J Appl Genet 49(3), 2008, pp. 237–250

Original article

Gene expression profiling in skeletal muscle of Holstein-Friesian bulls with single-nucleotide polymorphism in the myostatin gene 5'-flanking region

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Abstract. Myostatin (GDF-8) is a key protein responsible for skeletal muscle growth and development, thus mutations in the *mstn* gene can have major economic and breeding consequences. The aim of the present study was to investigate myostatin gene expression and transcriptional profile in skeletal muscle of Holstein-Friesian (Black-and-White) bulls carrying a polymorphism in the 5'-flanking region of the *mstn* gene (G/C transversion at position -7828). Real-time qRT-PCR and cDNA microarray revealed significantly lower *mstn* expression in muscle of bulls with the CC genotype, as compared to GG and GC genotypes. The direct comparison of skeletal muscle transcriptional profiles between the CC genotype and GG and GC genotypes resulted in identification of genes, of which at least some can be putative targets for myostatin. Using cDNA microarray, we identified 43 common genes (including *mstn*) with significantly different expression in skeletal muscle of bulls with the CC genotype, as compared to GG and GC genotypes, 15 of which were upregulated and 28 were downregulated in the CC genotype. Classification of molecular function of differentially expressed genes revealed the highest number of genes involved in the expression of cytoskeleton proteins (9), extracellular matrix proteins (4), nucleic acid-binding proteins (4), calcium-binding proteins (4), and transcription factors (4). The biological functions of the largest number of genes involved: protein metabolism and modification (10), signal transduction (10), cell structure (8), and developmental processes (8). The main identified signaling pathways were: Wnt (4), chemokines and cytokines (4), integrin (4), nicotine receptor for acetylocholine (3), TGF-beta (2), and cytoskeleton regulation by Rho GTPase (2). We identified previously unrecognized putatively myostatin-dependent genes, encoding transcription factors (EGR1, Nf1b, ILF1), components of the proteasomal complex (PSMB7, PSMD13) and proteins with some other molecular function in skeletal muscle (ITGB1BP3, Pla2g1b, ISYNA1, TNFAIP6, MST1, TNNT1, CALB3, CACYBP, and CTNNA1).

Keywords: cattle, DNA microarray, myostatin, polymorphism, skeletal muscle, transcriptome.

Introduction

Myostatin (GDF-8) is a key protein responsible for skeletal muscle growth and development. As a negative regulator, it inhibits proliferation and terminal differentiation of myogenic cells, thus limiting the final number of muscle cells and myofibers at birth (Oksbjerg et al. 2004).

In cattle, myostatin is considered to be one of the most important factors responsible for meat productivity traits. It is mainly based on the fact that hypermuscularity in Belgian Blue cattle (the so-called double-muscle phenotype) is a result of nt821(del11) mutation in exon 3 in the *mstn* gene (Grobet et al. 1997; McPherron and Lee 1997). Double-muscled animals had, on average, a 20% higher skeletal muscle mass, as compared with normal cattle. The mutation mentioned above is a deletion of 11 nucleotides, resulting in a shift of the reading frame and appearance of the stop

Received: April 4, 2008. Revised: June 25, 2008. Accepted: July 3, 2008.
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codon, UGA. The product of the mutated gene is a biologically inactive, 273-amino-acid (aa) peptide. This mutation was also identified in other cattle breeds: Parthenaise, Asturiana, Rubia Gallega, and Polish Red cattle; however, in Polish Red the double-muscle phenotype has never been found (Klauzińska et al. 2000).

Since the identification of the key role of myostatin in skeletal muscle growth and development, the possible consequences of other polymorphisms in *mstn* have been intensively investigated. At present, 9 other mutations in exons of the *mstn* gene are known, among which 6 are destructive mutations resulting in extreme forms of muscle hypertrophy, and the remaining 3 are missense mutations (Kambadur et al. 1997; Grobet et al. 1998; Dunner et al. 2003; Marchitelli et al. 2003). These mutations were found in different cattle breeds, but they do not occur in the Holstein-Friesian (HF) cattle. There is evidence of *mstn* mutations causing hypertrophy not only in cattle, but also in the mouse and dog (McPherron et al. 1997; Szabo et al. 1998; Mosher et al. 2007). Of special interest is the single-nucleotide polymorphism in intron 1 of *mstn* described by Schuelke et al. (2004), resulting in gross muscle hypertrophy in a child. Those authors for the first time described the possibility of single-nucleotide polymorphism (g.IVS1+5 G→A mutation) in an intron of the *mstn* gene, causing gross changes in skeletal muscle mass and strength. Another important mutation is G to A transition in the 3'-UTR of *mstn* in Telex sheep, which creates a target site for mir1 and mir206, microRNAs (miRNAs) that are highly expressed in skeletal muscle. This causes translational inhibition of the myostatin gene and hence contributes to the muscular hypertrophy of Texel sheep (Clop et al. 2006). Recently the same research team has described 2 single-nucleotide polymorphisms in the *mstn*, having significant association with muscle depth of commercial Charollais sheep (Hadjipavlou et al. 2008).

The separate groups of *mstn* polymorphisms are mutations in the 5'-flanking region of this gene. Changes in this region can increase or decrease *mstn* gene expression in skeletal muscles, affecting muscle growth and development. Surprisingly, only a few mutations in this region have been reported, having a very low frequency. Jeanplong et al. (2000) described mutation BTAFJ in the 5'-flanking region of the *mstn* gene, which is probably a region acting as a transcription silencer. Crisa et al. (2003) described 2 polymorphisms in the bovine *mstn* gene 5'-flanking region: transversions T/A at position -371 and G/C at -805 (in relation to codon ATG). However, the influence of these mutations on *mstn* expression in the muscle has not been studied. Lu et al. (2007) found 6 mutations in the 5'-regulatory region of the myostatin gene in Yorkshire pigs, but only 2 of them (located at –435 and –447 relative to ATG) were postulated to have real influence on early growth traits. The –447 site is very close to the E9 box (–441), which is considered to be an important element in the regulation of myostatin expression.

