Animal (2018), **12:9**, pp 1912–1920
doi:10.1017/S1751731117003329

animal

In vitro supplementation with the porcine plasma product, betaGRO®, stimulates activity of porcine fetal myoblasts and neonatal satellite cells in a divergent manner

M. A. Vaughn, K. J. Phelps and J. M. Gonzalez†

Department of Animal Sciences and Industry, Kansas State University, 1424 Claflin Rd., 226 Weber Hall, Manhattan, KS 66506, USA

(Received 22 June 2017; Accepted 2 November 2017; First published online 6 December 2017)

Two separate experiments were conducted to evaluate the effect of betaGRO® supplementation on in vitro *porcine fetal myoblasts (PFM) and porcine satellite cells (PSC) proliferation, fusion and myotube thickness. The PFM and PSC were isolated from the* m. longissimus dorsi *of day 60 of gestation fetuses and piglets within 24 h of birth, respectively. Proliferation assays were conducted as 4 × 3 factorial arrangements with time of culture (24, 48, 72, 96 h) and media treatment (standard porcine media supplemented with 10% (vol/vol) fetal bovine serum (HS); HS without 10% fetal bovine serum (LS); and LS supplemented with 10 mg/ml betaGRO® (BG)) as main effects. Fusion and myotube growth assays were conducted as 2 × 2 factorial designs with serum concentration (HS or LS), and betaGRO® inclusion (0 or 10 mg/ml) as main effects. There was a treatment × time interaction and betaGRO® × serum interactions for proliferation, fusion and myotube thickness of PFM (*$P < 0.01$*). At all-time points, HS and BG-PFM had greater proliferation rates compared LS (*$P < 0.01$*). The HS treatment had greater proliferation rates than BG (*$P < 0.02$*) except at 72 h of culture (*$P = 0.44$*). When betaGRO® was added to LS media, fusion percentage and myotube thickness decreased (*$P < 0.01$*), while fusion percentage increased (*$P < 0.01$*) and myotube thickness was unaffected (*$P = 0.63$*) when betaGRO® was added to HS media. There were treatment × time and betaGRO® × serum interactions for proliferation rate and fusion rate of PSC, respectively (*$P < 0.01$*). At all-time points, HS had greater proliferation rates than LS and BG (*$P < 0.01$*), and LS had greater proliferation rates than BG (*$P < 0.02$*). When betaGRO® was added to LS and HS media, fusion percentage increased for both media types (*$P < 0.01$*). There was no betaGRO® × serum interaction (*$P = 0.63$*) for PSC myotube thickness; however, betaGRO® supplemented myotubes were thicker (*$P < 0.01$*) than non-betaGRO® supplemented myotubes. These two experiments indicate* in vitro *betaGRO® supplementation stimulates divergent responses based on the age of cell examined.*

Keywords: betaGRO®, differentiation, porcine fetal myoblasts, porcine satellite cells, proliferation

Implications

These findings suggest betaGRO® supplementation may enhance both myogenesis *in utero* and postnatal muscle hypertrophy. *In utero,* betaGRO® may help increase the muscle progenitor cell pool, thus possibly enhancing the establishment of muscle fiber number or the satellite cell pool. Postnatally, betaGRO® may stimulate increased protein synthesis by enhancing satellite cell fusion and signaling of the phosphatidylinositol 3-kinase pathway.

Introduction

Efficient growth and development of skeletal muscle of swine plays a key role in the overall efficiency of pork production. Muscle is a dynamic tissue accounting for ~40% of the body mass and 65% of fetal glucose usage (Yates *et al.*, 2012). Prenatal myogenesis encompasses the proliferation, commitment and differentiation of embryonic and fetal myoblasts into muscle fibers (Biressi *et al.*, 2007; Bentzinger *et al.*, 2012). In mature animals, proliferation and fusion of satellite cells into existing muscle fibers provide additional myonuclei, which provide the DNA template for protein synthesis (Moss and Leblond, 1971; Dodson *et al.*, 1987). The transition from paired box transcription factor-7 (Pax7) to myogenic factor-5 (Myf-5) characterizes the developmental maturity of proliferative myogenic cells (Zammit *et al.*, 2006). In addition, there are a subclass of daughter cells that co-express Pax7 and Myf-5, which exhibit advanced proliferative characteristics (Kuang *et al.*, 2007; Li *et al.*, 2011). This transcriptional transition is influenced by a variety of factors, including endocrine signaling factors.

† E-mail: johngonz@ksu.edu

Insulin-like growth factors are structurally similar to insulin and stimulate cellular activity through binding of the insulin-like growth factor-1 receptor. The role of IGF-1 has been thoroughly investigated and plays a central role in proliferation and differentiation of satellite cells *in vitro* and *in vivo* (Engert *et al*., 1996; Stitt *et al*., 2004). Supplementation of IGF-1 to porcine satellite cells (PSC) increased the abundance of proteins associated with protein synthesis and decreased proteins of degradation involved in the phosphatidylinositol 3-kinase (PI3-K) pathway (Rommel *et al*., 2001; Stitt *et al*., 2004; Han *et al*., 2008). Therefore, these findings implicated that IGF-1 mediates muscle protein synthesis through PI3-K pathway signaling.

Supplementation of porcine plasma during the nursery phase improves ADG and G : F (Coffey and Cromwell, 1995; de Rodas *et al*., 1995; Everts *et al*., 2001). de Rodas *et al*. (1995) found porcine plasma possesses elevated levels of IGF-1 and Tran *et al*. (2014) reported procine plasma stimulated proliferation of jejunal epithelial cells *in vitro*, suggesting porcine plasma can directly affect cellular activity during tissue development. The IGF-1 in traditional porcine plasma is largely complexed to IGF-binding proteins, which modulates the availability of IGF-1 to the target tissue (Baxter, 2000). The next generation porcine plasma product, betaGRO®, undergoes proprietary processing that enriches and releases IGF-1 from the IGFBPs; thus, improving its bioavailability which will likely elicit direct effects on target tissues (Magpantay *et al*., 2016). Therefore, the objective of the current studies were to determine the effects of betaGRO® supplementation *in vitro* on porcine fetal myoblast (PFM) and PSC proliferation, differentiation and protein synthesis.

Material and methods

The experimental procedures were approved by the Kansas State University Institutional Animal Care and Use Committee.

