

# Single nucleotide polymorphisms in the upstream regulatory region alter the expression of myostatin

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**Abstract** The expression of the gene encoding myostatin (*MSTN*), the product of which is a negative regulator of skeletal muscle growth and development in mammals, is regulated by many cis-regulatory elements, including enhancer box (E-box) motifs. While E-box motif mutants of *MSTN* exhibit altered expression of myostatin in many animal models, the phenotypes of these mutations in chicken are not investigated. In this study, we cloned and sequenced the full encoded DNA sequence of *MSTN* gene and its upstream promoter region in Wenshang Luhua chicken breed. After analysis of the sequence, 13 E-box motifs were identified in the *MSTN* promoter region, which were denoted by E1 to E13 according to their positions in the region. Although many single nucleotide polymorphisms (SNPs) were revealed in the *MSTN* promoter region, only two SNPs were in the E-boxes, i.e., the first nucleotide of the E3 and the fifth nucleotide of E4. The effects of these two polymorphisms on the expression of *MSTN* gene were explored both with *MSTN*-GFP reporter constructs in vitro and real-time PCR in vivo. The results suggested that the E-boxes in the chicken *MSTN* promoter region are involved in the regulation of myostatin expression and the polymorphisms in E3 and E4 altered the expression of myostatin.

**Keywords** Myostatin · Promoter · E-box · Expression · Single nucleotide polymorphisms

## Introduction

Myostatin (*MSTN*, also known as growth differentiation factor 8) is a member of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily (Sharma et al. 1999), which is well recognized as a negative regulator of skeletal muscle mass in mammalian species (Elliott et al. 2012). Mutations in *MSTN* acquired during early development may cause both hypertrophy and hyperplasia in adult skeletal muscle fibers (McPherron et al. 1997; Hamrick et al. 2000; Gentry et al. 2011). In addition to muscle, the expression of *MSTN* occurred in many tissues, e.g., liver, spleen, kidney, lung, and placenta etc. (Garikipati et al. 2006; Mitchell et al. 2006; Arai and Nishiyama 2007; Peiris et al. 2010; Jiao et al. 2011; Zheng et al. 2011), which suggests that the myostatin may have additional functions other than myogenesis (Argiles et al. 2012). In chicken, *MSTN* is expressed ubiquitously and most strongly in the testis and ovary (Kubota et al. 2007), and myostatin seems to have some important roles in the development. The deficiency of myostatin induced by the treatment using myostatin-specific small-interfering RNA delays cell differentiation and causes significant alterations in the cellular morphology of chicken embryonic myotubes (Sato et al. 2006).

The *MSTN* gene is highly polymorphic (Stratil and Kopečný 1999; Hill et al. 2010; Sjakste et al. 2011; Han et al. 2012; Sanchez-Ramos et al. 2012; Zhu et al. 2012), and many polymorphisms are breed specific. Seven single nucleotide polymorphisms (SNPs) in *MSTN* were identified in broiler and/or layer chicken lines (Baron et al. 2002), and four SNPs were identified in the *MSTN* of Bian chick (Zhang et al. 2011a; Zhang et al. 2012). Further studies on the growth traits show that the SNPs in chicken *MSTN* may

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affect the abdominal fat weight and percentage, breast muscle weight and percentage, birth weight, and adult weight (Gu et al. 2004; Zhang et al. 2011a, b, 2012). At the same time, in many species, polymorphisms in the non-coding region of *MSTN* can also cause similar effects comparing with the phenotypes of the mutations in the *MSTN* coding region (Clop et al. 2006; Zhang et al. 2012), especially those in the enhancer box (E-box) motif (CANNTG), which is considered as one of the critical regulatory components in muscle gene expressions (Spiller et al. 2002; Salerno et al. 2004; Du et al. 2007; Funkenstein et al. 2009). Moreover, E-box has been shown to participate in the regulation of many myogenic factors (Dechesne et al. 1994; Jane et al. 2002; Heidt et al. 2007), e.g., the E-box promoter elements are suggested to induce the expression of cathepsin B gene during human myoblast differentiation (Jane et al. 2002).

