THE EFFECT OF FETAL GROWTH RESTRICTION AND SEX ON THE DEVELOPMENT AND FUNCTION OF ADIPOSE TISSUE

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Declaration

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 the test.

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Publications Arising From This Thesis

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Related Publications

1. McMillen IC, MacLaughlin SM, Muhlhausler BS, Gentili S, Duffield JA and Morrison JL. Developmental Origins of Adult Health and Disease: The Role of Periconceptional and Fetal Nutrition. *Basic & Clinical Pharmacology & Toxicology.* 2008 102:82-89.

2. Muhlhausler BS, Duffield JA, McMillen IC. Increased Maternal Nutrition Increases Leptin Expression in Perirenal and Subcutaneous Adipose Tissue in the Postnatal Lamb. *Endocrinology.* 2007 Dec;148(12):6157-63.

3. Muhlhausler BS, Duffield JA, McMillen IC. Increased maternal nutrition stimulates Peroxisome Proliferator Activated Receptor-γ (PPAR-γ), adiponectin and leptin mRNA expression in adipose tissue before birth. *Endocrinology*. 2007 Feb;148(2):878-85.

4. Muhlhausler BS, Adam CL, Findlay PA, Duffield JA, McMillen IC. Increased maternal nutrition alters development of the appetite-regulating network in the brain. *FASEB J.* 2006 Jun;20(8):1257-9.

5. McMillen IC, Edwards LJ, Duffield J, Muhlhausler BS. Regulation of leptin synthesis and secretion before birth: implications for the early programming of adult obesity. *Reproduction.* 2006 Mar;131(3):415-27.

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Abstract

A world-wide series of epidemiological studies has demonstrated that there is an association between being born small and the risk of visceral obesity, a more central deposition of subcutaneous fat and insulin resistance in adult life. In the lamb, intrauterine growth restriction (IUGR) results in a low birth weight and an increased visceral fat mass by 45d of postnatal life. In this thesis I have investigated the effect of IUGR on adipose tissue development and function during fetal and early postnatal life in the sheep. IUGR was induced by removal of the majority of endometrial caruncles in non pregnant ewes prior to mating which resulted in the subsequent placental restriction of fetal growth (PR). Fetal blood samples were collected from 116d gestation and visceral perirenal adipose tissue (PAT) collected from PR and control fetuses at 145d. In lambs IUGR was defined as a birth weight less than 2 standard deviations below the mean of a cohort of singleton Merino lambs. Blood samples were collected throughout the first 3 weeks of life and PAT and subcutaneous adipose tissue (SAT) was collected at 21 d. It was determined whether IUGR alters the expression of genes which regulate adipogenesis (IGF1, IGFR1, IGF2, IGFR2, PPARγ, and RXRα), adipocyte metabolism (LPL, G3PDH, GAPDH) and adipokine signalling (leptin, adiponectin) in adipose tissue depots before and after birth using qRT-PCR.

PR fetuses were hypoglycaemic, hypoinsulinaemic, hypoxic, and had a lower body weight than Control fetuses. The expression of both IGF1 and leptin mRNA in PAT, the major fetal adipose depot, was lower in the PR fetuses, although

there was no difference in the expression of other adipokine or adipogenic genes in PAT between PR and control fetuses. Thus restriction of placental and hence fetal substrate supply results in decreased IGF1 and leptin expression in fetal visceral adipose tissue which may alter the functional development of the perirenal fat depot and contribute to altered leptin signalling in the growth restricted newborn and the subsequent emergence of an increased visceral adiposity.

At 21d of postnatal life there was no increase in the relative mass of perirenal or subcutaneous fat in IUGR lambs compared with controls. Thus, this study has investigated the effect of IUGR on the development of adipose tissue prior to the development of an obese phenotype.

At 21d of life there was a sex specific effect of IUGR on the expression of PPARγ and leptin mRNA in perirenal visceral fat such that PPARγ and leptin mRNA expression was decreased in male IUGR lambs, but not females. Interestingly PAT mass was greater in females than males, independent of birth weight. Plasma insulin concentrations during the first 24h after birth predicted the size of the adipocytes and expression of adiponectin in visceral adipose tissue in both males and females at 21d. Thus, the nutritional environment before, and immediately after birth, may program adipocyte growth and gene expression in visceral adipose tissue. The differential effect of sex and birth weight on PPARγ and leptin expression in visceral fat may be important in the subsequent development of visceral obesity and the insulin resistant phenotype in later life.

At 21d of life there was no difference between Control and IUGR lambs in the relative mass of subcutaneous fat, or the expression of PPARγ, RXRα, leptin, adiponectin, LPL, G3PDH, and GAPDH in subcutaneous fat at 21d of life. We have shown that the growth of the subcutaneous fat depot is related to plasma glucose, insulin and leptin concentrations, and to the development of perirenal fat. Thus, in contrast to perirenal adipose tissue, the postnatal, but not the fetal nutritional environment, programs subcutaneous adipocyte growth and gene expression. This thesis speculates that there may be a factor secreted from visceral fat that influences the development of the subcutaneous fat depot.

At 21d of life there was also an effect of sex, but not IUGR, on the expression of IGF mRNA in adipose tissue. Male lambs had a higher expression of IGF1 mRNA in both PAT and SAT, and a higher expression of IGF1R and IGF2R in SAT compared with female lambs. It is likely that these differences in IGF mRNA levels reflect sexual dimorphism of the GH-IGF axis. When male and female lambs were combined there was a higher expression of IGF1 mRNA in SAT compared with PAT, and a higher expression of IGF2, IGF1R and IGF2R mRNA in PAT compared with SAT. These differences in IGF mRNA expression provide a potential mechanism to explain the sex and depot specific variations in mitogenic potency of IGF1 and proliferative capacities of preadipocytes, the regional variation in adipocyte metabolism, and the difference in incidence of visceral obesity between men and women in adult life.

List of Tables and Figures

Commonly Used Abbreviations

A	Acrp30	adipocyte complement-related protein of 30kDa
	AGA	average for gestational age
	AgRP	agouti-related protein
	ANOVA	analysis of variance
	ATP	adenosine triphosphate
B	BMI	body mass index
	bp	base pairs
C	cAMP	cyclic adenosine monophosphate
	CART	cocaine-amphetamine related transcript
	cDNA	complementary deoxyribonucleic acid
	C/EBP	CCAAT/enhancer binding protein
	CRL	crown rump length
	cT	comparative threshold
	CV	coefficient of variation
D	d	days
	DEXA	dual emission x-ray absorptiometry
	DM	dry matter
	DNA	deoxyribonucleic acid
E	ELISA	enzyme linked immunosorbent assay
F	FFA	free fatty acids
G	G3PDH	glycerol-3-phosphate dehydrogenase
	GAPDH	glyceraldehyde-3-phosphate dehydrogenase
	GA	gestational age
	GH	growth hormone

	GHRH	growth hormone releasing hormone
	GLUT	glucose transporter
H	h	hours
	HBW	high birth weight
	HDL	high density lipoprotein
	HOMA-IR	homeostasis model assessment to predict insulin resistance
I	IGF	insulin-like growth factor
	IGFBP	insulin-like growth factor binding protein
	IGFR	insulin-like growth factor receptor
	IR	insulin receptor
	i.m.	intramuscular
	i.v.	intravenous
	IUGR	intra-uterine growth retardation
J	JAK	janus kinase
K	kDA	kilodalton
	KO	knock out
L	LBW	low birth weight
	LDL	low density lipoprotein
	LGA	large for gestational age
	LPL	lipoprotein lipase
M	MAPK	mitogen-activated protein kinase
	MDI	methylisobutylxanthine, dexamethasone and IGF1 / insulin
	ME	metabolisable energy
	MRI	magnetic resonance imaging
	mRNA	messenger ribonucleic acid

N	NADH	nicotinamide adenine dinucleotide
	NEFA	non-esterified fatty acid
	NPY	neuropeptide Y
O	*Ob*	*obese* gene
	OB-Rb	leptin receptor type b
P	PAT	perirenal adipose tissue
	PI	ponderal index
	PO_2	partial pressure of oxygen
	POMC	pro-opiomelanocortin
	PPARγ	proliferator-activated receptor-γ
	PPRE	peroxisome proliferator response elements
	PR	placentally restricted
Q	qRTPCR	quantitative real-time polymerase chain reaction
R	RPLP0	acidic ribosomal protein large subunit P0
	RNA	ribonucleic acid
	RXR	retinoid-X receptor
S	SAT	subcutaneous adipose tissue
	SD	standard deviation
	SEM	standard error of the mean
	SES	socioeconomic status
	SGA	small for gestational age
	STAT	signal transducers and activators of transcription
T	TSW	time suckling withheld
	TZD	thiazolidinedione
U	UCP	uncoupling protein
	US	United States (of America)

	UTR	untranslated region
V	VAT	visceral adipose tissue
WXYZ		

1. Literature Review

1.1 Fetal Growth Restriction and Low Birth Weight

A range of fetal, placental and maternal factors can result in a perturbation or restriction of fetal growth, including gene defects, chromosomal abnormalities, small placental size or poor placental function, maternal smoking, parity and maternal age, maternal alcohol or drug abuse and altered maternal substrate concentrations (Robinson *et al.*, 1994; Wollmann, 1998; Bryan & Hindmarsh, 2006). Restriction of fetal growth results in a low birth weight, with low birth weight newborns defined as being either small-for-gestational-age (SGA) and / or intra-uterine growth restricted (IUGR).

Being small for gestational age (SGA) is commonly considered a birth weight below the 10th percentile for gestational age. This definition includes IUGR newborns, newborns with a birth weight in the lower normal birth weight range and newborns where there has been a wrong assessment of gestational age (Wollmann, 1998). In the past IUGR newborns have been defined as having a birth weight and/or birth length below the 10th percentile for GA with a pathologic restriction of fetal growth due to adverse genetic or environmental influences (Chatelain *et al.*, 1998; Wollmann, 1998). This definition, however, is similar to the definition for SGA, and doesn't include a determination of the fetal growth pattern. IUGR newborns may be further classified by their anthropometric measurements, as either asymmetrically or symmetrically growth restricted. These growth patterns reflect the timing of inadequate intrauterine nutrition. The asymmetric type of growth restriction develops when oxygen or substrate supply

to the fetus is reduced during the last trimester of pregnancy. Growth restriction during this timeframe generally results in “adaptive brain sparing”, a redistribution of blood flow to the brain and other key organs (Peeters *et al.*, 1979; Desai *et al.*, 1996), which results in only a slightly reduced length and head circumference, but a significantly reduced weight and therefore a reduced ponderal index (body weight $kg^{1/3}$/height cm *1000) (PI) (Kramer *et al.*, 1989). This type of restriction achieves clinical relevance with increasing substrate demands during the last trimester (Gluckman *et al.*, 1990) and occurs on a continuum with increasing disproportionality with increasing severity of growth restriction (Kramer *et al.*, 1989). The symmetric type of growth restriction results from an adverse environment present from early gestation, and generally results from an intrinsic fetal problem such as genetic or other congenital disease, infection or toxic effects. The growth of the head, femur and abdomen is proportionally reduced and this can lead to a wrong assessment when determining GA by ultrasound (Wollmann, 1998).

In 2001 a panel of thirteen researchers called the International Small for Gestational Age Advisory Board (Lee *et al.*, 2003) redefined SGA and IUGR so that SGA referred specifically to the size of an infant at birth, while the term intrauterine-growth restricted (IUGR) could be reserved to refer specifically to a diminished growth velocity of the fetus as documented by at least 2 intrauterine growth assessments. Therefore, SGA was defined as infants whose birth weight and/or length is at least 2 SD below the mean (< 2 SD) for gestational age, which is approximately the third percentile (Alkalay AL, 1998; Wollmann, 1998), and could be further classified as SGA_W (low birth weight), SGA_L (low birth length), or

SGA_{WL} (low birth weight and length), subject to available data, allowing for the determination of different types of growth restriction (Lee *et al.*, 2003). Criticism of this definition of SGA would be that without adequate training of assessors, length at birth is not as reproducible a parameter as weight , and in the past has not been routinely measured (Gibson *et al.*, 2003). By these definitions of SGA and IUGR, a child who is born SGA has not necessarily suffered from IUGR, and infants who are born after a short period of IUGR are not necessarily SGA (Lee *et al.*, 2003).

Given the temporal discrepancies in the definitions of SGA and IUGR it is important to be careful when interpreting studies of 'SGA' and 'IUGR' newborns. Further to this, it is important to be aware that the range of birth weights that categorise the SGA and IUGR newborn is different between different populations and geographic areas due to factors like altitude, racial characteristics, socioeconomic status and methodologic factors like the accurate assessment of GA.

1.2 The Fetal Origins of Obesity and Insulin Resistance

1.2.1 The Association Between Low Birth Weight, Visceral Obesity and the Development of Insulin Resistance

During 2001-2002 16% of US children (aged 2 through 19 years) (Hedley *et al.*, 2004) and 5% of Australian primary school children were considered obese (BMI $\geq$ 30 kg/m^2). Moreover, 25-50% of obese children go on to become obese adults, who are at greater risk for obesity related metabolic disorders (2002). Interestingly, a range of epidemiological studies have reported that a low birth

weight (LBW), in these studies defined as either SGA (<10th percentile for gestational age) or IUGR (weight or length at/or below two standard deviations of the mean for gestational age and gender of the relevant study population) is associated with an increase in the incidence of childhood obesity (Gray *et al.*, 2002; Cole, 2004), particularly a more central fat distribution (Ong *et al.*, 2000).

In children born SGA an increased adiposity is evident by 2 to 47 months of age. These children have a lower body mass index (BMI: weight(kg)/height(m^2)), but a greater percent body fat than children born average for gestational age (AGA) (Hediger *et al.*, 1998). In a study that compared Caucasian babies with Indian babies, who tend to be smaller and thinner at birth, it was shown that Indian babies had preserved their subcutaneous fat mass and that poor intrauterine growth predicted a higher central adiposity at 8 years of age (Bavdekar *et al.*, 1999; Yajnik, 2000).

The relationship between early growth patterns and obesity in adulthood was demonstrated in a French cohort of 21-27 year olds who had been either IUGR or normally grown at birth (Jaquet *et al.*, 2000). There was no difference between these two groups in body weight or BMI, however percentage body fat mass, determined by bioelectrical impedance analysis, was significantly higher in subjects born with IUGR. In the recent study of Parsons and colleagues on a 1958 British birth cohort, the cohort was divided into birth weight quintiles and BMI at 7, 11, 16, 23 and 33 years of age were compared. It was shown that the shape of the relationship between birth weight and body mass index changed with age, from a linear shape to a J shape in women and tending towards a J

shape in men (Parsons *et al.*, 2001). When Parsons and colleagues examined the relationship between birth weight and postnatal growth (defined as percentage of adult height achieved at age 7) on the incidence of obesity (defined as BMI>30) it was shown that those individuals who had a low birth weight and had achieved more of their adult height by age 7 had an increased prevalence of obesity. Therefore both birth weight, and postnatal growth are implicated in the development of an increased relative fat mass in childhood and adult life. This relationship between birth weight, postnatal growth and an increased disposition for obesity, particularly a more central fat distribution, is evident as early as 5 years of age (Ong *et al.*, 2000).

It has been proposed that perturbations of signaling by hormones that play a key role in the maintenance of body fat mass, such as leptin or insulin, may underlie the development of an increased adiposity in low birth weight individuals. A modified glucose metabolism in SGA infants is evident in human infants as early as 48 hours of life. Bazaes and colleagues (Bazaes *et al.*, 2003) found that SGA infants had lower plasma leptin concentrations, and were more insulin sensitive as determined by Homeostasis Model Assessment (HOMA, an estimate of steady state beta cell function and insulin sensitivity), and had higher plasma FFA levels than AGA infants at 48 hours after birth. Increased insulin sensitivity during early postnatal life has also been associated with postnatal growth patterns. Of infants born SGA (SGA: $<10^{th}$ percentile) and AGA, children with the highest weight gain velocity up to two months of age were of the lowest birth weight and / or were the most insulin sensitive, where birth weight and insulin sensitivity influenced postnatal weight gain independently (Gray *et al.*, 2002).

This implicates an important role for intrauterine nutrient supply in the development of increased insulin sensitivity. It does not, however, rule out postnatal influences in the progression from a more insulin sensitive to an insulin resistant state. An increased insulin resistance has been observed in association with a higher BMI as early as 8 years of life in low birth weight children (Bavdekar *et al.*, 1999), and there is a strong association between low birth weight and the insulin resistance (Bavdekar *et al.*, 1999; Veening *et al.*, 2002) and type 2 diabetes (Veening *et al.*, 2002) in adult life.

1.2.2 The Association Between Low Birth Weight, Subcutaneous Adiposity and the Development of Insulin Resistance

Whilst there has previously been a strong focus on the association between LBW and an increased visceral adiposity in childhood (Ong *et al.*, 2000), there is a collection of more recent studies that have investigated the association between LBW and a greater central subcutaneous fat deposition. It has been shown that in 7-12 year old American school children a low birth weight is associated with a proportionally more central subcutaneous fat deposition as measured by a ratio of skinfold thicknesses, with increasing trunk fat compared with fat on the extremities. It was shown that birth weight accounted for 2-8% of the variance in relative subcutaneous fat distribution at school age (Malina *et al.*, 1996).

LBW has also been shown to be associated with a more truncal distribution of subcutaneous fat during adolescence. In a population of 13-17 year old males adjusted birth weight was related to the ratio of subscapular:triceps skinfold thicknesses, however, in females this relationship was not significant (Labayen *et*

al., 2006). In contrast, in a separate study of 14-16 year old adolescent females adjusted birth weight was significantly negatively associated with the ratio of subscapular:triceps skinfold thickness. This study went on to show that for every kilogram decrease in birth weight, subscapular skinfold thickness increased by 7% (Barker *et al.*, 1997). The dissimilarities between these two studies may be attributed to the size of the sample population or to the parameters by which birth weight was adjusted. The inverse relationship between birth weight and subscapular:triceps skinfold thickness in adolescent females was not significant when birth weight was adjusted for pubertal stage, age, SES, gestational age, physical activity and height (n=198), whereas it was significant when adjusted only for BMI and SES (n=348).

In a study of 229 adult women from the 1979 Amsterdam Growth and Health Longitudinal Study it was shown that there were associations between birth weight, and measures of skinfold thickness and waist circumference, when corrected for adult body mass, indicating that in women a lower birth weight was associated with a higher adult subcutaneous fat mass, a higher waist circumference, and a more truncal fat distribution. The same trend was observed in men, however only the relationship between male birth weight and adult waist-to-hip ratio was significant (te Velde *et al.*, 2003).

A study of twins that investigated the association between birth weight and adult body composition whilst controlling for maternal and genetic influences, showed that in 18-34 year old adult twin males, the lighter twin at birth was shorter and lighter as an adult, but, when adjusted for body mass, had a higher waist-to-hip-

ratio, more subcutaneous fat, and less lean body mass, compared to the heavier sibling (Loos RJ *et al.*, 2001). **I**n a similar study of birth weight and body composition in 18-34 year old adult twin females, twins who were lighter at birth were shorter, by 3.3 cm per kg of lower birth weight, and slightly lighter, by 1.13 kg per kg of lower birth weight, as adults than were heavier twins, although they did not show marked differences in body composition when the waist to hip ratio was adjusted for body mass (Loos RJ *et al.*, 2002).

The relationship between an increased abdominal visceral fat mass and the increased incidence of insulin resistance and type 2 diabetes is well established (Busetto, 2001; Despres, 2006). Recent research has further shown that an increased deposition of abdominal subcutaneous fat is also associated with insulin resistance in the adolescent and adult population. A study of 14-25yr old Asian-Indians in New Delhi showed that for both sexes, fasting insulin concentrations correlated siginificantly with BMI, % body fat, waist circumference, the individual and sum of biceps, triceps, subscapular and suprailiac skinfold thicknesses, central and peripheral skinfold thickness, and that these correlations were stronger in males. The relationship between central skinfold thickness and fasting insulin concentrations and HOMA-IR was stronger than that of peripheral skinfold thickness, and remained significant when peripheral skinfold thickness was adjusted for. Thus in postpubertal Asian-Indian children there is a high prevalence of insulin resistance that correlates with overweight, abdominal obesity, high subcutaneous truncal adiposity, and excess body fat. (Misra A *et al.*, 2004) .

In adults 20 years and older truncal skinfold thickness has been shown to independently predict type-2 diabetes mellitus (Butler *et al.*, 1982). Furthermore, in a study of 30-60 year old males and females with type 2 diabetes mellitus deep subcutaneous adipose tissue at L4-5 level as quantitated by MRI, was associated with both peripheral insulin resistance measured using total glucose disposal during euglycaemic hyperinsulinaemic clamp at 40mU/min/m body surface area of insulin, and hepatic insulin resistance, measured as 'basal endogenous glucose production*fasting plasma insulin production' at 40mU/min/m body surface area of insulin. When males and females were analysed separately these relationships only remained significant in males, who had a greater ratio of superficial:deep subcutaneous fat mass, highlighting the sex differences in abdominal fat distribution and peripheral and hepatic insulin resistance (Miyazaki *et al.*, 2002).

Whilst there have been a series of studies which have shown that there is an association between low birth weight, increased postnatal growth rate, a higher subcutaneous fat mass and an increased truncal distribution of subcutaneous adiposity, it is not clear how the increase in subcutaneous fat mass is programmed as a consequence of restricted substrate supply in utero. It it possible that the programming effect of fetal substrate restriction results from a perturbation in the early differentiation and growth of subcutaneous fat mass, and/or an altered handling of lipid and glucose metabolism.

There are a number of mechanisms proposed to explain the development of an increased fat mass in adult life after exposure to a limitation of placental

substrate supply in fetal life. It has been proposed that alterations in the fetal growth-promoting hormones as a result of poor fetal substrate supply might alter the differentiation and function of adipose tissue, predisposing an individual to an increased deposition of visceral and/or central truncal fat.

1.3 Insulin-like Growth Factors and Fetal Growth

Insulin-like growth factor-1 (IGF1) and Insulin-like growth factor-2 (IGF2) make up 90% of the total extractable molecular insulin-like noninsulin material in serum (Froesch *et al.*, 1985) and have a considerable structural similarity to insulin (Rinderknecht & Humbel, 1978; Humbel, 1990; O'Dell & Day, 1998). As expected, there is evidence for an interaction between the IGFs and insulin at each others receptors (Froesch *et al.*, 1985). The IGF receptor-1 (IGF1R) is structurally very similar to the insulin receptor (IR), and binds IGF1, IGF2 and insulin with affinities in that order of magnitude (Nielsen, 1992; O'Dell & Day, 1998). The IGF receptor-2 (IGF2R) binds IGF2 for internalization and degradation, and also binds mannose-6-phosphate (Nielsen, 1992; O'Dell & Day, 1998).

IGF1, originally known as somatomedin C, and IGF2, originally known as somatomedin A, are monomeric plasma proteins 70 and 67 amino acids in length. They occur in blood plasma at concentrations of 20-80 nM, existing in both the free and bound form, and at lower concentrations in most if not all tissues of the body (Daughaday, 1989; Daughaday & Rotwein, 1989; Humbel, 1990).

The growth promoting properties of IGF1 and IGF2 are well established. They are both stimulators of DNA synthesis and cell replication in a variety of embryo cultures and cell lines (Zapf *et al.*, 1978). IGF infusion in hypophysectomised rats results in an increased body weight, bone width and in the incorporation of radiolabeled thymidine into costal cartilage (Schoenle *et al.*, 1985).

The effect of a loss of circulating IGF1 on growth is evident in the pygmies of Central Africa who have low basal levels of IGF1 that do not increase in response to exogenous GH administration, and have a small stature that can't otherwise be explained by nutritional or environmental factors (Zapf *et al.*, 1978). Interestingly one incidence of a homozygous partial deletion of IGF1 has been reported in an adolescent male. The IGF1 deletion resulted in significantly reduced IGF1 concentrations that consisted of truncated protein (25 instead of 70 amino acids) and elevated basal and peak serum GH concentrations, presumably as a result of loss of inhibition of hypothalamic GHRH release by IGF1. At birth (37 weeks), this patient had symmetric growth retardation, with a weight of 1.4 kg (3.9 SD below the mean in normal subjects), a length of 37.8 cm (5.4 SD below the mean), and a head circumference of 27 cm (4.9 SD below the mean). Throughout infancy and childhood, severe growth failure continued (Woods *et al.*, 1996). Further studies showed normal serum IGF-binding protein-3, acid-labile subunit, and GH-binding activity (Woods *et al.*, 1997). Treatment with recombinant IGF resulted in improved linear growth and normalized circulating GH and insulin levels (Camacho-Hubner *et al.*, 1999). This severe IUGR of this patient provides direct evidence that IGF1 plays a critical role in both fetal and postnatal development, independently of GH, and is confirmed by mouse

knockout studies in which homozygous IGF1-null mice have a fetal growth failure, evident from day 13.5 of gestation, and after birth continue to grow with a retarded rate in comparison with wild-type littermates to become 30-60% of normal weight as adults (Baker *et al.*, 1993; Liu *et al.*, 1993).

Numerous clinical studies have shown a relationship between IGFI concentrations and current fetal or birth weight. At 15-23 weeks of gestation fetal IGF1 concentrations (n=20) were lower than cord blood and adult values, and were correlated with placental weight, fetal body weight and length at that age (Ashton *et al.*, 1985). At 27 weeks gestation SGA fetuses with evidence of placental insufficiency have been shown to have a lower cord serum IGF1 than either SGA fetuses with no evidence of placental insufficiency or AGA fetuses (Langford *et al.*, 1995). At birth, birth cord serum IGF1 concentrations are related to body weight (Gluckman *et al.*, 1983; Verhaeghe *et al.*, 1993), and are reduced by 40% in SGA newborns (SGA definition not specified) and increased by 28% in large-for-gestational-age (LGA) newborns (in the absence of maternal gestational diabetes) (Verhaeghe *et al.*, 1993). Similarly, at birth cord plasma IGF1 concentrations have been shown to be related to body weight, length and head circumference (Geary *et al.*, 2003b).

The relationship between IGF2 concentrations, fetal growth and birth weight is less clear. In a small study (n=20) of fetuses aged 15-23 weeks there was no correlation found between IGF2 and placental weight, body weight or length was found (Ashton *et al.*, 1985). In support of the lack of a relationship between circulating IGF2 and birth weight a larger study (n = 206) found that cord serum

IGF2 concentrations did not correlate with either size at birth or IGF1 values (Gluckman *et al.*, 1983). In contrast, in a large study of 538 cord serum samples collected during the third trimester and at birth, IGF2 concentrations were shown to be sixfold to tenfold higher than those of IGF1 and were 8% to 10% ($p < 0.001$) higher in LGA than in average weight for gestational age (AGA) and SGA newborns (Verhaeghe *et al.*, 1993). In an additional study of 987 term singleton newborns, it was found that cord plasma IGF2 concentrations were also related to birth weight, length and head circumference (Geary *et al.*, 2003b). While there are inconsistencies between studies in observing a relationship between IGF2 concentrations and fetal growth, the importance of IGF2 for fetal growth has been demonstrated in a mouse model with a null mutation for IGF2 (IGF2 KO). Heterozygous IGF2 KO mouse embryos contain approximately 10-fold less IGF2 mRNA transcripts than homozygous wild type mouse embryos and are 50% smaller at birth, but after birth heterozygous IGF2 KO mice have a similar postnatal rate of weight increase when compared to the homozygous wild type mice (DeChiara *et al.*, 1990). Interestingly, an allelic association between an *ApaI* polymorphism in the 3'UTR of *IGF2* mRNA with body mass index and IGF2 levels in a cohort of 2600 middle-aged men has been described. Men that had the polymorphism associated with higher serum IGF2 concentrations also had a lower weight (approximately 4kg) and BMI (O'Dell *et al.*, 1997). Whilst this study did not determine whether the weight loss was a result of changes in musculature or adiposity it implicates IGF2 in the regulation of body mass.

1.4 Insulin-like Growth Factors and Adipose Tissue Development

IGF1 is one of the most important growth factors in the development of adipose tissue. The experimental induction of a global IGF1R deficiency in mice produced growth restriction, and a dramatic reduction in the growth of adipose tissue relative to that of other organs (Holzenberger *et al.*, 2001). At physiologic levels IGF1 stimulates both proliferation and differentiation of preadipocytes in cell culture, and along with cAMP and glucocorticoids, has been shown to be one of the most consistently required agent for induction of adipose differentiation in both serum-containing and serum-free media (Gregoire *et al.*, 1998; Soret *et al.*, 1999). IGF1 mRNA is expressed and IGF1 protein is secreted from porcine preadipocyte cultures (Doglio *et al.*, 1987; Gaskins *et al.*, 1990). In the epididymal fat of 7 week old rats IGF1 mRNA is expressed at levels similar to those measured in the liver and IGF1 protein content per ng of adipose tissue is also high (Peter *et al.*, 1993). At 6 weeks postnatal age it has been shown that 75% of circulating murine IGF1 is hepatic in origin (Butler *et al.*, 2002), which suggests that adipose tissue IGF1 does not contribute significantly to the pool of circulating IGF1, but must have a predominantly autocrine or paracrine role. Further evidence for the paracrine control of adipogenesis by IGF comes from the observation that an increase in fat cell size often precedes an increase in fat cell number (Faust *et al.*, 1978) and from subsequent in vitro studies of IGF1 secretion and action on human and rat adipocytes (Lau *et al.*, 1990; Considine *et al.*, 1996a; Marques *et al.*, 1998; Marques *et al.*, 2000). In one notable study, preadipocyte proliferation was prevented by stripping IGF1 from culture medium conditioned with adipose tissue extracted from the inguinal and epididymal fat pads of rats fed a high fat diet (Marques *et al.*, 2000). The paracrine role of

tissue IGF1 is most likely through interaction with cell surface IGF1-receptors (Dai *et al.*, 1992).

IGF1 also plays a role in adipocyte differentiation in vitro and in vivo (Smith *et al.*, 1988; Ramsay *et al.*, 1989; Wright & Hausman, 1995; Rajkumar *et al.*, 1999; Holzenberger *et al.*, 2001). Once cells reach confluence they become growth arrested, and committed to terminal differentiation. Differentiation can then be synchronously induced using MDI (methylisobutylxanthine, dexamethasone and either IGF1 or supraphysiological levels of insulin) or randomly induced in clusters by fetal bovine serum. After induction by MDI growth arrested preadipocytes go through one round of clonal expansion prior to a significant increase in the expression of peroxisome proliferator-activated receptor-γ (PPARγ), a prominent adipogenic transcription factor, marking the entry of the cell into terminal differentiation. PPARγ then acts as a transcription factor to initiate transcription of the regulatory genes involved in lipid accumulation and glucose metabolism, particularly lipoprotein lipase (LPL) and the adipokines leptin and adiponectin (Reul *et al.*, 1997; Gregoire *et al.*, 1998; Picard & Auwerx, 2002) (See Figure 1.1 from Avram MM et al. *J Am Acad Dermato.l* 2007;56:472-92).

Figure 1.1 Progression of 3T3-L1 preadipocyte differentiation.

Major identified events of preadipocyte differentiation, including growth arrest, postconfluent mitosis, and clonal expansion, are presented chronologically. Areas labelled by gene names represent periods of gene expression during the differentiation program. A representation of the distinct stages of differentiation , very early, early, intermediate and late, is also provided. *C./EBP*, CCAAT.enhancer binding protein; *DEX*, dexamethasone; G_D, growth arrest; *MIX*, methylisobutylxanthine; *PPAR*, peroxisome proliferator-activated receptor. (Figure and text directly copied from Avram MM et al. *J Am Acad Dermato.I* 2007;56:472-92)

1.5 PPARγ, Obesity and Insulin Sensitivity

There are many modulators of adipocyte differentiation, most notably the nuclear receptor PPARγ plays a major role in the induction of growth arrest and terminal differentiation (Picard & Auwerx, 2002). PPARγ is a receptor that functions as an adipogenic transcription factor once it heterodimerises with retinoid-X receptor (RXR) and. The PPARγ-RXR heterodimer binds to sequences on DNA peroxisome proliferator response elements (PPRE) (Auwerx, 1999) to initiate transcription of the regulatory genes involved particularly in lipid metabolism, but also in glucose metabolism during terminal differentiation, when the adipocyte acquires insulin sensitivity. Three different splice variants of PPARγ have been identified in the human genome. PPARγ1 and PPARγ2 have also been identified in the mouse genome. These differ by 30 amino acids in the NH2 terminus.

Obesity (BMI = 43.5 ± 3.1 kg/m^3) has been shown to be associated with an increased ratio of PPARγ2 : 18S mRNA, but not PPARγ1 : 18S mRNA expression in subcutaneous adipocytes of both men and women compared with lean subjects (BMI = 23.3 ± 0.6 kg/m^3). Adipocytes from women also had an increased expression of both PPARγ1 and PPARγ2 when compared to adipocytes from men of a similar BMI. A low-calorie diet was found to acutely downregulate adipose tissue PPARγ expression in subcutaneous adipocytes of obese humans, suggesting that PPARγ may be responsible, at least in part, for the alterations in adipocyte number and function in obesity (Vidal-Puig *et al.*, 1997). While there is a possible role for PPARγ expression in the pathogenesis of altered adipocyte number and function in adult obesity it is not known whether a sub-optimal

nutritional environment before birth programs altered expression of PPARγ or other regulatory genes within the period of postnatal adipocyte differentiation, which might contribute to an increased disposition to obesity and the development of insulin resistance in later life.

Enhanced insulin sensitivity has been associated with PPARγ activity in humans and rats (Hegarty *et al.*, 2004). Treatment of obese and diabetic adults with high-affinity synthetic partial agonists of PPARγ in adipose tissue (thiazolidinediones; TZDs) (Lehmann *et al.*, 1995), results in enhanced insulin sensitivity (64). TZD treatment ameliorates hyperglycaemia and hyperinsulinamia, increases LPL mRNA expression in adipose tissue, increases the synthesis and secretion of adiponectin (Combs *et al.*, 2002), and decreases the synthesis and secretion of leptin. These biological effects are accompanied by an increased mass of white adipose tissue consisting of more small, newly differentiated cells and fewer enlarged white adipocytes (Picard & Auwerx, 2002).

