Prolonged underfeeding of sheep increases myostatin and myogenic regulatory factor Myf-5 in skeletal muscle while IGF-I and myogenin are repressed

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Abstract

The IGF axis is nutritionally sensitive *in vivo* and IGFs stimulate myoblast proliferation and differentiation *in vitro*, while myostatin inhibits these processes *in vitro*. We hypothesised that underfeeding would reversibly inhibit the myogenic activity of satellite cells *in vivo* together with decreased IGF-I and increased myostatin in muscle. Satellite cell activity was measured indirectly from the expression of proliferating cell nuclear antigen (PCNA) and the myogenic regulatory factors (MRFs), MyoD, Myf-5 and myogenin. Young sheep were underfed (30% of maintenance) and some killed after 1, 4, 12, 17, 21 and 22 weeks. Remaining underfed animals were then re-fed a control ration of pellets and killed after 2 days, and 1, 6 and 30 weeks.

Expression of PCNA and MRFs decreased during the first week of underfeeding. This coincided with reduced IGF-I and myostatin mRNA, and processed myostatin. Subsequently, Myf-5, MyoD, myostatin mRNA and processed myostatin increased, suggesting that satellite cells may have become progressively quiescent. Long-term underfeeding caused muscle necrosis in some animals and IGF-I and MRF expression was increased in these, indicating the activation of satellite cells for muscle repair. Re-feeding initiated rapid muscle growth and increased expression of PCNA, IGF-I and the MRFs concurrently with decreased myostatin proteins.

In conclusion, these data indicate that IGF-I and myostatin may work in a coordinated manner to regulate the proliferation, differentiation and quiescence of satellite cells *in vivo*.

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Introduction

Muscle wasting during prolonged nutritional deficit is an adaptive response that allows the mobilisation of protein to sustain life, and reduces the metabolic demand in a physiologically constrained system. Following the formation of skeletal muscle from embryonic and fetal myoblasts, a population of specialised myoblasts termed satellite cells provides the additional myonuclei required by individual muscle fibres as they increase in size during post-natal development. The incorporation of myonuclei from satellite cells into muscle fibres is an obligatory event to increase fibre size (Moss & LeBlond 1971, Mitchell & Pavlath 2001); however, it is unknown how nutritional status affects the myogenic activity of satellite cells in growing animals.

Satellite cell activity in post-natal muscle may be evaluated by using the relative expression of muscle transcription factors as markers of specific myogenic events (Yablonka-Reuveni & Rivera 1994, Cornelison & Wold 1997). The MyoD family of transcription factors (MRFs) is expressed exclusively in myogenic cells and members of the family are expressed in a sequence that corresponds temporally with myoblast or satellite cell proliferation, withdrawal from the cell cycle, terminal differentiation and myofibre formation. Myf-5 and MyoD are expressed in a cell cycle-dependent manner during proliferation and early differentiation (Cornelison & Wold 1997, Kitzmann & Fernandez 2001). Later during differentiation, the expression of myogenin increases before fusion of satellite cells into multi-nucleated myotubes or existing myofibres (Florini *et al.* 1991, Rosenthal & Cheng 1995). The expression of MyoD and myogenin then declines following the incorporation of new myonuclei into myofibres; however, they may be induced in satellite cells of mature muscle in response to specific environmental stimuli (Grounds *et al.* 1992, Cornelison & Wold 1997).

The insulin-like growth factors (IGF-I and -II) have metabolic, mitogenic and anti-apoptotic effects in a range of cell types, and may act in either an endocrine or

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paracrine manner (Le Roith *et al.* 2001). In sheep, circulating levels of IGFs in plasma are regulated by nutrition (Oldham *et al.* 1999); however, the expression of IGF-I in skeletal muscle is relatively insensitive to short-term fasting (Oldham *et al.* 1996). Proliferation and differentiation of myoblasts *in vitro* is increased by treatment with IGFs (Rosenthal *et al.* 1994, Napier *et al.* 1999) and they are also considered to be survival factors, protecting myoblasts from apoptosis (Napier *et al.* 1999).

Myostatin is an inhibitor of muscle growth and development belonging to the greater transforming growth factor-beta (TGF-β) family, for which roles in the postnatal remodelling of muscle have yet to be established (Lee & McPherron 1999). Like other TGFs, myostatin is translated into a precursor protein that is proteolytically cleaved to yield the N-terminal, latency-associated peptide (LAP) and the C-terminal, processed peptide before secretion from the cell (Thies *et al.* 2001). Myostatin mRNA is unchanged following short-term fasting in pigs (Ji *et al.* 1998) or reduced food intake in mice (Carlson *et al.* 1999), although expression is increased during disuse atrophy of muscle in rodents (Carlson *et al.* 1999, Wehling *et al.* 2000). In culture, myostatin reversibly inhibits the proliferation of myoblasts (Thomas *et al.* 2000, Taylor *et al.* 2001) and expression increases as myoblasts differentiate and fuse (Kocamis *et al.* 2001, Rios *et al.* 2001). There is also a sharp increase in myostatin expression at the onset of secondary muscle fibre formation in fetal cattle (Oldham *et al.* 2001). Collectively, these studies suggest that myostatin regulates the activity of myogenic cells during both proliferation and differentiation.

We hypothesised (i) that the expression of MRFs, as markers of satellite cell activity, would decrease during prolonged underfeeding and be restored with re-feeding, and (ii) that the changes in MRF expression would be associated with increased myostatin and reduced IGF-I during underfeeding and then reduced myostatin and increased IGF-I expression during re-feeding. The hypotheses were tested by measuring the molecular expression of MRFs in muscle from growing lambs that were chronically underfed and subsequently re-fed a control ration of pellets, and relating these data to the expression of IGF-I and myostatin in muscle.

Materials and Methods

Animals

Female Romney sheep (8 months of age, $n=75$) were randomly allocated to one of two treatment groups. They were trained to eat sheep pellets (60% lucerne, 30% barley, 5% linseed, 5% molasses; Country Harvest Stockfeed, Hamilton, New Zealand) during the 3 weeks preceding the experiment. Sheep in the control group ($n=17$) then received an individual ration of pellets that was increased daily by 200 g whenever all the feed was consumed, while those in the underfeeding/re-feeding group (UR, $n=58$) received an individual ration of pellets calculated to be 30% of a maintenance diet. Following 22 weeks of underfeeding and serial slaughters (described below) those animals remaining on UR ($n=22$) were returned to the control intake of an increased allocation whenever all the feed was consumed, and re-fed during a 30-week recovery period. Approval for this study was obtained from the Animal Ethics Committee of Ruakura Research Centre.

