THE ROLE OF GROWTH FACTORS IN THE REGENERATION OF SKELETAL MUSCLE

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Abstract

The overall aim of this thesis was to examine myostatin and components of the insulin-like growth factor (IGF) axis during regeneration, to determine whether these factors were temporally regulated during regeneration, whether the absence or presence of growth hormone (GH) affected their levels, and lastly, whether the administration of exogenous IGF-II enhanced muscle regeneration. A histological approach was utilised, to determine specific effects on individual tissue types (regenerating muscle fibres, survivor muscle fibres, undamaged muscle fibres, and connective tissue) within damaged muscle. Regeneration was induced by injection of notexin, a myotoxin, into muscle.

The first experiment tested the hypothesis that the IGFs and their receptors are regulated during muscle regeneration, and that the levels of these components are regulated by GH. The experimental model used was the GH-deficient *dw/dw* rat in which damage and subsequent regeneration were induced by a single intramuscular injection of notexin, then either GH- or saline-administered during the regeneration period. IGF-I and –II mRNA were assessed by *in situ* hybridisation, and binding determined by *in vitro* incubations with ^{125}I-IGF competed with unlabelled homologous IGF (*specific binding*). The presence of IGF binding proteins (IGFBPs) was determined by comparison of the specific binding of ^{125}I-IGF-I with the residual binding of ^{125}I-IGF-I following competition with des(1-3)IGF-I (which has a greatly reduced affinity for IGFBPs relative to IGF-I). Results of the localisation studies

revealed up-regulation of IGF-I and –II specific binding in regenerating fibres at the time of myotube formation, and indicated the presence of IGFBPs in damaged muscle tissues at the same timepoint. IGF-I and -II mRNAs were significantly up-regulated in regenerating muscle fibres concurrent with myotube formation and enlargement, while IGF-I mRNA was also elevated in regenerating muscle fibres at the time of muscle precursor cell proliferation. IGF-I mRNA was elevated in connective tissue of damaged, relative to undamaged, muscle during early regeneration. GH administration increased bodyweight, and the weights of damaged and undamaged muscles. GH administration did not affect the level of specific binding when ^{125}I-IGF-I was used as the ligand, or the level of IGFBPs as determined by competition of ^{125}I-IGF-I binding with unlabelled IGF-I versus unlabelled des(1-3)IGF-I, however GH administration did result in increased specific binding of ^{125}I-IGF-II to all damaged muscle tissues, relative to muscles from saline-treated animals. GH did not affect IGF-I or –II mRNA levels in damaged muscle tissues. In summary, this trial showed that all components of the IGF axis examined showed temporal regulation following muscle damage, and that GH administration significantly increased the binding of ^{125}I-IGF-II to damaged, but not undamaged, muscle tissues.

The second hypothesis tested in this thesis is that the negative regulator of muscle growth, myostatin, is regulated during muscle regeneration, and that its levels are decreased in muscles undergoing enhanced growth due to the administration of GH. The temporal regulation of myostatin protein was assessed by immunohistochemical staining of regenerating muscle sections of Sprague-Dawley rats, and of GH-deficient *dw/dw* rats. The effect of GH on myostatin protein levels was determined by

comparing myostatin protein levels in saline- versus GH-treated *dw/dw* rats. Myostatin immunostaining is present in the cytoplasm of fast muscle fibres, and is absent from the connective tissue of undamaged muscles, however following notexin injection, abundant myostatin immunostaining was observed at early timepoints in connective tissue, and high intensity immunostaining was observed in both fast and slow necrotic muscle fibres. Myostatin protein was absent from muscle precursor cells at the time of proliferation, and fusion to form new myotubes. Myostatin then gradually appeared in the muscle fibres undergoing enlargement. GH administration did not affect the temporal regulation, or level, or myostatin immunostaining observed. These findings suggest a role for myostatin in the regulation of muscle regeneration, including possible effects on connective tissue deposition.

The third hypothesis tested was that administration of IGF-II peptide during muscle regeneration would advance the onset of muscle precursor cell proliferation and differentiation. This was tested by implanting miniosmotic pumps fitted with catheters and filled with either IGF-II (to deliver 3.48 ug IGF-II/day) or vehicle (equal volume), in the subcutaneous compartment of Sprague-Dawley rats, then inducing damage and regeneration adjacent to the site of peptide of release. Results of the immunohistochemical analysis of MyoD, myogenin and developmental myosin heavy chain proteins in damaged muscle sections showed that IGF-II administration resulted in a delay in the onset of muscle precursor cell proliferation and differentiation, as compared to vehicle only controls. *In vitro* incubations using ^{125}I-IGF-I as the ligand showed no difference in the IGF binding capacity of day 1 tissues, indicating that the delay in early regeneration was not caused by down-regulation of the Type 1 IGF

receptor in response to administered IGF-II. Cross-sectional areas of regenerating muscle fibres on day 7 showed that late regeneration of muscle fibres was enhanced relative to the control, vehicle-only group. The period in which the administration of IGF-II enhanced muscle regeneration coincides with the time that IGF-II mRNA is elevated in regenerating muscle fibres, as shown in the first trial, suggesting that a greater endogenous production of IGF-II is associated with enhanced regeneration.

In conclusion, the results of these studies indicate that changes in myostatin and components of the IGF axis are associated with muscle regeneration, and that the levels of these components are differentially regulated depending on tissue type. These studies suggest that IGF-II may be an effective therapeutic agent in regenerating skeletal muscle, pending refinement of the administration protocol.

Preface

Some of the work contained in this thesis has been published in the following internationally refereed publications:

Kirk, S.; Oldham, J.; Kambadur, R; Sharma, M.; Dobbie, P.; Bass, J. 2000. Myostatin regulation during skeletal muscle regeneration. *Journal of Cellular Physiology 184:* 356-363.

Kirk, S.; Whittle, M.; Oldham, J.; Dobbie, P.; Bass, J. 1996. GH regulation of the Type 2 IGF receptor in regenerating skeletal muscle of rats. *Journal of Endocrinology:* 81-91.

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List of Abbreviations

bFGF	Basic fibroblast growth factor
BME	Beta-mercaptoethanol
bp	Basepair
BSA	Bovine serum albumin
C	Celsius
Ci	Curie
DAB	3,3-diaminobenzidine
DAR-B	Donkey anti-rabbit immunoglobulin, biotinylated
DEPC	Diethyl pyrocarbonate
dMHC	Developmental myosin heavy chain
DTT	Dithiothreitol
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
GDF	Growth and differentiation factor
GH	Growth hormone
HGF	Hepatocyte growth factor
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IL-6	Interleukin-6
Kb	Kilobase
LAP	Latency associated protein
LIF	Leukaemia inhibitory factor

LTBP	Latent transforming growth factor-beta binding protein
M	Molar
MEF-2	Muscle-specific enhancer factor-2
MHC	Myosin heavy chain
MPC	Muscle precursor cell
MRF	Myogenic regulatory factor
mRNA	Messenger ribonucleic acid
NDS	Normal donkey serum
NSS	Normal sheep serum
o	ovine
PDGF	Platelet-derived growth factor
rh	Recombinant human
RNA	Ribonucleic acid
SA-B-HRP	Streptavidin-biotin-horseradish peroxidase
SAM-B	Sheep anti-mouse immunoglobulin, biotinylated
SEM	Standard error of the mean
SSC	Sodium saline citrate buffer
TBS	Tris buffered saline
TBST	Tris buffered saline + Tween
TBSTB	Tris buffered saline + Tween + BSA
TGF-β	Transforming growth factor-beta
TNF-α	Tumour necrosis factor- alpha
XAR	X-OMAT-AR5

This Thesis Is Dedicated To

William James Powers

1935-1996

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Muscle tissue is responsible for most types of body movement. There are three types of muscle, identifiable by their structure and contractile properties: skeletal muscle, smooth muscle, and cardiac muscle. In this thesis, skeletal muscle will be examined. As the name indicates, skeletal muscle is responsible for the movement of the skeletal framework, as well as having a role in the maintenance of posture.

Skeletal muscle has a great capacity for regeneration, ie. the restoration of structure and function, following injury. This thesis addresses the hypothesis that growth factors regulate the regeneration of skeletal muscle. To provide sufficient background for the ensuing work, the composition of intact muscle will first be described, followed by a review of the processes that occur when muscle is damaged and undergoes regeneration. Subsequently, a review of the growth factors involved in the regulation of muscle growth and regeneration will be given, and an explanation of the overall aim and experimental hypotheses of this thesis.

1.2 An overview of muscle

Skeletal muscle is composed of four types of tissue- muscle fibres, connective tissue, nerves and blood vessels- all of which are necessary for the integrity and proper functioning of muscle.

1.2.1 Muscle fibres

The basic unit of skeletal muscle is the muscle fibre. Muscle fibres are cylindrical, and can extend either partially or wholly down the length of a muscle. It is the coordinated contraction of these fibres that generates force and movement (see Section 1.3). Within individual fibres are numerous myonuclei, the majority of which reside just inside the plasmalemma of the muscle fibre in mature muscle (Allbrook 1973). Myonuclei are post-mitotic, rendering them unable to participate in processes requiring cellular replication, such as growth and regeneration. Muscle cells that are, however, capable of replication, termed satellite cells, reside just to the exterior of the fibre, between the plasmalemma of the muscle fibre and its surrounding basal lamina. Satellite cells normally exist in a quiescent state, however, they can be activated in certain conditions to undergo mitosis in order to provide the additional muscle precursor cells (MPC) required for the growth maintenance and regeneration of postnatal skeletal muscle (this will be discussed in more detail in Section 1.5).

1.2.2 Connective tissue

Connective tissue plays a number of key roles in skeletal muscle: a) the provision of strength and structure, b) as a means of attaching muscle to bone, which is necessary for the generation of movement, and c) as a reservoir for growth factors, for connective tissue is a large component of the extracellular matrix surrounding muscle fibres, and d) as a medium through which metabolites are exchanged between muscle fibres and capillaries (Bloom & Fawcett 1975). As shown in Figure 1-1, three types of connective tissue are found within skeletal muscle: epimysium, perimysium and the endomysium. The epimysium envelopes the muscle, and is connected to the perimysium, which surrounds fascicles, or bundles of muscle fibres. The endomysium surrounds individual muscle fibres, and it is the portion that lies adjacent to the muscle fibre that is referred to as the basal lamina.

Connective tissue is composed primarily of a protein- and carbohydrate-rich matrix, including these components: collagen, elastin, fibronectin, laminin, proteo- and glycosaminoglycans. Collagen is a major component of connective tissue, and there exist numerous collagen types that are associated with skeletal muscle. Types I and III collagens are key components of the epimysium and perimysium, respectively, while Types IV and V are predominant in the endomysium (Bailey *et al.* 1979; Duance *et al.* 1977; Foidart *et al.* 1981).

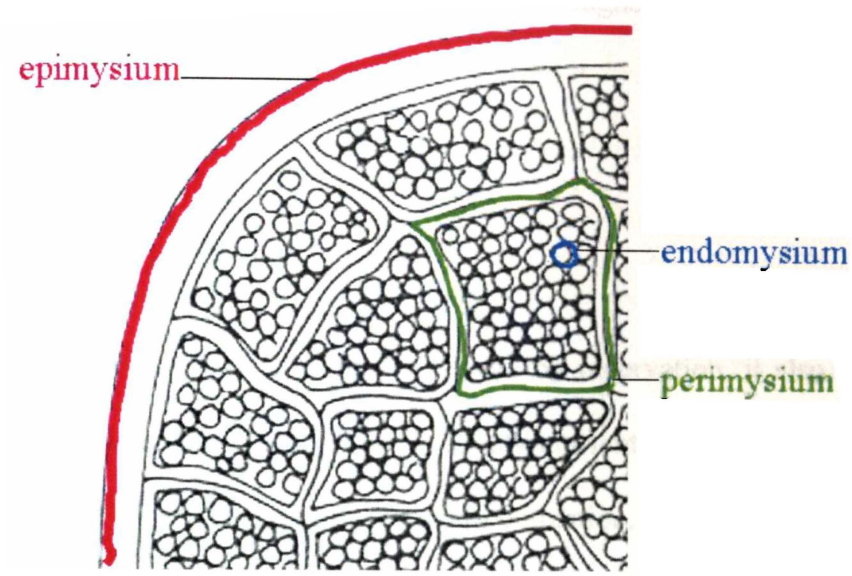


Figure 1-1. Connective tissue elements within skeletal muscle.

Shown is a cross-section of muscle with the endomysium (blue) surrounding individual muscle fibres, the perimysium (green) surrounding muscle fibre bundles, or fascicles, and the epimysium (red) surrounding the entire muscle. Redrawn from Lehto (1983).

1.2.3 Nerves

Innervation is essential to the functioning of muscle, as it is through the neuromuscular junction, formed where muscle meets nerve, that chemical signals are sent which trigger muscular contractions. Nervous input into muscles is via a main nerve trunk, which then bifurcates within the muscle so that each fibre is innervated. The majority of muscle fibres are innervated by a single motoneuron near to the centre of the fibre (Sanes & Lichtman 1999). Conversely, motoneurons innervate more than

one fibre (Engel 1994). The grouping of the motoneuron with the muscle fibres it innervates is referred to as a "motor unit" (Bodine-Fowler 1994). The muscle fibres of a motor unit are relatively uniformly distributed throughout normal, undamaged muscle (Bodine-Fowler 1994).

1.2.4 Blood vessels

Not only does proper functioning of muscle depend on innervation, it also depends upon adequate vascularisation. Blood vessels supply nutrients and carry away waste generated by muscle fibres during both rest and contraction. The patterning of the vascular system within individual muscles is highly varied, as muscles can be supplied by either single or multiple vessels (Jerusalem 1994). A common feature in muscles, however, is that within the perimysial space, arteries undergo remarkable branching. These arteries then lead to a succession of smaller vessels (arterioles, terminal arterioles and capillaries) that are located within the endomysium and in close apposition to muscle fibres, and it is through these smaller vessels that adequate nutrient supply to the muscle is accomplished.

1.3 Muscle fibre composition

As muscle fibres are the basic unit of skeletal muscle, an overview of their composition is integral to an understanding of the regeneration process. In adult muscle, the majority (80%) of muscle fibre volume is occupied by myofibrils, which contain the repeating contractile units known as sarcomeres (see Figure 1-2).

Sarcomeres, the specific units responsible for force generation, are composed of four types of protein filaments: thick, thin, nebulin and titin. These filaments are attached to, or in register with Z-discs. The main components of the thin filaments are actin, troponin and tropomyosin, while the thick filaments are composed of myosin, myomesin, as well as C-, H- and M-proteins (Schiaffino & Reggiani 1996). Within the proteins that comprise the thick and thin filaments there is considerable diversity due to the presence of numerous isoforms, and it is this variation in isoforms that determines the contractile properties of muscle fibres (Schiaffino *et al.* 1989).

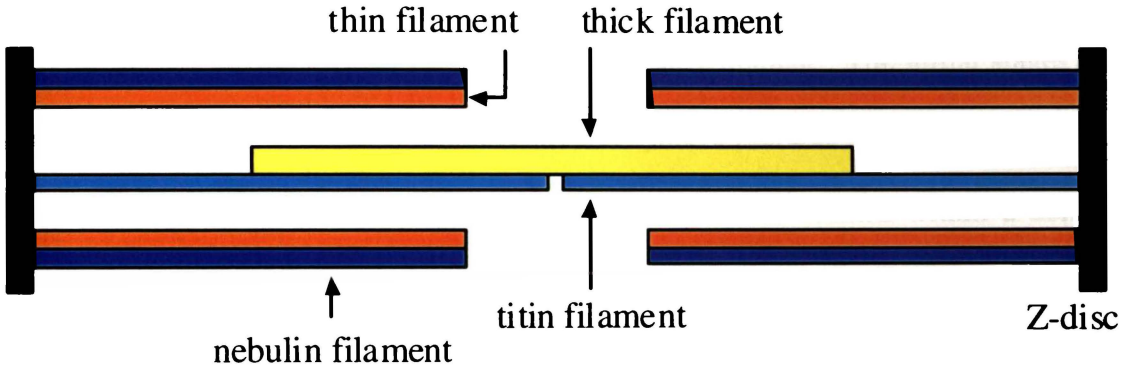


Figure 1-2. Structure of a sarcomere.

Redrawn from Schiaffino and Reggiani (1996).

Muscle fibres are characterised on the basis of their contractile properties, as either slow (Type 1) or fast (Type 2) fibres, and on the basis of their metabolic properties as either oxidative or glycolytic. As suggested by the latter terms, oxidative fibres utilise oxygen in their generation of energy, while glycolytic fibres utilise an anaerobic pathway. As such, oxidative fibres have a greater number of surrounding capillaries than do glycolytic fibres (Viscor *et al.* 1992). At present there are 4 identified muscle

fibre types in rats- slow oxidative (Type 1), fast oxidative (Type 2A), and two fast glycolytic (Type 2B, 2X) (Schiaffino *et al.* 1989). Different muscles of the body contain varying proportions of these fibre types, giving rise to muscles that have been specially adapted for different functions such as postural control, running, etc. Muscles with a high proportion of glycolytic fibres are especially suited for short bursts of intense energy, while muscles with a high proportion of oxidative fibres are better suited to long periods of lower intensity use.

1.4 Muscle development

Skeletal muscle is first formed during embryonic development. The initial stages of muscle development occur in specialised mesenchymal structures near the neural tube, called somites (see Figure 1-3). Within somites, MPC are located within a dorsal region referred to as the dermomyotome. These MPC embark on one of two possible courses: a) migration from the dorsal-medial (epaxial) region of the dermomyotome into the myotome, which eventually becomes part of the back musculature, or b) migration from the ventrolateral (hypaxial) region of the dermomyotome into the trunk and developing limb areas to form the muscles of the trunk and limb, respectively (reviewed by Hawke & Garry 2001). These MPC proliferate to form additional MPC, which then either fuse with other mononucleate MPC to form cylindrical muscle fibres, the basic units of skeletal muscle, or supplement existing fibres through the addition of more nuclei. Nuclei that have fused are post-mitotic.

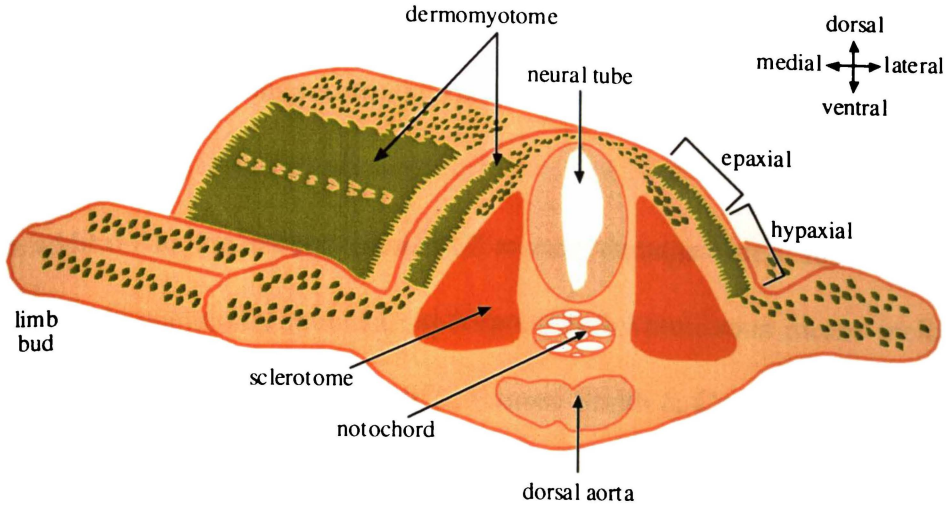


Figure 1-3. Embryonic origins of muscle.

A somite consists of the sclerotome and dermomyotome. The dermomyotome in turn is composed of the dermatome, which goes on to become the dermis, and the underlying myotome, which gives rise to muscle. The medial and lateral divisions of the myotome give rise to epaxial and hypaxial muscles, respectively. Figure revised from Hawke and Garry (2001).

1.5 Muscle damage and regeneration

1.5.1 General overview

Regeneration of skeletal muscle occurs naturally in response to a number of conditions, including injury such as muscle strain, crush injury or freezing. Similarly, muscle regeneration plays a key role in myopathies such as Duchenne's muscular dystrophy, a heritable condition affecting young males that leads ultimately to a substantial reduction in life span. In Duchenne's muscular dystrophy, a subsarcolemmal protein (dystrophin) is lacking, leading to repeated cycles of muscle fibre necrosis and regeneration (Karpati & Carpenter 1989). Damaged fibres are

replaced by fat and connective tissue rather than muscle (Liu *et al.* 1993b; McGeachie & Grounds 1999), thus causing loss of muscle function (Cohen *et al.* 1982).

In addition to the natural causes of muscle damage listed above, skeletal muscle damage is commonly induced experimentally by numerous means including muscle grafting (Carlson & Gutmann 1975; Hansen-Smith & Carlson 1979), and exposure to toxins (notexin, taipoxin) (Harris *et al.* 2000) or local anaesthetic (bupivacaine) (Carlson 1976). Toxin-induced regeneration will be discussed in more detail in Section 3.1. Irrespective of the means of damage, successful skeletal muscle regeneration consists of a number of coordinated, conserved processes: cellular infiltration of damaged muscle and phagocytosis of muscle fibre debris, revascularisation, proliferation of satellite cells to give rise to MPC, fusion of MPC to form myotubes, enlargement and maturation of regenerating myotubes, re-innervation, and remodelling of connective tissue (Bodine-Fowler 1994; Grounds 1991; Hansen-Smith & Carlson 1979; Kami *et al.* 1993). Section 1.5 looks at these processes in detail, while Section 1.7 considers the input of specific growth factors in modulating these processes.

1.5.2 Phagocytosis

The initial events that occur following muscle damage are: a) the disruption of myogenic structural protein organisation and myofibril retraction away from the site of damage (reviewed by Tidball 1995), and b) damage to the plasmalemma (reviewed by Grounds 1991). Subsequent to this there is de-regulation of calcium homeostasis,

leading to the free entry of calcium into the damaged muscle fibre (Armstrong 1990). High intracellular calcium interferes with the normal functioning of mitochondria (Grounds 1991; Nakayama *et al.* 2001), and leads to the activation of calcium-dependent proteases, known as calpains, which then degrade myofibrillar and other muscle proteins (Evans *et al.* 1984; Koh & Tidball 2000). In addition to these intracellular effects, is the activation of the lytic complement pathway, as indicated by the presence of complement components (Orimo *et al.* 1991; Sewry *et al.* 1987) and the membranolytic C5b-9 complement membrane attack complex (Engel & Biesecker 1982) on necrotic muscle fibres *in vivo*. The activation of this pathway contributes to the removal of necrotic debris from damaged muscle fibres (Grounds 1991).

Muscles can either be fully or partially damaged. In the case of incomplete damage of a muscle fibre, a new plasmalemma is synthesised at the junction of damaged and undamaged muscle that effectively seals off the undamaged muscle fibre from the damaged fibre (Carpenter & Karpati 1989; Papadimitriou *et al.* 1990). In most forms of muscle damage the basal lamina survives, where it then goes on to function as the scaffolding for new muscle formation (Sanes 1994).

Subsequent to muscle damage there is a remarkable increase in inflammatory and phagocytic cells within the lesion (Pimorady-Esfahani *et al.* 1997; Tidball 1995). It is believed that chemotactic factors attract these cells to damaged areas, as damaged muscle is chemotactic for polymorphonuclear leucocytes and macrophages (Robertson *et al.* 1993a), and the generation of complement reaction products stimulates cellular infiltration and the phagocytosis of necrotic muscle fibre debris (Engel & Biesecker

1982). Polymorphonuclear leucocytes appear first at the lesion, a response that has been shown to occur within 30 minutes, and to peak at 12 hours, following bupivacaine-induced myonecrosis (Orimo *et al.* 1991). Macrophages subsequently appear within damaged muscle, and are comprised of three distinct populations (McLennan 1996). Of these three populations, there is variability in their time of appearance (from three hours to 1-2 days following freeze-lesioning), as well as in function, for some macrophages have a clear association with necrotic tissue whereas others have none (McLennan 1996), suggesting that macrophages may perform additional functions to phagocytosis within damaged muscle (McLennan 1996; Merly *et al.* 1999). Alternative functions for macrophages include the enhancement of MPC chemotaxis and/or proliferation through the production of growth factors, as macrophages are known to secrete platelet-derived growth factor (PDGF), leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), and transforming growth factor-ß (TGF-ß) (Robertson *et al.* 1993a). Indeed, PDGF (AB and BB isoforms), LIF, bFGF, and TGF-ß are all chemotactic for MPC (Robertson *et al.* 1993a), while PDGF (BB isoform) and bFGF are mitogenic for MPC, indicating that macrophages likely function in the stimulation of MPC chemotaxis and proliferation (Robertson *et al.* 1993a). Mouse and turkey satellite cells co-cultured with macrophages exhibit enhanced proliferation and delayed differentiation relative to control satellite cell cultures (Merly *et al.* 1999).

1.5.3 Satellite cells and muscle precursor cells (MPC)

Satellite cells, which give rise to the MPC necessary for new fibre formation during muscle regeneration are, as stated in Section 1.2.1, normally located in the compartment between the basal lamina (also referred to as the basement membrane) and the plasmalemma (also referred to as the plasma membrane) as shown in Figure 1-4. On isolated, non-regenerating rat muscle fibres, satellite cells are present at a frequency of 2-3 per 100 myonuclei (Bischoff 1986a). Quiescent satellite cells typically have a heterochromatic nucleus, and a scant cytoplasm with few organelles (Hawke & Garry 2001). As is the case for the embryonic and foetal myoblasts that give rise to muscle during embryonic development, satellite cells are generally believed to derive from somites (Schultz & McCormick 1994), although recent studies suggest that at least some satellite cells may be derived from embryonic dorsal aorta (De Angelis *et al.* 1999).

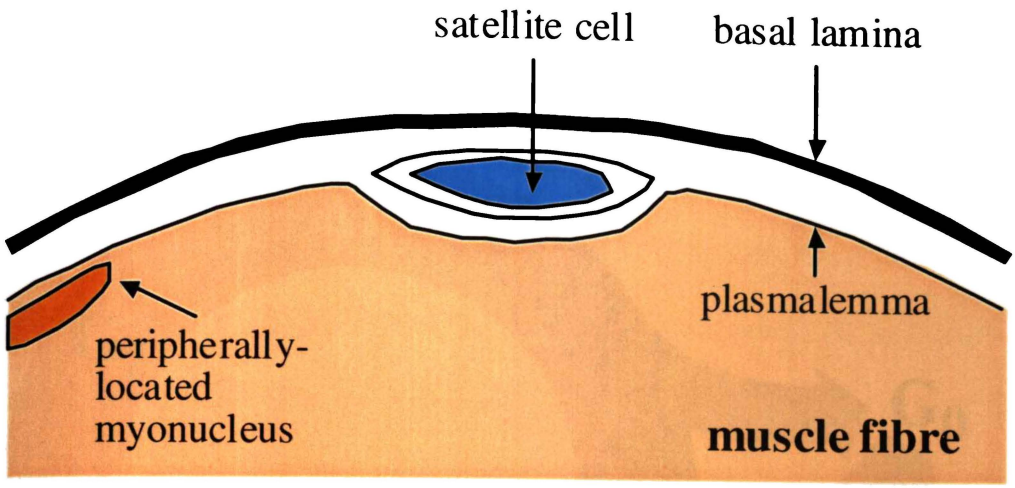


Figure 1-4. Location of satellite cells.

Satellite cells are located between the plasmalemma of the muscle fibre, and the surrounding basal lamina. Post-mitotic myonuclei of muscle fibres are peripherally located but within the plasmalemma of muscle fibres. Redrawn from Chambers and McDermott (1996).

1.5.3.1 Activation

Satellite cells normally exist in a quiescent state, known as the G_0 phase of the cell cycle. The cell cycle consists of the following sequential phases: gap 1 (G_1), synthesis (S), gap 2 (G_2), and mitosis (M), as shown in Figure 1-5, while G_0 is a state of quiescence. In order for satellite cells to produce additional MPC, they must first be activated to leave G_0 and enter the G_1 phase of the cell cycle (Hulleman & Boonstra 2001; Tatsumi *et al.* 1998). Once in G_1, cells must pass a restriction point (R) after which point they are committed to the phases of DNA synthesis (S phase), G_2 and mitosis (M phase) (Dou *et al.* 1993; Hulleman & Boonstra 2001; Pardee 1974). Satellite cell activation is often referred to as "cell cycle commitment", whereas

passage through the restriction point in G_1 is referred to as "cell cycle progression" (Johnson & Allen 1993). Following M phase, MPC may undergo another round of replication, differentiate, or become quiescent by re-entering the G_0 phase.

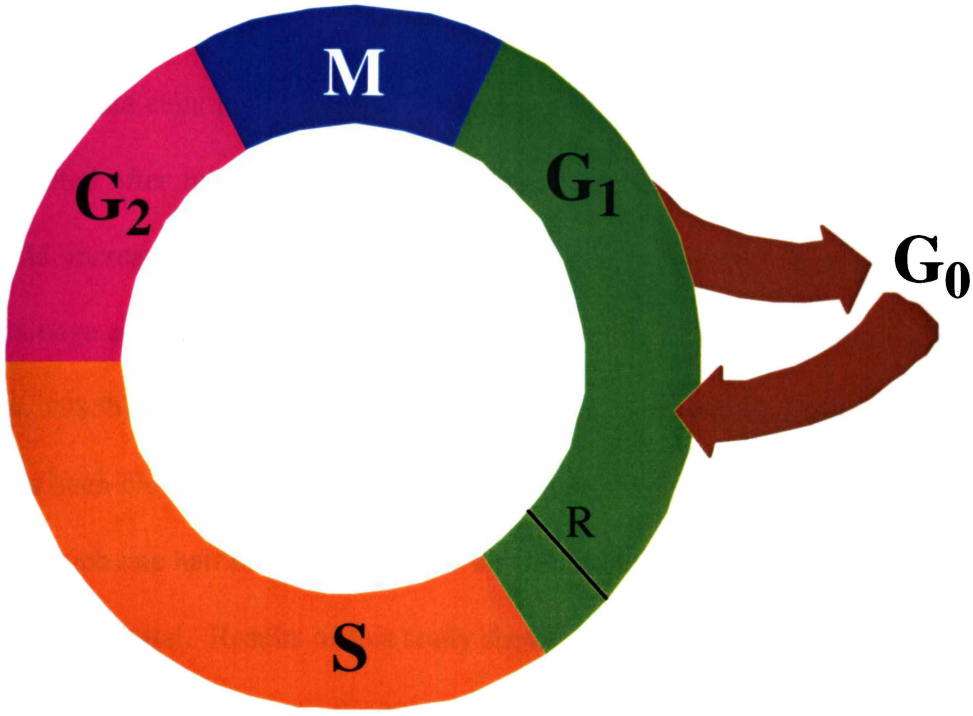


Figure 1-5. Diagram of the cell cycle.

Satellite cells normally exist in the quiescent state (G_0), but can be activated to re-enter the cell cycle in order to produce the MPC necessary for growth and regeneration. Activated satellite cells first enter the G_1 growth phase, and then must pass the growth factor-dependent point termed the restriction point (R) in order to progress onto the synthesis (S) phase in which DNA replication takes place. Cells then progress into the second growth phase (G_2), and finally undergo mitosis (division) in M phase. Redrawn from Chambers and McDermott (1996).

1.5.3.2 Migration

Once activated, satellite cells migrate to the site of injury (reviewed by Bischoff 1994). Activated satellite cells are able to move out of their characteristic position

between the basal lamina and plasmalemma (Grounds 1999). Satellite cells migrate to damaged areas from undamaged areas, creating a gradient in the number of satellite cells with distance from the site of injury (Klein-Ogus & Harris 1983; Schultz *et al.* 1985a). Migration of satellite cells can occur in a number of ways: longitudinally along a muscle fibre, perpendicular to the fibre axis, and between muscles. Migration of satellite cells longitudinally along muscle fibres has been shown in fibre segments isolated after injury, whereby the damaged portion of muscle fibres showed a four-fold increase in the number of satellite cells, while the region furthest from the damage contained one-third the number of satellite cells in control muscles (Schultz *et al.* 1985b). The occurrence of MPC migration perpendicular to the muscle fibre axis has been cleverly demonstrated using a longitudinally-split autograft model in the rat, in which one half of the muscle was devitalised by freeze damage, while the other half remained vital. Results of this study showed that regenerated muscle fibres formed in the devitalised half, with the MPC being contributed by the live half of the autograft (Phillips *et al.* 1990). This study therefore showed that MPC can travel considerable distances without constraint by the endomysium or perimysium, and is in keeping with the observation of myoblast migration across the basal lamina during normal muscle development (Hughes & Blau 1990). Studies of the migration of MPC between nearby muscles in mice and rats has led to seemingly contradictory results, with both positive (Morgan *et al.* 1987b; Partridge & Sloper 1977; Watt 1982; Watt *et al.* 1987) and negative (Ghins *et al.* 1984; Schultz *et al.* 1986) reports of a host contribution to the regeneration of allografted muscle. The conflict in these reports appears to be due to species differences (Phillips *et al.* 1990), whereby there is considerable input from nearby host muscle in mice (Morgan *et al.* 1987b; Partridge & Sloper 1977; Watt

1982; Watt *et al.* 1987), but not in rats (Ghins *et al.* 1984; Schultz *et al.* 1986) under ordinary circumstances. The formation of a physical bridge between the epimysia of neighbouring muscles is sufficient to allow migration from neighbouring muscles in rats to occur (Schultz *et al.* 1986), suggesting that while MPC can apparently traverse the epimysium of mice, they are unable to in rats due presumably to its increased thickness (Phillips *et al.* 1990). It has also been suggested that the distances for satellite cells to travel within the rat, but not the mouse, are too great to allow for satellite cell contributions from adjacent muscle (Ghins *et al.* 1984; Phillips *et al.* 1990).

Because of the notable increase in MPC in regenerating muscle, some researchers have considered additional sources of MPC other than satellite cells. A possible source is myoid cells, which are located in the thymus and express MyoD and myogenin mRNA (Grounds *et al.* 1992). Myoid cell levels are decreased in the thymus after muscle damage, and are lower than normal during dystrophy-induced chronic damage (Wong *et al.* 1999). Bone marrow cells have recently been shown to contribute to regeneration, however this contribution is minimal relative to total nuclei within regenerated muscle, and occurs late in the regeneration process (Ferrari *et al.* 1998). Therefore, although a contribution to skeletal muscle regeneration by non-satellite cell derived MPC may occur in some instances, the absolute numbers of non-satellite cell MPC involved in regeneration appears relatively minor at present.

1.5.3.3 Proliferation

The proliferation stage of muscle regeneration consists of the division of satellite cells to form MPC that will then go on to form the regenerating muscle fibres. As stated in Section 1.5.3.1, cellular proliferation involves progression through at least one entire cell cycle (G_1 to M), thereby creating additional MPC. These daughter MPC will then either progress on to terminal differentiation, or undergo additional proliferation (Grounds & McGeachie 1989). A more in depth examination of the cell cycle and regulators of the cell cycle will be undertaken in Section 1.7, in reference to growth factor control of these processes.

Not all satellite cells appear to enter into the cell cycle during regeneration, based on the reported observation of differentiation-associated markers prior to proliferation markers (Rantanen *et al.* 1995). This has led to the assertion that a population of satellite cells exists which is already committed to immediate terminal differentiation without preceding cell division (Rantanen *et al.* 1995).

Heterogeneity in the proliferating satellite cell population has been reported for rat muscle cells grown in culture. Specifically, two distinct populations, separable on the basis of phenotype and proliferation rate were reported (Molnar *et al.* 1996), thus expanding the potential range of regeneration efficiencies by differences in the inherent properties of the satellite cells themselves.

1.5.3.4 Fusion

Once sufficient proliferation has taken place, MPC undergo terminal differentiation, and so exit from the cell cycle. As such, the cell is no longer capable of re-entering the cell cycle and proliferating. Terminal differentiation marks the time when the appearance of many proteins typical of mature muscle, such as sarcomeric proteins, takes place, and when fusion to form myotubes occurs. Myotubes are immature muscle fibres. They normally form within the tube-like structure of the empty basal lamina, which serves as a scaffolding of sorts for the newly-formed myotubes (Caldwell *et al.* 1990). In instances where there is no basal lamina, such as after mincing of muscle, myotube formation can still occur, but the level of organisation at earlier, but not later, timepoints is rather poorer than the organisation of myotubes within pre-existing basal lamina tubes (Caldwell *et al.* 1990). The generation of mechanical forces along a muscle may play an important role in the orientation of regenerating muscle fibres and their basal lamina tubes (Caldwell *et al.* 1990).

Five distinct types of fusion may take place during skeletal muscle regeneration: MPC to MPC, MPC to myotube, myotube to myotube, myotube to muscle fibre, and MPC to muscle fibre (Robertson *et al.* 1990; Robertson *et al.* 1993b). Electron microscopic evidence for fusion includes the formation of small gaps in the apposed myogenic cells, coalescence of the cytoplasm of the adjoining cells and, in some types of fusion, the union of the free ends (Robertson *et al.* 1990). In the case of myotube to myotube fusion, union usually occurs at multiple sites along the lateral borders of the apposed

myotubes (Robertson *et al.* 1990), while for MPC or myotube fusion to the sealed, damaged ends of muscle fibres, fusion occurs at the "stump" (end) region, and gives rise to sarcoplasmic extensions or "buds" (Robertson *et al.* 1993b). Collagen or other extracellular material deposited amongst sarcoplasmic extensions could give rise to fibres with a "split" appearance (Robertson *et al.* 1993b), a phenomenon that has been reported often in muscle regeneration literature (Ontell 1986).

1.5.3.5 Maturation

Following myoblast fusion to form myotubes, a period of muscle fibre enlargement and maturation commences. This period is completed when the mature muscle phenotype has been re-established. The primary events occurring during this period are re-innervation (addressed in Section 1.5.4) and cytoplasmic enlargement, including the synthesis of new contractile units, or sarcomeres. Considerable alteration in the pattern of myosin heavy chain (MHC) isoform expression occurs during this period, and will be addressed in Section 1.6.2. Although the fibre enlargement during regeneration bears resemblance to muscle fibre hypertrophy (defined as the enlargement of muscle fibre cytoplasm) the two processes are, strictly speaking, distinct from one another.

1.5.4 Innervation

Innervation is the connection of motoneurons to muscle fibres via the formation of synapses and motor endplates. The re-establishment of innervation is essential for the

growth and maturation of regenerating muscle fibres, as shown by studies comparing the regeneration of muscle in the presence or absence of innervation. The early stages of muscle regeneration up to the time of early myotube formation occur normally in the absence of innervation, but thereafter diverge so that denervated muscles are growth retarded, have lower isometric tension values, express alternate myosin isoforms, have abnormal sarcotubular morphology, and eventually undergo atrophy (Bodine-Fowler 1994; Sesodia & Cullen 1991; Whalen *et al.* 1990).

Denervated muscle can be reinnervated by either the original neurons, or by axon outgrowth from neighbouring muscles. Studies of the reinnervation of grafted muscles have shown that the former is the predominant means of muscle reinnervation, and that the latter tends only to occur if there has been damage to the epimysium of the neighbouring muscle(s) (Klueber 1987).

The basal lamina plays an integral role in the reinnervation of muscle. New neuromuscular junctions (where nerve terminals meet the basal lamina) form predominantly at the original synaptic sites on the basal lamina (Marshall *et al.* 1977). The basal lamina contains signals that direct reinnervation to specific sites (Sanes *et al.* 1978), and this process occurs regardless of the presence of the myofibre (Sanes *et al.* 1978). In damaged muscle, the pattern of distribution of muscle fibres innervated by motoneurons (referred to as "motor units") is altered relative to undamaged muscle. In undamaged muscle, muscle fibres served by a single motoneuron are reasonably uniformly distributed throughout the muscle, however following reinnervation there

are notable groupings of muscle fibres within a given motor unit (Bodine-Fowler 1994).

The normal state of the neuromuscular junction is single innervation by motoneurons. However, in the early stages of innervation following nerve damage, polyneuronal innervation occurs, whereby neuromuscular junctions are innervated by multiple motoneurons. In order to return the neuromuscular junctions to a "normal" state, synapses are eliminated through a process of gradual removal of its component parts (Culican *et al.* 1998). In mice, this process occurs within two weeks of reinnervation (Rich & Lichtman 1989).

1.5.5 Vascularisation

Revascularisation is a critical process for the effective regeneration of skeletal muscle, for without the timely re-establishment of vascularisation, excessive fibrotic tissue deposition and muscle cell death can result (Borisov *et al.* 2000). Revascularisation begins near to the time of the onset of necrotic debris phagocytosis (Roberts & McGeachie 1990). In grafted muscle undergoing regeneration, revascularisation begins at the periphery of the muscle, then proceeds inward towards the centre of the muscle (Roberts & McGeachie 1990). Revascularisation occurs rapidly, with functional blood vessels established within 2 days of the onset of the revascularisation process, although the complete re-establishment can take 14 days in a heavily damaged muscle, such as that resulting from the transplantation model (Roberts & McGeachie 1990).

1.5.6 Connective tissue deposition

During muscle regeneration there are changes in the connective tissue, or extracellular matrix, compartment. Following contusion damage to rat muscle, the sequence of changes in the connective tissue compartment begins with disruption of the extracellular matrix (Stauber *et al.* 1990) and an increased widening of the interstitial spaces due to the deposition of extracellular matrix components (Stauber *et al.* 1990). Also associated with this early phase is fibroblast proliferation (Hurme *et al.* 1991; Stauber *et al.* 1990), for fibroblasts produce many of the extracellular matrix components, such as collagens and fibronectin (Hurme *et al.* 1991; McMinn 1967). Capillary formation within the connective tissue compartment also occurs as an early event (Hurme *et al.* 1991; Jarvinen 1975). In the next phase, while satellite cells are proliferating and forming new myotubes, phagocytosis of unnecessary and/or surplus proteins occurs (Hurme *et al.* 1991). During this early stage, the connective tissue compartment is very fragile, however after new fibre formation, the connective tissue becomes denser and attains a greater tensile strength (Hurme *et al.* 1991) and, at later stages of fibre maturation, the endomysial and perimysial compartments appear thickened relative to those of undamaged muscle (Hurme *et al.* 1991). The connective tissue strength appears to be related to the collagen isoforms expressed, with first Type III collagen, which is associated with plasticity, then Type I collagen, which provides tensile strength (Hurme *et al.* 1991).

Muscle regeneration is enhanced by the presence of the basal lamina (Kami *et al.* 1993; Vracko & Benditt 1972), but is hindered by excessive connective tissue formation. Excessive connective tissue can develop after severe trauma (Carlson

1968; Phillips *et al.* 1990), forming a dense barrier that regenerating muscle fibres must penetrate (McMinn 1967).

1.6 Gene expression in skeletal muscle

1.6.1 Myogenic regulatory factors (MRFs)

The myogenic regulatory factors (MRFs) play an important role in the commitment of cells to the myogenic lineage, as well as their subsequent differentiation to form mature skeletal muscle (Rudnicki & Jaenisch 1995; Sabourin *et al.* 1999). Four MRFs have been identified thus far: MyoD, myf-5, myogenin and MRF4. Expression of each of these factors in non-muscle cells results in conversion to a myogenic phenotype (Braun *et al.* 1989b; Choi *et al.* 1990; Edmondson & Olson 1989; Rhodes & Konieczny 1989; Wright *et al.* 1989). Targeted gene disruption experiments in mice support the importance of MRFs for normal muscle development, as disruption of myogenin and MRF-4 leads to perinatal lethality due to major defects in skeletal muscle (Nabeshima *et al.* 1993; Patapoutian *et al.* 1995). In the case of gene disruption of either MyoD or myf-5, normal muscle develops, however simultaneous disruption of both MyoD and myf-5 results in perinatal lethality (Braun *et al.* 1992; Rudnicki *et al.* 1992; Rudnicki *et al.* 1993). Thus there exists some measure of redundancy in the MRFs, by virtue of the ability of MyoD or myf-5 to compensate for loss of the other MRF.

MRFs have a coordinated and sequential pattern of expression during satellite cell activation, proliferation, and differentiation. In quiescent satellite cells, it was

previously held that there was no expression of MRFs (Smith *et al.* 1994), however activation of myf-5 gene expression within quiescent mouse satellite cells (Beauchamp *et al.* 2000), and the presence of myf-5 protein in quiescent C2 muscle cells (Kitzmann *et al.* 1998) have recently been reported. Upon satellite cell activation, either MyoD or myf-5 mRNA is expressed, followed by frequent co-expression of both MyoD and myf-5, as observed at both the mRNA and protein level (Cornelison & Wold 1997; Kitzmann *et al.* 1998). Myogenin and MRF-4 mRNA expression occur subsequent to the expression of MyoD and myf-5 mRNAs (Cornelison & Wold 1997), with the expression of myogenin mRNA coincident with early differentiation (Smith *et al.* 1994; Yoshida *et al.* 1998). In recent years, MyoD expression has been routinely used in muscle regeneration studies as a marker for MPC proliferation, and myogenin expression as a marker for the entry of MPC into the differentiation pathway (Floss *et al.* 1997; Jin *et al.* 2000; Merly *et al.* 1999).

MRFs exert effects on muscle cells through the formation of heterodimers with E-proteins (reviewed by Puri & Sartorelli 2000), as shown in Figure 1-6. E-proteins are the gene products of E2A (E12 and E47) (Brennan & Olson 1990; Lassar *et al.* 1991; Murre *et al.* 1989), and HEB, a gene that is highly homologous to E2A (Hu *et al.* 1992). MRF-containing heterodimers bind to a region referred to as the E-box, which has the consensus sequence CANNTG, and is present in the promoter and enhancer regions of many muscle-specific genes. The enhancement of muscle differentiation by MRFs is positively regulated by MEF-2 (Molkentin & Olson 1996), and negatively regulated by the proteins Id ("inhibitor of differentiation") (Jen *et al.* 1992), twist (Hamamori *et al.* 1997; Hebrok *et al.* 1994), I-mf (Chen *et al.* 1996), Mist1

(Lemercier *et al.* 1998), MyoR (Lu *et al.* 1999), and ZEB (Postigo & Dean 1997). An additional level of control is present in that the MRFs exert positive feedback on themselves as well as regulate the levels of other family members (Braun *et al.* 1989a; Rudnicki *et al.* 1992; Thayer *et al.* 1989).

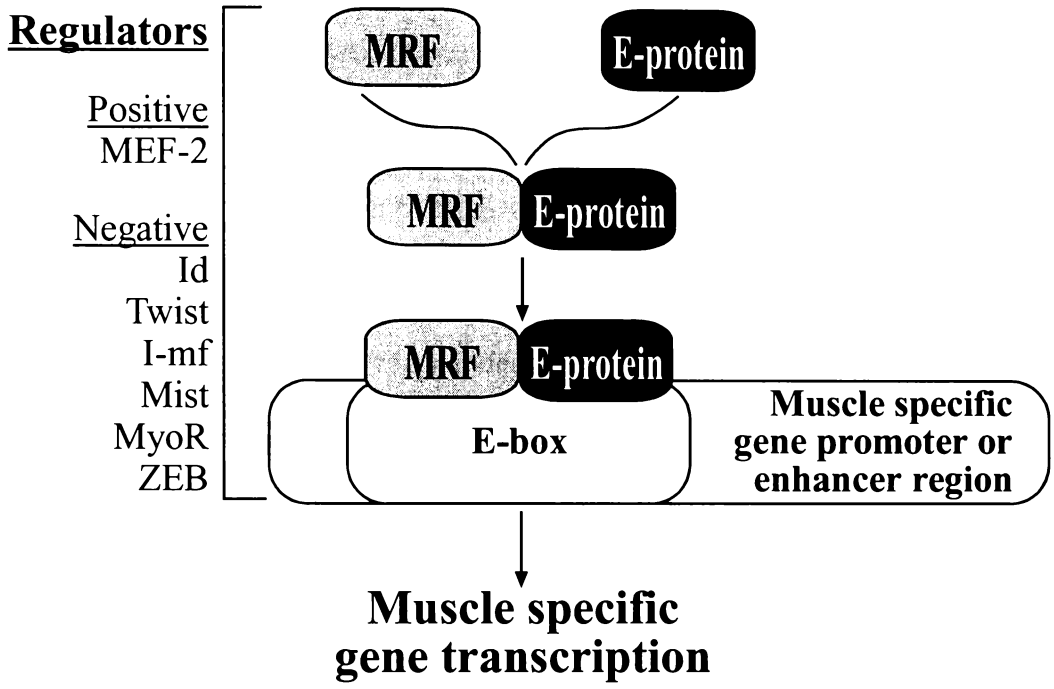


Figure 1-6. Control of gene transcription by MRFs.

MRFs bind to E-proteins, which then recognise and bind to a sequence (CANNTG) referred to as an "E-box" in the promoter region of muscle specific genes. The process of MRF/E-protein complex formation and binding to the promoter region is positively regulated by MEF-2, and inhibited by Id, Twist, I-mf, Mist, MyoR and ZEB.

1.6.2 Myosin heavy chains (MHCs)

As discussed in Section 1.3, muscle fibres have distinct fibre types that are determined by their contractile and metabolic properties. The determination of specific MHC isoforms is considered the most accurate determinant of fibre type (Kelly & Rubenstein 1994). There exist over 10 identified MHC isoforms, many of which have an extremely restricted expression profile. For instance, neonatal and embryonic MHCs are expressed during embryonic development, but are not normally present in the adult (Condon *et al.* 1990). Adult rat muscle, like the muscle of many mammals, normally contains four MHC isoforms- I, IIa, IIb, and IIx -with very small but detectable amounts of α-cardiac MHC (Dunn & Michel 1997).

During regeneration, newly regenerated muscle fibres re-express neonatal and embryonic MHCs, two isoforms that together are referred to as developmental MHC (dMHC) (Davis *et al.* 1991; Sartore *et al.* 1982). The dMHC in the regenerating fibres is then replaced by either fast and/or slow MHC isoforms (Yoshimura *et al.* 1998). Fibre type, and thus MHC isoform expression, at this point is highly dependent on the innervation status of the regenerating muscle fibres, so that fibres that are regenerating in the absence of nerve ("aneural") remain as fast (type II) fibres, and express only fast MHC isoforms as shown in Table 1-1 (Whalen *et al.* 1990; Yoshimura *et al.* 1998). This is in contrast to the situation for fibres that regenerate in the presence of nerve ("innervated"), which can become either fast or slow muscle fibres (predominantly slow), as indicated by the presence of either fast or slow MHC isoforms (Davis *et al.* 1991; Whalen *et al.* 1990; Yoshimura *et al.* 1998)(see Table 1-1).

Table 1-1. Effect of innervation on MHC expression.

The effect of innervation status of grafted muscle on the pattern of MHC isoform expression by regenerating muscle fibres. Abbreviations: dMHC ("dev"), fast MHC ("fast"), slow MHC ("slow"). Adapted from Yoshimura *et al.* (1998).

Innervation status	MHC expression pattern
Innervated	Dev → dev + fast → fast ↘ dev + fast + slow → fast + slow → slow
Aneural	Dev → dev + fast → fast

1.7 Growth factors in muscle regeneration

Growth factors and hormones modulate the processes of skeletal muscle growth and regeneration. A summary of the findings for the major growth factors involved in muscle growth, and their effects on the key processes of muscle regeneration, are presented in Table 1-2. As shown (Table 1-2), individual growth factors may affect either single or multiple processes. Much of what is currently known about the effects of individual growth factors on the steps of myogenesis have been determined by *in vitro* studies, and it is largely unknown whether these same actions occur *in vivo*. While *in vitro* studies are useful, one must bear in mind that tissue culture conditions are quite different to the complex *in vivo* environment that exists during muscle regeneration (Grounds 1991).

Naturally occurring growth factors, including fibroblast growth factor (FGF), platelet-derived growth factors (PDGFs), hepatocyte growth factor (HGF), insulin-like growth factors (IGFs), transforming growth factors (TGFs), leukaemia inhibitory factor (LIF), interleukin-6 (IL-6), epidermal growth factor (EGF), and myostatin can be produced locally- either within the same cell that they act upon (autocrine production) or within a cell from the adjacent tissue (paracrine production)- or they can be produced elsewhere within the body (endocrine production). The unique patterns of growth factor production and localisation during muscle regeneration provide a further means of modulating growth factor activity.

A key aspect of growth activity within regenerating muscle is the coordination of expression of multiple growth factors, for there is frequent co-expression of different factors. Growth factor activity can be altered by the presence of other growth factors, as has been clearly shown *in vitro* by the enhancement of IGF-I-stimulated proliferation by the addition of either basic(b)FGF or EGF (Doumit *et al.* 1993), and the inhibition of PDGF-BB stimulated proliferation by the addition of TGF-β (Cook *et al.* 1993). Furthermore, it has been suggested that not only is the absence/presence of a growth factor important for eliciting a response, so too is the growth factor gradient that is established within the regenerating muscle (Bischoff 1997).

Table 1-2. Effects of growth factors on regeneration processes.

The effects listed below have been determined by *in vitro* studies.

