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SHORT COMMUNICATION

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Regulation of muscle protein synthesis in an *in vitro* cell model using *ex vivo* human serum

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Abstract

Human serum embodies the integrated systemic response to any condition or perturbation, which may regulate muscle protein synthesis (MPS). Conditioning of medium with human serum represents a physiologically relevant method of regulating MPS *in vitro*. The primary purpose of the study was the development of a model using *ex vivo* human serum to condition medium and regulate MPS in *in vitro* skeletal muscle cells. Four young healthy men reported to the laboratory after an overnight fast and were fed with 0.33 g (kg body mass)$^{-1}$ whey protein. Blood samples were taken before (Fasted) and 60 min postprandial (Fed). Fully differentiated C2C12 skeletal muscle cells were nutrient and serum deprived for 1 h and subsequently treated with medium conditioned with Fasted or Fed *ex vivo* human serum (20%) for 4 h. The MPS was measured using the surface sensing of translation technique and activation of mTOR, P70S6K and 4EBP1 by Western blot. Fasted and fed *ex vivo* human serum increased MPS ($P < 0.05$). Although a strong effect ($\eta^2 = 0.36$) for increased MPS in Fed relative to Fasted was observed, this was not statistically significant ($P > 0.05$). Activation of mTOR, P70S6K and 4EBP1 was significantly increased after treatment with Fed compared with Fasted *ex vivo* human serum ($P < 0.05$). Here, we developed and optimized the conditions for culture of C2C12 skeletal muscle cells, measurement of MPS and signalling in medium conditioned by *ex vivo* human serum. Furthermore, the functionality of the model was demonstrated by comparison of the response to medium conditioned by Fasted and Fed *ex vivo* human serum.

KEYWORDS
muscle protein synthesis, serum, skeletal muscle

1 | INTRODUCTION

Numerous studies have measured muscle protein synthesis (MPS) *in vivo* using stable isotope tracers of amino acids (AAs) with heavy carbon (^{13}C), deuterium (^{2}H) or nitrogen (^{15}N) motifs. The incorporation of these labelled AAs into muscle protein allows for the calculation of a fractional synthetic rate (FSR) of MPS (Rennie et al., 1982; Yarasheski, Smith, Rennie, & Bier, 1992). More recently, $^{2}H_2O$ incorporation techniques have been used to calculate the FSR in more free-living conditions (Wilkinson et al., 2014). However, these techniques are invasive, require multiple skeletal muscle biopsies, pose technical challenges in the analyses and are expensive. Evidence from *in vitro* platforms offers an attractive alternative and can provide prior justification for translation to *in vivo* human experimentation.

Classically, *in vitro* skeletal muscle platforms are cultured and differentiated in growth-stimulating media such as Dulbecco's modified Eagle's medium (DMEM) supplemented with animal serum. Dulbecco's modified Eagle's medium is a nutrient-rich medium, with supraphysiological concentrations of several potent regulators of MPS, such as the essential AAs (EAAs), including leucine. Stimulating MPS in a background medium containing DMEM with high concentrations of these regulators is probably a confounding influence. Thus, removal of these regulators from any background medium potentially represents a better model to study factors affecting MPS *in vitro*.

New Findings

- **What is the central question of this study?**
  Can medium conditioned by *ex vivo* human serum regulate muscle protein synthesis in skeletal muscle cells *in vitro*?
- **What is the main finding and its importance?**
  This study demonstrates that medium conditioned by *ex vivo* human serum can regulate muscle protein synthesis in skeletal muscle cells *in vitro* via the mammalian Target of Rapomycin (mTOR) pathway, and this can be regulated differentially by fed and fasted *ex vivo* human serum.

Conditioning of culture medium represents a model for modulating MPS in skeletal muscle *in vitro*. Typically, this is achieved by the addition of a factor, nutrient or compound to a background medium. Translation of this approach to the *in vivo* human model is potentially limited, because the physiological relevance of adding compounds to medium in concentrations or forms that the cell is unlikely to encounter is questionable. Human serum *ex vivo* from diverse populations and in different nutrition and exercised conditions represents a potential model to 'condition' culture medium and modulate MPS *in vitro*. This *ex vivo/in vitro* approach represents a potential model for studying the regulation of MPS in skeletal muscle cells.

The primary purpose of this study was the development of a new model using *ex vivo* human serum to condition medium and regulate MPS in *in vitro* skeletal muscle. In order to achieve this, a number of secondary objectives had to be realized, including the development of an appropriate background medium, preconditioning of muscle cells, and determination of the optimal serum concentration and time course of treatment in which to study MPS. Finally, to demonstrate the functionality of the model, postprandial *ex vivo* human serum after protein feeding is compared with fasted serum.

