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The Central Role of Myostatin in Skeletal Muscle and Whole Body Homeostasis

Elliott, B., Renshaw, D., Getting, S. and MacKenzie, R.

REVIEW

The central role of myostatin in skeletal muscle and whole body homeostasis

B. Elliott, D. Renshaw, S. Getting and R. Mackenzie

Infection & Immunity Group, Department of Human & Health Science, School of Life Sciences, University of Westminster, London, UK

Abstract

Myostatin is a powerful negative regulator of skeletal muscle mass in mammalian species. It plays a key role in skeletal muscle homeostasis and has now been well described since its discovery. Myostatin is capable of inducing muscle atrophy via its inhibition of myoblast proliferation, increasing ubiquitin-proteasomal activity and downregulating activity of the IGF–Akt pathway. These well-recognized effects are seen in multiple atrophy causing situations, including injury, diseases such as cachexia, disuse and space flight, demonstrating the importance of the myostatin signalling mechanism. Based on this central role, significant work has been pursued to inhibit myostatin's actions *in vivo*. Importantly, several new studies have uncovered roles for myostatin distinct from skeletal muscle size. Myostatin has been suggested to play a role in cardiomyocyte homeostasis, glucose metabolism and adipocyte proliferation, all of which are examined in detail below. Based on these effects, myostatin inhibition has potential to be widely utilized in many Western diseases such as chronic obstructive pulmonary disease, type II diabetes and obesity. However, if myostatin inhibitors are to successfully translate from bench-top to bedside in the near future, awareness must be raised on these non-traditional effects of myostatin away from skeletal muscle. Indeed, further research into these novel areas is required.

Keywords adipogenesis, cachexia, cardiomyocyte homeostasis, glycolysis, muscle atrophy.

Introduction

More than a decade has passed since the initial identification of myostatin by McPherron *et al.* (1997), but the area still receives a significant amount of attention; new publications continue to emerge in both traditional and unexpected fields, demonstrating both the depth and dynamic nature of this field. The importance of myostatin is well established in regulating skeletal muscle homeostasis in a wide variety of conditions (e.g. Gonzalez-Cadavid *et al.* 1998, Gonzalez-Cadavid & Bhasin 2004, Costelli *et al.* 2008), as we

will discuss in the following sections. Indeed, it is the powerful role of myostatin in the regulation of the size of muscle mass that has resulted in it receiving so much initial attention. To the best of the authors' knowledge, every situation where muscle mass levels are altered is coupled with published examples of alterations in myostatin levels either systemically or at the tissue level.

Where various stimuli of a different nature lead to a similar phenotypic effect, it is plausible to hypothesize a 'bottleneck' in inter- or intracellular signalling must exist at some level. Understanding the common

mechanisms involved during all types of muscle mass loss is necessary and will result in future work to develop treatments targeting this hypothetical bottle-neck. If a common pathway in signalling can be iden-tified and targeted, it would prove beneficial in a wide variety of conditions, both pathological and environ-mental, in the prevention of muscle atrophy, as seen during disuse, space flight, injury, disease, cachexia and ageing. As reviewed below, increased myostatin expression is seen in all these homeostatic perturba-tions; however, the key question to be answered is of cause and effect. Does myostatin cause muscle mass alterations or is it altered in response? Throughout this review, evidence will be presented to support the working hypothesis that myostatin represents a central regulating factor in alterations in skeletal muscle homeostasis by multiple mechanisms.

Further, recent findings have hinted that myostatin may perform more diverse roles away from its tradi-tional control over the size of skeletal muscle mass; suggesting exciting new research directions and appli-cations may emerge in the future. Specifically, evi-dence has been presented for myostatin signalling playing a significant role in the regulation of glycolysis (Chen *et al.* 2010), adipogenesis (Feldman *et al.* 2006) and cardiomyocyte homeostasis (Bish *et al.* 2010), all significant in modern 'Western diseases' such as type II diabetes, cachexia and heart disease. As is often the case with early exciting findings, the picture emerging is more complex than originally thought. As we move forward towards the use of myostatin inhibitors in clinical settings, an understanding of the role of myo-statin in these non-traditional sites of action will help shape understanding of potential negative side effects that future treatments may otherwise unexpectedly present with, as well as shape novel areas of transla-tional research.

Here, we briefly review the initial 10 years of research into myostatin with the aim of introducing the non-muscle physiologist to the depth and breadth of research surrounding myostatin before discussing emerging findings, and finally, we will discuss possible future directions. Throughout this review, the use of the term 'muscle' should be considered to refer to skeletal muscle, unless otherwise noted. Furthermore, unless otherwise noted, 'myostatin' is in reference to the bioactive C-terminus peptide.

The first decade

Myostatin (originally named growth and differentia-tion factor-8; GDF-8) is a member of the GDF family, a subgroup of the transforming growth factor β family of proteins. Myostatin was first characterized by McPherron *et al.* (1997), while generating strains of GDF negative (−/−) mice to characterize phenotypic functions and developmental expression patterns. Myostatin −/− mice were noted to be larger than wild-type mice, with a 30% increase in body mass. This difference in size was demonstrated to be the result of significant muscular hypertrophy, with indi-vidual muscles 200–260% larger than wild-type ani-mals. Expression of myostatin RNA appeared to be primarily restricted to muscle tissue, with a small sig-nal from adipose tissue (McPherron *et al.* 1997).

The presence of myostatin homologues was identi-fied by Southern blot analysis in multiple mammalian species, including humans (McPherron *et al.* 1997). It was quickly confirmed that myostatin was relevant in other mammalian species. Multiple groups simulta-neously identified that the excessive muscle growth seen in Belgium Blue cattle was because of a naturally arising mutation in the myostatin-coding gene (Grobet *et al.* 1997, Kambadur *et al.* 1997, McPherron & Lee 1997). In a similar finding, whippet dogs with exces-sive muscle growth were found to have a heterozygous naturally occurring mutation (Mosher *et al.* 2007). This finding was interesting that these dogs were well known by the racing community to have greater exer-cise performance than wild-type animals, demonstrat-ing increased muscle mass owing to myostatin deletion was functional, a finding that has limited par-allels in humans (Seibert *et al.* 2001). In an early in-terventional study, sheep were subjected to reduced food intake to induce muscle atrophy. Myostatin mus-cle protein increases mirrored changes in muscle mass, suggesting some coupling mechanism between myosta-tin and muscle levels (Jeanplong *et al.* 2003).