Growth factor	Migration	Proliferation	Differentiation	Reference
bFGF	↑			Suzuki *et al.* (2000)
	↑	↑		Robertson *et al.* (1993a)
			↓	Olwin & Rapraeger (1992)
EGF		↑ or ↔[1]		Doumit *et al.* (1993)
		↔		Lim & Hauschka (1984)
		↑	↓	Lim & Hauschka (1984)
		↔	↔	Florini *et al.* (1986)
	↔			Bischoff (1997)
HGF		↑		Allen *et al.* (1995)
		↑	↓	Miller *et al.* (2000)
		↑		Tatsumi *et al.* (1998)
	↑			Suzuki *et al.* (2000)
	↑			Bischoff (1997)
IGF-I		↑	↑ or ↓[2]	Florini *et al.* (1986)
		↑	↑	Ewton *et al.* (1994)
	↑			Suzuki *et al.* (2000)
		↑	↑	Allen & Boxhorn (1989)
IGF-II		↑	↑ or ↓[2]	Florini *et al.* (1986)
			↑	Ewton *et al.* (1994)
			↑	Rosenthal *et al.* (1994)
		↑	↑	Ewton *et al.* (1998)
IL-6		↑		Austin & Burgess (1991)

[1] when in the presence of serum or other growth factor(s), but no effect in serum-free media

[2] promotes differentiation at low concentrations, inhibits differentiation at high concentrations (biphasic response).

[3] inhibits at high cell density, enhances at low cell density.

Table 1-2. Effects of growth factors on regeneration processes (continued).

Growth factor	Migration	Proliferation	Differentiation	Reference
LIF		↑		Austin & Burgess (1991)
		↑		Austin *et al.* (1992)
	↑			Robertson *et al.* (1993a)
Myostatin		↓		Thomas *et al.* (2000)
		↓		Rios *et al.* (2001)
PDGF-AA		↔		Ye *et al.* (1996)
	↔		↑ or ↓ [3]	Webb & Lee (1997)
	↔	↔		Robertson *et al.* (1993a)
PDGF-AB		↑		Ye *et al.* (1996)
	↑		↑ or ↓ [3]	Webb & Lee (1997)
	↑	↔		Robertson *et al.* (1993a)
PDGF-BB		↑		Ye *et al.* (1996)
	↑		↑ or ↓ [3]	Webb & Lee (1997)
	↑	↑		Robertson *et al.* (1993a)
TGF-α		↑		Austin & Burgess (1991)
		↑		Austin *et al.* (1992)
TGF-β		↓	↓	Allen & Boxhorn (1989)
			↑	Zentella & Massague (1992)
	↑			Bischoff (1997)

[1] when in the presence of serum or other growth factor(s), but no effect in serum-free media.
[2] promotes differentiation at low concentrations, inhibits differentiation at high concentrations (biphasic response).
[3] inhibits at high cell density, enhances at low cell density.

1.7.1 Insulin-like growth factors

The role of IGFs in the regeneration of skeletal muscle was pursued in this thesis because of the potent stimulatory effects of IGFs on muscle growth *in vitro* (see Table 1-2) (Adams & McCue 1998) and *in vivo* (Bark *et al.* 1998; Coleman *et al.* 1995). The existence of the insulin-like growth factors was first proposed by Salmon and Daughaday in 1957, with the "somatomedin hypothesis," namely that GH acts through a second factor that is secreted into the circulation (Salmon & Daughaday 1957). This second factor was initially called "somatomedin", and is now known as insulin-like growth factor-I (IGF-I). Insulin-like growth factor-II (IGF-II), previously known as "multiplication stimulation activity" (MSA) (Pierson & Temin 1972) and "non-suppressible insulin-like activity-2" (NSILA) (Rinderknecht & Humbel 1978b), was subsequently identified and shown to be highly homologous to IGF-I. IGFs are so named because they are structurally related to insulin (Blundell *et al.* 1983).

1.7.1.1 IGF-I and -II peptide synthesis

IGF-I

Rat IGF-I is encoded by a single gene that contains six exons. Two of these exons (3 and 4) encode the mature IGF-I molecule (Butler *et al.* 1994). Transcription and differential splicing of messenger RNA yield IGF-I transcripts with numerous permutations of the 5'- and 3'-termini. Variations in both these regions lead to alterations in the signal peptide and carboxy-terminal extension peptide (E-peptide) sequences of the preproIGF-I peptide that is first synthesised (Steenbergh *et al.* 1997). Variation in the signal peptide sequence is believed to affect the processing and

secretion of IGF-I (Gilmour *et al.* 1992), while variation in the 3'-terminal sequences is associated with distinct patterns of expression, and possibly function (McKoy *et al.* 1999). Both the signal- and E-peptides are cleaved in order to produce mature IGF-I. Recent studies indicate that the cleaved E-peptide, on its own, contains mitogenic activity (Tian *et al.* 1999).

Mature IGF-I is comprised of 70 amino acids (MW 7,646) arranged in a single chain, with three disulfide bonds linking six cysteine residues (Rinderknecht & Humbel 1978a). There are four "domains" within IGF-I, extending from amino to carboxy-termini in the order, B-C-A-D. IGFs share 38-40% homology with insulin in the A and B regions, however insulin does not contain a D-region (Adams *et al.* 1983; Whitfield *et al.* 1984). IGF-I peptides also share a high (>92%) degree of sequence homology amongst mammals (Foyt & Roberts 1991; Steenbergh *et al.* 1997).

IGF-II

The gene that encodes rat IGF-II contains six exons (Steenbergh 1997; Dull, 1984). The prepro-IGF-II molecule, from which the signal and E-terminal peptides are cleaved to produce the mature peptide, is encoded by exons 3-6 in rodents (Soares *et al.* 1986; Steenbergh *et al.* 1997). As for IGF-I, numerous transcripts are produced from the IGF-II gene as a result of different promoter usage, alternative splicing of the 5' sequences, and by alternative polyadenylation of 3' sequences. The expression of variant IGF-II transcripts, which may have diverse properties of stability and/or translatability (Rosen *et al.* 1993), are differentially regulated during *in vitro* myogenesis (Rosen *et al.* 1993).

Mature IGF-II contains 67 amino acids (mol wt 7,471) (Rinderknecht & Humbel 1978b) arranged in a single chain with three disulfide bonds, and has a similar domain structure to IGF-I. Human IGF-I and –II peptides are over 60% homologous at the amino acid level (Whitfield *et al.* 1984). The IGF-II peptide sequence has been shown to be highly conserved, with greater than 93% homology amongst mammals (Foyt & Roberts 1991; Steenbergh *et al.* 1997; Whitfield *et al.* 1984).

1.7.1.2 IGF receptors

Type I IGF receptor

The actions of IGFs are both mediated and modulated by binding to receptors. IGFs can bind to three receptors: the Type I IGF receptor, Type II IGF receptor, and the insulin receptor, all of which are found in muscle cells (Beguinot *et al.* 1985; Virkamaki *et al.* 2001). The binding affinity of the Type I IGF receptor is greatest for IGF-I, less for IGF-II, and least for insulin (Florini *et al.* 1996). The Type I IGF receptor is a tetrameric glycoprotein, composed of two alpha and two beta chains. The alpha-chains are entirely extracellular in location, and are responsible for ligand binding. The alpha-chains are linked to the transmembrane beta-chains via disulphide bonds in the extracellular compartment (reviewed by Butler *et al.* 1998). Like the receptors for a number of other growth factors, the Type I IGF receptor functions as a ligand-activated tyrosine-specific protein kinase (Ullrich *et al.* 1986). Within the cytoplasmic compartment, the beta chains have a tyrosine kinase enzymatic domain that includes an ATP binding site (Werner *et al.* 1991). Ligand binding stimulates

autophosphorylation of the intracellular domain, and enables the receptor to phosphorylate protein substrates (reviewed by Butler *et al.* 1998).

Insulin receptor

The insulin receptor has a similar structure to the Type I IGF receptor, and functions as a ligand-activated tyrosine-specific kinase receptor. The precursor molecules for the insulin and Type I IGF receptors are of a nearly identical length (approximately 1340 amino acids), are 84% homologous within the beta chains, and roughly 65% homologous in the regions surrounding the cysteine rich region of the alpha chains (Werner *et al.* 1991). The affinity of the insulin receptor for its ligand is: insulin>> IGF-II> IGF-I (Florini *et al.* 1996).

Alternative splicing of the insulin receptor mRNA gives rise to two isoforms, A and B, of the insulin receptor (Moller *et al.* 1989). As indicated above, the affinity of the insulin receptor for IGF-II is normally relatively low (Florini *et al.* 1996), however it has recently been shown that the insulin receptor isoform A, which is present in foetal tissues and some cancers, binds IGF-II with an affinity close to that of insulin, and on a par with the binding of IGF-II to the Type I IGF receptor (Frasca *et al.* 1999).

Type II IGF receptor

The Type II IGF receptor is a single chain polypeptide that contains an extracellular domain comprised of 15 cysteine-rich repeating units, a hydrophobic transmembrane helix and a short cytoplasmic sequence (Lobel *et al.* 1988). Unlike the insulin and Type I IGF receptors, the Type II IGF receptor does not have intrinsic tyrosine kinase

activity (Roth 1988). The affinity of the Type II IGF receptor for its ligand is IGF-II>>> IGF-I, and it does not bind insulin (Ewton *et al.* 1987; Florini *et al.* 1996; Rechler & Nissley 1985; Rosenfeld *et al.* 1987). The Type II IGF receptor is identical to the cation-independent mannose-6-phosphate receptor (Braulke *et al.* 1988; Morgan *et al.* 1987a; Roth *et al.* 1987), and it binds IGF-II and proteins containing mannose-6-phosphate moieties through two distinct binding sites on the receptor (Braulke *et al.* 1988). Proteins containing M6P moieties, including both leukaemia inhibitory factor (LIF) and lysosomal enzymes, are normally internalised and degraded (Blanchard *et al.* 1999; Kornfeld 1992), but in the case of the M6P-containing latent TGF-β, binding to the Type II IGF receptor leads to activation of TGF-β (Ghahary *et al.* 1999a; Ghahary *et al.* 2000). Additionally, retinoic acid can bind to Type II IGF receptor sites other than those used for IGF and M6P binding, resulting in altered Type II IGF receptor distribution within the cell (Kang *et al.* 1998; Kang *et al.* 1997). The Type II IGF receptor is predominantly intracellular in localisation, with only 5-10% of the receptor located on the cell surface (Kornfeld 1992). Binding of retinoic acid to the Type II IGF receptor results in enhanced binding and endocytosis of exogenous lysosomal enzymes (Kang *et al.* 1998; Kang *et al.* 1997), as well as enhanced sorting of lysosomal enzymes (Kang *et al.* 1997). These studies thus show that the Type II IGF receptor interacts with numerous ligands, however, knockout experiments in mice clearly show that the majority of growth-related interactions of the Type II IGF receptor during foetal development are with IGF-II (Ludwig *et al.* 1996). This is inferred by the complete rescue of the overgrowth phenotype in Type II IGF receptor null mice by concomitant disruption of the IGF-II gene (Ludwig *et al.* 1996).

A cleaved form of the Type II IGF receptor is present in the circulation (Causin *et al.* 1988), and is secreted from cells in culture (Clairmont & Czech 1991; Scott *et al.* 1996). This soluble form of the receptor is able to bind IGF-II with high affinity, and appears to inhibit IGF-II, but not IGF-I, activity (Scott & Weiss 2000).

1.7.1.3 IGF binding proteins

The IGF binding proteins (IGFBPs) are a group of proteins that modulate IGF action and are present both in the circulation and within tissues. Amongst the functions ascribed to the IGFBPs are extension of the IGF half-life, localisation of IGFs to certain cell types, and modulation of IGF binding to cell surface receptors (MacGregor & Parkhouse 1996). Through these functions, IGFBPs may either potentiate or inhibit IGF action. Inhibition is proposed to result from the association of IGFs with IGFBPs in solution, thereby making the IGF unavailable for binding to the receptor. Potentiation is believed to result from associations of IGFBPs with extracellular matrix or cell surface proteins, accompanied by a decrease in binding affinity, which allows the IGF to be released and to partake in receptor binding (Bach *et al.* 1994; Wood 1995).

There are six identified high-affinity IGF binding proteins (IGFBPs), named IGFBP-1 to -6 (Bach *et al.* 1994). IGFBP-3 is the main binding protein in the circulation, and is present predominantly in a GH-dependent 150kDa complex with IGF-I or –II, and an acid labile 85kDa subunit. IGFBP-3 binds >95% of circulating IGF-I and –II with high affinity (Cohick & Clemmons 1993). *In vitro* studies have shown that a number

of IGFBPs are produced within muscle cells, with proliferating myoblasts producing predominantly IGFBPs-2, -4, and -6 (Ernst *et al.* 1992; Ewton & Florini 1995). Differentiated muscle cells produce IGFBPs-4, -5, and -6 (Ewton & Florini 1995; James *et al.* 1993; Rotwein *et al.* 1995; Silverman *et al.* 1995), with increasing and decreasing amounts of IGFBPs –3 and –2, respectively, as differentiation proceeds (Ernst *et al.* 1992; Johnson *et al.* 1996).

The affinities of IGFBPs for IGF-I and IGF-II differs; thus IGFBPs-2 and –5, and -6 have a greater affinity for IGF-II relative to IGF-I (Baxter 2000; Hossner *et al.* 1997), while IGFBPs-1, -3 and –4 bind IGF-I and –II with equal affinity (Cohick & Clemmons 1993; Hossner *et al.* 1997).

In addition to the high affinity IGFBPs, there are four recently identified IGFBP-related proteins (IGFBP-rP-1 to –4) that also bind IGFs. The IGFBP-rPs are highly homologous to IGFBPs in the N-terminal region, however they do not contain the C-terminal region within which the IGFBPs share homology (Baxter *et al.* 1998; Kim *et al.* 1997). The binding affinity of IGFBP-rPs for IGFs is reduced relative to the affinity of IGFBPs for IGFs (Oh *et al.* 1996).

1.7.1.4 IGF gene knockout experiments

The important role of IGF peptides and receptors in regulating foetal growth has been clearly shown by knockout experiments, in which the gene of interest is disrupted. IGF-I knockout mice are 60% of normal weight at birth, and exhibit a generalised

muscular dystrophy that is particularly evident in the diaphragm, heart and tongue (Liu *et al.* 1993a; Powell-Braxton *et al.* 1993). IGF-I knockout mice have a high rate of perinatal mortality (over 95%), and those that do survive remain smaller in size postnatally (Liu *et al.* 1993a; Powell-Braxton *et al.* 1993). The mouse IGF-II gene is subject to imprinting, such that the paternal allele is active while the maternal allele is silent in virtually all embryonic tissues (DeChiara *et al.* 1991). Inactivation of the paternal IGF-II allele leads to phenotypically normal, fertile mice that are also 60% of normal weight at birth, with persistent low weight postnatally (DeChiara *et al.* 1990). Mice with an inactivated IGF-II gene are viable, indicating that although IGF-II protein has profound effects on size, it is not essential for embryonic development (DeChiara *et al.* 1990; DeChiara *et al.* 1991). Simultaneous knockout of both IGF-I and IGF-II genes results in mice that weigh only 30% of normal weight at birth (Liu *et al.* 1993a), thus indicating that IGF-I and IGF-II have additive effects on growth. Most of the biological effects of the IGFs are thought to be mediated through binding to the Type I IGF receptor (Ludwig *et al.* 1996). In keeping with this, inactivation of the Type I IGF receptor results in mice that are 45% of normal weight at birth (Liu *et al.* 1993a; Ludwig *et al.* 1996). Mice with a disrupted Type I IGF receptor have smaller muscles due to hypoplasia (decreased tissue cell number) (Liu *et al.* 1993a), and die at birth (Ludwig *et al.* 1996). In mice, but not humans, the Type II IGF receptor is oppositely imprinted to IGF-II, so that the maternal allele is transcriptionally active while the paternal allele is silent (Haig & Graham 1991; Ogawa *et al.* 1993). The Type II IGF receptor in mice is proposed to act, at least in part, as a "sink" for IGF-II during foetal development (Haig & Graham 1991), as Type II IGF receptor knockout mice are subject to foetal overgrowth (approximately 130%

of normal weight at birth (Lau *et al.* 1994). Taken together, these gene knockout experiments indicate that the IGFs and their receptors are key determinants of foetal growth.

1.7.1.5 IGFs in skeletal muscle

1.7.1.5.1 IGF expression

IGF-I and –II peptides are first expressed during early foetal development, and are subsequently regulated in a temporal fashion. IGF-I mRNA levels in muscle are highest during foetal and early neonatal development, then decline to approximately 25% of early neonatal levels by postnatal day 50 in the rat (Adamo *et al.* 1989). In contrast, IGF-II mRNA is expressed at a high, constant level throughout rat foetal development, but is undetectable in muscle and all tissues, other than brain, in the adult (Beck *et al.* 1988; Soares *et al.* 1985). The decrease in IGF-II mRNA levels in most tissues of the rat at birth is accompanied by a decrease in circulating levels of IGF-II (Cohick & Clemmons 1993). The decrease in circulating levels of IGF-II is observed in rodents, but not humans, postnatally (Cohick & Clemmons 1993; Steenbergh *et al.* 1997). The temporal patterns of expression during development suggest that these peptides play a role in foetal muscle development.

Just as IGF-I and –II are temporally regulated in muscle tissue during development, they are also regulated in distinctive patterns in muscle cells during *in vitro* myogenesis. In cultured C2 muscle cells, the expression of IGF-I mRNA is normally low during myoblast proliferation (Tollefsen *et al.* 1989a; Tollefsen *et al.* 1989b), but

increases 6-10 fold within 48-72 hours of a switch to differentiation medium (Tollefsen *et al.* 1989a). The expression of IGF-II mRNA is also low during C2 myoblast proliferation and increases during differentiation, with 25 times higher levels at 96 hours after the switch to differentiation medium (Tollefsen *et al.* 1989b). Both IGF-I and IGF-II peptides are secreted from C2 muscle cells concurrent with the increase in IGF transcription during differentiation (Tollefsen *et al.* 1989a; Tollefsen *et al.* 1989b). The cognate receptors for IGF-I and –II are also regulated during myogenesis, with detectable levels of Type I IGF receptors in proliferating BC3H-1 myoblasts, followed by a decrease in receptors during differentiation (Rosenthal *et al.* 1991). The Type II IGF receptor, which is non-abundant in proliferating C2 myoblasts, increases 6-fold during early differentiation, and remains high thereafter (Tollefsen *et al.* 1989b). IGFBPs are undetectable in proliferating myoblasts, but increase substantially within 16 hours of the switch to differentiation medium. The increase in IGFBPs during differentiation is accompanied by secretion of IGFBP from the muscle cells (Tollefsen *et al.* 1989a).

1.7.1.5.2 IGF action

IGFs exert pleiotropic effects on skeletal muscle cells. Studies have established a role for the IGFs in eliciting anabolic effects, including suppression of protein degradation (Janeczko & Etlinger 1984), enhanced amino acid uptake (Merrill *et al.* 1977), induction of hypertrophy (Adams & McCue 1998; Coleman *et al.* 1995), and the stimulation of myoblast proliferation and differentiation. It is the enhancement of myoblast proliferation and differentiation by IGFs that is most relevant to the work contained within this thesis.

Enhancement of myoblast proliferation involves an increase in the number of myoblasts traversing the cell cycle. As discussed in Section 1.5.3.1, the cell cycle consists of the phases G_1, S, G_2 and M, while G_0 is the state of quiescence. Growth factors participate in different phases of the cell cycle, and it is the G_1 phase of the cell cycle where pivotal growth factor regulation of cellular proliferation occurs (Hulleman & Boonstra 2001). There are two phases of growth factor dependence in G_1, first of which is the transition from G_0 to G_1 (activation) (Hulleman & Boonstra 2001; Zumstein & Stiles 1987), while the second phase is the progression through G_1 and the growth factor-dependent restriction (R) point, after which time cells are no longer dependent on growth factors for cell cycle progression (Hulleman & Boonstra 2001; Pardee 1974). To date, only hepatocyte growth factor (HGF), a factor found in crushed muscle extract, has been shown to be capable of activating satellite cells (Tatsumi *et al.* 1998). IGFs have been clearly established as progression factors, that is they are capable of progressing cells through the restriction point (Chakravarthy *et al.* 2000; Zhang *et al.* 1999) so that they are committed to completion of the cell cycle (Hulleman & Boonstra 2001). IGFs stimulate the proliferation of both transformed myoblasts and primary cultures of satellite cells *in vitro* (Dodson *et al.* 1985; Doumit *et al.* 1993; Florini *et al.* 1977; McFarland *et al.* 1993).

IGF-I is more potent than IGF-II in eliciting the proliferative response in myogenic cells (Ballard *et al.* 1986; Ewton *et al.* 1994), and early researchers recognised that the order of potency in stimulating proliferation (IGF-I> IGF-II> insulin) mirrored the order of affinity of the ligands for the Type 1 IGF receptor (Ewton *et al.* 1987). In confirmation of this, studies in chicken satellite cells show that IGF-I and -II are

bound to the Type I IGF receptor during IGF-stimulated proliferation (Duclos *et al.* 1991).

The mitogenic activity of IGF-I on myoblasts is enhanced by the presence of a co-factor, referred to as mitogenic competence factor, that is present in horse serum (McWade *et al.* 1997; McWade *et al.* 1995; Napier *et al.* 1999). Addition of horse serum to cultured L6 myoblasts that have previously undergone IGF-stimulation of proliferation allows the myoblasts to continue responding to the IGF with increased proliferation, whereas that responsiveness is lost without horse serum (Napier *et al.* 1999). The identity of mitogenic competence factor is unknown, but is not believed to be TGF-β, PDGF-BB, FGF, or IGFBPs (McWade *et al.* 1997; McWade *et al.* 1995).

The literature to date has tended to focus on the mitogenic effects of IGF-I, and not those of IGF-II, however that is not to say that IGF-II is a poor mitogen. Perhaps some of the most compelling evidence that this is not the case comes from recent oncogenic research, in which high levels of IGF-II have been associated with the uncontrolled cellular proliferation typical of the malignant state (Khandwala *et al.* 2000). This association has also been shown to occur in rhabdomyosarcomas, a skeletal muscle tumour (Minniti *et al.* 1994), and may be partially explained by the observation of reduced cell cycling time, through a diminished G_1 checkpoint, in IGF-II overexpressing myoblasts (Zhang *et al.* 1999). Such a scenario could give rise to the malignant state due to the importance of cell cycle checkpoints in making certain that all the necessary processes have been completed before progression to the next stage (Zhang *et al.* 1999). As discussed previously, IGF-II predominantly uses the

Type I IGF receptor to elicit a mitogenic effect, however IGF-II has also recently been shown to effectively stimulate cell cycle progression via the A isoform of the insulin receptor as well (Frasca *et al.* 1999; Scalia *et al.* 2001). The insulin receptor isoform A is present in foetal muscle and in some cancers (Frasca *et al.* 1999), however it is unknown whether this represents a significant mitogenic pathway in undamaged and/or regenerating adult muscle. Taken together, these studies indicate that not only IGF-I, but also IGF-II, is a potent regulator of myoblast proliferation.

IGFs are unusual growth factors, in that they are able to stimulate both proliferation and differentiation, as many growth factors that stimulate proliferation inhibit differentiation, and vice versa (Florini *et al.* 1996). The stimulation of myogenesis by IGFs has been reported for cell lines and primary cultures of satellite cells (Allen & Boxhorn 1989; Engert *et al.* 1996; Ewton *et al.* 1994; Greene & Allen 1991), and is concentration-dependent, with stimulation of myogenesis at low concentrations and inhibition at supraphysiological levels (Florini *et al.* 1986; Florini & Magri 1989).

IGFs interact predominantly with the Type I IGF receptor to elicit a myogenic response (Ewton *et al.* 1987), although involvement of the Type II IGF receptor is also suggested by studies showing that an IGF-II analog with high affinity for Type II IGF receptors stimulates differentiation (Rosenthal *et al.* 1994). The amount of Type I IGF receptor impacts on the speed with which myogenesis occurs, with overexpression leading to an increased rate of myogenesis, and functional inactivation resulting in delayed differentiation *in vitro* (Cheng *et al.* 2000; Quinn & Haugk 1996; Quinn *et al.* 1994). While these studies show that the Type I IGF receptor modulates

differentiation, *in vitro* studies also indicate that it is not a critical component, as serum-induced differentiation of cells in culture can still occur in the absence of a functional Type I IGF receptor (Cheng *et al.* 2000).

IGF-I and IGF-II are not equivalent in their stimulation of differentiation (Ewton *et al.* 1994; Florini *et al.* 1993). IGF-I has a greater potency than IGF-II- that is lesser amounts of IGF-I are required to stimulate differentiation- when administered to cultured myoblasts, however IGF-II effects a greater absolute stimulation of differentiation (Florini *et al.* 1993). The difference between IGF-I and –II in the stimulation of differentiation *in vitro* appears to be due at least in part to the greater stimulation of proliferation by IGF-I relative to IGF-II, for when the mitogenic effects of IGF-I on cultured L6A1 myoblasts are suppressed, the stimulation of differentiation by IGF-I approaches that of IGF-II (Ewton *et al.* 1994).

Proliferation and differentiation are normally considered mutually exclusive events, and the IGFs are unique in their ability to stimulate both processes. The mechanism by which IGFs achieve this involves cell cycle regulatory proteins, so a review of cell cycle control by regulatory proteins, and in particular the retinoblastoma gene product (Rb), is essential. The retinoblastoma (Rb) gene product is a primary controller of the cell cycle. Rb is a ubiquitously expressed protein containing 16 potential sites for phosphorylation, and it is the phosphorylation state of these sites that determines Rb activity (Driscoll *et al.* 1999). Rb protein plays an important role at the restriction point in G_1, by determining whether cells can undergo DNA synthesis (reviewed by Hatakeyama & Weinberg 1995). The active (hypo-phosphorylated) form of Rb

suppresses transcription of the genes necessary for DNA synthesis through inhibition of E2 promoter binding factor (E2F)-containing complexes, while the inactive (hyper-phosphorylated) form of Rb does not have growth suppressor activity (La Thangue 1994). The phosphorylation state of Rb protein is mediated by cyclins (A, D1 and E) and cyclin-dependent kinases (cdks), so that up-regulation of cyclins and cdks is associated with hyperphosphorylation of Rb, and subsequent cellular proliferation (Hatakeyama & Weinberg 1995; Sherr 1994).

The state of Rb phosphorylation and cyclin/cdks also plays a part in the decision by cells to exit the cell cycle following mitosis. Active (hypo-phosphorylated) Rb promotes cell cycle exit and terminal myogenic differentiation (Gu *et al.* 1993), while high levels of cyclins and cdks inhibit this process both directly through effects on Rb phosphorylation state, and through a mechanism independent of Rb (Skapek *et al.* 1996). So important is the effect of Rb phosphorylation on terminal myogenic differentiation, that expression of the phosphorylated, inactive form of Rb by terminally differentiated SV40T antigen-transfected C2C12 cells allows re-entry into the cell cycle (Gu *et al.* 1993). However, re-entry of non-transfected, "normal" terminally differentiated cells back into the cell cycle does not occur because pRB is refractory to rephosphorylation in response to growth factors in terminally differentiated cells, in agreement with the inability of muscle fibre nuclei to re-enter the cell cycle (Gu *et al.* 1993). The progression of cells from the proliferative to differentiated state is marked by an increased incidence of apoptosis, or programmed cell death, as shown for C2 muscle cells in culture (Stewart & Rotwein 1996). Apoptosis is a normal feature of mammalian skeletal muscle development (McArdle

et al. 1999). Upregulation of the cdk inhibitor, $p21^{WAF1/CIP1}$, and dephosphorylation of Rb are critical regulatory events for the establishment of the apoptosis-resistant state (Walsh & Perlman 1997), a state that allows nuclear number to be retained during differentiation (Stewart & Rotwein 1996).

Studies of the early and late effects of IGFs on muscle cells show that at early timepoints, IGF upregulates the expression of cyclin D1 and cdk4, which consequently leads to hyperphosphorylation of Rb (Rosenthal & Cheng 1995). This inactive form of Rb allows proliferation, in response to IGFs, to take place. Concurrent with the early enhancement of Rb phosphorylation, is a down-regulation of myogenin expression, an event that occurs independently to the effects on cell cycle proteins (Rosenthal & Cheng 1995). Myogenin plays a critical role in differentiation by controlling the transcription of muscle specific genes (Myer *et al.* 1997), and in regulating the extracellular environment in which myoblast fusion takes place, as indicated by the report that wild-type myoblasts rescue the ability of myogenin -/- myoblasts to fuse *in vivo* (Myer *et al.* 1997). Regulation of myogenin gene expression by IGFs is biphasic, whereby IGFs inhibit myogenin gene transcription during proliferation, then enhance myogenin gene transcription during later stages (Adi *et al.* 2000; Rosenthal & Cheng 1995). The inhibition of myogenin gene expression during proliferation inhibits differentiation (Adi *et al.* 2000; Myer *et al.* 1997; Rosenthal & Cheng 1995), while the later increase promotes differentiation (Adi *et al.* 2000; Rosenthal & Cheng 1995). The differential effects of IGFs on myogenin transcription occur via two distinct regions on the myogenin gene promoter, with inhibition occurring via promoter sequences located in the –145 to –9 region of the myogenin

gene, and enhancement occurring in the –1,565 to –375 region of the the myogenin gene (Adi *et al.* 2000). Effects of IGF on myogenin gene transcription via two distinct sites in the promoter region thus explain how IGFs can both stimulate and inhibit myogenin gene transcription (Adi *et al.* 2000). The coordinated control of cell cycle and muscle cell regulatory factors describes a refined mechanism by which the IGFs control both proliferation and differentiation processes.

In addition to the above-mentioned effects on muscle cell proliferation and differentiation, both IGFs act as survival factors during muscle cell differentiation in culture, by preventing apoptosis (Stewart & Rotwein 1996). In cultured IGF-II antisense C2 cells, the switch to differentiation medium is associated with markers of apoptosis in over 90% of the cells, however administration of either exogenous IGF-I or IGF-II is able to prevent this through interaction with the Type I IGF receptor (Stewart & Rotwein 1996). IGF-II similarly prevents apoptosis *in vivo*, as demonstrated by the reduction in programmed cell death in the muscle of *mdx* mice after crossing with IGF-II overexpressing transgenic mice (Smith *et al.* 2000).

1.7.1.6 IGFs in regenerating skeletal muscle

The levels of IGFs and their receptors are differentially regulated during the regeneration process. In homogenised regenerating muscle of young rats following bupivacaine-induced damage, IGF-I mRNA levels are elevated 6-fold relative to undamaged control muscle on days 5 and 10 of regeneration, then decline to approximately the same level as control muscle by day 15 post-injection (Marsh *et al.*

1997). Results of the same study showed that IGF-II mRNA levels are elevated 60- and approximately 30-fold on days 5 and 10, respectively, and that IGF-II mRNA levels approach control values by day 15 (Marsh *et al.* 1997). In contrast, Type I IGF receptor mRNA levels are elevated approximately 2- and 6-fold on days 5 and 10 of regeneration, respectively, then decline to control muscle levels by day 15 (Marsh *et al.* 1997). The Type II IGF receptor mRNA was not measured in the same study, but in a separate study of regenerating muscle following notexin-injection, Type II IGF receptor mRNA was detected but its levels were not significantly different to that of the contralateral control muscle (Levinovitz *et al.* 1992). Thus, IGF-I and –II, and their cognate receptors, are regulated differentially throughout regeneration, presumably as a reflection of their differing functions within regenerating muscle.

During regeneration, the tissue type specific pattern of IGF-I expression is altered. Undamaged muscle fibres do not show specific localisation of IGF-I protein, however after damage, IGF-I protein is observed in satellite cells, intramuscular nerves and blood vessels (Jennische & Hansson 1987; Jennische *et al.* 1987; Keller *et al.* 1999). Macrophages in damaged heart muscle following myocardial infarct express IGF-I (Matthews *et al.* 1999), however macrophages in regenerating skeletal muscle were not found to contain IGF-I protein (Keller *et al.* 1999).

The pattern of expression by different cell/tissue types during regeneration suggests that IGF-I may be performing a number of different functions within regenerating muscle. IGF-I stimulates the chemotaxis *in vitro* of skeletal myoblasts (Suzuki *et al.* 2000), vascular smooth muscle cells (Bornfeldt *et al.* 1994), and nerve cells

(Puglianello *et al.* 2000). However, studies of murine muscle following denervation and devascularisation indicate that in regenerating muscle, IGF-I has varying effects (Lefaucheur *et al.* 1996). In these studies, immune neutralisation of IGF-I during regeneration resulted in a two-fold increase in the number of capillaries, indicating an enhancement of muscle revascularisation, and a 50% increase in the number of macrophages in the damaged area (Lefaucheur *et al.* 1996). Immune neutralisation of IGF-I during muscle regeneration also resulted in 80% and 30% decreases in regenerating fibre number and diameter, respectively, by day 11 of regeneration (Lefaucheur & Sébille 1995c). Thus, by inference, the studies of Lefaucheur and colleagues show that in regenerating muscle, IGF-I may have a net negative effect on revascularisation and phagocytic processes (Lefaucheur *et al.* 1996), as well as a positive effect at later timepoints (Lefaucheur & Sébille 1995b), likely through effects on proliferation and differentiation. Similarly, IGF-I administration during laceration-induced regeneration resulted in a 3.5-fold increase in the number of regenerated myofibres, increased regenerated myofibre diameter, and increased muscle strength (Menetrey *et al.* 2000). The inferred inhibition of vascularisation reported by Lefaucheur and Sebille (1996) is in contrast to the marked enhancement of smooth muscle cell proliferation and migration following intraarterial injury *in vivo*, in smooth muscle cells that have been engineered to overexpress IGF-I (Zhu *et al.* 2001). The reason for these inconsistencies is unknown but may in part be due to the impact of other regeneration processes on vascularisation, or the fact that anti-IGF-I antibody rather than supplemental IGF-I was used for the studies by Lefaucheur and colleagues.

IGF-II, which is low or absent from rat muscle fibres postnatally (Levinovitz *et al.* 1992), is temporally regulated after damage in regenerating muscle cells (Levinovitz *et al.* 1992), and in areas enriched with neuromuscular juctions (Pu *et al.* 1999b). Vascular tissues contain IGF-II protein during regeneration, but strictly at constitutive levels (Keller *et al.* 1999), suggesting that IGF-II is not specifically regulated by vascular tissues during regeneration. As was the case for IGF-I, macrophages in regenerating skeletal muscle have not been shown to contain IGF-II, although IGF-II is present within macrophages in multiple sclerotic lesions (Gveric *et al.* 1999). Thus it is apparent from the patterns for IGF-I and –II expression that there are both similarities and differences in the expression patterns of the two peptides during skeletal muscle regeneration. To date, there have been no studies to test the effect of administered IGF-II on skeletal muscle regeneration, so it is not known if the effect of altered IGF-II levels during muscle regeneration is similar to that of altered IGF-I.

As mentioned above, both IGF-I and –II are specifically expressed in nervous tissue during muscle regeneration. IGF-II is often referred to a neurotrophic factor for two reasons: a) IGF-II expression is restricted to the brain in normal postnatal rats (Beck *et al.* 1988), and b) IGF-II markedly enhances nerve regeneration (Glazner *et al.* 1993; Near *et al.* 1992). IGF-I has also been shown to promote nerve regeneration (Zhuang *et al.* 1996). A consideration of the roles of IGFs in nerve regeneration is pertinent, in that the innervation status affects the state of the regenerating muscle fibres, in particular after the first 5-7 days of regeneration (Whalen *et al.* 1990).

The IGF axis in connective tissue of regenerating skeletal muscle has not been well characterised to date. IGF-I mRNA is up-regulated in fibroblasts of damaged heart following myocardial infarct (Matthews *et al.* 1999), and in the hypertrophic scar tissue that develops following thermal injury (Ghahary *et al.* 1995). Treatment of dermal fibroblasts with IGF-I results in increased collagen synthesis (Ghahary *et al.* 1995), which suggests that increased IGF-I levels in damaged tissues may be associated with increased connective tissue. Recently, the mechanism by which IGF-I modulates the extracellular matrix has been shown to involve IGF-I induction of latent TGF-β (Ghahary *et al.* 2000). Latent TGF-β is then activated through binding to the Type II IGF receptor, and it is the activated TGF-β that then causes fibroblasts to increase matrix deposition (Ghahary *et al.* 1999b).

1.7.1.7 Regulation of IGFs

1.7.1.7.1 Growth hormone

Growth hormone (GH), a protein produced by the anterior lobe of the pituitary gland, has a dramatic effect on growth. Resistance to GH, or lack of sufficient pituitary GH concentrations, are associated with the dwarf phenotype in rodents and humans (Charlton *et al.* 1988; Hull & Harvey 1999). Administration of exogenous GH promotes also muscle growth in rodents and farm animals (Beach & Kostyo 1968; Beerman *et al.* 1990; Pell & Bates 1987). The activity of GH is greatly influenced by nutritional status, for a low-plane of nutrition is associated with increased circulating levels of GH (Breier *et al.* 1988a), and decreased hepatic binding of GH in steers (Breier *et al.* 1988b).

The pattern of GH secretion is controlled by two hypothalamic peptides, the stimulatory factor, GH-releasing hormone (GHRH), and the inhibitory peptide, somatostatin (SRIF) (Wagner *et al.* 1998). The pattern of GH secretion differs between males and females, with dissimilarities in the nadir value and length, and GH pulse length and frequency (Jansson *et al.* 1985). The masculine pattern of GH secretion in rats is typified by narrow GH pulses at a frequency of one every 3-4 hours, and extended periods with low to undetectable nadir values (Jansson *et al.* 1985). The feminine pattern of GH secretion in rats has prolonged GH pulses, an irregular pattern of pulsatility, and higher nadir values than male rats (Jansson *et al.* 1985). The response to GH is sexually dimorphic, with differences in hepatic gene expression, including the cytochrome p450 (Sundseth *et al.* 1992) and major urinary protein (MUP) families (McIntosh & Bishop 1989) and in somatic growth responses (Jansson *et al.* 1985). These differential growth and gene expression patterns are the result of the different patterns of GH secretion between males and females (Jansson *et al.* 1985), with the length of the nadir being a key determinant of the dimorphic effect (Waxman *et al.* 1991). GH receptor signalling involves the Janus kinase/signal transducer and activation of transcription (JAK-STAT) pathway (Carter-Su *et al.* 1994; Gouilleux *et al.* 1995), and recent studies have shown that the STAT5b protein is a major determinant of the sexually dimorphic response of liver and growth rate to GH pulsatility (Udy *et al.* 1997). The pattern of GH pulsatility is regulated at least in part by testosterone, as exposure to testosterone can alter the pattern of pulsatility from a feminine to a masculine pattern (Painson *et al.* 2000).

The potent effects of GH on anabolic growth and nitrogen retention (Ambler *et al.* 1993) have led to studies regarding its use to enhance wound healing (Belcher & Ellis 1990; Kowalewski & Yong 1968) and muscle regeneration (Ullman *et al.* 1990). The latter aspect, the enhancement of muscle regeneration by GH, will be addressed in CHAPTER 4.

The "somatomedin hypothesis", of 1957 (Salmon & Daughaday), postulated that the effects of growth hormone (GH) were mediated by a factor that was secreted into the circulation, a factor that is now known as IGF-I. Liver is a major contributor to circulating levels of IGF-I in response to GH (Yakar *et al.* 1999), giving rise to approximately 75% of circulating IGF-I levels in mice (Sjogren *et al.* 1999). Until recently, the dogma has been that circulating IGF-I is the principle mediator of GH effects on postnatal growth and development, however recent studies using the Cre/*lox* recombination system to specifically delete the *igf1* gene in liver clearly show that normal postnatal growth occurs even when there is a substantial reduction in circulating levels of IGF-I (Sjogren *et al.* 1999; Yakar *et al.* 1999). Furthermore, normal growth occured despite a reported compensatory increase in circulating GH, due presumably to the lack of negative feedback by circulating levels of IGF-I (Sjogren *et al.* 1999; Yakar *et al.* 1999). These reports thus suggest that autocrine/paracrine production of IGF-I is likely to be critical for normal postnatal growth and development (Sjogren *et al.* 1999; Ueki *et al.* 2000; Yakar *et al.* 1999). IGF-I mRNA is increased in a number of tissues in response to supraphysiological levels of exogenous GH, including skeletal muscle (Isgaard *et al.* 1989), adipose tissue (Vikman *et al.* 1991), and bone growth plate (Isgaard *et al.* 1988). Furthermore,

GH receptor levels in skeletal muscle change coordinately with IGF-I mRNA levels during mouse development, suggesting that GH may interact directly with skeletal muscle to direct autocrine/paracrine synthesis of IGF-I (Shoba *et al.* 1999). In cultured C2C12 muscle cells, direct regulation of IGF-I levels by GH has recently been shown (Sadowski *et al.* 2001), thus providing unequivocal evidence of a direct effect of GH on IGF-I production in non-hepatic cells. Not only does this study provide evidence of GH regulation of IGF-I in muscle, it also suggests an effect of GH on the Type I IGF receptor, via the suppressor of cytokine signalling-2 (SOCS-2) protein (Sadowski *et al.* 2001), which has been shown to interact directly with the Type I IGF receptor both *in vitro* and *in vivo* (Dey *et al.* 1998). It should be noted that although GH has been shown to directly regulate IGF-I in cultured muscle cells (Sadowski *et al.* 2001), GH can also exert a mitogenic effect directly on cultured pro-B Ba/F3 cells independently of IGF-I (Baixeras *et al.* 2001).

In contrast to the effect of GH on hepatic IGF-I expression, GH generally does not induce expression of hepatic IGF-II when administered *in vivo* (Lewis *et al.* 2000; Turner *et al.* 1988). This is consistent with the observation that hepatic IGF-II levels are high in the rat foetus and low postnatally (Norstedt *et al.* 1988; Park & Buetow 1991), while the growth response to GH is absent during foetal development but is present postnatally (reviewed by Bass *et al.* 1992).

The association of GH with stimulation of IGF-II production in skeletal muscle is less clear than the association of GH with muscle IGF-I production. Neonatal pigs administered GH for 7 days do not have increased skeletal muscle IGF-II mRNA

(Lewis *et al.* 2000), however adult rats implanted with GH-secreting, pituitary-derived GH_3 cells for 80 days show a six-fold increase in IGF-II mRNA in muscle (Turner *et al.* 1988). Further evidence that GH may not control skeletal muscle IGF-II production is seen in the greater induction of IGF-II mRNA in the muscles of hypophysectomised (GH-deficient) rats relative to normal (GH-replete) rats in response to work-induced hypertrophy (DeVol *et al.* 1990). Therefore, GH regulates IGF-I in the liver and also to some extent in other tissues, however it does not appear to regulate IGF-II in the same manner.

1.7.1.7.2 IGF receptor downregulation by IGFs

The first suggestions of a link between IGFs and Type I IGF receptor levels were the observations that a) in many tissues the Type I IGF receptor levels decrease during the time that IGF-I levels increase during postnatal development in the rat (Werner *et al.* 1989), and b) IGF-II mRNA levels increase while Type I IGF receptor levels decrease during muscle cell differentiation (Rosenthal *et al.* 1991). It has since been shown that both IGF-I and –II induce down-regulation of the Type I IGF receptor (Rosenthal & Brown 1994), an effect that occurs via interaction with the Type I, but not the Type II, IGF receptor (Rosenthal & Brown 1994; Rosenthal *et al.* 1994). IGF-I and –II down-regulate Type I IGF receptor levels by decreased receptor gene transcription, while IGF-II has also been shown to increase degradation of the Type I IGF receptor protein (Hernandez-Sanchez *et al.* 1997; Rosenthal & Brown 1994).

1.7.2 Myostatin

Myostatin, also known as growth and differentiation factor-8 (GDF-8), is a member of the transforming growth factor-beta (TGF-β) superfamily. The TGF-β family consists broadly of TGF-βs, activins, inhibins, bone morphogenetic proteins (BMPs), and Mullerian-inhibiting substance. TGF-β family members are associated with wide ranging effects on numerous cell types, including the regulation of cell growth, cell differentiation, and matrix deposition. Myostatin, identified in 1997 by McPherron and colleagues (McPherron *et al.* 1997), is a potent negative regulator of skeletal muscle mass. Myostatin has been proposed to act as a "chalone" (Lee & McPherron 1999), a concept that was originally proposed by Bullough (Bullough 1965). Chalones are molecules that are produced by a specific tissue that inhibit its growth, so that local and/or systemic concentrations of the chalone reflect the mass of the tissue where it was produced (Bullough 1965; Lee & McPherron 1999).

1.7.2.1 Peptide synthesis

The myostatin gene is located on chromosome 1 in mice (Szabo *et al.* 1998), and on chromosome 2 in humans and cattle (Gonzalez-Cadavid *et al.* 1998; Kambadur *et al.* 1997; Smith *et al.* 1997), and is composed of 3 exons and 2 introns (Gonzalez-Cadavid *et al.* 1998; Jeanplong *et al.* 1999; McPherron *et al.* 1997). Myostatin nucleotide and amino acid sequence is highly conserved across species, with 93% homology between bovine and murine amino acid sequences (Kambadur *et al.* 1997). Full-length myostatin protein, which in mice consists of 376-amino acid residues,

shares many common structural features with other TGF-β family members, including: a carboxy(C)-terminal region containing a conserved pattern of cysteine residues, a signal sequence for secretion, and a proteolytic processing site (McPherron *et al.* 1997). As suggested by the latter two features, myostatin is further processed before it is capable of exerting biological effects. This begins with the dimerisation of full-length TGF-β polypeptides, via disulfide bonds, soon after translation (Gleizes *et al.* 1997). Precursor TGF-β molecules are then proteolytically cleaved in the Golgi apparatus to yield the N-terminal latency-associated protein (LAP) portion, and the C-terminal mature peptide (Gleizes *et al.* 1997; Okada *et al.* 1989). The LAP and mature TGF-β homodimers associate with each other in a non-covalent manner, and it is this complex that is secreted from cells. A similar situation occurs for myostatin (Thies *et al.* 2001), however in the case of myostatin the processed, mature form of myostatin (ie, not associated with LAP) is also found within the circulation of humans (Gonzalez-Cadavid *et al.* 1998), indicating that in at least some cases myostatin is released from the latent complex prior to secretion. Association of myostatin with the LAP protein renders it inactive (Thies *et al.* 2001), and it is only upon dissociation from the LAP protein that myostatin is rendered active, and able to interact with its receptor (Thies *et al.* 2001). The LAP portion of TGF-β has been shown to be important not only for receptor binding capability, but also to function in the folding and secretion of TGF-β, and perhaps in the trafficking of TGF-β to target cells (Gleizes *et al.* 1997; Gray & Mason 1990; Lee & McPherron 2001; Thies *et al.* 2001). These aspects of the LAP portion of myostatin have not been investigated as yet, however the importance of the LAP for myostatin function has been clearly demonstrated by two studies, even though the results are apparently conflicting. The

first report concerns transgenic mice produced with a myostatin pro domain construct, which had 22-44% greater carcass weight and larger fast fibre diameters, as compared to control mice (Yang *et al.* 2001). This study clearly suggests an inhibition of myostatin action by the LAP protein. In the second study, the *Cmpt* mouse is reported to carry a deletion in the LAP portion of the myostatin gene, and to exhibit a hypermuscularity directly linked to the myostatin gene (Szabo *et al.* 1998). Therefore, if one presumes that the deletion in the LAP portion inhibits LAP function rather than enhances it, this report infers that the LAP portion of the myostatin gene acts to enhance myostatin function. The apparently conflicting findings of these two studies may reflect different functions of the LAP protein, whereby the mutation in the *Cmpt* mouse affects a different function of the LAP protein, such as myostatin protein folding, to the function that is affected by LAP overexpression. Clearly further studies are required in this area, particularly in identifying the specific effect of the deletion in the *Cmpt* mouse, in order to resolve this discrepancy.

1.7.2.2 Receptors

TGF-β family members bind to receptors containing a cysteine-rich extracellular region, and an intracellular kinase domain with predicted serine/threonine specificity (Heldin *et al.* 1997). This group of receptors consists of Type I and Type II receptors, both of which contain kinase activity (Heldin *et al.* 1997). TGF-β family members bind to characteristic combinations of TGF-β, activin, and BMP Type I and II receptors, and binding to both Type I and II receptors is prerequisite for signal generation (Heldin *et al.* 1997). In the case of myostatin, substantial biological effects

are exerted both *in vivo* and *in vitro* through binding to the activin Type II receptor B (Act RIIB) (Lee & McPherron 2001). Activin Type II receptor A (Act RIIA) similarly binds dimerised mature myostatin, but at a much lower level than Act RIIB (Lee & McPherron 2001). The role of Act RIIB as the primary receptor regulating myostatin activity is indicated by the presence of increased muscle mass similar to that of the myostatin knockout mice, in transgenic mice expressing a dominant-negative form of Act RIIB (Lee & McPherron 2001).

The signalling mechanisms that take place downstream of receptor activation have not been elucidated for myostatin, however a family of proteins called Smads are known to function in the intracellular signalling pathways for other TGF-β family members. It is believed that Smads undergo conformational changes after activation by receptors, form complexes with other Smads, then translocate to the nucleus to modulate the transcription of target genes (Heldin *et al.* 1997).

1.7.2.3 Myostatin in muscle

1.7.2.3.1 Myostatin expression

Myostatin expression occurs early in development, and this time of onset appears to be well-conserved across diverse species. In mice, the first observation of myostatin mRNA in the myotome compartment of developing somites is on day 9.5 post-coitum (p.c.), with widespread expression in nearly all somites by day 10.5 p.c. (McPherron *et al.* 1997). Similarly, bovine and porcine embryos begin to express myostatin mRNA on days 29 and 21, respectively (Ji *et al.* 1998; Kambadur *et al.* 1997). Piscine species

first express myostatin in post-hatch larvae, at the time of early myogenesis (Rodgers *et al.* 2001). Myostatin expression continues to be associated with the development of skeletal muscle at later gestational stages (Ji *et al.* 1998; McPherron *et al.* 1997; Oldham *et al.* 2001), with the expression of myostatin mRNA in bovine hindlimb muscle increasing during early embryonic development to a peak on day 90 (approximately 6-fold birth levels), followed by a sharp decrease and steady low levels thereafter through the remainder of gestation (Oldham *et al.* 2001). Myostatin levels appear to be important for prenatal development, for a positive correlation between low birth weight and elevated myostatin expression has been shown for pigs (Ji *et al.* 1998).

Myostatin continues to be expressed postnatally, although at lower levels than prenatally (Ji *et al.* 1998), and with considerable variation between different muscles (McPherron *et al.* 1997). Much of the variation in myostatin expression between muscles has been attributed to variations in fibre type (Sakuma *et al.* 2000), however this association is not clearcut. Murine M. *soleus*, which is composed primarily of Type I fibres, does not contain myostatin mRNA; rather, Type IIb MHC isoform was found to correlate positively with myostatin mRNA (Carlson *et al.* 1999). In a study of the association of myostatin mRNA with fibre type in different fish species, myostatin was found to be associated with either fast fibres only, slow fibres only, or with both fast and slow fibres, depending on the species (Roberts & Goetz 2001). These variations may have been attributable to differential locomotion between the fish species studied (Roberts & Goetz 2001). It is apparent that this is an area of

research worthy of more attention in order to determine the exact nature of the relationship between myostatin and muscle fibre type.

In contrast to the situation observed during embryonic development in which myostatin expression is strictly limited to muscle and progenitor muscle cells, postnatally myostatin is expressed in a much wider range of tissues. Myostatin has been identified in the Purkinje fibres and cardiomyocytes of adult heart (Sharma *et al.* 1999), in adipose tissue (McPherron *et al.* 1997), and the mammary gland (Ji *et al.* 1998) of mammals. In brook trout, which express 2 isoforms of myostatin, one isoform is found in muscle, and in brain tissues including optic lobes, the hindbrain, and hypothalamus (Roberts & Goetz 2001). The second isoform is localised within the ovaries, and is expressed during ovulation, suggesting some sort of a reproductive role for myostatin in fish (Roberts & Goetz 2001).

1.7.2.3.2 Myostatin action

As stated above, myostatin is a key negative controller of skeletal muscle mass, as is clearly shown by the enhancement of muscle size in Piedmontese and Belgian Blue double-muscled cattle, animals which lack a functional myostatin protein (Grobet *et al.* 1997; Kambadur *et al.* 1997; McPherron & Lee 1997). In Piedmontese cattle the non-functional myostatin protein is due to a missense mutation causing a cysteine to tyrosine substitution in the coding region (Kambadur *et al.* 1997; McPherron & Lee 1997), while in Belgian Blue cattle the non-functional myostatin is due to an 11-bp deletion in the coding sequence, resulting in a premature stop codon (Grobet *et al.* 1997; Kambadur *et al.* 1997; McPherron & Lee 1997). The disruption of the

myostatin gene in mice yields muscles with 2-3 times greater mass due to both hypertrophy and hyperplasia (McPherron *et al.* 1997). The hypermuscular compact (*Cmpt*) mouse, which is the result of a selection programme for high carcass protein content (Varga *et al.* 1997), has a deletion in the myostatin gene (Szabo *et al.* 1998). These studies thus support a strong inhibitory role for myostatin in controlling muscle growth.

Cell culture studies have provided valuable insight into the mechanism by which myostatin inhibits muscle growth. Studies in our laboratory showed that the addition of myostatin protein to cultured C2C12 myoblasts resulted in blocks at two points in the cell cycle: the G_1 to S, and G_2 to M, transitions (Thomas *et al.* 2000). These findings suggest that the enhancement of muscle mass observed in double-muscled cattle and myostatin knockout mice is due, at least in part, to de-regulation of the myoblast cell cycle in the absence of functional myostatin (Thomas *et al.* 2000). Myostatin inhibits protein synthesis which, combined with the inhibition of myoblast proliferation, could account for the presence of both hyperplasia and hypertrophy in myostatin knockout mice (McPherron *et al.* 1997; Taylor *et al.* 2001). No effect of myostatin on either protein degradation or apoptosis was found in the same study (Taylor *et al.* 2001).

