Research Note: Increased myostatin expression and decreased expression of myogenic regulatory factors in embryonic ages in a quail line with muscle hypoplasia

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ABSTRACT Genetic selection of quail for a low body weight for more than 80 generations established a low-weight (**LW**) Japanese quail line that has been previously characterized to have a muscle hypoplasia phenotype. The aim of this study is to investigate the relationship of temporal expression levels of *myostatin* (***Mstn***) and *myogenic regulatory factors* (***MRFs***) with hypoplastic muscle growth in the LW line. During embryonic day (**E**) 13 to 15, gain of embryo weight was 2-fold lower ($P < 0.001$) in the LW line than that in the random bred control (**CON**). Gains in body weight and pectoralis muscle weight from hatch to posthatch day (**P**) 28 were also significantly lower ($P < 0.01$) in the LW line but increased by 4-fold ($P < 0.05$) during P42 to P75. PCR analysis showed that expression levels of *Mstn* were greater in the LW at embryonic stage (E12 to E14, $P < 0.05$), but there was no difference after hatch. In addition, expression levels of *Pax7* and *myogenin* (***MyoG***) at E12 were 23-fold ($P < 0.05$) and 3.4-fold ($P < 0.05$) lesser in the LW line, respectively. At E14, expression of *Pax3*, *Pax7*, and *MyoG* gene was 3.5-fold ($P < 0.05$), 6.5-fold ($P = 0.065$), and 4.4-fold ($P < 0.01$) less than that in the CON. Taken together, high expression levels of *Mstn* and low expression of *MRFs* during embryonic stages can be associated with development of muscle hypoplasia and delayed muscle growth in the LW quail line. These data provide evidence that genetic selection for a low body weight resulting in an avian model with muscle hypoplasia has altered the expression profiles of myogenic factors.

Key words: myostatin, myofiber number, quail, muscle development, myogenic regulatory factor

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INTRODUCTION

Myostatin (***MSTN***), also known as growth/differentiation factor-8, is abundantly expressed in skeletal muscle and is a crucial negative regulator of skeletal muscle mass (Lee, 2004). Overexpression of *MSTN* showed decreased muscle mass, fiber sizes, and myonuclear numbers in mice (Reisz-Porszasz et al., 2003) and muscle atrophy phenotype in rats (Amirouche et al., 2009). On the other hand, recent studies showed that lack of functional *MSTN* in chicken and quail results in muscle hyperplasia (Kim et al., 2020b; Lee et al., 2020).

Alternative splicing variants (*Mstn-A* to *Mstn-E*) of the *Mstn* gene are found in diverse avian species (Shin et al., 2015). Among the variants, exogenous overexpression of an alternative splicing variant, *Mstn-B*, in transgenic quail increased muscle weight with hyperplasia by inhibiting proteolytic cleavage of pro-MSTN (Shin et al., 2015; Chen et al., 2019). Recently, differential expression of splicing variants of *Mstn* has been associated with hypertrophic muscle growth in a heavy weight (**HW**) quail line (Kim et al., 2020a). However, temporal expression of *Mstn* variants has not been investigated in a low-weight (**LW**) quail line with muscle hypoplasia (Choi et al.,

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2014). These 2 quail lines were developed by selectively breeding at a 4-wk body weight over 80 generations at The Ohio State University (Nestor et al., 1983).

The muscle of the LW quail was characterized by fewer numbers of total myofibers without differences in myofiber size than the random bred control (**CON**) quail line (Choi et al., 2014). Although myogenic regulatory factors (**MRFs**) are critical regulators in proliferation of myocytes and formation of myofibers (Collins et al., 2009), the potential association of expression of MRFs with different characteristics of muscle growth between the CON and LW lines has not been studied. Therefore, the objective of the present study was to compare temporal expression patterns of MRFs including *Mstn* and to relate their expression patterns with different characteristics of muscle growth between the CON and LW quail lines.

MATERIALS AND METHODS

Use of Animal and Study Approval

The LW quail line used in this present study has been developed at The Ohio State University by selectively breeding for lower body weight (**BW**) at 4-wks than the CON line for over 80 generations (Nestor et al., 1983). All fertile quail eggs and animals (the CON and LW lines) used in this study were obtained from the Ohio Agricultural Research and Development Center of The Ohio State University. All the animal care and experiments were approved by The Ohio State University Institutional Animal Care and Use Committee (protocol no.: 2013A00000041). The eggs were incubated and turned through a 90-arc rotation every 2 h, and all animals had free access to food and water after hatch. Quail were sacrificed by CO_2 inhalation followed by cervical dislocation as guided by Institutional Animal Care and Use Committee protocol. BW and pectoralis major muscle (**PM**) tissue weights were calculated from embryonic day (**E**) 9 to E15 every 2 d, postnatal day (**P**) 1 to P7 every 3 d, and P7 to P75 every 7 d. For RNA extraction, PM tissues were sampled and snap-frozen in liquid nitrogen and stored at −80°C until used for analysis. Only male quail were used in this study because egg laying in hens caused a fluctuation of BW due to approximately 9 g of egg weight (Yang et al., 2013).

