IGF1 stimulates greater muscle hypertrophy in the absence of myostatin in male mice

Alexander Hennebry¹, Jenny Oldham¹, Tea Shavlakadze², Miranda D Grounds², Philip Sheard³, Marta L Fiorotto⁴, Shelley Falconer¹, Heather K Smith⁵, Carole Berry¹, Ferenc Jeanplong¹, Jeremy Bracegirdle¹, Kenneth Matthews¹, Gina Nicholas¹, Mônica Senna-Salerno¹, Trevor Watson¹ and Christopher D McMahon¹

¹AgResearch Ltd, Hamilton, New Zealand

²School of Anatomy, Physiology & Human Biology, The University of Western Australia, Crawley, Western Australia, Australia

³Department of Physiology, University of Otago, Dunedin, New Zealand

⁴USDA/ARS Children's Nutrition Research Center, Baylor College of Medicine, Houston, Texas, USA

⁵Department of Exercise Sciences, University of Auckland, Auckland, New Zealand

Correspondence should be addressed to C McMahon

Email
mcmahonc@xtra.co.nz

Abstract

Insulin-like growth factors (IGFs) and myostatin have opposing roles in regulating the growth and size of skeletal muscle, with IGF1 stimulating, and myostatin inhibiting, growth. However, it remains unclear whether these proteins have mutually dependent, or independent, roles. To clarify this issue, we crossed myostatin null (*Mstn*^{-/-}) mice with mice overexpressing *Igf1* in skeletal muscle (*Igf1*⁺) to generate six genotypes of male mice; wild type (*Mstn*^{+/+}), *Mstn*^{+/-}, *Mstn*^{-/-}, *Mstn*^{+/+;Igf1}⁺, *Mstn*^{+/-;Igf1}⁺ and *Mstn*^{-/-;Igf1}⁺. Overexpression of *Igf1* increased the mass of mixed fibre type muscles (e.g. *Quadriceps femoris*) by 19% over *Mstn*^{+/+}, 33% over *Mstn*^{+/-} and 49% over *Mstn*^{-/-} ($P < 0.001$).

By contrast, the mass of the gonadal fat pad was correspondingly reduced with the removal of *Mstn* and addition of *Igf1*. Myostatin regulated the number, while IGF1 regulated the size of myofibres, and the deletion of *Mstn* and *Igf1*⁺ independently increased the proportion of fast type IIB myosin heavy chain isoforms in *T. anterior* (up to 10% each, $P < 0.001$). The abundance of AKT and rpS6 was increased in muscles of *Mstn*^{-/-} mice, while phosphorylation of AKT^{S473} was increased in *Igf1*⁺ mice (*Mstn*^{+/+;Igf1}⁺, *Mstn*^{+/-;Igf1}⁺ and *Mstn*^{-/-;Igf1}⁺). Our results demonstrate that a greater than additive effect is observed on the growth of skeletal muscle and in the reduction of body fat when myostatin is absent and IGF1 is in excess. Finally, we show that myostatin and IGF1 regulate skeletal muscle size, myofibre type and gonadal fat through distinct mechanisms that involve increasing the total abundance and phosphorylation status of AKT and rpS6.

Key Words

- skeletal muscle
- AKT
- growth
- myofibre hypertrophy
- myofibre hyperplasia

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Introduction

Two factors play critical but opposing roles in regulating the growth and size of skeletal muscle. The growth hormone, insulin-like growth factor-1 (GH-IGF1) axis

stimulates growth and accounts for 83% of postnatal growth and the increased muscle size (hypertrophy) in young mice (Lupu *et al.* 2001). Locally produced IGF1

is now considered to have the predominant influence on growth of tissues and organs as was convincingly demonstrated when liver-specific deletion of *Igf1* failed to inhibit growth of mice despite a 75% reduction in the concentration of IGF1 in blood (Sjogren *et al.* 1999, Yakar *et al.* 1999). Thus, local delivery of IGF1 to skeletal muscle is essential for growth (Shavlakadze *et al.* 2010). The benefits of *Igf1* overexpression on adult muscles in homeostasis and ageing muscles are debated: while two studies reported that viral or transgenic overexpression of *Igf1* targeted to skeletal muscle reduced the extent of sarcopenia in mice aged up to 24 months (Barton-Davis *et al.* 1998, Musaro *et al.* 2001), more recent studies using transgenic *Igf1* mice aged up to 28 months found that IGF1 alone did not prevent the loss of mass and strength of old muscles (McMahon *et al.* 2014). In contrast to IGF1, myostatin inhibits muscle growth. In the absence of myostatin, the mass of skeletal muscles is increased two- to three-fold more than that in wild-type mice during development (McPherron *et al.* 1997). Despite the importance of IGF1 and myostatin during growth, their precise molecular interactions in regulating the critical early events of postnatal growth are unclear.

Myostatin and IGF1 activate common and independent signal transduction pathways. It is well documented that myostatin binds and activates activin type 2B receptors to promote phosphorylation of Smad2/3 (Rebbapragada *et al.* 2003), while IGF1 activates the IGF1 receptor and phosphorylates PI3 kinase (PI3K) and AKT (Rommel *et al.* 2001). The PI3K/AKT pathway is common to both factors, and there is considerable cross-regulation between the two, wherein myostatin inhibits AKT to block IGF1-induced hypertrophy of myotubes (Morissette *et al.* 2009), while IGF1 inhibits myostatin-induced activation of Smad3 (Retamales *et al.* 2015). Given that both factors are important regulators of muscle development, it remains unclear if both the absence of myostatin and excess IGF1 would increase the growth of skeletal muscle more than either condition alone. For example, the absence of myostatin during foetal development increases the number of myofibres, while the diameter of myofibres is increased in postnatal muscle (McPherron *et al.* 1997, Amthor *et al.* 2009). Furthermore, experimental antagonism to block the function of myostatin using antibodies (Latres *et al.* 2015), follistatin, which binds to and antagonises myostatin (Amthor *et al.* 2004), or antagonists of the activin2B receptor, also induces hypertrophy of myotubes *in vitro* and postnatal myofibres (Lee & McPherron 2001). However, when myostatin

activity is blocked, hypertrophy still requires the presence of IGF1 receptors (Kalista *et al.* 2012, Winbanks *et al.* 2012). Therefore, it appears that IGF1 is critical for enabling the actions of myostatin on muscle development.