In direct conflict with the evidence that an increased PPARγ activity results in an increased insulin sensitivity in humans, a decreased PPARγ activity has also been associated with improved insulin sensitivity. A relatively common Pro12Ala substitution on chromosome 3p25 of the PPARγ2 gene has been identified (Yen *et al.*, 1997) (frequency 0.12), and results in a decreased affinity of PPARγ2/RXRα heterodimers to PPRE of target genes. The 12Ala allele was associated with lower fasting insulin levels, BMI and higher insulin sensitivity in middle-aged non-diabetic individuals (Deeb *et al.*, 1998). Interestingly, it has been shown that the effects of the PPARγ2 polymorphism on lipid metabolism

and insulin sensitivity interact with size at birth, such that in individuals with the 12Ala allele a low birth weight is associated with higher total cholesterol, LDL, and non-HDL cholesterol concentrations (Eriksson *et al.*, 2003), and in individuals homozygous for the 12Pro allele a low birth weight was associated with insulin resistance (Eriksson *et al.*, 2002).

Further evidence that suggests a decrease in PPARγ expression results in an increase in insulin sensitivity has been found in the heterozygous PPARγ deficient mouse, which displays an improved insulin sensitivity as determined by hyperinsulinemic euglycemic glucose clamp, and an increased level of leptin mRNA in epididymal fat (Miles *et al.*, 2000). This conflicting evidence raises the possibility that administration of TZDs may not provide an accurate representation of the innate *in vivo* response to a pathological increase in PPARγ activity.

It has been proposed that the effect of an altered PPARγ expression or activation on insulin sensitivity might be mediated through its modulation of the expression of leptin and adiponectin. Perturbations in signalling by these adipocytokines, and other hormones that play a key role in the maintenance of body fat mass, such as insulin, may also be implicated in the changes in the development of adipose tissue in IUGR fetuses.

1.6 Leptin and the Regulation of Fat Mass

The 16kDa polypeptide hormone, leptin, was cloned in 1994 (Zhang *et al.*, 1994) and shown to play an important regulatory role in the maintenance of energy homeostasis. Transgenic Ob^{-}/Ob^{-} mice that are null for leptin are hyperphagic, morbidly obese, hyperglycaemic, hyperinsulinaemic and insulin resistant, and these symptoms can all be reversed by administration of recombinant leptin. Furthermore, administration of leptin to wild-type mice produces a loss of appetite, and subsequent weight loss of adipose tissue with sparing of lean body mass (Halaas JL *et al.*, 1995).

The mechanism by which leptin is thought to maintain a set body fat mass is through modulation of appetite and energy expenditure. An intake of excess energy substrate results in an increased fat mass, and a corresponding increase in adipocyte leptin mRNA abundance and circulating leptin levels (Maffei M & al., 1995). In the reverse situation, when energy intake is insufficient to meet total energy requirements, lipolysis occurs, resulting in an increase in circulating free fatty acids (FFA) available for oxidation, a decrease in lipid stores, and a corresponding decrease in adipocyte leptin mRNA abundance and circulating leptin levels. These changes in circulating leptin are detected by central leptin receptors (Mercer JG *et al.*, 1996) and lead to alterations in the levels of energy intake and expenditure such that a constant body fat mass is maintained across long time periods in any individual. Leptin receptors are splice variants of members of the cytokine-receptor superfamily. The long-form splice variant of the leptin receptor (OB-Rb) has been localized in several areas of the brain that are associated with feeding behaviour and energy balance, including the arcuate and

paraventricular nuclei of the hypothalamus, and the ventromedial and lateral areas of the hypothalamus (Mercer JG *et al.*, 1996). OB-Rb has also been detected in low levels in a range of other tissues including the liver, pancreatic beta cells and multilocular and unilocular adipose tissue (Bray GA & DA, 1997). Stimulation of OB-Rb results in activation of the Janus kinase (JAK)/Signal transducers and activators of transcription 3 (STAT3) pathway within neurones of the arcuate nucleus and results in downregulation of neuropeptide Y/agouti-related protein (NPY/AgRP) synthesis and stimulation of pro-opiomelanocortin/cocaine-amphetamine related transcript (POMC/CART) synthesis (Tartaglia *et al.*, 1995; Vaisse *et al.*, 1996). NPY, AgRP, POMC, and CART in turn are secreted within the paraventricular nucleus of the hypothalamus and induce neuronal stimulation of an appropriate metabolic response to changes in fat mass and leptin secretion. NPY and AgRP act to stimulate food intake and decrease sympathetic outflow, whereas POMC is cleaved into a number of peptides, including α-melanocyte stimulating hormone, which decrease appetite and increase energy expenditure (Schwartz *et al.*, 1996). Therefore activation of Ob-Rb receptors by leptin ultimately results in a decrease in appetite and an increase in energy expenditure, and the restoration of fat mass and circulating leptin levels.

Leptin also has peripheral actions, stimulating fatty acid oxidation and release in rat adipocytes in vitro (William Jr *et al.*, 2002) and stimulating fatty acid oxidation in rat adipocytes and skeletal muscle in vivo, but with no concomitant rise in plasma circulating fatty acid or β-hydroxybutyrate concentrations (Shimabukuro *et al.*, 1997). Leptin also directly inhibits insulin stimulated de novo fatty acid

synthesis (William Jr *et al.*, 2002). Therefore leptin acts to increase the use of fatty acids as substrates, both directly, by increasing fatty acid oxidation in adipose tissue and skeletal muscle, and indirectly by inhibiting insulin stimulated fatty acid storage mechanisms. Interestingly, when hyperleptinaemia is induced in normal rats, the subsequent depletion of skeletal muscle fatty acid stores is associated with a dramatic increase in insulin sensitivity as measured by hyperinsulinaemic euglycaemic clamp, and acute lipid infusion is no longer able to induce the development of the insulin resistance (Dube *et al.*, 2007).

Leptin is expressed predominantly in adipocytes, (Zhang *et al.*, 1994) and in the adult, leptin is a signal of body fat mass such that the amount of leptin mRNA expressed per adipocyte correlates with the lipid content and the size of individual adipocytes (Lonnqvist *et al.*, 1997; Chilliard *et al.*, 2001). Leptin is secreted into the systemic circulation by adipocytes at levels that are positively correlated with total body fat mass in the rodent, human, sheep and cow (Maffei *et al.*, 1995; Masuzaki *et al.*, 1995; Moinat *et al.*, 1995; Considine *et al.*, 1996b; Blache D *et al.*, 2000; Delavaud *et al.*, 2000; Ehrhardt *et al.*, 2000). A high proportion of body fat therefore results in a high level of circulating plasma leptin (Clapham JC *et al.*, 1997; Chessler SD *et al.*, 1998).

Circulating leptin levels reflect total body fat mass and adipocyte size, but leptin levels have also been shown to reflect substrate availability. In the human and rat there is a diurnal rhythm in plasma leptin concentrations that occurs in both sexes. In rats the rhythm is circadian and consists of a daytime trough and a peak in the early dark period that is controlled by the suprachiasmatic nucleus of

the hypothalamus, and has been shown to be independent of feeding behaviour (Kalsbeek A *et al.*, 2001). In humans it is less clear whether the fluctuations which are present in leptin concentrations throughout the day are circadian in nature or more related to meal times (Blache D *et al.*, 2000). In sheep, fluctuations in leptin concentrations are not circadian, rather they are directly related to feeding times with peaks in leptin concentrations occurring around 2-8 hours after food intake (Marie *et al.*, 2001).

While plasma leptin levels increase with increasing fat mass in both obese and normal weight adults (Maffei *et al.*, 1995; Considine *et al.*, 1996b; Clapham JC *et al.*, 1997; Chessler SD *et al.*, 1998) it has been shown that adults who were born IUGR (birth weight < 3rd percentile of the local distribution for gestational age and gender established from a population based-registry; n=26) have a relatively lower level of circulating leptin in comparison to normal birth weight adults at the same body weight and waist to hip ratio. Unadjusted leptin concentrations in IUGR adults were not different from that of control adults (n=25), however IUGR adults had a higher BMI, a higher % body fat as measured by bioelectrical impedance analysis, and higher mean fasting serum insulin concentrations, factors which are known to have a positive effect on leptin concentrations. Therefore, when the effect of a higher body fat content and circulating insulin level was considered in a multivariate analysis, leptin concentrations were relatively lower in IUGR adults (Jaquet *et al.*, 2001a).

Relatively low serum leptin concentrations have been previously reported to predict weight gain in Pima Indians (Ravussin E *et al.*, 1997). Interestingly, in

IUGR adults with relatively low levels of circulating leptin an increase in body weight and BMI was evident between 21 and 24 years of age, but did not occur in normal birth weight adults (Jaquet *et al.*, 2001a). In an expanded IUGR cohort of the same research group (n=236) there was also a relative increase of BMI between 6 and 20 years of age in comparison with normal birth weight adults (Leger *et al.*), consistent with the findings at 24 years of age. This raises the possibility that events that occurred in utero which result in a LBW might be associated with a change to the functional capacity of adipose tissue, such that basal adipocyte leptin production is reduced. Therefore, during times of excess energy intake relatively greater increases in fat deposition and leptin levels would be required to produce a set reduction in appetite, resulting in a predisposition to the development of obesity. Furthermore, in the presence of relatively lower circulating leptin there would be a decreased stimulation of fatty acid utilisation in adipose tissue and skeletal muscle resulting in a predisposition for the development of both adipocyte hypertrophy and insulin resistance.

A lower serum leptin concentration is also present in IUGR fetuses and SGA newborns. In humans, fetal leptin concentrations have a positive relationship with gestational age and fetal body weight (Jaquet et al., 1998; Cetin I et al., 2000). At birth cord blood leptin levels, which directly reflect circulating leptin levels in the newborn (Harigaya A et al., 1997), also display a positive relationship with birth weight (Schubring C et al., 1997; Ong KKL et al., 1999) and ponderal index (Lepercq J et al., 2001) in the normal birth weight range. The Mexican birth cohort of Bazaes and colleagues (Bazaes et al., 2003) demonstrated that leptin levels in SGA infant were much lower than in AGA infants at 48 hours after birth,

and in a French cohort leptin levels in IUGR infants were lower than that of control infants at 3d postnatal life (Jaquet et al., 1999). It is therefore possible that in low birth weight newborns, leptin levels at birth, or during early infancy, might be an important feedback modulator of substrate supply, specifically acting to initiate appetite in the newborn, or independently, lower leptin levels might reflect a reduced level of expression of leptin mRNA as a consequence of dysfunctional preadipocyte proliferation and differentiation, producing a functional immaturity of the leptin – fat mass axis.

While a low weight is associated with lower serum leptin around the time of birth and in adult life, at the end of the one year of life a cohort of French children who were born IUGR have been shown to have significantly higher serum leptin values than normal children regardless of BMI and sex, and the normal relationship between leptin concentrations and BMI was not present at this time. The IUGR children also experienced a significant level of catch up growth during the first year of life, but still had a lower body weight and BMI at one year (Jaquet *et al.*, 1999). The authors speculated that there might be a relative leptin resistance during the first year of life to allow the catch up growth to occur, however in children born SGA a greater percent body fat is evident by 2 months of age (Hediger *et al.*, 1998) therefore it is more likely that the higher leptin reflects an increased body fat deposition during the first year of life, indicating that the period of catch up growth is associated with excess substrate supply, and suggesting that leptin is not causal in the process of enhanced growth during postnatal life. The difference in circulating leptin levels between IUGR and control children tended to disappear during the second year of life (Jaquet *et al.*, 1999).

1.7 Adiponectin and Insulin Sensitivity

Adiponectin, also called Adipocyte complement-related protein of 30kDa (Acrp30) or AdipoQ, is an adipocyte derived plasma protein of 244 amino acids (Scherer *et al.*, 1995) that stimulates glucose and fatty acid metabolism in skeletal muscle. In cultured myocytes as well as in isolated muscle, treatment with the globular domain of adiponectin improves both fatty acid utilization, and glucose transport (Fruebis *et al.*, 2001; Tomas *et al.*, 2002; Yamauchi *et al.*, 2002b, a; Combs *et al.*, 2004) by enhanced translocation of glucose transporter 4 (GLUT4) molecules to the cell membrane, and a reduction in the basal and insulin-stimulated rates of glycogen synthesis in muscle cells (Ceddia *et al.*, 2005). In mice in vivo, gAcrp30, a truncated form of adiponectin containing the globular domain caused a reduction in the normal postprandial rise in glucose, FFA and triglycerides following a high fat/sucrose meal. gAcrp30 was also shown to increase clearance of circulating fatty acids after an i.v. injection of Intralipid indicating that the mechanism of the reduction in circulating fatty acids was not through decreased gastrointestinal absorption. Furthermore, mice fed a high fat diet over a 6 month period had a reduced weight gain at the same appetite level when infused with gAcrp30 for the duration of the diet, and this was associated with relatively lower levels of circulating fatty acids (Fruebis *et al.*, 2001) A similar reduction in adipose tissue mass and adipocyte size at the same level of caloric intake has been shown in a transgenic mouse model overexpressing adiponectin in adipose tissue (Bauche *et al.*, 2007).

Adiponectin is produced predominantly in adipocytes, however, in contrast to leptin adiponectin is secreted into the circulation at levels that are inversely related to the mass of adipose tissue, BMI (Arita *et al.*, 1999), the degree of central adiposity, fasting plasma insulin concentrations and insulin resistance (as determined through HOMA, and the euglycaemic hyperinsulinaemic clamp) (Matsubara *et al.*, 2002; Ryan *et al.*, 2003; Tschritter *et al.*, 2003), and also plasma leptin concentrations (Ryan *et al.*, 2003). It has been shown that plasma adiponectin is lower in obese children (Diamond *et al.*, 2004), and that hypoadiponectinemia at 13 years of age is a strong and independent correlate of insulin resistance, β-cell dysfunction, visceral adiposity, and the metabolic syndrome (Bacha *et al.*, 2004). In adults sex- and race-adjusted plasma adiponectin is inversely related to obesity and insulin resistance (Weyer *et al.*, 2001; Steffes *et al.*, 2004). Adiponectin mRNA expression in adipose tissue has also been shown to be decreased in obesity with Ob^{-}/Ob^{-} mice expressing 70-90% less adiponectin mRNA than lean mice and adipocytes from obese humans (n = 4) expressing 50-80% less adiponectin mRNA than lean humans (Hu *et al.*, 1996). Interestingly it has also been suggested that plasma adiponectin is reduced in patients with coronary artery disease (Ouchi *et al.*, 1999; Hotta *et al.*, 2000), further implicating adiponectin in the development of the Metabolic Syndrome.

A number of studies have reported an association between genetic polymorphisms in the adiponectin gene and variations in the incidence of obesity and insulin resistance suggesting that there is a genetic link between adiponectin and metabolic diseases (Hara *et al.*, 2002; Kondo *et al.*, 2002; Stumvoll *et al.*,

2002; Ohashi *et al.*, 2004). A recent study of monozygotic and dizygotic twins has shown, however, that while there is a major genetic component influencing plasma adiponectin concentrations, there is evidence for a nongenetic influence of birth weight on plasma adiponectin and the expression of Adiponectin receptor type-2 (AdipoR2) in skeletal muscle (Storgaard *et al.*, 2007).

Correspondingly, in two adult cohorts of 2277 Japanese men and women aged between 35-66 years and 1059 French men and women aged between 21-35 years, it was shown that low birth weight was related to low serum adiponectin concentrations independently of current BMI (Jaquet *et al.*, 2006; Tamakoshi *et al.*, 2006). The SGA adults in the French cohort also had a higher blood glucose concentration at 2h after a 75g oral glucose tolerance test (OGTT), and a higher fasting insulinaemia, insulin-to-glucose ratio and insulin area under the OGTT curve in comparison to adults born with a normal birth weight indicating that SGA adults who have lower adiponectin are more insulin resistant than AGA adults (Jaquet *et al.*, 2006). Serum adiponectin concentrations have also been shown to be lower in SGA newborns in comparison with AGA newborns (Kamoda *et al.*, 2004). In a study of 16 SGA children it was shown that at around 7 years of age SGA children had a lower level of serum adiponectin than short AGA children (n=20) at the same age, height, weight and BMI (Kamoda *et al.*, 2007). At 8 years of age SGA children who experienced catch up growth from birth to approximately 8 years of age had lower serum adiponectin than SGA children who did not experience any catch up growth (Cianfarani *et al.*, 2004). The lower adiponectin of LBW infants is not in direct conflict with the evidence for a relatively increased insulin sensitivity during the early postnatal period (Gray *et*

al., 2002; Bazaes *et al.*, 2003). In fact a reduction in glucose uptake and fatty acid oxidation in myocytes could provide an explanation for the relative adiposity of SGA infants after a period of catch up growth (Hediger *et al.*, 1998; Bavdekar *et al.*, 1999; Yajnik, 2000). While SGA has been associated with lower levels of circulating adiponectin, the effect of reduced fetal growth on the expression of adiponectin mRNA in adipose tissue has not been investigated.

Adiponectin is induced during adipocyte differentiation and its secretion in vitro has been shown to be stimulated by supraphysiological concentrations of insulin and IGF1 (Scherer *et al.*, 1995; Hu *et al.*, 1996; Halleux *et al.*, 2001). Adiponectin mRNA expression is induced by PPARγ as shown by the presence of a PPRE on the promoter region of the human adiponectin gene (Iwaki *et al.*, 2003), and the increase in adiponectin mRNA expression in mouse adipocytes (Maeda *et al.*, 2001) and in circulating adiponectin in normal, insulin resistant, and diabetic humans and obese and diabetic mice after administration of a PPARγ agonist (TZD) (Maeda *et al.*, 2001; Combs *et al.*, 2002; Yu *et al.*, 2002). Given that supraphysiological concentrations of insulin and IGF1 induce adipocyte differentiation via the induction of expression of PPARγ mRNA it can be concluded that the effect of supraphysiological concentrations of insulin and IGF1 on adiponectin secretion is mediated through an increase in PPARγ activity.

1.8 The Roles of LPL, G3PDH and GAPDH in Adipocyte Metabolism

1.8.1 LIPOPROTEIN LIPASE (LPL)

Lipoprotein lipase (LPL) is located on the luminal surface of the endothelial capillary wall and determines the amount of hydrolysis of the triacylglycerol content of circulating chylomicrons and very low density lipoproteins and therefore the fatty acid uptake in a particular tissue. This is distinct from hormone-sensitive lipase which is located inside adipocytes and is responsible for fatty acid release in response to glucagon. The physiologically active form of LPL is a homodimer enzyme with cofactor apolipoprotein C2 and is formed in the endoplasmic reticulum then translocated to its site of action (Braun & Severson, 1992).

LPL is predominantly produced in adipose tissue, myocytes and cardiomyocytes (Semenkovich *et al.*, 1989a). LPL expression is an early sign of adipocyte differentiation, with LPL mRNA occurring spontaneously during the end of growth arrest and early clonal expansion, the time point at which the expression of PPARγ induces terminal differentiation of the committed preadipocyte (Gregoire et al., 1998; Picard & Auwerx, 2002). PPARγ activity is known to stimulate LPL mRNA production as the LPL promoter region contains a PPRE, and TZD administration results in an increase in adipocyte LPL mRNA production (Schoonjans *et al.*, 1996). The effect of PPARγ activity on LPL mRNA expression may not translate directly through to changes in LPL activity however, as PPARγ activation by TZDs has also been shown to decrease LPL activity (Ranganathan & Kern, 1998), and a decreased PPARγ activity due to the Pro12Ala polymorphism of PPARγ2 is associated with lower LPL post-heparin plasma

activity in patients with diabetes or coronary artery disease (Schneider *et al.*, 2002).

Insulin has been shown to increase LPL mRNA levels in adipocytes without changing the rate of gene transcription suggesting that insulin increases LPL mRNA stability (Semenkovich *et al.*, 1989b). Further to that, there is evidence that insulin resistance is associated with altered LPL regulation. 26 adult nondiabetic individuals (12 women and 14 men) with a wide range of whole-body insulin-mediated glucose uptake displayed postprandial triglyceride increases, and steady-state plasma glucose concentrations during insulin suppression test as an insulin resistance index, were inversely correlated to adipose tissue LPL mRNA levels as well as to adipose tissue LPL heparin-releasable activity (Panarotto *et al.*, 2002). While there is some evidence for an association between insulin resistance and altered LPL mRNA expression in adipocytes (Panarotto *et al.*, 2002), most studies describe an association between obesity and insulin resistance and altered LPL activity (Zechner *et al.*, 2000; Mead *et al.*, 2002).

1.8.2 GLYCEROL-3-PHOSPHATE DEHYDROGENASE (G3PDH)

The cytosolic and mitochondrial glycerol-3-phosphate dehydrogenase enzymes act to regenerate oxidized nicotinamide adenine dinucleotide (NAD^+) from its reduced form NADH, which is produced during glycolysis (Figure 1.2, from JM Berg, JL Tymoczko, L Stryer. *Biochemistry,* 5^{th} Edition, WH Freeman and Company, 2002). Glycerol-3-phosphate is also utilised in intracellular triacylglycerol synthesis.

Figure 1.2 Glycerol-3-Phosphate Shuttle

Electrons from NADH can enter the mitochondrial electron transport chain by being used to reduce dihydroxyacetone phosphate to glycerol 3-phosphate. Glycerol 3-phosphate is reoxidized by electron transfer to an FAD prosthetic group in a membrane-bound glycerol 3-phosphate dehydrogenase. Subsequent electron transfer to Q to form QH_2 allows these electrons to enter the electron-transport chain (From JM Berg, JL Tymoczko, L Stryer. *Biochemistry* 5th Edition. WH Freeman and Company, 2002. Figure 18.37)

1.8.3 GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (GAPDH)

Glyceraldehyde 3-phosphate dehydrogenase (abbreviated as GAPDH, or less commonly as G3PDH) is an enzyme that catalyzes the sixth step of glycolysis (Figure 1.3. From http://www.unm.edu/~lkravitz/Exercise%20Phys/glycolysis.html Date accessed 26-11-07) and thus serves to break down glucose for energy and carbon molecules. In addition to this long established metabolic function, GAPDH has recently been implicated in several non-metabolic processes, including transcription activation, initiation of apoptosis, and ER to Golgi vesicle shuttling.

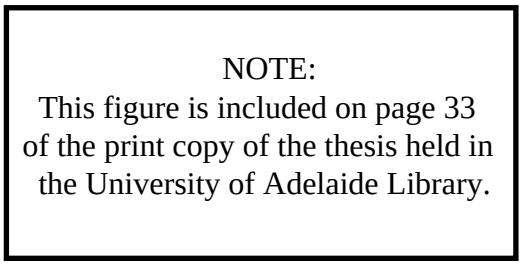

Figure 1.3 The glycolytic pathway.

Step 6 of the glycolytic pathway is catalysed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (From http://www.unm.edu/~lkravitz/Exercise%20Phys/glycolysis.html Date accessed 26-11-07).

1.9 The Experimental Induction of IUGR in Animal Models

Models of maternal undernutrition have been shown to produce fetal growth restriction. Maternal protein restriction in rats or maternal caloric restriction during pregnancy in rats (Ozanne *et al.*, 1996; Vickers *et al.*, 2000; Vickers *et al.*, 2001), and guinea-pigs (Kind *et al.*, 2003; Kind *et al.*, 2005) have been shown to reduce birth weight by reducing the available maternal nutrient supply to the fetus. The protein deprived rat dam has been used predominantly as a model to investigate the effects of low birth weight on the development of hypertension and have been shown to develop varying levels of hypertension, whereas maternal caloric restriction has been used more commonly to determine the effect of low birth weight on adiposity and the development of insulin resistance (Bertram & Hanson, 2001; Armitage *et al.*, 2004).

Several different experimental approaches have been used to produce placental insufficiency that results in fetal growth restriction and reduced birth weight in small and large animal models (McMillen *et al.*, 2001). The primary methods include vascular occlusion or ligation of uterine blood flow in rats, guinea-pigs and sheep (Lafeber *et al.*, 1984; Boyle *et al.*, 1996), placental infarction by repetitive embolisation in pregnant ewes (Clapp *et al.*, 1981; Block *et al.*, 1990; Murotsuki *et al.*, 1997), or the placentally restricted sheep model, in which the majority of visible endometrial caruncles are removed from the bicornuate uterus of the non-pregnant ewe. In this model the number of placentomes formed is markedly reduced and there is a resultant decrease in maternal to fetal transfer

of oxygen and nutrients with a subsequent restriction of fetal growth (McMillen *et al.*, 2001).

In the placentally restricted (PR) sheep model, PR fetuses are chronically hypoxaemic, hypoglycaemic and have increased blood lactate concentrations and, usually, no change in fetal arterial pH (Owens *et al.*, 1989; Robinson *et al.*, 1994). The changes in fetal blood gas status and nutrient supply in PR sheep fetuses are similar to those measured in cordocentesis studies of SGA human infants (Economides *et al.*, 1991). As is also the case in human IUGR, in the chronic hypoxaemia of experimentally induced placental restriction in the sheep fetus there is a redistribution of blood flow to the brain, heart and adrenal glands, from the gastrointestinal, renal and peripheral vascular beds (Yaffe *et al.*, 1987; Bocking *et al.*, 1988; Jansen *et al.*, 1989; Rurak *et al.*, 1990), and results in the maintenance of the relative growth of key fetal organs such as the brain and heart. Therefore, similar to the IUGR infant, experimental restriction of placental growth by carunclectomy results in an asymmetrical pattern of fetal growth restriction whereby body weight is reduced to a greater extent than crown–rump length or girth (Owens *et al.*, 1994; Robinson *et al.*, 1994; Kind *et al.*, 1995).

Whilst there is a redistribution of blood flow in the sheep fetus, there are no differences in mean arterial blood pressure (MAP) between normally grown and growth restricted sheep fetuses during late gestation (Robinson et al., 1983; Edwards et al., 1999). There is, however, a loss of the direct relationship between blood pressure and the mean gestational arterial PO_2 in the placentally restricted group, in comparison with control animals (Edwards et al., 1999) and it has been

shown that at 1 year of age the systolic blood pressure of normally grown and PR lambs (n = 35) is inversely related to the weight or ponderal index of the lamb at birth in an analysis that adjusts for current shoulder height (McMillen et al., 2001). It has been suggested that the redistribution of fetal cardiac output during chronic hypoxaemia is dependent on an increase in circulating catecholamines in PR fetuses, identified between 110-140d gestation. While both circulating noradrenaline and adrenaline are higher, the response to acute hypoxaemia in the PR fetus is to suppress adrenaline secretion, whereas in control fetuses the response is to increase circulating adrenaline (Simonetta et al., 1997).

In the rat there is evidence that brain sparing during fetal nutrient restriction appears to be related to an altered handling of glucose in peripheral tissue such as the lung and muscle, mediated through insulin insensitivity at the cellular level. Fetal growth restriction as a result of bilateral uterine arterial ligation in pregnant dams was associated with a diminished lung mass, and decreased glucose transporter 1 (GLUT1) mRNA expression, total glucose transporter protein and cellular glucose uptake, compared with sham-operated controls. Brain mass was unaffected by fetal growth restriction and there was no change in GLUT1 mRNA expression or protein levels and glucose uptake was unchanged in brain tissue (Simmons *et al.*, 1992). Treatment of cultured pneumocytes and myocytes with supraphysiological doses of insulin or IGF-I for 24h increased GLUT1 mRNA expression, GLUT1 protein and glucose uptake in control fetuses but had less or no effect in growth restricted fetuses.

1.10 IUGR and the Development of Postnatal Adiposity in Animal Models

The effect of a reduced fetal weight as a result of maternal caloric restriction (70% of ad libitum intake) on adiposity has been investigated in the guinea pig. At 60d gestation (term ≈ 69d gestation) the absolute mass of perirenal, retroperitoneal and interscapular fat depots were lower, and the mass of interscapular and retroperitoneal fat as a percentage of body weight was higher in the restricted fetuses compared with fetuses of mothers fed ad libitum. When the volume density of multilocular and unilocular adipocytes within the perirenal and interscapular adipose tissue depots was determined it was shown that the weight of multilocular fat relative to fetal weight in the perirenal and interscapular depots was not different. The weight of unilocular fat relative to fetal weight was increased in the interscapular fat depot of restricted fetuses, but was unchanged in the perirenal fat depot despite an increase in the mean unilocular lipid locule size (Kind *et al.*, 2005). This suggests that there is increased lipid accumulation in the perirenal adipose tissue that consists of single lipid locules.

At birth, in the guinea pig offspring of sows receiving 70% of ad libitum intake have a reduced weight and length, the males had an increased feed intake when measured between 50 and 80 days of life, and at 115d of life males had an increased mass of retroperitoneal fat relative to body weight, but no difference in total relative fat weight providing evidence for a more central distribution of body fat in lower birth weight guinea pig offspring of calorie restricted sows (Kind *et al.*, 2003).

The effect of a reduced birth weight as a result of maternal caloric restriction on adiposity has also been investigated in the rat. Low birth weight (LBW) rat pups that result from a reduced nutrient supply (30% of ad libitum intake) to dams during pregnancy, had an increased feed intake from weaning until puberty (22–40 days), and during postpuberty (60–80 days), and mature adulthood (100–125 days) (Vickers *et al.*, 2000). LBW rats went on to develop a greater mass of retroperitoneal and gonadal fat, and had a higher circulating plasma leptin and insulin, but not glucose concentration at 125d (4 months) (Vickers *et al.*, 2000) and 190d of life (~ 6.3 months) (Vickers *et al.*, 2001) when compared with controls. When exposed to a hypercaloric post-weaning diet LBW rats had catch up growth to achieve the same adult body weight as control pups that consumed a standard post-weaning diet (Vickers *et al.*, 2001).

Experimental induction of a lower birth weight in rats by uteroplacental insufficiency through bilateral uterine arterial ligation in pregnant dams on day 19 of gestation was associated with a more rapid postnatal growth rate, catching up to sham-operated control rats at 7d postnatal life at which time there was a significant increase in perirenal, gonadal and omental fat pads (Simmons *et al.*, 2001).

The relationship between early fetal and postnatal growth and adiposity has also been demonstrated in the sheep. In a recent study by Greenwood it was shown that low birth weight (LBW) male Suffolk-(Finn-Dorset) lambs (<2.9kg) from multiple litters had a higher proportion of body fat than high birth weight (HBW>4.3kg) lambs when examined at the same body weight (20kg) at autopsy.

Interestingly, these authors reported that the LBW lambs had a markedly higher feed intake during the first ten days of life when compared to HBW lambs and that this hyperphagia persisted for the first three weeks of life (Greenwood *et al.*, 1998). In the adult Leicester-Merino (~2.3 years) a LBW induced by placental embolisation from 120d gestation to birth resulted in an increase in the absolute and relative mass of abdominal fat. Interestingly total body fat content as determined by dual emission x-ray absorptiometry (DEXA) was not different between the LBW and control sheep (Louey *et al.*, 2005), which suggests that there is a redistribution of fat to the abdominal depot. Consistent with previous studies the LBW lambs also displayed an increased postnatal growth rate up to postnatal week 8. It should be noted, however, that in this study the lambs born with a LBW were also born ~ 6d earlier than control lambs and that the effect of gestational age on birth weight was not determined (Louey *et al.*, 2005).

Experimental induction of placental restriction (PR) to fetal sheep results in lambs that are lighter, shorter, thinner, and have a lower BMI at birth (De Blasio *et al.*, 2007b). PR lambs display a significantly higher level of growth and an increased absolute perirenal fat mass and perirenal fat mass relative to body weight is present at 45 days of life (De Blasio *et al.*, 2007a). There have been no studies in animals of any species to date that have investigated the effect of low birth weight on subcutaneous fat mass at any age.

1.11 IUGR and the Development of Postnatal Insulin Resistance in Animal Models

Birth weight has been shown to influence glucose metabolism in both early postnatal life and adulthood in rats, guinea-pigs, pigs and lambs (Clarke *et al.*, 2000; Poore & Fowden, 2002; Kind *et al.*, 2003; Wolf, 2003). In female Wistar rat pups maternal undernutrition (50% of ad libitum diet) during the second half of pregnancy (gestation = 22 days) resulted in a reduced birth weight, postnatal catch up growth and a reduced pancreatic islet insulin detectable on the day of birth and at 3 weeks gestation (Garofano *et al.*, 1997, 1998). This provides a mechanism to explain the finding of a decreased plasma insulin and increased plasma glucose evident at 3 and 11 weeks of postnatal life in a different study of the same model (Holemans *et al.*, 1996). Maternal protein restriction during pregnancy has also been shown to result in a reduced pancreatic insulin content in rats at 21d of gestation (Dahri *et al.*, 1991).

In the 15 month old male adult offspring of protein deprived rat dams there was a degree of insulin resistance determined by euglycaemic-hyperinsulinaemic clamp and a reduction in insulin stimulated glucose uptake in isolated soleus muscle that was not attributed to changes in insulin receptor or GLUT4 mRNA expression (Ozanne *et al.*, 2003). Interestingly at 3 months of age adult male offspring of protein restricted rat dams have an increased basal glucose uptake in isolated tibialis anterior muscle associated with no change in plasma insulin but an increased insulin receptor and GLUT4 protein content of plasma membranes in low-protein muscles, and an insulin stimulated glucose uptake that is not different from control muscles (Ozanne *et al.*, 1996). Assuming no differences in

fibre composition between soleus and anterior tibialis this suggests that there is an initial increased insulin sensitivity in the skeletal muscle of offspring of protein restricted rat dams with a subsequent downregulation of insulin receptor from the cellular membrane and insulin resistance during mature adulthood. Insulin receptor number has also been shown to be decreased in the adipocyte at 6 weeks of age in offspring of protein restricted dams (Shepherd *et al.*, 1997), providing evidence for an increased insulin sensitivity of the adipocyte during early postnatal life.

