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MSTN genotypes in Thoroughbred horses influence skeletal muscle gene expression and racetrack performance

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Summary

Myostatin, encoded by the *MSTN* gene, is a member of the TGF-β superfamily that regulates skeletal muscle development. A *MSTN* SNP significantly associated with Thoroughbred horse racing phenotypes has recently been identified as well as significant reductions in Thoroughbred skeletal muscle gene expression for three transcripts 400–1500 base pairs downstream of the *MSTN* gene following a period of training. Together, these findings indicate that *MSTN* genotypes may influence *MSTN* gene expression. To investigate this, *MSTN* mRNA expression was measured in biopsies from the middle gluteal muscle from 60 untrained yearling Thoroughbreds (C/C, $n = 15$; C/T, $n = 28$; T/T, $n = 17$) using two independent real-time qRT-PCR assays. *MSTN* gene expression was also evaluated in a subset ($N = 33$) of these animals using samples collected after a ten-month period of training. A significant association was observed between genotype and mRNA abundance for the untrained horses (assay I, $P = 0.0237$; assay II, $P = 0.003559$), with the C/C cohort having the highest *MSTN* mRNA levels, the T/T group the lowest levels and the C/T group intermediate levels. Following training, there was a significant decrease in *MSTN* mRNA (-3.35-fold; $P = 6.9 \times 10^{-7}$), which was most apparent for the C/C cohort (-5.88-fold, $P = 0.001$). These data demonstrate the tight relationship between phenotype, genotype and gene expression at the *MSTN* gene in Thoroughbred racehorses.

Keywords equine, exercise, gene expression, myostatin, skeletal muscle, training

Myostatin has profound effects on skeletal muscle mass in a range of mammalian species (Schuelke 2004; Mosher 2007). The Thoroughbred horse has an unusually large skeletal muscle mass (>55% of total body mass) compared to other species (30–40% of total body mass; Gunn 1978), and a key role for the *myostatin* (*MSTN*) gene in the growth and development of Thoroughbred skeletal muscle for racing performance has been observed (Binns *et al.* 2010; Hill *et al.* 2010; Tozaki 2010).

A novel single nucleotide polymorphism (SNP) (ECA18 g.66493737C/T), identified within the first intron of the equine *MSTN* gene, has been found to have a highly significant association with racing performance in Thoroughbreds (Hill 2010). Homozygotes for the C-allele (i.e. C/C) have been shown to compete preferentially in shorter-distance races, while C/T horses are best suited to middle-distance races and T/T horses tend to excel in longer distance races. A significant association has also been found between *MSTN* genotype and body composition, with C/C male horses having a significantly greater body mass-to-height ratio than C/T or T/T horses. This is consistent with the observations that sprinters are generally more muscular and compact than individuals that are more suited to racing over longer distances (Hill *et al.* 2010).

In a functional context, a separate study identified three mRNA sequences with significantly decreased expression following training that were located within a region between 1500 and 400 bp downstream of the *MSTN* gene (McGivney 2010). These results support the hypothesis that alterations in *MSTN* gene expression are involved in the

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adaptive response to training in Thoroughbred skeletal muscle. In the current study, we have investigated whether the g.66493737C/T SNP is associated with differential expression of the *MSTN* gene in Thoroughbred skeletal muscle.

Sixty untrained Thoroughbred yearling horses (24 males, 36 females; mean age, 20 months) were subdivided according to their g.66493737C/T genotype as follows: C/C (n = 15), C/T (n = 28) and T/T (n = 17). All animals were raised on the same farm for the previous two to three months and were destined for flat racing with the same trainer. In addition, a subset of 33 animals from the initial group (11 males, 22 females; mean age, 30 months) provided a comparative cohort of trained samples (C/C, n = 10; C/T, n = 13; T/T, n = 10). These trained horses undertook a regular exercise regime with the same trainer for 10 months. Owing to limitations determined by the general practice of initiating the training of flat racehorses at two years of age, it was not possible to sample untrained age-matched controls.

