Brief neonatal nutritional supplementation has sex-specific effects on glucose tolerance and insulin regulating genes in juvenile lambs

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BACKGROUND: The nutritional plane and composition during fetal life can impact upon growth and epigenetic regulation of genes affecting pancreatic β -cell development and function. However, it is not clear whether β -cell development can be altered by nutritional factors or growth rate after birth. We therefore investigated the effect of neonatal nutritional supplements on growth, glucose tolerance, and pancreatic development in lambs.

METHODS: Newborn lambs were randomized to daily nutritional supplements, calculated to increase macronutrient intake to a similar degree as human breast milk fortifier, or an equivalent volume of water, for 2 wk while continuing to suckle ewe milk. Intravenous glucose tolerance test (IVGTT) was performed at 4 mo of age, and pancreata collected for molecular analysis.

RESULTS: Supplemented lambs had slower weight gain than controls. In supplemented lambs, insulin response to IVGTT was increased in males but decreased in females, compared to same sex controls, and was unrelated to growth rate. mRNA expression of key genes in β -cell development showed sexually dimorphic effects. Epigenetic change occurred in the promoter region of *PDX1* gene with decreased suppression and increased activation marks in supplemented lambs of both sexes.

CONCLUSION: Nutritional interventions in early life have long-term, sex-specific effects on pancreatic function.

Nutrition during intrauterine life is the main determinant of fetal growth (1), a key factor in pancreatic development (2,3) and a powerful modulator of epigenetic regulation of genes affecting insulin synthesis and secretion (4). After birth, the “gold standard” of nutrition in healthy term infants is breast milk. However, some infants receive formula feeds, which have a greater macronutrient content than breast milk (5), and growth-restricted babies are often given supplemental calories, macronutrients, or micronutrients to meet their nutritional needs. This frequently is achieved by the use of

breast milk fortifier, which increases carbohydrate and protein content *per* unit volume when added to breast milk, in conjunction with specific vitamin and mineral supplements (6). Although there are some reports of the effects of nutritional supplementation on growth (7) and cognitive development (8), their effects on later metabolic function are unknown.

Studies of metabolic dysfunction after postnatal nutritional interventions have often focused on associations between the rate of neonatal weight gain and later outcomes such as obesity and insulin resistance (9,10). For example, in studies of formula feeding vs. breast feeding, or comparisons between high and low protein containing infant formulas, rates of obesity and altered insulin dynamics have largely been attributed to a difference in growth rates between groups, assuming over-nutrition, accelerated growth rates, and change in body composition to be causative factors (11–13). One difficulty with this assumption, however, is the poor correlation between energy intake and growth rate in the immediate postnatal period, particularly after intrauterine insult (14). It may be that intrauterine factors influence both early growth rate and later metabolism more strongly than postnatal caloric intake, and that dietary composition *per se* influences the development of physiological pathways and organ development independently of early growth rate.

In animal experiments, both the quality and quantity of the postnatal diet have been shown to alter pancreatic development. In rats, a low protein, isocaloric diet post weaning was associated with lower serum concentrations of insulin after feeding and altered expression of several pancreatic islet cell genes that encode for proteins related to insulin synthesis and secretion (15). Altering the quantity of milk intake during the suckling period by random assignment of male rat pups to small, medium (control) or large litter size resulted in an early and sustained defect in glucose responsiveness of pancreatic insulin secretion in both the large and small litter groups compared with controls, and differential expression of several islet cell genes through to adulthood (16). However, rats are altricial species, developmentally immature at birth compared

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with humans and most other mammals, so that it is not possible to relate the developmental effects of postnatal nutrition in rats to humans.

To further understand how manipulations of dietary quality and quantity in early postnatal life might impact on pancreatic development and function without the added confounders of nutritional compromise before or after birth, we investigated, in healthy term lambs, the effects of nutritional supplements based on human breast milk fortifier, adjusted for ewe milk composition, given for the first 2 wk after birth, on growth, milk intake, glucose tolerance, and key genes in pancreatic β -cell development and insulin synthesis.

RESULTS

Milk Intake and Growth

Forty-two singleton lambs commenced the study; 20 were supplemented (S) and 22 served as controls (C) (Table 1). During the 2-wk period of supplementation (the first 2 wk after birth), growth velocity was lower in S than C lambs ($P = 0.006$). In the subset of 17 lambs (8 C and 9 S) that underwent measurement of milk intake during the second week after birth, there was a trend toward S lambs having lesser volume, but not caloric, intake than C, and males a lesser intake than females (group effect $P = 0.06$, sex effect $P = 0.06$, no group $\times$ sex interaction) (Table 1). Protein intake was increased in supplemented lambs of both sexes, despite the trend toward lesser volume (Table 1). There was no difference in weight between S and C lambs, and males were heavier than females at each time point.