The effects of *MSTN* E-box motif mutants on the expression of myostatin are well recognized in many animal models, including sheep, fish, mouse, and bovine (Spiller et al. 2002; Salerno et al. 2004; Du et al. 2007; Funkenstein et al. 2009), while the number and position of E-boxes show great varieties among different species. In the bovine *MSTN* upstream region, ten E-boxes locate in three clusters (Spiller et al. 2002), and seven E-boxes have been identified in murine *MSTN* promoter region, among which the E-box 5 (E5) plays a role in regulating the promoter activity in murine (Salerno et al. 2004). However, little is known about the distribution and function of E-boxes in chicken *MSTN* promoter region. Some SNPs in the *MSTN* upstream region have been described in chicken (Zhang et al. 2012), while none of them is in the E-box motif.

The Wenshang Luhua chicken breed (*Gallus gallus*) originally lived in a few ranges in Shandong province of China, mainly in Wenshang county, and it has been recognized as a domestic bird with good economic traits. As a result of competition by exotic breeds, Wenshang Luhua chicken is now an endangered breed. In this study, we sought to identify the E-box motifs in Wenshang Luhua chicken *MSTN* promoter region, determine the SNPs in the E-boxes and investigate the potential effects of these SNPs on the *MSTN* expressions.

## Materials and Methods

**Cloning *MSTN* and its upstream region.** The chickens (breed name: Wenshang Luhua, *G. gallus*) used in this study were bought from Poultry Institute, Shandong Academy of Agricultural Science, China. All chickens were housed, fed, and sacrificed as per the local standards for animal welfare issues. This study was approved by the Animal Welfare Committee of Shandong University (ECAESDUSM 20123010). The genomic DNA was isolated from the leg

of 22 hatched 11-d chickens using the TIANamp Genomic DNA kit (Tiangen, Beijing, China). The full encoded *MSTN-Wlc* gene (*MSTN* gene in Wenshang Luhua chicken) flanking regions of each exon was amplified and cloned as described previously (Gu et al. 2004). The 2.3-kb wild-type *MSTN* upstream promoter fragment was amplified by PCR performed in a 50- $\mu$ l reaction mixture containing 1  $\mu$ g of genomic DNA, with the following PCR parameters: 33 cycles of (94°C for 40 s, 55°C for 40 s, and 72°C 5 min). The primers were Upper-F and Upper-R listed in Table 1. The PCR products were sequenced by Boya Biotechnology Company (Shanghai, China).

**Bioinformatical analysis.** The sequence of *MSTN-Wlc* was compared with the database at the National Center for Biotechnology Information (NCBI) by using BLAST. The promoter sequence of *MSTN-Wlc* was analyzed with Mat Inspector (Cartharius et al. 2005) to predict potential E-box, myocyte enhancer factors 2 (MEF2) factor, glucocorticoid receptor (GRE), muscle-specific Mt-binding site (MTBF) and progesterone response element (PRE). All the currently available promoter sequences of vertebrates *MSTN* were retrieved from NCBI database to conduct the phylogenetic analyses. The nucleoside sequences of the promoters were completely aligned by ClustalX (Chenna et al. 2003) and phylogenetic reconstruction of the promoter sequences was carried out by using distance/neighbor joining (NJ) programs in MEGA v.3.1 software (Kumar et al. 2004). The interior branch length supports were from 1,000 replicates.

