# BRIEF COMMUNICATION: Effects of restricted fetal nutrition *in utero* on mTOR signalling in ovine skeletal muscle

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## INTRODUCTION

High prolificacy (twins and triplets) is a desirable trait in sheep under intensive management systems. However, multiple-born lambs have lower birth weights resulting from placental insufficiency (McCoard, 1997), and carryover detrimental effects on lean deposition, feed efficiency, and lifelong performance compared to singletons (Greenwood *et al.*, 1998; De Blasio *et al.,* 2007), suggesting differential nutritional programming *in utero*.

We and others have shown that decreased birth weight in multiple-born lambs is associated with restricted skeletal muscle hypertrophy or myofibre size, but not number, reduced DNA accumulation, reduced MyoD-positive satellite cells (activated satellite cells) and protein synthesis (Greenwood *et al.,* 1999; McCoard *et al.,* 2001). However, the molecular mechanisms involved remain to be elucidated.

The mammalian target of rapamycin (mTOR) signalling pathway is critical both for sensing nutrient availability, fetal myogenesis, activation of myogenic satellite cells and for nutrient-stimulated muscle growth in both monogastrics and ruminants (Zhu *et al.,* 2004; Du *et al.,* 2005; Suryawan *et al.,* 2008). The downstream targets of mTOR are proteins that control cell size, gene expression, mRNA translation and metabolism. Inhibition of mTOR leads to arrest of cells in the G1 phase of the cell cycle, suggesting mTOR may be involved in regulating muscle hypertrophy (Wang & Proud, 2006), a process dependent on protein synthesis and satellite cell activity.

The objective was to determine whether restricted skeletal muscle hypertrophy in twin fetuses in late gestation is associated with changes in translational capacity and/or efficiency and mTOR signalling.

## MATERIALS AND METHODS

This study used *M. semitendinosus* samples collected from singleton and twin fetuses at 140 days gestation, collected as previously described (Kenyon *et al.,* 2009). Briefly, a commercial flock of oestrus-synchronised Romney ewes with a live weight of 60.8 ± 0.2 kg and a body condition score of 3.02 ± 0.03 (1 = Emaciated; 5 = Obese) were mated to one of four Suffolk rams. Twin and singleton pregnancies were determined at 50 days post-insemination by transabdominal ultrasonographic examination. All ewes were fed on a maintenance pasture-only nutritional regimen from Day 1 post-insemination until 140 days of pregnancy, and grazed such that the total increase in maternal body weight during pregnancy approximately equalled the expected conceptus mass at term, that is the ewes maintained conceptus free weight.

At Day 140 of pregnancy, five twin-bearing ewes and ten singleton-bearing ewes were euthanised, fetal weight and crown-rump length recorded, and the *M. semitendinosus* excised, weighed and snap frozen in liquid nitrogen and stored at -80°C until analysis.

Total RNA and DNA was extracted from frozen muscle tissue from singleton and twin fetuses using Tri-reagent (Invitrogen, Auckland, New Zealand) according to the manufacturer's instructions. RNA and DNA were quantified using a NanoDrop Spectrophotometer ND-1000 (Nanodrop Technologies, Wilmington, Delaware, USA). Protein was isolated using lysis buffer (50 mM Tris, pH 7.6; 250 mM NaCl; 5 mM EDTA; 0.1% Nonidet P-40; Complete (Roche Molecular Biochemicals, Mannheim, Germany) protease inhibitor). The muscle extracts were centrifuged to pellet the cell debris, and the supernatants were frozen at -80 °C. Bradford reagent (Bio-Rad, Hercules, California, USA) was used to estimate total protein content (Bradford, 1976).

Equal amounts of protein were separated electrophoretically using SDS-PAGE and transferred to nitrocellulose membranes using the iBlot system (Invitrogen). Membranes were probed with primary antibodies against total ribosomal protein S6 (RPS6), phospho-S6 (Ser235/236), total 4EBP1, phospho-4EBP1 (Thr70 and Ser65), and total eIF4E and phospho-eIF4E (Ser209), and

horseradish peroxidise linked goat anti-rabbit secondary antibody (Cell Signalling Technology, Beverly, Massachusetts, USA). Blots were visualised using the enhanced chemiluminescence method. Image/J software (available at rsb.info.nih.gov/ij/) was used to obtain densities of respective immunoreactive bands. A mean per animal was calculated from values of triplicate blots. Data were analysed for rank effects using the Mixed procedure in SAS (2002).

