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Key signalling factors and pathways in the molecular determination of skeletal muscle phenotype

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The molecular basis and control of the biochemical and biophysical properties of skeletal muscle, regarded as muscle phenotype, are examined in terms of fibre number, fibre size and fibre types. A host of external factors or stimuli, such as ligand binding and contractile activity, are transduced in muscle into signalling pathways that lead to protein modifications and changes in gene expression which ultimately result in the establishment of the specified phenotype. In skeletal muscle, the key signalling cascades include the Ras-extracellular signal regulated kinase-mitogen activated protein kinase (Erk-MAPK), the phosphatidylinositol 3′-kinase (PI3K)-Akt1, p38 MAPK, and calcineurin pathways. The molecular effects of external factors on these pathways revealed complex interactions and functional overlap. A major challenge in the manipulation of muscle of farm animals lies in the identification of regulatory and target genes that could effect defined and desirable changes in muscle quality and quantity. To this end, recent advances in functional genomics that involve the use of micro-array technology and proteomics are increasingly breaking new ground in furthering our understanding of the molecular determinants of muscle phenotype.

Keywords: hyperplasia, hypertrophy, meat quality, muscle fibres, skeletal muscle

Introduction

Importance of muscle fibre phenotype

The key constituents of muscle are muscle fibres, and their associated extracellular matrix in which are located the vascular supply, collagenous component and adipose tissue (intramuscular fat) of muscle. Muscle phenotype conferred by fibres is central to the quantity and quality of meat production. Quantity is the outcome of muscle fibre hyperplasia and hypertrophy. Hyperplastic growth *in utero* is primarily dependent on myocyte proliferation and differentiation (Oka *et al*., 2002). Post-natal growth is mainly the result of hypertrophy or enlargement of existing and replaced muscle fibres. Quality is a more complex trait and is assessed by a variety of objective and subjective measurements, such as colour, pH, tenderness, odour and juiciness. It is well established that fibre type composition is a key determinant of both meat quantity and quality. In the pig, in particular, favourable meat traits such as tenderness and colour, have been found to closely associate with the greater abundance of oxidative fibres (Klont *et al*., 1998; Karlsson *et al*., 1999; Chang *et al*., 2003; Maltin *et al*., 2003; Wood *et al*., 2004). Red or highly oxidative muscles possess higher lipid concentration which is associated with more tender meat (Hocquette *et al*., 1998; Wood *et al*., 1999 and 2003). Fast-glycolytic fibres (see later for details), on the other hand, being relatively the largest fibre type are a major fibre type for muscle hypertrophy as well as in the predisposition of pale, soft and exudative (PSE) pork independent of *ryanodine receptor 1* mutation.

In the living animal, muscle phenotype is a major factor in the functional integrity of mobility and movement, as well as of thermoregulation, which in turn governs its quality of life. A wide range of disease conditions often adversely affect muscle phenotype and lead to debilitating muscle wasting or atrophy. For instance, in human obesity, triglyceride accumulation in muscle (intramuscular fat) appears to be related to the development of insulin resistance and type 2 diabetes (Morio *et al*., 2001; Kelley *et al*., 2002; Wolfe, 2006). Hence, knowledge of the molecular events that affect muscle phenotype is of fundamental, agricultural, welfare and biomedical importance. By understanding the signalling processes and gene targets that effect muscle phenotype, strategic approaches, such as the use of marker-assisted selection (Beuzen *et al*., 2000), dietary manipulation (Casser-Malek *et al*., 2004) or even pharmacological targeting of novel effector genes, could be developed to improve muscle quality and quantity.

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This review endeavours to bring together recent advances in our understanding of the signalling pathways, and molecular mechanisms of several major growth promoting factors that affect muscle fibre phenotype relevant to farm animal production. Given that the subject area covered is wide, its purposes are to provide the reader with a general overview of the molecular aspects of muscle phenotype determination in the live animal and to highlight avenues for future research exploitation.

What is muscle fibre phenotype?

Post-natal muscle is a highly heterogeneous syncytial tissue, comprising muscle fibres and extracellular matrix, with the ability to rapidly undergo biochemical and physical fibre changes in response to appropriate external stimuli, such as nervous and hormonal stimulations, to adapt to the accompanying functional demands imposed on it (Caiozzo, 2004). The feature of functional plasticity indicates that skeletal muscle is highly amenable to changes in coordinated gene expression. Although the extracellular matrix of muscle, as exemplified by the orderly arrangement of endomycium, perimycium and epimycium, is a key determinant of meat quality (Fang *et al*., 1999; McCormick, 1999), this review is focussed on the regulation of muscle fibre phenotype. The physical and biochemical characteristics of muscle fibres can be conveniently defined under three distinct but overlapping categories: fibre number, fibre size and fibre type (Figure 1). Fibre types reflect differences in their biochemical (metabolic) and biophysical properties that arise from differences in coordinated expression of muscle gene isoforms (Schiaffino and Reggiani, 1996).

A feature of muscle fibre phenotype regulation that will be apparent is that the same signalling factor or pathway (such as IGF-1) can often have multiple phenotypic effects with regard to its influence on fibre number, size and type.

Muscle formation and regeneration

In vertebrates, most skeletal muscles are derived from muscle progenitor or precursor cells present in somites, which arise by segmentation of the paraxial mesoderm, located on either side of the notochord and neural tube in the early embryo. As it develops, each somite can be divided into the epaxial dermomyotome, which gives rise to the epaxial muscles, and into the hypaxial dermomyotome, from which derives the rest of the body and limb muscles. The first muscle mass to form, under the dermomyotome, is the myotome, which contributes to the trunk muscles. The early developmental events surrounding myogenesis are under intense molecular scrutiny (Buckingham, 2001). Myogenic cell fate specification is acquired from the activation of myogenic determination genes, namely the basic helix-loop-helix (bHLH) proteins encoded by *Myf-5* and *MyoD*. The other two members of the myogenic bHLH gene family, *myogenin* and *Mrf4*, are implicated, along with *MyoD*, in the subsequent activation of muscle-specific genes during myogenic differentiation. MEF-2 family, characterised by the presence of a MADS-box motif, also plays an important role in muscle differentiation (Buckingham *et al*., 2003). Prior to the activation of *Myf-5* or *MyoD*, signallings by Wnts, produced by the dorsal neural tube and surface ectoderm, and hedgehog (Hh) proteins, from the notochord and floor plate of the neural tube, are needed to serve as early triggers or inductive signals of myogenesis (Tajbakhsh and Buckingham, 2000). Hh signalling plays an additional role in committing progenitor cells to the slow-twitch lineage, a process specified by Blimp-1, originally identified as a factor that promotes B-cell maturation (Baxendale *et al*., 2004).

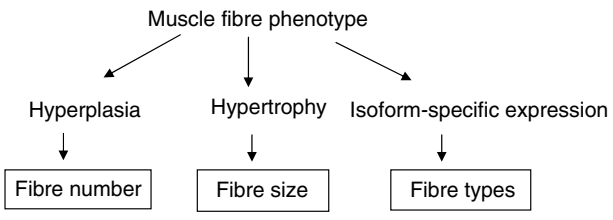

Figure 1 Categories of skeletal muscle fibre phenotype.

In limb muscle development, muscle progenitor cells opposite the limb buds must first delaminate from the hypaxial dermomyotome and migrate into the limb region. A number of homeobox proteins have been found to be essential for this migrational process (e.g. Pax3, c-met and its ligand hepatocyte growth factor (HGF)/scatter factor, and Lbx1) (Buckingham *et al*., 2003). Upon arriving at their destination, they begin to express MyoD and Myf-5 to mark the onset of myogenesis.

After appropriate rounds of cell proliferation, myoblasts leave the cell cycle and fuse to form multinucleated myotubes. The homeobox proteins of *Mox2* and *Msx1*, and several growth factors, including insulin-like growth factor (IGF)-1 and -2 (Morali *et al*., 2000), fibroblast growth factors (FGFs), bone morphogenic proteins (BMPs) and platelet-derived growth factors, variously contribute to the regulation of proliferation and differentiation (Zorzano *et al*., 2003). The first muscle fibres that appear are known as primary fibres (around gestation day 35 in the pig and 12.5 days in the mouse), around which subsequent secondary fast fibres form at the time when innervation begins to be established (beginning at day 50 to day 87 in the pig and day 15 in the mouse) (Picard *et al*., 2002; Buckingham *et al*., 2003; Da Costa *et al*., 2003; Caiozzo, 2004).

As post-mitotic cells, skeletal muscle fibres are unable to undergo self-replication. Damaged fibres are replaced with newly synthesised fibres through the proliferation, differentiation and subsequent fusion of muscle satellite cells (myoblasts). Satellite cells constitute a reservoir of undifferentiated muscle precursor cells, located between the basal lamina and the sarcolemma, that are activated in response to muscle injury or growth stimuli to proliferate and ultimately fuse to generate new fibres. Muscle satellite cells are characterised by the expression of myostatin,

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a transforming growth factor (TGF)-β like factor implicated in limiting muscle growth, c-met, Myf-5 and Pax-7 (Buckingham, 2001; Buckingham *et al*., 2003). It appears that not all muscle satellite cells are derived from somitic cells. There exists other population(s) of muscle precursor cells in skeletal muscle, the adult muscle stem cells, with haematopoietic and myogenic potentials (Asakura, 2003; Relaix, 2006). These adult muscle stem cells express haematopoietic markers, such as c-kit, CD45, CD34 and Sca1 (markers that are absent on satellite cells) but not myogenic markers like Pax7 and Myf-5 (present in satellite cells) (Asakura, 2003; Polesskaya *et al*., 2003). Recent work suggests that during growth and regeneration, adult stem cells proliferate and undergo phenotypic conversion, which involves Wnt signalling, into myogenic satellite cells (Asakura, 2003; Polesskaya *et al*., 2003). It is increasingly apparent that many of the regulatory genes involved in embryonic myogenesis are also required in post-natal muscle fibre formation by satellite cells.