2 | METHODS

2.1 | Ethical approval

The study was approved by the local ethics committee at the University of Limerick (EHSREC_2013_01_13) and conformed to the standards set by the *Declaration of Helsinki*, except for registration in a database. Four young healthy male participants, (23 ± 3.6 years of age, weighing 75.9 ± 10.1 kg, 1.77 ± 0.06 m tall, with body mass index 24.3 ± 3.3 kg m^{-2} and 16.4 ± 3.3% body fat) agreed to participate in the study, gave informed written consent and completed the intervention trial.

2.2 | Study design

Participants reported to the laboratory having fasted overnight (>10 h). A clinical nurse inserted a cannula in the antecubital vein, and a blood sample was collected as baseline (t = 0 min) in S-Monovette® EDTA and S-Monovette® Serum gel tubes (Sarstedt, Wexford, Ireland). Subsequently, participants consumed 0.33 g (kg body mass)$^{-1}$ of a whey protein beverage (WP) in 400 ml (7.6%, w/v). Blood samples were collected at 0 min (baseline) and 60 min postprandial. Plasma was used to determine the AA profile and humoral biomarkers, whereas serum was used for cell culture experiments.

2.3 | Amino acid analysis

The plasma AA profile was determined as previously described using the Agilent 1200 RP-UPLC system (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with an Agilent 1260 binary pump and a G1367C automated liquid handling system (Power-Grant et al., 2016). Chemstation software (Agilent Technologies Inc.) was used for data acquisition. Separation of AAs was carried out using a C_{18} ZORBAX rapid resolution column (4.6 mm × 50 mm, 1.8 μm; Agilent Technologies Inc.) thermostated at 40°C. Quantitative analysis was performed by the external standard method, using Agilent AA standard (Agilent Technologies Inc.). Standard AAs were measured in both unspiked and spiked pool plasma, and recoveries were calculated.

2.4 | Metabolic/humoral biomarker analysis

Plasma glucose concentrations were analysed using a glucose assay colorimetric kit (STA-60; Cell Biolabs, Inc., San Diego, CA, USA), following the supplier's instructions. Plasma cocnentrations of insulin and Insulin-like Growth Factor-1 (IGF-I) were determined using a commercial kit (Merck Millipore). Bead readings were performed on a MAGPIX™ Multiplex reader and data processed using Bio-Plex Manager™ MP.

2.5 | Cell culture

C2C12 skeletal muscle cells obtained from Sigma-Aldrich (91031101; Sigma-Aldrich) were cultured in DMEM (D6429; Sigma-Aldrich), supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin/streptomycin (P0781; Sigma-Aldrich) and 1 mM L-glutamine (G7513; Sigma-Aldrich), at 5% CO_2 in a humidified air atmosphere at 37°C. At 70% confluence, cells were differentiated in DMEM supplemented with 2% horse serum (H1270; Sigma-Aldrich) as previously described (Murphy, Kiely, Jakeman, Kiely, & Carson, 2016). Before serum treatment, cells were changed to DMEM AA- and serum-free medium (US biological, Salem, MA, USA) containing 1 mM sodium pyruvate (GE Healthcare, Thermo-Fisher), 1% (v/v) penicillin/streptomycin solution, 1 mM L-glutamine, 6 mM D-glucose (Sigma-Aldrich) and 34 mM NaCl (Sigma-Aldrich) (pH adjusted to 7.3.).

2.6 | Cell viability

2.6.1 | Crystal violet

Cells were seeded and differentiated in 96-well plates, and the assay was carried out as previously described (Navarro et al., 2014). Cells were washed with AA-free medium and changed to AA-free medium for 1 h. One hundred microlitres of different concentrations of serum

(0, 10, 20, 50 and 100%) diluted in AA-free medium was added to each well for 1–4 h. After treatment, cells were washed with 200 μl PBS per well (preheated at 37°C), incubated with 50 μl per well of 0.2% (w/v) Crystal Violet in 2% (v/v) ethanol for 20 min at room temperature, and washed three times with milli-Q water (300 μl per well) to remove excess dye. One hundred microlitres per well of 1% (w/v) SDS was added, and plates were and placed in a plate shaker for 10 min before reading the absorbance at 560 nm in a plate reader (Bio-Tek Synergy HT, Winooski, VT, USA).