Glucose Tolerance at 4 mo of Age

Intravenous glucose tolerance tests (IVGTT) were completed successfully on 39 of the 42 lambs at 4 mo of age (juvenile, just before puberty).

Glucose area under the curve to 60 min (AUC_{60}) was less in S male lambs than all other groups (group effect $P = 0.03$, group $\times$ sex $P = 0.04$) (Table 2). Glucose AUC_{180} was also lesser in S lambs (group effect $P = 0.04$).

Table 1. Growth and milk intake in control and supplemented lambs

	Control		Supplemented	
	Mn = 14	Fn = 8	Mn = 10	Fn = 10
Birth weight (kg)*	6.4 $\pm$ 0.2	5.5 $\pm$ 0.2	6.5 $\pm$ 0.1	5.9 $\pm$ 0.3
Weight day 4 (kg)*	7.4 $\pm$ 0.2	6.3 $\pm$ 0.3	7.3 $\pm$ 0.3	6.4 $\pm$ 0.3
Weight day 8 (kg)*	9.1 $\pm$ 0.3	7.6 $\pm$ 0.4	8.8 $\pm$ 0.3	7.9 $\pm$ 0.3
Weight 2 wk (kg)**	11.5 $\pm$ 0.3	9.6 $\pm$ 0.3	11.3 $\pm$ 0.3	10.0 $\pm$ 0.4
Growth velocity 0–2 wk (g.kg ⁻¹ .day ⁻¹)†	46 $\pm$ 1	46 $\pm$ 2	43 $\pm$ 1	40 $\pm$ 2
4 mo weight (kg)**	37 $\pm$ 1	33 $\pm$ 1	39 $\pm$ 1	33 $\pm$ 2
	Mn = 3	Fn = 5	Mn = 5	Fn = 4
Milk intake (ml.kg ⁻¹ .day ⁻¹)	197 $\pm$ 6	210 $\pm$ 13	169 $\pm$ 5	195 $\pm$ 17
Caloric intake (kcal.kg ⁻¹ .day ⁻¹)	213 $\pm$ 7	227 $\pm$ 14	223 $\pm$ 7	257 $\pm$ 22
Protein intake (g.kg ⁻¹ .day ⁻¹)†	11.8 $\pm$ 0.3	12.6 $\pm$ 0.8	15.8 $\pm$ 0.5	17.4 $\pm$ 1.6

Values are mean $\pm$ SEM. Milk intake was measured during the second week after birth.

* $P < 0.01$, ** $P < 0.001$ for difference between males (M) and females (F). † $P < 0.01$ for difference between control and supplement groups.

Insulin AUC_{60} was greater in S males but lesser in S females compared to C lambs (Group $\times$ sex $P = 0.03$, Figure 1). Insulin AUC_{180} also was least in S females (Table 2). Similarly, when glucose and insulin AUC_{60} were expressed as a ratio, values were least in S males and greatest in S females (group $\times$ sex $P = 0.04$) (Table 2).

Neither glucose nor insulin AUC at any time point were related to lamb growth velocity in the first 2 wk of life (data not shown).

Postmortem Findings

Twenty-one lambs (13 male, C $n = 6$, S $n = 7$; 8 female, C $n = 3$, S $n = 5$) underwent postmortem after the glucose tolerance test at 4 mo of age. Males were heavier than females (28 ± 1 vs. 24 ± 1 kg, $P = 0.03$) and had greater lung and kidney weights when expressed as % body weight (data not shown). Supplementation did not alter organ weights relative to body weight.

mRNA Expression in the Pancreas

Supplementation decreased *PDX1* (pancreatic and duodenal homeobox 1), *SLC2A2* (glucose transporter 2) and *IGF2* (insulin-like growth factor 2) mRNA levels in S males, but not females, compared with same sex controls (all $P < 0.01$) (Figure 2).

Table 2. Intravenous glucose tolerance tests at age 4 mo in control and supplemented groups

	Control		Supplement	
	Mn = 12	Fn = 8	Mn = 9	Fn = 10
Baseline glucose (mmol.l ⁻¹)	3.5 $\pm$ 0.1 ^{b*}	3.8 $\pm$ 0.1 ^a	3.9 $\pm$ 0.1 ^a	3.8 $\pm$ 0.1 ^a
Glucose AUC_{60} (mmol.min.l ⁻¹)	619 $\pm$ 17 ^a	582 $\pm$ 16 ^a	511 $\pm$ 13 ^{b*}	580 $\pm$ 32 ^a
Glucose AUC_{180} (mmol.min.l ⁻¹)†	964 $\pm$ 52	889 $\pm$ 54	770 $\pm$ 74	834 $\pm$ 65
Baseline insulin (ng.min.ml ⁻¹)	0.07 $\pm$ 0.02	0.12 $\pm$ 0.02	0.07 $\pm$ 0.02	0.06 $\pm$ 0.02
Insulin AUC_{60} (ng.min.ml ⁻¹)†	68 $\pm$ 7	90 $\pm$ 16	99 $\pm$ 23	56 $\pm$ 8
Insulin AUC_{180} (ng.min.ml ⁻¹)	131 $\pm$ 11	146 $\pm$ 22	140 $\pm$ 23	103 $\pm$ 9*
Glucose AUC_{60} :Insulin AUC_{60} †	10.4 $\pm$ 1.1	7.7 $\pm$ 1.1	8.1 $\pm$ 1.9	12.2 $\pm$ 1.7

* $P < 0.05$ for difference from subgroups denoted by a different letter. † $P < 0.05$ for group effect. * $P < 0.05$ for Group $\times$ sex interaction. AUC, area under the curve.