Low birth weight rats (bilateral uterine arterial ligation) that are fatter then sham-operated control rats develop mild fasting hyperglycemia between 7 and 10 weeks of age, which progressively worsened until the end of the experiment at 26 weeks of life. Fasting hyperglycaemia was also present between 7 and 15 weeks of age but then declined to levels that were no longer elevated at 26 weeks of life. Intraperitoneal glucose tolerance test showed a 50% reduction in glucose stimulated insulin release at 1 week of life which declined to almost no response at 26 weeks. I.V. insulin tolerance tests between weeks 1 and 15 demonstrated a progressive development of insulin resistance, and after week 15 of postnatal life the decline in fasting insulin concentrations and glucose stimulated insulin release was shown to be associated with a decrease in relative beta-cell mass of the pancreas (Simmons *et al.*, 2001).

As young adult males (90d) LBW (maternal caloric restriction, 70% of ad libitum intake) guinea-pigs display hyperinsulinaemia and an increased insulin-to-glucose ratio (Kind *et al.*, 2003). In a study of low (LBW <1.47 kg) and high (HBW

>1.53 kg) birth weight piglets, male LBW piglets displayed a higher rate of postnatal growth during suckling (birth to 1 month) whereas females displayed a higher rate of postnatal growth between 3-12 months of age. There was no difference in growth rates during 1-3 months of age between the LBW and HBW piglets for either sex. LBW pigs displayed a decreased glucose intolerance at 12 months of age as determined by i.v. glucose tolerance test (Poore & Fowden, 2002).

In lambs it has been shown that at one and six months of age “light” twin lambs (4.1 ± 0.3 kg) had a greater glucose tolerance than their “heavy” twin (5.1 ± 0.1 kg) (Clarke *et al.*, 2000). In particular, in the postnatal lamb it has been shown that insulin sensitivity of glucose metabolism, circulating amino acids and free fatty acids as measured by hyperinsulinaemic euglycaemic clamp has an inverse relationship with birth weight in both PR and control lambs, such that the a lower birth weight corresponds with a higher whole body insulin sensitivity at 45 days of postnatal life (De Blasio *et al.*, 2007a).

1.12 IGFs in the Placentally Restricted (PR) Sheep Fetus

There is a relationship between circulating IGF1 protein and fetal body weight is present in fetal sheep (Carr *et al.*, 1995). Reductions in fetal sheep plasma glucose, insulin and IGF1, but not IGF2 have also been reported in late gestation following varying periods of periconceptional and gestational maternal undernutrition in different breeds of sheep (Bauer *et al.*, 1995; Lee *et al.*, 1997; Gallaher *et al.*, 1998). In the Welsh Mountain fetal sheep plasma IGF1 increases

between 90-135d gestation. In this breed, fetuses of ewes maintained at 70% of daily maintenance requirements from 26 days of gestation had a lower plasma IGF1, and insulin concentration in comparison with fetuses of adequately maintained ewes (Osgerby *et al.*, 2002).

Circulating plasma IGF1 is reduced in the placentally restricted (PR) sheep model (Owens *et al.*, 1994; Kind *et al.*, 1995). In a cohort of PR and control fetuses (n=20) at 121d gestation IGF1 protein in fetal arterial blood was in the range of 63-176 ng/ml and was related to both placental and fetal weight with an r^2 of approximately 0.40. Circulating IGF1 protein was also related to the expression of IGF1 mRNA in the fetal liver, kidney and skeletal muscle, but not lung or heart. IGF2 protein in arterial blood was in the range of 423-921 ng/ml, but was not related to placental and fetal weight, or tissue IGF mRNA. Consequently, in PR fetuses at 121d gestation both circulating IGF1 protein and IGF mRNA in the liver, kidney and skeletal muscle are reduced, in comparison with control fetuses (Kind *et al.*, 1995). In a separate study IUGR fetuses that were 50% smaller than control fetuses also displayed a reduced hepatic expression of IGF1 mRNA (Rhoads *et al.*, 2000).

A reduction in fetal liver IGF1 mRNA levels has also been shown in fetal sheep at 115-120d gestation in response to 24h hypoxaemia produced by an experimental reduction in uterine blood flow. The hypoxaemia did not alter IGF1 mRNA levels in either kidney, muscle, lung or thymus tissue, or IGF2 mRNA tissue levels (McLellan *et al.*, 1992). While PR reduces the expression of IGF1 mRNA in fetal

liver, kidney and skeletal muscle, the effect of placental restriction on the expression of IGF1 and IGF2 mRNA in fetal adipose tissue is currently unknown. Given the evidence for an autocrine action of IGF1 and IGF2 on adipose tissue growth, and the evidence for reduced expression of IGF1 mRNA in other key fetal tissues, it is likely that there will be a reduced expression of IGF genes in the adipose tissue of placentally restricted fetal sheep.

1.13 Altered Nutritional Status In Utero and Changes in Adipocyte PPARγ, Leptin and Adiponectin mRNA Expression

The effect of maternal caloric restriction coincident with the period of maximal placental growth (28–80 d gestation), followed by refeeding on the expression of PPARγ in fetal sheep adipose tissue has been determined (Bispham et al., 2005). In animal models, caloric restriction isolated to early gestation is more commonly associated with the development of cardiovascular risk factors in adult life and is therefore less commonly used as a model for the development of obesity and diabetes. It has previously been shown that a 50% maternal caloric restriction during the first half of pregnancy followed by subsequent realimentation does not reduce birth weight in the lamb, but does result in the altered development of organs at birth such as the liver and heart (Vonnahme *et al.*, 2003). 50% maternal caloric restriction from 1 to 30 days gestation followed by a maintenance diet until birth resulted in no change in birth weight or postnatal growth rate to one year of life, but increased pulse pressure and produced a leftward shift in the baroreflex function curve (Gardner *et al.*, 2004). Maternal caloric restriction (60% maintenance requirements) from 28–80 d gestation

followed by either a maintenance (100%) or an ad libitum diet (150%) to 140d gestation did not reduce fetal body weight but increased perirenal adipose tissue compared to an ad libitum diet. The expression of PPARγ mRNA in fetal perirenal adipose tissue was not significantly different between the nutrient restricted and ad libitum fed groups, however the authors stated that the expression tended to be higher in the adipose tissue of fetuses in the nutrient restricted group (Bispham *et al.*, 2005).

It has also been shown that pregnant ewes consuming a maintenance diet (100%) from conception to 114d gestation, followed by an ad libitum diet (155%) from 115-139d gestation have fetuses that are not larger, and do not have more fat than fetuses of ewes fed at maintenance level throughout pregnancy (Muhlhausler *et al.*, 2002). At 140d gestation the relative expression of PPARγ mRNA in the perirenal adipose tissue of the ad libitum fed fetuses, however, was significantly increased, and was positively related to mean fetal plasma glucose concentrations during the 135-140d gestation (Muhlhausler *et al.*, 2007). It is not known, however, whether placental insufficiency, which produces a nutritional environment throughout pregnancy that is inadequate to maintain growth predominantly during late gestation, programs an altered expression of adipocyte PPARγ during fetal life, which might have consequences for the timing and magnitude of the proliferation and differentiation processes during adipose tissue development.

1.14 Adipose Tissue Development in the Fetal Sheep

During development, adipose tissue depots begin as small clusters of lipid droplets which are surrounded by extensive stroma and a network of capillaries. As development progresses lipid accumulates in the droplet clusters and they increase in size while the number of clusters remains relatively constant (Ailhaud *et al.*, 1992). There are detectable adipose tissue depots present before birth in humans (Merklin, 1973), pigs (Hausman & Richardson, 1982) and sheep (Alexander, 1978), however in these different species there are differences in the amount and distribution of adipose tissue that is deposited before birth and adipose tissue makes up a higher proportion of total body weight in the human infant (~16%) compared to the newborn lamb (~1.5%) (Fowden, 1995)

In development, preadipocytes differentiate into two, morphologically and functionally different types of adipose tissue, unilocular and multilocular adipocytes (Klaus, 1996). Unilocular adipocytes consist of a single lipid inclusion and eccentrically located nucleus, and the main function of these adipocytes is lipid storage. Multilocular adipocytes contain a number of small lipid locules (multilocular lipid inclusions), have an abundance of mitochondria and are the site of non-shivering thermogenesis, which is important for the maintenance of body temperature in newborn mammals. Thermogenic function is adrenergically stimulated and is attributed to a proton translocator in the inner mitochondrial membrane called uncoupling protein 1 (UCP-1). UCP1 acts to uncouple the mitochondrial respiratory chain, resulting in the generation of heat instead of ATP for cellular fuel (Klaus, 1996).

In the human infant the visceral depots consist primarily of brown adipocytes while the subcutaneous depot is predominantly comprised of white adipocytes. In fetal life the visceral adipose tissue of the sheep is composed of predominantly multilocular, mitochondria rich adipocytes, classified on the basis of their molecular and morphometric characteristics (Gemmell & Alexander, 1978). Multilocular adipose tissue is also found in the interscapular, subscapular, axillary and suprasternal depots (Klaus, 1996). In the fetal sheep, adipose tissue from both the perirenal and subcutaneous sites develop from preadipocytes containing few mitochondria that begin to accumulate lipid at about 70d of gestation. Increases in adipose tissue mass relative to body mass continues to occur up to about 110d gestation (Alexander, 1978). Between 80-90d gestation perirenal adipose tissue develops into brown fat via a proliferation of mitochondria characterised by numerous distinct cristae, whereas subcutaneous adipose tissue develops into white fat with few mitochondria containing fewer cristae. While the size of lipid locules within the visceral and subcutaneous fat depots increase during late gestation (Gemmell & Alexander, 1978) there is no further increase in adipose tissue mass relative to body weight before birth (Alexander, 1978), at which time both multilocular and unilocular cells have developed and are present in visceral and subcutaneous depots (Gemmell *et al.*, 1972).

1.15 Leptin in the Fetal Sheep

Leptin and leptin receptor mRNA has been detected in the fetal liver, skeletal muscle, kidney heart and placenta (Buchbinder *et al.*, 2001), and leptin mRNA has also been detected in fetal perirenal adipose tissue of sheep as early as 70d

gestation (Ehrhardt *et al.*, 2002). In a separate study of fetal sheep, leptin mRNA was also detected in perirenal adipose tissue at 90 and 140d of gestation with the level of expression being higher at the older gestational age. Leptin mRNA expression was strongly related to fetal body weight, and to fetal plasma leptin concentrations (Yuen *et al.*, 1999), relationships that are also present during adult life. It has also been shown in fetal sheep that circulating leptin levels are directly related to the size of the lipid locules present within the unilocular component of perirenal adipose tissue at 140d gestation (Muhlhausler *et al.*, 2002), which suggests that circulating leptin concentrations are a signal of fetal adipocyte lipid content rather than adipose tissue mass per se. Infusion of leptin into fetal sheep produces a decrease in the proportion of unilocular fat within the perirenal adipose tissue, and a decrease in adipocyte leptin mRNA levels (Yuen *et al.*, 2003). This provides evidence that leptin can act to regulate the lipid storage capacity of fat before birth. Leptin gene expression has also been shown to be higher in unilocular than in multilocular adipose tissue in the adult rat (Klaus S *et al.*, 1991).

In the pregnant ewe, 50% maternal caloric restriction during late gestation (115-147d gestation) reduced fetal glucose and insulin, but does not reduce fetal weight, fetal fat mass, or levels of plasma leptin and leptin mRNA expression in perirenal adipose tissue. In this study there was a relationship between fetal fat mass and plasma leptin levels (Yuen *et al.*, 2002). Similarly, an increased maternal nutrient intake (155% of maintenance requirements) during late gestation increases fetal glucose and insulin concentrations, but does not increase fetal weight, fetal fat mass or plasma leptin concentrations (Muhlhausler

et al., 2002). It is possible that the nutritional interventions utilised in these studies did not produce effects on fetal weight and other outcomes due to a compensatory changes in maternal metabolism that reduced the effect of nutritional manipulation on fetal substrate supply.

The effect of placental insufficiency on circulating leptin concentrations has been determined in fetal sheep. Placental insufficiency was induced by surgical vascular occlusion, which chronically reducted uterine blood flow to 50% of normal, from 115-138d gestation, and reduced fetal body weight and length. Placental insufficiency increased circulating fetal plasma leptin concentrations, which were inversely related to fetal body weight and to placental weight (Buchbinder *et al.*, 2001). Higher circulating leptin concentrations and higher adipose tissue leptin mRNA levels were also detected in fetuses of ewes kept at high altitude, who were therefore hypoxemic (Ducsay *et al.*, 2006). To date, the effect of placental insufficiency on the expression of leptin mRNA in adipose tissue has not been determined, but given the evidence for an effect on circulating leptin and the relationship between plasma leptin concentrations and leptin mRNA levels that has been previously reported in the fetal sheep it is likely that the expression of leptin mRNA in perirenal adipose tissue would be altered by placental insufficiency during late gestation.

In Chapter 2 I have therefore hypothesised that a decrease in fetal substrate supply as a consequence of experimentally induced placental insufficiency will result in a decreased expression of IGF mRNA in perirenal adipose tissue of the growth restricted fetus at 140-145d gestation. I further

hypothesised that the decreased expression of IGF genes would be associated with an altered pattern of expression of adipogenic, lipogenic and adipokine genes including PPARγ and retinoic X receptor (RXR)α, leptin, adiponectin, LPL, glycerol-3-phosphate dehydrogenase (G3PDH), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). I have determined the extent to which the expression of these genes in fetal adipose tissue are related to plasma glucose, insulin, leptin and NEFA concentrations measured during late gestation, between 116-145d gestation (term = 150d).

1.16 LBW and the Development of Adipose Structure and Function and Adipocyte PPARγ, Leptin and Adiponectin mRNA Expression in Adipose Tissue in the Postnatal Lamb

Early postnatal life is a period of marked growth and development of adipose tissue during which the rate of weight gain of the perirenal adipose depot is greater than the liver, lung, heart, kidney and adrenal (Clarke *et al.*, 1997). In both perirenal and subcutaneous depots the size of lipid locules increase during the first 2-3 weeks of life, and the percentage of multilocular adipose tissue decreases as all depots take on the molecular and morphometric characteristics of mature white adipose tissue (Gemmell *et al.*, 1972). The majority of studies on the development of adipose tissue in the sheep have focused on the perirenal depot in the late gestation fetus and in the neonate.

After birth mean perirenal adipose tissue weight remains stable over the first two days of life and then increases as a consequence of an increase in lipid content by 30d postnatal age (Clarke *et al.*, 1997). The expression of PPARγ mRNA relative to GAPDH in the perirenal fat of sheep appears to decrease during the first day of life followed by an increase to day 7 of postnatal life to levels similar to those present at birth (Lomax *et al.*, 2007). The profile of leptin expression is similar to that of PPARγ. Leptin mRNA levels and circulating leptin levels both decline during the first 6 hours after birth and then gradually increase during the first 7 days of life, in parallel with increased lipid deposition (Bispham *et al.*, 2002). There is a continued increase in total perirenal adipose tissue mass between 7 and 30 days of life, but despite this. adipocyte leptin mRNA and circulating leptin levels decrease to levels similar to those measured in newborns (Bispham *et al.*, 2002). Concomitantly there is a decline in UCP mRNA and UCP activity between 7 and 30 days of life, thus by around 21-30 days there is a complete differentiation of the perirenal adipose tissue depot to unilocular white adipocyte tissue (Clarke *et al.*, 1997; Mostyn *et al.*, 2003). Therefore during this postnatal period there appears to be a disruption in the relationship between leptin expression and adipocyte lipid content / fat mass, and this raises the possibility that that leptin levels during this developmental period may not directly reflect fat mass, but might be an important feed-back modulator of substrate supply, contributing to the initiation of either appetite, thermogenesis, or insulin sensitivity, and influencing the development of the homeostatic systems involved in the regulation of energy homeostasis.

In the adult sheep the relationship between leptin and fat mass is restored, with mean lifetime plasma leptin concentrations related to abdominal fat mass and total body fat content (DEXA) at 2.3 years (Louey *et al.*, 2005), and at 3 years of age basal plasma leptin concentrations correlated with the ratio of fat/fat-free mass (Gopalakrishnan *et al.*, 2004). In adulthood there is no difference in basal circulating leptin levels between LBW (following placental embolisation) and control sheep , or between calorie restricted LBW sheep and controls. Interestingly, in LBW adult sheep (after caloric restriction), intravenous noradrenaline administration stimulates an increase in plasma leptin concentrations, an effect which is not present in control sheep (Gopalakrishnan *et al.*, 2004).

In Chapter 3 I have therefore hypothesised that the experimental induction of low birth weight in the lamb by placental restriction of fetal growth will result in an altered pattern of expression of adipogenic, lipogenic and adipokine genes in perirenal adipose tissue at 21d of postnatal life. I have therefore investigated the extent to which changes in growth of the adipocyte and the expression of RXRα, PPARγ, leptin, adiponectin, LPL, G3PDH, and GAPDH are determined by birth weight and nutritional status during the transition into postnatal life as summarised by the plasma insulin, leptin and glucose concentrations measured within 24h of birth and by postnatal factors, including the postnatal growth rate and circulating hormonal and nutrient levels during the first three weeks of life.

In Chapter 4 I have also determined the effect of low birth weight in the lamb on expression of adipogenic, lipogenic and adipokine genes in the subcutaneous adipose tissue at 21d of postnatal life. I have investigated the extent to which changes in growth of the adipocyte and the expression of RXRα, PPARγ, leptin, adiponectin, LPL, G3PDH, and GAPDH are determined by birth weight and nutritional status during the transition into postnatal life as summarised by the plasma insulin, leptin and glucose concentrations measured within 24h of birth and by postnatal factors, including the postnatal growth rate and circulating hormonal and nutrient levels during the first three weeks of life.

Furthermore, in the lamb, the development of a dissectible depot of subcutaneous adipose tissue during early postnatal life is preceded by the development of the visceral fat depot in fetal life, therefore I have investigated whether there are associations between the growth and development of the visceral depot with the development of the subcutaneous depot during the first 3 weeks of life.

1.17 IGFs in the Placentally Restricted (PR) Postnatal Lamb

After birth plasma IGFI levels remain stable for 0-2 days, and then rise between 3-7 days of postnatal life, after which point there is no longer a relationship with birth weight. By 12h after birth IGF2 concentrations become stable at concentrations similar to that of the adult ewe (Gluckman *et al.*, 1983; Daughaday & Rotwein, 1989). After weaning, at 3 months lambs receiving a

balanced diet grew faster than lambs receiving a diet limiting for both energy and protein, and for that 6 week period had twofold higher circulating concentrations of IGF-I (Rhoads *et al.*, 2000). In a separate study, plasma levels of IGF1, but not IGF2 were decreased by fasting for 3 days at 4 months and 2 years of postnatal life (Hua *et al.*, 1995), and at 6 months of life plasma and tissue levels of IGF1 protein, and levels of kidney mRNA expression were also decreased by fasting for 5 days (Hua *et al.*, 1993). There was no effect of nutrition on liver or skeletal muscle IGF1 mRNA at 6 months of postnatal life (Hua *et al.*, 1993; Rhoads *et al.*, 2000).

Similarly, in the low birth weight (LBW) lambs of Greenwoods' study (Greenwood *et al.*, 2002), a subset of lambs were fed milk replacer at either restricted or ad libitum levels between d 12 and 38 of postnatal age. In this study, the concentrations of plasma IGFI were increased by the higher level of nutrition, but were not related to birth weight. The hepatic expression of IGF1 mRNA was also not affected by nutritional level or birth weight at any given body weight (5, 7.5, 10, 15, or 20 kg) during the same period (Rhoads *et al.*, 2000). Together, these studies suggest that the elevated levels of plasma IGF1 might not result from increased IGF production by the liver in the postnatal lamb.

It has been shown in the postnatal lamb that plasma IGF1 concentrations are related to the lipid content of perirenal adipose tissue from birth to 30d postnatal life (Clarke *et al.*, 1997), providing evidence that the relationship between plasma IGF1 and current nutrition is mediated through changes in the expression of IGF1 in adipose tissue at this age. Given the established autocrine/paracrine role of

IGF1 in adipose tissue development, a reduced adipocyte IGF1 mRNA expression and plasma concentration during fetal life when the majority of brown adipocytes are developing, coupled with an increased adipocyte IGF1 mRNA expression and plasma concentration during early postnatal life when the majority of white adipocytes are developing, could provide a mechanism for altered adipose depot development in the LBW lamb. It is not currently known, however, whether the expression of IGF1 mRNA in adipose tissue in the postnatal lamb is influenced by birth weight.

In Chapter 5 I have therefore hypothesised that a low birth weight will result in an altered expression of IGF1 mRNA and its receptor IGF1R mRNA in perirenal and subcutaneous adipose tissue in the lamb at 21d of postnatal life. I have therefore determined the extent to which the expression of IGF1, IGF2, IGF1R and IGF2R mRNA in both perirenal and subcutaneous adipose tissue are determined by birth weight and by postnatal factors, including the postnatal growth rate and circulating hormonal and nutrient levels during the first three weeks of life.

2. Placental restriction of fetal growth decreases IGF1 and leptin mRNA expression in the perirenal adipose tissue of the late gestation fetal sheep

2.1 SUMMARY

Placental restriction (PR) of fetal growth results in a low birth weight and an increased visceral fat mass in postnatal life. We have investigated whether PR alters expression of genes which regulate adipogenesis (IGF1, IGF1R, IGF2, IGF2R, PPARγ, RXRα), adipocyte metabolism (LPL, G3PDH, GAPDH) and adipokine signalling (leptin, adiponectin) in visceral adipose tissue before birth. PR was induced by removal of the majority of endometrial caruncles in non pregnant ewes prior to mating. Fetal blood samples were collected from 116d gestation and visceral perirenal adipose tissue (PAT) collected from PR and control fetuses at 145d. PAT gene expression was measured by qRT-PCR. PR fetuses had a lower weight (PR 2.90 ± 0.32 kg; Control, 5.12 ± 0.24 kg; $P<0.0001$), mean gestational arterial PO_2 ($P<0.0001$), plasma glucose ($P<0.01$) and insulin concentrations ($P<0.02$), than Controls. The expression of IGF1 mRNA in PAT was lower in the PR fetuses (PR 0.332 ± 0.063; Control 0.741 ± 0.083; $P<0.01$). Leptin mRNA expression in PAT was also lower in PR fetuses (PR 0.077 ± 0.009; Control, 0.115 ± 0.013; $P<0.05$), although there was no difference in the expression of other adipokine or adipogenic genes in PAT between PR and control fetuses. Thus restriction of placental and hence fetal substrate supply results in decreased IGF1 and leptin expression in fetal visceral

adipose tissue which may alter the functional development of the perirenal fat depot and contribute to altered leptin signalling in the growth restricted newborn and the subsequent emergence of an increased visceral adiposity.

2.2 INTRODUCTION

A world-wide series of epidemiological and clinical studies has demonstrated that there are associations between the patterns of growth in fetal and early postnatal life and the risk of insulin resistance, type 2 diabetes and obesity in adult life (Barker *et al.*, 1993; McCance *et al.*, 1994; Lithell *et al.*, 1996; Leger *et al.*, 1997; McKeigue *et al.*, 1998; Bavdekar *et al.*, 1999; Jaquet *et al.*, 2000; McMillen & Robinson, 2005). Small-for-gestational-age (SGA: birth weight and/or length < 2 standard deviations below the mean for gestational age), (Alkalay AL, 1998; Wollmann, 1998), infants have low circulating insulin-like growth factor-1 (IGF1) concentrations and a reduced body fat mass at birth (Enzi *et al.*, 1981), and then undergo a period of accelerated postnatal growth during the first few years of life (Fitzhardinge & Steven, 1972; Albertsson-Wikland *et al.*, 1993), with a relative increase in body fat mass from as early as 2 -12 months of age (Hediger *et al.*, 1998) and truncal fat mass during childhood (Ong *et al.*, 2000) and in adult life (Law *et al.*, 1992; Parsons *et al.*, 2001). An accelerated postnatal growth rate is associated with an early increased insulin sensitivity, followed by the emergence of insulin resistance (Curhan *et al.*, 1996; Whincup *et al.*, 1997; Bavdekar *et al.*, 1999; Eriksson *et al.*, 2001; Veening *et al.*, 2002; Levy-Marchal *et al.*, 2004), and in particular, insulin resistance of adipose tissue in later life (Jaquet *et al.*, 1998;

Jaquet *et al.*, 1999; Phillips *et al.*, 1999; Jaquet *et al.*, 2000).

IGF1 is one of the most important growth factors in the development of adipose tissue. The experimental induction of a global IGF1-receptor (IGF1R) deficiency in mice produces growth restriction, and reduces the growth of adipose tissue relative to that of other tissues (Holzenberger *et al.*, 2001). At physiologic levels, IGF1 stimulates both proliferation and differentiation of preadipocytes in cell culture, and along with cAMP and glucocorticoids, has been shown to be required for induction of adipose tissue differentiation in both serum-containing and serum-free media (Gregoire *et al.*, 1998; Soret *et al.*, 1999). It has been shown that after experimental induction of differentiation growth arrested preadipocytes go through clonal expansion prior to a significant increase in the expression of proliferator-activated receptor-γ (PPARγ), a prominent adipogenic transcription factor when heterodimerised with retinoid-X-receptor-α (RXRα). This initiates the transcription of the regulatory genes involved in lipid accumulation and glucose metabolism, particularly lipoprotein lipase (LPL), leptin and adiponectin and marks the entry of the cell into terminal diffferentiation (Gregoire *et al.*, 1998; Picard & Auwerx, 2002).

In species such as the sheep or pig, adipogenesis and lipogenesis occur before birth as in the human, and low birth weight offspring also grow faster in the postnatal period and have a higher proportion of body fat than their higher birth weight counterparts in later life (Greenwood *et al.*, 1998; Poore & Fowden, 2004; Louey *et al.*, 2005). In a cohort of lambs born after experimental restriction of

placental growth, there was an increase in relative visceral fat mass and a relationship between visceral adiposity and the action of insulin to suppress circulating free fatty acids at 6 weeks of age (De Blasio *et al.*, 2007a). In the fetal sheep, body weight is also related to circulating IGF1 concentrations (Owens *et al.*, 1994; Carr *et al.*, 1995; Kind *et al.*, 1995), and to hepatic IGF1 mRNA expression (Rhoads *et al.*, 2000). Experimental restriction of placental and hence fetal growth in the sheep reduces both circulating IGF1 protein and IGF mRNA expression in fetal tissues such as the liver, kidney and skeletal muscle (Kind *et al.*, 1995). Whilst there is evidence for a significant autocrine/paracrine role of IGF1 in adipose tissue proliferation (Faust *et al.*, 1978; Lau *et al.*, 1990; Peter *et al.*, 1993; Considine *et al.*, 1996a; Marques *et al.*, 1998; Marques *et al.*, 2000; Butler *et al.*, 2002) and differentiation, the effect of placental restriction on the expression of IGF1 or insulin-like growth factor-2 (IGF2) or on the ligand and clearance receptors, IGF1R and IGF2R in fetal adipose tissue, is not known. We have therefore used the placentally restricted (PR) fetal sheep as a model to test the hypothesis that poor fetal growth results in a decreased expression of IGF1 mRNA in perirenal adipose tissue, the major fetal adipose depot at 140-145d gestation. We further hypothesise that PR of fetal substrate supply will result in an altered pattern of expression of adipogenic, lipogenic and adipokine genes in the visceral adipose tissue before birth. We have therefore investigated the effect of PR on the expression of IGF1, IGF2, IGF1R, IGF2R, PPARγ and RXRα, LPL, glycerol-3-phosphate dehydrogenase (G3PDH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), leptin, and adiponectin in fetal perirenal adipose tissue.

2.3 METHODS

2.3.1 ANIMALS AND SURGERY

All procedures were approved by The University of Adelaide Animal Ethics Committee. Fifteen Merino ewes were used in the study. Nine non-pregnant ewes underwent surgery to remove the majority of endometrial caruncles from the uterus, leaving 3 to 8 caruncles in each horn in order to induce experimental restriction of placental and fetal growth (Robinson *et al.*, 1979).

Surgery was performed on control (n=6) and carunclectomised (n=9) pregnant ewes under aseptic conditions between 109 and 124 days (d) of gestation (term = 147 $\pm$ 3 d) with general anaesthesia induced by sodium thiopentane (1.25 g i.v.; Pentothal; Rhone Merieux, Pinkenba, Qld, Australia) and maintained with 2.5-4% (v/v) halothane (Fluothane; ICI, Melbourne, Vic, Australia) in oxygen. Vascular catheters were implanted in a maternal jugular vein, a fetal carotid artery and jugular vein, and the amniotic cavity as previously described (Edwards *et al.*, 2001). Catheters were filled with heparinised saline, and the fetal catheters were exteriorized through an incision made in the ewes' flank. During surgery, ewes and fetuses received a 2 ml i.m. injection of antibiotics (procaine penicillin [250 mg/ml], dihydrostreptomycin [250 mg/ml], and procaine hydrochloride [20 mg/ml]; Penstrep Illium; Troy Laboratories, Smithfield, NSW, Australia). Ewes were housed in individual pens in rooms with a 12 h light / dark cycle with lights on at 7

am and a daily temperature range between 19 and 22 °C. Ewes were fed once daily at 1100 h with 1 kg lucerne chaff (85% dry matter (DM), metabolisable energy (ME) content = 8.3 MJ/kg DM) and 0.3 kg concentrated pellets containing: straw, cereal, hay, clover, barley, oats, lupins, almond shells, oat husks and limestone (90% DM, ME content = 8.0 MJ/kg DM; Johnson & Sons, Kapunda, SA, Australia). This diet provided 100 % of the energy requirements for the maintenance of a ewe bearing a singleton pregnancy, as specified by the Ministry of Agriculture, Fisheries and Food, England (1975). Water was provided ad libitum. Animals were allowed to recover from surgery for at least 4 d before collection of fetal and maternal blood samples commenced.

2.3.2 Blood Sampling Protocol

Fetal arterial (3.5 ml) blood samples were collected between 0800 and 1100 h, before the ewes were fed, three times each week between 116 and 140 d of gestation. Blood samples were centrifuged at 1500g for 10 min and plasma separated into aliquots and stored at -20°C. At times fetal blood samples could not be collected due to technical problems (primarily related to blocked vascular catheters). Fetal arterial blood (0.5 ml) samples were also collected for the measurement of arterial blood gas status (ABL 520 blood gas analyzer; Radiometer, Copenhagen, Denmark).

2.3.3 Tissue Collection

Ewes were killed between 140 and 145 days of pregnancy with a lethal overdose of sodium pentobarbitone (Virbac Pty Ltd, Peakhurst, NSW, Australia). Fetuses

were delivered by hysterectomy, weighed, and killed by decapitation. Fetal perirenal adipose tissue (PAT) was dissected and weighed, and samples from control (n=6) and PR (n=8 out of 9) fetuses were frozen in liquid nitrogen and stored at -80°C for subsequent gene analysis.

2.3.4 PLASMA NUTRIENT AND HORMONE ASSAYS

2.3.4.1 Non-Esterified Fatty Acids (NEFAs)

Plasma NEFAs were measured by an *in vitro* enzymatic colorimetric method (Wako Pure Chemicals Industries Ltd, Osaka, Japan). The method relies upon the acylation of coenzyme A by the fatty acids in the presence of added acyl-CoA synthetase. The acyl-CoA thus produced is oxidised by added acyl-CoA oxidase with generation of hydrogen peroxide. Hydrogen peroxide, in the presence of peroxidase permits the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA) with 4-aminoantipyrine to form a purple coloured adduct which can be measured colorimetrically at 550nm (Konelab 20, Program Version 6.0 automated analysis system, Thermo Fisher Scientific, Suwanee, USA). The sensitivity of the assay was 0.25 mEq/l and the intra- and inter assay coefficients of variation (CVs) were both < 10 %.

2.3.4.2 Glucose Enzymatic Analysis

Plasma glucose concentrations were measured by enzymatic analysis using hexokinase and glucose-6-phosphate dehydrogenase to measure the formation of NADH photometrically at 340 nm (Konelab 20, Program Version 6.0

automated analysis system, Thermo Fisher Scientific, Suwanee, USA). The sensitivity of the assay was 0.5 mmol/l and the intra- and inter assay CVs were both < 5%.

2.3.4.3 Insulin Radioimmunoassay

Fetal plasma insulin concentrations were measured in control (n=6) and carunclectomised (n=6) fetuses using a commercial kit (Phasadeph radioimmunoassay kit; Pharmacia & Upjohn, Uppsala, Sweden). The detection range of the assay was 1.5-240 μU ml^{-1}. Guinea pig anti-insulin antisera and [^{125}**I**]human insulin (100μl) were added to plasma samples (100μl), which were then incubated for 2 h at room temperature before the addition of 2 ml of sheep anti-guinea pig immunoglobulin G. Samples were allowed to stand at room temperature for a further 30 min before being centrifuged at 1500 X g for 10 min as described previously (Edwards *et al.*, 2001). The inter- and intraassay CVs were < 10%.

2.3.4.4 Leptin ELISA

Plasma leptin concentrations were measured in control (n=6) and carunclectomised (n=6) fetuses using a competitive ELISA (Kauter *et al.*, 2000). Briefly, an ELISA plate was pre-incubated with recombinant bovine leptin in 50 μL of 0.1 M bicarbonate buffer and blocked with 200 μL of 5 % skim milk in ELISA buffer. Chicken anti-recombinant bovine leptin antiserum (50 μL) was added to the wells, followed by the addition of samples (100 μL) in duplicate. Following an

overnight incubation at 37 °C, a biotinylated phosphatase-Strepavidin conjugate (Amrad Biotech, Boronia, Vic, Australia) was added, incubated for 1 h, and the plate developed wih p-nitrophenylphosphate disodium salt hexahydrate. The sensitivity of the assay was 0.25 ng/ml and the intra- and inter- assay CVs were both $<$ 15.0 %.