Six animals in the control group were killed at the onset of the experiment. Animals from the UR treatment ($n=6$) were then killed serially at 1, 4, 12, 17, 21 and 22 weeks into the experiment. The remaining UR animals were returned to the control intake of pellets, and serial slaughters ($n=5$ or 6) were undertaken at 2 days, and 1, 7 and 30 weeks following the change. The latter re-fed groups were identified as 22·3, 23, 29 and 52 weeks of UR respectively. Control animals were killed together with animals on UR at 22 weeks and at 52 weeks ($n=5$ or 6). At slaughter, semitendinous muscle (M. semitendinosus) was dissected and weighed. Muscle samples were frozen in isopentane chilled with liquid nitrogen for immunohistochemistry and others homogenised for preparation of total RNA.

RNA isolation and Northern analyses

Muscle samples were homogenised on ice in Trizol reagent (GIBCO BRL, Gaithersburg, MD, USA) for 30 s at 13 500 r.p.m. using an Ultra Turrax homogeniser (Janke & Kunkel GmbH, Germany). Debris was removed by centrifugation for 10 min at 10 000 ***g*** and total RNA was isolated using the Trizol protocol (GIBCO BRL). RNA was re-suspended in diethyl pyrocarbonate-treated water and the final concentration determined by measuring absorbance at 260 nm. Ten micrograms of total RNA from each sample were separated on a 1·2% formaldehyde-agarose gel and transferred to uncharged nylon membrane (Hybond-N; Amersham Pharmacia Biotech) by capillary action. Membranes were cross-linked using UV radiation and stained with methylene blue to verify the uniformity of loading and transfer.

A description of the oligonucleotide primers used to produce cDNA probes for myostatin, myogenin and MyoD has previously been published (Kambadur *et al.* 1997, Oldham *et al.* 2001) and primer sequences for IGF-I, proliferating cell nuclear antigen (PCNA) and Myf-5 are presented in Table 1. Sequences of PCR fragments have been submitted to Genbank for ovine PCNA (Accession Number AF416380), ovine myogenin (Accession Number AF433651) and ovine Myf-5 (Accession Number AF434668). First-strand cDNA synthesis was performed using a Superscript II Pre-Amplification kit (GIBCO BRL) and 5 μg total RNA from ovine skeletal muscle. PCR was carried out according to the manufacturer's protocol, with 2 μl of the reverse transcriptase reaction

Table 1 Primer sequences and sizes of the PCR amplification products used as Northern hybridisation probes. Sequences for PCR primer design were obtained from the following Genbank Accession Numbers: bovine IGF-I (X15726), human PCNA (XM_009571.3) and bovine Myf-5 (M95684)

	Sequence	Size (bp)
Product		
IGF-I	5′-CTGTTAAACACCAGAAGACC-3′ 5′-ATGCCACAGATGGAATCTTG-3′	521
PCNA	5′-GCCTTCTGGTGAATTTGCACG-3′ 5′-AGATCCTTCTTCATCCTCG-3′	366
Myf-5	5′-TGGATCCGCGAGTGGCTGCTTTCGG-3′ 5′-CTGGATCCTGGAGAGGCAATCC-3′	588

(94 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min) for 35 cycles and a final extension of 5 min at 72 °C. Radiolabelled cDNA probes were prepared using [α-^{32}P]dCTP and the Rediprime II Labeling kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Membranes were pre-hybridised in Church and Gilbert buffer (0·5 M Na_2HPO_4, pH 7·2, 7% SDS, 1 mM EDTA) for 2 h and then hybridised overnight in fresh buffer with the appropriate radiolabelled probe. Following hybridisation, they were washed for 15 min in each of 2 × SSC/0·5% SDS and 1 × SSC/0·5% SDS and exposed against X-Omat AR film (Eastman Kodak Company, Rochester, NY, USA). Pre-hybridisation, hybridisation and washing temperatures were 55 °C for all probes with the exception of MyoD, which was processed at 60 °C. Optical density of the bands on autoradiographs was measured using Gel Doc 2000 (Bio-Rad, Richmond, CA, USA). Out of multiple transcripts detected for IGF-I, the 7·5 kb transcript was selected for densitometric analysis. In the interests of having comparisons made between membranes and autoradiographs, RNA samples from two control animals were repeatedly run as controls on each membrane.

Western analysis of myostatin proteins

Muscle samples (100 mg) were homogenised in 1 ml PBS buffer (Oxoid Ltd, Basingstoke, Hants, UK) with Complete Protease Inhibitor (Roche Diagnostics GmbH, Mannheim, Germany) at 13 500 r.p.m. for 30 s on ice. The homogenate was centrifuged at 10 000 ***g*** for 15 min at 4 °C to remove tissue debris. The total protein concentration of the supernatant was estimated, using BSA as a standard, with the DC Protein Assay (Bio-Rad). The protein extract was boiled for 5 min with 1:1 volume of loading buffer (125 mM Tris–HCl, pH 6·8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0·05% bromophenol blue) and then 10 μg total protein from each sample (n=39) were separated by SDS-PAGE (10%). After electrotransfer, nitrocellulose blots were stained with Ponceau S to confirm equal loading and uniformity of transfer, and then destained in Tris-buffered saline (TBS) with Tween (TBST) (0·05 M Tris, pH 7·4, 0·1 M NaCl, 0·1% Tween 20 (Sigma-Aldrich, St Louis, MO, USA)) and blocked in TBST containing 5% fat-free dry milk at 4 °C overnight.

The immunostaining procedure has previously been validated in our laboratory (Sharma *et al.* 1999). Membranes were incubated with primary antibody diluted 1:2000 for 2 h at room temperature. They were washed four times for 5 min each in TBST and further incubated with a 1:2500 dilution of anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Promega, Madison, WI, USA) for 1 h at room temperature. Membranes were washed four times further in TBST and HRP activity detected by chemiluminescence using Renaissance Western Blot Reagent (Perkin-Elmer Life Sciences, Boston, MA, USA). Band intensities on X-Omat AR film (Eastman Kodak) were quantified by densitometry (Bio-Rad GS 690) using MultiAnalyst software (Bio-Rad).

Immunohistochemistry of myostatin and MRFs

Muscle samples were sectioned (8 μm) at − 16 °C using a Cryocut 1800 (Leica, Heidelberg, Germany), air-dried and placed at − 20 °C until use. Serial sections were mounted with adjacent cut surfaces upright to assist the localisation of myostatin and Myf-5 to the same cells. For myostatin detection, the same antibody was used as in the Western analysis. This antibody has previously been optimised for immunohistochemical use in both sheep and rat tissues (Sharma *et al.* 1999, Kirk *et al.* 2000) and the procedure was modified for use with cryosections.