Progenitor cell isolation

At day 60 ± 2 of gestation, sows were transported to the Kansas State University Meats Laboratory (Manhattan, KS, USA) and euthanized by electrical stunning followed by exsanguination. After ~15 min, the whole reproductive tracts of the sows were removed and transported to the laboratory for removal of fetuses. Based on crown-to-rump length, whole left *m. longissimus dorsi* muscles (LM) were collected from the smallest (SM), median (ME) and largest (LG) male fetuses of litters of three randomly chosen sows. Muscles were utilized for PFM isolation with a total of nine pools ($n = 3$/size category). Within 24 h of birth, whole left LM were collected from male piglets nearest to the average weight of the litter for PSC isolation ($n = 3$). The methods of Li *et al*. (2011) were be followed for myoblast isolation with slight modifications. Briefly, muscles were excised of all visible connective tissue and minced with sterilized surgical scissors. The tissue was digested with 0.8 mg/ml of pronase XIV (Sigma Aldrich, St. Louis, MO, USA) in phosphate buffered saline (PBS; Corning, Corning, NY, USA) for 45 min at 37°C. To remove any residual pronase, samples were centrifuged at 1500 × **g** for 4 min, liquid was decanted, and resulting slurry was resuspended in 40 ml of PBS, shaken and centrifuged again. This process was repeated four times. The resulting slurry was resuspended in PBS, shaken, and centrifuged at 500 × **g** for 10 min; this step was repeated three times. After each 500 × **g** centrifugation step, supernatant containing myoblasts were retained and cells were pelleted via centrifugation at 1500 × **g** for 10 min. Cells were resuspended in PBS and passaged sequentially through 70- and 40-μm filters. After filtration, final myoblast pellets were resuspended in growth media (GM) consisting of high-glucose Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (vol/vol) fetal bovine serum (GE Healthcare, Pittsburgh, PA, USA), 2% (vol/vol) porcine serum (PS; Sigma Aldrich), 100 U penicillan/ml, 100 μg of strepmycin/ml, and 20 μg of gentamicin/ml. Cells were plated on 100 mm^2 culture dishes (Eppendorf, Hauppauge, NY, USA) for 24 h to allow all viable myogenic progenitor cells to attach to the dish.

Dishes were rinsed of all additional cell debris, and cells were scraped from the culture dishes and cryopreserved in GM containing 10% volume dimethyl sulfoxide (Sigma Aldrich). Cells were stored in liquid nitrogen vapor until needed for experiments.

Proliferation assay

Parallel cultures of progenitor cells were seeded at a density of 1×10^3 cells/cm^2 on tissue culture treated 12-well culture plates (Eppendorf) coated with 5 μg/cm^2 entactin-collagen IV-laminin (ECL; Millipore, Billerica, MA, USA). Each proliferation assay day, two culture plates were utilized to seed a pool of progenitor cells on six wells. Cells in two of the six wells were treated with one of three treatments 12-h post-plating: high serum which consisted of GM (HS), low serum which consisted of GM without 10% fetal bovine serum (LS), and LS supplemented with 10 mg/ml betaGRO® (BG). Experiments were replicated three times.

Treatments after 24, 48, 72, and 96 h were applied, proliferation assay cultures were immunostained for Pax7, Myf-5 and bromodeoxyuridine (BrdU; Sigma Aldrich). For detection of cells traversing S-phase, BrdU, a thymidine analog, was added to media 2 h before immunostaining (Allen *et al*., 1979). Temporal characterization of the muscle lineage markers was conducted as previously described (Li *et al*., 2011). Briefly, cells were fixed with 4% (wt/vol) formaldehyde (Polysciences, Warrington, PA, USA) in PBS. Cultures were incubated in 0.5% Triton-X 100 (Fisher Scientific, Waltham, MA, USA) in PBS for 10 min to permeabilize the nuclear membrane, followed by a 7-min incubation in 4 N hydrochloric acid (Fisher Scientific) to denature the DNA for BrdU immunostaining. Cultures were incubated in 5% horse serum (Fisher Scientific) in 0.2% Triton-X in PBS (blocking solution) for 30 min to block non-specific antigen binding. Cultures were incubated with a primary antibody solution

https://doi.org/10.1017/S1751731117003329 Published online by Cambridge University Press

containing, 1 : 250 anti-BrdU (Santa Cruz Biotechnology, Dallas, TX, USA), 1 : 10 supernatant anti-Pax7 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA), and 1 : 50 anti-Myf-5 (Santa Cruz Biotechnology) diluted in blocking solution for 1 h. Cells were incubated with goat anti-rat AlexaFluor 594 (1 : 1000; Invitrogen), goat anti-mouse AlexaFluor 488 (1 : 1000; Invitrogen) and goat anti-rabbit AlexaFluor 633 (1 : 1000; Invitrogen) to detect BrdU, Pax7, and Myf-5, respectively. Hoechst 33324 (Invitrogen) was utilized at 10 μg/ml for detection of nuclei.

Cells were visualized using an Eclipse TI-U microscope equipped with an X-Cite 120XL epiflouresence illumination system (Nikon Instruments Inc., Melville, NY, USA). Photomicrographs were captured using a Nikon DS-QiMc digital camera (Nikon Instruments Inc.). Five-microscopic fields at 200-fold magnification were analyzed per well for a total of 10 microscopic fields analyzed utilizing NIS Elements Imaging Sofware (Basic Research, 3.3; Nikon Instruments Inc.). Proliferation rate was determined as the percentage of total nuclei that immunostained positive for BrdU at each time point. In addition, cells were classified into three categories based on Pax7 and Myf-5 expression: cells only expressing Pax7 (Pax7+), only expressing Myf-5 (Myf-5+), and cells co-expressing Pax7 and Myf-5 (Pax7+/Myf-5).

Differentiation assay

Progenitor cell seeding density and plate characteristics were the same as described previously. Each pool of progenitor cells was seeded on 16 wells. All progenitor cells were maintained in GM for 96 h before application of treatments, at which time half of the 16 wells were treated with either HS or LS media. Within each serum type, half of the wells were treated with 0 (BGN) or 10 mg/ml betaGRO® (BGP) porcine plasma. Treatments were replenished 48 h after initial application, and cultures were immunostained 96 h after application of treatments.

Cultures were fixed with 4% (wt/vol) formaldehyde (Polysciences) in PBS. Cultures were incubated in 5% horse serum, 0.2% Triton-X 100 in PBS for 30 min to block non-specific antigen-binding sites, incubated for 1 h with a primary antibody solution containing 1 : 2 supernatant anti-sarcomeric myosin (MF20; Developmental Studies Hybridoma Bank), and incubated with 1 : 1000 goat anti-mouse AlexaFluor 594 (Invitrogen). Hoechst 33324 (Invitrogen) was included at 10 μg/ml for the detection of nuclei. Following immunostaining cultures were imaged as described above. Fusion rate was calculated as the number of nuclei present in multinucleated sarcomeric myosin positive myotubes, divided by the total number of nuclei in the photomicrograph.