Finally, we described a polymorphism in the 5'-flanking region of the bovine *mstn* gene – the G/C transversion at position –7828 (relative to ATG) (Jank et al. 2006). The most frequent genotype was GC (43.6%), followed by CC (34.2%) and GG (22.2%). This polymorphism appeared functional, since it influenced the expression of myostatin in bovine muscles (Jank et al. 2006). The concentration of the active form of myostatin (26-kDa) in *semitendinosus* muscle of CC homozygous bulls was the lowest, whereas expression of this cytokine in GC heterozygotes was the highest. In the present study we used real-time qRT-PCR and DNA microarrays to investigate the differences in *mstn* expression and transcriptional profile in skeletal muscle of 12-month-old HF bulls with different variants of the *mstn* gene 5'-flanking region. Since myostatin plays a crucial role for muscle differentiation and development during embryogenesis as well as in postnatal life, such an approach could help in identifying myostatin-dependent downstream regulated genes.

Materials and methods

Animals and sampling

The animals used for this study were 12-month-old HF (Black-and-White) bulls. They were born in herds yielding 6000–7000 kg of milk in lactation. The bulls were housed in tie-stalls, and fed silage, hay and concentrate *ad libitum* up to the age of 12 months. All bulls were then slaughtered after 24-hour fasting. Basing on the methodology described in our previous work (Jank et al. 2006), we identified animals with genotypes CC, GG and GC of the above-mentioned polymorphism. Samples for RNA isolation were taken from 3 bulls of every genotype (a total of 9 unrelated bulls were sampled), but from each animal were took 2 independent samples (from separate portions of *m. semitendinosus*). Sampling of larger groups of animals was limited by the rare occurrence of the CC genotype (we were able to identify only 3 CC bulls

among those slaughtered for the purpose of the present experiment). Samples of *semitendinosus* muscle were taken immediately after slaughtering, flash-frozen in liquid nitrogen, and kept in deep freeze (at –80°C) until RNA isolation. All of the procedures carried out with the use of 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 β-actin and GAPDH probes, as well as 241 negative controls containing only binding buffer (3xSSC) and 384 empty spots. The total number of spots printed is 19 200, and they are organized in 48 subarrays, each with 20×20 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 (compared genotype pairs CC-GC, CC-GG, GG-GC) in one tube and 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 genes used will be explained with the following notation, in which 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 $y_i \in \{c_1, c_2\}$ 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 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 $j = 1,\dots d$), 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 small value (e.g. below the 0.05 threshold) 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 $j = 1,\dots d$), 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 expression of the fold difference is given by the term $|\log \mu_1 - \log \mu_2|$, 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 qRT-PCR

Expression of the myostatin gene was checked by qRT-PCR with the following primers: GDF8-F 5'-GGT ATT TGG CAG AGC ATT GAT-3' and GDF8-R 5'-ATC TAC TAC CAT GGC TGG AAT-3' (Oldham et al. 2001), as well as 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 a 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 at 57°C for 10 s, 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^{-1}); and cooling step at 40°C for 30 s. For *gapdh*, annealing was at 57°C. Results are presented as the ratio of *mstn* to *gapdh* expression.

Results

Gene expression in *semitendinosus* muscle of bulls with various *mstn* genotypes

Analysis of *mstn* expression with the use of real-time qRT-PCR technique (Figure 1) revealed a significantly lower relative expression of this gene in bulls with the CC genotype (0.041±0.001), as compared to GG and GC genotypes (0.048±0.0005 and 0.048±0.002, respectively; $P < 0.05$). The expression of *mstn* in bulls with GG and GC genotypes did not differ. When *mstn* expression was evaluated by using values obtained from a microarray study (Table 1, position 29), the differences for genotype pairs CC-GG and CC-GC reached 52% and 40%, respectively. These results are in accordance with the real-time qRT-PCR results, namely bulls with the CC genotype have the lowest expression of *mstn*, in comparison with GG and GC genotypes. The expression of *gapdh* did not differ between genotypes (data not shown).

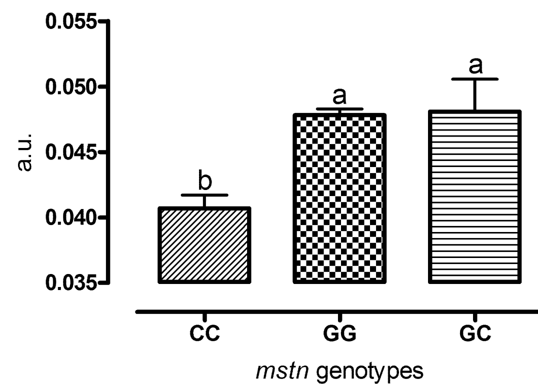

Figure 1. Relative expression of *mstn* in *semitendinosus* muscle of Holstein-Friesian (Black-and-White) bulls with various genotypes at G/C substitution at position -7828 in the 5'-flanking region of the *mstn* gene. The data presented (in arbitrary units ± SEM) were obtained from quantitative real-time qRT-PCR analysis, as a result of dividing *mstn* expression by *gapdh* expression. Bars with different superscripts differ significantly ($P < 0.05$); n = 9 (3 animals × 3 muscle samples).

