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MUSCLE SPECIFIC EXPRESSION OF REGULATORY FACTORS IN CATTLE SELECTED FOR HIGH AND LOW MUSCLING

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SUMMARY

This paper reports an investigation of the allele-specific expression of myostatin in the *semitendinosus* and *longissimus dorsi* muscles in a population of animals selected for high or low muscling, including animals heterozygous for the *nt821(del11)* loss of function myostatin polymorphism. In addition, expression of follistatin, myogenin, and *MYOD*, genes that also affect muscle growth and development were studied. Animals that were heterozygous for the *nt(821)del11* loss of function polymorphism expressed higher amounts of total myostatin but lower amounts of the functional (wild-type) allele compared to homozygous wild-type animals. The level of *MYOD* expression was greater in the wild-type high muscling line compared to the wild-type low muscling line. These findings demonstrate an up-regulation of total myostatin expression in cattle heterozygous for a non-functional myostatin allele compared to homozygous wild-type cattle, presumably due to the role of negative feedback in these cattle which express less wild-type myostatin than their homozygous wild-type counterparts. The findings also show that selection for divergence in muscling score can influence expression levels of other muscle regulatory genes such as *MYOD*.

INTRODUCTION

There is continuing interest in the use of the double muscled phenotype in cattle as a method of increasing the amount of saleable beef, or retail beef yield from carcases (O'Rourke *et al.* 2006, 2009). However, the double muscled phenotype of cattle is also associated with negative attributes such as decreased fertility and calving difficulties (Arthur 1995), hence less extreme phenotypes with increased muscling but without fertility and calving difficulties are more desirable for use in the beef industry.

Several mutations have been identified within the *bovine* myostatin gene that disrupt the function of the myostatin protein and, therefore, give rise to the double muscling phenotype. One such mutation, common in the Belgian Blue breed, is caused by an 11-bp deletion of nucleotides 821-831 inclusive and is referred to as *nt821(del11)*. The *nt821(del11)* polymorphism results in a frame shift and subsequent premature stop codon in the bioactive carboxy-terminal domain of the gene, a domain which is highly conserved in the TGF-β superfamily (Grobet *et al*. 1997).

The objective of this project was to assess if there is any differences in the level of gene expression of myostatin and other genes that also affect muscle growth and development in animals selected for differences in their levels of muscling, including a genotype heterozygous for the *nt821(del11)* mutation .

MATERIALS AND METHODS

The experimental animals were obtained from the NSW Department of Primary Industries low and high muscling selection lines. These lines originated at the Elizabeth Macarthur Agricultural Institute, Camden, NSW in 1998 from 140 females selected from 260 Angus x Hereford F1 female progeny based on muscle score (O'Rourke *et al*. 2006, 2009). Allocation to the high and low lines was based on individual muscle scores. Further divergence of the selection lines was achieved by

mating the high muscle line females with high muscle score bulls and the low muscle line females with low muscle score bulls. Forty-four 2003-born steers were used to study the effects of selection for muscling and of the polymorphisms in the myostatin gene. The steers were divided into 3 separate groups for the analyses: high muscling line with wild-type myostatin (n=14), high muscling line heterozygous for the *nt821(del11)* myostatin polymorphism (n=11), and low muscling line with wild-type myostatin (n=19). The steers were slaughtered at 25 months of age and live weight, carcass, and yield characteristics measured, as reported previously (Cafe *et al*. 2006). At slaughter, *semitendinosus* and *longissimus dorsi* muscle samples were collected, snap frozen in liquid nitrogen, and stored at - 80°C.

