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Characterization of myostatin gene (*MSTN*) of Pekin duck and the association of its polymorphism with breast muscle traits

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ABSTRACT. Myostatin, encoded by the *MSTN* gene, is a negative regulator of muscle growth, and its expression level in muscle tissue is closely correlated with muscle growth and satellite cell proliferation. To identify the characteristics of the Pekin duck *MSTN* gene and the relationship between its polymorphism and breast muscle traits in Pekin duck, cDNA cloning and analysis and the expression pattern in breast muscle development and polymorphism were performed using molecular cloning, quantitative real-time reverse-transcription polymerase chain

reaction, and molecular marker technology. The results showed that a 1320-bp sequence, including a 93-bp 5'-UTR, 1128-bp CDS, and 99-bp 3'-UTR, was obtained, and two alternative splicing isoforms were detected. The alternative splicing isoforms encoded 375- and 251-amino acid residues. The amino acid sequence of Pekin duck MSTN was similar to other vertebrates and exhibited the highest similarity to chicken. The expression pattern of *MSTN* in breast muscle tissue showed a tendency to increase, except for a slight decrease at 6 weeks. Three single nucleotide polymorphisms were found in the Pekin duck *MSTN* gene by cDNA sequencing from different individuals. The T129C had significant association with breast muscle thickness, and the T952C had significant association with the fossilia ossis mastodi length. This study reveals the molecular characteristics of the Pekin duck *MSTN* gene and the relationship of its polymorphism with breast muscle traits in Pekin duck. Therefore, it can provide some useful basic understanding of MSTN functions.

Key words: Pekin duck; Breast muscle; *MSTN*; Gene expression

INTRODUCTION

The transforming growth factor-β superfamily includes a large group of proteins that are responsible for the growth and development of tissues (Kollias and McDermott, 2008). Myostatin (MSTN), also known as growth differentiation factor 8, belongs to this superfamily. It is highly conserved and possesses all of the structural components common to the family: 9 invariant cysteine residues, an "RXXR" furin-type proteolytic processing site, and a bioactive C-terminal domain (Lee, 2004). A high level of *MSTN* gene expression is detected in skeletal muscle, but a low level of *MSTN* expression is also detected in other tissues including adipose tissue and heart (McPherron et al., 1997; Sharma et al., 1999). Inactivation of the *MSTN* gene in mice (McPherron et al., 1997; Nishi et al., 2002; Mendias et al., 2008) or mutations in the bovine (Kambadur et al., 1997; McPherron and Lee, 1997), canine (Mosher et al., 2007), ovine (Du et al., 2007; Boman and Vage, 2009; Boman et al., 2009, 2010), equine (Dall'Olio et al., 2010; Hill et al., 2010), and human (Schuelke et al., 2004) genes results in a similar phenotype of increased muscle growth. Therefore, it is believed that MSTN is a negative regulator of skeletal myogenesis, and its function is highly conserved during evolution. Functionally, MSTN not only regulates the proliferation and differentiation phases of myoblast growth but also controls the activation and further proliferation of muscle stem cells, which are known as satellite cells (McCroskery et al., 2003).

Many studies have investigated the characteristics or polymorphisms of the *MSTN* gene in different species (Funkenstein et al., 2009; Sjakste et al., 2011; Han et al., 2012; Pan et al., 2012; Zhang et al., 2012a). Zhang et al. (2012b) detected a mutation in exon 1 of the Bian chicken *MSTN*, which had a significant effect on the body weight. Tripathi et al. (2012) found that transient silencing of the *MSTN* gene in chicken embryo fibroblast cells could increase the muscle mass in the transgenic animals. In duck, the alternative splicing sites and developmental expression patterns of the *MSTN* genes were studied during embryo muscle development (Huang et al., 2011).

