Early life determinants of Beta-cell function in the sheep

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If we knew what it was we were doing, it would not be called research, would it?

Albert Einstein

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STATEMENT OF ORIGINALITY AND AUTHENTICITY

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree. I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. The author acknowledges that copyright of published works contained within this thesis resides with the copyright holder(s) of those works. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Signed,

Siti Aishah Sulaiman

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TABLE OF ABBREVIATIONS AND BIOCHEMICAL NAMES

ACTB	Beta cytoskeletal actin / β-actin
ADIPOQ	Adiponectin
ADP: ATP	Adenosine diphosphate: Adenosine triphosphate ratio
AGR	Absolute growth rate
ANOVA	Analysis of variance
arb. unit	Arbitrary unit
BMI	Body mass index
BUVL	Bilateral uterine artery ligation
CACNA1D	L-Type voltage-gated Ca^{2+} channel subunit
cDNA	Complementary DNA
CON	Control group
CpG island	Cytosine-Guanine base pairing rich regions
CRL	Crown-rump length
CSIRO	Commonwealth Scientific and Industrial Research Organisation
Ct	Cycle threshold
CUG	Catch-up growth
DAB	3,3'-Diaminobenzidine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	Deoxyribonucleotide triphosphate
DPP-IV	Enzyme dipeptidyl peptidase IV
FGR	Fractional growth rate
FOXA2	Forkhead box protein A2

GCK	Glucokinase
GIR	Glucose infusion rate
GLP1	Glucagon-like-peptide 1
HAT	Histone acetyltranferase
HDAC	Histone deacetylase
HEC	Hyperinsulinaemic euglycaemic clamps
HMT	Histone methyltransferase
HOMA-IR	Homeostasis model, calculation of insulin sensitivity
IGF	Insulin-like growth factor
IGF1	Insulin-like growth factor 1
IGF1R	Type 1 insulin-like growth factor receptor
IGF2	Insulin-like growth factor 2
IGF2R	Type 2 insulin-like growth factor receptor
IMVS	Institute Medical and Veterinary Science, Adelaide, Australia
INS	Insulin
INSR	Insulin receptor
IUGR	Intrauterine growth restriction
IUGR+Ex-4	IUGR lambs treated with exendin-4 as neonates
IUGR+Veh	IUGR lambs treated with vehicle as neonates
IVGTT	Intravenous glucose tolerance test
KCNJ11	A subunit of ATP-sensitive K^+ channel
KRB/BSA	Krebs Ringer buffer supplemented with bovine serum albumin
LB-Broth	Luria-Bertani broth
miRNA/miR	Micro ribonucleic acid, microRNA
mRNA	Messenger ribonucleic acid

MTPN	Myotrophin
NADH	Reduced form of nicotinamide adenine dinucleotide
NSW	New South Wales
OCT	Optimum cutting temperature embedding substrate
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDX1	Pancreatic duodenal homeobox-1
PIK3CB	PI-3-kinase, catalytic subunit beta
PIK3R1	PI-3-kinase, regulatory subunit 1
PR	Placental restriction
QC	Quality control
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RT PCR	Real Time PCR
SA	South Australia
s.c.	Subcutaneous
SGA	Small for gestational age
SLC2A2	Glucose transporter 2 / Glut2
SLC2A4	Glucose transporter 4 / Glut4
T2D	Type 2 Diabetes
TCA cycle	Tricarboxylic acid cycle,
USA	United States of America
V	Voltage
V_d	Volume density

PUBLISHED PEER REVIEWED JOURNAL ARTICLE AND CONFERENCE PRESENTATIONS ARISING FROM THIS THESIS

Neonatal exendin-4 reduces growth, fat deposition and glucose tolerance during treatment in the intrauterine growth-restricted lamb

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Neonatal exendin-4 intervention treatment normalises islet insulin secretion and expression of its molecular determinants in the intrauterine growth restricted lambs

Siti A Sulaiman, Kathryn L Gatford, Miles J De Blasio, Saidatul N Mohammad, Julie A Owens.

August 2011: *Faculty of Health Sciences 2011 Postgraduate Research Conference*, Adelaide, Australia. (Poster Presentation by Siti A Sulaiman)

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November 2011: *7th Asia Pacific Congress in Maternal Fetal Medicine*, Kuala Lumpur, Malaysia. (Poster presentation by Siti A Sulaiman)

Neonatal exendin-4 treatment in the twin IUGR lamb normalises *in vitro* islet insulin secretion and expression of its molecular determinants

Siti A Sulaiman, Kathryn L Gatford, Miles J De Blasio, Saidatul N Mohammad, Julie A Owens.

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Neonatal Exendin-4 Treatment Increases β-cell Mass and Alters Islet Gene Expression in the IUGR Lamb

SA Sulaiman, KL Gatford, SN Mohammad, ML Harland, MJ De Blasio, RA Simmons, JS Robinson, JA Owens

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Neonatal exendin-4 treatment increases insulin secretion, beta-cell mass and decreases fat deposition in the IUGR lamb

Siti A Sulaiman, Kathryn L Gatford, Saidatul N Mohammad, M Lyn Harland, Miles J De Blasio, Rebecca A Simmons, Julie A Owens

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Intervention strategies against programming of diabetes following IUGR

KL Gatford, SN Mohammad, SA Sulaiman, ML Harland, MJ De Blasio, AL Fowden, JS Robinson, JA Owens

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Effects of exendin-4 on the growth-restricted twin lamb

KL Gatford, SA Sulaiman, MJ De Blasio, TA How, ML Harland, SN Mohammad, JA Owens

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ABSTRACT

Low birth weight or intrauterine growth restriction (IUGR) consistently predict increased risk of Type 2 diabetes (T2D) through impairment of glucose tolerance, insulin resistance and inadequate insulin secretion in humans (1, 2), as well as in many experimental studies in other species (3, 4). IUGR due to insufficient supply of fetal nutrients, decreased oxygen supply and elevated exposure to stress hormones are thought to 'program' the impairment of β-cell mass, function and plasticity which then contributes to development of diabetes later in life, as observed in humans (5-7) and animals (4, 8). Interestingly, administration of the glucagon-like-peptide 1 (GLP1) analogue exendin-4 to neonatal IUGR rats normalised subsequent β-cell mass and insulin secretion and prevented later development of T2D (9), thus providing a possible intervention strategy to prevent T2D following IUGR. However, there are differences in the timing of pancreatic and β-cell development between species and therefore in the developmental stages during exposure to IUGR and neonatal interventions. In humans and sheep, most pancreatic and β-cell development occurs before birth (10-17). In contrast, rodents undergo later development of β-cells than sheep or humans, with the majority of pancreatic remodelling occurred at postnatal ages (18-20). It is therefore necessary to test the efficacy of neonatal exendin-4 treatment in animal models such as sheep that share similar profile of pancreatic development and growth with humans (9, 21). Therefore, this thesis will address the effects of IUGR on β-cell mass and function, expression of their molecular determinants, as well as epigenetic modifications, and the possible involvement of altered circulating adiponectin abundance and expression in adipose tissue in the young lamb from birth to 16 d of age. The efficacy of neonatal exendin-4 treatment as a postnatal intervention to prevent these adverse effects of IUGR on metabolic outcomes will also be assessed.

Here, natural twin pregnancies were used as a model of IUGR in progeny and unrestricted singleton lambs as the controls. In each twin set, sibling twin lambs with high and low birth weights were alternately allocated to either vehicle or exendin-4 treatment. Effects of IUGR due to twinning and of neonatal exendin-4 treatment of the twin lambs on neonatal growth, pancreatic β-cell *in vivo* and *in vitro* insulin secretory function, β-cell mass and islet expression of key regulatory genes including microRNAs, epigenetic regulators, and adiponectin, and on adiponectin abundance were analysed.

IUGR due to twinning reduced size at birth and increased neonatal growth, without altering insulin sensitivity, *in vivo* insulin action, β-cell mass or islet mRNA expression of β-cell mass molecular determinants when compared to CON lambs. However, *in vitro* glucose-stimulated insulin secretion was increased in the IUGR twin lamb relative to controls (+420%, P = 0.081), consistent with up-regulation of islet mRNA expression of *GCK* in this group (+80%, P = 0.017), thus suggesting up-regulated β-cell function at this age. Interestingly, IUGR twin lambs also had increased islet mRNA expression of *DNMT3B* relative to CON lambs (+96%, P = 0.027), which is responsible for *de novo* DNA methylation (22, 23). Islet mRNA expression of *GCK* was positively correlated with that of *DNMT3B* in the IUGR twin group, suggesting that altered islet *GCK* mRNA expression and β-cell function after IUGR may occur in part via epigenetic changes that may persist throughout life. In conjunction with enhanced β-cell function, up-regulation of adiponectin mRNA expression in omental fat (+72%, P = 0.008) and increased circulating adiponectin levels (P = 0.012) were also observed in the IUGR twin lamb group. Omental adiponectin mRNA expression and circulating adiponectin correlated positively with insulin secretion and β-cell mass

in combined control and IUGR twin lamb groups, suggesting that this adipokine may play a role in regulating neonatal insulin secretion.

Daily exendin-4 treatment of IUGR twin lambs during neonatal life prevented accelerated neonatal growth or catch up growth (CUG) and fat accumulation (-57%, $P < 0.001$), and normalised *in vitro* insulin secretion and *GCK* and *DNMT3B* mRNA expression in their islets, relative to vehicle-treated IUGR twins. This may retain adaptive capacity of β-cell function for later life. Glucose tolerance of twin IUGR lambs was impaired during exendin-4 treatment (+156%, $P = 0.003$) reflecting decreased insulin sensitivity (-46%, $P = 0.002$) in this group, despite having normal *in vivo* insulin secretion. This may be due to central actions of exendin-4 to inhibit food intake and insulin sensitivity (24-26). β-cell mass in IUGR twin lambs treated with exendin-4 tended to be higher than in their IUGR counterparts (+28%, $P = 0.083$), and consistent with this, islet mRNA expression of *IGF1* and *IGF2R* was increased in this group (+62%, $P = 0.058$ and +63%, $P = 0.005$ respectively) when compared to controls. Moreover, in the IUGR+Ex-4 lambs, islet mRNA expression of *PDX1* correlated positively with that of *IGF1R*, while *IGF1* mRNA expression correlated positively with β-cell volume density, which may suggest hyperplastic effects of the *IGF* axis on β-cell mass during exendin-4 treatment. Despite the profound reduction in visceral fat mass induced by neonatal exendin-4 treatment, circulating adiponectin concentrations were not reduced in exendin-4-treated lambs, possibly due to up-regulation of adiponectin expression in subcutaneous fat in these animals (+91%, $P = 0.007$). Nevertheless, the reduction in fat accumulation and normalised *in vitro* β-cell action of IUGR lambs during neonatal exendin-4 treatment suggest that neonatal exendin-4 might have beneficial effects on insulin-regulated glucose homeostasis in later life. These outcomes also demonstrate the biological activity of exendin-4 for the

first time in the sheep, at least in the context of individuals who had undergone growth-restriction before birth.

In conclusion, IUGR due to twinning induced CUG, early life up-regulation of *in vitro* β-cell insulin secretion and islet expression of its determinant, *GCK*, but did not alter *in vivo* insulin action, glucose tolerance or β-cell mass in young lambs at 16 d of age. These metabolic and molecular changes may be partly mediated by increases in circulating adiponectin and its expression in omental fat, as part of an adipose tissue response during neonatal fat deposition. Consistent with our hypothesis, neonatal exendin-4 treatment prevented this IUGR-induced CUG and decreased visceral fat deposition, increased 2^{nd} phase insulin secretion *in vivo*, normalised *in vitro* insulin secretion and islet expression of its determinant, *GCK*, at the end of treatment in the IUGR twin lambs. Although exendin-4 treatment only tended to increase β-cell mass in young IUGR lamb, the up-regulation of islet expression of β-cell mass determinants after 16 days of exendin-4 treatment may suggest beneficial effects of exendin-4 to subsequently expand β-cell mass. This may protect the exendin-4-treated IUGR individual from a need to increase β-cell function, and preserve the capacity of β-cells for later plasticity of insulin secretion in response to the development of insulin resistance with ageing. Hence, a long term investigation is required to address how these changes following IUGR and neonatal exendin-4 treatment at 16 d of age will affect β-cell function and mass and insulin action and their regulation in the IUGR sheep to adulthood.

CHAPTER 1

1 CHAPTER 1 INTRODUCTION

1.1 OVERVIEW

Type 2 diabetes (T2D) is one of the major metabolic diseases, with worldwide prevalence of approximately 380 million people, and its prevalence is predicted to increase two-fold by 2035 (27). A previous study of 80-year-old Swedish men revealed that low birth weight (< 3000g) accounted for 18% of diabetes prevalence in the population (28). Consistent with this, low birth weight or intrauterine growth restriction (IUGR) predict increased risk of T2D through impairment of glucose tolerance, insulin resistance and inadequate insulin secretion in humans (1, 2), as well as in animal studies (3, 4). Since insulin secretion varies inversely with insulin sensitivity to maintain adequate insulin action (29), the failure of β-cells to up-regulate insulin secretion to compensate for the insulin resistance in T2D (30), suggests that there are some defects in β-cell mass and function and their adaptation following IUGR. This indicates that during IUGR, early life environment factors including limited oxygen and nutrient availability that restrict fetal growth may program β-cell mass, function and insulin action in later life.

