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Effect of stocker management program on beef cattle skeletal muscle growth characteristics, satellite cell activity, and paracrine signaling impact on preadipocyte differentiation

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Abstract

The objective of this study was to determine the effect of different stocker management programs on skeletal muscle development and growth characteristics, satellite cell (SC) activity in growing-finishing beef cattle as well as the effects of SC-conditioned media on preadipocyte gene expression and differentiation. Fall-weaned Angus steers (n = 76; 258 ± 28 kg) were randomly assigned to 1 of 4 stocker production systems: 1) grazing dormant native range (NR) supplemented with a 40% CP cottonseed meal-based supplement (1.02 kg · steer^{-1} · d^{-1}) followed by long-season summer grazing (CON, 0.46 kg/d); 2) grazing dormant NR supplemented with a ground corn and soybean meal-based supplement fed at 1% of BW followed by short-season summer grazing (CORN, 0.61 kg/d); 3) grazing winter wheat pasture (WP) at high stocking density (3.21 steers/ha) to achieve a moderate rate of gain (LGWP, 0.83 kg/d); and 4) grazing winter WP at low stocking density (0.99 steers/ha) to achieve a high rate of gain (HGWP, 1.29 kg/d). At the end of the stocker (intermediate harvest, IH) and finishing (final harvest, FH) phases, 4 steers / treatment were harvested and *longissimus* muscles (LM) sampled for cryohistological immunofluorescence analysis and SC culture assays. At IH, WP steers had greater LM fiber cross-sectional area than NR steers; however, at FH, the opposite was observed ($p < 0.0001$). At IH, CORN steers had the lowest Myf-5+:Pax7+ SC density ($p = 0.020$), while LGWP steers had the most Pax7+ SC ($p = 0.043$). At FH, CON steers had the highest LM capillary density ($p = 0.003$) and their cultured SC differentiated more readily than all other treatments ($p = 0.017$). At FH, Pax7 mRNA was more abundant in 14 d-old SC cultures from HGWP cattle ($p = 0.03$). Preadipocytes exposed to culture media from proliferating SC cultures from WP cattle isolated at FH had more PPARγ ($p = 0.037$) and less FABP4 ($p = 0.030$) mRNA expression compared with NR cattle. These data suggest that different stocker management strategies can impact skeletal muscle growth, SC function, and potentially impact marbling development in growing-finishing beef cattle.

Keywords: Beef stocker cattle, Dormant native range, Marbling development, Satellite cell activity, Skeletal muscle growth, Winter wheat pasture

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Background

Stocker production systems provide a useful tool to increase uniformity in BW and promote skeletal growth of small-framed beef cattle [1,2]. Millions of fall-weaned calves are wintered on dormant native range (NR) or winter wheat pasture (WP) in the Great Plains region of the United States every year. These two stocker production systems result in different rates of BW gain [3] and can ultimately influence feedlot performance and final quality grade [4]. Cattle fed lower concentrations of carbohydrates and allowed slower rates of BW gain exhibit greater proportions of type 1, oxidative skeletal muscle fibers at the expense of type 2, glycolytic fibers [5–9]. Both oxidative muscle fibers and intramuscular (IM) adipocytes are commonly found located near a well-developed vascular supply [10–13]. Within the perimysium, IM adipocytes develop in close proximity to muscle fibers and their resident muscle stem cells, called satellite cells (SC), which play a critical role in postnatal muscle growth [14,15]. This close spatial arrangement supports the possibility that marbling development could be directly influenced by skeletal muscle fibers, SC, or both through paracrine signaling mechanisms. We hypothesized that changes in the stocker management strategy including diet composition and rate of BW gain during the stocker phase would influence skeletal muscle metabolism, capillary development (angiogenesis), and SC mitotic activity as well as the skeletal muscle microenvironment to impact marbling. Our objective was to examine the effect of stocker management system and rate of BW gain during the stocker period on skeletal muscle growth characteristics, SC activity, and their subsequent paracrine signaling effects on preadipocyte differentiation and lipid filling *in vitro*.

Materials and Methods

Before the initiation of this research, all procedures relating to animal care, handling, and sampling were reviewed and approved by the Oklahoma State University Institutional Animal Care and Use Committee (AG-50-372 and AG-09-15).

Animals and management

A detailed description of the experimental procedures for the feeding and management portions of this study as well as stocker growth and feedlot performance and carcass characteristic data has been previously published in a companion paper (Exp. 2; [16]). Briefly, fall-weaned Angus steers (n = 76; 258 ± 28 kg; 265 ± 20 d of age) from the Oklahoma State University cow herd were randomly assigned to 1 of 4 stocker production systems: 1) grazing dormant NR supplemented with a 40% CP cottonseed meal-based supplement (1.02 kg · steer^{-1} · d^{-1}) followed by long-season (132 d) summer grazing (CON, 0.46 kg/d); 2) grazing dormant NR supplemented with a ground corn and soybean meal-based supplement fed at 1% of BW followed by short-season (50 d) summer grazing (CORN, 0.61 kg/d); 3) grazing winter WP at high stocking density (3.21 steers/ha) to achieve a moderate rate of gain (LGWP, 0.83 kg/d); and 4) grazing winter WP at low stocking density (0.99 steers/ha) to achieve a high rate of gain (HGWP, 1.29 kg/d). Steers continued to graze until the average BW of the treatment group reached approximately 375 kg. Steers were adapted to a common finishing diet and fed until they reached a backfat thickness of at least 1.18 cm. At the conclusion of the stocker (intermediate harvest, IH) and finishing phase (final harvest, FH), 4 steers / treatment were transported to the Oklahoma State University Food and Agriculture Products Center for slaughter (total n = 32). Steers were equally stratified across treatments by known sire when selecting steers for harvests to minimize genetic variation among stocker treatments.

