Published December 8, 2014

Proteomic analysis of ovine muscle hypertrophy[1]

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ABSTRACT: Two-dimensional electrophoresis was used to investigate the effects of a QTL for muscle hypertrophy on sarcoplasmic protein expression in ovine muscles. In the Belgian Texel breed, the QTL for muscle hypertrophy is localized in the myostatin-encoding gene. Based on microsatellite markers flanking the myostatin gene, we compared the hypertrophied genotype with the normal genotype. The average age of the sheep was 3 mo. Among the 4 muscles studied, in the hypertrophied genotype only the vastus medialis was normal, whereas the semimembranosus, tensor fasciae latae, and LM were hypertrophied. In the hypertrophied genotype, these muscles showed upregulation of enzymes involved in glycolytic metabolism together with oxidative metabolism in LM. Certain chaperone proteins, including glutathione S-transferase-Pi, heat shock protein-27, and heat shock cognate-70, were also more highly expressed, probably due to increased use of energetic pathways. Expression of the iron transport protein transferrin was increased. Alpha-1-antitrypsin was the only protein showing a similar pattern of expression (i.e., less expressed) in all 4 muscles of the hypertrophied genotype. It is suggested that transferrin and alpha-1-antitrypsin may interact to reinforce myogenic proliferative signaling.

Key words: α1-antitrypsin, myostatin, sheep, skeletal muscle, sarcoplasmic protein, transferrin

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J. Anim. Sci. 2006. 84:3266–3276
doi:10.2527/jas.2006-162

INTRODUCTION

Belgian strains of Texel sheep present a QTL on chromosome 2 with a major effect on muscle development. The QTL results in improved carcass conformation, increased muscle weight and thickness, decreased fat content, and higher fast-twitch myosin proportion and muscle hyperplasia (Laville et al., 2004; Hamelin et al., 2005). This double-muscled trait is economically interesting because it is not associated with lower sensory meat qualities. The QTL is localized in the chromosomal region of the myostatin-encoding gene (Marcq et al., 2002), a good candidate to explain hypertrophy. Myostatin loss-of-function mutations cause double-muscling in mice, cattle, and humans (Lee and McPheron, 1999; Tobin and Celeste, 2005). Because sequencing of the coding region of the gene did not show polymorphism in hypertrophied lambs, further studies were initiated to identify alternative candidate genes, including proteins with differential expression in hypertrophied muscles.

Two-dimensional (**2D**) electrophoresis allows simultaneous separation of thousands of proteins. Use of 2D electrophoresis to study sarcoplasmic proteins is limited, but this fraction (approximately 30% of total proteins) contains the majority of proteins involved in metabolism and signal transduction pathways, which are of interest in understanding regulatory mechanisms.

In double-muscled cattle, the degree of hypertrophy varies with muscle (Ménissier, 1980). In double-muscled Charolais bulls, Dumont (1980) described hypertrophy of semimembranosus (**SM**) and hypotrophy of vastus medialis (**VM**). Thus, to investigate hypertrophy, protein expression of muscles with different levels of hypertrophy should be analyzed by comparing hypertrophied and normal genotypes.

The current study aimed to determine the effect of the Belgian Texel QTL on sarcoplasmic protein expres-

[1]Acknowledgments: This project was supported by grants from the French Research Agency: GENANIMAL and from APIS-GENE. Muriel Hamelin benefited from fellowship of the Région Auvergne and INRA.

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Received March 21, 2006.
Accepted July 24, 2006.

sion in 4 Texel hypertrophied or nonhypertrophied muscles, differing by their fiber type composition.

MATERIALS AND METHODS

Animals and Muscle Samples

The experiment was conducted in accordance with national regulations for humane care and use of animals in research. License, procedures, and holding facilities were approved by the French Veterinary Services (certificate of authorization of experiment on living animals no. 87.848 delivered to E. Laville).

To focus the study on a QTL effect independent of polygenic Texel or Romanov background, we used crossbred lambs originating from an F_2 crossing between Romanov (**RR**) ewes and double-muscled Belgian Texel (**TT**) rams. Animals were genotyped using 2 microsatellite markers flanking the myostatin gene to distinguish whether the locus originated from the Texel ram or Romanov ewe (Laville et al., 2004). To match commercial weight, 15 homozygous TT and 17 homozygous RR lambs were slaughtered at a fixed BW of 33 kg for females and 39 kg for males. After slaughter, carcasses were dressed according to commercial practices. Thirty minutes after slaughter, the LM, VM, SM, and tensor fasciae latae (**TL**) muscles were resected, weighed, and sampled in the middle part of the muscle mass.

The VM, SM, and TL muscles are localized in the proximal segment of the hind legs. These muscles were chosen according to contractile and metabolic type and according to degree of development in the hypertrophied genotype. A previous study (Hamelin et al., 2005) reported that SM, TL, and LM were heavier in the TT genotype and that the VM muscle presented a normal weight. The LM and SM muscles are conventionally classified as fast-twitch red, whereas TL is fast-twitch white muscle, and VM is slow-twitch red muscle. To confirm these traits, muscles were weighed and typed by histochemical method. Samples dedicated to electrophoresis were frozen in liquid nitrogen and then reduced to a fine powder under liquid nitrogen using a mortar and a mechanical pestle and stored at –80°C until protein extraction was performed. Samples dedicated to fiber typing were frozen in isopentane cooled with liquid nitrogen and stored at –80°C until analysis. Two-dimensional electrophoresis was performed on 5 TT and 5 RR lambs, with 3 males and 2 females in each group randomly selected from the 15 TT and 17 RR lambs.

Fiber Typing

Transverse cryosections (10-μm thick) were prepared according to the methods of Sayd et al. (1998). One section was stained using the myosin ATPase method after preincubation at pH 4.45 to define 3 fiber types: I, IIA, and IIB (Brooke and Kaiser, 1970). The next section was stained for succinate dehydrogenase activity (Nachals et al., 1957) to split the IIB type into 2 groups (i.e., more oxidative IIB and less oxidative IIB). Typing was conducted on an average of 300 fibers per muscle.

