Expression profiles of myostatin and calpastatin genes and analysis of shear force and intramuscular fat content of yak *longissimus* muscle

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ABSTRACT: The main objective of this study was to reveal the expression profiles of two negative regulators, myostatin (*MSTN*) and calpastatin (*CAST*) genes, of skeletal muscle growth in highland yaks (*Bos grunniens*). mRNA levels of both genes were quantified in different yak tissues by semi-quantitative RT-PCR to reveal the tissue expression pattern, and real-time quantitative RT-PCR was employed to compare the mRNA levels of *MSTN* and *CAST* in *longissimus* muscles of yaks at different ages and adult Yellow cattle. Intramuscular fat (IMF) content, tenderness and pH of *longissimus* muscle of yaks at different ages and of adult Yellow cattle were also measured. The results showed that *MSTN* and *CAST* expressions have tissue specificity and both exhibited a high level in *longissimus* muscle and a low level in adipose tissue. Yak calves had lower mRNA levels of both *MSTN* and *CAST* in *longissimus* muscle compared with adult yaks. The analysis of meat quality traits of *longissimus* muscle showed that the shear forces of raw *longissimus* muscle of yak calves were significantly lower than those of adult yaks and Yellow cattle, no significant difference was found between adult yaks and Yellow cattle of similar age. IMF content in *longissimus* muscle was lower in yaks than in Yellow cattle. Although yaks were smaller in body size than Yellow cattle, adult yaks showed lower levels of *MSTN* and similar level of *CAST* mRNA in *longissimus* muscle compared to Yellow cattle. These data indicate that the expression of both *MSTN* and *CAST* in *longissimus* muscle differs between adult yaks and yak calves, and the yak *longissimus* muscle shows a lower IMF content compared to cattle.

Keywords: *Bos grunniens*; meat trait; muscle growth; hypoxia; intramuscular fat

The yak (*Bos grunniens*) is a unique bovine species adapted to the hypoxic environment of Qinghai-Tibet plateau, the roof of the world. The total population is thought to number around 14 million, of which 13 million are distributed in China (Wiener et al., 2003). Yak meat is of high quality, less polluted than meat produced on ordinary farms and the most important protein source for local Tibetan farmers (Wiener et al., 2003). However, yaks are much smaller in body size than other bovine species, approximately 120 to 220 kg for male yaks (Cai and Wiener, 1995) and > 250 kg for male Yellow cattle at 2 to 2.5 years of age. The molecular basis for the muscle growth has not been documented in yaks. In recent years, it has been reported that different cattle breeds show different muscle transcriptional profiles (Wang et al., 2005). Many candidate genes have been presented for muscle growth and meat quality in cattle and other domestic animals (de Koning et al., 1999; Damon et al., 2006; Hoashi et al., 2008), but

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they are nearly unavailable in yaks. The yak shows great similarities of amino acid sequences with domestic cattle (*Bos taurus*) in proteins assayed (Zheng et al., 2008; Bai et al., 2010; Zhang et al., 2010). Thus, the yak would be a suitable model to study the influences of genetic or environmental factors on performances in bovine species. Myostatin (*MSTN*) and calpastatin (*CAST*) are candidate genes related to muscle growth and tenderness (Koohmaraie, 1996; Barnoy et al., 1997), both of them are negative regulators of skeletal muscle growth. Based on the much smaller body size of yaks relative to other bovine species, we hypothesized that their expressions of *MSTN* and *CAST* in skeletal muscles might be different from those of other bovine species. The inhibition of *MSTN* in skeletal muscle can increase muscle mass and reduce fat mass in mice (Guo et al., 2009). Additionally, intramuscular fat has been reported to affect both meat tenderness and quality (Geay et al., 2001). Therefore, the study of *MSTN* and *CAST* expression is of importance for elucidating the molecular basis of meat quality. In this study we compared the expressions of *MSTN* and *CAST* in *longissimus* muscles of Jiulong yak and cattle, and measured some properties (shear force, fat content and pH) of meat in order to address the molecular basis and characteristics of muscle growth and property in yaks.

