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ROLE OF GROWTH FACTORS IN THE REGULATION OF SKELETAL MUSCLE MASS, FIBRE TYPE AND FIBRE SIZE

A thesis submitted in partial fulfilment of the

requirements for the degree of

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by

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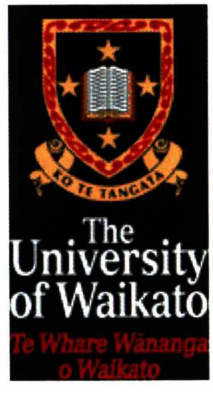

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ABSTRACT

Growth factors play an important role in the regulation of the growth and development of many tissues including muscle. Muscle is derived from somitic cells that differentiate to form myoblasts. These myoblasts then fuse to form myofibres. As these myofibres mature they take on the morphological, biochemical and physiological properties of mature muscle fibres. It is the plasticity of muscle fibres with respect to these properties that gives skeletal muscle its remarkable ability to adapt to different conditions of loading and usage. The aim of this thesis was to determine the role of growth factors in regulating muscle fibre type and fibre size in two different models of altered muscle mass.

Bovine muscle development was examined in normal foetuses (NM) and in double-muscled Belgian Blue foetuses (DM) carrying a mutation in the gene for myostatin. Muscle fibre type and fibre size were characterised using semi-automated computer-assisted image analysis after myofibrillar ATPase histochemical staining. Plasma IGF levels were measured, IGF-1 mRNA was measured using *in situ* hybridisation and IGF receptor binding was analysed by receptor autoradiography. Myostatin mRNA was analysed using semi-quantitative RT-PCR.

Skeletal muscle hypertrophy and atrophy were induced in adult rabbits by immobilisation of the hind limb with muscles in a lengthened and shortened position respectively. Fibre type and fibre size changes were analysed as described above and myostatin mRNA expression levels were measured using Northern blot analysis and semi-quantitative RT-PCR.

Results showed there was a biphasic change in the percentage of type 1 muscle fibres in both NM and DM, with the percentage initially declining, then increasing again. The percentage of type 1 fibres was consistently lower in DM. Circulating levels of IGF-1 increased with gestational age and levels of IGF-1 and IGF-2 were not different between NM and DM. IGF-1 mRNA expression in skeletal muscle declined with age in a pattern that was unrelated to changes in fibre type. Levels of IGF-1 mRNA were lower in DM during late gestation and this decrease was

temporally linked to a reduced area of type 1 fibres in DM at this stage of development. Myostatin mRNA declined with gestational age, and levels of the mutant mRNA were higher in DM.

In induced hypertrophy, myostatin mRNA expression was lower than in controls and in atrophy, myostatin mRNA was elevated at 2 days but not at 6 days. There were no changes in fibre type or fibre size over the treatment period. Myostatin protein was evenly localised to all muscle fibre types.

In conclusion, IGF-1 appears to be unrelated to changes in fibre type during bovine muscle development, but may play a role in the regulation of fibre size. The DM condition is associated with a change in fibre type, but the role of myostatin in mediating this change remains unknown. Changes in myostatin mRNA in response to induced hypertrophy and atrophy strongly suggest an important role for myostatin as a negative regulator of skeletal muscle mass in postnatal animals.

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ABBREVIATIONS

The International System of Units (SI) was used for all weights and measures.

(c)DNA	(complementary) deoxyribonucleic acid
β-ME	β-mercaptoethanol
aa	amino acids
ATP	adenosine - triphosphate
bp	base pairs
BSA	bovine serum albumin
Ci	curie
cpm	counts per minute
CRL	crown rump length
DAB	diaminobenzidine
DEPC	diethlypyrocarbonate
DH_20	deionised water
DM	double muscled
DTT	dithiothreitol
GH	growth hormone
IGF	insulin-like growth factor
MHC	myosin heavy chain
MRF	myogenic regulatory factors
mRNA	messenger RNA
nm	nanometer
NM	normal muscled
OD	optical density
PCR	polymerase chain reaction
RT	room temperature or reverse transcription
sed	standard error of the difference
sem	standard error of the mean
TA	M. *tibialis anterior*
TGF	transforming growth factor
VL	M. *vastus lateralis*
VM	M. *vastus medialis*

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To everyone else in the Kirton Wing, a great big thank you for so much help in so many ways.

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Dedicated to

Murray Kerr
1934-1996

and

Ella Murray Martyn
1995-

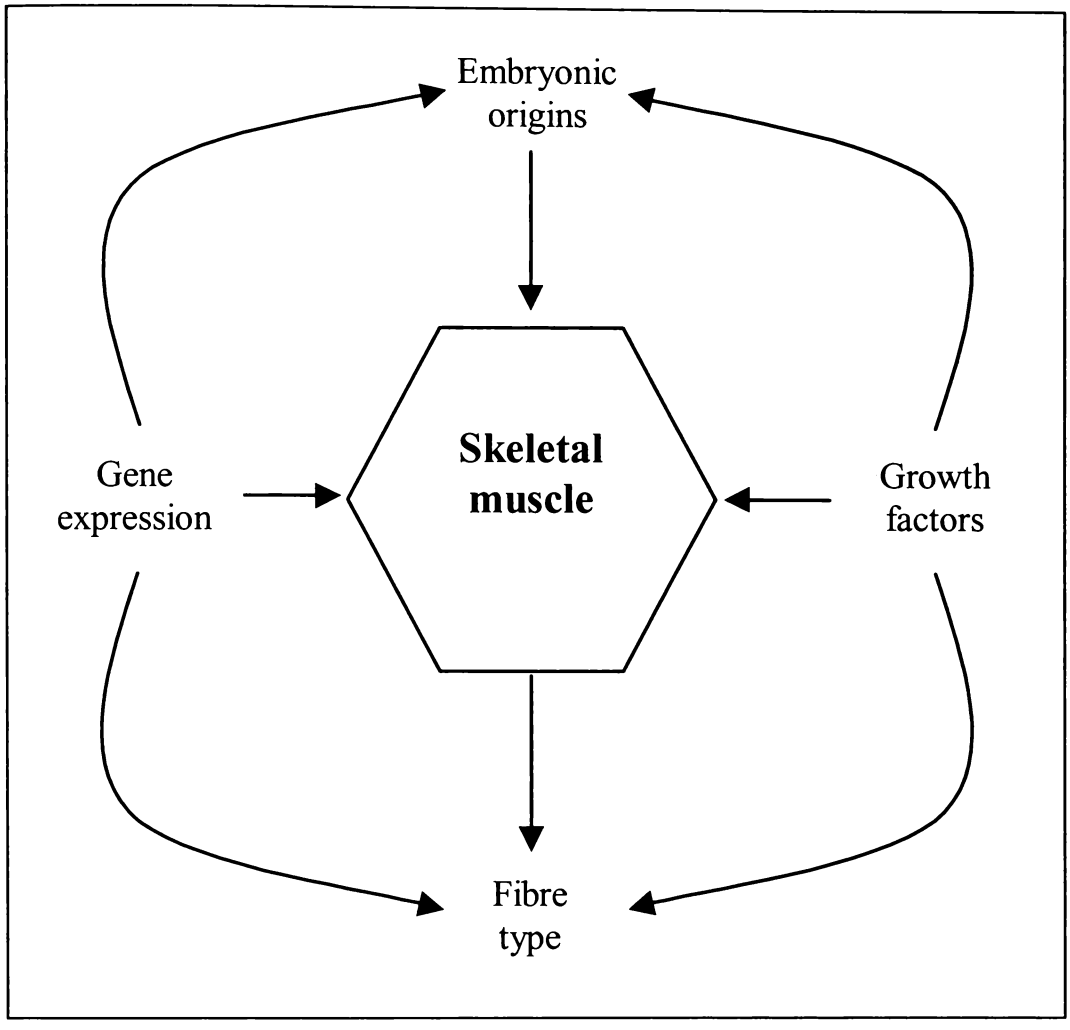
CHAPTER ONE

INTRODUCTION

Skeletal muscle performs a number of important tasks in the body. These may be as diverse as providing a rapid burst of speed or for the sustained maintenance of posture. This broad range of activities make very different demands upon muscles in terms of the need to carry out a range of metabolic and contractile functions.

The process of muscle development and the ongoing remodelling of muscle tissue that occurs throughout life, provides opportunities for investigation of the co-ordinate regulation of growth. This thesis examines the hypothesis that growth factors are involved in the regulation of skeletal muscle growth both during development and in a remodelling situation, with a specific focus on muscle fibre type and fibre size. In order to set the context for the experimental work undertaken, I will start this introduction by giving a general outline of what a muscle is and how it is formed. The basis of muscle fibre type diversity will be introduced and with it a discussion of the measurement of fibre type. The genetic regulation of skeletal muscle development and of fibre type determination will be discussed, as will the major growth factor families involved in muscle growth. In the final section, some animal models in which muscle growth is altered will be introduced and the rationale behind the selection of the specific models employed in this study will be given. A diagram showing the interrelationships of the various themes of this thesis is given in Figure 1.1.

Figure 1.1 Diagram showing the interrelationships of the different sections of this thesis



1.1 What is skeletal muscle?

Skeletal muscle is composed of various tissue types including muscle fibres, connective tissue, nerves, blood vessels and intramuscular fat. This section will give a broad overview of the structure and function of the main components of skeletal muscle, the developmental aspects of which will be follow in more detail later in this introduction.

1.1.1 Muscle fibres

Muscle fibres, which comprise the bulk of a muscle, are syncytia containing many post-mitotic nuclei within a single membrane, the sarcolemma. Individual muscle fibres are made up of repeating units known as sarcomeres. Skeletal muscle causes movement by contraction of the muscle fibres. The basic contractile unit of muscle is the sarcomere and this will be discussed in more detail later. Muscle fibres can be classified into different fibre types on the basis of their contractile and biochemical properties (Kelly and Rubenstein, 1994) and these will also be discussed in further detail in Section 1.3.

1.1.2 Connective tissue

Connective tissue is an important and complex component of skeletal muscle. The main protein component of connective tissue in muscle is collagen and this may occur either as discrete fibres or as specialised sheets known as basement membranes. Collagen fibres are found in connective tissue in association with large polysaccharides such as hyaluronic acid and proteoglycans, such as heparan sulphate and chondroitin, which make up the ground substance or extracellular matrix (Bailey and Light, 1989). Connective tissue gives both strength and elasticity to the muscles. It serves to anchor muscle to bone via specialised tendons and the extracellular matrix is a reservoir of growth factors for muscle growth and development (Taipale and Keski-Oja, 1997).

1.1.3 Nerves

Innervation is essential to the establishment and maintenance of correct muscle function and will be discussed later (Section 1.2.5). Muscle fibres are innervated by axons that arise from motor neurons. These axons branch on entering a muscle such that they connect to all fibres within a motor unit. The point at which the axon contacts a muscle fibre is known as the motor end plate (Swatland, 1984).

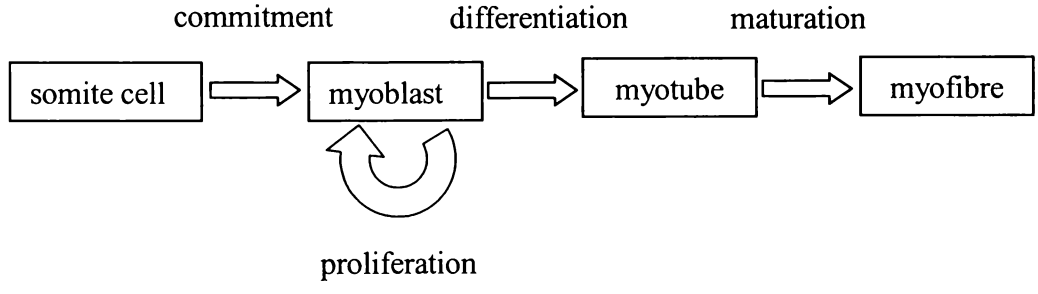
Although blood vessels and fat cells also contribute to the composite structure that is skeletal muscle, these will not be discussed further as they are of lesser importance to the overall theme of this thesis.

Muscle is a complex cellular environment and all of its component cell types are subject to an array of regulatory effects. The regulatory effects of greatest relevance to this study are growth factor environment and motor activity, but others include external temperature and nutritional state (Dauncey and Gilmour, 1996).

1.2 Development of skeletal muscle

The development of skeletal muscle, or myogenesis, proceeds through four broad developmental stages, summarised in Figure 1.2. This shows skeletal muscle originating from a pluripotent somitic stem cell, which then commits to the myoblast lineage, differentiates into a myotube and undergoes maturation to become a mature muscle fibre (Dauncey and Gilmour, 1996).

Figure 1.2 Diagram of main stages of muscle development showing the different types of cells involved at each stage. (Redrawn from Dauncey and Gilmour, 1996).

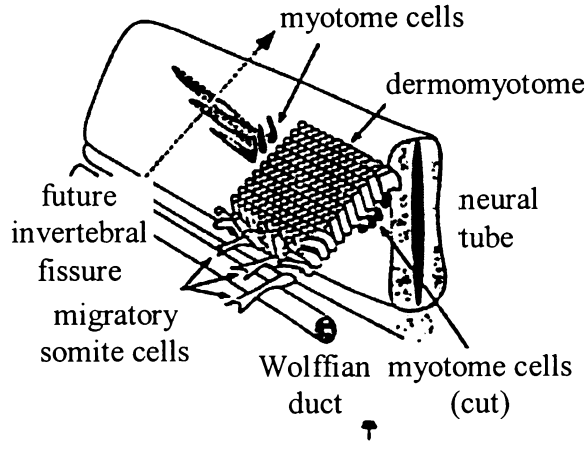


This thesis specifically examines the genetic and hormonal regulation of muscle fibre size and fibre type and this review will therefore have a greater emphasis on the latter two stages of muscle development as shown in Figure 1.2, namely differentiation and maturation. Throughout this introduction this schematic will be used to provide common temporal points of reference to tie together the diverse processes which take place during muscle development.

1.2.1 Embryonic origins of skeletal muscle

Vertebrate skeletal muscle is derived from pluripotent mesodermal cells (Hauschka, 1994; Ontell, 1982). During embryo development the cells of the mesoderm become arranged into paired segmented blocks, the somites. The somites are then compartmentalised such that the ventral region forms a loose network of cells called the sclerotome and the dorsal-lateral somite region becomes the structure known as the dermomyotome (Figure 1.3). The dermomyotome retains its epithelial character and it further subdivides to form the dermatome that gives rise to the dermis and the myotome. The myotome becomes a specialised layer of post-mitotic myocytes which lie beneath the dermomyotome and gives rise to skeletal muscle. Expansion of the myotome comes from the addition of cells from the rostral and caudal surfaces of the dermomyotome (Kahane *et al.*, 1998). The medial myotome produces the epaxial muscles of the back, while the lateral myotome and the lateral portion of the dermomyotome produce the hypaxial muscle, including the muscles of the body wall. Limb muscles are produced from the lateral surface of the dermomyotome only from the release of undifferentiated myogenic cells (Brand-Saberi and Christ, 1999). Current thinking is that these progenitor cells are committed to the myogenic lineage and they then migrate to the limb buds to form the appendicular muscles (Hauschka, 1994).

Figure 1.3 Diagram showing a developing somite, the dermomyotome and the underlying myotome layer (from Hauschka, 1994).



A number of signalling molecules such as *Pax-3*, *Sonic hedgehog* and the *wnt* family have a profound effect on muscle development and patterning and a more detailed discussion on the expression of some of these molecules can be found in Section 1.4.1 describing gene expression in muscle. The next section will describe the fate of myoblasts that have migrated from the somites and the processes involved in the formation of skeletal muscle

1.2.2 Myotube formation

Within the limb bud, myogenic progenitor cells that have migrated from the dermomyotome exit the cell cycle and undergo terminal differentiation. These terminally differentiated myoblasts then line up end-to-end and fuse rapidly to form myotubes that grow and ultimately extend from tendon to tendon. This first formed population of myotubes are known as primary myotubes. Primary myotubes originate from the early myoblast population (Miller and Stockdale, 1986). In the avian hind limb, these developing myotubes first appear as dorsal and ventral muscle masses. These masses further segregate into muscles of the dorsal and ventral thigh, shank and foot muscles and finally individual muscle masses are segregated (Kardon, 1998). A study of the ontogeny of primary myotubes has shown that fibres that originated as primary myotubes still persisted in the muscle of mature rats (Zhang and McLennan, 1998), yet there is also evidence for the selective degradation of primary myotubes during myogenesis (Fidzianska and Goebel, 1991). Whether the embryonic origin of the myoblast population from which the primary myotubes are formed is a factor in determining their ultimate fate is not known.

After the completion of primary myogenesis there is a delay during which primary myotubes mature and acquire their own basal lamina (Ross *et al.*, 1987a). Secondary myoblasts then proliferate on the surface of the primary myotubes and fuse to form myotubes in the vicinity of the endplate region (Duxson *et al.*, 1989). These secondary myotubes use the primary myotubes as a scaffold for their formation. The developing secondary myotubes are attached to the founding primary myotube by interdigitating processes that insert deeply into the primary myotube (Duxson *et al.*,

1989). The secondary myotubes grow until they too extend tendon to tendon and then separate from the primary myotube and acquire their own basal lamina. Much of the early work characterising this biphasic pattern of myotube formation has been carried out in rats (Harris *et al.*, 1989) and mice (Ontell and Kozeka, 1984). In large mammals such as humans and sheep, a tertiary phase of myogenesis occurs with a further population of myotubes forming which use the secondary myotubes as a scaffold (Draeger *et al.*, 1987; Maier *et al.*, 1992; Wilson *et al.*, 1992).

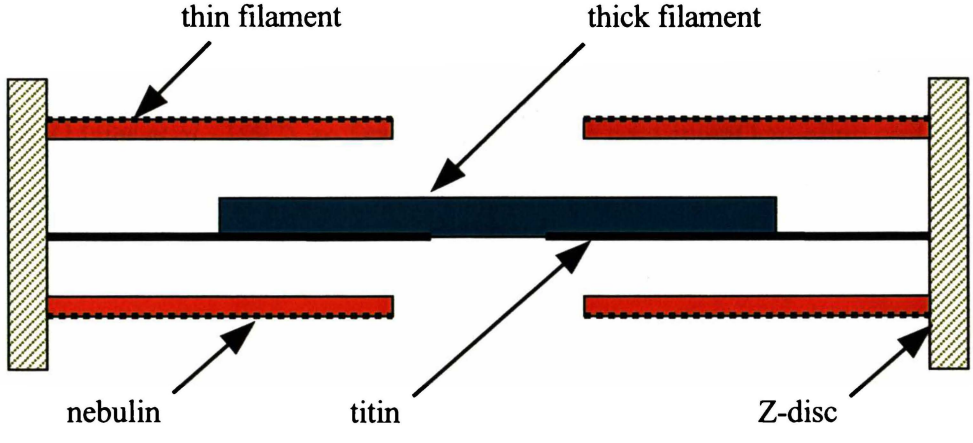
The morphology of newly formed myotubes is characterised by the central location of the nuclei, the presence of relatively few myofibrils and no basal lamina. As individual myotubes mature and accumulate myofibrils, the location of the myonuclei changes from a central position to a peripheral one, just underneath the sarcolemma and a connective tissue sheath, the endomysium or basal lamina, is formed (Ross *et al.*, 1987a). An additional population of myoblasts remains outside the sarcolemma, but within the basal lamina or basement membrane of the fibre. These cells are known as satellite cells and they represent a pool of stem cells which can become activated and proliferate in response to a requirement to increase the DNA content of a muscle fibre (Mauro, 1961). This can occur during normal growth (Moss and Leblond, 1971), or as a result of muscle injury (Bischoff, 1975; Schultz *et al.*, 1985).

An important theoretical concept which underlies our understanding of the need to balance increased cytoplasmic growth with additional nuclei is that of the DNA unit (Cardasis and Cooper, 1975; Cheek *et al.*, 1971; Hall and Ralston, 1989). This theory suggests that each nucleus is able to control a fixed volume of cytoplasm and that during growth and in remodelling situations, nuclear number is adjusted to maintain a constant protein to DNA ratio. This ratio then provides useful information about the mechanism of changes in muscle growth with respect to hyperplasia, where the ratio of protein to DNA decreases and hypertrophy, where the ratio increases.

1.2.3 Myofibrillogenesis and sarcomere assembly

After formation of the myotubes, the newly formed muscle fibres undergo a process of maturation during which they increase in both cross-sectional area and in length. The increase in fibre area is associated with the accumulation of additional myofibrils, which comprise the contractile apparatus of the muscle and the process of fibre lengthening occurs via the assembly of new myofibrils into sarcomeric units, a process that occurs at the myotendinous junction. The actual process of myofibrillogenesis includes the synthesis of the thin filaments (predominantly actin) and the thick filaments (predominantly myosin). The process by which the myofibrils are aligned into the characteristic banding pattern as defined by the sarcomeres remains unclear. It is commonly held that sarcomeric proteins, most likely including titin and nebulin, are laid down initially to form the basis for the registration of the sarcomeres. The thin filaments are attached to α-actinin in the Z-line via an association with nebulin and the thick filaments attached to the M-line via an association with titin (reviewed by Franzini-Armstrong and Fischman, 1994). This structure is shown in the following figure, Figure 1.4.

Figure 1.4. Structure of a sarcomere and its main protein components. (Redrawn from Schiaffino and Reggiani, 1996).



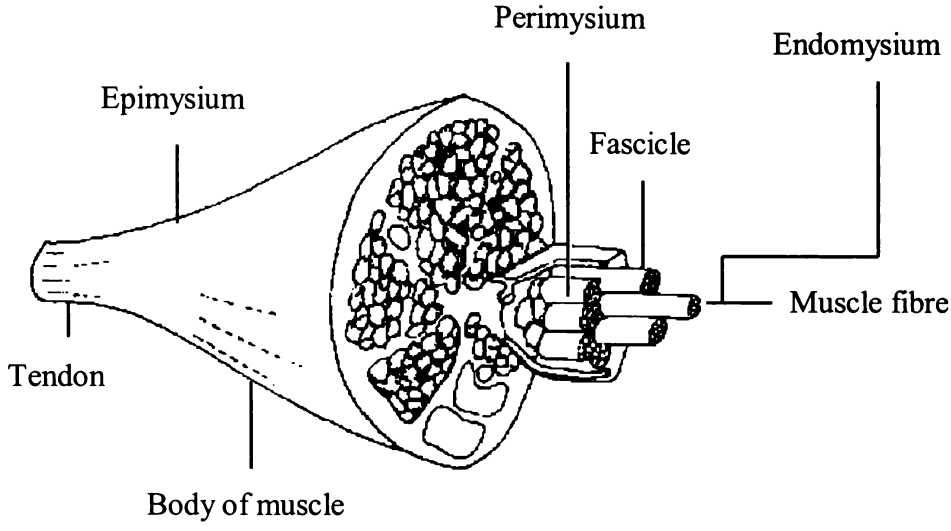
At the end of the muscle fibres, myofibrils also attach to the plasma membrane through an α-actinin-vinculin complex (Dix and Eisenberg, 1990). Individual

myofibres connect to the muscle tendons by means of a large number of interdigitating projections. Here also new sarcomeres are added to muscle fibres both during development and in response to stretch (Tabary *et al.*, 1972; Williams and Goldspink, 1971; Williams and Goldspink, 1973; Williams *et al.*, 1986). This process enables muscle fibres to lengthen with minimal disruption to the force generating capability of the muscle and also enables the properties of the muscle fibres to be modified in response to different metabolic and contractile requirements (Williams *et al.*, 1986).

1.2.4 Connective tissue formation

Concomitant with the formation of muscle fibres, a connective tissue network is laid down within and around the developing muscle. In most muscles, cells of this tissue component are derived from a different embryonic source to that of the muscle fibres, namely the somatopleural mesoderm (reviewed in McLennan, 1994). A connective tissue sheath known as the endomysium comprises the basal lamina that surrounds each individual fibre (Bailey and Light, 1989). Bundles of muscle fibres are contained within the epimysium and these make up what is known as a fascicle. Finally, the entire muscle is covered in an outer layer of connective tissue, the perimysium (Bailey and Light, 1989) (Figure 1.5). In developing bovine muscle, the epimysial connective tissue framework is established around 140 days gestation (Robelin *et al.*, 1991; Stickland, 1978). The distribution of skeletal muscle fibre types grouped within a single fascicle in general recapitulates that found in the entire muscle (Maier *et al.*, 1992), so fascicles represent a convenient sampling unit for the investigation of fibre type.

Figure 1.5 Schematic drawing of muscle showing different types of connective tissue (redrawn from Bailey and Light, 1989)



The development of muscle tendons, the connective tissue structures which connect muscle to bone, occurs during the formation of myotubes. This process has been examined in the avian hind limb where it has been shown that three tendon primordia are formed, one each between the muscle masses of the thigh and shank, the shank and the foot and distal to the foot muscles (Kardon, 1998). These primordia then form the tendons of insertion and the tendons of origin for the thigh, shank and foot muscles, with additional insertion tendons of the foot deriving from distally remote sources (Kardon, 1998). This study of the avian hind limb showed a strong temporal and spatial relationship between myogenesis and tendon morphogenesis which had not previously been described.

1.2.5 Innervation of skeletal muscle

At the same time as myoblasts are undergoing terminal differentiation and myotubes are being formed, neuronal growth is occurring and the innervation of muscle fibres

is taking place to form functional motor units. During development, primary myotubes segregate into dorsal and ventral pre-muscle masses (Kardon, 1998). This process can proceed in the absence of innervation, although the final muscle mass is dramatically reduced indicating that the very earliest stages of myogenesis are innervation independent (Butler *et al.*, 1982). The formation of secondary myotubes has been considered to be dependent upon innervation (Ross *et al.*, 1987b), although there is more recent evidence to show formation of secondary fibres can occur in denervated or paralysed muscles (reviewed by McLennan, 1994). In the continued absence of innervation, both primary and secondary myotubes degenerate (Ross *et al.*, 1987b; Wilson and Harris, 1993). Fibre type specialisation can occur in the absence of innervation, as was demonstrated when wing buds, transplanted to the pelvic cavity, developed their normal fibre type pattern (Laing and Lamb, 1983).

Differences in the temporal pattern of muscle fibre and nerve development give rise to the characteristic mosaic pattern of fibre types within a muscle. As all fibres within a motor unit have the same contractile and metabolic properties, it can be inferred from the mosaic pattern that the motor units themselves are intermingled (reviewed in Kelly and Rubenstein, 1994). The number of fibres in a motor unit is quite large – from 120 to 165 in rabbit muscle (Clark, 1931) and there is a correspondingly large number of axon branches which occur within the well defined nerves (reviewed in Swatland and Cassens, 1974). Branching of terminal axons is less frequent, but in double-muscled cattle there is a much higher frequency of branched terminal axons than is seen in normal animals (Swatland, 1973). The plasticity of muscle fibres with respect to fibre type was demonstrated by cross-innervation experiments where innervation by a foreign nerve caused an alteration in muscle fibre properties (Buller *et al.*, 1960).

Neurogenic regulation of muscle fibre development is of fundamental importance in muscle growth. The temporal relationships between myogenesis, innervation and the establishment and maintenance of skeletal muscle fibre type remain an interesting area for future investigations.

1.3 Skeletal muscle fibre type

Some skeletal muscles are redder in colour, while others have a paler appearance and this colour difference is a property of the muscle fibres themselves and not due to differences in blood content. Red fibres were shown to be slower contracting than white fibres (Ranvier, 1874, cited by Kelly and Rubenstein, 1994). Slow red fibres are found in those muscles involved in continuous activity and they are more fatigue resistant (reviewed by Close, 1972). Histological studies have also revealed heterogeneity of fibre type properties: red fibres are thin and dark and contain many mitochondria and fat droplets, while white fibres are thicker with a clear appearance and fewer mitochondria and fat droplets (reviewed by Close, 1972). Enzyme histochemistry has been used to classify different fibre types (Dubowitz and Pearse, 1960; Engel, 1962), as has myosin heavy chain immunohistochemistry composition (Pierobon-Bormioli *et al.*, 1981; Robelin *et al.*, 1993; Schiaffino *et al.*, 1986).

The following section will cover the embryonic origin of fibre type diversity and the process of fibre type transformation that occurs both during development and in response to external or intrinsic stimuli. I will also review the various ways in which fibre type can be analysed and give some examples of applications in which fibre type data is used.

1.3.1 The developmental origin of fibre type diversity

Skeletal muscle fibre type diversity originates from the establishment of separate populations of myoblasts. It has been shown by clonal analysis that myoblasts represent a heterogeneous pool of cells (Bonner and Hauschka, 1974; Miller, 1992). Myoblasts that are isolated from early embryonic muscle tissue predominantly form short fat myotubes *in vitro* with few nuclei and which are very particular with regard to growth factor requirements. Myoblasts which are isolated from later foetal muscle tissue predominantly differentiate to form thin myotubes with more nuclei, which are less particular as to growth factor conditions (Cho *et al.*, 1993; Hauschka, 1974). A third population of myoblasts, isolated from late gestation or post-natal muscle tissue are derived from satellite cells (Hauschka, 1994). These three populations of cells will be referred to as early myoblasts, late myoblasts and satellite cells

respectively. A number of properties further define these myoblast populations, for example late myoblasts only express Δ7 integrin on their cell surface (George-Weinstein *et al.*, 1993). Other cell surface markers define satellite cell populations (Cornelison and Wold, 1997) and there are differences in growth factor sensitivity also, for example TGF-E inhibits the differentiation of late but not early myoblasts (Cusella-DeAngelis *et al.*, 1994).

Although separate populations of myoblasts have been shown to occur *in vitro*, it was for a time unclear whether these separate populations of cells contributed to the formation of both primary and secondary myotubes. *In vivo* labelling studies have since shown that single populations of myoblasts do indeed contribute to both primary and secondary fibres (Evans *et al.*, 1994; Zhang and McLennan, 1998).

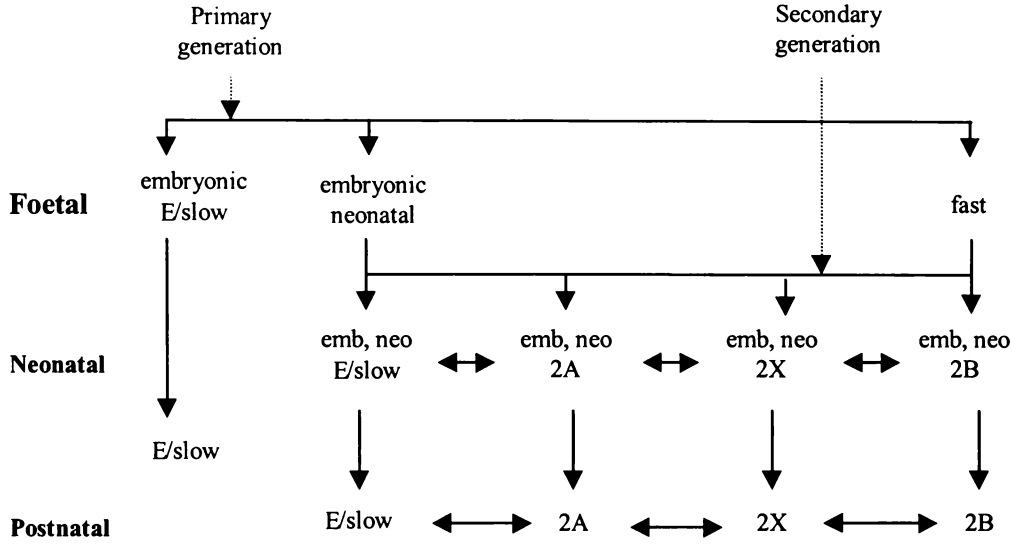
1.3.2 Fibre type transition

Individual muscle fibres undergo an ordered series of myosin heavy chain (MHC) isoform transitions during development and throughout postnatal life. Primary myotubes initially express the developmental isoforms embryonic MHC (MHC_{emb}) and neonatal MHC (MHC_{neo}) as well as slow/E MHC (MHC_{slow}) (Dhoot, 1986; Harris *et al.*, 1989; Narusawa *et al.*, 1987). The recent study of Zhang and McLennan (1998) has also described primary myotubes expressing fast MHC isoforms, with some myotubes having mixed fast and slow MHC composition. In their study, using BrdU labelling, primary myotubes expressing slow MHC were shown to preferentially mature into type 1 fibres and primary myotubes expressing fast MHC preferentially matured into type 2b fibres. The MHC isoform(s) expressed by primary myotubes is a function of the myoblast population from which they were derived. The developmental history of myotubes may therefore define a subset of MHC isoforms that may be expressed in postnatal life (for review see Gunning and Hardeman, 1991).

Secondary myotubes in developing rat muscle initially express only the developmental isoforms of MHC (Harris *et al.*, 1989). Their subsequent fate depends upon whether the developing muscle in which they reside is likely to attain the eventual phenotype of a predominantly slow or predominantly fast twitch

muscle. In mixed muscles, the majority of secondary myotubes develop into fast fibres, but in slow muscles such as the soleus, they may undergo a transition from the developmental isoforms to slow MHC (Dhoot, 1986; Narusawa *et al.*, 1987). Also in accordance with the overall fibre type composition of the muscle there may be a further transformation toward expression of fast MHC isoforms (Figure 1.6).

Figure 1.6 MHC isoform transitions in rat hind limb muscle showing phenotypic changes in three major phases of development (redrawn from De Nardi *et al.*, 1993).



In adult animals fibre type transformation still proceeds, with fast type 2a fibres in the rat soleus progressively transforming to slow fibres (Butler-Browne and Whalen, 1984). MHC isoform transitions also occur in response to induced atrophy or hypertrophy (Goldspink *et al.*, 1992; Loughna *et al.*, 1990; Periasamy *et al.*, 1989; Roy *et al.*, 1997), altered motor activity (Maier *et al.*, 1988) and regeneration (Sartore *et al.*, 1982).

1.3.3 Measurement of fibre type

Mature muscle fibres exhibit a range of properties which define their fibre type. This section will introduce a number of these properties and describe the ways in which they are used to define muscle fibre type. This will be followed by a review of the

advantages and disadvantages of different fibre typing methodologies. The property of muscle fibres that is held to most definitively determine their fibre type is the MHC isoform(s) expressed (Kelly and Rubenstein, 1994). In addition to the expression of specific MHC isoforms, muscle fibres can be classified on the basis of the level of myosin ATPase activity (high or low, Engel, 1962), the enzymes used in carbohydrate metabolism (oxidative or glycolytic, Dubowitz and Pearse, 1960) or the contraction speed of the fibres (fast, intermediate or slow twitch, Barany, 1967).

1.3.3.1 Myosin heavy chain expression

In all more than ten MHC isoforms have been identified, a number of which are restricted temporally or anatomically in their distribution and these are listed in Table 1.1.

Table 1.1 Correlations of MHC isoforms with histochemically defined fibre types (from Kelly and Rubenstein, 1994).

Fibre type	MHC isoform	Location
I	Cardiac E	Slow twitch skeletal
Superfast extraocular	IIeom	Extraocular muscle (EOM)
IIm	IIm	Branchial arch muscles, except humans
IIa/IIb/ IIx(d)	IIa/IIb/ IIx(d)	Predominant fast fibres. In leg and trunk and diaphragm
Ic	I>IIa, emb, neo	May be transitional types in fibres undergoing changes
IIc	I<IIa, emb, neo	Early development and regeneration; also adult EOM
?	embryonic	
?	Neonatal/Perinatal	Perinatal period, adult EOM
?	Slow tonic	EOM, tensor tympani, some intrafusal fibres
?	Cardiac Δ	Some jaw muscles

Immunohistochemical localisation of the MHC isoforms requires antibodies specific to the isoform being investigated. Even if the appropriate antibodies are available, they can be very expensive and this may preclude their widespread use. An advantage of this method of fibre typing, however, is its potential use for the analysis of valuable fixed archival muscle samples.

A more recent method of analysing MHC isoform expression is through the use of molecular biology techniques such as *in situ* hybridisation (Aigner and Pette, 1990; Dix and Eisenberg, 1988; Russell and Dix, 1992) and RT-PCR (D'Costa *et al.*, 1993; Peuker and Pette, 1997; Tanabe *et al.*, 1999; Uber and Pette, 1993; Wright *et al.*, 1997). Many of the genes for MHC isoforms have been cloned in a range of animal species and analyses can be performed at the level of tissue sections, or on RNA extracted from whole muscles or even single muscle fibres.

1.3.3.2 Myofibrillar ATPase activity

Fibre type analysis by myofibrillar ATPase activity has been carried out for many years. It is commonly used in human muscle pathology (Dubowitz and Brooke, 1973) and in meat animals (Ashmore and Doerr, 1971; Picard *et al.*, 1992; Russell and Oteruelo, 1983; Solomon and Dunn, 1988).

The activity of the myofibrillar ATPase enzyme enables fibre types to be defined on the basis of the relative stability or lability of the enzyme following pre-incubation in different pH solutions. The histochemical basis for the staining reaction depends upon the enzymatic liberation of free inorganic phosphate from endogenous ATP substrate added to the incubation mixture. In the presence of calcium, this forms calcium phosphate. The calcium is substituted for cobalt during a cobalt chloride incubation step and this then reacts with ammonium polysulphide to form an insoluble black precipitate, cobaltous sulphide (Dubowitz and Brooke, 1973). In fibres with high mATPase activity there is intense localisation of this insoluble black product and these fibres are identified as type 2b, fibres with intermediate intensity are type 2a and fibres with low mATPase activity are type 1 fibres.

The use of computer-assisted image analysis has meant this black precipitate has the advantage of enabling semi-automatic quantification of fibre type, due to the high

contrast between the different fibre types. A number of variations to this technique and some limitations to its use will be introduced in Chapter 3, which covers the optimisation of ATPase staining methodology. Discussion at this point will therefore be restricted to the interpretation of this technique in relation to other classification systems.

1.3.3.3 Fibre typing using metabolic enzyme activity

Two histochemical approaches have been taken to identifying metabolic activity of different fibre types. These are to stain for enzymes of the oxidative or tricarboxylic acid pathway, or for enzymes of the glycolytic pathway. In the former, succinate dehydrogenase activity, or the localisation of NADH-tetrazolium reductase (NADH-TR) identifies fibres with an oxidative metabolic pathway and in the latter phosphorylase activity is used as an indicator of glycogen metabolism (Dubowitz and Brooke, 1973; Dubowitz and Pearse, 1960). NADH-TR brings with it a risk of false positive results due to association of the enzyme substrate with mitochondria and the sarcoplasmic reticulum. Phosphorylase activity is reciprocal to that of oxidative enzymes and it generates a continuous spectrum of coloration dependent on the enzyme activity.

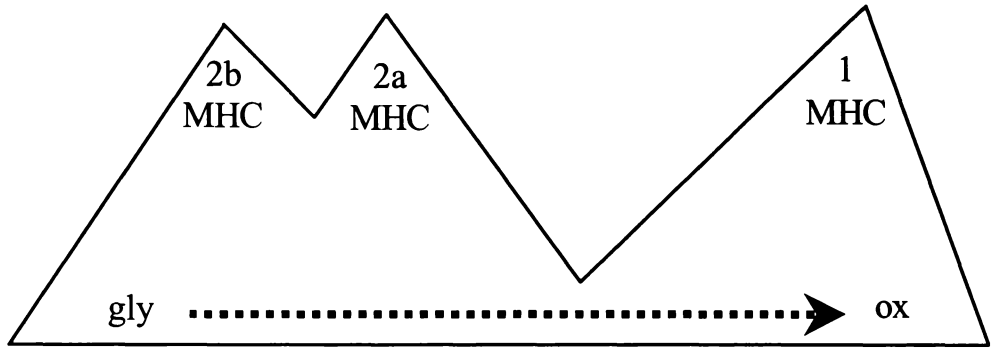
1.3.3.4 Interpretation of fibre typing results

An underlying assumption of fibre typing is that a specific mATPase staining reaction corresponds to the presence of a specific MHC isoform, as well as to contraction speed and possibly to metabolic enzyme activity. Is this assumption valid? Does the interpretation of fibre typing results depend upon this assumption being valid?

The availability of specific antibodies against myosin heavy chain isoforms and the assertion by Kelly and Rubenstein (1994) that MHC composition is the most definitive descriptor of fibre type, suggests that MHC immunohistochemistry should be the benchmark technique to which others should be compared. The identification of at least ten MHC isoforms strongly suggests that in principle classification systems limited to three fibre types may be limited in value. However, given the temporal and spatial restriction in MHC distribution, muscles may not express more

than three isoforms at any one time. One important finding that has arisen from MHC immunohistochemistry is the appearance of hybrid fibres. These fibres contain more than one MHC isoform, with an example being the type 2c fibres which are hybrids containing type 1 and type 2a MHC isoforms (Gauthier and Lowey, 1979). The ability to identify hybrid fibres is particularly important when considering fibres undergoing MHC isoform transformation. The existence of hybrid fibres and the concept of fibre type transformation suggests that the levels of MHC isoforms expressed in an individual fibre may represent a continuum, from none to exclusive. This is illustrated in Figure 1.7.

Figure 1.7. Diagram showing MHC isoforms as the most discretely distributed properties of fibre types at the apex of the triangles representing each fibre type, with the more variable property represented at the bottom (Redrawn from Hauschka, 1994)



Fibres with high mATPase activity exhibit variable metabolic activity from highly glycolytic to mixed oxidative-glycolytic metabolism. Even within the two high mATPase subtypes 2a and 2b, there is a relatively low correlation between mATPase activity and metabolic enzyme activity (Dubowitz and Brooke, 1973; Nemeth and Pette, 1980; Spurway, 1981). In mature muscle, fast contracting fibres may be highly variable in their metabolic activity, whereas slow fibres are exclusively oxidative although to a greater or lesser extent (Kelly and Rubenstein, 1994). This relationship also breaks down when considering developing muscle. Early post-natal muscle is slow contracting, yet stains intensely for mATPase activity, suggesting a fast fibre type (Guth and Samaha, 1972). Interpretation of

mATPase staining results in developing muscle must therefore be made with this consideration in mind.

In summary, the most important consideration in choosing a method for analysing muscle fibre type is recognising what information is being sought and why it is required. MHC isoform composition is best identified by immunohistochemistry, gene expression by PCR or *in situ* hybridisation. The mATPase method defines fibres on the basis of mATPase enzyme activity. If information is required on metabolic activity or contraction speeds of muscle fibres, then it is appropriate to use the methods and the systems of nomenclature that define these properties. All of these methods have a role to play in terms of the information they provide with respect to the physiology and biochemistry of muscle. With due attention to the interpretation of results from each of these methods they will continue to have a place in muscle biology.

1.3.4 Applications of fibre type analysis

The basis of much of the early interest in muscle fibre typing lies in human muscle pathology. A number of conditions exist in which the normal fibre type composition of muscle is perturbed. These include nemaline myopathy in which type 1 fibres are enlarged and more numerous and also congenital fibre type disproportion in which type 1 fibers are reduced in size (Miike *et al.*, 1986). In fascioscapulohumeral muscular dystrophy, type 2c fibres are affected in a process which resembles myofibre regeneration (Lin and Nonaka, 1991) and in congenital myotonic dystrophy there are discrepancies in both fibre size and fibre type distribution (Farkas-Bargeton *et al.*, 1988). The muscular dystrophy mutant mouse (mdx) is an animal model of human Duchenne muscular dystrophy and in the soleus of mdx mice there is an increase in fast glycolytic fibres at the expense of slow oxidative fibres (Marshall *et al.*, 1989).

In the context of this study which includes an analysis of fibre type in cattle, it is appropriate to also note the correlation between fibre type and meat quality (Ashmore, 1974). In cattle, organoleptic properties of meat such as juiciness and tenderness tend to be correlated with increased proportions of fast glycolytic fibres,

although results are not always clear-cut (Ashmore, 1974; Melton *et al.*, 1975; Totland *et al.*, 1988). One study of lambs selected for meat colour, found the redder, slow muscles had greater juiciness and flavour than the muscles with more white fibres (Valin and Touraille, 1982). Another meat quality issue in which fibre type composition plays a role is dark-cutting beef, with losses in carcass value of up to 20% estimated to occur due to this condition. Cattle at risk for dark-cutting have a higher proportion of oxidative muscle fibres (Zerouala and Stickland, 1991).

This section has given an introduction to fibre type diversity and the role that the MHCs play in defining fibre type. The following section will focus on the expression of other muscle-specific genes as well as the MHCs and examine the co-ordinate way in which gene expression is regulated in skeletal muscle.

1.4 Gene expression in muscle

This section will focus primarily on the transcriptional regulation of three groups of genes, the expression of which overlaps both temporally and spatially. The groups that will be reviewed are (i) those involved in positional signalling or the patterning of muscle organisation, (ii) the myogenic regulatory factors (MRFs) and (iii) the MHCs, all of which play specific roles in myogenic determination and in the specification of skeletal muscle fibre type.

There are many common aspects to the transcriptional regulation of muscle-specific genes. This includes the presence of several enhancers that are found in almost all of the muscle-specific genes that have been extensively studied. Both muscle specific promoters and the enhancers that elevate their transcriptional activity may contain several different muscle control elements. Major muscle control element families include the MEF1/E-box, A/T rich, MEF2, CarG/SRE and M-CAT families (reviewed by Hauschka, 1994). Binding to these muscle control elements represents an important control point in expression of these genes.

1.4.1 Genes involved in positional signalling

The patterning of the limb bud musculature is highly dependent upon positional cues which are derived from a variety of axial and lateral tissue sources (Buffinger and Stockdale, 1994; Buffinger and Stockdale, 1995; Stern *et al.*, 1995).