Myostatin also appears in pigs, with detectable mRNA levels varying both foetally and postnatally, suggesting a role in development (Ji *et al.* 1998). Adult pigs subjected to inflammatory viral infections, where muscle loss is seen, demonstrate increased myo-statin expression (Escobar *et al.* 2004), a result that creates an interesting causative question. Is myostatin increased in response to viral inflammation resulting in muscle atrophy or does viral inflammation cause muscle mass loss with results in a lower basal myosta-tin level? This question is addressed in the following section in detail (New directions – Inflammation).

Myostatin appears to also play a role in fish (Weber *et al.* 2005), where upwards of 16 homologues are noted (Rodgers & Garikipati 2008), occasionally with roles outside of skeletal muscle (Biga *et al.* 2004). We see therefore that the role of myostatin is well main-tained in mammalian species, with perturbations in various species coupled with myostatin alterations, but appears to demonstrate an evolutionary split in function between mammals and fish, a point noted previously (Rodgers & Garikipati 2008).

Myostatin acts directly upon myocytes; stimulation of myotubes with recombinant myostatin *in vitro* results in decreased myotube diameter (McFarlane *et al.* 2006), suggesting a direct catabolic or anti-anabolic effect. Myofibre damage followed by remodelling and increased satellite cell activity is induced by the snake venom notexin. Injection of notexin in rats was shown to increase myostatin protein levels during the 5 days post-remodelling period, returning to baseline after 7 days (Mendler *et al.* 2000). In a similar histological study, myostatin was located in necrotic fibres after injury by notexin (Kirk *et al.* 2000). Taken together, these data suggest myostatin is involved in the remodelling process from start to finish, first as a step in the induction of necrosis, then in the promotion of new fibre formation during fibre replacement. Further, identified increases in myostatin mRNA with muscle mass loss induced by both burn injury and dexamethasone injection in rats parallel loss of muscle mass (Lang *et al.* 2001, Salehian *et al.* 2006), the effects of which are partially inhibited by glutamine injection (Salehian *et al.* 2006). Dexamethasone has similar effects in C2C12 cells, increasing myostatin protein expression and reducing myotube diameter (Artaza *et al.* 2002).

Interesting links exist between models of human disease and myostatin, suggesting the involvement of myostatin in cachexic muscle loss. While chronic alcohol consumption alone does not appear to increase muscle myostatin mRNA levels (Molina *et al.* 2006), a rat model of liver cirrhosis shows decreases in intramuscular myosin heavy chain and MyoD expression as well as increased myostatin protein levels in samples taken from gastrocnemius (Dasarathy *et al.* 2004). Serum myostatin increases coupled with skeletal muscle atrophy is also seen in a mouse model of Addison's disease (Hosoyama *et al.* 2005). Chronic hypoxic exposure in rats, as a model of chronic obstructive pulmonary disease (COPD), results in elevated myostatin protein levels as well as reduced body and individual muscle mass, the effect of which is maintained under pair feeding conditions (Hayot *et al.* 2010). In mice challenged with implanted tumour masses, cachexia results along with increases in myostatin protein levels at 7 days post-tumour implantation (Costelli *et al.* 2008), suggesting myostatin may play a causative role in cachexic loss of muscle mass.

Another example of variation in musculation is the sexual dysmorphism seen in mammalian species. Differences in expression of myostatin protein levels in male and female mice post-puberty mirror the differences in muscle mass seen, with wild-type (+/+) male mice showing significant body and muscle mass differences over females post-puberty, as well as decreased myostatin expression (McMahon *et al.* 2003a, Old-

ham *et al.* 2009), suggesting myostatin could be playing a causal role in sexual dysmorphism. Interestingly, myostatin −/− mice show opposite dysmorphic muscular hypertrophy, with female muscle mass showing the greatest pubic increases (Gentry *et al.* 2010), demonstrating that removing myostatin reduces (but does not remove) sexual dysmorphic differences. Interestingly, the findings of Oldham *et al.* (2009) demonstrated the inhibitory effects of myostatin were caused by altered myostatin levels, not altering inhibitory propeptide levels. Alternatively, in elderly humans, males demonstrate higher concentrations of myostatin protein than females (Gruson *et al.* 2011). However, while Oldham *et al.* (2009) utilized Western blot, Gruson *et al.* (2011) used an immunoassay that recognizes both the mature peptide and the inhibitory propeptide, potentially confounding results.

Human findings

The first report suggesting myostatin may be playing a role in human muscle homeostasis came from Gonzalez-Cadavid *et al.* (1998) who demonstrated a negative correlation between serum myostatin levels and muscle mass in healthy individuals as well as those with HIV, both with and without cachexia. Myostatin elevation in response to chronic disorders is also seen in COPD (Hayot *et al.* 2010).

It was also demonstrated that 25 days of 'head down' bed rest, commonly used to model the physiological effect of microgravity, resulted in significant loss of lean body mass and serum myostatin concentrations that were significantly elevated by 12% from baseline (Zachwieja *et al.* 1999). Negative correlations were again noted between lean body mass and serum myostatin concentrations (Zachwieja *et al.* 1999). In another model of disuse atrophy, hip arthroplasty is coupled with increased myostatin mRNA expression 5 days post-surgery (Reardon *et al.* 2001).

Mixed evidence for myostatin elevations are seen in sarcopenia with some authors noting no relationship between myostatin mRNA levels and muscle mass (Marcell *et al.* 2001, Ratkevicius *et al.* 2011), while others show a correlation between increased circulating myostatin levels and the level of muscle mass loss in sarcopenic patients (Schulte & Yarasheski 2001, Yarasheski *et al.* 2002, Leger *et al.* 2008). Indeed, further evidence for myostatin playing a role in ageing are provided by a study in aged mice where myostatin inhibition results in increased muscle fibre cross-sectional area and grip strength (Siriett *et al.* 2007). Interestingly, genetic polymorphisms of myostatin have been identified in elderly females that parallel loss of muscle mass (Seibert *et al.* 2001). If some individuals are more sensitive to alterations in myostatin

expression, then this may help explain this variation in findings.

With fortuitous timing, a newborn child was identified in Germany as carrying a myostatin null mutation after being noted to have an unusually high level of muscle mass at birth (Schuelke *et al.* 2004). Besides increased muscular development, the child developed physiologically and mentally normally. Subsequent individuals with similar myostatin deficiencies have since been identified, however all with various other genetic mutative disorders (Gonzalez-Freire *et al.* 2009, Prontera *et al.* 2009, Meienberg *et al.* 2010). The presence of such myostatin −/− humans confirms myostatin's role is maintained.

