Research Note

Muscle hypertrophy in heavy weight Japanese quail line: Delayed muscle maturation and continued muscle growth with prolonged upregulation of myogenic regulatory factors

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ABSTRACT The objective of this study was to compare the temporal expression of myosin heavy chain (MyHC) isoforms, Pax7, and myogenic regulatory factors (MRF) between heavy weight (HW) and random bred control (RBC) Japanese quail lines during muscle development to better understand the mechanisms leading to increased skeletal muscle mass in the HW quail line selected for a greater BW at 4 wk of age separated from RBC quail. Expression of neonatal MyHC isoform began at 3 and 7 d posthatch in RBC and HW quail lines, respectively. In the RBC quail line, adult MyHC isoform, as a marker for muscle maturation, was expressed at 28 d posthatch with sustained expression through 75 d posthatch, whereas this protein was detected only at 75 d posthatch in the HW quail line. Moreover, Pax7 expression continued from embryonic ages to 14 d posthatch in the HW quail line and to 7 d posthatch in the RBC quail line. These expression patterns of MyHC isoforms and Pax7 in the HW quail line were accompanied by delayed muscle maturation and prolonged growth compared with the RBC quail line. Temporal expressions of the primary MRF showed that higher expression levels of MyoD and Myf-5 were observed at 9 and 11 d embryo in the HW quail line compared with the RBC quail line ($P < 0.05$). The HW quail line exhibited approximately 2 times greater average levels of myogenin expression from 7 to 75 d posthatch ($P < 0.05$) than the RBC quail line. Prolonged upregulation of these primary and secondary MRF during muscle development is associated with delayed maturation and continued muscle growth, which consequently would permit muscle hypertrophic potentials in the HW quail line compared with the RBC quail line.

Key words: muscle growth, myosin heavy chain isoform, Pax7, myogenic regulatory factor, heavy weight quail

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INTRODUCTION

A dramatic increase in growth performance and muscle mass can be achieved through genetic selection of food animals, including various poultry species (Rehfeldt et al., 2000). The heavy weight (**HW**) Japanese quail line selected for a higher BW at 4 wk of age showed greater growth rate compared with the random bred control (**RBC**) quail line (Choi et al., 2013a). At 6 wk of age, the HW quail line had approximately 2 and 3 times greater BW and pectoralis major muscle weight (**PMW**) compared with the RBC quail line, respectively (Choi et al., 2013a). This greater PMW seen in the HW quail line is mainly associated with muscle fiber hypertrophy, especially a higher percentage of type IIB fibers and greater area of type IIA fibers compared with the RBC quail line, rather than muscle fiber hyperplasia (Choi et al., 2013b).

Dramatic changes were observed in muscle characteristics during growth, which are natural concomitant effects of genetic selection (Rehfeldt et al., 2000). As myosin is the major protein that determines the main functions of skeletal muscle, including contraction speed and metabolic characteristics (Schiaffino and Reggiani, 1994; Choi and Kim, 2009), the transition of myosin heavy chain (**MyHC**) isoforms is often monitored as a marker for skeletal muscle growth and maturation (Bandman, 1999). In broiler chickens selected by their higher BW and PMW, embryonic to neonatal and neonatal to adult MyHC isoform transition is occurring faster, and this transition rate of MyHC isoform is related to faster muscle maturation compared with Leghorn chickens (Lee et al., 2012).

Both muscle fiber hyperplasia and hypertrophy are largely determined by expression time and levels of Pax7 and myogenic regulatory factors (**MRF**; de Almeida et al., 2010; Al-Musawi et al., 2011; Ropka-Molik