Differences in gene expression in *semitendinosus* muscle of bulls with various *mstn* genotypes

In order to search for putative myostatin target genes in the bovine skeletal muscle, we compared the transcriptional profile for the CC genotype (of decreased *mstn* expression) with those for GG and GC genotypes. The comparison revealed 43 genes, which significantly ($P < 0.05$) differed between CC and the other genotypes (GG and GC) (Table 1). Expression of these genes did not significantly differ between GG and GC genotypes. The group of genes identified consisted of 15 genes upregulated in CC and 28 downregulated in CC animals.

Hierarchical clustering of identified genes in GENESIS software revealed a clear clustering in

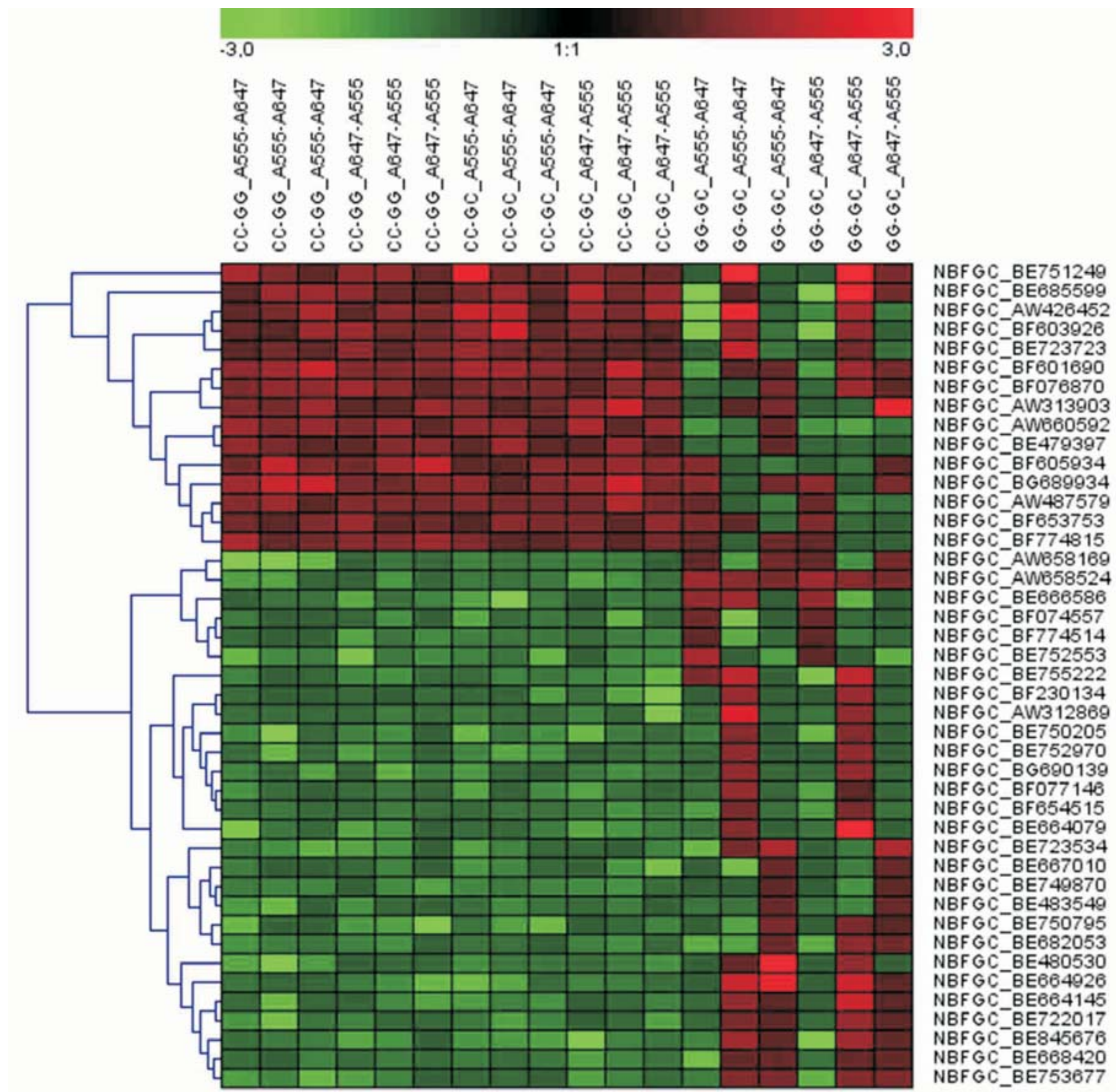

Figure 2. Hierarchical clustering of identified genes, which revealed a clear clustering of replicate experiments (comparison of genotypes CC-GG and CC-GC) and their dye swaps

replicate experiments (comparison of genotype pairs CC-GG and CC-GC) and their dye swaps (Figure 2). There was no repeatability of differences in gene expression in individual comparisons between GG and GC genotypes. In that case, only the repeatability of dye swaps was observed.

Using *Panther* software, we evaluated molecular functions in biological processes and metabolic pathways of genes identified in this study. Classification according to molecular function revealed the largest group of genes coding for cytoskeletal proteins (TCAP, MYH3, MYH8, MYO5A, Myl6, Mlc1a, CTNNA1, TNNT1, KTN1). The other identified groups consisted of genes encoding extracellular matrix proteins (TNFAIP6, Mfap4, COL1A2, COL6A1, COL6A3), nucleic acid-binding proteins (MYOG, EGR1, Nfib, ILF1), calcium-binding proteins (MST1, Myl6, CALB3, Mlc1a), transcription factors (MYOG, EGR1, Nfib, ILF1), receptor proteins (PAQR7, CD5, PTPD), and proteases (PSMB7, MST1, USP21) (Figure 3).

