

## The influence of undernutrition during gestation on skeletal muscle cellularity and on the expression of genes that control muscle growth

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(Received 7 July 2003 – Revised 12 November 2003 – Accepted 13 November 2003)

We examined the effects of two levels of gestational undernutrition (50% and 40% of *ad libitum*) on postnatal growth rate, skeletal muscle cellularity and the expression of genes that control muscle growth, in the offspring at weaning. The results showed that the rat pups born to mothers fed the 50% diet during gestation and a control diet during lactation had an increased postnatal growth rate compared with the pups fed the more restricted diet (40% of *ad libitum*). Surprisingly, the growth rate of the control group (*ad libitum*) was intermediate between the 50% and 40% groups. The restricted diets did not alter the number of muscle fibres in the semitendinosus muscle of the offspring but the number of muscle nuclei was reduced by 16% in the 40% group compared with the control group. In the 50% group, the lightest pups at birth (L) had elevated muscle insulin-like growth factor (IGF)-1, IGF binding protein (BP)-5 and proliferating cell nuclear antigen (PCNA) mRNA compared with the L pups from both the control and 40% groups. The heaviest pups at birth (H) in the 50% group had increased levels of IGFBP-4, PCNA and M-cadherin mRNA compared with both the control and 40% groups. Levels of IGF-1 receptor, myostatin and MyoD mRNA did not correlate with postnatal growth. Both H and L pups from the 40% group had reduced muscle IGF-1 mRNA but all other transcripts examined were similar to control levels. The results suggest that the increased postnatal growth rate, which accompanied milder fetal undernutrition (50%), may be due to a more active local muscle IGF system and increased muscle-cell proliferation.

### Gestation: Undernutrition: Skeletal muscle: Satellite cells: Insulin-like growth factor

The early stages of pregnancy exert a major influence on the postnatal growth rate of an individual and their health status into adulthood. Human epidemiological studies have shown that fetal undernutrition is associated with cardiovascular disease and type 2 diabetes in adult life (Barker *et al.* 1993a,b; Mi *et al.* 2000). Other studies carried out in various mammalian species have shown that the growth rate of an animal directly correlates with the number of skeletal muscle nuclei and/or fibres (Dwyer *et al.* 1994; Greenwood *et al.* 1999). In most mammals, skeletal muscle fibres form prenatally; thus the total number of muscle fibres, which constitute a given muscle, is normally fixed by the time of birth. Postnatally, skeletal muscles grow by hypertrophy, i.e. by increasing the diameter of existing fibres rather than hyperplasia (formation of new fibres). Muscle-fibre hypertrophy requires both increased protein synthesis and the recruitment of mononucleated muscle precursor cells known as satellite cells. Upon activation, satellite cells proliferate, differentiate and fuse with the existing fibres, thus donating nuclei to the growing fibres (for a review, see Hawke & Garry, 2001).

The number of muscle fibres that constitute a skeletal muscle can be influenced prenatally by maternal nutrition. Numerous studies carried out in a range of mammalian species have shown that maternal undernutrition during gestation can significantly reduce the number of both muscle fibres and nuclei in the offspring (Bedi *et al.* 1982; Wilson *et al.* 1988; Ward & Stickland, 1991; Dwyer *et al.* 1994, 1995; Greenwood *et al.* 1999). As more muscle fibres and/or muscle nuclei correlate with an increased postnatal growth, it appears that maternal nutrition during gestation has a major influence on the growth potential of an animal. Dwyer *et al.* (1995) identified a critical period during gestation when maternal undernutrition influenced muscle-fibre formation in the offspring. In the guinea-pig, maternal feed restriction during the first half of gestation induced the same 25% reduction in muscle-fibre number in the offspring as feed deprivation throughout the whole gestation period, whereas maternal undernutrition during late gestation had no effect. In the rat, maternal undernutrition during gestation produces the same reduction in muscle-fibre number in the offspring

**Abbreviations:** H, heaviest pup of each litter at birth; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; IGF-1R, insulin-like growth factor-1 receptor; L, lightest pup of each litter at birth; PCNA, proliferating cell nuclear antigen.

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as undernutrition during gestation and lactation (Wilson *et al.* 1988). These observations emphasise the importance of the early stages of pregnancy on muscle-fibre formation in the offspring. However, the molecular mechanisms by which maternal nutrition influences myogenesis are unclear.