Total RNA extraction from muscle was carried out by homogenising 100mg of tissue in Tri-reagent and isolating the RNA as per the manufacturer's instructions (Ambion Inc., USA). RNA cleanup and an on-column DNase treatment of extracted RNA was carried out to remove residual genomic DNA and other non-RNA impurities with RNesay Mini columns (Qiagen, Germany). First-strand cDNA synthesis was carried out with the Omniscript cDNA synthesis kit (Qiagen, Germany) with the addition of an 18SrRNA gene-specific primer (5'-ACACGCTGAGCCAGTCAGT-3'). Quantification of gene expression was carried out on a Rotorgene 3000 (Corbett Research, Australia). Each sample was assayed in triplicate. Real-time PCR reactions were carried out using a reagent containing 0.5 units Amplitaq Gold DNA polymerase (Applied Biosystems, USA), 1 x PCR Gold buffer (Applied Biosystems, USA), 200nM dNTPs (Invitrogen, USA) and 1 x SYTO9 fluorescent dye (Invitrogen, USA). Quantification of gene expression was carried out for total myostatin, *nt821(del11)* myostatin, wild-type myostatin, myogenin, *MYOD*, and follistatin. Reference genes assayed were *RPL19* and *18SrRNA*. Table 1 shows the primer sequences, the length of the amplified products and the Genbank accession numbers for each of the primers used.

Table 1. Forward and reverse primers for real-time PCR assays

Gene	Forward primer (5' – 3')	Reverse primer (5' – 3')	Length (bp)	Genbank accession no.
Total Myostatin	accttcccagaaccaggagaa	tcacaatcaagcccaaaatctct	101	AF019622
Myostatin wild-type allele	tcttgctgtaaccttcccagaac	acagcatcgagattctgtggagt	124	AF019622
Myostatin *nt821(del 11)* allele	tcttgctgtaaccttcccagaac	acgacagcatcgagattctgtca	121	AF019622
Myogenin	ggcagcgcactggagttt	ccgctgggagcagatgat	52	AF433651
MYOD	aactgttccgacggcatgat	gacaccgcagcgctcttc	128	X62102
Follistatin	gggcagatctattggattgg	cctctgccaaccttgaagtc	114	BC133637
18SrRNA	cggtcggcgtcccccaactt	gcgtgcagccccggacatctaa	103	M10098
RPL19	caactcccgccagcagat	ccgggaatggacagtcaca	76	AY158223

For each gene, cycle thresholds (Cts) were determined for the cDNA samples and a standard curve generated from 7 consecutive 2-fold dilutions of pooled cDNA. Normalised relative quantitation was carried out using qBase (Hellemans *et al*. 2007). One-way Analysis of Variance (ANOVA) of the normalised real-time PCR data was carried out for the 2 muscle types and the 3 muscling lines within muscle type, using the statistical package R (R Foundation for Statistical Computing, Austria). Tukey multiple comparison tests were carried out if a significant ANOVA result was observed for a particular muscle type.

RESULTS AND DISCUSSION

Mean normalised relative expression of follisatin, myogenin, *MYOD*, total myostatin, myostatin wild-type, *nt821(del11)* myostatin are presented in Table 2. There were no significant differences between muscles or between the muscling lines for the reference genes.

Table 2. Mean (S.E.) normalised relative expression of genes for the three muscling lines

Gene	High muscling wild-type (n=14)	High muscling Heterozygote (n=11)	Low muscling wild-type (n=19)
	M. longissimus dorsi		
Follistatin	3.13 (0.260)	3.10 (0.270)	3.21 (0.200)
Myogenin	6.62 (0.584)	6.41 (0.667)	6.82 (0.460)
MYOD	3.88 (0.363)	4.21 (0.604)	2.94 (0.260)
Total Myostatin	3.90 (0.350)	4.48 (0.509)	3.20 (0.352)
Wild-type Myostatin	3.87 (0.366) a	2.25 (0.239) b	3.17 (0.359) ab
nt821(del11) Myostatin	na	2.26 (0.199)	na
	M. semitendinosus		
Follistatin	3.32 (0.338) a	2.25 (0.222) b	3.07 (0.226) ab
Myogenin	4.19 (0.313)	3.26 (0.495)	4.05 (0.388)
MYOD	3.97 (0.264) a	3.09 (0.382) ab	2.77 (0.254) b
Total Myostatin	7.50 (0.521) a	9.82 (0.654) b	7.64 (0.562) a
Wild-type Myostatin	7.17 (0.555) a	4.86 (0.496) b	7.58 (0.578) a
nt821(del11) Myostatin	na	4.79 (0.198)	na

Within rows, mean values with different letters differ significantly ($P<0.05$). na = not applicable.

