Research Note: Effect of selection for body weight on the adipogenic conversion of turkey myogenic satellite cells by Syndecan-4 and its covalently attached N-glycosylation chains

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ABSTRACT Adult myoblasts, satellite cells, will proliferate, and differentiate into myotubes in vitro. However, changes in environmental and nutritional conditions will result in the satellite cells differentiating into adipocyte-like cells synthesizing lipids. Prior research has shown that levels of N-glycosylation and heparan sulfate can promote or prevent the adipogenic conversion of myogenic satellite cells. Syndecan-4, an N-glycosylated heparan sulfate proteoglycan, has been shown to play key roles in satellite cell proliferation and migration. The objective of the current study was to determine if syndecan-4, and syndecan-4 N-glycosylation and heparan sulfate chain levels altered the conversion of satellite cells to an adipogenic cell fate and if growth selection affected the response of the satellite cells. Over-expression of syndecan-4, syndecan-4 without N-glycosylated chains but with its heparan sulfate chains attached, syndecan-4 without heparan sulfate chains with its N-glycosylation chains, and syndecan-4 without N-glycosylation and heparan sulfate chains was measured for lipid accumulation in pectoralis major muscle satellite cells isolated from the Randombred Control line 2 (**RBC2**) and 16 wk body weight (F line) turkeys. The F line was selected from the RBC2 line for only 16 wk body weight. Results from this study demonstrated that wild type levels of syndecan-4 and its covalently attached N-glycosylation chains play a key role in regulating the conversion of pectoralis major muscle satellite cells to an adipogenic lineage while selection for body weight was not a major contributing factor in this conversion.

Key words: adipogenesis, N-glycosylation, glycosaminoglycan, heparan sulfate, syndecan-4

INTRODUCTION

Research on the pectoralis major (**p. major**: breast muscle) muscle in broilers has identified the presence of adipocytes by histological analysis (Velleman et al., 2010, 2014a). The adipocytes are located in the perimysial connective space between muscle fiber bundles and within muscle fiber bundles in the endomysial area in chicks undergoing an immediate posthatch feed restriction (Velleman et al., 2010, 2014a), and with thermal stress in fast-growing meat-type broilers (Baziz et al., 1996; Lu et al., 2007) and turkey poults (Velleman, 2014; Clark et al., 2017). The presence of intramuscular fat within broiler and turkey breast muscle is of concern to the industry as the breast muscle is the most economically valuable muscle due to its widespread consumer appeal from its low-fat content and absence of intramuscular fat. Intramuscular fat, as in other livestock species, can arise in the perimysial

connective tissue space located between the muscle fiber bundles from adipocytes likely produced by fibroblasts (Smith and Johnson, 2014). However, fat depots within the muscle fiber bundle endomysial connective space are unique from the perimysial fibroblast derived fat depots. A likely cell type responsible for fat depots within the broiler breast muscle endomysial connective area is the adult myoblast stem cell population also known as satellite cells.

Satellite cells, first discovered by Mauro (1961), are an undifferentiated pool of myogenic stem cells located between the basal lamina and the sarcolemma of skeletal muscle fibers. Posthatch growth and repair of the broiler p. major muscle is dependent on the proliferation and fusion of adult myoblasts, satellite cells, to existing muscle fibers. After hatch, muscle growth occurs through the enlargement, hypertrophy, of existing muscle fibers when the satellite cells fuse with and donate their nuclei to the existing muscle fiber (Stockdale and Holtzer, 1961; Moss and LeBlond, 1971). Although satellite cells represent a pool of stem cells for muscle growth and repair, they are a heterogeneous population of cells (Kuang and Rudnicki, 2008) that can differentiate into other cell types with appropriate stimuli

(Velleman et al., 2010, 2014a). Thus, satellite cells are a multipotential stem cell population with plasticity to commit to myogenesis or alternative differentiation programs such as adipogenesis (Asakura et al., 2001; Shefer et al., 2004, Vettor et al., 2009).

