Hypoxia further exacerbates woody breast myopathy in broilers via alteration of satellite cell fate

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ABSTRACT Woody breast (**WB**) condition has created a variety of challenges for the global poultry industry. To date, there are no effective treatments or preventative measures due to its unknown (undefined) etiology. Several potential mechanisms including oxidative stress, fiber-type switching, cellular damage, and altered intracellular calcium levels have been proposed to play a key role in the progression of the WB myopathy. In a previous study, we have shown that WB is associated with hypoxia-like status and dysregulated oxygen homeostasis. As satellite cells (**SC**) play a pivotal role in muscle fiber repair and remodeling under stress conditions, we undertook the present study to determine satellite cell fate in WB-affected birds when reared in either normoxic or hypoxic conditions. Modern random bred broilers from 2015 (n = 200) were wing banded and reared under standard brooding practices for the first 2 wk post-hatch. At 15 d, chicks were divided in 2 body weight-matched groups and reared to 6 wk in either control local altitude or hypobaric chambers with simulated altitude of 6,000 ft. Birds were provided ad libitum access to water and feed, according to the Cobb recommendations. At 6 wk of age, birds were processed and scored for WB, and breast samples were collected from WB-affected and unaffected birds for molecular analyses (n = 10/group). SCs were isolated from normal breast muscle, cultured in vitro, and exposed to normoxia or hypoxia for 2 h. The expression of target genes was determined by qPCR using $2^{-\Delta\Delta Ct}$ method. Protein distribution and expression were determined by immunofluorescence staining and immunoblot, respectively. Data were analyzed by the Student's t test with significance set at $P < 0.05$. Multiple satellite cell markers, myogenic factor (**Myf**)-5 and paired box (**PAX**)-7 were significantly decreased at the mRNA and protein levels in the breast muscle from WB-affected birds compared to their unaffected counterparts. Lipogenic-and adipogenic-associated factors (acetyl-CoA carboxylase, **ACCα**; fatty acid synthase, **FASN**, malic enzyme, **ME**; and ATP citrate lyase, **ACLY**) were activated in WB-affected birds. These data were supported by an in vitro study where hypoxia decreased the expression of Myf5 and Pax7, and increased that of ACCα, FASN, ME, and ACLY. Together, these data indicate that under hypoxic condition, SC change fate by switching from a myogenic to an adipogenic program, which explains at least partly, the etiology of the WB myopathy.

Key words: woody breast, hypoxia, fatty acid metabolism, myogenesis, satellite cell

INTRODUCTION

Genetic selection for high growth rate, body weight, and enhanced breast muscle yield in broiler chickens has enabled the poultry industry to produce more food and support the livelihoods of billions of people worldwide (Vaarst et al., 2015; Lilburn et al., 2019; Mourad et al., 2019). Parallel to these significant progresses, several undesirable changes have occurred including increased incidence of metabolic disorders such as fat deposition, leg problems, and muscle myopathies (Kuttappan et al., 2016; Wideman, 2016).

Woody breast (**WB**) is a metabolic condition that has a significant impact on modern broilers and is imposing a heavy economic burden on the poultry industry worldwide (Greene et al., 2020a). It is already present in many countries including USA,

France, Finland, Italy, Spain, Brazil, UK, and Japan (Sihvo et al., 2013; Mudalal et al., 2015; Russo et al., 2015; Alnahhas et al., 2016; de Brot et al., 2016; Cemin et al., 2018; Huang and Ahn, 2018; Kawasaki et al., 2018), and is emerging on a global scale. Many factors contribute to the adverse economic impact of WB phenotype including (1) on-farm culling and mortality, (2) lower meat yield due to decreased protein content, and increased fat and collagen content, (3) down-grading and condemnation at processing, (4) reduced protein functionality in further processed products, and (5) rejection from human consumption due to negative effects on visual appearance and overall consumer acceptance (Kuttappan et al., 2012; Mudalal et al., 2015; Petracci et al., 2015; Soglia et al., 2016; Tijare et al., 2016; Morey et al., 2020).

Although the exact etiology of WB myopathy is still unknown, omics studies indicated several potential contributing factors including hypoxia and oxidative stress, intracellular calcium, and muscle fiber type switching (Mutryn et al., 2015; Abasht et al., 2016; Greene et al., 2020a). In addition to hardening of the affected muscle area, pale color, surface hemorrhaging and the presence of gelatinous fluid on the muscle surface, WB is histologically and microscopically characterized by infiltration of immune and fat cells (Sihvo et al., 2013). However the cellular origin of the adipocyte-like cells is still unknown. de Almeida Mallmann et al. (2019) have shown abundant infiltration of adipose cells in WB-affected muscle and suggested that they originated from bone marrow adipose tissue. Meloche et al. (2018), on the other hand, have reported an increased population of myogenic stem cell types in WB-affected muscles. Velleman's group indicated that satellite cell-mediated regeneration of muscle is suppressed in WB myopathy (Velleman, 2019).

Muscle satellite cells (**SC**), stem cells located between the basement membrane and sarcolemma of muscle fiber (Moss and Leblond, 1971), are self-renewing mesenchymal cells that enable hypertrophy, maintenance, and repair of damaged skeletal muscle (Geiger et al., 2018). It has been shown that SC are multipotent and can be induced by various extrinsic factors and environment to follow myogenic, adipogenic, or osteogenic pathways (Asakura et al., 2001; Wang et al., 2015a; Liu et al., 2017).