2.6.2 | Real Time-Glo™ MT cell viability assay

Cells were seeded and differentiated in 96-well plates as described in the previous subsection. Cells were washed with AA- and serum-free medium and changed to AA-free medium for 1 h. Amino acid- and serum-free medium conditioned with different concentrations of serum (0, 10, 20, 50 and 100%) was added to each well. The Real Time-Glo™ MT Cell viability assay was carried out following the manual's instructions (Promega Corporation, Madison, WI, USA), and bioluminescence was measured to determine the number of viable cells by measuring the reducing potential of cells and thus metabolism.

2.7 | Muscle protein synthesis

Measurement of MPS using the surface sensing of translation (SUnSET) technique developed by Goodman et al. (2011) was optimized in C2C12 myotubes. After differentiation and maturation (7 days), C2C12 myotubes were nutrient deprived in AA- and serum-free medium for 0.5–4 h to determine the appropriate nutrient deprivation time. Cells were subsequently nutrient deprived for 1 h and treated with human serum (0, 10, 20, 50 or 100%).

2.8 | Protein extraction and western blotting

Cellular protein lysates were obtained by placing cells on ice, aspirating the medium and washing three times with cold phosphate-buffered saline (C-40232; PromoCell, Brennan & Co., Stillorgan, Co. Dublin, Ireland). Lysis buffer [50 mM Tris–HCl pH 7.4, 50 mM NaF, 10 mM $Na_4P_2O_7$, 100 mM NaCl, 5 mM EDTA 1% (v/v) Triton X-100, 10 μM phenylmethanesulfonyl fluoride, 10 μM sodium orthovanadate, 1 μM pepstatin A and 1.5 μg aprotinin ml^{-1}] was added to each well, scraped and collected in tubes. Lysates were placed on ice for 20 min before centrifugation at 130*g*, 4°C for 15 min.

Protein lysates (30 μg) and ladders Precision Plus Protein™ Dual Color Standards, 500 μl (#1610374, Fannin Ltd, Bio-Rad, Leopardstown, Dublin, Ireland) were loaded on 4–15% linear gradient SDS-PAGE precast gels (Mini-Protean TGX Stain-free; Bio-Rad 456-8083). After electrophoresis, gels were activated by ultraviolet light for stain-free technology using the UVITEC Cambridge Imaging system (UVITEC; Cambridge, UK). Total protein measurements were obtained by stain-free gel technology using the UVITEC imaging system. Whole-lane band densitometry was quantified for puromycin and total protein using NineAlliance UVITEC software. Proteins were electroblotted onto a 0.2 μm nitrocellulose membrane using the semi-dry transfer technique (Trans-blot® Turbo™; Bio-Rad). Membranes were blocked with TBST [0.05% (v/v) Tween-20 in Tris-buffered saline] containing 5% bovine serum albumin for 1 h at room temperature. Membranes were washed four times with TBST and incubated with primary antibody at 4°C overnight. All primary antibodies were diluted 1:1000 in 5% bovine serum albumin in TBST: mTOR (#2972S), pmTOR, P70S6K, pP70S6K, 4 EB-P1, p4EBP1, β-actin (#2972S, #5536S, #2708S, #9234S, #9644S, #2855S, #3700S; Cell Signaling, Bioke, Leiden, The Netherlands) and puromycin (MABE343 anti-puromycin, clone 12D10 mouse monoclonal; Merck Millipore Limited, Carrigtwohill, Co. Cork, Ireland). After incubation, membranes were washed four times and incubated with the secondary antibody green rabbit (926-32211 IRDye 800 CV goat anti-rabbit IgG; LI-COR Biosciences UK Ltd, Cambridge, UK) for all primary antibodies with the exception of puromycin, which was incubated with goat anti-mouse IgG2a-specific (LI-COR Biosciences UK Ltd, Cambridge, UK).

2.9 | Statistical analysis

GraphPad Prism v7.03 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. Data were tested for normality (Shapiro–Wilk test) and homogeneity of variances (Levene's test). One-way and two-way ANOVA with a Bonferroni correction for multiple comparisons was used for pooled serum and cell viability experiments, respectively. Student's paired *t* tests were used to analyse MPS and signalling responses to fed and fasted *ex vivo* human serum. The level of significance was set at 95% ($P < 0.05$).

3 | RESULTS

Differentiated myotubes were AA and serum deprived for between 0.5 and 4 h to assess MPS and signalling at different time points of nutrient deprivation. Muscle protein synthesis was reduced within 30 min of nutrient deprivation by ~80%, and activation of mTOR (~50%) and P70S6K (~95%) were significantly reduced within 30 min of nutrient deprivation and remained suppressed for up to 4 h; however, activation of 4EBP1 (~75%) wss not significantly reduced until 1 h after nutrient deprivation (data not shown). These results suggest that 1 h of nutrient deprivation is sufficient to depress activation of the mTOR signalling pathway and MPS. Based on these findings, subsequent experiments were carried out after 1 h of nutrient deprivation.