**Construction of *MSTNP<sup>W</sup>-GFP*: the green fluorescent protein (GFP) expression vectors driven by the upstream promoter sequences of *MSTN*.** The 2.3-kb *MSTN* promoter fragment was cloned into the T vector system (Promega, Madison, WI) to generate TMSTNP, which was subsequently served as the template for generating other *MSTN* constructs. The PCR parameters were 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min 30 s. The primers were T-F and T-R (Table 1). The GFP gene was amplified from Cam35S-GFP vector (Invitrogen, Carlsbad, CA) using the PCR (30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min ) with the GFP-F and GFP-R primers (Table 1). The PCR was performed to obtain *MSTN* promoter-GFP fusion fragment using *MSTN* promoter fragment and GFP gene as templates, and T-F and GFP-R as primers (Table 1). The PCR parameters were 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 3 min 30 s. The vector pcDNA3.1 (-) (V795-20, Invitrogen) was digested with MluI and BamHI to remove cytomegalovirus (CMV) promoter, and the *MSTN* promoter-GFP fusion fragment with the same digestion was subsequently inserted in the vector to construct *MSTNP<sup>W</sup>-GFP* expression vector.

**Table 1.** Primers used in this study

Primer	Sequence (5'→3')
Upper-F	GGCACTATTATCCAGGTTTCC
Upper-R	GCACAGTCCGTCTTTTCA
T-F	GGCCAGATATACGCGTTTGGCACTATTATCCAGGTTTC
T-R	GCTCACCATGGTGTGATCTTACAGTCACTGCAGCTTTTGG
GFP-F	GACACCATGGTGAGCAAGGGCGAGGAGCTGTT
GFP-R	AGATATCCCCGGATCCTCACTGCAGGAATTCGATTTCAAAGATCTA
E4-F <sup>a</sup>	ATGAGAACATCAAGGTTAGACAACCAAGCCACAGCCTGGAACACACCGAGCACAATACAT
E4-R	CCTTGATGTTCTCATTTGAGCCAAATCCACATGTA
E3-R <sup>a</sup>	CCTTGATGTTCTCATTTGAGCCAAATCCACATGTA
RT-GFP-F	CTCGTGACCACCCTGACCT
RT-GFP-R	TGTAGTTGCCGTCGTCCTTG
RT-MS-F	ATGAGACTGGACGAGATCTTGCT
RT-MS-R	CCGTTTCGGTGTGTCTGTAAGTC
RT-AC-F	CTGATGGTCAGGTCATCACCAT
RT-AC-R	CCACAGGACTCCATACCCAAGA

<sup>a</sup> The *underlined sequences* indicate the E-boxes, i.e., E3-box in primer E3-R and E4-box in primer E4-F, and the mutation sites are labeled with *hollow boxes*

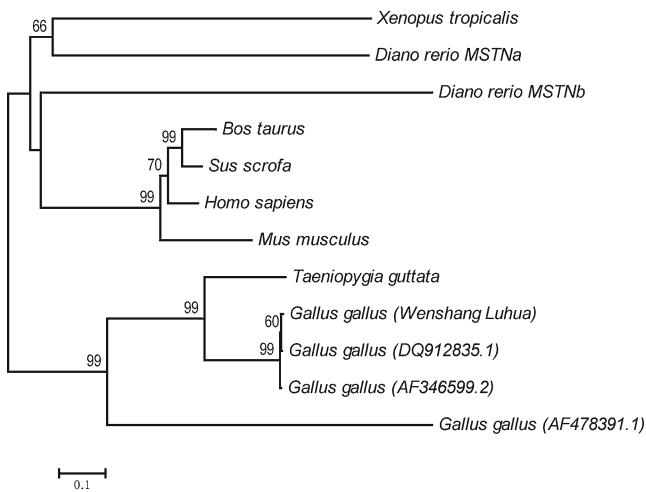
*Construction of vectors MSTNP<sup>M4</sup>-GFP and MSTNP<sup>M3,4</sup>-GFP.* Vectors MSTNP<sup>M4</sup>GFP and MSTNP<sup>M3,4</sup> were constructed with site-specific mutagenesis using the vector MSTNP<sup>W</sup>-GFP as the template. To construct vector MSTNP<sup>M4</sup>-GFP containing full-length *MSTN* promoter with a point mutation in E4-box (CACACG, the sequence of wild-type E4-box is CACATG), PCR was performed in a 50- $\mu$ l reaction mixture containing E4-F and E4-R as primers (Table 1). The PCR parameters were 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 4 min 30 s. The expression vector carrying full-length *MSTN* promoter (8.5 kb) with both mutated E3 (GAAATG, the sequence of wild-type E3-box is CAAATG) and E4-boxes (MSTNP<sup>M3,4</sup>-GFP) was constructed using PCR with primers E4-F and E3-R (Table 1). All the vectors were sequenced to confirm the presence of correct sequences.

