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Review

Impact of manipulations of myogenesis *in utero* on the performance of adult skeletal muscle*

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The possibility that early fetal programming affects health or disease status in adult life has been considered in relation to tissues such as the cardiovascular system but not with respect to skeletal muscle. Since muscle mass and function are important for life, it is pertinent to ask whether events during the development of muscle *in utero* can affect the performance of the tissue in later life. This review discusses the factors that influence muscle performance, outlines the current understanding of myogenesis and examines how manipulations alter myogenic outcome after birth. The performance of muscle is determined by the number, type and size of the muscle fibres, these in turn being affected by a number of factors, and the evidence indicates that the proportions of types of muscle fibre have a heritable component. The formation of muscle occurs early in embryogenesis and it appears that the major impacts on myogenesis are associated with extremes of treatment or embryo manipulations. The impact of extremes of treatment or embryo manipulations on myogenesis is seen in the secondary fibres whereas primary fibres appear to be insensitive or protected. Overall, the opportunities for manipulation of myogenesis *in utero* to improve adult performance are limited.

There is a growing interest in the way in which fetal programming can influence the performance of an adult in later life. Attention has been focussed on tissues relevant to major diseases such as the cardiovascular system (Barker, 1996), but skeletal muscle, one of the major components of the body, has been largely ignored. The aims of this review are to outline the main performance parameters in adult muscle, to provide a general overview of muscle development and then to consider whether myogenic programming may be influenced *in utero* and, if so, how changes in myogenesis might affect adult performance.

Factors that determine muscle performance in adults

Skeletal muscle represents about 40% of body mass and is used not only for locomotion but also acts as a reservoir of amino acids that can be called upon in times of physiological stress or disease. Skeletal muscle as meat in livestock species also serves as an important source of dietary protein and micronutrients. The functional units of differentiated muscle are the muscle fibres, which are long, cylindrical multinucleated cells. The performance of muscle is related to its mass and its functional capacity in terms of its ability to generate strength, speed and duration of movement.

Muscle mass and strength

Muscle mass depends on the number and size of the muscle fibres. In general, the number of muscle fibres is set at birth (Rowe and Goldspink, 1969) and hyperplasia does not occur to any significant extent during postnatal mammalian growth, and some studies indicate that this may be true for avian and fish species (Battram and Johnston, 1991; Remignon *et al.,* 1995). Consequently, any postnatal muscle growth in mammals relies largely on protein accretion leading to muscle fibre hypertrophy. Protein accretion depends on the balance between protein synthesis and protein degradation, with accretion rates highest in young growing animals and declining with age. The mechanism of both protein synthesis and degradation has been widely discussed and the factors that influence muscle protein accretion, for example, nutrition, insulin, innervation and growth factors are well recognized (for review, see Grizard *et al.,* 1999).

Muscle mass contributes to body shape and the conformation of a carcase is important in terms of the quality and value of meat animals. Generations of selective breeding for meat production have resulted in highly muscled, lean animals, although the genetic mechanism that underpins this remains to be fully elucidated. The size of a muscle is

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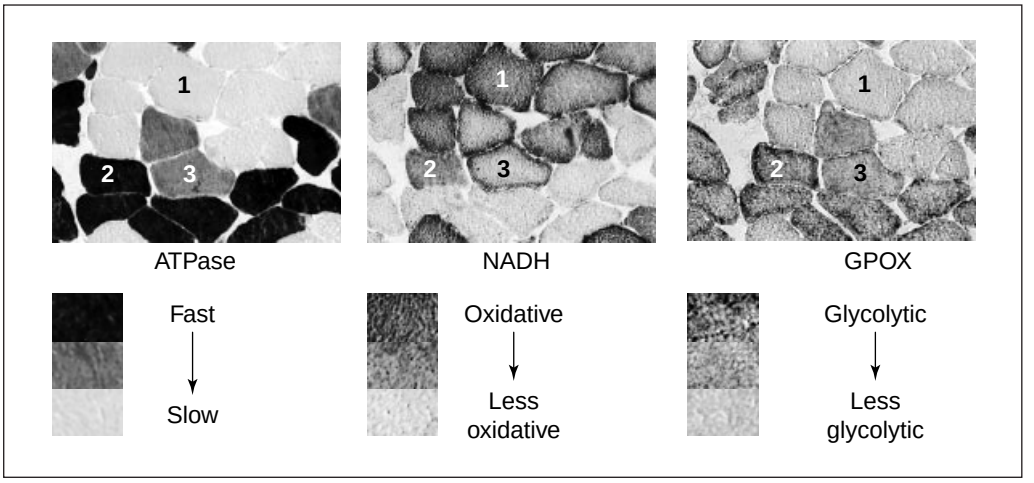

Fig. 1. A simple muscle fibre-type classification based on the density of the reaction product generated by the use of three staining reactions on transverse sections of muscle is shown. Formalin-insensitive Ca^{2+}-activated ATPase activity (ATPase) at pH 9.4 was used to indicate contractile speed, nicotinamide adenine dinulceotide (NADH) was used to demonstrate mitochondrial activity and α-glycerophosphate dehydrogenase (GPOX) indicated glycolytic activity. The density of the reaction product is graded, allowing the fibres to be classified according to the reaction intensity. Thus, slow-twitch oxidative (SO) fibres (1) have little reaction product for ATPase, consistent with their slow speed of contraction, but show a dense product for NADH, highlighting their oxidative metabolism. In contrast, fast-twitch oxidative glycolytic (FOG) fibres (2) have a fast contractile speed and both aerobic and anaerobic metabolism, and therefore have a positive reaction for all three staining regimens. Fast-twitch glycolytic (FG) fibres (3) have a high speed of contraction owing to their anaerobic metabolism, and therefore have positive staining for both ATPase and GPOX but less reaction product for NADH.

important in relation to its strength and there is a close relationship between the cross-sectional area of a muscle and its ability to generate force (Fenn and Marsh, 1935). The importance of muscle strength has been highlighted by the finding that mid-life grip strength is a good predictor of long term mortality, independent of body mass index (Rantanen *et al.,* 2000). The authors interpreted these results as indicating that early life influences on muscle strength may have long-term implications for mortality. Thus, factors that impinge on myogenic processes *in utero* to increase muscle cross-sectional area (for example, increases in the number or size of fibres) and ultimately lead to increased strength in the adult, may have benefits for survival.

Muscle fibre type proportions

Muscle fibres vary considerably in their morphological, biochemical and physiological properties. These properties are used to classify the fibres into at least two main fibre types: type I or slow-twitch oxidative (SO) fibres used for maintenance of posture or for endurance exercise; and type II or fast-twitch fibres used for more rapid bursts of activity. The fast-twitch fibres are divided into at least two classes: fast-twitch glycolytic (FG) and fast-twitch oxidative glycolytic (FOG), on the basis of their contractile and metabolic activities (Fig. 1).

The individual muscles in mature limbs contain a mixture of the different fibre types; very few muscles comprise solely one type. Therefore, muscles generically described as slow-twitch muscles, such as soleus, actually contain a proportion of fast-twitch fibres. As well as variations among muscles within an individual, there is also considerable variation among individuals and among breeds (Fig. 2). This variation in the proportions of fibre type may be one of the factors that determine the performance of the muscle in the adult, either in terms of athletic performance in humans (Gollnick *et al.,* 1972) or of meat production in livestock. Evidence from pigs and cattle indicate that muscle fibre type proportions may influence the eating quality of meat (Karlsson *et al.,* 1993; Koch *et al.,* 1995; Zamora *et al.,* 1996; Maltin *et al.,* 1997, 1998a,b; Vestergaard *et al.,* 2000) but the precise relationships between fibre type and sensory evaluation are not clear. Several studies have shown a positive relationship between SO fibre frequency and either tenderness or juiciness (for example, see Valin *et al.,* 1982; Ockerman *et al.,* 1984; Maltin *et al.,* 1998b), whereas other data link the proportion of fast-twitch fibres with tenderness (Koch *et al.,* 1995). In contrast, fast-twitch glyoclytic fibres may be associated with toughness. Muscles from callipyge lambs tend to be tougher than those from normal lambs (Duckett *et al* 2000) and show an increased frequency of fast-twitch glycolytic fibres and a lower proportion of fast-twitch oxidative glycolytic fibres (Carpenter *et al.,* 1996). Although it is clear that fibre

type proportions play some role in determining meat eating quality, further work is necessary to evaluate the precise relationship between this characteristic of living muscle and its quality as meat.