Sections were thawed to room temperature and fixed in phosphate-buffered formalin (phosphate-buffered saline (Oxoid), and 1·3% formaldehyde (BDH Laboratory Supplies, Poole, Dorset, UK)). Endogenous peroxidase activity was quenched by incubation in 3% H_2O_2 in TBS (0·05 M Tris, pH 7·4, 0·1 M NaCl) and non-specific background blocked by incubation in TBST with 3% donkey serum. Sections were then incubated overnight at 4 °C with primary antiserum in TBST with 1·5% donkey serum. Biotinylated donkey anti-rabbit IgG (Amersham Pharmacia Biotech) diluted to 1:500 in TBST was the secondary antiserum and streptavidin-biotin conjugated to HRP (Amersham Pharmacia Biotech), also diluted to 1:500 in TBST, was the tertiary complex. Immunoreactivity was visualised using 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and the sections were counterstained with Nuclear Fast Red (G T Gurr Limited, London, UK).

Antisera were used at 1:5000 for myostatin and 1:500 for Myf-5 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), while non-immune rabbit serum (Dako Corporation, Carpinteria, CA, USA) was used as a negative control at matched protein concentrations. Myogenin

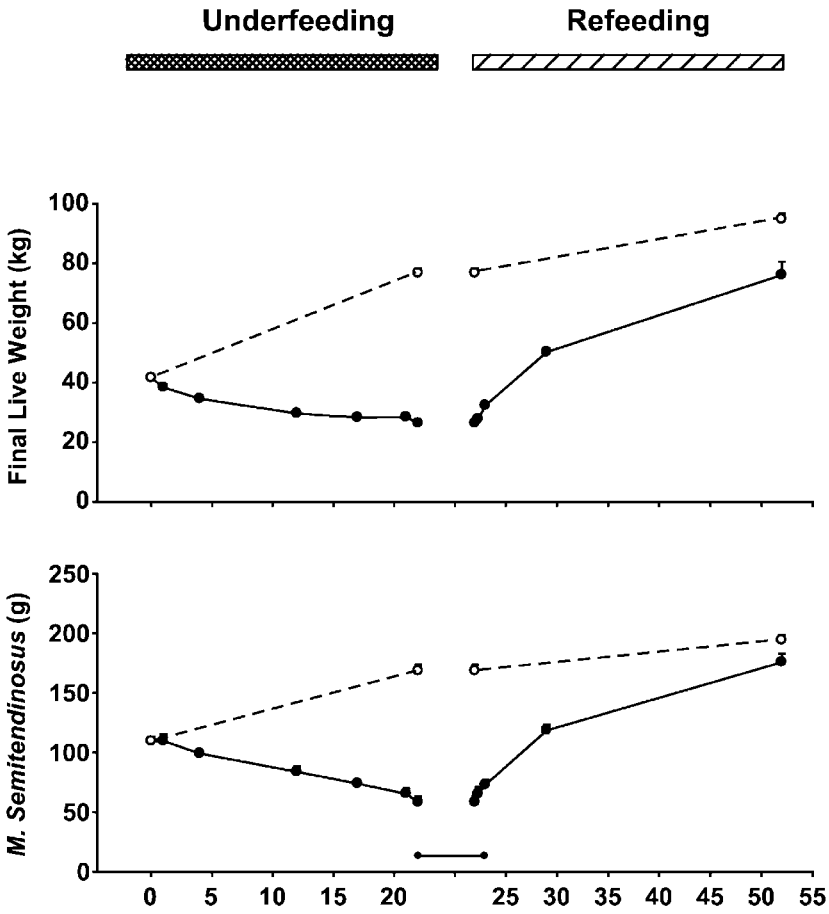

Figure 1 Upper panel: final body weights of lambs that were underfed for 1–22 weeks and then fed a control ration for 2 days to 30 weeks (UR; ●). Control (C; ○) animals were fed freely throughout the experiment. Lower panel: weights of M. semitendinosus in UR (●) and C (○) lambs killed at various times throughout underfeeding and re-feeding. Final data points for underfeeding are repeated as initial data points for re-feeding, as indicated by the horizontal line between the Underfeeding and Refeeding sides of the lower panel. (Means ± pooled S.E.M., $n=5$ or 6 per group.)

antiserum (Santa Cruz Biotechnology) was used at 1:100 for 1 h at room temperature and sections were counter-stained with Mayer's haematoxylin.

Statistics

The optical density data from Northern and Western procedures were used in a residual maximum likelihood analysis with independent comparisons made between sequential time points in control or UR treatments, or between control and UR treatments at 22 and 52 weeks. Light microscopy assessment of muscle sections from all animals identified a number that showed necrosis. Independently, gene expression data from the same animals were statistically identified as outlier points. On the basis of these two measurements, the gene expression data from four animals showing extensive muscle damage (one from each group at 21, 22, 22·3 and 23 weeks) were excluded from the statistical analyses of treatment effects. The expression of genes and immunohistochemical observations for these animals are presented separately.

Results

Body and muscle weights

Figure 1, upper panel, shows the final live weights of control and UR animals throughout the experiment. In Figs 1–3 and 5, the final data points for the underfeeding phase of UR are presented again on the same graph as the initial points for re-feeding, in order to assist the interpretation of underfeeding and re-feeding effects separately.

After 22 weeks of underfeeding, UR animals were 36% lighter than the pre-treatment control group and were 34% of well-fed controls at the same age. Re-feeding increased the mass of previously underfed animals to 80% of age-matched controls by the end of the experiment. Mass of M. semitendinosus was reduced by 23% after 12 weeks of underfeeding ($P<0·001$) and at the end of underfeeding was 53% of initial controls and 35% of well-fed controls at the same age (Fig. 1, lower panel). With re-feeding, compensatory growth was rapid and the mass of M. semitendinosus in previously underfed lambs was increased by 25% after 1 week of the increased food intake ($P<0·05$). By the end of the experiment, the mass in the re-fed lambs was 90% of well-fed controls at the same age.

MRF and PCNA mRNA

PCNA is a co-factor for DNA polymerase that is expressed during S phase of the proliferative cycle, and therefore also expressed in activated satellite cells (Yablonka-Reuveni & Rivera 1994). We have used PCNA expression to indicate overall changes in proliferation in skeletal muscle, and then used expression of the muscle specific MRFs (Grounds *et al.* 1992, Yablonka-Reuveni & Rivera 1994, Cornelison & Wold 1997) to indicate the activity of satellite cells more specifically. The molecular expression of PCNA, Myf-5, MyoD and myogenin in control animals all decreased significantly during the experiment, particularly during the first phase (Fig. 2; $P<0·001$ for each). Levels of PCNA, Myf-5 and MyoD expression were then without further change, although myogenin mRNA continued to decline during the second phase of the experiment ($P<0·05$).