*Cell culture and transfection.* Human embryonic kidney (HEK) 293T cells were used in the transfection assays. The cells were plated at a density of  $4 \times 10^4$  cells/well in 24-well cell culture plates and incubated for 18–24 h. The transfection was conducted using DNAfect transfection reagent (CoWin Bioscience, Menlo Park, CA) according to its standard protocol with minor modifications. Plasmid DNA (0.8  $\mu$ g) was diluted in 25  $\mu$ l serum-free medium high-glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco, Carlsbad, CA) to prepare the transduction complexes. The serum-free high-glucose DMEM was used as transfection medium and the incubation time for the cells with complexes was 4–8 h. After transfection, the old

medium was aspirated and 500  $\mu$ l fresh high-glucose DMEM supplied with 10% fetal bovine serum was added, the cells were cultured for additional 48 h, then collected. RNA was extracted from cells using RNAsimple total RNA extract kit (Tiangen) and stored at -20°C for further use.

*Real-time PCR to detect the expression of GFP in vitro.* Real-time PCR was performed to detect the expression of GFP in the transfected cells. The RNA was reverse-transcribed to obtain the cDNA and the expression of GFP was monitored using GFP-specific primers (RT-GFP-F and -R, Table 1) and SYBR Green dye-based detection. The real-time PCR was carried out in a 20- $\mu$ l reaction mixture comprised of 10  $\mu$ l 2 $\times$  SyBr Green Mix, 0.4  $\mu$ l of each primer (10  $\mu$ M), and 2  $\mu$ l template with the following parameters: 95°C for 10 min, followed by 40 cycles of (95°C for 15 s and 60°C for 60 s). The Ct method was used to compare the relative levels of expression of GFP, using a calibration factor of  $2^{(-\Delta\Delta Ct)}$ . The relative starting quantities were calculated by comparison with the sample derived from MSTNP<sup>W</sup>-GFP-transfected cells as the standard. All values were normalized to those for  $\beta$ -actin (ACTB) mRNA (ACTB-specific primers were purchased from CW Biotech, Beijing China), which served as the internal control. All of the samples were measured in triplicates.

*Real-time PCR to detect the expression pattern of MSTN in vivo.* To explore the in vivo function of the E-boxes upstream of *MSTN*, we extracted RNA from the leg of 22



**Figure 1.** Phylogenetic analysis of upstream sequences of *MSTN* from 12 species/breeds. The bar indicated the evolutionary distance. The numbers on branch nodes were percentages of 1,000 sets of bootstrap supports.

hatched 11-d chickens, and monitored the expression pattern of the *MSTN* using real-time PCR. The cDNA was obtained by reverse transcribing the total RNA as described above. The real-time PCR was carried out in 50  $\mu$ l reaction mixtures comprised of 25  $\mu$ l 2X Sybr Green Mix, 1  $\mu$ l primer F (10  $\mu$ M), 1  $\mu$ l primer R (10  $\mu$ M), and 1  $\mu$ l template with the following parameters: 95°C for 10 min, followed by 40 cycles of (95°C for 15 s and 60°C for 60 s). The primers for *MSTN* expression were RT-MS-F and RT-MS-R, and the primers for  $\beta$ -actin as the internal control were RT-AC-F

**Figure 2.** Sequence of the 2.3-kb region upstream of *MSTN-Wlc*. The sequences corresponding to the E-boxes are shown double-underlined and bold. The 13 E-boxes were denoted by E1 to E13, with E1 being the farthest upstream of the ATG start codon that is marked with dot beneath. The predicted MEF2 are labeled with shadow boxes and the GRE are in hollow boxes.