Figure 1. Insulin AUC_{60} in supplemented and control lambs. Control group represented by dark bars, supplemented group represented by light bars. M, male; F, female; C, control; S, supplemented; group $\times$ sex interaction $P = 0.03$

Figure 2. mRNA expression in the pancreas. Values are expressed as fold change in mRNA levels in supplemented males (M) and females (F) relative to the same sex control group (represented by the horizontal line) and normalized to the Bestkeeper index with 95% confidence intervals. Thus, mRNA levels are significantly different from same-sex controls at the 5% level if the 95% confidence intervals do not cross 1.

mRNA levels of *INSULIN* and *GLUCOKINASE* were increased in S females, but not males, compared with same sex controls ($P < 0.01$ for both).

Supplementation did not alter mRNA levels of *IGF1*.

Epigenetic Marks in the Promotor Region of the *PDX1* Gene

The promotor region of the *PDX1* gene was studied to clarify whether the observed changes in mRNA levels in response to supplementation (found on real-time PCR analysis) were regulated by a change in epigenetic status of *PDX1*. In the promotor region of the *PDX1* gene, DNA methylation, a repressor mark, was decreased in S animals of both sexes compared with C (% enrichment methylated genomic DNA:total genomic DNA Control M 11.02 ± 1.2 , F 9.16 ± 2.2 ; Supplemented M 7.83 ± 1.2 , F 5.36 ± 1.02 ; group effect $P = 0.02$). Enrichment of *H3K27me3*, another repressor mark, was greatest in C females (sex effect $P = 0.03$, sex $\times$ group interaction $P = 0.04$) (Figure 3a). *H3K9* acetylation, an activating mark, showed greatest % fold enrichment in S females (sex effect $P = 0.01$) (Figure 3b), while another activating mark, *H3K4me3* acetylation, was not different between groups or sexes (Figure 3c).

DISCUSSION

Brief postnatal nutritional supplementation of healthy term lambs resulted in epigenetic change in pancreatic β cells, and sex specific alteration in mRNA expression of genes related to β cell development and function. This confirms that early postnatal nutrition is a powerful modulator of epigenetic change, and may alter organ development in ways that affect long-term metabolic outcomes.

The nutritional intervention in this study was designed to result in a percentage increase in macronutrients equivalent to that seen when human milk fortifier is added to expressed breast milk, adjusted for the differing compositions of human and ewe milk. There are robust data associating *intrauterine* nutrition with pancreatic development and later function. In growth restricted human fetuses, pancreatic β cell fraction, islet vascularization, and *in vivo* β cell responsiveness have

Figure 3. % fold enrichment of (a) H3K27me3, (b) H3K9Ac, and (c) H3K4me3 ChIP/genomic DNA. Control group C represented by dark bars, supplemented group S represented by light bars. * represents sex effect $P < 0.05$; † represents sex by group interaction $P < 0.05$.

been shown to be reduced (17). Studies in rodents have shown a clear association between fetal growth restriction and β cell dysfunction, dependent on the type, timing, and duration of the fetal insult (15–18). In particular, both maternal caloric restriction and a maternal low protein, isocaloric diet resulted in decreased β cell mass in the offspring, but via different mechanisms. In fetuses of dams fed a low-energy diet, decreased β cell mass was due to reduced neogenesis indicated by fewer cells positive for Ngn3 and Pdx-1 (transcription factors that are involved in the β cell lineage), whereas the low-protein diet reduced offspring β cell mass by impairing islet vascularization and β cell proliferation (19). Beta cell mass was also reduced in fetal sheep that were growth restricted after either placental restriction via removal of placental implantation sites (20) or maternal exposure to a warm environment (21). However, it is also important to note that nutritional interventions in pregnant ewes can also result in altered glucose tolerance in the offspring independent of changes in birth weight (22).