It is well accepted that muscle-fibre formation and post-natal growth are largely regulated by insulin-like growth factor (IGF)-1. Definitive evidence comes from studies of transgenic animals and gene-expression analyses. Mice lacking IGF-1 have severely underdeveloped muscle tissue (Powell-Braxton *et al.* 1993) while the up regulation of the IGF-1 gene correlates with increased muscle growth and hypertrophy (Goldspink *et al.* 1995; Yang *et al.* 1997). In addition, specific overexpression of IGF-1 in skeletal muscle induces both muscle hypertrophy (Coleman *et al.* 1995) and hyperplasia (Mitchell *et al.* 2002). The mechanism by which IGF-1 induces muscle-fibre hypertrophy involves a combination of satellite-cell activation and protein synthesis (Barton-Davis *et al.* 1999). Dwyer & Stickland (1992) have shown that systemic IGF-1 levels were reduced in both mothers and fetuses following maternal undernutrition, suggesting that impaired myogenesis could be the consequence of lowered levels of IGF-1. However, hepatic IGF-1 levels do not correlate well with development and body growth (Sjögren *et al.* 1999; Yakar *et al.* 1999); thus, IGF-1 produced by muscle may be a more potent regulator of muscle growth via its autocrine and paracrine actions. However, little is known about the influence of maternal undernutrition on the local muscle IGF system. It is generally accepted that most IGF-1 actions are mediated through the IGF-1 receptor (IGF-1R) and the expression of its gene is nutritionally regulated in various species. For example, Matsumura *et al.* (1996) reported that IGF-1R transcript levels were increased in the skeletal muscle of 1-week-old growing chickens, following 5 d of undernutrition. Similar starvation-induced up regulation of the IGF-1R mRNA was also observed in the skeletal muscle of adult and ageing rats (Tomita *et al.* 2001), suggesting that dietary restriction could augment the local tissue response to IGF-1 by increasing the number of IGF-1R expressed in this tissue. However the effects of maternal undernutrition on skeletal muscle IGF-1R mRNA in the offspring are unknown. *In vivo*, IGF-1 is also associated with a family of six IGF-binding proteins (IGFBP). The main IGFBP expressed in skeletal muscle are IGFBP-4 and IGFBP-5. Their exact functions in this tissue are unclear but gene-expression experiments have shown that IGFBP-4 mRNA levels are higher during myoblast proliferation while IGFBP-5 mRNA, which cannot be detected in myoblasts, is highly increased in IGF- and insulin-stimulated myotube differentiation (Ewton & Florini, 1995). It is also accepted that IGFBP can either inhibit or facilitate the binding of IGF-1 to its receptor and therefore contribute to the fine local regulation of IGF-1 actions (for a review, see Florini *et al.* 1996).

Myostatin is another important regulator of muscle growth, as mutations in the myostatin gene induce the double muscle phenotype in cattle (McPherron & Lee, 1997). To date it is not clear whether the myostatin gene

is nutritionally regulated and whether it plays a role in the postnatal muscle-growth retardation caused by prenatal undernutrition.

In the present study we therefore examined the influence of two levels of maternal undernutrition on the gene expression of myostatin, IGF-1, IGF-1R and the two major muscle IGFBP (IGFBP-4 and IGFBP-5) in the offspring's skeletal muscle. We also examined general cell proliferation and satellite-cell activity by measurements of proliferating cell nuclear antigen (PCNA), M-cadherin and MyoD mRNA. PCNA is required for DNA replication and is expressed in activated satellite cells (Bravo *et al.* 1987; Johnson & Allen, 1993). M-cadherin is a trans-membrane protein that anchors satellite cells to the sarcolemma (Donalies *et al.* 1991) and the transcript can be detected in quiescent as well as active satellite cells whereas mRNA for the myogenic factor MyoD is only detected in activated satellite cells (Grounds *et al.* 1992; Cornelison & Wold, 1997). By studying these parameters the aim of the present study was to understand the mechanisms whereby nutritional status in early life influences long-term growth potential.

## Materials and methods

### Animals

A total of eighteen virgin female Wistar rats ( $220 \pm 5$  g) were individually housed with males in wire-bottomed cages. On the day a copulation plug was found, the females were isolated and assigned to one of three nutritional groups. The control group ( $n$  6) was given free access to food (standard rat chow, RM3; SDS Ltd, Betchworth, Surrey, UK) throughout gestation and lactation. The two other groups were given a daily-calculated restricted diet corresponding to either 50% ( $n$  6) or 40% ( $n$  6) of the amount eaten by the controls throughout the gestation period. From the last 2 d of gestation for the 40% group and the day of parturition for the 50% group, the rats were given a daily-calculated amount of food that was equal to the amount of food eaten by the control group. The mothers from the 40% group were given this rehabilitation diet from the last 2 d of gestation as pilot trials showed high mortality in pups born to mothers starved until parturition. The rehabilitation diet was maintained throughout lactation in both the 50% and 40% groups. The litters used in the present study contained between ten and fifteen live pups at birth. All animals had free access to water and were maintained in a light-, temperature- and humidity-controlled environment (14 h–10 h light-dark cycles;  $20 \pm 2^\circ\text{C}$ ; 45% relative humidity). The weight of the mothers and pups were recorded every morning. The heaviest (H) and lightest (L) pups of each litter at birth were marked and their weights were individually monitored daily throughout lactation. The H and L pups were monitored individually to reduce intra-litter variations, as placental blood flow and transfer of nutrients differ between small and large littermates in rodents, with the larger littermates receiving an improved nutrient supply than the smaller ones *in utero* (Saintonge & Rosso, 1981). On the twenty-first day postpartum, six

pups from each litter, including the H and L pups at birth plus four other pups chosen at random, were killed by CO<sub>2</sub> inhalation. The semitendinosus muscles were dissected and rapidly frozen in freezing isopentane for subsequent histological analyses; the gastrocnemius muscles were frozen in liquid N<sub>2</sub> for molecular biology analyses. All samples were collected within 10 to 15 min of slaughter and were stored at -80°C until further analyses.