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et al., 2011; Schiaffino and Reggiani, 2011). Pax7 is expressed in myogenic and satellite cells and is involved in the control of developmental processes (Mansouri et al., 1999; Seale et al., 2000). The primary MRF, MyoD and Myf-5, are required for determination of myoblasts from myogenic precursor cells and for acquisition of the myoblast phenotype (Cossu et al., 1996; Seale and Rudnicki, 2002). Secondary MRF, such as myogenin, are expressed last during the time associated with myoblast fusion and regulated terminal differentiation (Smith et al., 1994; Seale and Rudnicki, 2002). Temporal expressions of these proteins in selected animals for their BW are linked to temporal transition of the MyHC isoforms, and thus are associated with skeletal muscle growth and ultimate muscle mass (Lee et al., 2012; Riuzzi et al., 2012). However, temporal expressions of MyHC isoforms, Pax7, and MRF in the HW quail line compared with the RBC quail line have not been elucidated. Therefore, the aim of this study was to compare the expression time and levels of MyHC isoforms, Pax7, and MRF including MyoD, Myf-5, and myogenin between the HW and RBC quail lines during muscle development to better understand the mechanisms leading to increased skeletal muscle mass in the HW quail line compared with the RBC quail line.

MATERIALS AND METHODS

Birds and Muscle Samples

Fertile eggs from the RBC and HW quail lines were obtained from the Ohio Agricultural Research and Development Center of the Ohio State University in Wooster. Eggs were incubated in a hatchery (type 65Hs, Masalles, Spain). At 9, 11, 13, and 15 d embryo, eggs were randomly removed from the incubator, and 3 embryos from each line (total 12 embryos per each line) were extracted with forceps. After hatching, quail from each line were reared in heated Petersime battery brooders. Quail were maintained in temperature controlled cages with free access to food and water categorized by each line. Chicks were fed a commercial diet in accordance with the NRC (1998). At 0, 3, 7, 14, 21, 28, 42, and 75 d posthatch, 6 quail from each line (total 48 quail per each line) were randomly selected. All the experiments were approved by the Institutional Animal Care and Use Committee at the Ohio State University (protocol no. 2013A00000041). Quail were individually euthanized by CO_2 inhalation following standard procedures (FASS, 1999). Muscle samples were taken from the entire right pectoralis major muscles, and then immediately frozen in liquid nitrogen and stored at –80°C for the Western blot analysis and quantitative real-time PCR.

Western Blot Analysis

Pectoralis major muscle samples were homogenized in ice-cold 1 × lysis buffer (62.5 m*M* Tris, 5% SDS) with a polytron homogenizer (Fisher Scientific, Pittsburgh, PA) at the lowest setting using 3 × 4 s bursts and combined with 2 × Laemmli buffer (Bio-Rad Laboratories, Hercules, CA) containing 62.5 m*M* Tris, 1% SDS, 5% 2-mercaptoethanol, 12.5% glycerol, and 0.05% bromophenol blue. Proteins were separated by SDS-PAGE using a mini-PROTEAN system (Bio-Rad Laboratory, Hercules, CA), and then were stained with Coomassie brilliant blue and used as a control of protein loading for Western blot analysis (Supplemental Figure S1; http://dx.doi.org/10.3382/ps.2013-03844). After SDS-PAGE, proteins were wet-transferred to a 0.45 μm pore polyvinylidine fluoride (**PVDF**) membrane (Millipore, Billerica, MA). Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline-Tween (TBST; 20 m*M* Tris, 150 m*M* NaCl, pH 7.4, plus 0.15% Tween 20) for 30 min at room temperature. Membranes were incubated overnight at 4°C in 5 different primary antibodies: 2E9 antibody (neonatal MyHC isoform, 1:1,000 dilution; a gift from Everett Bandman, University of California, Davis), AB8 antibody (adult MyHC isoform, 1:1,000 dilution; a gift from Everett Bandman), Pax7 antibody (1:500 dilution; Developmental Studies Hybridoma Bank, Iowa City, IA), MyoD antibody (1:1,000 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA), and Myf-5 antibody (1:500 dilution; Sigma-Aldrich Co., St Louis, MO). Membranes were then washed in TBST for 1 h and incubated with horseradish peroxidase-conjugated secondary anti-mouse IgG (1:5,000 dilution; Santa Cruz Biotechnology Inc.) or secondary anti-rabbit IgG (1:5,000 dilution; Santa Cruz Biotechnology Inc.) in TBST for 1 h at room temperature. Membranes were washed for 1 h in TBST followed by detection with ECL Plus (GE Healthcare, Piscataway, NJ). The membranes were exposed to Bio-Max x-ray film (GE Healthcare) for visualization of the neonatal MyHC isoform, adult MyHC isoform, Pax7, MyoD, and Myf-5 proteins. For comparison of all time points between the 2 quail lines, one quail per each time point (9, 11, 13, and 15 d embryo, and 0, 3, 7, 14, 21, 28, 42, and 75 d posthatch) was used. Several membranes for RBC and HW quail protein samples were exposed on the same x-ray films at several different times to get a linear range of Western blot signals or maximum exposure to detect minor signals at specific time points. Based on these data with several exposure times, specific time points showing obvious differences were selected. Three samples per each specific time point were used for the purpose of quail line comparison. Each protein band intensity at specific time points (neonatal MyHC: 3 and 75 d posthatch; adult MyHC: 28 and 42 d posthatch; Pax7: 7 and 14 d posthatch; MyoD: 9 and 11 d embryo, and 7 d posthatch; Myf-5: 9 and 11 d embryo, and 3 and 7 d posthatch) was calculated by subtracting the background within the area measured in each band from the total band intensity (Kodak 1-D Image Analysis Software, Eastman Kodak Company, Rochester, NY).

