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RESEARCH ARTICLE | *Role of Fetal Programming and Epigenetic Regulation on the Development of Endocrine and Metabolic Alterations*

Maternal methyl donor and cofactor supplementation in late pregnancy increases β-cell numbers at 16 days of life in growth-restricted twin lambs

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Sulaiman SA, De Blasio MJ, Harland ML, Gatford KL, Owens JA. Maternal methyl donor and cofactor supplementation in late pregnancy increases β-cell numbers at 16 days of life in growth-restricted twin lambs. *Am J Physiol Endocrinol Metab* 313: E381–E390, 2017. First published July 5, 2017; doi:10.1152/ajpendo.00033.2017.—Restricted growth before birth (IUGR) increases adult risk of Type 2 diabetes by impairing insulin sensitivity and secretion. Altered fetal one-carbon metabolism is implicated in developmental programming of adult health and disease by IUGR. Therefore, we evaluated effects of maternal dietary supplementation with methyl donors and cofactors (MMDS), designed to increase fetal supply, on insulin action in the spontaneously IUGR twin lamb. In vivo glucose-stimulated insulin secretion and insulin sensitivity were measured at *days 12–14* in singleton controls (CON, $n = 7$ lambs from 7 ewes), twins (IUGR, $n = 8$ lambs from 8 ewes), and twins from ewes that received MMDS (2 g rumen-protected methionine, 300 mg folic acid, 1.2 g sulfur, 0.7 mg cobalt) daily from 120 days after mating (~0.8 of term) until delivery (IUGR+MMDS, $n = 8$ lambs from 4 ewes). Body composition and pancreas morphometry were assessed in lambs at *day 16*. IUGR reduced size at birth and increased neonatal fractional growth rate. MMDS normalized long bone lengths but not other body dimensions of IUGR lambs at birth. IUGR did not impair glucose control or insulin action at *days 12–14*, compared with controls. MMDS increased metabolic clearance rate of insulin and increased β-cell numerical density and tended to improve insulin sensitivity, compared with untreated IUGR lambs. This demonstrates that effects of late-pregnancy methyl donor supplementation persist until at least the third week of life. Whether these effects of MMDS persist beyond early postnatal life and improve metabolic outcomes after IUGR in adults and the underlying mechanisms remain to be determined.

sheep, intrauterine growth restriction; one-carbon metabolism; pancreas; insulin

SMALL SIZE AT BIRTH and intrauterine growth restriction (IUGR) consistently predict an increased risk of Type 2 diabetes mellitus (T2D) in humans (39, 59). This relationship persists when length of gestation is corrected for (24), and is substantial, with small size at birth accounting for ~18% of the lifetime risk of T2D in one population study (10). Impaired sensitivity and inadequate insulin secretion are both implicated in the increased risk of T2D after IUGR in human cohorts (39). Restriction of placental growth and function is a major cause of IUGR in humans (47), and when induced experimentally in other species, it reduces insulin sensitivity and secretion in offspring (17, 42, 49). Epigenetic mechanisms are proposed to partly underlie the impact of perturbations of the environment in fetal life on adult health and disease, including on insulin action (48). In the rat, IUGR induced surgically by uterine artery ligation in late pregnancy reduces the abundance of methyl donors and alters expression and activity of the enzymes that regulate histone acetylation and methylation and DNA methylation in fetal and postnatal offspring (34). These changes are associated with epigenetic changes and altered gene expression in tissues, including liver (12–14), pancreatic islets (43), and brain (26, 27) in late gestation, at birth, and in young progeny. Impaired glucose control that worsens with aging in the IUGR rat can be explained, at least in part, by progressive epigenetic modifications occurring in pancreatic islets at the gene promoter of *Pdx1*, a regulator of β-cell replication and function, and consequent reduced *Pdx1* expression and impaired insulin secretion (43).

Consistent with the suggestion of altered one-carbon metabolism pathway as a mechanism for effects of IUGR on later metabolism, feeding of diets deficient in folate (a one-carbon donor) and/or vitamin B_{12} (a required cofactor for methionine synthase) to Wistar rat dams and progeny increased visceral obesity of progeny from young adulthood onward (31). Also, in rats, periconceptional maternal methyl deficiency impaired glucose tolerance, with evidence of insulin resistance, in male but not female progeny (35). In sheep, periconceptional maternal deficiency of dietary sulfur (required for ruminal synthesis of one-carbon donors cysteine and methionine; 50) and cobalt (required for ruminal synthesis of vitamin B_{12}, 41), reduced maternal circulating concentrations of folate, methionine, and vitamin B_{12}, and elevated maternal circulating concentrations of homocysteine (50). Embryos transferred from these ewes into recipients on *day 6* after fertilization, and, hence, exposed to methyl deficiency during the periconceptional period only, had increased weight, fatness, and blood pressure and impaired insulin sensitivity as adults in males but not females, compared with progeny from embryos of control dams (50). Epidemiological evidence also indicates that adverse postnatal consequences follow reduced supply of one-carbon donors and cofactors required for their metabolism during pregnancy. Low maternal plasma vitamin B_{12} concentrations at 18 wk of pregnancy predicted greater HOMA-IR, an index of insulin resistance, in 6-yr-old children (61).

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In addition to the adverse effects of one-carbon donor deficiency, a systematic review and meta-analysis showed that folic acid supplementation in healthy pregnant women reduced the risk of having preterm birth and IUGR (62). In experimental animal models of IUGR, maternal folic acid supplementation throughout pregnancy prevented the adverse outcomes of IUGR in the offspring, specifically for hepatic metabolism and gene regulation (32, 33). Potential mechanisms for beneficial effects of one-carbon donor supplementation include improved fetal nutrient supply and, hence, reduced homocysteine and increased methyl group supply for nucleotide synthesis and methylation of DNA and histones, although these have not been measured in these animal models. Some methyl donors, such as betaine and folic acid, may also act as antioxidants (8, 33). In a meta-analysis of supplementation trials, folate intake of pregnant women was positively correlated with birth weight, with a similar trend ($P = 0.08$) for placental weight (11), while maternal vitamin B_{12} deficiency is associated with maternal adiposity and insulin resistance in human cohorts (28, 30). MMDS supplements may, therefore, potentially program progeny through maternal or placental responses, as well as direct effects on fetal nutrient supply. Nevertheless, there is good evidence that maternal one-carbon donor supplementation induces epigenetic effects involving altered DNA methylation in progeny, consistent with increased fetal supply of one-carbon donors as an important mechanism underlying programming by MMDS. Expression of the agouti viable yellow (A^{vy}) allele in mice induces yellow hair pigmentation and is associated with postnatal hyperphagia and obesity (58). Feeding these mice a methyl-supplemented diet (control diet plus folic acid, betaine, choline, and vitamin B_{12}) increased DNA methylation at the A^{vy} locus, reduced expression of the agouti allele and obesity in the first generation of progeny, and also prevented intergenerational amplification of obesity when dams were fed a high-fat diet (58).