Considerable attention has been paid to determining the role of myostatin in muscle atrophy and sarcopenia, with the expectation that because myostatin is a negative regulator of muscle growth, that the levels of myostatin would be increased in cases of muscle loss. Indeed, this inverse relationship of myostatin to muscle loss has been

observed for chronic disuse atrophy (Reardon *et al.* 2001), muscle loss due to space flight (Lalani *et al.* 2000), and weight loss associated with AIDS (Gonzalez-Cadavid *et al.* 1998). Elevated myostatin alone, however, is not sufficient for loss of muscle mass as surmised by the observation that rats undergoing atrophy due to hindlimb unloading continued to express high levels of myostatin even following periodic bouts of weight bearing sufficient to prevent muscle loss (Wehling *et al.* 2000). Further studies will undoubtedly be aimed at determining the factors and/or conditions that modulate the levels of myostatin associated with atrophy and muscle wasting.

1.7.2.4 Myostatin in regenerating skeletal muscle

The potent regulation of muscle growth observed in response to myostatin meant that keen interest quickly developed in determining the role of myostatin in skeletal muscle regeneration. Following is a summarisation of the key findings regarding the localisation and pattern of expression of myostatin mRNA during muscle regeneration. In rodents, myostatin protein is temporally-regulated during muscle regeneration (Mendler *et al.* 2000; Sakuma *et al.* 2000), with predominantly lower levels of myostatin protein beyond the necrotic stage (Mendler *et al.* 2000; Sakuma *et al.* 2000). The levels of myostatin mRNA and protein are strikingly different during the early stages of muscle regeneration, suggesting that perhaps circulating levels of myostatin alter the level of myostatin protein detected in muscle (Mendler *et al.* 2000). An intriguing study by Yamanouchi et al (2000) reported the localisation of myostatin mRNA in fibroblasts within regenerating skeletal muscle, then showed that skeletal muscle-derived fibroblasts expressed myostatin mRNA *in vitro* in response to

the addition of crushed muscle extract. Crushed muscle extract (Bischoff 1986b) is known to contain growth factor activity, including HGF (Tatsumi *et al.* 1998). HGF is capable of activating quiescent MPC (Allen *et al.* 1995), but whether it is this growth factor/cytokine or another that is responsible for invoking transcription of the myostatin gene in fibroblasts during regeneration, is unknown. This observation paves the way for interesting research into the interactions of myostatin with other growth factors, an area that is especially important in terms of muscle regeneration given the intricacies of growth factor expression at this time.

In muscular dystrophy, where continual rounds of muscle fibre degeneration and regeneration occur, myostatin may play a role. This has recently been investigated in two models of murine muscular dystrophy: a) in *mdx* mice, which lack dystrophin and bear resemblance to Duchenne's muscular dystrophy (Cavanna *et al.* 1988; Hoffman *et al.* 1987), but do not lose muscle strength as occurs in humans with Duchenne's muscular dystrophy (Tanabe *et al.* 1986); and b) in *gsg* -/- mice, which lack γ-sarcoglycan and bear likeness to limb girdle muscular dystrophy (Zhu *et al.* 2000). Analysis of three hindlimb muscles showed that myostatin mRNA is markedly reduced in these dystrophic strains relative to normal (wild type) mice (Zhu *et al.* 2000), suggesting an association of myostatin with muscular dystrophy.

The association of myostatin with innervation status is pertinent to a discussion of muscle regeneration. In rats, denervation of a fast-type muscle resulted in a slight increase in myostatin protein, while denervation of a slow-type muscle resulted in a small decrease in myostatin protein (Sakuma *et al.* 2000). Myostatin expression thus

changes slightly with denervation, and these changes are altered, or determined, by the fibre-type composition of muscle. The effects of myostatin on the reinnervation of muscle have not been explored, however based on reports of improved nerve regeneration with neutralisation of TGF-β1 (Davison *et al.* 1999) this may be an area worthy of future attention.

1.7.2.5 Regulation of myostatin

1.7.2.5.1 Follistatin

Follistatin was originally isolated from ovarian follicular fluid, and as its name would suggest, suppresses follicle stimulating hormone (FSH) production by cultured pituitary cells (Robertson *et al.* 1987). Follistatin binds to activin (Phillips 2000), another TGF-β family member that binds to the Act RIIB receptor (Gray *et al.* 2000), and so inhibits activin function (deWinter *et al.* 1996). Similarly, follistatin has been shown to also bind to dimerised mature myostatin, and so inhibit the binding of myostatin to the Act RIIB receptor (Lee & McPherron 2001). Follistatin over-expressing transgenic mice have extreme muscling, muscling that is significantly greater than that seen in myostatin knockout mice (Lee & McPherron 2001). This supports a role for follistatin as a potent inhibitor of myostatin function, and suggests that it may also inhibit another ligand in addition to myostatin (Lee & McPherron 2001).

1.7.2.5.2 MRFs

Analysis of the promoter region located upstream of the human myostatin gene has identified the presence of a binding site (E-box) for the MRF family of muscle-specific transcription factors (reviewed in Section 1.6.1) (Ferrell *et al.* 1999). Because some MRFs, ie myf-5 and MyoD, are present during early embryogenesis, as is myostatin, it is conceivable that the MRFs enhance the expression of myostatin at these early timepoints. Such a hypothesis is supported by the observation of coincidental increases in MyoD and myostatin mRNAs in the hindlimb muscles of foetal cattle during the stages of primary and secondary fibre formation (Oldham *et al.* 2001). Certainly, further studies to elucidate the relationship between MRFs and regulation of myostatin will be vital to understanding the mechanism of myostatin expression in normal and damaged muscle.

1.7.2.5.3 Myostatin

Double-muscled Belgian Blue cattle lack a functional myostatin gene product due to a deletion that results in a premature stop codon (Kambadur *et al.* 1997). When the expression of myostatin mRNA from double-muscled Belgian Blue cattle is compared to that of normal-muscled cattle, significantly greater levels are found in double-muscled cattle (Oldham *et al.* 2001). This suggests that a negative feedback mechanism by myostatin that is normally present, is non-operational in the double-muscled Belgian Blue cattle, thus giving rise to the higher levels of myostatin expression (Oldham *et al.* 2001). Further studies to corroborate this have not, as yet, been carried out.

1.8 Summary

The role of the insulin-like growth factors in skeletal muscle regeneration has not been fully explored. In particular, there is a noticeable lack of information on changes in the Type II IGF receptor population alongside changes in the other components of the IGF axis during regeneration. Most investigations up to now have involved a non-histological approach, where the inputs of individual tissues to overall changes in IGF and receptor levels are indistinguishable. Such an approach ignores the facts that a) growth factor responses may vary depending on distance from the injury site, and b) important changes in the IGF axis may occur in different compartments. For this reason, the work contained in this thesis utilises a histological approach, so that the growth factor activities of different tissues within damaged muscle can be analysed.

Growth hormone is a potent stimulator of muscle growth, suggesting that it could enhance muscle regeneration, however the majority of studies thus far have concentrated solely on its regulation of IGF-I, rather than on all components of the IGF axis. Furthermore, GH effects may vary depending on tissue type within the damaged muscle, an aspect that has been overlooked to date. In the following studies, GH effects on all components of the IGF axis, within the different tissues that comprise damaged muscle, will be examined.

Myostatin presented itself as a newly identified protein with a potent negative effect on muscle growth, however nothing was known of its role or expression during skeletal muscle regeneration. Thus, the localisation of myostatin in damaged and

regenerating muscle tissues will be examined herein, and a determination made of whether myostatin levels are affected in a situation of enhanced growth during muscle regeneration, such as that due to GH administration.

Lastly, IGF-I is the better studied of the two IGFs, and its effect on regeneration has been examined by various means. The same, however, has not been done for IGF-II, which shares many of the same actions, but is not identical to, IGF-I.

1.9 The Aim of this Thesis

The aim of this thesis is to characterise components of the IGF axis, and myostatin, during skeletal muscle regeneration, and to determine whether the level of these factors is altered by the administration of GH. Finally, the effect of IGF-II administration on the rate of skeletal muscle regeneration will be determined.

1.9.1 Achieving the Aim of the Thesis

The aim of this thesis will be achieved by testing the following individual hypotheses:

1.9.1.1 Hypothesis 1 (Chapter 4)

The IGFs and their receptors are regulated during muscle regeneration, and the level of IGF expression and binding in regenerating muscle is regulated by GH. This will be tested by comparing regeneration and the IGF-axis components in GH- versus saline-treated GH-deficient *dw/dw* rats.

1.9.1.2 Hypothesis 2 (Chapter 5)

A negative regulator of growth, myostatin, is temporally regulated during muscle regeneration, and its levels are decreased in muscles undergoing enhanced growth due to the administration of GH. The temporal regulation of myostatin protein during muscle regeneration will be examined in Sprague-Dawley rats, and in the GH-deficient *dw/dw* rat. The effect of GH on myostatin protein levels will be tested by comparing saline- versus GH-treated *dw/dw* rats.

1.9.1.3 Hypothesis 3 (Chapter 6)

Administration of IGF-II during skeletal muscle regeneration enhances the rate of skeletal muscle regeneration. This will be tested by comparing regeneration in Sprague-Dawley rats receiving a continuous infusion of IGF-II over the site of muscle damage, to Sprague-Dawley rats receiving a continous infusion of vehicle only.

CHAPTER 2

GENERAL MATERIALS AND METHODS

This chapter describes the main methodologies employed for the studies contained in Chapters 4, 5, and 6, and lists the source of materials utilised.

2.1 Materials

2.2 Antibodies and Detection System Reagents

2.2.1.1 Primary antibodies

MyoD antisera (mouse anti-recombinant mouse MyoD; PharMingen Clone MoAb 5.8A) was purchased from BD Biosciences (Franklin Lakes, NJ, USA).

Myogenin antisera (rabbit anti-rat, catalog number sc-576) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Developmental myosin heavy chain (dMHC) antisera (mouse anti-rat; NCL-MHCd) was purchased from Novocastra Laboratories Ltd. (Newcastle upon Tyne, UK).

Slow myosin heavy chain (slow MHC) antisera (mouse anti-human; clone NOQ7.1.1A) was purchased from Chemicon International (Temecula, CA, USA).

Fast myosin heavy chain (fast MHC) antisera (mouse anti-rabbit; clone MY-32) was purchased from Sigma Chemical Company (St. Louis, MO, USA).

Myostatin antibody (rabbit anti-bovine) was produced in-house and has been fully characterised (Sharma *et al.* 1999).

2.2.1.2 Negative controls

The following antisera/sera were purchased from DAKO Corporation (Carpinteria, CA, USA) for use as negative controls:

Normal rabbit immunoglobulin fraction (product x0903)

Normal rabbit serum, whole (x0902)

Normal mouse immunoglobulin fraction (product x0931)

Alpha-lactalbumin antisera (rabbit anti-human; A0579)

2.2.1.3 Detection system reagents

The following immunohistochemistry detection system reagents were purchased from Amersham Pharmacia Biotech (Auckland, New Zealand):

Sheep anti-mouse whole antibody, biotinylated (SAM-B; RPN1001)

Donkey anti-rabbit whole antibody, biotinylated (DAR-B; RPN1004)

Streptavidin-biotin-horseradish peroxidase (SA-B-HRP; RPN 1051)

2.3 Radioisotopes

(α-^{35}S)-labelled uridine-5'-triphosphate was purchased from Amersham International (Amersham, UK).

Na^{125}Iodide was purchased from New England Nuclear (Boston, MA, USA).

2.4 Peptides and hormones

Purified ovine (o) IGF-II was donated by Dr. Lloyd Moore (AgResearch, Wallaceville, NZ).

Recombinant human (rh) IGF-II was donated by Eli Lilly and Company (Indianapolis, IN, USA)

Recombinant human (rh) IGF-I was donated by Ciba-Geigy Corporation (Basel, Switzerland).

Recombinant human amino terminal methionyl IGF-I ((rh)N-Met IGF-I) was donated by Dr. BD Burleigh (IMC/Pitman-Moore, Terre Haute, IN, USA).

Desamino-(1-3) IGF-I (des(1-3)IGF-I) and desamino-(1-6) IGF-II (des(1-6)IGF-II) were purchased from Gro-Pep Pty (Adelaide, Australia).

Bovine insulin was purchased from Sigma Chemical Company (St. Louis, MO, USA).

2.5 Chemicals and other supplies

BDH (Poole, Dorset, UK)

Iso-pentane (Analar grade); eosin Y; acetic anhydride, acetic acid; aluminium sulphate; calcium chloride; chromium potassium sulphate; formaldehyde; Paramat

paraffin wax; sodium hydroxide; sodium citrate; sodium chloride; hydrogen peroxide; Tween-20; aluminium sulphate; ethylene glycol; glacial acetic acid; magnesium sulphate; magnesium chloride; sodium di-hydrogen orthophosphate; disodium hydrogen orthophosphate; paraformaldehyde; D.P.X. mountant; sodium iodate; sodium bicarbonate; sodium thiosulphate; ethanol; ammonium acetate; triethanolamine

Sigma (St. Louis, MO)

Toluidine Blue O, C.I. 52040; diethyl pyrocarbonate (DEPC); Trizma® hydrochloride; Trizma® base; bovine serum albumin (BSA); 3,3'-diaminobenzidine (DAB) tablets; phenylmethylsulfonyl fluoride; Triton X-100; citric acid; Type II-S trypsin from porcine pancreas; aluminium potassium sulphate; thymol; aminopropyltriethoxysilane; proteinase K; dextran sulphate; beta-mercaptoethanol (BME)

Promega Corporation (Madison, WI, USA)

Promega transcription kit, RNase-free Dnase

Oxoid Ltd. (Basingstoke, Hampshire, UK)

Phosphate buffered saline (PBS) tablets

Biolab Scientific (Auckland, NZ)

Esco Polysine™ slides; Esco coverslips

Life Technologies (Gaithersberg, MD, USA)

Formamide

International Merchants (Wellington, New Zealand)

Home Pro 4.8 mm cork tiles

Andrew Industrial Ltd. (Auckland, NZ)

Xylene

Ajax Chemicals Ltd. (Sydney, Aust.)

Haematoxylin (C.I. 75290)

George T. Gurr, Ltd. (London, UK)

Nuclear fast red (CI. 60760)

DAKO Corporation (Carpinteria, CA, USA)

PAP pen

Eastman Kodak (Rochester, NY, USA)

X-OMAT-AR5 (XAR) film

Amersham Pharmacia Biotech (Auckland, New Zealand)

Dipping chamber for photographic emulsion; LM-1

Miles Inc. (Elkhart, IN, USA)

Tissue-Tek® O.C.T. Compound

Bayer Diagnostics (Mulgrave, Victoria, AUST)

Tissue-Tek Unicassettes

Roche Molecular Biochemicals (Switzerland)

Yeast tRNA; degraded herring sperm DNA

Ethicon Inc. (Somerville, NJ, USA)

3-0 silk suture thread, 7-0 braided silk suture

National Veterinary Supplies, Ltd. (Christchurch, NZ)

Rompun®(xylazine hydrochloride) 2%; ketamine hydrochloride (100 mg/ml)

Ilford (Aust.) Pty. Ltd. (Mt. Waverley, Victoria)

Phenisol X-ray developer

2.6 Animals

The rats used for the ensuing experiments were housed in the Ruakura Small Animal Colony, where they were kept at constant temperature with a natural day/night cycle, and provided *ad libitum* food and water both before and during the trials. Rats were normally housed in groups of 3 or more within plastic cages, but were housed singly during the duration of the trial work.

Following all surgical manipulations, rats were kept in a warm (28-29°C) room until normal movement and activity was observed. Rats were then returned to a 20-22°C room where they were then monitored regularly throughout the duration of the trial.

All trial work described in this thesis was conducted in accordance with the AgResearch animal ethics policy. Each trial was approved by an institutional animal ethics committee that has representatives from the New Zealand Veterinary Association, the SPCA, MAF, and local authorities as mandated by New Zealand animal welfare legislation: the Animal Welfare Act 1999 and the Animals Protection Act 1960.

2.7 Tissue handling

2.7.1 Sacrifice and dissection

Rats were sacrificed with CO_2 gas followed by cervical dislocation, then the right and left *biceps femoris* muscles dissected out, weighed, and muscle samples taken for histological purposes.

2.7.2 Tissue processing

Cross-sectional and longitudinal samples were obtained from the most heavily damaged area, to be both frozen and formalin-fixed. Considerable attention was given to developing methods that would allow tissues to lie flat while being either fixed or frozen, so as to optimise for cross-sectional representations of the entire muscle.

2.7.2.1 Frozen samples

Frozen samples were taken for receptor autoradiography and immunohistochemistry purposes. For frozen samples, the muscle pieces were adhered to small rectangles of cork tile by placing a few drops of Tissue-Tek® O.C.T. Compound on the cork prior to laying the muscles samples upon it. The piece of cork was then stabbed with a scalpel, allowing the cork to be held firmly in a horizontal position during the freezing stage, and immersed in a container of melting iso-pentane. Melting iso-pentane was obtained by sitting a container of iso-pentane within a larger container of liquid nitrogen, freezing the iso-pentane until solid, then removing the iso-pentane from the liquid nitrogen until a sufficient volume had melted to cover the piece of muscle and cork. The muscle sample was held under the melting iso-pentane until the overt bubbling had subsided, and the tissue had taken on a whitish appearance. The muscle and attached cork were then rapidly wrapped in foil, and placed on dry ice until transfer into a –80°C freezer for storage.

2.7.2.2 Formalin-fixed samples

2.7.2.2.1 Solutions

Formalin fixative	37-40% formaldehyde solution	10 mls
	Phosphate buffered saline tablets	1 tablet
	Distilled water	90 mls

2.7.2.2.2 Procedure

Samples for formalin-fixation were cut as for frozen sections, then placed on pieces of stiff cardboard to keep them flat during fixation. The samples were submerged for 18 hours in formalin fixative. Tissues were removed and rinsed in 0.02% diethyl pyrocarbonate (DEPC)-treated water that had been autoclaved, then placed in marked histology cassettes (Tissue-Tek Unicassettes). The fixed tissues were processed overnight on the "routine overnight" programme in a Jung TP 1050 fully enclosed vacuum tissue processor (Leica, Cambridge Instruments GmbH, Heidelberg, West Germany). At the finish of the cycle, tissues were immediately transferred into a container of melted paraffin wax (Paramat). The tissues were then embedded in blocks of paraffin wax, using a Thermolyne Histo-Centre II-N embedding machine (Barnstead Thermolyne, Doubuque, Iowa).

2.7.3 Sectioning

2.7.3.1 Frozen tissues

Frozen tissue blocks were sectioned on a Cryocut 1800 (Reichert-Jung, Cambridge Instruments GmbH, Heidelberg, West Germany), at a thickness of 8 or 10 microns, for

receptor autoradiography or immunohistochemistry, respectively. Orientation and histology were examined by staining with a 1% aqueous stock of toluidine blue for 10 seconds, dabbing off the excess, then viewing under an Olympus BH-2 microscope at low magnification. Experimental sections were placed on Esco Polysine™ slides, and left to dry at air temperature. Sections were either stored at –20°C (if they were to be used within a week) or –80°C (if they were to be stored longer than one week).

2.7.3.2 Formalin-fixed, paraffin-embedded tissues

Formalin-fixed, paraffin-embedded tissue blocks were sectioned at 8 microns on a Leitz Type 1212 microtome (Ernst Leitz, GmbH, Wetzlar, Germany). Ribbons of tissue were floated out on a 42°C DEPC-water bath, and then the sections collected on Polysine™ slides. Sections were smoothed onto slides by resting slides on the side of the water bath (less than 5 minutes), then dried by placing slides on 60°C heating plates fitted with bars to provide an approximate 8 mm gap between slide and plate. Cut slides were stored in boxes fitted with spacers, unless they were to be used immediately.

2.8 Histological staining of tissue sections

Histological staining using haematoxylin and eosin was carried out in order to distinguish nuclei and cytoplasm, respectively, in muscle sections.

2.8.1 Solutions

The composition of the following solutions was sourced from a standard histology text (such as Lillie 1965).

Gill's haematoxylin*	Haematoxylin (C.I. 75290)	4.0 g
	Sodium iodate	0.4 g
	Aluminium sulphate	35.2 g
	Distilled water	710 ml
	Ethylene glycol	250 ml
	Glacial acetic acid	40 ml
Mayer's haematoxylin	Haematoxylin (C.I. 75290)	1 g
	Aluminium potassium sulphate	50 g
	Distilled water	1000 ml
	Sodium iodate	0.2 g
Nuclear fast red#	Aluminium sulphate, hydrated	5 g
	Distilled water	100 ml
	Nuclear fast red (C.I. 60760)	0.1 g
	Crystal of thymol	
Scott's tap water	Sodium bicarbonate ($NaHCO_3$)	2.0 g
	Magnesium sulphate ($MgSO_4$)	20.0 g
	Distilled water	1000 ml
	Crystal of thymol	
Eosin (1% solution)	Eosin Y (C.I. 45380)	10 g
	Distilled water	1000 ml
	Acetic acid, 5% aqueous	2.0 ml
	Crystal of thymol	

*Dissolve the aluminium sulphate in water with heat, then add the haematoxylin. Leave overnight, then add the ethylene glycol and acetic acid.

#Dissolve the aluminium sulphate in hot water, then sprinkle in the stain. Add thymol.

2.8.2 Procedure

Formalin-fixed, paraffin-embedded sections were prepared as described above, then stained as follows:

Procedure	Time
1. 60°C oven	10 minutes
2. deparaffinise in xylene	2 x 10 minutes
3. 100% ethanol	5 minutes
3. 95% ethanol	2 minutes
5. 70% ethanol	2 minutes
6. rinse in distilled water	5 minutes
7. Gill's haematoxylin (1:1 in distilled water)	90 seconds
8. tap water	until clear
9. Scott's tap water	2 minutes
10. tap water	2 minutes
11. 0.5% eosin Y (diluted in distilled water)	1 minute
12. tap water	Until clear, plus 2 minutes
13. 50% ethanol	3 dips
14. 70% ethanol	3 dips
15. 95% ethanol	30 seconds
16. 100% ethanol	2 minutes
17. 100% ethanol	5 minutes
18. xylene	5 minutes
19. xylene	3 minutes
20. mount coverslips with DPX mountant	

2.9 Immunohistochemistry

Immunohistochemistry is a technique that utilises the specificity of an antibody for its antigen in order to detect proteins at the cellular level. Immunohistochemistry involves use of a "primary" antibody, directed against the protein of interest, followed by a detection method that amplifies the signal so that the protein can be visualised at

the light microscope level. The detection method used in these studies is based on a labelled avidin-biotin (LAB) methodology (Boenisch 1989; Giorno 1984), which uses the high affinity of (strept)avidin for biotin to amplify the signal (Hsu *et al.* 1981). Thus, the "secondary" antibody is conjugated to biotin that the "tertiary" complex binds to because it contains avidin. The tertiary complex contains the enzyme horseradish peroxidase, which is then reacted with a substrate, 3,3'- diaminobenzidine (DAB), to yield a brown end-product. Immunostained sections are then counterstained with an appropriate histological stain for visualisation and/or quantitation purposes.

2.9.1 Solutions

1% paraformaldehyde*	PBS tablet	1 tablet
	Paraformaldehyde	1 g
	NaOH	to pH
	Distilled water	to 100 ml
10 mM Citrate buffer, pH 6.0	10 mM sodium citrate	428 ml
	10 mM citric acid	72 ml
0.1% Trypsin	Trypsin	0.05 g
	1% $CaCl_2$ solution	6 ml
	distilled water	44 ml
5 M sodium chloride	Sodium chloride	58.44 g
	Distilled water	200 ml
1 M Tris, pH 7.4	Trizma hydrochloride	132.2 g
	Trizma base	19.4 g
	Distilled water	1 litre
Tris-buffered saline (TBS) (1 litre)	1 M Tris, pH 7.4	50 ml
	5 M sodium chloride	20 ml
	distilled water	930 ml

TBS + Tween (TBST) (1 litre)	TBS Tween-20	999 ml 1 ml
Quench (10 mls)	30% hydrogen peroxide TBST	1 ml 9 ml
1.3% Formalin	40% formaldehyde distilled water PBS tablet	10 ml 90 ml 1
TBST + BSA (TBSTB)	TBST Bovine serum albumin	10 ml 20 mg
(NDS/NSS) Blocking solution	Serum (NDS or NSS) TBSTB	1 ml 9 ml
3,3'-diaminobenizidine (DAB)	Sigma DAB/buffer tablet set to make 5 mls Distilled water	1 tablet set 5 mls
TBSTB + 1.5% serum	TBSTB Serum (NDS or NSS)	985 ul 15 ul

*Note on paraformaldehyde fixative preparation: Add paraformaldehyde to 70 mls water, then heat until dissolved, but do not let the temperature of the solution exceed 70°C. Add a few drops of 1 M NaOH as necessary to dissolve the paraformaldehyde. Chill on ice immediately after the paraformaldehyde has gone into solution, then add NaOH to take to the pH to 7.4, and add water to 100 mls.

2.9.2 Procedure

The basic procedure followed is that of Hsu *et al.* (1981), with modifications that were unique to the antibody employed, thus the individual procedures are given below for each. In brief, paraffin-embedded tissues on Polysine™ microscope slides were briefly heated to soften the paraffin, then deparaffinised and rehydrated. Frozen tissues were fixed in paraformaldehyde, acetone or formalin to preserve histology and, in some cases, antigenicity. A hydrophobic wax pen (PAP pen) was used to contain solution during immunohistochemistry by drawing a ring around each tissue section. A quenching step with a hydrogen peroxide containing solution was performed to

eliminate endogenous peroxidase activity in tissue sections, followed by washes and a blocking step to prevent non-specific binding of antibody to tissue components. Tissue sections were incubated with primary antibody, then following washing to remove excess antibody, incubated with secondary antibody, which was raised against the species that the primary antibody was produced in, then biotinylated. After further washing, tissue sections were incubated with a tertiary complex consisting of streptavidin-biotin-horseradish peroxidase (SA-B-HRP). Following washing, the horseradish peroxidase was incubated with its substrate, DAB, to form a brown end-product, then the sections counterstained in either nuclear fast red or haematoxylin. Sections were then dehydrated and cleared, and coverslips placed over the tissue sections using D.P.X. mountant. Sections were visualised on a brightfield microscope to determine the cellular localisation of the protein of interest.

2.9.2.1 Developmental Myosin Heavy Chain (dMHC)

For frozen tissue sections. A formalin fixation step following the primary antibody step was added to this procedure in order to improve the histology, which is suboptimal when acetone is used as the fixative.

Bring frozen sections to room temperature in a wrapped, closed box	
Cold acetone	2 minutes
Air dry	
Encircle sections with PAP pen	
TBS	5 minutes
Quench	5 minutes
TBS	5 minutes
NSS blocking	20 minutes
dMHC Ab diluted 1:60 in blocking solution	1 hour
TBST	2 x 5 minutes
Post-fix in 1.3% formalin at room temperature	10 minutes
TBST	2 x 5 minutes
SAM-B 1:200 in TBSTB with 1.5% NSS	30 minutes
TBST	2 x 5 minutes
SA-B-HRP 1:200 in TBSTB	30 minutes
TBS	2 x 5 minutes
DAB	6 minutes
Distilled water	2 minutes
Mayer's haematoxylin	1 minute 20 seconds
Tap water	Until clear, plus 2 minutes
Dehydrate, clear in xylene, and mount coverslips with D.P.X. mountant.	

2.9.2.2 MyoD

As indicated below, it is necessary to use freshly cut frozen sections for anti-MyoD immunohistochemistry. The blocking step, and the addition of BSA and serum to diluents, was found to be detrimental to MyoD immunohistochemistry by reducing signal intensity and were therefore eliminated from the procedure for MyoD. Removal of these components results in slightly elevated, but acceptable, background (non-specific) signal that did not interfere with the detection of MyoD immunostaining in nuclei (as discussed in Section 6.3.3).

Frozen tissue sections must be freshly cut, ie within 4 hours (and NOT re-frozen after placing on slides)	
Encircle sections with PAP pen	
1% paraformaldehyde	15 minutes
TBS	5 minutes
Quench	5 minutes
TBS	5 minutes
MyoD diluted 1:80 in TBST	90 minutes
TBST	2 x 5 minutes
SAM-B 1:100 in TBST	30 minutes
TBST	2 x 5 minutes
SA-B-HRP 1:200 in TBSTB	30 minutes
TBS	2 x 5 minutes
DAB	6 minutes
Distilled water	2 minutes
Mayer's haematoxylin	1 minute 20 seconds
Tap water	Until clear, plus 2 minutes
Dehydrate, clear in xylene, and mount coverslips with D.P.X. mountant.	

2.9.2.3 Myogenin

For frozen tissues.

Bring frozen tissue sections to room temperature in a wrapped, closed box	
Encircle sections with PAP pen	
1.3% formalin	10 minutes
TBS	5 minutes
Quench	5 minutes
TBS	5 minutes
NDS blocking	20 minutes
Myogenin antibody diluted 1:200 in blocking solution	1 hour
TBST	2 x 5 minutes
DAR-B 1:200 in TBSTB with 1.5% NDS	30 minutes
TBST	2 x 5 minutes
SA-B-HRP 1:200 in TBSTB	30 minutes
TBS	2 x 5 minutes
DAB	6 minutes
Distilled water	2 minutes
Mayer's haematoxylin	1 minute 20 seconds
Tap water	Until clear, plus 2 minutes
Dehydrate, clear in xylene, and mount coverslips with D.P.X. mountant.	

2.9.2.4 Myostatin

For formalin-fixed, paraffin-embedded sections.

60°C oven	10 minutes
Deparaffinise and rehydrate tissue sections	As per Section 2.8.2, steps 2-6
TBST	5 minutes
Quench	10 minutes
TBST	5 minutes
NDS blocking	30 minutes
Myostatin antibody diluted 1:100 in blocking solution	30 minutes
TBST	2 x 5 minutes
DAR-B 1:200 in TBSTB with 1.5% NDS	30 minutes
TBST	2 x 5 minutes
SA-B-HRP 1:200 in TBSTB	30 minutes
TBS	2 x 5 minutes
DAB	6 minutes
Distilled water	2 minutes
Nuclear fast red	15 seconds
Tap water	Until clear, plus 2 minutes
Dehydrate, clear in xylene, and mount coverslips with D.P.X. mountant.	

2.9.2.5 Slow myosin heavy chain (slow MHC)

For formalin-fixed, paraffin-embedded sections. The immunostaining achieved with this antibody was enhanced by the addition of a microwave antigen retrieval step (Shi *et al.* 1991) to the staining procedure. In the microwave antigen retrieval procedure, formalin-fixed, paraffin-embedded tissues are microwaved in the presence of a metal solution, which can result in a more intense immunostaining signal.

60°C oven	10 minutes
Deparaffinise and rehydrate tissue sections	As per Section 2.8.2, steps 2-6
TBST	5 minutes
Microwave antigen retrieval	10 min in citrate buffer on high power (1000 Watts) 15 minutes cooling down in the same solution at room temperature
TBST	5 minutes
Quench	10 minutes
TBST	5 minutes
NSS blocking	30 minutes
Slow MHC antibody diluted 1:100 in blocking solution	30 minutes
TBST	2 x 5 minutes
SAM-B 1:200 in TBSTB with 1.5% NDS	30 minutes
TBST	2 x 5 minutes
SA-B-HRP 1:200 in TBSTB	30 minutes
TBS	2 x 5 minutes
DAB	6 minutes
Distilled water	2 minutes
Nuclear fast red	15 seconds
Tap water	Until clear, plus 2 minutes
Dehydrate, clear in xylene, and mount coverslips with D.P.X. mountant.	

2.9.2.6 Fast myosin heavy chain (fast MHC)

For formalin-fixed, paraffin-embedded tissues.

60°C oven	10 minutes
Deparaffinise and rehydrate tissue sections	As per Section 2.8.2, steps 1-6
TBST	5 minutes
Quench	10 minutes
TBST	5 minutes
NSS blocking	30 minutes
Fast MHC antibody diluted 1:500 in blocking solution	30 minutes
TBST	2 x 5 minutes
SAM-B 1:200 in TBSTB with 1.5% NDS	30 minutes
TBST	2 x 5 minutes
SA-B-HRP 1:200 in TBSTB	30 minutes
TBS	2 x 5 minutes
DAB	6 minutes
Distilled water	2 minutes
Nuclear fast red	15 seconds
Tap water	Until clear, plus 2 minutes
Dehydrate, clear in xylene, and mount coverslips with D.P.X. mountant.	

2.9.3 Controls

The specificity of staining for all antibodies was determined by immunostaining with a) diluent only for the primary antibody step, and b) with a matched concentration of normal immunoglobin or serum (as appropriate), from the same species as the primary antibody. In the case of myostatin antibody, the irrelevant class-matched antibody α-lactalbumin was run as an additional control to confirm specificity of staining. Staining was not observed for any of these negative controls.

2.10 Receptor autoradiography

2.10.1 Introduction

Receptor autoradiography was used to determine the binding sites for IGF within muscle tissue sections, and was carried out in accordance with the method of Elliott *et al.* (1992). In short, the method consists of the incubation of tissue sections with radiolabelled IGF (*total binding*), and competition of the radiolabelled IGF binding with unlabelled homologous IGF (*non-specific binding*). Non-specific binding is subtracted from total binding to determine the *specific binding.*

IGF binding is characterised by competition with unlabelled peptides (IGF-I, IGF-II, des(1-3)IGF-I, des(1-6)IGF-II, and/or insulin). The binding affinities of the IGF receptors for these peptides are as follows:

Type I IGF receptor	IGF-I = des(1-3)IGF-I > IGF-II >> insulin
Type II IGF receptor	IGF-II = des(1-6)IGF-II >>> IGF-I (does not bind insulin)

Des(1-3) IGF-I and des(1-6) IGF-II, so named because they lack the first 3- and 6-amino acid residues from the amino-terminus of the mature IGF-I sequence, respectively, have a very low affinity for IGFBPs (Francis *et al.* 1993; Szabo *et al.* 1988). Because of the greatly reduced affinity for IGFBPs, these peptides can be used to distinguish binding to the receptor, from binding to IGFBPs. Thus, when either des(1-3) IGF-I or des(1-6) IGF-II is used as a competing peptide, radiolabelled IGF binding to receptors will be displaced, but radiolabelled IGF binding to IGFBPs will not be displaced.

2.10.2 Solutions

Pre-incubation buffer	0.17 M Tris-HCl, pH 7.4	
Incubation buffer	0.17 M Tris-HCl, pH 7.4 1% BSA 5 mM $MgCl_2$ 1 mM phenylmethylsulphonyl flouride Iodinated hormone (400,000 cpm/ml)	
Post-incubation buffer	0.17 M Tris-HCl, pH 7.4 0.25% BSA 0.01% Triton X-100	
Neutral buffered formalin	37-40% formaldehyde solution	100 mls
	Distilled water	900 mls
	$NaH_2PO_4 \bullet H_2O$	4.5 g
	Na_2HPO_4	6.5 g

2.10.3 Procedure

2.10.3.1 Incubation

Frozen muscle sections were cut at 8 micron thickness at 16-18°C, thaw-mounted onto Polysine™ slides, then immediately placed in a –20°C freezer. Sections to be incubated were stored not longer than one week. At the time of incubation, sections were slowly brought up to room temperature in a closed slide box, and then encircled with a PAP pen, which leaves behind a hydrophobic line of wax. Sections were incubated for 10 minutes in pre-incubation buffer. Excess pre-incubation buffer was aspirated, and then 50 ul of incubation buffer containing iodinated hormone (prepared according to the iodogen method (Salacinski *et al.* 1981) as previously applied to IGF

(Hodgkinson *et al.* 1987)) with or without competing hormones was placed over the sections. Competing hormones were used at 1 μg/ml for (rh) N-Met IGF-I, oIGF-I, (rh) IGF-II, des(1-3) IGF-I, des (1-6) IGF-II, and bovine insulin at 10 ng/ml. Each slide contained one pair of sections for the determination of total binding, and one or more pairs that were incubated with iodinated peptide plus cold, unlabelled competing hormone. Sections were placed in a humidified slide chamber, and incubated for 2 hours at room temperature. Incubation buffer was then aspirated, and the unbound ligand washed away in two 5-minute baths in post-incubation buffer. The sections were subsequently fixed in neutral buffered formalin for 10 minutes, then washed in distilled water and dried.

2.10.3.2 Macroautoradiographs

The dried slides were apposed to X-Omat-AR5 film for a period not exceeding 11 days, to generate a macroautoradiograph. The images obtained on the developed film were used to determine the length of exposure of the emulsion-coated slides, and to qualitatively assess the nature of the ligand binding.

2.10.3.3 Microautoradiographs

Developer	Phenisol	1 part
	Distilled water	4 parts
Stop solution	0.5% acetic acid	
Fix solution	Sodium thiosulphate	30g
	Distilled water	To 100 mls

Slides were coated with liquid photographic emulsion for microautoradiographic purposes, by dipping the slides in a dipping chamber filled with molten emulsion. The reverse side of the slides were wiped with a clean tissue, then the slides immediately placed on an ice-cooled metal tray until the emulsion had solidified. Slides were then placed in slide boxes containing dessicant, and placed in a light-tight darkroom box for 24 hours, until the dessicant was replaced with a fresh supply. The slides were then wrapped with black polythene to omit all light, at which time they were removed to a 4°C refrigerator until development time.

The silver halide crystals deposited in the emulsion by the ^{125}I-labelled ligand were developed to form silver grains according to the manufacturers recommendations for LM-1 photographic emulsion. The procedure in brief is as follows. All solutions were first brought to 20°C, and were maintained at that temperature throughout the development process. Slides were placed in developer for 5 minutes, then in stop solution for one minute to end development. Slides were fixed for 8 minutes in fix solution, then washed in running tap water for 15 minutes, followed by two-fifteen

minute baths in distilled water. Slides were then air-dried until histological staining was carried out, at which time sections were rehydrated by placing in water for 5 minutes. Haematoxylin and eosin histological staining was then performed as per Section 2.8.2, steps 7-20.

2.11 Radiographic grain and nuclear quantification

Image analysis was performed to quantitate nuclei after immunohistochemical experiments (CHAPTER 6), and autoradiographic signal resulting from *in situ* hybridisation (CHAPTER 4) and receptor autoradiography experiments (CHAPTER 4 and CHAPTER 6). Three analysis and quantification systems were used during the course of the work contained in this thesis. The automated Visilog system was used for the analysis of radiographic grains resulting from *in situ* hybridisation with radioactive probes as carried out in CHAPTER 4. This system was chosen for use due to the high grain density in the *in situ* hybridisation experiments, whereas the lower resultant grain densities of receptor binding experiments in CHAPTER 4 were suited to manual grain counting using the Image system. The ScionImage system was used for all quantitation in CHAPTER 6. This system was the only system of the three that would enable quantitation of chromogenic signals, such as those resulting from immunohistochemistry and histological staining. The ScionImage system was used for manual counting of grains resulting from receptor autoradiography in CHAPTER 6 as this system superseded the Image system for grain counting purposes. In all cases, sites for analysis were determined from random microscope stage coordinates, as generated from within Microsoft Excel (Microsoft Corp., Auckland, NZ).

2.11.1 Visilog system

For hybridised sections, grain densities were determined for video images of microautoradiographs as obtained through an Olympus BX-50 light microscope with attached camera adaptor (CMA-D7CE, Sony Corporation, Japan) and CCD video camera (AVC-D7CE, Sony Corporation). Images were captured onto an IBM compatible 386 computer equipped with a MVP-AT machine vision processor (Matrox, Dorval, Quebec, Canada), Trinitron monitor (Sony Corporation) and Visilog DOS version 3.6 software (Noesis, Jouy-en-Josas, France). With this system, silver grains were detected following gray image processing to find local intensity maxima. Values obtained by this method correlate highly (r=0.99) with grain density values obtained by manual grain counting (Ord *et al.* 1993).

2.11.2 Image system

Manual grain counting of *in vitro* incubated slides was performed using an Olympus BH2 microscope fitted to a monochrome video camera unit (Cohu Inc., San Diego, CA). Neutral density filters were used to optimise for radiographic grain detection. Images for analysis were captured onto a Macintosh *IIfx* computer equipped with a DT2255 Quick Capture frame grabber card, and the public domain NIH Image programme (this can be downloaded at http://rsb.info.nih.gov/nih-image/).

2.11.3 ScionImage system

For the analysis of chromogenic signals and manual grain counting, digital images were captured using the CMS700 image analysis system (Scion Corporation, Frederick, MD, USA) which is comprised of an MTI DAGE 330 CCD colour video camera, frame grabber card (CG-7, Scion Corporation), and 786 x494 pixel, 24-bit camera control unit. Digital images were relayed to a 200 MHz Pentium II computer equipped with ScionImage software (which can be downloaded from the internet at http://www.scioncorp.com). Images were saved as TIFF files, then manually counted within ScionImage.

2.12 Image acquisition

Photomicrographic images, as contained within this thesis, were captured using the ScionImage system, as described in Section 2.11.3. Images were first saved as TIFF files in ScionImage, then as JPEG files within the PhotoEditor programme of Microsoft Office 2000 (Microsoft Corporation). Files were converted into JPEG format in order to reduce file size and so allow insertion of images into Word documents.

2.13 Statistics

Data was analysed for overall effects of treatment and time by ANOVA using GenStat 4.2 software. A modification of this method, REML, was used when indicated for the statistical analysis of unbalanced groups. Students' T-test was employed for statistical comparisons between individual groups, and the Tukey ("Honest") Significant Difference used when appropriate for multiple pairwise comparisons. Values shown are means ± S.E.M.

CHAPTER 3

REGENERATION MODELS

This chapter describes two models of skeletal muscle regeneration, notexin-induced and muscle grafting-induced, and the results obtained using these models in the rat. The notexin-induced regeneration model was subsequently used for the studies in Chapters 4, 5, and 6.

3.1 Notexin-induced regeneration model

3.1.1 Introduction

As discussed earlier in Section 1.5, there are a number of ways in which to damage muscle in order to initiate regeneration. Table 3-1 lists the key methods by which muscle has been experimentally damaged in published reports of skeletal muscle regeneration. Although the key events of MPC activation, proliferation and fusion occur regardless of the means of damage, the method used to damage muscle can result in extensive and/or prolonged damage to the basal lamina (Lawson-Smith & McGeachie 1997; Lefaucheur & Sébille 1995b; Trupin 1979), vascular (Hansen-Smith & Carlson 1979; Lefaucheur & Sébille 1995b) and nervous supplies (Yoshimura *et al.* 1998), and may result in the appearance of excessive fibrosis (Vracko & Benditt

1972). Further, satellite cells are killed by some methods such as freezing (Ghins *et al.* 1984; Schultz *et al.* 1986), and preserved by others (Harris 1989). These factors may alter the timing (Grounds & McGeachie 1990) and/or efficiency (Ghins *et al.* 1984; Lefaucheur & Sébille 1995b; McGeachie & Grounds 1987) of regeneration.

Table 3-1. Methods for damaging skeletal muscle.

INJECTED SUBSTANCES	**REFERENCE**
Snake toxins (notexin, taipoxin)	Harris *et al.* (1975) Harris & Maltin (1982)
Local anaesthetic (bupivicaine)	Hall-Craggs (1974)
Cardiotoxin	Ishii & Lo (2001)
SURGICAL MANIPULATIONS	
Muscle grafting	Hansen-Smith & Carlson (1979)
Denervation/devascularisation	Anderson (1991)
Ischaemia following glycogen depletion	Jennische (1986)
Crush injury	Mitchell *et al.* (1992)
Freeze injury	Schultz *et al.* (1986)
Mincing of muscle	Trupin (1979)

For the work described in this thesis, the injection of notexin was chosen as the means of inducing muscle damage, although for the purposes of the final experimental chapter, muscle grafting was considered, and this will be discussed later in Section 3.2. The reasons for choosing notexin for this work were: a) notexin-induced muscle damage and regeneration were well-characterised (Harris 1989; Harris *et al.* 1975) and notexin had been used for many regeneration studies (Davis *et al.* 1991; Klein-Ogus & Harris 1983; Preston *et al.* 1990), and b) it did not involve extensive surgical manipulations, and so was suitable for the large trials described herein, c) notexin

injection results in minor damage to microvascularisation (Harris 1989; Harris *et al.* 1975; Lefaucheur & Sébille 1995b) and basal lamina (Lefaucheur & Sébille 1995b), and reinnervation occurs quickly (Harris *et al.* 2000; Whalen *et al.* 1990).

Notexin, a fraction of the Australian tiger snake venom (*Notechis scutatus scutatus*), is a phospholipase A_2 consisting of 119 amino acids and seven disulphide bridges (Halpert & Eaker 1975; Halpert *et al.* 1976). Notexin contains both neurotoxic and myotoxic activites. As a neurotoxin, notexin acts at the presynaptic level, causing the depletion of the neurotransmitter acetylcholine from motor nerve terminals within one hour of injection (Harris *et al.* 2000). The block of neuromuscular transmission at the motor nerve terminal caused by notexin becomes irreversible (Mebs 1989), and is followed by the degeneration of the motor nerve terminal and of the axonal cytoskeleton (Harris *et al.* 2000). The neurotoxic effect of notexin results in denervation of 70% of muscle fibres in rat M. *soleus* by 24 hours (Harris *et al.* 2000). The myotoxic effect of notexin is exerted through binding to the sarcolemma, causing small lesions in the membrane, loss of ion gradients, subsequent hypercontraction and myofibre degeneration (Dixon & Harris 1996). Notexin binds to the sarcolemma of both glycolytic and oxidative muscle fibres, although slow oxidative fibres are more susceptible to the myotoxic effects of notexin (Dixon & Harris 1996).

In rats, a 2 μg quantity of notexin has typically been used for regeneration studies (Harris *et al.* 1975; Pluskal *et al.* 1978). The notexin in published studies has been administered variously, including subcutaneous injection (Harris *et al.* 1975; Pluskal *et al.* 1978), injection into the intermuscular space of the hindlimb (Harris & Johnson

1978), and intramuscular injection (Lefaucheur & Sébille 1995b; Sharp *et al.* 1993). Each mode of administration has been shown to effect damage sufficient for the study of regeneration, however intramuscular injection is recommended for animals that are larger than 100g, due to problems with the diffusion of notexin through larger muscle when injected subcutaneously or into the epimysial compartment (Davis *et al.* 1991). The aim of the following trial was to determine the timing of regeneration events in GH-deficient *dw/dw* rats, and to optimise sampling times for the experiments described in CHAPTER 4 and CHAPTER 5.

3.1.2 Materials and Methods

Twenty-eight male *dw/dw* rats were castrated at 28 days of age. Castration was performed in this experiment, as this was a preliminary trial for the studies contained in CHAPTER 4 and CHAPTER 5. The reasons for castration in these studies will be addressed in more detail in CHAPTER 4. At 60 days of age and time 0 in the trial, rats were weighed, then anaesthetised with an intraperitoneal injection of 3 mg pentobarbitone sodium B.P.(Vet.) (Sagital, May and Baker Ltd., Dagenham, England) per 100g bodyweight. The fur was trimmed from the area over the right M. *biceps femoris,* and a small incision made over the muscle. Using a 50µl syringe (Hamilton Co., Reno, Nevada, USA) with an attached 25G x 16mm needle, 2µg (in 10 µl) of notexin (Venom Supplies Pty. Ltd., Tanunda, South Aust) was injected intramuscularly into the right M. *biceps femoris*. A small dot of India ink (Stephen's, Dunedin, NZ) was placed on the surface of the muscle to mark the site of notexin injection. The wound was closed with 2 stitches using 3-O silk (Ethicon, Somerville,

NJ, USA). Rats were moved to a warm recovery room, and when fully alert and active, moved into a standard temperature room. Rats were provided water and food *ad libitum* throughout all pre- and post-operative phases of the trial. At the time of sacrifice rats were weighed, then killed by CO_2 gas and cervical dislocation. Right *biceps femoris* muscles were carefully dissected out and weighed, then formalin-fixed and paraffin-embedded (as per Section 2.7.2.2) for histological examination. The timepoints for sacrifice (days 1,2,3,5,10 and 15) were chosen to encompass the anticipated periods of MPC proliferation and fusion, and to include a later timepoint in which the fibres were enlarged but where regenerating fibres were still discernable from fibres that survived notexin treatment.

3.1.3 Results

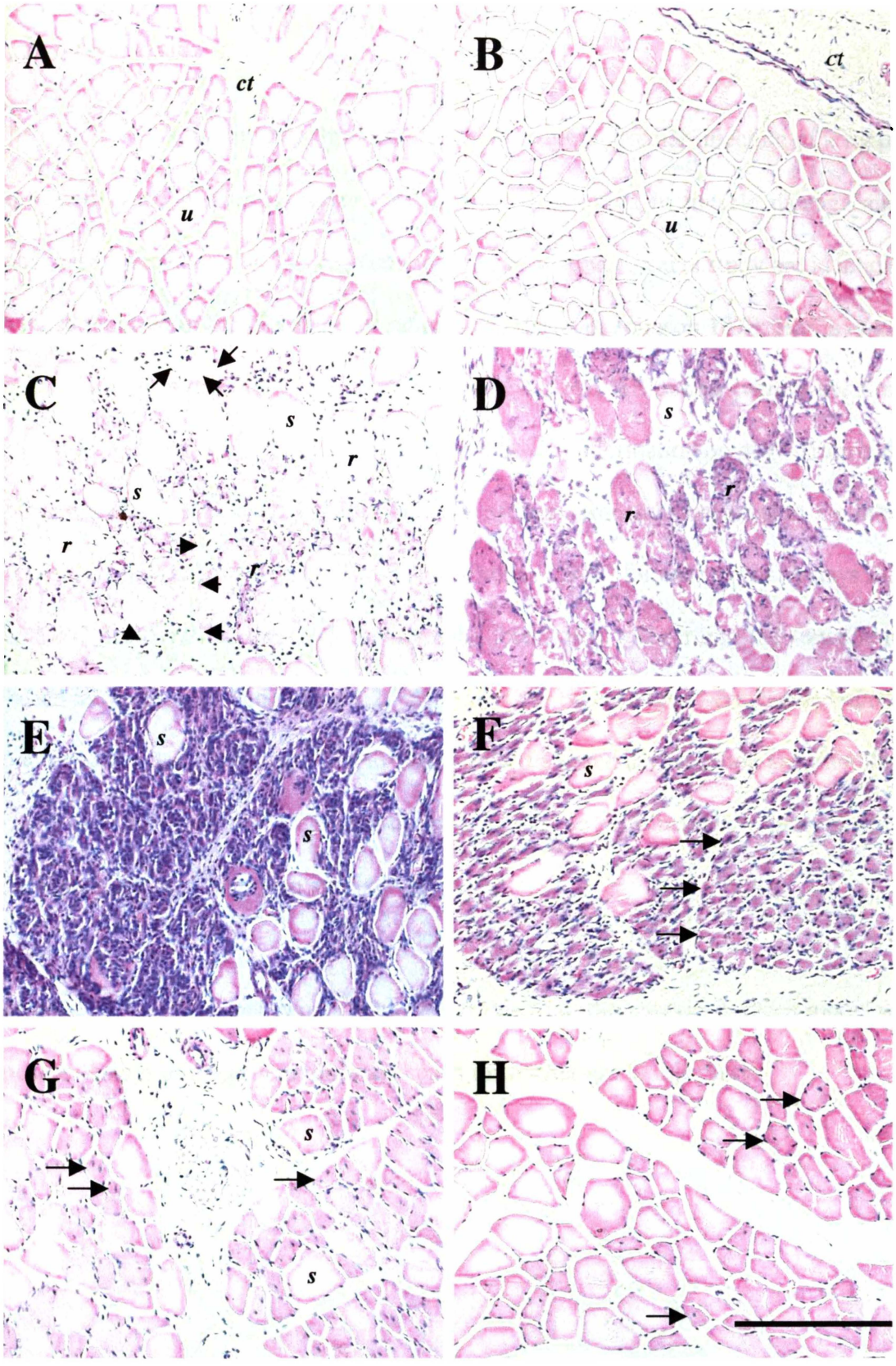
A gross assessment of damaged muscles following notexin-injection showed that not all of the fibres in the injected M. *biceps femoris* underwent damage and regeneration. At the site of notexin injection, all fibres were destroyed (referred to in CHAPTER 6 as the "core of damage"), however further out from this area, "regenerating fibres" were interspersed with "survivor fibres" (fibres that did not undergo necrosis and regeneration following notexin injection), as shown in Figure 3-1C. Lastly, further out from this area of interdigitating regenerating and survivor fibres was a complete absence of damage (Figure 3-1B). In this area, the fibres are referred to as "undamaged fibres". Undamaged fibres from notexin-injected muscles (Figure 3-1B) were indistinguishable from the undamaged fibres of the non-injected control muscle (Figure 3-1A).