Sarcoplasmic Protein Extraction

Two-dimensional electrophoresis was performed on 5 TT and 5 RR SM, LM, and TL muscles and 4 TT and 4 RR VM muscles. Gels were made in triplicate. The sarcoplasmic fraction was obtained using a subcellular fractionation method adapted from Pietrzak et al. (1997). The extraction buffer consisted of 50 m*M* KCl, 4 m*M* $MgCl_2$, 20 m*M* Tris, 2 m*M* EDTA, 1% (wt/vol) dithiolthreitol, and 5 m*M* Pefabloc (Fluka, Buchs, Switzerland) at pH 7. Muscle samples (150 mg) were added to 1.5 mL of extraction buffer in an Eppendorf tube containing a glass bead. Homogenization was performed in a Retsch MM2 ball mill (Retsch, Haan, Germany) for 1 h at 4°C. Extracts were centrifuged at $10{,}000 \times g$ for 15 min at 6°C, and the supernatant was collected. Samples were frozen in liquid nitrogen and stored at –80°C.

Two-Dimensional Electrophoresis

Immobilized pH gradient isoelectric focusing (**IEF**) was carried out in a Protean IEF cell (BioRad, Hercules, CA), using BioRad ReadyStrip, 17 cm, pH 5 to 8. Proteins were loaded onto the strips for analytical (110 μg) or preparative (300 μg) gels. Proteins were loaded by inclusion of an adequate volume of extract in a buffer consisting of 7 *M* urea, 2 *M* thiourea, 2% (wt/vol) 3-[(3-cholamidopropyl)dimethylammonio], 5 m*M* Pefabloc, 0.2% (wt/vol) DL-dithiothreitol, and 0.2% carrier ampholytes. The strips were rehydrated overnight. For the subsequent IEF, the voltage was increased gradually to 8,000 V until a total of 80,000 V hours. The strips were immediately frozen and stored at –20°C until further use. Before SDS PAGE, the strips were equilibrated for 15 min followed by a further 20 min in a solution of 6 *M* urea, 30% (vol/vol) glycerol, 2% (wt/vol) SDS, and 50 m*M* Tris successively supplemented with 1% (wt/vol) DTT or 2.5% (wt/vol) iodoacetamide, and bromophenol blue as a dye. Sodium dodecyl sulphate PAGE was performed in a protean IIxi cell (BioRad) on 12% polyacrylamide gels at 15 mA per gel until the dye track reached the end of the gels. Analytical and preparative gels were silver-stained according to the protocol described by Yan et al. (2000).

Image Analysis

Gel images were acquired through a GS-800 (BioRad) imaging densitometer and analyzed using PDQuest software (BioRad). After automated detection and matching, highly saturated or ill-defined spots were manually removed. Across-gel matching of retained spots was inspected and corrected when necessary. Spots were normalized by expressing the relative quan-

tity of each spot as the ratio of the individual spot to the total number of spots retained. Relative quantities were expressed in parts/million parts. For 1 sample and 1 spot, the mean of the 3 values (corresponding to the gels in triplicate) was calculated.

Identification of Spots of Interest by Mass Spectrometry

Spots were excised from the gels using pipette tips. Gel pieces were placed into a 1.5-mL microcentrifuge tube and destained for 2 min with a solution containing 30 m*M* KFe and 100 m*M* sodium thiosulphate, and the gel pieces were washed 3 times in purified water (18 Mohms) for 10 min. The spots were then washed twice for 30 min with 100 µL of 25 m*M* NH_4HCO_3 with 5% acetonitrile for 30 min, followed by 100 µL of 25 m*M* NH_4HCO_3, 50% acetonitrile (vol/vol) and dehydrated in acetonitrile. Gels spots were completely dried using a Speed Vac (Thermoelectron, Waltham, MA) before trypsin digestion. The dried gel volume was evaluated, and 3 volumes of trypsin (10 ng/µL; V5111, Promega, Madison, WI) in 25 m*M* NH_4HCO_3 were added. Digestion was performed at 37°C over 5 h. The gel pieces were precipitated by centrifugation, the supernatant was discarded, and 8 to 12 µL of acetonitrile (depending on the gel volume) was added to extract the peptides. The mixture was sonicated for 5 min and then centrifuged.

For matrix-assisted laser desorption/ionization–time of flight (**MALDI-TOF**) mass spectrometry analysis, 1 µL of supernatant was loaded directly onto the MALDI target. The matrix solution (5 mg/mL of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% trifluoroacetic acid) was immediately added and allowed to dry at room temperature. Peptide masses were determined in positive-ion reflector mode in a Voyager DE-Pro model MALDI-TOF mass spectrometer (PerSeptive BioSystems, Framingham, MA). External calibration was performed with a standard peptide solution (Proteomix, LaserBio Labs, Sophia-Antipolis, France). Internal calibration was performed using peptides resulting from auto-digestion of porcine trypsin, with protonated masses of 842.509, 1045.564, and 2211.104 Da. Peptides mass fingerprints were compared with mammalian databases [NCBI (http://www.ncbi.nlm.nih.gov/Database/) and SWISS-PROT (http://www.expasy.org/sprot/)] using MASCOT and ProFound software [http://www.matrixscience.com and http://prowl.rockefeller.edu, respectively (both last accessed Aug. 15, 2006)]. The search criteria used were 1 missing trypsin cleavage site, partial methionine oxidation, partial carbamidomethylation of cysteine, and mass deviation lower than 30 ppm. We required at least 5 matched peptides per protein for identification and used the ProFound and MASCOT probabilistic score and the accuracy of the experimental-to-theoretical isoelectric point (**pI**) and molecular weight (**mw**).

Table 1. Effect of Texel genotype on muscle weights

Muscle weight,[1] g	Means ± SE		QTL effect[3]
	TT[2]	RR[2]	Test
LM	554 ± 62	445 ± 75	***
SM	304 ± 32	249 ± 29	***
TL	45 ± 10	56 ± 8	*
VM	45 ± 8	45 ± 11	NS

[1]SM = Semimembranosus; TL = tensor fasciae latae; VM = vastus medialis (n = 15 TT and 17 RR).
[2]TT = Texel genotype; RR = Romanov genotype.
[3]GLM analysis, adjusted to carcass weight.
NS = $P > 0.10$; *$P < 0.05$; ***$P < 0.001$.