On the basis of these previous findings, we hypothesized that increasing maternal circulating concentrations of one-carbon donors and cofactors during rapid fetal growth and high nutrient demand in late pregnancy would partially prevent adverse effects of a constrained fetal environment due to twinning on glucose metabolism and insulin action in young offspring. This period was selected both because relatively rapid fetal growth after the plateau in placental growth in sheep means that nutrient supply is most likely to be limiting during this period (2) and because studies in rodents have identified late gestation as a period when progeny metabolism is vulnerable to programming by restricted fetal nutrition, including via epigenetic processes (43, 49). Therefore, we compared growth, glucose metabolism, and its determinants in singleton control lambs and in naturally growth-restricted twin lambs from unsupplemented ewes and ewes fed a methyl donor and cofactor supplement from 0.8 of gestation until delivery at term.

MATERIALS AND METHODS

All procedures were approved by the University of Adelaide Animal Ethics Committee (M-2007-84) and were conducted as per Australian guidelines (38).

Animals and treatments. Animal care and treatment of the control and IUGR groups have been described in detail previously (19). Briefly, natural twinning was used to induce IUGR in time-mated Australian Merino ewes. Three groups of ewes and their progeny were studied; singleton-bearing ewes (control, CON, $n = 7$), twin-bearing ewes (IUGR, $n = 8$ ewes), and twin-bearing ewes supplemented with methyl donors plus cofactors in the last month of gestation (maternal methyl donor supplement, IUGR+MMDS, $n = 4$ ewes). Two ewes allocated to supplementation as twin pregnancies on the basis of ultrasound at approximately *day 60* of gestation subsequently delivered singletons, and no data from these animals was included in the study. All ewes were fed 1 kg of Rumevite pellets daily (Ridley AgriProducts, Melbourne, Australia), plus 2 kg of lucerne chaff daily at 0900, and additional lucerne chaff was fed at 1500, according to demand, to provide ad libitum access to chaff. Feed offered and feed refusals were weighed daily. The IUGR+MMDS group were fed the maternal dietary supplement with ~1 cup of lucerne chaff between 0800 and 1000, from *day 120* of gestation (g120) until the day of delivery, with the remaining feed given after the feed containing the supplement was entirely consumed. The supplement consisted of rumen-protected methionine (2 g/day, Mepron, Evonik Degussa, Hanau, Germany), folic acid (300 mg/day, Sigma-Aldrich, St. Louis, MO), sulfur (1.2 g/day), and cobalt (0.7 mg/day as 5% dustless cobalt). The amounts of folic acid and rumen-protected methionine fed in the MMDS were based on doses fed previously in dairy cattle, where they approximately doubled maternal plasma and liver folate concentrations (20), respectively, and increased methionine flux through the transmethylation pathway, which produces the methyl donor SAM, by ~20% (45). Sulfur and cobalt were added to ensure that availability of these trace elements did not limit ruminal bacterial synthesis of sulfated amino acids or vitamin B_{12}, respectively (50). Ewes were monitored at least three times daily throughout lambing. All ewes delivered live-born lambs, and no ewes or lambs were excluded, except the two supplemented ewes and their lambs that were identified at birth as singleton pregnancies. All CON singleton lambs (CON; $n = 7$ lambs: 3 males and 4 females), one twin IUGR lamb from each twin pair from unsupplemented ewes ($n = 8$ IUGR lambs; 6 males and 2 females), and both lambs of twin pairs from supplemented ewes ($n = 8$ IUGR+MMDS lambs; 5 males and 3 females) were included in the present study, and metabolic outcome data are complete for all lambs. The other twin lamb from each nonsupplemented ewe received neonatal exendin-4 treatment as reported in a different study, and the IUGR group and singleton control groups in the present paper are the same animals reported in that paper as IUGR+Veh and CON lambs, respectively (19). As reported previously, we alternately allocated the heavy and light twins to treatment or vehicle, and both twins were reared together by the ewe for all twin pairs (19). All lambs (singletons and twins) were supplemented with whey protein (Resource Beneprotein instant protein powder; Nestle, Rhodes, NSW, Australia) given orally in two equal feeds (0900–1000 and 1600–1700), commencing at 1.25 $g \cdot kg^{-1} \cdot day^{-1}$ on *day 4* and increasing to 5 $g \cdot kg^{-1} \cdot day^{-1}$ on and after *day 7*. This supplement was calculated to increase protein availability by 25% above that available from ewe's milk (1, 53, 54, 60) during this period of maximal catch-up growth in IUGR lambs (6) to remove potential constraint due to competition for maternal milk supply. Although total milk and nutrient yields are higher in milk from ewes suckling twin compared with singleton lambs, the nutrients available to each lamb are, nevertheless, lower because these must be shared (54). All lambs were weighed on the day of birth and then every 2 days throughout the study. Additional measures of lamb size (crown-rump length, shoulder height, long bone lengths, skull width and length, and abdominal and thoracic circumferences) were made at birth and then every 4 days, and absolute (AGR) and fractional (FGR) growth rates from birth to *day 16* for each measure fitted by linear regression (7).