The immediate posthatch period is the time for maximal satellite cell mitotic activity (Halevy et al., 2000; Mozdziak et al., 2002). During this time, satellite cells are sensitive to temperature (Halevy et al., 2001; Loyau et al., 2013; Pietsun et al., 2013) and nutritional regimes (Halevy et al., 2000; Velleman et al., 2010, 2014a,b; Kornasio et al., 2011). Halevy et al. (2000) showed that the timing of a 2-d feed deprivation immediately following hatch affected the accretion of muscle mass. The closer the 2-d feed deprivation was to hatch, the greater the effect on muscle growth. In support of these findings, Velleman et al. (2014a,b) placed newly hatched broiler chicks on a 20% feed restriction either the first or second week after hatch. The results from this study demonstrated that feed restrictions during the first week posthatch negatively impacted the morphological structure of the p. major muscle and increased intramuscular adipose deposition within the muscle fiber bundles. In contrast, ad libitum feeding the first week after hatch and followed by feed restriction the second week after hatch eliminated morphological changes in p. major muscle organization and intramuscular fat deposition.

An important factor in the regulation of satellite cell function is the extracellular matrix (**ECM**). The ECM provides structural support needed to maintain the shape and function of tissues. The ECM, especially ECM proteoglycans, functions in the regulation of satellite cell signal transduction pathways (Velleman and Song, 2017), and their proliferation and differentiation (Velleman et al., 2007). In particular the membrane associated heparan sulfate proteoglycan, syndecan-4, has been shown to modulate the migration of turkey p. major satellite cells through RhoA signal transduction (Shin et al., 2013) and their proliferation (Velleman et al., 2007). Syndecan-4 has an extracellular domain to which heparan sulfate and N-glycosylation are attached to the core protein, a transmembrane domain, and a cytoplasmic domain. In the turkey p. major muscle, syndecan-4 has 3 heparan sulfate chains and 2 N-glycosylation chains. Grassot et al. (2017) reported that heparan sulfate and N-glycosylation levels lead to early fat deposition in murine muscle. Overall this study demonstrated that decreased levels of N-glycosylation and higher amounts of heparan sulfate promoted adipogenic differentiation rather than a myogenic pathway for satellite cells. This is not the only report suggestive of an important role for N-glycosylation in tissue development including cell migration (Lock et al., 2008; Janik et al., 2010) and cell adhesion (Gu et al., 2012).

To further pursue the mechanistic interaction of heparan sulfate glycosaminoglycan and N-glycosylation chains in the conversion of satellite cells from a myogenic to adipogenic lineage and the effect of growth selection, syndecan-4 was the focus in the current study because of its critical function in satellite cell mediated myogenesis. Selection for increased breast muscle mass yield in turkeys has been associated with increased expression of adipogenic genes (Velleman, 2014). In the turkey p. major muscle, syndecan-4 heparan sulfate and N-glycosylation chains affect the proliferation and differentiation of satellite cells as demonstrated by site directed mutagenesis of the syndecan-4 heparan sulfate and N-glycosylation chains (Zhang et al., 2008; Song et al., 2011; Velleman and Song, 2017). Thus, using syndecan-4 site directed mutants specific for the heparan sulfate and N-glycosylation chains, p. major satellite cells from a 16 wk body weight selected line (**F**) and a randombred turkey line (**RBC2**), from which the F line was originally selected, were transfected with wild type syndecan-4, syndecan-4 without any heparan sulfate chains, syndecan-4 without N-glycosylation chains, and syndecan-4 lacking both the heparan sulfate and N-glycosylation chains to determine the effect of these chains on the conversion of satellite cells to an adipogenic fate. The RBC2 and F lines have been well characterized with regard to their body weight and p. major muscle mass accretion through 16 wk of age (Lilburn and Nestor, 1991), the proliferation and differentiation of p. major muscle satellite cells (Velleman et al., 2000), and the functional impact of syndecan-4 expression (Velleman et al., 2018). Beginning at hatch, body weight and p. major muscle weight are significantly greater in the F line than the RBC2 line (Lilburn and Nestor, 1991). The increased p. major muscle growth in the F line is supported by the satellite cells proliferating and differentiating at a faster rate (Velleman et al., 2000). Furthermore, syndecan-4 expression plays an important role in modulating satellite cell proliferation and differentiation in an age-dependent manner in both lines as shown by Velleman et al. (2018).