2.3.5 ISOLATION OF RNA, PRODUCTION OF CDNA AND QRT-PCR

RNA was extracted from 100 mg perirenal adipose tissue (Tri Reagent, Prod T9424, Sigma) from 8 fetuses in the PR group and 6 fetuses in the Control group. RNA was treated for genomic DNA contamination using Ambion Dnase1 and after enzyme deactivation, the RNA was run through a secondary purification process using the RNeasy Mini Kit (QIAGEN, Basel, Switzerland). The quality and concentration of the RNA was determined by measuring the absorbance at 260 and 280 nm, and RNA integrity was confirmed by agarose gel electrophoresis. cDNA was then synthesised using the purified RNA ($\approx$ 5 μg) and Superscript 3 reverse transcriptase (Invitrogen Australia Pty Limited, Mount Waverley, Australia) with random hexamers.

The relative expression of PPARγ, RXRα, LPL, G3PDH, GAPDH, leptin, and adiponectin mRNA transcripts (Muhlhausler *et al.*, 2007) were measured for each fetus by quantitative real time reverse transcription-PCR (qRT-PCR) using the Sybr Green system in an ABI Prism 7900 Sequence Detection System (PE Applied Biosystems, Foster City, CA). Each qRT-PCR reaction well (5 μl total volume) contained: 2.5 μl 2x Sybr Green Master Mix (PE Applied Biosystems,

Foster City, CA); 0.25 μl of each primer giving a final concentration of 450 nM, 1.0 μl of molecular grade H_20 and 1.0 μl of a 1:10 dilution of the stock template.

The relative expression of IGF1, IGF2, IGF1R and IGF2R mRNA transcripts (MacLaughlin *et al.*, 2007) were measured for each fetus by qRT-PCR using the Sybr Green system in an ABI Prism 7300 Sequence Detection System (PE Applied Biosystems, Foster City, CA). Each qRT-PCR reaction well (10 μl total volume) contained: 6 μl 2x Sybr Green Master Mix (PE Applied Biosystems, Foster City, CA); 1ul of each primer giving a final concentration of 450 or 900 nM, 2.0 μl of molecular grade H_20 and 1.0 μl of a 50 ng/ul dilution of the stock cDNA template.

Primers for each transcript were designed with the aid of Primer Express software (PE Applied Biosystems, Foster City, CA) and where possible one primer of each pair was positioned over a splice site to prevent amplification of any residual genomic DNA. For each transcript RT-PCR was performed using the appropriate primers to ensure the amplification of a single amplicon of the expected size. The product was viewed on an ethidium bromide stained electrophoresis gel. Each amplicon, which was designed to be approximately 200 bp in length, was sequenced to ensure the authenticity of the DNA product and qRT-PCR melt curve analysis was performed to demonstrate amplicon homogeneity. Controls containing no reverse transcriptase were also used. For the qRT-PCR measurements, the cDNA primer concentrations were equivalent for all genes and the amplification efficiencies were 0.997 – 0.999. A constant

amount of cDNA equating to 10ng of total RNA was used for each qRT-PCR measurement and four technical replicates were performed for each gene. For all qRT-PCR runs the cycling conditions consisted of 40 cycles of 95 °C for 15 min and 60 °C for 1 min.

The abundance of each mRNA transcript was measured and expression relative to that of Acidic Ribosomal Protein P0 (ARP-P0) was calculated using the comparative threshold cycle (C_t) method (Q-gene qRT-PCR analysis software) (Muller *et al.*, 2002).

2.3.6 STATISTICAL ANALYSES

The effects of placental restriction on fetal size, plasma hormone and nutrient concentrations, the absolute and relative weights of perirenal fat, and the expression of adipose genes were determined using a Students unpaired t test. Relationships between fetal size, plasma hormone and nutrient concentrations or fat mass and perirenall adipose tissue (PAT) gene expression were determined using linear regression and partial correlation analyses. There was no effect of gestational age on any of the outcomes reported in this study. All data are presented as the mean ± SEM. A probability of <5% ($P<0.05$) was taken as the level of significance in all analyses.

Table 2.1: Primer sequences for qRT PCR

Primer Name (Accession Number)	Forward 5' - 3'	Reverse 5' - 3'	Amplicon size (bp)
RPLP0 (AF013214)	caaccctgaagtgcttgacat	aggcagatggatcagcca	220
18S rRNA (AY779625)	gtaacccgttgaaccccatt	ccatccaatcggtagtagcg	151
PPARg (AY179866)	atgtctcataatgccatcaggtt	gataacaaacggtgatttgtctgtc	225
RXRa (DQ100361)	cattttcgacagggtgctg	cttggcgaaccttcctgg	220
Leptin (NM173928)	atctcacacacgcagtccgt	ccagcaggtggagaaggtc	202
Adiponectin (NM174742)	atcaaactctggaacctcctatctac	ttgcattgcaggctcaag	232
LPL (M16966)	taccctgcctgaagtttccac	cccagtttcagccagactttc	302
G3PDH (BT020681)	gctttggcgacaacacca	agctgctcaatggactttcc	208
GAPDH (U85042)	cctggagaaacctgccaagt	gccaaattcattgtcgtacca	226
IGF1 (DQ152962)	ttggtggatgctctcgagttc	agcagcactcatccacgattc	116
IGF2 (M89789)	gcttcttgccttcttggcctt	tcggtttatgcggctggat	151
IGF1R (AY162434)	aagaaccatgcctgcagaagg	ggattctcaggttctgggcatt	104
IGF2R (AF327649)	gatgaaggaggctgcaaggat	cctgatgcctgtagtccagctt	103

2.4 RESULTS

2.4.1 THE EFFECT OF PLACENTAL RESTRICTION ON ARTERIAL PO_2, PLASMA GLUCOSE AND INSULIN CONCENTRATIONS, FETAL GROWTH AND FAT MASS

Mean arterial PO_2 ($P < 0.001$), plasma glucose ($P < 0.01$) and plasma insulin concentrations ($P < 0.02$) were lower in PR compared with control fetuses during late gestation (116-145d) (Table 2). There was no effect, however of placental restriction on either the mean plasma NEFA or leptin concentrations during late gestation (Table 2). PR fetuses had a lower weight ($P < 0.0001$), crown rump length (CRL) ($P < 0.0001$), and absolute mass of perirenal fat ($P < 0.01$) at 140-145d gestation compared with controls, but there was no difference in the relative mass of perirenal fat between the 2 groups (Table 2).

2.4.2 IGF1, IGF2, IGF1R AND IGF2R mRNA EXPRESSION IN FETAL PAT

IGF1 mRNA expression was significantly lower ($P<0.01$) in perirenal adipose tissue (PAT) from PR fetuses compared with Controls at 140-145d gestation (Figure 1A). IGF1 mRNA expression was directly related to arterial PO_2 ($r^2=0.61$, $P<0.001$), mean plasma glucose ($r^2= 0.47$, $P < 0.05$) and insulin concentrations ($r^2=0.42$, $P < 0.05$) during late gestation. Partial correlation analysis showed that these relationships were interdependent.

There was no difference in the expression of IGF2, IGF1R, or IGF2R mRNA in PAT from PR and Control fetal sheep (Table 3). When data from both treatment groups were combined, the expression of IGF2 mRNA ($r^2=0.32$, $P < 0.05$) and

IGF2R mRNA (r^2=0.34, P < 0.05) were each directly related to the expression of PPARγ mRNA.

2.4.3 LEPTIN, ADIPONECTIN, PPARγ, RXRα, LPL, G3PDH AND GAPDH MRNA IN FETAL PAT

Leptin mRNA expression in PAT was lower (P < 0.05) in PR fetuses when compared with Controls (Figure 1B) and was directly related to the absolute mass of perirenal fat (r^2=0.35, P < 0.01). Leptin and PPARγ mRNA expression were also directly related (r^2=0.55, P < 0.01).

There was no difference in the expression of adiponectin, PPARγ, RXRα, LPL, G3PDH and GAPDH mRNA in PAT between the PR and Control groups at 140-145d gestation (Table 2). Adiponectin mRNA expression was strongly related to G3PDH mRNA expression (r^2=0.64, P < 0.001) and PPARγ mRNA expression was related to the expression of both RXRα (r^2=0.31, P < 0.05) and adiponectin mRNA (r^2=0.35, P < 0.05).

Table 2.2 Fetal weight, crown rump length, perirenal fat mass and mean fetal arterial PO_2, and plasma NEFA, glucose, insulin and leptin concentrations (116-144d), in PR and Control fetuses.

FETAL OUTCOME	CONTROL (n = 6)	PR (n = 6-9)
Gestational Age (days)	144.8 ± 0.2	143.6 ± 0.7
Fetal Weight (kg)	5.13 ± 0.24	2.90 ± 0.32 ****
Fetal Crown Rump Length (cm)	60.6 ± 1.5	48.6 ± 1.6 ****
Absolute mass of perirenal fat (g)	21.6 ± 1.8	13.4 ± 1.3 **
Relative mass of perirenal fat (g/kg)	4.20 ± 0.29	4.53 ± 0.57
Mean arterial PO_2 (mmHg)	21.8 ± 1.3	13.8 ± 0.5 ***
Mean plasma glucose (mmol/L)	1.01 ± 0.03	0.68 ± 0.07 **
Mean plasma insulin (uU/ml)	8.65 ± 0.76	5.16 ± 0.83 *
Mean plasma NEFA (uEq/ml)	0.054 ± 0.004	0.053 ± 0.004
Mean plasma leptin (ng/ml)	2.28 ± 0.31	1.96 ± 0.23

* denotes a significant difference between control and PR group ($P < 0.05$). ** denotes a significant difference between control and PR group ($P < 0.01$). *** denotes a significant difference between control and PR group ($P < 0.001$). **** denotes a significant difference between control and PR group ($P < 0.0001$).

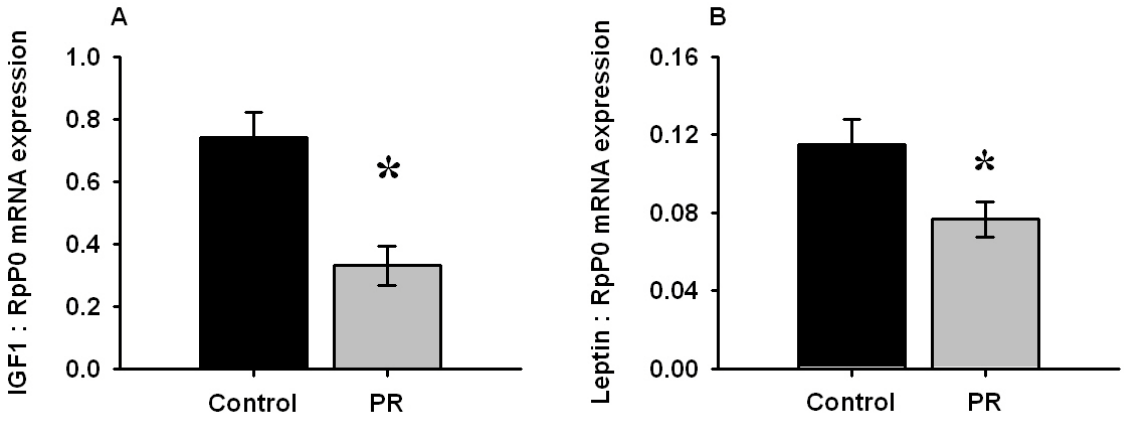

Figure 2.1 The expression of IGF1 and Leptin mRNA in fetal perirenal adipose tissue in Control fetuses.

There was a signficantly lower expression of (A) IGF1mRNA and (B) Leptin mRNA in the perirenal adipose tissue of PR fetuses (shaded bar) compared to Control fetuses (closed bar) at 140-145d gestation. * denotes significance.

Table 2.3: IGF1, IGF2, IGF1R, IGF2R, PPARγ, RXRα, Adiponectin, LPL, Glycerol-3-Phosphate Dehydrogenase (G3PDH) and Glyceraldehyde-3-Dehydrogenase (GAPDH) mRNA expression in perirenal adipose tissue in Control and PR fetal sheep.

Transcript (mean normalised expression)	CONTROL (n = 6)	PR (n = 8)
IGF2	0.81 ± 0.10	0.66 ± 0.05
IGF1R	0.005 ± 0.000	0.004 ± 0.000
IGF2R	0.004 ± 0.000	0.003 ± 0.000
PPARγ	1.16 ± 0.14	0.94 ± 0.06
RXRα	0.07 ± 0.01	0.05 ± 0.00
Adiponectin	2.49 ± 0.36	2.79 ± 0.29
LPL	0.28 ± 0.03	0.31 ± 0.05
G3PDH	0.53 ± 0.07	0.66 ± 0.08
GAPDH	1.91 ± 0.20	1.84 ± 0.10

2.5 DISCUSSION

In this study we have investigated the extent to which the expression of the insulin-like growth factors (IGF1, IGF2), their receptors (IGF1R, IGF2R) and other genes involved in adipogenesis, (PPARγ, RXRα) adipocyte metabolism (G3PDH, GAPDH) and adipokine signalling (leptin, adiponectin) are regulated by placental substrate supply during fetal life. We have demonstrated that the expression of IGF1 and leptin mRNA in adipose tissue were each decreased in response to placental restriction of fetal substrate supply.

2.5.1 THE EFFECT OF PLACENTAL RESTRICTION ON NUTRIENT SUPPLY AND FETAL GROWTH

Consistent with previous studies of the placentally restricted (PR) sheep model, PR fetuses in the current study were chronically hypoxaemic, hypoglycaemic and hypoinsulinaemic. The changes in fetal blood gas status and nutrient supply in PR sheep fetuses are similar to those measured in cordocentesis studies of SGA human infants (Economides *et al.*, 1991). In the current study the absolute mass of perirenal fat in PR fetuses at 140d gestation was significantly lower than that of control fetuses, but when fat mass was expressed relative to body weight there was no difference in fat mass between PR and control fetuses at 140d gestation. In a previous study it was shown that PR lambs have an increased perirenal and visceral fat mass relative to body weight at 45 days of postnatal life (De Blasio *et al.*, 2007b). Furthermore, there was an inverse relationship between the insulin sensitivity of glucose, circulating amino acids and free fatty acid metabolism and

birth weight in both PR and control lambs, such that a lower birth weight corresponded with a higher whole body insulin sensitivity at 45 days of postnatal life (De Blasio *et al.*, 2007a).

2.5.2 The Decrease in IGF1 mRNA Expression in Fetal Perirenal Adipose Tissue After Placental Restriction of Fetal Growth

We have shown that PR results in a decreased expression of IGF1, but not IGF2, IGF1R or IGF2R mRNA expression in fetal perirenal adipose tissue at 140-145d gestation. It has previously been reported that IGF1 mRNA expression is decreased in the fetal liver, kidney and skeletal muscle, and that circulating IGF1 concentrations are decreased in PR, compared to control fetuses at 121d gestation (Owens *et al.*, 1994; Kind *et al.*, 1995). It is currently unknown whether IGF1 mRNA expression in adipocytes is directly related to protein expression, however in hepatocytes IGF1 has been shown to be readily synthesised and secreted, with little preformed or stored (Mayer & Schalch, 1983; Schwander *et al.*, 1983). It is therefore possible that the decreased expression of IGF1 mRNA in the perirenal adipose tissue of PR fetuses may have consequences for the stimulation of the proliferation and differentiation of perirenal adipose tissue. At physiologic levels IGF1 stimulates both proliferation and differentiation of preadipocytes in cell culture, and along with cAMP and glucocorticoids, is required for induction of differentiation in both serum-containing and serum-free media (Gregoire *et al.*, 1998; Soret *et al.*, 1999). Furthermore, preadipocyte proliferation can be prevented by stripping IGF1 from adipose tissue conditioned culture medium (Marques *et al.*, 2000). It would therefore be predicted that PR

lambs would, at parturition, have a decreased number of adipocytes and therefore might have an altered capacity to respond to the transition from a limited to an abundant nutritional environment.

It has been shown previously that the induction of terminal differentiation of preadipocyte cultures by IGF1, or supraphysiological concentrations of insulin acting at the IGF1-receptor (Mur *et al.*, 2003) results in a marked increase in the expression of PPARγ mRNA (Reul *et al.*, 1997; Gregoire *et al.*, 1998; Picard & Auwerx, 2002). PPARγ in a heterodimer with RXRα acts as a transcription factor to initiate transcription of the regulatory genes involved in lipid accumulation and metabolism, particularly LPL, leptin and adiponectin, which all have functional peroxisome proliferator response elements (PPREs) in their promoter regions (Schoonjans *et al.*, 1996; Hollenberg *et al.*, 1997; Iwaki *et al.*, 2003). There is evidence that the effect of IGF1R signalling on PPARγ mRNA expression may be via the p38MAPK downstream signaling pathway (LeRoith *et al.*, 1995), which modulates the transcriptional activity of C/EBPβ, an adipocyte differentiation factor that induces PPARγ mRNA expression (Aouadi *et al.*, 2006). Whilst IGF1 expression in perirenal adipose tissue was decreased in PR fetal sheep, there was no effect of PR on PPARγ mRNA expression in this tissue. Interestingly, however, the expression of IGF2 related to the expression of PPARγ mRNA in PAT at 140-145d gestation, which may indicate that during fetal life IGF2 binding at the IGF1R significantly contributes to the regulation of PPARγ mRNA expression in adipose tissue. Alternatively PR may act to alter the expression of the IGF binding proteins and hence influence IGF bioavailability within the

perirenal adipose tissue. There was no effect of PR on the expression of RXRα, adiponectin LPL, G3PDH, or GAPDH in fetal perirenal adipose tissue although there was a positive relationship between PPARγ and adiponectin mRNA indicating that there may be functional activation of the PPRE in the promoter region of the adiponectin gene in perirenal adipose tissue of PR and normally grown fetal sheep in late gestation.

2.5.3 The Decrease in Leptin mRNA Expression in Fetal Perirenal Adipose Tissue After Placental Restriction of Fetal Growth

There was a significant decrease in the expression of leptin mRNA in the perirenal adipose tissue of PR fetal sheep at 140-145d gestation. It has previously been shown that the expression of leptin mRNA and the size of lipid locules in the perirenal adipocytes were increased in the hyperglycaemic sheep fetuses of overnourished ewes (Muhlhausler *et al.*, 2007). Therefore, the reduction in leptin mRNA expression in the PR fetus could reflect a decrease in lipid content of the fetal perirenal adipose tissue.

The leptin gene promoter region contains C/EBP (He *et al.*, 1995; Isse *et al.*, 1995; Taniguchi *et al.*, 2002), SP1, cAMP, glucocorticoid (Gong *et al.*, 1996), and hypoxia (Ambrosini *et al.*, 2002; Grosfeld *et al.*, 2002) response elements in addition to the PPRE. In the present study, there was a relationship between the expression of leptin and PPARγ mRNA in perirenal fat, which could reflect either direct activation of the PPRE in the promoter of the leptin gene, or indirect

activation of both the leptin and PPARγ promoters by the same transcription factor, such as C/EBP

In contrast to adult life, there was no relationship between the expression of leptin mRNA in adipose tissue and circulating plasma leptin concentrations in the fetal sheep at 140-145d gestation. It has been suggested that leptin may be derived from other fetal tissues, such as the liver and lung (Ehrhardt *et al.*, 2002), or from the placenta or maternal circulation (Bispham *et al.*, 2003). It is possible that after the transition to postnatal life and the induction of adipose tissue as the main source of circulating leptin that the reduction in leptin mRNA expression in the adipose tissue of lambs which were placentally restricted might result in a functional immaturity of the leptin-fat mass axis with consequences for appetite regulation, lipid metabolism and insulin sensitivity. Interestingly, evidence for a reduction in basal circulating leptin and higher relative increases in BMI have been described in adults who were born IUGR (birth weight < 3rd percentile) (Leger *et al.*, 1998; Jaquet *et al.*, 2001a).

2.5.4 SUMMARY

In summary, we have demonstrated that the expression of both IGF1 and leptin mRNA in fetal adipose tissue is decreased by placental restriction of fetal substrate supply. It is possible that a reduced expression of IGF1 mRNA in adipose tissue is associated with a direct reduction in the concentration of IGF1 available for paracrine activation of differentiation in developing adipose tissue, and therefore the decreased expression of IGF1 mRNA in the perirenal adipose

tissue of PR fetuses might have consequences for the timing and magnitude of the proliferation and differentiation processes during adipose tissue development. The reduction in leptin mRNA expression in perirenal adipose tissue of fetal sheep raises the possibility that events that occurred *in utero* which result in a growth restricted fetus may result in a functional immaturity of the leptin – fat mass axis with consequences for the regulation of lipid metabolism and insulin sensitivity during postnatal life.

3. Intra-uterine growth restriction (IUGR) and the sex specific programming of Peroxisome Proliferator Activated Receptor γ (PPARγ) and Leptin mRNA expression in perirenal adipose tissue in the lamb.

3.1 SUMMARY

A world-wide series of epidemiological studies has demonstrated that there is an association between being born small and the risk of visceral obesity and insulin resistance in adult life. We have investigated the effect of intrauterine growth restriction (IUGR), defined as a birth weight less than 2 standard deviations below the mean of a cohort of singleton Merino lambs on gene expression in visceral fat in the lamb at 21d of age, before the emergence of an obese phenotype. Perirenal fat mass was greater in females than males, independent of birth weight. Plasma insulin concentrations during the first 24h after birth predicted the size of the adipocytes and expression of adiponectin in visceral adipose tissue in both males and females at 21d. In males, PPARγ and leptin expression in perirenal visceral fat were significantly lower in IUGR compared to Control birth weight lambs at 21d. Thus the nutritional environment before and immediately after birth programs adipocyte growth and gene expression in visceral adipose tissue. The differential effect of sex and birth weight on PPARγ and leptin expression in visceral fat may be important in the subsequent development of visceral obesity and the insulin resistant phenotype in later life.

3.2 INTRODUCTION

A world-wide series of epidemiological and clinical studies has demonstrated that there are associations between the patterns of growth in fetal and early postnatal life and the risk of insulin resistance, type 2 diabetes and obesity in adult life (Barker *et al.*, 1993; McCance *et al.*, 1994; Lithell *et al.*, 1996; Leger *et al.*, 1997; McKeigue *et al.*, 1998; Bavdekar *et al.*, 1999; Jaquet *et al.*, 2000; McMillen & Robinson, 2005). Insulin resistance in those born small for gestational age (SGA $<10^{th}$ centile for gestational age) is markedly amplified by obesity during adulthood (Hales *et al.*, 1991; Valdez *et al.*, 1994; Lithell *et al.*, 1996; Jaquet *et al.*, 2000). Furthermore, whilst SGA infants have a reduced body fat mass at birth (Enzi *et al.*, 1981), most SGA infants undergo a period of accelerated postnatal growth, termed 'catch-up' growth, during the first few years (Fitzhardinge & Steven, 1972; Albertsson-Wikland *et al.*, 1993) and show a relative increase in body fat mass from as early as 2 -12 months of age (Hediger *et al.*, 1998). Infants who were growth restricted *in utero* and then experienced postnatal catch-up growth also have a higher body mass index (BMI), fat mass and truncal fat distribution during childhood (Ong *et al.*, 2000) and in adult life (Law *et al.*, 1992; Parsons *et al.*, 2001). Whilst catch-up growth is associated with increased insulin sensitivity, people who are thin at birth and develop obesity in childhood or adulthood have the highest risk of insulin resistance in later life (Curhan *et al.*, 1996; Whincup *et al.*, 1997; Bavdekar *et al.*, 1999; Eriksson *et al.*, 2001; Veening *et al.*, 2002; Levy-Marchal *et al.*, 2004). There is also evidence

that development of obesity after fetal growth restriction is associated with insulin resistance of adipose tissue (Jaquet *et al.*, 1998; Jaquet *et al.*, 1999; Phillips *et al.*, 1999; Jaquet *et al.*, 2000).

Peroxisome Proliferator-Activated Receptor (PPAR)γ is a nuclear hormone receptor that plays a central role in the regulation of adipogenesis and lipogenesis and control of the synthesis and secretion of adipokines such as adiponectin, which in turn regulate peripheral insulin sensitivity. Activation of PPARγ also enhances peripheral insulin sensitivity by reducing circulating FFA levels (Ferre, 2004), through induction of lipoprotein lipase (LPL) and promotion of triglyceride storage. Interestingly, there are also interactions between the effects of Pro12Ala polymorphisms of the Peroxisome Proliferator-Activated Receptor (PPAR)γ2 gene, birthweight and insulin resistance implicating a potential role for this gene in the pathway from fetal growth restriction to adult obesity (Eriksson *et al.*, 2002; Jaquet *et al.*, 2002; Eriksson *et al.*, 2003). Whilst the level of expression and activity of PPARγ may be important in determining the association between the perinatal environment and insulin resistance, it is not known, however, whether a sub-optimal nutritional environment before birth programs altered expression of PPARγ or other regulatory genes within the adipocyte.

In the sheep or pig, adipogenesis and lipogenesis occur before birth as in the human, and low birth weight offspring also grow faster in the postnatal period and have a higher proportion of body fat than their higher birth weight

counterparts in later life (Greenwood *et al.*, 1998; Poore & Fowden, 2004; Louey *et al.*, 2005). In a study on a cohort of lambs born after experimental restriction of placental growth, there was an increase in relative visceral fat mass and a positive relationship between the action of insulin to suppress circulating free fatty acids and the mass of visceral fat at 6 weeks of age (De Blasio *et al.*, 2007a). We have therefore used the newborn lamb as a model to test the hypothesis that intra-uterine fetal growth restriction (IUGR) in association with rapid postnatal growth results in an altered pattern of expression of adipogenic, lipogenic and adipokine genes in visceral fat before the emergence of the obese phenotype. In order to generate a cohort of low birth weight lambs that would fit the classification of IUGR, we extended the normal birth weight distribution by experimental induction of placental growth restriction from conception (McMillen *et al.*, 2001). We have therefore investigated the extent to which changes in growth of the adipocyte and the expression of retinoic X receptor (RXR)α, PPARγ, leptin, adiponectin, LPL, glycerol-3-phosphate dehydrogenase (G3PDH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) are determined by IUGR as summarised by birth weight and the plasma insulin, leptin and glucose concentrations measured within 24h of birth and by postnatal factors, including the postnatal growth rate and circulating hormonal and nutrient levels during the first three weeks of life.

3.3 METHODS

3.3.1 ANIMALS AND SURGERY

All procedures were approved by The University of Adelaide Animal Ethics Committee. Twenty-two Merino ewes were used in the study. Six non-pregnant ewes underwent surgery to remove the majority of endometrial caruncles (>70) from the uterus, leaving 3 to 8 caruncles in each horn in order to induce experimental restriction of placental and fetal growth (Robinson *et al.*, 1979).

At around 110d gestation all ewes were brought into the Central Animal House and were fed once daily at 1000 h with 1 kg lucerne chaff (85% dry matter (DM), metabolisable energy (ME) content = 8.3 MJ/kg DM) and 0.3 kg concentrated pellets containing: straw, cereal, hay, clover, barley, oats, lupins, almond shells, oat husks and limestone (90% DM, ME content = 8.0 MJ/kg DM; Johnson & Sons, Kapunda, SA, Australia). Ewes with a twin pregnancy were provided with an additional 0.2 kg of concentrated pellets. This diet provided 100 % of the energy requirements for the maintenance of a pregnant ewe bearing a singleton or twins, as specified by the Ministry of Agriculture, Fisheries and Food, England (1975). After parturition, an additional 1 kg lucerne chaff was offered at 1500 h to the ewe. The time of birth was recorded and the day of birth was taken to be Day 1 (when birth occurred overnight Day 1 was taken to start at 0900 h.) All ewes and lambs were housed under a 12 h light / dark cycle with lights on at 7 am and a daily temperature range between 19 and 22 °C.

3.3.2 BLOOD SAMPLING PROTOCOL, GROWTH MEASUREMENTS AND POST MORTEM

Lambs were weighed and crown-rump length measured daily between 1000 - 1400 h. Venous blood samples were collected in chilled tubes after approximately 60 min of non-suckling on alternate days between 0900 h – 1300 h, beginning on Day 1. The time that the ewes' teats were withheld from the lamb before a jugular venous blood sample was taken was recorded for each sample, and denoted as Time Suckling Withheld (TSW) (Table 1). There was no difference in TSW during both day 1, and weeks 1, 2, and 3 between birth weight groups or sex (Table 3.1). All blood samples were centrifuged at 1500xg for 10 min, and plasma separated into aliquots and stored at -20°C. On day 21, lambs were killed with an overdose of sodium pentobarbitone (Virbac Pty Ltd, Peakhurst, NSW, Australia) and all perirenal, retroperitoneal and omental adipose tissue was dissected and weighed. Samples of perirenal/retroperitoneal and omental adipose tissue were frozen in liquid nitrogen and stored at -80°C for subsequent gene analysis for subsequent gene analysis and fixed in 0.4% paraformaldehyde buffer for histological analysis.

Table 3.1: The mean amount of time that lambs were prevented from suckling prior to collection of venous blood samples during wks 1-3 of age.

Collection Period	Time suckling prevented (min)			
	Control	**Control**	**IUGR**	**IUGR**
	FEMALE (n = 6)	**MALE (n = 8)**	**FEMALE (n = 5)**	**MALE (n = 4)**
Day 1	68 ± 13.6	50 ± 16.7	77.5 ± 12.5	48.3 ± 25.2
Week 1	66.5 ± 6.5	55.3 ± 11.8	58.5 ± 11.8	51.0 ± 9.9
Week 2	59.8 ± 3.7	64.3 ± 3.7	56.2 ± 1.9	63.5 ± 10.8
Week 3	69.4 ± 7.1	63.9 ± 4.8	72.2 ± 7.0	61.0 ± 8.7

3.3.3 PLASMA NUTRIENT AND HORMONE ASSAYS

3.3.3.1 Non-Esterified Fatty Acids (NEFAs)

Plasma NEFAs were measured by an *in vitro* enzymatic colorimetric method (Wako Pure Chemicals Industries Ltd, Osaka, Japan). The method relies upon the acylation of coenzyme A by the fatty acids in the presence of added acyl-CoA synthetase. The acyl-CoA thus produced is oxidised by added acyl-CoA oxidase with generation of hydrogen peroxide. Hydrogen peroxide, in the presence of peroxidase permits the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA) with 4-aminoantipyrine to form a purple coloured adduct which can be measured colorimetrically at 550nm (COBAS MIRA automated analysis system, Roche Diagnostica, Basel, Switzerland). The

sensitivity of the assay was 0.25 mEq/l and the intra- and inter assay coefficients of variation (CVs) were both < 10 %.

3.3.3.2 Glucose Enzymatic Analysis

Plasma glucose concentrations were measured by enzymatic analysis using hexokinase and glucose-6-phosphate dehydrogenase to measure the formation of NADH photometrically at 340 nm (COBAS MIRA automated analysis system, Roche Diagnostica, Switzerland). The sensitivity of the assay was 0.5 mmol/l and the intra- and inter assay CVs were both < 5%.

3.3.3.3 Insulin Radioimmunoassay

Plasma insulin concentrations were measured using a radioimmunoassay (Linco Research, Inc., Missouri, USA). The sensitivity of the assay was 0.1 ng/ml. Samples (10 µl) were assayed in duplicate and added to borosilicate glass tubes with 100 µl of hydrated ^{125}I-Insulin and guinea-pig anti-rat insulin antibody and incubated overnight at 4°C. Precipitating reagent (1ml) was added and tubes were centrifuged for 25 min at 2000xg, then aspirated and the total radioactivity in the precipitate was counted. The intra and inter assay CVs were both <10%.

3.3.3.4 Leptin ELISA

Plasma leptin concentrations were measured using a competitive ELISA previously validated for use in sheep plasma (Kauter *et al.*, 2000). Briefly, an ELISA plate was pre-incubated with recombinant bovine leptin in 50 µl of 0.1 M bicarbonate buffer and blocked with 200 µl of 5 % skim milk in ELISA buffer. Chicken anti-recombinant bovine leptin antiserum (50 µl) was added to the wells,

followed by the addition of samples (100 μL) in duplicate. Following an overnight incubation at 37 °C, a biotinylated phosphatase-Strepavidin conjugate (Amrad Biotech, Boronia, Vic, Australia) was added, incubated for 1 h, and the plate developed with p-nitrophenylphosphate disodium salt hexahydrate. The sensitivity of the assay was 0.5 ng/ml and the intra- and inter- assay CVs were <16%.

3.3.4 ADIPOSE TISSUE HISTOLOGY

Tissues were fixed in 4% paraformaldehyde buffer, washed and embedded in paraffin wax. Sections were cut (4 -5 μm) and stained with Hematoxylin and Eosin. Standard point counting techniques were used with Video Image Analysis using Video Pro software (Leading Edge, Adelaide, SA, Australia) to measure the area of 100 adipocytes within each fat depot (Muhlhausler *et al.*, 2002).

3.3.5 ISOLATION OF RNA, PRODUCTION OF CDNA AND QRT-PCR

RNA from perirenal adipose tissue (≈ 600mg) was isolated using Trizol reagent (Invitrogen Australia Pty Limited, Australia) and chloroform. RNA was treated for genomic DNA contamination using Ambion Dnase1, and after enzyme deactivation the RNA was run through a secondary purification process using the RNeasy Mini Kit (QIAGEN Pty Ltd -Australia, Doncaster, Australia). RNA integrity was confirmed by agarose gel electrophoresis. cDNA was then synthesised using the purified RNA (≈ 5 μg), Superscript 3 Reverse Transcriptase (Invitrogen Australia Pty Limited, Australia) and random hexamers

The relative expression of PPARγ, RXRα, leptin, adiponectin, LPL, G3PDH and GAPDH mRNA transcripts in perirenal/retroperitoneal adipose tissue (representative of visceral adipose tissue) were measured by quantitative real time reverse transcription-PCR (qRT-PCR) using the Sybr Green system in an ABI Prism 7900 Sequence Detection System (PE Applied Biosystems, Foster City, CA) (Gibson *et al.*, 1996; Heid *et al.*, 1996). All primers were designed with the aid of Primer Express (PE Applied Biosystems, Foster City, CA) software and where possible one primer of each pair was positioned over a spilce site to prevent amplification of any residual genomic DNA. For each transcript RT-PCR was performed using the appropriate primers (Table 2.1) to ensure the amplification of a single amplicon of the expected size. The product was viewed on an ethidium bromide stained electrophoresis gel. Each amplicon, which was designed to be approximately 200bp in length, was sequenced to ensure the authenticity of the DNA product and qRT-PCR melt curve analysis was performed to demonstrate amplicon homogeneity. Controls containing no reverse transcriptase were also used. For the qRT-PCR measurements, the primer concentrations were equivalent for all genes and the amplification efficiencies were 0.997 – 0.999. A constant amount of cDNA equating to 10ng of total RNA was used for each qRT-PCR measurement and four technical replicates were performed for each gene.

