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Effect of Growth Selection on Adipogenic Gene Expression During the Development of the Turkey Breast Muscle

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Abstract: The effect of growth selection in turkeys was studied for its effect on the expression of adipogenic genes in the pectoralis major (p. major) muscle both *in vivo* and *in vitro* in a random bred control line (RBC2) and a subline F selected long-term from the RBC2 for only increased 16-wk body weight. The expression of the adipogenic transcription factors peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer binding protein α (C/EBPα) and stearoyl-CoA desaturase (SCD) were measured in p. major muscle tissue isolated from 18 and 24 d old embryos and 1 and 16-wk old RBC2 and F line turkeys and *in vitro* with adult myoblasts (satellite cells) isolated from RBC2 and F line turkey p. major muscle tissue during proliferation and differentiation. Both *in vivo* and *in vitro*, the F line expressed significantly higher levels of PPARγ, C/EBPα and SCD. The data from the current study provides novel information about the effect of growth selection in turkeys on the potential of fat formation *in vivo* in the p. major muscle and the possible transdifferentiation of myogenic satellite cells to an adipogenic lineage.

Key words: Adipogenesis, muscle, satellite cells, transdifferentiation, Turkeys

INTRODUCTION

Skeletal muscle develops from the embryonic proliferation and differentiation of myoblasts to multinucleated myotubes that then differentiate into muscle fibers. Continued skeletal muscle growth after hatch is dependent on the activation, proliferation and differentiation of the adult myoblast (satellite cells) population of cells. Satellite cells were first identified by Mauro (1961) as being located between the basement membrane and sarcolemma of skeletal muscle fibers. Muscle growth following hatch is through the process of hypertrophy. Satellite cells are responsible for the hypertrophy of existing muscle fibers by fusing and donating their nuclei leading to an increase in muscle fiber size through increased protein synthesis (Moss and LeBlond, 1971). In poultry, the immediate posthatch period is the time of maximal satellite cell activity (Halevy *et al*., 2000; Mozdziak *et al*., 2002).

During the immediate posthatch period especially the first wk posthatch, satellite cells are responsive to extrinsic factors that can alter their cell fate including but not limited to nutritional regime (Halevy *et al*., 2000; Velleman *et al*., 2010; Velleman *et al*., 2014a). Satellite cells are multipotential mesenchymal stem cells that can commit to alternative differentiation programs other than myogenesis (Asakura *et al*., 2001; Shefer *et al*., 2004). Velleman *et al*. (2014a,b) using a 20% feed restriction during the first wk after hatch showed altered morphological structure of the pectoralis major (p. major) muscle, the expression of key myogenic and adipogenic genes and increased fat deposition. In contrast, administering the feed restriction during the second week posthatch eliminated the morphological and fat deposition changes in the p. major muscle and differences in gene expression.

Since satellite cells are the cell type responsible for posthatch muscle growth and the poultry breeding industry has placed a major emphasis in selection for increase posthatch growth rate and breast muscle yield, the question arises as to how selection for growth and increased breast muscle yield will affect satellite cell fate especially in terms of transdifferentiating to an adipogenic lineage. Thus, the objective of the present study was to determine the effect of growth selection on the expression of adipogenic genes in p. major satellite cell cultures *in vitro* and *in vivo* during both embryonic and posthatch development. To study the effects of growth selection a Randombred Control Line 2 (RBC2) turkey and a 16-wk body weight line (F) line turkey were used. The F line was selected long-term from the RBC2 line for only 16-wk body weight (Nestor, 1977) and has significantly increased higher p. major muscle weight than the RBC2 line (Lilburn and Nestor, 1991). Thus, differences between the lines are only due to selection for 16-wk body weight and not genetic background differences. The expression of the adipogenic transcription factors peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer binding

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protein α (C/EBPα), and stearoyl-CoA desaturase (SCD) were measured. The expression of these adipogenic transcription factors in myoblasts has been shown to activate the transdifferentiation of myoblasts to an adipogenic lineage (Hu *et al*., 1995; Yu *et al*., 2006).

MATERIALS AND METHODS

Birds and tissue collection: Pectoralis major muscle tissue was isolated from male RBC2 turkeys and F line turkeys. Both lines of turkeys are maintained at the Poultry Research Center of The Ohio State University/Ohio Agricultural Research and Development Center, Wooster, OH. From the RBC2 line a turkey genetic line (F) has been selected for only increased 16-wk body weight (Nestor, 1977) and has significantly higher p.major muscle weight than the RBC2 line (Lilburn and Nestor, 1991). At 18 and 24 d of embryonic development and 1 and 16-wk of age posthatch, p.major muscle samples were removed and quicken frozen in liquid nitrogen. Samples were stored at -70°C until used.