MATERIALS AND METHODS

Satellite Cells

Satellite cells were isolated from the p. major muscle of 7 wk posthatch male randombred control 2 (RBC2) line and F line turkeys from generation 15 as described in Velleman et al. (2000). Isolated cells from 30 turkeys of each line were expanded and stored in liquid nitrogen until use for experiments. The RBC2 line represents a 1967 commercial turkey that has been maintained at The Ohio State University, Ohio Agricultural Research and Development Center Poultry Research Unit without conscious selection for any traits. The F line turkeys were derived from the RBC2 line by selecting for only increased 16-wk body weight (Nestor, 1977) and has been continually selected based upon this single trait for over 50 generations. Thus, gene expression differences are only due to selection for 16-wk body weight and not genetic background differences.

Satellite Cell Culture

Seven wk posthatch p. major muscle satellite cells from the RBC2 and F lines were plated and transfected with syndecan-4 N-glycosylated no-chain mutant with (S4-N0) or without heparan sulfate chains (S4-S0N0), syndecan- 4 without heparan sulfate chains and containing N-glycosylation chains (S4-S0), wild type syndecan-4 (S4), or pCMS-EGFP empty vector (pCMS: control) as described by Song et al. (2010) to over express each of the syndecan-4 constructs. Over-expression of these syndecan-4 constructs in turkey p. major satellite cells was confirmed in Song et al. (2011). At 72 h of proliferation and at 48 h of differentiation, cell cultures were removed and stored at −70°C until analysis.

In brief, satellite cells were plated using gelatin-coated cell culture plates (Greiner Bio-one, Monroe, NC) in Dulbecco's Modified Eagle Medium (**DMEM**; Sigma-Aldrich, St. Louis, MO) containing 10% chicken serum (Sigma-Aldrich), 5% horse serum (Sigma-Aldrich), 1% antibiotic/antimycotic (Corning, Corning, NY), and 0.1% gentamicin (Omega Scientific, Tarzana, CA) in a 37.5°C 5%CO2/95% air incubator. After 24 h attachment, the cells were transfected with 1 μl Optifect (Invitrogen) and 0.6 μg plasmid DNA from S4, S4-S0, S4-N0, S4-S0N0, or pCMS-EGFP empty vector per well according to the manufacturer's protocol. After 5 h of transfection, the medium was changed to feeding medium containing McCoy's 5A medium (Sigma-Aldrich, St. Louis, MO), 10% chicken serum, 5% horse serum, 1% antibiotic/antimycotic, and 0.1% gentamicin. Feeding medium was changed daily until 72 h post-transfection or until the cells reached 60% confluency. Differentiation was induced by changing the feeding medium to a low serum medium containing DMEM, 3% horse serum, 1% antibiotic/antimycotic, 0.01 mg/mL porcine gelatin (Sigma-Aldrich), 0.1% gentamicin, and 1.0 mg/mL bovine serum albumin (Sigma-Aldrich). The differentiation medium was changed every 24 h until 48 h of differentiation. At 72 h post-transfection during proliferation and at 48 h of differentiation, cell cultures were removed from the incubator with the media removed, and stored at −70°C until analysis.