Quantitative Real-Time PCR

Pectoralis major muscle samples at 0, 3, 7, 14, 28, 42, and 75 d posthatch from the RBC and HW quail lines were used (6 samples per each time point; total 42 samples per each line). Total RNA was isolated from muscle tissues by homogenizing with Trizol (Invitrogen Life Technology, San Diego, CA) as described in our previous report (Shin et al., 2010). The RNA concentration were measured with a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA), and RNA quality was assessed from gel electrophoresis and normalized accordingly. Complementary DNA was produced by reverse transcription using 500 ng of total RNA for each sample following the instructions for manufacturer of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen Life technology). The thermal cycles of the reverse transcription reaction were 65°C for 5 min, 37°C for 52 min, and 79°C for 15 min. Relative mRNA expression levels of myogenin in the pectoralis major muscle between the 2 quail lines were assessed by quantitative real-time PCR (**qPCR**). Sequences for forward and reverse primers were 5′-CTG CCC AAG GTG GAG ATC CT-3′ and 5′-GGG TTG GTG CCA AAC TCC AG-3′, respectively. For the qPCR reaction, equal amounts of cDNA from each muscle sample of the 2 quail lines acted as templates with reagents: AmpliTaq Gold polymerase (Applied Biosystems), GeneAmp 10 × PCR Buffer (containing 100 m*M* Tris-HCl, pH 8.3), and 500 m*M* KCl. The amplification of the products was duplicated (25 μL each) and detected by SYBR green. The qPCR reaction was performed by using the following thermal conditions: 95°C for 10 min, 40 cycles of 94°C for 15 s, 60°C for 40 s, and 72°C for 30 s, with an additional 82°C for 33 s. The comparative $2^{-\Delta Ct}$ method for relative quantification was used to calculate the relative gene expression. Ribosomal protein 13 (RPS13) was used to normalize the qPCR calculation (Serr et al., 2011; Yang et al., 2013).

Statistical Analysis

General linear model procedure was performed for the association between the RBC and HW quail lines using the SAS software (SAS Institute, 2009). Significant differences in values between the 2 quail lines were detected by the probability difference (PDIFF), and the mean values were separated at the level of 5%. Results for the 2 quail lines are presented as least squares means together with the SE.