Postnatal growth of skeletal muscles in mice is primarily due to hypertrophy, with increasing net protein content associated with elongation and expansion of the cross-sectional area of myofibres, because the number of myofibres is fixed before birth (Rowe & Goldspink 1969, Ontell *et al.* 1984, Timson & Dudenhoefter 1990, White *et al.* 2010). Hypertrophy of myofibres occurs when the balance of protein synthesis and degradation is altered to increase protein accretion. Protein synthesis involves the PI3K/AKT/mTOR pathway, downstream of which is p70S6k, which regulates phosphorylation of 4EBP1 and rpS6 (Dever 2002, Proud 2004, Ruvinsky & Meyuhas 2006, Laplante & Sabatini 2012, Morita *et al.* 2015). To initiate protein synthesis, the repressor protein 4EBP1 becomes phosphorylated, which allows it to disengage from the translation initiation factor eIF4E on the 5' cap-end of nascent mRNA to enable assembly of the eIF4F complex (Dever 2002, Proud 2004, Ruvinsky & Meyuhas 2006, Laplante & Sabatini 2012, Morita *et al.* 2015). In addition, phosphorylated rpS6 promotes translation of mRNAs that encode ribosomal proteins and other translation factors, which leads to an increased cellular capacity for protein synthesis (Proud 2004, Magnuson *et al.* 2012). Myostatin inhibits protein synthesis via inhibiting p70S6k and rpS6 (Trendelenburg *et al.* 2009, Welle *et al.* 2009), while IGF1 stimulates phosphorylation of 4EBP1 and rpS6 (Shen *et al.* 2005, Gao *et al.* 2015). Thus, the two factors, having opposing effects, could act in an additive or synergistic manner via common or distinct pathways to establish muscle mass.

To elucidate such potential actions, we investigated the role of IGF1 on the growth of skeletal muscle in the presence and absence of myostatin. Our aims were to determine (1) if increased IGF1 would increase the mass of skeletal muscles when the myostatin gene was removed, and (2) what effect increased IGF1 may have on myofibre number, size and type, on body composition and on downstream signalling targets.

Materials and methods

Transgenic mice

This research was approved by the Ruakura Animal Ethics Committee (Application 1161). Generation of *Mstn*^{-/-}

mice (where all tissues lack expression of *Mstn*) has been previously described (McPherron *et al.* 1997). We obtained a breeding pair of these mice as a gift from S-J Lee (Johns Hopkins University School of Medicine, Baltimore, MD, USA). In Study 1, male wild-type mice that express myostatin (*Mstn*^{+/+}) and *Mstn*^{-/-} mice were used (see below). Study 2 used transgenic *Igf1* (C2:Ea) mice generated at the European Molecular Biology Laboratory, Rome, Italy by N Winn and N Rosenthal: the MLC/*Igf1* (C2:Ea) expression construct was generated by cloning the mouse *Igf1* (C2:Ea) cDNA sequences into the previously described skeletal muscle-specific expression cassette containing the myosin light chain (MLC) 1/3 promoter, a SV40 polyadenylation signal, followed by the MLC1/3 enhancer sequence (Rosenthal *et al.* 1989, Musaro *et al.* 2001, Shavlakadze *et al.* 2010). In these MLC/*Igf1* transgenic mice, the IGF1 is most highly expressed in fast-twitch myofibres in the order 2B, 2X and 2A (Shavlakadze *et al.* 2005). The *Igf1* (C2:Ea) (FVB background) and *Mstn*^{-/-} (C57BL/6J background) mice were maintained as separate colonies at the Ruakura campus of AgResearch Ltd. The F1 generation from a cross of *Igf1* (C2:Ea) and *Mstn*^{-/-} founders was used to produce six genotypes in the F2 offspring: *Mstn*^{+/+} (no *Igf1* transgene), *Mstn*^{+/-}, *Mstn*^{-/-}, *Mstn*^{+/+}:*Igf1*⁺, *Mstn*^{+/-}:*Igf1*⁺ and *Mstn*^{-/-}:*Igf1*⁺ for Study 2. Only male mice were used for Study 2, and all mice, including *Mstn*^{+/+}, had the same FVB × C57BL/6J background.

Mice were housed 2–4 per cage in a temperature-controlled (21°C) and light-controlled (12h:12h, light:darkness cycle) animal facility at Ruakura Research Centre. Mice had *ad libitum* access to a standard rodent chow (Specialty Feeds, Glen Forest, Western Australia; protein 19.4% (by weight), total fat 4.8%, crude fibre 4.8% and digestible energy 14 MJ ME/kg) and water.

Study 1. Quantification of *Igf1Ea* mRNA in muscles of *Mstn*^{+/+} and *Mstn*^{-/-} mice during postnatal growth from 4 to 20 weeks of age

Male *Mstn*^{+/+} and *Mstn*^{-/-} pups were killed at 4, 8, 12 and 20 weeks (*n*=6 mice per genotype, per age). *Quadriceps* muscles were extracted, and mRNA was prepared for quantitation of *Igf1Ea* via qPCR (see below).

Study 2. Phenotype analyses for six genotypes during early postnatal growth (sampled at 12 weeks of age)

Male pups with or without the *Igf1* (C2:Ea) transgene that had a matching myostatin genotype (*Mstn*^{+/+}, *Mstn*^{+/-} or

Mstn^{-/-}) within litters were included in the study. Mice were weighed weekly, and grip strength was measured before mice were killed at 12 weeks of age, and many tissues were sampled for molecular analyses as outlined below (*n*=8 per genotype). Mice were not fasted before death and were killed using CO₂ asphyxiation followed by cervical dislocation. At death, a sample of blood was collected via cardiac puncture into a tube containing EDTA. Plasma was harvested and stored at -20°C before assay of IGF1.

Grip strength (Study 2 only)

The grip strength of each mouse was assessed 2 days before death using the Muromachi grip strength metre (model MK-380M, Muromachi Kikai Co., Tokyo, Japan). This metre has a mesh pad on which a mouse is placed and can grip with all paws. Each mouse was placed on the mesh pad (attached to a force transducer) and pulled backwards by the base of the tail until release, for 5 consecutive attempts. The highest and lowest scores were removed, and the grip strength value for each mouse was recorded as the mean of the 3 remaining scores. The mean grip strength was then expressed relative to body mass (Force (kg)/BW (kg)).