Myostatin. Expression of total myostatin, myostatin wild-type, and myostatin *nt821(del11)* was higher in *M. semitendinosus* than in *M. longissimus dorsi* ($P<0.0001$) in all 3 muscling lines. This finding is consistent with previous studies that have shown greater myostatin expression in muscle tissue with a higher proportion of glycolytic fibres (Bass *et al.* 1999). There was no significant difference in expression of the myostatin wild-type allele between the high muscling wild-type and low muscling wild-type groups for both *M. semitendinosus* ($P= 0.85$) and *M. longissimus dorsi* ($P=0.30$). Total myostatin expression was higher in heterozygotes than homozygous wild-type animals. Despite the differences in the range of expression levels there was no significant difference in mean expression of the myostatin *nt821(del11)* and wild-type alleles in either the *M. semitendinosus* ($P=0.89$) or *M. longissimus dorsi* ($P=0.98$) from the heterozygote animals. However, heterozygote animals expressed lower levels of the functional wild-type allele than the homozygous normal animals. Since the myostatin *nt821(del11)* heterozygote line has only one copy of the wild-type allele, it would be expected that cattle from this line would express this allele at half the level as that of the wild-type lines. However, the level of expression of the wild-type allele in the myostatin *nt821(del11)* heterozygote cattle was greater than half that of the high muscling wild-type and the low muscling wild-type lines. This finding was more pronounced for *M. semitendinosus* than *M. longissimus dorsi.*

MYOD. There was no significant difference in the expression of *MYOD* between the two muscle types. There was however, a difference within the three muscling lines in expression in *M. semitendinosus*. The expression of *MYOD* in *M. semitendinosus* was significantly higher in the

high muscling wild type line than the low muscling wild type line (*P*=0.007). *MYOD* is a marker of satellite cell proliferation (Grounds *et al*. 1992) and increased *MYOD* expression appears to have been indirectly impacted by selection for high muscling. Hence, this may indicate that the low muscling wild-type line had less satellite cell activity than the high muscling wild-type line at the time of sampling.

Myogenin. Myogenin expression was higher in *M. longissimus dorsi* than *M. semitendinosus* (P<0.0001), but there were no significance differences between the three muscling lines.

Follistatin. Follistatin expression tended to be lower in the heterozygote animals compared to the high muscling wild-type animals, although the only significant difference was due to higher expression in the high muscling wild-type line compared with the high muscling heterozygote line for the *M. semitendinosus* (Table 2, *P*=0.049). Along with the findings for myostatin, this suggests a possible negative feedback loop whereby the lack of functional myostatin transcript in the heterozygotes results in an up-regulation of transcription of both the functional and non-functional myostatin alleles. The regulatory trigger for expression of myostatin may be myostatin itself, as found for transforming growth factor-β (TGF-β) by Kim *et al*. (1990). Alternatively, an intracellular/autocrine feedback loop may operate, as suggested for interactions between insulin-like growth factors and TGF-β (Bosche *et al*. 1995). It is possible that an endocrine growth factor stimulated by myostatin may then regulate expression of myostatin mRNA (Oldham *et al*. 2001).

CONCLUSIONS

There is an up-regulation of total myostatin expression in cattle heterozygous for a non-functional myostatin allele compared to homozygous wild-type cattle. Selection for divergence in muscling score can influence expression levels of other muscle regulatory genes such as *MYOD*.

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