Figure 3-1 provides a histological description of the regeneration events following notexin injection, as determined in haematoxylin and eosin stained muscle sections. The appearance of undamaged muscle fibres of non-injected control (Figure 3-1A) and notexin-injected muscles (Figure 3-1B) was in contrast to the oedemaceous and fragmented appearance of damaged muscle one day after notexin-injection (Figure 3-1C). As shown in Figure 3-1C, phagocytic and inflammatory cells were prevalent in day 1 damaged tissues, both in interstitial areas and within necrotic fibres, although many necrotic fibres were still non-infiltrated at this time. Day 2 tissues were more markedly hypercellular than were day 1 tissues, particularly within the necrotic fibres (Figure 3-1D), as necrotic debris was being phagocytosed and removed at this time. In day 3 notexin-injected muscle (Figure 3-1E), damaged areas contained a high density of mononucleate cells, of which a high proportion were presumed to be MPC, because: i) this is the appearance of regenerating muscle just prior to MPC fusion to form myotubes, and ii) these mononucleate cells have the generally scant cytoplasm typical of MPC (Harris *et al.* 1975; Moss & LeBlond 1970; Ontell 1973). The density of nuclei in regenerating fibre areas was higher on day 3 than at any other timepoint during regeneration (Figure 3-1; E versus C, D, F-H). Necrotic debris was still present on day 3, although in considerably smaller amounts than on days 1 and 2. Day 5 damaged muscle (Figure 3-1F) contained abundant myotubes, which are identifiable in cross-section by their centrally-located nucleus and small perimeter of cytoplasm (Figure 3-1F) and in longitudinal section as ladder-like arrangements of nuclei with surrounding cytoplasm. A few fibres still contained necrotic debris on day 5. The regenerating myotubes were increased in diameter by day 10 (Figure 3-1G), but still often contained a centrally-located nucleus and were smaller than survivor fibres, thus

allowing easy distinction of the two types of fibres. However by day 15 (Figure 3-1H), it is difficult to distinguish regenerating from survivor and undamaged fibres as shown in (Figure 3-1A) and (Figure 3-1B), respectively, due to the movement of regenerating muscle fibre myonuclei to a peripheral location, and increased regenerating fibre diameter. The regeneration process, as assessed histologically, was consistent between animals, and minimal fibrosis occurred during regeneration.

Figure 3-1. Muscle regeneration in the *dw/dw* rat.

(A) Non-injected, control muscle contains undamaged muscle fibres (u) and connective tissue (ct); (B) undamaged muscle fibres of the notexin-injected damaged muscle (injected on day 0), are located within the M. *biceps femoris*, but a long distance from the site of notexin-injection; (C) day 1 damaged muscle shows interspersed regenerating (r) and survivor (s) fibres, and the presence of oedema (arrowheads); (D) day 2 damaged muscle; (E) day 3 damaged muscle contains a greater number of mononucleate cells relative to day 2; (F) day 5 damaged muscle contains numerous myotubes (arrows indicate a fascicle that is filled with new myotubes); (G) day 10 damaged muscle with enlarged myotubes as indicated by the arrows; (H) day 15 damaged muscle shows many regenerating myotubes (arrows) which have become more difficult to discern from undamaged (u) and survivor fibres (s) of notexin-injected muscles as shown in (B) and (C), respectively, due to their large diameter. Sections have been stained with haematoxylin and eosin, and images captured at 25x magnification (bar=200 microns).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3.1.4 Discussion

The results of this preliminary trial showed that the intramuscular injection of 2 μg of notexin into the M. *biceps femoris* of *dw/dw* rats resulted in muscle damage and subsequent regeneration. The regeneration events observed in this study are identical to those reported in the literature for rat muscle (Harris & Johnson 1978; Harris *et al.* 1975), with a similar onset of timing of phagocytic cell infiltration (within the first day), a profusion of MPC present on day 3, and myotube formation on day 5 (Harris & Johnson 1978; Harris *et al.* 1975). The bulk of these processes occurred to the later end of the time ranges given by Harris and Johnson (1978) for each event (ie days 2-3 for MPC proliferation, days 3-5 for myotube formation). Regarding the timepoints chosen in the present study (days 1,2,3,5,10 and 15), two changes were decided upon: a) to bring the day 10 timepoint up to 9 days so as to lessen the sampling gap after day 5, and b) to bring the day 15 timepoint up to day 13, so as to be able to more readily distinguish regenerating from survivor fibres.

Because of the consistent results achieved in this trial, the notexin-induced model of muscle damage and regeneration was chosen for further use in this thesis.

3.2 Muscle-grafting regeneration model

3.2.1 Introduction

For the purposes of the final experiment, the ideal model of regeneration would exhibit widespread damage to all fibres, synchronous damage and regeneration processes, consistency between and within animals, and have an early onset and peak of MPC proliferation and fusion processes. The reasons for establishing these criteria are that they would a) allow for some non-histological quantitation of results, b) increase the probability of detecting an effect of administered IGF-II on regeneration, and c) not necessitate too lengthy a period of peptide administration. To achieve these aims two models of muscle regeneration were considered: a) notexin-induced muscle regeneration, and b) the muscle grafting-induced regeneration model.

The muscle-grafting induced model of muscle regeneration is very well characterised (Carlson 1986), and has been used extensively for muscle regeneration studies (Smythe *et al.* 2001; White *et al.* 2000). In the muscle-grafting model, the muscle is removed from its natural bed and all vascular and nervous supplies severed. The muscle is then placed in either the same, or an alternate position, in the same animal (autografting) or in a host animal (allografting). In the field of muscle regeneration research, the most prevalent model of muscle-grafting involves the transplantation of the M. *extensor digitorum longus* onto the anterior side of the M. *tibialis anterior*. In this case, the proximal and distal tendons of the M. *extensor digitorum longus* are attached by means of sutures to the tendon of the M. *quadriceps femoris* and M. *tibialis anterior*, respectively.

The grafted M. *extensor digitorum longus* undergoes complete regeneration of all muscle fibres, bar a small number of surviving fibres at the periphery of the muscle, which survive due to the availability of growth factors and nutrients in the surrounding environment (Grounds & McGeachie 1999). As shown in Figure 3-2, regeneration proceeds in a gradient from the exterior to the interior of the M. *extensor digitorum longus* (Carlson 1986), again as a result of greater nutrient and growth factor supply from the surrounding environment (Grounds & McGeachie 1999), and the re-establishment of a vascular supply from the outside which works its way inward with time (Bodine-Fowler 1994). This gradient results in a heterogenous population of cells and events within the M. *extensor digitorum longus*, for at certain timepoints regenerated myotubes are present in the periphery of the muscle while necrotic material is still present in the centre of the muscle. Innervation begins to take place in the second week following grafting of rat M. *extensor digitorum longus* (Carlson 1986), and as such is delayed relative to the reinnervation of muscle damaged by notexin.

The aim of this study is to determine whether the muscle grafting model is suitable for use in CHAPTER 6 by assessing: a) the variability in regeneration using this model, and b) the approximate peak of myotube fusion.

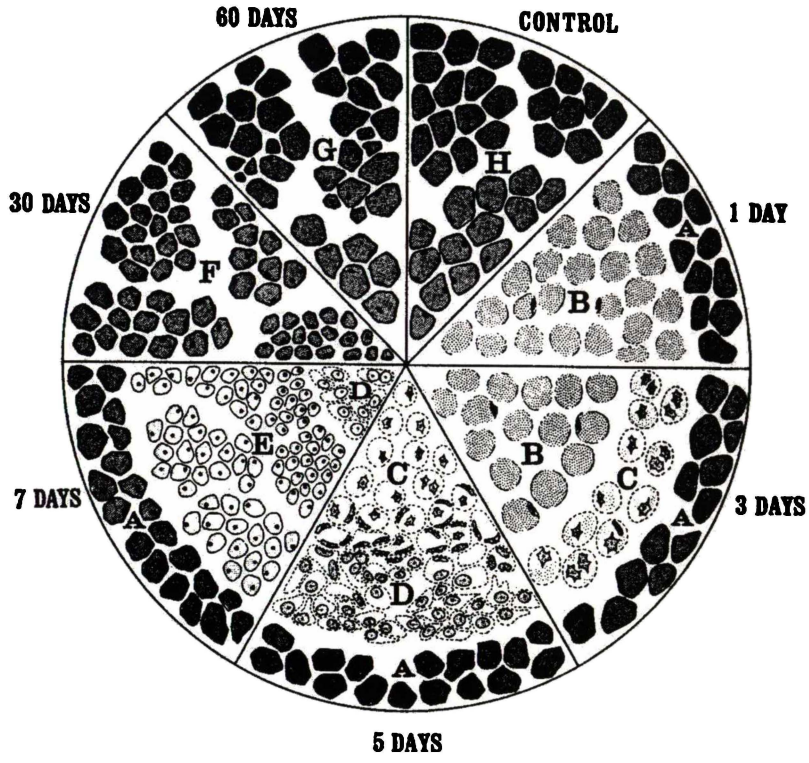


Figure 3-2. Diagram of muscle regeneration following grafting.

Diagrammatic representation of a cross-sectional view of a M. *extensor digitorum longus* following grafting, with the times for each stage in the rat indicated. Letter codes are as follows: (A) surviving muscle fibres; (B) original muscle fibres that are in a state of ischaemic necrosis; (C) muscle fibres that have been infiltrated by phagocytic cells; (D) MPC and new myotubes; (E) enlarged muscle fibres containing contractile elements; (F) enlarged and maturing regenerating muscle fibres; (G) mature regenerated muscle fibres; (H) normal control muscle fibres. Reprinted (with minor modifications) from Carlson (1986).

3.2.2 Materials and Methods

Thirty-three male Sprague-Dawley rats (aged 6 weeks) were used for this experiment, three of which were unoperated controls (t=0), and the remainder sacrificed at timepoints up to 14 days post-muscle grafting (n=3 per timepoint). Rats were balanced for weight at the outset of the trial. On the day of muscle grafting surgery

(day 0), rats were anaesthetised with a solution of 5 mg/ml Rompun® (xylazine hydrochloride), 37.5 mg/ml ketamine hydrochloride in sterile water (0.2 ml per 100g bodyweight), then an incision made in the skin over the M. *extensor digitorum longus*. After the M. *extensor digitorum longus* was located, a length of 7-0 braided silk suture (Ethicon) was slipped under the proximal myotendinous junction of the M. *extensor digitorum longus*, and firmly tied onto it with 3 double reef knots. This procedure was followed for the distal end of the M. *extensor digitorum longus*. The proximal end of the M. *extensor digitorum longus* tendon was subsequently severed with fine scissors, and the M. *extensor digitorum longus* laid over the M. *tibialis anterior*. The uncut ends of the suture threads on the proximal M. *extensor digitorum longus* were pulled through underneath the distal tendon of the M. *quadriceps femoris*, and firmly knotted. The distal tendon of the M. *extensor digitorum longus* was next cut with fine scissors, and the full length of the M. *extensor digitorum longus* laid flat upon the superficial side of the M. *tibialis anterior*. The uncut ends of the suture thread on the M. *extensor digitorum longus* were then tied around the distal tendons of the M. *tibialis anterior*, taking care to attach the M. *extensor digitorum longus* at its normal length.

3.2.3 Results

The grafted muscles showed profound histological changes with time, as shown in Figure 3-3. A larger degree of variation in the regeneration process was noted in these muscles, relative to the notexin model just described in Section 3.1, however the following provides a summary of the key events. By day 1 after grafting, numerous

phagocytic and inflammatory cells were interspersed throughout the grafted muscles (Figure 3-3B), and this pattern was present in day 2 muscles as well. Oedema, as indicated by enlarged pericellular and interstitial spaces, was apparent at early timepoints (Figure 3-3B). By days 2 and 3 a band of cells approximately 2-3 cells wide near the periphery of the M. *extensor digitorum longus* had been invaded by phagocytic cells, while the remainder of the cells in the more central portion of the M. *extensor digitorum longus* were non-infiltrated. By day 4 the band of phagocytosis had enlarged to approximately 4-6 fibres deep in some animals, and the density of mononucleate cells in that band had increased relative to day 3. A number of surviving muscle fibres around the very outside of the M. *extensor digitorum longus* were easily discernable from day 4 onwards. Myotube fusion was noted as early as day 5, but was not observed consistently until days 6 and 7. In day 6 and 7 muscles, the regenerated myotubes were generally restricted to a band approximately 8 cells wide near the periphery of the muscle, with the centre of the muscle still present as non-infiltrated/non-phagocytosed muscle fibres. By day 8, the regenerating myotubes were slightly larger in size, as shown in Figure 3-3D. Necrotic fibres were observed in the centre of the grafted muscles up through day 11, but were not seen in day 14 muscle. Day 14 muscle contained numerous enlarged myotubes (Figure 3-3E), some of which were similar in diameter to survivor fibres.

The sequence of regeneration events given in Figure 3-3 summarises the general regeneration pattern observed in these grafted muscles, however considerable variation relative to that seen in the previously described notexin-induced model (as described in Section 3.1) was observed. This variation appeared to have been due, at

least in part, to the deposition of substantial amounts of fibrotic tissue in many of the grafted muscles. Accumulations of fibrotic (connective) tissue within and to the exterior of the grafted muscle were noted from day 3 onward. These accumulations were not consistently deposited within and around the regenerating muscles, so that within the same timepoint, grafted muscles often contained vastly different amounts of connective tissue/fibrotic material. This is shown for two grafted muscles from day 8 animals in Figure 3-4.

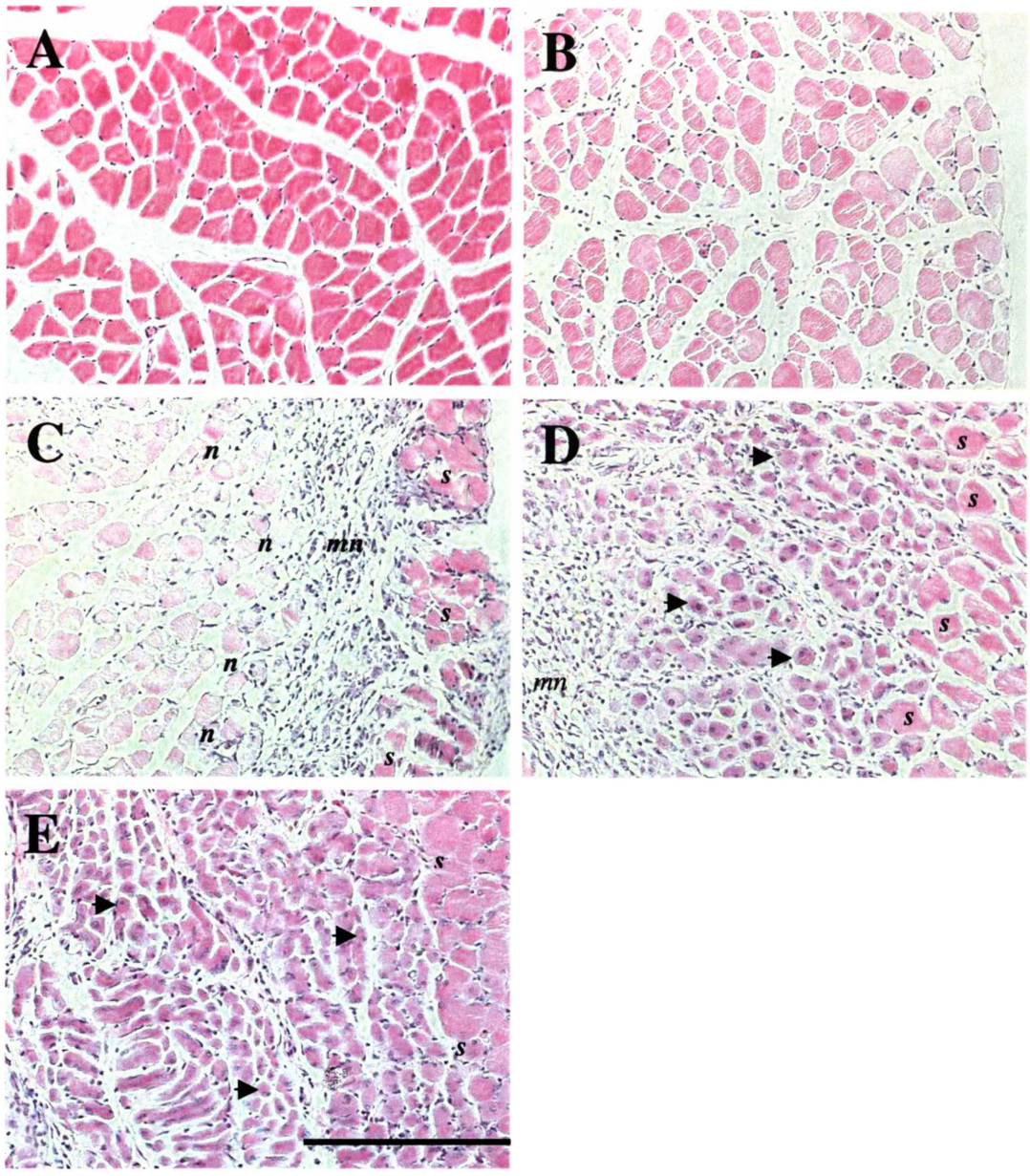


Figure 3-3. Regeneration of grafted muscle.

Histology of rat M. *extensor digitorum longus* following grafting on day 0. (A) shows undamaged, unoperated muscle at time 0. (B) M. *extensor digitorum longus* on day 1 following grafting shows scattered phagocytic and inflammatory cells throughout the muscle and the presence of oedema. (C) Grafted muscle on day 5 contains some survivor fibres (s) along the periphery of the muscle, necrotic fibres (n) in the core, and between these are mononucleate cells (mn) prior to the onset of fusion. (D) Grafted muscle at day 8 contains myotubes (arrowheads) between areas of mononucleate cells and survivor fibres. (E) Grafted M. *extensor digitorum longus* at 14 days contains enlarged myotubes (arrowheads) which are approaching the size of survivor fibres. Images are of haematoxylin and eosin stained cross-sections of M. *extensor digitorum longus* taken at 25x magnification (bar =200 microns).

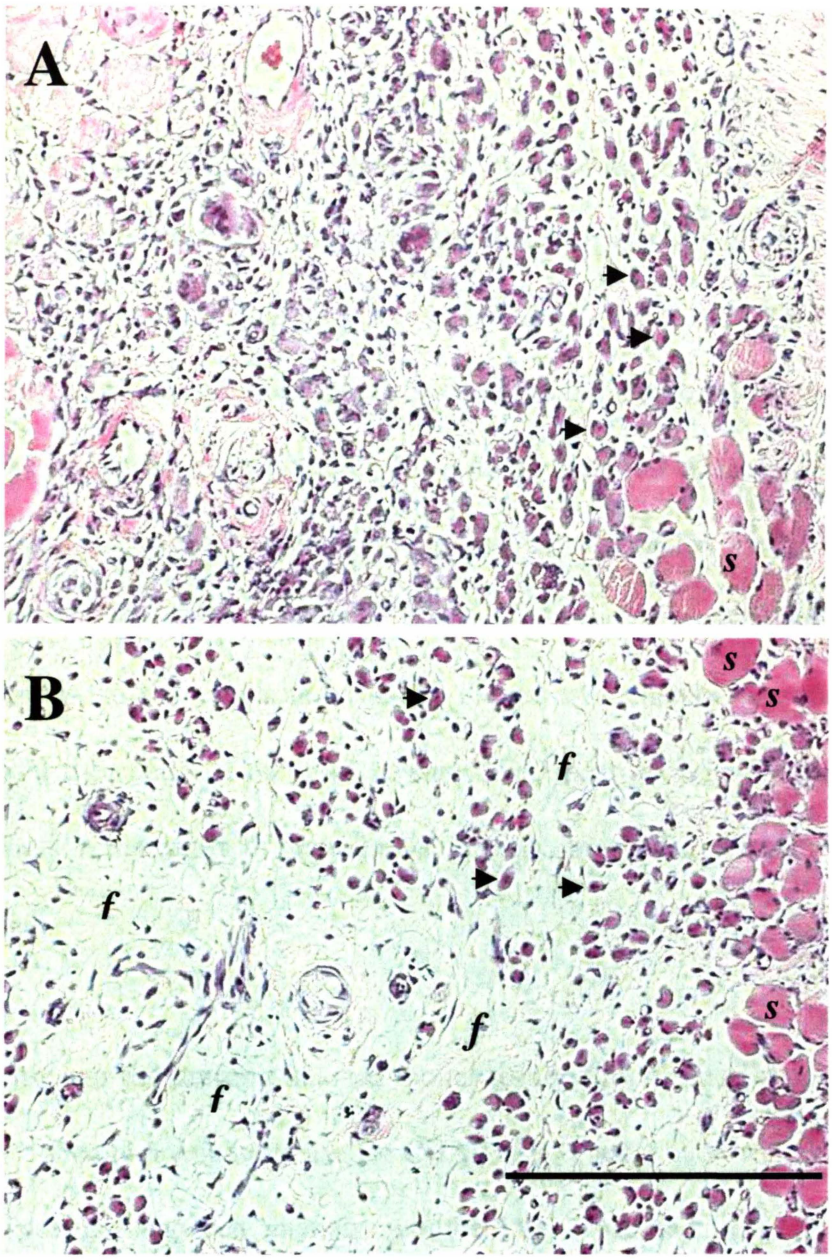


Figure 3-4. Fibrotic tissue deposition in grafted muscles.

(A) and (B) are photomicrographs of grafted M. *extensor digitorum longus* from two separate animals, on day 8 following surgery. (A) shows an area of damaged muscle that contains low amounts of fibrotic tissue, while (B) shows muscle at a similar stage of regeneration (early myotube stage; arrowheads indicate myotubes) but in the presence of high amounts of fibrotic tissue (*f*). Images are of haematoxylin and eosin stained formalin-fixed, paraffin-embedded tissue sections taken at 25x magnification (bar=200 microns).

3.2.4 Discussion

In the present study, the sequence of regeneration events normally seen after muscle grafting occurred, including the persistence of survivor fibres along the periphery of the regenerating muscle (Carlson 1986; Grounds & McGeachie 1999), and the gradient of regeneration from the exterior to the interior of the muscle (Carlson 1986). The timing of regeneration events such as the onset of muscle fibre phagocytosis on days 2-3, and the beginning of new myotube formation on days 6-7, were similar to the times reported for rat M. *extensor digitorum longus* (Carlson 1986). However, in contrast to the events indicated in Figure 3-2, whereby necrotic fibres were absent from the centre of the graft beyond day 7 post-grafting, the persistence of necrotic fibres in the centre of grafted muscles was observed up through day 11 in the present trial.

Although the grafting-induced model resulted in adequate damage and regeneration, two factors preclude its use for CHAPTER 6, namely the variability in fibrotic tissue deposition between animals and the late onset and peak of myotube formation. Fibrotic/connective tissue deposition can hamper muscle regeneration by forming a dense barrier to new fibres (McMinn 1967), therefore the presence of fibrous accumulations can alter not only the quality, but also the timing, of regeneration. Secondly, although the timing of the onset of myotube formation was similar to that reported by others for grafted rat M. *extensor digitorum longus* (Carlson 1986), the peak of myotube formation was obviously much later than days 6-7. This was

deduced by the fact that on days 6-7 there was still considerable necrotic tissue present in the centre of the grafted muscle. For the purposes of CHAPTER 6, the peak of myotube formation needed to occur prior to days 6-7, and over a relatively brief period in order to reduce inter-animal variation. The reason for the time requirement was that the pump being used to administer the peptide had a maximum administration period of 7 days, and because the effect of the administered peptide on MPC fusion was being tested, the bulk of MPC fusion had to occur within that time. For these reasons, the muscle-grafting induced model of regeneration was not used, and the notexin-induced model chosen for use instead for CHAPTER 6.

CHAPTER 4

THE EFFECT OF GH ON THE IGF AXIS DURING SKELETAL MUSCLE REGENERATION

4.1 Introduction

Skeletal muscle growth is affected by its hormonal environment. Growth hormone (GH) and the insulin-like growth factors (IGFs) have been shown to have a role in muscle growth and development (Dodson *et al.* 1985; Etherton & Kensinger 1984; Merrill *et al.* 1977), as described in Section 1.7.1. Growth hormone enhances normal postnatal muscle growth, at least in part, by augmenting skeletal muscle protein synthesis (Fryburg & Barrett 1993). The means by which GH exerts its effect on skeletal muscle is not fully understood, although much attention has been focussed on endocrine, as well as autocrine/paracrine, production of IGF-I (Isgaard *et al.* 1988; Isgaard *et al.* 1989; Sara & Hall 1990; Vikman *et al.* 1991). Previous animal studies

have suggested that GH may improve wound healing (Jorgensen & Andreassen 1987; Pessa *et al.* 1985). GH has been used in several regeneration studies, and similar to the case for normal postnatal muscle, has been found to enhance the weight of regenerated muscle (Ullman *et al.* 1989). Additionally, the GH receptor is expressed in regenerating skeletal muscle of rats (Jennische & Andersson 1991), further supporting a possible role for GH in regeneration.

Hypophysectomised rats have been used previously to examine the effect of GH on muscle growth and regeneration (Sommerland *et al.* 1989; Ullman & Oldfors 1991). In this study, the *dw/dw* rat was used to examine the effects of GH. The *dw/dw* rat has a selective autosomal recessive defect that results in greatly reduced (6-10% of normal) levels of pituitary GH, and reduced bodyweight (approximately 40% less at 3 months) relative to its normal counterpart, the Lewis rat (Charlton *et al.* 1988). Male and female *dw/dw* rats have barely detectable levels of circulating GH, but maintain the sexually dimorphic pattern of GH release typical of normal rats, only reduced in amplitude (Legraverend *et al.* 1992). Administration of GH to *dw/dw* rats results in a dramatic growth response, with approximately 2.5- to 4.0-fold greater daily weight gain following GH treatment, relative to untreated *dw/dw* rats (Charlton *et al.* 1988). *Dw/dw* rats have normal concentrations of the other anterior pituitary trophic hormones (LH, TSH, prolactin and ACTH), unlike hypophysectomised rats, which lack all anterior pituitary hormones (Charlton *et al.* 1988). This feature of a selective GH-deficiency, made the *dw/dw* rat a preferable model to the hypophysectomised rat for the study of a GH effect on muscle regeneration. The *dw/dw* rat is fertile, and thus can be maintained as a self-sustaining breeding colony (Crawford *et al.* 1994). Male

dw/dw rats achieve sexual maturity, based on plasma testosterone, and testis and seminal vesicle growth, on days 42-63 (Crawford *et al.* 1994).

In this study, a histological approach rather than one examining the overall changes in muscle, has been used. The reasons for this are: a) muscle tissues respond differentially to stimuli, as exemplified by a study of normal ovine muscle showing an effect of nutrition on IGF-I binding to connective tissue but not muscle fibres (Oldham *et al.* 1996); and b) the response of muscle fibres may be different depending on the proximity to damage. Therefore in this study IGF expression and binding will be examined in connective tissue ("connective tissue"), and in muscle fibres that are regenerating ("regenerating fibres"), interspersed with regenerating muscle fibres ("survivor fibres"), and in fibres that are within the notexin-injected muscle but are removed from the damage and not interspersed with regenerating fibres ("undamaged fibres").

4.1.1 Aim of this chapter

The aim of this chapter is to test the hypotheses that the IGFs and their receptors are regulated during muscle regeneration, and that the level of IGF expression and binding in regenerating muscle is regulated by GH.

4.2 Materials and Methods

4.2.1 Animals

The *dw/dw* GH-deficient rats used for this study were a gift of Dr. I.C.A.F. Robinson, National Institute of Medical Research (Mill Hill, London, UK). Fifty male *dw/dw* rats were grown to 28 days of age, then castrated under general anaesthetic. Prepubertal castration of these *dw/dw* rats was performed in order to remove endogenous testosterone. The reason for removing testosterone relates to the administration of GH. As discussed in Section 1.7.1.7.1, testosterone induces a masculine pattern of GH secretion (Painson *et al.* 2000), a pattern associated with greater somatic growth relative to the feminine pattern (Jansson *et al.* 1985). Castration was therefore performed in this trial in order to decrease the somatic growth response to endogenous GH. The reason that male rats were used was that this trial was part of a larger study in which the effects of testosterone on regeneration of a testosterone-responsive muscle (M. *levator ani*) was examined.

At 60 days of age, and weighing approximately 120 g, the rats were anaesthetised and a small incision made over the right M. *biceps femoris.* The rats then received one intramuscular notexin injection (2.0 μg) in the right M. *biceps femoris* ("injected muscle"; as described in Section 3.1). Contralateral muscles, which were not injected with notexin ("non-injected") were used as regeneration controls. Rats were divided into a GH-treated group which received a single daily subcutaneous injection of human N-methionyl GH (kindly provided by Genentech, So. San Francisco, CA; 200 μg in 0.1 ml/100 g body weight daily), while the control group received an equal

volume of saline vehicle (0.1 ml/100 g body weight daily). Animals were weighed and sacrificed at days 1, 2, 3, 5, 9 and 13 post-notexin injection (n=4 or 5 per treatment group per day).

In order to separate out the effects of notexin and GH, a second trial was carried out in which *dw/dw* rats received identical GH and saline volumes to those used in the first trial, but no notexin. Body weights were recorded on a daily basis up to day 10 (n=5/6 per group).

4.2.2 Tissue Sampling

Muscle samples for autoradiographic receptor studies were frozen (see Section 2.7.2.1), then stored at -70°C. Muscles for *in situ* hybridisation experiments were formalin-fixed and paraffin-embedded according to the method given in Section 2.7.2.2. Formalin-fixed, paraffin-embedded sections were stained with haematoxylin and eosin, by the method described in Section 2.8, for examination of morphological changes during regeneration.

4.2.3 Incubations and receptor autoradiography

In vitro incubations to determine IGF-I and -II binding capacity were carried out according to the methods given in Section 2.10. Notexin-injected muscle from all animals (n=4), and non-injected muscles from half (n=2) of each treatment group on days 3, 5 and 13 were examined. Days 1 and 2 were omitted as the tissues were at a

predominantly necrotic/phagocytic stage, in order to focus more specifically on the effects of GH and regeneration during the stages of MPC proliferation and differentiation. Pairs of sections were incubated with either ^{125}I-(rh)IGF-I or ^{125}I-(o)IGF-II for a determination of total binding, and the binding competed with excess unlabelled homologous IGF for a determination of non-specific binding. Des (1-3)IGF-I was utilised as a competing hormone for ^{125}I-IGF-I in order to determine whether, and at what times, binding proteins were a significant component of the specific ^{125}I-IGF-I binding observed. Other competing hormones for IGF-I were 1 μg/ml (rh)IGF-II and 10 ng/ml bovine insulin. For IGF-II, competing hormones included 1 μg/ml rh-N-met-IGF-I, 1 μg/ml des (1-3)IGF-I, 1 μg/ml des (1-6)IGF-II, and 10 ng/ml bovine insulin.

The binding of radiolabelled IGF-I and -II to notexin-injected muscle sections was uniform, except for the following two exceptions, as described here for ^{125}I-IGF-I, in which exceedingly high, isolated binding was noted. Firstly, a small subset of regenerating fibres (estimated to be less than 0.5% of all regenerating fibres) had high level binding which was effectively displaced by cold IGF-I and by des(1-3)IGF-I (Figure 4-1). This binding was not due to chemographic effects (interaction of tissue with emulsion, resulting in grain deposition) (Rogers & John 1969), as determined by the presence of visibly elevated signal in macroautoradiographs, corresponding to the location of these individual fibres. Because of the very low frequency and the distorting effect of the exceedingly high signal on the data obtained, these fibres were omitted from the quantitation of IGF-I binding. There was no association of these high signal fibres with GH-treatment, although there was some association with

regeneration time in that the fibres were occasionally present in day 3 and 5 muscle, but were never observed in day 13 muscle. A second case of aberrant signal was observed in cells with the appearance of polymorphonuclear leucocytes (also known as neutrophils), which have the morphological characteristics of a lobular, horseshoe-shaped nucleus and an abundant cytoplasm (Alberts *et al.* 1994), and are phagocytic. The presumed polymorphonuclear leucocytes were observed in regenerating fibres, and less frequently in connective tissue/interstitial spaces, and had high total binding of ^{125}I-IGF-I which was not displaced by unlabelled IGF-I or des(1-3)IGF-I (Figure 4-1), indicating that the binding was non-specific. These cells were thus also omitted from the quantitative analysis of ^{125}I-IGF-I to tissue sections.

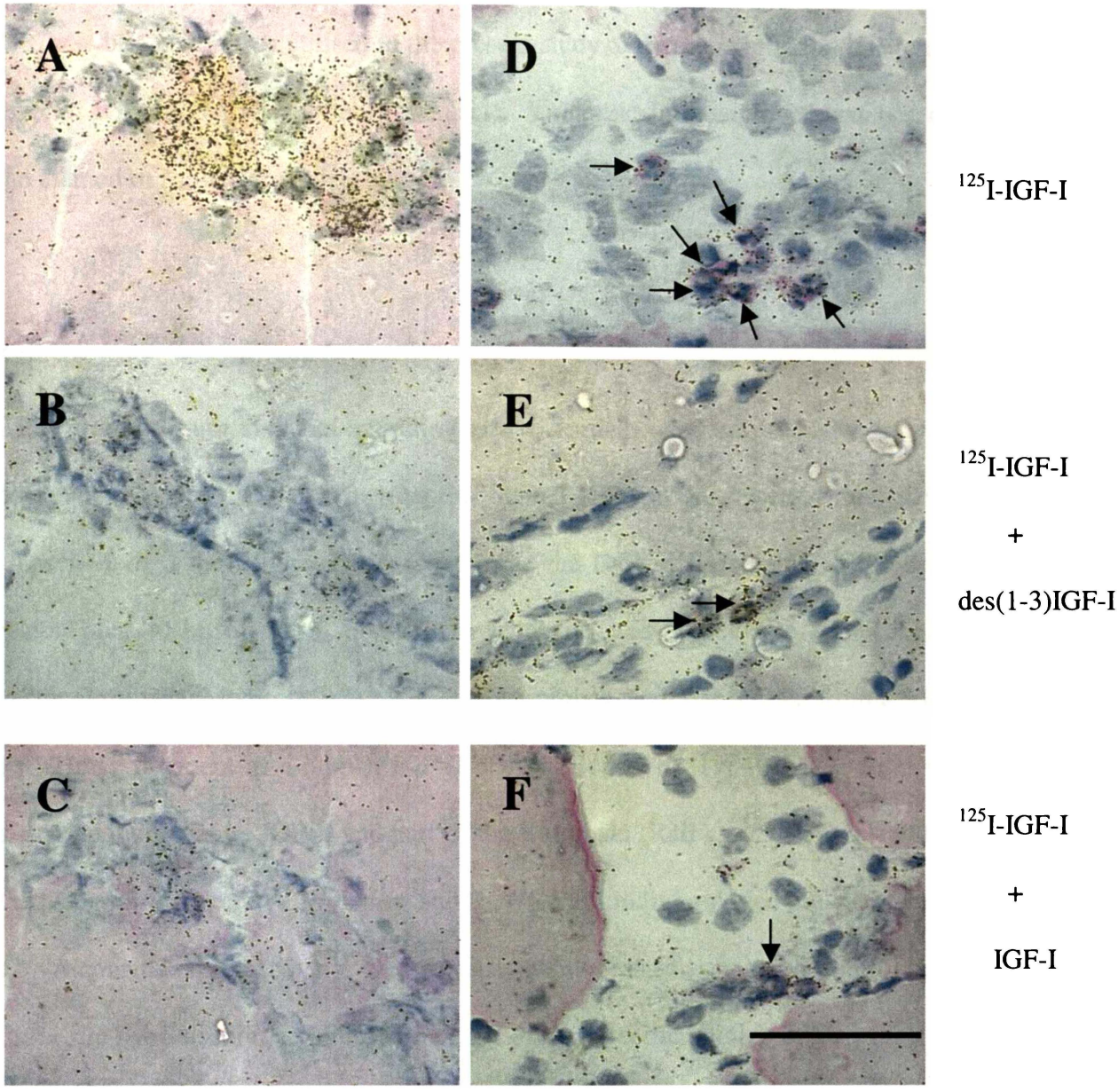


Figure 4-1. Unusual binding of ^{125}I-IGF-I within damaged muscle.

The binding of ^{125}I-IGF-I in notexin-injected muscle was unusually high in some regenerating muscle fibres (A-C), and presumed polymorphonuclear leucocytes (D-F). In the small subset of muscle fibres showing extraordinarily high total binding (A), the ^{125}I-IGF-I is effectively displaced by both competition with unlabelled des(1-3)IGF-I (B), and unlabelled IGF-I (C; NSB), and is therefore specific. In contrast however, high total binding (D) to presumed polymorphonuclear leucocytes (arrows) is not effectively displaced by competition with either des(1-3)IGF-I (E) or unlabelled IGF-I (F; NSB), and is thus non-specific. Magnification 100x, bar=50 microns.

4.2.4 In situ hybridisation

In situ hybridisation was utilised in the present study to determine the localisation and level of IGF mRNA in regenerating muscle. The procedure employed was based on the method of Molenaar *et al.* (1992).

4.2.4.1 IGF RNA probes

IGF-I probes for use in *in situ* hybridisation experiments were transcribed from a 203-base pair (bp) Sau3A/Sau3A fragment of human IGF-I cDNA inserted into pUC 13 (Jansen *et al.* 1983). The cDNA, containing the D- and E-domains of the coding region plus 40 bp of the 3' non-coding sequence, was cloned into the pGemini-3 vector (Promega, Madison, WI). IGF-II RNA probes were transcribed from a 534-bp Pst1/Hinf1 fragment of a human IGF-II cDNA (Bell *et al.* 1984) that codes for the signal peptide, mature IGF-II and part of the E-domain (Rall *et al.* 1987), cloned into the pGemini-3 vector. A. Molenaar and R. Wilkins kindly provided the IGF-I and –II cDNA constructs, respectively. Sense transcripts were used as negative control probes for *in situ* hybridisation, to indicate the level of non-specific binding of the probe.

Nuclease free MQ water		4.5 µl
Transcription buffer	5x concentrate	4.0 µl
DTT	100 mM	2.0 µl
rATP, rCTP, rGTP	10mM mix	3.0 µl
rUTP	500 µM	0.5 µl
RNasin	50 Units	1.0 µl
Purified DNA template	200-500 ng	2.0 µl
^{35}S-UTP	1000Ci/mmol	2.0 µl
RNA polymerase (T7 or SP6)	50 units	1.0 µl

Probe synthesis was performed according to the Promega Riboprobe protocol with minor modifications. All reagents as listed above, were supplied in a Promega transcription kit, except for uridine-5'-(α-^{35}S)thiotriphosphate (^{35}S-UTP; Amersham International, Amersham, UK) which was used to radioactively label the probes. All water used for probe synthesis and *in situ* hybridisation procedures was treated with 0.1% DEPC, then autoclaved, in order to avoid RNase contamination. Similarly, glassware was treated with DEPC-water, and then autoclaved. The probe synthesis reaction was performed at 37°C for 1 hour, with a further 0.5 ul of SP6 RNA polymerase added to applicable tubes halfway through the reaction. After this reaction, 1.5 µl of RNase-free DNase (Promega) was added to degrade the DNA template, followed by a 20 minute incubation at 37°C. At this time, the reaction tube was placed on ice, and the probe precipitated by the addition of: 10 µl 7.5 M ammonium acetate (BDH), 2 ul of 10mg/ml tRNA (from *Escherichia coli* strain HB101; Boehringer Mannheim, Auckland, NZ), and 2.2 volumes of 100% ethanol. The reaction tube was placed in a -20°C freezer for at least 30 minutes, then spun at 13,000 revolutions per minute in a benchtop centrifuge (Jouan S.A., St. Herblain, France) for 20 minutes. The supernatant was discarded, and the remaining pellet washed three times with 75% EtOH containing DEPC-water. All visible ethanol solution was removed, then the remainder left to evaporate off for up to 20 minutes. The probe was resuspended in 20 µl of 10mM 1,4-dithiothreitol (Boehringer Mannheim), with one 5-minute incubation in a 65°C water bath to aid in resuspension. Counts were taken from 1µl of probe placed in liquid scintillation fluor (Beckman, Alphatec Systems Ltd, NZ), and the cpm/µl of probe determined (normally greater than 1x 10^6 cpm/µl).

4.2.4.2 Tissue sections

Paraffin-embedded tissues from all animals in the 3, 5, 9 and 13 day groups were sectioned to a thickness of 7 μm, floated out on DEPC-treated water, and placed on slides coated with 3-aminopropyltriethoxysilane (Sigma). Two serial sections were placed on each slide, one for antisense probe, and the other for sense probe. Duplicate sections were assayed for each animal.

4.2.4.3 Solutions

Proteinase K solution	DEPC- water	80 mls
	1 M calcium chloride	200 µl
	1 M Tris, pH 7.2	20 ml
	20 mg/ml Proteinase K (add last)	10 µl
Acetylation solution pH 8.0	triethanolamine	1.32 mls
	DEPC- water	100 mls
	Acetic anhydride (add just before use)	1 ml
20 x SSC	Sodium chloride	175.32 g
	Sodium citrate	88.23 g
	Milli Q water	To 1 litre
	DEPC (only for pre-hyb steps)	0.2 ml
Premix hybridisation buffer	5 M sodium chloride	0.3 ml
	Deionised formamide	5.0 ml
	20x SSC	1.0 ml
	10 mg/ml yeast tRNA	0.2 ml
	10 mg/ml degraded herring DNA	1.0 ml
Hybridisation buffer	Premix hybridisation buffer	780 µl
	BSA (20 mg/ml)	20 µl
	50% dextran sulphate	200 µl
	DTT	Heaped spatula tip
Wash solution 1	2 X SSC	
	50% formamide	
	10mM β-mercaptoethanol (BME)	760 µl/litre
Wash solution 2	0.2 X SSC	
	10mM BME	760 µl/litre
Wash solution 3	70% ethanol	
	BME	760 µl/litre
RNase treatment *enzymes added after heating	RNase A (35 mg/ml)	14.3 ul
	RNase T1 (3.5 mg/ml)	3.4 ul
	2 x SSC	100 ml

4.2.4.4 Section pre-treatment

Slides were incubated at 60°C for 10 minutes in order to soften the paraffin, then deparaffinised by two successive 10 minute incubations in xylene. Sections were rehydrated by passage through a graded series of alcohols (100%, 100%, 95%, 70%) for 5 minutes each, then placed in DEPC-water for 3 minutes. The muscle sections were treated with 0.2N HCl for 10 minutes at room temperature to remove basic proteins, then washed in 2xSSC for 15 minutes. A 15 minute incubation in proteinase K solution at 37°C was performed to enhance section permeability to the probe, followed by acetylation of amino groups, via two 5 minute incubations at room temperature in acetylation solution (solution used immediately after the addition of acetic anhydride), to reduce non-specific binding of the probe to the tissue sections. Sections were incubated in 2 x SSC for 5 minutes, then placed in 100% ethanol baths for 5 minutes each, until the ethanol solution was clear, not cloudy. Sections were then air dried at room temperature.

4.2.4.5 Hybridisation

Sections were encircled with ADOS™ rubber cement (Para Rubber, Hamilton, NZ), to contain the radioactive probe during the hybridisation step. Probes were boiled for 3 minutes to reduce non-specific interactions, briefly spun, then diluted to 30,000 cpm/µl in hybridisation buffer. The hybridisation mixture was spread evenly across the tissue sections using the long edge of the pipette tip, and any remaining bubbles in the solution removed by quickly passing a gas flame over the surface. Slides were

placed on racks in air-tight chambers equilibrated with 50% formamide, 2 x SSC and hybridised overnight at 55°C.

4.2.4.6 Post-hybridisation washes

Slides were removed from the chambers in a ventilated hood, and placed in wash solution 1, for 15 minutes at 55°C, then rinsed in Wash Solution 2 for 15 minutes at room temperature. Sections were then incubated in RNase solution for 45 minutes at 37°C to destroy non-specifically bound probe, and any residual rubber cement removed from the slides at this point. Sections were rinsed in Wash Solution 2 three times for 5 minutes each, then transferred to Wash Solution 3 for 5 minutes, followed by 95% ethanol for 5 minutes. The slides were then air-dried.

4.2.4.7 Autoradiography and Emulsion

Macroautoradiographs were generated by apposing the slides against XAR film (Eastman Kodak) for 3 days. Slides were then coated with NTB-2 (Eastman Kodak) photographic emulsion, exposed for 10.5 weeks, developed and counterstained with haematoxylin and eosin as per Section 2.8.

4.2.5 Grain counting

Radiographic grains in hybridised and incubated sections were quantitated in the following fibre/tissue types within the notexin-injected muscle: a) regenerating fibres, b) survivor fibres, c) undamaged fibres, and d) connective tissue (excluded from IGF-

II mRNA grain counting due to low levels in connective tissue). The latter three tissue types, which were defined earlier in Section 3.1.3, were chosen in addition to regenerating fibres for the following reasons: a) it was considered that undamaged fibres from notexin-injected muscle may have different growth factor activities from survivor fibres as the latter are intermixed with the regenerating fibres; and b) previous studies have shown high levels of IGF-II receptor in skeletal muscle connective tissue (Oldham *et al.* 1993). Day 1 and 2 tissues were excluded from binding and mRNA grain counting as the damaged fibres were predominantly at a necrotic, not regenerative, stage. Also, survivor and regenerating fibres could not be definitively identified in incubated day 13 sections, so they were not counted. IGF binding and mRNA were quantitated in undamaged fibres and connective tissue of non-injected muscle (n=2 per treatment group per day for incubated sections; n=4 per treatment group per day for *in situ* hybridised sections).

For hybridised sections, grain densities were determined using the Visilog system (see Section 2.11.1). Grain densities were determined for six sites per slide each, and in three sites per slide for undamaged fibres as the latter group had less variable levels than did connective tissue, survivor and regenerating fibres. Specific IGF-I and -II mRNA was determined by subtracting the values from areas in sense-probed sections from the values in matched areas of antisense-probed sections.

4.2.6 Statistical analysis

Values are presented as means ± S.E.M. ^{125}I-IGF-I and -II binding was analysed to determine the effects of treatment and the relative tissue levels by the method of restricted (residual) maximum likelihood (REML). Tukey ("Honest") Significant Difference was used for significance comparisons between individual time x tissue values for ^{125}I-IGF binding. Statistical analyses of IGF-I and -II mRNA levels to determine the effects of treatment, time, and fibre or tissue type were carried out on log-transformed values using ANOVA. Muscle and body weights, following adjustment for initial liveweight, were analysed using ANOVA. Comparisons of the IGF mRNA levels of different tissues at individual timepoints were performed using a paired t-test, to determine whether the difference in values was significantly different from zero.

For the analysis of the IGFBP component of ^{125}I-IGF-I binding, des(1-3)IGF-I competed binding was compared with unlabelled IGF-I competed binding by using Student's t-test to determine if the difference of the two values was significantly different from zero. If the difference between the two resultant binding levels was significantly different to zero, the presence of IGFBPs was indicated.

4.3 Results

4.3.1 Body and muscle weights

Body weights changed significantly ($p<0.001$) after notexin injection (Figure 4-2), with a decrease in weights up to day 5 followed by a steady weight gain after this time. The initial decrease in body weights suggested a systemic effect of notexin in addition to the local effect. To test this, additional rats (n=5/6 per group) of the same line and mean weight on day 0 were administered equal amounts of GH or saline vehicle to the amounts used in this trial. Body weights were recorded and are also shown in Figure 4-2, and indicate a steady weight gain from day 0 in the absence of notexin, thereby confirming the existence of a systemic effect of notexin in addition to its local effect. Growth hormone treatment was not sufficient to overcome the systemic effect of notexin on body weights as there was no significant difference in weights with and without GH up to day 5 in the present trial. Growth hormone administration to notexin-injected rats eventually resulted in 14% higher body weight ($p<0.001$ overall), with increases in the GH-treated group relative to the saline group on days 9 ($p<0.05$) and 13 ($p<0.001$).

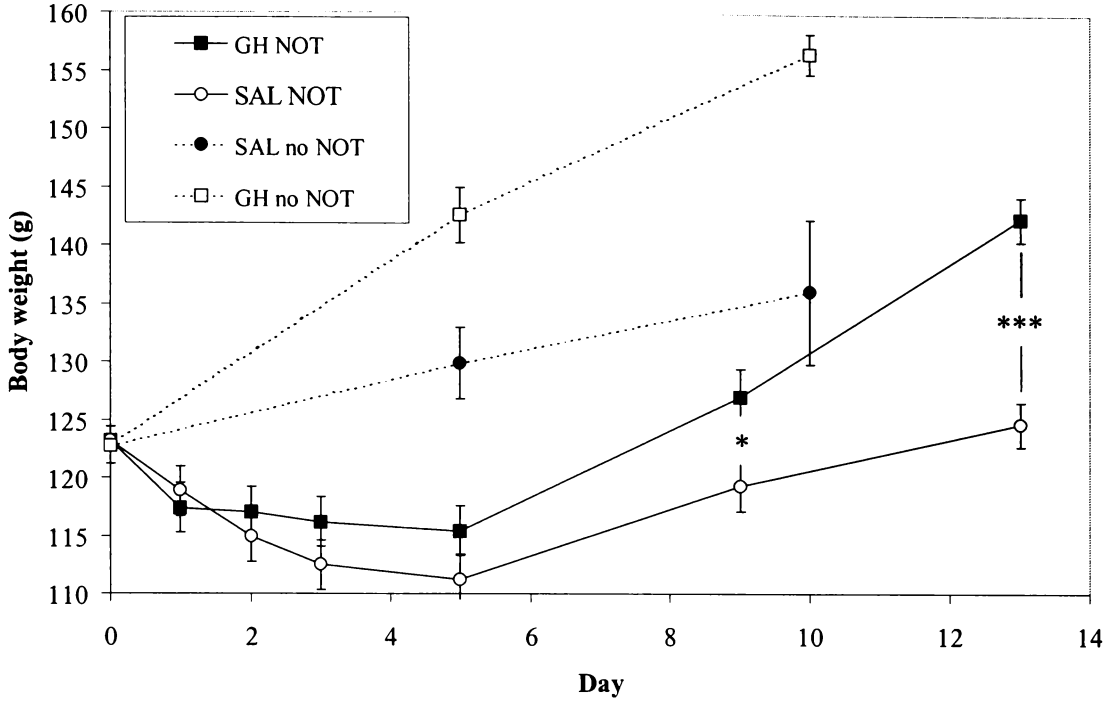


Figure 4-2. Effect of GH on body weight of *dw/dw* rats.

Effects of daily GH (200 µg/100g body weight) and notexin (NOT; one i.m. injection, day 0) on body weights of *dw/dw* rats. Values represent the mean weights ± S.E.M., *n*= 4/5 per group. Significance is shown for the saline-treated (SAL NOT) versus GH-treated (GH NOT) notexin-injected rats (*p<0.05, ***p<0.001; ANOVA).

Weights of notexin-injected and non-injected M. *biceps femoris* changed significantly over days 1-13 ($p<0.001$; Figure 4-3). Similar to changes in bodyweight, non-injected muscle weights declined from days 1-5, while notexin-injected muscle weights declined from days 1-9, then increased. Weights of notexin-injected and non-injected M. *biceps femoris* were increased in GH-treated animals by 15% and 18%, respectively, relative to saline group animals ($p=0.007$ and $p=0.002$, respectively). Specific increases in muscle weight due to GH were seen on day 1 ($p<0.05$, notexin-injected and non-injected), day 9 ($p<0.05$, non-injected) and day 13 ($p<0.05$, notexin-injected; $p<0.001$, non-injected).

Day	1	2	3	5	9	13
Non-injected	*	NS	NS	NS	*	***
Injected	*	NS	NS	NS	NS	*

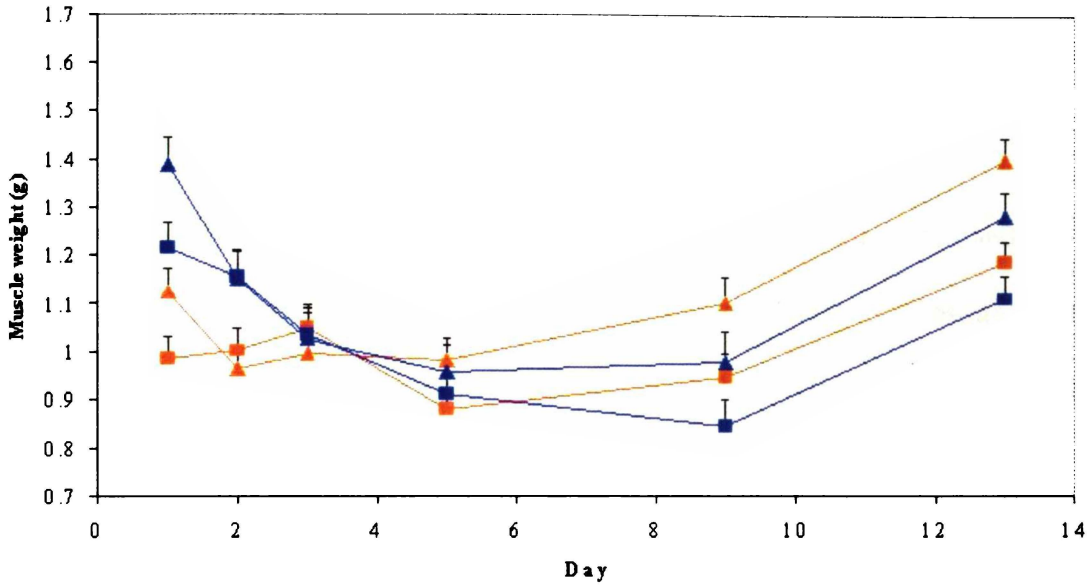


Figure 4-3. Effect of GH on muscle weight of *dw/dw* rats.

Effect of daily GH (200 µg/100g body weight) on the weights of notexin-injected (blue) and non-injected (orange) M. *biceps femoris*. Values are the means ± S.E.M., *n*=4/5 per group. Asterisks indicate the significance of the saline (■) versus GH-treated (▲) muscle weight comparison for either notexin-injected or non-injected muscle (* p<0.005, ***p<0.001; ANOVA).