Statistics

Statistical analysis was performed using SAS (SAS Inst. Inc., Cary, NC). The GLM procedure was applied to test the differences between mean muscle weight values of the 2 genotypes (TT and RR). The model used was $X_{lmn} = \mu + sx_m + h_l + (b_m \times CW_{lmn}) + E_{lmn}$, where X = performance of the *n*th individual of the *l*th QTL haplotype and the *m*th sex; μ = the general mean; h_l = the fixed effect of the *l*th QTL genotype, with *l* varying from 1 to 2; sx_m = the fixed effect of the *m*th sex, with *m* varying from 1 to 2; b_m = the regression coefficient of carcass weight on character for an individual of the *m*th sex; CW_{lmn} = the carcass weight of the *n*th individual, of the *l*th QTL haplotype and the *m*th sex; and E_{lmn} = the random residual value, $\sim N(O, \sigma^2_E)$.

The ANOVA procedure was used to test for the significance ($P < 0.05$) of the fixed effect of muscle on fiber type percentage. Where significant effects were found, Student's *t*-tests were used to identify differences between pairs of muscles at the 5% significance level.

Concerning 2D electrophoresis, the resulting set of averaged spot quantities was submitted independently for each muscle to 1-way ANOVA. An ANOVA at $P < 0.05$ indicated that the spot was significant.

RESULTS

Muscle Weight

Means and genetic effects on muscle weights are presented in Table 1. Weights of SM, TL, and LM were greater in the TT genotype than in RR muscles. There was no between-genotype difference in weight of VM muscle.

Fiber Typing

The muscle effect on fiber type percentage is presented in Table 2. Compared with VM, the TL, SM, and LM were predominantly fast-twitch (IIB). Among this group, the TL presented the greatest rate of fast-twitch white fibers (IIB). The LM was characterized by a greater rate of red fast-twitch IIA fibers, whereas the SM had a greater rate of slow-twitch red fibers (I). The

Table 2. Effect of muscle type on fiber type percentage

	Muscle[2]			
Fiber type[1]	LM	SM	TL	VM
I	$9.5^{a} \pm 3.9$	$13.6^{b} \pm 4.5$	$7.1^{c} \pm 3.7$	$23.4^{d} \pm 4.7$
IIA	$24.5^{a} \pm 7.8$	$18.5^{b} \pm 7.9$	$18.9^{b} \pm 4.9$	$18.6^{b} \pm 3.4$
IIB	$66^{a} \pm 7.7$	$68.9^{a} \pm 9$	$74^{b} \pm 6.4$	$58^{c} \pm 4.4$
IIBo	$23.3^{a} \pm 10.5$	$24.8^{a} \pm 10.5$	$27^{a} \pm 10.4$	$35.4^{b} \pm 6.4$
IIBno	$42.7^{a} \pm 7.7$	$43.1^{a} \pm 13.9$	$47^{a} \pm 8.6$	$22.6^{b} \pm 3.7$

[a–d]Within a row, means with different superscripts are significantly different at $P < 0.05$.

[1]I = Fiber type I; IIA = fiber type IIA; IIB = fiber type IIB; IIBo = fiber type IIB more oxidative; and IIBno = fiber type IIB less oxidative. IIBo and IIBno are subdivisions of IIB.

[2]SM = Semimembranosus; TL = tensor fasciae latae; and VM = vastus medialis (n = 15 TT and 17 RR).

VM presented a greater rate of slow-twitch red fibers and a predominantly oxidative metabolism (I and fiber type IIB more oxidative).

Two-Dimensional Electrophoresis and Mass Spectrometry

Using MALDI-TOF mass spectrometry, we identified 63 spots expressed differentially between genotypes. Differential protein expression between genotypes is presented independently for each muscle because protein redundancy was low (Tables 3, 4, 5, and 6, Figure 1). Many of the common proteins evidenced in the different muscles corresponded to different isoforms (see spot labels in the Tables).

LM. We identified 22 differentially expressed spots in the LM. Ten were associated with glycolytic metabolism and matched to 5 different proteins: enolase, phosphoglucomutases (3 spots), glycerol-3-phosphate dehydrogenases (2 spots), pyruvate kinase 3 and triosephosphate isomerases (3 spots). Excluding one of the triosephosphate isomerase isoforms, all these proteins showed greater expression in the TT genotype. They included 2 that were related to Krebs' cycle and oxidative phosporylation in mitochondria; i.e., 3 spots of succinate dehydrogenase and the NADH dehydrogenase whose role is to allow NAD+ cofactors to regenerate. Also showing greater expression in the TT were the alpha type 1 and beta type 7 proteasome subunits and the constitutive chaperone protein heat shock cognate 70 involved in myosin head stabilization. Expression of the iron transporter transferrin was also greater in the TT genotype. Three fragments of ovine prepro serum albumin and the alpha-1-antitrypsin, a serine protease inhibitor, were less expressed in the TT genotype.

Semimembranosus. We identified 22 differentially expressed spots in the SM. Similar to the LM, expression of proteins associated with glycolytic metabolism was greater in the TT genotype: enolase (4 spots including a fragment), pyruvate kinase (4 spots including 2 fragments), lactate dehydrogenase B, 3 fragments of glycogen myophosphorylase, and 6 spots including 5 fragments matching to creatine kinase. Two spots more highly expressed in the TT genotype were chaperone proteins: the small heat shock protein 27 kDa and the glutathione S-transferase Pi. Similarly to the LM, expression of alpha-1-antitrypsin was less and the iron transporter transferrin was greater in the TT genotype.