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from the cell cultures at 72 h post-transfection and 48 h of differentiation using RNAzol RT (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. The cDNA was synthesized using moloney murine leukemia virus reverse transcriptase (**M-MLV**; Promega, Madison, WI). In brief, the RNA-primer mix consisting of 1 μg of total RNA, 1 μL of 50 μM Oligo d(T)20 (Eurofins Genomics, Louisville, KY), and nuclease-free water up to 13.5 μL was incubated at 80°C for 5 min, and then cooled on ice. Then 11.5 μl of the reaction mix containing 5 μL of M-MLV reverse transcription

5X buffer, 1.0 μL 10 mM deoxynucleoside triphosphate mix, 0.25 μL RNasin (40 U/μL), 1 μL M-MLV (200 U/μL), and 4.25 μl nuclease-free water was then added to the cooled RNA-primer mix. The mixture was incubated at 55°C for 60 min, and then heated at 90°C for 10 min to stop the reaction. Twenty-five microliters of nuclease-free water was then added to the cDNA.

Real Time Quantitive PCR Analysis of Gene Expression

Real-time quantitative PCR was performed with a DNA Engine Opticon 2 real-time system (BioRad, Hercules, CA) using the DyNAmo Hot Start SYBR Green qPCR kit (Thermo Scientific, Waltham MA). The PCR reaction consisted of 1 μL cDNA, 5 μL 2X master mix, 0.5 μl primer mix (250 nM of each of the forward and reverse primers), and 3.5 μl nuclease free water up to 10 μL. The primers for peroxisome proliferator-activated receptor gamma (PPARγ) were: forward primer 5′-CCA CTG CAG GAA CAG AAC AA-3′ and reverse primer 5′-CTC CCG TGT CAT GAA TCC TT-3′ (GenBank accession number NM_001001460). The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were: forward primer 5′-GAGGGTAGTGAAGGCTGCTG-3′ and reverse primer 5′-CCACAACACGGTTGCTGTAT-3′ (GenBank accession number U94327.1). For both of the genes, the cycling parameters were denaturation at 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and at 72°C for 30 s with a final elongation of 72°C for 5 min. The melting curve program was 52 to 95°C, 0.2°C per read, and a 1 s hold. The PCR products were analyzed on a 1% agarose gel to check the amplification specificity. Standard curves were constructed with serial dilutions of purified PCR products from each gene. The PCR products were purified by agarose gel electrophoresis using the QI-Aquick Gel Extraction Kit (Qiagen, Valencia, CA). The amount of sample cDNA for each gene was determined by comparing the results to the syndecan-4 standard curve, and then normalized to GAPDH expression. The PCR products were verified by DNA sequencing for specificity.

Quantitation and Imaging of Lipid Accumulation

Lipid accumulation in satellite cell cultures was measured by AdipoRed (Lonza Inc., Walkersville, MD) at 72 h of proliferation and 48 h of differentiation. Satellite cells were cultured as described. AdipoRed quantitation was performed according to the manufacturer's protocol. In brief, plates were removed from the cell culture incubator, the media was removed, and wells were rinsed with 1 mL phosphate buffered saline (**PBS**: 171 mM NaCl, 3.0 mM KCl, 2.0 mM KH_2PO_4 and 10 mM Na_2HPO_4, pH 7.08). One mL of PBS was added

to each cell well and the blank wells as a control for AdipoRed incorporation. To each cell culture well and the control well, 30 μl AdipoRed was added and mixed by pipette. Plates were incubated with the AdipoRed for 15 min at room temperature and read on a Fluorskan Ascent FL scanner (Thermo Fisher Scientific, Pittsburgh, PA) with an excitation of 485 nm and emission of 538 nm. Experiments were repeated four times with four replicates per experiment.