Quantitative Real-Time PCR and RT-PCR

Total RNA was isolated from the PM tissues using Trizol reagent (#15596; Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and cDNA was synthesized by Moloney murine leukemia virus reverse transcriptase (#28025; Invitrogen) with RNaseOUT recombinant ribonuclease inhibitor (#10777019; Invitrogen). To quantify relative fold changes in gene expression, qPCR was performed with AmpliTaq Gold polymerase (#N8080241; Applied BioSystems) and SYBR green I as a detection dye for a dissociation curve on the ABI 7500 (Applied BioSystems, Foster City, CA). The qPCR condition was 95°C for 10 min followed by 40 cycles of 94°C for 15 s, 53°C for 45 s, 72°C for 40 s, and 82°C for 32 s. The expression levels were normalized to those of endogenous *Rps13*, and the data were analyzed using the ΔΔCt method (Livak and Schmittgen, 2001).

For RT-PCR, DNA Taq-polymerase (#M0273; New England BioLabs, Ipswich, MA) was used. The PCR products were amplified for 26 cycles for *Rps13* and for 37 cycles for both *Mstn-A* and *Mstn-B* when a relatively low expression of *Mstn-B* can be amplified enough to be detected. The PCR condition was denaturation at 95°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s. The PCR products were separated in 1.5% agarose gel electrophoresis. *Rps13* was used as an internal control for qPCR and RT-PCR, and all primer sets used in this study were from our previous studies (Shin et al., 2009; Kim et al., 2020b).

Statistical Analysis

All data were expressed as means ± SEM ($n \geq 4$). The data were analyzed using the Graphpad PRISM software, version 6.02 (Graphpad Software, La Jolla, CA). For all comparisons in this study, multiple *t* tests were conducted. A P value < 0.05 was considered a statistically significant difference.

RESULTS AND DISCUSSION

The present study further confirmed that the BW of the LW line was 61.1% less than that of the CON quail at 4 wks of age (CON vs. LW, 80.3 g vs. 31.3 g). To compare temporal changes in muscle growth of 2 different lines of quail, gains of BW and PM weight (**PMW**) for age intervals were presented (Figure 1). The weight gains during embryonic periods were lower in the LW line than those in the CON line (Figure 1A). Especially, the gains of embryo weights from E13 to E15 were more than 2-fold less in the LW than those in the CON (0.81 g vs. 1.91 g, $P < 0.001$). During all posthatch periods up to 28 d of age, gains of BW and PMW in the LW line were less than those of the CON line ($P < 0.001$) (Figures 1B and 1C). During the period of P28-P42, gains of BW and PMW were not significantly different between the 2 lines of quail. From P42 to P75, the LW line had greater gains of BW and PMW than the CON line (for BW gain, 9.3 g vs. 36.4 g, $P < 0.05$, and for PMW gain, 2.3 g vs. 5.5 g, $P < 0.001$). Taken together, the LW quail have significantly less weight gain from embryonic ages to P28, similar gain at P28-P42, and greater gain at P42-P75.

Our previous study characterized the LW quail as a new avian model with muscle hypoplasia (Choi et al., 2014). Because myofiber numbers are determined around fetal/early posthatch ages in avian (Moore et al., 2005), comparing expression of MRFs involved in myofiber formation during embryo development between 2 lines of quail would provide a link to the muscle fiber hypoplasia of the LW quail.

MSTN has been known to regulate myofiber numbers and muscle mass in chickens and quail (Chen et al., 2019;

