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Abstract
Myostatin, a member of the transforming growth factor-beta superfamily, is a negative regulator of skeletal muscle growth. Cattle with mutations that inactivate myostatin exhibit a remarkable increase in mass of skeletal muscle called double muscling that is accompanied by an equally remarkable decrease in carcass fat. Although a mouse knockout model has been created which results in mice with a 200% increase in skeletal muscle mass, molecular mechanisms whereby myostatin regulates skeletal muscle and fat mass are not fully understood. Using suppressive subtractive hybridization, genes that were differentially expressed in double-muscled vs. normal-muscled cattle embryos were identified. Genetic variation at other loci was minimized by using embryonic samples collected from related Piedmontese x Angus dams or Belgian Blue x Hereford dams bred to a single sire of the same breed composition. Embryos were collected at 31-33 days of gestation, which is 2-4 days after high-level expression of myostatin in the developing bovine embryo. The suppressive subtraction resulted in 30 clones that were potentially differentially expressed, 19 of which were confirmed by macroarray analysis. Several of these genes have biological functions that suggest that they are directly involved in myostatin's regulation of skeletal muscle development. Furthermore, several of these genes map to quantitative trait loci known to interact with variation in the myostatin gene.

Keywords
bovine, gene expression, macroarray, myostatin

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Characterization of gene expression in double-muscled and normal-muscled bovine embryos

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Summary

Myostatin, a member of the transforming growth factor-β superfamily, is a negative regulator of skeletal muscle growth. Cattle with mutations that inactivate myostatin exhibit a remarkable increase in mass of skeletal muscle called double muscling that is accompanied by an equally remarkable decrease in carcass fat. Although a mouse knockout model has been created which results in mice with a 200% increase in skeletal muscle mass, molecular mechanisms whereby myostatin regulates skeletal muscle and fat mass are not fully understood. Using suppressive subtractive hybridization, genes that were differentially expressed in double-muscled vs. normal-muscled cattle embryos were identified. Genetic variation at other loci was minimized by using embryonic samples collected from related Piedmontese × Angus dams or Belgian Blue × Hereford dams bred to a single sire of the same breed composition. Embryos were collected at 31–33 days of gestation, which is 2–4 days after high-level expression of myostatin in the developing bovine embryo. The suppressive subtraction resulted in 30 clones that were potentially differentially expressed, 19 of which were confirmed by macroarray analysis. Several of these genes have biological functions that suggest that they are directly involved in myostatin's regulation of skeletal muscle development. Furthermore, several of these genes map to quantitative trait loci known to interact with variation in the myostatin gene.

Keywords bovine, gene expression, macroarray, myostatin.

Introduction

Culley (1807) first described the double-muscled phenotype in cattle. Increased skeletal muscle mass in double-muscled cattle, which occurs prenatally, is primarily because of hyperplasia (an increase in muscle fibre number) coupled with a small amount of hypertrophy (increased myofibre diameter) (Swatland & Kieffer 1974). Belgian Blue and Piedmontese double-muscled cattle have an ~15–30% increase in skeletal muscle mass, in addition to increased birth weight, rib eye area, feed efficiency, and improved retail product yield (Hanset 1986). However, double-muscled cattle have problems with dystocia, decreased female fertility, and lower stress tolerance (Hanset 1991). Because of these management problems, there has been limited use of double-muscled cattle in commercial beef production in the United States. Furthermore, the extremely low level of intramuscular fat deposition typically results in down grading of the meat in the current marbling-based grading systems, which relegates meat from double-muscled cattle to niche markets.

In 1997, myostatin was identified by virtue of homology to conserved regions within transforming growth factor-β (TGFB) superfamily genes (McPherron *et al.* 1997). To determine the biological function of this new TGFB superfamily member, knock-out mice were produced. Myostatin null mice have a dramatic ~200% increase in skeletal muscle mass, which is due to myofibre hyperplasia (McPherron *et al.* 1997). Myostatin must be proteolytically processed to be active, and nine cysteine residues are necessary for proper folding and homodimerization (Berry *et al.* 2002), but the molecular mechanisms whereby myostatin regulates skeletal muscle growth remains to be elucidated.