A common cause of poor fetal growth, or IUGR, that restricts fetal supplies of oxygen and nutrients is impaired placental growth and/or function (31). In the sheep, surgically-induced restriction of placental growth (PR) from before mating produces low birth weight offspring and these PR lambs have impaired insulin sensitivity and blunted basal and glucose-stimulated insulin action at 1 year of age (8, 32). Impaired β-cell function is the primary cause of this inadequate insulin secretion, which occurs despite increases in β-cell mass in these 1 year old PR offspring (8). Similarly, experimentally-induced PR late in pregnancy in the rats produces progeny with low birth weight and normal insulin action at 1 week of age, but who later develop diabetes

as 26 weeks olds (3, 4). Impaired β-cell function with later reduction in β-cell mass are also implicated as causing the decreased postnatal insulin secretion of these PR rat progeny (3, 4). Importantly, in these PR rat progeny, expression of *PDX1*, a master regulator of pancreatic β-cell mass and function, becomes much lower than that in controls with postnatal aging in association with loss of β-cell function and β-cell mass (9, 21, 33). Moreover, *PDX1* expression is epigenetically down-regulated in PR rat progeny (21), which suggests that epigenetic programming of the developing β-cell during IUGR programs later β-cell mass and function possibly by silencing expression of *PDX1*. Thus, substrate and nutrient deprivation to the fetus due to IUGR and poor placental growth may in part induce epigenetic changes to the important genes that persist and maintain altered expression throughout life. Interestingly, administration of the GLP1 analogue exendin-4 to neonatal PR rats normalised subsequent β-cell mass and insulin secretion and prevented later development of T2D (9) and this was at least partially due to normalisation of *PDX1* expression (21).

Application of exendin-4 as an intervention strategy following IUGR to prevent development of T2D in humans requires additional investigation to these studies in the PR rat, since there are differences in the timing of pancreatic and β-cell development between species. In humans and sheep, most pancreatic development takes place before birth, with β-cells present as early as at 0.25 of gestation (10-17). In contrast, rodents undergo later development of β-cells than sheep or humans, with the majority of pancreatic remodelling occurring at postnatal ages (18-20). Thus, despite the positive outcomes of neonatal exendin-4 treatment in IUGR rats (9, 21), evaluation of exendin-4 treatment as a potential intervention to prevent development of T2D following IUGR in humans also requires evaluation of this intervention in animal models such sheep, that share similar timing of pancreatic development and growth as humans. Therefore, this

chapter will review the effects of IUGR on T2D and its determinants, insulin secretion and sensitivity, and describe β-cell mass and function and their key regulatory molecular determinants. Finally, I will discuss epigenetic modifications and adiponectin production, and how these may regulate insulin secretion, and its adaptation and plasticity following IUGR, and their contributions to development of T2D. The role of exendin-4 as a potential postnatal intervention strategy to prevent these adverse effects of IUGR on β-cell mass and function will also be discussed.

1.2 PREVALENCE OF TYPE 2 DIABETES (T2D)

Currently, the worldwide prevalence of T2D is approximately 380 million people and it is estimated to increase to 592 million people by 2035 (27). In Australia alone, more than 1.1 million people or 3.9% of the population, are reported to have T2D, with the same number again currently undiagnosed or in the early stages of diabetes (34). Critically, diabetes is also reported to be one of the top 10 leading causes of death in Australia, accounting for 2.9% of the total deaths in 2012, an increase from 2.8% in 2007, and 2.6% in 2003 (35), thus indicating a rise of this epidemic disease burden in the Australian population. The increasing prevalence of T2D in Australia is a major economic burden on the health budget. In 2008-2009, $1.5 billion was spent on diabetes in Australia (36) while $548 billion was spent worldwide in the management of the disease (27). Moreover, T2D has been recognised as one of the main causes of heart and renal diseases, stroke, foot ulceration, gangrene and also visual impairment up to and including blindness (37). Developing effective measures to slow this epidemic has therefore become a national and international priority.

1.3 T2D AND INSULIN ACTION

T2D is a result of impaired insulin action, due to the failure of insulin secretion to increase appropriately in response to increasing demand or insulin resistance (29). Pancreatic endocrine β-cells produce and secrete insulin to act on skeletal muscle and adipocytes, stimulating these tissues and cells to increase their glucose uptake from the blood and thus reducing the blood glucose level (29). Insulin also acts on the liver where it suppresses gluconeogenesis as well as promoting glucose storage as glycogen (29). Normally, insulin secretion varies inversely with insulin sensitivity to maintain adequate insulin action (Figure 1.1, 29). However, in T2D, insulin secretion fails to up-regulate adequately to compensate for insulin resistance (30), which suggests that there are defects in β-cell mass and function and their adaptability or plasticity in T2D.

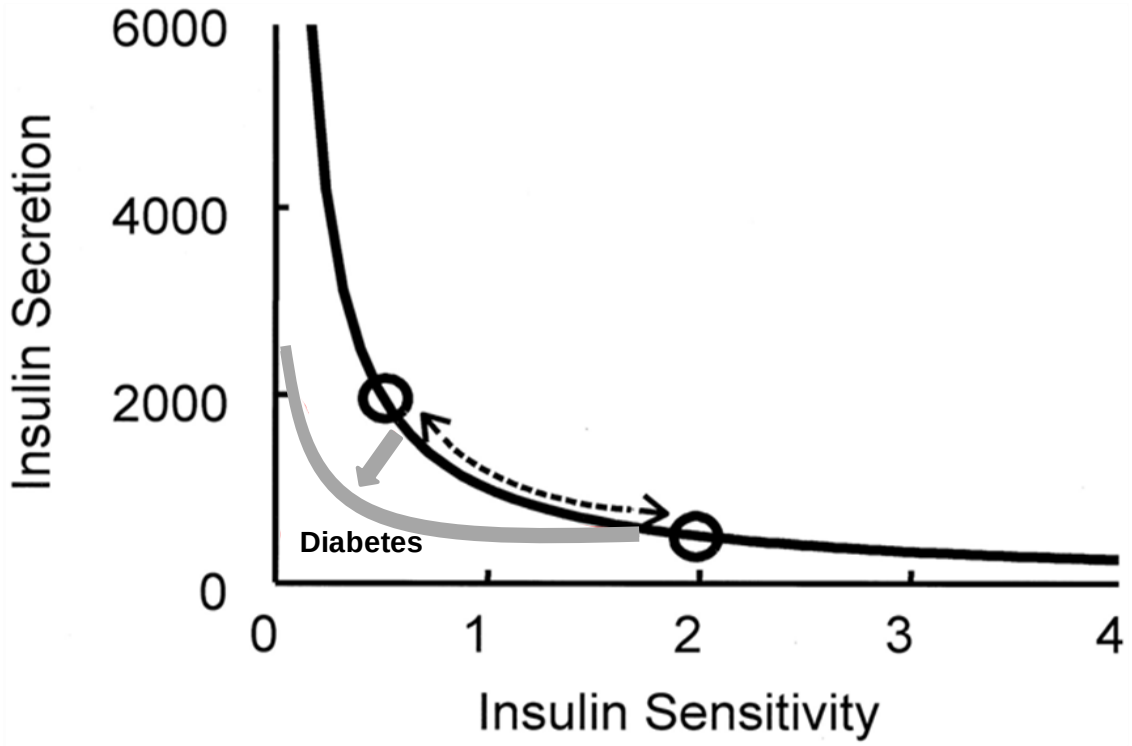

Figure 1.1 Hyperbolic relationship between insulin sensitivity and insulin secretion, modified from (29).

β-cells normally adjust their insulin secretion for different levels of insulin sensitivity to maintain adequate insulin action in a hyperbolic relationship (black line). However, in the case of diabetes, insulin secretion fails to up-regulate adequately to compensate for insulin resistance (grey line).

1.4 SIZE AT BIRTH AND T2D

IUGR refers to poor fetal growth due to restricted nutrients and oxygen supply during gestation, which prevents the fetus from achieving its growth potential (38). Since human studies often lack direct measures of fetal growth, many studies use small for gestational age (SGA), defined as having a birth weight less than 10^{th} percentile at a given gestational age (39), as a surrogate marker of IUGR, while some studies that lack gestational age data have used low birth weight as an indicator of IUGR. In a study of 64-year-old men, those who were lighter at birth had poorer glucose tolerance, and increased fasting plasma glucose and insulin, which are indicators of insulin resistance (40). Subsequently, systematic reviews have confirmed these associations between increased risks of impaired glucose tolerance and diabetes and lower birth weights in humans (1, 2, 41), independent of gestational length (42). This effect is significant, as low birth weight (< 3000 g) accounted for 18% of the diabetes prevalence in 80-year-old Swedish men, and their risk of diabetes decreased by 53% for every 1 kg increase in their birth weight (28). As fetal growth rates and size are substantially dependent on nutrients and oxygen supply during gestation, these associations between IUGR and later T2D may reflect the effects of an adverse early life environment on development of β-cell function, mass and their adaptation in later life (43).

1.5 EARLY LIFE ORIGINS OF IMPAIRED INSULIN ACTION

Hales and Barker (1992) proposed the 'Thrifty phenotype hypothesis' or 'Fetal origins of adult disease hypothesis' whereby prenatal deprivation causes the fetus to adapt to survive by slowing growth and preserving critical organs and tissues at the expense of others, including the β-cells in the pancreas that make insulin and the organs that respond to insulin (44). This process is also referred to as 'fetal programming of

adult disease', when these insults occur at critical stages of development and initiate changes that then persist throughout life (44, 45, reviewed by, 46, 47). These fetal survival adaptations however, become detrimental when postnatal nutrition is greater than that of the nutritional environment that occurred prenatally (48).

Although the term 'programming' describes the process of an insult at critical stages of development that induces changes which persist throughout life, the term 'developmental plasticity' is more appropriate to describe this process in context of biological and metabolic consequences (reviewed by, 46, 47). The formal definition of 'developmental plasticity' is the ability of a single genotype to produce more than one alternative form of structure, physiological state or behaviour in response to environmental conditions (47). Thus, Bateson and colleagues (2004) proposed that individuals who have poor nutrition in early life and develop a small-sized phenotype, the expected adult life outcomes may be variable (48). However, they are predicted to be worse when the postnatal environment has far greater nutritional availability than that experienced prenatally (48). This general model is in agreement with the 'thrifty phenotype hypothesis', whereby the prenatal fetal adaptation may induce adverse consequences in the offspring when there is a relative excess in postnatal nutrition (reviewed by, 46, 47). Nevertheless, it is important for evolutionary and reproductive survival that fetal adaptations in the prenatal environment occur, and not all of those responses to the prenatal environment are predictive of postnatal outcomes (46). It is also unknown whether these responses are 'adaptive' or 'predictive', but they did result in the programming of decreased functional capacity (46). The fact that T2D emerges in IUGR individuals with impaired insulin secretion and the onset of insulin resistance is in adulthood suggests that restored or excess nutrition after birth may expose any defects in insulin action following IUGR (49).

To assess the relative contributions of insulin secretion and insulin sensitivity to impaired insulin action in low birth weight or IUGR individuals, a systematic review of 48 studies revealed that low birth weight consistently predicted insulin resistance or reduced insulin sensitivity (17 out of 22 studies). These include studies where insulin sensitivity was measured indirectly using calculation of insulin sensitivity or directly using the "gold-standard technique" to measure insulin sensitivity; the hyperinsulinaemic-euglycaemic clamp (HEC, 1). One example is a study that assessed insulin sensitivity using the HEC in 28 SGA 9-year-old prepubertal children, compared to 22 born appropriate for gestational age (AGA) children, in which SGA children were insulin resistant when compared to AGA children, although they did have normal insulin action (50). In a younger cohort of 55 SGA and 13 AGA children from birth to 3 years of age, an indirect calculation of insulin sensitivity using the homeostasis model (HOMA-IR) was used (7). In this study (7), SGA neonates were initially more insulin sensitive immediately after birth, but then developed insulin resistance and reduced insulin action by 1 year of age when compared to the AGA group. Despite the differences in the measurement of insulin sensitivity methods, both studies reported that insulin resistance emerges during childhood (7).

In contrast to the positive relationship between birth weight and insulin sensitivity, at least after the first year of life, the relationships between insulin secretion and low birth weight identified in the systematic review were less consistent, with negative (16 studies), positive (6 studies) or no association (7 studies) variously reported (1). These variable findings may reflect effects of both demand and capacity on insulin secretion. Thus, lower plasma insulin may be due to either high insulin sensitivity (in which case it is adequate to meet demand) or β-cell secretory dysfunction (when low insulin may reflect insufficient secretion relative to demand), and measures

of basal or stimulated insulin secretion do not differentiate appropriate from inadequate insulin secretion. An accurate measurement of insulin secretion therefore requires independent measures of insulin secretion and sensitivity, so that insulin secretion can be calculated relative to the sensitivity, and this is known as insulin disposition (29).

Limited studies of insulin disposition after IUGR are available, and these suggest that insulin disposition is impaired after IUGR. For example, in one study comparing 20 SGA and 20 AGA 19 year-old Caucasian men, insulin secretion was measured by intravenous glucose tolerance test (IVGTT) and insulin sensitivity by HEC (6). In this study, SGA men had normal insulin sensitivity and insulin secretion when compared to AGA men (6). When insulin secretion was expressed relative to insulin sensitivity, however, insulin disposition was 30% lower in SGA men compared to AGA men (6). This suggests that insulin secretion is impaired following IUGR and that this impairment can be present before insulin resistance emerges. In contrast, the authors of a second study which compared 26 SGA and 25 AGA 25 year-old young adults, which used the same techniques to independently measure insulin secretion and sensitivity, reported that the SGA young adults had normal insulin disposition, despite being insulin resistant (5). This suggests that a β-cell compensatory increase of insulin secretion may have occurred to maintain normal insulin action in the SGA group (5).

The effect of IUGR on insulin disposition has also been studied in children. One study compared between 28 SGA and 22 AGA 9 year-old Caucasian children, and also used the same techniques as the adult studies described above to independently measure insulin secretion and sensitivity (50). In this study, SGA children had normal insulin secretion and disposition when compared to AGA children, despite being insulin resistant (50). In a younger cohort of 55 SGA and 13 AGA children studied from birth to 3 years of age, insulin secretion was also measured by IVGTT and insulin sensitivity

by HOMA-IR (7). In this study, SGA neonates were initially more insulin sensitive than AGA neonates immediately after birth, but developed insulin resistance by 1 year of age (7). Consequently, these SGA children had lower insulin disposition compared to the AGA group by 3 years of age (7). These contradictory results from two studies in children may reflect their methodological differences in measuring insulin sensitivity, but may also be due to the early postnatal catch-up growth (CUG) that the younger cohort of SGA children exhibited (7) to a greater extent than the older cohort. Therefore, reduced insulin disposition in younger cohort (7) may reflect a greater postnatal challenge to insulin secretory capacity of increased nutrient intake and growth. Long-term increased demand for insulin secretion due to increased nutrient intake can exhaust insulin secretory capacity, and depending on its extent, may interact with prenatal programming of impairment to cause insulin secretory deficits to emerge at varying times in early life (43, 51). IUGR may also cause defects in β-cell function (52) and/or impair the capacity to increase β-cell function and mass in response to insulin resistance (53). The nature of the precise defects induced by IUGR is unclear, as is their molecular and cellular basis, but there are several known processes that may be affected including reduced β-cell function and mass due to reduced expression of their molecular determinants (43).

