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Original article

Comparison of skeletal muscle transcriptional profiles in dairy and beef breeds bulls

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Abstract. A cDNA microarray (18 263 probes) was used for transcriptome analysis of bovine skeletal muscle (*m. semitendinosus*) in 12-month-old bulls of the beef breed Limousin (LIM) and the typical dairy breed Holstein-Friesian (HF, used as a reference). We aimed to identify the genes whose expression may reflect the muscle phenotype of beef bulls. A comparison of muscle transcriptional profiles revealed significant differences in expression of 393 genes between HF and LIM. We classified biological functions of 117 genes with over 2-fold differences in expression between the examined breeds. Among them, 72 genes were up-regulated and 45 genes were down-regulated in LIM vs. HF. The genes were involved in protein metabolism and modifications (22 genes), signal transduction (15), nucleoside, nucleotide and nucleic acid metabolism (13), cell cycle (9), cell structure and motility (9), developmental processes (9), intracellular protein traffic (7), cell proliferation and differentiation (6), cell adhesion (6), lipid, fatty acid and steroid metabolism (5), transport (5), and other processes. For the purpose of microarray data validation, we randomly selected 4 genes: *trip12*, *mrps30*, *pycrl*, and *c-erbb3*. Real-time RT-PCR results showed similar trends in gene expression changes as those observed in microarray studies. Basing on results of the present study, we proposed a model of the regulation of skeletal muscle growth and differentiation, with a principal role of the somatotropic pathway. It may explain at least in part the development of muscle phenotype in LIM bulls. We assume that the growth hormone directly or indirectly (through IGF-1) activates the calcium-signaling pathway with calcineurin, which stimulates myogenic regulatory factors (MRFs) and inhibits early growth response gene. The inhibition results in indirect activation of MRFs and impaired activation of TGF-beta1 and myostatin, which finally facilitates terminal muscle differentiation.

Keywords: microarray, skeletal muscle, Limousin, Holstein-Friesian, cattle, gene expression, transcriptional profile, transcriptome.

Introduction

There are some studies of the biological mechanisms involved in the expression of meat quality characteristics (Lefaucheur et al. 1991; Harper 1999), showing joint effects of various factors – such as breed, age, gender, feeding – on sensory attributes (flavour, colour, texture) and biological characteristics of the muscles (collagen, fibers, lipids). Various cattle breeds or genotypes differ in muscle characteristics due to marked differences in their physiology (Hocquette et al. 2006). Late-maturing beef breeds (e.g. Limousin) deposit more muscles and less fat, compared to dairy breeds (Holstein), dual-purpose (e.g. Polish Red) or early-maturing beef breeds (Hereford). Breed differences reported in the literature are thus often confounded with differences in somatic maturation time, and hence fatness (Chambaz et al. 2003). It has been demonstrated that beef breeds (Limousin, Hereford) are characterized by lower collagen content, compression, and shear force in raw and cooked meat, respectively, compared to a dairy breed (Holstein) (Monson et al. 2004).

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There is an increasing number of studies on the growth and metabolism of skeletal muscle in cattle, where the DNA microarray method is applied. These include studies concerning age-dependent changes in the bovine skeletal muscle transcriptomic profile (Sadkowski et al. 2006), identification of differentially expressed genes in distinct skeletal muscles in cattle (Yu et al. 2007), development of bovine longissimus muscle from 2 different beef cattle breeds (Lehnert et al. 2007), transcriptome analysis of 2 bovine muscles (oxidative and glycolytic) during ontogenesis (Sudre et al. 2003), transcriptional profiling of skeletal a muscle from 2 breeds of cattle (Wang et al. 2005), and gene expression profiling of muscle tissue in Brahman steers during nutritional restriction (Byrne et al. 2005). There are also studies concerning the effect of double-muscled phenotype (mutation nt821 (del11)) in the *mstn* gene on transcriptomic profile in bovine embryos (Potts et al. 2003) and fetuses (Cassar-Malek et al. 2007). Very recently cDNA microarrays have been used for analysis of bovine skeletal muscle transcriptome in relation to polymorphism in the 5'-flanking region of the *mstn* gene (Sadkowski et al. 2008).

In the present study, the cDNA microarray technique was used to compare the gene expression profiles of skeletal muscle in bulls of a dairy breed (Holstein-Friesian) and a beef breed (Limousin), and thus to identify genes responsible for phenotypic differences between these breeds.

Holsteins (HF) are a typical dairy breed, early maturing. Carcass quality in this breed is relatively poor. HF bulls have a lower dressing percentage than beef bulls, but tend to marble well, since fat accumulates inside the muscle instead of outside, and can produce prime carcasses (Dvorak 1991).

Limousin (LIM) breed originated in the Limousin region of South-West France. It is a typical beef breed, with a large proportion of muscle and low proportion of fat. This breed is noted for having a high yield of lean meat in the carcass. Its meat is finely textured, tender, and low in saturated fats and cholesterol. The breed is intermediate in maturity between British and most other European breeds (Chambaz et al. 2003).

Materials and methods

Animals and sampling

The experimental group was composed of 30 bulls: 15 Holstein-Friesian (HF; Polish Black-and-White type), and 15 Limousin (LIM). The bulls were not related. LIM bulls were born in beef herds, while HF bulls in dairy herds, where all the animals were artificially reared on milk, calf pellets and hay. At the age of 2–3 months, the bulls were transferred to the Institute Farm in Jastrzebiec near Warsaw. The bulls were housed in a loose barn from the age of 3 months until slaughter. After the transfer, the animals were fed ad libitum a total mixed ration (TMR) consisting of corn silage (75%), concentrates (20%), and hay (5%). The animals had free access to water. At the age of 12 months, all bulls were slaughtered after 24-hours fasting, in the local abattoir. The carcasses were chilled for 24 hours at 4°C, and dissected into lean, fat and bone (Oprządek et al. 2001). Mean body weight and major carcass traits of bulls of the examined breeds are shown in Table 1.

Table 1. Major carcass traits of Holstein Friesian (HF) and Limousin (LIM) bulls slaughtered at the age of 12 months (n = 15)

Breed	Mean body weight (kg)		Carcass dressing percentage (%)		Percentage of lean in valuable cuts (%)	
	LSM	SE	LSM	SE	LSM	SE
HF	381	32.1	50.94^{B}	0.90	68.99^{B}	0.60
LIM	366	45.1	59.25^{A}	1.30	78.45^{A}	0.90

Within columns, values with different superscripts differ significantly at $P < 0.01$. LSM = least squares mean, SE = standard error

Basing on the methodology described in our previous work (Jank et al. 2006), we identified animals with genotypes CC, GG and GC for the polymorphism in the 5'-flanking region of the myostatin gene (G/C substitution at position -7828 relative to ATG). The polymorphism influences transcript and protein level of myostatin – a key factor involved in the regulation of skeletal muscle growth. Our recent study (Sadkowski et al. 2008) revealed essential differences in transcriptional profile of skeletal muscle between *mstn* genotypes. For this reason, in the present study, animals with the same *mstn* genotype were selected. We chose genotype CC, because only this genotype occurred in LIM bulls. Sampling of a larger group of animals was limited by the rare occurrence of CC genotype in HF bulls. Samples for RNA isolation were taken from 3 bulls of each breed (in total, 6 bulls were sampled), but from each animal, 2 independent samples were taken from separate portions of *m. semitendinosus*. The muscle samples were taken immediately after slaughtering, and frozen in liquid nitrogen. All of the procedures carried out with the use of the animals were approved by the Local Ethics Commission (permission No. 3/2005).