Resistance exercise is a well-defined stimulus for muscular hypertrophy (Kraemer *et al.* 2002), and not surprisingly, is coupled with altered myostatin expression. Nine weeks of a resistance training designed to build muscle mass resulted in reduced myostatin mRNA in muscle (Roth *et al.* 2003) and 10 weeks of a similar programme resulted in decreased serum myostatin (Walker *et al.* 2004). In contrast, Willoughby showed increases in both muscle mRNA and serum protein levels of myostatin after 12 weeks of resistance training (Willoughby 2004a,b). This increase may have been because of timing of sampling, as Willoughby (2004a,b) took samples immediately post the final training session, whereas Walker *et al.* (2004) waited 2 days post the final session to ensure a basal level was examined. Indeed, the acute response to a single bout of isometric exercise in rats demonstrates a rapid increase in myostatin mRNA levels 30 min to 6 h post-exercise, returning to baseline levels 24 h post-exercise (Peters *et al.* 2003), a finding that has yet to be paralleled in experiments on human participants. Furthermore, after 2 weeks cast immobilization significant myostatin mRNA decreases 24 h and 6 weeks post-return to mobility are seen (Jones *et al.* 2004) and 21 weeks of resistance training results in

reduced myostatin mRNA expression 48 h post the final training session (Table 1; Hulmi *et al.* 2007). Different resistance training protocols have been shown to have different effects on myostatin levels (Heinemeier *et al.* 2007), perhaps unsurprisingly, as it is recognized that muscle-building exercise is a complex, often oversimplified task (Kraemer *et al.* 2002). Indeed, it would appear that the effect of resistance exercise is dose-dependent as light resistance exercise (20% 1RM, four sets, 15–30 reps) does not alter mRNA myostatin levels in healthy, untrained males and females (Manini *et al.* 2011).

Taken together, these data suggest resistance exercise may induce an acute increase in myostatin activity to promote cellular remodelling (Peters *et al.* 2003, Willoughby 2004b), followed by a chronic adaptive response of decreased basal expression (Hulmi *et al.* 2007), facilitating a hypertrophic phenotype. To the best of the author's knowledge, no acute time course of myostatin expression post-resistance training has been completed.

The effect of endurance exercise on myostatin is less well defined in human studies. Six months of endurance exercise training decreases serum and muscle myostatin protein levels in middle-aged men (Hittel *et al.* 2010), and 9 weeks of endurance exercise decreases muscle myostatin mRNA levels in maintenance haemodialysis patients (Kopple *et al.* 2006). The response of muscle myostatin mRNA varies by trained state; endurance-trained cyclists show a small decrease in muscle myostatin mRNA three hours after maximal resistance training, while resistance-trained power lifters show no changes (Coffey *et al.* 2006). Unfortunately, this study did not directly compare between resistance- and endurance-trained athletes at baseline, which would have made for an interesting comparison.

If animal work in endurance training is examined in lieu of human findings, we see that chronic endurance

Table 1 Effect of resistance training on serum myostatin levels

	Sessions per week × weeks	Sets × reps (1RM%)	Sample timing post-final session	Myostatin effect	% Increase	Measured
Willoughby (2004b)	3 × 12	3 × 6–8 (85–90%) 3 × 10–12 weeks 1–3 (80%)	Immediate	↑↑↑	56%	Protein & mRNA
Walker *et al.* (2004)	2 × 10	4 × 8–10 weeks 4–6 4 × 6–8 weeks 7–10	48 h post	↓↓↓	20%	Protein
Jones *et al.* (2004)	3 × 6	Resumption of weight bearing following disuse	48 h post	↓↓↓	48%	mRNA
Hulmi *et al.* (2007)	2 × 21	5 × 10 (at 10RM)	48 h post	↓↓↓	48%	mRNA

↑↑↑ indicates an increase in myostatin level; ↓↓↓ indicates a decrease.

training in rats induces a decrease in muscular myostatin mRNA, while a single acute bout does not (Matsakas *et al.* 2006). Counter to this, 14 days voluntary running wheel activity (>4000 m day^{-1}) has no effect on muscle myostatin mRNA expression in rats (Bodell *et al.* 2009), nor does 5 weeks of treadmill running (1 h day^{-1}, 21 m min^{-1}) in mice (Lehti *et al.* 2007). A potential hypothesis explaining this difference would be exercise modality. Matsakas *et al.* (2006) utilized swim training, which has been shown to have varying effects on fibre-type conversion and enzymatic expression when directly compared to wheel running in mice (Matsakas *et al.* 2010).

While acute starvation (40 h) does not appear to alter myostatin expression, either in serum or from muscle biopsy (Larsen *et al.* 2006), 18 months post-biliopancreatic diversion surgery in morbidly obese individuals does demonstrate a decrease in myostatin mRNA expression from muscle biopsy (Milan *et al.* 2004). The result of Larsen *et al.* (2006) is perhaps unsurprising as no alterations were seen in any of the measured pathways of either catabolism (atrogin, murf) or synthesis (IGF-1), suggesting perhaps the timeline investigated was insufficient to induce a response. It also should be noted that the subject pool used in the study by Larsen *et al.* (2006) was small ($N = 6$, $3 = \male$, $3 = \female$).

Mechanisms of actions

With the rapid build-up of knowledge outlining the importance of myostatin in the regulation of skeletal muscle with various atrophic and hypertrophic stimuli, the key research direction was to understand how myostatin-controlled muscle homeostasis, as the ability to mediate such a powerful regulator, would have clear clinical benefits in a wide variety of conditions.

Myostatin is expressed primarily by myotubes *in vitro*, where it is primarily found in the nucleus (Artaza *et al.* 2002). As noted earlier, myostatin circulates as an endocrine hormone and can be measured in serum, with serum levels correlating with muscle mass in healthy and cachexic HIV patients (Gonzalez-Cadavid *et al.* 1998). Upon atrophic stimulation of mature myotubes by dexamethasone, myostatin is released into the cytoplasm of the cell (Artaza *et al.* 2002). After propeptide cleavage, the active C-terminus of myostatin forms a homodimer under normal conditions (Taylor *et al.* 2001). Myostatin is then held in an inactive state by its propeptide (Hill *et al.* 2003, Jiang *et al.* 2004). Myostatin can then be released from the cell (Artaza *et al.* 2002) and act in an autocrine or paracrine manner.