1.6 DETERMINANTS OF β-CELL FUNCTION, MASS AND PLASTICITY

1.6.1 Regulation of β-cell function, mass and plasticity

The endocrine β−cells of the pancreas are able to vary the total capacity for insulin secretion (termed plasticity) in response to insulin demand through two routes, either by changing the number or size (mass) of β-cells or by altering intrinsic β-cell insulin secretory function, including glucose sensing (44). β-cell mass can be increased

by neogenesis (formation of new β-cells), replication (division of existing β-cells) or hypertrophy (increase in size of existing β-cells), or reduced by apoptosis (Figure 1.2). A range of transcription and growth factors regulate these processes and with insulin-like growth factor-2 (*IGF2*, 54, 55) and pancreatic duodenal homeobox-1 (*PDX1*, 55, 56, 57) in particular having major roles in regulation of β-cell mass. In addition to PDX1 and IGF2, β-cell mass can be increased by other regulatory factors, such as insulin (*INS*) and its receptor (*INSR*, 58) and insulin-substrate 2 (*IRS2*, 59) and RAC-β serine/threonine protein kinase (*AKT2*, 60).

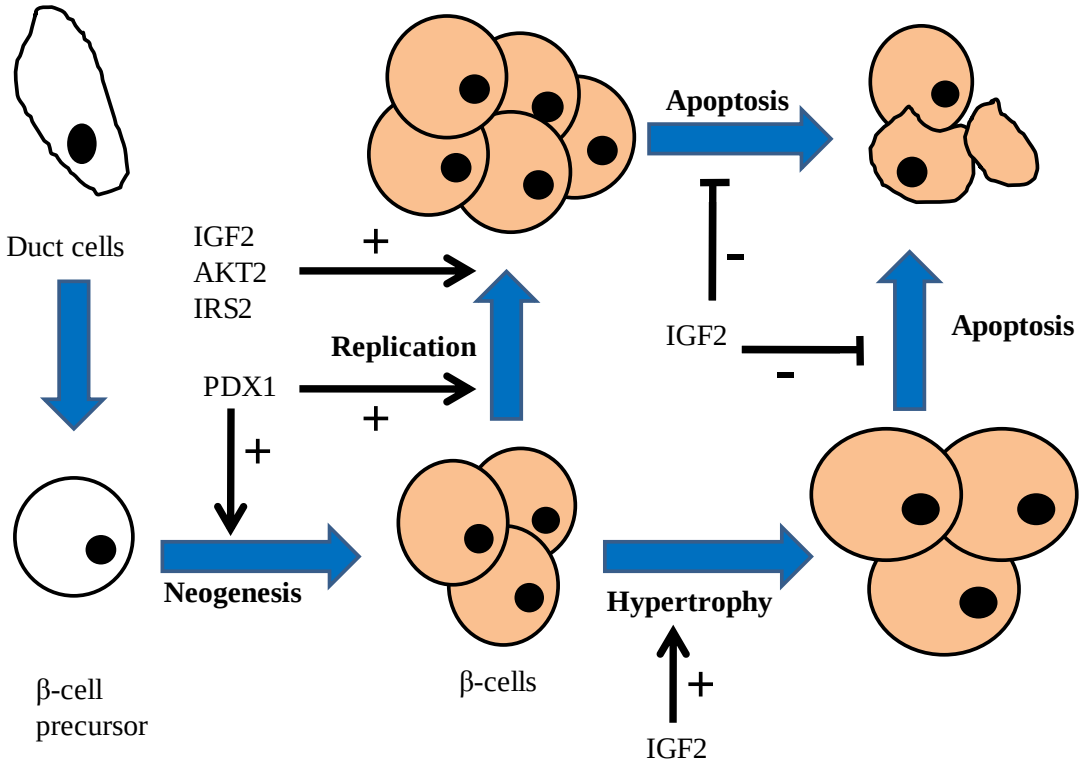

Figure 1.2 Regulation of β-cell mass and its molecular determinants.

→ indicates positive regulation and ⊥ , indicates inhibition. IGF2 = insulin-like growth factor-2, PDX1 = pancreatic duodenal homeobox-1, IRS2 = insulin-substrate 2 and AKT2 = RAC-β serine/threonine protein kinase.

Regulation of β-cell function is determined by glucose uptake and metabolism, and activation of downstream pathways that result in insulin secretion (Figure 1.3). Glucose is transported into the β-cell by glucose transporter 2 (*SLC2A2*), and is phosphorylated by glucokinase (*GCK*, 61). Then, a series of metabolic steps in the cytoplasm and mitochondria increase the ADP:ATP ratio in cytoplasm, leading to membrane depolarisation and opening of calcium channels which in turn promotes exocytosis of insulin containing vesicles (61). Because *PDX1* directly up-regulates β-cell expression of *INS* (62), *SLC2A2* (63) and *GCK* (64), increases in *PDX1* expression can effectively up-regulate insulin secretion, and decreased *PDX1* expression after IUGR, shown to date in the PR rat (21), is likely to impair β-cell function. Other mediators such as *KCNJ11* (a subunit of the ATP-sensitive K^+ channel) and *CACNA1D* (a subunit of the L-Type voltage-gated Ca^{+2} channel) are also important to β-cell function as their gene expressions strongly correlated with impaired β-cell secretory function in an ovine model of IUGR (8).

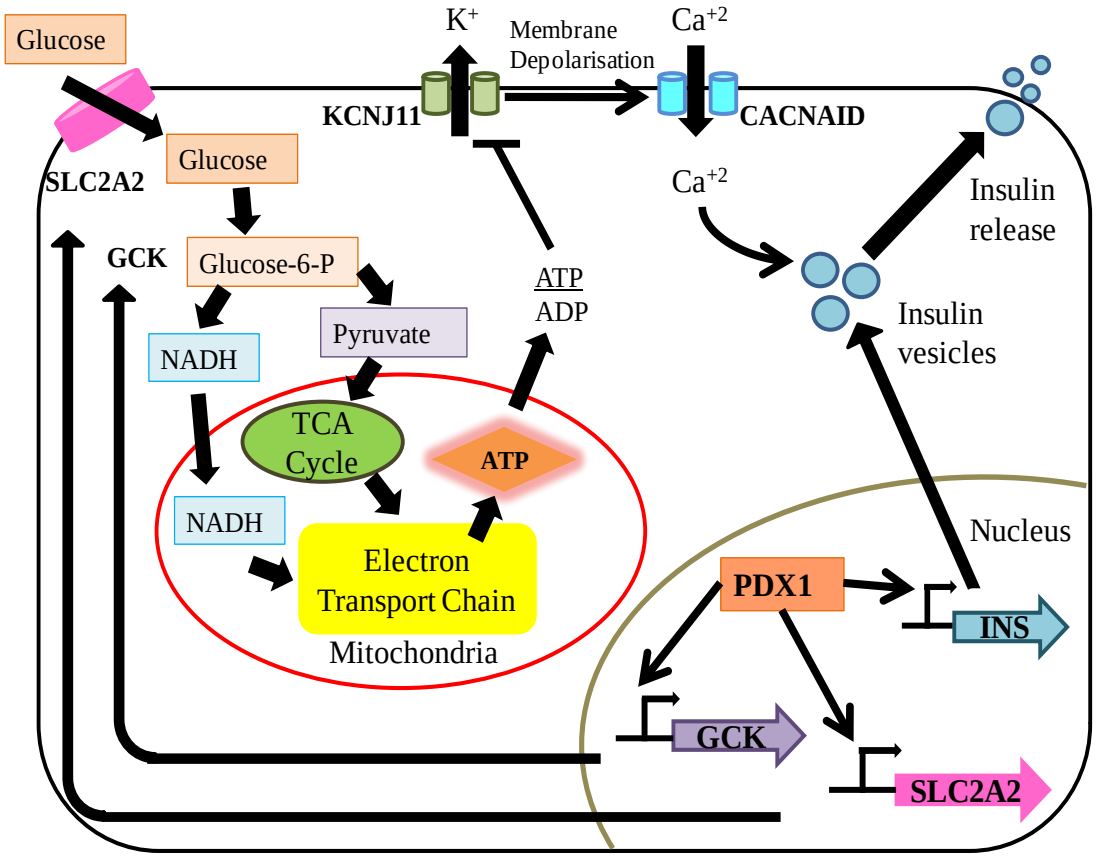

Figure 1.3 Regulation of glucose-stimulated insulin secretion in pancreatic β-cells, adapted from (61).

➡ indicates movement of molecules, → indicates positive regulation and ⊥, indicates inhibition, *SLC2A2* = glucose transporter 2 gene, *GCK* = glucokinase gene, *INS* = insulin gene, KCNJ11 = a subunit of the ATP-sensitive K^+ channel, CACNA1D = a subunit of the L-Type voltage-gated Ca^{+2} channel, NADH = Reduced form of nicotinamide adenine dinucleotide, TCA cycle = Tricarboxylic acid cycle, ADP = Adenosine diphosphate, ATP = Adenosine triphosphate.

1.6.2 *PDX1*

PDX1 is a transcription factor which acts prenatally and postnatally as a master regulator of pancreatic and β-cell development and function (56). Homozygous *PDX1* knockout (*PDX1*$^{-/-}$) mice exhibit an absence of pancreatic development during gestation and these mice die a few days after birth (65), which indicates that *PDX1* is essential for prenatal β-cell development. Heterozygous *PDX1* knockout mice (*PDX1*$^{+/-}$) are viable, but have an impairment in plasticity of glucose-stimulated insulin secretion compared to *PDX1*$^{+/+}$ mice, despite having normal fasting glucose, insulin content and β-cell mass (66). This was demonstrated by cross breeding *PDX1*$^{+/-}$ mice with insulin resistant mice (*GLUT4*$^{+/-}$) to assess the β-cell response in the presence of insulin resistance (66). *PDX1*$^{+/-}$*GLUT4*$^{+/-}$ progeny had impaired *in vivo* glucose-stimulated insulin secretion and glucose tolerance with progressive increases in fasting blood glucose with ageing, despite an increased β-cell mass when compared to control mice and mice heterozygous for only one of the two defects (66). These results indicate that a deficiency of *PDX1* expression leads to failure of compensatory increases in β-cell secretory function in response to insulin demand or resistance, although β-cell mass did increase when challenged (66). In a study with multiple cross-breeding of heterozygous *PDX1*$^{+/-}$ mice with different types of insulin resistant mice, the SLC2A2 protein was almost completely absent from the islets of all mice that only carried a single allele for *PDX1* (67). This suggests that a deficiency of *PDX1* may at least in part impair glucose-stimulated insulin secretion via down-regulation of *SLC2A2*. Moreover, *PDX1* directly regulates expression of *INS* (62) and *GCK* (64), both determinants of insulin secretion by β-cells. Together these studies show that *PDX1* expression is not only important for regulating prenatal β-cell development and growth, but it is also important for postnatal β-cell maintenance and adaptation.

This capacity of the pancreas and β-cell to adapt to changes in insulin demand is critical in maintaining insulin action. Understanding how these processes and their underlying molecular determinants can be influenced by substrate deprivation and IUGR before birth is required. In addition to potential changes in the processes regulating plasticity, there is also limited evidence that severe IUGR reduces the β-cell mass at birth in humans. In a study of 6 IUGR compared to 10 AGA infants, IUGR infants had smaller islets and a reduced percentage of insulin producing β-cells in the pancreas when compared to the AGA group (68). Similar effects of IUGR on β-cell mass have been reported in other species. Experimentally-induced IUGR in sheep reduced pancreatic mass in late gestation fetuses (143 days gestational age), and fetal weight correlated positively with absolute β-cell mass (8). In rats, IUGR offspring from placentally-restricted pregnancies had progressive reduction in β-cell mass with ageing relative to offspring from control pregnancies (4), consistent with their progressive silencing of *PDX1* expression, leading to onset of frank diabetes by adulthood (21, 33). As yet, however, how IUGR causes impairment of β-cell mass, function and their molecular determinants requires further and more extensive verification.

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1.6.3 MicroRNA

Recently, microRNAs (miRNA) have been recognised as a new class of molecular regulators of β-cell development and insulin secretion. MicroRNAs are short single-stranded non-coding RNAs (18-22 nucleotides in length) which are able to negatively regulate gene expression by binding to complementary sites in the target messenger RNA (mRNA) at the 3' untranslated regions (69). MicroRNAs are synthesised in the nucleus and exported out to the cytoplasm where the enzyme Dicer cuts the double-stranded miRNAs into single-stranded miRNA (Figure 1.4, 70). One

strand of the miRNAs is incorporated into a complex with the Argonaute 2 protein to form a RNA-induced silencing complex (RISC). The RISC complex binds to the target mRNA and can lead to two different outcomes. A perfect complementary binding between miRNA and its target mRNA will lead to mRNA degradation, while 'incomplete matching' binding will lead to translational repression of the mRNA (70). Since one miRNA can target many genes (71), the idea of miRNAs forming coordinated regulatory networks (72, 73) to stimulate changes in expression of multiple genes is promising and may explain their regulatory role.

