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Expression analysis of myostatin and its receptor during the growth of adipose and muscle tissues in Meishan pig

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ABSTRACT

Myostatin (*MSTN*), mainly expressed in muscle, inhibits the growth of skeletal muscle and regulates the formation of adipose tissue. This study examined the expression of *MSTN*, activin-receptor-type II B (*ACVR2B*), peroxisome proliferator-activated receptor (*PPAR*) *g* and adipocyte specific fatty acid binding protein (*aP2*) in *longissimus* muscle, back fat and abdominal fat at the stages of 35-day, 2-month, 3-month, 4-month and 6–month Meishan pig respectively, using quantitative real-time PCR methods. Our results showed that the expressions of *MSTN* and its receptor (*ACVR2B*) were lower in adipose tissue (back fat and abdominal fat) than in muscle at all stages. Both *MSTN* and its receptor had different expression patterns in *longissimus* muscle, back fat and abdominal fat. *MSTN* had a weak correlation with *aP2* and *PPARg* in *longissimus* muscle.The expression of *MSTN* had a positive correlation with *aP2* and *PPARg* in back fat, however that of *MSTN* had a negative correlation with *aP2* and *PPARg* in abdominal fat. Results indicated that those 3 tissues might respond differently at growing stages to MSTN signaling.

Key words: Adipose tissue, ACVR2B, Expression pattern, MSTN, Meishan pig

Fat tissue is mainly composed of adipocytes in the mammalian. As mammalian grows, adipose cells increase in different parts of body including back, abdominal and muscle (Gerbens 2004). Adipose cells are differentiated from preadipocites in the lipogenesis environment and during this process many proteins are involved, such as PPARγ which is an important transcription factor for adipogenesis, and aP2 which is a marker of adipocyte (Rosen 2000, Haunerland and Spener 2004).

As a member of transforming growth factor-β (TGF-β) superfamily, MSTN is primarily expressed in muscle and plays an important role in myogenesis (Thomas *et al.* 2000, Lee 2002). However, McPherron *et al.* (1997) found that *MSTN* could also be detected in fat tissue, indicating that it might have some functions in adipogenesis. In the regulation signaling of MSTN, it always firstly binds to its receptor (ACVR2B), and then activates relative gene expressions on TGF-β like signaling pathway (Rebbapragada *et al.* 2003).

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In vivo, *MSTN* over-expression in the skeletal muscle of transgenic mice has more body fat-mass than wild-type one (Reisz-Porszasz *et al.* 2003). Additionally, its overexpression in adipose tissue results in small and immature adipocytes for transgenic mice (Feldma 2006). Conversely, *MSTN* deletion could reduce adiposity in *MSTN* mice (McPherron *et al.* 2002, Guo *et al.* 2009). Research on 3T3-L1 pre-adiposities found that MSTN inhibited its differentiation though regulating CCAAT/enhancer binding protein (C/EBP) α and PPARγ (Kim *et al.* 2001). These findings proved that MSTN plays an important role in the fat mass.

Patruno *et al.* (2008) showed that the pattern of *MSTN* expression seemed to retain its myogenic inhibitory in pig skeletal muscles. In addition, Allen *et al.* (2008) showed that obesity might be accompanied by perturbations in expression of the MSTN signaling system. However, little information is reported about the expression of *MSTN* with the growth of pig adipose tissue. As the knowledge on the expression pattern of *MSTN* in fat tissue could help us recognize the probable influence of MSTN on the growth of adipose tissue, it is of importance to know the expression pattern. In addition, the expression patterns of *PPARγ* and *aP2* might reflect the growth of adipose tissue in transcriptional level as they are usually used as markers of fat (Rosen 2000, Haunerland and Spener 2004, Luo *et al.* 2009).

Thus, in the present research, we used real-time PCR to

detect the expressions of *MSTN* and *ACVR2B* in the *longissimus* muscle, back fat and abdominal fat of Meishan pig, a typical fatty type breed, at the stage of respectively 35-day, 2-month, 3-month, 4-month and 6-month; and then examined the expression of *PPARγ* and *aP2* to reflect adipogenesis in those tissues. Our results provided information available to recognize probable influence of MSTN on the growth of adipocyte tissue.

MATERIALS AND METHODS

Experimental animals and samples: Meishan pigs of different periods (35-day, 2-month, 3-month, 4-month and 6-month) were humanely slaughtered at the slaughterhouse according to protocols approved by Biological Studies Animal Care and Use Committee, Hubei Province, P R, China. The tissue samples were immediately removed with care from *longissimus* muscle, subcutaneous adipose and abdominal adipose, and then these samples were frozen in liquid nitrogen, and stored at –70 °C for further use.