### Histology

The semitendinosus muscles were sectioned (10 µm) in the mid-belly region and stained with toluidine blue, haematoxylin and eosin, or Hoechst 33258 (Sigma-Aldrich Co. Ltd, Gillingham, Dorset, UK) for the determination of muscle cross-sectional area, fibre number and nuclei number respectively. The total number of muscle fibres and nuclei per muscle cross-sectional area were determined using the Kontron image analysis software (Carl Zeiss, Oberkochen, Germany).

### RNA isolation

Total RNA was extracted from the gastrocnemius muscles of the H and L pups from six litters in each nutrition group using Tri-Reagent (Sigma-Aldrich Co. Ltd.). The RNA was dissolved in diethylpyrocarbonate-treated water and quantified by spectrophotometry using the Gene Spec I (Naka Instruments, WEB Scientific Ltd, Crewe, UK). The integrity of the RNA was checked by formaldehyde gel electrophoresis and the visualisation of intact 18S and 28S ribosomal RNA bands under UV light.

### Reverse transcription

Total RNA (1 µg) from each sample was reverse transcribed using the Ominiscript RT kit (Qiagen, Crawley, UK) with 5 pmol of random hexamer primers (Invitrogen, Paisley, UK) and 0.5 units of RNase inhibitor (Roche, Basel, Switzerland) in 20 µl reaction volume.

### Real-time quantitative polymerase chain reaction

The real-time PCR analyses were performed using protocols developed by Owino *et al.* (2001) and Hameed *et al.* (2003). The relative quantification of amplified target sequences was performed as described by Hameed *et al.* (2003). All primers used for the real-time PCR are listed in Table 1. Primers for rat IGF-1, IGF-1R and MyoD mRNA were designed by Owino *et al.* (2001) and primers for IGFBP-4, IGFBP-5, PCNA, myostatin and M-cadherin mRNA were designed using the Omega software version 2.0 (Oxford Molecular (Accelrys Ltd), Cambridge, UK). All primers were synthesised by MWG-Biotech (Ebersberg, Germany). Real-time PCR reactions were performed in a Roche Lightcycler (Basel, Switzerland) with 2 µl cDNA product from the reverse-transcription reaction using SyBR green detection (Qiagen, Crawley, UK). SyBR green fluorescence increases when bound to double-stranded DNA, allowing the detection of newly formed DNA at the end of each real-time PCR cycle. The relative concentration of the target sequences in control and feed-restricted samples were calculated by the Roche Lightcycler software (analysis version 3.3.40) from a standard curve created with serial dilutions of standard DNA (target sequence of interest), which were included with each run. The relative concentrations of target sequences in each run were expressed as copies per µg total RNA. All PCR products were checked for specificity and purity from a melting-curve profile performed by the Lightcycler software at the end of each run. The PCR products were further checked for size by agarose gel electrophoresis. They were subsequently sequenced at the Advanced Biotechnology Centre (Imperial College London, London, UK) and checked by BLAST analyses (NCBI, Bethesda, MD, USA) to ensure homology of the amplification products to the mRNA of interest.

### Statistical analyses

All results were analysed by Levene's test for homogeneity of variance and differences between the control, 50% and

**Table 1.** Real-time polymerase chain reaction primer sequences from 5' to 3'

Primer	Sequence 5' to 3'	Product size (bp)	Accession number
IGF-1 forward	GCTTGCTCACCTTTACCAG	300	M17335
IGF-1 reverse	AAGTGTA CTTCCTTCTGAGTCT	—	—
IGF-1R forward	GCATTGACATCCGCAACGA	207	NM_052807
IGF-1R reverse	ACTGTGAGGTTCCGGAAGA	—	—
IGFBP-4 forward	CAGTGCCCATGATCACAG	236	X81582
IGFBP-4 reverse	TGTTTGGGGTGGAAGTTG	—	—
IGFBP-5 forward	GATCGCAGAAAGAAGCTGAC	272	NM_012817
IGFBP-5 reverse	GTCCACACACCAGCAGATGC	—	—
PCNA forward	CTTAGCACTAGTATTTGAAGCACC	143	NM_022381
PCNA reverse	GTGCAAATTCACCAGATGG	—	—
Myostatin forward	GCTCTTTGGAAGATGACGATT	99	AF019624
Myostatin reverse	CATTTGGGCTTTCCATCC	—	—
M-cadherin forward	ATGTGCCACAGCCACATCG	238	M74541
M-cadherin reverse	TCCATACATGTCCGCCAGCC	—	—
MyoD forward	GGAGACATCCTCAAGCGATGC	104	M84176
MyoD reverse	AGCACCTGGTAAATCGGATTGG	—	—

IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; PCNA, proliferating cell nuclear antigen.

