

## RESEARCH

# Rates of myogenesis and myofiber numbers are reduced in late gestation IUGR fetal sheep

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## Abstract

Intrauterine growth-restricted (IUGR) fetuses are born with reduced skeletal muscle mass. We hypothesized that reduced rates of myogenesis would contribute to fewer and smaller myofibers in IUGR fetal hindlimb muscle compared to the normally growing fetus. We tested this hypothesis in IUGR fetal sheep with progressive placental insufficiency produced by exposing pregnant ewes to elevated ambient temperatures from 38 to 116 days gestation (dGA; term = 147 dGA). Surgically catheterized control (CON,  $n = 8$ ) and IUGR ( $n = 13$ ) fetal sheep were injected with intravenous 5-bromo-2'-deoxyuridine (BrdU) prior to muscle collection (134 dGA). Rates of myogenesis, defined as the combined processes of myoblast proliferation, differentiation, and fusion into myofibers, were determined in biceps femoris (BF), tibialis anterior (TA), and flexor digitorum superficialis (FDS) muscles. Total myofiber number was determined for the entire cross-section of the FDS muscle. In IUGR fetuses, the number of BrdU<sup>+</sup> myonuclei per myofiber cross-section was lower in BF, TA, and FDS ( $P < 0.05$ ), total myonuclear number per myofiber cross-section was lower in BF and FDS ( $P < 0.05$ ), and total myofiber number was lower in FDS ( $P < 0.005$ ) compared to CON. mRNA expression levels of cyclins, cyclin-dependent protein kinases, and myogenic regulatory factors were lower ( $P < 0.05$ ), and inhibitors of the cell cycle were higher ( $P < 0.05$ ) in IUGR BF compared to CON. Markers of apoptosis were not different in IUGR BF muscle. These results show that in IUGR fetuses, reduced rates of myogenesis produce fewer numbers of myonuclei, which may limit hypertrophic myofiber growth. Fewer myofibers of smaller size contribute to smaller muscle mass in the IUGR fetus.

## Key Words

- ▶ skeletal muscle
- ▶ myogenesis
- ▶ myoblast
- ▶ myonuclear domain
- ▶ fetal programming

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## Introduction

Two distinct phases of myogenesis *in utero* establish fetal skeletal muscle mass. After the scaffold of primary myofibers is established during the embryonic period, secondary myogenesis occurs from the proliferation and fusion of fetal myoblasts to increase myofiber number during mid-gestation (Lee *et al.* 2013).

Total myofiber number is established prior to birth, as has been demonstrated in mice (Rowe & Goldspink 1969), piglets (Wigmore & Stickland 1983), sheep (Fahey *et al.* 2005b), and humans (Widdowson *et al.* 1972). Myogenesis continues to support muscle hypertrophic growth by adding myonuclei to existing secondary myofibers

during late gestation and into early postnatal life (Moss & Leblond 1971, White *et al.* 2010, Gokulakrishnan *et al.* 2017). Slower rates of myogenesis and/or fewer myoblasts entering the cell cycle *in utero* can have lasting effects on muscle mass throughout the lifespan by both reducing the number and size of myofibers.

Several factors regulate fetal myogenesis, including insulin, IGFs, nutrients, and oxygen availability (Brown 2014). Conditions in human pregnancy that reduce nutrient and oxygen delivery to the fetus, such as placental insufficiency, result in an intrauterine growth-restricted (IUGR) fetus with less muscle mass than in normally growing fetuses (Padoan *et al.* 2004). A particularly relevant sheep model of placental insufficiency and IUGR produced by exposing pregnant ewes to elevated ambient temperatures mimics the human IUGR condition with 'fetal brain sparing' at the expense of the growth of skeletal muscle and splanchnic organs (Bell *et al.* 1987, Galan *et al.* 1999). In this model, placental insufficiency begins early in gestation and is progressive (Arroyo *et al.* 2008), such that nutrient and oxygen restriction to the fetus occurs concurrently with the period of secondary myogenesis (Du *et al.* 2010, Lee *et al.* 2013, Brown 2014). By late gestation, fetal muscle weights relative to fetal body weight, *in vivo* muscle protein fractional synthetic rates, muscle protein accretion rates, and myofiber cross-sectional areas are lower compared to normally growing fetal lambs, indicating impaired hypertrophic growth of the myofiber (Yates *et al.* 2016, Rozance *et al.* 2018). In addition, fetal myoblasts within muscle cross-sections collected from IUGR muscle at late gestation express less proliferating cell nuclear antigen (PCNA), Ki-67, and myogenin, indicating that fewer myoblasts are undergoing proliferation and differentiation (Yates *et al.* 2014, Soto *et al.* 2017). Whether reduced rates of fetal myogenesis, as defined by the process of myoblast proliferation, differentiation, and fusion into myofibers *in vivo*, contribute to reduced myofiber number, myonuclear number, and slower hypertrophic myofiber growth in the IUGR fetus has not been determined.

In this study, we tested the hypothesis that rates of myogenesis are reduced in the IUGR fetus. This was accomplished using 5-bromo-2'-deoxyuridine (BrdU), which is a thymidine analog that is selectively incorporated into cellular DNA during the synthesis (S) phase of the cell cycle. BrdU was intravenously infused into late gestation control and IUGR fetal sheep to trace the number of myoblasts that had proliferated, differentiated, and fused into the myofiber to become myonuclei during the labeling period. To further define the cellular processes in skeletal

muscle that might be impacted by placental insufficiency, we measured mRNA expression of myogenesis regulators in fetal muscle biopsies. We determined the expression of the primary regulators of cell cycle progression, including cyclins, cyclin-dependent protein kinases (CDKs), and cell cycle inhibitors. To determine whether IUGR increased rates of cell death, we measured the mRNA expression of genes that regulate apoptosis and the protein expression of cleaved caspase 3. We also measured the mRNA expression of muscle regulatory factors and other myokines known to regulate myoblast differentiation, including myostatin and irisin. Finally, we measured myonuclear number per myofiber and the total number of myofibers at the level of the mid-belly of the flexor digitorum superficialis (FDS) hindlimb muscle to determine whether lower rates of myogenesis earlier in gestation produced overall fewer numbers of myofibers with fewer myonuclei and thus the decreased muscle mass that is characteristic of the IUGR fetus.

## Materials and methods

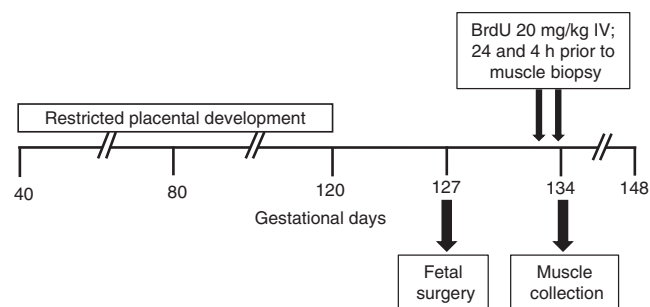
### Animal care, surgical procedure, and study design

Pregnant Columbia-Rambouillet mixed-breed sheep were studied at the University of Colorado Perinatal Research Center using protocols approved by the Institutional Animal Care and Use Committee (#77617(10)1E). The Perinatal Research Center is accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC) International. Pregnant sheep were randomly assigned to either an environmental chamber that exposed sheep to temperatures that cycled between 40°C and 35°C every 12 h with 35–40% humidity from 38 to 116 days gestation (dGA, term=147 dGA) to produce placental insufficiency and intrauterine growth restriction (IUGR group;  $n=13$ ) (Bell *et al.* 1987, Rozance *et al.* 2018) or to an environmental chamber at 21°C for 24 h with 35–40% humidity from 43 to 120 dGA (CON group;  $n=8$ ). In the environmental chambers, sheep were kept in individual pens alongside other sheep. After environmental treatment, all sheep were housed in normal ambient temperatures and humidity for the remainder of the studies. All sheep were given *ad libitum* access to water. Maternal feed intake was similar between sheep in CON and IUGR groups (Rozance *et al.* 2018). Fetuses in the study were all singletons except for one triplet fetus in the IUGR group. The triplet fetus (fetal weight: 1466 g) was included in the analysis because it was not an outlier

for any physiological or anthropometric parameters measured within the IUGR group.