Myotube width assay

Progenitor cells were plated in the same arrangement as the differentiation assay and maintained in GM for 96 h. Media was replaced with LS media to induce differentiation for an additional 96 h, at which time media was replaced with LS media supplemented with 10 μM cytosine β-D-arabinofuranoside (Sigma Aldrich) for 24 h to eliminate any proliferating mononuclear cells (Hinterberger and Barald, 1990). Cells were subsequently exposed to treatments described for the differentiation assay for 72 h, after which cultures were immunostained with MF20 (Developmental Studies Hybridoma Bank). Cultures were immunostained and imaged as described for the differentiation assay. To determine myotube width, a minimum of 50 measurements per well were taken perpendicular to the length of each myotube, using the length measurement tool in NIS Elements Imaging Software (Nikon Instruments Inc.).

Protein expression analysis

The culture conditions described in the myotube width analysis were followed until application of treatments. Following elimination of mononuclear cells, cultures were treated with the treatments used in the myotube width analysis for 180 min, after which protein was extracted according to methods of Hidalgo *et al.* (2012). Briefly, cells were scraped from the bottom of each well in 300 μl of lysis buffer (50 M Tris-HCL, 150 M NaCl, 1 M EDTA, 0.5% Triton-X 100, 1 M phenlymethanessulfonyl fluoride, 1 M $NaVO^4$; Fisher Scientific) containing a complete protease inhibitor (Sigma Aldrich) and homogenized through a 21-gauge needle. Homogenates were centrifuged at 14 000 × **g** for 15 min at 4°C. Sample protein concentrations were quantified using Pierce's BCA Protein Assay Kit (Thermo Scientific).

Three micrograms of protein was heated in 4 × sample loading buffer (40% vol/vol glycerol, 4% vol/vol β-mercaptoethanol, 0.08% wt/vol SDS; Fisher Scientific) at 95°C for 3 min. Samples were loaded onto four 7.5% separating polyacrylamide gels with 3.5% stacking gels and separated at 40 mA per gel. Proteins were transferred to a nitrocellulose membrane (Amersham Hybond-ECL; GE Healthcare) using blotting paper saturated with transfer buffer (25 mM Tris, 192 mM glycine and 5% vol/vol methanol) and a Semi-dry Transfer Unit (Hoefer, Holliston, MA, USA). Blots were incubated with 5% nonfat dry milk in TBS-T (10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween-20) for 30 min at room temperature to block non-specific antigen sites. After the nitrocellulose membranes were blocked, the first blot was incubated with an p-mTOR, p-AKT (Ser^{473}), p-FOXO-1 and p-4EBP-1 antibodies (1 : 1000, 1 : 2000, 1 : 500 and 1 : 1000, respectively, in 1% nonfat dry milk in TBS-T; Cell Signaling, Beverly, MA, USA). The second blot was incubated with total mTOR, total AKT antibody, total FOXO-1 and total 4EBP-1 (1 : 1000 in 1% nonfat dry milk in TBS-T; Cell Signaling). The third blot was incubated with an p-S6 kinase, p-AKT (Thr^{308}; 1 : 1000 in 1% nonfat dry milk in TBS-T; Cell Signaling) and MAFbx antibodies (1 : 500, in 1% nonfat dry milk in TBS-T; Santa Cruz Biotechnology). The fourth blot was incubated with total S6 kinase (1 : 1000 in 1% nonfat dry milk in TBS-T; Cell Signaling) and MURF-1 antibodies (1 : 2000, in 1% nonfat dry milk in TBS-T; Santa Cruz Biotechnology) overnight at 4°C. Following primary antibody incubation, blots were incubated with an anti-rabbit horse radish peroxidase linked secondary antibody (1 : 1000 in 5% nonfat dry milk in

https://doi.org/10.1017/S1751731117003329 Published online by Cambridge University Press

TBS-T; Cell Signaling) at room temperature for 1 h. Blots were developed using an enhanced chemiluminescence kit (ECL Plus; Amersham, Pittsburgh, PA, USA) and visualized using the ChemiDoc-It 415 Imaging System (UVP, Upland, CA, USA). Band intensities were quantified using VisionWorksLS Image Acquisition and Analysis Software (UVP). Values are reported as abundance of total and phosphorylated protein that was equalized to a pooled sample on each blot and as a ratio of protein phosphorylation (normalized phosphorylated band intensity/normalized total band intensity × 100).

Statistical analyses

The proliferation assay was analyzed as a $3 \times 3 \times 4$ factorial arrangement, with repeated measures. Fixed effects included fetus size, treatment (Trt) and day of culture (Time). The random effect was pool of cells. There were no fetus size interactions with Trt or Time; therefore, data was pooled and the final model was a 3×4 factorial arrangement with Trt and Time as the main effects. Differentiation, protein synthesis and western blot data were analyzed as a $3 \times 2 \times 2$ factorial design. Fixed effects were fetus size, media serum type (SER) and betaGRO® supplementation status (BET), with pool of cells as the random effect. There were also no fetus size interactions with serum and BG; therefore, data was pooled and analyzed as a 2×2 factorial arrangement. Statistical analyses were performed using the GLIMMIX Procedures of SAS 9.4 (Cary, NC, USA). Pair-wise comparisons between the least square means of the factor levels, were computed using the PDIFF option of the LSMEANS statement. Differences were considered significant at $\alpha \leqslant 0.05$.

Results

Porcine fetal myoblasts

There were Trt × Time interactions for proliferation rate, percentage of Pax7+, and the percentage of Pax7+/Myf-5+ PFM ($P \leqslant 0.05$; Table 1); however, there was no interaction ($P = 0.21$) for the percentage of Myf-5+ PFM. At all-time points, HS-PFM had a greater percentage of proliferating cells than LS-PFM ($P \leqslant 0.02$). High serum-PFM had a greater percentage of proliferating cells than BG-PFM at all-time points ($P \leqslant 0.02$), except hour 72 ($P = 0.44$). Porcine fetal myoblasts supplemented betaGRO® had a greater percentage of proliferating cells than LS-PFM at all-time points ($P < 0.01$), except hour 24 ($P = 0.09$).

At 24 h, BG-PFM had a greater percentage of Pax7+ compared with HS- and LS-PFM ($P \leqslant 0.02$), which did not differ from each other ($P = 0.75$). At 48 and 72 h, percentage of Pax7+ PFM did not differ among all treatments ($P \geqslant 0.12$). At 96 h, LS-PFM had a greater ($P = 0.03$) percentage of Pax7+ cells compared with HS-PFM, and BG-PFM did not differ in percentage of Pax7+ cells compared with HS- and LS-PFM ($P \geqslant 0.28$). At all-time points HS- and BG-PFM had a greater percentage Pax7+/Myf-5+ cells compared with LS-PFM ($P < 0.01$) and did not differ from each other ($P = 0.10$).