40% groups were analysed by one-way ANOVA followed by Tukey's *post hoc* test. Results were considered statistically significant when  $P < 0.05$  and were considered as trends when  $0.05 \leq P \leq 0.1$ .

### Ethical considerations

The animal work was approved by the Royal Veterinary College ethics and welfare committee and was carried out under the Home Office Animals (Scientific Procedures) Act 1986.

## Results

### Body weights

The body weights of the pregnant rats fed either the 50% or 40% restricted diets were reduced throughout gestation by 23.0 and 27.6% respectively when compared with the control group fed *ad libitum*. The body weights of the mothers from the restricted-diet groups remained lower than the controls throughout the lactation period, being 20.3 and 22.6% lower shortly after parturition and 13.5 and 12.0% lower than the controls at weaning for the 50% and 40% restricted groups respectively (data not shown).

Table 2 shows that, at birth, the average pup weight from the 40% group was lower than the average pup weight from the control group. The average birth weight from the 50% group tended to be lower than that for the control group ( $P = 0.115$ ) but was not different from that of the 40% group ( $P = 0.841$ ). At weaning the average pup weight from the 40% group was no longer different from the control group and there were no differences between the control and 50% groups. However, the average pup body weight at weaning was increased in the 50% group compared with the 40% group (Table 2). Similar variations in body weights at birth and at weaning were found for the H pups. The body weight at birth of the L pups was reduced in both restricted groups (50% and 40%) when compared with the control group, but there were no differences between the 50% and 40% groups. At weaning, the body weight of the L pups from the 40% group tended to

be lower than the L pups from both the control group ( $P = 0.067$ ) and the 50% group ( $P = 0.062$ ), but there were no differences between the 50% group and the control group. Analyses of growth rates showed that the pups from the 50% group grew more efficiently postnatally than the pups from the 40% group, their weights being significantly increased by about 15% on average per d. The average growth rate in the 50% group tended to be increased when compared with the control group ( $P = 0.11$ ). The average growth rate in the 40% group was comparable with controls (Table 2). It is interesting to note that the average growth rate in the control group (2.02 (SEM 0.07) g/d) was intermediate between the 50% (2.21 (SEM 0.05) g/d) and the 40% (1.88 (SEM 0.07) g/d) groups (ANOVA,  $P = 0.008$ ). Similar growth-rate variations were found for both the L and H pups from each litter. The daily growth rates of the L and H pups from the 50% group were increased by 16 and 18% respectively when compared with the L and H pups from the 40% group.

### Muscle fibres and nuclei

At 21 d postpartum, the total number of muscle fibres per cross-sectional area was not altered in the semitendinosus muscle of the pups from the nutritionally restricted groups (Fig. 1 (A)). The total number of muscle nuclei per cross-sectional area was reduced by about 16% in the pups from the 40% restricted diet group when compared with the control group (Fig. 1 (B)). There was a trend towards a reduced nuclei number in the 40% compared with the 50% group ( $P = 0.10$ ) but nuclei numbers were similar between the control and 50% groups (Fig. 1 (B)).

Similar variations in muscle-fibre and nuclei numbers were observed at 21 d for the L and H pups except that the total number of nuclei was not lower in the L pups from the 40% restricted group compared with the L pups from the control group (data not shown).

There were no differences in the average semitendinosus muscle cross-sectional areas, being 4.3, 4.83 and 4.46 mm<sup>2</sup> for the control, 50% and 40% groups respectively (data not shown).

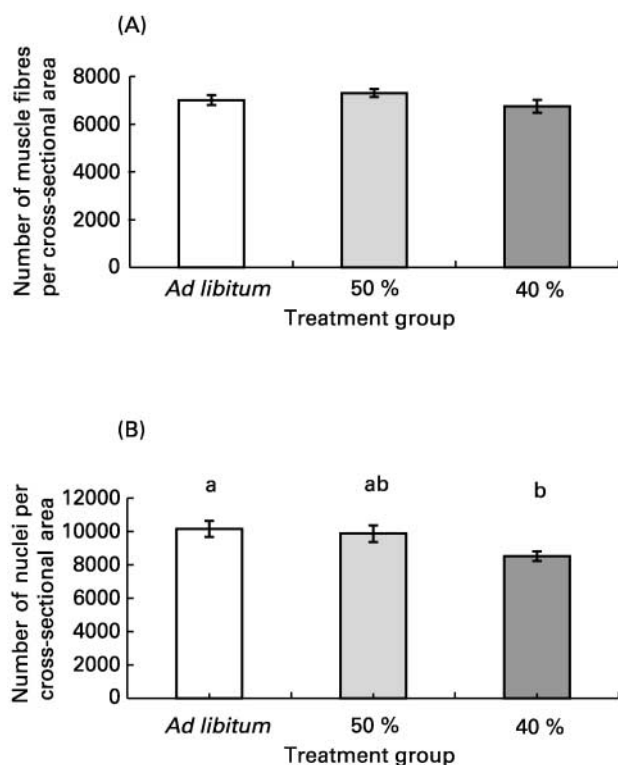
**Table 2.** Effects of maternal undernutrition on body weights and growth rates of the offspring (Mean values and standard errors of the mean for six pups per treatment group)