Lipid containing cells in the satellite cell cultures were detected with Oil Red O (Sigma-Aldrich) staining at 72 h of proliferation and 48 h of differentiation. Plates were removed from the incubator, media removed and the cells were fixed in 500 μl of 10% formalin for 5 min. The initial 10% formalin was removed and replaced with an equal volume of fresh 10% formalin for at least 1 h at room temperature. Cells were then washed with 60% isopropanol then allowed to dry completely. Once dry, 200 μl 0.5% Oil Red O was added to each well for 10 min. The plates were then washed under gentle running water for about 5 min until all the non-specific Oil Red O was removed. All plates were allowed to dry before 250 μl of 2 μg/mL of 4', 6-diamidino-2-phenylindole (DAPI; Biotium, Hayward, CA) was added to each well for 20 min to stain cellular nuclei. The DAPI was removed and each well was washed with PBS. Labeled cells were stored in PBS at 4°C until imaging with an Olympus IX70 fluorescent microscope (Olympus America, Mellville, NY) and a QImaging digital camera (QI Imaging, Burnaby, Canada) using CellSens software (Olympus America).

Statistical Analysis

The MIXED procedure of SAS (2010, SAS Institute Inc., Cary, NC) was used to determine differences between treatments. For quantification of intracellular lipid by AdipoRed staining, data were analyzed as a complete block design. The cell culture wells were considered the experimental unit and the data was blocked across experiments consisting of 4 wells for each treatment. For quantification of gene expression, arbitrary units were analyzed as a complete randomized design. Total RNA was pooled across multiple wells, therefore each experimental plating was considered the experimental unit. For both intracellular lipid and gene expression analyses, the least square means (lsmeans) statement was used to determine means and standard error of the means. Differences were considered significant with a $P \leq 0.05$.

RESULTS AND DISCUSSION

Turkey RBC2 and F line p. major muscle satellite cells were transfected with the empty pCMS vector (control), S4, S4-S0, S4-N0, and S4-S0N0. Lipid accumulation was measured at 72 h post transfection during proliferation and at 48 h of differentiation. At 72 h of proliferation in both the RBC2 and F
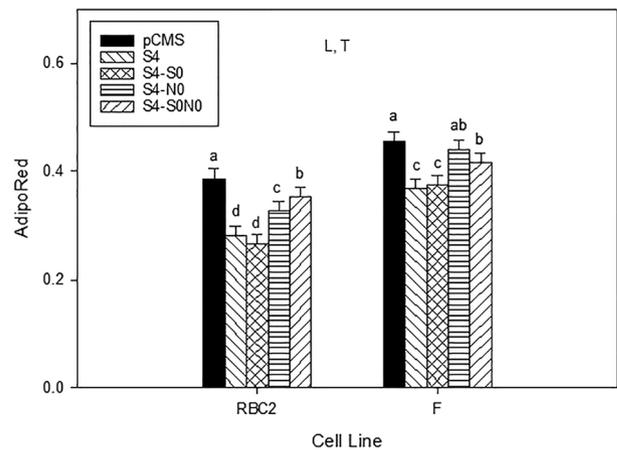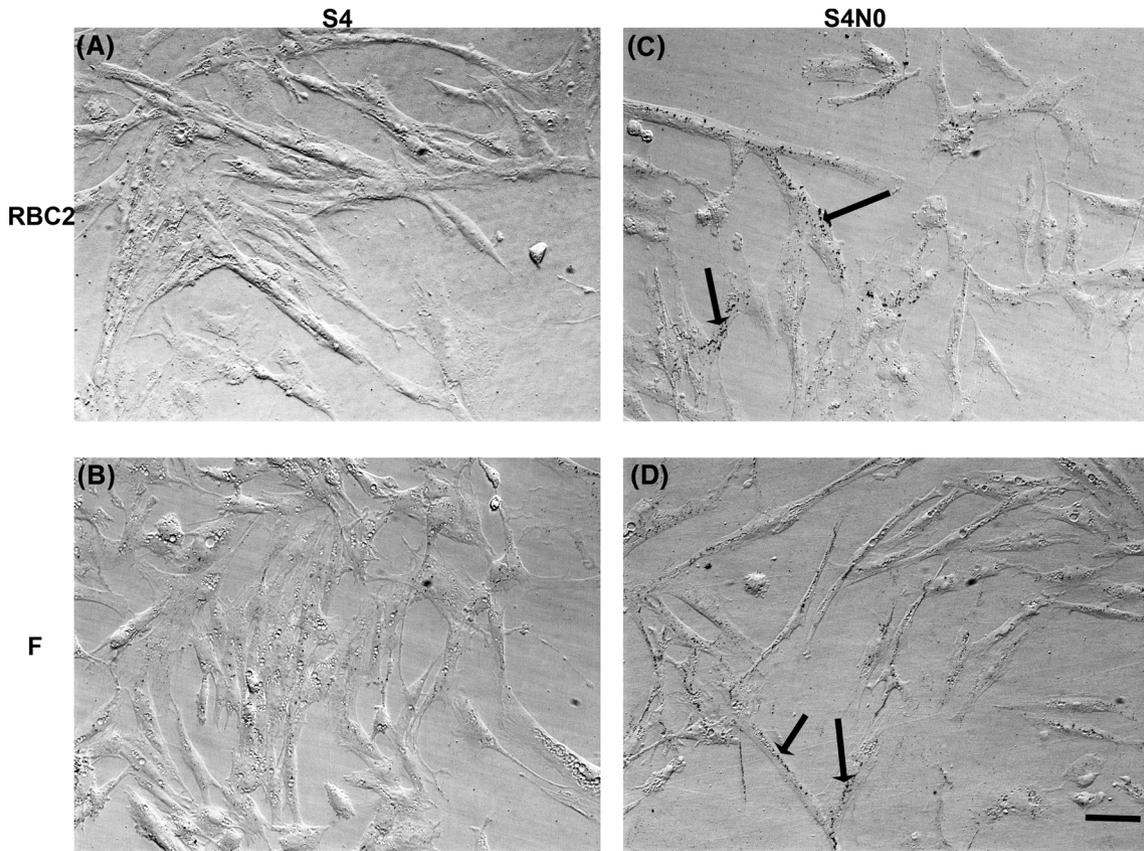