PCNA expression in UR lambs was reduced by 1 week of underfeeding ($P<0·001$) and remained low until after 17 weeks, at which time levels progressively increased to greater than those of controls by 22 weeks ($P<0·001$). Expression of Myf-5 was reduced from initial levels by 1 week of UR ($P<0·001$) and gradually increased after this time until 21 weeks. By 22 weeks, Myf-5 expression had decreased ($P<0·001$) to levels that were not different from controls. MyoD expression during UR was decreased by 1 week ($P<0·001$); however, the drop was reversed by 4 weeks ($P<0·001$) and levels were then maintained until a further decrease at 22 weeks ($P<0·001$). Myogenin expression was reduced by 1 week of UR ($P<0·001$) and remained low for the duration of underfeeding to be less than controls at week 22 ($P<0·05$).

After increasing the feed allocation to UR lambs, PCNA expression showed a sharp peak at 23 weeks (1 week following the change) before falling again ($P<0·001$) to be steady but still higher than that of controls at 52 weeks ($P<0·01$). Myf-5 ($P<0·01$) and MyoD ($P<0·001$) expression in UR lambs was increased by 2 days of re-feeding and showed a gradual decrease after this time to levels not different from controls at the end of the experiment. Re-feeding also caused an increase in myogenin expression to a peak at 29 weeks with a return to control levels by the end of the experiment ($P<0·001$).

IGF-I and myostatin mRNA

In control animals, IGF-I mRNA decreased and myostatin mRNA increased during the experiment, largely as a result of changes during the second phase (Fig. 3; $P<0·05$, $P<0·01$ respectively). With UR, IGF-I mRNA was lower than in pre-treatment controls after 1 week of restricted intake ($P<0·001$) and then remained low for the duration of underfeeding. By week 22, however, levels were not different from those of control animals. Levels of myostatin mRNA were transiently reduced by 1 week of UR and then restored to pre-treatment values by 4 weeks (both changes $P<0·001$), after which there was little change in expression until week 22. At week 22, expression was less than at 21 weeks of UR ($P<0·05$) and also lower than in controls at week 22 ($P<0·001$).

During re-feeding of UR lambs, there was an increase in expression of IGF-I to a peak at 29 weeks ($P<0·001$) before decreasing to control levels by 52 weeks ($P<0·001$). Myostatin mRNA was also increased by 22·3 weeks ($P<0·01$) and showed a peak in expression at 29 weeks ($P<0·001$). However, levels in UR were lower than controls at the conclusion of the experiment ($P<0·001$).

Myostatin proteins

In order to determine the relative importance of post-transcriptional regulation of myostatin mRNA in response to nutritional intake, a Western analysis of myostatin protein levels in M. semitendinosus was undertaken. A representative blot shows the three forms of myostatin detected by the antiserum and the relative sizes of precursor, LAP and processed myostatin in sheep muscle (Fig. 4A). Myostatin immunoreactivity was also localised in muscle cryosections, predominantly to presumptive satellite cells with no immunostaining in muscle fibres (Fig. 4B). Satellite cells were tentatively identified by their shape and apposition to fibres, and then further by immunostaining with Myf-5 antiserum (Fig. 4B).

Western analysis of myostatin protein in muscle showed temporal patterns that differed from myostatin mRNA. Precursor myostatin and LAP decreased during the experiment in control animals ($P<0·01$) while there was no significant change in the levels of processed myostatin (Fig. 5). During UR, precursor myostatin increased through to 4 weeks of underfeeding and decreased again by 12 weeks ($P<0·05$), with no change after this time. LAP increased by 1 week ($P<0·05$) and declined again between 4 and 12 weeks ($P<0·05$). There was also an increase in LAP from 17 to 21 weeks ($P<0·05$). In contrast, processed myostatin initially decreased and then progressively increased; however, only the final change between 21 and 22 weeks was

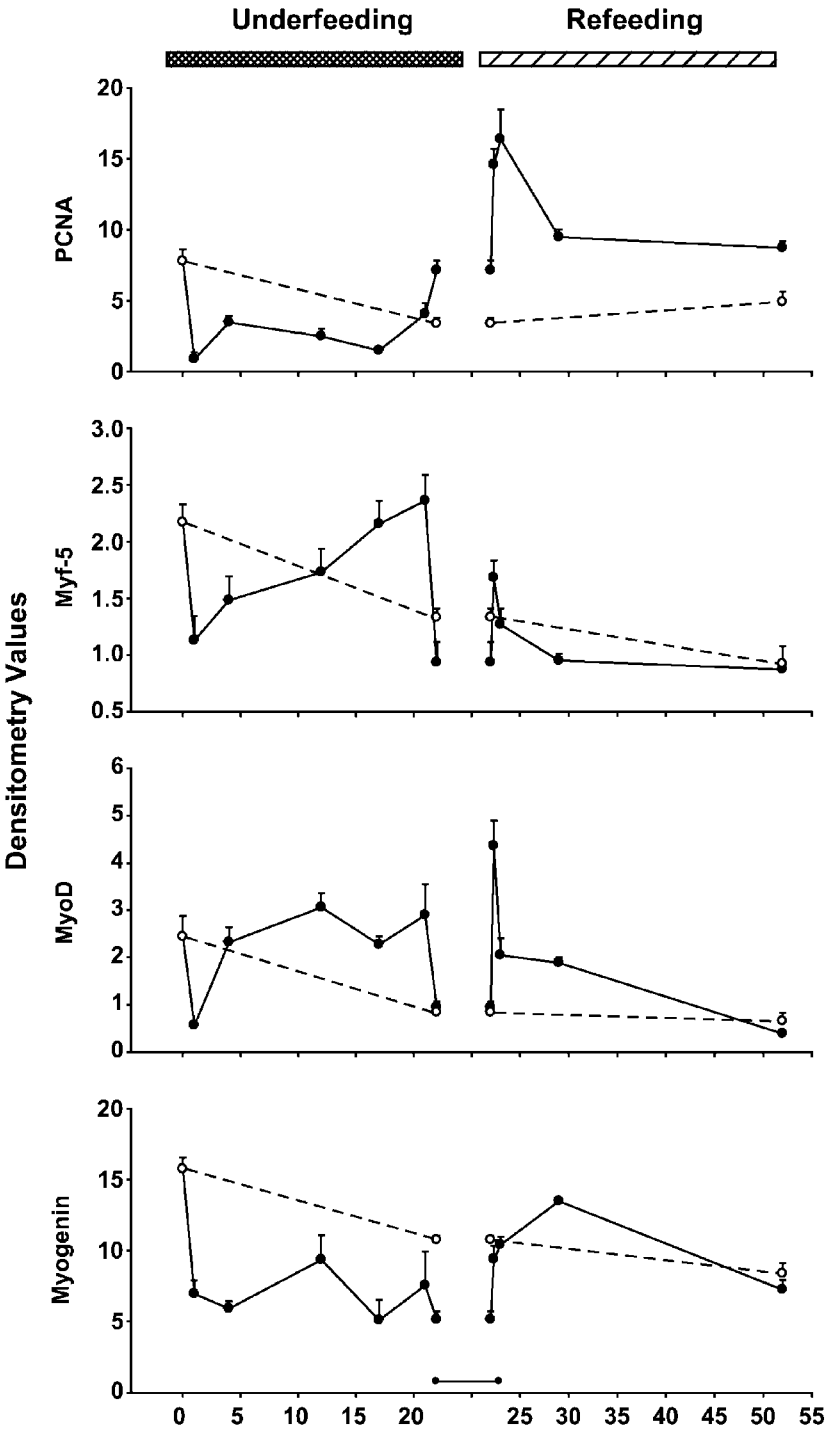

Figure 2 Northern blot analysis of mRNA for PCNA, Myf-5, MyoD and myogenin during underfeeding and re-feeding (UR; ●) or control intake (C; ○). Final data points for underfeeding are repeated as initial data points for re-feeding, as indicated by the horizontal line between the Underfeeding and Refeeding sides of the lower panel. (Means ± S.E.M., $n = 4–6$ per group.)