Diet ...		<i>Ad libitum</i>		50% restricted		40% restricted	
		Mean	SEM	Mean	SEM	Mean	SEM
Body weight at birth (g)	A	6.63 <sup>a</sup>	0.33	5.68 <sup>ab</sup>	0.17	5.43 <sup>b</sup>	0.40
	L	6.27 <sup>a</sup>	0.28	4.93 <sup>b</sup>	0.15	4.86 <sup>b</sup>	0.42
	H	7.43 <sup>a</sup>	0.32	6.43 <sup>ab</sup>	0.22	6.05 <sup>b</sup>	0.30
Body weight at weaning (g)	A	49.03 <sup>ab</sup>	1.76	52.12 <sup>a</sup>	0.88	44.81 <sup>b</sup>	1.94
	L	49.82 <sup>*</sup>	1.19	49.93 <sup>*</sup>	1.48	42.78 <sup>*</sup>	2.97
	H	52.73 <sup>ab</sup>	1.69	56.71 <sup>a</sup>	1.46	47.30 <sup>b</sup>	1.95
Growth rate (g body weight/d)	A	2.02 <sup>ab</sup>	0.07	2.21 <sup>a</sup>	0.05	1.88 <sup>b</sup>	0.07
	L	2.07 <sup>ab</sup>	0.04	2.14 <sup>a</sup>	0.065	1.80 <sup>b</sup>	0.13
	H	2.16 <sup>ab</sup>	0.07	2.39 <sup>a</sup>	0.07	1.96 <sup>b</sup>	0.09

A, average pups; L, lightest pups at birth from each litter; H, heaviest pups at birth from each litter.

<sup>a,b</sup> Mean values within a row with unlike superscript letters were significantly different ( $P < 0.05$ ) (Tukey's *post hoc* test).

\*  $P < 0.039$  (between-groups ANOVA analysis).



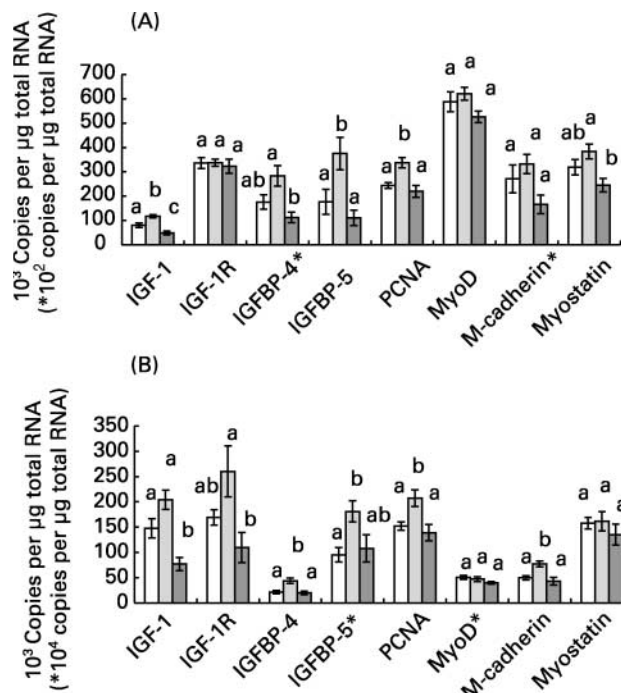
**Fig. 1.** Effects of maternal undernutrition on skeletal muscle fibre (A) and nuclei (B) numbers in the semitendinosus muscle of the offspring at weaning. Results are mean values, with standard errors of the mean represented by vertical bars ( $n$  6 per treatment group). <sup>a,b</sup> Mean values with unlike superscript letters were significantly different ( $P < 0.05$ ) (Tukey's *post hoc* test between control (*ad libitum*), 50% and 40% groups).

#### Maternal nutrition and gene expression

**The insulin-like growth factor system.** Real-time PCR analyses showed that the IGF-1 mRNA levels were up regulated and down regulated in the L pups from the 50% and 40% groups respectively when compared with the L pups from the control group (Fig. 2 (A)). IGF-1 mRNA levels were also higher in the L pups from the 50% group compared with the L pups from the 40% group. A similar trend was observed in the H pups where the IGF-1 mRNA levels tended to be increased in the 50% group ( $P = 0.09$ ) compared with the control group but were down regulated in the 40% group compared with control levels (Fig. 2 (B)). IGF-1 mRNA levels were higher in the H pups from the 50% group compared with those from the 40% group.

The IGF-1R mRNA levels were unaltered by maternal nutritional restriction in the L pups. However, IGF-1R mRNA levels were reduced in the H pups from the 40% group compared with the H pups from the 50% group but there were no differences between the control *v.* the 50% group and the control *v.* the 40% group (Figs. 2 (A) and (B)).