Muscle and fat pad collection (Study 2 only)

Following euthanasia, the *Quadriceps femoris* (*quadriceps*), *B. femoris*, *gastrocnemius*, *plantaris*, *soleus*, *Extensor digitorum longus* (EDL) and *Tibialis anterior* (*T. anterior*) muscles were excised from both hind limbs, along with the heart and weighed. The soleus is a slow-twitch muscle. The *T. anterior*, *plantaris* and EDL are fast-twitch muscles, and the *quadriceps*, *B. femoris* and *gastrocnemius* muscles have a mixed myofibre composition (Hamalainen & Pette 1993, Augusto *et al.* 2004). Because mouse muscles are small, we had to use multiple muscles for the analyses. For Study 1, we used the *quadriceps* for qPCR and for Study 2, we used the *T. anterior* for muscle fibre typing, the *gastrocnemius* for qPCR and Western analyses and the *quadriceps* for measuring the amount of IGF1 in skeletal muscle.

The length of the tibia was measured and used to normalise the mean masses of skeletal muscles and the heart as previously described (White *et al.* 2016). The gonadal fat pads were excised and weighed and were expressed as a per cent of body mass. The gonadal fat pads correlate highly with total body fat (Rogers & Webb 1980).

Morphometric analysis and histology (Study 2 only)

The *T. anterior* muscles were cut transversely in the mid-belly region, mounted in OCT compound and frozen in isopentane cooled in liquid nitrogen. Cryosections (8 µm) were collected onto glass slides and immunostained with a rabbit anti-laminin antibody (L9393, Sigma) (dilution 1:300). A digital image of the entire transverse muscle section was produced by tiling non-overlapping individual images acquired using a Nikon Eclipse Ti microscope and a CoolSNAP EZ digital camera. The resolution allowed the total number of myofibres to be counted from 3 to 5 mice per genotype. The minimal myofibre diameter was calculated from automated digital capture of an average of 2900 myofibres per *T. anterior* muscle using FoveaPro software (Reindeer Graphics, Inc.) for 5–8 mice per genotype. This method has the advantage of being semi-automated and captures a large number of myofibres for analysis. The method also copes with myofibres oriented obliquely by treating each myofibre as a cylinder, wherein the minimal diameter is the least affected parameter (Briguet *et al.* 2004). The myofibre diameters were binned into 5 µm intervals from 10 to 100 µm using the Frequency function in Microsoft Excel to produce a frequency distribution for each group.

RNA extraction and quantitative (q) PCR (Studies 1 and 2)

Total RNA was extracted from the *quadriceps* muscle (Study 1) and from the *gastrocnemius* muscles (Study 2) using Trizol reagent (Invitrogen), and complementary DNA (cDNA) was reverse transcribed (RT) from 2 µg total RNA using qSCRIPT (Quanta BioSciences, Maryland, USA) as described previously (Smith *et al.* 2014). The qPCR was carried out using a Lightcycler 2.0 (Roche Diagnostics) and Roche Faststart DNA master PLUS SYBR Green I mix (Roche Diagnostics). The primers for *Pgc1A*, *Pgc1B*, *Mef2c*, *Igf1Ea*, *Igf1* transgene, *Igf1* receptor and binding proteins and myostatin are presented in Table 1. Standard curves were established by serial dilution of a pool of cDNA obtained from each sample, and results were normalised to the concentration of ssDNA in the RT samples (Lundby *et al.* 2005).

Western blot analyses (Study 2 only)

Protein was extracted from the *gastrocnemius* muscle, and Western blotting was performed as previously

described (Smith *et al.* 2014). Membranes were incubated with rabbit anti-AKT1/2/3 (1:10,000, #8312, Santa Cruz Biotechnology), rabbit anti-phospho AKT^{S473} (1:500, #7985, Santa Cruz Biotechnology), rabbit anti-4EBP1 (1:500, #R113, Santa Cruz Biotechnology), rabbit anti-phospho-4EBP1 (1:2000, Thr37/46, #236B4, Cell Signaling Technology), rabbit anti-rpS6 (1:1000, #2217, 5G10, Cell Signaling Technology) and rabbit anti-phospho-rpS6 (1:1000, Ser235/236, #2211, Cell Signaling Technology), and the abundance of each protein was detected with enhanced chemiluminescence. GAPDH was used as a loading control (mouse anti-rabbit GAPDH (1:10,000, #RDI-TRK5G4-6C5, Research Diagnostics, Inc., NJ, USA). The optical densities of each immunoreactive band were captured with a densitometer (GS 800, Bio-Rad Laboratories (NZ) Pty Ltd) and analysed using Quantity One software (Bio-Rad Laboratories (NZ) Pty Ltd).

Assessment of myosin heavy chain (MyHC) isoforms (Study 2 only)

Changes in the composition of MyHC in *T. anterior* muscles were determined electrophoretically as described previously (Smith *et al.* 2014). Despite improvements in separation of the bands using an 8 M urea/2 M thiourea buffer in which the samples were loaded, the 2A and X bands typically do not separate as cleanly in mouse skeletal muscle as they do in rat skeletal muscle (Blough *et al.* 1996). A 50:50 mixture of mouse *soleus* and *EDL* muscle were run in the gels to act as a marker to help identify MyHC bands.

IGF1 assay (Study 2 only)

Concentrations of IGF1 protein were assayed in homogenates from *quadriceps* muscles and in plasma using a mouse/rat IGF1 ELISA kit (#MG100, Quantikine ELISA, R&D Systems).

Statistical analysis

All data were analysed with GenStat v17 using the general procedure of ANOVA with variations in myostatin copy number (*Mstn*^{+/+}, *Mstn*^{+/-}, or *Mstn*^{-/-}) and IGF1 (*Igf1*, or *Igf*⁺) as main effects along with their interaction. *Post hoc* multiple comparisons were performed using Tukey's method (GenStat 2003). Data are presented as means along with the standard error of the mean (S.E.M.), or standard error of the differences between means (S.E.D.).

Table 1 Primers used for qPCR (Study 1 and 2).