MATERIAL AND METHODS

Animal sampling

The experimental Jiulong yak herd was reared at Jiulong County of Sichuan Province, at 3500 m a.s.l. It included yak male calves (0.5 ± 0.1 year, n = 6) and adult male yaks (4.4 ± 0.6 years, n = 10). The yaks grazed on natural pasture without feed supplementation. Within 30 min after slaughter, *longissimus* muscle samples were taken from each yak at the position between the last thoracic spine and the third lumbar spine of right carcass. Heart, liver, spleen, lung, kidney and adipose tissues were also taken from each yak. *Longissimus* muscle samples of Chinese Yellow cattle (4.8 ± 0.9 years, male, n = 8) living at a low altitude were taken for comparison. All of the samples were promptly frozen and stored at –80°C until analysis. This experiment was conducted according to the guidelines of Chinese government for the use of experimental animals including animal welfare and conditions.

Analysis of mRNA levels

Semi-quantitative RT-PCR was employed to assay mRNA levels of *MSTN* and *CAST* genes in tissues of yaks randomly chosen out of the ten adult male yaks mentioned above. The housekeeping gene, β-actin, was used as internal control. Total RNA was extracted from tissues using TRIzol reagent (Invitrogen, Auckland, New Zealand). cDNA was synthesized by reverse transcription from 1 μg of total RNA as described in the manufacturer's protocol (Fermentas Life Science). PCR amplifications were performed in standard conditions: denaturation at 94°C for 2 min, then 33 cycles of 94°C for 30 s, 52~54°C (annealing temperature) for 30 s and 72°C for 1 min. Primers were designed using Primer Premier 5.0 software according to the sequences of corresponding genes of yak or cattle in GenBank (*Bos taurus* β-actin: BT030480, F: CATCCGCAAGGACCTCTAC, R: ATGCCAATCTCATCTCGTTTT, fragment size 340 bp; *Bos taurus MSTN*: GQ184147, F: AAAGAGGGGCTGTGTAATGC, R: ATGGTAATGACCGTTTCCGT, fragment size 260 bp; *Bos grunniens CAST*: FJ483833, F: CGTGCCTCGGACCTCTAT, R: CGTCTTTATCCTTGGCTTCT, fragment size 254 bp). PCR products were examined by 1% agarose gel electrophoresis, and band intensity was estimated by Quantity One software of gel imager (Versa Doc 1000, Bio-Rad, Hercules, USA). The relative mRNA level is expressed as the ratio of intensity between the bands of a target gene and the β-actin gene.

In order to reveal their age difference and species differences in expression, quantitative real-time RT-PCR was developed to compare *MSTN* and *CAST* mRNA levels in *longissimus* muscle of yak calves (n = 6), adult yaks (n = 10) and Chinese Yellow cattle (n = 8). The RT-PCR amplification mixture (25 μl) contained 1 μl of RT reaction mix, 12.5 μl of SYBR® Premix Ex Taq ™ (2×) (TaKaRa, Dalian, China), 0.5 μl of 10 μmol/l each of primers (as listed above) and addition of ultra-pure water to 25 μl. Reactions were run on a fluorescence temperature iCycler (Bio-Rad, Hercules, Usa). The PCR conditions were as follows: one cycle of 30 s at 95°C; 45 cycles of 10 s at 95°C, 10 s at the annealing temperature of the primers, 30 s at 72°C. The samples in the RT-PCR were run in duplicate. The threshold cycle (C_T) resulting from RT-PCR was analysed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). Changes in the expression of