Folate, vitamin B_{12}, and homocysteine concentrations during pregnancy and in neonates. Blood was collected by jugular venipuncture from ewes at g119, g135, and on the day of delivery, and from neonates on the day of birth where possible (within 24 h of birth for all neonates that were able to be sampled). Blood was collected into lithium heparin tubes on ice and centrifuged, and plasma was stored

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at −80°C for later analysis. Plasma vitamin B_{12} and folate were measured using a commercially available kit (Simultrac Radioassay vitamin B_{12} [^{57}Co]/Folate [^{125}I] kit; MP Biomedicals), as described previously for sheep (50). Plasma homocysteine was analyzed by a Hitachi 912 automated sample system, using a Diazyme homocysteine enzymatic assay (Hcy) kit (Microgenics, Lidcombe, Australia), which had an intra-assay CV of <3%.

In vivo measures of insulin secretion, sensitivity, and action. On *day 4*, catheters were inserted into the lamb's femoral artery and vein under general anesthesia, induced, and maintained by fluothane inhalation anesthetic (Independent Veterinary Supplies, South Australia, Australia), as described previously (6). All lambs received an intramuscular injection of sedative and analgesic (Xylazil, 0.05 ml/kg, Lyppards, Victoria, Australia) before the surgery and an intramuscular injection of antibiotic (1 ml Norocillin SA, Lyppards, Victoria, Australia) after the surgery and then daily for 3 days postsurgery. Arterial blood was sampled and catheters were flushed every second morning before supplement feeding. Glucose tolerance and glucose-stimulated insulin secretion were measured during an intravenous glucose tolerance test (IVGTT; 0.25 g glucose/kg) at *day 14*), and indices of glucose tolerance and insulin secretion (fasting and change in glucose and insulin concentrations, glucose, and insulin areas under the curve and their ratios, overall and during the first and second phase of insulin secretion) were calculated as described previously (6, 16). The whole body insulin sensitivity of glucose metabolism was measured by hyperinsulinemic-euglycemic clamp (2 mU insulin·kg^{-1}·min^{-1}, 120 min) at *day 12* (16). The insulin sensitivity of glucose metabolism, the metabolic clearance rate (MCR) of insulin, and basal and maximal insulin disposition indices (IDI) were calculated as described previously (16).

Analysis of plasma insulin and metabolites. Plasma insulin concentrations were measured in duplicate by a double antibody, solid-phase radioimmunoassay using a commercially available kit (human insulin-specific RIA, HI-14K; Linco Research, St. Charles, MO), as described previously for sheep (19). The intra-assay coefficients of variation (CV) for the insulin assay were 7.0% and 5.0%, and interassay CV were 9.4% and 17.8% for QC samples containing 10.1 and 36.0 mU/l insulin, respectively (n = 12 assays). Plasma glucose concentrations were measured by colorimetric enzymatic analysis on a Hitachi 912 automated metabolic analyzer using Roche/Hitachi glucose/HK kits (Roche Diagnostics, Mannheim, Germany).

Post-mortem. Lambs were humanely killed by overdose of pentobarbital sodium at *day 16*. Organs (liver, kidneys, lungs, and heart), muscles (semitendinosus, gastrocnemius, soleus, tibialis, extensor digitorum longus, biceps femoris, vastus lateralis, and biceps), and dissectable fat depots (left and right perirenal fat, left and right retroperitoneal fat, and omental fat) were dissected and weighed for each lamb (6). Dissected muscle and visceral fat weights were calculated as the sum of weights of these muscles and fat depots, respectively (6).

Pancreas immunostaining and morphometric analysis. Each pancreas was rapidly dissected and weighed, before sampling of a representative mixed aliquot. Each pancreas sample was fixed for 48 h in 4% paraformaldehyde before embedding in paraffin wax. One section per block (animal) was immunostained to detect insulin-positive cells, and counterstained with hematoxylin and eosin to allow nuclei to be counted, and morphometric analysis of β-cells was performed in 20 fields of view per sheep selected by random-systematic sampling, as previously described (17). The number of fields counted was based on β-cell volume density and variation between fields and was validated in our previous studies to give a SE of <10% within individuals in lambs at similar ages (17). Additional measures of in vivo β-cell function were calculated by dividing total, first phase, and second phase of glucose-stimulated insulin secretion and basal and maximal IDI by β-cell mass (19).

Statistical analysis. Data for nonrepeated measures on each animal were analyzed by the mixed models procedure of SPSS for effects of treatment (fixed effect) and including dam as a random (block) effect in the model, to account for the common maternal environment in twins. Neonatal growth patterns and glucose, insulin, and insulin: glucose ratios overall and during the first phase (0–30 min) and second phase (30–210 min) of insulin secretion during the IVGTT were analyzed by repeated measures for effects of treatment (between factor), time (within factor), and interactions, and including dam as a random (block) effect in the model to account for the common maternal environment in twins. Within each model, we then compared groups by the LSD method, based on a priori questions to determine: *1*) effects of IUGR (CON cf. IUGR groups), *2*) effects of maternal methyl donor supplement (MMDS) in IUGR lambs (IUGR cf. IUGR+MMDS groups), and *3*) to assess whether MMDS restored outcomes to those of controls (CON cf. IUGR+MMDS groups).

