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

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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.

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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 procoince plasma stimuli 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 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 from the smallest (SM), median (ME) and largest (LG) male fetuses of litters of three randomly chosen sows. 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 penicillin/ml, 100 μg of streptomycin/ml, and 20 μg of gentamicin/ml. Cells were plated on 100 mm² 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 × 10³ cells/cm² on tissue culture treated 12-well culture plates (Eppendorf) coated with 5 μg/cm² 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

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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 33342 (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 epiillumination system (Nikon Instruments Inc., Melville, NY, USA). Photomicrographs were captured using a Nikon D5-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 Software (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 GM. Cultures were immunostained with MF20 (Developmental Studies Hybridoma Bank), and incubated with 1:1000 goat anti-sarcomeric myosin (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 phenylmethanesulfonyl fluoride, 1 M NaVO₄; 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 gel 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 (Ser473), 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 antibodies (1: 1000, 1: 2000, 1: 500 and 1: 1000, respectively, in 1% nonfat dry milk in TBS-T; Cell Signaling). The third blot was incubated with an p-S6 kinase, p-AKT (Thr308, 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, blot were incubated with an anti-rabbit horse radish peroxidase linked secondary antibody (1: 1000 in 5% nonfat dry milk in 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.).
betaGRO® stimulates myogenesis in vitro

There were Trt × Time interactions for PSC proliferation rate, percentage of Myf-5+/Myf-5+ PFMs (P < 0.01; Table 3); however, Myf-5+ and Pax7+/Myf-5+ percentages were not affected by the interaction (P > 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 > 0.16).

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 ≥ 0.16). At 24 h there was a greater percentage of Myf-5+ PFM compared with all other time points (P < 0.03), which did not differ from each other (P ≥ 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 < 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 < 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 Trt × 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.
Table 1  Effect of betaGRO® supplementation in vitro on porcine fetal myoblast (PFM) proliferation rate and temporal expression of Pax7 and Myf-5

<table>
<thead>
<tr>
<th>Treatment</th>
<th>P-value</th>
<th>Treatment</th>
<th>P-value</th>
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<tbody>
<tr>
<td></td>
<td>HS</td>
<td>LS</td>
<td>BG</td>
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<tr>
<td>Proliferation rate (%)</td>
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<tr>
<td>24 h</td>
<td>49.0a</td>
<td>28.3b</td>
<td>35.9b</td>
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<tr>
<td>48 h</td>
<td>46.5a</td>
<td>23.2b</td>
<td>35.2c</td>
</tr>
<tr>
<td>72 h</td>
<td>44.7a</td>
<td>13.7b</td>
<td>46.9a</td>
</tr>
<tr>
<td>96 h</td>
<td>39.5a</td>
<td>7.8b</td>
<td>31.7c</td>
</tr>
<tr>
<td>Pax7  (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>0.4a</td>
<td>0.6a</td>
<td>2.8b</td>
</tr>
<tr>
<td>48 h</td>
<td>0.5</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>72 h</td>
<td>0.2</td>
<td>1.7</td>
<td>0.2</td>
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<tr>
<td>96 h</td>
<td>0.3a</td>
<td>2.3b</td>
<td>1.3b</td>
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<tr>
<td>Myf-5 (%)</td>
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<td>24 h</td>
<td>31.9</td>
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<td>48 h</td>
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<td>96 h</td>
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<td>29.2</td>
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<tr>
<td>Pax7+/Myf-5 (%)</td>
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<tr>
<td>24 h</td>
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<tr>
<td>96 h</td>
<td>68.9a</td>
<td>25.3b</td>
<td>59.6a</td>
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</tbody>
</table>

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 penicillin/ml, 100 µg of streptomycin/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

<table>
<thead>
<tr>
<th>Treatment2</th>
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<tr>
<td>LS</td>
<td>HS</td>
<td>BGN</td>
<td>BGP</td>
<td>SEM</td>
<td>SER × BET</td>
<td>SER</td>
<td>BET</td>
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<td>Porcine fetal myoblasts</td>
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<tr>
<td>Fusion (%)</td>
<td>56.8a</td>
<td>46.2b</td>
<td>40.8c</td>
<td>51.6d</td>
<td>3.6</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Myotube width (µm)</td>
<td>34.4a</td>
<td>30.4b</td>
<td>32.9c</td>
<td>33.2ab</td>
<td>1.0</td>
<td>&lt;0.01</td>
<td>0.12</td>
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<td>Porcine satellite cells</td>
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<tr>
<td>Fusion (%)</td>
<td>24.1a</td>
<td>43.3b</td>
<td>30.7c</td>
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<td>&lt;0.01</td>
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<td>Myotube width (%)</td>
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<td>35.8</td>
<td>1.7</td>
<td>0.63</td>
<td>&lt;0.01</td>
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</table>

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 penicillin/ml, 100 µg of streptomycin/ml, and 20 µg of gentamicin/ml. Treatments included 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.

