Role of MicroRNA in Early Life Placental Programming of Insulin Resistance and Metabolic Health

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‘I love fools’ experiment, I am always making them’ – Charles Darwin

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STATEMENT OF ORIGINALITY AND AUTHETICITY

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Signed,

Himawan Harryanto

Date:

TABLE OF ABBREVIATIONS AND BIOCHEMICAL NAMES

Acox1	Acyl-CoA oxidase 1, palmitoyl
AdipoR2	Adiponectin Receptor 2
AMC	Adelaide Microarray Centre
ANOVA	Analysis of Variance
BMI	Body Mass Index
BUVL	Bilateral uterine artery vessel ligation
cDNA	complementary DNA
Cbl	E3 ubiquitin-protein ligase
Ccdc88a	Coiled-coil domain containing 88A
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DMR	Differentially Methylated Region
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	Deoxyribonucleotide triphosphate
FFA	Free Fatty Acid
Foxo1	Forkhead box protein O1
Foxo3	Forkhead box protein O3
HEC	Hyperinsulinaemic euglycaemic clamp
Hk2	Hexokinase 2
IGF-1	Insulin-like Growth Factor 1

IPA	Ingenuity Pathway Analysis
IPGTT	Intraperitoneal glucose tolerance test
IR	Insulin Receptor
Irs1	Insulin receptor substrate 1
Irs2	Insulin receptor substrate 2
IVGTT	In Vitro Glucose Tolerance Test
IUGR	Intrauterine Growth Restriction/Retardation
LNA	Locked Nucleic Acid
µg	microgram
microRNA/miRNA/miR	Micro ribonucleic acid
Mon2	MON2 homolog
mRNA	Messenger ribose nucleic acid
p110β	PI-3-kinase, catalytic, beta polypeptide
p85α	PI-3-kinase, regulatory subunit 1
PASW	Predictive Analytics SoftWare
PI	Ponderal Index or Placental Insufficiency
Pparα	Peroxisome proliferative activated receptor, alpha
PR	Placental Restriction or placentally restricted
RNA	Ribonucleic acid
RT	reverse transcribed
RT-PCR	Real Time Polymerase Chain Reaction
SA	South Australia
SEM	Standard error of mean

Slc2a4	Solute carrier family 2, member 4
T2DM	Type 2 Diabetes Mellitus
UNICEF	United Nations Children's Fund
WEHI	Walter and Eliza Hall Institute of Medical Research
WHO	World Health Organization

CONFERENCE PRESENTATIONS ARISING FROM THIS THESIS

‘**Placental Restriction Alters Insulin Actions and microRNAs Expression in Insulin Sensitive Tissues of Adult Offspring in the Rat’**
H. Harryanto, E. M. Jamil, B. L. Summers-Pearce, P. A. Grant, M. J. De Blasio, S. Moretta, W-C. Kong, K. L. Gatford, M. Dziadek, M. E. Wlodek, J. A. Owens

August 2012
ESA-SRB Annual Scientific Meeting 2012
Oral presentation on ESA Orals: Diabetes

‘Role of Hepatic miR-142-3p and miR-144 in Development of Non-Alcoholic Fatty Liver Disease in Placentally Restricted Adult Sheep Offspring’
Himawan Harryanto, Miles J. De Blasio, Patricia A. Grant, Kathryn L. Gatford, Julie A. Owens

July 2011
2nd RNAi Australia Meeting
Oral presentation

‘Placental Restriction Alters MicroRNAs Expression in Skeletal Muscle, Liver and Adipose Tissue Young and Adult Offspring in the Sheep’
H. Harryanto, M. J. De Blasio, P. A. Grant, K. L. Gatford, J. S. Robinson, J. A. Owens

August 2010
ESA-SRB Annual Scientific Meeting 2010
Oral presentation on ESA Orals: Early life programming & IUGR

‘IUGR Alters rno-miR-16, -18a and -142-3p in Day 260 Omental Fat BUVL Rat Offspring Tissue’
H. Harryanto, B. L. Summers-Pearce, M. J. De Blasio, S. Moretta, T. A. How, P. A. Grant, J. A. Owens

August 2010
ESA-SRB Annual Scientific Meeting 2010
Poster presentation

'Role of microRNAs in Placental Programming of Skeletal Muscle Insulin Resistance'
H. Harryanto, M. L. Harland, P. A. Grant, M. J. De Blasio, K. L. Gatford, J. S. Robinson, J. A. Owens

Presented on:

October 2009
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Poster Presentation
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ESA-SRB Annual Scientific Meeting 2009
Oral presentation on ESA Orals: Metabolism & Obesity

June 2009
ASMR Medical Research Week®
SA Scientific Meeting 2009
Poster Presentation

ABSTRACT

Intrauterine growth restriction (IUGR) is associated with insulin resistance and diabetes, particularly later in adult life. Placental restriction is a common cause of IUGR, this condition induces insulin resistance and/ or insulin deficiency and consequently, impaired glucose tolerance in offspring, in the sheep and rat. Reduced expression of insulin signalling genes and that of their key metabolic targets in insulin sensitive tissues and of some molecular determinants of pancreatic *β*-cell insulin secretion and mass, contributes to impaired insulin action in offspring, in experimental and human IUGR. However, the underlying molecular mechanisms whereby IUGR alters the molecular profile of insulin sensitive and secreting tissues in later life are largely unknown. The studies described in this thesis examine the potential role of microRNAs (miRNAs) in the developmental programming of impaired insulin action in IUGR offspring. MiRNAs are short single-stranded RNAs (22 nucleotide in length), which are able to reduce the translation and/or abundance of mRNA and protein of targets. Each miRNA is predicted to regulate the abundance of many targets in co-ordinated networks to modify function, providing a potentially powerful pathway for developmental programming to influence later phenotype.

Here, IUGR was induced by restricting placental growth and development surgically in sheep (pre-conception removal of most implantation sites) or in the rat (ligation of uterine blood vessels in late gestation). In each species, the effect of placental restriction and IUGR on miRNA expression and expression of key predicted targets, including that of insulin signalling and key metabolic genes in the insulin sensitive tissues: liver, skeletal muscle and adipose (perirenal in sheep and omental in rat), in

adult offspring were characterised. The effect of placental restriction on pancreatic miRNA expression in adult offspring was also determined in the sheep.

Placental restriction and IUGR mostly increased miRNA expression in insulin sensitive tissues of the adult sheep and in a sex specific manner, suggesting the potential for increased repression of the translation or abundance of their molecular targets and related functions. The liver, followed by skeletal muscle and adipose tissue, showed the greatest susceptibility in terms of numbers of miRNAs with altered expression following placental restriction. In males, placental restriction increased hepatic expression of eight miRNAs by ~1.5-3.5-fold, with differential expression of four independently confirmed by qPCR (hsa-miR-1, hsa-miR-21, hsa-miR-142-3p and hsa-miR-144). Each of these four miRNAs was predicted to target molecules involved in insulin signalling, metabolism and hepatic disease. The latter included *p85α, Pparα, Igf1, Foxo3* and *Acox1*, all exhibiting reduced hepatic expression (~2.3-4.0 fold) following placental restriction in males, with the abundance of hsa-miR-1, hsa-miR-142-3p and hsa-miR-144 correlating negatively with *Acox1* expression. Thus, placental restriction co-ordinately alters hepatic expression of miRNAs and predicted targets related to non-alcoholic fatty liver disease (NAFLD) in adult male offspring in sheep. Reduced hepatic expression of *Pparα* (regulates lipid catabolism), and *Acox1* (peroxisomal fatty acid β-oxidation) is characterised to promote the development of NAFLD, increasingly common following fetal growth restriction in humans, and miRNAs may partly mediate this prenatal programming of NAFLD.

In the sheep, placental restriction increased vastus lateralis expression of seven miRNAs by ~1.23-2.04 fold, with differential expression of two independently

confirmed by qPCR (hsa-miR-17-5p and hsa-miR-376b). Both of these two miRNAs were predicted to regulate *Pparα* expression, which tended to negative correlate with that of hsa-miR-376b (r = -0.617, *P*-value: 0.052) and hsa-miR-17-5p (r = -0.533, *P*-value: 0.087), in placentally restricted female offspring. Placental restriction also increased perirenal fat hsa-miR-451 expression in female offspring. This miRNA is predicted to regulate a network that is involved in lipid metabolism, molecular transport and small molecule biochemistry in adipose tissue. Furthermore, perirenal fat expression of hsa-miR-451 was correlated positively with *in vivo* insulin sensitivity of free fatty acids in control offspring (r = 0.687, *P* = 0.020, n = 11), but not in placentally restricted offspring.

In the rat, placental restriction impaired insulin secretion in adult offspring, while insulin sensitivity was enhanced in young adult offspring, which then disappeared with aging, particularly in females. Placental restriction and IUGR also mostly increased miRNA expression in the insulin sensitive tissues of older adult offspring and usually in a sex specific manner, with omental fat the most affected, followed by skeletal muscle and liver. Placental restriction and IUGR reduced hepatic expression of the insulin signalling molecule, *p110β* in female offspring and that of related molecules, *Slc2a2* and *Igf1*, in male offspring. Placental restriction also increased hepatic rno-miR-126 expression, in female offspring and is predicted to target molecules involved in lipid metabolism, molecular transport and small molecule biochemistry. Placental restriction and IUGR reduced omental fat expression of *Irs1*, *Irs2* and *Slc2a4* in male offspring and increased that of rno-miR-18a, rno-miR-142-3p, rno-miR-19b, rno-miR-21, rno-miR-20b and mmu-miR-106a. The latter are predicted to target insulin signalling but also small molecule biochemistry, lipid

metabolism and its regulation, including similar pathways to those targeted by IUGR altered miRNAs in liver of adult offspring in sheep. Expression of omental fat rno-miR-18a was found to be negatively correlated with *Slc2a4* in placentally restricted offspring overall (r = -0.451, *P* = 0.040).

Placental restriction also alters the pancreatic expression of three miRNAs in adult sheep offspring, hsa-miR-339-5p in males, rno-miR-331* in females and hsa-miR-513a-3p in both sexes. Furthermore, the predicted molecular and functional targets of these differentially expressed miRNAs and predicted functional outcomes following placental restriction mirror the previously reported sex differences in *β*-cell insulin secretory function and mass in the placentally restricted adult sheep. We found that hsa-miR-339-5p was predicted to regulate PLEKHH1 and PAK6, proteins which are essential to maintain the development of the pancreas and/or differentiation of islets. Down-regulation of both rno-miR-331* and hsa-miR-513a-3p in the pancreas of placentally restricted female offspring would be expected to indirectly up-regulate *Pdx1* expression, and potentially contribute to the increased number of *β*-cells per islet they exhibit.

Overall, placental restriction and IUGR mostly increase abundance of miRNAs in key insulin sensitive tissues of adult offspring in both sheep and the rat, with sex-specific and tissue-specific differences. Nevertheless, the networks targeted by miRNAs differentially expressed following IUGR in such tissues, share common functions and pathways, both across tissues and species, including small molecule biochemistry, lipid metabolism, carbohydrate metabolism and molecular transport. Of interest, regardless of the tissues and species, placental restriction generally increased

expression of miR-142-3p, miR-1, miR-21 and miR-17-5p. Of note, expression of hsa-miR-1 and hsa-miR-21 were each up-regulated in both liver and vastus lateralis in sheep offspring. These up-regulations of common miRNAs expression could be due to alteration of epigenetic mechanism affected by placental restriction, such as DNA methylation, or common systemic regulations of their expression that has been 'programmed' due to placental restriction.

Therefore, placental restriction of fetal growth does alter expression of miRNAs and their networks involving insulin signalling and metabolism in key insulin sensitive tissues in the adult. The mechanism underlying this and the extent to which they contribute to overall developmental programming of metabolic dysfunction warrant for future investigation.

Chapter 1

Introduction

1.1 Overview of the thesis

Intrauterine growth restriction or retardation (IUGR) refers to fetal growth that has been constrained by an inadequate substrate supply *in utero*, causing the individual to not reach their growth potential (ACC/SCN, 2000). IUGR is difficult to detect accurately; hence the term of small-for-gestational age (SGA) is commonly used as a surrogate (Abitbol & Rodriguez, 2012). SGA is defined as any infant born with a birth weight below the 10th percentile of the recommended sex-specific birth weight based on gestational age reference curves (WHO, 1995; de Onis & Habicht, 1996). Worldwide, IUGR contributes to at least 10-15% of all pregnancies (Zhang *et al.*, 2010). In Australia, 17% of indigenous infants born at term were classified as SGA, higher than the expected 10% (Roberts & Lancaster, 1999); however, it is unclear how many of these were IUGR offspring. The main identifiable contributors to IUGR development include placental insufficiency, pre-eclampsia and malnutrition during pregnancy (Ergaz *et al.*, 2005). IUGR is characterised by reduced placental delivery of oxygen and substrates to the fetus, which adapts to these conditions by redistributing blood flow and undergoing a range of metabolic and endocrine changes that ultimately slow growth, reduce demand and promote survival, particularly of the most essential organs (Owens *et al.*, 1987).

This adverse intrauterine environment and consequent fetal adaptations may have numerous other consequences. Hence, IUGR has been linked to the development of metabolic diseases in the offspring's later life, including heart disease, high blood pressure and type 2 diabetes (Barker, 1998). A systematic review and meta-analysis shows consistent associations between birth weight and type 2 diabetes (Whincup *et al.*, 2008). The development of type 2 diabetes generally requires a development of

insulin resistance and the failure of insulin secretion to adapt sufficiently for maintaining good glucose control (Prentki & Nolan, 2006). The development of adult onset diabetes following IUGR may be due to the reduced insulin signalling expression in major insulin sensitive tissues, liver and/or skeletal muscle (Jaquet *et al.*, 2000; Jaquet *et al.*, 2001b; Ozanne *et al.*, 2006; De Blasio *et al.*, 2007a; Jensen *et al.*, 2007; De Blasio *et al.*, 2012) and reduced insulin secretion (Simmons *et al.*, 2001; Jensen *et al.*, 2002; Gatford *et al.*, 2008), all observed in both humans and in experimental IUGR in other animal species. However, it is not well understood why these changes underpinning impaired insulin action occur in later life following IUGR; the mechanisms involved are also unclear. Altered microRNAs (miRNAs) expression and action in key tissues responsible for insulin action is one of the possible mechanisms that may link an intrauterine environment which restricts growth to alter the expression of insulin signalling genes.

MiRNAs are highly conserved short, single-stranded, non-coding RNAs, about 22 nucleotides in length (Bushati & Cohen, 2007). Each miRNA can target and regulate multiple target mRNAs by binding to sites in the 3' untranslated region (UTR) or elsewhere; up to 50% of mRNAs are estimated to be targeted by miRNAs, enabling the formation of powerful regulatory networks (Chi *et al.*, 2009; Thomson *et al.*, 2011; Yang *et al.*, 2011). In addition, miRNAs have a high proportion of CpG islands, compared with coding genes (Saini *et al.*, 2007). This suggests that miRNAs are potentially more susceptible to early life programming through epigenetic modifications of their genes that may persist in the long term, particularly altered DNA methylation (Chuang & Jones, 2007). MiRNAs have been shown to regulate insulin sensitivity in insulin-sensitive tissues, liver and skeletal muscle, and insulin secretion

in the pancreas. Expression of some miRNAs in these tissues is shown to be deregulated in the type 2 diabetes condition (Poy *et al.*, 2007; Guay *et al.*, 2011; Melkman-Zehavi *et al.*, 2011; Rottiers & Naar, 2012; Fernandez-Hernando *et al.*, 2013). Therefore, the present thesis describes studies which aim, firstly, to examine whether placental restriction of fetal growth alters the expression of miRNAs in insulin-sensitive and secreting tissues. The second aim is to examine whether differentially expressed miRNAs target insulin signalling or related pathways and in doing so, contribute to impaired insulin action in the offspring following IUGR, induced experimentally in the sheep and the rat.

1.2 IUGR and subsequent metabolic disease

1.2.1 Consequences of IUGR

IUGR is mostly caused by placental insufficiency or nutrient deficiency. As a result, the offspring are born with an asymmetrical condition, where brain growth is being spared at the expense of other organs (Kliegman *et al.*, 2007). The development of IUGR can be detected during pregnancy only by serial measurement of fetal growth via ultrasound to estimate the fetal size, but not fetal health (Miller *et al.*, 2008). It is common for IUGR newborns to have a low birth weight, of <2,500 g or below the lowest 10th percentile of weight at gestation (Abitbol & Rodriguez, 2012). IUGR has been proposed to reflect an adverse intrauterine environment that both restricts fetal growth and 'programs' impaired insulin action and glucose control in later life (Gatford *et al.*, 2010). A major cause of IUGR is placental insufficiency (PI), a common pregnancy complication, whereby placental delivery of oxygen and nutrients does not adequately meet the needs of the developing fetus (Ergaz *et al.*, 2005; Baschat *et al.*, 2007). Because of the fetal metabolic, hormonal and physiological adaptations to a restricted substrate supply (Fowden & Forhead, 2004), PI is proposed to induce cellular and molecular changes in the fetus that have persistent effects on the phenotype which affects the risk of diseases, such as diabetes (Gatford *et al.*, 2010).

Throughout the world, low birth weight babies account for around 14% of born infants and for 60-80% of neonatal mortality (Bang *et al.*, 2002). In 2000, it was estimated that IUGR affected approximately 24% (or 30 million) newborns per year in developing countries, with Central Asia the most affected region (ACC/SCN, 2000). During the first two years of life, IUGR newborns typically undergo catch-up growth by accumulating excess fat or adipose tissue (Hales & Ozanne, 2003); this affects

their future adult muscle strength, body size and composition. However, catch-up growth is not enough to compensate for prenatal growth restriction, as IUGR adults are shorter, lighter and weaker strength than non-IUGR controls (Martorell *et al.*, 1998), and it does not reverse prenatal programming of poor later health, in fact, exacerbating this.

1.2.2 Type 2 diabetes mellitus (T2DM): association with IUGR

T2DM is a result of impaired insulin action. It is a product of impaired insulin sensitivity or resistance in key insulin-sensitive tissues that control blood glucose levels (skeletal muscle and liver), and the failure of insulin secretion to adapt to and compensate for this impairment. The World Health Organization (WHO) predicted that, by 2030, about 180 million people worldwide will develop a diabetic condition, with 90% suffering from T2DM. Furthermore, diabetes is also associated with increased risk of retinopathy, neuropathy, kidney failure, heart disease and stroke. WHO estimates that, during 2005-2015, China will lose about US$558 billion in annual revenue as a result of diabetes and related diseases (WHO Fact Sheet no. 312, January 2011).

Exposure to famine during gestation, such as those exposed to the 1945 Dutch 'Winter Hunger', increased the incidence of impaired glucose tolerance in adult life, suggesting the association between deprivation *in utero* and later poor health of offspring to be causal in nature (Roseboom *et al.*, 2006). Adults born with a low birth weight have been directly shown, using hyperinsulinaemic euglycaemic clamps or minimal modelling in intravenous glucose tolerance test, to have whole body insulin resistance, (Newsome *et al.*, 2003). Furthermore, the main tissue site of this insulin resistance was shown to be in the skeletal muscle, with reduced peripheral insulin-

stimulated glucose uptake among young adults born with a low birth weight (Jaquet *et al.*, 2000; Jaquet *et al.*, 2001b; Hermann *et al.*, 2003). This reduction of insulin-stimulated glucose uptake is partly due to a reduction of the proximal insulin signalling gene and protein expression in the skeletal muscle, liver and adipose tissues (see discussion in section 1.2.3). On the other hand, young men born with low birth weight have altered skeletal muscle fiber composition and size, which may contribute to impaired peripheral insulin sensitivity and their development of whole-body insulin resistance (Jensen *et al.*, 2007). These studies provide a potential molecular framework for explaining why individuals born with a low birth weight are at an increased risk of developing type 2 diabetes.

1.2.3 IUGR alters insulin sensitivity

1.2.3.1 Skeletal muscle

Skeletal muscle is one of the two major organs or tissues that acutely respond to insulin to control blood glucose. Insulin sensitive organs or tissues respond to insulin stimuli through their insulin signalling cascade, which initiates numerous processes (Figure 1.1). These processes include the translocation of GLUT-4 into the cell membrane to enable glucose uptake by skeletal muscle fibres, activation of glycogen synthase for storing excess glucose as glycogen and indirect initiation of protein synthesis necessary for regulating cell growth, via the MAPK pathway.

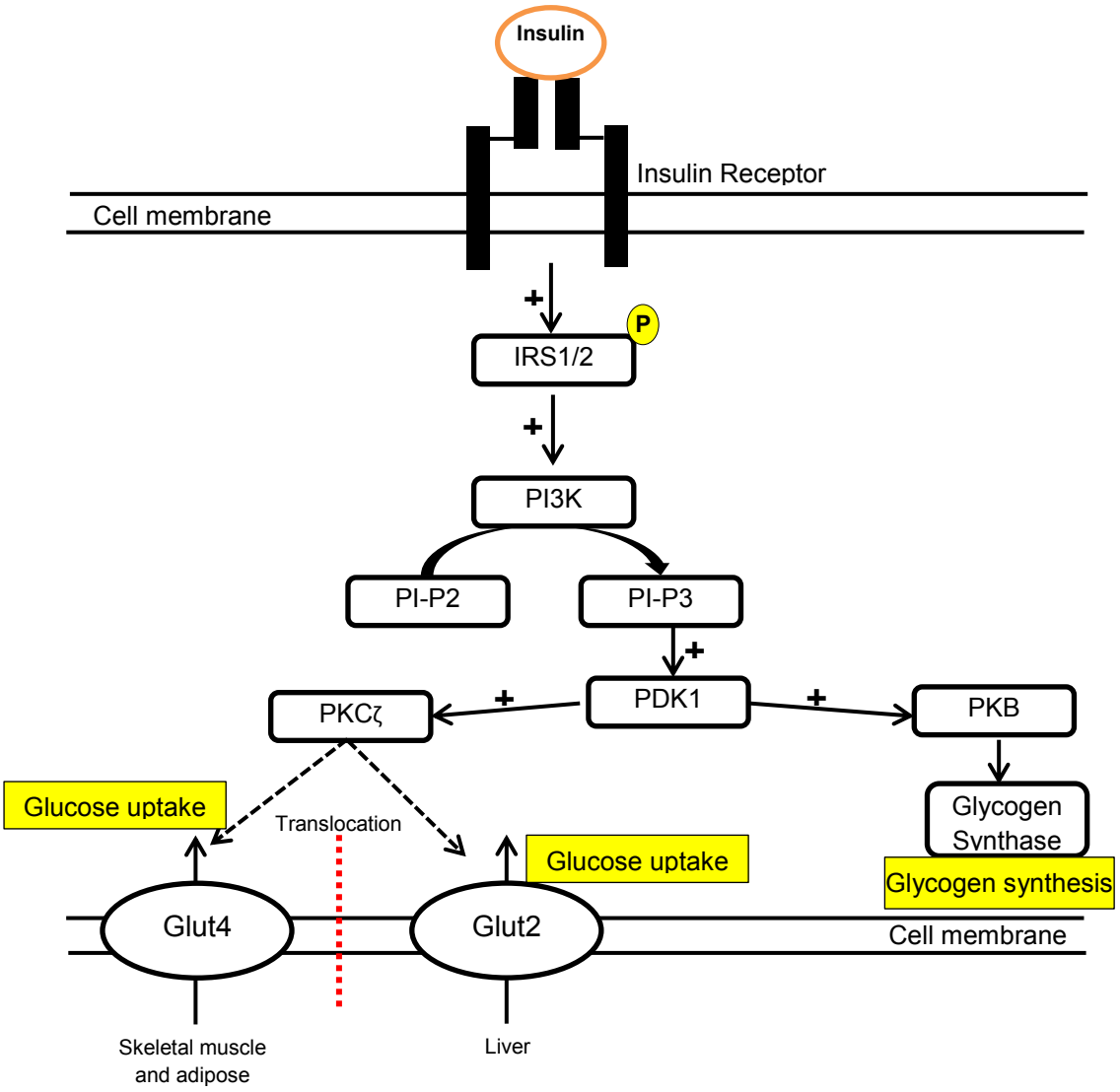


Figure 1.1. Simplified outline of insulin signalling pathway in skeletal muscle, liver and adipose tissues.

In the presence of insulin, insulin receptor phosphorylates IRS1 and IRS2, activates phosphoinositide 3-Kinase (PI3K) which phosphorylates phosphatidylinositol 4,5-biphosphate (PI-P2) into phosphatidylinositol 3,4,5-triphosphate (PI-P3). PI-P3 activates phosphoinositide dependent protein kinase 1 (PDK1). PDK1, together with protein kinase C-zeta (PKCζ), translocates glucose transporters (GLUT4, in skeletal muscle and adipose; GLUT2 in liver), to facilitate glucose uptake from blood stream into the cells. PDK1, together with protein kinase B (PKB), activates a pathway to release glycogen synthase enzyme. This enzyme is responsible to convert excess glucose into a storage form, glycogen.

Depending on the proportion of myofibrillar adenosine triphosphate (myo-ATPase) on each muscle fibres, skeletal muscle can be divided into two major fibre types, types 1 and 2 (Reiser *et al.*, 1985). Myo-ATPase is an important protein in the muscle and is responsible for muscle contraction. Type 1 muscle fibres, a slow twitch muscle, is significantly more insulin sensitive than type 2 muscle fibres, a fast twitch. In human, the type 2 muscle fibre is divided into two sub-groups, 2a and 2b. Type 2a is fast twitch, but is still fairly responsive towards insulin, whereas type 2b respond poorly towards insulin (Spangenburg & Booth, 2003). Furthermore, the type 1 muscle fibre is more sensitive in terms of insulin-stimulated glucose uptake, which is likely due to its a higher GLUT-4 content than type 2b muscle fibres (Gaster *et al.*, 2000). Vastus lateralis, which is located on the proximal part of the human lower limb, is known to have a mix of muscle fibres types 1 and 2, while the soleus muscle, located on the distal part of the human lower limb, is known to have a higher proportion of the type 1 muscle fibre (Edgerton *et al.*, 1975).

Humans

One of the studies on this subject, has shown that skeletal muscle exhibits weak insulin-stimulated glucose uptake among low birth weight subjects, compared with normal birth weight subjects (Hermann *et al.*, 2003). Phillips *et al.* (1996) reported that there is no correlation between birth weight and glycogen synthase activity from muscle biopsies of women aged around 50. However, Jaquet *et al.* reported less of an increase of GLUT-4 expression in the muscle of IUGR-born young adults following insulin stimulation, compared to control adults, as measured quantitatively by RT-PCR (Jaquet *et al.*, 2001b). This study showed that proximal or nearest molecules of the insulin signalling pathway would be affected in the muscle of IUGR-born

offspring, rather than the molecules further down in the pathway. The latter also exhibit reduced protein expression of PKCzeta and Akt1, and increased protein expression of IRS1, p85alpha and p110beta in skeletal muscle in the insulin stimulated state (Jensen *et al.*, 2008). A reduction of *Glut4* mRNA expression has also been observed in the adipose tissue of IUGR young adult men (Jaquet *et al.*, 2001b).

In sheep, IUGR increased expression of insulin receptor, *p85α* and *Akt2* in the skeletal muscle of the sheep fetus; IUGR was induced by exposing the pregnant ewes to elevated ambient temperature from about 38-day gestation age (Thorn *et al.*, 2009). Placentally restriction in sheep, reduced mRNA expression of insulin receptor, *Irs1, Akt2, Glut4, Gsk3α* and *Gys1* in the skeletal muscle of juvenile IUGR offspring (De Blasio *et al.*, 2012). Therefore, onset of peripheral insulin resistance following placental restriction occurs early in postnatal life in the sheep, however the mechanism responsible and the molecular basis is unknown.

1.2.3.2 Liver

The liver is another key insulin-sensitive organ, albeit with a slightly different function and role. The liver's default function is to convert glycogen into glucose, this process is halted when the blood sugar level and, hence, insulin levels are high, leading to glucose uptake and storage as glycogen. To date, there are no human studies examining the gene expression of insulin signalling in the liver of IUGR offspring. However, stably isotopically labelled glucose studies in young adults of low birthweight, suggests that hepatic insulin resistance is not present or merges later, after peripheral insulin resistance (Jensen *et al.*, 2002; Brons *et al.*, 2008). Therefore,

animal models have been utilised as alternatives for studying the insulin signalling gene and protein expression levels in the liver IUGR offspring. IUGR in the rat decreased phosphorylation of IRS-2 and Akt-2 in the liver of 8-week old offspring (Vuguin *et al.*, 2004). In sheep, placental restriction does not alter hepatic expression of proximal insulin genes and related molecules among juvenile offspring (De Blasio *et al.*, 2012). However, it is yet to be determined whether IUGR or placental restriction would affect the gene or protein expression of insulin signalling in old adult offspring.

1.2.3.3 Adipose

Fat or adipose tissues are broadly categorized into two groups, subcutaneous and visceral fat. Subcutaneous fat functions as insulation for the body and as a reserve energy source, whereas visceral fats are attached near various organs, such as the gut system and between skeletal muscles (Marks & Miller, 2006). One sub-group of visceral fat is omental fat, found closely associated with the gut. In humans, IUGR offspring have more central body fat mass due to an increase of the omental fat mass (Tarantino *et al.*, 2007; Tchernof, 2007). Both human and animal IUGR studies observed an increase in overall adipose tissue fat weight as the consequences of catch-up growth, in the offspring postnatally (De Blasio *et al.*, 2007a). Adipose tissues are insulin-sensitive and able to store excess glucose in response to insulin, as lipid. Some studies have examined insulin sensitivity and the expression of insulin signalling pathways in adipose tissue of IUGR offspring, as described below.

A study conducted in Hagenau (France), by collecting abdominal adipose tissue biopsies from 24-year old human IUGR and controls, found reduced *Glut4* mRNA

expression following insulin-stimulation in IUGR (Jaquet *et al.*, 2001b). Other studies found that IUGR-born subjects developed an excess of adipose tissues, lower serum leptin levels and a significantly reduced serum adiponectin (Jaquet *et al.*, 2006; Coelho *et al.*, 2007); therefore, IUGR was shown to alter adipocyte endocrine function (Jaquet *et al.*, 2001a). No studies have been conducted in IUGR animal models, whether placental restriction would reduce insulin signalling expression in the adipose tissues. Table 1.1 shows a summary of known insulin signalling expression altered in the insulin sensitive tissues.

Table 1.1. Summary of insulin signalling molecules altered in skeletal muscle, liver and adipose tissues in intrauterine growth restricted offspring.

Tissue	Subject	Age	Insulin signalling expression	Ref.
Skeletal muscle	Human	20 years	↓ *Glut4* mRNA	(Jaquet *et al.*, 2001b)
		19 years	↓ PKCζ and AKT1 protein ↑ IRS1, p85α & p110β protein	(Jensen *et al.*, 2008)
		19 years	↓ p85α, p110β & PKCζ protein	(Ozanne *et al.*, 2005)
	Sheep	134 dGA	↓ *Insr*, *p85α* & *Akt2* mRNA	(Thorn *et al.*, 2009)
		43 days	↓ *Insr*, *Irs1, Akt2, Glut4, Gsk3α* & *Gys1* mRNA	(De Blasio *et al.*, 2012)
	Rat	0 day	↓ *Insr* mRNA	(Germani *et al.*, 2008)
Liver	Rat	7 weeks	↓ IRS2 & AKT2 protein	(Vuguin *et al.*, 2004)
Adipose	Human	20 years	↓ *Glut4* mRNA	(Jaquet *et al.*, 2001b)

↓: reduced expression; ↑: increased expression; dGA: day gestation age, sheep term at ~147 dGA

1.2.4 IUGR alters insulin secretion

IUGR in humans characterised by insulin deficiency later in life (Jensen *et al.*, 2002). This impairment in insulin secretion has also been observed among IUGR animal models. In rats, placental restriction impaired glucose-dependent insulin secretion and reduced *β*-cell mass in juvenile offspring, when compared with controls (Simmons *et al.*, 2001). In sheep, placental restriction impaired glucose-stimulated

insulin production in juvenile offspring (De Blasio *et al.*, 2007a), as well as impairing the insulin secretory capability of the *β*-cells and insulin secretion in young adult offspring (Gatford *et al.*, 2008). The latter occurred together with reduced pancreatic expression of the L-type voltage-gated Ca^{2+} channel and of the Kir6.2 subunit of the K_{ATP}-channel (Gatford *et al.*, 2008), which is responsible for regulating insulin secretion (Navarro-Tableros *et al.*, 2007).

Placental restriction in the rat reduces *Pdx1* expression, in the pancreas, persistently from fetal to adult offspring (Stoffers *et al.*, 2003). Pdx1 is a duodenal homeobox 1 transcription factor necessary for pancreatic development and *β*-cell differentiation; its reduced expression in the pancreas may partly account for loss of *β*-cell function and mass. Furthermore, placental restriction in rat, increased *Fgfr1* and reduced *Gch1*, *Pcsk5* and *Vgf* expression in pancreatic islet of offspring at 7-weeks of age (Thompson *et al.*, 2010). Gch1 (GTP cyclohydrolase 1) has been directly implicated in diabetes-associated endothelial dysfunction due to its role in nitric oxide synthesis (Meininger *et al.*, 2004). This down-regulation of *Gch1* expression in the pancreatic islet has been proposed to play a role in the loss of *β*-cell function observed following placental restriction (Thompson *et al.*, 2010). Reduced islet Vgf expression may also contribute via reduced vascularisation, as seen in some models of IUGR in rodents (Boujendar *et al.*, 2003; Ham *et al.*, 2009). However, what caused these change of genes expression yet to be determined.

1.3 Experimental placental restriction of fetal growth: IUGR

In order to analyse the effect of IUGR on gene expression from the insulin sensitive and insulin secreting tissues in the offspring, animal studies need to be used, since it is not ethical to impose placental restriction or feasible to collect the key tissues from human subjects. Numerous studies have examined the expression of various insulin signalling genes in the insulin sensitive tissues and insulin secreting genes in the pancreas, but none have examined how these alterations occurred in the first place. MicroRNAs have been shown to play important roles in regulating the expression of genes involved in insulin signalling and insulin secretion more generally, but not in IUGR to date (as discussed later). Therefore, experimental placental restriction in non-human species will enable investigation of microRNA genes expression in insulin sensitive and insulin secreting tissues, and their relationship to insulin action, later in adult life.

1.3.1 Sheep model of placental restriction

In sheep, placental restriction is induced by removing of the majority of placental implantation sites from the uterus of non-pregnant ewes (Robinson *et al.*, 1979). This reduces the number of placentomes formed during subsequent pregnancy and, hence, reduces placental size and function (Harding *et al.*, 1985) (Figure 1.2). Placental restriction impaired insulin secretion before birth (Owens *et al.*, 2007a), reduces the body weight of offspring at birth (De Blasio *et al.*, 2007b), and increases insulin action in the young lamb (De Blasio *et al.*, 2007a). On the molecular level, placental restriction reduces the expression of key proximal insulin signalling in the fetal skeletal muscle (Thorn *et al.*, 2009), reduces the expression of IGF-I in fetal perirenal adipose tissue (Duffield *et al.*, 2008), reduces the expression of insulin

signalling and glucose transporter genes in skeletal muscle (De Blasio *et al.*, 2012) and increases pancreatic expression of IGF-I and IGF-II on the 43rd day of age (Gatford *et al.*, 2008). However, there yet has to be a study examining these gene expressions among adult sheep offspring.

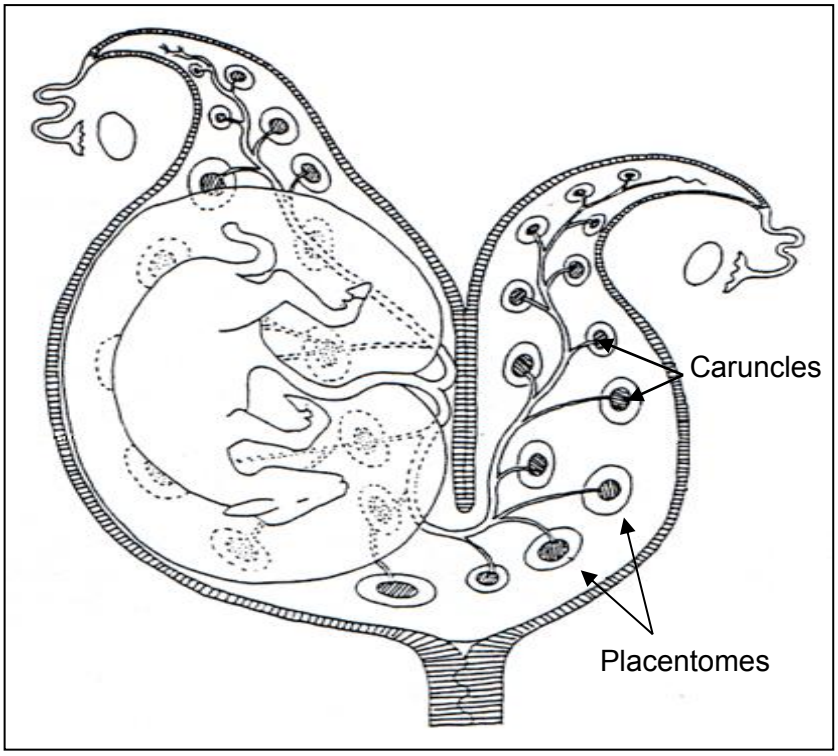


Figure 1.2. Schematic diagram of sheep placentation.
Caruncles are sites where individual placentomes form the placenta. Removing most of these caruncles reduces placental size and function, causing IUGR. *(Modified from (Harding et al., 1985)).*

1.3.2 Rat model of placental restriction

Initially, placental restriction in rats was induced by a single uterine artery ligation (Wigglesworth, 1964). This meant that one horn of the uterus contained the IUGR offspring, while the other horn (which did not undergo artery ligation) contained the control offspring. However, this method was later modified into bilateral uterine artery vessel ligation (BUVL), which involved ligation of uterine arteries with two silk ligatures near the lower end of both uterine horns (Neitzke *et al.*, 2008) (Figure 1.3). The control offspring for this method were from other dams that underwent an identical surgery procedure without the ligation. As a result of this BUVL surgery, the rat offspring are born with a low birth weight (Wadley *et al.*, 2008), are less insulin sensitive (Neitzke *et al.*, 2008) and are at increased risk of developing type 2 diabetes in postnatal life (Simmons *et al.*, 2001). BUVL surgery also reduced both *β*-cell number and function contribute to insulin deficiency in the IUGR adult (Simmons, 2007a; Park *et al.*, 2008).

The rat reaches maturity more rapidly than sheep and humans. This enables a more feasible study of longer term outcomes with a much larger number of offspring available for investigation. Moreover, the rat has more genomic and bioinformatics information available than the sheep, where it is only recently that sheep miRNA sequences have been increasingly identified and characterised (Caiment *et al.*, 2010; Sheng *et al.*, 2011; Cox *et al.*, 2012; Zhang *et al.*, 2013).

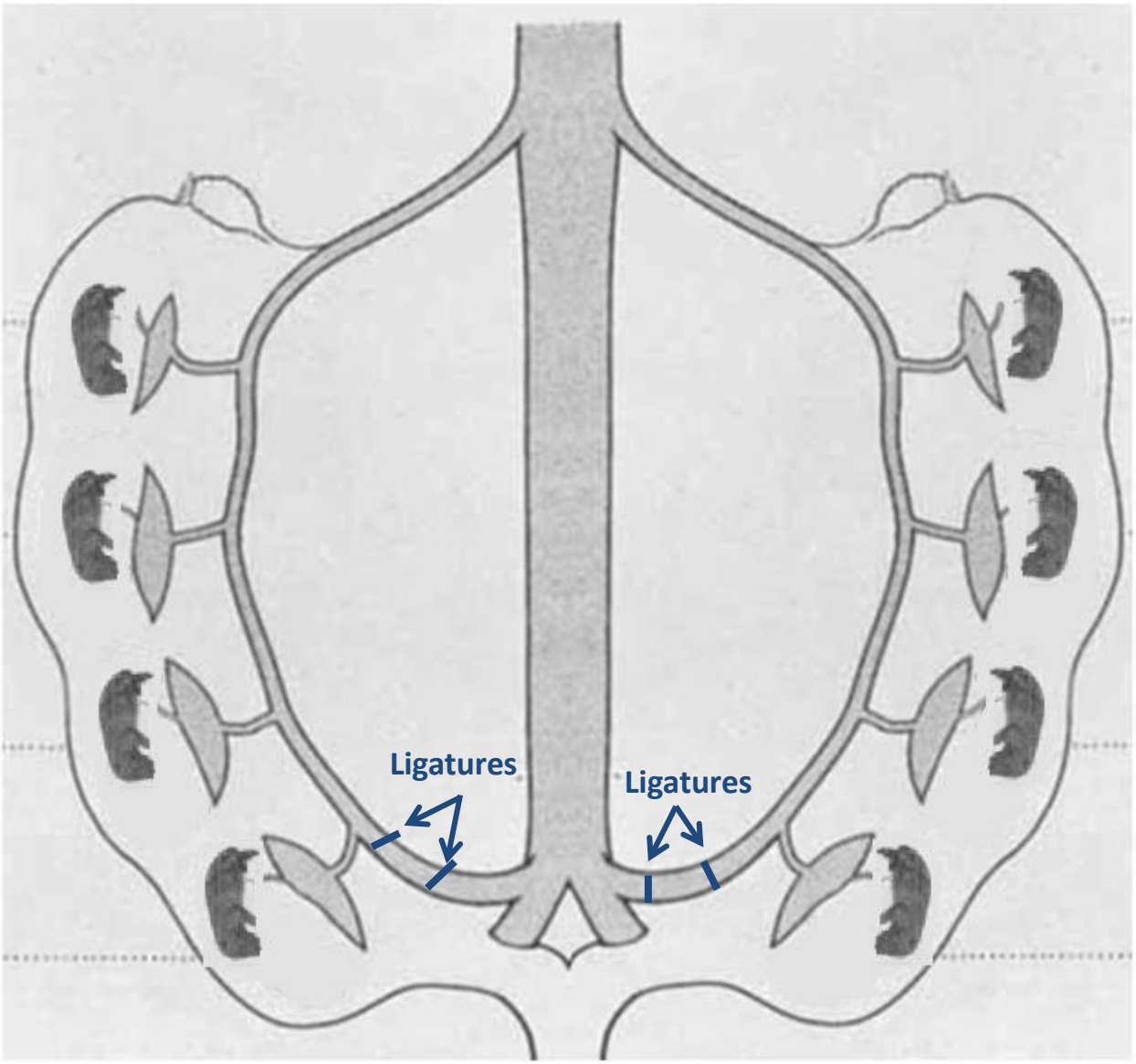


Figure 1.3. Schematic diagram of BUVL surgery in rats at day 18 following mating.

Ligations on both uterine arteries restrict the supply of blood and nutrients from the dam to the placenta. This diagram shows the location of ligations uterine arteries, which causes the fetus to receive less oxygen and nutrients, leading to growth restriction. (Adapted from (Wigglesworth, 1974).

1.3.3 Consequences of experimental IUGR

Experimental IUGR has been shown to impair glucose tolerance, insulin secretion and sensitivity in both sheep and rat offspring. When offspring were cross-fostered, studies reported that placental restriction in the rat impaired glucose tolerance from as early as 7 weeks and persisted to 26 weeks of age (Simmons *et al.*, 2001; Vuguin *et al.*, 2004). In another study in the rat, where offspring were not cross-fostered, placental restriction did not impair glucose tolerance (Wadley *et al.*, 2008). This suggests that in the rat cross-fostering may interact with placental restriction to impair glucose tolerance in the offspring. Furthermore, placental restriction in the rat impaired insulin secretion earlier and to a greater extent among the male offspring (Simmons *et al.*, 2001; Vuguin *et al.*, 2004; Siebel *et al.*, 2008; Wadley *et al.*, 2008).

Placental restriction in sheep induces foetal hypoglycaemia from mid-gestation, hypoxemia, together with reduced plasma insulin (Owens *et al.*, 2007b), insulin-like growth factors (Owens *et al.*, 1994), and increased plasma cortisol and catecholamine (Harding *et al.*, 1985; Forhead *et al.*, 2008). Placental restriction in the sheep also induces age-related loss of insulin secretion from fetal life onwards, eventually impairing the insulin secretory function of the *β*-cell in young adult IUGR males (Gatford *et al.*, 2008). The latter results in impaired *in vivo* insulin secretion relative to sensitivity, despite increased *β*-cell mass (Gatford *et al.*, 2008).

Reduction of in expression of key genes in insulin signalling or secretory pathways , in insulin sensitive and insulin secreting tissues (see Section 1.2.3 and 1.2.4), may partly responsible for the impaired insulin action in both sheep and rat IUGR

offspring. However, the underlying causes of these altered molecular profiles are poorly understood.

1.4 Programming and epigenetic mechanisms

1.4.1 Definition of programming and epigenetics

'Programming' is defined as a phenomenon whereby a disturbance of the fetal environment at a critical stage of functional differentiation may persistently alter the functional development of key tissues and organs, with longer-term consequences and increased risk of disease in later life (Barker, 1992). This may be mediated by alterations in the number and the relative proportion of different cell types within a tissue, as well as by changes in the intrinsic functional capacity of those cells which persists (Robinson *et al.*, 2000; Fowden & Forhead, 2004).

Epigenetic mechanisms may play a key important role in 'programming', as they can persistently alter gene expression and potentially modify function throughout life. 'Epigenetic' is a term describing information that can be passed to the progeny through the genome without changing its DNA sequence (Gluckman *et al.*, 2007). Epigenetic mechanisms include DNA methylation, genomic imprinting and chromatin remodelling. DNA methylation involves the addition of a methyl donor group to the cytosine 5' position of the cytosine in a CpG dinucleotide mediated by DNA methyltransferases (DNMTs) (Chuang & Jones, 2007). This latter process usually represses downstream genes expression.

Genomic imprinting uses a similar mechanism to DNA methylation, but only the paternal or maternal allele of a particular gene is expressed. A classic example of genomic imprinting is the expression of *Igf2*; its expression is controlled by a downstream gene, called *H19*. *H19* is methylated only at the differentially methylated region (DMR) of the paternal allele; therefore, downstream enhancers at the paternal

allele are free to interact with the *Igf2* promoter region. At the maternal allele, the *H19* DMR region is not methylated, allowing CTCF (a DNA binding protein) to bind at the DMR region, thus insulating the maternal *Igf2* from downstream enhancers (Engel *et al.*, 2006).

1.4.2 The developmental origins of health and disease hypothesis

The developmental origins of health and disease hypothesis states that environmental factors, such as maternal under-nutrition during pregnancy, may programme the risk for adverse adult life health outcomes, such as cardiovascular disease, obesity and metabolic syndrome (McMillen *et al.*, 2008). The link between low birth weight, later onset diabetes and cardiovascular disease (CVD) was first noted by Professor David Barker in 1992 (Barker, 1992; Hales & Barker, 1992). Another study showed fetal growth restriction or at least, low birthweight, to be associated with coronary heart disease in later adult life (Barker *et al.*, 1993). A similar correlation between low birth weight and coronary heart disease in adult life was reported among men and women born between 1934 and 1935 in South India (Stein *et al.*, 1996). Birthweight has also been shown to be inversely related to development of glucose intolerance and type 2 diabetes later in life (Newsome *et al.*, 2003; Harder *et al.*, 2007; Whincup *et al.*, 2008; Gatford *et al.*, 2010). As low birthweight largely reflects poor fetal growth, these associations suggest that fetal substrate deprivation is linked to poor metabolic and cardiovascular health in later life or programming of offspring physiology.

Epigenetic mechanisms are likely to play a role in the metabolic development of disease among IUGR offspring (Waterland & Michels, 2007). Studies have shown

that the IUGR may alter the molecules that underpin insulin sensitivity and secretion in adult offspring in humans and other species. Pancreatic *Pdx1* expression, an enzyme which regulates insulin secretion, is reduced in rat IUGR offspring via altered histone modifications, followed by later alterations in DNA methylation (Park *et al.*, 2008). Furthermore, placental restriction alters genome wide DNA methylation in the pancreatic islets of IUGR offspring at 7 weeks of age, mostly at CpG islands (25%), CG clusters (25%) and promoter regions (20%) (Thompson *et al.*, 2010). However, in the latter, most differential methylation occurs in the intergenic regions (Thompson *et al.*, 2010), where numerous non-coding genes, such as those for miRNAs are located, implicating them in altered molecular and expression of islets following placental restriction. Therefore, epigenetic mechanisms, including those affecting microRNAs, could be an important pathway contributing to the development of metabolic diseases in later life of IUGR offspring.

1.5 MicroRNA

1.5.1 Definition and role of microRNA

MicroRNAs (miRNAs) are short, single-stranded, non-coding RNA, about 22 nucleotides in length. Most miRNA genes are conserved in mammalian species (Figure 1.4) and a complete review on miRNA biogenesis was compiled in (Winter *et al.*, 2009). Varying outcomes can be caused by repression of the targets' abundance and translation into protein, initiated by the RNA-induced silencing complex (RISC), formed by association between the miRNA and the Argonaute protein, which binds to sites in the 3' untranslated region (UTR) of the target mRNA, and depending on the extent of homology between miRNA and its target mRNA. Most exhibit partial homology, which causes translational repression of mRNA, while perfect homology leads to a cleavage of the target mRNA (Figure 1.4). Some studies have highlighted the role of miRNAs in the development of an organ (Wienholds & Plasterk, 2005; Nguyen & Frasch, 2006), a cancer or tumour (Weber *et al.*, 2006; Lee *et al.*, 2008; Nikiforova *et al.*, 2008; Yan *et al.*, 2008), as well as metabolic diseases (Poy *et al.*, 2004; Plaisance *et al.*, 2006; Kato *et al.*, 2007; Poy *et al.*, 2007; Walker, 2008; Williams, 2008). An increasing number of algorithms (most are reviewed in (Mazière & Enright, 2007)) have been developed to predict the mRNAs targeted by miRNAs, although few have been experimentally verified to date.

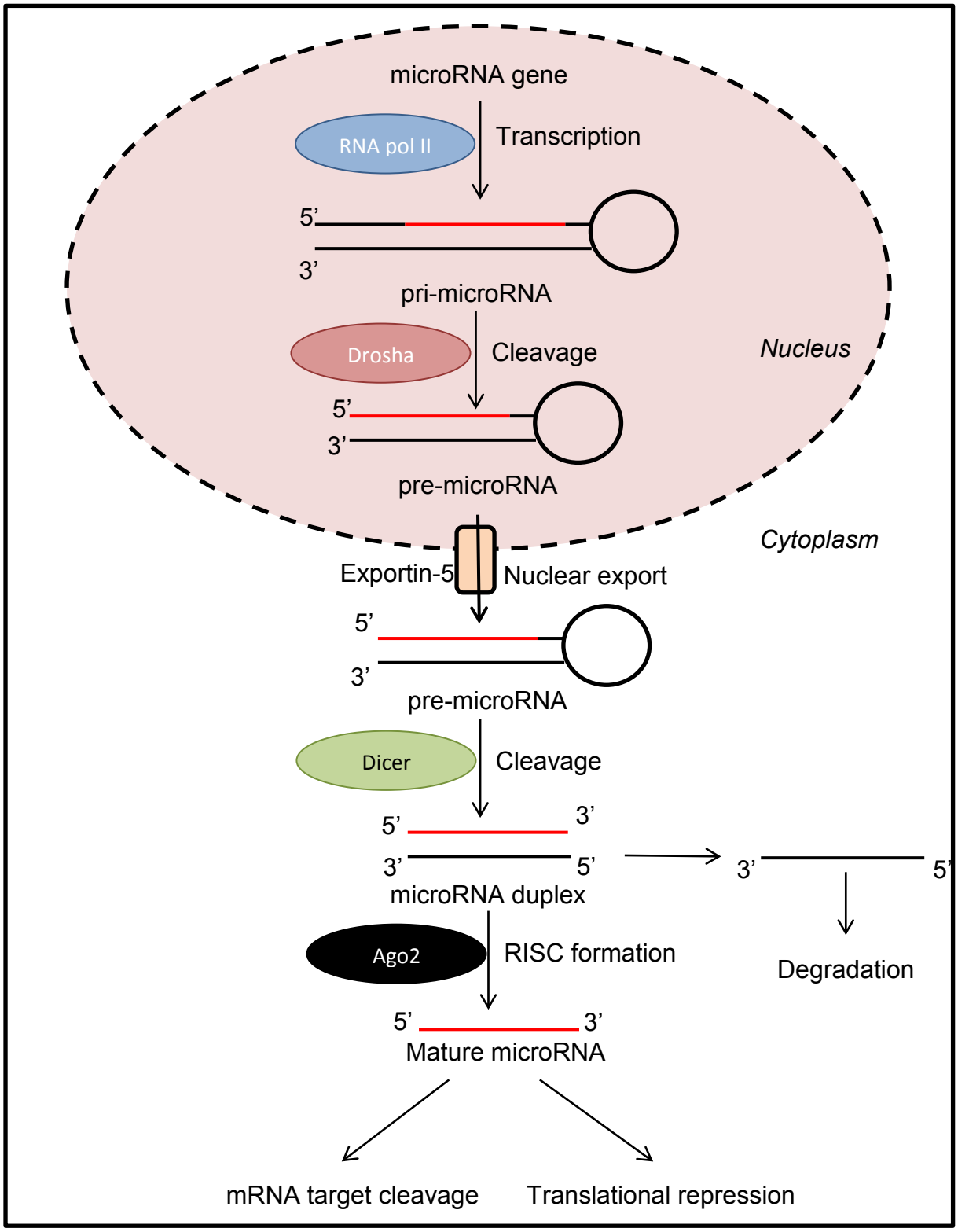


Figure 1.4. MicroRNA biogenesis.
miRNA genes are transcripted by RNA polymerase II, through modification by Drosha endonuclease, translocation to cytoplasm and Dicer endonuclease. A mature miRNA strand associates Argonaute 2 (Ago 2) protein to form a RISC complex. In a mammalian system, a partial homology formed with its target mRNA leads to translational repression, whereas in plant systems, a perfect homology leads to mRNAs cleavage.

Most miRNAs are clustered together and some are able to target from two to several hundred mRNAs (Royo *et al.*, 2006); leading to the hypothesised that miRNAs are able to form a regulatory network (Fazi & Nervi, 2008; Chowdhury *et al.*, 2013). Regulation of miRNA expression varies, depending on where the miRNA genes are located. MiRNAs whether they are clustered or not, occur either within the introns of the host genes or intergenically. Majority of miRNAs are found in the intronic region of a host genes, and are usually regulated by the same factors that influence transcription of the host gene, with additional other factors that affect their generation. Where else, intergenic miRNAs are found to have independent promoter elements (Hinske *et al.*, 2010).

1.5.2 Role of programming and epigenetics in microRNA gene expression

In order to gain better understanding of their primary structure, some human miRNA genes have been analysed for the presence of polyadenylation signals, transcription start sites and CpG (Saini *et al.*, 2007). CpG islands were specifically investigated in a 10kb (kilo bases) limit either upstream or downstream of miRNA genes. It is interesting to note that there were 111 predicted CpG islands among 82 analysed intergenic miRNA genes. This was confirmed in another study (Inouchi *et al.*, 2007), which found human miRNA genes to contain a higher CpG content (47.3% on average), compared with the whole human genome (41%). Furthermore, uteroplacental insufficiency has been shown to alter DNA methylation by reducing the mRNA expression of components involved in one-carbon metabolism in the liver of rat offspring (MacLennan *et al.*, 2004).

Therefore, it is possible that placental restriction and other causes of IUGR, low birth weight, and long-term diseases are able to persistently alter miRNA expression through alteration in the epigenetic state, which leads to altered abundance or translation of insulin signalling or related molecules or insulin secretory related molecules' mRNA transcripts that they may target. This means that epigenetic modifications by early life events might influence miRNA expression and function in the long term and could so contribute to programming. The long-term impact of prenatal deprivation on tissue expression of miRNAs in offspring has been little investigated to date.

1.5.3 miRNA regulation in insulin signalling

Despite each miRNA potentially targeting numerous mRNAs (Brown & Sanseau, 2005), currently there are not many involved in insulin signalling and type 2 diabetes, whether directly or indirectly, that have been experimentally validated to date. The manipulation of miRNA abundance by knockdown or transfection to reduce or increase their expression, respectively, shows that several miRNAs directly alter either expression of proteins related to insulin signalling or its targets or insulin-stimulated glucose uptake itself (summarised in Table 1.2). One study showed that, by transfecting miR-145 in colorectal cancer cell lines, one of its target, IRS-1 protein levels decreased, as determined by western blot (Shi *et al.*, 2007). When dme-miR-278 was deleted in *Drosophila*, these mutants were observed to have elevated circulating sugar and development of insulin resistance, postulating that this particular miRNA promotes insulin sensitivity (Teleman *et al.*, 2006)..

Table 1.2. Validated and hypothetical predicted miRNA targets involved in insulin signalling and type 2 diabetes

miRNA	Target	Function	Ref.
miR-145	IRS1	(unknown)	(Shi *et al.*, 2007)
miR-29	(unknown)	Inhibition of glucose uptake	(He *et al.*, 2007)
miR-278	(unknown)	Inhibition of glucose-stimulated insulin signalling	(Teleman *et al.*, 2006)
miR-375	Myotrophin		(Kato *et al.*, 2007)
miR-9	OneCut2 transcription factor	Inhibition of insulin secretion	(Poy *et al.*, 2004)
miR-192	E-box repressor	Inhibition of glucose-stimulated insulin release	(Plaisance *et al.*, 2006)
miR-142	GLUT4, HSL, fatty acid-binding protein aP2, PPAR-µ2	TGFβ-induced matrix protein collagen Co11a1 & a2 Adipocyte differentiation	(Esau *et al.*, 2004)
miR-33a/b	IRS2	Reduced IRS2 mRNA expression	(Davalos *et al.*, 2011)
miR-144	IRS1	Reduced IRS1 mRNA and protein expression	(Karolina *et al.*, 2011)

(modified from (Muhonen & Holthofer, 2009) and (Dehwah *et al.*, 2012)

1.5.4 miRNA regulation in insulin secretion

MiRNAs may potentially play a key role in insulin impairment, as recent studies have shown miRNA to be involved, directly or indirectly, in regulating insulin synthesis *in vitro* (Table 1.3). It has been suggested that pancreatic miR-30d plays a role in insulin gene expression through a regulation of epithelial to mesenchymal transition by targeting vimentin (Ozcan, 2009). Pancreatic miR-375 is known to regulate glucose-stimulated insulin secretion and *β*-cell differentiation (El Ouaamari *et al.*, 2008). Pancreatic miR-9 could play an important role in insulin secretion, as *in vitro* analysis reported that miR-9 was highly expressed during glucose-dependent insulin secretion (Ramachandran *et al.*, 2011). Moreover, it has been shown that knocking down the expression of miR-24, miR-26, miR-182 and miR-148 in the pancreatic *β*-cells reduces promoter activity and insulin mRNA levels (Melkman-Zehavi *et al.*, 2011). This indicates that miRNAs may play a role in positive feedback in regulation of insulin production. A functional analysis of miR-19b highlighted its ability to inhibit

insulin 1 expression in MIN6 *β*-cells (Zhang *et al.*, 2011). Lastly, increased expressions of miR-9 in pancreatic *β*-cells have been shown to reduce glucose-dependent insulin secretion by reducing Sirt1 protein expression in mice (Ramachandran *et al.*, 2011).

In the young IUGR lamb resulting from placental restriction, pancreatic expression of the L-type voltage-gated calcium channel and of the Kir6.2 subunit of the K_{ATP}-channel was reduced (Gatford *et al.*, 2008). These key molecular mediators in pathways for Ca^{2+} and K^{+} flux controlling insulin secretion (Zomer *et al.*, 2010) are novel candidates for PR-induced defects in insulin secretion. *In vitro* analysis has demonstrated that the Kir6.2 mRNA expression increased upon blocking the expression of miR-124a2 in MIN6 *β*-cells (Baroukh *et al.*, 2007). In fact, mutations in either of the K_{ATP}-channel subunits in humans have been shown to increase the diabetes risk (Flanagan *et al.*, 2009). All of these studies suggested that miRNA could play an important role in regulating insulin production and secretion in the pancreatic *β*-cells and potentially in the alterations seen following placental restriction.

Table 1.3. Known microRNAs involved in regulation of the pancreas mass and function.

miRNA	Pancreatic sites	Function	Ref.
miR-30d	Human islets	Regulates insulin gene expression	(Ozcan, 2009)
miR-7, miR-9, miR-375 & miR-376	Human islets	Highly expressed during human pancreatic islet development	(Joglekar *et al.*, 2009)
miR-9	*β*-cells cell lines (β-TC-6)	Highly expressed during glucose-dependent insulin secretion	(Ramachandran *et al.*, 2011)
miR-24, miR-26, miR-182 & miR-148	Cultured *β*-cells	Regulates insulin promoter activity and insulin mRNAs levels	(Melkman-Zehavi *et al.*, 2011)
miR-19b	Mice pancreatic progenitor cells	Regulates insulin 1 expression, by targeting transcription factor NeuroD1	(Zhang *et al.*, 2011)
miR-124a2	*β*-cells cell lines (MIN6)	Indirectly regulates *Pdx-1* expression	(Baroukh *et al.*, 2007)
miR-7	Human pancreas	Potential role in the endocrine cell differentiation and/or function	(Correa-Medina *et al.*, 2009)
miR-34a	*β*-cells cell lines (MIN6B1)	Inhibition of the expression of VAMP2, reducing insulin released	(Lovis *et al.*, 2008)
miR-29a & b	*β*-cells cell lines (MIN6)	Affecting insulin release, by reducing expression of *β*-cells specific MCT1 transporter	(Pullen *et al.*, 2011)

Currently, no studies of miRNA expression in the pancreas, islets or *β*-cells of sheep have been conducted, whereas pancreatic and of a lesser extent, islet miRNAs expression in humans and rats has been characterised (Table 1.4). Highly expressed pancreatic miRNAs in humans and rats are let-7a, let-7b and let-7c. Pancreatic let-7 has been proposed to play an important regulatory role in glucose-induced insulin secretion in mice; however, the function of let-7 in the pancreas is still unknown (Frost & Olson, 2011). In humans, the highly expressed pancreatic miRNAs across development and maturation include hsa-miR-26a and hsa-miR-16. Since miRNAs are known to be conserved across animal species (Ha *et al.*, 2008), we expect that many highly expressed pancreatic miRNAs in sheep will be identical in sequence and abundance to those in humans and rats.

Table 1.4. Highly expressed pancreatic microRNAs in humans and rats.

Rosero et al. 2010 (humans) (Rosero *et al.*, 2010)		Szafranska et al. 2007 (Szafranska *et al.*, 2007)	Larsen et al. 2011 (Larsen *et al.*, 2011)
10 wga	**22 wga**	**Humans-older adult**	**Rat (pool E20 to P2)**
hsa-miR-126	hsa-miR-26a	hsa-let-7a	miR-298
hsa-miR-19b	hsa-miR-126	hsa-let-7c	miR-494
hsa-miR-26a	hsa-miR-16	hsa-let-7b	miR-292-5p
hsa-miR-125b	hsa-miR-125b	hsa-miR-26a	miR-503
hsa-miR-92	hsa-miR-19b	hsa-miR-21	miR-290
hsa-miR-16	hsa-miR-24	hsa-let-7d	miR-320
hsa-miR-30c	hsa-miR-30c	hsa-miR-200c	let-7c
hsa-miR-200c	hsa-miR-26b	hsa-miR-148a	miR-327
hsa-miR-125a	hsa-miR-142-3p	hsa-let-7f	miR-185
hsa-miR-24	hsa-miR-199a	hsa-miR-29c	let-7b
hsa-miR-99a	hsa-miR-100	hsa-miR-16	let-7a
hsa-miR-214	hsa-miR-99a	hsa-miR-217	
hsa-miR-20a	hsa-miR-20a	mmu-miR-217	
hsa-miR-218	hsa-miR-191	hsa-miR-30a-3p	
hsa-miR-484	hsa-miR-125a	hsa-miR-29a	
hsa-miR-594	hsa-miR-30a-5p	hsa-let-7g	
hsa-miR-26b	hsa-miR-140	hsa-miR-200b	
hsa-miR-127	hsa-miR-130a	hsa-miR-141	
hsa-miR-130a	hsa-miR-127	hsa-miR-27b	
hsa-miR-335	hsa-miR-30d	hsa-miR-26b	

wga: week of gestational age; E20: embryonic age day 20; P2: postnatal age day 2; hsa: *Homo sapiens*; mmu: *Mus musculus*.

1.6 Summary

Placental restriction and IUGR has been shown to alter epigenetic state of key functional and regulatory genes in offspring, in fetal and later life. MicroRNAs are small non coding RNAs able to co-ordinately regulate transcription of many genes and are highly susceptible to epigenetic disruption. They are known to target genes in major metabolic pathways and also insulin action, including insulin signalling and secretion, in skeletal muscle, adipose, liver and pancreatic tissues. However, little is known as to whether placental restriction and IUGR affects the expression of microRNAs in insulin sensitive tissues or in the pancreas of offspring, later in adult life. Therefore, these studies will determine if placental restriction alters microRNA expression in the insulin sensitive and secreting tissues of IUGR offspring in two species, the sheep and rat. The predicted gene and functional pathways targeted by microRNAs altered by placental restriction will then be identified and the relationship of the abundance of microRNAs and their molecular and physiological targets assessed.

1.7 General hypothesis

Placental restriction of nutrients and oxygen delivery to the fetus and IUGR alter the expression of non-coding microRNAs and protein-coding genes in the insulin sensitive tissues, skeletal muscle, liver and adipose tissue of offspring, as well as in the pancreas of offspring, impairing insulin signalling, sensitivity and secretion.

1.7.1 Specific hypothesis

Some of these altered expression microRNAs are able to target insulin signalling and their molecular mediators in the skeletal muscle, liver and adipose tissue of IUGR sheep and rat offspring. Altered microRNAs in the pancreas are able to target molecules involved in regulating insulin secretion of IUGR sheep offspring.

1.8 Significance of the project

By using complementary placental restriction induced IUGR models in two different species, the sheep and the rat, this study will determine whether microRNAs play an important role in the later life development of insulin resistance. This study will also examine whether microRNA could play a role in the alterations in insulin production and secretion in the pancreas of IUGR offspring. Lastly, this study will examine the relationship of altered microRNA expression in these key tissues, involved in insulin action to the altered metabolic state of the IUGR individual. An understanding of how placental restriction affects the expression of microRNAs in insulin action may identify novel opportunities in targeting diagnosis, prevention and treatment of impaired metabolic health of the IUGR offspring.

1.9 Proposed studies

1.9.1 Overall aim

This study will determine the consequences of placental restriction for expression of microRNAs in key glucoregulatory tissues of offspring during postnatal life; it will demonstrate how these consequences vary with age and sex. Moreover, the present study will assess the relationship of altered expression of miRNAs to that of their predicted targets and key molecules in glucose uptake and metabolism and insulin signalling or secretion in such tissues.

1.9.1.1 Aim 1

To analyse miRNA expression in the liver, skeletal muscle and adipose tissues of placental restricted IUGR sheep offspring, and their associations with their predicted targets and measures of metabolic state and insulin action. Please refer to Figure 1.5 and 1.7 for experimental flow diagrams.

1.9.1.2 Aim 2

To analyse miRNA expression in the liver, skeletal muscle and adipose tissues of placental restricted IUGR rat offspring, and their associations with their predicted targets and measures of metabolic state and insulin action. Please refer to Figure 1.6 and 1.7 for experimental flow diagrams.

1.9.1.3 Aim 3

To analyse whether placental restriction alters miRNA expression in the pancreas of offspring In the sheep, known to have impaired insulin secretion. Please refer to Figure 1.5 and 1.7 for experimental flow diagrams.

1.10 Experimental flow diagram

Detailed description of the design and methods used for the sheep study can be found in the materials and methods section of Chapter 2 (Section 2.2) and 4 (Section 4.2). Details of the design and methods used for the rat study can be found in the materials and method section on Chapter 3 (Section 3.2). The number of animals used can be found on Table 1.5 and 1.6.

1.10.1 Surgical treatment for animal models

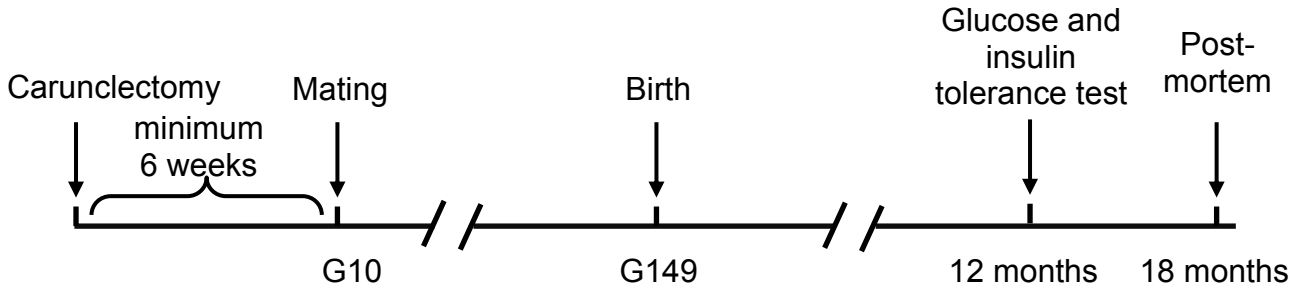


Figure 1.5. Schematic flow diagram of treatment for the sheep IUGR model used in the present thesis

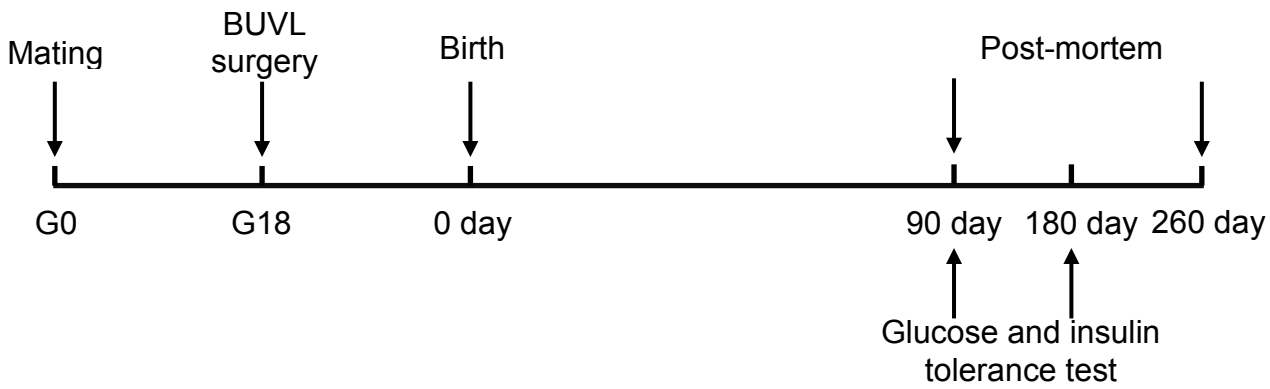


Figure 1.6. Schematic flow diagram of treatment for the rat IUGR model used in the present thesis

1.10.2 Schematic experimental flow diagram

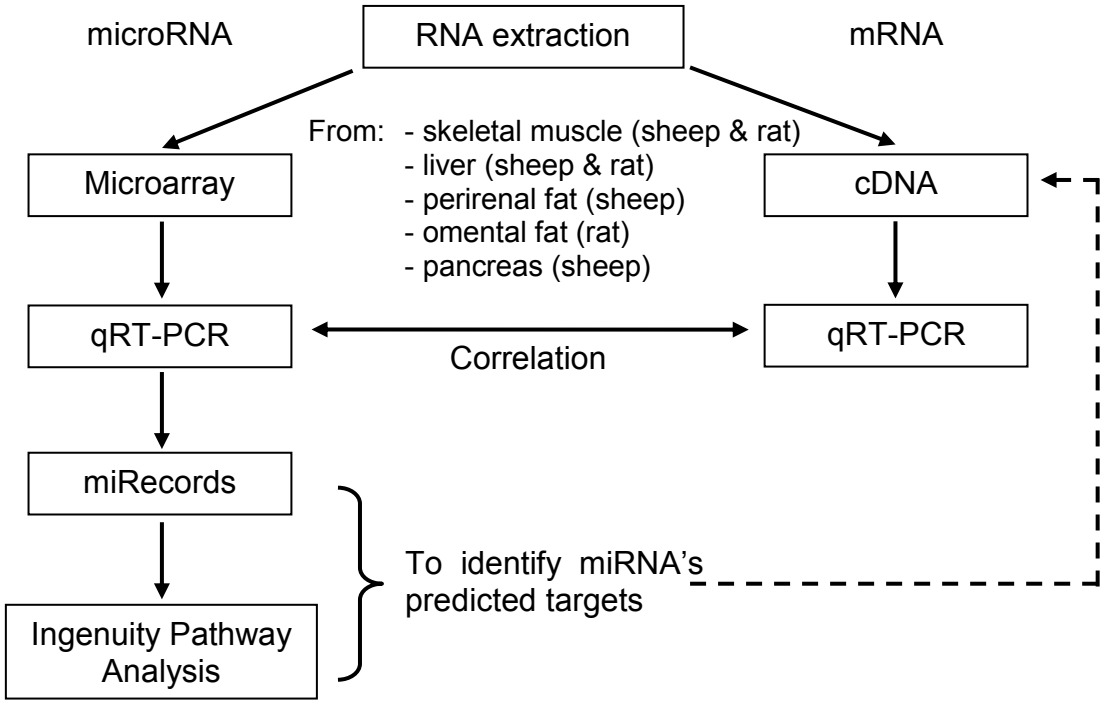


Figure 1.7. Experimental flow diagram for analysing miRNA and predicting targets gene expression.

Table 1.5. A summary of the experiments and number of sheep used in Chapter 2 and 4.

Chapter 2				
Control/Placentally restricted	Control (16)		PR (14)	
Male/female	Male (8)	Female (8)	Male (6)	Female (8)
Size at birth	8	8	6	8
Size at 18 months	8	8	6	8
Body composition	8	8	6	8
Plasma glucose	8	8	6	5
Plasma insulin	8	8	6	5
Insulin sensitivity (glucose)	8	8	6	5
Insulin sensitivity (cholesterol)	8	8	6	5
Insulin sensitivity (triglycerides)	8	8	6	5
Insulin sensitivity (free fatty acids)	8	8	6	5
Exiqon miRNA microarray	8	8	6	8
miRNA qRT-PCR	8	8	6	8
Predicted target qRT-PCR	8	8	6	8
Chapter 4				
Fasting plasma insulin	8	7	4	6
Exiqon miRNA microarray	8	7	4	6
miRNA qRT-PCR	8	7	4	6

Table 1.6. A summary of the experiments and numbers of rats used in Chapter 3.

Chapter 3						
Control/BUVL/Sham	Control (28)		BUVL (30)		Sham (24)	
Male/Female	Male	Female	Male	Female	Male	Female
Size at birth	15	13	14	16	12	12
Rate of growth weight	15	13	14	16	12	12
Cohort 1						
Post-mortem at day 90	7	5	6	8	4	4
Exiqon miRNA microarray	7	5	6	8		
miRNA qRT-PCR	7	5	6	8		
Predicted target qRT-PCR	7	5	6	8		
Cohort 2						
Glucose tolerance (day 90 and 180)	8	8	8	8	8	8
Insulin tolerance (day 90 and 180)	8	8	8	8	8	8
Post-mortem at day 260	8	8	8	8	8	8
Exiqon miRNA microarray	8	8	8	8		
miRNA qRT-PCR	8	8	8	8		
Predicted target qRT-PCR	8	8	8	8		

Chapter 2

Placental restriction alters microRNA expression in insulin sensitive tissues of the adult sheep: relationship to insulin signalling and metabolism

2.1 Introduction

Intrauterine growth restriction (IUGR) is associated with an increased risk of diabetes in later adult life (as systematically reviewed in (Newsome *et al.*, 2003; Whincup *et al.*, 2008)), as well as low birth weight (< 2,500 grams); this can account for about 18% of type 2 diabetes prevalent among the population (Eriksson, 2007). In developed countries, placental insufficiency during pregnancy is a significant contributor to IUGR, which occurs in 3-7% of the population and more in some communities (Romo *et al.*, 2009). Due to the pre-birth substrate supply largely determining the fetal substrate supply, as well as the growth rate and size at birth, IUGR has been proposed to reflect an adverse intrauterine environment that restricts fetal growth and 'programs' impaired insulin action and glucose control in later life (Gatford *et al.*, 2010). A major cause of IUGR is placental insufficiency (PI), a common complication of pregnancy, whereby the placental delivery of oxygen and nutrients does not adequately meet the needs of the developing fetus (Ergaz *et al.*, 2005; Baschat *et al.*, 2007). Together with the fetal metabolic, hormonal and physiological adaptations to a restricted substrate supply (Fowden & Forhead, 2004), this PI is proposed to induce cellular and molecular changes, which persistently impact the phenotype that affect the risk of diseases, such as diabetes (Gatford *et al.*, 2010).

Among humans and other species, IUGR is shown to be characterised by impaired insulin secretion and sensitivity (Jaquet *et al.*, 2000; De Blasio *et al.*, 2007a), along with altered expression and abundance of insulin signalling and related molecules in the insulin sensitive tissues (Jaquet *et al.*, 2001b; Oak *et al.*, 2006; Thorn *et al.*, 2009). Importantly, experimental placental restriction of fetal growth in the sheep (De

Blasio *et al.*, 2007a) impairs insulin sensitivity and secretion in its offspring. Moreover, experimental placental restriction of fetal growth in the rat impairs insulin secretion (Neitzke *et al.*, 2008), and in some but not all studies, insulin sensitivity, resulting in type 2 diabetes (Simmons *et al.*, 2001; Vuguin *et al.*, 2004; Wlodek *et al.*, 2005; Wadley *et al.*, 2008). The molecular basis of insulin sensitivity changes seen among IUGR adults of other species has, to some extent, been determined mostly in insulin-sensitive tissues. However, the responsible mechanisms are not completely understood.

IUGR in humans appears to be characterised by an increased insulin sensitivity at birth, which subsequently disappears and reverses during childhood or early adult life, with the onset of insulin resistance (Gatford *et al.*, 2010). Among insulin-resistant IUGR young adult men, the *Glut4* mRNA expression is reduced in skeletal muscle and adipose tissue (Jaquet *et al.*, 2001b). Paradoxically, the skeletal muscle expression of the PI3K units, p85α and p110β, is increased in the insulin-stimulated state, but reduced in the basal state; this suggests that the Akt pathway may be the initial molecular site of an initial onset of insulin resistance among young men of low birth weight (Jensen *et al.*, 2008). IUGR in sheep, induced by exposing pregnant ewes to elevated ambient temperature during early to mid-gestation, increased the expression of the insulin receptor, *p85α* and *Akt2*, in skeletal muscle of the fetus (Thorn *et al.*, 2009). This is consistent with placental restriction and IUGR enhancing insulin sensitivity in the sheep fetus before birth (Limesand *et al.*, 2007; Owens *et al.*, 2007a). Subsequently, these insulin sensitivity changes, induced by placental insufficiency before birth, are reversed in early postnatal life with an emergence of insulin resistance in the young lamb (De Blasio *et al.*, 2012). Consistent with this, this

study also reported a reduced expression of the insulin receptor, *Irs1, Akt2, Glut4, Gsk3α* and *Gys1* mRNA in the skeletal muscle of juvenile sheep offspring who were IUGR due to placental restriction (De Blasio *et al.*, 2012).

To date, there has been no investigation of the role microRNAs (miRNAs) play in placental programming of these molecular changes related to altered insulin sensitivity and glucose metabolism among offspring. MiRNAs are short, single-stranded, non-coding RNAs, about 20-23 nucleotides in length (Bushati & Cohen, 2007), able to bind to sites in 3' untranslated region (UTR) of multiple target mRNAs, leading to translational repression or cleavage of mRNA. As miRNAs regulate the expression of multiple targets, often within the same or related pathways, they can form regulatory networks and become influential mediators of early life programming of the later phenotype (Ambros, 2004). Recently, various miRNAs have been identified as potential regulators of insulin signalling; the miRNAs expression can be altered within tissues key to the insulin action in type 2 diabetes (Ferland-McCollough *et al.*, 2010; Rottiers & Naar, 2012) (as summarised in Table 1.2). It is unknown whether placental restriction and IUGR alter the miRNA expression in key insulin-sensitive tissues, liver and skeletal muscle, as well as in adipose tissue, particularly the miRNAs expressions that target insulin signalling and related molecules.

As placental restriction in humans is difficult to study directly and invasively, the present study investigates the effects of placental restriction on glucose tolerance, insulin sensitivity and its underlying determinants from before birth and postnatally among the sheep (De Blasio *et al.*, 2007a; Owens *et al.*, 2007a; Owens *et al.*, 2007b; De Blasio *et al.*, 2010). Placental restriction in sheep induces a sequence of prenatal

events and consequences similar to that observed for human placental insufficiency (Harding *et al.*, 1985; De Blasio *et al.*, 2007b). These events include fetal growth restriction, fetal hypoglycaemia from mid-gestation, hypoxaemia, reduced plasma insulin (Owens *et al.*, 2007b), insulin-like growth factors (Owens *et al.*, 1994) and increased plasma cortisol and catecholamine (Harding *et al.*, 1985; Forhead *et al.*, 2008). Placental restriction in sheep can be surgically induced by removing the majority of visible endometrial caruncles prior to mating. In the sheep, these caruncles are sites where the cotyledons form during pregnancy, thus reducing the number of attachment sites before pregnancy and the number of cotyledons formed during subsequent pregnancy; in turn, this limits placental size and function (Robinson *et al.*, 1979; Owens *et al.*, 1987).

Therefore, the present research hypothesises that placental restriction (and, hence, IUGR) alters the expression of miRNAs in major insulin-sensitive tissues, skeletal muscle, liver and adipose tissue; this includes expression of miRNAs known or predicted to target insulin signalling and its molecular targets in adult offspring. This study aimed to determine the effect of IUGR on miRNA expression in the skeletal muscle, liver, and adipose tissue in adult sheep, to identify their predicted targets, and to determine what pathways and phenotype are predicted to be affected by the differential expression of such miRNAs. In addition, this study examined the associations between differentially expressed miRNAs and the expression of predicted molecular targets on insulin action in specific tissues, as well as their associations with measures of insulin sensitivity. Since males are more susceptible to perinatal programming of metabolic and cardiovascular homeostasis (Sugden &

Holness, 2002), the outcomes were examined in males and females both separately and overall.

2.2 Materials and Methods

2.2.1 Restriction of placental and fetal growth

All procedures carried out in this study were approved by the University of Adelaide Animal Experimentation and Ethics Committee and complied with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (NHMRC, 2004). The animals used in this study are a subset of the previously described cohort (Owens *et al.*, 2007b). The tissues collected were from sheep, of which 16 were control (8 males, 8 females) and 14 were placentally restricted (6 males, 8 females). The overall experimental design has been described previously (Owens *et al.*, 2007b). The placental growth was restricted by removing the majority of visible endometrial caruncles (implantation sites) from non-pregnant Merino ewes (Robinson *et al.*, 1979) (obtained from the South Australian Research and Development Institute, Turretfield). Surgery was performed under aseptic conditions, with general anaesthesia induced by intravenous injection of sodium thiopentone (1.25 g/ml; Boehringer Ingelheim, NSW, Australia), and maintained by inhalation of 2% to 4% halothane in oxygen. A 10 cm mid-line incision was made along the abdomen and the uterus was exteriorised. Each uterine horn was opened along its length from the cervix to near the utero-tubular junction. The majority of endometrial caruncles were removed; leaving four visible caruncles in each horn of the bicornuate uterus.

Post-surgery, the ewes were kept under observation for 4 to 7 days, before being returned to the field. The control ewes were un-operated. After a recovery period of

at least 10 weeks, both the restricted ewes and un-operated control ewes were mated, with pregnancies confirmed by ultrasound at 50 to 80 days gestation. From 90 days gestational age, the pregnant ewes were housed in individual pens within animal holding rooms, with a 12-h light/12-h dark cycle; they were fed lucerne chaff once daily, with water available *ad libitum*. Pregnant and lactating ewes also received 150g of oats daily. Lambs were housed in pens with their mothers from birth and weaned at 90 days. All lambs were housed with a 12-h light/12-h dark cycle and fed lucerne chaff once daily, with water available *ad libitum*. Their weight and body dimensions were measured both at birth and subsequently, as reported previously (Owens *et al.*, 2007a; Owens *et al.*, 2007b).

2.2.2 Measurement of insulin sensitivity of glucose, amino acid and lipid metabolism

As previously reported, the hyperinsulinaemic euglycaemic clamp (HEC) was used to measure insulin secretion and sensitivity of glucose metabolism, circulating free fatty acids (FFAs) and α-amino nitrogen (Gatford *et al.*, 2004; Owens *et al.*, 2007b); these measurements were taken at 373 days of age, after a 12-hour overnight fast. Arterial blood was sampled (2ml) at 10, 5, and 0 min prior to commencing the clamp for determining the fasting blood glucose, as well as plasma insulin and FFA concentrations. Human insulin (Actrapid, Novo Nordisk) was infused at 2 $mU.kg^{-1}.min^{-1}$ IV from 0 min, for a duration of 2 h. Arterial blood (0.2ml) was sampled at 5 min intervals via the clamp and blood glucose was rapidly measured using a HemoCue glucometer (HemoCue AB, Sweden). At 15 min, an intravenous infusion of glucose (25% dextrose) commenced at 2 $mg.kg^{-1}.min^{-1}$, with the rate being adjusted every 5 min in order to restore and maintain euglycaemia. Plateau rates of glucose

infusion reached during the second hour of the clamp were termed steady-state glucose infusion rates. For the purpose of measuring plasma glucose and insulin, additional blood samples (2 ml) were collected from the arterial catheter at 10 and 0 min before, and 60, 75, 90, 105, and 120 min after commencing the insulin infusion.

The insulin sensitivity of glucose metabolism was determined as the mean glucose infusion rate from 60 to 120 mins during the HEC, divided by the mean plasma insulin concentration measured every 15 mins during the second hour of infusion.

The insulin sensitivity of circulating FFA was calculated as the percentage change from fasting plasma FFA concentrations to those of the second hour of the HEC, corrected for the plateau plasma insulin (FFA_{60-120}).

2.2.3 Measurement of glucose tolerance and insulin secretion in vivo

Glucose tolerance was assessed with an intravenous glucose tolerance test (IVGTT), at 375 days of age, after a 12-hour overnight fast. Detailed experimental procedures have been described previously (Owens *et al.*, 2007b). Briefly, the arterial blood (2ml) was sampled at 5, 3, and 0 min prior to commencing IVGTT for the purpose of determining the fasting blood glucose and plasma insulin. Next, a bolus of 0.25g glucose (25% dextrose)/kg live weight was infused intravenously over a 1 min period; the start time was recorded as the start of the bolus infusion. Arterial blood (2ml) was sampled at 2, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, and 210 min after the administration of glucose. Blood glucose was rapidly measured using a HemoCue glucometer (HemoCue AB, Sweden). The remaining blood was centrifuged and the plasma stored for subsequent measurement of plasma insulin at

each time point. Glucose tolerance was measured as the area under the plasma glucose curve.

Insulin secretion during IVGTT was calculated as the area under the plasma insulin concentration curve and in relation to the glucose area under the curve. Areas under the curve were determined using the Sigma Scan Pro v4 software package (Jandel Scientific Software, San Jose, CA, USA).

2.2.4 Plasma metabolites and insulin

The plasma free fatty acid (FFA) concentrations were measured using the COBAS MIRA automated sample system using the NEFA-C Free Fatty Acid assay kit (NovoChem, Jacksonville, FL, USA) and quality controls of QC1 and 2 (Bio-Rad, Australia). Each plasma FFA concentration was measured in single assays, with an intra-assay coefficient variation of < 4%.