Fibre number: mediators of muscle hyperplasia

It is generally regarded that by birth, an animal, such as the pig, would have nearly the same number of muscle fibres as in adulthood (Wigmore and Stickland, 1983). Therefore, the extent of fibre number formation or hyperplasia during foetal development will have a major bearing on muscle growth potential. In post-natal muscle, fibre number may not necessarily be constant. Periodic repair and replacement of damaged fibres are necessary to maintain functional integrity, a process performed by satellite cells that can proliferate and fuse with damaged fibres or fuse to form new fibres (Goldring *et al*., 2002). The relative contribution of satellite cells to the formation of new fibres (hyperplastic growth) and to existing fibres (hypertrophic growth) is not clear, but is likely to be dependent on the nature of the inductive signals. Fibre number determination can be affected by any factor that plays a role in embryonic or post-natal myogenesis through myoblast specification, proliferation and/or differentiation. The main signalling factors to be considered that affect fibre number are IGF-1 and -2, myostatin and nutrition.

IGF-1 and IGF-2 activate Erk-MAPK pathway of cell proliferation (hyperplasia)

Growth factors such as IGFs and FGF by virtue of their ability to stimulate cell proliferation are regarded as potent agents that can affect fibre number, pre- and post-natally (Bass *et al*., 1999). Indeed, daily injections of growth hormone (GH) in the sow during early pregnancy has been shown to enhance foetal fibre number (Rehfeldt *et al*., 2001), an effect likely to be mediated by IGF-1. IGF-1 and -2 stimulate both muscle cell proliferation and differentiation through the interaction with the type 1 IGF receptor (IGF1R), insulin receptor exon 11- (IR-A) and insulin receptor exon11 + (IR-B), all of which are transmembrane tyrosine kinase receptors (Denley *et al*., 2005). These activated receptors initiate two major signalling cascades (Figures 2 and 3). The type 2 IGF receptor (IGF-2R) (known also as mannose-6-phosphate receptor) and a family of high affinity IGF binding proteins (IGFBPs) 1 to 6 modulate the availability of IGF-1 and IGF2 to bind to receptors. IGF-2R has no intrinsic signalling transduction capability and serves to sequester IGF-2 from potential receptor interactions and to internalise and degrade IGF-2 (Denley *et al*., 2005). Liver is the main endocrine source of IGF-1 (IGF-1Ea), which is induced by GH. Additionally, damaged, stretched or load-bearing muscles are local sources IGF-1 and IGF-2 which induce proliferation and hypertrophy in an autocrine / paracrine manner (Adams, 2002). Besides the major IGF-1Ea isoform, a spliced variant with a carboxyl terminus different from IGF-1Ea, named MGF (mechano growth factor), is rapidly inducible in skeletal muscle and appears to be an early trigger for satellite cell proliferation (Yang and Goldspink, 2002). The autocrine / paracrine muscle production of IGF-2 during muscle differentiation and regeneration was found to participate in a positive feedback loop to further enhance muscle differentiation (Erbay *et al*., 2003; Wilson *et al*., 2003) and regeneration (Kirk *et al*., 2003). Although both IGF-1 and -2 are clearly important to muscle growth, their relative contribution to cell proliferation, differentiation and hypertrophy is uncertain.

IGF-1 (as well as insulin and IGF-2) binding activates the receptor tyrosine kinase (IGF1R or insulin receptor), which subsequently recruits insulin receptor substrate 1 (IRS-1), a non-enzymatic docking protein that propagates the signal to two crucial signalling pathways, the mitogen-activated protein kinase (Erk-MAPK) pathway, via Ras-Raf-MEK-Erk (Rommel *et al*., 1999; Zimmermann and Moelling, 1999; Glass, 2003a) and the phosphatidylinositol 3'-kinase (PI3K)-Akt1 pathway (Glass, 2003b; Vollenweider, 2003). The Ras-Erk MAP kinase (Erk-MAPK) pathway, a serine/threonine phosphorylation cascade, is responsible for cell proliferation and plays an important role in hyperplastic growth (Figure 2). The PI3K-Akt1 pathway is a major route to muscle differentiation and hypertrophy (see later section). *Ras* is a membrane bound GTPase, and cycles between an inactive *Ras*-GDP to an active *Ras*-GTP. Expression of oncogenic Ras inhibits myogenic differentiation (Mitin *et al*., 2001) in part by promoting cell proliferation via the MAP kinase pathway. Interestingly, activated Ras was also found to induce the expression of slow myosin heavy chain (MyHC), although the downstream mechanism that leads to this fibre type-specific effect is not understood (Murgia *et al*., 2000). Both Erk-MAPK and PI3K-Akt1 pathways are necessary and complement each other in mediating post-natal muscle growth (Haddad and Adams, 2004).

Myostatin inhibits cell proliferation

Myostatin (Mst), also known as growth and differentiation factor 8 (GDF-8), is a secreted negative regulator of muscle

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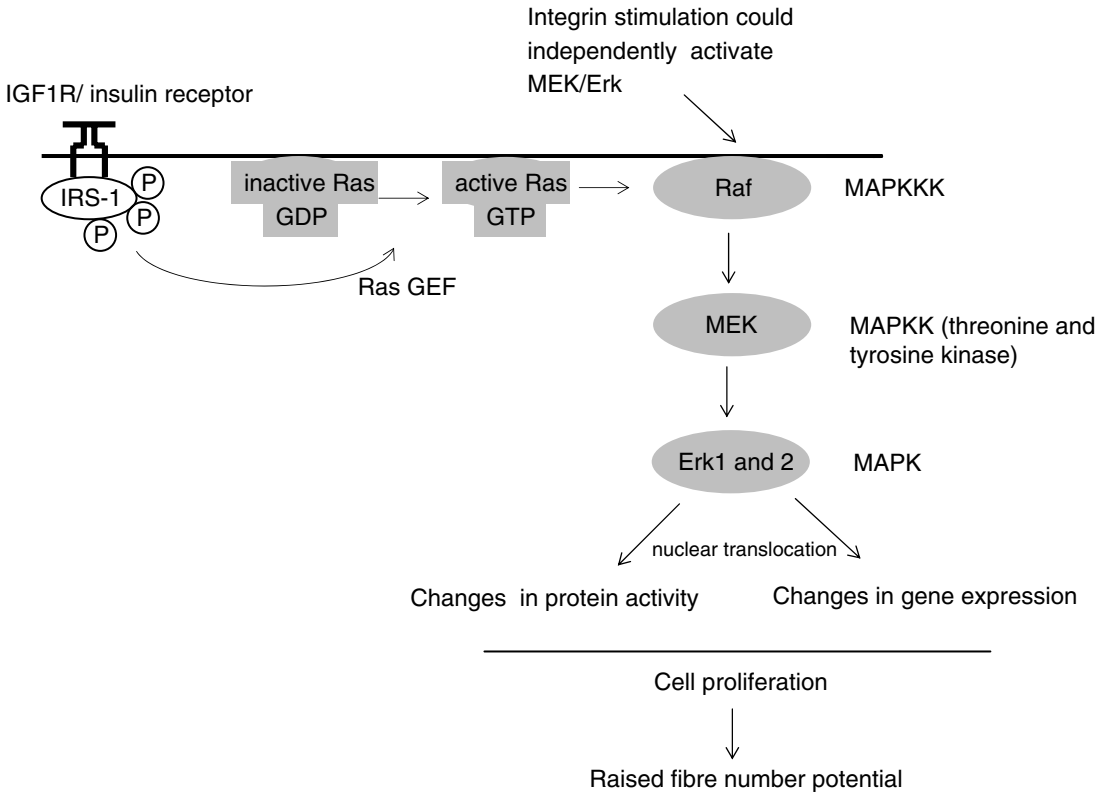

Figure 2 The IGF-induced Erk-MAPK proliferation signalling pathway of hyperplasia. IGF-1, IGF-2 or insulin binding to tyrosine kinase receptor triggers a phosphorylation cascade that leads to transcriptional and protein modifications, culminating in increase cell proliferation. Integrin can also activate the Erk-MAPK pathway independent of tyrosine kinase receptor binding. Note that activated Ras can also activate the PI3K pathway.

mass that belongs to the transforming growth factor (TGF)-β superfamily (Kocamis and Killefer, 2002). Its biological significance is demonstrated in several breeds of double-muscling cattle (e.g. Belgian Blue and Piedmontese) and *Mst*-null mice as dramatic increase of muscularity through fibre hypertrophy and hyperplasia (Grobet *et al.*, 1997; McPherron and Lee, 1997; Marchitelli *et al.*, 2003). The Belgian Blue carries a naturally occurring homozygous 11-bp deletion in the coding region of *Mst* whereas the Piedmontese possesses a homozygous G → A point mutation that changes the cysteine residue in *Mst* to a tyrosine (Kambadur *et al.*, 1997). The porcine *Mst* gene has been molecularly cloned and characterised, but a mutation that confers the enlarged muscle phenotype has not been described (Ji *et al.*, 1998). Mst arrests muscle cells in the G_1 and G_2 phases of the cell cycle, through the up-regulation of cyclin-dependent kinase (cdk) inhibitor *p21* and down-regulation of cdk-2, thereby inhibiting cell proliferation (Thomas *et al.*, 2000). This inhibition is mediated, at least in part, through the p38 MAPK stress response pathway (Philip *et al.*, 2005) (Figure 4). Mst also appears to directly inhibit muscle differentiation by interfering with the activity of MyoD (Langley *et al.*, 2002). Muscle wasting conditions, such through disease or disuse atrophy, are associated with elevated Mst in affected muscles (McCroskery *et al.*, 2003). A primary effect of *Mst* inactivation in post-natal growth is an increase of satellite cell proliferation and differentiation (McCroskery *et al.*, 2003). GH, mediated through IGF-1, was shown to inhibit *Mst* expression in skeletal muscles and C2C12 myotubes (Liu *et al.*, 2003). Conversely, *Mst* expression in porcine embryonic myogenic cells up-regulates the production of IGF binding protein-3 (IGFBP-3), which reduces the activities of IGF-1 and -2 (Kamanga-Sollo *et al.*, 2003). These results suggest that the Mst and IGF signalling pathways are closely connected in an antagonistic manner.

