneration trial:

ANOVA was carried out to confirm that the differences over time were due to the treatment not random variation. Student's *t*-tests were carried out using the pooled standard deviation to determine significant differences in Mighty expression between wild-type and knock-out at each time point. Student's *t*-tests were also carried out to determine significant changes in Mighty expression within each genotype compared to the uninjured control values. For MyoD expression Student's *t*-tests were carried out to determine significant changes in expression levels within each genotype compared to the contra-lateral controls of the same time point.

Satellite cell isolations:

Student's *t*-test was carried out to determine if there was a significant change in Mighty expression between wild-type and myostatin-null quiescent satellite cells, and between wild-type and myostatin-null activated satellite cells. A Mann-Whitney test was also carried out on each data set.

Reserve cell and myotube enrichments:

Student's *t*-test was used to determine significance differences in gene expression between myotubes and reserve cells of the same cell type. Student's *t*-test was used to determine if there was a significant change in gene expression between control C_2C_{12} cells and Mighty over-expressing clones of the same enrichment (e.g.: C_2C_{12} myotubes compared to Clone 7 myotubes).

Chapter Three

Results

3.1 Characterisation of isolated satellite cells

To determine that the cells isolated by Percoll gradient (Section 2.2.1) were myogenic in origin, the isolated satellite cells were immediately fixed and then stained for MyoD. The immunocytochemistry showed that 92% of the cells were positive for MyoD. The negative control did not show any non-specific background staining; representative images are shown in Figure 3.1 A and E. The position of cell nuclei was determined by DAPI stain, as in Figure 3.1 B and F.

To determine that the cells isolated by Matrigel adherence were myogenic in origin the protocol for isolation of activated satellite cells (Section 2.2.2) was followed. After the cells were allowed to attach and proliferate for a further 24 hours in an 8-well chamber slide, they were fixed and stained for desmin. The immunocytochemistry showed that desmin was expressed by the majority of cells in the culture as seen in Figure 3.1 C. The position of cell nuclei was determined by DAPI stain, as in Figure 3.1 D. The percentage of myogenic cells has been determined several times in the laboratory, and is usually 80-85%.

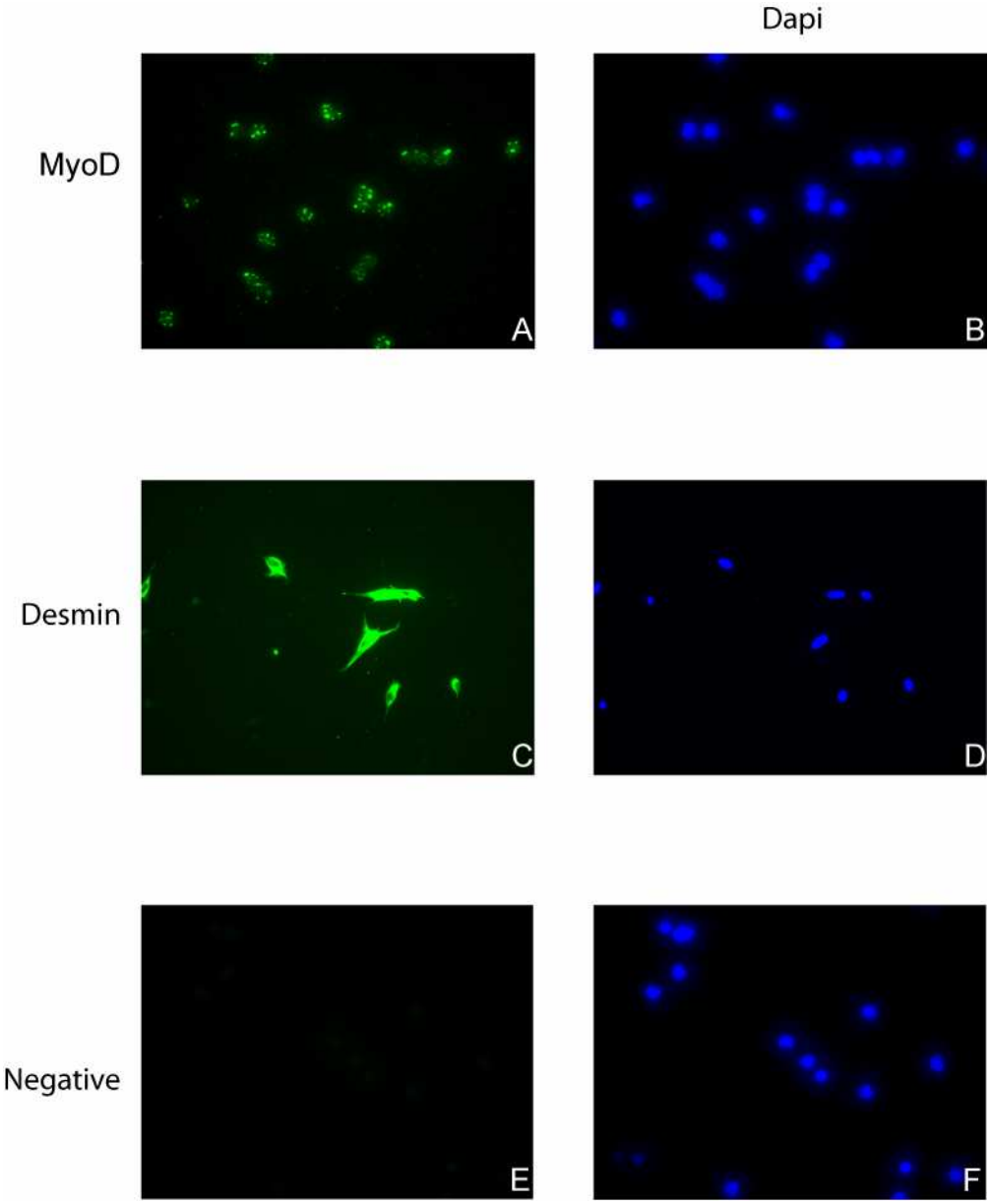

Figure 3.1: Characterisation of isolated satellite cells.
Cells isolated by Percoll gradient were fixed and stained for MyoD (A) and DAPI (B). Cells isolated by Matrigel adherence were fixed and stained for Desmin (C) and DAPI (D). Negative control (E) and DAPI (F) were carried out by omission of the primary antibody to ensure that non-specific antibody binding was not present. Microscope magnification: 400x.

3.2 Expression of Mighty in quiescent and activated satellite cells of wild-type and myostatin-null mice.

To determine if Mighty was present in quiescent and activated satellite cells from wild-type and myostatin-null mice, a 35 cycle PCR for Mighty was carried out. The results indicated that Mighty was present in both quiescent and activated satellite cells from both wild-type and myostatin-null mice. A semi-quantitative analysis of the levels of Mighty was carried out by decreasing the cycle number of the PCR. Decreasing the cycle number to 28 showed that the quiescent satellite cells from wild-type and myostatin-null mice expressed less Mighty than activated satellite cells from wild-type and myostatin-null mice. No difference in Mighty expression in quiescent cells of wild-type and myostatin-null mice was observed, as determined by Student's *t*-test and Mann-Whitney tests, as shown in Figure 3.2 A. Decreasing the PCR cycle number to 22 cycles allowed the levels in activated satellite cells from wild-type and myostatin-null to be measured. No difference in the expression of Mighty in wild-type and myostatin-null activated satellite cells was observed, as determined by Student's *t*-test and Mann-Whitney tests, as seen in Figure 3.2 B.

Determination of Mighty levels in quiescent and activated satellite cells from wild-type mice was also carried out by Western Blot, as described in Section 2.2.11. Levels of Mighty protein in wild-type quiescent satellite cells were undetectable and increased dramatically as satellite cells became activated, as shown in Figure 3.2 C.

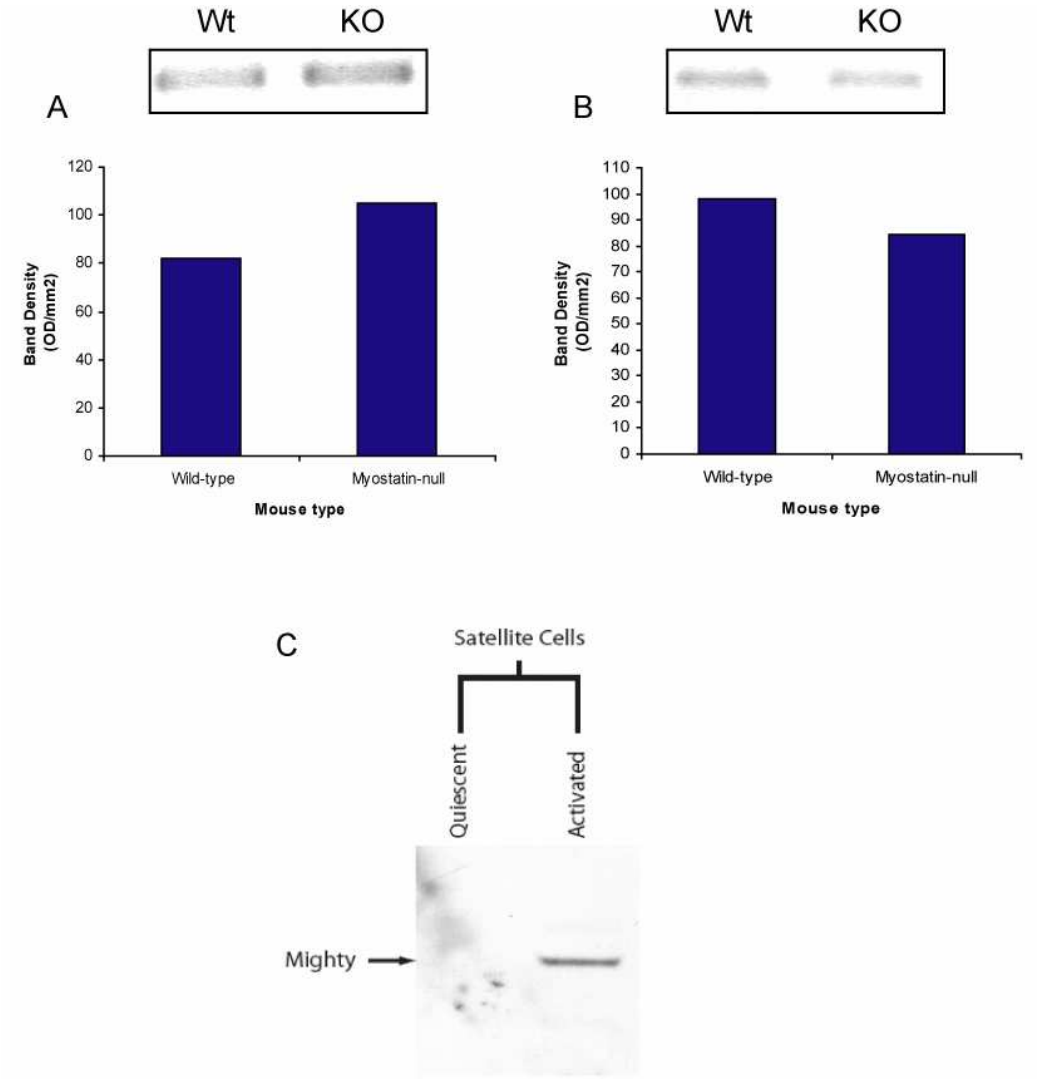

Figure 3.2: Mighty RNA levels in quiescent and activated satellite cells
A) Mighty levels in quiescent satellite cells from wild-type (Wt) and myostatin-null (KO) mice. There was no significant expression difference between the two mouse types by Student's *t*-test and Mann-Whitney test. B) Mighty levels in activated satellite cells from wild-type (Wt) and myostatin-null (KO) mice. There was no significant difference in Mighty expression between the two genotypes as determined by Student's t-test and Mann-Whitney test. PCR was normalised to total RNA. Values represent the average of two PCRs. C) Mighty protein levels are up-regulated during activation in wild-type mice (normalised to total protein levels).