A schematic of the study design is shown in Fig. 1. Late gestation pregnant sheep underwent a surgical procedure for fetal and maternal vascular catheter placement according to methods previously published (Rozance *et al.* 2018). Briefly, sheep were fasted for 24 h and thirsted for 12 h prior to surgery. A superficial maternal vein was used to administer diazepam (0.2 mg/kg) and ketamine (20 mg/kg) and sheep were maintained on isoflurane inhalation anesthesia (2–4%) for the surgical procedure. The fetal lamb was exposed by maternal laparotomy and hysterotomy. Catheters were placed in the external iliac artery, the distal inferior vena cava, and the external iliac vein. A 3-mm transit time ultrasonic blood flow transducer was positioned around the external iliac artery. The catheters and flow probe were tunneled subcutaneously to the maternal flank. Sheep recovered for a minimum of 6 days after surgery. A metabolic study performed on the day of muscle collection included hindlimb blood flow, substrate uptake rates by the hindlimb, arterial plasma hormone concentrations, and protein metabolic rates, and those results were previously published (Rozance *et al.* 2018). Fetal plasma arterial insulin, IGF-1, and cortisol concentrations were measured by an ELISA as described previously (Soto *et al.* 2017). Plasma norepinephrine concentrations were measured using HPLC as described previously (Brown *et al.* 2012).

Twenty-four hours prior to muscle collection, a dose of BrdU (Sigma-Aldrich) diluted in 0.9% NaCl was administered by IV bolus directly into the fetus at a dose of 20 mg/kg estimated fetal weight. The dose was repeated



**Figure 1**

Schematic design. Pregnant sheep were housed in an environmental chamber with elevated temperatures (40°C for 12 h; 35°C for 12 h; 35–40% humidity) for 70 days of gestation to produce placental insufficiency and IUGR. Control pregnant sheep were housed in an environmental chamber (21°C for 24 h; 35–40% humidity) for the same number of days. Six days after fetal vascular catheters were surgically placed, BrdU was administered directly to the fetus by intravenous (IV) bolus injection 24 h and 4 h prior to muscle biopsy.

4 h prior to muscle collection to ensure that the presence of BrdU was maintained throughout the entire 24-h dosing period. The dose and timing of BrdU bolus administration was determined based on previous publications (Shah *et al.* 1997, Greenwood *et al.* 1999b, Herdrich *et al.* 2010, Yu *et al.* 2015, Gokulakrishnan *et al.* 2017).

### Fetal skeletal muscle collection

After conclusion of the metabolic study, ewes received diazepam (0.2 mg/kg) and ketamine (20 mg/kg) intravenously and fetuses were delivered via maternal laparotomy and hysterotomy. The biceps femoris muscle was exposed and a biopsy was obtained from the anesthetized fetus and immediately frozen in liquid nitrogen for RNA and protein analysis. Intravenous pentobarbital sodium (Fatal Plus; Bortech Pharmaceuticals, Dearborn, MI, USA) was administered to both the mother and the fetus, after which the fetus was weighed. The biceps femoris (BF), FDS, and tibialis anterior (TA) in the fetal hindlimb were weighed. Portions of the BF and TA mid-bellies and the entire cross-section of the FDS mid-belly were placed on corkboard thinly coated with optimal cutting temperature media, frozen in liquid nitrogen-cooled isopentane for 60 s, and stored at –80°C.

### Immunohistochemistry

#### BrdU immunostaining

Cryopreserved BF, TA and FDS muscles were sectioned (10 µm) as previously described (Brown *et al.* 2016). All antibodies used in this study were diluted in Permeabilization/Blocking Solution (P/BS; phosphate buffered saline, 0.01% v/v Triton X-100, 0.12% w/v BSA, 0.12% w/v non-fat dry milk) with 5% normal goat serum. Sections were incubated with anti-BrdU rat monoclonal IgG2a (1:400; Abcam), anti-dystrophin mouse monoclonal IgG2b (1:250; MANDRA1, Developmental Studies Hybridoma Bank, Iowa City, IA, USA), and counterstained with DAPI nuclear stain (1:1000; Sigma-Aldrich). Primary antibodies were detected with goat anti-rat polyclonal IgG conjugated to Alexa Fluor 568 (1:500; Thermo Fisher Scientific) and donkey anti-mouse polyclonal IgG (H+L) conjugated to Cy2 (1:250; Jackson ImmunoResearch Laboratories, Inc.). Images were quantified using the Count and Measure module within the CellSens Dimension Imaging Software (Olympus America, Inc.). For each muscle type, two sections were analyzed with a minimum of 1000 fibers per section. BrdU<sup>+</sup> nuclei were expressed as a ratio to total nuclei to determine the BrdU labeling index

among all cell types within muscle. DAPI-stained nuclei within the dystrophin-stained sarcolemma were classified as myonuclei. BrdU<sup>+</sup> nuclei within the sarcolemma represented myoblasts that underwent proliferation, differentiation, and fusion to become myonuclei (Fig. 2A). Therefore, the number of BrdU<sup>+</sup> myonuclei normalized to either total myonuclei or myofiber number within a section represented the rate of myogenesis, or myoblast proliferation, differentiation, and fusion, during the BrdU labeling period. The total number of myonuclei per fiber cross-section was calculated to determine myonuclear number.