Tensor Fasciae Latae. We identified 11 differentially expressed spots in the TL. Amongst them, 5 were proteins associated with glycolytic metabolism, 4 were more expressed in the TT genotype: fructose-1,6-biphosphatase 2, glycerol-3-phosphate dehydrogenase 1, enolase, and creatine kinase. Biphosphoglycerate mutase was less expressed in the TT genotype. Expression of actin alpha 1, which is the monomeric form of actin polymer filament, was also greater in the TT genotype. We found 2 fragments of prepro serum albumin, one more expressed and one less expressed in the TT genotype. We identified 4 proteins that were less expressed in the TT genotype. Two were related to mitochondria and oxidative metabolism: the ATP synthase F1 beta subunit and the heat shock protein 60kDa. Alpha-1-antitrypsin was less expressed in the TT genotype.

Vastus Medialis. We identified 8 differentially expressed spots in the VM. Four were involved in glycolytic metabolism: the glycogen myophosphorylase (fragment), the triosephosphate isomerase (fragment), and the phosphoglycerate kinase, which were less expressed in the TT genotype; and a fragment of lactate dehydrogenase-A, which was more expressed in the TT genotype. An immunoglobulin heavy-chain C region was also more expressed in the TT. We identified 2 chaperone proteins, glutathione S-transferase-Pi, which was less expressed in TT and Antiquitin 1, which was more expressed in TT. Similar to the other 3 muscles, expression of alpha-1-antitrypsin was lower in TT.

DISCUSSION

Posttranslational Modifications and Protein Fragments

The same protein can be found in different spots at different pI and mw locations within a muscle, between different muscles, or both. The different pI and mw locations of a same protein on gel maps could correspond to different isoforms. Isoform expression can result from the expression of different genes; be triggered by posttranslational modifications such as oxidation, glycosyl-

Table 3. Proteins differentially expressed between Texel (n = 5) and Romanov (n = 5) genotypes in LM

Spot	Accession number[1]	Protein ID[1]	Mw (kDa)/pI theoretical[2]	Mw (kDa)/pI estimate[2]	Matched peptide[3]	Sequence coverage,[3] %	Relative abundance TT/RR[4]
Energy metabolism: Glycolytic							
1	gi|57086343	Enolase	47–55/6.4–8.4	47/6.6	11	23	1.8
2	gi|1942567	Phosphoglucomutase Chain B	61.5/6.62	60/7.1	18	34	2.0
3	gi|538558	Phosphoglucomutase	61.5/6.58	63/7.7	9	18	2.3
4	gi|31980726	Phosphoglucomutase 2	61.5/6.30	62/6.8	17	31	2.0
5	gi|61867146	Pyruvate kinase 3	58/7.2–7.9	50/6.3	21	33	2.9
6	gi|61888856	Triosephosphate isomerase	27/6.45	30/6.75	8	39	1.6
7	gi|59858493	Triosephosphate isomerase 1	27/6.45	30/7.2	7	33	–2.7
8	gi|59858493	Triosephosphate isomerase 1	27/6.45	30/7.5	14	56	–2.7
9	gi|59857727	Glycerol-3-phosphate dehydrogenase 1	38/6.42	40/6.8	9	25	3.0
10	gi|59857727	Glycerol-3-phosphate dehydrogenase 1	38/6.42	40/7.0	13	38	1.9
Energy metabolism: Oxidative							
11	gi|1364245	NADH dehydrogenase	23.5/5.71	29/6.1	5	27	2.3
12	gi|284649	Succinate dehydrogenase (ubiquinone)	73/7.27	64/6.55	11	20	1.9
13	gi|284648	Succinate dehydrogenase (ubiquinone)	73/7.27	62/6.7	19	33	2.1
14	gi|284649	Succinate dehydrogenase (ubiquinone)	73/7.27	62/6.65	16	30	1.9
Contractile apparatus-associated protein							
15	gi|56385	Heat shock Cognate 70	71/5.43	70/5.35	22	45	1.9
Detoxification							
16	gi|30582133	Proteasome subunit. alpha type 1	30/6.51	36/6.8	9	39	3.0
17	gi|38051889	Proteasome subunit. beta type 7	30/8.13	34/6.3	12	31	1.9
Regulator							
18	gi|57526646	Alpha-1-anti trypsin	46/5.83	60/5.1	13	29	–2.1
19	gi|29135265	Transferrin	78/6.75	75/6.9	16	24	2.7
Other							
20	gi|57164373	Pre-pro serum albumin, ovine fgt[5]	69/5.80	54/5.5	24	38	–2.4
21	gi|57164373	Pre-pro serum albumin, ovine fgt	69/5.80	30/5.5	15	22	–2.6
22	gi|57164373	Pre-pro serum albumin, ovine fgt	69/5.80	53/5.8	14	22	–2.3

[1]Protein names and accession numbers were taken from the NCBI database (http://www.ncbi.nlm.nih.gov/Database/).
[2]Mw and pI, theoretical (recorded in the NCBI database) and estimated (calculated from the spot position on the gel).
[3]Number of peptides that matched the protein sequence and total percentage of sequence coverage.
[4]Relative abundance TT/RR = greatest average quantity/lowest average quantity. A minus sign indicates that relative abundance of the protein was lower in the TT genotype.
[5]fgt = Protein fragments.

ation, phosphorylation, or proteolytic cleavage; or both. One isoform may be more expressed in one muscle, whereas for another muscle it may be another isoform. We observed that isoforms differentially expressed between genotypes showed similar patterns of between-muscle expression, as was the case with the enolase proteins. This phenomenon is probably related to functional abilities of muscles for which one isoform may be predominant. Different protein isoforms present different functional properties. For example, it was found that troponin T isoforms may be related to slow- or fast-twitch fiber types (Bouley et al., 2005).