Time affected proliferation rate and percentage of Myf-5+ and Pax7+/Myf-5+ PFM ($P < 0.01$), but did not affect ($P = 0.13$) percentage of PFM that were Pax7+. The percentage of proliferative PFM was less at 96 h compared with other time points ($P < 0.01$), which did not differ ($P \geqslant 0.16$). At 24 h there was a greater percentage of Myf-5+ PFM compared with all other time points ($P \leqslant 0.03$), which did not differ from each other ($P \geqslant 0.22$). At 48 and 72 h, there was a greater percentage of Pax7+/Myf-5+ PFM than at 24 and 96 h ($P < 0.01$), which did not differ from each other ($P = 0.38$). The percentage of Pax7+/Myf-5+ PFM did not differ ($P = 0.27$) at 48 and 72 h.

There was a Trt effect for percentage of proliferating cells, percentage of Myf-5+ PFM, and Pax7+/Myf-5+ PFM ($P \leqslant 0.03$), but Trt did not affect ($P = 0.12$) the percentage of Pax7+ PFM. The HS-PFM had a greater percentage of proliferating cells compared with both the LS and BG treatments ($P < 0.01$), and BG-PFM had a greater ($P < 0.01$) proliferation percentage compared with LS-PFM. The LS-PFM had a greater percentage of Myf-5+ PFM compared with the HS- and BG-PFM ($P \leqslant 0.03$), which did not differ ($P = 0.55$). The LS treatment had less Pax7+/Myf-5+ PFM than HS- and BG-PFM ($P < 0.01$), which did not differ ($P = 0.31$).

There were SER × BET interactions for fusion percentage and myotube width of ($P < 0.01$; Table 2). When cultured in the presence of HS, BGP increased ($P < 0.01$) fusion percentage compared with BGN cells; however, in the presence of LS, BGP decreased ($P < 0.01$) fusion percentage. When cultured in the presence of LS, BGP decreased ($P < 0.01$) myotube width compared with BGN, but betaGRO® status did not influence myotube width in the presence of HS media ($P = 0.57$).

There was an effect of SER on fusion percentage ($P < 0.01$), with HS-PFM having a greater ($P < 0.01$) fusion percentage than LS-PFM. In contrast, SER did not affect myotube width ($P = 0.12$). There was no BET effect on fusion percentage ($P = 0.97$). There was a BET effect for myotube width ($P < 0.01$), with BGP-PFM forming myotubes with decreased ($P < 0.01$) width compared with BGN myoblasts.

Porcine satellite cells

There were Time × Trt interactions for PSC proliferation rate and percentage of Pax7+ cells ($P \leqslant 0.05$; Table 3); however, Myf-5+ and Pax7+/Myf-5+ percentages were not affected by the interaction ($P \geqslant 0.09$). Overall, at all-time points the HS-PSC had a greater proliferation rate than the LS- and BG-PSC ($P < 0.01$), and the LS-PSC had a greater ($P < 0.01$) proliferation rate than the BG-PSC. At 24 h of culture, BG-PSC had a greater percentage of Pax7+ cells than HS- and LS-PSC ($P < 0.03$). For the remainder of the time points, all treatments did not differ in Pax7+ percentage ($P \geqslant 0.16$).

Time affected all proliferation characteristics assessed ($P < 0.01$). The proliferation rate of PSC was greater at 24 h compared with 48, 72, and 96 h ($P < 0.01$), which did not differ from each other ($P \geqslant 0.79$). At 24 h, there was a greater percentage of Pax7+ PSC compared with all other time points ($P < 0.01$); 48 h PSC had a greater percentage of

https://doi.org/10.1017/S1751731117003329 Published online by Cambridge University Press

Table 1 *Effect of betaGRO® supplementation* in vitro *on porcine fetal myoblast (PFM) proliferation rate and temporal expression of Pax7 and Myf-5*[1]

	Treatment				*P*-value		
	HS	LS	BG	SEM	Trt × time	Time	Trt
Proliferation rate[2] (%)							
24 h	49.0[a]	28.3[b]	35.9[b]	2.9	<0.01	<0.01	<0.01
48 h	46.5[a]	23.2[b]	35.2[c]				
72 h	44.7[a]	13.7[b]	46.9[a]				
96 h	39.5[a]	7.8[b]	31.7[c]				
Pax7 + [3] (%)							
24 h	0.4[a]	0.6[a]	2.8[b]	0.7	0.05	0.13	0.12
48 h	0.5	0.2	0.1				
72 h	0.2	1.7	0.2				
96 h	0.3[a]	2.3[b]	1.3[a,b]				
Myf-5 + [4] (%)							
24 h	31.9	41.3	31.8	3.5	0.21	0.01	0.03
48 h	21.4	35.7	25.8				
72 h	20.5	39.3	21.6				
96 h	25.6	34.3	29.2				
Pax7 + /Myf-5 + [5] (%)							
24 h	59.6[a]	40.8[b]	60.3[a]	3.7	<0.01	<0.01	<0.01
48 h	74.4[a]	51.8[b]	70.8[a]				
72 h	76.2[a]	38.2[b]	75.8[a]				
96 h	68.9[a]	25.3[b]	59.6[a]				

Trt = treatment effect.
[a,b,c]means within a time period with different superscripts differ.
[1]PFM isolated from the *m. longissimus dorsi* muscle of male fetuses at day 60 of gestation. Myoblasts were cultured in one of three treatments with following as a base media: 2% porcine serum in Dulbecco's Modified Eagle Medium supplemented with 2% porcine serum, 100 U penicillan/ml, 100 μg of strepmycin/ml, and 20 μg of gentamicin/ml. Treatments included high serum media containing 10% fetal bovine serum (HS), low serum media consisting of only base media (LS), or LS media supplemented with 10 mg/ml betaGRO® (BG). Parallel cultures were immunostained daily.
[2]Cultures were exposed to 5 μM bromodeoxyuridine (BrdU) and immunostained. Percentage calculated as the number of BrdU positive PFM divided by the total number of PFM present within 10 representative photomicrographs.
[3]Represents the percentage of all PFM that solely immunostained Pax7 positive within 10 representative photomicrographs.
[4]Represents the percentage of all PFM that solely immunostained Myf-5 positive within 10 representative photomicrographs.
[5]Represents the percentage of all PFM that co-expressed Pax7 and Myf-5 within 10 representative photomicrographs.