In the L pups, the levels of IGFBP-4 mRNA were not different between the control and 40% groups but there was a trend towards an increase in the 50% group compared with the controls ( $P = 0.08$ ). IGFBP-4 mRNA levels were reduced in the 40% group relative to the 50%



**Fig. 2.** Effects of maternal undernutrition on skeletal muscle gene expression in the gastrocnemius muscle of the offspring at weaning. The gene-expression analyses were performed by real-time PCR at weaning on cDNA made from total RNA extracted from the gastrocnemius muscle of the lightest (A) and heaviest (B) pups from each litter at birth. Results are mean values, with standard errors of the mean represented by vertical bars ( $n$  6 per treatment group). <sup>a,b,c</sup> Mean values with unlike superscript letters were significantly different ( $P < 0.05$ ) (Tukey's *post hoc* test between control (*ad libitum*; □), 50% (■) and 40% (■) groups). IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; PCNA, proliferating cell nuclear antigen.

group (Fig. 2 (A)). In the H pups, IGFBP-4 mRNA levels were higher in the 50% group compared with both the control and 40% groups but were unchanged in the 40% group relative to control (Fig. 2 (B)). The IGFBP-5 mRNA levels were up regulated in both the L and H pups from the 50% group relative to control levels but were similar to controls in the 40% restricted group (Fig. 2). IGFBP-5 mRNA levels were reduced in the L pups from the 40% group relative to the 50% group and there was a trend towards a reduction in the 40% group relative to the 50% group in the H pups ( $P = 0.07$ ).

**Cell proliferation and differentiation.** PCNA mRNA levels were up regulated in both the L and the H pups from the 50% group relative to control but were unchanged in the 40% group when compared with the control group (Fig. 2). PCNA mRNA levels were reduced in the both the L and H pups from the 40% group compared with the 50% group (Fig. 2). M-cadherin levels were up regulated in the H pups from the 50% group relative to both the control and 40% groups but only tended towards significance in the L pups ( $P = 0.055$  for 50% *v.* 40%; Figs. 2 (A) and (B)). The MyoD mRNA levels were unchanged in all treatments and animals examined (Figs. 2 (A) and (B)).

Levels of myostatin mRNA in the L pups were elevated in the 50% group relative to the 40% group but there were

no differences between the control *v.* 40% groups and the controls *v.* 50% groups (Fig. 2). In the H pups, myostatin levels were unaffected by the three levels of maternal nutrition examined (Fig. 2).

## Discussion

In the present study we examined the effects of two levels of reduced food intake during gestation on the postnatal growth rate, skeletal muscle cellularity and the transcription of genes that are known to regulate muscle growth, in rat offspring. The present results showed that pups born to mothers that were mildly undernourished (50% of *ad libitum*) had an increased daily growth rate compared with those exposed to more severe gestational undernutrition (40% of *ad libitum*), but surprisingly the growth rate of pups fed *ad libitum* was intermediate between the 50% and 40% restricted groups. There was also a trend towards an increased growth rate in the pups from the 50% group compared with those fed *ad libitum*. These results suggest that there was not a linear correlation between maternal feed intake during gestation and postnatal growth rate in the offspring. Rather, mild gestational undernutrition may have beneficial effects on postnatal growth in this situation. Although the two levels of maternal undernutrition examined (50% and 40% of *ad libitum*) did not affect the number of muscle fibres that formed in the offspring, the 40% of *ad libitum*-restricted diet induced long-term effects on muscle cellularity, as the number of muscle nuclei was reduced at weaning, *i.e.* 3 weeks after rehabilitation, when compared with the control group. Wilson *et al.* (1988) previously reported a decreased number of muscle fibres in rats undernourished during gestation; however in their studies, the pregnant rats were more severely undernourished (30% of *ad libitum*) which could explain the impact on muscle-fibre number observed in that study. Rats are very immature at birth and muscle-fibre formation continues until about the time of weaning (Rayne & Crawford, 1975; Condon *et al.* 1990). This raises the possibility that only the most severe gestational undernutrition has lasting effects on muscle-fibre number in the offspring and perhaps a milder nutritional restriction does not affect muscle-fibre numbers prenatally or may allow the newborn rats to 'catch up' with unrestricted animals in terms of muscle-fibre formation following rehabilitation during the lactation period. In the present study, we chose not to restrict the animals further than 40% as under this regimen animal mortality was increased.

Greenwood *et al.* (1999) demonstrated that the number of muscle nuclei rather than the number of fibres correlated with postnatal growth in sheep. This was not confirmed in the present study where pups from the 40% group, which had fewer muscle nuclei at weaning, had similar growth rates up to weaning compared with the control group. Greenwood *et al.* (1999) showed that the differences in DNA content between well-fed and underfed fetuses increased with age, suggesting that, as in sheep, the differences in muscle nuclei numbers and body weights we observed between the three diets examined may be more marked when the rats are fully grown.

Little is known about the signalling mechanisms by which maternal nutrition influences muscle growth.