Based on an apparent mw lower than the theoretical, some of the spots identified were protein fragments (Table 3, 4, 5, and 6). Most of these were overrepresented in the SM of TT. Many fragments were derived from glycolytic proteins (pyruvate kinase, glycogen myophosphorylase). Due to its high mw and basic pI, the whole protein of glycogen myophosphorylase could not be visualized on our 2D gels, and thus only fragments were evidenced. Previous studies using 2D electrophoresis have also observed protein fragments in muscles sampled very early after slaughter (Lametsch et al., 2002; Morzel et al., 2004). The authors explained the presence of fragments as being due to early proteolysis after slaughter as well as probable intra vitam proteolysis. It is possible that the greater abundance of fragments may be a consequence of increased oxidation levels. Nikawa et al. (2002) have shown that proteolysis can be induced intra vitam by exercise and that the concomitant increased use of the respiratory chain is a natural source of reactive oxygen species production and, consequently, oxidative stress. Some sarcoplasmic proteins such as enolase and creatine kinase are particularly prone to oxidation, as described in oxidative stress-induced diseases (Castegna et al., 2002a,b). However, fragments were not identified in all muscles (i.e., only in the SM and VM) and did not appear to be related to a particular genotype or degree of hypertrophy. Given that a majority of intact glycolytic enzymes were found to be overrepresented in the TT genotype, we may also hypothesize that the overrepresentation of glycolytic

Table 4. Proteins differentially expressed between Texel (n = 5) and Romanov (n = 5) genotypes in semimembranosus

Spot	Accession number[1]	Protein ID[1]	Mw (kDa)/pI theoretical[2]	Mw (kDa)/pI estimate[2]	Matched peptide[3]	Sequence coverage,[3] %	Relative abundance TT/RR[4]
Energy metabolism: Glycolytic							
23	gi|27806645	Enolase 1	47/6.44	41.5/5.3	14	41	2.5
24	gi|27806645	Enolase 1	47/6.44	41.5/5.2	15	45	2.4
25	gi|57086343	Enolase 3	47/7.58	41/6.7	14	42	1.9
26	gi|57086343	Enolase 3 fgt[5]	47/7.58	27/5.6	7	21	1.9
27	gi|57163939	Glycogen myophosphorylase fgt	97/6.65	35/6.65	8	9	2.0
28	gi|57163939	Glycogen myophosphorylase fgt	97/6.65	35/7.0	13	12	1.9
29	gi|57163939	Glycogen myophosphorylase fgt	97/6.65	35/6.6	12	11	3.9
30	gi|332864188	Pyruvate kinase	58/7.2–7.9	50/6.3	20	34	2.0
31	gi|2117873	Pyruvate kinase	58/7.2–7.9	55/7.8	20	39	1.9
32	gi|33286422	Pyruvate kinase fgt	58/7.2–7.9	40/5.9	12	23	2.2
33	gi|31416989	Pyruvate kinase fgt	58/7.2–7.9	40.5/6.0	16	31	2.0
34	gi|59858383	Lactate dehydrogenase B	37/6.02	40.5/6.5	20	55	2.0
35	gi|4838363	Creatine kinase M fgt	43/6.63	29/6.9	13	22	1.9
36	gi|6729828/gi|66920	Creatine kinase Chain A/M fgt	43/6.63	33/7.0	11	28	1.9
37	gi|6729828/gi|66920	Creatine kinase Chain A/M fgt	43/6.63	33/6.9	8	21	1.9
38	gi|4838363	Creatine kinase M fgt	43/6.63	17/6.0	7	15	2.8
39	gi|4838363	Creatine kinase M fgt	43/6.63	29/7.3	11	23	2.3
40	gi|4838363	Creatine kinase M	43/6.63	35.5/7.6	16	33	2.0
Contractile apparatus-associated protein							
41	gi|61553385	Heat shock protein 27 kDa	23/6.0–6.2	29/6.7	9	44	2.4
Detoxification							
42	gi|6013379	Glutathione S-transferase Pi	24/7.65	25/6.9	9	63	2.5
Regulator							
18	gi|57526646	Alpha-1-anti-trypsin (A1AT)	46/5.83	60/5.1	13	29	–2.0
19	gi|29135265	Transferrin	78/6.75	75/6.9	16	24	3.6

[1]Protein names and accession numbers were taken from the NCBI database (http://www.ncbi.nlm.nih.gov/Database/).
[2]Mw and pI, theoretical (recorded in the NCBI database) and estimated (calculated from the spot position on the gel).
[3]Number of peptides that matched the protein sequence and total percentage of sequence coverage.
[4]Relative abundance TT/RR = greatest average quantity/lowest average quantity. A minus sign indicates that relative abundance of the protein is lower in the TT genotype.
[5]fgt = Protein fragments.

enzyme fragments reflects increased initial levels of entire protein.

Energetic Metabolism and Related Proteins

Most of the proteins differentially expressed between genotypes were involved in energy metabolism. An increase in enzyme concentration will lead to an acceleration of the production of energetic molecules. Different metabolic pathways were involved.

Creatine kinase, which is an enzyme essential to a rapid replenishment of cellular ATP stocks, was more highly expressed in the SM and TL muscles of TT animals; Okumura et al. (2005) found it more highly expressed in glycolytic muscles, where it ensures fast energy refill.

Most of the proteins identified as more highly expressed in TT than RR genotype were enzymes involved in carbohydrate degradation. They included triosephosphate isomerase, glycerol-3-phosphate dehydrogenase, and biphosphoglycerate mutase, which are all involved in shifts in the main pathway of glycolysis. Thus, the reduced triosephosphate isomerase and biphosphoglycerate mutase expression in LM and TL muscles of TT animals can be interpreted as overactivation of the main pathway of glycolysis. The reverse activity of glycerol-3-phosphate dehydrogenase means that its greater expression in TT animals remained unexplained, but is possibly evidence of increased mobilization of triglycerides.

The lower abundance of phosphoglycerate kinase in the VM muscle of TT suggests that an enhanced glycolytic pathway is related to muscle development but not to gene polymorphism. The greater abundance of enzymes involved in glycolytic metabolism in hypertrophied muscles was in accordance with a previous study showing a greater level of the myosin heavy-chain fast isoform in muscles of the TT genotype (Laville et al., 2004) and another study on the muscle proteome of a myostatin deletion in cattle (Bouley et al., 2005). Working on total protein extract, the authors mainly observed modifications in contractile apparatus toward a fast-twitch potential. This result was supported by an upregulation of the glycolytic enzyme phosphoglucomutase. Our subcellular compartment fractionation method enabled us to visualize more proteins involved in metabolism and signaling, which are masked by myofibrillar proteins in total extract analysis.