One of the genes involved in positional signalling is *Pax-3*, a member of the paired type homeobox family of transcription factors (Strachan and Read, 1994). Expression of this family of genes is developmentally regulated and in the case of *pax-3*, it is switched on in myogenic cells of the somites, migratory muscle precursor cells and myoblasts of developing limbs (Goulding *et al.*, 1994). *Splotch* mutant mice lack a functional gene for *pax-3* and they contain no limb muscles, although trunk muscle formation is normal. This suggests that *pax-3* expressing cells migrating from the dermomyotome are necessary for the development of limb muscles (Franz *et al.*, 1993).

Co-ordinate expression of the gene for hepatocyte growth factor/scatter factor (HGF/SF) and its tyrosine kinase receptor *c-met*, has been shown to be essential for the migration of myogenic precursor cells from the somite to the limb buds (Bladt *et al.*, 1995; Brand-Saberi *et al.*, 1996). The *c-met* receptor is expressed in the cells of the dermomyotome, while its ligand HGF/SF is expressed in the proximal limb bud. Mice carrying a null mutation in the *c-met* gene have a phenotype very similar to the splotch mutant (Bladt *et al.*, 1995) and it has been suggested that the *c-met* gene may be regulated by *pax-3* (Yang *et al.*, 1996a). The mode of action of HGF/SF appears to be through stimulating the release of myogenic cells from the dermomyotome (Brand-Saberi *et al.*, 1996).

Another gene involved in patterning of early vertebrate myogenesis is Sonic hedgehog (*Shh*). *Shh* is expressed in committed myogenic precursor cells after their migration to the limb buds, but before expression of the MRFs (Deprez *et al.*, 1998). Ectopic expression of the *Shh* gene results in increased expression of *pax-3*, *MyoD* and myosin and in hypertrophy of the limb (Deprez *et al.*, 1998), suggesting that *Shh* functions through increasing myoblast proliferation.

In addition to the genes mentioned here, there are a number of others with important roles in positional signalling. These include the *wnt* family, *noggin*, *BMP4* and *FGF* and their role in limb muscle organisation has been recently reviewed (Blagden and Hughes, 1999; Johnson and Tabin, 1997).

1.4.2 Myogenic regulatory factors (MRFs)

Identification of a family of basic helix-loop-helix (bHLH) proteins, called the myogenic regulatory factors (MRFs) represented a major breakthrough in the understanding of mechanisms controlling myogenesis (Hauschka, 1994; Olson, 1990; Olson, 1994). These factors were identified on the basis of the ability of any of the MRF genes to induce non-myogenic cells to express muscle specific proteins (Braun *et al.*, 1989; Davis *et al.*, 1987; Edmondson and Olson, 1989; Rhodes and Konieczny, 1989). The MRFs (myf5, MyoD, myogenin and MRF-4) are part of a large family of transcription factors involved in the regulation of growth and differentiation in a wide variety of tissues.

The MRFs bind to products of the E2A gene eg E12 or E47 and these heterodimers bind to a consensus DNA binding sequence, the E-box motif (CAANTG), present in muscle-specific enhancers (Buskin and Hauschka, 1989). Formation of these heterodimers can be inhibited by another HLH protein, Id, (inhibitor of differentiation) which binds to E-proteins and prevents dimerisation with the MRFs (Benezra *et al.*, 1990). Retinoblastoma protein (RB) on the other hand, binds to the DNA-bound MRF-E2A heterodimer to stabilise the complex and maintain RB in a non-phosphorylated and inactive state (Gu *et al.*, 1993). T-antigen and cellular oncogene products seem to have an opposite effect on myogenesis to that of the MRFs and this is thought to arise through disruption of the association between RB and MRFs (Gu *et al.*, 1993). Once formed however, this transcriptionally active complex of MRF+E-protein+RB is thought to be an important control point in myogenesis. Formation of this complex is dependent upon the external environment including the concentrations of growth factors and oncogene products.

Expression of the MRF genes is not limited to developing muscle. In adult rat muscle MRF4 occurs in the greatest abundance. Myf-5 mRNA is undetectable in

adult muscle and the relative abundance of MyoD and myogenin is associated with muscle fibre type (reviewed by Hauschka, 1994). In predominantly fast twitch muscles, MyoD predominates and in slow twitch muscles, myogenin is most abundant (Hughes *et al.*, 1993). Changes in MRF expression occur in adult muscle in response to hypertrophy and atrophy, in a muscle fibre type dependent manner (Loughna and Brownson, 1996) and myf-5 and MRF-4 expression is increased in response to stretch and electrical stimulation (Jacobs-El *et al.*, 1995). MyoD can be used as a marker for satellite cells in mature skeletal muscle, where its expression is neurally regulated (Koishi *et al.*, 1995). This evidence collectively supports the suggestion that in addition to their role in early myogenesis, the MRFs may also contribute to the ongoing maintenance of muscle-specific gene expression.

1.4.3 Myosin heavy chain (MHC) gene expression

Many of the structural proteins that make up skeletal muscle are synthesised in a number of isoforms and the MHCs are no exception. The genes that code for the MHC protein isoforms, as for all the myofibrillar protein isoforms, share a high degree of sequence homology, indicating a common evolutionary origin. In humans the MHC genes are found in two clusters, one on chromosome 14 which codes for the $MHC_{\beta/slow}$ and MHC_{α} isoforms and another on chromosome 17 which codes for MHC_{emb}, MHC_{neo} and two fast MHC genes (reviewed in Schiaffino and Reggiani, 1996)

Regulation of MHC gene expression can occur at the levels of transcription, mRNA stability (Cox and Buckingham, 1992) and translation (Lyons *et al.*, 1990). The transcriptional regulation of MHCs has much in common with other structural proteins in muscle. Much of the work in this area has been on other muscle-specific genes, in particular creatine kinase, myosin light chains and troponin isoforms (reviewed in Hauschka, 1994) and these studies have focussed on common regulatory elements: muscle specific enhancers, promoters and control elements. The interactions of the MRF family of transcription factors with MHC gene expression have been described in the previous section and will not be discussed further. Other transcription factors which impact upon MHC gene expression

include MAPF2 and serum response factor (SRF) which bind to the CarG/SRE enhancer and MEF2 and RSRF which bind to the MEF2 enhancer. All of this work has been reviewed most comprehensively by Hauschka (1994) and in summary, it was concluded that the transcriptional response of each MHC gene is dependent upon the activity of common control elements.

The following section will introduce another level of regulation of the myogenesis programme and will describe the role of two important growth factor families in muscle development.

1.5 Growth factor regulation of muscle development

The growth factor milieu plays a critical role in determining the fate of myoblasts. Myoblast proliferation *in vitro* is induced by the presence of some growth factors, while the signal for myoblasts to exit the cell cycle and become terminally differentiated is associated with low growth factor conditions. This review will focus in particular on two growth factor families involved in muscle development, the insulin-like growth factors (IGFs) and the transforming growth factor β superfamily (TGF-βs).

1.5.1 Insulin-like growth factors (IGFs)

The role of the IGFs in muscle development has been intensively investigated. There are two IGFs, IGF-1 and IGF-2 and their respective receptors, as well as a family of 6 IGF binding proteins and a further 4 low-affinity binding proteins (IGFBP-rP1-4) (Rechler, 1997). Human IGF-1 is a 70 amino acid, 7649 dalton peptide (Rindernecht and Humbel, 1978a) and human IGF-2 is a 67 amino acid, 7471 dalton peptide (Rindernecht and Humbel, 1978b).

1.5.1.1 IGF production

The primary source of the IGFs in the circulation is the liver (Schwander *et al.*, 1983). IGF-1 production is under the influence of growth hormone which stimulates the release of IGF-1 into the circulation (Green *et al.*, 1985; Mathews *et al.*, 1986; Zapf *et al.*, 1981). IGF-2 production is not considered to be growth hormone

dependent (Sara and Hall, 1990). Significant quantities of IGFs are synthesised in tissues other than liver, including muscle (D'Ercole *et al.*, 1984; Mathews *et al.*, 1986), suggesting an autocrine/paracrine role for the IGFs. Recent reports using the *Cre/loxP* recombination system have deleted the *igf1* gene from the liver of mice resulting in a 75% reduction in circulating IGF-1 levels (Sjögren *et al.*, 1999; Yakar *et al.*, 1999), but no effect on body size. These studies have provided very strong new evidence in support of the importance of the paracrine/autocrine role for IGF-1.

The *igf1* gene is found on human chromosome 12 and consists of at least 6 exons (Jansen *et al.*, 1983). Alternative splicing produces multiple mRNA transcripts. The mature IGF-1 peptide purified from human plasma contains 4 domains A-D, while cDNA sequences from liver contain three different E peptide coding sequences (Rotwein, 1986). One specific E peptide isoform of IGF1 (IGF-1Eb), appears to be specifically up-regulated in response to stretch induced muscle hypertrophy in rabbits (Yang *et al.*, 1996b).

The *igf2* and *insulin* genes are found in close proximity to each other on the short arm of human chromosome 11 (Bell *et al.*, 1984). The human *igf2* gene comprises 8 exons of which exons 5-7 encode the IGF-2 precursor and the 3' untranslated region. *igf2* is an imprinted gene and only the paternal allele is normally expressed (reviewed by O'Dell and Day, 1998). There are four different promoter regions (P1-4) for *igf2* (van Dijk *et al.*, 1991) and there is developmental and tissue specificity with respect to transcription from the different promoters. In adult liver, IGF-2 transcribed from the P1 promoter exhibits biallelic expression whereas in fetal tissues, where promoters P3 and P4 are used, the gene is imprinted (Vu and Hoffman, 1994).

There is a high degree of sequence homology among species in the genes for both IGF-1 and IGF-2. Human, bovine and porcine IGF-1 are identical and the rat and mouse sequences differ by three and four amino acids respectively. Human and bovine IGF-2 sequences differ by three amino acids (reviewed in Sara and Hall, 1990).

Expression of both of the IGF genes shows strong developmental and tissue specific regulation. Both are expressed during fetal and early postnatal life but IGF-2 is far more abundant (Sara and Hall, 1990). In adult rats, IGF-1 is abundant in liver but at low levels in other tissues, while IGF-2 mRNA is abundant in brain only (Murphy *et al.*, 1987).

1.5.1.2 IGF receptors

The biological effects of the IGFs and other growth factors are elicited by their binding to specific cellular receptors. There are two specific IGF receptors and the IGFs also bind to the insulin receptor (Rechler and Nissley, 1985). The IGF-1 receptor is a 300 kDa heterotetramer consisting of two α and two β subunits. The affinity of the type 1 receptor for the IGF family can be described as IGF-1≥IGF-2>>>insulin (Rechler and Nissley, 1985). The type 1 receptor is considered to be of greatest importance in transducing growth factor signals. Evidence for this comes from knockout mice where an *Igf1r*(-/-) mutation results in a more dramatically reduced birthweight than either the IGF-1 or IGF-2 knockouts (Liu *et al.*, 1993). The type 1 IGF receptor utilises a tyrosine kinase signalling pathway. Ligand binding to the α-subunit induces tyrosine kinase activity in the β-subunit and this in turn leads to the activation of the events of the downstream signalling cascade (Rechler and Nissley, 1985).

The IGF-2 receptor is a 220 kDa monomer, consisting of a single transmembrane domain, an extracellular domain with fifteen repeating motifs and a small cytoplasmic domain. Its affinity for the IGFs is: IGF-2>>>IGF-1=insulin (Rechler and Nissley, 1985). The role of the IGF-2 receptor in the signal transduction pathways of the IGFs remains unknown, but it is generally accepted that the actions of all the IGFs are mediated through binding to the IGF-1 receptor. The IGF-2 receptor has been shown to be identical to the mannose-6-phosphate receptor which is associated with protein degradation via the targeting of lysosomal enzymes (MacDonald *et al.*, 1988; Morgan *et al.*, 1987). A role for this receptor in the metabolism of IGF-2 has been proposed and this has been supported by an observation that antibodies which blocked the IGF-2 receptor caused a 90% reduction in IGF-2 degradation in L6 myoblasts (Keiss *et al.*, 1987). The

establishment of a null mutation in the *Igf-2/Mpr* gene surprisingly resulted in a high birthweight mouse, but almost all mutants died due to generalized organomegaly, heart abnormalities and oedema. In a double cross between surviving mutants and the *igf2*(-/-) line, the phenotype was completely rescued (Ludwig *et al.*, 1996). This further supports the notion that the type 2 IGF receptor has a major role at least in the embryo in regulating IGF-2 levels (Wang *et al.*, 1994).

1.5.1.3 IGF binding proteins

The IGFs are found in the circulation bound to specific high affinity IGF binding proteins (IGFBPs). These binding proteins serve to protect the IGFs from degradation, thus increasing their half life and they aid in the delivery of IGF to specific tissues. The majority of the effects result in potentiation of IGF activity. IGFBP-4 however has been shown to be inhibitory in its actions (reviewed in Florini *et al.*, 1996), as has IGF-BP-3 (Rechler, 1997). In addition to the six high affinity IGFBPs, four low-affinity IGFBP related proteins (IGFBP-rPs) have also been identified (Kim *et al.*, 1997). Collectively these make up the IGFBP superfamily of proteins which may function in an IGF-dependent or IGF-independent manner to regulate growth.

The IGFBPs are synthesised in a tissue-specific manner. The major IGFBP in skeletal muscle appears to be IGFBP-5 and its secretion is rapidly up-regulated at the onset of differentiation in muscle cells *in vitro* (Tollefsen *et al.*, 1989). This BP has been identified in abundance *in vitro* and *in vivo* with IGFBP-4 and –6 also present in cultured myoblasts. IGFBP-2 was detected in turkey myoblasts *in vitro* and in embryonic skeletal muscle of chickens, but not in postnatal rat muscle (reviewed in Florini *et al.*, 1996). These studies suggest that the IGFBPs may have a number of different functional roles in the regulation of muscle growth.

1.5.1.4 Biological actions of IGFs in skeletal muscle

The role of the IGFs as important regulators of myogenic development has been demonstrated through gene targeting studies (De Chiara *et al.*, 1990; Liu *et al.*, 1993), cell culture studies (Ewton *et al.*, 1994; Quinn and Haugk, 1996) and *in vivo* expression analysis (Gerrard *et al.*, 1998; Listrat *et al.*, 1994).

There is an apparent paradox in the biological actions of the IGFs on skeletal muscle, in that they are able to stimulate both proliferation and differentiation of myoblasts, two mutually exclusive activities. These two activities are however separated on a temporal basis and it appears that IGFs are initially associated with the inhibition of differentiation (Rosenthal and Cheng, 1995) and that the differentiation stimulating activity is induced subsequently (Ewton and Florini, 1981). The mechanisms by which IGFs act to inhibit differentiation include the down-regulation of myogenin expression and the maintenance of RB in its phosphorylated state, possibly by the up-regulation of cyclinD1 and cdk4 as regulators of RB phosphorylation (Rosenthal and Cheng, 1995).

The inhibition of differentiation by IGFs is functionally equivalent to a stimulation of proliferation and this has been demonstrated in myoblasts through their ability to effect a generalised increase in metabolic activity. This includes the stimulation of amino acid and glucose uptake, increased protein synthesis and decreased protein degradation (Dodson *et al.*, 1996). Although all members of the IGF family have general anabolic actions in muscle cells, there is evidence to suggest that an additional "mitogenic competence factor" is also required for these actions to take place (McWade *et al.*, 1995; Napier *et al.*, 1999).

It is the ability of IGFs to stimulate myogenic differentiation that has been the focus of most research interest, particularly given that other growth factors such as the TGF-βs and FGFs strongly inhibit differentiation (Allen and Boxhorn, 1989; Allen *et al.*, 1984; Vaidya and Rhodes, 1989). The stimulation of differentiation by IGF-1 occurs in a biphasic manner, with low concentrations (0.5 – 20 ng/ml) being stimulatory, but higher concentrations (>100 ng/ml) reducing differentiation to almost control levels, although not inhibiting it (Florini *et al.*, 1986). The primary mechanism through which IGF-1 is proposed to effect an increase in differentiation is through the induction of myogenin (Florini and Ewton, 1990), this being the opposite response to that observed when IGF-1 is active in the inhibition of differentiation (Rosenthal and Cheng, 1995).

The response of muscle cell lines to exogenous IGF is variable and this is associated with the endogenous IGF production. Differentiation of myoblasts is associated with

an increase in the autocrine expression of IGF-2 (Florini *et al.*, 1991b). Subsequently, the autocrine production of IGF-2 was shown to down-regulate the IGF-1 receptor in myoblasts undergoing differentiation (Rosenthal *et al.*, 1991). The IGF-2 receptor has been shown to be co-ordinately up-regulated along with IGF-2 in association with differentiation (Tollefsen, 1989)

The actions of IGFs both *in vitro* and *in vivo* can be moderated by other growth factors. A study of proliferation and differentiation of satellite cells in culture revealed that the differentiation-inhibiting action of TGF-β could not be counteracted by IGF-1 or FGF, but the proliferation depressing effect of TGF-β could not inhibit the mitogenic action of FGF. The greatest proliferative response occurred in the presence of both IGF-1 and FGF (Allen and Boxhorn, 1989). Thyroid hormone also has a profound effect on the IGF axis through regulation of the expression of the type 1 IGF receptor (Moreno *et al.*, 1997) and the IGFBPs (Nanto-Salonen *et al.*, 1991).

The biological actions of IGFs are by no means restricted to the prenatal period. In skeletal muscle of postnatal animals, the IGFs continue to play an important role. IGF-1 has been shown to be associated with changes in muscle mass due to hypertrophy or atrophy (Adams and Haddad, 1996; Czerwinski *et al.*, 1994; De Vol *et al.*, 1990; Vandenburgh *et al.*, 1991). IGF-2 mRNA is significantly decreased in muscles undergoing atrophy after space flight, but IGF-1 is not affected (Shivji *et al.*, 1999). The IGFs are also involved to a significant extent in the regeneration of skeletal muscle post-injury (Keller *et al.*, 1999; Kirk *et al.*, 1996; Ullman *et al.*, 1989). Two quantitative trait loci (QTLs) with major effects on skeletal and cardiac muscle mass have been identified which map to the *igf2* locus in pigs (Jeon *et al.*, 1999; Nezer *et al.*, 1999), demonstrating a role for the *igf2* locus in postnatal muscle development.

Skeletal muscle fibre type is also modulated by the IGFs in post-natal animals. Over-expression of IGF-1 in mouse skeletal muscle results in a transformation to more oxidative fibre types (Coleman *et al.*, 1995) and hypophysectomy in rats, which reduces circulating IGF-1 levels, causes a decrease in the proportion of slow oxidative fibre types (Ayling *et al.*, 1989).

In ovine skeletal muscle, IGF receptor abundance is altered during postnatal life and is affected by nutritional status, particularly in connective tissue (Oldham *et al.*, 1996a; Oldham *et al.*, 1996b). Circulating levels of IGFs increase after birth in the lamb (Bass *et al.*, 1994), while in skeletal muscle, mRNA expression levels and receptor abundance decline (Oldham *et al.*, 1996a), suggesting a greater emphasis on an endocrine rather than autocrine/paracrine mode of action in the postnatal period.

1.5.2 Transforming growth factor-β (TGF-β) super-family

The (TGF-β) super-family comprises a number of sub-families of growth factors grouped together on the basis of structural similarities. These include the activins/inhibins, bone morphogenetic proteins, Muellerian inhibiting substance, Vgr's, growth and differentiation factors (gdf's) and the type family TGF-β's. This review will focus on three mammalian isoforms of TGF-β (β1-β3) and their role in myogenesis and on another more recently identified member of the TGF-β family, myostatin.

There are a number of features that are common to all the TGFs in spite of considerable divergence within the amino acid sequences. These common features include the presence of a hydrophobic signal sequence, a post-translational modification which cleaves the latent precursor form and the presence of seven conserved cysteine residues in the most highly homologous C-terminal region of the peptide (McPherron and Lee, 1995). TGF-βs are synthesised in a latent form and cleavage of a latency-associated peptide (β1-LAP) from the inactive precursor is a prerequisite to activation of TGF-β. β1-LAP is the N-terminal remnant of the precursor TGF-β peptide (Miyazono *et al.*, 1993). Dimerisation of the C-terminal 112 amino acid peptide through disulphide bonding then constitutes activation of the peptide (Miyazono *et al.*, 1993).

Myostatin (GDF-8) shares greatest sequence homology with Vgr-1 (45% in the C-terminal region (McPherron *et al.*, 1997)) and it is likewise produced in a latent precursor form. In transfected CHO cells, recombinant murine precursor myostatin is proteolytically cleaved to give a mature 15 kDa peptide (McPherron *et al.*, 1997).

In bovine skeletal muscle extract an unprocessed precursor form of approximately 55 kDa has been identified, as well as a processed form of approximately 28 kDa (Somers, 1999).

1.5.2.1 TGF receptors

In common with the IGFs, biological responses to TGFs are elicited via binding to trans-membrane receptors which tranduce a signal to the nucleus via a complex signalling pathway. In the case of the TGFs most cell types exhibit three cell surface receptors, types I, II and III (Miyazono *et al.*, 1993). The action of TGF-β in inhibiting myogenesis appears to be mediated through the type II TGF-β receptor (Olson, 1993). The type II receptor has a threonine/serine kinase domain (Lin *et al.*, 1992) and this signalling pathway is common to other members of the TGF family. The receptor for myostatin and its signal transduction pathway has not yet been identified.

1.5.2.2 TGF binding proteins

Active TGF-β peptides bind to both soluble and membrane bound proteins in associations that are independent of signal transduction pathways. These binding proteins may act as reservoirs of TGF-β, they may serve to inhibit TGF action or they may serve to mop up excess TGF in tissues (Miyazono *et al.*, 1993). In bovine skeletal muscle extract, myostatin has been found to associate with glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase III and one other as yet unidentified protein (Somers, 1999). The association of myostatin with these two proteins may be indicative of a role for the growth factor in regulation of metabolic pathways in muscle (Somers, 1999).

1.5.2.3 Biological actions of TGFs in skeletal muscle

Expression of TGFs occurs in a wide variety of tissues. In mammalian skeletal muscle, the predominant isoforms are TGF-βs 1-3. TGF-β is an extremely potent inhibitor of myogenic differentiation. *In vitro*, TGFs can substitute for serum and block differentiation of myoblasts, but unlike the highly mitogenic actions of serum, this inhibition can occur without stimulating proliferation (Hu and Olson, 1990). Within the limb bud, TGF-β inhibits late myoblasts but not early myoblasts and this

has been proposed as a mechanism for the regulation of skeletal muscle development (Cusella-DeAngelis *et al.*, 1994). This mechanism allows for the formation of primary fibres from post-mitotic embryonic myoblasts, which may in turn stimulate a wave of proliferation of late myoblasts destined to form secondary fibres.

The action of TGF-β in inhibiting myogenesis appears to be mediated through binding to the type II TGF-β receptor, as described. As myoblasts undergo terminal differentiation and begin fusion, cell surface receptors for TGF-β are down-regulated. Under culture conditions which permit cells to withdraw from the cell cycle and differentiate to produce muscle-specific proteins, but not to undergo fusion, TGF-β receptors are not down-regulated (Hu and Olson, 1990). This observation suggests that the down-regulation of TGF-β receptors is associated with fusion rather than the expression of muscle-specific proteins.

Expression of TGF-β1 has been identified in connective tissue of developing muscles (Ignotz and Massagué, 1986) and it has been proposed that those muscle fibres which form adjacent to TGF-β1 expressing connective tissue are likely to mature into fast fibres. In the absence of TGF-β1, slow fibres form (McLennan, 1993). This evidence is consistent with a previously proposed autocrine/paracrine mechanism for TGF-βs in embryonic development (Pelton *et al.*, 1991).

Studies using cardiac muscle cell culture have identified TGF-β as a growth factor with the ability to up-regulate expression of tissue-specific foetal genes (Parker *et al.*, 1991), including the gene for β-cardiac MHC, which is identical to the gene for slow MHC (Lompre *et al.*, 1984).

1.5.2.4 Role in postnatal animals

In postnatal animals, members of the TGF-β family are expressed in skeletal and in cardiac muscle (Lafaytis *et al.*, 1991) and their localisation at the neuromuscular junction suggests a significant function in neuromuscular communication (Toepfer *et al.*, 1999). In damaged and regenerating muscle, TGF-β2 is up-regulated and may have a role in myotube formation *in vivo* (McLennan and Koishi, 1997). TGF-β1 is

increased in a number of muscular dystrophy conditions where it may be associated with increased synthesis and accumulation of extracellular matrix (Bernasconi *et al.*, 1999; Iannaccone *et al.*, 1995; Yamazaki *et al.*, 1994).

Expression of myostatin has been shown in a number of different muscles in adult cattle (Kambadur *et al.*, 1997), as well as in pigs (Ji *et al.*, 1998) and in mice (Carlson *et al.*, 1998; McPherron *et al.*, 1997). The expression of myostatin was up-regulated in response to muscle wasting in adult human skeletal muscle (Gonzalez-Cadavid *et al.*, 1998) and atrophy (Carlson *et al.*, 1999; Carlson *et al.*, 1998; Shivji *et al.*, 1999).

1.5.3 Other growth factors

This review has focussed on IGFs and the TGF-β super-family, but there are a number of other growth factors with important roles in muscle development. Members of the fibroblast growth factor (FGF) family for example have been shown to have a role in aspects of myogenesis. *In vitro* FGFs stimulate proliferation and inhibit differentiation (Maley *et al.*, 1995). Basic FGF is increased in regenerating myotubes after crush injury in mice (Anderson *et al.*, 1995) and a recent study has demonstrated changes in FGF expression in response to induced skeletal muscle hypertrophy (Mitchell *et al.*, 1999).

The thyroid hormones 3,5,3'-triiodothyronine (T_3) and thyroxine (T_4) have been well studied with respect to muscle development. Thyroid hormones are proposed to have a role in the regulation of muscle specific transcription factors (Muscat *et al.*, 1994), as well as an effect on the expression of genes for contractile proteins. Specifically, hyperthyroidism has been shown to be associated with a transition from slow to fast MHC isoforms (Izumo *et al.*, 1986; van der Linden *et al.*, 1996).

Earlier in this introduction (Section 1.4.1) hepatocyte growth factor/scatter factor (HGF/SF) was discussed with respect to its role in patterning early vertebrate myogenesis. HGF/SF has also been identified in adult muscle, where its expression is up-regulated in regenerating muscle (Jennische *et al.*, 1993).

Having now considered a number of the regulatory influences that have an effect on muscle growth, with a focus on the growth factors examined in this thesis, the final section will introduce the some of the major animal models in which muscle mass has been manipulated.

1.6 Models of altered skeletal muscle mass

Models of altered skeletal mass have the potential to contribute greatly to our understanding of muscle development. These models fall into two categories. The first are genetic models that may be either natural mutations, or be created by using transgenic methods. The second are alterations in skeletal mass induced by experimental manipulation. Within these two categories, two specific models will be discussed in some detail as they form the basis for the experimental studies described in this thesis. These are the double-muscling condition in cattle, arising from a natural mutation in the gene for myostatin and the experimental induction of skeletal muscle hypertrophy and atrophy as a result of immobilisation of muscles in lengthened and shortened positions respectively.

1.6.1 Genetic models

1.6.1.1 Double muscling

The double muscling or muscular hypertrophy condition in cattle has been observed for many years. One of the earliest descriptions comes from Culley (1807). Animals exhibiting this condition have various names in different countries and may also be known as culard, doppelender or a groppa doppia. The Belgian Blue breed, which has been used in this study, has been systematically selected for the expression of double muscling to a point where it is now considered to be fixed in many herds.

The genetic basis for the double muscling condition remained unknown until relatively recently. It was known to be caused by a single gene, *mh*, which was "partially recessive", given that homozygous mutants exhibit extreme hypertrophy, while heterozygotes have intermediate characteristics, but are closer in appearance to the homozygous normal (Hanset and Michaux, 1985). The *mh* gene was mapped to bovine chromosome 2 (Charlier *et al.*, 1995). More recently it was shown that

there is an 11 bp deletion mutation in the gene for myostatin in Belgian Blue cattle (Grobet *et al.*, 1997; Kambadur *et al.*, 1997) and a single bp substitution in the Piedmontese breed (Kambadur *et al.*, 1997). A series of further allelic mutations in myostatin has also been identified in other double-muscled breeds of cattle (Grobet *et al.*, 1998), with these mutations ranging from predicted loss of function of the protein, to conservative amino acid substitution, one silent DNA sequence variant and four polymorphisms in intronic sequences.

The 11 bp deletion mutation in the Belgian Blue results in a frame shift and the appearance of a premature stop codon (Kambadur *et al.*, 1997). This results in a truncated protein which does not include the sequence deemed to mediate essential function of the protein (McPherron *et al.*, 1997). In the Piedmontese, a conserved cysteine residue is converted to tyrosine and this is also likely to affect the function of the protein. The myostatin gene has been mapped to the interval containing the locus for the *mh* gene, strongly suggesting it is the gene responsible for the double-muscling condition (Smith *et al.*, 1997).

In addition to the extreme increase in musculature exhibited by these animals, there are other phenotypic characteristics typical of double-muscled (DM) breeds. In the Belgian Blue these characteristics include a thin skin, reduced skeletal mass, virtual absence of sub-cutaneous fat and a decrease in connective tissue giving the meat a coarser appearance (Boccard, 1981). These properties of increased leanness and improved tenderness due to less connective tissue, are consistent with greater consumer demand for 'high value' cuts of meat such as are produced by these animals. However, reproductive efficiency is also reduced, with an extremely high incidence of Caesarean deliveries due to dystocia and there are difficulties associated with reduced tolerance to stress in these animals. These problems have limited full exploitation of the breed's potential for the production of high quality meat.

Within the Belgian Blue breed, differences in muscle fibre number, fibre size and fibre type proportions have made these animals a useful model for the investigation of bovine muscle development. The increased musculature is apparent during prenatal development with an increase in body weight, but there is no associated

increase in crown rump length at the same gestational age (Ashmore *et al.*, 1974; Hanset *et al.*, 1976). The extreme muscular hypertrophy arises for the most part from an increased number of fibres in these animals (Swatland and Kieffer, 1974). This increase in muscle fibre number occurs in all muscles but the degree of muscular hypertrophy has been shown to be highly variable upon slaughter of mature animals, depending on the anatomical location of specific muscles (Shahin and Berg, 1985). In the more superficial muscles, those with a large surface area such as the M. *cutaneous trunci* and the M. *latissimus dorsi*, the hypertrophy is most dramatic, while in deeper muscles, close to the bone, there is in fact reduced muscle mass relative to normal animals. Examples of these reduced muscles include the diaphragm and the M. *vastus medialis* (Boccard, 1981). These muscles therefore have a smaller fibre diameter to accommodate the increased number of fibres within the smaller mass (Ouhayon and Beaumont, 1968).

In addition to increases in muscle fibre number, changes in muscle fibre size also have the potential to contribute to increased muscle mass. Literature describing changes in muscle fibre area in DM cattle does not give a clear-cut picture of fibre size changes in DM animals. While there is general agreement that increases in fibre area contribute little or nothing to the overall increase in muscle mass either during foetal development or postnatally (Ashmore *et al.*, 1974; West, 1974), there is some evidence to the contrary. Holmes et al (1972) showed that "white" (type 2) fibres were larger in double-muscled animals at all postnatal ages, whereas "red" (type 1) fibres were in general smaller than normal during early postnatal life, but not at slaughter. Relative differences in the size of "red" fibres between double-muscled and normal animals varied however according to individual muscles.

Differences in fibre type proportions between double-muscled and normal animals are more widely acknowledged. The general observation is that there is a greater proportion of type 2b (fast glycolytic) fibres in the double-muscled animals. This has been shown both during foetal development (Ashmore *et al.*, 1974) and postnatally (Holmes and Ashmore, 1972). Indeed the ratio of red to white fibres has been proposed as a potential index for the double-muscling condition (West, 1974). The most sensitive measure of genotypic differences in fibre type proportions in this

study was found to be the percentage of area classified as a particular fibre type (West, 1974). This measure combines information on fibre type proportions and the average area of those fibres and essentially magnifies the differences highlighted by the independent measurements.

The differences in muscle development that exist within the double-muscling condition made it a useful model for the investigation of the role of growth factors in muscle development. In particular, *in vitro* studies have focussed on the proliferative ability of myoblasts from double-muscled and normal animals and on the effects of conditioned media and serum derived from these two genotypes (Gerrard and Judge, 1993; Quinn *et al.*, 1990). The IGFs have become implicated in such studies as a family of growth factors that may be associated with the double-muscled condition. Conditioned media from double-muscled fibroblast cultures contained soluble growth factor activity (Quinn *et al.*, 1990) which was possibly due to IGFBP secretion (Quinn, personal communication to Dr Bass) and the mitogenic activity in double-muscled serum was postulated to be due to increased circulating levels of IGFs (Gerrard and Judge, 1993).

Circulating levels of a number of growth factors have been measured in double-muscled animals in an effort to identify whether the double-muscling condition was associated with an endocrine imbalance. Reduced levels of IGF-1, T_3, T_4 and testosterone have been demonstrated in Belgian Blue bulls, relative to light muscled Holsteins (Istasse *et al.*, 1990). The same study showed no consistent differences in levels of growth hormone and insulin, while a separate study showed growth hormone and insulin to be reduced in double-muscled animals (Arthur *et al.*, 1990). Another report describes elevated levels of T_3 and T_4 in double-muscled cattle (Strath *et al.*, 1982). Clearly the endocrine regulation of the double-muscling condition remains an area which requires further investigation.

Given the strong evidence in support of an autocrine/paracrine role for the IGFs in muscle development, local production of IGF-2 has been analysed in muscle of developing calves using Northern blot and *in situ* hybridisation (Gerrard and Grant, 1994; Listrat *et al.*, 1999). These studies have both shown an elevated period of high expression of IGF-2 in association with peak secondary fibre formation. In the

Northern analysis the peak of IGF-2 expression was delayed in double-muscled animals relative to normal animals (Gerrard and Grant, 1994) which may be associated with an inhibition of differentiation, and a prolonged period of myoblast proliferation.

1.6.1.2 IGF transgenics

There are a number of genetic models of altered IGF expression that have shown the profound effect this family of growth factors has on muscle development. IGF-1, type 1 IGF receptor (Liu *et al.*, 1993) and IGF-2 (De Chiara *et al.*, 1990) knockout mice all have reduced muscle development. Mice lacking IGF-1 or the type 1 receptor die at birth due to breathing difficulties and have essentially no functional muscle (Liu *et al.*, 1993).

Over-expression of IGF-1 in transgenic mice resulted in increased muscle mass as a result of hypertrophy of all muscle fibres. In these transgenic mice there was also a transformation towards more oxidative fibre types (Coleman *et al.*, 1995). In a different transgenic model, over-expression of IGF-1 was not able to prevent unloading induced atrophy of skeletal muscle in mice (Criswell *et al.*, 1998).

1.6.1.3 Myostatin knockout

The myostatin knockout mouse was produced after a gene sequence was identified from mouse genomic DNA for a potentially new member of the TGF-β super family, which was called *GDF-8* (McPherron *et al.*, 1997). This gene was then disrupted by homologous targeting in embryonic stem cells. The resultant homozygous mutant mice exhibited extreme muscular hypertrophy, which arose due to an increase in both the number and the average size of muscle fibres (McPherron *et al.*, 1997). On the basis of the proposed role for the GDF-8 gene in the negative regulation of skeletal muscle mass, it was renamed myostatin.

1.6.2 Experimental models of hypertrophy

Experimentally induced alterations in muscle growth permit the investigation of temporal changes in factors such as gene expression which occur in association with increases or decreases in muscle mass.

1.6.2.1 Stretch

Passive stretch is a very potent stimulus for muscle growth and can be experimentally induced by two different procedures.

The first experimental model involves the immobilisation of a limb in a plaster cast, with one functional group of muscles held at greater than normal resting length. This model will be explored in some detail as it the method that was chosen for the induction of skeletal muscle hypertrophy in Chapter 6 of this thesis.

Stretch which is induced by immobilisation of a muscle in a lengthened position, results in a rapid increase in muscle mass (Goldspink, 1977b; Williams and Goldspink, 1973). Unlike models which involve surgical ablation of a synergist muscle, which is one from within a functional group of muscles, this increase in mass is not thought to be associated with inflammation due to trauma (Armstrong *et al.*, 1979). It has been reported to be predominantly due to the increase in the number of sarcomeres in series along the length of the muscle fibres (Williams and Goldspink, 1971; Williams and Goldspink, 1973). This process in many ways recapitulates the developmental process of muscle growth where muscle fibres likewise increase in length by the assembly of new sarcomeric units at the fibre ends (Dix and Eisenberg, 1990). This assertion is further supported by studies which show the expression of embryonic or slow MHC isoforms at the myotendinous junction of stretched muscle fibres (Dix and Eisenberg, 1990; Kennedy *et al.*, 1988; Yang *et al.*, 1997).

IGF-1 mRNA expression is dramatically increased in stretched muscles and it was also shown that the muscle fibres undergoing fibre type transformation express high levels of IGF-1 mRNA (McKoy *et al.*, 1999; Yang *et al.*, 1997). The role of IGF-1 in skeletal muscle adaptation to increased loading has been recently reviewed (Adams, 1998). There is also a strong correlation between fibre type and expression of MRFs in stretched muscles, with fast twitch muscles showing an increase in myogenin mRNA and slow twitch muscles showing an increase in MRF-4 (Loughna and Brownson, 1996). A combination of stretch and electrical stimulation resulted in increased expression of MRF-4 and *myf-5* in fast twitch adult rat muscle (Jacobs-El *et al.*, 1995).

The other stretch model which is widely used applies to birds and involves the application of a weight to the wing of a chicken or quail to provide a stretch stimulus to the M. *anterior latissimus dorsi* (ALD) (Alway *et al.*, 1989; Barnett *et al.*, 1980) or M. *patagialis* (Czerwinski *et al.*, 1994). This model induces a rapid increase in muscle mass which is sustained for several weeks (Antonio and Gonyea, 1993; Barnett *et al.*, 1980; Laurent *et al.*, 1978; Winchester *et al.*, 1991). There is abundant evidence for increases in fibre area in this model (Alway *et al.*, 1990; Antonio and Gonyea, 1993; Holly *et al.*, 1980). Fibre type transformation in the wing weighting models is not so clear-cut. The ALD muscle used for most of these studies is 90% slow-β fibres (Alway *et al.*, 1989) and studies variously show either no change in fibre type (Antonio and Gonyea, 1993), or a switch to increased numbers of fast α-fibres (Holly *et al.*, 1980; Sola *et al.*, 1973). There is also some disagreement in the literature as to whether or not fibre number is increased with stretch. There is considerable evidence in support of an increase in fibre number from the avian wing-weighting models (Alway *et al.*, 1989; Alway *et al.*, 1990; Kennedy *et al.*, 1988; McCormick and Schultz, 1992), but others have found no increase (Barnett *et al.*, 1980; Gollnick *et al.*, 1983b). Relatively fewer studies have focussed on molecular mechanisms which underlie the increase in muscle mass, but it has been shown that IGF-1 mRNA is increased in wing weighted muscles (Czerwinski *et al.*, 1994), as are serum response factor and MyoD mRNA (Carson and Booth, 1999).

Although the stretch models are less well characterised than the synergist ablation model to be described next, there is sufficient evidence to suggest they have much to offer yet with respect to understanding the mechanisms involved in regulating changes in skeletal muscle mass.

1.6.2.3 Synergist ablation/tenotomy

Ablation (removal of a synergistic muscle), or tenotomy (severing of a synergist tendon) are surgical procedures designed to put extra load onto the remaining muscles within a functional group. These remaining muscles then undergo compensatory hypertrophy in response to the requirement to produce extra work. There has been considerable debate in the literature as to whether the compensatory hypertrophy induced in these models is in fact true work-induced hypertrophy

(Mackova and Hnik, 1973). The rapid increase in weight in the first few days after surgery is attributed to inflammation associated with the surgery (Armstrong *et al.*, 1979). A further complication in the use of this model is the suggestion that a component of the subsequent sustainable increase in weight arises because the tension generated by the antagonistic muscles group is distributed over fewer muscles in the affected group. Denervation of antagonistic muscle groups to eliminate the stretch component prevented hypertrophy in the remaining synergists (Mackova and Hnik, 1973).

Muscles which have undergone hypertrophy in response to synergist ablation have increased fibre diameters of both fast and slow fibres, with a greater increase in type 2b fibres than in 2a or type 1 (James, 1979). There are also an increase in the proportions of slow type 1 fibres (Baldwin *et al.*, 1982; Roy *et al.*, 1982; Roy *et al.*, 1997). Increased satellite cell activity is a prerequisite for the increase in muscle mass to occur in response to synergist ablation (Schiaffino *et al.*, 1976; Snow, 1990).

Some analysis of the role of growth factors in the regulation of muscle mass in compensatory hypertrophy models has been undertaken. IGF-1 and IGF-2 gene expression is up-regulated in response to functional overload in rats (Adams and Haddad, 1996; De Vol *et al.*, 1990). In a similar study, no difference was found in the expression of myogenin or MyoD with overload treatment (Mozdziak *et al.*, 1998).

1.6.2.3 Exercise

A number of weight lifting models have been evaluated for comparison with exercise induced hypertrophy in humans (Timson, 1990). The most well characterised model involved training cats to flex their wrists against increasing resistance (Gonyea and Ericson, 1976). In this model there was an increase in muscle mass and the mean diameter of all fibre types was increased but there was no effect on fibre type composition (Gonyea, 1980). There was also an increase in fibre number due to longitudinal splitting of fibres, as had been reported in previous studies of functional overload in rats (Hall-Craggs, 1970).

1.6.3 Experimental models of atrophy

1.6.3.1 Shortened immobilisation

This model of induced atrophy was used in the current study because it was completely complementary to the stretch induced hypertrophy experiment and enabled within animal comparisons of the effects of immobilisation of muscles at greater or less than resting length. Immobilisation of muscles at less than resting length induces rapid atrophy of muscles due to a decrease in protein synthesis and increased protein degradation (Goldspink, 1977a; Goldspink, 1977b). Muscle mass is lost through a reduction in the number of sarcomeres along the length of a muscle fibre and there is a reduction in the cross-sectional area of the fibres (Williams and Goldspink, 1973).

1.6.3.2 Space flight

Weightlessness induces rapid atrophy of skeletal muscle as has been shown in astronauts (Booth and Criswell, 1997; Edgerton *et al.*, 1995; Widrick *et al.*, 1999) and rats (Martin *et al.*, 1988). Given that the force generating capacity of a muscle fibre is proportional to its cross-sectional area, this has significant implications with regard to astronaut strength. Studies have been undertaken in rats to investigate the mechanisms which regulate this loss in muscle mass (Backup *et al.*, 1994) and in humans to investigate the types of exercise which best minimise these effects (Booth and Criswell, 1997). There has been particular interest in the role of growth factors in mediating this loss in muscle mass and it has been shown that myostatin mRNA expression is dramatically increased during space flight (Shivji *et al.*, 1999) and IGF-2 mRNA is decreased (Shivji *et al.*, 1999).

1.6.3.3 Hind-limb suspension

Given the difficulty associated with sending rats into space and the intense interest in minimising space induced atrophy, considerable effort has been directed towards developing a model which mimics the effects of microgravity. The hindlimb suspension model for the induction of skeletal muscle atrophy was devised by Musacchia (1980). Rodents are suspended in a harness so the hind limbs are non-weight bearing and this induces significant muscle atrophy. This model has a more

pronounced effect on slow muscle fibres, which have an anti-gravity role in the maintenance of correct posture. Slow muscle fibres have reduced cross-sectional area and are fewer in number as a result of hind limb suspension (Templeton *et al.*, 1988). This result is similar to that found in hind limb immobilisation. A study was carried out with the specific aim of identifying which of these two models more accurately represented muscle loss due to space-flight (Fitts *et al.*, 1986). The conclusion reached was that the primary difference between the two models was the elimination of load bearing or isometric contractions in the suspension model and that this most closely resembled the microgravity condition.

1.6.4 Justification for the animals models used in this study

Why were the double-muscling and the hindlimb immobilisation animal models selected for this investigation of skeletal muscle hypertrophy? In part these decisions were based on practicalities. The decision to use the double-muscled animals was based on existing information that suggested the potential of this model for investigating different stages of myogenic development – proliferation differentiation and maturation. Collaboration with two Belgian Blue breeders, Mr Bruce Worsnop and Ms Wendy Hopper and access to the facilities and expertise of the Molecular Embryology group at Ruakura Agricultural Centre meant that this investigation was practical.

The hind-limb immobilisation model was chosen based on evidence from the literature suggesting that this model gives a rapid change in muscle mass and a change in fibre type composition in adult animals. This made the model very complementary to the investigations that were being carried out in the double-muscled developmental model. Experimental animals for the study were readily available and the hind-limb immobilisation procedure avoided surgical intervention thereby eliminating potential difficulties in interpretation of results with respect to the contribution of inflammation and trauma. Furthermore, as normal muscle function is dependant upon groups of muscles or synergists, working together in functional groups to generate movement and opposed in their actions by antagonistic groups, this model allows all of the muscles within the lengthened group to be used

for analysis. Additionally, as the muscles of the antagonist functional group are under no tension and therefore undergo atrophy, these atrophied muscles can be studied also. A further advantage although not exploited in the current study, is that the casts can be removed, allowing the regression of the muscles to be investigated as sarcomeres are lost and the muscles returns to their normal size (Tabary *et al.*, 1972; Williams and Goldspink, 1973). Finally, the model presented very real possibilities with respect to the investigation of growth factor regulation of fibre type.