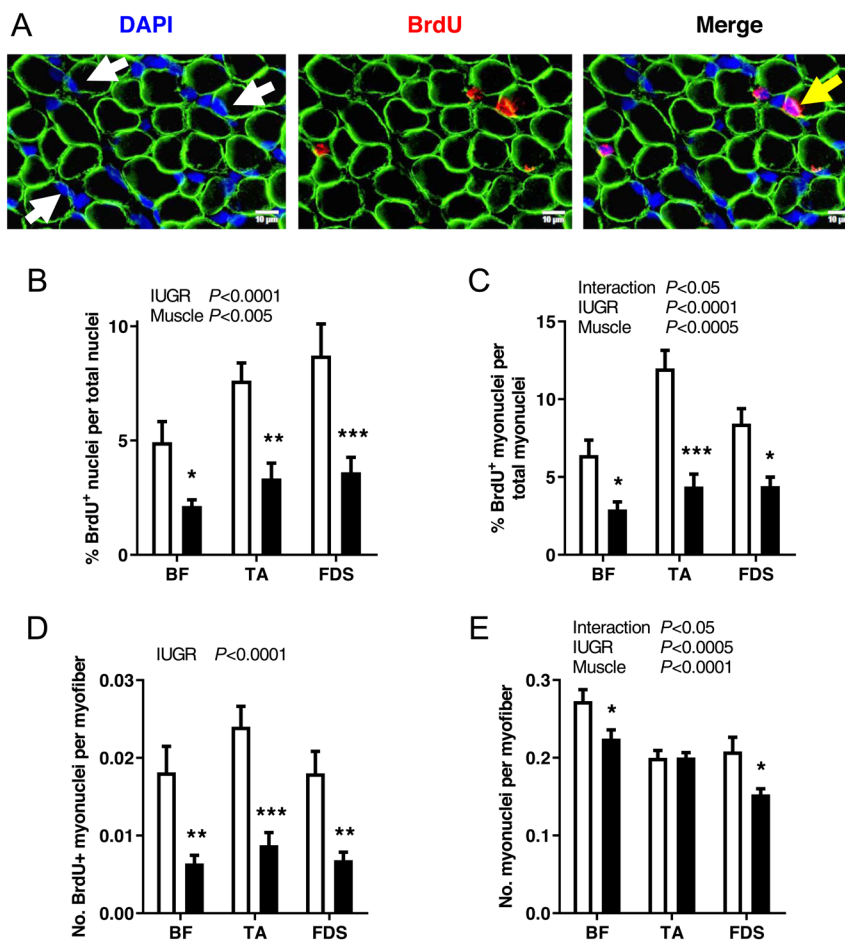
### Total myofiber number and myonuclear domain quantification

The FDS muscle was used to determine total myofiber number because it is encased in fascia, thus allowing for clear delineation of the cross-sectional borders of the muscle mid-belly. The FDS muscle at mid-belly was incubated with anti-laminin rabbit polyclonal IgG (1:100, Sigma-Aldrich) and anti-dystrophin mouse monoclonal IgG2b (1:250). Primary antibodies were detected with goat

anti-rabbit conjugated to Alexa Fluor® 488 (1:250; Thermo Fisher Scientific) and donkey anti-mouse polyclonal IgG conjugated to Cy2 (1:250). The entire section was imaged (Fig. 3A) and particle analyzer function (ImageJ, US National Institutes of Health) was used to quantify the total myofiber number, total area of the section composed of myofibers, and average myofiber area. In ImageJ, the FDS images were converted into an 8-bit binary image (i.e. 256 shades of gray), and a standard minimum (0 gray scale level) and maximum (4-9 gray scale levels) threshold was manually set to exclude non-fibers and was applied uniformly across all images. The myonuclear domain, or the cytoplasmic area per myonucleus, was calculated by dividing the average myofiber area by the number of myonuclei per myofiber cross-section in the FDS muscle.

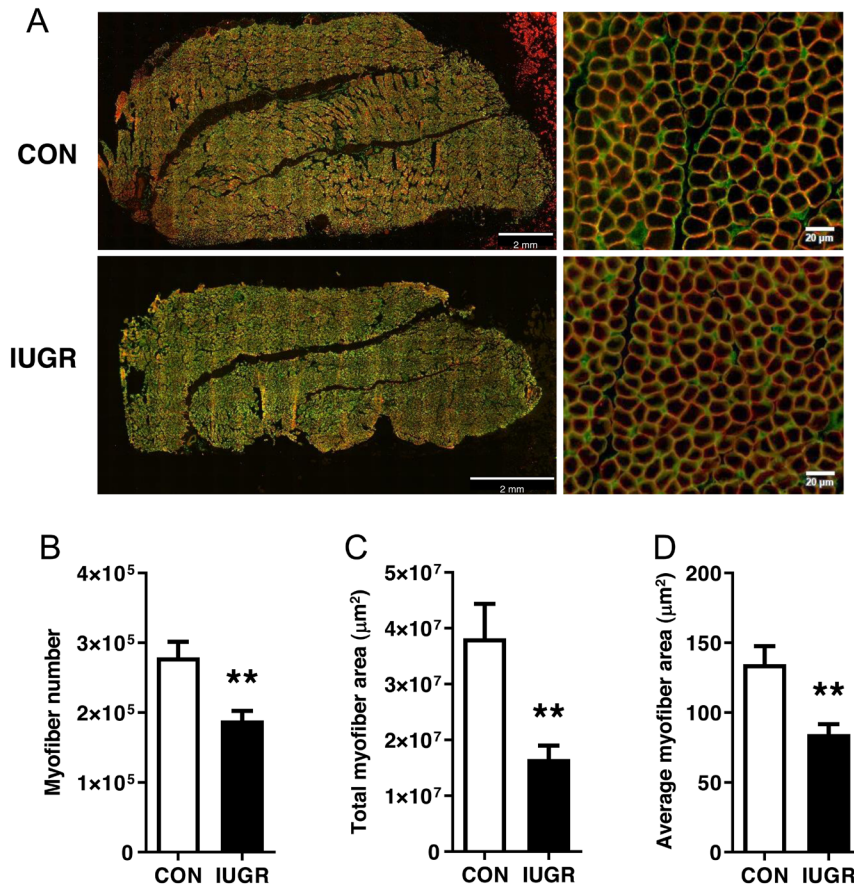
### RNA analysis

RNA was extracted from BF muscle (100 mg) using TRIzol LS (Invitrogen) and homogenization. To separate nucleic acids and proteins, the homogenate was mixed with chloroform and centrifuged at 12,000 *g* at 4°C for 15 min.



**Figure 2**

BrdU immunostaining. (A) Representative sections of biceps femoris (BF) muscle showing myofiber plasma membrane (anti-dystrophin, green), nuclei (DAPI, blue), and BrdU<sup>+</sup> nuclei (anti-BrdU, red). Myonuclei within the confines of the myofiber and BrdU<sup>+</sup> myonuclei are identified by white and yellow arrows, respectively. Results are shown for (B) BrdU labeling index, (C) percent of BrdU<sup>+</sup> myonuclei per total myonuclei, (D) number of BrdU<sup>+</sup> myonuclei per myofiber cross-section, and (E) myonuclei per myofiber cross-section in BF, tibialis anterior (TA), and flexor digitorum superficialis (FDS) muscles from CON (white bar, *n* = 8) and IUGR (black bar, *n* = 12) fetuses. Values represent mean ± s.e.m. Two-way ANOVA results are shown; \**P* < 0.05, \*\**P* < 0.005, \*\*\**P* < 0.0005 by Bonferroni *post-hoc* test comparisons.

**Figure 3**

Myofiber number. (A) Representative sections of flexor digitorum superficialis (FDS) muscle showing the basal lamina (anti-laminin, green) and myofiber plasma membrane (anti-dystrophin, red). (B) Total number of myofibers, (C) total area occupied by myofibers in the muscle cross-section, and (D) average size of individual myofibers in FDS muscle from CON (white bar,  $n = 8$ ) and IUGR (black bar,  $n = 12$ ) fetuses. Values represent mean  $\pm$  s.e.m. \*\* $P < 0.005$  by Student's *t*-test.

