Impact of maternal undernutrition around the time of conception on factors regulating hepatic lipid metabolism and microRNAs in singleton and twin fetuses

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Submitted 22 December 2014; accepted in final form 31 August 2015

Hepatic lipid accumulation is regulated partly by AMP-activated protein kinase (AMPK), which stimulates fatty acid β-oxidation through the inhibition of malonyl-CoA synthesis.
The rate of fatty acid β-oxidation is also regulated by pyruvate dehydrogenase kinase (PDK)2 and PDK4 (Fig. 1) (44). Additionally, fatty acid metabolism in the liver is regulated by peroxisome proliferator-activated receptor-α (PPARα), which stimulates transcription of factors regulating fatty acid transport and mitochondrial fatty acid β-oxidation, including carnitine palmitoyltransferase-1 (CPT-1) and PDK4 (10).

It has also been proposed that impairment of mitochondrial function can lead to the accumulation of lipid metabolites such as diacylglycerol and ceramides, resulting in the development of insulin resistance (45). PPARγ coactivator-1α (PGC-1α) subunit is a transcriptional coactivator that plays an important role as a metabolic regulator by stimulating mitochondrial biogenesis and increasing the expression of factors regulating mitochondrial fatty acid β-oxidation (Fig. 1) (37). Additionally, phosphorylation of 3-phosphoinositide-dependent protein kinase-1 (PDPK-1), which is stimulated following activation of the insulin receptor and phosphatidylinositol-3 kinase (PI3K), results in the phosphorylation of the atypical protein kinase Cζ (PKCζ), which also plays a role in hepatic lipid synthesis (42).

MicroRNAs (miRs) are small (~22 nucleotides) species of noncoding RNA that act as posttranscriptional regulators through perfect or nearly perfect binding of the 7nt “seed” sequence of the miR to the 3′-untranslated region (UTR) of the target transcript that results in translational repression or degradation of the target mRNA (3). Recent studies have shown that a number of miRs play an essential role in regulating the abundance of factors within the fatty acid β-oxidation pathway (1, 13) and that dysregulated expression of specific miRs in the liver, muscle, and/or fat has been shown to result in a number of features of the metabolic syndrome and type 2 diabetes in a range of in vivo and in vitro experimental models (9, 47). There is also evidence that miRs may play an important role in mediating the programming effects in the offspring exposed to the effects of poor maternal nutrition before pregnancy and during gestation (14, 40). There have been no studies, however, on whether the expression of miRs that play a role in hepatic lipid metabolism may be altered after exposure to poor maternal nutrition in the periconceptional or preimplantation periods.

Therefore, in the current study we have investigated the separate effects of maternal undernutrition in the periconceptional period (PCUN; for ≥2 mo before and 6 days after conception) or preimplantation period (PIUN; for 6 days after conception) on the mRNA expression and protein abundance of factors regulating fatty acid β-oxidation in the liver of fetal sheep in singleton and twin pregnancies. We have also determined the impact of PCUN or PIUN on the expression of candidate miRs that may play a role in the regulation of protein abundance of the key factors within the lipid metabolism pathway. We hypothesized, based on our findings on the abundance of insulin-signaling and gluconeogenic molecules in the fetal liver of the PCUN and PIUN groups (26), that exposure to PCUN and PIUN would result in decreased abundance of fatty acid β-oxidation molecules in singletons but increased abundance of fatty acid β-oxidation molecules in twins.

MATERIALS AND METHODS

All procedures were approved by the University of Adelaide Animal Ethics Committee and by the Primary Industries and Resources South Australia Animal Ethics Committee (26, 49).

Nutritional Management

South Australian Merino ewes were fed a diet that consisted of lucerne chaff and pellets containing cereal hay, lucerne hay, barley, oats, almond shells, lupins, oat bran, lime, and molasses (Johnsons & Sons, Kapunda, South Australia, Australia), as described previously (26, 49). All ewes received 100% of the nutritional requirements to provide sufficient energy for the maintenance of a nonpregnant ewe as defined by the Agricultural and Food Research Council (Energy and Protein Requirements of Ruminants) in 1993.