Plasma triglycerides and total cholesterol were measured in duplicate using enzymatic colorimetric analysis, according to the manufacturer instructions: Unimate TRIG, Roche Diagnostics for triglycerides; and CHOL, Roche Diagnostics for cholesterol. Both plasma triglycerides and cholesterol concentrations were measured in single assays, with an intra-assay coefficient variation of < 4%.

Plasma insulin was measured in duplicate using a commercially available radioimmunoassay kit (Pharmacia, Uppsala, Sweden). For insulin assays, the intra- and inter-assay coefficient variations were 3.7% and 5.9%, respectively.

2.2.5 Post-mortem of sheep offspring

At 18 months of age, the sheep offspring were killed via an intravenous administration of an overdose of barbiturates: Pentobarbitone sodium 325 mg/mL (Lethabarb, Virbac Australia Pty Ltd, Peakhurst, NSW, Australia). The offspring organs and tissues (including liver, various skeletal muscles and adipose depots) were removed, weighed and measured. Tissue weight measurements were recorded in grams and percentage of body weight. A sample from each organ was snap-frozen in liquid nitrogen and stored at -80°C for subsequent analysis. This study used the tissues of the vastus lateralis muscle from the right hind limb, the ventral lobe of the liver, and the perirenal fat.

2.2.6 RNA extraction

Total RNA was extracted from skeletal muscle, liver and adipose tissues (~100 mg) using TRIzol extraction methodology (Invitrogen, Australia). The extraction was performed according to manufacturer's instructions and a Precellys®24 Lyse (Bertin Technologies, France) was used for homogenisation. Following homogenisation, insoluble materials were removed via centrifugation at 12,000 rpm for 10 mins at 4˚C; the supernatant was transferred into a fresh tube. Next, chloroform was added to the supernatant and mixed, then centrifuged for 30 mins at 12,000 rpm at 4˚C. Following phase separation, the aqueous phase was transferred into a new tube and isopropyl alcohol was added; this was followed by overnight incubation at -20˚C. Following incubation, the pellet was collected via a 30 min centrifugation at 12,000 rpm at 4˚C. The pellet was washed with 75% ethanol and pelleted further via a 30 min centrifugation at 12,000 rpm at 4˚C. The pellet was then dissolved in RNase-free water and stored at -80˚C.

The quantity of total RNA was determined by using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Australia) to measure the absorbency at 260 nm. Only samples with 260/280 and 260/230 ratios of ~2.0 and 1.8-2.2, respectively (indicative of no protein, phenol or co-purified co-contaminants), were selected for further analyses. The quality of RNA was assessed via a separation of ribosomal 18S and 28S stained bands, which were visualised using UV light with ethidium bromide on agarose (1%) gel. Only RNA with a 28S:18S ratio of approximately 2:1 was considered to be intact and selected for further analyses.

2.2.7 Microarray hybridization

This study used exiqon miRCURY LNA ™ microRNA Arrays v8.1 (for vastus lateralis and liver) and v11 (for perirenal fat). Array v8.1 contains ~1500 miRNA probes, which have been cloned and confirmed in over 50 animal species, based on the miRBase library v8.1. Array v11 contains ~1700 probes specific to highly conserved human, rat and mouse miRNAs, based on miRBase library v11. Thus, the expression of miRNAs in sheep major insulin target tissues, liver, skeletal muscle and adipose tissues was characterised using probes to miRNAs that are completely or extensively conserved across many mammalian species (http://www.exiqon.com/microrna-microarray-analysis-microrna-array). Upon identifying any differentially expressed miRNAs or highly abundant miRNAs of interest, literature was searched to determine whether sheep sequences have currently been defined and to assess their homology with human and other species (see Supplement Table S2.9). Small-interfering RNA (siRNA) probes of bacterial origin with no known homology to any mammalian species were included as negative controls. Twelve captured probes of small nuclear

RNA (such as U6-snRNA), which are known to be present in mammalian species, were included as positive controls. All spotted arrays of Exiqon microarray slides were prepared by the Adelaide Microarray Centre (based at University of Adelaide, SA, Australia). Each probe was plotted in duplicate and two spotted arrays were printed per one slide.

Total RNA (5 µg) was labelled by the ligation of fluorescently modified RNA, as previously described by Thompson *et al.* (Thomson *et al.*, 2004). For the purpose of this study, eight individual biological replicates were used to compare control (n = 16; 8 males and 8 females) and PR (n = 16; 8 males and 8 females, 2 replicates of placentally restricted males were repeated to complete the use of slides) within each sex. To reduce the effects of dye bias, four dye swap experiments were performed, so that half of the arrays were carried out using the opposite configuration. Samples from both control and placentally restricted sheep tissues were randomly paired within each sex. The labelling reaction contained 5µg total RNA, 10X Iglori buffer (1mM ATP, 500mM HEPES pH 7.8, 35mM DTT, 200mM $MgCl_2$, and 100mg/mL BSA), 100% DMSO (dimethyl sulfoxide), and either 500ng of 5'-phosphate-cytidyl-uridyl-Cy3-3' or 5'-phosphate-cytidyl-uridyl-Cy5-3' (Cy3 or Cy5, Dharmacon USA), with 20 units T4 RNA ligase 1 (New England Biolabs Inc., USA), in a total volume of 10µl. The labelling reaction was incubated at 4ºC for 2 hours in dark.

Labelled-RNA was precipitated with a solution containing 40µl DEPC water, 5µl of 3M sodium acetate pH 5.5, 1µl 20mg/ml glycogen (Invitrogen, Melbourne, Australia), and 150µl 100% ethanol. The RNA was placed on ice for 10 mins, and then

centrifuged at 13,000rpm for 10 mins at 4ºC. The supernatant was removed, the pellet washed with 150µl 75% ethanol and re-pelleted via centrifugation at 13,000rpm for 5 mins. The ethanol was removed and the pellet was dried at 65ºC for a few minutes. The paired samples were re-dissolved in hybridisation buffer and mixed together.

Labelled-RNA was hybridised to the arrays at 60ºC in Corning hybridisation chambers overnight, in darkness. Following incubation, the array was washed twice in a solution of 0.5 X standard sodium citrate and 0.01% sodium dodecyl sulfate for 2 mins at 60 ºC, followed by a final wash in 0.2 X standard sodium citrate for 2 mins. The slides were scanned at 10µm (micron) resolution with a Genepix 4000B Scanner (Molecular Devices, CA, USA) using photomultiplier tube (PMT) settings, which balanced the channel histograms.

2.2.8 Computational microarray analysis

Foreground and background median pixel intensity values were extracted from scanned images for both channels (Cy3, Cy5) using the Spot v3 plug-in (CSIRO, Australia) for R (the R Foundation for Statistical Computing, Vienna, Austria). Following background subtraction, the foreground intensities were $\log^2$ transformed, and a single ratio (Cy5/Cy3) value was obtained for each probe. The ratio values were normalised within the LIMMA plug-in (WEHI, Australia) for R, using the global loss normalisation routine.

The background was calculated as the average absolute value of seven negative probes $\pm$ 2 SD units. Any miRNA probe value above background was analysed

further. The data was analysed using Benjamini-Hochberg multiple comparisons to generate an adjusted *P*-value (stringent). MiRNAs with an adjusted *P*-value < 0.05 were prioritised for further bioinformatic analysis. In addition, the method used previously by Stekel (Stekel, 2003) was applied, where the number of expected false positive results is divided by the number of genes analysed on the array (hence, 1/1535 for v.8.1 and 1/1787 for v.11); any miRNA with an unadjusted *P*-value less than 0.000651 and 0.000560, respectively, was also prioritised for further analysis (Stekel, 2003; Herrera *et al.*, 2009).

2.2.9 qRT-PCR analysis of miRNA expression

RNA, 5µg, was reverse transcribed on a GeneAmp PCR System 9700 using the TaqMan MicroRNA Assays (Applied Biosystems, Australia). Water substituted for reverse transcriptase samples served as the negative control. Assay-specific cDNA and probes were combined with the TaqMan 2X Universal PCR Master Mix, No AmpErase UNGb (Applied Biosystems, Australia). The real-time PCR amplification was performed in triplicate using a Corbett RotorGene 6000 (Qiagen, Australia). The resulting data was analysed using Rotor-Gene software v1.7.

Gene expression was calculated using the ΔΔCt method (Livak & Schmittgen, 2001). The NormFinder algorithm was used to identify the optimum reference gene out of a group of candidate genes, based on their Ct values (Andersen *et al.*, 2004). The candidate genes comprised let-7a, let-7f, miR-26b, miR-92 and average of miR-26 and miR-92 ((Ct miR-26b + Ct miR-92) / 2). The NormFinder algorithm identified the average Ct value of miR-26b and miR-92 as the optimal reference for liver and adipose tissue, and let-7f as the optimal reference gene for skeletal muscle.

Normalisation was achieved by dividing the Ct value of the miRNA of interest by the average Ct values of miR-26b and miR-92 or let-7f, to obtain a ΔCt value. The ΔCt values for each miRNA were converted to linear form using $2^{-(\Delta Ct)}$, prior to commencing statistical analysis (Livak & Schmittgen, 2001).

2.2.10 Identification of predicted targets of differentially expressed miRNAs

The predicted targets for each miRNA were determined using the miRecords database (http://mirecords.biolead.org) (Xiao *et al.*, 2009), which provides 11 different algorithm databases, including miRanda, PicTar and TargetScan. The number of predicted targets for each miRNA was limited by requiring them to be commonly predicted by at least by three of the algorithms mentioned above.

Multiple miRNAs are able to target the same mRNA by binding at the 3' UTR of the target mRNA, resulting in synergistic and more potent repression of the expression of the target gene and its protein (Bushati & Cohen, 2007). Hence, this enabled the identification of common predicted targets between multiple miRNAs in the same tissue.

2.2.11 Ingenuity Pathway Analysis

Ingenuity Pathway Analysis 7.0 (IPA) (Redwood City, CA, USA) was used to analyse each differentially expressed miRNA and its targets, including the 'common' predicted targets identified through miRecords. IPA is a database which is able to identify the biological mechanisms or metabolic pathways and functions most relevant to given experimental genes of interest and tissue of origins (Ingenuity®

Systems, www.ingenuity.com). For this study's purpose of generating a network, the IPA criteria used were based on mouse, human and rat data sources, including published data derived from tissues and cells or from cell lines related to those studied here (skeletal muscle, liver or adipose tissue). Most of the miRNAs found to be of interest to this study now possess known sequences for the sheep and show complete homology with the human and the rat (and, often, the mouse). Although the sequences of regions containing miRNA targets in the sheep 3'untranslated regions of genes are yet to be characterised and compared to those in other species, many already appear to be conserved across these species.

In order to identify the networks predicted to be closely regulated by each miRNA or group of miRNAs, each network was limited to a maximum of 35 molecules, a score > 4, which indicated a 1 in 10,000 chance that the genes were part of a network due to random chance (Ingenuity, 2004). The most significant networks (in terms of selected miRNA targeted components) were chosen for further gene expression analysis (see below). Official permission to use figures and analyses performed by this software was obtained from Ingenuity Systems (Redwood City, CA, USA).

2.2.12 qPCR analysis of expression of insulin signalling molecules, related molecules and predicted microRNA targets

Total RNA (10 µg), was treated with a TURBO DNA-free^{TM} kit according to the manufacturer's instructions (Ambion Inc., Austin, USA), in order to remove any contaminating DNA. Next, the RNA was reverse transcribed according to the manufacturer's instructions (Invitrogen, Melbourne, Australia). Briefly, 2 µg of RNA was incubated with 500 ng random hexamers (GeneWorks, Adelaide, Australia), 10

nM dNTPs (Invitrogen, Melbourne, Australia), and RNase free water (added to make up the total volume to 13 µl) at 65ºC for 5 min, then placed on ice for at least 1 min. Subsequently, 1 µl of Superscript III RT (Invitrogen, Melbourne, Australia) and 200µl Rnasin (Promega, Sydney, Australia) were added, followed by incubation for 5 min at 25ºC, 60 min at 50ºC, and 15 min at 70ºC using a GeneAMP PCR system 9700 thermocycler (Applied Biosystems, Melbourne, Australia). The negative controls were prepared by omitting Superscript III RT from the reaction mix. The cDNA was stored at -20˚C for future analysis.

Initially, cDNA was diluted at 1/500; a quantitative PCR analysis was performed using a Corbett RotorGene 6000 and real master mix (5 Prime; Quantum, Brisbane, Australia) with SYBR green as the fluorescence detector, as previously described (De Blasio *et al.*, 2012). Quantitative PCR was performed with an initial 2 min hold cycle at 95°C, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. The primers were designed using Primer3 (Rozen & Skaletsky, 2000) (Supplement Table S2.1) against either the ovine sequence where available or the bovine sequence. Gene expression was analysed using RotorGene software v1.7 and the relative standard curve method of quantitation. Expressions of all coding genes were normalised against the reference gene *β-actin*, which was unaltered by placental restriction. The qPCR analysis did not confirm that the expression of miR-451 was differentially expressed following placental restriction in the perirenal fat; therefore, expression of insulin-signalling genes and related molecules were analysed only in the liver and vastus lateralis.

2.2.13 Statistical Analyses

The effects of placental restriction (between factor – two levels: control and placentally restricted) and sex (between factor – two levels: male and female), as well as the effects of interaction of PR and sex, on offspring birth size and adult body composition, were assessed via an analysis of variance (ANOVA), using Predictive Analytics SoftWare (PASW) for Windows (Version 17; SPSS Inc., Chicago, IL, USA).

ANOVA (PASW) was also used to assess the effects of placental restriction (between factor – two levels: control and placentally restricted), sex (between factor – two levels: male and female), and time (repeated measures factor – 22 levels), and the interactions of the above, on blood glucose and plasma insulin before and during IVGTT in adult offspring.

Furthermore, ANOVA (PASW) was used to assess the effects of placental restriction (between factor – two levels: control and placentally restricted) and sex (between factor – two levels: male and female), as well as their interactions, on various summative indices of glucose homeostasis, insulin secretion, insulin sensitivity and plasma lipid.

Lastly, ANOVA (PASW) was used to assess the effects of placental restriction (between factor – two levels: control and placentally restricted) and sex (between factor – two levels: male and female), and their interactions, on tissue expression of miRNAs (measured by qPCR), insulin signalling, related and target molecules Associations between the abundance of miRNAs and of predicted targets or functional measures were assessed using Pearson's correlation and non-linear

regression (PASW). All data is presented as the mean ± SEM, unless otherwise stated. A *P*-value < 0.05 was considered to be statistically significant.

2.3 Results

2.3.1 Effect of placental restriction on size at birth, postnatal growth and body composition in offspring

Placental restriction was found to reduce birth weight by 35% ($P < 0.001$ for all) and body mass index at birth by 12% ($P = 0.006$), compared with control offspring (see Table 2.1). Placental restriction in females reduced body mass index at birth by 21% ($P = 0.004$), compared with control offspring (Table 2.1). Placental restriction in males did not alter body dimensions at birth.

Overall, placental restriction did not alter body dimensions in adult offspring (Table 2.1). However, placental restriction in males increased their adult shoulder height by 9% ($P = 0.012$), compared with control male offspring (Table 2.1). Placental restriction did not alter body dimensions in female adult offspring, compared with control female offspring.

Placental restriction increased ventral liver weight relative to body weight by 20% in overall adult offspring ($P = 0.027$), compared with controls (Table 2.1); and by 29% ($P = 0.050$) in males, compared with controls (Table 2.1). Compared with the control group, placental restriction in females did not alter ventral liver weight or ventral liver weight relative to body weight.

2.3.2 Effect of placental restriction on metabolic state, glycaemia, lipidaemia and insulin abundance and sensitivity

Placental restriction did not alter fasting blood glucose, plasma insulin or plasma metabolites in adult offspring, except for the tendency to increase plasma cholesterol

(*P* = 0.10) (see Table 2.2). In females, placental restriction tended to reduce plasma cholesterol by 34% (*P* = 0.091), compared with control offspring; plasma cholesterol was not altered in male offspring.

For female offspring, placental restriction improved glucose tolerance late during IVGTT (*P* < 0.05) and did not alter plasma insulin during IVGTT (see Figure 2.1). Overall, placental restriction did not alter whole-body insulin sensitivity of glucose metabolism or circulating FFA in adult offspring (Table 2.2).

Fasting blood glucose correlates negatively with ponderal Index (PI) at birth in control males (r = 0.766, *P* = 0.026) and females (r = 0.714, *P* = 0.036) (see Supplement Figure S2.2). Plasma FFA correlates negatively with PI at birth in placentally restricted males (r = 0.446, *P*-value: 0.074) (Supplement Figure S2.2), while plasma cholesterol correlates positively with PI at birth in control female offspring (r = 0.865, *P* = 0.011) (Supplement Figure S2.3).

2.3.3 Effect of placental restriction on expression of insulin signalling and glucose transporters in tissues of offspring

Overall, placental restriction did not alter the expression of proximal insulin-signalling molecules in liver of adult offspring (Figures 2.2 and 2.3); however, it did reduce the hepatic expression of *Slc2a2* (Fold change = -1.9, *P* = 0.025) (see Figure 2.2). In general, placental restriction also reduced the hepatic expressions of *Acox1* (Fold change = 1.4, *P* = 0.034) and *Igf1* (Fold change = -2.7, *P* = 0.001), compared with controls (Figure 2.2).

Placental restriction in males reduced the hepatic expression of *Acox1* and *Igf1* (Fold change = -2.6 to -4.0, $P < 0.05$), compared with controls (Figure 2.2). The placental restriction did not alter hepatic expression of any proximal insulin-signalling molecules.

Furthermore, placental restriction did not alter the expression of proximal insulin-signalling molecules in the vastus lateralis of adult offspring (see Figure 2.3).

2.3.4 MicroRNA expression in ovine tissues

The microarray analysis detected 740 miRNAs in sheep liver, with the top 10 most highly expressed miRNAs being similarly abundant in both control and placentally restricted offspring (Supplement Figure S2.1A). The top 10 most highly expressed miRNAs in the livers of both sexes were similar: sbi-miR-164c, hsa-miR-363*, hsa-miR-122a, hsa-miR-494, hsa-miR-164f, hsa-miR-451, let-7a, let-7c, mmu-miR-711, and cbr-miR-231 (Supplement Figure S2.1A). Only two of the top 10 highly expressed miRNAs (miR-363* and miR-122a) have known sequences in sheep and show complete homology with human and rat miRNAs (Caiment *et al.*, 2010) (Supplement Table S2.9).

The microarray analysis detected 420 miRNAs in the sheep vastus lateralis, with the top 10 most highly expressed miRNAs similarly abundant in both control and placentally restricted offspring (Supplement Figure S2.1B). The top 10 most highly expressed miRNAs in the vastus lateralis of both sexes were similar: hsa-miR-363*, sbi-miR-164c, mml-miR-133a, hsa-miR-206, hsa-miR-133a, hsa-let-7c, hsa-miR-1, aga-miR-1, mmu-miR-298, and aga-miR-1 (Supplement Figure S2.1B). Five of the

top 10 highly expressed miRNAs (miR-206, miR-133a, miR-1, let-7c and let-7a), as detected here, have known sheep sequences and show complete homology with human and rat miRNAs (Caiment *et al.*, 2010) (Supplement Table S2.9).

The microarray analysis detected 527 miRNAs in sheep perirenal fat, with the top 10 most highly expressed miRNAs being similarly abundant in both control and placentally restricted offspring, except for hsa-miR-451 (Supplement Figure S2.1C). The top 10 most highly expressed miRNAs in the perirenal fat in both sexes were similar: hsa-miR-1826, hsa-miR-143, hsa-miR-126, hsa-miR-451, hsa-let-7c, hsa-let-7f, hsa-let-7a, mmu-let-7a, hsa-miR-29a, and hsa-miR-933 (see Supplement Figure S2.1C). Seven of the top 10 highly expressed miRNAs (miR-126, -7c, let-7f, let-7a, miR-29a, miR-26b, and let-7d) have known sheep sequences and show complete homology with human and rat miRNAs (Caiment *et al.*, 2010) (Supplement Table S2.9).

2.3.5 Placental restriction alters expression of miRNAs in liver of offspring

Based on the microarray, overall placental restriction in adult sheep offspring increased the hepatic expression of four miRNAs (namely, hsa-miR-600, hsa-miR-212, hsa-miR-144, and hsa-miR-1) by ~1.39-1.84 fold, and reduced that of hsa-miR-30b by 1.26 fold (see Table 2.3). The qPCR confirmed that placental restriction increased hepatic expression of hsa-miR-144 and tended to increase that of hsa-miR-212 gene expression in overall adult offspring (Table 2.4). In males only, placental restriction increased the hepatic expression of eight miRNAs (hsa-miR-142-3p, hsa-miR-134, hsa-miR-600, hsa-miR-1, hsa-miR-21, hsa-miR-597, hsa-miR-144, and hsa-miR-17-5p) by ~1.54-3.50 (Table 2.3); qPCR confirmed this for hsa-miR-1,

hsa-miR-21, hsa-miR-142-3p, and hsa-miR-144 (Table 2.4). The sheep sequences for up-regulated miRNAs are known and show complete homology with human and rat miRNAs (Supplement Table S2.9), with the exception of miR-30b, whose sheep sequence is not yet known.

In adult offspring, ventral liver weight was compared with the hepatic expression of several miRNAs. The expression of hsa-miR-1 positively correlated with ventral liver weight in absolute terms and was relative to body weight in placentally restricted offspring (r = 0.847, $P < 0.001$ & r = 0.721, $P = 0.003$, n = 13, respectively), but not in control offspring (r = 0.076, $P = 0.394$ & r = -0.305, $P = 0.134$, n = 15, respectively) (see Supplement Figures S2.5a and S2.5b). Hepatic hsa-miR-142-3p expression positively correlated with ventral liver weight in placentally restricted offspring (r = 0.723, $P = 0.003$, n = 13), but not in control offspring (r = 0.008, $P = 0.254$, n = 15) (Supplement Figure S2.5c). Finally, hepatic hsa-miR-212 expression negatively correlated with ventral liver weight relative to body weight in both control and placentally restricted offspring (r = -0.620, $P = 0.007$, n = 15 & r = -0.545, $P = 0.027$, n = 13, respectively) (Supplement Figure S2.5d).

In adult male offspring, ventral liver weight was compared with the hepatic expression of several miRNAs. The hepatic hsa-miR-21 expression correlated negatively with ventral liver weight in absolute terms and was relative to body weight in control male offspring (r = -0.680, $P = 0.032$ & r = -0.680, $P = 0.032$, n = 8), but did not correlate with the above factors in placentally restricted male offspring (r = 0.284, $P = 0.322$ & r = 0.120, $P = 0.424$, n = 5, respectively) (Supplement Figures S2.5e and S2.5f). Hepatic hsa-miR-17-5p expression correlated negatively with ventral liver

weight and was relative to body weight in control male offspring (r = -0.679, *P* = 0.032 & r = -0.652, *P* = 0.040, n = 8, respectively), but did not correlate in placentally restricted male offspring (Supplement Figures S2.5g and S2.5h).

2.3.6 Predicted targets of differentially expressed hepatic miRNAs following placental restriction in adult sheep

In general, most of the differentially expressed miRNAs in the liver of placentally restricted adult offspring are predicted to target similar pathways and functions; namely, lipid metabolism, small molecule biochemistry, carbohydrate metabolism, and cell cycle or growth and proliferation, as well as some targeting insulin signalling and action (see Table 2.6 and Figure 2.4).

Hsa-miR-142-3p was predicted to target 291 molecules (Supplement Table 2.6) and the IPA analysis identified a network based on these targets; this network is involved in lipid metabolism and hepatic cellular growth and proliferation (Table 2.6 and Figure 2.4A). It is possible that hsa-miR-142-3p could indirectly regulate *Irs1* and *Gsk3b* expression, as each of these interacts with molecules (*Foxo1* and *Sgk1*, respectively) predicted to be directly regulated by hsa-miR-142-3p (Table 2.5).

Hsa-miR-1 was predicted to target 244 molecules (Supplement Table 2.6) and the IPA analysis identified a network based on these targets; this network plays a role in lipid metabolism, small molecule biochemistry and carbohydrate metabolism (Table 2.6 and Figure 2.4B). Hsa-miR-1 is predicted to regulate *Igf1* expression (Table 2.5). Placental restriction reduced the hepatic *Igf1* gene (Fold change: -3.99, *P* < 0.05) expression, predominantly among placentally restricted male offspring (Figure 2.2).

Moreover, the hepatic *Igf1* and hsa-miR-1 expression were negatively correlated in adult male offspring (r = -0.560, *P*-value = 0.023).

Hsa-miR-21 was predicted to target 408 molecules (Supplement Table S2.6) and the IPA analysis identified a network based on these targets; this network is involved in lipid metabolism, small molecule biochemistry and carbohydrate metabolism (Table 2.6 and Figure 2.4C). Hsa-miR-21 is predicted to regulate *p85α* expression (Table 2.5); however, placental restriction did not alter *p85α* mRNA expression (Figure 2.2). Correlation was not found between hepatic hsa-miR-21 and *p85α* expressions, not between control or placentally restricted offspring, nor in males or females.

Hsa-miR-144 was predicted to target 262 molecules (Supplement Table S2.6) and the IPA analysis identified a network based on these predicted targets; this network is involved in gene expression lipid metabolism and the cell cycle (Table 2.6 and Figure 2.4D). Some of the predicted targets of hsa-miR-144 include *Pparα* and *Foxo1* (Table 2.5). Placental restriction in male offspring tended to reduce hepatic *Pparα* expression (Fold change = -2.28, *P* = 0.068, Figure 2.2). In addition, hepatic expression of hsa-miR-144 and *Pparα* correlated negatively in both placentally restricted male offspring (r = -0.797, *P* = 0.053) and control male offspring (r = -0.696, *P* = 0.036).

The remaining differentially expressed miRNAs (hsa-miR-134, hsa-miR-600, hsa-miR-212 and hsa-miR-30b) in the liver of placentally restricted offspring were predicted to target 221, 164, 156 and 175 molecules, respectively (Supplement Table

S2.6). However, IPA analysis did not identify any networks based on these predicted targets.

The above eight miRNAs (hsa-miR-142-3p, hsa-miR-134, hsa-miR-600, hsa-miR-1, hsa-miR-21, hsa-miR-597, hsa-miR-144, and hsa-miR-17-5p) did not share any common predicted targets. However, IPA analysis of their combined predicted targets identified seven networks, which are mainly involved in lipid metabolism, molecular transport and small molecule biochemistry (Supplement Table S2.5 and Figure S2.4).

A subset of these differentially expressed miRNAs (the differential expression following placental restriction confirmed by qPCR) in the liver of adult male offspring – hsa-miR-142-3p, hsa-miR-1, hsa-miR-21, and hsa-miR-144 – was predicted to regulate 99 common targets (Supplement Table S2.3). Only one network was generated based on the IPA analysis of these 99 common targets (Figure 2.5); this network is involved in hepatic system disease, inflammatory disease and the development of liver hepatitis (Score = 4, focus molecule = 2). The network consists of only three molecules, which are *Acox1*, *Pparα* and *Adipor2* (Figure 2.5). *Acox1* and *Pparα* are directly predicted to be targeted by four up-regulated miRNAs following placental restriction – hsa-miR-1, hsa-miR-21, hsa-miR-142-3p, and hsa-miR-144 (see Supplement Table S2.5). Correspondingly, placental restriction in males reduced the *Acox1* mRNA expression (fold change: -2.64, $P < 0.05$), tended to reduce the *Pparα* mRNA expression (fold change: -2.28, $P = 0.068$) (see Figure 2.2), and did not alter the *Adipor2* mRNA expression in the liver (Figure 2.2).

Overall, in adult offspring, hepatic expressions *Adipor2, Acox1* and *Pparα* correlated with each other (summarised in Figure 2.5). Similar correlations between hepatic expressions of these molecules in the network were observed in both male and female offspring (summarised in Figure 2.5). However, it was only among male offspring that this study observed linear negative correlations between *Pparα* and *Acox1* gene expressions and that of hsa-miR-144, hsa-miR-1 and hsa-miR-142-3p in the liver (summarised in Figure 2.6). This is consistent with the latter's direct contribution to the reduced *Pparα* and *Acox1* gene expression seen in male offspring following placental restriction.

In males, the hepatic expression of hsa-miR-21 correlated negatively with fasting plasma FFA in control offspring (r = -0.797, P = 0.029, n = 6), but not in placentally restricted offspring (r = 0.497, P = 0.197, n = 5) (see Supplement Figure S2.6). Other correlations between hepatic miRNAs expression and plasma lipids were not found in females or in overall offspring. Furthermore, correlations between hepatic miRNAs expression and any measures of glucose tolerance or insulin sensitivity of glucose metabolism were not found in control or in placentally restricted offspring.

2.3.7 Placental restriction alters the expression of miRNAs in vastus lateralis of offspring

Based on the microarray results, placental restriction increased the vastus lateralis expression of seven miRNAs in female offspring only (hsa-miR-142-3p, hsa-miR-21, hsa-miR-101, hsa-miR-17-5p, dme-miR-278, hsa-miR-376b, and hsa-miR-324-3p) by ~1.23-2.04 fold (Table 2.3); qPCR confirmed the differential expression of hsa-miR-17-5p and hsa-miR-376b gene (Table 2.4). Placental restriction did not alter any

miRNA expression in vastus lateralis in offspring overall or in males. Five of these up-regulated miRNAs (miR-142-3p, miR-21, miR-376b, miR-324-3p, and miR-101) have known sequences in the sheep, and four of these miRNAs (miR-142-3p, miR-21, miR-376b, and miR-324-3p) show complete homology with human and rat miRNAs (Supplement Table S2.9), whereas miR-101 did not display any homology.

2.3.8 Predicted targets of differentially expressed vastus lateralis miRNAs following placental restriction in adult female sheep

The differentially expressed miRNAs in the vastus lateralis of placentally restricted offspring (hsa-miR-142-3p, hsa-miR-21, hsa-miR-101, hsa-miR-17-5p, dme-miR-278, hsa-miR-376b, and hsa-miR-324-3p) were predicted to target 241, 336, 243, 209, 199, 37 and 338 molecules, respectively (Supplement Table S2.7). However, the IPA analysis did not identify any networks based on these predicted targets.

The above differentially expressed miRNAs did not share any common predicted targets. However, upon combining their predicted targets, the IPA predicted these miRNAs to regulate one network, which is involved in lipid metabolism, molecular transport and small molecule biochemistry (see Supplement Table 2.4 and Figure S2.4).

The two miRNAs hsa-miR-17-5p and hsa-miR-376b, whose differential expression in vastus lateralis of adult female offspring was confirmed by qPCR, were predicted to target 566 and 312 molecules, respectively (Supplement Table 2.7). However, in placentally restricted female offspring, negative linear correlations were observed between *Pparα* and hsa-miR-376b (r = -0.617, *P*-value: 0.052) (Figure 2.7A), and

between *Pparα* and hsa-miR-17-5p (r = -0.533, *P*-value: 0.087) (see Figure 2.7B). No correlations were observed between expressions of hsa-miR-21 and with *Pparα* in any of the offspring.

2.3.9 Placental restriction altered expression of miRNAs in the perirenal fat of offspring and its predicted targets

Based on the microarray results, placental restriction increased the perirenal fat expression of hsa-miR-451 in female offspring only by 2.20 fold, although this was not confirmed by qPCR (see Tables 2.3 and 2.4). The sequence of miR-451 in the sheep is not yet known. Placental restriction did not alter the expression of any miRNAs in perirenal fat overall or in male offspring.

Hsa-miR-451 is predicted to target 44 molecules (Supplement Table 2.8). The IPA analysis suggested that hsa-miR-451 targets a network involved in lipid metabolism, molecular transport, and small molecule biochemistry in adipose tissue (Table 2.7 and Figure 2.8). There was no correlation in offspring between perirenal fat expression of hsa-miR-451 and circulating plasma lipids. The perirenal fat expression of hsa-miR-451 correlated positively with insulin sensitivity of FFAs in control offspring (r = 0.687, *P* = 0.020, n = 11), but not in placentally restricted offspring (Supplement Figure S2.7).

2.4 Discussion

The present study pioneers the finding that placental restriction alters miRNA expression in key insulin-sensitive and glucoregulatory tissues of adult offspring, in a specific sex and tissue pattern. The liver, followed by skeletal muscle and adipose tissue, displays the greatest susceptibility in terms of altered miRNA expression following placental restriction. In these tissues, placental restriction up-regulated miRNA expression, suggesting increased repression of the translation or abundance of their molecular targets and related functions, including insulin signalling and action. Thus, the predicted molecular targets of these differentially expressed miRNAs in insulin-sensitive tissues of adult offspring following placental restriction included insulin-signalling molecules, such as *Irs1* and *p85*α. Their predicted targeted networks comprise additional insulin-signalling and related molecules, such as glucose transporters (Tables 2.6 and 2.7), as well as network functions, such as carbohydrate metabolism (Table 2.6).

It is worthy of note that, following placental restriction, the predicted molecular targets and networks of differentially expressed miRNAs in insulin-sensitive tissues have other functions in common, which are known to indirectly affect insulin signalling and metabolic homeostasis, including lipid metabolism, small molecule biochemistry, and carbohydrate metabolism. The present study also shows that placental restriction alters expression of some of the key predicted direct and indirect molecular targets of differentially expressed miRNAs, particularly in liver. Further evidence that placental restriction alters miRNAs and their regulatory networks is in the significant associations between the expression of miRNAs and some of their key targets. In the present study's subset of animals, placental restriction reduced size at birth, but did

not alter insulin secretion, insulin action of glucose, or the lipid status of adult offspring. Here, thinness at birth (in terms of ponderal index) was associated with increased fasting blood glucose in adult offspring and elevated fasting plasma FFA in male adult offspring; this indicates the possible emergence of associated impaired metabolic control after IUGR. Nevertheless, the alterations in abundance of miRNAs and their targets in insulin-sensitive tissues appear to be unlikely secondary consequences of impaired metabolic homeostasis. Together, these outcomes support a significant role that miRNAs and their networks play in mediating part of the early impact of placental restriction on insulin action and metabolism in older offspring. Additionally, in the tissue affected the most, the liver, placental restriction up-regulates miRNAs, affecting networks and functions that modulate susceptibility to developing non-alcoholic fatty liver disease (NAFLD). This suggests a role in the increased risk of NAFLD observed among IUGR children and adolescents, particularly with regards to obesity (Alisi *et al.*, 2011a).

In the present study, the expression of miRNAs in major insulin target tissues (liver, skeletal muscle and adipose tissues) in sheep was characterised using probes to miRNAs which are completely or extensively conserved across many mammalian species. In 2009, only four *Ovis aries* miRNAs were listed in the miRBase (Liu et al 2009); currently, 55 miRNAs are listed in the miRBase. In addition, a further 31 *Ovis aries* miRNAs have recently been identified and sequenced, with some miRNAs reported to be the most abundant miRNAs in the skeletal muscle and liver in other species (discussed below) (Sheng *et al.*, 2011). Supplement Table S9 lists miRNAs that were among the top 10 most abundant in insulin-sensitive tissues or that were differentially expressed following placental restriction, and whose sequences are now

known for the sheep (Caiment *et al.*, 2010). Many, although not all, of the alterations in miRNA expression in various sheep tissues detected here by array, were also detected by qPCR. For some miRNAs, qPCR was not accurate, compared with miRNA microarray or direct sequencing; while, for others, apparent differences may reflect interference by other miRNAs or RNA species (Ach *et al.*, 2008; Git *et al.*, 2010). Quantitation of miRNAs poses particular challenges, which include their short length, lack of common sequence (such as a poly(A) tail), constituting only a small percentage of the total RNA species present, the generation of diverse forms (including precursor miRNAs), and variations in sequence length and post-transcriptional modifications (Pritchard *et al.*, 2012). Such differences could be resolved by direct sequencing and analysis of protein expression.

For the most abundant miRNAs in liver and vastus lateralis, all miRNAs with known sheep sequence were identical to both humans and rats. For the most abundant miRNAs in adipose tissue, seven out of nine miRNAs with known sheep sequences were identical to both humans and rats; two miRNAs with known sheep sequences, miR-143 and miR-101, were not identical to both humans and rats (Supplement Table S2.9). For the differentially expressed miRNAs with known sheep sequences, all miRNAs in both the liver and vastus lateralis had sequences identical to those in humans and rats, except for miR-101 (Supplement Table S2.9).

The present study shows that several of the most highly expressed miRNAs in the liver, skeletal muscle, and adipose tissue are consistent with those reported in other species. Based on microarray, hsa-miR-122a was one of the highly expressed miRNAs in the liver (Supplement Figure S2.1). MiR-122a/miR-122 has been reported

as the most abundant hepatic miRNA in humans (Girard *et al.*, 2008), rodents (Lynn, 2009), and recently in sheep (Sheng *et al.*, 2011); the sequence was the same across these species. MiR-122 has been shown to regulate networks that are involved in lipid metabolism, cell differentiation and hepatic circadian regulation and reduced hepatic miR-122 expression occurs in subjects with NAFLD, liver cirrhosis and hepatocellular carcinoma (Tsai *et al.*, 2012).

As detected by microarray, hsa-miR-133a, hsa-miR-206, and hsa-miR-1 were among the top 10 most abundant miRNAs in the vastus lateralis (Supplement Figure S2.1). For these three miRNAs, the sequences were similar in both humans and rodents. These miRNAs have previously been reported to be highly expressed in skeletal muscle in the sheep (Sheng *et al.*, 2011) and the mouse (Chen *et al.*, 2006; McCarthy, 2008). Inhibition of expression of these three miRNAs reduces cell differentiation of C2C12 myoblast *in vitro* by down-regulating the p180 subunit of DNA polymerase expression, which is involved in DNA synthesis (Kim *et al.*, 2006; Goljanek-Whysall *et al.*, 2012). Importantly, these three miRNAs form an important regulatory network with YY1 in skeletal myogenesis in the mouse (Lu *et al.*, 2012).

In adipose tissue, hsa-miR-143, hsa-miR-126 and hsa-miR-29a were amongst the most abundant miRNAs observed in the sheep (Supplement Figure S2.1). The sequences for miR-126 and miR-29a in the sheep are identical to those in humans and rats, but not for miR-143 (Supplement Table S2.9). MiR-126 has been suggested as an important regulator of inflammation in the white adipose tissue of obese humans (Arner *et al.*, 2012). Thus, miR-126 controls the release of chemokine ligand 2 from human adipocytes and macrophages, *in vitro* (Arner *et al.*, 2012). Expression

of miR-29a has been shown to be up-regulated in 3T3-L1 adipocyte, upon exposure to hyperglycaemia which suggests that altered miRNAs expression is related to the development of type 2 diabetes (Herrera *et al.*, 2010). MiR-143 has also been shown to be highly expressed in, and to be an important regulator of, adipocyte differentiation in both humans (Esau *et al.*, 2004) and mice (Xie *et al.*, 2009).

A few of the most highly expressed miRNAs in the examined sheep tissues have not been previously reported as the most abundant in these tissues, either in sheep or other species. Here, this study found that hsa-miR-363* and sbi-miR-164c are the most highly expressed miRNAs in sheep liver and skeletal muscle. Hsa-miR-363* belongs to the miRNA* star strand class, which can work with its partner guide strand in a synergic manner to help mediate regulatory activity in various settings (Ro *et al.*, 2007; Okamura *et al.*, 2008; Yang *et al.*, 2011). However, currently there is no known role of either hsa-miR-363* or hsa-miR-363 in any tissue in any species. Sbi-miR-164c is a miRNA belonging to *Sorghum bicolor,* a grass species; it is found only in plants, suggesting that it may represent detection of another novel miRNA in the sheep. In the current study, hsa-miR-1826 was the most highly expressed miRNA in the sheep perirenal fat; however, miRBase recently classified this miRNA as a fragment of 5.8S rRNA, which is not a miRNA.

In terms of miRNA expression, the liver was the tissue most affected by placental restriction. Here, most of the differentially expressed miRNAs either target or are predicted to target insulin signalling or related molecules directly, as well as pathways and functions able to indirectly influence insulin action. Based on microarray, placental restriction in adult offspring overall altered hepatic expression

of five miRNAs, with four (hsa-miR-600, hsa-miR-212, hsa-miR-144, and hsa-miR-1) increasing by 40% to 84%, and one (hsa-mir-30b) reducing by 26% (see Table 2.3). MiR-144 and miR-1 have known sheep sequences, which are homologous to those in humans and rats. MiR-30b expression in the liver has been shown to be up-regulated in a rodent model of liver repair (Chaveles *et al.*, 2012), and in hepatocyte cell lines in response to polyphenols, which have beneficial effects on metabolic disorders (Arola-Arnal & Blade, 2011). Therefore, down-regulation of mir-30b following placental restriction suggests increased vulnerability to damage and metabolic dysfunction in adult offspring following placental restriction.

In regards to other miRNAs that were up-regulated in the liver of adult offspring, little is known of the actions of miR-600, whereas miR-212 has actions in other gastrointestinal tissues. However, a role in the liver has not been described, while up-regulation of miR-1 could protect against hepatic carcinogenesis by acting through endothelin-1 (Li *et al.*, 2012). MiR-144 was predicted to target IRS1, and its expression is up-regulated in the liver and other insulin sensitive tissues in a rodent model of type 2 diabetes, as well as in peripheral blood cells in human type 2 diabetes (Karolina *et al.*, 2011). The latter suggests that placental restriction may increase susceptibility to hepatic insulin resistance in adult sheep offspring, partly by up-regulation of hepatic miR-144 expression, as well as by other pathways discussed below. However, this current study did not find IRS1 as one of the predicted targets of hsa-miR-144 in the liver, therefore the expression of *Irs1* was not analysed further.

The present study has shown that *Igf1* gene expression was reduced in the liver of placentally restricted male offspring, which is a consistent finding in offspring

following placental restriction (Duffield *et al.*, 2008; Gentili *et al.*, 2009). In human IUGR, *Igf1* expression in the blood normally increased during early age for catch-up growth and reduced in adults as catch-up growth ceased (Kyriakakou *et al.*, 2009). Hsa-miR-1 was predicted to regulate *Igf1* gene expression; here, however, hepatic hsa-miR-1 and *Igf1* expression did not correlate in any offspring. This indicates that other factors may contribute to altered hepatic *Igf1* expression following placental restriction, including altered growth hormone axis activity, which varies in sheep in a sex specific manner (Gatford *et al.*, 2004; De Blasio *et al.*, 2007a; De Blasio *et al.*, 2010), or other epigenetic mechanisms, such as the histone code (Sanchez *et al.*, 2009).

Adult male offspring were particularly affected, as placental restriction up-regulates the hepatic expression of eight miRNAs (hsa-miR-142-3p, hsa-miR-134, hsa-miR-600, hsa-miR-1, hsa-miR-21, hsa-miR-597, hsa-miR-144, and hsa-miR-17-5p), while none were affected in the adult female offspring (Table 2.3). While these miRNAs did not share any common predicted targets, their collective targets were part of eight networks involved in similar functions and pathways, including lipid metabolism, molecular transport, small molecule biochemistry, as well as cell signalling, growth, proliferation and death (Table 2.6).

qPCR confirmed the increased up-regulation of hepatic expressions of four miRNAs (hsa-miR-142-3p, hsa-miR-1, hsa-miR-21, and hsa-miR-144). Consistently, expressions of three of these (hsa-miR-144, hsa-miR-1, and hsa-miR-142-3p) negatively correlated with *Acox1* gene expression in the liver of adult male offspring (Figure 2.6). *Acox1* gene expression is regulated by *Adipor2* and *Pparα* (Figure 2.5).

Placental restriction reduced both *Acox1* and *Pparα* (but not *Adipor2)* expression in the liver of adult male offspring (Figure 2.2). No correlations were found between expressions of these miRNAs and *Acox1* in adult females or combined offspring. Hepatic expression of *Acox1, Pparα* and *Adipor2* correlated with each other in both adult females and combined offspring (Figure 2.5). However, placental restriction reduced *Acox1* mRNA expression only in combined offspring, not in adult females, and did not alter either *Pparα* or *Adipor2* expression in these offspring (Figure 2.2). This suggests that miRNAs and *Pparα* are mainly responsible for the down-regulation of *Acox1* expression in the liver of males.

Impaired PPAR-alpha expression, results in a reduced expression of *Acox1,* which leads to an accumulation of very long fatty acids in the liver of Shionogi mice (Harano *et al.*, 2006). IUGR is now recognised as a major risk factor for the development of NAFLD in children (Alisi *et al.*, 2011c). NAFLD occurs when fat is deposited in the liver, not due to excessive consumption of alcohol, and it is related to the metabolic syndrome and insulin resistance (Clark & Diehl, 2003; Diehl *et al.*, 2003). Diet-induced NAFLD in the rat is associated with altered hepatic miRNA expression, including that of miRNAs targeting the lipid metabolism pathway (Alisi *et al.*, 2011b). However, none of the miRNAs reported previously are changed in the sheep liver following placental restriction. Therefore, this study proposes that placental restriction in sheep is able to reduce *Acox1* gene expression in the liver via altered expression and actions of miRNA networks, which make the male offspring particularly susceptible to NAFLD.

Mir-21, which was up-regulated in liver of placentally restricted adult male offspring, repressed PPARα protein level in human HuH7 hepatocyte cell lines (Kida *et al.*, 2011). PPARα is a transcription factor which regulates lipid metabolism in the liver (Kersten *et al.*, 1999). Increased hepatic expression of hsa-miR-21 in placentally restricted adult male offspring would reduce the removal of fatty acids from the liver, which may lead to the development of NAFLD (Alisi *et al.*, 2011a).

In the vastus lateralis of placentally restricted offspring, microarray showed that only seven miRNAs (hsa-miR-142-3p, hsa-miR-21, hsa-miR-101, hsa-miR-17-5p, dme-miR-278, hsa-miR-376b, and hsa-miR-324-3p) were up-regulated in female offspring. However, only two were confirmed by qPCR to be significantly up-regulated (hsa-miR-17-5p, and hsa-miR-376b), while another two miRNAs (hsa-miR-21 and hsa-miR-324-3p) showed a trend for up-regulation ($P < 0.10$). For miR-21, miR-324-3p and miR-376b, the sequences are now known in the sheep and share similar homology to humans and rats; however, the miR-17-5p sequence in the sheep is not yet known (Supplement Table S2.9). Although *Pparα* and *p85α* expression were predicted to be regulated by a couple of differentially expressed miRNAs, the present study did not find that placental restriction altered their gene expression. The current study found a trend for negative correlation between hsa-miR-17-5p and *Pparα* expression only in placentally restricted female offspring (r = -0.533, *P*-value: 0.087) (see Figure 2.7B). Although hsa-miR-376b was not predicted to target *Pparα* expression, this study also found a negative correlation trend between hsa-miR-376b and *Pparα* expression only in placentally restricted female offspring (r = -0.617, *P*-value = 0.052) (see Figure 2.7A). Moreover, the current study did not find any correlation between hsa-miR-376b and hsa-miR-17-5p with *p85α* expression in

vastus lateralis. However, these up-regulated miRNAs would affect at the protein expression level, as miRNAs are known to affect translation (Bushati & Cohen, 2007).

In the muscle, only the role of hsa-miR-21 was experimentally verified, and was found to regulate PPARα protein expression in the human smooth muscle cell line (Sarkar *et al.*, 2010). *In vitro* alterations of hsa-miR-21 expression resulted in lead to the opposite expression of the PPARα protein (Sarkar *et al.*, 2010). However, the current study did not find any correlation between the expression of hsa-miR-21 and *Pparα* expression in the vastus lateralis of female offspring. Further studies are required to examine whether placental restriction alters PPARα protein expression in the skeletal muscle of these offspring.

Placental restriction increased hsa-miR-451 expression in the perirenal fat of female offspring. Currently, there is no known role of hsa-miR-451 in adipose tissue. However, the current study found positive correlations between perirenal fat hsa-miR-451 and insulin sensitivity of FFAs in control offspring only ($r = 0.687$, $P = 0.020$, $n = 11$). This suggests a novel role of hsa-miR-451 in regulating insulin sensitivity in relation to circulating FFAs.

Placental restriction in offspring has been closely associated with the development of type 2 diabetes later in life, caused either by deficiency in insulin-sensitive tissues or by lower insulin production (Simmons *et al.*, 2001; Oak *et al.*, 2006; Simmons, 2007a; Germani *et al.*, 2008). A recent review postulates that pancreatic, skeletal muscle, hepatic, and adipose miRNAs play significant roles in the development of types 1 and

2 diabetes among rat and mice (Guay *et al.*, 2011). However, the present study did not detect any of the listed miRNAs to be differentially expressed. Although miRNA sequences are thought to be conserved throughout mammalian species (Piriyapongsa *et al.*, 2007), the expression between species would be affected by numerous factors; such as: location on the genomes, expression of the host genes and other effects of cis- or trans-regulatory elements (Hon & Zhang, 2007; Wang *et al.*, 2008b). Nevertheless, an awareness of the role of miRNA in different species would provide a novel understanding or treatment of types 1 or 2 diabetes in humans.

There are several candidate mechanisms for ways that miRNAs expressions are altered in various tissues following placental restriction. Some of the miRNA genes are encoded in the intron of the host genes; therefore, these miRNAs expressions are subjected to the same regulation as the host genes, including regulation by hormones and transcription factors. IUGR in humans reduced the thyroid hormone of umbilical cord blood and the cortisol hormone in the amniotic fluid (Nieto-Diaz *et al.*, 1996). Placental restriction in sheep reduced the plasma thyroid hormone and was associated with postnatal growth among young offspring (De Blasio *et al.*, 2006). Therefore, alteration of these circulating hormones could alter the expression of various genes, including miRNAs. The expression of transcription factors can be altered through placental restriction. IUGR in pigs reduced hepatic *Pgc1α* expression, a transcription co-activator, in the liver of piglets (Liu *et al.*, 2012). This transcription co-activator regulates the genes involved in cellular cholesterol homeostasis and the development of type 2 diabetes (Ek *et al.*, 2001). Expression of *Pgc1α* was altered in the skeletal muscle of placentally restricted fetal and juvenile rat offspring; this was

speculated to contribute to these offspring's development of insulin resistance (Lane *et al.*, 2003).

The epigenetic mechanism is another possible mechanism that alters the miRNAs expression (Chuang & Jones, 2007). The presence of CpG islands has been found upstream from the hsa-miR-1 gene in colorectal carcinoma cell lines; hypermethylation at these sites reduces hsa-miR-1 expression. Of note, three miRNAs (hsa-miR-142-3p, hsa-miR-21, and hsa-miR-17-5p) were differentially expressed in both the liver and skeletal muscle following placental restriction, albeit in males for the liver and in females for skeletal muscle (Table 2.3). This suggests that they may be particularly susceptible to deregulation by placental restriction, possibly via epigenetic modification or altered activity of regulators operating in both of the above tissues. Mir-142 and its alternate forms, including miR-142-3p, form a mutually inhibitory regulatory loop with p300 in the cardiac muscle in the mouse (Sharma *et al.*, 2012). The miR-21/STAT3 pathway is well established, with STAT3 directly activating miR-21 (Kim *et al.*, 2012). Of interest, STAT3 in immune cells is regulated by miR-17-5p together with other miR-17 cluster family members. At the same time, STAT3 binds to the miR-17-92 cluster promoter and facilitates expression in a variety of cell types, establishing the potential for positive feed-forward loops (Kohanbash & Okada, 2012). However, it has not yet been examined whether these upstream regulators of commonly deregulated miRNAs are also altered in adults following IUGR.

2.5 Conclusion

This study has reported that an early insult, such as placental restriction, enables up-regulation of miRNAs expression in insulin-sensitive tissues (liver, skeletal muscle, and adipose) later in adult sheep; some of the expressions varied with the sex of the offspring. Bioinformatic analyses showed that some of these miRNAs are predicted to target insulin-signalling molecules, both directly and indirectly. The present study also identified hsa-miR-144, hsa-miR-1, and hsa-miR-142-3p as potential regulators of the *Acox1* gene expression in the liver, specifically among male offspring. The down-regulation of the *Acox1* expression may contribute to development of NAFLD in placentally restricted offspring. Furthermore, placental restriction up-regulates expression of hsa-miR-17-5p in the vastus lateralis of female offspring, and correlates negatively with *Pparα* expression, one of its predicted targets. Placental restriction also up-regulates the expression of hsa-miR-451 in the perirenal fat in female offspring, and correlates positively with the insulin sensitivity of FFAs in control offspring. This suggests that hsa-miR-451 may be important in regulating insulin sensitivity relative to circulating FFAs.

Overall, placental restriction up-regulates miRNAs expression in insulin-sensitive tissues (particularly in the liver) concomitant with the onset of hepatic insulin resistance; moreover, it is consistent with the miRNA networks' early role in programming these phenotype aspects in the offspring.

2.6 Tables and Figures

Table 2.1. Effect of placental restriction and sex on size at birth and adult weight and body composition

	Male		Female		*P*-value (ANOVA)		
	Control (n = 8)	PR (n = 6)	Control (n = 8)	PR (n =8)	PR	S	PR X S
Birth							
Weight (kg)	5.66 ± 0.37	4.01 ± 0.32	5.19 ± 0.14	3.03 ± 0.24	<0.001	0.016	ns
Body mass index (kg/m^2)	16.05 ± 0.74	15.7 ± 0.74	17.12 ± 0.72	13.49 ± 0.72	0.006	ns	0.068[1]
Ponderal index (kg/m^3)	28.30 ± 1.22	31.25 ± 1.34	31.25 ± 1.88	28.70 ± 1.53	ns	ns	0.084[2]
Adult (18 month)							
Weight (kg)	61.81 ± 1.99	61.28 ± 3.01	54.04 ± 1.82	48.03 ± 2.29	ns	<0.001	ns
Shoulder height (cm)	69.68 ± 0.72	75.60 ± 1.21	68.83 ± 0.80	64.57 ± 2.43	ns	0.017	0.036[3]
Body mass index (kg/m^2)	40.40 ± 0.76	42.61 ± 3.50	38.93 ± 1.27	37.00 ± 1.76	ns	ns	ns
Ponderal index (kg/m^3)	32.34 ± 0.82	34.54 ± 3.87	32.97 ± 1.55	32.29 ± 2.30	ns	ns	ns
Vastus lateralis (g)	233 ± 27	219 ± 15	198 ± 11	212 ± 30	ns	ns	ns
(%BW)	0.38 ± 0.04	0.35 ± 0.02	0.37 ± 0.02	0.45 ± 0.06	ns	ns	ns
Total liver (g)	934 ± 122	1110 ± 27	911 ± 19	839 ± 39	ns	0.046	0.090[4]
(%BW)	1.50 ± 0.19	1.83 ± 0.09	1.70 ± 0.06	1.76 ± 0.08	ns	ns	ns
Liver ventral (g)	309 ± 17	397 ± 41	254 ± 7	237 ± 13	0.094	<0.001	0.015[5]
(%BW)	0.50 ± 0.03	0.66 ± 0.07	0.47 ± 0.02	0.50 ± 0.03	0.027	0.023	ns
Perirenal fat (g)	84 ± 12	100 ± 23	186 ± 34	188 ± 30	ns	0.002	ns
(%BW)	0.26 ± 0.04	0.35 ± 0.12	0.70 ± 0.11	0.73 ± 0.11	ns	<0.001	ns

Values are means ± SEM; n, number of animals; %BW, percentage relative to body weight. * $P < 0.05$, compared with controls. PR: placental restriction (treatment); S: sex. [1]BMI at birth was similar in control and PR males ($P = 0.472$) but smaller in PR females than in control females ($P = 0.004$). [2]PI at birth was similar in control and PR males ($P = 0.132$) and also similar in control and PR females ($P = 0.307$). [3]Shoulder height at 18 month of age was greater in PR males than in control males ($P = 0.012$) but similar in control and PR females ($P = 0.235$). [4]Total liver weight was similar in control and PR males ($P = 0.244$) and also similar in control and PR females ($P = 0.114$). [5]Ventral liver weight was greater in PR males than in control males ($P = 0.050$) but similar in control and PR females ($P = 0.247$)

Table 2.2. Effect of placental restriction on glucose tolerance, insulin secretion, insulin sensitivity and plasma lipid in adult sheep offspring

	Male		Female		*P* value (ANOVA)		
	Control (n = 8)	PR (n = 6)	Control (n = 8)	PR (n = 8)	PR	S	PR X S
Fasting blood glucose (mmol/l)	3.88 ± 0.19	3.86 ± 0.13	3.69 ± 0.13	3.40 ± 0.09	ns	0.033	ns
Fasting plasma insulin (mU/l)	2.26 ± 0.32	2.07 ± 0.25	1.79 ± 0.08	2.18 ± 0.22	ns	ns	ns
Insulin sensitivity (glucose) mg.l.mU^{-1}.kg^{-1}.min^{-1}	0.049 ± 0.010	0.043 ± 0.001	0.045 ± 0.011	0.061 ± 0.010	ns	ns	ns
Insulin sensitivity (FFAs)							
Absolute change (meq/mU)	-0.007 ± 0.002	-0.007 ± 0.001	-0.008 ± 0.001	-1.015 ± 0.111	ns	ns	ns
% change	-1.07 ± 0.16	-1.00 ± 0.10	-1.02 ± 0.11	-1.01 ± 0.16	ns	ns	ns
Plasma cholesterol (mmol/l)	0.92 ± 0.07	0.95 ± 0.08	1.19 ± 0.16	0.78 ± 0.07	0.100	ns	0.068*
Plasma triglycerides (mmol/l)	0.18 ± 0.03	0.23 ± 0.02	0.18 ± 0.03	0.20 ± 0.03	ns	ns	ns
Fasting plasma FFA (meq/l)	0.60 ± 0.09	0.66 ± 0.05	0.80 ± 0.06	0.62 ± 0.06	ns	ns	ns

Values are means ± SEM; n, number of animals; PR: placental restriction (treatment); S: sex; ns: not significant. *Fed plasma cholesterol concentrations were similar in control and PR males (*P* = 0.826) but tended to be higher in control females than in PR females (*P* = 0.091).

Table 2.3. Placental restriction alters expression of microRNAs in liver, vastus lateralis and perirenal fat tissue in adult sheep offspring

Liver

Sex	miRNAs	Fold Change	qPCR		Accession No.
			Fold Change	*P*-value	
Male	**hsa-miR-142-3p**	3.50	3.02	0.033	MIMAT0000434
	hsa-miR-134	1.65		ns	MIMAT0000447
	hsa-miR-600	2.10		nd	MIMAT0003268
	hsa-miR-1	1.88	2.77	0.040	MIMAT0000416
	hsa-miR-21	2.22	2.22	0.046	MIMAT0000076
	hsa-miR-597	2.41		nd	MIMAT0003265
	hsa-miR-144	2.74	2.47	0.035	MIMAT0000436
	hsa-miR-17-5p	1.54		ns	MIMAT0000070
Female	**-**				
Combined	**hsa-miR-600**	1.68		nd	MIMAT0003268
	hsa-miR-212	1.65	1.61	ns	MIMAT0000269
	hsa-miR-144	1.84	2.27	0.027	MIMAT0000436
	hsa-miR-1	1.39		0.067	MIMAT0000416
	hsa-miR-30b	-1.26		ns	MIMAT0000420

Vastus lateralis

Male	**-**				
Female	**hsa-miR-142-3p**	2.04		ns	MIMAT0000434
	hsa-miR-21	1.86	1.80	0.062	MIMAT0000076
	hsa-miR-101	1.85		nd	MIMAT0000099
	hsa-miR-17-5p	1.37	1.90	0.006	MIMAT0000070
	dme-miR-278	1.60		nd	MIMAT0001511
	hsa-miR-376b	1.44	2.38	<0.001	MIMAT0002172
	hsa-miR-324-3p	1.23	1.47	0.086	MIMAT0000761
Combined	**-**				

Perirenal Fat

Male	**-**				
Female	**hsa-miR-451**	2.20		ns	MIMAT0001631
Combined	**hsa-SNORD118**	-1.33		nd	-

MiRNAs significantly differentially expressed determined by LIMMA analysis (in bold, adjusted $P < 0.05$; non-bold: unadjusted $P < 0.000651$ and 0.000560 for liver and fat respectively). MiRNAs are listed with their miRBase accession numbers and their human chromosomal location. hsa: *Homo sapiens*; dme: *Drosophila melanogaster*. ns: no significant difference between miRNAs expression of control and placentally restricted offspring (qPCR, two-way ANOVA). nd: not detected by qPCR.

Table 2.4. Quantitative analysis on expression of microRNAs in insulin sensitive tissue in adult sheep offspring following placental restriction

as

Linearised	Male		Female		2-way ANOVA		
(qPCR)	Control (8)	PR (5)	Control (7)	PR (8)	PR	S	PR x S
Liver							
hsa-miR-142-3p	0.419 ± 0.104	0.967 ± 0.147	0.352 ± 0.101	0.324 ± 0.001	0.026	0.003	0.015[1]
hsa-miR-134	0.004 ± 0.002	0.001 ± 0.001	0.001 ± 0.001	0.001 ± 0.001	ns	ns	ns
hsa-miR-1	0.019 ± 0.006	0.039 ± 0.007	0.010 ± 0.003	0.010 ± 0.003	0.067	0.001	0.060[2]
hsa-miR-21	1.14 ± 0.36	2.04 ± 0.33			ns		
hsa-miR-144	0.001 ± 0.001	0.003 ± 0.001			0.031		
hsa-miR-17-5p	0.074 ± 0.012	0.111 ± 0.013			0.061		
Vastus lateralis							
hsa-miR-142-3p			2.59 ± 0.75	3.79 ± 0.58	ns		
hsa-miR-21	8.87 ± 1.13	8.01 ± 1.78	4.07 ± 1.46	6.22 ± 0.93	ns	0.019	ns
hsa-miR-17-5p			0.19 ± 0.04	0.33 ± 0.02	0.005		
hsa-miR-376b			0.002 ± 0.001	0.005 ± 0.001	0.001		
hsa-miR-324-3p			0.103 ± 0.019	0.146 ± 0.016	ns		
Perirenal fat							
hsa-miR-451	1.74 ± 0.56	3.10 ± 1.81	2.45 ± 0.66	3.86 ± 2.45	ns	ns	ns

MiRNAs expression were analysed quantitatively by qPCR. MiRNA expression in the liver and perirenal fat were normalised against average ΔCt of miR-26b and miR-92, where in vastus lateralis, miRNA expressions was normalised against ΔCt of let-7f. [1]Placental restriction reduced hepatic expression of hsa-miR-142-3p in male offspring (P = 0.009), but was similar in both control and placentally restricted offspring (P = 0.884). [2]Placental restriction tended to reduce hepatic expression of hsa-miR-1 in male offspring (P = 0.069), but was similar in both control and placentally restricted offspring (P = 0.945). Data presented as mean ± SEM.

Table 2.5. Predicted targets in insulin signalling and related pathways of miRNAs that are differentially expressed following placental restriction

Tissue	Sex	miRNA	Expression in PR	Targets	
				Direct	Indirect
Liver	Male	miR-142-3p	up	FOXO1	IRS1
				SGK1	GSK3B
		miR-1	up	IGF1	
		miR-21	up	p85α	
				PPARα	
		miR-144	up	PPARα	
				FOXO1	
				mTOR	
Vastus Lateralis	Female	miR-17-5p	up	PPARα	

Direct predicted targets were predicted by at least 3 different algorithms, listed on miRBase. Indirect targets were predicted by IPA, through association of other molecules, for example IRS1 is indirectly regulated by miR-142-3p, by regulating FOXO1 expression.

Table 2.6. IPA networks predicted to be targeted by differentially expressed miRNAs in liver of placentally restricted adult offspring

microRNA	Molecules in Network		Score	Focus Molecules	Top Functions
	Predicted by miRNAs	Associated molecules			
miR-1	CCND1, CLOCK, CREBL2, DDX5, FN1, GCLC, HMGCR, IGF1, MET, RARB	ACADVL, APOA2, APOH, ASL, C5, CYP4A11, CYP4A14, DBI, EHHADH, FADS2, FGB, HGD, HGF, HNF1A, ICAM1, IDH1, LEP, LIPC, MDM2, NCAM1, NR3C1, PAH, PPARA, SERPINA1, TGFB1	11	10	Nutritional Disease, Lipid Metabolism, Small molecule biochemistry
miR-21	ABCD3, ALDH1A1, CREBL2, HNRNPK, NCOA2, PER2, PIK3R1, PPARA, RHOB, STAT3, THRB	ACADVL, ALAS1, APOA2, APOH, ASL, CD36, CSNK1D, CXCL3, CYP4A11, CYP4A14, CYTB, EHHADH, ELF3, G6PC, HNF1A, IGF1, IGF1R, IL1B, IRF1, LEP, lipid, NR1I2, PAH, PER1	10	11	Lipid Metabolism, Small Molecule Biochemistry, Carbohydrate Metabolism
miR-142-3p	ANK3, BACH1, BCL2L1, CLND12, CXADR, FOXO1, GNAQ, HMGA1, NR3C1, RAB11A, TGFBR1, WASL	ADH1C (includes EG:126), AGT, AKT1, APOC3, CYP7A1, DBI, EGF, EGFR, HNF4A, IDH1, IGFALS, IGFBP1, IRS1, JUN, MAPK8, ONECUT1, PPARG, PPARGC1A, RPS6KB1 SPP1, SULT2A1, TAT, UGT1A1	12	12	Cellular Growth and Proliferation, Cancer, Lipid Metabolism
miR-144	ABCA1, ATP2B1, ESRRG, FGA, FOS FOXO1, NFE2L2, NR2F2, PPARA, PTP4A1α, RARB, SEL1L, TRFC	AHR, ASL, ATF2, CEBPA, CPB2, CYP7A1, ELF3, EP300, FGB, GATA6, HNF1A, IL1B, IL1RN, LOC100129193, NCOA2, NQO1, NR0B2, ORM2, PAH, PCK1, SLC17A2, SOCS2	6	13	Gene Expression, Lipid Metabolism, Molecular Transport
miR-597 (Network 1)	ABCA1, ABCB11, CYP1B1, ESRRG, FOXA3, IDI1, INSR, LDLR, PDK2, PIM1, PRKAA1,PRKCE, SCD, SHB	ACLY, AHR, CYB5A, CYP3A4, CYP51A1, CYP8B1, GCK, GPAM, IL1B, IL1RN, INSIG1, INSIG2, lithocholic acid, NR0B2, PCK1, PCSK9, PTPN1, SCD2, SREBF1, TAT, tauroursodeoxycholic acid	13	14	Lipid metabolism, Molecular transport, Small molecule biochemistry
miR-597 (Network 2)	GAB2, HGF, IGF1R, IL10, KITLG, MAPK1, NCAM1, SMAD2, THRB	AGT, AKT1, BCL2L1, COL1A1, EGF, EGFR, GRB2, ICAM1, IGF1, IRS1, IRS2, JUN, MAPK3, MAPK8, MYC, PIK3R1, PPARG,PTPN11,SMAD7,SRC,STAT3,TGFB1,TNF,VCAM1	6	9	Cancer, Cellular Growth and Proliferation, Cellular development
miR-17-5p (Network 1)	CCND1, CDKN1A, KAT2B, KLF9, MECP2, PDGFRA, PTEN, RB1, RBL1, RBL2, SKI, SMAD7, TIMP2, VEGFA, ZBTB7A	acetaldehyde, ACTG2, AR, CDK4, COL1A1, COL1A2, GHR, HDAC1, IGF1, MT1F, MYC, PDGFC, SMAD2, SMAD4, SMARCA2, SOCS3, SP1, SP3, TGFB1, TP53	13	15	Cellular Growth and Proliferation, Gene Expression, Cell cycle
miR-17-5p (Network 2)	ABCA1,ACSL4,CLOCK,CRY2, DDX5,HIFIA,IL25,IRF1,LDLR,PDE3B PPARA, SGMS1, STAT3, YES1	ACADVL, ACSL5, DBI, EHHADH, FADS1, FADS2, GCK, HGD, HMGCS1, IFNG, LEP, LIPC, NR3C1, PCSK9, PIGR, SC4MOL, SC5DL, SCARB1, SCD2, SREBF1	12	14	Lipid metabolism, Small molecule biochemistry, Molecular transport

List of targets of networks that are predicted by IPA to be affected by differentially expressed miRNAs in the liver of placentally restricted offspring. Associated molecules are ones that directly interact with predicted targets. Each network was limited to a maximum of 35 molecules and a score of > 4, indicating that there was a less than 1 in 10,000 chance that the genes were part of a network due to random chance. The functions of the networks listed are based on the IPA database in the liver.

Table 2.7. IPA network predicted to be targeted by differentially expressed miRNAs in adipose of placentally restricted adult offspring

microRNA	Molecules in Network		Score	Focus Molecules	Top Functions
	Predicted by miRNAs	Associated molecules			
miR-451	ACACA, AKT1, CEBPB, GPAM, HK2, IL1B, INSR, LEP, MAPK1, MIF, NAMPT, PPARA, PPARGC1A, PRKCA, RXRA	CASP3, CFD, DLK1, EGF, FN1, FOXC2, glycerol,histamine, HSD11B1, IRS1, LIF, norepinephrine, PRKAR1A , PTPRA, SCD, SLC2A4, SREBF1, UCP1, VEGFA, vitamin A	13	16	Lipid metabolism Molecular transport Small molecule biochemistry

List of targets of networks that are predicted by IPA to be affected by differentially expressed hsa-miR-451 in perirenal fat of placentally restricted offspring. Associated molecules are ones that directly interact with predicted targets. Each network was limited to a maximum of 35 molecules and a score of > 4, indicating that there was a less than 1 in 10,000 chance that the genes were part of a network due to random chance. The functions of the networks listed are based on the IPA database in the adipose tissue.

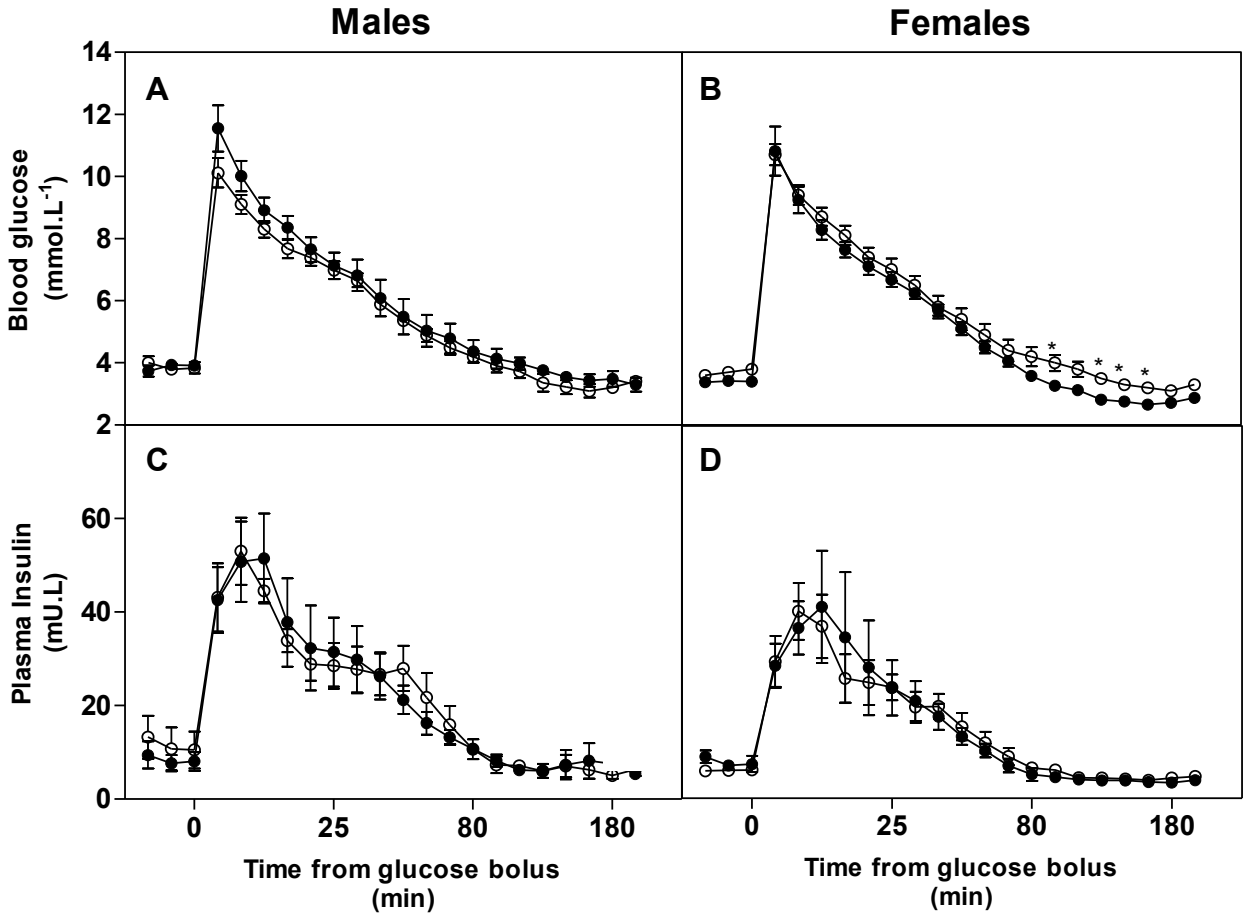


Figure 2.1. The effect of placental restriction on blood glucose and insulin during intravenous glucose tolerance test in adult sheep offspring

Blood glucose (A and B) and plasma insulin (C and D) were measured during IVGTT. A glucose bolus of 0.25 g glucose (25% dextrose)/kg was administered at 0 min. Control: white circle; placentally restricted: black circle. Data are presented as mean ± SEM, determined by addition of time as a repeated measure factor (22 levels) by ANOVA (Effect of treatments: plasma glucose, $P = 0.881$; plasma insulin, $P = 0.736$ *: $P < 0.05$ for control compared with placentally restricted.

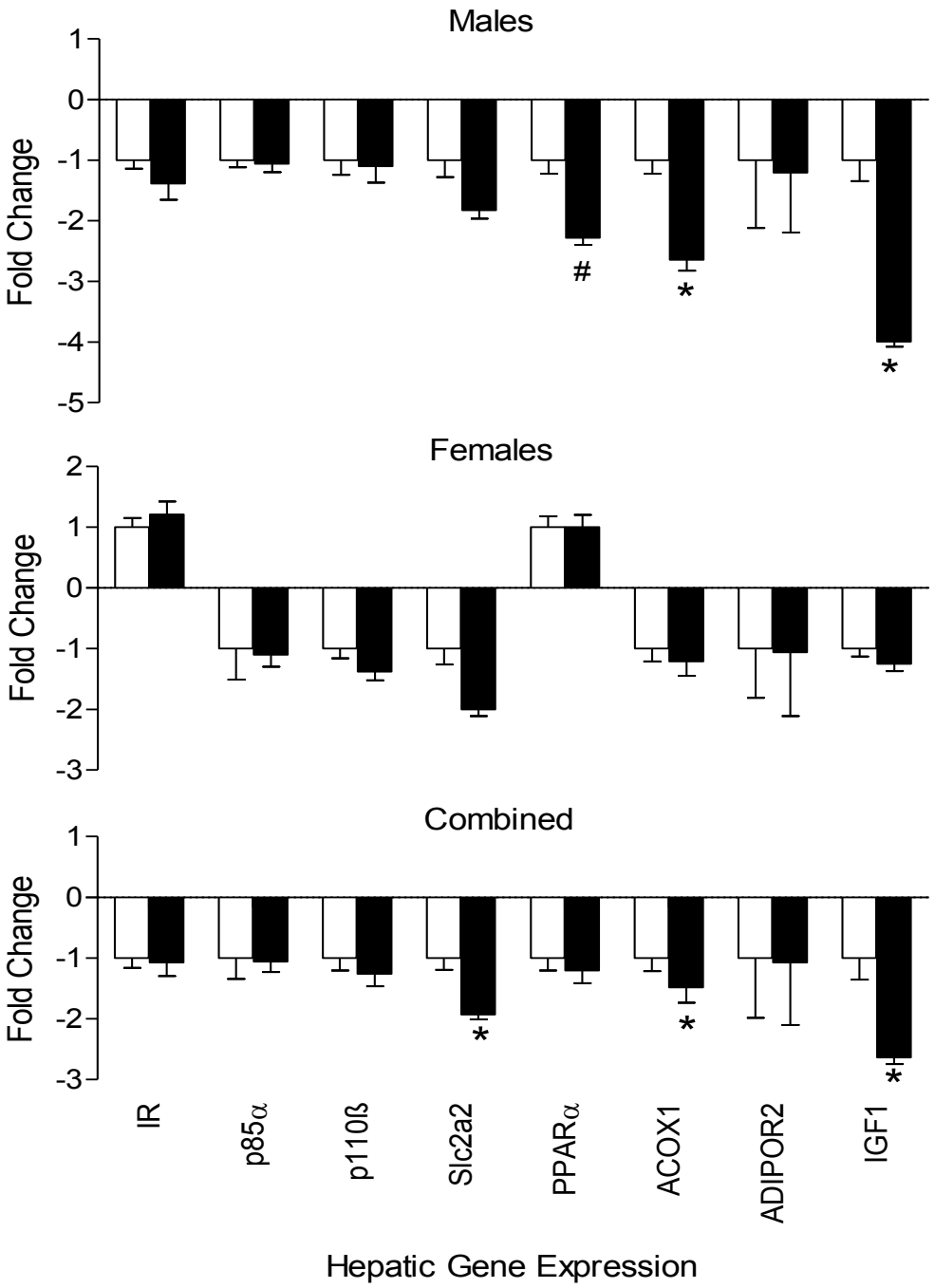


Figure 2.2. Effect of placental restriction on hepatic expression of selected insulin signalling and related genes

Gene expression was normalised against β-actin. Controls shown as white bars, placentally restricted shown as black bars. Data shown as mean ± SEM. *: $P < 0.05$; #: $P < 0.10$ (two-way ANOVA, placental restriction sex and their interaction; placental restriction only, *Slc2a2*: $P = 0.025$, *Acox1:* $P = 0.034$ and *Igf1*: $P = 0.001$; Placental restriction x sex, *Igf1:* $P = 0.006$; where male $P = 0.006$, but female is not significant)

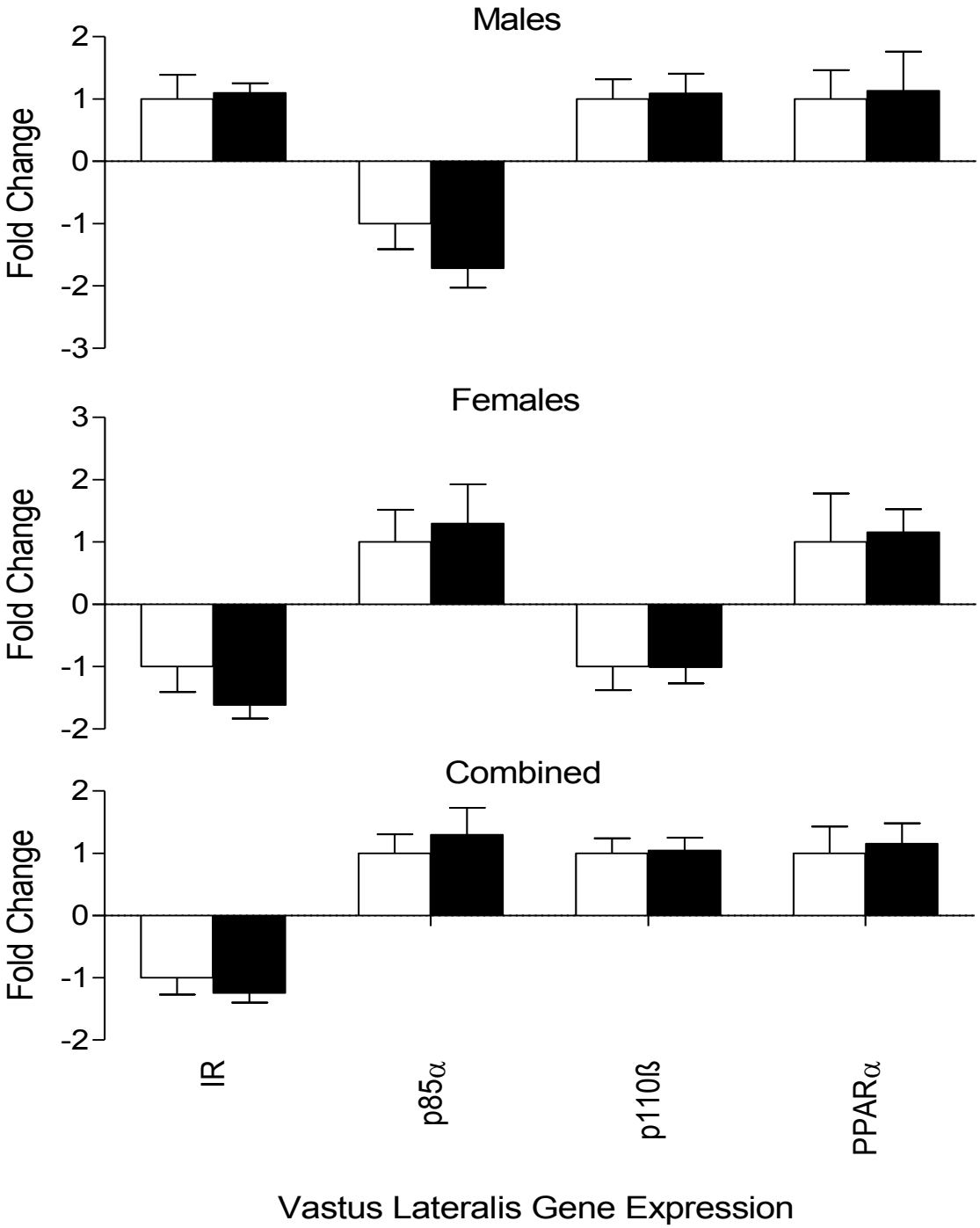
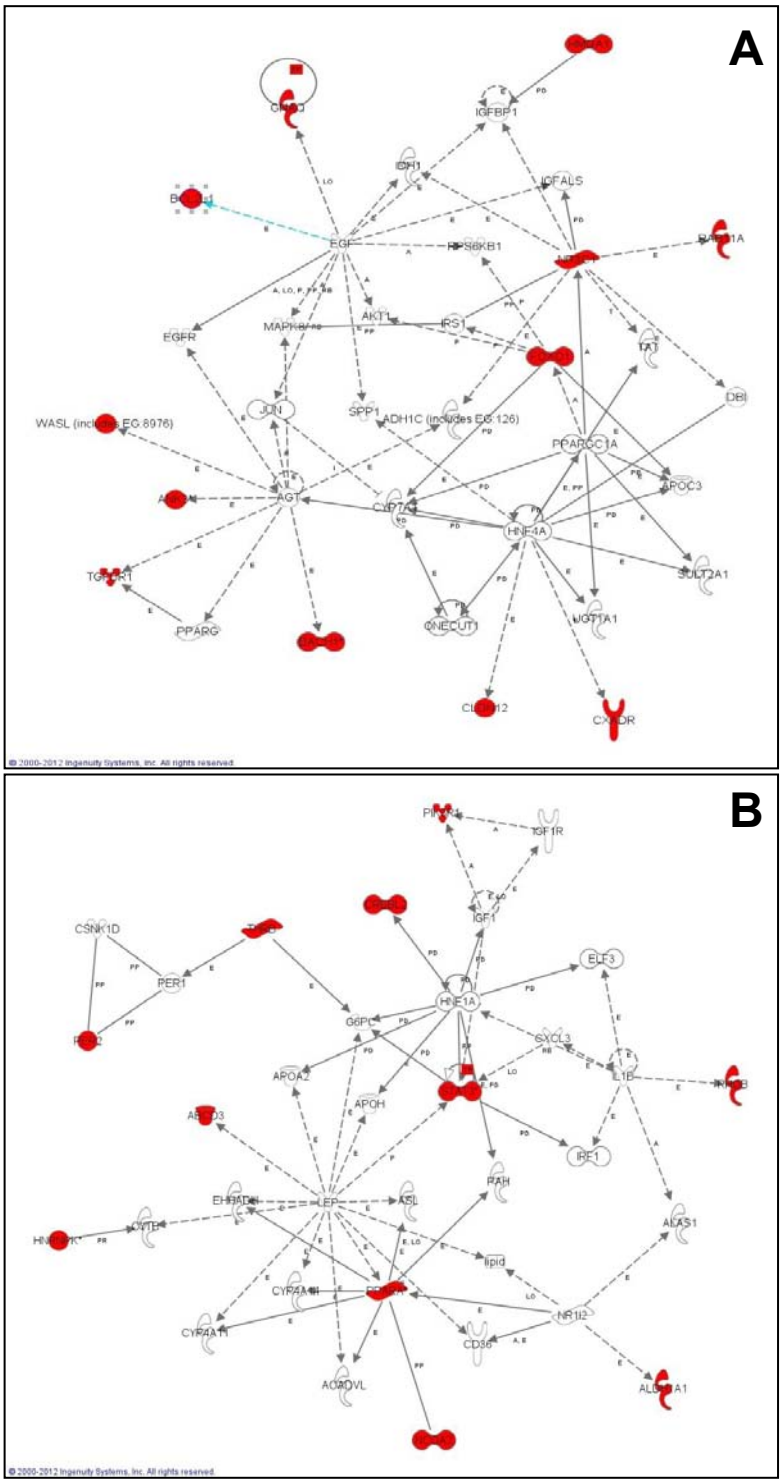


Figure 2.3. Effect of placental restriction on expression of selected insulin signalling and related genes in vastus lateralis

Gene expression was normalised against β-actin. Controls shown as white bars, placentally restricted shown as black bars. Data shown as mean ± SEM (two-way ANOVA of placental restriction, sex and their interaction).