The aqueous phase was removed, and total RNA was isolated using RNeasy Mini Kit (Qiagen). Quantity of RNA was determined with a spectrophotometer (Nanodrop 1000). RNA (2  $\mu$ g) was reverse transcribed using Superscript III and Oligo dT 18–20 (Invitrogen) at 50°C for 1 h. Real time PCR (Lightcycler 480 II; Roche Life Science) on a 1:10 dilution was performed in triplicate using standard curves for relative quantification between groups as previously described (Benjamin *et al.* 2017). Primers were developed and validated for all real-time PCR assays and are either previously published (Soto *et al.* 2017) or shown in Table 1. mRNA expression was normalized to the average of three reference genes: *ACTIN*, *S15*, and *RPL37A*.

### Protein analysis

Biopsies of BF collected from the anesthetized fetus were powdered using mortar and pestle and incubated for 1 h at 4°C in lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA, 2.5 mM Na pyrophosphate, 20 mM NaF) with 0.004% v/v phosphatase inhibitor II/III and 0.01% v/v protease inhibitor (Sigma-Aldrich). Lysate was homogenized using a probe sonicator (VC50 Vibra-Cell;

Sonics and Materials, Danbury, CT, USA) and then centrifuged at 17,000  $g$  at 4°C for 30 min and supernatant was stored at –80°C. Protein quantification, gel electrophoresis, and transfer to nitrocellulose membranes was performed in duplicate as previously described (Brown *et al.* 2009). Membranes were incubated with primary rabbit polyclonal IgG antibodies p130 and caspase 3 (1:1000 in Tris-buffered saline (TBS) with 0.1% v/v Tween 20 and 5% w/v BSA; Cell Signaling Technology). Actin mouse monoclonal IgG (1:10,000; MP Biomedicals, Santa Ana, CA, USA) was used as a loading control.

For nuclear enrichment preparations from the BF, Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Reagents were used per manufacture protocol (Product # 78835). Protein (15  $\mu$ g) was separated by gel electrophoresis and transferred to membranes and blocked as previously described (Brown *et al.* 2016). Membranes were incubated with primary rabbit monoclonal IgG antibody p21 (1:1000 in TBS with 0.1% v/v Tween 20 and 5% w/v BSA; Cell Signaling Technology). Lamin C (1:1000; Millipore) was used to verify nuclear extraction and to control for loading differences.

**Table 1** Primers used for real-time qPCR assays.

Symbol	Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Accession number	Amplicon length (bp)	Source
RBL2	RB Transcriptional Corepressor Like 2	AGAGATCCAACCCAGGCTA	TTGTTCTGCTCAATAACAGAT	NM_001098073.1	176	<i>Bos taurus</i>
CCND1	Cyclin D1	ACTACTCTGACCCGCTTCT	TTGGAGAGGAGTGTCTCGAT	NM_053056.2	253	<i>Homo sapiens</i>
CCNA2	Cyclin A2	CCTGAACTGCAAGTTGAA	GGTGAAGTCCAGGAGACA	NM_001237.2	216	<i>Homo sapiens</i>
CDK2	Cyclin Dependent Kinase 2	GGTGTACCAGTACTGCCA	CGCAGAGCATCCATGAATT	BT020790.1	158	<i>Bos taurus</i>
CDK4	Cyclin Dependent Kinase 4	ATTTCTTCATGCAACTGCCA	CCAACACTCCACATGTCCAC	NM_000075.2	216	<i>Homo sapiens</i>
CDK6	Cyclin Dependent Kinase 6	GCATGTGATTAACACACA	GAGTCCAATCACGTCCAAGA	NM_001259.5	285	<i>Homo sapiens</i>
BCL2	Apoptosis Regulator Bcl-2	GGAGCTGATGGCCCTAGCA	TGAGCAGTGCCCTCAGAGACA	NM_001166486	68	<i>Bos taurus</i>
BAD	BCL2 Associated Agonist Cell Death	CGATATGGCCGGCAACTC	GCTCTCGGGGAGGAA	NM_001035459	75	<i>Bos taurus</i>
APAF1	Apoptotic Peptidase Activating Factor 1	TTCACTCCAGTGGCCCTGTTG	CAAAAGCCCACTGCCAAAT	NM_001191507	101	<i>Bos taurus</i>
CASP3	Caspase 3	CCATGTGAAGAAAGGAATCAITT	CCCTCTGAAGAAACTTGTCTAAT	AF068837	77	<i>Ovis aries</i>
CASP8	Caspase 8	TGGCTGCCCTCAAGTTCCT	GGAAATGATCAAGGCCATCCTT	NM_001045970	81	<i>Bos taurus</i>
CASP9	Caspase 9	GAGTTGCCTCGCTTTGG	CTCGATCATGTCGGAGTGA	NM_001205504	63	<i>Bos taurus</i>
CDKN1A	CDK Inhibitor 1A	GAGGACCACTTGGACCTGT	TCTGCGTTTGGAGTGTGAGA	NM_001098958.1	146	<i>Bos taurus</i>
CDKN1B	CDK Inhibitor 1B	GCTTGCCGAGTTCTACTAC	CATTTTCTTCTGTTCTGTTGG C	NM_004064.2	273	<i>Homo sapiens</i>
RB1	RB Transcriptional Corepressor 1	CATGCGCTTGAGGTTGTA	GGTTGCTTGTGCTCTGAAT	NM_000321.1	436	<i>Homo sapiens</i>
TP53	Tumor Protein 53	ACGACCGGAACACCTTTAGACACA	ACCACGAGAGTCTCCAGTGTGAT	FJ85523.1	166	<i>Ovis aries</i>
MSTN	Myostatin	ACGATCCAGAGAGATGACAGCA	ATCAGACTCCGTGGCATGTAAT	NM_001009428.1	97	<i>Ovis aries</i>
FST	Follistatin	TGCATCTCAAGGCCAGATGTAA	ATTAGTCTGGTCCACCACCATGT	NM_175801.2	126	<i>Bos taurus</i>
FND5	Irisin	CCAGTAACGTACCCGTCA	CCTGGATGAAGCCGAGCAT	BC149873.1	135	<i>Bos taurus</i>
RPL37A	Ribosomal Protein L37a	ACCAAGAAGTTCGGAACTCGT	GGCACCAACCACTACTGTTT	XM_027965159	192	<i>Ovis aries</i>

## Statistical analysis

The study was not designed *a priori* to measure differences between sexes; thus, determination of sex differences was limited to screening for a fixed effect of sex for each variable, and none were significant. A Student's *t*-test or Mann-Whitney test was used when CON and IUGR groups were compared directly (Prism 6, GraphPad Software). If the analysis was performed among different muscle types within the fetal hindlimb, a two-way ANOVA was used to determine the main effects of group (CON, IUGR) and muscle type (FDS, TA, BF), and their interaction. Bonferroni *post hoc* test was performed to determine the differences among groups. Correlations between circulating hormones and BrdU-labeled myonuclei and/or myofiber number were determined using Pearson's correlation. Correlations were determined for all fetuses pooled, as variability in both hormone concentrations and myogenesis rates also could be related to muscle size in CON as well as IUGR fetuses. Norepinephrine, insulin, and IGF-1 were log transformed for correlation analysis.  $P < 0.05$  was considered statistically significant.