At the end of an acclimatization period, ewes were randomly assigned to one of three feeding regimes, as described previously (26, 49). The control ewes (C; n = 12) received 100% of the nutritional requirements

![Fig. 1. Molecular signaling pathways regulating fatty acid β-oxidation and mitochondrial biogenesis. Phosphorylation of AMP-activated protein kinase (AMPK) phosphorylates acetyl-CoA carboxylase (ACC), which inhibits malonyl-CoA synthesis. Malonyl-CoA inhibits carnitine palmitoyltransferase 1 (CPT-1), which facilitates the transport of fatty acids into the mitochondria, which is the first and rate-limiting step in mitochondrial fatty acid β-oxidation. Pyruvate dehydrogenase kinase (PDK)2 and PDK4 also stimulate fatty acid β-oxidation by inhibiting glucose oxidation while favoring fatty acid β-oxidation. Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) subunit regulates fatty acid β-oxidation by stimulating the expression of fatty acid β-oxidation regulators and mitochondrial biogenesis. p-AMPK and p-ACC, phosphorylated AMPK and ACC, respectively.](http://ajpendo.physiology.org/Download/10.1152/ajpendo.00600.2014)
requirements from 84.5 ± 7.0 days prior to mating until 6 days after
matting. Ewes in the periconceptional undernutrition (PCUN) group
(n = 13) received 70% of the control allowance from 76.8 ± 4.0 days
prior to mating until 6 days after mating. Ewes in the preimplantation
undernutrition (PIUN) group (n = 9) received 70% of the control diet
from mating until 6 days after mating. All of the dietary components
were reduced by an equal amount in the restricted diet. From 7 days
after conception, all ewes were fed 100% of nutritional requirements.

Mating, Surgery, Fetal Outcomes, and Postmortem

Ewes were released in a group every evening with rams of proven
fertility that were fitted with harnesses and marker crayons. Ewes
were housed individually the following morning, and the occurrence
of mating was confirmed by the presence of a crayon mark on the
ewe’s rump. The day of mating was defined as day 0. Ewes were
weighed weekly after the feeding regime was commenced until
postmortem at 135–138 days of gestation. The body weight of the
ewes in the PCUN group was significantly lower compared with the
body weight of the C and PIUN groups during the preconceptional
period (49). Pregnancy and fetal number were estimated by ultrasound
at 40–80 days gestation, and the nutritional intake for the ewes was
adjusted for gestational age and fetal number.

Surgery was performed in the pregnant ewes (C: n = 12; PCUN:
n = 13; PIUN: n = 9), carrying singletons (C: n = 6; PCUN: n = 8;
PIUN: n = 3), or twins (C: n = 6; PCUN: n = 5; PIUN: n = 6) under
general anesthesia between 105 and 110 days gestation, as described
previously (7). In brief, vascular catheters were inserted into a fetal
carotid artery and jugular vein, a maternal jugular vein, and the
amniotic cavity. Vascular catheters were inserted into only one fetus
in twin pregnancies. All catheters were filled with heparinized saline,
and fetal catheters were exteriorized through an incision in the ewe’s
flanks.

All ewes carrying fetuses used in this study (n = 34) were
humanely euthanized with an overdose of pentobarbitone sodium
between 135 and 138 days gestation, and the utero-placental unit was
delivered by hysterotomy. In four ewes carrying twin fetuses, fetal
health declined prior to postmortem and/or a twin fetus died within 24
h of the postmortem; in these instances, fetal tissues were not
collected. Fetuses (singleton: C n = 6, PCUN n = 8, PIUN n = 3;
twin: C n = 11, PCUN n = 8, PIUN n = 11) were weighed and
euthanized by decapitation. Crown rump length and body weight were
measured, and liver samples were collected and snap-frozen in liquid
nitrogen. Samples were then stored at −80°C for further molecular
analyses. Details of the number of animals included in the study for
the range of analyses are provided in Table 1.

Quantification of mRNA Expression

RNA was extracted from ~50 mg of liver tissue using Trizol
reagent (Invitrogen, Groningen, The Netherlands) from singleton and
twin fetuses (Table 1). The relative expression of mRNA transcripts of
AMPKα1, AMPKα2, PGC-1α, PPARα, PDK2, PDK4, PKCζ, and the housekeeper gene cyclophilin was measured by quantitative real-
time reverse transcription-PCR (qRT-PCR) using the SYBR Green
system in an ABI Prism 7500 Sequence Detection System (Applied
Biosystems, Foster City, CA), as described previously (26).

Primer sequences were validated for use in the sheep in this
(Table 2) or in prior studies (32, 36). The abundance of each mRNA
transcript was measured, and their expression relative to cyclophilin
was calculated using the comparative threshold cycle (Ct) method
(Q-gene qRT-PCR analysis software).