Cryohistological analysis

Samples from the left *longissimus* muscle (LM) between the 9th and 13th ribs were collected from each steer and embedded in optimal cutting temperature compound (VWR International, West Chester, PA, USA) in metal molds on liquid nitrogen–cooled metal blocks and stored at –80℃ before cryosectioning. Serial, 10-μm-thick sections were cut using a Leica CM 1950 and mounted on positively charged glass slides (Superfrost Plus; VWR International). Slides were stored at –20℃ until immunofluorescence (IF) staining could be performed.

Immunofluorescence staining and antibodies

Cryosections were fixed and stained as previously described [17,18] with slight modifications. Cryosections were fixed in 4% paraformaldehyde (4% PFA; USB Corporation, Cleveland, OH, USA) in PBS (Invitrogen, Carlsbad, CA, USA) for 10 min at room temperature (RT), followed by a 10 min PBS rinse. Sections were incubated with 5% horse serum (HS; Invitrogen), 2% BSA (MP Biomedical, Solon, OH, USA), 0.2% Triton-X100 (Thermo Fisher Scientific, Fairlawn, NJ, USA) in PBS for 30 min at RT to block non-specific antibody binding. Antibodies and their respective dilutions are described below. After blocking solution was removed, cryosections were incubated in primary and secondary antibodies for 1 h and 30 min, respectively. Following both primary and secondary antibody exposure, sections were rinsed 3 times for 5 min each. Finally, cryosections were incubated in 1 μg/mL 4',6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific) for 1 min followed by 2 brief PBS rinses. Slides were cover-slipped with mounting media (Aqua Mount; Lerner Laboratories, Pittsburgh, PA, USA) and thin glass cover slips (VWR International) and left

to dry at 4℃ overnight. All cryosections were analyzed within 48 h of IF staining.

SC cultures were IF stained following the procedure for cryosections described above with slight modifications [17]. After fixation, cultures used to assess SC activity were rinsed twice with PBS, incubated in 0.5% Triton-X100 in PBS for 10 min, and then incubated for 7 min in 4 N hydrochloric acid (Thermo Fisher Scientific) before incubation in a blocking solution as described above. All SC cultures were stored in 1 mL of PBS and imaged within 48 h after IF staining.

Primary antibodies utilized for analysis of LM cryosections were mouse anti-chicken Pax7 hybridoma cell supernatant (Pax7; 1:10 dilution), mouse anti-type 1 Myosin hybridoma cell supernatant (BA-D5; 1:100 dilution), mouse anti-type 2 Myosin hybridoma cell supernatant (SC-71; 1:100 dilution; Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA, USA), rabbit polyclonal anti-C-terminus human Myf-5 (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and un-conjugated rabbit anti-Ulex Europaeus Agglutinin-1 (UEA; 1:50 dilution; EY Laboratories, San Mateo, CA, USA). Primary antibodies were detected using the following secondary antibodies at a 1:1,000 dilution: Alexa Fluor 488-conjugated goat anti-mouse IgG1, Alexa Fluor 488-conjugated goat anti-rabbit IgG heavy and light chain, Alexa Fluor 546-conjugated goat anti-mouse IgG1, and Alexa Fluor 633-conjugated goat-anti mouse IgG2b (Invitrogen).

SC cultures were stained with anti-Pax7 hybridoma cell supernatant (1:5 dilution) and rat IgG2a anti-BrdU (1:250 dilution; Santa Cruz Biotechnology). Differentiated SC cultures were IF stained with anti-Pax7 hybridoma cell supernatant (1:5 dilution; DSHB) and mouse anti-sarcomeric Myosin IgG2b hybridoma supernatant (MF-20; 1:5 dilution; DSHB). Primary antibodies were detected using Alexa Fluor 488-conjugated goat anti-mouse IgG1 and Alexa Fluor 546-conjugated goat anti-rat IgG2b (Invitrogen) secondary antibodies diluted 1:1,000.

Microscopy and digital imaging

Immunofluorescence-stained cryohistological slides were imaged at 200-fold magnification using an inverted fluorescence microscope (Nikon Eclipse, Ti-E; Nikon Instruments Inc., Melville, NY, USA) equipped with a UV light source (Nikon Intensilight Inc.; C-HGFIE). Images were captured with a CoolSnap ES2 monochrome camera and analyzed using NIS Elements Imaging software (Basic Research, 3.10; Nikon Instruments Inc.).

Five random images were taken of cryosections from the LM of each steer. The Myf-5+, Pax7+, and Myf-5+:Pax7+ SC populations in each image were identified, counted, and densities expressed on a square millimeter basis. The proportion of type 1 and type 2 muscle fibers was determined in every image for each steer. All UEA+ capillaries in each image were enumerated and densities expressed on a square millimeter basis. The cross-sectional area of each fiber in each image was measured using NIS Elements software (Nikon Instruments Inc.) and expressed on a square micron basis. The total number of DAPI-stained cells in each image was also enumerated to determine nuclear density on a square millimeter basis.

All digital photomicrographs of SC and preadipocyte cell cultures were taken and analyzed using the microscopy equipment described above equipped with extra-long working distance objectives. Ten representative images from each steer at each time point were taken at 200-fold magnification.

