Research Note: Association of temporal expression of myostatin with hypertrophic muscle growth in different Japanese quail lines

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ABSTRACT Myostatin (**MSTN**) negatively regulates in muscle growth and development. Among alternative splicing isoforms of avian MSTN, MSTN-A has anti-myogenic activities and MSTN-B functions as a pro-myogenic factor. In this study, different lines of Japanese quail were used: a random bred control (**RBC**) and a heavy weight (**HW**) quail line with muscle hypertrophy. The objectives of the current study are to compare temporal expression of the MSTN isoforms in *pectoralis major* muscle (**PM**) between 2 quail lines and to relate *MSTN* expression with temporal changes in muscle growth and total amounts of DNA in PM. Gains of body weight (**BW**) and PM weight were greater until post-hatch day (**D**) 28 ($P < 0.001$), and the fold increases in total DNA contents of PM were greater in the HW line compared with the RBC line during D7 to D28 ($P < 0.05$). PCR analysis showed that MSTN-A expression was greater at 14 D (E14) of embryonic age ($P < 0.01$), D7 ($P = 0.052$), and D14 ($P < 0.01$) in the RBC line compared with the HW line. At D28 and D75, expression of MSTN-A was greater in the HW line compared with the RBC line ($P < 0.05$). *MSTN*-B expression was barely detectable from E14 to D14 and measurable from D28 to D75 in the muscle of both lines. Ratios of the *MSTN*-B/-A form ranging from 0.15 to 0.29 indicate a minor expression of the B form. Taken together, the lesser expression levels of *MSTN*-A at E14, D7, and D14 are associated with the fast growth of PM, and greater MSTN-A expression at D28 and D75 are associated with a slowdown of PM growth in the HW line. These data indicate a negative association of MSTN expression with PM growth and provide a scientific basis for potential usage of MSTN expression as a selection marker for greater muscle growth in poultry.

Key words: myostatin, hypertrophy, HW quail, muscle development, MSTN isoforms

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INTRODUCTION

Myostatin (**MSTN**) is well-known to have inhibitory effects on muscle growth which were demonstrated by increased fiber size and fiber number in muscles of *MSTN* knockout (**KO**) mice (McPherron et al., 1997). The inhibitory role of MSTN in muscle development was further confirmed in cattle, dogs, and humans, which have mutations in the *MSTN* gene and exhibit a double muscling phenotype (Grobet et al., 1997; Schuelke et al., 2004; Mosher et al., 2007). Recently, knockdown of myostatin expression in chickens by shRNA resulted in a 15% increase in body weight at 6 wk of age (Bhattacharya et al., 2019).

Antimyogenic activities of MSTN can be achieved by processing of promyostatin (**pro-MSTN**) to mature myostatin. Myostatin is initially formed as a pro-MSTN, which undergoes 3 proteolytic processing events to generate the biologically active mature MSTN (Wolfman et al., 2003; McFarlane et al., 2005). The mature MSTN dimers are capable of binding to the receptors, resulting in generation of antimyogenic signals. Although several approaches successfully inactivated MSTN and increased muscle mass or inhibited muscle wasting, natural mechanisms or endogenous factors that regulate maturation processes of pro-MSTN during muscle development have not been clearly demonstrated.