Quantification of Protein Abundance

The protein abundance of total AMPK, total phosphorylated (p-)
AMPK (Thr172), AMPKα1, AMPKα2, p-AMPKα1 (Ser485), PGC-
1α, PDK-1, P-PDK-1 (Ser241), PKCζ, PDK2, PDK4, ACC, p-ACC
(Ser79), and CPT-1 was determined using Western blotting, as
described previously (26). Briefly, liver samples (~100 mg) from
singleton and twin fetuses (Table 1) were homogenized in lysis buffer,
and protein content of the clarified extracts was quantified using
bicinchoninic acid protein assay. Prior to Western blot analysis,
samples (10 μg of protein) were subjected to SDS-PAGE and stained
with Coomassie blue reagent (Thermo Fisher Scientific, Rockford, IL)
to ensure equal loading of the proteins. Equal volumes and concentra-
tions of protein were subjected to SDS-PAGE. The membranes
were blocked with 5% BSA in Tris-buffered saline with 0.1%
Tween-20 (TBS-T) at room temperature for 1 h and then incubated
overnight with primary antibody in TBS-T against total AMPK, total
p-AMPK (Thr172), AMPKα1, AMPKα2 (Ser485), AMPKα2, PGC-
1α, ACC, p-ACC (Ser79), PDK-1, p-PDK-1 (Ser241), p-PKCζ
(Thr410) (Cell Signaling Technology, Danvers, MA), PKCζ, CPT-1
(Santa Cruz biotechnology, Santa Cruz, CA), PDK2 (Epitomics,
Burlingame, CA), and PDK4 (Abcam, Cambridge, UK). Membranes
were washed, and bound antibody was detected using anti-rabbit (Cell
Signaling Technology) horseradish peroxidase-conjugated secondary
IgG antibodies at room temperature for 1 h. Enhanced chemilumines-
cence reagents SuperSignal West Pico Chemiluminescent Substrate
(Thermo Fisher Scientific) and ImageQuant LAS 4000 (GE Health-
care, Rydalmere, New South Wales, Australia) were used to detect the
protein-antibody complexes. AlphaEaseFC (Alpha Innotech, Santa
Clara, CA) was used to quantify the protein bands.

Table 1. No. of animals from each treatment group in singleton and twin pregnancies used in each set of analyses

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PCUN, periconceptional undernutrition; PIUN, preimplantation undernutrition.

Table 2. Primer sequences for quantitative RT-PCR

<table>
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<th>Reverse Primer Sequence</th>
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<td>5′- CATTGCGCCATGAGAAAGATGCGA-3′</td>
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<tr>
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<tr>
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<td>5′- GCCATGCTGCTGCTGCTGCTGCTG-3′</td>
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<td>PKCζ</td>
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<td>5′- GCCATGCTGCTGCTGCTGCTGCTG-3′</td>
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<tr>
<td>Reverse</td>
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<td>5′- GCCATGCTGCTGCTGCTGCTGCTG-3′</td>
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</tr>
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</table>

PDK2 and -4, pyruvate dehydrogenase kinase 2 and 4, respectively.
Clara, CA) was utilized to quantify specific bands of the target proteins.

**Statistical Analyses**

*mRNA expression and protein abundance*. All data are presented as means ± SE. All data were analyzed using the Statistical Package for the Social Sciences Software (SPSS, Chicago, IL). Two-way analysis of variance (ANOVA) was used to determine the effects of maternal nutritional treatment (PCUN and PIUN) and fetal number (singleton or twin) on mRNA expression and protein abundance in the liver. When there was no interaction between the effects of nutritional treatment and fetal number, the data from singleton and twin fetuses were combined for presentation of the effects of nutritional treatment. When there was an interaction between the effects of nutritional treatment and fetal number, data from singletons and twins were split and the effects of nutritional treatment determined using one-way ANOVA. Duncan’s post hoc test was used to determine the level of significant difference in mean values between nutritional treatment groups. A probability level of 5% (P < 0.05) was taken as significant.

**miR expression.** MiR data were obtained using next-generation small-RNA sequencing, as described previously (17). Candidate miRs altered by either PCUN and/or PIUN were identified using the following criteria: a threshold for a fold difference of expression of miRs between the PCUN or PIUN treatment groups relative to controls was set at 1.5 or <0.67 with a threshold of >100 reads/million or at 1.2 or <0.83 with a threshold of >10,000 reads/million, where the relative standard deviation was <50% among animals within a treatment group. Selected miRs based on these criteria from data mapped to the human miRBase were then cross-checked with the corresponding miRs mapped to the bovine miRBase. miRs were then selected as high-confidence “candidates.” Using the stringent threshold criteria described above, we have previously identified 23 miRs with altered expression in either the PCUN or PIUN groups relative to controls (26).

The correlation of the expression of all candidate miRs with the abundance of proteins within the lipid metabolism signaling pathway found to be changed in this study was then determined using linear regression analysis (SPSS). Candidate miRs were also analyzed using Targetscan to identify 8mer, 7mer-m8, or 7mer-1A matches between the seed sequence of the candidate miRs within the 3’-UTR of the putative mRNA targets within the lipid metabolism signaling pathway that are conserved across species.