Satellite cells and culture: Satellite cells were isolated from the p. major muscle of 7-wk-old male RBC2 and F-line turkeys (Velleman *et al*., 2000). Satellite cells were plated in gelatin-coated cell culture plates (Greiner Bio-one, Monroe, NC, USA) in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, MO) containing 10% chicken serum (Gemini BioProducts, West Sacramento, CA), 5% horse serum (Gemini BioProducts), 1% antibiotic/antimycotic (Gemini BioProducts) and 0.1% gentamicin (Gemini BioProducts) in a 37.5°C 5%CO_2/95% air incubator. After 24 h, the cells were cultured in feeding medium composed of McCoy's 5A medium (Sigma-Aldrich, St. Louis, MO) with 10% chicken serum, 5% horse serum, 1% antibiotic/antimycotic and 0.1% gentamicin. Medium was changed every 24 h until the cells reached 60 to 65% confluency at approximately 72 h of proliferation. Differentiation was then induced by changing the medium to a low-serum medium composed of DMEM containing 3% horse serum, 1% antibiotic/antimycotic, 0.1% gentamicin, 0.1% gelatin (Sigma-Aldrich), and 1 mg/mL bovine serum albumin (BSA:Sigma-Aldrich) and medium was changed every 24 h until 72 h of differentiation.

Every 24 h during proliferation and differentiation, cultures were viewed with an Olympus XI 70 microscope (Olympus America, Melville, KY) with digital images recorded with a QI Imaging (QImaging, Burnaby, BC, Canada) camera system. The cell culture plates were then rinsed with PBS, air dried for 15 min and were stored at -80°C until use.

RNA isolation and real-time quantitative PCR: Total RNA was isolated from F and RBC2 satellite cells grown in cell culture or from p. major muscle tissue at 18 and 24 d of embryonic development and from 1 and 16-wk old F and RBC2 line birds. The RNA was extracted using RNAzol RT (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer's instructions. The concentration of the isolated RNA was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA). Moloney murine leukemia virus reverse transcriptase (MMLV: Promega, Madison, WI) was used to synthesize the cDNA. In brief, 1 µg of total RNA, 1 µL of 50 µM oligo dT (Operon, Huntsville, AL) and nuclease-free water to 13.5 µL total reaction volume was incubated at 70°C for 5 min, then cooled. A reaction mixture volume of 11.5 µL containing 5 µL of 5X MMLV buffer, 1 µL of 200 U/µL MMLV reverse transcriptase, 1 µL 10 mM dNTP, 0.25 µL of 40U/µL RNasin and nuclease-free water up to 11.5 µL was added to each tube. The samples were then incubated 50°C for 60 min and then heated at 90°C for 10 min to inactivate the reaction mixture. The reaction mixture was then diluted with 25 µL of nuclease free water for use.

Real-time quantitative PCR (RT-qPCR) was performed according to the manufacturer's instruction using DyNAmo Hot Start SYBR Green (Finnzymes, Ipswich, MA). Each reaction contained 2 µL of cDNA, 10 µL of 2X master mix, 1 µL of a 1:1 mix of 10 µM forward and reverse primers and 7 µL of nuclease free water. Reactions were run using a DNA Engine Opticon 2 real-time machine (Biorad, Hercules, CA). The PPARγ primer sequences (GenBank accession number NM_001001460) were: forward primer 5'-$_{805}$CCACTG CAGGAACAGAACAA$_{824}$-3' and reverse primer 5'-$_{1053}$CTC CCGTGTCA GAATCCTT$_{1034}$-3'. The SCD primer sequences (GenBank accession number NM_204890) were: forward primer 5'-$_{152}$CACGGGTGACCAAGA ATGGG$_{171}$-3' and reverse primer 5'-$_{483}$CAGGGGCA GTGTAGCTTT GT$_{464}$-3' and C/EBPα (GenBank accession number NM_001031459.1) forward primer 5'-$_{728}$CAGTGGACAAGAACAGCAACGA$_{749}$-3' and reverse primer 5'-$_{955}$CCTTCACCAGCGAGCTTTCG$_{936}$-3'. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer sequences (GenBank accession number U94327) were: forward primer 5'-$_{504}$GAG GGT AGT GAA GGC TGC TG$_{523}$-3' and reverse primer 5'-$_{703}$CCA CAA CAC GGT TGC TGT AT$_{684}$-3'. Amplification specificity was confirmed by resolving randomly selected samples from all RT-qPCR reactions on a 1% agarose gel. Standard curves were produced using serial dilutions of purified PCR products for each gene. Sample concentrations were determined by comparing results to the appropriate standard curve. Data were then normalized to average GAPDH expression, the cycle threshold of which remained constant. For the p. major tissue isolation, RNA was pooled from 5 birds from each line with 3 separate reverse transcription reaction run and each RT-qPCR was performed using 3 replicates per