Gene	Primer sequences	Size (bp)	Annealing (°C)	GenBank accession
<i>Pgc1A</i>	Fwd: GCAGAGACAAATGTGCTTCG Rev: GGAGGAGTTGTGGGAGGAGT	269	60	XM_006503779.3
<i>Pgc1B</i>	Fwd: CCAGACCAGCCTCAGTTCCAGA Rev: TTGGGGAGCAGGCTTTCACTC	164	60	NM_133249.2
<i>Mef2c</i>	Fwd: GTGTTGCTCAAGTACACCGAG Rev: CTGGACACTGGGATGGTAACT	260	60	NM_001170537.1
<i>Igf1Ea</i>	Fwd: GCTTGCTCACCTTTACCAGC Rev: AAATGTACTTCCTTCTGGGTCT	301	60	NM_001111275.2
<i>Igf1</i> (C2:Ea) ^a	Fwd: ACTGACATGCCCAAGACTCAG Rev: ATTCCACCACTGCTCCCATTC	283	60	Shavlakadze et al. (2010)
<i>Igf1r</i>	Fwd: CTGCGGCGATGAAGAGAAGAAAA Rev: TACCGGTGCCACGTTATGATGATT	546	60	NM_010513.2
<i>IgfBP3</i>	Fwd: AATGGCCGCGGGTTCTGC Rev: TTCTGGGTGTCTGTGCTTTGAG	269	60	NM_008343.2
<i>IgfBP4</i>	Fwd: CCATCCAGGAAAGCCTGCAG Rev: TGGAAGTTGCCGTTGCGGTACAG	298	60	NM_010517.3
<i>IgfBP5</i>	Fwd: TGCCTCAACGCCCGAGCTACG Rev: ACAAACTTGGACTGGGTCTCAGC	200	60	NM_010518.2
<i>IgfBP6</i>	Fwd: CCCCAGAGAGAACGAAG Rev: CACAGTTTGGCACATAGAG	307	60	NM_008344.3
<i>Mstn</i>	Fwd: ACCCATGAAAGACGGTACAAG Rev: TCATCACAGTCAAGCCCAAAG	288	60	NM_010834.3

^aFwd primer is common to exon 4, while the reverse primer is for the SV40 enhancer for the transgene.

Results

Study 1

There was significantly less *Igf1Ea* mRNA (the principal endogenous *Igf1* transcript) in *quadriceps* muscles of *Mstn*^{-/-} mice at 4 ($P<0.01$) and 8 ($P<0.01$) weeks (about half the amount) compared with *Mstn*^{+/+} mice, with concentrations of *Igf1Ea* mRNA reducing after this age in both strains (as muscle growth slows down) to similar concentrations by 20 weeks of postnatal growth (Fig. 1).

Figure 1

Concentrations of mRNA in muscles of wild-type (*Mstn*^{+/+}) and myostatin null (*Mstn*^{-/-}) mice (Study 1). Arbitrary concentrations of endogenous *Igf1Ea* mRNA in *quadriceps* muscles of *Mstn*^{+/+} and *Mstn*^{-/-} mice from 4 to 20 weeks (Study 1, $n=6$ per genotype). Asterisks and symbols indicate significant differences between the strains at each age (** $P<0.01$; * $P<0.1$).

Therefore, we anticipated that skeletal muscles would grow to a greater size in *Mstn*^{-/-} mice if the concentration of *Igf1Ea* IGF1 was increased locally, using transgenic *Igf1* (C2:Ea) mice (Study 2).

Study 2

To confirm that the *Igf1* transgene was being expressed in muscles of the *Mstn*^{+/+}:*Igf1*⁺, *Mstn*^{+/-}:*Igf1*⁺ and *Mstn*^{-/-}:*Igf1*⁺ mice, we quantified *Igf1* mRNA in the *gastrocnemius* muscles and observed that it was 20-fold higher ($P<0.001$) than in the *Mstn*^{+/+}, *Mstn*^{+/-} and *Mstn*^{-/-} muscles (Fig. 2A). In contrast, there was a main effect of overexpression of *Igf1* which tended ($P=0.07$) to decrease concentrations of myostatin mRNA in the *gastrocnemius* muscles of mice transgenic for *Igf1* (there was no mRNA for the exon 3 coding region of myostatin in *Mstn*^{-/-} mice) (Fig. 2B).

Sequential removal of myostatin together with overexpression of *Igf1* had a greater than additive effect on the rate of weight gain (*Mstn*×*IGF1* interaction, $P<0.001$), than the effect of each gene alone. Rates of weight gain over the 12 weeks were least in *Mstn*^{+/+} mice and strikingly highest in *Mstn*^{-/-}:*Igf1*⁺ mice (Fig. 3A and B). The increase in body mass could be attributed to the corresponding greater growth of skeletal muscles (Fig. 3C and E). There was no difference among genotypes in body length (nose to anus) or tibia length (Table 2) and so there was no contribution to the differences in mass due

Figure 2

Concentrations of mRNA in muscles of wild-type and transgenic mice (Study 2). (A) Arbitrary concentrations of the *Igf1* transgene in the *gastrocnemius* muscles of *Mstn*^{+/+} (*M*^{+/+}), *Mstn*^{+/-} (*M*^{+/-}), *Mstn*^{-/-} (*M*^{-/-}), *Mstn*^{+/+};*Igf1*⁺ (*M*^{+/+};*I*⁺), *Mstn*^{+/-};*Igf1*⁺ (*M*^{+/-};*I*⁺) and *Mstn*^{-/-};*Igf1*⁺ (*M*^{-/-};*I*⁺) mice (*n*=8 per genotype). Unlike letters denote significance (^{a,b}*P*<0.001). (B) Arbitrary concentrations of *Mstn* mRNA in *gastrocnemius* muscles of the six genotypes of mice. Concentrations of myostatin mRNA were reduced (*P*<0.001) with elimination of copy number and tended (*P*=0.07, main effect) to be reduced in muscles of *Igf1Ea* transgenic mice.

to allometric organ growth (other than that of skeletal muscle). The mass of the gonadal fat pad (corrected to body mass) was correspondingly reduced (Fig. 3D), and the mass of the heart (corrected to tibia length) was not affected by the genotypes (Fig. 3F). The combined action of these two genes on skeletal muscle growth was synergistic. Notably, the sizes of *quadriceps* (Fig. 3C) as well as the *gastrocnemius*, *B. femoris*, *T. anterior*, *EDL* and *Plantaris* muscles at 12 weeks of age (corrected to tibia length) were increased by up to 2-fold in *Mstn*^{-/-} mice, and there was a ~20–30% increase in the mass of those muscles in all mice expressing the *Igf1* transgene alone (Table 2). However, when combined (*Mstn*^{-/-};*Igf1*⁺), there was nearly a 3-fold increase across fast-twitch muscles (Table 2).