**RESULTS**

There was no difference in either the absolute liver weight (C: 106.2 ± 5.4 g; PCUN: 121.6 ± 5.7 g; PIUN: 107.6 ± 5.0 g) or liver weight relative to body weight (C: 24.7 ± 0.8 g; PCUN: 26.1 ± 1.1 g; PIUN: 24.4 ± 1.0 g) between treatment groups. Similarly, there was no difference in fetal weight (C: 4.3 ± 0.2 kg; PCUN: 4.6 ± 0.2 kg; PIUN: 4.4 ± 0.02 kg) between either treatment groups (27).

**Expression and Abundance of Factors Regulating Cellular Energy Homeostasis and Fatty Acid β-Oxidation**

There was no difference in PPARα mRNA expression, AMPKα2 mRNA or protein abundance, or total AMPK, total p-AMPK (Thr172), and ACC protein abundance in the PCUN or PIUN groups compared with controls in singletons and twins (data not shown).

The hepatic mRNA expression of PGC-1α (P < 0.05) and PDK2 (P < 0.01) (Fig. 2), but not the protein abundance (data not shown), was lower in the PCUN and PIUN groups compared with controls in singleton and twin fetuses. The hepatic AMPKα1 mRNA expression was lower (P < 0.05), whereas AMPKα1 protein abundance was higher (P < 0.01) in the PIUN group compared with controls (Fig. 3). There was no difference, however, in the protein abundance of phosphorylated AMPKα1 (Ser485) in the fetal liver in the PIUN or PCUN groups compared with controls (Fig. 3). Hepatic PDK4 mRNA expression (P < 0.01) and protein abundance (P < 0.01) were each lower in the PCUN and PIUN groups (Fig. 4), and the protein abundance of CPT-1 was also lower (P < 0.01) in these groups compared with controls (Fig. 5).

The protein abundance of PDKP-1 but not p-PDKP-1 (Ser241) was lower (P < 0.05) in the fetal liver in the PCUN group compared with controls (Fig. 6). The hepatic protein abundance of PKCδ, but not its phosphorylated form (Thr410), was lower (P < 0.01) in the PCUN and PIUN groups compared with controls (Fig. 6).

![Fig. 2](http://ajpendo.physiology.org/) The hepatic expression of PGC-1α and PDK2 mRNA in singleton and twin fetuses in late gestation. Expression of PGC-1α mRNA (A) and PDK2 mRNA (B) in the periconceptional (PCUN) and preimplantation undernutrition (PIUN) groups compared with controls in singletons and twins. Different letters denote significant differences between treatment groups.
Fig. 3. The hepatic mRNA expression of AMPKα1 and protein abundance of AMPKα1 and p-AMPKα1 (Ser485) in singleton and twin fetuses in late gestation. Expression of AMPKα1 mRNA in singletons (A) and twins (B), protein abundance of AMPKα1 in singletons and twins (C), and abundance of p-AMPKα1 (Ser485) in singletons (D) and twins (E) in the PCUN and PIUN groups compared with controls. Different letters denote significant differences between treatment groups.
**Twins.** Hepatic mRNA expression of AMPKα1 was not different in the PCUN and PIUN groups compared with controls (Fig. 3). However, the protein abundance of AMPKα1 was higher \( (P < 0.01) \) in the PIUN group, and the protein abundance of p-AMPKα1 (Ser\(^{485}\)) was also higher \( (P < 0.01) \) in both the PCUN and PIUN groups compared with controls (Fig. 3).

Hepatic PDK4 mRNA expression was lower \( (P < 0.01) \) in the PCUN and PIUN groups (Fig. 4). However, the protein abundance of PDK4 was higher \( (P < 0.001) \) only in the PIUN group compared with controls (Fig. 4). The protein abundance of CPT-1 was also higher \( (P < 0.05) \) in the PIUN group compared with controls (Fig. 5).

There was a trend \( (P = 0.055) \) toward an increase in the hepatic protein abundance of PDPK-1 in the PIUN group, and the protein abundance of p-PDPK-1 (Ser\(^{341}\)) was higher \( (P < 0.05) \) in the PCUN and PIUN groups compared with controls (Fig. 6). The hepatic protein abundance of PKCζ, but not phosphorylated PKCζ (Thr\(^{410}\)), was higher \( (P < 0.05) \) in the twin fetuses in the PCUN and PIUN groups (Fig. 6) compared with controls.