In continuum of our previous research where we have shown that WB myopathy is associated with dysmetabolism of oxygen homeostasis and activation of hypoxia pathway (Greene et al., 2020a) as well as alteration of fatty acid profile (Cauble et al., 2020), we undertook the present study to determine the fate of SC in WB muscle using both in vivo and in vitro approaches.

MATERIALS AND METHODS

Birds, Housing and Diets

All animal experiments were approved by the University of Arkansas Animal Care and Use Committee (protocol number 18088) and were in accordance with the recommendations in NIH's Guide for the Care and Use of Laboratory Animals. Birds from the 2015 modern random bred (Orlowski et al., 2017; Tabler et al., 2020), which is composed of broiler packages offered by three broiler genetics companies and have been blended homogenously over five generations of random mating, were maintained at the University of Arkansas. At day of hatch, chicks were vent sexed and males were wing banded and reared under standard brooding practices for two weeks at local altitude. At 15 d, chicks were divided in two body weight-matched groups and reared to 6 wk in either control local altitude (**LA**, 427 m, 1,400 ft) or a simulated high-altitude challenge (**HA**, 1,829 m, 6,000 ft) in a hypobaric chamber. The hypobaric chamber simulates a set altitude above sea level and creates a hypoxic environment from a decrease in the partial pressure of oxygen by operating under a partial vacuum. An altitude of 6,000 ft was chosen to induce a hypoxic environment without inhibiting growth of the broilers or inducing pulmonary hypertension (ascites) syndrome. Both LA and HA chambers were set up identical to each other with litter shavings floor pens, hanging feed cans and a nipple water line. Ventilation was set to maintain airflow at 17 m^3m (600 cfm). Both chambers were warm-room brooded with temperature gradually decreased from 32°C on d 1 to 3, to 31°C on d 4 to 6, 29°C on d 7 to 10, 27°C on d 11 to 14, and 24°C for day 15 through day 42. The photoperiod was maintained at 24 h light. Feed and water were provided ad libitum throughout the study. Birds were individually weighed weekly. All daily and weekly tasks were conducted at altitude. Access to the hypobaric chamber was through an airlock, which allowed for air pressure equilibrium. At 6 wk of age, birds (100 birds/group) were processed in the pilot processing plant at the University of Arkansas and evaluated for the WB myopathy and compression force of the cranial breast lobe as described previously (Orlowski *et al.*, 2018; Greene *et al.*, 2019; Cauble et al., 2020). Breast tissues were collected from WB-affected and unaffected HA birds (n = 10/group), snap frozen in liquid nitrogen, and stored at −80°C for further molecular and biochemical analyses.

SC Culture

Ross 708 broiler SC was previously isolated by Dr. S. G. Velleman (The Ohio State University, Wooster, OH) as described previously (McFarland et al., 1997; Harding et al., 2016; Clark et al., 2018). Briefly, cells were cultured in 6 well-plates containing Dulbecco's Modified Eagle Medium (Life Technologies, Grand Island, NY) with 10% chicken serum (Sigma-Aldrich, St. Louis, MO), 5% horse serum (Sigma-Aldrich, St. Louis, MO), 1% antibiotic/antimycotic (Life Technologies, Grand Island, NY), and 0.1% gentamicin (Life Technologies, Grand Island, NY) at 38°C in a 5% CO_2/95% O_2 humidified incubator. At exponential phase of growth (~80%), cells were exposed to hypoxia by placing

the plates into gas-tight modular hypoxic chamber (1% $O_2/5\%$ $CO_2/94\%$ N_2; Coy Laboratory Products, Inc, MI) for 2h. The control cells were maintained at normoxic conditions (5% $CO_2/95\%$ O_2). The duration of hypoxia exposures was based on pilot and previous studies (Latil et al., 2012).

RNA Extraction, cDNA Synthesis and Quantitative Real-Time PCR

Total RNA extraction, cDNA synthesis, and quantitative real-time PCR were performed as previously described (Greene et al., 2019). Oligonucleotide primers used for chicken lipogenic and adipogenic genes (acetyl CoA carboxylase alpha, ACC-α; fatty acid synthase, FASN; ATP citrate lyase, ACLY; malic enzyme, ME; stearoyl-CoA desaturase-1, **SCD-1**; sterol regulatory element-binding protein 1 and 2, **SREBP-1/2**) and the housekeeping ribosomal 18S were previously described (Nguyen et al., 2015; Flees et al., 2017; Greene et al., 2020b). For satellite cell markers and myogenic transcription factors, pair box protein 3 (PAX-3), PAX-7, myogenic factor **(Myf-5)**, myogenin **(MyoG)**, and myogenic regulatory factor (**MRF**)-4), the oligonucleotide sequences are summarized in Table 1. Relative expressions of target genes were normalized to the expression of r18S RNA and calculated by the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). For the in vivo trial, unaffected birds were used as calibrator; while, for the in vitro trial, SC under normoxia were used as calibrator.