In respect of the biological function of the gene products, the largest number of genes were involved in protein metabolism and modification (10) (Figure 4). These included 5 genes whose products were involved in proteolysis (PSMB7, PSMD13, USP21, CACYBP, ITGA2B), 4 in protein modification (CSNK1G2, MAPKAP1, DAPK1, PTPD), and 1 in protein complex formation (TCAP). A large group was also formed by genes whose products were involved in signal transduction (CSNK1G2, TNFAIP6, Pla2g1b, PAQR7, Mfap4, MST1, CD5, COL6A1,

Table 1. List of genes overlapping in a comparison of muscle transcriptional profiles for genotype pairs CC-CG and CC-GG of the *mstn* gene in 12-month-old Holstein-Friesian bulls; n = 6 (3 animals × 2 muscle samples).

No.	MSU gene ID	Gene name (GenBank Accession Number)	E value[1]	Fold change[2]	P value[3]
1	2	3	4	5	6
1	NBFGC_BF601690	troponin T type 1 (skeletal, slow) [Bos taurus] (TNNT1) (NP_776899.1)	9.00E-55	1.77	0.01041
2	NBFGC_BF603926	integrin beta 1 binding protein 3 [Bos taurus] (ITGB1BP3) (NP_001073111.1)	1.00E-100	1.76	0.01041
3	NBFGC_BE751249	collagen, type I, alpha 2 [Bos taurus] (COL1A2) (NP_776945.1)	1.00E-04	1.60	0.01041
4	NBFGC_BG689934	PREDICTED: similar to collagen, type VI, alpha 1 isoform 1 [Bos taurus] (COL6A1) (XP_588755.3)	5.00E-52	1.54	0.01041
5	NBFGC_AW660592	PREDICTED: similar to casein kinase I gamma 2 [Bos taurus] (CSNK1G2) (XP_582453.3)	0.00E+00	1.51	0.01631
6	NBFGC_AW426452	PREDICTED: similar to myosin, heavy chain 3, skeletal muscle, embryonic isoform 1 [Bos taurus] (MYH3) (XP_871374.2)	4.00E-64	1.43	0.01631
7	NBFGC_BF605934	PREDICTED: similar to alpha 3 type VI collagen isoform 5 precursor isoform 1, partial [Bos taurus] (COL6A3) (XP_609132.3)	4.00E-74	1.37	0.01631
8	NBFGC_BF076870	death-associated protein kinase 3 [Bos taurus] (DAPK1) (NP_001094594.1)	7.00E-65	1.37	0.02472
9	NBFGC_BF774815	cyclin-dependent kinase 2-interacting protein [Bos taurus] (CINP) (NP_001092595.1)	1.00E-114	1.34	0.02497
10	NBFGC_BE479397	PREDICTED: similar to interleukin enhancer binding factor 1 [Bos taurus] (ILF1) (XP_610873.3)	6.00E-92	1.33	0.02497
11	NBFGC_BE685599	myogenin (myogenic factor 4) [Bos taurus] (MYOG) (NP_001104795)	3.00E-98	1.31	0.02497
12	NBFGC_BE723723	myosin, light chain 4, alkali; atrial, embryonic [Bos taurus] (Mlc1a) (NP_001068617.1)	7.00E-75	1.28	0.02497
13	NBFGC_AW313903	calbindin 3 (vitamin D-dependent calcium-binding protein) [Bos taurus] (CALB3) (NP_776682.1)	3.00E-30	1.24	0.02497
14	NBFGC_AW487579	catenin, alpha [Bos taurus] (CTNNA1) (NP_001073106.1)	4.00E-35	1.24	0.03605
15	NBFGC_BF653753	kinectin 1 [Bos taurus] (KTN1) (XP_617792.3)	2.00E-18	1.22	0.03704
16	NBFGC_BE750795	phospholipase A2, group IB [Bos taurus] (Pla2g1b) (NP_777071.1)	5.00E-65	–1.78	0.00395
17	NBFGC_BE750205	progestin and adipoQ receptor family member VII [Bos taurus] (PAQR7) (NP_001033642.1)	1.00E-104	–1.75	0.00395
18	NBFGC_BE752553	PREDICTED: similar to developmental myosin heavy chain neonatal isoform 2 [Bos taurus] (MYH8) (XP_870972.1)	0.00E+00	–1.64	0.00395
19	NBFGC_BE480530	early growth response 1 [Bos taurus] (Egr1) (NP_001039340.1)	3.00E-27	–1.61	0.00395
20	NBFGC_BE845676	nuclear factor I/B [Bos taurus] (Nfib) (NP_001069572.1)	9.00E-54	–1.58	0.00395
21	NBFGC_BE722017	ubiquitin specific peptidase 21 [Bos taurus] (USP21) (NP_001039841.1)	2.00E-23	–1.57	0.00639
22	NBFGC_BE666586	tumor necrosis factor, alpha-induced protein 6 [Bos taurus] (TNFAIP6) (NP_001007814.1)	1.00E-153	–1.55	0.00649
23	NBFGC_AW658169	proteasome beta 7 subunit [Bos taurus] (PSMB7) (NP_001033616.1)	1.00E-132	–1.55	0.00649
24	NBFGC_BE664079	PREDICTED: similar to protein tyrosine phosphatase delta isoform 23 [Bos taurus] (PTPD) (XP_881620.2)	7.00E-19	–1.53	0.00649
25	NBFGC_BE664145	calcyclin-binding protein [Bos taurus] (CACYBP) (NP_001029981.1)	3.00E-38	–1.53	0.00649
26	NBFGC_BE752970	CD5 antigen (p56-62) [Bos taurus] (NP_776324.1)	1.00E-34	–1.53	0.00649
27	NBFGC_AW658524	fasting-induced protein [Bos taurus] (DEPP) (NP_001039980.1)	9.00E-48	–1.51	0.00649
28	NBFGC_BE664926	proteasome 26S non-ATPase subunit 13 [Bos taurus] (PSMD13) (NP_001019703.1)	5.00E-21	–1.50	0.01041
29	NBFGC_BE749870	myostatin [Bos taurus] (GDF8) (BC134563.1)	0.00E+00	–1.46	0.01041
30	NBFGC_BF077146	myoinositol 1-phosphate synthase A1 [Bos taurus] (ISYNA1) (NP_001039497.1)	0.00E+00	–1.46	0.01041
31	NBFGC_BF774514	follistatin [Bos taurus] (FST) (NP_786995.2)	0.00E+00	–1.42	0.01041
32	NBFGC_BE753677	myosin-binding protein H [Bos taurus] (MyBP-H) (NP_001068903.1)	9.00E-18	–1.38	0.02497

Table 1 cont.