## Results

### Fetal weights and physiological parameters

Physiological measurements of fetuses included in this study have been previously published (Rozance *et al.* 2018) but are shown in Table 2 for completeness. IUGR fetuses had lower body weights ( $P < 0.0005$ ) and lower hindlimb BF ( $P < 0.0001$ ), TA ( $P < 0.0005$ ), and FDS ( $P < 0.0005$ ) muscle weights compared to CON fetuses at 134 dGA. Arterial plasma glucose concentrations and blood oxygen content, pH,  $P_aO_2$ , and  $S_aO_2\%$  were lower in IUGR fetuses compared to CON ( $P < 0.05$ ). Circulating plasma insulin ( $P < 0.005$ ) and IGF-1 ( $P < 0.005$ ) concentrations were lower and norepinephrine concentrations were higher ( $P < 0.005$ ) in IUGR fetuses compared to CON (Table 2).

### Myogenesis *in vivo*

The BrdU dose was administered based on an estimated fetal weight at the time of surgery. Because fetal weight in the IUGR group was over-estimated, IUGR fetuses received a larger dose per kg than CON based on actual weight at the time of animal necropsy (IUGR:  $23.3 \pm 1.5$  vs CON:  $18.3 \pm 0.7$  mg/kg/dose;  $P < 0.05$ ). Despite receiving a higher dose of BrdU per kg, the BrdU labeling index, or the ratio of BrdU<sup>+</sup> nuclei per total nuclei, was lower in BF ( $P < 0.05$ ), TA ( $P < 0.005$ ), and FDS ( $P < 0.0005$ )

**Table 2** Fetal weights and physiological measurements.

	Control	IUGR	P value
Gestational age (days)	134.4 ± 0.4	134.1 ± 0.3	0.5
Male fetus (%)	50	50	
Fetal weight (kg)	3.3 ± 0.1	2.0 ± 0.2	<0.0005
Biceps femoris (BF; g)	18.1 ± 0.7	9.9 ± 1.0	<0.0001
Tibialis anterior (TA; g)	4.0 ± 0.3	2.1 ± 0.3	<0.0005
Flexor digitorum superficialis (FDS; g)	3.1 ± 0.3	1.6 ± 0.2	<0.0005
Fetal blood gas measurements			
Blood O <sub>2</sub> content (mM)	3.3 ± 0.2	1.9 ± 0.3	<0.01
Blood pH	7.4 ± 0.0	7.3 ± 0.0	<0.05
Blood P <sub>a</sub> CO <sub>2</sub> (mmHg)	50.4 ± 0.7	51.5 ± 0.7	0.3
Blood P <sub>a</sub> O <sub>2</sub> (mmHg)	20.6 ± 0.7	14.6 ± 0.9	<0.0005
Blood Hgb (mM)	6.9 ± 0.2	6.9 ± 0.3	0.9
S <sub>a</sub> O <sub>2</sub> (%)	48.3 ± 1.8	26.6 ± 3.2	<0.0005
Fetal arterial plasma substrate and hormone concentrations			
Glucose (mM)	1.0 ± 0.3	0.7 ± 0.1	<0.005
Lactate (mM)	2.0 ± 0.1	2.6 ± 0.2	0.06
Insulin (ng/mL)	0.40 ± 0.09	0.14 ± 0.02	<0.005
IGF-1 (ng/mL)	108.2 ± 15.2	43.6 ± 9.5	<0.005
Cortisol (ng/mL)	20.2 ± 5.3	25.3 ± 5.3	0.5
Norepinephrine (pg/mL) <sup>a</sup>	617 ± 253	4313 ± 1960	<0.005

Values are means ± s.e.m. Control *n* = 8; IUGR *n* = 13. *P* values from Student's *t*-tests or Mann–Whitney test are shown.

<sup>a</sup>Statistical analysis on log-transformed values. Data from this cohort of animals were previously published (Rozance *et al.* 2018).

IUGR muscle compared to CON (Fig. 2B). To assess the rate of myogenesis, we counted the number of nuclei that were BrdU<sup>+</sup> and resided within the sarcolemma. This measurement represented those myoblasts that had proliferated, differentiated, and fused to become differentiated myonuclei during the BrdU-labeling period. The percent of BrdU<sup>+</sup> myonuclei per total nuclei was lower in BF (*P* < 0.05), TA (*P* < 0.0005), and FDS (*P* < 0.05) IUGR muscle compared to CON (Fig. 2C). Similarly, the number of BrdU<sup>+</sup> myonuclei per myofiber cross-section was lower in BF (*P* < 0.005), TA (*P* < 0.0005), and FDS (*P* < 0.005) IUGR muscle compared to CON (Fig. 2D).

Myonuclear number, or the total number of myonuclei per myofiber cross-section, was lower in BF (*P* < 0.05) and FDS (*P* < 0.05) IUGR muscles, respectively, compared to CON, but not the TA (Fig. 2E). The ratio of myonuclei to total nuclei was similar between IUGR and CON groups for all muscles (BF: 0.29 ± 0.02 IUGR, 0.29 ± 0.02 CON; TA: 0.28 ± 0.02 IUGR, 0.26 ± 0.02 CON; FDS: 0.24 ± 0.01 IUGR, 0.28 ± 0.02 CON), indicating that the numbers of myonuclei were reduced to a similar degree as other nuclei within IUGR muscle.

### Myofiber number and size

Total myofiber number across the entire FDS mid-belly was 32% lower in IUGR compared to CON (*P* < 0.005) (Fig. 3B). Total area of the section that was composed of

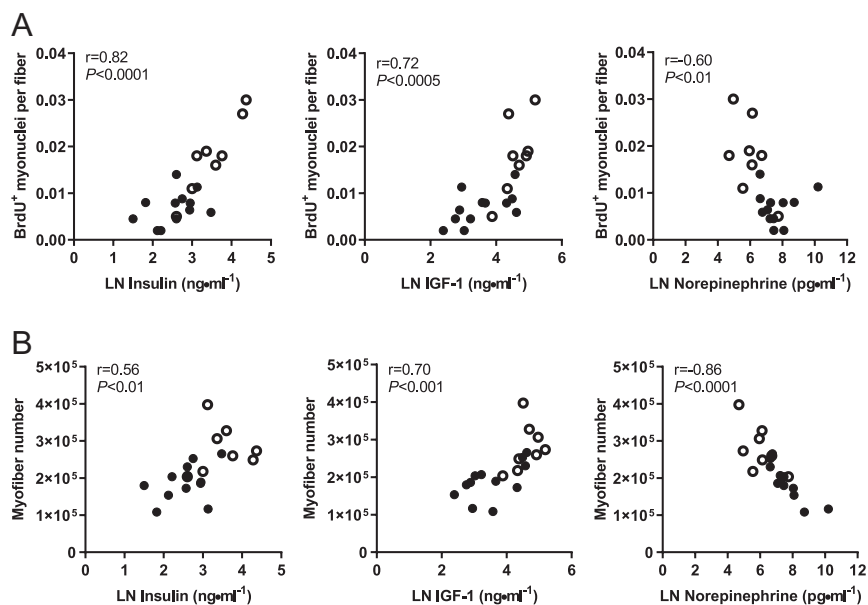
myofibers and average myofiber cross-sectional area were lower by 57 and 37%, respectively, in IUGR compared to CON (*P* < 0.005) (Fig. 3C and D). The myonuclear domain, or the cytoplasmic area per myonucleus, was similar between groups (IUGR: 572 ± 58 vs CON: 667 ± 66 μm<sup>2</sup>).