A
IGFBP1
IDH1
IGFALS
EGF
RPS6KB1
EGFR
MAPK1/3
AKT1
IRS1
JUN
SPP1
ADH1C (includes EG:126)
PPARGC1A
AGT
CYP7A1
HNF4A
APOC3
SULT2A1
UGT1A1
ONECUT1
PPARG
DBI
B
IGF1R
IGF1
CSNK1D
PER1
ELF3
HNF1A
G6PC
CXCL3
APOA2
APOH
IRF1
EHHADH
LEP
ASL
PAH
ACAS1
lipid
CYP4A11
CD36
NR1I2
ACADVL

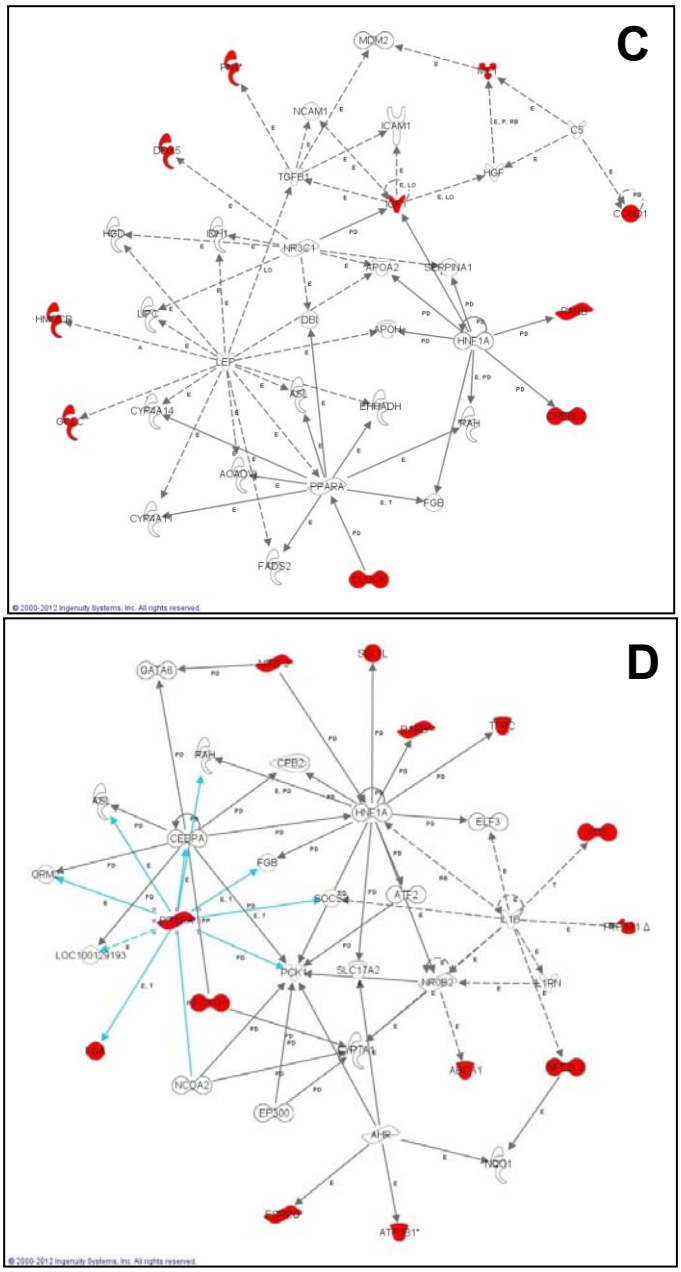


Figure 2.4. Molecular networks predicted to be regulated by differentially expressed miRNAs in the liver of placentally restricted offspring

Expression of hsa-miR-142-3p, hsa-miR-1, hsa-miR-21 and hsa-miR-144 are up-regulated in liver of adult male sheep following placental restriction. IPA generated one network for each of hsa-miR-142-3p **(A)**, hsa-miR-1 **(B)**, hsa-miR-21 **(C)** & hsa-miR-144 **(D),** based on liver tissue database only, limited to maximum 35 molecules and score > 4. The direct targets of the miRNAs (highlighted in red) include PPAR-alpha for hsa-miR-144 (Network D), a transcription factor responsible for lipid metabolism. *Straight lines: direct interaction; Dotted lines: indirect interaction; PD: protein-DNA binding; PP: protein-protein binding; E: expression; LO: localisation; RB: regulation of binding; L: proteolysis; A: activation; P: phosphorylation; T: transcription. Complete list of molecules in each network can be found on Table 2.6.*

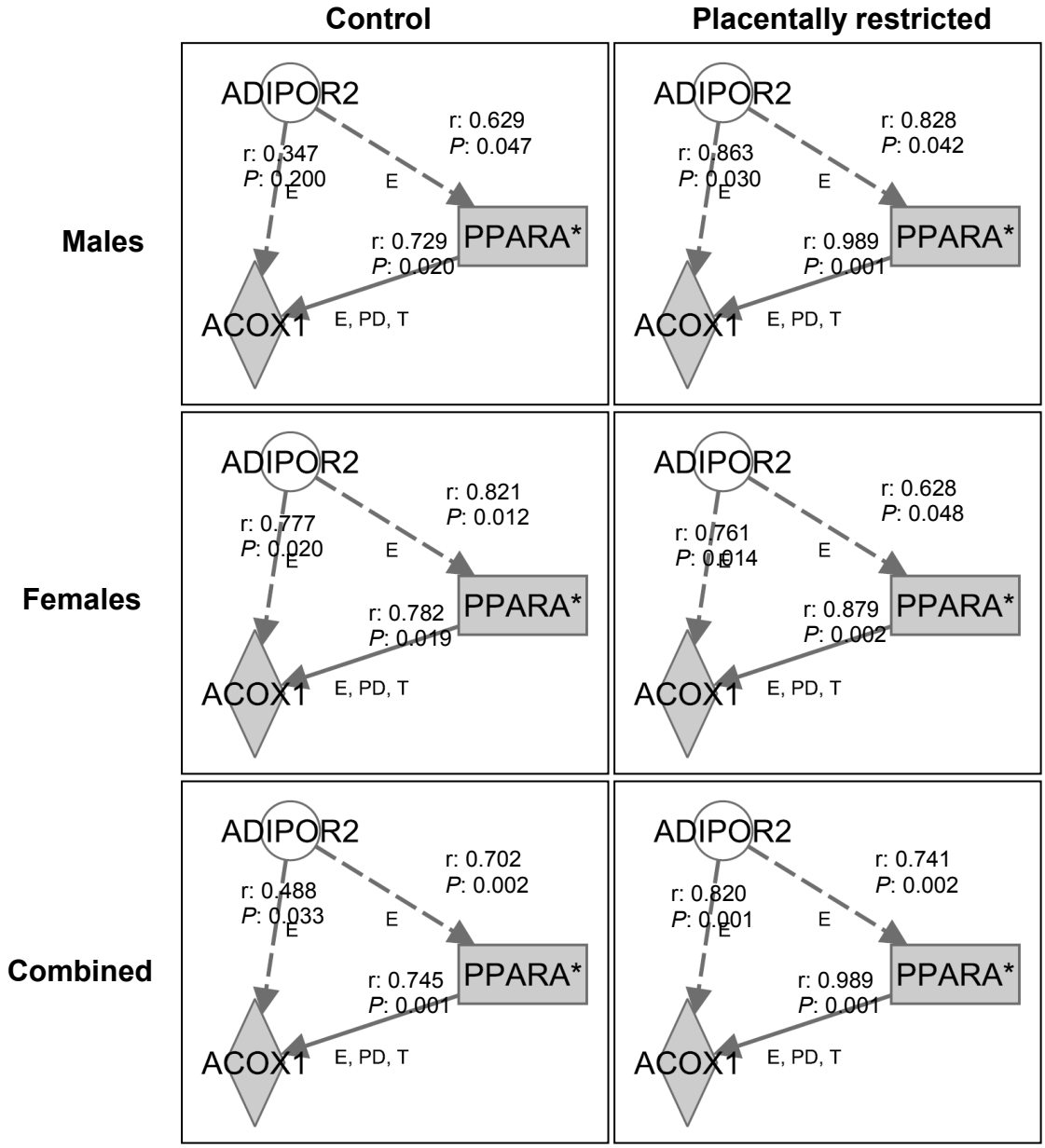


Figure 2.5. Associations between hepatic expression of molecules in the network that is predicted to be targeted by four up-regulated microRNAs in liver of placentally restricted adult offspring

Genes marked in *grey* are predicted to be targeted by differentially expressed miRNAs. *E*: expression; *PD*: protein-DNA binding; *T*: transcription; *dashed arrow*: indirect interaction; *solid arrow*: direct interaction. Network was generated by Ingenuity Pathway Analysis. The correlations were determined by Pearson's correlation.

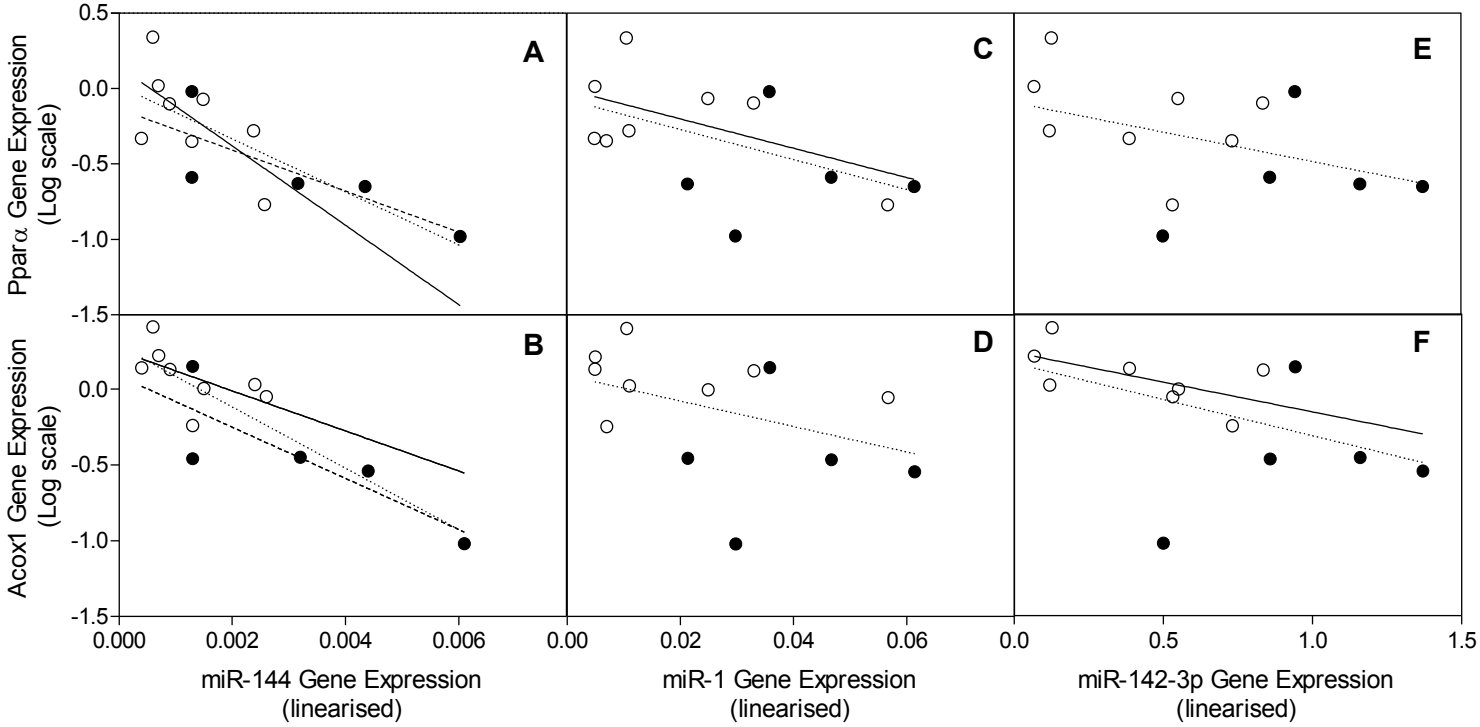


Figure 2.6. Hepatic expression of hsa-miR-144, hsa-miR-1 and hsa-miR-142-3p and that of *Pparα* and *Acox1* genes are inversely correlated in adult male offspring

Hsa-miR-144 versus **(A)** *Pparα* gene expression, Control, r = -0.664, *P*-value: 0.036; Placental restricted (PR), r : -0.798, *P*-value: 0.053; Combined, r = -0.779, *P*-value: 0.001; **(B)** *Acox1* gene expression, Control, r = -0.566, *P*-value: 0.072; PR, r : -0.836, *P*-value: 0.039; Combined, r = -0.858, *P*-value < 0.001. Hsa-miR-1 versus **(C)** *Pparα* gene expression, Control, r = -0.549, *P*-value: 0.079; Combined, r = 0.268, *P*-value: 0.035; **(D)** *Acox1* gene expression, Combined, r = -0.518, *P*-value: 0.078. Hsa-miR-142-3p versus **(E)** *Pparα* gene expression, Combined, r = -0.423, *P*-value: 0.075; **(F)** *Acox1* gene expression, Control, r = -0.586, *P*-value: 0.063; Combined, r = -0.610, *P*-value: 0.013. White circle: control; black circle: PR; continuous line: control linear correlation; dotted line: PR linear correlation; spaced dotted line: combined linear correlation.

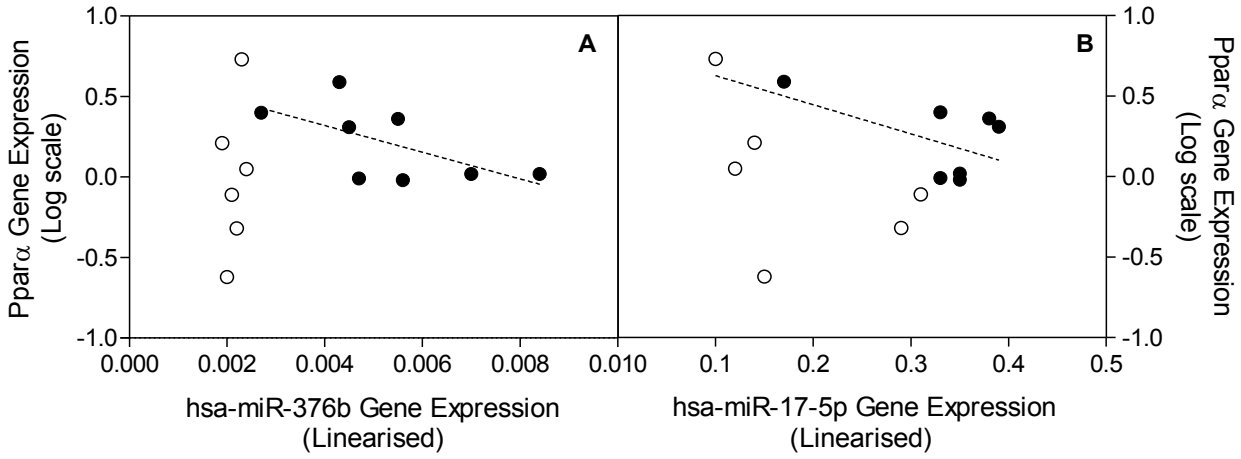


Figure 2.7. Vastus lateralis expression of miR-376b and miR-17-5p and that of PPARα in adult male offspring

(A) miR-376b versus *Pparα* gene expression, PR, r = -0.617, *P*-value: 0.052. **(B)** miR-17-5p versus *Pparα* gene expression, PR, r = -0.533, *P*-value: 0.087. White circle: control; black circle: placental restricted; dotted line: PR linear correlation.

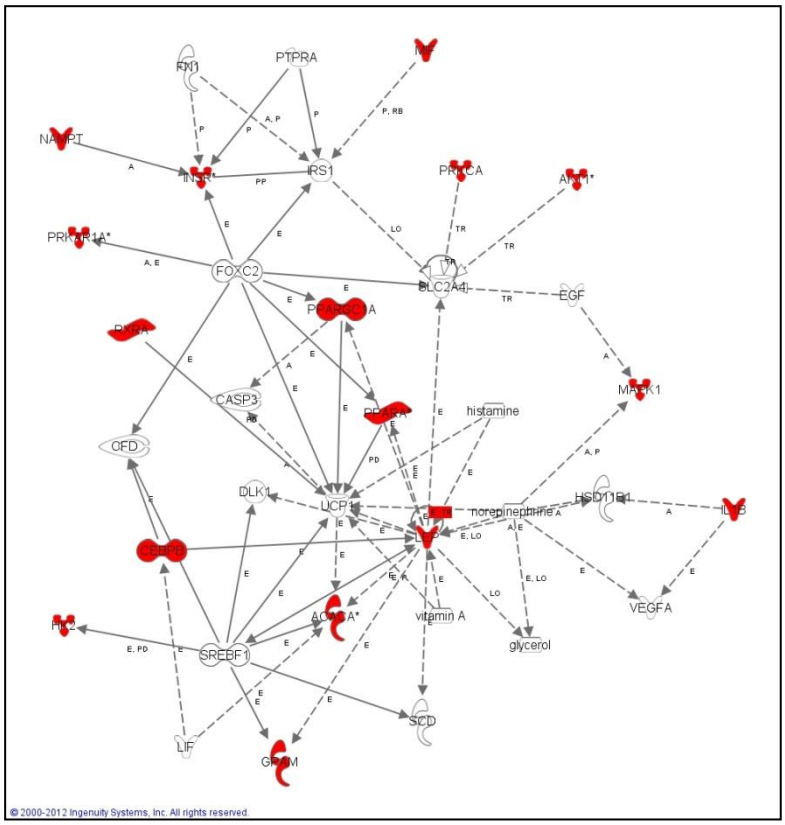


Figure 2.8. Molecular networks predicted to be regulated by hsa-miR-451 in the perirenal fat of placentally restricted adult offspring

Expression of hsa-miR-451 is up-regulated in the perirenal fat of adult female sheep following placental restriction. The direct targets (highlighted in red) include leptin (LEP) and protein kinase C alpha (PRKCα), which indirectly regulate the expression of insulin-responsive glucose transporter 4 (SLC2A4). IPA generated one network, based on adipose tissue database only, limited to a maximum of 35 molecules with a score > 4. *Straight lines: direct interaction; Dotted lines: indirect interaction; PD: protein-DNA binding; PP: protein-protein binding; E: expression; LO: localisation; RB: regulation of binding; L: proteolysis; A: activation; P: phosphorylation; T: transcription. Complete list of molecules in each network can be found on Table 2.7.*

2.7 Supplementary Tables and Figures

Supplement Table S2.1. Primers sequences used to measured gene expression by qPCR

Gene	Amplicon (bp)	Forward Primer 5' – 3'	Reverse Primer 5' – 3'	GenBank accession no.
β-actin, ovine	180	ATGTACCCTGGCATCGCA	ATCCACATCTGCTGGAAGGTGG	U39357.1
p85α, bovine	69	CTTCAAAAACTGAAGCAGACAGTGA	GGTGCGAACTGCTCTGCAA	NM_174575
Pparα, ovine	134	CGTGTGAACATGACCTAGAAG	ACGAAGGGCGGATTGTTG	AY369138.1
Acox1, bovine	99	TGCATTCATCCAAAGGACTC	CATAAGTGGCTGTGGTTTCC	NM_001035289
Adipor2, bovine	109	AGGTCGGGAGCGTCTTGTAG	CATGAACCCCTCATCTTCCTGAG	NM_001040499
Foxo3, bovine	126	TGTTCAATGGGAGCTTGGA	CCACGTTCAAACCAACAACA	XM_002690117.1
Igf1, ovine	87	CAGGTGAAGATGCCAGTCACA	GCTGAAGGCGAGCAAGCA	NM_001009774.2

Supplement Table S2.2. List of molecules which are involved in the networks generated by IPA, specific to the liver

microRNA	Molecules in Network		Score	Focus Molecules	Top Functions
	Predicted by miRNAs	Associated molecules			
miR-1	CCND1, CLOCK, CREBL2, DDX5, FN1, GCLC, HMGCR, IGF1, MET, RARB	ACADVL, APOA2, APOH, ASL, C5, CYP4A11, CYP4A14, DBI, EHHADH, FADS2, FGB, HGD, HGF, HNF1A, ICAM1, IDH1, LEP, LIPC, MDM2, NCAM1, NR3C1, PAH, PPARA, SERPINA1, TGFB1	11	10	Nutritional Disease, Lipid Metabolism, Small Molecule Biochemistry
miR-21	ABCD3, ALDH1A1, CREBL2, HNRNPK, NCOA2, PER2, PIK3R1, PPARA, RHOB, STAT3, THRB	ACADVL, ALAS1, APOA2, APOH, ASL, CD36, CSNK1D, CXCL3, CYP4A11, CYP4A14, CYTB, EHHADH, ELF3, G6PC, HNF1A, IGF1, IGF1R, IL1B, IRF1, LEP, lipid, NR1I2, PAH, PER1	10	11	Lipid Metabolism, Small Molecule Biochemistry, Carbohydrate Metabolism
miR-142-3p	ANK3, BACH1, BCL2L1, CLND12, CXADR, FOXO1, GNAQ, HMGA1, NR3C1, RAB11A, TGFBR1, WASL	ADH1C (includes EG:126), AGT, AKT1, APOC3, CYP7A1, DBI, EGF, EGFR, HNF4A, IDH1, IGFALS, IGFBP1, IRS1, JUN, MAPK8, ONECUT1, PPARG, PPARGC1A, RPS6KB1, SPP1, SULT2A1, TAT, UGT1A1	12	12	Cellular Growth and Proliferation, Cancer, Lipid Metabolism
miR-144	ABCA1, ATP2B1, ESRRG, FGA, FOS, FOXO1, NFE2L2, NR2F2, PPARA, PTP4A1α, RARB, SEL1L, TRFC	AHR, ASL, ATF2, CEBPA, CPB2, CYP7A1, ELF3, EP300, FGB, GATA6, HNF1A, IL1B, IL1RN, LOC100129193, NCOA2, NQO1, NR0B2, ORM2, PAH, PCK1, SLC17A2, SOCS2	6	13	Gene Expression, Lipid Metabolism, Molecular Transport

Supplement Table S2.3. List of common molecules which were predicted to be targeted by four microRNAs differentially expressed in liver of placentally restricted adult offspring

Symbol Field	Refseq Field
ABHD2	NM_007011
ACOX1	NM_007292
ADAM12	NM_003474
AFAP1	NM_021638
APAF1	NM_013229
AQP4	NM_001650
ATM	NM_000051
ATRN	NM_139321
BACH1	NM_001011545
BAG5	NM_001015048
BRWD1	NM_033656
BTBD7	NM_001002860
BTBD9	NM_001099272
C17orf51	XM_378661
C18orf1	NM_001003674
CACNB2	NM_000724
CAMKK2	NM_153500
CASP10	NM_001230
CCPG1	NM_004748
CD59	NM_000611
CDKN2B	NM_004936
CLCC1	NM_001048210
COPA	NM_004371
CREB5	NM_001011666
CUGBP2	NM_001025076
DCUN1D4	NM_001040402
DCX	NM_000555
DIAPH2	NM_006729
DST	NM_015548
DTNA	NM_001390
DYRK1A	NM_001396
EMR2	NM_013447
ENAH	NM_018212

Symbol Field	Refseq Field
ERBB4	NM_0010425
ERC1	NM_015064
ERLIN2	NM_007175
FKTN	NM_0010798
FMNL3	NM_175736
FOXN3	NM_0010854
FOXO3	NM_001455
GAS7	NM_003644
GCNT2	NM_001491
GIT2	NM_057169
GJC1	NM_0010803
H2AFV	NM_138635
HNRNPC	NM_0010774
ICA1L	NM_138468
IKZF2	NM_0010795
KCNJ16	NM_170741
LYCAT	NM_0010022
MCC	NM_0010853
MGC13057	NM_0010425
MYST3	NM_0010994
NFAT5	NM_006599
NSL1	NM_015471
NT5DC3	NM_016575
NTRK2	NM_0010070
PALM2	NM_053016
PAPD5	NM_0010402
PCDH11X	NM_032968
PCDH11Y	NM_032971
PCDH7	NM_032456
PDE4B	NM_0010373
PFKFB2	NM_006212
PODXL	NM_0010181
POGZ	NM_207171

Symbol Field	Refseq Field
PPARA	NM_001001928
PPP1CB	NM_002709
PRELP	NM_002725
PSD3	NM_015310
QKI	NM_006775
RAB3IP	NM_001024647
RBM33	NM_001008408
RBM9	NM_001031695
REPS2	NM_001080975
RIPK5	NM_015375
SERBP1	NM_001018067
SHANK2	NM_133266
SLC26A7	NM_052832
SLC6A15	NM_018057
SLC7A2	NM_001008539
SLC7A6	NM_001076785
SMAD2	NM_001003652
SMAD5	NM_001001419
SNX1	NM_148955
SRGAP3	NM_001033117
SS18	NM_005637
STOM	NM_004099
TCF4	NM_001083962
TMEM1	NM_003274
TNPO1	NM_002270
TNRC6B	NM_001024843
TPM3	NM_152263
TULP4	NM_001007466
UBQLN1	NM_053067
WHSC1	NM_001042424
ZAK	NM_133646
ZC3HAV1	NM_020119
ZNF295	NM_001098403

Supplement Table S2.4. IPA networks for combined predicted targets of miRNAs differentially expressed in vastus lateralis of placentally restricted adult female offspring

microRNA	Molecules in Network		Score	Focus Molecules	Top Functions
	Predicted by miRNAs	Associated molecules			
7 miRNAs Female (array)	ABCA1,ACSL4,CAV3,CEBPB,CHRNE,DLAT,FABP3,ME1, PGAM1,PIK3R1,PPARA,PTGS2,THRB	GABPA,IRS1,PPARG,PPARGC1A,PPARGC1B,RORA,SLC2A	10	13	Lipid metabolism, Molecular transport, Small molecule biochemistry

Supplement Table S2.5. IPA networks for combined predicted targets of miRNAs differentially expressed in liver of placentally restricted adult male offspring

microRNA	Network ID #	Molecules in Network		Score	Focus Molecules	Top Functions
		Predicted by miRNAs	Associated molecules			
8 miRNAs Male (array)	1	ABCD2,ABCD3,ALDH3A2,C1QA,CCND1,CHKA, CXCL12FBXO21,FGA,FOS,GCH1,FOXO1,GPD2,HGF, IDI1,HMGA1,HMGCR,HSPA5,IGFBP1,INSR,LDLR,LIFR, LIPG,ME1,MMD,NCOA2,NPC1,NRF1,PPARA,PRKCE, SCD,SLC25A1,STAT3,THRB,ZNF236		30	35	Lipid metabolism, Molecular transport, Small molecule biochemistry
	2	ACSL4,ACVR2B,ADPGK,CLOCK,CRY2,WEE1,DNAJB9, EZH2,FOXA3,HAS2,HSF2,KLF13,NR2C1,PAFAH1B2, PER2,PER3,PICALM,SEL1L,SLC1A2,SLC30A5,TFAIP1, TNFRSF21	CRY1,PER1	16	22	Behaviour, Nervous system and Connective tissue development and function
	3	ABCB11,ATP1B1,BCL2L1,CREBL2,ETV6,GCLC,HAX1, HSP90B1,IFNAR1,IL10,M6PR,MORF4L2,NFE2L2,NR2F2, PCBP1,PFN2,PTPLAD1,ARB,RBBP7,SMAD6,TFRC	ABCB4,BHMT,CCRN4L,CYP2E1,GBE1,GSTM4,Gstp1, HNF1A, HSD3B1,PCK1,RORC,SLCO1A2,SLCO1B3,Sult1d1	9	21	Lipid metabolism, Molecular transport, Small molecule biochemistry
	4	AHCY,ALDH1A1,ATP2B1,CAMK2N1,CDKN1A,CYP1B1, ESRRG,FZD7,HUNK,JAG1,KLF9,OLFML1,PLXN2,RBL2, SHB,SMAD7,TGFBR1,TGFBR2,TIPARP	ACOX1,AHR,Ces2b/Ces2c,COL1A1,CYP1A1,CYP1A2, CYP2B6,CYP4A11,GOT1,Hsd3b4,IL6,INSIG2,NR1I2,PPARG, TGFB1,TNF	7	19	Lipid metabolism, Small molecule biochemistry, Vitamin and mineral metabolism
	5	ABCA1,DDX5,FAM107B,IGF1,MAFF,MAP3K5,NFE2L2, NR3C1,PAX8,PCSK6,PCYOX1,PDE38,PPP6R3,RBA11A, SGK1,SHOC2,USP38	AHR,APOA1,CYP3A4,DIO1,GSTA5,GSTM5,NR1I2,NR1I3, PCK1,PPARD,PPARGC1A,RORA,RORC,STAT5A	7	17	Cell signalling, Lipid metabolism, Molecular transport
	6	ACSL1,BCL2,CDKN1A,FOXO1,IGF1R,IL12A,IRF1,MBNL2, NFE2L2,PTEN,SON,STAG1,STAT3,THRB,TIRAP	IRF2,IRS1,IRS2,NCOA1,SOCS1,SREBF1,SREBF2,STAT1, THRA,THRSP,TOB1,TP53	7	16	Gene expression, Organism survival, Cell death
	7	AKT1S1,CCND1,CLDN12,CXADR,FASLG,GAB1,GAB2, ITGB1,MET,MTOR,PDK2,PRLR,RHPN2,RPTOR,SKI,SMA2 TSC22D1,YES1	APOC3,CDH1,CLDN2,CTNNB1,CYP7B1,CYP881,EGFR,FAS GRB2,HNF4A,MAPK8,PPARGC1A,PTPN11,Slco1a1,SMAD4, SRC,UGT1A1	7	18	Cancer, Cellular development, Cellular growth and proliferation
4 miRNAs Male (qPCR)	1	ABCD2,ABCD3,ACSL4,ADPGK,CCND1,CLOCK,CXCL12 FBXO21,FGA,FOS,FOXO1,GCH1,GPD2,HMGCR,HSF2, HSP90B1,KLF13,LIFR,ME1,MMD,MORF4L2,NCOA2, NFE2L2,NR2C1,PAFAH1B2,PCBP1,PER2,PICALM,PPARA PTPLAD1,SEL1L,SLC25A1,STAT3,THRB,ZNF236		41	35	Gene expression, Lipid metabolism, Molecular transport
	2	AHCY,ALDH1A1,ATP2B1,ESRRG,FAM107B,FZD7,GCLC HAX1,JAG1,NFE2L2,NR2F2,PCSK6,SMAD7,TGFBR1, TGFBR2,TIPARP,USP38	AHR,AKR1A1,ANG,CELA1,Ces2a,Cml1/Nat8b,CYP2C8, ENTPD5,EPHX1,Gsta4,GSTA5,NQO1,NR1I2,NR1I3,PAPSS2, PPARD,TGFB1,UGT2B17	10	17	Cell signalling, Cancer, Cell death
	3	ABCA1,BCL2,CLDN12,CREBL2,CXADR,DDX5,FASLG, GAB1,HMGA1,IGF1,MET,NR3C1,PRLR,RARB,SGK1,TFRC	APOA2,CDH1,CLDN2,CTNNB1,DBI,F11,GJB1,HNF1A,HNF4A IGFBP1,IRS1,LIPC,MAPK8,OAT,SERPINA1,SLCO1A,VTN	10	17	Cancer, Cellular growth and proliferation, Lipid metabolism
	4	AKT1S1,BCL2L1,CCND1,IL12A,MBNL2,MTOR,PTEN,SKI, SON,STAG1	CDKN1A,IRF2,IRF1,SMAD4,TOB1,TP53	7	10	Cell death, Cellular development, Cellular growth and proliferation

Supplement Table S2.6. List of the predicted targets of miRNAs differentially expressed miRNAs in liver of placentally restricted adult male offspring

hsa-miR-142-3p				
ACSL4	CUGBP2	MARCKS	RGL2	TWF1
ADAMTS3	CXADR	MARK3	RHOBTB3	USP33
ADCY9	DCUN1D4	MBD6	RICTOR	USP6NL
AFF1	DIRC2	ME1	RLF	USP9X
AFF2	DMTF1	MGAT4A	RNF126	UTRN
AKT1S1	DPY19L4	MLL	ROCK2	UTX
ANK3	DST	MLLT1	RPE	UTY
ANKRD11	EDEM3	MMD	S1PR3	VAMP3
ANKRD46	EGR2	MMP16	SEMA6D	VPS24
ANKS1A	EHF	MOBKL3	SGK1	WASL
APC	EML4	MON2	SGMS1	WHDC1
ARID5B	EPM2AIP1	MORF4L1	SLC1A3	XPO1
ARL15	ERC1	MORF4L2	SLC30A9	ZBTB41
ARNTL	ERG	MRFAP1	SLC35F5	ZEB1
ARRDC3	FAM44B	MYH9	SLC37A3	ZEB2
ASB7	FAM46A	MYLK	SLC38A4	ZFP91
ASH1L	FAM80B	NBPF3	SLC7A11	ZNF217
ATF7IP	FBXO21	NR2C1	SLCO4C1	ZNF641
ATG16L1	FBXO3	NUMBL	SMG1	ZNF644
BACH1	FGF23	OSBP	SNF1LK	ZNF676
BACH2	FKBP1A	OSBPL3	SNF1LK2	ZNF827
BAZ1A	FMNL2	PAFAH1B2	SOX11	
BCL2L1	FNBP1L	PATZ1	SOX6	
BCLAF1	FNDC3A	PCAF	SP8	
BHLHB9	FOXO4	PCGF3	SPNS1	
BRWD3	GAB1	PCMTD2	SPRED1	
BTBD7	GFI1	PCSK1	STAM	
C10orf56	GNB2	PDE4B	STAU1	
C12orf53	GTF2A1	PDSS2	STRN3	
C14orf24	HECTD1	PELI1	STX12	
C18orf25	HGS	PICALM	SYN2	
C1orf9	HMGA2	PIK3R6	SYPL1	
C20orf112	HMGB1	PMAIP1	TAOK1	
C5orf24	IL1RAPL1	PNO1	TARDBP	
C9orf72	INPP5A	PPFIA1	TESK2	
CALM1	ITGAV	PPP1R2	TFG	
CCDC6	ITGB8	PPP1R3A	TGFBR1	
CCNT2	JMJD1C	PPP3CA	TIPARP	
CFL2	KLF13	PRLR	TJP1	
CHRNE	KLF4	PTPN23	TM2D2	
CLCN5	KLHDC1	PUM1	TMEM110	
CLDN12	KLHDC6	PURB	TMEM132E	
CLTA	LCOR	RAB11A	TMEM200B	
CNNM1	LRRC1	RAB11FIP2	TMEM55B	
COL24A1	LRRC3B	RAB1A	TMEM59	
COPS7A	LRRC59	RAB2A	TMTC3	
CPEB2	MAN1A2	RAB40C	TNKS	
CREB5	MAP3K11	RAC1	TP53INP2	
CRK	MAP3K7IP2	RARG	TRPS1	
CRTAM	MAP4K3	RGAG1	TSEN34	

hsa-miR-1				
A2BP1	CREM	INPP5F	PTBP1	UBE2H
ABHD2	CTNND2	JOSD1	PTPLAD1	UBE4A
ADAR	CXorf23	KCNA5	PUM2	UBQLN1
ADPGK	DACH1	KCND2	QKI	UBR5
AKAP12	DDX5	KCNE1	RAB11FIP2	USP33
ANKIB1	DLC1	KIAA2022	RARB	UST
ANKRD29	E2F5	KIF2A	RASA1	VAMP2
ANXA4	EAF1	LASP1	RBM47	VAMP4
AP1G1	EFNB2	LIN7C	RGAG1	VGLL4
API5	EIF2C1	LOC285636	RIT2	WDR1
ARCN1	EIF4E	LPPR4	RNF145	WDR40B
ARHGAP21	EML4	MAB21L1	RNF19A	WDR48
ARHGEF18	ETS1	MAGI2	RNUXA	WDR61
ASH2L	FAM155A	MAP1A	RPE	WNK3
ATP6V1A	FBXL10	MAP3K7IP3	RSBN1	XPNPEP3
ATP6V1B2	FBXO33	MAP4K3	SEC22B	YWHAQ
AZIN1	FLJ38973	MATR3	SEC62	YWHAZ
BACH2	FN1	MEOX2	SEC63	ZBTB4
BAG4	FNDC3A	MET	SEMA6D	ZFP36L1
BCL11A	FNDC3B	MEX3C	SERP1	ZFP91
BDNF	FOXP1	MIPOL1	SETBP1	ZIC4
BET1	FRAS1	MLL5	SFRP1	ZMAT3
BHLHB5	FUBP1	MMD	SFRS1	ZNF217
BLCAP	FZD7	MNT	SFRS10	ZNF236
BOLL	GCH1	MON2	SFRS9	ZNF264
BPNT1	GCLC	MPP5	SH3BGRL3	ZNF800
BSCL2	GDAP1L1	MUM1L1	SH3TC2	
BSN	GJA1	MYLK	SLC25A1	
BTAF1	GLCCI1	MYST3	SLC31A1	
C11orf61	GNPDA2	NCL	SLC38A2	
C1orf96	GNPNAT1	NCOA3	SLC39A10	
C4orf34	GNPTAB	NECAB1	SLC44A1	
C7orf58	GOLPH3	NETO2	SMEK2	
C9orf82	GPD2	N-PAC	SPRED1	
CCDC141	GPR85	NRP1	SULF1	
CCND1	H3F3B	OSBPL7	SYT1	
CCND2	HACE1	OTX2	TAGLN2	
CD2AP	HDAC4	PAFAH1B1	TBC1D15	
CDK6	HIAT1	PALLD	TGIF2	
CDK9	HIVEP2	PCDH17	THBS1	
CENTB2	HMBOX1	PDCD10	TIMP3	
CHSY1	HMG2L1	PDIK1L	TKT	
CLCN3	HMGCR	PFTK1	TMCC1	
CLOCK	HNRNPU	PGM2	TMEM135	
CLTC	HOXB4	PHF6	TMOD2	
CNN3	HSP90B1	PLAG1	TNKS2	
COL4A3	HSPD1	POGK	TRAPPC3	
CORO1C	IGF1	PREX1	TRIM2	
CPEB1	IGFBP5	PRIC285	TRPS1	
CREBL2	IHPK2	PRKACB	TWF1	

Supplement Table S2.6. *Continued*

hsa-miR-21						
ABCD2	CBX4	FBXL2	LRRC57	PIK3R1	SETD8	TRIM2
ABCD3	CCDC121	FBXO11	LUM	PIP5K3	SETX	TRIM33
ABHD2	CCDC88A	FCHO2	LYRM7	PITX2	SFRS3	TRIM9
ACBD5	CCDC93	FEM1C	MALT1	PLAA	SGK3	TRMT6
ACVR2A	CCL1	FGF1	MAP3K7IP3	PLAG1	SKI	TRPM6
ADNP	CCL20	FLJ14213	MAPK10	PLCB1	SLC12A6	TRPM7
AKAP6	CCRL1	FLJ45139	MAPRE1	PLEKHA1	SLC22A15	TSNAX
ALDH1A1	CCRL1	FRS2	MATN2	PM20D2	SLC2A13	TSPAN2
ALX1	CCT5	FUBP1	MATR3	POM121	SLC35F5	TSPO
ANGPTL5	CD47	FZD6	MBNL1	PPARA	SLC7A6	TXNDC4
ARHGAP24	CD69	GABRB2	MDGA2	PPP1R3A	SMAD7	UBE2D1
ARHGEF12	CDC25A	GALNT12	MED21	PPP1R3D	SMARCD1	UBE2D3
ARHGEF7	CENPP	GANC	MED28	PPP3CA	SMURF2	UBE4A
ARID4A	CENPQ	GARNL1	MEGF9	PRKG1	SNED1	UBR3
ARL1	CEP97	GATAD2B	MGC70863	PRPF39	SOCS5	VHL
ARL4D	CFL2	GK5	MPRIP	PRPF4B	SOX2	WDR22
ARMC8	CHD7	GLCCI1	MRPL9	PRRG4	SOX5	WDR68
ASF1A	CHIC1	GLDN	MSH2	PRTFDC1	SOX6	WWC2
ASPN	CLCA3	GLIS2	MTAP	PSRC1	SOX7	WWP1
ATP2B4	CLEC4A	GNG2	MTMR12	PTGER3	SPG20	XCL1
ATRN	CNOT6	GPC4	MTPN	PURB	SPON1	XKR6
ATXN10	CNOT8	GPR180	NARG2	RAB11A	SPRY1	YAP1
BAHD1	CNTFR	GPR64	NAT13	RAB11FIP2	SPRY2	YOD1
BBS12	COMMD8	HAX1	NBEA	RAB22A	SRI	ZADH2
BCL11A	COX15	HBP1	NCOA2	RAB6A	SSFA2	ZBTB38
BCL2	CPEB3	HNRNPK	NEGR1	RAB6C	ST3GAL6	ZCCHC3
BDH2	CREB5	HNRNPU	NEK11	RAGE	ST6GAL1	ZDHHC17
BEST3	CREBL2	IL12A	NFIB	RASA1	STAG2	ZFPM2
BMP3	CRIM1	IPO11	NKIRAS1	RASGRP1	STAT3	ZNF10
BMP8A	DLX2	ITGB8	N-PAC	RBM22	STK38L	ZNF200
BMPR2	DMD	JAG1	NPTN	RBMS3	STK40	ZNF208
BNC2	DNAJC16	JHDM1D	NSL1	RCCD1	SYT14L	ZNF295
BOLL	DNM1L	JPH1	NTF3	RECK	TAF5	ZNF367
BRCC3	E2F3	KBTBD6	NUPL2	RHOB	TAGAP	ZNF431
BRD1	EGLN1	KCNA1	OLFM3	RMND5A	TBX2	ZNF576
BTBD3	EHD1	KCNA3	OSR1	RNF103	TESK2	ZNF592
BTG2	EIF1AX	KIAA0564	PABPC5	RNF111	TET1	ZNF654
C12orf12	EIF2C2	KIAA1012	PAG1	RNF6	TGFBI	ZNF704
C17orf39	ELF2	KIAA1333	PAN3	RP2	TGFBR2	ZNF708
C3orf57	ELF2	KIAA1468	PBRM1	RPS6KA3	THPO	ZRANB1
C4orf16	ELOVL7	KIAA2026	PCBP1	RSAD2	THRB	
C4orf33	EPHA4	KLHDC5	PCGF5	RSBN1	TIAM1	
C5orf41	EPM2A	KRIT1	PCSK6	RTKN2	TIMP3	
C7	FAM107B	LANCL1	PDCD4	SACM1L	TLR4	
CA1	FAM133A	LEMD3	PDLIM5	SALL3	TNFRSF10B	
CADM2	FAM13A1	LOC150786	PDZD2	SATB1	TNPO1	
CALCB	FAM46A	LOC153364	PELI1	SCML2	TNRC6B	
CAPN2	FAM63B	LONRF2	PELI2	SCN8A	TNS1	
CASC4	FASLG	LPA	PER2	SCRN1	TOR1AIP2	
CASKIN1	FBXL17	LRAT	PHF14	SETD6	TRAPPC2	

Supplement Table S2.6. *Continued*

hsa-miR-144					hsa-miR-134			
ABCA1	CPEB2	ID4	PHF20L1	TBL1XR1	ABHD2	FLG2	MYCN	SPATA18
ABCC5	CPEB3	IDH2	PHTF2	TFAP4	ABI2	FNIP1	MYST2	SPTAN1
ACBD3	CTDSPL2	KBTBD8	PKNOX1	TFRC	ACCN2	FSTL5	NIPA1	SSX7
ACSL4	CUGBP2	KCND2	PLAG1	TJP1	ACSL1	FYTTD1	NRF1	STAT5B
AEBP2	CUGBP2	KIAA0232	PPARA	TMED5	AFF3	GINS1	OPCML	STK40
AFF4	CXCL12	KIAA0240	PPP1R12A	TMEM55A	ANKRD50	GLDN	OSBP	STRBP
AHCY	DCBLD2	KIAA0423	PPP1R16B	TRIB1	BACH1	GLRA3	PATZ1	STXBP1
ALDH1A3	DDEF1	KIF2A	PPP2R2A	TRIM2	BAGE4	GLRA3	PAX8	SUMO3
ALS2	DDIT4	KLF12	PPP3R1	TRIO	BAGE5	GNAL	PCDH11X	SUV420H1
ANK2	DGCR2	KPNA1	PTCH1	TSHZ3	BANF1	GNAO1	PDE5A	SYT14
APP	DLG5	LGR4	PTEN	TSPAN12	BCL9L	GNE	PGM2L1	SYT14L
APPBP2	DMD	LHX2	PTGFRN	TTN	BEST1	GOLPH3	PGM3	TCF21
ARFGEF1	DR1	LIFR	PTP4A1	UBE2D2	BMP3	GPR45	PHF8	TEDDM1
ARHGAP26	DYRK1A	LIN54	PUM2	UBR3	BNC2	HIC2	PHLPPL	TICAM2
ARID1A	E2F8	LSM14A	PURA	USP38	C12orf5	HOOK3	PISD	TIRAP
ARID2	EDAR	MAGI2	PURB	USP47	C1orf21	IL16	PKD2	TMEM184B
ARID5B	EIF5	MAML3	QKI	VKORC1L1	C1orf32	IL17RD	PLD1	TMPRSS11A
ARRDC3	EMP1	MAP7D1	RAB14	WDFY3	C3orf58	IL8RB	PPP1R12A	TSC22D1
ASPN	ERBB4	MAPK6	RAB1A	WIF1	C6orf120	ITGB1	PPP1R7	TTBK1
ATP2B1	ESRRG	MARCKS	RAB5A	XYLT1	C9orf5	ITGB8	PSD3	UBE2N
ATP2B2	ETS1	MARK1	RAP2C	ZCCHC2	CACHD1	KIAA0802	PUM2	USP31
ATRX	FAM126B	MBNL1	RARB	ZDHHC17	CAND1	KLHL6	RAB27A	USP9X
ATXN1	FAM60A	MBNL2	RBM12	ZEB2	CBX6	KPNA3	RAD50	USP9Y
BACH2	FAM76B	MED12L	RGL1	ZFHX4	CCDC141	KRAS	RELT	VEGFA
BCL2L11	FBN2	MED14	RGS16	ZFX	CCL16	KTELC1	REPS1	VGLL4
BNC2	FBXL3	MITF	RIN2	ZNF207	CDH9	LAIR1	RFC5	VPS36
BRPF1	FBXO32	MPP5	RIPK5	ZNF238	CISD2	LCMT2	RHOQ	VTI1A
BTBD14B	FBXO32	MTPN	RNF111	ZNF777	CLCC1	LIMK2	RNF103	WIPF1
C10orf141	FBXW11	MXRA5	SALL1	ZNF800	CLSTN2	LPHN3	RUNX1T1	WNK3
C12orf34	FGA	MYO1E	SCN1A		CMPK1	LRRC1	SEC11A	XKR6
C16orf72	FLRT2	MYST3	SEC24A		CNTD2	LSM12	SERPINE1	ZBTB1
C1orf77	FMR1	NARG1	SEL1L		COL2A1	LVRN	SERTAD2	ZDHHC9
C3orf58	FNDC3A	NAV3	SEMA6A		CPEB2	MAGI2	SFRS2	ZFPM2
CACHD1	FOS	NCOA7	SEMA6D		CPEB4	MAGIX	SFRS2B	ZMAT2
CADM2	FOSB	NDRG4	SENP7		CREBZF	MAP3K7IP1	SFXN1	ZMAT5
CALCRL	FOXO1	NEUROD1	SETD5		CSNK1G3	MAP4K4	SGMS1	ZNF219
CAMSAP1L1	FRAP1	NFE2L2	SLC12A2		DACH1	MAT2A	SH2B3	ZNF354C
CAMTA1	FUBP3	NLK	SLC4A4		DIO2	MBNL1	SH3TC2	ZNF562
CAPZA1	FZD4	NPTX1	SLC7A11		DMN	MBTD1	SLC14A1	ZNF711
CAV3	FZD6	NR2F2	SLITRK4		DOCK5	MED13	SLC16A4	
CBX1	GATA3	NRK	SMPD3		DSCAM	MED13L	SLC25A34	
CCDC85A	GCNT2	ONECUT2	SOBP		DST	MEIS2	SLC25A5	
CCDC88A	GDF10	OTUD4	SON		EIF3J	MLL2	SLC46A1	
CCNT2	GLCCI1	PAFAH1B1	SORCS3		EML4	MMP24	SLC5A7	
CD28	GLTSCR1	PANK1	SPRED1		EPHA7	MPZL1	SLFN11	
CDC2L6	GOLGA4	PAPOLG	ST18		ETF1	MRS2	SLFN13	
CDH11	HDHD2	PAPPA	ST6GALNAC3		FAM168B	MS4A2	SMAD6	
CDH2	HNRNPU	PCDH18	STAG1		FAM19A5	MTMR10	SMARCA1	
CDH5	HSF2	PDE4D	STARD8		FAM91A1	MTMR2	SMARCAD1	
COL10A1	ICK	PDS5B	TAL1		FCRL5	MXRA5	SMU1	

Supplement Table S2.6. *Continued*

hsa-miR-600				hsa-miR-212			hsa-miR-30b			
ABCA10	DYDC2	OSBP	TUBGCP5	ACVR2B	HMGA2	RASA1	ACTC1	ELL2	NEDD4L	TBC1D10B
AEBP2	ETNK1	PAFAH1B1	UBR3	ADAMTS5	HN1	RDX	ACTR1A	ELMOD2	NFAT5	TBC1D15
ALDH3A2	EYA2	PCAF	VGLL3	AMD1	HNRNPM	RGS7BP	ACVR1	EPC2	NFIB	TIA1
ANKRD45	EZH2	PCF11	VPS13D	ANP32A	HNRNPU	RICS	ADAM19	ERG	NRIP1	TMCC1
ARFIP1	F2RL2	PDE4A	WFDC12	ARHGEF11	KCNA6	SALL1	ADAMTS3	ERLIN1	OMG	TMEFF1
ARIH2	FAM134B	PHTF2	WRN	ARID2	KCNJ12	SAP30L	ARID4A	FAM152A	OTUD4	TMEM47
ARL1	FAM152A	PKN2	ZBTB24	ARMC1	KCNK2	SATB2	ARID4B	FAM43A	PAPD4	TMEM87A
ARL5B	FAM40B	PKP2	ZMPSTE24	BNC2	KIAA0265	SEC24A	ARL6IP6	FOXD1	PER2	TNRC4
ATP11C	FAM73A	PLS1	ZNF136	BOLL	LASS2	SEC62	ATG5	FOXG1	PFN2	TNRC6A
BEX1	FAM81A	PLXNA2	ZNF323	BRCA1	LIN28B	SEMA4G	ATP2B1	FRMPD1	PHTF2	UBE2F
BEX4	FAR1	PRND	ZNF396	BRI3	LRRC55	SEMA6A	ATP2B2	FYCO1	PNN	UBE2I
BEX5	FBXL17	PTPLAD2	ZNF605	BTG2	LRRFIP1	SETD5	B3GNT5	GALNT1	PPP1R12A	UBE2J1
BLZF1	FOXO1	PTPRD	ZNF776	C14orf147	LSM11	SGK3	BAZ2B	GALNT2	PPP3R1	UNC5C
C10orf118	FRY	RAD51L1	ZNF830	C14orf43	MAPK1	SIRT1	BCL11B	GALNT3	PRDM1	USP37
C10orf46	FTSJ1	RBBP9		C14orf43	MECP2	SLC6A1	BCL9	GIGYF2	PSMD7	VKORC1L1
C1orf130	GDAP1	REPIN1		C1QL1	MEX3B	SOX11	BECN1	GLCE	PTGFRN	WDR7
C1orf181	GLI3	RFPL1		C5orf13	MEX3C	SOX4	BNC1	GLDC	PTP4A1	XPR1
C1orf213	GLRX5	RHOBTB3		CALU	MMP16	SOX5	BNIP3L	GNAI2	R3HDM1	YTHDF3
C1orf93	IGFBP1	RHPN2		CDC2L6	MYCBP2	SOX6	BRD1	GRB10	RAB10	YWHAZ
C1QTNF7	IKZF2	RICTOR		CFL2	NCALD	SPRY1	C12orf30	GTF2H1	RAB22A	ZCCHC2
C22orf9	IL24	RP6-213H19.1		CNIH	NEIL2	SRGAP3	C20orf108	HLX	RAD23B	ZDHHC21
C3orf15	INA	SEC24A		CREB5	NFAT5	STX16	C4orf34	HOXA1	RAP2C	ZFAND5
C3orf58	IPO8	SH3PXD2A		CSDE1	NFYA	TCF7L1	C9orf72	IHPK3	RASD1	ZNF280B
C5orf4	ISOC1	SHOC2		CTDSPL2	NMNAT2	TIMM9	CADPS	IL1A	REEP3	ZNF644
C6orf167	JARID1A	SHPRH		DAZAP2	NOVA1	TJAP1	CALCR	ITGA6	RHOB	
C9orf6	KBTBD11	SLAMF8		DCUN1D3	NPAS4	TLN2	CAMK2D	JAKMIP2	RIPK5	
CBFA2T2	KCNE3	SLC25A21		DNAJA2	NR4A2	TMEFF1	CAMTA1	JOSD1	RRAD	
CCK	KCNJ2	SLC2A10		DNAJB14	OLFM1	TRIM2	CAPZA1	KCNJ12	RUNX1	
CCPG1	KCTD3	SLC38A2		DPYSL3	OSBPL8	UNQ1887	CCDC117	KIAA1576	SCYL3	
CDC2L6	KDELC2	SLITRK5		DUSP9	PAIP2	USP9X	CCDC43	KIAA1622	SEC23A	
CDK2AP1	KIAA0157	SPSB1		DYNC1LI2	PAPOLA	VDAC2	CCNK	KLHL20	SEMA3A	
CDK6	KIAA0831	SRP19		DYNLL2	PCDH10	WT1	CCNT2	LARGE	SETD3	
CEP97	KIAA1598	STK4		EIF4A2	PCGF3	YWHAG	CELSR3	LIN28	SGCB	
CFTR	KLF9	SUV420H1		EP300	PDE7B	ZCCHC11	CENTB2	LIN28B	SH3RF1	
CGGBP1	LOC51149	TBL1XR1		ETNK1	PFTK1	ZEB2	CFL2	LPHN3	SIRT1	
CIAO1	LONRF2	TCF12		FAM152A	PHF12	ZNF238	CHD7	LPPR4	SLC38A2	
CKS2	LPAR1	THAP9		FAM76B	PHF20L1	ZNF644	CHMP2B	LRRC17	SLC4A7	
CLASP2	LYPD6	TIMM8A		FAM91A1	PNN		CHST1	LRRC8D	SLC6A6	
CLIP4	MAP1LC3B	TLL2		FEM1C	POM121		COL13A1	LYCAT	SNAI1	
COG3	MKI67	TMEM17		FKBP2	PPM1G		CPNE8	MAML1	SNX16	
CSNK1G3	MTMR6	TMEM194A		FOXA1	PPP1R12A		DCUN1D3	MAT2A	SOCS3	
CYP4F3	MYEF2	TMEM57		FOXO3	PPP2R5C		DDAH1	MBNL3	SOX9	
DCLRE1B	MYO5A	TMF1		GATA2	PRICKLE2		DDIT4	MEX3B	SPEN	
DDX58	NAIF1	TMPRSS11D		GMFB	ProSAPiP1		DLL4	MIER3	SSX2IP	
DIRAS3	NARG1L	TNRC6B		GTDC1	PRPF4B		DNAJC13	MKRN3	ST8SIA4	
DLGAP2	NEK3	TRIM52		H2AFZ	PRRT2		DNMT3A	MMD	STAC	
DLX6	NEK4	TRIM59		H3F3B	PSMA2		DOLPP1	MTDH	STK35	
DNAJB14	NOVA1	TRUB1		HBEGF	PSMD12		EAF1	NAALADL2	STK39	
DTNA	NPY2R	TTC23		HIC2	PTBP2		EDEM3	NECAP1	STX2	
DUSP14	NRG1	TTC37		HMG2L1	RAB15		EIF5A2	NEDD4	SYPL1	

Supplement Table S2.7. List of the predicted targets of miRNAs differentially expressed in vastus lateralis of placentally restricted adult female offspring

hsa-miR-142-3p						
ACSL4	CCNT2	FNBP1L	ME1	PURB	STX12	ZFP91
ACVR2A	CD1D	FNDC3A	MGAT4A	RAB11A	SYN2	ZFR
ADAMTS3	CFL2	FNTB	MLL	RAB11FIP2	SYPL1	ZNF217
ADCY9	CHRNE	FOXF2	MLLT1	RAB1A	TAOK1	ZNF641
AFF1	CLCN5	FOXO1	MMD	RAB2A	TARDBP	ZNF644
AFF2	CLDN12	FOXO4	MMP16	RAB40C	TBC1D2B	ZNF676
AKT1S1	CLTA	GAB1	MOBKL3	RAC1	TESK2	ZNF827
ANK3	CNNM1	GFI1	MON2	RARG	TFG	
ANKRD11	COL24A1	GNAQ	MORF4L1	RGAG1	TGFBR1	
ANKRD46	COPG	GNB2	MORF4L2	RGL2	TIPARP	
ANKS1A	COPS7A	GTF2A1	MRFAP1	RHOBTB3	TJP1	
APC	CPEB2	HECTD1	MTMR9	RICTOR	TM2D2	
ARID5B	CREB5	HGS	MYH9	RLF	TMEM110	
ARL15	CRK	HMGA1	MYLK	RNF126	TMEM132E	
ARNTL	CRTAM	HMGA2	NBPF3	ROCK2	TMEM200B	
ARRDC3	CUGBP2	HMGB1	NR2C1	RPE	TMEM55B	
ASB7	CXADR	IL1RAPL1	NUMBL	S1PR3	TMEM59	
ASH1L	DCUN1D4	INPP5A	OSBP	SEMA6D	TMTC3	
ATF7IP	DIRC2	ITGAV	OSBPL3	SGK1	TNKS	
ATG16L1	DMTF1	ITGB8	PAFAH1B2	SGMS1	TP53INP2	
ATP2A2	DPY19L4	ITPKB	PATZ1	SH3GLB1	TRPS1	
BACH1	DSCR6	ITPR3	PCAF	SLC1A3	TSEN34	
BACH2	DST	JMJD1C	PCGF3	SLC30A9	TWF1	
BAZ1A	EDEM3	KLF13	PCMTD2	SLC35F5	USP33	
BCL2L1	EGR2	KLF4	PCSK1	SLC37A3	USP6NL	
BCLAF1	EHF	KLHDC1	PDE4B	SLC38A4	USP9X	
BHLHB9	EML4	KLHDC6	PDSS2	SLC7A11	UTRN	
BRWD3	EP400	LCOR	PELI1	SLCO4C1	UTX	
BTBD7	EPM2AIP1	LIFR	PICALM	SMG1	UTY	
C10orf56	ERC1	LRRC1	PIK3R6	SNF1LK	VAMP3	
C12orf53	ERG	LRRC3B	PMAIP1	SNF1LK2	VPS24	
C14orf24	FAM44B	LRRC59	PNO1	SOX11	WASL	
C18orf25	FAM46A	MAN1A2	PPFIA1	SOX6	WDR5B	
C1orf9	FAM80B	MAP3K11	PPP1R2	SP8	WHDC1	
C20orf112	FBXO21	MAP3K7IP	PPP1R3A	SPNS1	XPO1	
C5orf24	FBXO3	MAP4K3	PPP3CA	SPRED1	ZBTB41	
C9orf72	FGF23	MARCKS	PRLR	STAM	ZCCHC14	
CALM1	FKBP1A	MARK3	PTPN23	STAU1	ZEB1	
CCDC6	FMNL2	MBD6	PUM1	STRN3	ZEB2	

Supplement Table S2.7. *Continued*

hsa-miR-21						
ABCD2	CCDC121	FEM1C	MALT1	PLAA	SGK3	TRMT6
ABCD3	CCDC88A	FGF1	MAP3K7IP	PLAG1	SKI	TRPM7
ABHD2	CCDC93	FLJ14213	MAPK10	PLCB1	SLC12A6	TSNAX
ACBD5	CCL1	FLJ45139	MAPRE1	PLEKHA1	SLC22A15	TSPAN2
ACVR2A	CCL20	FRS2	MATN2	PM20D2	SLC2A13	TSPO
ADNP	CCRL1	FUBP1	MATR3	POM121	SLC35F5	TXNDC4
AKAP6	CCT5	FZD6	MBNL1	PPARA	SLC7A6	UBE2D1
ALDH1A1	CD47	GABRB2	MDGA2	PPP1R3A	SMAD7	UBE2D3
ALX1	CD69	GALNT12	MED21	PPP1R3D	SMARCD1	UBE4A
ANGPTL5	CDC25A	GANC	MED28	PPP3CA	SMURF2	UBR3
ARHGAP24	CENPP	GARNL1	MEGF9	PRKG1	SNED1	VHL
ARHGEF12	CENPQ	GATAD2B	MGC70863	PRPF39	SOCS5	WDR22
ARHGEF7	CEP97	GK5	MPRIP	PRPF4B	SOX2	WDR68
ARID4A	CFL2	GLCCI1	MRPL9	PRRG4	SOX5	WWC2
ARL1	CHD7	GLDN	MSH2	PRTFDC1	SOX6	WWP1
ARL4D	CHIC1	GLIS2	MTAP	PSRC1	SOX7	XCL1
ARMC8	CLCA3	GNG2	MTMR12	PTGER3	SPG20	XKR6
ASF1A	CLEC4A	GPC4	MTPN	PURB	SPON1	YAP1
ASPN	CNOT6	GPR180	NARG2	RAB11A	SPRY1	YOD1
ATP2B4	CNOT8	GPR64	NAT13	RAB11FIP2	SPRY2	ZADH2
ATRN	CNTFR	HAX1	NBEA	RAB22A	SRI	ZBTB38
ATXN10	COMMD8	HBP1	NCOA2	RAB6A	SSFA2	ZCCHC3
BAHD1	COX15	HNRNPK	NEGR1	RAB6C	ST3GAL6	ZDHHC17
BBS12	CPEB3	HNRNPU	NEK11	RAGE	ST6GAL1	ZFPM2
BCL11A	CREB5	IL12A	NFIB	RASA1	STAG2	ZNF10
BCL2	CREBL2	IPO11	NKIRAS1	RASGRP1	STAT3	ZNF200
BDH2	CRIM1	ITGB8	N-PAC	RBM22	STK38L	ZNF208
BEST3	DLX2	JAG1	NPTN	RBMS3	STK40	ZNF295
BMP8A	DMD	JHDM1D	NSL1	RCCD1	SYT14L	ZNF367
BMPR2	DNAJC16	JPH1	NTF3	RECK	TAF5	ZNF431
BNC2	DNM1L	KBTBD6	NUPL2	RHOB	TAGAP	ZNF576
BOLL	E2F3	KCNA1	OLFM3	RMND5A	TBX2	ZNF592
BRCC3	EGLN1	KCNA3	OSR1	RNF103	TESK2	ZNF654
BRD1	EHD1	KIAA0564	PABPC5	RNF111	TET1	ZNF704
BTBD3	EIF1AX	KIAA1012	PAG1	RNF6	TGFBI	ZNF708
BTG2	EIF2C2	KIAA1333	PAN3	RP2	TGFBR2	ZRANB1
C12orf12	ELF2	KIAA1468	PBRM1	RPS6KA3	THPO	
C17orf39	ELOVL7	KIAA2026	PCBP1	RSAD2	THRB	
C3orf57	EPHA4	KLHDC5	PCGF5	RSBN1	TIAM1	
C4orf16	EPM2A	KRIT1	PCSK6	RTKN2	TIMP3	
C4orf33	FAM107B	LANCL1	PDCD4	SACM1L	TLR4	
C5orf41	FAM133A	LEMD3	PDLIM5	SALL3	TNFRSF10B	
C7	FAM13A1	LOC15078	PDZD2	SATB1	TNPO1	
CA1	FAM46A	LOC15336	PELI1	SCML2	TNRC6B	
CADM2	FAM63B	LONRF2	PELI2	SCN8A	TNS1	
CALCB	FASLG	LPA	PER2	SCRN1	TOR1AIP2	
CAPN2	FBXL17	LRAT	PHF14	SETD6	TRAPPC2	
CASC4	FBXL2	LRRC57	PIK3R1	SETD8	TRIM2	
CASKIN1	FBXO11	LUM	PIP5K3	SETX	TRIM33	
CBX4	FCHO2	LYRM7	PITX2	SFRS3	TRIM9	

Supplement Table S2.7. *Continued*

hsa-miR-101					
ABCC5	CDK6	FLRT3	MBNL1	PRPF4B	STAG2
ABLIM3	CDYL	FMR1	MED14	PTCH1	STC1
ACCN2	CLCN3	FOS	MEF2D	PTGS2	STK4
ACVR2B	COL10A1	FZD4	MEPCE	PUM2	STMN1
ADCY6	CPEB2	FZD6	MET	PURB	SUB1
ADRB1	CPEB3	GJA1	MITF	RAB14	SULT4A1
AEBP2	CTCF	GLCCI1	MOBKL3	RAB5A	TAL1
ANKRD17	CTTNBP2	GLTSCR1	MPP5	RANBP9	TGFBR1
ANKS1A	CUGBP2	GNB1	MPPE1	RAP1B	TLK2
AP1G1	DAG1	GOLGA4	MTMR2	RAP2C	TMEM161B
APP	DCBLD2	GPR85	MYCN	RAPGEF5	TNRC6B
ARHGAP26	DDIT4	GZF1	MYRIP	RAPH1	TP63
ARID1A	DDX3X	HAS2	NARG1	RASD2	TRIB1
ASPN	DIP2B	HNRNPF	NDFIP1	RBBP7	TRIM2
ATP2B2	DLG5	HOXD4	NEUROD1	REV3L	TSC22D1
ATRX	DMD	ICK	NLK	RGS1	TSHZ3
ATXN1	DNMT3A	IGFBP5	NOVA1	RIN2	TULP4
BAZ2A	DR1	IKZF2	NXPH1	RIPK5	TULP4
BCL9	DUSP1	IKZF4	OTUD4	RNF111	TXNDC4
BICD2	DYRK1A	KBTBD8	PAFAH1B1	RNF38	UBE2D1
BTBD3	EDEM3	KCNA1	PANK1	ROBO2	UBE2D2
BZW2	EED	KCNH7	PAPOLG	RUNX1	UBE2Q1
C10orf83	EIF5	KIAA0152	PCDH18	SACM1L	USP38
C12orf34	ELF5	KIAA0182	PCDH8	SCN2A	USP47
C16orf72	EMP1	KIAA0423	PDE4A	SEL1L	VKORC1L1
C1orf52	EPB41L1	KIAA1244	PDE4D	SELI	ZBTB33
C20orf20	ERBB2IP	KIAA1370	PDS5B	SEMA3G	ZBTB41
C3orf58	ESCO1	KIAA1409	PHF20L1	SH2B3	ZCCHC2
CACNB2	ETNK1	KIF1A	PHF6	SHANK2	ZFAND3
CADM1	ETV5	KPNA3	PHLDA1	SLC12A2	ZFHX4
CAMSAP1L1	EXOC5	KPNB1	PHTF2	SLC19A2	ZNF207
CAMTA1	EYA1	LASS2	PIK3C2B	SLC1A1	ZNF24
CAV3	EZH2	LONRF1	PIP5K3	SLC30A7	ZNF385B
CBFA2T2	FAM108B1	LRCH2	PKNOX1	SLC4A4	ZNF532
CCDC18	FAM152A	LRRC4	PLXNB1	SMARCA1	ZNF618
CCDC88A	FAM78A	LRRFIP2	POGZ	SNF1LK2	ZNF746
CCNJ	FBN2	LRRN1	POMGNT1	SOCS2	ZNRF2
CDC2L6	FBXW11	MAGI1	PPFIA1	SOCS5	
CDH11	FEM1C	MAGI2	PPP1R12A	SOX9	
CDH5	FGA	MAK	PPP2R5E	SPRED1	
CDK5R1	FLRT2	MARK1	PRKAA1	ST18	

Supplement Table S2.7. *Continued*

hsa-miR-17-5p					
ABHD2	DYNC1LI2	MAP3K14	PCDHAC2	SENP1	YTHDF3
ACSL4	EGLN3	MAP3K2	PEX5L	SERP1	ZFP91
APBB2	EIF2C1	MAP3K8	PFN2	SGMS1	ZNF148
ARHGAP1	EIF4H	MAP3K9	PGBD5	SKI	ZNF238
ARHGAP12	EIF5A2	MAPRE1	PHTF2	SLC22A23	ZNF25
ARID4A	ELK3	MASTL	PKD2	SMAD7	ZNF362
ARID4B	ENPP5	MAT2B	PKIA	SMOC2	ZNF512B
ARL4C	EPHA4	MED12L	PKNOX1	SOX4	ZNF800
ATAD2	EREG	MGEA5	PLAGL2	SPOPL	ZNFX1
ATP1A2	ETV1	MKRN1	PPARA	SPRY4	
BAMBI	EZH1	MTMR3	PPP3R1	SSH2	
BCL11B	F3	MYO1D	PPP6C	SSX2IP	
BICD2	FAM126B	NAPEPLD	PRR16	STAT3	
BTBD7	FAM134A	NBEA	PRRG1	STK38	
BTG3	FAM46C	NBL1	PTPN3	SUMF1	
C11orf30	FAM57A	NCOA3	PTPN4	SUV420H1	
C19orf2	FAM60A	NEDD4L	PURB	TMCC1	
C5orf41	FBXL5	NETO2	RAB10	TMEM127	
C7orf43	FBXW11	NEUROG1	RAB11FIP1	TNFRSF21	
CALD1	FGD5	NKIRAS1	RAB22A	TNKS2	
CAMK2N1	FLJ31818	NPAS2	RAB5B	TNRC6A	
CAMTA1	FNDC3B	NPAS3	RAP2C	TOPORS	
CCNG2	FOXL2	NR4A2	RAPGEFL1	TP53INP1	
CDC37L1	GAB1	NR4A3	RASL11B	TRIM3	
CDKN1A	GABBR2	NTN4	RB1	TRIP10	
CEP57	GNB5	ORMDL3	RBBP7	TRIP11	
CEP97	GOLSYN	OTUD4	RBL1	TSG101	
CFL2	HBP1	PAPOLA	RBL2	TSHZ3	
CHD9	HIF1A	PBX3	RGL1	TXNIP	
CLIP4	HLF	PCAF	RGMA	UBE2Q2	
CMPK1	HMGB3	PCDHA10	RHOV	UBR5	
CNOT7	INOC1	PCDHA12	RNF128	ULK1	
COL4A3	ITGB8	PCDHA13	RP5	USP3	
CRIM1	KIAA0922	PCDHA3	RPS6KA5	USP46	
CRY2	KLF12	PCDHA4	RSBN1	VANGL1	
DCBLD2	KLHL20	PCDHA5	RSRC2	VEGFA	
DDX5	LACE1	PCDHA6	RUNX1	VSX1	
DMTF1	LAPTM4A	PCDHA7	SALL1	WAC	
DPYSL2	LIMA1	PCDHA8	SASH1	WDFY3	
DPYSL5	LIMK1	PCDHAC1	SEMA4B	WDR37	

Supplement Table S2.7. *Continued*

dme-miR-278					hsa-miR-376b
abd-A	CG1572	CG3860	crm	nAcRalpha-96Aa	ANK2
alphaTub84B	CG15835	CG5010	Cyp6a14	NetB	ATP6V1G1
Asph	CG16798	CG5098	dah	nito	BMPR2
Atpalpha	CG16833	CG5278	dap	Nop56	BRUNOL4
att-ORFB	CG17352	CG5669	DDB1	oa2	BRWD1
B52	CG17562	CG5674	desert	OstStt3	COPS7A
bab1	CG17744	CG5687	didum	par-1	CRISPLD2
brat	CG18170	CG5830	dob	Pde8	CUX2
Bsg25D	CG18223	CG5899	DopR2	Pif1A	DSCAML1
CG10068	CG18431	CG5934	dpr10	plx	EBF2
CG10263	CG18812	CG6017	drm	pros	EIF2C2
CG10731	CG1910	CG6123	Dscam	ptc	EIF4B
CG10804	CG1968	CG6665	dx	qkr54B	FAM131B
CG11190	CG2006	CG6672	Ect4	Rab3	FBXO11
CG1136	CG2680	CG6783	epsin-like	Rac1	FERMT2
CG11597	CG30043	CG6897	Ets65A	Rad17	GPC6
CG11779	CG3036	CG6923	ewg	rad201	GPR180
CG11889	CG3075	CG7144	ex	RhoGAPp190	GRAMD3
CG12105	CG31191	CG7518	faf	Rlip	HNRNPA0
CG12609	CG31286	CG7852	fry	Rpd3	IKZF2
CG12701	CG3156	CG7872	Ggamma30A	RpI12	KHDRBS3
CG12814	CG31828	CG8271	gl	rst	KIAA0494
CG13175	CG32062	CG8290	Gld	Sas10	MACROD2
CG13436	CG32199	CG8407	hig	Sema-1a	NFKBIZ
CG13569	CG32500	CG8726	HLHmdelta	SF2	PDIA6
CG13624	CG32529	CG8726	Hr4	sl	PEX12
CG13705	CG32556	CG9007	Indy-2	Socs36E	PLEKHA5
CG13784	CG3257	CG9130	kel	sol	RAB3IP
CG14017	CG32594	CG9133	king-tubby	spt4	RBMS1
CG14053	CG32662	CG9339	LSm-4	su(w[a])	RYBP
CG14245	CG32736	CG9339	lz	Syn	SCOC
CG14330	CG33248	CG9411	mab-2	Tapdelta	SETD7
CG14442	CG33528	CG9505	mei-217	Tom40	SIDT2
CG14669	CG3355	CG9743	Mes2	Tsp66E	SLC6A1
CG14681	CG33785	CG9813	meso18E	twin	SYT16
CG14714	CG33981	chif	micr	Vago	TMTC4
CG14762	CG34140	chp	Mob1	Vinc	VPS54
CG14881	CG3638	Clk	mRpL37	wun2	
CG15083	CG3651	coro	mura	zfh1	
CG15203	CG3711	cpo	mus81		

Supplement Table S2.7. *Continued*

hsa-miR-324-4p						
ABCA1	CKB	FAM62A	INOC1	MMP15	RAB11FIP1	SUDS3
ABCG1	CLEC16A	FAM71C	INPPL1	MMP2	RAB5C	SUPT6H
ACVRL1	CNIH2	FBXO40	JRK	MMP24	RAB5C	SYNGR1
ADAMTS17	CNNM4	FLJ36031	KCNMA1	MRPL19	RAGE	SYT12
ADARB1	CNOT6L	FLJ45224	KIAA0152	MSN	RARG	TBC1D2B
ADO	CNR1	FOXJ3	KIAA0265	MTERFD2	RBED1	TEGT
AKT2	COL22A1	FOXN3	KIAA0427	MTHFR	RER1	TFE3
ALPK3	COL7A1	FOXP1	KIAA1147	MXI1	RGS14	TMBIM1
ALS2CL	CRHR1	FTH1	KIF5C	MYLK2	RIMS3	TMEM101
AP4S1	CRMP1	FURIN	KIF7	MYO5A	RIMS4	TMEM109
ARHGAP11A	CSAD	FZD4	KLHDC6	NCKIPSD	RNF112	TMEM129
ARHGAP25	CTDSP1	G3BP2	KLHL18	NDST2	RNF121	TNFRSF10D
ARID3B	CYP19A1	GABRA4	KLHL22	NEURL	RNF149	TOB2
ARL16	CYP4F11	GALNT10	KSR2	NIPSNAP1	RPRML	TOMM20
ATP2B2	DACT1	GARNL4	LAIR1	NKIRAS2	RYK	TOX4
ATRX	DAG1	GATA2	LAIR1	NRP1	SAP18	TPPP
BAHD1	DEPDC5	GATAD2B	LDLRAD2	OAF	SDF2	TRAF7
BAZ2A	DGCR2	GBGT1	LDLRAP1	OLA1	SELE	TRPC1
BCL11A	DKFZp761E198	GFAP	LGALS8	OLA1	SENP2	TRPV3
BCL2L11	DLST	GHITM	LHFPL4	OTUD4	SETD4	TSPAN15
BICD2	DNALI1	GIT1	LIMD2	OXSR1	SFPQ	TTLL12
C13orf34	DPY19L3	GJB2	LOC51057	P15RS	SFXN5	VANGL1
C1orf159	DSC1	GLP1R	LOC51057	PADI1	SH3BP2	VPRBP
C1orf21	DUSP1	GPATCH8	LOC646174	PCBP4	SH3TC2	WASF2
C20orf112	DYNC1LI2	GPR114	LPHN1	PCTP	SHANK2	WDR44
C20orf54	EDAR	GPR124	LPPR2	PDE2A	SHC4	WDR5B
C22orf15	EFEMP2	GRIN1	LRRFIP2	PDRG1	SHISA3	WNT3A
C2CD2L	EFNB2	GSG1L	LUC7L2	PEX19	SHROOM3	WWOX
C2orf16	EIF4G1	HBP1	LYNX1	PGAM1	SLC25A35	YTHDC1
C9orf25	ERC1	HDAC5	MAP3K7IP1	PHF17	SLC34A2	ZNF3
CADM4	ESAM	HEATR6	MAP3K7IP2	PI16	SMAD7	ZNF346
CALCRL	ESR1	HECW1	MAPK1	PIK3R1	SORCS1	ZNF502
CAMKV	ETF1	HELZ	MAPKAPK2	PLAGL2	SORCS3	ZNF518B
CARM1	ETS2	HES2	MBD6	PLXND1	SOX11	ZNF532
CCDC69	ETV5	HHAT	MDGA1	PNMA5	SRGAP3	ZNF696
CD109	ETV6	HIC2	MEF2D	PNPLA4	SRR	ZNF711
CD47	FAM126A	HNRNPA3	MEMO1	POLR3H	SRRM2	ZNF783
CDH5	FAM155B	HTR2C	MEP1A	PRKACB	SSH2	ZZEF1
CHD4	FAM168B	IGF2	MEPCE	PSCD3	STAG2	
CHFR	FAM26E	IGF2BP1	MGEA5	PTPRT	STIP1	
CIT	FAM29A	IL10RA	MKI67	QSOX2	STK32B	

hsa-miR-17-5p									
ABCA1	C7orf43	EGLN3	GAB1	KPNA2	NBL1	PGBD5	RHOV	SSFA2	UNKL
ABHD2	CALD1	EGR2	GABPB2	KPNA3	NCOA3	PHF1	RNF128	SSH2	USP3
ACSL4	CAMK2N1	EHD3	GAN	L3MBTL3	NEDD4L	PHF6	RNF145	SSX2IP	USP31
ADAM9	CAMTA1	EIF2C1	GATAD2B	LACE1	NETO2	PHTF2	RNF6	ST6GALNAC3	USP46
AKT3	CC2D1A	EIF4H	GBF1	LAPTM4A	NEUROG1	PITPNA	RP5-10	ST8SIA2	USP6
ALS2CR13	CCDC100	EIF5A2	GIGYF1	LDLR	NFAT5	PKD1	RPS6KA5	STAT3	VANGL1
ALX4	CCDC128	ELAVL2	GLIS3	LDLRAP1	NHLH1	PKD2	RRM2	STK38	VEGFA
ANK2	CCND1	ELK3	GNB5	LHX6	NIPA1	PKIA	RSBN1	STYX	VPS26A
ANKRD13C	CCND2	ENPP5	GOLSYN	LHX8	NKIRAS1	PKN2	RSRC2	SUMF1	VSX1
ANKRD28	CCNG2	EPAS1	GOSR1	LIMA1	NPAS2	PKNOX1	RUFY2	SUV420H1	WAC
ANKRD50	CCNJ	EPHA4	GPR6	LIMK1	NPAS3	PLAGL2	RUNX1	SYAP1	WDFY3
ANKRD9	CCNT2	EPHA5	GRHL2	LPGAT1	NPAT	PLCB1	S1PR1	TAL1	WDR37
ANUBL1	CD69	EPHA7	GRM7	LRIG1	NPLOC4	PLEKHM1	SACS	TANC1	WEE1
APBB2	CDC37L1	EREG	HAS2	LYPD6	NR2C2	PLEKHO2	SALL1	TBC1D20	WNK3
APP	CDKN1A	ETF1	HBP1	M6PR	NR2E3	PLS1	SAPS3	TBC1D8B	XRN1
ARHGAP1	CENPO	ETV1	HDAC4	MAP3K11	NR4A2	PLXNA1	SAR1B	TET1	YES1
ARHGAP12	CEP57	EZH1	HECA	MAP3K12	NTN4	PPARA	SASH1	TGFBR2	YOD1
ARHGAP26	CEP97	F3	HIF1A	MAP3K14	NUP35	PPP2R2A	SCRT2	TGOLN2	YPEL2
ARHGEF18	CFL2	FAM102A	HLF	MAP3K2	OBFC2A	PPP3CA	SEMA4B	TIMP2	YTHDF3
ARHGEF3	CHAF1A	FAM126B	HMGA2	MAP3K3	OCRL	PPP3R1	SENP1	TLE4	ZBTB4
ARID4A	CHD9	FAM130A2	HMGB3	MAP3K5	ORMDL3	PPP6C	SFMBT1	TMCC1	ZBTB47
ARID4B	CLIP4	FAM134A	HN1	MAP3K8	OSR1	PREX1	SFRS2	TMEM127	ZBTB6
ARL4C	CLOCK	FAM134C	HPS5	MAP3K9	OTUD4	PRR15	SGMS1	TMEM168	ZBTB7A
ATAD2	CMPK1	FAM13C1	HRBL	MAP7	PAF1	PRR16	SH3PXD2A	TMUB2	ZBTB9
ATG16L1	CNOT6	FAM40B	IKZF4	MAPK4	PAFAH1B1	PRRG1	SKI	TNFAIP1	ZDHHC1
ATL3	CNOT7	FAM45A	IL25	MAPK9	PAK7	PTEN	SLC16A9	TNFRSF21	ZDHHC9
ATP1A2	CNR1	FAM46C	IL8	MAPRE1	PANX2	PTGFRN	SLC17A7	TNFSF11	ZFP91
ATXN1	COL4A3	FAM57A	INOC1	MARK4	PAPOLA	PTHLH	SLC1A2	TNKS1BP1	ZFPM2
BAHD1	CRIM1	FAM60A	INTS6	MASTL	PBX3	PTPN3	SLC22A23	TNKS2	ZFYVE26
BAMBI	CRK	FASTK	INTS6	MAT2B	PCAF	PTPN4	SLC24A4	TNRC6A	ZFYVE9
BCL11B	CRY2	FAT2	IRF1	MCL1	PCDHA1	PURB	SLC2A4	TNRC6B	ZHX2
BCL2L11	CYP26B1	FBXL11	ITCH	MECP2	PCDHA10	RAB10	SLC2A4RG	TOPORS	ZNF148
BCL2L2	DAZAP2	FBXL5	ITGB8	MED12L	PCDHA11	RAB11FIP1	SLC30A3	TP53INP1	ZNF217
BHLHB3	DCBLD2	FBXO21	JAK1	MEX3D	PCDHA12	RAB22A	SLC40A1	TP53INP2	ZNF236
BICD2	DDHD1	FBXW11	JAZF1	MFAP3L	PCDHA13	RAB30	SLITRK3	TRIM3	ZNF238
BMPR2	DDX5	FCHO2	JOSD1	MGEA5	PCDHA2	RAB5B	SMAD5	TRIM36	ZNF25
BNC2	DERL2	FEM1C	KBTBD8	MINK1	PCDHA3	RAP2C	SMAD7	TRIP10	ZNF280B
BRMS1L	DIP2A	FGD5	KCNJ10	MKRN1	PCDHA4	RAPGEF4	SMOC1	TRIP11	ZNF362
BTBD10	DLGAP2	FJX1	KIAA0494	MLL4	PCDHA5	RAPGEFL1	SMOC2	TRPV6	ZNF367
BTBD7	DMTF1	FLJ31818	KIAA0513	MORF4L1	PCDHA6	RASD1	SNIP	TSG101	ZNF512B
BTG3	DNAJB9	FLT1	KIAA0922	MTF1	PCDHA6	RASL11B	SNX16	TSHZ3	ZNF697
C10orf46	DPYSL2	FNBP1L	KIAA1128	MTMR3	PCDHA7	RB1	SOCS6	TUSC2	ZNF800
C11orf30	DPYSL5	FNDC3A	KIF23	MYCN	PCDHA8	RB1CC1	SOX4	TWF1	ZNFX1
C14orf28	DUSP2	FNDC3A	KIF3B	MYO1D	PCDHA9	RBBP7	SP8	TXNIP	
C16orf72	DUSP8	FNDC3B	KIF5A	MYT1L	PCDHAC1	RBL1	SPOPL	UBE2Q2	
C19orf2	DYNC1LI2	FOXJ2	KLF11	NANOS1	PCDHAC2	RBL2	SPRY4	UBE2W	
C1orf63	DYRK1A	FOXL2	KLF12	NAP5	PDE3B	REEP3	SPTY2D1	UBFD1	
C1orf9	E2F1	FRMD6	KLF9	NAPEPLD	PDGFRA	REPS2	SR140	UBR5	
C2CD2	E2F3	FRMD6	KLHL20	NAT12	PEX5L	RGL1	SRPK1	ULK1	
C5orf41	E2F5	FURIN	KLHL28	NBEA	PFN2	RGMA	SS18L1	UNK	

Supplement Table S2.7. *Continued*

hsa-miR-376b					
ACSL1	EIF2C2	KBTBD6	PDE8A	SNX27	ZNF833
ACSL5	EIF4B	KCNT2	PDIA6	SORBS2	ZNF84
ADARB1	ELOVL2	KHDRBS3	PEX12	SORCS3	
ADNP	ELP4	KIAA0157	PGAP1	SORD	
AHSA2	ENAH	KIAA0494	PHF17	SP1	
AMAC1	ENOX2	KIAA1147	PI15	ST6GALNAC3	
ANK2	ENPP3	KIAA1407	PLAG1	STARD13	
ANK2	EZR	KIAA1804	PLEKHA5	SULF1	
ANKRD28	FAM120C	KIAA2026	PLK4	SYT16	
AQR	FAM131B	KLF12	POLK	TERF1	
ARMC10	FAM23B	KLF15	PON2	TGFA	
ATP2B1	FAM98A	KPNA4	PPP1R10	TGFBRAP1	
ATP6V1G1	FBXO11	LAMC1	PRKACB	TIAL1	
BCL2L11	FERMT2	LARP4	PRLR	TIFA	
BHLHB2	FGFR1	LIPH	PRMT8	TLR2	
BMPR2	G6PC2	LMBR1	PRSS35	TMEM100	
BNC1	GABRB2	LOC130576	PSMD10	TMEM2	
BPTF	GABRG1	LRPPRC	PYROXD1	TMTC4	
BRUNOL4	GAL3ST4	LUZP2	QKI	TNIP3	
BRWD1	GALNT17	MACROD2	RAB1A	TRIM13	
C10orf129	GALNTL2	MBD5	RAB3IP	TRIM22	
C14orf106	GCG	MEGF11	RALA	TRIM60	
C16orf44	GCLC	MGA	RBMS1	TSPAN9	
C21orf66	GLE1	MIB1	RFTN2	UBE2D2	
C4orf41	GLIPR1	MT1X	RFX4	UST	
CAPZA1	GPC6	MTMR12	RFX4	VHLL	
CASP9	GPR180	MTMR2	RHBDL2	VPS26A	
CBFB	GRAMD3	MTRF1	ROCK2	VPS29	
CCDC47	GRIK2	NAP5	RP1	VPS54	
CD1C	GRIK5	NAV3	RPS6KA3	WBSCR19	
CDC2L6	HDAC9	NDST1	RYBP	WDR22	
CEBPB	HELLS	NEDD4	SALL1	WIPI2	
CFTR	HES5	NEK11	SATB1	YEATS4	
CH25H	HFE	NFIB	SCOC	ZBTB43	
CHD9	HGF	NFKBIZ	SERPINB9	ZBTB7A	
COL14A1	HNRNPA0	NFX1	SETD7	ZCCHC7	
COPS7A	HRNR	NIP7	SGCD	ZFHX3	
CRISPLD2	HSPB7	NIPBL	SGMS1	ZFR	
CSNK1G3	IARS2	NLGN4X	SIDT2	ZKSCAN1	
CUGBP2	IGF1R	NPNT	SLC2A10	ZMYND11	
CUX2	IKZF2	NRP1	SLC30A9	ZNF107	
DACH1	IL20RA	NUP160	SLC34A2	ZNF180	
DDHD1	IL22RA2	NUPL1	SLC4A10	ZNF189	
DLAT	IL7	OPALIN	SLC6A1	ZNF254	
DLX5	IMPG2	P53AIP1	SLC6A15	ZNF439	
DSCAML1	INSIG2	PAK7	SLC7A11	ZNF545	
DSE	INTS2	PANX3	SLC7A2	ZNF548	
DZIP1	IQGAP2	PCDH17	SLFN11	ZNF566	
EBF2	ISCA1L	PCDHB12	SLITRK6	ZNF662	
EIF2A	JOSD1	PCDHB9	SMARCA2	ZNF80	

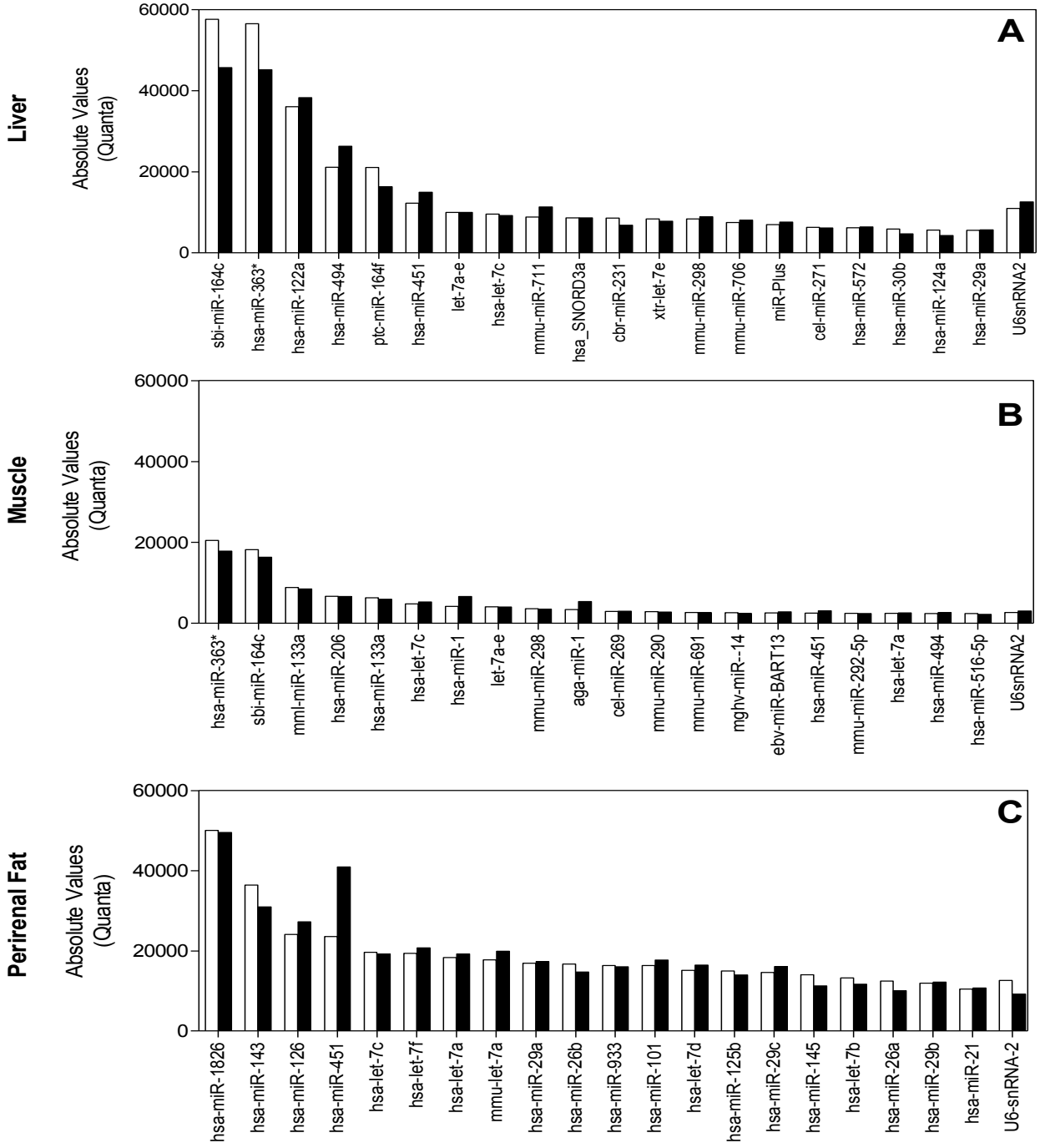
Supplement Table S2.8. List of the predicted targets of differentially expressed hsa-miR-451 in perirenal fat of placentally restricted adult offspring

hsa-miR-451			
ACADSB	DBX2	MEX3C	TSC1
AKTIP	DTX3L	NRARP	TTN
ATF2	EREG	OSR1	USP46
C11orf30	FMNL3	PMM2	VAPA
CAB39	GADL1	PRICKLE2	WDFY2
CDKN2B	GALK2	SAMD4B	YTHDF2
CMTM6	GRSF1	SEC23IP	ZIC3
CTNNBIP1	KIF24	ST8SIA4	ZNF644
CXCL16	MBP	TBC1D9B	

Supplement Table S2.9. List of sheep, human and rat sequences for most abundant or differentially expressed microRNAs following placental restriction in sheep

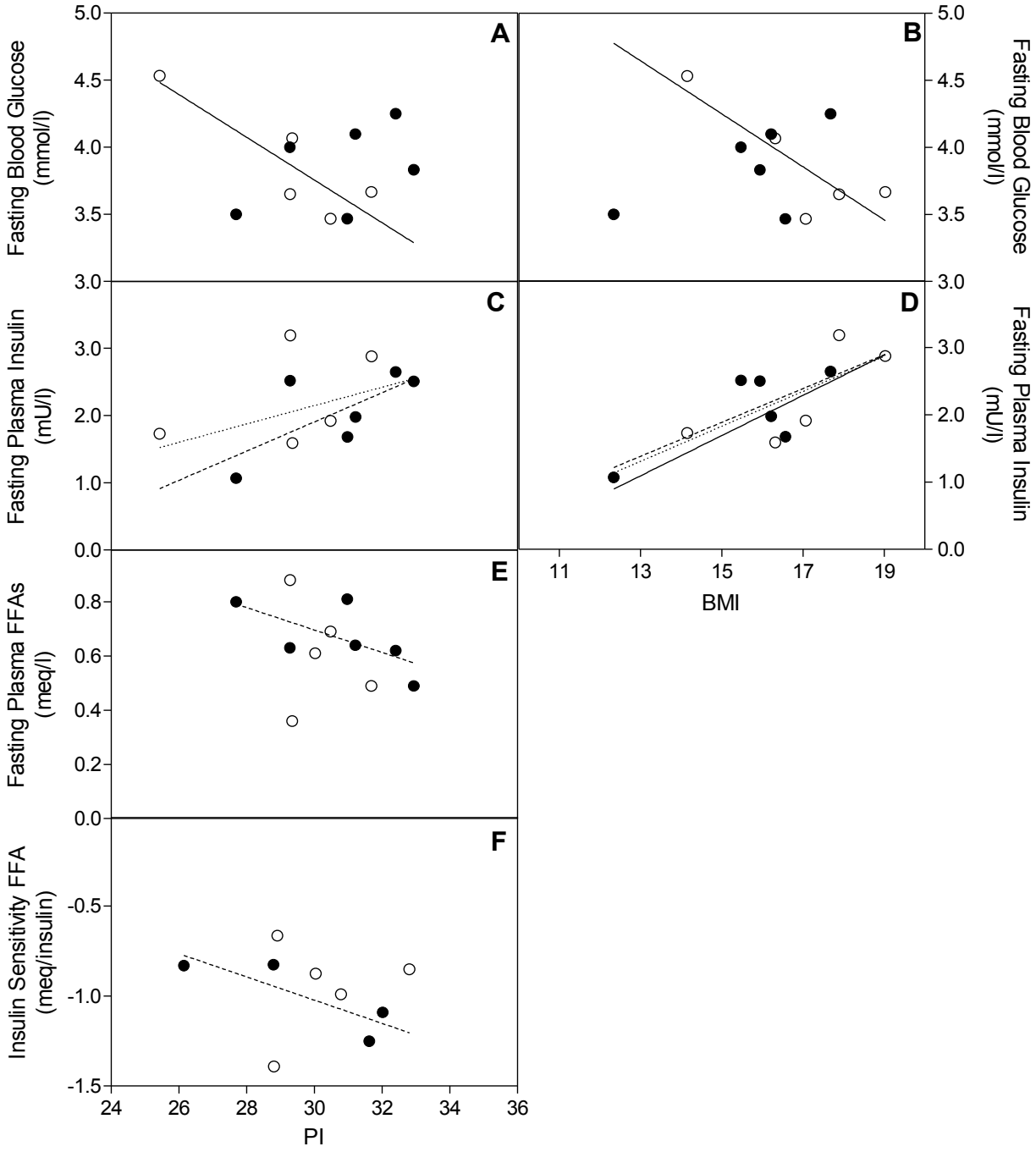
Most abundant	Sheep	Human	Rat
Liver			
miR-363*	cggguggaucacgaugcaauuu	cggguggaucacgaugcaauuu	cggguggaucacgaugcaauuu
miR-122a	uggagugugacaaugguguuug	uggagugugacaaugguguuug	uggagugugacaaugguguuug
Skeletal Muscle			
miR-206	uggaauguaaggaagugugugg	uggaauguaaggaagugugugg	uggaauguaaggaagugugugg
miR-133a	uuggucccuucaaccagcugu	uuuggucccuucaaccagcug	uuuggucccuucaaccagcug
miR-1	uggaauguaaagaaguauguau	uggaauguaaagaaguauguau	uggaauguaaagaaguauguau
let-7c	ugagguaguagguuguaugguu	ugagguaguagguuguaugguu	ugagguaguagguuguaugguu
let-7a	ugagguaguagguuguauaguu	ugagguaguagguuguauaguu	ugagguaguagguuguauaguu
Adipose			
miR-143#	caguguucuacaguccguc	ugagaugaagcacuguagcuc	ugagaugaagcacuguagcuca
miR-126	cguaccgugaguaauaaugcga	ucguaccgugaguaauaaugcg	ucguaccgugaguaauaaugcg
let-7c	ugagguaguagguuguaugguu	ugagguaguagguuguaugguu	ugagguaguagguuguaugguu
let-7f	ugagguaguagauuguauaguu	ugagguaguagauuguauaguu	ugagguaguagauuguauaguu
let-7a	ugagguaguagguuguauaguu	ugagguaguagguuguauaguu	ugagguaguagguuguauaguu
miR-29a	acugauuucuuuugguguucaga	acugauuucuuuugguguucag	acugauuucuuuugguguucag
miR-26b	uucaaguaauucaggauagguu	uucaaguaauucaggauaggu	uucaaguaauucaggauaggu
miR-101#	gggaaugucaagaagaauguau	uacaguacugugauaacugaa	uacaguacugugauaacugaa
let-7d	agagguaguagguugcauaguu	agagguaguagguugcauaguu	agagguaguagguugcauaguu
Differentially expressed			
Liver			
miR-142-3p	guaguguuuccuacuuuaugg	uguaguguuuccuacuuuaugga	uguaguguuuccuacuuuaugga
miR-134	ugugacugguugaccagagggg	ugugacugguugaccagagggg	ugugacugguugaccagagggg
miR-1	uggaauguaaagaaguauguau	uggaauguaaagaaguauguau	uggaauguaaagaaguauguau
miR-21	uagcuuaucagacugauguuga	uagcuuaucagacugauguuga	uagcuuaucagacugauguuga
miR-144	ggauaucaucauauacuguaag	ggauaucaucauauacuguaag	ggauaucaucauauacuguaagu
miR-212	accuuggcucuagacugcuuacu	accuuggcucuagacugcuuacu	accuuggcucuagacugcuuacug
Skeletal Muscle			
miR-142-3p	guaguguuuccuacuuuaugg	uguaguguuuccuacuuuaugga	uguaguguuuccuacuuuaugga
miR-21	uagcuuaucagacugauguuga	uagcuuaucagacugauguuga	uagcuuaucagacugauguuga
miR-101#	gugacugacaggcugcccuggc	caguuaucacagugcugaugcu	ucaguuaucacagugcugaugc
miR-376b	ggauauuccuucuauguuu	cguggauauuccuucuauguuu	guggauauuccuucuaugguua
miR-324-3p	ccacugccccaggugcugcugg	acugccccaggugcugcugg	ccacugccccaggugcugcugg
Endogenous control			
miR-26a	uucaaguaauccaggauaggcu	uucaaguaauccaggauaggcu	uucaaguaauccaggauaggcu
miR-92	uauugcacuugucccggccugu	uauugcacuugucccggccugu	uauugcacuugucccggccugu

Known sheep miRNA sequences are identical in human and rat, except for those that marked with #. MiRNA sequences from the sheep were obtained from Caiment et al. 2010 (Caiment *et al.*, 2010), and human and rat miRNA sequences were obtained from miRBase. *: the minor star miRNA molecule class.



