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Maternal Nutrient Restriction During Late Gestation and Early Postnatal Growth in Sheep Differentially Reset the Control of Energy Metabolism in the Gastric Mucosa

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Fetal growth restriction followed by accelerated postnatal growth contributes to impaired metabolic function in adulthood. The extent to which these outcomes may be mediated centrally within the hypothalamus, as opposed to in the periphery within the digestive tract, remains unknown. In a sheep model, we achieved intrauterine growth restriction experimentally by maternal nutrient restriction (R) that involved a 40% reduction in food intake through late gestation. R offspring were then either reared singly to accelerate postnatal growth (RA) or as twins and compared with controls also reared singly. From weaning, all offspring were maintained indoors until adulthood. A reduced litter size accelerated postnatal growth for only the first month of lactation. Independently from postnatal weight gain and later fat mass, R animals developed insulin resistance as adults. However, restricted accelerated offspring compared with both the control accelerated and restricted restricted offspring ate less and had higher fasting plasma leptin as adults, an adaptation which was accompanied by changes in energy sensing and cell proliferation within the abomasum. Additionally, although fetal restriction down-regulated gene expression of mammalian target of rapamycin and carnitine palmitoyltransferase 1-dependent pathways in the abomasum, RA offspring compensated for this by exhibiting greater activity of AMP-activated kinase-dependent pathways. This study demonstrates a role for perinatal nutrition in the peripheral control of food intake and in energy sensing in the gastric mucosal and emphasizes the importance of diet in early life in regulating energy metabolism during adulthood. (Endocrinology 152: 2816–2826, 2011)

The low birth weight newborn, especially when experiencing rapid postnatal growth, can be at greater risk of developing the metabolic syndrome later in life (1, 2). This relationship can reflect long-term fetal and postnatal adaptations to the nutritional environment (3), although the consequences for the regulation of energy homeostasis have yet to be established. Fetal growth is greatest over the final quarter of gestation, which is the time when endocrine functions linked to maturation of the hypothalamus are established (3, 4). It is also the period during which changes in maternal diet can have the greatest effect on birth weight in both sheep (5) and humans (6).
Nutritionally programmed changes in the control of appetite due to interactions between trophic, energetic, hormonal, and epigenetic factors (7) have been described in both rodents (8, 9) and sheep (10), and these may ultimately determine the long-term regulation of energy balance. They involve changes in leptin sensitivity (11) and expression of a range of hypothalamic neuropeptides, including proopiomelanocortin, neuropeptide Y (NPY), and the melanocortin 4 receptor (MC4R) (12). To date, these observations in sheep have been established after nutritional challenges specifically targeted during organogenesis of the fetal hypothalamus (i.e. early- to midgestation). Such early interventions do not, however, affect birth weight or postnatal growth (10). The influences of maternal nutrient restriction (R) during late gestation on the long-term control of food intake and metabolic health, therefore, remain to be fully established.

Currently, little is known about the potential programming outcomes in peripheral organs that also control appetite and regulate whole body energy homeostasis. This is surprising, considering the critical influence the perinatal period can have on the development of the gastrointestinal tract, a major complex organ actively involved in the control of energy balance (13). Indeed, the development of the stomach in utero is partly dependent upon fetal swallowing of amniotic fluid (14), which is regulated centrally and is confined to periods of fetal breathing that are, in turn, influenced by maternal energy intake (15) and neuroendocrine factors such as NPY (14). Immediately after birth, the gastrointestinal tract undergoes a pronounced transformation exhibiting rapid growth and a marked increase in acid production (16), at a time when milk intake and composition can both determine gastric barrier function (17). However, the extent to which changes in maternal food intake can contribute to long-term changes in gut function remains to be fully established.