1.7 Scope of this thesis

Many previous studies have shown that a number of growth factors, including the IGFs, are involved in regulating changes in skeletal muscle mass, fibre type and fibre size. However, the temporal and spatial associations of specific growth factors with individual muscle fibres during changes that occur in normal development or in response to altered loading have received much less consideration. In addition, functional analysis of the role of the recently identified growth factor, myostatin, in adult skeletal muscle, is only beginning to be addressed. This thesis puts forward and tests the following hypotheses, in order to explore the relationship of both IGFs and myostatin with individual muscle fibres, in two different models of altered skeletal muscle mass, fibre type and fibre size:

Hypothesis 1: That the relative differences in size between M. *vastus medialis* and M. *vastus lateralis* in adult DM compared to NM, are present during foetal development and arise as a result of genetic differences.

Hypothesis 2: That differences in fibre type and fibre size contribute to overall differences in muscle mass between DM and NM.

Hypothesis 3: That growth factors are involved in the regulation of skeletal muscle fibre type and fibre size in DM and NM animals

Hypothesis 4: That myostatin is involved in the regulation of skeletal muscle mass in response to experimentally induced hypertrophy and atrophy

Hypothesis 5: That myostatin regulates changes in skeletal muscle mass through altered mRNA/protein abundance at different sites within a muscle.

CHAPTER TWO

MATERIALS AND METHODS

2.1 INTRODUCTION

The first part of this chapter presents information on less commonly known materials and reagents used in this thesis. Secondly the animals models used are described and thirdly the experimental protocols employed are outlined. Where these protocols are not widely used, or include modifications to standard procedures, methods are described in detail and justification for any changes made are given.

2.2 MATERIALS

2.2.1 Peptides and hormones

The following growth factors were donated to the Growth Physiology programme: purified ovine (o) IGF-2 by Dr L.G. Moore, (AgResearch, Wallaceville, NZ); recombinant human (rh) IGF-2 by Eli Lilly and Company, (Indianapolis, IN, USA), rh amino-terminal methionyl IGF-1 by Dr B.D. Burleigh, (I.M.C./Pitman-Moore, Terre Haute, IN, USA) and rh IGF-1 from Ciba-Geigy Corporation, (Basel, Switzerland). Des(1-3)-IGF-1 and des(1-6)-IGF-2 were purchased from Gro-Pep Pty, (Adelaide, Australia). Bovine insulin was from Sigma Chemical Co. (St Louis, MO).

2.2.2 Antibodies

Slow MHC antisera NOQ7.1.1A (mouse anti-human, monoclonal) was from Chemicon (Temecula, CA, USA).

Fast skeletal MHC antisera MY32 (mouse anti-rabbit, monoclonal) was purchased from Sigma Chemical Company (St Louis, MO, USA).

Embryonic MHC antisera 2B6 (mouse anti-rat, monoclonal) was a gift from Dr NA Rubinstein, (University of Pennsylvania Medical Centre, PA, USA).

Myostatin antisera (rabbit anti-bovine polyclonal, purified IgG fraction) was produced in the Growth Physiology group by Pete Fowke and Greg Somers.

IGF-1 antisera (rabbit anti-human, polyclonal) was purchased from Gro-Pep Pty Ltd.

IGF-1 antisera for radioimmunoassay: anti-recombinant human amino terminal methionyl IGF-1, was purchased from IMC/Pitman Moore.

IGF-2 antisera for radioimmunoassay (mouse anti-rat monoclonal) was purchased from Amano Pharmaceutical Company Ltd (Nagoya, Japan).

2.2.3 Probe template for *in situ* hybridisation

Human IGF-1 cDNA was obtained from M Jansen. It consisted of a 203 bp *Sau* 3A/*Sau* 3A insert in pUC 13 (Jansen *et al.*, 1983). The cDNA encodes part of the A domain of mature IGF-1, the E domain found in unprocessed IGF-1 and a small stretch of 3' untranslated region. The 203 bp cDNA sequence was subcloned into pGEM 3 by A Molenaar (Dairy Science Group, AgResearch) and used for riboprobe synthesis.

2.2.4 Isotopes

^{35}S-dUTP, ^{32}P-dCTP and ^{125}I radionuclide were obtained from NEN Research Products, (Boston, MA, USA).

2.2.5 Other chemicals

Ajax Chemicals (NSW, Australia)
Safe Solvent

Amersham Pharmacia Biotech (Uppsala, Sweden)

Hybond-N+ membrane; biotinylated donkey anti-rabbit antibody; biotinylated sheep anti-mouse antibody; streptavidin-biotin-horseradish peroxidase enzyme complex.

BDH Ltd (Poole, Dorset, UK)

2-Amino-2-methyl-propan-1-ol; ammonium polysulphide, calcium chloride, chloroform; citric acid; cobalt chloride, DePeX mounting medium, ethanol, formaldehyde, glycerol; hydrochloric acid, hydrogen peroxide, isopentane, methanol, paraformaldehyde, Paramat wax; potassium acetate; sodium chloride, *di*-sodium hydrogen orthophosphate; sodium hydroxide; sucrose; Tween 20;

DAKO (Carpintaria, CA, USA)

Normal rabbit IgG, normal mouse IgG.

Eastman Kodak Company (Rochester, NY, USA)

Kodak X-Omat AR film, NTB-2 liquid photographic emulsion, D-19 photographic developer, X-Omat fixer.

Life Technologies (Gaitherberg, MD, USA)

Agarose (ultra PURE™ and LMP low melting point); ethidium bromide; formamide; sodium citrate; sodium dodecyl sulphate (SDS); 100mM dNTPs; Taq DNA polymerase; 10X PCR buffer; RNase H; T3 and T7 RNA polymerase; TRIzol® reagent; Superscript™ Pre-amplification system; RadPrime DNA labelling system.

Promega (Madison, WI, USA)

Wizard PCR prep kits, Promega transcription kit.

ProSciTech (Queensland, Australia)

Sodium cacodylate

Roche Molecular Biochemicals (Switzerland)

Adenosine-triphosphate (ATP), degraded herring sperm DNA, Quickspin columns, yeast tRNA.

Sigma Chemical Company (St Louis, MO, USA)

Bovine serum albumin (BSA), dextran sulphate, diethyl pyrocarbonate (DEPC), β-mercaptoethanol (βME); polyvinvlpyrrolidine (PVP), proteinase K, Tris base.

Smith & Nephew (UK)

Dynacast Pro 5cm conformable fibreglass casting tape

2.3 COMPOSITION OF SOLUTIONS

2.3.1 *In situ* hybridisation buffers

Premix hybridisation buffer	5 M NaCl	0.3 ml
	formamide (AR)	5.0 ml
	20XSSC	1.0 ml
	yeast tRNA	0.2 ml
	degraded herring DNA (10mg/ml)	1.0 ml
Hybridisation buffer:	Pre-mix hybridisation buffer	780 µl
	BSA (20 mg/ml)	20 µl
	50% dextran sulphate	200 µl
	DTT	large blob
Washing solution 1:	2XSSC	
	10 mM β-ME	760 µl/l
Washing solution 2:	50% formamide	
	2XSSC	
	10 mM β-ME	760 µl/l
RNase treatment:	RNase A (35 mg/ml)	43 µl
	RNase T1 (3.5 mg/ml)	11 µl

2.3.2 Northern hybridisation solution

Pre-hybridisation buffer:	5X SSC
	50% deionised formamide
	5X Denhardts solution
	1% SDS
	25 mg/ml salmon sperm DNA

2.3.3 RT-PCR buffers

RT reaction mixture 1:	Total RNA (1µg/µl)	5 µl
	$Oligo(dT)_{12-18}$	1 µl
	DEPC water	6 µl
RT reaction mixture 2:	10XPCR buffer	2 µl
	25 mM $MgCl_2$	2 µl
	10 mM dNTP mix	1 µl
	0.1 M DTT	2 µl
PCR reaction mixture:	10x PCR buffer	5 µl
	2 mM dNTPs	5 µl
	primer S	2 µl
	primer AS	2 µl
	Taq polymerase	0.25µl
	water (no DEPC)	33.75µl
	cDNA from RT reaction	2 µl

2.3.4 Immunohistochemistry buffers

Tris buffered saline (TBS):	0.05 M Tris 0.15 M NaCl pH = 7.4
Citrate buffer: (for antigen unmasking)	10 mM sodium citrate 10 mM citric acid pH = 6.0
Blocking solution: (NS/TBS)	TBS 10% normal sheep serum
Washing buffer:	TBS 0.05% Tween-20
Chromagen	712 µl 0.2 M phosphate buffer 2.137 ml dH_2O 150µl DAB 7.5 µl 30% H_2O_2 7.5 µl nickel/cobalt intensifier

2.3.5 Receptor autoradiography buffers

Pre-incubation buffer:	0.17 M Tris-HCl at pH 7.4	
Incubation buffer:	0.17 M Tris-HCl at pH 7.4 1% BSA 5 mM $MgCl_2$ 1 mM phenylmethylsulphonyl fluoride iodinated hormone (20 000 cpm/ml)	
Post-incubation buffer:	0.17 M Tris-HCl at pH 7.4 0.25% BSA 0.01% Triton X-100	
Neutral buffered formalin:	100 ml 900 ml 4.5 g 6.5 g	37-40% formaldehyde solution dH_2O $NaH_2PO_4.2H_2O$ Na_2HPO_4

2.3.6 Fibre typing buffers

Fixative:	Formaldehyde (37-40% solution) 200 mM sodium cacodylate 68 mM $CaCl_2$ 340 mM sucrose dH_20	68 ml 16 g 3.77 g 58.2 g 500 ml
Pre-incubation buffer:	0.1 M potassium acetate	pH 5
Incubation solution:	0.1M AMIP (1M stock) 18mM $CaCl_2$ (1M stock) 2.7mM ATP dH_20	30ml 1.8ml 489 mg 300 ml
Tris rinse:	100mM Tris 18mM $CaCl_2$ dH_20	6.06 g 940 mg 500 ml

2.4 ANIMALS

2.4.1 Fibre type optimisation study

Tissue samples were collected from the M. *longissimus dorsi* of three Angus steers aged 30 months, slaughtered at the Ruakura Abattoir. Rabbit M. *tibialis anterior* was from the experiment described in Chapter 6 and ovine M. *semitendinosus* was provided by Dr Jenny Oldham.

2.4.2 Bovine muscle hypertrophy study

DM foetuses were produced using standard super-ovulation and embryo transfer techniques. The donor cows were pure-bred DM Belgian Blues and they were inseminated with semen from pure-bred highly muscled DM Belgian Blue bulls. As was stated in Chapter 1, the double-muscling condition is fixed in the Belgian Blue breed and the phenotype of the parents is a major predictor of double muscling in pure bred offspring. This approach maximised the production of highly muscled foetuses. Embryos were recovered seven days after insemination and were transferred to Hereford x Friesian recipient heifers.

Normal muscled (NM) foetuses were produced using either artificial insemination of heifers from a Hereford x Friesian recipient herd, or using embryo transfer into recipients from this herd. Embryos were derived from abattoir-sourced ovaries, using *in vivo* production techniques (Lu *et al.*, 1990). Friesian semen was used to generate NM foetuses. This approach resulted in NM controls being generated from a pool of NM beef and/or dairy breeds.

All of the recipients were grazed in a single mob to minimise any effects of environment on foetal development. Recipients were slaughtered at 120, 160, 210 and 260 days gestation and foetuses removed.

Five foetuses were produced at each gestational age for both breeds except for the 210 and 260 day Belgian Blues when 3 foetuses were produced. Foetuses were weighed and crown-rump length measured from the top of the head, at a point

between the ears, to the start of the tail. Blood samples were taken by cardiac puncture and plasma was separated and stored at -20°C until assayed. M. *vastus lateralis* and M. *vastus medialis* were dissected, cleaned of excess connective tissue and weighed. Sub-samples from the midpoint of both muscles were frozen in isopentane cooled in liquid nitrogen for receptor analyses, fibre typing, immunohistochemistry (IHC) and RNA extraction. Additional tissue pieces were placed in histocasettes and fixed in neutral buffered formaldehyde for *in situ* hybridisation and IHC.

2.4.3 Induced hypertrophy and atrophy study

Ten female Californian rabbits aged 4 months, weighing approximately 2.5 kg, were randomly assigned to 2 day treatment groups or 6 day treatment groups (n=5 per group). Rabbits were anaesthetised using a mixture of ketamine hydrochloride (Rompun, Bayer AG, Leverkusen Germany) (35 mg/kg) and xylazine hydrochloride (5 mg/kg) (Parnell Laboratories, Auckland), administered intramuscularly. Left or right hind limbs were cast in full plantar-flexion. This position causes the muscles running down the front of the limb (M. *tibialis anterior*, M. *extensor digitorus longum*) to be held in an extended position. Muscles running down the back of the limb are at less than resting length and are in a state of disuse. Muscles in this group include the M. *plantaris*, M. *soleus* and M. *gastrocnemius*. Limbs were immobilised using Dynacast Pro conformable fibreglass casting tape (Smith & Nephew, UK). Animals were checked every day for signs of chewing damage to the casts and on the morning of day six, two rabbits were found to have caused sufficient damage to their casts, so that the limb was no longer fully immobilised. These animals were excluded from the analyses, so the 6 day treatment group had a final n=3. At the end of the treatment periods rabbits were euthanased with an overdose of sodium pentobarbitone (South Island Chemicals, Christchurch). Casts were removed and the lengthened M. *tibialis anterior* (TA) and the disused M. *plantaris* muscles were dissected out and cleaned of excess connective tissue. Resting lengths of muscles were taken and the muscles were weighed.

These studies were all carried out with the approval of the Ruakura Animal Ethics Committee.

2.5 EXPERIMENTAL METHODS

2.5.1 IGF-1 and IGF-2 radioimmunoassays

Recombinant human IGF-1 (Ciba-Geigy, Basel, Switzerland) and IGF-2 (Eli Lilly Co, Indianapolis IN, USA), were radiolabelled using Iodogen as previously reported (Hodgkinson *et al.*, 1989). The peptides were iodinated to a specific activity of 900Ci/mmol and purified using reversed-phase Sep-Pak (Waters Associates, Milford, MA, USA). IGF-1 and IGF-2 were dissociated from IGF binding proteins using FPLC extraction that routinely has a recovery of greater than 80%.

Radioimmunoassay for IGF-1 and IGF-2 was carried out as previously reported (Hua *et al.*, 1995). The IGF-1 antibody was generated against a recombinant human terminal methionyl IGF-1 (IMC/Pitman Moore) and the IGF-2 antibody was a mouse monoclonal against rat IGF-2 (Amano Pharmaceutical Co. Ltd). The IGF-1 assay cross reacts <1% with IGF-2 and <0.1% with insulin and the IGF-2 assay cross reacts <1% with IGF-1 and <0.1% with insulin. Within and between assay coefficients of variation were 8 % and 8 % respectively for IGF-1 and 8 % and 9 % respectively for IGF-2.

2.5.2 Protein to DNA ratios

Alterations in the ratio of protein to DNA gives useful information about the way in which cells are growing. An increase in the ratio is an indication of an accumulation of cytoplasmic protein such as might occur with cellular hypertrophy. Standard assays were used for both protein determination and DNA (Labarca and Paigen, 1980).

2.5.3 General molecular biology protocols

Most molecular biology protocols were carried out according to methods described in standard reference texts (Sambrook *et al.*, 1989). Methods that have been modified in-house are described in the following section.

2.5.3.1 *In situ* hybridisation

2.5.3.1.1 Introduction

In situ hybridisation allows the cellular localisation of mRNA to tissue sections, via the hybridisation of labelled antisense riboprobes to the target mRNA species.

2.5.3.1.2 Method

The IGF-1 probe was transcribed from a 203 bp Sau3A sequence containing the coding region for IGF-1 D- and-E domains and 40 bp of the 3′ non-coding sequence, cloned into the BamHI site of a pGEM3 vector. This allowed for synthesis of both sense and anti-sense probes, the former being used as a control for non-specific hybridisation.

2.5.3.1.2.1 Probe synthesis

nuclease free MQ water		4.5 µl
Transcription buffer	5 X concentrate	4.0 µl
DTT	0.1 M	2.0 µl
rATP, rCTP, rGTP	10 mM mix	3.0 µl
rUTP	500µM	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

A Promega transcription kit was used for all components. All glassware was DEPC treated and sterile disposables were used throughout to avoid RNase contamination. Transcription was carried out in a waterbath at 37°C for 1 hour. A further 1 µl of SP6 RNA polymerase was added to the sense probe after 30 minutes to increase the efficiency of transcription. After the transcription reaction was completed, 1 µl of

RNase free DNase was added and the mixture was incubated at 37°C for 20 minutes to degrade the DNA template.

The sense and antisense probes were purified using phenol:chloroform extraction. Chloroform/isoamyl alcohol was mixed 1:1 with phenol and vortex mixed. Twenty µl of this mixture was added to the probes with vortexing. Tubes were centrifuged briefly to separate phases. The upper aqueous phase (~20µl) was carefully pipetted off to a new tube. The probes were precipitated by the addition of 1µl DEPC tRNA (10mg/ml), 7µl ammonium acetate (7.5M) and 62.5µl ice cold absolute ethanol. This was mixed well and left at -20°C for at least 30 mins. After centrifugation at 1500 rpm for 20 minutes the supernatants were removed. RNA pellets were washed in 75% ethanol in DEPC water and resuspended in 20 µl 10 mM DTT.

2.5.3.1.2.2 Pretreatment of slides

Slides were dewaxed in two changes of xylene and rehydrated through decreasing ethanol concentrations (100%, 95%, 70%, prepared with DEPC water), then rinsed in DEPC water. The slides were treated with 0.2N HCl for 10 minutes at room temperature to remove basic proteins. Slides were washed in 2XSCC for 30 minutes at RT, then treated with proteinase K (30U/µl) in 200mM Tris pH 7.2 and 2 mM $CaCl_2$ to increase permeability of the sections to the probe. Slides were incubated in proteinase solution for 15 minutes at 37°C. Acetylation of amino groups was carried out in a mixture of 1.32 ml triethanolamine in 98 ml DEPC MQ. (pH 7–8). Immediately before use, 1 ml acetic anhydride was added, mixed and poured over slides. Slides were incubated for 5 minutes. The solution was recovered and, after the addition of another 1 ml acetic anhydride, was reused for second 5 minute incubation.

2.5.3.1.2.3 Hybridisation

Slides were pre-hybridised by applying hybridisation buffer without probe to sections for 30-45 minutes in a Hybaid oven (Middlesex, UK) at 55°C. Hybridisation steps were carried out in a sealed container equilibrated with 50% formamide, 50% 2XSSC.

Probes were boiled for 3 minutes as a precaution against non-specific adhesion and spun down briefly. One µl of probe was added to 400 µl of liquid scintillation fluor (Beckman, Alphatech Systems, NZ) and counted in a scintillation counter. The probe was diluted in hybridisation buffer to give 1000 cpm/µl, then very thoroughly vortexed. A further 1 µl of the diluted probe was then counted as before. Dilutions were aimed at having sense counts slightly higher than anti-sense and were adjusted if necessary. The correctly diluted probes were then applied to the sections, after the pre-hybridisation solution was aspirated off. Slides were hybridised overnight in a Hybaid oven at 55°C, in a sealed container equilibrated with 50% formamide, 50% 2XSSC.

2.5.3.1.2.4 Washing

Slides were washed in washing buffer 1 (see Section 2.1.2.3)

2.5.3.1.2.5 RNase treatment

Unhybridised ssRNA template was degraded by incubation in an RNase bath. The RNase enzymes (see Section 2.1.2.3) were added to 1.5 ml of 2XSSC and this was added to a further 300 ml of 2XSSC, preheated to 37°C. Slides were incubated at 37°C for 30 mins. To avoid cross contamination of hybridisation steps with RNase enzymes, a separate set of glassware was maintained for this step.

2.5.3.1.2.6 Detection

After drying slides were exposed to XAR film (Kodak) in an X-ray cassette at room temperature for 4 days. Films were developed in an automatic processor. Slides were then coated with liquid photographic emulsion (NTB-2, Kodak) to enable localisation of probe hybridisation at the cellular level. Exposure was for 50 days at 4°C. Slides were then developed and lightly counterstained with haematoxylin and eosin.

2.5.3.2 Extraction of total RNA from tissue

This was carried out according to the manufacturer's instructions given for TRIzol®. Briefly, 400 mg of tissue was homogenised in 4 ml of TRIzol®, using a

Polytron homogeniser (Janke and Kunkel, IKA-Laborteknik, Staufen, Germany). Chloroform was added at the rate of 200 µl per ml of TRIzol®. The homogenate was centrifuged at 10 000 x g for 15 minutes in a Sorvall RC5C using an SS34 rotor. The upper aqueous phase was removed to a fresh centrifuge tube. Two mls of isopropanol were added to the aqueous phase, mixed and left to incubate for 10 minutes at RT. The samples were centrifuged again at 10 000xg for 10 minutes, to pellet the RNA. The RNA pellet was washed in 70% ethanol in DEPC treated water and centrifuged at 7 500 x g for 5 minutes. The pellet was dried and solubilised in DEPC water. RNA integrity was checked by running 2µg on a denaturing agarose/formaldehyde gel containing ethidium bromide (Sambrook *et al.*, 1989) and visualising the gel on a trans-illuminator (UVP, Upland, CA). The RNA concentration was determined by measuring the optical density at 260 nm in a spectrophotometer.

2.5.3.3 Northern analysis

Northern analysis is used for the quantitation of mRNA levels. The process involves size fractionation of RNA on a denaturing 1% formaldehyde-agarose gel and transfer to a nylon membrane. The RNA is then immobilised on the membrane by UV cross-linking and hybridised using a labelled complementary probe. Details of the methodology used in this study can be found in standard molecular biology texts (Sambrook *et al.*, 1989).

Fifteen µg of total RNA was run on a gel as described above and transferred to Hybond N+ membrane (Amersham). The pre-hybridisation and hybridisation solutions for the Northern blots was 5X SSC, 50% formamide, 5X Denhardts solution, 1% SDS and 0.25mg/ml salmon sperm DNA. The membranes were pre-hybridised in this solution for 2 hrs at 42°C. The bovine myostatin cDNA probe was random primed [^{32}P] labelled and purified using Quickspin columns (Roche Molecular Biochemicals). The labelled probe was added and hybridised overnight. Membranes were washed at 50 °C for 15 minutes each with 2X SSC/0.1%SDS then 0.2X SSC/0.1%SDS, before exposure to XAR film.

2.5.3.4 Reverse transcription-polymerase chain reaction (RT-PCR)

2.5.3.4.1 Introduction

The initial step in the RT-PCR process is the synthesis of single stranded cDNA from total RNA. Target cDNA can be amplified using specific oligonucleotide primers, under amplification conditions that are determined for each primer pair. This technique can be used for the amplification of low abundance mRNA species and, with careful attention to controls, can be used for the quantification of relative abundance of mRNA in tissues.

2.5.3.4.2 Method.

2.5.3.4.2.1. First strand cDNA synthesis.

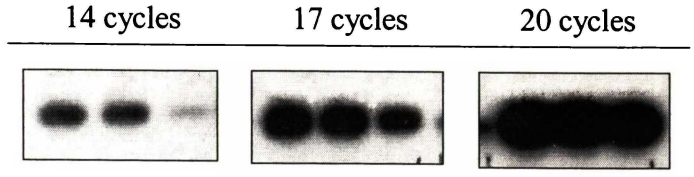
First strand cDNA synthesis was primed using oligo(dT) to hybridise to the 3' poly(A) tail found in most eucaryotic mRNA. Five μg of total RNA was added to oligo(dT). The reaction was incubated for 10 minutes at 70°C. The reagents for RT reaction mixture 2 (see Section 2.1.2.5) were added and the mixture incubated at 42°C for 5 minutes. One μl of Superscript II RT enzyme was added then incubated at 42°C for 50 minutes. The reaction was terminated by heating to 70°C for 15 minutes and then chilled on ice. The final step was to treat with 1 μl of RNase H at 37°C for 20 minutes. RT reactions were stored at -70°C until used.

2.5.3.4.2.2. Polymerase chain reaction (PCR).

For each 50 μl PCR reaction, components were assembled as described in section 2.2.2.5. PCR was carried out under conditions that were established for each primer set, in a PTC-100™ Programmable Thermal controller (MJ Research Inc, Watertown, MA, USA).

PCR conditions were as follows: denaturing at 94°C for 1 minute; annealing at 50°C for 30 secs, extension at 72°C for 1 minute. These steps were repeated for 14 cycles, this number of cycles having been identified as within the linear range for myostatin primers in foetal bovine muscle (Figure 2.1). After the 14 cycles were completed there was a further extension step of 72°C for 5 minutes. At this point the reactions could be held at 4°C overnight if necessary.

Figure 2.1. RT-PCR after 14, 17 and 20 cycles. The three samples were from normal bovine muscle at 90, 160 and 260 days gestation.



2.5.3.4.2.3 Primer sequences

Bovine myostatin:

513-bp cDNA. Genbank accession number AF019761

5'-GGTATTTGGCAGAGTATTGAT-3'

5'-GTCTAC-TACCATGGCTGGAAT-3'

Ovine ubiquitin activating enzyme:

264-bp cDNA. Genbank accession number AF088855

5'-TTGGATCCCCA-CCWGAGAARTCCATCCC-3'

5'-TTGAATT-CGGYAGGCCCARG-TCACRCA-3'

Single bands were amplified for each primer pair and to verify that PCR products were the expected fragments, cDNA was amplified with each pair of primers for 35 cycles and run on a low-melting point agarose gel. Fragments were excised from the gel, purified using the Wizard kit (Promega) and directly sequenced using an ABI automated sequencer (Model No 377). Sequence alignments were performed using DNA Lasergene software (DNA STAR, Madison, WI, USA)

2.5.3.5 Southern Blotting

Southern Blotting refers to the transfer of DNA from an agarose gel to a nylon or nitrocellulose membrane. The DNA is then immobilised on the membrane by UV cross-linking (Stratalinker, Stratagene, La Jolla, CA, USA) and hybridised using a labelled complementary probe. Probes were prepared by random-primed [^{32}P] labelling of myostatin and uae cDNA. Probes were purified using Quickspin columns (Roche Molecular Biochemicals). After overnight hybridisation at 42°C,

membranes were washed twice with 5X SSC/0.1%SDS at 42°C for 15 minutes each wash, twice with 2X SSC/0.1%SDS at 55°C for 15 minutes each wash, then twice with 0.2X SSC/0.1%SDS at 55°C, before exposure to XAR film.

2.5.4 Immunohistochemistry

2.5.4.1 Introduction

Immunohistochemistry (IHC) allows the cellular localisation of proteins or other molecules to tissue sections, via the binding of specific antibodies to the target molecule. In indirect IHC, which is the technique used throughout this study, a secondary antibody carrying a biotin label binds the target antibody. A tertiary complex containing streptavidin, biotin and horseradish peroxidase enzyme is then added, which binds to the biotin label. The final detection step consists of adding a substrate for the horseradish peroxidase enzyme that produces a coloured end product.

2.5.4.2 Methods

2.5.4.2.1 Sample preparation

Muscle samples were placed in histocasettes, then fixed for 12-24 hours in freshly prepared 10% neutral buffered formalin. Samples were washed in dH_20, then transferred into 70% ethanol and stored at 4°C until processed into Paramat paraffin wax (BDH Chemicals, NZ). Processing into wax was carried out in an automatic tissue processor (Reichart Jung, Leitz Wetzlar, Germany). Wax infiltrated tissues were then embedded in moulds prior to sectioning. Sections were cut with a Reichart microtome, at 7 μm and mounted in pairs on Polysine™ slides (Esco, Oakridge, New Jersey, USA).

Prior to incubations, slides were placed at 60°C for 30 minutes until the wax melted, to ensure adhesion of sections to slides. Sections were then dewaxed in two changes of xylene and rehydrated through decreasing ethanol concentrations (100%, 95%, 70%), finishing in buffer (TBS or PBS).

2.5.4.2.2 Slow MHC (1A)

Immunostaining for the slow MHC isoform required antigenic unmasking using the following protocol. After pre-incubation in TBS, slides were transferred to citrate buffer (pH 6.0) and microwaved on full power for 10 minutes. Slides were then cooled in the buffer and blocked for 1.5 hrs in NS/TBS. Primary antibody (1:300 in NS/TBS) was incubated overnight at 4°C in a humidified slide chamber. Slides were washed for 3x5 minutes in washing buffer and secondary antibody (biotinylated sheep anti-mouse) (1:300 in NS/TBS) was applied for 30 mins at RT. After washing in TBS-Tween, the tertiary complex (1:300 in NS/TBS) was incubated for 30 minutes at RT. After final washes, diaminobenzidine (DAB) enzyme substrate at 10 mg/ml, with intensifier, was added to the slides for 5 minutes. Slides were then rinsed in PBS then tap water and counterstained for 30 seconds in Nuclear Fast Red before dehydrating and mounting. Serial sections, incubated with normal mouse IgG at the same concentration as the primary antibody, served as negative controls.

2.5.4.2.3 Fast MHC (MY32)

The protocol for MY32 was the same as for slow MHC except that antigenic unmasking was not required. The primary antibody was used at 1:1200 dilution in embryonic tissues, 1:600 in adult bovine muscle and 1:200 in rabbit muscle. The secondary antibody and tertiary complex were used at 1:200. No counterstain was used. Serial sections, incubated with normal mouse IgG at the same concentration as the primary antibody, served as negative controls.

2.5.4.2.4 Embryonic MHC (2B6)

The protocol for embryonic MHC was as for fast MHC except that the primary antibody was used at 1:500 dilution and secondary antibody and tertiary complex at 1:200. No counterstain was used. Serial sections, incubated with normal mouse IgG at the same concentration as the primary antibody, served as negative controls.

2.5.2.4.5 IGF-1

A different protocol was required for IGF-1 as it is a rabbit polyclonal antibody, not a mouse monoclonal, as were the MHC antibodies. The blocking reagent was 10% normal donkey serum in TBS and this solution was also used as antibody diluent.

The primary antibody was used at 1:1000 dilution. Secondary antibody (biotinylated donkey anti-rabbit, Amersham) was used at 1:200 dilution and the tertiary complex at 1:300. Slides were lightly counterstained with Nuclear Fast Red. Serial sections, incubated with normal rabbit IgG at the same concentration as the primary antibody, served as negative controls.

2.5.4.2.6 Myostatin

The protocol used for myostatin was as for IGF-1 except that the primary antibody was used at 1:250 dilution. Slides were lightly counterstained with haematoxylin. Serial sections, incubated with normal rabbit IgG at the same concentration as the primary antibody, served as negative controls.

2.5.5 *In vitro* autoradiography

2.5.5.1 Introduction

In vitro autoradiography is a technique for the localisation and characterisation of ligand binding sites in tissues. The availability of radiolabelled ligands and the specificity of the ligand-receptor interaction has enabled the technique to be widely used in receptor analyses. The methodology allows for these analyses to be carried out at both a macro-autoradiographic level, where images on X-ray film are analysed densitometrically and at a micro-autoradiographic level, where the cellular localisation of ligand binding can be identified following exposure against liquid photographic emulsion. This technique was used in this study for the localisation and quantification of IGF-1 and IGF-2 binding to muscle samples from DM and NM bovine foetuses at four gestational ages. The binding sites of the radiolabelled tracer were characterised on the basis of competitive displacement by heterologous peptides. For characterisation of binding to the type 1 IGF receptor, sections were incubated with tracer in the presence of excess unlabelled IGF-2, des(1-3) IGF-1 and insulin. The following peptides bind to the type 1 receptor with decreasing affinity as shown: IGF-1=des(1-3)IGF-1>IGF-2>insulin. Affinity for the type 2 IGF receptor can be described as follows: IGF-2=des(1-6)IGF-2>>>IGF1=insulin. Des(1-3)-IGF-1 and des(1-6) IGF-2 are included because they have equal affinity for the IGF receptors but with much lower affinity to IGF binding proteins. Therefore IGF tracer

binding which cannot be displaced by these truncated variants is most likely to be to binding proteins (Ballard *et al.*, 1990; Luthi *et al.*, 1992; Ross *et al.*, 1989).

2.5.5.2 Methods

Receptor autoradiography was carried out for the localisation of IGF-1 and IGF-2 binding sites according to a previously published method (Elliott *et al.*, 1992). Two pairs of 8 μm cryostat sections were cut at -16°C from frozen blocks of muscle, then thaw-mounted onto a gelatinised slide for each incubation treatment. A well was made around each pair of sections using a wax pen (Pap-Pen, Daido Sangyo, Japan), to contain the buffer solutions. Pre-incubation buffer (see section 2.2.2.1) was applied to the slides for 10 minutes at RT. This was then replaced with incubation buffer containing ^{125}I-rh IGF-1 or ^{125}I-o IGF-2 (20,000 cpm per pair of sections) in the absence or presence of excess unlabelled competing hormones. One pair of sections, for the determination of total binding, was incubated with iodinated hormone alone. The other pair of sections, used for the determination of non-specific binding, was incubated with tracer plus excess unlabelled competing hormone. Specific binding was calculated as the difference in binding between the two pairs of sections. Competing hormones used were rhN-Met IGF-1, rh IGF-2 and des(1-3)-IGF-1 at 1 μg/ml and bovine insulin at 10 ng/ml.

Sections were incubated with the radiolabelled tracer for 2 hours at RT in a humidified slide chamber, then the incubation buffer was aspirated off. Slides were washed twice for 5 minutes in post-incubation buffer at 4°C, then fixed for 5 minutes in neutral buffered formalin at 4°C and rinsed in distilled water for 5 minutes.

After drying, slides were exposed against X-Omat AR5 film (Kodak) for 4 days for IGF-1 and 2 days for IGF-2. Liquid NTB-2 photographic emulsion (Kodak) was applied to the sections under safelight conditions and exposed at 4°C in a slide case containing dessicant for 8 days. The slides were placed in Kodak D-19 developer for 3.5 minutes at 18-20°, rinsed for 30 seconds in dH_20 and fixed in X-Omat fixer for 9 minutes. After rinsing in running tap water for 1 hour, the slides were stained with

Ehrlich's haematoxylin and eosin, then dehydrated through increasing concentrations of ethanol, mounted with DePeX and examined under the microscope.

2.5.6 Fibre typing

2.5.6.1 Introduction

Histochemical fibre typing is carried out for the identification of different fibre types in skeletal muscle, on the basis of the activity of myofibrillar actomyosin ATPase (mATPase) enzyme under specific incubation conditions.

2.5.6.2 Method

For mATPase histochemistry, 10μm cryostat sections were cut at -16°C and thaw-mounted onto uncoated slides. Slides were air dried for up to 4 hours and then stored at -80°C and used within one week. Slides were brought to room temperature in a humidified chamber, fixed for 5 minutes exactly as described (Guth and Samaha, 1969) then rinsed and transferred to pre-incubation buffer for 10 minutes at pH 5.0. Pre-incubations were carried out at room temperature in a room maintained between 18 and 22°C. The rest of the staining protocol was also as described by Guth and Samaha. Slides were rinsed in a jet of tap water if required to dislodge any surface black precipitate, dehydrated through graded alcohols, cleared in Saf-Solvent and mounted in DePeX.

2.5.7 Image analysis

2.5.7.1 Introduction

This section aimed to set up a computer assisted image analysis system for the semi-automated analysis of skeletal muscle fibre type and fibre area.

2.5.7.2 Method

Slides were prepared under the conditions described in the previous section, for optimal staining intensity of all histochemical fibre types. Slides were examined using an Olympus BH2 microscope with a monochrome video camera mounted (Cohu Inc, San Diego, CA, USA). Neutral density filters were used to reduce light

intensity. A Macintosh *IIfx* computer with a DT2255 Quick Capture frame grabber card (Data Translation, Marlboro, MA, USA) was used for image analysis using the public domain NIH Image programme (developed at the US National Institutes of Health and available from the Internet at http://rsb.info.nih.gov/nih-image/).

2.5.8 Photomicrography

Digital images were captured using a proprietary image analysis system (CMS700, Scion Corporation, Frederick, MD, USA). This system consisted of a Dage 330 colour CCD video camera with a camera control unit (786x494 pixels x 24 bit resolution) and a frame grabber card (CG-7, Scion Corp). The system was operated on a 200 mHz Pentium computer, running ScionImage, the PC version of the NIH Image software package described above. This programme can be downloaded from the Internet at http://www.scioncorp.com. Images were saved as TIFF files then imported into image processing software packages as required.

2.5.9 Statistical Analysis

Statistical analysis was carried out using two-way analysis of variance for comparison of differences between age and breed in Chapters 4 and 5, and between treatment and time in Chapter 6. This approach also allowed for analysis of interactions between the two main effects of age and breed or treatment and time. Breed or treatment effects at individual ages or time points were analysed for significant differences using t-test analysis. RT-PCR and Northern blot analyses of mRNA levels were carried out after normalisation to levels of expression of a control housekeeping gene.

CHAPTER THREE

OPTIMISATION OF METHODOLOGY FOR SKELETAL MUSCLE FIBRE TYPING

3.1 INTRODUCTION

The series of experiments described in this chapter were designed to establish optimal conditions for skeletal muscle fibre typing in bovine muscle sections using the histochemical localisation of myofibrillar actomyosin ATPase (mATPase) activity.

Analysis of skeletal muscle fibre type is a complex area as reviewed in Chapter 1. The histochemical staining for mATPase activity remains a routinely used procedure for the identification of fibre types in skeletal muscle in meat animals. The widely accepted nomenclature of type 1, type 2a and type 2b fibre types is based on this staining protocol, describing low, intermediate and high mATPase activity respectively, after incubation in alkaline conditions (Brooke and Kaiser, 1970). Irrespective of the widespread use of this technique, the literature contains many modifications to the procedure that can profoundly influence the staining result. Alterations in staining patterns following pre-incubation of tissue sections at different pH levels has led to the concept of acid and alkaline stabile and labile forms of the mATPase enzyme (Brooke and Kaiser, 1970; Guth and Samaha, 1969; Padykula and Herman, 1955). Other studies have examined the influence of ionic composition and buffering agent (Matoba and Gollnick, 1984) and incubation time and temperature (Gollnick *et al.*, 1983a) on the staining reaction. Some differences also occur between species in response to a time course of staining (Gollnick *et al.*, 1983a). As a final consideration, the mATPase activity of different muscle fibre

types also responds differently to formaldehyde fixation (Guth and Samaha, 1969; Hayashi and Freiman, 1966).

The documented variations in results that arise from the use of different histochemical staining protocols suggested there was a need to develop a standardised procedure. The primary aim of this study was to develop a method, based on the widely used method of Guth and Samaha (1969), which would optimise the identification type 1, type 2a and type 2b fibres in bovine M. *longissimus dorsi*. One aim of this method development study was to establish a protocol that would enable accurate fibre typing to be carried out using image analysis software. For this purpose it was necessary to have the greatest degree of contrast possible between the different fibre types. Other aims were to develop a procedure for computer-assisted, semi-automated quantitative analysis of skeletal muscle fibre type percentages and average fibre cross-sectional areas and finally to test whether a modified version of the procedure was suitable for use in species other than bovine.

3.2 MATERIALS AND METHODS

3.2.1 Tissue collection

M. *longissimus dorsi* was collected from Angus steers aged 30 months (n=3), slaughtered at the Ruakura Abattoir. Muscle samples were collected from the widest portion of the muscles (the mid-belly), at right angles to the direction of the muscle fibres. Tissue pieces of approximately 1 cm^3 were snap frozen in liquid nitrogen. Ovine M. *semitendinosus* tissue samples were provided by Dr Jenny Oldham and the rabbit M. *tibialis anterior* samples came from the experiment described in Chapter 6. These tissue samples were frozen in isopentane cooled in liquid nitrogen.

3.2.2 Optimisation of histochemical staining procedure

For investigation of the effects of pH and formalin fixation on myosin ATPase histochemistry, 22 consecutive 10μm cryostat sections were cut at -16°C and thaw-mounted onto uncoated slides. Slides were air dried at room temperature for up to 4

hours and then stored at -80°C and used within one week. Eleven pre-incubation buffers were prepared as follows: 0.1M potassium acetate at pH 3.5, 4.0, 4.2, 4.3, 4.35. 4.6, 5.0, 6.0 and 0.1M 2-amino-2-methyl-1-propanol (AMIP) at pH 7.8, 9.4 and 10.4. These pH values were chosen to cover the most commonly published protocols used for the demonstration of acid- and alkali-stabile staining patterns (Brooke and Kaiser, 1970; Gollnick *et al.*, 1983a; Guth and Samaha, 1969).

Eleven alternate slides from the series of 22 slides were fixed in 0.2M sodium cacodylate buffer (as described in Section 2.2.6) for 5 minutes (Guth and Samaha, 1969). One fixed and one unfixed slide was transferred to each pre-incubation treatment for 10 minutes for pH 6.0 and below and 15 minutes for the remainder. Pre-incubations were carried out at room temperature in a room maintained between 18 and 22°C. The rest of the staining procedure was carried out according to the protocol of Guth and Samaha (1969) (as described in Section 2.4.6.2).

3.2.3 Validation of modification to staining method

3.2.3.1 Comparison with routine ATPase staining

To provide quantitative evidence in support of the improved result from the modified staining reaction established in the previous section, quantitative image analysis was carried out on identical sites after staining of M *longissimus dorsi* muscle according to the routine and the modified protocols. All fibres were analysed for each procedure to give average fibre cross-sectional area and fibre type percentages for both protocols.

3.2.3.2 Immunohistochemistry

For correlation of modified histochemical staining with immunohistochemical localization of MHC isoforms, another series of slides with consecutive 10 μm cryostat sections was prepared from bovine M. *longissimus dorsi.* These sections were thaw-mounted onto Polysine™ slides and were fixed for 5 minutes at 4°C in 4% neutral buffered formaldehyde fixative freshly prepared from paraformaldehyde. The fixative was rinsed off in 0.1% glycine, the slides were rinsed again in Tris buffered saline (TBS) and they were then air-dried. Slides were stored at -18°C and

used within one week of cutting. Slides were brought to room temperature in a humidified chamber, then pre-incubated for 5 minutes in TBS, followed by 30 minutes blocking with 10% normal sheep plasma in TBS (NSP/TBS). The following monoclonal antibodies were used: MY32 (specific for fast MHC isoforms) and 1A (specific for slow MHC isoforms). Details of antibodies and immunohistochemical staining protocols are given in Section 2.4.4.2.

An additional slide from the series of consecutive sections was thaw-mounted onto an uncoated slide and stained using the modified mATPase histochemical staining procedure.

3.2.3.3 Comparison of fibre typing data with data from literature

After the optimum conditions for fibre typing were identified from the study described in Section 3.2.2, M. *longissimus dorsi* sections from 3 animals were incubated according to the modified procedure. These sections were analysed according to the semi-automated image analysis system that had been developed. Data on average percentage, average area and total proportion of each fibre type were calculated and compared with data extracted from the literature on the same fibre type parameters, taken from the same muscle and from animals of similar age, breed and gender.

3.2.3.4 Modified staining reaction in other animal species

To test the versatility of the modified staining procedure in other species, serial sections of rabbit M. *tibialis anterior* and ovine M. *semitendinosus* were incubated according to the modified and the routine procedures.

3.2.4 Image analysis

Image analysis was carried out to compare fibre typing results in modified and routine stained mATPase sections of adult bovine M. *longissimus dorsi*, according to the procedure described in section 2.4.7.2.

In order to optimise the efficiency of the image analysis procedure, a macro routine was written for use within the NIH Image programme. This macro can be found in

Appendix One. Fibre size measurements were calibrated against a stage micrometer in the horizontal and vertical directions and the pixel aspect ratio adjusted to compensate for rectangular pixel shape. Optical density (OD) measurements were not calibrated. Analyses were carried out on all fibres within distinct fascicles of each muscle, until approximately 200-300 fibres were analysed per section. Data files containing fibre cross-sectional area and OD measurements were transferred to a spreadsheet and plots were prepared of OD values against size. On the basis of this visual representation of OD, threshold values were selected for the automated assignment of fibres to a fibre type grouping. Average size, proportions and percentage areas of each fibre type were calculated for each site and then for each muscle.

3.2.5 Photomicrography

All sections in the pH-fixation series of slides were examined using an Olympus BX50 microscope. A site was found which could be identified in every section of the pH-fixation series. Digital images were captured using the Scion CMS-700 image analysis system as described in Section 2.4.7.2. Each fibre type in each section was scored, on a scale of 1 to 3 according to the intensity of the staining reaction.

Serial sites were identified in the series of sections used for ICC correlation with mATPase histochemistry and digital images from these slides were captured as described in Section 2.4.8.

3.3. RESULTS

3.3.1 Optimisation of mATPase staining protocol

In tissue sections which were fixed prior to pre-incubation the same pattern of staining was demonstrated at all pH levels above 4.35. Type 2b fibres had the highest mATPase activity, type 2a fibres had intermediate activity and type 1 fibres had the lowest. The distinction between type 2a and type 2b fibre sub-types was most pronounced between pH 4.6 and 6.0. At pH 4.0, mATPase activity was

activated in type 1 fibres, was abolished in 2a fibres and was moderate in type 2b fibres. At pH 3.5, mATPase activity was completely abolished in all fibres (Plates 3.1-3.3, left panel and Table 3.1).

In unfixed tissue sections at pH 4.6 and above, mATPase activity was consistently inhibited in type 1 fibres, but it was difficult to distinguish the type 2 fibre sub-types. Histology was poor at pH 9.4 and 10.4. At pH 4.35 the type 2 sub-types could just be discerned. At pH 4.2 the mATPase activity of the type 1 fibres was activated giving a strong reaction, with an intermediate reaction in type 2a and 2b fibres. At 4.0 activity was abolished in type 2a and 2b fibres but still present in type 1. All activity was abolished in unfixed sections at pH 3.5 (Plates 3.1-3.3, right panel and Table 3.1).