Supplement Figure S2.1. Twenty most highly expressed miRNAs detected in insulin sensitive tissues by microarray, in control and placentally restricted sheep offspring

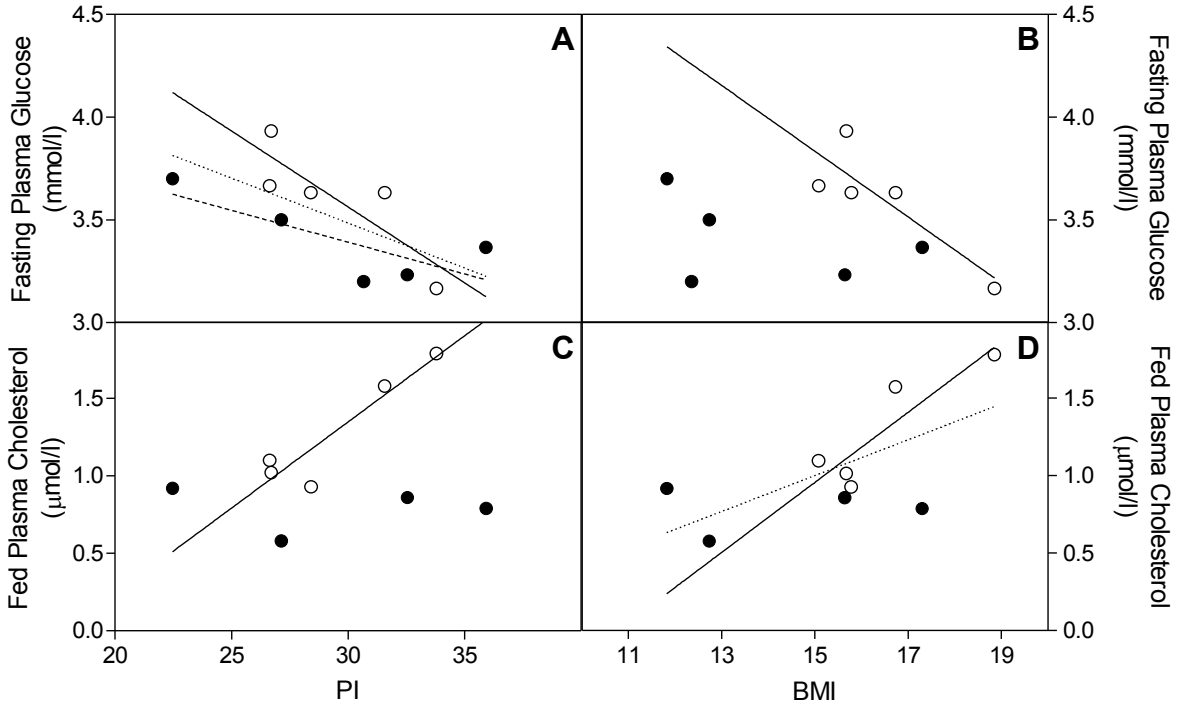
Tissue specific miRNAs, as reported from other animals, were detected as highly expressed miRNAs from each tissue. In vastus lateralis, expression of miR-1, miR-133a and miR-206 were amongst the highly expressed miRNAs (A). In liver, expression of miR-122a was amongst the highly expressed miRNAs (B). In perirenal fat, expression of miR-143 was amongst the highly expressed miRNAs. U6-snRNA2 shown as a positive control. Controls shown as white bars, placentally restricted shown as black bars.



Supplement Figure S2.2. Associations between plasma metabolites in adult male offspring and size at birth

Adult male offspring: Fasting blood glucose and **(A)** PI in controls (r = -0.875, *P*-value = 0.026, n: 5), and **(B)** BMI in controls (r = -0.848, *P*-value = 0.035, n: 5) at birth. Associations were not significant in placentally restricted offspring. Fasting plasma insulin and **(C)** PI in placentally restricted (r = 0.693, *P*-value = 0.063, n: 4), and combined offspring (r = 0.465, *P*-value = 0.075, n: 9), and **(D)** BMI in controls (r = 0.759, *P*-value = 0.068, n: 5), in placentally restricted (r = 0.741, *P*-value = 0.046, n:

4) and in combined offspring (r = 0.749, *P*-value = 0.004, n: 9). Fasting plasma FFAs and **(E)** PI in placentally restricted offspring (r = -0.683, *P*-value = 0.074, n: 4). Association was not significant in control offspring. Insulin sensitivity FFAs and **(F)** PI in placentally restricted offspring (r = -0.848, *P*-value = 0.076, n: 4). Association was not significant in control offspring. White circle: control; black circle: PR; solid line: control; Dashed line: placental restriction linear correlation; Dotted line: combined linear correlation; BMI: Body Mass Index; PI: Ponderal Index.

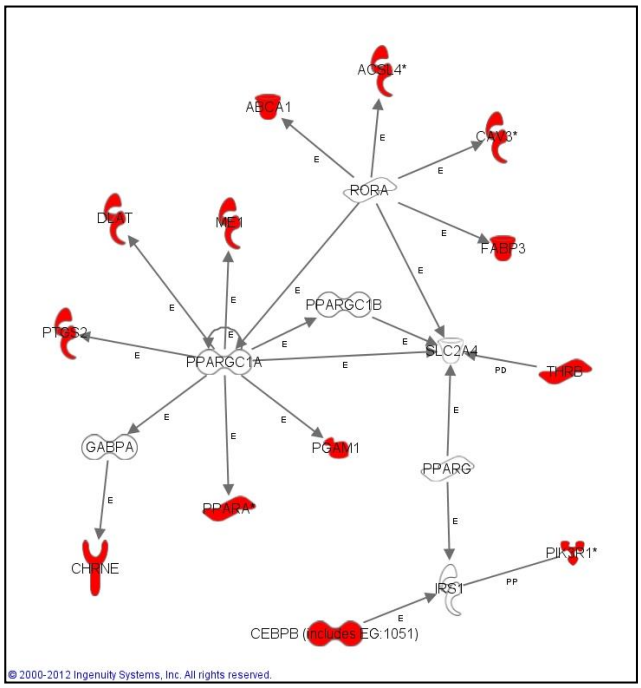


Supplement Figure S2.3. Associations between plasma metabolites in adult female offspring and size at birth

Adult female offspring: Fasting blood glucose and **(A)** PI in controls (r = -0.845, *P*-value = 0.036, n: 5), placentally restricted (r = -0.778, *P*-value = 0.061, n: 5) and in combined offspring (r = -0.695, *P*-value = 0.013, n: 10). Fasting blood glucose and **(B)** BMI in controls (r = -0.858, *P*-value = 0.031, n: 5). Association was not significant in placentally restricted offspring. Fed plasma cholesterol and **(C)** PI in controls (r = 0.930, *P*-value = 0.011, n: 5). Association was not significant in placentally restricted offspring. Fed plasma cholesterol and **(D)** BMI in controls (r = 0.892, *P*-value = 0.021, n: 5) and in combined offspring (r = 0.654, *P*-value = 0.028, n: 9). White circle: control; black circle: PR; solid line: control; Dashed line: PR linear correlation; Dotted line: combined linear correlation; BMI: Body Mass Index; PI: Ponderal Index.

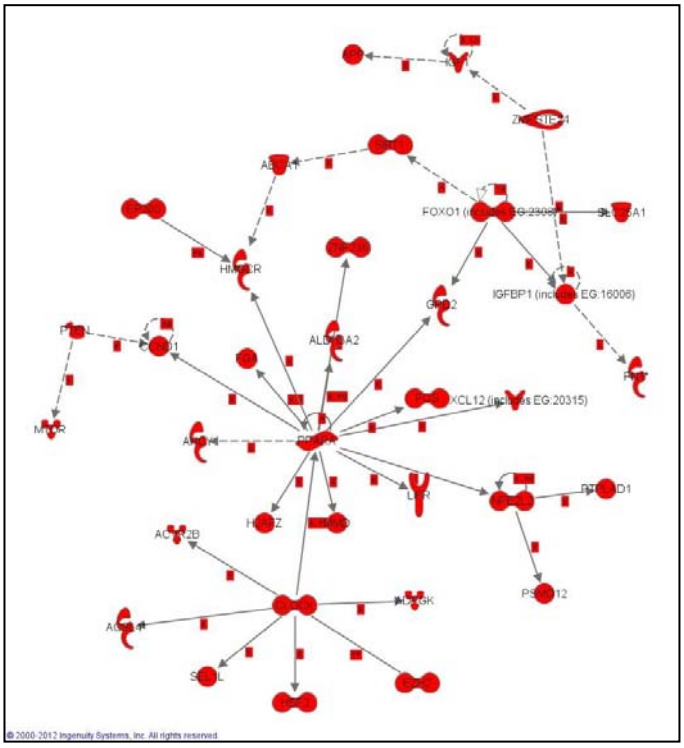
Supplement Figure S2.4. Molecular networks of combined targets predicted to be regulated by differentially expressed miRNAs in tissues of placentally restricted offspring

Predicted network of combined targets from seven differentially expressed miRNAs in liver of placentally restricted adult female offspring
Network 1

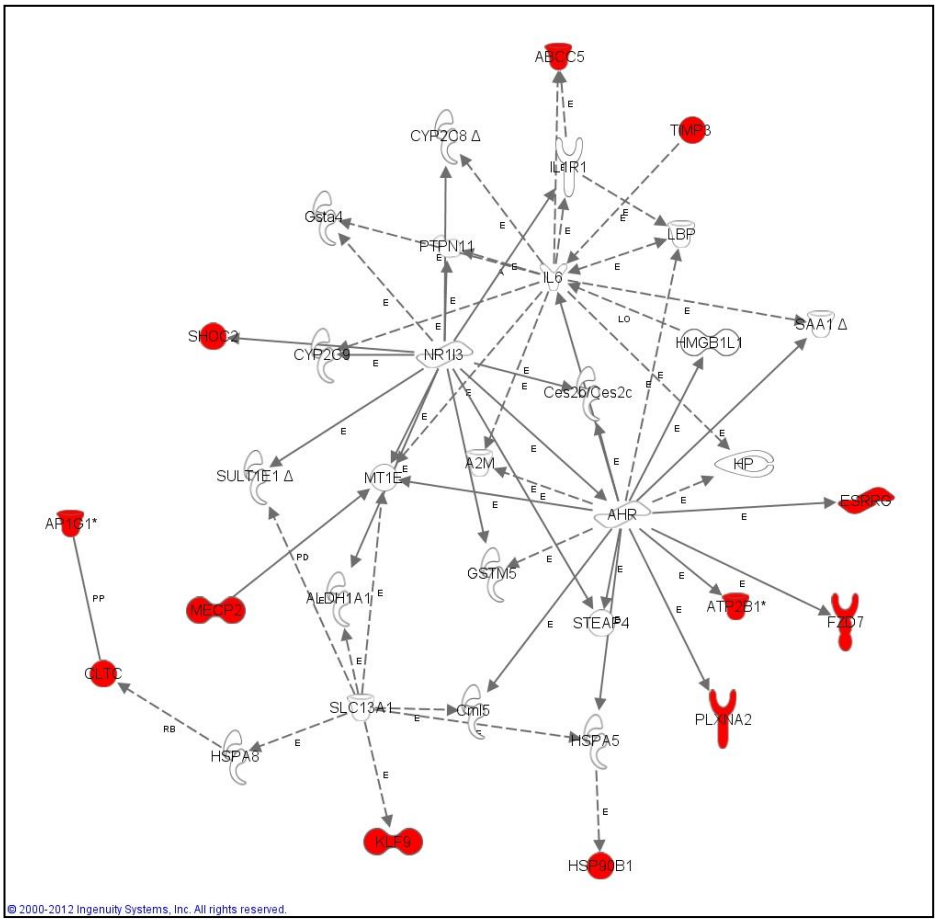


Predicted network of combined targets of 4 differentially expressed miRNA in liver of placentally restricted adult offspring

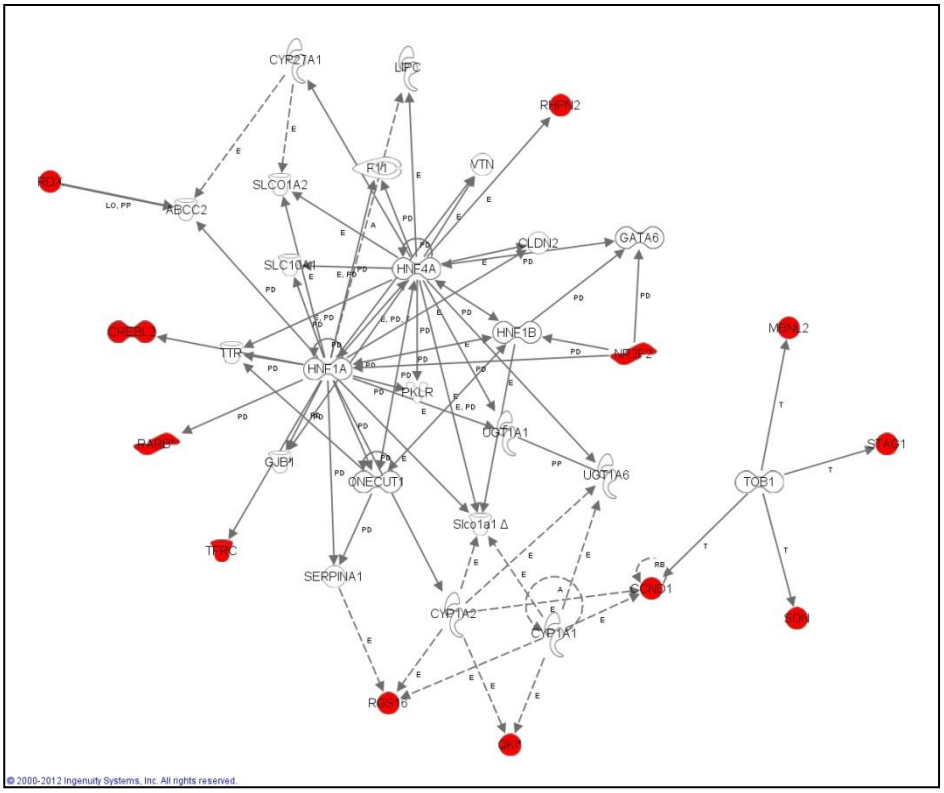
Network 1



Network 2



Network 3


CYP27A1
LIPC
VTN
SLCO1A2
ABCC2
CLDN2
GATA6
HNF4A
HNF1B
TTR
HNF1A
PKLR
UGT1A1
UGT1A6
GJB1
ONECUT1
Slco1a1 Δ
SERPINA1
CYP1A2
CYP1A1
TOB1
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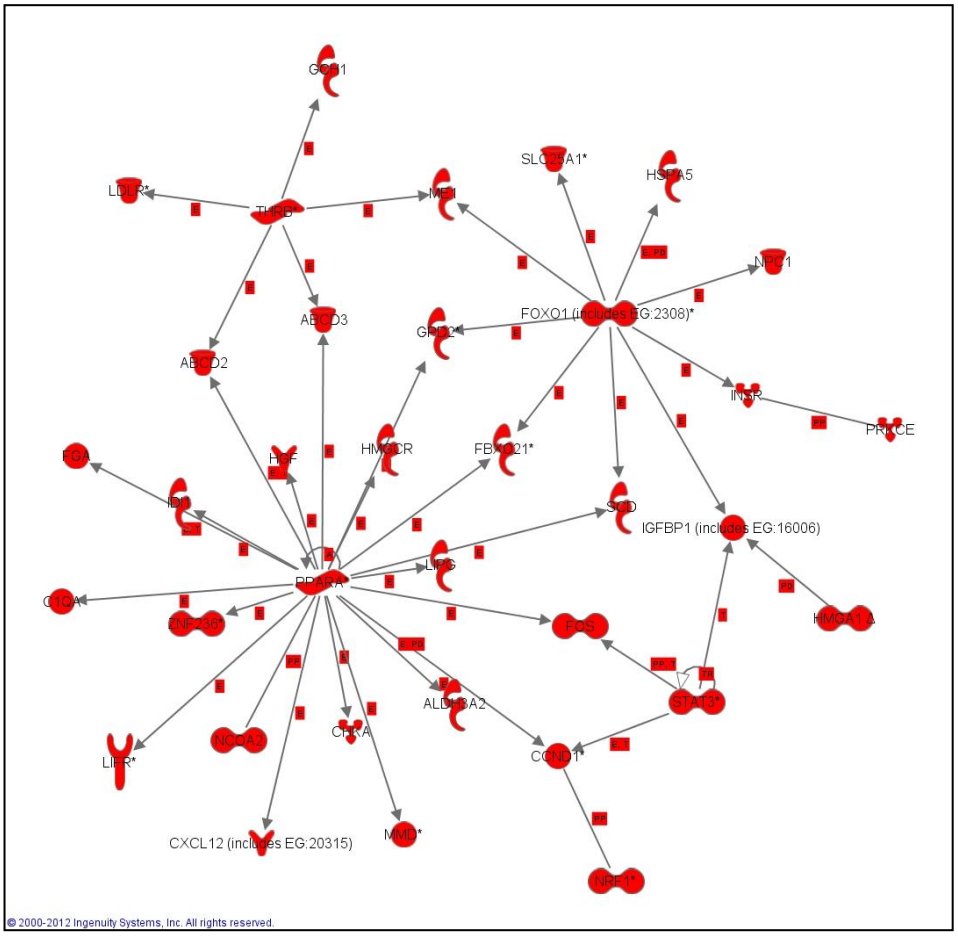
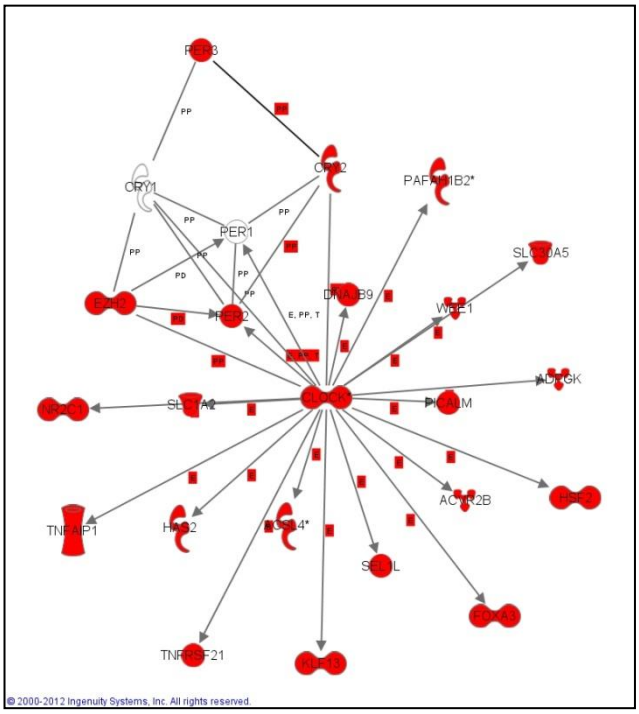
Predicted networks of combined targets of 8 differentially expressed miRNAs in liver of placentally restricted adult male offspring

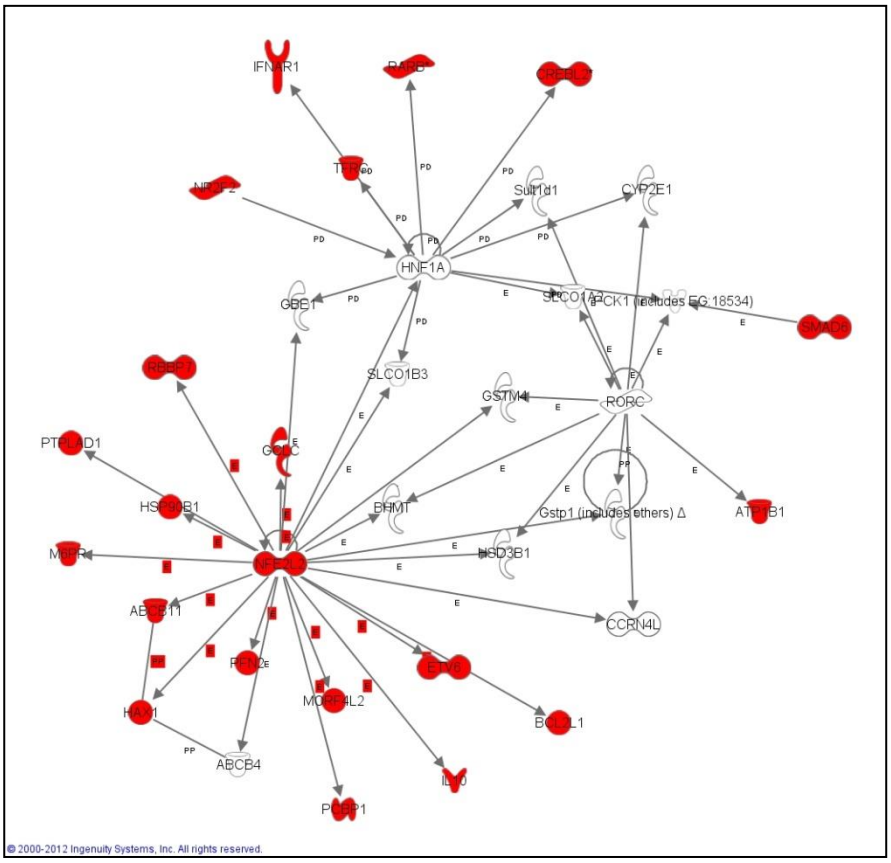
Network 1



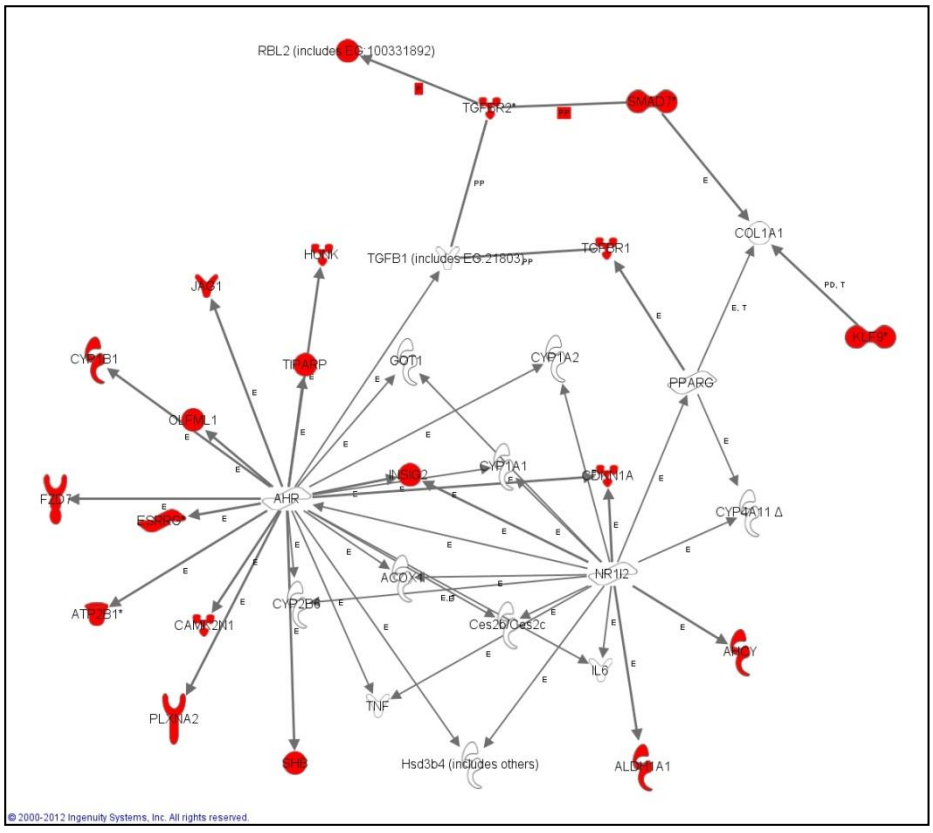
Network 2



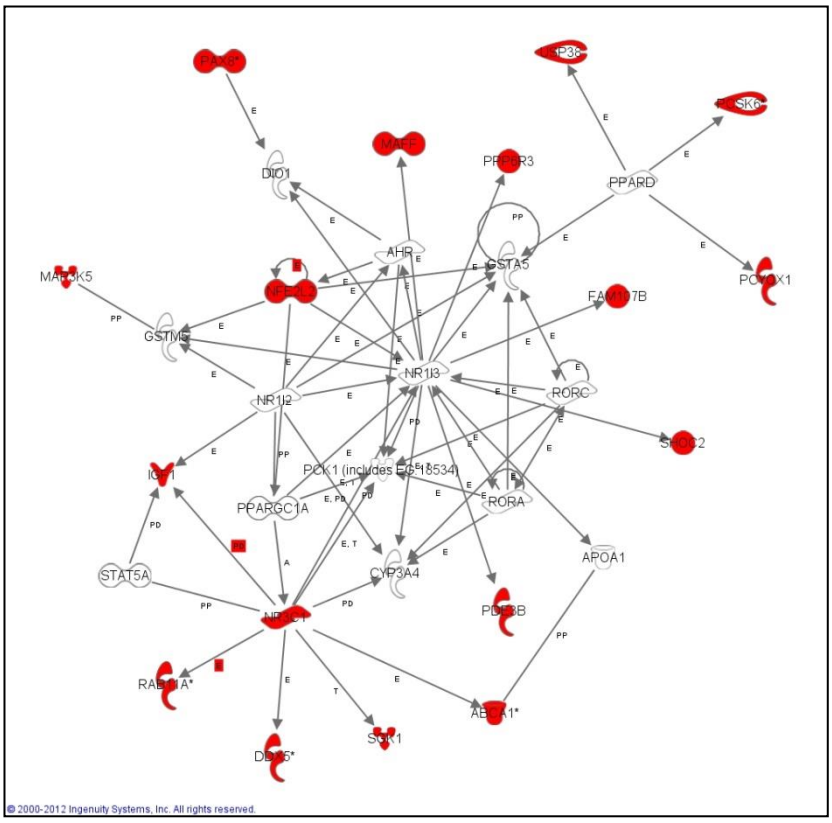
Network 3



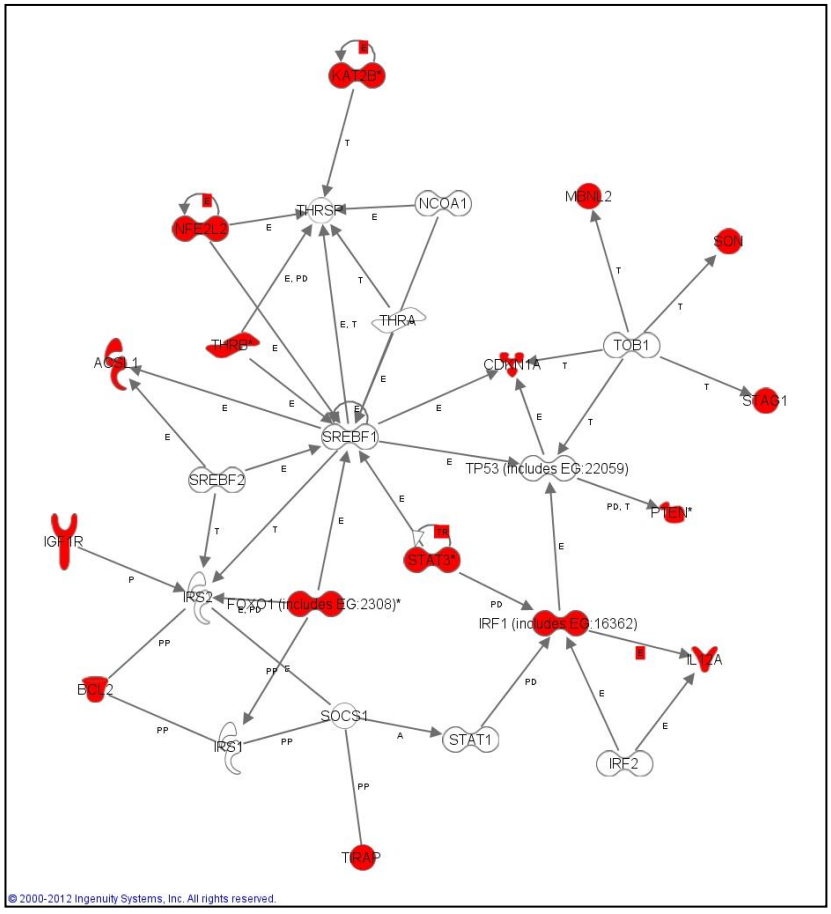
Network 4



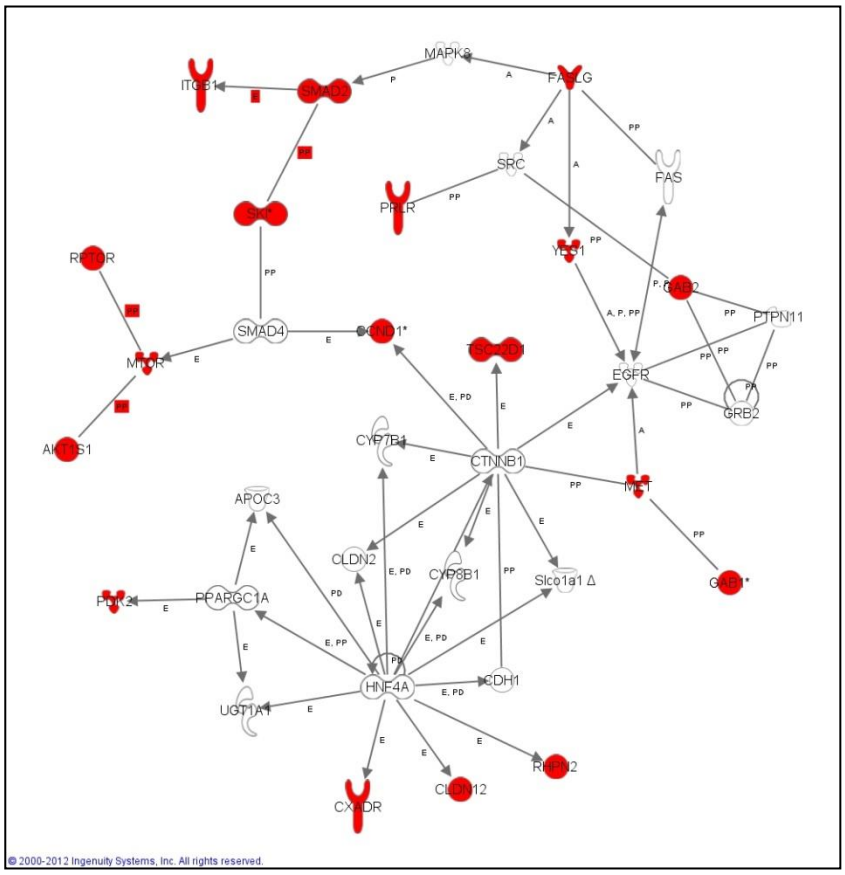
Network 5



Network 6



Network 7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SMAD2
FASLG
SRC
FAS
PRLR
SMAD4
YES1
PTPN11
EGFR
GRB2
CYP7B1
CTNNB1
MET
APOC3
CLDN2
CYP8B1
PPARGC1A
HNF4A
CDH1
UGT1A1
CLDN12
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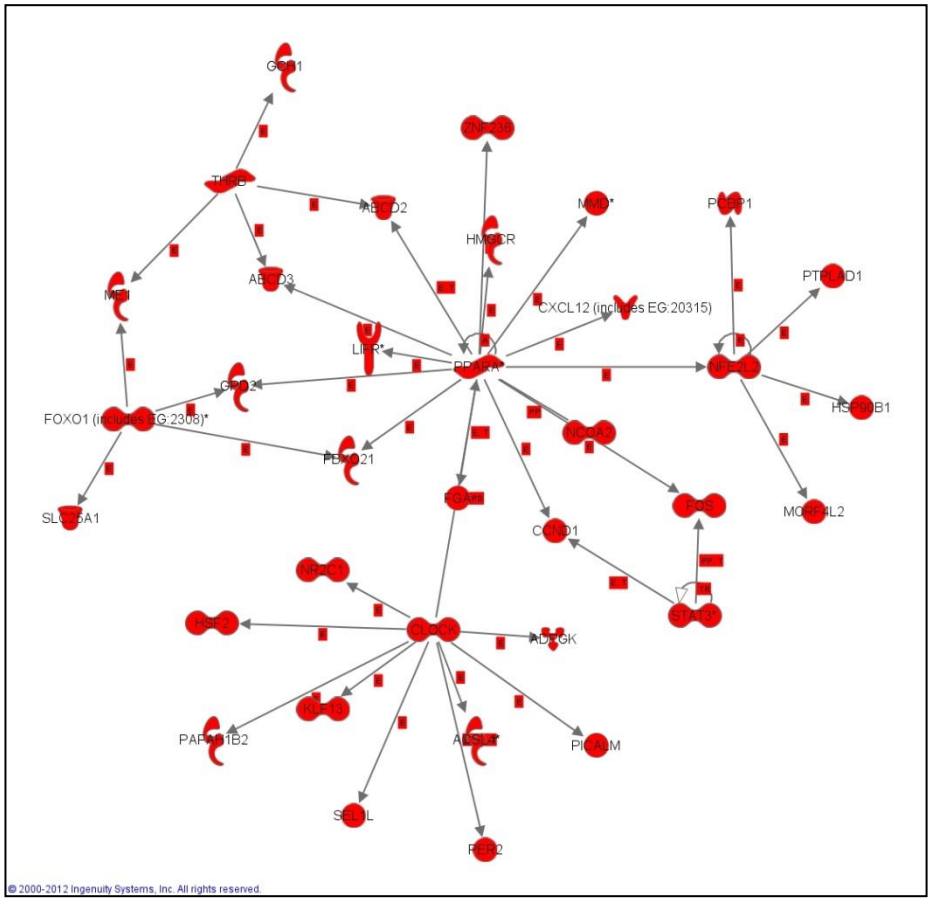
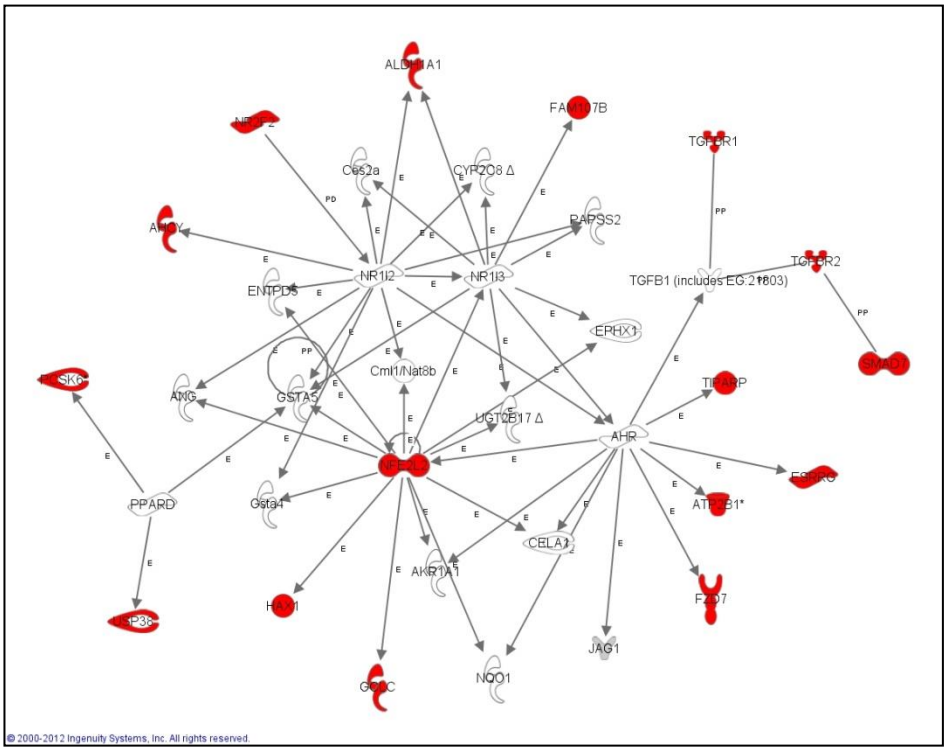
Predicted networks of common targets from 4 of differentially expressed miRNAs in liver of placentally restricted adult male offspring

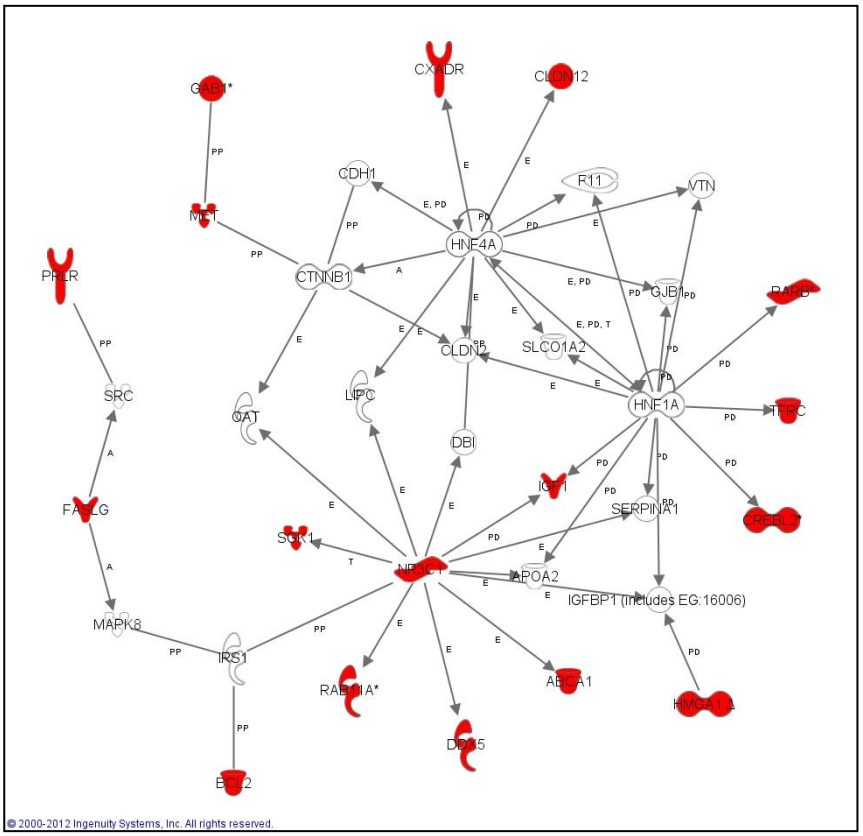
Network 1



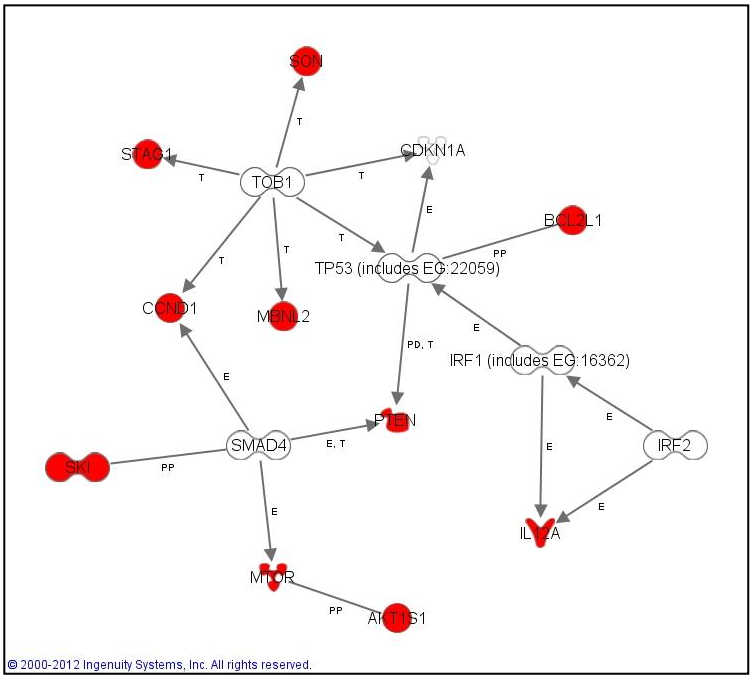
Network 2



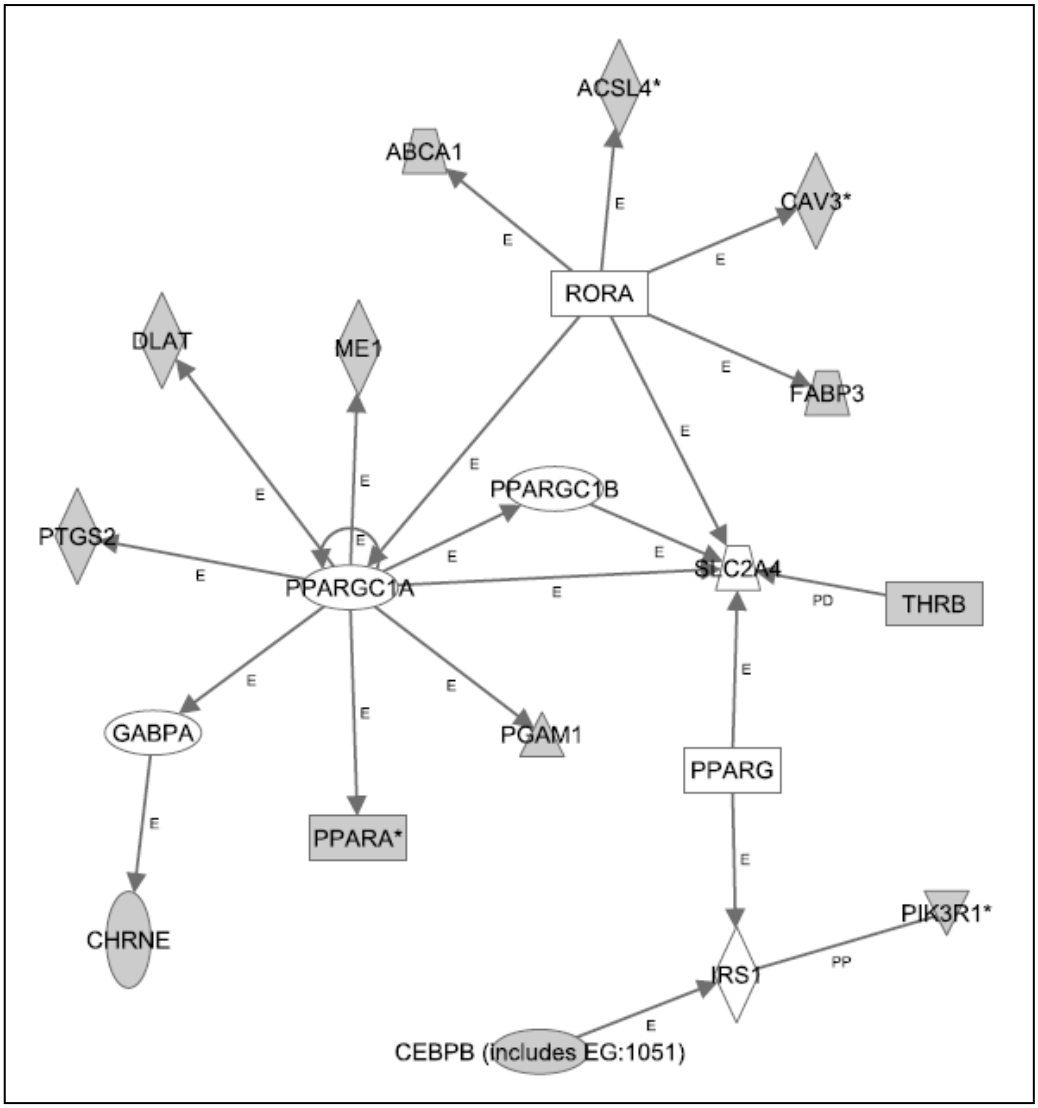
Network 3

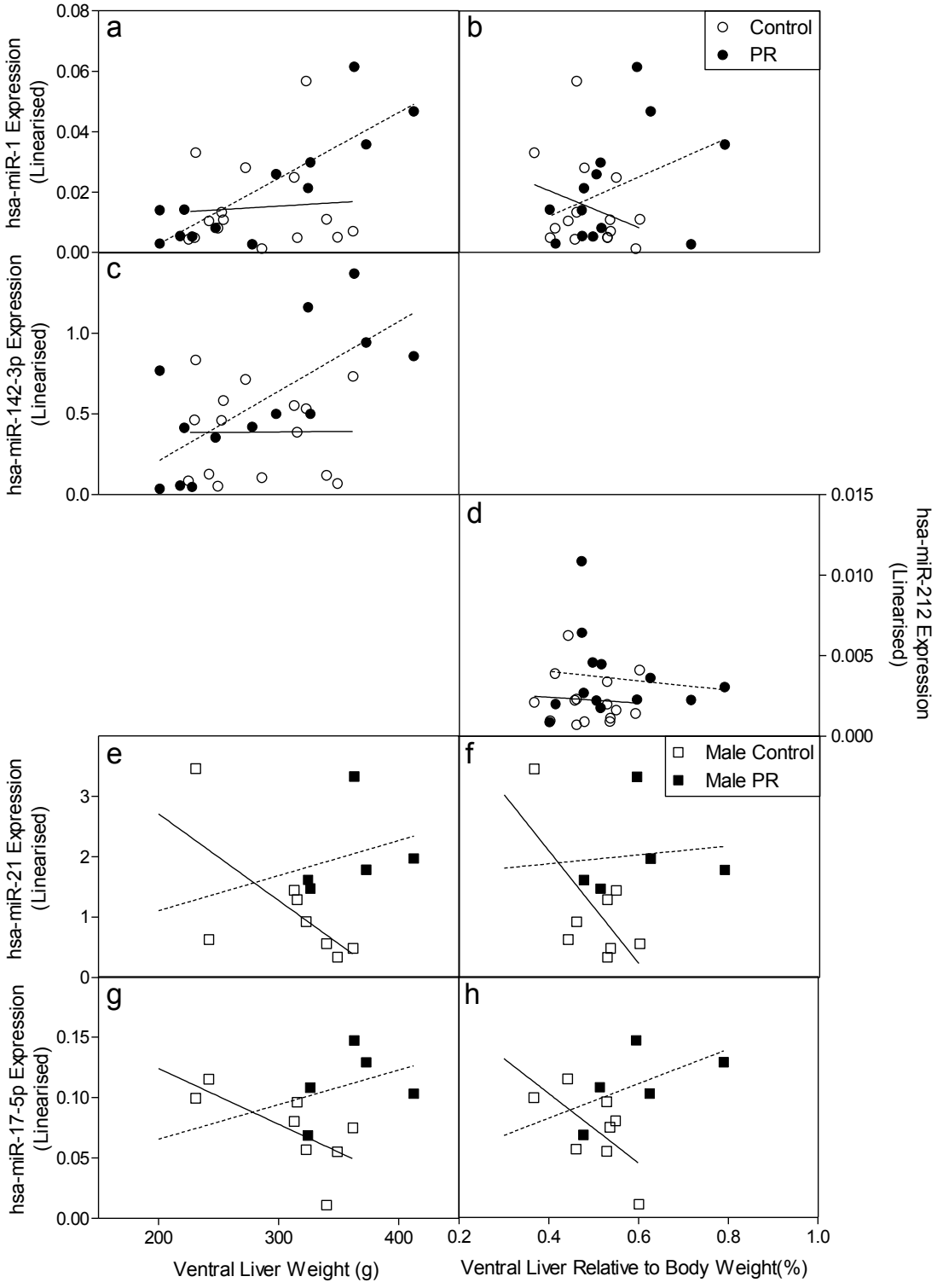


Network 4



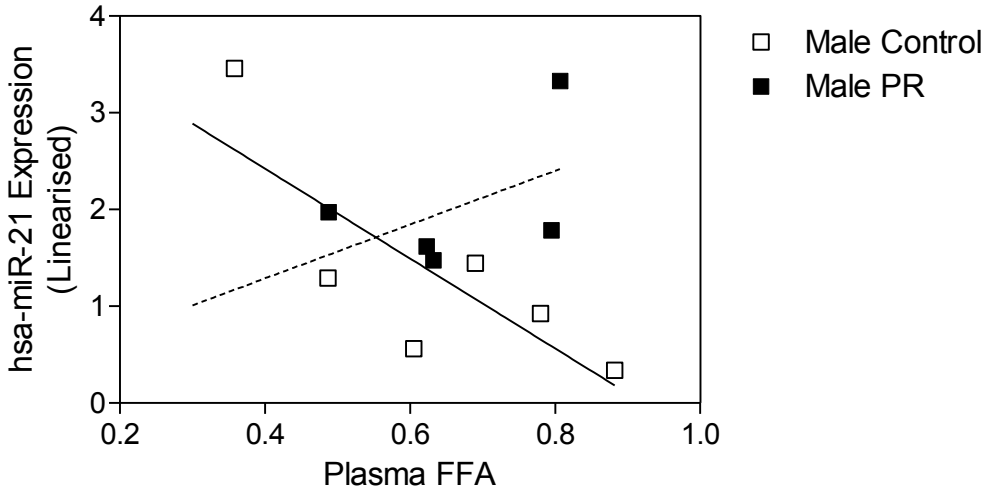
Predicted networks of common targets from 7 of differentially expressed miRNAs in vastus lateralis of placentally restricted adult female offspring





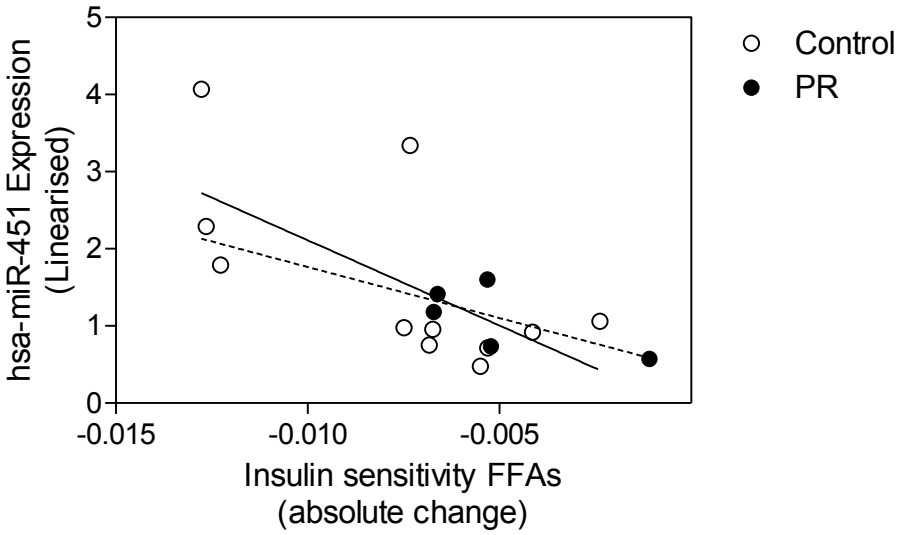
Supplement Figure S2.5. Associations between ventral liver fat weight and differentially expressed miRNAs in liver of placentally restricted adult offspring

Adult offspring: hepatic hsa-miR-1 and (a) ventral liver weight in placentally restricted (r = 0.847, *P* < 0.001, n: 13), and (b) ventral liver relative to body weight in placentally restricted (r = 0.721, *P* = 0.003, n: 13). Associations were not significant in control offspring. Hepatic hsa-miR-142-3p and (c) ventral liver weight in placentally restricted (r = 0.723, *P* = 0.003, n: 13), and no association was significant in control offspring. Hepatic hsa-miR-212 and (d) ventral liver relative to body weight in both control and placentally restricted offspring (r = -0.620, *P* = 0.007, n = 15 & r = -0.545, *P* = 0.027, n = 13, respectively).
Adult male offspring: hepatic hsa-miR-21 and (e) ventral liver weight in controls (r = -0.680, *P* = 0.032, n: 8), and (f) ventral liver relative to body weight (r = -0.680, *P* = 0.032, n: 8), and no association was significant in placentally restricted offspring. Hepatic hsa-miR-17-5p and (g) ventral liver weight in controls (r = -0.679, *P* = 0.032, n: 8), and (f) ventral liver relative to body weight (r = -0.652, *P* = 0.040, n: 8), and no association was significant in placentally restricted offspring. Overall Control: white circle; Overall PR: black circle; Male Control: white bars; Male PR: black bars; continuous line: control linear correlation; dotted line: PR linear correlation.



Supplement Figure S2.6. Associations between plasma free fatty acids and differentially expressed hsa-miR-21 in liver of placentally restricted adult male offspring

Association of plasma FFAs and hsa-miR-21 expression in liver controls (r = -0.797, *P* = 0.029, n = 6), but not in placentally restricted offspring (r = 0.497, *P* = 0.197, n = 5). Male Control: white box; Male PR: black box; solid line: control linear correlation; dotted line: PR linear correlation.



Supplement Figure S2.7. Associations between insulin sensitivity of plasma free fatty acids and differentially expressed hsa-miR-451 in perirenal fat of placentally restricted adult offspring

Association of insulin sensitivity of plasma FFAs and hsa-miR-451 expression in perirenal fat of controls (r = 0.687, *P* = 0.020, n: 11), but not in placentally restricted offspring. Control: white circle; PR: black circle; solid line: control linear correlation; dotted line: PR linear correlation.

Chapter 3

Placental restriction alters insulin action and expression of microRNAs and their targets in insulin sensitive tissues of adult rat offspring

3.1 Introduction

Intrauterine growth restriction (IUGR) and low birth weight are associated with an increased risk of developing type 2 diabetes in later life (Kaijser *et al.*, 2009). In developed countries, placental insufficiency during pregnancy is a common cause of IUGR (Ergaz *et al.*, 2005), which occurs in 3-7% of the total population (Romo *et al.*, 2009). The IUGR and low birth weight adult exhibits whole body and initially peripheral insulin resistance and consistent with this, down regulated expression of insulin signalling genes and their key molecular targets in skeletal muscle (Ozanne *et al.*, 2005; Vaag *et al.*, 2006) and fat (Jaquet *et al.*, 2000; Jaquet *et al.*, 2001b). However, the mechanisms that contribute to the molecular basis of insulin resistance in IUGR are poorly understood. IUGR is substantially due to an inadequate supply of substrates *in utero* (Romo *et al.*, 2009), which can metabolically 'program' the phenotype of the offspring long term. 'Programming' is defined as a phenomenon whereby disturbance of the fetal environment at a critical stage of functional differentiation may persistently alter the functional development of key tissues and organs, with longer term consequences that include an increased risk of diabetes in later life (Barker, 1992; Barker, 1998).

Low birth weight or IUGR in humans is consistently associated with various markers of insulin resistance in adults with onset between childhood and adolescence or early adult, suggesting that this is a primary defect underlying their development of impaired glucose tolerance and diabetes (Newsome *et al.*, 2003). Typically, type 2 diabetes develops when insulin secretion is insufficient to compensate for the level of insulin resistance present in an individual. Importantly, experimental placental restriction of fetal growth in the sheep (De Blasio *et al.*, 2007a) impairs insulin

sensitivity and secretion in 6-week old offspring. Experimental placental restriction of fetal growth in the rat also impairs insulin secretion (Neitzke *et al.*, 2008) and in some but not all studies, insulin sensitivity, resulting in type 2 diabetes (Simmons *et al.*, 2001; Vuguin *et al.*, 2004; Wlodek *et al.*, 2005; Wadley *et al.*, 2008). The molecular basis of the changes in insulin sensitivity in the IUGR adult in humans and other species has been studied to some extent, mostly in the insulin sensitive tissues, but the mechanisms responsible are still unclear.

In IUGR young adult men who are insulin resistant, *Glut4* mRNA expression is reduced in skeletal muscle and adipose tissue (Jaquet *et al.*, 2001b). In low birth weight men who have not yet developed insulin resistance, skeletal muscle insulin-stimulated protein expression of PKCζ and AKT1 and their phosphorylation is reduced (Jensen *et al.*, 2008). Paradoxically, their skeletal muscle expression of the PI3K units, p85α and p110β, is increased in the insulin stimulated state, although reduced in the basal state, suggesting that the Akt pathway may be the molecular site of initial onset of insulin resistance in young men of low birth weight (Jensen *et al.*, 2008). In sheep, IUGR induced by exposing the pregnant ewes to elevated ambient temperature in early to mid-gestation, increased the expression of insulin receptor, *p85α* and *Akt2* in skeletal muscle of the fetus (Thorn *et al.*, 2009). This is consistent with placental restriction and IUGR enhancing insulin sensitivity in the fetus before birth in the sheep (Limesand *et al.*, 2007; Owens *et al.*, 2007a). Subsequently, similar to that seen in human IUGR, these changes in insulin sensitivity, induced by placental insufficiency before birth, reverse in early postnatal life, with the emergence of insulin resistance in the young lamb (De Blasio *et al.*, 2012). Consistent with this, we have recently reported reduced expression of the

insulin receptor, *Irs1, Akt2, Glut4, Gsk3α* and *Gys1* mRNA in the skeletal muscle, but not liver, of juvenile sheep IUGR offspring (De Blasio *et al.*, 2012).

In the rat, placental restriction of fetal growth can also induce insulin resistance in later life (Simmons *et al.*, 2001; Vuguin, 2007; Raab *et al.*, 2009), albeit less consistently. In several studies, experimental restriction of fetal growth in the rat did not alter whole-body or peripheral insulin sensitivity, but did induce hepatic insulin resistance, in young adult offspring (Vuguin *et al.*, 2004; Raab *et al.*, 2009). In another study, placental restriction in the rat did not alter glucose tolerance or insulin secretion in older offspring, but did induce whole-body insulin resistance and elevated intra-muscular triglycerides in adult female offspring (Wadley *et al.*, 2008). The molecular basis of the latter is not known, but placental restriction does reduce insulin receptor mRNA expression in skeletal muscle of the neonate (Germani *et al.*, 2008); whether this persists in the adult has not been studied.

In human IUGR, use of isotopically-labelled glucose, showed evidence of insulin resistance in the insulin sensitive tissues and enhanced hepatic insulin sensitivity (Jensen *et al.*, 2002), whereas another study in slightly older subjects, reported impaired hepatic insulin sensitivity, rather than peripherally (Brons *et al.*, 2008). Consistent with the latter, placental restriction in the rat also reduces insulin-stimulated IRS2 and AKT2 phosphorylation (Vuguin *et al.*, 2004). In the sheep, placental restriction does not alter hepatic expression of proximal insulin genes and related molecules in juvenile offspring (De Blasio *et al.*, 2012), but is associated with reduced hepatic *Slc2a2* mRNA expression in adult offspring (Chapter 2). While these outcomes suggest that the impact of placental restriction of fetal growth on insulin

action in liver and time of onset may differ between species, this may be partly attributable to variation in the ages at which offspring have been studied as well as development across species.

In general, the mechanisms by which placental restriction and IUGR alter long term gene and/or protein expression of proximal insulin signalling molecules and their molecular targets of insulin sensitive tissues are largely unknown. One candidate mechanism which may be involved is microRNAs (miRNAs) (Poy *et al.*, 2007; Ferland-McCollough *et al.*, 2010), which are short, single stranded, non-coding RNAs, approximately 20-23 nucleotides in length (Bushati & Cohen, 2007). MiRNAs bind to sites in 3' untranslated region (UTR) of many target mRNAs, leading to translational repression or cleavage of mRNA. Therefore they are thought to be able to co-ordinately regulate many biological processes (Ambros, 2004).

MiRNA gene expression is potentially highly regulated by epigenetic mechanisms, particularly DNA methylation (Weber *et al.*, 2007), being characterised by a greater abundance of CpG sites and islands compared to other genes in the human genome (Saini *et al.*, 2007). Furthermore, placental restriction and IUGR in the rat alters expression of CpG binding protein 2, a key component for DNA methylation, in the lungs of weanling offspring and in a sex specific manner (Joss-Moore *et al.*, 2011). Uteroplacental insufficiency in the rat also reduces hepatic expression of methionine adenosyltransferase and cystothionine-β-synthase which are enzymes involved in one-carbon metabolism, a pathway important for DNA methylation, in weanling offspring (MacLennan *et al.*, 2004). Whether this altered epigenetic capacity is accompanied by altered microRNA expression in the insulin sensitive tissues of

IUGR offspring is unknown. Recently, various miRNAs have been identified as potential regulators of type 2 diabetes and insulin signalling (Ferland-McCollough *et al.*, 2010; Rottiers & Naar, 2012) (summarised on Table 1.2). If placental restriction and IUGR alter expression of these and other miRNAs that target and influence molecules that underpin insulin action, this may contribute to insulin resistance and other underlying molecular changes seen in IUGR offspring.

The consequences of placental restriction for long term phenotype of offspring and its molecular basis can be readily studied in the rat. Utero-placental insufficiency can be induced by bilateral uterine artery vessel ligation (BUVL) of pregnant dams in late gestation inducing IUGR. The resulting IUGR offspring have reduced birth weight and develop early onset impaired insulin secretion, with later onset of insulin resistance and diabetes in the adult seen in some, but not all studies (Simmons *et al.*, 2001; Lane *et al.*, 2003; Vuguin *et al.*, 2004; Neitzke *et al.*, 2008; Siebel *et al.*, 2008; Wadley *et al.*, 2008; Raab *et al.*, 2009). Some of the variability in outcomes of experimental placental restriction in the rat for insulin sensitivity and glucose control may be due to differences in study designs such as the strain of rat used, whether cross-fostering occurred or whether sham operated controls were used, if offspring were selected based on birth weight outcome, or the sex and age of offspring studied.

We therefore characterised the effect of placental restriction of fetal growth in the rat on the ontogeny of insulin sensitivity, secretion and glucose control in male and female offspring, in early to mature adulthood, and compared this with sham and un-operated controls. We further determined the effect of placental restriction and IUGR

on expression of key proximal insulin signalling molecules and selected molecular mediators in the key insulin sensitive tissues such as liver, skeletal muscle and adipose, in young and older adult offspring. We also concomitantly examined the effect of placental restriction and IUGR on expression of miRNAs in these tissues to assess their involvement and to identify significant miRNA molecular networks that may be affected. We then analysed expression of relevant and influential targets within these networks, and if these related to relevant aspects of offspring phenotype, particularly insulin action.

3.2 Materials and Methods

3.2.1 Animal treatment

All surgical and experimental procedures performed in this project were approved by The University of Adelaide Animal Ethics Committee which complies with the *Australian code of practice for the care and use of animals for scientific purposes* (NHMRC, 2004). All rats were housed under a 12:12h light-dark cycle in a temperature controlled room. Female rats were fed a pregnancy diet from two weeks before and throughout pregnancy (Digestible energy: 13.9 $MJ.kg^{-1}$; Protein: 19 %; Total fat: 4.8%; Crude fibre: 4.8%; Folate: 2.0%; Specialty Feeds, Western Australia), then standard rat chow after delivery, with water *ad libitum*. Daily vaginal bioimpedence readings were taken to confirm oestrus cycle stage (Wlodek *et al.*, 2005). Rats were mated overnight and pregnancy was confirmed by the presence of sperm in the vaginal smear. Pregnant rats were housed in single cages at day 1 of mating throughout pregnancy and with their pups until weaning. After weaning, offspring were fed standard rodent chow diet with water available *ad libitum*. Offspring bodyweight, nose-rump length and abdominal circumference were measured from postnatal day (PD) 3 onwards.

Placental restriction in the pregnant Wistar/Lewis rat was induced surgically by bilateral uterine artery vessel ligation (BUVL) at day 18 of pregnancy as previously described, after fasting overnight (Wlodek *et al.*, 2005). Briefly, anaesthesia was induced with 0.1 mL of Illium Xylazil-20 (20 mg/ml) and 0.1 mL of Ketamine (100 mg/ml) (Lyppard Australia Ltd., Adelaide, Australia) injected intravenously into the tail vein. The abdominal area was shaved around the umbilicus and swabbed with 70% Ethanol (EtOH). A 2 cm mid-line incision was made along the abdomen and each

uterine horns were placed on a swab dampened with 0.9% sterile saline. Ligation was made with surgical silk around the left and right uterine arteries near the distal end (cervix) of the uterus (Wlodek *et al.*, 2005). Sham operated dams underwent the same surgical procedure at day 18 of pregnancy, however without ligation of the arteries. All surgical procedures were performed under aseptic conditions.

Litters were culled to 8 offspring (4 males, 4 females) on PD 3. Two cohorts were used for this study, Cohort 1 (studied as young adults only for body composition at post-mortem at PD 90 and miRNAs expression in the insulin sensitive tissues) and Cohort 2 (studied both as young and older adults for glucose tolerance, insulin secretion and insulin sensitivity at PD 90 and 180 and for body composition, expression of proximal insulin signalling and related molecules, and miRNAs and their predicted targets in the insulin sensitive tissues at PD 260). The number of animals studied were as follows: Cohort 1: male control n = 7, female control n = 5, male PR n = 5, female PR n = 8, male sham = 4, female sham = 4; Cohort 2: n = 8 for each groups, male control, female control, male PR, female PR, male sham and female sham. Offspring from both Cohorts were weekly from PD 3 to PD 90, while only Cohort 2 offspring were weighed at the additional ages of PD 180 and 260.

3.2.2 Intra-peritoneal glucose tolerance test (IPGTT)

Glucose control and insulin secretion in offspring were tested using an intra-peritoneal glucose tolerance test (IPGTT), adapted from a previously published protocol (Hua *et al.*, 2008). Briefly, at PD 90 ± 3 and PD 180 ± 3 rats were fasted overnight (12-16 hours), with free access to water. Rats were injected with glucose (1 $mg.g^{-1}$ bodyweight; Sigma, St. Louis, MO, USA) into the intra-peritoneal cavity over

30 seconds. Blood (200 - 300 µl) was obtained from the tail vein, before (-5 and 0 min), and after (5, 10, 15, 30, 45, 60, 90 and 120 min) administration of glucose. Heparinised blood was centrifuged and plasma was stored at -20°C for later analyses of blood glucose and insulin.

Plasma glucose was measured on a Hitachi 912 automated analyser with Glucose HK kits (Roche Diagnostics, NSW, Australia). Plasma insulin was measured using the Sensitive Rat Insulin RIA kit (Millipore Corporation, Billerica, MA, USA). The intra- and inter-assay coefficient of variation for the insulin assay at were 9.5% and 20.9%, respectively.

Fasting plasma glucose and plasma insulin concentrations were calculated as the mean values taken prior to glucose administration during the IPGTT. Glucose area under the curve (AUC) was calculated as the area under the plasma glucose profile during the IPGTT above the fasting plasma glucose concentrations, and used as a measure of glucose tolerance. The maximum increase in plasma insulin (Δinsulin) was calculated as the maximal plasma insulin achieved during IPGTT minus fasting plasma insulin. Insulin AUC was similarly calculated, as the area of the plasma insulin profile during the IPGTT above the fasting plasma insulin concentrations. Insulin secretion relative to the glucose stimulus was calculated as the insulin AUC divided by glucose AUC.

3.2.3 Intra-peritoneal insulin tolerance test (IPITT)

Plasma glucose following insulin administration was measured for each rat offspring at PD 96 ± 2 and PD 185 ± 2. Rats were fasted for 1-2 hours, with free access to

water. Rats were injected with insulin (0.75 $mU.g^{-1}$ bodyweight; Actrapid human insulin, Novo Nordisk, Bagsvaerd, Denmark), into the intra-peritoneal cavity over 30 seconds. Blood was collected, from the tail vein, before (-5 and 0 min), and after (5, 10, 15, 30, 45, 60, 90 and 120 min) administration of insulin. Plasma glucose concentration was measured on Hitachi 912 automated analyser with Glucose HK assay kits (Roche Diagnostics, NSW, Australia). Measurement of insulin sensitivity was calculated by glucose area above the curve (AAC). AAC plasma glucose was calculated as the area above the plasma glucose profile and below plasma glucose during the IPITT. The decrease in glucose was calculated as the difference between maximum plasma glucose after the insulin infusion and the minimum plasma glucose reached during the ITT.

3.2.4 Insulin action

Insulin action was assessed as insulin disposition or insulin secretion relative to sensitivity. It reflects whether insulin secretion is adequate to maintain glucose control given one's insulin sensitivity (Gatford *et al.*, 2004). Two insulin disposition indices (DI) for basal insulin action were calculated. Basal DI_{AAC} was calculated as the fasting insulin in the IPGTT multiplied by the glucose AAC from 0-120 min during the IPITT. Basal $DI_{\Delta glucose}$ was calculated as the fasting insulin in the IPGTT multiplied by the magnitude of the decrease in the IPITT. Two DI for maximal insulin action were also calculated. Maximal DI_{AAC} was calculated as the Δinsulin in the IPGTT multiplied by the glucose AAC from 0-60 min during the IPITT. The maximal $DI_{\Delta glucose}$ was calculated as the Δinsulin in the IPGTT multiplied by the magnitude of the decrease in glucose in the IPITT.

3.2.5 Post-mortem

Offspring were anaesthetised by inhalation of halothane (5% in oxygen) and blood (10-15 mL) was collected by cardiac puncture into heparinised syringes. Blood was placed on ice until centrifuged at 2,400 x g for 15 mins at 4°C, and plasma collected and stored at -20°C for subsequent analyses. Offspring were killed by decapitation. Tissues were dissected, weighted and snap-frozen on liquid nitrogen and stored at -80°C for later analyses. A range of tissues and organs were collected with skeletal muscle of the right hind limb, liver and omental fat used for analysis of this study

3.2.6 Total RNA extraction

Samples from liver, skeletal muscle of right hind limb and omental fat (~100 mg each) were homogenised with Precellys® 24 Lyse at 6,500 rpm (liver and muscle: 1 x 20 secs; fat: 2 x 20 secs with 15 secs interval) (Bertin Technologies, France) using TRIzol extraction methodology (Invitrogen, Australia). Following homogenization, insoluble materials were then removed by centrifugation at 12,000 rpm for 10 mins at 4˚C, and the supernatant was then transferred into a fresh tube, mixed with chloroform then centrifuged for 30 mins at 12,000 rpm at 4˚C. Following phase separation, the aqueous phase was transferred into a new tube and isopropyl alcohol was added followed by incubation overnight at -20˚C. Following incubation, the pellet was collected by centrifugation for 30 mins at 12,000 rpm at 4˚C, then washed with 75% ethanol and pelleted by centrifugation at 12,000 rpm for 30 minutes at 4˚C. The pellet was dissolved in RNase-free water.

The quantity of total RNA was determined by measuring the absorbance at 260 nm using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Australia). Only

samples with no contaminants and intact RNA were selected for further analysis. This was assessed by using a NanoDrop spectrophotometer, with those having 260/280 and 260/230 ratio of ~2.0 and 1.8 - 2.2 respectively, indicating no protein, phenol and co-purified co-contaminants, being selected for further analyses. Quality of RNA was also assessed by separation of the ribosomal 18S and 28S stained bands were visualised by UV light with ethidium bromide on agarose (1%) gel. Only RNA with a 28S:18S ratio of approximately 2:1 was considered as intact.

3.2.7 MicroRNA Microarray

The microarray platform used was miRCury LNA® Exiqon miRNA microarray v11 (Denmark) printed by The Adelaide Microarray Centre (The University of Adelaide). The probes in the arrays were based on miRBase library v11. On the arrays, 350 miRNAs probes were specific for rat miRNAs, out of 1,789 probes. Total RNA of 2 - 6 µg was used in each array with eight comparisons between un-operated controls against BUVL offspring, for each sex. For Cohort 1, some of the animals were used twice, this was done to reach the eight comparisons of animals for microarray analysis. For Cohort 2, animals were assigned randomly with eight comparisons between un-operated controls against BUVL offspring. To eliminate the dye bias effect, a dye swap was performed for the second half of the array comparison. RNA was labelled by the ligation of fluorescently modified RNA, as previously described (Thomson *et al.*, 2004). The RNA was then allowed to hybridise to the microarray slides overnight in the dark at 56°C. The slides were then washed and scanned with a GenePix 4000B scanner (Molecular Devices, CA, USA). Detailed experimental procedures were described earlier in Chapter 2, in Sections 2.2.7 and 2.2.8.

Microarray statistical analyses were performed with R software with LIMMA method (The R Foundation for Statistical Computing, Vienna, Austria) which normalised the data inter- and intra-slides. The P-value was adjusted for false discovery rate with two methods, the Benjamini-Hochberg for multiple testing for correction, and a second, based on the number of expected false positive, divided by the number of genes analysed on the microarray plate (1/1789 = 0.000559) (Stekel, 2003). Any miRNA reported with an unadjusted *P*-value < 0.000559 was considered as significant and further analysed. Another FDR was calculated based on the number of rat miRNA probes on the array (1/350 = 0.00286).

3.2.8 qPCR analysis of miRNA expression

RNA (5 µg) was reverse transcribed on a GeneAmp PCR System 9700 using the TaqMan MicroRNA Assays (Applied Biosystems, Australia). Water substituted for reverse transcriptase samples, served as the negative control. Assay-specific cDNA and probes were combined with TaqMan 2X Universal PCR Master Mix, No AmpErase UNGb (Applied Biosystems, Australia), and real-time PCR amplification was performed in triplicates using an ABI Prism 7900 HT sequence detection system (Applied Biosystems, Australia). Data were quantitated using SDS software 2.1 and $\Delta\Delta C_t$ method (Livak & Schmittgen, 2001). NormFinder algorithm was used to identify the optimum reference gene out of a group of candidate genes, based on the Ct values (Andersen *et al.*, 2004). The candidate genes were let-7a, let-7f, miR-26b, miR-92 and average of miR-26 and miR-92 ((Ct miR-26b + Ct miR-92) / 2). NormFinder algorithm identified that the average Ct value of miR-26b and miR-92 was the optimal reference gene. As there is no definitive reference gene for miRNA analysis, averaging two or more miRNAs Ct values has been shown to be more

stable (Andersen *et al.*, 2004). Expression of miR-26b and miR-92 were not affected by BUVL. Expression of miRNA genes were therefore normalised against the average Ct value of miR-26b and miR-92; the Ct value of miRNA of interest was divided by the average Ct values of miR-26b and miR-92, to give its normalised ΔCt value. These ΔCt values for each miRNA were converted to a linear form using $2^{-(\Delta Ct)}$ prior to statistical analysis.

3.2.9 Bioinformatic Analyses

The predicted targets for each miRNA were determined by miRecords database (http://mirecords.biolead.org) (Xiao *et al.*, 2009), which provides 11 different algorithm databases, including miRanda, PicTar and TargetScan. The number of predicted targets for each miRNA was limited by requiring that they be commonly predicted by at least by three of the algorithms mentioned above. Each differentially expressed miRNA and its targets, including the predicted targets 'commons' to all differentially expressed miRNAs, were analysed by Ingenuity Pathway Analysis 7.0 (IPA) (Redwood City, CA, USA). IPA is a software and database that is able to identify the biological mechanisms or metabolic pathways and functions most relevant to the given experimental genes of interest and tissue of origin, based on the evidence base in the publications in Pubmed and Medline (*Ingenuity Pathway Analysis)* For this study, the IPA criteria used to generate these were based on mouse, human and rat data sources and restricted only to the relevant tissue and cell types for each data set (skeletal muscle, liver and adipose tissues). Core analyses were used in IPA to identify networks which were predicted to be regulated by differentially expressed miRNAs, with each network limited to a maximum 35 molecules and a score > 4. The latter indicates that a network was not predicted due

to random chance, with less than a 1 in 10,000 chance that the genes were part of a network due to random chance (Ingenuity, 2004). Official permission to use figures and analyses generated by this software was obtained from Ingenuity Systems (Redwood City, CA, USA). Predicted targets and key molecules in significant networks were then selected for further analysis.

3.2.10 qPCR analysis of insulin signalling and related molecules and predicted miRNA target gene expression

Total RNA (10 µg) was treated with a TURBO DNA-free™ kit to remove any contaminating DNA, according to the manufacture's instruction (Ambion Inc., Austin, TX, USA). RNA was then reverse transcribed according to the manufacturer's instructions (Invitrogen, Melbourne, Australia). Briefly, 2 µg of RNA was incubated with 500 ng of random hexamers (GeneWorks, Adelaide, Australia), 10 nM dNTPs (Invitrogen, Melbourne, Australia) and RNase free water to make up the total volume to 13 µl at 65ºC for 5 minutes, then placed on ice for at least 1 min. Superscript III RT (1 µl) (Invitrogen, Melbourne, Australia) and 200 µl Rnasin (Promega, Sydney, Australia) was added and incubated for 5 min at 25ºC, 60 min at 50ºC, and 15 min at 70ºC, using a GeneAMP PCR system 9700 thermocycler (Applied Biosystems, Melbourne, Australia). Negative controls were prepared by omitting Superscript III RT from the reaction mix. The cDNA was stored at -20˚C for future analysis.