Critically, both centrally, i.e. in the hypothalamus and in peripheral tissues, the control of food intake is regulated through common energy-sensing pathways that integrate cellular energy concentration (i.e. AMP:ATP ratio) and endocrine signals. For example, the leptinaemic signal to reduce food intake only occurs at high cellular concentrations of ATP (18, 19). Conversely, in the gastric mucosa, the hunger hormone ghrelin is only synthesized when ATP concentrations are depleted (20). This endocrine energy-sensing cross talk is governed by two proteins, AMP-activated kinase (AMPK) and the mammalian target of rapamycin (mTOR), which are also closely linked in the regulation of oxidative and inflammatory processes (19, 21, 22). The activity and intensity of AMPK and mTOR responses are dependent on mitochondrial activity that involves the transcription factors carnitine palmitoyltransferase protein (CPT1) (23) and peroxysome proliferator-activated receptor (PPARγ) that favor fatty acid transport and β-oxidation into the mitochondria. In addition, the contents of mitochondria are regulated, in part, by the mitochondrial biogenitor PPARγ coactivator 1 (PGC1α) (24). Furthermore, although it has been established that ghrelin production in the stomach is regulated by mTOR-dependent pathways (20, 22), whether this can be set in early life is unknown. The extent to which exposure to a chronic low caloric nutritional environment during late gestation, a critical period in the acquisition of fetal endocrine functions (3, 25), can program tissue energy sensitivity was a further focus of the present study.

Materials and Methods

Animals and experimental design

All animal procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act, 1986 with approval from the Local Ethics Committee of the University of Nottingham (Nottingham, UK).

Experimental design

A summary of the animal protocol is illustrated in Supplemental Fig. 1, published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org.

Dietary intervention in pregnancy

At 100 d of gestation (dGA), 26 Bluefaced Leicester cross Swaledale twin bearing sheep were individually housed, and on dGA 110, they were randomly allocated to one of two nutrition groups until normal delivery at term (~145 ± 2 d). Thus, a control (C) group of nine pregnant sheep was fed to completely meet their metabolisable energy (ME) requirements (i.e. from 0.56 MJ/kg·body weight0.75 at dGA 110 increasing to 0.72 MJ/kg·body weight0.75 at dGA 130) (26), whereas the remaining 17 pregnant sheep were all R and fed a diet 60% of this amount. Each pregnant sheep was weighed on a weekly basis before feeding, after which their total food requirements were adjusted as necessary. The diet compromised a mix of 40% concentrated pellets and 60% straw nuts (Manor Farm Feeds, Oakham, UK) that had an estimated ME content of 12.6 MJ/kg and a crude protein content (nitrogen × 6.25) of 162 g/kg and 8.6 MJ/kg and a crude protein content of 69 g/kg dry matter, respectively. In addition, all pregnant sheep had free access to a mineral block to ensure adequate micronutrient supply.

Lactation

All mothers were fed to fully meet their ME requirements throughout lactation. Within 12 h of birth, each pair of twins was either separated, with only one twin being reared by its mother to accelerate postnatal growth, or reared together by their mother as twins (R). All nine animals successfully completed the study in each of the control accelerated (CA) and restricted accelerated (RA) groups (CA: four females, five males; RA: seven females, two males). Both offspring born to restricted restricted
(RR) mothers were kept together with their mothers through to weaning, but only one twin from each mother was subsequently placed in an obeseogenic environment (see below, six females and two males). Offspring were weaned twice a week during the first month of postnatal life and once a week thereafter until weaning. For ethical considerations intended to reduce the number of animal used in such studies, the remaining twin of the R pair was not used in this experiment but allocated to an independent study (see Supplemental Fig. 1).

Postweaning
All offspring were weaned at 3 months of age. All were raised in the same restricted indoor environment thereafter to promote obesity (27) and weighed monthly.

Fractional growth rate (FGR) from birth to 40 d of age and from 3 to 7 months of age was determined in each animal using the following formula:

\[
\text{FGR 1–40 d} = \frac{\text{weight at 40 d} - \text{weight at birth}}{\text{weight at birth}}
\]

\[
\text{FGR 3–7 months} = \frac{\text{weight at 7 months} - \text{weight at 3 months}}{\text{weight at 3 months}}
\]

Blood samples (5 ml) were collected in the morning before feeding at 3, 7, and 16 months of age from each animal, collected from the jugular vein into heparinized and K$_{2}$EDTA-coated tubes. The plasma was immediately separated by centrifugation (2500 g × 10 min at 4 C) and stored at −80 C until analysis. Additional blood sampling over a 24-h period, at 2, 4, 8, and 24 h after feeding, was undertaken at 16 months of age. In addition, glucose tolerance tests were undertaken on all offspring at 8 and 17 months of age after an overnight fast and the area under the curve (AUC) calculated (10).