3.3.2 Modified versus routine mATPase staining comparison

Results from this validation experiment show the modified staining procedure to generate the same results as the routine stain with respect to fibre type percentage and fibre area measurements. Charts showing comparisons of OD values versus fibre area demonstrate that there is a greater spread in the range of fibre type intensities in the modified procedure, making it easier to identify threshold values at which to delineate fibre type groupings (Figure 3.1).

Plates 3.1-3.3

Photomicrographs showing the effect of pH and fixation on mATPase staining reaction in serial sections from adult bovine M. *longissimus dorsi*. Plate 3.1 shows pH 3.5-4.3, Plate 3.2 shows pH 4.35-6.0, Plate 3.3 shows pH 7.8-10.4. Panels on left shows fixed sections, panels on right shown unfixed sections. 1 = type 1 fibres, a = type 2a fibres, b = type 2b fibres, asterisks (Plate 3.1) indicate non-serial sections. Bar = 50 μm.

Plate 3.1. Effect of pH and fixation on mATPase staining.

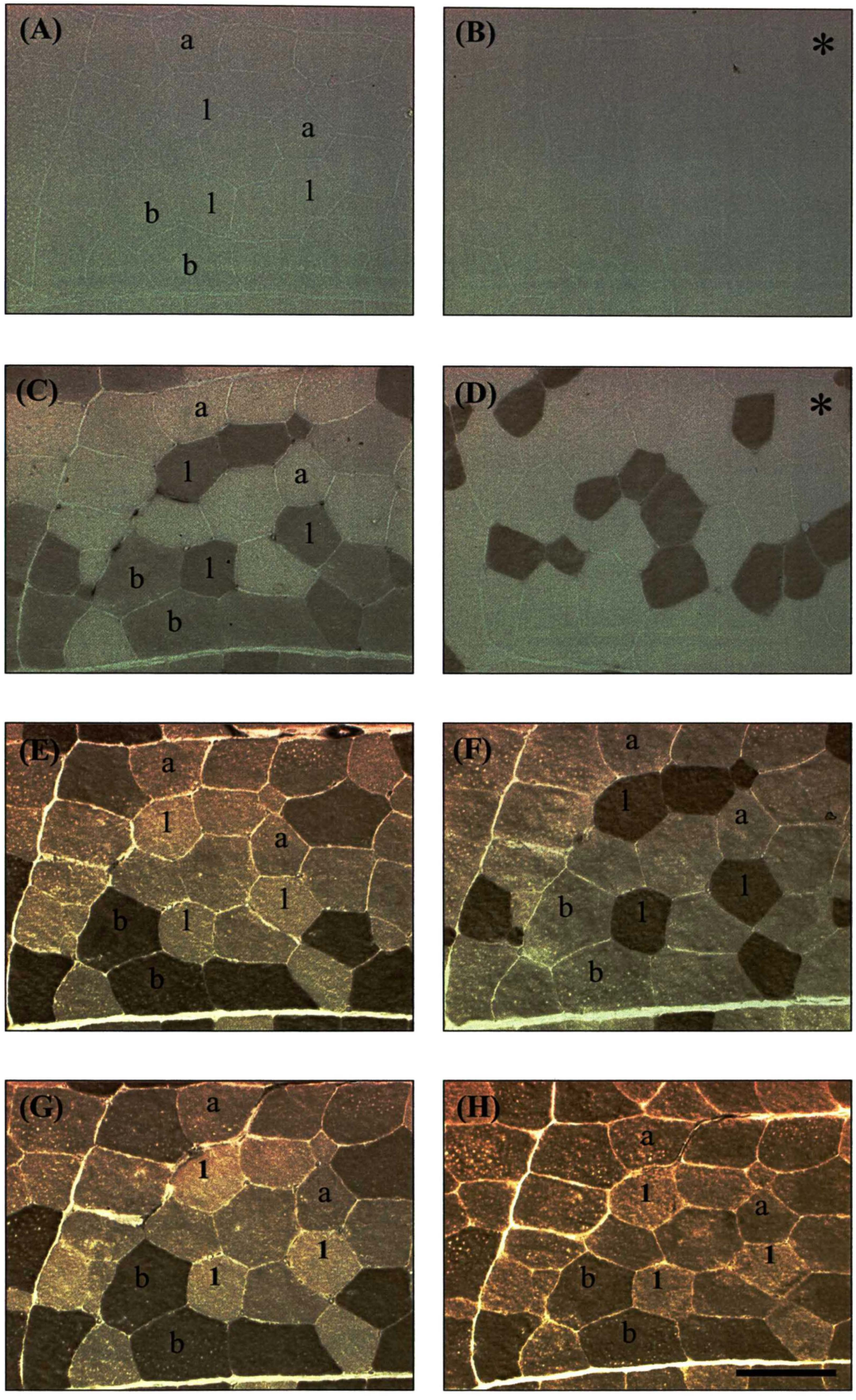


Plate 3.2. Effect of pH and fixation on mATPase staining.

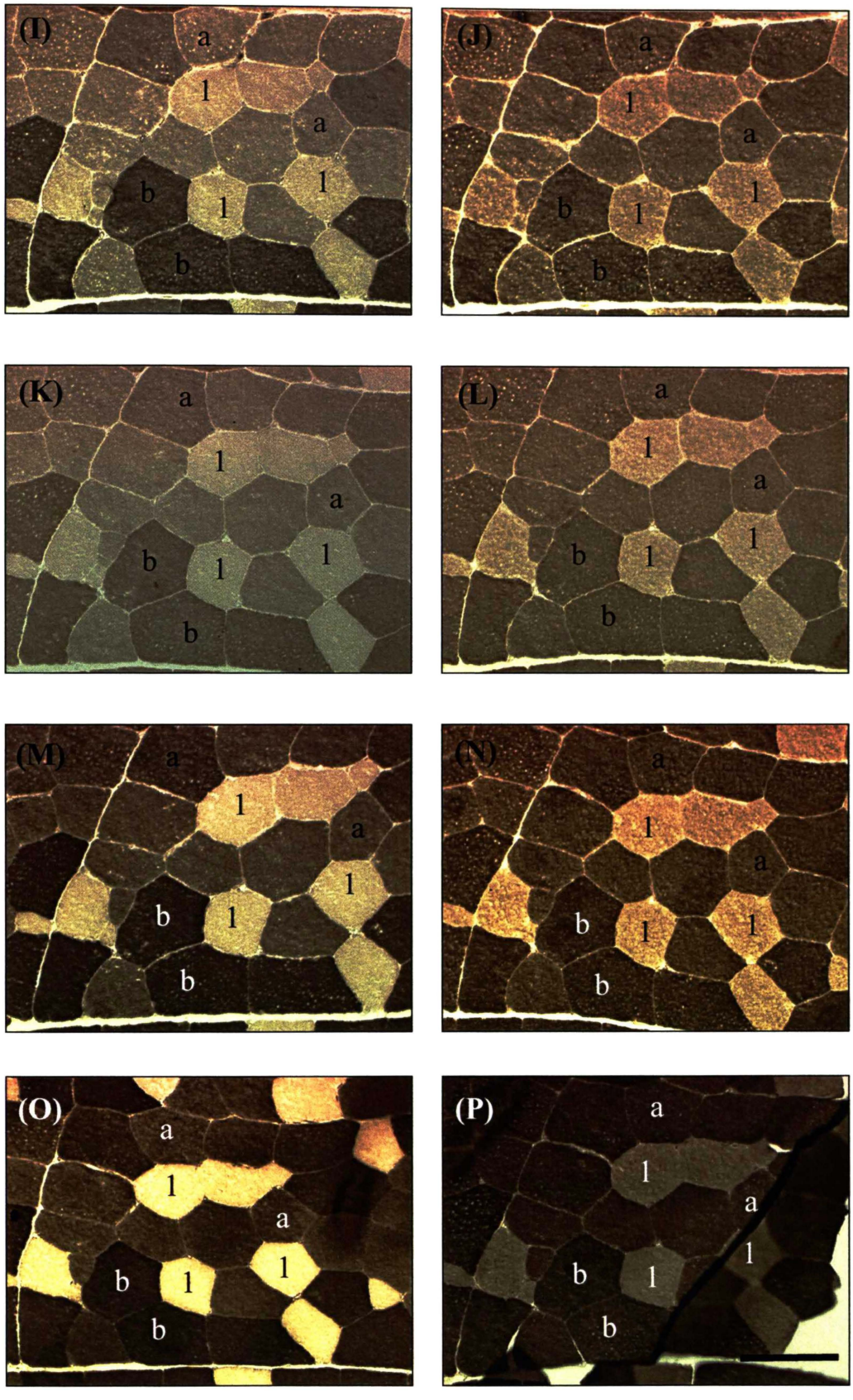


Plate 3.3. Effect of pH and fixation on mATPase staining reaction

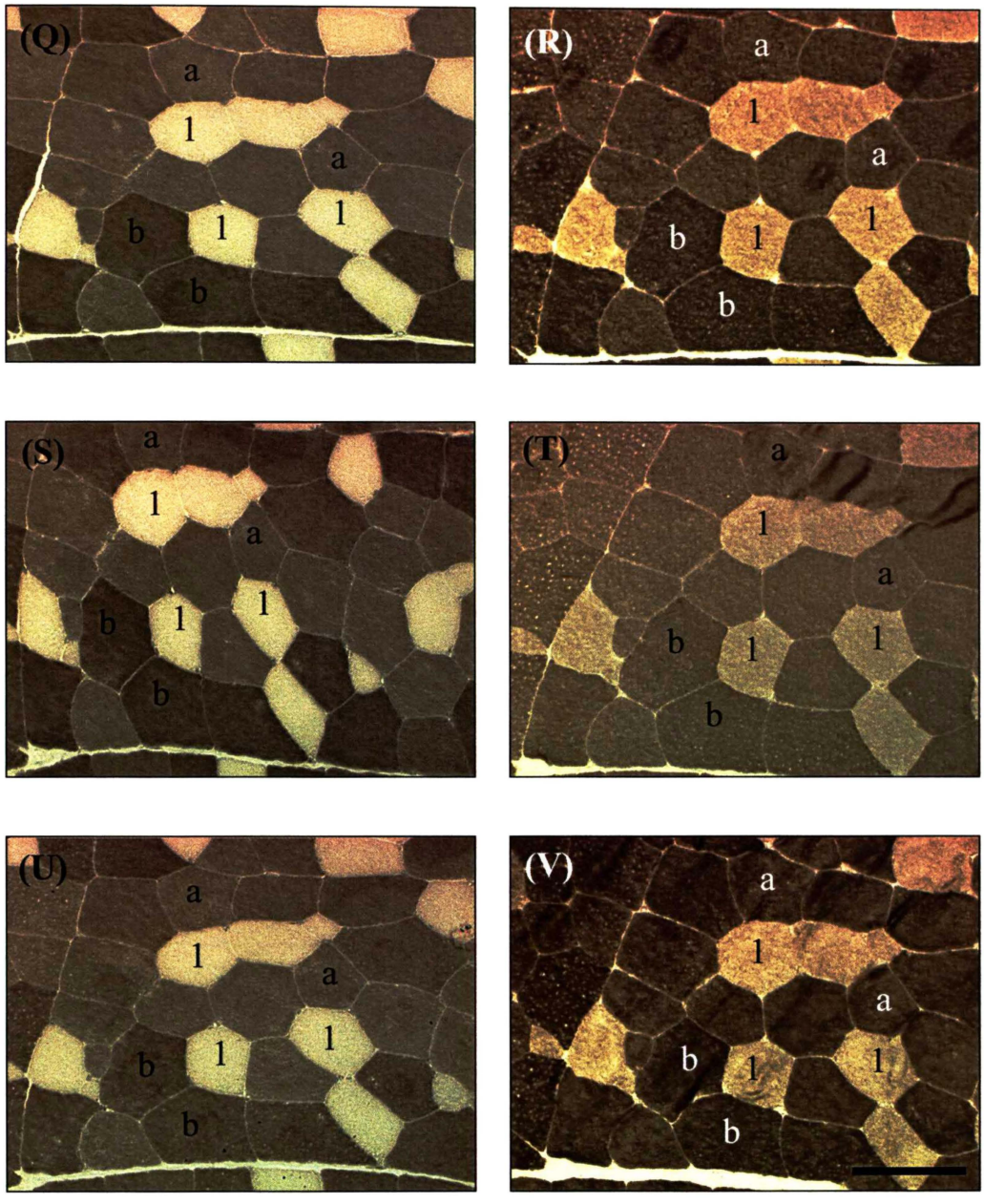


Table 3.1: Intensity of histochemical staining reactions of different skeletal muscle fibre types in fixed and unfixed bovine M longissimus dorsi sections after pre-incubation at different pH. The boxed region defines optimum staining conditions.

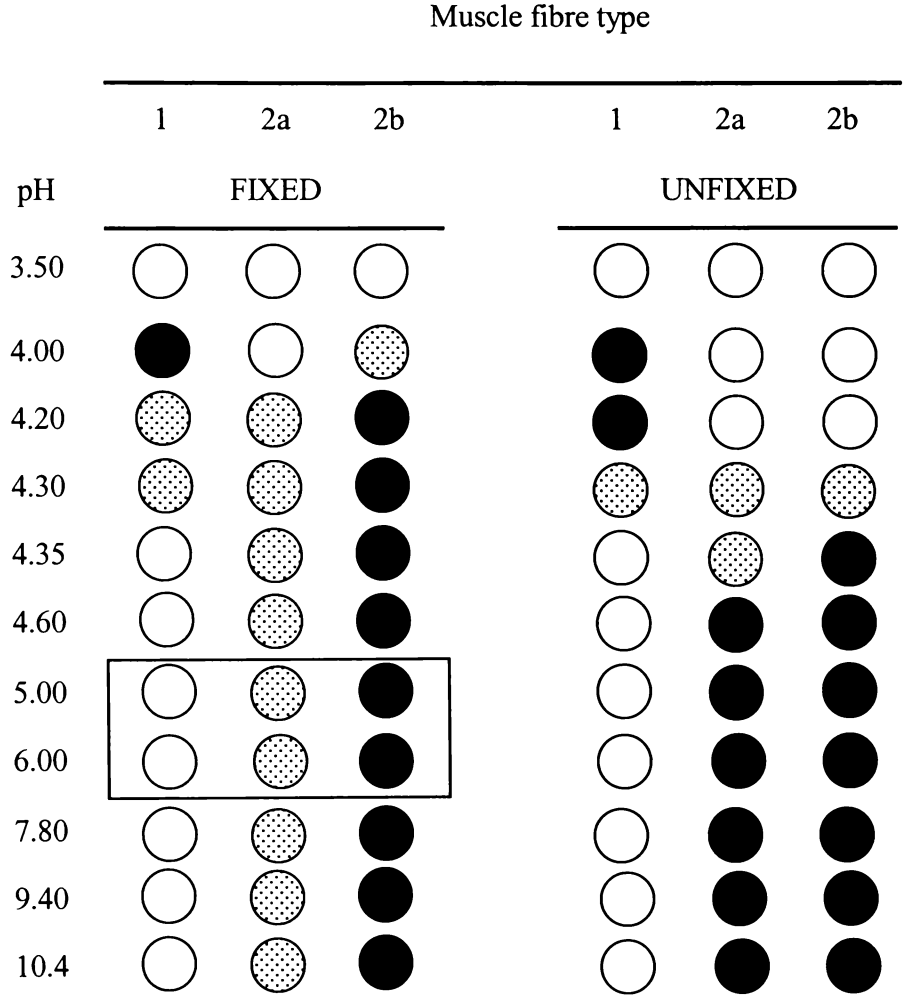
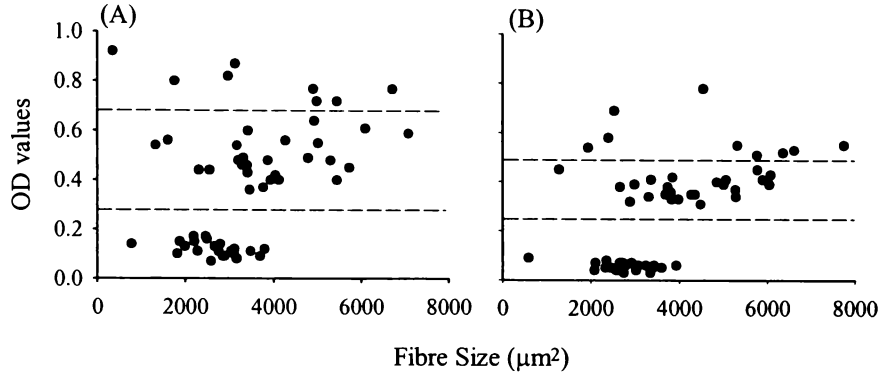


Figure 3.1 Comparison of modified (A) and routine (B) mATPase staining reactions, showing greater range of staining intensities in (A). Equations for assignment of fibre type are shown under each chart.



=IF(F12<0.3, "type 1", IF(F12<0.7, "type 2a", IF(0.69<F12, "type 2b")))

=IF(F12<0.3, "type 1", IF(F12<0.5, "type 2a", IF(0.49<F12, "type 2b")))

3.3.3 Immunohistochemistry

Immunohistochemical staining for myosin heavy chain isoforms gave results which were consistent with the mATPase staining reaction (Plate 4.4), with antibody 1A giving a strong positive reaction in type 1 fibres (A) and antibody MY32 staining type 2a and 2d/x fibres (B). The modified ATPase reaction is shown in (C), where 2a and 2d/x fibres can be distinguished in the basis of staining intensity.

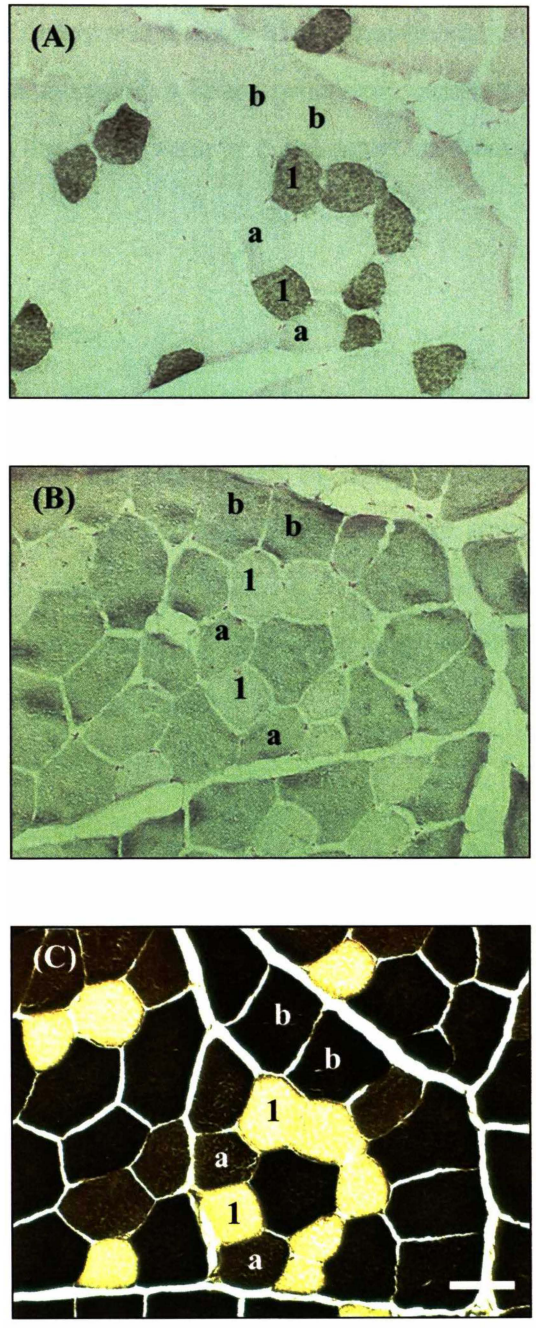
3.3.4 Comparison with existing fibre typing literature

The study has demonstrated that in bovine M *longissimus dorsi*, type 2b fibres have the largest mean fibre area and make up both the greatest proportion and the greatest area of the muscle (Table 3.2). Type 2a fibres are intermediate in both fibre size, proportions and percentage area and type 1 fibres are smallest and least abundant (Table 3.2). This data is entirely consistent with the results of others that have shown the same fibre type characteristics in M *longissimus dorsi* from animals of similar age, breed and gender (Table 3.2).

Plate 3.4

Photomicrographs showing IHC localisation of slow MHC (A), fast MHC (B) and modified mATPase (C) staining in serial sections of adult bovine M. *longissimus dorsi*. 1 = type 1 fibres, a = type 2a fibres, b = type 2b fibres. Bar = 50 μm.

Plate 3.4 Serial sections showing MHC and mATPase staining.



3.3.5 Use of modified procedure in other species

Both rabbit M *tibialis anterior* and ovine M *semitendinosus* gave an improved result after staining with the modified ATPase procedure, relative to the routine pH 9.4 protocol, although in the ovine sample it remained to distinguish type 2 subtypes (Plate 3.5).

3.3.6 Image analysis system

The procedure described for the semi-automated analysis of muscle fibre size and optical density combines the capabilities of image analysis software with data manipulation using a spreadsheet and allows for data from approximately 1500 fibres to be processed comfortably within a day including data analysis time. The image analysis software allows for the clear and unambiguous labelling and measuring of individual fibres (Figure 3.2).

Plate 3.5

Photomicrographs of rabbit M. *tibialis anterior* (A, B) and ovine M. *semitendinosus* (C, D) after modified (A, C) and routine (B, D) mATPase staining. 1 = type 1 fibres, a = type 2a fibres, b = type 2b fibres, 2 = type 2 fibres. Bar = 50 μm.

Plate 3.5 mATPase staining in rabbit and ovine muscle.

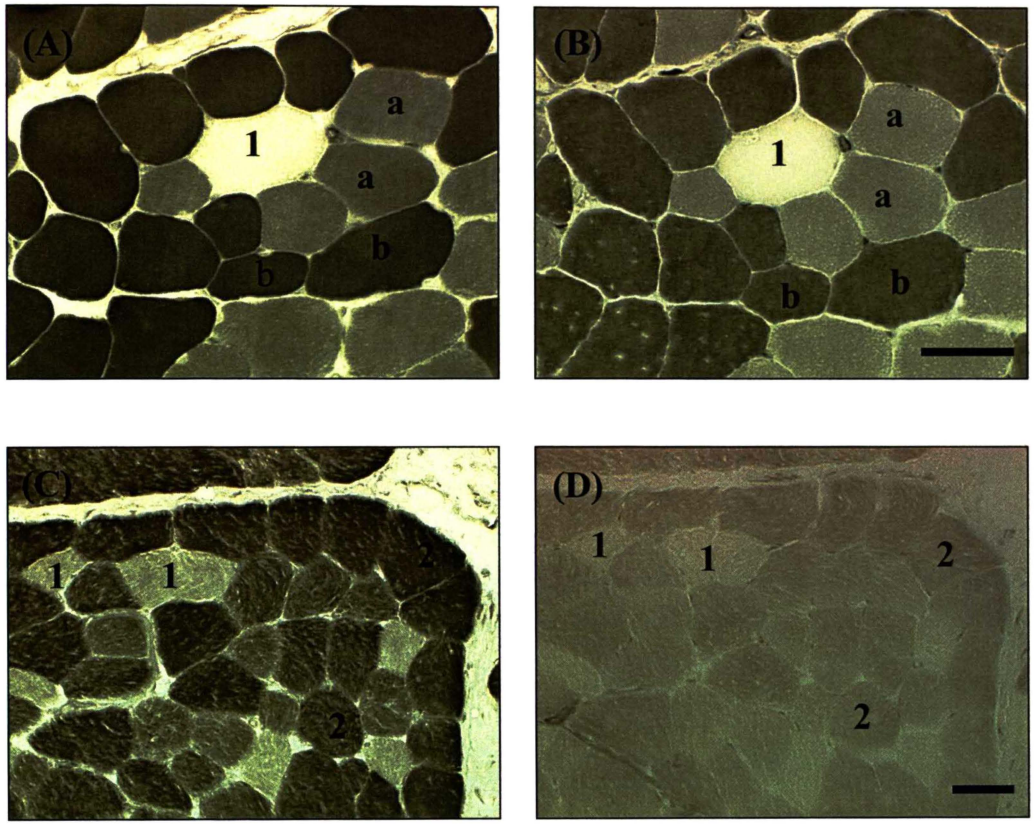


Figure 3.2 Computer assisted image analysis

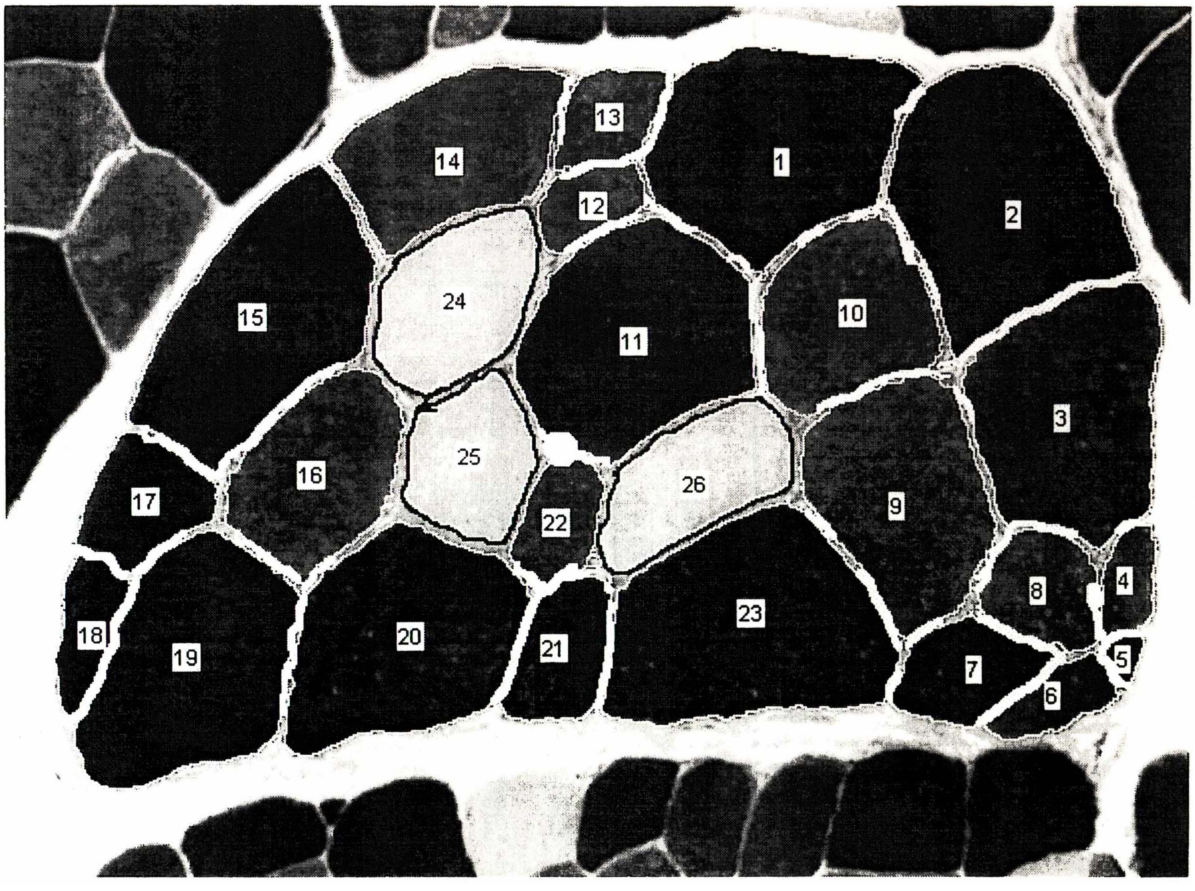


Table 3.2: Comparison of fibre type parameters of bovine M. *longissimus dorsi* as analysed in this study using semi-automated computer assisted image analysis, with data from the literature in animals of similar age and breed.

			Mean fibre area (mm^2)			Fibre type (%)			Fibre area (%)		
Reference	Breed	Age	type 1	type 2a	type 2b	type 1	type 2a	type 2b	type 1	type 2a	type 2b
This study	Angus steers	30 months	1787	2452	3151	25	35.7	39.1	17.6	33.4	49
Solomon, 1986[1]	Angus bulls	10-17 months	2642	3394	4276	23.1	33.3	43.6	nd	nd	nd
Siedeman, 1986[2]	mixed breed steers	16 months	2100	2380	3270	27.2	31.6	41.2	21.6	28.3	50.1
Clancy, 1986[3]	Friesian X steers	26 months	2250	3211	4729	25.9	20.9	53.3	15.4	18	66.6
		average	2194.8	2859.3	3856.5	25.3	30.4	44.3	18.2	26.6	55.2
		sd	355.1	518.1	770.1	1.7	6.5	6.3	3.1	7.8	9.9

[1] (Solomon *et al.*, 1986), [2] (Seideman and Crouse, 1986), [3] (Clancy *et al.*, 1986).

3.4. DISCUSSION

The correlation between mATPase enzyme histochemistry and MHC immunohistochemistry described in this study, supports the contention that mATPase staining does reflect MHC composition. Fibres identified as type 1 by mATPase histochemistry express slow MHC and type 2 fibres express fast MHC isoforms. The underlying assumption that this correlation holds true has always been implicit in the interpretation of fibre typing results (Kelly and Rubenstein, 1994). However, is it important to be aware that conclusions as to MHC isoform expression cannot be inferred from histochemical fibre typing. As an example, the mATPase staining reaction in rabbit M. *tibialis anterior* identified three fibre types, which would be identified as type 1, 2a and 2b on the basis of staining intensity for this reaction (Brooke and Kaiser, 1970). Immunohistochemical analyses have shown that rabbit M. *tibialis anterior* does not contain the type 2b MHC isoform, but the 2d/x isoform instead (Aigner *et al.*, 1993; Hämäläinen and Pette, 1993). This emphasises the need to restrict interpretations of fibre type analysis to consideration of the specific properties of muscle fibres that are being investigated.

In all the modifications to the various staining procedures that have appeared over the years, very little attention has been given to the effect of formalin fixation. The dogma for many has been that ATPase enzyme activity is very sensitive to the effects of formalin, so fixation should be avoided (Padykula and Herman, 1955). However, it has been shown that the addition of sucrose to the fixative can result in the retention of a high level of enzyme activity and a further increase in activity can be achieved with the addition of enzyme substrate in the fixative (Hayashi and Freiman, 1966). This is thought to occur due to the role of the substrate in protecting the active site of the enzyme during the fixation process (Hayashi and Freiman, 1966). This study investigated the interaction between pH and fixation in an mATPase staining procedure based on the method of Guth and Samaha (1969).

The results of the modified ATPase staining procedure described here agree with and extend the results described by Guth and Samaha (1969), where it was suggested that fixation before pre-incubation in acid gave a staining pattern similar

to unfixed tissue incubated in alkaline conditions. The current study has extended this observation to show that at pH 4.0 in fixed sections the characteristic reversal of staining does indeed occur. This phenomenon occurs at pH 4.2 in unfixed tissues. The so-called alkali-stabile fast 2b fibres retain their ATPase activity at pH 4.2 after fixation. At the microscopic level, histology is improved with fixation and with the optimum combination of fixation and pH, the distinction between fast fibre sub-types is more apparent.

The results presented in this chapter suggest that the concept of acid and alkali stable forms of mATPase enzymes needs to be revised to take account of the effect of fixation. The alkali-stable mATPase activity in type 2b fibres is stable at pH levels as low as 4.2 if the tissues are previously fixed whereas the so-called acid stable ATPase in type 1 fibres is activated in a narrow range of pH levels between 4.0 and 4.2 depending on fixation.

The main advantages of mATPase staining over MHC immunohistochemistry are that three fibre types can be identified on a single section and that there is a high degree of contrast in the staining reaction which is amenable to computer-assisted image analysis. With the modified protocol in particular, a full characterisation of fibre types can be carried out using a single buffer. Pre-incubation in potassium acetate buffer at pH 4.0 and at pH 5.0 following fixation will demonstrate both the classical reversal of staining and the routine mATPase staining reaction, giving optimum histological quality for both. Limitations to the modified procedure are similar to those described in other ATPase staining protocols. This histochemical reaction is extremely sensitive to variations in pH, temperature and time of incubation and in order to ensure consistency of results it is essential that careful attention be paid to these aspects of the procedure.

3.4.1 Conclusions

The procedure described in this study for modified mATPase staining, combined with the computer-assisted semi-automated image analysis of muscle fibre size and optical density which was developed, provides an effective system for the accurate analysis of muscle fibre type in a range of species.

CHAPTER FOUR

SKELETAL MUSCLE DEVELOPMENT IN NORMAL AND DOUBLE-MUSCLED CATTLE

4.1. INTRODUCTION

Cattle raised for meat production spend approximately one third of their lives *in utero* (Johnson, 1974; Swatland, 1984) and this period of development is critical in determining the growth potential of an animal. It is the mass of skeletal muscle that is of greatest interest to meat producers and this is largely a product of muscle fibre number and muscle fibre size. These factors are controlled by a series of events including myoblast proliferation, myotube formation and maturation of the mature muscle fibres, reviewed in Chapter 1 (see Figure 1.2). A number of regulatory factors can influence each of these stages of muscle development, and one of these factors is the genetic background, or breed of an animal. It is the effect of breed on skeletal muscle development that is the focus of this chapter. The first section will examine prenatal development of two skeletal muscles in different breeds of cattle. The second part will investigate changes in muscle fibre type and fibre size during the prenatal development of muscles in different breeds of cattle.

4.1.1 Bovine muscle development

Breed has been reported to influence a number of aspects of muscle development in cattle including muscle fibre type, fibre size and carcass composition (La Flamme *et al.*, 1973; Luckett *et al.*, 1975; Solomon *et al.*, 1986). Within the double-muscled (DM) cattle breeds, muscle fibre number is also increased (Ashmore, 1974). This is

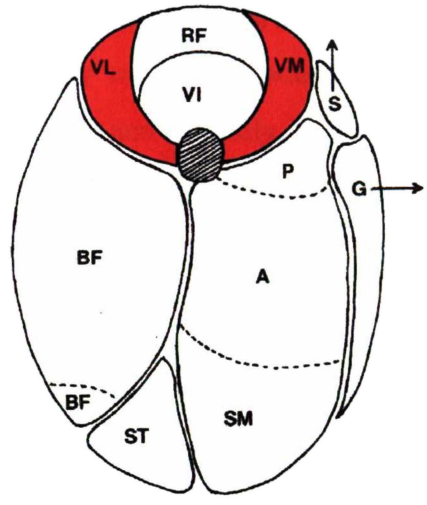
associated with a generalised increase in muscle size and a corresponding improvement in meat yield in DM animals (Boccard, 1981). The genetic basis of the DM condition in Belgian Blue animals, describing the mutation in the gene for myostatin in these animals, has been described in detail in Chapter 1 (Section 1.6.1) and will not be considered further in this chapter.

The extent of the muscular hypertrophy exhibited by DM animals has been reported to vary among different muscles in a number of DM breeds (Boccard and Dumont, 1974; Butterfield, 1966; Rollins *et al.*, 1969). Muscles with a large surface area tend to be the most enlarged, while deeper muscles tend to be reduced in size, relative to NM. As all of these muscles have increased muscle fibre numbers, it follows that the reduced muscles must have a smaller fibre size (Ouhayon and Beaumont, 1968). No similar studies have been carried out in prenatal animals to see if these differences in muscle mass and fibre size are present during development.

The first part of the work described in this chapter was undertaken to investigate the development of the M. *vastus lateralis* which in adult DM animals is 15% heavier than in NM and M. *vastus medialis* which is 38% lighter in DM than NM. These two muscles form part of the *quadriceps* group along with M. *rectus femoris* and M. *vastus intermedius*, where they serve to extend the hindlimb, by pulling on the patella (Swatland, 1984). The arrangement of these muscles can be seen in Figure 4.1, with the M. *vastus lateralis* located on the lateral surface of the thigh and the M. *vastus medialis* on the medial surface.

The first part of this study tests the hypothesis that the relative differences in growth which exist between different muscles of adult DM animals, when compared to muscles of normal animals, arise due to the genetic differences between the two breeds. The hypothesis was tested by analysing the prenatal development of the M. *vastus lateralis* and the M. *vastus medialis* in normal animals and in DM Belgian Blue animals. This second part of this study examined muscle fibre type and fibre size in NM and DM animals and this section will be introduced next.

Figure 4.1 Arrangement of hindlimb muscles around the femur (shaded). The M. sartorius (S) indicates the anterior direction, while the M. gracilis (G) indicates the medial direction. Muscles of the quadriceps group are the M. vastus lateralis (VL), M. vastus intermedius (VI), M. rectus femoris (RF) and M. vastus medialis (VM). Other muscles are the M. biceps femoris (BF), M. semitendinosus (ST), M. pectineus (P), M. adductor (A) and M. semimembranosus (SM) (From Swatland, 1984).



4.1.2 Fibre type in developing bovine muscles

Fibre type composition has important implications in farm animals because of its association with meat quality (Ashmore, 1974; Cassens, 1977). Histochemical fibre typing using myofibrillar ATPase (mATPase) activity is a widely used method for the classification of muscle fibre types in meat animals. DM cattle have a different composition of histochemical fibre types to NM, both during prenatal development (Ashmore *et al.*, 1974) and in the postnatal animal (Holmes and Ashmore, 1972), where it has been reported that there are increased proportions of type 2 muscle fibres and fewer type 1 fibres.

In addition to mATPase histochemical staining, myosin heavy chain (MHC) immunohistochemistry has also been widely used in the investigation of bovine

muscle development. The sequence of MHC isoform transitions which myotubes undergo as they mature is an important determinant of skeletal muscle fibre type (see Chapter 1, Figure 1.6) and this will be explored in more detail in this chapter. The relationship between the numbers of primary myotubes formed, the MHC isoforms they express and the fibre type composition of mature muscles is not yet fully understood (Dhoot, 1986; Whalen *et al.*, 1984; Zhang and McLennan, 1998). There appear to be two possible mechanisms by which the numbers of primary myotubes and/or the MHC isoform they express can be modulated. Firstly, in human muscle a number of primary myotubes undergo a natural degeneration process (Fidzianska and Goebel, 1991) and secondly, in developing rat muscle fibres, slow MHC is selectively degraded and replaced with a fast MHC isoform (Dhoot, 1986). Both of these mechanisms represent ways in which fibre type proportions, as defined by MHC isoform expression, can be altered during development.

Immunohistochemistry using antibodies against developmental MHC isoforms has enabled changes in MHC isoform expression to be identified during normal bovine development (Picard *et al.*, 1995b; Picard *et al.*, 1994; Robelin *et al.*, 1993) and in DM animals (Picard *et al.*, 1995a). This latter study reported developmental differences in MHC expression between NM and DM, with DM tending to express more immature isoforms at the same gestational age during the first two thirds of foetal life (Picard *et al.*, 1995a).

This study aimed to investigate differences in muscle development between NM and DM animals using mATPase histochemistry for quantitative analysis of muscle fibre type and fibre size and MHC isoform immunohistochemistry for a qualitative analysis of developmental differences between the two breeds.

4.1.3 Fibre size in developing bovine muscles

In normal bovine muscle, fibre size varies according to fibre type. Type 2b fibres have the largest cross-sectional area, type 2a fibres are intermediate and type 1 fibres

are the smallest. In DM cattle muscle fibre size is altered relative to NM, with both increases and decreases in fibre size being reported. These variations in fibre size are related to differences in fibre type and animal age (Ashmore *et al.*, 1974; Holmes and Ashmore, 1972). In postnatal animals there is an increase in the size and frequency of type 2b fibres in DM animals (Holmes and Ashmore, 1972) and this contributes to the overall increase in the "whiteness" of meat from these animals. In muscles from DM animals that are decreased in size relative to normal animals, average muscle fibre size is smaller (Ouhayon and Beaumont, 1968).

The second part of this study, examining changes in skeletal muscle fibre type and fibre size in NM and DM muscle, was undertaken in order to gain a better understanding of the effect of breed on these properties of muscle fibres. It also examined the contribution that these properties of muscle fibres make to overall muscle growth and development.

4.1.4 Aims of this chapter

The aims of this study were as follows:

(i) to test the hypothesis that there was a genetic basis for the relative differences in size between M. *vastus lateralis* and M. *vastus medialis* in adult NM and DM animals by analysing the growth of these muscles during prenatal development and

(ii) to test the hypothesis that differences in fibre type composition and fibre size contributed to overall differences in skeletal muscle mass between NM and DM foetuses by quantitative and qualitative analysis of changes in fibre type and fibre size during prenatal development.

4.2. MATERIALS AND METHODS

4.2.1 Animal data.

NM and DM calves were generated as described in Section 2.3.2. Foetuses at 120, 160, 210 and 260 days gestation were collected after slaughter of the recipient cows, weighed and crown rump length (CRL) measurements taken. The M. *vastus lateralis* and M. *vastus medialis* were dissected out of one hind limb from each animal and then weighed. A five mm slice was taken through the mid-belly of the muscle, at right angles to the direction of the muscle fibres. These samples were stored at –80°C for fibre typing and immunohistochemistry. There were five foetuses in each of the age groups for the normal animals and in the 120 and 160 day groups for the DM and three in the 210 and 260 day groups for the DM. These gestational ages were selected to cover the late stages of primary myotube formation which is nearing completion by 120 days (Robelin *et al.*, 1991; Stickland, 1978), a period of active secondary fibre formation at around 160 days (Stickland, 1978) and a period when all fibres were undergoing hypertrophic growth (210-260 days).

4.2.2 Protein:DNA ratios

Protein and DNA levels in muscles from each animal were analysed using standard laboratory techniques as described in Section 2.4.2. Data is presented as a ratio of protein (mg/g muscle):DNA (mg/g muscle) versus gestational age. Values are least squares means adjusted for sex within age groups, ± standard error of the mean (sem).

4.2.3 Fibre typing

Muscle fibre typing was carried out according to a modification of the myosin ATPase method of (Guth and Samaha, 1969), as described in Section 2.4.6. This procedure results in a staining pattern identical to that of unfixed sections

preincubated at pH 9.4, but with improved histology (see Chapter 3 for validation of methodology).

4.2.4 Immunohistochemistry

Immunohistochemistry was carried out on serial cryostat sections, fixed in neutral buffered formaldehyde as described in Section 2.4.4. Slides were incubated using antibodies specific for fast MHC (MY32), slow/β-cardiac MHC (1A) and embryonic MHC (2B6). The protocols used for this study are as described in Section 2.4.4.

4.2.5 Image analysis

Three sections stained for mATPase activity were analysed from each group for fibre type proportions and average area for each fibre type. All fibres within each of five fascicles were analysed for each animal, giving a total of 200-300 fibres per muscle. The rationale behind sampling entire fascicles to enable a more accurate assessment of fibre type proportions was based on reports suggesting that the fibre type composition of fascicles recapitulates that of entire muscles (Maier *et al.*, 1992). Quantitative image analysis was carried out using the NIH Image system for the Macintosh, as described in section 2.4.7.2. For photographic purposes, digital images were captured from one representative animal for each age and breed, using the ScionCorp CMS-700 image analysis system as described in Section 2.4.8.

4.2.6 Statistical analysis

Statistical analysis of body weight, CRL, muscle weight, fibre type and fibre size was carried out using analysis of variance with age and breed as the main effects. Foetal sex was included as a covariate in analysis of body weight, CRL, muscle weight and protein:DNA ratio but was never significant. Data was log-transformed

before analysis as required and back transformed for presentation of results. Values are presented as means, errors are sem.

4.3 RESULTS

4.3.1 Foetal data

Measurements of body weight and crown rump length were taken to demonstrate differences in size between the DM and NM foetuses at the same gestational age. Body weights increased significantly with age in both breeds ($p \leq 0.001$) and were greater in the DM foetuses relative to NM ($p \leq 0.001$) (Figure 4.2, A). There was a significant increase in CRL with gestational age ($p \leq 0.001$) and CRL was greater overall in NM foetuses($p \leq 0.01$) (Figure 4.2, B)

Both M. *vastus lateralis* and M. *vastus medialis* weights showed a highly significant increase with increasing gestational age ($p \leq 0.001$) and both muscles were significantly larger in the DM animals, relative to NM ($p \leq 0.001$) (Figure 4.3).