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Avian MSTN has been found in several isoforms by alternative splicing of *MSTN* mRNA (Shin et al., 2015). In chickens, turkeys, and quail, *MSTN*-A and *MSTN*-B isoforms are dominant isoforms in muscle among 5 isoforms (MSTN-A to MSTN-E). Overexpression of the quail *MSTN*-A form in the quail myogenic cell line (**QM7**) inhibited myogenic differentiation. The quail MSTN-B form encodes only 129 amino acids by alternative splicing and consequently creating a premature stop codon and consists of only a half of MSTN prodomain (Shin et al., 2015). In the mouse, the MSTN prodomain is known to bind to mature MSTN and to inhibit mature MSTN (Walker et al., 2016). The MSTN prodomain enhanced myogenic differentiation in vitro and also increased muscle mass in transgenic animals with overexpression of the MSTN prodomain (Yang et al., 2001; Lee, 2012). Interestingly, the quail *MSTN*-B form can enhance myogenic differentiation of QM7 cells by binding to the N-terminal part of the pro-MSTN and inhibiting processing of the pro-MSTN to mature MSTN protein. Therefore, the MSTN-B form in the avian species has been suggested to be a promyogenic factor. However, the temporal expression of MSTN-A and MSTN-B forms in avian muscle tissue has yet to be investigated. In the current study, expression of MSTN-A and MSTN-B forms was compared between a heavy weight (**HW**) quail line with a hypertrophic muscle growth and a random-bred control line (Choi et al., 2013). In addition, differences in temporal expression of MSTN forms between 2 different genetic quail lines were related to differences in characteristics of muscle growth and maturation.

MATERIALS AND METHODS

Experimental Birds and Animal Usage

All the animal care and experiments were approved by The Ohio State University Institutional Animal Care and Use Committee (protocol no. 2013A00000041). Fertile eggs of random bred control (**RBC**) and HW quail lines were obtained from the Ohio Agricultural Research and Development Center of The Ohio State University. Quail was sacrificed by CO_2 inhalation followed by cervical dislocation as guided by IACUC protocol (protocol no. 2013A00000041). The eggs were incubated with turning through a 90 arc rotation every 2 h. Body weight and *Pectoralis major* muscle (**PM**) tissues weight were calculated until 75 D of postnatal, and for RNA extraction, PM tissues were sampled at different developmental stages: embryonic day (**E**) 14 and postnatal day (**D**) 1, 7, 14, 28, and 75. At each time point, collected samples were snap-frozen in liquid nitrogen and stored at −80°C until used for analysis. Only male quail was used in this study.

DNA Concentration, RT-PCR, and Quantitative Real-time PCR

For DNA concentration, the whole right PM tissue was used for the analysis according to the method described in a previous study (Choi et al., 2013). Total RNA was isolated using Trizol (Life Technologies Inc.) according to the manufacturer's instructions. Quantity and quality of RNA were assessed by Nanodrop 1000 (Nanodrop Technology). Approximately 1 μg of RNA was reverse-transcribed to cDNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen) with a thermocycler, 65°C for 5 min, 37°C for 52 min, and 70°C for 15 min, and 1 μg of cDNA was used for RT-PCR or quantitative real-time PCR (**qPCR**). To quantify folds of gene expression, qPCR was performed in a thermocycler, the ABI 7500 (Applied BioSystems) using AmpliTaq Gold polymerase (Applied BioSystems) with the condition, 95°C for 10 min followed by 40 cycles of 94°C for 15 s, 55°C for 45 s, 72°C for 40 s, and 82°C for 32 s with primer sets; MSTN, F: 5′-GGTATCTGGCAGAGTATTGATGT-GAA and R: 5′-CAAAATCTCTGCGGGACCGT. For RT-PCR, DNA Taq-polymerase (New England BioLabs) was used. To avoid PCR saturation and get a linear amplification (Oh et al., 2011), PCR products were amplified for 26, 28, 30 cycles for ribosomal protein S13 (***Rps13***) at denaturing 95°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s with specific primer sets; F: 5′-AAGAAGGCTGTTGCTGTTCG and R: 5′-GGCA-GAAGCTGTCGATGATT and for 30, 32, 34 cycles for *MSTN* at denaturing 95°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 30 s with specific primer sets: F: 5′-AGCACCTAACATTAGCAGGGACGT and R: 5′-TTCACATCAATACTCTGCCAGATACC. The PCR products were separated by 2% agarose gel electrophoresis and stained by ethidium bromide, and images of PCR products on gels at 26 cycles for *Rps13* and 34 cycles for *MSTN* were captured by a gel imaging system (FOTO/Analyst Express, Fisher Scientific). *Rps13* was used as an internal control for qPCR and RT-PCR. The expression levels were normalized to those of endogenous *Rps13*, and the data were analyzed using the ΔΔCt method (Livak and Schmittgen, 2001). Densitometry analysis of the specific bands was performed using NIH ImageJ software.