Myostatin mRNA is first detected at very low levels in 16 days bovine embryos, but at much higher levels at

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29 days of gestation (Kambadur *et al.* 1997). Myostatin expression in early embryos is primarily restricted to the myotomal compartment of the somite, which will give rise to myoblasts (Gagniere *et al.* 1999). Later in embryonic development, myostatin is expressed in all skeletal muscles and its expression is seen throughout development (Lee & McPherron 1999).

Myostatin has been reported to decrease myoblast proliferation (Thomas *et al.* 2000; Rios *et al.* 2001; Taylor *et al.* 2001). Myoblasts treated with myostatin appear to exit the cell cycle and enter into a quiescent state. In addition, myostatin inhibits terminal differentiation of myoblasts (Artaza *et al.* 2002; Rios *et al.* 2002). Furthermore, myostatin represses *MYOD1* mRNA expression, which is important for myogenic commitment (Oldham *et al.* 2001). In total, myostatin appears to have several downstream targets, whose role may be to regulate skeletal myoblast cell cycle progression and terminal differentiation.

In this study, suppressive subtractive hybridization (SSH) was used to identify genes that are differentially expressed in double-muscled vs. normal-muscled embryos. These genes may be downstream targets of myostatin and/or interact with myostatin genetic variants.

Materials and methods

Embryo collection

Two sets of dams were used to produce embryos. The first set included 21 Piedmontese × Angus cross cows that were heterozygous for the A to G transition mutation in myostatin that causes double muscling in the Piedmontese breed (Kambadur *et al.* 1997). Calves were early weaned from the dams shortly before dams were synchronized with two Prostaglandin F (PGF) injections 12 days apart. They were superovulated using FSH injections for four consecutive days, and artificially inseminated using semen from a single Piedmontese × Angus bull previously determined to be heterozygous for the myostatin mutation. Pregnant dams ($n = 16$) were slaughtered 2–4 days after the initial expression of myostatin in the developing embryo (Kambadur *et al.* 1997), on day 30, 31, or 32 post-insemination. The reproductive tract was promptly removed after death and embryos ($n = 52$) dissected from the tract and snap frozen in liquid nitrogen. Myostatin genotypes of the embryos were determined by amplification and sequencing of RT-PCR products as described (Kambadur *et al.* 1997).

The second set of dams included 29 Belgian Blue × Hereford females heterozygous for the 11 bp deletion causing myostatin inactivation in the Belgian Blue breed. Dams ($n = 29$) were synchronized and inseminated similar to the first set with semen from a single Belgian Blue × MARC III bull that was heterozygous for the myostatin inactivating mutation, but these dams were not superovulated. Pregnant dams ($n = 21$) were slaughtered on day 31 ($n = 7$), 32 ($n = 10$), or 33 ($n = 6$) post-insemination, and embryos ($n = 23$) collected as above. Embryos were genotyped for the 11 bp deletion as described (Kambadur *et al.* 1997).

RNA processing

Ribonucleic acid was extracted using the RNeasy midi kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Briefly, tissue (~150 mg) from two normal- (32 days old) and two double-muscled embryos (31 and 32 days old) was homogenized in the presence of RLT buffer (Qiagen) with a Polytron (Brinkman Instruments, Westbury, NY, USA) for 45 s at 15 000 rpm. Protein was degraded by digestion with proteinase K (20 mg/ml; Sigma, St Louis, MO, USA) at 55 °C for 20 min. The RNA sample was bound to an RNeasy midi column (Qiagen) and genomic DNA was degraded by in-column DNase1 (20 μl) digestion at room temperature for 15 min. The RNA on the column was subsequently eluted in RNase-free water (50 μl). PolyA mRNA was purified from total RNA with an Oligotex Mini Spin Column (Qiagen).