Figure 4.2 Body weight (A) and crown rump length (B) of NM (●) and DM (○) foetuses at 4 gestational ages (n = 3-5 per group). Values are least squares means ± sem. Data are back-transformed after log transformation for analysis. ** = p≤0.01, * = p≤0.05, ns = not significant

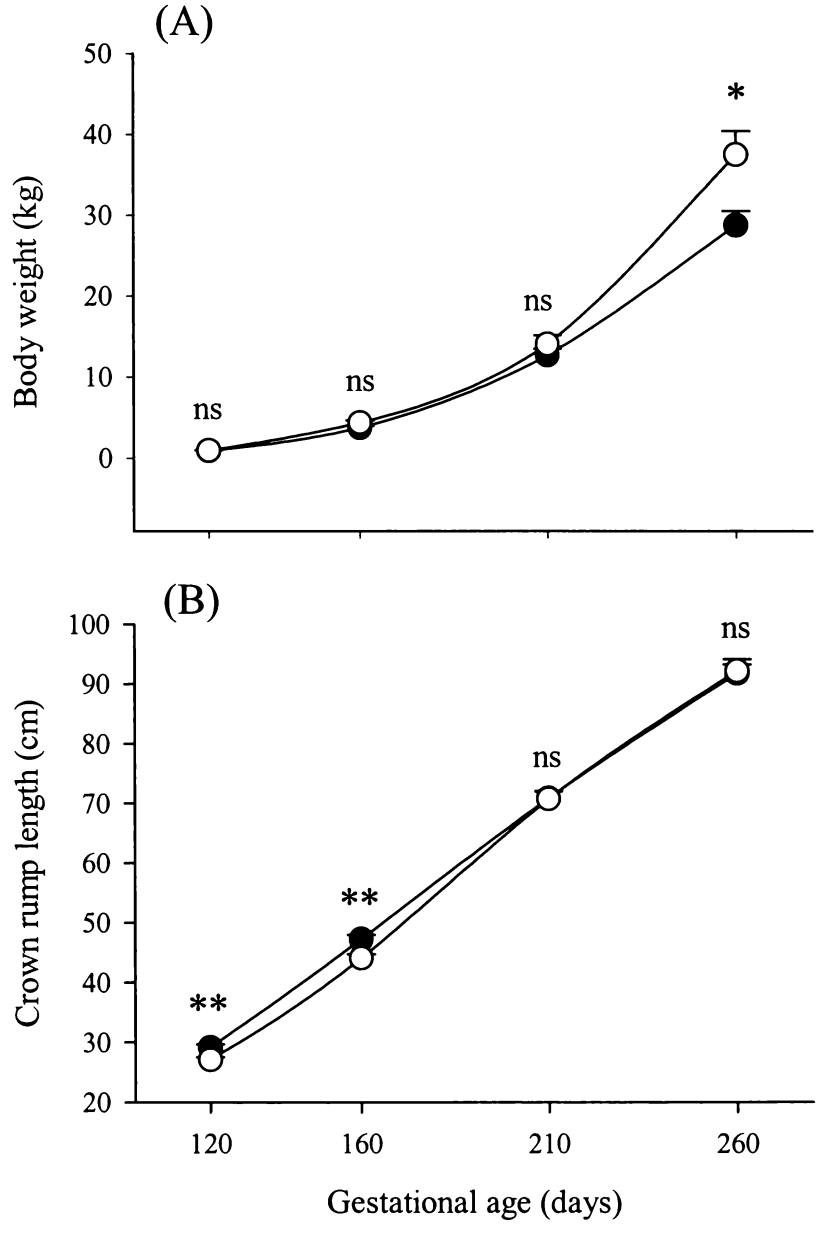
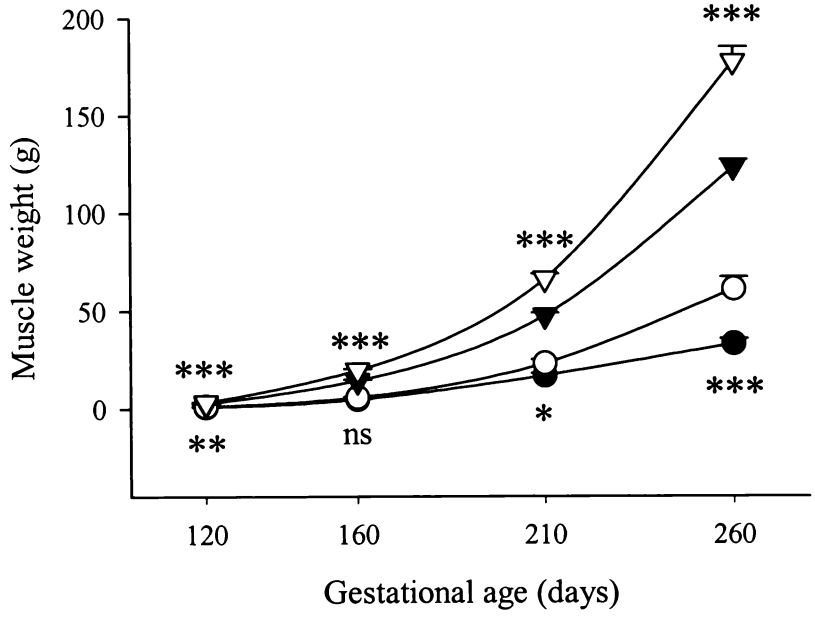


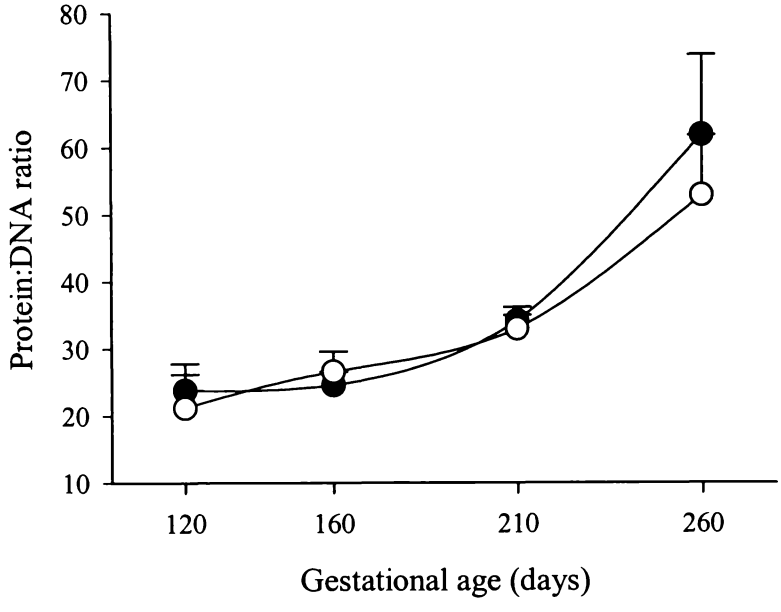
Figure 4.3 Weights of M. vastus lateralis (▼) and M. vastus medialis (●) from DM (open symbols) and NM (closed symbols) foetuses at 4 gestational ages (n = 3-5 per group). Values are least squares means, adjusted within groups for body weight and sex ratio, ± sem. Data are back-transformed after log transformation for analysis. *** = p≤0.001, ** = p≤0.01, * = p≤0.05, ns = not significant



4.3.3 Protein:DNA ratio

The protein:DNA ratio can be taken as an indication of the volume of cytoplasm controlled by a nucleus, ie, the size of the DNA unit (Cheek *et al.*, 1971). The protein:DNA ratio increased with increasing gestational age for both NM and DM ($p \leq 0.001$) (Figure 4.4). This indicates that the size of the DNA unit gets larger with gestational age, ie each nucleus acquires the capacity to control a larger volume of cytoplasm. There was no significant difference between the two breeds in the size of the DNA unit at any gestational age measured.

Figure 4.4 Protein to DNA ratio in NM (●) and DM (○) M. *vastus lateralis* plotted against gestational age. Values are least squares means ± sem, n=3-5.



4.3.4 Fibre typing and image analysis

4.3.4.1 Muscle fibre type

Qualitative analysis of sections stained using mATPase histochemistry showed similar fibre morphology between NM and DM at all gestational ages. There was a noticeable difference between 120 and 160 days gestation in the morphology of the presumptive primary myotubes, from being large and vacuolated to more closely resembling mature myofibres. A few fibres with the morphology of primary myotubes remained at 160 days gestation and the numbers of these were similar between NM and DM. An example of one of these fibres can be seen in Plate 4.1. The smaller size of the type 1 fibres in the DM muscles was easily seen at 260 days gestation (Plate 4.1).

The percentage of type 1 fibres per fascicle in both DM and NM showed a biphasic pattern of change throughout development (Figure 4.5), with numbers decreasing between 120 and 160 days and then increasing again between 210 and 260 days ($p \leq 0.001$). It can be seen that the DM muscles have a greater net gain in percentage type 1 fibres from an initial 7% ending up at 9%. NM muscles started at 19% and finished on 16%. There were consistently fewer type 1 muscle fibres per fascicle in DM than NM ($p \leq 0.001$), but this difference was smaller at 260 days gestation (Figure 4.5).

Plate 4.1

Photomicrographs showing mATPase staining in M. *vastus lateralis* at four gestational ages: 120 days (A, B), 160 days (C, D), 210 days (E, F) and 260 days (G, H) gestation. NM in panels on left (A, C, E, G), DM in panels on right (B, D, F, H). A and B stained after pre-incubation at pH 4.2, C-G stained after pre-incubation at pH 5.0, according to modified histochemical staining procedure. Dotted lines indicated primary myotubes, open arrows indicate type 1 fibres, closed arrow (D) indicates possible degenerating fibre. Bar = 50μm.

Plate 4.1. mATPase staining reaction in M. *vastus lateralis*.

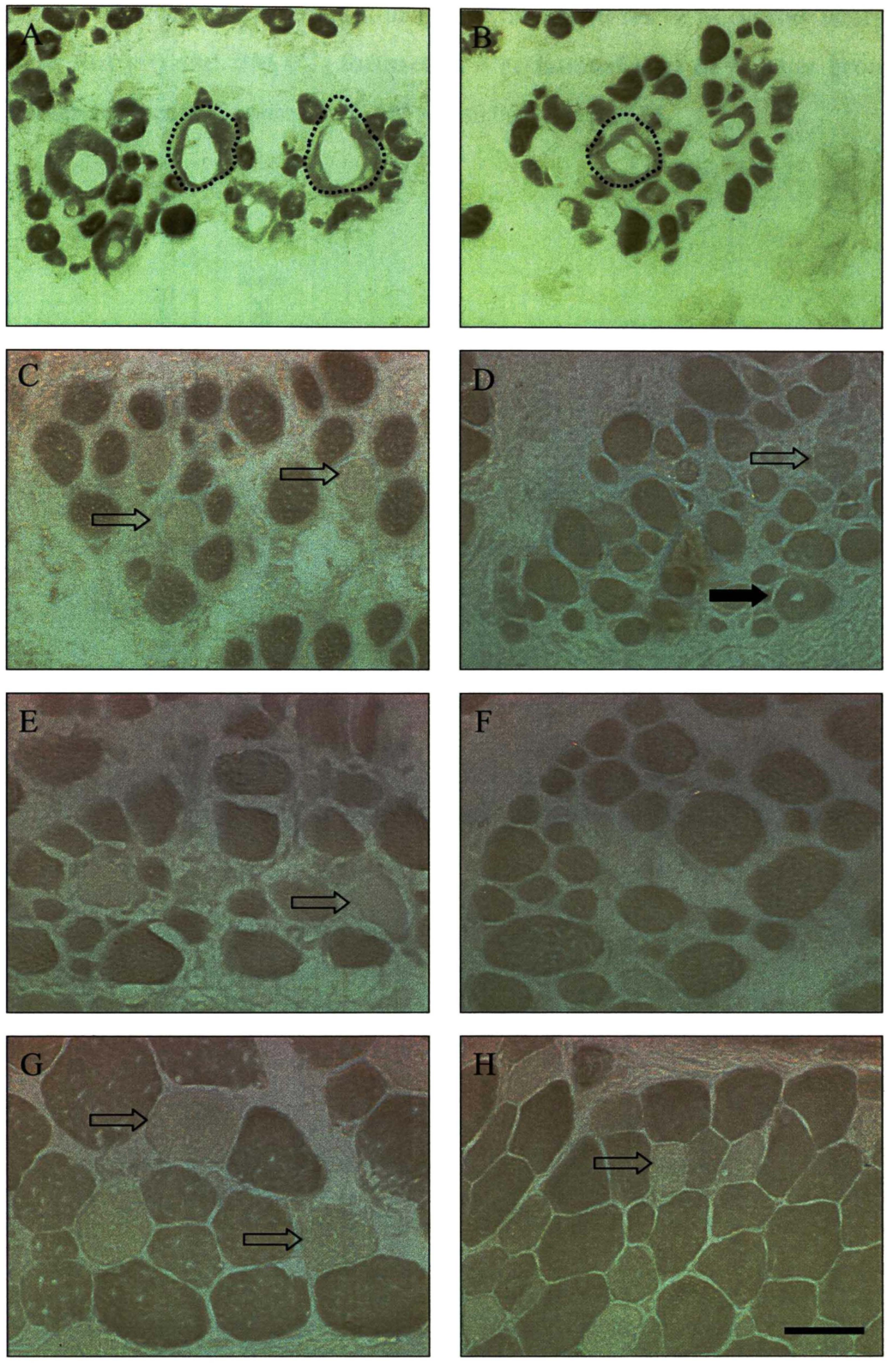
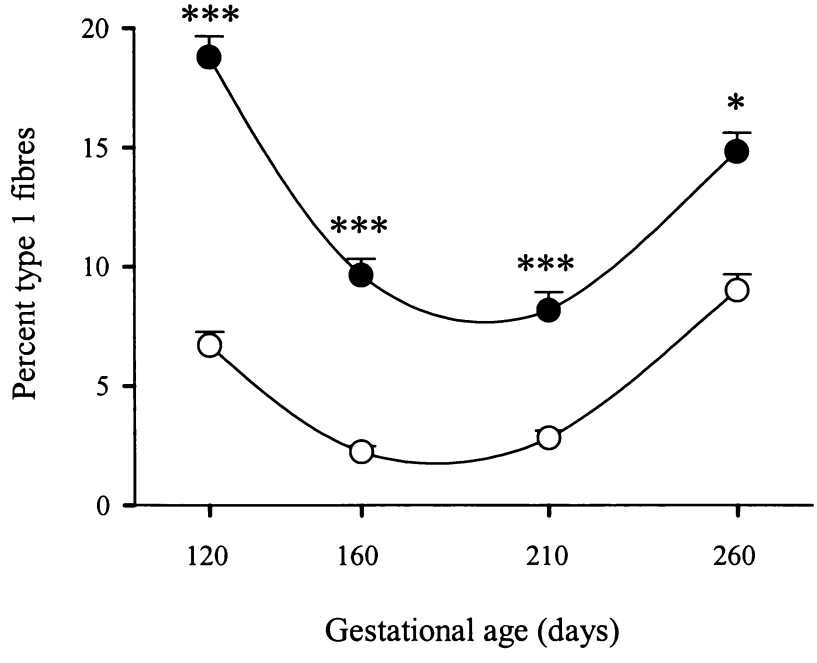


Figure 4.5 Average percentages of type 1 muscle fibres in M. vastus lateralis from NM (●) and DM (○) foetuses at 4 gestational ages (n = 3 per group). Values are means ± pooled sem. *** = p≤0.001, * = p≤0.05.



4.3.4.2 Number of fibres per fascicle

The total number of muscle fibres per fascicle was not significantly different between ages or between DM and NM (Table 4.1).

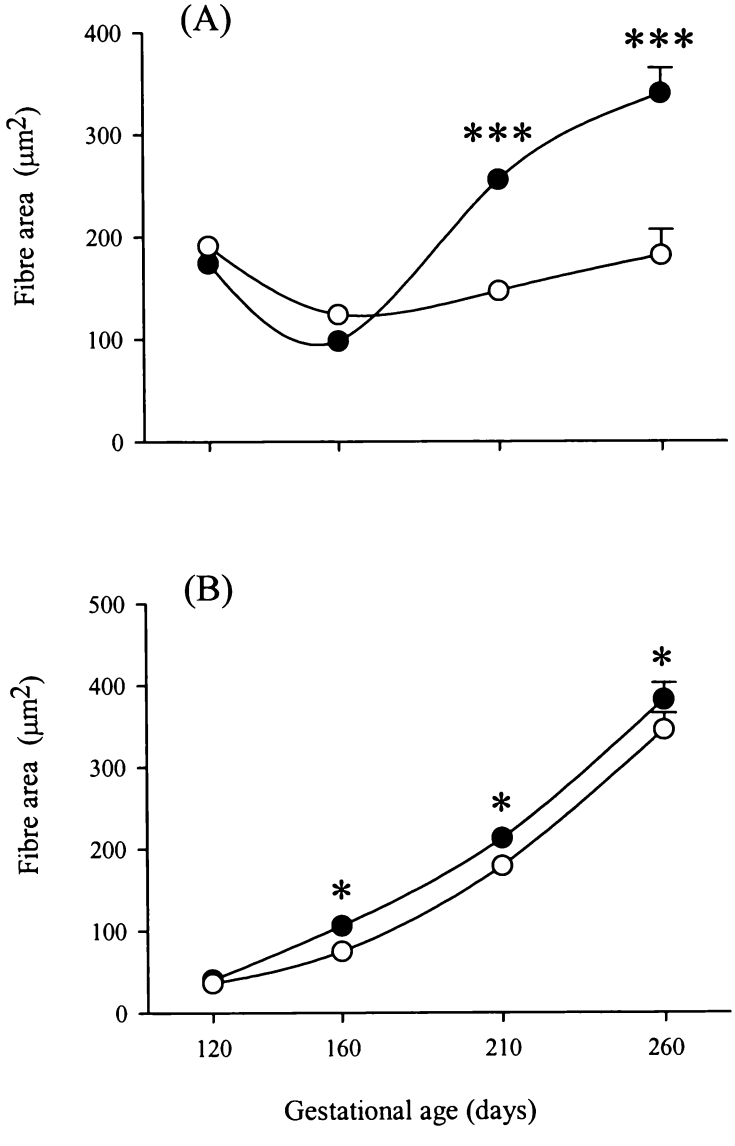
Table 4.1. Total number of muscle fibres per fascicle in M. *vastus lateralis* from NM and DM foetuses at 4 gestational ages (n = 3 per group).

Gestational Age	Breed		pooled sed	significance
	NM	DM		
120	58.5	56.6	4.1	ns
160	67.2	78.3	4.1	ns
210	65.2	54	4.1	ns
260	58.5	67.1	4.1	ns
pooled sed	5.8	5.8		
significance	ns	ns		

4.3.4.3 Muscle fibre size

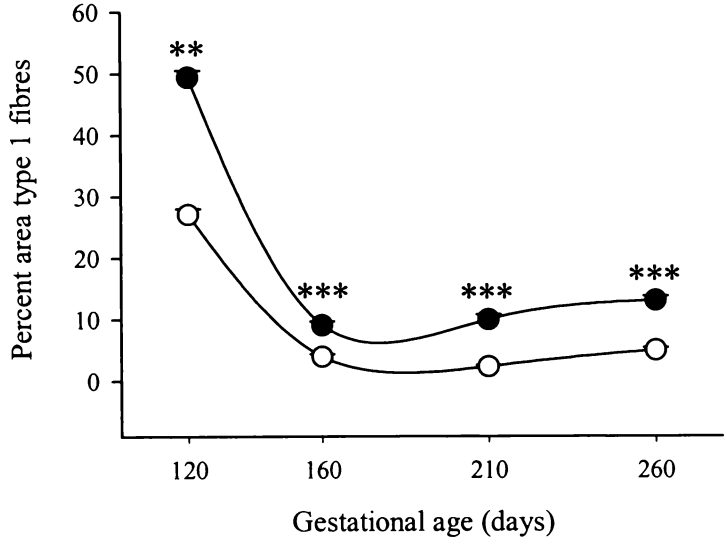
The average area of type 1 fibres in both DM and NM decreased from 120 to 160 days gestation (Figure 4.6 A), as their morphology changed from that of primary myotubes with a central region devoid of myofibrils, to more mature muscle fibres surrounded by developing secondary fibres. By 210 days fibres of NM muscles markedly increased in size as they continued to mature, while DM fibres had only very small increases in size, failing to regain the size they had previously attained at 120 days. By main effects analysis type 1 fibres were significantly smaller overall in DM than NM ($p \leq 0.001$), due to the differences in size at 210 and 260 days (Figure 4.6 A). The average area of type 2 muscle fibres increased with age ($p \leq 0.001$) and was less in DM than NM ($p \leq 0.05$). This effect was consistent across all age groups (Figure 4.6 B).

Figure 4.6 Average cross sectional area of type 1 (A) and type 2 (B) muscle fibres in M. vastus lateralis NM (●) and DM (○) foetuses at 4 gestational ages (n = 3 per group). Values are means ± pooled sem. A t-test was used for the calculation of significant differences within time periods. *** = p≤0.001, * = p≤0.05.



One of the most sensitive measures of overall fibre type composition is total % area given over to a specific fibre type (see Chapter 1). The proportion of total area given over to type 1 fibres declined rapidly between 120 and 160 days gestation in both NM and DM, then remained relatively constant throughout the remainder of the period studied. The net effect of the changes in size and proportions of type 1 fibres in the DM animals was that the muscle overall had a significantly lower proportion of total area given over to type 1 fibres at all gestational ages ($p \leq 0.001$) (Figure 4.7).

Figure 4.7 Total percentage of area of type 1 fibres calculated from average fibre number per fascicle multiplied by average fibre area. M. *vastus lateralis* from NM (●) and DM (○) foetuses at 4 gestational ages (n = 3 per group). Values are least squares means ± sem. Data was log transformed for analysis. *** = $p \leq 0.001$, ** = $p \leq 0.01$.



4.3.5 Immunohistochemistry

This study has shown a similar pattern of MHC expression in both DM and DM at 120 days gestation, with both presumptive primary and presumptive secondary fibres staining positively for embryonic MHC (Plate 4.2). All primary fibres were also positive for slow MHC and all secondary fibres were also positive for fast MHC. At 160 days gestation the pattern was similar, but there were a number of fibres in NM that were negative for embryonic MHC (Plate 4.3). At 210 days a number of presumptive secondary fibres in the DM were negative for embryonic MHC and others were positive for slow MHC (Plate 4.4). Some presumptive secondary fibres were positive for all MHC isoforms. No fibres were observed in NM that were positive for all MHC isoforms. At 260 days, immunostaining in DM muscle was essentially the same as 210 days, but in NM, all fibres were negative for embryonic MHC (Plate 4.5).

Plate 4.2-4.5.

Photomicrographs showing myosin heavy chain (MHC) immunohistochemistry in M. *vastus lateralis* at 120 days (Plate 4.2), 160 days (Plate 4.3), 210 days (Plate 4.4) and 260 days gestation (Plate 4.5). NM in panels on left (A, C, E), DM in panels on right (B, D, F). Embryonic MHC (A, B), slow MHC (C, D) and fast MHC (E, F). Dotted lines and black arrows indicate type 1 fibres, open arrows (Plate 4.4) indicate fibres positive for all MHC isoforms, Bar = 50 μm.

Plate 4.2. Myosin heavy chain immunohistochemistry at 120 days gestation.

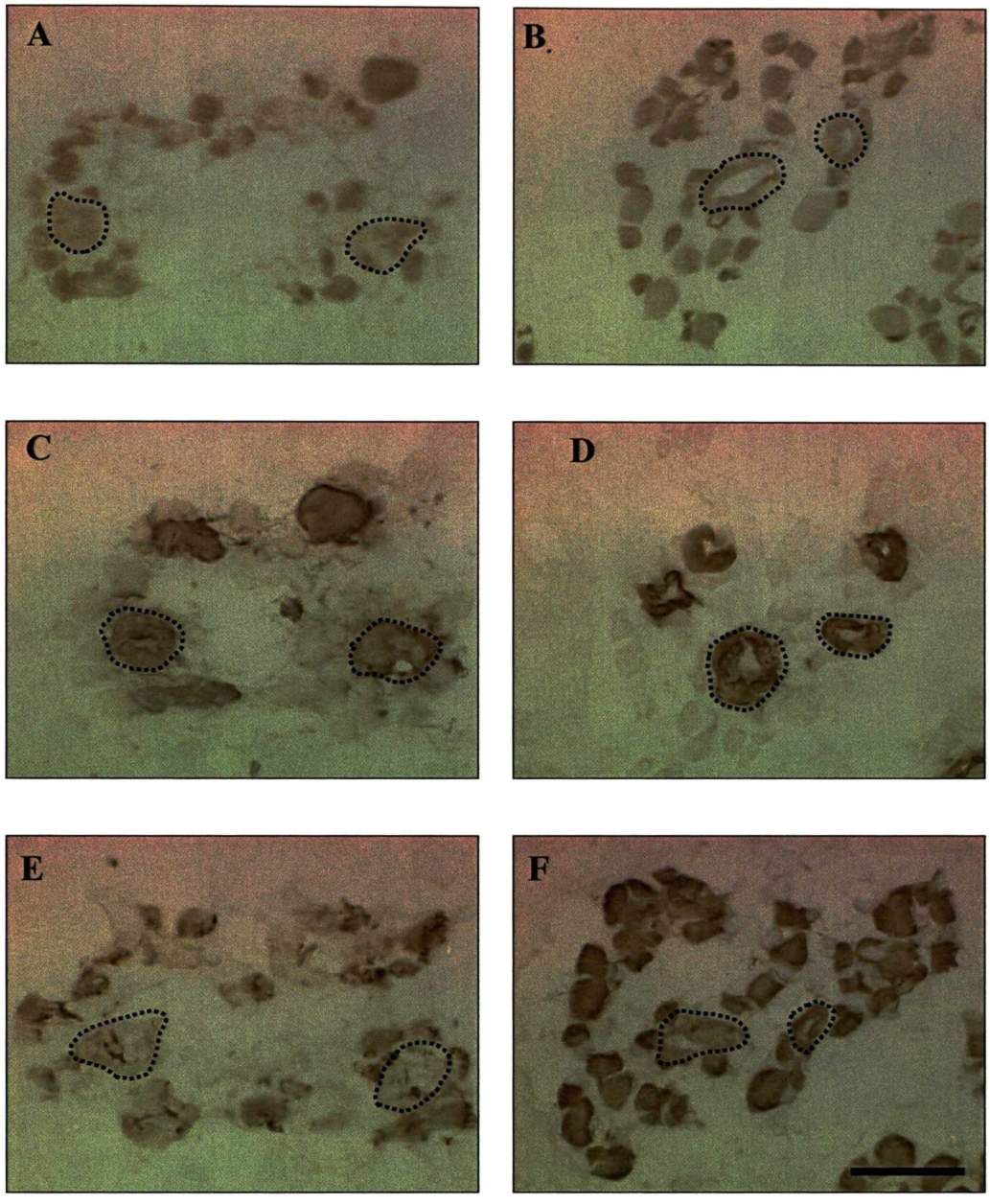


Plate 4.3. Myosin heavy chain immunohistochemistry at 160 days gestation.

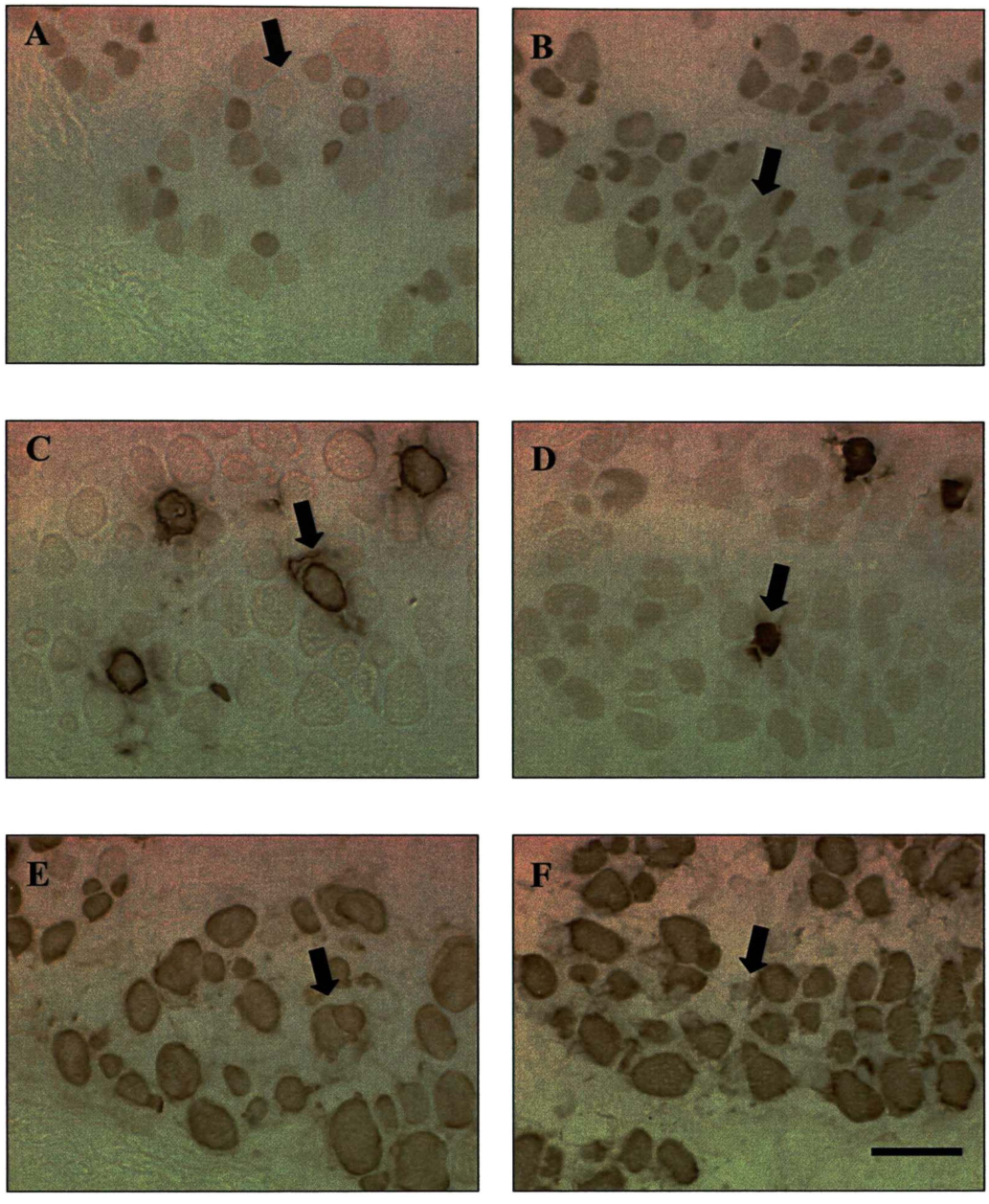


Plate 4.4. Myosin heavy chain immunohistochemistry at 210 days gestation.

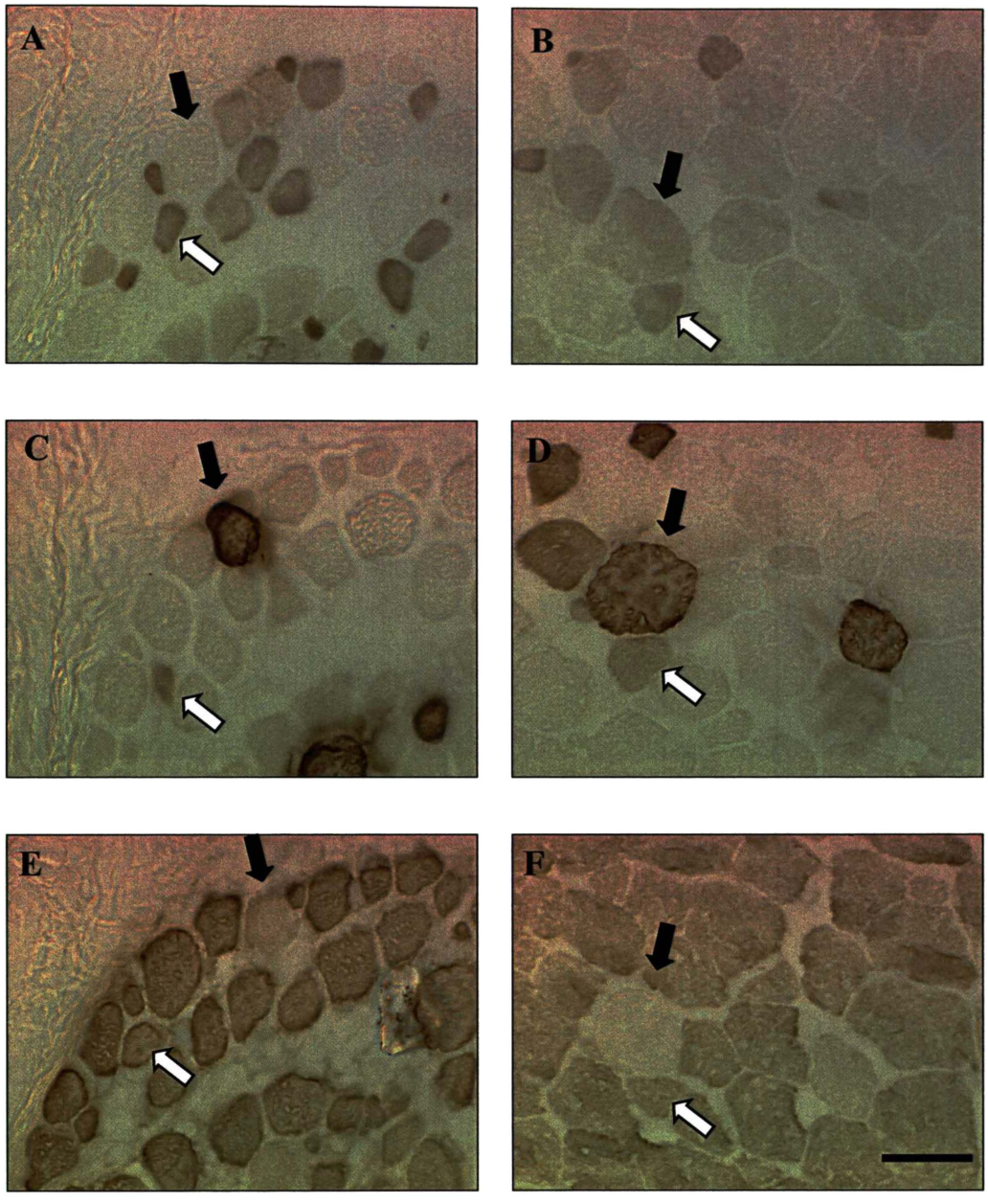
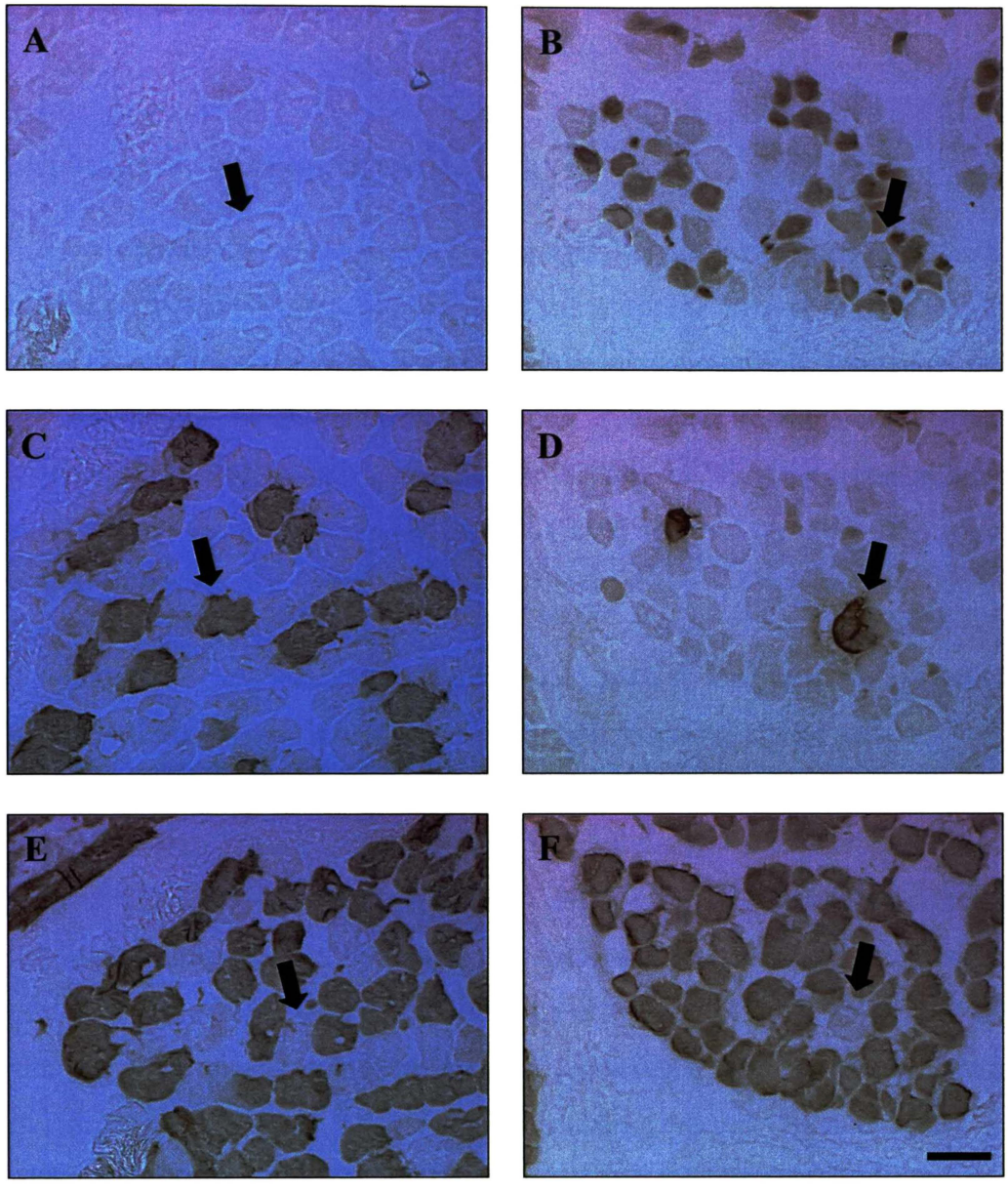


Plate 4.5. Myosin heavy chain immunohistochemistry at 260 days gestation.



4.4 DISCUSSION

4.4.1 Muscle development in NM and DM

This study has shown an increase in body weight, but not crown rump length in DM foetuses relative to NM. The implication of this is that the increase in body weight is predominantly associated with increased muscle mass, not with a change in skeletal size and this is consistent with previously published results during prenatal development of DM animals (Swatland and Kieffer, 1974). An increase in muscle mass with no increase in skeletal size has been reported in adult DM animals (Boccard, 1981).

Skeletal muscle mass was also increased in DM foetuses relative to NM, in both the M. *vastus medialis* and the M. *vastus lateralis*. In postnatal DM animals the M. *vastus medialis* is hypotrophied, or reduced in size, relative to NM animals (Boccard, 1981). This result suggests that the difference in muscle weight between DM and NM is not due to a direct genetic effect on muscle development and that postnatal environmental effects may be implicated in the development of this difference. In humans, injury to, or diseases of the knee joint, may be associated with atrophy of the M. *vastus medialis* and hypertrophy of the M. *vastus lateralis* (Speakman and Weisberg, 1977). Animals exhibiting extreme muscular hypertrophy have some abnormalities in stance and in the anatomy of the front limbs and hocks (Kieffer *et al.*, 1972). Possibly the M. *vastus medialis* in DM animals undergoes atrophy during postnatal life as a result of this postural abnormality which arises due to an interaction between genotype and environment.

In this study the protein:DNA ratio increased with increasing gestational age in the M. *vastus lateralis*. This result was predictable given that the muscle fibres are undergoing substantial hypertrophy during this period of development and a higher protein:DNA ratio is consistent with increased hypertrophy. However, a previous study has reported a decrease in the protein:DNA ratio in bovine M. *semitendinosus*

during gestation (Godfredson *et al.*, 1990) and concluded that this decrease was associated with increased proliferation of satellite cells during late gestation. The differences found during muscle development between these two studies may reflect a different growth impetus for M. *semitendinosus* and the M. *vastus lateralis*, as has been described for a number of bovine muscles during development (Johnson, 1974). Postnatally, the protein:DNA ratio has been shown to increase in bovine muscle up to 300 kg liveweight as a result of increased fibre hypertrophy (DiMarco *et al.*, 1987), so the increasing ratio in the M. *vastus lateralis* suggests that this muscle is relatively faster maturing than the M. *semitendinosus*.

4.4.2 Muscle fibre type

The percentage of type 1 muscle fibres initially declines with gestational age, then increases again. As mentioned in the introduction to this chapter, two possible mechanisms exist which could explain the initial reduction in the numbers of type 1 fibres and these will be discussed with respect to the results presented. In developing human muscle, a proportion of primary myotubes degenerate between 16 and 20 weeks gestation (Fidzianska and Goebel, 1991), a time period which coincides with the decrease in the percentage of type 1 fibres seen in the current study. This would have the net effect of decreasing the percentage of type 1 fibres, as seen in this study. This mechanism would still allow for a further increase in the numbers of newly formed secondary fibres without affecting the total number of muscle fibres within a fascicle, which was shown to be relatively constant throughout this study. Degenerating myotubes have the appearance of primary myotubes (Fidzianska and Goebel, 1991) and this may explain the presence of apparent primary myotubes at 160 days gestation, reportedly after primary myotubes have attained the morphology of mature myofibres (Robelin *et al.*, 1991; Stickland, 1978).

An alternative mechanism for the reduction in the percentage of type 1 fibres may be that a proportion of type 1 fibres were transformed from type 1 to type 2 fibres (Whalen *et al.*, 1984). This possibility could not be directly tested in this study, as

individual fibres could not be followed through gestation. However, this mechanism would not allow for the accumulation of any further secondary fibres within a fascicle, when the number of fibres per fascicle remained constant. Our histological evidence and that of others (Stickland, 1978; Swatland and Kieffer, 1974), showing an abundance of small myofibres up until at least 160 days gestation, suggests secondary fibre myogenesis is still occurring at this stage of development.

During late gestation the percentage of type 1 fibres increases in both NM and DM. A similar observation to this has been previously made in developing ovine muscle (Maier *et al.*, 1992), where the percentage of type 1 fibres was 10% at 76 days gestation, (equivalent to 210 days in the bovine) increasing to 70% in the adult. Maier *et al* (1992) suggested that the majority of the fibres transforming to slow MHC initially expressed an adult fast MHC isoform. This was not, however, supported by Picard *et al* (1994), who suggested that in predominantly fast twitch muscles, all the slow fibres originated from primary generation myotubes. Although this current study did not investigate temporal changes in MHC isoform changes in individual fibres, this remains an interesting area for future investigation to determine whether those fibres that express slow MHC isoforms in late gestation are indeed the original population of primary myofibres.

The pattern of changes in fibre type proportions described in this study was similar in both NM and DM, although in the DM the percentage of type 1 fibres was consistently lower than in the NM. This result had been previously reported both during prenatal development (Ashmore *et al.*, 1974) and in adult animals (Holmes and Ashmore, 1972). On that basis it appears that the initial decrease in type 1 fibres and the subsequent increase again are unrelated to the DM condition. The overall fibre type composition is, however, affected by the mutation and this will be further addressed in the next chapter.

An interesting observation that arose out of the quantification of fibre type proportions was that the number of muscle fibres per fascicle remained relatively

constant throughout the period of gestation studied for both breeds. A study of ovine muscle development suggested that muscle growth occurs by an increase in fascicle number rather than an increase in the number of fibres per fascicle (Sivachelvan and Davis, 1986). The mechanism by which this occurs is still unclear. There is possibly *de novo* formation of new fascicles or alternatively, the existing perimysium could be remodelled to give new, smaller fascicles. The first possibility was favoured by Sivachelvan and Davis (1986), but they offered no histological evidence showing fascicles at different stages of maturity.

This current study supports an increase in the number of fascicles in DM, on the basis of increased numbers of muscle fibres but a constant number of fibres per fascicle. At each gestational age examined in this study, all fascicles appeared to be at a similar stage of maturity. From these observation it could be concluded that the increased numbers of fascicles in DM might be established at the time the connective tissue framework is laid down, at approximately 140 days gestation. The mechanism by which fascicle number increases during development remains unknown.

This study used myosin heavy chain immunohistochemistry for the qualitative assessment of changes in patterns of embryonic, slow and fast MHC isoform localisation. In the earliest periods of gestation covered by this study, differences in MHC isoform expression between NM and DM were restricted to a population of fibres in NM that were negative for embryonic MHC at 160 days. As this is a developmental isoform, expression of which is lost as fibres mature, this suggests a relatively more advanced stage of development in the NM at this gestational age. Picard *et al* (1995a) identified 90 and 130 days gestation as the gestational ages during which developmental differences were most marked between NM and DM. At 210 days in the current study, some DM fibres began to lose expression of embryonic MHC and others began to express slow MHC. A population of fibres exists in DM which express all MHC isoforms. Picard *et al.* (1995a) recorded this same observation and they also described a number of fibres that were negative for

all isoforms. The current study extended the period of gestation a further 50 days beyond that of Picard *et al.* (1995a) and it demonstrated that at 260 days gestation NM no longer expresses embryonic MHC although it is still relatively abundant in DM. In summary, results from this section have shown that muscle from DM foetusus is delayed in its development relative to NM foetuses, with respect to the expression of MHC isoforms.

4.4.3 Muscle fibre size

This investigation showed an initial decrease in the average area of primary myotubes from 120 to 160 days gestation as they developed into mature myofibres. A similar result has been previously reported in bovine muscle (Stickland, 1978), but the validity of measuring muscle fibre size in histological sections in this way has been questioned (McLennan, 1994). In the current study, it is recognised that differences in the extent of contraction of muscle fibres can have an impact of fibre cross sectional area, but for the purposes of analysing relative differences between samples treated identically, the method is considered to be appropriate.

After the initial decrease in fibre size that occurred in both breeds, fibres in muscles from NM foetuses began to enlarge, but fibres from DM foetuses did not. This result suggests that during late gestation, some hypertrophic stimulus induces growth in type 1 fibres of NM only, while in DM, either this stimulus is not present, or the muscle fibres are unable to respond. Other studies have shown that the size of type 1 fibres is the same in DM and NM in early gestation (up to approximately 180 days) (Ashmore *et al.*, 1974), while between 4 and 26 weeks postnatally, type 1 fibres were smaller in the DM. By 66 weeks there was again no difference (Holmes and Ashmore, 1972). The current study has defined the time period during which the initial difference in size of type 1 fibres develops as being between 160 and 210 days gestation. The significance of this fibre type specific difference in muscle fibre area

between DM and NM will be considered further in the next chapter in relation to growth factor expression.