### Relationships between circulating hormone concentrations and myogenesis rates

Fetal plasma circulating growth factors insulin and IGF-1 correlated positively and norepinephrine correlated negatively with both the number of BrdU<sup>+</sup> myonuclei per myofiber and total myofiber number in the FDS muscle (*P* < 0.01; Fig. 4A and B). Fetal plasma cortisol concentrations correlated negatively with BrdU<sup>+</sup> myonuclei (*r* = −0.57, *P* < 0.01), but were not associated with myofiber number. Similar relationships were observed in the BF and TA muscles between the number of BrdU<sup>+</sup> myonuclei and insulin concentrations (BF: *r* = 0.62, *P* < 0.005; TA: *r* = 0.64, *P* < 0.005), IGF-1 concentrations (BF: *r* = 0.65, *P* < 0.005; TA: *r* = 0.67, *P* < 0.005), and norepinephrine concentrations (BF: *r* = −0.64, *P* < 0.005; TA: *r* = −0.73, *P* < 0.0005).

### Expression of mRNA and proteins related to cell cycle progression, apoptosis, and myogenesis

The relative expression levels of mRNA and proteins that regulate cell cycle activity and myoblast differentiation

**Figure 4**

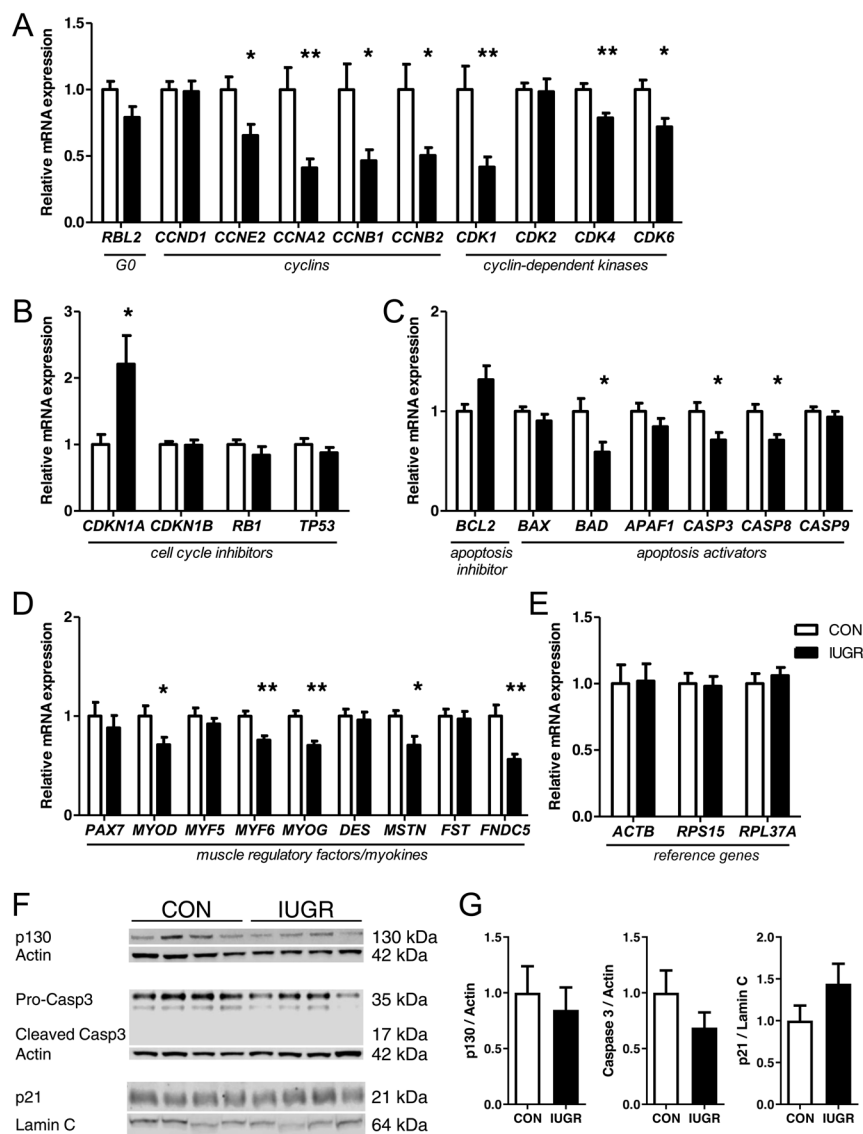
Correlations between circulating plasma hormones and indices of myogenesis in the flexor digitorum superficialis muscle. Fetal plasma insulin, IGF-1, and norepinephrine are correlated with (A) BrdU<sup>+</sup> myonuclei and (B) myofiber number. IUGR fetuses (closed circles,  $n = 12$ ) and CON fetuses (open circles,  $n = 8$ ) with correlation coefficients and  $P$  values are shown. The natural log for hormone concentrations was used for analysis.

in IUGR and CON muscle biopsies are shown in Fig. 5. The mRNA and protein expression of *RBL2* (p130), which is a marker of cells in G0 phase of the cell cycle, was similar between IUGR and CON groups (Fig. 5A and F). mRNA expression levels of the cyclins *CCNA2*, *CCNB1*, *CCNB2*, and *CCNDE2*, and the cyclin-dependent protein kinases *CDK1*, *CDK4*, and *CDK6*, were lower in IUGR compared to CON ( $P < 0.05$ ), but expression levels of *CCND1* and *CDK2* were similar between groups (Fig. 5A). Of the cell cycle inhibitors evaluated, *CDKN1A* (encoding p21) expression was two-fold higher in IUGR compared to CON ( $P < 0.05$ ; Fig. 5B). However, total nuclear protein level of p21 was similar between groups (Fig. 5F). mRNA expression levels of genes that function to activate apoptosis were either similar (*BCL2*, *BAX*, *APAF*, *CASP9*) or lower in IUGR compared to CON (*BAD*, *CASP3*, *CASP8*;  $P < 0.05$ ) (Fig. 5C). Pro-caspase 3 protein expression was similar between groups and the cleaved caspase 3 product was not detectable for either group (Fig. 5F). mRNA expression levels of the muscle regulatory factors *MYOD*, *MYF6*, and *MYOG* were lower in IUGR compared to CON ( $P < 0.05$ ), though expression levels of *PAX7* and *MYF5* were similar between groups (Fig. 5D). Expression of *MSTN*, a negative regulator of skeletal muscle growth, was lower in IUGR versus CON ( $P < 0.05$ ) and expression of *FST*, an inhibitor of *MSTN* and thus an activator of muscle growth, was similar between groups (Fig. 5D). Expression of *FNDC5* (encoding the pro-myogenic factor irisin) was reduced by 44% in IUGR compared to CON muscle ( $P < 0.005$ ; Fig. 5D).