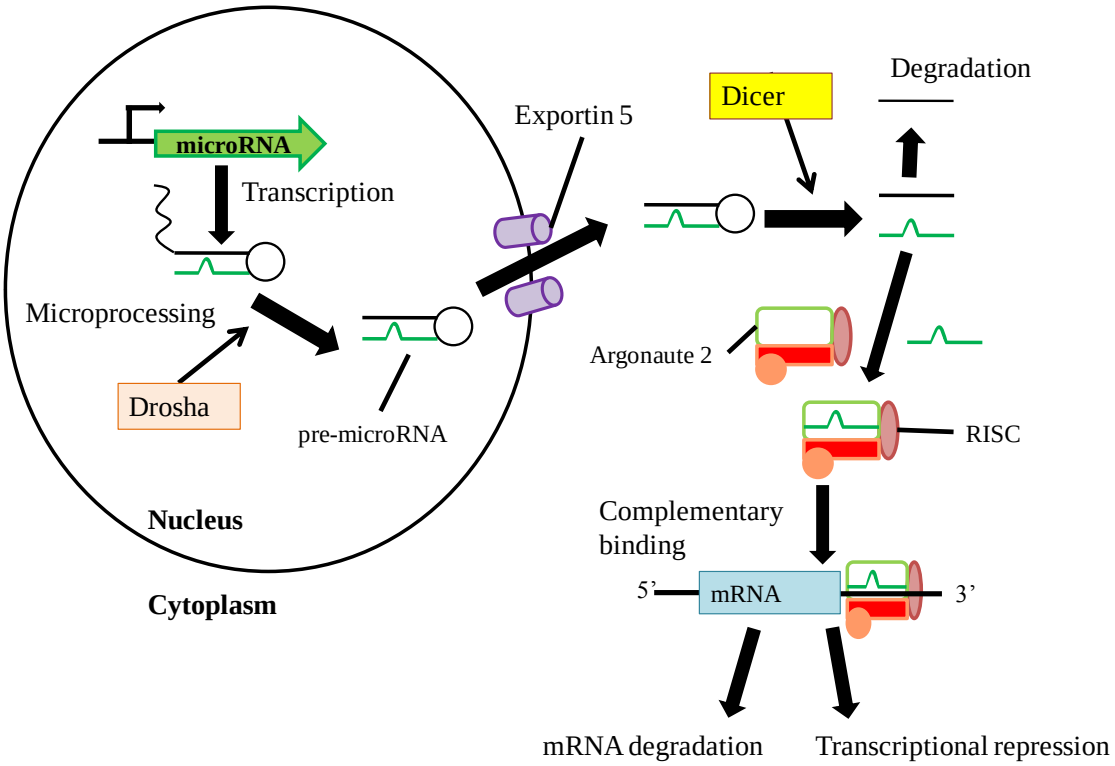

Figure 1.4 Bio-synthesis of microRNAs, adapted from (70).

➡ indicates molecular process and ⟶ indicates enzyme action, RISC = RNA-induced silencing complex.

Several miRNAs have been shown to be important in β-cell development and function. One of these miRNAs is miRNA-124a (miR-124a), which is abundantly expressed in islets (74) and targets *FOXA2*, a transcriptional activator of the *PDX1* gene (75). Over-expression of miR-124a in insulin-producing cell lines suppressed *FOXA2* expression and subsequently reduced the mRNA expression of its downstream targets, *PDX1* and *INS* (75). Another miRNA that is also abundantly expressed in islets is miRNA-375 (miR-375), which down-regulates mRNA expression of the Myotrophin (*MTPN*) gene required for exocytosis of insulin vesicles (76, 77). A third miRNA candidate regulator of β-cell development and function is miRNA-7 (miR-7), which is also highly expressed in endocrine islets (77). Inhibition of miR-7 using antisense miRNA in mice embryos reduced insulin synthesis and β-cell number in newborn pups, suggesting that this miRNA is important for pancreatic development (78). Importantly, these mice develop impaired glucose tolerance postnatally (78), which suggests that suppression of miR-7 in early life has permanent effects to lead to these adverse effects postnatally. Whether gene expression of these miRNAs is altered following IUGR and contributes to any change in *PDX1* expression as part of the development of diabetes is still unknown, however, and therefore requires further investigation.

1.6.4 Epigenetic regulation of β-cell mass and function

Programming of β-cell development in early life by restriction of placental growth and substrate supply to the fetus, leading to functional impairment later in life, may occur in part via epigenetic changes that persist and maintain altered gene expression throughout life. Epigenetics describes processes including the modification of chromosomes and associated structures that change gene expression without altering the nucleotide sequence of DNA (79-81). These epigenetic modifications can be stable

and heritable from one cell generation to the next (79). Epigenetic modifications explain the variability of gene expression between different cell types even though each shares the same genome (80, 81). Within chromosomes, DNA strands are looped around histone protein complexes to form nucleosomes, which are the base units of chromatin (Figure 1.5, 82, 83). These nucleosomes are usually closely packed together (heterochromatin) and chromatin state determines the state of gene expression. A loosely packed chromatin (euchromatin) provides an accessible region for transcription factors to bind to DNA and initiate gene expression (84, 85). Thus any mechanisms that can change the state of nucleosome packing will change gene expression.

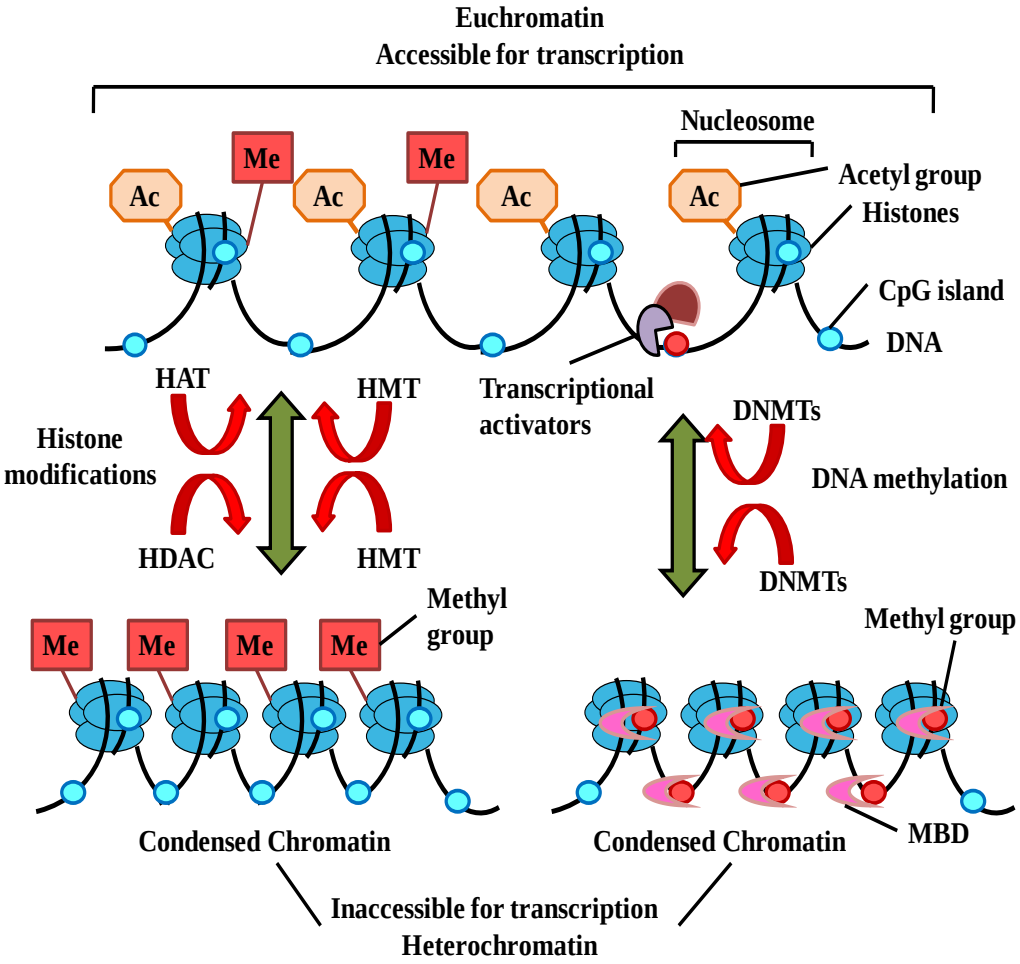

Figure 1.5 Schematic diagram of gene expression regulation by epigenetic mechanisms, adapted from (85-87).

Reversible epigenetics modifications on chromatin change its state from heterochromatin (closed) to euchromatin (open), through the actions of enzymes such as histone acetyltransferase (HAT), histone deacetylase (HDAC) and histone methyltransferase (HMT). Methylation of DNA sequences on cytosine-guanine base pairing rich regions (CpG islands) is facilitated by DNA methyltransferase (DNMTs) enzymes which promote binding of methyl binding domain (MBD) proteins to further silences the genes. ⟺, indicates reversible process and ↷, indicates enzyme action.

There are many types of epigenetic marks, however, the best characterised are histone protein modifications by acetylation and methylation and DNA methylation (85-87). Acetylation of histone proteins usually occurs at the ε-amine group of the N-terminal tails of H3K and H4K proteins, and is regulated by the histone acetyltransferase (HAT) enzyme that adds acetyl groups and by histone deacetylase (HDAC) enzymes that remove them (Figure 1.5, 85, 86, 87). Acetylation of histone loosens the structure of nucleosomes (promotes a euchromatin state) and thus provides an accessible area of the DNA for transcription factors to initiate gene expression (85-87). Conversely, removal of the acetyl groups on histone proteins favours the formation of heterochromatin and silences the gene (85-87). In contrast to histone acetylation, methylation of histone proteins has variable effects on the status of the genes depending on the location of methylation on the histone proteins (Figure 1.5, 85). Transfer of mono-(me1), di-(me2) and tri-(me3) methyl groups to lysine (K) in different locations within the histone proteins by the histone methyltransferase (HMT) promote either euchromatin or heterochromatin status (85, 88). For example, mono- or di-methylation of histone 3 residue K 4 (H3K4me1 or H3K4me2) are markers for euchromatin, whereas mono-methylation of histone 3 residue K 9 (H3K9me1) is a marker of heterochromatin (88). These combinations of histone modifications are often referred to as the histone codes and describe the position and type of the modification at differing histone proteins and their amino acid residues (86, 87).

DNA methylation involves the transfer of a methyl group by DNA methyltransferase enzymes (DNMTs) to a cytosine base of the DNA adjacent to a guanosine base (CpG site), and usually occurs in cytosine-guanine base pairing rich regions (CpG islands, 89, 90). There are 3 known DNMTs (22, 91), which are DNA methyltransferase 1 enzyme (DNMT1), DNA methyltransferase 3A (DNMT3A) and

3B (DNMT3B). DNMT1 maintains methylation patterns during cell replication (22, 91) and is primarily involved during DNA repair and maintenance (reviewed by, 23, 92, 93). DNMT3A and DNMT3B cause *de novo* methylation to establish and maintain genomic methylation patterns (22, 23) and thus determine DNA methylation status of the genes throughout life. Methylation of the CpG island is usually tissue-specific and reflects the status of specific genes; either transcriptionally active or repressed (89, 90). Highly methylated CpG islands usually promote the binding of methyl-CpG-binding domain protein (MBD) and block the binding of transcriptional activators, thus silencing gene expression (86, 87), while low methylation levels in CpG islands allows association of the promoter with gene transcriptional activators and actives gene expression (Figure 1.5, 94).

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association of the promoter with gene transcriptional activators and activates gene expression (Figure 1.5, 94).

DNA methylation and histone modifications can be affected by nutrient availability during gestation and may be involved in β-cell growth, development and function. In mice, supplementing diets of females with methyl donors before and during pregnancy permanently increased the methylation status of the *axin fused* gene in the offspring (95). This led to a change in phenotype, because methylation of *axin fused* gene in offspring correlated negatively with the kinkiness of their tails (95). This suggests that nutrient availability during prenatal life can induce epigenetic changes in the gene and lead to a different phenotype later in life; a concept termed epigenetic programming. Thus, epigenetic state and expression of genes that are involved in β-cell growth, development and function might be altered due to the reduction of maternal nutrient and oxygen supply leading to IUGR, and epigenetic programming might underlie effects of IUGR on β-cell growth, development and function. Consistent with this theory, in experimentally-induced IUGR rats IUGR triggered progressive epigenetic silencing of the *PDX1* promoter region leading to onset of diabetes (21). A restricted environment during late gestation in these rats reduced histone acetylation by HDAC, promoting tighter packing of the chromatin and thus blocking transcription, associated with a 50% reduction in *PDX1* expression at birth (21). After birth, the continuing loss of histone acetylation promoted the methylation of histone proteins which progressively further suppressed *PDX1* expression. By adulthood, DNA methylation had occurred, which caused permanently reduced *PDX1* expression (21). To what extent IUGR could lead to permanent changes in epigenetic status of β-cell genes in non-rodent species including humans is still unknown, and requires investigation.

1.6.5 Adiponectin regulation of β-cell mass and function

Adiponectin is a hormone secreted by adipose tissues which modulates metabolism, including insulin sensitivity and adiposity (96, 97). Adiponectin promotes insulin sensitivity by stimulating fatty acid oxidation and glucose uptake into muscle and liver (98, 99). Adiponectin is present in cord blood (100, 101) and is produced by the placenta and fetus (102, 103), with its concentration higher in cord blood and newborns compared to adults in humans (101, 104). Adiponectin can be detected in the serum and plasma in multiple forms including in a full complex globular form, andless complex forms as high, medium or low molecular weight adiponectin (105). In previous epidemiological studies low adiponectin concentrations are associated with increased risks of cardiovascular diseases such as diabetes (106-108) and obesity (109). More specifically, the high molecular weight form of adiponectin in the plasma is correlated positively with glucose tolerance in humans (110).