DNA microarrays

Bovine DNA microarrays used in our study were based on EST libraries owned by the National Bovine Functional Genomics Consortium (NBFGC), and were printed and provided by the Center for Animal Functional Genomics (Michigan State University, USA). The slides contain 18 263 unique transcripts, coming from the NBFGC library and representing various bovine tissues in different physiological states. Each NBFGC microarray contains also 96 spots of bovine b-actin and GAPDH probes, as well as 241 negative controls containing only binding buffer (3×SSC) and 384 empty spots. The total number of spots printed is 19 200, and they are organized in 48 subarrays, each with 2020 spots (Suchyta et al. 2003).

RNA isolation and validation

Total RNA from muscle sample was isolated by using a total RNA kit (A&A Biotechnology, Poland) according to the manufacturer's protocol. Isolated RNA samples were dissolved in RNase-free water, and RNA quantity was measured with the use of NanoDrop (NanoDrop Technologies, USA). The samples with an adequate amount of RNA were treated with DNase I to eliminate DNA contamination. Subsequently, the samples were purified by using RNeasy MiniElute Cleanup Kit (Qiagen, Germany). The samples were again analyzed with a BioAnalyzer (Agilent, USA) to measure final RNA quality and integrity (Sadkowski et al. 2006).

Labeling of probes

Total RNA (10 µg) was reverse-transcribed by using SuperScript Plus Indirect cDNA Labeling kit (Invitrogen, USA) according to the manufacturer's protocol. Single-strand cDNA was labeled with Alexa 555 or Alexa 647 dyes (Invitrogen, USA). The efficiency of dye incorporation was measured by using NanoDrop. Afterwards, the samples were randomly paired in one tube, and then hybridized.

Hybridization

Before hybridization, the NBFGC microarray cDNA slides were prehybridized by rinsing them 2 times in 0.1% SDS for 2 min in the RT reaction mixture. Afterwards, slides were boiled in MiliQ H_2O for 3 min, rinsed with ice-cold ethanol for 30 s, and dried immediately. Hybridization was performed by using an automatic hybridization station HybArray12 (PerkinElmer, USA). Slides were fixed in hybridization chambers, and after o-ring conditioning, probes were added. Hybridization of slides was performed according to the 18-hour hybridization protocol provided by the manufacturer of microarrays. After hybridization, slides were automatically washed.

Hybridization, signal detection, quantification, and analysis

Acquisition and analysis of hybridization intensities were performed by using a microarray scanner ScanArray Express HT and ScanArray Express software (PerkinElmer, USA). Mean spot intensity values were automatically normalized (LOWESS method) by ScanArray Express software, and used for further analyses. For data visualization, Panther (Thomas et al. 2003) and Pathway Architect (Stratagene, USA) software were used.

Selection of the most differently expressed genes

In this work, we decided to select the most differently expressed genes in the groups compared, based on some group comparison measures. The methods of ranking of genes used will be explained with the following notation: x_i, y_i, (where $i = 1, 2\ldots n$), represent data related to the n samples tested in a microarray experiment, where $x_i = [x_{i,1}, x_{i,2},\ldots,x_{i,d}] \in R^d$ denotes the vectors of expression of d genes (transcripts) measured for sample i, and $\{c_1, c_2\}$ denotes membership associated with sample i. In this study, we used as elements of vectors x_i the mean pixel intensity for a spot, as measured by the chip scanner.

Prior to the actual gene ranking stage, we preprocessed our data in order to ensure equal mean intensity of each sample. Technically, we multiplied each of the vectors x_i, (where $i = 1, 2\ldots n$), by a rescaling factor defined as $avg(x_1)/avg(x_i)$, where $avg(x_j) = \frac{1}{d}\sum_k x_{j,k}$, thus rescaling its intensity to the intensity of sample 1.

For ranking of genes, we used the Wilcoxon statistic and the fold change. Gene selection based on the Wilcoxon statistic (Polanski and Kimmel, 2007) requires that for each fixed gene j (where $j = 1,\ldots d$), a nonparametric rank test is performed, comparing 2 groups of samples, $\{x_{i,j} : y_i = c_1\}$ against $\{x_{i,j} : y_i = c_2\}$. This gives a P value, whose

significant value (i.e. $P < 0.05$) indicates that expression of gene j for classes c_1 and c_2 should be considered different. Sorting the list of genes by increasing P values, places the most differently expressed genes on top of this list.

Selection of differently expressed genes by using the fold change requires that for each gene j, (where $j = 1,\ldots d$), the ratio of mean expressions of this gene for classes c_1 and c_2 is computed. If we denote the mean expression of a given fixed gene for classes c_1 and c_2 as μ_1 and μ_2, respectively, then a convenient formula of the fold difference is $|\log \mu_1 - \log \mu_2|$, which produces high values if either of the means exceeds the other. Sorting the list of genes by decreasing values of this measure gives the most differently expressed genes on top of the list.

Real-time RT-PCR

Expression of validated genes was checked by real-time RT-PCR, using the following primers: MRPS30-F, 5'-CGA GTT GAT GCT GTG CGA TAC-3'; MRPS30-R, 5'-ATG GGA GTT TGT CTG GCT TAC-3'; TRIP12-F, 5'-TAC CCA AAG GCT AAC CCA CC-3'; TRIP12-R, 5'-CAC AGG AGA AAG TGA AGC GTT-3'; PYCRL-F, 5'-GTC TGT GAA GGG ACC AAC AAG; PYCRL-R, 5'-AGG TGA AGA AAT GGA CTC TGG-3'; c-erbB3-F, 5'-ACG CCT GGC ATC AGA ATC ATC G-3'; c-erbB3-R, 5'-ACC ATT GAC ATC CTC TTC CTC TAA CC-3'; GAPDH-F, 5'-ATG AGA TCA AGA AGG TGG TG-3'; and GAPDH-R, 5'-CGT ACC AGG AAA TGA GCT TG-3' (primer sequences designed in Primer3 software, basing on sequence BC102589 from the GenBank). We used LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Germany) according to the following procedure: Mg^{2+} added at a final concentration of 1.5 mM; preincubation step at 95°C for 10 min; amplification step (40 cycles) including denaturation at 95°C for 10 s, annealing for MRPS30 and TRIP12 at 63°C, while PYCRL and c-erbB3 at 57°C for 10 s, respectively, and extension at 72°C for 10 s; melting curve including denaturation at 95°C for 0 s, annealing at 65°C for 15 s, continuous melting at 95°C for 0 s (slope = 0.1°C/s); and cooling step at 40°C for 30 s. For *gapdh*, annealing was at 57°C. Results are presented as the ratio of *mrps30*, *trip12*, *pycrl*, *c-erbB3* to *gapdh* expression.