Table 2 *Effect of betaGRO® supplementation* in vitro *on differentiation capacity and myotube width of porcine fetal myoblasts and satellite cells*[1]

	Treatment[2]					*P*-value		
	LS		HS					
	BGN	BGP	BGN	BGP	SEM	SER × BET	SER	BET
Porcine fetal myoblasts								
Fusion[2] (%)	56.8[a]	46.2[b]	40.8[c]	51.6[d]	3.6	<0.01	<0.01	0.97
Myotube width[3] (μm)	34.4[a]	30.4[b]	32.9[c]	33.2[a,b]	1.0	<0.01	0.12	<0.01
Porcine satellite cells								
Fusion[2] (%)	24.1[a]	43.3[b]	30.7[c]	38.5[d]	2.5	<0.01	0.58	<0.01
Myotube width[4] (%)	36.0	40.6	29.9	35.8	1.7	0.63	<0.01	<0.01

SER = serum effect; BET = betaGRO® effect.
[a,b,c]means within a row with different superscripts differ.
[1]Porcine fetal myoblasts and satellite cells isolated from the *m. longissimus dorsi* muscle of male fetuses at day 60 of gestation or within 24 h after birth, respectively. Myoblasts were cultured in one of four treatments with following as a base media: 2% porcine serum in Dulbecco's Modified Eagle Medium supplemented with 2% porcine serum, 100 U penicillan/ml, 100 μg of strepmycin/ml, and 20 μg of gentamicin/ml.
[2]LS = base media with 0% fetal bovine serum; HS = base media with 10% fetal bovine serum; BGN = 0 mg/ml betaGRO® added to media; BGP = 10 mg/ml betaGRO® added to media.
[3]Percentage of all PFM that have incorporated into multinucleated sarcomeric myosin positive myotubes.
[4]Average width of 100 multinucleated myotubes from 10 representative photomicrographs.

https://doi.org/10.1017/S1751731117003329 Published online by Cambridge University Press

Table 3 *Effect of betaGRO® supplementation* in vitro *on porcine satellite cell (PSC) proliferation rate and temporal expression of Pax7 and Myf-5*[1]

	Treatment				*P*-value		
	HS	LS	BG	SEM	Time × Trt	Time	Trt
Proliferation rate[2] (%)							
24 h	58.5[a]	46.3[b]	20.7[c]	1.1	0.05	<0.01	<0.01
48 h	56.1[a]	36.9[b]	15.4[c]				
72 h	59.6[a]	38.3[b]	11.7[c]				
96 h	56.6[a]	42.1[b]	9.7[c]				
Pax7 + [3] (%)							
24 h	2.4[a]	3.5[a]	6.4[b]	0.8	0.02	<0.01	0.46
48 h	1.6	3.1	1.4				
72 h	0.6	0.3	0.4				
96 h	0.1	0.9	0.3				
Myf-5 + [4] (%)							
24 h	10.3	15.0	19.8	3.2	0.09	<0.01	<0.01
48 h	9.5	26.9	29.7				
72 h	14.9	25.5	37.8				
96 h	15.3	24.7	28.0				
Pax7 + /Myf-5 + [5] (%)							
24 h	85.2	76.9	72.2	3.0	0.12	<0.01	0.03
48 h	84.3	71.1	68.1				
72 h	81.9	69.6	61.2				
96 h	83.1	72.7	71.5				

Trt = treatment effect.
[a,b,c]means within a time period with different superscripts differ.
[1]Porcine satellite cells isolated from the *m. longissimus dorsi* muscle of male piglets within 24 h of birth. Satellite cells were cultured in one of three treatments with following as a base media: 2% porcine serum in Dulbecco's Modified Eagle Medium supplemented with 2% porcine serum, 100 U penicillan/ml, 100 μg of strepmycin/ml, and 20 μg of gentamicin/ml. Treatments included high serum media containing 10% fetal bovine serum (HS), low serum media consisting of only base media (LS), or LS media supplemented with 10 mg/ml betaGRO® (BG). Parallel cultures were immunostained daily.
[2]Cultures were exposed to 5 μM bromodeoxyuridine (BrdU) and immunostained. Percentage calculated as the number of BrdU positive PFM divided by the total number of PSC present within 10 representative photomicrographs.
[3]Represents the percentage of all PSC that solely immunostained Pax7 positive within 10 representative photomicrographs.
[4]Represents the percentage of all PSC that solely immunostained Myf-5 positive within 10 representative photomicrographs.
[5]Represents the percentage of all PSC that co-expressed Pax7 and Myf-5 within 10 representative photomicrographs.

Pax7+ PSC than cells at 72 and 96 h ($P < 0.01$), which did not differ from each other ($P = 0.94$). At 24 h, there were fewer Myf-5+ PSC compared with all other time points ($P < 0.01$), which did not differ from each other ($P \geqslant 0.07$). At 24 h, the percentage of the Pax7+/Myf-5+ PCS was greater than at 48 and 72 h ($P \leqslant 0.01$), and did not differ compared ($P = 0.09$) to 96 h. At 72 h there were fewer Pax7 +/Myf-5+ PSC than at the 48 and 96 h ($P \leqslant 0.01$), which did not differ from each other ($P = 0.33$).

The main effect of Trt affected proliferation rate, percentage of Myf-5+ PSC, and the percentage of Pax7+/Myf-5+ PSC ($P \leqslant 0.03$); however, Trt did not affect percentage of Pax7+ PSC ($P \geqslant 0.06$). The PSC exposed to the HS treatment had greater proliferation rate compared with all other treatments ($P < 0.01$), and LS-PSC had a greater ($P < 0.01$) proliferation rate than BG-PSC. The HS-PSC had a decreased percentage of Myf-5+ PSC compared with LS- and BG-PSC ($P < 0.01$), which did not differ from each other ($P = 0.06$). The HS-PSC had a greater percentage of Pax7+/Myf-5+ PSC compared with LS and BG treatments ($P \leqslant 0.04$), which did not differ from each other ($P = 0.29$).

There was a SER × BET interaction for fusion rate of PSC ($P < 0.01$; Table 2). Porcine satellite cells cultured in LS-BGP had a greater fusion rate compared with LS-BGN ($P < 0.01$), and the HS-BGP had a greater ($P < 0.01$) differentiation capacity compared with HS-BGN; however, the difference was more pronounced in the LS treatment. Serum did not affect differentiation capacity ($P = 0.06$). There was a BET effect for differentiation capacity ($P < 0.01$). The BGP-PSC had an increased ($P < 0.01$) percentage of cells that fused into multinucleated myotubes compared with BGN-PSC.

There was no SER × BET interaction for myotube width ($P \geqslant 0.13$). Serum affected myotube width ($P < 0.04$). The LS-PSC had a greater ($P < 0.01$) myotube width compared with HS-PSC. There was a BET effect on myotube width when BGP-PSC had a greater ($P < 0.01$) myotube width than BGN-PSC.