Each qRT-PCR reaction (5 μl total volume) contained: 2.5 μl 2x Sybr Green Master Mix (Applied Biosystems); 0.25 μl of each primer giving a final concentration of 450 nM, 1.0 μl of molecular grade H_20 and 1.0 μl of a 1:10

dilution of the stock template. The cycling conditions consisted of 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. At the end of each run, a dissociation melt curve was obtained.

The abundance of each mRNA transcript was expressed relative to that of Acidic Ribosomal Protein Large Subunit P0 (RPLP0) and calculated using Q-gene qRT-PCR analysis software (Muller *et al.*, 2002). The abundance of each mRNA transcript was also expressed relative to that of an additional reference gene, 18S ribosomal RNA. Correlational data of relative mRNA expression that could not be replicated when using both reference genes was not included and therefore only abundance relative to RPLP0 is reported.

3.3.6 STATISTICAL ANALYSIS

3.3.6.1 Definition of Intra-uterine Growth Restriction (IUGR)

A frequency distribution curve of birth weights of control Merino singleton lambs from a separate cohort (n=45) of animals born during the past 5 years was used to determine the birth weight cut off for IUGR in this population. The mean birth weight (± SD) of the singleton cohort was 5.63 ± 0.67 kg. Newborn lambs in the current study were classified as IUGR when their birth weight was greater than 2 SD below the cohort mean, equivalent to the 3rd percentile (IUGR: < 4.3 kg, n = 9) or normally grown if their birth weight was within 2 SD on either side of the mean (Control: 4.5 – 6.7 kg, n = 14). Using these criteria, 3 male and 3 female placentally restricted singleton lambs, a male and female pair of twin siblings and one female growth restricted control singleton were allocated into the IUGR

group. The Control group consisted of all control singleton lambs. Therefore there were 4 males and 5 females in the IUGR group and 8 males and 6 females in the Control group.

3.3.6.2 Calculation of Body Mass Index, Percentage daily growth rate and Visceral fat mass

The Body Mass Index (BMI) for each lamb was calculated (weight/crown-rump length^2). Daily growth rate (%) was calculated as body weight gained per day as a percentage increase from the previous days' body weight ((BodyWeight$_n$/ BodyWeight$_{n-1}$ x100)-100)%, where n equals the number of days after birth, between 1 and 21. The daily growth rate for each week was calculated as the mean of the daily growth rates for that given week. The total body weight gain (%) was calculated as body weight on Day 21 as a percentage increase of the body weight on Day 1 (BodyWeight$_{Day21}$/BirthWeight x 100)-100)/20. Visceral fat mass was calculated as the total of perirenal, retroperitoneal and omental fat.

3.3.6.3 Statistical tests

Parametric analyses were used in this study. The effects of IUGR and sex on the absolute and relative weights of perirenal and omental fat, size of perirenal and omental adipocytes, levels of plasma insulin, glucose, NEFA and leptin, and on expression of adipogenic genes were determined using two-way analysis of variance (ANOVA). Linear regression analysis also showed that there was no relationship between the TSW and either plasma insulin, glucose, NEFA or leptin concentrations on Day 1, or during weeks 1, 2, and 3. The Duncan's multiple range test was used post hoc to identify the differences between mean values.

Relationships between measures of visceral fat mass, adipocyte cell size or gene expression and either measures of prenatal growth (birth weight) or postnatal growth (postnatal growth rate and plasma hormonal and nutrient concentrations) were determined using linear regression analyses. All data are presented as the mean ± SEM and a probability of <5% ($P<0.05$) was taken as the level of significance.

3.4 RESULTS

3.4.1 THE EFFECT OF IUGR ON BIRTH WEIGHT, PLASMA NEFA DURING THE FIRST 24HR AND POSTNATAL GROWTH

IUGR lambs were lighter and shorter at birth ($P < 0.001$) (Table 3.2) and had a higher daily growth rate during week 1 ($P < 0.001$) (Figure 3.1) and week 2 ($P < 0.05$), but not week 3. There was no effect of sex on birth weight and there was an inverse relationship between birth weight and the daily growth rate during week 1 for both male ($r^2=0.58$, $P < 0.01$) and female ($r^2=0.52$, $P < 0.02$) lambs (Figure 3.1). At birth, there were no differences between male and female lambs in either weight or length.

IUGR lambs had lower plasma NEFA concentrations during the first 24h compared to Control lambs ($P < 0.05$) (Table 3.2) and there was a direct relationship between birth weight and plasma NEFA during the first 24h in both male and female lambs (Table 3.3). In female lambs there was a positive relationship between birth weight and plasma insulin concentrations during the

first 24h after birth (Table 3.3). The daily growth rate decreased ($P < 0.05$) between week 1 and 3 in both Control and IUGR groups (Figure 3.1). At 21d, IUGR lambs were lighter ($P < 0.001$) and shorter ($P < 0.05$) than Control lambs and there were no differences between male and female lambs in weight or length (Table 3.2).

3.4.2 Plasma NEFA, Glucose, Insulin and Leptin Concentrations During Weeks 1-3

During weeks 1-3, plasma NEFA concentrations were lower in IUGR than Control lambs. Plasma insulin and glucose concentrations were related throughout the first 3 weeks of life (mean concentrations; $r^2=0.41$, $P < 0.001$). Plasma insulin, but not glucose, concentrations were higher in males than females, in both the IUGR and Control groups (Table 3.2). There were no differences in circulating leptin concentrations between IUGR and Control lambs, or between males and females (Table 3.2).

Table 3.2: Size at Birth and Plasma Glucose and Insulin Concentrations During the First 24h After Birth and the 3 week Postnatal Period

	Control	**Control**	**IUGR**	**IUGR**
	FEMALE (n = 6)	**MALE (n = 8)**	**FEMALE (n = 5)**	**MALE (n = 4)**
Birth weight (kg)	5.65 ± 0.10	6.02 ± 0.18	3.78 ± 0.21*	3.85 ± 0.16*
Birth CRL (cm)	47.3 ± 0.8	47.4 ± 1.1	42.3 ± 1.3*	43.0 ± 0.4*
Birth BMI (kg/m^2)	0.25 ± 0.00	0.27 ± 0.01	0.21 ± 0.01*	0.21 ± 0.01*
24h Plasma NEFA (mEq/l)	0.98 ± 0.11	0.87 ± 0.09	0.70 ± 0.10*	0.65 ± 0.13*
24h Plasma insulin (ng/ml)	2.00 ± 0.31	2.57 ± 0.28 †	1.05 ± 0.35	2.65 ± 0.88 †
24h Plasma glucose (ng/ml)	5.52 ± 0.38	6.32 ± 1.18	4.34 ± 0.58	5.32 ± 0.61
Mean plasma NEFA (mEq/l)	0.71 ± 0.09	0.61 ± 0.06	0.50 ± 0.08*	0.47 ± 0.06*
Mean plasma insulin (ng/ml)	1.79 ± 0.25	2.31 ± 0.19 †	1.43 ± 0.18	2.69 ± 0.69 †
Mean plasma glucose (mMol/l)	5.73 ± 0.26	5.90 ± 0.28	5.66 ± 0.17	6.00 ± 0.41
Mean plasma leptin (ng/ml)	6.74 ± 0.33	5.18 ± 0.50	5.39 ± 0.60	5.67 ± 1.37
Total growth rate (wk 1-3) (%)	7.17 ± 0.27	6.65 ± 0.26	8.01 ± 0.23*	7.56 ± 0.22*
Body weight at 21d (kg)	12.94 ± 0.19	13.43 ± 0.23	9.82 ± 0.53*	9.69 ± 0.51*
CRL at 21d (cm)	66.9 ± 1.3	66.1 ± 2.2	63.0 ± 1.7*	59.3 ± 1.0*
BMI at 21d (kg/m^2)	0.29 ± 0.01	0.31 ± 0.02	0.24 ± 0.01*	0.27 ± 0.01*

Superscripts indicate significant differences between mean values in the Control and IUGR groups (*) or between mean values in the male and female groups (†).

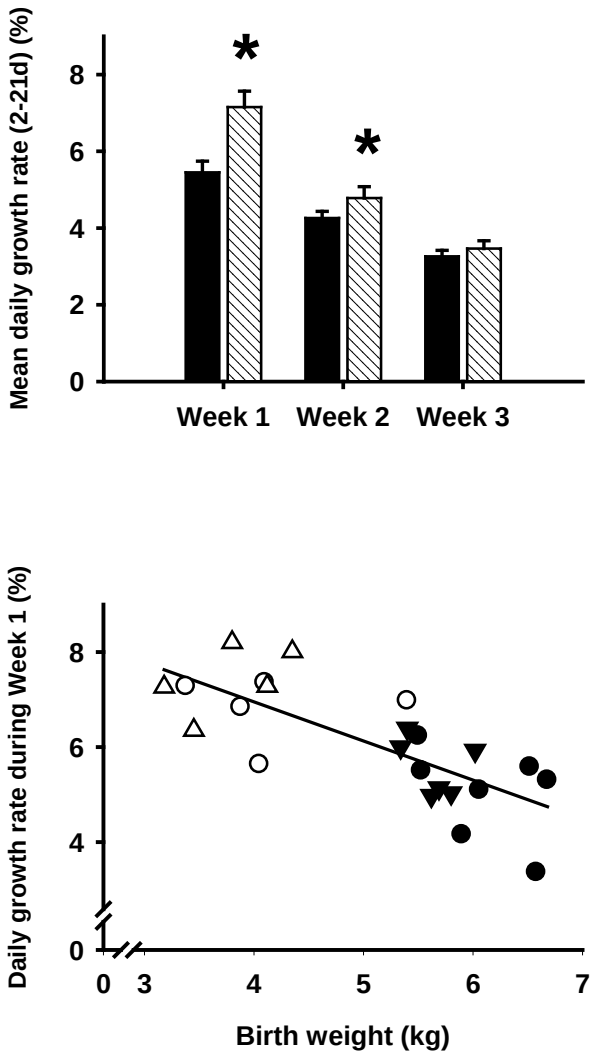

Figure 3.1 Percentage daily growth rate in Control and IUGR neonates.

Upper panel: The daily growth rate (% daily increase in body weight) during weeks 1-3 in Control (black vertical bars) and IUGR (white vertically striped bars) lambs.

Lower panel: The significant relationship ($P < 0.0001$, $R^2 = 0.53$) between birth weight (kg) and the average daily growth rate during week 1 (%) in Control male (closed circles), Control female (closed triangles), IUGR male (open circles), and IUGR female (open triangles) lambs.

Table 3.3: Relationships Between Measures of Growth and Perirenal Fat Mass, and Plasma Insulin or NEFA Concentrations During the First 24h of Life in Female and Male Lambs

	FEMALE		MALE	
	Plasma Insulin during 24h after birth (n=9)	**Plasma NEFA during 24h after birth (n=11)**	**Plasma Insulin during 24h after birth (n=10)**	**Plasma NEFA during 24h after birth (n=12)**
Birth Weight (kg)	y = 0.559x - 1.054 r^2 = 0.46, P < 0.05	y = 0.183x - 0.024 r^2 = 0.45, P < 0.01	NS	y = 0.140x + 0.055 r^2 = 0.35, P < 0.05
Daily Growth Rate Wk 1 (%)	y= -0.569x + 5.162 r^2 = 0.55, P < 0.05	NS	NS	y = -0.155x + 1.683 r^2 = 0.56, P < 0.01
At 3 weeks of age				
Relative Perirenal Fat Mass (g/kg)	NS	y = 0.073x - 0.181 r^2= 0.65, P < 0.01	y = 0.248x - 0.043 r^2= 0.43 , P < 0.05	NS
Size of Perirenal Adipocytes (μm^2)	y = 0.004x - 3.089 r^2 =0.85, P < 0.001	NS	y = 0.002x - 0.751 r^2 = 0.40, P < 0.05	NS
PPARγ mRNA Expression (y)	NS	IUGR; y=2.4x + 0.24, r^2 = 0.98, P < 0.002 Control; y =1.6x + 0.12, r^2 = 0.73, P < 0.03	NS	NS
Adiponectin mRNA Expression (y)	NS	NS	NS	y = 0.697x + 9.99 r^2 = 0.61, P < 0.01
LPL mRNA Expression (y)	NS	NS	NS	y = -0.548x + 3.01 r^2 = 0.57, P < 0.01
G3PDH mRNA Expression (y)	NS	NS	NS	y =1.16x + 1.88 r^2 = 0.33, P < 0.05

3.4.3 VISCERAL FAT MASS AND ADIPOCYTE SIZE

At 21d, IUGR lambs had a lower ($P < 0.05$) absolute mass of perirenal (male 101 ± 19; female 134 ± 15 g), but not omental fat (male 77 ± 7; female 65 ± 15 g), when compared with Control lambs (perirenal, male 137 ± 12; female 190 ± 23; omental, male 81 ± 10; female 73 ± 8 g). There was no difference, however, in the relative perirenal or omental fat mass between IUGR and Control lambs (Figure 3.2). At 21d, both absolute and relative perirenal fat mass were lower ($P < 0.05$) in males than females (Figure 3.2). There was a relationship between the relative perirenal fat mass at 21d and plasma insulin concentrations during the first 24h after birth in males and with plasma NEFA concentrations during the first 24h after birth in females (Table 3.3). The relative perirenal fat mass in female lambs was also directly related to mean plasma glucose (r^2=0.51, P<0.05) and insulin concentrations (r^2=0.52, P< 0.05) during weeks 1-3.

There were no differences in the mean size of perirenal or omental adipocytes between IUGR and Control lambs, or between males and females (Figure 3.2). The mean size of the perirenal adipocytes was directly related to either the relative perirenal fat mass in females (r^2=0.43, $P < 0.05$) or the absolute perirenal fat mass in males (r^2=0.39, $P < 0.05$). Plasma insulin concentrations during the first 24h after birth predicted the mean size of the perirenal adipocytes at 21d in both male and female lambs (Figure 3.3 and Table 3.3).

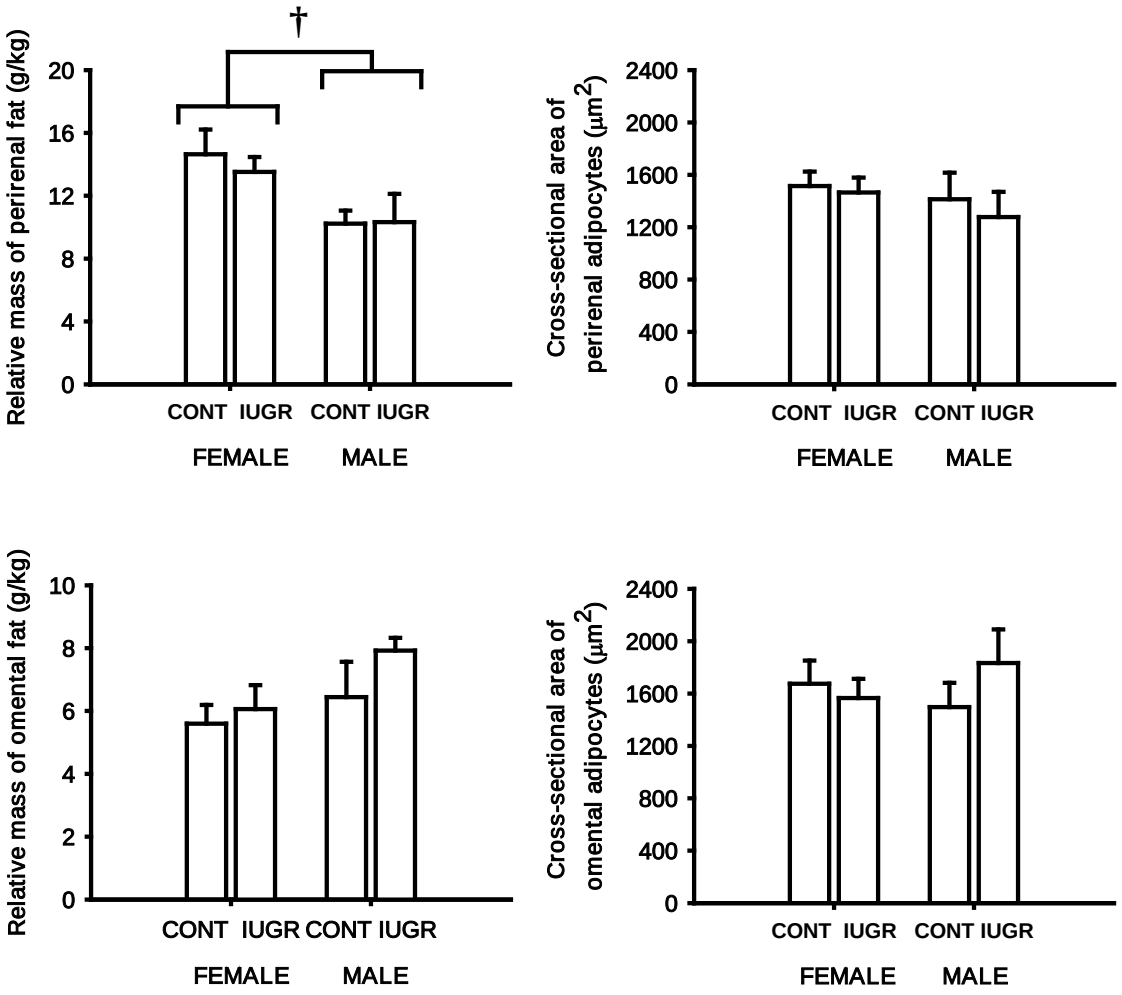

Figure 3.2 Relative perirenal and omental fat mass and the size of perirenal and omental adipocytes at 21d of life in Control and IUGR lambs.

Upper Panel: The relative mass of perirenal fat (left hand side) and mean cross sectional area of perirenal adipocytes (right hand side) in male and female Control (closed bars) and IUGR (open bars) lambs at 21 d. There was a higher relative fat mass in female lambs (†, $P < 0.05$) compared with males in both the IUGR and Control groups.

Lower Panel: The relative mass of omental fat (left hand side) and mean cross sectional area of omental adipocytes (right hand side) in male and female Control (closed bars) and IUGR (open bars) lambs at 21 d.

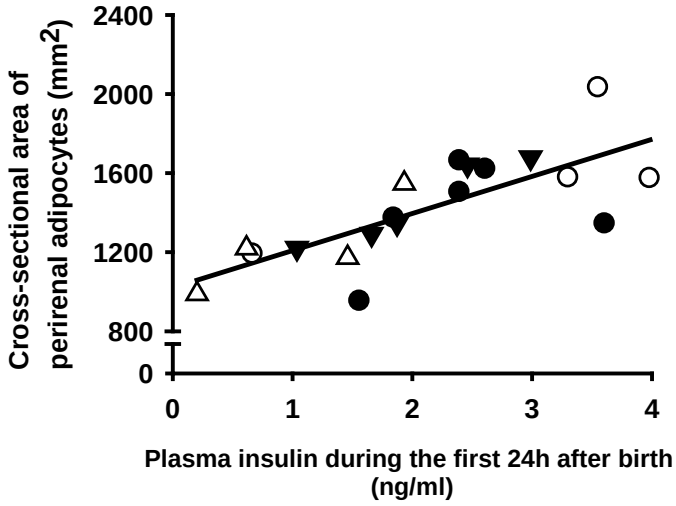

Figure 3.3 The relationship between plasma insulin concentrations during the first 24h after birth and the mean cross-sectional area of perirenal adipocytes at 21d.

The relationship between plasma insulin concentrations during the first 24 h after birth and the mean cross-sectional area of perirenal adipocytes at 21 d in both male Control (closed circles) and IUGR (open circles) lambs, and female Control (closed triangles) and IUGR (open triangles) lambs. The relationship is independent of birthweight.

3.4.4 RXRα, PPARγ AND LEPTIN mRNA EXPRESSION

There was no effect of either IUGR or sex on RXRα mRNA expression in perirenal fat (Table 3.4). There was a significant interaction (*P*<0.05), however, between the effects of IUGR and sex on both PPARγ and leptin mRNA expression (Figure 3.4).

Male lambs

PPARγ and leptin mRNA levels were significantly lower in the perirenal fat of IUGR male lambs when compared with Control male lambs ($P < 0.05$) (Figure 3.4) and there was a direct relationship between birth weight and each of PPARγ mRNA (r^2=0.37, $P < 0.05$) and leptin mRNA expression (r^2=0.53, $P < 0.01$). In contrast to female lambs, PPARγ mRNA expression was not related to plasma NEFA concentrations during the first 24h after birth (Table 3.3), or to relative perirenal fat mass.

Female lambs

In female lambs there was no difference in PPARγ and leptin mRNA expression in perirenal fat between the IUGR and Control groups (Figure 3.4), and no relationship between PPARγ and leptin mRNA with birthweight. PPARγ and leptin mRNA levels in perirenal fat were, however, directly related to each other (r^2=0.54, $P < 0.01$). PPARγ mRNA expression was directly related to plasma NEFA during the first 24h after birth in both IUGR an Control female lambs (Table 3.3). PPARγ mRNA expression was also related to mean insulin

concentrations during weeks 1-3 (r^2=0.41, $P < 0.05$) and to the relative mass of perirenal fat in both the IUGR (y=3.78x +6.40, r^2=0.93, $P < 0.01$) and Control lambs (y=6.97x + 2.85, r^2=0.85, $P < 0.01$), and these relationships were interdependent. Leptin expression was related to the mean size of the perirenal adipocytes (r^2=0.50, $P < 0.05$) in females.

Table 3.4 RXRα, Adiponectin, LPL, Glycerol-3-Phosphate Dehydrogenase (G3PDH) and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) mRNA expression in perirenal adipose tissue.

Target Gene : Reference Gene	Control	Control	IUGR	IUGR
	FEMALE (n = 6)	MALE (n = 8)	FEMALE (n = 5)	MALE (n = 4)
RXRα : RPLP0	0.18 ± 0.01	0.20 ± 0.02	0.18 ± 0.02	0.17 ± 0.01
Adiponectin : RPLP0	7.64 ± 1.04	8.76 ± 01.53	7.10 ± 1.68	8.50 ± 0.65
LPL : RPLP0	1.37 ± 0.22	1.89 ± 0.47	1.39 ± 0.43	1.77 ± 0.25
G3PDH : RPLP0	3.00 ± 0.41	2.95 ± 0.30	2.82 ± 0.51	2.07 ± 0.36
GAPDH : RPLP0	1.36 ± 0.07	1.32 ± 0.10	1.43 ± 0.16	1.29 ± 0.28

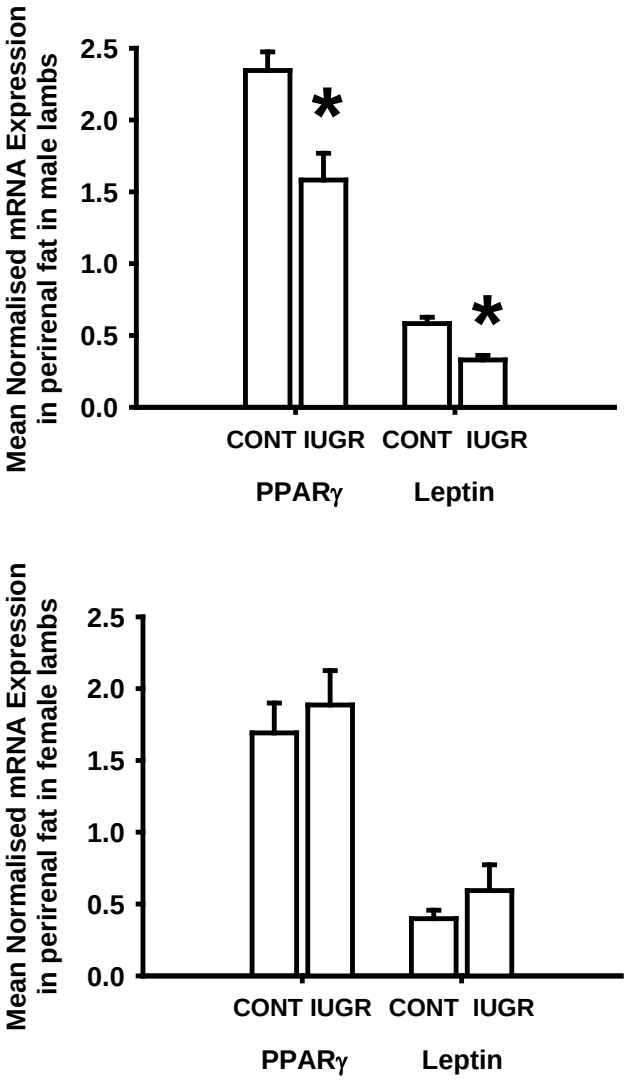

Figure 3.4 The expression of PPARγ and Leptin mRNA in perirenal adipose tissue at 21d of life in Control and IUGR lambs.

Upper Panel: Peroxisome Proliferator-Activated Receptor-γ (PPARγ) and Leptin mRNA expression in perirenal fat in Control male (closed bars) and IUGR male (open bars) lambs. PPARγ mRNA (*, $P < 0.05$) and leptin mRNA levels (*, $P < 0.001$) were significantly lower in the perirenal fat of IUGR male lambs when compared with Control male lambs.

Lower Panel: PPARγ and Leptin mRNA expression in perirenal fat in Control female (closed bars) and IUGR female (open bars) lambs at 21d.

3.4.5 ADIPONECTIN, LPL, G3PDH, AND GAPDH MRNA EXPRESSION

There was no effect of either IUGR or sex on the levels of adiponectin, LPL, G3PDH and GAPDH mRNA expression in perirenal fat (Table 3.4). Adiponectin mRNA and LPL mRNA levels in perirenal fat were also each related to plasma insulin concentrations during the first 24 h after birth (adiponectin r^2=0.28, $P < 0.05$; LPL r^2=0.28, $P < 0.05$) and to the daily growth rate during week 1 (adiponectin r^2=0.27, $P < 0.05$; LPL r^2=0.18, $P < 0.05$).

Female lambs

In female lambs, adiponectin and LPL mRNA expression in perirenal fat were strongly related to each other (r^2=0.78, $P < 0.001$; Figure 3.5) and to G3PDH mRNA expression (adiponectin r^2=0.71, $P < 0.01$; LPL r^2=0.84, $P < 0.0001$). In female lambs, plasma insulin concentrations during the first week after birth predicted G3PDH mRNA levels in perirenal fat at 21d (r^2=0.42, $P < 0.05$), whereas expression of GAPDH mRNA was related to plasma insulin concentrations at 19-21d (r^2=0.38, $P < 0.05$).

Male lambs

In males, as in the females, adiponectin and LPL mRNA expression in perirenal fat were also strongly related to each other (r^2=0.79, $P < 0.0001$; Figure 3.5) and to G3PDH mRNA expression (adiponectin r^2=0.37, $P < 0.05$; LPL r^2=0.35, $P < 0.05$). In contrast to the female lambs, there was no relationship between plasma insulin concentrations during the first week after birth and G3PDH mRNA levels in perirenal fat at 21d. There was, however, a relationship between plasma NEFA during the first 24h after birth and the expression of adiponectin mRNA, LPL mRNA and G3PDH mRNA in the male lambs (Table 3.3). The relationship

between adiponectin and LPL mRNA expression remained significant ($P<0.002$) when the effect of plasma NEFAs was controlled for in the analysis.

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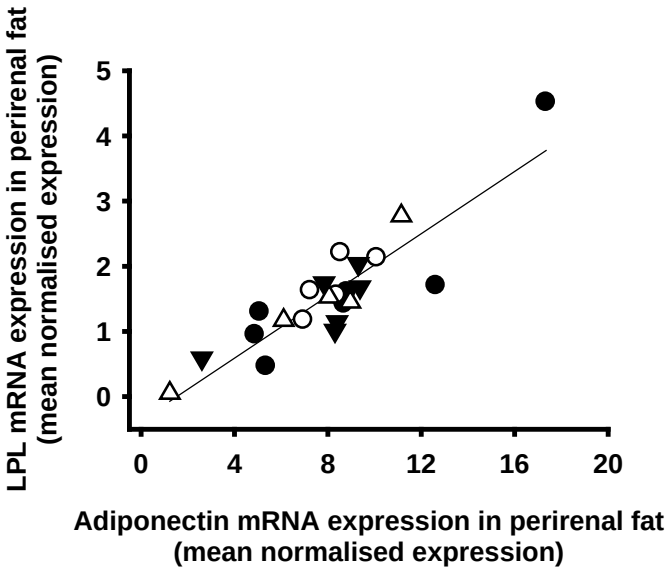

Figure 3.5 The relationship between Adiponectin and LPL mRNA expression in perirenal fat at 21d in the lamb.

Adiponectin and LPL mRNA expression in perirenal fat were strongly related in both male (Control, closed circles; IUGR, open circles) and female (Control, closed triangles; IUGR open triangles) lambs, independent of birthweight ($y=0.269x - 0.542$, $r^2=0.86$, $P < 0.0001$).

3.5 DISCUSSION

In this study we have investigated the extent to which changes in growth of the adipocyte and the expression of PPARγ and other adipogenic and lipogenic genes are determined by intra-uterine growth restriction, the postnatal growth rate and circulating hormonal and nutrient levels during the first three weeks of life, and sex. We have found that the there is a significant interaction between the effects of IUGR and sex on both PPARγ and leptin mRNA expression. We have also found that the immediate postnatal nutritional environment, represented by plasma insulin and NEFA concentrations during the first 24 h of life, plays an important role in the growth and functional development of perirenal visceral adipose tissue. Furthermore, there is a differential effect on sex on relationships between these measures of the early nutritional environment and adipogenic and lipogenic gene expression in perirenal fat in male and female lambs.

3.5.1 THE EFFECT OF IUGR ON BIRTH WEIGHT

In the current study, we determined the impact of being IUGR (< 4.3 kg; less than 2 SD below the cohort mean, equivalent to the 3rd centile) on adipogenic and lipogenic gene expression in Merino lamb perirenal visceral adipose tissue. There are a number of methods used in the literature to achieve a low birth weight in order to study the effects of intra-uterine growth restriction on neonatal growth and development. Because of the placental growth restriction that occurs in prolific ewes, investigators have used ewes carrying one or more fetuses to

compare postnatal growth and development in a range of birth weights. An elegant series of studies by Greenwood and Bell (Greenwood *et al.*, 1998, 2000a; Greenwood *et al.*, 2000b; Ehrhardt *et al.*, 2003; Greenwood *et al.*, 2004) have used the birth weight range generated by Suffolk x (Finnsheep x Dorset) ewes carrying singleton and multiple pregnancies to compare the growth responses in low and high birth weight lambs (<2.9kg and >4.3kg respectively) to different nutritional regimes after birth. Louey and colleagues (2005) investigated the long term consequences of fetal growth restriction on postnatal growth and adiposity using a model of LBW induced in either single or twin lambs by placental embolisation and compared these lambs to control singleton lambs. A recent study by De Blasio et al (2007) also used a cohort of placentally restricted lambs and compared postnatal growth rates and adiposity in these lambs and control counterparts. In the current study multiple pregnancy and uterine carunclectomy was used to restrict placental substrate supply and extend the low birth weight range of our lamb cohort.

As expected, the IUGR lambs in the present study were smaller and thinner at birth and had a higher daily fractional growth rate in the first two weeks of life compared with Control lambs. Despite growing faster, the IUGR lambs had not caught up to their Control counterparts in terms of weight or length by 21d. These findings are consistent with previous studies which reported that LBW lambs had a higher growth rate than higher birth weight lambs during the first month after birth and did not catch up in body weight when compared to higher

birth weight counterparts until around 2 months of age (Greenwood *et al.*, 1998; Louey *et al.*, 2005; De Blasio *et al.*, 2007b).

3.5.2 The Effect of IUGR on Circulating Plasma NEFA Concentrations

IUGR lambs had lower plasma NEFA concentrations during the first 24h after birth and throughout the 3 week postnatal period. There was also a direct relationship between birth weight and plasma NEFA concentrations. This suggests that the reduced fetal substrate supply that leads to a low birth weight may also lead to altered NEFA handling during postnatal life. This may be through a reduced lipolytic capacity of adipose tissue, or an increased NEFA uptake or oxidation. Alternatively, the lower plasma NEFA concentration in IUGR lambs could result from a decreased feed intake, however this is unlikely given evidence from a previous study of LBW male Suffolk-(Finn-Dorset) lambs which found they had an increased feed intake that persisted for the first three weeks of life (Greenwood *et al.*, 1998), or a lower fat content of the colostrum of ewes carrying LBW lambs (Le Dividich *et al.*, 1991; Le Dividich *et al.*, 1994).

3.5.3 The Effect of IUGR and Sex on Postnatal Levels of Plasma Insulin and NEFA, and the Development of Perirenal Adipose Tissue

There was no difference between IUGR and Control lambs in the relative mass of perirenal or omental adipose tissue present at 21 days of life, or between the mean size of perirenal or omental adipocytes. In previous studies, lambs that were IUGR were found to have an increase in the relative mass of perirenal and

total visceral fat at 6 weeks of age when compared to control counterparts (De Blasio *et al.*, 2007a), a higher total body fat content than their higher birthweight counterparts when measured at the same body weight (20kg) 40-50d after birth (Greenwood *et al.*, 1998) and a higher abdominal fat mass at 2 years of age (Louey *et al.*, 2005). In SGA human infants, catch-up growth also influences adipose tissue growth as is evidenced by an increased BMI during the first year of life (Jaquet *et al.*, 1999) and a higher fat mass and truncal fat distribution during childhood (Ong *et al.*, 2000) and in adult life (Law *et al.*, 1992; Leger *et al.*, 1997; Leger *et al.*, 1998; Jaquet *et al.*, 2001b; Parsons *et al.*, 2001). Therefore this study has determined whether there was an effect of prenatal and postnatal growth rates and the postnatal nutritional environment on the growth and functional development of unilocular white perirenal adipose tissue in male and female lambs at 3 weeks of life, which is predisposed to a subsequent increase in mass, and the development of the obese phenotype.