Satellite cell isolation and culture

SC were isolated from the LM of each steer at intermediate and FH using a previously described differential centrifugation method [19–21] with only minor modifications. A 200-g sample of the left LM from between the 9th and 13th ribs of each steer was excised, placed in a sterilized stainless-steel tray, and transported to the cell culture laboratory where all visible fat was removed under a laminar flow hood. Each LM sample was ground using a sterilized meat grinder and a total of 96 g of tissue was used for SC isolation. Ground muscle was incubated in Pronase (Calbiochem, San Diego, CA, USA) diluted to 1 μg/mL in Earle's Balanced Salt Solution supplemented 100 U penicillin/mL, 100 μg streptomycin/mL, and 20 μg of gentamicin/mL (AB + G; Invitrogen) for 40 min at 37℃ with gentle mixing by hand every 10 min. After differential centrifugation, pellets containing the mononucleated cells were resuspended in 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) warmed to 37℃. Cell suspensions from all 3 rounds of differential centrifugation from each steer were combined and filtered with 100 and 40 μm Steriflip filters (Millipore, Billerica, MA, USA) to remove any remaining tissue debris. The filtered cell suspension was centrifuged at 1,500 ×g and the resulting cell pellet was resuspended in cold (4℃) 10% dimethylsulfoxide (Sigma Aldrich, St. Louis, MO, USA) in 10% FBS in DMEM supplemented with AB + G, and then distributed evenly into Nunc cryotubes (Thermo Fisher Scientific) on ice. Cell suspensions enriched with SC were kept at –80℃ for 16 h and then stored in liquid nitrogen until analysis.

To determine the effect of stocker system on SC proliferative activity, SC isolated from steers harvested at the end of the stocker and finishing phases were plated at equal densities on 24-well tissue culture plates coated with growth factor reduced Matrigel (BD Biosciences, Bedford, MA, USA) diluted to 1 mg/mL in DMEM. Parallel cultures were grown for 24, 48, 72, and 96 h post-plating in 10% FBS in DMEM containing Ab + G. All SC cultures were maintained in a water-saturated environment at 37℃ with

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5% CO_2 and received fresh media every 48 h. To determine the proportion of mitotically active cells, SC cultures were incubated in media containing 1.5 mg/mL bromodeoxyuridine (BrdU; Invitrogen) for 2 h prior to fixation and IF staining for BrdU and Pax7 as described above. The total number of mitotically active SC (Pax7+;BrdU+) cells was determined and expressed as a proportion of total DAPI+ cells.

For analysis of differentiation (fusion) capacity, SC were plated at equal densities and grown on reduced growth factor Matrigel-coated 24-well plates in 10% FBS in DMEM supplemented with AB + G until the cultures reached confluence. Culture media was replaced every 48 h. On d 8, the FBS concentration in the culture media was reduced to 2% and cultures were maintained for another 6 d to allow maximum differentiation to occur. On d 14, SC cultures were fixed, IF stained, and imaged as described above. Differentiation capacity (fusion percentage) was determined by enumerating the total number of DAPI+ cells within sarcomeric Myosin+ multinucleated myotubes as a proportion of all DAPI+ cells/image.

Satellite cell culture RNA isolation and gene expression analysis

Total RNA was isolated from differentiated SC cultures 14 d post-plating. Total RNA was isolated using the Absolutely RNA Nanoprep Kit (Agilent Technologies, Santa Clara, CA, USA) per manufacturer's instructions including the on-column DNase treatment step to remove contaminating genomic DNA. Immediately after isolation total RNA was quantified using a spectrophotometer (Nanodrop-1000; Thermo Fisher Scientific) and stored at −80℃ until reverse transcription into cDNA and amplification was performed. Reverse transcription and amplification of 10 ng of total RNA was performed using Quantitect Whole Transcriptome Kit (Qiagen; Valencia, CA, USA) following manufacturer's instructions for a high yield reaction. Immediately after reverse transcription and amplification, total cDNA was quantified using a spectrophotometer (Nanodrop-1000; Thermo Fisher Scientific) and samples were stored at −20℃ until gene expression analysis was performed.

Species and gene specific primers were designed using exonic sequences from Ensembl Genome Browser or National Center for Biotechnology Information (NCBI) using Primer3 software package [22]. Each primer was designed to be intron spanning to prevent amplification of contaminating genomic DNA. Specificity of each primer set was evaluated by comparing primer sequences to the database of GenBank using the blast tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Complementarity of forward and reverse primer sequences for each primer pair was evaluated using OligoAnalyzer 3.1 (Integrated DNA Technologies, Coralville, IA, USA). Primers used for RT-PCR are listed in Table 1 including their accession number or the references from which they were ob-

Table 1. Primer sequences used for quantification of relative gene expression of satellite cell and 3T3-L1 preadipocyte cell cultures