Suppressive subtractive hybridization

Forward and reverse SSH (PCR-select cDNA subtraction; ClonTech, Palo Alto, CA, USA) was completed according to the manufacturer's protocol with 1 μg mRNA as starting material. Double-stranded cDNA was digested with *Rsa*I, and adaptors were ligated to the cDNA ends. Hetero-hybrids of the tester and driver were generated by an initial hybridization of tester and driver cDNA at 68 °C. In order to further enrich for differentially expressed sequences, a second hybridization was also completed. Differentially expressed cDNA was PCR amplified with PCR reaction buffer (1×), dNTPs (10 mM), PCR primer 1 (10 mM), and Advantage cDNA polymerase mix (50×). The PCR conditions consisted of 94 °C for 5 min followed by 28 cycles of 94 °C for 30 s, 66 °C for 30 s, and 72 °C for 1.5 min. A secondary PCR amplification was completed with PCR reaction buffer (1×), nested PCR primer 1 (10 μM), nested PCR primer 2R (10 μM), dNTPs (10 mM), and Advantage cDNA polymerase mix (50×). Secondary PCR conditions included 12 or 15 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 1.5 min.

DNA isolation

The final PCR products were subcloned into pT-Advantage (ClonTech) according to the manufacturer's protocol. Clones were plated on LB agar in the presence of ampicillin (50 mg/ml). Plasmid DNA was isolated by alkaline lysis (protocol partially adapted from Marra *et al.* 1997). Inserts size was estimated by excision with *Eco*RI restriction endonuclease digestion and size separated on a 1% agarose gel.

Sequencing and analysis

A total of 58 plasmids (31 from the forward subtraction and 27 from the reverse subtraction) were sequenced with an ABI 3700 sequence analyzer (Applied Biosystems Inc., Foster City, CA, USA). Clone identification was predicted by BLAST analysis against GenBank NR database. Complementary DNA clones were randomly assigned numbers and putative gene names were given to clones with >80% homology to a known gene.

cDNA macroarray

In order to maximize the identification of genes isolated by SSH, a macroarray was generated by spotting PCR amplified cDNA onto a nylon membrane. The PCR programme included 2 min at 95 °C, 30 cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 2 min, followed with a final extension at 72 °C for 5 min.

Amplified cDNA clones were manually spotted in duplicate onto membranes (BioRad, Hercules, CA, USA) using hand-held multiprint equipment (V & P Scientific, San Diego, CA, USA). The cDNA on the blot was denatured with 0.5 N NaOH/3 M NaCl solution for 5 min, neutralized with 6× SSC solution for 5 min, and cross-linked (120 000 μJ) to the blot with a UV Stratalinker 2400 (Stratagene, La Jolla, CA, USA).

Radiolabelled target was generated using a mixture of PCR amplified cDNA from the 58 clones that had been sequenced (50 ng/μl) in order to insure that all unique clones had been identified or from cDNA generated from homozygous wild-type and homozygous double-muscled embryos. The target mixture included 10× reaction buffer (1×), random primers (50 μM) (Ambion, Austin, TX, USA), α^{32}P dCTP (15 μCi), and exo-Klenow enzyme (0.5 unit). Radiolabelled probes were purified with Bio-spin columns (BioRad). Standard methods for hybridization were used. Macroarray blots were exposed to a phosphoimaging screen for 1 h and scanned on a phosphoimager (Molecular Dynamics, Piscataway, NJ, USA). ImageQuant (Amersham, Piscataway, NJ, USA) was used to determine signal intensity.

COMPASS and BLAST analysis of differentially expressed clones

Putative chromosomal location of the identified clones was predicted by COMPASS (Ma *et al.* 1998), which has been shown to accurately predict chromosomal location and gene identification via comparative mapping of cattle and humans (Rebeiz & Lewin 2000).

Radiation hybrid panel

Gene specific PCR primers (Table 1) were screened across a bovine radiation hybrid panel (Womack *et al.* 1997) and a bovine somatic cell hybrid panel (Womack & Moll 1986) to determine chromosomal location. Statistical analysis of chromosomal location of the genes was completed in collaboration with Dr James Womack and Dr Srivinas Kata, Texas A & M University. Genes that could not be unambiguously assigned to a single chromosomal location using the radiation hybrid panel were mapped on the somatic cell hybrid panel.