Western Blot

Immunoblot for satellite cell cultures, and breast muscle samples were performed according to previously published papers by our group (Flees et al., 2017; Nguyen et al., 2017; Greene et al., 2019). The following primary polyclonal antibodies diluted to 1:1000 were used: rabbit polyclonal anti-HIF-1α (#LS-C287203), rabbit anti-phospho ACCα^{Ser79} (#3661), rabbit anti-ACCα (#3662), rabbit anti-ACLY (#LS-C290517), rabbit anti-ME (#ARP48511_P050), rabbit anti-

FASN (#NB400-114), mouse anti-PAX-7 (#sc-81648), and rabbit anti-Myf-5 (#ab139523). All the antibodies were purchased from Cell Signaling (Danvers, MA), except the anti-FASN antibody was from Novus Biologicals (Littleton, CO), the anti-ME from Aviva Systems Biology (San Diego, CA), the anti-ACLY and anti-HIF1α antibodies from LSBio (Seattle, WA), the anti-PAX7 from Santa Cruz Biotechnology (Dallas, TX), and the anti-Myf5 from Abcam (Cambridge, MA). The HRP-conjugated secondary antibodies (dilution 1:5000) were from Santa Cruz Biotechnology (Dallas, TX). A prestained molecular weight marker (Precision Plus Protein Dual Color) was used as a standard (BioRad, Hercules, CA). The signal was visualized by enhanced chemiluminescence (ECL plus; GE Healthcare Bio-Sciences, Buckinghamshire, UK), and captured by FluorChem M MultiFluor System (Proteinsimple, Santa Clara, CA). Image acquisition and analysis were performed by AlphaView software (Version 3.4.0, 1993 −2011, Proteinsimple,Santa Clara, CA).

Immunofluorescence

Immunofluorescence staining was performed as previously described (Dridi et al., 2012; Greene et al., 2020b). Briefly, SC were grown in chamber slides and fixed with methanol for 10 min at −20°C, then permeabilized with Triton-X 100. Cells were incubated with serum-free protein block (Dako, Carpinteria, CA) for 1 hour at room temperature, then incubated with anti-HIF1α, anti-PAX7, anti-Myf5, anti-ACLY, anti-FASN, anti-ME, anti-SREBP1 or anti-SREBP2 (1:200, in Antibody Diluent, Dako, Carpinteria, CA, overnight at 4°C) for mono-staining or a combination of anti-PAX7 with anti-Myf5 for double-labeling immunofluorescence. Signal was visualized with DyLight 488- or 594-conjugated secondary antibody (Thermo Fisher Scientific, Grand Island, NY). Slides were cover slipped with Vectashield with 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA), and images were obtained and analyzed using Zeiss Imager M2 and AxioVision software (Carl Zeiss Microscopy, Pleasanton, CA).

Table 1. Oligonucleotide primers for real-time qPCR.

Gene	Accession number[1]	Primer sequence (5' → 3')	Orientation	Product size (bp)
PAX7	NM-205065	AGGCTCCGATGTCGAATCAG	Forward	55
		GCGGCGCTGCTTCCT	Reverse	
PAX3	NM_204269	GCCTCACCAGCCCCAAA	Forward	57
		GGCTCCAGACCTCCAGTCAA	Reverse	
Myf5	NM_001030363	CCTCATGTGGGCTTGCAAA	Forward	59
		CCTTCCGCCGGTCCAT	Reverse	
MRF4	D10599	GCATGATGATGGACCTTTTCG	Forward	61
		CCATTCTCCCCGTCCAAGTA	Reverse	
MyoG	NM_204184	GGAGAAGCGGAGGCTGAAG	Forward	62
		GCAGAGTGCTGCGTTTCAGA	Reverse	