Differentiated myotubes were nutrient deprived for 1 h before carrying out cell viability assays for 0.5–4 h using 10–100% pooled fasted human serum obtained from four healthy men (Table 1). Cell adherence, as measured by the Crystal Violet assay, was significantly reduced after 2 h in 100% serum and after 4 h in 50% serum compared with the control conditions (Figure 1a). Cell viability measured by the Real Time MT assay was significantly decreased after 1 h in the 100 and 50% serum conditions compared with control conditions (Figure 1b). Treatment of cells with a lower serum concentration (10 or 20%) after 1 h of nutrient deprivation led to no negative effect on cell adherence (all time points; Figure 1a) or cell viability (all time points; Figure 1b) compared with control conditions. These data provide evidence that treatment with a lower serum concentration (10–20%) has no negative

TABLE 1 Humoral biomarkers and amino acid profile of plasma samples

Parameter	0 min	60 min	Change (from 0 to 60 min)
Insulin (pM)	67 ± 31	112 ± 44	45 ± 28
GLP-1 (pM)	39 ± 8	63 ± 15	25 ± 9
GIP (pM)	8.3 ± 3.3	20.8 ± 5.7	12.5 ± 3.6
Growth hormone ($\mu g\ l^{-1}$)	0.30 ± 0.22	0.21 ± 0.17	0.09 ± 0.20
IGF-1 ($\mu g\ l^{-1}$)	116 ± 49	109 ± 17	8 ± 34
Glucose (mmol l^{-1})	3.7 ± 0.3	3.2 ± 0.5	0.5 ± 06
Leucine ($\mu mol\ l^{-1}$)	128 ± 18	342 ± 58	215 ± 41
Isoleucine ($\mu mol\ l^{-1}$)	59 ± 10	194 ± 39	135 ± 29
Valine ($\mu mol\ l^{-1}$)	232 ± 29	406 ± 56	174 ± 28
Arginine ($\mu mol\ l^{-1}$)	79 ± 13	117 ± 18	38 ± 8
Branched chain amino acids ($\mu mol\ l^{-1}$)	419 ± 57	943 ± 154	524 ± 97
Essential amino acids ($\mu mol\ l^{-1}$)	965 ± 82	1895 ± 208	930 ± 134

Data represent means ± SD (n = 4). Glucagon-like Peptide-1 (GLP-1); Gastric Inhibitory Polypeptide (GIP).

effects on cell viability during treatment up to 4 h; therefore, the higher serum concentration (20%), which is closer to the *in vivo* conditions experienced by skeletal muscle, was used for subsequent treatments.

After 1 h of nutrient deprivation, C2C12 cells were treated for 4 h with AA- and serum-free medium (control) or medium conditioned with 20% *ex vivo* pooled fasted serum for up to 4 h. Repeated-measures ANOVA revealed a statistically significant effect ($P < 0.01$, $F = 14.65$) of *ex vivo* human serum on MPS above the control conditions after 2 h and a further increase after 4 h ($P < 0.01$) of treatment (Figure 2a). This suggests that 4 h of treatment is superior to observe a detectable change in MPS. Activation of mTOR, P70S6K and 4EBP1 was significantly increased after 2 and 4 h of treatment with *ex vivo* human serum; however, activation of P70S6K was further enhanced after 4 h (Figure 2b–d). These results demonstrate that *ex vivo* human serum can condition medium to elicit a stimulatory effect on MPS mediated by signalling through the mTOR pathway. To activate signalling and stimulate MPS sufficiently, 4 h of treatment with *ex vivo* human serum was performed for all subsequent experiments.