Dwyer & Stickland (1992) showed that maternal undernutrition reduced circulating IGF levels in both mother and pups, but more recent studies have demonstrated that normal systemic IGF-1 levels are not required for normal postnatal growth and that the local IGF system may be more important (Sjögren *et al.* 1999; Yakar *et al.* 1999). After birth, skeletal muscles grow by the hypertrophy of existing fibres and IGF-1 has been shown to promote muscle-fibre hypertrophy by a combination of protein synthesis and activation of satellite cells (Barton-Davis *et al.* 1999). We therefore performed gene-expression analyses for the local muscle IGF system and satellite-cell activity in the offspring.

### *Insulin-like growth factor system and satellite-cell activity in the 40% restricted group*

Early fetal undernutrition to 40% of *ad libitum* followed by 3 weeks of postnatal nutritional rehabilitation was accompanied by reduced local muscle IGF-1 mRNA in both the L and H pups of the 40% group compared with the control group. The decreased IGF-1 mRNA levels observed in the gastrocnemius muscle correlated with a reduced number of nuclei in the semitendinosus muscle of the H pups but not the L pups suggesting that there was not a strong correlation between IGF-1 mRNA levels and nuclei numbers at this particular time point of postnatal growth.

Maternal undernutrition to 40% of *ad libitum* did not alter the muscle IGF-1R, IGF-1BP4 and IGF-1BP5 mRNA levels, when compared with the control group. It was suggested in previous reports (Tomita *et al.* 2001) that the increase in IGF-1R mRNA, which followed a few days of starvation, was a mechanism used to augment the local skeletal muscle response to IGF-1. In the present study, however, the results did not show evidence for a similar mechanism taking place in the muscle of growing rats that were nutritionally restricted to 40% of control during gestation, when compared with those fed *ad libitum*.

IGF-1 is known to promote both muscle-cell proliferation and differentiation. In order to assess overall cell proliferation, we measured the transcription of the PCNA gene. PCNA is required for DNA replication and cell-cycle progression (Bravo *et al.* 1987; Jaskulski *et al.* 1988). PCNA is expressed in proliferating satellite cells and detection of its mRNA has previously been used to measure satellite-cell activation (Johnson & Allen, 1993; Allen *et al.* 1995). Unlike IGF-1 mRNA, the levels of PCNA transcripts were similar between the control and 40% restricted animals. As fewer muscle nuclei in the H pups from the 40% group must be the consequence of decreased cell proliferation the present results show that this was not reflected at the levels of PCNA transcripts at this time point of postnatal growth. This suggests that either reduced cell proliferation may have occurred earlier in development and/or in earlier stages of postnatal growth, or that the differences between the control and 40% groups are too small to be detected at one time point in postnatal growth, but may be prolonged over time. The levels of M-cadherin and MyoD transcripts in the 40% group were similar to control levels, suggesting that no significant

differences in satellite-cell activity were detected in the offspring's skeletal muscle at 21 d postpartum.

#### *Insulin-like growth factor system and satellite-cell activity in the 50 % group*

Pups from the 50 % group had higher daily growth rates than the pups fed a more restricted diet (40 % of *ad libitum*) and there was also a trend towards improved postnatal growth in the 50 % group compared with the pups fed *ad libitum*. The accelerated growth rate observed in the 50 % group compared with the 40 % group correlated with a marked increase in muscle IGF-1 mRNA levels at weaning in both the L and H pups. In the L pups IGF-1 mRNA levels were also greater in the 50 % group compared with the control group; however the difference was not as marked as in the 50 % *v.* the 40 % group. There was a trend towards increased IGF-1 mRNA levels in the 50 % group compared with the control group in the H pups. Taken together, these data suggest that only the marked increase in skeletal muscle IGF-1 mRNA levels observed between the 50 % and 40 % groups correlated with significantly increased body growth in both the largest and smallest littermates.

As in the 40 % group, muscle IGF-1R mRNA levels were unchanged in the 50 % group compared with the control group. However IGF-1R mRNA levels were significantly increased in the H pups but not in the L pups of the 50 % group compared with the 40 % group. These data indicate possible increased local muscle sensitivity to IGF-1 in response to mild gestational undernutrition in the largest littermates only. However the present data do not show a strong correlation between body growth and increased local sensitivity to IGF-1 in skeletal muscle, at this time point of postnatal growth.

The present results also illustrate variations in the transcription rate of the two major muscle IGFBP. The exact functions of IGFBP-4 and IGFBP-5 on modulating the IGF actions are unclear but evidence suggests that they contribute to the 'fine tuning' of IGF-1-induced muscle-cell proliferation and differentiation (for a review, see Florini *et al.* 1996). Gene-expression analyses carried out *in vitro* indicate that IGFBP-4 plays a role in myoblast proliferation, while IGFBP-5 mRNA, which is not detected in myoblasts, is largely up regulated during myotube differentiation (Ewton & Florini, 1995). A proposed role for IGFBP-4 is to sequester excess IGF-1, whereas IGFBP-5 can both inhibit and enhance IGF-1 actions due to its ability to interact with the extracellular matrix (Ewton *et al.* 1998). IGFBP-4 and IGFBP-5 transcription profiles were slightly different in the H and L pups. In the H pups, the transcription profile of IGFBP-4 matched that of PCNA and M-cadherin suggesting that in the largest littermate, IGFBP-4 may modulate the IGF-1 actions towards more cell proliferation and satellite-cell activation. It is more difficult to predict the function of IGFBP-5 in the H pups from its transcription profile; however there may be more muscle-cell differentiation in the 50 % group compared with the control group. In the L pups, differences in IGFBP-4 expression between the three nutritional groups were not as marked as in the H pups. In contrast,