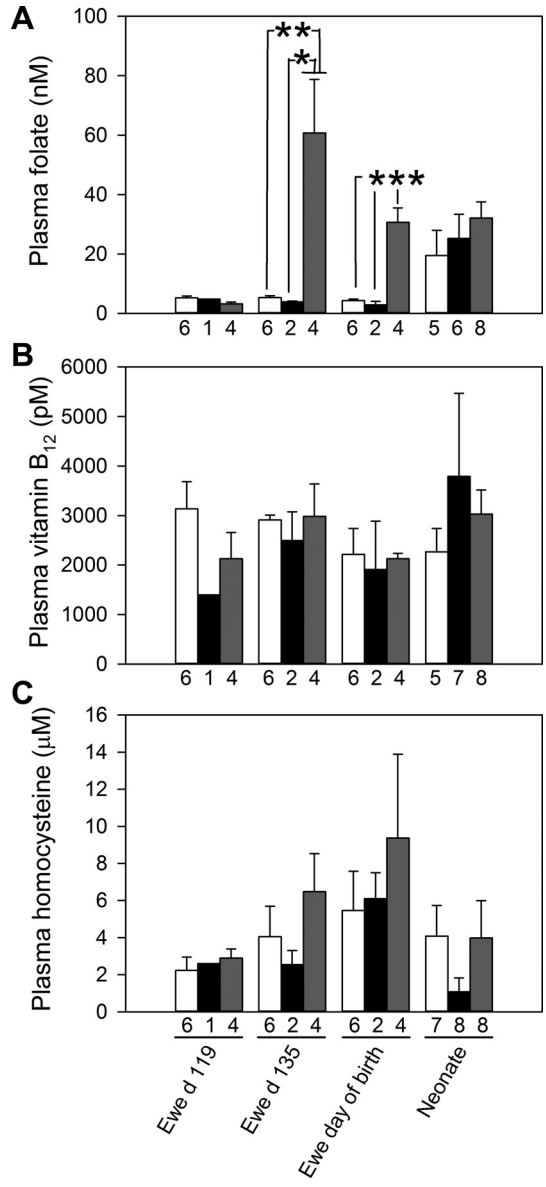

Fig. 1. Effects of IUGR and maternal methyl donor supplement in late pregnancy on plasma concentrations of folate (*A*), vitamin B_{12} (*B*), and homocysteine (*C*) in mothers before supplementation at *day 119* of pregnancy, after 3.5 wk of supplementation at d 135 of pregnancy, and in mothers and offspring on the day of birth. CON (white bar; n = 7), IUGR (black bar; n = 8) and IUGR+MMDS (gray bar; n = 8). Data are expressed as means ± SE, and sample numbers included at each time point are indicated below each bar. Specific contrasts: *P < 0.05, **P < 0.01, ***P < 0.001.

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Lamb sex was not included in statistical models due to limited animal numbers, and because previous studies have found few effects of sex or interactions between sex and IUGR on metabolic outcomes in lambs at 21, 30 and 43 days of age (5, 7, 16, 37).

RESULTS

One-carbon pathway and cofactor abundance. Maternal plasma folate before and during supplementation and on the day of birth was unaltered by twinning ($P > 0.05$ for each time) and increased by MMDS at *day 135* of gestation ($P < 0.05$ compared with unsupplemented twin-bearing ewes, $P < 0.01$ compared with control ewes, Fig. 1) and on the day of birth ($P < 0.001$ compared with both groups, Fig. 1). Maternal plasma vitamin B_{12} and homocysteine and plasma folate, vitamin B_{12}, and homocysteine concentrations in newborn offspring did not differ between groups (all $P > 0.05$, Fig. 1).

Size at birth, neonatal growth, and body composition. IUGR induced by twinning reduced size at birth in terms of weight, abdominal and thoracic circumferences, body mass index, and long bone and skull lengths, compared with the singleton CON group (each $P < 0.05$, Table 1). MMDS in late gestation partially normalized bone growth of twin IUGR lambs before birth, resulting in long bone and skull lengths at birth in IUGR+MMDS lambs that were not different to those of the CON group (Table 1). Metacarpal and metatarsal bones in IUGR+MMDS lambs were longer than those of IUGR lambs (each $P < 0.05$, Table 1). Twinning increased AGR for shoulder height and abdominal circumference (each $P < 0.05$), but not crown-rump length, compared with the CON group (Table 1). Twin IUGR lambs also had greater FGR for weight, abdominal, and thoracic circumference (each $P < 0.05$), but

Table 1. *Effects of IUGR and maternal methyl donor supplement in late pregnancy on size at birth, neonatal growth, and body size and composition at 16 days of age in lambs*

	CON	IUGR	IUGR+MMDS	Significance (Treatment Effect)
Number of ewes	7	8	4	
Gestational age at birth	148.7 ± 0.6	148.6 ± 0.5	150.8 ± 0.8	0.076
Number of lambs	7	8	8	
Size at birth				
Birth weight, kg	6.01 ± 0.21	4.82 ± 0.17*	4.74 ± 0.24*	< 0.001
Crown rump length, cm	56.3 ± 1.4	54.6 ± 1.1	55.3 ± 1.5	NS
Shoulder height, cm	44.0 ± 0.7	40.1 ± 0.8*	42.3 ± 0.6	0.008
Radius-ulna length, cm	13.7 ± 0.3	13.3 ± 0.3	13.6 ± 0.3	NS
Metacarpal length, cm	11.1 ± 0.2	10.3 ± 0.1*	11.0 ± 0.2†	0.015
Tibia length, cm	14.0 ± 0.2	13.1 ± 0.2*	13.4 ± 0.2	0.024
Metatarsal length, cm	12.7 ± 0.1	12.0 ± 0.2*	12.7 ± 0.2†	0.035
Skull width, cm	6.61 ± 0.12	6.36 ± 0.05	6.38 ± 0.08	NS
Skull length, cm	13.3 ± 0.2	12.8 ± 0.1*	13.3 ± 0.1	0.060
Abdominal circumference, cm	40.1 ± 0.4	35.1 ± 0.9*	36.5 ± 1.1*	< 0.001
Thoracic circumference, cm	39.5 ± 0.5	35.7 ± 0.7*	36.9 ± 0.8*	0.004
Body mass index, kg/m	19.2 ± 1.1	16.3 ± 0.8*	15.5 ± 0.6*	0.040
Neonatal growth rates				
AGR_{weight}, g/day	309 ± 29	327 ± 14	313 ± 12	NS
FGR_{weight}, %/day	5.17 ± 0.48	6.90 ± 0.39*	6.73 ± 0.42*	0.023
$AGR_{crown\ rump\ length}$, g/day	0.76 ± 0.11	0.95 ± 0.07	0.80 ± 0.07	NS
$FGR_{crown\ rump\ length}$, %/day	1.37 ± 0.21	1.77 ± 0.16	1.47 ± 0.14	NS
$AGR_{shoulder\ height}$, g/day	0.39 ± 0.03	0.51 ± 0.04*	0.44 ± 0.04	0.086
$FGR_{shoulder\ height}$, %/day	0.89 ± 0.06	1.17 ± 0.19	1.06 ± 0.10	NS
$AGR_{abdominal\ circumference}$, g/day	0.47 ± 0.07	0.78 ± 0.04*	0.56 ± 0.06†	0.004
$FGR_{abdominal\ circumference}$, %/day	1.18 ± 0.19	2.25 ± 0.17*	1.56 ± 0.18†	0.001
$AGR_{thoracic\ circumference}$, g/day	0.519 ± 0.053	0.629 ± 0.057	0.543 ± 0.048	NS
$FGR_{thoracic\ circumference}$, %/day	1.31 ± 0.13	1.78 ± 0.18*	1.48 ± 0.14	NS
Size at *day 16*				
Body weight, kg	11.0 ± 0.5	10.1 ± 0.3	9.9 ± 0.3	NS
Crown rump length, cm	68.7 ± 1.9	70.3 ± 0.7	68.6 ± 1.3	NS
Shoulder height, cm	50.5 ± 0.7	48.6 ± 0.5*	49.6 ± 0.6	NS
Radius-ulna length, cm	15.9 ± 0.3	15.3 ± 0.2	16.1 ± 0.3	0.098
Metacarpal length, cm	12.3 ± 0.4	11.9 ± 0.2	13.0 ± 0.4	NS
Tibia length, cm	15.9 ± 0.3	15.3 ± 0.1	16.0 ± 0.3	NS
Metatarsal length, cm	14.3 ± 0.4	14.0 ± 0.4	15.2 ± 0.6	NS
Skull width, cm	7.14 ± 0.08	7.07 ± 0.08	7.02 ± 0.09	NS
Skull length, cm	15.2 ± 0.2	14.7 ± 0.1	14.7 ± 0.2	NS
Abdominal circumference, cm	48.2 ± 1.4	48.2 ± 0.6	45.8 ± 1.1	NS
Thoracic circumference, cm	47.9 ± 1.1	46.0 ± 0.9	45.6 ± 0.9	NS
Body mass index, kg/m	23.3 ± 1.2	20.4 ± 0.4*	21.0 ± 0.4	0.074
Body composition at *day 16*				
Summed muscles, g	263 ± 13	226 ± 8*	220 ± 10*	0.037
Summed muscles, %	2.42 ± 0.07	2.25 ± 0.04*	2.25 ± 0.05*	0.082
Visceral fat, g	132 ± 19	118 ± 11	116 ± 9	NS
Visceral fat, %	1.19 ± 0.17	1.16 ± 0.09	1.18 ± 0.09	NS