Recent micro-array investigations further found that *Mst*-null mice exhibited raised Wnt4 expression which stimulated satellite cell proliferation (Steelman *et al.*, 2006). The use of both micro-array and proteomic analyses showed a clear fibre phenotype switch in *Mst*-null muscles from slow to fast-twitch fibres (Bouley *et al.*, 2006; Steelman *et al.*, 2006) (see section IGF-1, β_2-agonist and myostatin-null signal fast fibre phenotype). Mst has been recently found to promote adipogenesis in C3H 10T(1/2) cells which appears to be associated with adipocyte lineage commitment (Artaza *et al.*, 2005). However, in committed bovine preadipocytes, Mst clearly suppresses differentiation of preadipocytes into adipocytes (Hirai *et al.*, 2007).

Nutrition on fibre number and characteristics

Nutrition in animal growth is a complex subject of major importance, which due to its enormity cannot be fully

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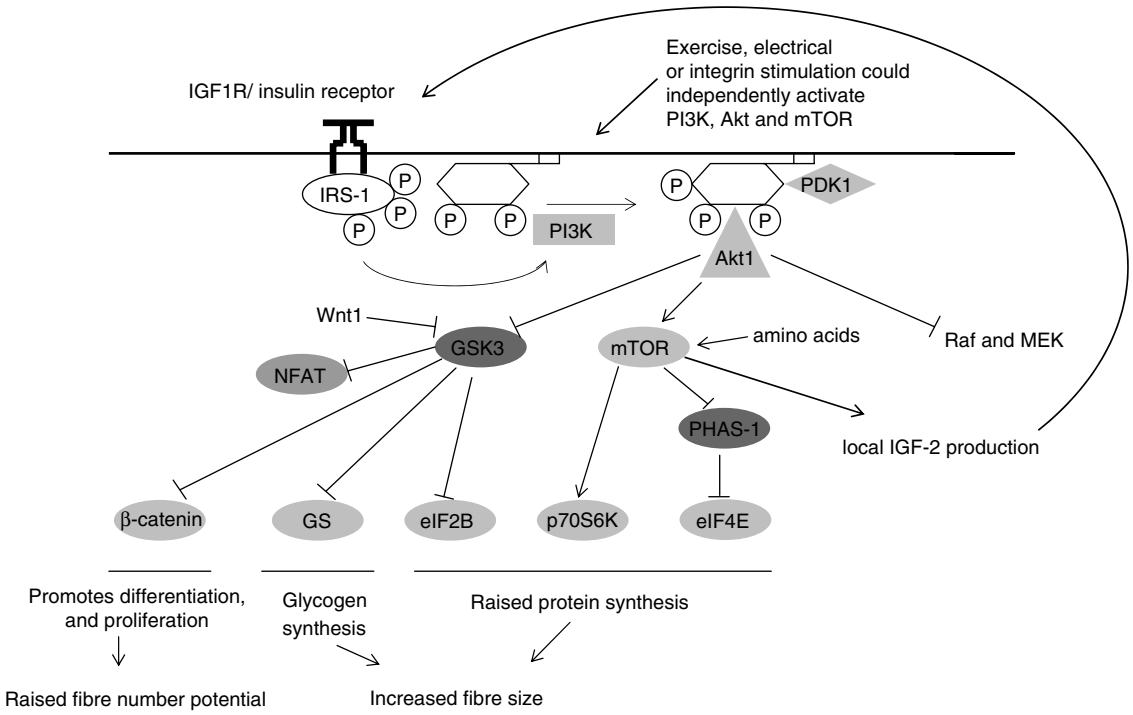

Figure 3 The IGF-induced PI3K-Akt1 signalling pathway of muscle differentiation and hypertrophy. It affects a range of cellular processes, including the promotion of protein synthesis, glycogen synthesis, and cell proliferation and differentiation, that culminate in muscle hypertrophy. mTOR can be activated by amino acids (nutrients) and induce local IGF-2 production which, in turn, acts in a positive feedback manner. The PI3K cascade can be independently activated by exercise and by integrin stimulation (see also integrin stimulation of Erk-MAPK pathway, Figure 2). Activated Akt1 via inhibiting Raf and MEK phosphorylation is involved in inhibitory crosstalk with the Erk-MAPK pathway. Selected abbreviations: PI3K = phosphatidylinositol 3′-kinase, PDK1 = phosphatidylinositol-dependent protein kinase 1, GSK3β = glycogen synthase kinase 3 β, mTOR = mammalian target of rapamycin, GS = glycogen synthase, and p70S6K = ribosomal protein S6 kinase. Note that Akt, GSK3β, mTOR, p70S6k and PHAS-1 are serine/threonine kinases.

addressed in the present review. Maternal nutrition is undoubtedly a key factor in foetal growth and its subsequent survival which manifests its effects through a complex signalling network that includes IGFs (Hornick *et al.*, 2000; Maak *et al.*, 2001; Wu *et al.*, 2006). However, in the context of foetal fibre number, the precise role of maternal nutrition is not entirely clear. Earlier work suggests that doubling food intake during early sow pregnancy increases the number of secondary fibres in the newborn, which would confer greater potential for post-natal growth (Dwyer *et al.*, 1994). More recent work, however, could not reproduce this finding (Nissen *et al.*, 2003).

Connected to nutrition, mild dietary restriction on beef cattle has little apparent effect on muscle characteristics or meat quality (Casser-Malek *et al.*, 2004). However, dietary changes during weaning in calves appear to have an appreciable effect on increasing the number of oxidative fibres (Picard *et al.*, 1995). Under moderate protein and energy dietary restriction (20% less protein and 7% less energy) in young growing pigs, we recently found, with the use of a porcine cDNA muscle micro-array, significant increase in the accumulation of intramuscular fat, which could have production implications on meat quality (Da Costa *et al.*, 2004). A similar dietary restriction study has been reported on Brahman cattle but no data on meat characteristics are provided (Byrne *et al.*, 2005). Lamb and beef animals raised on grass showed greater flavour intensity in comparison with grain-fed animals because of a higher accumulation of 18:3 polyunsaturated fatty acids from a grass diet (Wood *et al.*, 1999).

Fibre size: mediators of muscle hypertrophy/atrophy

Post-natal muscle growth is primarily a function of enlargement and elongation of existing fibres as a result of net protein synthesis, known as muscle hypertrophy (Figure 1) (Glass, 2003b). Conversely, loss of muscle mass as a consequence of disease or muscle inactivity is primarily due to a reduction in fibre size, described as muscle atrophy (Glass, 2003b). Muscle differentiation is a prerequisite to hypertrophy and, as detailed below, the same factors are often involved in both processes (differentiation and hypertrophy). In this section, we consider some well known signalling factors and pathways that mediate muscle hypertrophy or atrophy (IGF-PI3K pathway, contractile activity, p38 MAP kinase pathway, synthetic β_2 adrenergic agonists, anabolic steroids and *ski* proto-oncogene.