There are fewer reports relating *postnatal* nutrition alone to metabolic outcomes. Increasing postnatal caloric intake in rats reduced glucose-stimulated insulin secretion after weaning and

resulted in differential expression of a range of islet cell genes involved in insulin regulation, including reduced mRNA levels of the insulin gene (16). Altering nutritional quality by giving an isocaloric, high carbohydrate milk formula to rats in the neonatal period resulted in hyperinsulinemia and increased glucokinase and Slc2a2 protein content, which persisted into adulthood (23). However, the developmental immaturity of rats at birth makes meaningful comparisons with humans and other mammals difficult. In sheep, which have an intrauterine developmental trajectory more similar to humans, pancreatic development and remodeling occur in late gestation and continue into the postnatal period; hence, we hypothesized that neonatal nutritional manipulation in lambs would result in epigenetic change and mRNA expression of genes involved in insulin synthesis and secretion. There are a number of potential target genes in which epigenetic change could result in altered glucose-insulin axis function. The transcription factor *PDX1* is one of the most important in the development of the pancreas (24). Postnatally, *PDX1* is selectively expressed in the β cells and plays a key role in β cell growth and development. Insulin-like growth factors (IGF)-1 and -2 also influence islet development, with IGF-2 involved in islet cell proliferation and survival during the fetal and neonatal period and IGF-1 having a similar effect in childhood (25). Insulin synthesis and secretion occur primarily in response to post-prandial increases in blood glucose concentration, although amino acids such as leucine and arginine also stimulate insulin release. Glucose is transported into the β cell by facilitated diffusion through the glucose transporter protein Slc2a2 (GLUT2) and phosphorylated by glucokinase, inducing oscillations in the ATP/ADP ratio. This alters plasma membrane permeability within the β cell and insulin is secreted. Epigenetic change in any combination of these factors could, therefore, affect insulin action and glucose disposal. We found altered protein, mRNA and evidence of epigenetic change in several of these target genes. However, the main stimulus for this remains unclear. One possibility is a direct effect of pancreatic exposure to increased dietary protein via the portal circulation, given that protein intake has been shown to alter pancreatic development in rodent studies, and protein intake was increased by approximately 60% in the supplemented animals in our study.

We chose the first 2 wk of life as the period of nutritional supplementation because previous studies have indicated that growth rate during this time is associated with later adverse health outcomes (26), and we anticipated that supplemented lambs would grow more rapidly. However, contrary to our expectation, the supplemented lambs in our study had decreased, rather than accelerated, growth during the period of supplementation. Provision of the supplement, which was given separately from ewe milk as all lambs were allowed to suckle freely from their ewe, did not, in fact, increase caloric intake compared with controls despite increasing protein intake. This is probably explained by the trend towards decreased voluntary intake of ewe milk, particularly in males. Taken together, these observations suggest that an altered dietary composition may be responsible for the observed

changes in metabolic outcomes, as neither caloric intake nor growth were different between groups. This and other studies have shown that in the immediate postnatal period, nutritional intake and growth rate are not necessarily well correlated, particularly after intrauterine insult, so that growth rate cannot necessarily be assumed to be a valid proxy for nutritional intake (14). Relationships between accelerated neonatal growth and later metabolic disease may, therefore, reflect the presence of pre-existing metabolic derangement, rather than postnatal “overnutrition” *per se*, and the composition of postnatal diet, as well as caloric intake, may be additive to intrauterine insult in determining long-term outcomes.

One of the most striking findings of our study was the sexual dimorphism in both mRNA expression of key genes and insulin response to a glucose load. Sex-specific responses to early life environmental influences increasingly are being recognized in both humans and animals. Moreover, it has been demonstrated that the development of impaired glucose homeostasis in young adult rats exposed to maternal low protein diet during gestation and lactation involved different mechanisms in males and females (27,28). While the female rat offspring were relatively more insulin deficient, with impaired glucose tolerance likely to be due to the reduction in the number and size of islets and β cell mass, the male offspring were more insulin resistant, but with no difference in glucose tolerance compared to control animals despite basal insulin concentrations being twofold higher. In sheep, impaired glucose tolerance in offspring of ewes undernourished in the periconceptional period occurred earlier and was more pronounced in females (22), whereas fat mass in adulthood was increased only in male offspring (29). Human studies investigating long-term outcomes of nutritional enrichment after preterm birth or intrauterine growth restriction have also shown sexually dimorphic outcomes in neurodevelopment (30), weight gain (31), blood pressure (32), and adiposity (9) in infancy and childhood. However, there are fewer studies investigating alterations of diet in term babies. One study of term babies born small for gestational age found neurodevelopmental outcomes were worse in girls in the supplemented group at 9 mo of age, despite a positive effect on growth, although developmental differences between groups did not appear to persist at later follow up (30). The mechanisms underlying these dimorphic responses remain unclear.

The insulin response to glucose load was increased in supplemented males and decreased in supplemented females compared with same sex controls. In supplemented males, this resulted in a lower glucose AUC₆₀, suggesting that the increased insulin secretion was due to intrinsic factors, rather than compensating for peripheral insulin resistance. However, the decreased AUC insulin in supplemented females was not associated with an increased glucose AUC₆₀, which raises the possibility that peripheral insulin sensitivity might be increased. Although we specifically investigated the effect of nutritional supplements on pancreatic development and function, they may also have resulted in other sexually dimorphic effects on metabolism.