Measurement of food intake
At 16 months of age, for 2 wk before the end of the study, all offspring were housed individually indoors in United Kingdom Home Office designated floor pens (3 m$^2$) to monitor food intake and appetite. Each animal, daily energy intake was assessed through weighed intake and food refusal when offered sufficient energy for the 24-h period based on a mix of low (straw nuts, 8.9 MJ/kg) and 800 g of high (concentrate pellets, 12.6 MJ/kg) energy-dense food.

Physical activity
The level of spontaneous physical activity at adulthood was determined using uniaxial accelerometers (Actiwatch; Linton Instrumentation, Diss, UK) (10). A ratio between physical activity and food intake was calculated at 16 months of age as previously described (10).

Body composition
Total body fat, fat free mass, and bone mineral density was determined at 8 and 16 months of age when the animal was sedated (im injection of 1.5 mg · kg$^{-1}$ ketamine with 0.1 mg · kg$^{-1}$ xylazine) and scanned in a transverse position using a Lunar DPX-L (fast-detail whole body smartscan).

Postmortem analysis
At 17 months of age, the animals were humanely euthanized by electrical stunning and exsanguination after an overnight fast. All the major organs were weighed and representative samples snap frozen in liquid nitrogen and stored at −80 C as was the entire hypothalamus of each animal (10).

Laboratory analysis

**Plasma metabolite and hormones**
Glucose, nonesterified fatty acid and triacylglycerol concentrations were determined by colorimetric assays (Randox, Crumlin, UK). Insulin was assayed using an ovine-specific ELISA (Mericodis; Diagnostics Ltd., Milton Keynes, UK), and leptin was determined by a radio-immunoassay (10) as was cortisol (Diagnostic Products Corporation coat-a-count; Siemens, Camberley, UK).

**Gene expression**
This was determined by real-time RT-PCR. Total RNA was extracted from entire hypothalamus, abomasum, omental, and sc adipose tissue using the Chomczynski and Sacchi method (28, 29) with deoxyribonuclease treatment (Rneasy Plus mini kit; QIAGEN, Crawley, UK). It was then reverse transcribed (Superscript II reverse transcriptase; Invitrogen Ltd., Pasley, UK) and cDNA amplified on a real-time thermocycler (Quantica; Techne, Inc., Barloword Scientific Ltd., Stone, UK) using SYBR green based Taq polymerase reaction mix (ABSolute blue QPCR SYBR green; Thermo Scientific, Epsom, UK). Product specificity for every pair of primers was confirmed by sequencing and the efficiency of the primer sets, within the range of requested DNA amplification, assessed and optimal efficiency established (2.00 ± 5%). Hypothalamus were analyzed for insulin receptor, leptin receptor (ObR), tyrosine-protein phosphatase nonreceptor (PTP1B), adiponectin receptors (AdnR) (AdnR1 and AdnR2), suppressor of cytokine signaling, ghrelin receptor, melanocortin receptors (MC4R and MC3R), agouti-related peptide, NPY, fat mass and obesity-associated protein (FTO), AMPKα, and mTOR. Abomasal samples were analyzed for ghrelin, leptin, AMPKα2, acetyl coenzyme A carboxylase (ACCα), dinucleotide methyl transferase type I, FTO, AMPKα, FTO, mTOR, CPT1, ObR, insulin receptor, uncoupling protein 2 (UCP2), PPARγ, PGC1α, and sirtuin (SIRT1). Omental and sc adipose tissues were analyzed for leptin, adiponectin, IL-6, IL-18, TNFα, toll-like receptor 4, glucose-related peptide 78, FTO, and macrophage chemoattractant protein 1. Ribosomal 18S RNA and ribosomal protein large P0 were used as housekeeping genes. Gene expression was determined by using the 2$^{-\Delta\DeltaCT}$ calculation.

**Protein abundance**
AMPKα and phospho-AMPKα were analyzed by immunoblotting. Total proteins were extracted from abomasal samples (30) using ice-cold lysis buffers (Sigma, Hertfordshire, UK) and protease inhibitors. After reduction and denaturation with sodium dodecyl sulfate at 100 C, 10 μg of protein extracts were separated by vertical, polyacrylamide-based electrophoresis. After migration and electrical protein transfer, nitrocellulose membranes were blocked overnight and blotted with antibodies for either AMPKα, phospho-AMPKα (catalog no. 23A3 and Thr172, respectively; Cell Signaling, Hitchin, UK), or β-actin (Abcam, Cambridge, UK). Relative protein abundance was then determined using chemiluminescence (Immobilon Western Chemiluminescence; Millipore, Watford, UK) and the image analyzed (Aida Image Analyzer; Raytek Scientific Ltd., Sheffield, UK).
Maternal R in late gestation induced IUGR. Influence of a 40% R on birth weight of (A) siblings and (B) standardized Z score for birth weight. Values are mean ± sem and significant differences between groups, *** P < 0.001.