There is, however, no report on the characteristics of post-hatch Pekin duck *MSTN* and its association with breast muscle traits in post-hatching Pekin ducks. Therefore, the improved knowledge of the full-length clone, expression pattern, and alternate splicing of *MSTN* and its association with breast muscle traits will enhance our understanding of this gene. In our previous study (Xu et al., 2012), the genes differentially expressed in breast muscle of 2- and 6-week-old Pekin ducks were separated and identified using the suppression subtractive hybridization technique. Significant differences in *MSTN* mRNA levels were observed between the two breast development stages, suggesting its potential role as a candidate gene for breast developmental traits. To confirm the function of the Pekin duck *MSTN* gene and to further investigate its characteristics, the cDNA of the gene was cloned, and the expression pattern during breast development was analyzed by quantitative real-time RT-PCR (qRT-PCR). The single nucleotide polymorphisms (SNPs) and their association with economic traits of Pekin duck were examined in our present study. The results of this paper should provide further understanding of the molecular mechanism of the *MSTN* gene in Pekin duck.

MATERIAL AND METHODS

Animal and tissue sampling

Fifty healthy Pekin ducks (5 male and 5 female ducks, aged postnatal day 1, and 2, 4, 6, and 8 weeks old, respectively) were selected to collect breast muscle samples for qRT-PCR analysis. All samples were collected aseptically and immediately stored in liquid nitrogen until use.

At 6 weeks of age, 368 healthy Pekin ducks were selected to measure breast width and fossilia ossis mastodi length (FL) using a vernier caliper (precision = 0.01 cm) and to measure breast muscle thickness (BMT) by supersonic scan (precision = 0.01 cm). At the same time, venous blood was collected from the jugular vein to extract genomic DNA.

Total RNA extraction and 1st cDNA synthesis

Total RNA was isolated from all samples using an RNAiso plus kit (TaKaRa, Dalian, China). The RNA quality was analyzed by 1.0% agarose gel electrophoresis and spectrophotometric absorption at 260 nm in a Nanodrop ND-1000® spectrophotometer (NanoDrop, USA).

The total RNA of breast muscle were treated with DNase I recombinant (Roche, Shanghai, China) and reverse transcribed to 1st cDNA using a PrimeScript RT reagent kit (TaKaRa) following manufacturer instructions.

qRT-PCR analysis

The acquired single-stranded cDNA was diluted three times and assayed as the template by qRT-PCR using MSTN-1 primers (Table 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize variations in the amount of starting material. qRT-PCR was performed with the SYBR® Premix *Ex Taq*™ II (TaKaRa) in an iQ™ real-time PCR detection system (Bio-Rad) in triplicates. Dissociation curve analysis was performed after each qRT-PCR experiment to ensure that there was only one product.

Table 1. Information of primers used in this study.

Primer name	Primer sequence	Tm (°C)	Product length (bp)
GAPDH	5'-GCCCAGAACATTATCCCAG-3'	55.0	94
	5'-AGCCCATTCCGGTAAAGC-3'	57.5	
MSTN-1	5'-CTGGTATTTGGCAGAGTATTG-3'	52.9	108
	5'-CTCGTCCATTCTCATCAAAAG-3'	54.9	
MSTN-2	5'-AGTCAGCCCACAGAGAACG-3'	55.3	1041
	5'-CCCGCAACGATCTACAACC-3'	58.6	
MSTN-3	5'-GATTTTGGCCTTGACTGCGATG-3'	64.4	176
	5'-TGAGTGTGCGGGTATTTCTGCA-3'	63.8	
MSTN-4	5'-AGGCACTGGTATTTGGC-3'	50.6	139
	5'-TGGTCCTGGGAAAGTTAC-3'	49.6	
MSTN-5	5'-AGTCAGCCCACAGAGAACG-3'	55.3	125
	5'-TGTTCCAGGCGCAGTTTG-3'	58.3	
MSTN-6	5'-AAGCCGTCTCTCAGATTGC-3'	54.9	327
	5'-GTTAGGTGCTTGTTCCAGG-3'	52.7	
MSTN-7	5'-ACCAAGCGAATCCTAGAG-3'	52.6	262
	5'-ATGTTGGCAATGCCTAGC-3'	53.9	

GAPDH = glyceraldehyde-3-phosphate dehydrogenase; MSTN = myostatin; Tm = melting temperature.