IGF activates PI3K-Akt1 pathway of hypertrophy

The IGF-activated PI3K-Akt1 signalling pathway (Figure 3) is widely regarded as the primary route to skeletal muscle differentiation and hypertrophy (Coolican *et al.*, 1997; Jiang *et al.*, 1998; Glass, 2003b). Phosphatidylinositol 3′-kinase

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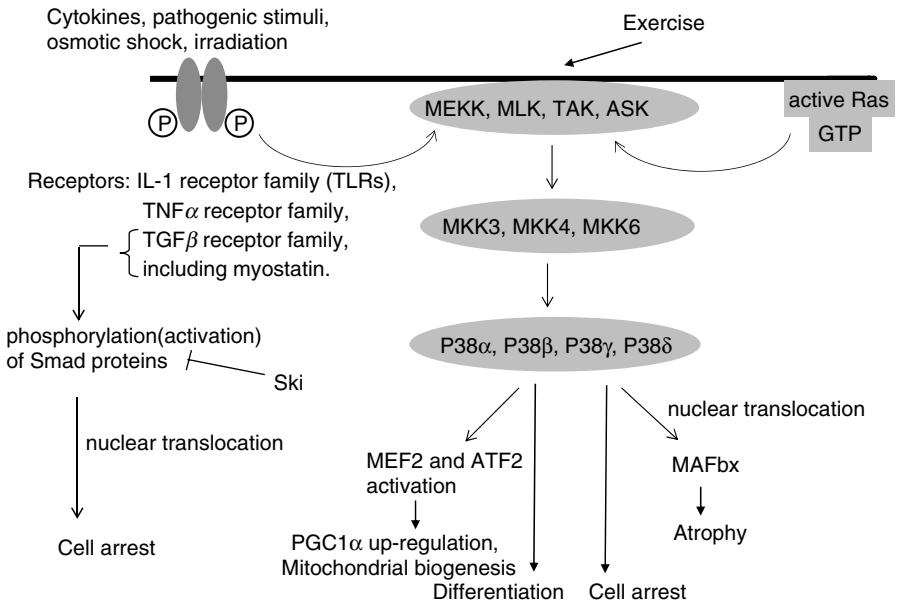

Figure 4 The p38 MAPK stress response pathway of cell arrest and atrophy. It is activated by a variety of stimuli, including exercise and activated Ras, and through different receptor families. It mediates a range of effects: cell arrest, differentiation, atrophy as well as mitochondrial biogenesis. Additionally, TGFβ receptors readily signal through Smad proteins which also lead to cell arrest. *Ski* functions to inhibit Smad signalling thus relieving cell arrest and promoting proliferation. Selected abbreviations: IL-1 = interleukin 1, TLRs = toll-like receptors, TNF α = tumour necrosis factor α, and TAK = TGFβ-activated kinase.

(PI3K) is a dimeric lipid kinase that catalyses the phosphorylation of membrane-bound phosphatidylinositol (4,5)-bisphosphate (PIP_2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). PIP_3 provides a membrane-binding site for Akt1 (also known as protein kinase B) a serine/threonine kinase, and phosphatidylinositol-dependent protein kinase 1 (PDK1) (Figure 3). Akt1 is phosphorylated by PDK1, and once activated Akt1 phosphorylates a number of substrates that are responsible for a range of growth processes, which include protein synthesis, glycogen synthesis, muscle differentiation and cell proliferation. A key target of activated Akt1 is the phosphorylation of mTOR (mammalian target of rapamycin), a serine/threonine kinase, which in turn activates a number of downstream effectors including ribosomal protein S6 kinase (p70S6K) and eIF-4E, both of which mediate translation initiation and appear to play important roles in muscle hypertrophy. mTOR has also been shown to mediate myogenesis by a process that is independent of its kinase activity, although the exact mechanism involved is unclear (Erbay and Chen, 2001). During differentiation, mTOR was found to induce the local production of IGF-2, and it seems that the myogenic effect of mTOR is mediated in part through a positive feedback loop with IGF-2 (Erbay *et al.*, 2003). There is evidence that mTOR can also be activated by amino acids, independent of Akt1 (Parkington *et al.*, 2003) and that muscles of nutrient-restricted pregnant cows showed reduced phosphorylation activation of mTOR (Du *et al.*, 2005).

Glycogen synthase kinase 3β (GSK3β), a serine/threonine kinase, is another major substrate of activated Akt1 that modulates muscle hypertrophy (Figure 3). Like mTOR, GSK3β can also be phosphorylated independent of Akt1. Activation of protein kinase A or C is able to lead to the phosphorylation of GSK3β (Jope, 2003). Unstimulated (dephosphorylated) GSK3β is active and its phosphorylation activity appears to act as a general repressor on its substrates. Many substrates of GSK3β have been identified, including glycogen synthase, c-Jun, cyclin D1, β-catenin, nuclear factors of activated T-cells (NFATs), and Notch2 (Masuda *et al.*, 1998; Espinosa *et al.*, 2003). The inhibition of GSK3β by phosphorylation, leads to dephosphorylation activation of glycogen synthase, resulting in glycogen synthesis, and to activation of eIF-2B, which promotes protein synthesis (Rommel *et al.*, 2001). A primary effect of GSK3β inhibition on muscle is the promotion of hypertrophy (Rommel *et al.*, 2001; Vyas *et al.*, 2002). Clearly, with its wide range of targets, GSK3β can mediate a host of other effects. GSK3β inhibition, by lithium chloride, improves glucose uptake of muscle cells, which makes it a pharmacological target for the treatment of diabetes (MacAulay *et al.*, 2003). Wnt signalling leads to the inhibition of GSK3β, an integral component of the linear cascade that results in the stabilisation of β-catenin, a co-activator necessary for cell proliferation (Novak and Dedhar, 1999; Giles *et al.*, 2003) and myogenic differentiation (Martin *et al.*, 2002; Petropoulos and Skerjanc, 2002; Shi *et al.*, 2002). Hence the inhibition of GSK3β facilitates cell proliferation, muscle differentiation (via β-catenin) and, subsequently, hypertrophy.

Contractile activity and integrins

Muscle hypertrophy can be an adaptive response to load-bearing exercise, which stimulates the local expression of

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IGF-1, a potent inducer of muscle hyperplasia and hypertrophy. There is also growing evidence that key signalling intermediates in the Erk-MAPK and PI3K-Akt1 pathways can be activated independent of growth factor stimulation (Figures 2 and 3). Integrins are heterodimeric ($\alpha\beta$) transmembrane adhesion proteins that link the actin cytoskeleton to the extracellular matrix, and participate in a vast array of signalling events including mechanical sensing, cell growth and cell migration. Stimulation of β_3 integrin by a synthetic Arg-Gly-Asp ligand in feline cardiomycytes led to activation of PI3K, mTOR, p70S6K and MEK/Erk (Balasubramanian and Kuppuswamy, 2003). It appears that in rats exposed to certain treadmill running regimes, and in certain isolated muscles subjected to passive stretch, Akt1 can be independently activated (Sakamoto *et al*., 2003). mTOR, but not Akt1, was also found to be activated in rat hindlimb muscles subjected to 6 hours of high frequency electrical stimulation (Parkington *et al*., 2003). Hence exercise or electrical stimulation can variously activate the Erk-MAPK and PI3K-Akt1 pathways as well as the p38 MAPK cascade (see next section) in the regulation of muscle development.

p38 MAP kinase pathway of cell arrest and atrophy

p38 MAP kinase represents another MAPK signal transduction pathway that is activated by cellular stress (e.g. heat shock, oxidative stress and certain cytokines) as well as insulin (Conejo *et al*., 2001; Lee *et al*., 2002). The p38 MAPK pathway is intriguing in that it appears to mediate a range of cellular processes in muscle besides fibre size (Figure 4). There are four members of the p38 MAP kinase family: p38 MAPK-α, -β, -γ and -δ. The dual specificity kinases, MKK3 and MKK6, are involved in p38 MAP kinase activation, with MKK3 activating p38 MAPKα and β, and MKK6 activating all four p38 MAPK isoforms (Lee *et al*., 2002) (Figure 4). A variety of receptor families (including interleukin-1 [IL-1], tumour necrosis factor α [TNFα] and TGFβ) signal through this cascade. Signalling of p38 MAPK has been reported to be essential for terminal differentiation (myoblasts fusion into myotubes), by enhancing the expression of *MyoD*, *MEF2A*, *MEF2C*, sarcomeric muscle genes and cdk inhibitor *p21* (Cabane *et al*., 2003; Wu *et al*., 2000c). p38 MAPK has the added role of inhibiting the Erk-MAPK pathway thereby inducing cell cycle arrest during muscle differentiation (Lee *et al*., 2002). It is interesting to note that the Erk-MAPK pathway is also inhibited by activated Akt1 through the prevention of Raf and MEK phosphorylation (Rommel *et al*., 1999; Zimmermann and Moelling, 1999)(Figure 3). TNF-α stimulated p38 MAPK signalling up-regulates *MAFbx* mRNA expression, which codes for a major E3 ubiquitin ligase responsible for muscle atrophy (Li *et al*., 2005) (see section on markers of atrophy and hypertrophy). Exercise induced p38 MAPK signalling has been shown to stimulate *PGC-1*α expression which leads to mitochondrial biogenesis hence promoting oxidative capacity (Akimoto *et al*., 2005). Furthermore, we recently found that the p38 MAPK pathway performs an important role in the activation of the fast oxidative-glycolytic MyHC 2x promoter in skeletal muscle (J.D. Meissner *et al*., unpublished data). Hence, depending on the nature of p38 MAPK stimulation, different phenotypic outcomes, such as cell arrest, atrophy, terminal differentiation or raised oxidative capacity, can result from this pathway (Figure 4).

Synthetic β_2 adrenergic agonists

β_2 adrenergic receptor agonists (β_2-agonists), such as clenbuterol, cimaterol and fenoterol, are potent agents for muscle hypertrophy as well as fibre type switch from slow/I to fast fibres (Ryall *et al*., 2002). They are also important regulators of T-cells development in the thymus (Blanco *et al*., 2003), and were primarily developed for use as smooth muscle bronchodilators (Ryall *et al*., 2002). A feature of β adrenergic receptors signalling, through the binding of catecholamines, is triglyceride hydrolysis, a process that has been harnessed in animal production to produce leaner carcasses. Ractopamine ('Paylean', Elanco), a β_1- and β_2.agonist, is a commercial compound sold in the USA as a promoter of lean growing pigs (Mills *et al*., 2003). β_2-adrenergic receptor but not β1 adrenergic receptor is responsible for mediating the hypertrophic effect of clenbuterol (Hinkle *et al*., 2002). The growth promoting efficacy of β_2-agonists appears to show animal species variation; ruminants display the greatest and broiler chickens the least response, with pigs occupying an intermediate position (Mersmann, 1998). Although the effects of β-agonists on meat quality are somewhat equivocal, the overall picture, in particular in the pig, is that many β-agonists decrease intramuscular fat and increase shear force or toughness (Dunshea *et al*., 2005).