Gene	Accession or citation		Primer sequence (5' to 3')	Product size (bp)	Annealing temp. (°C)
Satellite cell cultures					
Bovine 18S rRNA[1)]	DQ222453	FWD	GACACGGACAGGATTGACAG	243	60.0
		REV	CGGACATCTAAGGGCATCAC		
Pax7	ENSBTAT00000019924	FWD	GGGCTCAGATGTGGAGTCAG	85	57.0
		REV	CATCTACACCCGAGAGGAGC		
Myf-5	ENSBTAT00000038612	FWD	CAGCGTCTACTGTCCTGATGT	182	60.5
		REV	TTCTCAACCTGCAACTCCAG		
Myogenin	ENSBTAT00000007923	FWD	CTCCCATAGCGCCTCCTG	162	60.0
		REV	CAGACGCCCACAATCTGC		
Myostatin	NM_001001525	FWD	GTTTGGCTTGGCGTTACTCA	178	60.0
		REV	TTCCTTCTGCTCGCTGTTCT		
IGF-1	NM_001077828	FWD	ATCACATCCTCCTCGCATCT	131	60.5
		REV	CTGTCTCCGCACACGAACT		
3T3-L1 preadipocyte cultures					
Bovine 18S rRNA	Granfar et al., 2005	FWD	CTTAGAGGGACAAGTGGCG	107	62.0
		REV	ACGCTGAGCCAGTCAGTGTA		
PPARγ	Taylor-Jones et al., 2002	FWD	GCTGTTATGGGTGAAACTCTG	351	60.0
		REV	ATAAGGTGGAGATGCAGGTTC		
FABP4	Guan et al., 2002	FWD	GGGATTTGGTCACCATCCG	204	60.0
		REV	CCAGCTTGTCACCATCTCG		

tained [23–25].

To quantify relative mRNA abundance, PCR reactions were performed using a Bio-Rad IQ5 Optical Detection system and software (BioRad Laboratories, Inc., Hercules, CA, USA). Reactions were mixed in 96-well plates with each well containing 7 μL of RT2 SYBR Green Fluor FAST Mastermix (Qiagen), 0.23 μL of a 25 μm forward and reverse primer mix (Integrated DNA Technologies), 2.77 μL PCR Certified Water (Teknova; Hollister, CA, USA), and 10 ng of cDNA. Gene expression assays were performed using the following cycling conditions: a 10 min hold at 95℃ and 40 cycles of 95℃ for 10 s, optimum annealing temperature for each primer set (Table 1) for 30 s, with a 72℃ extension for 30 s. Following amplification, melt curve analysis was performed to verify the specificity of the reaction. For each gene, all reactions displayed a single melt peak temperature, indicating that a unique product was amplified. Bovine 18S ribosomal RNA (B18S) was used as the reference gene for normalization. The relative quantification of mRNA expression was computed using the procedure as described previously [26]. Briefly, the threshold cycle (Ct) values of each gene were converted to quantitative expression values relative to the sample with the lowest Ct value using the PCR reaction efficiency factor of each gene. The quantitative expression value was then divided by the quantitative expression value of B18S the reference gene to calculate normalized mRNA expression. This procedure allowed statistical analysis of linear mRNA expression values relative to B18S after adjusting for differences in reaction efficiency.

Satellite cell-conditioned culture media and preadipocyte differentiation

Embryonic mouse preadipocytes (3T3-L1; ATCC, Manassas, VA, USA) were plated at a density of 4.5×10^4 cells/cm^2 and cultured for 6 d in 10% calf serum (CS; HyClone)/DMEM with AB + G. Culture media was changed every 48 h. After 6 d in culture when 3T3-L1 preadipocytes were confluent, media was replaced with SC-conditioned media from either 96 h (proliferative) or 14 d (differentiated) SC cultures collected at 96 h and 14 d post-plating, respectively. After 48 h of exposure to SC-conditioned media, the 3T3-L1 preadipocyte cultures were fixed and stained with the neutral lipid marker, Oil red O (ORO; Sigma Aldrich) as previously described [27,28]. Cultures were incubated in the ORO staining solution for 2 min at RT, rinsed twice briefly in PBS, and incubated in DAPI for 1 min followed by 2 brief PBS rinses. The proportion of total DAPI+ 3T3-L1 preadipocytes exhibiting lipid accumulation and ORO positivity was determined.

Total RNA was isolated from 3T3-L1 preadipocytes cultures after 48 h of exposure to SC-conditioned media using the GE Healthcare RNA MiniSpin kit (Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions. Total RNA was quantified, reverse transcribed, and amplified exactly as described above for SC. Gene expression analysis was conducted as described above for SC except that 5 ng of cDNA was used. Primers used for gene expression analysis of 3T3-L1 preadipocytes were obtained from the literature [23–25] and are listed in Table 1.

Statistical analysis

All data were analyzed using the GLIMMIX procedure of SAS (V9.2, SAS Institute, Inc., Cary, NC, USA). For all analyses, treatment was the fixed effect and the Satterthwaite adjustment was used to correct degrees of freedom. Steer (n = 4 / treatment) served as the experimental unit for all analyses as all grazing steers consumed their individual maximum amount of forage each day during the stocker phase [16,29–31]. Proportional data such as % muscle fiber type, % proliferative SC, % SC fusion, and % ORO+ 3T3-L1 cells were subjected to analysis of variance using the events/experiments syntax for binomial distributions with an R-side covariance structure. All continuous variables were subjected to analysis of variance using the least square means syntax for Gaussian distributions with an R-side covariance structure. Least square means were separated using the PDIFF option of SAS and considered significantly different when $p \leq 0.05$. Tendencies for differences among treatment least square means were declared when $0.0501 \leq p \leq 0.1000$.

Results and Discussion

Determining how stocker production program including differing diet composition and rate of BW gain during the stocker phase impacts skeletal muscle development and growth characteristics, SC activity, and the ability of factors released by beef cattle SC to influence preadipocyte differentiation *in vitro* were the objectives of this study.