higher IGFBP-5 in the L pups from the 50 % group compared with both the control and 40 % groups correlated with more cell proliferation (PCNA) but no significant difference in satellite-cell activity (M-cadherin). IGFBP-5 has been shown to promote fibroblast-induced inhibition of satellite-cell growth in Duchenne muscular dystrophy (Melone *et al.* 2000) and IGF-1 has also been shown to promote the proliferation of other cell types including fibroblasts (Clemmons & van Wyk, 1981). Therefore the present data, which show higher levels of IGFBP-5 and PCNA mRNA in the L pups of the 50 % group *v.* both the control and 40 % groups but no significant difference in M-cadherin expression, suggest that IGFBP-5 may modulate more IGF-1-induced-proliferation of non-muscle cell types in the smallest littermates (L pups) than in the largest ones (H pups). This theory would be consistent with previous observations by Clelland (2001) who reported an increase in intramuscular connective tissue in small littermates compared with large littermates. However, these conclusions remain speculative as the IGFBP modulate the IGF-1 actions at the protein level; therefore mRNA levels may not reflect biological activities.

The overall changes in IGF-1 and IGFBP gene expression in the skeletal muscle of the growing rats from the 50 % group reflect a more active local IGF system in particular when compared with the 40 % group. Interestingly, the intermediate growth rate observed in the control group compared with the 50 % and 40 % groups also correlated with intermediate levels of IGF-1, IGFBP-4 and IGFBP-5 mRNA. Because IGF-1 induces muscle-fibre hypertrophy through a combination of satellite-cell activation and protein synthesis (Barton-Davis *et al.* 1999), the present results suggest that the increased growth rate observed in the pups from the 50 % group compared with the 40 % group may be due to increased local muscle IGF activity.

Increased IGF-1 mRNA levels in the 50 % group compared with the 40 % group correlated with increased PCNA mRNA expression suggesting that there was more cell proliferation in the muscle of growing rats from the 50 % group when compared with the pups fed a more restricted diet. This increased cell proliferation was accompanied by significantly increased satellite-cell activity in the H pups and a trend towards increased satellite-cell activity in the L pups ( $P=0.055$ ), which was reflected by increased M-cadherin mRNA levels but not MyoD. As PCNA is not specific to satellite cells and muscle is composed of various cell types, we cannot exclude that there may be more proliferation of 'non-muscle' cells in the muscle of pups from the 50 % restricted group compared with those fed *ad libitum* and 40 % of *ad libitum*. The 'non-muscle' cells could include cells of the extracellular matrix including adipose tissue. Although we did not quantify it, we did not observe a noticeable increase in connective tissue at this stage of growth in the 50 % group but previous studies have demonstrated an increase in intramuscular connective tissue in animals that had been undernourished prenatally (Clelland 2001).

Myostatin is a well-characterised inhibitor of muscle growth (McPherron & Lee, 1997), but there is no evidence

to date that its gene is nutritionally regulated. As maternal undernutrition altered the postnatal growth rate of the offspring from the 50 % group, we tested whether gestational undernutrition could also alter the rate of transcription of the myostatin gene. The results from the present study showed that myostatin mRNA was similar to control levels in both the 40 % and 50 % restricted group. Myostatin mRNA levels were higher in the 50 % group compared with the 40 % group in the L pups but were unchanged in the H pups. Higher myostatin levels in the L pups of the 50 % group correlated with the lack of a marked increase in M-cadherin mRNA that occurred in the H pups from the same nutrition group. Increased myostatin and no significant change in M-cadherin in the L pups as opposed to no change in myostatin and increased M-cadherin in the H pups further emphasises that although both the L and H pups from the 50 % group have increased postnatal growth rates compared with the 40 % group, there may be less myogenesis taking place in the smallest littermates than in the largest littermates of the 50 % group. However, our myostatin data do not bring strong evidence that myostatin plays an important role in regulating the postnatal growth rates of gestationally undernourished pups.

Somewhat surprisingly, the present study shows that there was not a linear correlation between maternal food intake and postnatal growth rate in the offspring. A mild gestational undernutrition to 50 % of *ad libitum* had a positive effect on the postnatal growth rate of the offspring when compared with more severely restricted animals, whereas the growth rate of animals fed *ad libitum* was intermediate between mild and more severe starvation. Overall, the increased growth rate in the 50 % group was accompanied by a more active local muscle IGF system as well as increased muscle-cell proliferation and a trend towards increased satellite-cell activity.

### Acknowledgements

The authors are grateful to the BSU staff at the Royal Veterinary College and Professor Goldspink's staff at the Royal Free Hospital for their technical assistance. The authors would also like to thank Ms Helen Hunt for her help with the image analysis. The present study was funded by the Wellcome Trust.

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