RESULTS AND DISCUSSION

As shown in Figure 1, the HW quail line had distinct temporal expression patterns of MyHC isoforms in the pectoralis major muscle compared with the RBC quail line. The 2E9 antibody, which specifically detects neonatal MyHC isoform, reacted with MyHC in the HW quail line from 7 through 75 d posthatch (Figure 1A-1), whereas neonatal MyHC isoform in the RBC quail line was detected from 3 to 75 d posthatch. This initial view of temporal expression patterns from one individual per time point for all time points encouraged us to focus on the specific time point that showed obvious differences between the 2 quail lines; and further compare 3 individuals per each line. Comparison of the quail lines at 3 ($P < 0.001$) and 75 ($P < 0.05$) d posthatch (Figure 1A-2) showed that the expression level of neonatal MyHC isoform was significantly higher in the RBC quail line compared with the HW quail line. Comparison of temporal expression of adult MyHC isoform for all time points by Western blot analysis using the AB8 antibody revealed that expression began at 28 d posthatch in the RBC quail line with sustained expression through 75 d posthatch. In the HW quail line, adult MyHC isoform was only expressed at 75 d posthatch (Figure 1B-1). Comparing the quail lines, after overexposure of the x-ray film from 0.5 to 3 min, the adult MyHC isoform in the HW quail line could be barely detected, although significantly lower expression levels of this protein were observed in the HW quail line at 28 ($P < 0.001$) and 42 ($P < 0.001$) d posthatch compared with the RBC quail line (Figure 1B-2).

Stabilization of the adult phenotype is associated with the time of skeletal muscle maturation (Bandman and Bennett, 1988). Especially, the time window of adult MyHC isoform and transition time from neonatal to adult MyHC isoform can be used as markers for the time of muscle maturation (Bandman and Bennett, 1988). Broilers selected for their greater PMW not only exhibit earlier expressions of neonatal and adult MyHC isoforms, but also show faster muscle growth rate and faster maturation of muscle fibers compared with Leghorns at the same age (Reddish et al., 2005; Lee et al., 2012). In the comparison of the quail lines, the HW quail line showed greater BW and PMW during all age groups from 6 d embryo to 75 d posthatch (Choi et al., 2013a). Unlike broilers, lower growth rate of the pectoralis major muscle was observed in the HW quail line between 0 to 4 d posthatch (2.1- vs. 2.7-fold) compared with the RBC quail line (Choi et al., 2013a). Thereafter, the HW quail line showed the most rapid growth rate of PMW (33.0- vs. 12.9-fold) between 4 to 15 d posthatch coinciding with the most rapid DNA accretion (10.3- vs. 4.0-fold) compared with the RBC quail line (Choi et al., 2013a). Results from the current study support these findings that delayed expressions of neonatal and adult MyHC isoforms in the HW quail line are associated with slower muscle growth at the early posthatch period and delayed muscle maturation compared with the RBC quail line. Delayed maturation of muscle may allow for retention and maintenance of more myogenic progenitor cells that could eventually proliferate and differentiate to donate more nuclei into myofibers, resulting in a greater accretion of DNA that was found in the HW quail line. In this regard, pro-