Muscle biopsies were obtained from the gluteus medius muscle, total RNA was isolated, and real-time qRT-PCR was performed as previously described (McGivney 2010). qRT-PCR was performed in duplicate for untrained samples (n = 60) and repeated for a subset of n = 33 trained samples (from the same individuals) using two independent assays (Table 1). Using the PLINK software package (Purcell 2007), the linear regression model was used to evaluate the association between genotype and gene expression. Student's t-test was used to identify significant differences in mRNA abundance among cohorts.

A significant ($P < 0.05$) association was observed between *MSTN* genotype and gene expression for both assays in the untrained cohort. The C/C cohort had significantly ($P < 0.01$) higher *MSTN* mRNA levels than the C/T and T/T cohorts (Fig. 1a,b). There was no significant difference in gene expression among the three *MSTN* genotypes in samples post-training. However, when all genotypes were considered together, there was a highly significant reduction in *MSTN* mRNA (−3.35-fold; $P = 6.9 \times 10^{-7}$) in post-training samples compared to the untrained samples. The greatest reduction in *MSTN* mRNA was observed for the C/C cohort followed by the C/T and T/T groups (Fig. 2).

In other species, muscle hypertrophy, as a result of a dysfunctional myostatin protein, manifests in a dose-dependent fashion; for example, mice heterozygous for a null mutation have intermediate muscle mass compared to homozygotes (McPherron *et al.* 1997). Further evidence for a dose-dependent effect of myostatin comes from the studies of the whippet dog breed, where animals homozygous for a 2-bp non-sense deletion mutation in *MSTN* show extreme hypermuscularity and are not raced, while heterozygotes have superior racing ability (Mosher 2007). These findings indicate that lower levels rather than the absence of myostatin may be optimal for athletic performance (Mosher 2007).

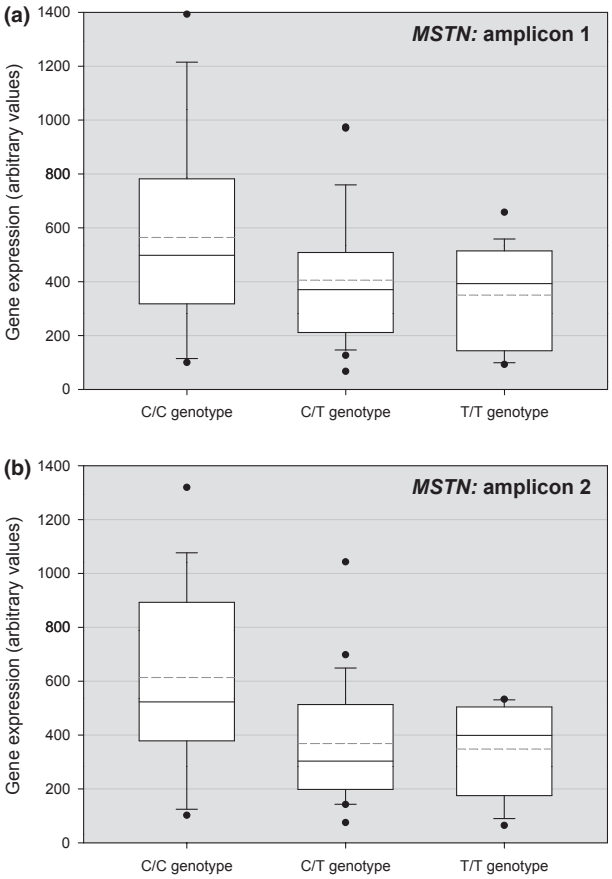

Figure 1 *MSTN* mRNA expression in untrained skeletal muscle samples. The C/C (n = 15) cohort had significantly ($P < 0.01$) higher expression levels than the C/T (n = 28) and T/T (n = 17) cohorts. Real time qRT-PCR was performed in duplicate using two independent *MSTN* primer pairs (a) spanning exons 1 and 2 (exon 1/2) and (b) spanning exons 2 and 3 (exon 2/3).