1	2	3	4	5	6
33	NBFGC_BE667010	integrin alpha 2b [Bos taurus] (ITGA2B) (NP_001014929.1)	1.00E-152	–1.37	0.02497
34	NBFGC_BF074557	PREDICTED: similar to myosin Va, partial [Bos taurus] (MYO5A) (XP_615219.3)	3.00E-06	–1.37	0.02497
35	NBFGC_BE682053	myosin, light chain 6, alkali, smooth muscle and non-muscle [Bos taurus] (Myl6) (NP_786974.1)	2.00E-84	–1.36	0.02497
36	NBFGC_BE723534	macrophage stimulating 1 (hepatocyte growth factor-like) [Bos taurus] (MST1) (NP_001069145.1)	1.00E-132	–1.35	0.02497
37	NBFGC_BG690139	casein alpha-S2 [Bos taurus] (csn1s2) (NP_776953.1)	2.00E-96	–1.34	0.02497
38	NBFGC_BE483549	microfibrillar-associated protein 4 [Bos taurus] (Mfap4) (NP_001073686.1)	1.00E-51	–1.29	0.02497
39	NBFGC_BE755222	telethonin (titin-cap) [Bos taurus] (TCAP) (NP_001014915.1)	3.00E-84	–1.25	0.03605
40	NBFGC_BE668420	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor) [Bos taurus] (ITGA4) (NP_777173.1)	0.00E+00	–1.20	0.03605
41	NBFGC_BF230134	mitogen-activated protein kinase-associated protein 1 [Bos taurus] (MAPKAP1) (NP_001075072.1)	1.00E-107	–1.20	0.03704
42	NBFGC_BF654515	hsp70-interacting protein [Bos taurus] (CHIP) (NP_001039596.1)	1.00E-23	–1.19	0.03737
43	NBFGC_AW312869	apoptosis-inducing factor, mitochondrion-associated, 2 [Bos taurus] (AIFM2) (NP_001035646.1)	7.00E-22	–1.19	0.03737

[1] *E* value = expectation value, which indicates the probability of generation of an identical score for the sequence chosen (presumed to be the basis of query identification), resulting from random matching of our query with other sequences available in the GenBank. The lower *E* value (closer or equal to 0, reflecting the lower probability of random score generation), the higher reliability of the BLAST score, and sequence identification is closer to the bone.

[2] Fold change = value describing the average level of increase or decrease in gene expression in the CC genotype, as compared to GG and GC genotypes. Positive and negative values correspond to upregulation and downregulation of gene expression in genotype CC with lowered *mstn* expression, respectively.

[3] We rejected the null hypothesis if $P \leq 0.05$ (Wilcoxon non-parametric rank test).

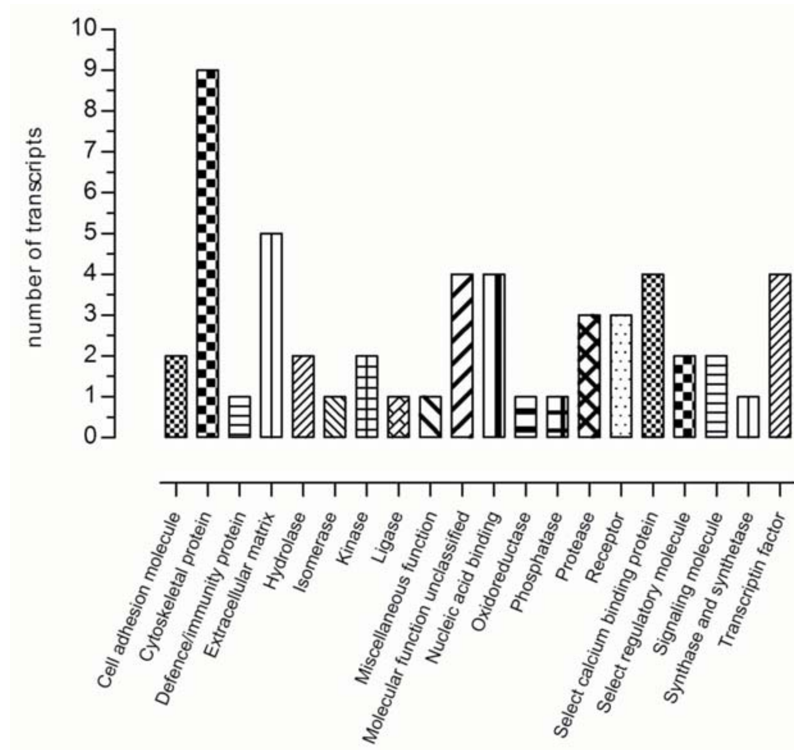

Figure 3. Molecular function of genes overlapping in a comparison of muscle transcriptional profiles for genotype pairs CC-CG and CC-GG of the *mstn* gene; n = 6 (3 animals × 2 muscle samples), evaluated with Panther software.