Results

Identification of genes expressed differentially between dairy and beef breeds bulls

Our analysis revealed significant differences in expression of 393 genes between LIM and HF bulls. Over 2-fold changes were found in the case of 117 genes. Among them, 72 genes were up-regulated and 45 genes were down-regulated in LIM as compared to HF. The highest differences in expression were observed in the up-regulated genes *odc, clim2, znf414, nckx3, kctd20, c10orf28, sgsh, mondoa, pthr15242* and *bgn*, and the down-regulated genes *lap, egr4, yipf2, ca11, pycrl, bac ch240-89p8, hbxip, mrp-s6, clone kb1552d7* and *klk10* (Table 2).

Validation of microarray results by real-time RT-PCR

For the purpose of microarray data validation, we have randomly selected 4 genes: *trip12* (position 25 in Table 2), *mrps30* (position 67), *pycrl* (position 77), *c-erbb3* (position 83). Real-time RT-PCR results showed similar trends in gene expression changes as we observed in microarray studies (Table 3). Namely, expression of *mrps30* was very significantly higher ($P < 0.001$) in LIM (0.271±0.018) than in HF (0.077±0.004), and expression of *trip12* was significantly higher ($P < 0.01$) in LIM (0.011±0.0007) than in HF (0.002±0.0004) (Figure 1). Conversely, the expression of *c-erbb3* was significantly lower ($P < 0.05$) in LIM (0.005±0.001) than in HF (0.010±0.002). Expression of *pycrl* in LIM (0.020±0.003) and HF (0.024±0.004) was not statistically significant. The fold changes for particular genes estimated by using cDNA microarray and real-time RT-PCR techniques were comparable (Table 3).

Functional characteristics of genes differing in expression between HF and LIM

Using Panther software (Thomas at al. 2003) it was possible to classify the identified genes according to biological processes they were involved in. This analysis revealed 3 main groups of genes involved in the following biological processes: protein metabolism and modifications (22 genes), signal transduction (15 genes), and nucleoside, nucleotide and nucleic acid metabolism (13 genes) (Figure 2). The genes involved in protein metabolism and modifications, included 9 involved in proteolysis (*trip12, becn1, rps27a, ubl7, ubd, fbx3, klk10, shap* and *cul1*), 6 involved in biosynthesis

Table 2. Comparison of the gene expression profile of skeletal muscle (*m. semitendinosus*) of 12-month-old Limousin and Holstein-Friesian (Polish Black-and-White type) bulls, i.e. typical beef and dairy cattle, respectively. Only genes with fold change over 2.00 are shown. In each breed, n = 6 (3 individuals × 2 muscle samples)

No.	GenBank ID	Gene Name	E value	Fold change	P value
1	2	3	4	5	6
1	BC146218	Bos taurus ornithine decarboxylase (ODC) gene	0.00E+00	+3.34	0.004
2	XM_604843	PREDICTED: Bos taurus similar to CLIM2	0.00E+00	+2.93	0.016
3	NM_001038175	Bos taurus similar to zinc finger protein 414 (ZNF414)	1.00E-77	+2.91	0.016
4	XM_868421	PREDICTED: Bos taurus similar to potassium-dependent sodium-calcium exchanger NCKX3	8.00E-166	+2.84	0.016
5	XM_592890	PREDICTED: Bos taurus similar to K+ channel tetramerization protein (KCTD20)	5.00E-55	+2.79	0.037
6	XM_001501230	PREDICTED: Equus caballus similar to growth inhibition and differentiation related protein 86 (C10orf28)	0.00E+00	+2.78	0.037
7	XM_615532	PREDICTED: Bos taurus similar to heparan sulfate sulfamidase transcript variant 1 (SGSH)	0.00E+00	+2.69	0.025
8	XM_001251579	PREDICTED: Bos taurus similar to MondoA	0.00E+00	+2.66	0.025
9	XM_001489761	PREDICTED: Equus caballus similar to CTD-binding SR-like protein rA9 (KIAA1542 - SPLICING FACTOR. ARGININE/SERINE RICH 2.RNAP C-TERM INTERACTING PROTEIN (PTHR15242))	7.00E-69	+2.65	0.025
10	NM_178318	Bos taurus biglycan (BGN)	5.00E-176	+2.59	0.025
11	NM_001076941	Bos taurus hypothetical protein LOC616005 (C1orf164)	0.00E+00	+2.56	0.004
12	NM_001046225	Bos taurus progestin and adipoQ receptor family member VI (PAQR6)	0.00E+00	+2.56	0.004
13	XM_534172	PREDICTED: Canis familiaris similar to Transmembrane 9 superfamily protein member 2 precursor (p76)	0.00E+00	+2.56	0.010
14	NM_000163	Homo sapiens growth hormone receptor (GHR)	4.00E-94	+2.55	0.016
15	NM_001046256	Bos taurus myotubularin-related protein 9 (MTMR9)	0.00E+00	+2.54	0.016
16	BT021816	Bos taurus TNFAIP3 interacting protein 1 (TNIP1)	0.00E+00	+2.54	0.010
17	NM_001075190	Bos taurus hypothetical LOC505124	0.00E+00	+2.53	0.010
18	BC102491	Bos taurus ribosomal protein S27a (RPS27A)	0.00E+00	+2.51	0.004
19	NM_001098910	Bos taurus similar to Efs1	0.00E+00	+2.48	0.004
20	AY528252	Bos taurus cullin 1 mRNA (CUL-1)	0.00E+00	+2.48	0.004
21	XR_028496	PREDICTED: Bos taurus similar to A-kinase anchor protein 13 (AKAP13)	0.00E+00	+2.48	0.046
22	XM_613279	PREDICTED: Bos taurus similar to LRTS841 (KIAA1822L - THYROID HORMONE UPREGULATED/GENE 5 RELATED)	0.00E+00	+2.46	0.046
23	NM_001075585	Bos taurus similar to RIB43A domain with coiled-coils 1 (Ribc1)	0.00E+00	+2.43	0.025
24	XR_027678	PREDICTED: Bos taurus similar to plexin A3 (Plxna3)	0.00E+00	+2.43	0.025
25	XM_592231	PREDICTED: Bos taurus similar to thyroid hormone receptor interactor 12. transcript variant 1 (TRIP12)	0.00E+00	+2.42	0.016
26	NM_001015573	Bos taurus ubiquitin-like 7 (bone marrow stromal cell-derived) (UBL7)	0.00E+00	+2.40	0.016
27	NM_001075630	Bos taurus similar to vesicle-associated membrane protein 1 - Synaptobrevin-1 (VAMP1)	0.00E+00	+2.38	0.016
28	Y09207	Bos taurus MHC class 1 protein molecule D18.3	0.00E+00	+2.37	0.010
29	XM_848577	PREDICTED: Canis familiaris similar to casein kinase 1. alpha 1 isoform 1. transcript variant 2 (CSNK1A1)	0.00E+00	+2.36	0.037
30	BC114736	Bos taurus XPA binding protein 2 (XAB2)	0.00E+00	+2.35	0.037
31	NM_001076514	Bos taurus similar to mitochondrial ribosomal protein L21 (MRPL21)	0.00E+00	+2.34	0.025
32	XM_864245	PREDICTED: *Bos taurus* similar to Golgi complex autoantigen golgin-97, transcript variant 2 (golgin-97)	0.00E+00	+2.34	0.025
33	X64124	*Bos taurus* DNA for SINE sequence Bov-tA (BTBOV1)	1.00E-51	+2.34	0.016
34	XM_864692	PREDICTED: *Bos taurus* similar to dynein, cytoplasmic, heavy polypeptide 1, transcript variant 3 (DNCH1)	0.00E+00	+2.33	0.016
35	XM_876610	PREDICTED: *Bos taurus* similar to neuropilin-1, transcript variant 9 (NRP1)	0.00E+00	+2.33	0.004
36	NM_001013586	*Bos taurus* minichromosome maintenance complex component 3 (MCM3)	0.00E+00	+2.29	0.046