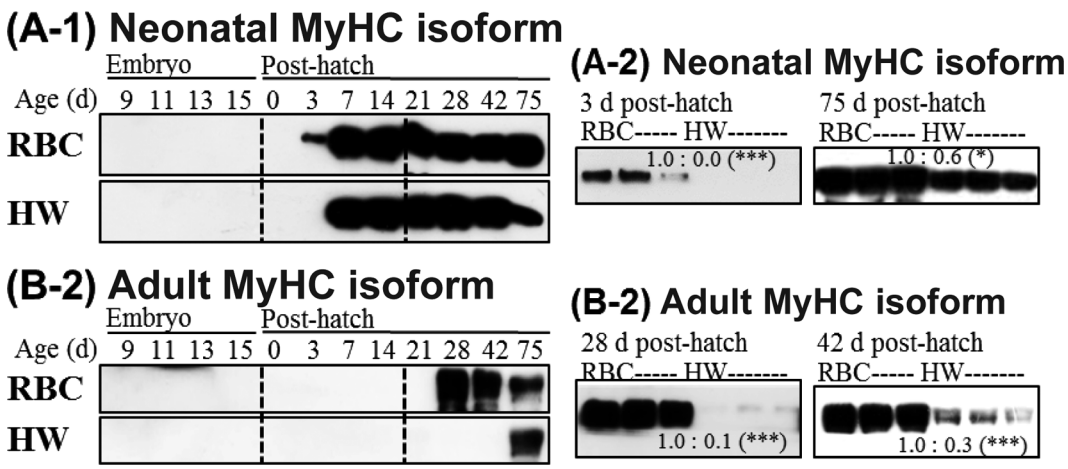

Figure 1. Comparison of expression time and levels of different myosin heavy chain (MyHC) isoforms in the random bred control (RBC) and heavy weight (HW) quail lines. Time point comparison of neonatal MyHC isoform (A-1) and adult MyHC isoform (B-1) between the RBC and HW quail lines (1 quail per each time point). Quail line comparison of neonatal MyHC isoform at 3 and 75 d posthatch (A-2) and adult MyHC isoform at 28 and 42 d posthatch (B-2; 3 quail per each time point and each quail line). Level of significance: $^{*}P < 0.05$; $^{***}P < 0.001$.

longed proliferation followed by consequent processes of myogenesis could be tested by examining temporal expression of markers, specific to each step of myogenesis.

Rapid muscle growth coincides with a high increase in total DNA mass (Merly et al., 1998) as well as satellite cell proliferation, indicated by higher expression level and prolonged expression of Pax7 (Seale et al., 2000; Neill et al., 2009). Greater expression level of Pax7 protein was observed at 1 d posthatch compared with the expression levels at 3 and 6 d posthatch in chickens (Halevy et al., 2004). Lee et al. (2012) reported that broiler chickens with greater hypertrophic potential exhibited significantly greater expression levels of Pax7 at 19 d embryo and 1 d posthatch compared with the Leghorn chickens. In this study, comparison of temporal expression of Pax7 showed that expression continued to 14 d in the HW quail line and to 7 d posthatch in the RBC quail line (Figure 2A-1). Comparison of the quail lines (Figure 2A-2) demonstrated that no significant difference was observed between the 2 quail lines at 7 d posthatch ($P > 0.05$), whereas the HW quail line showed a significantly higher expression level compared with the RBC line at 14 d posthatch ($P < 0.001$). Pax7 belongs to a member of the paired-box family of transcription factors that play important regulatory roles during muscle growth (Mansouri et al., 1999), evidenced by the finding that Pax7 (−/−) mice showed a 50% reduced BW with a significantly thinner cross-sectional area of muscle fibers at 7 d of age compared with wild-type littermate mice (Seale et al., 2000). Pax7 is downregulated in differentiated muscle cells, and upregulation of Pax7 prevented myogenic differentiation (Seale et al., 2000). However, premature myogenic differentiation leads to depletion of progenitor cells and causes muscle hypotrophy (Schuster-Gossler et al., 2007). In addition, neonatal MyHC isoform is initially expressed in differentiated muscle cells (Picard et al., 2002). Thus, expression time and levels of Pax7 are linked to temporal expression of developmental MyHC isoforms as well as the time of differentiation and skeletal muscle maturation (Lee et al., 2012; Choi et al., 2014). In the current study, Pax7 expression persisted longer in the HW quail line; this result is associated with delayed expression of neonatal MyHC isoform as well as delayed muscle maturation compared with the RBC line. Considering delayed muscle maturation accompanied by prolonged expression of Pax7 and correlative increase in total DNA mass in the HW quail line, it is hypothesized that patterns of temporal expression of the MRF, as markers of myogenic potentials, may differ between the HW and RBC quail lines.

Seale et al. (2000) reported that Pax7 is co-expressed with MyoD and Myf-5 during myoblast proliferation. Expression of these MRF persists to myogenic differentiation during muscle growth (Le Grand and Rudnicki, 2007). MyoD plays a vital role in the differentiation potential of myoblasts (Sabourin et al., 1999), whereas Myf-5 is required for the regulation of the proliferation rate and homeostasis (Le Grand and Rudnicki, 2007; Ustanina et al., 2007). Temporal expressions of the primary MRF including MyoD and Myf-5 are graphically displayed in Figure 2B-1 and 2C-1, respectively. Expressions of MyoD and Myf-5 continued from embryonic ages up to 7 and 3 d posthatch in the 2 quail lines, respectively. Higher expression levels of MyoD (Figure 2B-2) and Myf-5 (Figure 2C-2) were observed in the HW quail line at 9 and 11 d embryo compared with the RBC quail line ($P < 0.05$); moreover, greater band intensities of Myf-5 were observed in the HW quail line at 3 ($P < 0.001$) and 7 ($P < 0.05$) d posthatch compared with the RBC quail line. These high expression levels of primary MRF occurred with increased myoblast proliferation and differentiation (de Almeida et al., 2010), which in turn influence muscular hypertrophy and hyperplasia in animals (Langley et al., 2002). Therefore, an increased expression of primary MRF in the HW