**Relationship Between miRs and the Abundance of the Proteins Regulating Fatty Acid ß-Oxidation**

In twins only, there was an inverse relationship between the expression of miR-126-5p and the protein abundance of AMPKα1 \( (P < 0.01, r^2 = 0.74) \), let-7g-5p \( (P < 0.01, r^2 = 0.72) \) and miR-335-5p with the protein abundance of PDK-1 \( (P < 0.05, r^2 = 0.62) \), and miR-379-3p \( (P < 0.01, r^2 = 0.82) \) and miR-148a-3p \( (P < 0.05, r^2 = 0.55) \) with the protein abundance of PDK4 (Table 3). Using the Targetscan software, we found that a number of the identified miRs were predicted to regulate the expression of a range of factors relevant to hepatic lipid metabolism, as summarized in Table 4.

Interestingly, there was a positive relationship between the expression of miR-106b-5p and the abundance of AMPKα1 \( (P < 0.01, r^2 = 0.46) \), miR-19a-3p \( (P < 0.01, r^2 = 0.75) \) and miR-19b-3p \( (P < 0.01, r^2 = 0.51) \) with the protein abundance of AMPKα2 and between the expression of miR-30a-5p \( (P < 0.01, r^2 = 0.42) \), and miR-30e-5p \( (P < 0.01, r^2 = 0.48) \) with the protein abundance of PDK4 in singletons and twins when
that there was a downregulation of hepatic PGC-1α/H9251 during the 4-mo-old postnatal lamb, although we found which we investigated the effects of periconceptional undernutrition on protein abundance of either PGC-1α or PDK2. It has been suggested that during fetal life the expression of the key factors controlling mitochondrial biogenesis mediators is regulated by changes at the transcriptional level, whereas after birth mitochondrial differentiation occurs rapidly, and this is associated with the emergence of regulation at the posttranscriptional level (5). Therefore, any impact of periconceptional undernutrition on protein abundance and mitochondrial number may not emerge until after the transition to postnatal life. PGC-1α also acts to upregulate hepatic phosphoenolpyruvate carboxykinase C expression (41). PDK2 also promotes gluconeogenesis by inhibiting complete glucose oxidation through inactivation of PDC, thus conserving the three-carbon substrate for glucose production (16). Therefore, a decrease in PGC-1α and PDK2 expression may each contribute to a decrease in hepatic gluconeogenic capacity in postnatal life.

Singletons. In singleton fetuses, we found that hepatic AMPKα1 mRNA expression was lower, whereas its protein abundance was higher in the absence of a change in the phosphorylated form of AMPKα1 (Ser485) in PIUN fetal liver after exposure to maternal undernutrition around the time of conception, this phenotype may not emerge until later in life, as described in prior studies (31, 39, 46).

Impact of PCUN and PIUN on Fatty Acid β-Oxidation and Lipid Synthesis Signaling Factors

The hepatic mRNA expression of PGC-1α and PDK2 was lower in singletons and twins in both the PCUN and PIUN groups compared with controls. PGC-1α is a marker of mitochondrial biogenesis and function, which has been shown to be decreased in the liver of overweight and obese insulin-resistant individuals (15). It has also been shown that PDK2 acts to inhibit pyruvate dehydrogenase complex (PDC) and thus inhibits glucose oxidation, which can then result in an increase in fatty acid β-oxidation (44). There was no impact, however, of exposure to PCUN or PIUN on the protein abundance of either PGC-1α or PDK2. It has been suggested that during fetal life the expression of the key factors controlling mitochondrial biogenesis mediators is regulated by changes at the transcriptional level, whereas after birth mitochondrial differentiation occurs rapidly, and this is associated with the emergence of regulation at the posttranscriptional level (5). Therefore, any impact of periconceptional undernutrition on protein abundance and mitochondrial number may not emerge until after the transition to postnatal life. PGC-1α also acts to upregulate hepatic phosphoenolpyruvate carboxykinase C expression (41). PDK2 also promotes gluconeogenesis by inhibiting complete glucose oxidation through inactivation of PDC, thus conserving the three-carbon substrate for glucose production (16). Therefore, a decrease in PGC-1α and PDK2 expression may each contribute to a decrease in hepatic gluconeogenic capacity in postnatal life.