3.3 Effect of muscle injury on body and muscle weights

The weight of the mice that had notexin-induced damage of the *tibialis anterior* (TA) muscle was taken twice. An initial weight was taken on the day of injury and a final weight was taken on the day of euthanasia. As shown in Figure 3.3, a decrease in weight during the first three days of the trial was observed in both wild-type and myostatin knock-out mice. The wild-type mice lost more weight than the myostatin-null mice. The myostatin-null mice appeared to regain any weight lost more rapidly than the wild-type mice. By day 28 post-injury the myostatin-null mice were significantly heavier ($p=0.003$) than the wild-type mice.

The weight of both the damaged TA and the contra-lateral control muscles were taken at the time of excision. The weight of the notexin-damaged TA of the wild-type mice increased during the early stages of regeneration, becoming significantly heavier at day-three post-injury ($p=0.03$), as illustrated in Figure 3.4 A. The weight of the notexin-damaged TA of the myostatin-null mice also increased during the first days of regeneration, and was significantly greater ($p=0.04$) at day-two post-injury, as seen in Figure 3.4, B. In both mouse models the weight of the damaged TA returned to normal by day-seven post-injury and mirrored the changes seen in the contra-lateral control until day-28 post-injury. The wild-type mice showed an increase in the damaged TA weight at day-28 post-injury, a change that was not seen in the myostatin-null mice.

Haematoxylin and eosin staining of control TA sections showed that both wild-type (Figure 3.5 A) and myostatin-null (Figure 3.5 B) had healthy muscle with fibres surrounded by blue nuclei. Staining of tissue at day-one post-injury

revealed large areas of damaged tissue in both the wild-type (Figure 3.5 C) and myostatin-null (Figure 3.5 D) muscle. The extent of muscle damage is similar between both wild-type muscle and myostatin-null muscle, indicating that notexin was an efficient inducer of necrotic damage in the muscle regardless of the genetic background of the mouse.

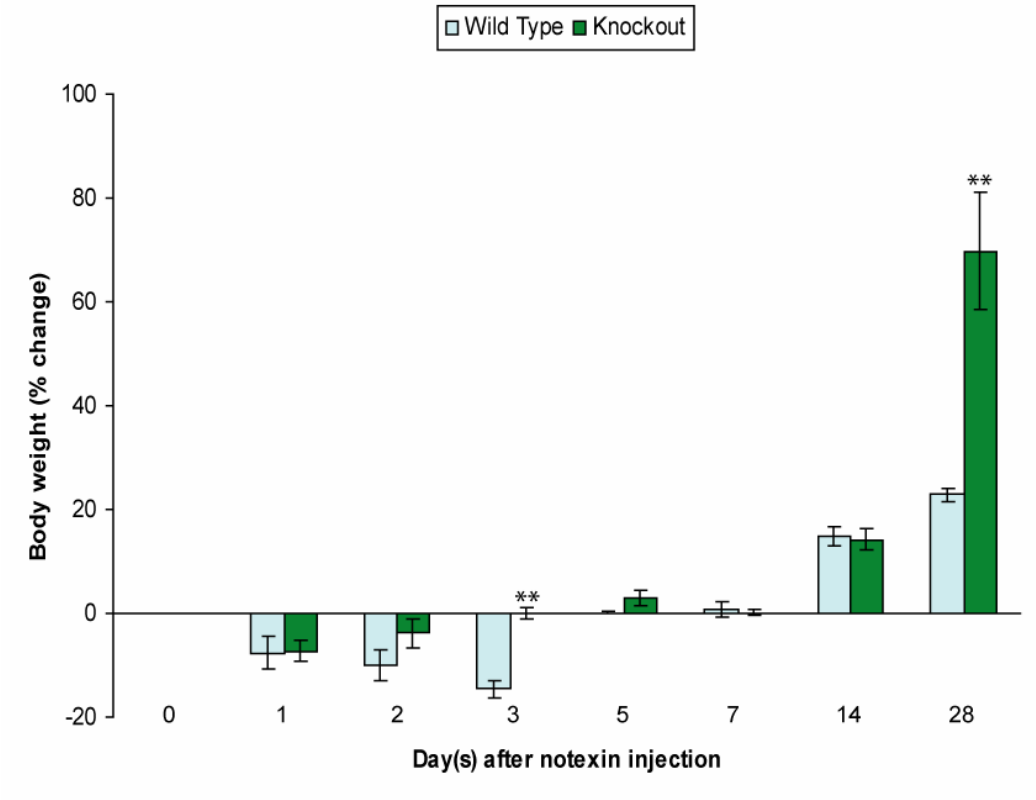

Figure 3.3: Body weight changes during muscle regeneration following notexin injection
Over the initial days of regeneration both wild-type (light bars) and myostatin-null mice (dark bars) lost body weight. By the end of the trial, the mice had regained this lost weight and had grown further. The myostatin-null mice grew to a greater extent than their wild-type counterparts. Values represent the mean of 5 animals ± SEM. Weight change is relative to the animal's weight at day-zero. **p<0.01 by Student's *t*-test.

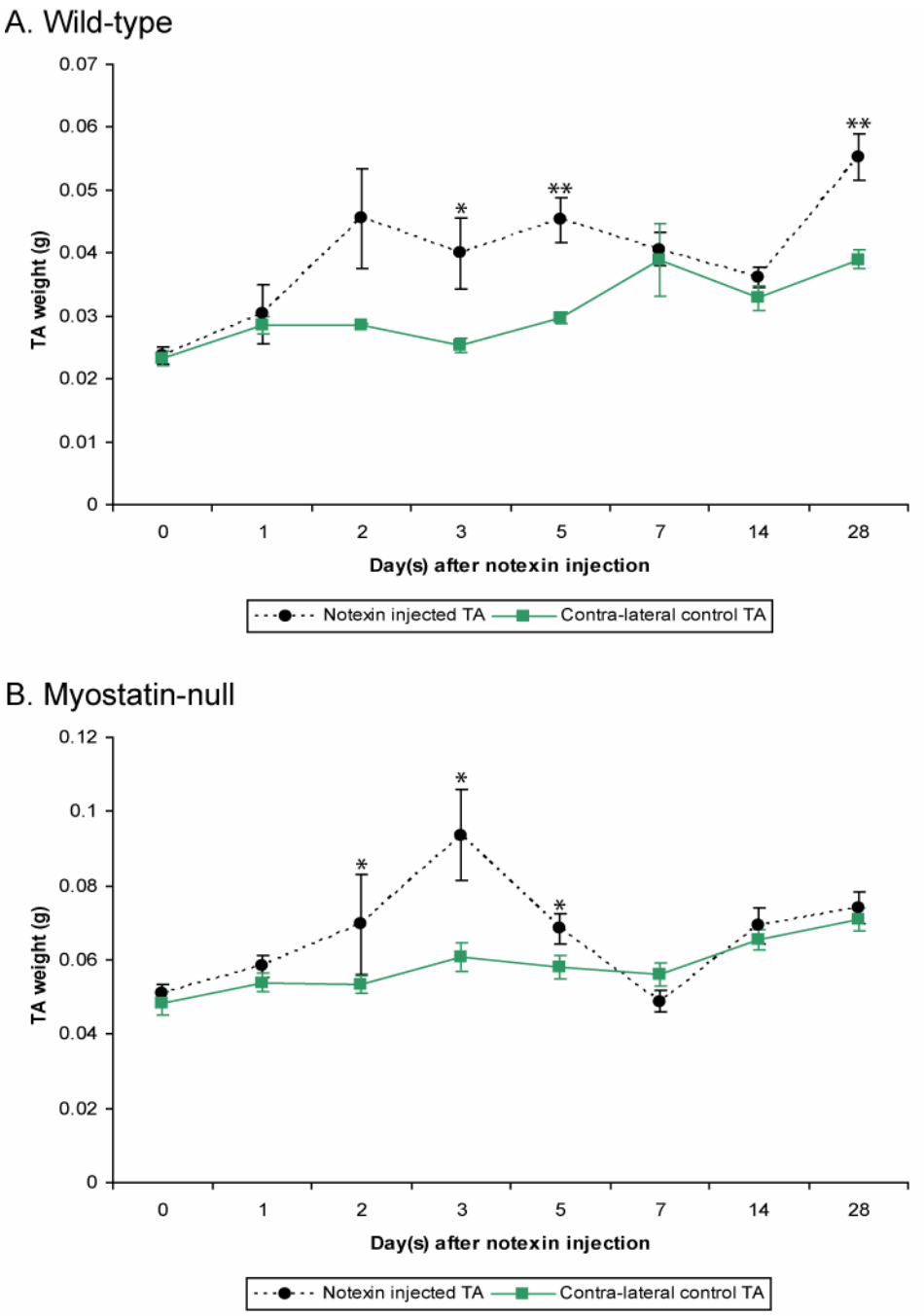

Figure 3.4: Changes in weight of the *tibialis anterior* during regeneration following notexin injection

A) Wild-type mice showed an increased weight of the injected TA by three days post-injury. The weight of the TA remained higher until day seven post-injury when it returned to be similar to the contra-lateral control. B) Myostatin-null mice showed an increased TA weight by day-two post-injury. As in wild-type mice, the weight of the myostatin-null TA returned to that of the contra-lateral control by day-seven post-injury. Each value represents the average of 5 animals ± SEM. *p<0.05, **p<0.01 by Student's *t*-test against the contra-lateral control at the same time point.

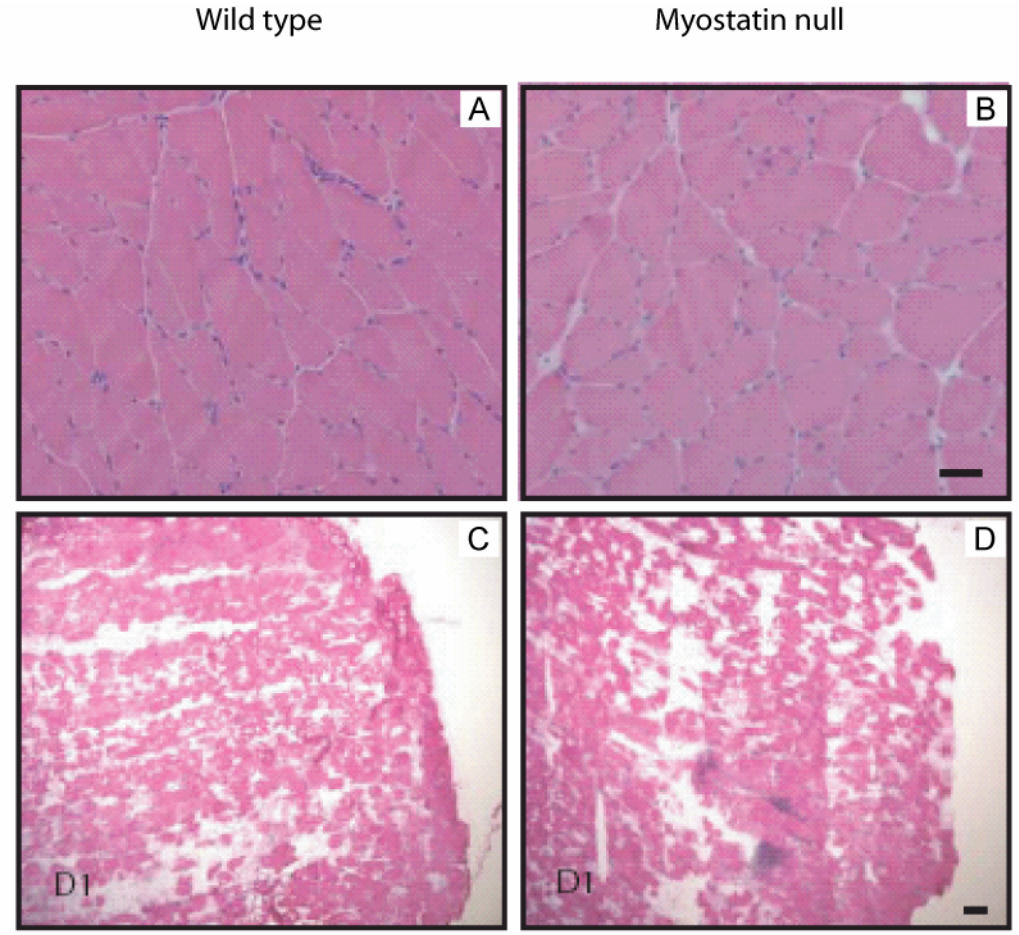

Figure 3.5: Notexin-induced damage of mouse *tibialis anterior* muscle
Haemotoxylin and eosin staining of uninjured muscle from wild-type (A) and myostatin-null (B) mice showed healthy tissue. Staining of the TA tissue taken from wild-type (C) and myostatin-null (D) mice at day-one (D1) post-injury showed similar levels of damage had occurred in both mouse genotypes

3.4 Mighty was up-regulated during regeneration

Control and notexin-injected muscles were either frozen in liquid nitrogen (n=3) for RNA analysis or in Tissue Tek O.C.T Compound (Sakura) (n=2) for immunocytochemistry of Mighty (Section 2.2.9.). RNA extraction and cDNA synthesis was carried out as described (Sections 2.2.5 and 2.2.6).