Table 2 cont.

1	2	3	4	5	6
37	XM_594628	Bos taurus eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein) (EEF1D)	0.00E+00	+2.29	0.046
38	XM_594628	PREDICTED: Bos taurus hypothetical LOC540389. transcript variant 1	0.00E+00	+2.27	0.037
39	NM_001083723	PREDICTED: Bos taurus hypothetical LOC534327	0.00E+00	+2.27	0.037
40	NM_001075221	Bos taurus similar to chromosome 17 open reading frame 37 (C17orf37)	0.00E+00	+2.27	0.004
41	NM_001098909	PREDICTED: Bos taurus hypothetical LOC508503, transcript variant 1	0.00E+00	+2.26	0.004
42	XM_612243	PREDICTED: Bos taurus hypothetical LOC532997	0.00E+00	+2.26	0.046
43	XM_001252118	PREDICTED: Bos taurus similar to coiled-coil-helix-coiled-coil-helix domain containing 8 (CHCHD8)	0.00E+00	+2.22	0.010
44	XM_582133	PREDICTED: Bos taurus hypothetical LOC505788	0.00E+00	+2.20	0.010
45	XM_606342	PREDICTED: Bos taurus similar to GLIS family zinc finger 2 (Glis2)	0.00E+00	+2.20	0.025
46	NM_174806	Bos taurus glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2) (GOT2)	0.00E+00	+2.20	0.025
47	NM_001075353	Bos taurus similar to Inter-alpha-trypsin inhibitor heavy chain H1 precursor (ITI heavy chain H1) (Inter-alpha-inhibitor heavy chain 1) (Inter-alpha-trypsin inhibitor complex component III) (Serum-derived hyaluronan-associated protein) (SHAP)	0.00E+00	+2.20	0.016
48	NM_001075706	Bos taurus similar to CG3295-PA	0.00E+00	+2.18	0.037
49	NM_001083647	Bos taurus programmed cell death 4 (PDCD4)	0.00E+00	+2.17	0.037
50	CU179667	Pig DNA sequence from clone CH242-291A15 on chromosome 4	9.00E-44	+2.17	0.010
51	XM_580972	PREDICTED: Bos taurus similar to Apolipoprotein B48 receptor (APOB48R)	0.00E+00	+2.16	0.037
52	X15609	Bovine mRNA for monoamine oxidase type A (MAO-A)	0.00E+00	+2.12	0.004
53	NM_174177	Bos taurus scinderin (SCIN)	0.00E+00	+2.12	0.004
54	XM_001252804	PREDICTED: Bos taurus similar to PP3111 protein	0.00E+00	+2.12	0.004
55	NM_001177	Homo sapiens ADP-ribosylation factor-like 1 (ARL1)	5.00E?32	+2.11	0.004
56	NM_001037454	Bos taurus similar to MON1 homolog B	0.00E+00	+2.10	0.010
57	NM_001033627	Bos taurus beclin 1 (BECN1)	0.00E+00	+2.10	0.010
58	NM_001075898	Bos taurus similar to Alpha-1-syntrophin (59 kDa dystrophin-associated protein A1, acidic component 1) (Pro-TGF-alpha cytoplasmic domain-interacting protein 1) (TACIP1) (Syntrophin 1)	0.00E+00	+2.09	0.010
59	XM_581363	PREDICTED: Bos taurus similar to chromodomain helicase DNA binding protein 1, transcript variant 1 (CHD1)	0.00E+00	+2.09	0.037
60	XM_593633	PREDICTED: Bos taurus similar to heat shock 70-kD protein 12B (HSPA12B)	0.00E+00	+2.08	0.037
61	XM_603849	PREDICTED: Bos taurus similar to MTH1a (p26)	0.00E+00	+2.07	0.016
62	AC013476	Homo sapiens BAC clone RP11-525G12 from 2	1.00E-16	+2.06	0.016
63	NM_007361	Homo sapiens nidogen 2 (osteonidogen) (NID2)	8.00E-53	+2.06	0.004
64	XM_610565	PREDICTED: Bos taurus hypothetical LOC532056	0.00E+00	+2.06	0.004
65	BC102178	Bos taurus similar to 6-phosphogluconate dehydrogenase (decarboxylating). mRNA (6PGD)	0.00E+00	+2.05	0.046
66	Z83826	Human DNA sequence from clone RP3-473B4 on chromosome X. Contains 3' end of LOC159091 gene. Two novel genes, a high-mobility group protein pseudogene, and three CpG islands	5.00E-18	+2.05	0.046
67	XM_001251581	PREDICTED: *Bos taurus* similar to mitochondrial 28S ribosomal protein S30 (S30mt) (programmed cell death protein 9) (MRPS30)	0.00E+00	+2.04	0.037
68	NM_001034216	*Bos taurus* phosphatidylinositol glycan anchor biosynthesis. class S (PIGS)	0.00E+00	+2.04	0.025
69	NM_001046321	*Bos taurus* mitochondrial ribosomal protein L44 (MRPL44)	0.00E+00	+2.03	0.025
70	BC122795	*Bos taurus* Ig kappa chain (IGKC)	0.00E+00	+2.00	0.016
71	NM_001046502	*Bos taurus* solute carrier family 22 (organic cation transporter). member 5 (SLC22A5)	0.00E+00	+2.00	0.016
72	BT021198	*Bos taurus* coiled-coil domain containing 8 (CCDC8)	0.00E+00	+2.00	0.010
73	NM_001076058	*Bos taurus* similar to lysosomal acid phosphatase precursor (LAP)	3.00E-75	–3.12	0.037
74	NM_001040497	*Bos taurus* early growth response 4 (EGR4)	0.00E+00	–2.94	0.004

Table 2 cont.