Table 3. Composition of human breast milk, ewe milk, and fortifier

	Preterm human breast milk per 100 ml	Breast milk 100 ml + human milk fortifier	% increase with human milk fortifier	Ewe milk per 100 ml	Ewe milk 100 ml + ewe milk fortifier	% increase with ewe milk fortifier
Energy (kcal)	72	88	22	108	132	22
Protein (g)	1.35	2.2	63	6.0	9.8	63
Fat (g)	4.3	4.3	0	7.0	7.0	0
Carbohydrate (g)	7.2	10.5	46	5.3	7.7	45
Vitamin A (IU)	214	647	201	276	832	201
Vitamin D (IU)	1.6	5.05	215	7.2	22.5	213
Vitamin C (mg)	4.2	16.2	286	4.16	16.06	286

The composition of ewe milk fortifier is based on the percentage increase of components provided with human breast milk fortifier. Breast milk composition taken from Foodworks (Version 5) 1998–2007, New Zealand (Xyris Software (Australia) Pty Ltd, Australia). Ewe milk composition information obtained from smallstock.info website (New Zealand). The far right hand column represents the % increase in composition of 100 ml ewe's milk supplemented with the ewe milk fortifier.

We chose to look at epigenetic changes in the promotor region of the *PDX1* gene because of its ubiquity in β cell development and insulin production (33). Also, manipulation of the protein content in the postnatal diet in rodents has been found to alter both *PDX1* expression and activation (34). The increased insulin mRNA expression that we found in supplemented female lambs is consistent with the decreased repressor marks and increased activator marks observed. However, as similar hypomethylation without altered insulin mRNA expression was observed in supplemented males, additional epigenetic modifications to those we examined are likely to have occurred. We examined both methylation and histone modifications in the *PDX1* promoter but there may be other epigenetic mechanisms operating, such as epigenetic modifications in other loci that have downstream effects on the expression of *PDX1*, for example, through activity of microRNAs, that we have not examined. While the interactions between epigenetic change, mRNA expression and pancreatic function are clearly complex, it is likely that dietary composition, and in particular protein content, is a key moderator of pancreatic development. The differences in gene expression between sexes in the Control groups, the different relationships between epigenetic marks on *PDX1* and gene expression between the sexes, and an interaction between sex and supplementation, all suggest that nutrition is a powerful environmental factor that affects the development of metabolic pathways differently in males and females.

Limitations of the Study

In this study, we standardized as far as possible confounding factors such as gestation length and ewe and lamb health. However, further information about the effect of the high protein, high calorie supplement on neonatal growth and metabolism could have been gained from measuring metabolic rate and body composition in the lambs. Estimates of ewe milk composition were taken from published data, but we did not measure composition directly in the ewes in our study. We also did not measure the non-nutritive components of ewe milk, to see if concentrations of hormones such as leptin and adipokines, which may have affected satiety in the lambs, were similar amongst groups. Milk intake was measured, but observation and recording of lamb feeding behaviour may have

yielded additional information about timing and frequency of spontaneous feeds. Similarly, formal measurement of insulin sensitivity may have aided interpretation of the glucose tolerance tests. Longer-term studies looking at the duration of effect on the glucose-insulin axis beyond juvenile life will be important. Finally, it was beyond the scope of this study to measure the influence of nutritional supplements on the gut microbiome, but there is an increasing body of evidence suggesting that neonatal nutrition influences the composition of the microbiome, which in turn affects the development of metabolic pathways (35). Further studies of neonatal supplementation would be enriched by inclusion of outcome measures assessing effects on the gut microbiome.

Implications for Practice

In healthy term lambs, nutrition continues to influence pancreatic development via epigenetic change after birth in ways that affect later glucose–insulin axis function. We wished to determine the effect of supplementation in healthy, normally grown lambs as a first step to determine the effects of enhanced nutrition on the described outcomes independent of other variables. The experiment we describe does not compare a milk substitute with mother's milk and should not be extrapolated as such, but nutritional supplementation in addition to mother's milk that resulted in an altered dietary composition. Clearly, it would be of interest to investigate the impact of nutritional supplementation and dietary composition in other groups, such as growth restricted, preterm or twin lambs. While it is difficult to directly extrapolate these data to other species, it is likely that in humans also, nutritional manipulations in early postnatal life may alter later pancreatic function. This may be particularly relevant to infants born preterm or after nutritional insult, and requires further investigation. It is clear that neonatal dietary manipulation results in different physiological responses in males and females, so that for babies in whom neonatal nutritional supplementation is required, management plans may have to be sex specific.

Understanding how neonatal nutrition influences immediate and later pancreatic function, and further study into the reversibility of these effects, will contribute to the development of interventions to ameliorate adverse metabolic outcomes.