Abbreviations: MRF4, myogenic regulated factor 4; Myf5, myogenic factor 5; MyoG, myogenin; PAX, paired box.
[1]Accession number refer to Genbank (NCBI).
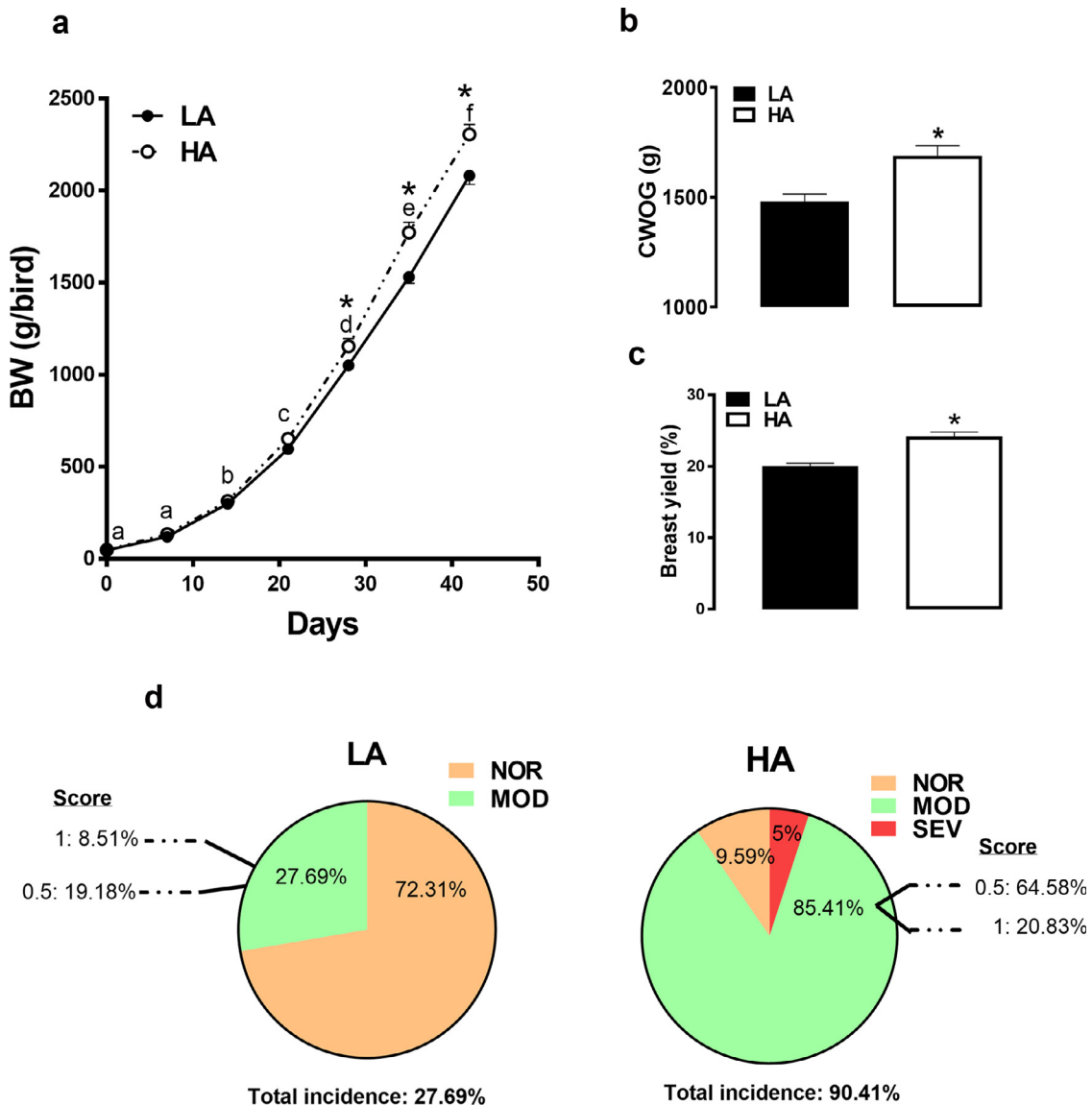
Figure 1. Effects of high altitude (hypoxia) on growth performance and woody breast incidence in broiler chickens. Birds were divided in two body weight-matched groups and reared from 2 to 6 wk in either control local altitude (LA, 427 m, 1400 ft) or high altitude challenge (HA, 1,829 m, 6,000 ft) in hypobaric chambers. Body weight (BW) (A), CWOG (B), and breast yield (C) were recorded. At 6 wk of age, WB was scored as previously described (Greene et al., 2019) (D). Data are presented as mean ± SEM (n = 100/group). *denotes significant difference compared to the LA at the same age and different letters indicate significant difference between age at $P < 0.05$.

Statistical Analyses

For body weight, data were analyzed by two-way repeated measure ANOVA with age and altitude as factors. For the rest of growth performances, mRNA abundances, and protein expression, data were analyzed by Student "t" test. Graph Pad Prism software (version 7.0 for Windows, La Jolla, CA) was used. Differences were considered significant at $P < 0.05$.

RESULTS

High Altitude Induces WB Incidence and Alters CS Fate in Broilers

Performance and WB incidence in birds raised in LA vs. birds raised under HA are shown in Figure 1.