The impact of IUGR on adiponectin levels in early postnatal life is unclear. In a study of 20 SGA and 20 AGA newborn humans, both groups had similar plasma adiponectin concentrations in neonatal blood (100). Similarly, plasma adiponectin concentration in cord blood was similar between SGA and AGA newborns in a larger cohort of 50 SGA and 50 AGA individuals (111). Conversely, two other studies in similar (49 SGA and 41 AGA) or smaller (28 IUGR and 34 AGA) cohorts of neonates have reported that plasma adiponectin concentrations were lower in the SGA groups (112, 113). These discrepancies in the findings may be due to the facts that all the above studies did not consistently classify IUGR, with some defining SGA as having birth weight below the 3^{rd} percentile (100, 113, 114) or 10^{th} percentile (112), or having birth weight less than 2.5 kg (111). Subsequently, plasma adiponectin was reduced in SGA children aged 6-8 years old together and these SGA children had greater visceral

adiposity and insulin resistance compared to AGA children (115, 116). Plasma adiponectin varies inversely with weight gain and body fat mass in non-obese children (117) and adults (106). Therefore, adiponectin deficiency in IUGR neonates and young children may be a predisposing factor for later development of visceral adiposity in life. Interestingly, in a study of SGA prepubertal children, serum adiponectin was increased in lean SGA children compared to lean AGA children (118). In the same study, overweight SGA children had reduced serum adiponectin and were more insulin resistant than to lean SGA (118), consistent with the inhibition of adiponectin production in response to increasing weight or fat mass as seen in normal individuals (109). Therefore, adiponectin deficiency in early life and in childhood may predict the risk of adiposity which in turn contributes to development of insulin resistance and increased risk of T2D following IUGR.

In addition to potential effects via adiposity, adiponectin directly up-regulates insulin secretion and action. Treating mouse islets with adiponectin stimulated insulin secretion via increasing exocytosis of insulin granules as well as enhancing *INS* mRNA expression (119). Similarly, incubating mouse islets *in vitro* with two doses of adiponectin (2.5 and 5 $ug.ml^{-1}$) together with glucose for 1 h increased glucose-stimulated insulin secretion when compared to islets incubated in glucose alone (120). In the same study, incubation of mouse islets *in vitro* with 3 doses of adiponectin (2.5, 5 and 10 $ug.ml^{-1}$) for 24 h increased mRNA expression of *PDX1* (120), which may suggest that *PDX1* is a target molecule or pathway for induction of insulin secretion by adiponectin in β-cells. In islets from insulin-resistant mice, adiponectin inhibited insulin secretion at low concentrations of glucose and stimulated insulin secretion at high glucose concentrations (121). This suggests that adiponectin action on β-cells is influenced by glucose levels and the presence of insulin resistance. Together these

findings imply that adiponectin is not just involved in insulin resistance and adiposity, but may also play role in regulating insulin secretion, and reduced adiponectin abundance may therefore contribute to the impairment in β-cell function following IUGR.

1.7 EFFECTS OF EXPERIMENTAL IUGR DUE TO RESTRICTED PLACENTAL FUNCTION ON INSULIN ACTION

Poor placental growth or function is one of the most common causes of human IUGR in developed countries (122). The placenta is an organ that connects mother and fetus, delivering the essential components for fetal growth, from maternal to fetal blood. Restriction of placental growth and function impairs delivery of nutrients and oxygen to the fetus, which then leads to IUGR (reviewed by, 123). Experimental models of restricted placental function (PR) have therefore been used to explore effects of IUGR on insulin action in progeny.

Several of the earliest studies in animal models of PR assessed the effects of PR in rats on the development of diabetes in the offspring in later life. In these studies, PR was induced in rats by bilateral uterine artery ligation (BUVL) in the mother on day 19 of gestation (term 22 days) to restrict oxygen and nutrient supply to fetuses in late pregnancy (4, 124). The severity of PR effects on the fetuses depends on their proximity to the ligation sites (124). Progeny that survived and were included in postnatal studies have therefore experienced varying levels of growth restriction in the gestation, which is reflected on their birth weight and size. Fetal consequences of PR-induced growth restriction for Sprague-Dawley rats included reduced plasma glucose, oxygen and insulin concentrations with evidence of acidosis (125, 126), consistent with effects of IUGR on nutrient and hormone concentrations in human fetuses (127, 128).

Sprague-Dawley PR rat progeny were smaller and lighter at birth, but gained more weight after weaning and eventually surpassed control progeny in body weight (4). With aging, these Sprague-Dawley PR progeny became obese and developed diabetes with progressive development of impaired glucose tolerance and insulin secretion (4). Importantly, this impaired insulin secretion was present in neonates and worsened with ageing (4), indicating that β-cell secretory defects were present early in postnatal life following IUGR. In contrast, PR did not induce obesity or insulin resistance in Wistar-Kyoto adult rats, despite the progeny also having lighter birth weights and impaired glucose-stimulated insulin secretion compared to progeny of control pregnancies (129, reviewed by, 130, 131). The reasons for these differences in the metabolic effects of PR between the two rat strains are unclear, although possibilities include genetic susceptibility to the prenatal challenge and differences in litter sizes and use of cross-fostering between studies (130-133).

PR can also be experimentally induced in sheep and leads to similar metabolic consequences as occur after human IUGR. In sheep, the majority of visible endometrial caruncles (attachment sites) in the uterus are surgically removed before pregnancy to induce PR (134, 135). These sites allow the embryo to attach to the uterus and form the placenta (134, 135). Surgical reduction in the number of placental attachment sites restricts placental growth and causes restriction of nutrient and oxygen delivery to the fetus and its subsequent growth (134, 135). These PR fetal sheep had lower plasma glucose and evidence of hypoxia and acidosis (136-140) consistent with effects on the fetus of PR in rats (125, 126) and IUGR in humans (127, 128). Moreover, the PR fetal sheep has impaired glucose or arginine-stimulated insulin secretion (at 120 and 140 gestational age respectively), but no change in insulin sensitivity or insulin disposition (140). In small sheep fetuses that have less β-cell mass, enhanced glucose-stimulated

insulin disposition is observed with normal insulin sensitivity (8, 140), indicating that an increase in β-cell function occurs to compensate for the low number of β-cells. These findings suggest that PR and IUGR can induce changes in insulin secretion and β-cell function, which are present in prenatal life. In young IUGR lambs, in the presence of catch up growth following IUGR, basal but not maximal insulin disposition was negatively correlated with birth weight, (8, 32, 141), which further suggests an enhancement of β-cell function in the basal state as a compensatory mechanism to enhance insulin action after IUGR in growing lambs. With aging, lower basal and maximal insulin disposition was observed in IUGR adult sheep, even though there was a compensatory increase in β-cell mass (8, 32). This indicates that β-cell function failed to respond to the increase in insulin demand, due to developing insulin resistance in adulthood in PR sheep.

Intriguingly, twin pregnancies in sheep can also be used as a natural model of IUGR due to competition for placental implantation sites which results in moderate prenatal deprivation of oxygen and nutrients, and consequently caused lower birth weight in comparison to singleton sheep (142, 143). Similar to IUGR/PR effects, twinning in sheep reduced insulin, IGFs and amino acids supply to the fetuses (143). However, limited studies of postnatal glucose control and insulin action in twin lambs are available. One study of twin lambs characterised lighter twins and heavier twins according to their birth weight and found that lighter twins had greater glucose tolerance and insulin sensitivity but slower growth compared to heavier twins at 6-month-old (144). These metabolic differences were however absent by 1 year of age when their body weights were similar between the groups (144). In a different study, the authors compared twin lambs to singleton lambs and showed that twin lambs had normal glucose tolerance, insulin secretion and sensitivity in comparison to singleton

lambs at 10 months of age (145). Importantly in this study (145), weight gain during growth was positively associated with insulin secretion from birth to weaning, and negatively associated with insulin secretion from weaning to 10 months of age in all lamb groups, similar to previously observed relationships in PR lambs (8, 32, 141). These moderate implications of IUGR due to twinning on postnatal glucose control and insulin action in the twin lambs and adults are influenced by the weight gain in first few months of life in these twins (145). It is possible that competition for milk supply restricts postnatal catch-up growth and at least partially protects twins from the adverse metabolic effects of IUGR coupled with accelerated neonatal growth. It is therefore possible that if milk availability is increased for twin lambs, more substantial effects of IUGR on postnatal growth and insulin action may be evident.

Although both of these experimental models (rat and sheep) give insight into how PR may program the development of metabolic disease in later life, there are important differences in the timing of pancreatic and β-cell development between species. In humans, sheep and pig, the first insulin-positive cells are detected in fetuses at about 25% of term gestation and islets are present in mid-gestation (10-14). β-cell functional maturation and pancreatic remodelling, in which β-cells mature from a fetal to adult-type phenotype, mainly occurs before birth in humans and sheep (10-14). This maturation of β-cells involves the shift in β-cell insulin secretion from being responsive to amino acids such as arginine, to being more glucose-responsive (16). In contrast, the rat pancreas develops later in gestation compared to humans and sheep, with the first insulin positive cells able to be detected at ~60% of gestation and pancreatic remodelling occurring at ~10-17 d postnatal age (16, 18-20). These differences in the timing of pancreatic and β-cell functional maturation between humans, sheep and rats may contribute to different outcomes of PR and IUGR between species. PR and IUGR

are likely to impose greater effects on the β-cell or pancreas and its determinants when the insults occur at critical developmental periods during gestation, and may not affect processes that happen postnatally in rodents.

1.8 SEX SPECIFIC IUGR EFFECTS ON POSTNATAL INSULIN SECRETION AND ACTION

Previous studies in humans (146-148) and in other species (32, 149) have reported that there are sex-specific effects on perinatal programming of growth, metabolic and cardiovascular outcomes including insulin secretion and action, in which males are more susceptible to these IUGR effects compared to females. One example is a study of 20 year-old young adults, where IUGR men had lower insulin sensitivity compared to control men, but there was no difference in insulin sensitivity between control and IUGR women (146). Similarly, in animal studies of IUGR rats induced by protein restriction during gestation, adult IUGR males had greater insulin response to glucose when compared to control males at 20 weeks of age, indicative of insulin resistance (149), with no differences in females. In an older cohort of these IUGR rats, at 11 months of age, IUGR males but not females were less glucose tolerant when compared to control groups (150). Similar outcomes are also observed in adult PR sheep at 1 year of age, where adult PR males had normal insulin sensitivity but impaired insulin action compared to control males, and adult PR females were more insulin sensitive but had normal insulin action (32). Importantly, these sex-specific IUGR effects in PR sheep are absent in young lambs at 45 d of age (141), in which the glucose tolerance, insulin sensitivity, secretion and action outcomes of PR did not differ between sexes (141). In young lambs at 21 d of age, females were fatter than males, with no effect of PR on relative fat mass (151). Adiponectin expression did not differ

between PR and control or between sexes at this age, although some sex-specific effects of PR on fat were evident, as *PPARγ* and leptin expression in perirenal fat in males only (151). In general, these studies suggest that effects of PR on metabolic outcomes including insulin action and adiponectin expression are not sex-specific in neonatal life.

1.9 NEONATAL EXENDIN-4 AS AN INTERVENTION AFTER IUGR

Understanding the factors that regulate expression of *PDX1* has led to development of experimental intervention strategies aiming to prevent development of diabetes after IUGR. Glucagon-like-peptide-1 (GLP1) is a hormone secreted by enteroendocrine cells of the gut as part of the insulin secretory response after meal ingestion, and its receptor is expressed by β-cells (152-154). *In vitro* incubation with GLP1 (10 nM) together with glucose for 1 h in the insulin-producing rat cell line RIN 1046-38 increased glucose-stimulated insulin production when compared to cells incubated with glucose alone (155). Longer *in vitro* GLP1 incubation (24 h) increased mRNA expression of *INS* and biosynthesis of INS protein in insulin-producing cell lines of mice (156) and rats (157). Importantly, *in vitro* incubation with GLP1 in rat insulin-producing cell lines (RIN 1046-38) increased mRNA expression of *PDX1* as early as 1 h after incubation and by 2 h, PDX1 protein level was higher than in controls (158). This suggests that GLP1 promotes *INS* gene expression and insulin secretion and this may be in part via PDX1 (Figure 1.6). In rats GLP1 treatment *in vivo* increased β-cell neogenesis (159) and β-cell replication (159-161), and decreased β-cell apoptosis (161), thus suggesting that GLP1 can increase β-cell mass (Figure 1.6). In diabetic humans, intravenous infusion of GLP1 increased insulin secretion and decreased fasting glucose and glucagon secretion as well as decreasing gastric emptying

(reviewed by, 153, 162, 163) however, subcutaneous injection of GLP1 only transiently increased insulin secretion and decreased plasma glucose (reviewed by, 153, 164). This is partly due to short half-life of GLP1, which is 1.5 - 2 min in human plasma *in vitro*, due to cleavage of GLP1 by the enzyme dipeptidyl peptidase IV (DPP-IV), which removes the two N-terminal amino acids residue and inactivates the peptide (165). Long life analogues of GLP1 including exendin-4 have therefore been developed as therapeutics for treating T2D.

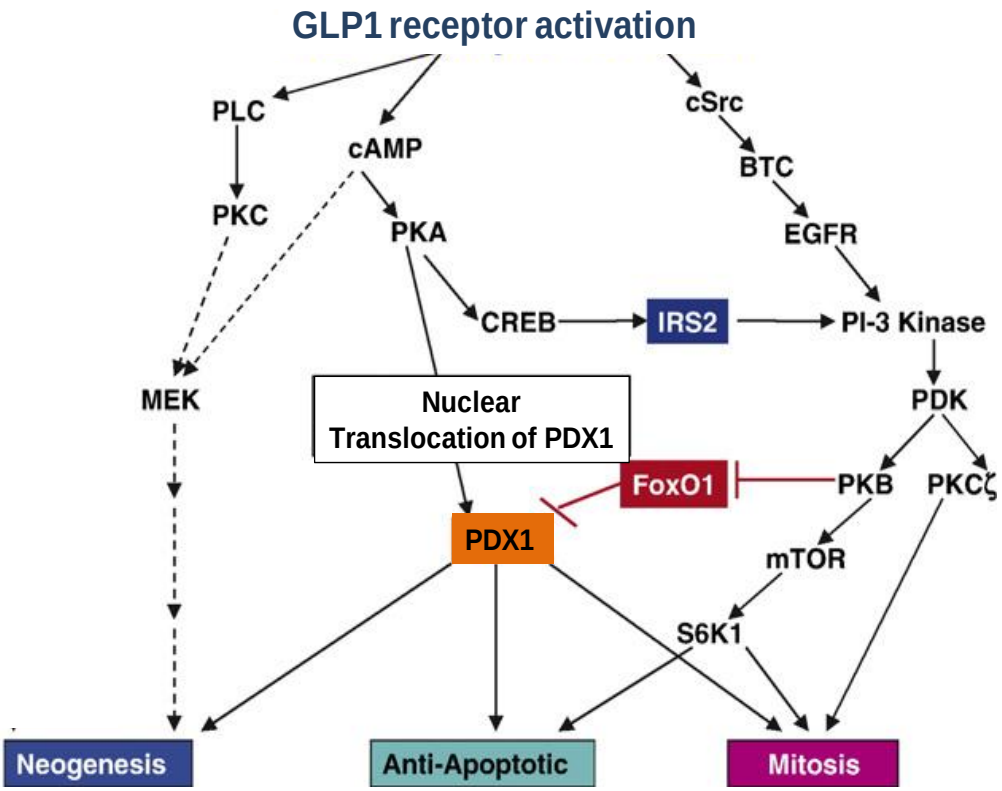

Figure 1.6 Potential pathways for action of GLP1 to stimulate or increase β-cell mass via *PDX1*, modified from (154).