Figure 1. Lipid accumulation in F and Randombred Control Line 2 (RBC2) satellite cells at 72 h of proliferation after transfection with expression constructs for: pCMS-EGFP (pCMS; control), wild type syndecan-4 (S4), syndecan- 4 without heparan sulfate chains and containing N-glycosylation chains (S4-S0), and syndecan-4 N-glycosylated no-chain mutant with (S4-N0) or without heparan sulfate chains (S4-S0N0). Bars without a common letter within cell line are statistically significant ($P \leq 0.05$). Errors bars are the standard error of the mean.

lines, overexpression of wild-type S4 and the S4-S0 constructs decreased the amount of lipid in satellite cells compared to the control transfected with the empty pCMS vector (Figure 1). The amount of lipid did not differ between the S4 and S4-S0 constructs. Overexpression of both S4 constructs without the N-glycosylation chains increased the amount of lipid in the RBC2 and F line satellite cells compared to the S4 and S0 constructs. Oil red O staining of the satellite cells at 72 h of proliferation is depicted for the RBC2 and F line S4 and S4-N0 constructs in Figure 2. The arrows highlight the lipid accumulation within the myotubes of the S4-N0 transfected myotubes in each line.

Similar to the period of satellite cell proliferation, at 48 h of differentiation overexpression of S4 and S4-S0 significantly decreased lipid levels compared to the control empty pCMS vector in both the RBC2 and F lines (Figure 3). In the RBC2 line, overexpression of S4-S0 and S4-S0N0 increased lipid levels to an amount similar to the control empty pCMS vector. However, in the F line satellite cells, overexpression of the S4-S0N0 without both the heparan sulfate glycosaminoglycan chains and N-glycosylation chains increased lipid amount to a greater level than those measured in the control empty pCMS vector. The S4-N0 construct containing the heparan sulfate chains but lacking the N-glycosylation chains increased lipid concentrations to those observed in the control empty pCMS vector, but greater than those in the overexpression with the S4 and S4-S0 constructs.