Myostatin binds to its receptor, the transmembrane receptor protein activin receptor type IIB (ARIIB), and also shares limited binding with activin receptor II (Rebbapragada *et al.* 2003). Upon myostatin binding, ARIIB homodimerizes with either activin receptor-like kinase-4 or -5 to induce signalling in the internal cellular environment. The role of myostatin appears to be greater in type II (fast) fibres, as perturbation of C2C12 cells with dexamethasone induces increases in myostatin expression to a greater level in cells that also stain positive for myosin heavy chain type two (Artaza *et al.* 2002). Indeed, myostatin expression is primarily localized in type II 'fast twitch' cells (McPherron & Lee 1997, Allen & Unterman 2007). Further, the hypertrophy seen with myostatin $-/-$ mice is greatest in type II muscle fibres (McPherron *et al.* 1997, personal obervations), and inhibition of myostatin activity results in greater hypertrophy in type II fibres over type I (Lawlor *et al.* 2011). Finally, murine type II muscle appears to have greater expression of ACTRIIB mRNA (Allen & Unterman 2007), providing a further explanation for the above-mentioned findings.

An early hypothesis was for a direct link between growth hormone (GH) and myostatin. Indeed, Liu *et al.* (2003) noted GH addition to C2C12 cells resulted in decreased myostatin expression, and inhibition of GH had the opposite effect. However, treatment of pigs with GH does not appear to change muscle myostatin level (Ji *et al.* 1998). Treatment of GH-deficient adults with GH does reduce myostatin mRNA expression, but not the bioactive protein levels (Liu *et al.* 2003). Furthermore, the myostatin response to muscle overload in adult hypophysectomized rats is not altered (Yamaguchi *et al.* 2006), suggesting the relationship seen by Liu *et al.* (2003) was indirect in nature.

Loss of muscle mass *in vivo* may either be as a result of inhibition of synthesis, an increase in degradation or some combination of the two. Myostatin activity appears to inhibit the Akt-mTOR pathway, upregulate activity of the ubiquitin-proteasomal mechanism and prevent synthesis by inhibition of satellite cell replication and translocation, examined separately below.

Myostatin & satellite cell activity

As myotubes are both multinucleated and terminally differentiated, the only source of new nuclei during hypertrophy of mature myotubes is fusion of new myoblasts from satellite cells. It was quickly confirmed that one mechanism of myostatin was to inhibit myoblast proliferation. C2C12 myoblasts cultured in standard growth media (Dulbecco's modified Eagle's media + 10% foetal bovine serum) in the presence of myostatin do not replicate, and further, this inhibition

of myoblast proliferation is dose-dependent (Thomas *et al.* 2000, Taylor *et al.* 2001). These effects appear to be via a prevention of Pax7–MyoD co-localization, maintaining satellite cells in quiescence. Increases in recombinant myostatin protein levels result in decreased co-localization, maintaining satellite cells in quiescence and preventing proliferation (McFarlane *et al.* 2008).

Further to the direct effects of myostatin on proliferation, myostatin appears to have the ability to inhibit fusion of myoblasts into differentiated myotubes. Cloned C2C12 cells overexpressing myostatin have a reduced differentiation response (Rios *et al.* 2002), which appears to result from inhibition of MyoD activity (Langley *et al.* 2002). MyoD is a key rate-limiting step in the formation of mature myotubes, it being necessary for myoblast differentiation (Megeney *et al.* 1996). The effect of myostatin on MyoD appears to be mediated via the common SMAD signalling pathway, similar to other TGF-β family members. Specifically, in response to myostatin binding to its receptor, SMAD2 and SMAD3 are phosphorylated, inducing binding with SMAD4, allowing the entire transcription factor complex to translocate and block MyoD production (Zhu *et al.* 2004, Allen & Unterman 2007), thereby subsequently reducing the ability of new myoblasts to proliferate and fuse.

However, it should be noted that the effect of myostatin on satellite cell activity may not be a necessary step in muscular hypertrophy. Myostatin treatment appears to have no effect on myoblast proliferation *in vitro*. Further, myostatin −/− mice do not show significantly increased number of satellite cells over wild-type mice, as measured by Pax7-positive staining during immunohistochemistry (Amthor *et al.* 2009). The authors may have potentially overreached with the significance of this conclusion however, as their Pax7 methodology measured only number of satellite cells but gave no information on the more important

ratio of activation to quiescence. These findings remind us that the effect of myostatin on muscle mass is multipathway-dependent, as seen in Figure 1 below.

Finally, once proliferation has taken place, new myoblasts must fuse to produce mature myotubes. Myoblasts co-cultured in differentiation media with myostatin (100 ng mL^{-1}) show reduced fusion, the effect of which is rescued by the inhibition of myostatin (Nozaki *et al.* 2008).

Increased proteasomal activity

The ubiquitin-proteasomal system is a key pathway for the identification of proteins for ubiquitination, either for the rapid targeted removal of signalling protein or for the normal protein turnover. Cachexic loss of muscle mass in chronic conditions often coexists with elevated proteasomal activity and subsequently increased protein degradation (reviewed by Mitch & Goldberg 1996). Muscle tissue has at least two specific ubiquitin ligases downstream of FoxO1, murf and atrogin, identified by Bodine *et al.* (2001). Myostatin stimulation (10 μg mL^{-1}) of C2C12 cells results in a decrease in myotube diameter by 57%, with up-regulated atrogin (150% increase above control) but not murf. Further, myostatin stimulation in the presence of siRNA for FoxO1 results in no change in atrogin expression (McFarlane *et al.* 2006), demonstrating the FoxO1-dependent nature of this mechanism. The myostatin promoter region contains FoxO1 binding sites, and activation of these by FoxO1 significantly increases myostatin expression, where blocking of FoxO1 activity inhibits myostatin production in both myoblasts and myotubes (Allen & Unterman 2007). Furthermore, this signalling pathway is independent of the above-mentioned SMAD signalling pathway, C2C12 myoblasts with inhibited SMAD signalling retain normal FoxO1 activity, and vice versa (Allen & Unterman 2007). This ability of myostatin to regulate
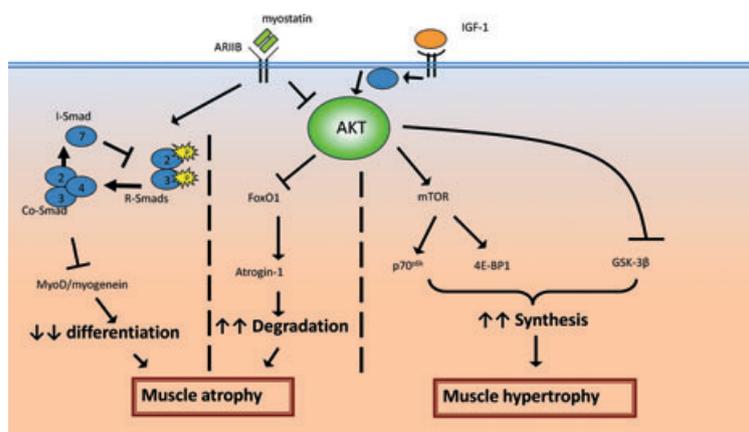

Figure 1 Three separate intracellular signaling pathways by which myostatin is known to alter muscle homeostasis.

proteasomal activity is also independent of the (below described) Akt pathway as Akt −/− mice produce atrogin and murf normally in response to myostatin increases (Goncalves *et al.* 2010).