DISCUSSION

We have shown for the first time that maternal undernutrition during the periconceptional and the preimplantation periods results in significant changes in the mRNA expression and/or protein abundance of factors regulating fatty acid β-oxidation (Fig. 1) and lipid synthesis in the late-gestation fetal sheep liver and that these effects were different in singletons and twins. There was no effect of either PCUN or PIUN on fetal weight or on the absolute or relative liver weight. This is consistent with our previous study (27) and other studies that have investigated the impact of nutritional perturbations around the time of conception (7, 39, 46). There is evidence that changes in maternal nutrition during pregnancy have an impact on hepatic lipid storage in postnatal rather than in prenatal life (23, 31). In particular, Mouralidarane et al. (31) found that after exposure to a maternal high-fat diet throughout prenatal life (23, 31). In particular, Mouralidarane et al. (31) found that after exposure to a maternal high-fat diet throughout pregnancy, hepatosteatosis and a clear NAFLD phenotype emerged at 12 mo rather than 3 or 6 mo after birth in the rat offspring. We also note that in a previously published paper in which we investigated the effects of periconceptional undernutrition in the 4-mo-old postnatal lamb, although we found that there was a downregulation of hepatic PGC-1α in the postnatal lamb, the changes in triglyceride staining in the livers of the PCUN lambs were not significant at this age (34). We also note that the concentrations of circulating free fatty acids are low in the fetal lamb compared with either the postnatal or adult lamb, and therefore, substrate for hepatic triglyceride storage is relatively limited before birth. Consistent with this relative lack of available substrate, in the present study, preliminary staining for hepatic triglycerides showed no difference in the density of staining between the PCUN, PIUN, and control groups (unpublished observations). We conclude that although hepatic gene changes are present within the fetal and postnatal liver after exposure to maternal undernutrition around the time of conception, this phenotype may not emerge until later in life, as described in prior studies (31, 39, 46).

Fig. 5. The hepatic protein abundance of CPT-1 in singleton and twin fetuses in late gestation. Protein abundance of CPT-1 in singletons (A) and twins (B) in the PCUN and PIUN groups compared with controls. Different letters denote significant differences between treatment groups.
Fig. 6. The hepatic protein abundance of 3-phosphoinositide-dependent protein kinase-1 (PDPK-1), p-PDPK-1 (Ser241), and PKCζ in singleton and twin fetuses in late gestation. Protein abundance of PDPK-1, p-PDPK-1 (Ser241), and PKCζ in singletons (A–C) and twins (D–F) in the PCUN or PIUN groups compared with controls. Different letters denote significant differences between treatment groups.
Table 3. Relationship between candidate microRNAs and the abundance of the factors regulating fatty acid β-oxidation in the fetal liver in late gestation (correlations)

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<td>Twin only</td>
<td>Twin only</td>
</tr>
<tr>
<td>hsa-miR-335-5p</td>
<td>$y = -0.5 \times 10^{-4} + 11.739$</td>
<td>Twin only</td>
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</tr>
<tr>
<td>hsa-miR-379-3p</td>
<td>Twin only</td>
<td>Twin only</td>
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<tr>
<td>hsa-miR-148a-3p</td>
<td>Twin only</td>
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AMPKα1, AMP-activated protein kinase-α; PDK-1, 3-phosphoinositide-dependent protein kinase-1. hsa- denotes that the data were mapped to human miRBase.

It is possible that there is a downregulation of different miRs in the liver of the singleton and twin fetus as a consequence of exposure to early embryonic undernutrition. The phosphorylation level of intracellular AMPK has been shown to be a relatively transient measure reflecting the current cellular energy status (28), and therefore, the findings of the present study suggest that the hepatocyte energy status in late gestation may be relatively normal in the PCUN and PIUN singletons.

The hepatic mRNA expression and protein abundance of PDK4 as well as the protein abundance of CPT-1 were lower in both the PCUN and PIUN groups compared with controls. In mice with a PDK4-null mutation, gluconeogenesis is decreased, and although these animals have better glucose tolerance, there is also an increase in circulating nonesterified fatty acid (20). In contrast, another study on the PDK4-null mouse found that PDK4 knockout resulted in a decreased rather than increased accumulation of fat in the liver. In this latter study, however, there was an increase in hepatic PGC-1α and PPARα expression together with a decrease in fatty acid synthase and ACC (18). Additionally, a decrease in CPT-1 and the subsequent decrease in fatty acid β-oxidation would be expected to result in hepatic triglyceride accumulation, which is strongly associated with insulin resistance and metabolic syndrome (43). Therefore, a decrease in the expression and abundance of PDK4 and CPT-1 protein abundance, together with the decrease in PGC-1α and PDK2 expression present in the fetal livers in both the PCUN and PIUN groups, may result in a potential decrease in fatty acid β-oxidation capacity, leading to intrahepatic fat accumulation in postnatal life when there is an increase in circulating free fatty acid concentrations in the lamb.