1	2	3	4	5	6
75	XM_593996	PREDICTED: Bos taurus similar to YIPF2 protein	0.00E+00	–2.88	0.046
76	BC123827	Bos taurus carbonic anhydrase-related XI protein. mRNA (CA11)	0.00E+00	–2.88	0.046
77	NM_001014906	Bos taurus pyrroline-5-carboxylate reductase-like (PYCRL)	0.00E+00	–2.82	0.037
78	AC150524	Bos taurus BAC CH240-89P8 [Children's Hospital Oakland Research Institute Bovine BAC Library (male)]	2.00E-17	–2.58	0.037
79	NM_001034517	Bos taurus hepatitis B virus x interacting protein (HBXIP)	0.00E+00	–2.54	0.016
80	BC042752	Homo sapiens mitochondrial ribosomal protein S6 (MRP-S6)	1.00E-22	–2.33	0.016
81	AP003471	Homo sapiens genomic DNA, chromosome 8q23, clone: KB1552D7	7.00E-24	–2.29	0.046
82	NM_001075890	Bos taurus similar to Kallikrein 10 precursor (protease serine-like 1) (normal epithelial cell-specific 1) (KLK10)	0.00E+00	–2.26	0.046
83	XR_028216	PREDICTED: Bos taurus similar to receptor tyrosine-protein kinase erbB 3 precursor (c-erbB3) (tyrosine kinase-type cell surface receptor HER3)	0.00E+00	–2.25	0.010
84	AB002152	Capra hircus mRNA for stem cell factor (SCF)	0.00E+00	–2.21	0.010
85	NM_001077101	Bos taurus hypothetical protein MGC137645	0.00E+00	–2.20	0.010
86	XM_001502467	PREDICTED: Equus caballus similar to glycogen synthase kinase 3 beta, transcript variant 1 (GSK3B)	0.00E+00	–2.19	0.004
87	XM_597823	Bos taurus phospholipase A2, group IVB (cytosolic) (PLA2G4B)	2.00E-43	–2.18	0.004
88	XM_527380	PREDICTED: Pan troglodytes glucagon-like peptide 1 receptor (GLP1R)	5.00E-05	–2.18	0.046
89	AB081095	Bos taurus bcnt. h-type bcnt genes (CFDP1)	0.00E+00	–2.17	0.046
90	XM_593299	PREDICTED: Bos taurus similar to Cdc6-related protein (CDC6)	0.00E+00	–2.10	0.046
91	NM_004834	Homo sapiens mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4), transcript variant 1	3.00E-39	–2.10	0.037
92	XM_613515	PREDICTED: Bos taurus 5-lipoxygenase, transcript variant 1 (ALOX5)	0.00E+00	–2.09	0.037
93	AB055289	Macaca fascicularis brain cDNA, clone:QflA-12135, similar to human progestin and adipoQ receptor family member VI (PAQR6)	1.00E-113	–2.08	0.025
94	XM_864237	PREDICTED: Bos taurus similar to KIAA0369 doublecortin-like kinase 1 (DCAMKL1)	0.00E+00	–2.08	0.025
95	NM_174210	Bos taurus uncoupling protein 3 (mitochondrial, proton carrier) (UCP3)	0.00E+00	–2.07	0.004
96	XM_859845	PREDICTED: Canis familiaris similar to transcriptional regulating factor 1 isoform 3, transcript variant 3 (TRERF1)	1.00E-165	–2.07	0.010
97	BC010019	Homo sapiens mediator of RNA polymerase II transcription, subunit 8 homolog (S. cerevisiae). mRNA (MED8)	1.00E-64	–2.07	0.046
98	NM_001042535	Homo sapiens centaurin gamma 3 (CENTG3), transcript variant 2	0.00E+00	–2.06	0.016
99	NM_001035038	Bos taurus MYG1 protein (C12orf10)	3.00E-163	–2.06	0.016
100	AC150752	Bos taurus BAC CH240-288E20 [Children's Hospital Oakland Research Institute Bovine BAC Library (male)]	2.00E-17	–2.06	0.046
101	XM_615197	PREDICTED: Bos taurus similar to caspase recruitment domain family, member 10 (CARD10)	0.00E+00	–2.05	0.046
102	NM_001038143	Bos taurus glutamate-cysteine ligase, modifier subunit (GCLM)	0.00E+00	–2.05	0.037
103	AL034344	Human DNA sequence from clone RP1-118B18 on chromosome 6p24.1-25.3. Contains FOXC1 gene for forkhead box C1 gene, 3' end of GMDS gene for GDP-mannose 4 6-dehydratase, and four CpG Islands	3.00E-19	–2.04	0.046
104	BC102438	Bos taurus similar to ubiquitin D. mRNA (UBD)	3.00E-143	–2.04	0.004
105	NM_174258	Bos taurus caldesmon 1 (CALD1)	2.00E-154	–2.04	0.004
106	NM_001034274	Bos taurus mitochondrial ribosomal protein L17 (MRPL17)	3.00E-88	–2.04	0.010
107	NM_005647	Homo sapiens transducin (beta)-like 1X-linked (TBL1X)	2.00E-24	–2.04	0.025
108	NM_001014851	Bos taurus cysteine-rich with EGF-like domains 1 (CRELD1)	2.00E-68	–2.03	0.025
109	NM_001034697	Bos taurus tubulin beta 4 (TUBB4)	0.00E+00	–2.03	0.025
110	NM_001037621	Bos taurus similar to CG3625-PB	2.00E-60	–2.02	0.016
111	XM_001254003	PREDICTED: Bos taurus similar to Trip11 protein	0.00E+00	–2.02	0.016
112	XM_528904	PREDICTED: Pan troglodytes kelch-like 15 (KLHL15)	6.00E-44	–2.01	0.010
113	BC109898	Bos taurus UBX domain containing 1 (UBXD1)	0.00E+00	–2.01	0.037
114	NM_001075517	Bos taurus similar to mammary tumor virus receptor 2 (predicted) (Mtvr2)	0.00E+00	–2.01	0.010
115	XM_535764	PREDICTED: Canis familiaris similar to disrupted in renal carcinoma 2 (DIRC2)	5.00E-116	–2.00	0.004

Table 2 cont.

1	2	3	4	5	6
116	XR_027700	PREDICTED: Bos taurus similar to PTPL1-associated RhoGAP 1 (Arhgap29)	0.00E+00	–2.00	0.004
117	XM_001144906	PREDICTED: Pan troglodytes similar to F-box protein 3. transcript variant 2 (FBX3)	4.00E-21	–2.00	0.025

Table 3. Validation of the expression of selected genes by real-time RT-PCR. In each breed, n = 6 for cDNA microarray (3 individuals × 2 different tissue samples); and n = 9 for real-time RT-PCR (3 individuals × 3 different tissue samples)

Gene name	Fold change	
	cDNA microarray	real-time RT-PCR
MRPS30	+3.48	+3.53
TRIP12	+2.35	+7.58
PYCRL	–2.48	–1.52
c-erbB3	–2.04	–2.33