Maturation and ageing

In addition to the variation among individuals, the types and sizes of fibre in muscle change with age (Caccia *et al.,* 1979; Eddinger *et al.,* 1985; Monemi *et al.,* 1998; Hughes and Schiaffino, 1999). As a muscle matures, the fibres hypertrophy and muscle mass increases. At the same time, there is a tendency for the functional area of slow fibres to increase with age (Fig. 3) (Caccia *et al.,* 1979; Eddinger *et al.,* 1985), consistent with the observed increase in relative endurance (Aoyagi and Shephard, 1992). In humans, there is a decline in muscle mass and force generation from about the age of 50 or 60 (Aoyagi and Shephard, 1992; Harris, 1997) as a result of a reduction in the number of muscle fibres (Aoyagi and Shephard, 1992) and a reduction in the synthetic rates of specific muscle proteins (Proctor *et al.,* 1998). Similar changes are observed in other species. There are ethnic differences in the rate of muscle loss with ageing, with the rate lower in black women than in white women (Aloia *et al.,* 2000). It is unclear whether this finding is the result of ethnic differences in the genetic regulation of initial muscle mass through the number or size of muscle fibres or in the control of muscle loss.

Training

The proportions of the different types of fibre in a muscle not only change with age but also show a considerable degree of plasticity in response to stimuli such as training (Gollnick *et al.,* 1972; Abernethy *et al.,* 1990). Indeed, the adaptations of muscle to the stresses of physical exercise underpin the current wealth of different training regimens used to maximize the athletic performance of muscle in animals and humans. Different types of training appear to lead to different adaptations with respect to both myosin heavy chain (MHC) isoform expression and metabolism within the muscle, such that the muscles of sprint- and endurance-trained athletes are reported to have different fibre-type characteristics (Costill *et al.,* 1976). Sprint training tends to decrease the proportion of SO fibres and increase the proportion of FOG fibres. In contrast, endurance training tends to convert FG fibres to FOG fibres and to enhance the proportion of SO fibres. These effects of training illustrate the capacity of differentiated muscle to undergo fibre type transformations. There is evidence that these transformations are triggered by subtle changes in innervation. The mechanisms by which the neuronal firing patterns respond to training and the neuronal signals lead to changes in muscle gene expression are not fully understood, although a calcium-dependent, calcineurin signalling pathway has been implicated (Olson and Williams, 2000).

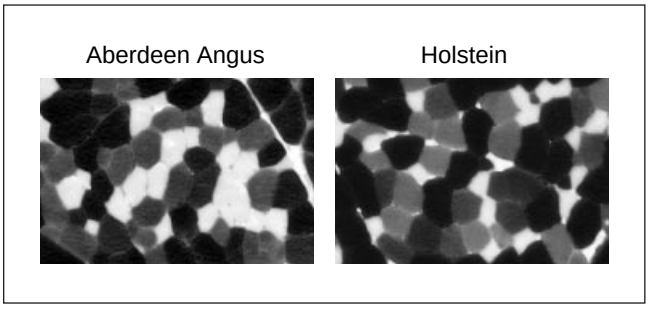

Fig. 2. Differences in muscle fibre-type proportions and fibre size between Aberdeen Angus and Holstein breeds of cattle. Transverse sections through the *m longissimus lumborum* of different breeds of cattle of the same gender and age on the same feeding regimen reacted to demonstrate ATPase activity and show the variation in fibre-type proportions. Comparison between these two examples demonstrates the differences in fibre type and size between genotypes. Scale bar represents 100 μm.

Inheritance

The concept of heritable differences in muscle fibre characteristics is not new (Joubert, 1956; Saltin, 1973). Since there are incentives to select and train individual humans or other animals with the optimum physical characteristics to excel in a specific athletic event, there is considerable interest in the potential heritability of performance parameters and fibre composition. Evidence from both humans and racehorses indicates that fibre characteristics, particularly those relating to SO fibre types and endurance exercise, are heritable. Estimates of heritablility in humans range from 99.5% in one small study on twins (Komi *et al.,* 1977) to approximately 30% in athletes (Thibault *et al.,* 1986). Larger population studies from North America indicate that about 45% of the phenotypic variance in SO fibre proportions is associated with inherited factors (Simoneau and Bouchard, 1995).

Heritability in racehorses is similar to that reported for humans and is approximately 35% (More O'Ferrall and Cunningham, 1974). The different proportions of SO fibres in the various equine breeds have been related to their selection over time for divergent performance characteristics (Snow, 1983). Consequently, heavy Hunters selected for endurance have a high proportion of SO fibres (31%), whereas Thoroughbreds bred for sprint speed have a low proportion of SO fibres (13%). These types of data do not come from strict experimental breeding protocols and it could be argued that adaptive factors such as training had played some role in the aetiology of these differences. However, in studies in inbred strains of mice, the heritability of the proportions of types of muscle fibre, the total number of fibres and the relative size of slow and fast fibres was highly significant (Nimmo *et al.,* 1985). Indeed, the data showed that 57% of the variation in muscle composition could be accounted for by a genetic component. In livestock, it has been suggested that heritable differences in muscle fibre