Protein expression

There were only SER × BET interactions for the abundance of phosphorylated AKT-Ser^{473}, ratio of phosphorylated AKT-Ser^{473}, and the abundance of total 4EBP following 180-min treatment period ($P \leqslant 0.02$; Table 4). When cultured

https://doi.org/10.1017/S1751731117003329 Published online by Cambridge University Press

Table 4 *Effect of serum type and betaGRO® exposure on differentiated satellite cell phosphatidylinositol 3-kinase (PI3-K) pathway signaling*[1]

	Treatment							
	LS		HS		*P*-value			
	BGN	BGP	BGN	BGP	SEM	SER × BET	SER	BET
PI3-K signaling[2]								
AKT								
Total (AU)	1.00	0.86	0.97	1.36	0.16	0.14	0.19	0.49
Ser^{473} phosphorylated (AU)	0.43[a]	0.95[b]	1.75[c]	1.19[b]	0.11	<0.01	<0.01	0.87
Ser^{473} phosphorylated ratio	43[a]	139[b]	213[c]	89[a,b]	20.7	<0.01	0.02	0.53
Thr^{308} phosphorylated (AU)	1.70	2.23	2.12	2.29	0.64	0.78	0.72	0.60
Thr^{308} phosphorylated ratio	182	264	253	169	51.1	0.14	0.82	0.99
mTOR								
Total (AU)	1.25	1.22	1.30	1.38	0.14	0.67	0.46	0.87
Ser^{2448} phosphorylated (AU)	1.11	1.54	1.42	1.60	0.23	0.61	0.46	0.23
Ser^{2448} phosphorylated ratio	98	131	114	127	17.5	0.58	0.73	0.24
S6 Kinase								
Total (AU)	0.52	0.71	0.75	1.04	0.17	0.77	0.13	0.18
Thr^{389} phosphorylated (AU)	0.57	0.88	0.73	1.24	0.13	0.47	0.09	0.02
Thr^{389} phosphorylated ratio	145	143	153	151	37.5	0.99	0.84	0.96
4EBP								
Total (AU)	1.61[a]	1.20[b]	1.07[b]	1.40[a,b]	0.13	0.02	0.19	0.75
$Thr^{37/46}$ phosphorylated (AU)	1.20	1.45	1.19	1.34	0.22	0.80	0.77	0.31
$Thr^{37/46}$ phosphorylated ratio	85	134	124	110	14.6	0.06	0.60	0.25
FOXO								
Total (AU)	1.39	1.13	1.51	1.64	0.35	0.53	0.34	0.84
Ser^{319} phosphorylated (AU)	0.78	0.96	0.81	1.05	0.08	0.66	0.44	0.02
Ser^{319} phosphorylated ratio	79	104	84	93	13.2	0.46	0.73	0.13
MAFbx (AU)	1.29	2.01	1.90	1.46	0.47	0.25	0.95	0.77
MURF-1 (AU)	1.44	1.39	1.17	1.36	0.20	0.57	0.49	0.74

LS = base media with 0% fetal bovine serum; HS = base media with 10% fetal bovine serum; BGN = 0 mg/ml betaGRO® added to media; BGP = 10 mg/ml betaGRO® added to media; SER = serum effect; BET = betaGRO® effect; AKT = protein kinase B; mTOR = mammalian target of rapamycin; S6 Kinase = p70 S6 kinase; 4EBP = eukaryotic translation initiation factor 4E binding protein; FOXO = forkhead box O1; MAFbx = muscle atrophy F-box; MURF-1 = muscle ring finger-1; AU = arbitrary unit.
[a,b,c]Means within a row with different superscripts differ.
[1]Porcine satellite cells isolated from the *m. longissimus dorsi* muscle of male fetuses within 24 h after birth. Satellite cells were cultured in one of four treatments for 180 min post-differentiation with following as a base media: 2% porcine serum in Dulbecco's Modified Eagle Medium supplemented with 2% porcine serum, 100 U penicillan/ml, 100 μg of strepmycin/ml, and 20 μg of gentamicin/ml.
[2]Values are reported as abundance of total and phosphorylated protein that was equalized to a pooled sample on each blot and as a ratio of protein phosphorylation (normalized phosphorylated band intensity/normalized total band intensity × 100).

in the presence of HS the BGP-PSC had decreased abundance and ratio of phosphorylated AKT-Ser^{473} compared with BGN-PSC ($P \leqslant 0.01$); however in the presence of LS, BGP-PSC had a greater abundance and ratio of phosphorylated AKT-Ser^{473} compared with BGN-PSC ($P = 0.01$). When in the presence of HS the BGP-PSC had an increased abundance of total 4EBP; however, in the presence of LS the BGP-PSC had a decrease in the abundance of 4EBP ($P = 0.02$).

Serum affected the abundance and ratio of phosphorylated AKT-Ser^{473} ($P \leqslant 0.02$), but did not affect any of the other PI3-K pathway signaling proteins assessed ($P \geqslant 0.09$). The HS-PSC had a greater abundance and ratio of phosphorylated AKT-Ser^{473} compared with LS-PSC ($P \leqslant 0.02$). There was a BET effect for the abundance of phosphorylated S6 Kinase and FOXO ($P = 0.02$), but BET did not affect any of the other PI3-K pathway signaling proteins ($P \geqslant 0.13$). The BGP-PSC media had a greater abundance of phosphorylated S6 Kinase and FOXO compared with BGN-PSC ($P = 0.02$).

Discussion

Myoblasts are responsible for replenishing their own population and terminally differentiating into muscle fibers to contribute the DNA template needed for protein synthesis (Bentzinger *et al.*, 2012). Satellite cell activity is modulated through the transition of transcriptional signals, that begins with elevated Pax7 expression levels in quiescent satellite cells and progresses through the expression of the myogenic regulator factors as the satellite cells begin to proliferate and commit to terminal differentiation (Zammit *et al.*, 2006; Biressi and Rando, 2010). Li *et al.* (2011) categorized *in vitro* proliferative satellite cells into three sub-classes of cells that express Pax7, Myf-5, and co-express Pax7 and Myf-5. Cells that solely express Pax7 are activated and are less proliferative than cells that co-express Pax7 and Myf-5, which are the most proliferative of the three populations of myogenic cells. As Pax7 expression subsides, the rate of

https://doi.org/10.1017/S1751731117003329 Published online by Cambridge University Press

proliferation is lessened, and cells that solely expressing Myf-5 are committed to myogenic differentiation (Zammit *et al*., 2006). Therefore, these markers can be utilized to identify the effects of treatment on porcine myoblast proliferation. The current study focused on the potential of betaGRO® supplementation to stimulate fetal and neonatal skeletal muscle development *in vitro* and serves as a potential intervention to improve skeletal muscle development during both stages of development.