Quantitation of mRNA for target genes was performed using primers designed against rat sequences using Primer3 (Rozen & Skaletsky, 2000) (Supplement Table S3.1) and followed by real-time PCR using a Corbett RotorGene 6000. Briefly, cDNA was diluted to 1/500 and mixed with real master mix (5 Prime; Quantum, Brisbane,

Australia) with SYBR green as the fluorescent probe for detection, as described previously (De Blasio *et al.*, 2012). An initial 2-min hold cycle at 95 °C followed by 40 cycles of 95 °C for 15 sec and 60 °C for 60 sec was used. Primers were designed against rat sequences. Gene expressions were analysed using RotorGene software v1.7 and the relative standard curve method of quantitation. Expression of all genes were normalised against the reference gene *β-actin*, which was unaltered by placental restriction.

3.2.11 Statistical Analyses

The effects of treatments (between factor; three levels: control, sham and BUVL) or BUVL (between factor; two levels: control and BUVL) on litter size and weight at 3 days of age were assessed by analysis of variance (ANOVA) (PASW for Windows Version 17; SPSS Inc., Chicago, IL, USA).

The effects of treatments (between factor; three levels: control, sham and BUVL) or BUVL (between factor; two levels: control and BUVL), sex (between factor; two levels: male and female) and age (repeated measures factor; six levels: PD 3, PD 7, PD 14, PD 21, PD 40 and PD 90; specific comparison analyses were performed individually for PD 180 and PD 260 with Bonferonni adjustment), on postnatal growth, in terms of bodyweight were assessed by ANOVA (PASW for Windows Version 17; SPSS Inc., Chicago, IL, USA).

The effects of treatments (between factor; three levels: control, sham and BUVL) or BUVL (between factor; two levels: control and BUVL), sex (between factor; two levels: male and female), age (repeated measures factor; two levels: young adult, 90

days and older adult, 180 days) and time (repeated measures factor; 9 and 10 levels for IPGTT and IPITT, respectively) on plasma glucose or plasma insulin were assessed by ANOVA (PASW for Windows Version 17; SPSS Inc., Chicago, IL, USA).

The effects of treatments (between factor; three levels: control, sham, BUVL) or BUVL (between factor; two levels: control, BUVL), sex (between factor; two levels: male, female) and age (repeated measures factor; two levels: young adult, 90 days, older adult, 180 days) on various summative indices of glucose homeostasis, insulin secretion and sensitivity were assessed by ANOVA (PASW for Windows Version 17; SPSS Inc., Chicago, IL, USA).

The effects of treatments (between factor; three levels: control, sham and BUVL) or BUVL (between factor; two levels: control and BUVL), sex (between factor; two levels: male and female) and age (between factor; two levels: young adult, 90 days and older adult, 180 days) on body composition were assessed by ANOVA (PASW for Windows Version 17; SPSS Inc., Chicago, IL, USA). The effects of treatments on fat depots weights and relative to body weight were assessed by repeated measures (factors: three levels; retroperitoneal fat, omental fat and visceral fat) by ANOVA (PASW for Windows Version 17; SPSS Inc., Chicago, IL, USA).

The effects of BUVL (between factor; two levels: control and BUVL) and sex (between factor; two levels: male and female) on tissue expression of miRNAs (measured by qPCR) and insulin signalling and related molecules were assessed by ANOVA (PASW for Windows Version 17; SPSS Inc., Chicago, IL, USA).

Associations between the abundance of miRNAs and that of predicted targets or functional measures were assessed by Pearson's correlation (PASW for Windows Version 17; SPSS Inc., Chicago, IL, USA). All data are presented as the mean ± SEM, unless otherwise stated. A *P*-value < 0.05 was considered statistically significant.

3.3 Results

3.3.1 Effects of surgery and BUVL on size at birth and postnatal growth in offspring

In combined offspring regardless of age, surgical treatments reduced nose-rump length at birth, compared to controls (P = 0.003) (Table 3.1). Specific comparisons showed that BUVL reduced nose-rump length by 4% (P = 0.001) and body weight by 6% (P < 0.001), compared to controls (Supplement Table S3.2). BUVL also reduced body weight by 13% (P = 0.037), compared to sham offspring (Supplement Table S3.2). Sham surgery did not alter any body dimensions, compared to controls (Supplement Table S3.2). All body dimensions assessed at post-mortem increased with age (P < 0.05) (Table 3.1).

Surgical treatments reduced body weight of offspring from PD 3 to PD 90 (P < 0.001, repeated measures ANOVA), compared to control offspring (Table 3.1) and similarly in males and females. Surgical treatments also reduced body weight of offspring at PD 180, compared to control offspring (P = 0.002) (Table 3.1), but not at PD 260 (Table 3.1). Specific comparisons showed that BUVL reduced body weight at PD 3 by 25% (P < 0.001), PD 7 by 22% (P < 0.001), PD 14 by 17% (P < 0.001), PD 21 by 16% (P < 0.001), PD 40 by 13% (P = 0.001), PD 90 by 9% (P = 0.002) and PD 180 by 12% (P = 0.001), compared to controls and similarly in males and females (Figure 3.1 & Supplement Table S3.2).

BUVL reduced body weight at PD 3 by 17% (P < 0.001), PD 7 by 20% (P < 0.001), PD 14 by 11% (P < 0.001) and PD 21 by 10% (P = 0.009), compared to sham offspring (Figure 3.1 & Supplement Table S3.2). In females, BUVL reduced body

weight at PD 21 by 17% (P = 0.007), compared to sham (Supplement Table S3.2). In males, BUVL did not alter body weight at PD 21 (P = 0.289), compared to sham offspring (Supplement Table S3.2). BUVL did not affect body weight at other ages, compared to sham, both in males and females (Supplement Table S3.2).

Sham surgery only reduced body weight at PD 3 by 9% (P = 0.037), compared to controls (Figure 3.1 & Supplement Table S3.2) and similarly in males and females. Sham surgery did not reduced body weight at other ages, compared to controls.

Surgical treatments reduced body mass index compared to controls in young adult offspring (P = 0.029, quadratic) (Table 3.1). Specific comparisons showed that BUVL reduced body mass index of young adult offspring (P = 0.017), when compared to control offspring (Supplement Table S3.2). Sham surgery did not alter any body dimensions of offspring when compared to either control or BUVL offspring (Supplement Table S3.2).

In older adult offspring, surgical treatments reduced nose-rump length compared to control offspring (P = 0.007, quadratic) (Table 3.1). Specific comparisons showed that BUVL reduced nose-rump length by 3% in older adult offspring (P = 0.005), compared to controls, mainly females, where it was reduced by 6% (P = 0.005) (Supplement Table S3.2). BUVL did not alter any other body dimensions of offspring compared to controls. Sham surgery increased nose-rump length compared to controls in older adult offspring by 2% (P = 0.032) (Supplement Table S3.2). In males, sham surgery reduced abdominal circumference by 6% (P = 0.020), compared to controls (Supplement Table S3.2). In females, sham surgery did not

alter any body dimensions, compared to controls. BUVL did not alter any body dimensions of offspring compared to sham offspring, both in males and females (Supplement Table S3.2).

3.3.2 Effects of BUVL on body composition in offspring

3.3.2.1 Combined ages

Overall, weights of organs and tissues increased with age ($P < 0.05$), except for the adrenals (Table 3.2). When compared to controls, surgical treatment reduced absolute weight of the heart ($P = 0.001$, quadratic), spleen ($P = 0.002$, linear), kidneys ($P = 0.001$, quadratic), liver ($P = 0.049$, linear) and lungs ($P = 0.021$, linear), but did not affect their relative weights (Table 3.2). Regardless of age, surgical treatments altered brain weight relative to body weight, differently in males and females ($P = 0.004$) (Table 3.2). Surgical treatments also altered heart weight relative to body weight ($P = 0.006$) and lungs weight relative to body weight ($P = 0.023$), differently with age and sex of offspring (Table 3.2). In males, surgical treatments reduced testes weight, compared to controls ($P = 0.005$) (Table 3.2). In males, surgical treatments did not alter any other organ or tissue weights, including relative to body weight. In females, surgical treatments altered adrenals weight relative to body weight ($P = 0.035$) and brain weight relative to body weight ($P = 0.004$), compared to controls (Table 3.2).

Specific comparisons shown that BUVL reduced weight of the heart by 11% ($P = 0.001$), spleen by 9% ($P = 0.002$), kidneys by 10% ($P < 0.001$), liver by 7 %($P = 0.017$) and lungs by 7% ($P = 0.029$), when compared to control offspring, but did not affect these relative to body weight (Supplement Table S3.3). BUVL did not alter any

other organ and tissue weights, including relative to body weight. In males, BUVL reduced testes weight by 9% (*P* = 0.002), compared to controls (Supplement Table S3.3). In females, BUVL increased adrenal weight relative to body weight by 50% (*P* = 0.018) and brain weight relative to body weight by 16% (*P* = 0.002), compared to controls (Supplement Table S3.3). BUVL did not alter any organ or tissue weights, compared to sham offspring (Supplement Table S3.3). Sham surgery reduced spleen weight by 8% (*P* = 0.002), lungs weight by 6% (*P* = 0.007) and visceral fat weight relative to body weight by 12% (*P* = 0.042), compared to control offspring (Supplement Table S3.3). In males, sham surgery reduced spleen weight by 10% (*P* = 0.024) and spleen weight relative to body weight by 11% (*P* = 0.032) (Supplement Table S3.3). In females, sham surgery did not alter any organ and tissue weights, compared to control offspring. BUVL surgery did not alter adiposity fat weight but tended to alter in term of relative adiposity to body weight, differently with sex and age of the offspring, compared to control offspring (repeated measures of BUVL surgery x age x sex, *P* = 0.056), where affected mostly in older adult (Supplement Figure S3.1A & B).

3.3.4.2 Young adult

Overall, surgical treatments reduced kidney (*P* = 0.021, quadratic) and lungs weight (*P* = 0.026, linear), compared to control offspring, but did not affect these relative to body weight (Table 3.3). Surgical treatment did not affect organ or tissue weights differently with sex of the offspring.

Specific comparisons showed that BUVL reduced heart weight by 8% (*P* = 0.030), kidneys weight by 8% (*P* = 0.004), liver weight by 10% (*P* = 0.039) and increased

brain weight relative to body weight by 9% (*P* = 0.003), compared to controls (Supplement Table S3.4). BUVL did not alter any other of organ or tissue weights, including relative to body weight. In male offspring, BUVL reduced testes weight by 4% (*P* = 0.041), compared to controls (Supplement Table S3.4), but did not alter any other organ or tissues weights, including relative to body weight (Supplement Table S3.4). BUVL did not alter any organ or tissue weights compared to sham offspring (Supplement Table S3.4).

Sham surgery reduced spleen weight by 10% (*P* = 0.011), lungs weight by 15% (*P* = 0.006) and increased brain weight relative to body weight by 6% (*P* = 0.003), compared to controls (Supplement Table S3.4). In males, sham surgery reduced spleen weight by 16% (*P* = 0.025), increased retroperitoneal fat weight by 21% (*P* = 0.031) and increased visceral fat weight relative to body weight by 23% (*P* = 0.041), compared to control offspring (Supplement Table S3.7). In females, sham surgery only increased brain weight relative to body weight by 9% (*P* = 0.002), compared to control offspring (Supplement Table S3.4).

3.3.4.3 Older adult

Overall, surgical treatments reduced heart (*P* = 0.019, quadratic), spleen (*P* = 0.011, linear) and kidneys weight (*P* = 0.016, quadratic), compared to control offspring, but did not affect these relative to body weight (Table 3.4). Surgical treatments reduced retroperitoneal fat (*P* = 0.040, linear) and visceral fat weight relative to body weight (*P* = 0.025, linear), compared to control offspring (Table 3.4). In males, surgical treatments reduced absolute weight of the testes (*P* = 0.020), compared to controls (Table 3.4). In females, surgical treatments linearly reduced spleen (*P* = 0.014) and

increased brain weight relative to the body weight (P = 0.006), compared to controls (Table 3.4). Surgical treatments did not alter any other organ or tissue weights, including relative to body weight.

Specific comparison analysis shown that BUVL reduced heart by 10% (P = 0.014), spleen by 11% (P = 0.003), kidneys by 10% (P = 0.012) and soleus weight by 29% (P = 0.037), compared to controls (Supplement Table S3.5). In males, BUVL reduced testes by 10% (P = 0.017) and soleus weight by 29% (P = 0.023), compared to controls (Supplement Table S3.5). In females, BUVL increased adrenals by 50% (P = 0.008) and brain weight relative to the body weight by 16% (P = 0.007), compared to controls (Supplement Table S3.5). BUVL did not alter any other organ or tissue weights, including relative to body weight, compared to controls. BUVL did not alter any other organ or tissue weights of offspring, including relative to body when compared to sham offspring (Supplement Table S3.5).

Sham surgery reduced spleen weight by 9% (P = 0.020), retroperitoneal fat weight by 26% (P = 0.011), omental fat weight relative to body weight by 18% (P = 0.047), visceral fat weight by 24% (P = 0.015) and visceral fat weight relative to body weight by 23% (P = 0.008), compared to control offspring (Supplement Table S3.5). In males, sham surgery only reduced testes weight by 15% (P = 0.014) and retroperitoneal fat weight relative to body weight by 31% (P = 0.022) (Supplement Table S3.5). In females, sham surgery increased brain weight relative to body weight by 7% (P = 0.002) (Supplement Table S3.5). Sham surgery did not alter any other organ or tissue weights of offspring, including relative to body weight when compared to controls. BUVL surgery tended to alter relative adiposity to body weight differently

with sex of the offspring, compared to control offspring (repeated measures of BUVL surgery x sex, *P* = 0.075). BUVL reduced relative adiposity in males, whereas increased relative adiposity in females (Supplement Figure S3.1C & D).

3.3.3 Effect of surgery and BUVL on glucose control and insulin action with aging

Fasting glycaemia and glucose tolerance

Following administration of glucose (IPGTT), fasting plasma glucose rose rapidly to peak at ~15 min later, and then fell but remained above fasting levels up to 90 min after glucose administration in all offspring (Figure 3.2A & B). Following glucose administration, surgical treatments did not alter plasma glucose at any time points, compared to control offspring. Specific comparisons did not show any difference of plasma glucose between treatment groups.

Surgical treatments reduced fasting plasma glucose compared to control offspring, differently with age and sex of offspring (*P* = 0.007, linear) (Table 3.5). Fasting plasma glucose was higher in male offspring than in females, regardless of age and surgical treatment (*P* = 0.007) (Table 3.5). Fasting plasma glucose also increased with age overall (*P* = 0.001) (Table 3.5).

Specific comparisons showed that BUVL reduced fasting plasma glucose by 10 (*P* = 0.015), compared to control offspring (Supplement Table S3.6). Sham surgery reduced fasting plasma glucose by 8% (*P* = 0.019), compared to control offspring (Supplement Table S3.6). Sham surgery did not alter fasting plasma glucose compared to BUVL offspring.

Glucose tolerance in terms of various indices deteriorated with age ($P < 0.05$) (Table 3.5). Similarly, BUVL or Sham surgery did not alter any glucose tolerance indices in offspring compared to controls or to each other, overall or in any interactions with other factors (Table 3.5).

Fasting insulin and insulin secretion

Plasma insulin peaked at ~5 min after administration of glucose, then fell gradually, but remained above fasting levels up to 90 min after glucose administration (Figure 3.2C & D). Surgical treatments altered the plasma insulin response to glucose ($P = 0.001$, linear), compared to control offspring (repeated measures). Specific comparisons showed that BUVL reduced the plasma insulin response to glucose by 37% ($P = 0.002$), compared to controls. BUVL did not alter the plasma insulin response to glucose, compared to sham offspring. Sham surgery reduced the plasma insulin response to glucose by 33% ($P = 0.005$), compared to controls.

Surgical treatments reduced all insulin secretion indices, compared to control offspring ($P < 0.05$, linear) (Table 3.5). Surgical treatments altered fasting plasma insulin differently in male and female offspring ($P = 0.047$) (Table 3.5). In addition, insulin secretion in terms of AUC insulin: glucose changed with age in offspring ($P = 0.030$) (Table 3.5). Female offspring also had reduced fasting plasma insulin ($P = 0.007$), maximum plasma insulin ($P < 0.001$) and fasting plasma insulin: glucose ($P < 0.001$), compared to male offspring (Table 3.5).

Specific comparisons showed that BUVL reduced all insulin secretion indices by 14 to 49%, compared to control offspring ($P < 0.05$) (Supplement Table S3.6). BUVL also reduced fasting plasma insulin by 40% ($P < 0.009$) and fasting insulin:glucose by 45% ($P = 0.004$), when compared to sham offspring (Supplement Table S3.6). Sham surgery reduced maximum plasma insulin by 30% ($P = 0.019$), the change in plasma insulin in response to glucose by 34% ($P = 0.015$) and AUC insulin by 40% ($P = 0.001$), compared to control offspring (Supplement Table S3.6).

Insulin sensitivity of glucose metabolism

Following administration of insulin (IPITT), plasma glucose initially rose rapidly to peak at ~5 min, and then fell to a nadir at 30-60 minutes, followed a gradual increased to reach pre-insulin plasma glucose concentrations, in most animals by 120 min (Figure 3.2E & F). Following insulin administration, surgical treatments did not alter plasma glucose, compared to control offspring (Repeated measures with factor of 8). Specific comparisons did not show any difference of plasma glucose between treatment groups.

Surgical treatments reduced insulin sensitivity, in terms of AAC glucose during IPITT, compared to control offspring ($P < 0.001$, linear) (Table 3.5). Basal plasma glucose prior to IPITT increased with age ($P = 0.003$) and differently in males and females ($P = 0.023$) (Table 3.5).

Specific comparisons showed that BUVL reduced the minimum plasma glucose following insulin by 18% ($P = 0.012$) and increased the decrease in plasma glucose following insulin by 31% ($P = 0.024$) and the AAC glucose following insulin by 24% (P

= 0.007), compared to control offspring (Supplement Table S3.6). In females, BUVL also reduced the minimum plasma glucose following insulin by 32% compared to controls (*P* = 0.011) (Supplement Table S3.6). Sham surgery did not alter any insulin sensitivity indices compared to control or BUVL offspring (Supplement Table S3.6).

Insulin action

Surgical treatments reduced basal DI_{AAC} compared to control offspring (*P* = 0.022, linear) (Table 3.5). Basal DI_{AAC} also changed with age differently in male and female offspring (*P* = 0.023) (Table 3.5). Specific comparisons showed that BUVL reduced basal DI_{AAC} compared to control offspring (*P* = 0.008) (Supplement Table S3.6). Sham surgery did not alter any insulin action indices compared to control or BUVL offspring (Supplement Table S3.6).

3.3.4 Effect of surgical and BUVL on glucose tolerance, insulin secretion, sensitivity and action in young adult offspring

Fasting glycaemia and glucose tolerance

Surgical treatments altered the plasma glucose response to glucose administration (*P* = 0.045, linear), compared to control offspring (repeated measures) (Figure 3.3A & B). In males, plasma glucose at 15, 30, 45 and 90 mins was reduced in sham offspring (*P* < 0.05), compared to control offspring. In females, sham surgery did not alter plasma glucose compared to control offspring. BUVL did not alter plasma glucose following glucose administration, compared to sham and control offspring.

Surgical treatments reduced fasting plasma glucose compared to control offspring (*P* = 0.001, linear) (Table 3.6). Specific comparisons showed that BUVL reduced fasting

plasma glucose by 22% compared to control offspring (*P* = 0.005) (Supplement Table S3.7), but did not alter other glucose tolerance indices compared to control offspring (Supplement Table S3.7). BUVL did not alter fasting plasma glucose or any glucose tolerance indices compared to sham offspring (Supplement Table S3.7).

Sham surgery reduced fasting plasma glucose by 20%, compared to control offspring (*P* = 0.008) (Supplement Table S3.7), but did not alter any glucose tolerance indices compared to control offspring (Supplement Table S3.7)

Fasting insulin and insulin secretion

Surgical treatments altered the plasma insulin response to glucose in young adult offspring (*P* = 0.029, linear), compared to controls (repeated measures), and similarly in male and female offspring (Figure 3.3C & D). Specific comparisons did not show any differences of the plasma insulin response to glucose during IPGTT between treatment groups.

Surgical treatments reduced fasting plasma insulin (*P* = 0.005, linear), maximum plasma insulin following glucose (*P* = 0.031, linear), the change in plasma insulin following glucose (*P* = 0.009, linear), the AUC insulin (*P* = 0.048, linear) and fasting plasma insulin: glucose (*P* = 0.033, linear), compared to control offspring (Table 3.6). In males, surgical treatments reduced fasting insulin (*P* = 0.008, linear) and fasting plasma insulin: glucose (*P* = 0.036, linear), compared to control offspring (Table 3.6). In females, surgical treatments did not alter any insulin secretion indices.

Specific comparisons shown that BUVL reduced fasting plasma insulin by 52% (*P* = 0.006), maximum plasma insulin concentration following glucose by 33% (*P* = 0.044)

and the change in plasma insulin by 37% (P = 0.009), compared to control offspring (Supplement Table S3.7). In males, BUVL reduced fasting plasma insulin by 62% (P = 0.010), compared to male control offspring (Supplement Table S3.7). In females, BUVL did not alter insulin secretion indices, compared to control offspring.

BUVL reduced fasting plasma insulin by 51% (P = 0.001) and fasting plasma insulin:glucose by 44% (P = 0.001), compared to sham offspring (Supplement Table S3.7). In males, BUVL reduced fasting plasma insulin by 55% (P = 0.001) and fasting plasma insulin: glucose by 54% (P = 0.001) compared to sham offspring (Supplement Table S3.7). In females, BUVL did not alter any insulin secretion measured compared to sham offspring (Supplement Table S3.7).

Sham surgery reduced maximum plasma insulin following glucose by 37% (P = 0.027), the change in plasma insulin by 45% (P = 0.011) and the AUC insulin by 33% (P = 0.034) compared to control offspring (Supplement Table S3.7) and similarly in male and female offspring.

Insulin sensitivity of glucose metabolism

Surgical treatments did not alter the plasma glucose response to insulin, compared to control offspring (repeated measured) (Figure 3.3E & F). Specific comparisons did not show any differences of the plasma glucose response to insulin during the IPITT between treatment groups.

Surgical treatments reduced minimum plasma glucose reached following insulin (P = 0.043, linear) (Table 3.6) and altered the minimum plasma glucose concentration,

differently in males and females (P = 0.042) (Table 3.6). Specific comparisons showed that BUVL reduced the minimum plasma glucose by 17% (P = 0.002), the decrease in plasma glucose by 32% (P = 0.041) and increased the AAC glucose by 33% (P = 0.034), compared to control offspring (Supplement Table S3.7), similarly in males and females. Sham surgery did not alter any indices of insulin sensitivity of glucose metabolism, compared to control or BUVL offspring (Supplement Table S3.7).

Insulin action

Surgical treatments did not alter any insulin action indices compared to control offspring (Table 3.6). However, specific comparison showed that BUVL reduced the insulin action in the fasting state (basal DI_{AAC}) by 42% (P = 0.048), compared to control offspring (Supplement Table S3.7). BUVL reduced insulin action in the fasting state (basal DI_{AAC}) by 37% (P = 0.023), compared to sham offspring (Supplement Table S3.7). In males, BUVL reduced insulin action in the fasting state (basal DI_{AAC}) by 52% (P = 0.021), compared to sham offspring (Supplement Table S3.7). In females, BUVL did not alter any insulin action indices, compared to sham offspring. Sham surgery did not alter any insulin action indices, compared to control offspring.

3.3.5 Effect of surgical and BUVL on glucose tolerance, insulin secretion, sensitivity and action in older adult offspring

Fasting glycaemia and glucose tolerance

Surgical treatments did not alter the plasma glucose response to glucose administration, compared to control offspring (repeated measures) (Figure 3.4A & B) or any glucose tolerance indices compared to control offspring (Table 3.7 &

Supplement Table S3.8). BUVL also did not alter any glucose tolerance indices, compared to sham offspring (Supplement Table S3.8).

Fasting insulin and insulin secretion

Surgical treatment altered the total plasma insulin response to glucose (P = 0.018, linear), compared to control offspring (repeated measures) (Figure 3.4C & D). Specific comparisons showed that BUVL reduced the total plasma insulin following glucose administration by 43% (P = 0.018), compared to control offspring. In males, plasma insulin at 90 mins was reduced in BUVL (P < 0.05), compared to control offspring. In females, plasma insulin at 5, 15, 60 and 90 mins was reduced in BUVL (P < 0.05), compared to control offspring. Sham surgery did not alter the total plasma insulin response to glucose, compared to control offspring. However, in males, plasma insulin at 90 mins was reduced in sham offspring (P < 0.05), compared to control offspring. In females, plasma insulin at 15, 60 and 90 mins was reduced in sham offspring (P < 0.05), compared to control offspring. BUVL did not alter the total plasma insulin response to glucose, compared to sham offspring. However, only in females, plasma insulin at 5 mins was reduced in BUVL (P < 0.05), compared to sham offspring.

Surgical treatments reduced fasting plasma insulin, the maximum plasma insulin, the change in plasma insulin following glucose and the AUC insulin (P < 0.05, linear), compared to control offspring (Table 3.7).

Specific comparisons showed that BUVL reduced the fasting plasma insulin by 46% (P = 0.009), the maximum plasma insulin by 44% (P = 0.016), the change in plasma

insulin following glucose by 48% (*P* = 0.018), the AUC insulin by 52% (*P* = 0.008), the fasting plasma insulin: glucose by 44% (*P* = 0.043) and the AUC insulin:glucose by 53% (*P* = 0.015), compared to control offspring (Supplement Table S3.8) and similarly in male and female offspring. Sham surgery did not alter any insulin secretion indices, compared to control and BUVL offspring.

Insulin sensitivity of glucose metabolism

Surgical treatments did not alter the plasma glucose response to insulin, compared to control offspring (repeated measures) (Figure 3.3E & F). Specific comparisons did not show any differences in the plasma glucose response to insulin at any time points between treatment groups.

Surgical treatments did not alter any indices of insulin sensitivity of glucose metabolism, compared to control offspring (Table 3.7). BUVL did not alter any indices of insulin sensitivity of glucose metabolism, compared to sham offspring (Supplement Table S3.8).

Insulin action

Surgical treatments did not alter any indices of insulin action in older adult offspring, compared to controls (Table 3.7). However, specific comparisons showed that BUVL reduced the maximal DI_{AAC} by 33% (*P* = 0.045), compared to sham offspring (Supplement Table S3.8). BUVL and sham surgery did not alter indices of insulin action, compared to control offspring (Supplement Table S3.8).

3.3.6 Effects of BUVL on expression of insulin signalling and related molecules in insulin sensitive tissues of offspring

3.3.6.1 Liver

BUVL reduced hepatic *Slc2a2* gene expression (Fold change = -2.23, *P* = 0.030) in older offspring and reduced hepatic *Slc2a2* (Fold change = -2.55, *P* = 0.043) and *Igf1* (Fold change = -3.01, *P* = 0.010) gene expression in older male offspring only (Figure 3.9). BUVL also reduced hepatic *p110β* (Fold change = -3.15, *P* = 0.008) gene expression in older female offspring only (Figure 3.5).

3.3.6.2 Skeletal muscle

BUVL tended to reduce skeletal muscle expression of the insulin receptor (Fold change = -3.00, *P* = 0.066) and *p110β* (Fold change = -2.38, *P* = 0.064) in older male offspring only (Figure 3.6) and did not alter expression of any insulin signalling or related genes in female offspring.

3.3.6.3 Omental fat

BUVL tended to reduce omental fat expression of *Irs1* (Fold change = -4.85, *P* = 0.078), *Irs2* (Fold change = -3.65, *P* = 0.075), *Slc2a4* (Fold change = -5.69, *P* = 0.057) and *Pparα* (Fold change = -5.48, *P* = 0.055) in older male offspring only (Figure 3.7) and increase that of *Igf1* (Fold change = 2.57, *P* = 0.037) in older female offspring (Figure 3.7).

3.3.7 Effects of BUVL on expression of microRNAs and their predicted targets in insulin sensitive tissues of offspring

BUVL did not alter microRNA gene expression in the insulin sensitive tissues of young adult offspring (data not shown), but did increase gene expression of several miRNAs in liver, skeletal muscle and omental fat of older adult offspring (Table 3.8). For most of differentially expressed miRNAs in older adult offspring, their expressions were reduced in younger adult following placental restriction (Supplement Table S3.16 and Supplement Figure S3.4). In general, the abundance of the most highly expressed miRNAs appeared to increase with age in these tissues (Supplement Figure S3.2), particularly in adipose tissue.

3.3.7.1 Liver miRNAs and predicted targets

BUVL reduced hepatic expression of the star strand miRNA, mmu-miR-199b* in older male offspring (Fold change: -2.11, P = 0.009), but tended to increase this in older female offspring (Fold change: 1.67, P = 0.067) (Table 3.9). BUVL increased hepatic rno-miR-126 gene expression in older female offspring (Fold change: 1.42, P = 0.015) (Table 3.9). MiRecords did not identify any predicted targets for mmu-miR-199b*, but predicted that rno-miR-126 would target 189 molecules (Supplementary Table S3.10). Rno-miR-126 was predicted to target *Irs1* gene expression, a molecule involved in insulin signalling (Table 3.10), but BUVL did not alter hepatic *Irs1* gene expression in older female offspring (Figure 3.5). IPA analysis identified two networks based on the predicted targets of rno-miR-126, with the most significant network was being involved in lipid metabolism, molecular transport and small molecule biochemistry. The second network was involved in haematological system,

development and function, tissue morphology and inflammatory response (Table 3.11 and Figure 3.8).

3.3.7.2 Skeletal muscle microRNAs and predicted targets

BUVL increased rno-miR-451 expression in skeletal muscle of older offspring (Fold change: 1.83, *P* = 0.024) (Table 3.9) and tended to increase this in female BUVL offspring (Fold change: 1.90, *P* = 0.099). MiRecords identified 755 predicted targets (Supplementary Table S3.11) for rno-mIR-451, including *Irs2* and *hexokinase* in the rat and the insulin receptor in humans (Table 3.10). BUVL did tend to reduce skeletal muscle expression of the insulin receptor in older male offspring (Fold change: -3.00, *P* = 0.066) (Figure 3.6). IPA analysis of the predicted targets of rno-miR-451 identified one network involved in carbohydrate metabolism, molecular transport and small molecule biochemistry (Table 3.11 and Figure 3.9). In female BUVL offspring, rno-miR-451 expression was positively correlated with soleus weight and relative to body weight (r = 0.747, *P* = 0.017 and r = 0.759, *P* = 0.014, respectively) and with vastus lateralis absolute weight (r = 0.771, *P* = 0.013). No correlation between abundance of this miRNA and soleus weight occurred in the remaining treatment groups.

3.3.7.3 Omental fat microRNAs and predicted targets

BUVL increased omental rno-miR-16, rno-miR-18a and rno-miR-142-3p (adjusted *P*-value < 0.05) expression based on microarray analyses, with this confirmed by qPCR for rno-miR-18a (Table 3.9). BUVL tended to increase omental expression of rno-miR-19b, rno-miR -21, rno-miR -20b, hsa-miR-106a and mmu-miR-106a based on

microarray (unadjusted *P*-value < 0.000559), which was confirmed by qPCR for rno-miR-19b, rno-miR-21, rno-miR-20b and mmu-miR-106a (Table 3.9).

Rno-miR-16 was predicted to target 246 molecules (Supplementary Table S3.12). IPA identified one network involving these predicted targets, which involved had top functions in lipid metabolism, molecular transport and small molecule biochemistry (Figure 3.10A and Table 3.11). Hsa-miR-106a and mmu-miR-106a were predicted to target 237 and 178 molecules, respectively (Supplementary Table S3.12). IPA identified similar networks for both of these predicted targets, which were involved in lipid metabolism, molecular transport and small molecule biochemistry (Figure 3.10B & C and Table 3.11).

Rno-miR-18a was predicted to target 78 molecules (Supplementary Table S3.12), which included some molecules involved in insulin signalling, such as insulin receptor, *p85α* and *Slc2a4* (Table 3.10). However, expression of rno-miR-18a was found to be negatively correlated only with *Slc2a4* in placentally restricted offspring overall (r = -0.451, *P* = 0.040). IPA analysis did not identify any network based on the predicted targets of rno-miR-18a.

Rno-miR-142-3p was predicted to target 75 molecules (Supplementary Table S3.12), included *M6pr* and *Prkcı* which regulate *Slc2a4* expression (Table 3.10). No correlation found between rno-miR-142-3p and *Slc2a4* expression. IPA analysis did not identify any network based on the predicted targets of rno-miR-142-3p.

Rno-miR19b was predicted to target 288 molecules (Supplementary Table S3.12), included *Irs1* and *Irs2* (Table 3.10), but no correlation was found between rno-miR-19b and *Irs1* or *Irs2* expression. IPA analysis did not identify any network based on the predicted targets of rno-miR-19b.

Rno-miR-21 and rno-miR-20b were predicted to target 132 and 388 molecules respectively (Supplementary Table S3.12), however IPA did not identify any networks based on their predicted targets, in adipose tissue and cells. Six of the differentially expressed omental fat rat miRNAs (rno-miR-18a, rno-miR-142-3p, rno-miR-19b, rno-miR-21, rno-miR-20b and mmu-miR-106a) did not have predicted targets in common. On the other hand, when their individual predicted targets were combined, IPA identified one commonly targeted network involving genetic, nutritional disease and endocrine system disorders (Supplement Figure S3.3).

Omental expression of several miRNAs and measures of omental fat mass were associated in female, but not in male nor combined regardless of sex, offspring. Expression of rno-miR-16 was positively correlated with omental fat absolute weight and relative to body weight in female control offspring (r = 0.711, *P* = 0.024 & r = 0.852, *P* = 0.004), but negatively correlated with omental weight in female BUVL offspring (r = -0.643, *P* = 0.043) (Figure 3.11a & b). Omental expression of rno-miR-19b positively correlated with omental fat absolute weight and relative to body weight in female controls (r = 0.665, *P* = 0.036 & r = 0.819, *P* = 0.006), but negatively correlated with these in female BUVL offspring (r = -0.769, *P* = 0.013 & r = -0.608, *P* = 0.055) (Figure 3.11c & d). Omental expression of rno-miR-21 tended to positively correlate with omental fat weight and relative to body weight in female controls (r =

0.518, *P* = 0.094 & r = 0.819, *P* = 0.006), but negatively correlated with omental fat absolute weight and relative to body weight in female BUVL offspring (r = -0.830, *P* = 0.005 & r = -0.590, *P* = 0.062) (Figure 3.11e &f). Omental expression of rno-miR-20b was negatively correlated with omental fat absolute weight and relative to body weight in female BUVL offspring (r = -0.724, *P* = 0.033 & r = -0.606, *P* = 0.074), but was not correlated with these in female controls (Figure 3.11g & h). Omental expression of rno-miR-142-3p was positively correlated with omental fat weight and relative to body weight in female controls (r = 0.778, *P* = 0.020 & r = 0.644, *P* = 0.059), but negatively correlated with these in female BUVL offspring (r = -0.560, *P* = 0.074 & r = -0.716, *P* = 0.023) (Figure 3.11i & j).

3.4 Discussion

Here we show that in the rat, placental restriction alters insulin sensitivity, as well as insulin secretion and overall insulin action, in adult offspring, and differently with age and sex (Supplement Table S3.13). Placental restriction impairs insulin secretion in adult offspring as has been consistently seen before, while insulin sensitivity is enhanced in young adult offspring, which then disappears with aging, mainly in females. There was also evidence for onset of increased adiposity in older adult female offspring, but this was reversed in males, following placental restriction. Placental restriction up-regulated microRNAs (miRNAs) expression in insulin sensitive tissues (liver, skeletal muscles and omental fat tissue) of older adult offspring, particularly in the omental fat. Overall, miRNA expression in these tissues increased with age in the adult rat, particularly in adipose tissue, suggesting that this age related change was accelerated by placental restriction.

In adipose tissue and to some extent in the other tissues, up-regulated expression of miRNAs occurred concomitantly with down-regulation of insulin signalling and related molecules, particularly in male offspring. Expression of miRNAs were associated and with some of key predicted targets, including insulin signalling and related molecules or pathways known to indirectly affect lipid metabolism. Together, the evidence is consistent with miRNAs as mediators of placental programming of phenotype in offspring long term, particularly in the onset of insulin resistance and altered adiposity. It also suggests a novel role for placental programming of increased activity of miRNAs in adipose tissue in some of these outcomes, notably that of the *miR-17-92* polycistronic cluster, miR-21 and miR-143, known to regulate

adipogenesis and obesogenesis, in the eventual onset of central obesity, as seen in IUGR offspring (De Blasio *et al.*, 2007b).

While sham surgery affected a few measures of glycaemia, insulin secretion and sensitivity, this was mainly in young adult offspring (Supplement Table S3.13). Placental restriction mostly had more widespread and marked effects on such outcomes, which were therefore attributable to placental restriction per se. On this basis, fasting glycaemia and glucose tolerance in adult offspring were not affected by placental restriction, but insulin secretion was substantially reduced and increasingly with age. In contrast, insulin sensitivity was modestly enhanced by placental restriction, mainly in young adult offspring, which disappeared with aging, mainly reflecting outcomes in females. The net outcome of placental restriction for insulin action was a modest reduction in basal insulin disposition in young adult offspring that did not persist. Sex differences were evident, as insulin secretion was impaired by placental restriction earlier and to a greater extent in male offspring, while the enhanced insulin sensitivity occurred mainly in female offspring (Supplement Table S3.14 and S3.15).

Consistent with the pattern of these sex specific and age related responses of insulin sensitivity in offspring to placental restriction, older males showed trends to reduced hepatic and skeletal muscle expression of some proximal insulin signalling molecules and glucose transporter, while females did not, with the exception of hepatic expression of *p110β* (Figure 3.5). This suggests that insulin resistance in these key glucoregulatory tissues was just beginning to develop in older adult offspring and earlier in males. This sex specific pattern was also seen in omental fat, with trends to

reduce expression of proximal insulin signalling and related molecules seen in older male, but not female, offspring following placental restriction.

Here we also show for the first time that placental restriction in the rat increased expression of various miRNAs in these insulin sensitive tissues overall and/or in both males and females, with only one miRNA altered in liver and skeletal muscle, but seven increased in omental fat. These up-regulated miRNAs following placental restriction are predicted to target proximal insulin signalling molecules and related molecules, consistent with their contribution to altered expression of the latter. Importantly, the up-regulated miRNAs were also commonly predicted to target lipid metabolism and other pathways that can affect insulin signalling indirectly, suggesting that miRNAs networks may indeed play a role in onset of insulin resistance later in life following placental restriction. Of interest, placental restriction in the rat increased skeletal muscle triglycerides, known to impair insulin sensitivity, in older adult offspring in a previous study (Wadley *et al.*, 2008).

Effect of placental restriction on glucose tolerance, insulin sensitivity and insulin action

Some previous studies, in which rats were cross-fostered, reported that placental restriction impairs glucose tolerance early on from 7 weeks and persisted to 26 weeks of age (Simmons *et al.*, 2001), compared to sham offspring (Vuguin *et al.*, 2004). In another study however, placental restriction in the rat did not impair glucose tolerance, compared to sham offspring (Wadley *et al.*, 2008). Of note, the latter did not use cross-fostering, which in itself appears to substantially impair glucose tolerance when the outcomes for actual glucose tolerance measures in controls

alone are compared, as well as those for placentally restricted offspring, in two studies that used the same strains, methodology and from the same laboratory (Siebel *et al.*, 2008; Wadley *et al.*, 2008). This suggests that cross-fostering itself may interact with placental restriction to alter glucose tolerance in the rat. Here, the current study also showed that placentally restricted adult offspring did not develop impaired glucose tolerance, compared to either sham or un-operated controls (Supplement Table S3.13), consistent with studies where cross-fostering was not used.

The current study showed that insulin secretion was impaired by placental restriction earlier and to a greater extent in male offspring, consistent with most previous studies (Simmons *et al.*, 2001; Vuguin *et al.*, 2004; Siebel *et al.*, 2008; Wadley *et al.*, 2008). This impairment was present overall in young adults, specifically in young adult males and in both older adult males and females offspring, following placental restriction. This is broadly consistent with the impaired insulin secretion following placental restriction in the rat reported previously (Simmons *et al.*, 2001; Vuguin *et al.*, 2004; Wadley *et al.*, 2008). However, other studies did not find any impaired insulin secretion in placentally restricted older female offspring (Siebel *et al.*, 2008), albeit where cross-fostering may itself have had an impact. Here, sham surgery also impaired insulin secretion in terms of some indices, but in fewer than compared to placentally restricted offspring, and mainly in young adult offspring and not later (Supplement Table S3.13).

Here, insulin sensitivity in offspring was modestly enhanced by placental restriction, mainly in young adult offspring, which disappeared with aging (Supplement Table

S3.13). This suggests that in the absence of an additional challenge such as cross-fostering, placental restriction in the rat initially increases insulin sensitivity, which is evident in young adult offspring, but less so in males, and that this is lost with aging. Previous studies had shown that placental restriction followed by cross-fostering impaired whole-body insulin sensitivity in 7 weeks of age offspring (Simmons *et al.*, 2001) or did not affect this in older adult offspring (Siebel *et al.*, 2008). Placental restriction in the rat, in the absence of cross-fostering did not alter whole-body insulin sensitivity in male 8 weeks of age offspring (Vuguin *et al.*, 2004) or in older adult offspring (Wadley *et al.*, 2008). However, the latter studies did report impaired hepatic insulin sensitivity in male offspring (Vuguin *et al.*, 2004) and signs of whole-body insulin resistance in adult female offspring (Wadley *et al.*, 2008). In contrast, the current study found enhanced whole body insulin sensitivity in young adult offspring, mainly in females, with this diminishing with age (Supplement Table S3.13). The differences in outcomes may reflect variations in methods as well as strains used, but nevertheless suggests that placental restriction may accelerate the age related loss of insulin sensitivity seen normally.

Expression of miRNAs in insulin sensitive tissues

The tissue specific patterns of miRNA expression found were consistent with previous reports. Five of the ten most highly expressed miRNAs in each of the three tissues studied in young and older adult rat offspring, were common across the ages and comparable to previous reports in the rat (Tzur *et al.*, 2009; Herrera *et al.*, 2010). These miRNAs were: Omental fat: miR-143, -451, -29 and let-7a, -7c; Liver: miR-29a, -363*, -21, 122 and let-7a; and Muscle: miR-1, -30c, 29a, 133a and 206). The ten most highly expressed miRNAs in these tissues in un-operated control and

placentally restricted offspring were also similar (Supplement Figure S3.2). Furthermore, several miRNAs that are both highly expressed and essential for tissue specific development in humans and rats were amongst the ten most highly expressed miRNAs seen in the current study; in skeletal muscle (miR-1, miR-133a and miR-206) (Chen *et al.*, 2006; McCarthy, 2008), liver (miR-122) (Girard *et al.*, 2008) and adipose tissue (miR-143) (Esau *et al.*, 2004) (Supplement Figure S3.2).

The most highly expressed miRNA in the skeletal muscle was rno-miR-378, however the role of miR-378 in skeletal muscle of any species is yet to be determined. However, miR-378 has been shown to target *Igf1r* in cardiomyocyte cells (Knezevic *et al.*, 2012) and played a role during myoblast differentiation by targeting the myogenic repressor, MyoR (Gagan *et al.*, 2011). MiR-378 also correlates with and predicts gains in skeletal muscle mass gain in response to exercise in young men (Davidsen *et al.*, 2011). In the current study, the most highly expressed miRNA in the liver was rno-miR-30b-5p. Its function in the liver of any species is still yet to be determined, although increased expression of miR-30b has been associated with hepatocellular carcinoma (Huang *et al.*, 2012). Here, the most highly expressed miRNA in omental fat was rno-miR-125b-5p. Expression of rno-miR-125b-5p is reduced during *in vitro* adipogenic differentiation assessed in adipose-derived stem cells (Tang *et al.*, 2009). Furthermore, miR-125b-5p, together with other miRNAs, has been shown to play important roles in regulating human adipose-derived stem cells differentiation into an osteoblast lineage, by targeting cell osteogenic differentiation, self-renewal, signal transduction and cell cycle control (Zhang *et al.*, 2012). However, the predicted targets of rno-miR125b-5p have not been experimentally validated.

Overall, miRNAs expression from the insulin sensitive tissues examined in this study, increased with age in the adult rat and particularly in adipose tissue (Table 3.8). This suggests maturational and aging related changes in enzymes and pathways that affect production and breakdown of miRNAs. Increased expression of the let-7 family occurs in skeletal muscle of old, compared to younger men (Drummond *et al.*, 2011). In mice however, reduced expression of a number of miRNAs in adipocytes of 6 month old adults compared to 1 month old juvenile mice has been reported, together with reduced abundance of miRNA post-transcriptional processing machinery, with reduced *Dicer* mRNA expression (Mori *et al.*, 2012). This study found that adipose tissue expression of 10 miRNAs expression, including let-7e, miR-125a, miR-125b, miR-351, miR-24, miR-30c, miR-99b, miR-103, miR-145 and miR-181d, were reduced with aging (Mori *et al.*, 2012). In contrast, in the current study, this omental expression of these 10 miRNAs was increased in old, compared to young adult offspring (data not shown). The expression of the most abundant miRNAs in omental fat, also increased in older compared to young adult rats, which suggests that placental restriction may accelerate the effect of aging on miRNA expression seen in controls in the current study.

Placental restriction altered miRNAs expression in insulin sensitive tissues

Placental restriction of fetal growth did not alter expression of miRNAs in insulin sensitive tissues in younger adult offspring, but did increase that of several miRNAs in older adult offspring. For the miRNAs that were up-regulated in older adult offspring, expressions levels were usually reduced in younger adult following placental restriction, although there were no significant differences, compared to control offspring (Supplement Table S3.16 and Supplement Figure S3.4).

Several of these miRNAs were differentially expressed following placental restriction studied and were located in three different clusters. In the rat, rno-miR-126 (cluster 1) was differentially expressed in the liver, rno-miR-451 (cluster 2) was differentially expressed in skeletal muscle and several miRNAs (cluster 3 to 6) were differentially expressed in omental fat. Expression of other cluster members were not significantly altered by placental restriction, although microarray analysis showed that the levels of their other cluster members were up-regulated or changed in a similar direction, albeit not significantly (Supplement Table S3.9). This indicates that differences in downstream processing or translocation to the cytoplasm may be operative within clusters for different miRNAs (Ohrt *et al.*, 2012).

In the liver, mmu-miR-199b* expression was reduced in male offspring, while that of rno-miR-126 expression was increased in female offspring, by placental restriction. Mmu-miR-199b* is belonging to the minor star molecule class (miR*), where studies show that this miRNA* species could work with their partner guide strands in a synergic manner and mediate regulatory activity in various settings (Ro *et al.*, 2007; Okamura *et al.*, 2008; Yang *et al.*, 2011). However, currently, there is no known role of mmu-miR-119b* nor its complementary major strand, mmu-miR-199b, in the liver and other tissues. Over expression of hepatic miR-126 has been shown to reduce IRS-1 protein expression *in vitro* (Ryu *et al.*, 2011), however hepatic *Irs-1* gene expression at least was not affected by placental restriction in this study. Based on IPA analysis, our study showed that miR-126 could regulate two networks, both of which are involved in lipid metabolism and tissue morphology in liver. Over-expression of gankyrin in the liver of zebra fish, a protein that is consistently over-

expressed in human cancer, increased fatty acid accumulation in the liver (Her *et al.*, 2011). The same study also reported that over-expression of gankyrin, increased miR-126 expression in the liver of zebra fish (Her *et al.*, 2011). Further studies are required to determine whether up-regulation of rno-miR-126 affects fatty acid accumulation in the liver of placentally restricted offspring.

Expression of miR-126 is regulated by the transcription factors ETS-1 and ETS-2 in endothelial cells (Harris *et al.*, 2010). An *In vitro* transfection study showed that these transcription factors bind to the promoter region (71 and 100 bp) upstream of miR-126 gene and promote its up-regulation (Harris *et al.*, 2010). Expression of miR-126 could also be epigenetically regulated, as miR-126 is found to have a CpG island on the intron of the host gene, EGFL7, in human cancer cell lines (Saito *et al.*, 2009). However, whether rno-miR-126 gene expression is similarly regulated by a CpG island in the rat is yet to be determined. These potential origins of altered expression in the liver following placental restriction could be assessed in future by examining the expression of ETS-1 and ETS-2 and methylation of CpG sites in promoter regions in offspring.

Insulin-like growth factors (IGFs), including IGF1, have been shown to play a major role in 'catch-up' growth, following re-oxygenation, in the embryo of hypoxia-induced growth restricted zebra fish (Lynn *et al.*, 2007). In sheep, placental restriction has been reported to reduce hepatic *Igf1* mRNA expression in fetal growth restricted offspring (Gentili *et al.*, 2009). We have reported that placental restriction reduced hepatic *Igf1* mRNA expression in the young adult sheep, predominantly in males but not in females (Chapter 2, Figure 2.2). In the rat, placental restriction reduced hepatic

Igf1 mRNA expression in neo-natal rat offspring (Allagnat *et al.*, 2010) and serum IGF-1 levels at both day 0 and day 21 offspring, both in males and females (Fu *et al.*, 2009). The latter study also reported that placental restriction increased DNA methylation at the CpG sites at the exon 1 and 2 of IGF-1 genes, persistently from day 0 to day 21 only in male offspring (Fu *et al.*, 2009). In this study, we found that placental restriction reduced hepatic *Igf1* mRNA expression in males but not in females, of older adult rat offspring (Figure 3.5). Thus overall, the evidence presents in Chapter 2 and here shows for the first time placental restriction reduces hepatic *Igf1* mRNA expression in males but not in females, later in adult life. The reduced hepatic *Igf1* mRNA expression observed in this study, could be due to persistence of the increased DNA methylation at the CpG sites, reported by others in younger offspring (MacLennan *et al.*, 2004).

Placental restriction of fetal growth also increased rno-miR-451 gene expression in skeletal muscle of older adult offspring. Rno-miR-451 has been predicted to regulate insulin receptor, *Irs2* and *Hk2* expression (Table 3.10), and here, placental restriction tended to reduce insulin receptor gene expression in male offspring at least (Figure 3.6). Based on IPA analysis, rno-miR-451 is predicted to regulate a network that is involved in carbohydrate metabolism, molecular transport and small molecule biochemistry, all important in skeletal muscle function. In young men who showed a low response in terms of increasing muscle mass to training exercise, skeletal muscle mir-451 expression was up-regulated in comparison with high responders, with failure to up-regulate IGF-1 expression, which is predicted to be targeted by miR-451 through mTOR and is consistent with this limiting their response (Davidsen *et al.*, 2011). Here, placental restriction up-regulated skeletal muscle rno-miR-451

expression in adult male offspring, which may contribute to the reduced skeletal muscle mass they also exhibited.

Placental restriction increased omental fat expression of six miRNAs, rno-miR-18a, rno-miR-142-3p, rno-miR-19b, rno-miR-21, rno-miR-20b and mmu-miR-106a in older adult offspring. Several of these up-regulated miRNAs were predicted to target insulin signalling or related molecules, with rno-miR-18a targeting the insulin receptor, *p85α* and *Slc2a4*, rno-miR-19b targeting *Irs1* and *Irs2* and mmu-miR-106a targeting *Slc2a4*. Consistent with this, placental restriction tended to reduce expression of insulin receptor and *Irs1* in omental fat in older adult offspring and that of *Irs1*, *Irs2* and *Slc2a4* in older adult males. Furthermore, IPA analysis found that rno-miR-16, hsa-miR-106a and mmu-miR-106a were predicted to target and regulate similar and overlapping networks, involved in lipid metabolism, molecular transport and small molecule biochemistry, indicating a potentially wide impact on adipocyte function in offspring.

Three of the differentially expressed miRNAs in omental fat, miR-18a, miR-19b and miR-20b in omental fat are located in the highly conserved *miR-17-92* polycistronic cluster in humans (He *et al.*, 2005). This miRNA cluster is essential for adipocyte differentiation and triglycerides accumulation, with increased expression a necessary adjunct to the actions of hormonal that initiate differentiation (Wang *et al.*, 2008a). In the rat, rno-miR-18a and rno-miR-19b are located on chromosome 15, while rno-miR-20b is located on chromosome X, showing that the *miR-17-92* polycistronic cluster is not necessarily conserved across mammalian species.

Two more of the miRNAs that are up-regulated in omental fat following placental restriction, rno-miR-21 and rno-miR-142-3p, have been found to play a role or are implicated in the development of adipose tissue, affecting adipogenesis and obsesogenesis in rodents and humans. MiR-21 regulates pre-adipocyte proliferation and adipogenic differentiation through modulation of TGF-β signalling in human adipose tissue derived mesenchymal stem cells (Evans *et al.*, 1993), appearing to act through STAT3 (Kim *et al.*, 2012). MiR-21 has been shown to regulate both key processes, with increased expression inhibiting stem cell proliferation, but promoting adipogenic differentiation and lipid accumulation, in human adipose tissue-derived mesenchymal stem cells (Kim *et al.*, 2012). A high fat diet in male adult mice increases miR-21 and miR-142-3p expression in white adipose tissue, consistent with the wider evidence for their actions in adipogenesis and obesity (Chartoumpekis *et al.*, 2012).

In the current study, omental expression of rno-miR-21, rno-miR-142-3p and other miRNAs with known roles in adipocyte biology, such as rno-miR-18a, rno-miR-19b and rno-miR-20b, was associated with fat mass in older female offspring, but not in males. Furthermore, there was a biphasic association of fat mass with miRNA expression in control and placentally restricted offspring, with fat mass increasing with increased expression in controls up to a point, then a subsequent modest decline with further increases in miRNA expression (Figure 3.11). In control females, omental fat mass was positively associated with expression of these miRNAs, consistent with their increased expression in expanded adipose tissue in long term diet-induced obesity in mice (Chartoumpekis *et al.*, 2012).

In contrast, omental rno-miR-21 expression was negatively correlated with omental fat mass in placentally restricted older adult female offspring (Figure 3.11E & F). Overall rno-miR-21 expression was higher in the placentally restricted offspring (Table 3.9), suggesting an increased promotion of adipocyte differentiation in mature adipocyte, then able to expand their lipid storage. Overall however, omental fat mass in combined older adult female offspring shows a biphasic association with expression of these adipocyte regulatory miRNAs (Figure 3.11). Of note, early on in diet-induced obesity in mice, miR-21 is down-regulated, allowing expansion of adipocyte numbers, followed by increased expression and increased adipocyte size and lipid content, once numbers plateau (Kim *et al.*, 2012). Furthermore, other studies show that down-regulation of miR-21 is needed for clonal expansion of adipocyte numbers (Kim *et al.*, 2009; Xie *et al.*, 2009), suggesting its up-regulation following placental restriction may limit this and potentially being to explain the biphasic pattern seen here.

Thus, the further increases in expression of such miRNAs following placental restriction may expand fat mass through differentiation and lipid accumulation, but only to a point, after which their up-regulation may limit clonal expansion and the ability to expand adipocyte number. Assessment of the impact of placental restriction on adipocytes cell size and number, as well as their time courses in relation to omental expression of rno-miR-21 and others miRNAs would assist in unravelling the mechanistic basis for these divergent patterns. Similar associations were not seen in male offspring overall, although a similar pattern was present in control, but not placentally restricted male offspring (data not shown). Overall, the altered expression of these miRNAs in omental fat and their relationships with adiposity in placentally

restricted female offspring may be evidence for a potential novel role of peripheral miRNA networks in the programming of central obesity, one that may add to consequences of impaired appetite control, reported previously (Remacle *et al.*, 2004). Of interest, placental restriction tended to alter adiposity differently with age and sex, with females showing increased relative adiposity as they aged, while males showed further reduced adiposity (P = 0.056, Supplement Figure S3.1A & B).

Placental restriction also up-regulated omental rno-miR-18a expression in older adult offspring, a miRNA which is predicted to target *Slc2a4* gene expression. Consistent with this, placental restriction tended to reduce *Slc2a4* gene expression in male offspring only (Figure 3.7) and rno-miR-18a and *Slc2a4* gene expression were negatively correlated in placentally restricted male offspring (r = -0.451, P = 0.040), but not in controls. *Slc2a4* codes for the glucose transporter type 4 (Glut 4), which is responsible for insulin-regulated glucose transport into adipocytes. Therefore, up-regulation of rno-miR-18a expression in omental fat may reduce the abundance of Glut4 and the capacity for insulin stimulated glucose uptake for lipid synthesis and storage and other pathways following placental restriction in male offspring and could potentially explain their reduced adiposity, also seen in the current study (Table 3.4)

Sex differences in the phenotypic outcomes of early life programming as seen here are common, with males often affected adversely and earlier, compared to females, as seen here for a range of outcomes. The underlying mechanisms are still poorly understood, but may include interactions of the divergent hormonal and physiological states that result from sexual differentiation from before birth and effects of or on the sex chromosomes themselves (Bryan *et al.*, 2002; Menger *et al.*, 2010). Placental

and epigenetic pathways may be involved, with sex differences in placental molecular and functional profiles in various tissues evident in several species and in their response to prenatal perturbation. Sex differences in epigenetic machinery, such as expression of DNMTs, also exist in some tissues and may add to the variable outcomes seen. There is evidence for the latter in placental restriction (Fu *et al.*, 2004; MacLennan *et al.*, 2004; Fu *et al.*, 2009) and similar processes may contribute to the outcomes reported for insulin action and metabolism following placental restriction.

3.5 Conclusion

This study has shown that placental restriction alters insulin sensitivity, as well as insulin secretion and overall action, in adult offspring, and differently with age as well as sex. Placental restriction impairs insulin secretion in adult offspring, while insulin sensitivity is enhanced in young adult offspring, which then disappears with aging, particularly in females. Placental restriction also up-regulates expression of miRNAs in the insulin sensitive tissues such as liver, skeletal muscles and adipose tissue, and only in older adult offspring. Many of the differentially expressed miRNAs following placental restriction were predicted to target insulin signalling and related molecules and alterations in expression of the latter were consistent with onset of miRNA mediated changes in insulin action in each tissue.

In liver, up-regulated rno-miR-126 and key predicted targets were associated with pathways involving lipid metabolism, molecular transport and small molecule biochemistry. In skeletal muscle, up-regulated rno-miR-451 and key predicted targets were associated with pathways involving carbohydrate metabolism, molecular transport and small molecule biochemistry. In omental fat, up-regulated miRNAs and key predicted targets were associated with pathways involving lipid metabolism, molecular transport and small molecule biochemistry, consistent with previous reports of altered adipocyte triglycerides. Placental restriction up-regulated expression of miRNAs in omental fat, many have known to have major roles in adipogenesis and obesogenesis. The altered expression of these miRNAs in omental fat and their relationships with adiposity in older adult female offspring may be evidence for a potential novel role of peripheral miRNA networks in the programming

of central obesity, one that may add to consequences of impaired appetite control, reported previously.

In male offspring, placental restriction up-regulated rno-miR-18a in omental fat and was negatively correlated with *Glut4* mRNA expression, which may reduce the capacity for insulin stimulated glucose uptake for lipid synthesis and storage and partly explain their reduced adiposity. Overall, placental restriction up-regulates miRNAs expression in insulin sensitive tissues and particularly omental fat, concomitant with onset of insulin resistance and obesity. This is consistent with an early role for miRNA networks in the programming of these aspects of phenotype in offspring.

3.6 Tables and Figures

Table 3.1. The effect of surgical treatments on body weight and dimensions of offspring

	Male			Female			*P*-value (ANOVA)						
	Control	BUVL	Sham	Control	BUVL	Sham	T	A	S	A x T	A x S	T x S	A x T x S
Combined age	(15)	(14)	(12)	(13)	(16)	(12)	(control v BUVL v sham)						
Nose-rump length (mm)	282 ± 5	277 ± 6	282 ± 5	242 ± 4	229 ± 3	242 ± 4	0.003	< 0.001	< 0.001	ns	< 0.001	ns	ns
Abdominal circumferance (mm)	198 ± 8	190 ± 7	197 ± 6	150 ± 5	145 ± 4	157 ± 5	ns	< 0.001	< 0.001	ns	0.001	ns	ns
Body Mass Index (kg.m²)	6.9 ± 0.3	6.9 ± 0.2	7.0 ± 0.1	5.0 ± 0.2	4.5 ± 0.2	4.8 ± 0.1	ns	0.049	< 0.001	ns	< 0.001	ns	ns
Ponderal Index (kg.m³)	22.9 ± 0.9	22.1 ± 0.7	23.8 ± 0.5	24.1 ± 0.7	21.7 ± 0.5	20.8 ± 0.9	ns	0.022	0.001	ns	ns	ns	ns
Body weight (g)	190 ± 20	193 ± 22	167 ± 20	122 ± 12	107 ± 11	99 ± 10	ns	< 0.001	< 0.001	ns	< 0.001	ns	ns

	Male			Female			*P*-value (ANOVA)		
	Control	BUVL	Sham	Control	BUVL	Sham	T	S	T x S
PD 90	(7)	(6)	(4)	(5)	(8)	(4)	(control v BUVL v sham)		
Nose-rump length (mm)	265 ± 3	255 ± 5	259 ± 3	225 ± 3	220 ± 3	233 ± 11	ns	< 0.001	ns
Abdominal circumferance (mm)	169 ± 4	164 ± 4	170 ± 3	132 ± 1	131 ± 3	136 ± 2	ns	< 0.001	ns
Body Mass Index (kg.m²)	7.4 ± 0.2	7.0 ± 0.3	7.1 ± 0.1	4.4 ± 0.1	3.9 ± 0.1	4.3 ± 0.1	0.029	< 0.001	ns
Ponderal Index (kg.m³)	24.0 ± 0.6	25.5 ± 1.1	24.5 ± 0.8	23.2 ± 0.5	22.1 ± 0.8	21.1 ± 2.6	ns	0.01	ns
PD 260	(8)	(8)	(8)	(8)	(8)	(8)			
Nose-rump length (mm)	282 ± 2	294 ± 4	293 ± 2	252 ± 2	237 ± 4	246 ± 2	0.007	< 0.001	0.094
Abdominal circumferance (mm)	224 ± 5	210 ± 6	210 ± 3	161 ± 5	160 ± 6	168 ± 3	ns	< 0.001	0.060
Body Mass Index (kg.m²)	6.6 ± 0.5	6.8 ± 0.2	6.9 ± 0.2	5.4 ± 0.5	5.1 ± 0.2	5.1 ± 0.2	ns	< 0.001	ns
Ponderal Index (kg.m³)	22.0 ± 1.5	23.0 ± 0.8	23.5 ± 0.6	21.4 ± 1.5	21.4 ± 0.8	20.7 ± 0.6	ns	0.029	ns
Body weight									
PD 3	9 ± 0	7 ± 0	8 ± 0	8 ± 0	6 ± 0	7 ± 0	< 0.001	0.003	ns
PD 7	16 ± 0	13 ± 1	15 ± 1	14 ± 0	11 ± 1	14 ± 1	< 0.001	0.003	ns
PD 14	32 ± 1	27 ± 1	30 ± 0	29 ± 1	23 ± 1	29 ± 1	< 0.001	0.001	ns
PD 21	51 ± 1	45 ± 1	47 ± 1	47 ± 1	37 ± 2	45 ± 1	< 0.001	< 0.001	ns
PD 40	166 ± 6	153 ± 8	163 ± 6	150 ± 3	123 ± 4	135 ± 3	0.001	< 0.001	ns
PD 90	423 ± 10	394 ± 13	408 ± 10	256 ± 4	224 ± 6	241 ± 5	0.029	< 0.001	ns
PD 180	557 ± 14	504 ± 21	514 ± 15	297 ± 5	249 ± 12	276 ± 6	0.002	< 0.001	ns
PD 260	584 ± 44	586 ± 27	593 ± 16	340 ± 10	284 ± 12	308 ± 6	ns	< 0.001	ns

Values expressed as means ± SEM and number of animals within brackets. BUVL: bilateral uterine artery vessel ligation; A: age (day 90 or 260); T: Surgical treatments (control, BUVL or sham); S: sex (male or female); ns: not significant. *P*-values are for linear contrasts, which were the most significant with surgical treatments as factors (un-operated, sham and BUVL), except for: Combined age nose-rump length (*P* = 0.001, quadratic); PD 90 body mass index (*P* = 0.022, quadratic); PD 260 nose-rump length (*P* = 0.008, quadratic).

Table 3.2. The effect of surgical treatments on organs and tissues weight on combined age offspring

Combined Age	Male			Female			*P*- value (ANOVA) (control v BUVL v sham)						
	Control (15)	BUVL (14)	Sham (12)	Control (13)	BUVL (16)	Sham (12)	T	A	S	A x T	A x S	T x S	A x T x S
Heart (g)	1.4 ± 0.1	1.3 ± 0.0	1.3 ± 0.0	0.9 ± 0.0	0.8 ± 0.0	0.9 ± 0.0	0.001	< 0.001	< 0.001	ns	0.002	ns	0.087
(%BW)	0.27 ± 0.02	0.25 ± 0.01	0.25 ± 0.01	0.30 ± 0.02	0.30 ± 0.01	0.30 ± 0.01	ns	< 0.001	< 0.001	ns	ns	ns	0.006
Spleen (g)	0.92 ± 0.02	0.85 ± 0.03	0.83 ± 0.03	0.55 ± 0.01	0.49 ± 0.02	0.52 ± 0.02	0.002	0.098	< 0.001	ns	< 0.001	ns	ns
(%BW)	0.18 ± 0.01	0.17 ± 0.01	0.16 ± 0.01	0.18 ± 0.01	0.19 ± 0.01	0.18 ± 0.01	ns	< 0.001	< 0.001	ns	0.049	ns	ns
Adrenals (g)	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.06 ± 0.05	0.08 ± 0.06	0.08 ± 0.06	ns	ns	ns	ns	0.069	ns	ns
(%BW)	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	ns	ns	< 0.001	ns	0.048	0.074[1]	ns
Kidneys (g)	3.4 ± 0.1	3.2 ± 0.1	3.3 ± 0.1	1.8 ± 0.1	1.6 ± 0.1	1.8 ± 0.1	0.001	< 0.001	< 0.001	ns	0.052	ns	ns
(%BW)	0.69 ± 0.13	0.64 ± 0.09	0.63 ± 0.06	0.60 ± 0.01	0.61 ± 0.01	0.63 ± 0.02	ns	< 0.001	0.004	ns	0.044	ns	ns
Liver (g)	20.6 ± 0.6	20.1 ± 0.9	20.1 ± 0.9	10.4 ± 0.4	8.6 ± 0.2	9.6 ± 0.4	0.049	< 0.001	< 0.001	ns	0.001	ns	ns
(%BW)	4.12 ± 0.85	3.98 ± 0.66	3.78 ± 0.36	3.39 ± 0.15	3.36 ± 0.10	3.31 ± 0.12	ns	< 0.001	< 0.001	ns	ns	ns	ns
Lungs (g)	1.7 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.2 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	0.021	< 0.001	< 0.001	ns	0.087	ns	ns
(%BW)	0.34 ± 0.02	0.32 ± 0.02	0.37 ± 0.01	0.37 ± 0.04	0.42 ± 0.01	0.37 ± 0.01	ns	< 0.001	< 0.001	ns	ns	ns	0.023
Brain (g)	2.2 ± 0.0	2.1 ± 0.1	2.1 ± 0.0	1.9 ± 0.0	1.9 ± 0.0	1.9 ± 0.0	ns	0.012	< 0.001	ns	0.084	ns	ns
(%BW)	0.43 ± 0.02	0.41 ± 0.02	0.41 ± 0.02	0.63 ± 0.02	0.73 ± 0.02	0.67 ± 0.02	ns	< 0.001	< 0.001	ns	ns	0.004[2]	ns
Uterus (g)				0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	ns	< 0.001	ns	ns			
(%BW)				0.17 ± 0.01	0.17 ± 0.01	0.19 ± 0.01	ns	< 0.001	ns	ns			
Testes (g)	3.5 ± 0.1	3.2 ± 0.1	3.1 ± 0.1				0.005	0.046	ns	ns			
(%BW)	0.70 ± 0.04	0.63 ± 0.03	0.59 ± 0.04				ns	< 0.001	ns	ns			
Fat depots													
Retroperitoneal fat (g)	11.6 ± 2.0	10.9 ± 1.9	10.6 ± 1.4	4.8 ± 0.8	3.9 ± 0.7	3.7 ± 0.6	ns	< 0.001	< 0.001	ns	< 0.001	ns	ns
(%BW)	2.16 ± 0.34	1.95 ± 0.24	1.88 ± 0.17	1.47 ± 0.21	1.42 ± 0.21	1.23 ± 0.18	ns	< 0.001	< 0.001	ns	0.068	ns	ns
Omental fat (g)	6.9 ± 1.2	6.3 ± 1.2	6.8 ± 1.1	3.7 ± 0.4	3.1 ± 0.4	3.1 ± 0.3	ns	< 0.001	< 0.001	ns	< 0.001	ns	ns
(%BW)	1.28 ± 0.18	1.12 ± 0.16	1.20 ± 0.15	1.17 ± 0.10	1.16 ± 0.10	1.04 ± 0.10	ns	< 0.001	ns	ns	0.001	ns	ns
Visceral fat (g)	18.6 ± 3.1	17.2 ± 3.1	17.3 ± 2.4	8.2 ± 1.1	7.2 ± 0.9	6.9 ± 0.8	ns	< 0.001	< 0.001	ns	< 0.001	ns	ns
(%BW)	3.44 ± 0.52	3.07 ± 0.39	3.08 ± 0.31	2.64 ± 0.25	2.58 ± 0.29	2.27 ± 0.24	ns	< 0.001	0.001	ns	0.006	ns	0.087

Values expressed as means ± SEM and number of animals within brackets. BUVL: bilateral uterine artery vessel ligation; A: age (day 90 or 260); T: Surgical treatments (control, BUVL or sham); S: sex (male or female); ns: not significant; %BW: percentage to the body weight. [1]Surgical treatments did not alter adrenals weight relative to body weight across male offspring (P = 0.833), but linearly reduced in female treatment offspring than in female control offspring (P = 0.035). [2]Surgical treatments did not alter brain weight relative to body weight across male offspring (P = 0.578), but linearly reduced in female treatment offspring than in female control offspring (P = 0.004). Contrasts for polynomial fit for P-values based on the surgical treatments as factors (un-operated, sham and BUVL), Heart weight (P = 0.003, quadratic); spleen weight (P = 0.005, linear); kidneys weight (P = 0.001, quadratic); liver weight (P = 0.086, linear); lungs weight (P = 0.015, linear); testes weight (P = 0.004, linear); females adrenals weight relative to body weight (P = 0.035, linear); females brain weight relative to body weight (P = 0.004, linear).

Table 3.3. Effect of surgical treatments on organ and tissue weights in young adult offspring

PD 90	Male			Female			*P*-value (ANOVA) control v BUVL v sham		
	Control (7)	BUVL (6)	Sham (4)	Control (5)	BUVL (8)	Sham (4)	T	S	T x S
Heart (g)	1.2 ± 0.0	1.2 ± 0.0	1.2 ± 0.0	0.9 ± 0.1	0.7 ± 0.0	0.8 ± 0.0	0.054	< 0.001	ns
(%BW)	0.27 ± 0.01	0.28 ± 0.01	0.28 ± 0.01	0.35 ± 0.04	0.31 ± 0.01	0.31 ± 0.01	ns	0.001	ns
Spleen (g)	0.87 ± 0.02	0.81 ± 0.05	0.73 ± 0.02	0.56 ± 0.02	0.51 ± 0.03	0.56 ± 0.02	ns	< 0.001	ns
(%BW)	0.20 ± 0.00	0.19 ± 0.01	0.17 ± 0.01	0.21 ± 0.01	0.22 ± 0.01	0.22 ± 0.01	ns	< 0.001	ns
Adrenals (g)	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	ns	0.009	ns
(%BW)	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.01	ns	< 0.001	ns
Kidneys (g)	3.2 ± 0.1	3.0 ± 0.1	3.0 ± 0.1	1.7 ± 0.1	1.5 ± 0.1	1.7 ± 0.1	0.021	< 0.001	ns
(%BW)	0.73 ± 0.02	0.72 ± 0.03	0.70 ± 0.02	0.63 ± 0.02	0.63 ± 0.02	0.65 ± 0.03	ns	< 0.001	ns
Liver (g)	19.3 ± 0.7	18.0 ± 1.0	17.5 ± 0.5	10.1 ± 0.4	8.6 ± 0.2	9.2 ± 0.9	0.072	< 0.001	ns
(%BW)	4.36 ± 0.15	4.32 ± 0.30	4.11 ± 0.07	3.79 ± 0.19	3.65 ± 0.05	3.58 ± 0.25	ns	0.001	ns
Lungs (g)	1.6 ± 0.1	1.5 ± 0.1	1.4 ± 0.0	1.3 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	0.026	< 0.001	ns
(%BW)	0.36 ± 0.02	0.36 ± 0.03	0.34 ± 0.01	0.47 ± 0.04	0.43 ± 0.01	0.38 ± 0.02	ns	< 0.001	ns
Brain (g)	2.1 ± 0.0	1.9 ± 0.2	2.1 ± 0.1	1.9 ± 0.0	1.8 ± 0.0	2.0 ± 0.0	ns	ns	ns
(%BW)	0.47 ± 0.00	0.05 ± 0.05	0.48 ± 0.02	0.70 ± 0.01	0.78 ± 0.02	0.76 ± 0.01	ns	< 0.001	ns
Uterus (g)				0.4 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	ns		
(%BW)				0.07 ± 0.04	0.08 ± 0.03	0.15 ± 0.02	ns		
Testes (g)	3.3 ± 0.1	3.0 ± 0.1	3.1 ± 0.1				0.062		
(%BW)	0.75 ± 0.01	0.72 ± 0.03	0.73 ± 0.01				ns		
Fat depots									
Retroperitoneal fat (g)	4.4 ± 0.3	5.0 ± 0.8	5.3 ± 0.3	2.4 ± 0.3	1.9 ± 0.2	1.7 ± 0.1	ns	< 0.001	ns
(%BW)	0.99 ± 0.07	1.17 ± 0.15	1.25 ± 0.05	0.88 ± 0.10	0.80 ± 0.05	0.67 ± 0.04	ns	< 0.001	ns
Omental fat (g)	2.7 ± 0.3	2.5 ± 0.1	3.0 ± 0.4	2.5 ± 0.2	2.0 ± 0.2	2.1 ± 0.3	ns	0.008	ns
(%BW)	0.61 ± 0.05	0.59 ± 0.03	0.71 ± 0.10	0.93 ± 0.06	0.85 ± 0.07	0.81 ± 0.11	ns	0.001	ns
Visceral fat (g)	7.1 ± 0.5	7.5 ± 0.9	8.4 ± 0.5	4.9 ± 0.5	4.3 ± 0.4	4.3 ± 0.7	ns	< 0.001	ns
(%BW)	1.60 ± 0.10	1.76 ± 0.15	1.96 ± 0.09	1.81 ± 0.15	1.64 ± 0.10	1.48 ± 0.13	ns	ns	ns

Values expressed as means ± SEM and number of animals within brackets. BUVL: bilateral uterine artery vessel ligation; T: Surgical treatments (control, BUVL or sham); S: sex (male or female); ns: not significant; %BW: percentage to the body weight. Contrasts for polynomial fit for *P*-values based on the surgical treatments as factors (un-operated, sham and BUVL), Kidneys weight ($P = 0.028$, quadratic); lungs weight ($P = 0.012$, linear).

Table 3.4. Effect of surgical treatments on organ and tissue weights in rat in older adult offspring

PD 260	Male			Female			*P*-value (ANOVA) (control v BUVL v sham)		
	Control (8)	BUVL (8)	Sham (8)	Control (8)	BUVL (8)	Sham (8)	T	S	T x S
Heart (g)	1.5 ± 0.1	1.3 ± 0.1	1.4 ± 0.0	0.9 ± 0.0	0.8 ± 0.0	0.9 ± 0.0	0.019	< 0.001	ns
(%BW)	0.30 ± 0.03	0.23 ± 0.01	0.24 ± 0.01	0.27 ± 0.01	0.29 ± 0.01	0.29 ± 0.01	ns	0.001	ns
Spleen (g)	0.97 ± 0.03	0.87 ± 0.04	0.87 ± 0.04	0.54 ± 0.02	0.47 ± 0.02	0.50 ± 0.02	0.011	< 0.001	ns
(%BW)	0.17 ± 0.02	0.15 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	ns	ns	ns
Adrenals (g)	0.08 ± 0.02	0.08 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	ns	ns	ns
(%BW)	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.00	0.03 ± 0.01	0.02 ± 0.01	ns	0.001	0.033[1]
Kidneys (g)	3.6 ± 0.1	3.4 ± 0.1	3.5 ± 0.1	1.9 ± 0.0	1.7 ± 0.1	1.9 ± 0.1	0.016	< 0.001	ns
(%BW)	0.65 ± 0.06	0.57 ± 0.02	0.59 ± 0.01	0.57 ± 0.01	0.59 ± 0.01	0.61 ± 0.02	ns	ns	ns
Liver (g)	21.7 ± 0.6	21.7 ± 1.1	21.5 ± 1.1	10.6 ± 0.6	8.7 ± 0.3	9.8 ± 0.3	ns	< 0.001	ns
(%BW)	3.91 ± 0.39	3.72 ± 0.17	3.61 ± 0.11	3.14 ± 0.15	3.07 ± 0.12	3.17 ± 0.10	ns	< 0.001	ns
Lungs (g)	1.8 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.2 ± 0.0	1.2 ± 0.1	1.1 ± 0.0	ns	< 0.001	ns
(%BW)	0.32 ± 0.04	0.28 ± 0.01	0.30 ± 0.02	0.31 ± 0.05	0.41 ± 0.02	0.37 ± 0.01	ns	0.011	ns
Brain (g)	2.2 ± 0.0	2.2 ± 0.1	2.2 ± 0.0	2.0 ± 0.0	1.9 ± 0.0	1.9 ± 0.0	ns	< 0.001	ns
(%BW)	0.40 ± 0.04	0.37 ± 0.01	0.36 ± 0.01	0.58 ± 0.02	0.67 ± 0.02	0.62 ± 0.01	ns	< 0.001	0.022[2]
Uterus (g)				0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	ns		
(%BW)				0.17 ± 0.01	0.21 ± 0.02	0.20 ± 0.01	ns		
Testes (g)	3.6 ± 0.1	3.3 ± 0.1	3.1 ± 0.2				0.020		
(%BW)	0.66 ± 0.07	0.57 ± 0.03	0.53 ± 0.03				ns		
Skeletal muscles									
Left soleus (g)	0.21 ± 0.01	0.15 ± 0.02	0.17 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.066	< 0.001	ns
(%BW)	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.01	ns	ns	ns
Left vastus lateralis (g)	1.39 ± 0.27	1.49 ± 0.28	1.47 ± 0.74	0.69 ± 0.05	0.70 ± 0.04	0.85 ± 0.08	ns	< 0.001	ns
(%BW)	0.26 ± 0.07	0.25 ± 0.04	0.24 ± 0.04	0.20 ± 0.01	0.25 ± 0.01	0.28 ± 0.02	ns	ns	ns
Fat depots									
Retroperitoneal fat (g)	17.9 ± 1.6	15.3 ± 2.2	13.2 ± 1.3	6.3 ± 0.9	5.9 ± 0.9	4.7 ± 0.6	0.078	< 0.001	ns
(%BW)	3.19 ± 0.35	2.54 ± 0.26	2.20 ± 0.17	1.84 ± 0.25	2.05 ± 0.28	1.51 ± 0.20	0.040	< 0.001	ns
Omental fat (g)	10.6 ± 0.9	9.1 ± 1.4	8.6 ± 1.1	4.4 ± 0.4	4.2 ± 0.4	3.6 ± 0.4	ns	< 0.001	ns
(%BW)	1.86 ± 0.15	1.52 ± 0.17	1.44 ± 0.16	1.31 ± 0.13	1.48 ± 0.08	1.15 ± 0.12	ns	0.013	ns
Visceral fat (g)	28.6 ± 2.4	24.4 ± 3.6	21.8 ± 2.3	10.7 ± 1.0	10.1 ± 1.1	8.2 ± 0.8	ns	< 0.001	ns
(%BW)	5.05 ± 0.48	4.05 ± 0.41	3.64 ± 0.30	3.15 ± 0.26	3.53 ± 0.29	2.66 ± 0.25	0.025	< 0.001	ns

Values expressed as means ± SEM and number of animals within brackets. BUVL: bilateral uterine artery vessel ligation; T: Surgical treatments (control, BUVL or sham); S: sex (male or female); ns: not significant; %BW: percentage to the body weight. [1]Surgical treatments did not alter adrenals weight relative to body weight across male offspring (*P* = 0.773), but linearly reduced in female treatment offspring than in female control offspring (*P* = 0.014). [2]Surgical treatments did not alter brain weight relative to body weight across male offspring (*P* = 0.563), but linearly increased in female treatment offspring than in female control offspring (*P* = 0.006). Contrasts for polynomial fit for *P*-values based on the surgical treatments as factors (un-operated, sham and BUVL), Heart weight (*P* = 0.013, quadratic); Spleen weight (*P* = 0.021, linear); Kidneys weight (P = 0.007, quadratic); Retroperitoneal fat weight relative to body weight (*P* = 0.013, linear); Visceral fat weight relative to body weight (*P* = 0.008, linear); Testes weight (*P* = 0.006, linear); Females adrenals weight relative to body weight (*P* = 0.023, quadratic); Females brain weight relative to body weight (*P* = 0.005, quadratic)

Table 3.5. Effect of surgical treatments on glucose tolerance, insulin secretion, sensitivity and action in offspring

Combined Age	Male			Female			*P*-value (ANOVA) (control v BUVL v sham)						
	Control (16)	BUVL (16)	Sham (16)	Control (16)	BUVL (16)	Sham (16)	T	A	S	A x T	A x S	T x S	A x T x S
Glucose tolerance													
Fasting plasma glucose ($mmol.L^{-1}$)	6.8 ± 0.4	5.8 ± 0.2	5.8 ± 0.2	5.8 ± 0.1	5.6 ± 0.2	5.6 ± 0.2	0.007	< 0.001	0.007	0.001[1]	ns	ns	ns
Maximum plasma glucose ($mmol.L^{-1}$)	13.5 ± 0.7	13.4 ± 1.1	12.6 ± 0.8	12.7 ± 1.1	12.3 ± 0.7	11.5 ± 0.6	ns	< 0.001	0.098	ns	ns	ns	ns
ΔPlasma glucose ($mmol.L^{-1}$)	6.8 ± 0.6	7.6 ± 0.9	6.8 ± 0.7	7.0 ± 1.1	6.8 ± 0.6	5.9 ± 0.6	ns	0.001	ns	ns	ns	ns	ns
AUC glucose, 0-90min ($mmol.L^{-1}$)	348 ± 25	364 ± 30	370 ± 43	315 ± 40	361 ± 20	286 ± 34	ns	0.037	ns	ns	ns	ns	ns
Insulin secretion													
Fasting plasma insulin ($\mu g.L^{-1}$)	0.81 ± 0.10	0.40 ± 0.06	0.61 ± 0.07	0.25 ± 0.02	0.14 ± 0.02	0.21 ± 0.02	< 0.001	ns	< 0.001	ns	ns	0.047[3]	ns
Maximum plasma insulin ($\mu g.L^{-1}$)	2.38 ± 0.49	1.97 ± 0.27	2.38 ± 0.24	1.75 ± 0.36	1.58 ± 0.20	1.75 ± 0.21	0.002	ns	0.012	ns	ns	ns	ns
ΔPlasma insulin ($\mu g.L^{-1}$)	2.74 ± 0.46	1.49 ± 0.21	1.77 ± 0.20	2.32 ± 0.37	1.40 ± 0.17	1.62 ± 0.21	0.001	ns	ns	ns	ns	ns	ns
AUC insulin, 0-90min ($\mu g.L^{-1}$)	94 ± 15	52 ± 9	57 ± 6	79 ± 9	51 ± 8	48 ± 4	< 0.001	ns	ns	ns	ns	ns	ns
Fasting insulin:glucose ($\mu g.mmol^{-1}$)	0.12 ± 0.02	0.08 ± 0.01	0.11 ± 0.01	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.008	ns	< 0.001	ns	ns	ns	ns
AUC insulin:glucose ($\mu g.mmol^{-1}$)	0.24 ± 0.05	0.19 ± 0.02	0.14 ± 0.03	0.29 ± 0.04	0.17 ± 0.03	0.22 ± 0.05	0.015	0.030	ns	ns	ns	ns	ns
Insulin sensitivity													
Basal plasma glucose ($mmol.L^{-1}$)	7.25 ± 0.15	7.22 ± 0.20	6.87 ± 0.14	7.01 ± 0.08	7.31 ± 0.19	7.17 ± 0.15	ns	0.003	ns	ns	0.023	ns	ns
Minimum plasma glucose ($mmol.L^{-1}$)	4.61 ± 0.07	4.56 ± 0.09	4.16 ± 0.25	4.68 ± 0.41	3.17 ± 0.36	4.15 ± 0.46	0.066	ns	ns	ns	ns	0.054[4]	ns
Decrease plasma glucose ($mmol.L^{-1}$)	-2.5 ± 0.2	-2.8 ± 0.2	-2.7 ± 0.3	-2.7 ± 0.4	-4.1 ± 0.4	-3.0 ± 0.4	0.064	ns	0.03	ns	0.094	ns	ns
AAC glucose, 0-60min ($mmol.L^{-1}$)	96 ± 10	105 ± 9	99 ± 8	119 ± 9	162 ± 8	147 ± 10	0.023	< 0.001	< 0.001	ns	ns	ns	ns
Insulin action													
Basal DI_{AAC}, 0-60min ($\mu g.mmol.min.L^{-2}$)	61.5 ± 8	42.5 ± 9	54 ± 7	35 ± 6	18 ± 4	30 ± 5	0.022	ns	< 0.001	ns	0.023	ns	ns
Basal $DI_{\Delta glucose}$ ($\mu g.mmol.min.L^{-2}$)	1.79 ± 0.29	1.42 ± 0.37	1.49 ± 0.18	0.77 ± 0.15	0.50 ± 0.10	0.59 ± 0.11	ns	ns	< 0.001	ns	ns	ns	ns
Maximal DI_{AAC}, 0-60min ($\mu g.mmol.min.L^{-2}$)	197 ± 23	156 ± 26	177 ± 27	243 ± 38	216 ± 25	231 ± 42	ns	0.006	0.008	0.054[2]	ns	ns	ns
Maximal $DI_{\Delta glucose}$ ($\mu g.mmol.min.L^{-2}$)	5.13 ± 0.85	3.83 ± 0.69	4.68 ± 0.69	5.01 ± 1.15	5.69 ± 0.82	4.77 ± 1.06	ns	ns	ns	ns	ns	ns	ns

Values expressed as means ± SEM and number of animals within brackets. BUVL: bilateral uterine artery vessel ligation; A: age (day 90 or 180); T: Surgical treatments (control, BUVL or sham); S: sex (male or female); ns: not significant. [1]Surgical treatments linearly reduced fasting plasma glucose at PD 90 compared to control offspring (P = 0.002), but was similar across treatment at PD 180 (P = 0.815). [2]Maximal DI_{AAC} was similar across treatment group both at PD 90 and PD 180 (P = 0.273 & P = 0.209, respectively). [3]Surgical treatments linearly reduced fasting plasma insulin both in male and female offspring than in controls (P = 0.003 & P = 0.002, respectively). [4]Surgical treatments did not alter minimum glucose in male offspring compared to control male offspring (P = 0.002), but linearly reduced in female treatment offspring than in female control offspring (P = 0.046). Contrasts for polynomial fit for P-values based on the surgical treatments as factors (un-operated, sham and BUVL), Fasting plasma glucose (P = 0.004, linear); Fasting plasma insulin (P < 0.001, linear); Maximum plasma insulin (P = 0.001, linear); Δinsulin (P < 0.001, linear); AUC insulin (P < 0.001, linear); Fasting plasma insulin:glucose (P = 0.002, linear); AUC insulin:glucose (P = 0.004, linear); AAC glucose (P = 0.006, linear); Basal DI_{AAC} (P = 0.006, linear).