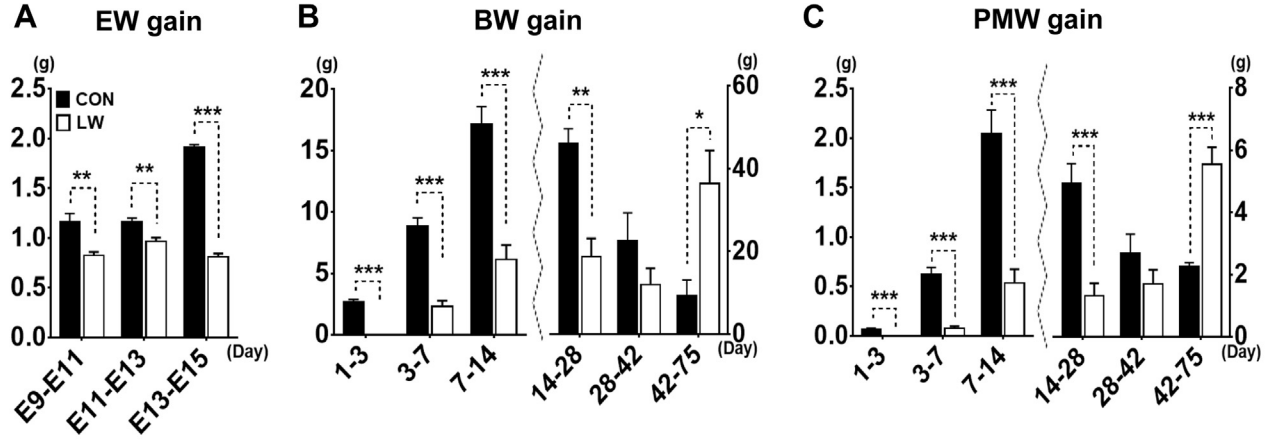

Figure 1. Comparison of embryo weight (EW), body weight (BW), and pectoralis major muscle (PM) weight. (A) EW gain. (B) and (C) BW and PM weight (PMW) muscle gain and gain fold. The value was shown by weight gains of body and PM during development. Multiple *t* test was used for statistical analysis by the Graphpad PRISM 6.02 program. Values presented as means ± SEM (n = 6). Black square: the CON line. White square: the LW line. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Abbreviations: E, embryonic day; P, postnatal day.

Kim et al., 2020b; Lee et al., 2020). Among alternative mRNA splicing variants (*Mstn-A* to *Mstn-E*) in avian *Mstn*, *Mstn-A*, a dominant form, had an inhibitory effect on proliferation of quail myogenic cells, fusion rate, and myotube formation *in vitro* (Shin et al., 2015). Our recent study showed that the heavy-weight quail line (**HW**) had a muscle hypertrophic phenotype with low expression levels of *Mstn* in E14 compared with the CON (Kim et al., 2020a). Opposite of the antimyogenic function of *Mstn-A*, the *Mstn-B* form has promyogenic function (Shin et al., 2015). Quantitative PCR data from the present study show that combined levels of *Mstn-A* and *Mstn-B* expression were greater in the LW at E12 and E14 (CON vs. LW, 5.3 vs. 11.2 at E12 and 3.2 vs. 15.3 at E14), but there were no differences after hatching (Figure 2A). In addition, RT-PCR was used