Table 4. Primer and probe sequences for housekeeping genes

Gene and Genbank Accession ID	Primers and Probes	Sequences
<i>Cyclophilin A</i> ; Accession ID: AY251270.1	Forward	5'-GTA CTG GTG GCA AGT CCA TCT-3'
	Reverse	5'-CAG GAC CTG TAT GCT TCA GAA TGA-3'
	Probe	6FAM-ATG GCG AGA AAT TTG-MGBNFQ
<i>ACTB</i> ; Accession ID: NM_001009784.1	Forward	5'-ACC AGT TCG CCA TGG ATG ATG-3'
	Reverse	5'-CCG GAG CCG TTG TCA AC-3'
	Probe	6FAM-ACG AGC GCA GCA ATA T-MGBNFQ
<i>GAPDH</i> ; Accession ID: NM_001190390	Forward	5'-GGG CTG CTT TTA ATA CTG GCA AA-3'
	Reverse	5'-CAT GTA GAC CAT GTA GTG AAG GTC AA-3'
	Probe	6FAM-CAT CGT TGC CAT CAA TG-MGBNFQ
<i>YWHAZ</i> ; Accession ID: AY970970.1	Forward	5'-GAG GGT CGT CTC CAG TAT TGA G-3'
	Reverse	5'-TTC TCG AGC CAT CTG CTG TTTT-3'
	Probe	6FAM-CAG CAC CTT CCG TCT TT-MGBNFQ
<i>HPRT1</i> ; Accession ID: EF078978	Forward	5'-AGG TGT TTA TTC CTC ATG GAC TAA TTA TGG-3'
	Reverse	5'-CAC CCA TCT CCT TCA TCA CAT CTC-3'
	Probe	6FAM-ACA GGA CCG AAC GAC TG-MGBNFQ
<i>RPL19</i> ; Accession ID: AY158223.1	Forward	5'-CAA AAA CAA GCG GAT TCT CAT G-3'
	Reverse	5'-GCT TCT TGC GAG CCT TGT CT-3'
	Probe	6FAM-AAC ATA TCC ACA AGCTGA A-MGBNFQ
<i>PDX1</i> exon 1	Forward	5'-CCG GAC ATC TCC CCA TAC GA-3'
	Reverse	5'-GGT GGA GGT GCG CCA T-3'
	Probe	6FAM-CCG CTC GCC GAC GAC-MGBNFQ
<i>PDX1</i> promoter	Forward	5'-GGC ACA GCG GAG CCT AT-3'
	Reverse	5'-GCC ACC CCT GCA GCT T-3'
	Probe	6FAM-CCG CGC CGA GCT GT-MGBNFQ
<i>IGF-1</i> ; Accession ID: M31734	Forward	5'-CTT CCG GAG CTG TGA TCT GA-3'
	Reverse	5'-TGA GCG GGC CGA CTT G-3'
	Probe	6FAM-CTG TGC GCC TCT CAA G-MGBNFQ
<i>IGF-II</i> ; Accession ID: M89788	Forward	5'-CGA GGC ATC CAG CGA TTA G-3'
	Reverse	5'-TAG ATG GTG TCA CTT GGC AGA ATT-3'
	Probe	6FAM-AGT GAG CCA AAG TGT C-MGBNFQ
<i>SCL2A2</i> ; Accession ID: AJ318925	Forward	5'-GAA GAA TCA AAG CCC TGT TGGT-3'
	Reverse	5'-TTC GAA AAC CCC ATC AAG AGA-3'
	Probe	6FAM-CAA ACA TTC TTT CAT TAG TTG GA-MGBNFQ
<i>INSULIN</i> ; Accession ID: AH005355S1	Forward	5'-GCA GAA GCG TGG CAT CGT-3'
	Reverse	5'-TTA CAG TAG TTC TCC AGC TGG TAG AGA-3'
	Probe	6FAM-AGC AGT GCT GCG CC-MGBNFQ
<i>GLUCOKINASE</i> ; Accession ID: NM_001102302.1	Forward	5'-TGT GAG GTT GGC ATG ATT GTG-3'
	Reverse	5'-TCT GCA TCT CCT CCA TGT AGC A-3'
	Probe	6FAM-CAC GGG CTG CAA CG-MGBNFQ
18S	Eukaryotic 18S rRNA endogenous VIC/MGB Probe and Primers (Applied Biosystems).	

FAM/VIC, fluorescent reporter dyes bound to the TaqMan probe; MGBNFQ, molecular-groove binding non-fluorescence quencher.

METHODS

All procedures used in this experiment were approved by the Animal Ethics Committee, University of Auckland (R628).