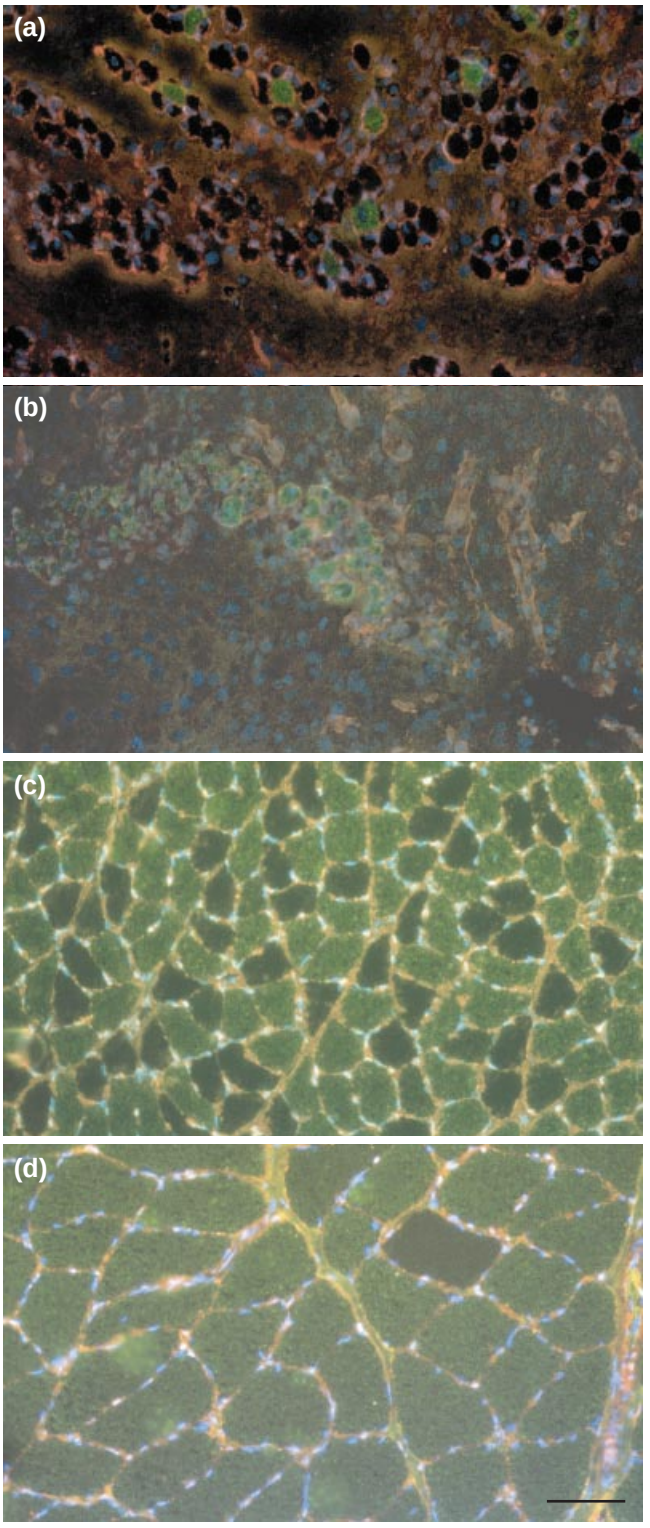

Fig. 3. The functional area of muscle fibres expressing slow myosin heavy chain (MHC) isoform increases with age. Transverse sections of muscle samples labelled immunocytochemically to show the localization of slow MHC isoform (green) and laminin (red) and nuclei (blue). The samples illustrate the increase in slow fibre functional area with age in rats from the fetus to the mature adult. Slow myosin immunoreactivity is shown in: (a) the dorsal muscle groups of the lower leg from a rat fetus at day 19 of gestation; (b) the dorsal muscle groups of the lower leg from a 3-day-old neonatal rat; (c) the soleus muscle of a weanling rat (19 days old); and (d) the soleus muscle of a 1-year-old rat. Scale bar represents 50 μm.

characteristics explain part of the variation in eating quality. For example, Duroc pigs, known for producing meat of high eating quality have a higher proportion of SO fibres in their muscles than do Large White pigs, which produce meat of lower eating quality. Fibre-type analysis of pure- and cross-bred animals showed that the SO fibre characteristics of Duroc animals are heritable and indicate that a single gene or gene cluster is responsible (Maltin *et al.,* 1998a). Similarly, it has been suggested that the observed variation in eating quality between Aberdeen Angus and Holstein cattle may relate to genotypic differences in SO fibre proportions and metabolic characteristics (Lobley *et al.,* 2000; Maltin *et al.,* 2001). Although such a difference between cattle bred for meat and milk production, respectively, may not be so surprising, it does perhaps indicate that selection for product diversity has also selected for divergence in muscle metabolic and contractile properties.

If the heritability of the proportions of muscle fibre types is high, then this is not merely relevant to the selection and training of elite athletes, it may be important in relation to the predisposition of an individual to disease conditions. For example, the suggestion that SO fibre frequency is inversely related to fatness and that at least 40% of the variability in fatness is explained by variation in muscle fibre type (Wade *et al.,* 1990) raises some interesting questions as to the aetiology and heritability of obesity. In addition, back pain with concomitant loss of muscle strength (Hultman *et al.,* 1993) and endurance (Roy *et al.,* 1990) has been shown to be associated with a significantly higher frequency and percentage area of FG fibres in the paraspinal muscles of patients with low back pain than in those of controls (Mannion *et al.,* 1997). Although this finding may explain the symptoms of low back pain, it is also possible that the heritability of fibre characteristics may increase susceptibility to the condition.

The performance of muscle in adult animals appears to depend on the number, type and size of the fibres that comprise that muscle. The evidence indicates that, although muscle responds to stimuli such as training, a considerable part of the inter-individual variation, particularly in SO fibres, may be due to heredity, although the genetic components and regulators remain unknown. The key questions are what controls the growth and diversity of fibre types of muscle and are these systems sensitive to impact or manipulations during development that might alter the adult tissue outcome? Given that there is a huge range of size and fibre-type composition of skeletal muscles to fulfil a large range of physiological requirements, the basic process and principles controlling the early development of muscle must be capable of generating muscles with the required diversity of structure and function.

Muscle development

Discussion of the processes leading to the formation of myogenic cells often uses the terms specification, determination, commitment and differentiation in a number of different contexts. For the purposes of this review, specification is the sequence of events that gradually restricts the fate of the pluripotent mesodermal cells and makes them responsive to signals that lead to their establishment as myogenic precursors cells. Determination, also referred to as commitment, is also a multistep process, at the end of which the cells are determined (usually irreversibly) and have acquired a myogenic identity. Determined cells then differentiate at their final location and start to express a full range of myogenic markers.

Somitogenesis

One of the earliest steps in the development of vertebrate embryos is somitogenesis (for review, see Rawls *et al.,* 2000) during which epithelial somites form from the anterior end of the segmental plate. During development the somites mature and become compartmentalized to form the sclerotome, myotome and dermomyotome (Fig. 4).

It is well accepted that all vertebrate skeletal muscles (except those of the head) originate from mesodermal precursor cells in the somites, but few studies acknowledge that the specification of the pool of cells that will give rise to skeletal muscle may occur long before somitogenesis. Embryonic cells with the potential to form muscle are present during gastrulation (George-Weinstein *et al.,* 1996, 1997) and, when dissociated cells from the epiblast layer of primitive streak-stage embryos were cultured at high density in serum-free medium, they were shown to express a muscle-specific transcription factor and to differentiate into skeletal muscle (George-Weinstein *et al.,* 1996). These observations point to a basic genetic specification of some muscle precursor cells and to a possible opportunity to alter myogenic outcome by early embryonic manipulation. However, although some of the epiblast cells may function as founder cells for the myogenic lineage, most epiblastic cells are pluripotent, and hence the major commitment to the myogenic lineage appears to occur in the mesodermal somites. In this context, it is of interest that, in zebrafish, separate slow and fast muscle precursor cells have been identified before somite formation (Devoto *et al.,* 1996).

Myogenic regulatory factors

The discovery in the late 1980s of a factor, the ectopic expression of which resulted in a stable, heritable conversion of non-muscle cells to a myogenic lineage, was a landmark in the understanding of muscle development. Subsequent studies revealed a family of four myogenic regulatory factors (MRFs); myoD, myf5, myogenin and MRF4 (myf6), which can each activate skeletal muscle differentiation when introduced into non-muscle cells. The MRFs are a group of basic helix–loop–helix (bHLH) transcription factors that play a central regulatory function in the skeletal muscle development programme. The function of these four related but distinct MRFs has now been largely worked out through the use of gene knockout experiments (for review, see Ludolph and Konieczny, 1995). The outcome of these experiments is that myoD and myf5 appear to be important for the formation and survival of myoblasts, having redundant function in myoblast specification, whereas myogenin and either myoD or MRF4 seem to be involved in the control of the terminal differentiation in the myotubes (Rawls *et al.,* 1995). Although unravelling the role of the individual MRFs has been complicated by redundancy and crossregulation, studies using triple-mutant mice (lacking myoD, myogenin and MRF4) have revealed that normal numbers of myoblasts form in these mice but differentiation occurs neither *in vivo* nor in mutant-derived myoblasts *in vitro* (Valdez *et al.,* 2000). This finding indicates that although myf*5* can activate myogenesis in transfection assays and trigger myoblast specification *in vivo* when expressed alone, in the absence of other MRFs *in vivo*, it cannot act autonomously to maintain the myogenic programme.

Myogenic identity

Signals from the neural tube and notochord are important in the control of muscle development, but recently their precise role in the molecular events leading to myogenic commitment and differentiation has started to become clear. Early studies showed that diffusible signals from the dorsal neural tube and the dorsal ectoderm act to organize the dermomyotome (Spence *et al.,* 1996). Signals from the dorsal neural tube induced myf5 expression, whereas those from the dorsal ectoderm induced the expression of myoD (Cossu *et al.,* 1996). More recently molecular messengers have been identified. Sonic hedgehog (Shh), a secreted glycoprotein produced by the notochord and floor plate, has been shown to have an essential inductive function in the early activation of myf5 leading to the determination of epaxial dermomyotomal cells to myogenesis (Borycki *et al.,* 1999). Members of the Wnt family can induce myogenesis (Tajbakhsh *et al.,* 1998). Wnt-1 expressed in the dorsal neural tube preferentially activates Myf5, whereas Wnt-7a expression in the dorsal ectoderm leads to myoD activation. In addition, combined Shh and Wnt-1 signals can induce the expression of the transcription factor Pax3 in the dorsal lip of the dermomyotome (Stern *et al.,* 1995). Somitic myogenesis is also under a negative regulation (Reshef *et al.,* 1998) possibly via bone morphogenic protein 4 (BMP4) signalling, which inhibits the activation of myoD and myf5 in dermomyotomal cells expressing Pax3. BMP4 signalling may be modulated through the action of the BMP antagonist Noggin, which is expressed in the dorsal lip of the dermomyotome (McMahon *et al.,* 1998). The expression of Noggin is, in turn, induced by Wnt-1 produced in the dorsal neural tube (Reshef *et al.,* 1998). These pathways are clearly