The signalling events of β_2-agonists leading to muscle hypertrophy is poorly understood. There is evidence to suggest that clenbuterol induces local muscle production of IGF-1, which mediates hypertrophy (Awede *et al*., 2002). More recent work, however, could not detect sustained local production of IGF-1 in rat muscles treated with clenbuterol but a reduction in the expression of components of the ubiquitin-proteasome pathway was found (Yimlamai *et al*., 2005). The use of clenbuterol in meat production or in the treatment of muscle wasting conditions, however, has been impaired by reports of possible side effects. Pigs treated with anabolic doses of clenbuterol showed immunosuppressive effects, with raised T-cell apoptotic index (Blanco *et al*., 2003) and testicular degeneration (Blanco *et al*., 2002). In the pig, the hypertrophic effect of clenbuterol on muscle was short-lived after drug withdrawal, which could render its use ineffective in the growth promotion of food animals (Sillence *et al*., 2002). In rats, clenbuterol was found to cause significant cardiac and skeletal muscle necrosis (Burniston *et al*., 2002).

Anabolic steroids

Testosterone is an important male hormone whose effects on body composition are to increase muscle mass and

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reduce fat, effects that are not dissimilar to β_2-agonists (Kutscher *et al.*, 2002). Testosterone administration is associated with hypertrophy of type 1 and 2 fibres, and increases in satellite cell number in humans and some animals (Sinha-Hikim *et al.*, 2003; Chen *et al.*, 2005). In porcine satellite cells, however, the effect of testosterone on proliferation rate is equivocal (Doumit *et al.*, 1996). Anabolic androgenic steroids (stanozolol and nandrolone), made notorious by their misuse in sports, are structural synthetic derivatives of testosterone, designed to maximise anabolic and reduce androgenic (male sexual) effects. Outside the European Union, where anabolic steroids are used in beef production, meat quality can be adversely affected by a small but significant rise in shear force (Dunshea *et al.*, 2005). Little is known about the anabolic steroid-induced signalling mechanisms that regulate muscle mass. One of the primary anabolic effects of androgens may be their ability to stimulate localised IGF-1 production in skeletal muscle (Chen *et al.*, 2005).

Ski

Ski is a nuclear proto-oncogene that is essential for embryonic development. It is expressed in most post-natal tissues. Like IGF-1, it exhibits dual functions in promoting cell proliferation and muscle differentiation. In addition to transforming chicken embryo fibroblasts, *Ski* can induce cells derived from quail embryonic body wall to undergo myogenesis. Lines of transgenic mice carrying chicken *Ski* cDNAs under the control of murine sarcoma virus (MSV) long terminal repeat (LTR) preferentially express high levels of Ski mRNA and protein in skeletal muscle, even though MSV-LTR is usually active in other tissues (Sutrave *et al.*, 1990 and 2000). This near exclusive skeletal muscle expression of *Ski* is associated with hypertrophy of fast glycolytic 2b fibres, without increase in fibre number or nuclear number (Sutrave *et al.*, 1990). The hypertrophic effect of *Ski* on 2b fibres is associated with reduced protein degradation rates (Costelli *et al.*, 2003). It could be that *Ski* over-expression is deleterious to cells, which would account for its restricted distribution of expression in MSV-LTR-Ski transgenic mice, and an absence of expression in skeletal-α-actin promoter-driven *Ski* transgenic mice (Sutrave *et al.*, 2000). Indeed, satellite cells isolated from muscles of MSV-LTR-Ski transgenic mice showed accelerated deterioration in termination differentiation with increasing age (Charge *et al.*, 2002).

Unlike other oncogenes, the over-expression of the wild type *c-Ski* is sufficient to cause transformation (Prunier *et al.*, 2003). The oncoproteins from *Ski* and the related *SnoN* (*ski*-related novel gene) are able to interact with a variety of transcription regulatory complexes, including histone deacetylase complexes (HDACs) and tumour suppressors (Ueki and Hayman, 2003). The TGF-β signalling pathway has been identified as a key interacting site of *Ski*. The regulation of cell growth and differentiation by TGF-β is mediated by the Smad proteins, which are important tumour suppressors. TGF-β signalling is initiated when the bound ligand induces the formation of a heteromeric complex comprising type I and type II serine/threonine receptors. Type II receptor transphosphorylates type I receptor, which in turn phosphorylates Smad2 and Smad3. Activated Smad2 and Smad3 form heterodimers with Smad4 and translocate into the nucleus where they interact with a host of complexes to bring about transcriptional activation or repression of specific genes (Figure 4). *Ski* was recently found to directly interact with Smad2, Smad3 and Smad4, and to block the phosphorylation of Smad2 and Smad3 by activated TGF-β type I receptor (Prunier *et al.*, 2003; Ueki and Hayman, 2003). Therefore, a mechanism of the transforming ability of *Ski* (and *SnoN*) is the repression of Smad function, whose inactivation prevents TGF-β-induced cell cycle arrest. The up-regulation of *Ski* in proliferating satellite cells points to its possible role in mediating cell proliferation in the regeneration of damaged fibres (Soeta *et al.*, 2001). The mechanisms behind the effects of *Ski* on muscle differentiation and hypertrophy are unknown. One speculation is that Ski inhibits the signalling of myostatin, a member of the TGF-β superfamily, thereby enhancing cell proliferation and differentiation (Costelli *et al.*, 2003) (Figure 4). *Ski* might be developed as a candidate marker for hypertrophic growth in marker-assisted selection.

Fibre types: coordinated isoform-specific expression

The plasticity of muscle fibres is not confined to its ability to undergo changes in fibre size. Physiological and biochemical properties can show wide variations between individual fibres, and such variations are further subjected to modulations by external stimuli. Traditionally, classification of muscle fibre types is based on differences in a number of biochemical parameters between fibres (Gil *et al.*, 2001; Zierath and Hawley, 2006). Succinate dehydrogenase (SDH) histochemical staining, for example, is able to differentiate fibres into two or three different types, based on the relative amount of the enzyme present in each fibre. As SDH is an integral component of the citric acid cycle, strongly positive fibres are classified as oxidative fibres. Another commonly cited histochemical staining method depends on the overall myosin adenosine triphosphatase (ATPase) activity in each fibre (Brooke and Kaiser, 1970; Bancroft and Gamble, 2002). Myosin ATPase activity originates from myosin heavy chain (MyHC), the principal sacromeric protein component of the thick myofilament. Differential myosin ATPase staining is due to differences in susceptibility to pH between different MyHC isoforms. Since myosin ATPase is highly sensitive to pH change, this staining method is intrinsically prone to variability in results. Histochemical methods, such as SDH and myosin ATPase stainings, are invaluable in describing the biochemical profile of individual fibres. However, they are less reliable in the objective determination of fibre types. Different histochemical stains often provide slightly

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different classification outcome of individual fibres, which in the past had made findings of association studies between fibre types and meat quality traits variable and even contradictory (Essén-Gustavsson, 1993; Klont *et al.*, 1998; Lefaucheur *et al.*, 2004).

Definition of fibre types. A recent major advance in farm animal muscle research has been the development of an objective approach to muscle fibre typing based on the identity of the primary MyHC isoform expressed in each fibre (Chang *et al.*, 1993 and 1995; Chang and Fernandes, 1997). MyHCs are encoded by a highly conserved multigene family, of which eight isoforms are known in mammals (2a, 2x, 2b, embryonic, perinatal, slow/β, extraocular and α), each with its own myosin ATPase activity and each encoded by a distinct gene (Weiss and Leinwand, 1996). In pre-natal mammalian muscles, the embryonic, perinatal and slow/β/type I MyHC isoforms represent the three dominant skeletal muscle fibre types in the developing foetus. Shortly after birth, the post-natal MyHC isoforms (2a, 2x and 2b) replace the expression of embryonic and perinatal MyHC genes. Thus, in post-natal muscles of pigs, dogs and rodents, there are four major fibre types (Figure 5) characterised by the expression of the slow/β/type I, 2a, 2x and 2b MyHC gene isoforms (Schiaffino and Reggiani, 1996; Wu *et al.*, 2000b). In cattle and horses, MyHC 2b fibres are effectively absent (Chikuni *et al.*, 2004; Maccatrozzo *et al.*, 2004). Based on the MyHC approach, post-natal muscle fibres in animals can be resolved by immunocytochemistry or *in situ* hybridisation into three or four major types, depending on animal species. Metabolic, biochemical and biophysical characteristics, such as oxidative and glycolytic capacities, fibre size, colour, and glycogen and lipid contents, have been found to vary between MyHC fibre types (Schiaffino and Reggiani, 1996; Klont *et al.*, 1998; Karlsson *et al.*, 1999) (Table 1). The slow/β and fast 2b fibres, also known as slow oxidative (red) and fast glycolytic (white) respectively, represent two extreme metabolic profiles. Slow MyHC fibres are characterised by slow isoform contractile proteins, high levels of myoglobin, high volumes of mitochondria, high oxidative capacity, high lipid contents and high capillary density. Favourable meat traits such as colour and, in the pig in particular, tenderness have been found to closely associate with the greater abundance of red or highly oxidative fibres (Klont *et al.*, 1998; Karlsson *et al.*, 1999; Chang *et al.*, 2003; Maltin *et al.*, 2003; Wood *et al.*, 2004). There is a general perception that leaner meat, especially pork, contains reduced intramuscular fat, resulting in increased toughness and reduced succulence (Dunshea *et al.*, 2005). Red muscles possess higher lipid concentration (intra- and inter-fibre fat) which is associated with more tender / juicy meat (Hocquette *et al.*, 1998;Wood *et al.*, 1999 and 2003).