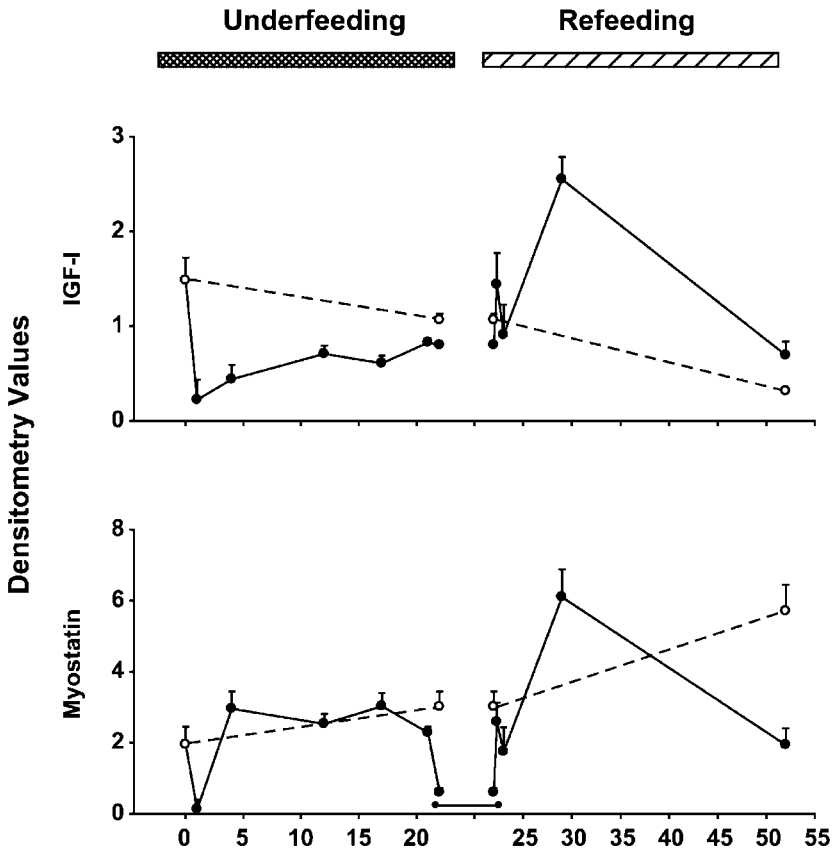

Figure 3 Northern blot analysis of mRNA for IGF-I and myostatin during underfeeding and re-feeding (UR; ●) or control intake (C; ○). Final data points for underfeeding are repeated as initial data points for re-feeding, as indicated by the horizontal line between the Underfeeding and Refeeding sides of the lower panel. (Means ± S.E.M., $n=4$–6 per group.)

significant ($P<0{\cdot}05$). There was no difference between controls and UR at 22 weeks for precursor, LAP or processed myostatin.

Re-feeding caused an immediate decrease in precursor myostatin ($P<0{\cdot}001$) and LAP ($P<0{\cdot}05$), levels subsequently returning to be not different from controls at the end of the experiment. In contrast, processed myostatin increased transiently at 23 weeks ($P<0{\cdot}05$) and returned to levels that remained less than those of controls at the conclusion of the experiment ($P<0{\cdot}01$).

Underfeeding results in muscle damage and regeneration

Four UR sheep killed between 21 and 23 weeks showed expression in a number of the candidate genes that was uncharacteristic of the remainder of the treatment group. Figure 6A shows the gene expression profile of the group underfed for 22 weeks and highlights one animal (*) with a level of IGF-I expression that was identified initially as an outlier data point. The elevated expression of that and other genes was then confirmed histologically as being associated with damaged muscle (Fig. 6C). Molecular expression of IGF-I, myogenin and Myf-5 was most prominently affected by muscle damage – showing 2- to 8-fold increases in expression over the respective group averages. In contrast, expression of MyoD and myostatin mRNA and myostatin proteins was not different in muscles showing necrosis.

Discussion

This experiment describes the response of muscle *in vivo* to severe and prolonged feed restriction and the subsequent restoration of adequate nutrition, in order to test the hypotheses that (i) expression of MRFs in satellite cells will be reversibly repressed by underfeeding, and (ii) this will be associated with a decrease in IGF-I and increased myostatin. The main findings are that the MRFs are affected differentially by prolonged underfeeding and the expression of myostatin and IGF-I in skeletal muscle is regulated by nutrition. Further, we report that myostatin