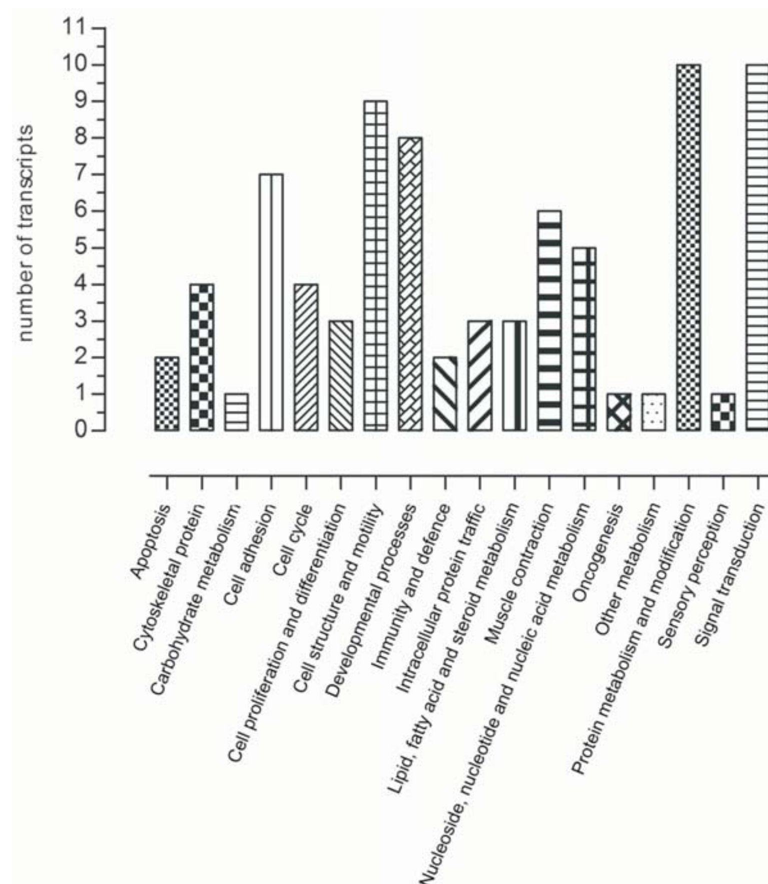

Figure 4. Biological role of genes overlapping in a comparison of muscle transcriptional profiles for genotype pairs CC-CG and CC-GG of the *mstn* gene; n = 6 (3 animals × 2 muscle samples), evaluated with Panther software

COL6A3, PTPD). Products of the remaining identified genes were involved in cell structure (TCAP, CSNK1G2, CTNNA1, KTN1, COL1A2, COL6A1, COL6A3, ILF1, PTPD), developmental processes (MYOG, MYH3, MYH8, GDF8, CSNK1G2, CD5, ILF1, PTPD), adhesion (ITGA2B, ITGA4, Mfap4, COL1A2, COL6A1, COL6A3, PTPD), contractile activity (MYH3, MYH8, Mlc1a, Myl6, MyBP-H, TNNT1) and nucleoside, nucleotide and nucleic acid metabolism (CSNK1G2, MYOG, EGR1, Nfib, ILF1) (Figure 5). Among the genes involved in signal transduction, the largest group was represented by 6 genes encoding proteins with a function in cell communication (Mfap4, MST1, CD5, COL6A1, COL6A3, PTPD). In the case of developmental processes, the dominant category were the genes whose products were involved in mesoderm development (MYH3, MYH8, GDF8, MYOG, CD5, ILF1).

The analysis of the role of the identified genes in various pathways revealed that the largest number of gene products were involved in the Wnt signaling pathway (MYH3, MYH8, CSNK1G2, CTNNA1), integrin signaling pathway (ITGA2B, ITGA4, COL6A1, COL6A3), inflammatory chemokines and the cytokines pathway (MYH3, MYH8, ITGA4, COL6A3), Parkinson disease pathway (PSMB7, PSMD13, CSNK1G2, CHIP), nicotine receptor for acetylocholine pathway (MYH3, MYH8, MYO5A), TGF-beta pathway (GDF8, ILF1), and cytoskeleton regulation by Rho GTPase (MYH3, MYH8) (Figure 5).

To localize the products of gene expression on metabolic pathways in skeletal muscle fibers, we used Pathway Architect software (Stratagene, USA). A result of this analysis is the a network describing the relations between myostatin (cyan color), products of the identified genes that can be putative myostatin targets (yellow) and the gene products with commonly known functions within the cell (red) (Figure 6). Except the well-known direct inhibitory effect of myostatin on myogenin and Myf5 or binding of intracellular myostatin by follistatin and telethonin, it was also possible to show other genes that can be indirectly myostatin-dependent, i.e. various types and isoforms of collagen, cytoskeleton proteins, cal-