Table 5. Proteins differentially expressed between Texel (n = 5) and Romanov (n = 5) genotypes in tensor fasciae latae

Spot	Accession number[1]	Protein ID[1]	Mw (kDa)/pI theoretical[2]	Mw (kDa)/pI estimate[2]	Matched peptide[3]	Sequence coverage,[3] %	Relative abundance TT/RR[4]
		Energy metabolism: Glycolytic					
43	gi|61839453	Bisphosphoglycerate mutase	30/6.0	32/6.6	10	41	–1.6
44	gi|29792061	Enolase 1	47/6.4–7.0	49.5/6.25	8	18	2.4
45	gi|2154755	Fructose-1.6-bisphosphatase 2	37/6.84	39/7.0	5	13	1.9
10	gi|59857727	Glycerol-3-phosphate dehydrogenase 1	38/6.42	40/7.0	13	38	1.9
46	gi|4838363	Creatine kinase M	43/6.63	45/7.2	8	22	3.0
		Energy metabolism: Oxidative					
47	gi|1374715	ATP synthase F1. beta subunit	51/4.97	52/5.05	13	38	–1.9
48	gi|1334284	Heat shock protein 60 kDa	61/5.71	60/5.15	8	27	–2.5
		Contractile apparatus-associated proteins					
49	gi|33563240	Actin. alpha 1	42/5.23	46/5.5	8	26	2.1
		Regulator					
50	gi|57526646	Alpha-1-anti trypsin	46/5.83	60/5.0	11	26	–2.1
		Other					
51	gi|57164373	Prepro serum albumin, Ovine fgt[5]	69/5.80	55/5.8	26	41	8.4
52	gi|57164373	Prepro serum albumin, Ovine fgt	69/5.80	55/5.95	23	39	–2.2

[1]Protein names and accession numbers were taken from the NCBI database (http://www.ncbi.nlm.nih.gov/Database/).
[2]Mw and pI, theoretical (recorded in the NCBI database) and estimated (calculated from the spot position on the gel).
[3]Number of peptides that matched the protein sequence and total percentage of sequence coverage.
[4]Relative abundance TT/RR = greatest average quantity/lowest average quantity. A minus sign indicates that the relative abundance of the protein is lower in the TT genotype.
[5]fgt = Protein fragments.

The switch in hypertrophied muscle metabolism toward a more glycolytic pattern can be related to the lower level of capillary supply previously observed in the TL and SM muscle of the TT genotype animals (Hamelin et al., 2005). This hypothesis is consistent with the effects of chronic hypoxia described by Lopez-Barneo et al. (2001) because hypoxia upregulates glucose degradation.

Four mitochondrial proteins that are involved in the respiratory chain and oxidative phosphorylation were visualized. Heat shock protein 60 kDa is a chaperone protein produced in precursor form in cytoplasm and then translocated into mitochondria. It stabilizes proteins and facilitates folding and protein assembly in the mitochondrial matrix (Liu and Steinacker, 2001). Its expression is increased in skeletal muscle after pro-

Table 6. Proteins differentially expressed between Texel (n = 4) and Romanov (n = 4) genotypes in vastus medialis

Spot	Accession number[1]	Protein ID[1]	Mw (kDa)/pI theoretical[2]	Mw (kDa)/pI estimate[2]	Matched peptide[3]	Sequence coverage,[3] %	Relative abundance TT/RR[4]
		Energy metabolism: Glycolytic					
53	gi|57163939	Glycogen myophosphorylase fgt[5]	97/6.65	35/6.9	13	16	–2.4
54	gi|217590	Lactate dehydrogenase-A fgt	34/8.17	15.5/7.15	7	20	1.9
55	gi|61873464	Phospho-glycerate kinase	44.5/8.02	42/6.2	5	16	–2.3
56	gi|59858493	Triosephosphate isomerase 1 fgt	27/6.45	20/5.5	10	48	–3.2
		Detoxification					
57	gi|6013379	Glutathione S-transferase Pi	24/7.65	24/7.3	5	44	–2.0
58	gi|25108887	Antiquitin 1	55/6.24	55/5.8	13	22	2.0
		Regulator					
18	gi|57526646	Alpha-1-antitrypsin	46/5.83	60/5.1	13	29	–2.7
		Other					
59	gi|109029	Immunoglobulin heavy-chain C region	34/6.07	53/6.05	5	36	3.2

[1]Protein names and Accession numbers were taken from the NCBI database (http://www.ncbi.nlm.nih.gov/Database/).
[2]Mw and pI, theoretical (recorded in NCBI database) and estimated (calculated from the spot position on the gel).
[3]Number of peptides that matched the protein sequence and total percentage of sequence coverage.
[4]Relative abundance TT/RR = greatest average quantity/lowest average quantity. A minus sign indicates that the relative abundance of the protein is lower in the TT genotype.
[5]fgt = Protein fragments.