4.3.2 Morphological changes during muscle regeneration

Histological examination of haematoxylin and eosin stained sections showed that the occurrence of regeneration events in this trial was similar to that reported for *dw/dw* rats in Section 3.1.3. In brief, mononucleate cells presumed to be MPC were most abundant in day 3 tissues, while considerable MPC fusion was observed on day 5.

Additional myotube formation, and myotube enlargement occurred after day 5. GH-treatment did not affect the quality or timing of muscle regeneration as determined by histological assessment of haematoxylin and eosin stained muscle sections.

4.3.3 IGF-I specific binding

The relative levels of IGF-I binding to histological zones of notexin-injected and non-injected muscle from saline-treated animals are shown in Table 4-1. Results show that there were highly significant differences in binding between the different tissue types ($p<0.001$), and that the pattern of binding to the different tissue types examined changed significantly over time ($p=0.018$). IGF-I binding to undamaged muscle fibres of the non-injected muscle was low at all timepoints, as was binding to the undamaged muscle fibres in the notexin-injected muscle on days 3 and 5. In contrast, regenerating fibres had high level binding on day 3, which rose further to peak at day 5. ^{125}I-IGF-I binding to day 5 regenerating muscle fibres was significantly greater than the binding to all other tissue types on day 5 ($p<0.001$). ^{125}I-IGF-I binding to survivor fibres was not significantly different from the binding to undamaged muscle fibres of notexin-injected and non-injected muscles on days 3 and 5. The binding of ^{125}I-IGF-I to connective tissue was similar to the binding to regenerating fibres on day 3, then declined to day 5 values. The pattern and level of ^{125}I-IGF-I binding to connective tissue of notexin-injected muscle was similar to the ^{125}I-IGF-I binding to connective tissue of non-injected muscle. ^{125}I-IGF-I binding at day 13 following notexin-injection was high in undamaged muscle fibres and connective tissue of notexin-injected muscle, and in connective tissue from the non-injected muscle. The binding

of ^{125}I-IGF-I to connective tissue of non-injected muscle was significantly greater than the binding of ^{125}I-IGF-I to muscle fibres of non-injected muscle ($p<0.05$) on day 13. GH did not have a significant effect on ^{125}I-IGF-I binding, as shown in Figure 4-4.

Table 4-1. ^{125}I-IGF-I binding to muscle from saline-treated rats.

The values shown for ^{125}I-IGF-I specific binding in notexin-injected and non-injected muscles from saline-treated rats were determined by receptor autoradiography. Grain densities (grains/μm^2) were determined by subtracting non-specific from total binding values for each section. Means as determined by the REML method are shown for survivor fibres (S), regenerating fibres (R), undamaged fibres (U), and connective tissue (C). Means were determined from three animals unless otherwise indicated (§ n=2 and ¶ n=4). Regenerating and survivor fibres were not distinguishable in day 13 sections, and so were not quantitated (N/A; not available). Significance is shown for comparisons within time-points (different letters indicate significance at the $p<0.05$ level; all other comparisons were not significant).

	NOTEXIN-INJECTED				NON-INJECTED		
	S	R	U	C	U	C	Ave S.E.M.
Day							
3	0.0070^{a}	0.0262^{a}	0.0036^{a}	0.0230^{a}	0.0102$^{\S a}$	0.0264$^{\S a}$	0.0065
5	0.0126^{b}	0.0430^{a}	0.0052$^{\P b}$	0.0129$^{\P b}$	0.0051$^{\S b}$	0.0022$^{\S b}$	0.0042
13	N/A	N/A	0.0267^{ab}	0.0277^{ab}	0.0078$^{\S b}$	0.0370$^{\S a}$	0.0091

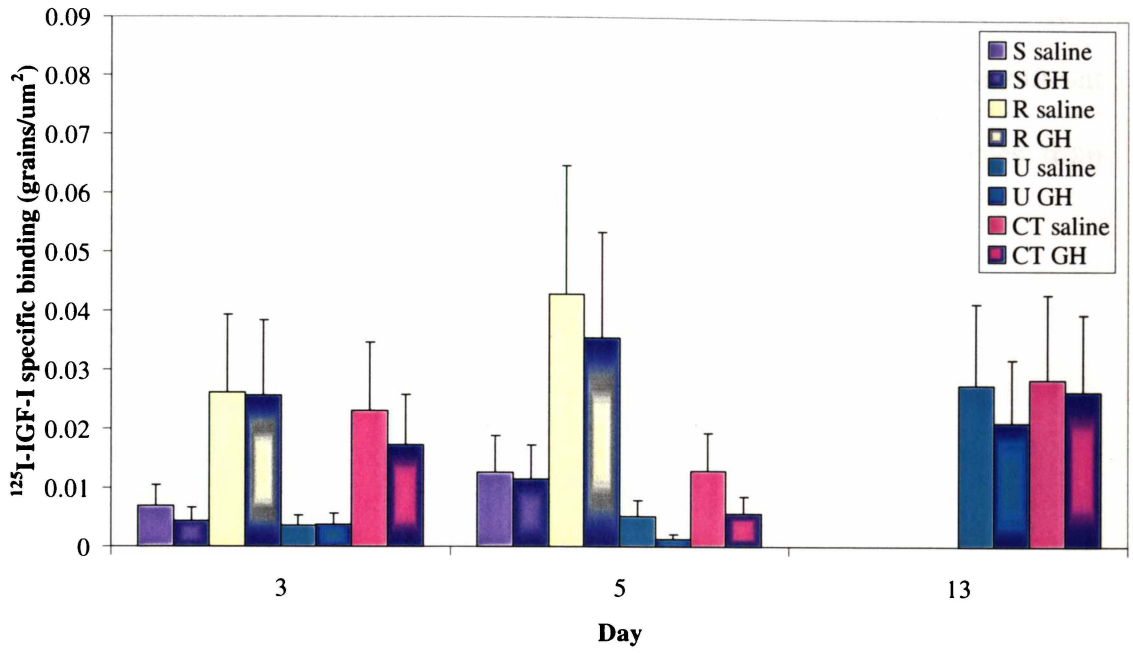


Figure 4-4. Effect of GH on ^{125}I-IGF-I specific binding.

^{125}I-IGF-I specific binding values were determined by receptor autoradiography for tissues within notexin-injected muscle. Grain densities (grains/μm^2) were determined by subtracting non-specific from total binding values for each section. Means ± S.E.M. as determined by the REML method are shown for survivor fibres (S), regenerating fibres (R), undamaged fibres (U), and connective tissue (C). There was no significant effect of GH treatment on ^{125}I-IGF-I binding. Three animals were analysed per group except for the following groups, in which 4 animals were analysed: day 3 CT (GH), day 5 CT (GH and saline), and day 5 U (saline).

4.3.4 IGF-II specific binding

Table 4-2 shows the relative distribution of ^{125}I-IGF-II binding in the histological zones of notexin-injected and non-injected muscles from saline-treated animals. ^{125}I-IGF-II binding in regenerating fibres changed significantly with time ($p<0.05$), with peak levels on day 5. No other tissue types, from either notexin-injected or non-injected muscles, showed change over time. The ^{125}I-IGF-II binding in regenerating fibres was significantly higher than in connective tissue, undamaged fibres or survivor

fibres of notexin-injected muscle ($p<0.001$), and was higher than in undamaged muscle fibre and connective tissue ($p<0.01$) on day 5. There was no significant difference in ^{125}I-IGF-II binding at any timepoint between the other tissue types, from either notexin-injected or non-injected muscles.

Table 4-2. ^{125}I-IGF-II binding to muscle from saline-treated rats.

The values shown for ^{125}I-IGF-II specific binding in notexin-injected and non-injected M. *biceps femoris* from saline-treated rats were determined by receptor autoradiography. Grain densities (grains/μm^2) were determined by subtracting non-specific from total binding values for each section. Means as determined by the REML method are shown for survivor fibres (S), regenerating fibres (R), undamaged fibres (U), and connective tissue (C). Means were determined from four animals unless otherwise indicated (‡ n=2 and ¶ n=3). Regenerating and survivor fibre values were not determined for day 13 (N/A). Significance is shown for comparisons within time-points (different letters indicate significance at the $p<0.001$ level; all other comparisons were not significant).

	NOTEXIN-INJECTED				NON-INJECTED		
	S	R	U	C	U	C	Ave S.E.M.
Day							
3	0.0342¶	0.1242¶	0.0731	0.0381	0.0250§	0.0363§	0.0440
5	0.0650^{b}	0.2256^{a}	0.0350^{b}	0.0375^{b}	0.0388$^{\S b}$	0.0363$^{\S b}$	0.0267
13	N/A	N/A	0.0206	0.0275	0.0125§	0.0300§	0.0069

The effect of GH on IGF-II binding in notexin-injected muscle is shown in Figure 4-5. A significant effect of GH on IGF-II binding was observed on day 5 ($p<0.01$ overall), whereby levels in all notexin-injected muscle tissues were elevated with GH ($p<0.001$, regenerating fibres; $p<0.05$, connective tissue, survivor and undamaged fibres). Notexin-injected muscle tissues responded to GH, whereas non-injected muscle tissues did not ($p<0.05$ for the GH x tissue interaction). No difference in IGF-II binding due to GH was noted on either day 3 or 13.

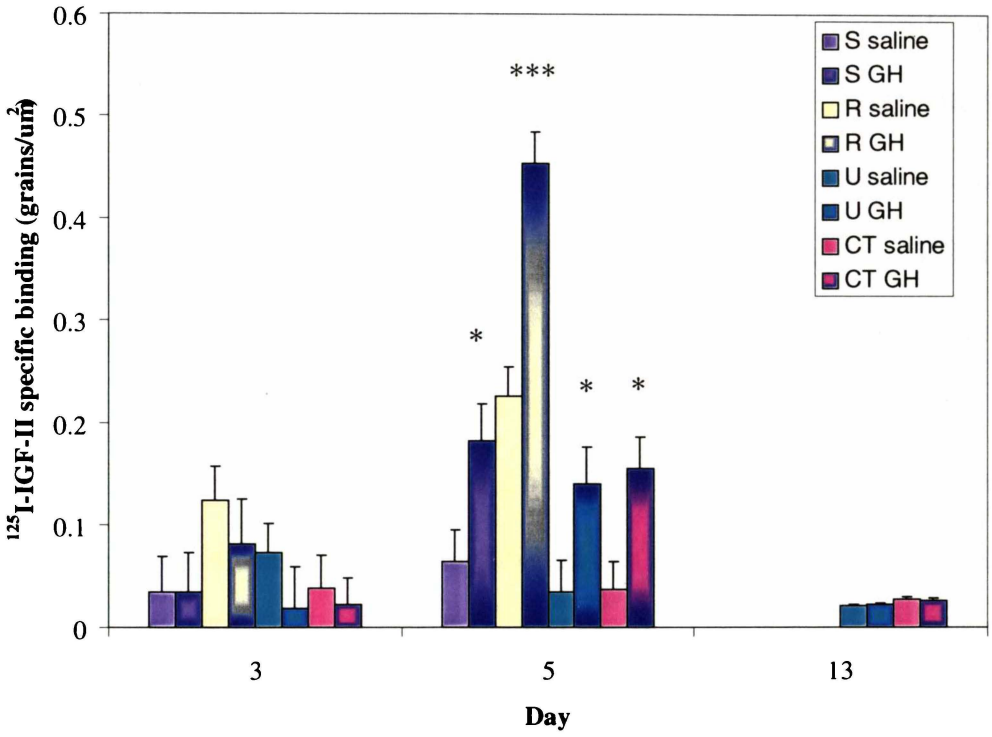


Figure 4-5. Effect of GH on ^{125}I-IGF-II specific binding.

^{125}I-IGF-II specific binding values were determined by receptor autoradiography for tissues within notexin-injected muscle. Grain densities (grains/μm^2) were determined by subtracting non-specific from total binding values for each section. Means ± S.E.M. as determined by the REML method are shown for survivor fibres (S), regenerating fibres (R), undamaged fibres (U), and connective tissue (C). The number of animals analysed per tissue type is 3-4, except for all day 3 GH-treated values, where $n=2$. Significance is shown for the GH versus saline comparison (*, $p<0.005$, ***, $p<0.001$).

4.3.5 IGFBPs

A qualitative assessment of total binding, and competition by unlabelled-IGF-I and des(1-3)IGF-I, indicated that binding proteins may be present within certain tissues at distinct times during the regeneration process in notexin-injected muscle. Since the presence of significant levels of IGFBPs could affect the assessment of Type I IGF receptor levels in these regenerating tissues, an initial analysis was carried out to determine at what time, and in which tissues, significant levels of IGFBPs were present. One animal per treatment per timepoint was analysed, the results of which showed that there was no significant difference between the des(1-3)IGF-I and unlabelled IGF-I competed binding of ^{125}I-IGF-I in the different tissue types of day 3 and 13 notexin-injected muscles. This indicated that the binding of ^{125}I-IGF-I was to receptor and not IGFBPs in these tissues on days 3 and 13. A similar analysis of day 5 tissues from the two treatment groups indicated, however, that IGFBPs were a significant component of the total binding of ^{125}I-IGF-I at this timepoint. Quantitation of ^{125}I-IGF-I binding competed with unlabelled des(1-3)IGF-I was thus carried out for a larger number of the day 5 animals, in all of the histological tissue types of notexin-injected muscle that showed a significant level of specific binding, namely connective tissue, regenerating and survivor muscle fibres, in order to separate out the effect of IGFBP from receptor. Figure 4-6 shows the results of this analysis, whereby specific binding of ^{125}I-IGF-I (which indicates binding to both BP and receptor) is compared with binding following competition with des(1-3)IGF-I (which indicates binding to receptor). Each day 5 tissue examined, namely connective tissue, regenerating and survivor muscle fibres, has a significant component of binding protein present

($p<0.001$). There was no effect of GH on IGFBPs, either overall or on individual tissues from day 5.

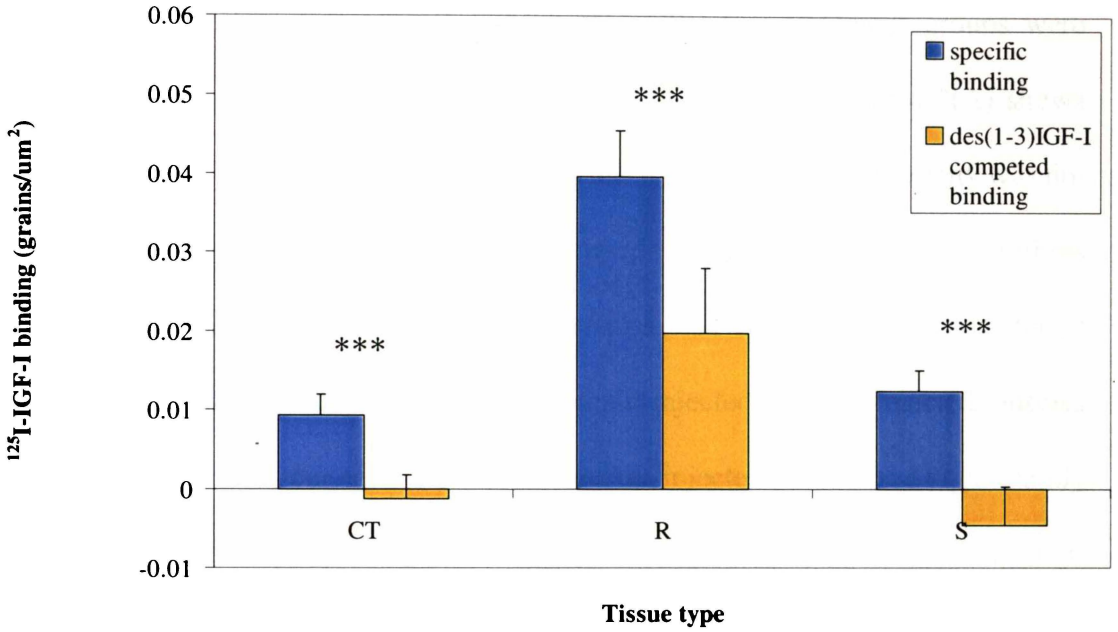


Figure 4-6. IGFBPs in damaged muscle tissues.

Specific binding versus ^{125}I-IGF-I binding competed with des(1-3)IGF-I in day 5 notexin-injected muscle from saline- and GH-treated rats. ^{125}I-IGF-I binding density was determined from tissue sections following IGF-I receptor autoradiography, as detailed in Section 2.11. Treatment groups were combined as there was no significant effect of GH on ^{125}I-IGF-I binding competed with either unlabelled IGF-I or des(1-3)IGF-I. Values shown represent the means ± S.E.M, n=6 for regenerating (R) and survivor (S) fibres, n=8 for connective tissue (CT). Significance shown is for comparisons within a tissue (*, $p<0.001$).**

4.3.6 IGF-I mRNA

IGF-I mRNA levels in tissue types of notexin-injected and non-injected muscles were unaffected by the administration of GH, therefore the two treatment groups were pooled together for a combined analysis of IGF-I expression. Figure 4-7(A) shows that the level of IGF-I mRNA as determined by autoradiographic grain density was not significantly different, overall or at individual timepoints, in undamaged muscle fibres from notexin-injected versus non-injected muscles. In contrast, connective tissue IGF-I mRNA levels were different between notexin-injected and non-injected muscles (Figure 4-7B), whereby connective tissue in notexin-injected muscle had significantly greater IGF-I mRNA levels on day 3 relative to that of connective tissue from non-injected muscle ($p=0.013$). Connective tissue IGF-I mRNA levels were not significantly different at subsequent timepoints (days 5, 9 or 13).

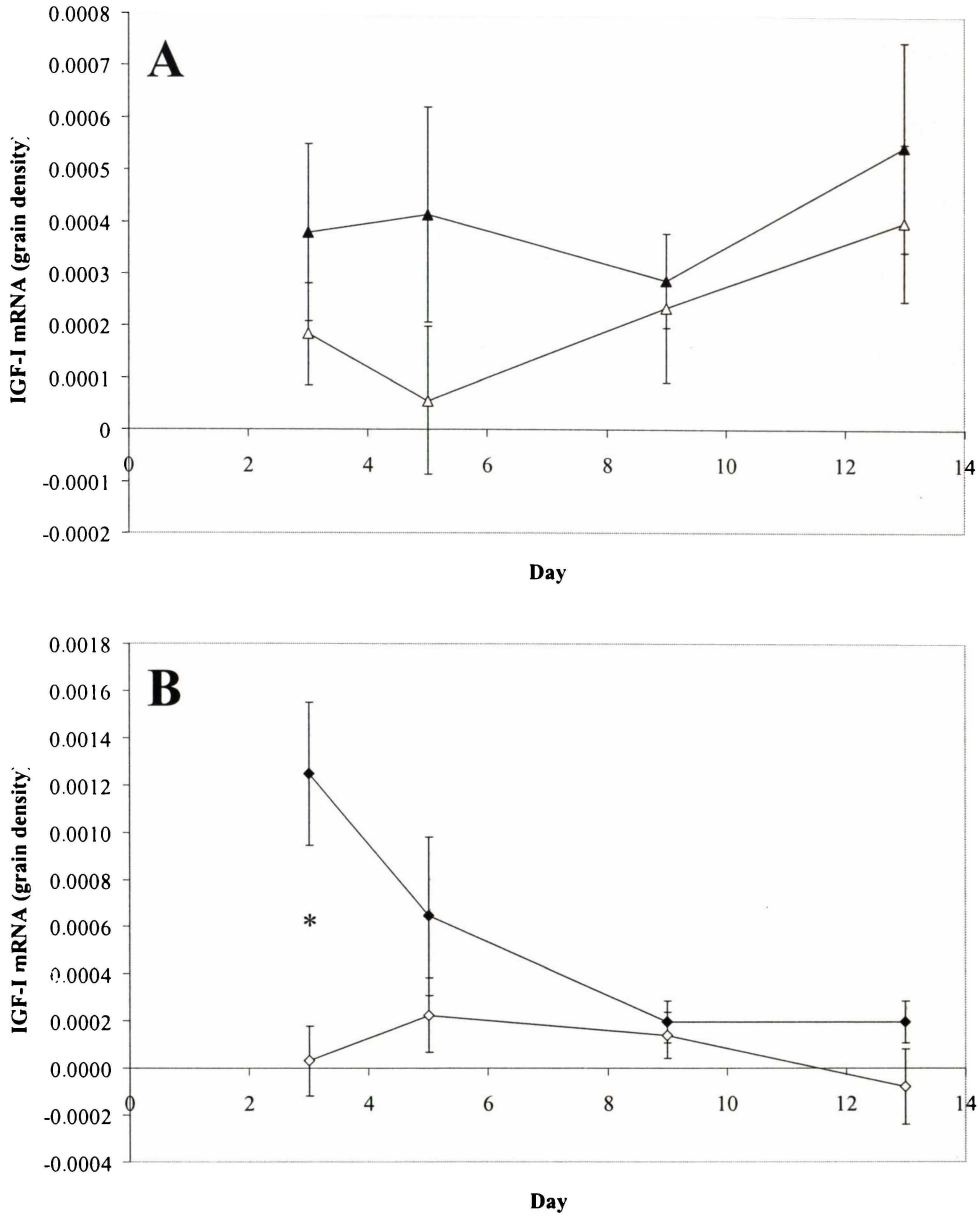


Figure 4-7. IGF-I mRNA levels in damaged and undamaged muscles.

IGF-I mRNA in undamaged muscle fibres (A) and connective tissue (B) of non-injected (open symbols) and notexin-injected (solid symbols) muscles following notexin injection at time 0. IGF-I mRNA levels are expressed as grain densities following quantitation of *in situ* hybridised muscle sections as described in Section 4.2.4. Values shown are the means ± S.E.M. of the pooled treatment groups (ANOVA). Signficance is shown for notexin-injected versus non-injected muscle connective tissue on day 3 (*, p=0.013), however all other timepoints for both connective tissue and undamaged muscle fibres are not significant (NS).

In notexin-injected muscles, a comparison of IGF-I mRNA expression in connective tissue, regenerating, survivor and undamaged muscle fibres identified a distinct pattern of temporal regulation ($p<0.001$ overall; Figure 4-8). Regenerating muscle fibres had greatly increased levels of IGF-I mRNA as early as day 3 ($p<0.001$ relative to undamaged muscle fibres). IGF-I mRNA levels in regenerating fibres peaked on day 9 (Figure 4-8 and Figure 4-9), after substantial myotube formation had occurred, with peak levels that were 16-times that of undamaged muscle fibres. IGF-I mRNA levels decreased within regenerating fibres after day 9 to day 13 levels, but remained greater than undamaged muscle fibres levels ($p<0.001$). The increase in IGF-I mRNA observed in regenerating muscle fibres did not appear to be related to changes in nuclear density, as the peak in nuclear density in regenerating areas occurred on day 3 (refer to Section 3.1.3), while peak levels of IGF-I mRNA occurred on day 9, and were still substantially elevated on day 13 (Figure 4-8), when the nuclear density was greatly decreased relative to day 3.

Survivor fibres also showed an early increase in the expression of IGF-I mRNA, with a significantly higher mRNA level relative to undamaged fibres on day 3 only ($p<0.01$). Survivor fibres from day 5 onward, and connective tissue throughout the regeneration period, did not contain significantly different levels of IGF-I mRNA when compared to undamaged muscle fibres.

DAY	3	5	9	13
R vs U	***	***	***	***
S vs U	**	NS	NS	NS
CT vs U	NS	NS	NS	NS

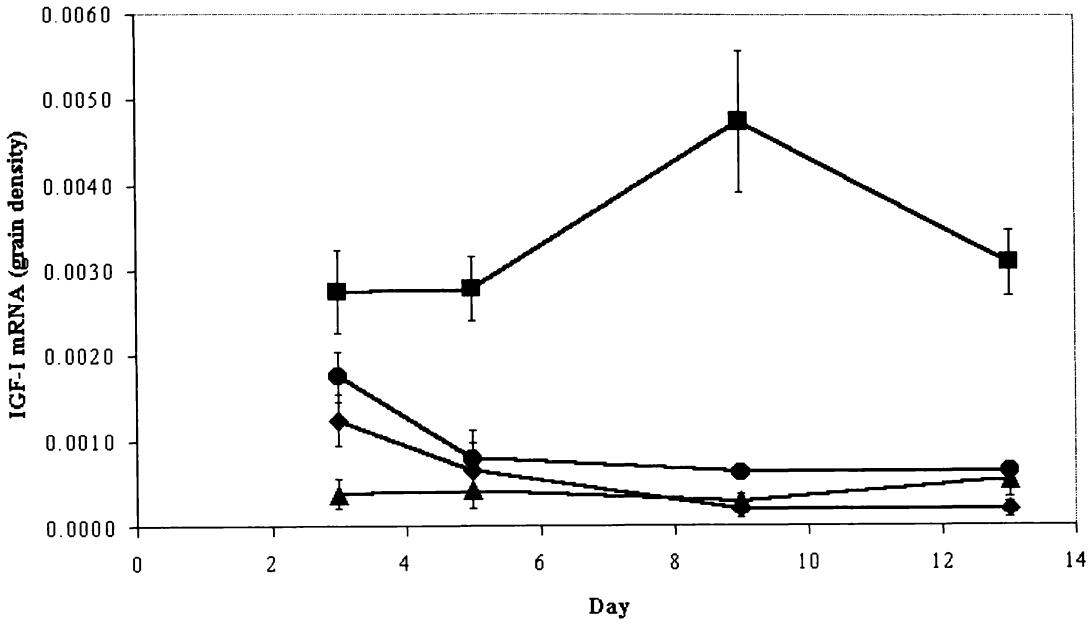


Figure 4-8. IGF-I mRNA levels in damaged muscle tissues.

Grain densities, as determined by grain counting following *in situ* hybridisation, are shown for connective tissue (◆), regenerating (■), survivor (●), and undamaged (▲) muscle fibres. Significance levels for the comparison of regenerating fibres (R), connective tissue (CT), and survivor fibres (S) with undamaged (U) muscle fibres are shown above (, $p<0.01$; ***, $p<0.001$; NS, not significant).**

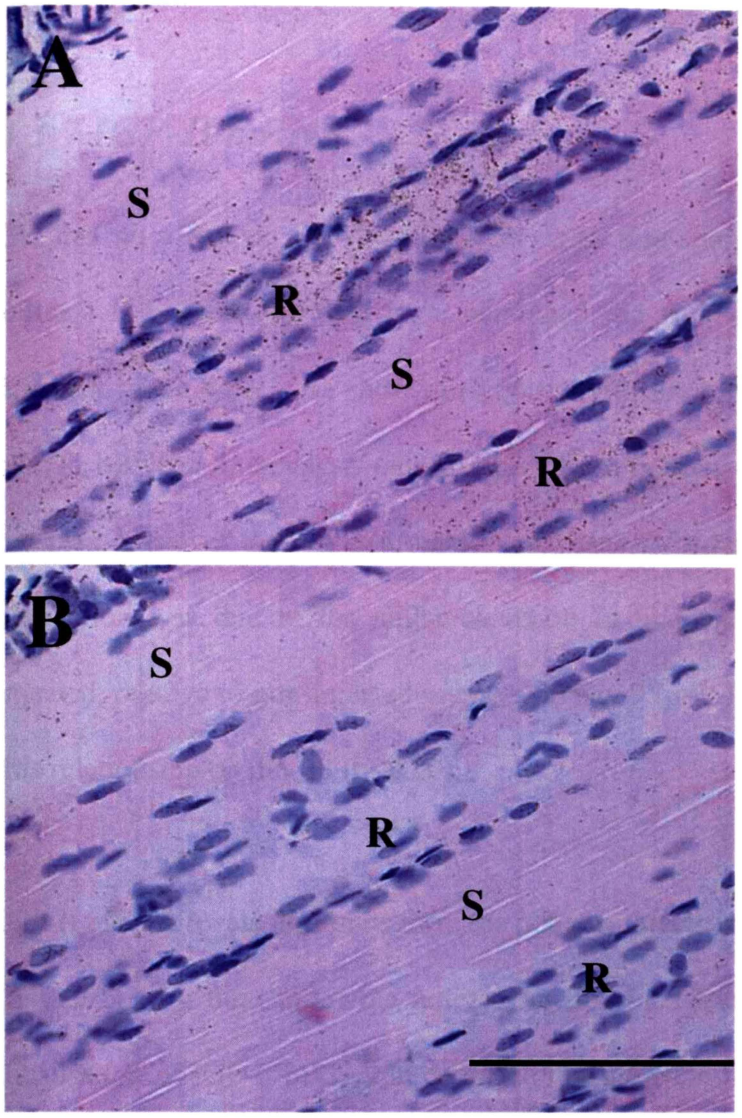


Figure 4-9. Localisation of IGF-I mRNA in regenerating muscle fibres.

Photomicrographs of radiographic grains after *in situ* hybridisation using antisense (A) and sense (B) ^{35}S-IGF-I RNA probes. (A) Regenerating muscle fibres of notexin-injected muscle 9 days after insult show elevated IGF-I mRNA in regenerating fibres (R) relative to survivor fibres (S). (B) A matched sense-probed section contains very few radiographic grains. Sections have been counterstained with haematoxylin and eosin, magnification is 50x, bar =100 μm.

4.3.7 IGF-II mRNA

GH had no effect on IGF-II mRNA levels in this study, so GH and saline treated animals IGF-II mRNA data were combined for the analysis of IGF-II mRNA in different tissue types. As shown in Figure 4-10, there was no significant difference overall between undamaged muscle fibres from notexin-injected versus non-injected muscles, however there was a significant tissue by time interaction ($p<0.05$). This interaction was due to the slight elevation in undamaged fibre IGF-II mRNA in injected, relative to non-injected muscles on day 9 ($p<0.10$), followed by a reversal of this relationship on day 13 ($p<0.10$).

IGF-II mRNA levels in tissues from the notexin-injected muscle changed significantly ($p<0.001$) with time following notexin injection (Figure 4-11). Distinct patterns of localisation of IGF-II mRNA in damaged muscle were immediately apparent (Figure 4-11), with the highest mRNA levels in regenerating fibres (Figure 4-11 and Figure 4-12). Regenerating muscle fibre IGF-II mRNA levels were not significantly different to that of survivor and undamaged muscle fibres on day 3, but were significantly elevated on day 5, 9, and 13, as indicated in Figure 4-11. IGF-II mRNA in undamaged muscle fibres was not significantly different from survivor fibre IGF-II mRNA except for on day 13, when survivor fibre levels were elevated relative to undamaged muscle fibre levels ($p<0.05$).

As was the case for IGF-I mRNA (refer to Section 4.3.6), increases in IGF-II mRNA were discordant with increases in nuclear density during regeneration, as peak levels of IGF-II mRNA occurred on day 9, and not on day 3, when the nuclear density is greatest (refer to Section 3.1.3).

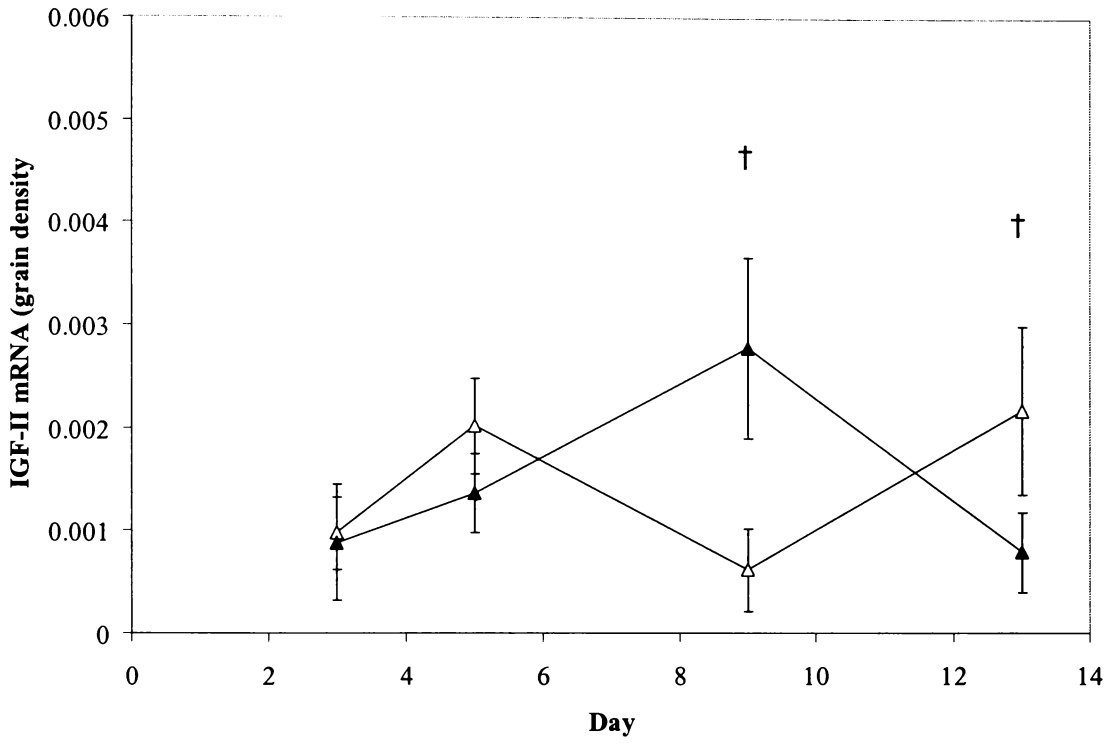


Figure 4-10. IGF-II mRNA levels in undamaged muscle fibres.

IGF-II mRNA levels were determined for non-injected (open symbols; *n*=8) and notexin-injected (solid symbols; *n*=7/8) muscles following notexin injection at time 0. IGF-II mRNA levels are expressed as grain densities following quantitation of *in situ* hybridised muscle sections as described in Section 4.2.4. Values shown are the means ± S.E.M. of the pooled treatment groups (ANOVA). Significance at the p<0.10 level is indicated by (†).

DAY	3	5	9	13
R vs S	NS	*	***	**
S vs U	NS	NS	NS	*
R vs U	NS	*	**	**

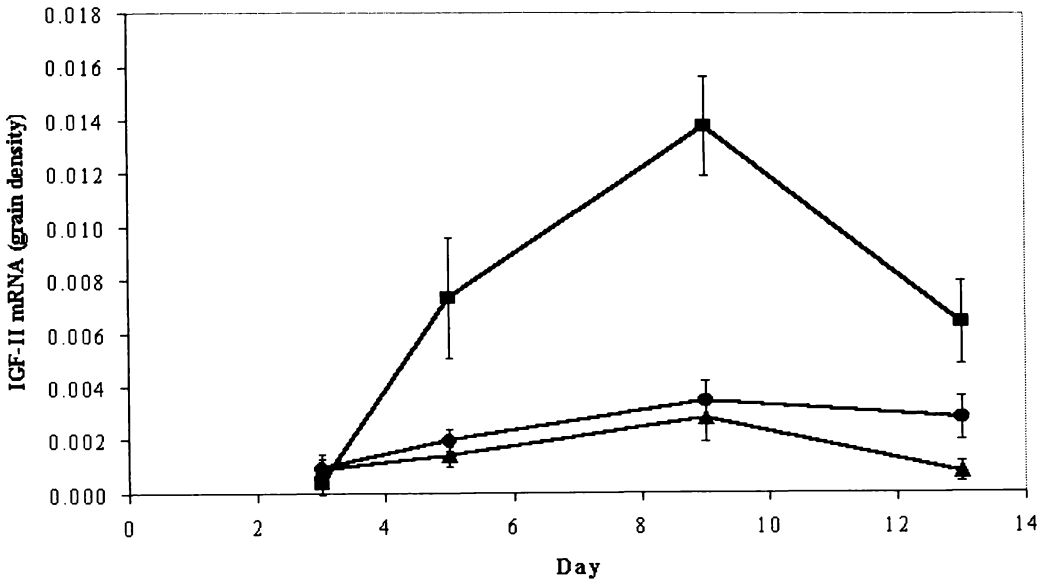


Figure 4-11. IGF-II mRNA levels in damaged muscle tissues.

IGF-II mRNA expression in notexin-injected muscle sections, as determined by *in situ* hybridisation. Grain density was determined as the difference between antisense- and sense-probe densities for each fibre type in each section. Undamaged fibres (▲, *n*=7/8); survivor fibres (●, *n*=7/8); regenerating fibres (■, *n*=7/8). Values shown are the means ± S.E.M. of the pooled treatment groups (ANOVA). Significance is shown for the between tissue type comparisons (*p<0.05, **p<0.01, ***p<0.001).

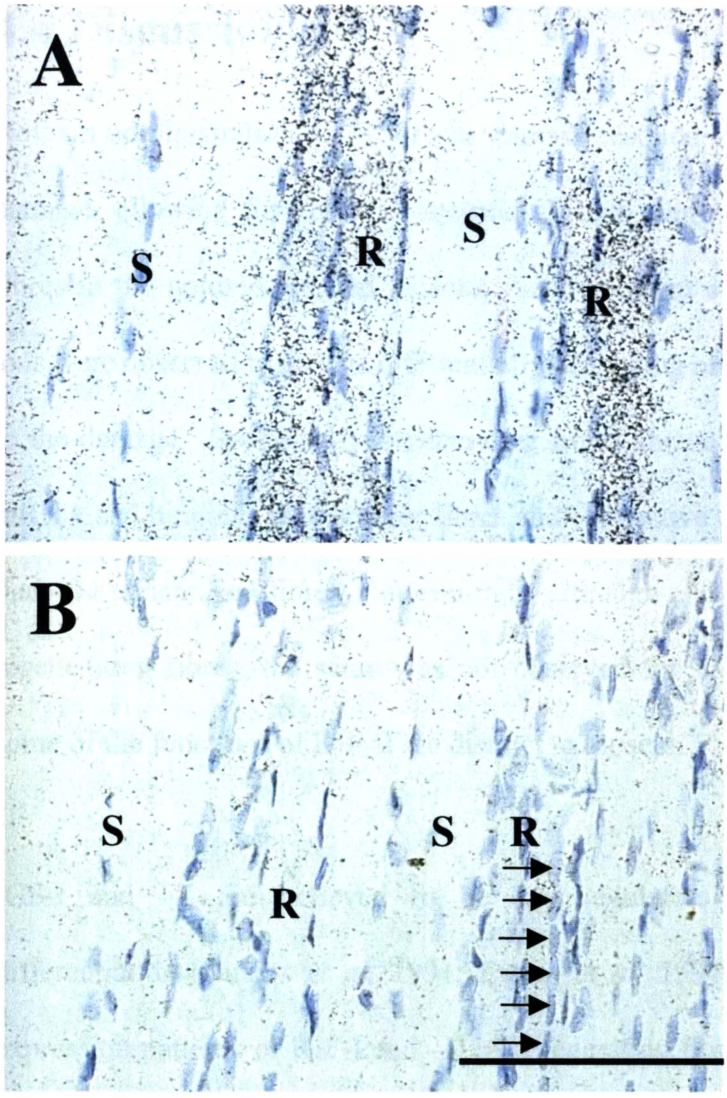


Figure 4-12. Localisation of IGF-II mRNA in regenerating muscle fibres.

Photomicrograph of radiographic grains after *in situ* hybridisation using ^{35}S-labelled antisense (A) and sense (B) IGF-II RNA probes. (A) Regenerating muscle fibres of notexin-injected muscles 9 days after injection show elevated IGF-II mRNA in regenerating fibres (R) relative to survivor fibres (S). (B) A matched sense-probed section contains very few radiographic grains. Arrows show a series of MPC that are lined up end-to-end, indicative of myotube formation. Sections have been counterstained with haematoxylin and eosin, magnification is 50x, bar =100 μm.

4.4 Discussion

Notexin administration to M. *biceps femoris* resulted in a heterogeneous pattern of damage, allowing for separate analysis of both non-regenerating and regenerating fibres in the notexin-injected muscle. In the present study, components of the IGF axis were observed to change differentially depending on the tissue type and proximity to the damage. Specifically, regenerating fibres showed the greatest changes in IGF mRNA and binding, and survivor fibres and connective tissue showed greater changes than did undamaged fibres. Interestingly, although elevated IGF-I was noted in non-regenerating fibres, the same was not observed for IGF-II, suggesting that at least some of the functions of IGF-II are distinct to those of IGF-I in these tissues.

IGF-I and –II are believed to be key regulators of MPC proliferation and differentiation (Duclos *et al.* 1991; Ewton *et al.* 1994; Florini *et al.* 1991a). The expression patterns of IGF-I and –II in regenerating fibres as observed in the present study are fully consistent with such a regulatory role for the IGFs, because IGF levels were elevated during the phases of MPC proliferation and differentiation. While IGF-I and –II expression patterns were generally similar in that they were massively up-regulated during regeneration and had peak levels during late differentiation (day 9), there were also key differences between the two IGFs. Specifically, IGF-I was up-regulated at the time of MPC proliferation, whereas up-regulation of IGF-II mRNA was not apparent until the onset of myotube formation. A similar pattern of IGF-I and –II mRNA regulation, whereby IGF-I levels increase prior to IGF-II levels, has been reported for regenerating rat M. *soleus* and M. *extensor digitorum longus* (Levinovitz

et al. 1992). Both IGF-I and –II mRNA levels reached maximal levels during late myotube fusion, suggesting an important role for both IGFs in the stimulation of differentiation, as has been shown for cultured muscle cells (Florini *et al.* 1991a; Florini *et al.* 1991b). Autocrine induction of IGF-II is essential for the induction of differentiation, as shown by studies using an IGF-II antisense construct to block autocrine IGF-II production (Florini *et al.* 1991b). Both IGFs elevate myogenin mRNA as a part of the stimulation of myogenesis (Florini *et al.* 1991a; Florini *et al.* 1991b), thereby promoting the expression of muscle-specific gene expression via MRFs (Kaushal *et al.* 1994). In addition to the effects of IGFs on muscle specific gene expression via the MRFs (Kaushal *et al.* 1994), IGFs also function to protect cultured muscle cells from apoptosis as they exit the cell cycle and undergo terminal differentiation (Stewart & Rotwein 1996). A similar function has recently been shown *in vivo*, whereby skeletal muscle programmed cell death in dystrophic *mdx* mice was prevented by IGF-II overexpression (Smith *et al.* 2000). These studies would suggest that the elevated IGFs observed in regenerating muscle during differentiation could be serving more than one function, and that the consequences of a lack of IGF at this point can be quite severe.

In the present study, ^{125}I-IGF-I binding was highest in regenerating muscle fibres. ^{125}I-IGF-I binding, which constitutes the cumulative binding to both the Type I IGF receptor and IGFBPs, was elevated on day 3 but then rose further to day 5 levels, concomitant with the onset of differentiation. Elevated Type I IGF receptor binding in regenerating muscle has been similarly reported for the M. *extensor digitorum longus* of hypophysectomised rats (Jennische & Matejka 1992). What is of interest is that the

levels on day 5 were higher than the day 3 levels, which is contrary to the reported down-regulation of the Type I IGF receptor during muscle cell differentiation (Rosenthal *et al.* 1991). This discrepancy can be reconciled, however, by taking into account the presence of IGFBPs, as indicated by the *in vitro* incubations utilising des(1-3)IGF-I as the competing hormone (Ross *et al.* 1989). Competition with des(1-3)IGF-I showed that a very significant component (approximately 50%) of the day 5 ^{125}I-IGF-I specific binding is comprised of binding to IGFBPs. Binding proteins were not observed in the *in vitro* incubated sections from day 3, thereby suggesting that the day 3 specific binding values are comprised solely of binding to the Type I IGF receptor. Thus, in terms of the Type I IGF receptor alone, there may be a decrease in specific binding from day 3 to day 5, in the present trial. These findings are thus in agreement with the reported decrease in Type I IGF receptor levels during muscle cell differentiation *in vitro* (Rosenthal *et al.* 1991). Studies of cultured muscle cells have shown that the amount of Type I IGF receptor affects the speed with which myogenesis occurs, whereby increased receptor levels lead to an increased rate of myogenesis (Quinn & Haugk 1996; Quinn *et al.* 1994), while functional inactivation of the receptor results in delayed differentiation (Cheng *et al.* 2000). Thus, the down-regulation of receptor levels at differentiation may be a mechanism by which the rate of differentiation is controlled *in vivo*.

In the current study, binding of ^{125}I-IGF-II to regenerating muscle fibres was increased during the myotube stage, concomitant with the up-regulation of IGF-II mRNA. A positive association of IGF-II mRNA and Type II IGF receptor up-regulation during myogenesis has also been shown in culture for C2 muscle cells (Szebenyi & Rotwein

1991; Tollefsen *et al.* 1989b). Whether the simultaneous up-regulation of IGF-II mRNA and receptor are functionally related is unknown. Evidence for IGF-II action through the Type II IGF receptor comes from studies in which an IGF-II analogue with high affinity for the Type II IGF receptor was found to stimulate differentiation (Rosenthal *et al.* 1994), however additional evidence of such an action is lacking. Based primarily on results from knockout studies, the Type II IGF receptor is proposed to act as a "sink" for IGF-II by binding and internalisation of the growth factor (Haig & Graham 1991), however such a function would seem unlikely as it would directly counteract the up-regulation of IGF-II during differentiation. The Type II IGF receptor has numerous activities other than growth factor binding and internalisation, including activation of TGF-β (Ghahary *et al.* 1999a; Ghahary *et al.* 2000), and the trafficking and targeting of lysosomal enzymes (Kornfeld 1992). It has been proposed that in the adult the primary function of the Type II IGF receptor is the maintenance of lysosomal activity (Wang *et al.* 1994). Lysosomal activation has been reported to occur in developing primary rat muscle culture, denervated muscle and in muscular dystrophy (Libelius & Tagerud 1989). During muscle cell differentiation in culture, the increase in Type II IGF receptor number occurs alongside an increased proportion of lysosomal enzyme (β-hexosaminidase) in the intracellular versus extracellular compartments (Szebenyi & Rotwein 1991), suggesting that the Type II IGF receptor may function during differentiation to increase the efficiency of lysosomal enzyme targeting (Szebenyi & Rotwein 1991).

In the present study, IGFBPs, as determined by the *in vitro* binding of ^{125}I-IGF-I competed with unlabelled IGF-I and des(1-3)IGF-I, were observed only in day 5

connective tissue, regenerating and survivor fibres. In day 5 connective tissue and survivor fibres, IGFBPs appear to account for the majority of the observed ^{125}I-IGF-I binding, as competition of ^{125}I-IGF-I binding with des(1-3)IGF-I, failed to displace ^{125}I-IGF-I binding. This finding of a significant contribution of IGFBPs to regenerating muscle tissues is supported by a recent study of IGFBP expression and localisation in post-ischaemic muscle, which showed that IGFBPs-4, -5, and –6 are up-regulated relative to control muscle on day 4 of regeneration (Jennische & Hall 2000). The IGFBP expression reported by Jennische and Hall (2000) was found to be cell-type specific, with IGFBP-4 expressed predominantly in connective tissue, IGFBP-5 in regenerating cells, and IGFBP-6 in muscle cells and connective tissue, thus indicating IGFBP up-regulation in the same tissues that IGFBPs were reported in in the present study. The actions of IGFBPs can be either inhibitory or stimulatory, with inhibition of IGF action by IGFBPs primarily through reduced interaction with the Type I IGF receptor (reviewed by Hossner *et al.* 1997). Stimulation of IGF action by IGFBPs is thought to be due to formation of IGF-IGFBP complexes that associate with cell surfaces, often with a concomitant reduction in the affinity of IGFBP for IGF (Hossner *et al.* 1997). IGFBP-4 acts predominantly as an inhibitor of IGF-stimulated proliferation (Bayes-Genis *et al.* 2001; Duan & Clemmons 1998), IGFBP-5 as a potentiator of IGF-induced proliferation and an inhibitor of IGF-stimulation of differentiation (James *et al.* 1996), and IGFBP-6 as an inhibitor of IGF-II induction of muscle cell differentiation (Bach *et al.* 1994). IGFBPs thus constitute additional mechanisms for fine-tuning the actions of IGFs during skeletal muscle regeneration. The differential localisation of IGFBPs in regenerating muscle may allow for

inhibitory effects on some tissues, such as connective tissue, alongside stimulatory actions on other tissues such as muscle.

In the present trial an effect of GH on IGFBP levels, as determined by the *in vitro* binding of ^{125}I-IGF-I competed with unlabelled IGF-I and des(1-3)IGF-I, was not observed in M. *biceps femoris*. Similarly, skeletal muscle IGFBP-3 mRNA in pigs is unaffected by GH administration (Dunaiski *et al.* 1999), as is the case for an IGFBP (presumed to be IGFBP-4) in the same strain of dwarf rats as used in the present study (Lemmey *et al.* 1997). These findings are in contrast, however, to the observation of increased IGFBP-3 protein (42-48 kDa band), and presumably IGFBP-1 and/or –2 protein (28-32 kDa band), in response to GH in *dw/dw* rats (Lemmey *et al.* 1997), as well as the up-regulation of IGFBP-5 mRNA in hypophysectomised rats in response to GH administration (Gosteli-Peter *et al.* 1994). It is possible that the differences between the latter studies and the present study relate to sampling time, which in the present study was approximately 24-hours post GH injection. IGFBP mRNA levels have been shown to vary with time following GH administration, as exemplied by the increased 28-32kDa band (presumed to be IGFBP-1 and/or –2) by Western blot at 4 hours, but not at 8 or 24 hours, following GH treatment (Lemmey *et al.* 1997).

In the present study a low level of IGF-II mRNA over connective tissue was observed. Human foetal connective tissue expresses high levels of IGF-II mRNA, suggesting that it may provide a source of paracrine IGF-II for developing tissues (Han *et al.* 1987). However, in adult sheep, IGF-II mRNA is undetectable in connective tissue (Hodges *et al.* 1992), a finding that supports our current observation. Low levels of IGF-II mRNA in connective tissue were noted at all stages of the regeneration process,

and were in contrast to the increased levels of IGF-I mRNA observed during early regeneration (day 3). IGF-I mRNA is transiently expressed in the healing wound (Steenfos & Jansson 1990), and is produced by fibroblasts in wound chambers (Steenfos *et al.* 1990) and *in vitro* (Clemmons 1984). IGF-I protein in connective tissue during regeneration could serve as a local source of IGF-I for regenerating muscle fibres, or as a source of IGF-I protein for fibroblasts. During regeneration, IGF-I may stimulate the proliferation of fibroblasts, as occurs *in vitro* in response to IGF-I (Cook *et al.* 1988), and/or stimulate the synthesis of collagen, the major structural component of connective tissue (Kelley *et al.* 1990). IGF-I stimulates collagen synthesis by lung-derived fibroblasts *in vitro* (Goldstein *et al.* 1989), a process that likely occurs in regenerating muscle as a part of the repair of intramuscular connective tissue and/or the synthesis of scar tissue.

The rats used in this trial responded to exogenous GH with increased body weight, notexin-injected and non-injected muscle weights, an observation which is in accord with the growth enhancing effect of GH on body and skeletal muscle weights previously reported for normal and regenerating muscle (Clark *et al.* 1985; Ullman *et al.* 1989). However, despite these effects of GH, analysis of *in situ* hybridised notexin-injected and non-injected M. *biceps femoris* failed to reveal an effect of GH on IGF-I or -II mRNA levels in any of the fibre types examined. In contrast to our findings, porcine muscle has increased IGF-I mRNA in response to GH administration (Brameld *et al.* 1996), and skeletal muscle IGF-I and -II mRNA is increased in response to GH-stimulated hypertrophy in GH3-tumour implanted rats (Turner *et al.* 1988). It is possible that the discrepancy between this report and the present one is

that the sampling times used in this study may have missed an induction of IGF mRNA, for peak levels of tissue IGF-I occur 12 hours after GH injection in hypophysectomized rats (D'Ercole *et al.* 1984). It would be interesting to examine IGF mRNA in regenerating muscle at earlier timepoints relative to the daily GH administration, ie. within 6-8 hours of GH treatment.

Just as an effect of GH was not observed for IGF-I and –II mRNA, an effect of GH on ^{125}I-IGF-I binding was not observed. The lack of an effect of GH on ^{125}I-IGF-I binding is supported by the demonstration that in hypophysectomised and normal rats, identical patterns of ^{125}I-IGF-I binding relative to regeneration stage are seen in both animals (Jennische & Matejka 1992). This indicates that the GH status does not significantly affect ^{125}I-IGF-I binding during regeneration.

In contrast to the above-mentioned lack of effect of GH on IGF mRNA and ^{125}I-IGF-I binding, this study showed that binding of ^{125}I-IGF-II to damaged muscle tissues is sensitive to GH. The fact that the growth promoting effect of GH was observed in both notexin-injected and non-injected muscles, but that the effect of GH on ^{125}I-IGF-II binding was observed in only the notexin-injected muscle, suggests that the GH effect on ^{125}I-IGF-II binding was related to the regeneration process. Additionally, the effect of GH on ^{125}I-IGF-II binding within the notexin-injected muscle tissues showed temporal variation, with a significant effect observed on day 5, but not on days 3 and 13. The presence of antibodies to human GH following 9 days of GH administration to hypophysectomised rats has been reported, and it has been suggested that this could result in a decreased growth response (Groesbeck & Parlow 1987). This could explain

the lack of effect of GH on ^{125}I-IGF-II binding after 13 days of GH treatment in the present trial, although this is considered unlikely as no such effect on growth rates following long term GH treatment was observed in this strain of dwarf rats. The difference between the two sets of observations may relate to the animal model used. The function of the up-regulated ^{125}I-IGF-II binding in response to GH in notexin-injected muscle tissues may involve modulation of lysosomal enzyme transport and uptake by the Type II IGF receptor (Kornfeld 1992; Roth 1988), as previously discussed in this section. Growth hormone administration to both hypopituitary dwarfs and normal subjects results in elevated lysosomal activity in polymorphonuclear leucocytes (Rovensky *et al.* 1985), thus suggesting that in the present study the increased binding to the Type II IGF receptor in GH-treated notexin-injected muscle tissues may be related to alterations in lysosomal enzyme trafficking.