Longissimus muscle developmental and growth characteristics

Intermediate Harvest. At the conclusion of the stocker phase, cattle that grazed winter WP and achieved the highest rates of BW gain (HGWP = 1.29 kg/d and LGWP = 0.83 kg/d) had greater LM skeletal muscle fiber cross-sectional area compared with cattle that grazed dormant NR and achieved lower rates of gain during the stocker phase (CON = 0.46 kg/d and CORN = 0.61 kg/d; $p < 0.0001$; Table 2). Steers on the CON treatment that grazed dormant NR and had the lowest rate of BW gain during the stocker period tended to have more type 1, oxidative muscle fibers ($p = 0.0572$), fewer type 2, glycolytic muscle fibers ($p = 0.0572$), and greater capillary density ($p = 0.0781$) in their LM at the conclusion

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Table 2. Skeletal muscle fiber cross-sectional area, fiber type, and capillary and nuclear density in the *longissimus* muscle of beef cattle from different stocker management programs at the conclusion of the stocker and finishing phases

Variable	Stocker management program				SEM	*p*-value
	CON	CORN	LGWP	HGWP		
Intermediate harvest						
Fiber area (μm^2)	1,902.06^{c}	1,850.04^{c}	2,458.69^{a}	2,159.87^{b}	38.09	< 0.0001
Type 1 muscle fibers (%)	31.62^{x}	28.01^{xy}	26.79^{y}	28.00^{xy}	1.35	0.0572
Type 2 muscle fibers (%)	68.38^{x}	71.99^{xy}	73.21^{y}	72.00^{xy}	1.35	0.0572
Capillaries/mm^2	66.60^{x}	49.40^{y}	48.15^{y}	58.55^{y}	5.62	0.0781
Nuclei/mm^2	674.00	629.65	660.50	658.40	19.19	0.4234
Final harvest						
Fiber area (μm^2)	3,015.05^{b}	3,209.15^{a}	2,927.39^{b}	2,471.57^{c}	56.04	< 0.0001
Type 1 muscle fibers (%)	27.82^{xy}	26.77^{y}	31.13^{x}	29.40^{xy}	1.23	0.0720
Type 2 muscle fibers (%)	72.18^{xy}	73.23^{y}	68.87^{x}	70.60^{xy}	1.23	0.0720
Capillaries/mm^2	67.30^{a}	45.95^{b}	41.30^{b}	50.05^{b}	5.10	0.0034
Nuclei/mm^2	598.15^{a}	585.25^{ab}	542.95^{b}	572.90^{ab}	18.41	0.1862

^{a,b}Least square means within a row lacking a common superscript differ ($p \leq 0.05$).

^{x,y}Least square means within a row lacking a common superscript tend to differ ($0.0501 \leq p \leq 0.10$).

SEM, standard error of the LS mean.

CON, 40% crude protein cottonseed meal-based supplement on dormant native range; CORN, corn and soybean meal-based supplement on dormant native range; LGWP, reduced rate of gain on wheat pasture; HGWP: high rate of gain on wheat pasture.

of the stocker phase compared with cattle from all other treatments (Table 2). Nuclear densities in the LM of cattle from the 4 stocker programs were similar at IH ($p = 0.4234$; Table 2). Densities of SC in the LM expressing only Myf-5 ($p = 0.87$) did not differ among treatment (Table 3). However, at the conclusion of the stocker phase, steers that grazed NR assigned to the CORN treatment had the lowest densities of Pax7+ ($p = 0.0429$) and Myf-5+:Pax7+ ($p = 0.0203$) SC in their LM compared with steers from other treatments (Table 3).

Final Harvest. At the conclusion of the finishing phase, LM fiber cross-sectional area was greatest in CORN steers that grazed NR during the stocker period compared with those that grazed winter WP at a high rate of gain (HGWP), with the CON and LGWP steers being similar and intermediate ($p < 0.0001$; Table 2). Steers with the highest rate of gain during the stocker period (HGWP; 1.29 kg BW gain/d) had the smallest LM fiber cross-sectional area at FH ($p < 0.0001$; Table 2). By FH, the LM of CON steers had similar proportions of type 1 (oxidative) and type 2 (glycolytic) muscle fibers compared with all other treatments ($p = 0.0720$). Yet, at the end of the finishing phase, capillary density

Table 3. Skeletal muscle satellite cell densities in the *longissimus* muscle of beef cattle from different stocker management programs at the conclusion of the stocker and finishing phases

Variable	Stocker management program				SEM	*p*-value
	CON	CORN	LGWP	HGWP		
Intermediate harvest						
Myf-5+ cells/mm^2	80.95	88.90	85.50	81.85	6.32	0.8020
Pax7+ cells/mm^2	21.95^{ab}	17.40^{b}	25.35^{a}	20.15^{ab}	1.97	0.0429
Myf-5+:Pax7+ cells/mm^2	6.95^{a}	1.75^{b}	5.90^{a}	5.60^{a}	1.22	0.0203
Final harvest						
Myf-5+ cells/mm^2	90.20	87.50	86.25	88.60	7.33	0.9841
Pax7+ cells/mm^2	17.65	21.35	21.70	20.35	3.17	0.7997
Myf-5+:Pax7+ cells/mm^2	5.15	4.50	5.20	4.90	1.34	0.9820

^{a,b}Least square means within a row lacking a common superscript differ ($p \leq 0.05$).

SEM, standard error of the LS mean.