The mass of the *soleus*, which is predominantly slow twitch (Augusto *et al.* 2004), was also increased, although to a lesser degree (less than two-fold increase) than muscles expressing the fast 2X and 2B MyHCs (Table 2). In contrast, deletion of the myostatin gene biased the composition of myofibres of one of these muscles, the *T. anterior*, towards type 2B MyHC (6% more for *Mstn*^{+/-} and 10% more for *Mstn*^{-/-}, *P*<0.001, Fig. 4A and B). *Igf1* overexpression also increased the bias of myofibre type composition towards 2B MyHC by 7% (*P*<0.001, Fig. 4A and B). By contrast, the abundance of type 2A and X (these bands did not separate during electrophoresis) were reduced from 27.9±0.6% in *Mstn*^{+/+} controls by 20% in *Mstn*^{+/-} and 36% in *Mstn*^{-/-} (*P*<0.001). In addition, *Igf1* overexpression also decreased the abundance by 26% (*P*<0.001). Type 1 MyHC was not discernible in the *T. anterior*.

To ascertain whether the increase in muscle growth was due to increased numbers of myofibres during development (myofibre hyperplasia) and/or hypertrophy, we counted and measured the minimal diameter of each myofibre within transverse sections collected from the middle of the *T. anterior* muscle. Removal of myostatin alone (*Mstn*^{-/-}, *Mstn*^{-/-};*Igf1*⁺) increased the number of myofibres (*P*<0.001, Fig. 4C) with no effect on myofibre diameter (Fig. 4D), while addition of *Igf1* alone (*Mstn*^{+/+};*Igf1*⁺, *Mstn*^{+/-};*Igf1*⁺, *Mstn*^{-/-};*Igf1*⁺) increased their diameter, but not myofibre number (*P*<0.001, main effect) (Fig. 4C and D). The distribution of the diameters of myofibres was unimodal and showed a shift to the right with sequential removal of myostatin and addition of *Igf1* (Fig. 4E). Despite the increase in muscle size, the function, as assessed by grip strength, although greater overall (Fig. 4F), was not altered when normalised to body mass (Fig. 4G).

We measured concentrations of IGF1 protein in *quadriceps* muscles and blood to determine if IGF1 was getting into circulation to affect other tissues. Concentrations were increased (~40 fold, *P*<0.001) in *quadriceps* muscles of *Igf1* transgenic mice (Fig. 5A). However, there was no change in circulating concentrations of IGF1 (Fig. 5B). To determine if the lack of change in circulating concentrations of IGF1 was due to changes in expression of the IGF1 receptor, or of binding proteins, we measured the mRNA expression of *Igf1r* and *Igf1bp3–6*. There was a main effect of the *Igf1* transgene to reduce concentrations of *Igf1r* mRNA (*P*<0.001), *Igf1bp3* mRNA (*P*<0.05), *Igf1bp4* mRNA (*P*<0.1) and *Igf1bp6* mRNA (*P*<0.05) in *gastrocnemius* muscles (Fig. 5C, D, E and F). By contrast,

Figure 3

Body mass, muscle mass and growth dynamics of wild-type and transgenic mice (Study 2). (A) Body mass (mean $\pm$ S.E.M.) of *Mstn*^{+/+} (*M*^{+/+}), *Mstn*^{+/-} (*M*^{+/-}), *Mstn*^{-/-} (*M*^{-/-}), *Mstn*^{+/-};*Igf1*⁺ (*M*^{+/-};*I*⁺), *Mstn*^{-/-};*Igf1*⁺ (*M*^{-/-};*I*⁺) and *Mstn*^{+/-};*Igf1*⁺ (*M*^{+/-};*I*⁺) mice from 4 to 12 weeks of age ($n=8$ per genotype). (B) Average rate of daily growth of mice from 4 to 12 weeks of age. (C) Mass of the quadriceps muscle (mean $\pm$ S.E.M.) normalised to tibia length and (D) mass of the gonadal fat pad (mean $\pm$ S.E.M.) normalised to body mass at 12 weeks of age. (E) Four of the six genotypes of mice at death (top) and with skin removed (bottom) to show the extensive hypertrophy of the hind limb muscles. (F) Mass of the heart (mean $\pm$ S.E.M., normalised to tibia length) for the six mouse genotypes. Unlike letters denote significance (at least $P<0.05$).

concentrations of *Igf1* mRNA were increased ($P<0.001$ in *gastrocnemius* muscles in the absence of myostatin (Fig. 5G).

Total AKT was increased 2.5-fold in the absence of myostatin alone (Figs 6 and 7A, $P<0.001$, main effect), while IGF1 alone increased the abundance of pAKT^{S473}

Table 2 Mass (mg) of 6 different muscles corrected to length of tibia (mm) for the 6 genotypes of mice that differed in (systemic) deletion of *Mstn* and/or addition of the *Igf1* transgene expressed specifically in skeletal muscle throughout development (sampled at 12 weeks – Study 2).

	<i>Mstn</i> ^{+/+}	<i>Igf1</i> ⁺	<i>Mstn</i> ^{+/-}	<i>Mstn</i> ^{+/-} ; <i>Igf1</i> ⁺	<i>Mstn</i> ^{-/-}	<i>Mstn</i> ^{-/-} ; <i>Igf1</i> ⁺	<i>n</i>	<i>P</i>		
								<i>Mstn</i>	<i>Igf1</i>	<i>Mstn</i> $\times$ <i>Igf1</i>
Gast	7.2 $\pm$ 0.2 ^a	8.9 $\pm$ 0.3 ^b	8.5 $\pm$ 0.1 ^b	10.6 $\pm$ 0.3 ^c	14.3 $\pm$ 0.5 ^d	20.7 $\pm$ 0.8 ^e	8	0.001	0.001	0.001
<i>B. fem</i>	9.6 $\pm$ 0.6 ^a	11.7 $\pm$ 0.4 ^a	10.9 $\pm$ 0.2 ^a	14.9 $\pm$ 0.6 ^b	17.4 $\pm$ 1.0 ^c	28.2 $\pm$ 1.5 ^d	8	0.001	0.001	0.001
<i>T. ant</i>	2.8 $\pm$ 0.1 ^a	3.5 $\pm$ 0.1 ^b	3.2 $\pm$ 0.1 ^a	4.5 $\pm$ 0.2 ^c	4.9 $\pm$ 0.3 ^c	7.6 $\pm$ 0.4 ^d	8	0.001	0.001	0.001
EDL	0.64 $\pm$ 0.13 ^a	0.83 $\pm$ 0.14 ^a	0.66 $\pm$ 0.02 ^a	0.89 $\pm$ 0.04 ^b	1.08 $\pm$ 0.06 ^b	1.75 $\pm$ 0.05 ^c	8	0.001	0.001	0.01
Soleus	0.38 $\pm$ 0.02 ^a	0.42 $\pm$ 0.03 ^a	0.41 $\pm$ 0.02 ^a	0.49 $\pm$ 0.03 ^b	0.59 $\pm$ 0.03 ^c	0.68 $\pm$ 0.06 ^d	8	0.01	0.001	0.637
Plantaris	0.94 $\pm$ 0.04 ^a	1.17 $\pm$ 0.02 ^b	1.11 $\pm$ 0.03 ^a	1.53 $\pm$ 0.07 ^b	2.19 $\pm$ 0.08 ^c	3.09 $\pm$ 0.17 ^d	8	0.001	0.001	0.001
Tibia (mm)	18.5 $\pm$ 0.2	18.1 $\pm$ 0.1	18.3 $\pm$ 0.3	18.4 $\pm$ 0.2	18.3 $\pm$ 0.2	18.2 $\pm$ 0.2	8	0.98	0.49	0.55

The probability (*P*) is given for the main effects of myostatin (*Mstn*) and (IGF1) genotypes and their interaction on muscles mass.