Short cycle PCRs for Mighty were carried out on wild-type TA cDNA template from an uninjured six week old wild-type mouse (Figure 3.6 A). Densitometric analysis showed that there was a linear increase in product density between 24 and 28 cycles (Figure 3.6 B), hence a PCR cycle number of 26 was used to analyse Mighty levels in the regenerating muscle.

As seen in Figure 3.6 C, the levels of Mighty in undamaged TA were significantly higher ($p=0.001$) in the myostatin-null mice than in the wild-type mice. Mighty cDNA levels in both mouse models stayed fairly constant over the days immediately after injury. Mighty levels peaked in both mouse types at day-five post-injury. At day-five post-injury significantly more Mighty was present in the myostatin-null than in the wild-type samples ($p=0.002$). At day-seven after injury the Mighty cDNA levels in the myostatin-null mice were still elevated and are again significantly increased when compared to the wild-type levels ($p=0.00001$). At day-seven post-injury Mighty levels had dropped in the wild-type samples, although they remain higher than at day-zero ($p=0.028$). By day-14 the levels of Mighty in the myostatin-null mice have also decreased. Both mouse models showed an increase in Mighty at day-28 post-injury when compared to the day-zero values ($p=0.001$).

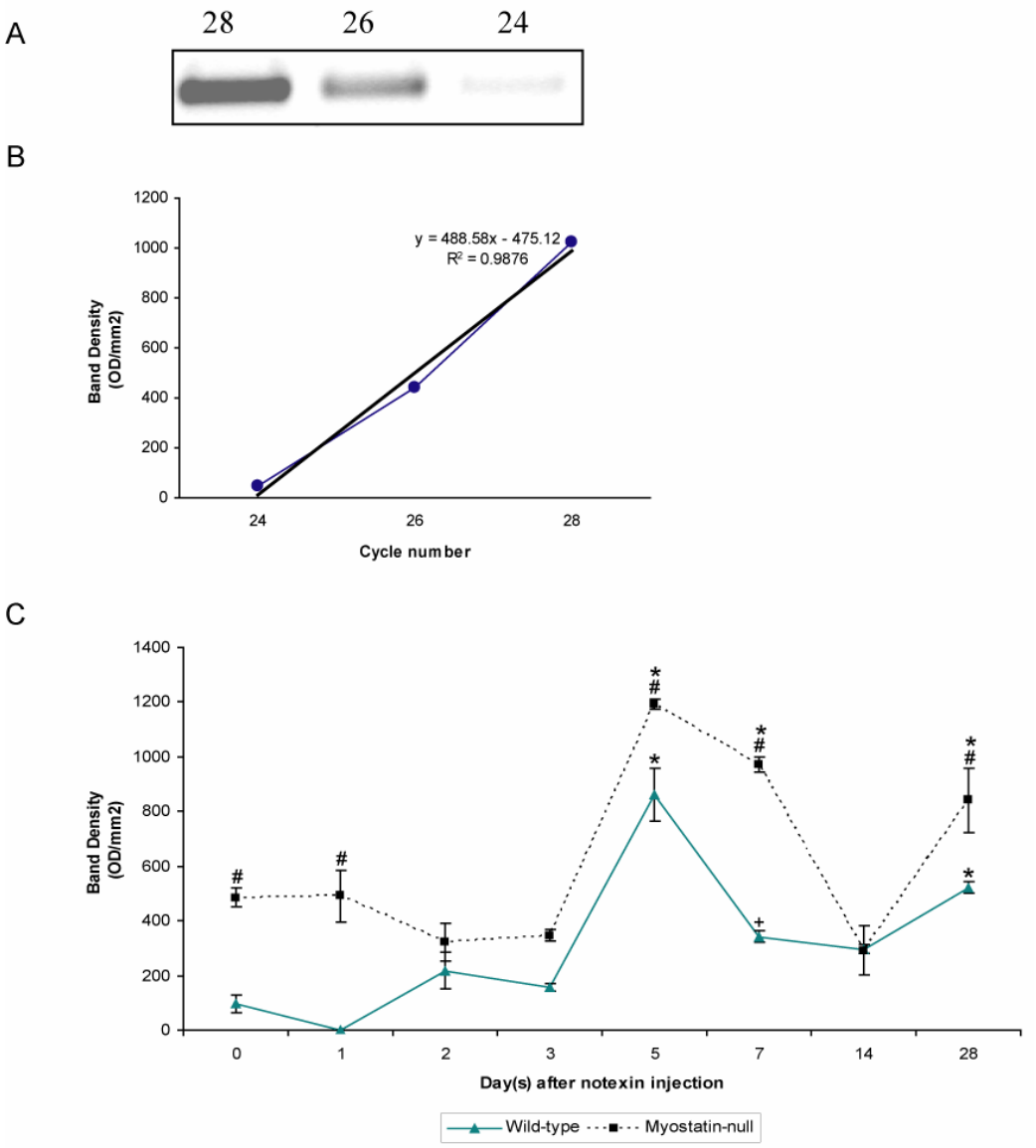

Figure 3.6: Mighty levels were increased during regeneration following notexin injection

A) Ethidium bromide stained PCR products of cycle numbers 24, 26 and 28.

B) PCR product increased linearly as cycle numbers were increased, indicating that the PCR was in the linear amplification stage.

C) Mighty levels were increased in the injured TA of both wild-type and myostatin-null mice. Myostatin-null mice had a higher starting level of Mighty than wild-type mice and had an extended peak of Mighty expression between day-five and day-seven post-injury that was not seen in wild-type mice. Each value represents the average Mighty level of three animals ± SEM. #p<0.001 by Student's *t*-test when compared to wild-type values. +p<0.05, *p<0.001 by Student's *t*-test when compared to day-zero of the same genotype.

Immunocytochemistry for Mighty was carried on sections of tissue from damaged TA. Undamaged TA from myostatin-null mice had higher levels of Mighty than undamaged wild-type TA, determined visually, as seen in Figure 3.7, A and B. At day-one post-injury there was no major change in the levels of Mighty in either wild-type (Figure 3.7 C) or myostatin-null (Figure 3.7 D) tissue. By day-five post-injury there had been an increase in Mighty in the wild-type tissue (Figure 3.7 E) and in myostatin-null samples (Figure 3.7 F) there was an increase in the number of nuclei present within the muscle. At day-seven post-injury, wild-type muscle had a large increase in the number of nuclei present within the muscle and protein levels of Mighty appeared to remain high (Figure 3.8 A), though cDNA levels had decreased by this stage. The myostatin-null tissue still showed high levels of Mighty at day-seven post-injury (Figure 3.8 B). Tissues from day-14 post-injury (Figure 3.8 C and D) had reduced levels of Mighty. Wild-type tissue at this time had more nuclei present than the myostatin-null tissue. By day-28 post-injury the levels of Mighty protein had decreased in both the wild-type and the myostatin-null tissue, although levels remained higher than in the controls. Mighty levels in the myostatin-null tissue at day-28 post-injury (Figure 3.8 F) were higher than those seen in the wild-type tissue (Figure 3.8 E).

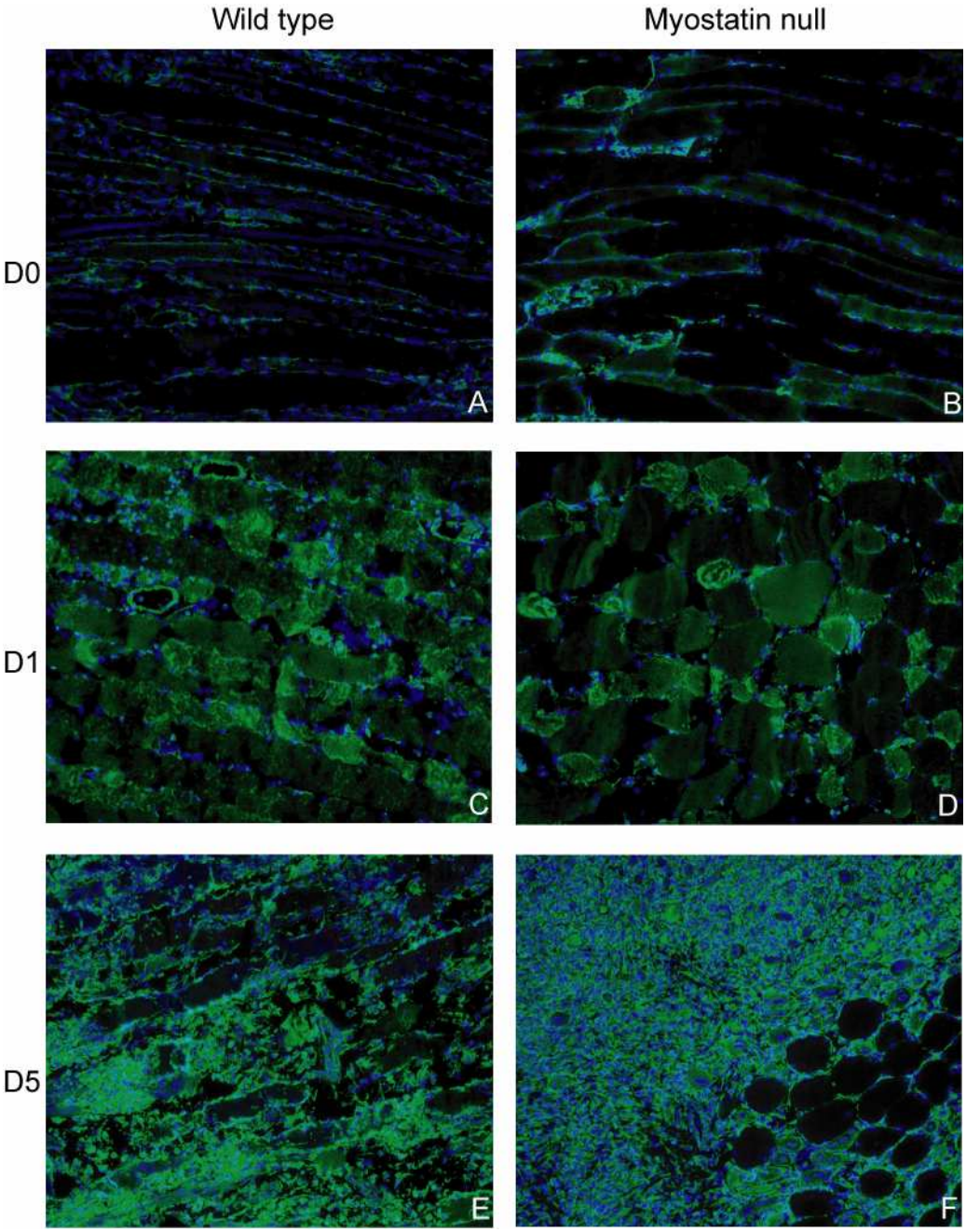

Figure 3.7: Mighty protein expression increased during early regeneration following notexin injection

Uninjured muscle did not express high levels of Mighty. Myostatin-null (B) muscle showed higher levels of Mighty than wild-type (A). At day-one post-injury (C and D) there was little change in Mighty levels. By day-five post-injury myostatin-null muscle (F) showed greater numbers of infiltrating cells (indicated by an increase in blue nuclei, as determined visually) within the muscle than did the wild-type (E). By day-five post-injury both genotypes showed an increase in Mighty levels. Microscope magnification: 200x