Statistical Analysis

All data were expressed as means ± SEM ($n \geq 3$). The data were analyzed using Graphpad Prism software, version 6.02. For all comparisons in this study, multiple *t*-tests were conducted. *P*-value, $P < 0.05$, was considered a statistically significant difference.

RESULTS AND DISCUSSION

The 2 genetic lines of quail used in the current study originated from the same population of quail (Nestor et al., 2002, 2012). The HW quail were developed at The Ohio State University by selectively breeding for their greater body weight compared with the RBC line for more than 80 generations (Nestor et al., 1983). Our previous studies described characteristics of the HW quail line providing a new avian model for muscle hypertrophy with no significant differences in the total fiber

numbers between the RBC and HW lines (Choi et al., 2014a, b). In the current study, visual differences in sizes of bodies and PM between RBC and HW lines were presented in Figures 1A and 1C, and the gains of BW and PM weight were compared between RBC and HW lines (Figures 1B, 1D, and 1E). The rates of body weight gain were greater in the HW line compared with the RBC line. Especially, the body weight gained 11 g in RBC and 43 g in the HW line from D7 to D14 ($P < 0.001$), and 44 g in RBC and 117 g in the HW line from D14 to D28 ($P < 0.001$). The rate of PM weight gains also showed similar patterns with body weight: D0 to D7: 0.4 g vs. 1.3 g ($P < 0.001$), D7 to D14: 0.6 g vs. 3 g ($P < 0.001$), D14 to D28: 2.5 g vs. 9 g ($P < 0.001$) (RBC vs. HW). These detailed characteristics of temporal growth provide a muscle developmental model with which *MSTN* expression can be related, along with expression of myogenic markers.

To investigate temporal expression of *MSTN*, 2 different methods were employed for measuring combined expression of *MSTN*-A and *MSTN*-B forms by qPCR and for separately detecting the 2 forms based on size differences (443 vs. 300 bps) by RT-PCR followed by gel electrophoresis and densitometry analysis. The primer sets used for 2 methods were described in Figure 2A and the materials and methods section. Previous studies showed greater embryo weights and expression of a proliferation marker MyoD in the muscle of HW embryos than the RBC at E14 (Berkes and Tapscott, 2005; Choi et al., 2014a), suggesting a more proliferative potential of myoblasts in the HW line. Given the antiproliferative function of MSTN by downregulating *MyoD* expression in muscle cells (Thomas et al., 2000; Langley et al., 2002), higher MyoD expression and lower *MSTN* expression in the HW muscle at E14 ($P < 0.01$) (Figure. 2B) could be related to the greater embryo weight at E14 compared with the RBC line (Choi et al., 2014a).

Right after hatching, chicks before access to food have been under stressful conditions such as the hatching process which demands for high energy expenditure (Lee et al., 2009). The chicks at D0 actively mobilize fatty acid from both adipose tissue and yolk to supply energy for survival (Lee et al., 2009; Chen et al., 2014). This stress condition causes a temporal halt in growth of the chicks that may inhibit expression of *MSTN* in PM of both RBC and HW lines (Figures 2B and 2C). Similar low expression of *MSTN* in muscle also has been shown in hatchlings of chickens and ducks (Duan et al., 2016; Dou et al., 2018).