Body weight of HA birds was significantly higher compared to that of LA counterparts on days 28, 35, and 42 (Figure 1A). Chilled with-out-giblets and breast yield were significantly higher in HA compared to LA birds (Figure 1B, C). Total incidence of WB is higher in the HA (90.41%) compared to LA (27.6%) (Figure 1D). When analysed by category, HA-birds manifest 85.4 % moderate (**MOD**) and 5% severe (**SEV**) WB compared to 27.6% MOD and absence of SEV in their LA counterparts. Using SEV WB-affected and unaffected breast tissues from HA birds, molecular and biochemical analyses show that the expression of PAX7 and Myf5 was significantly down regulated at the mRNA and protein levels in WB-affected compared to unaffected-birds (Figure 2A-cC. The mRNA abundances of *PAX3* were also significantly decreased in the breast of
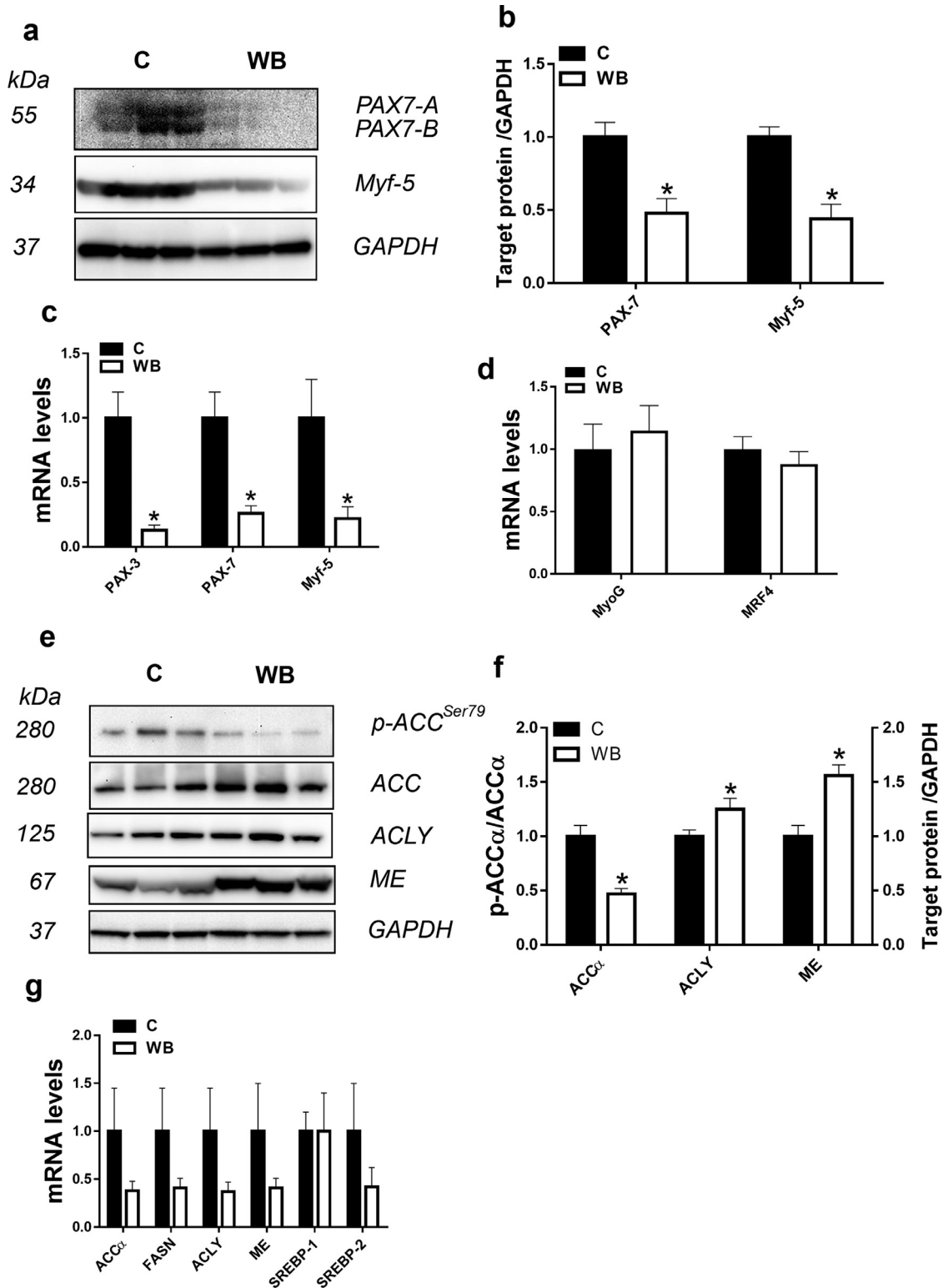
Figure 2. The expression profile of satellite cell- and adipogenic- markers in woody breast muscle. Protein levels were measured by Western blot (A, B, E, F), and mRNA abundances were determined by qPCR (C, D, G) using $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). Data are presented as mean ± SEM (n= 10/group). *denotes significant difference of treated (affected) compared to the control (unaffected) group at $P < 0.05$. Abbreviations: ACC, acetyl-CoA carboxylase; ACLY, ATP citrate lyase; C, control; FASN, fatty acid synthase; ME, malic enzyme; Myf5, myogenic factor 5; MyoG, myogenin; MRF4, myogenic regulatory factor 4; PAX, paired box; SREBP, sterol regulatory element binding protein; WB, woody breast.