The effect of syndecan-4 overexpression, its attached heparan sulfate glycosaminoglycan, and N-glycosylation chains on the expression of PPARγ was measured in the RBC2 and F line satellite cells at 72 h of proliferation and 48 h of differentiation (Figure 4).
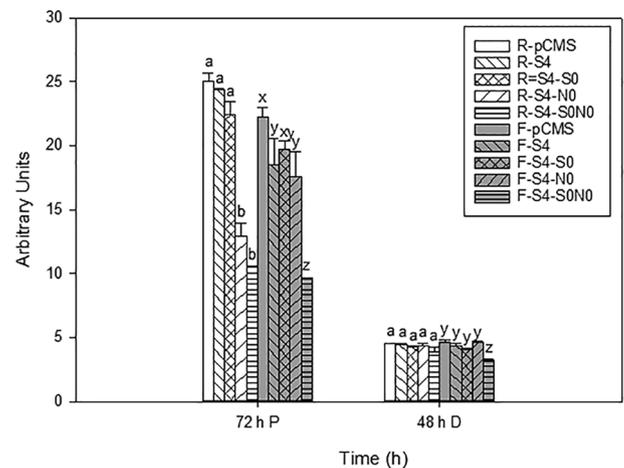
Figure 2. Oil Red O staining representative images of the syndecan-4 (S4) and syndecan-4 N-glycosylated no-chain mutant (S4-N0) at 72 h of proliferation in the F and Randombred Control Line 2 (RBC2) satellite cell cultures. (A) RBC2 line S4; (B) F line S4; (C) RBC2 line S4-N0; and (D) F line S4-N0. Arrows highlight oil red O staining within the myotubes. Scale bar = 50 μm.
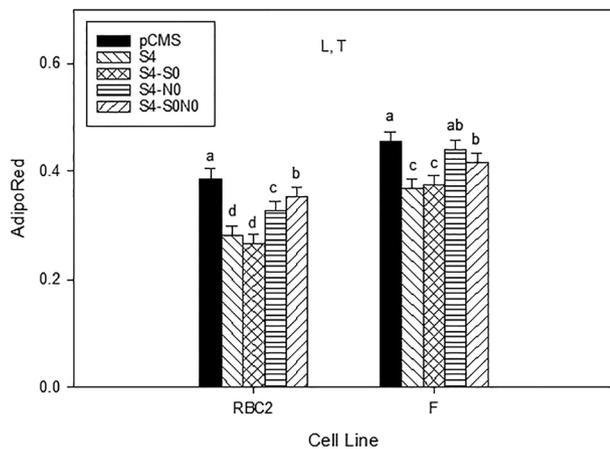
Figure 3. Lipid accumulation in F and Randombred Control Line 2 (RBC2) satellite cells at 48 h of differentiation after transfection with expression constructs for: pCMS-EGFP (pCMS; control), wild type syndecan-4 (S4), syndecan-4 without heparan sulfate chains and containing N-glycosylation chains (S4-S0), and syndecan-4 N-glycosylated no-chain mutant with (S4-N0) or without heparan sulfate chains (S4-S0N0). Bars without a common letter within cell line are statistically significant ($P \leq 0.05$). Errors bars are the standard error of the mean.

Figure 4. Expression of peroxisome proliferator-activated receptor gamma (PPARγ) in F and Randombred Control Line 2 (RBC2) satellite cells at 72 h of proliferation and 48 h of differentiation for satellite cells transfected with syndecan-4 N-glycosylated no-chain mutant with (S4-N0) or without heparan sulfate chains (S4-S0N0), syndecan-4 without heparan sulfate chains and containing N-glycosylation chains (S4-S0), wild type syndecan-4 (S4), or pCMS-EGFP empty vector (pCMS: control). Bars without a common letter within cell lines are statistically significant ($P \leq 0.05$). Errors bars are the standard error of the mean.