As the HW line was established by selectively breeding for 4-week body weight (Nestor et al., 1983), BW and PMW gains were greater in the HW line during the period of D7 to D28 compared with the RBC line ($P < 0.001$) (Figure 1). In this period, fold increases in total amounts of DNA in PM were greater in the HW line ($P < 0.05$) (Figure 1F), suggesting higher rates of cell proliferation in the HW line. Relatively lower expression of *MSTN* in the HW line than the RBC line at D7

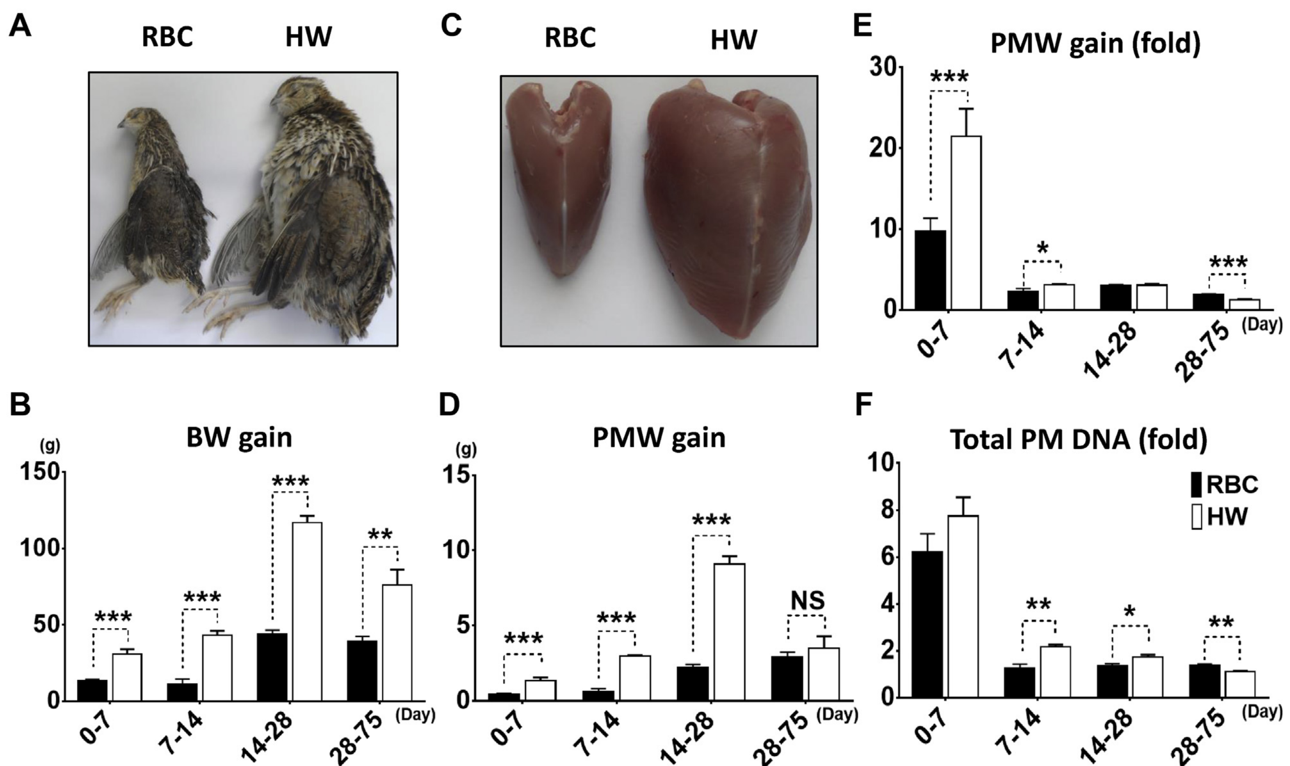

Figure 1. Comparison of body weight (BW) and *pectoralis major* muscle (PM) weight. (A and C) Visual differences of body and breast muscle at 42 D posthatch. (B and D) BW and PM weight (PMW) muscle gain. The value was shown by weight gain of body and PM during development, respectively. (E) PMW gain fold. (F) Fold changes of amount of total PM DNA. Multiple *t*-test was used for statistical analysis by the Graphpad PRISM 6.02 program. Values present means ± SEM (n = 5). Black square: the RBC lines and White square: the HW lines. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. NS, no significance; RBC, random bred control; HW, heavy weight.