**Immunohistochemistry**

**Tissue fixation**

Snap-frozen abomasal samples that had been stored at −80 C were fixed. Before fixation, each sample was gradually thawed by first placing it at −20 C for 24 h and then for 12 h at 4 C. They were fixed with 10% saline formaldehyde solution and embedded in paraffin wax with 5-μm microtome sections used for subsequent analysis. Hematoxylin and eosin staining was performed to confirm that tissue integrity was maintained.

**Cell proliferation**

These sections were also immunostained to determine the relative expression of the proliferating cell nuclear antigen (PCNA) (Abcam) (27). Staining was carried out on the Bondmax histology system using Bond Polymer Refine Detection System (DS9800; Vision Biosystems, Mount Waverley, Australia) and Bond software version 3.4A. Briefly, slides are heated and stained as follows: 5 min on a peroxide block, 15 min with primary antibody, 8 min with secondary antibody, 10 min with 3,3-diaminobenzidine, and 5-min counterstaining with hematoxylin and eosin. Negative slides were run in parallel, with the exclusion of the primary antibody. Slides were imaged using a Nikon Eclipse 90i microscope (Nikon, Surrey, UK) with charge-coupled device high-speed color camera (Micropublisher 3.3RTV; Qimaging, Surrey, British Columbia, Canada) and analyzed using Volocity 4 (version 4.2.1; Improvision, Coventry, UK) quantification software. To ensure uniformity of staining using the automated protocol, all animals were analyzed at the same time with the investigator blinded to nutritional group.

**Detection of apoptosis**

Terminal transferase-mediated 2’-deoxyuridine, 5’-triphosphate nick end labeling (TUNEL) was determined using a fluorescence Nikon Eclipse 90i microscope using a similar protocol to that described above.

**Statistical analysis**

Statistical analysis of the data were performed using PASW statistics software (version 17.02; IBM, Chicago, IL). Kolmogorov-Smirnoff tests were realized on every parameter analyzed to determine the Gaussian distributions of the variables. The influence of maternal nutrition (R vs. C) or early postnatal growth (RA vs. RR) was determined, according to parametric distribution, using unpaired Student’s t or Mann-Whitney U tests. Data are expressed as mean values with their se. To address the limitations of multiple testing, statistical trend was accepted with a 95% interval of confidence (P < 0.05), and significance was accepted with a confidence interval of 99% (P < 0.01). Each variable was tested for gender. Only body weight and fat mass after birth differed between males and females, and no other factors were found to be affected. Moreover, comparison for each variable between groups for females only demonstrated similar outcomes.

**Results**

**Birth weight and postnatal growth**

A 40% caloric restriction in late gestation had no effect on length of gestation.
Body composition and metabolic parameters

Offspring born to C and R mothers exhibited similar total body composition as determined by dual-energy x-ray absorptiometry scanning at 8 and 16 months, and there was no difference between groups in the weight of visceral fat depots (i.e., omental, perirenal, and pericardial) at postmortem (Table 1). At 8 months of age, RA offspring exhibited signs of insulin resistance with an enhanced insulin, but not glucose, AUC, which was maintained up to 16 months of age when both the RA and RR offspring were insulin resistant compared with C. Fasting blood samples were analyzed to assess whether the circulating metabolic profile was programmed by prenatal diet, and R animals exhibited raised plasma insulin, but reduced triacylglycerol, only as adults (Table 2).

Characteristics of energy homeostasis in the adult offspring

With increased age and fat mass, plasma leptin rose in all groups. However, in the adults, fasting plasma leptin further increased in RA offspring (Table 2), a difference that persisted over the 24-h sampling period (Fig. 3A).