By contrast, fast MyHC 2b fibres are the largest of the four fibre types with fast isoform contractile proteins, low amounts of myoglobin and mitochondria, high glycolytic capacity (high glycogen store), low lipid contents and low capillary density. The fast MyHC 2a and 2x fibres are intermediate fast oxidative-glycolytic fibres. Fast 2a fibres are more closely related to slow/I fibres, and fast 2x are more similar to fast 2b fibres (Table 1). Fast glycolytic fibres, in particular 2b fibres, are major contributors of hypertrophic growth and of rapid fall in muscle pH *post mortem*, associated with the formation of PSE pork. Hence in the modern pig, hypertrophic growth potential (meat quantity) from an abundance of MyHC2b and 2x fibres comes at a cost to

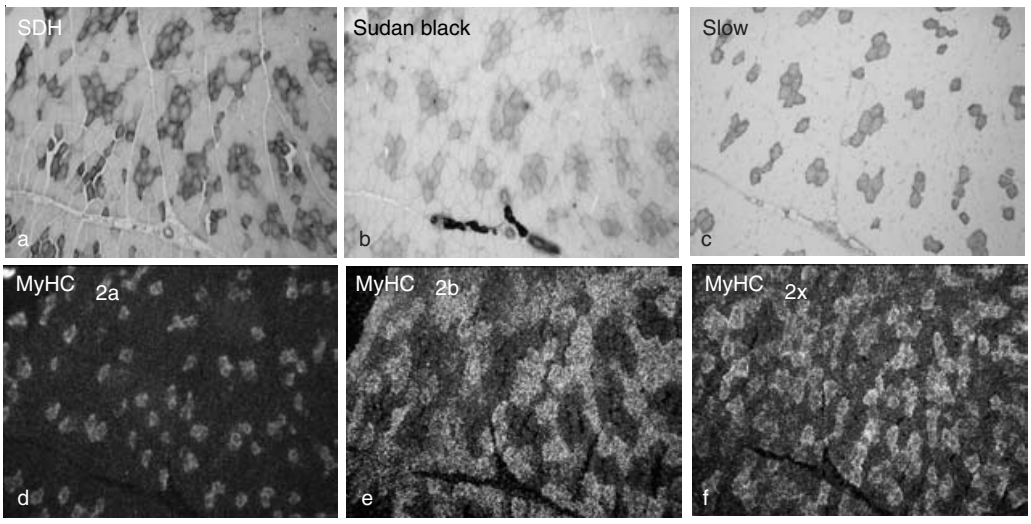

Figure 5 Typing of porcine muscle fibres. Serial sections of the *psoas* of a 22-week-old pig to illustrate the combined use of histochemistry, immunocytochemistry and *in situ* hybridisation to identify the four major post-natal MyHC fibre types. Succinate dehydrogenase (SDH) staining (a), sudan black staining (b), NOQ7.5.4D (slow) monoclonal antibody binding (c), and *in situ* hybridisations with MyHC isoform-specific fast 2a probe (d), fast 2b (e) and fast 2x probe (f) were performed. SDH and Sudan Black positive fibres closely mirrored each other and corresponded mainly to slow and fast 2a fibres. Note the presence of inter-fibre adipose tissue on panel b.

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Table 1 *Metabolic profiles of MyHC fibre types*

	MyHC			
	Slow/beta	2a	2x	2b
Type	Slow-oxidative	Fast oxidative-glycolytic	Fast oxidative-glycolytic	Fast glycolytic
Size	++	(++)	(++)	+++
Glycogen	(+)	(++)	(++)	+++
Lipids	+++	++	(+)	(+)
Fatigue	Resistant	Intermediate	Intermediate	Sensitive
PSE[†]	Resistant	Intermediate	Intermediate	Prone

[†] Pale, soft exudative (PSE) meat quality, independent of ryanodine receptor mutation. The abundance of MyHC 2b fibres in a normal pig is a contributory factor to PSE. ( ) indicates possibly variable levels or data not fully established in the pig.

meat quality (Chang *et al*., 2003). On the other hand, the absence of MyHC 2b fibres in cattle is a likely explanation for the lack of a PSE problem in beef. Some beef studies have linked an abundance of fast-twitch fibres (defined by histochemical detection) to improved tenderness. The interpretation of fast-twitch fibres should be made with care as it is presently clear that in the absence of MyHC 2b fibres there are strictly speaking no fast-glycolytic fibres in bovine muscle (Geay *et al*., 2001; Maltin *et al*., 2003). Consequently, in a technical sense, bovine muscle may have higher oxidative potential than its porcine counterpart.

In addition to temporal regulation, each MyHC isoform is subjected to specific spatial control, such that the number and distribution of fibres expressing each isoform often vary between anatomical muscles, e.g. *soleus* and *longissimus thoracis et lumborum* (also referred by some as *longissimus dorsi*). Fibre type composition varies between muscles according to their functional adaptation. Postural muscles are under continual use and comprise a high proportion of oxidative fibres. Muscles that are periodically used for intensive activities like sprinting possess large numbers of fast fibres. A further degree of fibre type heterogeneity is the presence of a small number of hybrid MyHC fibres, usually presented as a mixture of two MyHC isoforms (slow/2a, 2a/2x, or 2x/2b) (Sant'ana Pereira *et al*., 1995; Pette and Staron, 2000). Thus fibre population in muscle is a continuum of pure and hybrid fibres that can be altered in the fast-to-slow or slow-to-fast direction under appropriate stimulatory conditions (Schiaffino and Reggiani, 1994).

Distinctive biochemical and biophysical differences between fibre types point to a coordinated programme of fibre-type or isoform specific expression (Hallauer and Hastings, 2002). However, compared with our knowledge of muscle hypertrophy, much less is known about the molecular mediators of fibre type specific expression. Coordinated fibre type specific expression requires the orchestrated regulation of a large number of gene isoforms consistent with the fibre phenotype. Gene family and differential splicing are features of muscle genes (Schiaffino and Reggiani, 1996). Understanding the complexities of fibre type specific expression, requires insights into the signalling pathways that coordinate the temporal and spatial distribution of expression of subsets of muscle gene isoforms. The emerging picture of fibre type specific regulation is that it is governed by multiple signalling pathways and factors rather than a single pathway (Spangenburg and Booth, 2003). These pathways and factors almost inevitably have additional cellular functions, such as proliferation, differentiation and hypertrophy, in addition to modulating fibre type specific expression.

IGF-1, β_2-agonists and myostatin-null signal fast fibre phenotype

As highlighted earlier, the major phenotypic effects of IGF-1 and -2 are cell proliferation, muscle differentiation and hypertrophy. Such properties of IGF-1 have been shown to be beneficial to ageing, atrophic, and dystrophic muscles (Barton-Davis *et al*., 1998; Lynch *et al*., 2001; Musarò *et al*., 2001). IGF-1 has the additional effect of converting fibres to a fast glycolytic phenotype, as evident by raised expression of glycolytic enzymes in IGF-1-transfected C2C12 myotubes (Semsarian *et al*., 1999), by modest rise in fast 2b fibres in transgenic mice carrying muscle IGF-1 isoform driven by a rat myosin light chain (MLC)-1/3 promoter (Musarò *et al*., 2001) and by raised type 2a and 2b fibres at the expense of slow fibres in IGF-1-treated dystrophic mice (Lynch *et al*., 2001). As previously indicated, clenbuterol (a β_2-agonist) treated rats (Ryall *et al*., 2002) as well as myostatin-null mice (Steelman *et al*., 2006) not only showed muscle hypertrophy but also slow-to-fast fibre conversion. The mechanism responsible for the slow-to-fast fibre type switch, however, remains elusive. In general, in the pig and ruminants, it appears that growth hormone (indirectly IGF-1) and β-agonists may negatively affect meat quality, as shown by increased shear force or sensory perception (Dunshea *et al*., 2005). Such effects may be connected to increased fast glycolytic (MyHC 2b or 2x) fibres but because fibre typing in meat quality association studies had been almost exclusively based on histochemical methods, the changes in MyHC 2b/2x fibres in the pig or MyHC 2x fibres in cattle have not been determined.