WB-affected birds compared to their unaffected counterparts (Figure 2C). The expression of myogenic factors (MyoG and MRF4) remained unchanged between the groups (Figure 2D). Concomitantly, the phosphorylated levels of ACCα at Ser79 site were significantly decreased (Figure 2E). The protein, but not the mRNA levels of ACLY and ME were significantly increased in WB-affected muscle compared to unaffected birds (Figure 2E-G). The expression of the transcription factors *SREBP-1* and
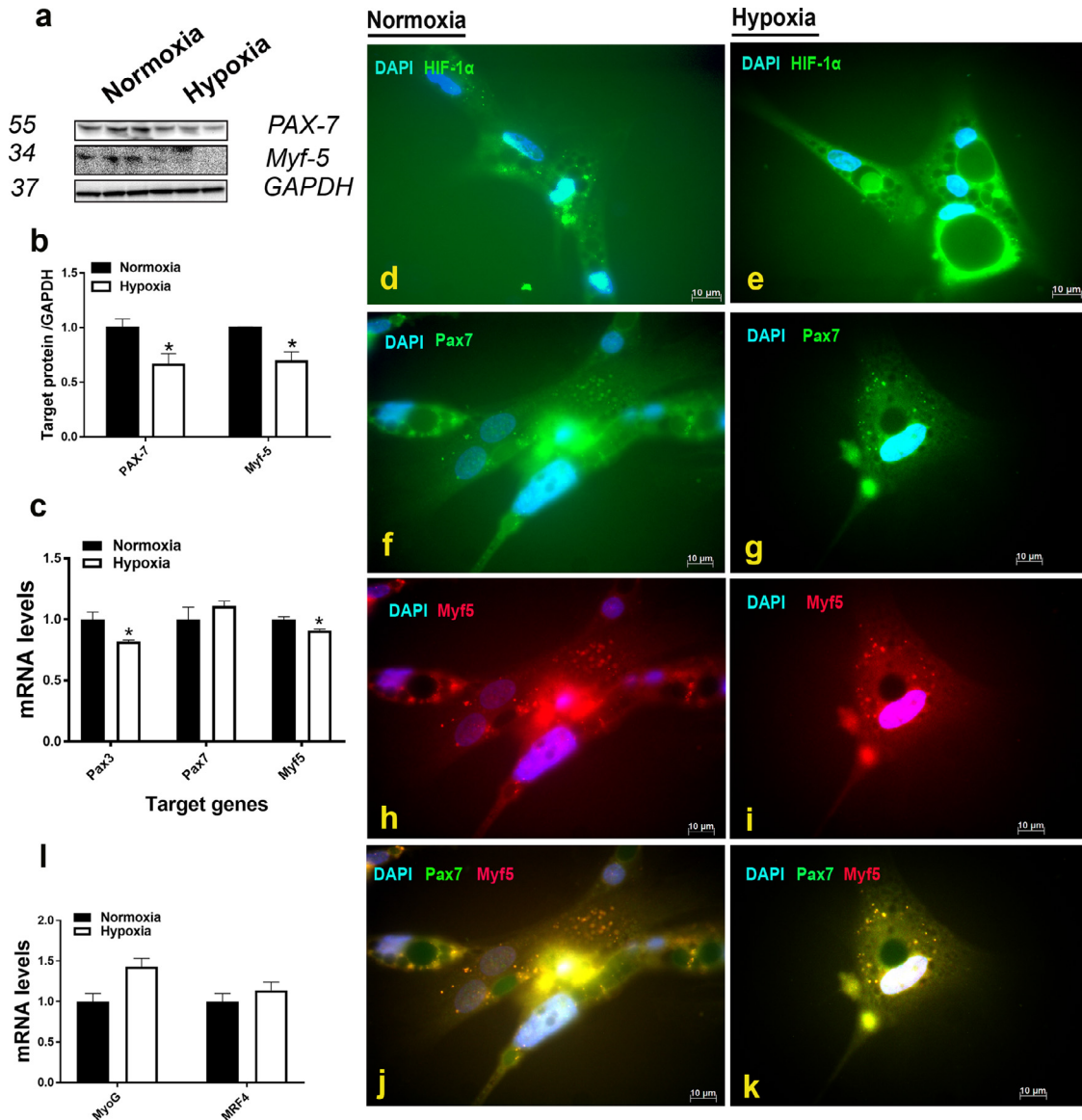
Figure 3. Effects of hypoxia exposure (2 h) on the expression of satellite cell markers in primary satellite cells. Protein levels were measured by Western blot (A, B), and immunofluorescence staining (D-K, scale bars = 10 μm), and mRNA abundances were determined by qPCR (C, L) using $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). Data are presented as mean ±SEM (n= 6/group). *denotes significant difference of treated (hypoxic) compared to the control (normoxic) cells at P < 0.05. Abbreviations: HIF-1α, hypoxia inducible factor 1 alpha; Myf5, myogenic factor 5; MyoG, myogenin; MRF4, myogenic regulatory factor 4; PAX, paired box.

SREBP-2 gene did not differ between the groups (Figure 2G).

Hypoxia Exposure Alters the Fate of Chicken Primary SC

Short exposure (2 h) to hypoxic conditions, as confirmed by HIF-1α activation (Figure 3D, E), down regulates the expression of PAX7 and Myf5 at both the mRNA and protein levels (Figure 3A-C). The expression of the myogenic factors (MyoG and MRF4) did not differ between hypoxic and normoxic SC (Figure 3L). Mono- and double-immunofluorescence staining supports these data and further shows that PAX7- and Myf5-positive cells are decreased in hypoxia compared to normoxia (Figure 3F-K). Phosphorylated levels of ACCα at Ser79 site was significantly reduced in hypoxic

compared to normoxic SC (Figure 4A, B). Protein levels of FASN, but not ME, were significantly induced in hypoxic compared to normoxic SC (Figure 4A, B). Immunofluorescence staining support these data and show an increase of lipogenic proteins along with increased levels of both transcription factors SREBP-1 and 2 (Figure 4D-M). As for proteins, mRNA abundances of ACCα, FASN, and ACLY, but not ME were up regulated in hypoxic compared to normoxic cells (Figure 4C). The expression of SCD-1 and SREBP-1 gene was not affected by 2 h-hypoxia exposure (Figure 4C).