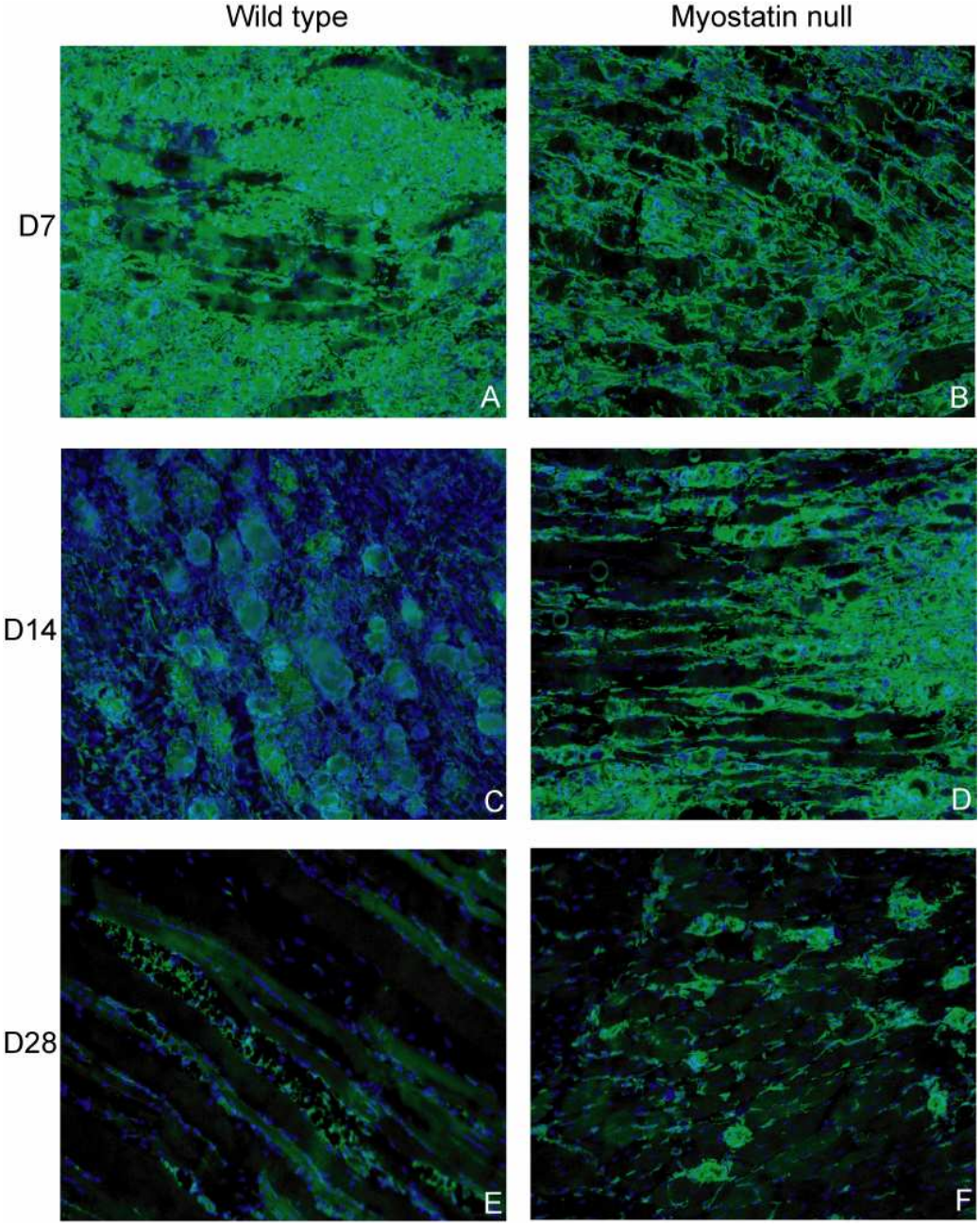

Figure 3.8: Mighty protein expression during late regeneration following notexin injection

At day-seven post injury both wild-type (A) and myostatin-null (B) tissue still had high levels of Mighty protein, determined visually. During the later stages of regeneration (day-14 and day-28 post injury) Mighty levels are still elevated in the myostatin-null mice (D and F) compared to the wild-type (C and E) at the same stage of regeneration. Microscope magnification: 200x

3.5 MyoD levels were up-regulated during regeneration

MyoD is one of the MRFs which is up-regulated shortly after satellite cell activation in injured muscle (Cooper *et al*., 1999) and is responsible for the induction of transcription of many myogenic genes. Mighty expression in relation to this gene was analysed to determine where Mighty might fit in the hierarchy of transcription factor expression during regeneration.

Short cycle PCRs for MyoD (24, 26, and 28 cycles) were carried out on wild-type TA cDNA template from an uninjured six-week-old mouse (Figure 3.9 A). Densitometric analysis showed that there was a linear increase in product density for MyoD between 24 and 28 cycles (Figure 3.9 B) and thus 26 cycles was chosen to use for MyoD analysis in the regenerating muscle.

MyoD levels were up-regulated during early regeneration. In wild-type mice the levels began to increase at day-two post-injury and were significantly up-regulated at day-five post-injury ($p=0.02$). By day-seven post-injury the MyoD levels in the damaged muscle had returned to those seen in the contra-lateral control muscle (Figure 3.10 A). In myostatin-null mice, the levels of MyoD in uninjured muscle are significantly higher than those in wild-type uninjured muscle ($p=0.0004$). MyoD levels in the damaged muscle did not increase over those in the contra-lateral control until day-three post-injury ($p=0.03$). The levels remain high at day five post-injury ($p=0.03$) before decreasing at day-seven post-injury to the levels seen in the contra-lateral controls (Figure 310, B).

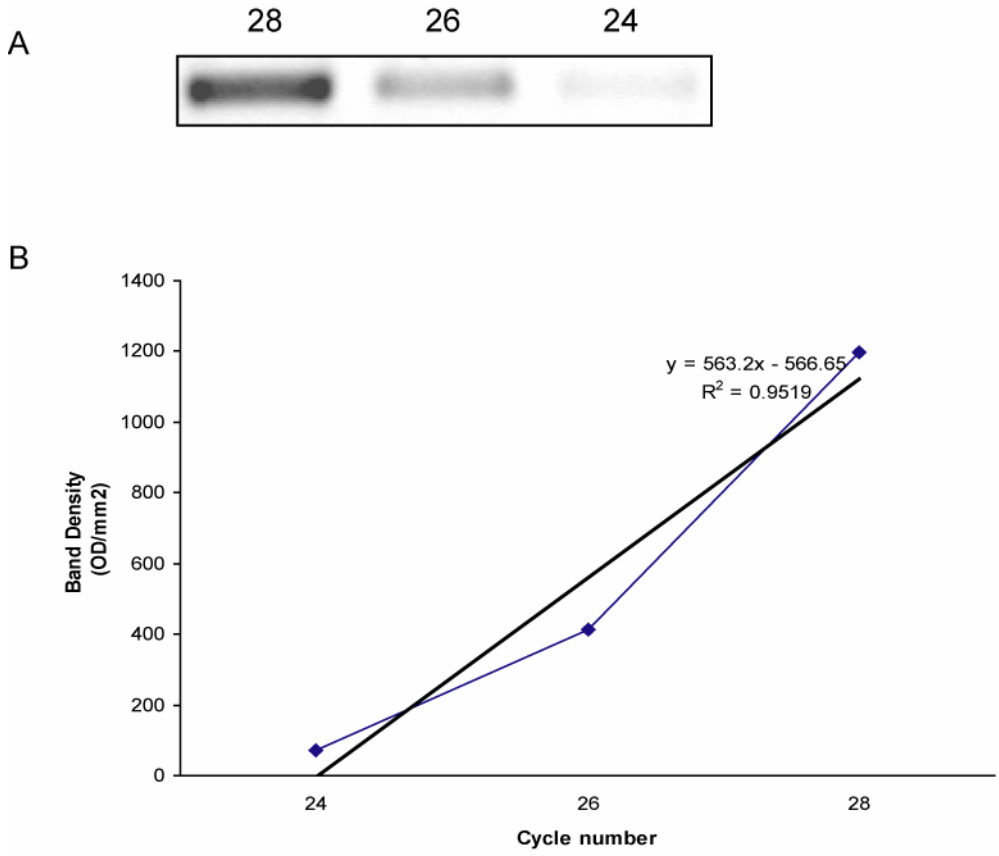

Figure 3.9: MyoD PCRs were linear between 24 and 28 cycles
Ethidium bromide staining of MyoD PCR products (A) between 24 and 28 cycles indicates that the PCR was in the linear amplification stage as seen in (B).

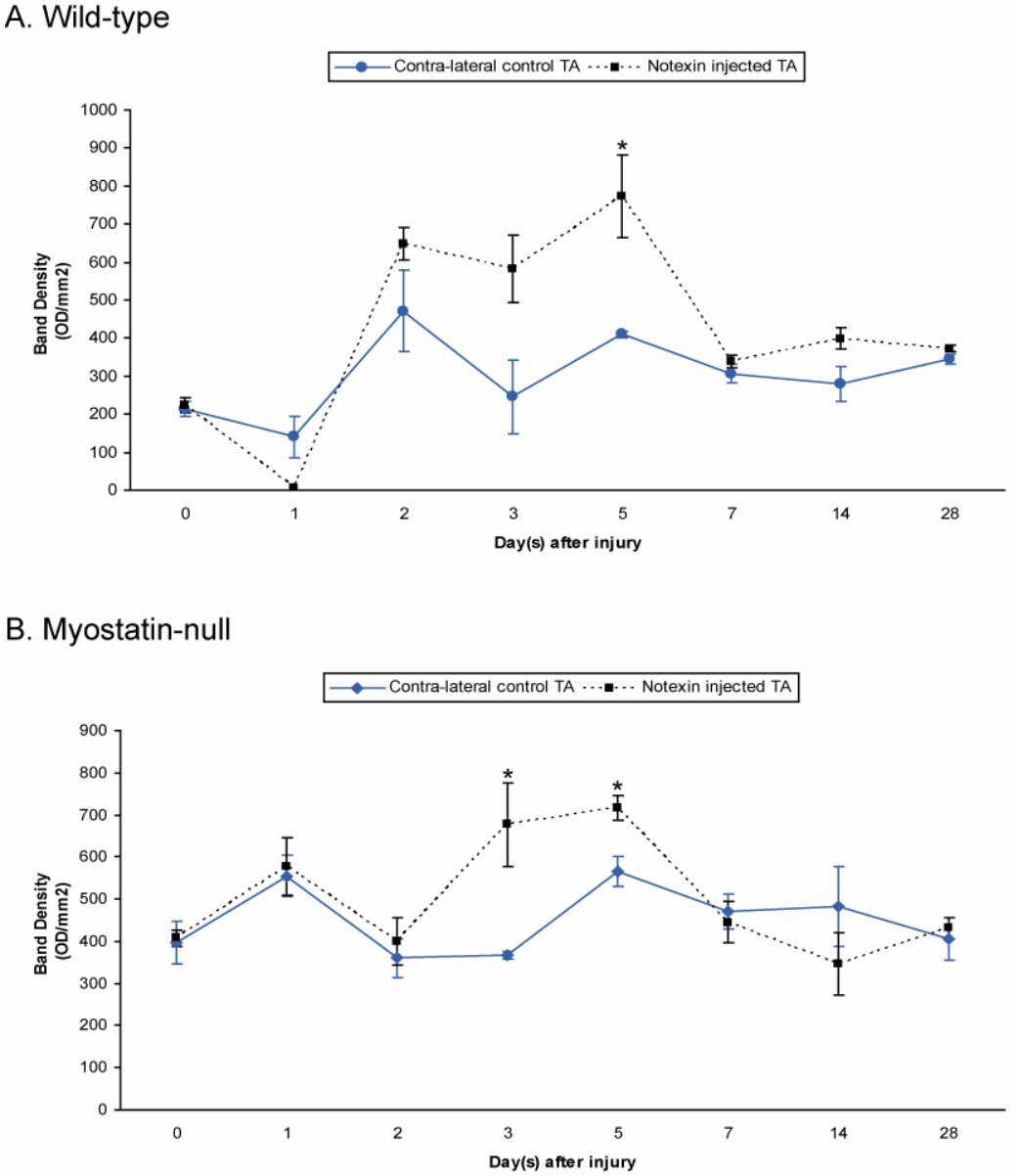

Figure 3.10: MyoD levels were up-regulated during early regeneration following notexin injection

A) In wild-type mice MyoD levels in the injured muscle began to be up-regulated at day-two post-injury, although the levels were not significantly increased until day-five post-injury. MyoD levels returned to those of the contra-lateral control at day-seven post-injury. B) In myostatin-null mice MyoD levels were higher in undamaged muscle than in the wild-type. MyoD levels were elevated at three days post-injury in the damaged muscle and remain high at day-five. By day-seven post-injury the levels of MyoD had decreased to those in the contra-lateral control. Each value represents the average MyoD level of 3 animals ± SEM. *$p<0.05$ by Student's *t*-test when compared to contra-lateral control of the same time point and genotype.