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TTGGCACTATTATCCAGGTTCCCACTCAGGGGTAGATTTCCTTTTATATTCAGCTCTTAGTCTGATTAAATATAAAATAGCTTCATTATGACATCTTTGTGTATAACATTTAA
AAGAACTGCCAAAACAAGAACCAACCAAGCTTTGTAAGTACGCTGAACTGGGTAGTGATTTCAAGATCTTTACGTGAATCATCTATCCACGGTCCATAAAGATGTTTT
CTTTCGAGCACCTGTAGCGTACTGGCCTCTGAAAGTACAAGATACTAAGAGTCTGCTTAAACAGTATGCTATGAACATAGCATGCAAACTCAGATCTCTCTAACCTGTAGT
E1
CCAGTGAACCATACCACATATTGAGAGCACTTACAAGAGCACCTGTGTAAAAAGAAATGCTCAGGATATAAAGCAGTCAAAATGCTTCTTACATGTGGATTGGCTCAAATGAG
E2 E3
AACATCAAGGTTAGACAACCAAGCCACAGCCTGGAACACATGAGCACAATACATTTATGCTTACATCAACTTTACACCATGGAAGGACAATCAATTCATCAAACTACAGAT
E4
TTATGTTACTGGTGTAGCTGAATATCAGGCTCAGTTCTGTGACATGTAGTCAATACCCATACCCCTGGAAGGCAATACAAAATAACAAATGACCAGGCAGAAAAGAGCTCT
E5
TAGATTGTTCTGATCCACATCTTCTGTGCGAGTAACATCTTCTATATGAACACATGCAAAACAAATCCCTGTGTGTGCCACACCTTTTCATGTGTGTTTCATAATGAAAAGAAACA
E6 E7
AAGGAAAAAAGAGATCAGTGATAAAGTACATCATGGTCCCACTCGATAGGCAAACTGCAAGTATTTTGCATGTAATTCAGGATTTGTGTTGTTCTAAACATACCGGACCC
CCCCCCCACCTCCCAAGTGAATAAATCAATTTTTTTTTTTTTTTTGTAGCAGCATGCCAATGTCTATATAATGTAAGATTTTATAAGCCATGACCTGTAATTAAG
E8
ACAATCCTGTCTAGGAGCATCTCGTGCAGAAAGCACTACTAAAACATAGTTTTAAAAATCTAAGTGGGATAATACAGAGCCTACAGATGCTTGGTGAAGCAAAAACACACTATTA
E9
CTTCAGTTAGCATAATACAGAGGTATCCTGAACTGAAATAAATAGGATATGAAAAAGATTTACTGACAGAAAGGATTTAAATGGTTTATGCTTTTACAACCTTCAGTAAATAGG
AAAAAGCAAGCTGGACTATTTCTGTGTAATATTAGAGTTAAGTAGCTCATGATATTCCTTAAAAATACCTCTCCTACTAAGGTCTCTCAGTATCTAATTTGCTGCCAGGATTTTG
CTGACAAGGCAAACTTGCTTTACTTAATAAGCGTCCACACTTCAGTAAATGAATTTTGTACTAAAGGGTCCAATAGTTAGCACTTATAGTCCACAGCTGTACAAAATGTTTATTCCT
E10
GCTCACCTAGTACCTATCAGGAAAACCTATCATGATTTCTGAAATCTGAGCTGCTTAATGCAGCTGAACCTGTTGAACAGCATGGATTCCTGTTGTCATGATTTTATATGTT
E11
ATTTTTTCCCTCCTCCTAACAGAAATCCCTCAGAATTTTCTGAGGTAGTACAAACTTTGAGCCACAATAGTGATAGAATCCTAAAGGAACCTAAAAGAGAGCTGCTGCCATA
TTCATAGTCAACTATGCTTCAGTGTATTTAAGAATGATAGTGTCTTCCAGCACTGCTGCCATAGTACTTGGAAATATATCTTTTTCAGTATGTGAAGACGTATCCTCTAC
GAAGCCACAGATAAATCAGTTACCCCTGGCTGTAACCAATGCTGTCTAGTGACTGTGATCGACAGGGCTTTAACCTCTGACAGCTAGATTCATGTTGGGACAACAACCAAT
E12
CGTGGTTTTGACGACATGAGCCTAATCAAAGTTGGAGTATAAAGGCCCTTGGCATATATAAGGCACACCAGTGTGGCAAGCCGCTCTCAGATTCATTGCTGTGCAGGATC
E13
TGTTTTGAACTGAAAGAAAAGGGGAAAGGGAGGGGGGGAAAAAGGCAAAAAGCTGCAGTACTGTAAGATCATGAAAAGCTAGCAGTCTATGTTTTATTTTACCTG