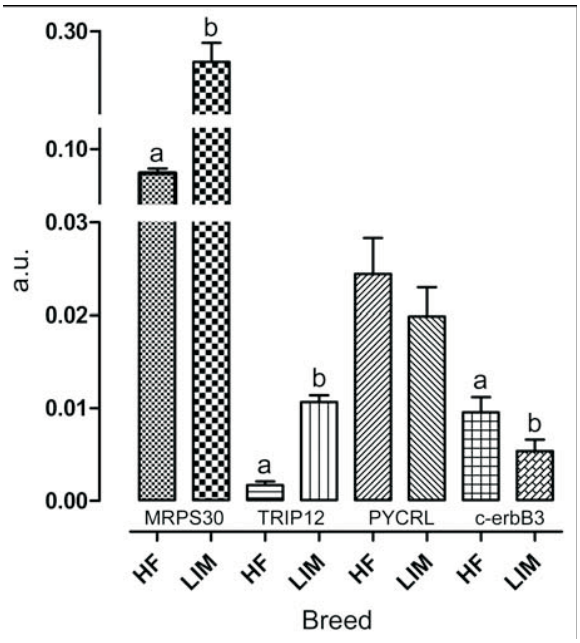

Figure 1. Expression of *mrps30*, trip12, pycrl and *c-erbb*3 genes in *m. semitendinosus* of Holstein-Friesian (HF) and Limousin (L) bulls analyzed by real-time RT-PCR. The results were obtained by dividing the expression of genes mentioned above by *gapdh* expression, and are presented in arbitrary units. Bars with different superscripts differ significantly ($P< 0.05$). In each breed, n = 9 (3 individuals × 3 different tissue samples).

(*eef1d, mrpl21, mrpl17, pdcd4, gclm* and *mrp-s6*), 6 involved in protein modifications (*gsk3b, plxna3, map4k4, dcamkl1, csnk1a1* and *c-erbb3*), and a gene involved in protein folding (*kctd20*). Among genes involved in signal transduction, 7 were involved in surface receptor signal transduction (*efs1, glp1r, paqr6, plxna3, centg3, ghr* and *c-erbb3*), 5 involved in intracellular signaling cascade (*tacip1, gsk3b, odc, csnk1a1* and *card10*), and 3 involved in cell communication (*bgn, scf* and *nrp1*). Among genes involved in nucleoside, nucleotide and nucleic acid metabolism: 7 genes were responsible for mRNA transcription (*egr4, chd1, clim2, lap, xab2, monado* and *glis2*), 5 for DNA metabolism (*trerf1, xab2, csnk1a1, mcm3* and *cdc6*), and single genes were involved in pre-mRNA processing (*kiaa1542*), RNA catabolism (*trerf1*), and tRNA metabolism (*pp3111*). The molecular functions of 28 genes were unclassified and the remaining genes belonged to the following classes: amino acid metabolism (3), apoptosis (4), carbohydrate metabolism (3), cell adhesion (6), cell cycle (9), cell proliferation and differentiation (6), cell structure and motility (9), developmental processes (9), homeostasis (1), immunity and defense (2), intracellular protein traffic (7), lipid, fatty acid and steroid metabolism (5), neuronal activities (2), oncogenesis (3), phosphorus metabolism (1), protein targeting and localization (1), sulfur metabolism (2), and transport (5).

Gene interactions

Pathway Architect software was used to show interactions between genes identified by using the DNA microarray technique (at least 2-fold changes in expression) and genes differentially expressed in muscle described up to now in literature. It was possible to identify the genes converging signals from other genes involved in various metabolic and signal pathways. On the basis of Pathway Architect visualization, we hypothesized that *odc*, *ubd*, *gsk3b*, *c-erbb3*, *nrp1*, *rps27a*, *cul1*, *cdc6*, *mcm3* and *ghr* are key converging genes in skeletal muscle, as their expression significantly differed between HF and LIM (Figure 3). We assumed that they include putative genes involved in the appearance of phenotypic features of the examined beef breed (Limousin).

Discussion

During the past few decades, advances in molecular genetics have led to the identification of genes that affect meat quality in farm animals (Mullen

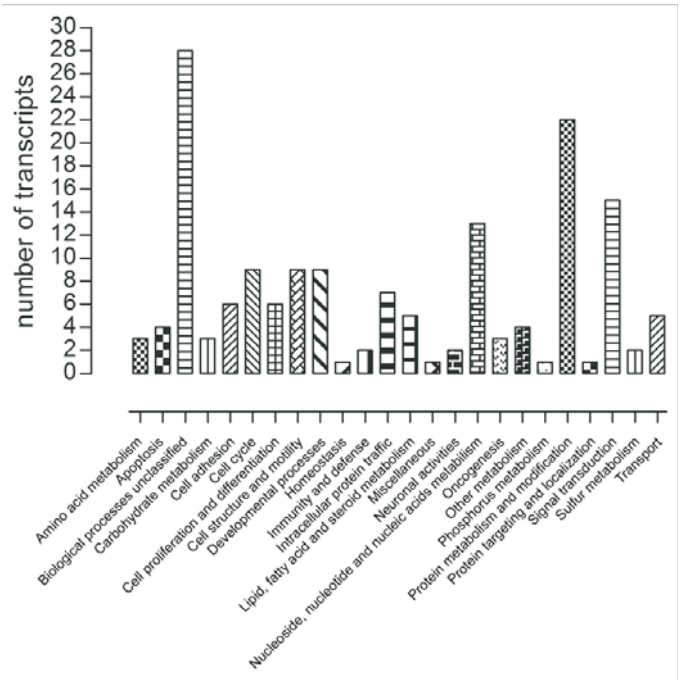

Figure 2. Classification of genes differing in expression between LIM and HF muscle according to their biological processes (Panther software).