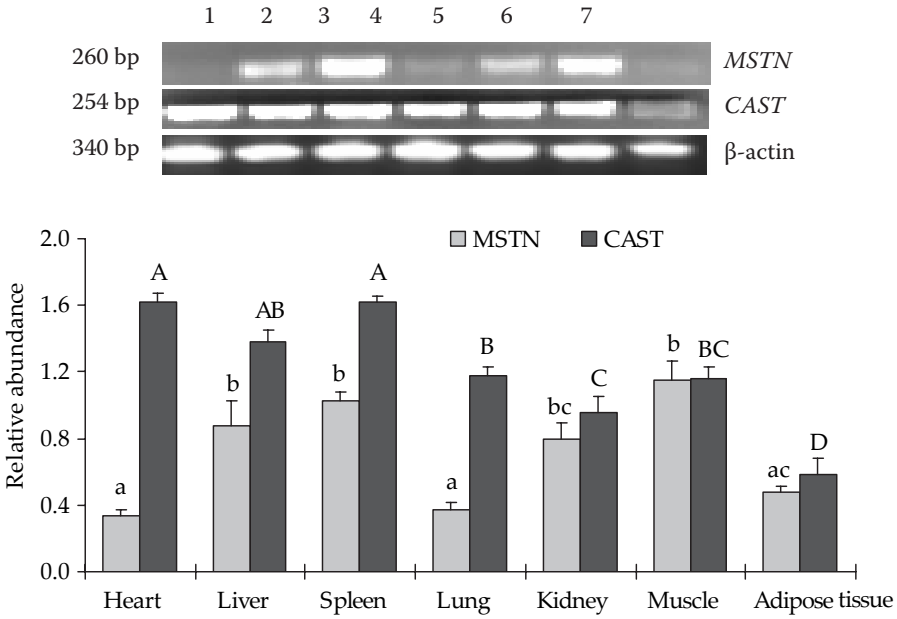

Figure 1. The tissue expression profiles of *MSTN* and *CAST* genes in yak. (A) Electrophoresis of RT-PCR products on 1% agarose gel, 1–7: heart, liver, spleen, lung, kidney, muscle and adipose tissue; (B) Relative mRNA level of *MSTN* and *CAST*

Compared among the same gene, different capital letters (used in *CAST*) and small letters (used in *MSTN*) indicate $P < 0.05$. β-actin mRNA level is used to compute the relative mRNA abundance
The data are averages of five adult yaks

MSTN and *CAST* were normalized to the β-actin expression level in the same sample.

Analysis of meat shear force, pH and intramuscular fat content

Longissimus muscles of yak calves (n = 6), adult yaks (n = 10) and Chinese Yellow cattle (n = 8) were used in the analysis. Frozen *longissimus* muscles were thawed at 4°C and round cores 1.27 cm in diameter were removed parallelly to the muscle fibre. Meat shear force was measured before and after 24 h aging at 4°C using TA.XT plus Texture Analyser (Stable Micro Systems Ltd., Godalming, UK) by shearing perpendicularly to the fibre direction of the cores. The crosshead speed was set at 200 mm/min, each core was sheared once and results were averaged of three repetitions. pH values of the intact *longissimus* muscle samples before and after 24 h aging at 4°C were measured using a pH meter (Mettler-Toledo, Inc., Columbus, USA). The intramuscular fat (IMF) content of *longissimus* muscles was measured using the standard Soxhlet extraction method.

Statistical analysis

Data were analysed using Statistical Package for the Social Science (SPSS 11.5). Values were expressed as mean ± SE. The expression patterns of genes were assayed by one-way ANOVA, and the significance of gene expressions between yak and cattle was evaluated using an independent-sample t-test and the significance level was set at $P < 0.05$.