Results

Embryo collection

This experiment utilized Piedmontese cross animals, which carried one copy of an A to G transition mutation that inactivates myostatin (Kambadur *et al.* 1997). Fifty-two embryos (average 3.2 per pregnant cow) ranging from 30 to 32 days post-insemination were collected. This strategy was predicted to minimize background genetic variation and its impact on gene expression measurements, as embryos from all genotypic classes (0, 1 or 2 double-muscled alleles) would be generated from a limited genetic background. However, genotyping of the 52 embryos revealed the presence of 18 homozygous normal, 34 heterozygous, and 0 homozygous double-muscle embryos (deviation from a 1 : 2 : 1 ratio, $\chi^2 = 17.4$). It should be noted that no abnormal or reabsorbing embryos were encountered during dissection of the reproductive tracts.

It is assumed that the failure to obtain the predicted 1 : 2 : 1 ratio of genotypes was related in some way to the superovulation procedure. To avoid this complication, the experiment was repeated by artificial insemination of synchronized cows that were not superovulated. Additional Piedmontese animals were not available, so Belgian Blue cross animals, which carried one copy of an 11 bp deletion that inactivates myostatin (Kambadur *et al.* 1997) were used. Twenty-three embryos were produced, with five homozygous normal, 14 heterozygous, and four homozygous double-muscled embryos. These embryos did not deviate from the expected 1 : 2 : 1 ratio ($\chi^2 = 1.036$).

Gene	Primer sequences	Annealing
ATP5H	5′-CCTCACGGAGCCTACCATCA-3′ 5′-CACGGGCCCTGACTACANTT-3′	59
MLL2	5′-GTACAGAAGGCAAGCGACAG-3′ 5′-CAGAGAGGTACATGCTGCAG-3′	61
HMGA2	5′-ACTTGCAAAGACCTACCTCCA-3′ 5′-AACCATCTCTCTTCCAGCCG-3′	64

Table 1 Primer information for genes mapped with radiation hybrid and somatic cell hybrid panel.

Differential expression

BLAST analysis of sequence data obtained from 58 unique cDNA clones isolated using suppressive subtractive hybridization indicated that there were 30 differentially expressed genes. Eleven genes were isolated from wild-type cDNA that had been subtracted with double-muscle cDNA, suggesting that expression for these genes was at a greater level in the wild type embryos than in the double-muscled embryos. Nineteen genes were identified from double-muscled cDNA that had been subtracted with wild-type cDNA, suggesting that they were expressed at a higher level in the double-muscled embryos.

To verify the results of the subtractive hybridization, macroarray analysis of gene expression was performed with the 30 cDNA identified by SSH. These arrays were probed with radiolabelled cDNA from either wild-type or double-muscled embryos. Nineteen genes were found to have at least a twofold difference in expression levels of wild-type and double-muscled embryos (Table 2). The remaining

Table 2 Genes identified by suppression subtraction hybridization.

EST name	Symbol[1]	Homology to human (%)	Myostatin response[2]	Chromosomal location	GenBank accession number
DMA1	*DDX17*	82	0.67	5[4]	CF195394
DME6	*SALL1*	89	2.50*	18[3]	CB603720
DME7	*SET*	95	1.01	8,11[4]	CB603721
DMD11	*RAB2*	90	1.36	14[6]	CB603719
DMA2	*KIAA0697*	85	22.80*	6[4]	CB603713
DMA4	*ACTB*	90	1.87	3[4]	CF195395
DMA5	*RPL18*	83	14.17*	7[7]	CB603714
DMA12	*TF*	98	0.64	1[8]	CB603712
DMB9	Unique	–	2.43*	–	CB603717
DMB11	*SOD1*	90	2.03*	3[9]	CB603716
DMD1	*HBE1*	88	3.52*	15,25[4]	CB603718
DMA7	*MTND5*	87	1.64	13,18[4]	CB603715
DMG1	*TMSB10*	98	2.44*	2[4]	CB603722
WTB5	*MLL2*	94	0.16*	5[3]	CB603729
WTC2	*ATP5H*	95	0.24*	5[3,5]	CB603732
WTH3	*HMGA2*	90	0.24*	5[3]	CB603739
WTE5	*RAF1*	91	0.19*	22[8]	CB603735
WTH10	*RPL3*	99	0.29*	5[10]	CB603738
WTB8	*EEF1A1*	98	1.03	6[4]	CB603731
WTF4	*RPS5*	87	0.52	9[4]	CB603736
WTA3	*AFP*	86	0.91	6,17[4]	CB603724
WTA4	Unique	–	0.24*	–	CB603725
WTA5	*RPL11*	90	0.23*	15,1[4]	CB603726
WTA6	*TUBB*	93	0.19*	18[4]	CB603727
WTA9	*RPS9*	94	1.46	7,18[4]	CB603728
WTA10	*HBZ*	98	0.26*	29,5[4]	CB603723
WTB6	*TUBGCP6*	89	0.21*	22,17[4]	CB603730
WTD4	Unique	–	0.41*	–	CB603733
WTF8	*PTMA*	90	1.10	7[4]	CB603737
WTE11	*RPS3*	85	0.35*	6[7]	CB603734