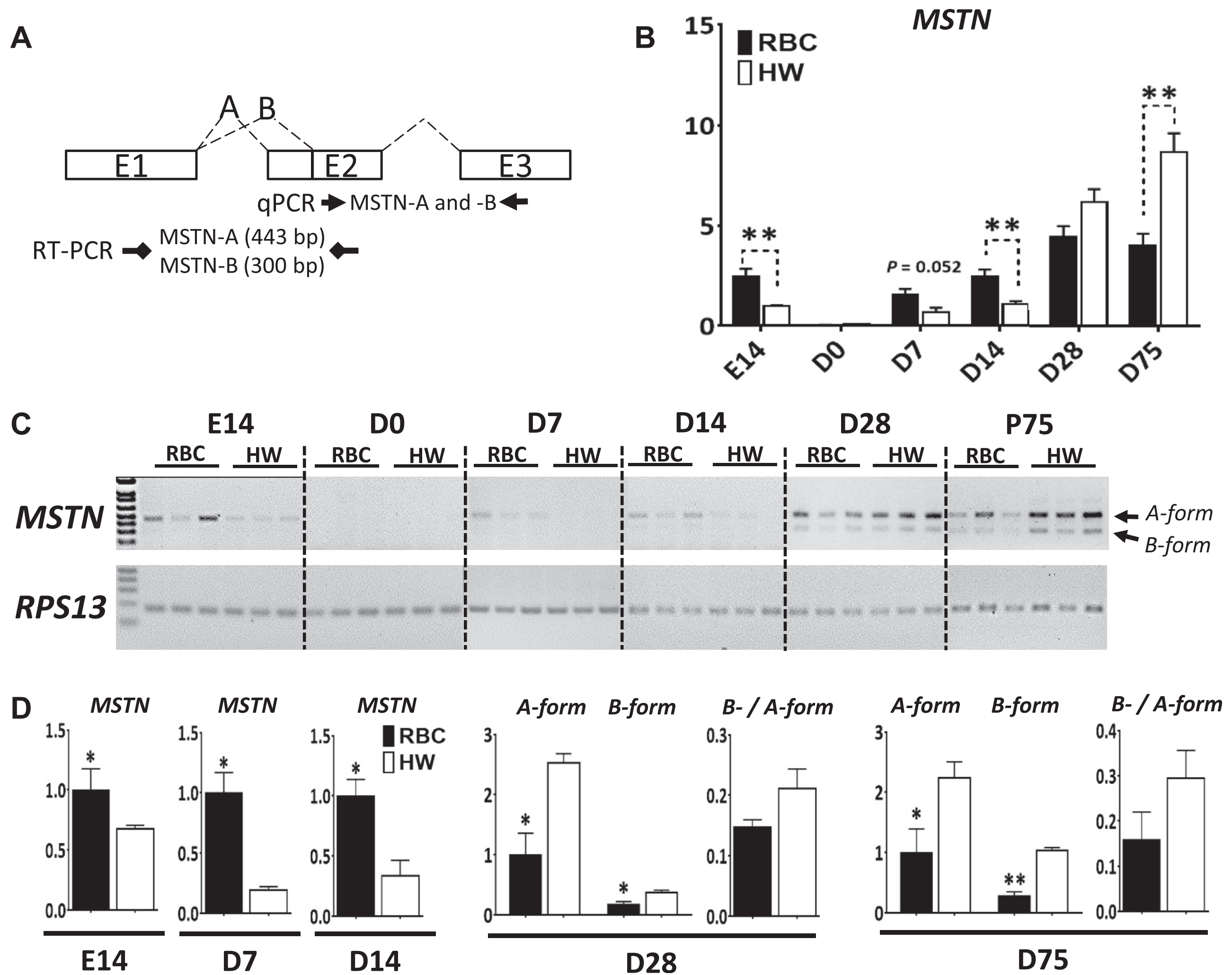

Figure 2. Comparisons of expression levels of myostatin (*MSTN*). (A) Schematic diagram shown the loci of specific primer sets for analysis expression levels of *MSTN* by RT-PCR and qPCR. The primer sets were designated to analyze *MSTN*-A or *MSTN*-B form only among isoforms (A–E form). (B) Quantitative analysis of gene expression levels, *MSTN*-A and *MSTN*-B form during development by qPCR (n = 4). (C) Gel electrophoresis of *MSTN* by RT-PCR. The expression of *MSTN*-A and *MSTN*-B form in RBC and HW lines at E14, P1, P7, P14, P28, and P75 during development by RT-PCR (n = 3). *RPS13* was used as an internal control for qPCR and RT-PCR, both. (D) Densitometry analysis of *MSTN* or *MSTN*-A, *MSTN*-B form. The densitometry analysis was performed using NIH ImageJ software. Multiple *t*-test was used for statistical analysis by the Graphpad PRISM 6.02 program. Values present means ± SEM. Black square: the RBC lines and White square: the HW lines. *: $P < 0.05$ and **: $P < 0.01$. RBC, random bred control; HW, heavy weight.

and D14 ($P < 0.05$) (Figure 2) may allow active cell proliferation and myogenic differentiation, consequently resulting in greater PMW in the HW line.

During the period of D28 to D75, fold increases in PMW gain and total PM DNA were significantly greater in the RBC line than the HW line (Figures 1E and 1F). These data suggest greater cell proliferation per unit of muscle in the RBC line, which is accompanied with a similar gain, but a greater fold change, of PMW (2.9 g of gain from 3.3 g to 6.2 g) in the RBC line from D28 to D75 compared with the HW line (3.5 g of gain from 13.5 g to 17.0 g) ($P < 0.01$). Relatively greater expression of MSTN may slow down cell proliferation and muscle growth in the HW line; whereas, lower expression of MSTN in the RBC line may activate cell proliferation with accompanied increase in muscle growth at the later stage.