RESULTS

Semi-quantitative RT-PCR assay of expression profiles of *MSTN* and *CAST* genes

The semi-quantitative RT-PCR analysis of gene expressions in adult yak tissues (n = 5) revealed that the expressions of *MSTN* gene were lower in heart, lung and in most cases in adipose tissue than in the other tissues examined, while the highest mRNA levels of *CAST* gene were observed in heart, liver and spleen, and the lowest level in adipose tissue

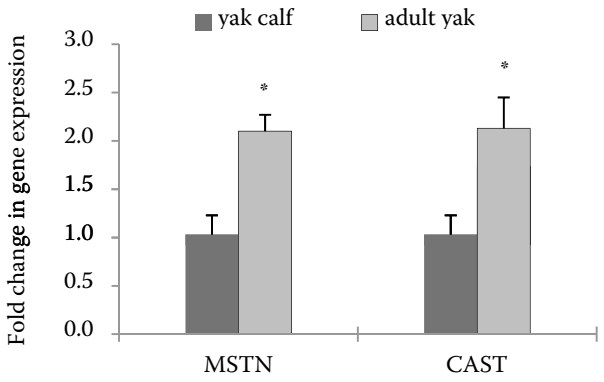

Figure 2. Quantitative real-time PCR analysis of *MSTN* and *CAST* genes in the *longissimus* muscle of yak calf and adult yak

Fold changes of *MSTN* and *CAST* normalized to the β-actin gene expression and relative to yak calf expression level $2^{-\Delta\Delta C_T}$ are shown

Error bars represent the standard error, $^*P < 0.05$

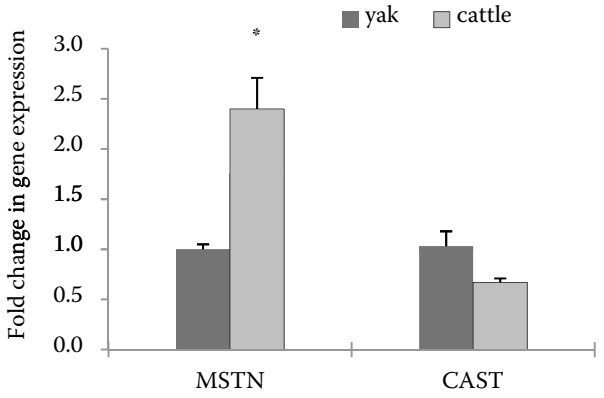

Figure 3. Quantitative real-time PCR analysis of *MSTN* and *CAST* genes in the *longissimus* muscle of adult yak and cattle

Fold changes in *MSTN* and *CAST* amounts normalized to the β-actin gene expression and relative to adult yak expression level $2^{-\Delta\Delta C_T}$ are shown

Error bars represent the standard error, $^*P < 0.05$, compared between the same gene of yak (n = 10) and cattle (n = 8)

(Figure 1). The tissue expression pattern also differed between the two genes, especially in heart and lung. However, in *longissimus* muscle it showed a similar level.

Real time RT-PCR assay of mRNA levels in *longissimus* muscle of adult cattle and yaks at different ages

The real-time quantitative RT-PCR assay showed that in yak *longissimus* muscle both *MSTN* and *CAST* mRNA levels of adult yaks (n = 10) were nearly twice higher than those of yak calves ($P < 0.05$, Figure 2). The *longissimus* muscles of adult yaks (n = 10) contained a lower mRNA level of *MSTN* ($P < 0.05$) and a similar mRNA level of *CAST* as compared with those of cattle (Figure 3). *MSTN* expression in the *longissimus* muscle of adult cattle was more than twice higher than that of adult yaks.

Meat quality measurements

Shear forces of the raw *longissimus* muscle of yak calves, before or after aging, were both significantly lower than those of adult yaks and cattle, no significant difference was found between adult yaks and cattle of similar age. However, the tenderization rate of yak calf *longissimus* muscle was relatively lower than that of adult yak or cattle (Figure 4). In *longissimus* muscle, yak calves, adult yaks and cattle had similar pH and the extent of pH decline after aging (Figure 5). IMF contents in *longissimus* muscle were 1.00 ± 0.19%, 1.47 ± 0.23% and 3.22 ± 0.46% for yak calves, adult yak and cattle, respectively ($P < 0.05$).