In conclusion, this study has identified specific increases in IGF-I and -II mRNA, IGF receptor binding, and indicated changes in the abundance of IGFBPs, during skeletal muscle regeneration. GH administration resulted in increased muscle weight of both notexin-injected and non-injected muscles, and elevated binding of IGF-II, but no changes in the histology of the notexin-injected muscle. This study suggests involvement of the IGF axis in skeletal muscle regeneration, and that GH may modulate the regeneration process in part through the IGF axis.

CHAPTER 5

LOCALISATION OF MYOSTATIN DURING MUSCLE REGENERATION

5.1 Introduction

While the focus of the previous chapter was IGFs, the focus of this chapter is myostatin. The study of both IGFs and myostatin was chosen because a) the coordinated expression of different growth factors is an integral part of muscle regeneration (Grounds 1991), and b) the balance of both positive (IGFs) and negative (myostatin) regulators, at least in part, determines net muscle growth.

Myostatin, and the current body of literature concerning its role in muscle growth, was reviewed in Section 1.7.2. Key studies discussed therein showed that an absence of myostatin leads to a substantial increase in muscle weight in mice (McPherron *et al.* 1997), while *in vitro,* the addition of myostatin results in an inhibition of myoblast proliferation (Thomas *et al.* 2000).

These studies strongly indicate a role for myostatin as a negative regulator of muscle growth, and raise the question of whether myostatin is differentially regulated in a situation of enhanced growth, such as following GH-stimulation; and whether levels of myostatin are temporally-regulated during regeneration, thereby suggesting a role for this protein during muscle regeneration.

5.1.1 Aim of this Chapter

The aim of the present study is to determine whether a negative regulator of growth, myostatin, is temporally regulated during muscle regeneration, and whether its levels are decreased in muscles undergoing enhanced growth due to the administration of GH.

5.2 Materials and Methods

5.2.1 Animals

Twenty-one intact male Sprague-Dawley rats were used to determine regeneration in normal animals. Intact (non-castrated) rats were used for these normal rats, and for the study contained in CHAPTER 6, because GH was no longer being administered, thus testosterone was no longer considered a confounding factor (refer to Section 4.2.1). At 60 days of age and weighing approximately 330g, these animals were anaesthetised with a solution of 5 mg/ml Rompun® (xylazine hydrochloride), 37.5 mg/ml ketamine hydrochloride in sterile water (0.2 ml per 100g bodyweight), and the right M. *biceps femoris* injected with 2 μg notexin, as described in Section 4.2.1. The

dwarf (*dw/dw*) rats used for this study were a subset (n=3/timepoint), chosen at random, of the animals studied in CHAPTER 4.

5.2.2 Tissue sampling

Normal rats were sacrificed, as described in Section 2.7.1, on days 1, 2, 3, 5, 9, and 13 (n=3/timepoint). Contralateral muscles were used as regeneration controls, and three normal rats were sacrificed on day 0 as additional regeneration controls. Muscle samples were formalin-fixed for 18 hours prior to paraffin-embedding. Sections were cut to a thickness of 7 microns then placed on slides coated with 3-aminopropyltriethoxysilane (Sigma-Aldrich, St. Louis, MO, USA).

5.2.3 Immunohistochemistry

Antibodies used for this study were the following: rabbit anti-bovine myostatin antibody, raised against amino acid sequence 201-370, which recognises the precursor and processed forms of myostatin protein (Sharma *et al.* 1999); the irrelevant antibody, rabbit anti-human α- lactalbumin; and non-immune rabbit immunoglobulin fraction were used to verify the specificity of myostatin immunohistochemistry; the monoclonal antibodies mouse anti-slow muscle myosin (slow MHC), and mouse anti-fast muscle myosin (fast MHC) which recognises all Type II fibres, were used together to determine fast and slow fibre-types in muscle sections. The myostatin antibody used in these studies does not cross-react with other TGF-β superfamily members, as determined by Western blot analysis of normal and Belgian Blue skeletal muscle

extracts (Sharma *et al.* 1999). Immunohistochemistry was performed according to the method described in Section 2.9.

5.2.4 Statistics

Muscle and body weights were adjusted for initial liveweight, then analysed using ANOVA, to determine the effects of notexin on muscle and body weights over the trial period.

5.3 Results

5.3.1 Characteristics of the regenerating muscle

The regeneration of notexin-injected muscle in both normal and *dw/dw* rat muscle was similar, although the regeneration in normal rats tended to occur at an earlier time. This reduction in regeneration time was determined by analysis of histological sections which showed that a greater proportion of damaged fibres were at the myotube stage in normal rat muscle relative to *dw/dw* rat muscle on days 3 and 5, as shown for day 3 in Figure 5-1.

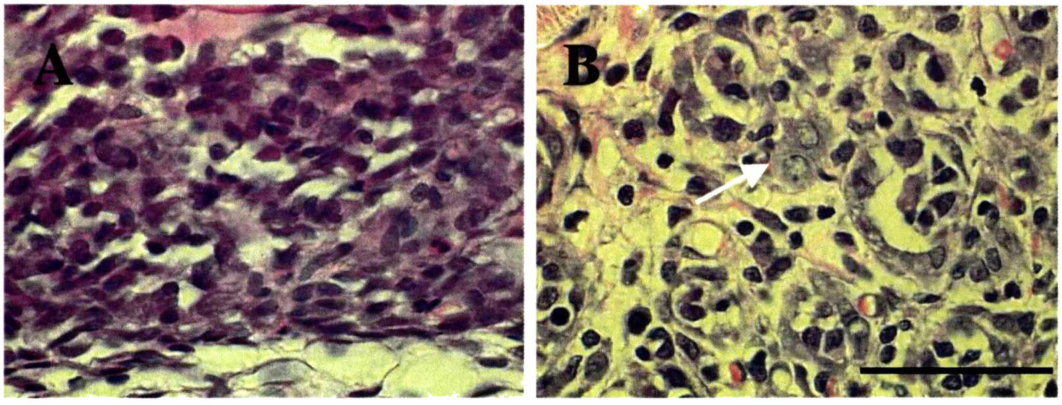


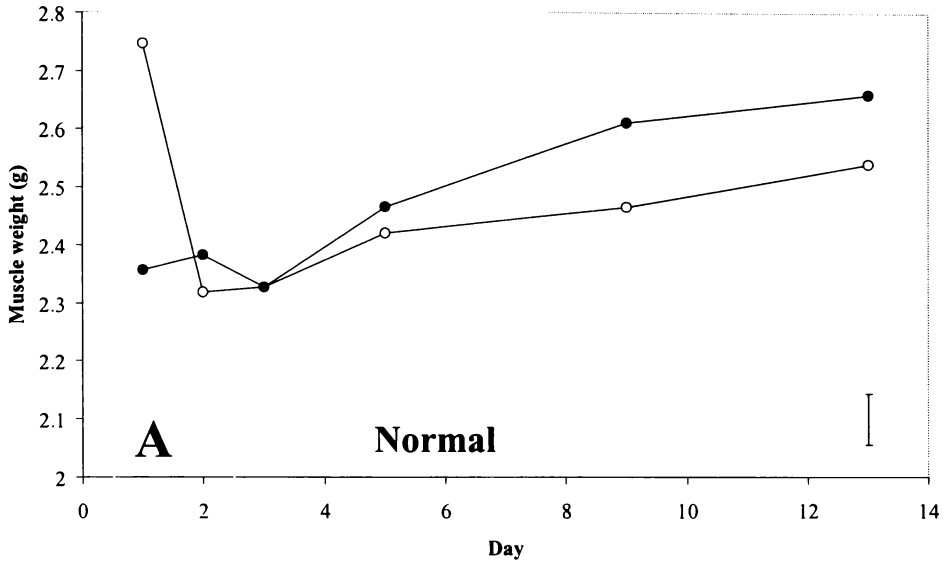
Figure 5-1. Regenerating muscle of *dw/dw* and normal rats.

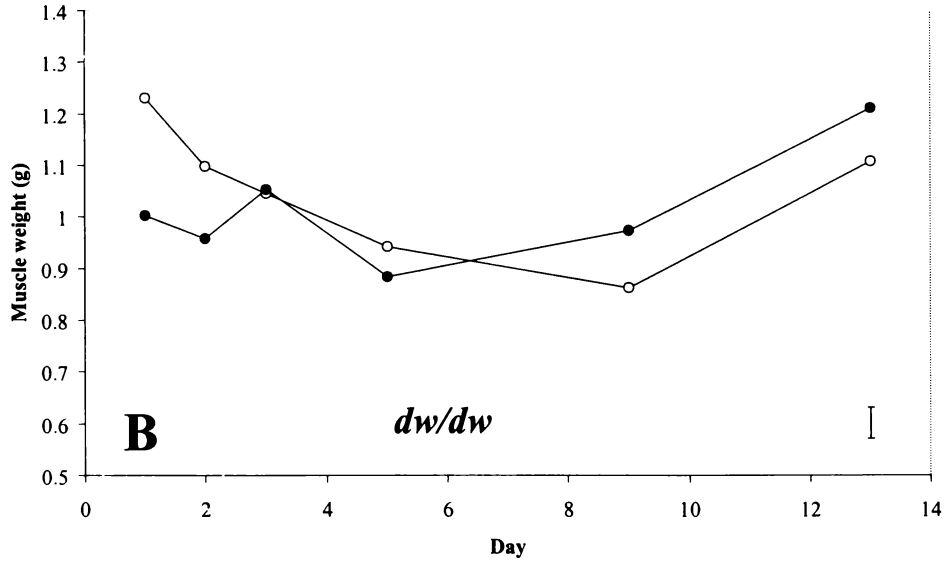
Cross-sections of *dw/dw* (A) and normal (B) rat M. *biceps femoris* on day 3 following notexin-injection. Notexin-injected muscle from normal rats (B) contains occasional immature myotubes (white arrow), in contrast to notexin-injected muscle from *dw/dw* rats (A), which does not show signs of MPC fusion into myotubes. Haematoxylin and eosin stained, magnification=100x, bar = 50 microns.

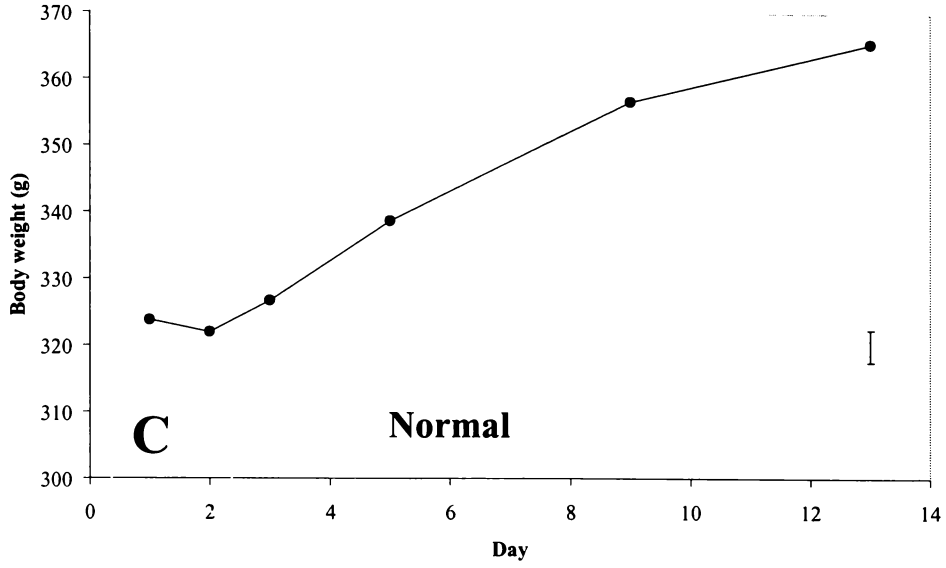
Notexin-injected muscle weights from normal rats are given in Figure 5-2, alongside the muscle weights for the subset of saline-treated *dw/dw* rats from CHAPTER 4 that were used in this study. Notexin-injected muscle weights from normal rats changed significantly over time ($p=0.005$), with lowest levels on days 2 and 3, followed by an increase after that time (Figure 5-2A). In *dw/dw* rats, notexin-injected muscle weights increased from day 9 onward ($p<0.001$, Figure 5-2B). The highly significant ($p<0.001$) changes in weight of both notexin-injected and non-injected muscles of the normal rat (Figure 5-2A), and non-injected muscles of the *dw/dw* rat (Figure 5-2B), were similar in pattern to the changes in body weight in the respective animals (Figure 5-2C, D), although this was not the case for the notexin-injected muscles of *dw/dw* rats (Figure 5-2B). A pronounced systemic effect of notexin was observed in *dw/dw* rats, as evidenced by decreased bodyweight up to day 5 (Figure 5-2; see also Section 4.3.1), in contrast to normal rats which showed little sign of a systemic effect of notexin (Figure 5-2) with bodyweights that only decreased marginally between days 1-2, then increased.

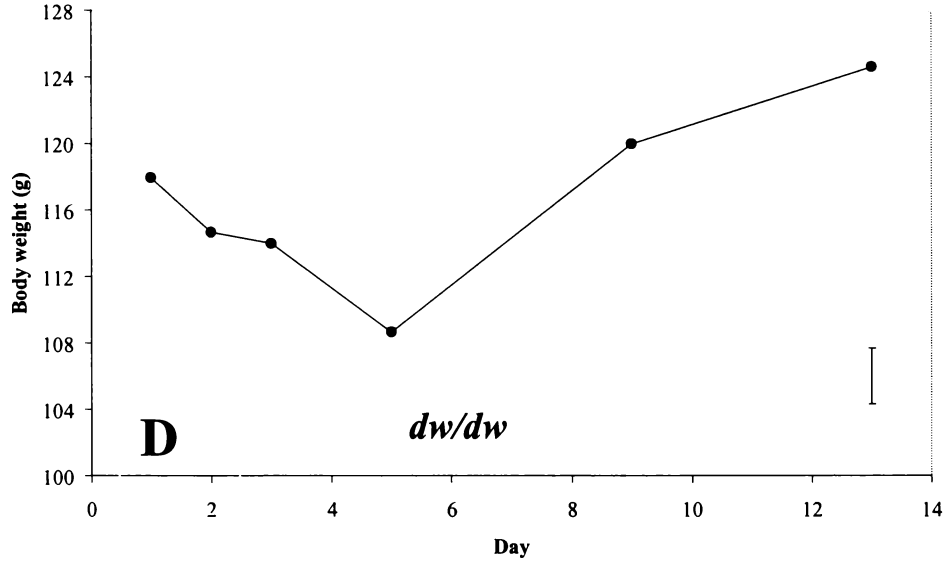
Figure 5-2. Muscle and body weights of normal and *dw/dw* rats.

Muscle (A,B) and body weights (C,D) following injection of notexin on day 0 in normal (A,C) and saline control *dw/dw* rats (B,D). A, B: Weights of notexin-injected and non-injected muscles are shown by the open and solid circles, respectively. Values represent the means, the error bar equals one SEM (n=3 per group).


Muscle weight (g)
2.8
2.7
2.6
2.5
2.4
2.3
2.2
2.1
2
A
Normal
0
2
4
6
8
10
12
14
Day


Muscle weight (g)
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
B
dw/dw
0
2
4
6
8
10
12
14
Day


370
360
350
340
330
320
310
300
Body weight (g)
C
Normal
0
2
4
6
8
10
12
14
Day


128
124
120
116
112
108
104
100
Body weight (g)
D
dw/dw
0
2
4
6
8
10
12
14
Day

5.3.2 Myostatin distribution within muscle tissue

In non-injected normal and *dw/dw* rat muscle, myostatin was localised in the muscle fibre cytoplasm (Figure 5-3A,B, respectively), and was absent from the connective tissue (Figure 5-3A). Sections immunostained with non-immune rabbit IgG showed no immunostaining over connective tissue or muscle (Figure 5-3D), as was the case for all negative control sections in these studies. A similar distribution of myostatin to non-injected muscle was also observed for notexin-injected muscle, with three exceptions, all of which occurred during early damage and regeneration. Firstly, myostatin was observed in interstitial areas and connective tissue in heavily damaged areas at early timepoints following notexin injection (Figure 5-3C; Table 5-1). Interstitial and connective tissue myostatin was highest on day 1 and then declined until day 3 when it was absent. Secondly, although myostatin was mainly found in the cytoplasm of muscle fibres, it was occasionally also observed in the nuclei of fibres up to day 5. Lastly, myostatin was observed within some phagocytic cells during early damage (data not shown), which possibly resulted from phagocytosis of necrotic tissue containing myostatin (Figure 5-3C,E,F).

5.3.3 Myostatin localisation in necrotic and regenerating muscle fibres

Distinct temporal variation in the intensity of myostatin immunostaining was observed in regenerating muscle fibres of both normal and *dw/dw* rats (Table 5-1). Necrotic fibres contained high levels of myostatin protein on day 1 following damage (Figure 5-3C). Myostatin protein within necrotic fibres then reached peak levels, and in the majority of fibres observed, stayed elevated until the necrotic debris was removed (Figure 5-3F; Table 5-1). A slight decrease from peak levels of myostatin immunostaining in necrotic fibres was noted in some normal rat necrotic fibres that were heavily infiltrated by phagocytes, but not in the necrotic fibres in the *dw/dw* rat (Table 5-1, day 3). Mononucleate cells located in regenerating areas at the end of phagocytosis, when activated satellite cells are most abundant (Koishi *et al.* 1995), did not contain myostatin (Figure 5-3F). Similarly, myotubes initially did not contain myostatin protein (Figure 5-3G). There was then a slight but noticable increase in the intensity of myostatin immunostaining in regenerating myotubes (Figure 5-3H), an increase that occured more rapidly in normal rats than in *dw/dw* rats (Table 5-1). Specifically, some larger regenerating myotubes of the normal rat had increased myostatin at day 5, whereas the regenerating myotubes of *dw/dw* rats did not show an increase in myostatin until day 9.

Figure 5-3. Myostatin localisation in regenerating muscle.

Myostatin localisation in non-injected (A, B) and notexin-injected (C-H) muscle. Notexin-injected muscle sections show the progression through necrosis and regeneration as follows: (C) and (D) are of early necrosis; (E) from mid to late necrosis; (F) from late necrosis/early regeneration; (G) from early to mid regeneration, at the onset of myotube formation; (H) from late regeneration. Sections were immunostained with anti-myostatin antibody (A-C, E-H) or matched normal rabbit non-immune immunoglobulin (D) then counterstained with nuclear fast red. Myostatin was localised in the muscle fibre cytoplasm in non-injected normal (A) and *dw/dw* (B) muscle. During muscle necrosis, high intensity myostatin localisation was observed in connective tissue (ct, C) and necrotic muscle fibres (black arrowheads; C, E, F), but was absent from the mononucleated cells present during late necrosis (mn, F). Newly-formed myotubes (white arrowheads, G) also lacked myostatin protein, but showed increased intensity of myostatin immunostaining after further development (arrows, H). Negative control sections (D) had no immunostaining. Bar=100 microns.

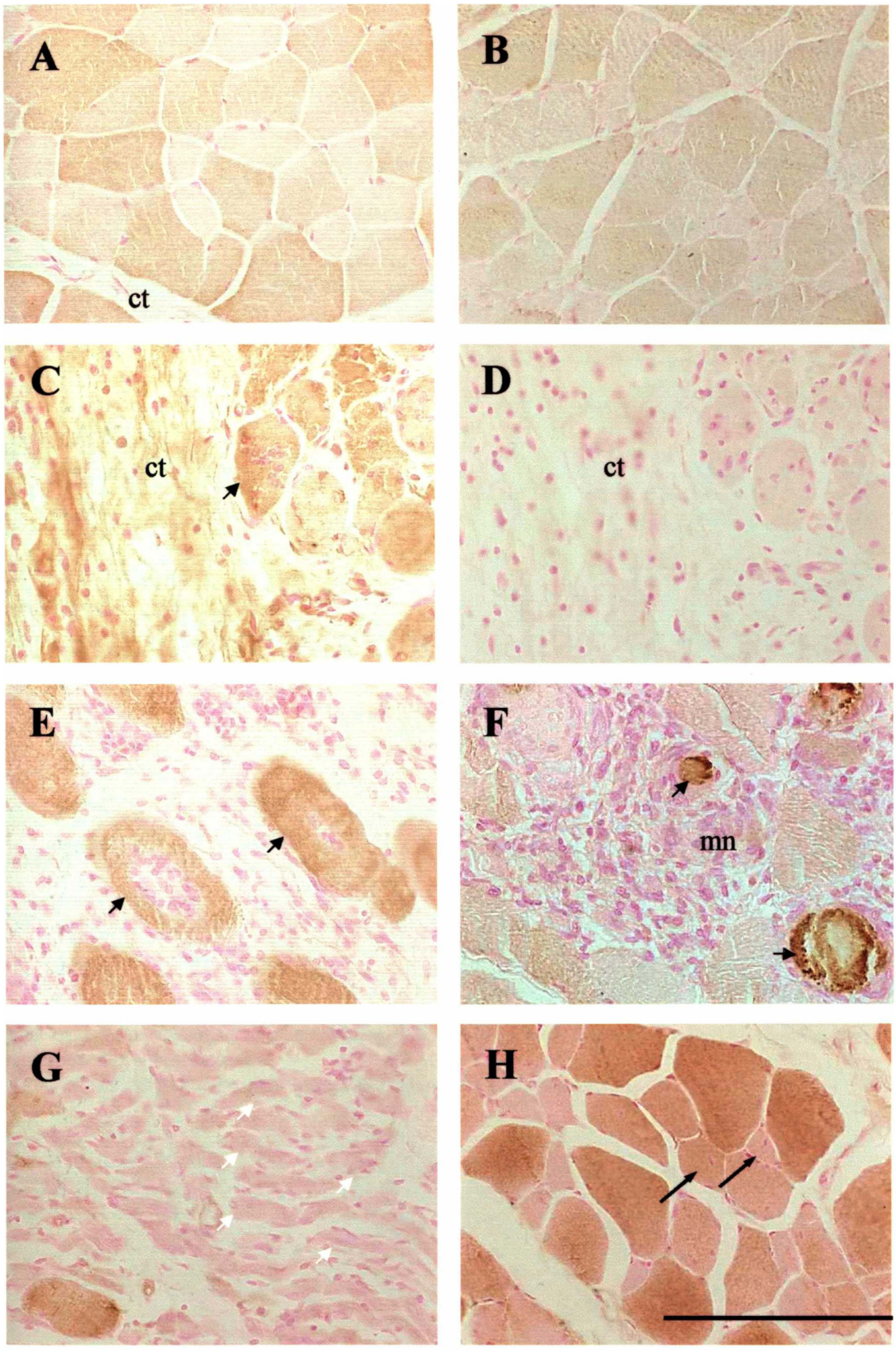

A
ct
B
C
ct
D
ct
E
F
mn
G
H

Table 5-1. Myostatin immunoreactivity in damaged muscle tissues.

Shown below are the relative levels of myostatin protein in skeletal muscle tissue types during damage and regeneration in normal and *dw/dw* rats as determined by immunohistochemistry.

	Damage			Regeneration		
				Peak of myotube formation ↓		
Day	1	2	3	5	9	13
Necrotic fibres						
normal	+++	+++	++	NM	NM	NM
dw/dw	+++	++++	++++	NM	NM	NM
Regenerating myotubes						
normal	NM	NM	-	++	++	+
dw/dw	NM	NM	-	-	+	++
Survivor fibres						
normal	++++	++++	++++	+++	++	+++
dw/dw	++	++	+	+	++	++
Connective tissue/ Interstitium						
normal	++	+	-	-	-	-
dw/dw	+++	+	-	-	-	-

The intensity of myostatin immunostaining was determined on a scale of - to ++++, where ++++ represents the highest intensity.
NM= "not measured" due to low or negligible quantities of the tissue type.

Myostatin expression has been shown by others (Carlson *et al.* 1999) to be fibre-type specific, as determined by the presence of myostatin mRNA in fast (Type IIb) fibres, and absence from slow (Type I) fibres. Therefore the association of myostatin with muscle fibre-type in notexin-injected and non-injected muscle was examined. Serial sections of non-injected rat muscle were immunostained with anti-myostatin, anti-slow MHC and anti-fast MHC to reveal the presence of myostatin protein in fast, but not slow, fibres (Figure 5-4, A-C). Immunostaining of notexin-injected muscle with the same panel of antibodies, however, showed the presence of myostatin within not only fast fibres, but also slow fibres (Figure 5-4, D-F). This indicates that during muscle fibre damage there is altered fibre-type localisation of myostatin protein.

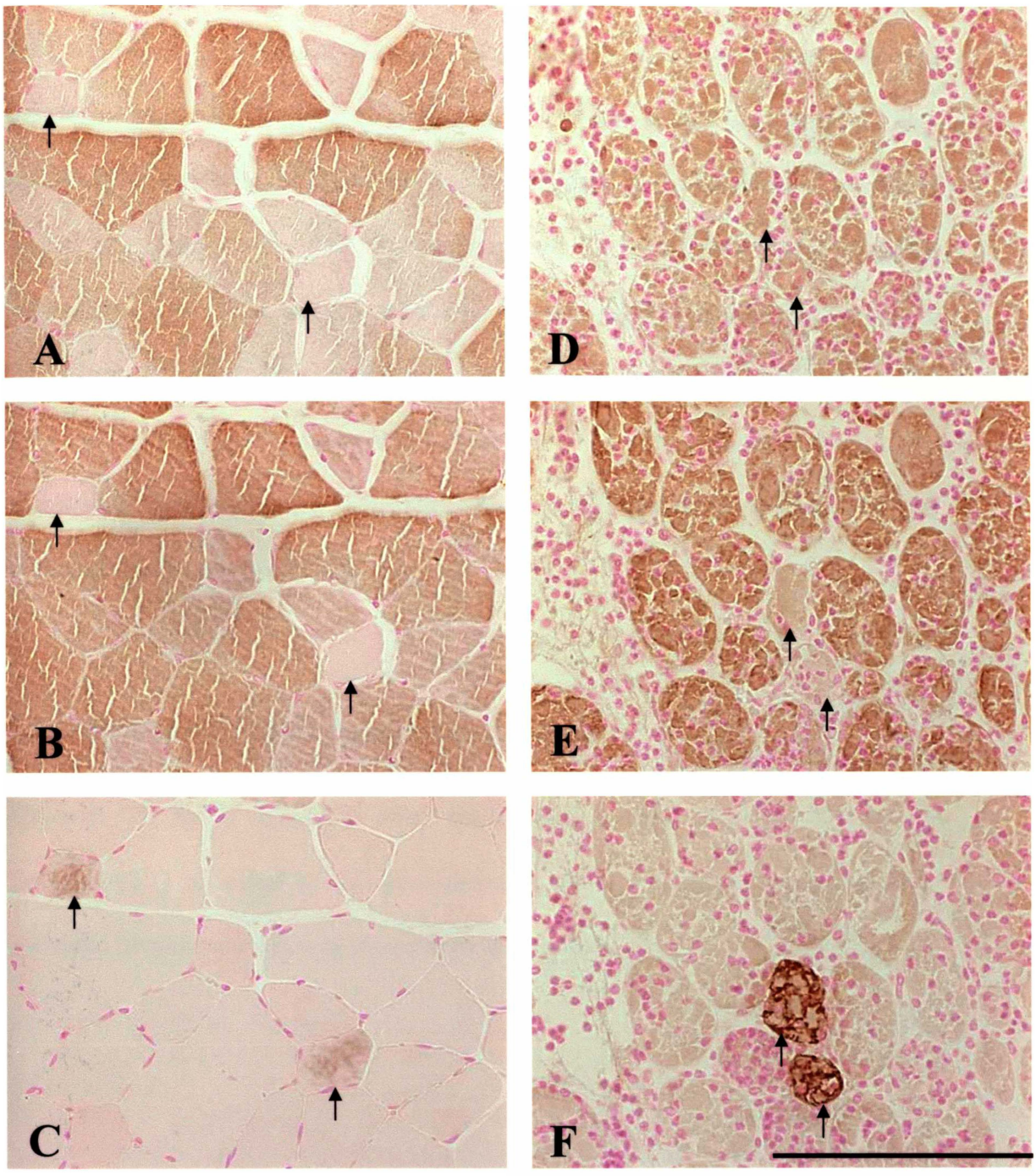


Figure 5-4. The association of myostatin with muscle fibre-type.

Sections (A-C) are from non-injected muscle, while sections (D-F) are from notexin-injected muscle. Serial sections of normal rat muscle were immunostained with antibodies to myostatin (A, D), fast MHC (B, E), and slow MHC (C, F) as detailed in Section 2.9. Positive immunostaining with anti-slow MHC, and negative immunostaining with anti-fast MHC, was used to identify slow muscle fibres (arrows). Myostatin immunostaining was absent from slow muscle fibres of non-injected (A), but not notexin-injected (D), muscle. Bar=100 microns.

5.3.4 Myostatin localisation in survivor fibres

Temporal regulation of myostatin levels was observed in the survivor fibres of normal and *dw/dw* rats. Specifically, myostatin immunostaining in survivor fibres increased immediately after notexin treatment when compared with non-injected muscle in normal rats (Table 5-1). This was not observed in the survivor fibres of *dw/dw* damaged muscles. Both normal and *dw/dw* rat survivor fibres showed a decrease in the intensity of myostatin immunostaining on days 3-5, and a subsequent increase in the intensity of myostatin immunostaining on days 9-13. The changes in the intensity of myostatin immunostaining in survivor fibres during regeneration tended to be later in normal rats than in *dw/dw* rats.

5.3.5 Effect of GH on myostatin protein

Although GH had a significant effect on the muscle mass of regenerated muscle (Section 4.3.1) no effect of GH-administration on the intensity or distribution of myostatin immunostaining was observed in these tissues.

5.4 Discussion

This study describes the localisation of myostatin in the regenerating muscles of normal and *dw/dw* rats. High levels of myostatin protein were observed in necrotic fibres and connective tissue during the damage phase, followed by a marked decline in myostatin concurrent with new fibre synthesis. Survivor fibres showed a notably different pattern of myostatin regulation from regenerating fibres.

Histological analysis showed that the muscle regeneration that occurred in the GH-deficient *dw/dw* rats was delayed relative to that of normal rats. Interestingly, the pattern of myostatin immunostaining in necrotic fibres/regenerating myotubes was similarly altered, as the decrease in the intensity of myostatin immunostaining observed in both normal and *dw/dw* rats took place earlier in the notexin-injected muscle of normal rats (day 3) relative to *dw/dw* rats (day 5). This observation of a similar pattern of myostatin localisation and expression in two strains of rats may indicate the importance of maintaining this pattern of myostatin expression in order to effectively regenerate damaged skeletal muscle.

High levels of myostatin were associated with the earliest stages of the muscle repair process, when necrosis and phagocytosis were the primary activities taking place. Similarly, increased myostatin protein has been reported for M. *extensor digitorum longus* and M. *soleus* on days 1 and 3 following notexin-induced damage (Mendler *et al.* 2000). In the present study, not only was the elevated myostatin protein observed

in notexin-injected muscle fibres, it was also observed at high levels within connective tissue and interstitial areas. In a separate regeneration study, Yamanouchi *et al.* (2000) reported the presence of myostatin mRNA in presumed fibroblasts, and showed that when exposed to crushed muscle extract, cultured fibroblasts from rat muscle increased expression of myostatin mRNA. This shows that a cell type that is normally associated with connective tissue is responsive to one or more factor(s) in damaged muscle, and that this results in the synthesis of myostatin. Whether this is the means by which connective tissue-associated myostatin protein is increased, or whether it is due to an influx of myostatin from the circulation is not known. Myostatin protein has been identified in the circulation, and levels therein found to be increased in humans with AIDS-associated muscle wasting (Gonzalez-Cadavid *et al.* 1998), however changes, if any, in circulating levels of myostatin, associated with regeneration, have not as yet been determined. That connective tissue might function as a "reservoir" for myostatin in damaged muscle might be expected, given that other TGF-β family members have been shown to bind to extracellular matrix components (Munger *et al.* 1997). A proposed function for myostatin located within connective tissue is the enhancement of connective tissue deposition, as TGF-β both enhances the synthesis, and down-regulates the proteolysis, of extracellular matrix components (Gleizes *et al.* 1997), and double-muscled cattle, which lack functional myostatin (Grobet *et al.* 1997; Kambadur *et al.* 1997; McPherron & Lee 1997), have decreased amounts of connective tissue (Arthur 1995). A second possibility is that high levels of myostatin protein within damaged muscle fibres and/or connective tissue may serve as a chemoattractant for phagocytes and inflammatory cells, or conversely as an inhibitor

of their proliferation, as has been reported for TGF-β (Adams *et al.* 1991; Ranges *et al.* 1987; Reibman *et al.* 1991; Wahl *et al.* 1987; Wahl *et al.* 1988).

Of additional interest during this time was the altered association of myostatin expression with fibre type. Specifically, myostatin protein in control muscles was confined to a subset of fast fibres, however during early regeneration this pattern changed so that slow fibres also contained myostatin protein. Notexin affects muscle fibre innervation (Harris *et al.* 2000), and innervation in turn has a potent effect on fibre type (Yoshimura *et al.* 1998). As discussed in Section 1.5.4, this is demonstrated by the observation that regenerating muscle fibres lacking innervation become fast in fibre type, and that the return to a slow fibre type is not possible until innervation has been restored (Yoshimura *et al.* 1998). It may therefore be postulated that myostatin is localised within slow fibres of damaged muscle because of acute sensitivity of the myostatin regulatory elements and/or pathways to changes in innervation. Muscle denervation has been shown to have varying effects on myostatin protein levels as determined by Western blot analysis, with decreased myostatin protein in M. *soleus* (a slow-type muscle), and increased myostatin in M. *gastrocnemius* and M. *plantaris* (fast-type muscles) (Sakuma *et al.* 2000). Because the direction of changes in myostatin expression (decreased myostatin in slow muscle) reported by Sakuma *et al.* (2000) for denervated muscle are opposite to those observed in the present study (increased myostatin in slow muscle fibres), it appears unlikely that the cause of the altered association of myostatin with fibre type in the present study is muscle denervation. This would be worthy of subsequent study, however, because the total protein levels as assessed by Western blot analysis in the Sakuma *et al.* (2000) study

are the sum total of changes in a number of histological tissue types, and may not represent specific changes in slow, damaged fibres alone.

Myostatin is a key controller of C2C12 muscle cell proliferation *in vitro*, via inhibition of cell cycle progression (Thomas *et al.* 2000). Consequently, one might expect that myostatin levels would be low during a time of high MPC proliferation, thus allowing proliferation to proceed unhindered. Indeed, that is the pattern observed in this study, wherein myostatin immunostaining levels were low to non-existent in regenerating areas during the time of MPC proliferation, and absent from myotubes. Although slightly elevated from proliferation- and fusion-associated myostatin levels, myostatin protein in post-fusion fibres undergoing enlargement was still very low. Satellite cell proliferation, as indicated by the presence of MyoD (Koishi *et al.* 1995), is elevated in notexin-damaged rat muscle during the time of post-fusion fibre enlargement (Mendler *et al.* 1998). Although the period of fibre enlargement during regeneration is distinct from muscle hypertrophy, there are certain similarities, including increased satellite cell replication in order to maintain a constant nuclear domain (Goldberg *et al.* 1975; Hikida *et al.* 1997; Snow 1990). Myostatin has been reported to have a regulatory role in hypertrophic processes, as indicated by the presence of both hypertrophy and hyperplasia in myostatin null mice (McPherron *et al.* 1997), as well as hypertrophy, not hyperplasia, in mice expressing a dominant-negative form of myostatin (Zhu *et al.* 2000). The observation of low myostatin during the phase of muscle fibre enlargement in the present study thus fits well with a role for myostatin in the regulation of hypertrophic processes (McPherron *et al.* 1997; Zhu *et al.* 2000).

In the survivor fibres of normal but not *dw/dw* notexin-injected muscle there was a slight increase in myostatin immunostaining immediately after notexin treatment. This was followed by a decrease and then a rise in survivor fibre myostatin in both normal and *dw/dw* survivor fibres, however these changes occured later in the normal rats as compared to the *dw/dw* rats. The earlier changes in the pattern of myostatin immunostaining in *dw/dw* survivor fibres did not appear to be associated with their lack of GH, as GH treatment failed to delay the pattern seen in *dw/dw* rats. Furthermore, the earlier pattern of changes in myostatin immunostaining in *dw/dw* rats did not appear to be associated with changes in muscle weight, as the *dw/dw* rats showed a delay in muscle growth relative to normal rats. This delay in muscle growth may have been because the area of damage was relatively greater in the *dw/dw* rats as their muscle weights were less than half those of the normal rat muscle weights, yet they had identical doses of notexin. The greater weight of normal rats relative to *dw/dw* rats is also likely to account for the presence of a pronounced systemic effect of notexin in *dw/dw* rats, but not normal rats, due to the greater relative concentration of notexin per gram of bodyweight in the *dw/dw* rats.

This study did not identify altered myostatin immunostaining associated with GH-induced muscle growth in *dw/dw* rats. This is similar to the findings of Ji *et al.* (1998), where GH administered to growing pigs did not alter the abundance of myostatin mRNA. There is no evidence of direct regulation of myostatin expression by GH, however a possible inter-relationship between myostatin and the GH/IGF axis is suggested by recent research. IGF-I regulates the expression of myogenin, a

myogenic regulatory factor (MRF) involved in the terminal differentiation of skeletal muscle cells (Florini *et al.* 1991a), and the related MRF, MyoD (Lawlor & Rotwein 2000). MRFs bind to E-boxes, and this binding is enhanced by the muscle-specific enhancer factor-2 (MEF-2) (Li & Capetanaki 1994). Sequence analysis has identified both an E-box and a MEF-2 binding site in the upstream region of the bovine myostatin gene (Jeanplong *et al.* 1999), thereby suggesting that IGF-I could indirectly affect myostatin expression via MRFs. GH administration can lead to up-regulation of locally produced IGF-I in muscle (Isgaard *et al.* 1989), however in the *dw/dw* rats in this study, GH administration did not affect the levels of IGF-I or myostatin in muscle (see CHAPTER 4). Therefore, the results of the present study can neither confirm nor discount an association of IGF-I with myostatin expression.

The results from these studies indicate a role for myostatin in the regeneration of skeletal muscle, and suggest that it may serve more than one function during this process, including possible functions as a chemotactic agent and/or as a regulator of connective tissue deposition.

CHAPTER 6

EFFECT OF IGF-II ON SKELETAL MUSCLE REGENERATION

6.1 Introduction

Skeletal muscle regeneration is a process that figures into many physiological situations, including sports injuries (Armstrong *et al.* 1991), reconstructive surgery (Arnold *et al.* 1994), and severe trauma. In these situations it is important that regeneration proceeds in a timely and efficient manner, and that the full complement of muscle is regenerated in order to restore full function. For this reason there is interest in developing therapeutics capable of enhancing the regeneration of skeletal muscle.

As discussed in detail in Section 1.5, skeletal muscle regeneration consists of a series of sequential steps. Therapeutic agents can be developed to target one or more of these steps. A number of peptides and growth factors have been used to date in regeneration studies. A summary of the relevant studies is given in Table 6-1,

showing the administered growth factor(s) and their effects on major regeneration processes. It is clear that administration of individual growth factors can, and often does, affect more than one step of the regeneration process (Lescaudron *et al.* 1999; Menetrey *et al.* 2000; Miller *et al.* 2000).

Two opposing therapeutic approaches have been taken in the studies listed in Table 6-1, one of which is to increase growth factor levels by administering the peptide directly, and the other is to eliminate the naturally occurring peptide of interest by administering an antibody directed against it (Lefaucheur *et al.* 1996; Lefaucheur & Sébille 1995c)). Antibodies have the advantage of an often longer half-life within the body, however they carry the disadvantage of causing both enhancing and inhibitory effects when administered (Dijiane *et al.* 1985; Massart *et al.* 1993), thus potentially clouding the assertions made regarding the activity of the peptide of interest *in vivo*.

As shown in Table 6-1, significant effects of growth factor administration on the regeneration process have been reported for both single injection (Lefaucheur & Sébille 1995a) and continuous infusion (Barnard *et al.* 1994) methods, although continuous infusion of growth factor is frequently used due presumably to concerns that a single injection of peptide would not provide peptide at later timepoints, when it might have a biological effect, due to loss from the regenerating area.

Table 6-1. Effect of growth factors on muscle regeneration.

Reference	Growth factor	Delivery method	Effect of growth factors on regeneration processes					
			Phagocytosis	MPC proliferation	MPC fusion	Strength	Fibre number or density	Fibre area
Peptide administration								
Mitchell *et al.* (1996)	bFGF	MII and CR	NR	NR	NR	NR	↔	NR
Menetrey *et al.* (2000)	bFGF IGF-I NGF	MII	NR	NR	NR	↑ ↑ ↔	↑ ↑ ↔	↑ ↑ ↔
Miller *et al.* (2000)	HGF	MII early MII late	NR	↑*	↓*	NR	↓ or ↔ ↔	↓ ↔
Lescaudron *et al.* (1999)	MIP 1-β VEGF	*In vitro* conditioning	↑ ↑	↑ ↑	↔ ↑	NR	↔ ↑	NR
Barnard *et al.* (1994)	LIF	CR	↑#	NR	NR	NR	↑	↑
Lefaucheur & Sebille (1995a)	bFGF	SII	NR	↑	NR	NR	↑	NR
Antibody administration								
Lefaucheur & Sebille (1995c)	IGF-I bFGF TGF-β1	SII	NR	NR	NR	NR	↓ ↓ ↑	↓ ↓ ↓
Lefaucheur *et al.* (1996)	IGF-I bFGF TGF-β1	SII	↑ ↓ ↑	NR	NR	NR	NR	NR

*assayed *in vitro*.

effect indicated but not measured

Abbreviations: LIF, leukaemia inhibitory factor; HGF, hepatocyte growth factor; bFGF, basic fibroblast growth factor; NGF, nerve growth factor; MIP-1β, macrophage inflammatory protein 1-beta; MPC, muscle precursor cell; NR, not reported; MII, multiple intramuscular injection; SII, single intramuscular injection; CR, continuous release; ↑, enhancement of regeneration process; ↓, inhibition of regeneration process, ↔, no significant effect.

In the previous two chapters, temporal and histological regulation of growth factor expression during the regeneration of skeletal muscle was reported. Among these changes was a dramatic up-regulation of IGF-II mRNA in newly-formed myotubes (CHAPTER 4). Further, the increased IGF-II mRNA was in regenerating muscle fibres, but not survivor fibres, connective tissue, or undamaged muscle fibres, suggesting that the provision of high amounts of IGF-II locally is important for skeletal muscle regeneration, and that the IGF-II protein is likely to function in an autocrine/paracrine fashion during regeneration. In the elegant study of Stewart *et al.* (1996), the overexpression of autocrine IGF-II by C2 myoblasts resulted in an advancement of the onset of differentiation. This study combined with earlier findings (CHAPTER 4) raised the possibility that if exogenous IGF-II were administered locally during skeletal muscle regeneration, that the timing of myogenic differentiation could be advanced.

In CHAPTER 4, an up-regulation of IGF-II at the time of MPC fusion was observed, but not an upregulation of IGF-II concurrent with the period of MPC proliferation. Studies by others (Bach *et al.* 1995; McFarland *et al.* 1993; Minniti *et al.* 1995) however have clearly shown an effect of IGF-II on myoblast proliferation, via interactions with the Type I IGF receptor (Ewton *et al.* 1987). These *in vitro* studies suggested that if exogenous IGF-II were supplied at the time of MPC proliferation, it might positively affect that step. Therefore, in this trial the effect of IGF-II on the process of MPC proliferation, as well as fusion, will be examined.

The IGF axis, which includes not only the growth factors IGF-I and IGF-II, but receptors and binding proteins as well, is tightly regulated by way of positive and negative feedback loops between the various components. For instance, GH down-regulates its own secretion via negative feedback (Hashimoto *et al.* 2000), while IGF-I, IGF-II and high concentrations of insulin down-regulate IGF-II at the transcriptional level (Magri *et al.* 1994). Similarly, IGF-II down-regulates the Type I IGF receptor, via transcriptional down-regulation and increased receptor degradation (Rosenthal & Brown 1994; Rosenthal *et al.* 1991). Such an IGF-II-induced down-regulation of the Type I IGF receptor could have a significant effect on the timing of differentiation of myogenic cells, as suggested by studies showing an advancement of differentiation with Type I IGF receptor overexpression (Quinn & Haugk 1996; Quinn *et al.* 1994), or a delay with functional inactivation of the Type I IGF receptor (Cheng *et al.* 2000).

As stated in the introduction (Section 1.6.1), two members of the MRF family of transcriptional regulators, MyoD and myogenin, are frequently used as markers of myogenic proliferation and differentiation processes. MyoD is localised within proliferating MPC, while myogenin is one of the earliest markers of differentiation. These markers have been employed in the ensuing study in order to precisely determine the effects of administered IGF-II on proliferation and early differentiation events. Developmental MHC (dMHC), an embryonic/neonatal form of MHC that is not normally expressed in adult muscle, but is expressed during regeneration, is used in this study as a later marker of differentiation, for dMHC has been shown to persist in damaged rat muscle until at least 7 days after damage (St. Pierre & Tidball 1994).

6.1.1 Aim of this chapter

The aim of this chapter is to test the hypothesis that administration of IGF-II during skeletal muscle regeneration advances regeneration. This will be determined by examining:

a) MPC proliferation and differentiation

b) muscle fibre size.

6.2 Materials and Methods

6.2.1 Alzet pumps

Two methods were considered for the administration of IGF-II, namely a single intramuscular injection or continuous infusion. Single injections of polypeptide during regeneration have been used with success by others (Lefaucheur & Sébille 1995a), however continuous polypeptide infusion was preferred, as it is unlikely that a single dose of polypeptide would remain throughout the 7-day time period under examination. A sampling period of up to 7 days was chosen so as to include the periods of MPC proliferation, MPC fusion and muscle fibre enlargement during notexin-induced regeneration in these Sprague-Dawley rats.

Localised, as opposed to systemic, peptide administration was chosen because previous work (see CHAPTER 4) showed high levels of IGF-II within regenerating fibres, suggesting that local production of IGF-II is important during muscle regeneration. The options available for the local delivery of peptide included

impregnated polymers (Langer & Folkman 1976; Mitchell *et al.* 1996), microspheres (Lee *et al.* 1997), and miniosmotic pumps (Alzet pumps; Alza Corporation, Mountain View, CA). Miniosmotic pumps were chosen for use in this study for the following reasons: a) they are the best characterised of the three options; b) Alzet miniosmotic pumps have been frequently used to administer IGF-II (Conlon *et al.* 1995; Shaar *et al.* 1989; Spencer *et al.* 1996), including over periods of 14 days (Conlon *et al.* 1995), and often with a significant treatment effect of IGF-II (Conlon *et al.* 1995; Shaar *et al.* 1989). A seven-day Alzet miniosmotic pump that was suitable for implantation in rats (Model 1007D) was therefore chosen for this study. Pumps were fitted with single lumen, vinyl catheters (SV55 tubing, inner diameter 0.80mm, outer diameter 1.20mm; Dural Plastics and Engineering, Dural, NSW, Australia) so that they did not lie directly over the damaged/regenerating muscle (possibly causing further damage). Catheters were cut to length then fitted with a "cuff" to ensure that the catheter stayed in place. Cuffs were made by attaching 2 small rings of SV102 (inner diameter 1.40mm, outer diameter 1.90mm, single lumen vinyl tubing; Dural Plastics) approximately 2 mm apart, two-thirds of the way along the length of the catheter. The catheter was then autoclaved, allowing for 25% shrinkage. To attach the catheter tubing, the plastic portion of the flow moderator was crushed with scissors or pliers and disposed of. The catheter tubing was then attached to the end of the metal flow moderator tube, and secured with Loctite adhesive (Loctite Australia, Caringboh, NSW, Australia).

On the afternoon prior to surgery, both the pump assembly, and the flow moderator with attached catheter, were filled with a 0.29 ug/ul solution of recombinant human

IGF-II (OM-001, lot EJI-O01, GroPep Pty Ltd, Adelaide, Australia; reconstituted to 1mg/ml in 10mM acetic acid) diluted in RPMI 1640 media, or diluent only (controls). After filling each component (the pump and the flow moderator/catheter), the two parts were fitted together. If the pumps are being used with catheters, or if immediate peptide delivery is desired, the manufacturers recommend that the filled Alzet pumps be pre-incubated in saline for 4-6 hours at 37°C, or preferably overnight, prior to use (Alzet miniosmotic pump instruction and specification sheet). Therefore, for this study the filled pump and catheter assemblies were stored at 37°C in sterile 0.9% saline overnight, in order to start the pumps flowing.

The concentration of IGF-II used in this study was 0.29 ug/ul. The flow rate of the Alzet pump model 1007D was 0.5 ul/hr over a period of 7 days, therefore the IGF-II filled pumps used in this study delivered 0.145 ug IGF-II/hr, or 3.48 ug IGF-II/day. This particular IGF-II concentration was chosen because earlier work by Conlon *et al.* (1995) showed that administration of 5.87 ug IGF-II/g bodyweight/day (=2.93 ug/0.5 g bodyweight/day) from subcutaneously implanted Alzet pumps to young rats resulted in significant effects on body weight gain and feed conversion efficiency. The amount of tissue affected by injection of 10ul of notexin was unlikely to exceed 0.5 g, as deduced by the fact that injection of 10 ul of methylene blue dye into non-viable rat muscle affects only 0.11 g of tissue. Local administration of IGF-II would have a small amount of peptide loss due to diffusion to tissues other than the regenerating muscle, so that with these factors taken into account, the concentration used in this study approximates the concentrations used in the study by Conlon *et al.* (1995).