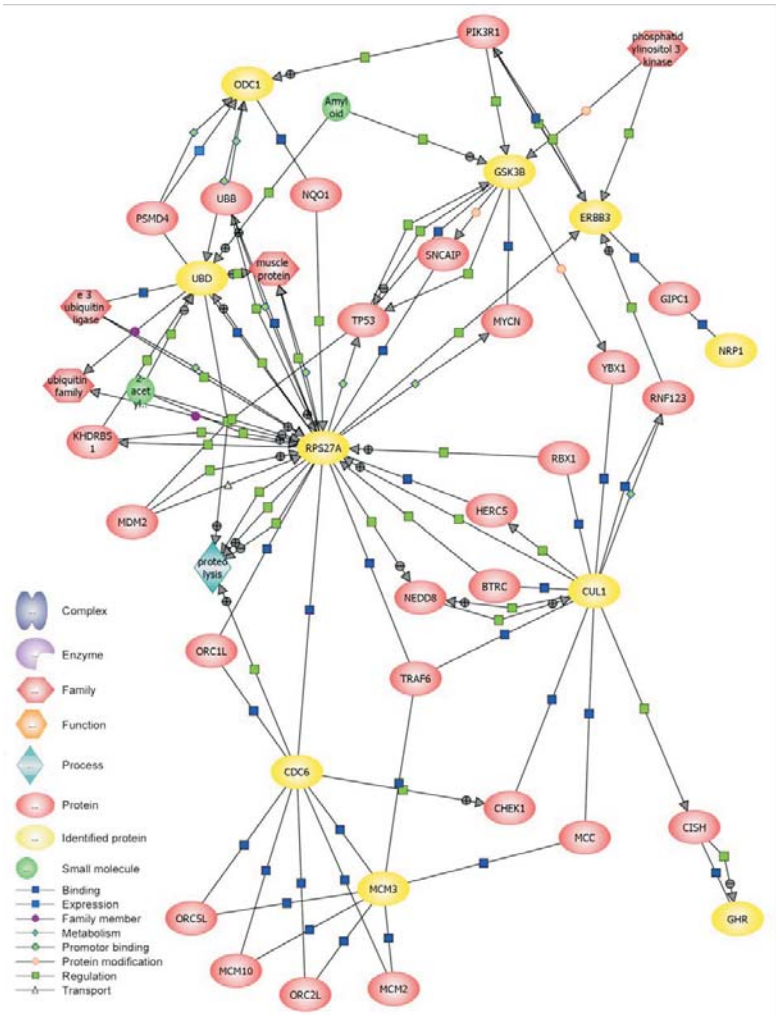

Figure 3. The network of interactions of *rps27a*, *odc1*, *gsk3b*, *ubd*, *nrp1*, *cul1*, *cdc6*, *mcm3*, *c-erbb3* and *ghr* with well-known genes involved in the regulation of skeletal muscle growth and metabolism (Pathway Architect software).

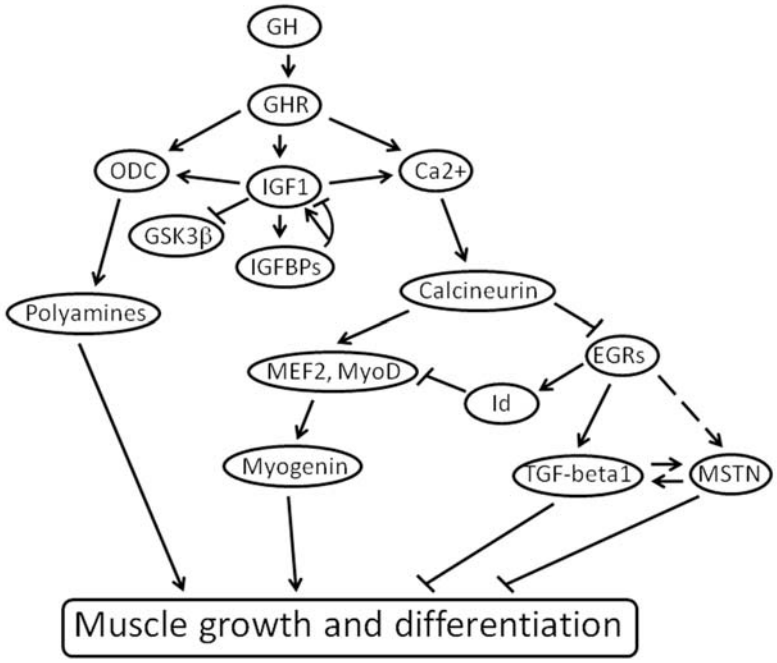

Figure 4. Hypothetical model of the regulation of skeletal muscle growth and differentiation with a principal role of the somatotropic pathway. Positive regulation is indicated by arrows, whereas inhibition is represented by blunt-ended lines.

et al. 2006; Gao et al. 2007; Świtoński, 2008). In cattle, important genes affecting beef quality have been reported (Coffey 2007; Hocquette et al. 2007; Oprządek et al. 2007). Validation of DNA tests for quantitative beef quality traits have been performed for their use in cattle breeding practice (Dekkers, 2004; Van Eenennaam et al. 2007). For meat tenderness, several gene markers have been found, e.g. the gene encoding calpain I and its inhibitor – calpastatin (Lonergan et al. 1995; Juszczuk-Kubiak et al. 2004a; Juszczuk-Kubiak et al. 2004b; Page et al. 2004; White et al. 2005; Casas et al. 2006; Schenkel et al. 2006; Rosochacki et al. 2008). Furthermore, the genes encoding leptin (Kononoff et al. 2005; Nkrumah et al. 2005; Schenkel et al. 2005), thyreoglobulin (Barendse et al. 2004), DGAT1 (Thaller et al. 2003), growth hormone (Grochowska et al. 2001; Beauchemin et al. 2006), growth hormone receptor (Maj et al. 2004; Maj et al. 2006; Tatsuda et al. 2008), STAT5A (Flisikowski et al. 2003), and myostatin (Wheeler et al. 2002), were associated with beef quality traits. Another group of important candidate genes for muscle growth, not yet exploited in cattle, are those encoding myogenic regulatory factors (MRFs).

However, there is a limited number of data on transcriptional profiling in skeletal muscle of various cattle breeds. They concern the development of bovine longissimus muscle from 2 different beef cattle breeds (Hereford, Piedmontese) (Lehnert et al. 2007) or the gene expression profiles of skeletal muscle tissue from 2 breeds of cattle (Wang et al. 2005). The latter publication is of special interest, since it compares Japanese Black (JB) cattle (typical beef breed bred in Japan) with Holstein cattle. The authors identified a group of genes up-regulated in JB cattle, including genes involved in lipid metabolism (unsaturated fatty acid synthesis, fat deposition) and thyroid hormone pathway, whereas the genes up-regulated in Holstein cattle were responsible mainly for skeletal muscle contraction and energy metabolism. However, the number of identified significant genes is rather low as compared to our study (67 elements representing 24 individual genes). The reason for that could be that all hybridizations were performed on a muscle/fat microarray, with a number of probes 2-fold lower than on the microarray used in our study.

In the present study, cDNA microarrays revealed significant differences ($P < 0.05$) in expression of 393 genes between HF and LIM bulls. Table 2 includes only 117 genes with over 2-fold changes. Among them, 72 genes were up-regulated whereas 45 genes were down-regulated in LIM in comparison with HF. Classification according to their biological functions revealed 3 dominant groups of genes, involved in protein metabolism and modifications, signal transduction, or nucleoside, nucleotide and nucleic acid metabolism (Figure 2). This may suggest that differences in the metabolism of skeletal