Neonatal growth rates were fitted by linear regression to measures of size between birth and *day 16*. AGR, absolute growth rate; FGR, fractional growth rate; CON, control; IUGR, intrauterine growth rate; MMDS, methyl donors and cofactors. NS, $P > 0.1$. *Significantly different from CON ($P < 0.05$). †Significantly different from IUGR ($P < 0.05$).

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not for crown-rump length or shoulder height (each $P > 0.05$), compared with CON lambs (Table 1). Neonatal AGR and FGR for long bone lengths did not differ between CON and twin IUGR lambs (data not shown). MMDS of twin IUGR pregnancies in late gestation generally resulted in growth rates that were intermediate between CON and IUGR, and decreased growth rates for abdominal circumference relative to the untreated IUGR group (each $P < 0.05$), to growth rates similar to those of CON lambs (Table 1). At *day 16* of age, IUGR lambs had caught up in most measures of body weight and size, but remained shorter (shoulder height, $P < 0.05$) and thinner (BMI, $P < 0.05$, Table 1) than CON lambs. IUGR+MMDS lambs did not differ in size from either IUGR or CON lambs at *day 16* (Table 1). Organ weights of liver, kidneys, and heart in absolute terms and relative to body weight at *day 16* were similar between groups (data not shown). IUGR reduced absolute and relative summed muscle mass compared with CON (each $P < 0.05$), and this was not restored by maternal late-gestation methyl donor supplementation (Table 1). Absolute and relative weights of visceral fat did not differ between groups (each $P > 0.05$, Table 1).

Insulin secretion, sensitivity, and action. Fasting glucose and insulin concentrations and their ratio, and overall, first phase and second phase in vivo insulin secretion were similar between untreated IUGR lambs compared with CON lambs (each $P > 0.1$, Table 2). Similarly, MMDS did not alter any of these measures of glucose controls and insulin secretion in comparison to untreated IUGR lambs or to CON lambs (each $P > 0.1$, Table 2), except that the increase in blood glucose after the glucose bolus and $AUC_{glucose}$ during the first phase of insulin secretion were lower in IUGR+MMDS only when compared with CON lambs (Table 2). Relative and absolute insulin secretion did not differ between groups (Table 2). Insulin sensitivity was not altered by treatment, but it tended to be greater in IUGR+MMDS lambs than in CON ($P = 0.070$) or in untreated IUGR lambs ($P = 0.086$, Table 2). The MCR of insulin was greater in IUGR+MMDS than in IUGR lambs ($P = 0.023$, Table 2). Insulin action, measured as insulin disposition indices in the fasting or glucose-stimulated states, did not differ between groups (Table 2). Across the whole of the IVGTT, and within the first phase of insulin secretion, plasma glucose changed with time (each $P < 0.001$, Fig. 2*A*), and did not differ between groups, but the pattern of change in glucose across time differed between groups (time × group interaction: $P = 0.004$). Plasma insulin (Fig. 2*B*) also changed with time throughout the IVGTT ($P < 0.001$), and within the first ($P < 0.001$), but not in the second phase ($P = 0.4$) of insulin response. Plasma insulin was lower in IUGR+MMDS than in CON lambs overall during the IVGTT ($P = 0.021$) and during the second phase of insulin secretion ($P = 0.037$), but not during the first phase of insulin secretion ($P > 0.2$). The ratio of plasma insulin to glucose (Fig. 2*C*), an index of insulin secretion, similarly changed with time throughout the IVGTT overall ($P < 0.001$), and within the first ($P < 0.001$), but not the second phase ($P > 0.2$) of insulin response. The plasma insulin:glucose concentration ratio was also lower in IUGR+MMDS than in CON lambs during the IVGTT (Fig. 2*C*, $P = 0.025$) and during the second phase of insulin secretion ($P = 0.023$), but not during the first phase of insulin secretion ($P > 0.2$).