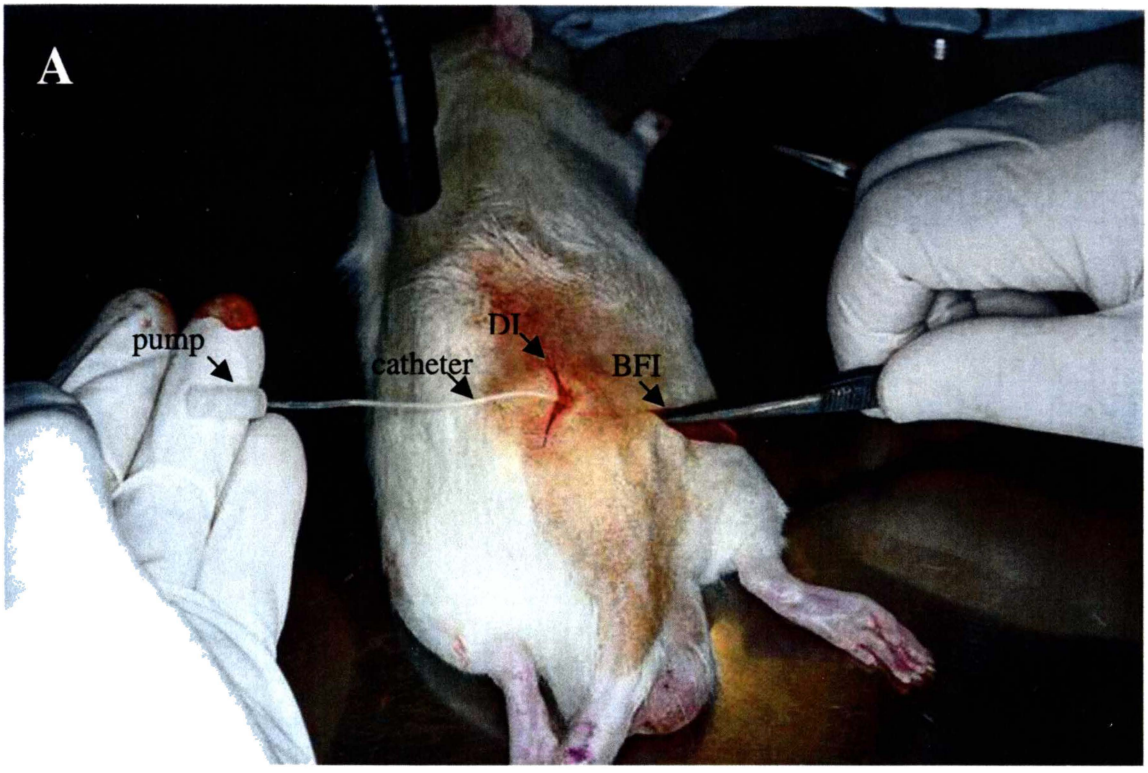
6.2.2 Surgical procedures

Seventy-five male Sprague-Dawley rats were housed in the Small Animal Colony of Ruakura Agricultural Research Centre. At approximately 7 weeks of age, 70 rats were anaesthetised with a solution of 5 mg/ml Rompun® (xylazine hydrochloride), 37.5 mg/ml ketamine hydrochloride in sterile water (0.2 ml per 100g bodyweight) then operated on to inject notexin and implant the Alzet pumps. Surgical manipulations began with the making of two incisions: one on the dorsal region on the right side of the body and slightly anterior to the M. *biceps femoris*, and the second directly over the M. *biceps femoris*. These two incisions were referred to as "DI" ("dorsal incision") and "BFI" ("*biceps femoris* incision"), respectively. The catheter portion of the pump/catheter assembly was fed through the DI out through the BFI as shown in Figure 6-1 A and B. The pump was sited just to the side of the DI (Figure 6-1B), and the DI closed with 9mm autoclips (Clay Adams, Becton-Dickinson, Franklin Lakes, NJ) (Figure 6-1C). The catheter was secured in place with polypropylene suture thread (Prolene blue monofilament with attached RB-1 taper needle, Johnson and Johnson, Janssen-Cilag Pty. Ltd., Newmarket, Auckland, New Zealand) within the cuff (see above) and approximately 1 cm above that, then the end of the catheter cut at an angle so that the angled end sat directly over the muscle (Figure 6-1 D and E). A loop was placed in the catheter, to allow the rat freedom of movement without dislodging the pump/catheter assembly (Figure 6-1 E). Figure 6-1 F shows the microsyringe (50 µl gastight microsyringe, 005229, SGE, Melbourne, Australia) with attached Luer-Lok microlance needle (26 gauge x 13mm, Becton-Dickinson) and 11.5mm long vinyl tubing sleeve (0.028mm inner diameter, 0.046mm outer diameter,

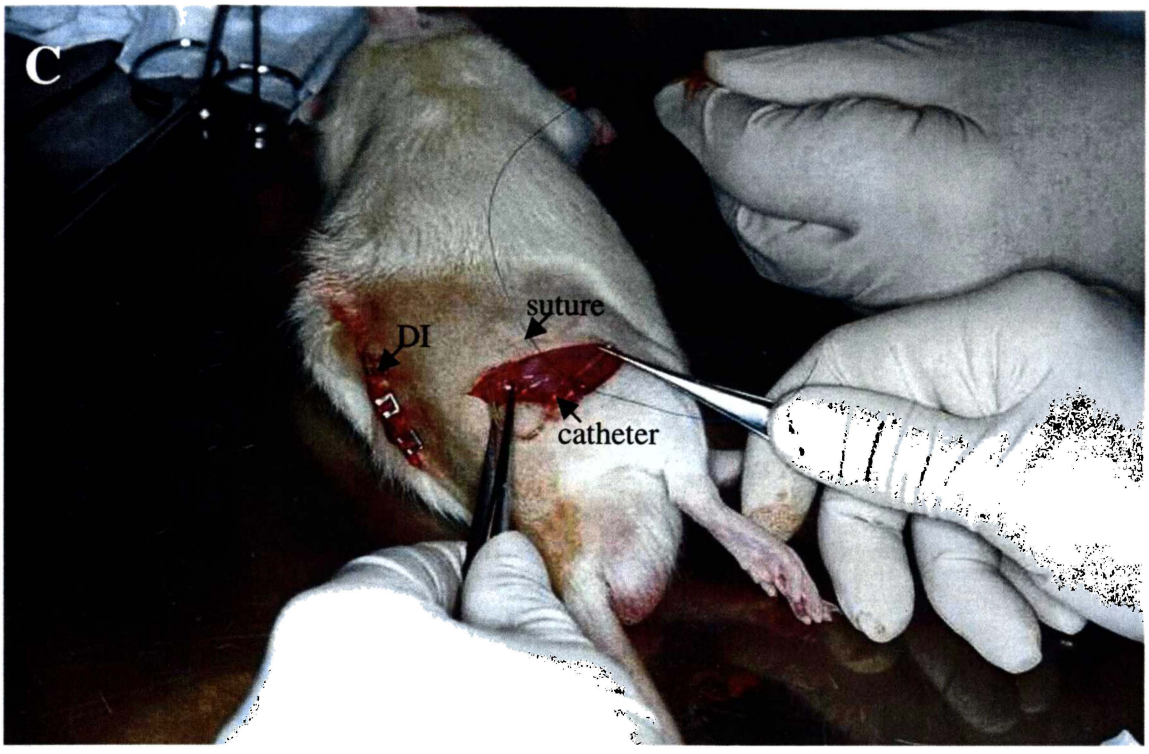
VX028, No 6129; Becton-Dickinson) that was used to administer the notexin. The sleeve was fitted onto the needle to prevent the notexin from being delivered too deep in the muscle, as it was critical in this trial to have the regeneration occurring as close as possible to the site of IGF-II/diluent administration. The 10ul volume of notexin was injected into the right M. *biceps femoris* directly under the angled end of the catheter tubing (Figure 6-1 G). Finally, the BFI was closed with wound clips (Figure 6-1 H). Rats were kept in a warm room until actively moving (approximately 45 minutes). Rats were walking normally and bearing weight on both legs without favouritism, as well as eating and drinking, within 2.5 hours of surgery.

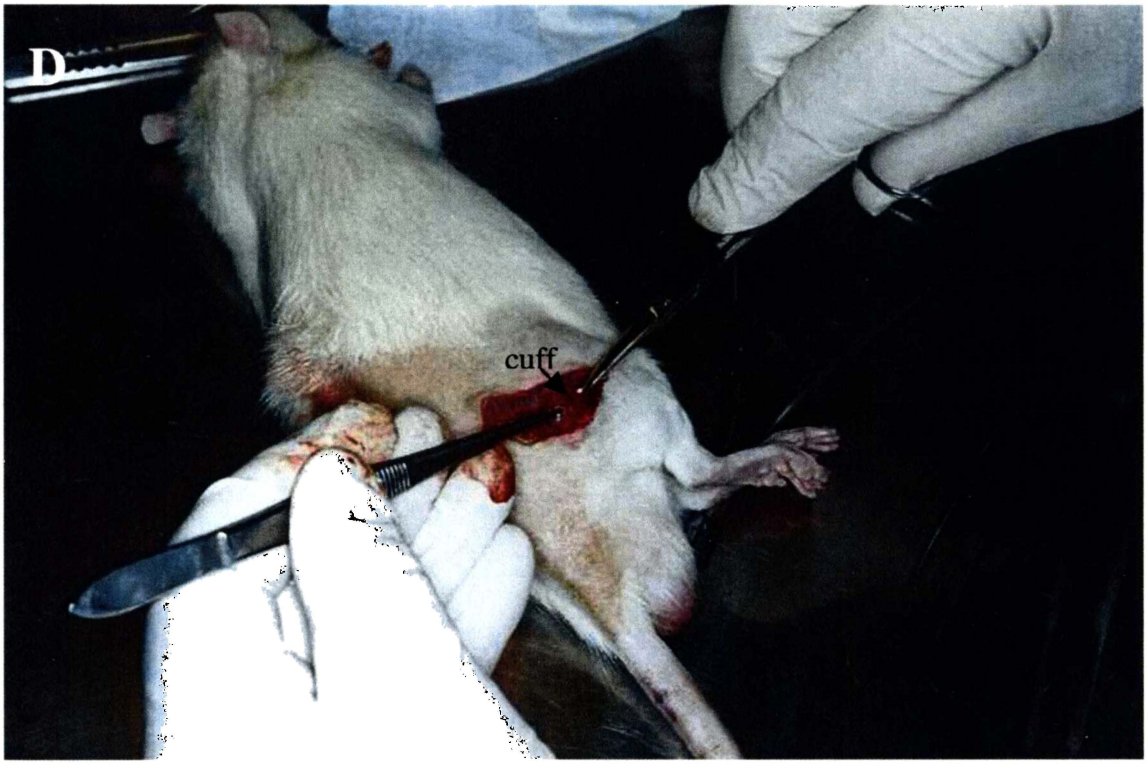
Figure 6-1. Alzet pump implantation and notexin injection.

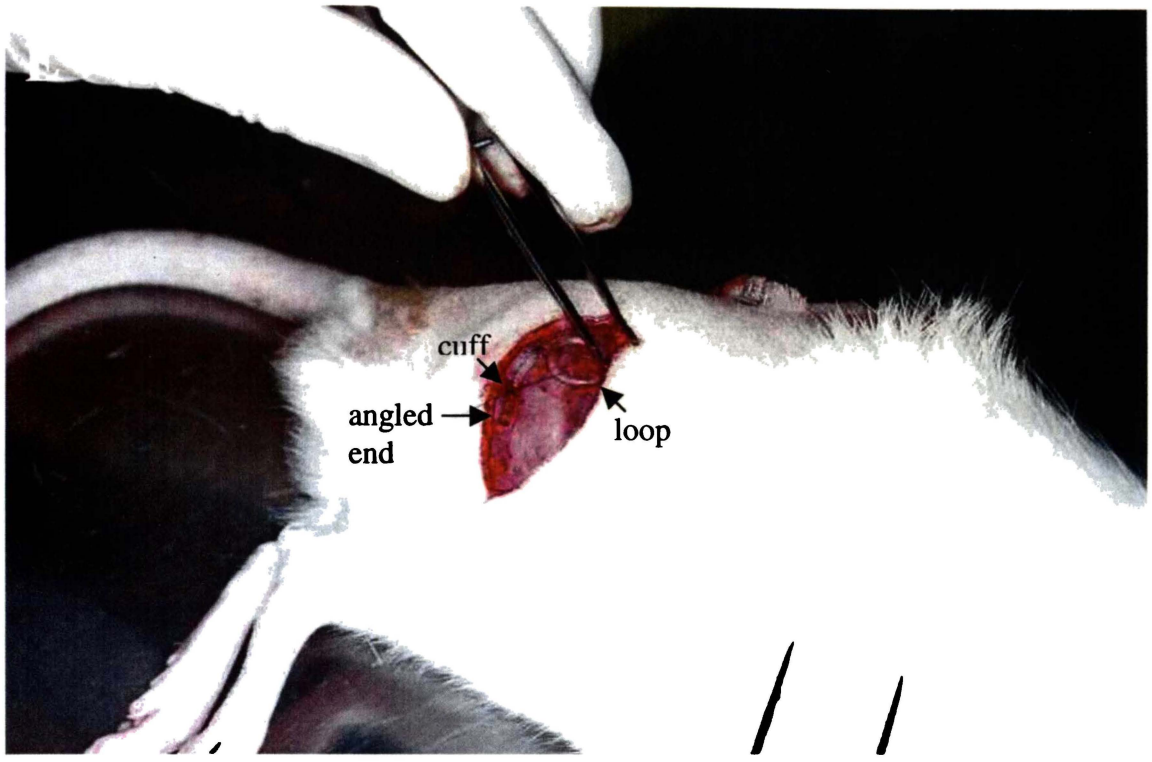
(A) The catheter attached to the Alzet pump ("pump") was fed through from the dorsal incision (DI) and out through the incision over the M. *biceps femoris* (BFI). (B) The pump was sited just to the side of the (DI), then (C) the DI closed with wound clips. (D, E) The pump and catheter assembly was secured in place, and the notexin injected (F, G) using a modified needle/microsyringe apparatus. (H) Finally, the BFI was closed with wound clips.

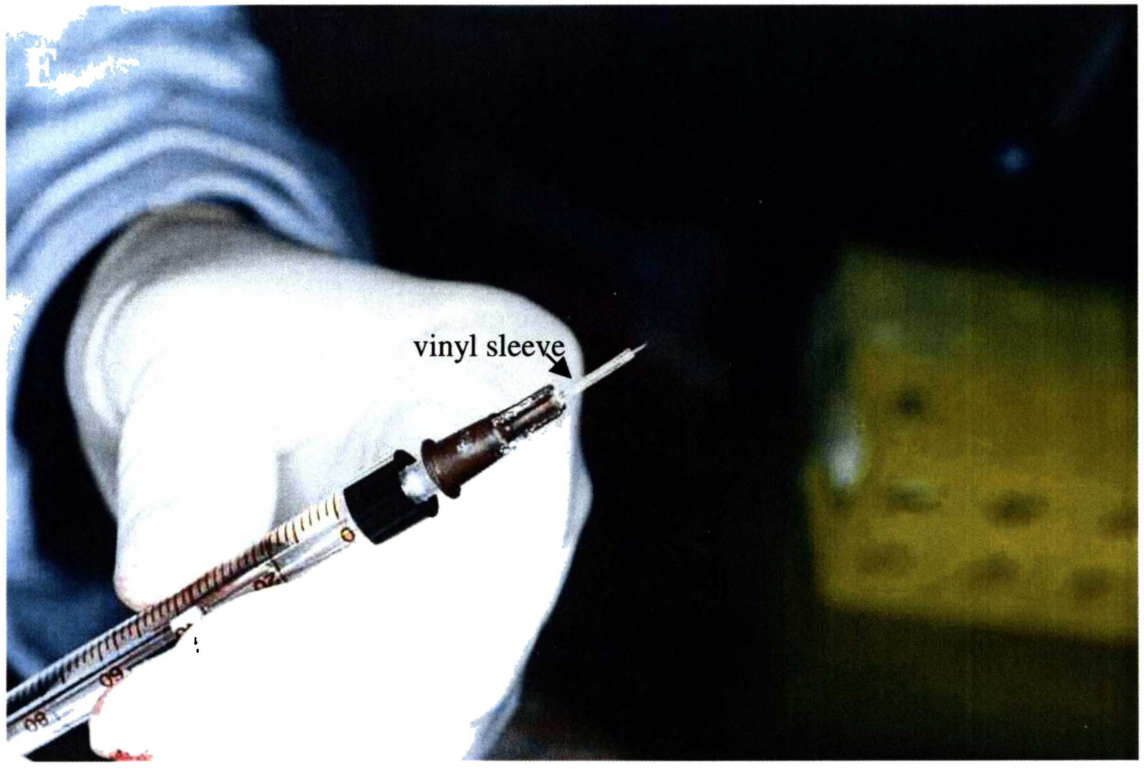

A
pump
catheter
DI
BFI

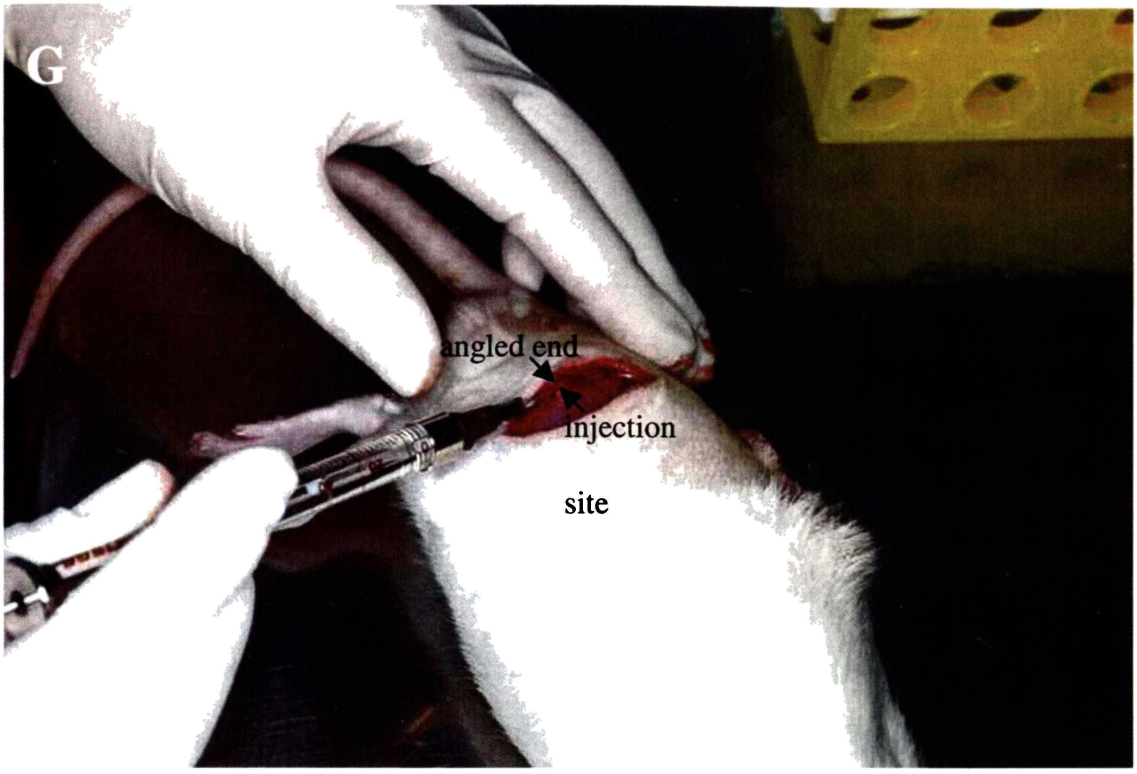

B
pump
catheter


C
suture
DI
catheter


D
cuff


cuff
angled
end
loop


vinyl sleeve


G
angled end
injection
site


DI
BFI

6.2.3 Sampling and tissue processing

Animals were sacrificed by CO_2 gas followed by cervical dislocation on days 0-7, 5 rats per treatment per timepoint. Prior to dissection of the leg muscles, the Alzet pumps were carefully removed from the flow moderator plus catheter assembly. Using a marker pen (Stephen's Vivid permanent waterproof marker, Bic NZ Ltd, Auckland, New Zealand), the site of peptide delivery/notexin injection was marked on the right M. *biceps femoris*. The catheter assembly and associated sutures were then cut away, and both right and left *biceps femoris* muscles dissected away from the hindlimb. Muscle tissue was both frozen for immunohistochemistry, and formalin-fixed, paraffin-embedded for routine histological staining, as described in Section 2.7.

6.2.4 Immunohistochemistry

Immunohistochemistry was utilised to identify the presence of myogenic proteins (MyoD, myogenin and dMHC), as markers of proliferation and differentiation, in regenerating muscle sections. The reason for choosing a microscopic approach over methods such as Northern or Western analysis, where tissues are ground up prior to analysis, is that the former method allows for the study of areas of similar damage. The core of damage (refer to Section 3.1.3) was chosen for examination in all facets of this trial because of its proximity to the site of peptide administration. The core of damage represents the site where notexin is most abundant, ie. the site of injection. This site was purposefully located just below the site of peptide infusion, in order to optimise for peptide effects on regenerating muscle tissue.

Cross-sections of regenerating muscle tissue were carefully sectioned in order to obtain an optimal core of damage. These tissue sections were then incubated with MyoD, myogenin and dMHC antibodies according to the protocol listed in Section 2.9. Nuclei that were positive or negative for a given marker were quantitated, using the ScionImage system (refer to Section 2.11.3). Five areas, generated by random coordinates, were counted for each animal. These values were then used to yield the percent positive nuclei per total nuclei.

6.2.5 Fibre area

The purpose of this analysis was to determine the size of regenerated fibres at the last timepoint (day 7). Images of regenerating muscle were captured from the core of damage in sections that had been immunostained with dMHC antibody. The captured images (one per animal) were obtained using the Olympus BX-50 microscope fitted with a 5.0x photo eyepiece and 10x objective, which was attached to the ScionImage system (as described in Section 2.11.3). To determine regenerating fibre size in the ScionImage programme, individual fibres were outlined using the freeform selection tool, then the "measure" command used to generate the fibre area (in pixels). The counted fibres were numbered and the data recorded for each individual fibre. The number of regenerating fibres analysed per animal ranged from 31 to 89. A conversion factor of $2.46 \times 10^{-7} mm^2$ /pixel was used to convert the data to actual numbers.

6.2.6 Type 1 receptor autoradiography

Receptor autoradiography was performed, as detailed in Section 2.10, on frozen muscle sections taken one day after notexin-injection. Muscle sections were incubated with ^{125}I- rh IGF-I alone or in the presence of unlabelled competing peptides including the following: 1 μg/ml (rh) N-Met IGF-I, 1 μg/ml des(1-3) IGF-I, 1 μg/ml rh IGF-II, or 10 ng/ml bovine insulin. Macroautoradiographic analysis of the incubated section images on XAR film showed no difference in the displacement of ^{125}I-IGF-I binding by unlabelled des(1-3)-IGF-I versus unlabelled IGF-I, indicating that the binding observed was to receptor and not binding protein. Grain counting to determine Type I IGF receptor levels was performed as detailed in Section 2.11.3.

6.2.7 Statistics

Body and muscle weights were adjusted for initial liveweight, then analysed by ANOVA for the overall effect of time and treatment. Values shown are means ± SEM.

MyoD, myogenin, and dMHC data were log-transformed then analysed by Student's t-test for differences at individual timepoints, or by ANOVA to determine overall effects. Day 1 values were omitted from myogenin and dMHC overall analyses due to high "zero" components in the data sets. Values shown for MyoD, myogenin and dMHC are least squares means, and the errors shown are SEMs.

Fibre area and receptor autoradiography data were analysed by Student's t-test, and the values given are means ± SEM.

6.3 Results

6.3.1 Body and muscle weights

The body weights of notexin-injected rats changed significantly ($p<0.001$) over time, with a decline to day 2 values, then a steady increase in weight over the remaining days (Figure 6-2). By days 6/7, bodyweights were similar to day 0 (pre-treatment) values. There was no significant effect of IGF-II on body weight, and no interaction of IGF-II treatment with time.

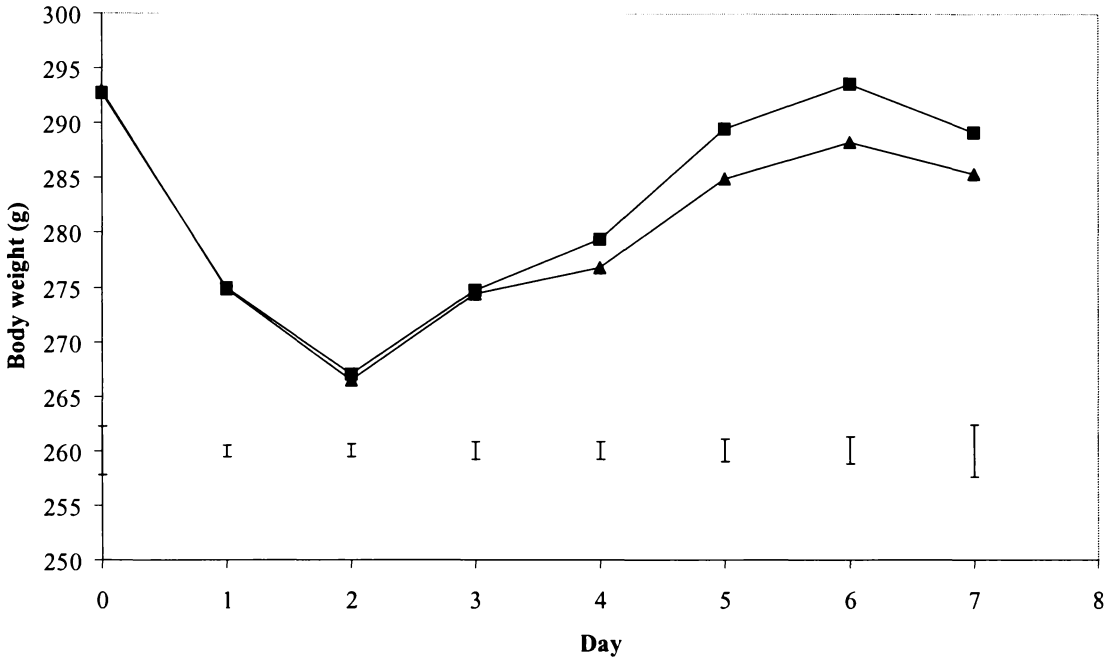


Figure 6-2. Effect of IGF-II on body weight.

Body weights of IGF-II treated and control rats following administration of notexin at time 0 are shown. Control (■) and IGF-II treated (▲) values are means (n=4-5), bars indicate one SEM.

Non-injected muscle weights changed significantly (p=0.004; Figure 6-3) over time following notexin administration. Non-injected muscle weights decreased between days 0 and 1, remained low until day 4, and then gradually increased through to day 7. There was no significant difference in non-injected muscle weights between IGF-II and control groups at any timepoint, or overall, nor any interaction of treatment with time.

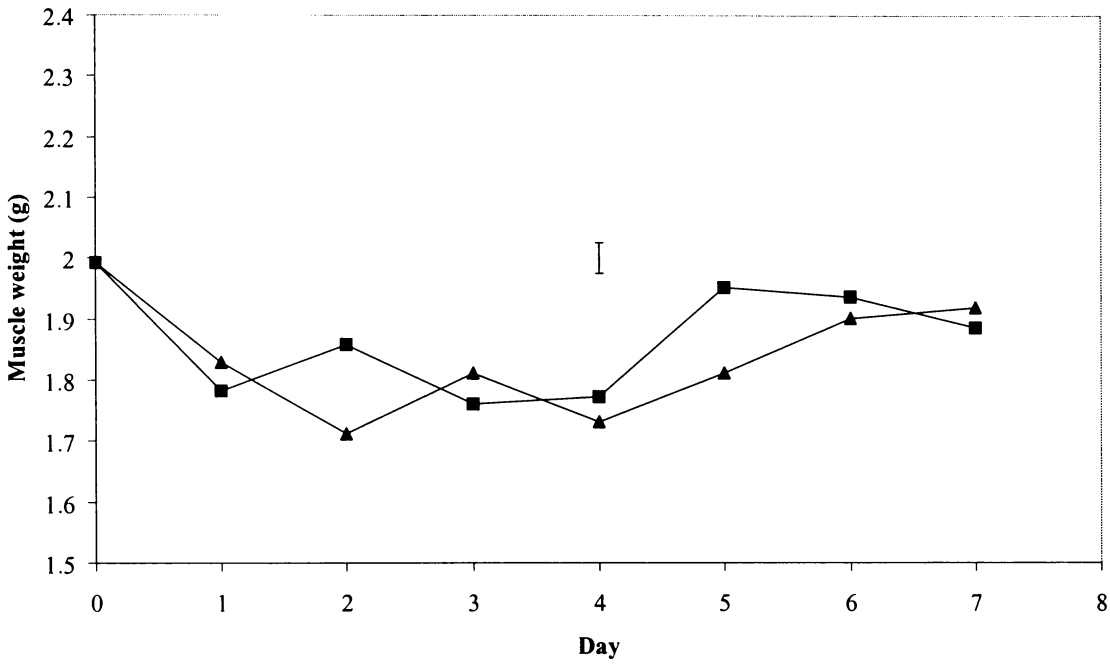


Figure 6-3. Effect of IGF-II on non-injected muscle weights.

Non-injected M. *biceps femoris* weights following administration of notexin at time 0. Control (■) and IGF-II treated (▲) values are means (n=4-5), and the bar indicates one SEM.

Notexin-injected muscle weights changed significantly (p<0.001; Figure 6-4) over time after damage on day 0. Notexin-injected muscle weights showed an initial increase on day 1, a sharp decline to day 2 values, then little change through to day 7. As was the case for non-injected muscle weights, there were no differences in notexin-

injected muscle weights between IGF-II and control groups at any timepoint, or overall, and no interaction of time versus treatment.

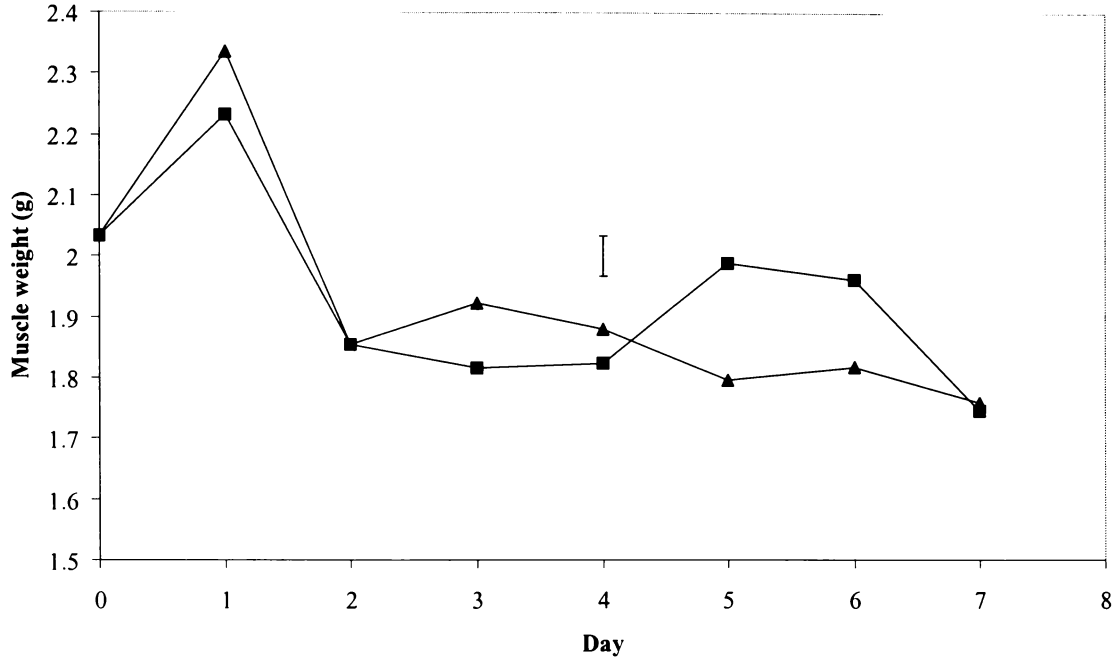


Figure 6-4. Effect of IGF-II on notexin-injected muscle weights.

Notexin-injected M. *biceps femoris* weights following administration of notexin at time 0. Control (■) and IGF-II treated (▲) values are means (n=4-5), and the bar indicates one SEM.

6.3.2 Muscle histology

The timecourse of muscle regeneration, within the core of damage in animals that were not treated with IGF-II, is depicted in Figure 6-5. In undamaged (day 0) animals fibres are whole, and contain low numbers of mononucleate cells (Figure 6-5A), however by day 1 in notexin-injected control muscle, substantial numbers of infiltrating phagocytic and inflammatory cells are observed both within and outside of muscle fibres (Figure 6-5B). Approximately 50% of damaged muscle fibres in control

animals showed signs of phagocytic infiltration, and considerable necrotic fibre cytoplasm fragmentation was observed. Day 2 control muscles (Figure 6-5C) contained large accumulations of mononucleate cells, with low to moderate amounts of necrotic debris. Fusion was first observed on day 3 following notexin-injection (Figure 6-5D), with high levels of myotube formation occurring on both days 3 and 4, such that the bulk of the regenerated myofibres had been formed by day 4. Myotube enlargement occurred between days 5-7, with the myotube diameter at day 7 still less than that of survivor fibres (Figure 6-5E).

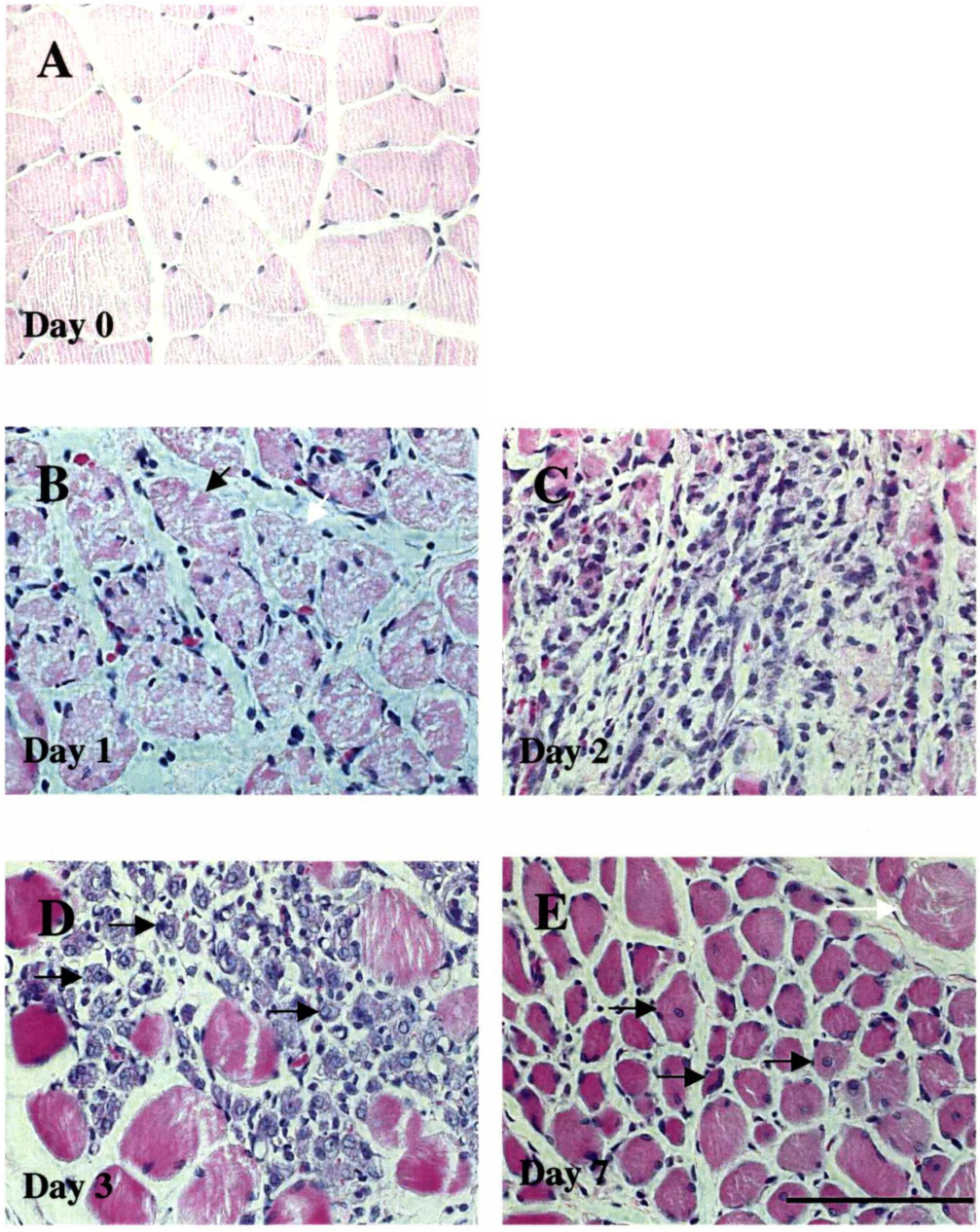


Figure 6-5. Muscle regeneration in control animals.

(A) shows undamaged (day 0) muscle fibres prior to notexin injection. On day 1 following notexin-injection (B), muscle fibres are both infiltrated (white arrow) or non-infiltrated (black arrow) by phagocytic cells. By day 2 of damage (C), mononucleate cells, often in the absence of necrotic debris, are seen in the heavily damaged areas, and by days 3 and 4 (D; day 3 shown) immature myotubes are evident (black arrows). (E) By day 7 after notexin-injection, regenerating myotubes (black arrows) have enlarged, but are still smaller in diameter than the survivor fibres (white arrow). Haematoxylin and eosin stain; magnification = 50x, bar = 100 microns.

Regeneration of the two treatment groups was compared histologically by examining haematoxylin and eosin stained sections of regenerating muscle. There was no histological difference in the rate of muscle regeneration between IGF-II and control groups on day 1 after damage, as both groups contained approximately 50% infiltrated fibres. However by day 2 there were obvious differences in the regenerating muscle, as shown in Figure 6-6. Day 2 control tissues had considerably higher numbers of mononucleate cells with the morphological appearance of MPC (refer to Section 3.1.3) present in the regenerating areas. Conversely, the IGF-II treatment group contained considerably fewer mononucleate cells, and of these mononucleate cells, many appeared to be phagocytic, as assessed by their cellular morphology (multi-lobed nuclei with extensive cytoplasm (polymorphonuclear leucocytes), or cells with a much greater cytoplasm to nuclear ratio than is found in MPC (macrophages)). Furthermore, the IGF-II treated regenerating muscle contained higher amounts of necrotic debris relative to the control group (Figure 6-6), consistent with the greater proportion of phagocytic cells present in the IGF-II treated muscle.

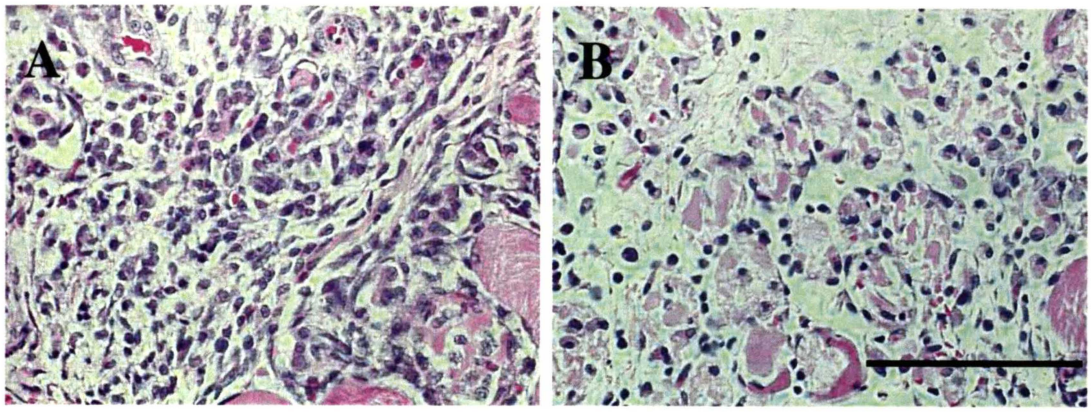


Figure 6-6. Histology of control and IGF-II-treated regenerating muscle.

Sections of control (A) and IGF-II treated (B) muscles on day 2 of regeneration are shown. Regenerating muscle sections of control rats (A) have high numbers of mononucleate cells with the morphological appearance of MPC, and sparse necrotic debris, while IGF-II treated (B) muscles have a high proportion of cells with the morphological appearance of phagocytes, and greater amounts of necrotic debris. Haematoxylin and eosin stain, magnification 50x, bar= 100 microns.

6.3.3 MyoD

MyoD was used as a marker of proliferating MPC in this study. Immunocytochemical staining of muscle sections showed changes in the number of nuclei positive for MyoD during the process of muscle regeneration. Specifically, MyoD positive nuclei were observed infrequently in undamaged (day 0) muscle sections. In regenerating muscle from day 1 onwards, MyoD protein was observed in mononucleate cells (Figure 6-7) either in the presence or absence of necrotic debris. The proportion of nuclei that were positive for MyoD protein was less than that observed for either myogenin or dMHC. MyoD protein was never observed in myotube nuclei, however after substantial myotube formation, MyoD(+) mononucleate cells were often observed closely aligned to myotubes, presumably in satellite cells.

Immunohistochemistry using this particular protocol did result in low level background staining, staining that was also observed in negative control sections (Figure 6-7), and so considered to be non-specific. This light background staining was easily distinguished from the specific nuclear staining of MyoD, and never interfered with the detection of MyoD positive nuclei.

Figure 6-8 shows the frequency of MyoD protein in the core of damage of regenerating muscle in treated and control rats over the period of days 1-4. This time period was chosen for an examination of MyoD, myogenin and dMHC levels because it included virtually all proliferation and differentiation events in these tissues. MyoD positive nuclei were counted, then expressed as a percentage of total nuclei, in order to determine the effect of IGF-II on the proliferation of MPC. The results of this quantitation show that there was a significant change over time ($p<0.001$), with an increase up to day 2 in the percentage of nuclei that contain MyoD protein, after which time levels decreased. On day 1 there is less MyoD protein in the IGF-II treated group relative to the control group ($p= 0.057$; Figure 6-7 and Figure 6-8).

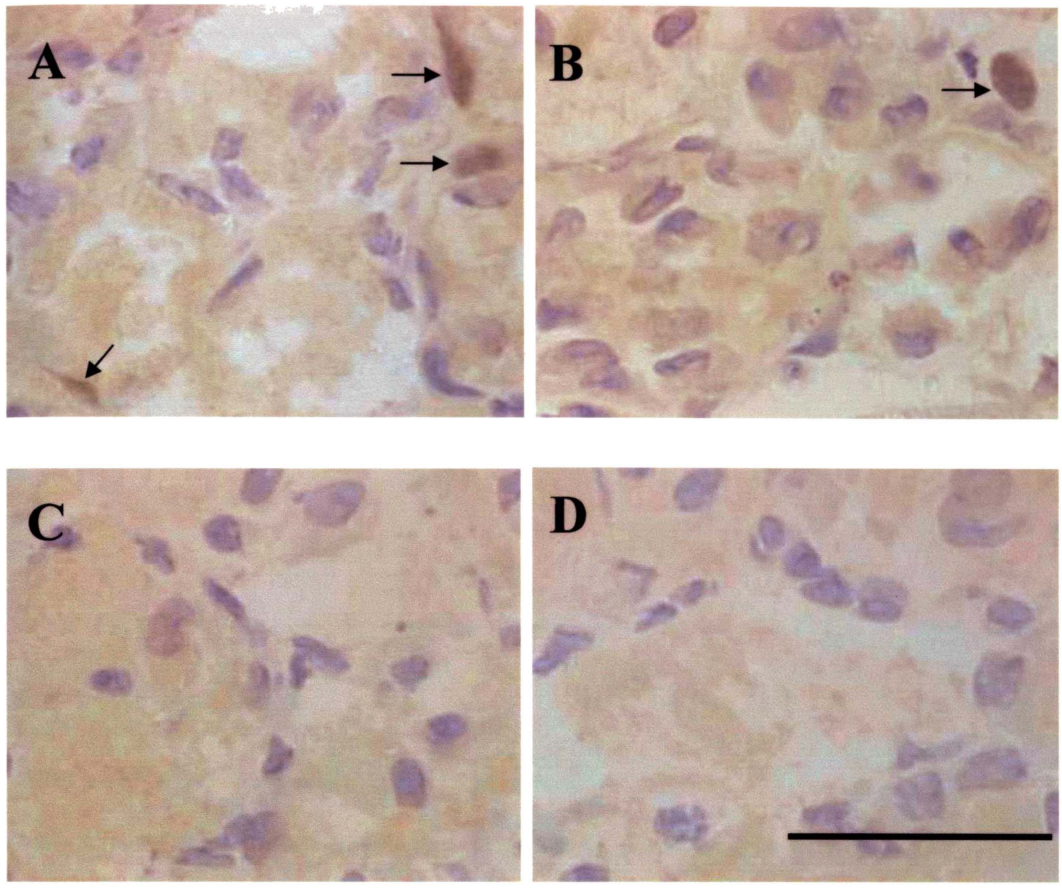


Figure 6-7. Localisation of MyoD protein.

MyoD immunohistochemistry in day 1 regenerating muscle of control (A) and IGF-II (B) treated animals. MyoD protein (arrows) was localised within nuclei in regenerating areas. Negative control sections (C,D) were devoid of nuclear staining. The proportion of nuclei that contained MyoD protein was greater (p=0.057) in control versus IGF-II-treated muscles. Sections were counterstained with haematoxylin. Magnification 100x, bar=40 microns.

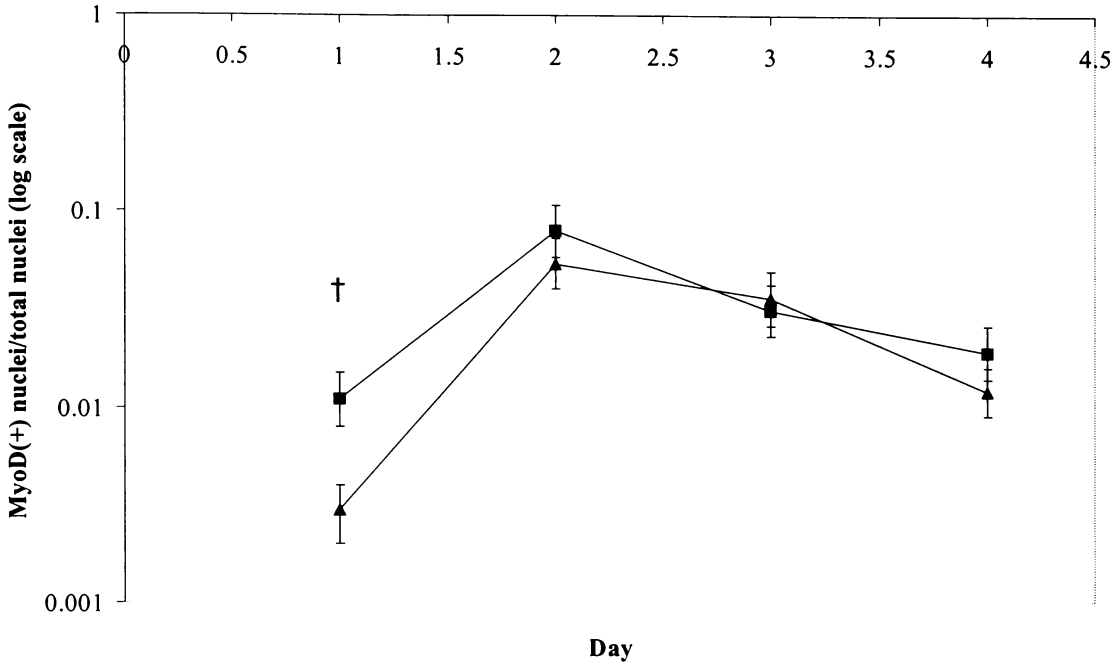


Figure 6-8. Proportion of MyoD positive nuclei.

MyoD protein expression in regenerating muscle of control (■) and IGF-II (▲) treated rats. Values are means (n=3-5) ± SEM. † indicates significance (p=0.057) between control and IGF-II treatment groups for the timepoint shown.

6.3.4 Myogenin

Myogenin protein was present only occasionally (approximately 1 per 250 nuclei) in undamaged (day 0) muscle. These myogenin positive nuclei were near the periphery of muscle fibres, suggestive of localisation within satellite cells. In damaged muscle, virtually all myogenin was contained within the nuclei of mononucleate MPCs (Figure 6-9). After fusion, myogenin protein was sometimes observed in very immature myotubes, within the cytoplasm and less often within the myotube nuclei. Larger, more mature myotubes did not contain myogenin protein.

Myogenin positive nuclei in mononucleate MPC in day 1-4 regenerating muscle were counted within the core of damage, and expressed relative to total nuclei, the results of which are shown in Figure 6-10. The percentage of nuclei that were positive for myogenin changed significantly over time ($p<0.001$) with an increase up to day 3, followed by a decrease to day 4 values. Minimal numbers of myogenin positive nuclei were observed in day 1 tissues, as only one of the five control muscles had non-zero values, while all IGF-II treated rat muscles yielded zero values when counted. Because of the low numbers of myogenin positive nuclei observed on day 1, these values were omitted from the overall statistical analysis of myogenin data. On day 2, prior to the peak in myogenin values, IGF-II treated muscle had significantly less ($p=0.034$) myogenin protein than control muscle (Figure 6-9 and Figure 6-10). No significant effect of IGF-II treatment on myogenin protein was observed on days 3 or 4.

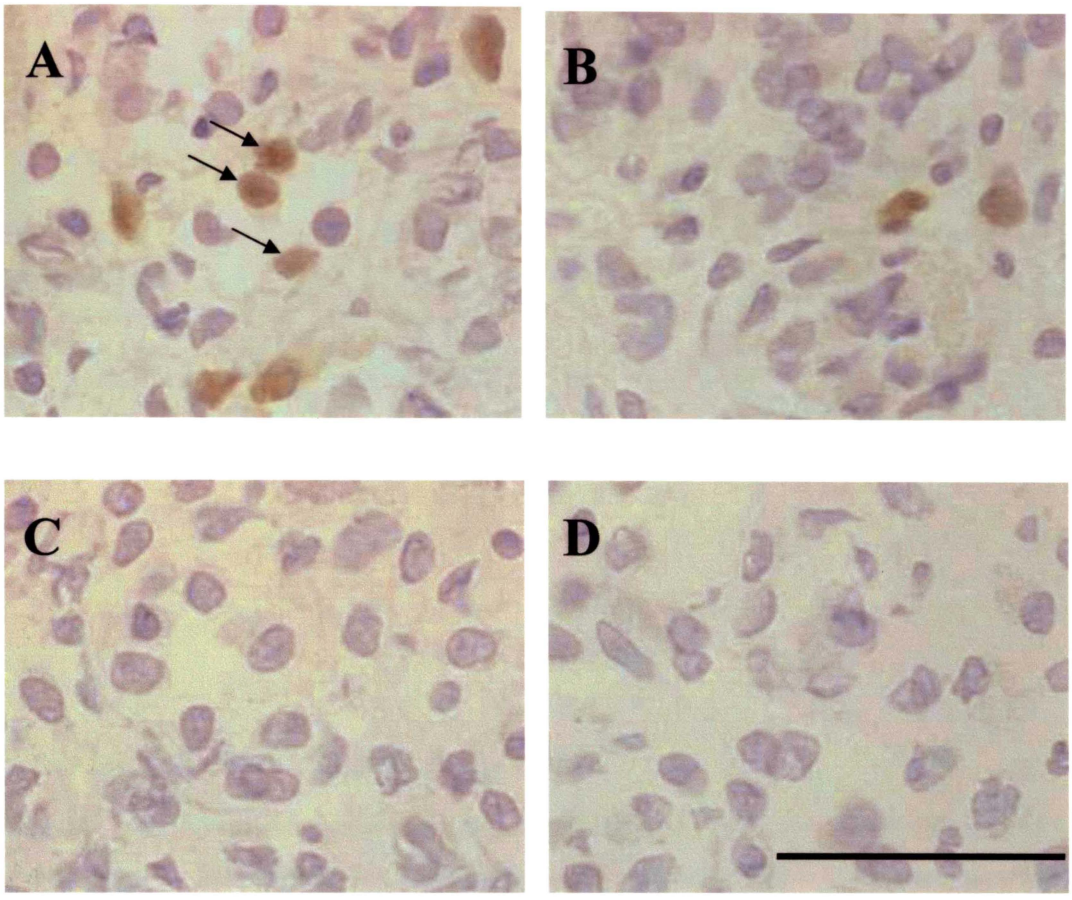


Figure 6-9. Localisation of myogenin protein.

Myogenin immunohistochemistry in muscle sections of control (A) and IGF-II- (B) treated animals 2 days after notexin treatment. Myogenin protein was localised almost exclusively within nuclei of mononucleate MPC (arrows indicate examples of myogenin positive nuclei) within regenerating areas in the first 4 days following damage. Negative control sections are shown in (C) and (D) for control and IGF-II animals, respectively. Sections were counterstained with haematoxylin. Magnification 100x, bar=40 microns.

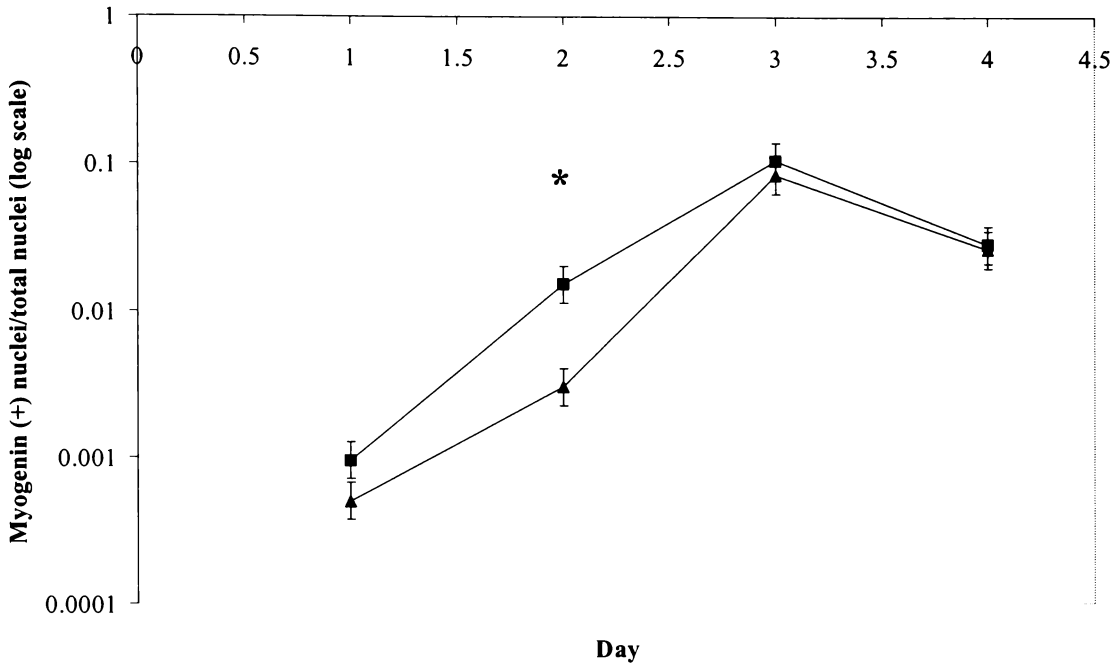


Figure 6-10. Proportion of myogenin positive nuclei.

Myogenin protein expression in regenerating muscle of control (■) and IGF-II (▲) treated rats on days 1-4 after notexin-injection. Values are means (n=3-5) ± SEM. * indicates significance (p=0.034) between control and IGF-II treatment groups for the timepoint shown.

6.3.5 DMHC

No dMHC protein was observed in undamaged fibres, or in day 1 damaged muscles. By days 2 and 3 however, dMHC was observed in individual nuclei in regenerating areas (day 3, Figure 6-11B). As regeneration proceeded, dMHC became localised within the cytoplasm, but not in the nuclei, of regenerating myotubes (Figure 6-11A). Immunostaining with dMHC antibody identified a very flattened morphology within the early regenerated myotubes. These early myotubes stained particularly intensely for dMHC protein. Immunostaining with the dMHC antibody within the core of damage clearly showed the presence of newly regenerated myotubes that appeared to

be using earlier, larger regenerated myotubes as a scaffold. For quantitation purposes, nuclei were considered dMHC positive if they fitted either of the following criteria: a) they were dMHC(+) mononucleate cells, or b) they were myonuclei contained within newly regenerated dMHC positive myotubes.