muscle between LIM and HF bulls concern first of all protein and nucleic acid turnover and the mechanisms of their control. A higher expression of genes involved in protein metabolism and modification in muscle of LIM bulls is in concordance with better feed conversion in this breed than in HF crosses. It has been shown that LIM and Hereford bulls required less DM (dry matter), CP (crude protein), UFV (feed unit for maintenance and meat production), and PDI (protein truly digestible in the small intestine) to gain 1 kg live weight than bulls of other breeds, including HF (Dymnicki et al. 2001). Among mechanisms involved in the regulation of muscle growth and development, we focused on the function of genes implicated in somatotropic pathway. Growth hormone (GH), the best known stimulator of growth, may act on skeletal muscle directly or indirectly, through insulin-like growth factors (IGFs) of hepatic and muscle origin (Moseley et al. 1992; Florini et. al. 1996; Klover and Hennighausen 2007; Velloso 2008). For this reason, exogenous bovine somatotropin (bST) is used to stimulate growth rate in beef cattle (Velayndhan et al. 2007). Administering bST to young, light-weight HF steers increased skeletal muscle growth and protein accretion, and reduced carcass fat content, resulting in a leaner product (Schlegel et. al. 2006). These features correspond to the phenotype of LIM bulls with a large proportion of muscle, low proportion of fat, and higher proportion of lean in valuable cuts (Table 1). Growth hormone receptor (GHR) is expressed in bovine embryos, fetus and adult muscle, and bovine liver (Lucy et al. 1998; Kölle et al. 2001; Grochowska et al. 2002; Listrat et al. 2005). Our results, showing elevated GHR expression in skeletal muscle of LIM bulls (Table 2), suggest that GHR expression is closely related to muscle phenotype in cattle. This can be confirmed by another study, showing a significantly higher expression of GHR in double-muscled bovine fetuses, compared to normal ones (Listrat et al. 2005). That study and yet another one (Liu et al. 2003) indicate a relationship between GH action and expression of myostatin (MSTN) in skeletal muscle. Mutation in the *mstn* gene is accompanied by an increased expression of GHR in double-muscled bovine fetuses (Listrat et al. 2005). On the other hand, GH exerts an inhibitory effect on MSTN expression in skeletal muscle (Liu et al. 2003). In our study, the elevated expression of GHR in the LIM muscle was accompanied by a significantly lowered level of MSTN protein (Sadkowski et al. 2008). MSTN expression by GH may be also inhibited indirectly by the action of IGF-1. It has been shown that IGF-1 decreases the level of the mature 26-kDa MSTN peptide in C2C12 mouse myoblasts stimulated to differentiate (Budasz-Świderska et al. 2005).

Important genes involved in the regulation of myogenesis by GH and MSTN are zinc-finger transcription factors from the Egr (early growth response) family (Iwaki et al. 1990; Hodge et al. 1998; Tourtellotte et al. 2001; Friday et al. 2003). The Egr family consists of Egr1, Egr2, Egr3 and Egr4, which are involved in cellular growth and differentiation. Our study revealed 3-fold lower expression of Egr4 in skeletal muscle of LIM in comparison with HF bulls (Table 2). Similarly, the decreased expression of Egr1 was observed in skeletal muscle of HF bulls with lowered MSTN content, dependent on polymorphism in the 5'-flanking region of the *mstn* gene (Sadkowski et al. 2008). It has been suggested that Egr1 may inhibit myoblast differentiation by activation of Id proteins, which in turn inhibit MEF2, MyoD, and consequently myogenin (Friday et al. 2003). The calcium-dependent phosphatase calcineurin plays an important stimulatory role in myoblast differentiation by inhibition of Egr1, and thus releasing of MEF2 and MyoD from its inhibitory influence (Friday et al. 2003). EGRs may also inhibit myoblast differentiation through stimulation of TGF-beta1 and MSTN expression, which are both negative regulators of skeletal muscle growth and differentiation (Liu et al. 1999; Budasz-Świderska et al. 2005; Sadkowski et al. 2008).

The involvement of calcium signaling in the regulation of muscle growth can be confirmed by up-regulation of the inositol hexaphosphate kinase 2 (*IHPK2*) gene. The product of this gene is the enzyme involved in formation of the multiphosphate homolog of inositol triphosphate, which is a messenger molecule that releases calcium from intracellular stores. In our study, IHPK2 expression in muscle was 1.95-fold higher in LIM than in HF bulls (not shown in Table 2), which may suggest a higher activity of calcium signaling in beef cattle.

The somatotropic pathway is regulated by the insulin-like growth-factor-binding proteins (IGFBPs), which can potentiate or inhibit IGF action by modulation of their bioavailability to receptors. There are reports suggesting that IGFBP3 and IGFBP5 may differ in their effect on skeletal muscle myogenesis. It has been shown that IGFBP3 support muscle development by the switch between myoblast proliferation and differentiation (Foulstone et al. 2003). On the other hand, overexpression of IGFBP5 results in increased neonatal mortality, whole-body growth

inhibition, and retarded muscle development (Salih et al. 2004). Our study revealed up-regulation of IGFBP3 (1.60-fold change) and down-regulation of IGFBP5 (1.34-fold change) in LIM muscle in comparison with HF (data not shown in Table 2). Thus, the higher IGFBP3/IGFBP5 ratio in LIM muscle may facilitate muscle differentiation in this breed.

Another important gene affected by the action of somatotropic pathway in skeletal muscle is glycogen synthase kinase 3 beta (GSK3β). This gene was significantly down-regulated (2.16-fold) in LIM as compared to HF muscle (Table 2, position 86). GSK3b is a distinct substrate of Akt and is inhibited by Akt phosphorylation (Glass 2005). Expression of a dominant-negative, kinase-inactive form of GSK3b and pharmacological inhibition of GSK3b induce dramatic hypertrophy in skeletal myotubes (Rommel et al. 2001; Vyas et al. 2002). It has been suggested that PI3K/Akt-pathway-dependent GSK3b inhibition may induce differentiation and hypertrophy, by stimulating protein synthesis independent of the mTOR pathway. The above effect is presumably associated with activation of the Wnt pathway that inhibits GSK3b (Glass 2005).

In our study, among up-regulated genes in LIM, the highest fold change concerns ornithine decarboxylase (ODC) (Table 2), which is a key and rate-limiting enzyme in polyamine biosynthesis. There are some pieces of evidence that hormones (GH, prolactin, insulin, glucocorticoids) and growth factors (EGF, IGF-1, IGF-2, PDGF, TGF-alpha) induce ODC in various types of cells, including myogenic cells (Blachowski et al. 1994; Borland et al. 1994; Gritli-Linde et al. 1997; Płoszaj et al. 2000). Polyamines are indispensable for cell proliferation, differentiation, and maturation. They also protect cells against apoptosis (Płoszaj et al. 2000). The antiproliferative and apoptotic effect of TGF-beta1 is associated with inhibition of ODC (Motyl et al. 1993; Grzelkowska et al. 1995; Motyl et al. 1996). Also growth inhibition of L6 myoblasts by orotic acid occurs with a significant reduction of ODC activity (Grzelkowska et al. 1993). Experiments with constitutively elevated levels of circulating GH in elderly transgenic mice, overexpressing bovine GH, revealed a high activity of ODC and polyamine levels in the examined tissues (Gritli-Line et al. 1997). A very high expression of ODC in LIM muscle, as compared to HF bulls, may indicate the significance of polyamine biosynthesis in somatotropic regulation of skeletal muscle development in beef cattle.

As a conclusion, we propose a model of the regulation of skeletal muscle growth and differentiation with the principal role of the somatotropic pathway (Figure 4). This model includes proteins whose genes were identified in our study (ODC, GSK3b, IGFBP3, IGFBP5, Egr4, GHR, calcineurin) and connects them with known pathways involved in muscle growth and differentiation. It may explain at least in part the development of muscle phenotype in LIM bulls. We assume that GH directly or indirectly (through IGF-1) activates the calcium-signaling pathway with calcineurin, which stimulates MRFs and inhibits EGRs. Inhibition of EGRs results in indirect activation of MRFs and impaired activation of TGF-beta1 and MSTN, which finally facilitates terminal muscle differentiation.

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