Feeding 0.33 g protein (kg body mass)$^{-1}$ resulted in a change in the concentration of circulating AAs and humoral regulators of MPS at 60 min postprandial (Table 1). After 1 h of nutrient deprivation, C2C12 cells were treated for 4 h with AA- and serum-free medium (control) or medium conditioned with 20% *ex vivo* fasted and 60 min postprandial [fed 0.33 g protein (kg body mass)$^{-1}$] serum from four healthy men. Responses to the conditioned medium are reported as the fed relative to the fasting conditions for each participant. Both fasted and fed *ex vivo* human serum significantly increased MPS above the control conditions (data not shown), confirming an effect of *ex vivo* human serum on MPS (as already observed in Figure 2). Although a strong effect ($\eta^2 = 0.36$) for an increase in MPS for the fed serum relative to the fasted serum was observed, this was not statistically significant ($P = 0.14$; Figure 3a). Activation of mTOR, P70S6K and 4EBP1 was significantly increased after treatment with both fasted and fed *ex vivo* human serum ($P < 0.05$; data not shown; already demonstrated in Figure 2). Activation of P70S6K and 4EBP1 was significantly enhanced ($P < 0.05$) by fed compared with fasted serum, with a large effect size observed for both ($\eta^2 = 0.78$ and $\eta^2 = 0.73$, respectively; Figure 3c,d). Although not statistically significant, a statistical trend was observed for increased activation of mTOR by fed compared with fasted serum ($P = 0.06$), also with a large effect size ($\eta^2 = 0.59$; Figure 3b). These results demonstrate that *ex vivo* fed and fasted human serum has the potential to condition medium differentially and regulate MPS mediated by signalling through the mTOR pathway in skeletal muscle cells.

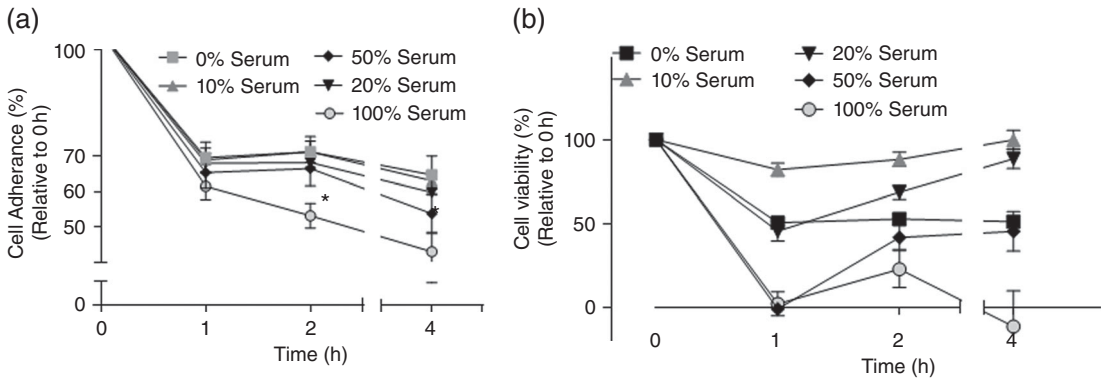

FIGURE 1 Cell adherence (a) and viability (b) of C2C12 myotubes exposed to increasing concentrations of fasted human serum (n = 4). Fully differentiated C2C12 myotubes were treated with amino acid- and serum-free medium conditioned with different concentrations of pooled fasted serum (0, 10, 20, 50 and 100%) for 1–4 h. (a) Cell adherence, expressed as the percentage of cell viability referred to the absorbance measurement obtained in control cells (2% Horse Serum–Dulbecco's modified Eagle's medium) without nutrient deprivation was measured using the Crystal Violet method. (b) Cell viability, expressed as the percentage of cell viability referred to the luminescence measured in control cells (2% Horse Serum–Dulbecco's modified Eagle's medium) without nutrient deprivation was measured using Real Time-Glo™ MT. Data are reported as means ± SEM. *$P < 0.05$ by one-way ANOVA