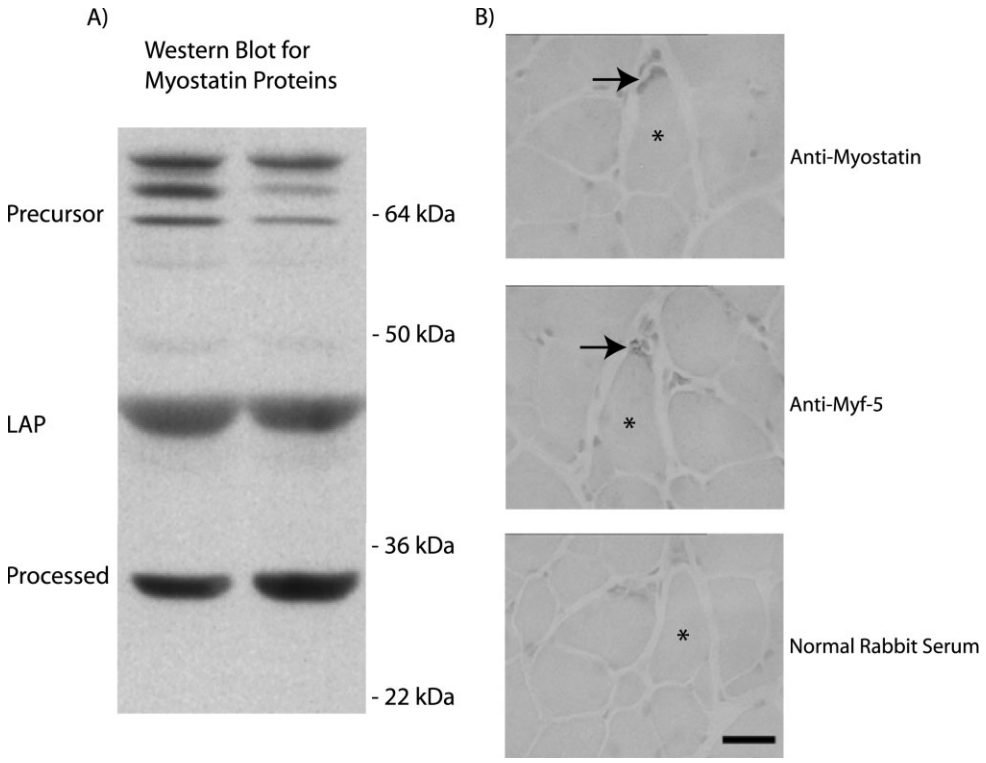

Figure 4 (A) Western blot of myostatin proteins in muscle with size and processing indicated. (B) Photomicrographs of myostatin and Myf-5 immunoreactivity in muscle cryosections, with normal rabbit serum as a negative control. The same muscle fibre in each section is identified by (*) and presumptive satellite cells are indicated by arrows. (Bar=25 μm.)

mRNA differs from the translated precursor myostatin, LAP and processed myostatin in terms of expression pattern in response to underfeeding and increasing age.

We also observed changes in gene expression from 8 to 20 months of age in well-fed controls that coincided with a slowing of growth as the animals approached physical maturity. Differences in satellite cell activity and MRF expression *in vitro* are reported for muscle from fetal and adult chickens (Yablonka-Reuveni & Patterson 2001). A recent study has also highlighted that changes in activity of porcine satellite cells between 1 week and 5 months of age are related to a pronounced decrease in their differentiation capacity while proliferation is largely unaltered (Mesires & Doumit 2002). In the current experiment, expression of Myf-5, MyoD and myogenin in control animals declined significantly between 8 and 20 months of age, suggesting that the relative size of the satellite cell population or the myogenic activity of satellite cells may have decreased during this time.

Interestingly, IGF-I and myostatin expression in well-nourished animals change after the age-related decrease in expression of Myf-5, MyoD and myogenin, suggesting that the developmental change in MRF expression is unlikely to be regulated directly by these growth factors. Our reporting of increased steady-state myostatin mRNA together with decreased translation of the protein in muscle as animals approach post-natal maturity lends support to suggestions made previously that myostatin may act as chalone in the regulation of muscle mass (Lee & McPherron 1999) and that myostatin protein may participate in an autoregulatory loop on mRNA expression (Oldham *et al.* 2001).

The response of muscle to nutritional deprivation alters as a consequence of both the severity and duration of restriction. Our data suggest that there are at least four phases of muscle adaptation to underfeeding in the current study and that gene expression is differentially regulated as the cumulative effect of underfeeding increases. Table 2 broadly classifies the response of muscle into immediate changes (weeks 0–4), followed by an intermediate period (weeks 12–21) and then a terminal phase during which a controlled response to underfeeding becomes deregulated (week 22) and will progress to necrotic muscle damage. The directional change of genes and proteins is indicated within each of these phases and during the muscle growth after increased dietary intake (weeks 22·3–52).

The earliest measured response of muscle to underfeeding was characterised by the reduced expression of all four

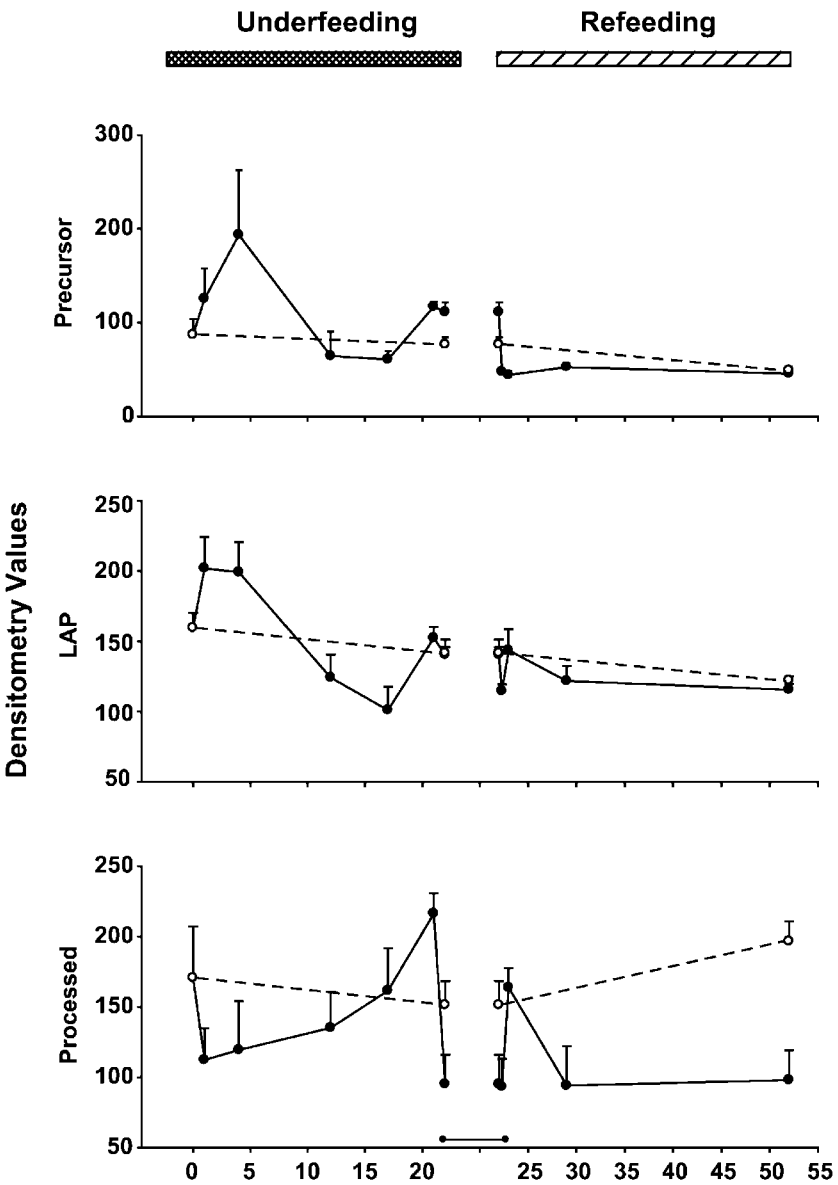

Figure 5 Western blot analysis of precursor, LAP and processed myostatin proteins in muscle during underfeeding and re-feeding (UR; ●) or control intake (C; ○). Final data points for underfeeding are repeated as initial data points, as indicated by the horizontal line between the Underfeeding and Refeeding sides of the lower panel. (Means ± S.E.M., $n=4$–6 per group.)