⇢, indicates more complex regulation and intermediates of MEK pathway, ⟶, indicates direct regulation, and ⊥, indicates inhibition.

Exendin-4 was first isolated from *Heloderma suspectum*, known as the Gila Monster, and is resistant to DPP-IV degradation, making it a long lasting analogue of GLP1 (166). The half-life of exendin-4 is ~ 9 h maximum in human plasma *in vitro* due to its capacity to form a compact secondary structure that prevents access of DPP-IV to degradation sites (167). Experimentally, *in vivo* exendin-4 treatment increases *PDX1* mRNA expression in mice (168) and rats (9), stimulates *in vivo* β-cell function (glucose-stimulated insulin secretion) in rats (9, 169), and increases β-cell mass in rats (9, 169). Exendin-4 also induces the differentiation of human duct lines to endocrine cells *in vitro* (170), thus imitating the functional effects of GLP1. Interestingly, *in vivo* exendin-4 treatment during neonatal life prevents development of diabetes in experimentally-induced IUGR rats (9). In this study (9), Sprague-Dawley strain of pregnant females had BUVL surgery at 19 d of gestation (term = 22 d) to experimentally induce PR of offspring. At birth, some PR rat offspring were treated with 1 nM exendin-4/kg of body weight s.c once daily for 6 days without any alteration to nutrition (9). PR rat offspring not given exendin-4 treatment had a progressive development of T2D with impaired glucose tolerance as young as 2 weeks of age, and had high fasting glucose, loss of β-cell mass and *PDX1* expression at 3 months of age (9). In contrast, PR rat offspring that had neonatal exendin-4 treatment had normal glucose tolerance at 2weeks of age, and this rescue persisted to adulthood, when these exendin-4-treated PR offspring had normal glucose control, β-cell mass and *PDX1* expression, similar to non-PR control offspring (9). Importantly, this prevention of diabetes was partially due to the restoration of *PDX1* expression (9) by exendin-4 treatment, by reversing neonatal epigenetic changes and preventing their progression to DNA methylation at the *PDX1* promoter (21, 33). This effectiveness of neonatal exendin-4 provides a significant possible intervention to restore the modification or

epigenetic programming of β-cell by IUGR. However, to what extent exendin-4 treatment can be applicable for humans to prevent development of T2D following IUGR is unknown. Answering this question requires this intervention to be tested in an animal model that has similar timing of pancreatic development as humans, such as the sheep.

1.10 SUMMARY

Programming of pancreatic β-cell mass and function following IUGR may occur via multiple mechanisms or pathways. These pathways include altered regulation of molecular determinants of β-cell mass and function as well as epigenetic regulation of these determinants that either reduce or increase their expression. Moreover, external regulation by adipocytes via adiponectin may also alter β-cell mass and function after IUGR. Increased postnatal nutrition and growth after IUGR may expose the impairments of insulin secretion and its plasticity in response to increased insulin demand and insulin resistance. Together, these defects contribute to an increased risk of developing T2D in adulthood in IUGR individuals. Neonatal eexendin-4 treatment in PR rats restored insulin action and prevented the development of T2D postnatally in this experimental model of IUGR. However, further development and evaluation of the potential efficacy of the exendin-4 intervention for humans requires this treatment to be tested in animal models that share similar timing of pancreatic development and growth as humans.

1.11 HYPOTHESIS AND AIMS

1.11.1 Hypothesis

IUGR due to twinning impairs insulin action, β-cell mass and function as well as gene expression of their key regulatory molecular determinants, and a neonatal intervention with the GLP1 analogue exendin-4 will restore these outcomes in the twin IUGR sheep.

1.11.2 General Aim

To investigate the mechanistic basis of impaired β-cell function and plasticity following IUGR due to twinning, and the efficacy of an intervention, neonatal treatment with the GLP1 analogue exendin-4 to overcome these in the twin IUGR sheep.

1.11.2.1 Aim 1

To determine whether IUGR due to twinning alters islet expression of key functional genes including those for microRNAs and epigenetic machinery that may regulate β-cell function and mass in neonatal lambs, and whether neonatal treatment of twin IUGR lambs with the GLP1 analogue exendin-4 can reverse these effects of IUGR.

1.11.2.2 Aim 2

To determine whether IUGR due to twinning alters islet expression of key functional genes including microRNAs and epigenetic mechanism that regulates β-cell function and mass in the neonatal lambs, and whether neonatal treatment of the lambs with the GLP1 analogue, exendin-4 can reverse these effects of IUGR.

1.11.2.3 Aim 3

To determine whether IUGR due to twinning alters adiponectin gene expression and circulating adiponectin concentrations, and their relationships with *in vivo* and *in vitro* β-cell function and β-cell mass in neonatal lambs, and whether neonatal treatment of twin IUGR lambs with the GLP1 analogue exendin-4 can normalise these outcomes after IUGR.

1.12 SIGNIFICANCE

This study will be the first to characterise the mechanistic basis of how poor growth before birth leads to impaired insulin action specifically via β-cell function and mass in a species in which the pancreas is mature at birth. The changes in the expression of the key regulatory molecular determinants of β-cell function and mass including microRNA and epigenetic mechanisms after IUGR will contribute to the understanding of how the postnatal effects of IUGR persist into adulthood. This study will also evaluate the efficacy and the underlying mechanisms for neonatal exendin-4 treatment as an intervention to prevent T2D following IUGR, for the first time in a species that shares similar timing of pancreatic β-cell development as humans.

CHAPTER 2

2 CHAPTER 2 MATERIALS AND METHODS

2.1 THEORETICAL FRAMEWORK

In this study, natural twin pregnancies were used as the model of placental restriction to induce IUGR, and unrestricted singleton lambs as the control group. In each twin set, sibling twin lambs with heavier and lighter birth weights were alternately allocated to either vehicle or exendin-4 treatment (Figure 2.1). All procedures in this study were approved by the University of Adelaide Animal Experimentation and Ethics Committee (Animal Ethics Approval Number: M-084-2007) and complied with the *Australia code of practice for the care and use of animals for scientific purposes* (171). Animals, treatment, growth measurements, *in vivo* and *in vitro* insulin action and β-cell mass methods have been published (172).

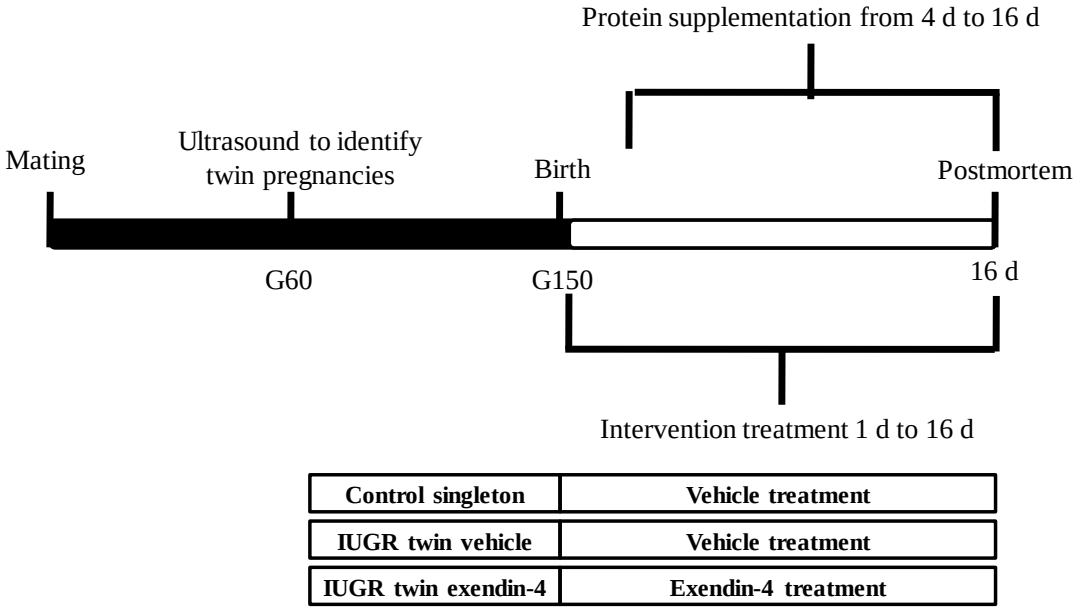

Figure 2.1 Schematic flow diagram and timeline of *in vivo* studies.

Aim 1: *In vivo* insulin-regulated glucose metabolism and *in vitro* insulin secretion

- Glucose tolerance at 14 d and insulin sensitivity test at 12 d of age
- β-cell mass and *in vitro* β-cell function at 16 d of age

Aim 2: Molecular and epigenetic determination – islet mRNA gene expression of key molecular determinants of β-cell function and mass, and of epigenetics regulatory machinery

Aim 3: Adiponectin expression and circulating levels, and their relationships with insulin secretion, β-cell function and mass.

2.2 ANIMALS AND TREATMENTS

2.2.1 Animals

Australian Merino ewes underwent a timed-mating program, and singleton and twin-bearing pregnancies were confirmed by ultrasound scanning at 60 days of gestation (G60). From G120 of gestational age, ewes were transferred to individual floor pens in animal holding rooms with a 12 h: 12 h light/dark lighting cycle, and fed lucerne chaff *ad libitum*, with water available *ad libitum*. Delivery occurred naturally at term and the lambs were housed in floor pens with their mothers throughout the study and allowed to suckle freely, with access to their mother's feed and water, except during experimental protocols as described later. In order to minimise the potential for limitation of neonatal growth by milk availability in twin progeny, we provided a protein supplement on a per body weight basis and calculated to increase protein availability by 25% above that available from milk in ewes suckling twin lambs (173-175) during this period of maximal catch-up growth in IUGR lambs (176). Lambs were supplemented with whey protein (Resource Beneprotein instant protein powder, Nestle) given orally in two equal feeds (at 0900 – 1000 h and 1600 – 1700 h), commencing at 1.25 $g.kg^{-1}.d^{-1}$ on 4 d of age and increasing to 5 $g.kg^{-1}.d^{-1}$ on and after 7 d of age. This supplement should have removed protein constraints due to competition for maternal milk supply, but may have changed dietary composition towards a higher protein content than would be seen in lambs suckling milk alone. Nevertheless, because the

supplement dose was calculated on a per body weight basis we expect that all lambs would have a similar nutrient composition in their total intakes. Unfortunately, for logistical reasons, it was not possible to measure lamb milk intake and feeding behaviour during the present study. Future studies should consider including these measures and alternate supplementary approaches including provision of whole milk or supplements with a similar balance of nutrients as would be obtained in sheep's milk.

2.2.2 Treatments

Ewes delivered 23 lambs, which included 7 singleton lambs (CON) and 8 sets of twin lambs. In each twin set, sibling twin lambs with heavier and lighter birth weights were alternately allocated to IUGR-Vehicle (IUGR+Veh) and IUGR-Exendin-4 (IUGR+Ex-4) groups. From 1 d of age, singleton lambs (CON, n = 7, Male = 3, Female = 4) and IUGR twin lambs (IUGR+Veh, n = 8, Male = 6, Female = 2) were injected s.c. daily with vehicle (0.5% methanol in 0.9% saline s.c.) and twin IUGR lambs (IUGR+Ex-4, n = 8, Male = 6, Female = 2) were injected s.c. daily with exendin-4 (1 $nmol.kg^{-1}$ s.c., Bachem, Buberndorf, Germany). This dose of exendin-4 treatment was the same as that used in a previous study in PR and control rats (9). Exendin-4 was prepared as a 5 nM stock in 0.5% methanol and 0.9% saline, and stored at -20ºC in single use aliquots (1 mL), which were thawed immediately prior to injection. For logistical reasons, and as we were interested in neonatal exendin-4 primarily as an intervention for use after IUGR, we did not include control lambs treated with exendin-4 in the present study. In control rats, neonatal exendin-4 treatment did not change β-cell mass, insulin secretion or action postnatally (9), thus indicating a specific action of exendin-4 after IUGR.

2.2.3 Measurement of size at birth and postnatal growth rates

Lambs were weighed at birth and then every 2 d throughout the study. Body weight, crown-rump length (CRL), shoulder height, lengths of tibia, metatarsal, radius/ulna and metacarpal bones, skull width and length, abdominal circumference, hind limb circumference (knee joint), hind limb circumference (between knee and hip), radius/ulna circumference and lower thoracic circumference were measured as described previously (141). Each of parameters was measured in duplicate for each age and then averaged. Body mass index (BMI) was calculated as weight/CRL^2 ($kg.cm^{-2}$). For each lambs, the absolute growth rate (AGR) was calculated by linear regression as the slope of the relationship between age and each size parameter (141). The fractional growth rate (FGR) was calculated as AGR divided by parameter size at birth (141).