Female lambs had a significantly greater relative perirenal fat mass compared to male lambs at 3 weeks of age, but there was no sex related difference in the mean size of the perirenal adipocytes. This suggests that the increased mass of the perirenal fat depot in females occured predominantly through hyperplastic, rather than hypertrophic mechanisms. It has been well described in previous literature that clonal expansion of growth arrested preadipocytes is predominantly modulated by stimulation of IGF1R by both plasma insulin and IGF1 (for reviews please see (Gregoire *et al.*, 1998; Avram *et al.*, 2007)), therefore an increased responsiveness of IGF1R in female perirenal

preadipocytes to either of these hormones is a good candidate mechanism to explain the increased adipose tissue mass in female lambs. Evidence from the current study that the perirenal adipose tissue of female lambs may be relatively more insulin sensitive than that of males is the presence of a relationship in female, but not male lambs, between plasma glucose and insulin concentrations during the first three weeks after birth and perirenal fat mass at 21d. Insulin action at the mature adipocyte stimulates GLUT4 mediated uptake of glucose and inhibits lipolysis. If the adipose tissue of male lambs was insulin resistant relative to the adipose tissue of female lambs, this would provide an explanation for the lower level of lipid deposition during the early neonatal period and the development over time of an increased store of perirenal visceral adipose tissue (Wajchenberg, 2000), in comparison to females. Interestingly, male lambs had higher plasma insulin concentrations from birth to 3 weeks compared with female lambs. A higher plasma insulin in males has also been described in the rat, which by adulthood had a higher visceral fat mass than the female rat (Clegg *et al.*, 2003).

3.5.4 The Effect of Plasma Insulin and NEFA Concentrations During the First 24h of Life on the Development of Perirenal Adipose Tissue

In both male and female lambs, plasma insulin concentrations during the first 24h after birth were related to the size of the perirenal adipocytes 3 weeks later, and in male, but not female lambs plasma insulin concentrations during the first 24h after birth were also related to the relative perirenal fat mass. This suggests

that the first day of life might be the time point at which the in vivo adipogenic effect of insulin on white adipocytes is most pronounced.

Fatty acids also play a role in the induction of adipocyte differentiation (Ailhaud *et al.*, 1995; Sampath & Ntambi, 2004; Madsen *et al.*, 2005), and in IUGR and Control female lambs there was a relationship between plasma NEFA concentrations during the first 24h after birth and both the expression of PPARγ mRNA and the relative perirenal fat mass at 3 weeks of life. Interestingly, the slope of these relationships were similar in both IUGR and Control females, but PPARγ mRNA expression on day 21 was higher when related to any given plasma NEFA concentrations on day 1 in the IUGR, compared to the Control group. One possibility is that in the female lamb, who has low plasma insulin concentrations, fatty acids may act either as transcriptional activators of PPARγ expression (Sampath & Ntambi, 2004, 2005) or bind as ligands to activate the nuclear receptor to result in enhanced insulin signalling and hyperplasia of the perirenal adipocytes during early postnatal life (Summary Figure 6.1). The direct relationship between PPARγ and leptin expression in perirenal fat in female lambs may be a consequence of the effects of PPARγ on perirenal fat mass, as leptin expression was also directly related to adipocyte cell size in these lambs.

3.5.5 The Sex Specific Effect of IUGR on the Expression of PPARγ and Leptin mRNA in Perirenal Adipose Tissue in the Postnatal Lamb

In this study in female lambs there was no effect of IUGR on the expression of PPARγ and leptin mRNA in perirenal adipose tissue at 3 weeks of life, however

in male lambs IUGR resulted in a reduced level of expression of PPARγ and leptin mRNA. In both males and females, plasma NEFA concentrations were lower in IUGR than in Control lambs, and plasma insulin concentrations were higher in males compared to females throughout the first 3 weeks of life. It is possible that in the presence of the relatively higher insulin concentrations in the IUGR male lamb, there is less activation of PPARγ or less gene expression when fatty acid concentrations are low (Summary Figure 6.2). A decrease in PPARγ mRNA expression in adipose tissue is associated with a decrease in adipose tissue and hepatic insulin sensitivity, particularly during periods of high caloric intake (He *et al.*, 2003). Therefore the reduced expression of PPARγ mRNA in the perirenal visceral fat depot of male IUGR lambs may be associated with insulin resistance of adipose tissue during the early postnatal period and further predispose male IUGR lambs to the subsequent development of an insulin resistant phenotype.

The reduced level of expression of leptin mRNA in perirenal adipose tissue has also been shown in placentally restricted fetal sheep at 140-145d gestation, in comparison with controls (Chapter 2). Therefore the restriction of placental substrate supply in male fetuses results in a change in the functional capacity of adipose tissue, such that basal adipocyte leptin production is reduced. While there is no reduction in circulating plasma leptin levels in IUGR male lambs it is possible that there is a functional immaturity of the leptin – fat mass axis which may have long term consequences for the regulation of lipid metabolism and whole body insulin sensitivity.

3.5.6 IUGR, ADIPONECTIN AND LPL MRNA EXPRESSION IN PERIRENAL ADIPOSE TISSUE IN THE POSTNATAL LAMB

In the human, plasma adiponectin concentrations are lower in SGA than AGA infants (Kamoda *et al.*, 2004), and in SGA infants, there is a greater decrease in circulating adiponectin in those infants who experience the highest weight gain between 1 and 2 years of age (Iniguez *et al.*, 2004). In the present study, plasma insulin concentrations during the first 24h after birth and the daily growth rate, predicted the level of adiponectin expression in perirenal fat in both male and female lambs at 3 weeks of age. Plasma NEFA concentrations during the first 24h after birth also predicted the expression of adiponectin mRNA in the perirenal adipose tissue in male lambs. Whether there is a longer term impact of the early low circulating plasma NEFA concentrations in IUGR male lambs on the subsequent level of adiponectin expression in the visceral fat after the initial postnatal growth period will be important to determine. We have also shown that there was a strong relationship between the expression of adiponectin and LPL in the perirenal fat in both male and female lambs. This is the first demonstration, to our knowledge, that adiponectin and LPL expression are related in visceral adipose tissue, either during development or in adult life.

3.5.7 SUMMARY

In summary, there are sex specific differences in the development of perirenal visceral adipose tissue during the early postnatal period which imply that the level of insulin sensitivity in perirenal adipose tissue might differ between male and female lambs. There was also a sex specific effect of intra-uterine fetal

growth restriction on both PPARγ and leptin mRNA expression, such that male IUGR lambs had a lower level of expression of both PPARγ and leptin, which may have long term consequences for the regulation of lipid metabolism and whole body insulin sensitivity. This study therefore describes mechanisms that provide explanations for the epidemiological evidence for an increased susceptibility to the development of insulin resistance in both males in comparison to females, and in small for gestational age compared to average for gestational age individuals. This study also suggests that nutritional substrate supply during the first day of life may have long term implications for the growth and functional development of visceral adipose tissue, which highlights the early postnatal period as a potentially important time for nutritional intervention to limit the adverse metabolic consequences of being small for gestational age.

4. IUGR and the expression of adipogenic and lipogenic genes in subcutaneous adipose tissue in male and female lambs in the early postnatal period: Evidence for cross-talk between adipose depots.

4.1 SUMMARY

We have investigated the effect of intrauterine growth restriction (IUGR), defined as a birth weight less than 2 standard deviations below the mean of a cohort of singleton Merino lambs on subcutaneous fat mass and gene expression in the lamb at 21d of age. We have shown in Chapter 3 in the same cohort that there was a sex specific effect of IUGR on the expression of PPARγ and leptin mRNA in perirenal visceral fat at 21d. In the current study we have found that there is no difference between Control and IUGR lambs in the relative mass of subcutaneous fat, or the expression of PPARγ, RXRα, leptin, adiponectin, LPL, G3PDH, and GAPDH in subcutaneous fat at 21d of life. We have also shown that the relative weight of the subcutaneous fat depot is related to plasma glucose, insulin and leptin concentrations, and to measures of perirenal adipose tissue development. Thus, in contrast to perirenal adipose tissue, it appears that the postnatal, but not the fetal nutritional environment, programs subcutaneous adipocyte growth and gene expression. This study suggests that there may be a factor secreted from visceral fat that influences the development of the subcutaneous fat depot.

4.2 INTRODUCTION

Recent studies have found that around 16% of US children (Hedley *et al.*, 2004) are obese (BMI $\geq$ 30 kg/m^2), and that 25-50% of obese children remain obese in adult life, and are at greater risk for obesity related metabolic disorders (2002). A range of epidemiological studies have reported that a restricted nutrient supply *in utero* results in a low birth weight (LBW) (Ravelli *et al.*, 1976; Brown *et al.*, 2002) and a faster postnatal growth rate that is associated with an increase in the incidence of childhood obesity (Ong *et al.*, 2000; Cole, 2004), in particular a greater central or visceral fat distribution (Ong *et al.*, 2000). Interestingly, it has also been shown that in 7-12 year old American schoolchildren a low birth weight is associated with a proportionally more central subcutaneous fat deposition as measured by a ratio of skinfold thicknesses, with birth weight accounting for 2-8% of the variance in relative subcutaneous fat distribution at school age (Malina *et al.*, 1996). The inverse relationship between birth weight and a more truncal distribution of subcutaneous fat has also been demonstrated in both adolescent males (Labayen *et al.*, 2006) and females (Barker *et al.*, 1997). In adult women a lower birth weight was associated with a higher subcutaneous fat mass and a more truncal fat distribution, whilst in adult men low birth weight was inversely related to their waist-to-hip ratio (te Velde *et al.*, 2003). A study of twins that investigated the association between birth weight and adult body composition whilst controlling for maternal and genetic influences showed that in adult males the lighter twin at birth was also shorter and lighter as an adult, but had more subcutaneous fat compared to his heavier sibling (Loos RJ *et al.*, 2001).

It has recently been shown that an increased deposition of abdominal subcutaneous fat is associated with insulin resistance in the adolescent and adult population. In postpubertal Asian-Indian children there is a high prevalence of insulin resistance that correlates with overweight, abdominal obesity, high truncal subcutaneous adiposity, and excess body fat. (Misra *et al.*, 2004) . In adults of 20 years and older truncal skinfold thickness has been shown to independently predict type-2 diabetes (Butler *et al.*, 1982). Further to that, in a study of male type-2 diabetics, deep subcutaneous adipose tissue at L4-5 level as quantitated by MRI, was associated with both peripheral and hepatic insulin resistance (Miyazaki *et al.*, 2002).

Despite this series of studies which demonstrates the association between low birth weight, increased postnatal growth rate, and a higher subcutaneous fat mass with an increased truncal distribution, it is not clear how the increase in subcutaneous fat mass is programmed as a consequence of restricted substrate supply in utero. As summarised earlier in this thesis, the nuclear receptor Peroxisome Proliferator-Activated Receptor-γ (PPARγ) is a modulator of adipocyte differentiation, having a major role in the induction of early growth arrest of adipocytes and in their terminal differentiation, whereas clonal expansion is predominantly modulated by plasma insulin and IGF1 (Gregoire *et al.*, 1998). PPARγ in combination with Retinoid-X-Receptor-α (RXRα), functions as a heterodimeric transcription factor, which binds to peroxisome proliferator response elements (PPRE) on DNA (Auwerx, 1999; Kersten *et al.*, 2000) to initiate transcription of the regulatory genes involved in lipid accumulation and metabolism, including adiponectin and leptin (Gregoire *et al.*, 1998; Combs *et al.*,

2002; Picard & Auwerx, 2002). It is therefore possible that a sub-optimal nutritional environment before birth may result in an altered expression of PPARγ and other regulatory genes during development, and thus have long term consequences for the emergence of obesity and insulin resistance in adult life.

There have been no studies in animals that have investigated the specific effect of low birth weight on subcutaneous fat mass at any age. In a study by Greenwood it was shown that low birth weight (LBW) lambs (<2.9kg), ate more, grew faster and had a higher proportion of body fat than high birth weight (HBW>4.3kg) lambs when examined at the same body weight (20kg) at autopsy (Greenwood *et al.*, 1998). Similarly, in a study of placentally restricted (PR) and control lambs at age 45d it was shown that there was an increased relative visceral fat mass and whole body insulin sensitivity in placentally restricted lambs compared with controls (De Blasio *et al.*, 2007a). Previously, in Chapter 3, I demonstrated that there was a reduction in the expression of PPARγ and leptin mRNA in the perirenal fat of IUGR male lambs (birth weight less than 2SD below the mean of control singleton lambs) compared with Control male lambs at 21days of life, thus implicating a role for the early nutritional programming of PPARγ mRNA expression in the visceral fat depot in the subsequent emergence of an insulin resistant phenotype in LBW male lambs (Chapter 3). I have therefore investigated the extent to which changes in growth of the subcutaneous adipocyte and the expression of retinoic X receptor (RXR)α, PPARγ, leptin, adiponectin, LPL, glycerol-3-phosphate dehydrogenase (G3PDH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) are determined by prenatal factors in male and female lambs as summarised by birth weight and

the plasma insulin, leptin and glucose concentrations measured within 24h of birth and by postnatal factors, including the postnatal growth rate and circulating hormonal and nutrient levels during the first three weeks of life. Finally, in the sheep the visceral fat depot emerges in fetal life before the development of subcutaneous adipose tissue in early postnatal life and I have therefore investigated whether there are relationships between the development of the visceral and subcutaneous adipose tissue depots during the first 3 weeks of life.

4.3 METHODS

4.3.1 ANIMALS AND SURGERY

All procedures were approved by The University of Adelaide Animal Ethics Committee. Twenty-two Merino ewes were used in the study. Six non-pregnant ewes underwent surgery to remove the majority of endometrial caruncles (>70) from the uterus, leaving 3 to 8 caruncles in each horn in order to induce experimental restriction of placental and fetal growth (Robinson *et al.*, 1979).

The methods of animal husbandry were as described in Chapter 3. Briefly, at around 110d gestation all ewes were brought into the Central Animal House and were fed a standard diet that provided 100 % of the energy requirements for the maintenance of a pregnant ewe bearing a singleton or twins, as specified by the Ministry of Agriculture, Fisheries and Food, England (1975). After parturition, an additional 1 kg lucerne chaff was offered at 1500 h to the ewe. The time of birth was recorded and the day of birth was taken to be Day 1 and when birth occurred

overnight Day 1 was taken to start at 0900 h. All ewes and lambs were housed under a 12 h light / dark cycle with lights on at 7 am and a daily temperature range between 19 and 22 °C.

4.3.2 Blood Sampling Protocol, Growth Measurements and Post Mortem

Lambs were weighed (kg) and crown-rump length (cm) measured daily between 1000 - 1400 h. Venous blood samples were collected in chilled tubes after approximately 60 min of non-suckling on alternate days between 0900 h – 1300 h, as previously described (Chapter 3). All blood samples were centrifuged at 1500g for 10 min and plasma separated into aliquots and stored at -20°C. On day 21, lambs were killed with an overdose of sodium pentobarbitone (Virbac Pty Ltd, Peakhurst, NSW, Australia) and all visible adipose tissue was dissected and weighed. This included the subcutaneous, perirenal, retroperitoneal, omental, pericardial, axillary and parametrial or epididymal fat depots. The subcutaneous fat collected and weighed was from the torso, between the cranial cervical vertebrae and the caudal sacral vertebrae, and around the proximal limbs. The adipose tissue surrounding the axillary lymph node was physically separate from subcutaneous fat and therefore collected separately. Samples of subcutaneous adipose tissue were collected consistently from the upper back, approximately 5cm left lateral to the 6^{th} thoracic vertebrae, and were frozen in liquid nitrogen and stored at -80°C for subsequent gene analysis and separate samples were also fixed in 0.4% paraformaldehyde buffer for histological analysis. Samples of perirenal fat were processed as described previously (Chapter 3).

4.3.3 PLASMA NUTRIENT AND HORMONE ASSAYS

Plasma NEFAs were measured by an *in vitro* enzymatic colorimetric method (Wako Pure Chemicals Industries Ltd, Osaka, Japan). The method relies upon the acylation of coenzyme A by the fatty acids in the presence of added acyl-CoA synthetase. The acyl-CoA thus produced is oxidised by added acyl-CoA oxidase with generation of hydrogen peroxide. Hydrogen peroxide, in the presence of peroxidase permits the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA) with 4-aminoantipyrine to form a purple coloured adduct which can be measured colorimetrically at 550nm (COBAS MIRA automated analysis system, Roche Diagnostica, Basel, Switzerland). The sensitivity of the assay was 0.25 mEq/l and the intra- and inter assay coefficients of variation (CVs) were both < 10 %.

Plasma glucose concentrations were measured by enzymatic analysis using hexokinase and glucose-6-phosphate dehydrogenase to measure the formation of NADH photometrically at 340 nm (COBAS MIRA automated analysis system, Roche Diagnostica, Switzerland). The sensitivity of the assay was 0.5 mmol/l and the intra- and inter assay CVs were both < 5%.

Plasma insulin concentrations were measured using a radioimmunoassay (Linco Research, Inc., Missouri, USA). The sensitivity of the assay was 0.1 ng/ml. Samples (10 µl) were assayed in duplicate and added to borosilicate glass tubes with 100 µl of hydrated ^{125}I-Insulin and guinea-pig anti-rat insulin antibody and incubated overnight at 4°C. Precipitating reagent (1ml) was added and tubes

were centrifuged for 25 min at 2000xg, then aspirated and the total radioactivity in the precipitate was counted. The intra and inter assay CV were both <10%.

Plasma leptin concentrations were measured using a competitive ELISA previously validated for use in sheep plasma (Kauter *et al.*, 2000). Briefly, an ELISA plate was pre-incubated with recombinant bovine leptin in 50 μl of 0.1 M bicarbonate buffer and blocked with 200 μl of 5 % skim milk in ELISA buffer. Chicken anti-recombinant bovine leptin antiserum (50 μl) was added to the wells, followed by the addition of samples (100 μL) in duplicate. Following an overnight incubation at 37 °C, a biotinylated phosphatase-Strepavidin conjugate (Amrad Biotech, Boronia, Vic, Australia) was added, incubated for 1 h, and the plate developed with p-nitrophenylphosphate disodium salt hexahydrate. The sensitivity of the assay was 0.5 ng/ml and the intra- and inter- assay CVs were <16%.

4.3.4 Adipose Tissue Histology

Tissues were fixed in 4% paraformaldehyde buffer, washed and embedded in paraffin wax. Sections were cut (4 -5 μm) and stained with Hematoxylin and Eosin. Standard point counting techniques were used with Video Image Analysis using Video Pro software (Leading Edge, Adelaide, SA, Australia) to measure the area of 100 adipocytes within each fat depot (Muhlhausler *et al.*, 2002).

4.3.5 Isolation of RNA, Production of cDNA and qRT-PCR

The methods used to isolate RNA from adipose tissue and determine the relative expression of mRNA transcripts have been previously described (Chapter 3).

Briefly, RNA from subcutaneous adipose tissue (≈ 600mg) was isolated using Trizol reagent (Invitrogen Australia Pty Limited, Mount Waverley, Australia) and chloroform, and purified using Ambion Dnase1 and the RNeasy Mini Kit (QIAGEN Pty Ltd -Australia, Doncaster, Australia). cDNA was synthesised from 5 μg RNA with Superscript 3 reverse transcriptase (Invitrogen Australia Pty Limited, Mount Waverley, Australia) and random hexamers. The relative expression of PPARγ, RXRα, leptin, adiponectin, LPL, G3PDH and GAPDH mRNA transcripts were measured by quantitative real time reverse transcription-PCR (qRT-PCR) using the Sybr Green system in an ABI Prism 7900 Sequence Detection System (PE Applied Biosystems, Foster City, CA). Each qRT-PCR reaction well (5 μl total volume) contained: 2.5 μl 2x Sybr Green Master Mix (Applied Biosystems); 0.25 μl of each primer giving a final concentration of 450 nM, 1.0 μl of molecular grade H_20 and 1.0 μl of a 1:10 dilution of the stock template. The cycling conditions consisted of 40 cycles of 95 °C for 15 min and 60 °C for 1 min. Primers for each transcript were designed with the aid of Primer Express (PE Applied Biosystems, Foster City, CA) and validated as previously described (Table 2.2). The amplification efficiency of all primers was 0.997 – 0.999. A constant amount of cDNA (1μl) was used for each qRT-PCR measurement and four technical replicates were performed for each gene.

The abundance of each mRNA transcript was measured and expression relative to that of Acidic Ribosomal Protein P0 (RPLP0) and separately to 18S ribosomal RNA was calculated using the comparative threshold cycle (C_t) method (Q-gene qRT-PCR analysis software) (Muller *et al.*, 2002). Correlational data of relative mRNA expression that could not be replicated when using both reference genes was discarded and abundance relative to RPLP0 is reported.

4.3.6 STATISTICAL ANALYSIS

4.3.6.1 Definition of Intra-uterine Growth Restriction (IUGR)

As described in Chapter 3, a frequency distribution curve of birth weights of control Merino singleton lambs from a separate cohort (n=45) of animals born during the past 5 years was used to determine the birth weight cut off for IUGR in this population. The mean birth weight (± SD) of the singleton cohort was 5.63 ± 0.67 kg. Newborn lambs in the current study were classified as IUGR when their birth weight was greater than 2 SD below the cohort mean, equivalent to the 3rd percentile (IUGR: < 4.3 kg, n = 9) or normally grown if their birth weight was within 2 SD on either side of the mean (Control: 4.5 – 6.7 kg, n = 14). Using these criteria, 3 male and 3 female placentally restricted singleton lambs, a male and female pair of twin siblings and one female growth restricted control singleton were allocated into the IUGR group. The Control group consisted of all control singleton lambs. Therefore there were 4 males and 5 females in the IUGR group and 8 males and 6 females in the Control group.

4.3.6.2 Calculation of Body Mass Index, Percentage daily growth rate and Total fat mass

The Body Mass Index (BMI) and daily growth rate (%) were determined as described in Chapter 3. Total fat mass was calculated as the total of subcutaneous, perirenal, retroperitoneal, omental, pericardial, axillary and parametrial or epididymal fat depots and relative mass of total fat, calculated as grams of fat relative to kg of total body weight. Visceral fat mass was calculated as the total of perirenal, retroperitoneal and omental fat.

4.3.6.3 Statistical tests

Parametric analyses were used in this study. The effects of IUGR and sex on the absolute and relative weights of subcutaneous and total fat, size of subcutaneous adipocytes, and on expression of adipogenic genes were determined using two-way analysis of variance (ANOVA). The Duncan's multiple range test was used post hoc to identify the differences between mean values. Relationships between measures of fat mass, adipocyte cell size or gene expression and either measures of prenatal growth (birth weight) or postnatal growth (postnatal growth rate and plasma hormonal and nutrient concentrations) were determined using linear regression analyses and partial correlation analyses. The expression of adipose genes in the visceral and subcutaneous depots in each animal were compared using a paired student's paired t-test. All data are presented as the mean ± SEM. A probability of <5% ($P<0.05$) was taken as the level of significance in all analyses.

4.4 RESULTS

4.4.1 SUBCUTANEOUS AND TOTAL FAT MASS AND SUBCUTANEOUS ADIPOCYTE SIZE

There was no effect of IUGR or sex on the absolute or relative mass of subcutaneous fat (Table 4.1) or on the mean cross sectional area of the subcutaneous adipocytes. The absolute mass of total fat in IUGR lambs was lower compared to Control lambs ($P = 0.054$), however there was no difference in the relative mass of total fat between Control and IUGR lambs, or between male and female lambs (Table 4.1).

Table 4.1: Visceral and Subcutaneous Fat Mass and the Cross Sectional Area of the Subcutaneous Adipocytes in Control and IUGR Male and Female Lambs at 3 weeks Postnatal Age

Fat Parameters	FEMALE		MALE	
	Control (n = 6)	IUGR (n = 5)	Control (n = 8)	IUGR (n = 4)
Absolute visceral fat mass (g)	256 ± 28	233 ± 46	237 ± 17	190 ± 16
Absolute subcutaneous fat mass (g)	343 ± 29	297 ± 43	299 ± 37	265 ± 42
Absolute total fat mass (g)	717 ± 76	588 ± 65 *	657 ± 50	512 ± 71 *
Relative visceral fat mass (g/kg body wt)	21.2 ± 1.6†	22.2 ± 2.8†	18.3 ± 1.5	17.4 ± 0.6
Relative subcutaneous fat mass (g/kg body wt)	26.4 ± 2.0	30.0 ± 3.9	22.1 ± 2.6	27.0 ± 3.6
Relative total fat mass (g/kg body wt)	55.1 ± 5.1	59.3 ± 4.5	48.8 ± 3.5	52.2 ± 5.7
Mean-cross sectional area of subcutaneous adipocytes (μm2)	1353 ± 165	1202 ± 134	1339 ± 232	1537 ± 242

Superscripts indicate significant differences between mean values in the Control and IUGR groups (*) or between mean values in the male and female groups (†).

4.4.2 THE EFFECT OF IUGR AND SEX ON GENE EXPRESSION IN SUBCUTANEOUS ADIPOSE TISSUE IN THE POSTNATAL LAMB.

There was no effect of either IUGR or sex on Leptin, PPARγ, RXRα, Adiponectin, LPL, G3PDH or GAPDH mRNA expression in subcutaneous fat at 21d (Table 4.2). In both male and female lambs, there was a direct relationship between adiponectin and LPL mRNA expression in the subcutaneous depot (male, r^2=0.83, P < 0.0001; female, r^2=0.55, P < 0.02; Figure 4.1) and similarly PPARγ and G3PDH mRNA expression in this depot were also directly related (male, r^2=0.49, P < 0.02; female, r^2=0.46, P < 0.05).

Male lambs

In male lambs the relative mass of subcutaneous fat at 21 d was related to plasma glucose concentrations during wk 1 (r^2=0.47, P < 0.02). Plasma leptin or plasma insulin concentrations during the first 24h after birth were each related to the cross sectional area of subcutaneous adipocytes 3 weeks later (plasma leptin, r^2=0.44, P < 0.05; plasma insulin, r^2=0.32, P < 0.05). At 21d, leptin mRNA expression in subcutaneous fat was directly related to the cross sectional area of subcutaneous adipocytes (r^2=0.54, P < 0.05).

The expression of PPARγ in subcutaneous fat in male lambs was inversely related to plasma leptin concentrations during the first 24 h after birth (r^2=0.37, P < 0.05), whereas adiponectin mRNA expression was inversely related to both plasma insulin (r^2=0.36, P < 0.052) and plasma glucose (r^2=0.37, P < 0.05) concentrations during the second week after birth.

Female Lambs

In female lambs, the relative mass of subcutaneous fat was related to plasma leptin concentrations during week 1 (r^2=0.40, $P < 0.05$), whereas the cross sectional area of the subcutaneous adipocytes and leptin mRNA expression were each related to plasma insulin concentrations during the 48h before tissue collection (adipocyte size: r^2=0.37, $P < 0.05$; leptin mRNA expression: r^2=0.63, $P < 0.01$).

PPARγ mRNA expression in subcutaneous fat in female lambs was directly related to plasma insulin concentrations during week 1 (r^2=0.42, $P < 0.05$).

Table 4.2: The Expression of Leptin, PPARγ, RXRα, Adiponectin, G3PDH and GAPDH mRNA in Subcutaneous Adipose Tissue in the Postnatal Lamb.

Target Gene : Reference Gene	FEMALE		MALE	
	ABW (n = 6)	LBW (n =4- 5)	ABW (n = 8)	LBW (n = 3)
Leptin : RPLP0	0.67 ± 0.19	0.46 ± 0.16	0.49 ± 0.06	0.43 ± 0.05
PPARγ : RPLP0	1.92 ± 0.28	1.79 ± 0.23	2.08 ± 0.21	1.54 ± 0.10
RXRα : RPLP0	0.21 ± 0.02	0.20 ± 0.03	0.20 ± 0.02	0.20 ± 0.02
Adiponectin : RPLP0	6.88 ± 1.01	5.72 ± 1.62	8.72 ± 1.80	7.87 ± 2.76
LPL : RPLP0	1.66 ± 0.35	2.05 ± 0.67	3.24 ± 1.04	2.05 ± 0.73
G3PDH : RPLP0	4.18 ± 0.69	3.35 ± 0.66	3.65 ± 0.45	2.49 ± 0.11
GAPDH : RPLP0	1.63 ± 0.40	1.95 ± 1.13	1.08 ± 0.09	2.45 ± 1.25

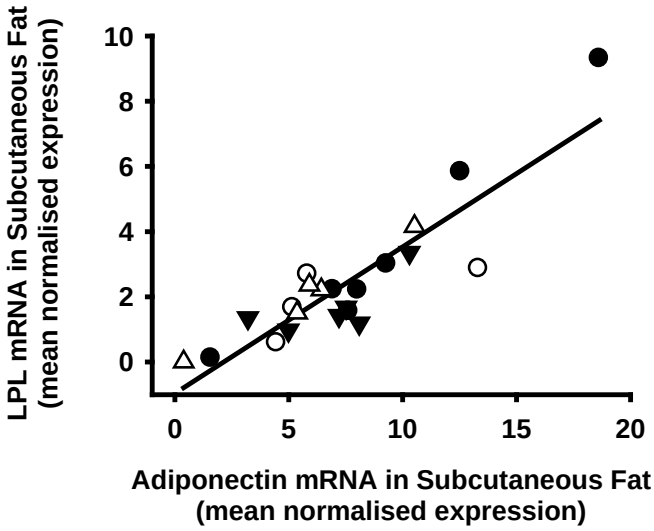

Figure 4.1 The relationship between Adiponectin and LPL mRNA expression in subcutaneous fat at 21d in the lamb.

Adiponectin and LPL mRNA expression in perirenal fat were strongly related in both male (Control, closed circles; IUGR, open circles) and female (Control, closed triangles; IUGR open triangles) lambs.

4.4.3 POTENTIAL INTERACTIONS BETWEEN THE SUBCUTANEOUS AND PERIRENAL ADIPOSE TISSUE DEPOTS IN THE POSTNATAL LAMB.

The mean size of subcutaneous adipocytes was strongly related to the relative mass of visceral fat (r^2=0.78, P < 0.0001). The expression of leptin mRNA in subcutaneous fat was also related to the relative mass of visceral fat (r^2=0.31, P < 0.01, Figure 4.2) and to the mean size of the perirenal adipocytes (r^2=0.31, P < 0.01, Figure 4.2). G3PDH mRNA expression in subcutaneous fat was also directly related to the size of the perirenal adipocytes (r^2=0.56, P < 0.05).

The expression of G3PDH mRNA was higher (P < 0.05) in subcutaneous fat (3.58 $\pm$ 0.30) than in perirenal fat (2.84 $\pm$ 0.20), but there was no difference in subcutaneous and perirenal mRNA expression levels for the remaining genes (Table 4.3). There was a direct relationship between the expression of G3PDH, leptin, adiponectin and LPL mRNA, but not PPARγ, RXRα, or GAPDH mRNA in the subcutaneous and perirenal depots (G3PDH (r^2=0.46, P < 0.001); leptin (r^2=0.23, P < 0.05); adiponectin (r^2=0.38, P < 0.01) and LPL (r^2=0.51, P < 0.001)).

4.4.3.1 PPARγ mRNA expression in perirenal fat and subcutaneous and total fat mass.

In male lambs the relative mass of total fat (r^2=0.42, P < 0.05) was inversely related to the expression of PPARγ mRNA in perirenal, but not subcutaneous fat at 21d (Figure 4.3). In contrast, in female lambs the relative mass of total fat (r^2=0.60, P < 0.01) was positively related to the expression of PPARγ mRNA in perirenal, but not subcutaneous fat at 21d (Figure 4.3).

Table 4.3: The Mean Expression of Leptin, PPARγ, RXRα, Adiponectin, LPL, G3PDH and GAPDH mRNA transcripts in Subcutaneous and Perirenal Adipose Tissue in the Postnatal Lamb.

Target Gene : Reference Gene	**Subcutaneous Adipose Tissue mRNA Expression (n = 21)**	**Perirenal Adipose Tissue mRNA Expression (n = 23)**
Leptin : RPLP0	0.52 ± 0.07	0.47 ± 0.04
PPARg : RPLP0	1.90 ± 0.10	1.94 ± 0.11
RXRa : RPLP0	0.20 ± 0.01	0.19 ± 0.01
Adiponectin : RPLP0	7.42 ± 0.86	8.48 ± 0.66
LPL : RPLP0	2.38 ± 0.43	1.72 ± 0.20
G3PDH : RPLP0	3.58 ± 0.30 *	2.84 ± 0.20
GAPDH : RPLP0	1.61 ± 0.31	1.35 ± 0.06

Superscripts indicate significant differences between mean values in the Subcutaneous and Perirenal depots (*).