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and RT-AC-R (Table 1). The data were analyzed as described above.

## Results

***MSTN* gene and its upstream region.** The sequence of *MSTN* gene in Wenshang Luhua chicken (*MSTN-Wlc*) showed very high similarities with sequenced *MSTN* genes in other breed, e.g., 98–100% identities. Since we focused on the promoter region of *MSTN-Wlc* (NCBI access no. KC405577), all the currently available upstream sequences of *MSTN* genes from different species were retrieved from NCBI database to carry out the phylogenetic analyses. As shown in the phylogenetic tree (Fig. 1), three sequenced promoters from *G. gallus*, including the one from Wenshang Luhua breed, were very close-related within a phylogenetic distance of approximately 1%, while one sequence from *G. gallus* (NCBI access no. AF478391.1) extruded from the group. The analysis also showed that these 12 promoter sequences could be divided into four deeply branching groups, which suggested extensive sequence variations in the upstream region of *MSTN* from different species. After further exploring the promoter sequence of *MSTN-Wlc*, several E-boxes, MEF2, and GRE were identified (Fig. 2). Interestingly, no MTBFs or PRE were predicted in *MSTN-Wlc* upstream, which commonly existed in several mammals' *MSTN* upstream (Du et al. 2007). This is consistent with the previous finding that the expression patterns of *MSTN* were different among chick,

yak, and pig (Sundaresan et al. 2008; Jiao et al. 2011; Zheng et al. 2011).

Considering the important role in regulating the expression of *MSTN* (Spiller et al. 2002; Salerno et al. 2004; Du et al. 2007), the E-boxes were further analyzed. There were 13 E-boxes in the 2.3-kb upstream region of *MSTN-Wlc*, which were denoted by E1 to E13 according to their positions in the region. The E1-box was farthest from the coding sequence, and E13-box was nearest to the coding sequence (Fig. 2). Furthermore, many SNPs were revealed in the *MSTN-Wlc* promoter region (data not shown), but only two SNPs were in the identified E-boxes, i.e., E3- and E4-box. Polymorphisms were present in the first nucleotide of the E3-box and in the fifth nucleotide of E4-box (Table 2).

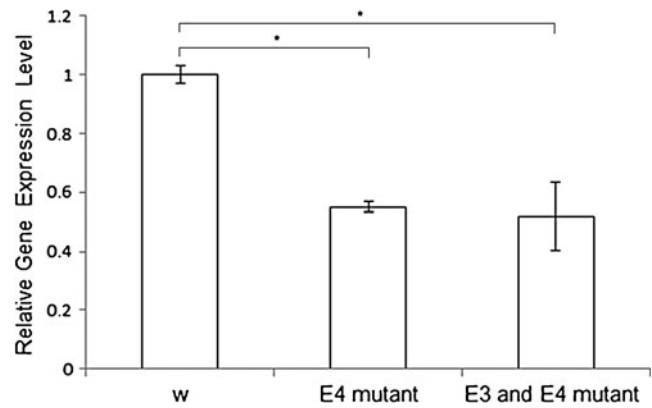
*The role of the MSTN-Wlc E-box polymorphism in regulating the expression pattern in vitro.* To further analyze the effects of the polymorphisms in the E-boxes of *MSTN-Wlc*, various upstream sequences were cloned into a GFP reporter construct, i.e., the vectors MSTNP<sup>W</sup>-GFP, MSTNP<sup>M4</sup>-GFP, and MSTNP<sup>M3,4</sup>-GFP, which carried the wild-type full-length upstream region, the upstream region with mutated E4-box, and the upstream region with both mutated E3- and E4-boxes, respectively. After transfection, the expression of GFP was monitored using real-time PCR. As shown in Fig. 3, the relative GFP expression level of the E4 mutant was about 55% of that of the wild-type, while the GFP expression level of the E3 and E4 mutants was 52% of that of the wild-type.