## Discussion

This study demonstrates that in IUGR fetuses, reduced rates of myogenesis *in vivo* produced fewer numbers of myonuclei and myofibers, which would contribute to smaller muscle mass. By identifying BrdU<sup>+</sup> myonuclei within the sarcolemma of the myofiber, we demonstrated that fewer myoblasts had proliferated, differentiated, and fused to form myonuclei during the BrdU labeling period in IUGR muscle compared to normally-growing controls. Expression levels of the muscle regulatory transcription factors *MYOD*, *MYF6* and *MYOG* were lower in IUGR muscle, as was the expression of *FNDC5* (encoding the myokine irisin), further indicating down-regulation of the myogenic program in IUGR skeletal muscle. The proliferation, differentiation, and fusion of satellite cells is an essential regulator of muscle fiber growth by hypertrophy (White *et al.* 2010, Blaauw & Reggiani 2014, Egner *et al.* 2016, Snijders *et al.* 2016, Dungan *et al.* 2019); thus, reduced myonuclear accumulation likely contributed to lower muscle weights and myofiber area. Total myofiber number also was lower in the IUGR fetus, indicating that reductions in myogenesis occur as early as mid-gestation when the bulk of secondary myofibers are formed. The mRNA expression levels of nearly all cyclins and CDKs that regulate cell cycle transition points were markedly reduced and markers of apoptosis were not increased in IUGR muscle biopsies, indicating that lower cell cycle activity as opposed to increased rates of apoptosis may have contributed to lower muscle mass. BrdU<sup>+</sup> myonuclei and myofiber number in CON and IUGR fetal muscle were



**Figure 5**

Fetal skeletal muscle mRNA and protein expression. mRNA expression of (A) cell cycle regulators, (B) cell cycle inhibitors, (C) apoptosis regulators, (D) muscle regulatory factors, (E) reference genes; and (F) representative Western blots and densitometry analysis of protein expression p130, caspase 3, and nuclear p21 from CON (white bar,  $n = 8$ ) and IUGR (black bar,  $n = 13$ ) fetal biceps femoris muscle. Values represent mean  $\pm$  s.e.m. \* $P < 0.05$ , \*\* $P < 0.005$  by Student's *t*-test.

positively correlated with circulating fetal plasma insulin and IGF-1 concentrations, and negatively correlated with norepinephrine concentrations, suggesting a role for hormones in the regulation of myogenesis in the fetus.

BrdU is a thymidine analog that is selectively incorporated into cellular DNA during the synthesis (S) phase of the cell cycle. Despite IUGR fetuses receiving a higher dose of BrdU per kg than control fetuses, they had a lower BrdU labeling index, indicating that the dose of ~40 mg/kg (20 mg/kg/dose) used in these studies was a flooding dose, given in excess of DNA synthesis rates. An alternative possibility is that the higher relative dose of BrdU given to IUGR fetuses inhibited cellular proliferation, as BrdU has been shown to inhibit myogenesis *in vitro* (Lawson-Smith & McGeachie 1998) and embryonic neurogenesis *in vivo* (Sekerkova *et al.* 2004). However,

it is unlikely that the slightly, albeit statistically, higher cumulative dose of BrdU given to IUGR versus control fetuses (~46 mg/kg versus ~36 mg/kg, respectively) inhibited cell proliferation because both doses were within reported BrdU dosing ranges for pregnant animals to evaluate myogenesis *in vivo* (Greenwood *et al.* 1999b, Gokulakrishnan *et al.* 2017) and much lower than those doses that exceeded 100 mg/kg previously shown to inhibit neurogenesis (Sekerkova *et al.* 2004, Taupin 2007, Hancock *et al.* 2009).

The extent to which specific deficits in myoblast proliferation versus the capacity of the myoblast to differentiate and/or fuse into the myofiber were not distinguished in this study. However, previous work indicates that reduced myoblast proliferation is a likely driver for reduced rates of myogenesis in IUGR.

We previously showed that in placental insufficiency induced by maternal heat exposure, IUGR fetal skeletal muscle had fewer Pax7<sup>+</sup> myoblasts that expressed Ki-67, a marker of cell proliferation (Soto *et al.* 2017). In the same IUGR model, fewer Pax7<sup>+</sup> myoblasts expressed both PCNA and myogenin, indicating fewer differentiated myonuclei as a result of reduced myoblast proliferation (Yates *et al.* 2014). Further evidence for the suppression of fetal myoblast proliferation as a result of placental insufficiency was shown in sheep bred to produce litters of multiple lambs of variable birthweight. Muscles collected from low-birth-weight lambs in these studies demonstrated less muscle DNA and fewer nuclei entering S phase compared to larger lambs (Greenwood *et al.* 1999a, 2000). In a rat model of fetal growth restriction induced by exposure of the fetus to maternal glucocorticoids, lower numbers of BrdU<sup>+</sup> myonuclei per myofiber and Pax7<sup>+</sup> myoblasts were demonstrated (Gokulakrishnan *et al.* 2017). Lower expression of muscle regulatory factors *MYOD*, *MYF6* and *MYOG* in IUGR muscle in the present study, which are transcription factors expressed in a sequential manner during the differentiation process (Braun & Gautel 2011), could be the direct result of reduced myonuclear accumulation from lower myoblast proliferation rates, or could be the result of intrinsic defects in the capacity for differentiation. Muscle-specific fusion proteins also have been identified that regulate the fusion process independent of differentiation (Sampath *et al.* 2018), which are yet to be explored in this model. Taken together, these results demonstrate that rates of myogenesis are exquisitely sensitive to conditions that produce IUGR; whether there are intrinsic deficits in differentiation and/or fusion capacity of the myocyte require further study.

Interestingly, we found reduced mRNA expression of *FNDC5* that encodes for irisin in IUGR fetal muscle. Irisin is a myokine that was first demonstrated to induce the expression of genes that result in non-shivering thermogenesis and enhance the browning of white adipose tissue (Bostrom *et al.* 2012). In addition, irisin has been shown to induce myogenic differentiation and stimulate muscle growth by activating myoblast proliferation, fusion and protein synthesis in both murine and human myotubes (Reza *et al.* 2017). Cord blood irisin concentrations have been shown to positively correlate with birth weight (Joung *et al.* 2015) and are lower in IUGR pregnancies (Caglar *et al.* 2014, Baka *et al.* 2015). Whether reductions in circulating irisin concentrations are a result of reduced muscle mass or reduced expression of irisin in the IUGR fetus, or how irisin might be involved in the

pathogenesis of impaired muscle development in IUGR is yet to be determined.

The addition of myonuclei, either during early development or later in life through the activation of satellite cells, is a key component of the hypertrophic process (Blaauw & Reggiani 2014). In the present study, myonuclear number, or the number of myonuclei per myofiber cross-section, was reduced in IUGR. Myonuclear number reflects the cumulative history of myonuclear accumulation during muscle development (Amthor *et al.* 2009), indicating that lower rates of fetal myogenesis are long standing in this model of IUGR. The reduction in myonuclear accumulation paralleled the reduction in the cytoplasmic area of the myofiber, as shown by similar myonuclear domains between CON and IUGR groups. In adult muscle, the myonuclear domain size is relatively constant under most physiological conditions (Allen *et al.* 1999), including in the hypertrophic response to mechanical overload (Egner *et al.* 2016) and after prolonged exercise training (Snijders *et al.* 2016). There are some conditions where hypertrophic growth exceeds the addition of myonuclei, resulting in an increased myonuclear domain, as previously demonstrated in myostatin-knockout mice (Amthor *et al.* 2009), models of overexpression of protein kinase B (*Akt*) (Blaauw & Reggiani 2014), and in mice during early postnatal life (White *et al.* 2010). However, in the case of the late gestation IUGR fetus, reductions in the cellularity and cytoplasmic area of the IUGR myofiber occur to similar degrees.