Inhibited Akt signalling

GH and IGF-1 promote protein synthesis via Akt, a powerful anabolic signalling nexus (Goncalves *et al.* 2010). Akt signalling induces muscular hypertrophy by multiple mechanisms (reviewed by Frost & Lang 2007). Myostatin reduces phosphorylated levels of Akt_{thr308} by $\approx 25\%$, as well as reducing phosphorylation ratio of several downstream signalling proteins (Amirouche *et al.* 2009). This mechanism is distinct from the above-mentioned SMAD signalling as inhibition of SMAD2 signalling in the presence of increased myostatin does not prevent Akt inhibition (Yang *et al.* 2007).

In parallel to the above findings, elderly (≈ 70 years of age) human participants with sarcopenia demonstrate increased myostatin levels as well as a reduced Akt phosphorylation/total Akt ratio (Leger *et al.* 2008). Opposite effects are seen post-resistance exercise, whereby myostatin protein levels from muscle biopsy are decreased and phosphorylation of Akt and its downstream pathway constituents is increased (Mascher *et al.* 2008).

It was previously hypothesized that FoxO1 and the Akt pathway shared a direct link by which phosphorylation of FoxO1 reduced activation of mTOR and its downstream 4E-BP1, thereby further reducing protein synthesis (Southgate *et al.* 2007). However, it should be noted that the publication by Southgate *et al.* (2007) has been withdrawn, no reason has currently been given. The current state of understanding of myostatin's effect on synthesis, degradation and satellite cell activity is presented in Figure 1.

Inhibition of myostatin

The above-reviewed papers demonstrate the breadth of situations where myostatin is involved in the process of muscle atrophy. With myostatin's involvement in such a range of different conditions, an attractive hypothesis would be that myostatin acts as a central regulator or common step in the muscle atrophy process. Such a hypothesis makes the potential of myostatin inhibitors clinically attractive as they could conceivably be useful in a wide range of conditions. Indeed, in patients with COPD, it has been demonstrated that peripheral muscle function correlates with disease progression (Bernard *et al.* 1998) as well as with quality of life and mortality (Swallow *et al.* 2007). If myostatin inhibition could slow or even prevent loss of muscle mass in cachexic and sarcopenic

populations, both quality of life and mortality would logically be expected to improve.

After propeptide cleavage, the active C-terminus of myostatin forms a homodimer under normal conditions (Taylor *et al.* 2001). The myostatin protein is then held in an inactive state by its propeptide (Hill *et al.* 2003, Jiang *et al.* 2004). As expected, overexpression of the myostatin propeptide *in vitro* leads to inhibition of myostatin activity (Thies *et al.* 2001). Titan-cap, a sarcomeric protein, is also capable of binding to and inactivating mature myostatin inside the cell, providing a mechanism of regulating myostatin activity prior to secretion (Nicholas *et al.* 2002).

As myostatin circulates as an endocrine hormone (Gonzalez-Cadavid *et al.* 1998, Zachwieja *et al.* 1999, Zimmers *et al.* 2002), an obvious target would be the removal of, or nullifying, serum myostatin. Indeed, GASP-1 (Hill *et al.* 2003), Follistatin-related gene (Hill *et al.* 2002) and follistatin (Zhu *et al.* 2004) all circulate systematically and bind to and inhibit myostatin activity. Unfortunately, the majority of these myostatin inhibitors act via multiple mechanisms, making them unsuited for use. For example, transgenic inhibition of follistatin causes perinatal death in mice and has significant phenotypic effects beyond muscle mass, as follistatin acts on several aspects of homeostasis via its effects on the activin family (Matzuk *et al.* 1995). It is worth noting that follistatin overexpression in myostatin −/− mice results in further increases in muscle mass over myostatin −/− mice alone (Lee 2007), which may be via the effects of follistatin on other TGF-β family members. Nakatani *et al.* (2008) successfully inhibiting myostatin expression in a mouse model of muscular dystrophy (MDX) with a transgenic derivative of follistatin, FS-1, which maintained its myostatin binding ability but had reduced activin binding activity. Subsequent FS-1-overexpressing mice had significantly increased muscle mass and an increase in individual muscle fibre size with no reported side effects. FS-1 mice also demonstrated increased strength and endurance (Nakatani *et al.* 2008), showing induced muscular hypertrophy was functional.

Inhibition of myostatin by serum circulating antibody in the MDX mice (a model of muscular dystrophy) results in increased muscle mass, enhanced grip strength and exercise performance, an increase in basal metabolic rate, but also an increase in the number of centrally located nuclei in skeletal muscle cells (Bogdanovich *et al.* 2002). This increase can either be interpreted as a sign of abnormal development or a higher rate of cellular turnover, suggesting more investigation is necessary. Counter to this, MDX myostatin −/− mice demonstrate no increase in centrally located nuclei at 15 months of age (Amthor *et al.* 2009).

Potentially explaining differences in findings are the different models used by either group. A constitutive MDX myostatin −/− mice is arguably less physiologically relevant as a research model than an MDX mouse model treated with a pharmacological myostatin inhibitor.

Whittemore *et al.* (2003) treated adult mice with an anti-myostatin monoclonal antibody to assess the effects of myostatin modulation in the adult animal. Results demonstrated increased muscle mass, increased muscle fibre cross-sectional area and importantly, increased functionality, as measured by grip strength. Finally, in a mouse model of limb-girdle muscular dystrophy, overexpression of a modified, serum-soluble, myostatin receptor prevented muscle atrophy, apparently via the SMAD signalling pathway, as it was noted that dystrophic mice had increased SMAD2 activity, whereby treated mice had levels similar to wild-type mice (Ohsawa *et al.* 2006). Inhibition of myostatin during disuse atrophy in mice leads to a protective effect, partially preventing muscle mass loss and offsetting loss of force production (Murphy *et al.* 2011), a finding with exciting applications in settings such as disuse atrophy and microgravity exposure.

Unfortunately, successful inhibition of myostatin in human work has yet to be conclusively demonstrated. Myostatin inhibition utilizing a human analogue of Whittemore's work (Whittemore *et al.* 2003), in patients with various muscular dystrophies, gives a dose-dependent increase in muscle fibre size, but no changes in patient strength or rate of disease progression (Wagner *et al.* 2008). Phenotypically, muscle mass increases were significant in one disease subgroup (Becker's muscular dystrophy), while all other groups were approaching significance. However, as the authors note, this trial was not powered to assess muscle mass increases, instead focussing on drug safety (Wagner *et al.* 2008).