### Table 1. Influence of fetal growth restriction and accelerated postnatal growth on insulin sensitivity and fat mass

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>CA</th>
<th>RA</th>
<th>RR</th>
<th>IUGR</th>
<th>Postnatal growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC insulin (a.u.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>38.3 ± 10</td>
<td>64.0 ± 11</td>
<td>32.6 ± 6.9</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>16</td>
<td>53.1 ± 15</td>
<td>107 ± 37</td>
<td>91.1 ± 47</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>AUC glucose (a.u.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1118 ± 121</td>
<td>1015 ± 189</td>
<td>1233 ± 227</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>602 ± 322</td>
<td>831 ± 345</td>
<td>803 ± 461</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Total fat mass (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>11.7 ± 7.2</td>
<td>15.3 ± 3.2</td>
<td>15.1 ± 3.2</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>18.2 ± 7.3</td>
<td>21.8 ± 7.0</td>
<td>20.0 ± 3.3</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Postmortem analysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total perirenal fat (g)</td>
<td>960 ± 162</td>
<td>1069 ± 240</td>
<td>939 ± 113</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Total omental fat (g)</td>
<td>1353 ± 162</td>
<td>1563 ± 381</td>
<td>1509 ± 195</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SEM. a.u., Arbitrary units. Full details of pre- and postnatal nutritional interventions are included in Materials and Methods.

### Table 2. Plasma concentrations of metabolites and hormones as measured at immediately prior to daily feeding at 0900 h in the growing offspring at 3, 8, and 16 months of age

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>CA</th>
<th>RA</th>
<th>RR</th>
<th>IUGR</th>
<th>Postnatal growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3.0 ± 0.17</td>
<td>2.8 ± 0.10</td>
<td>2.6 ± 0.10</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>3.4 ± 0.34</td>
<td>4.0 ± 0.18</td>
<td>3.0 ± 0.42</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>2.9 ± 0.25</td>
<td>2.8 ± 0.34</td>
<td>3.1 ± 0.09</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>NEFA (nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.76 ± 0.44</td>
<td>0.76 ± 0.31</td>
<td>0.78 ± 0.44</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1.34 ± 0.07</td>
<td>1.01 ± 0.11</td>
<td>1.27 ± 0.08</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>0.67 ± 0.05</td>
<td>0.48 ± 0.07</td>
<td>0.48 ± 0.08</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Triacylglycerol (mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.46 ± 0.06</td>
<td>0.38 ± 0.04</td>
<td>0.36 ± 0.09</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.18 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.18 ± 0.04</td>
<td>—</td>
<td></td>
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<tr>
<td>16</td>
<td>0.19 ± 0.02</td>
<td>0.09 ± 0.01</td>
<td>0.12 ± 0.02</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Cortisol (nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>34.1 ± 3.4</td>
<td>48.6 ± 13.8</td>
<td>49.2 ± 10.3</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>66.8 ± 18.0</td>
<td>62.1 ± 12.6</td>
<td>59.5 ± 10.3</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>38.8 ± 8.4</td>
<td>55.5 ± 13.9</td>
<td>45.0 ± 10.0</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.7 ± 0.11</td>
<td>0.5 ± 0.10</td>
<td>0.7 ± 0.10</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1.8 ± 0.22</td>
<td>2.4 ± 0.45</td>
<td>2.1 ± 0.40</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>3.8 ± 0.60</td>
<td>6.2 ± 0.94</td>
<td>3.7 ± 0.47</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Insulin (pM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>12.0 ± 1.7</td>
<td>12.0 ± 2.0</td>
<td>13.7 ± 1.7</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>25.8 ± 2.1</td>
<td>29.2 ± 2.0</td>
<td>27.5 ± 2.4</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>17.2 ± 2.1</td>
<td>25.8 ± 1.9</td>
<td>25.8 ± 6.8</td>
<td>&lt;0.05</td>
<td>—</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Full details of pre- and postnatal nutritional interventions are included in Materials and Methods. NEFA, Nonesterified fatty acid.
In addition, a significant positive correlation between plasma leptin with early growth was observed (CA: R² = 0.77, P = 0.001; RR: R² = 0.77, P = 0.001). In contrast, gastric ghrelin and leptin mRNA expression were not affected by either maternal diet or early postnatal growth (Fig. 3B and Table 3). Measurement of daily food intake and 24-h activity profiles demonstrated that the RA group consumed less food at 16 months of age (Fig. 3C), which is likely to be mediated by the raised plasma leptin in these animals compared with CA and RR groups (Fig. 3A). There were no differences in physical activity or the ratio of physical activity to food intake (Fig. 3D). Although in rodent models of diet-induced obesity reduced insulin sensitivity frequently arises from a chronic proinflammation that originates from hyperplastic adipose tissue, gene expression analysis in both the sc and omental fat depots in the present study was similar between groups as was adipocyte size, and there are no obvious signs of exaggerated crown-like structures in any samples (data not shown).