markers of proliferation and differentiation, together with reduced IGF-I and myostatin mRNA. The role of IGF-I as a mitogenic growth factor that also has the capacity to stimulate differentiation of myogenic cells and fibre hypertrophy (Le Roith *et al.* 2001) suggests that lower levels of IGF-I mRNA in the current experiment may have reduced myogenic activity. In particular, myogenin is thought to be a direct downstream target, initially being repressed by IGF-I and thereby inhibiting differentiation then subsequently being stimulated by IGF-I to promote differentiation (Rosenthal & Cheng 1995, Adi *et al.* 2000). IGF-I also has anabolic effects in muscle that occur via the suppression of protein degradation, increasing amino acid uptake and inducing hypertrophy (Janeczko & Etlinger 1984, Semsarian *et al.* 1999) and the suggestion has recently been made (Le Roith *et al.* 2001) that reduced IGF-I may be obligatory in allowing muscle fibre atrophy and protein mobilisation to proceed. Therefore, reduced

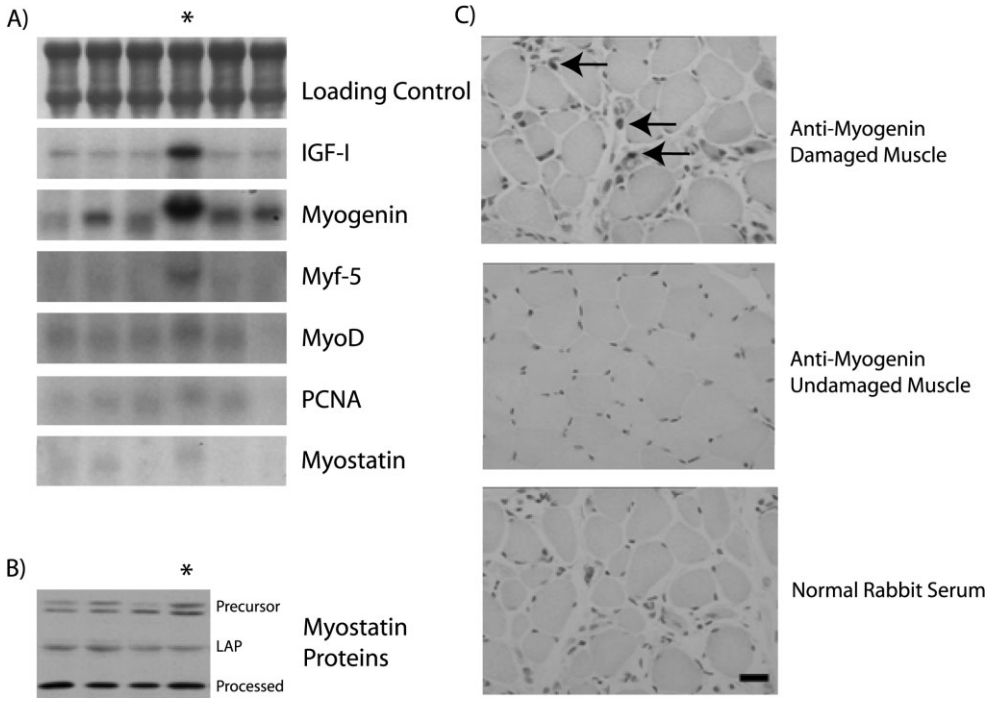

Figure 6 (A) Northern radiographs showing candidate gene expression for UR animals after 22 weeks of underfeeding and RNA blot stained with methylene blue to show 18S and 28S ribosomal bands as a loading control. An animal with muscle damage is indicated (*). (B) Western blot of myostatin proteins for four UR animals after 22 weeks of underfeeding, including an animal with muscle damage (*). (C) Photomicrographs of myogenin immunoreactivity in an animal with muscle damage after 22 weeks of underfeeding and an animal without muscle damage after 22 weeks of underfeeding. Normal rabbit serum was used as a negative control. Presumptive satellite cells are indicated by arrows. (Bar=25 μm.)

expression of IGF-I is likely to have been an influential factor in the decreased expression of MRFs and PCNA.

Levels of precursor myostatin and LAP proteins increased during the initial weeks of underfeeding. This is compatible with the role of myostatin as a specific inhibitor of muscle cell proliferation *in vitro* (Thomas *et al.* 2000, Rios *et al.* 2001, Taylor *et al.* 2001), although the decrease in mRNA was unexpected. Cell withdrawal from the proliferative cycle is dependent on the phosphorylation state of pRb (Rosenthal & Cheng 1995). Myostatin specifically up-regulates the expression of p21, a cyclin-dependent kinase inhibitor, and consequently represses the cyclin-dependent kinase activity that would otherwise ensure hyper-phosphorylation of pRb and continuation of the proliferative cycle (Thomas *et al.* 2000). Using immunohistochemistry we localised myostatin to mononucleated cells tightly apposed to the boundary of muscle fibres, and believe that these are satellite cells on which myostatin acts in an autocrine/paracrine manner to down-regulate proliferation.

Muscle wasting in men who are HIV positive is correlated with increased plasma and intramuscular levels of myostatin immunoreactive protein (Gonzalez-Cadavid *et al.* 1998), while growth-retarded piglets show greater levels of myostatin mRNA than normal littermates (Ji *et al.* 1998). Our data differ from these reports in that we do not show a simple relationship between myostatin mRNA, the translated precursor and processed proteins and a reduction in muscle mass. Further, while other studies have measured processed myostatin (Gonzalez-Cadavid *et al.* 1998, Sakuma *et al.* 2000) the current experiment shows that LAP and precursor myostatin differ substantially from processed myostatin in terms of a response to underfeeding.

The disparate results for myostatin mRNA and proteins suggest that there is more than one level of transcriptional and translational control operating. Previously, Ji *et al.* (1998) reported that myostatin mRNA did not alter with short-term fasting in pigs. Considering the complexity of the pattern of myostatin mRNA expression that we report, it is possible that immediate changes resulting from severe nutritional restriction were missed in their study. Species differences may also exist in the possible action of myostatin in post-natal muscle, as we detected no myostatin immunoreactivity in the cytoplasm of muscle fibres, although immunostaining of presumptive satellite cells was evident. Previously, the same antibody has been used for the immunodetection of myostatin in sheep myocardium

Table 2 Relative changes in myogenic regulatory factors, IGF-I and myostatin as a result of underfeeding

	Weeks 0–4	Weeks 4–21	Weeks 21–22	Weeks 21–23	Weeks 22–52
Myogenic markers					
PCNA	↓	↔	↑	↔	↑
MyoD	↓	↑	↓	↔	↑
Myf-5	↓	↑	↓	↑	↑
Myogenin	↓	↔	↔	↑	↑
Growth factors					
IGF-I	↓	↔	↔	↑	↑
Myostatin mRNA	↓	↑	↓	↔	↑
Processed myostatin	↓	↑	↓	↔	↑↓
Precursor and LAP myostatin	↑	↔	↔	↔	↓↑

(Sharma *et al.* 1999) and detection in undamaged cardiomyocytes was also relatively low. This is in contrast to myostatin localisation in rat muscle where immunoreactivity is clearly observed within muscle fibres (Kirk *et al.* 2000, Sakuma *et al.* 2000).