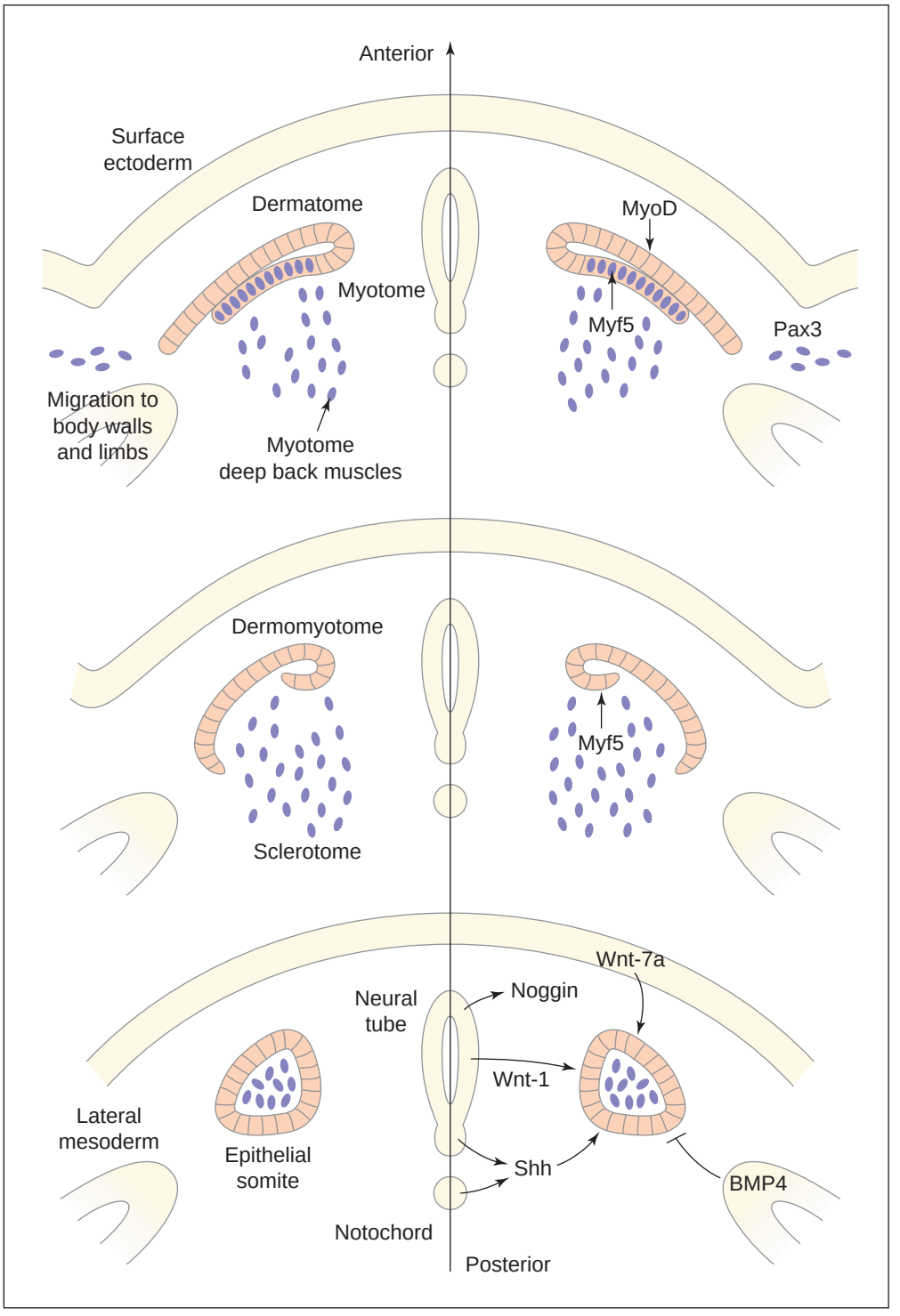

Fig. 4. Illustration of early muscle development. The left-hand side of the figure details the main structural changes during early muscle development; the right-hand side outlines some of the key regulators thought to be involved in myogenic specification, determination and migration of myogenic cells. The mesodermal somites form in a head to tail progression by segmentation of the mesodermal tissue on both sides of the neural tube. Newly formed somites consist of an epithelial ball of cells that becomes arranged into distinct cellular regions as a result of signal arising from surrounding tissues. The ventral region gives rise to the sclerotome from which the ribs and vertebrae form; the dorsal part of the somite forms the dermomyotome, which gives rise to muscles and dermis. Cells from the dorsomedial region of the dermomyotome develop first and eventually form the muscle of the back, whereas cells from the ventrolateral part develop after a

complex, but results so far indicate that, through the outlined signalling network involving at least Shh, Wnts, BMP4, Noggin and Pax3, precise temporal and spatial regulation can be achieved. (Fig. 4).

These signal pathways lead to determination of the myogenic cells and formation of myogenic precursors cells (MPCs), although a small number of cells are believed to undergo terminal differentiation to form myotomal muscle. Most of the cells remain in an undifferentiated state and, after appropriate migration, will divide and proliferate before the formation of fully differentiated muscle.

Cell migration

The formation of the limb musculature is particularly interesting because it involves obligatory migration of MPCs from the dermomyotome, where determination occurs, to the limb, where differentiation occurs. Migration depends on the activity of a number of gene products that block the myogenic programme and keep the MPCs in an undifferentiated state so that the onset of myogenesis in the muscles of the limbs and the body walls is delayed. Pax3 is essential for the migration of the MPCs (Goulding *et al.,* 1994), which represses the expression of myoD (Pourquié *et al.,* 1995), but is not necessary for the differentiation of limb muscle precursors (Daston *et al.,* 1996). Therefore, the switch from a Pax3-positive state to the expression of MRFs is a key step in the entrainment of the differentiation process (Pourquié *et al.,* 1995). The c-met ligand scatter factor and hepatocyte growth factor (SF/HGF) also plays a role in the migration of MPC, although not as a chemotactic signal as initially thought. SF/HGF that is expressed adjacent to the dermomyotome causes delamination of the migratory cells from the dermomytomal epithelium (Heymann *et al.,* 1996). Several candidate genes have been proposed to control the migration of the Pax3-positive MPCs (Büscher and Belmonte, 1999), but the genes and mechanism that regulate the progress and positioning of determined MPC from the dermomyotome to the limb remain unknown.

Proliferation versus differentiation

After the migration of MPCs into the limb buds, the cells undergo rounds of proliferation and increases in the number of cells. At some point, the cells withdraw from the cell cycle and begin the process of cell fusion and maturation. The events that determine the withdrawal from the cell cycle may be critical to the ultimate number of cells in the adult muscle and indeed may influence the number of fibres and hence muscle mass.

The mutual exclusivity of proliferation and differentiation in myogenesis is well recognized (Olson, 1992) and studies have now started to unravel the mechanism by which cell cycle activity blocks terminal differentiation. Myogenic differentiation is highly regulated and shows marked temporal organization (Walsh and Perlman, 1997). Under conditions of growth, myoblasts proliferate and do not express markers of differentiation such as myogenin. In the presence of growth factors: (i) inhibitor of differentiation (Id) is expressed and acts by dimerization with E12 (a member of the E-type family of ubiquitous bHLH transcription factors), which process prevents the heterodimerization between MRF and the E-type factors, hence preventing the binding to the E-box consensus sequence within the muscle-specific promoters; and (ii) protein kinase C is activated, which phosphorylates MRF and inhibits DNA-binding activity. Reduction or removal of mitogens leads to a decrease in Id, thus permitting the formation of bHLH heterodimers, which then bind to their DNA targets and initiate muscle-specific gene transcription (Fig. 5).