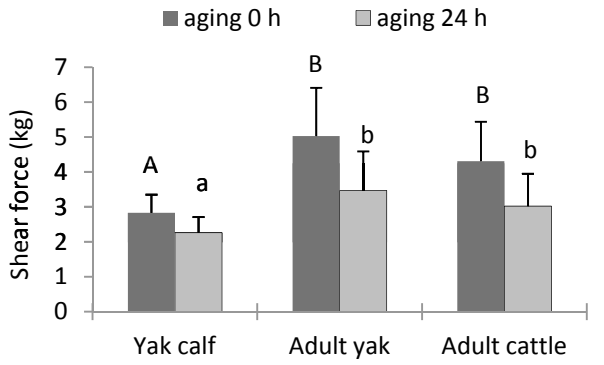

Figure 4. Shear force of meat samples from yak and cattle

Different capital or small letters indicate significant differences among the three groups before or after aging, respectively

Error bars represent the standard error.

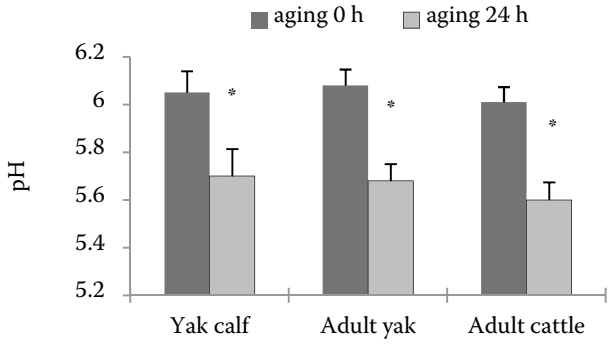

Figure 5. pH of meat samples from yak and cattle

Error bars represent the standard error, *$P < 0.05$, compared between pH of meat before and after 24 h aging

DISCUSSION

MSTN is a secreted growth factor predominately expressed in skeletal muscle that negatively regulates the skeletal muscle mass (Lee, 2004). *CAST* is an endogenous inhibitor of Ca^{2+}-dependent protease (Barnoy et al., 1997). Garikipati et al. (2006) reported the widespread expression of *MSTN* in over twenty tissues of rainbow trout and especially high level in spleen and eyes. In the present experiment, the expression of *MSTN* was also observed in all tissues examined. It is reported that the inhibition of *MSTN* in muscle and adipose tissue has a different influence on the fat mass of mouse (Guo et al., 2009), thus the expression pattern of *MSTN* might be linked to their tissue-specific functions. The lower level of both *MSTN* and *CAST* in the *longissimus* muscle of yak calf relative to adult yak (Figure 2) might imply that the *longissimus* muscle growth is less inhibited in yak calf compared to adult yak, which is in accordance with the faster growth rate of young animals. In this study we also observed that the *longissimus* muscle of adult yak contained a lower mRNA level of *MSTN* than in cattle and a similar level of *CAST*. Because both *MSTN* and *CAST* are negative regulators of skeletal muscle growth (Barnoy et al., 1997; Lee, 2004), we therefore suggest that *MSTN* and *CAST* in *longissimus* muscle at least are not key factors affecting the body size of yak (smaller than that of cattle). In addition, we observed a high level of *CAST* in yak heart and spleen (Figure 1). This might be related to the control of the normal structure and function of heart and spleen by preventing the uncontrolled activity of Ca^{2+}-dependent protease (Kar et al., 2007).