3.6 Mighty localisation in reserve cell populations

C_2C_{12} and Mighty over-expressing clones (Clone 7 and Clone 11) were subjected to a limited trypsinisation as described in Section 2.2.4. Mighty ICCs were carried out on isolated reserve cell populations to determine how Mighty is localised in these cells. Negative controls, as seen in Figure 3.11 A were carried out by omitting the primary antibody to ensure that non-specific binding was not present.

Mighty was localised primarily to the cytoplasm in the C_2C_{12} reserve cells, as seen in Figure 3.11 B, although some cells did show some peri-nuclear staining. The Mighty over-expressing clones (Figure 3.11, C and D) both showed high levels of Mighty. Mighty was localised primarily to the peri-nuclear regions of these cells, although some cells, as indicated by asterisks, did show more cytoplasmic Mighty.

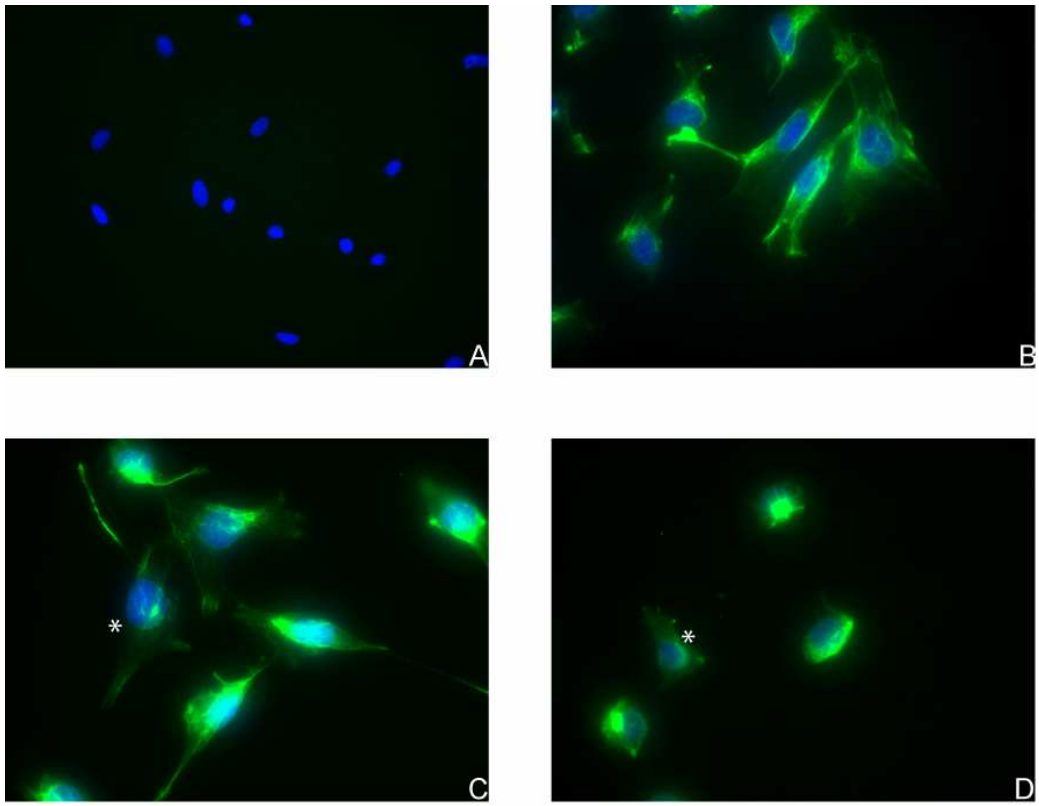

Figure 3.11: Mighty localisation in the reserve cell population
A) Negative control. Microscope magnification for negative control: 200x
Mighty localises mainly to the cytoplasm of C_2C_{12} reserve cells (B) although some peri-nuclear staining is observed. In the Mighty over-expression clones (Clone 7 (C) and Clone 11 (D)), Mighty localised mainly to the peri-nuclear regions of the cells, although some cells only showed cytoplasmic staining (asterisks). Microscope magnification for B, C and D: 400x.

3.7 Downstream targets of Mighty

Micro-array analysis of the gene expression of the Mighty clones in respect to the C_2C_{12} cell line has been carried out to examine how Mighty influences gene expression. Several possible targets of Mighty have been identified. Two genes which are down-regulated are Sca-1 and CD34. As both CD34 and Sca-1 appear to play roles in myogenic differentiation (Epting *et al*., 2004; Mitchell *et al*., 2005), the down-regulation of their expression by Mighty was further investigated.

3.7.1 Mighty decreased the levels of Sca-1 when over-expressed

Short cycle PCRs for Sca-1 were carried out on mixed C_2C_{12} cDNA template for 20, 22, 24, 26 and 28 cycles (Figure 3.12 A). Densitometric analysis showed that there was a linear increase in product density between 22 and 28 cycles as seen in Figure 3.12 B. Initially a 22 cycle PCR was carried out on the reserve cell and myotube cDNA, however no product was able to be visualised (results not shown). Thus 26 cycles, which is still within the linear amplification stage of the PCR, was used to analyse the Sca-1 levels of the reserve cell and myotube populations.

In normal C_2C_{12} cells, the expression of Sca-1 was greater in the reserve cell population than in the myotube population. This pattern of expression was also seen in the Mighty over-expressing clones (Clone 7 and Clone 11). Overall the levels of Sca-1 in the reserve cell and myotube populations were decreased compared to the C_2C_{12} control line, as seen in Figure 3.13 A ($p= 0.02$). Within the myotube population of the Mighty over-expressing clones, the levels of Sca-1 decreased by 60-70% ($p=0.002$ for Clone 7 and 0.005 for Clone 11). In the

reserve cell population of the Mighty over-expressing clones, the levels of Sca-1 decreased by 40-50% (p=0.01), as seen in Figure 3.13 B.

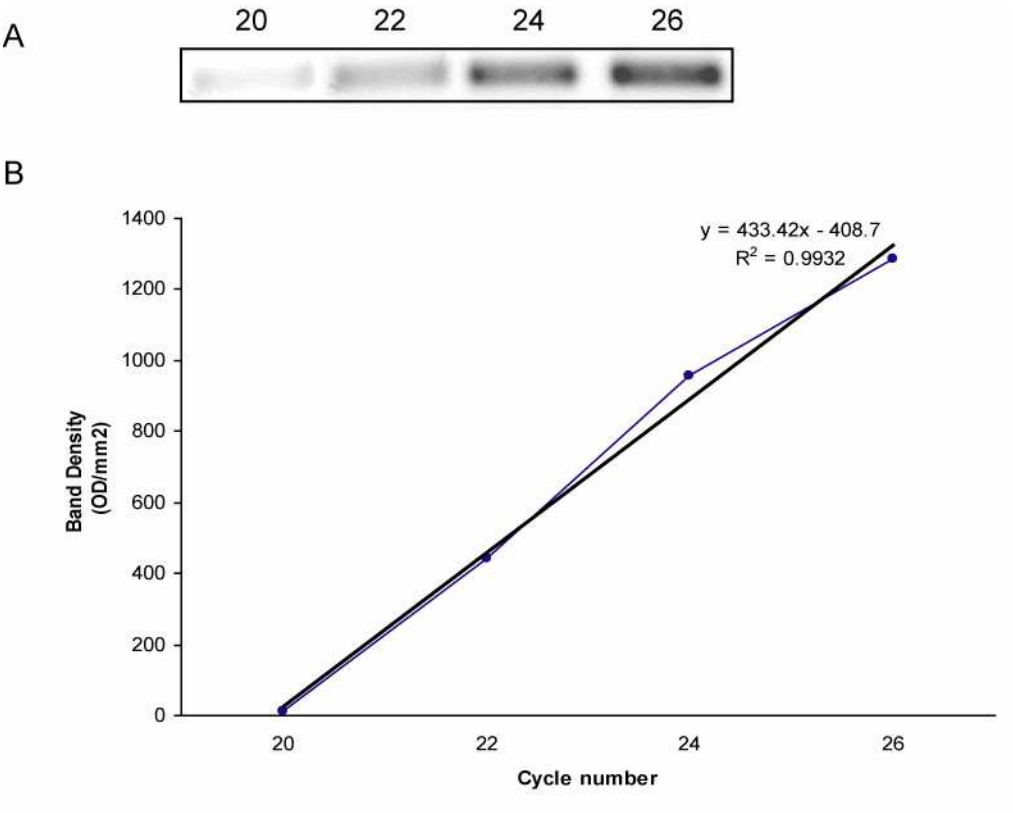

Figure 3.12: Sca-1 PCRs were linear between 20 and 28 cycles
Ethidium bromide staining of Sca-1 PCR products between 22 and 28 cycles (A) indicated that the PCR was in the linear amplification stage (B).