Table 1 Primers used for real-time qRT-PCR.

Target gene	Orientation	Location	Sequence
Myostatin (*MSTN*)	Forward primer (5′-3′)	Exon 1	TGACAGCAGTGATGGCTCTT
Myostatin (*MSTN*)	Reverse primer (5′-3′)	Exon 2	TTGGGTTTTCCTTCCACTTG
Myostatin (*MSTN*)	Forward primer (5′-3′)	Exon 2	TTCCCAAGACCAGGAGAAGA
Myostatin (*MSTN*)	Reverse primer (5′-3′)	Exon 3	CAGCATCGAGATTCTGTGGA
Titin (*TTN*)	Forward primer (5′-3′)	Exon 357	GCATGACACAACTGGAAAGC
Titin (*TTN*)	Reverse primer (5′-3′)	Exon 358	AACTTTGCCCTCATCAATGC

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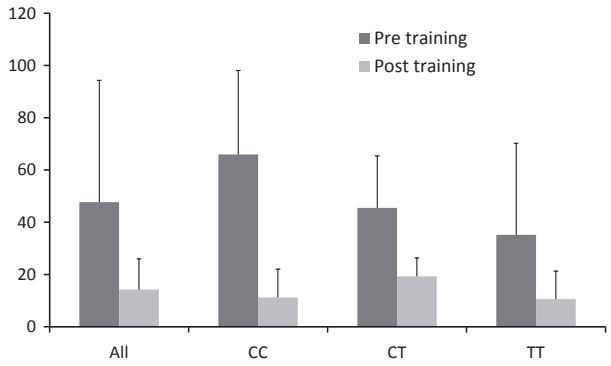

Figure 2 *MSTN* mRNA expression in untrained (n = 60; pre training; dark grey bars) and trained (n = 33; post training; light grey bars) skeletal muscle samples. There was a highly significant reduction in *MSTN* mRNA (−3.35-fold; $P = 6.9 \times 10^{-7}$) post training. The greatest reduction was observed for the C/C cohort (C/C, −5.88-fold, P = 0.001; C/T, −2.36-fold, P = 0.007; T/T, −3.31-fold, P = 0.001).

In the current study, it was surprising therefore to find that untrained samples with the C/C genotype, which is associated with sprint racing performance, displayed the highest levels of *MSTN* mRNA. However, while C/C individuals initially had the highest levels of *MSTN* mRNA, the greatest alteration in mRNA content was observed for this cohort (−5.88-fold) compared to C/T (−2.36-fold) and T/T horses (−3.31-fold) following training. Notably, following training, there was no genotype-specific variation in *MSTN* gene expression.

These findings suggest that myostatin may have multiple functions at different stages of development. For example, a range of non-hypertrophic functions have been proposed for myostatin, including regulation of Type IIb muscle fibre development (Hennebry 2009) and oxidative enzyme activity resulting from increased mitochondrial content (Amthor 2007). Although the data from the current study cannot identify the exact mechanisms by which variation in the expression of *MSTN* may affect athletic capabilities in Thoroughbred horses, it is likely that the reduction in *MSTN* gene expression during the course of training has a direct impact on muscle growth and development in Thoroughbred skeletal muscle. Furthermore, it seems that the influence of myostatin on muscle growth and differentiation may be more complex than simply acting as a limiter of muscle growth.

The influence of myostatin on skeletal muscle fibre Type IIb development is of particular interest in the context of the Thoroughbred, as Type IIb fibres have not previously been detected in Thoroughbred skeletal muscle. Also, the genes encoding a number of key signalling molecules that interact with myostatin are located within genomic regions that show evidence for recent strong selection in the Thoroughbred population (Gu 2009).

There is a significant relationship between g.66493737C/T genotypes and *MSTN* gene expression; however, this observed correlation disappears following training. This study further supports a central role for myostatin in equine skeletal muscle and represents a contribution to elucidating the mechanisms behind the regulation of myostatin and its influence on exercise-induced muscle development.

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