Clone and sequencing of Pekin duck *MSTN* coding sequence (CDS)

The Pekin duck *MSTN* CDS was cloned by RT-PCR by using the MSTN-2, MSTN-6, and MSTN-7 primers (Table 1). The PCR products were cloned into pMD-19T plasmid vector (TaKaRa) according to manufacturer instructions and transformed into *Escherichia coli* DH5α. The negative clones were sequenced by Sangon Biotech Co. Ltd. (Shanghai, China).

SNP screening and genotyping

The Pekin duck *MSTN* cDNA was cloned by RT-PCR using the MSTN-2 primers (Table 1) and then sequenced. The obtained sequences were aligned using SeqMan (DNAStar software) to screen SNPs, based on the differences between sequences. The restriction endonuclease sites that harbored an SNP were detected with the Primer Premier 5.0 software to design the genotyping protocols. Two specific primer pairs (MSTN-3 and MSTN-4) were designed to judge the genotypes by PCR-restriction fragment length polymorphisms (RFLP), and one specific primer pair (MSTN-5) was designed to assess the genotypes by asymmetry PCR-based single-strand conformation polymorphism (SSCP), as described by Zhang et al. (2008).

Association analysis

General linear model procedures of the SAS® software package (SAS Inst. Inc., Cary, NC, USA) were used to determine associations between genotypes and breast muscle traits, according to the following model: $Y_{ij} = \mu + S_i + G_j + \varepsilon_{ij}$, where Y_{ij} is the observed value of breast muscle traits, μ is the population mean, S_i and G_j are the sexual effects and genotype, respectively, and ε_{ij} is the random error. Values were considered significant at $P < 0.05$ and are reported as least-squares means ± standard error.

Statistical analysis

The relative gene expression level was determined by the comparative cycle threshold (CT) method. The Δ^{CT} value was calculated by subtracting the target CT of each sample from its GAPDH CT value. Gene expression at different stages was analyzed by one-way ANOVA followed by the Bonferroni test for pairwise comparison.

RESULTS

Molecular cloning and sequencing of Pekin duck *MSTN* mRNA

After sequencing and assembling of the fragments, the total coding sequence of *MSTN* was obtained and two alternative splicing isoforms were found (Figure 1). The first alternative splicing isoform (isoform 1) contains the 5'-untranslated regions (5'-UTR, 93 bp), CDS (1128 bp), and 3'-UTR (99 bp), and encodes 375-amino acid residues. The second alternative splicing isoform (isoform 2) loses 180 nucleic acids in the CDS and encodes 251-amino acid residues.