Type 2 fibres grew at a relatively constant rate throughout the time period studied and were consistently slightly smaller in DM than NM. In postnatal animals type 2 fibres have been shown to be larger in DM than NM (Holmes and Ashmore, 1972). As the relative differences in fibre type composition between DM and NM change from prenatal to postnatal life (Ashmore *et al.*, 1974; Holmes and Ashmore, 1972), it is possible that fibre size differences may also alter with developmental age. Although DM animals have increased muscle mass this is not necessarily associated with increased muscle fibre size (Ouhayon and Beaumont, 1968). This study provides further evidence that in the DM model of muscular hypertrophy, the increase in muscle mass is associated with muscle fibre hyperplasia rather than hypertrophy.

Information about fibre type percentage and average fibre area can be combined to give the total area of muscle classified as type 1 or type 2 fibres. This is a more sensitive measure of overall fibre type composition than measurement of either fibre type percentage or average fibre area in isolation (Holmes and Ashmore, 1972; West, 1974). In this study the total area of type 1 muscle fibres falls dramatically between 120 and 160 days then remains essentially level. The increase in the percentage of type 1 fibres in the DM at 210 and 260 days gestation is able to fully compensate for the smaller average fibre size, with no overall decrease in the total area. Care should be taken with respect to extrapolating conclusions about other fibre type properties from this data. As was discussed in Chapter 1, mATPase activity in developing muscle is not necessarily a reliable indicator of contraction speed or metabolic activity (Guth and Samaha, 1972). However, the very dramatic drop in area of type 1 fibres does warrant some consideration. Previous researchers, in interpreting similar data did propose that a transformation towards more glycolytic fibre types may have reflected an inability of the cardiovascular system to supply the excess musculature such that there was a compensatory shift towards a

more anaerobic type of metabolism (Ashmore *et al.*, 1974). This interpretation notwithstanding, it is reasonable to assume that this decline does indeed represent a substantial change in the metabolic pathway being employed by the muscles at this stage of development, towards the more glycolytic pathway that is found in adult animals. Further implications of this change will be discussed in Chapter 5.

4.4.4 Summary and conclusions

The first hypothesis that this chapter set out to test was that the differences in the extent of the muscular hypertrophy within DM animals relative to NM were genetically determined. This study has shown that the M. *vastus medialis* in DM animals is not reduced in mass relative to NM during prenatal growth. It was concluded that the reduced size of this muscle in postnatal DM animals may arise as a consequence of the reduced skeletal development of DM animals and the increased muscle mass, combining to cause a defect in posture which led to the muscle atrophy. The second part of this study examined the effect of breed on muscle fibre type composition and fibre size in NM and DM muscles during prenatal development. Type 1 muscle fibres exhibited a biphasic change in proportions with gestational age and numbers of type 1 fibres were consistently lower in DM. A number of different mechanisms may underlie this fibre type remodelling in both DM and NM animals during prenatal development and there appears to be a genetic basis to differences in fibre type composition between the two breeds. Type 1 fibres are smaller in DM than NM in late gestation only and type 2 fibres are smaller throughout gestation, suggesting differential regulation of muscle fibre size in NM and DM, depending on fibre type. Finally, this study showed that the pattern of MHC isoform localisation in DM muscle is indicative of a delay in development relative to NM.

CHAPTER FIVE

GROWTH FACTORS AND MUSCLE DEVELOPMENT IN NORMAL AND DOUBLE-MUSCLED CATTLE

5.1 INTRODUCTION

Growth factors play a key role in the proliferation and differentiation of muscle cells during development and in both the regulation of muscle fibre hypertrophy and fibre type transformation postnatally. The experiments described in this chapter characterise aspects of the IGF axis and also myostatin expression, during the development of normal and double-muscled animals. This chapter focuses specifically on the role of the IGFs and myostatin in the regulation of bovine skeletal muscle development, with respect to the temporal changes in fibre type and fibre size that were described in Chapter 4.

5.1.1 Growth factor and hormonal regulation of fibre type

The role of hormones and growth factors in fibre type determination during foetal development is not well defined. Some evidence supporting a role for growth factors in fibre type determination comes from genetic models of altered muscle mass. The IGF-1 transgenic mice have increased proportions of oxidative fibres (Coleman *et al.*, 1995) and as was described in Chapter 4, there is a significantly lower proportion of type 1 fibres in DM animals carrying a myostatin mutation compared to NM. Foetal hypophysectomy, resulting in a loss of growth hormone and a consequent decrease in circulating levels of IGF-1, impairs the normal pattern of type 2 to type 1 fibre type conversion (Hausman and Watson, 1994). Foetal

thyroidectomy causes an increase in the proportion of type 2 muscle fibers (Finkelstein *et al.*, 1991). There is evidence which suggests that TGF-β in developing connective issue may play a role in the regulation of fibre type (McLennan, 1993). TGF-β also influences the development of muscle pioneers cells in zebrafish embryos, where it acts to antagonise the activity of Hedgehog in inducing slow muscle precursors throughout the somite (Du *et al.*, 1997). As yet, no definitive association has been identified between myostatin expression and the determination of skeletal muscle fibre type, although as was described in the bovine development study in Chapter 4, there are significant differences in fibre type composition between normal animals and DM animals carrying a myostatin mutation.

During postnatal life there are many examples of hormonal control of skeletal muscle fibre type (reviewed by Cassar-Malek *et al.*, 1998; and Vigneron *et al.*, 1989) and Table 5.1 summarises much of this information. Hypophysectomy has been shown to cause a decrease in proportions of oxidative type 1 fibres in rats and this effect can be reversed by giving growth hormone (Ayling *et al.*, 1989). Thyroid hormone causes an increase in numbers of slow muscle fibres (Butler-Browne *et al.*, 1984; Gambke *et al.*, 1983; Gustafson *et al.*, 1985; Izumo *et al.*, 1986). In type 1 diabetes there is a decrease in the numbers and size of type 2A and 2B fibres (Klueber and Feczko, 1994) and this condition can be reversed by pancreatic islet transplantation (Medina-Sanchez *et al.*, 1994), suggesting that insulin may be a mediator of the fibre type transformation. In addition, the sex steroids, glucocorticoids and also beta adrenergic agonists all have differential affects on fibre type (see Table 5.1 for references). Postnatally, myostatin expression in pigs is lower in red muscle, which has a predominance of slow fibre types than in white, fast twitch muscle (Ji *et al.*, 1998). In mice, myostatin mRNA expression is strongly correlated with the type 2b MHC isoform and is undetectable in the soleus which contains only slow and type 2a MHC (Carlson *et al.*, 1999). In humans, myostatin

protein is reported to be equally abundant in both fast and slow muscle fibres (Gonzalez-Cadavid *et al.*, 1998).

5.1.2 Growth factor and hormonal regulation of fibre size

Growth factors play an important role in regulating fibre size, both during foetal development and in postnatal life. Excessive secretion of growth hormone in the rat leads to an increase in the size of type 1 but not 2a muscle fibres (Prysor-Jones and Jenkins, 1980) while endogenous growth hormone selectively increased the size of type 2 fibres in young rats (Aroniadou-Anderjaska *et al.*, 1996). Foetal hypophysectomy and thyroidectomy each cause a decrease in average muscle fibre area (Walker and Luff, 1995) indicating specific roles for the GH/IGF axis and thyroid hormone in muscle fibre hypertrophy. Other studies have shown IGF-1 to be associated with increased muscle fibre hypertrophy *in vivo* (Coleman *et al.*, 1995) and *in vitro* (Semsarian *et al.*, 1999a; Semsarian *et al.*, 1999b; Vandenburgh *et al.*, 1991). Type 1 diabetes is associated with selective atrophy of type 2b fibres (Klueber and Feczko, 1994). Glucocorticoids also induce fibre atrophy and beta agonists and the sex steroids induce skeletal muscle fibre hypertrophy (see Table 5.1 for references). The role of myostatin in regulating muscle fibre size remains unclear. In the myostatin knockout mice, average fibre area was increased (McPherron *et al.*, 1997) but in the myostatin mutant DM cattle, the effect on fibre size is variable according to age and fibre type (Ashmore *et al.*, 1974; Holmes and Ashmore, 1972). Table 5.1 summarises the information on growth factor and hormonal regulation of skeletal muscle fibre type and fibre size.

Table 5.1 Hormonal regulation of changes in muscle fibre type and size (modified from Cassar-Malek *et al.*, 1998)

	Fibre type changes	Fibre size changes
Growth hormone	↔[1]	↑[1,2]
IGF-1	↑type 1[3,4]	↑[3]
Thyroid hormone	↓↑[5]	?
Insulin	↑2A, ↓2B[6]	↓2A and 2B[6]
Beta agonists	↑type 2[7]	↑ type 2[7]
Anabolic hormones	↑2A[8]	↑2B, ↑2A[8]
Glucocorticoids	↓type 2[9,10]	↓ type 2[9.10]
TGFs, myostatin	↓type1 [11,12]	↑, ↓, ↔[11-13]

[1] (Prysor-Jones and Jenkins, 1980); [2] (Aroniadou-Anderjaska *et al.*, 1996); [3] (Coleman *et al.*, 1995); [4] (Ayling *et al.*, 1989); [5] (Izumo *et al.*, 1986); [6] (Klueber and Feczko, 1994); [7] (Criswell *et al.*, 1996); [8] (Egginton, 1987); [9,10] (Almon and Dubois, 1990; Falduto *et al.*, 1990); [11,12] (Ashmore *et al.*, 1974; Holmes and Ashmore, 1972); [13] (McPherron *et al.*, 1997).

5.1.3 The double-muscling condition

5.1.3.1 Muscle development in DM animals

The condition known as double-muscling (DM) in Belgian Blue cattle is caused by a mutation in the gene for myostatin (Grobet *et al.*, 1997; Kambadur *et al.*, 1997). This mutation eliminates the production of functional myostatin and results in extreme muscle hypertrophy. The increase in muscle mass is reported to arise as a result of hyperplasia (Gerrard and Judge, 1993; Ouhayoun, 1982; Swatland and Kieffer, 1974) and to some extent, hypertrophy (Holmes and Ashmore, 1972) of muscle fibres in these animals. DM animals are therefore a useful model for investigations into muscle development and this formed the basis for the study described in Chapter 4 of this thesis.

5.1.3.2 Growth factors in DM animals

Prior to the identification of the myostatin mutation in DM animals, the IGF axis was implicated in the development of the DM condition in cattle. Maximal expression of IGF-2 in muscle is delayed in DM cattle relative to NM (Gerrard and Grant, 1994). In conjunction with the increased proliferation of myoblasts in DM animals (Quinn *et al.*, 1990), this evidence was suggested to support a role for IGF-2 in the regulation of myoblast differentiation (Gerrard and Grant, 1994) as has been previously proposed (Tollefsen, 1989). It has also been reported that conditioned media from DM fibroblasts contains a factor, thought to be an IGF binding protein, which stimulates myoblast proliferation (Quinn *et al.*, 1990).

5.1.4 Aim of this chapter

The aim of this chapter was to test the hypothesis that the IGFs and myostatin are involved in mediating differences in skeletal muscle fibre type and fibre size in developing muscle of normal and double-muscled foetuses.

5.2 MATERIALS AND METHODS

5.2.1 IGF-1 and IGF-2 radioimmunoassays

Radioimmunoassays for IGF-1 and IGF-2 were carried out on plasma samples from all animals from each age group and each breed (3-5 per group) as described in Section 2.4.1. Data are presented as means ± pooled sem.

5.2.2 *In situ* hybridisation

In situ hybridisation was carried out using a human IGF-1 probe, according to the procedure described in Section 2.4.3.1. Muscle sections were cut from paraffin processed tissue blocks from three animals in each breed and each age group. After hybridisation, slides were exposed against XAR film for 4 days. XAR films were

analysed by densitometry to determine the levels of specific hybridisation by subtracting OD values for sense sections from anti-sense values. Data was log-transformed for overall analysis of variance. Comparisons within age were carried out using the pooled standard error.

For localisation of hybridisation at the cellular level, sections were coated with photographic emulsion as described in Section 2.4.3.1.2.6. Photomicrographs of developed sections were taken using the image analysis system described in Section 2.4.8.

5.2.3 RT-PCR.

Semi-quantitative RT-PCR was carried out according to the method given in Section 2.4.3.4. Briefly, specific primers were used for the amplification of myostatin and ubiquitin activating enzyme as housekeeping control gene. PCR products were run on an agarose gel and transferred to a nylon membrane. Random prime labelled probes for myostatin and ubiquitin activating enzyme (UAE) were used to hybridise to the membrane. After overnight hybridisation, membranes were exposed to XAR film and films were analysed on a densitometer. Analysis of variance was carried out using the optical density (OD) of the housekeeping gene as a covariate for myostatin. Data were square root transformed for analysis and are presented as back transformed least squares means ± sem.

5.2.4 IGF-1 immunohistochemistry

This experiment aimed to identify any changes in IGF-1 immunolocalisation with gestational age or between NM and DM animals. It also aimed to examine any fibre type specific differences in the level of IGF-1 peptide in muscle fibres. Immunolocalisation of IGF-1 was carried out on sections cut from tissue blocks from which serial sections had been used for MHC immunostaining. Sections were incubated with IGF-1 primary antibody according to the method given in Section

2.4.4.2.5. Serial sections were also incubated with slow MHC antibody according to the protocol in Section 2.4.4.2.2.

5.2.5 Receptor autoradiography

Histological autoradiography was carried out in order to characterise differences in receptor binding between DM and NM muscles at the four gestational ages investigated. Two pairs of cryostat sections were cut at 8 μm thickness and mounted on gelatine-chrome alum subbed slides. Eight slides were prepared for each animal for incubation with either IGF-1 or IGF-2 in the presence of tracer plus unlabelled homologous peptide and three competing heterologous peptides. The basis of the competitive displacement assay of receptor binding was described in Section 2.4.5. There were 5 animals per group for all the normal controls, 5 per group for 120 and 160 day DM and 3 per group for 210 and 260 day DM. The large number of slides involved and the long time intervals between collecting muscle samples meant it was not possible to carry out all the incubations simultaneously. Therefore the incubations were carried out to compare receptor binding levels between DM and NM within each time period. Incubations were carried out as described in Section 2.4.5. Slides were exposed to XAR film for 4 days and the resulting XAR film was used to estimate the exposure period for the NTB-2 emulsion. This was 24 days for IGF-1 and 10 days for IGF-2.

5.3 RESULTS

5.3.1 Plasma IGF assays

There were no overall differences between NM and DM in levels of IGF-1 or IGF-2, so data was pooled for overall analysis of effects of gestational age. Circulating levels of IGF-1 only increased with increasing gestational age ($p \leq 0.05$) (Figure 5.1,

A). Differences in levels of IGF-1 and IGF-2 between NM and DM at each gestational age were tested by *t-test.* Results showed plasma IGF-2 was lower in NM than DM at 120 days gestation ($p \leq 0.05$) (Figure 5.1, B).

5.3.3 IGF-1 *in situ* hybridisation

In situ hybridisation enables the localisation of mRNA at the cellular level. This study showed IGF-1 mRNA to be predominantly localised to muscle fibres rather than connective tissue, at all ages and in both NM and DM. Quantitative analysis of levels of hybridisation by densitometry of XAR images showed than mRNA levels declined with gestational age ($p \leq 0.01$) and that hybridisation was lower in DM than NM ($p \leq 0.05$) (Figure 5.2). Qualitative analysis of sections after emulsion autoradiography showed levels of hybridisation to be even across all muscle fibres. (Plate 5.1).

Figure 5.1 Levels of IGF-1 (A) and IGF-2 (B) circulating in the plasma of NM (●) and DM (O) foetuses at 4 gestational ages. Values are means ± average sem (n=3-5 per group). * = p≤0.05.

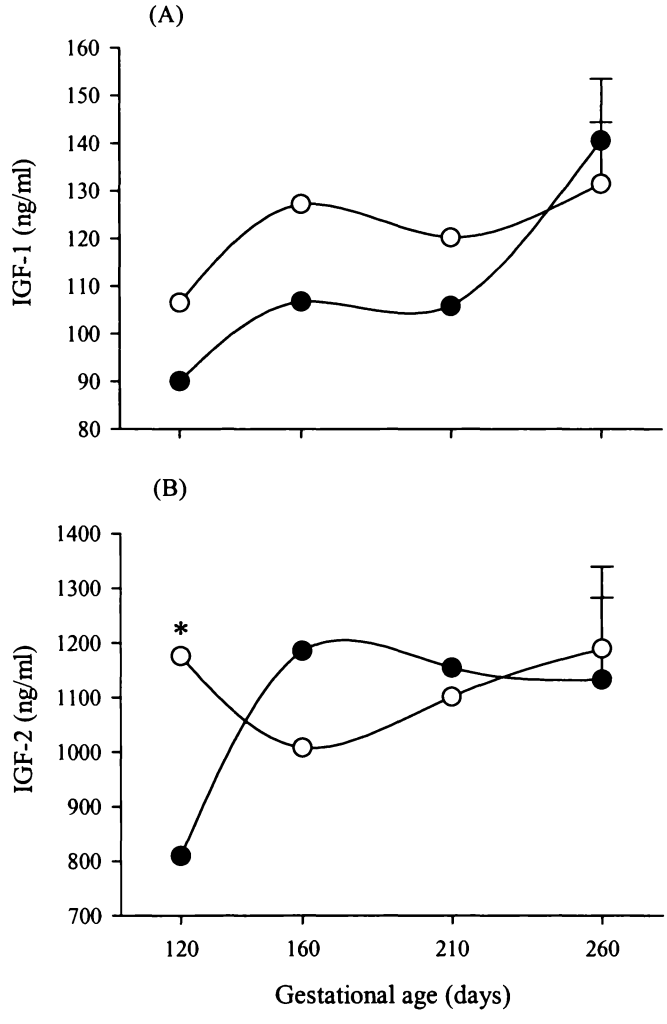


Figure 5.2 Levels of IGF-1 mRNA by quantitation of XARs after *in situ* hybridisation of labelled probe to NM (●) and DM (O) M. *vastus lateralis* at 4 gestational ages. Values are means ± sem (n=3 per group). * = p≤0.05.

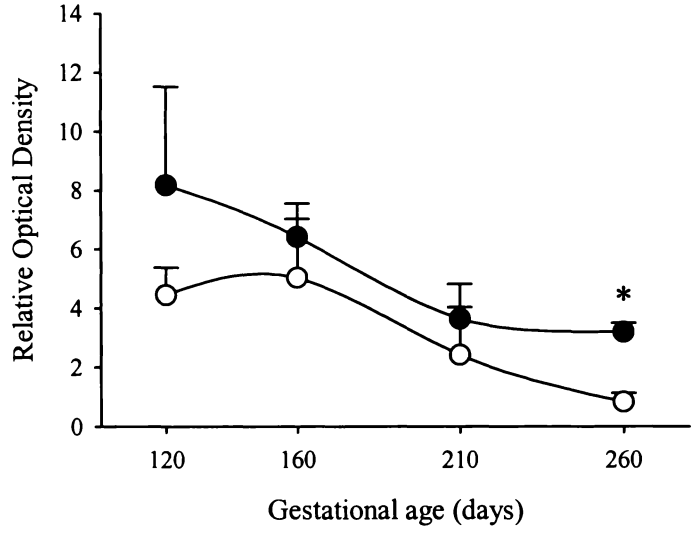


Plate 5.1

***In situ* hybridisation of IGF-1 probe to M. *vastus lateralis* at 120 days (A, B) and 160 days (C, D) gestation.**

Plate 5.2

***In situ* hybridisation of IGF-1 probe to M. *vastus lateralis* at 210 days (E, F) and 260 days (G, H) gestation.**

NM in panels on left (A, C, E, G), DM in panels on right (B, D, F, H). Upper panels in each pair show hybridisation with anti-sense probe and lower panels show hybridisation with sense probe as a control for non-specific hybridisation. Bar = 20 μm.

Plate 5.1 IGF-1 *in situ* hybridisation at 120 and 160 days gestation.

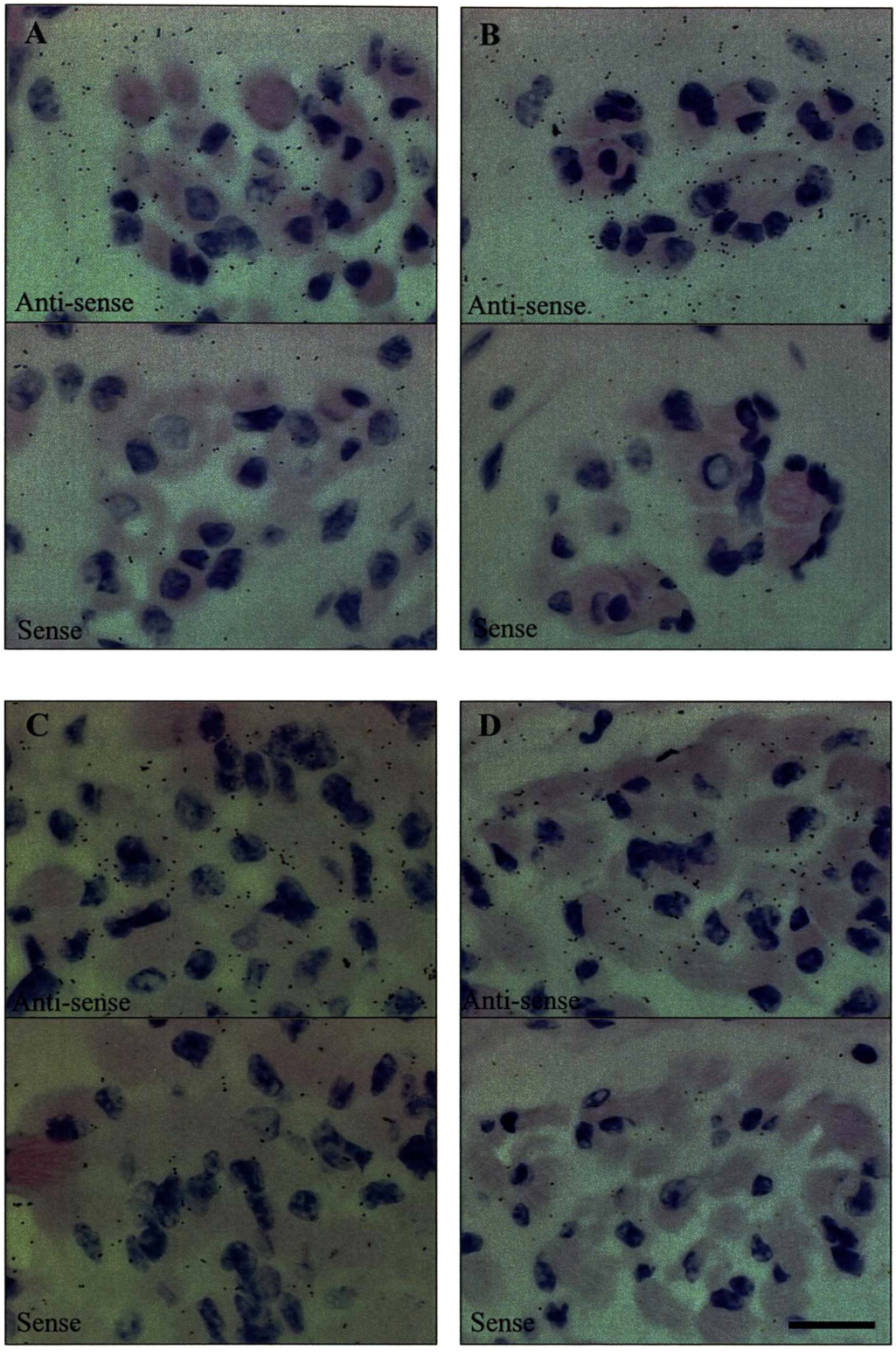
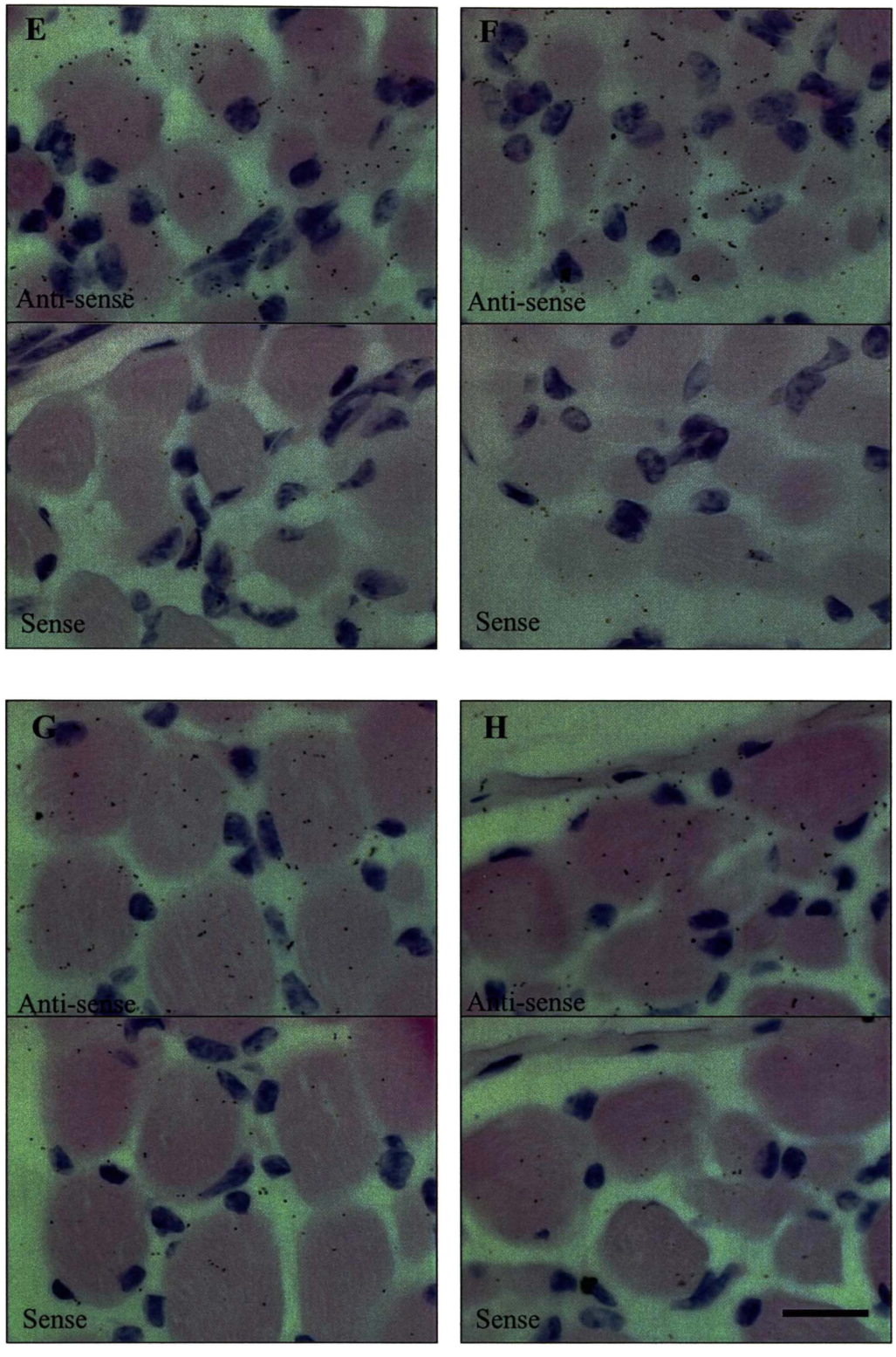


Plate 5.2 IGF-1 *in situ* hybridisation at 210 and 260 days gestation.



5.3.2 Semi-quantitative RT-PCR

Myostatin mRNA expression was measured by Southern blot analysis of PCR products after semi-quantitative RT-PCR. XAR films (Figure 5.3) were scanned on a densitometer and quantified as described in Section 2.4.3.4. Results showed that expression declined in NM with gestational age (Figure 5.4). Expression of the mutant myostatin mRNA was measured in the DM animals and was elevated in the DM relative to NM ($p \leq 0.05$). Comparison of differences at individual ages showed a statistically significant difference at 210 days ($p \leq 0.01$) (Figure 5.4).

Figure 5.3 shows XAR films of myostatin Southern blots after semi-quantitative RT-PCR, to give an indication of differences in mRNA levels. Intensity of bands was quantified by densitometry and results are presented in Figure 5.4, after normalisation with uae.

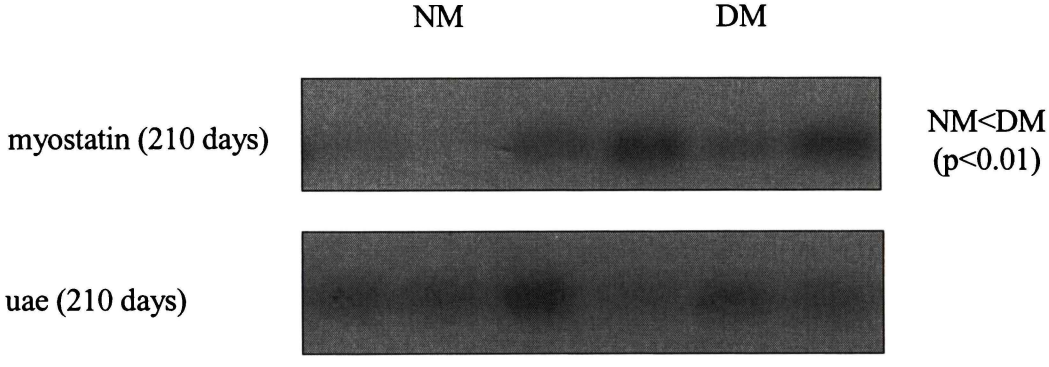
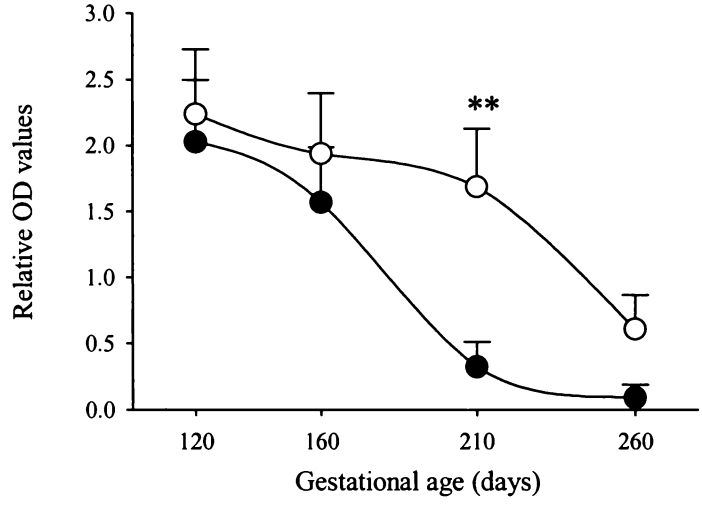


Figure 5.4 Levels of myostatin mRNA by RT-PCR of M. *vastus lateralis* from NM (●) and DM (O) foetuses at 4 gestational ages. Values are least squares means ± sem after normalisation with uae (n=3 per group). ** = p≤0.01.



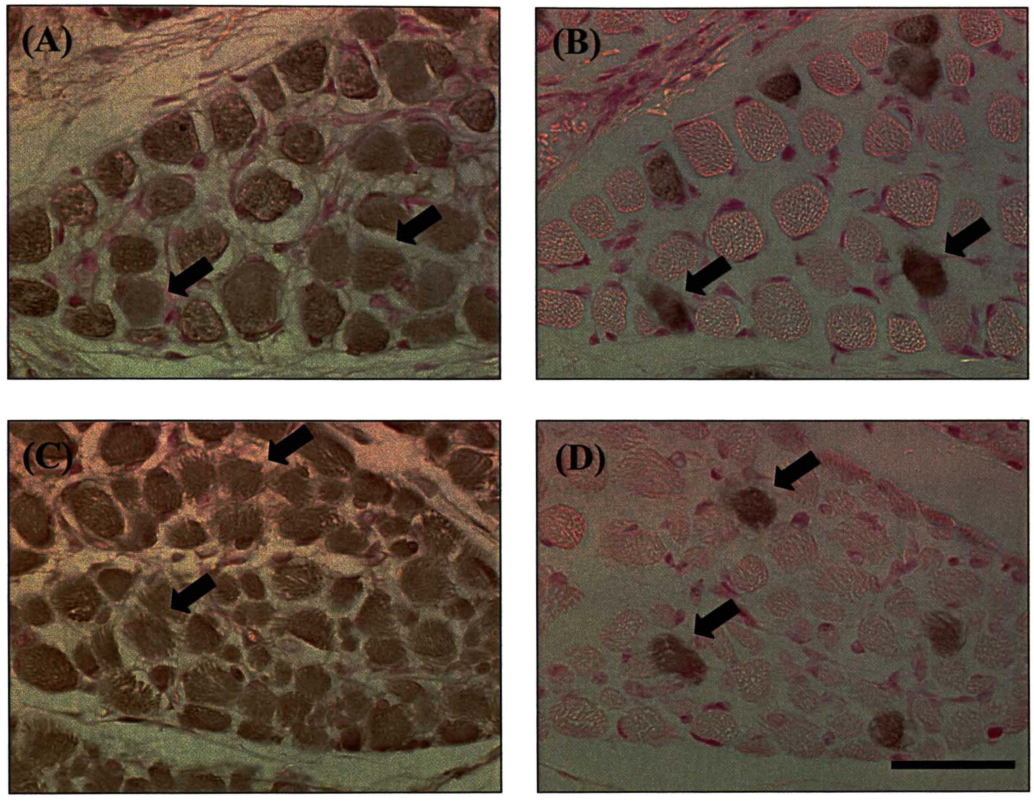
5.3.4 Immunohistochemistry

Immunohistochemistry was used to determine whether there were any differences in IGF-1 distribution with respect to fibre type. IGF-1 immunostaining was abundant in all muscle fibres, independent of fibre type as can been seen in serial sections stained for slow MHC (Plate 5.3).

Plate 5.3

Photomicrograph showing IGF-1 (A, C) and slow MHC (B, D) immunohistochemical localisation to serial sections of NM and DM M. *vastus lateralis* at 210 days gestation. Arrows indicate slow MHC positive fibres. Bar = 50 μm.

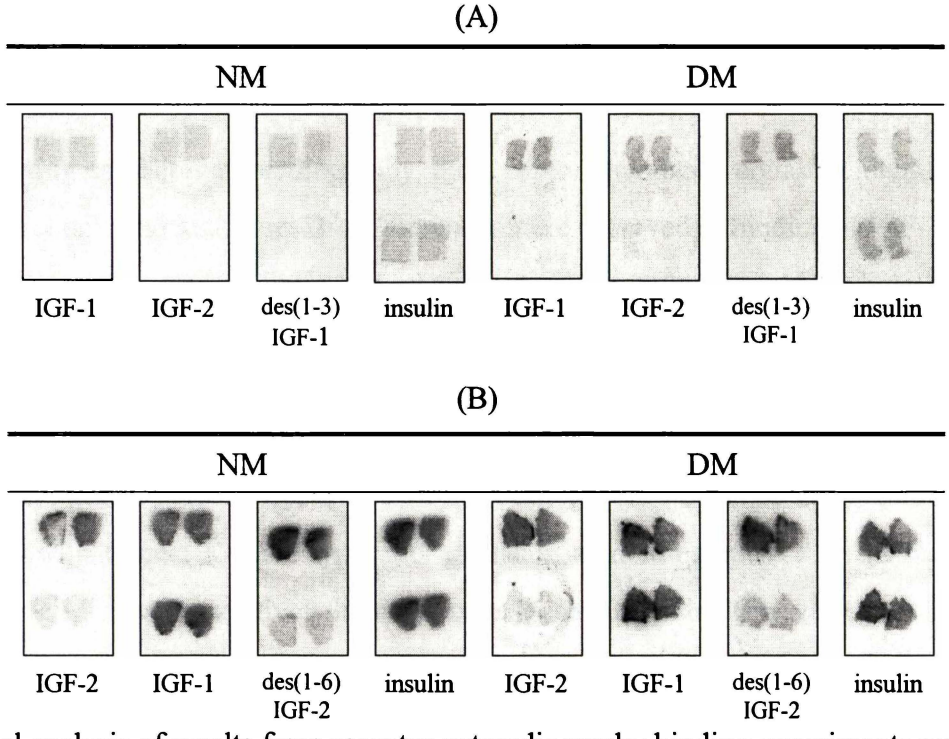
Plate 5.3. IGF-1 and slow MHC immunohistochemistry.



5.3.5 Receptor autoradiography

The capacity of the type 1 and type 2 IGF receptors was characterised on the basis of specific binding of tracer in an *in vitro* competitive displacement assay. Figure 5.5 shows XARs of displacement of IGF-1 and IGF-2 in the presence of all competing peptides used in this study. On the basis of the competitive displacement study it could be concluded that IGF-1 binding was predominantly to the type 1 receptor, not the type2 receptor, binding proteins or the insulin receptor. Likewise IGF-2 binding was predominantly to the type 2 IGF receptor. Specific binding was calculated as the difference between total binding, in the upper sections and non-specific binding in the presence of the homologous unlabelled peptide, in the lower sections.

Figure 5.5. Characterisation of (A) radiolabelled IGF-1 binding at 160 days and (B) IGF-2 binding at 120 days to sections from NM and DM animals. Upper sections show total binding and lower sections show non-specific binding in the presence of excess unlabelled peptide as shown.



Initial analysis of results from receptor autoradiography binding experiments was by densitometric analysis of XAR films. This data showed that there was no difference

between DM and NM in binding of radiolabelled IGF-1 to the type 1 IGF receptor at 120, 210 or 260 days gestation, but at 160 days, binding in DM was significantly higher than NM ($p \leq 0.01$), (Table 5.1). Although this experiment aimed to identify differences in IGF binding between NM and DM at different gestational ages and was not an ontogenetic study, binding was noticeably higher at 160 days gestation for both IGF-1 and IGF-2 (Table 5.2, Plates 5.3 and 5.4). The 160 day incubations were also carried out with lower specific activity tracers, relative to the other age groups (Table 5.2). Non-specific binding was very low as can be seen in the XARs.

Table 5.2 Specific binding of IGF-1 to type 1 IGF receptors in M. *vastus lateralis* (relative OD values). Values are means (n=3-5). ** = $p \leq 0.01$.

Age (days)	Specific activity (μCi/μg)	NM	DM	pooled sed	significance
120	166.3	4.0	2.9	0.5	ns
160	48.7	4.4	6.6	0.5	**
210	73.9	1.9	2.1	0.6	ns
260	73.9	0.7	0.8	0.6	ns

Following emulsion autoradiography, the receptor abundance could be identified at the level of individual fibres. No differences were observed in binding of IGF-1 with respect to fibre type. All fibres had relatively homogeneous levels of binding (Figure 5.7).

Specific binding of IGF-2 to the type 2 IGF receptor is only significantly different at 120 days gestation ($p \leq 0.05$), (Table 5.2). No differences were observed in binding of IGF-2 with respect to fibre type after emulsion autoradiography. As was seen for IGF-1, all fibres had relatively homogeneous levels of binding (Figure 5.8).

Table 5.3 Specific binding of IGF-2 to type 2 IGF receptors in M. *vastus lateralis* (relative OD values). Values are means (n=3-5). * = p≤0.05.

Age (days)	Specific activity (μCi/μg)	NM	DM	pooled sed	significance
120	144.5	17.6	12.3	2.0	*
160	33.1	43.7	41.8	2.0	ns
210	69.5	33.9	32.9	2.3	ns
260	69.5	22.1	23.5	2.3	ns

Plate 5.4

Autoradiographic silver grains showing localisation of radiolabelled IGF-1 to type 1 IGF receptors in M. *vastus lateralis* at three gestational ages 120 days (A, B), 210 days (C, D), 260 days (E, F) gestation. Slides from 160 days gestation were accidentally damaged. NM in panels on left (A, C, E), DM in panels on right (B, D, F). Bar = 20 μm.

Plate 5.5

Autoradiographic silver grains showing localisation of radiolabelled IGF-2 to type 2 IGF receptors in M. *vastus lateralis* at four gestational ages 120 days (A, B), 160 days (C, D), 210 days (E, F) and 260 days (G, H) gestation. NM in panels on left (A, C, E, G), DM in panels on right (B, D, F, H). Bar = 20 μm.

Plate 5.4 Radiographic binding of IGF-1.

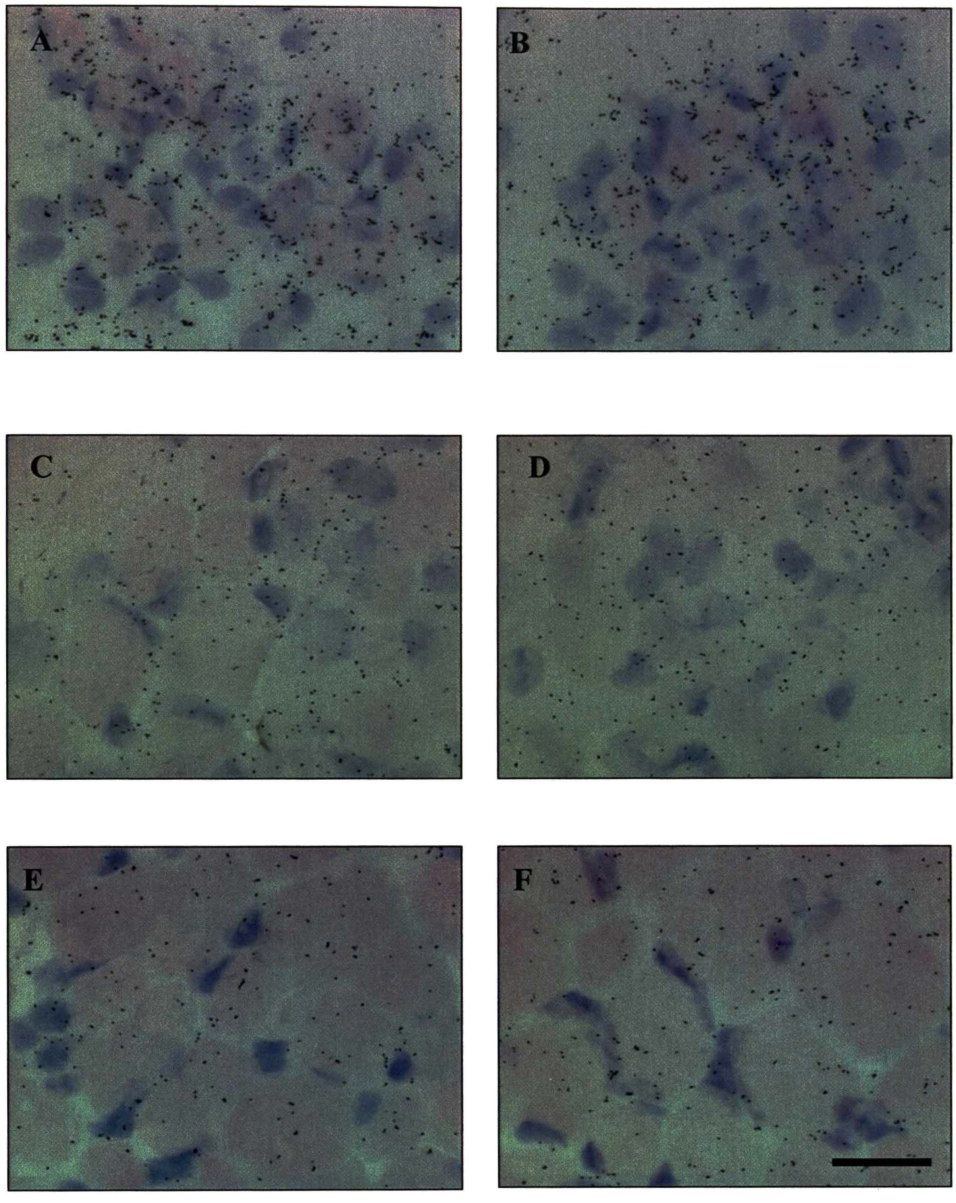
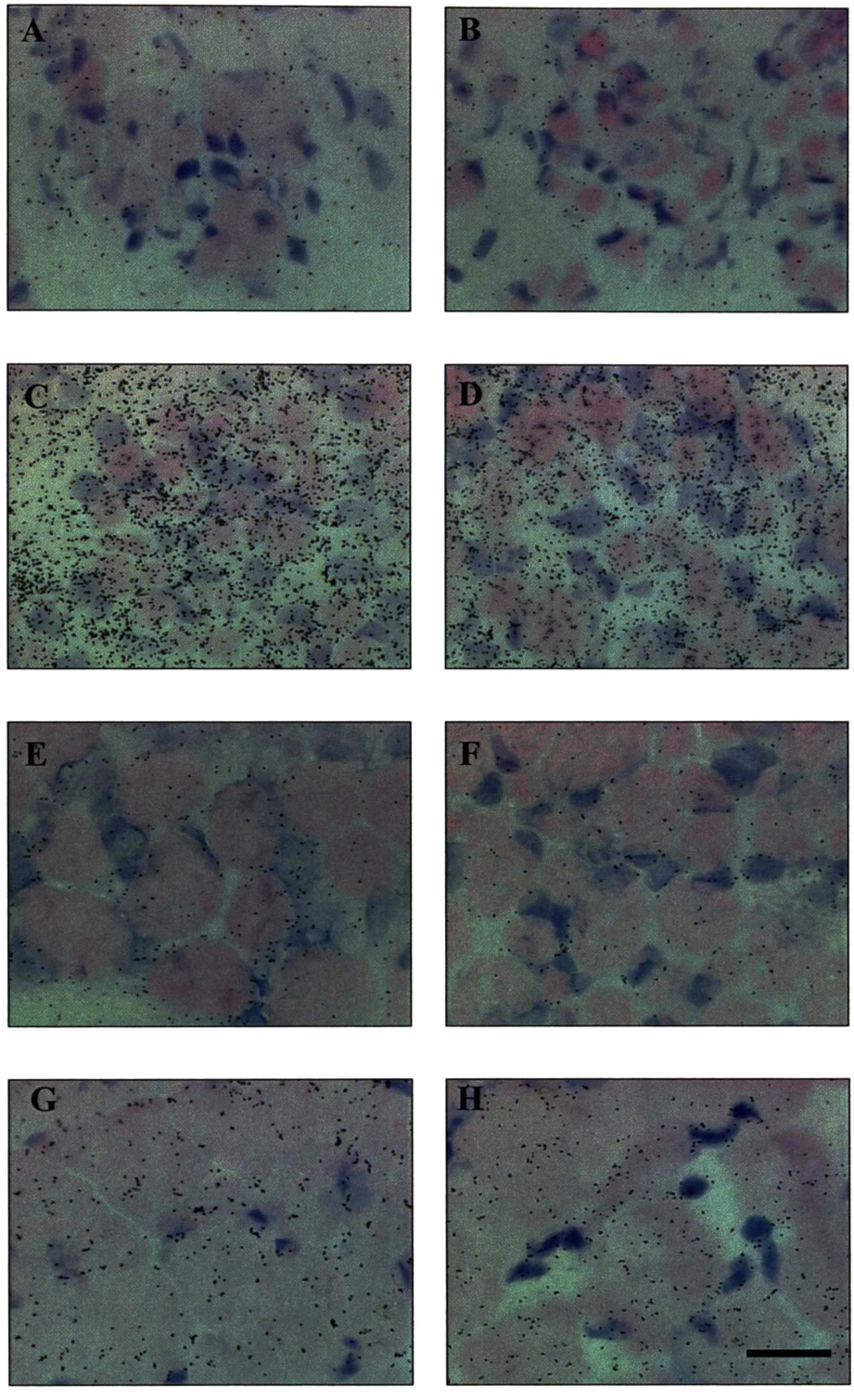


Plate 5.5 Radiographic binding of IGF-2.