Table 3.6. Effect of surgical treatments on glucose tolerance, insulin secretion, sensitivity and action in young adult offspring

PD 90	Male			Female			*P*-value (ANOVA) (control x BUVL x sham)		
	Control (8)	BUVL (8)	Sham (8)	Control (8)	BUVL (8)	Sham (8)	T	S	T x S
Glucose tolerance									
Fasting plasma glucose ($mmol.L^{-1}$)	7.2 ± 0.8	5.1 ± 0.2	5.3 ± 0.3	5.6 ± 0.1	4.9 ± 0.2	5.0 ± 0.1	0.001	0.027	ns
Maximum plasma glucose ($mmol.L^{-1}$)	13.3 ± 1.2	11.7 ± 0.9	11.6 ± 1.3	10.5 ± 0.9	9.9 ± 0.1	10.4 ± 0.9	ns	0.022	ns
ΔPlasma glucose ($mmol.L^{-1}$)	6.1 ± 0.9	6.6 ± 0.8	6.3 ± 1.2	5.0 ± 0.8	5.0 ± 0.2	5.4 ± 1.0	ns	ns	ns
AUC glucose, 0-90min ($mmol.L^{-1}$)	324 ± 14	351 ± 37	361 ± 87	216 ± 23	302 ± 24	314 ± 46	ns	0.090	ns
Insulin secretion									
Fasting plasma insulin ($\mu g.L^{-1}$)	0.79 ± 0.14	0.30 ± 0.05	0.67 ± 0.07	0.26 ± 0.04	0.20 ± 0.03	0.24 ± 0.03	0.005	< 0.001	0.029[1]
Maximum plasma insulin ($\mu g.L^{-1}$)	3.72 ± 0.84	1.90 ± 0.35	2.29 ± 0.21	2.02 ± 0.20	1.95 ± 0.33	1.34 ± 0.11	0.031	0.015	ns
ΔPlasma insulin ($\mu g.L^{-1}$)	2.93 ± 0.72	1.44 ± 0.23	1.62 ± 0.21	1.99 ± 0.28	1.66 ± 0.26	1.10 ± 0.10	0.009	ns	ns
AUC insulin, 0-90min ($\mu g.L^{-1}$)	93 ± 20	53 ± 6	66 ± 6	75 ± 12	71 ± 13	47 ± 4	0.048	ns	ns
Fasting insulin:glucose ($\mu g.mmol^{-1}$)	0.12 ± 0.03	0.06 ± 0.01	0.13 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.033	< 0.001	0.073[2]
AUC insulin:glucose ($\mu g.mmol^{-1}$)	0.28 ± 0.08	0.16 ± 0.03	0.25 ± 0.04	0.33 ± 0.07	0.24 ± 0.05	0.18 ± 0.03	0.095	ns	ns
Insulin sensitivity									
Basal plasma glucose ($mmol.L^{-1}$)	6.95 ± 0.21	7.01 ± 0.36	6.43 ± 0.12	7.01 ± 0.14	7.31 ± 0.38	7.03 ± 0.23	ns	ns	ns
Minimum plasma glucose ($mmol.L^{-1}$)	4.56 ± 0.23	4.58 ± 0.15	4.35 ± 0.21	4.80 ± 0.36	3.16 ± 0.21	3.90 ± 0.55	0.043	0.042	0.042[3]
Decrease plasma glucose ($mmol.L^{-1}$)	-2.2 ± 0.3	-2.4 ± 0.4	-2.1 ± 0.3	-2.8 ± 0.4	-4.2 ± 0.4	-3.1 ± 0.4	0.065	< 0.001	ns
AAC glucose, 0-60min ($mmol.L^{-1}$)	76 ± 9	88 ± 14	79 ± 7	101 ± 11	147 ± 16	133 ± 11	0.059	< 0.001	ns
Insulin action									
Basal DI_{AAC}, 0-60min ($\mu g.mmol.min.L^{-2}$)	54 ± 10	24 ± 5	50 ± 9	33 ± 10	27 ± 6	31 ± 4	0.076	0.072	ns
Basal $DI_{\Delta glucose}$ ($\mu g.mmol.min.L^{-2}$)	1.71 ± 0.42	1.35 ± 0.7	1.31 ± 0.20	0.87 ± 0.24	0.83 ± 0.11	0.73 ± 0.12	ns	0.044	ns
Maximal DI_{AAC}, 0-60min ($\mu g.mmol.min.L^{-2}$)	191 ± 29	143 ± 43	135 ± 25	198 ± 39	231 ± 34	147 ± 19	ns	ns	ns
Maximal $DI_{\Delta glucose}$ ($\mu g.mmol.min.L^{-2}$)	5.74 ± 1.3	3.08 ± 0.94	3.34 ± 0.60	5.61 ± 1.27	6.93 ± 1.31	3.37 ± 0.49	ns	ns	0.049[4]

Values expressed as means ± SEM and number of animals within brackets. BUVL: bilateral uterine artery vessel ligation; T: Surgical treatments (control, BUVL or sham); S: sex (male or female); ns: not significant. [1]Surgical treatments linearly reduced fasting insulin in male treatment offspring than in male control offspring (P = 0.008), but was similar across female offspring (P = 0.491). [2]Surgical treatments linearly reduced fasting insulin:glucose in male treatment offspring than in male control offspring (P = 0.036), but was similar across treatment group in female offspring (P = 0.805). [3]Surgical treatments did not alter minimum glucose across male offspring (P = 0.591), but was reduced in female treatment offspring than in female control offspring (P = 0.036). [4]Surgical treatments did not alter maximal $DI_{\Delta glucose}$ across male offspring (P = 0.139), but was tended to reduce in female treatment offspring than in female control offspring (P = 0.051). Contrasts for polynomial fit for P-values based on the surgical treatments as factors (un-operated, sham and BUVL), Fasting plasma glucose (P = 0.001, linear); Fasting plasma insulin (P = 0.002, linear); Maximum plasma insulin (P = 0.032, linear); Δinsulin (P = 0.017, linear); AUC insulin (P = 0.059, linear); Fasting plasma insulin:glucose (P = 0.027, linear); Minimum plasma glucose (P = 0.015, linear).

Table 3.7. Effect of surgical treatments on glucose tolerance, insulin secretion, sensitivity and action in older adult offspring

PD 180	Male			Female			*P*-value (ANOVA) (control x BUVL x sham)		
	Control (8)	BUVL (8)	Sham (8)	Control (8)	BUVL (8)	Sham (8)	T	S	T x S
Glucose tolerance									
Fasting plasma glucose ($mmol.L^{-1}$)	6.3 ± 0.3	6.5 ± 0.2	6.3 ± 0.1	6.1 ± 0.2	6.1 ± 0.2	6.2 ± 0.1	ns	ns	ns
Maximum plasma glucose ($mmol.L^{-1}$)	13.8 ± 0.9	15.1 ± 1.8	13.6 ± 0.9	14.8 ± 1.7	14.4 ± 0.7	12.5 ± 0.8	ns	ns	ns
ΔPlasma glucose ($mmol.L^{-1}$)	7.5 ± 0.9	8.5 ± 1.7	7.3 ± 0.9	9 ± 1.8	8.3 ± 0.6	6.3 ± 0.8	ns	ns	ns
AUC glucose, 0-90min ($mmol.L^{-1}$)	369 ± 45	377 ± 49	380 ± 22	403 ± 57	413 ± 17	258 ± 50	ns	ns	ns
Insulin secretion									
Fasting plasma insulin ($\mu g.L^{-1}$)	0.83 ± 0.14	0.49 ± 0.10	0.55 ± 0.12	0.25 ± 0.02	0.09 ± 0.03	0.17 ± 0.03	0.027	< 0.001	ns
Maximum plasma insulin ($\mu g.L^{-1}$)	3.08 ± 0.57	2.04 ± 0.38	2.46 ± 0.44	2.77 ± 0.66	1.25 ± 0.21	2.16 ± 0.36	0.032	ns	ns
ΔPlasma insulin ($\mu g.L^{-1}$)	2.51 ± 0.60	1.54 ± 0.38	1.92 ± 0.36	2.70 ± 0.73	1.16 ± 0.20	2.22 ± 0.32	0.028	ns	ns
AUC insulin, 0-90min ($\mu g.L^{-1}$)	94 ± 24	52 ± 16	50 ± 11	83 ± 14	33 ± 6	49 ± 8	0.006	ns	ns
Fasting insulin:glucose ($\mu g.mmol^{-1}$)	0.12 ± 0.03	0.08 ± 0.02	0.09 ± 0.02	0.04 ± 0.01	0.01 ± 0.00	0.03 ± 0.01	ns	< 0.001	ns
AUC insulin:glucose ($\mu g.mmol^{-1}$)	0.19 ± 0.06	0.11 ± 0.03	0.13 ± 0.03	0.26 ± 0.06	0.10 ± 0.02	0.26 ± 0.11	0.093	ns	ns
Insulin sensitivity									
Basal plasma glucose ($mmol.L^{-1}$)	7.59 ± 0.13	7.42 ± 0.14	7.32 ± 0.12	7.01 ± 0.1	7.31 ± 0.15	7.31 ± 0.55	ns	0.049	ns
Minimum plasma glucose ($mmol.L^{-1}$)	4.67 ± 0.08	4.55 ± 0.10	3.96 ± 0.47	4.55 ± 0.78	3.19 ± 0.68	4.40 ± 2.17	ns	ns	ns
Decrease plasma glucose ($mmol.L^{-1}$)	-2.9 ± 0.2	-3.1 ± 0.2	-3.4 ± 0.5	-2.7 ± 0.8	-4.1 ± 0.7	-2.9 ± 0.8	ns	ns	ns
AAC glucose, 0-60min ($mmol.L^{-1}$)	114 ± 14	121 ± 9	116 ± 11	145 ± 3	173 ± 7	164 ± 38	ns	< 0.001	ns
Insulin action									
Basal DI_{AAC}, 0-60min ($\mu g.mmol.min.L^{-2}$)	71 ± 13	59 ± 13	58 ± 10	38 ± 4	12 ± 3	27 ± 10	ns	< 0.001	ns
Basal $DI_{\Delta glucose}$ ($\mu g.mmol.min.L^{-2}$)	1.80 ± 0.44	1.49 ± 0.30	1.67 ± 0.30	0.60 ± 0.19	0.20 ± 0.07	0.45 ± 0.47	ns	< 0.001	ns
Maximal DI_{AAC}, 0-60min ($\mu g.mmol.min.L^{-2}$)	204 ± 37	170 ± 33	214 ± 42	304 ± 69	206 ± 37	348 ± 69	ns	0.024	ns
Maximal $DI_{\Delta glucose}$ ($\mu g.mmol.min.L^{-2}$)	4.40 ± 1.11	4.40 ± 1.00	6.00 ± 1.07	5.30 ± 2.15	4.60 ± 0.94	6.16 ± 5.66	ns	ns	ns

Values expressed as means ± SEM and number of animals within brackets. BUVL: bilateral uterine artery vessel ligation; T: Surgical treatments (control, BUVL or sham); S: sex (male or female); ns: not significant. Contrasts for polynomial fit for *P*-values based on the surgical treatments as factors (un-operated, sham and BUVL), Fasting plasma insulin (*P* = 0.009, linear); Maximum plasma insulin (*P* = 0.009, linear); Δinsulin (*P* = 0.008, linear); AUC insulin (*P* = 0.003, linear).

Table 3.8. Effect of bilateral uterine artery vessel ligation on miRNAs expression in liver, skeletal muscle and omental fat tissue in older adult offspring

Sex	miRNAs	Fold Change	qPCR		Accession No.	Chr.	Chromosomal location	miRNA Cluster	CpG Island
			Fold Change	*P*-value					
Liver									
Male	mmu-miR-199b*	-1.09	-2.11	0.009	MIMAT0000672	2	O/T; antisense		
Female	rno-miR-126	1.18	1.42	0.015	MIMAT0000832	3	O/T; sense	1	
	mmu-miR-199b*	1.21	1.67	0.067	MIMAT0000672	2	O/T; antisense		
Combined	-								
Skeletal Muscle									
Male	**rno-miR-451**	1.80	1.73	ns	MIMAT0001633	10	Intergenic	2	
Female	rno-miR-451	1.16	1.90	0.099	MIMAT0001633	10	Intergenic	2	
Combined	**rno-miR-451**	1.43	1.83	0.029	MIMAT0001633	10	Intergenic	2	
Omental Fat									
Male	-								
Female	-								
Combined	**rno-miR-16**	1.44	1.35	ns	MIMAT0000785	2	O/T; sense	3	
	rno-miR-18a	1.33	1.34	0.044	MIMAT0000787	15	Intergenic	4	
	rno-miR-142-3p	2.40	2.65	0.051	MIMAT0000848	10	Intergenic		
	rno-miR-19b	1.64	1.97	0.012	MIMAT0000788	15	Intergenic	4	
	rno-miR-21	1.62	1.49	0.051	MIMAT0000790	10	Intergenic		
	rno-miR-20b	1.42	1.85	0.018	MIMAT0003211	X	Intergenic	5	
	hsa-miR-106a	1.41	2.17	ns	MIMAT0000103	X	Intergenic	6	Present
	mmu-miR-106a	1.27	1.65	0.033	MIMAT0000385	X	O/T; sense	6	

MiRNAs significantly differentially expressed determined by LIMMA analysis (In bold, adjusted $P < 0.05$; non-bold: unadjusted $P < 0.000559$). miRNAs are listed with rat and mouse miRBase accession numbers and chromosomal location. rno: *Rattus norvegicus*; mmu: *Mus musculus*; hsa: *Homo sapiens*. O/T: overlapping transcript, located either at intron or exon of host gene. For hsa-miR-106a, CpG island was detected between 10 kb up and down stream of miRNA gene location, based on UCSC genome browser database (accessed on November 2010). List of cluster member can be found on Supplement Table S3.9.

Table 3.9. Quantitative analysis on effect of bilateral uterine artery vessel ligation on microRNAs expression in insulin sensitive tissues in older adult offspring

Linearised	Male		Female		two-way ANOVA		
(qPCR)	Control (8)	BUVL (8)	Control (8)	BUVL (8)	T	S	T x S
Liver							
mmu-miR-199b*	0.001 ± 0.000	0.000 ± 0.000	0.001 ± 0.000	0.001 ± 0.000	ns	ns	0.002[1]
rno-miR-126	3.14 ± 0.18	2.57 ± 0.24	2.58 ± 0.28	3.55 ± 0.21	ns	ns	0.002[2]
Skeletal muscle							
rno-miR-451	0.036 ± 0.011	0.061 ± 0.013	0.026 ± 0.012	0.051 ± 0.013	0.029	0.388	0.985
Omental fat							
rno-miR-16	8.98 ± 3.41	10.27 ± 1.92	9.24 ± 1.41	13.65 ± 2.17	ns	ns	ns
rno-miR-18a	0.007 ± 0.001	0.01 ± 0.005	0.005 ± 0.001	0.007 ± 0.001	0.044	0.094	ns
rno-miR-142-3p	5.05 ± 2.59	13.54 ± 12.90	2.44 ± 1.40	5.72 ± 2.00	0.051	0.082	ns
rno-miR-19b	0.66 ± 0.25	1.29 ± 0.34	0.81 ± 0.13	1.53 ± 0.23	0.012	ns	ns
rno-miR-21	2.57 ± 0.53	4.87 ± 1.62	2.81 ± 0.49	4.42 ± 0.73	0.051	ns	ns
rno-miR-20b	0.015 ± 0.004	0.033 ± 0.010	0.015 ± 0.003	0.033 ± 0.010	0.018	ns	ns
hsa-miR-106a	0.014 ± 0.004	0.033 ± 0.011	0.013 ± 0.001	0.106 ± 0.078	ns	ns	ns
mmu-miR-106a	0.043 ± 0.007	0.073 ± 0.022	0.032 ± 0.006	0.059 ± 0.009	0.033	ns	ns

MiRNAs expressions were analysed quantitatively by qPCR. MiRNA expression in the liver, skeletal muscle and omental fat were normalised against average ΔCt of miR-26b and miR-92. [1]Placental restriction reduced hepatic mmu-miR-199b* expression in male offspring (P = 0.009), but tended to increase in female offspring (P = 0.067). [2]Placental restriction increased hepatic rno-miR-126 expression in female offspring (P = 0.015), but similar in control and placentally restricted male offspring (P = 0.354).

Table 3.10. Predicted targets involved in insulin action and metabolic control of miRNAs differentially expressed following bilateral uterine artery vessel ligation

Tissue	Sex	miRNA	Expression in BUVL	Predicted Targets	
				Insulin Signalling	Others
Liver	Female	miR-126	up	IRS1 (h)	
Skeletal Muscle	Combined	miR-451	up	HK2 (r, h) INSR (h) IRS2 (r)	LEP (h) PPARA (h) PTPRE (r) RXRA (h)
Omental Fat	Combined	miR-18a	up	INSR (h) PIK3R1 (h) SLC2A4 (h) IGF1 (r, h)	ACOX1 (h) ADIPOR2 (h) FOXO1 (r, h) PPARA (h) VAMP2 (r)
		miR-142-3p	up		FOXO1 (h) M6PR (r) PRKCI (r)
		miR-19b	up	IRS1 (r) IRS2 (r)	ADIPOR2 (r) FOXO1 (r) IGF1 (r, h) PPARA (r, h) VAMP2 (r)
		mmu-miR-106a	up	SLC2A4 (r)	FOXO1 (r)
		hsa-miR-106a	up	PIK3R1 (h) SLC2A4 (h)	M6PR (h) PPARA (h)

r: predicted in rat; h: predicted in human.

Table 3.11. Networks and predicted direct and indirect molecular targets of differentially expressed miRNAs following bilateral uterine artery vessel ligation

Sex	miRNA	Associated Molecules		Score	Focus Molecules	Top Functions
		Direct	Indirect			
	Liver					
Female	miR-126 (Network 1)	CXCL12, DUSP10, INSIG1, LY6E, MID1IP1, NBR1, PRKCI,PROX1,RFC1,SCD	ACACA,ALDH2,ASL,ASS1,ATF7,ATP2A2,CEBPA,CNR1, DBI,DYNLL1,F9,FASN,G6PD,GATA6,GSTT1,GSTT2,HNF4A, MBL2,MGLL,MGST1,NFE2L2,NUDT7,OTC,PPARA,THRB	15	11	Lipid metabolism, Molecular transport, Small molecule biochemistry
	miR-126 (Network 2)	CD68, LTB, STAT1, VCAM1	APOA1,APOE,CCL13,CD68,CES1,Cyp2c29,CYP3A4,IFNG, IL6,IL10,IL1A,IL1B,JUN,PPARD,PPARG,SOCS1,SOCS3, TGFB1,TNF, TNFRSF1A	4	4	Hematological system development and function, Tissue morphology, Inflammatory response
	Skeletal muscle					
Combined	miR-451	EHHADH, PPP1R3C, PTPRE, SIX1	ALDOA, IRS1, NR4A1, UCP3	4	4	Carbohydrate metabolism, Molecular transport, Small molecule biochemistry
	Omental fat					
Combined	miR-16	ADRB2, MAP2K1, MAPK8, MLYCD, PDE3B	ACACA,ACADL,ACADM,ADRB3,CASP3,CD36,CEBPA,CFD, CPT1B,CYP19A1,EIF4EBP1,FABP4,FASN,FGF2,FOXC2, GHRL,IGF1,IRS2,JUN,LPL,MFN2,NCOA2,PPARA,PPARD, PPARG,PPARGC1A,RB1,SCD,UCP1,UCP3	5	6	Lipid metabolism, Molecular transport, Small molecule biochemistry
	miR-18	*--none--*				
	miR-142-3p	*--none--*				
	miR-19b	*--none--*				
	miR-21	*--none--*				
	miR-20b	*--none--*				
	hsa-miR-106a	F3, LDLR, PPARA, RB1, STAT3, TGFBR2	ACACA,ADRB3,AGT,CCL13,CD36,CEBPB,CEBPD,CPT1B, CYP19A1,DGAT1,DLK1,FASN,GPAM,HSD11B1,IGF1,IL6, JUN,LEP,LEPR,MAPK14,NRIP1,SMPD1,SMPD2,SOCS3, TGFB1,TNF,UCP1,UCP2,UCP3	5	6	Lipid metabolism, Molecular transport, Small molecule biochemistry
	mmu-miR-106a	F3, PDE3B, SLC2A4, STAT3	ACACA,ACLY,ACOX1,ADRB3,AGT,AKT1,AKT2,ANGPTL4, CCL13,CD36,CPT1B,DGAT1,ENPP2,FOXO1,GFPT1,GPAM, IDH1,IGF1,IRS2,LEP,LPL,NR1H3,PCK1,PPARA,PPARG, RELA,RETN,SOCS3,STAT1,TGFB1,UCP3	3	4	Lipid metabolism, Molecular transport, Small molecule biochemistry

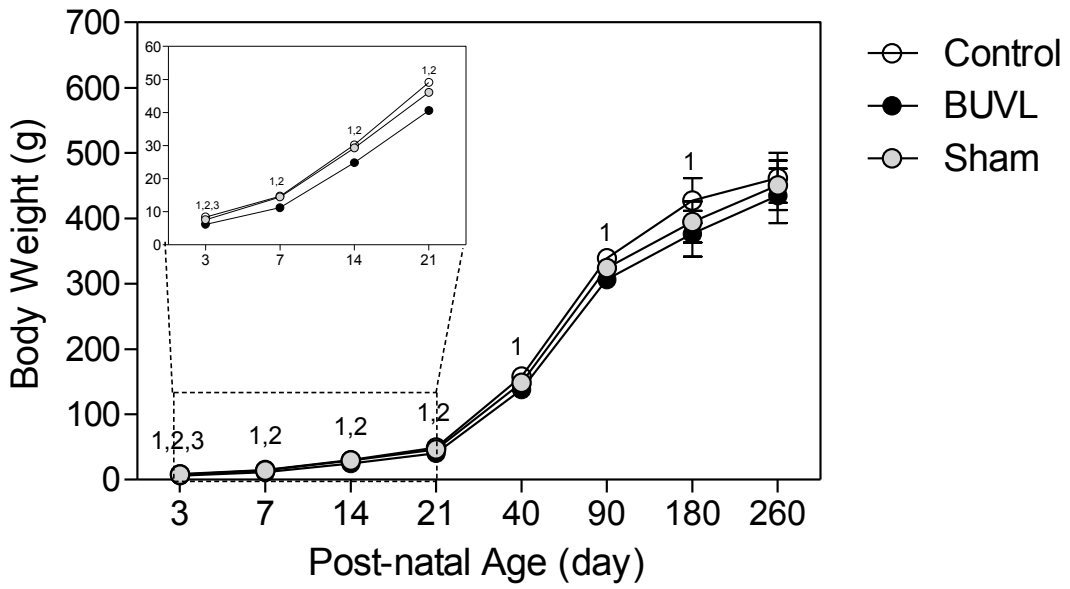


Figure 3.1. Effect of surgical treatments on postnatal growth of offspring

BUVL reduced body weight of offspring from PD 3 to 180, compared to control offspring. BUVL reduced body weight of offspring from PD 3 to PD 21, compared to sham offspring. Sham surgery reduced body weight of offspring only at PD 3, compared to control offspring. *P*-value was calculated by repeated measures ANOVA for PD 3 to PD 90, with age as factors ($P < 0.001$). Specific comparison analyses between treatment groups were performed for individual time points with Bonferonni adjustment. Control: white circle; PR: black circle; Sham: grey circle. 1: $P < 0.05$ for control compared with BUVL; 2: $P < 0.05$ for sham compared with BUVL; 3: $P < 0.05$ for sham compared with control.

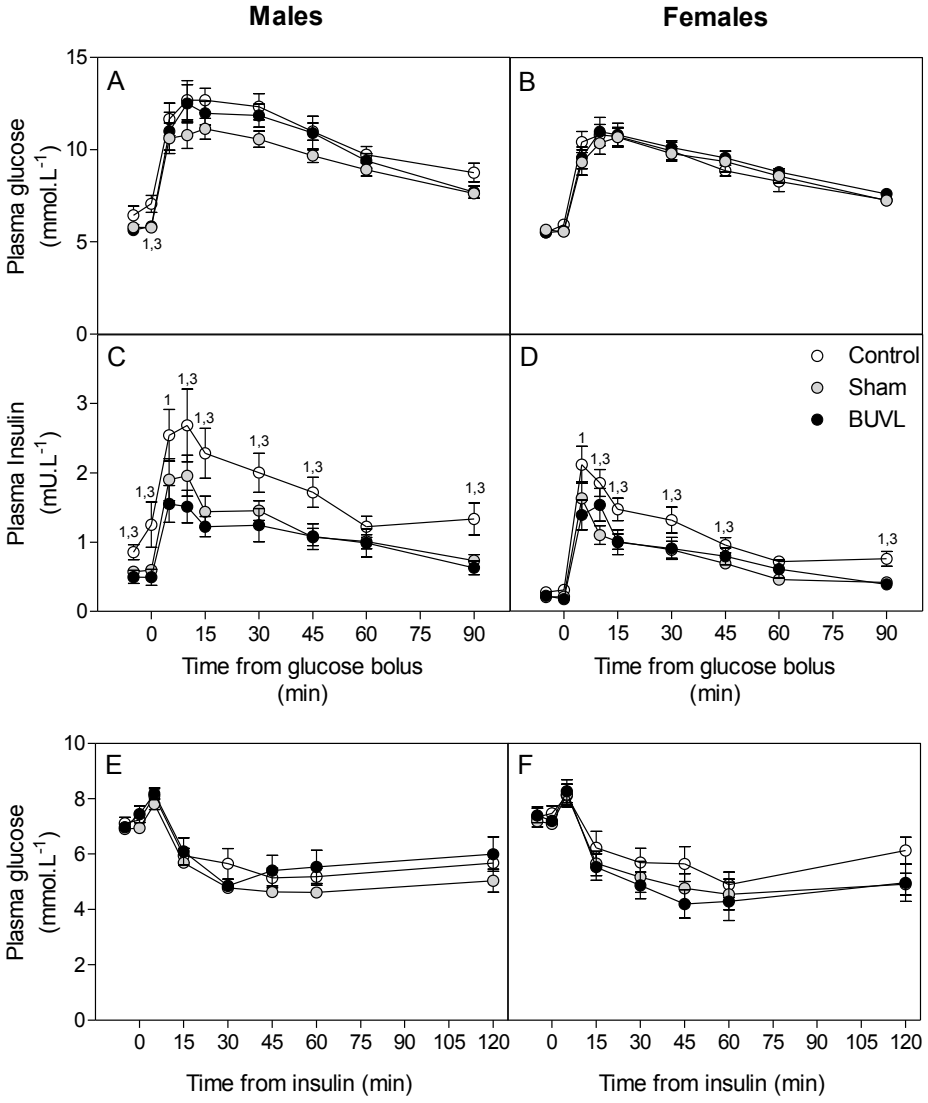


Figure 3.2. The effect of surgical treatments on plasma glucose and insulin secretion during intra-peritoneal glucose and insulin tolerance test in combined age offspring

Plasma glucose (A & B) and insulin levels (C & D) measured before and during IPGTT from baseline (-5 and 0 min) to 90 min, with the glucose bolus administered at 0 min. Plasma glucose (E & F) measured during IPITT from baseline (-5 and 0 min) to 120 min, with the insulin administered at 0 min. Control: white circle; BUVL: black circle; Sham: grey circle. Data are presented as mean ± SEM, determined by addition of time as a repeated measure factor (9 levels) by ANOVA (Effect of treatments: plasma glucose (IPGTT), P = 0.126; plasma insulin (IPGTT), P = 0.001; plasma glucose (IPITT), P = 0.315). Glucose dose for IPGTT: 1 mg.g^{-1} bodyweight; Insulin dose for IPITT: 0.75 mU.g-1 bodyweight. 1: P < 0.05 for control compared with BUVL; 2: P < 0.05 for sham compared with BUVL; 3: P < 0.05 for sham compared with control.

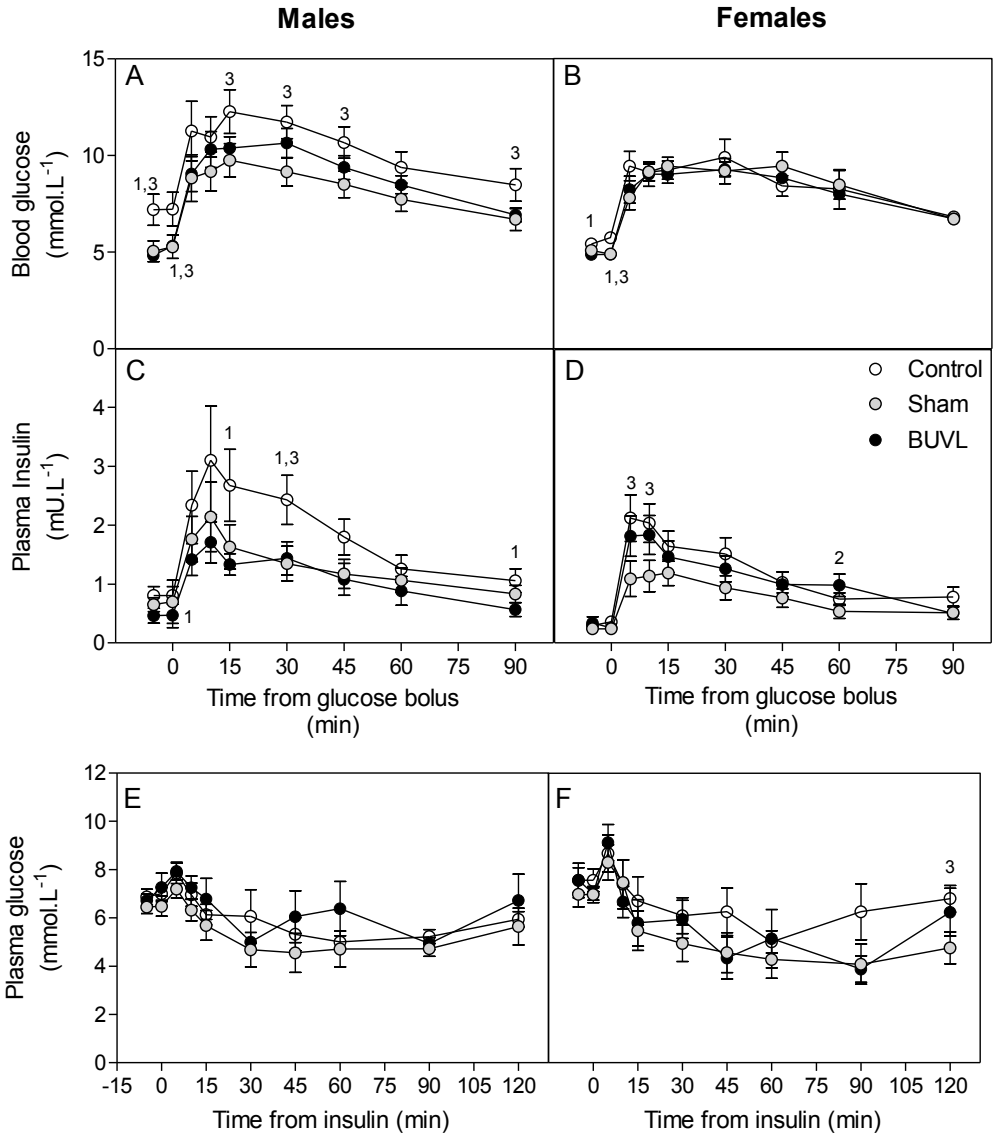


Figure 3.3. The effect of surgical treatments on plasma glucose and insulin secretion during intra-peritoneal glucose and insulin tolerance test in young adult offspring

Plasma glucose (A & B) and insulin levels (C & D) measured before and during IPGTT from baseline (-5 and 0 min) to 90 min, with the glucose bolus administered at 0 min. Plasma glucose (E & F) measured during IPITT from baseline (-5 and 0 min) to 120 min, with the insulin administered at 0 min. Control: white circle; BUVL: black circle; Sham: grey circle. Data are presented as mean ± SEM, determined by addition of time as a repeated measure factor (9 levels) by ANOVA (Effect of treatments: plasma glucose (IPGTT), P = 0.045; plasma insulin (IPGTT), P = 0.029; plasma glucose (IPITT), P = 0.110). Glucose dose for IPGTT: 1 mg.g^{-1} bodyweight; Insulin dose for IPITT: 0.75 mU.g-1 bodyweight. 1: P < 0.05 for control compared with BUVL; 2: P < 0.05 for sham compared with BUVL; 3: P < 0.05 for sham compared with control.

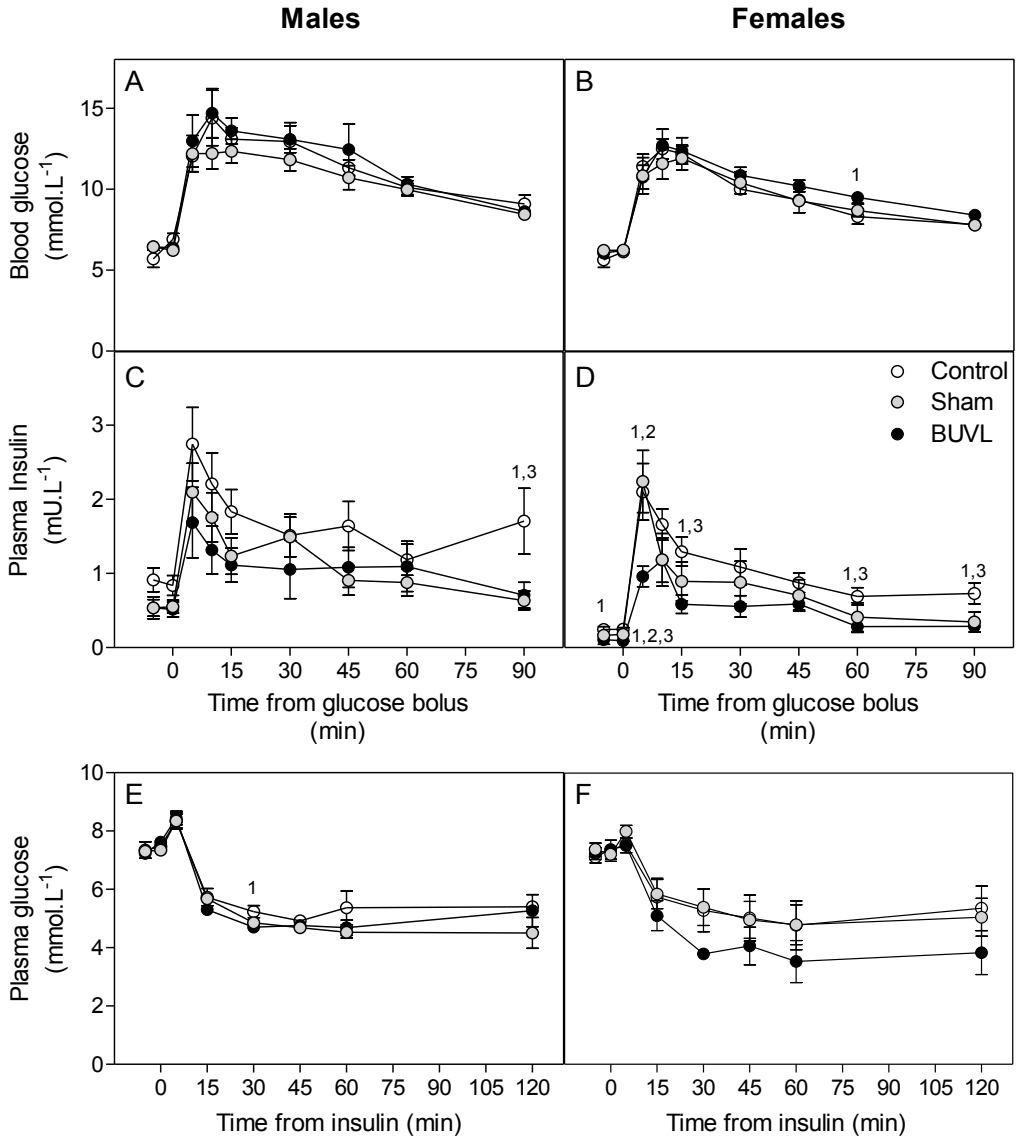


Figure 3.4. The effect of surgical treatments on plasma glucose and insulin secretion during intra-peritoneal glucose and insulin tolerance test in older adult offspring

Plasma glucose (A & B) and insulin levels (C & D) measured during IPGTT from baseline (-5 and 0 min) to 90 min, with the glucose bolus administered at 0 min. Plasma glucose (E & F) measured during IPITT from baseline (-5 and 0 min) to 120 min, with the insulin administered at 0 min. Control: white circle; PR: black circle; Sham: grey circle. Data are presented as mean ± SEM, determined by addition of time as a repeated measure factor (9 levels) by ANOVA (Effect of treatments: plasma glucose (IPGTT), P = 0.434; plasma insulin (IPGTT), P = 0.018; plasma glucose (IPITT), P = 0.156). Glucose dose for IPGTT: 1 mg.g^{-1} bodyweight; Insulin dose for IPITT: 0.75 mU.g-1 bodyweight. 1: P < 0.05 for control compared with PR; 2: P < 0.05 for sham compared with PR; 3: P < 0.05 for sham compared with control.

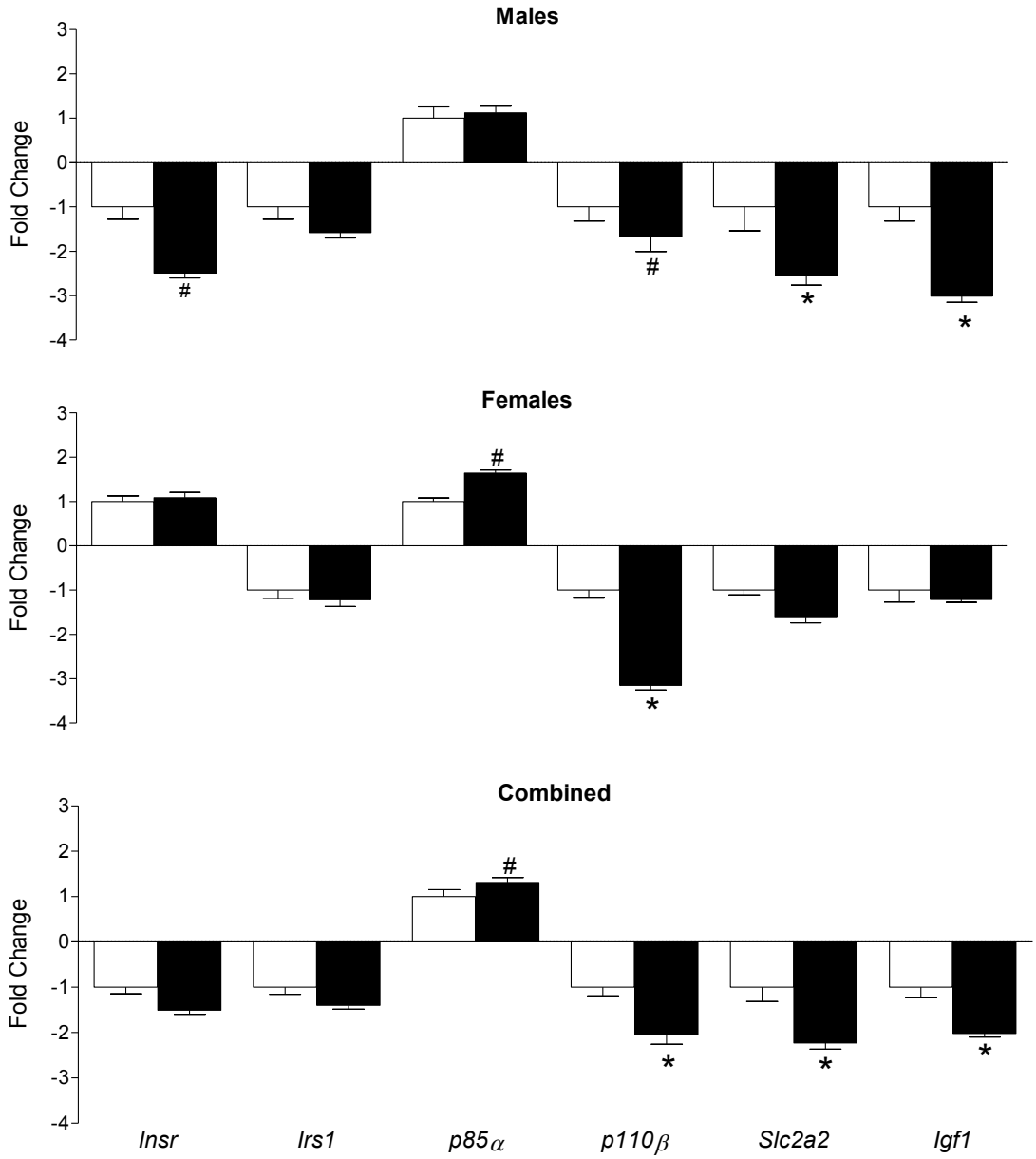


Figure 3.5. Effect of bilateral uterine artery vessel ligation on hepatic expression of insulin signalling and other predicted targets in older adult offspring

Gene expression was normalised to *β-actin* gene expression, which was unaltered by BUVL. Data shown as fold change ± SEM. Control: white bar; PR: black bar; * $P < 0.05$ compared to control; # $P < 0.10$.

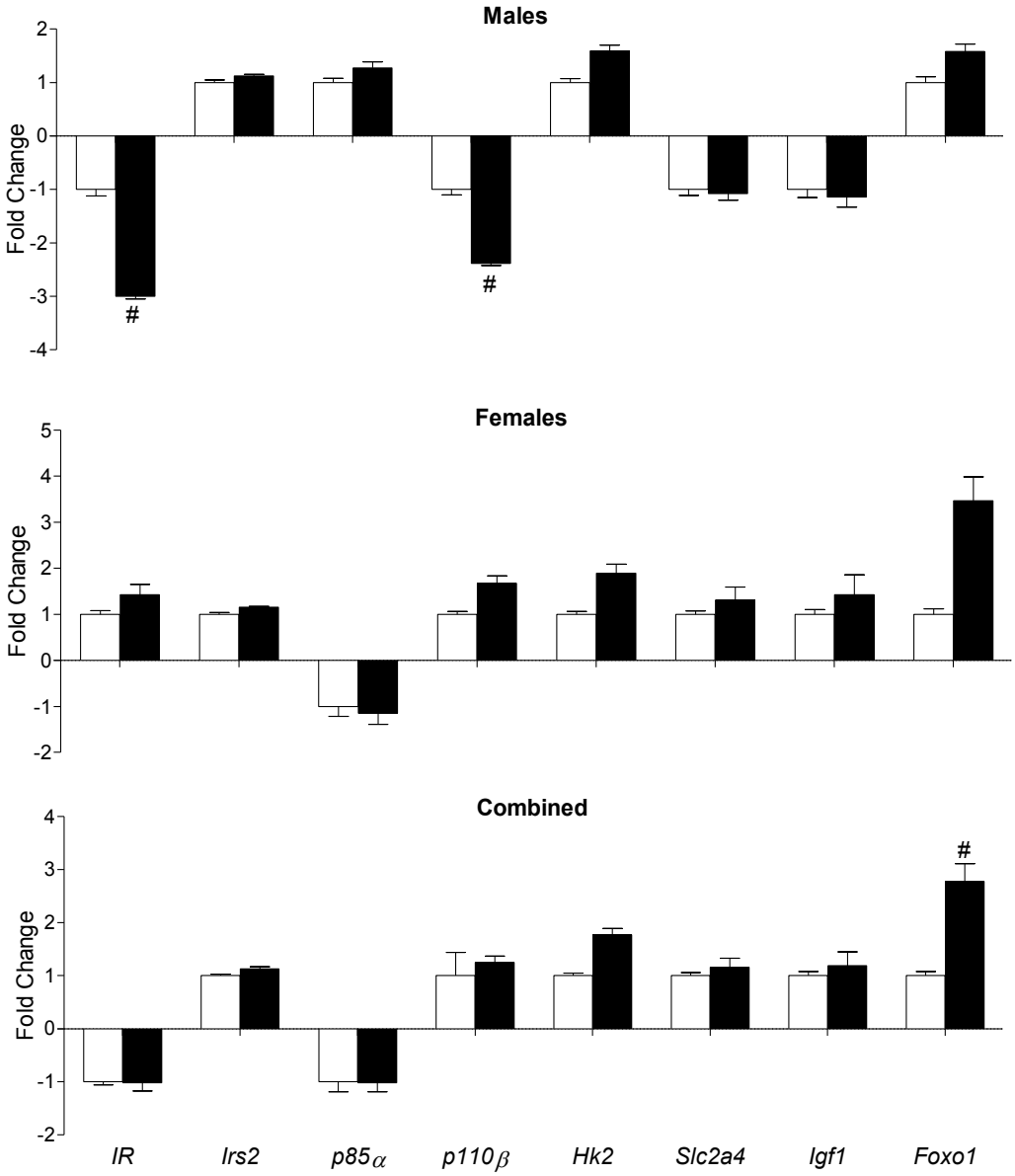


Figure 3.6. Effect of bilateral uterine artery vessel ligation on skeletal muscle expression of insulin signalling and other predicted targets in older adult offspring

Gene expression was normalised to *β-actin* gene expression, which was unaltered by BUVL. Data shown as fold change ± SEM. Control: white bar; PR: black bar; $^{\#}P <$ 0.10: compared to control.

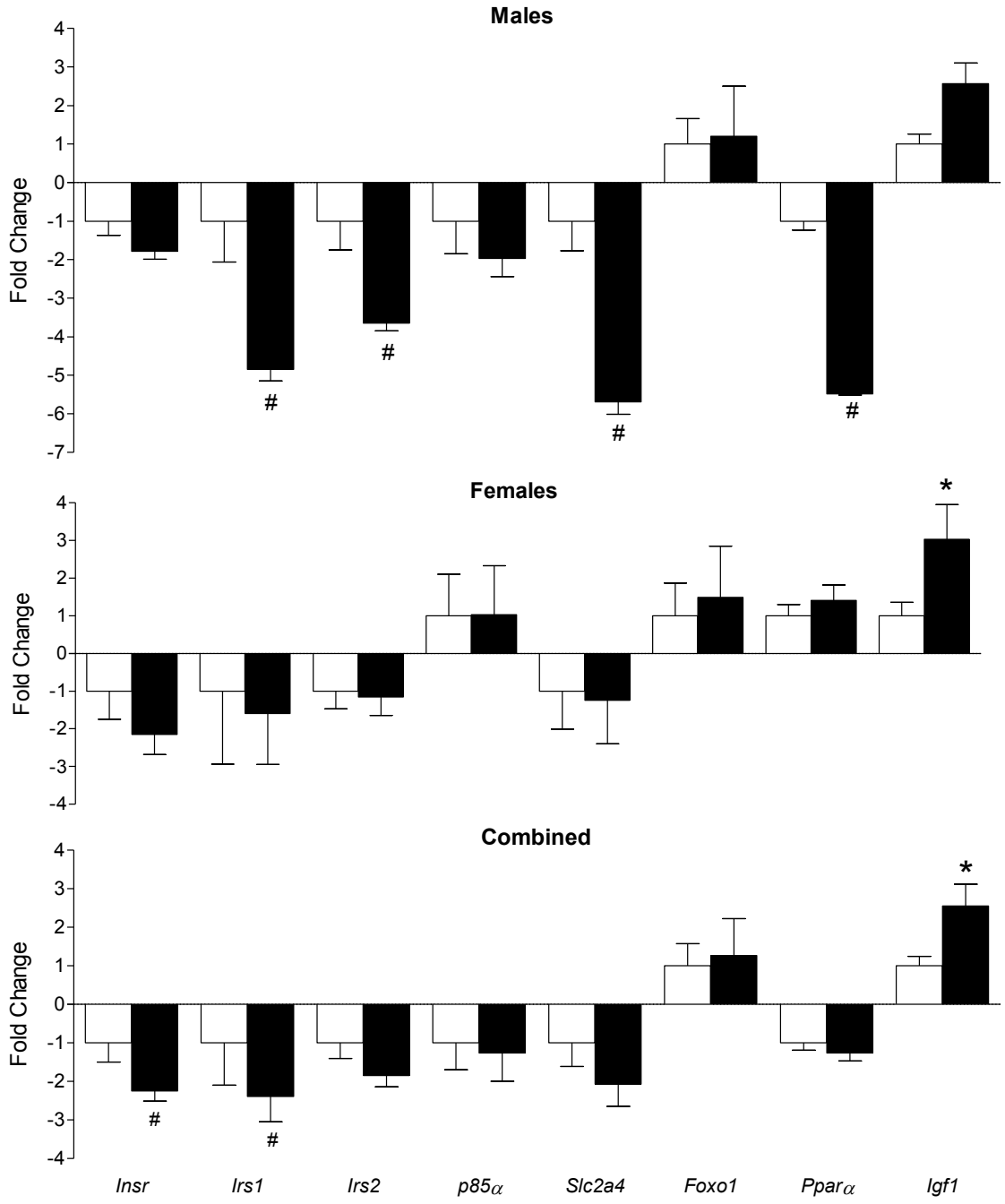


Figure 3.7. Effect of bilateral uterine artery vessel ligation on omental fat expression of insulin signalling and other predicted targets in older adult offspring

Gene expression was normalised to *β-actin* gene expression, which was unaltered by BUVL. Data shown as fold change ± SEM. Control: white bar; PR: black bar; * $P < 0.05$ compared to control; # $P < 0.10$.

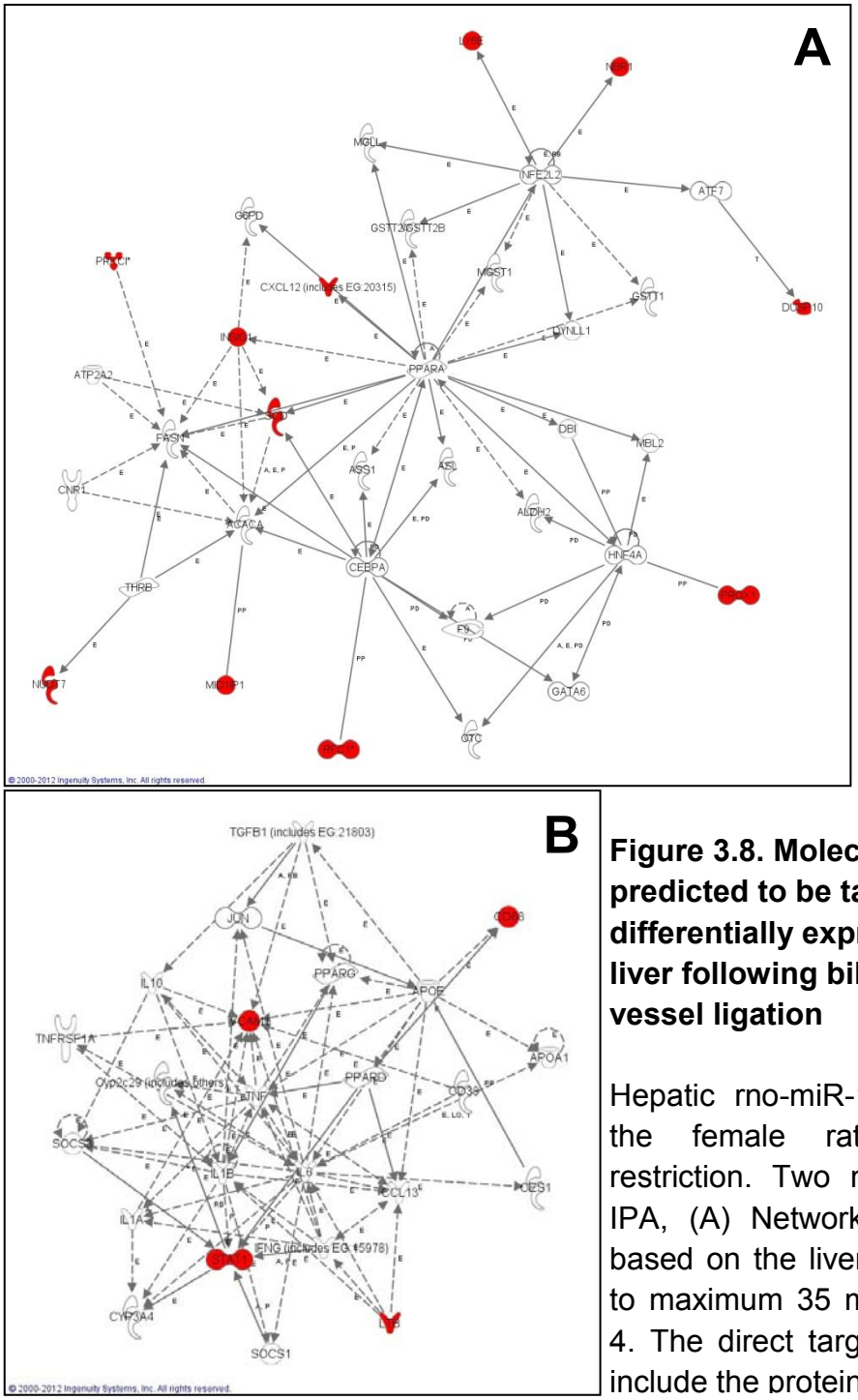


Figure 3.8. Molecular networks predicted to be targeted by the differentially expressed rno-miR-126 in liver following bilateral uterine artery vessel ligation

Hepatic rno-miR-126 is up-regulated in the female rat following placental restriction. Two networks generated by IPA, (A) Network 1 & (B) Network 2, based on the liver database only, limited to maximum 35 molecules and scores > 4. The direct targets (highlighted in red) include the protein kinase C I (PRKCI).

Straight lines: direct interaction; Dotted lines: indirect interaction; PD: protein-DNA binding; PP: protein-protein binding; E: expression; LO: localisation; RB: regulation of binding; L: proteolysis; A: activation; P: phosphorylation; T: transcription. (Complete list of molecules in the networks can be found on Table 3.11).

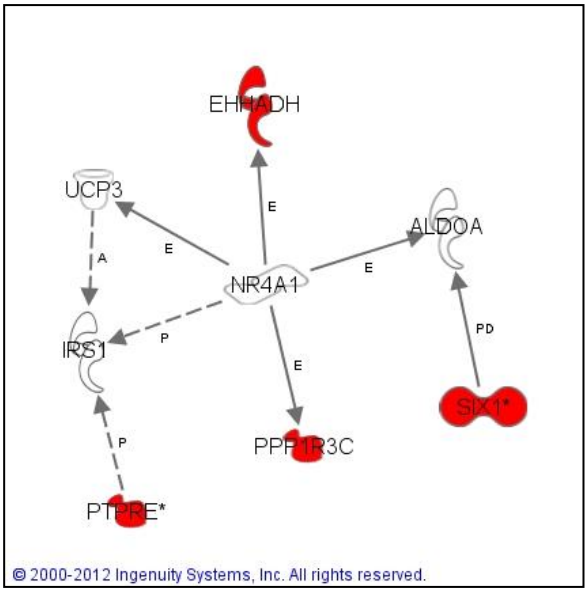
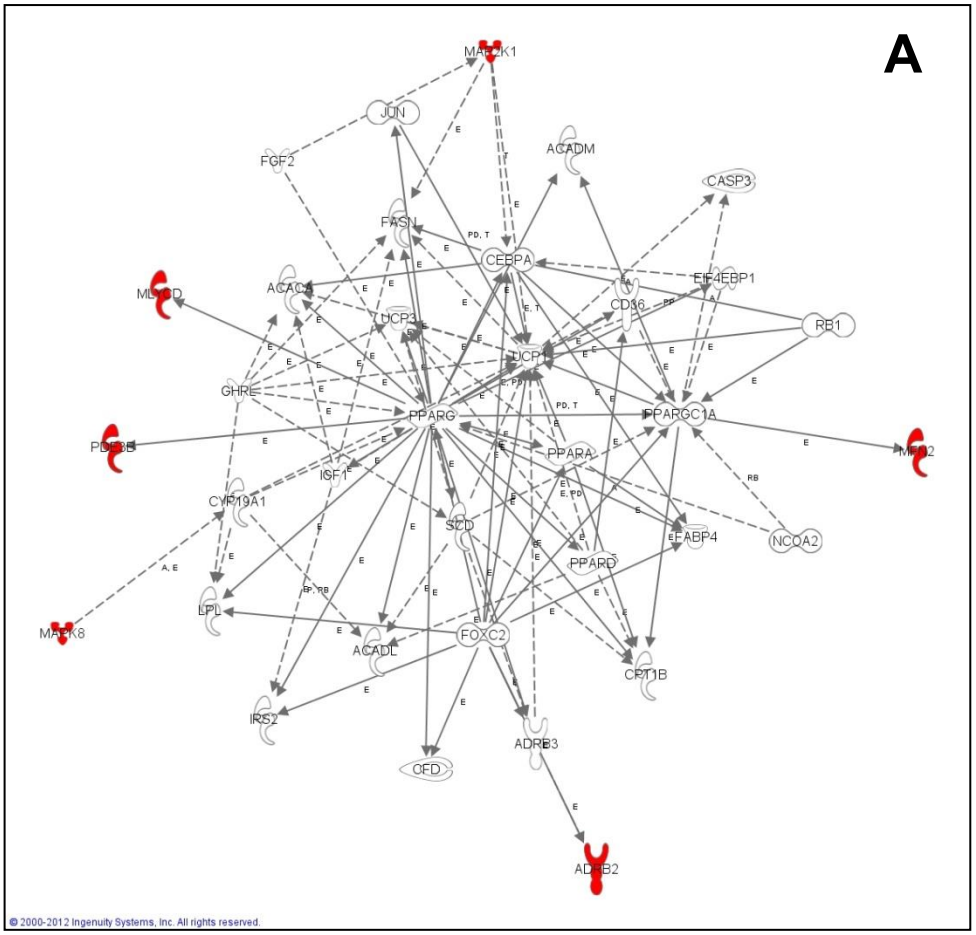


Figure 3.9. Molecular networks predicted to be targeted by the differentially expressed rno-miR-451 in skeletal muscle following bilateral uterine artery vessel ligation

Skeletal muscle rno-miR-1451 is up-regulated in the adult rat following placental restriction. The direct targets (highlighted in red) include the protein tyrosine phosphatise, receptor type E (PTPRE), which indirectly regulate IRS1 expression through phosphorylation. IPA generated a network, based on the skeletal muscle database only, limited to maximum 35 molecules and scores > 4. Straight lines: direct interaction; Dotted lines: indirect interaction; PD: protein-DNA binding; PP: protein-protein binding; E: expression; LO: localisation; RB: regulation of binding; L: proteolysis; A: activation; P: phosphorylation; T: transcription. (Complete list of molecules in the networks can be found on Table 3.11).


A
JUN
FGF2
ACADM
CASP3
FASN
CEBPA
EIF4EBP1
ACACA
CD36
RB1
UCP3
UCP1
GHRL
PPARG
PPARGC1A
PPARA
IGF1
CYP19A1
SCD
FABP4
NCOA2
PPARD
LPL
FOXC2
ACADL
CPT1B
IRS2
ADRB3
OFD
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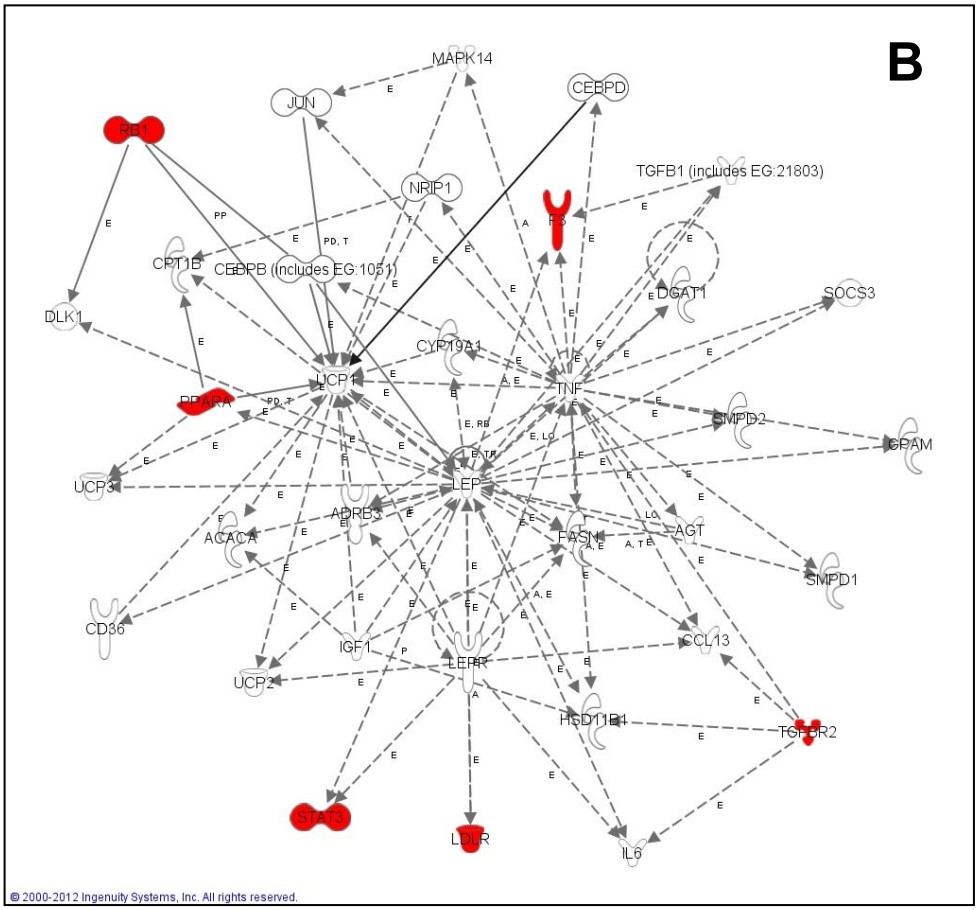
B
MAPK14
CEBPD
JUN
RB1
NRIP1
TGFB1 (includes EG:21803)
F3
CPT1B
CEBPB (includes EG:1051)
DGAT1
SOCS3
DLK1
CYP19A1
UCP1
TNF
PPARA
SMPD2
GPAM
UCP3
LEP
ADRB3
AGT
ACACA
FASN
SMPD1
CD36
CCL13
IGF1
LEPR
UCP2
HSD11B1
TGFBR2
STAT3
LDLR
IL6
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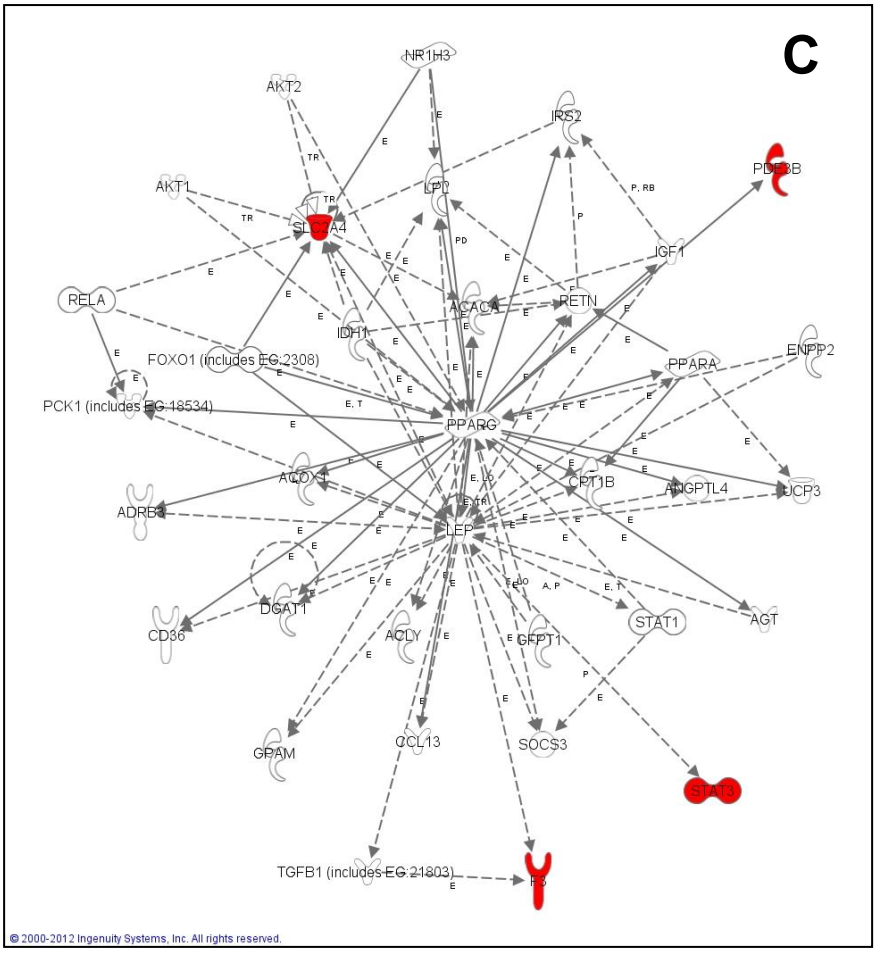


Figure 3.10. Molecular networks predicted to be targeted by the differentially expressed miRNAs in omental fat following bilateral uterine artery vessel ligation

Omental fat rno-miR-16, hsa-miR-106a and mmu-miR-106a are up-regulated in the adult rat following placental restriction. IPA generated one network for each of (A) rno-miR-16, (B) hsa-miR-106a and (C) mmu-miR-106a, based on the adipose tissue database only, limited to maximum 35 molecules and scores > 4. The direct targets (highlighted in red) include the insulin-responsive glucose transporter 4 (SLC2A4), for mmu-miR-106a, which facilitates glucose intake into adipocytes in response to insulin. Straight lines: direct interaction; Dotted lines: indirect interaction; PD: protein-DNA binding; PP: protein-protein binding; E: expression; LO: localisation; RB: regulation of binding; L: proteolysis; A: activation; P: phosphorylation; T: transcription. (Complete list of molecules in the networks can be found on Table 3.11).

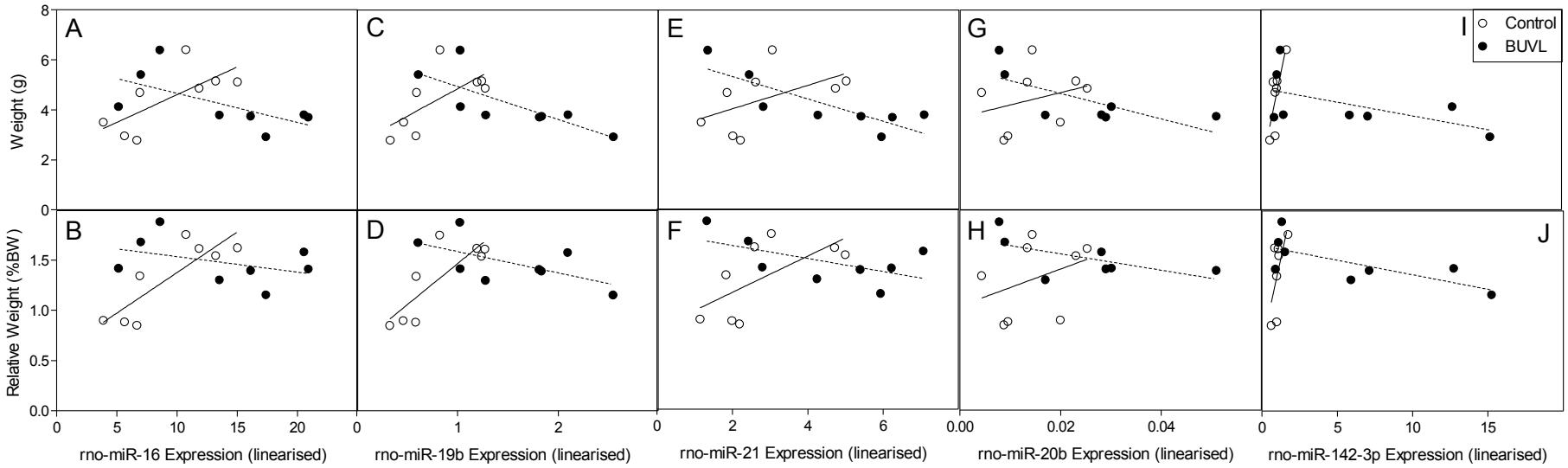


Figure 3.11. Effect of bilateral uterine artery vessel ligation on associations between differentially expressed miRNAs and tissue weight in omental fat in older adult female offspring

MiRNAs expressions were first linearised before correlations were performed. Correlations between omental weight and relative to body weight with rno-miR-16 (A & B), rno-miR-19b (C & D), rno-miR-21 (E & F), rno-miR-20b (G & H) and rno-miR-142-3p (I & J). Correlations were calculated with Pearson's correlation; with $P < 0.05$ considered as significant (refer to section 3.3.5.3 for the *P*-values). Control: white circle; PR: black circle; continuous line: control linear correlation; dotted line: BUVL linear correlation.

3.7 Supplement Tables and Figures

Supplement Table S3.1. Primers sequences used in analysis of genes expression

Gene	Amplicon (bp)	Forward Primer 5' – 3'	Reverse Primer 5' – 3'	GenBank accession no.
β-actin	63	GCTGATCCACATCTGCTGGAA	TCTGTGTGGATTGGTGGCTCTA	NM_031144
Irs1	124	AAACGCCCTTAAACTCGGAT	TGGAGGAAGCAAGCAGAAAT	NM_012969
Irs2	160	GGAAGTCTGTTCGGGTGTGT	CACATCTGCCTCAGTGTGCT	NM_001168633.1
IR	179	ATTCTTTCTACGCCTTGGAC	GTCTTCAGGGCAATGTCGTT	NM_017071.2
p85α	142	CCCTCAGTGGACTTGGATGT	CGGAGCTTGGTACTTCTTGG	NM_013005
p110β	142	CAGTGGAGACAGTGCGAACGTA	TCGGCAGTCTTGCCGTAGAG	NM_053481
Slc2a4	104	CATTCTCGGACGGTTCCTCAT	CCAAGGCACCCCGAAGAT	D28561
Slc2a2	161	CAACAACTCCGCACGCAAC	TCCAGAGGAACACCCAAAACA	NM_012879.2
Hk2	91	GGAGCTACCACGCACCCTAC	CCAGGAACTCCCCATGTTCT	NM_012735.1
Igf1	208	TCCGCTGAAGCCTACAAAGT	GATGTTTTGCAGGTTGCTCA	NM_178866
Pparα	188	GTGTCGAATATGTGGGGACAA	CGGACAGGCACTTGTGGAA	NM_013196
Ccdc88a	80	CAGTCACCCTGTGGTTCTCC	GCCTTAGCATCAGCCAGTTC	XM_223709.5
Mon2	141	CCATCTCTCTTTGCTGTTGC	TGCTCCCCATTCTCTCATTC	XM_001054664.2
Foxo1	95	TCCCTTACTACCGCGAGAACA	GTAGTGGCGCGGTACCTTCA	M87634
Cyclin D1	198	GGAGCAGAAGTGCGAAGAGG	TCGGGGCGGATAGAGTTGT	NM_171992.4

Supplement Table S3.2. Specific comparisons between surgical treatments on body composition

Specific comparisons	(control v BUVL)							(sham v BUVL)							(control v sham)						
	T	A	S	A x T	A x S	T x S	A x T x S	T	A	S	A x T	A x S	T x S	A x T x S	T	A	S	A x T	A x S	T x S	A x T x S
Combined age																					
Nose-rump lenght (cm)	0.001	< 0.001	< 0.001	ns	0.004	ns	0.094	0.037	< 0.001	< 0.001	ns	0.001	ns	ns	ns	< 0.001	< 0.001	ns	0.007	ns	ns
Abdominal circumferance (cm)	0.090	< 0.001	< 0.001	ns	0.001	ns	ns	0.095	< 0.001	< 0.001	ns	0.034	ns	ns	ns	< 0.001	< 0.001	ns	0.007	0.053	ns
Body Mass Index (kg.m²)	ns	ns	< 0.001	ns	< 0.001	ns	ns	ns	0.005	< 0.001	ns	< 0.001	ns	ns	ns	ns	< 0.001	ns	0.001	ns	ns
Ponderal Index (kg.m³)	ns	0.015	0.025	ns	ns	ns	ns	ns	0.097	< 0.001	ns	ns	ns	ns	ns	ns	0.021	ns	ns	ns	ns
Body weight (g)	0.001	< 0.001	< 0.001	< 0.001	< 0.001	ns	ns	ns	< 0.001	< 0.001	ns	< 0.001	ns	ns	ns	< 0.001	< 0.001	ns	0.006	ns	ns

	(control v BUVL)			(control v sham)			(sham v BUVL)		
	T	S	T x S	T	S	T x S	T	S	T x S
PD 90									
Nose-rump lenght (cm)	0.064	< 0.001	ns	ns	< 0.001	ns	ns	< 0.001	ns
Abdominal circumferance (cm)	ns	< 0.001	ns	ns	< 0.001	ns	ns	< 0.001	ns
Body Mass Index (kg.m²)	0.017	< 0.001	ns	ns	< 0.001	ns	ns	< 0.001	ns
Ponderal Index (kg.m³)	ns	0.020	ns	ns	ns	ns	ns	0.020	ns
PD 260									
Nose-rump lenght (cm)	0.005	< 0.001	0.068[1]	0.032	< 0.001	ns	ns	< 0.001	ns
Abdominal circumferance (cm)	ns	< 0.001	ns	ns	< 0.001	0.016[2]	ns	< 0.001	ns
Body Mass Index (kg.m²)	ns	< 0.001	ns	ns	< 0.001	ns	ns	< 0.001	ns
Ponderal Index (kg.m³)	ns	ns	ns	ns	0.094	ns	ns	0.002	ns
Body weight									
PD 3	< 0.001	0.001	ns	0.037	0.049	ns	< 0.001	0.037	ns
PD 7	< 0.001	0.002	ns	ns	0.016	ns	< 0.001	0.067	ns
PD 14	< 0.001	0.002	ns	ns	0.007	ns	< 0.001	0.025	ns
PD 21	< 0.001	0.001	ns	ns	0.012	ns	0.009	0.004	0.090[3]
PD 40	0.001	< 0.001	ns	ns	< 0.001	ns	ns	< 0.001	ns
PD 90	0.002	< 0.001	ns	ns	< 0.001	ns	ns	< 0.001	ns
PD 180	0.001	< 0.001	ns	0.057	< 0.001	ns	ns	< 0.001	ns
PD 260	ns	< 0.001	ns	ns	< 0.001	ns	ns	< 0.001	ns

[1]Nose-rump length at PD 260 was similar in BUVL and control males (P = 0.388), but shorter in BUVL females than in controls (P = 0.005). [2]Abdominal circumference at PD 260 was smaller in sham males than in control (P = 0.020), but similar in sham and control females (P = 0.269). [3]Body weight at PD 21 was similar in BUVL males than in sham (P = 0.289), but lighter in BUVL females than in sham (P = 0.007). A: age (day 90 or 260); T: surgical treatment (control, BUVL or sham); S: sex (male or female); ns: not significant. Specific comparison analyses for body weight were adjusted with Bonferonni.

Supplement Table S3.3. Specific comparisons for the effect of surgery and bilateral uterine artery vessel ligation on post-mortem organ and tissue weights in combined age rat offspring

Combined Age	*P*-value (ANOVA) (control v BUVL)							*P*-value (ANOVA) (sham v BUVL)							*P*-value (ANOVA) (control v sham)						
	A	T	S	A x T	A x S	T x S	A x T x S	A	T	S	A x T	A x S	T x S	A x T x S	A	T	S	A x T	A x S	T x S	A x T x S
Heart (g)	< 0.001	0.001	< 0.001	ns	0.005	ns	0.061	< 0.001	0.053	< 0.001	ns	0.096	ns	ns	< 0.001	0.072	< 0.001	ns	0.004	ns	ns
(%BW)	0.002	ns	< 0.001	ns	ns	ns	0.011	< 0.001	ns	< 0.001	ns	0.005	ns	ns	0.014	ns	0.003	ns	ns	ns	0.032
Spleen (g)	ns	0.002	< 0.001	ns	0.015	ns	ns	ns	ns	< 0.001	ns	0.006	ns	ns	0.050	0.002	< 0.001	ns	0.001	0.038[6]	ns
(%BW)	< 0.001	ns	ns	ns	ns	ns	ns	< 0.001	ns	< 0.001	ns	0.097	ns	ns	< 0.001	ns	0.039	ns	0.057	0.065[7]	ns
Adrenals (g)	0.059	ns	ns	ns	ns	ns	ns	0.075	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
(%BW)	ns	ns	< 0.001	ns	0.087	0.045[1]	ns	ns	ns	< 0.001	ns	ns	ns	ns	0.062	ns	< 0.001	ns	0.040	ns	ns
Kidneys (g)	< 0.001	< 0.001	< 0.001	ns	ns	ns	ns	< 0.001	0.050	< 0.001	ns	0.064	ns	ns	< 0.001	ns	< 0.001	ns	0.078	ns	ns
(%BW)	< 0.001	ns	0.002	ns	ns	ns	ns	< 0.001	ns	0.062	ns	0.003	ns	ns	0.003	ns	0.027	ns	ns	ns	ns
Liver (g)	0.001	0.017	< 0.001	ns	0.008	ns	ns	0.001	ns	< 0.001	ns	0.003	ns	ns	0.001	0.073	< 0.001	ns	0.013	ns	ns
(%BW)	0.001	ns	< 0.001	ns	ns	ns	ns	< 0.001	ns	< 0.001	ns	ns	ns	ns	0.003	ns	0.001	ns	ns	ns	ns
Lungs (g)	0.040	0.029	< 0.001	ns	ns	ns	ns	< 0.001	ns	< 0.001	ns	ns	ns	ns	0.001	0.007	< 0.001	0.066[3]	0.019	ns	ns
(%BW)	0.001	ns	0.001	ns	ns	ns	0.022	0.004	0.040	< 0.001	ns	0.060	ns	ns	0.011	ns	0.026	ns	ns	ns	0.073
Brain (g)	0.010	0.096	< 0.001	ns	ns	ns	ns	0.096	ns	0.005	ns	ns	ns	ns	0.007	ns	< 0.001	ns	0.052	ns	ns
(%BW)	< 0.001	0.092	< 0.001	ns	ns	0.004[2]	ns	< 0.001	ns	< 0.001	ns	ns	ns	ns	< 0.001	ns	< 0.001	ns	ns	ns	ns
Uterus (g)	< 0.001	ns		ns				< 0.001	0.073		ns				< 0.001	ns		ns			
(%BW)	0.005	ns		ns				< 0.001	ns		ns				0.035	ns		ns			
Testes (g)	0.003	0.002		ns				ns	ns		ns				ns	0.008		ns			
(%BW)	0.001	ns		ns				< 0.001	ns		ns				0.001	ns		ns			
Fat depots																					
Retroperitoneal fat (g)	< 0.001	ns	< 0.001	ns	< 0.001	ns	ns	< 0.001	ns	< 0.001	ns	0.003	ns	ns	< 0.001	0.056	< 0.001	ns	< 0.001	ns	ns
(%BW)	< 0.001	ns	0.001	ns	0.047	ns	0.099	< 0.001	ns	0.001	ns	ns	ns	ns	< 0.001	0.061	< 0.001	0.045[4]	0.048	ns	0.097
Omental fat (g)	< 0.001	ns	< 0.001	ns	< 0.001	ns	ns	< 0.001	ns	< 0.001	ns	0.001	ns	ns	< 0.001	ns	< 0.001	ns	< 0.001	ns	ns
(%BW)	< 0.001	ns	ns	ns	0.001	ns	0.084	< 0.001	ns	ns	ns	0.065	ns	ns	< 0.001	ns	ns	ns	0.002	ns	ns
Visceral fat (g)	< 0.001	ns	< 0.001	ns	< 0.001	ns	ns	< 0.001	ns	< 0.001	ns	0.001	ns	ns	< 0.001	ns	< 0.001	ns	< 0.001	ns	ns
(%BW)	< 0.001	ns	0.010	ns	0.006	ns	0.056	< 0.001	ns	0.014	ns	ns	ns	ns	< 0.001	0.042	0.001	0.037[5]	0.006	ns	0.081

BUVL: bilateral uterine artery vessel ligation; A: age (day 90 or 260); T: surgical treatment (control, BUVL or sham); S: sex (male or female); ns: not significant. [1]BUVL did not alter adrenals weight relative to body weight across male offspring (P = 0.616), but increased in female BUVL offspring than in female control offspring (P = 0.018). [2]BUVL did not alter brain weight relative to body weight in male offspring (P = 0.616), but increased in female BUVL offspring than in female control offspring (P = 0.002). [3]At PD 90, sham surgery reduced lungs weight in than in control offspring (P = 0.045), but was similar in sham and control offspring at PD 260 (P = 0.619). [4]At PD 90, sham did not alter retroperitoneal fat weight relative to body weight than in control offspring (P = 0.902), but at PD 260, reduced in sham offspring than in control offspring (P = 0.042). [5]At PD 90, sham did not alter visceral fat weight relative to body weight than in control offspring (P = 0.826), but at PD 260, reduced in sham offspring than in control offspring (P = 0.033). [6]Sham surgery reduced spleen weight in male offspring than in male control offspring (P = 0.024), but was similar in female sham and control offspring (P = 0.161). [7]Sham surgery reduced spleen weight relative to body weight in male offspring than in male control offspring (P = 0.032), but was similar in female sham and control offspring (P = 0.987).

Supplement Table S3.4. Specific comparisons for the effect of surgery and bilateral uterine artery vessel ligation on post-mortem organ and tissue weights in young adult offspring

PD 90	*P*-value (ANOVA)								
	(control v BUVL)			(sham v BUVL)			(control v sham)		
	T	S	T x S	T	S	T x S	T	S	T x S
Heart (g)	0.030	< 0.001	0.060[1]	ns	< 0.001	ns	ns	< 0.001	ns
(%BW)	ns	0.002	0.095[2]	ns	0.001	ns	ns	0.018	ns
Spleen (g)	ns	< 0.001	ns	ns	< 0.001	ns	0.011	< 0.001	0.012[4]
(%BW)	ns	0.030	ns	ns	0.001	ns	ns	0.001	0.054[5]
Adrenals (g)	ns	0.053	ns	ns	0.029	ns	ns	0.021	ns
(%BW)	ns	< 0.001	ns	ns	< 0.001	ns	ns	< 0.001	ns
Kidneys (g)	0.004	< 0.001	ns	ns	< 0.001	ns	ns	< 0.001	ns
(%BW)	ns	< 0.001	ns	ns	0.012	ns	ns	0.003	ns
Liver (g)	0.039	< 0.001	ns	ns	< 0.001	ns	0.079	< 0.001	ns
(%BW)	ns	0.002	ns	ns	0.008	ns	ns	0.008	ns
Lungs (g)	0.056	< 0.001	ns	ns	< 0.001	ns	0.006	< 0.001	ns
(%BW)	ns	0.001	ns	0.067	0.015	ns	0.036	0.006	ns
Brain (g)	ns	ns	ns	ns	ns	ns	ns	< 0.001	ns
(%BW)	0.003	< 0.001	0.068[3]	ns	< 0.001	ns	0.003	< 0.001	0.024[6]
Uterus (g)	0.071			ns			ns		
(%BW)	ns			ns			ns		
Testes (g)	0.041			ns			ns		
(%BW)	ns			ns			ns		
Fat depots									
Retroperitoneal fat (g)	ns	< 0.001	ns	ns	< 0.001	ns	ns	< 0.001	0.023[7]
(%BW)	ns	0.018	ns	ns	< 0.001	ns	ns	ns	ns
Omental fat (g)	0.078	0.085	ns	ns	0.006	ns	ns	0.064	ns
(%BW)	ns	< 0.001	ns	ns	0.039	ns	ns	0.013	ns
Visceral fat (g)	ns	< 0.001	ns	ns	< 0.001	ns	ns	< 0.001	ns
(%BW)	ns	ns	ns	ns	0.036	ns	ns	ns	0.013[8]

[1]Heart weight was similar in BUVL and control males (*P* = 0.589) but reduced in BUVL female than control females (*P* = 0.025). [2]Heart weight relative to the body weight was similar in both male and female of BUVL and control offspring (*P* = 0.684 & 0.154, respectively). [3]Brain weight relative to body weight was similar in BUVL and control males (*P* = 0.634) but reduced in BUVL female than control females (*P* = 0.003). [4]Spleen weight was reduced in sham males than control males (*P* = 0.003) but was similar in sham and control females (*P* = 0.977). [5]Spleen weight relative to body weight was reduced in sham males than control males (*P* = 0.017) but was similar in sham and control females (*P* = 0.627). [6]Brain weight relative to body weight was similar in sham and control males (*P* = 0.503) but greater in sham females than control females (*P* = 0.002). [7]Retroperitoneal fat weight was reduced in sham males than control males (*P* = 0.031) but was similar in sham and control females (*P* = 0.134). [8]Visceral fat weight relative to body weight was reduced in sham males than control males (*P* = 0.041) but was similar in sham and control females (*P* = 0.146).

T: surgical treatment (control, BUVL or sham); S: sex (male or female); ns: not significant.

Supplement Table S3.5. Specific comparisons for the effect of surgery and BUVL bilateral uterine artery vessel ligation on post-mortem organ and tissue weights of older adult offspring

PD 260	*P*-value (ANOVA)								
	(control v BUVL)			(sham v BUVL)			(control v sham)		
	T	S	T x S	T	S	T x S	T	S	T x S
Heart (g)	0.014	< 0.001	ns	0.070	<0.001	ns	ns	< 0.001	ns
(%BW)	ns	0.070	0.056[1]	ns	< 0.001	ns	ns	ns	0.088[10]
Spleen (g)	0.003	< 0.001	ns	ns	< 0.001	ns	0.020	< 0.001	ns
(%BW)	ns	ns	ns	ns	0.035	ns	ns	ns	ns
Adrenals (g)	ns	ns	ns	ns	ns	ns	ns	ns	ns
(%BW)	0.074	0.031	0.021[2]	ns	< 0.001	ns	ns	ns	0.090[11]
Kidneys (g)	0.012	< 0.001	ns	0.050	< 0.001	ns	ns	< 0.001	ns
(%BW)	ns	ns	ns	ns	ns	ns	ns	ns	ns
Liver (g)	ns	< 0.001	ns	ns	< 0.001	ns	ns	< 0.001	ns
(%BW)	ns	0.005	ns	ns	< 0.001	ns	ns	0.010	ns
Lungs (g)	ns	< 0.001	ns	ns	< 0.001	ns	ns	< 0.001	ns
(%BW)	ns	ns	0.034[3]	ns	< 0.001	0.096[9]	ns	ns	ns
Brain (g)	ns	< 0.001	ns	ns	< 0.001	ns	0.074	< 0.001	ns
(%BW)	ns	< 0.001	0.022[4]	0.055	< 0.001	ns	ns	< 0.001	0.079[12]
Uterus (g)	ns			ns			ns		
(%BW)	ns			ns			0.093		
Testes (g)	0.017			ns			0.014		
(%BW)	ns			ns			0.091		
Skeletal muscles									
Left soleus (g)	0.037	< 0.001	0.086[5]	ns	< 0.001	ns	ns	< 0.001	ns
(%BW)	ns	ns	0.081[6]	ns	ns	ns	ns	ns	ns
Left vastus lateralis (g)	ns	0.001	ns	ns	0.001	ns	ns	0.002	ns
(%BW)	ns	ns	ns	ns	ns	ns	ns	ns	ns
Fat depots									
Retroperitoneal fat (g)	ns	0.001	ns	ns	< 0.001	ns	0.011	< 0.001	ns
(%BW)	ns	0.003	ns	0.063	0.015	ns	ns	< 0.001	0.009[13]
Omental fat (g)	ns	< 0.001	ns	ns	< 0.001	ns	0.073	< 0.001	ns
(%BW)	ns	0.043	0.075[7]	ns	ns	ns	0.047	0.006	ns
Visceral fat (g)	ns	< 0.001	ns	ns	< 0.001	ns	0.015	< 0.001	ns
(%BW)	ns	0.003	0.078[8]	0.053	0.025	ns	0.008	< 0.001	ns

T: surgical treatment (control, BUVL or sham); S: sex (male or female); ns: not significant.