A key element in the switch from proliferation to differentiation is cyclin D1, which appears to inhibit myogenic transcription (Skapek et al., 1995), although this inhibition is reversed by retinoblastoma protein (pRb). pRb is required for the maintenance of the postmitotic state as myogenic cells lacking pRb undergo apoptosis at a high frequency during myogenesis (Wang et al., 1997).

Myogenin, an early marker of entry into the differentiation pathway, is not expressed until proliferating cells experience a low mitogen-containing environment but, despite apparent initiation of the differentiation process, myogenin-positive cells have been shown to retain the capacity to synthesize DNA. However, shortly after the onset of myogenin expression, the cells co-express the cell cycle inhibitor p21 and the ability to synthesize DNA is lost as the cells withdraw irreversibly from the cell cycle (Andres and Walsh, 1996).

Fibre-type diversity

As the specific proportion of types of fibre within differentiated muscle influences the muscle function, questions central to understanding tissue performance are: what is the origin of fibre-type diversity; how is it controlled at the cellular level; and are there opportunities to manipulate diversity to enhance performance?

Myoblasts that have withdrawn from the cell cycle and

significant delay and eventually migrate into the limb buds to form the musculature of the limbs and body wall. A working model of the proposed pathways has been developed on the basis of current knowledge. The model proposes that Shh from the notochord and the floor plate acts on the ventral domain of the newly formed epithelial somite inducing the formation of the scelerotome and acts on the dorso–medial region of the somite to induce the formation of the medial dermomyotome. Wnt-1 produced by the dorsal neural tube acts in conjunction with Shh on the dorsomedial region and activates myogenesis in the deep back muscle progenitors via a pathway that appears to be myf5-dependant. Wnt-7a from the dorsal ectoderm acts on the dorsolateral domain to specify the body muscle progenitors through a myoD-dependent pathway. In mice, the initial sites of myogenic regulatory factor activation appear distinct, but in chicks, it appears that a myoD-dependent pathway is activated first in the medial somite and this is followed by the activation of a myf5 pathway.

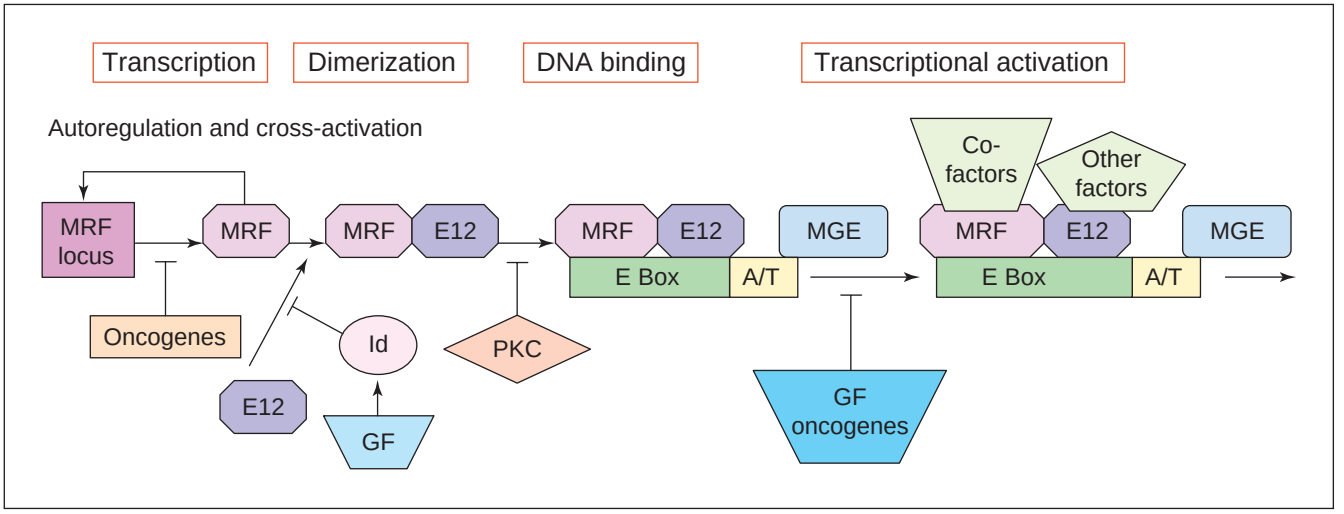

Fig. 5. Schematic representation of myogenic regulatory factor (MRF) regulation of muscle-specific gene expression (MGE). Transcription of each MRF locus may be influenced by positive autoregulation and cross-activation of other MRFs. MRFs form heterodimers with E12 to permit DNA binding at the E Box. Growth factors (GF) influence the formation of MRF–E12 dimers by maintaining high concentrations of the inhibitor Id, which binds E12, and by activating protein kinase C, which phosphorylates MRF and prevents DNA binding. Removal of GF permits heterodimer formation, and transcriptional activation leads to MGE. Activation is shown by arrows, inhibition is shown by bars.

are co-expressing myogenin and p21 start to express MHC and enter the final process of differentiation. The final stage in the formation of muscle fibres is biphasic: the production of new fibres through myoblast fusion occurs in two discrete phases. An initial wave of roughly synchronous fusion of myoblasts gives rise to a population of primary fibres. These primary fibres form early during myogenesis, in rats at about embryonic day 14 (Wigmore and Dunglison, 1998) or in humans between 6 and 8 weeks of gestation (Barbet *et al.,* 1991). Primary fibres are distributed throughout the muscle-forming areas in the limb and rapidly increase in size. In rats, chickens, mice and guinea-pigs, the primary fibres represent a small proportion of the final fibres, but they extend from tendon to tendon and form the shape of the muscle.

After primary fibre formation is complete, a second wave of myogenesis begins, and new fibres form on the surface of each primary fibre. These secondary fibres appear asynchronously over a period of days, between embryonic days 17 and 21 in rats (Wigmore and Dunglison, 1998) and between weeks 8 and 18 of gestation in humans (Barbet *et al.,* 1991). The secondary fibres form initially on the surface of the primary fibres, which they use as scaffolding to guide growth towards the tendons (Fig. 6). The number of secondary fibres associated with each primary fibre appears to vary: approximately twice as many secondary fibres per primary fibre occurring in large animals compared with small animals (Stickland and Handel, 1986; Ontell *et al.,* 1988). It has been suggested that these observations support the concept that the large number of fibres in larger animals is achieved through higher numbers of secondary fibres rather than increased numbers of primary fibres (Wigmore and Dunglison, 1998). However, the situation in large animals may be more complex. Studies in sheep and humans (Draeger *et al.,* 1987; Wilson *et al.,* 1992) indicate that at least three generations of myoblasts use the previous generation of myofibres as a scaffolding. Thus, further populations of cells would not only enhance the growth rate of the developing tissue but also contribute to the total number of fibres. This contention begs the question of whether the fundamental mechanisms underlying myogenesis in larger animals are the same as or more complex than those in laboratory animals.

During secondary fibre formation, the primary fibres continue to increase in size and to recruit nuclei. Primary myotubes absorb nuclei at all stages of development, including secondary fibre formation (Zhang and Mclennan, 1995). During the early part of secondary myogenesis, more myoblasts fuse with the primary than with the secondary fibres, but this process is reversed by the end of secondary myogenesis, ensuring that the sarcoplasmic to nuclear ratio is maintained throughout development, although it is unclear what determines whether a cell will fuse with a primary or a secondary fibre.

Initially, the two cohorts of myotubes express different MHC isoforms. In both pigs and rats, the earliest primary fibres express both slow and embryonic MHC but subsequently, in some of these fibres, this expression is lost and neonatal MHC is expressed. The secondary fibres express embryonic and neonatal MHC, although some of them later switch to slow MHC expression (Condon *et al.,* 1990; Lefaucheur *et al.,* 1995). Many of these changes occur at the time of the innervation that appears to regulate MHC expression (Lefeuvre *et al.,* 1996). Innervation of the primary fibres occurs before the onset of secondary fibre formation as pathfinder axons form multiple synapses along the length of the primary fibres. Secondary myotube

formation appears to occur around the primary myotube innervation sites (Duxson and Sheard, 1995) and, after embryonic day 17 in rats, this distribution is nonrandom (Wigmore *et al.,* 1996). A signal associated with innervation seems to stimulate the formation of secondary fibres on the surface of the primary fibres. The identity of the signal is unknown but candidates such as acetylcholine, calcitonin gene-related peptide, calcium flux and local changes in membrane potential have been proposed (Duxson and Sheard, 1995). As it is possible that variation in the number of secondary fibres may contribute to the variation in total number of fibres (Dwyer *et al.,* 1994), the exact nature and source of this signal is an important area for future research.