[1]Gene name was determined by the closet human homologue. Identity had to be greater than 80% over a distance of >60 bp.

[2]Response is reported as wild-type expression divided by double-muscled. Those genes with a greater than twofold difference in expression are denoted with an asterisk.

[3]Assigned to chromosomal location using a bovine radiation hybrid panel (Womack *et al.* 1997).

[4]Assigned to chromosomal location using COMPASS (Ma *et al.* 1998).

[5]Assigned to chromosomal location using a bovine somatic cell hybrid panel (Womack & Moll 1986).

[6]Band *et al*. (2000).

[7]Wang *et al*. (2001).

[8]Threadgill *et al*. (1991).

[9]Heuertz & Hors-Cayla (1981).

[10]Allan *et al.* (2001).

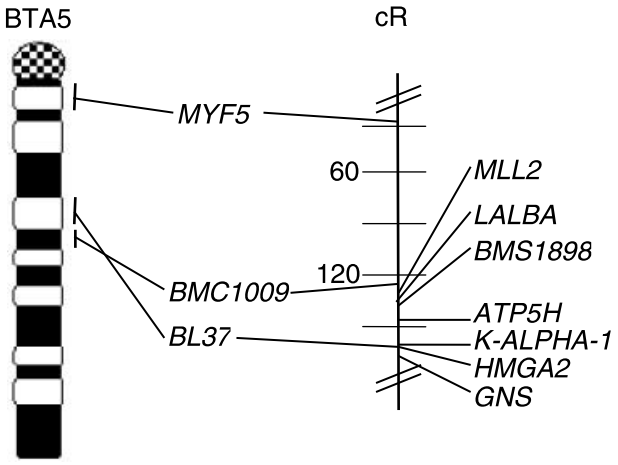

Figure 1 Physical and linkage map of *ATP5H, MLL2, HMGA2*, and associated markers. BTA5 represents the cytogenetic map of bovine chromosome 5. On the right hand side, radiation hybrid panel mapping results are shown, along with markers that were near each gene (Band *et al.* 2000).

11 clones were not differentially expressed (less than two-fold change). The mRNA expression level for 12 clones was greater in double-muscled vs. wild-type embryos, whereas the mRNA expression level of seven clones was greater in wild-type vs. double-muscled embryos.

Chromosomal location

Gene identification and chromosomal location (Table 2) were predicted for 27 clones using COMPASS (Ma *et al.* 1998). Comparative mapping suggested that *HMGA2, MLL2*, and *ATP5H* mapped on bovine chromosome 5 (BTA5), which also contains a myostatin interacting QTL for day 14 Warner–Bratzler shear force (Casas *et al.* 2000). Therefore, to more precisely map these loci, a radiation hybrid panel was screened with primers developed for these three genes (Table 1). *HMGA2* was located centromeric to glucosamine (*N*-acetyl)-6-sulphatase (*GNS*) and *MLL2* was positioned telomeric to alpha-lactalbumin B (LALBA; Fig. 1). Although *ATP5H* could not be assigned to a single chromosomal location using the radiation hybrid panel, it was positioned on BTA5 using a bovine somatic cell hybrid panel (Womack & Moll 1986) with 94% confidence.