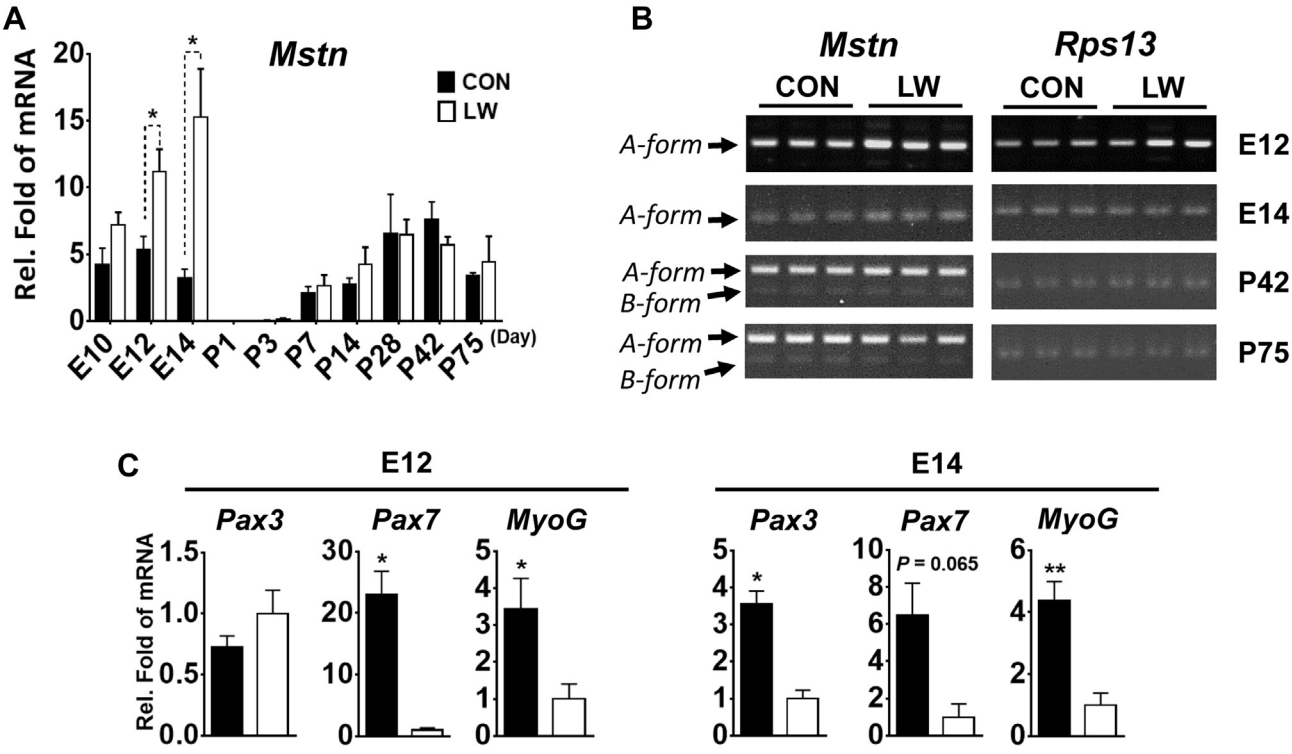

Figure 2. Comparisons of expression levels of *myostatin* (*Mstn*) and *myogenic regulatory factors* (*MRFs*). (A) Quantitative analysis of gene expression levels, *Mstn* during development by qPCR; n = 4. (B) Gel electrophoresis of *Mstn* by RT-PCR at E12, E14, P42, and P75. The expression of *Mstn* in CON and LW lines during development by RT-PCR; n = 3. (C) Quantitative analysis of *MRFs* expression levels by qPCR; n = 4. *RPS13* was used as an internal control for qPCR and RT-PCR. Multiple *t* test was used for statistical analysis by the Graphpad PRISM 6.02 program. Values presented as means ± SEM. Black square: the CON line and white square: the LW line. $^{*}P < 0.05$ and $^{**}P < 0.01$. Abbreviations: E, embryonic day, P, postnatal day.

with a specific primer set to amplify both *Mstn-A* and *Mstn-B* forms (Figure 2B). The PCR products were separated into 2 different sizes in agarose gel electrophoresis to access changes of *Mstn* variants during development. The results show that expression of the *Mstn-B* form was barely detected in the LW at P42 and P75 (Figure 2B) and may not significantly contribute to the total amounts of *Mstn* expression.

Although myofiber numbers are largely determined during the embryonic ages and hyperplastic muscle phenotype has been reported in animal models with a disruption of the *Mstn* gene, the *Mstn* expression during embryo development to link total number of myofiber has not been reported. In this regard, the current finding that higher *Mstn* expression during embryo development in the LW line is associated with muscle fiber hypoplasia is the first *in vivo* evidence showing a negative relationship of *Mstn* with myofiber formation. It will be interesting to further investigate whether *Mstn* expression in embryos is negatively associated with mammalian models with muscle hypoplasia.

Expression of MRFs such as *Pax3/7* and *MyoG* was further investigated. *Pax3/7* is expressed in quiescent and activated satellite cells in skeletal muscles, and *MyoG* is required for the myocyte fusion and myotube formation (Collins et al., 2009). The expression levels of *Pax3* were not different at E12 but 3.5-fold lower in the LW quail line at E14 (Figure 2C). *Pax7* and *MyoG* also expressed at lower levels in the LW at E12 and E14: for *Pax7*, 23-fold lower at E12 and 6.5-fold lower at E14; for *MyoG*, 3.4-fold lower at E12 and 4.4-fold lower at E14 (Figure 2C). A significantly low expression of these MRFs in the LW embryos may lead to reduced activation of satellite cells and myotube formation (Messina and Cossu, 2009), being potentially associated with muscle hypoplasia of the LW line.

A late catch-up in gains of BW and PMW from P42 to P75 in the LW line (Figures 1B and 1C) is mainly due to the delayed increase in myofiber size that is associated with greater expression levels of *MyoG* and a higher percentage of centered nuclei (Choi et al., 2014). However, there was no difference in *Mstn* expression between the 2 quail lines during the posthatch period, although there were significant differences in gains of PMW. There was a negative association of *Mstn* with growth of embryo and myofiber number in the LW quail line. These findings suggest that differential expression of *Mstn* may potentially contribute to embryonic muscle development when the myofiber is being formed, thereby influencing rates of muscle growth at posthatch ages. This explanation is partially supported by the recent findings of the involvement of *Mstn* in regulation of total myofiber numbers in chickens and quail with CRISPR/Cas-mediated indel mutations or shRNA-mediated knockdown of the *Mstn* gene (Bhattacharya et al., 2019; Kim et al., 2020a; Lee et al., 2020).