DISCUSSION

Broiler chickens play a key role in worldwide meat production and support the food security of billions of
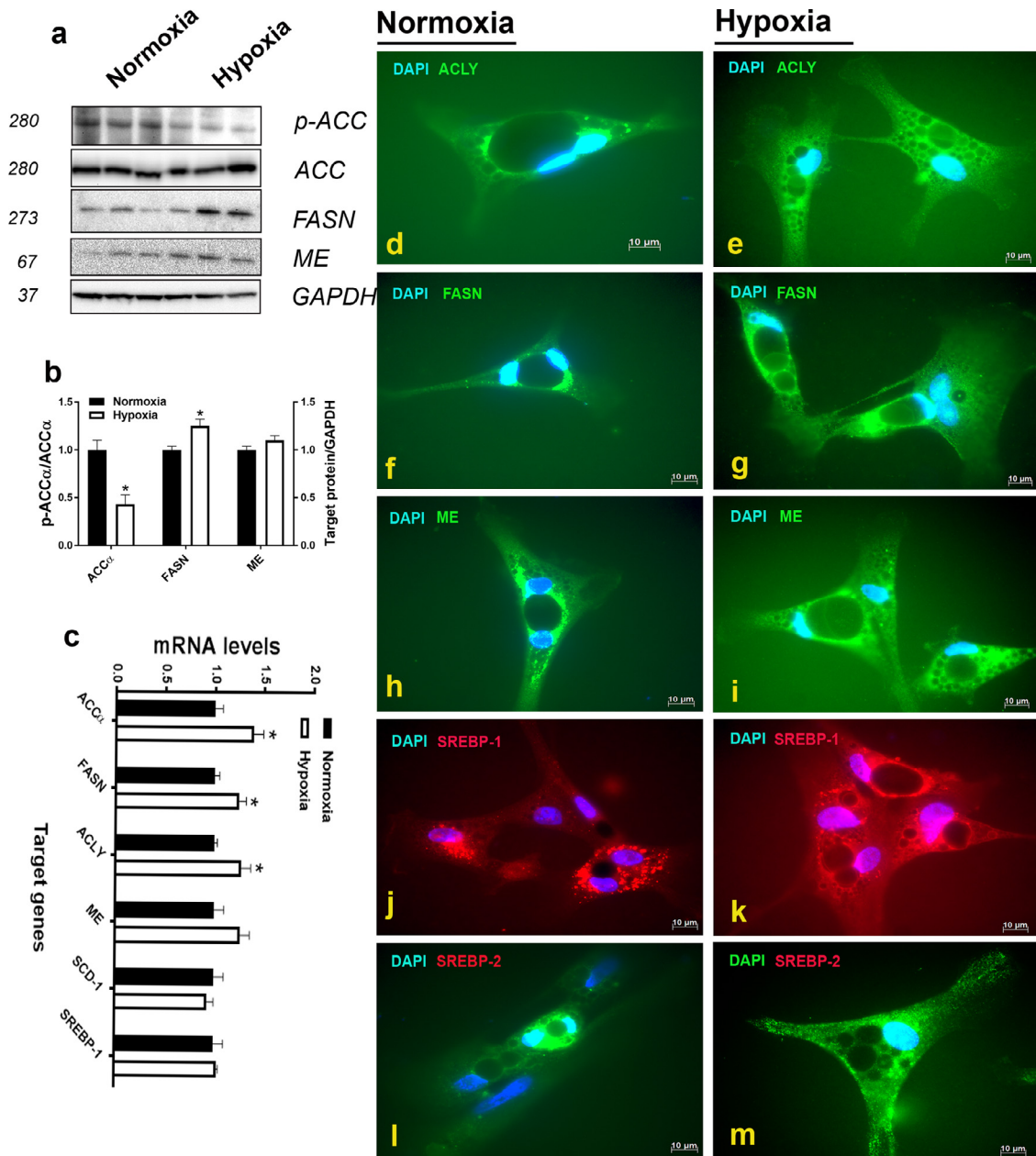
Figure 4. Effects of hypoxia exposure (2 h) on the expression of adipogenic markers in primary satellite cells. Protein levels were measured by Western blot (A, B), and immunofluorescence staining (D-M, scale bars = 10 μm), and mRNA abundances were determined by qPCR (C) using $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). Data are presented as mean ± SEM (n = 6/group). *denotes significant difference of hypoxic compared to the control (normoxic) cells at $P < 0.05$. Abbreviations: ACC, acetyl-CoA carboxylase; ACLY, ATP citrate lyase; FASN, fatty acid synthase; ME, malic enzyme.

peoples. However, the relatively new emerging muscle disorder WB has increased drastically on a global scale, and it is negatively impacting worldwide broiler meat production quality, and sustainability (Trocino et al., 2015). It constitutes a major animal health, welfare, and economic concern which are estimated to cost the poultry industry millions of dollars a year due to on-farm culling and mortality, down-grading and condemnation at processing, as well as rejection from human consumption (Petracci et al., 2019). The WB lesions are associated with clinical and histological changes including multifocal degeneration and necrosis, fiber fragmentation, hyalinization, swelling and fibrosis (Kuttappan et al., 2016), which in turn results in

palpable severe hardness of the breast muscle. Microscopic analyses showed also infiltration of immune and fat cells (de Almeida Mallmann et al., 2019), with a debatable and unclear origin. Although seminal omics, managerial, and nutritional work has been conducted, the etiology of WB is still not fully defined.

Breast muscles have a low concentration of myoglobin and are poorly vascularized (Lilburn et al., 2019), and our previous research indicated that WB in broiler chickens is associated with hypoxia-like status and dysregulated oxygen homeostasis (Greene et al., 2019; Greene et al., 2020a). Using the hypobaric chambers in the present study, the results clearly support our previous data (Greene et al., 2019) and demonstrate that

hypoxia is a cause, not a consequence, of WB myopathy. The WB myopathy in industry broiler flocks has proven to be erratic and unpredictable in both incidence and severity making it hard to understand the mechanism behind it. The hypobaric chambers when run at moderate altitudes could be used as a potential and highly relevant model to consistently trigger high incidences of WB for subsequent identification of mechanism-based strategies to reduce/prevent the myopathy.