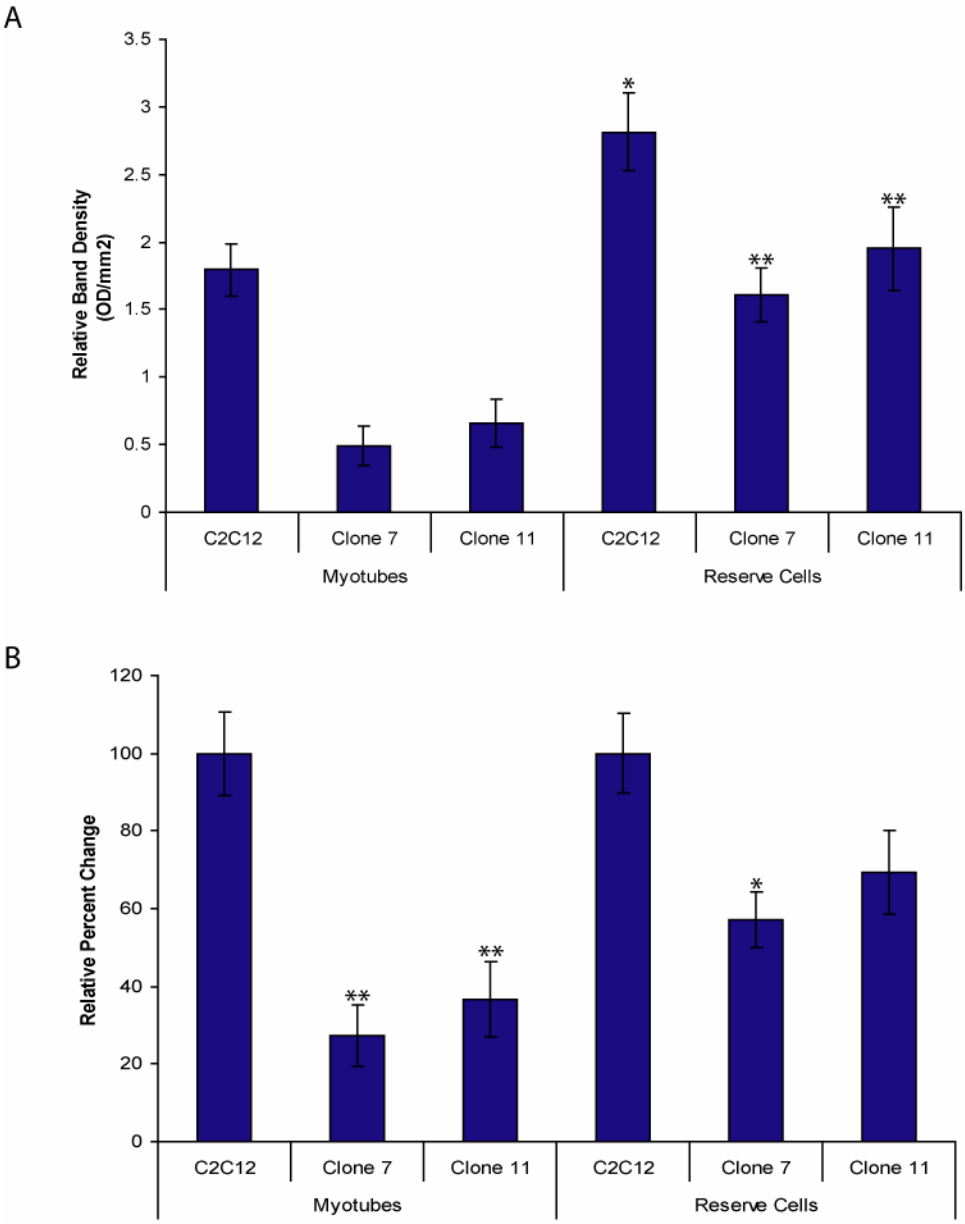

Figure 3.13: Sca-1 levels were decreased by Mighty over-expression
A) Levels of Sca-1 were greater in the C_2C_{12} reserve cell population than in the myotube population. This pattern of expression was also seen in the Mighty over-expression clones, but the overall values are decreased compared to the C_2C_{12} control. *$p<0.05$, **$p<0.01$ by Student's *t*-test when compared to myotubes of the same cell type. B) Sca-1 was decreased by 60-70% in the myotube population and by 40-50% in the reserve cell population of the Mighty clones. *$p<0.05$, *$p<0.01$ by Student's *t*-test when compared to C_2C_{12} of the same cell population. Values represent the average of four PCRs ± SEM.

3.7.2 Mighty decreased the levels of CD34 when over-expressed

Short cycle PCRs for CD34 were carried out on mixed C_2C_{12} cDNA template for 22, 24, 26 and 28 cycles (Figure 3.14 B). Densitometric analysis showed that there was a linear increase in product density between 22 and 28 cycles. Figure 3.14 B illustrates the linear change between 22 and 28 cycles. 24 cycles was used to analyse the reserve cell and myotube populations.

The two forms of CD34, truncated and quiescent, are both detectable in the C_2C_{12} myotube and reserve cell populations. The truncated form, which is expressed in quiescent satellite cells, was higher in the C_2C_{12} reserve cells than in the myotubes. The truncated form of CD34 is also more highly expressed in the reserve cells of the two Mighty over-expression clones (Clone 7, p=0.008 and Clone 11, p=0.02) but the overall levels were dramatically reduced from those seen in the C_2C_{12} reserve cells, as shown in Figure 3.15 A and B. Over-expression of Mighty caused a 95-98% decrease in truncated CD34 in the myotube population (Clone 7, p=0.0002 and Clone 11, p=0.0002) and a 80-90% decrease of truncated CD34 in the reserve cell population (Clone 7, p=7.8E-06 and Clone 11, p=6.2E-05).

The full-length transcript of CD34 is expressed in activated satellite cells. More full-length transcript was observed in the reserve cell population than in the myotube population of the C_2C_{12} cells. The same expression pattern was observed in Clone 7 (p=0.003) and Clone 11 (p=0.004) As with the truncated form, the overall levels of the full-length transcript were dramatically reduced in the Clone 7 and Clone 11 samples when compared to the C_2C_{12} controls (Figure 3.16 A). Mighty caused a 90-95% decrease of the full form of CD34 in the

myotube population (Clone 7, p=1.2E-05 and Clone 11, p=1.2E-05) and a 70-85% decrease in the reserve cell population (Clone 7, p=0.0001 and Clone 11, p=0.0008) of the Mighty over-expression clones (Figure 3.16 B).

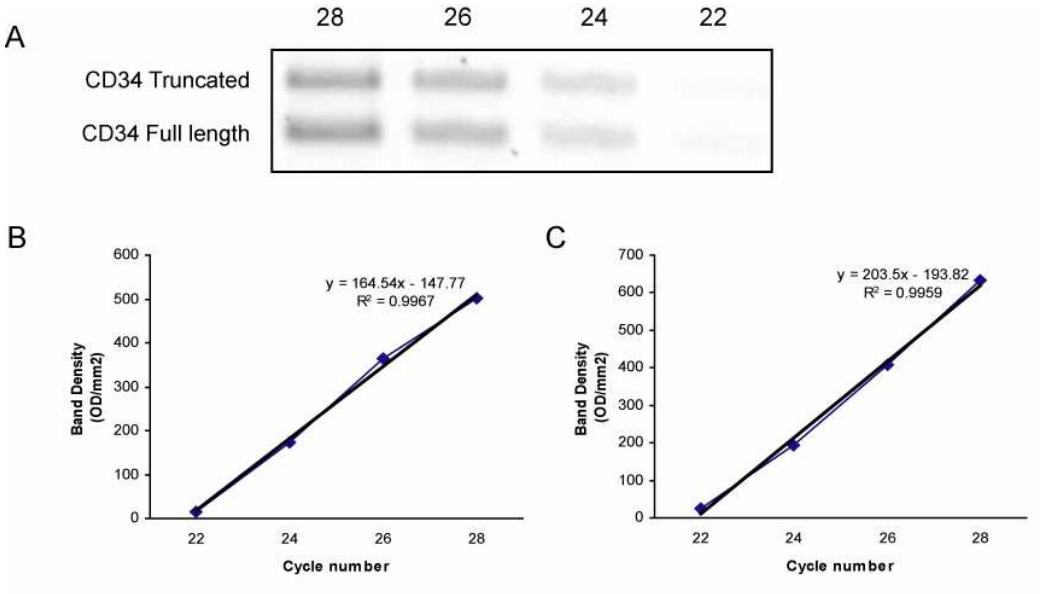

Figure 3.14: CD34 PCRs were linear between 22 and 28 cycles.
A) Ethidium bromide stained CD34 PCR products. B) CD34-truncated PCR product increased linearly as cycle numbers were increased, indicating that the PCR was in the linear amplification stage. C) CD34-full PCR product increased linearly as cycle numbers were increased, indicating that the PCR was in the linear amplification stage.

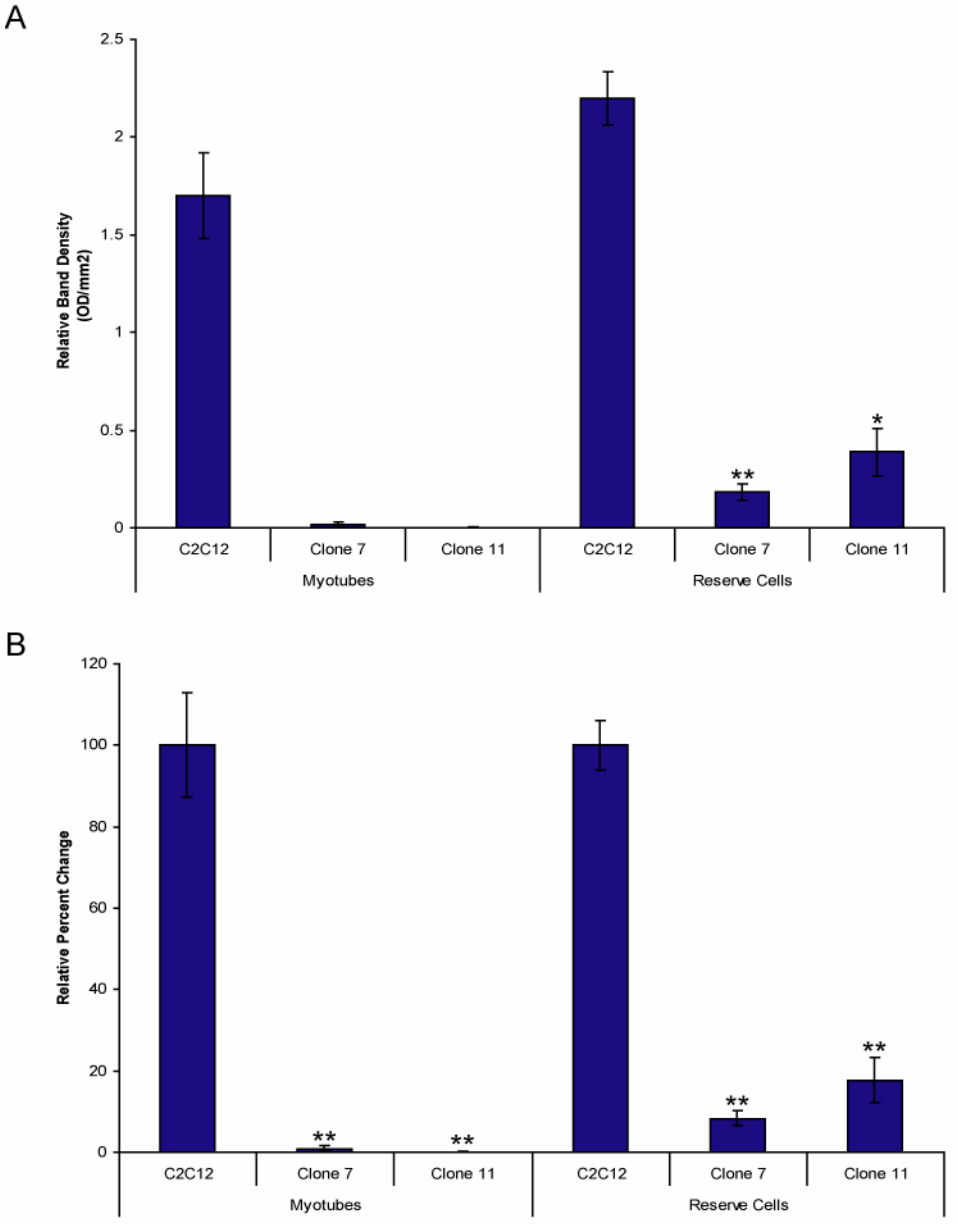

Figure 3.15: The truncated form of CD34 as decreased when Mighty was over-expressed.
A) C_2C_{12} reserve cell enrichment showed higher expression of the truncated form of CD34 than the myotube enrichment. This pattern was preserved in the Mighty over-expressing clones, but the overall values were decreased compared to the C_2C_{12} control. *$p<0.05$, **$p<0.01$ by Student's *t*-test when compared to myotubes of the same cell type. B) The percent decrease of the truncated form of CD34 was 95-98% in the myotube enrichments and 80-90% in the reserve cell enrichments of the Mighty clones. **$p<0.01$ by Student's *t*-test when compared to C_2C_{12} of the same cell population. Values represent the average of four PCRs ± SEM.