In recent years, research has been focused on the identification of transcriptional markers for meat traits (Wang et al., 2009; Miao et al., 2010). Differences in the gene expression in muscle from cattle breeds or porcine breeds with different fat deposition capacity have been reported (Lehnert et al., 2007; Liu and Gao, 2010). Guo et al. (2009) found that the inhibition of *MSTN* signalling in skeletal muscle resulted in increased lean mass, reduced fat mass and improved glucose metabolism. In this study, the *MSTN* level was exactly parallel to the IMF content in *longissimus* muscles of the three experimental groups (yak calves, adult yak and cattle). However, the correlation between *MSTN* and meat fat deposition in yak needs further study. On the other hand, hypoxia is the most specific environmental factor for yaks. It is now recognized that acclimation to severe hypoxia decreases the mitochondrial content of muscle fibres, and oxidative muscle metabolism is shifted towards a higher reliance on carbohydrates as fuel and intramyocellular lipid stores are reduced (Hoppeler et al., 2003). Our previous study showed that skeletal muscles of yak exhibited higher glycolytic energy metabolism than those of cattle (Lin et al., 2011). The lower IMF content of yak relative to cattle might be related to the hypoxic adaptation. It is reported that type I (slow) muscle fibre has a lower glycolytic capacity and contains a higher intracellular lipid content than type IIb (fast) muscle fibre (Henckel et al., 1997), and in mice *MSTN* could regulate the fibre-type composition during myogenesis, and a loss of *MSTN* leads to an increase in fast glycolytic fibres (Hennebry et al., 2009). Thus the lower *MSTN* level in the *longissimus* muscle of yak compared to Yellow cattle (Figure 3) might result in an increase in fast glycolytic fibres which have a lower lipid content. However, Chen et al. (2010) demonstrated that *MSTN* regulates glucose metabolism through the *AMPK* signal pathway, promoting glucose consumption and glucose uptake, and increasing glycolysis in skeletal muscle cells. This seems to be inconsistent with the present result, the reason is unclear. More studies concerning the relationship between *MSTN* and energy metabolism are required at cell and whole animal level. The lower IMF content in the *longissimus* muscle of adult yak compared to cattle could be an advantage of yak meat over beef for consumers having problems with obesity.

Tenderness is an economically important trait because it is associated with consumer satisfac-

tion. However, tenderness is a complex trait, determined by the contribution of connective tissue, sarcomere length determined pre-rigor and proteolysis rate during aging, as well as contributions from intramuscular fat and post-mortem energy metabolism (Warner et al., 2010). Jiulong yak is the biggest yak breed in bodyweight and mainly used for meat production, so we analysed two important parameters for meat traits (shear force and IMF), as well as the expression of *CAST* which is correlated with meat tenderness (Morgan et al., 1993). We found that the expression of *CAST* in *longissimus* muscle was significantly higher in adult yaks than in calves (Figure 2). *CAST* is an inhibitor for Ca^{2+}-dependent protease in cells, it can inhibit the degradation of muscle protein and has a negative influence on meat tenderness (Koohmaraie, 1996; Barnoy et al., 1997). Our result on *CAST* expressions in the *longissimus* muscle of yak is consistent with the increased meat shear force with age. However, because meat tenderness is a complex trait and is affected by many factors (Purslow, 2005), it is difficult to be conclusive about the *CAST* function in tenderization of yak meat based on the present experimental data. The shear force and pH values of yak *longissimus* muscle obtained in this experiment are in the normal range of bovine muscle (Ilian et al., 2001; Torrescano et al., 2003). A similar extent of *longissimus* muscle pH decline among yak calves, adult yaks and cattle after aging indicates similar at-death levels of muscle glycogen. Besides, the relationship between tenderness and metabolic capacity which affects muscle fibre types is not well defined (Renand et al., 2001; Nam et al., 2009), and more related researches are needed in yaks.

In conclusion, our experiment indicates that both *MSTN* and *CAST* are widespread in tissues of yak. Their expression in *longissimus* muscle differs between adult yaks and yak calves. Yaks contain lower IMF content, lower level of *MSTN* mRNA and similar level of *CAST* mRNA in *longissimus* muscles compared to Yellow cattle.

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