Overall, muscle hypoplasia in the LW quail line (Choi et al., 2014) is accompanied by the higher *Mstn* and lower MRFs expressions in embryonic stages. To the best of our knowledge, it is the first study showing a negative association of *Mstn* expression with a total number of myofibers. As a unique animal model with muscle hypoplasia, the LW line can be a valuable avian model to identify a new genetic network regulating myofiber formation and understanding its associated genetic diseases in muscle.

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DISCLOSURES

The authors declare no conflicts of interest.

REFERENCES

Amirouche, A., A. C. Durieux, S. Banzet, N. Koulmann, R. Bonnefoy, C. Mouret, X. Bigard, A. Peinnequin, and D. Freyssenet. 2009. Down-regulation of Akt/mammalian target of rapamycin signaling pathway in response to myostatin overexpression in skeletal muscle. Endocrinology 150:286–294.

Chen, P. R., Y. Suh, S. Shin, R. M. Woodfint, S. Hwang, and K. Lee. 2019. Exogenous expression of an alternative splicing variant of myostatin prompts leg muscle fiber hyperplasia in Japanese quail. Int. J. Mol. Sci. 20:1–17.

Choi, Y. M., Y. Suh, S. Shin, and K. Lee. 2014. Skeletal muscle characterization of Japanese quail line selectively bred for lower body weight as an avian model of delayed muscle growth with hypoplasia. PLoS One 9:e95932.

Collins, C. A., V. F. Gnocchi, R. B. White, L. Boldrin, A. Perez-Ruiz, F. Relaix, J. E. Morgan, and P. S. Zammit. 2009. Integrated functions of Pax3 and Pax7 in the regulation of proliferation, cell size and myogenic differentiation. PLoS One 4:e4475.

Kim, D. H., Y. M. Choi, Y. Suh, S. Shin, J. Lee, S. Hwang, and K. Lee. 2020a. Research Note: association of temporal expression of myostatin with hypertrophic muscle growth in different Japanese quail lines. Poult. Sci. 99:2926–2930.

Kim, G. D., J. H. Lee, S. Song, S. W. Kim, J. S. Han, S. P. Shin, B. C. Park, and T. S. Park. 2020b. Generation of myostatin-knockout chickens mediated by D10A-Cas9 nickase. FASEB J. 34:5688–5696.

Lee, S.-J. 2004. Regulation of muscle mass by myostatin. Annu. Rev. Cell Dev. Biol. 20:61–86.

Lee, J., D. H. Kim, and K. Lee. 2020. Muscle hyperplasia in Japanese quail by single amino acid deletion in MSTN propeptide. Int. J. Mol. Sci. 21:1504.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25:402–408.

Messina, G., and G. Cossu. 2009. The origin of embryonic and fetal myoblasts: a role of Pax3 and Pax7. Genes Dev. 23:902–905.

Moore, D. T., P. R. Ferket, and P. E. Mozdziak. 2005. Muscle development in the late embryonic and early post-hatch poult. Int. J. Poult. Sci. 4:138–142.

Nestor, K. E., W. L. Bacon, and A. L. Lambio. 1983. Divergent selection for egg production in Coturnix coturnix japonica. Poult. Sci. 62:1548–1552.

Reisz-Porszasz, S., S. Bhasin, J. N. Artaza, R. Shen, I. Sinha-Hikim, A. Hogue, T. J. Fielder, and N. F. Gonzalez-Cadavid. 2003. Lower skeletal muscle mass in male transgenic mice with muscle-specific overexpression of myostatin. Am. J. Physiol. Endocrinol. Metab. 285:E876–E888.

Shin, S., Y. Song, J. Ahn, E. Kim, P. Chen, S. Yang, Y. Suh, and K. Lee. 2015. A novel mechanism of myostatin regulation by its alternative splicing variant during myogenesis in avian species. Am. J. Physiol. Cell Physiol. 309:650–659.

Shin, J., S. G. Velleman, J. D. Latshaw, M. P. Wick, Y. Suh, and K. Lee. 2009. The ontogeny of delta-like protein 1 messenger ribonucleic acid expression during muscle development and regeneration: Comparison of broiler and Leghorn chickens. Poult. Sci. 88:1427–1437.

Yang, S., Y. Suh, Y. M. Choi, S. Shin, J. Y. Han, and K. Lee. 2013. Loss of fat with increased adipose triglyceride lipase-mediated lipolysis in adipose tissue during laying stages in quail. Lipids 48:13–21.