Finally, small interfering RNA (siRNA) approaches have been tested successfully in mice models. Kinouchi *et al.* (2008) developed and delivered successfully a siRNA targeting myostatin intravenously, resulting in reduced muscle myostatin protein expression and increased muscle mass. Alternatively, antisense-RNA interference is also possible, antisense-RNA targeting myostatin mRNA increases muscle mass in both normal and cachexic mice (Liu *et al.* 2008), showing obvious clinical applications.

While myostatin inhibition may soon prove to be a positive clinical tool in the prevention of muscle loss during disuse, cachexia and sarcopenia, a note of caution should be made. Myostatin −/− mice lost significantly greater muscle mass during hindlimb suspension disuse atrophy than wild-type mice (McMahon *et al.* 2003b), counter to the hypothesized

protective effects of reduced myostatin levels. Further, it was recently noted that some strains of myostatin −/− mice have increased rates of muscle degeneration and intramuscular lesion formation, where myostatin −/+ mice did not (Gentry *et al.* 2010), perhaps suggesting that partial inhibition, such as that which would be seen with pharmaceutical interventions, may prove safe. Indeed, to the best of the authors' knowledge, excluding the results of Bogdanovich *et al.* (2002) for the above-mentioned reasons, no reports of muscle pathology have been seen with *in vivo* myostatin inhibitory studies.

Role in cachexia & disease states

Is myostatin involved in cachexic loss of muscle mass? The answer must be considered to be yes. Since the identification of myostatin as muscle-specific regulator, increased myostatin expression has repeatedly been seen in cachexic patients secondary to HIV/AIDS, in cancer modelled in mice (Liu *et al.* 2008) and rats (Costelli *et al.* 2008), as well as in humans with severe COPD (Hayot *et al.* 2010) and heart failure (Gruson *et al.* 2011).

While it is clear that myostatin is involved in the cachexic loss of muscle mass in human diseases (Gonzalez-Cadavid *et al.* 1998, Hayot *et al.* 2010, Gruson *et al.* 2011), the more important question must be, 'are changes in myostatin observed during cachexia causative or secondary to muscle atrophy?'. The answer to this is less clear and difficult to investigate in humans. However, there is some evidence to suggest that myostatin is playing a causative role in cachexia.

First, the cause of cachexia itself is still unclear. A review by Wagner (2008) describing cachexia in patients with COPD presented evidence for various hypotheses including peripheral tissue hypoxia, systemic inflammation and hormonal imbalance potentially involving myostatin and elevated proteasomal activity. Indeed, the case for systemic inflammation is a strong one, as increased pro-inflammatory signalling induced by chronic disease clearly leads to cachexia-like atrophy in animal models of disease. Interleukin 6 overexpression in mice results in reduced muscle mass levels (Franckhauser *et al.* 2008). TNF-α infusion downregulates IGFBP-5 (Lang *et al.* 2006), while inhibition of TNF-α prevents inflammatory-induced inhibition of anabolic Akt pathway members such as 4EBP1, mTOR and ribosomal protein S6 (Lang & Frost 2007). The transgenic MIKK mouse overexpresses IκK in a muscle-specific manner, resulting in constantly activated pro-inflammatory NF-κB activity, demonstrates significant loss of muscle mass in mice (Cai *et al.* 2004). Finally, inhibition of TNF-α partially prevents muscle mass loss in cachexic rats

(Steffen *et al.* 2008). Multiple mechanisms have been suggested by which increased inflammation results in increased catabolism, discussed further below (New directions).

As previously mentioned, serum myostatin protein levels correlate with muscle mass levels in HIV patients with and without cachexia, as well as healthy normal individuals (Gonzalez-Cadavid *et al.* 1998). Overexpression of myostatin in mice also directly leads to muscle wasting in a manner that mirrors cachexia, both at the cellular level (McFarlane *et al.* 2006, 2008) and phenotypically (Zimmers *et al.* 2002). Further, this expression occurs at sites distal to myostatin expression, demonstrating the endocrine nature of its actions, as suggested by early work on human AIDS patients (Gonzalez-Cadavid *et al.* 1998, Zachwieja *et al.* 1999). Finally, muscle atrophy in a mouse model of cancer cachexia is offset by myostatin inhibition (Liu *et al.* 2008).

This hypothesized causative role of myostatin in cachexia will be confirmed with positive results from new studies currently utilizing myostatin inhibitors in animal, and hopefully soon, human clinical studies.

New directions

Inflammation regulation

The myostatin promoter gene expresses a NF-κB binding site (Ma *et al.* 2001), suggesting NF-κB is capable of inducing myostatin transcription. Indeed, concurrent increases in myostatin expression are seen with viral pneumonia infection of pigs (Escobar *et al.* 2004). Sustained NF-κB activity in mice causes significant muscle atrophy (Cai *et al.* 2004). Further, inflammatory mediators such as TNFα act in muscle to inhibit myogenic differentiation via NF-κB (Guttridge *et al.* 2000, Li & Reid 2000, Langen *et al.* 2001, 2004, Li & Schwartz 2001), upregulate atrogin and murf expression (Liu *et al.* 2003, Li *et al.* 2005), and inhibit the anabolic actions of the insulin-like growth factor-Akt pathway (Fernandez-Celemin *et al.* 2002), all similar mechanisms to myostatin actions (as reviewed above). Finally, inhibition of inflammatory processes prevents the above-mentioned catabolic processes (Costelli *et al.* 1993, Tessitore *et al.* 1994, Llovera *et al.* 1998, Lang *et al.* 2006, Lang & Frost 2007).

However, Lang *et al.* (2001) suggested lipopolysaccharide (LPS) injection into rats had no effect on myostatin, as 24 h post-LPS injection myostatin mRNA levels were unchanged. Stimulation of cultured myotubes with TNF-α also had no effect on myostatin mRNA 0.5–24 h post-stimulation (Bakker *et al.* 2005) or after 4 days of TNF-α stimulation (Larsen *et al.* 2008). Finally, attempts to induce NF-κB activity via myostatin stimulation in C2C12 myoblasts were unsuccessful (Bakker *et al.* 2005). Combined, these results suggest that the hypothesized inflammation–myostatin link was absent.