Unlike letters denote significant differences within rows (at least $P<0.05$).

B. fem, *B. femoris*; EDL, extensor digitorum longus; Gast, *gastrocnemius*; *T. ant*, *T. anterior*.

Figure 4

Myofibre phenotype of wild-type and transgenic mice (sampled at 12 weeks – Study 2). (A) A representative image of an SDS-PAGE gel showing the separation of myosin heavy chains (MyHC) in *T. anterior* muscles along with a control sample (ladder, L) from a 50:50 mixture of soleus and EDL muscles from mice for use as a reference. (B) Percent of MyHC 2B (mean $\pm$ S.E.M.) from the optical densities (ODs) in *T. anterior* muscles of *Mstn*^{+/+} (*M*^{+/+}), *Mstn*^{+/-} (*M*^{+/-}), *Mstn*^{-/-} (*M*^{-/-}), *Mstn*^{+/+;}*Igf1*^{+/+} (*M*^{+/+;}*I*^{+/+}), *Mstn*^{+/-;}*Igf1*^{+/+} (*M*^{+/-;}*I*^{+/+}) and *Mstn*^{-/-;}*Igf1*^{+/+} (*M*^{-/-;}*I*^{+/+}) mice ($n=8$ per genotype). (C) Number and (D) minimal diameter of myofibres (mean $\pm$ S.E.M.) in the *T. anterior* muscles of the six genotypes of mice. (E) Frequency distribution of the minimal diameter of myofibres (10 μm bins) in *T. anterior* muscles of the six genotypes of mice. (F) Uncorrected grip strength (mean $\pm$ S.E.M.) and (G) grip strength normalised to body mass of the six genotypes of mice. Unlike letters denote significance (at least $P < 0.05$).

by 1.7-fold overall (Figs 6 and 7B, $P=0.002$, main effect). The ratio of phosphorylated to total AKT was reduced by 50% in the absence of myostatin ($P < 0.05$, Fig. 7C). Total rpS6 was increased in the absence of myostatin alone by 1.4-fold ($P < 0.05$, Figs 6 and 7D), while there was no effect of either myostatin or IGF1 on phosphorylated rpS6 (Figs 6 and 7E) or the ratio of phosphorylated to total rpS6 (Figs 6 and 7F), or on total, phosphorylated or the ratio of phosphorylated to total 4EBP1 (Figs 6 and 7G, H and I).

To understand how the change in composition of myofibres was being regulated, we measured the mRNA expression of *Mef2c*, *Pgc1A* and *Pgc1B*. There was a main effect of *Igf1*⁺ to reduce expression of *Mef2c* mRNA (9.54 vs 12.43 ± 1.45 S.E.D., pooled means, $P < 0.05$) and *Pgc1A* (9.13 vs 13.41 ± 1.50 S.E.D., pooled means, $P < 0.01$) in *gastrocnemius* muscles of mice transgenic for *Igf1* (*Mstn*^{+/+;}*Igf1*^{+/+}, *Mstn*^{+/-;}*Igf1*^{+/+} and *Mstn*^{-/-;}*Igf1*^{+/+}) compared with non-*Igf1* transgenic mice (*Mstn*^{+/+}, *Mstn*^{+/-} and *Mstn*^{-/-}). Similarly, there was a tendency ($P=0.06$, main effect) for concentrations of *Pgc1B* mRNA to be reduced in *gastrocnemius* muscles of mice transgenic for *Igf1*

(10.5 vs 13.9 ± 1.8 S.E.D., pooled means) compared to non-*Igf1* transgenic mice.

Discussion

These data are unique in showing for the first time that growth of skeletal muscle is modulated by the combined actions of IGF1 and myostatin with each factor having a distinct role – myostatin regulates hyperplasia and IGF1 regulates hypertrophy of myofibres. It is likely that these two processes are temporally separated because the number of myofibres is largely fixed before birth (Rowe & Goldspink 1969, Timson & Dudenhoefter 1990), although myogenesis can continue in the *T. anterior* and *EDL* muscles for up to a week postnatally (Li *et al.* 2015). Therefore, the absence of myostatin throughout *in utero* development is likely to have increased the number of myofibres prenatally. By contrast, hypertrophy of myofibres, an expansion of myofibre length and cross-sectional area, occurs progressively postnatally concomitant with an increase in the number of myonuclei

Figure 5

Concentrations of IGF1 protein and expression of *Igf1Ea*, receptor and binding protein mRNA in wild-type and transgenic mice (sampled at 12 weeks – Study 2). (A) Concentrations of IGF1 protein in quadriceps muscles of *Mstn*^{+/+} (*M*^{+/+}), *Mstn*^{+/-} (*M*^{+/-}), *Mstn*^{-/-} (*M*^{-/-}), *Mstn*^{+/-};*Igf1*⁺ (*M*^{+/-};*I*⁺), *Mstn*^{-/-};*Igf1*⁺ (*M*^{-/-};*I*⁺) and *Mstn*^{-/-};*Igf1*⁺ (*M*^{-/-};*I*⁺) mice (*n*=8 per genotype). (B) Concentrations of IGF1 protein in plasma of the same six genotypes of mice. (C) Arbitrary concentrations of *Igf1r* receptor (*r*) mRNA, (D) *IgfBP3* mRNA, (E) *IgfBP4* mRNA, (F) *IgfBP6* mRNA and (G) *IgfBP5* mRNA in the *gastrocnemius* muscles of the six genotypes of mice. There was a main effect of the *Igf1Ea* transgene to reduce concentrations of *Igf1r* mRNA (*P*<0.001), *IgfBP3* mRNA (*P*<0.05), *IgfBP4* mRNA (*P*<0.1) and *IgfBP6* mRNA (*P*<0.05). Concentrations of *IgfBP5* mRNA were increased in *gastrocnemius* muscles of *Mstn*^{-/-} mice irrespective of the *Igf1Ea* transgene. Unlike letters denote significance (*P*<0.001).

within individual myofibres until adulthood (White *et al.* 2010). Given that expression of the *Igf1* transgene driven by the MLC promoter is activated after 10 days post-partum (Musaro *et al.* 2001), the increased production and availability of transgenic IGF1 in postnatal skeletal muscle likely exacerbated the increase in mass of these growing muscles: this booster effect is especially pronounced during the very rapid growth phase in the first 6 weeks after birth (Fig. 3A and B).