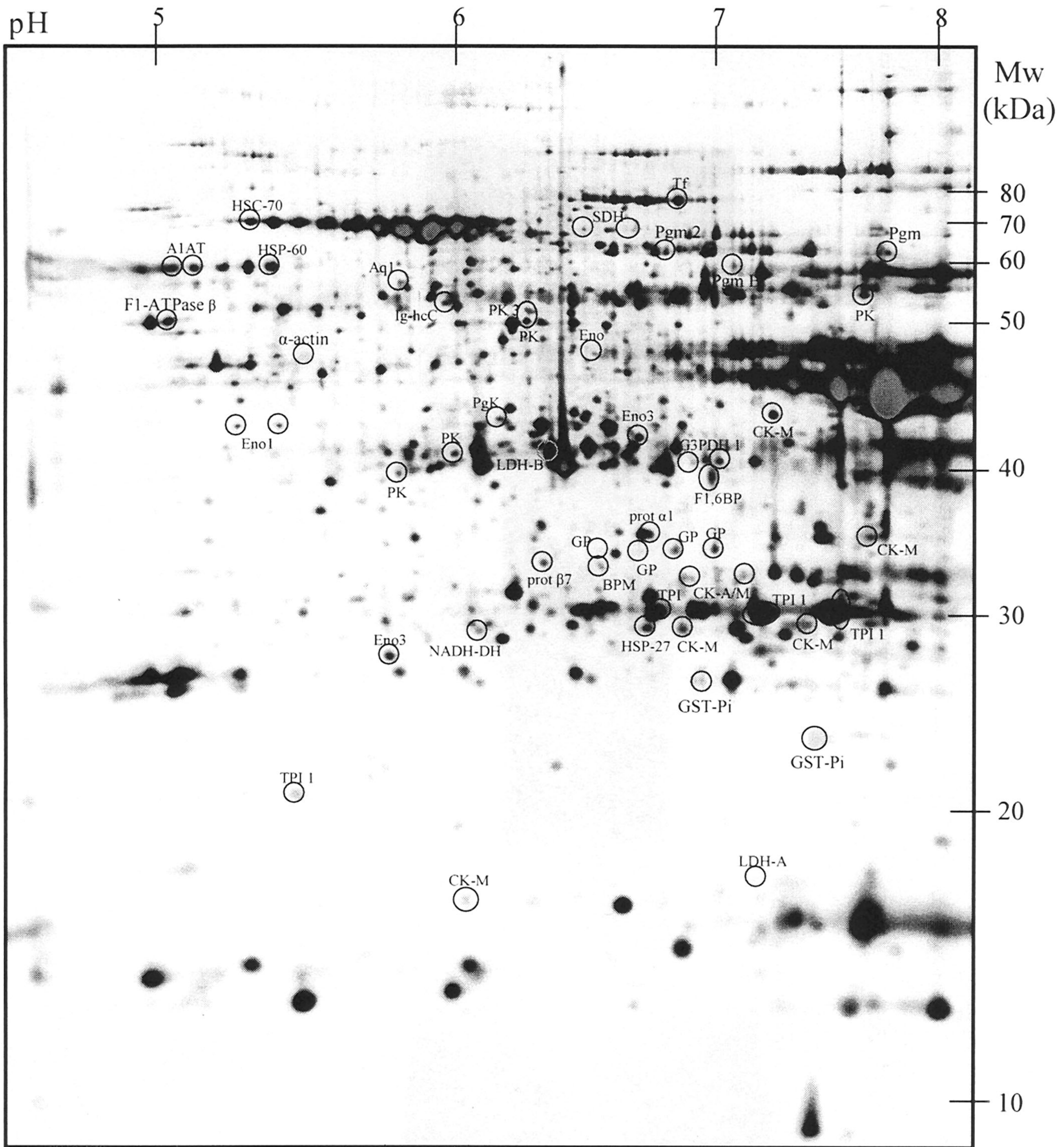

Figure 1. Representative 2-dimensional gel map of muscle sarcoplasmic proteins. The differentially expressed proteins between Texel genotype and Romanov genotype in the 4 muscles are encircled (spots corresponding to prepro serum albumin are not shown). Abbreviations: A1AT = Alpha-1-antitrypsin; Aq1 = Antiquitin 1; BPM = Biphosphoglycerate mutase; CK = Creatine Kinase; CK-M = Creatine Kinase M; CK-A/M = Creatine Kinase Chain A/M; Tf = Transferrin; Eno = Enolase; Eno1 = Enolase 1; Eno3 = Enolase 3; F1,6BP = Fructose-1,6-bisphosphatase 2; F1-ATPase β = ATP synthase F1, beta subunit; G3PDH 1 = Glycerol-3-phosphate dehydrogenase 1; GP = Glycogen myophosphorylase; GST-Pi = Glutathione S-transferase Pi; HSC-70 = Heat Shock Cognate 70; HSP-27 = Heat Shock Protein 27kDa; HSP-60 = Heat Shock Protein 60kDa; Ig-hcC = Immunoglobulin heavy-chain C region; LDH-A = Lactate dehydrogenase-A; LDH-B = Lactate dehydrogenase B; NADH-DH = NADH dehydrogenase; PgK = Phosphoglycerate Kinase; Pgm = Phosphoglucomutase; Pgm 2 = Phosphoglucomutase 2; Pgm B = Phosphoglucomutase Chain B; PK = Pyruvate kinase; PK 3 = Pyruvate kinase 3; prepro-SAO = prepro Serum Albumin, Ovine; Prot α1 = Proteasome subunit, alpha type 1; Prot β7 = Proteasome subunit, beta type 7; SDH = Succinate dehydrogenase (ubiquinone); TPI = Triosephosphate isomerase; TPI 1 = Triosephosphate isomerase 1; and α-actin = Actin alpha 1.

longed endurance training due to increased metabolic demand associated with oxidative fiber type (Liu and Steinacker, 2001). The ATP synthase F1 beta subunit (**F1-ATPase** β) is the catalytic part of the ATP synthase complex in the mitochondria. The F1-ATPase β catalyzes ATP synthesis starting from ADP + Pi, using the proton gradient as energy source. The lower expression of both heat shock protein 60 kDa and F1-ATPase β proteins in TL muscle of the TT genotype is consistent with the more glycolytic profile of this hypertrophied muscle. The NADH dehydrogenase and succinate dehydrogenase were more highly expressed in the LM of TT. The hypertrophied LM presented a peculiar feature in that proteins associated with both oxidative and glycolytic metabolism were upregulated. In comparison with SM and TL muscles, LM presented a more oxidative metabolism (Table 1). The LM is richer in type IIA fibers, which in normal sheep present more oxidative metabolism than type I fiber (Suzuki, 1971; Briand et al., 1981; Sayd et al., 1998). This would lead to the conclusion that muscle hypertrophy enhances the predominant metabolism in that muscle. If the muscle combines oxidative and glycolytic metabolisms, both would be enhanced. Because this feature has not previously been described, it would be useful to study enzyme expression in a hypertrophied muscle with a more oxidative profile, such as the supraspinatus.