*Role of E-box polymorphisms in regulating the expression pattern of MSTN-Wlc in vivo.* The expression level of the *MSTN-Wlc* gene in the leg of 22 hatched 11-d chickens was analyzed by real-time PCR. The results showed that the polymorphisms in E3- and E4-boxes affected the expression level in vivo. For the two genome types of E4-box (TC and CC types), two sets of expression data showing the variation in *MSTN-Wlc* expression levels were obtained (Fig. 4). The

**Table 2.** Polymorphisms in the first nucleotide of the E3-box and in the fifth nucleotide of E4-box of *MSTN-Wlc* promoter

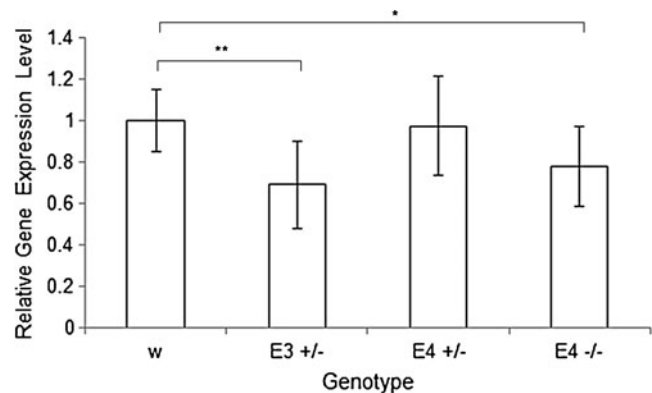
1st nucleotide of E3-box polymorphisms	Number of individuals (%) <sup>a</sup>	5th nucleotide of E4-box polymorphisms	Number of individuals (%) <sup>a</sup>
C/G	5 (22.73%)	C/C	4 (18.18%)
G/G	0 (0%)	T/C	5 (22.73%)
C/C	17 (77.27%)	T/T	13 (59.09%)

<sup>a</sup> The data represents polymorphisms genotypic distribution in 22 hatched-11-d Wenshang Luhua chicken as described in Materials and Methods



**Figure 3.** The effect of variations in the E-box sequences on the expression of *GFP* reporter gene in vitro. Expression vectors with the various E-box-containing sequences of *MSTN-Wlc* inserted upstream of the *GFP* gene were transfected into HEK 293T cells. The relative expression of *GFP* was measured by real-time PCR. On the x-coordinate of the plot, *W* represents the vector MSTNP<sup>W</sup>-GFP carrying the wild-type full-length *MSTN-Wlc* upstream sequence; *E4 mutant* represents vector MSTNP<sup>M4</sup>-GFP carrying full-length *MSTN-Wlc* upstream with the mutated E4-box (CACACG in the E4-box); *E3 and E4 mutant* represents vector MSTNP<sup>M3,4</sup>-GFP carrying full-length *MSTN-Wlc* upstream with the mutated E3- and E4-boxes (with both GAAATG in E3-box and CACACG in E4-box). Unpaired *t* test was employed for statistic analysis, \**P*<0.05.

results also indicated that chickens with the heterozygous genotype of E3-box and homozygous E4-box both had lower relative expression of *MSTN-Wlc* compared to that of wild-type (Fig. 4).