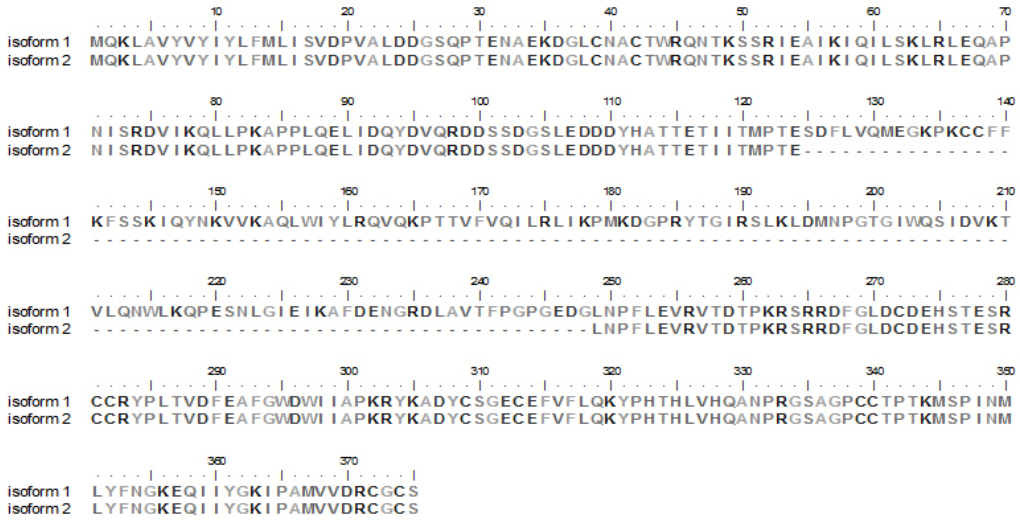

Figure 1. Alignment of *MSTN* alternative splicing isoforms in amino acid sequences.

The Pekin duck MSTN amino acid sequence shared 98.4, 92.5, 92.0, 91.2, 88.8, and 80% similarity with chicken, human, pig, rat, cattle, and goat amino acid sequences, respectively. This indicates that *MSTN* was highly conserved in molecular evolution. The phylogenetic tree was constructed using the BioEdit software (Figure 2) and showed that duck had a closer genetic relationship with chicken than with other species.

Expression pattern of *MSTN* during the development of chest muscle in Pekin duck

The expression of *MSTN* showed an ascending tendency from 1 day post-hatch to 4

weeks of age (Figure 3). *MSTN* expression levels at 4 and 8 weeks of age were significantly higher ($P < 0.05$) than at 2 weeks and 1 day post-hatch. It was noticed that the expression of *MSTN* decreased significantly at 6 weeks of age.

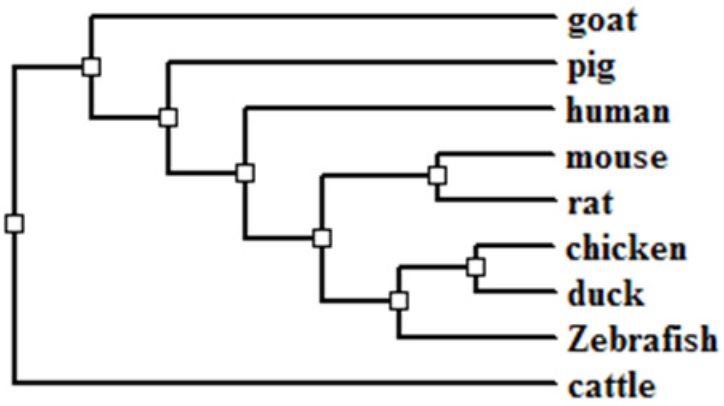

Figure 2. Phylogenetic tree of *MSTN* protein amino acid sequences.

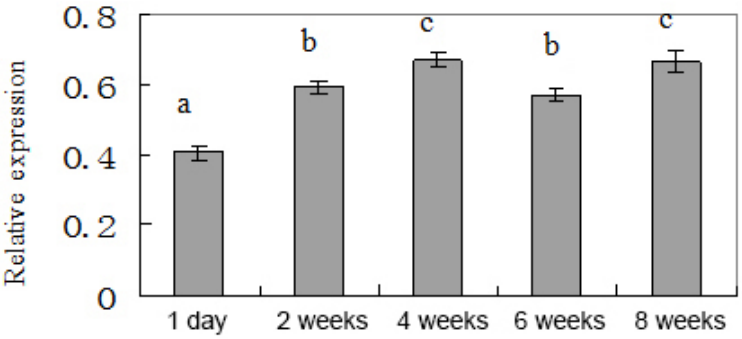

Figure 3. Expression of *MSTN* during the development of breast muscle in Pekin duck. Treatments headed by different letters denote significant difference ($P > 0.05$) and thereby the same letter denotes no significant difference ($P > 0.05$).