The effects of betaGRO® or porcine plasma on characteristics of PFM *in vitro* has not yet been evaluated; however, Tran *et al*. (2014) indicated porcine plasma increased proliferation of porcine intestinal epithelial cells. In proliferation assays of the current study, the HS treatment served as a positive control and betaGRO® was supplemented to the LS treatment, which served as a negative control. The BG-PFM exhibited proliferative characteristics more similar to HS-PFM than LS-PFM. Over the course of the proliferation assay, BG-PFM had a 19% greater proliferation rate compared with the LS-PFM. Additionally, the temporal expression profile of Pax7 and Myf-5 of BG-PFM was more similar to the HS-PFM than the LS-PFM. Over the course of the proliferation assay the BG-PFM had a 27% greater percentage of Pax7+/Myf-5+ PFM and 10% fewer Myf-5+ PFM compared with the LS-PFM. Therefore, these data indicate supplementation of betaGRO® stimulated PFM to retain Pax7 expression and maintain a proliferative state.

In rat neonatal cells, Engert *et al*. (1996) reported IGF-1 increases proliferation before stimulating differentiation. In contrast, the proliferation rate of BG-PSC was decreased 43% and 27% compared with HS- and LS-PSC, respectively. Also in contrast to the response of PFM, the expression of the transcription factors Pax7 and Myf-5 of BG-PSC was more similar to LS-PSC than HS-PSC. The BG- and LS-PSC had 15% and 11% fewer cells that were Pax7+/Myf-5+ compared with the HS treatment, respectively. In addition, BG- and LS-PSC had 16% and 11% more Myf-5+ PSC compared with HS-PSC, respectively. Therefore, treatment of proliferative PSC with BG resulted in PSC that were less proliferative and this was reflected by myogenic regulatory factor more favorable for terminal differentiation than proliferation.

Following proliferation, a subset of myogenic precursor cells undergo differentiation, contributing to the pool of muscle fibers prenatally or nuclei in existing muscle fibers postnatally (Moss and Leblond, 1971; Biressi *et al*., 2007). Insulin-like growth factor-1 been implicated in promoting myogenic differentiation (Schiaffino and Mammucari, 2011); however, PFM fusion rate was not impacted by the supplementation of betaGRO® and when supplemented to differentiated PFM, betaGRO® reduced myotube width by 5%. The accepted role of IGF-1 in skeletal muscle is to stimulate muscle hypertrophy through stimulation of the PI3-K pathway signaling (Engert *et al*., 1996; Schiaffino and Mammucari, 2011). Interestingly, the response of fetal myoblasts to transforming growth factor-β and topographical stimuli varies by age of the cell. Cusella-De Angelis *et al*. (1994) found transforming growth factor-β reduced differentiation in embryonic myoblasts but had not effect on fetal myoblasts. Evans *et al*. (1999) reported neonatal satellite cells only form fibers in cultures with thick groves, while fetal myoblasts can form fibers in cultures with thick and thin groves. Overall, these results indicate betaGRO® does not affect PFM fusion and reduces PFM protein synthesis.

In contrast to PFM, supplementation of PSC with betaGRO® increased myoblast fusion and myotube development, which is more consistent with the characteristic IGF-1 response. The BGP-PSC had a 33% greater differentiation capacity and 14% increase in myotube width compared with BGN-PSC. Similarly, Gardner *et al*. (2015) observed IGF-1 was a potent differentiation factor and Stitt *et al*. (2004) observed an 18% increase in myotube diameter when IGF-1 was supplemented to the C2C12 muscle cell line. Therefore, these results indicate betaGRO® stimulates satellite cell fusion and myotube protein synthesis to a similar magnitude as IGF-1 supplementation.

The feed supplement betaGRO® is the growth factor rich fraction of porcine plasma, that has undergone a process that results in elevated levels of bio-available IGF-1. The growth factor IGF-1 promotes myogenic differentiation and protein synthesis through binding to the IGF-1R to trigger a PI3-K pathway signaling phosphorylation cascade (Engert *et al*., 1996; Schiaffino and Mammucari, 2011). Supplementation of IGF-1 *in vitro* stimulated increased average myotube diameter, greater abundance of phosphorylated AKT and phosphorylated S6 kinase, and decreased abundance of total 4EBP-1 in murine cell lines (Rommel *et al*., 2001). The AKT branch-point also signals to decrease the extent of muscle atrophy through phosphorylation of FOXO and down-regulation of the ubiquitin ligases MAFbx and MURF-1 (Stitt *et al*., 2004). Ubiquitin ligases MAFbx and MURF-1 degrade myogenic regulatory factors to provide the appropriate expression of myogenic regulatory factors to promote myogenic differentiation, as well as, serve as the mechanism to clear oxidized proteins accumulated during the myogenic differentiation (Bell *et al*., 2016), therefore their inhibition is associated with reduced myoblast fusion (Gardrat *et al*., 1997). In the current study mTOR pathway signaling was evaluated in differentiated PSC after 180 min of treatment exposure.

Han *et al*. (2008) supplemented IGF-1 to PSC from 6 month old pigs for 15 to 180 min and observed that 100 ng/ml recombinant IGF-1 stimulated the production of the phosphorylated forms of AKT and mTOR, illustrating that IGF-1 supplementation begins to impact protein metabolism very rapidly (Han *et al*., 2008). At 180 min, the additional growth factors of the HS treatment stimulated 53% more phosphorylated AKT-Ser^{473}, which resulted in a 40% increase in the ratio of phosphorylated AKT-Ser^{473} compared with LS treatments. Interestingly, BGP-PSC had a 47% reduction of phosphorylated AKT-Ser^{473} in the presence of HS media, but a 55% increase in phosphorylation of the same protein in the presence of LS media. These findings suggest the full benefits of betaGRO® will be experienced in a nutrient poor environment. In addition, betaGRO® resulted in a 39% increase of phosphorylated S6 Kinase and a 21% increase of

https://doi.org/10.1017/S1751731117003329 Published online by Cambridge University Press

phosphorylated FOXO. While some proteins of the pathway were affected in a manner to explain the betaGRO® stimulated increase in myotube width, the increase in pathway phosphorylation for all proteins was not observed. It is hypothesized this occurred due to the myotubes not being serum starved before treatment exposure. Experiments of this nature illustrate the PI3-K pathway signaling is stimulated when serum starved myotubes are exposed to IGF-1 for 15 to 180 min before protein extraction (Rommel *et al*., 2001; Han *et al*., 2008).

Until the current investigation, research involving porcine plasma supplementation have focused on the overall gains, gut health and immunity (Coffey and Cromwell, 1995; de Rodas *et al*., 1995; Everts *et al*., 2001). Porcine plasma supplementation during the nursery phase increases ADG and feed intake (Everts *et al*., 2001), and stimulates the proliferation of intestinal epithelial cells, promoting gut health and nutrient absorption (Tran *et al*., 2014). Very little research emphasis has been placed on the impact of the growth factors available in porcine plasma. de Rodas *et al*. (1995) calculated that the amount of IGF-1 needed to elicit a biological response in dairy calves and stated it is not realistic to provide the needed levels of growth factors through porcine plasma. Even though the starting material for betaGRO® is porcine plasma the current study identifies that the enrichment and activation of growth factors elicits direct stimulation of myogenic progenitor cells. Moreover, betaGRO® elicits divergent effects on fetal and neonatal myogenic progenitor cells. These divergent effects suggest that betaGRO® is able to promote proliferation during prenatal myogenesis, which may positively affect muscle fiber number and satellite cell number establishment. Postnatally, betaGRO® stimulates satellite cell mediated muscle hypertrophy to modulate postnatal muscle fiber hypertrophy. Therefore, betaGRO® may have positive effects on muscle development and growth when fed *in vivo*.