Among 5 MSTN isoforms (A to E forms) in the avian, MSTN-A and MSTN-B form are major and second dominant forms in the muscle, respectively (Huang et al., 2011; Shin et al., 2015). Our previous in vitro study showed that binding of MSTN-B to MSNT-A causes a reduced production of mature MSTN from the MSTN-A, resulting in increases in muscle fiber length, diameter, and nuclei numbers (Shin et al., 2015). In the current study, PCR analysis to detect both forms of *MSTN* showed absence of *MSTN*-B expression from E14 to D7, barely detectable levels at D14 and measurable expression levels from D28 to D75 in the muscle of both lines (Figures 2C and 2D). This expression pattern indicates age-associated alternative splicing of *MSTN* in quail muscle. The greater expression levels of *MSTN*-A at D28 and D75 are associated with slowdown of PM growth in the HW line ($P < 0.05$). Ratios of *MSTN*-B to *MSTN*-A form ranging from 0.15 to 0.29 indicate minor expression of the B form over A. The role of MSTN-B form in regulation of muscle growth *in vivo* needs to be further studied by generating transgenic quail overexpressing B form.

Overall, the temporal expression of *MSTN* is negatively correlated with growth potential of PM muscle in the HW line. Greater rates of breast muscle growth and increasing DNA contents in whole PM of the HW quail are associated with lower *MSTN* expression from the embryonic age to posthatch day 14. *MSTN-B*, an alternative mRNA splicing isoform having promyogenic function in vitro, was expressed at very low levels from embryonic age to posthatch day 14 and appeared to be expressed thereafter, suggesting developmental regulation of splicing of *MSTN* mRNA. Taken together, low expression levels of *MSTN* in the HW quail line with muscle hypertrophy might function as a selection marker for body weight. The current study shows evidence that expression levels of alternative splicing variants of MSTN is age-related and that expression levels of MSTN-A and MSTN-B might contribute to regulation of muscle growth in quail. These findings provide new insight into use of expression of MSTN variants as a selection marker for a superior line of poultry with high muscle growth and a foundation for future studies focusing on function of MSTN on muscle hypertrophy using gene editing in quail.

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