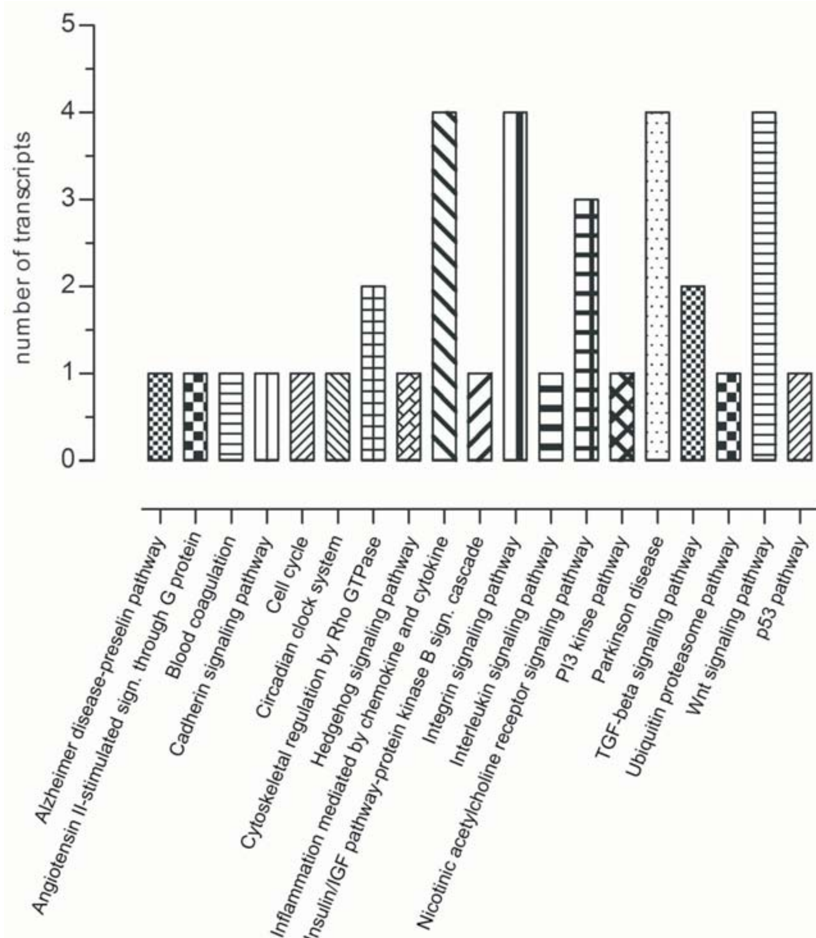

Figure 5. Signaling pathways of genes overlapping in a comparison of muscle transcriptional profiles for genotype pairs CC-CG and CC-GG of the *mstn* gene; n = 6 (3 animals × 2 muscle samples), evaluated with Panther software.

Figure 6. Network of mutual interactions in bovine skeletal muscle between myostatin (cyan), products of the identified genes that can be putative myostatin targets (yellow), and products of other genes described in literature (red). The analysis was performed by using Pathway Architect software.

cium-binding proteins, and proteins involved in muscle contractility as well as integrins.

Differences between genotypes concerned also genes that have not been described earlier as putative targets for myostatin, encoding early growth response 1 protein (EGR1), nuclear factor I/B (Nf1b), TNF-α-inducting protein (TNFAIP6), macrophage stimulating factor 1 (MST1), T-type troponin 1 (TNNT1), calbindin 3 (CALB3), calcyclin-binding protein (CACYBP), integrin-beta-1-binding protein 3 (ITGB1BP3), phospholipase A2 (Pla2g1b), myoinositol phosphate syntase A1 (ISYNA1), alpha-catenin (CTNNA1), and proteasome complex proteins (PSMB7 and PSMB13) (Table 1).

Discussion

There is an increasing number of studies on the growth and metabolism of skeletal muscle in cattle, where this method was applied. Among them, there are studies concerning age-dependent changes in the bovine skeletal muscle transcriptome (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 a skeletal 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). However, there is a limited number of data on myostatin-related transcriptional profiling in a bovine muscle. They concern the effect of double-muscled phenotype, i.e. mutation nt821 (del 11) on gene expression pattern in bovine embryos (Potts et al. 2003) and fetuses (Cassar-Malek et al. 2007). The present study is the first one where cDNA microarrays were used for analysis of bovine skeletal muscle transcriptome in relation to polymorphism in the 5'–flanking region of the *mstn* gene.

Results of the present study show that the examined polymorphism in the 5'-flanking region of the *mstn* gene is associated with significant differences in myostatin expression at the level of mRNA in skeletal muscle of bulls (Figure 1;

Table 1, position 29). It is consistent with our previous observation, showing the lowest level of mature myostatin protein (26 kDa) in homozygote CC in comparison with other *mstn* genotypes: GC and GG in *semitendinosus* muscle of HF (Black-and-White) bulls (Jank et. al. 2006). However, we cannot exclude that another polymorphism, tightly linked to the analyzed G/C polymorphism, is responsible for the observed effects.

It has been commonly accepted that the lack of myostatin protein or its nonfunctional structure strongly affects skeletal muscle phenotype by hyperplasia and hypertrophy of muscle fibers, which was shown in humans and other animal species (Joulia-Ekaza and Cabello 2007). For this reason, we can assume that *mstn* CC genotype, responsible for low *mstn* expression, may facilitate muscle growth in bulls. It should be pointed out that, as we found, the *mstn* CC genotype is the only one detected in Limousine cattle, which is a typical beef breed (not shown). On the other hand, it should be noted that the frequency of the CC genotype in HF cattle, which is a dairy breed, amounts to 34.2% (Jank el al. 2006). Again we cannot exclude that another polymorphism, tightly linked to the analyzed G/C polymorphism, is responsible for the observed effects. The predominance of the CC genotype in Limousine may suggest that the investigated *mstn* polymorphism is of importance for the skeletal muscle features in this beef cattle breed. In skeletal muscle of Limousine bulls the lowest level of active myostatin protein (26 kDa) and its precursor were also observed, when compared to Hereford and especially HF bulls (Sadkowski 2008). Since this observation concerned only the CC genotype in those cattle breeds, it can be concluded that beside the described polymorphism in the *mstn* gene, the expression of *mstn* is influenced by other breed-specific factors. Since the described polymorphism is located quite far in the 5'-flanking region of *mstn*, it is difficult to interpret the molecular mechanism responsible for *mstn* regulation.