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Calcineurin signals oxidative fibre type

Calcineurin (protein phosphatase 2B/PP2B) is an enzyme complex that comprises calcineurin A (CnA) catalytic subunit, calcineurin B (CnB) regulatory subunit and calcium-binding protein calmodulin (Schulz and Yutzey, 2004). It is a calcium dependent serine-threonine phosphatase that is widely distributed throughout the body. Calcineurin has been implicated in a wide variety of biological processes, including T-lymphocyte activation, vascular, neuronal and cardiac development and growth, and, more recently, skeletal muscle development (Crabtree, 2001; Bueno *et al.*, 2002; Horsley and Pavlath, 2002). In cardiac muscle, calcineurin signalling is necessary for cardiomyocyte maturation, heart chamber formation and cardiac hypertrophy (Schulz and Yutzey, 2004). In skeletal muscle, calcineurin appears to be needed in a number of key developmental processes, namely enhanced muscle cell differentiation, and in the fibre type context, conversion to slow (oxidative) muscle phenotype (Musarò *et al.*, 1999; Semsarian *et al.*, 1999; Bigard *et al.*, 2000; Delling *et al.*, 2000). It has been reported that activated calcineurin mediates the hypertrophic effect of IGF-1 (Musarò *et al.*, 1999; Semsarian *et al.*, 1999). However, there is compelling evidence, including transgenic and knock-out data, to show that calcineurin has no effect on muscle hypertrophy but that the hypertrophic effect of IGF-1 is mediated by the PI3K pathway as detailed earlier (Naya *et al.*, 2000; Bodine *et al.*, 2001b; Rommel *et al.*, 2001; Pallafacchina *et al.*, 2002). Calcineurin is activated by raised intracellular calcium, triggered by extracellular signals like nervous impulses, or hormonal input, such as IGF-1 stimulation. To date, few substrates, namely NFATs, MEF2s and PGC-1α, of calcineurin action in skeletal muscle are known.

NFATs. The best characterised calcineurin substrates are members of the phosphorylated NFAT (nuclear factor of activated T-cells) family of transcription factors. Five NFAT genes, each with a distinct cellular role, have been identified: NFATc1 (NFAT2/NFATc), NFATc2 (NFATp/NFAT1), NFATc3 (NFAT4/NFATx), NFATc4 (NFAT3), and NFAT5 (Delling *et al.*, 2000; Horsley and Pavlath, 2002). Several NFAT isoforms are expressed in skeletal muscle, each of which undergoes activation at specific stages of myogenesis. For instance, NFATc2 is activated only in new myotubes and plays a crucial role via IL-4 in mediating myoblast fusion (Horsley *et al.*, 2003). Dephosphorylation of NFATs unmasks their nuclear localisation signal, resulting in nuclear translocation, where they bind to NFAT-binding sites on their own or as co-operative complexes with other factors, such as calcineurin, AP1, MEF2 and GATA2/4, to activate gene transcription (Musarò *et al.*, 1999; Sugiura *et al.*, 2001; Schulz and Yutzey, 2004). Cultured muscle fibres showed NFAT nuclear translocation only when electrically stimulated at a slow muscle pattern, which results in high intracellular calcium (100 to 300 nM) but not at a fast muscle pattern that results in low intracellular calcium ($<$ 50 nM) (Liu *et al.*, 2001; Fraysse *et al.*, 2003). Nuclear NFATs are deactivated and re-located back to the cytoplasm through phosphorylation by several protein kinases, including GSK-3β (Figure 3), protein kinase A, p38 MAP kinase and casein kinase (Schulz and Yutzey, 2004). In T-cells, simultaneous activation of AP-1 (Jun/Fos) and NFAT is absolutely essential for cytokine transcriptional induction (Rao *et al.*, 1997). In skeletal muscle, however, only GATA2 has been shown to be a co-operative binding partner of NFATs (Paul and Rosenthal, 2002).

MEF2s. Members of the MEF2 (myocyte enhancer-binding factor 2) family belong to a class of transcription factors that are responsible for the activation of many muscle-specific genes with a conserved A/T-rich *cis*-acting regulatory element (Naya and Olson, 1999) and play a crucial role in p38 MAP kinase-mediated terminal muscle differentiation (discussed above). Like NFATs, MEF2 proteins form co-operative complexes with other factors, like members of the MyoD family, to regulate transcription. Calcineurin up-regulates the transcription of MEF2 genes (Wu *et al.*, 2000a). It can also dephosphorylate MEF2 directly and enhance its transactivational activity (Dunn *et al.*, 2001; Wu *et al.*, 2001). Hence members of the MEF2 family are transcriptional targets and protein substrates of calcineurin.

PGC-1α. Peroxisome proliferator-activated receptor-γ co-activator-1 (PPARγ co-activator 1α /PGC-1α), a ubiquitous transcriptional co-factor for nuclear receptors, is a potent inducer of mitochondrial biogenesis (Lin *et al.*, 2002). It was recently shown to be able to convert fast fibres to the slow phenotype when over-expressed in transgenic mice. Part of this effect is thought to be mediated through the action of calcineurin on PGC-1α as a substrate (see PPARs below) (Lin *et al.*, 2002). As a co-factor, PGC-1α is likely to exert its effects indirectly by modulating the expression of a specific group of downstream genes. Although all three substrates (NFATs, MEF2s and PGC-1α) are important mediators of calcineurin activation, none is exclusively expressed in skeletal muscle. Additional substrates not yet identified may well be involved in the signalling pathway. It is not known which regulatory target genes activated by NFAT, MEF-2 or PGC-1α are responsible for the phenotypic effects of calcineurin. Further work is needed to identify the genes regulated by the known and, possibly, other unidentified substrates of calcineurin.

Several endogenous calcineurin-specific inhibitors have been discovered. AKAP79, cain/cabin 1, and CBHP are ubiquitous factors and were found to inhibit NFAT function or translocation to the nucleus (Crabtree, 2001). More recently, two additional endogenous calcineurin-specific inhibitors (DSCR1/MCIP1 and ZAKI-4/DSCR1L1/MCIP2), highly expressed in striated muscles and brain, were found (Yang *et al.*, 2000; Rothermel *et al.*, 2001). DSCR1 expression is induced by calcineurin and hence forms a negative feedback loop to limit calcineurin activation

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(Yang *et al.*, 2000). Its over-expression in transgenic mice prevented cardiac hypertrophy (Rothermel *et al.*, 2001; Van Rooij *et al.*, 2004). In skeletal muscle, the role of ZAKI-4 is particularly relevant because, unlike DSCR1, it is responsive to thyroid hormone stimulation (Cao *et al.*, 2002). Thyroid hormone is a well known pleiotropic endocrine regulator of metabolism that modulates the transcription of a large number of genes, leading to increased metabolic rate, protein breakdown and muscle loss (Clement *et al.*, 2002). The importance of thyroid hormone, however, on glucose and lipid homeostasis has not been addressed. In hyperthyroidism, muscle loss is accompanied by increase of fast fibres at the expense of slow fibres (Caiozzo *et al.*, 1997). Triiodothyronine (T_3) with clenbuterol greatly enhances the slow to fast fibre type switch (Awede *et al.*, 2002). Such changes resemble the effects of chemical inhibitors of calcineurin, cyclosporine A or FK506, on muscle (Bueno *et al.*, 2002). It is possible that the effects of thyroid hormone on muscle loss and fast phenotype conversion is mediated through the inhibition of calcinuerin by ZAKI-4.

There is little doubt that the calcineurin-NFAT pathway is critical in muscle phenotype determination, in particular its roles in muscle differentiation and slow fibre conversion. However, as in other pathways, calcineurin signalling is not an absolute effect in that it does not activate all slow genes in all muscles. Some fast muscle genes, like MyHC 2b and SERCA1, are up-regulated in activated calcineurin transfected C2C12 cells (Swoap *et al.*, 2000). Transgenic mice over-expressing the activated calcineurin in skeletal muscle showed substantial slow fibre switch but only in certain muscles (Naya *et al.*, 2000).

PPARs and PGC-1α influence oxidative phenotype

Peroxisome proliferator-activated receptors (PPAR) α, γ and δ/β belong to an important family of nuclear hormone receptors (transcription factors) that regulate genes that are involved in lipid metabolism (Muoio *et al.*, 2002). PPARα, initially identified as the mediator of a class of compounds that induces peroxisomal proliferation in rodent liver, is most abundantly expressed in striated muscle (Muoio *et al.*, 2002). Its activation increases fatty acid β-oxidation in muscle and reduces body weight (Koh *et al.*, 2003). PPARγ is more highly expressed in adipose tissues (Koh *et al.*, 2003) and, in contrast to PPARα, stimulates adipocyte differentiation (Oberkofler *et al.*, 2002; Yu *et al.*, 2006). PPARδ is highly expressed in adipose tissue, heart and skeletal muscle. Recently, it was shown that activated PPARδ increases fatty acid oxidation in adipose tissue and muscle (Wang *et al.*, 2003) and that during starvation its expression is up-regulated in skeletal muscle (Holst *et al.*, 2003). PPARα, PPARγ and PPARδ bind to the same consensus sequence, suggesting that mechanisms must exist that regulate PPAR type specificity (Oberkofler *et al.*, 2002).

PPARγ co-activator 1α (PGC-1α), cloned from a brown fat cDNA library for its interaction with PPARγ, is a potent transcriptional activator that interacts with several nuclear hormone receptors, which include PPARs, steroid hormone receptors (glucocorticoid, oestrogen and mineralocorticoid), retinoic X receptor, thyroid hormone receptor (Oberkofler *et al.*, 2002), as well as MEF2 (Handschin *et al.*, 2003). PGC-1α stimulates mitochondrial function and number (biogenesis), and fatty acid oxidation in cardiac and skeletal muscles by the induction of mitochondrial and nuclear genes involved in energy production pathways, including PPARα and nuclear respiratory factor-1 (NRF-1) (Miura *et al.*, 2003). Therefore, the phenotypic effect of PGC-1α activation, in partnership with nuclear hormone receptors, including PPARs, in skeletal muscle is increased oxidative capacity.

Markers of atrophy and hypertrophy

Markers of atrophy and hypertrophy are relevant to farm animal production from the perspective of disease and welfare. Animals suffer from a wide range of infectious and non-infectious diseases that are often manifested as reduced weight gain or net weight loss. Markers that can readily measure muscle atrophy or hypertrophy could have veterinary applications.