The results of the quantitation of dMHC protein in regenerating muscle sections are shown in Figure 6-12. This figure shows that the percentage of dMHC positive nuclei changed significantly over time ($p<0.001$), with a sharp rise in dMHC positivity up to day 3, followed by a more gradual increase to day 4. Analysis of day 3 dMHC values revealed a significant ($p=0.047$; Figure 6-11, Figure 6-12) decrease in the percentage of dMHC positive nuclei in IGF-II treated muscles relative to control muscles, while days 2 and 4 showed no significant difference between the two treatment groups.

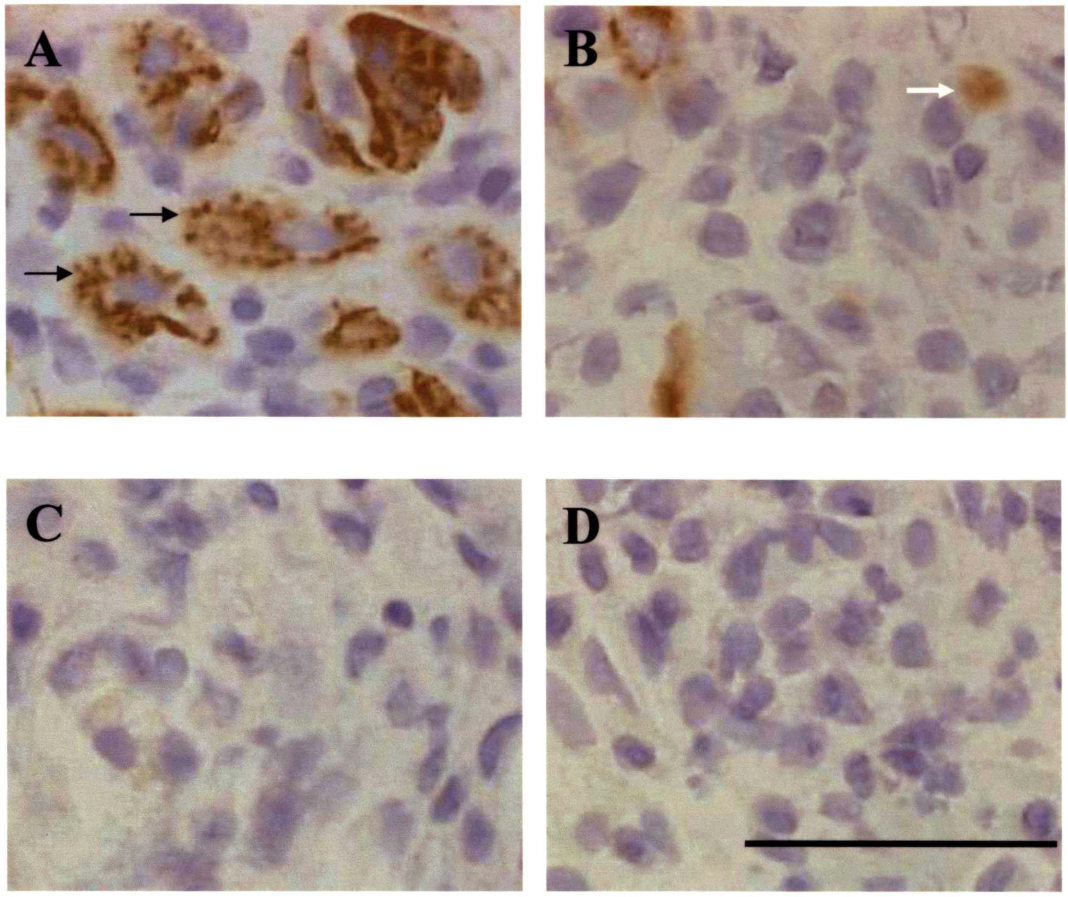


Figure 6-11. Localisation of dMHC protein.

Immunolocalisation of dMHC protein in regenerating muscle sections of control (A) and IGF-II- (B) treated animals 3 days after notexin injection. DMHC protein was detected in the nuclei of mononucleate cells during early regeneration (white arrow), and almost exclusively in the cytoplasm of myotubes (black arrows) in tissues that were further along in the regeneration timecourse. Negative control sections are shown in (C) and (D) for control and IGF-II animals, respectively. Sections were counterstained with haematoxylin. Magnification 100x, bar=40 microns.

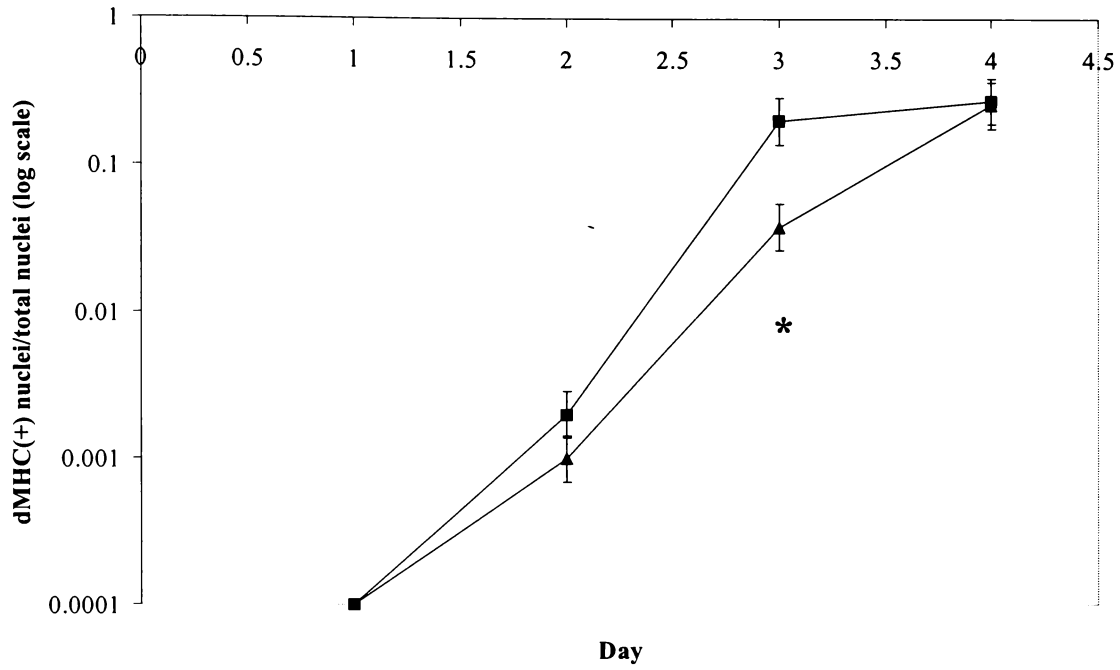


Figure 6-12. Proportion of dMHC positive nuclei.

DMHC protein expression in regenerating muscle of control (■) and IGF-II (▲) treated rats on days 1-4 after notexin-injection. Values are means (n=3-5) ± SEM. * indicates significance (p=0.047) between control and IGF-II treatment groups for the timepoint shown.

6.3.6 Muscle fibre size

Muscle fibre size was measured at the final sampling point to determine a) whether the treatment effect of IGF-II, as previously noted on days 1 (MyoD), 2 (myogenin), and 3 (dMHC), was still apparent at day 7, and b) whether the two groups had different rates of fibre enlargement in the newly-regenerated muscle fibres. Figure 6-13 shows the microscopic appearance of regenerated fibres in day 7 IGF-II treated and control groups. The results of the fibre size analysis (Figure 6-14) show that the IGF-II treated muscle contains significantly larger regenerated muscle fibres than did the control muscles on day 7 (p=0.0092).

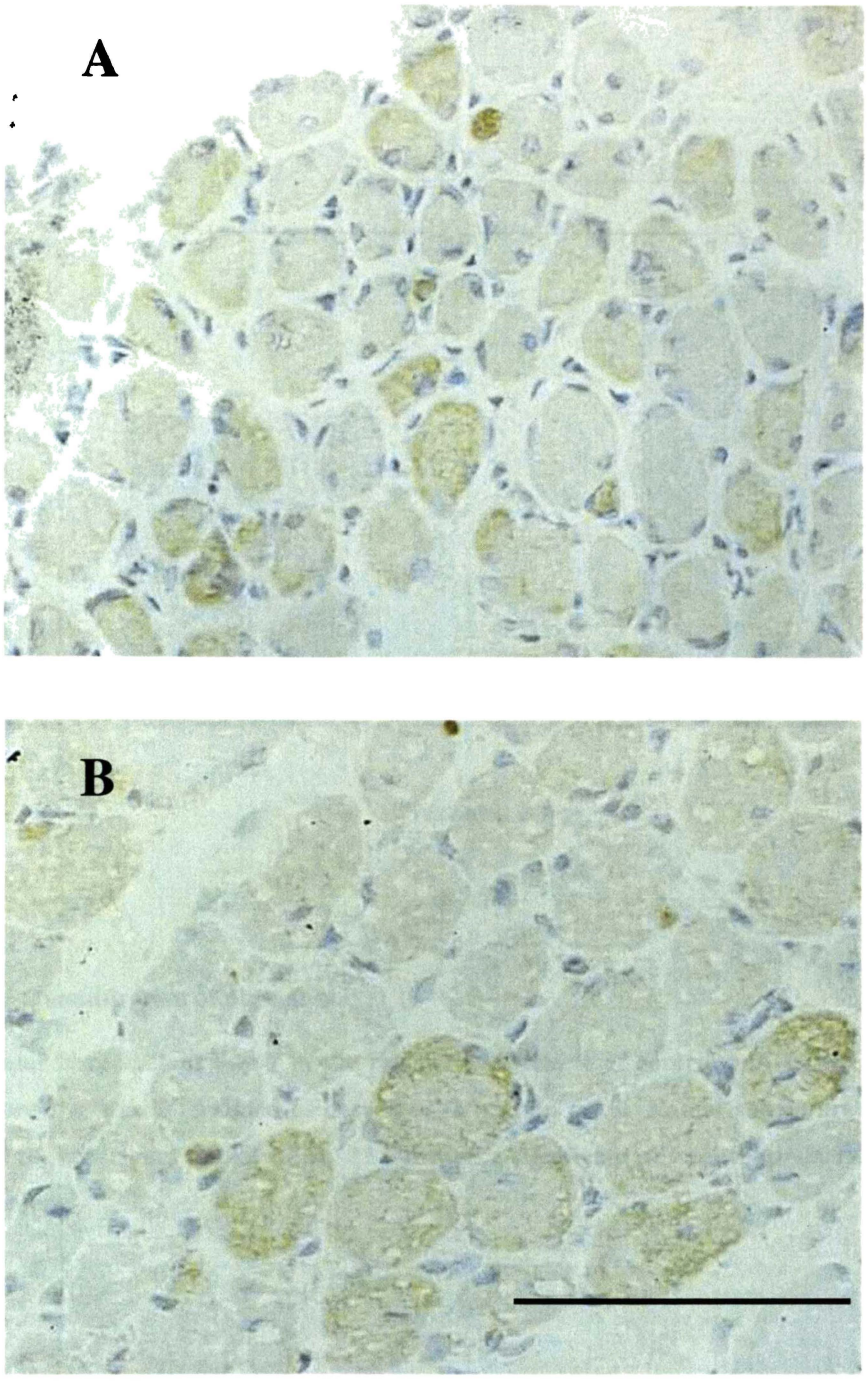


Figure 6-13. Effect of IGF-II treatment on the area of regenerating fibres.

Cross-sectional fibre area in control (A) and IGF-II-treated (B) regenerating muscle 7 days after injury. Fibre areas of IGF-II-treated muscles were significantly greater than those of control muscles. These sections have been immunostained with anti-dMHC antibody, then counterstained with haematoxylin. Magnification 50x, bar= 100 microns.

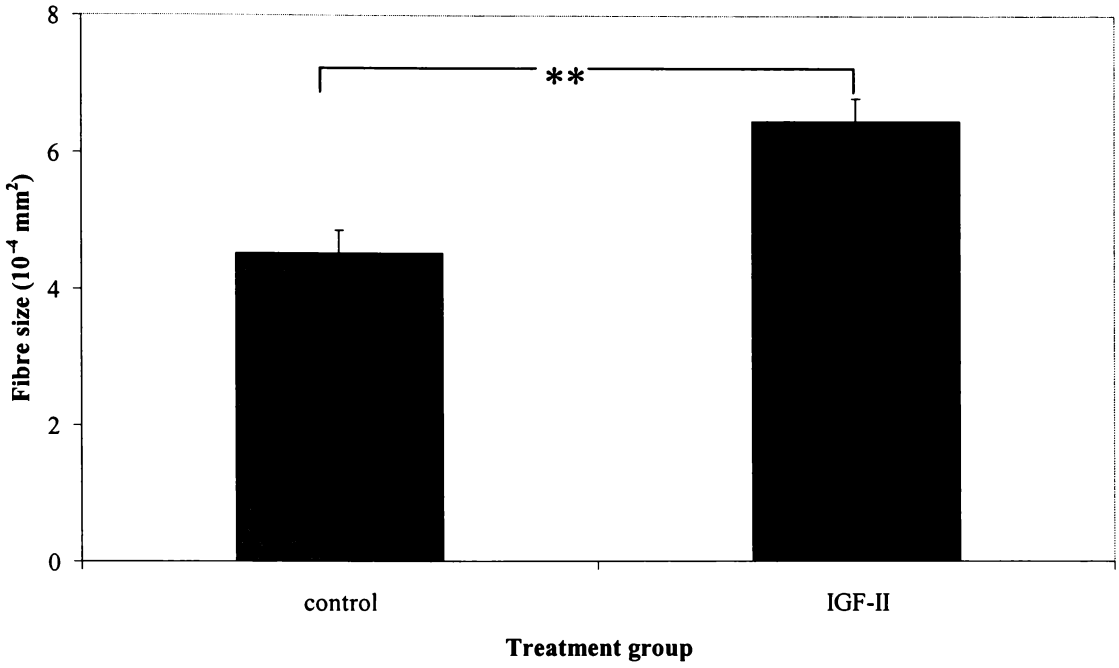


Figure 6-14. Quantification of fibre area.

Cross-sectional fibre area at day 7 in the regenerated muscle of control and IGF-II treatment groups. Fibre size was determined as described in Section 6.2.5. Values shown are the means (n=3/4), and the error bars show 1 SEM. ** indicates a significant difference (p=0.0092) between treatment groups.

6.3.7 Type 1 IGF receptor autoradiography

Receptor autoradiography was chosen to determine levels of Type I IGF receptor in regenerating muscle from control and IGF-II treatment groups on day 1. This timepoint was chosen because the earliest differences in the expression of myogenic markers in response to IGF-II were noted on day 1, suggesting a possible difference in

receptor levels on, or prior to, this time. The results of this analysis show that the Type I IGF receptor levels were greater in the control (0.247 ± 0.073 grains/ μm^2) than in the IGF-II-treatment (0.137 ± 0.063 grains/ μm^2) group however this difference was not significant (p=0.311; Figure 6-15).

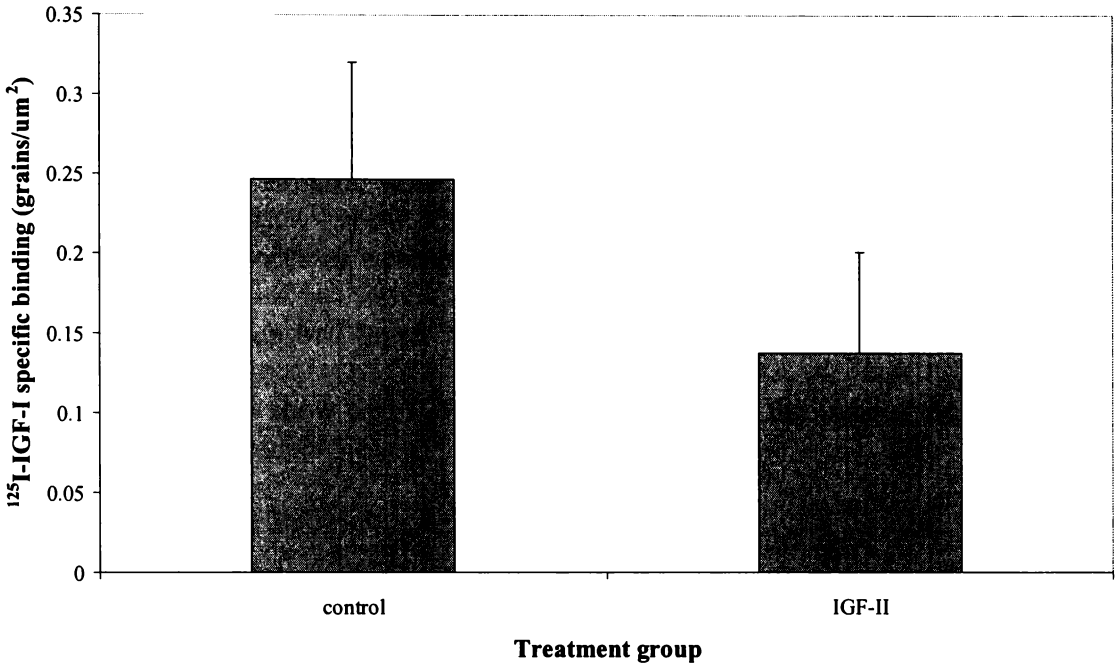


Figure 6-15. Specific binding of ^{125}I- IGF-I to day 1 damaged muscle.

Type I IGF receptor levels in regenerating muscles from control and IGF-II treatment groups on day 1 following notexin injection. Tissue sections were incubated with ^{125}I-labelled IGF-I, then the bound peptide visualised as silver grains in an overlying emulsion layer, as described in Section 2.10. Grains were counted as per Section 2.11.3 and used to generate the density of radiolabelled IGF-I in treated and control tissues. The Type 1 IGF receptor levels in treated and control tissues was not significantly different (p=0.311). Values shown are the means ± SEM.

6.4 Discussion

The aim of this study was to determine whether the processes of MPC proliferation and differentiation during skeletal muscle regeneration are altered by the continuous administration of IGF-II to the damaged muscle. The MRFs, MyoD and myogenin, were used as markers of proliferation and early differentiation, respectively, while dMHC was employed as a later marker of differentiation. This study clearly shows that the administration of IGF-II to regenerating muscle delayed both proliferation and differentiation. Furthermore, fibre size analysis showed that although proliferation and differentiation processes were delayed in the IGF-II-treatment group, by day 7 the IGF-II treatment group had larger regenerated muscle fibres relative to the control group. These results show that IGF-II had varying actions throughout the course of regeneration.

The present study identified induction times of less than 1 day for MyoD, 1-2 days for myogenin, and 2 days for dMHC protein expression in regenerating rat M. *biceps femoris*. Similarly, it is well established in the literature that MyoD expression precedes that of myogenin (Cornelison *et al.* 2000; Smith *et al.* 1994) and that myogenin expression precedes the expression of sarcomeric myosin heavy chain isoforms (Andres & Walsh 1996). Peak values for the number of nuclei positive for MyoD, myogenin, and dMHC were observed on days 2, 3, and 4, respectively. These observed patterns of MyoD, myogenin and dMHC protein induction and peak values are virtually identical to those reported by Yablonka-Reuveni and Rivera (1994) for activated satellite cells undergoing myogenesis on cultured rat muscle fibres. Our

results combined with those of Yablonka-Reuveni and Rivera (1994), support the existence of an approximate 24-hour lag between MyoD(+), myogenin(+), and dMHC(+) compartments for rat MPC and muscle fibres.

IGF-II has been firmly established as a proliferation and differentiation-enhancing peptide, with the evidence for this coming from a number of *in vitro* studies (Minniti *et al.* 1995; Prelle *et al.* 2000; Stewart *et al.* 1996). Given the body of evidence for positive effects of IGF-II on proliferation and differentiation in culture, the observation of an opposite effect in this regeneration experiment was surprising. Furthermore, the effects were of a highly consistent, sequential nature, with significant differences observed on day 1 for MyoD, day 2 for myogenin, and day 3 for dMHC, and the significant treatment effects always occurred prior to peak marker expression. This pattern, taken together with the fact that the effect of IGF-II treatment occurred as early as day 1, suggests that IGF-II may have affected a process up to, or on, day 1 following damage, and that this initial set-back of the regeneration process by IGF-II was simply carried on through until at least the stage of myotube fusion. One key possibility that was considered for these initial effects of IGF-II was down-regulation of the Type 1 IGF receptor.

IGF-II can down-regulate the Type 1 IGF receptor in muscle cells via transcriptional down-regulation and/or increased receptor degradation (Rosenthal & Brown 1994; Rosenthal *et al.* 1991). IGFs bind to, and exert effects through, the Type I IGF receptor during the stimulation of MPC proliferation and differentiation (Ewton *et al.* 1987; Rosenthal *et al.* 1994). Functional inactivation of the Type I IGF receptor

results in delayed differentiation in mouse C2C12 cells (Cheng *et al.* 2000). In the present trial, the level of Type I IGF receptor in day 1 regenerating muscle was measured in order to determine whether administration of IGF-II effected down-regulation of the Type 1 IGF receptor which may have in turn caused the delays in proliferation and differentiation. Results show that while the density of Type I IGF receptors is lower in the IGF-II treatment group relative to the control group, the difference is non-significant. These results suggest that rather than affecting Type 1 IGF receptor levels, IGF-II treatment may have affected another early process, such as phagocytosis.

The rate of phagocytosis that occurs within damaged muscle can affect subsequent phases of regeneration (Grounds 1991), for phagocytosis is a necessary event in the regeneration of skeletal muscle. A role for the IGF system in the modulation of inflammatory and phagocytic responses is supported by studies in which immune neutralisation of endogenous IGF-I during muscle repair resulted in enhanced macrophage infiltration (Lefaucheur *et al.* 1996). The means by which macrophages contribute to the regeneration process is not limited to just the removal of necrotic tissue, but rather includes the production of growth factors that are capable of affecting MPC (Grounds 1991; Layne & Farmer 1999). One such growth factor is tumour necrosis factor-alpha (TNF-α) (Renier *et al.* 1996), a growth factor that is believed to be responsible for muscle wasting in a number of pathological conditions, including cancer (Meadows *et al.* 2000). Monocytes and macrophages up-regulate TNF-α in response to exogenous IGF-I *in vitro* (Renier *et al.* 1996), and TNF-α inhibits IGF-I-induced stimulation of myogenesis in cultured C2C12 cells (Layne &

Farmer 1999). These findings thus lend support to the interaction of IGFs with phagocytic processes, and indeed a detailed examination of the different phagocytic cell populations within the 0-24 hour timeperiod following notexin injection and IGF-II administration in a future study would be of great interest.

This study was designed to address the hypothesis that administration of IGF-II results in a change in the onset of proliferation and/or differentiation processes within regenerating skeletal muscle. The pattern of control versus IGF-II treatment values observed in this study would seem to suggest that instead of a change in the onset of proliferation and differentiation events, an overall decrease in MPC proliferation and differentiation was observed. Two factors which cloud the interpretation of an overall decrease, however, are that: a) there is a lack of sampling points between days which would unequivocally establish when the absolute peaks of MyoD and myogenin expression occurred, as otherwise it cannot be ruled out that the peak values obtained were taken from different parts of their respective curves, and b) the dMHC values on day 4 are virtually identical, indicating that by day 4 the same total number of nuclei in control and IGF-II treatment groups had gone through the differentiation process. For this reason, it is reasonable to assert that there was a delay in the onset of proliferation and differentiation processes, but it cannot be stated whether there were changes in the overall numbers of MPC undergoing proliferation and differentiation processes.

This study identified an effect of IGF-II on both proliferation and differentiation events, thus raising the question of whether the delayed proliferation results in the

delayed differentiation, or in fact whether these two observations are completely independent of each other. This question could be addressed in an interesting series of experiments where IGF-II is pulsed at different times and durations, in order to see if effects are observed on single or multiple regeneration events.

This study showed a delay in MPC proliferation and fusion in muscles that received exogenous IGF-II, as well as a late effect whereby the regenerated fibres that received IGF-II were larger than the control fibres. The fact that the IGF-II treated fibres were larger at day 7 than the control group fibres was precisely the opposite result to what might have been anticipated given that the early regeneration was hindered. This suggests that the administered IGF-II had a pronounced effect on the regenerated muscle between days 4-7, when the primary process occurring was fibre enlargement. Likewise, an increase in muscle fibre diameter has been reported for laceration-damaged muscle treated with IGF-I peptide (Menetrey *et al.* 2000), and a decrease in fibre diameter observed following treatment of damaged muscle with anti-IGF-I antibodies (Lefaucheur & Sébille 1995b).

Strictly speaking, the period of fibre enlargement in regenerating muscle fibres is distinct from muscle hypertrophy, however both processes lead to increased muscle fibre size, thus warranting an examination of the role of IGFs in hypertrophy in relation to the present findings. It should be noted that the term "hypertrophy", which is classically defined as an increase in the protein to DNA ratio in the mature fibre, is also frequently used in the literature to refer to instances of increased protein with concomitantly increased DNA (Adams & McCue 1998; Barton-Davis *et al.* 1999).

Markers of hypertrophy as reported in the literature include increased muscle weight (Barton-Davis *et al.* 1998), fibre size (Coleman *et al.* 1995), and biochemical determinations of protein and DNA (Adams & McCue 1998).

A role for IGF-I in the induction of hypertrophy has been very clearly established by studies *in vitro* (Semsarian *et al.* 1999) and *in vivo* (Adams & McCue 1998; Barton-Davis *et al.* 1998; Coleman *et al.* 1995), with as much as a 66% increase in fibre area reported for IGF-I-transfected myotubes in culture (Semsarian *et al.* 1999). In this study there was a fairly similar increase (42%) in fibre area in response to exogenous IGF-II. Hypertrophy in response to IGF-I occurs via binding to the Type 1 IGF receptor (Semsarian *et al.* 1999), induction of calcineurin and the transcription factor, GATA-2, which then cooperate with selected NF-ATc1 transcription factor isoforms in order to activate genes responsible for the hypertrophic response (Musaro *et al.* 1999). Despite the fact that Type 1 IGF receptor levels increase after differentiation (Tollefsen *et al.* 1989a), hypertrophy occurs only if the exogenous IGF-I is administered prior to, but not after, the induction of differentiation (Semsarian *et al.* 1999). In the present trial IGF-II was administered continuously throughout proliferation, differentiation and enlargement, and was therefore present in high levels during the critical period for a hypertrophic response.

IGF-II has also been associated with hypertrophy, although certainly to a lesser degree than IGF-I, for at present there have been no studies looking directly at the effects of administered IGF-II on skeletal muscle hypertrophy. Levels of both IGF-I and IGF-II mRNA are elevated in rat M. *plantaris* and M. *soleus* subjected to compensatory

hypertrophy (DeVol *et al.* 1990), suggesting that both IGFs have a role in the induction of hypertrophy. Hypertrophy of cardiac tissue, which occurs by a similar mechanism to muscle hypertrophy in response to IGF-I (Musaro *et al.* 1999), has also been shown to occur in response to IGF-II (Adachi *et al.* 1994; Liu *et al.* 1996). Taken together, there is a strong body of evidence supporting a role for IGF-II in the enhancement of muscle fibre enlargement as seen in this study.

An additional factor that must be considered within the scope of the current study is re-innervation, and possible effects of IGF-II upon that process. Notexin injection results in denervation of 70% of the muscle fibres in rat M. *soleus* (Harris *et al.* 2000), and the ensuing functional innervation is complete by 7 days (Whalen *et al.* 1990). Sesodia and Cullen (1991) report that the regeneration of denervated and non-denervated rat M. *soleus* is identical up to 3-4 days following notexin injection, however after that time non-denervated muscles grow more rapidly than denervated muscles. IGF-II has positive effects on nervous tissue, as illustrated by studies in which IGF-II administration to damaged nerves results in increased motoneuron survival and enhanced nerve regeneration (Near *et al.* 1992; Pu *et al.* 1999a). This opens up the possibility that in the current study, IGF-II administration may have speeded up and/or enhanced the functional innervation of the regenerating muscle, thus allowing for more rapid growth of the regenerating muscle fibres.

In summary, this study clearly shows that local administration of IGF-II to regenerating skeletal muscle results in delayed MPC proliferation and differentiation events, however by late regeneration the muscle fibres formed in the presence of IGF-

II attain a larger size than control fibres. These findings indicate that IGF-II has pleiotropic effects within regenerating skeletal muscle, likely as a function of the unique environment present during the sequential steps that make up the whole of muscle regeneration. The use of IGF-II as a therapeutic agent for skeletal muscle regeneration may have potential, pending further investigation into the individual effects of IGF-II, and subsequent refinement of the administration protocol.

CHAPTER 7

FINAL DISCUSSION

Regeneration of skeletal muscle is a fascinating process involving a number of coordinate steps, each of them regulated, at least in part, by the presence of growth factors. Recent studies suggest a critical role for locally produced growth factors in the regulation of normal postnatal growth and development (Sjogren *et al.* 1999; Ueki *et al.* 2000; Yakar *et al.* 1999). Accordingly, in the present studies, the tissue levels of IGF mRNA and myostatin protein have been associated with the processes occurring concomitantly within the regenerating muscle. A histological approach was used throughout this thesis in order to assess the individual growth factor activities in damaged muscle tissues; the identified growth factor activities can then be related to the histological changes taking place at that particular time. Finally, in the last experimental chapter, the effect of IGF-II administration on muscle regeneration was examined, and the effects on key regeneration steps examined in detail.

7.1 Summary of Results

7.1.1 Muscle regeneration

7.1.1.1 Necrosis

Two of the earliest processes in the repair of muscle following damage are inflammation and phagocytosis of necrotic debris. The IGF axis was not examined during the stage of muscle necrosis, as the focus of work contained in this thesis is on MPC proliferation and fusion events, however, when myostatin was examined, it was apparent myostatin regulation was considerably altered during these early timepoints. The results presented here showed that during the stage of inflammation and phagocytosis, high levels of myostatin protein were present in connective tissue and necrotic fibres. Similarly, M. *extensor digitorum longus* and M. *soleus* have increased levels of myostatin protein, as determined by immunoblot analysis, on days 1 and 3 following notexin-induced damage (Mendler *et al.* 2000). Skeletal muscle-derived fibroblasts up-regulate myostatin expression in a dose-dependent fashion in response to crushed muscle extract (Yamanouchi *et al.* 2000), suggesting that the myostatin protein within interstitial areas, as observed in the present study, may be produced by connective tissue-associated fibroblasts. Other likely candidate sources for the increased myostatin protein within interstitial areas include necrotic fibres, which show markedly increased protein levels at this time; or the bloodstream, as increased circulating myostatin is associated with muscle wasting (Gonzalez-Cadavid *et al.* 1998). Regeneration is a process associated with significant changes in the connective tissue compartment (McMinn 1967; Phillips *et al.* 1990; Stauber *et al.* 1990), thus a

proposed role for the increased myostatin protein within connective tissue during necrosis is in the enhancement of connective tissue deposition. A role in connective tissue deposition is inferred by the presence of decreased connective tissue (Arthur 1995) in double-muscled cattle, which lack functional myostatin (Grobet *et al.* 1997; Kambadur *et al.* 1997; McPherron & Lee 1997). Alternatively, during the necrotic stage, the high myostatin protein present in muscle fibres and connective tissue may modulate phagocytic and inflammatory cell processes, as has been shown for the related factor TGF-β (Adams *et al.* 1991; Ranges *et al.* 1987; Reibman *et al.* 1991; Wahl *et al.* 1987; Wahl *et al.* 1988).

7.1.1.2 MPC proliferation

In the present growth factor localisation studies (CHAPTER 4 and CHAPTER 5), day 3 tissues of the *dw/dw* rat contained the greatest proportion (relative to other timepoints) of mononucleate cells with the histological appearance of MPC. This timepoint (day 3) therefore will be the focus of this section regarding MPC proliferation.

Survivor, regenerating and undamaged muscle fibres from notexin-injected muscles on day 3 were not significantly different from undamaged muscle fibres of non-injected muscles in terms of IGF-I and IGF-II binding, and IGF-II mRNA production, as was the case for connective tissue comparisons (CHAPTER 4). These results are in agreement with the virtually unchanged levels of IGF-II, and Type I and II IGF

receptor mRNA, relative to time zero controls, in M. *soleus* following notexin injection, as determined by solution hybridisation (Levinovitz *et al.* 1992).

These studies showed that, in contrast to the lack of change in other components of the IGF axis, differences were observed in IGF-I mRNA levels in regenerating fibres relative to undamaged muscle fibres (CHAPTER 4). Similarly, elevated IGF-I mRNA, as determined by solution hybridisation, has been reported for rat M. *soleus* on day 3 following notexin injection (Levinovitz *et al.* 1992). In histological studies, IGF-I immunoreactivity was found in satellite cells of normal and hypophysectomised rat M. *extensor digitorum longus* following ischaemia (Jennische *et al.* 1987) and taipoxin-induced (Jennische & Hansson 1987) injury. Given the role of IGF-I in the stimulation of cell proliferation, as discussed in Section 1.7.1.5.2, and the presence of the Type I IGF receptor on regenerating muscle fibres (CHAPTER 4), it is possible that IGF-I produced by regenerating muscle fibres acts in an autocrine manner to stimulate the proliferation of MPC during muscle regeneration.

The present studies showed that during the stage of MPC proliferation, IGF-I mRNA was also elevated in survivor fibres of notexin-injected muscle (CHAPTER 4). In regenerating rat M. *extensor digitorum longus*, IGF-I immunoreactivity was present in regenerating muscle fibres, but not in "surviving, undamaged" muscle cells (Jennische *et al.* 1987). The apparent discrepancy between the Jennische (1987) study and the present one is possibly due to the proximity of the "surviving, undamaged" fibres of the Jennische (1987) study to the damaged area. Following notexin injection, there is a decreasing gradient in activated satellite cell number from the damaged area of

notexin-injected muscle (Klein-Ogus & Harris 1983), so that "survivor" fibres (that are interspersed with the regenerating fibres) would be expected to have a greater satellite cell activity than "undamaged" fibres (which are located further away from the damage) (see Section 3.1.3). In the present study (CHAPTER 4), IGF-I mRNA was elevated in survivor, but not undamaged, muscle fibres, a pattern that suggests the IGF-I production in survivor fibres may be linked to satellite cell replication. Previous studies have treated all uninjured fibres as a single population (Jennische *et al.* 1987), however the results of the present study shows that the uninjured muscle fibre population is not homogenous.

The results of the present study showed that like regenerating and survivor fibres, connective tissue of notexin-injected muscles contained greater IGF-I mRNA than did non-injected muscles (CHAPTER 4). IGF-I may act in connective tissue to stimulate fibroblast proliferation and/or collagen synthesis by fibroblasts, as occurs *in vitro* in response to IGF-I (Cook *et al.* 1988; Goldstein *et al.* 1989), or it may be a source of locally-acting growth factor for damaged muscle tissues.

Myostatin protein was not observed in proliferating MPC in this study (CHAPTER 5). This finding is in contrast to that of Yamanouchi (2000), who reported occasional localisation of myostatin mRNA in mononucleated myogenic cells of regenerating rat femoral muscle two days after bupivacaine-induced damage, but is similar to the virtually undetectable levels of myostatin protein, by Western blot analysis, in rat muscle 1-14 days after bupivacaine-induced damage (Sakuma *et al.* 2000). The report that myostatin mRNA was only occasionally observed in mononucleated myogenic

cells of regenerating muscle (Yamanouchi *et al.* 2000), combined with the apparently undetectable levels for M. *tibialis anterior* (Sakuma *et al.* 2000), and the present report of no myostatin localisation in MPC (CHAPTER 5), suggest that myostatin does not play a critical role in the proliferation of MPC during skeletal muscle regeneration.

7.1.1.3 Myotube formation

In the present study, the period of myotube formation, the peak of which occurred on day 5 in regenerating muscle tissues of the *dw/dw* rat, was typified by dramatic increases in components of the IGF axis (CHAPTER 4). Specifically, IGF-I and -II binding capacity, and IGF-I and –II mRNAs, were significantly up-regulated in regenerating muscle fibres on day 5, and it was at this time that the presence of IGFBPs in regenerating muscle tissues was indicated by the *in vitro* binding of ^{125}I-IGF-I (CHAPTER 4). A similar elevation of IGF-I and –II mRNA, in homogenised tissue, during myotube formation has been shown for notexin-injected rat M. *soleus* (Levinovitz *et al.* 1992). Similarly, specific binding of IGF-I and Type I IGF receptor mRNA are both elevated in damaged rat muscles on day 5 following ischaemia/glycogen depletion and bupivacaine injection, respectively (Jennische & Matejka 1992; Marsh *et al.* 1997), and the Type II IGF receptor is co-ordinately up-regulated with IGF-II during muscle cell differentiation *in vitro* (Tollefsen *et al.* 1989b). However, such an elevation of Type -I and -II IGF receptors was not reported for regenerating rat M. *soleus* analysed by solution hybridisation (Levinovitz *et al.* 1992). The lack of change in the Levinovitz (1992) study is possibly due to dilution

of receptor levels in the homogenised muscle. IGFBPs, as mentioned above, were determined by the *in vitro* binding of ^{125}I-IGF-I to be present in day 5 tissues only, and to be localised within connective tissue, and regenerating and survivor fibres (CHAPTER 4). In the present study, competition of ^{125}IGF-I with unlabelled des(1-3)IGF-I and IGF-I suggested that IGFBPs account for virtually all of the IGF-I specific binding to connective tissue and survivor fibres, and approximately half of the IGF-I specific binding to regenerating muscle fibres, at day 5 (CHAPTER 4). In a detailed study of IGFBP mRNAs in regenerating rat skeletal muscle, the presence of IGFBPs-4 and –6 in connective tissue, IGFBP-5 in regenerating muscle fibres, and IGFBP-6 in uninjured muscle fibres was reported (Jennische & Hall 2000). IGFBPs may serve important modulatory functions within damaged muscle tissues, as they can either potentiate or inhibit IGF action (Bach *et al.* 1994; Wood 1995).

In the present study, myostatin protein was not observed in newly regenerating muscle fibres (CHAPTER 5). Similarly, in cultured C2C12 muscle cells, increased myostatin mRNA was not observed until day 4 following the switch to differentiation medium, when fusion was already well-established (Mendler *et al.* 2000). In rat M. *tibialis anterior* regenerating after bupivacaine-induced damage, myostatin protein is virtually undetectable by Western blot analysis during the stage of myotube formation (Sakuma *et al.* 2000). Results from another regeneration study, where both low and high intensity immunostaining were observed in newly formed myotubes of notexin-damaged M. *soleus* (a slow-type muscle) and M. *extensor digitorum longus* (a fast-type muscle) (Mendler *et al.* 2000), respectively, suggests that muscles vary considerably in the expression of myostatin at the time of myotube formation, leading

to the proposal by Mendler *et al.* (2000) that this variation may be modulated by the re-innervation status of the muscle.

7.1.1.4 Muscle fibre enlargement

Late regeneration, when the major event taking place was muscle fibre enlargement, persistent increases in IGF-I and –II message were observed in regenerating muscle fibres, and elevated IGF-II in survivor fibres relative to undamaged muscle fibres (CHAPTER 4). Similarly, IGF-I mRNA levels are elevated in homogenised M. *tibialis anterior* on day 15 following bupivacaine-induced damage (Marsh *et al.* 1997).

In this study, myostatin was observed in generally increasing amounts in regenerating and survivor muscle fibres undergoing enlargement and maturation (CHAPTER 5). Similarly, the stage of muscle fibre enlargement is associated with increased myostatin protein in homogenised rat M. *tibialis anterior* (Sakuma *et al.* 2000), and with increased myostatin mRNA in homogenised rat M. *extensor digitorum longus* (Mendler *et al.* 2000). The increased myostatin levels in these fibres may be related to the slowing down of cellular proliferation, relative to earlier timepoints.

7.1.2 Modulation of muscle regeneration

7.1.2.1 GH

The GH-deficient, dwarf *dw/dw* rat was used in these studies to examine the effect of GH on skeletal muscle regeneration. In order to eliminate the confounding effects of

endogenous GH on somatic growth in the trial, the *dw/dw* rats were castrated in order to remove testosterone. Exposure to testosterone alters the pattern of GH pulsatility from a feminine to a masculine pattern of GH secretion (Painson *et al.* 2000), and the masculine pattern is associated with a greater somatic growth response relative to the feminine pattern (Jansson *et al.* 1985). This strategy has been similarly undertaken by others (Gevers *et al.* 1995). It is possible that castration of the *dw/dw* rats in these experiments slightly altered the resultant regeneration processes, for exogenous testosterone has a stimulatory effect on satellite cell proliferation in castrated neonatal pigs (Mulvaney *et al.* 1988), and research suggests that during regeneration, elevated testosterone may impair phagocytic processes (Grounds 1987). However, the regeneration processes observed in castrated *dw/dw* rats in the present study were qualitatively similar to those reported in the literature for notexin-induced damage (Harris & Johnson 1978; Harris *et al.* 1975), with the only observed difference being a slightly extended timeframe for the regeneration process, which may be due to the *dw/dw* GH-deficient phenotype, and/or castration.

In this set of experiments, an effect of GH was observed on body and muscle weights, and on IGF-II binding. Similarly, a 25% increase in body weight, and significantly greater damaged and undamaged M. *extensor digitorum longus* weights were reported for GH-treated Sprague-Dawley rats following ischaemic necrosis (Ullman *et al.* 1989). The effect of GH on muscle weights was observed in both notexin-injected and non-injected control muscles, thus indicating that the enhancement of muscle weight was not related to regeneration. The effect of GH on IGF-II binding levels in all tissues of the notexin-injected muscle on day 5 only was unexpected. The Type II

IGF receptor has been postulated to be a negative regulator of IGF-II action, based primarily on developmental studies (Haig & Graham 1991). A few studies have indicated a signalling pathway for the Type II IGF receptor, via G-proteins (McKinnon *et al.* 2001; Okamoto & Nishimoto 1991), and a role for the Type II IGF receptor in muscle has been suggested by the report that an IGF-II analog with high selective affinity for the Type II IGF receptor, stimulates differentiation (Rosenthal *et al.* 1994). However, the Type II IGF receptor up-regulation in response to GH that occurred in this study was observed in all tissues, including connective tissue, making it unlikely to be a muscle differentiation-associated up-regulation. The primary role of the Type II IGF receptor in adults has been proposed to be the maintenance of lysosomal activity (Wang *et al.* 1994), and GH administration elevates lysosomal activity in polymorphonuclear leucocytes (Rovensky *et al.* 1985). This suggests that GH may modulate lysosomal activity in regenerating muscle tissues.

These studies did not identify changes in myostatin or other components of the IGF axis, including IGF-I mRNA, in response to GH. This was in contrast to other studies showing GH regulation of IGF-I mRNA in muscle cells *in vivo* and *in vitro* (Brameld *et al.* 1996; Butler *et al.* 1994; Sadowski *et al.* 2001), but was in keeping with previous studies using the same strain of rats where GH treatment did not affect levels of the Type I IGF receptor (Butler *et al.* 1994), and an earlier immunohistochemical study that showed IGF-I levels are unaffected by the GH status of the animal during regeneration (Sommerland *et al.* 1989). A number of studies now have implicated GH in having direct effects, ie. not mediated by IGF-I, on skeletal muscle by showing immediate up-regulation (within 5 minutes) of many GH-signalling proteins (Chow *et*

al. 1996; Sadowski *et al.* 2001). Furthermore, treatment of GH-responsive pro-B Ba/F3 cells with an anti-IGF-I antibody that blocks IGF-I action does not block the proliferative effects of GH (Baixeras *et al.* 2001). These reports combined with the present study thus provide evidence of GH effects that are distinct from those of IGF-I. The findings of the present study and that of Sommerland *et al.* (1989) may suggest that for regenerating muscle, the IGF-I mediated pathway (for GH action) is utilised to a lesser degree relative to normal, uninjured muscle and cultured muscle cells.

7.1.2.2 IGF-II

In the present study, the administration of IGF-II to regenerating skeletal muscle led to interesting, and unanticipated results, with a delay in the expression of MyoD (a marker of proliferating MPC), myogenin and dMHC (sequential markers of MPC differentiation) (CHAPTER 6). The effects on these markers of the regeneration process were consistent, with each step showing the same directional change, that is, a delay due to IGF-II administration. The fact that a delay in regeneration was apparent by day 1 following notexin-injection indicated that an early regeneration process was affected, or that perhaps there had been a down-regulation of the Type I IGF receptor in response to the administered IGF-II, as has been shown *in vitro* for BC3H-1 muscle cells (Rosenthal & Brown 1994; Rosenthal *et al.* 1991), which could account for the lack of a positive effect on regeneration by IGF-II. The latter hypothesis, of a down-regulation of Type I IGF receptor, was examined in day 1 tissues, however the receptor levels were found to be unchanged by the administration of IGF-II. Because the Type I IGF receptor levels were not down-regulated on day 1, it is postulated that

the administration of IGF-II to regenerating muscle impeded phagocytic and/or inflammatory processes, as those were the other major events occurring at that early time, and the rate of phagocytosis can affect ensuing phases of regeneration (Grounds 1991). A proposed inhibitory effect of IGF-II on phagocytic processes corresponds with the reported enhancement macrophage infiltration following immune neutralisaton of endogenous IGF-I (Lefaucheur *et al.* 1996). Certainly, an interesting experiment would be to determine if there are changes in the phagocytic population, with administered IGF-II, in the first 0-24 hours following notexin injection.

The most promising result from the trial examining the effect of IGF-II on skeletal muscle regeneration is the significant enhancement of muscle fibre size observed on day 7. This finding is particularly exciting given the inhibition of regeneration processes observed as late as day 4, implying that the effects of IGF-II between days 4-7 were potent. The enhancement of regeneration between days 4-7 may have been the result of stimulation of hypertrophic-like events as has been observed for cultured C2C12 cells stably transfected with IGF-I (Semsarian *et al.* 1999), and cultured rat cardiomyocytes administered IGF-II (Adachi *et al.* 1994). A second possibility is that the enhancement of muscle fibre size of day 7 was due to an augmentation of innervation by IGF-II. Administration of IGF-II to crushed sciatic nerve results in increased distance of motor axon regeneration (Near *et al.* 1992), and conversely, spontaneous nerve regeneration is inhibited by the presence of antiserum directed against IGF-II (Near *et al.* 1992). From 3-4 days following notexin injection, regeneration is more rapid in non-denervated muscles than in denervated muscles

(Sesodia & Cullen 1991), therefore an effect of IGF-II on nerve regeneration could lead to enhanced muscle fibre size by day 7.

An important feature of the trial testing the effects of IGF-II is that it has identified a "window" of time (days 4-7) during which IGF-II appears to be beneficial to the regeneration process in these trial animals (CHAPTER 6). This "window" of time is coincident with the natural pattern of IGF-II expression during regeneration, as determined in earlier studies (CHAPTER 4). The finding of an enhancement of regeneration processes with additional IGF-II suggests that a greater endogenous expression of IGF-II during regeneration is associated with improved muscle regeneration.

7.1.3 Achievement of the aims of the thesis

The aims of the thesis, as outlined in Section 1.9, were to test the following hypotheses:

1. The IGFs and their receptors are regulated during muscle regeneration, and the level of IGF expression and binding in regenerating muscle is regulated by GH.
2. A negative regulator of growth, myostatin, is temporally regulated during muscle regeneration, and its levels are decreased in muscles undergoing enhanced growth due to the administration GH.
3. Administration of IGF-II during skeletal muscle regeneration enhances the rate of regeneration.

As a result of the studies contained in this thesis, these hypotheses were tested. IGF-I and –II mRNAs, Type I and II IGF receptors, and myostatin protein were regulated during muscle regeneration, as reported in CHAPTER 4 and CHAPTER 5. GH administration regulated IGF-II binding to regenerating muscle, but did not affect other components of the IGF axis (CHAPTER 4) or myostatin protein levels (CHAPTER 5). Lastly, administration of IGF-II during skeletal muscle regeneration inhibited the early stages of muscle regeneration (up to myotube formation), but then enhanced the later stages of regeneration (CHAPTER 6).

7.2 Future Work

This work has highlighted a number of avenues worthy of pursuit, as described below.

7.2.1 IGF-II

In the present study, administered IGF-II inhibited MPC proliferation on day 1, inhibited MPC differentiation on days 2 and 3, but enhanced regenerating fibre size on day 7. These results leave the following questions unanswered:

a) Was there an early inhibitory effect on phagocytosis and/or MPC migration?

b) Was the inhibition of differentiation simply a follow on from the inhibition of proliferation, or did IGF-II have separate effects on both of these stages?

c) What is the optimal administration protocol for IGF-II?

d) Does the quantity of IGF-II delivered alter the effect of IGF-II?

To address (a) above, a similar trial to the one reported in CHAPTER 6, with a number of sampling times in the first 24 hours after injury, could be carried out, then a histological assessment of phagocytic processes and/or MPC migration performed. Such an investigation in an IGF-II knockout mouse would also be useful in determining the effects of IGF-II on these processes. If the histological assessment of IGF-II effects on phagocytosis and/or MPC migration showed an effect of IGF-II, the effects on migration could be further studied *in vitro* using a Boyden chamber (Takano & Nakagawa 2001).

To address (b) and (c) above, IGF-II would be administered at varying times, such as from day 2.5 onwards to determine whether IGF-II has a negative effect on MPC differentiation that is separate to its negative on MPC proliferation ((b) above). To determine the optimal administration time for IGF-II ((c) above), peptide would be administered on days 4-7, 5-7, and 6-7, histological sections taken, and fibre area determined to decide the optimal time to begin IGF-II administration. Similarly, the optimal duration of IGF-II administration would be determined in studies going beyond 7 days.

A much earlier *in vitro* study of the effects of IGF-II on L6 muscle cells showed that IGF-II stimulates myogenesis at low concentrations, and inhibits myogenesis at supraphysiological levels (Florini *et al.* 1986). A logical extension then, which would test (d) above, would be to administer different concentrations of IGF-II to regenerating muscle, to see if the amount of IGF-II delivered alters the effect of IGF-II on MPC proliferation and differentiation.

7.2.2 GH

In the present studies, GH treatment resulted in effects on Type II IGF receptor levels, but not on any other component of the IGF axis or myostatin. The effects of GH vary considerably depending on the administration regime used (Gevers *et al.* 1995; Jansson *et al.* 1985), therefore it would be of interest to test other dose and treatment regimes for GH, to see if these alterations result in additional changes in the IGF axis or myostatin.

As discussed, in the present study GH treatment resulted in up-regulation of IGF-II binding to all tissues, including connective tissue, of the notexin-damaged muscle. The Type II IGF receptor has a number of different functions, but in the adult, the primary function of this receptor is believed to be the maintenance of lysosomal activity (Wang *et al.* 1994). A logical set of experiments would thus be aimed at determining whether GH administration results in changes in lysosomal activity in regenerating muscle tissues. If GH is found to alter lysosomal activity during regeneration, GH could then be administered to an animal with a conditional gene knockout of the Type II IGF receptor in skeletal muscle, such as that generated using Cre/*lox* technology (Le & Sauer 2001). If an altered pattern of lysosomal enzyme processing resulting from GH treatment is present in regenerating muscles with a disrupted Type II IGF receptor, relative to muscles with a functional Type II IGF receptor, then the Type II IGF receptor would be implicated in having a role in lysosomal enzyme processing in GH-treated regenerating muscle tissues.

7.2.3 Myostatin

The present study identified high levels of myostatin within connective tissue of regenerating muscle in the first few days after muscle damage (CHAPTER 5). This localisation, in a tissue that it is normally absent from, suggests that myostatin has a specialised function that is specific to early regeneration processes, such as phagocyosis, MPC recruitment/chemotaxis and/or granulation tissue formation. To determine the functional significance of the myostatin in connective tissue, the myostatin knockout mouse (McPherron *et al.* 1997) would be an extremely useful model. Muscle damage could be induced in the knockout mice and their background strain (as a control), histological samples taken, and the following parameters examined: infiltration of phagocytes; rate of removal of necrotic debris; MPC migration into damaged areas; and connective tissue deposition. If results of knockout experiments indicate an effect of myostatin on MPC and phagocyte chemotaxis, these effects could then be examined *in vitro* using a Boyden chamber (Takano & Nakagawa 2001).

7.2.4 Other factors

In the present study, a histological approach was taken, with the presumption that different tissues within a notexin-damaged muscle may not be identical in their growth factor and receptor levels. This was supported, for instance, by the findings of elevated IGF-II mRNA in regenerating muscle fibres, relative to undamaged and survivor fibres (CHAPTER 4). An extension of the present study, in which the localisation of IGFs and myostatin was examined, is to use laser microdissection to

separate out the tissue types (regenerating, surviving, and undamaged muscle fibres, and connective tissue) of damaged muscle, isolate the RNA from each, and then to carry out a microarray analysis (Schena *et al.* 1995) of each sample. Such an approach could identify other candidate genes that may play a role in the damage and repair process in specific tissues. The use of microarray technology means that only a small sample quantity is required (Schena *et al.* 1995). The localisation and expression patterns of candidate genes during muscle regeneration would then be determined using an approach like that used for the studies in CHAPTER 4 and CHAPTER 5. If the candidate genes are temporally regulated during regeneration, suggesting a role in the process, the effect of the particular candidate gene product could be tested by either administration of the factor (such as carried out in CHAPTER 6), or elimination of the factor by antibody administration or by use of a gene knockout model.

7.3 Conclusion

In summary, the results presented in this thesis show that local growth factor expression is tightly regulated during skeletal muscle regeneration, and indicates roles for IGF-I, IGF-II, IGFBPs, the Type 1 and II IGF receptors, and myostatin in this process. The results of these studies suggest that IGFs may carry promise of improving muscle regeneration after injury.

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