IGF1 is required during embryonic and postnatal development (Baker *et al.* 1993, Liu *et al.* 1993, Lupu *et al.* 2001). Locally produced IGF1 (including skeletal muscle), rather than liver derived, is now understood

to have the predominant influence on postnatal growth (Sjogren *et al.* 1999, Yakar *et al.* 1999). However, there is relatively high expression of both *Igf1* and *Igf2* mRNA in skeletal muscle during gestation and, for example, in the semitendinosus muscle of pigs, both *Igf1* and *Igf2* were observed to peak just before parturition and then decline during postnatal life (Lee *et al.* 1993, Gerrard *et al.* 1998). Similarly, concentrations of *Igf1*, -2, and *Igf1r* mRNA are increased in skeletal muscles of rodents after birth and decline in postnatal life by about 1 month in mice (Fiorotto *et al.* 2014) and 2 months in rats (Adamo *et al.* 1989, Alexandrides *et al.* 1989, Shoba *et al.* 1999). Recently, IGF2 was reported to contribute to the excess muscle

Figure 6

Representative Western blots of proteins in *gastrocnemius* muscles from wild-type and transgenic mice (sampled at 12 weeks – Study 2). Representative Western blots of total AKT, phosphorylated (p) AKT^{S473}, total 4EBP1, p4EBP1^{T37/46}, total rpS6, p-rpS6^{S235/236} and GAPDH in *gastrocnemius* muscles of *Mstn*^{+/+} (*M*^{+/+}), *Mstn*^{+/-} (*M*^{+/-}), *Mstn*^{-/-} (*M*^{-/-}), *Mstn*^{+/+;Igf1} (*M*^{+/+;I}), *Mstn*^{+/-;Igf1} (*M*^{+/-;I}) and *Mstn*^{-/-;Igf1} (*M*^{-/-;I}) mice (*n*=8 per genotype).

development in *Mstn*^{-/-} mice because concentrations of *Igf2* mRNA were higher in muscles of *Mstn*^{-/-} compared with WT mice postnatally from 3 to 10 weeks of age

(Clark *et al.* 2015). While the increased concentrations of *Igf2* mRNA prevailed in skeletal muscles of *Mstn*^{-/-} mice, they progressively decline from birth in both *Mstn*^{-/-} and

Figure 7

Expression of total and phosphorylated proteins in wild-type and transgenic mice (sampled at 12 weeks – Study 2). Optical density of (A) total (t) Akt, (B) pAKT^{S473}, (C) p-AKT^{S473}/t-AKT, (D) t-rpS6, (E) p-rpS6^{S235/236}, (F) p-rpS6^{S235/236}/t-rpS6 (G) t-4EBP1, (H) p-4EBP1^{T37/46}, (I) p-4EBP1^{T37/46}/t-4EBP1 in *gastrocnemius* muscles of *Mstn*^{+/+} (*M*^{+/+}), *Mstn*^{+/-} (*M*^{+/-}), *Mstn*^{-/-} (*M*^{-/-}), *Mstn*^{+/+;Igf1} (*M*^{+/+;I}), *Mstn*^{+/-;Igf1} (*M*^{+/-;I}) and *Mstn*^{-/-;Igf1} (*M*^{-/-;I}) mice (*n*=8 per genotype). Unlike letters denote significance (^{a,b}*P*<0.001) and asterisks denote significance where indicated (**P*<0.05).

WT mice (Clark *et al.* 2015). By contrast, concentrations of *Igf1* mRNA were reduced in skeletal muscles of *Mstn*^{-/-} mice up to 3 weeks of age (Clark *et al.* 2015), and the differences between *Mstn*^{-/-} and WT were further diminished in mice older than 6 weeks (Kocamis *et al.* 2002, Clark *et al.* 2015). The reduced expression of *Igf1* mRNA in skeletal muscles of juvenile *Mstn*^{-/-} mice reported here (Study 1) suggested to us that further growth would occur in skeletal muscles if *Igf1* expression was increased during postnatal growth.

We observed that the actions of myostatin and IGF1 were quite specific with myostatin regulating hyperplasia and IGF1 regulating hypertrophy, at least in the *T. anterior* muscle. However, others have shown that the absence of myostatin alone increased both the number and size of myofibres of the *EDL*, *gastrocnemius* and *T. anterior* muscles (McPherron *et al.* 1997, Amthor *et al.* 2009). In addition, cultured primary myoblasts from *Mstn*^{-/-} mice form larger myotubes than those cultured from wild-type mice (Rodriguez *et al.* 2011), which supports the view that the absence of myostatin permits hypertrophy *in vitro*. These differences from the current study might be attributable to the different muscles selected and ages of mice that were assessed in other studies (2.8 months in our study and 3.2–7 months in the other studies), and may also be explained by differences in the expression of *Igf1* and *Igf1* receptors, which are crucial for antagonists of TGF- β family members to induce hypertrophy (Kalista *et al.* 2012). In addition, the role of IGF2 in regulating hypertrophy in *Mstn*^{-/-} mice cannot be overlooked as noted earlier (Clark *et al.* 2015) and may also explain the increase (~40%) in mass of *quadriceps* muscles in young adult mice when MSTN protein is reduced in skeletal muscles using the Cre/Lox recombinase system to block the expression of *Mstn* mRNA (Welle *et al.* 2007). We show that the greatly increased concentrations of IGF1 in the transgenic *Igf1*⁺ mice enhanced the hypertrophy of skeletal muscle at a younger age, which was particularly evident in *Mstn*^{-/-} mice.