## RESULTS AND DISCUSSION

Twin fetuses had 25% reduced body weights and 43% reduced *M. semitendinosus* weights compared to singletons (Table 1). Reduced muscle mass in twins compared to singletons was associated with a 30% lower protein to DNA ratio suggesting restricted myofibre hypertrophy (Table 1). Twins also had 32% less DNA and ~40% less total RNA compared to singles, indicative of fewer myonuclei and reduced ribosome number respectively and thus capacity for muscle growth (Table 1). Because 85% of total RNA is ribosomal RNA (Nader *et al*., 2005), and increased rRNA is an indicator of increased ribosome biogenesis (Camacho *et al*., 1990), an increase in total RNA is indicative of increased ribosome number. Furthermore, twin fetuses had a 16% lower protein to RNA ratio compared to singles, indicating that twins had reduced protein synthetic efficiency compared to single fetuses (Table 1). The findings of the present study are consistent with previous observations (Greenwood *et al.,* 1999; McCoard *et al.,* 2001), highlighting the importance of fetal nutrition on skeletal muscle growth and development *in utero*.

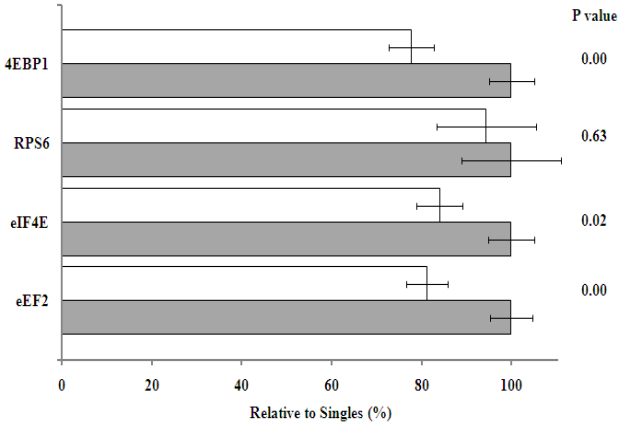
The mTOR pathway senses nutrient availability and is critical for nutrient-stimulated muscle growth, and proteins downstream of mTOR including 4EBP1, eIF4E, RPS6 and eEF2 control the initiation and elongation phases of mRNA translation (Wang & Proud, 2006). The relative content (µg protein$^{-1}$) of both the native and phosphorylated forms of all the proteins evaluated (eIF4E, 4EBP1, RPS6 and eEF2) was increased 144 to 222% in muscle from twin compared to single lambs, with the exception of RPS6-Ser235/236 which did not differ. However, the absolute abundance of these translational components per muscle was reduced 46 to 55% in twins compared to singles, suggesting decreased capacity for protein synthesis consistent with decreased muscle mass in twins. The ratio of phosphorylated to native protein for each of the targets did not differ, suggesting that the short-term activation of mRNA translation did not differ between singles and twins. However, mTOR also increases the translational capacity of the cell in the longer-term by increasing the number of ribosomes and other translation components (Wang & Proud, 2006). Thus, using total RNA as an estimate of ribosome number, we observed in twins a decrease in the abundance of total eEF2, 4EBP1, and eIF4E per ribosome (Figure 1), consistent with decreased translational capacity in muscle from twin compared to single fetuses. We suggest that long-term restriction of nutrient availability to twin compared to singleton fetuses in late gestation down-regulates mTOR signalling in late gestation, leading to reduced ribosome number and abundance of the translational machinery and ultimately leads to retarded myofibre hypertrophy and muscle mass.

These potential mechanisms warrant further investigation under differing feeding regimes to determine whether nutrient availability is a key driver or whether other environmental signals are involved. The ability of mTOR to link environmental cues to ribosome biogenesis provides

**TABLE 1:** Characteristics of *M. semitendinosus* in singleton and twin foetuses (n = 9 per group) from maintenance-fed ewes at 140 days gestation. Data are represented as the mean ± standard error of the mean.

| Characteristic | Singletons | Twins | P value |
|---|---|---|---|
| Body weight (kg) | 6.4 ± 0.2 | 4.8 ± 0.1 | <0.001 |
| *M. Semitendinosus* weight (g) | 11.4 ± 0.4 | 6.6 ± 0.5 | <0.001 |
| Total DNA per muscle (mg) | 34.9 ± 2.9 | 23.9 ± 2.1 | 0.008 |
| Total RNA per muscle (mg) | 15.7 ± 0.6 | 9.6 ± 0.7 | <0.001 |
| Total protein per muscle (mg) | 358 ± 24 | 182 ± 12 | <0.001 |
| Protein : RNA | 23.0 ± 1.2 | 19.1 ± 0.4 | 0.008 |
| Protein : DNA | 11.0 ± 1.2 | 7.9 ± 0.5 | 0.039 |

**FIGURE 1:** Total abundance of 4EBP1, RPS6, eIF4E and eEF2 per ribosome (µg protein.RNA$^{-1}$) in twin foetal lambs at 140 days gestation, expressed as a percentage relative to singletons. The graph shows the mean ± standard error, n = 9/treatment; Solid bars = Singletons; Open bars = Twins.



an efficient mechanism for skeletal muscle cells to alter their overall protein biosynthetic capacity.

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