2 Percentage of all PFM that have incorporated into multinucleated sarcomeric myosin positive myotubes.

3 Average width of 100 multinucleated myotubes from 10 representative photomicrographs.
PSC (Percentage of Pax7 treatments (P with LS- and BG-PSC (P + had a decreased percentage of Myf-51 age of Myf-5 not differ from each other (P 0.03), which did not differ from each other (P 0.01) proliferation rate than BG-PSC. The HS-PSC had a greater (P 0.01) proliferation rate than cells at 72 and 96 h (P 0.04). 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 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-Ser473, ratio of phosphorylated AKT-Ser473, and the abundance of total 4EBP following 180-min treatment period (P 0.02; Table 4). When cultured

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>P-value</th>
<th>Time × Trt</th>
<th>Time</th>
<th>Trt</th>
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<tr>
<td><strong>Proliferation rate (%)</strong></td>
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<tr>
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<td>58.5a</td>
<td>46.3b</td>
<td>20.7c</td>
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<td>96 h</td>
<td>56.6a</td>
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<td>9.7c</td>
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<tr>
<td><strong>Pax7 + (%)</strong></td>
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<td>24 h</td>
<td>2.4a</td>
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<tr>
<td>48 h</td>
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<td>1.4</td>
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<td>0.6</td>
<td>0.3</td>
<td>0.4</td>
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<tr>
<td>96 h</td>
<td>0.1</td>
<td>0.9</td>
<td>0.3</td>
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<tr>
<td><strong>Myf-5 + (%)</strong></td>
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<tr>
<td>24 h</td>
<td>10.3</td>
<td>15.0</td>
<td>19.8</td>
<td>3.2</td>
</tr>
<tr>
<td>48 h</td>
<td>9.5</td>
<td>26.9</td>
<td>29.7</td>
<td></td>
</tr>
<tr>
<td>72 h</td>
<td>14.9</td>
<td>25.5</td>
<td>37.8</td>
<td></td>
</tr>
<tr>
<td>96 h</td>
<td>15.3</td>
<td>24.7</td>
<td>28.0</td>
<td></td>
</tr>
<tr>
<td><strong>Pax7+/Myf-5 + (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>85.2</td>
<td>76.9</td>
<td>72.2</td>
<td>3.0</td>
</tr>
<tr>
<td>48 h</td>
<td>84.3</td>
<td>71.1</td>
<td>68.1</td>
<td></td>
</tr>
<tr>
<td>72 h</td>
<td>81.9</td>
<td>69.6</td>
<td>61.2</td>
<td></td>
</tr>
<tr>
<td>96 h</td>
<td>83.1</td>
<td>72.7</td>
<td>71.5</td>
<td></td>
</tr>
</tbody>
</table>

Trt = treatment effect.

1Porcine 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 penicillin/ml, 100 µg of streptomycin/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.

2Cultures were exposed to 50µM bromodeoxyuridine (BrdU) and immunostained. Percentage calculated as the number of BrdU positive PFMs divided by the total number of PSC present within 10 representative photomicrographs.

3Represents the percentage of all PSC that solely immunostained Pax7 positive within 10 representative photomicrographs.

4Represents the percentage of all PSC that solely immunostained Myf-5 positive within 10 representative photomicrographs.

5Represents the percentage of all PSC that co-expressed Pax7 and Myf-5 within 10 representative photomicrographs.
in the presence of HS the BGP-PSC had decreased abundance and ratio of phosphorylated AKT-Ser473 compared with BGN-PSC (P < 0.01); however in the presence of LS, BGP-PSC had a greater abundance and ratio of phosphorylated AKT-Ser473 compared with BGN-PSC (P = 0.02). 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-Ser473 (P < 0.02), but did not affect any of the other PI3-K pathway signaling proteins assessed (P > 0.09). The HS-PSC had a greater abundance and ratio of phosphorylated AKT-Ser473 compared with LS-PSC (P < 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 > 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). Liet 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