2.2.4 Insertion of vascular catheters

At 4 ± 1 d of age, catheters (1.52 mm OD x 0.86 mm ID, Biocorp Australia, Victoria, Australia) were inserted into the lambs' right femoral artery and vein under general anaesthesia, induced and maintained by Fluothane inhalation anaesthetic (Independent Veterinary Supplies, South Australia, Australia) as previously described (141). All lambs received an intramuscular injection of sedative and analgesic (Xylazil, 0.05 $mL.kg^{-1}$, Lyppards, Victoria, Australia) before the surgery and an intramuscular injection of antibiotic (1 mL of Norocillin SA injection, Lyppards, Victoria, Australia) after the surgery and then daily for 3 d post-surgery. Catheter patency was maintained by flushing the catheters with heparinised saline (500 $IU.mL^{-1}$) daily for 3 d after surgery and then every second d.

2.2.5 Post-mortem and tissue collection

At 16 ± 1 d of age, lambs (CON, n = 7, IUGR+Veh, n = 8 and IUGR+Ex-4, n = 8) were humanely killed by an overdose of sodium pentobarbitone (Pentobarbitone sodium Lethabarb, Lyppards, Victoria, Australia). Organs (liver and pancreas), muscles (semitendinosus, gastrocnemius, soleus, tibialis, extensor digitorum longus, biceps femoris, vastus lateralis, biceps), and dissectable visceral fat depots (left and right perirenal fat, left and right retroperitoneal fat and omental fat) were dissected and weighed for each lamb as previously described (141). Muscle and visceral fat weights were calculated as the sum of weights of these muscles and fat depots, respectively (141). A sample of each organ was fixed in 4% paraformaldehyde and 2 samples frozen in liquid nitrogen, and fat depots were placed in OCT compound (ProSciTech Pty Ltd, NSW, Australia) and frozen in liquid nitrogen, and stored at -80ºC for later analysis.

2.3 *IN VIVO* INSULIN ACTION

2.3.1 Immunohistochemical analysis of pancreas morphology

2.3.1.1 Staining of insulin-positive cells

Pancreas samples (CON, n = 7, IUGR+Veh, n = 8 and IUGR+Ex-4, n = 8) were fixed overnight in 4% paraformaldehyde and then washed 3 times in Phosphate buffered saline, PBS (Dulbeccos $Mg^{2+}Ca^{2+}$ free) over 3 d before storing in 70% ethanol at 4ºC. Tissues were embedded in paraffin and then cut (5 µm-thickness) using a Leica rotary microtome (Leica Microsystems, Illinois, USA), placed on slides (Superfrost Plus Slides, Menzel Glaser, Germany) and immuno-stained to detect insulin-positive cells as described previously (8). Briefly, each section was incubated with guinea pig anti-porcine insulin (DAKO Cytomation, Glostrup, Denmark) at 1:150 dilution as primary antibody, then goat biotin-conjugated F(ab) fragment of anti guinea pig IgG (Rockland Immunochemicals, Pennsylvania, USA) at 1:400 dilution as secondary

antibody, and finally, insulin-positive cells were visualised with 3,3'-Diaminobenzidine (DAB) Fast (Sigma-Aldrich, St. Louis, USA). Negative controls were incubated using the same procedure without the addition of primary antiserum. The insulin-positive cells were stained brown.

2.3.1.2 Morphometric analysis

Morphometric analyses were done with minor modifications of a previously published method (8). Briefly, each stained slide was visualised and captured at 40x resolution using NanoZoomer (Hamamatsu Co., Shizuoka, Japan). Random-systematic sampling was used to select 20 fields of view per section using NDPview software (Hamamatsu Co., Shizuoka, Japan), and this sample size was calculated to give a SEM less than 10%. Cell volume density (V_d) was quantitated by point-counting (204 points/field, V_d equals the number of insulin-positive cells as a proportion of test points on pancreas), and counting numbers of islets, small islets (<5 β-cells), and β-cells per islet in each field (0.293 mm^2/ field), using ImageJ software (USA). β-cell mass was calculated by multiplying V_d by pancreas mass.

2.3.2 Assessment of the insulin axis

2.3.2.1 In vivo insulin sensitivity

At 12 d ± 1 of age, hyperinsulinaemic euglycaemic clamps (HEC) were performed to measure insulin sensitivity of whole-body glucose metabolism as previously described (177). Briefly, lambs (CON, n = 7, IUGR+Veh, n = 8 and IUGR+Ex-4, n = 8) were fasted for 3 hours prior to the experiment with access to water *ad libitum*. Human insulin (2 $mU.kg^{-1}.min^{-1}$, Actrapid, Novo Nordisk A/S, Denmark) was infused continuously via venous catheter and arterial blood was sampled (0.2 mL) every 5 min for 120 min. Larger (2 mL) blood samples were collected before (-10, -5

and 0 min from start of insulin infusion) and at 15 min intervals in the last hour of the clamp (60, 75, 90, 105 and 120 min from start of insulin infusion), centrifuged and plasma stored for later measurement of plasma insulin. Blood glucose at each time point was measured using a HemoCue glucometer (HemoCue AB, Angelholm, Sweden). At 15 min after the start of insulin infusion, an intravenous infusion of glucose (25% dextrose, Baxter Health Care, NSW, Australia) was commenced at an initial rate of 2 $mg.kg^{-1}.min^{-1}$. The glucose infusion rate (GIR) was then adjusted every 5 min based on blood glucose concentrations, to restore and maintain euglycaemia calculated as the average of fasting blood glucose concentrations measured at -10, -5 and 0 min from the start of the insulin infusion. Steady-state plasma insulin concentration was calculated as the average plasma insulin in the 2^{nd} h of the HEC and the insulin sensitivity of glucose metabolism was calculated as the steady-state glucose infusion rate (glucose infusion rate averaged across the 2^{nd} h of the HEC), divided by the steady-state plasma insulin concentration (177).

2.3.2.2 In vivo glucose tolerance, insulin secretion and action

At 14 ± 1 d of age, an intravenous glucose tolerance test (IVGTT) was performed as previously described (177) to measure insulin secretion and *in vivo* glucose tolerance. Lambs (CON, n = 7, IUGR+Veh, n = 8 and IUGR+Ex-4, n = 8) were fasted for 3 h prior to the experiment with access to water *ad libitum*. Basal plasma glucose and insulin were calculated as the means of concentrations in samples collected 10 min, 5 min and 0 min before the administration of glucose. At 0 min, 0.25 g of glucose (as 25% dextrose, Baxter Health Care, NSW, Australia) per kg of bodyweight was administered as a bolus intravenously. Blood (2 mL) was sampled at every 5 min for the first 30 min, then every 10 min for the next 70 min, every 20 min for the next 80 min and then 30 min later until 210 min after the administration of glucose (177).

Blood samples were centrifuged, and plasma collected for subsequent measurement of glucose and insulin concentrations. Indices of glucose tolerance and insulin secretion were calculated as described previously (177). Basal post-hepatic insulin delivery rate was calculated as the fasting plasma insulin concentration before commencement of the IVGTT, multiplied by the MCR for insulin from the HEC (177). Maximal post-hepatic insulin delivery rate was calculated as the maximum plasma insulin concentration during the IVGTT, multiplied by the MCR for insulin from the HEC (177). Basal and maximal insulin disposition indices were calculated by multiplying insulin sensitivity by measures of post-hepatic insulin delivery rate in the basal and glucose-stimulated states, respectively (177).

2.3.2.3 Analysis of plasma hormones and metabolites

Blood samples were centrifuged at 4000 rpm for 10 min at 4ºC to collect plasma for subsequent measurements. Plasma insulin concentrations were measured in duplicate by a double-antibody, solid phase radioimmunoassay using a commercially available kit (Human insulin-specific RIA, HI-14K, Linco Research Inc., St Charles, MO, USA). Cross-reactivity of this assay is 100% with human insulin and 62% with bovine insulin (manufacturer's information) and would be expected to be similar for ovine and bovine given the 97% homology between bovine and ovine insulin protein sequences. The intra-assay coefficients of variation (CV) for the insulin assay were 7.2% and 5.3%, and inter-assay CV were 7.0% and 19.6% for QC samples containing 9.9 and 35.9 $mU.L^{-1}$ insulin respectively (n = 10 assays). Plasma glucose concentrations were measured in duplicate by colorimetric enzymatic analysis on a Hitachi 912 automated metabolic analyser using Roche/Hitachi Glucose/HK kits (Roche Diagnostics GmbH, Mannheim, Germany).

2.3.3 *In vivo* assessment of pancreatic β-cell function

Measurement of *in vivo* pancreatic β-cell function was determined by dividing measures of insulin action of total, 1st phase and 2nd phase glucose-stimulated insulin secretion, basal and stimulated insulin disposition by absolute β-cell mass and also by relative β-cell mass (β-cell mass as a percentage of bodyweight at post-mortem), to assess the β-cell functional secretory capacity in response to *in vivo* glucose stimulation (8).

2.4 *IN VITRO* ASSESSMENT OF PANCREATIC ISLET FUNCTION

2.4.1 Isolation and purification of pancreatic islets

Pancreatic islet isolation and purification were performed with a modification of previously described methods (178). Lamb pancreas (CON, n = 7, IUGR+Veh, n = 8 and IUGR+Ex-4, n = 8) was dissected, weighed and a large sample (~80% of total pancreas) quickly placed in ice cold Krebs-Ringer buffer, KRB (Sigma-Aldrich, NSW, Australia) with glucose. Cold pancreas were removed from buffer and rapidly homogenised with a scalpel blade, then 2.5 g of homogenised pancreatic tissue was incubated at 35ºC for 40 min with gentle shaking with 25 mL of collagenase solution (20 mg of collagenase (Sigma C9697) and 10 mg of DNAse (Sigma DN-25) dissolved in 50 ml of KRB without glucose, Sigma-Aldrich, NSW, Australia). The solution was then centrifuged at 50 *g* for 2 min at 4ºC and supernatant was discarded. Cell pellets were resuspended with KRB supplemented with 0.5 g of bovine serum albumin, KRB/BSA solution (Sigma-Aldrich, NSW, Australia) to stop the collagenase reaction. Aliquots (4 x 1 mL) were centrifuged and pellets were snap frozen in liquid nitrogen and stored at -80ºC for later RNA isolation. Additional aliquots (0.3 mL) were diluted with KRB/BSA (Sigma-Aldrich, NSW, Australia) and added to cytospin slides for later staining for insulin (refer 2.3.1.1). Other aliquots (2 mL) were cultured overnight at

37ºC in 95% O_2/5% CO_2 in 4 mL of RPMI 1640 media (Sigma-Aldrich, NSW, Australia). Pancreatic islet cultures were then washed with KRB/BSA (without glucose) (Sigma-Aldrich, NSW, Australia) and handpicked to obtain islets >100 µm in diameter for *in vitro* insulin secretion analysis (refer 2.4.2), with purity of islet isolations confirmed by immunostaining of islet aliquots as previously described (178).

2.4.2 *In vitro* insulin secretion

Static islet incubation and experiments were performed as previously described (172, 178). Briefly, for each animal and incubation condition, 0.25 mL of KRB/BSA/Forskolin solution (4 µl of 100 µM Forskolin in 20 mL of KRB/BSA solution and equilibrated with 95%O_2-5% CO_2) was added into 1.5 ml tubes in triplicates. Ten handpicked islets were added into each 1.5 mL tubes. Static incubations were performed at 37ºC for 1 h in 1.5 mL tubes containing either 0, 1.1, 11.1 mM glucose, or 15 mM KCl, or 11.1 mM glucose plus 5 mM lysine, 11.1 mM glucose plus 5 mM arginine, 1.1 mM glucose plus 10 mM leucine, or 11.1 mM glucose plus 10 µM epinephrine, and also at 0ºC for 1 h with 11.1 mM glucose. Islets were then centrifuged, supernatant collected for insulin analysis and DNA was ethanol-extracted from pellets and quantified by PicoGreen dsDNA Quantification kit (Invitrogen, Victoria, Australia). *In vitro* insulin secretion for each replicate was calculated as insulin concentration divided by DNA concentration. *In vitro* data for an animal was included in analyses provided that the insulin secretion in the incubations with KCl (test of maximal release) was greater than those obtained from incubations with epinephrine or at 0ºC (inhibitory quality controls). Due to technical difficulties with some preparations, *in vitro* insulin secretion data was obtained successfully for 5 CON, 5 IUGR+Veh and 6 IUGR+Ex-4 lambs.

2.5 PANCREATIC ISLET GENE EXPRESSION

2.5.1 Pancreatic islet RNA extraction

Lamb pancreatic islet RNA (CON, n = 6, IUGR+Veh, n = 8 and IUGR+Ex-4, n = 8) was extracted from 1 mL of isolated islets (refer 2.4.1) using miRNAeasy Mini Kit (Qiagen, Victoria, Australia) according to the manufacturer's recommendations. RNA was eluted using RNase free water and stored at -80ºC for later analysis.

2.5.2 RNA quantity and quality assessments

The quantity and purity of RNA in the sample was determined using a Nanodrop Spectrophotometer. Samples were considered to be sufficiently pure if the ratio of OD_{260}:OD_{280} was > 1.7. The quantity of RNA was calculated using the following equation: OD_{260} x 40 x Dilution factor = RNA concentration ($\mu g.mL^{-1}$). RNA integrity was further confirmed by gel electrophoresis in 1% Agarose gels (Progen Biosciences, Brisbane, Australia) and visualisation of 18S and 28S ribosomal sub-units as follows. Each sample (5 µL) was mixed with 2 µL of loading buffer and run with pre-stained 1% Agarose (Progen Biosciences, Brisbane, Australia) gel with 0.5 $mg.mL^{-1}$ Ethidium Bromide (Sigma-Aldrich, NSW, Australia) for 45 min at 90 V. RNA size marker, SPPI/EcoRI (GeneWorks, SA, Australia) was used to determine each product size. Bands were viewed under ultraviolet light and photographed using a digital camera (DC120, Kodak, Eastman, Rochester, USA). Sample with a 28S:18S ratio of approximately 2:1 was considered acceptable, and any degraded RNA sample was re-extracted.