sample for each gene. For the cell culture analyses, RNA was isolated from 3 separate experiments and RT-qPCR was performed using three replicates per sample for each gene.

Statistical analysis: All statistical analyses were performed using the PROC GLM of SAS (2010, SAS Institute Inc., Cary, NC) to determine differences between the RBC2 and F line at each sampling time. Differences between the means were evaluated using a Student's *t*-test. Differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

The expression of PPARγ, C/EBPα and SCD was measured at 18 and 24 d of embryonic development and 1 and 16-wk of age in the RBC2 and F lines. In general, the F-line had significantly higher expression of these genes, at 18 d of embryonic development and 1 and 16-wk of age (Fig. 1). None of the genes examined in the p. major muscle of the RBC2 line were at higher expression levels than the F line. Expression for each of the genes was highest at 18 d of embryonic development and decreased with age.

To determine if the expression of the adipogenic genes *in vivo* in the p. major was due in part to the satellite cell transdifferentiation, isolated satellite cells from the RBC2 and F line were cultured during proliferation and differentiation and the expression of PPARγ, C/EBPα and SCD were measured (Fig. 2). F line expression of PPARγ and C/EBPα at 48 h of proliferation was significantly higher (Fig. 2a-b). Expression of PPARγ at 24 and 72 h of differentiation was higher in the F line (Fig. 2a). Only at 48 h of differentiation did the RBC2 line have higher expression of PPARγ. Expression of C/EBPα was increased in the F line compared to the RBC2 at 72 h of proliferation and 24 h of differentiation (Fig. 2b). The expression of SCD was very low in the satellite cell cultures and was not above threshold levels until 24 h of differentiation and at this time the F line had increased expression of SCD compared to the RBC2 line (Fig. 2c).

In summary, the *in vivo* and *in vitro* results together showed that muscle type cells expressing myogenic genes can also express adipogenic genes and selection for growth increases adipogenic gene expression. The expression of PPARγ, C/EBPα and SCD have been shown to be associated with the transdifferentiation of myoblasts to an adipogenic lineage (Hu *et al*., 1995; Yu *et al*., 2006). In addition, the early expression of adipogenic genes during the embryonic period of development suggests the possible accumulation of adipocytes during both hyperplasia and hypertrophy stages of p. major muscle development in the turkey. As expected, the higher expression of the adipogenic genes during proliferation in both the RBC2 and F lines suggests that some of the satellite cells transdifferentiate to adipogenic-like cells rather than fuse to form multinucleated myotubes during differentiation. During proliferation the satellite cells are individual cells and not a multinucleated differentiated structure. During differentiation of the satellite cells into the multinucleated myotubes, expression of the