Singleton-bearing Romney ewes were identified by ultrasound scanning at 50 d. One week before expected lambing (140 d of gestation (day 140); term = day 147), ewes were brought into the feedlot and housed in individual pens with open mesh sides, adjacent to other sheep. Prior to birth, lambs were randomized to either the supplemented group (S; allocated to receive a calorie dense nutritional supplement from days 1–14 after birth (day 0) in addition to suckling freely from their mother) or to the control group (C; allowed to suckle freely from their mother, and given aliquots of water in similar volume to the nutritional supplement). The supplement consisted of a mixture of protein (NZMP Whey Protein Isolate 895, Fonterra New Zealand) and carbohydrate (Maltodextrin Dextrose Equivalence of 18.0, Unitech Industries Limited, Auckland, New Zealand) powders plus vitamins A, C and D (KaricareVitadol C, Nutricia, Auckland, New Zealand) reconstituted in the minimal amount of water possible (approximately 20 ml). It was formulated to provide approximately 25% increase in daily calorie intake and 66% increase in daily protein intake, based on the additional intakes achieved clinically when milk fortifiers are added to expressed human breast milk, adjusted for ewe milk composition (Table 3). The total amount of supplement given was dependent on estimated milk intake based on postnatal day and weight of the lamb. Supplement for day 1 to day 3 was based on birth weight and an intake of $100 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$; increased to an intake of $120 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ (calculated on the day 4 weight) on days 4 to 7 and to $150 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ (calculated on day 8 weight) from days 8 to 14. The supplement, or similar volume of water for the C group, was administered via a syringe with a teat in two to four aliquots per day.

Milk intake was measured in a subset of lambs during the second week after birth using a deuterium oxide (D_2O) dilution-technique (14).

Lambs were weighed at birth, on day 4 and day 8 and weekly thereafter until 12 wk of age. Growth velocity was calculated (36). At 2 wk after birth, lambs and ewes returned to the paddock and were brought in weekly for weighing. Weaning occurred at 12 wk and lambs were separated from the ewes at this time and subsequently reared in sex-segregated flocks. All lambs remained intact.

At 4 mo of age, lambs were brought into the feedlot and weighed, and an IVGTT was performed. Jugular venous catheters were inserted using local anesthesia (xylocaine 2%). Lambs were fasted overnight, but allowed free access to water. A baseline blood sample was taken and glucose bolus (0.5 g/kg of body weight of 50% glucose) was given intravenously at time 0. Blood samples were taken at baseline, and at 2, 5, 10, 15, 20, 30, 40, 50, 60, 80, 100, 120, and 180 min post-glucose bolus, and the plasma frozen until analysis. Plasma glucose concentrations were measured by autoanalyser (Hitachi 902 Automatic Analyser; enzymatic colorimetric assay). Plasma ovine insulin concentration was measured by radioimmunoassay (RIA), using ovine insulin as the standard (Sigma Chemical, St Louis, MO). Glucose tolerance was calculated by plotting plasma glucose and insulin concentrations against time and calculating the area under the curve by a triangulation method, in the first hour of the test (glucose AUC_{60} and Insulin AUC_{60}) to look for differences in first and second phase insulin release and for the whole duration of the test (glucose AUC_{180} and insulin AUC_{180}).

After the IVGTT, a subset of lambs underwent postmortem examination. Organs were weighed and measured, and samples of liver and pancreas were collected.

The pancreas was bisected longitudinally, so that each half contained head, body, and tail. One half of the pancreas was rapidly frozen in liquid nitrogen and stored at -80°C for further analysis.

RNA Isolation and RT-PCR

Whole pancreas was ground into powder in liquid nitrogen and well mixed in a container. Total RNA was extracted using TRIZOL (Invitrogen, Carlsbad, CA). RNA concentration was measured and the absorbance ratios 260/280 and 260/230 with ratios ≥ 1.9 considered acceptable purity. RNA gels were run to check the RNA integrity. The band density ratios for 28S rRNA and 18S rRNA were determined

using Image J software (National Institute of Health, Bethesda, MD) with ratios above 1.8 accepted as representing good quality intact RNA. Total RNA ($2.5 \mu\text{g}$) was treated with RNase-free DNase I (Invitrogen) to eliminate potential genomic DNA. First-strand cDNA was synthesized using SuperScript VILO cDNA Synthesis system (Invitrogen, Life Technologies).

Primer Express Software (Applied Biosystems, Foster City, CA) was used to design Taqman probe and primer for real-time PCR (Table 2). A BLAST search ensured that primers and probes were not designed from homologous regions that would encode for genes other than the target genes.