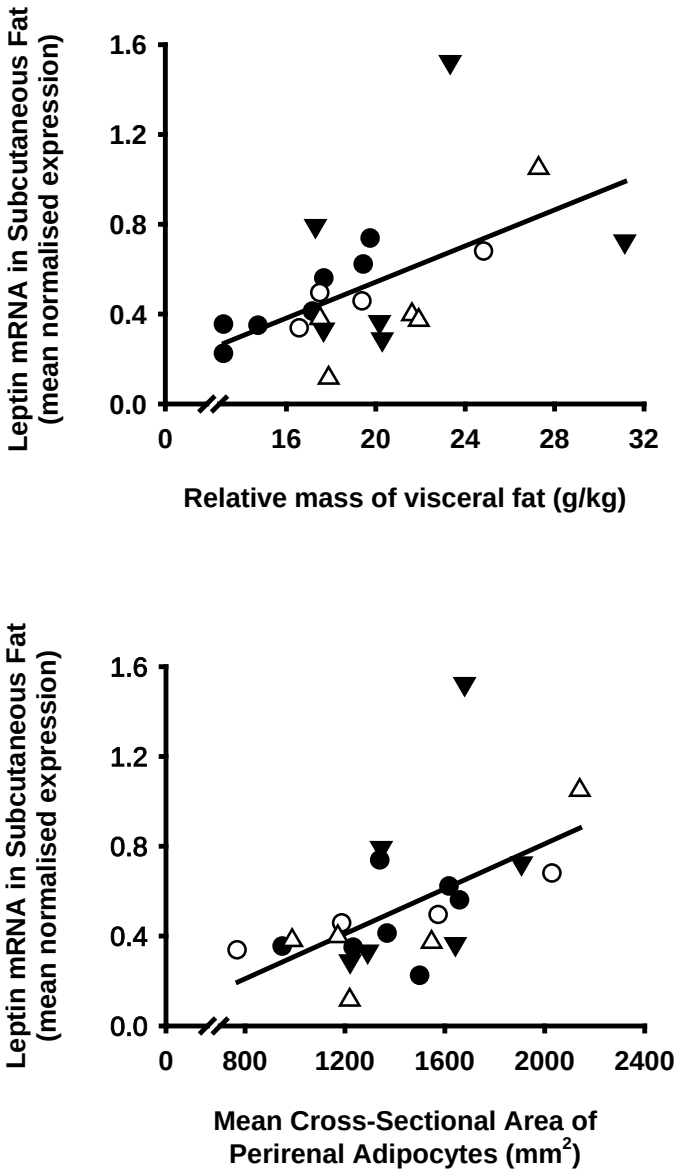

Figure 4.2 Relative perirenal and omental fat mass and the size of perirenal and omental adipocytes at 21d of life in Control and IUGR lambs.

Upper Panel: The expression of leptin mRNA in subcutaneous fat was related to the relative mass of visceral fat (r^2=0.31, P < 0.01) in both male (Control, closed circles; IUGR, open circles) and female (Control, closed triangles; IUGR open triangles) lambs.

Lower Panel: The expression of leptin mRNA in subcutaneous fat was related to the meancross-sectional area of perirenal adipocytes (r^2=0.31, P < 0.01) in both male (Control, closed circles; IUGR, open circles) and female (Control, closed triangles; IUGR open triangles) lambs.

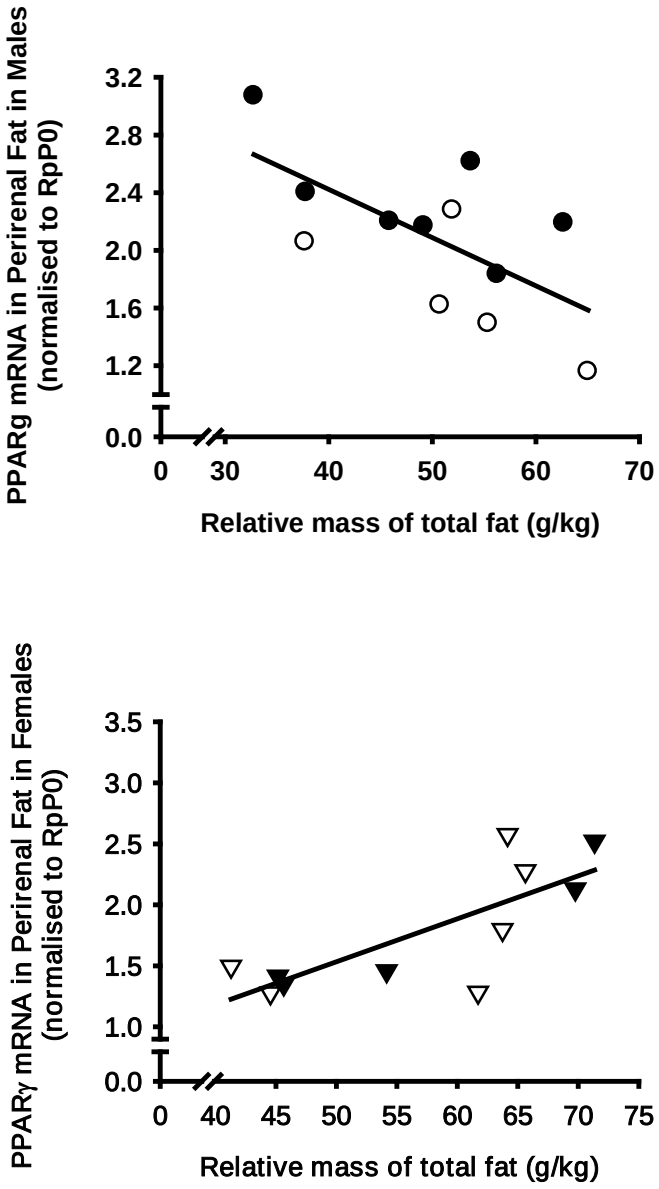

Figure 4.3 The sex specific relationships between the relative mass of total fat and PPARγ mRNA expression in perirenal fat in Control and IUGR lambs.

Upper Panel: The relative mass of total fat is inversely related to the expression of Peroxisome Proliferator-Activated Receptor-γ (PPARγ) mRNA in perirenal fat in male Control (closed circles) and IUGR (open circles) lambs.

Lower Panel: The relative mass of total fat is positively related to the expression of PPARγ mRNA in perirenal fat in female Control (closed triangles) and IUGR (open triangles) lambs.

4.5 DISCUSSION

In this study we have investigated the extent to which changes in growth of the subcutaneous adipocyte and the expression of PPARγ and other adipogenic and lipogenic genes are determined by prenatal and postnatal factors, including the postnatal growth rate and circulating hormonal and nutrient levels during the first three weeks of life. We have found that there is no effect of the fetal nutritional environment on the development of subcutaneous adipose tissue however, the growth and functional development of subcutaneous adipose tissue was related to measures of both the postnatal nutritional environment and the development of the perirenal adipose tissue depot, leading to the speculation that there may be a factor secreted from visceral fat that influences the development of the postnatal fat depots.

4.5.1 THERE WAS NO EFFECT OF IUGR OR SEX ON THE DEVELOPMENT OF SUBCUTANEOUS ADIPOSE TISSUE

There was no effect of either IUGR or sex on the mass of subcutaneous or total fat in lambs at 21d of life. This is in contrast to the visceral fat mass, which is increased in female, compared with male lambs in this study. In previous studies of IUGR lambs a higher total, and a higher visceral fat mass has been found at 45d of life and onwards (Greenwood *et al.*, 1998; Louey *et al.*, 2005; De Blasio *et al.*, 2007a). Therefore the present study has been conducted before the onset of the increased visceral adiposity in the IUGR lamb.

4.5.2 There Was No Effect of IUGR or Sex on the Expression of Adipogenic or Lipogenic Genes in the Subcutaneous Adipose Tissue of the Postnatal Lamb

There was also no effect of IUGR or sex on the expression of PPARγ, RXRα, leptin, adiponectin, LPL, G3PDH or GAPDH mRNA in subcutaneous fat at 21d. We reported in Chapter 3 that in perirenal fat there was a decreased expression of both PPARγ and leptin mRNA in male lambs, which suggests that the development of the perirenal fat depot is more sensitive to a decrease in fetal substrate supply. In both male and female lambs there was a direct relationship between adiponectin and LPL mRNA expression in the subcutaneous depot. This relationship is also present in perirenal adipose tissue, which suggests that the relationship results from a tissue-specific, but not a depot-specific mechanism. In adults a relationship between postheparin LPL activity and plasma adiponectin has been described and appears to influence triglyceride handling in hypoadiponectinemic states, including insulin resistance (von Eynatten *et al.*, 2004; De Vries *et al.*, 2005)

4.5.3 The Sex Specific Relationships Between Postnatal Nutrient and Hormone Levels and the Development of Subcutaneous Adipose Tissue in the Lamb

There were sex specific relationships between postnatal nutrient and hormone levels and the development of subcutaneous fat at 21 d. In male lambs plasma glucose concentrations during wk 1 were related to fat mass, and both plasma leptin and insulin concentrations during the first 24h after birth were related to the size of subcutaneous adipocytes. Interestingly plasma leptin concentrations

during the first 24 h after birth were also related to the expression of subcutaneous PPARγ mRNA. In female lambs plasma leptin concentrations during week 1 were related to the development of subcutaneous fat mass, while plasma insulin concentrations during week 1 were related to PPARγ mRNA expression in subcutaneous fat. Both the cross sectional area of the subcutaneous adipocytes and leptin mRNA expression were related to current plasma insulin concentrations in females. These relationships may provide some insight into the timing of the effect of nutritional factors on the level of expression of genes that influence adipose tissue differentiation and regulate insulin sensitivity, and highlight a potential role for sex-hormones in modulating these relationships. In males there was also a relationship between leptin mRNA expression in subcutaneous fat and the cross sectional area of subcutaneous adipocytes, indicating that the leptin-fat mass axis is functional in the subcutaneous adipocytes of male lambs at 21d of life.

4.5.4 Cross-talk Between the Perirenal and Subcutaneous Adipose Tissue Depots in the Lamb

There were relationships between the mass of perirenal fat and the development of subcutaneous adipose tissue in both male and female lambs. The mass of visceral fat was related to the mean size of subcutaneous adipocytes, and both the mass of visceral fat and the size of perirenal adipocytes were related to the expression of leptin mRNA in subcutaneous fat. Furthermore leptin, adiponectin and LPL mRNA expression in both the subcutaneous and perirenal depots were each related. This either reflects depot specific responses to the postnatal nutritional environment that occur in parallel, or alternatively it is possible that there is a factor secreted from visceral adipose tissue proportional to adipocyte

size and tissue mass that has an impact on the development of subcutaneous adipocytes and the development of the leptin fat mass axis in subcutaneous tissue. It may be this factor, which ultimately conveys the effect of the fetal nutritional environment from perirenal to subcutaneous adipocytes, and results in an increased subcutaneous adiposity in individuals who were born small for gestational age.

There was an impact of sex on the relationship between the expression of PPARγ mRNA in perirenal fat and total fat mass. This implies that if there is a factor secreted from perirenal fat that subsequently influences the development of other postnatal depots, including the subcutaneous depot, it is possible that its effect is modulated by sex hormones. The impact of sex on the relationship between PPARγ mRNA expression in perirenal fat and total fat mass may provide an insight into the mechanisms which determine the sex specific distribution of body fat that is apparent in adult life.

4.5.5 SUMMARY

In summary, we have shown that there is no effect of IUGR on the growth of the subcutaneous adipose tissue depot. There was, however, evidence for cross talk between the perirenal and subcutaneous depots which raises the possibility that the impact of the fetal nutritional environment on the development of perirenal adipose tissue may subsequently affect the development of subcutaneous adipose tissue and result in an increased subcutaneous adiposity in individuals who were small for gestational age.

5. The impact of IUGR and sex on the expression of insulin-like growth factor (IGF) mRNA in subcutaneous and visceral adipose tissue in the postnatal lamb.

5.1 SUMMARY

It was reported in Chapter 3 that IGF1 mRNA expression is relatively lower in perirenal adipose tissue in IUGR fetal sheep when compared to controls and it was speculated that this could have implications for the subsequent development of adiposity and adipose tissue insulin sensitivity. We have therefore investigated the effect of IUGR on adipose tissue IGF mRNA expression during early postnatal life in the sheep. As in chapters 3 and 4, IUGR was defined as a birth weight less than 2 standard deviations below the mean of a cohort of singleton Merino lambs. Blood samples were collected throughout the first 3 weeks of life and adipose tissue from the perirenal (PAT) and subcutaneous (SAT) depots were collected at 21 d of life. The expression of IGF1, IGF2, IGF1R and IGF2R in PAT and SAT was determined using qRT-PCR. At 21d of life there was an effect of sex, but not IUGR, on the expression of IGF mRNA in adipose tissue. Male lambs had a higher expression of IGF1 mRNA in both PAT and SAT, and a higher expression of IGF2R in SAT compared with female lambs, which may reflect sexual dimorphism of the GH-IGF axis. There was a higher expression of IGF1 mRNA in SAT compared with PAT, and a higher expression of IGF2, IGF1R and IGF2R mRNA in PAT compared with SAT in both male and female lambs. These differences in IGF mRNA expression provide a potential mechanism to explain the sex and depot specific variations in mitogenic potency of IGF1 and

proliferative capacities of preadipocytes, the regional variation in adipocyte metabolism, and the difference in incidence of visceral obesity between men and women in adult life.

5.2 INTRODUCTION

As discussed previously SGA infants have low circulating insulin-like growth factor-1 (IGF1) concentrations and a reduced body fat mass at birth (Enzi *et al.*, 1981), and then undergo a period of accelerated postnatal growth during the first few years of life (Fitzhardinge & Steven, 1972; Albertsson-Wikland *et al.*, 1993), with a relative increase in body fat mass from as early as 2 -12 months of age (Hediger *et al.*, 1998) and truncal fat mass during childhood (Ong *et al.*, 2000) and in adult life (Law *et al.*, 1992; Parsons *et al.*, 2001). An accelerated postnatal growth rate is associated with an initial increased insulin sensitivity, followed by the emergence of insulin resistance (Curhan *et al.*, 1996; Whincup *et al.*, 1997; Bavdekar *et al.*, 1999; Eriksson *et al.*, 2001; Veening *et al.*, 2002; Levy-Marchal *et al.*, 2004), and in particular, insulin resistance of adipose tissue in later life (Jaquet *et al.*, 1998; Jaquet *et al.*, 1999; Phillips *et al.*, 1999; Jaquet *et al.*, 2000)

IGF1 is one of the most important growth factors in the development of adipose tissue. The experimental induction of a global IGF1-receptor (IGF1R) deficiency in mice produces growth restriction, and reduces the growth of adipose tissue relative to that of other tissues (Holzenberger *et al.*, 2001). At physiologic levels, IGF1 stimulates both proliferation and differentiation of preadipocytes in cell culture, and along with cAMP and glucocorticoids, has been shown to be required for induction of adipose tissue differentiation in both serum-containing and serum-free media (Gregoire *et al.*, 1998; Soret *et al.*, 1999). It has been shown that after experimental induction of differentiation growth arrested preadipocytes go through clonal expansion prior to a significant increase in the expression of

proliferator-activated receptor-γ (PPARγ), a prominent adipogenic transcription factor when heterodimerised with retinoid-X-receptor-α (RXRα). This initiates the transcription of the regulatory genes involved in lipid accumulation and glucose metabolism, particularly lipoprotein lipase (LPL), leptin and adiponectin and marks the entry of the cell into terminal diffferentiation (Gregoire *et al.*, 1998; Picard & Auwerx, 2002).

In the sheep or pig, as in the human, low birth weight (LBW) offspring grow faster in the postnatal period, have a higher proportion of visceral (De Blasio *et al.*, 2007a), and total body fat than their higher birth weight counterparts in later life (Greenwood *et al.*, 1998; Poore & Fowden, 2004; Louey *et al.*, 2005), and an altered glucose metabolism and insulin sensitivity in early postnatal life (Clarke *et al.*, 2000; Poore & Fowden, 2002; De Blasio *et al.*, 2007a). We have therefore used the newborn lamb as a model to test the hypothesis that poor fetal growth in association with rapid postnatal growth results in an altered pattern of expression of IGF genes in visceral and subcutaneous fat before the emergence of an increase in visceral adiposity in these lambs. It was shown in chapter 2 that there is a reduced adipocyte IGF1 mRNA expression in the perirenal adipose tissue of placentally restricted fetal sheep during fetal life, when this tissue is comprised of brown adipocytes (Gemmell & Alexander, 1978; Klaus, 1996). After birth, in both perirenal and subcutaneous depots the size of lipid locules increases during the first 2-3 weeks of life, and the percentage of brown adipose tissue decreases as all depots take on the molecular and morphometric characteristics of mature white adipose tissue (Gemmell *et al.*, 1972). We have therefore investigated the extent to which changes in growth of the adipocyte and

the expression of IGF1, IGF2, IGF1R and IGF2R in perirenal and subcutaneous fat are determined by prenatal factors as summarised by birth weight, and by postnatal factors, including the postnatal growth rate and circulating hormonal and nutrient levels during the first three weeks of life.

5.3 METHODS

5.3.1 ANIMALS AND SURGERY, BLOOD SAMPLING PROTOCOL, GROWTH MEASUREMENTS AND POSTMORTEMS

All procedures were approved by The University of Adelaide Animal Ethics Committee. Twenty-two Merino ewes were used in the study. As described previously in this thesis, 6 non-pregnant ewes underwent surgery to remove the majority of endometrial caruncles (>70) from the uterus, leaving 3 to 8 caruncles in each horn in order to induce experimental restriction of placental and fetal growth (Robinson *et al.*, 1979).

Lambs were weighed (kg) and crown-rump length (cm) measured daily between 1000 - 1400 h. Venous blood samples were collected in chilled tubes after approximately 60 min of non-suckling on alternate days between 0900 h – 1300 h, as previously described (Chapter 3,4). All blood samples were centrifuged at 1500g for 10 min and plasma separated into aliquots and stored at -20°C. On day 21, lambs were killed with an overdose of sodium pentobarbitone (Virbac Pty Ltd, Peakhurst, NSW, Australia) and the perirenal/retroperitoneal and subcutaneous adipose tissue was dissected and weighed, and stored for further analyses as described in Chapters 3 and 4.

5.3.2 Plasma Nutrient and Hormone Assays and Adipose Tissue Histology

Plasma NEFAs, insulin, leptin and glucose concentrations were measured using specific assays as described in Chapters 3 and 4.

Tissues were fixed in 4% paraformaldehyde buffer, washed and embedded in paraffin wax. Sections were cut (4 -5 μm) and stained with Hematoxylin and Eosin. Standard point counting techniques were used with Video Image Analysis (VIA) using Video Pro software (Leading Edge, Adelaide, SA, Australia) to measure the area of 100 adipocytes within each fat depot (Muhlhausler *et al.*, 2002).

5.3.3 Isolation of RNA, Production of cDNA and qRT-PCR

The relative expression of IGF1, IGF2, IGF1R and IGF2R mRNA transcripts in perirenal (representative of visceral adipose tissue) and subcutaneous adipose tissue were measured by quantitative real time reverse transcription-PCR (qRT-PCR) using the Sybr Green system in an ABI Prism 7900 Sequence Detection System (PE Applied Biosystems, Foster City, CA) (Gibson *et al.*, 1996; Heid *et al.*, 1996). All primers were designed with the aid of Primer Express (PE Applied Biosystems, Foster City, CA) software and where possible one primer of each pair was positioned over a splice site to prevent amplification of any residual genomic DNA. For each transcript RT-PCR was performed using the appropriate primers (Table 2.2), and controls containing no reverse transcriptase were also used. A qRT-PCR melt curve analysis was performed to demonstrate amplicon homogeneity. For the qRT-PCR measurements, the primer concentrations were equivalent for all genes and the amplification efficiencies were 0.997 – 0.999. A

constant amount of cDNA equating to 10ng of total RNA was used for each qRT-PCR measurement and four technical replicates were performed for each gene.

Each qRT-PCR reaction (10 μl total volume) contained:: 6 μl 2x Sybr Green Master Mix (PE Applied Biosystems, Foster City, CA); 1ul of each primer giving a final concentration of 450 or 900 nM, 2.0 μl of molecular grade H_20 and 1.0 μl of a 50 ng/ul dilution of the stock template. The cycling conditions consisted of 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. At the end of each run, a dissociation melt curve was obtained.

The abundance of each mRNA transcript was expressed relative to that of Acidic Ribosomal Protein Large Subunit P0 (RPLP0) and calculated using Q-gene qRT-PCR analysis software (Muller *et al.*, 2002). The abundance of each mRNA transcript was also expressed relative to that of an additional reference gene, 18S ribosomal RNA. Correlational data of relative mRNA expression that could not be replicated when using both reference genes was not included and therefore only abundance relative to RPLP0 is reported.

5.3.4 Statistical Analyses

Using the criteria for the definition of IUGR, 3 male and 3 female placentally restricted singleton lambs, a male and female pair of twin siblings and one female growth restricted control singleton were allocated into the IUGR group. The Control group consisted of all control singleton lambs. Therefore there were 4 males and 5 females in the IUGR group and 8 males and 6 females in the Control group.

Parametric analyses were used in this study. Using the Statistical Package for Social Scientists, the effects of IUGR, sex and depot on the expression of IGF genes in adipose tissue were determined using a univariate analysis of variance (ANOVA). Relationships between measures of fat mass, adipocyte cell size or gene expression and either measures of prenatal growth (birth weight) or postnatal growth (postnatal growth rate and plasma hormonal and nutrient concentrations) were determined using linear regression analyses and partial correlation analyses. All data are presented as the mean ± SEM. A probability of <5% ($P<0.05$) was taken as the level of significance in all analyses.

5.4 RESULTS

5.4.1 THE EFFECT OF IUGR, SEX AND DEPOT ON IGF1 MRNA EXPRESSION IN ADIPOSE TISSUE IN THE POSTNATAL LAMB

There was no effect of IUGR on the expression of IGF1 mRNA in male and female lambs in either the perirenal adipose tissue (PAT) or subcutaneous adipose tissue (SAT) of lambs at 3 weeks of age (Table 5.1). Independent of size at birth, there was an effect of both sex and depot on the expression of IGF1 mRNA in adipose tissue at 3 weeks of age. Male lambs had a higher expression of IGF1 mRNA in PAT ($P < 0.05$) and tended to have a higher expression of IGF1 mRNA in SAT ($P = 0.06$), compared with female lambs (Table 5.1), while in each sex the expression of IGF1 was higher in SAT compared with PAT (males $P < 0.0001$, females $P < 0.0001$).

Male Lambs - IGF1 mRNA Expression in PAT

In male lambs the expression of IGF1 mRNA in PAT at 3 weeks of age tended to be related to plasma leptin concentrations during the first 24h after birth in male lambs ($P < 0.09$) (Figure 5.1 Upper Panel).

Female Lambs - IGF1 mRNA Expression in PAT

The expression of IGF1 in perirenal fat at 3 weeks of age was significantly related to plasma leptin concentrations during the first 24h after birth in female lambs (Figure 5.1 Lower Panel), In females IGF1 mRNA expression was also related to the expression of PPARγ (r^2=0.36, $P = 0.05$), LPL (r^2=0.40, $P < 0.05$), and G3PDH mRNA (r^2=0.41, $P < 0.05$) in perirenal fat.

Female lambs - IGF1 mRNA Expression in SAT

In females the expression of IGF1 in subcutaneous fat at 3 weeks of age was positively related to the expression of G3PDH (r^2=-0.46, $P < 0.05$) and IGF1R mRNA (r^2=0.64, $P < 0.01$).

5.4.2 The Effect of IUGR, Sex and Depot on IGF2 mRNA Expression in Adipose Tissue in the Postnatal Lamb

There was no effect of IUGR or sex on the expression of IGF2 mRNA in male and female lambs in either the perirenal adipose tissue (PAT) or subcutaneous adipose tissue (SAT) of lambs at 3 weeks of age (Table 5.1). There was a trend for an interaction between sex and depot ($P = 0.055$) therefore the effect of depot on IGF1R expression has been analysed separately for males and

females. For each sex the expression of IGF2 was higher in PAT compared with SAT (males $P < 0.0001$, females $P < 0.0001$).

Male lambs – IGF2 mRNA Expression in SAT

In male, but not female lambs, there was an inverse relationship between IGF2 and PPARγ mRNA expression in SAT (r^2=-0.38, $P < 0.05$).

Female Lambs - IGF2 mRNA Expression in PAT

In female, but not male lambs the expression of IGF2 in PAT was inversely related to mean plasma leptin concentrations during weeks 1 to 3 (r^2=-0.49, $P < 0.02$), and to plasma NEFA at post mortem (r^2=-0.51, $P < 0.02$).

Female Lambs - IGF2 mRNA Expression in SAT

In female lambs the expression of IGF2 mRNA in subcutaneous fat at 3 weeks of age was positively related to the daily growth rate during week 1 (r^2=-0.77, $P < 0.01$), and inversely related to plasma glucose (r^2=-0.45, $P < 0.05$) and insulin (r^2=-0.45, $P < 0.05$) concentrations during the first 24h after birth.

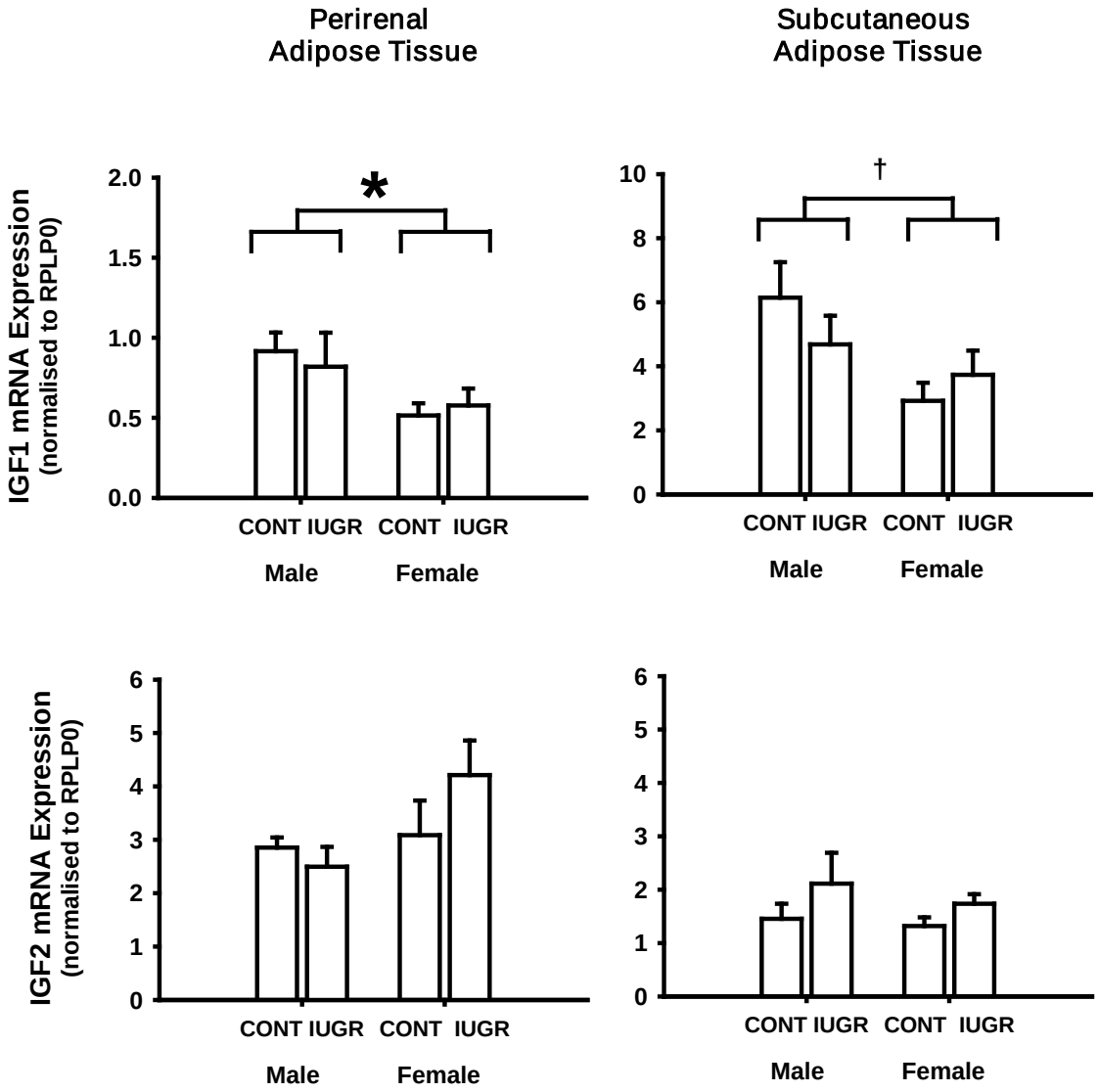

Figure 5.1 IGF1 and IGF2 mRNA expression in Control and IUGR male and female lambs in the Perirenal and Subcutaneous Adipose Tissue Depots.

Upper Panel: IGF1 mRNA expression in the PAT (left hand side) and SAT (right hand side) of male and female Control (closed bars) and IUGR (open bars) lambs at 21 d. Male lambs have a higher expression of IGF1 mRNA in PAT (*, $P < 0.05$) and tend to have a higher expression of IGF1 mRNA in SAT **(**†**,** $P = 0.06$), compared with female lambs. IGF1 mRNA expression is significantly higher in SAT compared with PAT ($P < 0.0001$).

Lower Panel: IGF2 mRNA expression in the PAT (left hand side) and SAT (right hand side) of male and female Control (closed bars) and IUGR (open bars) lambs at 21 d. IGF2 mRNA expression is significantly higher in PAT compared with SAT ($P < 0.001$).

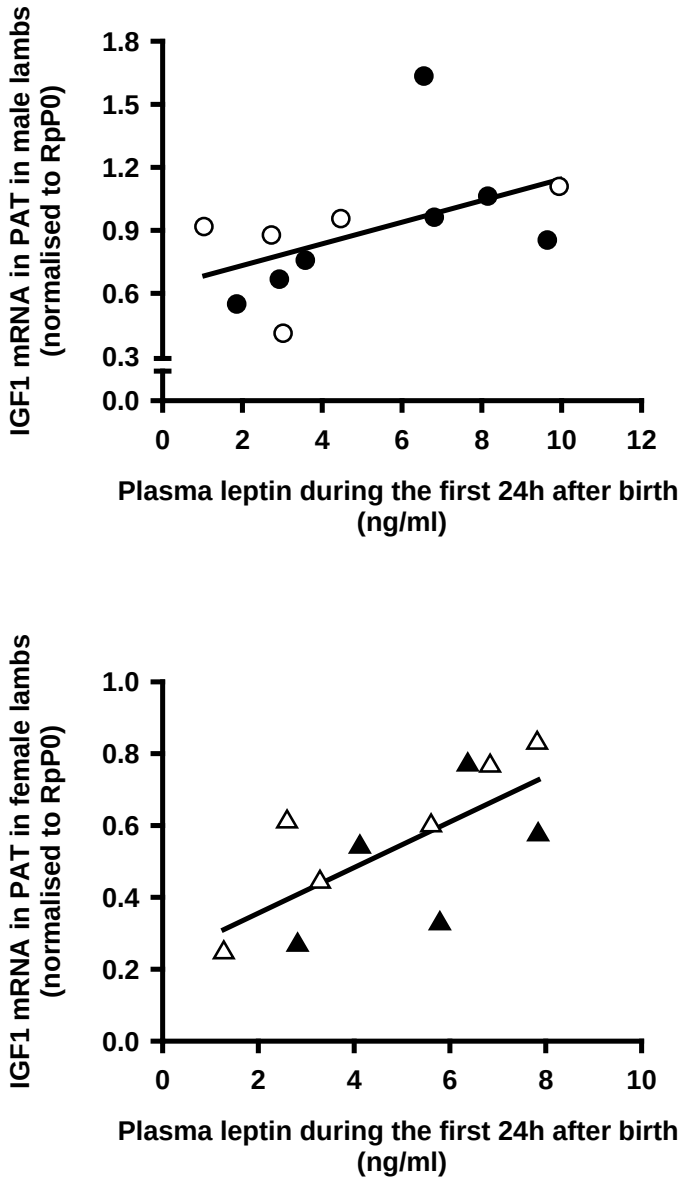

Figure 5.2 The relationships between plasma leptin during the first 24h after birth and the expression of IGF1 mRNA in perirenal fat in male and female lambs in the Control and IUGR groups.

Upper Panel: There was a trend for a relationship between plasma leptin concentrations during the first 24h after birth and IGF1 mRNA expression in perirenal fat at 21 d in male Control (closed circles) and IUGR (open circles) lambs (r^2=0.51, P = 0.09).

Lower Panel: There was a significant relationship between plasma leptin concentrations during the first 24h after birth and IGF1 mRNA expression in perirenal fat at 21 d in female Control (closed triangles) and IUGR (open triangles) lambs (r^2=0.49, P < 0.02).

5.4.3 The Effect of IUGR, Sex and Depot on IGF1R and IGF2R mRNA Expression in Adipose Tissue in the Postnatal Lamb

There was no effect of IUGR or sex on the expression of IGF1R mRNA in male and female lambs in either PAT or SAT in lambs at 3 weeks of age (Table 5.1). Independent of size at birth and of sex, there was an effect of depot on the adipose tissue expression of IGF1R mRNA at 3 weeks of age, such that IGF1R mRNA expression was higher in PAT compared with SAT ($P < 0.0001$) (Table 5.1).

There was no effect of IUGR on the expression of IGF2R mRNA in male and female lambs in either the PAT or SAT of lambs at 3 weeks of age (Table 5.1). In dependent of weight at birth, there was an effect of sex on the expression of IGF2R mRNA in subcutaneous, but not perirenal adipose tissue at 3 weeks of age, such that male lambs had a higher expression of IGF2R mRNA than female lambs ($P < 0.0001$) (Table 5.1), For each sex the expression of IGF2R was higher in PAT compared with SAT (males $P < 0.02$, females $P < 0.0001$).

Male lambs - IGF1R and IGF2R mRNA Expression in PAT

In the PAT of male, but not female lambs, IGF1R and IGF2R mRNA expression were directly related ($r^2=0.42$, $P < 0.05$). In addition, in male, but not female lambs, the expression of IGF2 mRNA was related to both IGF1R ($r^2=0.35$, $P < 0.05$) and IGF2R mRNA expression ($r^2=0.61$, $P < 0.001$). When the relationships between IGF1R, IGF2R and IGF2 mRNA expression were investigated using partial correlation analysis, only the relationship between IGF2R and IGF2 was independent ($r^2=0.42$, $P < 0.05$).

Male lambs - IGF1R and IGF2R mRNA Expression in SAT

In the SAT of male, but not female lambs, IGF1R and IGF2R mRNA expression were also directly related (r^2=0.66, P < 0.01). The expression of IGF1R mRNA in SAT was also positively related to plasma leptin concentrations during week 3 (r^2=0.37, P < 0.05), and inversely related to prevailing plasma glucose concentrations (r^2=-0.56, P < 0.01). The expression of IGF2R mRNA was positively related to the current daily growth rate (r^2=0.37, P < 0.05), and inversely related to both current plasma glucose (r^2=-0.38, P < 0.05) and NEFA concentrations (r^2=-0.44, P < 0.05).