The sheep model used in this study is one of early, progressive, and severe placental insufficiency with evidence of fetal hypoxemia and growth restriction early in gestation (Arroyo *et al.* 2008). Thus, in this model, nutrient and oxygen restriction to the fetus occurs concurrently with the period of secondary myogenesis, which produces the majority of myofibers (Du *et al.* 2010, Lee *et al.* 2013). When the entire cross-section of the FDS muscle mid-belly was examined, total myofiber number was lower in IUGR compared to CON. The finding of fewer myofibers in IUGR offspring also has been reported when maternal undernutrition is initiated during early gestation in sheep and other animals. For example, when pregnant sheep were diet restricted by 50% during early and mid-gestation, fetal myofiber number was reduced by late gestation and remained lower at 8 months of age (Zhu *et al.* 2004, 2006). In pregnant rat dams receiving 30% of an *ad libitum* diet during gestation, secondary myofiber number within the fetal soleus and lumbrical muscles was reduced (Wilson *et al.* 1988). Guinea piglets born to mothers who

had a 40% reduction in feed intake during the peak period of secondary myofiber formation had fewer myofibers within glycolytic muscle types (Dwyer & Stickland 1992, Dwyer *et al.* 1995). Muscle fiber density, however, was previously shown to be similar between control and IUGR fetal sheep in an ovine model of placental insufficiency (Yates *et al.* 2014). Thus, the phenotype of the IUGR fetus includes fewer bundles of fibers with similar amounts of connective tissue. Given that there is minimal increase in myofiber number postnatally (Rowe & Goldspink 1969, Widdowson *et al.* 1972, White *et al.* 2010), a reduction in total myofiber number in the fetus potentially limits attainment of normal skeletal muscle mass in adulthood.

Previously, we reported that cell cycle regulation was one of the primary pathways affected by IUGR in fetal skeletal muscle (Soto *et al.* 2017). In the present study, we found that expression levels of several CDKs and cyclins that regulate each of the transition points in the cell cycle, including first gap (G1) to S phase, S to second gap phase (G2), and G2 to mitosis (M), were downregulated in IUGR BF muscle biopsies. These results are consistent with an overall reduction of cellular proliferation in the IUGR muscle as demonstrated by a lower BrdU labeling index. Both mRNA and protein expression levels of *RBL2* (p130), a G0 marker, were not upregulated in IUGR muscle, arguing against cell cycle arrest in G0. Regulators of apoptosis, including *BAX*, *BAD*, *APAF1*, and *CASP 3*, 8, and 9 were not upregulated in IUGR muscle. In addition, there was no difference in protein expression of pro-caspase 3 and the cleaved caspase 3 product was not present in either group. While apoptosis has been implicated in the reduction of myonuclear number in adults under conditions of disuse resulting in muscle atrophy (Siu 2009, Calvani *et al.* 2013), it does not appear that increased rates of cell death is a factor contributing to lower muscle mass in IUGR.

Circulating fetal plasma insulin and IGF-1 concentrations were positively correlated and norepinephrine, a suppressor of insulin secretion in the fetus (Limesand & Rozance 2017), was negatively correlated with both the number of BrdU<sup>+</sup> myonuclei per myofiber and myofiber number. Insulin has been shown to play an important anabolic role in regulating fetal skeletal muscle growth via the PI3 kinase/Akt signaling pathway (Brown *et al.* 2009, Rhoads *et al.* 2016, Soto *et al.* 2017). Similarly, IGF-1 is mitogenic and has been shown to regulate both prenatal and postnatal skeletal muscle growth and regeneration (Liu *et al.* 1993, Powell-Braxton *et al.* 1993, Ten Broek *et al.* 2010) and to increase satellite cell proliferation (Chakravarthy *et al.* 2000). The regulation of myogenesis by IGF-1, however, is highly

complex, as both proliferation and differentiation are stimulated through the same IGF1R receptor (Rosenthal & Cheng 1995) and IGF-1 has been shown to promote muscle cell survival during myogenic differentiation through Akt-mediated induction of p21 (Lawlor & Rotwein 2000). p21 is a cyclin-dependent kinase inhibitor that arrests cell cycle progression in G1/S by associating with CDKs 2, 4, and 6, and cyclins D and E (Karimian *et al.* 2016). p21 is integral to myogenic differentiation (Guo *et al.* 1995) and can be regulated at transcriptional, post-transcriptional, and post-translational levels (Jung *et al.* 2010). We found an increase in *CDNK1A* mRNA expression but no increase in the active nuclear protein product p21 in IUGR muscle biopsies. Further studies are required to determine whether the downregulation of several cell cycle regulators is a mechanism via which lower concentrations of insulin and IGF-1 may reduce myoblast proliferation and/or the proliferation of other cell types within fetal muscle, or whether lower expression of several cyclins and CDKs, which can still be expressed in terminally differentiated myonuclei (Guo *et al.* 1995), simply reflects lower myonuclear number in IUGR muscle.

A limitation of this study was that mRNA and protein analyses were performed on whole muscle biopsies, which contain several cell types. The BrdU labeling index was lower in IUGR muscle, demonstrating that the reduction in proliferation rates likely affected many cell types within muscle, in addition to myonuclei, which make up about 25% of all nuclei in both CON and IUGR fetal muscle. Future studies using immunohistochemistry, flow cytometry, or other methods will be required to specifically identify cell type (e.g., fibroblasts, vascular cells, endothelial cells, and macrophages), along with exploration of the mechanisms that result in impairments in cellular proliferation in IUGR fetal skeletal muscle. Additionally, sex is known to affect fetal growth, with males being larger than females (Wallace *et al.* 2018). Testing for the effect of sex in the current study was limited due to lack of power to identify potential interactions between IUGR and sex. Investigation as to whether IUGR affects male and female skeletal muscle growth differently will be important for future studies. Finally, three different muscles were used from the fetal hindlimb for analysis, and there were minor differences in BrdU incorporation and myonuclear number among muscles. It is possible that skeletal muscles collected from different sites (forelimbs, diaphragm, lumbricals) might demonstrate different myogenic responses to placental insufficiency.

The timing of onset and severity of IUGR will have important implications for the growth potential of muscle postnatally. The sheep model used for these studies is one of long-standing and severe placental insufficiency that mimics 'early' human IUGR with onset <32 weeks gestation, as opposed to more mildly affected 'late' IUGR pregnancies (Savchev *et al.* 2014, Figueras *et al.* 2015). If the onset of oxygen and nutrient restriction occurs at the peak of myoblast proliferation and fusion to form myotubes, the likelihood of reductions in myonuclear and myofiber number will be greater (Fahey *et al.* 2005a,b). Fewer total myofibers established during gestation is likely one of the reasons why low birth weight from IUGR strongly predicts muscle mass later in adulthood (Brown & Hay 2016). Lower myonuclear numbers in IUGR fetal muscle at birth also might limit postnatal hypertrophic muscle growth. Muscle fiber diameter in growing animals has been directly correlated with myonuclear number (Allen *et al.* 1979), consistent with the concept of an upper limit in the myonuclear domain size or amount of cytoplasm that each myonucleus can support (Petrella *et al.* 2006). In 'late' occurring IUGR, however, we speculate that myofiber and myonuclear number may remain intact with the potential for compensatory postnatal hypertrophy (Prakash *et al.* 1993, Fahey *et al.* 2005a). Further evaluation into the consequences of reduced rates of myogenesis during fetal life and the impact on postnatal muscle growth capacity is warranted.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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