**Discussion**

The present study demonstrates that, in a large animal model known to share important similarities with humans in the outcomes of fetal programming, growth rate immediately after birth was an important determinant of adult energy homeostasis. Growth-restricted fetuses born to R mothers were, therefore, insulin resistant and exhibited specific impairments in the regulation of food intake that were accompanied with long-term changes in the activity of the abomasum.

**The animal model**

The strength of sheep as a model for the human lies in the fact that time courses of fetal and perinatal development are comparable, particularly in the establishment of hypothalamic neural networks and the rapid growth of adipose tissue during late gestation. It must, however, be noted that ruminant sheep differ from monogastric omn-
nivorous humans in their short-term control of feeding. Nevertheless, the fourth stomach chamber in sheep, the abomasum, acts as does the stomach of monogastric mammals and is the main site for ghrelin production (33), although there is limited information in sheep. The similarities between humans and sheep in the nutritional control of abomasal ghrelin synthesis clearly suggest a role in the control of food intake (34). Critically, during the early postnatal period when young sheep only feed on milk, an esophageal groove exists, and they only use their true stomach (i.e. the abomasum) to digest milk making them comparable with human infants. Both pregnant women and sheep are prone to maternal catabolism in the last third of gestation (5, 35), and this may act to maintain fetal and visceral fat mass, adipocyte morphology or gene expression. No change in hypothalamic expression of the AgRP or any other neurotransmitters was observed to influence the regulation of energy balance. Adult offspring born to R mothers ate less than C, an outcome that was abolished in the RR offspring, i.e. those in which early postnatal growth was reduced. In addition, RA offspring exhibited higher plasma leptin, which could partially explain their lower food intake. Future studies will now be needed to determine whether these adaptations are mediated by changes in leptin production from adipose tissue and/or the development of leptin resistance. We found no influence of perinatal weight gain on either total or visceral fat mass and insulin sensitivity and postnatal growth in the term growth restricted newborn are less clear. The results of the present study suggest that intrauterine growth restriction (IUGR) and rapid postnatal growth may not represent separate risk factors for insulin resistance later in life.

Despite having a modest effect on the onset of insulin resistance, weight gain in the first month of life specifically influenced the regulation of energy balance. Adult offspring born to R mothers ate less than C, an outcome that was abolished in the RR offspring, i.e. those in which early postnatal growth was reduced. In addition, RA offspring exhibited higher plasma leptin, which could partially explain their lower food intake. Future studies will now be needed to determine whether these adaptations are mediated by changes in leptin production from adipose tissue and/or the development of leptin resistance. We found no influence of perinatal weight gain on either total or visceral fat mass, adipocyte morphology or gene expression that would support a programmed response in white adipose tissue. No change in hypothalamic expression of the ObR or any other neurotransmitters was observed to indicate the development of central leptin resistance. Moreover, despite exhibiting enhanced gene expression for AMPK, mTOR, PTP1B, MC3R, and FTO in relation to IUGR, the activity of the hypothalamus remained surprisingly unaffected by postnatal growth. The hypothalamic changes we observed may, in fact, be secondary to those in
must, however, acknowledge that in both these experiments, the entire hypothalamus was used. This has been adopted by several other groups (40, 41), and future analysis may need to focus on specific regions within the different regulatory nuclei.

**IUGR reduces gastric energy sensitivity**

We have demonstrated the influence of the early life nutritional environment on programming of the gastrointestinal tract as gene expression of constitutive mTOR, plus SIRT1, CPT1, and UCP2 were all halved in offspring born to R mothers, irrespective of postnatal growth rate. These adaptations are likely to be critical as mTOR signaling integrates cellular pathways regulating gastric ghrelin synthesis and proinflammatory processes (42–44). When energy production increases within the gut, a parallel rise in mTOR, by inhibiting ghrelin synthesis, reduces food intake (20). Our findings, therefore, suggest that chronic energy restriction during late gestation reduces mTOR action in the stomach with the potential to either increase energy intake or enhance the rate of nutrient exchange across the gut.