Pancreas morphology and β-cell function. Absolute and relative pancreas weights, numbers of β-cells per islet, and absolute β-cell mass did not differ between treatments (Table 3). Relative β-cell mass tended to be greater in IUGR+MMDS than in CON lambs ($P = 0.060$) but did not differ between IUGR+MMDS and IUGR groups ($P > 0.1$, Table 3). β-cell volume density was 30% greater in IUGR+MMDS than CON

Table 2. *Effects of IUGR and maternal methyl donor supplement in late pregnancy on insulin action in lamb*

	CON	IUGR	IUGR+MMDS	Significance (Treatment Effect)
Number of animals	7	8	8	
Fasting				
Plasma glucose, mmol/l	6.47 ± 0.26	6.40 ± 0.11	6.48 ± 0.06	NS
Plasma insulin, mU/l	20.4 ± 6.0	15.4 ± 2.2	15.3 ± 2.8	NS
Increase postglucose bolus (change from fasting to peak levels)				
Plasma glucose, mmol/l	5.50 ± 0.32	5.10 ± 0.16	4.73 ± 0.11*	NS
Plasma insulin, mU/l	26.0 ± 6.5	32.5 ± 10.9	22.0 ± 4.6	NS
Glucose tolerance, AUC glucose, $mmol \cdot min^{-1} \cdot l^{-1}$				
Total	61.6 ± 5.8	55.8 ± 3.4	56.8 ± 10.6	NS
First phase	60.9 ± 5.4	54.8 ± 2.9	49.0 ± 3.6*	NS
Second phase	0.7 ± 0.5	1.0 ± 1.0	7.8 ± 7.6	NS
Absolute insulin secretion, AUC insulin, $mU \cdot min^{-1} \cdot l^{-1}$				
Total	587 ± 184	590 ± 181	334 ± 70	NS
First phase	499 ± 133	579 ± 180	328 ± 69	NS
Second phase	88 ± 61	12 ± 6	6 ± 4	NS
Relative insulin secretion, AUC insulin:AUC glucose, mU/mmol				
Total	10.8 ± 4.3	10.9 ± 3.6	6.6 ± 1.7	NS
First phase	8.9 ± 2.8	10.7 ± 3.5	7.0 ± 1.7	NS
Second phase	26.5 ± 25.9	0.3 ± 0.3	3.6 ± 3.6	NS
Insulin sensitivity, $mg \cdot l^{-1} \cdot mU^{-1} \cdot kg^{-1} \cdot min^{-1}$	0.097 ± 0.010	0.100 ± 0.011	0.135 ± 0.017	NS
MCR insulin, $ml \cdot kg^{-1} \cdot min^{-1}$	33.8 ± 4.0	27.1 ± 2.2	41.0 ± 4.6†	0.068
Basal IDI, $mg \cdot ml^{-1} \cdot kg^{-2} \cdot min^{-1}$	69.7 ± 31.2	39.5 ± 5.5	89.1 ± 20.5	NS
Maximal IDI, $mg \cdot ml^{-1} \cdot kg^{-2} \cdot min^{-2}$	138 ± 28	119 ± 27	226 ± 65	NS

Glucose and insulin area under the curve (AUC) were measured during an intravenous glucose tolerance test (IVGTT) (0.25 g glucose/kg) at *day 14*. First- and second-phase values for insulin and glucose were measured from 0 to 30 and from 30 to 210 min after glucose administration, respectively. Insulin sensitivity was measured during a hyperinsulinemic euglycemic clamp (2 mU $insulin \cdot kg^{-1} \cdot min^{-1}$) at *day 12*. MCR, metabolic clearance rate; IDI, insulin disposition indices. NS: $P < 0.1$. *Significantly different from CON ($P < 0.05$). †Significantly different from IUGR ($P < 0.05$).

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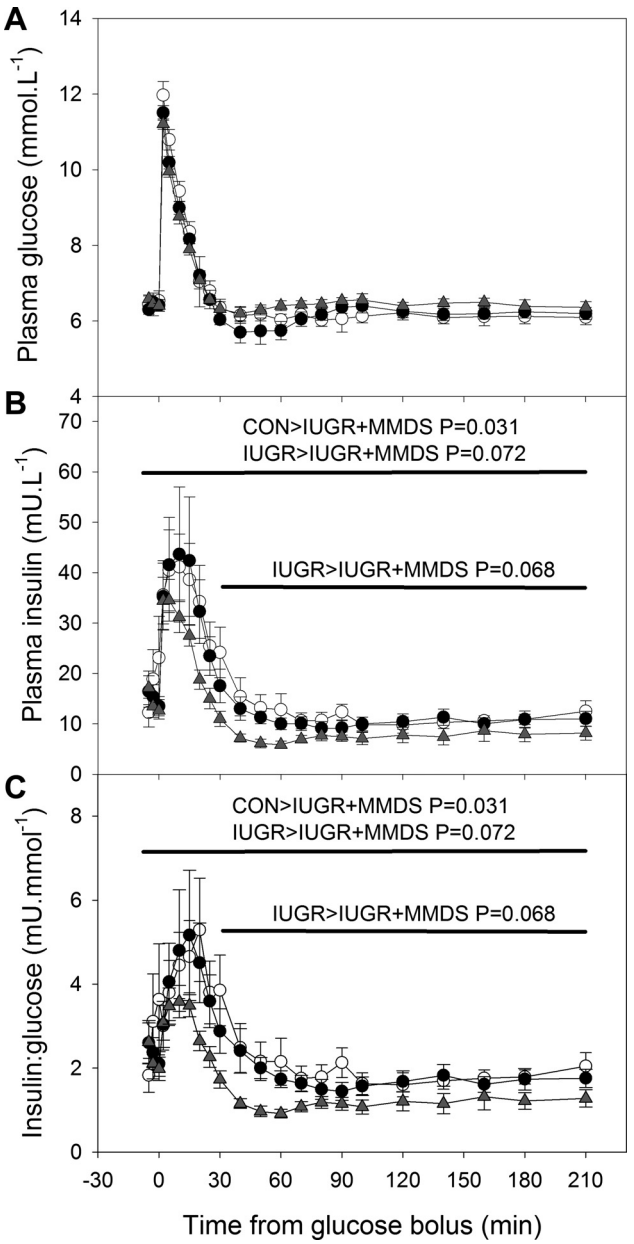

Fig. 2. Effect of IUGR and maternal methyl donor supplement in late pregnancy on plasma glucose (*A*), plasma insulin (*B*), and relative plasma insulin to plasma glucose ratio (*C*) during IVGTT of the young lambs. CON (○; $n = 7$), IUGR (●; $n = 8$), and IUGR+MMDS (gray triangles; $n = 8$). Data are expressed as means ± SE.

lambs ($P = 0.029$) but not different from IUGR lambs ($P > 0.1$, Table 3). IUGR+MMDS lambs had more β-cells per area of the pancreas than either CON ($P = 0.006$) or IUGR groups ($P = 0.015$, Table 3). Islet density and numbers of β-cells per islet (Table 3) and measures of β-cell function (insulin secretion or insulin disposition relative to β-cell mass, data not shown) did not differ between groups.