[1]Heart weight relative to body weight was similar in control and BUVL males (P =0.165) but tended to be heavier in BUVL females than in controls (P = 0.052). [2]Adrenals weight relative to body weight was similar in control and BUVL males (P = 0.890) but greater in BUVL females than in control females (P = 0.008). [3]Lungs weight relative to body weight was similar in control and BUVL males (P = 0.320) but tended to be heavier in BUVL females than in control females (P = 0.057). [4]Brain weight relative to body weight was similar in control and BUVL males (P = 0.550) but greater in BUVL females than in control females (P = 0.007). [5]Left hind soleus weight was lighter in BUVL males than in control males (P = 0.023) but similar in control and BUVL females (P = 0.748). [6]Left hind soleus weight relative to bodyweight was less in BUVL than control males (P = 0.050) but similar in control and BUVL females (P = 0.964). [7]Omental fat weight relative to body weight was similar in control and BUVL (males, P = 0.149; females, P = 0.312). [8]Visceral fat weight relative to body weight was similar in control and BUVL (males, P = 0.140; females, P = 0.355). [9]Lungs weight relative to body weight was similar in sham and BUVL males (P = 0.574) but tended to be heavier in BUVL females than in sham females (P = 0.073). [10]Heart weight relative to body weight was similar in control and sham (males, P = 0.248; females, P = 0.288). [11]Adrenals weight relative to body weight was similar in control and sham (males, P = 0.539; females, P = 0.287). [12]Brain weight relative to body weight was similar in control and sham males (P = 0.378) but greater in BUVL females than in control females (P = 0.002). [13]Retroperitoneal fat weight relative to body weight was reduced in sham males than in control males (P = 0.022) but similar in sham and control females (P = 0.127).

Supplement Table S3.6. Specific comparisons for the effect of surgery and bilateral uterine artery vessel ligation on IPGTT and IPITT of combined age offspring

Combined Age	*P*-value (ANOVA) (control v BUVL)							*P*-value (ANOVA) (sham v BUVL)							*P*-value (ANOVA) (control v sham)						
Specific comparisons	T	A	S	A x T	A x S	T x S	A x T x S	T	A	S	A x T	A x S	T x S	A x T x S	T	A	S	A x T	A x S	T x S	A x T x S
Glucose tolerance																					
Fasting plasma glucose (mmol.L^{-1})	0.015	0.036	0.019	0.004[1]	ns	ns	ns	ns	< 0.001	0.05	ns	ns	ns	ns	0.019	0.072	0.02	0.008[14]	0.091[16]	ns	ns
Maximum plasma glucose (mmol.L^{-1})	ns	< 0.001	ns	ns	ns	ns	ns	ns	< 0.001	ns	ns	ns	ns	ns	ns	0.007	ns	ns	ns	ns	ns
ΔPlasma glucose (mmol.L^{-1})	ns	0.001	ns	ns	ns	ns	ns	ns	0.014	ns	ns	ns	ns	ns	ns	0.018	ns	ns	ns	ns	ns
AUC glucose, 0-90min (mmol.L^{-1})	ns	0.001	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.09	0.062[15]	ns	ns	ns
Insulin secretion																					
Fasting plasma insulin (µg.L^{-1})	< 0.001	ns	< 0.001	ns	ns	0.018[2]	ns	0.009	ns	< 0.001	ns	ns	ns	0.078	0.065	ns	< 0.001	ns	ns	ns	ns
Maximum plasma insulin (µg.L^{-1})	0.003	ns	0.060	ns	ns	ns	ns	ns	ns	0.035	0.095[7]	ns	ns	ns	0.019	ns	0.022	ns	ns	ns	ns
ΔPlasma insulin (µg.L^{-1})	0.002	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.021[8]	ns	ns	0.067	0.015	ns	ns	ns	ns	ns	ns
AUC insulin, 0-90min (µg.L^{-1})	0.002	ns	ns	ns	ns	ns	ns	ns	0.063	ns	ns	ns	ns	0.06	0.001	ns	ns	ns	ns	ns	ns
Fasting insulin:glucose (µg.mmol^{-1})	0.006	ns	< 0.001	ns	ns	0.086[3]	ns	0.004	0.037	< 0.001	ns	ns	0.072[12]	0.047	ns	ns	< 0.001	ns	ns	ns	ns
AUC insulin:glucose (µg.mmol^{-1})	0.003	0.016	ns	ns	ns	ns	ns	ns	0.067	ns	ns	ns	ns	0.034	ns	ns	ns	ns	ns	ns	ns
Insulin sensitivity																					
Basal plasma glucose (mmol.L^{-1})	ns	ns	ns	ns	ns	ns	ns	ns	0.017	ns	ns	ns	ns	ns	ns	< 0.001	ns	ns	0.009[17]	0.023[19]	ns
Minimum plasma glucose (mmol.L^{-1})	0.012	ns	0.031	ns	ns	0.018[4]	ns	ns	ns	0.042	ns	ns	0.044[13]	ns	ns	ns	ns	ns	ns	ns	ns
Decrease plasma glucose (mmol.L^{-1})	0.018	ns	0.020	ns	ns	0.083[5]	ns	ns	ns	0.018	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
AAC glucose, 0-60min (mmol.L^{-1})	0.007	< 0.001	< 0.001	ns	ns	ns	ns	ns	< 0.001	< 0.001	ns	ns	ns	ns	0.094	< 0.001	< 0.001	ns	ns	ns	ns
Insulin action																					
Basal DI_{AAC}, 0-60min (µg.mmol.min.L^{-2})	0.008	ns	0.001	ns	0.023[6]	ns	ns	ns	ns	0.001	ns	0.016[11]	ns	ns	ns	ns	< 0.001	ns	ns	ns	ns
Basal $DI_{\Delta glucose}$ (µg.mmol.min.L^{-2})	ns	ns	0.001	ns	ns	ns	ns	ns	ns	< 0.001	ns	ns	ns	ns	ns	< 0.001	ns	ns	ns	ns	ns
Maximal DI_{AAC}, 0-60min (µg.mmol.min.L^{-2})	ns	ns	0.049	ns	ns	ns	ns	ns	0.015	0.018	0.016[9]	ns	ns	ns	ns	0.001	0.033	ns	0.072[18]	ns	ns
Maximal $DI_{\Delta glucose}$ (µg.mmol.min.L^{-2})	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.051[10]	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

A: age (day 90 or 180); T: surgical treatment (control, BUVL or sham); S: sex (male or female); ns: not significant. [1]Fasting glucose was reduced at PD 90 in BUVL compared to controls (P = 0.008), but similar at PD 180 in both BUVL and controls (P = 0.588). [2]Fasting insulin was reduced in both males and females BUVL compared to controls (P = 0.002 & 0.001). [3]Fasting insulin secretion relative to glucose was reduced in both males and females BUVL compared to controls (P = 0.017 & 0.034). [4]Minimum glucose was similar in male BUVL and controls (P = 0.651), but was reduced in female BUVL compared to controls (P = 0.011). [5]Decrease in glucose was similar in male BUVL and controls (P = 0.367), but was reduced in female BUVL compared to controls (P = 0.020). [6]Regardless of the treatment, males basal DI_{AAC} was reduced in PD 90 than in PD 180 (P = 0.035), but similar in females at both PD 90 and 180 (P = 0.391). [7]Maximum insulin was similar at PD 90 in sham and BUVL (P = 0.700), but tended to reduce in BUVL compared to sham offspring at PD 180 (P = 0.086). [8]Change in insulin was similar at PD 90 in sham and BUVL (P = 0.401), but reduced in BUVL compared to sham offspring at PD 180 (P = 0.033). [9]Maximal DI_{AAC} was similar at PD 90 in sham and BUVL (P = 0.304), but tended to reduce in BUVL compared to sham offspring at PD 180 (P = 0.095). [10]Maximal $DI_{\Delta Glucose}$ was tended to reduce at PD 90 in BUVL compared to sham offspring (P = 0.098), but similar at PD 180 (P = 0.237). [11]Regardless of the treatment, males basal DI_{AAC} was reduced in PD 90 than in PD 180 (P = 0.047) and tended to reduce in PD 90 than in PD 180, in females (P = 0.062). [12]In males, fasting insulin relative to glucose was reduced in BUVL compared to sham (P = 0.018) and tended to reduce in females (P = 0.092). [13]Minimum glucose was similar in BUVL and sham, at both males and female (P = 0.154 & 0.108, respectively). [14]At PD 90, fasting glucose was reduced in sham compared to controls (P = 0.013), but was similar in control and sham at PD 180 (P = 0.676). [15]AUC glucose was similar in sham and controls, at both PD 90 and PD 180 (P = 0.227 & 0.163, respectively). [16]Regardless of the treatment, in males fasting glucose was similar in PD 90 and PD 180 (P = 0.957), but in females was reduced at PD 90 compared to PD 180 (P = 0.001). [17]Regardless of the treatment, in males basal glucose was reduced in PD 90 compared to PD 180 (P < 0.001), but was similar in females (P = 0.416). [18]Regardless of the treatment, in males maximal DI_{AAC} was similar in PD 90 and PD 180 (P = 0.185), but in females was reduced at PD 90 compared to PD 180 (P = 0.003). [19]Regardless of age, sham tended to reduce basal glucose compared to control in males (P = 0.080), but was similar in females (P = 0.139).

Supplement Table S3.7. Specific comparison for the effect of surgery and bilateral uterine artery vessel ligation on IPGTT and IPITT of young adult offspring

PD 90	*P*-value (ANOVA)								
	(control v BUVL)			(sham v BUVL)			(control v sham)		
	T	S	T x S	T	S	T x S	T	S	T x S
Glucose tolerance									
Fasting plasma glucose (mmol.L^{-1})	0.005	0.056	ns	ns	ns	ns	0.008	0.031	ns
Maximum plasma glucose (mmol.L^{-1})	ns	0.019	ns	ns	ns	ns	ns	0.081	ns
ΔPlasma glucose (mmol.L^{-1})	ns	0.085	ns	ns	ns	ns	ns	ns	ns
AUC glucose, 0-90min (mmol.L^{-1})	ns	ns	ns	ns	ns	ns	ns	ns	ns
Insulin secretion									
Fasting plasma insulin (µg.L^{-1})	0.006	0.002	0.025[1]	0.001	< 0.001	0.004[3]	ns	< 0.001	ns
Maximum plasma insulin (µg.L^{-1})	0.044	0.007	ns	ns	0.096	0.066[4]	0.027	0.007	ns
ΔPlasma insulin (µg.L^{-1})	0.045	ns	ns	ns	ns	0.083[5]	0.011	0.08	ns
AUC insulin, 0-90min (µg.L^{-1})	ns	ns	ns	ns	ns	0.029[6]	0.034	ns	ns
Fasting insulin:glucose (µg.mmol^{-1})	ns	0.079	ns	< 0.001	< 0.001	0.002[7]	ns	< 0.001	ns
AUC insulin:glucose (µg.mmol^{-1})	ns	ns	ns	ns	ns	0.054[8]	ns	ns	ns
Insulin sensitivity									
Basal plasma glucose (mmol.L^{-1})	ns	ns	ns	ns	ns	ns	ns	0.083	ns
Minimum plasma glucose (mmol.L^{-1})	0.002	0.022	0.002[2]	ns	0.008	ns	ns	ns	ns
Decrease plasma glucose (mmol.L^{-1})	0.041	0.005	ns	0.059	0.001	ns	ns	0.026	ns
AAC glucose, 0-60min (mmol.L^{-1})	0.034	0.004	ns	ns	< 0.001	ns	0.084	< 0.001	ns
Insulin action									
Basal DI_{AAC}, 0-60min (µg.mmol.min.L^{-2})	0.048	ns	ns	0.023	ns	0.093[9]	ns	0.031	ns
Basal $DI_{\Delta glucose}$ (µg.mmol.min.L^{-2})	ns	ns	ns	ns	ns	ns	ns	0.013	ns
Maximal DI_{AAC}, 0-60min (µg.mmol.min.L^{-2})	ns	ns	ns	ns	ns	ns	0.079	ns	ns
Maximal $DI_{\Delta glucose}$ (µg.mmol.min.L^{-2})	ns	ns	ns	0.065	0.033	0.036[10]	0.050	ns	ns

T: surgical treatment (control, BUVL or sham); S: sex (male or female); ns: not significant.

[1]Fasting insulin concentration was less in BUVL than control males (*P* = 0.010) but similar in BUVL and control females (*P* = 0.383). [2]Minimum glucose concentration was similar in control and BUVL males (*P* = 0.532) but less in BUVL females than control females (*P* = 0.002). [3]Fasting insulin concentration was less in BUVL than sham males (*P* = 0.001) but similar in BUVL and sham females (*P* = 0.374). [4]Maximum insulin was similar in sham and BUVL males (*P* = 0.351) but tended to be less in BUVL females than sham females (*P* = 0.080). [5]Delta insulin was similar in sham and BUVL males (*P* = 0.566) but tended to be less in BUVL females than sham females (*P* = 0.057). [6]AUC insulin was similar in sham and BUVL males (*P* = 0.158) but tended to be less in BUVL females than sham females (*P* = 0.098). [7]Fasting insulin:glucose was less in BUVL than sham males (*P* = 0.001) but similar in BUVL and sham females (*P* = 0.508). [8]AUC insulin:glucose was tended to be less in BUVL than sham males (*P* = 0.068) but similar in BUVL and sham females (*P* = 0.323). [9]Basal DI_{AAC} was less in BUVL than sham males (*P* = 0.021) but similar in BUVL and sham females (*P* = 0.549). [10]Maximum $DI_{\Delta glucose}$ was similar in BUVL and sham males (*P* = 0.823) but less in BUVL females than sham females (*P* = 0.019).

Supplement Table S3.8. Specific comparison for the effect of surgery and bilateral uterine artery vessel ligation on IPGTT and IPITT of older adult offspring

PD 180	*P*-value (ANOVA)								
	(control v BUVL)			(sham v BUVL)			(control v sham)		
	T	S	T x S	T	S	T x S	T	S	T x S
Glucose tolerance									
Fasting plasma glucose (mmol.L^{-1})	ns	ns	ns	ns	ns	ns	ns	ns	ns
Maximum plasma glucose (mmol.L^{-1})	ns	ns	ns	ns	ns	ns	ns	ns	ns
ΔPlasma glucose (mmol.L^{-1})	ns	ns	ns	ns	ns	ns	ns	ns	ns
AUC glucose, 0-90min (mmol.L^{-1})	ns	ns	ns	0.052	ns	0.045[2]	ns	ns	0.098[3]
Insulin secretion									
Fasting plasma insulin (µg.L^{-1})	0.009	< 0.001	ns	ns	< 0.001	ns	0.080	< 0.001	ns
Maximum plasma insulin (µg.L^{-1})	0.016	ns	ns	0.085	ns	ns	ns	ns	ns
ΔPlasma insulin (µg.L^{-1})	0.018	ns	ns	0.033	ns	ns	ns	ns	ns
AUC insulin, 0-90min (µg.L^{-1})	0.008	ns	ns	ns	< 0.001	ns	0.017	ns	ns
Fasting insulin:glucose (µg.mmol^{-1})	0.043	< 0.001	ns	ns	ns	ns	ns	< 0.001	ns
AUC insulin:glucose (µg.mmol^{-1})	0.015	ns	ns	ns	ns	ns	ns	ns	ns
Insulin sensitivity									
Basal plasma glucose (mmol.L^{-1})	ns	0.015	0.098[1]	ns	ns	ns	ns	0.046	0.059[4]
Minimum plasma glucose (mmol.L^{-1})	ns	ns	ns	ns	ns	ns	ns	ns	ns
Decrease plasma glucose (mmol.L^{-1})	ns	ns	ns	ns	ns	ns	ns	ns	ns
AAC glucose, 0-60min (mmol.L^{-1})	ns	0.001	ns	ns	< 0.001	ns	ns	0.006	ns
Insulin action									
Basal DI_{AAC}, 0-60min (µg.mmol.min.L^{-2})	0.075	0.001	ns	ns	0.001	ns	ns	0.005	ns
Basal $DI_{\Delta glucose}$ (µg.mmol.min.L^{-2})	ns	< 0.001	ns	ns	< 0.001	ns	ns	< 0.001	ns
Maximal DI_{AAC}, 0-60min (µg.mmol.min.L^{-2})	ns	ns	ns	0.045	0.066	ns	ns	0.040	ns
Maximal $DI_{\Delta glucose}$ (µg.mmol.min.L^{-2})	ns	ns	ns	ns	ns	ns	ns	ns	ns

T: surgical treatment (control, BUVL or sham); S: sex (male or female); ns: not significant.

[1]Basal glucose was similar in both male and female BUVL than in control (*P* = 0.421 & 0.0.131, respectively). [2]AUC glucose was similar in sham and BUVL males (*P* = 0.962) but less in BUVL females than sham females (*P* = 0.0010). [3]AUC glucose was similar in sham and control males (*P* = 0.834) but tended to be less in female sham than control females (*P* = 0.076). [4]Basal glucose was similar in both male and female sham than in control (*P* = 0.158 & 0.204, respectively)

Supplement Table S3.9. Differentially expressed miRNAs which are known to be clustered on rat, mouse and human genome.

Cluster	miRNA members
1	miR-3567, miR-126
2	miR-144, miR-451
3	miR-15b, miR-16
4	miR-17-1, miR-18a, miR-19a, miR-20a, miR-19b-1, miR-92a-1
5	miR-17-2, miR-20b, miR-19b-2, miR-92a-2, miR-363
6	miR-106a, miR-18b, miR-20b, miR-19b-2, miR- 92a-2, miR-363

Supplement Table S3.10. List of the predicted targets of rno-miR-126 which was differentially expressed in liver following bilateral uterine artery vessel ligation

rno-miR-126					
LOC652956	Dcbld1	Rai1	Tbx4	Nbr1	Sema6d
LOC498369	Lpcat1	Abba-1	Mgat5b	Slc39a6	Nudt7
Pex5	RGD1565416	Gsx2	Rgr	LOC289482	Dcun1d4
LOC680726	Slc24a3	Lrrc47	Dock5	nod3l	RGD1311343
Mpp3	Slc25a25	Nol9	Tmed4	Dlst	Gnat2
Peli1	Kctd4	RGD1564387	Centd1	Scd1	Eml4
RGD1564943	RGD1564943	Otud1	Prox1	Serp1	Lad1
LOC682874	Rnf139	Pear1	Tnn	Anxa4	Adamts9
RGD1310950	Nol9	LOC690139	Trove2	Golph3	Spire1
RGD1564259	Pex5	Oxa1l	Sart3	Srd5a2	Tra2a
LOC688922	Dcbld1	RGD1310950	Hps4	Bad	Cacna1c
Crtc3	Abba-1	Mpp3	Dusp10	Insig1	Cdkl3
LOC691164	Rai1	Mpp3	Aqr	Qdpr	Rgs3
LOC683470	RGD1310016	RGD1306228	Zbtb24	Kit	Gria2
Prkci	RGD1564259	LOC691781	Isy1	Pik3r2	RGD1305020
Nudcd1	Camsap1	Thumpd1	Cd97	Bmpr1a	Slc20a2
LOC688452	RGD1565416	LOC498369	Polr3c	Ptprn2	Scn5a
RGD1305899	Flywch1	Rfc1	Xkr4	Stat1	Vcam1
LOC681858	Otud1	Camsap1	Thumpd1	Sulf1	Ret
Ltb	Crtc3	Rimbp2	MGC94199	Trak2	LOC501237
Mid1ip1	Gsx2	Zfp346	Zfp219	Ptpn21	LOC501335
Unc5c	Nudcd1	Aff4	Pdlim2	Tmlhe	Skp1a
Flcn	RGD1306228	Pof1b	Alx3	Zfp36	LOC680831
Kctd13	Slc24a3	Six4	Ly6e	Rnf138	LOC690413
Hnrnpa3	Prkci	Phf19	LOC500567	Cabin1	Cd68
Grinl1a	Lpcat1	Sp5	Gria2	Gas7	Cd68
Chp2	Mpp3	RGD1566320	Cyp4f18	Dcx	LOC501420
Mustn1	LOC501307	RGD1309394	Tusc4	Cxcl12	
LOC365157	Pear1	Hic2	Prmt2	Tm6sf1	
LOC680752	Rfc1	Slc43a2	Brf2	B4galt5	

The predicted targets were determined by the miRecords database, which provides 11 different algorithm databases, including miRanda, PicTar and TargetScan. The number of predicted targets was limited by requiring that they be commonly predicted by at least by three of the algorithms mentioned above.

Supplement Table S3.11. List of the predicted targets of rno-miR-145 which was differentially expressed in skeletal muscle following bilateral uterine artery vessel ligation

rno-miR-451											
A3galt2	Cep350	Ehhadh	Gpr88	Mtmr1	LOC685929	RGD1308612	Pisd	RGD1309707	Saps3	Stat2	Vof16
Aak1	Cep55	Ei24	Grid2	L3mbtl3	LOC686141	RGD1308775	Pitpnb	RGD1309995	Scml4	Stim1	Vps26b
Aard	Cep76	Eif2c1	Gsc	Lass6	LOC686286	Muc13	Pla2g6	RGD1310263	Scn3a	Stmn1	Vps36
Actbl2	Cetn2	Eif4a2	H1fx	Lemd3	LOC686530	Muc3a	Pld1	RGD1310311	Scn3b	Strbp	Wasf1
Actr3b	Cgn	Elavl2	Hap1	Lgals12	LOC686753	Myc	Plekha1	RGD1310423	Scyl1bp1	Syf2	Wdr21
Aff4	Cgn	Elk4	Hcrtr2	Lipa	LOC687381	Mycl1	Plekhk1	RGD1310553	Sec23ip	Syne1	Wdr22
Akap6	Chd1l	Eltd1	Hdac3	Lipc	LOC687978	Myo5c	Plxnc1	RGD1310810	Senp5	T2	Wdr41
Akr1b7	Chmp2b	En1	Heca	Lkap	LOC688115	Nae1	Pmm2	RGD1311345	Sepp1	Tacstd2	Wdr44
Alg10b	Chmp7	Enox1	Herpud1	Lman2l	LOC688119	Nbeal1	Pmp22	RGD1311357	Sfpq	Taf1	Wif1
Alpk3	Chn1	Erg	Hfe2	Lnx2	LOC688135	Nck2	Pou5f2	RGD1311364	Sfxn3	Taf9	Wipf3
Alpk3	Chrdl1	Etnk1	Hhip	LOC252831	LOC688261	Nfatc3	Ppcdc	RGD1311659	Sgcg	Tbc1d14	Wsb1
Ankfy1	Clcc1	Ext1	Hibch	LOC292666	LOC688578	Nfia	Ppm1k	RGD1311745	Sgip1	Zkscan17	Wsb1
Ankrd15	Cldn1	Fam20a	Hic2	LOC294154	LOC688585	Ngfr	Ppp1r14c	RGD1311805	Sgip1	Tcfap2e	Xkr5
Ankrd28	Cnot2	Fam20c	Hist3h2a	LOC305633	LOC688780	Nhlrc2	Ppp1r3c	RGD1559962	Sh2bpsm1	Tcfcp2l1	Xpo4
Ankrd50	Cntn4	Fam44b	Hk2	LOC306096	LOC688940	Nip7	Prkd3	RGD1560175	Sh3bp5	Tctex1d4	Ybx1
Ankrd52	Col24a1	Fam89a	Hmgcl	LOC306766	LOC689035	Nipsnap1	Tbl1x	RGD1560191	Siglec1	Tesk2	Yipf6
Anxa13	Col5a3	Fance	Hmgn1	LOC310958	LOC689117	Nkiras1	Prps2	RGD1560286	Sirpa	Tgfb2	Yme1l1
Aqp11	Cpeb2	Fcar	Hn1l	LOC360807	LOC689166	Nkx6-2	Psat1	RGD1560300	Sirt5	Tgfbr3	Ythdf1
Arf3	Cpt1a	Fcho2	Hnrnpa1	Tbx18	LOC689898	Nol10	Ptafr	RGD1560723	Six1	Tifa	Ythdf3
Arf6	Csde1	Fem1c	Hnrnpa3	RGD1565310	LOC689962	Nolc1	Ptms	RGD1560812	Slc12a2	Tjp2	Ywhae
Arhgef17	Csrp2bp	Fign	Homer1	LOC364281	LOC691770	Nppc	Ptpra	RGD1561694	Slc12a7	Tmc5	Ywhaz
Arhgef19	Ctnnal1	Fndc3b	Homer2	LOC367808	LOC691853	Nr3c1	Ptpre	RGD1561849	Slc22a8	Tmem110	Zan
Arid1b	Prpf39	Fnta	Hpgd	LOC497729	Loh11cr2a	Nrcam	Ptprj	RGD1562218	Slc24a3	Tmem130	Zbp1
Arl4c	Prnp	Foxo3a	Hpn	LOC498463	Lppr4	Nrip3	Rab22a	RGD1562317	Slc25a30	Tmem16a	Zbtb43
Arl5b	Cul2	Frmd5	Hps4	LOC498464	Lrfn3	Nsun4	Rab23	RGD1562532	Slc25a36	Tmem16f	Zc3h14
Arpc5	Cwc15	Fts	Hs1bp3	LOC498465	Lrrc14	Nub1	Rab9	RGD1562952	Slc2a13	Tmem19	Zc3h7b
Arpp21	Cxcl12	Fusip1	Hsbp1	LOC498468	Lrrk1	Nudt12	Rarb	RGD1563180	Slc31a2	Tmem196	Zdhhc15
Arsa	Cxcl12	G6pc	Hsd3b7	LOC498470	Lsm6	Nxph1	Rasip1	RGD1563342	Slc35f2	Tmem43	Zdhhc19
Asb11	Cyb561	Gabpa	Hspa5	LOC498471	Lyn	Ocm	Raver1	RGD1563510	Slc35f5	Tmem45b	Zfp189
Asph	Cybasc3	Galnt11	Htr5b	LOC498474	Lysmd3	Odf3	Rbbp5	RGD1563666	Slc38a2	Tmod3	Zfp251
Atg12	Dao1	Galntl2	Htr5b	LOC498480	Lzts2	Odz3	Rbm20	RGD1563945	Slc38a9	Tmx2	Zfp36l2
Atp6ap1	Dapk1	Gap43	Ifi204	LOC498973	Man1a	Omd	Rbm25	RGD1564040	Slc46a1	Tnfrsf1a	Zfp422
Atxn3	Dapp1	Gas7	Igf2bp2	LOC499531	Map4k3	Osr1	Rbx1	RGD1564093	Slc6a9	Tollip	Zfp423
Axud1	Dcx	Gata5	Il1f10	LOC499814	Mapk3	Otud4	Rer1	RGD1564149	Slc7a13	Tomm20	Zfp462
B3galnt1	Ddah1	Gbe1	Il1rl2	LOC500392	Marveld2	Pafah1b1	Rfxdc2	RGD1564200	Slit2	Top2a	Zfp644
Bat2d	Ddx4	Gbp5	Il6ra	LOC501503	Mcfd2	Palb2	RGD1304792	RGD1564319	Sms	Tpc1808	Zwint
Bdh1	Ddx58	Gca	Ing3	LOC678813	Megf8	Pan3	RGD1305211	RGD1564747	Snai3	Tpo1	Zxdc
Bnc1	Dmn	Gdf11	Iqgap1	LOC679082	Megf8	Panx1	RGD1305350	RGD1564854	Snap91	Tram1l1	
Brp44l	Dnajb12	Gfpt2	Irf6	LOC679094	MGC125086	Papd5	RGD1305560	RGD1565218	Snn	Trove2	
Cab39	Dnmt3b	Gfra1	Irs2	LOC679126	Midn	Parg	RGD1305593	Tbx1	Sorcs3	Tspan5	
Cap1	Dok4	Gfra2	Isca1	LOC679126	Mmrn2	Parp3	RGD1305697	RGD1565775	Sorl1	Uba6	
Cbara1	Dph2	Glce	Itpr1	LOC679474	Mobkl1b	Pax9	RGD1305725	RGD1566402	Sos2	Ube2v2	
Ccdc44	Dpp8	Gltpd2	Kcnv2	LOC680040	Prickle2	Pcdh9	RGD1306105	Rgnef	Sox21	Ubxd3	
Ccdc82	Dppa1	Gmpr	Kera	LOC680308	RGD1565247	Pdgfrb	RGD1306151	Rnasek	Sox9	Ulk2	
Ccdc91	Drd1a	Gnal	Kif11	LOC680704	Mpp6	Pdp2	RGD1307215	Rnf103	Sp100	Upf2	
Cd5	Dusp3	Gpm6a	Kif1b	LOC682905	Mrpl37	Pex26	Snx27	Rnf14	Sparc	Usp16	
Cdca7	Dusp6	RGD1565496	Kl	LOC682967	Mrpl47	Pfn4	Son	Rnf185	Spast	Usp26	
Cdca7l	Dusp8	Prom1	Klf4	LOC684233	Mrps2	Pgrmc1	RGD1308915	Rnmt	Spcs2	Usp6nl	
Cdk2	Dyrk2	Gpr174	Klhl23	LOC684272	Ms4a1	Pgs1	RGD1309106	Rras2	Spock3	Vcan	
Cdkn1c	Ebf3	Gpr176	Klhl24	LOC684322	Msh3	Phospho2	RGD1309188	Rspo1	St3gal6	Vdac1	
Cdkn2d	Ecel1	Gpr63	Klrg1	LOC684852	Msl31	Phtf2	RGD1309360	S1pr3	Stag1	Vezf1	
Cenpj	Eef1a1	Gpr64	Mtch1	LOC685837	Msrb3	Pirb	RGD1309621	S1pr5	Stat1	Vezf1	

The predicted targets were determined by the miRecords database, which provides 11 different algorithm databases, including miRanda, PicTar and TargetScan. The number of predicted targets was limited by requiring that they be commonly predicted by at least by three of the algorithms mentioned above.

Supplement Table S3.12. List of the predicted targets of miRNAs which were differentially expressed in omental fat following bilateral uterine artery vessel ligation

rno-miR-16			hsa-miR-106a				mmu-miR-106a		
Acvr2a	Gse1	Phf20l1	ACSL4	FAM13C1	PCDHA13	STAT3	Abca1	Hmgb3	Slain2
Adrb2	Gtpbp1	Pisd	AHCTF1	FAM57A	PCDHA2	STK38	Acsl4	Jazf1	Slc16a12
Ahcyl2	Herc6	Pklr	ALS2CR13	FCHO2	PCDHA3	SUV420H1	Ankib1	Kbtbd8	Slc25a36
Akap12	Hibadh	Pldn	ANKFY1	FJX1	PCDHA5	TANC1	Ankrd13c	Kif26b	Slc2a4
Akr1c12	Higd1a	Podxl	ANKRD50	FNDC3B	PCDHA6	TBC1D20	Ankrd17	Klf9	Slc40a1
Ankib1	Hoxa10	Ppp6c	APBB2	FOXL2	PCDHA7	TBC1D8B	Ankrd52	Kpna2	Slc6a5
Arpp21	Hoxc9	Pth	ARHGAP1	GAB1	PCDHAC2	TBC1D9	Arhgap12	Lace1	Snx16
Atp1b4	Hsd17b7	Pura	ARHGAP26	GABBR2	PEX5L	TGFBR2	Arhgap26	Laptm4a	Sos1
Atxn7l2	Iars	Pvrl1	ARHGEF3	GNB5	PHLPPL	TGOLN2	Arhgef3	Limk1	Ss18l1
B4galt7	Inhbc	Rab6a	ARID4A	GOLSYN	PHTF2	TIMP2	Arid4b	Map3k12	Stat3
Bag5	Irak2	Rbm34	ARL4C	GOSR1	PKD2	TMCC1	Atg7	Map3k14	Styx
Bcl2l2	Itpr1	Reln	ASF1A	HAS2	PKIA	TMEM127	Bambi	Map3k2	Syap1
Bdnf	Kcnc2	Ret	ATG16L1	HBP1	PKN2	TMUB2	BC013529	Map3k8	Tbcel
Btg2	Kcnv1	RGD1305422	ATL3	HLF	PKNOX1	TNFSF11	BC037034	Mar8	Timp2
Btrc	Kdr	RGD1305628	ATP1A2	HMGB3	PLAGL2	TNRC6B	Bicd2	Mex3d	Tle4
Capn6	LOC300308	RGD1307799	BAHD1	INTS6	PLS1	TOPORS	Bicd2	Mfap3l	Tmem168
Capza2	LOC679383	RGD1308059	BCL2L11	IRF1	POLQ	TP53INP1	Brms1l	Mkrn1	Tnfaip1
Carm1	LOC680856	RGD1308127	BHLHB3	ITGB8	PPARA	TRIM3	Btbd10	Morf4l1	Tnfrsf21
Cask	LOC682617	RGD1309762	BMPR2	KIAA0494	PPP2R2A	TRPV6	Btg3	Mtf1	Tsg101
Cc2d1b	LOC683571	RGD1311739	BNC2	KIAA0513	PPP3CA	TSG101	C80913	Mycn	Twf1
Ccdc19	LOC684921	RGD1560020	BTBD10	KIAA0922	PPP3R1	UBE2Q2	Ccdc128	Nedd4l	Txnip
Ccdc28a	LOC685233	RGD1561090	BTBD7	KIF26B	PPP6C	UBE2W	Ccnt2	Nek9	Ube3c
Ccdc80	LOC685957	RGD1564085	BTG3	KLHL20	PRRG1	UBE3C	Cep97	Neurog2	Ulk1
Cdc25a	LOC687620	RGD1564943	C14orf28	KPNA3	PTPN3	UNK	Cfl2	Nfat5	Unk
Cdc37l1	LOC688133	RGD1564964	C19orf2	LACE1	PTPN4	USP31	Cmpk1	Obfc2a	Usp46
Cdx2	LOC688135	RGD1566155	C1orf63	LAPTM4A	PURB	VLDLR	Col4a3	Oxr1	Vldlr
Cflar	LOC688211	RGD621352	C2CD2	LDLR	RAB11FIP1	VPS26A	Creb1	Pafah1b2	Wdr37
Clcn4-2	LOC690776	Rnf10	C5orf41	LHX6	RAP2C	WAC	Cry2	Pbx3	Wdr37
Cmtm1	Lrp2	Rnf138	CAMTA1	LIMK1	RAPGEF4	WDR37	Cxadr	Pcaf	Wdr42a
Col24a1	Luzp1	Rsbn1	CCDC128	LMO3	RASL11B	WEE1	Ddx5	Pcdha1	Wee1
Cops2	Lypla3	Rtn3	CCNG2	LOC283871	RB1	XRN1	Derl2	Pcdha10	Ythdf3
Csde1	Map1lc3b	Sar1a	CCNT2	MAP3K2	RBBP7	YOD1	Dirc2	Pcdha11	Zbtb41
Dclk1	Map2k1	Scoc	CD69	MAR8	RGL1	YPEL2	Dmtf1	Pcdha2	Zbtb44
Dd25	Mapk8	Sec61a1	CEP57	MAT2B	RHOC	YTHDF3	Dusp2	Pcdha4	Zfp148
Dek	Mapk9	Sep2	CFL2	MCL1	RNF128	ZBTB41	Dync1li2	Pcdha5	Zfp238
Dmtf1	Mfn2	Serinc3	CHD9	MED12L	RNH1	ZBTB6	Egln3	Pcdha6	Zfp367
Dpp9	MGC114483	Setd3	CLIP4	MEX3D	RP5	ZBTB7A	Eif4g2	Pcdha7	Zhx2
Dpy19l4	Mkks	Setd3	CMPK1	MGEA5	RPS6KA5	ZBTB9	Eif5a2	Pcdha8	
Dync1i1	Mlycd	Setd3	CREB5	MORF4L1	RSBN1	ZFP91	Elavl2	Pcdha9	
Dync1li2	Mobkl3	Sfrs18	CRIM1	MTMR3	RUFY2	ZFYVE26	Enpp5	Pcdhac1	
Dynlt3	Myt1l	Sgk1	CRK	MYCN	SACS	ZFYVE9	Epha4	Pcdhac2	
Eif2b2	N4bp1	Sh3bgrl2	CRY2	MYO1D	SAR1B	ZNF148	Epha5	Pde3b	
Esam	Nf1	Siah1a	DDHD1	MYT1L	SCN2A	ZNF238	Epha7	Pdgfra	
Esrrg	Nrn1	Slc11a2	DDX5	NAP5	SEMA4B	ZNF25	Ereg	Phtf2	
Fam113a	Nsg1	Slc25a22	DERL2	NAPEPLD	SENP1	ZNF362	Ezh1	Plekha3	
Fam116a	Nuak2	Smad7	DLGAP2	NAT12	SERP1	ZNF512B	F3	Ppp3r1	
Fermt2	Nup210	Socs6	DMTF1	NCOA3	SGMS1	ZNF704	Fbxo21	Pxk	
Fkrp	Nxph1	Spnb3	DPYSL2	NFAT5	SKI	ZNF800	Fbxo28	Rab5b	
Fzd1	Pafah1b1	Srp72	DPYSL5	NPAS2	SLC22A23		Fcho2	Rasd1	
Glb1l3	Pcdh9	Srp72l	DUSP2	NPLOC4	SLC25A27		Fgd5	Rassf2	
Glud1	Pcmt1	Srpr	DYNC1LI2	NR2C2	SLC40A1		Ficd	Rbbp7	
Gng11	Pde3b	Stx1a	EEA1	NR4A3	SLITRK3		Fnbp1l	Rbl2	
Stxbp3	Trhr	Wbp11	EGLN3	NUP35	SMOC1		Fndc3b	Rhov	
Tacc1	Trpv5	Wee1	EIF2C1	OCRL	SOBP		Foxb1	Rnf6	
Tacstd2	Txndc1	Wipi2	EIF4H	OSR1	SOX4		Frmd6	RP23-12I24.6	
Tarbp2	Ubap1	Wnt7a	ENPP5	PAFAH1B2	SPOPL		Furin	Rps6ka1	
Tasp1	Ube4b	Yt521	EPHA5	PAK7	SPRY4		Gbf1	Rps6ka5	
Tbp	Ubl5	Zbtb39	EREG	PAPOLA	SPTY2D1		Gcap14	Rragd	
Tbpl1	Umod	Zfhx4	ETV1	PBX3	SR140		Gosr1	Rsbn1	
Tgfbr3	Uqcrbl	Zfp105	F3	PCAF	SSH2		Gria2	Sar1b	
Tmcc1	Usp12	Zfp362	FAM102A	PCDHA1	SSX2IP		Hbp1	Sema4b	
Tmem33	Usp14	Zfp622	FAM126B	PCDHA10	ST6GALNAC3		Hif1a	Sema7a	
Tmem55a	Vat1	Zfp655	FAM134A	PCDHA12	ST8SIA2		Hlf	Sertad2	
		Zmym2							

Supplement Table S3.12. *Continued*

rno-miR-18a	rno-miR-142-3p	rno-miR-19b			
Akr1d1	Acsl4	Acsl1	Gulp1	Pcdhac1	Tnrc6b
Alpl	Aff2	Acsl4	Hbp1	Pcdhac2	Tor1aip2
Ankrd13c	Ank3	Adamts7	Hexb	Pde5a	Tor1b
Ap2b1	Arntl	Adnp	Hhip	Phf12	Trak2
Apon	Atp2a2	Aftph	Hspc159	Pomt2	Tspan2
Atxn1	Baz1a	Aldh3a2	Id4	Pou4f1	Ubl3
Bco2	Cask	Ankib1	Igf2r	Prom1	Vps37a
Ccl11	Dd25	Arc	Igfbp3	Psd3	Vps4b
Cirbp	Dirc2	Arfgef1	Inoc1	Rab18	Wdfy3
Crebl2	Fam114a1	Arfip1	Itfg3	Raf1	Wdr1
Crkrs	Fam44b	Arl6ip2	Itpr1	Rap1a	Wdr44
Ctgf	Fkbp1a	Armcx1	Jakmip1	Rapgef4	Wee1
Dd25	Fntb	Atp10a	Kcna4	Rbms1	Ythdf2
Dzip1l	Foxm1	Atp2c1	Kpna6	RGD1305269	Zbtb10
Enc1	Ghr	Atp6v1b2	Kpna6	RGD1305314	Zc3h8
Epb4.1l1	Gpr182	Bmpr2	Lipa	RGD1305350	Zfp110
Epn3	Grlf1	Brwd3	LOC30676	RGD1306880	Zfp426
Etv6	Gtf2h5	Btaf1	LOC31367	RGD1307235	Zfp445
Gng11	Hectd1	Cacna1c	LOC36088	RGD1308470	Zfp654
Gpr37	Il17d	Camsap1	LOC37846	RGD1308872	Zmynd11
Hibadh	LOC678709	Cast	LOC49835	RGD1309701	
Hif1a	LOC681252	Cbfb	LOC68013	RGD1309969	
Hmbox1	LOC681423	Cbln2	LOC68215	RGD1559432	
Hsf2	LOC686359	Cbx7	LOC68384	RGD1560601	
Ifit1lb	LOC686520	Ccdc51	LOC68419	RGD1561090	
Kpna6	Lrrc1	Ccdc88a	LOC68560	RGD1561758	
LOC302576	Map3k7ip2	Ccnl1	Lonrf1	RGD1561963	
LOC361230	Med20	Cd47	Lrch1	RGD1562339	
LOC493574	Mmd	Cdc26	Lrp2	RGD1563437	
Mdm1	Morf4l2	Cds1	Lrrcc1	RGD1564379	
MGC105830	Mtmr10	Cgn	Lycat	RGD1565775	
Myh7	Mtmr11	Chic1	Mageb16	RGD621098	
Nedd9	Ncam2	Chp	Mapk6	Rhebl1	
Orai3	Pawr	Chst1	Mbd6	Rhob	
Pck1	Pcaf	Cldn8	Mdm1	Rnf11l	
Pex3	Prlr	Clip1	Memo1	Rnf167	
Ptafr	Rai14	Cltc	MGC1128	Robo2	
Ptgfr	RGD1305560	Csmd1	MGC1247	Scn1b	
Rapgef4	RGD1305938	Cyb5d2	MGC1247	Sec11a	
RGD1562562	RGD1309701	Cyld	Mier1	Sec22a	
Rgs6	RGD1564327	Dbn1	Mier3	Sgcb	
Rims2	RGD1565502	Dcp2	Mon2	Sgk1	
Rras2	Rlf	Diap3	Mphosph9	Sh3d19	
Sdc4	S1pr3	Dpysl5	Mtmr10	Skil	
Sfrs18	Scyl1bp1	Dync1li2	Mycl1	Slc12a7	
Slc39a13	Sdccag1	E2f8	Mycn	Slc31a2	
Smad2	Serinc1	Efr3a	Nbeal2	Slc6a8	
Sntg1	Slc1a3	Ehbp1	Nptn	Slc9a1	
Spata1	Slc20a2	Ell2	Nr3c2	Slc9a6	
St3gal2	Slc2a13	Enc1	Nxf7	Smarcd2	
Tbccd1	Smpd4	Entpd5	Otud1	Snip	
Thex1	Stx12	Es22	Pcdha1	Snip1	
Tmprss2	Tgfbr1	F3	Pcdha10	Snx17	
Tnip2	Tmem33	Fam114a1	Pcdha11	Sphk2	
Txndc13	Tsen34	Fam124a	Pcdha12	Sstr3	
Zfand1	Xkr6	Fam152a	Pcdha13	St3gal5	
Zfp367		Fam43a	Pcdha2	Stau2	
Zfp804a		Fam69a	Pcdha3	Stau2	
		Fbxo8	Pcdha4	Stk38	
		Fibin	Pcdha5	Tfb2m	
		Flnc	Pcdha6	Tgif1	
		Fmr1	Pcdha7	Tgoln1	
		Foxp2	Pcdha8	Thsd7a	
		Golsyn	Pcdha9	Tmem196	

Supplement Table S3.12. *Continued*

rno-miR-21		rno-miR-20b				
Acat1	RGD1559960	Aadac	Fam13c1	Mfn2	RGD1305469	Tmem123
Alx1	RGD1561241	Acsl4	Fat2	Midn	RGD1306227	Tmem168
Ank2	RGD1562326	Adcy7	Fbxo39	Morf4l1	RGD1307493	Tmem16c
Arhgap24	RGD1563612	Adh7	Fcho2	Mospd1	RGD1307882	Tmem87b
Armcx1	RGD1565247	Adra1b	Fgd4	Mrps15	RGD1307882	Tnfaip1
Atrnl1	RGD1565421	Ahctf1	Fgfr1op2	Mtmr3	RGD1308087	Tob2
Bahd1	RGD1565591	Ampd3	Fhl4	Mycn	RGD1308127	Trim3
Bbs7	Rmnd5a	Ankib1	Fndc3b	Myo1d	RGD1309759	Trip10
Bhlhb3	Scnn1g	Ankrd13c	Frmd6	Nanos1	RGD1310335	Trip11
Caskin1	Slc12a1	Ankrd28	Fxr1	Nbl1	RGD1310712	Trpv6
Ccdc100	Slc16a4	Ankrd52	Gabbr2	Ncoa3	RGD1311188	Tsg101
Ccl22	Slc25a46	Arid4b	Glra2	Nedd4l	RGD1359108	Ttrap
Ccrk	Smad7	Arl4c	Glrx2	Nr4a3	RGD1560022	Twf1
Chic1	Sos2	Armc8	Golph3l	Nrbp	RGD1560755	Txnip
Cnksr2	Spn	Atad2	Golsyn	Nup35	RGD1561149	Ube3c
Ctnna1	Spna1	Bahd1	Gpr158	Oas1e	RGD1562373	Usp3
Cwf19l2	Stag2	Bhlhb3	Gpr6	Obfc2a	RGD1562629	Usp46
Dag1	Stat3	Bicd2	Gtdc1	Olfm3	RGD1562665	Uxs1
Elf2	Sult1c2a	Btbd10	Gucy1b3	Olr1	RGD1563296	Vcsa1
Elovl7	Tbc1d14	Btg3	Gzmb	Oxr1	RGD1563952	Vdac2
Eml5	Tbc1d15	Camk2n1	Gzmc	Pafah1b2	RGD1565310	Vhlh
Eny2	Tbpl1	Casc4	Has2	Panx1	Ripk3	Wasf1
Epm2aip1	Tc2n	Casp8	Hcrtr2	Pcaf	Rkhd1	Wbp7
Fbxo11	Tiam1	Cbara1	Hif1a	Pcdha1	Rnf138	Wdr42a
Fgd4	Tipin	Ccdc5	Hist1h4b	Pcdha10	Rnf181	Wee1
Gnrhr	Tmem106a	Cct6a	Hn1	Pcdha11	Rnf2	Xrn1
Gpr64	Vps54	Cd69	Htr5b	Pcdha12	Rpe	Xrn1
Hspa14	Wdr55	Cdc37l1	Igsf10	Pcdha13	Rps6ka3	Zbtb44
Ifrd1	Wwp1	Cdk2	Inoc1	Pcdha2	Rrm2	Zc3h14
Il17d	Zcchc3	Cercam	Ipo11	Pcdha3	Rsbn1	Zfp362
Ing3	Zdbf2	Chaf1a	Irak2	Pcdha4	Sar1b	Zfp367
Jag1	Zdhhc17	Chmp4c	Isoc1	Pcdha5	Scamp2	Zfp91
Kcnv1	Zdhhc9	Clcc1	Jak1	Pcdha6	Sema3c	Zfpm2
Klf6	Zfp367	Cmpk1	Jam2	Pcdha7	Sema4b	Zhx2
Lemd3		Coil	Klf9	Pcdha8	Sema7a	
Lhfpl3		Cops4	Kpna2	Pcdha9	Serp1	
LOC678905		Coro2b	Lactb2	Pcdhac1	Serpina5	
LOC679565		Cp	Laptm4a	Pcdhac2	Siat7F	
LOC682575		Crim1	Lbxcor1	Pde3b	Sike	
LOC683527		Crot	Lhx8	Pdzd3	Slc18a2	
LOC684297		Cryz	Limk1	Peli1	Slc19a2	
LOC684996		Cyb5d2	LOC300225	Pfn2	Slc22a23	
LOC687022		Dcun1d3	LOC303259	Phtf1	Slc24a2	
Lyrm2		Dd25	LOC306766	Pip4k2c	Slc25a36	
MGC114381		Ddhd1	LOC499331	Pirb	Slc5a7	
Moxd1		Ddx5	LOC500118	Pkd2	Slc6a15	
mrpl9		Derl2	LOC679811	Plekha3	Slc6a5	
Ntf3		Dip2a	LOC682704	Plscr3	Smad7	
Ola1		Dirc2	LOC684652	Ppp1r3b	Sorl1	
Paip2b		Dmn	LOC685548	Ppp2r2a	Spty2d1	
Pan3		Dmtf1	LOC686087	Ppp6c	Spz1	
Pbrm1		Dpyd	LOC686701	Prkx	Sqstm1	
Pcbp1		Dpysl5	LOC687705	Prrg1	Ssx2ip	
Peli1		Drd1a	LOC687750	Pthlh	St6galnac3	
Phf20l1		Dusp2	Lrrcc1	Ptpn21	Stk17b	
Pitx2		Dync1li2	Lzic	Ptpn3	Stx6	
Pja2		Dyrk4	Map3k12	Ptprj	Sult1c1	
Plcb1		E2f1	Map3k2	Pxk	Sv2b	
Plekha1		E2f5	Map3k8	Rab10	Tbxas1	
Rab11a		Egln3	Map3k9	Rab30	Tcfcp2l2	
Rasgrp1		Elavl2	Mapre1	Rab5b	Tes	
RGD1309362		Epha4	Mapre3	Rasl11b	Tgoln1	
RGD1310686		F3	Mat2b	Rbbp7	Tiam1	
RGD1359616		Fam134a	Mcm3	RGD1305455	Tle4	

The predicted targets were determined by the miRecords database, which provides 11 different algorithm databases, including miRanda, PicTar and TargetScan. The number of predicted targets was limited by requiring that they be commonly predicted by at least by three of the algorithms mentioned above.

Supplement Table S3.13. Summary of specific effects of placental restriction on metabolic control and insulin action in overall adult offspring

Overall	Combined age			Young adult			Older adult		
	Control vs:		Sham vs	Control vs:		Sham vs	Control vs:		Sham vs
	BUVL	Sham	BUVL	BUVL	Sham	BUVL	BUVL	Sham	BUVL
Glucose tolerance									
Fasting plasma glucose (mmol.L^{-1})	↓	↓		↓	↓				
Maximum plasma glucose (mmol.L^{-1})									
ΔPlasma glucose (mmol.L^{-1})									
AUC glucose, 0-90min (mmol.L^{-1})									
Insulin secretion									
Fasting plasma insulin (µg.L^{-1})	↓	↓	↓	↓		↓	↓		
Maximum plasma insulin (µg.L^{-1})	↓	↓		↓	↓		↓		
ΔPlasma insulin (µg.L^{-1})	↓	↓		↓	↓		↓		
AUC insulin, 0-90min (µg.L^{-1})	↓	↓			↓		↓		
Fasting insulin:glucose (µg.mmol^{-1})	↓		↓			↓	↓		
AUC insulin:glucose (µg.mmol^{-1})	↓						↓		
Insulin sensitivity									
Basal plasma glucose (mmol.L^{-1})									
Minimum plasma glucose (mmol.L^{-1})	↓			↓					
Decrease plasma glucose (mmol.L^{-1})	↑			↑					
AAC glucose, 0-60min (mmol.L^{-1})	↑			↑					
Insulin action									
Basal DI_{AAC}, 0-60min (µg.mmol.min.L^{-2})	↓			↓		↓			↓
Basal $DI_{\Delta glucose}$ (µg.mmol.min.L^{-2})									
Maximal DI_{AAC}, 0-60min (µg.mmol.min.L^{-2})									
Maximal $DI_{\Delta glucose}$ (µg.mmol.min.L^{-2})									

Supplement Table S3.14. Summary of specific effects of placental restriction on metabolic control and insulin action in male adult offspring

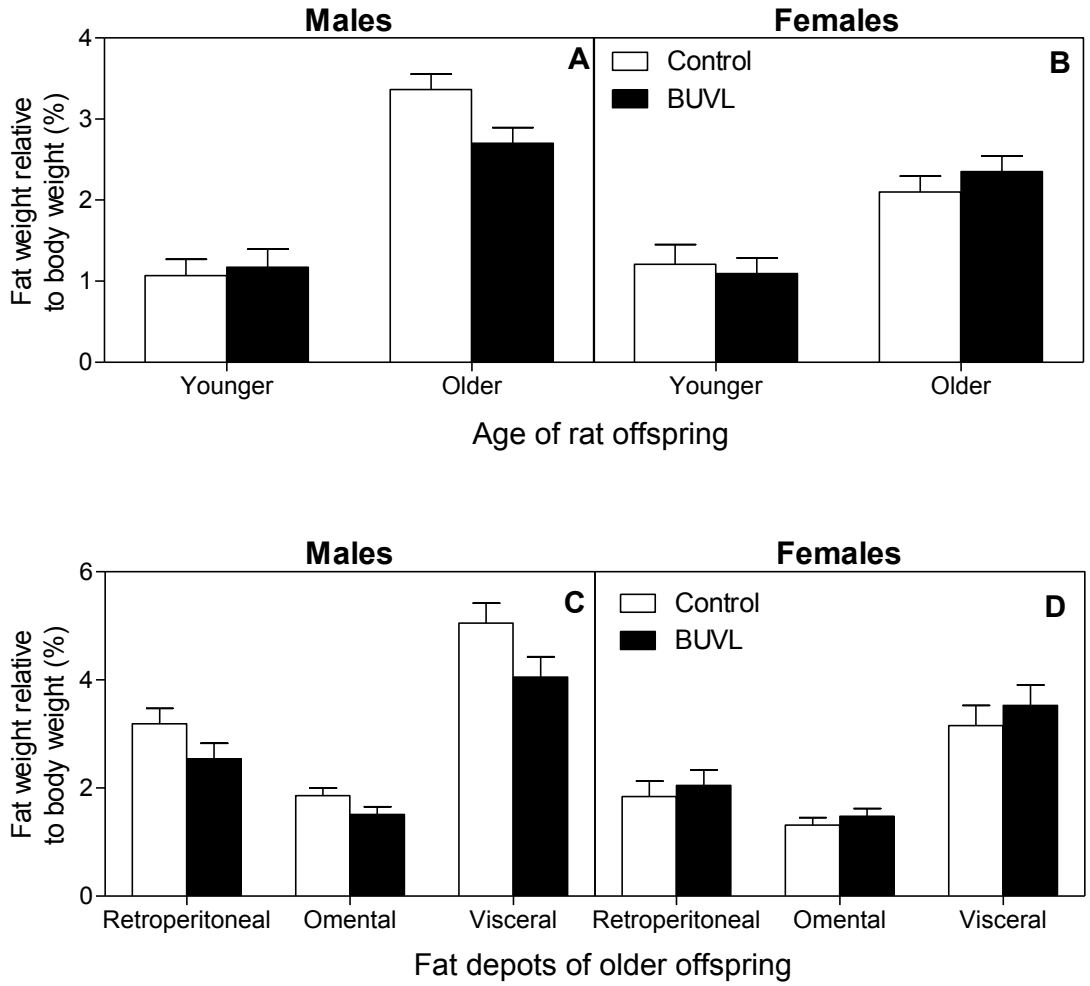
Male	Combined age			Young adult			Older adult		
	Control vs:		Sham vs	Control vs:		Sham vs	Control vs:		Sham vs
	BUVL	Sham	BUVL	BUVL	Sham	BUVL	BUVL	Sham	BUVL
Glucose tolerance									
Fasting plasma glucose (mmol.L^{-1})	↓	↓		↓	↓				
Maximum plasma glucose (mmol.L^{-1})									
ΔPlasma glucose (mmol.L^{-1})									
AUC glucose, 0-90min (mmol.L^{-1})									
Insulin secretion									
Fasting plasma insulin (µg.L^{-1})	↓	↓	↓	↓		↓	↓		
Maximum plasma insulin (µg.L^{-1})	↓	↓		↓	↓		↓		
ΔPlasma insulin (µg.L^{-1})	↓	↓		↓	↓		↓		↓
AUC insulin, 0-90min (µg.L^{-1})	↓	↓			↓		↓	↓	
Fasting insulin:glucose (µg.mmol^{-1})	↓		↓				↓		
AUC insulin:glucose (µg.mmol^{-1})	↓						↓		
Insulin sensitivity									
Basal plasma glucose (mmol.L^{-1})									
Minimum plasma glucose (mmol.L^{-1})									
Decrease plasma glucose (mmol.L^{-1})				↑					
AAC glucose, 0-60min (mmol.L^{-1})	↑			↑					
Insulin action									
Basal DI_{AAC}, 0-60min (µg.mmol.min.L^{-2})	↓			↓		↓			
Basal $DI_{\Delta glucose}$ (µg.mmol.min.L^{-2})									
Maximal DI_{AAC}, 0-60min (µg.mmol.min.L^{-2})									↓
Maximal $DI_{\Delta glucose}$ (µg.mmol.min.L^{-2})									

Supplement Table S3.15. Summary of specific effects of placental restriction on metabolic control and insulin action in female adult offspring

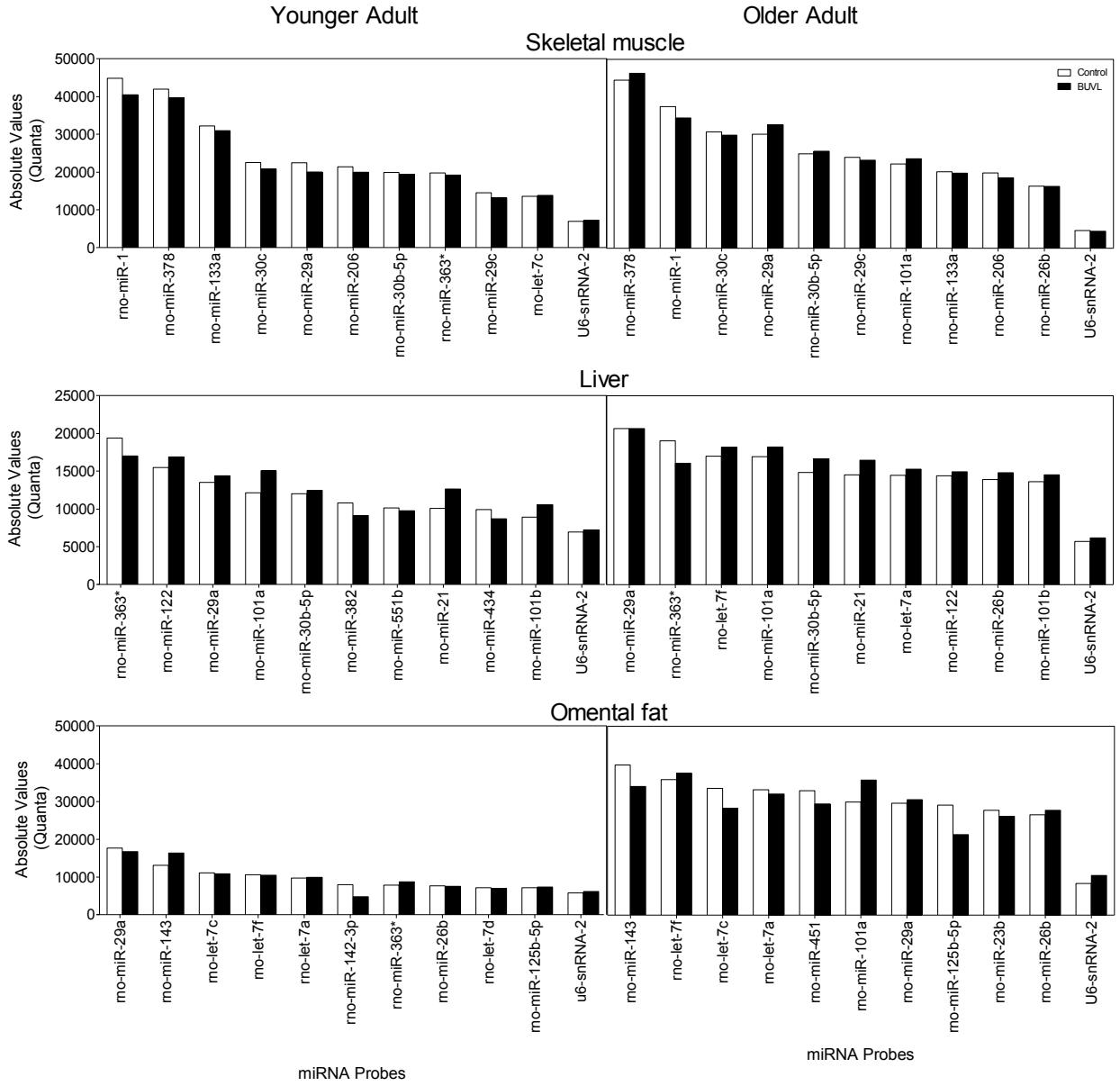
Female	Combined age			Young adult			Older adult		
	Control vs:		Sham vs	Control vs:		Sham vs	Control vs:		Sham vs
	BUVL	Sham	BUVL	BUVL	Sham	BUVL	BUVL	Sham	BUVL
Glucose tolerance									
Fasting plasma glucose (mmol.L^{-1})	↓	↓		↓	↓				
Maximum plasma glucose (mmol.L^{-1})									
ΔPlasma glucose (mmol.L^{-1})									
AUC glucose, 0-90min (mmol.L^{-1})								↓	
Insulin secretion									
Fasting plasma insulin (µg.L^{-1})	↓	↓	↓				↓		
Maximum plasma insulin (µg.L^{-1})	↓	↓		↓	↓		↓		
ΔPlasma insulin (µg.L^{-1})	↓	↓		↓	↓		↓		↓
AUC insulin, 0-90min (µg.L^{-1})	↓	↓			↓		↓	↓	
Fasting insulin:glucose (µg.mmol^{-1})	↓						↓		
AUC insulin:glucose (µg.mmol^{-1})	↓						↓		
Insulin sensitivity									
Basal plasma glucose (mmol.L^{-1})									
Minimum plasma glucose (mmol.L^{-1})	↓			↓					
Decrease plasma glucose (mmol.L^{-1})	↑			↑					
AAC glucose, 0-60min (mmol.L^{-1})	↑			↑					
Insulin action									
Basal DI_{AAC}, 0-60min (µg.mmol.min.L^{-2})	↓			↓					
Basal $DI_{\Delta glucose}$ (µg.mmol.min.L^{-2})									
Maximal DI_{AAC}, 0-60min (µg.mmol.min.L^{-2})									↓
Maximal $DI_{\Delta glucose}$ (µg.mmol.min.L^{-2})						↓			

Supplement Table S3.16. Fold change of miRNAs in younger adult offspring following bilateral uterine artery vessel ligation

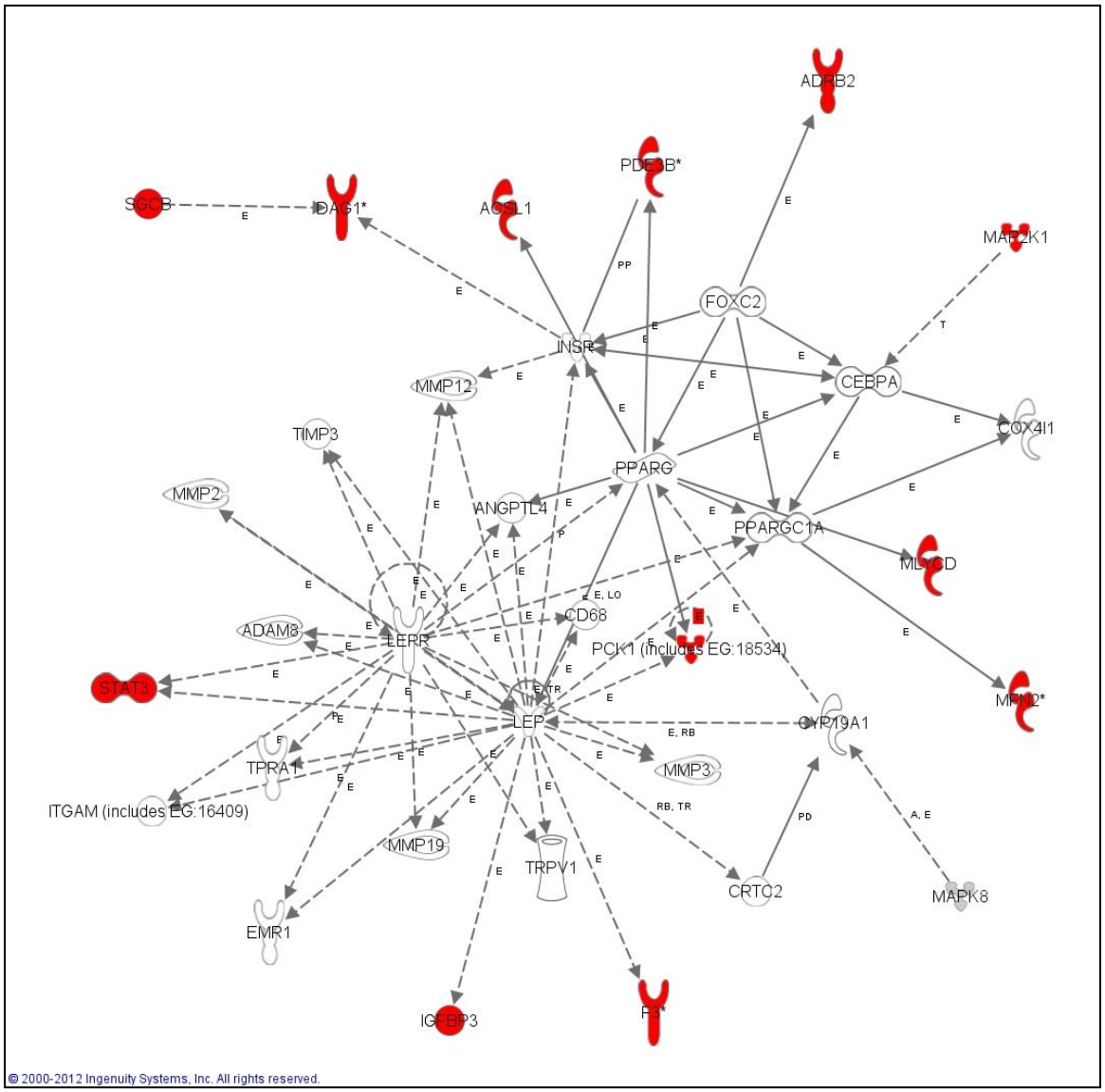
	miRNAs	Fold change
Liver		
Male	mmu-miR-199b*	-1.08
Female	rno-miR-126	1.09
	mmu-miR-199b*	1.28
Skeletal muscle		
Male	rno-miR-451	-1.11
Female	rno-miR-451	-1.08
Combined	rno-miR-451	-1.09
Omental Fat		
Combined	rno-miR-16	-1.09
	rno-miR-18a	1.02
	rno-miR-142-3p	-1.66
	rno-miR-19b	-1.05
	rno-miR-21	-1.02
	rno-miR-20b	-1.03
	hsa-miR-106a	-1.05
	mmu-miR-106a	-1.08



Supplement Figure S3.1. Comparison of relative adiposity relative to body weight BUVL altered overall relative adiposity measured in young and older rat offspring, in male (A) and female (B) offspring (repeated measures of age x surgical treatment x sex, $P = 0.056$). Effect of BUVL on individual fat depots relative to body weight in older adult offspring, in male (C) and female (D) offspring (repeated measures of surgical treatment sex x fat depots, $P = 0.075$). Control: white bar; BUVL: black bar.

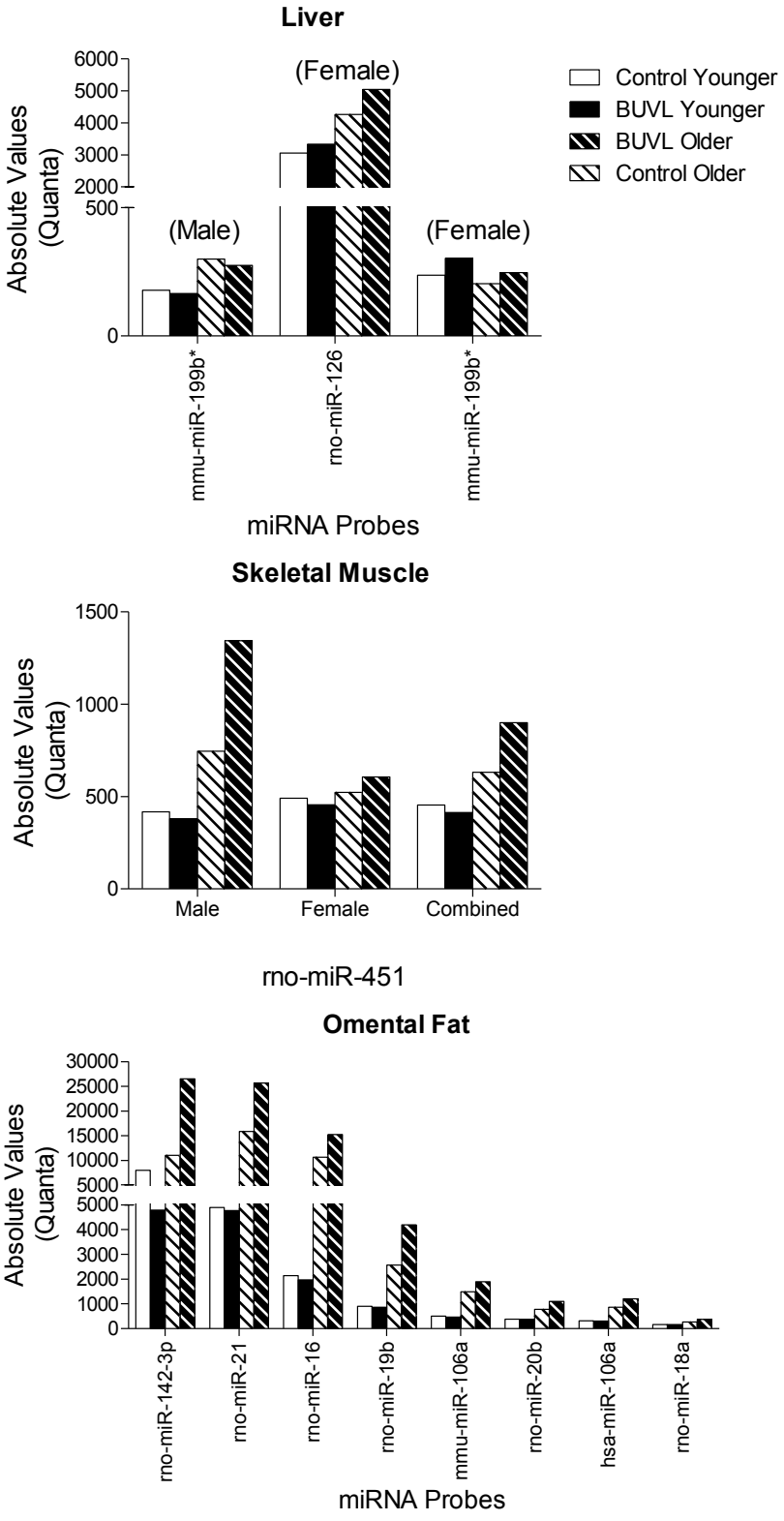


Supplement Figure S3.2. The ten most highly expressed miRNAs as in skeletal muscle, liver or omental fat of control and BUVL younger and older adult offspring
List of miRNAs were obtained based on the highest absolute values in control offspring, from each tissues. U6snRNA, ubiquitously expressed in any tissues, was included a positive control. Control: white bar; BUVL: black bar.



Associated Molecules		Score	Focus Molecules	Top Functions
Direct	Indirect			
ACSL1, ADRB2, DAG1, F3, IGFBP3, MAP2K1, MAPK8,MFN2, MLYCD, PCK1,PDE3B,SGCB, STAT3	ACSL1, ADRB2, ANGPTL4, CD68, CEBPA, COX4I1, CRTC2, CYP19A1, EMR1, FOXC2, INSR, ITGAM, LEP, LEPR, MMP2, MMP3, MMP12, MMP19, PPARG, PPARGC1A, TIMP3, TPRA1, TRPV1	12	13	Genetic Disorder, Nutritional Disease, Endocrine System Disorders

Supplement Figure S3.3. IPA network derived from the combined predicted targets of the six miRNAs differentially expressed in omental fat following bilateral uterine artery vessel ligation.



Supplement Figure S3.4. Effect of placental restriction on microRNA expression in insulin sensitive tissues in young and older adult offspring
Younger adult, Control: white bar, BUVL: black bar; Older adult, Control: white bar with dashed lines, BUVL: black bar with dashed lines.

Chapter 4

Effect of placental restriction on pancreatic microRNA expression in adult sheep offspring

4.1 Introduction

Individuals that undergo intrauterine growth restriction (IUGR) are more likely to develop insulin deficiency later in life (Jensen *et al.*, 2002), contributing to their increased risk of diabetes (Gatford *et al.*, 2010). Evidence in non-human species suggests that this development of glucose-stimulated insulin deficiency in the IUGR individual may be due to reduced pancreatic *β*-cell number, function or both (Simmons, 2007a; Gatford *et al.*, 2008; Park *et al.*, 2008). In adult IUGR sheep, impaired *β*-cell function is the major defect present (Gatford *et al.*, 2008), while in rodents, reduction in both *β*-cell number and function contribute to insulin deficiency in the IUGR adult (Simmons, 2007a; Park *et al.*, 2008). Reduced pancreatic or islet expression of several key regulatory and functional molecules following IUGR is implicated in this, but how such changes are induced and can be present long after the intrauterine insult is poorly understood. There is some evidence for altered epigenetic state, such as altered global and locus specific DNA methylation in various tissues in rodent IUGR offspring (MacLennan *et al.*, 2004; Park *et al.*, 2008), including pancreatic islets (Thompson *et al.*, 2010), providing a potential mechanism linking early exposures to later outcome. The latter study (Thompson *et al.*, 2010) implicates other candidates, possibly non-coding RNAs, in placental 'programming' of long term *β*-cell function, but this has not been investigated to date.

Low birth weight and IUGR are consistently associated with an increased risk of diabetes in adult life (Newsome *et al.*, 2003) with low birth weight accounting for approximately 18% of the population prevalence of type 2 diabetes (Eriksson, 2007). IUGR, is defined as failure to meet fetal growth potential, and commonly identified by being small for gestational age (SGA), a birth weight below the 10th percentile or less

than 2,500 grams at term (Kinzler & Kaminsky, 2007). In developed countries, placental insufficiency during pregnancy is a significant contributor to IUGR, which occurs in 3-7% of the total population (Romo *et al.*, 2009). Placental insufficiency is a common complication of pregnancy whereby placental delivery of oxygen and nutrient is inadequate for the needs of the developing fetus (Ergaz *et al.*, 2005; Baschat *et al.*, 2007), but can also have longer term consequences for offspring. Experimental placental restriction of fetal growth in the sheep (De Blasio *et al.*, 2007a) and rat (Simmons *et al.*, 2001; Wlodek *et al.*, 2005), impairs insulin secretion and sensitivity in offspring, which may lead to development of type 2 diabetes. In sheep, placental restriction induces age related loss of insulin secretion from fetal life onwards, eventually impairing insulin secretory function of the *β*-cell in young adult males (Gatford *et al.*, 2008). The latter results in impaired *in vivo* insulin secretion relative to sensitivity, despite increased *β*-cell mass (Gatford *et al.*, 2008). Furthermore, in the young IUGR lamb, pancreatic expression of L-type voltage-gated Ca^{2+} channel and of the Kir6.2 subunit of the K_{ATP}-channel is reduced, which may impair insulin secretion (Gatford *et al.*, 2008). These molecules and their associated pathways for Ca^{2+} and K^{+} flux that control insulin secretion (Zomer *et al.*, 2010) are novel candidates for PR-induced defects in insulin secretion. In fact mutations in either of K_{ATP}-channel subunits in human have been shown to increase diabetes risk (Flanagan *et al.*, 2009). However, how placental restriction alters the pancreatic expression of these genes long after birth is unclear.

In the rat, placental restriction reduces pancreas weight and *β*-cell mass (De Prins & Van Assche, 1982) and impairs *in vivo* glucose-dependent insulin secretion in juvenile offspring, before the onset of hyperglycaemia (Simmons *et al.*, 2001).

Placental restriction in the rat does not impair arginine-stimulated insulin secretion, suggesting a defect in glucose sensing in *β*-cells, in addition to their reduced *β*-cell mass (Simmons *et al.*, 2001). Placental restriction in the rat also reduces pancreatic islet *Pdx1* expression in the fetus, pre-weaning and in adult offspring (Stoffers *et al.*, 2003). Pdx1 is a duodenal homeobox 1 transcription factor that is required for pancreatic development and *β*-cell differentiation, and its reduced pancreatic expression in offspring following placental restriction may partly account for loss of *β*-cell function and mass. Placental restriction also alters expression of genes in pancreatic islets of the young rat, in association with altered methylation in CpG islands in their promoter regions (Thompson *et al.*, 2010). Specifically, placental restriction increased *Fgfr1* and reduced *Gch1, Pcsk5* and *Vgf* expression in pancreatic islets of the young rat (Thompson *et al.*, 2010). *Gch1*, GTP cyclohydrolase 1, has been directly implicated in diabetes-associated endothelial dysfunction through its role in nitric oxide synthesis (Meininger *et al.*, 2004). Down-regulation of *Gch1* has been proposed to play a role in the loss of *β*-cell function seen following placental restriction (Thompson *et al.*, 2010). Reduced islet *Vgf* expression may also contribute through reduced vascularisation, seen in some rodent models of IUGR (Boujendar *et al.*, 2003; Ham *et al.*, 2009). However, the mechanisms which are involved altered pancreatic expression of these key *β*-cell regulatory and functional genes in postnatal life following IUGR are still incompletely understood.

The altered fetal metabolic and endocrine environment of placental insufficiency appears to ‘program’ persistent alterations in body systems in postnatal life, via epigenetic and other pathways, leading to an increased risk of diseases such as diabetes in postnatal life, including the endocrine pancreas (Simmons, 2007b). DNA

methylation patterns influence gene expression and can persist through cell division. With DNA methyltransferase 1 (DNMT1) having a pivotal role in their maintenance and providing a mechanism for any changes persisting into postnatal life (Bird, 2002). Placental restriction in the rat alters hepatic one-carbon metabolism and global hepatic genomic DNA of cytosine methylation, at 21 days age in both male and female offspring (MacLennan *et al.*, 2004). In the rat, placental restriction affects global and locus specific DNA methylation in various tissues in the fetus or juvenile offspring after birth, in liver (MacLennan *et al.*, 2004), in lungs (Joss-Moore *et al.*, 2011) and in pancreatic islets (Park *et al.*, 2008). There is also increasing evidence for this in humans, with genome wide DNA methylation in cord blood cells varying with birth weight (Adkins *et al.*, 2012). In the rat, placental restriction induces hypermethylation of a CpG island located in the proximal promoter of Pdx1 locus in adulthood, resulting in permanent silencing of *Pdx1* expression (Park *et al.*, 2008). Furthermore, placental restriction altered genome wide DNA methylation in pancreatic islets of IUGR offspring at 7 weeks of age, mostly at CpG islands (25%), CG clusters (25%) and promoter regions (20%) (Thompson *et al.*, 2010). Altered pancreatic islet expression of several genes also correlated with altered methylation of intergenic sites upstream, with hypomethylation at *Fgfr1* and hypermethylation at *Gch1, Pcsk5* and *Vgf,* respectively (Thompson *et al.*, 2010). However, most differential methylation occurred in the intergenic regions (Thompson *et al.*, 2010), where many miRNAs genes are located, suggesting that miRNA expression and activity may also be affected in islets following placental restriction.

The miRNAs are non-coding regulatory RNAs that can inhibit translation or promote degradation of protein coding messenger RNA (mRNA). MiRNAs are short, single

stranded, non-coding RNAs, about 22 nucleotides in length. They usually bind to sites in the 3' untranslated region of the target mRNA, down regulating its abundance or at least its translation (Bushati & Cohen, 2007). Each miRNAs can target up to hundreds of genes, many is targeting common genes and forming regulatory networks. Placental programming of miRNA expression and activity may therefore affect such regulatory networks to affect function and phenotype in a co-ordinated and powerful fashion. Increasing evidence suggests that miRNAs are particularly susceptible to epigenetic regulation and potentially to early life programming via altered epigenetic state that may persist and impact long term (Chuang & Jones, 2007). MiRNAs genes can be located on introns of the host gene or on the intergenic regions. Expression of both, particularly intergenic miRNA genes are potentially regulated through methylation of CpG islands (Inouchi *et al.*, 2007), as miRNA genes contain more CpG content (47.3% on average), compared to the whole genome (41%), in humans (Saini *et al.*, 2007).

Recently, we have found that placental restriction of fetal growth in the sheep up-regulates the expression of several miRNAs in the liver, skeletal muscle and adipose tissue, that are predicted to target the abundance of various molecules involved in proximal insulin signalling and metabolic homeostasis in adult sheep and rat offspring (see Chapter 2 and 3). This is the first evidence to suggest that placental and IUGR programming of adult onset diseases or conditions, such as type 2 diabetes and insulin resistance are in part mediated by modulation of miRNA regulatory networks. To our knowledge, there has been no study thus far, examining pancreatic miRNA expression following placental restriction.

Because this question is difficult to directly and invasively study in humans, we have investigated the effects of placental restriction on insulin secretion and its underlying determinants from before birth and postnatally, in the sheep (De Blasio *et al.*, 2007a; Owens *et al.*, 2007a; Owens *et al.*, 2007b; De Blasio *et al.*, 2010). Placental restriction in the sheep induces a similar sequence of prenatal events and consequences to that seen in human placental insufficiency (Harding *et al.*, 1985; De Blasio *et al.*, 2007b). These include fetal growth restriction, fetal hypoglycaemia from mid-gestation, hypoxaemia, reduced plasma insulin (Owens *et al.*, 2007b), Insulin-like growth factors (Owens *et al.*, 1994), and increased plasma cortisol and catecholamine (Harding *et al.*, 1985; Forhead *et al.*, 2008). In the young adult IUGR sheep, we have reported evidence of reduced *β*-cell function and impaired *in vivo* insulin secretion in male offspring (Gatford *et al.*, 2008). This reduction of insulin secretion occurs despite increased *β*-cell mass, which is nevertheless insufficient to compensate for the loss of *β*-cell function and to therefore maintain overall insulin action in placentally restricted offspring (Gatford *et al.*, 2008). We propose that miRNA networks may play a role in the programming of insulin insufficiency in offspring born after IUGR. Therefore, we hypothesise that, placental restriction will alter pancreatic expression of miRNAs in adult sheep, which will be associated with impaired insulin secretory capacity and its determinants (Gatford *et al.*, 2008).

4.2 Materials and Methods

4.2.1 Restriction of placental and fetal growth

All animal experimental procedures are described in Section 2.2.1.

4.2.2 Measurement of insulin secretion in vivo

Insulin secretion was measured in 375 days of age in offspring, as described previously in Section 2.2.4. Briefly arterial blood was sampled (2 ml) 5, 3, and 0 min before the commencement of the intravenous glucose tolerance test (IVGTT) for the determination of fasting blood glucose and plasma insulin. A bolus of 0.25 g glucose (25% dextrose)/kg live weight was then infused intravenously over a 1 min period, and time zero was taken as the start of the bolus infusion. Arterial blood was sampled (2 ml) at 2, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, and 210 min after administration of glucose. Blood was centrifuged, and plasma collected for measurement of plasma glucose and insulin concentrations at each time point.

Plasma insulin concentrations were measured in duplicate using a commercially available radioimmunoassay kit (Phadeseph, Pharmacia-Upjohn Diagnostics AB, Sweden), as previously described in Section 2.2.4. Insulin secretion during the IVGTT was calculated as the area under the plasma insulin concentration curve versus time, and corrected for the plasma glucose area under the curve (refer to Chapter 2). Areas under the curve were determined using the Sigma Scan Pro v4 software package (Jandel Scientific Software, San Jose, CA, USA) (refer to Chapter 2).

4.2.3 Post-mortem of sheep offspring

Sheep offspring were humanely killed and tissues collected and processed at 18 months of age as described previously in Section 2.2.5.

Insulin immunostaining and morphomeric analysis of pancreatic sections to quantify *β*-cells and islets were as described in the previously published study (Gatford *et al.*, 2008).

4.2.4 Total RNA extraction

Total RNA was extracted from whole pancreatic tissue (~100 mg) collected at post-mortem using TRizol extraction methodology (Invitrogen, Australia), as described previously in Section 2.2.6.

4.2.5 Microarray hybridisation

The 6th Generation Exiqon miRCURY LNA ^{TM} microRNA Array (Exiqon, Denmark) was used for this analysis. This microarray contains more than 1,891 capture probes covering all human, mouse and rat microRNAs annotated in miRBase 16.0. Each probe was printed in duplicate and two spotted arrays were printed in one slide. Negative control probes were small-interfering RNA (siRNAs) of bacterial origin with no known homology to any mammalian species. Positive controls were twelve captured probes of small nuclear RNA, such as U6-snRNA, which is present on those mammalian species examined to date.

For this study, eight microarray replicates were used to compare control (n= 15; 8 males and 7 females) and placentally restricted (n= 10; 4 males and 6 females)

offspring, to have eight replicates, some duplications (1 control female, 4 placentally restricted males and 2 placentally restricted females) were used to enable comparison on a sex specific basis. Four dye swap experiments were performed so that half of the arrays were carried out using the opposite configuration. Microarray protocols were as previously described in Section 2.2.7 and 2.2.8.

Microarray raw data analysis was conducted as previously described in Section 2.2.8. Briefly, data was analysed by Benjamini-Hochberg multiple comparisons which identified significant differences in expression using an adjusted *P*-value (stringent). MiRNAs with an adjusted *P*-value < 0.05 were prioritised for further bioinformatic analysis. Secondly, data was analysed as described by Stekel (Stekel, 2003), which is based on modifying the unadjusted *P*-value as follows, the number of expected false positive results (in this study = 1) divided by the number of genes analysed on the array (2,454 genes) or 1/2,454 = 0.000407. Accordingly, any miRNA which had an unadjusted *P*-value less than 0.000407 was also prioritised for further analysis (Stekel, 2003; Herrera *et al.*, 2009). The top 20 miRNAs based on the smallest unadjusted *P*-value are listed in Supplement Table S4.1. Any miRNA whose expression was below the negative control threshold was not included for further analysis. This threshold was calculated based on the average expression value from 7 negative controls + (2 x standard deviation).

4.2.6 qRT-PCR analysis of microRNA expression

All the protocols were as previously described in Section 2.2.9. The endogenous control for normalization was selected using the NormFinder algorithm to pick the most stable genes, across treatment and sex (Andersen *et al.*, 2004). NormFinder

analysis showed that the combined average Ct values of miR-26b and miR-92 was the most stable, hence this was used as an endogenous control. Animal numbers: Control, n = 15 (8 males and 7 females) and placentally restricted, n= 10 (4 males and 6 females) offspring.

4.2.7 Identification of predicted targets of differentially expressed miRNAs

Identification of predicted targets was as previously described in Section 2.2.10.

A freezer malfunction resulted in a loss of RNA after the extraction had been performed and therefore subsequent analysis of gene expression of the predicted targets for any differentially expressed miRNA's was not possible.