SNP screening and genotyping

Three SNPs were found in the Pekin duck *MSTN* gene by alignment of the PCR sequences from different individuals. The first SNP was positioned at 129 bp for the T/C mutation (Figure 4) in the open reading frame (ORF). Genotyping of 368 Pekin ducks was tested by asymmetry PCR-SSCP, and three genotypes (AA, AB, BB) were found in this population (Figure 5). The second SNP was located at 708 bp for the T/C mutation (Figure 6) in the ORF; this SNP was genotyped by *Bgl*II-RFLP analysis. The 139-bp PCR product was digested into two fragments (113 and 26 bp) at the T allele (Figure 7 and Table 2). The third SNP was located at 952 bp for the T/C mutation (Figure 8) in the ORF and was genotyped by *Pst*I-RFLP

analysis. The 176-bp PCR product was digested into two fragments (155 and 21 bp) at the C allele (Figure 9).

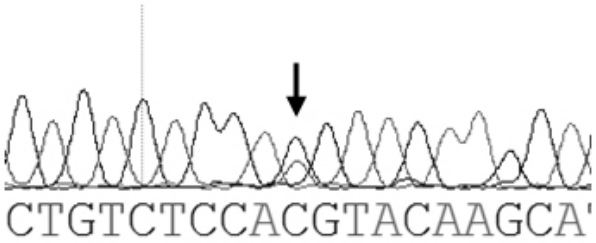

Figure 4. T/C mutation at the position of 129 bp of the open reading frame.

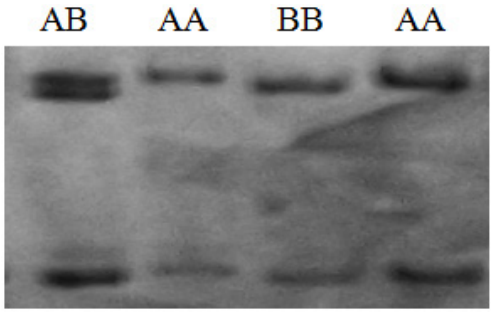

Figure 5. Results of asymmertry PCR-SSCP analysis.

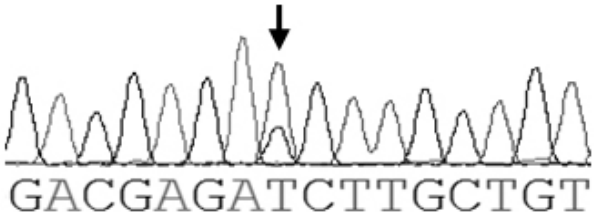

Figure 6. T/C mutation at the position of 708 bp of the open reading frame.

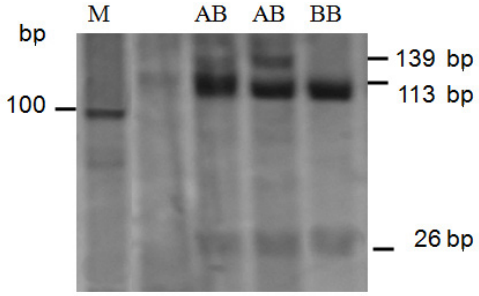

Figure 7. Results of *Bgl*II-RFLP.

Table 2. Genotypic frequencies, allelic frequencies, and Hardy-Weinberg equilibrium tests.

SNP	Observed genotypes (number)			Total	Allelic frequencies		χ^2 (HWE)
	AA	AB	BB		A	B	
T129C	58	185	41	284	0.53	0.47	26.85**
T708C	173	86	27	286	0.76	0.24	9.96**
T952C	-	72	201	273	0.13	0.87	6.30*

HWE = Hardy-Weinberg equilibrium. **The allelic frequencies were also not in HWE ($P < 0.01$). *The allelic frequencies were also not in HWE ($P < 0.05$).