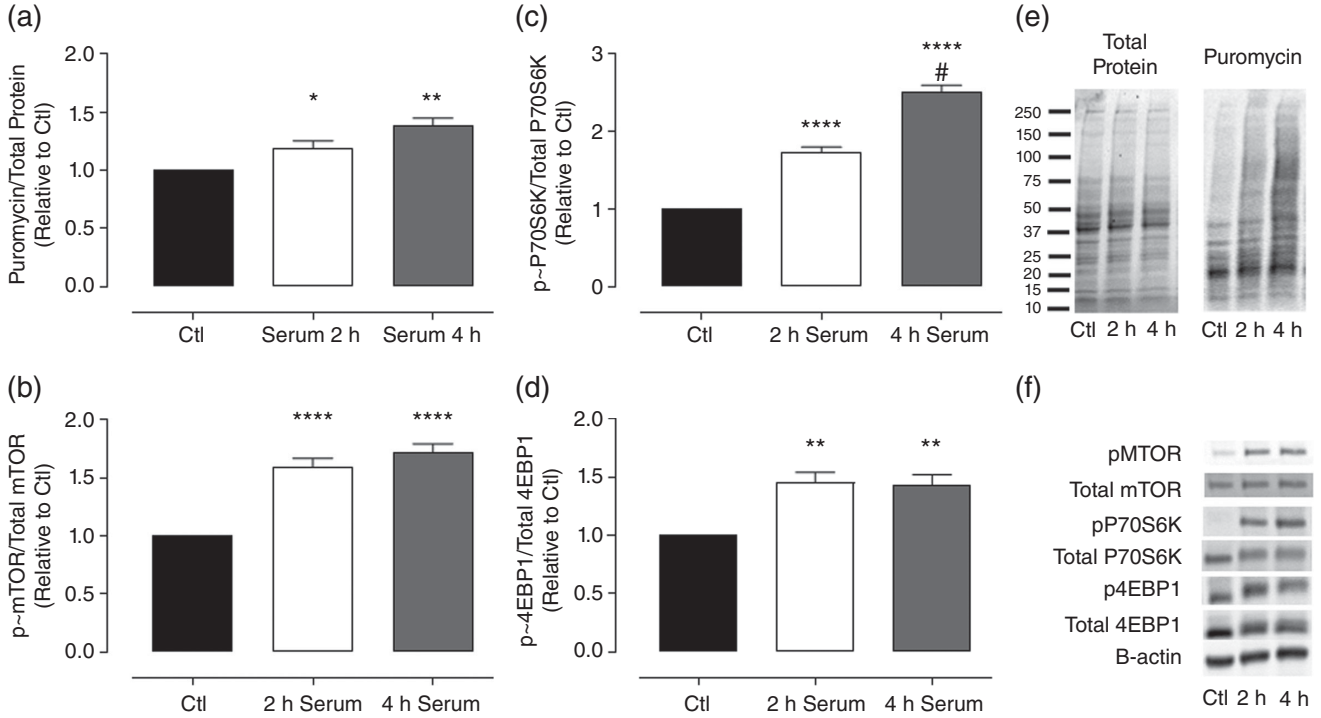

FIGURE 2 Muscle protein synthesis and signalling in C2C12 skeletal muscle cells treated 20% *ex vivo* fasted human serum ($n = 4$). Fully differentiated C2C12 myotubes were cultured in Dulbecco's modified Eagle's medium and 2% Horse Serum before nutrient deprivation in amino acid- and serum-free medium for 1 h. Cells were then treated with amino acid- and serum-free medium conditioned with pooled fasted serum (20%) for up to 4 h. (a) Muscle protein synthesis was measured by incorporation of puromycin (1 μM) incubated for the duration of the treatment. (b–d) Phosphorylation of mTOR (b), P70S6K (c) and 4EBP1 (d) relative to the total protein of interest was measured by Western blot. (e, f) Representative western blots of total protein and puromycin incorporation (e) and phosphorylated and total mTOR, P70S6K, 4EBP1 and β-actin (f). Abbreviation: Ctl, control. Data are reported as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{****}P < 0.0001$ by one-way ANOVA

4 | DISCUSSION

The primary purpose of this study was the development of a model using *ex vivo* human serum to condition medium and regulate MPS in *in vitro* skeletal muscle cells. In order to achieve this, a number of secondary objectives were realized, including the development of an appropriate background medium, the preconditioning of muscle cells, the optimal serum concentration and the time course of treatment in which to study MPS.

A background medium that does not independently stimulate MPS and can be conditioned appropriately to regulate MPS required development. Typically, cells are preconditioned before experimentation with appropriate medium, usually serum free, to synchronize cells and transition to a phase of growth arrest. Classically, this preconditioning step is ≥4 h in duration. However, information from Ham, Caldow, Lynch, & Koopman (2014) indicated that MPS signalling is switched off within 30 min of removal from growth medium in skeletal muscle. Therefore, we empirically tested the required duration of preconditioning of skeletal muscle cells in appropriate background medium (AA- and serum-free DMEM) to turn off both signalling and MPS. We found that MPS and signalling were sufficiently downregulated within 1 h of preconditioning and adopted this protocol for all subsequent experiments.

To achieve the primary aim of this paper, we needed to establish the appropriate concentration of *ex vivo* serum to condition medium for treatment of skeletal muscle. Previously, others have used *ex vivo* human serum in this way for other physiological outcomes, such as myoblast proliferation and metabolic gene expression (Bruckbauer & Zemel, 2011; Nguyen et al., 2014), using a wide range of serum concentrations (5–25%). Ideally, we wanted to replicate the interstitial concentrations of the key nutrient and hormonal regulators of MPS. We (McCormack, Cooke, O'Connor, & Jakeman, 2017) demonstrated that the interstitial concentrations of leucine and EAAs in response to a similar protein feeding protocol to the one used in this study [0.33 g protein (kg body mass)$^{-1}$] were ~70% of the plasma concentration. Therefore, a model that could use a serum concentration close to this was desirable. However, cell viability and adherence were negatively affected by high serum concentrations (50–100%) and were thus considered suboptimal (Figure 1a,b). Cell viability and adherence at 10 and 20% serum were optimal; therefore, the higher serum concentration (20%) was selected for subsequent experimentation.