5.4 DISCUSSION

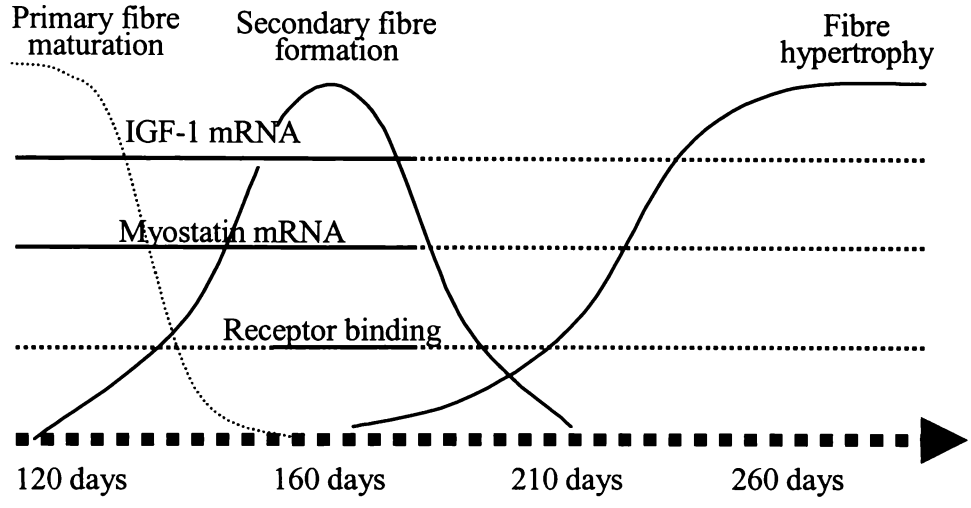
This chapter investigated the IGF axis and myostatin expression in developing muscle of normal and double muscled foetuses to elucidate possible roles for these growth factors in mediating differences in fibre type and fibre size between these foetuses. This was undertaken by describing the ontogeny of (i) IGF levels in the circulation, (ii) IGF-1 and myostatin mRNA expression and (iii) IGF receptor binding in skeletal muscle of normal muscled animals and of double-muscled foetuses carrying a mutation in the gene for myostatin.

The IGF axis has been relatively well characterized with respect to muscle development and a number of studies have identified roles for components of the IGF axis in the proliferation and differentiation of myoblasts and satellite cells (De Chiara *et al.*, 1990; Florini *et al.*, 1991a; Liu *et al.*, 1993). Before the identification of the myostatin mutation in DM animals, the IGFs were postulated to have a major role in the determination of the DM condition (Gerrard and Grant, 1994; Quinn *et al.*, 1990). A number of studies describing IGFs during normal bovine development have also been carried out (Boge *et al.*, 1995; Greene and Allen, 1991).

5.4.1 Growth factors in muscle

In NM foetuses the pattern of mRNA expression for IGF-1 and myostatin were very similar. For both of these genes, expression was highest from 120 to 160 days gestation and lower from 210 to 260 days. In DM animals IGF-1 declined more rapidly than in NM and myostatin declined more slowly. *In vitro* a high mitogen environment is associated with myoblast proliferation and low mitogen conditions promote differentiation (Florini and Magri, 1989; Florini *et al.*, 1996), so it is of interest to consider the stages of myogenesis which coincide with changes in levels of growth factor expression.

Figure 5.6 Summary of key changes in growth factor activity relative to stages of myogenic development in normal bovine muscle.



5.4.1.1 IGF-1

Plasma levels of IGF-1 increased with gestational age. This result is similar to previously reported data showing an increase in IGF-1 during bovine foetal development, which was strongly correlated with increasing foetal body weight (Holland *et al.*, 1997), leading to the conclusion that IGF-1 is related to foetal growth in cattle (Holland *et al.*, 1997). In the newborn calf there is also a correlation between circulating IGF-1 levels and birthweight (Breier *et al.*, 1988).

There was no temporal link between circulating levels of IGF-1 and specific stages of myogenic development. This suggests that it is the autocrine/paracrine action of IGF-1 rather than an endocrine role that is most important in the regulation of muscle development. This conclusion is supported by recent reports that a 75% reduction in circulating IGF-1 levels, as a result of tissue-specific deletion of the *igf1* gene in the liver, had no effect on postnatal body growth in mice (Sjögren *et al.*, 1999; Yakar *et al.*, 1999).

IGF-1 mRNA expression in muscle declines with increasing gestational age. This evidence, in conjunction with the increasing levels of circulating IGF-1, supports a shift in the balance of the endocrine and the paracrine/autocrine roles of IGF-1 during bovine muscle development. During the early stages of gestation examined in this study, covering the period of secondary myofibre formation, the autocrine/paracrine mode of action appears to dominate. This is consistent with the role of IGFs in regulating both the proliferation and differentiation of muscle cells. Later in development when the increase in muscle growth is associated with hypertrophy of muscle fibres, there appears to be a greater role for circulating levels of IGF-1.

IGF-1 mediates changes in skeletal muscle fibre type in a number of animal models. These changes include an increase in the proportion of type 1 fibres after hypophysectomy or over-expression of IGF-1 (Ayling *et al.*, 1989; Coleman *et al.*, 1995). There was no effect on fibre type in young adult mice and a decrease in the age related change in proportions of type 2b fibres in ageing mice after viral mediated expression of IGF-1 (Barton-Davis *et al.*, 1998). *In vitro*, transfection of a muscle cell line with IGF-1 induced an up-regulation of glycolytic enzyme activity, consistent with a change in myotube phenotype (Semsarian *et al.*, 1999a; Semsarian *et al.*, 1999b). In view of these variable effects of IGF-1 on fibre type, it was of interest to consider the possible role of the IGFs in mediating fibre type changes during bovine muscle development, presented in Chapter 4. In this study, the highest levels of IGF-1 (at 120 days gestation) are associated with the highest proportions of type 1 fibres. Levels of IGF-1 remain high however, while the proportions of slow fibres decreases between 120 and 160 days. The later period of gestation, from 210 to 260 days, when IGF-1 expression is at its lowest, coincides with a transformation in fibre type when a number of fibres appear to be switching from type 2 to type 1 fibres, described in Chapter 4. In a postnatal study of induced skeletal muscle hypertrophy, isoform switching from fast to slow MHC was associated with an up-regulation of IGF-1 expression (McKoy *et al.*, 1999; Yang *et al.*, 1996b; Yang *et al.*, 1997). In this current study, using *in situ* hybridisation, no difference in IGF-1

expression was detectable in any particular subset of fibres. It is possible that the mechanisms that regulate both MHC isoform switching and myotube hypertrophy during development may be different from that which operates in postnatal animals.

Comparing IGF-1 mRNA expression in DM with NM revealed that expression levels were lower in DM than NM. This difference was greatest at 260 days gestation. The decrease in levels of IGF-1 at this stage of development of the DM foetuses is temporally linked with the reduced fibre size of type 1 fibres in DM muscles at 210 and 260 days. The overall decrease in IGF-1 levels may also contribute to the reduced size of type 2 fibres throughout gestation in DM. This possibility is supported by a strong body of evidence showing a role for IGF-1 in inducing muscle fibre hypertrophy (Adams and McCue, 1998; Adams and Haddad, 1996; Coleman *et al.*, 1995; Semsarian *et al.*, 1999a; Vandenburgh *et al.*, 1991).

5.4.1.2 IGF-2

Levels of plasma IGF-2 did not change throughout the period of gestation covered in this study. At 120 days gestation levels of IGF-2 were lower in DM than NM. Previously reported results from normal bovine foetuses did show an increase in IGF-2 with gestational age (Holland *et al.*, 1997). In another study comparing circulating IGF-2 levels in DM and NM animals, there was also an increase with gestational age but no difference between breeds (Listrat *et al.*, 1999). Both of these studies covered a longer period of gestation than described here and this is likely to be the reason that the age effect was not significant in this experiment. The difference in plasma IGF-2 seen at 120 days between NM and DM may indicate that DM animals are delayed in reaching maximal circulating levels of IGF-2. There was a delay in reaching maximal IGF-2 mRNA expression in DM muscle relative to NM (Gerrard and Grant, 1994) and this was thought to contribute to the overall delay in muscle differentiation seen in DM animals (Robelin *et al.*, 1993). This delay in differentiation was proposed as a possible mechanism for the increase in fibre

numbers in DM, as the period of myoblast proliferation is extended, resulting in more myoblasts and therefore more muscle fibres (Gerrard and Grant, 1994).

Type 2 IGF receptor binding was lower in DM than NM at 120 days and was noticeably higher in both DM and NM at 160 days gestation. Both IGF-1 (Boge *et al.*, 1995) and IGF-2 (Pfuender *et al.*, 1995) receptor abundance is higher in bovine muscle during mid gestation relative to late gestation, consistent with results reported here. It has been previously shown by Northern analysis that the peak in IGF-2 mRNA expression which occurs at 150 days gestation in normal bovine muscle, is delayed until 170 days in DM (Gerrard and Grant, 1994). Other studies reported no differences in IGF-2 expression in muscle between DM and NM at any developmental age, although there were distinct genotypic differences in the developmental regulation of the multiple IGF-2 transcripts found (Listrat *et al.*, 1999). *In vitro,* the coordinated up-regulation of IGF-2 and its receptor during differentiation of muscle cells has lead to the suggestion that IGF-2 plays an autocrine role in the regulation of myoblast differentiation (Tollefsen, 1989). The results presented in this chapter showing increased type 2 IGF receptor binding, temporally linked with the previously reported increase in IGF-2 mRNA, provides evidence in support of a similar role for IGF-2 *in vivo* during bovine development.

5.4.1.3 Myostatin

This study has shown relatively high levels of myostatin expression in NM during the period of myogenic development covering maturation of primary myofibres and the formation of secondary myofibres (see Figure 5.6). In porcine muscle development, myostatin expression is increased in association with peak periods of primary and secondary myofibre formation (Ji *et al.*, 1998) and decreased at birth, when myogenic activity, differentiation and fusion are reduced. In the myostatin knockout mouse (McPherron *et al.*, 1997) and in DM cattle (Boccard, 1981), there is an increase in myoblast proliferation, resulting in more muscle fibres in the mature animal. Myostatin contains an amino acid signal sequence which signals that it is a secreted protein (McPherron *et al.*, 1997) and it is produced in muscle cells. On that

basis, it has been proposed that myostatin may have an autocrine or paracrine role in the regulation of myoblast proliferation or differentiation (Kambadur *et al.*, 1997).

The ontogeny of myostatin expression as reported in this study of the bovine M. *vastus lateralis,* shows high levels of expression concomitant with peak secondary fibre formation. This suggests that myostatin may have a role in the formation of secondary myofibres, as has been suggested during porcine muscle development (Ji *et al.*, 1998). A previous study in this laboratory, examining early bovine myogenesis in M. *semitendinosus*, showed myostatin gene expression levels peaked at 90 days gestation suggesting a role in the differentiation of early myoblasts and in primary myofibre formation (Oldham *et al.*, 1998). This study also identified a possible downstream target gene, MyoD, through which myostatin may regulate these stages of myogenesis (Oldham *et al.*, 1998).

Concomitant with the myotube maturation and formation, muscle fibre type proportions also change between 120 and 160 days gestation (see Chapter 4). The decreased proportions of type 1 fibres in DM muscle relative to NM are unlikely to be a direct result of the myostatin mutation alone, but there may be a downstream effect of the mutation on the transcriptional regulation of the MHC genes.

In the DM animals in this study, levels of expression of the mutant myostatin mRNA are higher than in the NM with the largest difference occurring at 210 days gestation. A similar result has been previously reported from this laboratory in the M. *semitendinosus* (Oldham *et al.*, 1998), where myostatin expression was elevated throughout gestation. As a consequence of the mutation in the myostatin gene in the DM, the myostatin mRNA codes for a protein that is not translated. It was therefore proposed (Oldham *et al.*, 1998) that a possible feedback inhibition mechanism may operate in the regulation of myostatin expression in normal animals, with elevated levels of myostatin in the circulation inducing a down-regulation of mRNA production. This mechanism could not operate in the DM animals due to the

absence of biologically active myostatin in these animals, so therefore myostatin mRNA abundance would remain elevated.

No obvious associations were identified in this study between myostatin expression and alterations in fibre size between DM and NM muscles. In the myostatin knockout mouse, fibre area is increased (McPherron *et al.*, 1997), while in DM animals a range of differences in fibre type have been reported. The role of myostatin in regulating fibre size remains unknown at this time.

5.4.2 Conclusions from this study

This chapter describes an investigation of the IGF axis and myostatin expression in developing muscle of normal and double muscled animals in order to elucidate possible roles for these growth factors in mediating differences in fibre type and fibre size between these animals. This study has shown ontogenetic changes in the IGF axis and in myostatin expression during normal bovine muscle development. It has also shown some differences in growth factor gene expression between muscle from normal animals and those with a mutation in the gene for myostatin. In considering these results, in conjunction with results from Chapter 4 describing changes in fibre type and fibre size in NM and DM animals, it can be concluded that:

(i) IGF-1 shifts from a predominantly autocrine/paracrine to a predominantly endocrine mode of action during foetal bovine muscle development,

(ii) IGF-1 may play a role in the regulation of fibre size during bovine development, but unlike the situation in postnatal animals, the regulation of fibre type appears to be independent of IGF-1,

(iii) IGF-2 may play a role in the differentiation of late myoblasts and the formation of secondary myofibres through a co-ordinated up-regulation of type 2 receptors and IGF-2 mRNA and

(iv) myostatin may be involved in the differentiation of late myoblasts and the formation of secondary myofibres, but its role in the regulation of muscle fibre type and muscle fibre size remains unknown.

CHAPTER SIX

INDUCED SKELETAL MUSCLE HYPERTROPHY AND ATROPHY IN RABBITS

6.1 INTRODUCTION

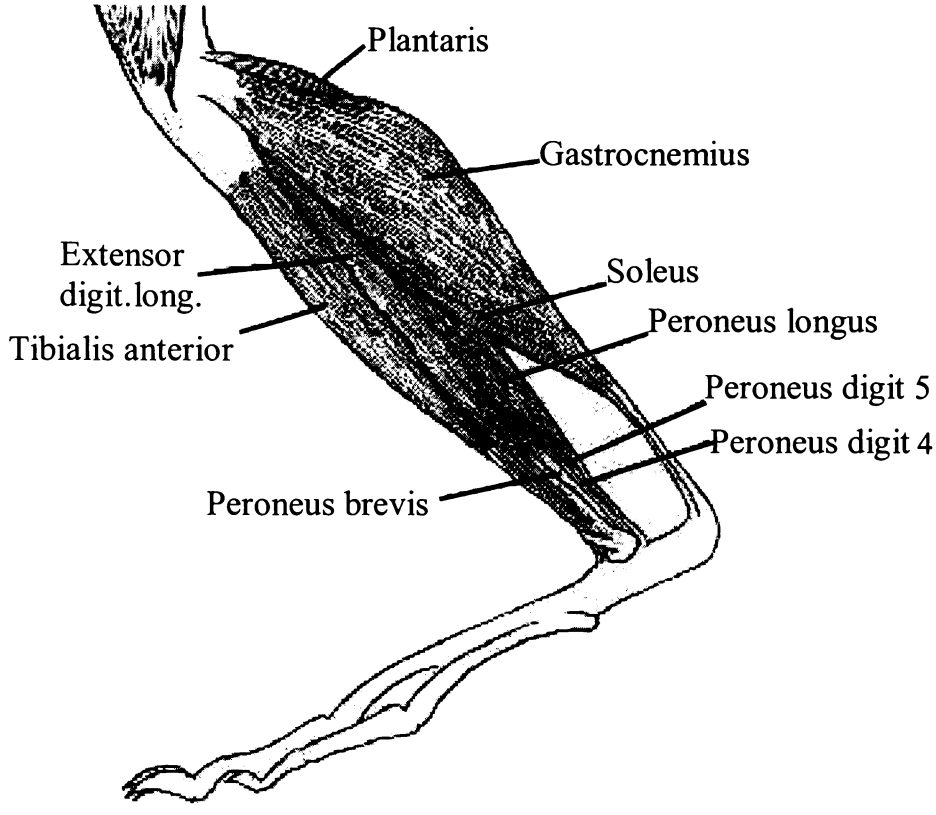
Skeletal muscle has the ability to adapt rapidly to changes in levels of activity. These adaptive responses may take the form of changes in muscle mass and muscle fibre type and they may be mediated by alterations in growth factor activity. Muscle mass can be manipulated using a number of experimental models as were reviewed in Chapter 1. The aim of this study was to investigate the role of growth factors in regulating skeletal muscle mass and fibre type, using a model of experimentally induced hypertrophy and atrophy in rabbits.

6.1.1 Anatomy of the rabbit hindlimb musculature

The lower hind limb of the rabbit consists of two functional groups of muscles that work in a synergistic/antagonistic relationship to each other. The group of muscles involved in flexing the foot upwards consists of M. *tibialis anterior*, M. *extensor digitorum longus* and M. *peroneus*. The group of muscles which are antagonist to the action of these muscles, are those responsible for extension of the foot, ie "pointing the toes". The muscles that make up this group are M. *plantaris*, M. *soleus* and M. *gastrocnemius*. These muscle groups are shown in Figure 6.1 in the rat, where the same functional groups exist as are found in the rabbit. The relationship of these muscle groups is such that in plantar-flexion, ie "toes pointed", the M. *tibialis anterior*, M. *extensor digitorum longus* and M. *peroneus* are stretched and the M.

plantaris, M. *soleus* and M. *gastrocnemius* are in a disuse position. In dorsi-flexion, the muscles are held in the opposite position.

Figure 6.1 Anatomy of the distal hindlimb of a rat (Chiasson, 1988)



6.1.2 Induced skeletal muscle hypertrophy and atrophy

Passive stretch, which is the application of tension without altering innervation status (Vandenburgh, 1987), is one of the most potent methods for the induction of increased skeletal muscle mass. Immobilization of muscles in a lengthened position is a form of passive stretch that in many ways recapitulates the process of muscle development that occurs during embryonic and neonatal growth. Muscle fibres are lengthened in response to stretch by the accumulation of newly formed myotubes at the myotendinous junction (MTJ) (Dix and Eisenberg, 1990). Fibre lengthening occurs concomitant with an accumulation of mRNA at the MTJ and the assembly of

new sarcomeric units at the ends of the muscle fibres (Dix and Eisenberg, 1990; Williams and Goldspink, 1971; Williams and Goldspink, 1973). In addition to the increase in muscle mass there is also a transformation towards more slow twitch fibres (Goldspink *et al.*, 1991; Gregory *et al.*, 1986; Matthews *et al.*, 1990) and in some reports, an increase in fibre number due to fibre splitting (Alway *et al.*, 1990). Protein synthesis and protein degradation rates are both increased (Laurent and Millward, 1980; Loughna *et al.*, 1986), resulting in some wasting of protein. The induction of skeletal muscle hypertrophy is dependent upon satellite cell activation as was demonstrated in a study in which muscle was irradiated prior to application of the stretch stimulus. This resulted in inactivation of the satellite cells and no hypertrophy took place (Phelan and Gonyea, 1997).

The adaptive response that is opposite to skeletal muscle hypertrophy, is atrophy or muscle wasting. As was reviewed in Chapter 1, atrophy is characterised by a decrease in muscle weight, a reduction in fibre cross-sectional area and a switch in fibre type towards more fast myosin heavy chain (MHC) (Allen *et al.*, 1996; Hikida *et al.*, 1997). Protein synthesis is decreased and protein degradation is increased in muscles undergoing atrophy (Booth and Kirby, 1992; Thomason *et al.*, 1989). There is also a reduction in the number and proliferative activity of satellite cells in muscles after hindlimb suspension (Darr and Schultz, 1989). One mechanism through which the number of myonuclei is suggested to be reduced in muscles undergoing atrophy is through increased apoptosis (Allen *et al.*, 1997).

Apoptosis is a process of programmed cell death which is involved in the remodelling of tissues both during development (Jeffs and Osmond, 1992) and postnatally in conditions such as mammary gland involution (Atwood *et al.*, 1995) and atresia of ovarian follicles (Chun *et al.*, 1996). The role of growth factors and hormones in both preventing and inducing apoptosis has been recently reviewed (Keiss and Gallaher, 1998).

6.1.2 Myostatin in muscle growth

Growth factors are involved in the regulation of many aspects of skeletal muscle growth both during development and postnatally, as was reviewed in Chapter 1. The transforming growth factor (TGF) super-family, most notably TGF-β, was included in that review, with respect to the role of this family of growth factors in the proliferation and differentiation of muscle cells (Allen and Boxhorn, 1987; Florini *et al.*, 1986). Myostatin is a recently identified member of the TGF-β super-family, which has been shown to negatively regulate muscle mass during development. This has been shown in both myostatin knockout mice (McPherron *et al.*, 1997) and in the double muscled cattle breeds (Kambadur *et al.*, 1997).

Myostatin mRNA expression is increased in muscle of low birth-weight piglets (Ji *et al.*, 1998) and in skeletal muscle undergoing atrophy in response to hindlimb suspension (Carlson *et al.*, 1998) and space flight (Shivji *et al.*, 1999). Serum and intramuscular levels of myostatin-related protein are increased in HIV-infected men with muscle-wasting (Gonzalez-Cadavid *et al.*, 1998). Although these reports describe myostatin expression in postnatal animals, its function in adult muscle remains unknown.

6.1.3 Aims of this chapter

This chapter tests the hypothesis that myostatin is involved in regulating changes in skeletal muscle mass in adult animals during induced hypertrophy and atrophy. This study also undertook to examine the abundance of myostatin mRNA and protein at different anatomical sites within hypertrophied muscles in order to identify possible mechanisms of action for myostatin in regulating skeletal muscle mass and fibre type.

6.2 MATERIALS AND METHODS

6.2.1 Animal data

The casting of the rabbit limbs was described in Chapter 2.2.3. Briefly, eight rabbits were randomly assigned to either a 2 day treatment group (n=5) or a 6 day treatment group (n=3). Rabbits were anaesthetized using a mixture of ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (5 mg/kg), administered intramuscularly. Hind limbs were cast in a fully plantar-flexed position using Dynacast Pro conformable fibreglass casting tape (Smith & Nephew, UK). The contralateral limb served as a within-animal control. The validity of using the muscles of the contralateral limbs as controls in this experimental model has been previously reported (Yang *et al.*, 1997). At the end of the treatment periods rabbits were euthanased with an overdose of sodium pentobarbitone. Casts were removed and the M. *tibialis anterior* (TA) and M. *plantaris* muscles were dissected free and cleaned of excess connective tissue. Muscle lengths were taken with muscles at their resting length using calipers and weights of muscles were recorded. This study was carried out with the approval of the Ruakura Animal Ethics Committee.

6.2.2 RNA analyses

6.2.2.1 RNA extraction

Total RNA was extracted from proximal, middle and distal sites of the M. *tibialis anterior* (n = 3 for each time period) according to manufacturers instructions for the use of TRIzol® (Gibco BRL). The M. *plantaris* muscle was cut into small pieces and snap frozen in isopentane cooled with liquid nitrogen. RNA was prepared from frozen tissue using TRIzol®.

6.2.2.2 Northern Blot Analysis

Northern Blot analysis was carried out on RNA samples extracted from M. *tibialis anterior* and M. *plantaris*. RNA samples were analysed from treated and control

limbs of three animals at each time period. X-AR films were scanned and optical density of bands was analysed as described for RT-PCR. Optical density values were statistically analysed as ratios of control to treated values to eliminate between animal variation and were log transformed. A one-way analysis of variance was carried out with treatment as the main effect. Values are means of OD ± sem (n=3).

6.2.2.3 RT-PCR

First strand cDNA synthesis was carried out using Superscript pre-amplification kit, according to the manufacturer's instructions (Gibco BRL). PCR amplification of myostatin cDNA was carried out according to a previously described protocol (Kambadur *et al.*, 1997). Ubiquitin activating enzyme (uae) mRNA was amplified from the same cDNA as a control. PCR products were electrophoresed on an agarose gel and transferred to Hybond N+ nylon membrane (Amersham Pharmacia Biotech). After overnight hybridization at 42°C with ^{32}P random prime labelled myostatin or uae cDNA probes, the membranes were washed and exposed against X-AR film (Kodak). X-AR films were scanned using a Bio-Rad Personal Densitometer (Bio-Rad, Hercules, CA, USA). Optical density of bands was measured using Bio-Rad Multi-Analyst software.

6.2.3 Immunohistochemistry

6.2.3.1 Myostatin

Pairs of 8 μm sections of M. *tibialis anterior* muscle were mounted on polysine slides (Esco). Slides were dewaxed and rehydrated in Tris buffered saline (TBS), treated with 1% hydrogen peroxide for 10 minutes and incubated in blocking solution (10% donkey serum in TBS), for 1 hour at room temperature. Blocking solution was tapped off and replaced with myostatin antibody at 1:250 dilution in blocking solution. The method of antibody production and its specificity for myostatin has been previously described (Sharma *et al.*, 1999). Details of the incubation procedure are given in Section 2.4.4.

6.2.3.2 Myosin heavy chain isoforms (MHCs)

For correlation of immunohistochemical localization of myostatin with MHC isoforms, serial sections were incubated with antibodies specific for fast MHC (MY32, Sigma Chemical Company) and slow MHC (1A, Chemicon) as described in Section 2.4.4.

6.2.4 Histochemical fibre typing for mATPase activity

Frozen sections were cut at 8 μm thickness from blocks of tissue taken from the midbelly of the TA. Sections were thaw mounted onto uncoated slides and used within one week of cutting. Slides were fixed according to (Guth and Samaha, 1969) and pre-incubated in 0.1M potassium acetate buffer at pH 5.0 for 10 minutes. The remainder of the procedure was also carried out according the protocol described in Section 2.4.6. This staining procedure identifies three distinct fibre types: type 2d/x which has been shown to be the predominant fast MHC isoform in the rabbit TA muscle (Aigner *et al.*, 1993; Hämäläinen and Pette, 1993) are the darkest staining, type 2a are of intermediate intensity and type 1 fibres are the lightest.

6.3 RESULTS

6.3.1 Muscle data

Muscle weights were taken to show the extent of muscle hypertrophy or atrophy following immobilisation of the hindlimb in a plantar-flexed position. The M. *tibialis anterior* muscles underwent significant hypertrophic growth in response to the stretch treatment, as evidenced by the overall 31% increase in muscle weights ($p \leq 0.001$), (Figure 6.2, A). There was no significant interaction between treatment and time as the magnitude of the weight increase was similar after both two and six days of stretch (26% and 34% respectively). The weight of the shortened M. *plantaris* muscle decreased by 7.8% overall, ($p \leq 0.001$). There was a 4.8% loss in weight relative to controls after 2 days and a larger 12.6% loss after 6 days, resulting

in a significant interaction between treatment and time ($p=0.004$) (Figure 6.2, B). Muscle lengths were recorded to determine whether changes in muscle mass were associated with changes in the length of muscle fibres (Figure 6.3). The stretched TA muscle lengths increased by 12.1% overall ($p=0.003$). The size of the effect at the individual time points was not significantly different (12% after 2 days and 14% after 6 days) (Figure 6.3, A). The atrophied plantaris showed no significant change in muscle length after either 2 or 6 days of treatment (Figure 6.3, B).

Figure 6.2 Muscle weights of (A) M. *tibialis anterior* and (B) M. *plantaris* after 2 and 6 days of immobilisation. Black bars are control muscles, grey bars are treated muscles. Values are means ± sem (n=3-5). *** = p≤0.001, ** = p≤0.01.

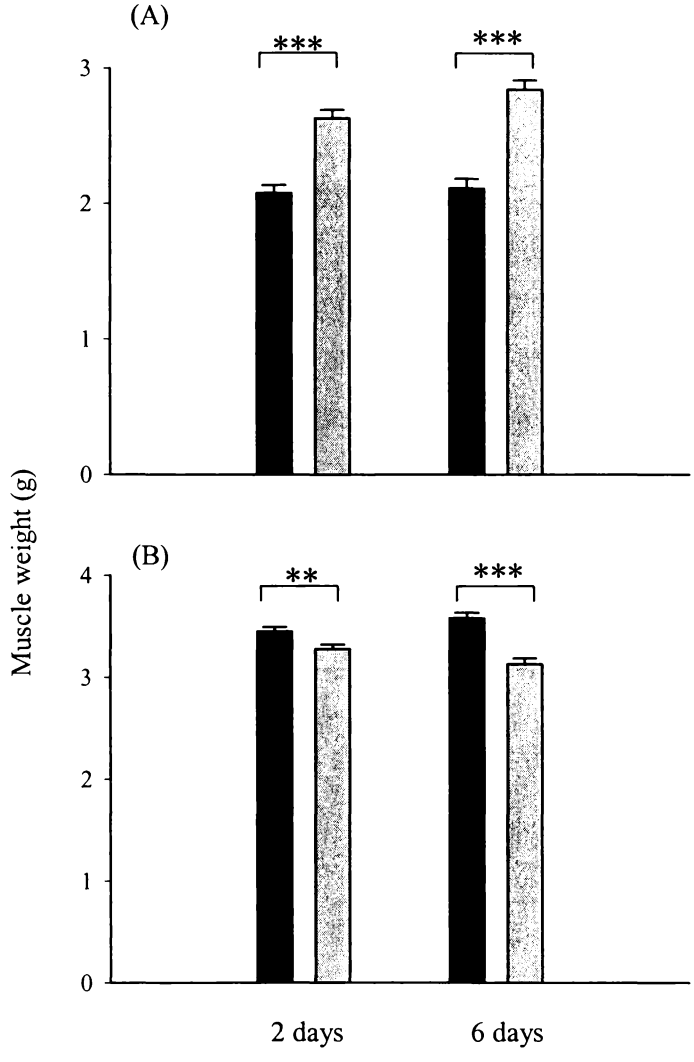
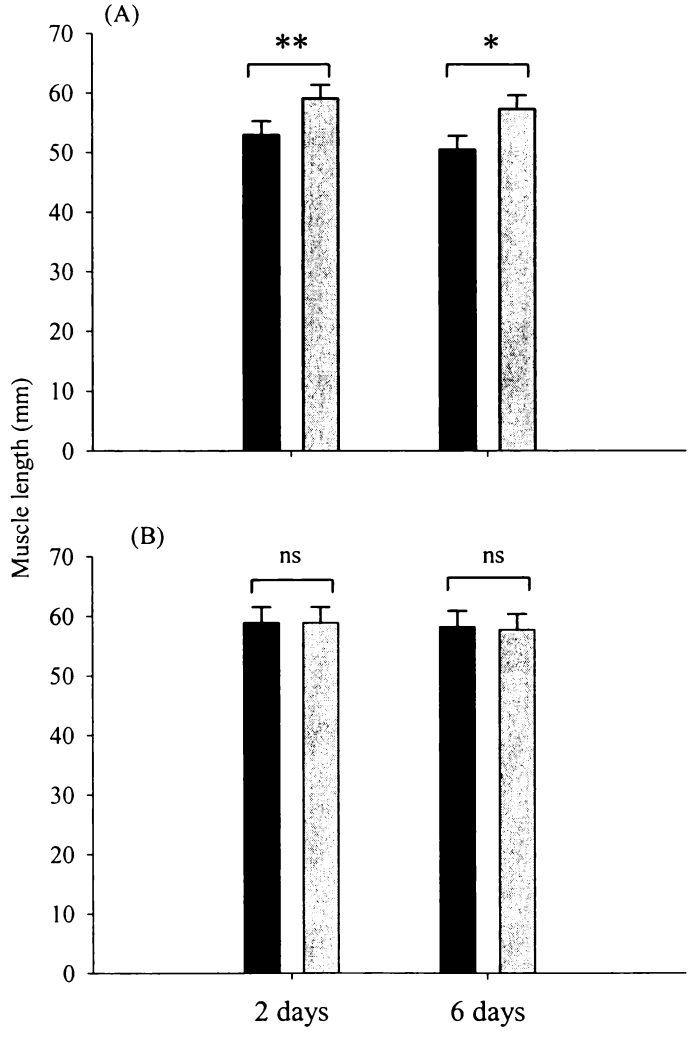


Figure 6.3 Muscle lengths of (A) M. *tibialis anterior* and (B) M. *plantaris* after 2 and 6 days of immobilisation. Black bars are control muscles, grey bars are treated muscles. Values are means ± sem (n=3-5). ** = p≤0.01, * = p≤0.05, ns = not significant.



6.3.2 Myostatin mRNA expression

To determine whether myostatin mRNA was associated with the regulation of stretch-induced skeletal muscle hypertrophy and disuse atrophy, mRNA expression levels were measured using Northern blot analysis and semi-quantitative RT-PCR.

Figure 6.4 Myostatin mRNA Northern blot XARs. Bands from control (C) and treated (T) muscles are shown for three animals except for 2 day M. tibialis anterior (2 animals). Ethidium bromide stained gel shows RNA loading.

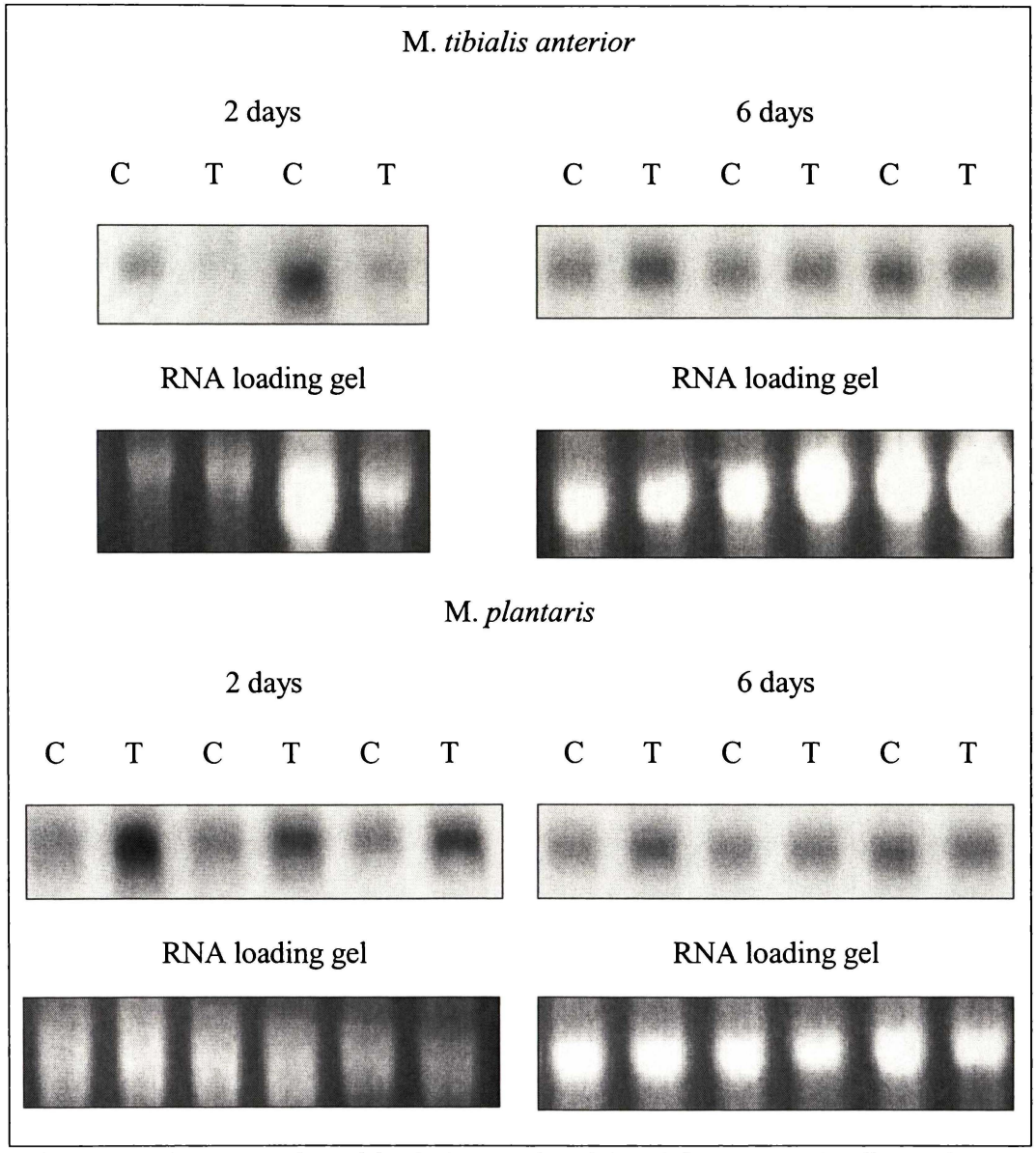
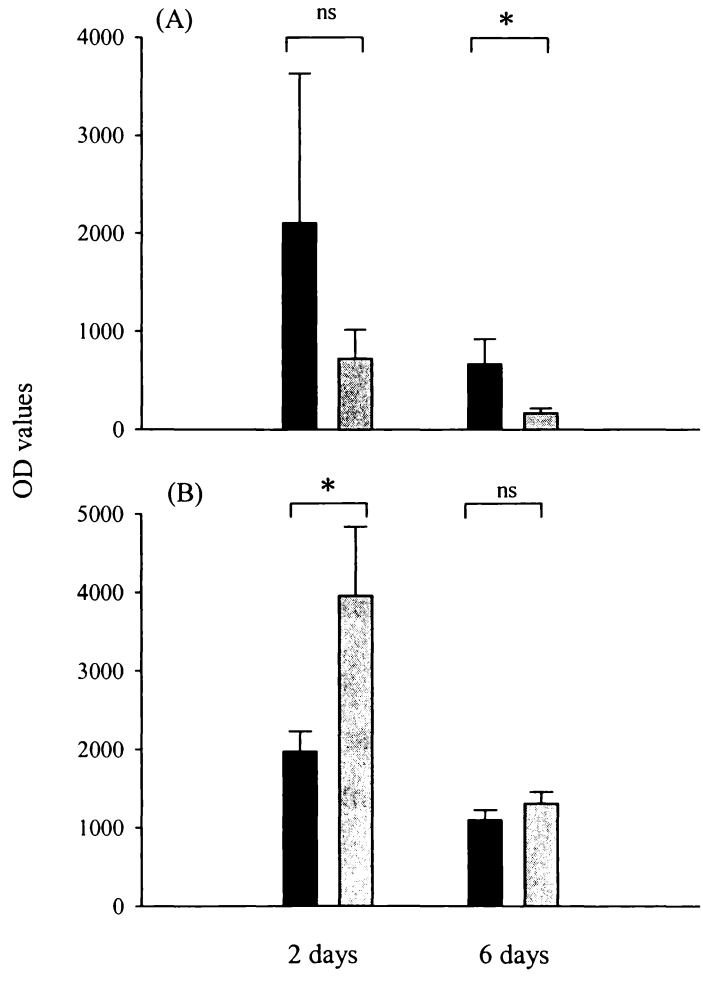


Figure 6.4 shows Northern blot XARs and 18S band from RNA loading gels. RNA from one of the 2 day TA samples was degraded so only 2 animals were included for

this group. One control lane also had very high RNA loading in this group. Figure 6.4 shows results of quantitative analysis after scanning of XARs. In the hypertrophied muscle, there was no significant difference after 2 days of treatment and there was a decrease in myostatin mRNA levels in stretched muscles after 6 days of treatment ($p \leq 0.0.05$). In the atrophied M. *plantaris* muscle, there was an increase in myostatin mRNA in the treated muscle after 2 days ($p \leq 0.05$) and no significant difference after 6 days.

Figure 6.5. Myostatin mRNA expression levels in (A) M. *tibialis anterior* and (B) M. *plantaris* by quantitative analysis of Northern blot. Black bars are control muscles, grey bars are treated. Values are means ± sem (n=3). * = $p \leq 0.05$.



RT-PCR analysis was carried out on the same RNA samples that were used for Northern analysis. Figures 6.6 and 6.7 show XAR images of myostatin mRNA expression in treated and control muscles after hypertrophy and atrophy respectively, compared to a housekeeping gene, uae. Results from quantitative analysis after densitometry of XAR images are shown in Figure 6.8.

Figure 6.6 XAR film of Southern hybridisation showing levels of myostatin and uae PCR products in M. *tibialis anterior* after 2 days of immobilisation in a lengthened position. Samples shown are taken from medial portion of muscles, distal and proximal samples not shown.

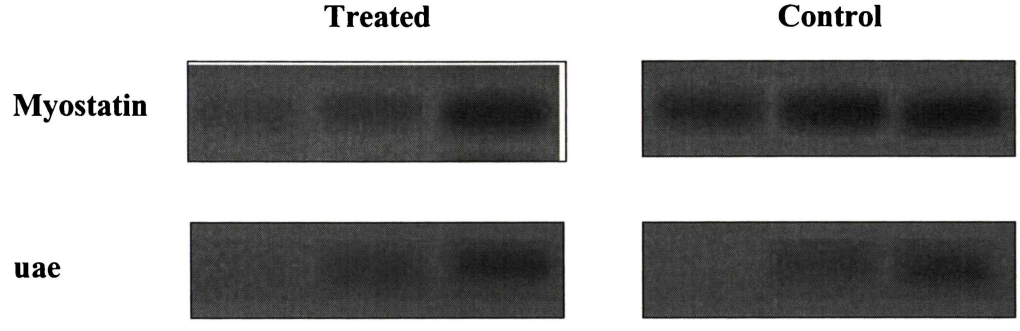
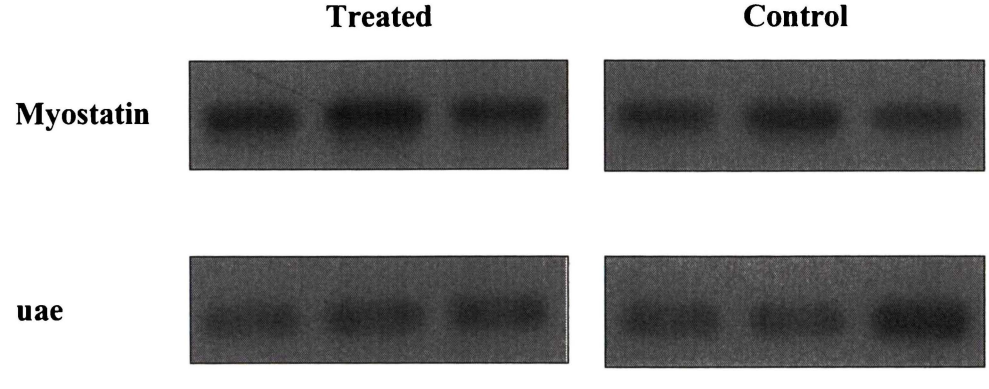


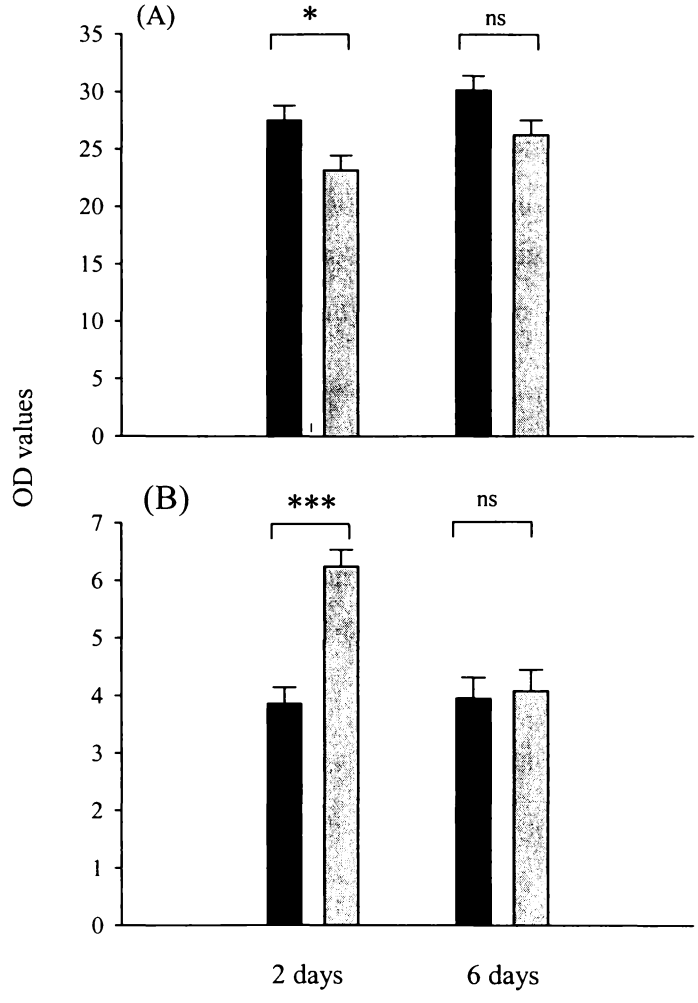
Figure 6.7 XAR film of Southern hybridisation showing levels of myostatin and uae PCR products in M. *plantaris* after 2 days of immobilisation in a shortened position.