As the period of underfeeding progressed, PCNA, myogenin and IGF-I expression continued to be low, suggesting a sustained repression of myogenic activity. However, Myf-5, MyoD and myostatin all increased from previous low levels and similarities became apparent between the expression patterns of MyoD and myostatin mRNA, and between Myf-5 mRNA and processed myostatin. Initial decreases in the expression of Myf-5 and MyoD confirmed their suitability as markers of proliferation in muscle cells (Yablonka-Reuveni & Rivera 1994, Kitzmann & Fernandez 2001); however, the subsequent patterns of expression of these MRFs suggest that they may control the transcription of genes involved in other aspects of muscle adaptation to prolonged malnutrition.

Until recently it was thought that quiescent satellite cells, in G0 of the cell cycle, did not express MRFs; however, Myf-5 expression has since been reported in quiescent satellite cells both *in vitro* and *in vivo* (Kitzmann *et al.* 1998, Beauchamp *et al.* 2000). The suggestion has also been made that cells entering G0 between mitotic divisions comprise a sub-population of reserve satellite cells (Schultz 1996). Therefore, our observation of increasing Myf-5 expression may be related to an increasing number of reserve satellite cells becoming quiescent in response to continued food deprivation. Levels of processed myostatin in muscle followed a similar pattern to Myf-5 and satellite cells showing myostatin and Myf-5 immunoreactivity were frequently found closely apposed as doubles, possibly indicative of recently completed cell division (Fig. 4B). It might be speculated that the maintenance of quiescence in satellite cells requires the continued presence of both Myf-5 and myostatin. Our evidence suggests that Myf-5 may be upstream of myostatin in the regulatory cascade, as a sharp peak of expression during re-feeding occurred after 2 days for Myf-5 and 1 week for processed myostatin (Figs 2 and 5). Exactly how Myf-5 might regulate the accumulation of processed myostatin in satellite cells is unknown. In the context of this experimental treatment, however, the patterns of expression for myostatin mRNA, precursor and LAP proteins do not support the concept of direct regulation of myostatin transcription or translation by Myf-5.

In contrast, our data and the published literature do provide evidence for a transcriptional relationship between MyoD and myostatin. A consensus binding site for MyoD has been identified on the myostatin promoter region in humans (Ferrell *et al.* 1999, Ma *et al.* 2001) and cattle (Sharma *et al.* 2000). So myostatin may act downstream of MyoD in the regulatory cascade, although increased MyoD expression in the absence of myostatin protein in double-muscled cattle also suggests that MyoD induction may be regulated by myostatin (Oldham *et al.* 2001). In myogenic cultures, MyoD expression is greatest during G1 of the cell cycle, the point at which differentiation is initiated (Kitzmann *et al.* 1998), and at which treatment of proliferating cells with myostatin reversibly blocks cell cycle progression via increased p21 (Thomas *et al.* 2000). Therefore, an increase in expression of myostatin and MyoD mRNA may be an integral step in the withdrawal

of cells from the proliferative cycle that subsequently persists to block progression of the cells in situations where differentiation is inappropriate, such as in a severely underfed state.

Myoblasts in culture do not irreversibly withdraw from the cell cycle until co-expression of p21 and myogenin (Andres & Walsh 1996), so the sustained repression of myogenin in the current experiment potentially allows for proliferation or differentiation pathways to be restored in satellite cells. There is also evidence that passage through the proliferative cycle may not be obligatory in the activation of certain populations of satellite cells (Moss & LeBlond 1971, Rantanen *et al.* 1995). Our data for MyoD and myostatin expression may suggest that a population of satellite cells, differing from those expressing Myf-5 in a quiescent state, are held at a pivotal point of increased MyoD and myostatin expression from which they may subsequently differentiate or return to proliferation with improved nutrition.

The final group of underfed animals was characterised by marked decreases in previously elevated expression of Myf-5, MyoD and myostatin mRNA, together with processed myostatin. At this point of the experiment, we speculate that pathways for the survival and preservation of muscle mass became deregulated, although this was not obvious from the histological observations of muscle until necrotic damage was present. It is possible that the reduction of Myf-5, MyoD and myostatin effectively removed any regulatory effect of these proteins on satellite cells in order to facilitate proliferation and differentiation in response to environmental cues for improved nutrition or the onset of muscle damage.

As observed in Fig. 5, animals showing necrotic damage of muscle fibres during late-stage underfeeding retained the capacity to initiate muscle repair, as characterised by increases in IGF-I, Myf-5 and myogenin. In various models of muscle damage and regeneration, both the IGFs and MRFs are expressed during early phases of muscle repair coincident with satellite cell proliferation, differentiation and myotube formation (Levinovitz *et al.* 1992, Launay *et al.* 2001).

An immediate induction of myogenic activity also followed the re-feeding of previously underfed lambs, as indicated by elevated mRNA expression of the MRFs, PCNA, IGF-I and myostatin. In addition to the mitogenic effects of IGF-I on myoblasts (Napier *et al.* 1999), overexpression in muscle *in vitro* and *in vivo* leads to fibre hypertrophy (Semsarian *et al.* 1999, Musaro *et al.* 2001) supporting the likelihood of IGF-I acting as a hypertrophic growth factor during the rapid regrowth of muscle in the current experiment. If myostatin is a regulatory component of satellite cell withdrawal from the proliferative cycle in G1, then the increase in myostatin mRNA expression as a result of re-feeding may reflect increased activity of satellite cells during the early stages of myogenic differentiation. This is supported by our data showing that myogenin expression is greatest during re-feeding at the same time as myostatin and observations *in vitro* that myostatin expression increases as cells differentiate (Kocamis *et al.* 2001, Rios *et al.* 2001).

In conclusion, this study has determined that chronic underfeeding reversibly represses the myogenic activity of satellite cells *in vivo*. We suggest that the adaptive response of muscle to prolonged underfeeding has several successive phases during which the expression of IGF-I, myostatin and the MRFs may change as the roles of these growth and transcription factors are modulated by nutrition. Finally, the complex nature of transcriptional and translational control of myostatin *in vivo* is highlighted and awaits further investigation.

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