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BGN</th>
<th>BGP</th>
<th>SEM</th>
<th>SER × BET</th>
<th>SER</th>
<th>BET</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PI3-K signaling</strong>&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>AKT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (AU)</td>
<td>1.00</td>
<td>0.86</td>
<td>0.97</td>
<td>1.36</td>
<td>0.16</td>
<td>0.14</td>
</tr>
<tr>
<td>Ser&lt;sup&gt;473&lt;/sup&gt; phosphorylated (AU)</td>
<td>0.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.75&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.11</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ser&lt;sup&gt;473&lt;/sup&gt; phosphorylated ratio</td>
<td>43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>139&lt;sup&gt;b&lt;/sup&gt;</td>
<td>213&lt;sup&gt;c&lt;/sup&gt;</td>
<td>89&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Thr&lt;sup&gt;389&lt;/sup&gt; phosphorylated (AU)</td>
<td>1.70</td>
<td>2.23</td>
<td>2.12</td>
<td>2.29</td>
<td>0.64</td>
<td>0.78</td>
</tr>
<tr>
<td>Thr&lt;sup&gt;389&lt;/sup&gt; phosphorylated ratio</td>
<td>182</td>
<td>264</td>
<td>253</td>
<td>169</td>
<td>51.1</td>
<td>0.14</td>
</tr>
<tr>
<td><strong>mTOR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (AU)</td>
<td>1.25</td>
<td>1.22</td>
<td>1.30</td>
<td>1.38</td>
<td>0.14</td>
<td>0.67</td>
</tr>
<tr>
<td>Ser&lt;sup&gt;2448&lt;/sup&gt; phosphorylated (AU)</td>
<td>1.11</td>
<td>1.54</td>
<td>1.42</td>
<td>1.60</td>
<td>0.23</td>
<td>0.61</td>
</tr>
<tr>
<td>Ser&lt;sup&gt;2448&lt;/sup&gt; phosphorylated ratio</td>
<td>98</td>
<td>131</td>
<td>114</td>
<td>127</td>
<td>17.5</td>
<td>0.58</td>
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<tr>
<td><strong>S6 Kinase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (AU)</td>
<td>0.52</td>
<td>0.71</td>
<td>0.75</td>
<td>1.04</td>
<td>0.17</td>
<td>0.77</td>
</tr>
<tr>
<td>Thr&lt;sup&gt;37/46&lt;/sup&gt; phosphorylated (AU)</td>
<td>0.57</td>
<td>0.88</td>
<td>0.73</td>
<td>1.24</td>
<td>0.13</td>
<td>0.47</td>
</tr>
<tr>
<td>Thr&lt;sup&gt;37/46&lt;/sup&gt; phosphorylated ratio</td>
<td>145</td>
<td>143</td>
<td>153</td>
<td>151</td>
<td>37.5</td>
<td>0.99</td>
</tr>
<tr>
<td><strong>4EBP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (AU)</td>
<td>1.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.40&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.13</td>
<td>0.02</td>
</tr>
<tr>
<td>Thr&lt;sup&gt;37/46&lt;/sup&gt; phosphorylated (AU)</td>
<td>1.20</td>
<td>1.45</td>
<td>1.19</td>
<td>1.34</td>
<td>0.22</td>
<td>0.80</td>
</tr>
<tr>
<td>Thr&lt;sup&gt;37/46&lt;/sup&gt; phosphorylated ratio</td>
<td>85</td>
<td>134</td>
<td>124</td>
<td>110</td>
<td>14.6</td>
<td>0.06</td>
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<tr>
<td><strong>FOXO</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (AU)</td>
<td>1.39</td>
<td>1.13</td>
<td>1.51</td>
<td>1.64</td>
<td>0.35</td>
<td>0.53</td>
</tr>
<tr>
<td>Ser&lt;sup&gt;319&lt;/sup&gt; phosphorylated (AU)</td>
<td>0.78</td>
<td>0.96</td>
<td>0.81</td>
<td>1.05</td>
<td>0.08</td>
<td>0.66</td>
</tr>
<tr>
<td>Ser&lt;sup&gt;319&lt;/sup&gt; phosphorylated ratio</td>
<td>79</td>
<td>104</td>
<td>84</td>
<td>93</td>
<td>13.2</td>
<td>0.46</td>
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<tr>
<td><strong>MAFbx (AU)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (AU)</td>
<td>1.29</td>
<td>2.01</td>
<td>1.90</td>
<td>1.46</td>
<td>0.47</td>
<td>0.25</td>
</tr>
<tr>
<td>MURF-1 (AU)</td>
<td>1.44</td>
<td>1.39</td>
<td>1.17</td>
<td>1.36</td>
<td>0.20</td>
<td>0.57</td>
</tr>
</tbody>
</table>

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.

<sup>a,b,c</sup>Means within a row with different superscripts differ.

<sup>1</sup>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 penicillin/ml, 100 µg of streptomycin/ml, and 20 µg of gentamicin/ml.

<sup>2</sup>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).
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. 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 has 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 myobute 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 myobute 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 myobute diameter when IGF-1 was supplemented to the C2C12 muscle cell line. Therefore, these results indicate betaGRO® stimulates satellite cell fusion and myobute 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 myobute 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-Ser473, which resulted in a 40% increase in the ratio of phosphorylated AKT-Ser473 compared with LS treatments. Interestingly, BGP-PSC had a 47% reduction of phosphorylated AKT-Ser473 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
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.

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References


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