Although it is still an area of active research, hypoxia is well known to induce reactive oxygen species production and results in skeletal muscle cell injury and damage (Lundby et al., 2003; Magalhães et al., 2005). In general, responding to injury, skeletal muscle undergoes a highly orchestrated degeneration and regenerative process that takes place at the tissue, cellular, and molecular levels (Yin et al., 2013). SC, which are a heterogeneous population of stem and progenitor cells, are key engines of muscle repair (Wang and Rudnicki, 2011; Chang and Rudnicki, 2014). They proliferate following muscle trauma and form new myofibers through a process similar to embryo muscle development. Many transcription factors including Pax-7, Myf-5, myogenic differentiation, myogenin, and MRF-4 control the myogenic program in SC (Chang and Rudnicki, 2014). Pax-7 is a transcription factor expressed by quiescent SC with myogenic activity, and inactivation of Pax7 results in severe depletion of these muscle stem cells. In adult mice, Pax7 expression appears specific to the satellite cell myogenic lineage (Seale et al., 2000). Myf-5, a member of basic helix-loop-helix transcription factors, is a myogenic regulatory factor and a marker of activated SC (Hernández-Hernández et al., 2017). In quiescent state, Myf-5 gene is transcribed in SC, but Myf-5 mRNA is sequestered in messenger ribonucleoprotein (**mRNP**) granules. These granules are dissociated upon satellite cell activation; thus, Myf-5 protein is the marker of satellite cell activation (Crist et al., 2012). The down regulation of both PAX7 and Myf5 expression at both the mRNA and protein levels, in our experimental conditions, indicate a loss of myogenic activity in SC in WB muscle and in hypoxic cells.

SC are multipotent cells, and depending on the stimuli and stress type, duration, and severity, they can follow myogenic, adipogenic, or osteogenic pathways (Asakura et al., 2001; Shefer et al., 2004; Wang et al., 2015a; Liu et al., 2017). As WB is characterized by infiltration of fat cells, we sought to evaluate here the potential activation of adipogenic program in SC under hypoxic conditions. Adipogenic/lipogenic program is tightly controlled by the key enzymes ACC, FASN, ME, and ACLY. ACLY is a cytosolic enzyme that catalyzes the cleavage of citrate into acetyl-CoA and oxaloacetate (Verschueren et al., 2019; Wei et al., 2019). ACC is a multi-subunit and biotin-dependent enzyme that catalyzes the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA, the rate limiting step in fatty acid synthesis (Abu-Elheiga et al., 1995; Abu-Elheiga et al., 2001). The main function of FASN is

to catalyze the synthesis of long chain fatty acids from malonyl-CoA via a series of decarboxylative Claisen condensation reactions (Smith, 1994; Jayakumar et al., 1995). ME (also known as decarboxylating malate dehydrogenase) catalyzes a reversible oxidative decarboxylation of malate to produce pyruvate (Chang and Tong, 2003; Jiang et al., 2013). The catalytic function of ACC is regulated by phosphorylation (inactive) and dephosphorylation (active) (Witters et al., 1988), and its reduced phosphorylated levels at Ser79 indicate that hypoxia activates ACC in both in vivo and in vitro studies. Concomitant with these changes, the upregulation of ACLY, FASN, and ME proteins in WB and hypoxic cells indicate that hypoxia alters the fate of SC and activates the adipogenic program. Furthermore, the upregulation of ME suggests that hypoxia may alter the fatty acid profile by reducing PUFA and increasing saturated FA as previously reported in WB (Cauble et al., 2020). A previous study reported that a hypoxia state caused by the supplementation of amino acids contributed to the formation of fatty acids, and eventually breast muscle myopathy (Livingston et al., 2019). Additionally, hypoxic conditions have been reported to limit the regenerative capacity of rodent muscle fibers by favoring the replacement of degenerated muscle fibers with lipid and fibrotic tissues (Hoppeler and Vogt, 2001).

Although the upstream mechanisms by which hypoxia activates adipogenic enzyme pathways are not known at this time point, it is possible that HIF-1α is a key mediator. The body's adaptive responses to several stressors including hypoxia is regulated by HIF-1α (Semenza, 2007), which coordinates gene expression relating to the reprogramming required for adaptation to stressors (Mylonis et al., 2019). During hypoxia, it has been shown that fatty acid synthesis is regulated by Akt- and HIF-1α-dependent activation of SREBP-1, which in turn up regulates the expression of FASN (Furuta et al., 2008). Our previous study showed an activation of Akt and HIF-1α in WB (Greene et al., 2020a), and the present data demonstrate, by immunofluorescence staining, an activation of HIF-1α and increased SREBP-1 in hypoxic SC. SREBP-1 is now well established as a key transcription factor for the transactivation of adipogenic genes such as FASN and ACCα (Hansmannel et al., 2006). Once it is activated through a proteolytic process in response to intracellular demands for lipids, the cleaved mature form of SREBP-1 translocate into the nucleus as a homodimer and stimulates the transcription of target genes by binding to the SREs in their promoters (Wang et al., 2015b). The absence of differential expression of SREBP-1 mRNA between WB-affected and unaffected birds and between hypoxic and normoxic SC indicates that SREBP-1 might be regulated at post-transcriptional and/or post-translational levels (Giandomenico et al., 2003; Sundqvist et al., 2005; Bengoechea-Alonso and Ericsson, 2009). Further studies using specific antibodies that cross react with chicken SREBP-1a/c and work in

immunoblot technique are warranted. The upregulation of SREBP-2 in hypoxic SC suggests that hypoxia alters the cholesterol metabolism and this needs further in-depth investigation.

In conclusion, results from the present in-vitro and in-vivo studies indicate that:

1) Hypoxia induces and exacerbates WB incidence in broilers and that the hypobaric chamber could be used as a highly relevant model to consistently induce and study the myopathy.

2) Hypoxia alters SC fate by activating the adipogenic rather than the myogenic program, which might explain, at least partially, the progression of WB myopathy and the origin of infiltrated fat cells. However, the potential contribution of bone marrow fat cells and dietary fat are not ruled out, and merit further investigations.

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DISCLOSURES

Author Mike Bedford is employed by the company AB Vista. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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