**Figure 4.** The correlation between variations in the upstream sequences and the expression of *MSTN-Wlc* in vivo. The expressions of *MSTN-Wlc* gene in 22 Wenshang Luhua chickens were analyzed by real-time PCR and correlated with their E3- and E4-box genotypes as described in Materials and Methods. On the x-coordinate of the plot, *W* represents wild-type of E3- and E4-boxes; *E3 +/-* represents heterozygous for E3-box [(C/G)AAATG in E3-box, five chickens]; *E4 +/-* represents heterozygous for E4-box [CACA(T/C)G in E4-box, five chickens]; *E4 -/-* represents homozygous for E4-box (CACACG in E4-box, four chickens). The number of individuals with polymorphisms is presented in Table 2. Unpaired *t* test was employed for statistic analysis. \**P*<0.05; \*\**P*<0.01.

## Discussion

In this study, the upstream promoter region of *MSTN* in Wenshang Luhua chicken was analyzed by cloning and sequencing analysis. Thirteen E-boxes were identified in the upstream of *MSTN-Wlc* and the polymorphisms of E-boxes were explored for the first time. Subsequently, analysis of the expression of endogenous *MSTN-Wlc* in vivo and *MSTN*-GFP reporters in vitro were both conducted. The effect of the E-boxes on expression in vitro is not identical to their effects in vivo, which may be due to the presence of other regulatory mechanisms of muscle tissues in vivo that are absent in cultured 293T cells, or the differences caused by an artificial reporter system. Nevertheless, both results indicated that the polymorphisms in E-box altered *MSTN* expression levels in chicken. Furthermore, chickens with the heterozygous genotype of the E3-boxes and homozygous E4-boxes had a significantly reduced expression of *MSTN-Wlc*. The heterozygosity of E4-box did not affect expression any further as the heterozygosity of E3-box did. Therefore, we suggest that in spite of that both E3- and E4-boxes could play roles in vivo, the E3-box might be the more important in regulating the expression of *MSTN-Wlc* versus E4-box. In addition, the GFP expression data also implies that both E3- and E4-boxes are involved in regulating the expression of *MSTN* in a cooperative manner.

The role of E-box motifs in the regulation of myostatin expression has been studied in many animal models, and different E-boxes are proposed to be involved in the regulation of *MSTN* expression in different species (Spiller et al. 2002; Salerno et al. 2004; Du et al. 2007; Funkenstein et al. 2009). The highly polymorphic nature of the myostatin gene and differences in the lengths of the upstream regions revealed in many studies might count for the variation in the multiplicity of the E-boxes. Eight, seven, and ten E-boxes were identified in the 1.2-, 2.5-, and 1.6-kb upstream regions of the ovine, murine, and bovine *MSTN* genes, respectively (Spiller et al. 2002; Salerno et al. 2004; Du et al. 2007). Among these E-boxes, the E6 in the bovine, E5 in the murine, and E3 and E5 together with E7 in ovine were found to be involved in the regulating the expression of *MSTN* (Spiller et al. 2002; Salerno et al. 2004; Du et al. 2007). From the results of this study, in Wenshang Luhua chicken, the E3- and E4-boxes were extruded from 13 identified E-boxes due to their regulating activities.

Due to the effects of myostatin on muscle mass, growth and other traits, the variations in *MSTN* are of great interest to animal breeding. Knowledge of *MSTN* null alleles and hypomorphs has been utilized to improve the selection of beef cattle and sheep (Georges 2010). A better understanding of the polymorphisms in *MSTN* in chicken will benefit the establishment of marker-assisted selection strategies for improving the agronomical traits. At the same time, our

observations need to be extended in larger sample sets, including other species of chicken. In the future study, the polymorphisms in the upstream region and *MSTN* expression might be correlated with some important agronomical traits, such as muscle mass, growth, and resistance to infections. Thus, it will help to address the applicability of the molecular and sequential data regarding myostatin in breeding programs in chicken.

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