Proteins Associated with Contractile Apparatus

The heat shock protein-27 (**HSP-27**) and heat shock cognate-70 (**HSC-70**) are chaperone proteins that promote cell survival during physiological stress (Thompson et al., 2003). The HSP-27 has been reported to play a central role in the structural and functional organization of the 3-dimensional intermediate filament structure and the actin microfilament system (Fischer et al., 2002) and may also act as a molecular chaperone. The HSP-27 exists in many isoforms (Scheler et al., 1999). Kim et al. (2004) reported that one isoform is more highly expressed in glycolytic muscle. This is in agreement with the results presented here, where the increased glycolytic metabolism in SM muscles of the TT genotype was associated with upregulation of HSP-27. The HSC-70 was more highly expressed in the LM of the TT genotype. It is involved in folding and assembly of myosin in striated muscle (Srikakulam and Winkelmann, 2004) and facilitates the proper folding of labile proteins, protects proteins from unfolding during stress, and prevents polymerization of already unfolded proteins (Welch, 1992). Actin alpha 1 corresponds to the monomeric form of actin filaments. Before polymerization, these monomers remain free in the cell cytoplasm. All 3 proteins Actin alpha 1, HSP-27, and HSC-70 are associated with the contractile apparatus. Their overexpression in hypertrophied muscles may indicate an increased need for material to promote myofibril assembly. This need may simply be due to the hypertrophic status of the muscles of 3-mo-old lambs still in growing phase. In addition, an enhanced metabolism as observed in hypertrophied muscles is known to produce cytotoxic compounds such as methylglyoxal (see Thornalley, 1996, for review) as well as cytoplasm acidification (lactate production), which can damage proteins and cell structures such as myofibrillar filaments. Consequently, these muscles present greater protein turnover, as indicated by the upregulation.

Detoxification

Glutathione S-transferase Pi (**GST-Pi**), which was more expressed in the TT genotype in the most hypertrophied muscle (SM) but less expressed in the less hypertrophied muscle (VM), plays a role in mechanisms of cellular detoxification and cellular resistance to oxidative damage (Lo Bello et al., 2001) by catalyzing the conjugation of glutathione to potentially toxic compounds such as reactive oxygen species. Gelfi et al. (2004) reported that GST-Pi was highly expressed in muscles of Tibetan people living at high altitude where blood oxygenation is more difficult.

The proteasome is involved in degradation of altered proteins. The greater relative abundance of 2 subunits of proteasome in the LM of the TT genotype would indicate an increase in altered proteins needing to be degraded. As suggested above, enhanced muscle metabolism produces a range of cytotoxic compounds (Bloomer and Goldfarb, 2004), and therefore the greater abundance of proteasome subunits and GST-Pi is consistent with the greater metabolism observed in hypertrophied TT muscles.

Regulators

Transferrin (**Tf**) is a protein that is normally synthesized in the liver. It binds iron and delivers it to cells. Lopez-Barneo et al. (2001), in a study on the effects of chronic hypoxia, reported that hypoxia induces in skeletal muscle an upregulated expression of glucose degradation enzymes and transporters, including Tf. In our study, the SM and LM of the TT genotype showed greater glycolytic enzyme expression in association with a less developed vasculature of the SM muscle (Hamelin et al., 2005). The more highly expressed Tf could therefore be interpreted as a mechanism to compensate the hypoxia generated by decreased blood supply or increased metabolic demand. Furthermore, Tf has also been identified in different species as a myotrophic factor stimulating the proliferation of myoblasts and satellite cells in culture (Ozawa, 1989; Graziadei, 1998). Moreover, Tf levels are greatest when the proliferative demands of fetal tissues are at a peak, indicating a mitogenic effect of Tf independent of its ability to supply iron to proliferating tissues (Meek and Adamson, 1985). Alpha-1-antitrypsin (**A1AT**) expression was less in all 4 muscles of the TT genotype studied. Alpha-1-antitrypsin is a member of the serine protease inhibitor protein family (serpins) whose main target is the

elastase (Travis and Salvesen, 1983). It can also inhibit muscle cystein proteinases such as cathepsin K, L, and S (Gettins, 2002). Graziadei (1998) provided evidence of another function for A1AT: because it has a strong affinity for the Tf membrane receptor, A1AT may compete directly with Tf for the receptor site and consequently inhibit erythroid and fibroblast proliferation by interfering with Tf-mediated iron uptake. To our knowledge, there have been no reports of such an effect on myoblasts or muscle cells. However, the combined effects of greater abundance of Tf in SM and LM of the TT genotype and lower abundance of A1AT could interact to a reinforce proliferative signaling. This hypothesis is supported by the hyperplasic status of TT hypertrophy (Hamelin et al., 2005) and the known effects of Tf on muscle development.

Others

Some proteins, such as the immunoglobulin heavy-chain C region and prepro serum albumin fragments, appear to be irrelevant in skeletal muscle. Immunoglobulins are usually present in the extracellular medium and on the membrane surface, and the overexpression in VM of the TT genotype of a single immunoglobulin region is difficult to interpret in terms of muscular function.

This study investigated the effect of the Texel QTL on the expression of sarcoplasmic proteins in 4 different muscles. Alpha-1-antitrypsin was the only protein identified to show a similar pattern of expression between TT and RR genotypes in the 4 muscles studied, independently of hypertrophic or muscle fiber type status. Because of its affinity to the Tf membrane receptor, the decrease in A1AT expression could interact with upregulated Tf expression to reinforce the Tf proliferative signal. Recently, Clop et al. (2006) have shown that the double-muscled Belgian Texel strain is a myostatin mutant. These authors described a mutation in the 3′UTR region of the myostatin gene creating a microRNA target leading to microRNA-mediated translational downregulation in the myostatin concentration contributing to muscular hypertrophy. The hypertrophic model of Texel muscle is thus probably a hyperplasic phenomenon that occurred in the fetal stage of development. Myostatin regulates muscle development by a very complex mechanism that remains only partially understood. Many myogenic factors are involved, including the Tf effect, whose interaction with A1AT should therefore be considered. In this perspective, further studies should be conducted to investigate the expression of these proteins or their corresponding genes at the fetal stage of proliferation when myostatin exerts its main effects. These studies should be associated to measures of the expression of myostatin and microRNA. Certain muscles as VM do not express the hypertrophic phenotype, suggesting more complex mechanisms of regulation.

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