DISCUSSION

Feeding twin-bearing ewes a methyl donor and cofactor dietary supplement throughout late pregnancy increased circulating folate concentrations in the pregnant ewe during supplementation. We were unable to detect increased methyl donor availability in neonates, probably due to limited numbers and variation in time between feeding of the last maternal supplement and neonatal sampling. Nevertheless, the progeny outcomes were broadly consistent with our hypothesis that feeding a methyl donor and cofactor supplement would ameliorate adverse effects of IUGR, as the supplement had beneficial effects on growth and pancreatic β-cells of progeny that persisted at least until the third week of life. Given the associations between maternal or fetal deficiency of one-carbon metabolites or cofactors and increased risks of adverse adult metabolic health outcomes in humans and in experimental paradigms (32, 50, 61), our findings suggest that maternal and/or fetal methyl donor availability during late pregnancy can program development and contribute to risks of adult disease and that supplementation may improve long-term outcomes for IUGR progeny.

Restriction of fetal growth due to spontaneous twinning reduced most measures of neonatal size at birth, consistent with reduced nutrient supply to twin compared with singleton sheep fetuses (56). This together with previous reports of divergence in fetal weights between singleton and twin ovine fetuses from ~100 days after conception (46) confirms that twinning provides a naturally occurring model of IUGR. In the present study, MMDS did not alter neonatal measures of soft tissue size, including weight, abdominal and thoracic circumferences, and BMI, except that MMDS consistently normalized long bone lengths at birth in IUGR lambs, suggesting that an increased supply of these nutrients may have increased fetal bone elongation during late gestation. Consistent with a positive effect of methyl donor availability on bone development, maternal dietary folate intake and folate status during pregnancy are positively related to bone density in children aged 6–10 yr in epidemiological studies (15, 52, 55). Similarly, maternal folic acid supplementation during gestation in rats increases bone mineral density of the femur in 4-mo-old offspring (25). Despite the increased long bone lengths in IUGR+MMDS lambs at birth in the present cohort, neonatal growth rates of long bone lengths did not differ between IUGR and IUGR+MMDS lambs, suggesting that the effect occurs during the supplementation period.

Consistent with a lack of effect of the methyl donor and cofactor supplement on birth weight, both IUGR and IUGR+MMDS lambs had accelerated neonatal fractional growth rates in terms of weight compared with CON lambs. Neonatal growth of abdominal circumference was faster in untreated IUGR in comparison to CON, while MMDS treatment abolished this effect. Visceral fat mass at 16 days of age was not altered by either IUGR or maternal diet, but we hypothesize that this effect of twinning or maternal diets on adiposity emerges with aging. In previous experimental studies of IUGR induced by removal of placental attachment sites and, hence, restricted placental function throughout pregnancy (PR), visceral fat was increased at 43 days of age, but not in younger animals at 21 days of age (6, 9). Percentage fat mass is one-third greater in twin sheep than singletons as adults (21), confirming that this paradigm of natural IUGR also increases later adiposity. Twinning reduced both absolute and relative muscle mass of lambs in the present study at 16 days, and this was not normalized by MMDS. We suggest that additional follow-up studies to later ages are merited to determine

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Table 3. *Effects of IUGR and maternal methyl donor supplement in late pregnancy on pancreas morphology*

	CON	IUGR	IUGR+MMDS	Significance (Treatment Effect)
Number of animals	7	8	8	
Pancreas morphology				
Pancreas weight, g	10.8 ± 1.5	8.5 ± 0.9	9.0 ± 0.7	NS
Pancreas, % of body weight	0.103 ± 0.019	0.085 ± 0.009	0.092 ± 0.009	NS
β-cell volume density	0.033 ± 0.005	0.040 ± 0.003	0.052 ± 0.008*	0.079
β-cell density, number/mm^2	660 ± 77	761 ± 86	1271 ± 183*†	0.012
β-cell mass, g	0.326 ± 0.038	0.345 ± 0.054	0.452 ± 0.066	NS
β-cell mass, % of body wt	0.0030 ± 0.0004	0.0034 ± 0.0005	0.0046 ± 0.0007	NS
Islet density, number/mm^2	66.3 ± 10.6	78.0 ± 11.5	94.6 ± 7.4	NS
β-cells/islet	10.9 ± 1.4	10.5 ± 1.3	13.4 ± 1.6	NS
% of islets with <5 β-cells	27.7 ± 6.3	23.8 ± 6.4	23.8 ± 3.9	NS

NS: $P > 0.1$. *Significantly different from CON ($P < 0.05$). †Significantly different from IUGR ($P < 0.05$).

whether MMDS ameliorates the increase in adiposity seen after IUGR, including that induced by twinning.

IUGR due to twinning did not alter glucose control and insulin action in young lambs, as we have previously reported (19). However, in older PR lambs (5, 6, 17, 42), there is a shift to insulin resistance following IUGR in association with catch-up growth, as observed in human IUGR (36, 39, 59), thus suggesting that insulin resistance does not develop in this model until between 2 and 6 wk after birth. MMDS in the present study increased MCR for insulin and tended to increase insulin sensitivity compared with IUGR lambs at 12 days of age, each suggesting greater insulin action at the insulin receptor. The trends for lower insulin concentrations throughout the IVGTT in IUGR+MMDS than IUGR lambs, both in absolute terms and relative to glucose, are also consistent with persistent programming of increased insulin action in IUGR lambs by MMDS during late pregnancy. Unfortunately, the power of the present study was less than originally planned as two supplemented ewes delivered singleton rather than twin lambs as originally predicted from ultrasounds in early gestation. These ewes and lambs were consequently removed from the study, leaving only four sets of twin lambs from supplemented ewes. Whether this will improve long-term metabolic control is unknown, given the evidence for a reversal from enhanced neonatal to impaired adult insulin sensitivity after human and experimental IUGR (18). A larger and long-term study is required to further define the metabolic consequences for offspring of maternal methyl donor and cofactor availability after IUGR.