Fetal growth restriction was also accompanied by a reduction in SIRT1, UCP2, and CPT1 gene expression, together with histological evidence of impaired gastric mucosal integrity. Taken together, these adaptations are indicative of a prooxidative status with SIRT1, UCP2, and CPT1 acting to reduce intracytoplasmic reactive oxygen species concentration (45), although the precise mechanisms involved remain an area of intense debate (46, 47). Importantly, our study provides novel mechanistic insights into how epidemiological findings of a link between low birth weight, gastroesophageal reflux, or carcinoma (48–50) may occur.

**Protective effect of postnatal growth restriction on later gastric function**

One further outcome in our study was that, despite gene expression of mTOR, SIRT1, UCP2, and CPT1 being down-regulated in all offspring born to R mothers, the

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**FIG. 4.** Perinatal programming of energy sensing in the adult offspring at 17 months of age. Influence of maternal nutrition on gene expression in the (A) on the adult hypothalamus and (B and C) abomasum. CA, Born to C-fed sheep and reared as a singleton; RA, born to R sheep and reared as a singleton; and RR, born to R sheep and reared as a twin. Values are mean ± SEM and significant differences between groups, CA and RA: *, P < 0.05; RA and RR: a vs. b, P < 0.05; a vs. c, P < 0.01.
adverse histological outcomes in terms of PCNA staining were not found in those animals, which did not increase their growth during early lactation. We propose that this apparent protective effect was related to adaptations in gene expression regulating gastric energy metabolism as indicated by overexpression of those involved in AMPK/ACC-related pathways. As a consequence, these offspring would be able to stimulate mitochondrial biogenesis after increased PGC1α and cellular energy intake and oxidation due to raised glucose transporter 1 and PPARγ. At the same time, enhanced leptin sensitivity induced by a higher ObR abundance would ultimately reduce prooxidative pathways and favor gastric integrity (44, 51). The mechanistic basis for differential epigenetic resetting as suggested by constitutive changes in gene expression remains largely unexplained, and control mechanisms appear to differ greatly between genes in the same tissue (52). Intriguingly, we found these transcriptional changes to be associated with dinucleotide methyl transferase type I and FTO, two factors that can regulate DNA methylation (53, 54). Furthermore, FTO has been established to encode a 2-oxoglutarate DNA demethylase and provides a link between energy-sensing pathways (55–57) that could include be further modulated by changes in short-chain fatty acid production (58), which are the main products of rumination.

In conclusion, restricted maternal food intake during late gestation, which is sufficient to induce IUGR, resulted in a long-term resetting of energy homeostasis. Small birth weight offspring were predisposed to insulin resistance, a response which was not amplified with rapid postnatal growth but was accompanied with long-term changes in the control of food intake. This was linked to a programmed adaptation in the abomasum but not in the hypothalamus. Critically, both fetal growth restriction and the timing of postnatal growth promotion in the IUGR offspring had a large impact upon mTOR and AMPK-dependent energy-sensing pathways, which could influence cell proliferation in the abomasum. These observations could be clinically relevant and suggest that very early nutrition should have a major long-term effect in the gastrointestinal organs involved in the control of food intake.

FIG. 5. Fetal IUGR and accelerated postnatal growth compromise gastric structure. Tissue remodeling as determined in the 17-month-old offspring. Representative examples (×10 magnification) of (A) PCNA and (B) TUNEL staining are shown together with relative intensity analysis. CA, Born to C-fed sheep and reared as a singleton; RA, born to R sheep and reared as a singleton; and RR, born to R sheep and reared as a twin. Values are mean ± SEM and significant differences between groups, RA and RR: a vs. b, P < 0.05.

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References
43. Osawa H 2008 Ghrelin and Helicobacter pylori infection. World J Gastroenterol 14:6327–6333
58. Song IS, Ha GH, Kim JM, Jeong SY, Lee HC, Kim YS, Kim YJ, Kwon TK, Kim NS 4 March 2011 Human ZNF312b oncogene is regulated by Sp1 binding to its promoter region through DNA demethylation and histone acetylation in gastric cancer. Int J Cancer 10.1002/jic.25871