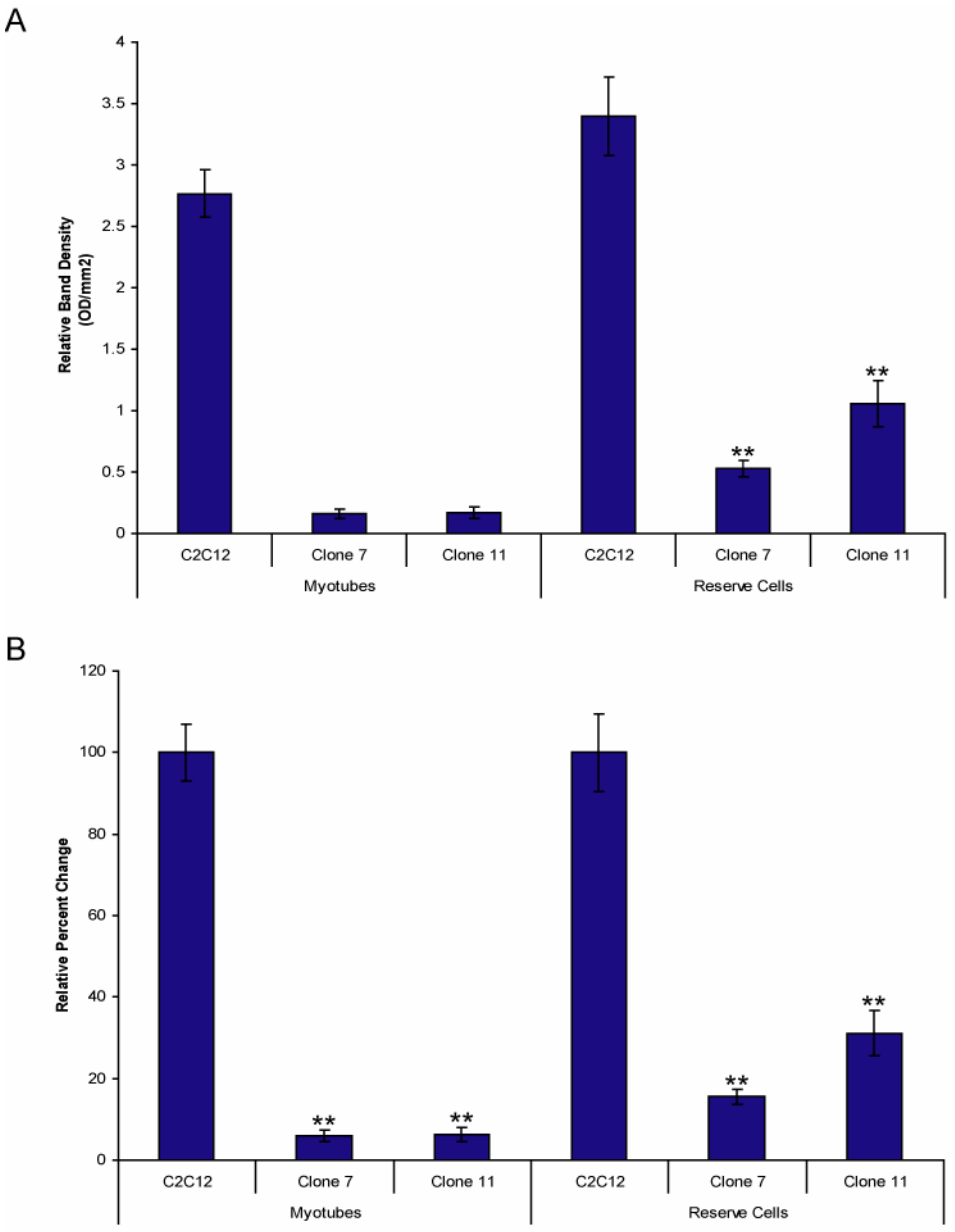

Figure 3.16: The full-length form of CD34 was decreased when Mighty was over-expressed.

A) C_2C_{12} reserve cell enrichment showed higher expression of the full form of CD34 than the myotube enrichment. This pattern was preserved in the Mighty over-expressing clones, but the overall values were decreased compared to the C_2C_{12} control. **p<0.01 by Student's *t*-test when compared to myotubes of the same cell type. B) The percent decrease of the full form of CD34 was 90-95% in the myotube enrichment and 70-85% in the reserve cell enrichments of the Mighty over-expression clones. **p<0.01 by Student's *t*-test when compared to C_2C_{12} of the same cell population. Values represent the average of four PCRs ± SEM.

Chapter Four

Discussion and Future Directions

4.1 Discussion

Satellite cells are the major myogenic precursors that contribute to post natal muscle growth and repair and were first identified by Katz (1961). Satellite cells activate, proliferate and differentiate into new muscle fibres in response to signals from their environment. Satellite cells are also capable of self-renewal to replenish the satellite cell niche and provide nuclei for future growth/repair needs. The myostatin-null mouse model has been shown to contain more satellite cells per fibre than wild-type mice. These satellite cells also have a greater propensity to activation and proliferation than cells isolated from wild-type mice (McCroskery *et al*., 2003). After muscle injury, myostatin-null mice have twice the number of myogenic cells, identified by MyoD, at the site of injury than wild-type mice. Healing is more complete, with less fibrosis and scar tissue formation when myostatin is absent (McCroskery *et al*., 2005). This evidence implicates myostatin as a negative regulator of satellite cells. One downstream target of myostatin, the novel gene called Mighty, is up-regulated in myostatin-null mice. Mighty over-expression results in enhanced differentiation and fusion of C_2C_{12} cells and a hypertrophy phenotype. The expression of Mighty during satellite cell activation and regeneration of skeletal muscle was investigated in this thesis.

To first participate in muscle growth or regeneration, satellite cells must leave quiescence and activate. This activation in response to growth factors such as

IGF's (Hill and Goldspink, 2003) is an essential step in the regeneration and growth program. Myostatin-null mice, which express higher levels of Mighty than wild-type mice, have a greater number of satellite cells that have a greater propensity to activation (McCroskery *et al*., 2003). We hypothesised that Mighty acts as a regulatory factor during this process and thus expected that activated satellite cells from myostatin-null mice would have more Mighty than those from wild-type mice. As shown in Figure 3.2 A, Mighty was expressed at a low level in quiescent satellite cells of both wild-type and myostatin-null mice. As expected, activated satellite cells had more Mighty cDNA than quiescent cells. However, the levels of Mighty between wild-type and myostatin-null mice were not significantly different by Mann-Whitney and Student's *t*-test. If Mighty is involved in the activation of satellite cells it is likely that peak levels occur during the first activation events. The satellite cells used in this experiment had already been cultured for 48 hours and it is possible that the peak of Mighty expression has been missed. Single fibre cultures would be a more accurate way of determining when the peak of Mighty expression occurs in relation to markers of satellite cell activation in both wild-type and myostatin-null satellite cells.

After activation, the regeneration of skeletal muscle requires migration of activated satellite cells to the site of injury, their proliferation, and then their commitment to terminal differentiation. Once committed, satellite cells will then fuse to other myoblasts or to existing fibres (Hawke and Garry, 2001). Myostatin-null mice show an improved regeneration phenotype, with less fibrosis and scar tissue formation (McCroskery *et al*., 2005). Mighty is hypothesised to enhance satellite cell commitment to the myogenic

differentiation programme, which results in the enhanced regeneration phenotype. To characterise Mighty expression during regeneration, wild-type and myostatin-null mice were used for notexin injection. On days 0, 1, 2, 3, 5, 7, 14 and 28 five mice from each genetic background were euthanised. Notexin is a cardiotoxin that is commonly used to induce muscle damage and regeneration. Notexin injection to the TA results in localised muscle damage and overall faster healing than crush injury (Lefaucheur and Sebille, 1995). Histologically, notexin injection causes consistent patterns of muscle injury over time and between rats that have different genetic backgrounds (Kirk *et al*., 1996: Kirk *et al*., 2000). Figure 3.5 shows that notexin induced comparable levels of damage in both mouse genotypes used in this study.

To determine the expression of Mighty during regeneration, a series of PCRs on undamaged wild-type mouse cDNA was carried out. The purpose of this was to determine the cycle numbers at which the PCR was in the linear phase of amplification. By analysing PCRs in the linear phase, the differences between samples can be semi-quantitatively determined. Levels of Mighty were significantly higher in the myostatin-null mice at day-zero (uninjured muscle) than in the wild-type mice. This difference was as expected and conforms to the differences seen in other experiments carried out. Mighty up-regulation at the messenger RNA level was not seen until day-five post-injury. Both wild-type and myostatin-null mice have significantly up-regulated Mighty cDNA at day-five compared to the undamaged day-zero levels. Most importantly, Mighty remained significantly up-regulated in myostatin-null mice at day seven post-injury compared to the wild-type mice.

Immunocytochemistry on sections of damaged tissue also revealed differences in the regeneration response of the two mouse genotypes. As seen in Figure 3.7, undamaged myostatin-null TA showed higher levels of Mighty protein expression, consistent with the cDNA data. By day-five post-injury the myostatin-null mice showed a large increase in the number of infiltrating nuclei present within the tissue, as well as an increase in the levels of Mighty protein. At day-seven post-injury Mighty protein levels within the wild-type tissue remained elevated, despite the decrease in cDNA levels described earlier. Mighty protein was persistent in the muscle longer than the transcript was. It is possible that Mighty protein levels in the myostatin-null background also persist beyond the down-regulation of transcription, but time points that would allow this to be determined were not taken. By day-14 post-injury Mighty protein levels in both mouse models had decreased, and at day-28 they were decreased further, although they had not yet decreased back to the levels seen in the undamaged controls.

MyoD is one of the four myogenic regulatory factors (MRFs) that are essential for myogenensis in the embryo. MyoD or Myf-5 are undetectable in the majority of quiescent satellite cells (Grounds *et al.*, 1992). As these cells become activated in response to injury, they will express either MyoD or Myf-5 (Cooper *et al*., 1999). By 48-hours post-injury, activated satellite cells co-express MyoD and Myf-5 and most go on to express Myogenin and MRF4 (Cornelison and Wold, 1997). During the regeneration time-course studied here, MyoD levels in wild-type mice started to increase at day two post-injury but were not significantly increased until day-five post-injury. In the myostatin-null mice, a similar pattern of MyoD expression was observed. MyoD did not increase over

the contra-lateral control values until day-three post-injury but the increase is significant at this stage. MyoD remains elevated until day seven post-injury, at which stage the levels decreased to those of the contra-lateral control.

The results displayed for the change in Mighty and MyoD message levels throughout regeneration are indicative of trend. During the regeneration process we found that two commonly used housekeeping genes, tubulin (McCroskery *et al*., 2003) and HPRT (hydroxanthine guanine phosphoribosyl transferase) (Rios *et al*., 2002) were both up-regulated (data not shown). GAPDH expression is also altered during regeneration (McCroskery *et al*., 2005). Several studies have shown that these commonly used genes are variably expressed in different tissues and proliferation or differentiation states. GAPDH and β-actin levels fluctuate during keratinocyte differentiation (Steele *et al*., 2002) and during arteriogenesis in the rabbit (Deindl *et al*., 2002), HPRT and β-tubulin levels vary in differentiating embryonic stem cell culture (Murphy and Polak, 2002), GAPDH expression is altered during T helper cell differentiation (Hamalainen *et al*., 2001) and in breast cancers when compared to control tissue (Tricarico *et al*., 2002). There is little consensus as to which gene should be examined as a housekeeper during muscle regeneration (McKinnell *et al*., 2005). When considering the extensive nature of the notexin-induced damage to the muscle, as shown in Figure 3.5, it is hardly surprising that genes which under normal conditions act as housekeeping genes are not suitable as internal controls. Structural proteins such as tubulin are required during muscle rebuilding and the increased cellular activity required to regenerate the muscle would require an increase in the molecules involved with energy generation, such as GAPDH (Vascotto *et al*., 2005), and the metabolic salvage of nucleotides, such as HPRT

(de Kok *et al*., 2005). To partially alleviate this problem, all cDNA synthesis reactions were carried out on equal amounts of total RNA as determined by spectroscopy using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies).