To develop the measurement of MPS in response to *ex vivo* human serum treatment, the SUnSET technique was adopted. The time course of treatment with *ex vivo* human serum and puromycin to observe a measurable effect had to be established. In our hands, we found little evidence of MPS as determined by puromycin incorporation when puromycin was incubated for the last 30 min of treatment as previously described (data not shown; Goodman et al., 2011). Our data demonstrate that puromycin incorporation is greater when included for the entirety of the treatment (up to 4 h) with *ex vivo* human serum.

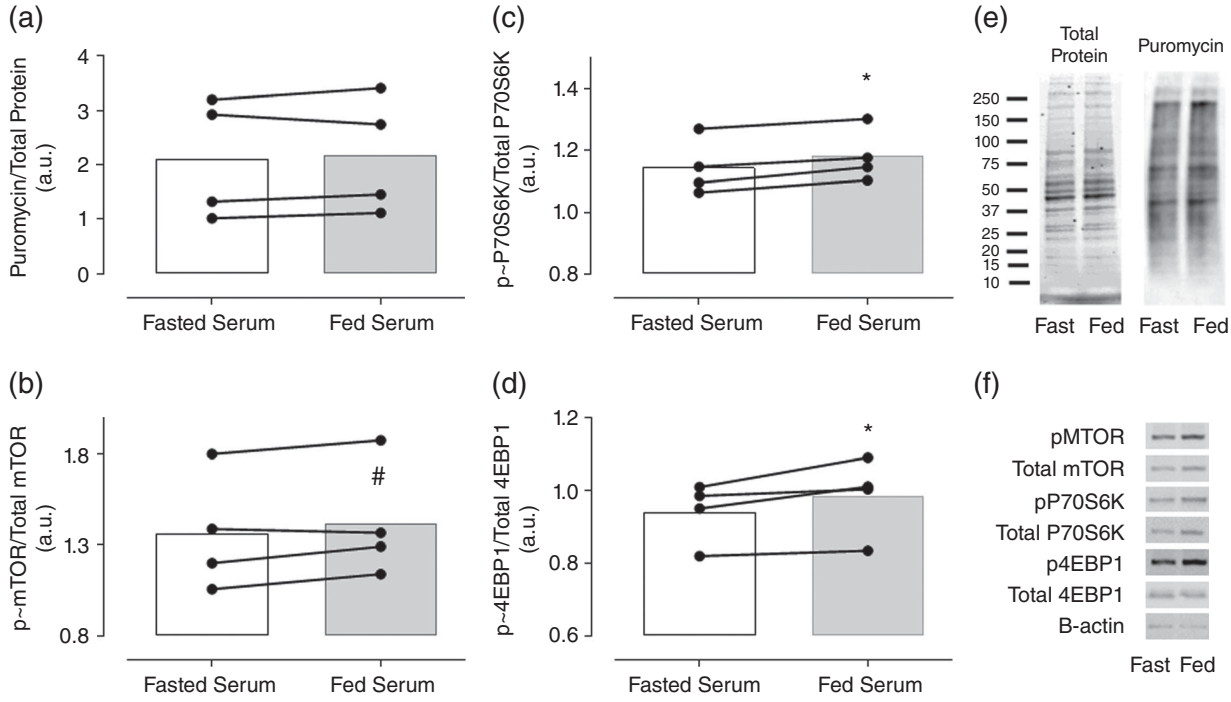

FIGURE 3 The effect of 20% *ex vivo* fasted and fed human serum on muscle protein synthesis and signalling in C2C12 skeletal muscle cells ($n = 4$). Fully differentiated C2C12 myotubes were cultured in Dulbecco's modified Eagle's medium and 2% Horse Serum before nutrient deprivation in amino acid- and serum-free medium for 1 h. Cells were then treated with amino acid- and serum-free medium conditioned with individual ($n = 4$) fasted and fed [60 min postprandial 0.33 g protein (kg body mass)$^{-1}$] serum (20%) for 4 h. (a) Muscle protein synthesis was measured by incorporation of puromycin (1 μM) incubated for the duration of the treatment. (b–d) Phosphorylation of mTOR (b), P70S6K (c) and 4EBP1 (d) relative to the total protein of interest was measured by Western blot. (e, f) Representative Western blots of total protein and puromycin incorporation (e) and phosphorylated and total mTOR, P70S6K, 4EBP1 and β-actin (f). Data are reported as means ± SEM. $^{*}P < 0.05$ and $^{\#}P < 0.1$ by paired samples and T-test