We also found that there was a decrease in hepatic PDKP-1 protein abundance in the absence of a change in phosphorylated PDKP-1 (Ser241) abundance in the PCUN group. Hepatic PDKP-1 deficiency is associated with glucose intolerance and dysregulation of hepatic insulin-regulated gene expression (30). Additionally, the protein abundance of PKCγ was decreased in singletons exposed to PCUN and PIUN, which would be associated with a decrease in lipid synthesis in the liver. However, there was an absence of change in the phos-
phosphorylation of PKCζ, which may be a consequence of the low circulating levels of free fatty acids present in the fetus and, therefore, low substrate supply for lipid synthesis in fetal life (36). In summary, the decreases in the key fatty acid β-oxidation regulators PGC-1α, PDK1, PDK2, and PDK4 and CPT-1 mRNA expression or protein abundance in the liver of the singleton fetuses exposed to maternal undernutrition during the periconceptional and/or preimplantation period may result in dysregulation of hepatic lipid metabolism and thus contribute to a glucose-intolerant phenotype in postnatal life (39, 46).

Twins. In the twin as in the singleton, we found that there was an increase in hepatic AMPKα1 abundance in the PIUN group, although in contrast to the singleton, the abundance of phosphorylated AMPKα1 (Ser485) was higher in the liver of twin fetuses in both the PCUN and PIUN groups compared with controls. We also found that there was a decrease in the hepatic expression of miR-126-5p and an inverse relationship between the expression of miR-126-5p and AMPKα1 protein abundance in the liver of the PIUN twin fetuses. Thus the increase in hepatic AMPKα1 protein abundance may be regulated by a downregulation of specific miRs in the preimplantation period, signaling a potential requirement to increase intracellular energy production after exposure to undernutrition in the preimplantation period. The increase in the activated phosphorylated form of AMPKα1 (Ser485) may be a consequence of this change or represent a response to a decrease in energy availability within the hepatocyte of twins in late gestation. Activation of AMPK has been shown to stimulate lipid oxidation and inhibit lipid synthesis in the skeletal muscle, adipose tissue, and liver (28). However, chronic activation of AMPKα1 in the liver of transgenic mice results in an increase in the expression of lipogenic genes such as sterol regulatory element-binding (SREB)-2 (50) and an increase in lipogenic gene expression and enhanced insulin sensitivity in white adipose tissue (21). Therefore, the effect of AMPKα1 activation in the liver may depend on the duration of activation and the prevailing level of nutrition supply.

Additionally, activation of PDK1 and subsequent activation of PKCζ plays a role in stimulating hepatic lipid synthesis through SREB-1c (42). Therefore, activation of PDK1 and an increase in protein abundance of PKCζ in twin fetuses exposed to PCUN and PIUN together with the increase in AMPKα1 activation may result in an increase in hepatic lipid synthesis and contribute to intrahepatic triglyceride accumulation in postnatal life. It is interesting that in this study we found a decrease in the hepatic expression of two miRs that each target PDK1-1 (miR-369-3p and miR-382-5p) in the PIUN (but not PCUN) twins and that there was an inverse relationship between the expression of miR-let-7g-5p and miR-335-5p with the hepatic PDK1-1 in twins. This suggests that exposure to maternal undernutrition in the periconceptional and preimplantation periods may act to program an upregulation of hepatic PDK1-1 abundance through the recruitment of a pool of specific miRs.

In contrast to singletons, there was an increase in the hepatic abundance of PDK4 and CPT-1 in twin fetuses exposed to PIUN. This suggests that, in the twin, there may be an increase in the capacity for fatty acid β-oxidation, which may be a compensatory mechanism in response to the increase in lipid synthetic capacity. It should also be noted, however, that an increase in PDK4 expression in postnatal life has been shown to be associated with insulin resistance and high plasma free fatty acids (2, 29). Interestingly, the expression of miR-379-3p and miR-148a-3p were each decreased in the liver of the PIUN twin fetus, and there was an inverse relationship between the expression of each of these miRs and hepatic PDK4 protein abundance. In addition, the hepatic expression of miR-122-5p was decreased in the PIUN and not in the PCUN twin fetuses, and PDK4 is an identified target of this miR. Again, this suggests that these miRs may be recruited specifically in response to exposure of the early twin embryo to maternal undernutrition.

Relationship Between Specific miRs Recruited by PCUN and PIUN and the Abundance of Factors Regulating Fatty Acid β-Oxidation and Lipid Synthesis