CON, 40% crude protein cottonseed meal-based supplement on dormant native range; CORN, corn and soybean meal-based supplement on dormant native range; LGWP, reduced rate of gain on wheat pasture; HGWP: high rate of gain on wheat pasture.

remained greater in the slow-growing, CON steers compared with those from all other treatments (p = 0.0034; Table 2). After finishing, the LGWP steers had 4% more type 1 fibers in their LM (p = 0.0720) compared with CORN steers (Table 2). The densities of total nuclei (p = 0.1862; Table 2) and all SC populations evaluated (Pax7+, Myf-5+, and Pax7:Myf-5+) in the LM were similar ($p \geq$ 0.7997; Table 3) among steers from different stocker production systems fed to a similar target backfat thickness.

The increase in oxidative muscle fiber types is consistent with what others have reported in cattle allowed reduced BW gain during the growing period [6,12,13]. At the conclusion of the finishing phase when cattle were fed to similar backfat thickness, the HGWP steers had the smallest LM areas and the highest USDA yield grades [16]. The reason for this is not apparent. Steers wintered on dormant NR supplemented with a followed by long-season summer grazing prior to finishing and allowed the lowest rates of BW gain during the stocker phase (CON) tended to develop the most oxidative muscle fibers with the greatest capillary density by IH and maintained the increased capillary density through the FH [16]. The CON stocker management system also resulted in steers with the highest ADG and gain:feed ratio during the finishing phase and suggests there may be a compensatory gain effect in these slow-growing steers that entered the feedlot at heavier BW [29]. Collectively, these data support previous work showing that the rate of BW gain during the stocker phase can influence feedlot performance and final carcass characteristics [32,33]. Combined with previous reports, the results from this study indicate that slower rates of BW gain in beef cattle during the stocker phase may promote the development of a skeletal muscle environment rich in vascular supply that may enhance IM marbling development compared with stocker systems where high rates of BW gain are achieved.

Skeletal muscle satellite cell activity *in vitro*

Intermediate Harvest. The stocker treatment did not affect the proportion of Pax7+:BrdU+ SC observed at 24, 48, 72, or 96 h post-plating in SC cultures from steers isolated at the conclusion of the stocker phase ($p \geq$ 0.1219; Table 4). Stocker management program also did not significantly alter the proportion of SC that fused into multi-nucleated myotubes in SC cultures isolated at IH (p = 0.1570; Table 5). The relative abundance of Pax7, Myf-5, Myogenin, Myostatin, and IGF-1 mRNA in 14 d-old differentiated SC cultures was also similar among treatment ($p \geq$ 0.3370; Table 5).

There was a marked increase in the density of SC co-expressing Pax7 and Myf-5 in the LM of CORN steers. Additionally, in culture SC from CORN steers at IH were the most proliferative SC at 96 h post-plating, and had the lowest fusion percentage at 14 d post-plating. Interestingly, their treatment counterparts ultimately went on to have the largest LM area at FH [16]. These data suggest that the CORN stocker management strategy may have resulted in greater SC proliferative capacity during the stocker phase and ultimately lead to enhanced SC-mediated hypertrophic growth of the LM after the cattle entered the feedyard for finishing. The reason for this apparent enhancement of what appears to be SC-mediated growth of LM fibers is not clear. It is possible that

Table 4. Mitotic activity over time in cultured skeletal muscle satellite cells of beef cattle from different stocker management programs at the conclusion of the stocker and finishing phases

Variable	Stocker management program				SEM	*p*-value
	CON	CORN	LGWP	HGWP		
Intermediate harvest						
Pax7+:BrdU+ cells (%)						
24 h	18.75	13.04	11.76	15.69	7.40	0.8772
48 h	17.86	22.58	19.23	24.44	7.84	0.9111
72 h	52.54	47.73	67.24	61.54	9.49	0.3897
96 h	61.04	80.95	77.78	63.33	7.65	0.1219
Final harvest						
Pax7+:BrdU+ cells (%)						
24 h	4.76	22.58	11.11	11.54	8.66	0.4276
48 h	22.73	30.77	40.00	23.53	9.36	0.4503
72 h	53.57	52.50	46.15	55.00	11.56	0.9041
96 h	68.06	71.55	82.61	65.75	7.13	0.4683

SEM, standard error of the LS mean.

CON, 40% crude protein cottonseed meal-based supplement on dormant native range; CORN, corn and soybean meal-based supplement on dormant native range; LGWP, reduced rate of gain on wheat pasture; HGWP, high rate of gain on wheat pasture.

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Table 5. Differentiation (fusion) capacity of cultured skeletal muscle satellite cells of beef cattle from different stocker management programs at the conclusion of the stocker and finishing phases

Variable	Stocker management program				SEM	*p*-value
	CON	CORN	LGWP	HGWP		
Intermediate harvest						
Fused, myotube nuclei (%)[1)]	20.49	12.90	14.23	17.40	3.03	0.1570
Final harvest						
Fused, myotube nuclei (%)[1)]	26.95[a]	18.71[b]	15.38[b]	17.67[b]	3.10	0.0173

[1)]Proportion of DAPI+ nuclei present inside sarcomeric Myosin+, multinucleated myotubes in 14 d-old satellite cell cultures.

[a,b]Least square means within a row lacking a common superscript differ ($p \leq 0.05$).

SEM, standard error of the LS mean.