RNA isolation and reverse transcription: Total RNA was extracted from the skeletal muscle, subcutaneous adipose tissue and abdominal adipose tissue samples of Meishan pig according to the protocol of TRIzol reagent manufacturer. RNA quality was checked by 1.5% agarose gel electrophoresis and stained with 1 μg/ml ethidium bromide.

One microgram of total RNA was treated with DNase I to remove the contaminating genomic DNA, it was reverse transcribed into cDNA using the reverse transcription kit according to the protocol. The cDNA stocks were stored at –70°C until analysis. β-actin which span an intron sequence, was used to verify the efficiency of the reverse transcription and to exclude genomic DNA contamination. The primer sequences were as follows: forward primer, 5'-CCAGGTCATCACCATCGG and reverse primer, 5'-CCGTGTTGGCGTAGAGGT (Luo *et al.* 2009).

Real-time PCR analysis: Quantitative real-time PCR was run on real-time PCR thermalcycle. Real-time PCR system consisted of 10.0 μl of 2 × SYBR Green qPCR mix, 0.5 μl of cDNA, 8.9 μl of double distilled water, and 0.6 ml of primer pairs (10 μM forward and 10 μM reverse) in a total volume of 20 μl. Negative controls were performed by replacing cDNA with double distilled water. The PCR parameters were as follows: denatured at 95 °C for 2 min, and then denatured at 95°C for 20 s, annealed at 58 °C for 20 s, and extend at 72°C for 20 s, which were performed for 45 cycles. The melting curve program was measured for its fluorescence with a heating rate of 2.5°C /s from 57°C to 95°C continuously. And all samples were measured in triplicate. The sequences of the quantitative real-time PCR primers were as follows: *MSTN* forward primer, 5'-CCAGAGAGATGACAGCAGTGATG and reverse primer, 5'-TTCCTTCCACTTGCATTAGAAGATC (Patruno *et al.* 2008). Primer sequences for *ACVR2B* included forward primer, 5'-ACAGATTACCTCAAGGGGAACA and reverse primer, 5'-CCTGGCTCAAACCGAACA (Zhang *et al.* 2008). Primer sequences for *PPARγ* included forward primer, 5'-AGCCCTTTGGTGACTT and reverse primer, 5'-AGGACTCTGGGTGGTT (Luo *et al.* 2009). Primer sequences for *aP2* included forward primer, 5'-CCTGGTACAGGTGCAGAA and reverse primer, 5'-TGTCGGGACAATACATCC (Luo *et al.* 2009). *β-actin* was used as the reference gene to normalize quantification of an mRNA target. The relative mRNA expression levels of target genes were calculated as fold changes of threshold cycle (Ct) value relative to reference using the $2^{-\Delta\Delta Ct}$ method. The Ct values of all the samples were calculated the mean Ct of triplicate reactions. ΔCt=Ct (target gene) - Ct (reference gene), the samples were normalized to the calibrator, ΔΔCt=ΔCt (target gene) - ΔCt (calibrator). Calibrator was an untreated control or a particular stage of development; it was the basis of comparative results (Livak and Schmittgen 2001). In the present study, the muscle sample of 2 month stage was selected as the calibrator sample to evaluate the putative differential mRNA expression of target gens before and after-ablactating development.

Data analysis: All the data were analyzed using SPSS software 13.0 for Windows. Data were presented as means±SE. Statistical differences in *MSTN*, *ACVR2B, aP2* and *PPARγ* in the back fat, abdominal fat and *longissimus* muscle of Meishan pig at different developmental stages were analyzed by one-way ANOVA and Tukey's post-tests. The pearson-coefficient correlation (r) of *MSTN* and *ACVR2B* expressions with *aP2* and *PPARγ* were calculated in 3 tissues, respectively. Results were considered significantly different at $P < 0.05$.