4.2.8 Ingenuity Pathway Analysis

The protocol is described in Section 2.2.11. Ingenuity Pathway Analysis (IPA) criteria used to identify significant networks included use of mouse, human and rat data sources and restricted to data derived from pancreatic tissue and related cells or cell lines. Each network was limited to a maximum 35 molecules, to create a network which was closely regulated by the predicted targets of differentially expressed miRNAs. A score > 4 indicated that a network was significant. A score of 4 indicated that there was a 1 in 10,000 chance that the genes were related in a network due to random chance (Ingenuity, 2004). Permission to use figures and analyses performed by this software was obtained from Ingenuity Systems (Redwood City, CA, USA).

An additional exploratory analysis was undertaken of the top twenty differentially expressed miRNAs, analysed using unadjusted *P*-values. These miRNAs were directly entered into IPA with both targets and networks identified by using the IPA

Knowledge database. Criteria used to identify significant networks included use of mouse, human and rat data sources and no restricted to any tissue or cell type. Each network was limited to a maximum 35 molecules, to create a network which was predicted to be closely regulated by the predicted targets of differentially expressed miRNAs. A score > 4 indicated that a network was significant.

4.2.9 Statistical analyses

Data are expressed as mean ± SEM, unless otherwise stated. The effects of placental restriction, sex and their interaction on birth weight, adult weight and various measures of insulin abundance and production and pancreatic miRNA expression were assessed by two-way analysis of variance (ANOVA) (PASW v17 for Windows, Chicago, Il, USA). One-way ANOVA was used to analyse the effects of placental restriction on these in males and females separately. Linear correlations between miRNA expression and fasting plasma insulin and other indices of insulin secretion or their determinants were assessed by Pearson's correlation (PASW v17). Non-linear correlations between miRNA expression and indices of insulin secretion or determinants were also assessed with curve estimations (PASW v17).

4.3 Results

4.3.1 Effect of placental restriction on birth weight, adult body weight and insulin secretion and β-cell

The effect of placental restriction on fasting insulin, related indices and on insulin secretion during an *in vivo* glucose tolerance test (IVGTT) in adult offspring has been previously reported for the whole cohort (Owens *et al.*, 2007b). Here, a subset was studied, in which placental restriction reduced birth weight (-25.6%, P = 0.001), but did not alter adult body weight, insulin secretion or related parameters at one year of age (Table 4.1). However, in males, fasting plasma insulin positively correlated with birth weight (r = 0.536, P = 0.020, n = 12). Placental restriction did not alter any of *β*-cells mass and function (Table 4.1).

4.3.2 Highly expressed miRNA in the sheep pancreas

Microarray analysis detected 975 miRNAs in the sheep pancreas, with the top ten most highly expressed miRNAs in the sheep pancreas being of similar abundance in both control and placentally restricted offspring (Figure 4.1). Out of these, the top ten most highly expressed miRNAs, in the pancreas in both sexes, were similar: mmu-miR-1937a, hsa-miR-642b, hsa-miR-720, hsa-miR-1897-5p, hsa-miR-1260b, hsa-miR-1280, hsa-miR-659 and mmu-miR-667 (Figure 4.1). Rno-miR-294 was amongst the most highly expressed in male offspring only, whereas rno-miR-1196 was amongst the highly expressed in female offspring only (Figure 4.1). IPA analysis did not find any predicted targets of these highly expressed miRNAs, based on the pancreas database. None of the sequence of the top ten most highly expressed miRNAs, in the pancreas of the sheep detected here, are known as yet.

4.3.3 Effect of placental restriction on pancreatic miRNAs expression

Based on microarray analysis, placental restriction altered pancreatic expression of three miRNAs, two of which were in a sex specific manner (Table 4.2) in adult sheep. The selection of these miRNAs was based on the unadjusted *P*-value less than 0.000407. Placental restriction increased pancreatic hsa-miR-339-5p (Fold change = 1.61, unadjusted. *P* = 0.000026) in male offspring only. MiR-339-5p is identical in sequence in the sheep, human and rat (Supplement Table S4.2). PR reduced pancreatic rno-miR-331* (Fold change = -2.67, unadjusted. *P* = 0.000192) in female offspring only. MiR-331* is identical in sequence in the sheep, human and rat (Supplement Table S4.2). Placental restriction reduced pancreatic hsa-miR-513a-3p expression (Fold change = -1.23, unadjusted. *P* = 0.000124) in offspring overall. MiR-513a-3p has not yet been sequenced in the sheep. qPCR analysis did not confirm these outcomes for pancreatic miRNA expression as detected by microarray (Table 4.3).

The top 20 differentially expressed miRNAs in the pancreas, based on microarray, are listed on Supplement Table S4.1. These miRNAs are selected based on the ascending order on adjusted *P*-value, from smallest to largest. Only miR-134*, miR-185* and miR-92b sequence are known in the sheep, and their sequences are identical to both in humans and rats (Supplement Table S4.2).

4.3.4 Predicted target networks of differentially expressed miRNAs

There were no known predicted targets of rno-miR-331* currently available in miRecords target databases. This is due to rno-miR-331* belonging to the minor star

molecule class (miR*), members of which were initially thought to be produced at lower levels than their partner guide strands and were assumed to have no regulatory activity (Ambros, 2004). The partner guide strand is normally processed further into a mature miRNA strand. However, new evidence shows that miRNA* species could work with their partner guide strands in a synergistic manner and mediate regulatory activity in various settings (Ro *et al.*, 2007; Okamura *et al.*, 2008; Yang *et al.*, 2011). Of note, rno-miR-331* and mmu-miR-669e-5p have an identical seed region (rno-miR-331*: 5'-g**gucuug**uuuggguuuguu-3';
mmu-miR-669e-5p: 5'-u**gucuug**ugugugcauguucau-3'). The seed region includes the 5' bases 2-7 of the microRNA and this region determines its targets and is used to predict these in various algorithms (Bushati & Cohen, 2007). Therefore, mmu-miR-669e-5p was used to generate predicted targets for rno-miR-331*.

Based on information from the miRecords database, hsa-miR-339-5p, rno-miR-331* and hsa-miR-513a-3p were predicted to target 355, 52 and 1,217 molecules, respectively (Supplement Table S4.3, predicted target for hsa-miR-513a-3p is not listed, due to large number of targets). IPA identified one network from 13 target molecules of rno-miR-331*, whose expression was reduced in the pancreas of female offspring (Figure 4.2). This network is involved in cell death, carbohydrate metabolism and small molecule biochemistry (Table 4.4). Of note, based on the miRecords database, rno-miR-331* is predicted to directly target *Kcnj11*.

IPA identified two networks involving predicted targets of hsa-miR-513a-3p (Figure 4.3), while no network was identified for hsa-miR-339-5p (the complete lists of molecules can be found on Table 4.4). The first network of hsa-miR-513a-3p

(Network A), included some direct predicted targets that are involved in the insulin signalling pathway (*Insr* and *Irs2*) (Table 4.4). The second network of hsa-miR-513a-3p (Network B) included some molecules involved in insulin secretion (*Ins*, *Ins1* and *Pdx1*) that are indirectly regulated by this miRNA (Table 4.4). Based on the IPA knowledge database, these networks were involved in cell death, carbohydrate metabolism, small molecule biochemistry and endocrine system disorders, development and function (Table 4.4). Of note, one of the networks based on predicted targets of hsa-miR-513a-3p, includes as direct targets, insulin signalling molecules, such as insulin receptor, IRS2 and others implicated as modulating *β*-cell function, such as leptin and IGF1 (Table 4.4). Two of the direct predicted targets of hsa-miR-339-5p are *Plekhh1* and *Pak6*, which are involved in the development of *β*-cell mass and function.

An exploratory analysis of the top twenty differentially expressed miRNAs, as identified from unadjusted *P*-values, identified one network involving predicted targets of seven of these miRNAs (Figure 4.4). The seven miRNAs for which data existed in the IPA Knowledge database were: hsa-miR-4298, hsa-miR-182, hsa-miR-513a-3p, mmu-miR-384-5p, hsa-miR-891a, hsa-miR-92b and hsa-miR-1284. Only miR-92b sequence is known for sheep, and its sequence is identical to both in humans and rat (Supplement Table S4.2). Some of the predicted targets are *Igf1r* (insulin-like growth factor 1 receptor) and *Insl5* (insulin-like peptide 5) (Table 4.5). This network is involved in hereditary disorders, neurological disease and ophthalmic disease (Table 4.5).

4.3.5 Associations between insulin secretion, β-cell measures and miRNA expression

Pancreatic hsa-miR-339-5p correlates negatively with *β*-cell density (r = -0.687, *P* = 0.010, n = 11) in control offspring (Figure 4.5A). Pancreatic hsa-miR-339-5p also positively correlated with *β*-cell number in placentally restricted offspring (r = 0.531, *P* = 0.046, n = 11) (Figure 4.5B). Pancreatic rno-miR-331* did not correlate with any *β*-cell indices.

Pancreatic rno-miR-331* tended to correlate negatively with maximum plasma insulin during insulin secretion (r = -0.631, *P* = 0.069, n = 7) and the change between basal and maximum plasma insulin (ΔPlasma insulin) (r = -0.654, *P* = 0.506, n = 7), in male control offspring only (Figure 4.6A & B).

Fasting plasma insulin relative to glucose tended to correlate positively with pancreatic hsa-miR-339-5p and rno-miR-331* expression in female control offspring only (r = 0.639; *P* = 0.061; n = 7 and r = 0.557; *P*= 0.097; n = 7, respectively), but not in placentally restricted offspring (Figure 4.7A & B).

4.4 Discussion

This study has shown for the first time that placental restriction alters the pancreatic expression of several microRNAs in adult sheep offspring and in a sex specific manner. Moreover, pancreatic miRNAs affected by placental restriction in adult offspring are predicted to target pathways and networks that underpin *β*-cell function and mass and endocrine system development and function, consistent with a role in partially mediating the sex specific outcomes for insulin secretion in the young adult sheep. Furthermore, the pancreatic abundance of two miRNAs was associated with insulin secretion and *β*-cell mass, in manners consistent with their known actions in the endocrine pancreas.

The top ten most abundant miRNAs in the sheep pancreas have not predicted targets generated as yet in miRecords, and IPA had no information for any of these miRNAs from studies based on pancreatic tissue or related cell lines. Let-7b, miR-16, let-7a, let-7c, miR-26a and miR-26b were reported to be most abundant in the whole pancreas of human and rat (Szafranska *et al.*, 2007; Rosero *et al.*, 2010; Larsen *et al.*, 2011). Four of these miRNAs, let-7a, let-7c, miR-26a and miR-26b, were also detected in the sheep pancreas, but were not amongst the highly expressed (Figure 4.1). In the sheep, based on expression as measured by microarray, let-7b is ranked at 144 (out of 975 miRNAs) and mir-16 is ranked at 849, whereas let-7a, let-7c, miR-26a and miR-26b expression were below the negative control based threshold.

Our current study shows that pancreatic hsa-miR-339-5p expression was increased 1.61 fold in placentally restricted adult male offspring (Table 4.2). Of note, hsa-miR-339a-5p is located at the host gene of an uncharacterised protein coding gene in the

human (C7orf50), which currently has no known role and function. Pancreatic miR-339 has been shown to play important roles in development of the endocrine pancreas and in islet differentiation *in vitro* (Chen *et al.*, 2011). Comparison of the expression profiles between undifferentiated hES-T3 cells and differentiated embryonic bodies, found that miR-339, together with miR-186 and miR-199a, were highly expressed in pancreatic islet-like cell clusters (Chen *et al.*, 2011). *Plekhh1* and *Pak6* were identified as targets of hsa-miR-339-5p (Chen *et al.*, 2011) and predicted target analysis in this study (data not shown). PLEKHH1 protein is involved in intracellular signalling, where PAK6 protein interacts with androgen receptor. Down-regulation of the expression of these targets was necessary to maintain the development of the pancreas and/or differentiation of islets (Chen *et al.*, 2011).

Up-regulation of hsa-miR-339-5p would be expected to down-regulate these proteins, and therefore increase islet mass and/or *β*-cell numbers in placentally restricted offspring. For the latter statement, this study found a positive correlation between pancreatic hsa-miR-339-5p with *β*-cell number in placentally restricted offspring. This would indicate that hsa-miR-339-5p could play an important role for *β*-cell development in placentally restricted offspring. Our previous study showed that while placental restriction did not increase *β*-cell mass in adult male offspring, it did increase with decreasing birth weight, consistent with a possible role for hsa-miR-339-5p (Gatford *et al.*, 2008). This study found a positive correlation between pancreatic hsa-miR-339-5p with *β*-cell number in placentally restricted offspring

Placental restriction reduced pancreatic rno-miR-331* in female offspring only, which IPA analysis suggested is able to indirectly regulate *Pdx1* expression, by controlling

Cflar expression (Table 4.4). CASP8 and FADD-like apoptosis regulator (Cflar) protein have been reported to increase *Pdx1* mRNA in pancreatic *β*-cells and insulin production in the mouse (Schumann *et al.*, 2007). Therefore, in female placentally restricted offspring, the reduction of rno-miR-331* expression may increase *Cflar* expression, which consequently may increase *Pdx1* expression. Placental restriction increased the number of *β*-cells per islet in female offspring, but did not alter their *β*-cell mass (Gatford *et al.*, 2008). Placental restriction also reportedly tended to increase pancreatic *Pdx1* expression (Gatford *et al.*, 2008), which could be partly due the reduction of rno-miR-331* expression in female offspring found in this study. However, rno-miR-331* is also predicted to regulate *Srebf2* expression (Table 4.4). Srebf2 is a transcription factor which has a negative effect on *Pdx1* mRNA expression in the mouse (Ishikawa *et al.*, 2008). However, this study did not find any correlation between pancreatic rno-miR-331* expression with any *β*-cell indices. Further analysis of the expression of these miRNA targets and their relationship to that of rno-miR-331* may assess these possibilities.

Rno-miR-331* was also predicted to regulate *Kcnj11* expression, a major subunit of Kir6.2 of the pancreatic ATP-sensitive potassium (K ATP) channels. In the pancreatic *β*-cell, this channel regulates insulin secretion in response to increases in ATP concentration (Flechtner *et al.*, 2007). Direct manipulation of KCNJ11 protein expression in an insulin-secreting cell line improved insulin secretion (MacFarlane *et al.*, 1999). Thus, in adult female placentally restricted offspring, reduced pancreatic expression of rno-miR-331* may enhance insulin secretion from *β*-cells by up-regulating *Kcjn11* expression. In adult females, placental restriction did not alter insulin secretion overall or per *β*-cell mass, in contrast to male offspring (Gatford *et*

al., 2008). Therefore, altered rno-miR-331* expression may exert a protective effect to maintain pancreatic *β*-cell functions, in female offspring following placental restriction.

Placental restriction reduced pancreatic hsa-miR-513a-3p in both male and female adult sheep (Table 4.2). Hsa-miR-513a-3p was predicted to regulate insulin receptor and *Xbp1* gene expression (Table 4.4). Based on IPA, these two molecules also regulate *Pdx1* expression (Figure 4.3). Insulin plays a major role in transcription, translation, ion flux, proliferation and survival of *β*-cell (Leibiger *et al.*, 2008), besides regulating *Pdx1* expression (da Silva Xavier *et al.*, 2000). In contrast, XBP1 protein decreases expression of rat *Pdx1* mRNA in male *β*-cells (Allagnat *et al.*, 2010). Therefore, the outcomes of altered hsa-miR-513a-3p expression in the pancreas of placentally restricted males and females for *β*-cell survival is unclear, as the reduced expression of hsa-miR-513a-3p would indirectly increases *Pdx1* expression, through increased insulin signalling. But at the same time, reduced hsa-miR-513a-3p expression would increase *Xbp1* expression which would suppress *Pdx1* expression. Future studies should investigate *Pdx1* expression in *β*-cells to confirm the relationship of hsa-miR-513a-3p expression to *Pdx1* and other targets, and to *β*-cells mass and function in placentally restricted offspring.

The down-regulation of both rno-miR-331* and hsa-miR-513a-3p in female of placentally restricted offspring would be expected to indirectly up-regulate *Pdx1* expression (Table 4.4). While placental restriction did not alter *β*-cell mass in female offspring, it did increase the number of *β*-cells per islet (Gatford *et al.*, 2008). IPA indicates that both of the two miRNAs exhibiting reduced pancreatic expression

following placental restriction in female offspring, rno-miR-331* and hsa-miR-513a-3p, regulate networks that are involved in carbohydrate metabolism, small molecule biochemistry and endocrine system development and function. This suggests that reduced pancreatic expression of these two miRNAs would have a significant positive impact on pancreatic development and function in female offspring, via multiple pathways and may help explain the sex differences in the latter following placental restriction. However, since this study did not find any correlation between pancreatic rno-miR-331* expression with any *β*-cell indices, would indicate that rno-miR-331* has no role in the development of *β*-cell. As miRNAs may be amongst those particularly susceptible to early life programming through altered DNA methylation through one-carbon metabolism pathway in the liver of 21 days age in both male and female placentally restricted rat offspring (MacLennan *et al.*, 2004). Thus the genes for rno-miR-331* and hsa-miR-513a-3p are located in intergenic regions, in both the rat and human, where most differential methylation occurred in pancreatic islets of rat offspring following placental restriction (Thompson *et al.*, 2010). Of note, in female control offspring, rno-miR-331* expression is correlated positively with fasting plasma insulin relative to glucose. This at least suggests a potential causal relationship in the sheep and one that is abolished by placental restriction. However, due to lack of power in the current study, further investigation with increased numbers of sheep is required to determine the impact of placental restriction on this association. One potential mechanism by which placental restriction may change miRNA expression long term is by altering methylation at or near miRNA genes.

Sex differences in outcomes of early life programming have been reported for a range of body systems and functions (Wells, 2007; Wells *et al.*, 2007). The origins of

this may include sex differences in susceptibility to the initiating insults *in utero*, with females and males differing in glucocorticoid sensitivity for example (Stark *et al.*, 2011). In addition, actions of sex hormones and sex-linked chromosomes may play a role (Gabory *et al.*, 2011). Sex specific differences in programmed outcomes may also arise from other sex differences postnatally, with hepatic miR-29b being highly expressed in female but not male rats, for example (Cheung *et al.*, 2009). Overall, the sex differences in pancreatic miRNA expression reported here in response to placental restriction, may be one of the factors that contributes to the sex differences in the responses of glucose-stimulated insulin production and *β*-cell mass.

In an exploratory study of the top twenty differentially expressed miRNAs, several were predicted to regulate *Igf1r* and *Insl5* (Table 4.5). Insulin-like 5, Insl5, was also predicted to be affected by hsa-miR-182 (increased pancreatic expression) and hsa-miR-513-3p (reduced pancreatic expression) (Figure 4.4). The insulin-like growth factor type 1 receptor, *Igf1r*, was predicted to be regulated by both up-regulated (hsa-miR-4298 and hsa-miR-182) and down-regulated (hsa-miR-513a-3p and hsa-miR-384-3p) miRNAs following placental restriction. Both *Igf1r* and *Insl5* play an important role in *β*-cell differentiation and proliferation (Cornu *et al.*, 2010; Burnicka-Turek *et al.*, 2012). Further study of increased numbers of sheep and independent measurement of expression of these miRNAs and their predicted targets is required to assess the impact if any of their putative altered pancreatic expression.

The current study has a number of limitations, in addition to the loss of RNA which precluded analysis of target expression. This study analysed miRNA expression in the whole pancreas, which consists of the exocrine and paracrine pancreas. The

exocrine pancreas consists of two main cell types that secrete bicarbonate ions and various digestive enzymes. Only about 2% is the endocrine pancreas (Silverthorn, 2004), which consists of groups of cells known as the islets of Langerhans. These islets are comprised of *α*-cells (secrete glucagon), *β*-cells (synthesise and secrete insulin) and *Δ*-cells (secrete somatostatin) (Silverthorn, 2004). Future studies should focus on isolating the islets and *β*-cells in adult offspring to specifically analyse their miRNA and target expression in response to placental restriction.

In addition, there is limited genomic and miRNA sequence information available for the sheep to date, with only 9,407 coding RNA transcript and 193 miRNAs have been sequenced (Cox *et al.*, 2012). The differentially expressed miRNAs in pancreatic sheep, miR-339-5p and miR-331*, do have identical sequences to those in human and rat however (Caiment *et al.*, 2010) (Supplement Table S4.2). Numerous microRNA genes are recognised to be phylogenetically conserved through mammalian genomes (Ambros, 2004) and to play similar roles in animal development (Wienholds & Plasterk, 2005). Several pancreatic miRNAs are known for their roles in development and function of the pancreas and endocrine pancreas, in rodents (Table 1.3). At least six miRNAs (miR-9, miR-24, miR-26, miR-182, miR-148 & miR-124a2) have been shown to modulate *β*-cell function or mass (Table 1.3). However, only pancreatic miR-24, miR-26 and miR-148 were detected as highly expressed miRNAs in the whole pancreas in humans (Table 1.4).

In the sheep, only pancreatic miR-24 and miR-148a were detected here in the whole sheep pancreas, although they were not the highly expressed miRNAs (expression rank no. 507 and 257, respectively). Many of the highly expressed miRNAs observed

from this study, such as mmu-miR-1937a, hsa-miR-720 and hsa-miR-642b have as yet undefined roles in biological processes and they have not been sequenced yet. These microRNAs could be novel pancreatic miRNA markers or only specific to the sheep pancreas. The most highly expressed pancreatic miRNAs in sheep found here differ from the twenty most highly expressed miRNAs in whole pancreas of humans (Szafranska *et al.*, 2007; Rosero *et al.*, 2010) and rats (Larsen *et al.*, 2011) (Table 1.4). The differences in pancreatic miRNA pattern between sheep and other mammalian species could be due in part to the ruminant physiology and associated digestion of sheep.

The origins of the altered pancreatic expression of miRNAs following placental restriction in the current study are unknown. A range of indirect evidence implicates epigenetic mechanisms including DNA methylation in this, but direct evidence is required to assess the extent of their involvement. Expression of miR-331 is regulated by p53, as miR-331 was increased in embryo of p53 -/- mice (Hosako *et al.*, 2009). p53 protein binds to DNA and regulates expression of several genes, which are involved in DNA repair and apoptosis (May & May, 1999). MiR-331 expression is also regulated by HMGA protein (High Mobility Group A), as miR-331 expression was reduced in embryonic fibroblasts isolated from *Hmga1* -/- mice (De Martino *et al.*, 2009). HMGA protein plays a key role in gene expression, by enhancing or suppressing of many transcriptional factors (Reeves & Nissen, 1990). The outcomes of both of these studies emphasise the overall role of miR-331 family, including rno-miR-331*, in regulating cell proliferation. To date, there is no study investigating HMGA protein expression in the placentally restricted offspring, nor how expression of hsa-miR-513 or hsa-miR-339a-3p expression is regulated.

4.5 Conclusion

In summary, this pilot study has shown that placental restriction alters microRNA expression in the pancreas of adult sheep offspring and in a sex specific manner. Furthermore, the predicted molecular and functional targets of these differentially expressed miRNAs and imputed outcomes following placental restriction mirror the previously reported sex differences in *β*-cell insulin secretory function and mass in the adult placentally restricted sheep. We found that increased hsa-miR-339-5p expression would be expected to increase islet mass and/or *β*-cell numbers in placentally restricted male offspring, consistent with the increases seen in these measures with decreasing birthweight. Down-regulation of both rno-miR-331* and hsa-miR-513a-3p in female placentally restricted offspring would be expected to indirectly up-regulate *Pdx1* expression, and potentially contribute to the increased number of *β*-cells per islet they exhibit. Overall these findings provide the rationale for further studies of the effect of placental restriction on the molecular profile of miRNAs and their predicted target in islets and *β*-cells. As well as, subsequent analysis of miRNAs expression in epigenetic state, including DNA methylation.

4.6 Tables and Figures

Table 4.1. Effects of placental restriction on growth and *in vivo* insulin and β-cell morphometry in adult sheep offspring

	Males		Females		*P* -value		
	Control (n: 8)	PR (n: 4)	Control (7)	PR (6)	PR	S	PR x S
Birth weight (kg)	5.64 ± 0.4	4.49 ± 0.1	5.18 ± 0.1	3.56 ± 0.47	0.001	0.074	ns
Adult body weight (kg)	46.66 ± 3.8	50.7 ± 3.2	38 ± 1.9	38.40 ± 2.51	ns	0.002	ns
Fasting insulin (mU/l)	10.57 ± 2.6	9.8 ± 2.1	5.96 ± 0.4	8.32 ± 0.78	ns	ns	ns
Maximum insulin (mU/l)	44.59 ± 6.4	61.8 ± 9.1	43.1 ± 4.8	42 ± 10.1	ns	ns	ns
ΔInsulin (mU/l)	34.02 ± 6.1	52 ± 7.8	37.1 ± 4.7	33.7 ± 10.4	ns	ns	ns
AUC insulin, 0-120 min (mU/l)	1306 ± 217	1654 ± 175	1114 ± 156	890 ± 277	ns	0.047	ns
Fasting insulin:glucose (mU/mmol)	2.82 ± 0.7	2.44 ± 0.5	1.62 ± 0.1	2.31 ± 0.19	ns	ns	ns
AUC insulin:glucose (mU/mmol)	6.36 ± 1.2	8.25 ± 2.1	5.32 ± 0.9	4.58 ± 1.25	ns	0.091	ns
β-cell number	322 ± 72	303 ± 58	201 ± 55	347 ± 32	ns	ns	ns
β-cell mass (g)	1.53 ± 0.47	1.22 ± 0.49	1.00 ± 0.32	1.15 ± 0.30	ns	ns	ns
β-cell mass relative to pancreas (%)	2.33 ± 0.68	1.65 ± 0.64	1.69 ± 0.49	2.04 ± 0.44	ns	ns	ns
β-cell density	10.5 ± 1.5	9.7 ± 1.1	10.0 ± 2.0	10.4 ± 1.3	ns	ns	ns
Basal insulin relative to β-cell mass	11.7 ± 5.4	7.2 ± 3.9	10.4 ± 2.2	17.7 ± 10.4	ns	ns	ns
Maximum insulin relative to β-cell mass	39 ± 22	42 ± 17	73 ± 17	28 ± 8	ns	ns	ns
Insulin secretion relative to β-cell mass	5.0 ± 3.4	4.7 ± 1.9	9.5 ± 2.8	8.1 ± 4.8	ns	ns	ns

Values are means ± SEM; n, number of animals; PR: placental restriction (treatment); S: sex;. The effect of PR and sex and their interaction on plasma insulin were assessed by two-way analysis of variance (ANOVA) (PASW v17), significance was $P < 0.05$, ns: not significant.

Table 4.2. Microarray analysis on the effect of placental restriction on pancreatic miRNA expression in adult sheep offspring

	microRNA	Microarray			Accession number	Chr.	CpG Island
		Fold Change	unadj. *P*-value	adj. *P*-value			
Male	hsa-miR-339-5p	1.61	0.000026	0.06	MIMAT0000764	7	Present
Female	rno-miR-331*	-2.67	0.000192	0.31	MIMAT0017033	7	-
Combined	hsa-miR-513a-3p	-1.23	0.000124	0.31	MIMAT0004777	X	-

MiRNAs significantly differentially expressed determined by LIMMA analysis (adjusted $P < 0.05$ and unadjusted $P < 0.000407$). miRNAs are listed with rat and human miRBase accession numbers and chromosomal location. rno: *Rattus norvegicus*; hsa: *Homo sapiens*. For hsa-miR-339-5p, CpG island was detected between 10 kb up and down stream of miRNA gene location, based on UCSC genome browser database (accessed on November 2010).

Table 4.3. qPCR analysis on the effect of placental restriction on pancreatic miRNA expression in adult sheep offspring

Linearised (qPCR)	Male						Fold Change	Female						Fold Change	2-way ANOVA		
	Control (8)			PR (4)				Control (7)			PR (6)				PR	S	PR x S
hsa-miR-339-5p	0.030	±	0.003	0.035	±	0.007	1.11	0.035	±	0.006	0.044	±	0.011	1.14	ns	ns	ns
rno-miR-331*	0.077	±	0.0123	0.071	±	0.009	-1.05	0.107	±	0.012	0.095	±	0.014	-1.15	ns	ns	ns
hsa-miR-513a-3p	*nd*			*nd*				*nd*			*nd*						

hsa: *Homo sapiens*; rno: *Rattus novergicus*; Unadj. *P*-value: un-adjusted *P*-value; Adj. *P*-value: adjusted *P*-value. The effects of placental restriction and sex and their interaction on pancreatic miRNA expression were assessed by two-way analysis of variance (ANOVA). For qPCR, miRNAs expression in the pancreas were normalised against average ΔCt of miR-26b and miR-92. Data presented as mean ± SEM. nd: not detected by qPCR.

Table 4.4. Networks and predicted molecules for pancreatic miRNAs differentially expressed in adult sheep offspring following placental restriction

microRNA	Network	Molecules in Network		Score	Focus Molecules	Top Functions
		Predicted by miRNAs	Associated molecules			
mmu-miR-669e-5p **or** rno-miR-331*	A	ACACA, BCL2, CASP9, CFLAR, CTNNB1, ERO1LB, FASN, KCNJ11, MAFB, MAPK14, PRKCB, SFRP1, SREBF2	ABCC8, CASP3, FAS, FOXA2, GCG, GCK, IFNG, IGF1, IL1RN, Ins1, INS, LEP, MYC, PDX1, SLC2A1, SLC2A2, TNF, VIP	3	13	Cell death, Carbohydrate metabolism, Small molecule biochemistry
hsa-miR-513a-3p	A	CTNNB1, IGF1, INSR, IRS2, LEP, LEPR, MAPK14, NR5A2, PPARA, RPS6KB1, SEL1L, SFRP1, SREBF1, SSTR1, STAT3	GCG, GCK, GFPT1, HGF, HNF1A, IFNG, Ins1, ONECUT1, PDX1, SLC2A2, SREBF2, VIP	6	16	Carbohydrate metabolism, Small molecule biochemistry, Endocrine system disorders
	B	BTC, INHBA, ISL1, KLF11, MAF, MRAS,PAX6, PCSK2, PLD1, SLC11A2, XBP1	FOXA2, GCG, IFNG, INS, Ins1, MYC, NEUROG3, PAX4, PCSK1, Pdx1, SLC2A1, SLC2A2, SOD2	3	11	Carbohydrate metabolism, Cell death, Endocrine system development and function

Expression of rno-miR-331* was reduced in placentally restricted female offspring. Expression of hsa-miR-513a-3p was reduced in placentally restricted combined offspring.

Table 4.5. Network and predicted molecules based on common predicted targets of seven pancreatic miRNAs differentially expressed in adult sheep offspring following placental restriction

microRNA	Molecules in network	Score	Top Functions
hsa-miR-4298 hsa-miR-182 hsa-miR-513a-3p hsa-miR-384-5p hsa-miR-819a hsa-miR-92b hsa-miR-1284	CABS1, CCDC18, CNR1, FAM24A, FFAR2, GPR19, GPR22, GPR52, GPR108, GPR110, GPR112, GPR125, GPR135, GPR137C, GPR37L1 HCRTR2, IGF1R, INSL5, LIAS, LMBR1L, OPN1LW OPN1MW/OPN1MW2, P2RY10, PLEKHG3 SF3B5, STOX2, TARBP1, TCTE3	16	Hereditary disorder, Neurological disease, Ophthalmic disease

These differentially expressed miRNAs are for combined offspring, and based on the seven out of the twenty that were in the IPA database.

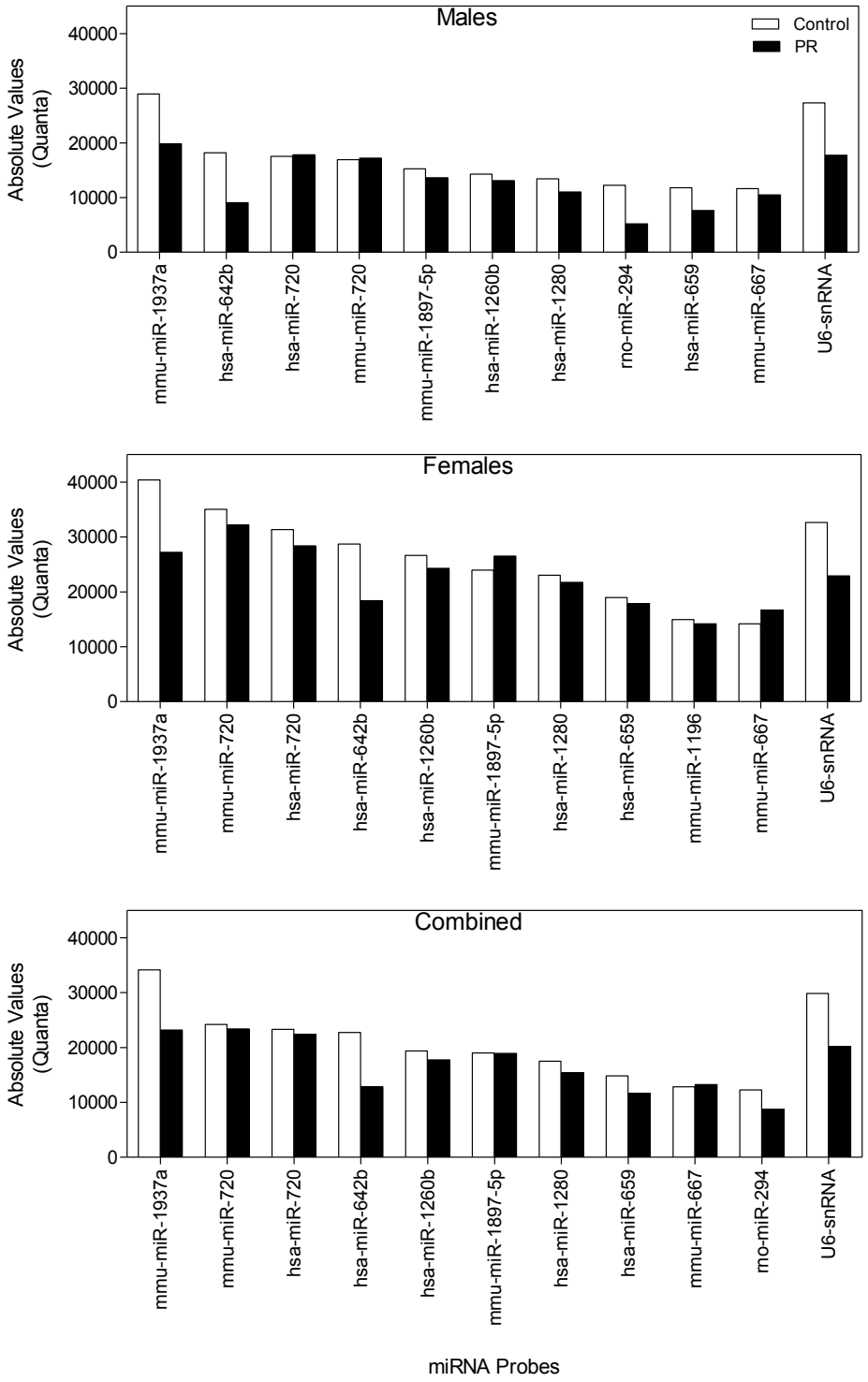


Figure 4.1. The top ten most highly expressed microRNAs in the adult sheep pancreas

These highly expressed miRNAs were selected based on the highest expression value on the microarray. U6-snRNA is ubiquitously expressed in all tissues and is a small non coding nuclear RNA component of an RNA-protein complex in the spliceosome.

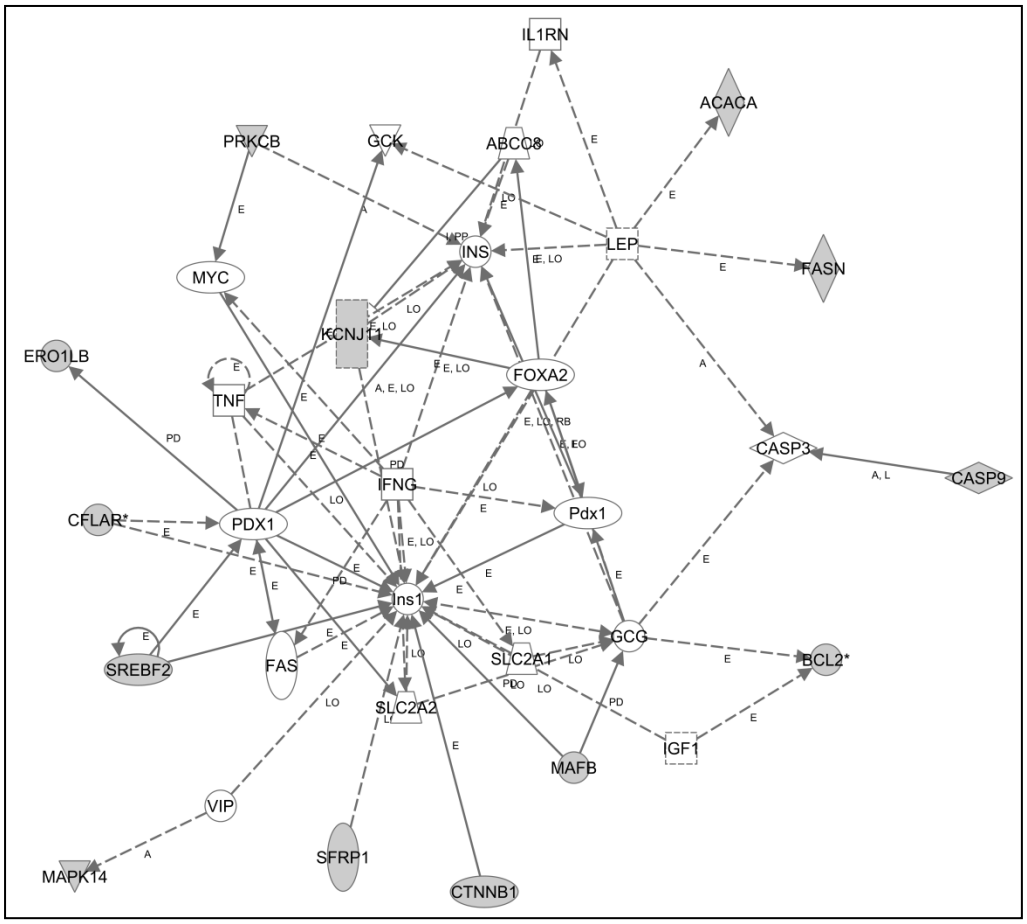


Figure 4.2. Network A was predicted to be targeted by rno-miR-331*

Pancreatic rno-miR-331* (homologous to with human and rat miR-331*) is down-regulated in the adult female sheep following placental restriction. The direct targets (highlighted in grey) include the potassium channel (KCJN11), which plays a role in insulin secretion. The network (score = 3, focus molecules = 13) generated by Ingenuity Pathway Analysis, based on the pancreas database only, limited to maximum 35 molecules and score > 4 (Table 4.4). The functions of this network are in cell death, carbohydrate metabolism and small molecule biochemistry. Straight lines: direct interaction; Dotted lines: indirect interaction; PD: protein-DNA binding; PP: protein-protein binding; E: expression; LO: localisation; RB: regulation of binding; L: proteolysis; A: activation; P: phosphorylation; T: transcription.

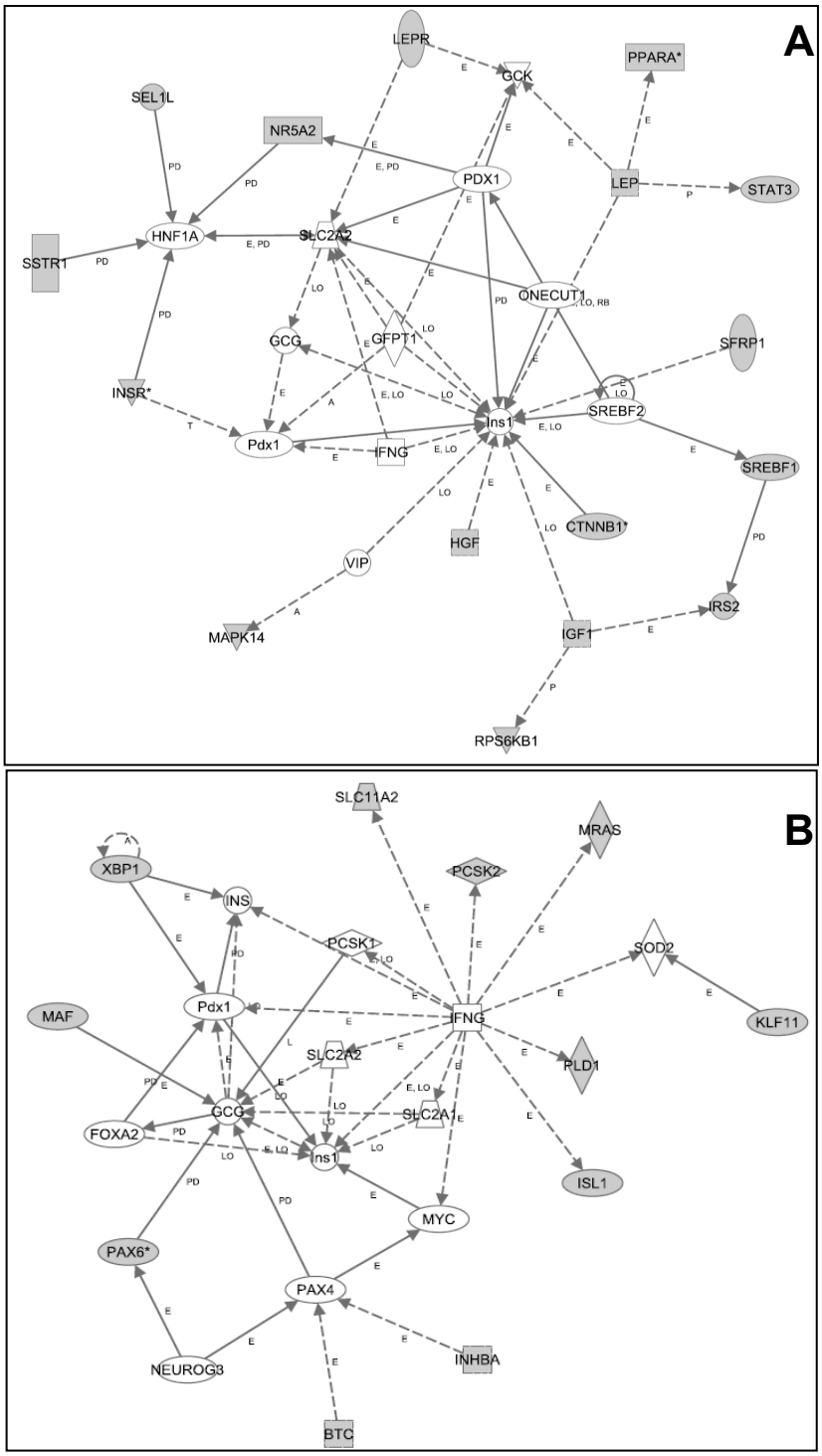


Figure 4.3. Network A and B were predicted to be targeted by hsa-miR-513a-3p

Pancreatic hsa-miR-513a-3p is down-regulated in the adult sheep following placental restriction. Network A (score = 6, focus molecules = 16) involves in carbohydrate metabolism and includes direct targets (highlighted in grey), such as IGF1, which indirectly regulates insulin secretion. Network B (score = 3, focus molecules = 11) involves in carbohydrate metabolism and endocrine system development and function, and includes XBP1, which directly regulates Pdx1 expression (Table 4.4). *See Figure 4.2 for legends.*

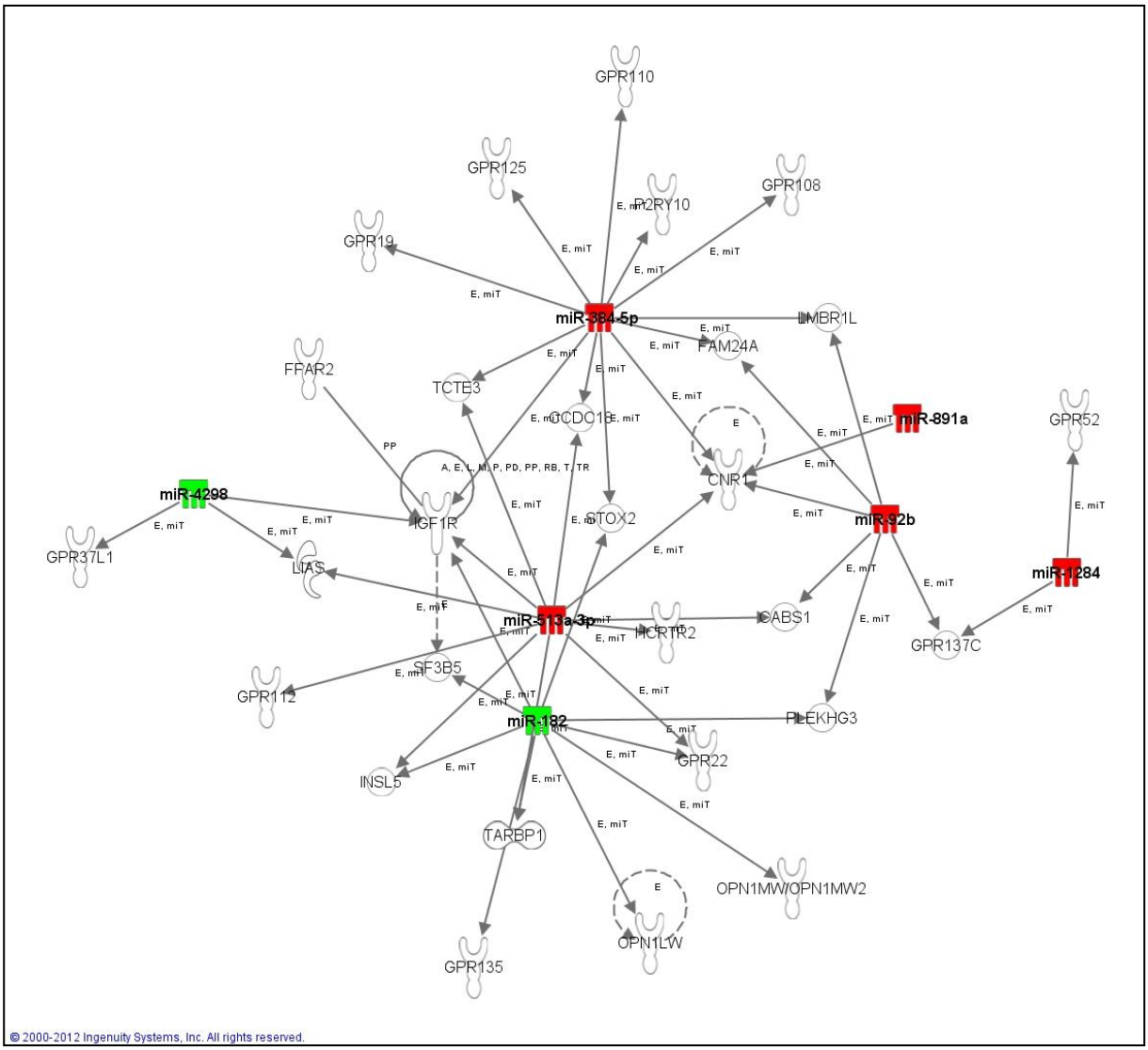


Figure 4.4. Network based on common targets of seven of the top twenty of differentially expressed miRNAs in pancreas of adult sheep offspring following placental restriction

Seven of the top 20 differentially expressed miRNAs in pancreas of adult offspring had predicted targets in the IPA database and with their targets formed a significant network (score = 16, focus molecule 7). The seven miRNAs are: hsa-miR-4298 (up), hsa-miR-182 (up), hsa-miR-513a-3p (down), mmu-miR-384-5p (down), hsa-miR-891a (down), hsa-miR-92b[#] (down) and hsa-miR-1284 (down). [#]: homologous with both human and rat miRNA sequence. Green indicates an up-regulated miRNA following PR and red indicates a down-regulated miRNA following placental restriction. In this network, *Igf1r* expression is predicted to be directly regulated by hsa-miR-4298, hsa-miR-182, hsa-miR-513a-3p and hsa-miR-384-3p. A list of the targets can be found in Table 4.5.

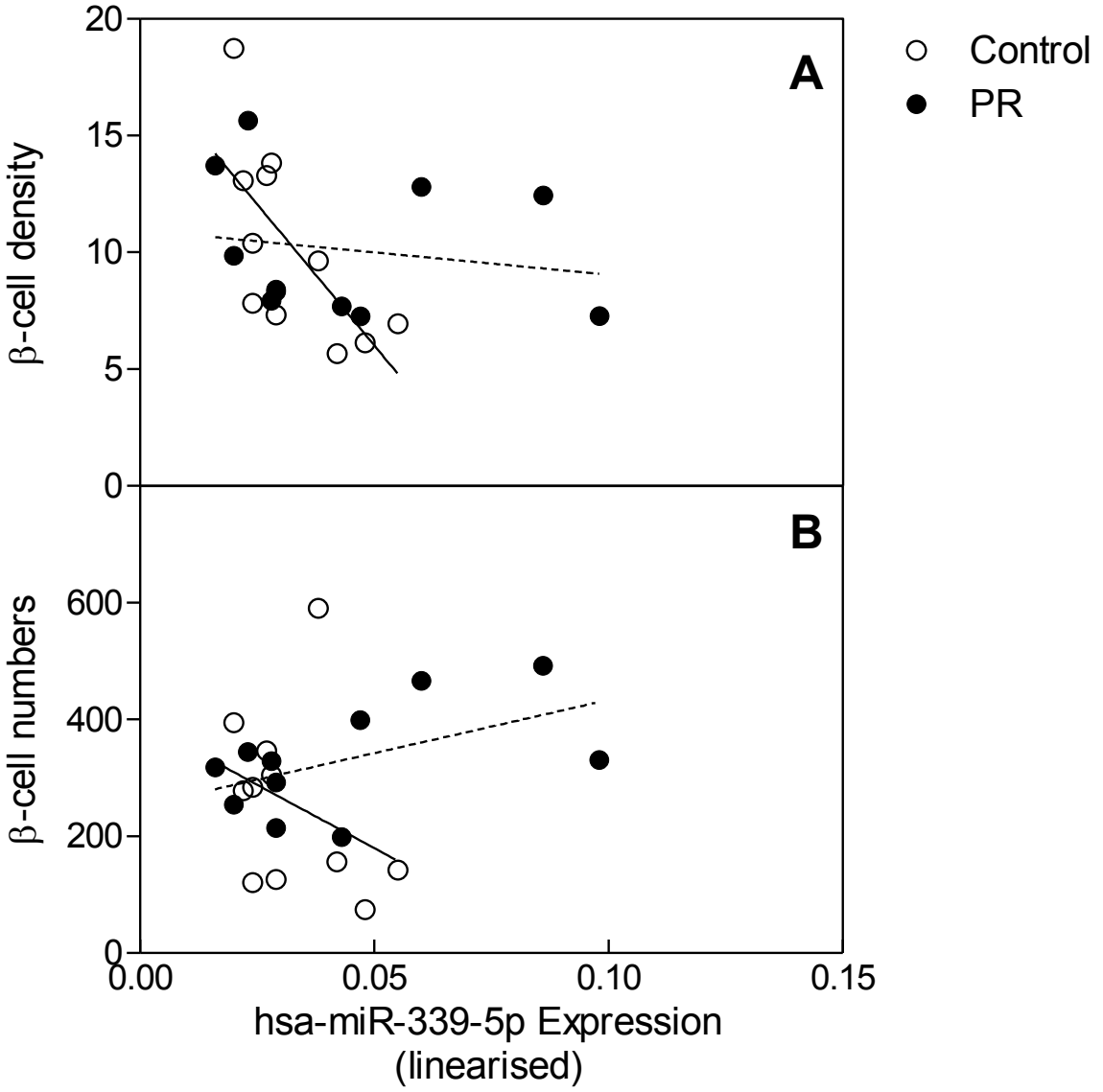


Figure 4.5. Associations between *β*-cell volume density and number and pancreatic expression of differentially expressed miRNAs in adult sheep offspring

(A) Pancreatic hsa-miR-339-5p correlates negatively with *β*-cell density in control offspring (r = -0.687, *P* = 0.010, n = 11), but not in placentally restricted offspring (r = -0.174, *P* = 0.304, n = 11). (B) Pancreatic hsa-miR-339-5p correlates positively with *β*-cell numbers in placentally restricted offspring (r = 0.531, *P* = 0.046, n = 11), but not in control offspring (r = -0.328, *P* = 0.163, n = 11). PR = placentally restricted.

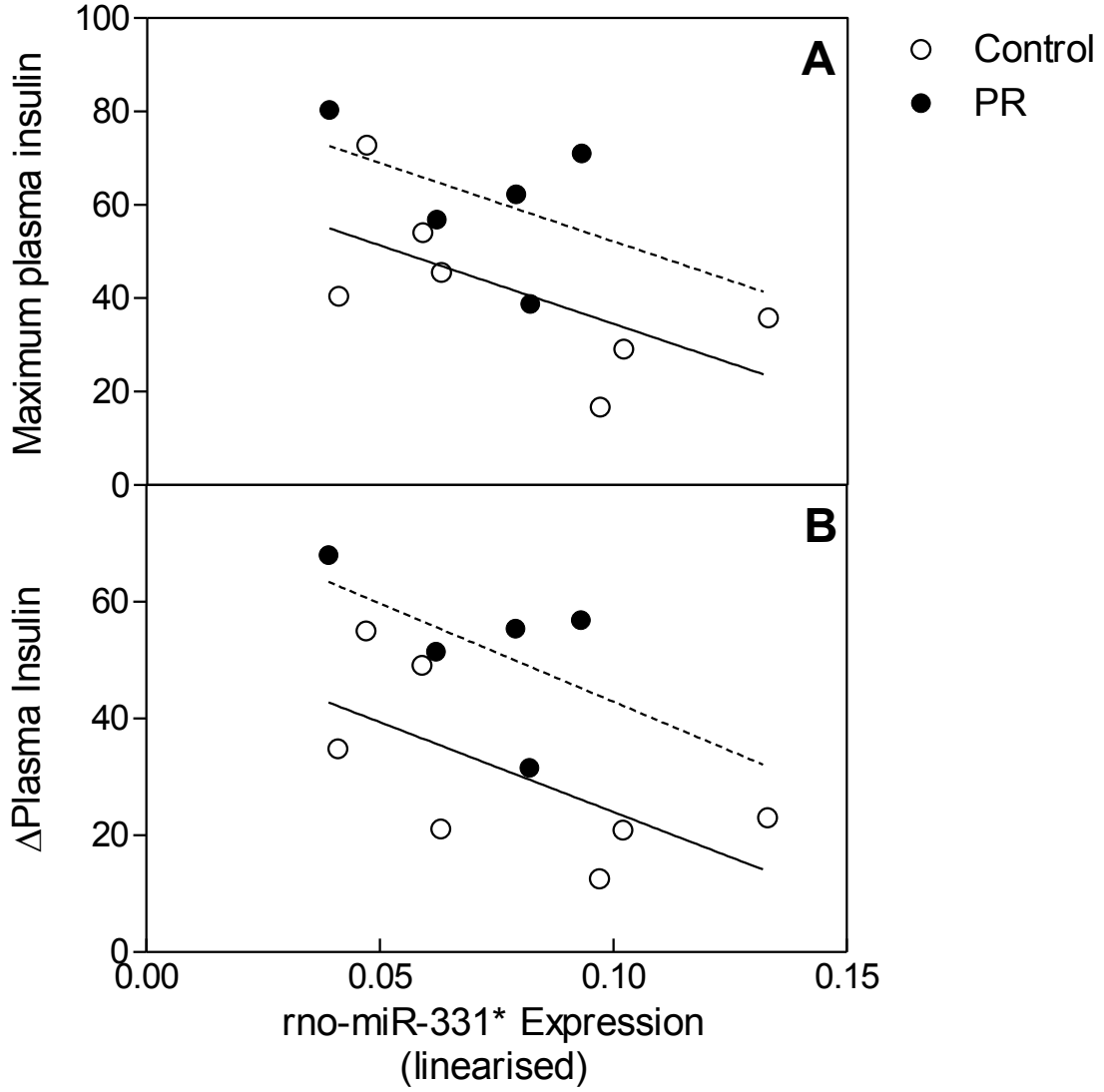


Figure 4.6. Associations between *in vivo* insulin secretion and pancreatic expression of differentially expressed miRNAs in adult male sheep offspring

(A) Pancreatic rno-miR-331* tended to correlate negatively with the maximum plasma insulin during insulin secretion in male control offspring (r = -0.631, *P* = 0.069, n = 7), but not in placentally restricted male offspring (r = -0.451, *P* = 0.223, n = 5). (B) Pancreatic rno-miR-331* tended to correlate negatively with the change of the plasma insulin during insulin secretion (ΔPlasma insulin) in male control offspring (r = -0.654, *P* = 0.506, n = 7), but not in placentally restricted male offspring (r = -0.534, *P* = 0.177, n = 5). PR = placentally restricted.

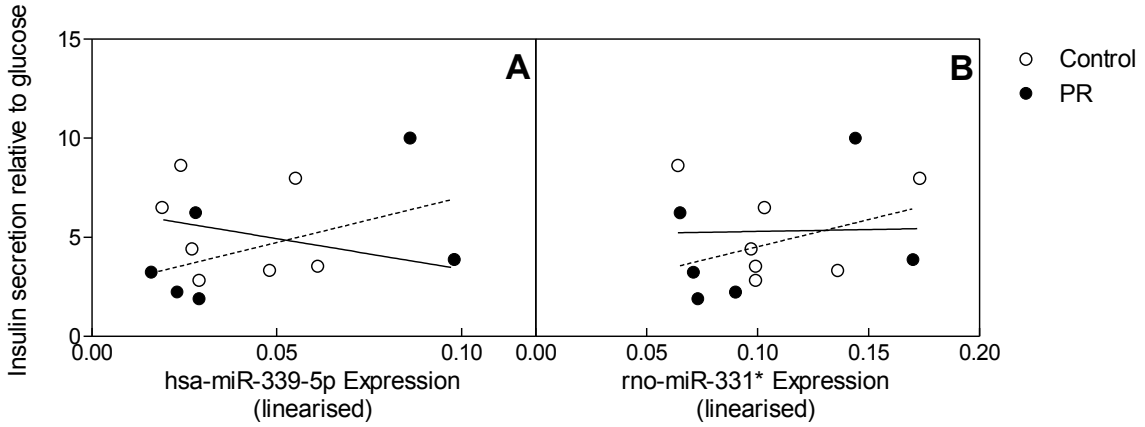


Figure 4.7. Associations between *in vivo* insulin secretion and pancreatic expression of differentially expressed miRNAs in adult female sheep offspring

(A) Pancreatic hsa-miR-339-5p tended to correlate positively with the fasting plasma insulin relative to glucose in female control offspring (r = 0.639, *P* = 0.061, n = 7), but not in placentally restricted female offspring (r = -0.440, *P* = 0.191, n = 6). (B) Pancreatic rno-miR-331* tended to correlate positively with the fasting plasma insulin relative to glucose in female control offspring (r = 0.557, *P* = 0.097, n = 7), but not in placentally restricted female offspring (r = -0.460, *P* = 0.179, n = 6). PR = placentally restricted.

4.7 Supplement Table

Supplement Table S4.1. The top twenty differentially expressed microRNAs in the pancreas of adult sheep

Male	Female	Combined
hsa-miR-339-5p#	**rno-miR-331*#**	**hsa-miR-513a-3p**
mmu-miR-3058*	mmu-miR-337-3p	hsa-miR-891a
mmu-miR-291b-5p	hsa-miR-654-3p	mmu-miR-1954
mmu-miR-1839-3p	mmu-miR-691	hsa-miR-182
hsa-miR-432	hsa-miR-4298	hsa-miR-1973
hsa-miR-92b#	hsa-miR-891a	hsa-miR-4298
mmu-miR-294*	mmu-miR-706	rno-miR-134*#
rno-miR-465	mmu-miR-2861	rno-miR-185*#
hsa-miR-3611	mmu-miR-1968*	mmu-miR-291b-5p
hsa-miR-320e	rno-miR-349	mmu-let-7c-1*
hsa-miR-3935	hsa-miR-299-5p	hsa-miR-1284
hsa-miR-452	mmu-miR-1949	hsa-miR-92b#
hsa-miR-3654	hsa-miR-148a*	mmu-miR-1937a
mmu-miR-1981*	hsa-miR-182	mmu-miR-298
hsa-miR-181a-2*	mmu-miR-193b	hsa-miR-4254
hsa-miR-299-5p	mmu-miR-381*	mmu-miR-1939
hsa-miR-1469	mmu-miR-429	rno-miR-3568
mmu-miR-1947*	hsa-miR-320a	hsa-miR-4325
rno-miR-672*	hsa-miR-125b	mmu-miR-719
hsa-miR-1284	rno-miR-770*	mmu-miR384-3p

MiRNAs are listed on ascending order, with the smallest *P*-value is listed on the top. MiRNAs which are highlighted in bold, have the unadjusted *P*-value less than 0.000407. *: the minor star miRNA molecule class, #: known miRNA sequence in the sheep, and is having identical sequence to both human and rat miRNAs.

Supplement Table S4.2. List of microRNAs sequences in sheep, human and rat

Differentially expressed	Sheep	Human	Rat
miR-134*	ugugacugguugaccagaggg	ugugacugguugaccagagggg	ugugacugguugaccagagggg
miR-185*	uggagagaaaggcaguuccuga	uggagagaaaggcaguuccuga	uggagagaaaggcaguuccuga
miR-92b	uauugcacucgucccggccucc	uauugcacucgucccggccucc	uauugcacucgucccggccucc
miR-339-5p	ucccuguccuccaggagcucacga	ucccuguccuccaggagcucacg	ucccuguccuccaggagcucacg
miR-331*	gccccugggccuauccuagaa	gccccugggccuauccuagaa	gccccugggccuauccuagaa
Endogenous control			
miR-26a	uucaaguaauccaggauaggcu	uucaaguaauccaggauaggcu	uucaaguaauccaggauaggcu
miR-92	uauugcacuugucccggccugu	uauugcacuugucccggccugu	uauugcacuugucccggccugu

Comparison of known sheep miRNA sequences against the similar miRNA in human and rat. All miRNA sequences in the sheep are identical to both human and rat. Sheep's miRNA sequences are obtained from Caiment et al. 2010 (Caiment *et al.*, 2010). *: the minor star miRNA molecule class.

Supplement Table S4.3. List of the predicted targets of miRNAs which were differentially expressed in the pancreas following placental restriction

hsa-miR-339-5p								
A1BG	C14orf101	CYP4F3	GABARAPL1	KRR1	NFIB	PSTPIP2	SLC2A12	TUBA1A
ABCC9	C15orf17	DAG1	GABRG1	LHFPL2	NNAT	PTP4A1	SLC2A4	UBE3A
ABLIM2	C15orf54	DHX40	GAL3ST4	LHX6	NOVA1	PTPRF	SLC44A1	UFM1
ABTB2	C16orf5	DNAJC7	GK5	LHX6	NPHS1	PURB	SLC4A10	UGDH
ACAN	C17orf65	DNAJC8	GLT1D1	LOC253012	NRXN1	RAB15	SLC7A8	UNC45B
ACBD7	C19orf54	DNMT3B	GNAI2	LOXL1	NTRK2	RAB39	SLC9A3R1	UNC5C
ACOX1	C1orf108	DOK6	GNRHR	LPHN1	NUAK1	RAB5C	SMPD3	UNC5CL
ADAMTS4	C1orf181	DTX3L	GPR110	LPIN3	NXPH1	RAB5C	SNX27	USF2
AGPAT3	C1orf212	DYRK1B	GPRIN2	LRP2BP	NXPH3	RAD23B	SORBS1	USP8
AHCYL2	C1QTNF2	E2F4	GRM3	LRP4	OGT	RASAL2	SORT1	VAT1
ALKBH1	C2orf18	EDEM1	GTF2I	LRRTM2	ONECUT2	RASSF1	SPARC	VCP
AMAC1	C2orf21	EFNB2	H6PD	MAGI2	OPRS1	RCP9	SPG7	VEGFA
AMAC1L2	C3orf18	EIF2AK2	HAP1	MAN1A2	OR7D2	RERE	SPTB	VIPR2
ANKRD49	C3orf58	EIF4B	HNRNPA1	MAP2K4	ORC1L	RFC5	SSH2	VPS13B
ANKZF1	C3orf63	ELAVL3	HOXA11	MAP3K7	PAFAH1B1	RGL1	SULT1E1	VSIG1
AP1S3	C6orf223	EMILIN2	HSD3B7	MAPRE1	PAICS	RHOF	SUMF2	WDR21C
APAF1	C6orf89	EMX2	IER5	MARK2	PAK6	RP11	SYNGAP1	WDR55
APOL6	CACNB2	EPB41L4A	IL11	MARK4	PAPPA	RP13	SYP	WDR81
ARHGAP29	CADM4	EPHA4	IL33	MDM1	PATE	RPL3L	TAF8	YWHAZ
ARL11	CAMLG	EPHB3	IRAK2	MDM2	PCYT1A	RUNDC3A	TAT	ZBED4
ARRDC3	CAPZB	ERLIN1	ITGA4	MECP2	PDCD2	SAPS2	TATDN3	ZBTB3
ARSD	CARD8	EXOSC6	JARID1B	MED17	PDCL	SAPS3	TFAP4	ZC3HAV1
ASGR2	CASKIN2	F11R	JOSD1	METTL7A	PDZD4	SATB2	THRA	ZDHHC22
ATP1B1	CCBP2	FAM108B1	JUNB	MLX	PFTK1	SCARB2	TIA1	ZNF202
ATP6V0D2	CCR7	FAM111A	KANK4	MNT	PGRMC2	SCARF1	TIMM50	ZNF280B
BACE1	CD274	FAM119B	KBTBD2	MOBKL1A	PIGR	SENP1	TM4SF20	ZNF329
BACH2	CDC42BPA	FAM19A2	KCNAB2	MOBKL2B	PIM2	SEPHS2	TMBIM1	ZNF385B
BAHD1	CDH1	FAM35A	KCNH3	MPL	PITX3	SERTAD3	TMEM127	ZNF480
BATF	CDK2	FAM5B	KCNJ4	MRPL30	PKP3	SERTAD4	TMEM169	ZNF491
BAZ2B	CENTB2	FAM83H	KCNJ4	MRPL49	PLEKHA7	SFMBT2	TMEM180	ZNF589
BCL2L11	CIC	FBF1	KCNMA1	MRTO4	PLEKHH1	SGCD	TMEM183A	ZNF595
BCL6	CIITA	FCRL1	KIAA0152	MS4A10	PLEKHH3	SGEF	TMEM183B	ZNF596
BLCAP	CLEC7A	FGFBP3	KIAA0323	MTMR3	PNMA2	SH3TC2	TMPRSS3	ZNF689
BMP1	CNNM4	FLJ35848	KIAA0427	MYO1C	PODXL	SHC1	TNRC6B	ZNF770
BRMS1L	CNO	FLT1	KIAA1712	N4BP1	PODXL	SIGLEC11	TPMT	ZNRF2
BRUNOL6	CNOT6	FOXN4	KIAA1754	NANOG	POMT2	SIX2	TRAPPC2	
BSN	COL29A1	FREM2	KIAA1967	NAV3	PPP1R14C	SLC11A1	TRIM13	
BTBD14B	CSF3	FUT1	KIF1B	NCSTN	PRAMEF3	SLC13A3	TRPC3	
C10orf141	CTDSPL	G3BP2	KLF11	NDUFV3	PRNPIP	SLC20A1	TSPAN31	
C13orf33	CUGBP2	GAB3	KLK7	NFASC	PSCD1	SLC25A45	TTLL3	

Supplement Table S4.3. *Continued*

rno-miR-331*		
Aim1	LOC314140	RGD1561145
Bud31	LOC364984	RGD1564005
Bzrap1	LOC364984	RGD1564454
Cdc42ep2	LOC680069	RGD1565482
Cdig1l	LOC682617	Ric8b
Creb3l2	LOC684112	Rnf150
Cspg4	LOC685957	Sema7a
Dclk1	LOC686868	Smad1
Dclre1b	Mafk	Smg5
Dgat1	Nptx1	Stam2
Eif2s1	Nrn1	Zfp513
Fam101b	Pabpn1	Zfp523
Fxr2	Phc2	
Gas7	Pon2	
Gli1	Ptp4a1	
Gnb2	Pvrl1	
Hr	RGD1307399	
Inppl1	RGD1310754	
Itm2c	RGD1310819	
Lmf2	RGD1311456	

Chapter 5

General Discussion

Intrauterine growth restriction (IUGR) is associated with and causes insulin resistance in adult life among both human and other species. This prenatally-induced insulin resistance appears to be, in part, due to reduced insulin signalling molecules expression in insulin sensitive tissues (Jaquet *et al.*, 2001b; Ozanne *et al.*, 2006; De Blasio *et al.*, 2012), impaired whole-body insulin sensitivity (Jaquet *et al.*, 2000; De Blasio *et al.*, 2007a; Jensen *et al.*, 2007; De Blasio *et al.*, 2012), and reduced insulin action (Simmons *et al.*, 2001; Jensen *et al.*, 2002; Gatford *et al.*, 2008). However, it is still unknown what causes this post-IUGR reduction in gene expression of insulin signalling molecules and insulin secretion. Recently, various microRNAs (miRNAs) have been shown to regulate insulin signalling genes mRNA or the protein abundant in insulin sensitive tissues and molecules involved in pancreatic insulin secretion. MiRNAs are short, single-stranded, non-coding RNA; they repress the target mRNA expression by binding to the 3' untranslated region (UTR) (Bushati & Cohen, 2007). In general, some miRNAs' expressions are susceptible to change in environmental milieu during fetal development. This change can be caused by lack of nutrients and/or oxygen, which causes IUGR and may persist into adult life (Saini *et al.*, 2007; Yajnik & Deshmukh, 2012).

The objective of the present thesis is to examine whether placental and fetal growth restriction in non-human species, specifically the sheep and the rat, could alter miRNA expression in insulin sensitive tissues (skeletal muscle, liver and adipose), and in the pancreas, the site of insulin secretion. Indeed, current studies have found that placental restriction increases various miRNA expression in insulin sensitive tissues among both sheep and rat adult offspring (Table 2.3 and Table 3.8), as well as in the pancreas of adult sheep offspring (Table 4.2). Pathway analysis of

differentially expressed miRNAs of the above tissues, predicted the role of specific miRNAs in relation to insulin sensitivity, insulin secretion and related metabolic diseases associated with the IUGR offspring. The experimental models used in these studies were the placentally-restricted sheep and rat. Previous studies have shown that placental and fetal growth restriction in each species usually results in offspring of low birth weight, followed by postnatal catch-up growth, and subsequent adult development of visceral obesity and insulin resistance, similar to that observed in human IUGR offspring (Simmons *et al.*, 2001; Owens *et al.*, 2007b; Vuguin, 2007; Raab *et al.*, 2009).

The present research reports, for the first time, that placental restriction alters miRNA expression in skeletal muscle, liver and adipose of offspring in both the adult sheep and rat, as well as in the pancreas of adult sheep. In the sheep, expression of miRNAs mostly increased in the liver of male placentally restricted offspring. In the rat, expression of miRNAs mostly increased in the omental fat of both male and female placentally restricted offspring. Some of these miRNAs whose expression in insulin sensitive tissues was altered by placental restriction and IUGR, were predicted to regulate insulin signalling gene expression (Table 1.2), and insulin secretion, in the pancreas (Table 1.3).

Some of the up-regulated miRNAs expression in insulin sensitive tissues of IUGR sheep and rat offspring are predicted to regulate insulin signalling molecules. In sheep liver, IUGR increased the expression of hsa-miR-21 and hsa-miR-142-3p, which were predicted to target the expression of *p85α* and *Irs1*, respectively (Table 2.5). The ovine sequence of miR-21 and miR-142-3p were found to be identical to

those in the human and the rat (Supplement Table S2.9); this may indicate that these two miRNAs potentially regulate *p85α* and *Irs1* expression in the sheep liver. Fetal growth restriction in the rat up-regulated hepatic rno-miR-126 expression (predicted to regulate the expression of *Irs1* in the liver), skeletal muscle rno-miR-451 expression (predicted to regulate the expression of insulin receptor, *Irs2* and *Hk2* in the skeletal muscle) and omental fat rno-miR-18a (predicted to regulate the expression of insulin receptor, *p85α* and *Slc2a4* in the adipose tissue – see Table 3.10). In the omental fat, expression of rno-miR-18a was found to negatively correlate only with *Slc2a4*, although, expression of *Slc2a4* was not altered by placental restriction. Therefore, further *in vitro* study is required to confirm their interaction.

The current study did not identify any miRNAs whose expression was altered by IUGR in the same sheep and rat offspring tissues. Thus, both species respond differently to regulating miRNA expression in skeletal muscle, liver and adipose tissue following IUGR. Fetal growth restriction in the sheep was induced before pregnancy, whereas fetal growth restriction in the rat was induced during late gestation. Therefore, different timing in the onset of placental restriction induced during gestation may have contributed to a different molecular mechanism regulating miRNAs expression later in life. Some light may be cast on this by the yet to be determined molecular mechanisms responsible for long-term changes in either species.

Interestingly, we found that some differentially expressed hepatic miRNAs following IUGR, both in the sheep (hsa-miR-142-3p, hsa-miR-1 and hsa-miR-144) and the rat

(rno-miR-126), were predicted to regulate lipid metabolism in the liver (Table 2.6 and Table 3.11); this may potentially lead to the development of non-alcoholic fatty liver disease (NAFLD) in placentally restricted offspring. IUGR is now recognised as a major risk factor for the development of NAFLD in children (Alisi *et al.*, 2011c). NAFLD occurs when fat is deposited in the liver, not due to excessive alcohol consumption, and it is related to the metabolic syndrome and insulin resistance (Clark & Diehl, 2003; Diehl *et al.*, 2003). Recent studies have shown that placentally restricted offspring in human and animal models have a higher risk of developing NAFLD (Nobili *et al.*, 2008; Thorn *et al.*, 2009; Yamada *et al.*, 2011). Our study showed that up-regulation of the above miRNAs, due to placental restriction, was associated with reduced expression of *Acox1* expression in male offspring. Further *in vitro* analysis, such as a luciferase reporter assay, is required to confirm the miRNA-mRNA interaction, and whether hsa-miR-142-3p, hsa-miR-1 and hsa-miR-144 would bind to the 3'UTR of *Acox1* mRNA. In the liver of the rat adult IUGR offspring, we observed an increased rno-miR-126 expression. Moreover, miR-126 has been associated with increased fatty acid accumulation in the liver of zebra fish (Her *et al.*, 2011), although the molecular target of miR-126 in the development of NAFLD is yet to be determined. Other studies have reported that IUGR in rats increases NAFLD, or fatty liver in offspring (Magee *et al.*, 2008; Torres *et al.*, 2010). It is suggested that fatty liver in the rat, as well as in newborn of IUGR zebra fish offspring, is programmed during gestation and is caused by a continual increase in lipogenic parameters as early as embryonic day 20 (Yamada *et al.*, 2011). Therefore, future studies are needed to investigate the hepatic lipid content in the liver of adult sheep and rat offspring.

In this study, we observed sex differences in skeletal muscle liver and adipose tissue of miRNA expression following placental restriction in both sheep and rat offspring. There are reports that about 10% of all miRNAs detected so far were located at the X-chromosome, and speculation that these additional miRNAs at the X-chromosome enhance immune response in females (Pinheiro *et al.*, 2011). Differences in miRNA profiles between sexes have previously been reported during embryogenesis and liver development in rodents (Cheung *et al.*, 2009; Ciaudo *et al.*, 2009). The expression of miR-29b was reported to be female-predominant in the liver of neonatal rats (Cheung *et al.*, 2009), while expression of the miR-302 family during mouse embryonic stem cell development was reported as male-specific miRNA (Ciaudo *et al.*, 2009). The differences in miRNA profiles between male and female offspring in insulin sensitive tissues and response to placental restriction, could be one factor contributing to different outcomes for insulin sensitivity among male and female IUGR offspring. An *in vitro* study has shown sex-specific miRNA expression in the embryonic stem (ES) cell (Ciaudo *et al.*, 2009), although the mechanistic basis is not fully understood. A recent study reports that fetal growth restriction could alter the binding ability of transcription factors, SP1, NF-kappaβ and Egr-1, in the kidney of IUGR rat offspring in a sex specific manner (Baserga *et al.*, 2010). In fact, IUGR reduced the binding ability of SP1 and NF-kappaβ to the 11beta-HSD2 promoter in males, but increased Egr-1 binding to the 11beta-HSD2 promoter in females (Baserga *et al.*, 2010). The same study reported that IUGR increases CpG methylation at promoter binding sites of the 11beta-HSD2 promoter and reduces trimethylation of histone tail H3K36 of 11beta-HSD2, in the kidney of Day 0 and Day 21 IUGR rat offspring. The binding ability variation of transcription factors between male and female IUGR offspring could potentially lead to variation of miRNA

expression between male and female IUGR offspring. Recently, some miRNAs expression has been shown to be potentially regulated by transcription factors (Fujita & Iba, 2008). Therefore, further studies are required: (1) to examine any of the observed differentially expressed miRNAs that could be regulated by transcription factors; and (2) whether the expression of these transcription factors could be regulated by placental restriction.

Sex-specific hormones have been shown to regulate miRNA expression in the liver of rat (Cheung *et al.*, 2009), as well as lipolysis in human adipocytes (Lundgren *et al.*, 2008). Prenatal testosterone in sheep has been shown to influence gene expression in insulin target tissues, such as *Irs-2* and *Pparγ* in the liver, *Irs-1 and Gsk3α* in the muscle and *Irs-2* and *Pi3k* in adipose tissue (Nada *et al.*, 2010). Placental restriction has been reported to increase the circulating thyroid hormone level (De Blasio *et al.*, 2006), however, it is not yet fully understood whether there are sex-specific hormonal response between male and female placentally restricted offspring.

Regarding the pancreas, the top 20 most abundant miRNAs in sheep pancreas differ from those reported previously in other mammalian species (Szafranska *et al.*, 2007; Rosero *et al.*, 2010; Larsen *et al.*, 2011). These differences could be due to the sheep being a ruminant, which may alter the hormonal and paracrine digestion system in comparison to other species, such as humans and rodents. In the pancreas of placentally restricted female sheep offspring, the down-regulation of both rno-miR-331* and hsa-miR-513a-3p occurs; these miRNAs are predicted to target *Pdx1,* and therefore, may up-regulate *Pdx1* expression (Figure 4.3), potentially contributing to an increased number of *β*-cells per islet in placentally restricted female

offspring (Gatford *et al.*, 2008). Thus, as Chapter 4 shows that IUGR in sheep increases the expression of pancreatic rno-miR-331* and hsa-miR-513-3p, these two miRNAs could regulate *Pdx1* expression indirectly through epigenetic mechanisms. In both male and female offspring, placental restriction up-regulates hsa-miR-339-5p expression in the pancreas. This miRNA is predicted to target PLEKHH1 and PAK6 proteins (Supplement Table S4.3) essential to maintaining the pancreas development and/or differentiation of islets.

Unfortunately, RNA degradation prevents the measuring of *Pdx1* expression in adult sheep offspring studied here. Pdx1 is a transcription factor important for regulating *β*-cell development and insulin production (Park *et al.*, 2008). However, a previous study in the rat has shown that placental restriction persistently reduces *Pdx1* expression in the *β*-cell from the 2-week-old offspring to the adult (Park *et al.*, 2008). This study has shown that placental restriction induces the reduction of islets/*β*-cell *Pdx1* expression caused by increased methylation at the *Pdx1* promoter (an epigenetic mechanism). Some miRNAs are reported to regulate pancreatic insulin production (Melkman-Zehavi *et al.*, 2011) and glucose metabolism (Lynn, 2009). In fact, pancreatic miR-375 was reported to play important roles in insulin secretion and pancreatic islet development (Poy *et al.*, 2004; El Ouaamari *et al.*, 2008; Poy *et al.*, 2009). In IUGR sheep, insulin production *in vivo* was not severely impaired due to the compensatory effect of increased *β*-cell mass, especially among male offspring (Gatford *et al.*, 2008).This could be due to placental restriction altering miRNA expression differently between male and female offspring. The limitation of our current study is that the pancreas tissue was analysed as a whole; therefore, RNA was obtained from various types of cells in the exocrine and endocrine pancreas. We

have previously shown that adult sheep male offspring following IUGR have impaired insulin secretory activity (Gatford *et al.*, 2008). Hence, the preferred RNA sample should be obtained from the isolated *β*-cell only, as these cells are responsible for insulin production.

Epigenetic mechanisms could influence miRNA expression, as epigenetic regulation in animal models is known to be affected by placental restriction (MacLennan *et al.*, 2004; Waterland & Michels, 2007; Thompson *et al.*, 2010). From our current studies, we postulate that placental restriction would alter the epigenetic state of miRNA gene expression in the insulin-sensitive and insulin secreting tissues. One of the epigenetic mechanisms, DNA methylation, is shown to be altered in the liver of placentally restricted rat offspring (MacLennan *et al.*, 2004; Holness, 2006). However, our current studies did not observe placental restriction to alter any miRNA expression in young rat offspring at post-natal day 90, indicating that there is/are other unknown factor(s) that could contribute to miRNA expression being altered during only later, not in the early age. Previous studies have reported loss of epigenetic regulation in older humans, such as loss of imprinting (Jelinic & Shaw, 2007) and increased DNA methylation in the promoter of COX7A1, leading to reduced COX7A1 mRNA expression in the skeletal muscle of older men (Ronn *et al.*, 2008). This indicates that the change of some miRNAs expression observed in the older (not the younger) rat offspring could be due to loss of epigenetic regulation, such as increased or lost DNA methylation and loss of histone tail modification. However, more studies are needed in order to examine compare the epigenetic regulation among younger and older animals in this study.

In general, miRNAs are known to play a major role in regulating glucose and lipid metabolism (Poy *et al.*, 2007; Ferland-McCollough *et al.*, 2010; Rottiers *et al.*, 2011). From these studies, we have found that placental restriction mostly up-regulates the expression of miRNAs in insulin sensitive tissues predicted to regulate many molecular targets involved in lipid metabolism, in both sheep and rat offspring. These expressions could subsequently reduce lipid metabolism and increase lipid storage in the skeletal muscle, liver and adipose tissue. Both human and animal IUGR offspring are reported to have higher total whole body fat mass and weight than control offspring, which contributes to the development of type 2 diabetes (Jaquet *et al.*, 2000; De Blasio *et al.*, 2007a; De Blasio *et al.*, 2007b; Germani *et al.*, 2008; Yamada *et al.*, 2011). Therefore, the increased expression of miRNAs in insulin sensitive tissues involved in lipid metabolism among IUGR offspring reported in these studies, could indirectly cause the development of type 2 diabetes. This can occur through the reduction of lipid metabolism and increased lipid storage in the skeletal muscle, liver and adipose tissue, as well as through impairing the insulin-signalling expression in respective insulin-sensitive tissues. However, further studies are required for the purpose of examining whether lipid metabolism in insulin sensitive tissues is affected by placental restriction of the adult offspring.

We have reported that, in the case of some miRNAs expressed differentially in insulin-sensitive tissues following placental restriction, the genomic locations were known to be clustered with other miRNAs (Table 3.8). Upon further examination, the expressions of their cluster members were not significantly different in insulin-sensitive tissues following placental restriction. This may reflect a limited power to detect significant differences or the presence of an additional post-transcriptional

regulation of clustered miRNAs, which may be able to fine-tune the abundance of miRNAs (Lee *et al.*, 2002; Ryazansky *et al.*, 2011). Dicer and Drosha together with other unknown factors, are speculated to selectively regulate miRNA expression (Kuehbacher *et al.*, 2007); however, the exact mechanism of this is still poorly understood. This could, therefore, indicate a selective novel mechanism for a particular miRNA gene from its cluster members to be processed into the next transcription stage. A previous study has shown that, among vertebrates, the promoter regions are evolutionarily conserved for some miRNAs genes (Fujita & Iba, 2008). Therefore, there is a possibility that some miRNAs are regulated by the same class of transcription factor (Wang *et al.*, 2008b).

A major problem currently facing miRNA research is the validation of expression outcomes assessed by microarray, which is normally done by qPCR. However, for some miRNAs, qPCR is not as accurate as miRNA microarray or direct sequencing. For other miRNAs, however, apparent expression differences analysed by qPCR may reflect interference by other miRNAs or RNA species (Ach *et al.*, 2008; Git *et al.*, 2010). The quantitation of miRNAs poses particular challenges, including their short length, lack of common sequences (such as a poly(A) tail), constituting only a small percentage of total RNA species present, generation of diverse forms (including precursor miRNAs), variations in sequence length, and post-transcriptional modifications (Pritchard *et al.*, 2012). Direct sequencing would help resolve such differences observed for both the microarray and qPCR analyses. For the current rat study, further analysis is required to investigate whether IUGR would alter miRNAs expression in the pancreas and/or *β*-cells, and whether they are comparable with the sheep study. *In vitro* analyses are needed to confirm whether differentially expressed

miRNAs following placental restriction, are able to alter the predicted target expression. Recently, a ChIP-on-chip microarray was designed for the purpose of specifically investigating miRNA promoter regions (Saito *et al.*, 2012). The ChIP-on-chip microarray is able to identify any miRNAs that could be epigenetically regulated. This technology would enable a better analysis for determining whether the expression miRNAs in sheep and rat are regulated by the same mechanism, such as transcription factors or the expression of host genes.

Only a handful of sheep miRNA sequences are currently known and confirmed (Caiment *et al.*, 2010; Sheng *et al.*, 2011; Cox *et al.*, 2012). In our sheep studies, some of the most abundant and differentially expressed miRNAs in sheep have the same sequences as in humans and rats (see Chapters 2 and 4). Up to 10 of the most abundant miRNAs observed in sheep liver, muscle and fat, have sequences comparable with other mammalian species, humans and rodents. This indicates the conservation of miRNA gene sequences in mammalian species and shows the validity of the assumption used in this study, that sheep and human miRNA sequences are identical.

Placental restriction had reduced the *Igf1* mRNA expression in the liver of adult sheep and rat offspring. Other studies show that placental restriction reduces *Igf1* mRNA expression in the liver of fetal sheep (Gentili *et al.*, 2009), and in neonatal and juvenile rat offspring (Fu *et al.*, 2009). However, there are no previous reports of the sex-specific effect on *Igf1* mRNA expression in young offspring. Sex-specific changes of *Igf1* mRNA expression in older IUGR offspring could be due to the sex-specific histone modification of *Igf1* in the liver of the juvenile rat (Fu *et al.*, 2009), which could

be worse among the older offspring. Since the liver is the major source of circulating IGF-1 (Yakar *et al.*, 1999). Future studies should examine whether reduced *Igf1* mRNA expression translates to a reduction in circulating IGF-1 among male offspring following placental restriction.

In conclusion, placental restriction is able to alter miRNA expression in a sex-specific manner in the skeletal muscle, liver and adipose tissue. These alterations of miRNA gene expression occur in the offspring only at a later age, in both the sheep and rat. Increased miRNA expression in insulin-sensitive tissues could lead to impairment of lipid metabolism, which contributes to the development of type 2 diabetes in later life. MiRNAs could contribute to the development of type 2 diabetes by not necessarily reducing insulin signalling expression, but by increasing fat mass and lipid content in IUGR offspring. We have shown that three miRNAs in the sheep (miR-142-3p, hsa-miR-1 and hsa-miR-144) and one in the rat (rno-miR-126) could play a vital role in the development of non-alcoholic fatty liver disease (NAFLD) among placentally restricted offspring. MiR-142-3p, hsa-miR-1 and miR-144 are shown to have a strong correlation with the *Acox1* expression in the liver of adult sheep offspring. The alteration of microRNA gene expression in specific tissue could be used as a novel targeted treatment for metabolic disease in offspring born following IUGR.

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