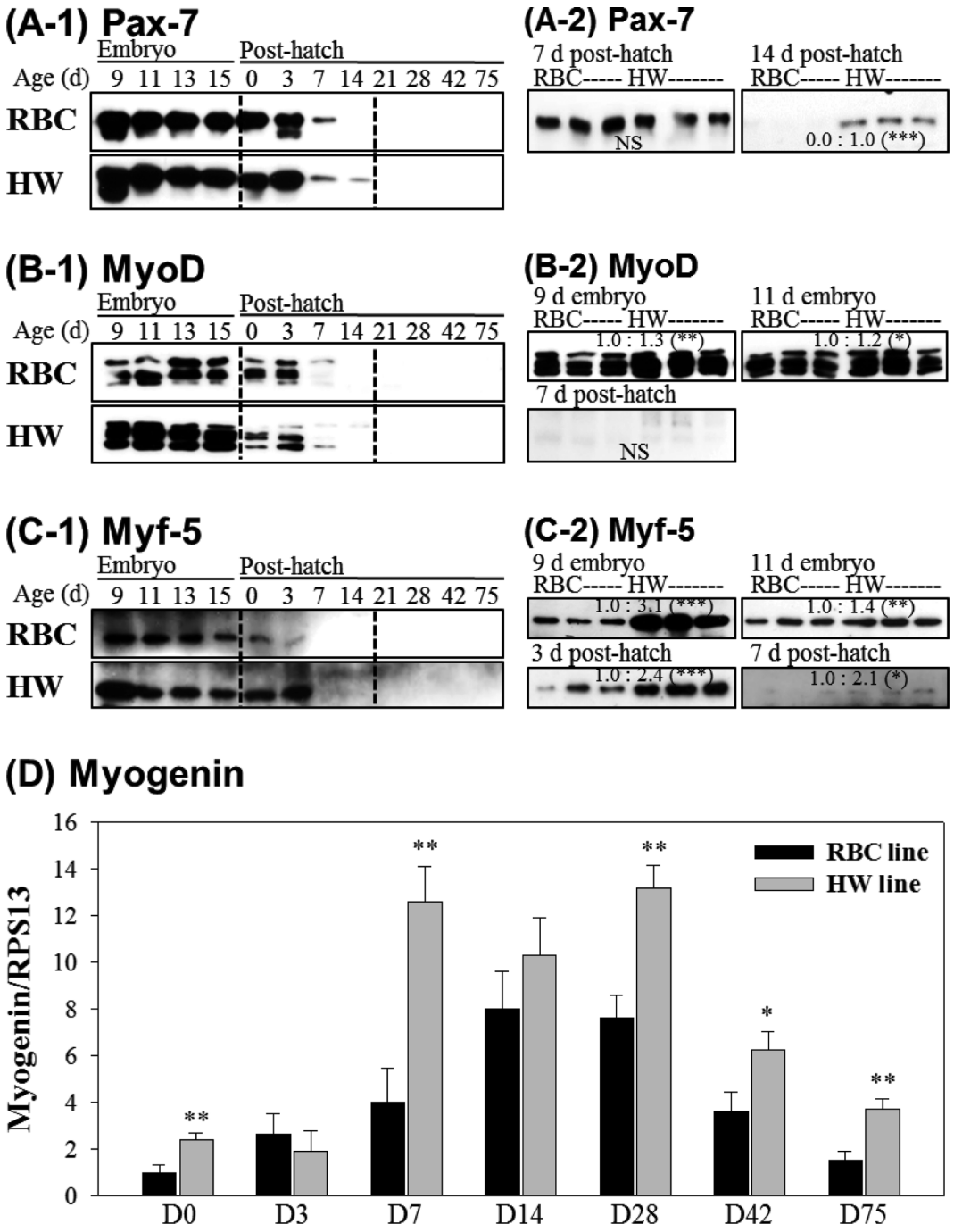

Figure 2. Comparison of expression time and levels of the different myogenic factors in random bred control (RBC) and heavy weight (HW) quail lines. Time point comparison of Pax7 (paired box 7; A-1), MyoD (myogenic differentiation; B-1), and Myf-5 (myogenic factor 5; C-1) between the RBC and HW quail lines (1 quail per each time point). Quail line comparison of Pax7 at 7 and 14 d posthatch (A-2), MyoD at 9 and 11 d embryo and 7 d posthatch (B-2), and Myf-5 at 9 and 11 d embryo and 3 and 7 d posthatch (C-2; 3 quail per each time point and each quail line). Expressions of myogenin mRNA in the RBC and HW quail lines at 0, 3, 7, 14, 28, 42, and 75 d posthatch (D). Expression levels of myogenin were measured by quantitative real-time PCR with avian ribosomal protein 13 (RPS13) as a control for normalization. Bars indicate SE. Level of significance: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

quail line during embryonic and early posthatch periods would provide greater growth potential in the HW quail line compared with the RBC quail line.

The MRF also have crucial functions regarding triggering the expression of structural proteins and generating the assembly of mature muscle fibers (Molkentin and Olson, 1996; Lin et al., 2006). Myogenin is one of the secondary MRF that acts as a key regulatory protein during muscle differentiation, evidenced by the finding that myogenin (−/−) mice die at birth due to a failure in assembling mature muscle fibers (Hasty et al., 1993). During postnatal periods, mice harboring a lower myogenin expression level showed a reduced body size compared with mice harboring a higher myogenin expression level (Knapp et al., 2006). Thus, expression levels of myogenin during muscle growth are associated