CON, 40% crude protein cottonseed meal-based supplement on dormant native range; CORN, corn and soybean meal-based supplement on dormant native range; LGWP, reduced rate of gain on wheat pasture; HGWP, high rate of gain on wheat pasture.

the combination of a higher starch content and lower overall gains of the CORN steers in the stocker phase stimulated IGF-1 production or increased blood glucose concentrations, which may help explain the differences in SC population heterogeneity (Table 3) in steers grazing NR compared with those entering the feedyard after high rates of gain during the stocker period (HGWP). Unfortunately, circulating blood glucose and IGF-1 concentrations were not measured on the cattle in this study; however, the data does provide a link between feeding regimen, SC transcriptional profile during the stocker period, and LM area at FH, where the HGWP steers had the smallest LM areas and highest USDA yield grade of the 4 stocker programs [16].

Final Harvest. SC mitotic activity as indicated by the proportion of Pax7+:BrdU+ SC over time in culture was not different among treatment in cultures of SC collected at the conclusion of the finishing phase ($p \geq 0.4276$; Table 4). In contrast to IH, the fusion capacity of steers from the CON stocker treatment collected at FH was at least 8% greater than that of SC from steers subjected to the other 3 stocker management systems ($p = 0.0173$; Table 5). The relative expression of Myf-5, Myogenin, Myostatin, and IGF-1 mRNA in differentiated SC cultures was not different among treatment ($p \geq 0.2299$; Table 6). However, SC cultures collected at the end of the finishing phase from HGWP steers expressed more Pax7 mRNA after 14 d in culture compared with those from all other treatments ($p = 0.03$; Table 6). The increased Pax7 mRNA abundance in differentiated SC cultures from steers on the

Table 6. Relative fold change in the expression of genes involved in myogenic regulatory pathways in differentiated (14 d in culture) skeletal muscle satellite cells of beef cattle from different stocker management programs at the conclusion of the stocker and finishing phases

Variable	Stocker management program				SEM	*p*-value
	CON	CORN	LGWP	HGWP		
Intermediate harvest						
Pax7	1.10	0.71	0.53	1.12	0.40	0.5801
Myf-5	1.23	1.92	1.22	2.19	0.49	0.3370
Myogenin	1.83	1.74	1.86	1.41	0.43	0.8317
Myostatin	0.03	0.45	0.18	0.09	0.25	0.5568
IGF-1	0.07	0.27	0.02	0.13	0.11	0.3491
Final harvest						
Pax7	0.22[b]	0.34[b]	0.43[b]	2.29[a]	0.48	0.0312
Myf-5	1.14	1.04	1.74	1.98	0.35	0.2299
Myogenin	1.82	2.44	1.46	2.31	0.50	0.5119
Myostatin	0.56	0.79	0.08	0.26	0.39	0.5952
IGF-1	0.17	0.25	0.20	1.71	0.68	0.2643

[a,b]Least square means within a row lacking a common superscript differ ($p \leq 0.05$).

SEM, standard error of the LS mean.

CON, 40% crude protein cottonseed meal-based supplement on dormant native range; CORN, corn and soybean meal-based supplement on dormant native range; LGWP, reduced rate of gain on wheat pasture; HGWP, high rate of gain on wheat pasture.

HGWP stocker program may indicate that there were more active, unfused SC remaining in the culture after 14 d. This phenomenon could indicate that the decreased LM area of the HGWP steers was a result of less SC mediated hypertrophy during the finishing period [16]. Combined, these data indicate that the stocker management program growing beef cattle are subjected to could alter the functionality and gene expression profiles of resident muscle SC populations and ultimately influence beef carcass characteristics, though the exact mechanisms of how this occurs are not yet clear.

Satellite cell-conditioned media and 3T3-L1 preadipocyte differentiation

Culture media from SC isolated from steers at the conclusion of the stocker and finishing phases was collected from proliferative SC at 96 h post-plating and differentiated SC cultures containing myotubes at 14 d post-plating. The SC-conditioned media was then used to determine whether SC-produced molecules secreted into the media could influence the adipogenic gene expression and differentiation of mouse 3T3-L1 preadipocytes into lipid-filled, Oil Red O+ adipocytes.

Intermediate Harvest. The relative expression of peroxisome proliferator-activated receptor-gamma (PPAR γ) and fatty acid binding protein 4 (FABP4) mRNA in 3T3-L1 mouse preadipocytes exposed to conditioned media from mitotically active or proliferative (96 h) SC cultures from steers at the conclusion of the stocker phase was not different among treatment ($p \geq 0.4924$; Table 7). However, preadipocytes exposed to media collected from proliferative SC from CORN steers exhibited the most lipid accumulation (ORO+) and those exposed to media conditioned by SC harvested from the HGWP steers had the least ($p = 0.0082$; Table 7). The relative expression of PPAR γ and FABP4 mRNA in preadipocytes exposed to conditioned media from differentiated (14 d) SC cultures from steers at the conclusion of the stocker phase were not different among treatment ($p \geq 0.1471$; Table 7). However, the preadipocytes exposed to conditioned media from SC cultures from the HGWP steers had the least lipid accumulation as demonstrated by the lowest proportion of ORO+ cells and those exposed to the differentiated cultures from CORN and LGWP steers accumulated the most lipid ($p = 0.0470$; Table 7).