Quantitative analysis of myostatin expression levels in the hypertrophied TA muscle showed a 14% reduction by overall main effects analysis ($p \leq 0.05$). After 2 days of stretch treatment, there was a decrease in myostatin mRNA expression in the treated muscle ($p \leq 0.05$) and there was no significant difference after 6 days. There was no difference between the two time periods in the size of the decrease (Figure 6.8, A). The atrophied plantaris muscle had higher levels of myostatin mRNA expression relative to untreated controls by main effects analysis ($p \leq 0.01$). Expression of myostatin mRNA was increased by 62% after 2 days of treatment ($p \leq 0.001$) and was not significantly different after 6 days (Figure 6.8, B).

Results from the two methods of RNA analysis have given very similar results, showing a small but significant decrease in myostatin mRNA after stretch-induced hypertrophy, which may be similar between 2 and 6 days of treatment. In the atrophy muscle, both analyses showed a larger increase in mRNA expression at 2 days, but no difference at 6 days.

Figure 6.8 RT-PCR analysis of myostatin mRNA expression in (A) M. *tibialis anterior* and (B) M. *plantaris*. Black bars are control muscles, grey bars are treated muscles. * = p≤ 0.05, *** = p≤0.001.



6.3.3 Myostatin ICC in hypertrophied muscle

Myostatin protein was present along the length of the muscle fibres of hypertrophied and control muscles. Plantaris was not included in the immunohistochemical analysis because of slow response time of fast twitch muscles to atrophy suggested differences would be difficult to detect within the time frame used. Both hypertrophied and control muscles showed high levels of myostatin immunostaining at the myotendinous junction (Plate 6.1). No obvious differences were found between hypertrophied and control muscles in the abundance or localization of myostatin protein using this qualitative technique. Comparison of myostatin immunostaining with serial sections incubated with an antibody against fast MHC showed myostatin staining to be equally abundant in both fast and slow muscle fibres (Plate 6.2, A, B, C).

Quantitative analysis of fibre type after histochemical staining for mATPase activity showed no significant differences in fibre type composition between treated and control muscles after 2or 6 days of treatment (Figure 6.9 A-C). There is however a trend towards more type 2d/x fibres and fewer type 1 and type 2a fibres.

Plate 6.1

Photomicrograph showing myostatin immunolocalisation to the myotendinous junction (MTJ) region of control (A) and stretched (C) muscle. (B) and (D) show corresponding negative control sections. Arrows indicate MTJ. Bar = 50μm.

Plate 6.1 Myostatin immunostaining at the myotendinous junction.

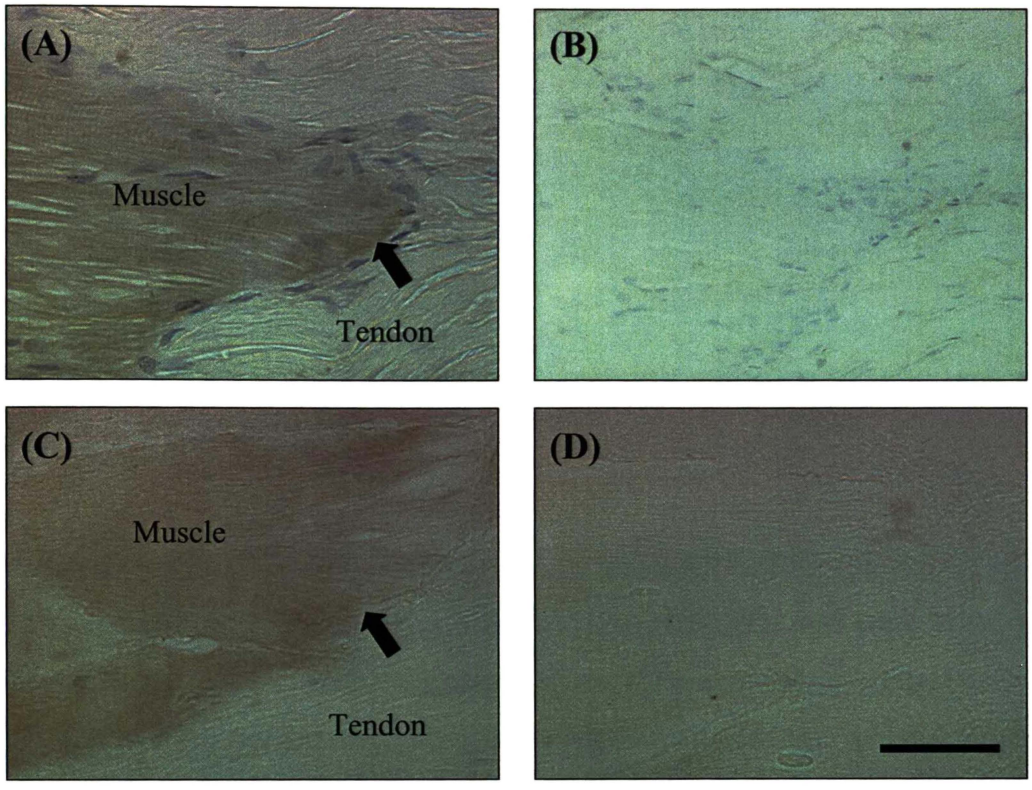


Plate 6.2

Photomicrograph showing myostatin (A), fast MHC (C) and slow MHC (E) immunolocalisation to serial sections of M. *tibialis anterior* from control animal. (B), (D) and (F) are corresponding negative control sections. Arrows indicate type 1/slow MHC positive fibres. Bar = 50μm.

Plate 6.2. Myostatin and myosin heavy chain immunolocalisation.

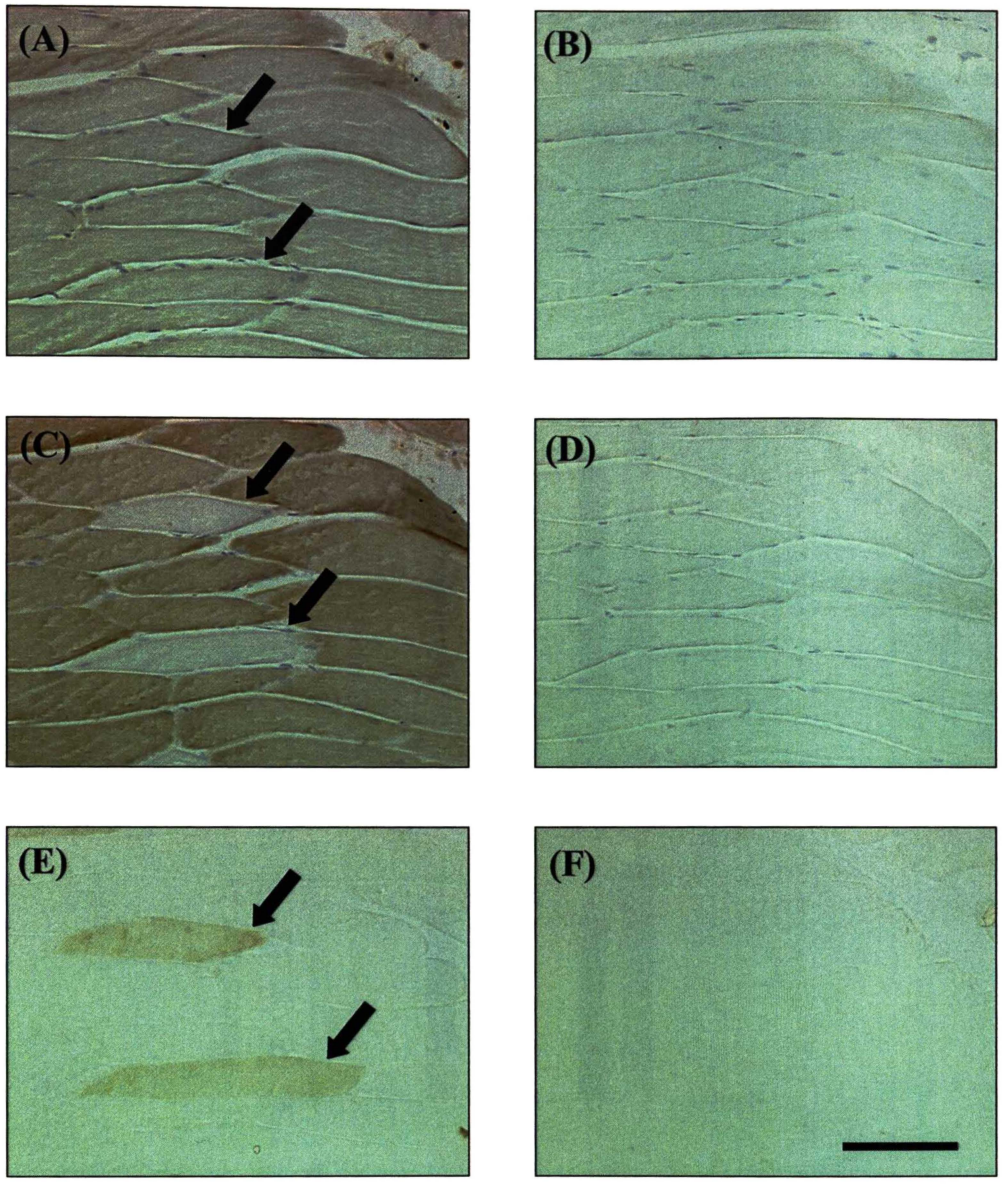
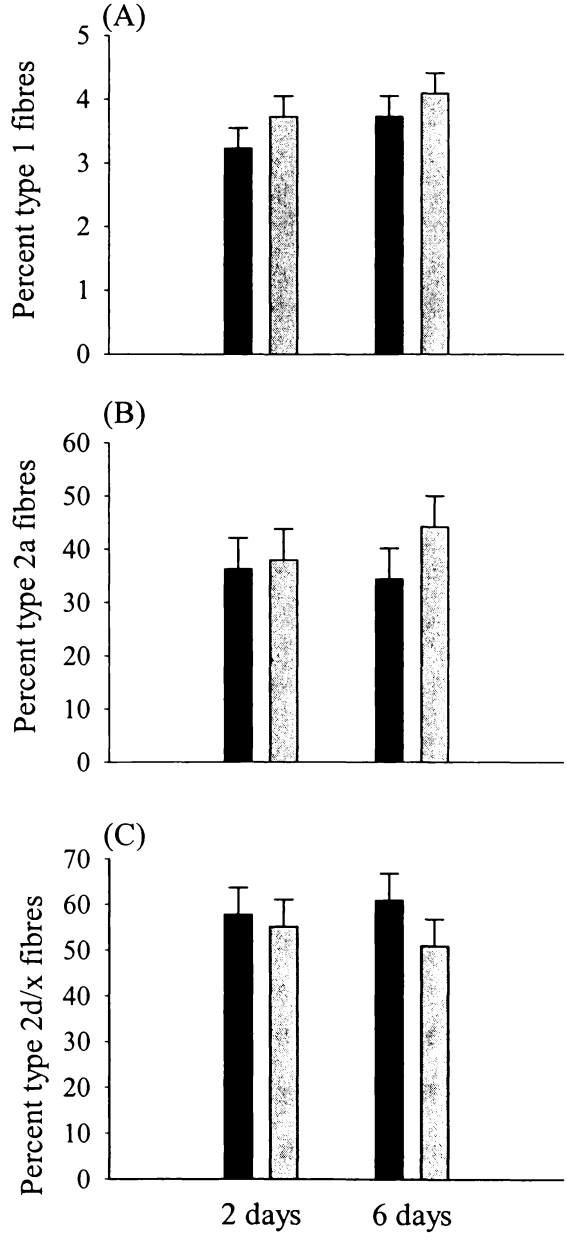
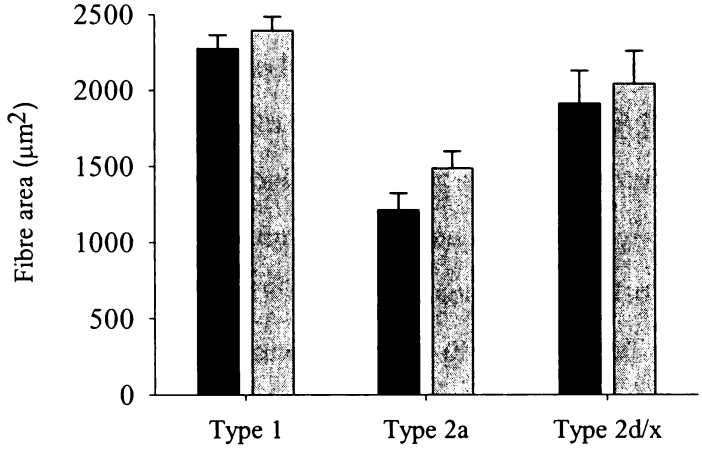


Figure 6.9. Percentage change in fibre type composition for (A) type 1 fibres, (B) type 2a fibres and (C) type 2d/x fibres in M. *tibialis anterior*. Black bars are control muscles, grey bars are treated muscles. All differences were non-significant.



Fibre area measurements were not affected by the stretch treatment in any fibre type, after 2 or 6 days of treatment (Figure 6.10).

Figure 6.10. Average muscle fibre area of M. *tibialis anterior*. Black bars are control muscles, grey bars are treated muscles. Values are means of 2 day and 6 day treatment groups. All differences were non-significant.



6.4 DISCUSSION

This study investigated changes in myostatin expression in response to immobilisation of muscles in lengthened or shortened positions. The results presented in this chapter show that myostatin expression is down-regulated in stretch-induced hypertrophy and up-regulated in disuse atrophy.

6.4.1 Effect of stretch on muscle mass

In this study the stretch treatment induced an increase in mass of the TA muscle but no significant change to muscle fibre type or fibre size. There was no significant difference in weight between 2 and 6 days suggesting that the peak period of hypertrophic growth must have occurred earlier than 2 days. Likewise myostatin mRNA expression levels were not different between 2 and 6 days in the TA. These results suggest that both muscle mass and myostatin expression have levelled out and it is possible that an earlier sampling period may have identified a change in myostatin expression levels in advance of change in muscle weight. Rapid changes in gene expression in response to hypertrophy stimulus are known to occur. Cellular oncogenes are possible candidates for inducing early events in the signalling cascade that leads to muscular hypertrophy and *c-fos* and *c-jun* expression are both up-regulated with hypertrophy within a time frame as short as one hour (Goldspink *et al.*, 1995; Osbaldeston *et al.*, 1995). MRF expression is also altered very rapidly (Jacobs-El *et al.*, 1995) with altered levels of MRF-4 and *myf*-5 mRNA being detected after 2 hours of combined stretch and electrical stimulation.

6.4.2 Effect of disuse on muscle mass

This study has also shown that myostatin mRNA expression is up-regulated in the shortened plantaris, during atrophy. Myostatin mRNA expression then returned to control levels while muscle mass continued to decline. This observation that the maximal change in myostatin mRNA expression precedes the maximal change in muscle mass, suggests that myostatin may play a functional role in inducing a down-regulation of skeletal muscle mass. The rapid increase in myostatin mRNA expression identified in this study occurred in a fast-twitch muscle undergoing disuse atrophy. This study has shown temporal differences in the pattern of change of myostatin expression in two fast twitch muscles undergoing hypertrophy and atrophy. It is well documented that muscles with a predominantly slow-twitch fibre type respond much more rapidly to atrophy (Brownson and Loughna, 1996;

Desplanches *et al.*, 1987; Loughna *et al.*, 1986). However a recent study has found myostatin mRNA to be undetectable in the slow twitch soleus in mice in either control muscles or muscles which had undergone atrophy as a result of hindlimb unloading (Carlson *et al.*, 1999).

6.4.3 Myostatin and changes in muscle mass

The mechanism by which myostatin regulates muscle mass is at present unknown. The down-regulation of myostatin expression in response to induced muscle hypertrophy in adult animals described in this study has not been previously reported. This result is consistent with the genetic models of deleted myostatin expression, namely the myostatin knockout mouse and the double-muscled cattle models, which are associated with increased muscle mass. A major contributor to the increase in muscle mass in the genetic models is an increase in muscle fibre number, proposed to arise as a result of increased myoblast proliferation (Holmes and Ashmore, 1972; McPherron *et al.*, 1997; Ouhayon and Beaumont, 1968; Swatland and Kieffer, 1974). Increased fibre number is not a feature of postnatal induced hypertrophy in mammalian models and it is therefore suggested that the mechanism of myostatin action in regulating muscle mass in postnatal animals may be different to that occurring in developmental models.

One feature of induced hypertrophy which does suggest a possible pathway in common with the genetic models of altered muscle mass is that there is an increase in satellite cell proliferation (Phelan and Gonyea, 1997; Snow, 1990; Winchester *et al.*, 1991). In the absence of satellite cell proliferation, hypertrophic growth in rat muscle is prevented (Phelan and Gonyea, 1997). Activated satellite cells fuse to form small myofibres at the ends of stretched muscles and these in turn fuse with and lengthen existing fibres (Dix and Eisenberg, 1990). The ends of stretched muscle fibres are also associated with increased MHC mRNA levels (Dix and Eisenberg, 1988; Dix and Eisenberg, 1990). If myostatin was involved in myotube formation and fusion, then its expression might be lower at the proximal and distal

ends of the muscles in stretched muscles where this activity was occurring. However, there were no differences found in myostatin gene expression in samples taken from proximal or distal ends of the muscle or from the muscle mid-belly.

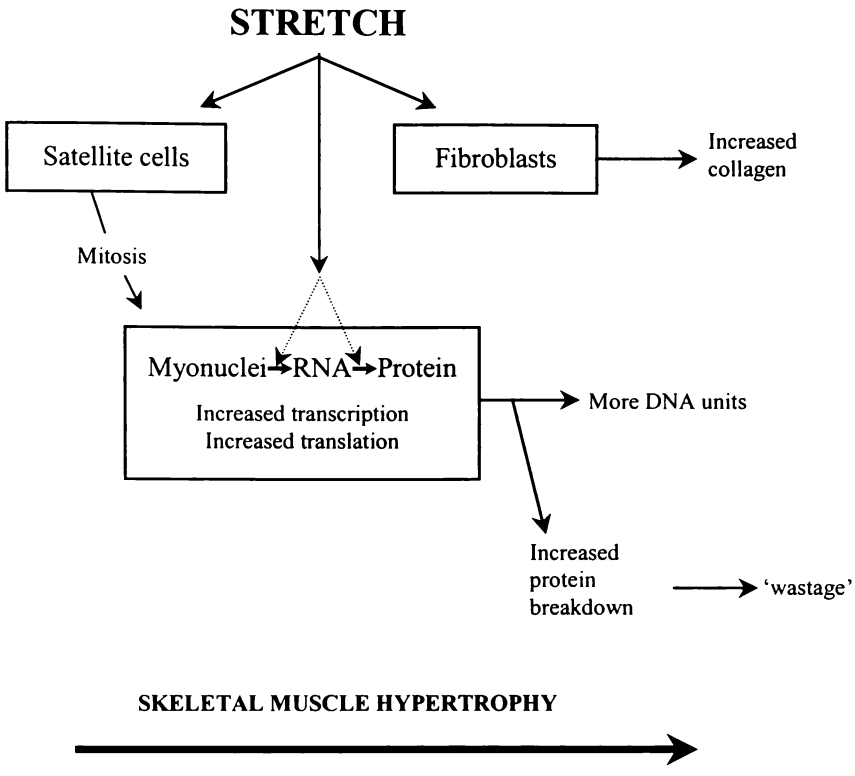
A number of studies have examined the incorporation of radiolabelled nucleotides in stretch-induced hypertrophy. Results from these studies have been inconclusive with respect to reported differences in the accumulation of radioactivity at the proximal and distal ends of muscle fibres. Some studies have reported increased incorporation at the proximal and distal ends of stretched muscles (Goldspink, 1971; Williams and Goldspink, 1973), while others were unable to identify differences in incorporation associated with the ends of muscle fibres (Barnett *et al.*, 1980; Winchester *et al.*, 1991). Data from this current study showing up-regulation of myostatin mRNA at sites from along the muscle length, suggests that those satellite cells that are activated in response to stretch may be recruited from along the length of the muscle fibres and then migrate to the fibre ends. The migration of satellite cells within muscle fibres and across the basal lamina towards sites of regeneration has been previously reported (Hughes and Blau, 1990; Watt *et al.*, 1994). It may be that the down-regulation of myostatin is a prerequisite for the proliferation of the satellite cells. This is consistent with observations from this laboratory that myostatin inhibits proliferation of muscle cells (unpublished data from this laboratory) and that the proliferation of muscle cells is increased in DM animals (Holmes and Ashmore, 1972; Ouhayon and Beaumont, 1968; Swatland and Kieffer, 1974) and in the myostatin knockout animals (McPherron *et al.*, 1997).

The results from this study showing an increase in myostatin expression in muscles undergoing disuse atrophy are supported by those of Carlson et al., (1999; 1998) and Shivji et al. (1999), where myostatin is up-regulated in response to hindlimb unweighting and space flight respectively. Apoptosis has been proposed as a mechanism by which the transcriptional capacity of muscle fibres may be modulated in the hindlimb unweighting model (Allen *et al.*, 1996), thereby maintaining a constant size of DNA unit (Cheek *et al.*, 1971). It is suggested therefore that the up-

regulation of myostatin may be associated with an increase in apoptosis in muscles undergoing remodelling as a result of skeletal muscle atrophy. Mechanisms by which growth factors modulate apoptosis may be related to their ability to influence the entry of a cell into the cell cycle (Keiss and Gallaher, 1998).

The potential roles for myostatin, as discussed in relation to the mediation of changes in skeletal muscle mass, have focussed on alterations in nuclear number via the mechanisms of satellite cell activation and apoptosis. An alternative possibility that should be considered is that myostatin may regulate the synthesis of muscle specific proteins. A constant DNA unit size is maintained in muscles undergoing hypertrophy and atrophy by alterations in the relative rates of protein synthesis and degradation, as well as by modulation of nuclear number as described in the previous section. Figure 6.10 shows key events associated with changes in protein synthesis and degradation in stretch hypertrophy, where both protein synthesis and degradation are increased, resulting in some 'wastage' of protein and possibly DNA (Laurent and Millward, 1980).

Figure 6.11 Diagram showing postulated key events during stretch induced hypertrophy (redrawn from Laurent and Millward, 1980).



Under conditions that induce atrophy, slow and fast twitch muscles have quite different response patterns. Slow twitch postural muscles exhibit a much greater response than fast twitch phasic muscles, but in both muscles, the atrophy is brought about by a small decrease in the fractional rate of protein synthesis and a large increase in the rate of protein degradation (Loughna *et al.*, 1986). The time course of these changes in protein degradation can be very rapid with a decrease shown within six hours of immobilisation (Goldspink, 1977b).

Low levels of myostatin may be associated with increased protein synthesis, as seen in the hypertrophy mode, and high levels of myostatin may be associated with decreased protein synthesis or increased rates of degradation as seen in the atrophy model. Immunohistochemical localisation of myostatin protein has identified high levels of the protein at the myotendinous junction of both stretched and control

muscles, where individual fibres terminate and interdigitate into the muscle tendon. The myotendinous junction is a very complex site where the muscle is anchored to the bone via the tendon in order to transmit force and so effect movement. The abundant myostatin protein at this location may be associated with a signal to terminate the synthesis of structural muscle proteins at this point.

Logical down-stream targets for myostatin in mediating the synthesis of muscle specific proteins are the myogenic regulatory factors (MRFs). MRFs are present in postnatal muscle in a fibre type specific pattern of localisation with MyoD being higher in fast twitch muscles and myogenin being higher in slow twitch muscles (Hughes *et al.*, 1993). Expression of the MRFs is altered in response to stretch and electrical stimulation in a fibre type specific manner (Jacobs-El *et al.*, 1995; Loughna and Brownson, 1996; Mozdziak *et al.*, 1998; Mozdziak *et al.*, 1999).

6.4.4 Myostatin and changes in muscle fibre type

This discussion has so far focussed on the role of myostatin in the regulation of skeletal muscle mass. The second part of this study was concerned with investigating a possible role for myostatin in regulating changes in muscle fibre type. The fibre type changes reported in this study were not significant, probably because of the short time frame. A similar study, also over six days of stretch, reported no significant increase in slow MHC in M. *tibialis anterior* (Yang *et al.*, 1997). In other studies using this model of induced skeletal muscle hypertrophy, there is however strong evidence for an eventual switch towards an increased proportion of slow fibres (Goldspink *et al.*, 1991; Gregory *et al.*, 1986; Matthews *et al.*, 1990). Differences in fibre type proportions have also been demonstrated in other models of muscle hypertrophy. For example, in the Belgian Blue cattle breed, where the increased muscle mass arises due to a mutation in the gene for myostatin, there is a reduction in the percentage of type 1 fibres per fascicle (see Chapter 4). In normal bovine muscle, it has been reported that myostatin mRNA levels are highly variable between muscles, independent of muscle fibre type (Kambadur *et al.*, 1997). In

porcine muscle, myostatin mRNA abundance is greater in white muscle than in red (Ji *et al.*, 1998) and in the mouse, myostatin mRNA concentration is strongly associated with muscles with a high proportion of type 2b fibres (Carlson *et al.*, 1999). The association between myostatin and carbonic anhydrase III (Somers, 1999), an oxidative enzyme that is abundant in slow muscle fibres, did suggest a basis for a possible fibre type specific pattern of localisation for myostatin. This same enzyme is also up-regulated in fast-twitch muscles undergoing hypertrophy in response to passive stretch (Brownson and Loughna, 1996; Laurila *et al.*, 1991), consistent with a transition towards a slower muscle fibre phenotype in this model.

Other growth factors reported to have a role in the regulation of fibre type include TGF-β1 which is implicated in the determination of fibre type in developing muscle (McLennan, 1993) and IGF-1, over-expression of which results in increased numbers of slow muscle fibres (Coleman *et al.*, 1995). In response to hypertrophy, fibres undergoing transformation from fast to slow MHC isoforms have high levels of a specific isoform of IGF-1 which is induced only in hypertrophied muscles (McKoy *et al.*, 1999; Yang *et al.*, 1996b).

In this study, immunohistochemistry was used to identify any specific association between myostatin and fibre type and it has shown in rabbit muscle that there is a homogeneous distribution of myostatin in all muscle fibre types. In human muscle, myostatin was also evenly localised to both fast and slow fibre types (Gonzalez-Cadavid *et al.*, 1998) and in developing porcine muscle there was no difference in myostatin expression between primary and secondary muscle fibres (Ji *et al.*, 1998). The regulation of fibre type in skeletal muscle is complex, involving factors such as hormonal and neural stimuli and this study has provided no evidence to suggest that myostatin is directly involved in the regulation of fibre type changes in postnatal rabbit muscle.

6.4.5 Conclusions

In conclusion, this study presents evidence in support of a functional role for myostatin in the regulation of skeletal muscle mass in response to stretch or shortening in adult animals. Two different mechanisms are discussed by which myostatin may regulate both increased and decreased muscle mass. In stretched muscles it is suggested that decreased myostatin expression permits the proliferation of satellite cells along the length of the muscle fibres. These satellite cells accumulate at the myotendinous junctions of muscle fibres where they are incorporated into existing fibres. In shortened muscles, increased myostatin expression may be associated with increased apoptosis, thereby reducing the number of myonuclei and maintaining a constant DNA unit size. The possibility that myostatin is involved in the regulation of protein synthesis and/or degradation is also discussed. This study has found no evidence in support of a role for myostatin in regulating changes in skeletal muscle fibre type in rabbit muscle.

CHAPTER SEVEN

GENERAL DISCUSSION

The overall aim of this thesis was to investigate the role of growth factors in regulating changes in skeletal muscle fibre type and fibre size. The IGF axis and myostatin expression were examined during bovine muscle development in animals from two different genetic backgrounds, normal (NM) and double-muscled (DM), a condition which is due to a mutation in the gene for myostatin. Myostatin expression was also examined in a model of experimentally induced hypertrophy and atrophy in rabbits. This final discussion will compare and contrast the different models of altered skeletal mass that were investigated, with respect to the role of growth factors in regulating changes in skeletal muscle fibre type and fibre size in these models. Areas of fundamental interest with respect to muscle development that have arisen from this study will also be discussed, along with possible future research directions.

7.1 Mechanisms of altered skeletal muscle mass in the two models

The increase in muscle mass in postnatal DM animals, relative to NM, is a consequence of an increased number of muscle fibres and an increase in the proportion and the size of the larger fast glycolytic fibres (Holmes and Ashmore, 1972; Ouhayon and Beaumont, 1968; Swatland and Kieffer, 1974). Fibre number is generally considered to be established during foetal development (Rowe and Goldspink, 1969; Stickland, 1978) and this study has shown that fibre type proportions are modulated throughout foetal development. Further fibre type changes also takes place postnatally (Holmes and Ashmore, 1972). The process of

skeletal muscle growth that occurs during foetal development in both the DM and NM includes the addition of new sarcomeres at the ends of the muscle fibres (Tabary *et al.*, 1972; Williams and Goldspink, 1971; Williams and Goldspink, 1973). This is the predominant mechanism by which muscle mass is increased in response to stretch (Dix and Eisenberg, 1990), making the stretch-induced hypertrophy model potentially useful for investigating the developmental regulation of muscle growth.

In considering the mechanism that regulated the decrease in muscle mass in postnatal M. *vastus medialis* in DM animals relative to NM, this study has provided an example of the complex way in which genotype and postnatal environment may interact to produce a specific phenotype. During foetal development it was found that the M. *vastus medialis* in DM animals is hypertrophied relative to NM, not atrophied as it is in adult animals. In this model it may be that the genetically regulated decrease in skeletal size (Arthur, 1995; Boccard, 1981), in conjunction with the increased muscle mass, combines to put excess pressure on the knee. This may then induce atrophy of the M. *vastus medialis* as has been reported in humans (Speakman and Weisberg, 1977). This phenotypic manifestation of reduced muscle mass may be a consequence of a genetic effect on skeletal development rather than a direct genetic effect on muscle development. The process of atrophy that the M. *vastus medialis* may subsequently undergo could share properties in common with disuse atrophy. These properties would include a reduced fibre size, with a preferential decrease in the proportions and the size of type 1 muscle fibres (Fitts *et al.*, 1986; Loughna *et al.*, 1996; Loughna *et al.*, 1990). The possibility that postnatal atrophy of the M. *vastus medialis* occurs could be investigated by serial biopsies of the muscle in DM calves during postnatal growth.

This investigation into the role of growth factors in regulating muscle fibre type and fibre size has provided further evidence in support of a role for the IGF axis in bovine myogenic development during the period of secondary myofibre formation. The relatively high level of myostatin expression during secondary myofibre

formation also suggests that this growth factor may have a role in the differentiation of the late myoblast population.

Myostatin is clearly implicated in the regulation of skeletal muscle mass in response to hypertrophy and atrophy in postnatal animals. Myostatin expression is decreased in muscles undergoing induced hypertrophy and two possible mechanisms have been put forward to explain how myostatin may negatively regulate muscle mass. The first mechanism was based on the hypothesis that myostatin expression may inhibit the proliferation of satellite cells *in vivo* and that down-regulation of myostatin therefore promoted satellite cell activation, leading to hypertrophy. This possibility is also consistent with low levels of myostatin during late gestation in the bovine development model, at a stage when most muscle growth is due to enlargement of existing fibres rather than new fibre formation (Swatland and Kieffer, 1974). This period of late gestation is associated with a rapid increase in the pool of satellite cells (Stockdale, 1992), again suggesting that low myostatin mRNA is permissive of increased satellite cell proliferation. This proposed mechanism of myostatin action, in inhibiting satellite cell proliferation, may therefore be common to both of the models investigated.

Incorporation of satellite cells is a way of maintaining a constant sized DNA unit in muscle which is undergoing hypertrophy (Cardasis and Cooper, 1975; Cheek *et al.*, 1971; Hall and Ralston, 1989). Changes in nuclear number must be balanced with alterations in the rate of protein synthesis and degradation, which are both increased with hypertrophy (Laurent and Millward, 1980). Myostatin may mediate this balance through effects on the synthesis of muscle specific proteins and this represents the second of the two possible mechanisms proposed through which myostatin may negatively regulate muscle mass.

Further evidence in support of a role for myostatin as a negative regulator of muscle growth comes from the observation in this study that myostatin expression is up-regulated in response to disuse. In disuse atrophy the transcriptional potential of

muscle fibres is modulated by decreasing myonuclear number through apoptosis (Allen *et al.*, 1997). Again two possible mechanisms were suggested through which myostatin might act in regulating this change in muscle mass. The first of these is through stimulating apoptosis and the second possibility is that myostatin is directly associated with either increasing protein degradation or reducing protein synthesis thereby contributing to a net loss in muscle mass. Both of these mechanisms have the potential to maintain a constant DNA unit size in muscle undergoing a change in mass.

7.2 Mechanisms of altered skeletal muscle fibre type in the two models

Some similarities can be found in comparing the biphasic change in proportions of type 1 fibres observed during bovine development with the experimental models of altered muscle mass in the rabbit. The initial decrease in the proportions of type 1 fibres that occurs in bovine muscle development also occurs in muscles undergoing atrophy (Allen *et al.*, 1996; Hikida *et al.*, 1997; Templeton *et al.*, 1988). During development, the initial decrease in the percentage of type 1 fibres may occur as a consequence of apoptotic degradation of muscle fibres (Fidzianska and Goebel, 1991) or switching from slow to fast MHC isoforms (Maier *et al.*, 1992). Apoptosis is also a feature of muscles undergoing atrophy in response to disuse, although only as a mechanism to decrease nuclear number as there is no change to fibre number (Allen *et al.*, 1995; Cardenas *et al.*, 1977). The atrophy model is also associated with a fibre type transformation towards increased proportions of fast fibres (Loughna *et al.*, 1996; Loughna *et al.*, 1990).

Although there are some similarities between the models investigated with respect to the declining percentage of type 1 fibre types, the mechanisms that induce the fibre type transformations may not be the same in the two models. With respect to the initial decrease in type 1 fibres in bovine muscle, levels of IGF-1 remained high throughout the period during which this apoptotic degradation/fibre type transformation is taking place, although the literature reports that IGF-1 protects

cells from apoptosis (Rodriguez-Tarduchy *et al.*, 1992). This would seem to indicate that either no protection from apoptosis was being given, or that the mechanism of decreased proportions of type 1 fibres was associated with fibre type transformation rather than degradation. Over-expression of the *igf1* gene also failed to prevent muscle atrophy in a hindlimb unloading study (Criswell *et al.*, 1998), although apoptosis was not specifically investigated in this study. Collectively, this evidence argues against a role for IGF-1 in protecting primary myotubes from apoptotic degradation.

Functionally, the decrease in the percentage of type 1 fibres may be associated in both models with a low requirement for the type of muscle activity employed in the maintenance of posture against gravity. The later developmental phase in which proportions of type 1 fibers increase in bovine muscle is comparable to the atrophy model where there is a similar transformation towards more type 2 fibres. The functional significance of this may be associated with preparation for the requirements of locomotion after birth. Postnatally an increased proportion of type 2 fibres is associated with increased fatigue resistance (Roy *et al.*, 1982), which would become important in the transition from the non-load-bearing conditions found *in utero* to those of standing and walking after birth.

The second phase of fibre type transition identified in developing bovine muscle, when there was an increase in the percentage of type 1 fibres, occurred during a period of relatively low IGF-1 mRNA expression. Fibres which are undergoing a transition from fast to slow MHC expression as a result of stretch induced hypertrophy express high levels of IGF-1 mRNA (McKoy *et al.*, 1999; Yang *et al.*, 1996b; Yang *et al.*, 1997). There was, however, no strong link between either the type 1-to-type 2 or the putative type 2-to-type 1 fibre type transformations and IGF-1 shown in this study of bovine muscle development. An alternative mechanism through which fibre type may be regulated is through expression of the MRFs

(Hughes *et al.*, 1993; Hughes *et al.*, 1999; Wheeler *et al.*, 1999), as will be discussed next in the context of the bovine development model.

The decreased proportion of type 1 fibres in DM muscles relative to NM throughout gestation raises the question of what role myostatin may play in regulating fibre type. One mechanism through which myostatin could regulate expression of the MHC genes is through transcriptional regulation at the promoter/enhancer level. Studies have shown effects of thyroid hormone (Wright *et al.*, 1999) and the MRFs (Carson and Booth, 1999; Wheeler *et al.*, 1999) on transcriptional regulation of MHC genes and these may represent possible downstream targets for myostatin action. Given that there are fewer slow MHC fibres in DM and therefore more fast fibres, the role of myostatin in normal bovine muscle development may be associated with enhanced slow MHC expression or down-regulation of fast MHC expression. The former possibility may be more likely in view of an earlier proposal that fast MHC isoforms represent the "default" genes and that a specific signal is required for the induction of slow MHC (Goldspink *et al.*, 1992; Whalen, 1985).

How does this interpretation of a role for myostatin in enhancing slow MHC expression during foetal development compare with the fibre type changes that occur in the postnatal hypertrophy model? There is a considerable temporal separation between the effects of hypertrophy on myostatin mRNA expression and fibre type differences. Changes in myostatin mRNA were evident even though fibre type differences never became significant. Furthermore, myostatin mRNA expression was decreased in the hypertrophy model, while the fibre type composition was tending towards more slow fibres as is consistently reported in the stretch model (Galler *et al.*, 1997; Holly *et al.*, 1980; Matthews *et al.*, 1990; Roman and Alway, 1995). In the two models examined therefore, a decrease in myostatin is associated with both a decrease and an increase in proportions of type 1 fibres. There is a suggestion that the slow MHC isoform which occurs in slow twitch muscles such as the M. *soleus*, may be different to the isoform which exists in predominantly fast twitch muscles such as the M. *tibialis anterior* (Goldspink *et al.*, 1992). The

developmental isoform of slow MHC may be different from the postnatal isoform, and both may respond to myostatin in a different manner. Alternatively, there may be different effects in the two models on downstream targets of myostatin action such as the MRFs, and it may be these changes that are responsible for mediating the differential response in fibre type. A recent report showing myostatin mRNA expression in mice to be strongly associated with the type 2b MHC isoform (Carlson *et al.*, 1999) is evidence in support of a fibre type specific role for myostatin in regulating changes in muscle mass.

7.3 Skeletal muscle fibre size

During bovine muscle development, presumptive primary myotubes underwent a decrease in fibre area between 120 and 160 days gestation, as has been previously reported in bovine and porcine foetuses (Stickland, 1978; Wigmore and Stickland, 1983). This change is associated with a change in fibre morphology that includes migration of the nuclei to a peripheral location within the muscle fibres. This would allow closer packing of the myofibrils and hence a smaller fibre area. There is no evidence to suggest that this decrease in fibre area is associated with a net decrease in myofibrillar protein, although there is apoptotic degradation of some primary myotubes in the human fetus (Fidzianska and Goebel, 1991). In disuse atrophy, reduced fibre size has been reported to be associated with a loss of muscle protein due to increased degradation (Booth and Criswell, 1997; Loughna *et al.*, 1986).

In analysing fibre size differences between DM and NM muscles, two main differences were apparent. Firstly, in NM muscle, after the initial decline in muscle mass, type 1 fibres increased in area again, but fibres from DM animals did not, remaining at essentially the same size for the remaining period of gestation. Secondly type 2 fibres were smaller in DM than NM throughout most of the period studied.

Evidence in support of a role for IGF-1 in inducing myofibre hypertrophy is very strong *in vitro* (Semsarian *et al.*, 1999a; Semsarian *et al.*, 1999b; Vandenburgh *et al.*, 1991) and *in vivo* (Adams and McCue, 1998; Coleman *et al.*, 1995). In the present study, lower levels of IGF-1 in DM animals may be associated with the decrease in type 1 fibre area in these animals during late gestation and with the decreased area of type 2 fibres. With respect to myostatin, low levels of myostatin expression in DM and NM were associated with the period of greatest muscle fibre hypertrophy, consistent with results during porcine muscle development (Ji *et al.*, 1998).

The stretch induced hypertrophy model in mammals is not associated with a rapid increase in fibre area and in atrophy it is the slow muscles that are affected to the greatest extent, with considerable sparing of fast fibre types (Booth and Criswell, 1997). Therefore the IGFs were not specifically targeted in this study with respect to the regulation of fibre size. Increased myostatin expression in hypertrophied muscle was associated with a trend towards increased fibre size, which was present in all fibre types.

The mechanisms responsible for the regulation of muscle fibre size are likely to be different between the two models of altered muscle mass and in the DM model at least, muscle fibre size may be related to local control by IGF-1.

7.4 Conclusions and future directions

In examining growth factor regulation of fibre type and fibre size in two different models of altered muscle mass, a number of conclusions can be drawn. Although both of the models investigated showed differences in muscle mass and there were some similarities with respect to alterations in fibre type, in general the mechanisms which regulate fibre type and fibre size changes appear to be different in developmental versus postnatal models.

This study has suggested that a greater understanding of the role of growth factors in the regulation of muscle fibre type will come from the elucidation of the mechanisms involved regulating MHC expression. The observed fibre type differences between DM and NM have lead to the hypothesis that myostatin may mediate fibre type through the transcriptional regulation of MHC genes. This hypothesis could be tested in primary NM or DM myoblast cultures by driving a reporter gene with MHC enhancer sequences to analyse the DNA elements that respond to myostatin signalling. Systematic deletions in the enhancer elements could then be used for identifying possible DNA binding sites that respond to myostatin signalling.

At the level of basic muscle biology, mechanisms that underlie developmental changes in MHC expression remain unknown. A fundamental question which has arisen from this study is, if there are fibres that undergo a type 1 to type 2 transition, do these same fibres then undergo a subsequent switch back to type 1 again. In order to test the hypothesis that these fibres are indeed the same, experiments involving the labelling of primary myotubes, for example with 5-bromodeoxyuridine, would allow the pattern of MHC isoform expression to be followed in labelled myotubes throughout foetal development.

The final and most exciting area of possible future investigation comes from the evidence presented here which suggests that myostatin is involved in the regulation of skeletal muscle mass in adult animals. This observation has lead to the hypothesis that myostatin may regulate both satellite cell proliferation and myonuclear apoptosis. Experiments to test this hypothesis could utilise the immobilisation model to investigate myostatin mRNA localisation in conjunction with the labelling of proliferating satellite cells and myonuclei undergoing apoptosis, possibly in experiments analogous to elegant single fibre studies of Allen *et al.* (1995). Investigations of this nature will certainly be required in order to gain some

understanding of the mechanisms which underlie the role that myostatin may play in mediating changes in skeletal muscle mass.

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APPENDIX ONE

Macro for semi-automated analysis of muscle fibre type and size

```
var Tag: string;

macro 'SetScale [\]';
begin
      SetScale(GetNumber('Enter Scale (pixels/um):
',1.235),'micrometer',1.025);

end;

macro 'Capture [F5]';
begin
  StartCapturing;
  StopCapturing;
   end;

macro 'Adjust [F6]';
begin
  SetDensitySlice(75,132);
  PutMessage('Make manual adjustments');
 end;

macro 'Measure [F7]';
begin
  SetOptions('area, mean density');
  Calibrate('Uncalibrated OD');
  LabelParticles(true);
  IncludeInteriorHoles(true);
  WandAutoMeasure(true);
  SetPrecision(2);
  Measure;
  PutMessage('Use wand to measure');
  ResetCounter;
  end;
```

```
macro 'Export results [F8]';
var Cnt: integer;
begin
  ShowResults;
  SetExport('Measurements');
  Tag:=GetString('Enter slide label: ');
  if rCount > 0 then
  begin
    Cnt := 1;
    repeat
      UpdateResults;
      Cnt := Cnt + 1;
    until (Cnt > rCount);
  end;
  Export(concat('HD-80:JKM:GP027:fibredat:', Tag));
  DisposeAll;
End
```

APPENDIX 2

Publications

Some of the work in this thesis has been presented in the following publications:

J Martyn, J Oldham, C Berry, S Kirk, B McGarry, W McMillan, J Bass. (1997) Characterisation of muscle development in foetuses with and without presumptive double-muscled phenotypes. *Proceedings NZ Society of Animal Production* **57**, 278-281.

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