Analysis of the pattern of expression of the MRFs during regeneration revealed that MyoD is slowly up-regulated in the days following injury to a peak at day-three post-injury. Myogenin also peaks at day-three post-injury, with Myf-5 and MRF4 expression being maximal at day-five post-injury (Yan *et al*., 2003). Cornelison and Wold (1997) showed that by 48 hours post-injury activated satellite cells express both MyoD and Myf-5. Kirk *et al*. (2000) described two phases of regeneration, the damage phase which occurs in the first three days following injury, in which expression of myostatin peaks; and a regeneration phase from day-five post-injury. Day five post-injury was described as the time at which peak myotube formation was occurring. *In vitro* myostatin negatively regulates proliferation of myoblasts (Thomas *et al.,* 2000) and its expression during the early phases of regeneration is consistent with a role in maintaining satellite cells in a non-proliferative state while necrotic tissue is removed by phagocytosis. Myostatin has been shown to negatively regulate the Mighty promoter (Bishop, 2005), and in the myostatin-null mice, the lack of myostatin resulted in an increase of Mighty expression in undamaged muscle compared to the wild-type mice. Mighty up-regulation in both wild-type and myostatin-null tissue during regeneration did not occur until day-five post-injury, the time when myotube formation is occurring. Events at this time involve fusion of myoblasts to each other and to existing myotubes, and Mighty up-regulation at this time point is consistent with a role for Mighty in inducing hypertrophy of

muscle via enhanced myoblast fusion and of myostatin negatively regulating Mighty. Interestingly, Mighty up-regulation occurs at the same time in both wild-type and myostatin-null tissues. This indicates that myostatin is involved with maintaining a basal level of Mighty expression in normal muscle tissue, and that the up-regulation seen at day-five post-injury is in response to other factors. MyoD is one factor that is capable of increasing transcriptional activity of the Mighty promoter, although this increase is slight (2-fold; Bishop, 2005). Other transcription factors that have binding sites within the Mighty promoter, such as Sp1, may be responsible for the increase in Mighty expression seen at day-five post-injury. Mighty has also been shown to specifically up-regulate mediators of cell cycle withdrawal, such as p21 and p27 (Bishop, 2005), thus expression of Mighty at the time of myotube formation is consistent with Mighty playing a role in enhancing differentiation. Myostatin expression is seen in regenerating myotubes from day-five post-injury (Kirk *et al*., 2000) and this increase is likely to result in the down-regulation of the Mighty transcript in the wild-type tissue. Further results from the FMG Laboratory indicate that Mighty is down-regulated as commitment to terminal differentiation occurs, thus the decrease in Mighty expression seen in both the wild-type and myostatin-null genotypes could be due to terminal differentiation of the satellite cells.

When C_2C_{12} cells are induced to differentiate, a proportion of the cells commit to terminal differentiation, while a different population withdraw from the cell cycle and do not differentiate. Isolation and culture of undifferentiated cells results again in the formation of two populations of cells; one differentiated, one undifferentiated (Yoshida *et al*., 1998). This reserve cell population is akin to the satellite cell population seen *in vivo*. As such it can act as an *in vitro* model

for the events occurring *in vivo*. Mighty, which is, as has been stated previously, a downstream target of myostatin, causes an enhanced fusion phenotype when over-expressed. Cultures of differentiated Mighty over-expression clones have larger myotubes and fewer reserve cells than are seen in normal C_2C_{12} culture. This observation has lead to the hypothesis that Mighty is involved in hypertrophy by enabling the recruitment of more reserve cells into the differentiation pathway. Two possible down-stream targets of Mighty, stem cell antigen-1 (Sca-1) and CD34, may be involved in this process.

Sca-1 was identified by micro-array analysis as being down-regulated in the Mighty over-expression clones. To further characterise this decrease, Sca-1 primers as described in Mitchell *et al*. (2005) were standardised on cDNA from C_2C_{12} cells. Analysis of the myotube and reserve cell populations revealed that in C_2C_{12} cells the level of Sca-1 was significantly higher in the reserve cell population than in the myotube population. This same pattern of expression was preserved in the Mighty over-expression clones. However the absolute levels were decreased from those seen in the C_2C_{12} populations, as was expected based on the micro-array data. Levels of Sca-1 decreased by 60-70% in the myotube population and by 40-50% in the reserve cell population. Epting *et al*. (2004) and Mitchell *et al*. (2005) proposed that expression of Sca-1 acts to keep a population of myoblasts in a slowly proliferating non-dividing state that is capable of giving rise to reserve cells. Sca-1 decrease by Mighty may result in a larger proliferative and differentiation competent myoblast population during growth and differentiation. This could result in myotube hypertrophy and in a smaller reserve cell population that is a characteristic of Mighty over-expression.

CD34 was also identified by micro-array analysis as being down-regulated by Mighty over-expression. To analyse this further, PCR primers as described by Beauchamp *et al*. (2000) were used. These particular primers give two PCR products, depending on the form of CD34 that is present. This allowed analysis of both truncated CD34 and full-length CD34 levels from the same PCR. Both the truncated form of CD34, which is expressed on quiescent satellite cells (Beauchamp *et al*., 2000), and the full-length form of the CD34 transcript were present in the reserve cell and myotube populations. Levels of both CD34 transcripts were significantly down-regulated in both the myotube and reserve cell populations by Mighty over-expression. These results for both CD34 full and CD34 truncated were as expected from the micro-array analysis.

CD34 may function in muscle to keep a subpopulation of proliferative cells from differentiating. This theory is substantiated by the recent results of Kitzmann *et al*. (2006), who showed that the reserve cell population was heterogeneous for CD34 expression and that inhibition of notch was specifically able to increase the fusion of CD34-negative cells. Notch inhibition by its antagonist numb causes proliferating myoblasts to commit to terminal differentiation (Conboy and Rando, 2002). The inhibition of notch by over-expressing numb or by use of a γ-secretase inhibitor resulted in an enhanced fusion index of the myoblasts and increased differentiation (Kitzmann *et al*., 2006). Consistent with this, our results indicate that down-regulation of CD34 by Mighty could result in a population of cells that are more sensitive to fusion and differentiation signals and more sensitive to signalling through the notch pathway.

4.2 Future Directions

4.2.1 Mighty and regeneration.

The results described in this thesis indicate that there is a large up-regulation of Mighty during the regeneration process. This up-regulation occurs after the induction of MyoD. Further characterisation of the major myogenic regulatory factors during regeneration needs to be carried out, so that Mighty's place in the hierarchy of regulatory factors can be determined. Once the timing of Mighty expression in relation to these other factors is known, the factors that are expressed before Mighty can be analysed to determine if they are capable of directly influencing the regulation of the Mighty promoter. It is already known that MyoD is capable of causing an increase in transcriptional activity of the Mighty promoter. Testing of the other transcription factors that have binding sites within the Mighty promoter to determine their ability to up-regulate or down-regulate Mighty during myogenensis and regeneration needs to be carried out. Furthermore, Mighty may be able to auto-regulate its own transcription, which is a possibility that also needs to be determined. Finally, the effect of positive regulators of satellite cell activation and proliferation such as HGF and IGF on Mighty transcription needs to be determined.

4.2.2 Mighty and Reserve Cells

Mighty has been shown in previous experiments to cause an increase in hypertrophy and myoblast fusion when over-expressed. This results in a smaller reserve cell population after complete differentiation than is seen in control C_2C_{12} cultures. Over-expression of Mighty also causes a decrease in two molecules that are considered markers of less differentiated, stem cell-like cells, CD34 and Sca-1. Firstly, the mechanism by which Mighty is able to negatively

regulate the promoters of these genes need to be determined. Secondly, the role of these molecules in causing the enhanced fusion phenotype needs to be resolved. Some possibilities have come to light from recent papers. If Sca-1 acts as a determining factor that allows cells to take the self-renewal pathway during differentiation, then a direct down-regulation of this factor may cause the phenotype seen in the Mighty over-expressers. CD34 has recently been shown to influence the sensitivity of cells to Notch signalling (Mitchell *et al*., 2005). It would be of interest to determine if the Notch signalling pathway is altered in the Mighty over-expression clones.

Appendix

Agarose

0.5 g UltraPure agarose (Invitrogen)

50 ml 1 x TAE buffer

Boiled until agarose is dissolved.

1 µl 10 mg/ml ethidium bromide per 50 ml Agarose.

Assay Buffer (2 x) for β-gal detection assay

2 ml 200 mM sodium phosphate buffer

20 µl 2 mM magnesium chloride

71.4 µl 100 mM β-mercaptoethanol

3.33 ml 1.33 µg/µl ONPG

MilliQ water to 10 ml

Collagenase (0.2%)

30 ml DMEM (no serum)

0.06 g Collagenase Type 1A (Sigma)

Dissolved then syringe filtered through a Millex GP 0.22 µm filter (Millipore)

Denaturing RNA agarose gel

0.6 g agarose

36 ml DEPC treated water

Boil to dissolve agarose then cooled to ~60^{o}C

In fume hood add:

5 ml 10 x MOPS

9 ml formaldehyde

DEPC (diethyl pyrocarbonate)-treated water

2 ml DEPC

2 l MilliQ water

Mixed overnight then autoclaved.

Differentiation medium

Dulbecco's modified Eagle medium (DMEM)

2% horse serum

DNA 1kb+ Ladder

90 µl 10 x DNA loading dye

810 µl MilliQ water

100 µl 1 µg/ml 1kb+ ladder

DNA Loading Dye (10 x)

10 ml 50% glycerol

2 ml 50 x TAE

Pinch of bromophenol blue

Eosin (1% solution)

10 g Eosin Y

1 l MilliQ water

2.0 ml acetic acid (5% aqueous)

1 crystal of thymol

Gill's Haematoxylin

4.0 g Haematoxylin

0.4 g sodium iodate

35.2 g aluminium sulphate

710 ml MilliQ water

250 ml ethylene glycol

40 ml glacial acetic acid

Matrigel

200 µl Matrigel

1800 µl DMEM

Bottom of plate is covered, excess is removed. Plate kept at 37^{o}C to allow Matrigel to set.

MOPS (10 x) Solution (3-[N-Morpholino]propane-sulfonic acid)

41.8% MOPS powder

50 mM sodium acetate

10 mM EDTA

PBS (Phosphate Buffered Saline)

1 PBS tablet (Oxoid)

100 ml MilliQ water

PBS (10x)

1 PBS tablet

10 ml MilliQ water

Percoll (90%)

9 parts Percoll solution

1 part 10 x PBS

Percoll (70%)

2.33 ml 90% Percoll

0.67 ml 1 x PBS

Percoll (40%)

1.33 ml 90% Percoll

1.67 ml 1 x PBS

Ponceau S stain

0.1% Ponceau S

0.1% Acetic acid

Make up to desired volume with MilliQ water

Protein loading buffer

3 parts 4 x NuPage sample buffer

1 part β-mercaptoethanol

Protein lysis buffer

50 mM Tris (pH 7.5)

250 mM sodium chloride

5 mM EDTA

0.1% NP-40

1 x Protease inhibitor

RNA Loading Dye (2 x)

2 ml MOPS (10 x)

2 ml deionised formaldehyde

5 ml deionised formamide

40 µl of 10% bromophenol blue

1 ml glycerol

100 µl of 0.5 M EDTA (pH 8.0)

40 µl of 10 mg/ml ethidium bromide

RNA running buffer

1 part 10 x MOPS

9 parts DEPC water

Satellite cell proliferation media

50 ml DMEM (2x)

8 ml bicarbonate solution

1 ml penicillin/streptomycin

20 ml FBS

10 ml HS

1 ml chicken embryo extract

Make up to 100 ml with MilliQ water

Scott's tap-water

2.0 g sodium bicarbonate

20.0 g magnesium sulphate

1 l MilliQ water

1 crystal of thymol

TAE (Tris-acetate EDTA) for DNA gels (50 x)

242 g Tris (base)

57.1 ml glacial acetic acid

100 ml 0.5 M EDTA (ph 8.0)

Made up to 1 l with MilliQ water

TBS (Tris buffered saline)

50 ml 1 M Tris

30 ml 5 M Sodium chloride

Made up to 1 l with MilliQ water

TBS-T (Tris buffered saline + Tween-20)

999 ml TBS

1 ml Tween-20

Western blocking buffer

0.15 g BSA

0.5 g PVP

0.5 g PEG

50 ml TBS-T

Western transfer buffer

25 mM Tris

190 mM glycine

20% methanol

20:2:1 fixative

20 parts 70% ethanol

2 parts 40% formaldehyde

1 part glacial acetic acid

75% ethanol

75 ml 100% ethanol

25 ml DEPC-treated water

0.35% Carageenen-lambda

0.175 g Carageenen from seaweed (Sigma)

50 ml PBS

10% serum

20 µl serum

1980 µl PBS

0.5% Triton X-100

250 µl Triton X-100

49.75 ml PBS

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