Previously, we have shown that the hepatic expression of miR-126-5p, let-7g-5p, miR-335-5p, and miR-379-3p was lower relative to controls in twin fetuses exposed to maternal undernutrition during the preimplantation period and that the expression of miR-148a-3p was lower relative to controls in singleton and twin fetuses exposed to preimplantation undernutrition (26). In the present study, we found that there was a negative relationship between the expression of miR-126-5p and the abundance of AMPKα1, the expression of let-7g-5p and miR-335-5p with the abundance of PDK1-1, and the expression of miR-379-3p and miR-148a-3p with the abundance of PDK4. Additionally, using target scan software, 12 out of the 23 candidate miRs previously identified to have altered expression in the PCUN or PIUN groups relative to controls in singleton or twin fetuses were predicted to regulate the key proteins in the lipid metabolism pathway. MiRs have been shown to repress protein translation without altering mRNA expression (3). Therefore, this study provides evidence that specific miRs were recruited by maternal undernutrition during the periconceptional and/or preimplantation period, which may underlie the programming of factors within the fatty acid β-oxidation and lipid synthesis pathway. It has been shown that there is altered expression of miR-126, miR-335, miR-379, and miR-148a in nonobese rats with type 2 diabetes (14), and the let-7 family has been shown to play an important role in maintaining glucose homeostasis and insulin sensitivity (9). Therefore, dysregulation of these miRs may also have an impact on hepatic insulin sensitivity and glucose tolerance. More importantly, alterations in miRs expression has been linked to the development of chronic liver disease such as nonalcoholic steatohepatitis (NASH), which is characterized by the accumulation of lipid droplets within the hepatocytes (25). We have shown that the expression of miR-146b-5p is upregulated in the singleton fetuses exposed to PCUN, the expression of miR-130a-3p is downregulated in twin fetuses exposed to PCUN and PIUN, and the expression of miR-122-5p and miR-126-5p is also downregulated in twin fetuses exposed to PIUN (26). Studies in models of NASH have shown that the expression of miR-146b was upregulated and the expression of miR-126 and miR-122 downregulated (4), and the expression of miR-130a was shown to be downregulated in hepatic steatosis (51). This suggests that PCUN and PIUN recruit a different subset of miRs in the singleton and twin fetus and that the alterations of these specific miRs may be implicated in the subsequent programming of hepatic insulin resis-
tance, dysregulation of hepatic lipid metabolism, and glucose intolerance in later life.

Summary

In summary, we have demonstrated that maternal undernutrition during the periconceptional and/or preimplantation period results in a decrease in the key fatty acid β-oxidation regulators PGC-1α, PDK-2, PDK-4, CPT-1, and PDKP-1 and PKCζ mRNA expression or protein abundance in the liver of the singleton fetuses, which may in turn contribute to a dysregulation of hepatic lipid metabolism and the glucose-intolerant phenotype in postnatal life. These changes may result in the programming of hepatic lipid metabolism that is highlighted by our recent studies, in which we found that exposure to maternal undernutrition in the periconceptional period resulted in decreased PGC-1α and PKCζ protein abundance in the liver of the singleton postnatal lamb at 4 mo of age (33, 34). In contrast to our findings in the singleton fetuses, however, there was an increase in the activation of PDKP-1 and the abundance of PKCζ in the twin fetuses, which may result in an increase in lipid synthesis in postnatal life. In contrast to singletons, there was also an increase in PDK4 and CPT-1 protein abundance in twins exposed to PIUN, suggesting that in the twin there may be an increase in the capacity for fatty acid β-oxidation as a compensatory response in the face of an increase in lipid synthetic capacity. We have also shown that maternal undernutrition during the periconceptional and/or preimplantation period programmed changes in specific miRs, which may underlie the alterations in AMPKα1, PDKP-1, and PDK4 abundance. The programmed changes in miRs in response to PCUN and/or PIUN may also have an impact in the development of NASH, insulin resistance, and glucose intolerance in later life.

These findings highlight that the impact of periconceptional or preimplantation nutrition is different in the singleton and twin fetuses and that this difference may be a consequence of the recruitment of a different suite of microRNAs. These differences suggest that the responses of the single and twin embryos to the impact of maternal undernutrition are in the context that there is a different expectation of the nutritional environment that will be experienced by the single and twin fetuses through gestation. These observations and those from our prior studies (26, 27) highlight the sensitivity of the embryo to the hormonal or nutritional cues of a twin pregnancy and the importance of those cues in determining the subsequent response of the embryo to maternal undernutrition and the programming of metabolic outcomes in the offspring. Importantly, this study also highlights the need for education of women of reproductive age about optimal nutritional intakes, given that most pregnancy is not diagnosed until at least 1 mo after conception.

ACKNOWLEDGMENTS

We gratefully acknowledge the research assistance provided by Anne Jurisevic, Laura O’Carroll, and Andrew Snell during the course of this study.

GRANTS

This study was supported by funding from the Australian Research Council (I. C. McMillen, C. T. Roberts, and S. K. Walker) and the National Health and Medical Research Council of Australia (I. C. McMillen and J. L. Morrison). C. T. Roberts was supported by a National Health and Medical Research Council Senior Research Fellowship (GNT1020749). J. L. Morrison was supported by a Fellowship from the South Australian Cardiovascular Research Network, fellowships from the Heart Foundation, and the National Health and Medical Research Council of Australia.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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