RESULTS AND DISCUSSIONS

Our results demonstrated that the expressions of *MSTN* (Fig. 1a) and *ACVR2B* (Fig. 2a) in *longissimus* muscle decreased gradually from the day 35th to the third month point, and then increased. MSTN binds to its receptor (ACVR2B) to negatively regulate myogenesis (Thomas *et al.* 2000, Rebbapragada *et al.* 2003). In addition, Patruno *et al.* (2008) showed that the pattern of *MSTN* expression seemed to retain its myogenic inhibitory in pig skeletal muscles. Furthermore, both *MSTN* (r, 0.24; 0.071) and *ACVR2B* (r, –0.149; –0.147) had a weak positive correlation ($r < 0.3$) with *aP2* and *PPARγ* in *longissimus* muscle which indicated that MSTN may not influence the adipogenesis in this tissue. Therefore, we conjecture that the decrease may be related to *longissimus* muscle growth before the third month point, and the increase may be related to the slowing down of *longissimus* muscle growth after the third month stage.

The present study also demonstrated that *MSTN* and its receptor could be detected in the back fat (Fig. 1b and 2b) and abdominal fat (Fig. 1c and 2c), but were lower than those in the *longissimus* muscle (Figs. 1a, 2a) at all periods. The

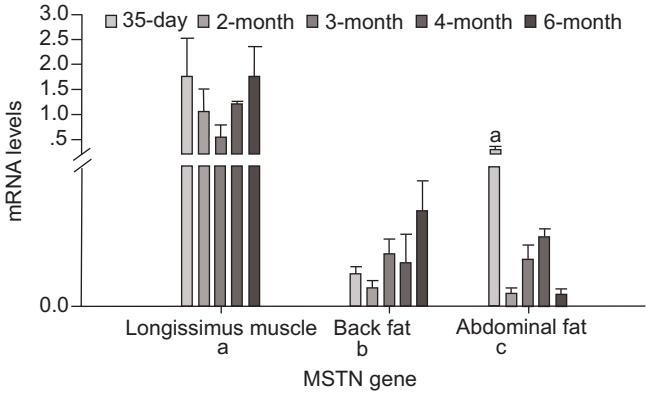

Fig 1. *MSTN* mRNA expression during the growth of Meishan pig *longissimus* muscle, back fat and abdominal fat. MSTN expression was measured by real-time PCR analysis. Data plotted represent the mean±SE (n = 4). Statistical differences of *MSTN* were analyzed in the same tissues. Values with different superscripts are significantly different (P < 0.05).

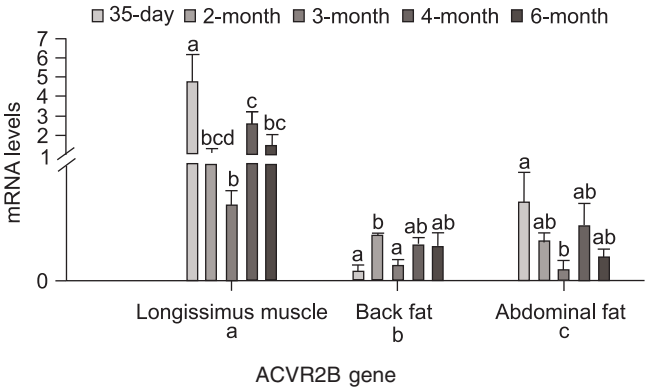

Fig 2. *ACVR2B* mRNA expression during the growth of Meishan pig *longissimus* muscle, back fat and abdominal fat. *ACVR2B* expression was measured by real-time PCR analysis. Data plotted represent the mean±SE (n = 4). Statistical differences of *ACVR2B* were analyzed in the same tissues. Values with different letter superscripts are significantly different (P< 0.05).

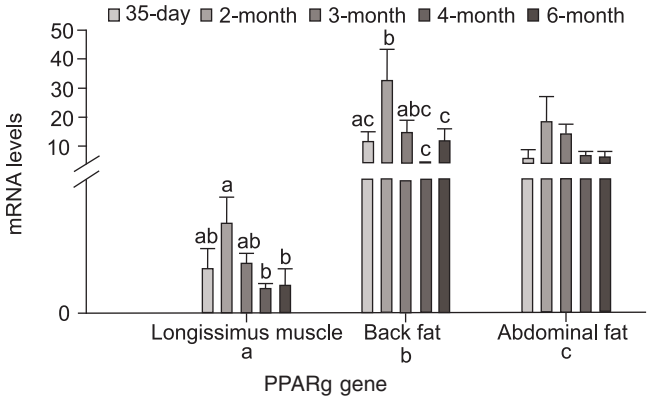

Fig 3. *PPARg* mRNA expression during the growth of Meishan pig *longissimus* muscle, back fat and abdominal fat. *PPARg* expression was measured by real-time PCR analysis. Data plotted represent the mean±SE (n = 4). Statistical differences of *PPARg* were analyzed in the same tissues. Values with different superscripts are significantly different (P< 0.05).