Final Harvest. Preadipocytes exposed to conditioned media from proliferative (96 h) SC isolated from HGWP steers had the

Table 7. Relative fold change in the expression of genes involved in adipogenic differentiation and lipid accumulation of 3T3-L1 preadipocytes exposed to conditioned media from mitotically active (96 h in culture) and differentiated (14 d in culture) satellite cells of beef cattle from different stocker management programs at the conclusion of the stocker and finishing phases

Variable	Stocker management program				SEM	*p*-value
	CON	CORN	LGWP	HGWP		
Conditioned media from mitotically active satellite cells						
Intermediate harvest						
PPARγ	1.07	1.14	1.08	1.10	0.04	0.5405
FABP4	0.93	0.88	0.93	0.91	0.03	0.4924
Oil Red O+ cells (%)	52.43^{ab}	56.04^{a}	51.81^{ab}	47.7^{b}	1.73	0.0082
Final harvest						
PPARγ	1.06^{b}	1.06^{b}	1.09^{ab}	1.16^{a}	0.03	0.0370
FABP4	0.94^{a}	0.95^{a}	0.92^{ab}	0.86^{b}	0.02	0.0295
Oil Red O+ cells (%)	57.12	56.35	53.56	54.02	1.50	0.2209
Conditioned media from differentiated satellite cells						
Intermediate harvest						
PPARγ	0.05	0.58	0.27	0.12	0.27	0.4666
FABP4	0.25	1.13	0.60	0.28	0.29	0.1471
Oil Red O+ cells (%)	72.09^{ab}	74.01^{a}	75.09^{a}	69.81^{b}	1.50	0.0470
Final harvest						
PPARγ	0.19	0.65	0.04	0.45	0.33	0.4610
FABP4	0.82	1.24	0.44	0.65	0.24	0.1499
Oil Red O+ cells (%)	69.18^{ab}	71.68^{a}	65.99^{bc}	63.55^{c}	1.66	0.0027

$^{a-c}$Least square means within a row lacking a common superscript differ ($p \leq 0.05$).

SEM, standard error of the LS mean.

CON, 40% crude protein cottonseed meal-based supplement on dormant native range; CORN, corn and soybean meal-based supplement on dormant native range; LGWP, reduced rate of gain on wheat pasture; HGWP, high rate of gain on wheat pasture.

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highest relative expression of PPAR γ mRNA (p = 0.0370) and lowest expression of FABP4 mRNA (p = 0.0295; Table 7). Interestingly, the media from the active SC cultures from steers on the CORN stocker treatment had the opposite effect on the PPAR γ and FABP4 expression of 3T3-L1 preadipocytes, although the proportion of ORO+ cells was similar among all treatments (p = 0.2209; Table 7).

Conditioned media from 14 d-old differentiated SC cultures collected from steers at FH did not alter preadipocyte expression of either PPAR γ or FABP4 ($p \geq 0.1471$). However, it did impact the proportion of lipid-filled (ORO+) cells. Preadipocytes exposed to the differentiated SC-conditioned media from HGWP steers exhibiting the fewest ORO+ or lipid-filled cells and the media collected from CORN steer derived SC cultures inducing the most adipogenic differentiation as evidenced by the greatest proportion of ORO+ lipid-filled cells (p = 0.0027; Table 7).

PPAR γ is considered an early marker of adipogenesis while FABP4 is a commonly used marker of later adipogenic differentiation [34]. Therefore, the greater abundance of PPAR γ mRNA and decreased FABP4 mRNA expression observed in 3T3-L1 preadipocytes exposed to 96 h-SC-conditioned media from HGWP steers indicates that these preadipocytes were not as far along in the adipogenic differentiation program compared with preadipocytes exposed to SC-conditioned media from CON and CORN steers with LGWP steers being intermediate.

Cattle grazing WP have a 70% lower acetate:propionate ratio compared with those grazing NR, which increases glucose availability compared to acetate [33]. It is known that IM adipocytes preferentially utilize glucose as the primary substrate for fatty acid synthesis [35]. Therefore, it is possible that the changes in acetate:propionate ratio during the stocker phase could alter IM adipocyte differentiation and have effects that last throughout the finishing phase. In addition, our previous work shows that the glucose-6-phosphate dehydrogenase (G6PDH) in the IM adipose tissue of HGWP cattle at the end of the finishing phase was significantly higher than that of LGWP cattle [31]. In addition, previous gene expression analysis work also suggests that there is a relationship between skeletal muscle growth rate and metabolism and IM tissue cellular differentiation and lipid synthesis [30]. Combined, these data support the notion that factors produced by skeletal muscle cells can influence adipogenic differentiation of mouse preadipocytes *in vitro* and could possibly influence beef cattle marbling development *in vivo*. Though determining exactly which signaling molecules the proliferative SC produced as well as the mechanism by which those products impacted the behavior of the preadipocytes will require further investigation. These data support the idea that skeletal muscle-derived factors can influence marbling adipocyte development and final carcass characteristics.

Overall, our results demonstrate that stocker management strategy and rate of BW gain during the stocker phase impact muscle fiber type and cross-sectional area, capillary density, as well as SC activity that may ultimately influence marbling development in growing beef cattle. In recent years, it has become clear that fetal nutrient availability impacts the metabolic profile of skeletal muscle, and results in life-long alternations in metabolism and growth [36,37]. Though this data indicates that stocker production regimen also programs later metabolism of beef cattle, some major questions remain regarding the cell signaling mechanisms responsible for the coordinated development of skeletal muscle and IM adipose tissue as well as how nutrition and management strategies imposed during the stocker phase can alter this relationship in growing beef cattle.

Competing interests

No potential conflict of interest relevant to this article was reported.

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Availability of data and material

Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions

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Ethics approval and consent to participate

Before the initiation of this research, all procedures relating to animal care, handling, and sampling were reviewed and approved by the Oklahoma State University Institutional Animal Care and Use Committee (AG-50-372 and AG-09-15).

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