Acknowledgments

Contribution no. 17-387-J of the Kansas Agricultural Experiment Station, Manhattan 66506. This study was sponsored by Puretein Biosciences, LLC.

References

Allen RE, Merkel RA and Young RB 1979. Cellular aspects of muscle growth: myogenic cell proliferation. Journal of Animal Science 49, 115–127.

Baxter RC 2000. Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. American Journal of Physiology, Endocrinology and Metabolism 278, E967–E976.

Bell RA, Al-Khalaf M and Megeney LA 2016. The beneficial role of proteolysis in skeletal muscle growth and stress adaptation. Skeletal Muscle 6, 16.

Bentzinger CF, Wang YX and Rudnicki MA 2012. Building muscle: molecular regulation of myogenesis. Cold Spring Harbor Perspectives in Biology 4, a008342.

Biressi S and Rando TA 2010. Heterogeneity in the muscle satellite cell population. Seminars in Cell Development and Biology 21, 845–854.

Biressi S, Tagliafico E, Lamorte G, Monteverde S, Tenedini E, Roncaglia E, Ferrari S, Ferrari S, Cusella-De Angelis MG, Tajbakhsh S and Cossu G 2007. Intrinsic phenotypic diversity of embryonic and fetal myoblasts is revealed by genome-wide gene expression analysis on purified cells. Developmental Biology 304, 633–651.

Coffey RD and Cromwell GL 1995. The impact of environment and antimicrobial agents on the growth response of early-weaned pigs to spray-dried porcine plasma. Journal of Animal Scieince 73, 2532–2539.

Cusella-De Angelis M, Molinari S, Le Donne A, Coletta M, Vivarelli E, Bouche M, Molinaro M, Ferrari S and Cossu G 1994. Differential response of embryonic and fetal myoblasts to TGF beta: a possible regulatory mechanism of skeletal muscle histogenesis. Development 120, 925–933.

de Rodas BZ, Sohn KS, Maxwell CV and Spicer LJ 1995. Plasma protein for pigs weaned at 19 to 24 days of age: effect on performance and plasma insulin-like growth factor I, growth hormone, insulin, and glucose concentrations. Journal of Animal Science 73, 3657–3665.

Dodson MV, Dodson EL, Martin MA, Brannon BA, Mathison DC and McFarland DC 1987. Optimization of bovine satellite cell-derived myotube formation in vitro. Tissue and Cell 19, 159–166.

Engert JC, Berglund EB and Rosenthal N 1996. Proliferation precedes differentiation in IGF-I-stimulated myogenesis. Journal of Cell Biology 135, 431–440.

Evans DJ, Britland S and Wigmore PM 1999. Differential response of fetal and neonatal myoblasts to topographical guidance cues in vitro. Development Genes and Evolution 209, 438–442.

Everts H, Nabuurs MJA, Margry R, van Dijk AJ and Beynen AC 2001. Growth performance of weanling pigs fed spray-dried animal plasma: a review. Livestock Production Science 68, 263–274.

Gardner S, Gross SM, David LL, Klimek JE and Rotwein P 2015. Separating myoblast differentiation from muscle cell fusion using IGF-I and the p38 MAP kinase inhibitor SB202190. American Journal of Physiology-Cell Physiology 309, C491–C500.

Gardrat F, Montel V, Raymond J and Azanza JL 1997. Proteasome and myogenesis. Molecular Biology Reports 24, 77–81.

Han B, Tong J, Zhu MJ, Ma C and Du M 2008. Insulin-like growth factor-1 (IGF-1) and leucine activate pig myogenic satellite cells through mammalian target of rapamycin (mTOR) pathway. Molecular Reproduction and Development 75, 810–817.

Hidalgo M, Le Bouffant R, Bello V, Buisson N, Cormier P, Beaudry M and Darribere T 2012. The translational repressor 4E-BP mediates hypoxia-induced defects in myotome cells. Journal of Cell Science 125, 3989–4000.

Hinterberger TJ and Barald KF 1990. Fusion between myoblasts and adult muscle-fibers promotes remodeling of fibers into myotubes invitro. Development 109, 139–148.

Kuang S, Kuroda K, Le Grand F and Rudnicki MA 2007. Asymmetric self-renewal and commitment of satellite stem cells in muscle. Cell 129, 999–1010.

Li J, Gonzalez JM, Walker DK, Hersom MJ, Ealy AD and Johnson SE 2011. Evidence of heterogeneity within bovine satellite cells isolated from young and adult animals. Journal of Animal Science 89, 1751–1757.

Magpantay VA, Cabigting SB, Angeles EP, Reyes FCC, Cardona JKM and Regaspi AFS 2016. Activated IGF-I supplementation during late gestation and lactation period affects sow and piglet performace. Philippine Journal of Veterinary and Animal Sciences 42, 136–142.

Moss FP and Leblond CP 1971. Satellite cells as the source of nuclei in muscles of growing rats. Anatomical Records 170, 421–435.

Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, Yancopoulos GD and Glass DJ 2001. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. Nature Cell Biology 3, 1009–1013.

Schiaffino S and Mammucari C 2011. Regulation of skeletal muscle growth by the IGF1-Akt/PKB pathway: insights from genetic models. Skeletal Muscle 1, 4.

Stitt TN, Drujan D, Clarke BA, Panaro F, Timofeyva Y, Kline WO, Gonzalez M, Yancopoulos GD and Glass DJ 2004. The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. Molecular Cell 14, 395–403.

Tran H, Bundy JW, Li YS, Carney-Hinkle EE, Miller PS and Burkey TE 2014. Effects of spray-dried porcine plasma on growth performance, immune response, total antioxidant capacity, and gut morphology of nursery pigs. Journal of Animal Science 92, 4494–4504.

Yates DT, Macko AR, Nearing M, Chen X, Rhoads RP and Limesand SW 2012. Developmental programming in response to intrauterine growth restriction impairs myoblast function and skeletal muscle metabolism. Journal of Pregnancy 2012, 631038.

Zammit PS, Partridge TA and Yablonka-Reuveni Z 2006. The skeletal muscle satellite cell: the stem cell that came in from the cold. Journal of Histochemistry and Cytochemistry 54, 1177–1191.

https://doi.org/10.1017/S1751731117003329 Published online by Cambridge University Press