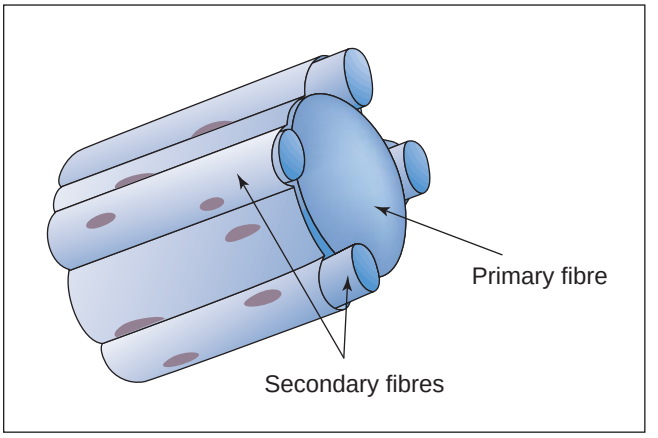

Fig. 6. Illustration of the formation of secondary muscle fibres on the surface of primary fibres.

Origin of myoblasts

The ordered nature of myogenesis has raised the question of whether the cells that contribute to primary and secondary fibre formation have a common or separate embryonic origin and whether, in turn, these different myogenic populations give rise to the fibre-type diversity seen in differentiated muscle. Several studies have demonstrated that the myogenic cells in the limb can be isolated and grouped into at least three populations: embryonic, fetal and adult (or satellite cells) (for example, Stockdale 1992). In human limb buds, for example, four phenotypically distinct populations of myoblasts have been identified at the beginning of primary myogenesis (Edom-Vovard *et al.,* 1999). These populations of myoblasts appear to predominate at different stages of development and are distinguished by their culture requirements, abilities to fuse, sensitivity to growth factors and the morphological nature of the myotubes they form. These various populations are likely to play different roles during myogenesis.

In mammals, the embryonic myoblasts, which are most numerous at the time of primary myogenesis, may be the progenitors of primary fibres and of the SO fibres in adults, whereas fetal and adult myoblasts contribute to secondary fibres and satellite cells in the adult. Retroviral marking of rat myoblasts at embryonic day 15 *in vivo* (Dunglison *et al.,* 1999) confirmed this contention by showing that embryonic myoblasts contribute only to the formation of primary fibres, all of which initially formed slow fibres, although some later converted to fast fibres. Dunglison *et al.* (1999) also clarified the fate of fetal and adult myogenic populations, which appeared to be pluripotent, fusing with both primary and secondary fibres to form either fast or slow fibres. However, the signalling mechanisms that regulate these processes are not understood fully.

In zebrafish, in which the embryonic origins of slow and fast fibres have been elucidated (Devoto *et al.,* 1996), the signal mechanisms underlying the determination of slow and fast fibres are being revealed. Slow-twitch fibres develop from presomitic cells adjacent to the notochord, whereas fast-twitch fibres are formed from the rest of the myotome. Members of the hedgehog family (HH) of proteins specifically induce slow-twitch fibres (Norris *et al.,* 2000). In contrast, fast fibre formation appears to be HH-independent. The precocious specification of slow fibres in zebrafish differs from that in mammalian embryos; however, as homologous mammalian HH genes have been reported, it is possible that similar transduction systems in mammals control the specification of slow fibre types.

Regulation and maintenance of fibre-type diversity and fibre size

The final stage in the establishment of adult muscle continues by fibre conversion and hypertrophy in the postnatal period and displays a characteristic diversity in the type and size of fibres present. Postnatal characteristics of muscle are more dependent on neural and growth factor influences than are prenatal characteristics. Studies have highlighted a new factor that appears to be central to muscle development and growth.

Calcineurin is a Ca^{2+}–calmodulin-regulated serine–threonine phosphatase that plays a pivotal role in the control of muscle signalling pathways regulating multiple muscle events including differentiation (Abbott *et al.,* 1998), hypertrophy (Dunn *et al.,* 1999; Musaro *et al.,* 1999; Semsarian *et al.,* 1999), regeneration (Abbott *et al.,* 1998) and determination of type of fibre (Chin *et al.,* 1998). The effects of activation of calcineurin in muscle are mediated almost invariably through subsequent activation of the nuclear factor of activated T cells (NFAT) family of transcription factors, which together with calcineurin, are highly expressed in muscle (Abbott *et al.,* 1998). Increased intracellular calcium concentrations through insulin-like growth factor I (IGF-I)- or nerve-mediated responses invoke activation of calcineurin, resulting in the binding and dephosphorylation of the NFATs (Rao *et al.,* 1997) (Fig. 7). This action unmasks two nuclear localization sequences, resulting in rapid nuclear translocation (Beals *et al.,* 1997). In the nucleus, NFATs associate with other transcription factors to activate transcription. For example, association with GATA-2 promotes muscle hypertrophy (Musaro *et al.,*

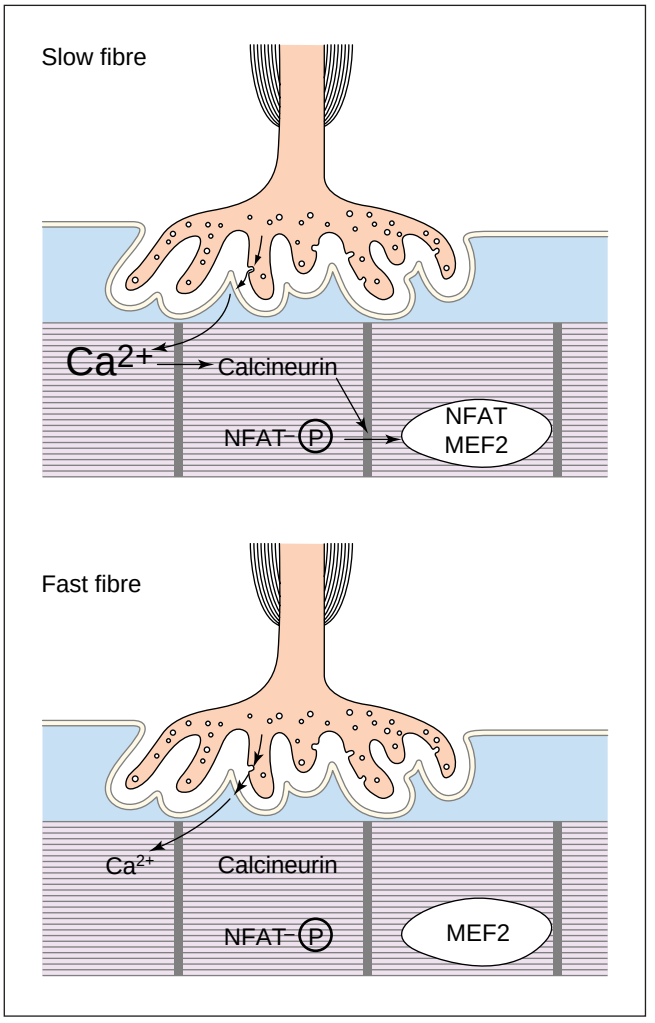

Fig. 7. Illustration of the regulation of calcineurin activity by calcium and the putative calcineurin-mediated determination of muscle fibre type. Diagrams represent motor endplates addressing (a) a slow muscle fibre and (b) a fast fibre. Calcineurin activity is regulated tightly by alterations in intracellular Ca^{2+} concentration. Slow fibre motorneurons are firing constantly, such that basal intracellular Ca^{2+} concentrations remain high (100–300 nmol l^{-1}) (Hennig and Lomo, 1985), and calcineurin remains active and dephosphorylates NFATs, which are translocated to the nucleus. In contrast, fast fibre neurones fire only sporadically, such that transient calcium spike values leave basal concentrations below 50 nmol l^{-1} (Westerblad and Allen, 1991), which is insufficient to activate calcineurin and thus NFATS remain phosphorylated. The presence of NFATs in the nucleus of slow fibres allows transcription of slow fibre-specific genes. Conversely, the absence of nuclear NFATs results in fast fibre-specific gene expression.