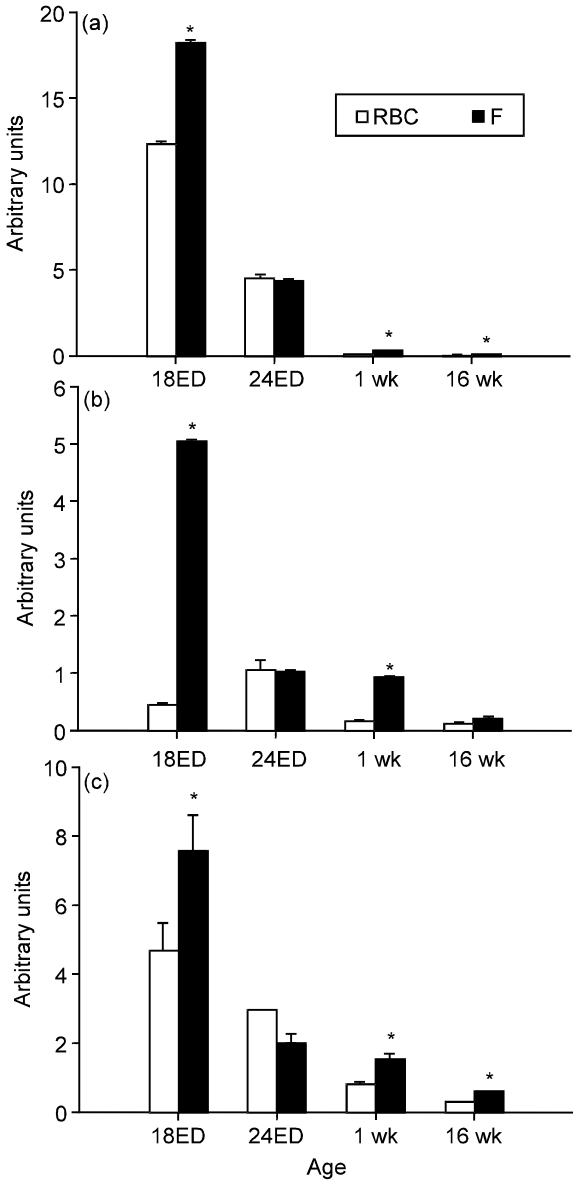

Fig. 1: Expression of (a) peroxisome proliferator-activated receptor gamma (PPARγ), (b) CCAAT/enhancer-binding protein alpha (C/EBPα) and (c) stearoyl-CoA desaturase (SCD) in Randombred Control Line 2 (RBC) and F Line pectoralis major muscle isolated at embryonic days (ED) 18 and 24 and 1 and 16 wk of age. Bars indicate the SEM. *Indicates a significant difference ($p<0.05$)

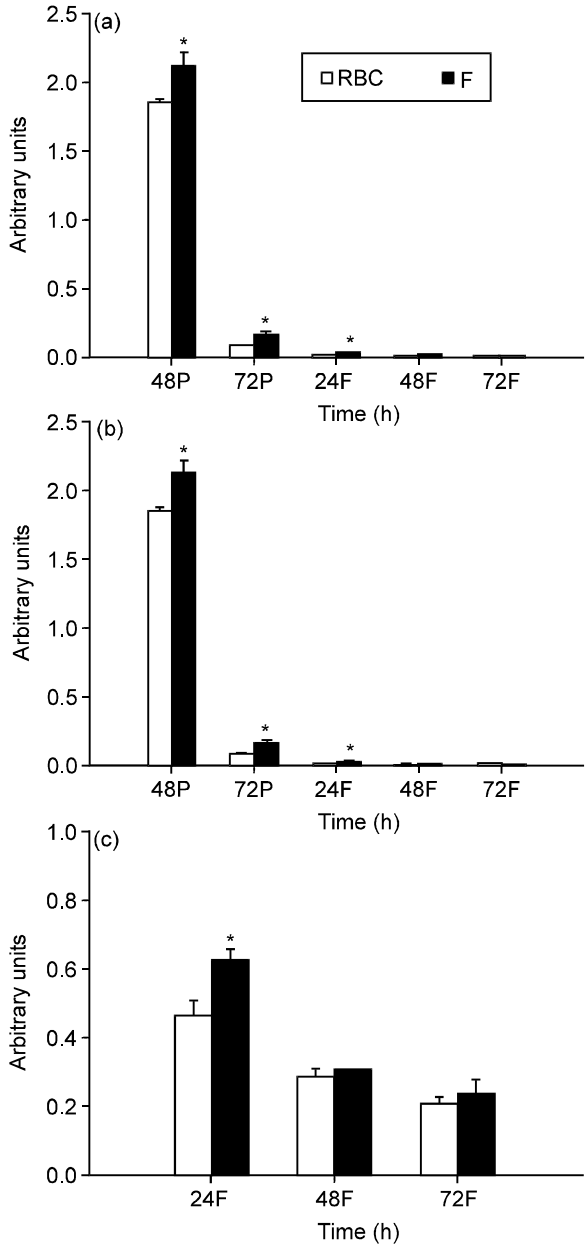

Fig. 2: Expression of (a) peroxisome proliferator-activated receptor gamma (PPARγ), (b) CCAAT/enhancer-binding protein alpha (C/EBPα) and (c) stearoyl-CoA desaturase (SCD) in Randombred Control Line 2 (RBC) and F Line pectoralis major muscle satellite cells during proliferation (P) and fusion into multinucleated myotubes during differentiation (F). Bars indicate the SEM. *Indicates a significant difference ($p<0.05$)

adipogenic genes was decreased compared to the expression levels measured during proliferation. Future research will need to focus on the possible transdifferentiation of the satellite cells during proliferation and the effect of growth selection in turkeys on p. major muscle adipocyte deposition.

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