Discussion

Failure to obtain double-muscled bovine embryos from super ovulated cows has two possible explanations. First, follicles carrying the double-muscled mutation may not have ovulated using a super-ovulation regime. This possibility is eliminated in that heterozygous embryos were obtained in the experiment. Alternatively, it is possible that homozygous double-muscled embryos, which lacked functional myostatin alleles and were obtained from super ovulated cows, died shortly after transfer. The reason for this death is not obvious.

The present study was designed to use the SSH method for identification and mapping of functional candidate genes for myostatin interacting alleles. In particular, we were interested in genes differentially expressed shortly after the first expression of myostatin in the developing bovine embryo. Comparisons of double-muscled and normal-muscled animals have revealed differences in gene expression, protein synthesis, energy homeostasis, and myofibre and adipose number (Hanset 1986; Oldham *et al.* 2001). Genes identified in this study appear to fall into general gene classes including transcription factors ($n = 5$), genes involved in protein synthesis and degradation ($n = 8$), cell proliferation ($n = 3$), or altered metabolism ($n = 4$).

The mRNA expression level of *Sall1* and high mobility group AT-hook 2 (*HMGA2*), which are both transcription factors, was decreased in embryos that were homozygous for the double-muscled mutation. *Sall1* is a spalt-like gene, which in *Drosophila* is a downstream target of decapentapalegic, a TGFB superfamily member (de Celis *et al.* 1996; Nellen *et al.* 1996). *HMGA2* is involved in fat cell proliferation, as *HMGA2* knockout mice have a decrease number of adipose cells (Anand & Chada 2000). In addition, inactivation of *HMGA2* in mice results in a pygmy phenotype, which indicates that *HMGA2* is required for normal cell proliferation (Zhou *et al.* 1995). Thus, decreased *HMGA2* expression could be a possible mechanism whereby myoblast and adipose proliferation is decreased in embryos with non-functional myostatin alleles.

RPL18, RPL3, RPL11, and *RPS3* are all involved in protein synthesis and degradation. Ribosomal protein L18 has been shown to inhibit double-stranded RNA protein kinase (PKR), which plays an important role in the regulation and inhibition of protein synthesis (Koromilas *et al.* 1992; Kumar *et al.* 1999). Also, ribosomal protein L3 expression is increased in mouse lines with higher fat deposition (Allan *et al.* 2001). The differential expression of these genes indicates that there may be differences in protein synthesis and degradation between wild-type and double-muscled embryos.

RAF1, thymosin beta-10 (*TMSB10*), and myeloid-lineage leukaemia (*MLL2*) are involved in regulation of cell proliferation. In C2C12 cells, *RAF1* has been shown to enhance the transcriptional activity of *MYOD1* (Gredinger *et al.* 1998). The decreased level of *RAF1* expression observed in wild-type animals may decrease MYOD1 functional activity, thus indicating a mechanism in addition to decreased *MYOD1* expression (Langley *et al.* 2002) whereby myostatin represses terminal differentiation. As myostatin is known to inhibit cell proliferation (Thomas *et al.* 2000), these genes may have an important role in the increased myoblast proliferation associated with this phenotype.

It has been reported that several chromosomes harbour suggestive QTL that contain alleles that interact with

myostatin. A suggestive interaction between alternative myostatin alleles and the genetic background on chromosome 5 was identified for Warner Bratzler shear force (WBS) at day 14 post-mortem (WBS14; Casas *et al.* 2000). Similar interactions with myostatin appear to exist on chromosome 8 and 14 for fat depth (Casas *et al.* 2000) and chromosome 4 for WBS at 3 days post-mortem (Casas *et al.* 2000, 2001). *MLL2*, *HMGA2* and *ATP5JD*, identified in this study, were physically mapped to bovine chromosome 5 very close to the WBS14-interacting QTL peak.

Despite the fact that SSH was successfully used to identify differentially expressed genes, one must be aware of potential limitation of suppressive subtractive hybridization analysis. First, 11 genes appeared to have escaped subtraction as they had lower than twofold change in gene expression. Thus, differential expression of all identified genes must be verified with an alternative method. Furthermore, embryos used in this study were obtained from crossbred cattle. The possibility exists that differences in genetic background were responsible for the observed changes in gene expression. For example, there are changes in fatty acid binding protein gene expression in pigs, which correlate with different genotypes (Gerbens *et al.* 2001).

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