1999) and association with myocyte enhancer factor 2 (MEF2) and Sp1 induces oxidative or slow fibre-specific *myoglobin* and *troponin* 1_{slow} gene expression (Chin *et al.,* 1998). However, calcineurin regulation of muscle differentiation does not involve NFATs (Friday *et al.,* 2000). Instead, calcium and calcineurin are required for the transcriptional activation of myogenin and the initiation of myogenesis (Friday *et al.,* 2000).

As essentially the same calcineurin signalling pathway appears to control both the type and size of muscle fibres, how are signals regulating fibre-type selective expression distinguished from those driving hypertrophy? This question may be resolved by examining the differences in the control of additional signalling pathways modulating calcineurin-regulated transcription or the different threshold concentrations for activating fibre-type expression versus hypertrophy. Furthermore, differences may occur in both the range and abundance of transcriptional activating partners (that is, MEF2, GATA, or AP1 for NFATs) permitting the activation of different sets of downstream target genes.

The hypertrophic processes also depend on the maintenance of the sarcoplasmic : nuclear ratio, which is achieved through the inclusion of nuclei derived from the satellite cells that are retained beneath the basal lamina of differentiated fibres. As these cells are formed during fetal myogenesis and act to regulate long-term hypertrophic potential, the accumulation of satellite cells during development may have critical impact on the realization of hypertrophic potential.

Factors impinging on muscle development to affect adult performance

Fetal growth and development are limited both by the intrauterine environment and by genetic potential. Experiments with gene knockouts and knockins and in cell culture have revealed a great deal about the mechanistic regulation of myogenesis. However, much less is known about how the intrauterine environment interacts with the genetic potential of the fetus to affect muscle development.

Nutrition

The impact of maternal nutrition on fetal growth or birth weight is well documented, although not all studies provide specific information on skeletal muscle. In general, severe manipulations of maternal nutrition (for example, starvation or substrate restriction) lead to a reduction in the number of fetal fibres (Wilson *et al.,* 1988; Dwyer and Stickland, 1992) which appears to be irreversible (Prakash *et al.,* 1993), whereas moderate undernutrition tends not to affect muscle development. However, the results of studies in which the number of fibres was reduced indicate that primary fibres are resistant to manipulation, whereas secondary fibres are preferentially affected (Ward and Stickland, 1991; Dwyer and Stickland, 1992). Similarly, in situations in which the number of fibres is increased, it is the number of secondary fibres that appears to change. For example, doubling maternal feed intake during a critical period (days 25–50 of gestation) increased the mean number of secondary fibres formed in pigs (Dwyer *et al.,* 1994). There are a few examples in which natural undernutrition occurs, such as the study by Aberle (1984) in

which fetal growth retardation owing to placental insufficiency resulted in a reduced number of secondary fibres. In sheep with a low birth weight, the small reduction in muscle mass was not accompanied by a reduction in the number of myofibres, indicating that the number of fibres was set before the effect of the growth retardation occurred (Greenwood *et al.,* 2000).

Several studies have examined the impact of specific restrictions or additions of nutrients. Protein restriction throughout gestation (Desai *et al.,* 1996), reduced body weight and muscle mass up to weaning, and restoration of normal nutrition only partially reversed these effects. However, imposing a protein restriction immediately after birth by cross-fostering has almost as great an effect on body weight and muscle mass as protein restriction from conception to weaning. This finding indicates that there is an early postnatal period during which muscle development is highly sensitive to nutritional insults. During the early postnatal period, maximum gut growth occurs because, with the loss of placental supply, a functional nutrient uptake system via the gut becomes essential. Consequently, any manipulation to take advantage of the apparent sensitivity of muscle during this period must acknowledge the potential of any impact on gut growth.

Maternal undernutrition can restrict the efficiency and size of the placenta, which may affect fetal–neonatal outcome because the placenta is crucial in providing an environment that supports optimal fetal growth through nutrient supply, waste removal, immune and pathogen protection and the production of hormones and growth factors, some of which may act to modulate fetal IGF-I. The importance of IGF-I in fetal development has been postulated for a long time (for review, see Gluckman, 1995) and IGF-I has also been implicated in fetal reprogramming. A study in sheep has shown that maternal periconceptual undernutrition reprogrammes IGF-binding protein 3 (IGFBP-3) and IGF-I regulation in the developing fetus, so that subsequent responses to undernutrition in late gestation are altered (Gallaher *et al.,* 1998). Although no information relating to skeletal muscle was presented in this report, the findings may explain other observations. IGF-I is a potent mitogen, implicated in the regulation of cell proliferation–differentiation and protein anabolism. Thus, any periconceptual reprogramming of IGF responsiveness owing to undernutrition might alter myogenesis and account for some of the observed reductions in the number of fibres after early undernutrition.

Thinness at birth has been linked to an increased risk of insulin resistance in adult life (Phillips *et al.,* 1994). The mechanistic basis of this relationship is not clear, but because muscle is a major target of insulin action it was speculated that reduced muscle growth *in utero* may alter developmental programming and perturb insulin action. However, fetal undernutrition was not associated with abnormal muscle histology or blood flow, and the metabolic performance of several key muscle enzymes was normal (Thompson *et al.,* 1997); rather it appeared that a low ponderal index (birthweight/length^3) was associated with increased muscle oxygen supply.

Vitamin A is essential for normal embryogenesis (for review, see Ross *et al.*, 2000). The biological activity of vitamin A depends on its conversion to retinoic acid and the enzymes thought to be responsible for retinoic acid production have been identified in the limbs and somites of rat embryos, demonstrating that vitamin A is converted to its active form in embryos (Bavik *et al.,* 1997). The function of retinoic acid in mammalian embryonic myogenesis is not extensively documented, but in chick limbs, it appears to play an important role in the control of muscle patterning (Duprez *et al.,* 1999). In regenerating axolotl limbs, retinoic acid appears to induce the expression of Shh (Torok *et al.,* 1999) and it is possible that similar responses occur in the early embryo to underpin the control of patterning (Ross *et al.,* 2000). Studies of myogenesis *in vitro* show that retinoic-acid-induced growth arrest and differentiation in myoblasts is mediated largely through retinoid X receptors (Downes *et al.,* 1994). Retinoic acid acts in a dose-dependent manner to reduce *myf5* gene transcription (Carnac *et al.,* 1993), whereas the expression of myoD in myoblasts and myogenin in clonal rhabdomyosarcoma cells appears to be inducible by retinoic acid (Arnold *et al.,* 1992; Alric *et al.,* 1998). Although addition of exogenous retinoic acid to myoblasts *in vitro* does influence myogenesis, how variations in maternal concentrations of vitamin A impact on muscle development and patterning in the embryo remains unclear.

Taken together, these data indicate that early myogenesis is well protected against nutritional insult, which may reflect a strategy to ensure neonatal viability in times of nutritional stress. The data also support the contention of programming of the number of primary fibres (and hence the number of SO fibres), but indicate that both the number of secondary fibres and early postnatal muscle growth are sensitive to nutrient manipulation.