Expression of PPARγ in both the RBC2 and F lines was only affected during 72 h of proliferation and not during differentiation except for the F line with the S4-S0N0 construct. For the RBC2 line, only the S4-N0 and S4S0N0 constructs altered PPARγ expression data by significantly decreasing the expression compared to

the control. For the F line satellite cells at 72 h of proliferation, overexpression of the S4, S4-N0, and S4-S0N0 constructs all significantly reduced PPARγ expression. The S4-S0 construct did not differ significantly from the

control pCMS empty vector. These results further support the potential importance of syndecan-4 N-glycosylation chains in lipid expression from myogenic satellite cells.

Taken together, these data are the first demonstration of the possible importance of a membrane-associated heparan sulfate proteoglycan, syndecan-4, in the conversion of p. major satellite cells to lipid expressing cells. Syndecan-4 playing a role in the conversion of satellite cells to an adipogenic cell fate would be a new function as previous studies have only reported syndecan-4 to be pivotal in the activation, self-renewal, regeneration, proliferation, and migration of satellite cells (Cornelison et al., 2001; Cornelison et al., 2004; Velleman et al., 2007; Tanaka et al., 2009; Shin et al., 2013). Thus, syndecan-4 functions in the development, growth, and maintenance of the p. major muscle through its regulation of satellite cell behavior. In turkey p. major satellite cells, syndecan-4 forms oligomers through its cytoplasmic core protein domain (Shin et al., 2012). The formation of syndecan-4 oligomers is necessary for the rearrangement of the cellular cytoskeleton and focal adhesion formation required for cell migration (Choi et al., 2008; Shin et al., 2012) and activation of RhoA signal transduction (Shin et al., 2013).

Results from the current study provide initial support for wild type syndecan-4 expression levels directly affecting satellite cell lipid synthesis. Interestingly, overexpression of wild type syndecan-4 decreased lipid accumulation compared to the control in both the F and RBC2 satellite cells during proliferation and differentiation. Absence of the attached glycosaminoglycan chains, while maintaining the N-glycosylation chains, did not further increase or lower lipid levels from overexpression of wild type syndecan-4. These data suggest that the syndecan-4 core protein may play a direct role in regulating the conversion of satellite cells to adipogenic producing cells. In addition to the syndecan-4 core protein association with lipid synthesis, the covalently attached N-glycosylation chains also play a role in regulating the amount of lipid produced by the satellite cells. In both the F and RBC2 lines during proliferation and differentiation, absence of the N-glycosylation chains increased lipid levels from those of the syndecan-4 overexpression to amounts similar to the control. These data suggest an interaction between the syndecan-4 core protein and N-glycosylation chains in regulating lipid synthesis from the satellite cells. Results from the current study are not the first to demonstrate the potential importance of syndecan-4 N-glycosylation chains in its biological function in satellite cells. Song et al. (2011) demonstrated that both the syndecan-4 N-glycosylated and glycosaminoglycan chains covalently attached to the core protein were required for regulating turkey satellite cell proliferation but not differentiation.

The change in adipocyte-like properties in the RBC2 and F line satellite cells may be associated with their conversion to an adipogenic lineage as supported by the work of Smith and Johnson (2014). Syndecan-4 and its covalently attached N-glycosylation chains when overexpressed reduced this possible conversion of the satellite cells. Intracellular lipid accumulation in satellite cells will result in intramuscular fat within the breast muscle. Thus, optimizing syndecan-4 expression can positively impact satellite cell mediated growth and potentially reduce intramuscular fat content in the breast muscle. Reducing intramuscular fat, especially within in the breast muscle, may positively impact consumer purchases since the demand for a low-fat inexpensive source of protein is a major factor in increased poultry consumption (OECD-FAO, 2015). Despite the changes in lipid levels with the various syndecan-4 constructs, selection for increased body weight with the F line satellite cells had no real impact on the cellular response to the syndecan-4 constructs.

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