The direct comparison of skeletal muscle transcriptional profiles between low *mstn* expression in the CC genotype and both GG and GC genotypes resulted in identification of genes that can be considered as putative targets of myostatin. We identified 43 common genes (including *mstn*) with significantly different expression in skeletal muscle of bulls with the CC genotype, as compared to GG and GC genotypes (Table 1), 15 of which were upregulated and 28 were downregulated in the CC genotype. Classification of molecular functions of identified genes revealed that the highest number of genes was involved in the expression of cytoskeleton proteins (9), then extracellular matrix proteins (4), nucleic acid-binding proteins (4), calcium-binding proteins (4), and transcription factors (4) (Figure 3). The biological functions of the largest number of genes involved: protein metabolism and modification (10) and signal transduction (10), cell structure and motility (8), and developmental processes (8) (Figure 4). The main identified signaling pathways were: Wnt (4), chemokines and cytokines (4), integrin (4), nicotine receptor for acetylocholine (3), TGF-beta (2), and cytoskeleton regulation by Rho GTPase (2) (Figure 5).

By placing the myostatin and differentially expressed genes in the network of relationship with others described in the literature, we were able to show direct and indirect relationship between *mstn* and the genes that can be considered as putative targets for myostatin. One of genes known to be directly inhibited by myostatin is myogenin, which was upregulated in bulls with lower *mstn* expression (Table 1). This effect is in accordance with the earlier described inhibitory effect of myostatin on the expression of myogenic regulatory factors (MRFs), including myogenin (Joulia et al. 2003; Joulia-Ekaza and Cabello 2007). Our results also indicate that expression of follistatin and telethonin (myostatin-binding proteins) is upregulated at higher *mstn* expression (Table 1), which may suggest the presence of a mechanism regulating the level of active myostatin within the cell. The expression of *mstn* and activity of myostatin protein are precisely regulated in the organism by many different factors, i.e. hormones and transcription factors (Budasz-Świderska et al. 2005), cytokines, transcription factors SMAD and FoxO (Allen and Unterman 2007), and auto-processing (McFarlane et al. 2005). Moreover, the activity of mature myostatin is regulated by its binding with intracellular (hSGT, titin-cap, follistatin) and extracellular (myostatin propeptide, GASP1, FRLG) binding proteins (Joulia-Ekaza and Cabello 2007).

We have to point out that some of the differentially expressed genes identified by us were previously described by Cassar-Malek et al. (2007), although those authors performed their study on muscle from bovine fetuses with the double-muscle phenotype, whereas our study was performed on 12-month-old bulls. In both studies the genes classified as putative targets of myostatin

turned out to be the genes coding for various types and isoforms of collagen, proteins of the troponin complex, myosin light and heavy chains, ubiquitin-dependent proteases, or genes involved in integrin signaling (Table 1).

We also identified some previously unrecognized putatively myostatin-dependent genes, encoding transcription factors: early growth response 1 (EGR1), nuclear factor I/B (Nf1b), interleukin enhancer-binding factor 1 (ILF1); or coding for proteins of the proteasomal complex: proteasome subunit beta 7 (PSMB7) and proteasome non-ATPase subunit 26S (PSMD13). The remaining genes related to myostatin expression level encode integrin-β-1-binding protein 3 (ITGB1BP3), phospholipase A2 (Pla2g1b), myoinositol phosphate synthase A1 (ISYNA1), TNF-α-inducing protein 6 (TNFAIP6), macrophage stimulating factor 1 (MST1), T-type troponin 1 (TNNT1), calbindin 3 (CALB3), calcyclin-binding protein (CACYBP), and alpha-catenin (CTNNA1). Among them, particularly interesting seems to be the gene of early growth response 1 (*egr1*), whose protein product regulates the expression of *tgf-beta1* (Liu et al. 1996). The mechanism of growth regulation by EGR1 is based on the coordinated TGF-beta 1 and fibronectin (FN1) regulation through direct binding and stimulation of their promoters (Liu et al. 1999). Protein products of these genes increase the expression of plasminogen activator inhibitor 1 (PAI1) through the TGF-beta1 pathway. FN1 and PAI1 increase cell adhesion and growth after being excreted from the cell. Other authors suggest that endothelial cell growth, angiogenesis, and tumor cell growth are closely EGR1-dependent (Fahmy et al. 2003). EGR1 plays also an important role in memory consolidation, since knock-out of *egr1* in mice resulted in impairment of long-term memory without any change in short-term memory (Bozon et al. 2003). The role of EGR1 in skeletal muscle has not been described yet. However, TGF-beta1, whose expression is regulated by EGR1, stimulates the expression of myostatin both in proliferating and in differentiating C2C12 myoblast cell culture (Budasz-Świderska et al. 2005). Knockdown of TGF-beta1 receptor II significantly decreases the level of precursor and active forms of myostatin in myoblasts and myotubes. On the other hand, it has been shown that myostatin increases TGF-beta1 expression both in proliferating and differentiating myoblasts (Budasz-Świderska et al. 2005).

Since EGR1 stimulates TGF-beta1 expression (Liu et al. 1999), it can be an important indirect link in interactions between myostatin and TGF-beta1 in the process of skeletal muscle growth regulation. Our results indicate that the low level of myostatin in the CC genotype bulls is accompanied by down-regulation of EGR1 expression (Table 1). The comparison of transcriptional profiles between skeletal muscle of a typical dairy cattle breed (HF) and typical beef cattle breed (Limousine) revealed not only a significant decrease in myostatin protein level, but also a 3-fold decrease in the expression of EGR4 (a transcription factor belonging to the same family as EGR1) in skeletal muscle of Limousine bulls (Sadkowski 2008). These data may suggest an important role of transcription factors belonging to the EGR family in the regulation of muscle growth.

Acknowledgments. This work was supported by grants no. 2PO6D03128 and PBZ-KBN-113/P06/2005 from the State Committee for Scientific Research, Warsaw.

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