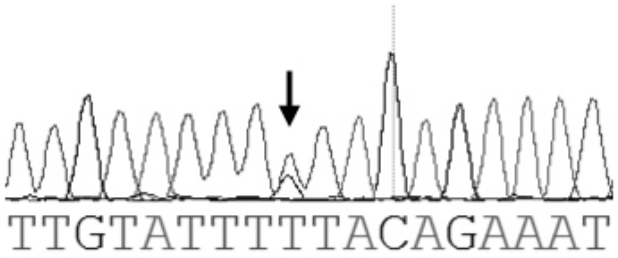

Figure 8. T/C mutation at the position of 952 bp of the open reading frame.

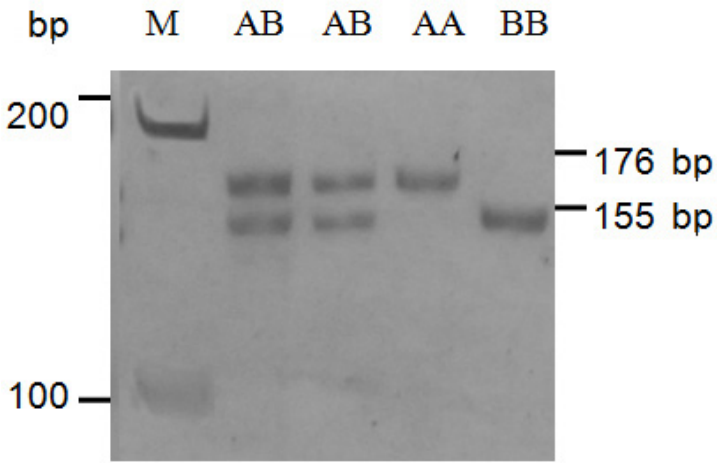

Figure 9. Results of *Pst*I-RFLP. The fragments of 21 bp moved out of the polyacrylamide gel.

Association analysis of the *MSTN* gene with breast muscle development in Pekin duck

Table 2 shows the results of genotypic frequencies, allelic frequencies, and Hardy-Weinberg equilibrium tests in the Pekin duck population. The distribution of the three genotypes was not within Hardy-Weinberg equilibrium in the population tested ($P < 0.05$).

The association of specific genotypes with breast muscle traits was analyzed by the

least-squares method as applied in the GLM procedure of SAS (SAS Institute Inc.) (Table 3). Three SNP mutation sites were found in the Pekin duck *MSTN* gene by cDNA sequencing. At the T129C SNP site, ducks with the BB genotype had significantly wider BMT (1.75 ± 0.11 cm) than those with the AA genotype (1.67 ± 0.12 cm; $P < 0.05$). At the T952C SNP site, ducks with the AB genotype had significantly longer FL (14.51 ± 0.71 cm) than those with the BB genotype (14.29 ± 0.76 cm; $P < 0.05$).

Table 3. Association of specific genotypes with breast development traits.

SNP	Genotypes	BW	FL	BMT
T129C	AA	10.58 ± 0.55	14.26 ± 0.78[a]	1.67 ± 0.12[a]
	AB	10.65 ± 0.56	14.34 ± 0.73[a]	1.70 ± 0.13[a]
	BB	10.62 ± 0.49	14.32 ± 0.85[a]	1.75 ± 0.11[b]
T708C	AA	10.63 ± 0.55	14.29 ± 0.76[a]	1.70 ± 0.12[a]
	AB	10.64 ± 0.55	14.41 ± 0.79[a]	1.69 ± 0.12[a]
	BB	10.68 ± 0.63	14.31 ± 0.63[a]	1.68 ± 0.15[a]
T952C	AB	10.70 ± 0.56	14.51 ± 0.71[a]	1.70 ± 0.13[a]
	BB	10.65 ± 0.55	14.29 ± 0.76[b]	1.71 ± 0.13[a]

BW = breast width; FL = length of fossilia ossis mastoid; BMT = breast muscle thickness. Values within the same SNP locus with different superscript letters indicate a significant difference between them ($P < 0.05$).