Counter to the above findings, increases in myostatin expression in a rat model of cancer cachexia are offset with pentoxifylline, an inhibitor of TNF-α (Costelli *et al.* 2008). Further, treatment of a rat model of rheumatoid arthritis with fenofibrate, to reduce systemic inflammation, both reduced systemic TNF-α levels and blunted increases in myostatin, atrogin and murf (Castillero *et al.* 2011). One potential explanation for the discrepancies in these findings may be due to an acute verses chronic timing model used. While Lang *et al.* (2001) investigated 24 h in the rat model, Costelli *et al.* (2008) and Castillero *et al.* (2011) investigated longer time periods (7 and 15 days, respectively).

To the best of our knowledge, all stimuli resulting in muscle atrophy involve both increased systemic inflammation and increased myostatin levels. Indeed, Frost & Lang (2005) noted that systemic inflammation by any cause appeared to result in muscle atrophy. If a direct myostatin–systemic inflammation link is confirmed, this link may prove an important link between inflammation and cachexia, as well as an easily targetable one. A greater understanding of the underlying reason behind the discrepancy in findings is first required.

Adipose activity

Myostatin may play a direct role in the regulation of adipose homeostasis. Indeed, McPherron *et al.* (1997) noted myostatin −/− mice had detectable myostatin RNA in adipose. Lin *et al.* (2002) and McPherron & Lee (2002) noted that myostatin −/− mice had significantly reduced levels of subcutaneous body fat, with Lin *et al.* (2002) further noting that myostatin −/− mice showed decreased leptin concentrations. Overexpression of the inhibitory myostatin propeptide, reducing myostatin activity, also results in reduced subcutaneous adipose tissue (Zhao *et al.* 2005). Two possible hypotheses arising could explain this phenotype. First, myostatin may act directly on adipocytes, promoting proliferation or differentiation in a reversed mechanism as in skeletal muscle. The alternative is that reduced fat mass may be secondary to increased metabolic demands of significantly increased muscle mass of myostatin −/− mice.

It would seem that the hypothesis for a direct link between myostatin and adipocytes is more likely as myostatin has been demonstrated to inhibit differentiation of primary culture of bovine pre-adipocytes (Hirai *et al.* 2007). Stolz *et al.* (2008) directly stimulated adipocytes *in vitro* with recombinant myostatin

to further explore this phenomena. Early results appeared promising, with myostatin regulating SMAD signalling in a similar manner as muscle, thereby inhibiting adipogenesis *in vitro* (Stolz et al. 2008). However, when Stolz *et al.* (2008) measured the effects of myostatin inhibition and overexpression in mice, findings showed no effect of either intervention on fat mass. Tissue-specific knockout of myostatin in muscle and fat reveals that muscle −/− mice are resistant to weight gain in response to high-fat diets, while adipose −/− mice respond to a high-fat diet as normal (Guo *et al.* 2009). However, on a chow diet, neither group has an altered weight gain response when compared to wild-type mice (Guo *et al.* 2009).

Examining the interaction between myostatin and adipocytes from the alternative direction, Feldman *et al.* (2006) demonstrated that dexamethasone-stimulated adipocytes, *in vitro,* are capable of producing and excreting myostatin. Further, this expressed myostatin acted in a paracrine manner, directly regulating the development of the adipocytes *in vitro* (Feldman *et al.* 2006).

Indeed, *in vivo* adipose tissue appears to alter or utilize myostatin signalling in an endocrine manner. Wild-type mice fed on a high-fat diet demonstrate elevated muscle myostatin expression 3, 9 and 12 weeks post the onset of high-fat intake, which the authors hypothesize, may be active in promoting adipogenesis (Lyons *et al.* 2010). Post-developmental myostatin −/− mice are partially protected from the effects of consuming high-fat diets, with reduced weight gain, no liver hypertrophy and reduced intramuscular fat deposits (Burgess *et al.* 2011). However, the increase in intra-abdominal fat deposits after high-fat diet was identical between wild-type and myostatin −/− mice (Burgess *et al.* 2011). Indeed, transgenic mice overexpressing myostatin during adipogenesis show resistance to diet-induced obesity (Feldman *et al.* 2006), which taken with the *in vitro* data above suggest that myostatin may be a necessary step in the accumulation of fat mass.

Conversely, myostatin overproduction in adult mice led to significant decreases in white adipose tissue (Zimmers *et al.* 2002). However, it should be noted that in Zimmers *et al.* (2002) experiments, myostatin overexpression was induced by the implantation of CHO cell tumours overexpressing myostatin, so the experimental animals had the stimuli of both excess myostatin and tumour load to enhance catabolism. When an identical experiment was attempted, but with excess myostatin provided by injection of recombinant protein, no alteration in fat mass was seen (Stolz *et al.* 2008).

A picture emerges therefore of myostatin and adipose tissue interaction, whereby increasing adiposity may upregulate myostatin expression. However, the role of myostatin overexpression on adipose tissue *in vivo* is less clear and warrants further investigation. There is difficulty *in vivo* in separating the effects of myostatin alteration into direct effects on adipocytes and indirect effects on muscle tissue with then alter fat mass via alterations of basal metabolic rate. Indeed, it is possible that the effects seen above are a result of secondary signalling downstream of myostatin, a point raised previously (Allen *et al.* 2011). If increasing adiposity *in vivo* does stimulate myostatin overexpression, this could conceivably result in the reduction of whole body muscle mass, further resulting in negative outcome for the obese individual.

Glucose metabolism

Skeletal muscle is the most metabolically demanding tissue in the human body, being responsible for 20–30% of energy expenditure at rest and up to 90% during intense exercise (Zurlo *et al.* 1990). Stimuli that alter whole body muscle mass would therefore have significant effects on substrate utilization, in terms of both total amount and proportion of fuel sources.

Increasing muscle mass by myostatin inhibition elevates basal metabolic rate (Bogdanovich *et al.* 2002) as would be expected. This has obvious and immediate clinical applications; treatment of type II diabetics by myostatin inhibition to altering muscle mass would hypothetically result in increased whole body energy consumption, reduce peripheral fat mass and potentially lower blood glucose. Indeed, these results have already been demonstrated in mice, where high-fat diets and myostatin inhibition by propeptide overexpression prevents reduced insulin sensitivity, elevated blood glucose and accumulation of fat mass seen in pair-overfeed wild-type mice (Zhao *et al.* 2005).