Effects of hormones, growth factors and environment

Hormones are central to the regulation of muscle growth and the regulation of fibre type (Walker and Luff, 1995). For example, administration of pig somatotrophin (pST) to sows early during gestation (between day 10 and day 24) increases the number of fetal muscle fibres (Rehfeldt *et al.,* 1993). Administration of pST later during gestation (between day 28 and day 48) not only alters embryonic development but also affects early postnatal growth of piglets (Kelley *et al.,* 1995). In the study of Kelley *et al.* (1995), embryonic survival, neonatal length, muscle mass and muscle cross-sectional area were increased, whereas back fat thickness was reduced, consistent with no change in birth weight. In addition, maternal uterine IGF-I expression appears to increase in treated sows, whereas fetal muscle myf5 expression is repressed and myogenin expression enhanced at day 41 of gestation. These findings indicate that myogenic hyperplasia is stimulated during somitogenesis and is consistent with the stimulation of Id

expression by pST (Mulvaney *et al.,* 1996). Single time point analyses are not particularly informative in a developmental situation; however, these findings do indicate an impact of pST treatment on myogenic gene expression programmes at the interface between proliferation and differentiation in the fetus with resultant changes in neonatal muscle. Although these data were not presented in these papers, information pertaining to treatment-related changes in muscle fibre might show an increase in the number of fibres in treated animals underlying the observed increase in muscle mass.

Experimentally induced hypothyroidism in pregnant mice induced a complete inhibition of postnatal muscle fibre type differentiation in the neonates (d'Albis *et al.,* 1987), whereas neonatal hyperthyroidism accelerated adult fast fibre type formation. Thus, there may be a window of sensitivity to stimuli early during development. Thyroid hormone also appears to work in conjunction with innervation mainly after birth (d'Albis *et al.,* 1990) to regulate fibre-type profiles and establish full realization of the phenotypic potential (White and Dauncey, 1999).

Synthetic β_2 adrenoceptor agonists that mimic the action of adrenaline have also been shown to affect myogenesis *in vivo*. Administration of clenbuterol to rats early during gestation reduces fetal weight and decreases secondary : primary fibre ratio, and leads to a reduction in muscle mass, protein, RNA and DNA content and total number of fibres after birth (Maltin *et al.,* 1990). These observations raise the question, pertinent to both animals and humans, as to whether high maternal concentrations of endogenous or exogenous β_2 agonists during early pregnancy have an effect on fetal myogenesis.

Further evidence detailing changes in myogenesis resulting from early embryonic insults supports the contention that events occurring before pregnancy is fully established can alter muscle growth and development, and also raises questions about the timing of myoblastic diversity. Two studies that used embryo transfer techniques (asynchronous transfer and co-culture, Maxfield *et al.,* 1998a,b) showed that preimplantation events could alter both the total number of fibres and the secondary : primary fibre ratio. The sensitivity of fetal myogenesis to procedures applied before implantation may support the concept that a pool of cells is specified to become muscle before the onset of somitogenesis. The mechanistic basis for these results is not clear, but it has implications in relation to technologies involving the manipulation of early embryos and may explain the fetal outcomes observed arising from the use of artificially produced or transferred embryos (Kruip and den Daas, 1997).

The embryo manipulation studies highlight again the observation that primary fibres appear to be resistant to manipulation. Thus, there is a body of data now indicating that not only are primary fibres genetically programmed, but that their programming does not appear to be affected by exogenous influences. From the point of view of the animal and in terms of the basic survival of the neonates, the protection of these fibres makes sense, as their presence in the limb musculature at least provides the means for the animal to maintain its position in gravity. Indeed, some animals such as the slow loris have few, if any, fast fibres (Ariano *et al.,* 1973).

It has been suggested that myostatin, a negative regulator of muscle mass, is important during primary and secondary fibre formation, as increased transcript concentrations have been noted around this period in pigs (Ji *et al.,* 1998). This suggestion is of interest because mutations in the myostatin gene are the cause of double muscling (resulting from an increased number and size of fibres) in cattle (McPherron and Lee, 1997), but it is not known whether the lack of myostatin affects both primary and secondary fibres in these animals. In mouse embryogenesis, myostatin is expressed in the somitic domain, which gives rise to skeletal muscle (Lee and McPherron, 1999), so it is not clear whether myostatin acts at this early stage to regulate the number of fibres, or whether later expression regulates prenatal fibre growth. Lee and McPherron (1999) proposed that, in its role to regulate muscle mass, myostatin functions as a 'chalone' (a secreted molecule that inhibits cellular function). Myostatin has to meet a number of functional prerequisites in order to be considered as a 'chalone', one of which is that myostatin should circulate *in vivo*. Although this possibility is explored, the intriguing observation that when fetuses with different genetic propensity for muscle development are twinned *in utero*, there is a reduction in the muscle mass but an apparent increase in the number of fibres in the heavier muscled fetus (Gerrard *et al.,* 1995) should be considered. If myostatin is a 'chalone' and is produced by the less muscled animals, then this might account for the reduction in muscle mass seen in the heavier muscled animals. Although the apparent increase in the number of fibres is not consistent with this hypothesis, close examination of the methods used to make this estimate (Gerrard and Judge, 1993) including the measurement of muscle circumference by tape measure, indicate that the estimate may be unreliable.

Transformations of fibre type and postnatal hypertrophy clearly affect the performance of the muscle in adults and, therefore, may be more sensitive to manipulation. Postnatal hypertrophy may also be influenced by innervation and thyroid hormone, since it appears that hypothyroidism early during postnatal life inhibits the incorporation of satellite cells into existing fibres, thereby reducing the growth potential of the muscle (Jacobs *et al.,* 1996). Thus, while some of the key factors (for example, hormones, innervation) that influence muscle in this sensitive postnatal period have been identified, a greater understanding of the postnatal regulation and maintenance of muscle characteristics is required to achieve targeted manipulation of muscle.

Genetic selection

Optimal muscle mass is often the commercial endpoint in agriculture. Animal breeding strategies (Robinson and Bradford, 1969) for meat production have focussed on

selecting for muscle mass and growth rate and, during this process, many have selected increases in the numbers and size of fibres, indicating that these traits are heritable. This heritability has been noted in commercial populations of pigs, in which divergent selection has produced populations with different muscle fibre proportions, particularly with respect to FG and SO fibres (Maltin *et al.,* 1997). Many 'improvements' in muscle have been made on the basis of achieving increased muscle mass in the adult. For example, the P-line quail (Coutinho *et al.,* 1993) has been developed through selection procedures for increased body weight, which has been achieved through an increased number of fibres. Increased numbers of fibres is associated with delayed somite formation and delayed expression of MRFs and MHC. Genetic selection is a powerful means by which to manipulate the performance potential of muscle. However, in most species this is not a rapid procedure and, in some cases, it is possible that selection has taken muscle to its full genetic potential. The rather arbitrary nature of the selection criteria (for example, muscle mass in livestock species rather than eating quality) is also a concern. If genetic 'improvement' of muscle is to be achieved, then greater understanding of the regulation of the performance parameters to be 'improved' is needed, so that detection and selection strategies for the key genes can be developed.

Conclusions and implications

Muscle mass and function are important for life. Muscle development starts early during embryogenesis and factors that impinge on the developing fetus can affect muscle development. The major impacts on myogenesis thus far identified appear to be associated with extremes of treatments or embryo manipulations, in which cases responses are seen predominantly in the secondary fibres and the primary fibres appear insensitive or protected. The mechanistic basis of this differential responsiveness is not known.

Many questions surround the relationship of muscle development with adult performance, not least of which is how the apparent heritability of fibre types affects performance and developmental sensitivity. Evidence indicates that the composition of muscle fibre types has a heritable component, particularly with respect to SO fibres that are largely derived from primary fibres. Is this proposed genetic control the underlying mechanism or reason for the insensitivity of primary fibres to insults? If the evidence of heritability is fully substantiated, then the evidence in humans and livestock species indicates that it would be beneficial to be able to select for or manipulate the numbers of SO fibres. Thus, the search for the genotypic basis of SO–primary fibre formation would become one of the Holy Grails in modern muscle biology.

This review has highlighted the limitations of opportunities for the manipulation of myogenesis to improve adult muscle performance. Most manipulations reported to date have not benefited adult muscle performance but have reduced the number of fibres or increased fatiguability. Although the number of muscle fibres can be increased through embryo manipulations or pST administration, it is doubtful whether this strategy, with the attendant problems of dystocia and welfare issues, is a wise one. Although early myogenesis is largely unaffected by all but extreme insults, early postnatal muscle growth may be susceptible to impacts that affect adult performance. As most muscle growth and fibre type transformations occur after birth, postnatal muscle growth and development is a key area of investigation for the future.

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