DISCUSSION

The quantitative traits are usually affected by many genes, and the effects of an individual gene only account for a small portion in the observed genetic variance. However, the *MSTN* gene seems to be a great negative regulator of skeletal muscle growth in mammals. Mutations of the *MSTN* gene result in increased muscle mass; in particular, the phenomenon known as "double muscling" found in several cattle breeds is associated with natural mutations in the *MSTN* gene coding sequence (Berry et al., 2002; Dunner et al., 2003; Casas et al., 2004). Mutations of the *MSTN* gene also affect the performance in poultry. Seventeen SNPs have been detected in the fowl *MSTN* gene, indicating that this gene is highly polymorphic. The *MSTN* gene polymorphisms have effects on multiple traits, and the mutations in regulatory regions have been shown to be associated with abdominal fat weight, abdominal fat percentage, birth weight, breast muscle percentage, and breast muscle weight in chickens (Gu et al., 2004). The mutations in the 5'-UTR of the *MSTN* gene have been reported to affect the breast meat percentage in Pekin duck (Lu et al., 2011). In this study, the mutations of the *MSTN* gene not only affected BMT but also the FL, indicating that the *MSTN* affected multiple traits in duck.

MSTN gene expression levels in muscle tissue are closely correlated with muscle growth and satellite cell proliferation (McPherron et al., 1997). The overexpression of *MSTN* in chicken leads to an exhaustion of the muscle progenitor population that ultimately results in muscle hypotrophy (Rehfeldt et al., 2008). During postnatal growth, the increased muscle mass is mainly due to muscle hypertrophy and is accompanied by the proliferative activity of satellite cells, thus resulting in an increased DNA content, and further provides the machinery for protein deposition (Rehfeldt et al., 2000). The expression of the *MSTN* gene increases after birth, which inhibits myoblast cell proliferation and differentiation, and maintains the satellite cells in a quiescent state by controlling cell cycle progression (Kambadur et al., 1997). *MSTN* expression showed a dramatic decrease at 1 day post-hatch in turkey skeletal muscle (Sporer

et al., 2011). This finding was consistent with the results of our study, in that the *MSTN* gene showed a high expression level during postnatal growth. It is noticed that the expression of *MSTN* decreased at 6 weeks of age, which agrees with the fact of the fastest breast muscle growth of Pekin duck at this stage. This finding also indicated that *MSTN* expression was precisely regulated during the postnatal skeletal muscle development. Treatment of chicken fetal myoblasts with the MSTN protein altered the expression of genes involved in myogenic differentiation, cell architecture, energy metabolism, signal transduction, and apoptosis (Yang et al., 2005), which regulated the balance between the proliferation and differentiation of embryonic muscle progenitors by promoting their terminal differentiation through the activation of p21 and MyoD (Velleman, 2007; Manceau et al., 2008). MicroRNAs (miRNAs) are small 19 to 24 nt regulatory RNAs that generally modulate gene expression through translational repression or by causing deadenylation and degradation of target mRNAs. The miR-1, miR-206, and miR-499 are involved in the expression regulation of the *MSTN* gene (Clop et al., 2006; Zhou et al., 2009; Li et al., 2011), and this created a bridge from miRNAs to the skeletal muscle development regulation.

The *MSTN* gene has different genetic characteristics in vertebrates: sole gene in mammals, gene duplication in fish, and alternative splicing in birds. Four alternatively spliced isoforms of *MSTN* mRNA have been found in skeletal muscle tissue of Pekin duck (Huang et al., 2011). The *MSTN*-a and *MSTN*-b isoforms have significantly higher expressions than *MSTN*-c and *MSTN*-d, suggesting that they play the major role during embryo muscle development. In this study, isoform 1 was found to be homologous with *MSTN*-a, and isoform 2 was homologous with *MSTN*-b. The *MSTN*-c and *MSTN*-d isoforms were not found in breast muscle tissue for the main reasons of very low expression level or different muscle development stages.

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