with muscle fiber hyperplasia as well as fiber hypertrophy (Al-Musawi et al., 2011). Results from quantitative real-time PCR (Figure 2D) showed no significant difference in expression levels of myogenin at 3 d posthatch between the HW and RBC quail lines (1.9 vs. 2.6, $P > 0.05$). Thereafter, the HW quail line showed more than 1.7-fold greater expression levels of myogenin from 7 to 75 d posthatch ($P < 0.05$) compared with the RBC quail line with the exception at 14 d posthatch (10.3 vs. 8.1, $P > 0.05$). This temporal expression of myogenin supports previous findings that the RBC quail line exhibited a greater muscle growth rate between 0 and 4 d posthatch, whereas the HW quail line showed faster rates of increase in total DNA content between 4 and 15 d posthatch (Choi et al., 2013a). Moreover, continuous muscle growth was detected in the HW quail line after 15 d posthatch compared with the RBC quail line (Choi et al., 2013a). In general, increased muscle DNA during the postnatal period involves muscle fiber hypertrophic growth, not muscle fiber hyperplasia, due to more nuclei donation into existing myofibers and myofibrillar protein accumulation in the myofibers (Merly et al., 1998). Therefore, upregulation of myogenin with high increases in muscle DNA within the HW quail line after 7 d posthatch lead to greater hypertrophic potential and continuous muscle growth compared with the RBC quail line.

Taken together, the results from this study suggest that delayed expressions of neonatal and adult MyHC isoforms could be related to delayed muscle maturation in the HW quail line compared with the RBC quail line. Prolonged upregulation of primary and secondary MRF is also associated with delayed maturation and continued muscle growth, which consequently leads to hypertrophic muscle growth potential in the HW quail line. These findings encourage identifying genetic factors responsible for muscle hypertrophy with changes in temporal expression of myogenic markers that could be a selection marker for superior poultry with greater growth rates and muscle mass.

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