The reciprocal decline in the mass of the gonadal fat pad with the increased lean mass is consistent with the loss of functional myostatin (Hamrick *et al.* 2006) and an increase in IGF1 (Fiorotto *et al.* 2003), which act in conjunction with each other to promote a lean phenotype. Myostatin directs the fate of precursor cells towards a lipogenic lineage (Artaza *et al.* 2005), while IGF1 promotes a myogenic fate via regulating Rho GTPase (Sordella *et al.* 2003). Therefore, the combined actions of loss of myostatin and excess IGF1 (in *Mstn*^{-/-}:*Igf1*⁺) are consistent with promoting the accretion of lean tissue and reduction of fat mass.

We observed that the absence of myostatin together with the presence of transgenic IGF1 altered the myofibre phenotype. These findings accord with those of others who showed that *Mstn*^{-/-} mice have more myofibres expressing type 2 MyHC in the *soleus* (type 2A) and *EDL* muscles (types 2B and 2X) (Girgenrath *et al.* 2005, Amthor *et al.* 2007) and that IGF1 directly upregulates the type 2B MyHC promoter (Shanely *et al.* 2009). Our data accord with the use of the MLC promoter, which is most active in muscles expressing type 2B MyHC and least in the type 1 myofibres (Donoghue *et al.* 1991, Musaro *et al.* 2001). The fast and slow twitch characteristics of skeletal muscle are determined by the composition of MyHC and these, in turn, are regulated by the transcriptional co-activator PGC1A, which promotes the development of type 1 myofibres when in excess (Lin *et al.* 2002) and of type 2B myofibres when absent (Handschin *et al.* 2007), and that of PGC1B, which promotes the development of type 2X myofibres (Arany *et al.* 2007). PGC1A acts in conjunction with the myocyte enhancer factor 2c (MEF2c) to promote the development of type 1 myofibres (Potthoff *et al.* 2007). In support of the switch from slow- to fast-twitch myofibres, the expression of *Mef2c* and *Pgc1A* mRNA was reduced in skeletal muscles of mice transgenic for IGF1. The tendency for reduced concentrations of *Pgc1B* mRNA in skeletal muscles of mice transgenic for IGF1 further supports the switch to type 2B myofibres.

We observed that grip strength (at 12 weeks of age) was unaltered among the genotypes when normalised to body mass. This observation is broadly consistent with that of others, who showed that loss of myostatin impaired the generation of force and that skeletal muscles were less resistant to fatigue (Amthor *et al.* 2007).

In seeking an explanation as to how the two factors co-ordinate the growth of skeletal muscle, we focused on the PI3k/AKT pathway. While IGF1 and myostatin signal primarily via the PI3K/AKT and Smad2/3 pathways, respectively, the former is common to both factors (Trendelenburg *et al.* 2009, Welle *et al.* 2009). We show here that the absence of myostatin increases the total abundance of AKT, while additional IGF1 increased its phosphorylation. In addition, we observed an increase in the abundance of total rpS6, but not that of phosphorylated rpS6, or in the ratio of phosphorylated to total rpS6. This contrasts with another report (Welle *et al.* 2009), wherein a greater abundance of phosphorylated rpS6 was observed, without a change in phosphorylated or total AKT in wild-type mice injected with an antibody to myostatin. The difference between the studies may be explained by the transient effect of an antibody to block myostatin in

the former study, compared with the constitutive absence of myostatin throughout development in the transgenic mice used here. Furthermore, the lack of difference in total and phosphorylated 4EBP1 in the current study agrees with the observations of others (Welle *et al.* 2009), but not with our previous study comparing wild-type and *Mstn*^{-/-} mice where we observed a higher abundance of total 4EBP1 and a lower ratio of phosphorylated to total 4EBP1 (Smith *et al.* 2014). This observation is not unexpected given that the mice were 12 weeks of age in the current study, an age at which the rate of growth of skeletal muscle was greatly reduced (Fig. 3A) and, therefore, it is understandable that the molecular mechanisms regulating muscle growth would be different. Therefore, we conclude that the greater size of skeletal muscles and reduction in fat in *Mstn*^{-/-}*Igf1*⁺ mice can be attributed to the enhanced activation of anabolic signalling via AKT. The increased abundance of rpS6 may help explain the greater rate of protein synthesis in skeletal muscle of *Mstn*^{-/-} mice (Welle *et al.* 2006) and hence hypertrophy observed in skeletal muscle of *Mstn*^{-/-} mice (McPherron *et al.* 1997, Amthor *et al.* 2009).

The lack of increased concentrations of IGF1 in circulation, despite increased concentrations in skeletal muscle, is consistent with an earlier report on the *Igf1* (C2:Ea) strain (Shavlakadze *et al.* 2010). We acknowledge that some transgenic IGF1 may be released into the circulation, but we were unable to differentiate between transgenic and endogenous forms to determine the proportion of transgenic and endogenous IGF1 in circulation. In addition, specific binding proteins are known to bind to and regulate the bioavailability of IGF1 (Clemmons 1998). We anticipated that expression of the binding proteins in skeletal muscle would be increased to potentially (1) reduce the anabolic actions of IGF1 because the expression of IGF1 receptors was reduced in skeletal muscle and (2) retain IGF1 in the muscle bed to prevent it from entering circulation. However, we observed reduced concentrations of binding proteins 3, 4 and 6 mRNA in muscles transgenic for IGF1. The exception was the expression of *Igfbp5* mRNA, which was increased in skeletal muscles of *Mstn*^{-/-} mice. Others have also reported higher expression of *Igfbp5* mRNA in skeletal muscles of *Mstn*^{-/-} mice (Clark *et al.* 2015). While the role of IGFBP5 is unclear, overexpression reduced birth weight and retarded the development of skeletal muscles (Salih *et al.* 2004). Therefore, it is likely that increased *Igfbp5* mRNA in skeletal muscles of *Mstn*^{-/-} mice (both control and *Igf1*⁺) may be acting to limit the excess growth of skeletal muscle induced by the absence of myostatin.

We conclude that myostatin regulates myofibre number, while IGF1 regulates myofibre size, at least in the *T. anterior* muscle, and that both conditions (*Mstn*^{-/-} and *Igf1*⁺) increase the proportion of fast 2B myofibres in hind limb muscles of young adult male mice. The combination of an increase in myofibre number and size is greater than the additive effects of these genes alone on muscle mass, which culminates in an extremely lean phenotype.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

A H, C D M, F J, H K S, M D G, P S, T S, M L F interpreted the data and wrote the manuscript. A H, J O, S F, C B, G N, J B, P S and M S S collected and analysed the data. C D M, K M and T W conducted the animal experiments.

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