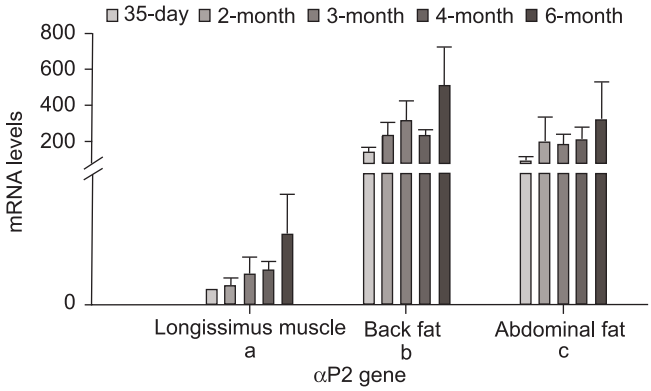

Fig 4. *αP2* mRNA expression during the growth of Meishan pig *longissimus* muscle, back fat and abdominal fat. *αP2* expression was measured by real-time PCR analysis. Data plotted represent the mean±SE (n = 4). Statistical differences of *αP2* were analyzed in the same tissues.

results were similar to the results of Allen *et al.* (2008).

We found that *MSTN* (Fig. 1b) showed an increased expression pattern, and *ACVR2B* (Fig. 2b) also increased gradually except the highest point at the stage of 2-month in the back fat. Allen *et al.* (2008) found that *MSTN* could significantly be increased in response to genetic and dietary obesity. The expression of *αP2* (Fig. 4b) also showed an increased expression pattern in the back fat, indicating that the obesity is increased with the growth of back fat, this is according to growth of adipose tissues. In addition, *MSTN* had a positive correlation with *αP2* (r = 0.783) and *PPARγ* (r = 0.481) in this tissue (P< 0.05), and *ACVR2B* had a positive correlation with *αP2* (r = 0.353, p = 0.181) and *PPARγ* (r = 0.348, p = 0.187). Thus, we could conjecture that the increased expression patterns of *MSTN* and *ACVR2B* in the back fat may be due to MSTN response to obesity in the growth period.

It is believed that MSTN can inhibit the differentiation of pre-adiposities through inhibiting PPARγ and CCAAT/enhancer-binding-protein (C/EBP) alpha (Kim *et al.* 2001). Our study found that the expressions of *PPARγ* (Fig. 3c) at 2-month and 3-month old were higher than that at the stage of 35-day, and *αP2* (Fig. 4c) was increased with the growth of abdominal fat tissue.Furthermore, *MSTN* had a negative correlation with *αP2* (r = –0187) and *PPARγ* (r = –0.292), and *ACVR2B* had a negative correlation with *PPARγ* (r = –0.028). However, the expressions of *MSTN* (Fig. 1c) and *ACVR2B* (Fig. 2c) at the stage of 35-day were higher than that at other stages (P< 0.05). Adiposities of abdominal fat tissue may has better growth at other stages than that at 35-day.

We noticed that the expression patterns of *MSTN* and its receptor in abdominal fat were not the same with that in the back fat, indicating that the role of MSTN in abdominal fat

may be different from that in back fat. Abdominal fat and back fat have different metabolic rates and depositions of triglyceride ratio. When deposition of triglyceride ratio is more than consumption, fat would be massed in tissue (Bjorntorp 1996, Gerbens 2004). Pethick *et al.* (2004) had shown that abdominal depot fat was prior to back fat. In addition, MSTN can influence metabolic. For example, the over expression of *MSTN* in adipose tissue resulted in the phenomenon that glucose uptake and glucose oxidation were increased significantly in adipose tissue of transgenic mice (Feldman *et al.* 2006). Therefore, the differences between the expression patterns of *MSTN* and *ACVR2B* in abdominal fat and back fat may be related to the two fat tissues' different metabolic characters.

We investigated the expression patterns of *MSTN* and its receptor in the growth period of adipose fat and muscle tissue of Meishan pig (fat type pig). Our results showed that *MSTN* and its receptor were much lower in the back fat and abdominal fat than that in *longissimus* muscle at all stages, and the expression patterns of *MSTN* and its receptor in *longissimus* muscle, back fat and abdominal fat were not the same, which may reflect the different responses to MSTN signaling of those tissues. Our research could help to recognize the probable influences of MSTN on the growth of adipose tissue.

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