Female lambs – IGF1R mRNA Expression in PAT

In female lambs, IGF1R and IGF2 mRNA expression in PAT at 3 weeks of age were directly related (r^2=0.80, P < 0.0001).

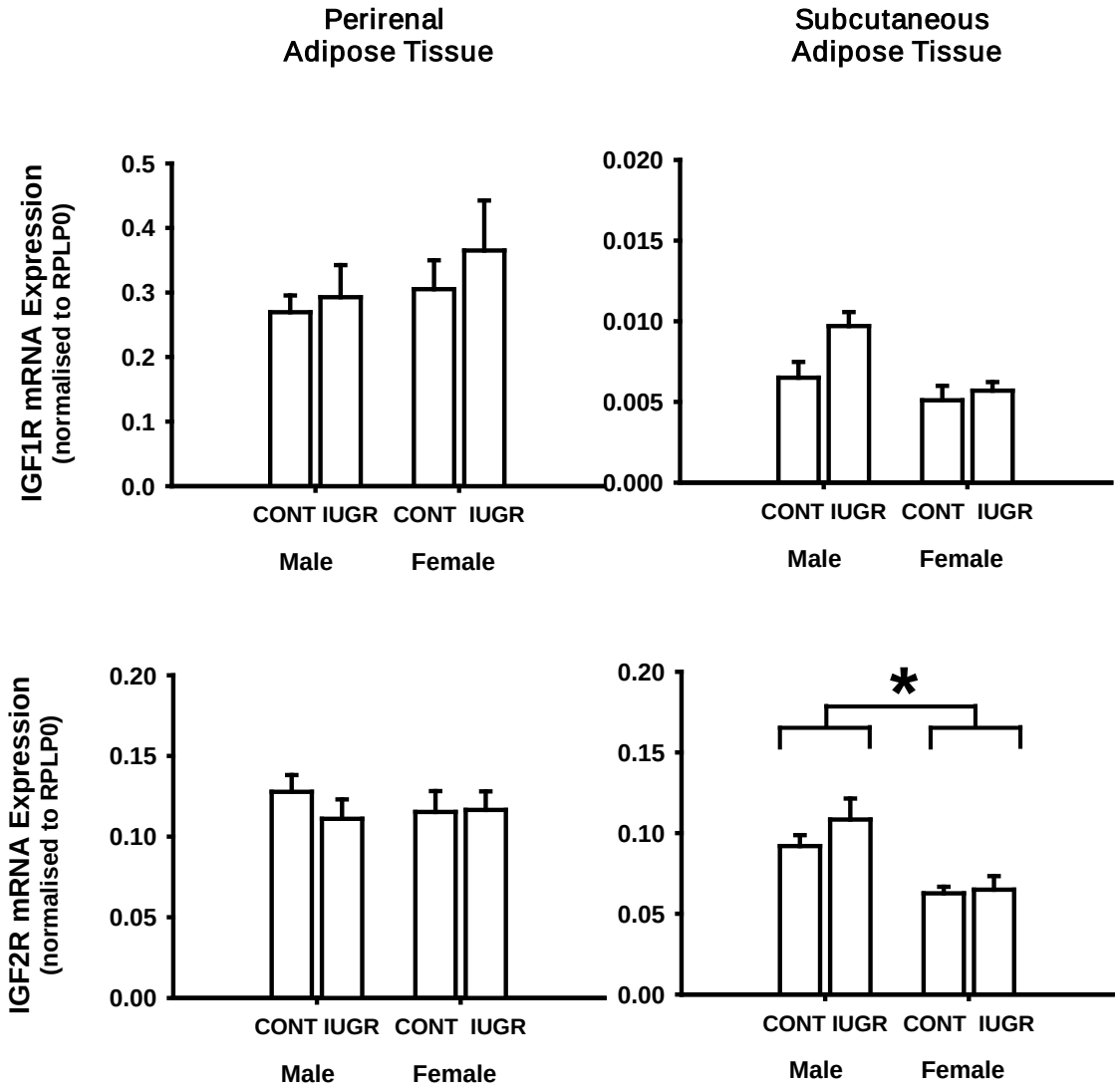

Figure 5.3 IGF1R and IGF2R mRNA expression in Control and IUGR male and female lambs in the Perirenal and Subcutaneous Adipose Tissue Depots.

Upper Panel: IGF1R mRNA expression in the PAT (left hand side) and SAT (right hand side) of male and female Control (closed bars) and IUGR (open bars) lambs at 21 d. IGF1R mRNA expression is significantly higher in PAT compared with SAT ($P < 0.0001$).

Lower Panel: IGF2R mRNA expression in the PAT (left hand side) and SAT (right hand side) of male and female Control (closed bars) and IUGR (open bars) lambs at 21 d. Male lambs have a higher expression of IGF2R mRNA in SAT **(*,** $P < 0.05$), compared with female lambs. IGF2R mRNA expression is significantly higher in SAT compared with PAT ($P < 0.05$).

5.5 DISCUSSION

5.5.1 THERE WAS NO EFFECT OF IUGR ON THE EXPRESSION OF IGF MRNA IN PERIRENAL AND SUBCUTANEOUS ADIPOSE TISSUE AT 3 WEEKS OF LIFE

There was no effect of IUGR on the expression of IGF1, IGF2, IGF1R or IGF2R mRNA in the perirenal fat of lambs at 3 weeks of age. This is in contrast to fetal life, where there is a reduction in IGF1 mRNA, but not IGF2, IGF1R or IGF2R mRNA expression in perirenal adipose tissue, the major fetal adipose tissue depot (Chapter 2). Therefore the effect of IUGR on IGF1 mRNA expression in perirenal fat that is observed during fetal life does not persist into postnatal life. This most likely reflects the importance of the prevailing nutritional environment in the regulation of the expression of IGF1 mRNA in the perirenal adipose tissue (Straus, 1994; Ketelslegers *et al.*, 1995) as the IUGR fetus undergoes a transition from a relatively low prenatal to a high postnatal nutritional environment.

5.5.2 IGF1 MRNA EXPRESSION IN ADIPOSE TISSUE IN THE POSTNATAL LAMB IS SEX AND DEPOT SPECIFIC

Interestingly there was an effect of sex on the expression of IGF1 mRNA expression in perirenal adipose tissue at 3 weeks of age. The expression of IGF1 mRNA in perirenal adipose tissue was higher in male than female lambs, and tended to be higher in the subcutaneous tissue in males compared with females. Sexual dimorphism of plasma IGF1 has been described previously in the lamb and adult sheep, where plasma IGFI is higher in rams than nonpregnant ewes (Van Vliet *et al.*, 1983; Gatford *et al.*, 1996). It is therefore possible that the

increase in plasma IGF in adult rams compared to ewes might be contributed to by an increased production and secretion of IGF1 by adipose tissue as a result of an increased basal transcription rate of IGF1 mRNA *(Nam & Marcus, 2000).*

We have previously shown that male lambs have a higher circulating plasma insulin concentration for any given glucose concentration during the first three weeks of life (Chapter 3). Both insulin and GH have been shown to be positive regulators of IGF1 secretion in both porcine (Chen *et al.*, 1996) and human preadipocyte cultures, but in human adipocytes in culture only insulin stimulates secretion of IGF1 (Wabitsch *et al.*, 2000). In the current study, however, there was no relationship between plasma insulin concentrations and IGF1 mRNA expression in perirenal fat in either male or female lambs, suggesting that the higher plasma insulin of male lambs does not directly explain the higher expression of IGF1 mRNA in perirenal adipose tissue. Interestingly, however, the plasma leptin concentration during the first 24h after birth was related to the expression of IGF1 mRNA in perirenal fat 3 weeks later in both male and female lambs, which may reflect a programming effect of early leptin concentrations on basal perirenal adipocyte IGF1 mRNA production.

We have also previously shown that male lambs have a lower relative mass of perirenal fat at 21d of life compared with female lambs, which we attributed to a decreased cell number rather than cell size (Chapter 3), and we proposed that this may be a result of a relatively lower insulin sensitivity of perirenal adipose tissue in males compared with females. It is possible that in males the relatively high plasma insulin concentration may alter the ratio of insulin : IGF1 signalling at

both the insulin receptor and IGF1R, such that there is a relative resistance to the actions of these anabolic factors with a consequent reduced proliferation of perirenal adipocytes.

In the current study, the increase in IGF1 mRNA in adipose tissue in male lambs may be evidence that there is sexual dimorphism of the GH-IGF axis present in the fat depots. At birth male newborns have higher umbilical cord serum levels of total and free testosterone and oestradiol (Simmons *et al.*, 1994), higher cord plasma GH concentrations, and lower IGF1 and IGF binding protein 3 (IGFBP3) concentrations (Geary *et al.*, 2003a). Sexual dimorphism of the plasma concentrations of components of the GH-IGF axis have also been observed in childhood (Fall *et al.*, 2000) and in later life (Hindmarsh *et al.*, 1999). Both testosterone and oestradiol have been shown to affect adipose tissue differentiation and function. 17β-oestradiol increases IGF1R protein content and the proliferation rate of preadipocytes, whereas dihydrotestosterone reduces IGF1R protein content of preadipocytes in a sex and depot specific manner (Roncari & Van, 1978; Dieudonne *et al.*, 2000). Testosterone has also been shown to exhibit lipolytic effects, by amplifying the lipolytic GH effect in adipose tissue (Yang *et al.*, 1995), and upregulating adipocyte androgen and β-3 adrenergic receptors (De Pergola *et al.*, 1990; Xu *et al.*, 1991). Oestradiol may have opposing lipogenic effects as it has been shown to downregulate GH-receptor signaling *in vitro* by inducing cellular expression of the negative-feedback signal, suppressor of cytokine signaling, in non-adipose cell lines (Leung *et al.*, 2003). There is some initial evidence that 17β-oestradiol increases PPARγ2 protein expression in rat preadipocyte and adipocyte cultures in a sex

and site dependent manner (Dieudonne *et al.*, 2000), and that estrogens promote overexpression of PPARγ1 by inducing production of prostaglandin J2 from arachidonic acid (Ma *et al.*, 1998). This is interesting given that we have previously shown that male, but not female IUGR lambs had a reduced expression of PPARγ mRNA in perirenal adipose tissue at 21d postnatal life, and may suggest that estrogens may be protective against the effect of IUGR on PPARγ mRNA expression in that depot. While male newborns also have higher umbilical cord serum levels of total and free testosterone and oestradiol (Simmons *et al.*, 1994), and both testosterone and oestradiol have been shown to affect adipose tissue differentiation and function, the role of sex steroids in the sexually dimorphic pattern of IGF1 secretion from nonhepatic tissues is unclear as a recent study of mice with an IGF1 deletion specific to the liver has suggested that the sexually dimorphic pattern of IGF1 secretion from nonhepatic tissues is not due to sex steroid activity, because castration did not alter GH-stimulated IGF secretion (Liu *et al.*, 2000).

In the present study there was evidence for nutritional regulation of the expression of IGF1R and IGF2R mRNA in male and female lambs. Given the inter-relationships in the expression of IGF1R, IGF2R and IGF2 in male and female lambs the suggestion of specific causal relationships between circulating nutrient and hormone levels and the mRNA levels of IGF2, IGF1R and IGF2R would be inappropriate. It appears, however, that in male lambs leptin promotes and glucose suppresses IGF1R mRNA expression in subcutaneous fat, while both glucose and NEFAs suppress IGF2R mRNA expression in subcutaneous fat. In female lambs it appears as though glucose and insulin may act to

suppress IGF2 mRNA expression in subcutaneous adipose tissue, while leptin and NEFAs may suppress IGF2 mRNA expression in perirenal adipose tissue.

In the current study IGF1 mRNA expression was higher in the subcutaneous than the perirenal fat depot, whereas IGF2, IGF1R and IGF2R mRNA expression were higher in the perirenal depot, in both male and female lambs. The depot specific differences in IGF receptor expression provide an explanation for the observed variation in mitogenic potency of IGF1 and proliferative capacities of preadipocytes derived from different fat depots (Wang *et al.*, 1989; Dieudonne *et al.*, 2000), and provides an explanation for the regional variation in metabolism observed between fat depots (Bouchard *et al.*, 1993; Blaak, 2001) as IGF1 and insulin have opposing effects on both insulin-stimulated glucose uptake and LPL activity in adipocytes (Oscarsson *et al.*, 1999; Frick *et al.*, 2000). The sex and depot specific differences in IGF receptor expression could also provide a potential mechanism for the higher incidence of metabolic syndrome in men in comparison with women (Despres, 1998; Lamarche, 1998), which is related to the more visceral pattern of adipose deposition (Wajchenberg, 2000), while females tend to deposit relatively more subcutaneous adipose tissue (Kotani *et al.*, 1994; Dua *et al.*, 1996; Havel *et al.*, 1996a; Havel *et al.*, 1996b; Legato, 1997).

5.5.3 SUMMARY

In summary, we have shown that there is no effect of IUGR on the expression of IGF mRNA in perirenal and subcutaneous adipose tissue at three weeks of life. There was, however, a sex and depot specific expression of IGF1, IGF2, IGF1R

and IGF2R mRNA in perirenal and subcutaneous adipose tissue, which provides a potential mechanism to explain the sex and depot specific variations in mitogenic potency of IGF1 and proliferative capacities of preadipocytes, the regional variation in adipocyte metabolism, and the difference in incidence of visceral obesity between men and women in adult life.

6. Summary, Conclusions and Future Directions

Intrauterine growth restriction has been shown to be associated with an acceleration of the postnatal growth rate and an initial increased whole body insulin sensitivity, followed by the emergence of an increased adiposity and whole body insulin resistance. In human and animal studies birth weight has been shown to be related to circulating concentrations of growth factors and adipokines, which suggests that substrate supply *in utero* may determine the level of expression of these factors in adipose tissue. During early postnatal life there appears to be an uncoupling of the leptin-fat mass axis and of the GH-IGF axis, where changes in nutrient levels result in greater fluctuations in circulating concentrations of both leptin and IGF1 than occur during adult life. Therefore, it seems than in the fetal and early postnatal period there is potential for nutritional factors to program these metabolic and endocrine axes, and in the presence of 'mismatched' fetal and postnatal nutritional environments there is the potential for such programming to lead to the development of metabolic dysregulation, including dyslipidaemia, hypercholesterolaemia, insulin resistance and cardiovascular outcomes. It was the purpose of this thesis to investigate the physiological adaptations of adipose tissue to a restricted fetal substrate supply that may subsequently manifest into pathophysiology of the metabolic axes in later life.

Experimental restriction of placental growth resulted in a hypoglycaemic, hypoinsulinaemic and hypoxic fetal nutritional environment between 116 and 145d gestation, and significantly reduced fetal growth resulting in a low body

weight. At postmortem in late gestation the PR fetuses had a lower absolute but not relative mass of perirenal fat compared to Control fetuses. IUGR did not affect the level of expression of PPARγ, RXRα, adiponectin, LPL, G3PDH, GAPDH, IGF2, IGF1R or IGF2R mRNA in fetal perirenal adipose tissue. A key finding of this thesis, however, is that there is a reduction in the expression of IGF1 and leptin mRNA in the fetal perirenal adipose tissue of PR fetuses in comparison to controls. Insulin-like growth factor-1 (IGF1) is involved in the induction of preadipocyte proliferation and differentiation to the mature adipocyte phenotype, and in the mature adipocyte plays a role in the regulation of adipocyte insulin sensitivity. It is possible that the reduced expression of IGF1 mRNA in the perirenal adipose tissue of PR fetuses is associated with a direct reduction in the concentration of IGF1 available for paracrine activation of differentiation in developing adipose tissue, and therefore the decreased expression of IGF1 mRNA in the perirenal adipose tissue of PR fetuses might have consequences for the timing and magnitude of the proliferation and differentiation processes during adipose tissue development. The reduction in leptin mRNA expression in perirenal adipose tissue of fetal sheep raises the possibility that events that occurred *in utero* which result in a growth restricted fetus may result in a functional immaturity of the leptin – fat mass axis with consequences for the regulation of lipid metabolism and insulin sensitivity during postnatal life.

In this thesis the newborn IUGR lamb, assessed by weight at birth, had a higher postnatal growth rate for the first two weeks of life and lower plasma NEFA concentrations during the first three weeks of life up to post mortem. There was no difference between IUGR and Control lambs in the relative mass of perirenal,

omental or subcutaneous adipose tissue present at 21 days of life, or in the mean size of perirenal, omental or subcutanous adipocytes. This thesis has determined whether there was an effect of the prenatal and postnatal nutritional environment and consequent growth patterns of fetuses and lambs on the growth and functional development of unilocular white perirenal adipose tissue in male and female lambs at 3 weeks of life, prior to the development of the obese phenotype.

In contrast to fetal life, there was no effect of IUGR on the expression of IGF1, mRNA in the perirenal fat of lambs at 3 weeks of age. Therefore there is not a programming effect of IUGR on basal IGF1 mRNA expression in perirenal fat that persists into postnatal life. As the IUGR fetus undergoes a transition from a relatively low to high nutritional environment at birth, the level of IGF1 mRNA expression in the adipose tissue of the postnatal lamb most likely reflects the importance of the nutrient regulation of IGF1 in this tissue. The regulation of IGF1 mRNA and protein expression by nutrients in hepatocytes has been well described in the literature (Straus, 1994; Ketelslegers *et al.*, 1995). This thesis provides evidence for an important effect of nutrition on the expression of IGF1 mRNA specifically in adipose tissue. There was also evidence for nutritional regulation of the expression of IGF1R and IGF2R mRNA in both perirenal and subcutaneous fat in male and female lambs.

Interestingly, the results of Chapter 5 show that there is a depot specific sexual dimorphism in the expression of IGF mRNA in perirenal and subcutaneous adipose tissue at 3 weeks of age. The expression of IGF1 mRNA in both

perirenal and subcutaneous adipose tissue tended to be higher in male than female lambs. Furthermore, the expression of IGF1R and IGF2R in subcutaneous adipose tissue (but not perirenal adipose tissue) was also higher in male compared with female lambs. A higher circulating IGF1 concentration in males compared with females has been described previously in the lamb and adult sheep (Van Vliet *et al.*, 1983; Gatford *et al.*, 1996). This increase may in part be due to the contribution of an increased synthesis of IGF1 mRNA in adipose tissue. These findings may reflect another component of the sexual dimorphism of the GH-IGF axis, as both testosterone and oestradiol have been shown to affect adipose tissue differentiation and function through modulation of GH at the adipocyte or adipocyte IGF expression and function. In Chapter 3 a higher circulating plasma insulin concentration for any given glucose concentration during the first three weeks of life was described in male compared with female lambs, which leads to the suggestion that plasma insulin levels may modulate IGF1 mRNA expression in adipose tissue. There was, however, no evidence for a relationship between plasma insulin concentrations at any age and IGF1 mRNA expression in the adipose tissue of either male or female lambs. In contrast, plasma leptin concentrations during the first 24h after birth were related to the expression of IGF1 mRNA in perirenal adipose tissue 3 weeks later in both male and female lambs, which could reflect a direct programming effect of early leptin concentrations on basal perirenal adipocyte IGF1 mRNA production.

It is important to acknowledge that this thesis has not investigated the adipose tissue mRNA levels of IGF binding proteins (IGFBP)s, which have been shown to

modulate IGF responsiveness by altering IGF1 bioavailability and interaction with IGF cell surface receptors (Sara & Hall, 1990). In particular, plasma IGFBP3 is the main binding protein for IGF1 in the postnatal sheep, prolonging the half-life of IGF1 from 10 to 545 min (Davis *et al.*, 1989; Gallaher *et al.*, 1992), and has been shown to inhibit the effect of IGF1 on lipogenesis and glucose oxidation in porcine adipose tissue culture (Walton *et al.*, 1989). IGFBP3 has also been shown to be higher in rams than nonpregnant ewes (Gatford *et al.*, 1996), which raises the possibility that there is also altered adipocyte mRNA expression of this IGFBP specifically, or other IGFBPs, which would have implications for the conclusions of this thesis. It would therefore be interesting to determine the adipocyte mRNA levels of IGFBPs in IUGR and Control, male and female lambs.

Another key finding of this thesis was the sex dependent effect of IUGR on the expression of PPARγ and leptin mRNA in perirenal adipose tissue at 3 weeks of postnatal life. In male, but not female lambs, IUGR resulted in a reduced level of expression of PPARγ and leptin mRNA in perirenal adipose tissue at that age. It has been shown that reduced adipocyte PPARγ mRNA expression decreases adipose tissue and hepatic insulin sensitivity (He *et al.*, 2003), therefore this thesis provides evidence that early nutritional programming of PPARγ mRNA expression in the perirenal fat depot may play a role in the subsequent emergence of an insulin resistant phenotype in LBW male lambs. It would therefore be interesting to determine the 'insulin sensitivity' of perirenal adipocytes from male IUGR and Control lambs, by assessing insulin-stimulated glucose uptake. It has also been previously shown that a reduced adipocyte leptin mRNA expression results in a relatively greater increase in adiposity during

early adulthood (Jaquet *et al.*, 2001a). This thesis therefore demonstrates that a reduction in *in utero* substrate supply results in a reduction in basal leptin mRNA expression in adipose tissue that is present both during fetal life and early postnatal life after the transition that occurs in this tissue from a predominantly brown to white adipose tissue. This may have implications for body composition during later life with a predisposition for an increased deposition of adipose tissue. There were also direct relationships between birth weight and both plasma NEFA concentrations in both male and female lambs, and PPARγ and leptin mRNA in only male lambs. This suggests that within the range of birth weights produced in this thesis there was not a minimal amount of nutrition required for fetal development, below which the effects of IUGR would manifest but rather that fetal nutrient levels across the continuum have an effect on the development of basal expression levels of PPARγ and leptin mRNA, and basal plasma NEFA concentrations, whether a result of altered fatty acid intake or handling.

After birth, in male lambs, plasma insulin concentrations during the first 24h after birth were related to the size of the perirenal adipocytes and relative perirenal fat mass, 3 weeks later. Therefore in males there appears to be an impact of insulin immediately after birth on the subsequent triglyceride storage capacity of individual perirenal adipocytes and the relative mass of the perirenal fat. Plasma insulin concentrations during the first 24h after birth were also strongly related to perirenal adipocyte size at 21d in female lambs. Whilst there was no difference in the mean size of the perirenal adipocytes between male and female lambs, the perirenal fat mass was significantly greater in females than males at 3 weeks

of age. This suggests that the increased growth of the perirenal fat depot in females occurs through a hyperplastic mechanism in combination with increased lipid deposition, when compared with males. It is therefore possible that adipogenesis may be relatively more insulin sensitive in females than in males during the early postnatal period. Furthermore, in females, but not males, plasma glucose and insulin concentrations during the first three weeks after birth were directly related to perirenal fat mass at 21d of life, and there were also relationships between either early or current plasma insulin concentrations and the respective expression of G3PDH and GAPDH in perirenal fat at 21d. Therefore, in females there appears to be an impact of insulin both immediately after birth and during the first 3 weeks of postnatal life on adipogenesis and lipogenic gene expression, providing further evidence that adipogenesis may be relatively more insulin sensitive in females than in males during early postnatal life.

In LBW and ABW females, the relative mass of perirenal fat mass at 21d was also related to plasma NEFA during the first 24h after birth. In this context, we also found in female lambs that PPARγ mRNA expression in perirenal fat was directly related to plasma NEFA concentrations during the first 24h after birth and to circulating insulin concentrations during weeks 1-3 in female lambs. Interestingly, the slope of this relationship was similar in both LBW and ABW females, but PPARγ mRNA expression on day 21 was higher when related to any given plasma NEFA concentrations on day 1 in the LBW, compared to the ABW group. One possibility is that in the female lamb, in the presence of low plasma insulin concentrations, fatty acids may act either as transcriptional

activators of PPARγ expression or bind as ligands to activate the nuclear receptor to result in enhanced insulin signalling and hyperplasia of the perirenal adipocytes during early postnatal life (Figure 6.1). The direct relationship between PPARγ and leptin expression in perirenal fat in female lambs may be a consequence of the effects of PPARγ on perirenal fat mass, as leptin expression was also directly related to adipocyte cell size in these lambs. The relationship between plasma leptin and NEFA concentrations during the first 3 weeks after birth in female lambs may also reflect the relatively greater adiposity and / or enhanced insulin sensitivity of the perirenal adipocyte in the female lamb.

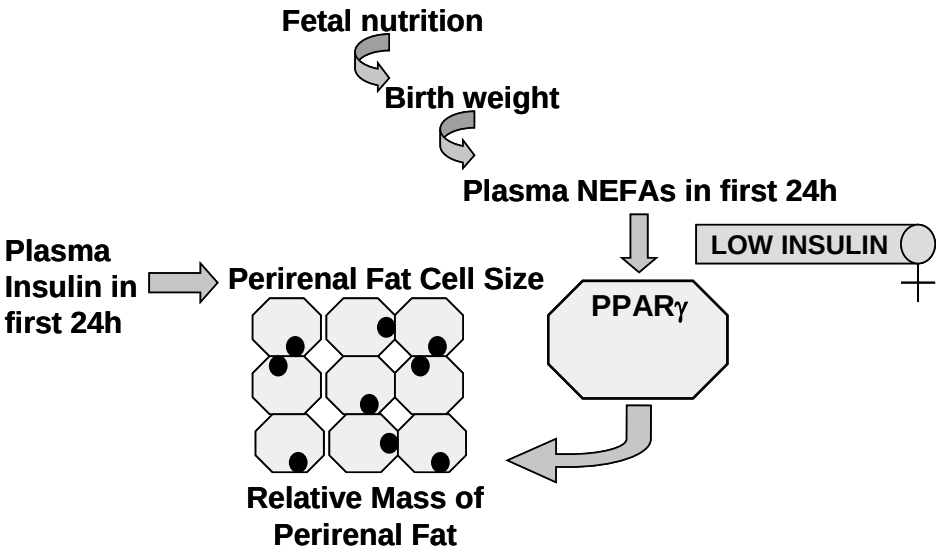

Figure 6.1 A summary diagram highlighting the effects of early insulin exposure on the development of perirenal fat and on PPARγ and leptin mRNA expression in the female lamb.

Decreased intrauterine substrate supply results in being born with a low birth weight (LBW) and lower circulating plasma NEFA concentrations during the first 24h after birth. In the female lamb, in the presence of a lower plasma insulin concentrations, plasma NEFA concentrations during the first 24h after birth drive the expression of PPARγ mRNA and the relative mass of perirenal fat at 21 days of life. This may occur through NEFA activation of PPARγ expression, to result in enhanced insulin signalling and hyperplasia of the perirenal adipocytes resulting in a greater relative mass of perirenal fat in female, compared with male lambs at 3 weeks of age. Plasma insulin during the first 24h after birth is directly related to perirenal adipocyte size at 3 weeks of age.

In contrast to female lambs, the expression of PPARγ mRNA and leptin mRNA in perirenal adipose tissue were each lower in LBW compared to ABW male lambs. In both LBW males and females, plasma NEFA concentrations were lower than in ABW lambs, although plasma insulin concentrations were higher in LBW males compared to LBW females throughout the first 3 weeks of life. It is possible that in the presence of the relatively higher insulin concentrations in the LBW male lamb, there is less activation of PPARγ or less gene expression when fatty acid concentrations are low (Figure 6.2).

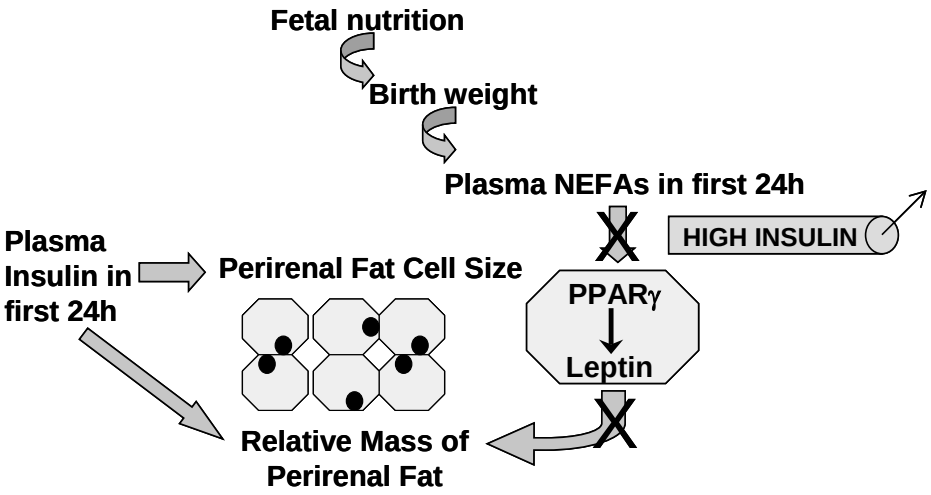

Figure 6.2 A summary diagram highlighting the effects of early insulin exposure on the development of perirenal fat and on PPARγ and leptin mRNA expression in the male lamb.

Decreased intrauterine substrate supply results in being born with a low birth weight (LBW) and lower circulating plasma NEFA concentrations during the first 24h after birth. In the male lamb, in the presence of a higher plasma insulin concentration, there is reduced NEFA activation of PPARγ and leptin gene expression. In males, plasma insulin concentrations at 24h after birth represent the predominant drive to both perirenal adipocyte size and relative perirenal fat mass at 3 weeks of age.

One mechanism to explain the relative differences in adipose tissue insulin sensitivity between male and female lambs that can be proposed from the results of this thesis, is that the increase in IGF mRNA expression in perirenal adipose tissue in male lambs results in an altered local concentration of IGF1 protein, and the altered concentrations of both IGF1 and plasma insulin result available for signalling at the insulin receptor and IGF1R is affected such that there is a reduced proliferation of perirenal adipocytes and therefore the lower mass of adipose tissue at 3 weeks of life. It would therefore be interesting to determine the 'insulin sensitivity' of perirenal adipocytes from male and female lambs, by assessing insulin-stimulated glucose uptake, and further to that to determine the impact of IGF1 concentrations on adipocyte insulin sensitivity.

In contrast to perirenal adipose tissue, where there is a decreased expression of both PPARγ and leptin mRNA in IUGR male lambs, the expression of PPARγ and leptin in subcutaneous adipose tissue was unaffected by IUGR, which suggests that the development of the perirenal fat depot is more sensitive to changes to the fetal nutritional environment. In both perirenal and subcutaneous adipose tissue there was no effect of IUGR on RXRα, adiponectin, LPL, G3PDH or GAPDH mRNA expression at 3 weeks of age. This thesis therefore provides no evidence for an effect of the fetal nutritional environment on the subsequent development of subcutaneous adipose tissue. The growth and functional development of subcutaneous adipose tissue was, however, related to measures of both the postnatal nutritional environment and the development of the perirenal adipose tissue depot. There were relationships between the mass of perirenal fat and the development of subcutaneous adipose tissue in both male and female

lambs. The mass of visceral fat was related to the mean size of subcutaneous adipocytes, and both the mass of visceral fat and the size of perirenal adipocytes were related to the expression of leptin mRNA in subcutaneous fat. Furthermore leptin, adiponectin and LPL mRNA expression in both the subcutaneous and perirenal depots were each related. This either reflects depot specific responses to the postnatal nutritional environment that occur in parallel, or alternatively it is possible that there is a factor secreted from visceral adipose tissue proportional to adipocyte size and tissue mass that has an impact on the development of subcutaneous adipocytes and the development of the leptin fat mass axis in subcutaneous tissue. It may be this factor, which ultimately conveys an effect of the fetal nutritional environment from perirenal to subcutaneous adipocytes, and results in an increased subcutaneous adiposity in individuals who were born small for gestational age.

In both the perirenal and subcutaneous fat depots there was a strong relationship between the expression of adiponectin and LPL, in both male and female lambs. This suggests that the relationship between adiponectin and LPL results from a tissue-specific, but not a depot-specific mechanism. This is the first demonstration, to our knowledge, that adiponectin and LPL expression are related in adipose tissue, either during development or in adult life.

In the current study the expression of G3PDH and the IGFs were depot specific. G3PDH and IGF1 mRNA expression was higher in the subcutaneous than the perirenal fat depot, whereas IGF2, IGF1R and IGF2R mRNA expression was higher in the perirenal depot, in both male and female lambs. The depot specific

differences in IGF receptor expression provide an explanation for the observed variation in mitogenic potency of IGF1 and proliferative capacities of preadipocytes derived from different fat depots (Wang *et al.*, 1989; Dieudonne *et al.*, 2000), and provides an explanation for the regional variation in metabolism observed between fat depots (Bouchard *et al.*, 1993; Blaak, 2001) as IGF1 and insulin have opposing effects on both insulin-stimulated glucose uptake and LPL activity in adipocytes (Oscarsson *et al.*, 1999; Frick *et al.*, 2000). Regional differences in G3PDH mRNA expression could also contribute to metabolic differences.

The sex and depot specific differences in IGF receptor expression also provide a potential mechanism for the higher incidence of metabolic syndrome in men in comparison with women (Despres, 1998; Lamarche, 1998), which is related to the more visceral pattern of adipose deposition (Wajchenberg, 2000), while females tend to deposit relatively more subcutaneous adipose tissue (Kotani *et al.*, 1994; Dua *et al.*, 1996; Havel *et al.*, 1996a; Havel *et al.*, 1996b; Legato, 1997). The sex specific effect of IUGR on both PPARγ and leptin mRNA expression, such that male IUGR lambs had a lower level of expression of both PPARγ and leptin in the perirenal adipose tissue depot, may further predispose male IUGR lambs to the subsequent development of an insulin resistant phenotype.

This thesis therefore describes mechanisms that provide explanations for the epidemiological evidence for an increased susceptibility to the development of insulin resistance in both males in comparison to females, and in small for

gestational age compared to average for gestational age individuals. This study also suggests that nutritional substrate supply during the first day of life may have long term implications for the growth and functional development of visceral adipose tissue, which highlights the early postnatal period as a potentially important time for nutritional intervention to limit the adverse metabolic consequences of being small for gestational age.

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