Neither IUGR due to twinning as previously reported (19) nor MMDS altered pancreatic mass in lambs at 16 days of age in the present study. However, MMDS in the last 1/5 of pregnancy (from 120 days after mating until term, ~28 days) increased the numbers of β-cells per area of pancreas, relative to both control and untreated IUGR lambs. Since the pancreas was a similar size in all groups, this implies that increased maternal methyl donor and cofactor supply during late pregnancy programmed increased total numbers of β-cells in these IUGR lambs, either directly through altered fetal supply of these nutrients or indirectly through changes in placental development or maternal metabolism. In surgically induced IUGR rats, progressive loss of β-cell mass with aging following IUGR (49) is consistent with the progressive epigenetic silencing of *Pdx1* expression in comparison to control offspring, and leads to onset of diabetes by adulthood (43, 44). PDX1 is an important regulator of β-cells, which contributes to increases in β-cell mass by replication, neogenesis of new β-cells, and transdifferentiation of pancreatic acinar cells into β-cells (3). Since epigenetic mechanisms, such as DNA methylation in utero, can be affected by maternal folate supplementation (40, 51), and *Pdx1* gene expression is altered by progressive epigenetic mechanisms in IUGR rats (43, 44), these findings suggest that maternal folate supplementation may increase *Pdx1* expression. Although it was not possible to measure *Pdx1* expression in the present study due to mRNA degradation in snap-frozen aliquots of pancreas, it is possible that similar mechanisms may enhance β-cell replication and formation of new cells and result in the increased β-cell number that we observed after MMDS in the present study of twin IUGR lambs. Other mechanisms may also be involved, since IUGR induced by PR did not reduce *Pdx1* expression in lambs at 43 days of age (17). We hypothesize that the greater numbers of β-cells in MMDS+IUGR lambs at 16 days of age would increase their capacity to expand β-cell mass and increase insulin secretion in response to demand in later life, for example, due to onset of insulin resistance. This may ameliorate the inadequate insulin action in postnatal life observed longer term after human IUGR (22, 36, 57) and in older IUGR offspring in sheep (17). Future investigations are needed to determine the mechanisms for the effects of maternal methyl donor and vitamin B_{12} supplementation on β-cell mass and function and to assess whether these improve insulin secretion into adult life.

Several limitations of the present study need to be acknowledged. First, for logistical reasons, it was not possible to measure lamb milk intake and feeding behavior in the present study. Lambs were supplemented with protein to prevent growth constraint due to potential limitation in maternal milk protein supply in twin litters (53, 54, 60). Because lambs were suckling to appetite, we expect that their consumption of ewe's milk might be reduced by supplementation, and this may restrict the capacity to compare the present results to those of studies in which lambs consumed only ewe's milk. Nevertheless, fortification of ewe's milk with a breast milk fortifier that increased protein, fat, carbohydrate, and energy content by approximately one-third did not reduce lambs' milk intake in the first 2 wk of life in either term-born or preterm-delivered lambs (4). Regardless of whether their intakes of ewe's milk were reduced, since all lambs in the present study were fed the same amount of whey protein per body weight, we assume that the dietary composition for all lambs within the present study should be similar between groups. It is possible that these

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groups of lambs might respond differently to supplements, however. Progeny metabolic responses to a different nutrient supplement, which increased protein intake by 63% and carbohydrate by 45% and did not alter fat content, differed between sexes in term-born lambs (23). In that study, neonatal supplementation during the first 2 wk of life increased glucose-stimulated insulin secretion and improved glucose tolerance in males, and it decreased glucose-stimulated insulin secretion without altering glucose tolerance in females, both studied as juveniles at 4 mo of age (23). There is also evidence from human clinical trials that increased protein content of the neonatal diet above levels available in breast milk accelerates infant growth and increases risks of later obesity and metabolic health (29). Therefore, we suggest that future studies consider use of artificial rearing on a milk replacer similar in composition to ewe's milk, to avoid both potential limitations to supply and potential adverse effects of altered nutrient composition in the neonatal diet. As mentioned previously, the limited number of ewes in the MMDS group and variability in the timing and limited number of neonatal blood samples able to be collected are also limitations of the study, although we have provided the first evidence in pregnancy that this supplement increases maternal circulating folate. Further studies are needed to confirm the effects of MMDS after IUGR on the offspring and their metabolic status and outcomes as young progeny and into adulthood.

In conclusion, maternal methyl donor and cofactor supplementation partially reversed effects of IUGR on prenatal long bone growth and neonatal soft tissue growth, increased numbers of β-cells per area of pancreas and tended to improve insulin sensitivity compared with untreated IUGR lambs, in the first 16 days of life. The presence of metabolic effects of maternal methyl donor supplementation in IUGR lambs persisting at least until the third week of postnatal life supports the hypothesis that prenatal methyl donor supply programs subsequent development. Whether MMDS is able to ameliorate adverse metabolic consequences of IUGR in later life and these responses persist or amplify with age and into adult life, as well as the underlying mechanisms, require study in longer-term cohorts.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

S.A.S., M.J.D.B., M.L.H., and K.L.G. performed experiments; S.A.S. and K.L.G. analyzed data; S.A.S., M.J.D.B., M.L.H., K.L.G., and J.A.O. interpreted results of experiments; S.A.S. and K.L.G. prepared figures; S.A.S. and K.L.G. drafted manuscript; S.A.S., M.J.D.B., K.L.G., and J.A.O. edited and revised manuscript; S.A.S., M.J.D.B., M.L.H., K.L.G., and J.A.O. approved final version of manuscript; K.L.G. and J.A.O. conceived and designed research.

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