Counter to the above findings, Zimmers *et al.* (2002) reported that mice who overexpressed myostatin demonstrated ~40–50% (muscle specific) loss in muscle mass. Interestingly, mice were hypoglycaemic as well as demonstrating loss of nearly all fat mass, despite no noted difference in caloric intake between overexpressing and wild-type mice. Reduced muscle mass resulting in hypoglycaemia is an unexpected paradox that requires further investigation. This finding was repeated *in vitro* in both C2C12 and L6 cell lines, stimulation of myotubes with myostatin results in a dose-dependent increase in glucose consumption, with 1.5–2.5 μg myostatin promoting a similar level of glucose consumption ($\approx$2.5 μmol mL^{-1}) as 100 nM insulin. Further, co-incubation with follistatin or the inhibitory myostatin propeptide blunted heightened consumption (Chen *et al.* 2010). One mechanism by
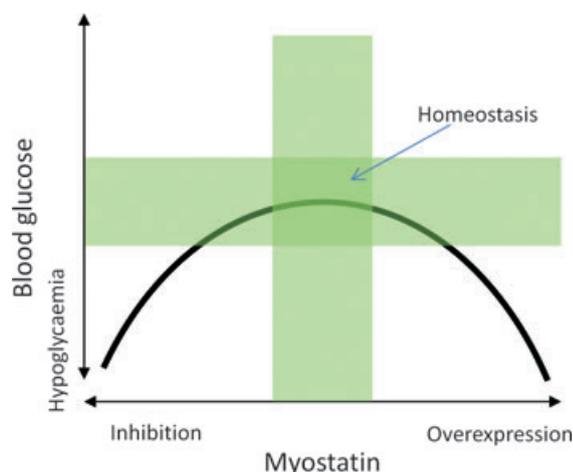
Figure 2 Conceptual presentation of the myostatin–hypoglycaemia paradox. Experimentally induced increases or decreases in systemic myostatin both result in hypoglycaemia. Green bars represent homeostasis in terms of blood glucose (horizontal) and serum myostatin (vertical), with overlap indicating homeostasis.

which myostatin stimulation induces hypoglycaemia is by increasing mRNA activity of several glucose-regulating proteins, including GLUT1 and GLUT4, IL6, hexokinase and phosphorylated adenosine triphosphate kinase (AMPK; Chen *et al.* 2010), thereby increasing cellular glucose uptake. One hypothesis is that this increase in energy demand is to provide energy acutely for catabolic processes, as catabolism is an energy intensive process.

Another potential hypothesis is that myostatin or some downstream signalling protein may act directly on metabolic pathways to alter the energy source. Indeed, it was recently shown that during human muscle atrophy, upregulated pathways were either cell cycle regulating (including myostatin) or energy metabolism affecting (Chen *et al.* 2007). Stimulation of C2C12 myotubes with myostatin protein results in increased GLUT1 and GLUT4 mRNA expression and increases the pAMPK:AMPK ratio (Chen *et al.* 2010). Increased GLUT4 mRNA expression and pAMPK protein levels are also seen in myostatin −/− mice (Zhang *et al.* 2011). Myostatin-stimulated myotubes show increased glucose consumption, but decreased total ATP level (Chen *et al.* 2010), suggesting that the effects of myostatin on AMPK and glucose are more likely due to a change in cellular energy state rather than a direct effect of myostatin on AMPK.

Combined, these results all suggest that a perturbation of myostatin in the whole body, either increasing or decreasing myostatin activity, results in increased glucose usage as a metabolic fuel source, resulting in hypoglycaemia (Fig. 2). From a clinical viewpoint, myostatin inhibition in the treatment of type II diabetes may present an exciting and useful intervention,

whereby myostatin overexpression to enhance cellular glucose uptake would not.

Cardiomyocytes homeostasis

Early evidence hinted that myostatin may not solely act in skeletal muscle. Sharma *et al.* (1999) identified the presence of myostatin in heart tissue in both mice and sheep, by both RT-PCR and immunocytochemistry. Indeed, with such structural and metabolic similarities, it is perhaps unsurprising that cardiomyocytes and myocytes share signalling mechanisms.

Myostatin mRNA levels are elevated in areas surrounding the damage post-infarct, suggesting a role in regulation of cardiomyocyte apoptosis after hypoxic injury (Sharma *et al.* 1999). Plasma myostatin concentration is elevated in non-cachexic human patients post-heart failure (Gruson *et al.* 2011). Myostatin-overexpressing mice also demonstrate cardiac atrophy with reduced left ventricular mass (Artaza *et al.* 2007). Indeed, it was recently demonstrated that myostatin overexpression results in decreased cardiac growth in the developing mouse, and this effect appears to be via inhibition of Akt (Bish *et al.* 2010), in a similar manner as myostatin's inhibition of Akt in muscle, reviewed above. Excitingly, a recent study took left ventricle cardiac biopsies from patients with ischaemic cardiomyopathy and age-matched controls. The authors showed no difference in myostatin protein expression from cardiac biopsies between ischaemic patients and controls, but did however demonstrate an increase in circulating myostatin levels (George *et al.* 2010). An interesting finding as chronic heart failure patients have been noted to suffer from cachexic loss of muscle mass (Anker *et al.* 1997). While speculative, it is interesting to hypothesize a causative role of myostatin increasing systemically post-heart failure resulting in cachexia in this population. If these same signalling mechanisms are maintained in the human, then future use of myostatin inhibitors will need to carefully investigate potential effects on the heart, as a foreseeable outcome would be cardiac hypertrophy, clinically problematic.

Outcomes

Myostatin has been well examined in the last decade, with significant gains in understanding of how it affects skeletal muscle, and more recently cardiac and adipose tissue. Smooth muscle appears to be the only contractile tissue to not express myostatin (Gonzalez-Cadavid *et al.* 1998). Research has advanced to the point where we are now regularly seeing animal studies of successful myostatin inhibition for the purpose of muscle hypertrophy (Whittemore *et al.* 2003,

Castillero *et al.* 2011, Murphy *et al.* 2011) and are now attempting *in vivo* clinical human studies targeting myostatin for the prevention of muscle mass loss in MDX (Wagner *et al.* 2008). Our understanding of cachexia and the role of myostatin during cachexic atrophy continues to evolve; the potential to link myostatin inhibitors currently under testing for MDX to cachexia would enable life prolonging and quality of life enhancing treatment for a number of terminal conditions, including cancer, HIV/AIDS, chronic heart and renal failure and COPD.

Finally, we are beginning to gain an understanding of the role of myostatin in other tissues, aside from its primary function in maintaining muscle mass homeostasis. With the ongoing and increasing prevalence of type II diabetes as a major problem for the world's population, any potential paths furthering our understanding of glucose homeostasis deserve further attention. As we have examined above, myostatin appears to play roles in both the regulation of energy homeostasis and adipose tissue proliferation. The current work focussing on the development of inhibitors of myostatin in human muscular dystrophic disorders could see exciting cross-over in the treatment of diabetes, metabolic syndrome and many other modern 'Western' diseases.

Conflict of interest

The authors have no conflict of interest and have nothing to disclose.

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