Real-Time PCR

Real-time PCR was carried out using ABI 7900HT Real-Time PCR system (Applied Biosystems). Standard curves for the target and reference genes (Ref) were included in the runs to calculate the amplification efficiencies of the genes ($E = 10^{-1/\text{slope}}$). Data were normalized to the geometric mean of the three most stably expressed reference genes. The relative expression ratios were calculated by a mathematical model (37):

$$\text{Ratio} = (\text{E}_{\text{target}})^{\Delta\text{Ct}_{\text{target}}(\text{control-sample})} / (\text{E}_{\text{ref}})^{\Delta\text{Ct}_{\text{ref}}(\text{control-sample})}$$

Analysis of Stably Expressed Reference Genes

We screened seven commonly used reference genes (*GAPDH*, *BETA ACTIN*, *CLYCOPHILIN A*, *HPRT1*, *YWHAZ*, *18S*, *RPL19*) to select a panel of three genes for use as a composite reference. Reference gene expression levels were calculated based on a cycle threshold (Ct). The stability of the expression of the reference genes was evaluated by inspection of the SD and coefficient of variance (CV) calculated for each gene. The range of expression stability of the candidate reference genes based on the inspection of SD and CV was (from the most stable to the least stable): *RPL19*, *GAPDH*, *HPRT1*, *YWHAZ*, *BETA ACTIN*, *CLYCOPHILIN*, *18S*. The Bestkeeper index (37) was calculated based on the geometric mean of the Ct values of the three most stably expressed reference genes, *RPL19*, *GAPDH*, and *HPRT1*, and was then used as a normalization factor in the analysis.

Sequencing Ovine PDX1

Due to the lack of sheep genomic sequence available, the bovine genome sequence which is highly homologous to the sheep (build *Bos taurus*_UMD_3.1/*bos* Tau6) and porcine genome sequence (SGSC Sscrofa 9.2/*sus*Scr2) were used as a base to map *PDX1* genes and its promoter region. Primers were designed against the conserved homologous regions and used to amplify ovine genomic DNA. The ovine *PDX1* promoter region identified was located 209 bp upstream of the transcriptional start site of the exon 1, and is CpG rich. Successfully amplified ovine genomic DNA from sequence -209 to $+420$ (GenBank accession number KP059125) was sent for sequencing and sequenced results were then aligned with bovine genome sequence using ClustalW 2.0 (European Molecular Biology Laboratory-European Bioinformatics Institute, Saffron Walden CB10 1SD, UK).

Primers used to sequence the ovine Pdx-1 were 5'-GGNCT GGCTTCTTAAGGC-3' and 5'-TATGGTTTAGTNCGACTCT-3'.

Methylation Analysis of Pdx-1 Promoter

Total genomic DNA was isolated from 100 mg of pulverized pancreatic tissues. The MethylminerTM methylated DNA Enrichment kit (Invitrogen, Life Technologies, Carlsbad, CA) was used to separate methylated genomic DNA from total genomic DNA. Briefly, genomic DNA was digested using the MSE1 enzyme (New England Biolabs (NEB), Ipswich, MA) to give an average size of less than 1,000 bp. One microgram of digested DNA was used in each IP sample and 10% IP volume of digested DNA was used as Input DNA. The methylated DNA was captured from digested whole genomic DNA via binding the MethylminerTM MBD-Biotin protein which coupled to Dynabeads M-280 Streptavidin. The methylated DNA was eluted from Dynabeads. Eluted methylated DNA was then cleaned and concentrated using Zymo Research ChIP DNA clean and concentrator kit (Zymo Research, Irvine, CA) before real time PCR analysis. Real time PCR was performed on Input DNA and compared with methylation-enriched DNA to establish relative differences in methylation

state. Primers used in the real time PCR for methylation analysis were *PDX1* promoter (Table 4), targeting from –179 to –81 base pairs relative to the transcriptional start site.

Chromatin Immunoprecipitation (ChIP)

Alterations of histones in the *PDX1* promoter were measured using Active Motif ChIP-IT™ Express Enzymatic kit (Active Motif, Australian Bioresearch, WA, Australia). Briefly, 140 mg of pulverized pancreatic tissues were fixed in 1% formaldehyde for 5 min. Sonication was performed to yield DNA fragments ranging in size from 200–1,500 bp. Thirty-eight micrograms of sheared chromatin were used in immunoprecipitation (IP) and 1% of IP volume was used as input DNA. IP was carried out overnight with either 3 µg of Histone H3 acetyl Lys9 pAb (Active Motif), Histone H3 trimethyl Lys27 pAb (Active Motif), Histone H3 trimethyl Lys4 (Active Motif), RNA pol II mAb (Active Motif), or normal rabbit IgG (Cell Signalling). RNase POL II was used as a positive control and rabbit IgG was used as a negative control. The resulting enriched DNA and the Input DNA were cleaned and concentrated using Zymo Research ChIP DNA clean and concentrator kit (Zymo Research) before real time PCR analysis. Real-time PCR was performed on input DNA as described above and compared with IP enriched DNA to establish relative differences in histone modifications. Primer used in real-time PCR for ChIP analysis was *PDX1* promoter (Table 4). IP data were normalized to both its input DNA and negative control.

Statistics

Data are presented as mean ± SEM.

Differences in growth and glucose tolerance between control and supplement groups were analyzed using ANOVA, with Fisher *post hoc* test.

Data for real-time PCR and epigenetic studies are presented as relative expression ratios of the male and female S groups relative to the same sex C group with 95% confidence intervals (CI).

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