Increased puromycin incorporation and MPS signalling was observed after 4 h of treatment compared with 2 h. Given that it is the rate of MPS that may differ between conditions, 4 h was therefore adopted for subsequent experimentation. These data demonstrate proof of principle that treatment of skeletal muscle cells with an appropriate background medium conditioned by *ex vivo* human serum (20%) can stimulate MPS as measured by puromycin incorporation and MPS activation via mTOR, P70S6K and 4EBP1. The effect of fasted *ex vivo* human serum on these processes is powerful, particularly when the concentrations of key regulators of MPS are at a nadir. This suggests that there is opportunity to impact these processes further when key regulators are more abundant in the circulation.

To demonstrate the functionality of the model, skeletal muscle cells were treated with *ex vivo* human sera containing different concentrations of key regulators of MPS manipulated by feeding protein (Table 1). Treatment of skeletal muscle cells with medium conditioned by fed serum increased P70S6K and 4EBP1 activation (Figure 3c,d). Fed serum displayed a trend ($P = 0.06$) to increase activation of mTOR relative to fasted serum, with a large effect size (Figure 3b). These data confirm the ability of the model to detect differential responses in MPS signalling to medium conditioned with *ex vivo* human serum in the fed and fasted conditions containing different concentrations of regulators of MPS. Treatment of skeletal muscle cells with medium conditioned by *ex vivo* fed serum had a large but not statistically significant effect on MPS compared with fasted serum (Figure 3a). The difference in the concentration of the key regulators of MPS between conditions (Table 1) might not have been sufficient to distinguish this response. For example, the difference in leucine as a potent stimulator of MPS between fed and fasted serum was ~200 μmol l^{-1}. At 20% concentration, this difference between fed and fasted is ~40 μmol l^{-1}. Rennie and colleagues reported that an increase of ~80 μmol l^{-1} in extracellular leucine is required to increase MPS *in vivo* (Bohe, Low, Wolfe, & Rennie, 2001; Rennie, 2007), suggesting that the differences reported here between fed and fasted serum are sufficient to stimulate MPS signalling further but do not result in a statistically measurable difference in MPS. It is also important to note the inter-individual responses evident. For MPS, the fed serum increased MPS relative to fasted serum in only three out of four participants. This impacted the aggregate effect of the group, and is the reason that such a large effect size is not supported by a statistically significant result.

A major strength of this *ex vivo–in vitro* approach is that human serum reflects the integrated systemic conditions. Therefore, treatment of skeletal muscle with *ex vivo* serum may be more physiologically relevant and therefore potentially more likely to translate to a human model. Culture medium can be conditioned accordingly to investigate the effects of different conditions, including nutrition, exercise and inflammation. One of the advantages of this approach for

nutrient interventions is that the key nutrient regulators that appear in the circulation have undergone human digestion, in contrast to enzymatically simulated gastrointestinal digestion models (Power-Grant et al., 2016). Furthermore, the humoral factors stimulated by feeding are also present. Therefore, the integrated effect of the nutrient and the physiological response to feeding is captured in the *ex vivo* serum sample and can elicit a pluripotent effect on MPS in skeletal muscle.

A limitation of the model described here is the concentration of the serum. C2C12 cells do not tolerate exposure to medium conditioned by high concentrations of serum over extended periods (4 h). Although we argue that this is a more physiologically relevant model compared with direct treatment with nutrient formulations or supraphysiological concentrations of key regulators, the interstitial concentrations of key regulators of MPS experienced by the muscle cell *in vivo* are not reached here (McCormack et al., 2017). Therefore, the translation of findings from this model to the *in vivo* conditions is less well known.

4.1 | Conclusions

In conclusion, in this study we describe the development of a model using *ex vivo* human serum to condition medium and regulate MPS in *in vitro* skeletal muscle cells, including the development of an appropriate background medium, the preconditioning of muscle cells, the optimal serum concentration and the time course of treatment in which to study MPS. This study provides proof of principle that *ex vivo* human serum can be used to condition medium to regulate MPS, mediated by signalling through the mTOR pathway.

AUTHOR CONTRIBUTIONS

B.P.C., P.M.J. and P.A.K.: conception, design, interpretation of data, drafting and revising the manuscript critically for important intellectual content. B.P., M.A.B., M.P., S.K.G., S.M.M.: data acquisition, analysis, interpretation and drafting and revising of the manuscript. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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