Biological markers that can predict the functional state of muscle are useful indicators in the diagnosis and monitoring of muscle conditions. Clearly, muscles that have undergone gross hypertrophy or atrophy would be apparent during examination. Muscles in the transitional process of remodelling are more difficult to recognise. Thus the use predictive biological markers would be valuable in identifying the underlying changes that are taking place in muscle.

E3 ubiquitin ligases as atrophic markers. Muscle atrophy is a consequence of a variety of conditions: denervation, injury, limb immobilisation, inactivity, glucocorticoid treatment, infection, diabetes, renal failure, cancer and ageing. In muscle atrophy, muscle loss is often the result of raised protein degradation and turn-over rather than simply due to reduced protein synthesis. Thus, atrophy is as much an active degradation process as a passive one from reduced stimulation of the anabolic (hyperplastic and hypertrophic) pathways. Of the different protein degradation pathways (Costelli *et al.*, 2003), the ATP-dependent ubiquitin-proteasome proteolytic system is thought to be of major importance in skeletal muscle (Dehoux *et al.*, 2003; Glass, 2003a). In this process, ubiquitin (Ub) is activated by an ubiquitin-activating enzyme (E1 family) and conjugated to a substrate protein by ubiquitin-conjugating enzyme (E2 family) in conjunction with ubiquitin protein ligase (E3 ligase family). E3 ligases, of which hundreds have been identified, confer substrate specificity (Glass, 2003a). During muscle atrophy, many of the genes encoding the Ub-proteasome pathway are up-regulated (Lecker *et al.*, 2004), including E2 (such as $E2_{14K}$ and UbcH2) and E3 (such as E3α / UBr1) genes (Li *et al.*, 2003; Lecker *et al.*, 2004). In particular, two E3 ubiquitin

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ligases, MuRF1 (muscle ring finger 1) and MAFbx (muscle atrophy F-box or atrogin-1) have been identified as mediators of muscle atrophy (Bodine *et al.*, 2001a; Dehoux *et al.*, 2003). MuRF1 and MAFbx are selectively induced in skeletal muscle and heart when subjected to a variety of atrophic conditions, such as denervation and glucocorticoid treatment. Their absence in null knock-out mice led to the preservation of muscle mass under atrophying conditions (Bodine *et al.*, 2001a). TNF-α stimulated proteolysis may be mediated through the increased expression of MAFbx and MuRF1 (Dehoux *et al.*, 2003; Glass 2005). MuRF1 contains three domains: a RING-finger domain, required for ubiquitin ligase activity, a B-box of unclear function, and a coil-coil domain, which may be required for heterodimerisation with a related protein MuRF2. Activation, by cachectic factors such as TNFα, of the NF-κB transcription pathway induces skeletal muscle atrophy, which is mediated in part by NF-κB on the up-regulation of MuRF1 (Glass, 2005). MuRF1 physically interacts with titin, which suggests a possible role for MuRF1 in titin turn-over (Centner *et al.*, 2001). MAFbx contains an F-box domain, characteristic of SCF family of E3 ubiquitin ligases. F-box E3 ligases usually bind post-translationally modified substrates, such as phosphorylation, and may target proteins involved in cell signalling. We recently found that in porcine congenital splayleg, a condition characterised with severe muscle weakness and fibre atrophy, MAFbx is abnormally up-regulated in relation to normal littermates (Ooi *et al.*, 2006).

FOXO proteins, a subgroup of the Forkhead family of transcription factors, have been recently identified as mediators that link the signalling pathways of muscle hypertrophy and atrophy. FOXO transcription factors are important for the induction of cell quiescence and apoptosis. FOXO factors are targets of activated Akt (Figure 3). Direct and multiple phosphorylation by Akt on FOXO factors leads to their displacement from the nucleus to the cytoplasm and to the inhibition of their transcriptional activities (Burgering and Medema, 2004). Under atrophic conditions, FOXO1 (Stitt *et al.*, 2004) and FOXO3 (Sandri *et al.*, 2004) have been shown to up-regulate the expression of *MAFbx* and *MuRF1*. Inhibition of FOXO activity that leads to the down-regulation of *MAFbx* and *MuRF1* is a necessary anti-atrophy step mediated by the PI3K-Akt1 pathway (Sandri *et al.*, 2004; Stitt *et al.*, 2004; Tesseraud *et al.*, 2007). This anti-atrophy effect can be triggered by insulin as ligand which has the dual roles of enhancing protein synthesis (Figure 3) and decreasing proteolysis via FOXO inhibition (Tesseraud *et al.*, 2007).

GATA-2 as a hypertrophic marker. Factors involved in signalling, muscle metabolism or sarcomeric structure that are associated with the process of muscle hypertrophy could potentially be used as hypertrophic markers. GATA-2 has been recently identified as a potentially useful candidate marker gene of muscle hypertrophy (Musarò *et al.*, 1999; Paul and Rosenthal, 2002). In mammals, the GATA zinc-finger transcription factor family comprises six gene members that can be divided into 2 sub-groups, based on structure and function (LaVoie, 2003). GATA-1/-2/-3 members are often associated with haematopoiesis and neural development. GATA-4/-5/-6 members are commonly associated with organ development, including heart, gut, blood vessels and parts of the genito-urinary system. With the exception of GATA-5, gene ablation of

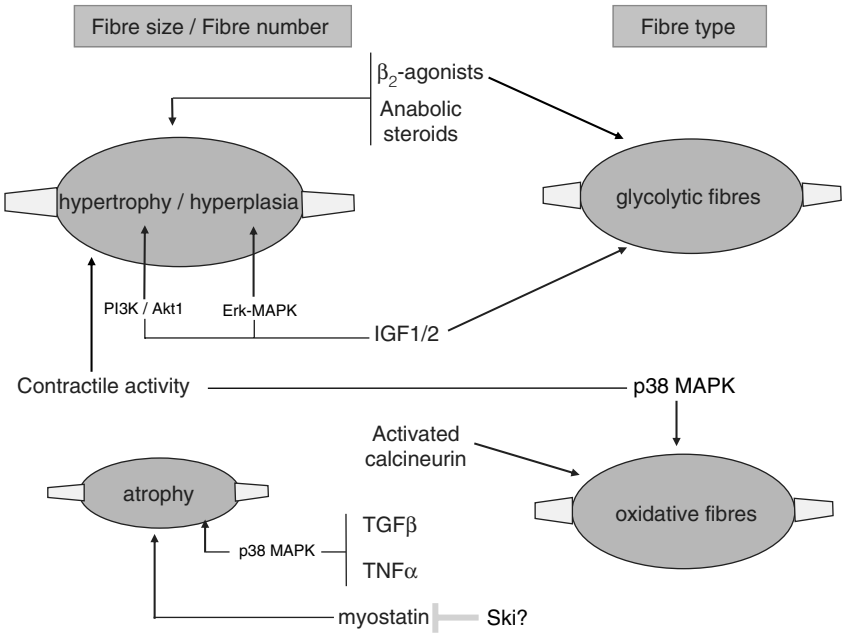

Figure 6 Summary actions of signalling factors and pathways on fibre size, fibre number and fibre types. p38 MAPK activation can result in a range of phenotypic outcome depending on the nature of activation. The proposed inhibition of myostatin by *Ski* has not been formally demonstrated.

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the GATA family members results in embryonic lethality. GATA-2 is necessary for mast cell development, and maintenance and expansion of multipotential progenitors and haematopoietic stem cells (Fujiwara *et al*., 2004). GATA-2 is usually absent or lowly expressed in skeletal muscle at all stages of development, but is induced in muscle cell culture and in vivo by IGF-1, during muscle regeneration after bupivacaine injection, and by exercise (Paul and Rosenthal, 2002; Sakuma *et al*., 2003). GATA-2 co-precipitates with calcineurin and NFATc1 (see below), suggesting it mediates its effect on muscle gene expression in conjunction with components of the calcineurin signalling pathway as a protein complex (Musarò *et al*., 1999; Sakuma *et al*., 2003). There is no direct evidence to show that GATA-2 is a hypertrophic factor, but its expression is closely associated with factors (e.g. IGF-1) and conditions (e.g. exercise, regeneration) that are involved in the process of hypertrophy and regeneration.

Conclusion

The determination of muscle phenotype (fibre number, size and fibre type) is highly complex and coordinated that requires the integration of several major signalling cascades (e.g. Erk-MAPK, p38 MAPK, PI3K-Akt and calcineurin signalling pathways), intracellular factors (transcription factors, like NFATs, and co-factors, such as PGC-1α) and extracellular factors (e.g. ligands, like myostatin and IGFs, and nutrition) (Figure 6). Enhanced hypertrophic growth, as exemplified in the pig, for greater lean meat production is associated with reduced intramuscular fat and increased accumulation of fast glycolytic fibres, the most common being the MyHC 2x and 2b fibres (Chang *et al*., 2003; Wood *et al*., 2004). Highly glycolytic fibres in the pig, however, are not conducive to the conferment of good meat quality traits, such as colour and water-holding capacity. In future farm animal production, improvements on meat quality could offer considerable economic attraction. At a fundamental level, one need is to discover key targets that mediate slow or oxidative fibre type switching. The recent introduction of the use of exon-expression arrays and ChIP-on-chip tiling arrays (Affymetrix) in functional genomics is likely to greatly accelerate our understanding of key molecular and signalling details in fibre phenotype determination. Identified target genes could be exploited through marker-assisted selection or by pharmacological / nutritional manipulation.

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