2.5.3 DNAse treatment

RNA samples were DNAse-treated to remove DNA contamination using Turbo DNA-free ambion kit (Life Technologies, Victoria, Australia) with few modifications

from the manufacturer's recommendation. Briefly, aliquots of 10 µL containing 10 µg of RNA was mixed with 2 µL of 10X Turbo DNAse I buffer and 1 µL of DNAse I and incubated at 37ºC for 30 min. DNAse inactivation reagent (2.5 µL) was added, mixed by flicking and incubated at room temperature for 2 min with occasional flicking. Samples were centrifuged at 10 000 *g* for 2 min at room temperature and supernatants were transferred to new tubes (DNAse-treated RNA). Samples were then measured for quantity and quality (refer to 2.5.2).

2.5.4 Reverse transcription

DNAse-treated RNA samples were reverse-transcribed using Applied Biosystems Conventional Thermocycler 9700 (Applied Biosystem, Warrington, UK) according to the manufacturer's instructions. For each reverse transcription reaction, two negative controls were used; one was the RNA negative control where no RNA was added and the other was the MasterMix Negative control where no Mastermix was added. Samples were then stored at -20ºC for later analysis.

2.5.5 Primer design

Using Primer Express 2.0 Software (Applied Biosystems, California, USA) and GenBank DNA and mRNA sequences, oligonucleotide primers for sheep were designed within the mature peptide region using ovine or bovine sequence where possible (Table 2.1 and 2.2). Primers were designed such that the generated amplicon overlapped an exon-intron boundary, based on previously published gene sequences for most genes (8), and based on predicted gene boundaries for *PIK3CB, PIK3R1, DNMT1, DNMT3A* and *DNMT3B* genes. Desalted purity primers were constructed by Sigma Genosys (Sigma Genosys, NSW, Australia) and Invitrogen (Invitrogen, Victoria, Australia).

Table 2.1 Primer details and sequences used for quantitative Real Time PCR of islet gene expression.

Gene	Function	Species	Predicted amplicon size (bp)	Forward primer sequence 5' → 3'	Reverse primer sequence 5' → 3'	GenBank accession No.
ACTB	β-actin, reference gene	Ovine	157	atgtaccctggcatcgca	atccacatctgctggaaggtgg	U39357.1
PDX1	β-cell master regulator	Bovine	97	cagagcccggaggagaacaag	cctggagatgtasttgttgaaaagg	XM583722
IGF1	Regulates β-cell mass	Ovine	64	gcttccggagctgtgatctg	gacttggcggccttgaga	NM001009774
IGF2	Regulates β-cell mass and function	Ovine	84	ggcggggagctggtggaca	tcggtttatgcggctggatggt	M89788
IGF1R	Regulates β-cell mass	Ovine	104	aagaaccatgcctgcagaagg	ggattctcaggttctggccatt	AY162434
IGF2R	Regulates β-cell mass	Ovine	90	atgaagctggactacaggcatca	gctcgccgtcctcagtttc	AF327649
INS	Regulates β-cell mass and function	Ovine	199	gagagcgcggcttcttctac	gcgggccaggtctagttaca	U00659.1
INSR	Regulates β-cell mass and function	Ovine	74	gcttcgaggctgcaccat	agctcagctgccaggttgtt	AY157728
GCK	Rate limiting for glycosis	Bovine	115	aagaccacgcaccagatgtactc	cttcatctgatgcttatccaggaa	XM_868629
SLC2A2	Controls glucose uptake into β-cells	Ovine	134	cgaaattgggaccatctcacat	caccgatagcacccctgagt	AJ318925
CACNA1D	Subunit of voltage-gated Ca^{2+} channel	Bovine	65	ttggcaagctgcaatcga	ggtgcggaggtgctcatagt	XM876930
KCNJ11	Subunit of ATP-sensitive K^{+} channel	Human	107	gatgccaacagcccactctac	ggtgatgccctggtttc	NM000525
PIK3CB	β catalytic subunit of PI-3-kinase	Bovine	114	ggagagtagaatatgtgtttggt	atggcaaccatctcctgttcat	XM613754
PIK3R1	Regulatory subunit 1 of PI-3-kinase	Bovine	69	cttcaaaaactgaagcagacagtga	ggtgcgaactgctctgcaa	NM174575
DNMT1	Maintains DNA methylation patterns	Ovine	145	gcaagccccccaaacc	gccctgtgcaaaataagatgtg	NM001009473
DNMT3A	Provides *de novo* DNA methylation	Bovine	129	gggagagggtgccactga	ggattcgatgttggtctccttc	AY271298
DNMT3B	Provides *de novo* DNA methylation	Bovine	149	aatacgtggcctccgaagtg	ggcttccaccaatcaccaagt	NM181813

2.5.6 Quantitative real time polymerase chain reaction

2.5.6.1 Preparation of cDNA

Amplification of cDNA was carried out using the primers for the gene of interest, Platinum *Taq* DNA polymerase (Invitrogen, Victoria, Australia) and an Applied Biosystems Conventional Thermocycler 9700 (Applied Biosystem, Warrington, UK) as previously described (8). Briefly, 5 µL of cDNA was mixed with a PCR master mix of 20 µL containing 2.5 µL of 10X buffer, 0.75 µL of $MgCl_2$ (50mM), 0.5 µL of dNTPs (10 nM), 0.125 µL of *Taq* Polymerase (5 IU. μL^{-1}), 0.5 µL each of forward and reverse primers (25 µM) and 15 µL of molecular grade water. Samples were run at PCR conditions of 94ºC for 15 min, and 35 cycles (94ºC for 30 seconds, 55ºC for 30 seconds, 72ºC for 1 min, and 72ºC for 7 min). PCR products were then confirmed as producing a single product by gel electrophoresis in a 2% Agarose gel for 45 min at 85 V, and visualisation to confirm production of a single band of the expected size (refer 2.5.2 for gel details) with pUC19 (GeneWorks, SA, Australia) used as a size marker.

2.5.6.2 Cloning reaction and plasmid standard extraction

Confirmed PCR product was cloned into StrataClone PCR Cloning Kit (Integrated Sciences, NSW, Australia) according to the manufacturer's instructions. Briefly, 2 µL of PCR product was mixed gently with 3 µL of StrataClone cloning buffer and 1 µL of VectorMix amp/kan and incubated at room temperature for 5 min to allow the ligation of PCR product into vector plasmid. One µL of this ligation mixture was then mixed gently into thawed StrataClone SoloPack Competent Cells and incubated at 4ºC for 20 min. Transformation of the competent cells was induced by heat-shock at 42ºC at 45 seconds and followed by cooling at 4ºC for 2 min. Then, 250

µL of Luria-Bertani, LB broth (10 g of Sodium chloride, 10 g of Tryptone, 5 g of yeast extract dissolved in deionised water up to 1L of final volume) was added to the transformation mixture and incubated at 37°C for 1 h with mixing at 200 rpm horizontally for competent cells to recover and grow. Hundred µL of the transformation mixture was sterile-plated in LB plus Ampicilin agar plate with 40 µL of 2% 5-bromo-4-chloro-3-inodlyl-β-D-galactopyranoside, X-gal (Sigma Aldrich, NSW, Australia) and incubated overnight at 37°C. At least 5 successful transformed white colonies per plate were selected and cultured in LB broth with Ampicillin (3 mL) overnight at 37ºC with shaking (200 rpm, half-horizontally). To determine the correct colonies with the appropriate size inserts (plasmid and cDNA insert), 5 µL of each culture was mixed with 20 µL of PCR master mix (refer 2.5.6.1) with addition of M13 universal reverse and forward primers and run at same PCR conditions (refer 2.5.6.1). Plasmids that contained the correct cDNA insert were isolated from competent cells using NucleoSpin Plasmid (Macherey Nagel, Duren, Germany) according to the manufacturer's instructions. Clean plasmid DNA was quantitated using a NanoDrop Spectrophotometer (refer 2.5.2) and stored at -20ºC.

2.5.6.3 Sequencing of cDNA inserts

Successful isolation of clean plasmid DNA for each gene of interest (refer 2.5.6.2) was further confirmed by sequencing of cDNA inserts using Applied Biosystems Big Dye Terminator version 3.1 (Life Technologies, Victoria, Australia). Briefly, 10 µL aliquots containing 1 µL of Big Dye, 2.5 µL of dilution buffer, 2.8 µM of primers, 4 µL of clean plasmid DNA and 1.5 µL of molecular grade water were run at PCR conditions of 96ºC for 30 seconds, and 30 cycles (96ºC for 10 seconds, 50ºC for 5 seconds, 60ºC for 4 min) on an Applied Biosystems Conventional Thermocycler 9700

(Applied Biosystem, Warrington, UK). Isopropanol (80 µL of 75%) was added to the samples which were incubated at room temperature for 15 min and centrifuged at 12000 *g* for 20 min at room temperature prior to discarding of supernatant. Isopropanol (250 µL of 75%) was added again and samples were centrifuged at 12000 *g* for 20 min at room temperature with supernatants being discarded. The cell pellet was allowed to air dry at 90 ºC for 1 min. Samples were then transported to the IMVS (SA, Australia) for sequencing with an Applied Biosystems 3730. Sequencing results were analysed by Chromas Software Version 2.33 (Technelysium Pty Ltd, Brisbane, Australia) and Genbank sequences. Samples that were 98-100% similar to the published sequence of the gene of interest at Genbank sequences were stored at -20ºC and subsequently used as the Real Time PCR standards.

2.5.6.4 Real Time PCR

Quantitative Real Time PCR (RT PCR) was performed on a Corbett RotorGene Real Time PCR 6000 (Corbett Life Sciences, San Francisco, USA) using 5 Prime MasterMix SyBR Rox (Quantum Scientific, NSW, Australia) according to manufacturer's recommendations. Briefly, each Real Time PCR tube contained 5 µL of sample cDNA and 15 µL of PCR reaction mix (9 µL of 2.5 X RealMasterMix SyBR solution, 5.6 µL of molecular grade water, 0.2 µL each of forward and reverse primers). Real Time PCR reaction was held at 95ºC for 2 min, followed by 40 cycles of (95ºC for 15 seconds, melting at 72-95ºC for 1 second, 60ºC for 45 seconds). Each sample was analysed in triplicate and each RT PCR plate included appropriate plasmid standards for the gene of interest, ranging from 1 µg to 1 pg per reaction tube, a water control and 2 types of negative cDNA control (refer 2.5.4).

2.5.6.5 Optimisation of Real Time PCR reactions

The optimum concentrations of primers were determined by preliminary experiments with serial dilutions of primer pairs and the gene standard dilutions. A primer concentration of 12.5 µM was selected as it was the lowest primer concentration at which the cycle threshold (Ct) of the standard dilutions versus the log known concentration of the standards formed a linear plot with efficiency range from 0.95-1.15 and was different from water and negative control templates. The *ACTB* gene was selected as the reference gene for normalisation of pancreatic islet gene expression as its expression did not differ between treatment groups. The optimum cDNA dilution for each primer pair was determined in preliminary experiments with a range of cDNA concentrations. The cDNA concentration at which the Ct increase was linear and most different from the water and negative control was selected. It was determined that a 1 in 10 dilution was required for the reference gene, *ACTB* and 1 in 5 dilution was used for analysis of all other genes. The absence of primer dimers and alternative PCR products in the PCR products was confirmed by the presence of a single dissociation peak (melting curve) and confirmed by gel electrophoresis and visualisation on a 2% agarose gel (refer 2.5.2 for gel) for 45 min at 85 V and pUC19 (GeneWorks, SA, Australia) were used as a size marker.

2.5.6.6 Quantification of pancreatic mRNA levels

Green fluorescence was measured at the end of each cycle (excitation wavelength of 490 nm and an emission channel of 605 to 610 nm) and after 40 cycles, a profile of fluorescence versus the cycle number was obtained. A fluorescence threshold was determined by the linear exponential phase of amplification of the known standard dilutions when the efficiency of the reaction falls into the range of 0.95-1.15. The cycle

number at which the amplification of the product crossed this threshold was determined and designated as the cycle threshold (Ct). These Ct values were used to determine the absolute expression of the amplified PCR product using the linear equation of the standard curve in each reaction and multiplied by the dilution factor before averaging the values for sample triplicates. The relative expression of the gene of interest was determined by normalising the absolute expression of that gene to the expression of the reference gene, *ACTB*. The expression of *ACTB* did not differ between the treatment groups.

2.6 PANCREATIC ISLET MICRORNA EXPRESSION

2.6.1 Pancreatic microRNA extraction, quantity and quality assessments

MicroRNA was extracted together with RNA using the miRNAeasy Mini Kit (Qiagen, Victoria, Australia) according to manufacturer's recommendation as described above (refer 2.5.1). The quality and quantity assessments were performed together with those for RNA (refer 2.5.2).

2.6.2 Exiqon microarray labelling

RNA labelling for Exiqon Microarrays using T4 RNA ligase (New England Biolabs Inc., Massachusetts, USA) were performed as previously described (179). Six µL of aliquots of 3 µg pancreatic islet RNA were mixed with 3 µL of labelling mix containing 1 µL of 10X Iglori buffer (1 mM ATP, 500 mM HEPES (pH 7.8), 35 mM DTT, 200 mM $MgCl_2$, 100 mg/ml BSA), 1 µL of 100% DMSO, and 1 µL of Cy3 (5'-phosphate-cytidyl-uridyl-Cy3-3', 500 $ng.\mu L^{-1}$) or Cy5 (5'-phosphate-cytidyl-uridyl-Cy5-3', 500 $ng.\mu L^{-1}$) dye (Trilink Biotechnologies, San Diego, USA). One µL of T4 RNA ligase (20000 $IU.mL^{-1}$, New England Biolabs Inc., Massachusetts, USA) was then

added to the mixture. Samples were then incubated for 2 h at 4°C in the dark. The labelling reaction was stopped and RNA was precipitated using 46 µL of Stop solution containing of 40 µL of DEPC-treated molecular grade water, 5 µL of 3 M sodium acetate (pH 5.2) and 1 µL of glycogen (20 $mg.mL^{-1}$). Ethanol (150 µL of 100%) was added and samples were vigorously vortexed and allowed to sit at room temperature for 10 min. Samples were then centrifuged at 13000 rpm for 10 min at 4°C, and the supernatant was discarded. The RNA pellet was mixed with 150 µL of 75% ethanol and centrifuged at 13000 rpm for 5 min at 4°C, and the supernatant was again discarded. The RNA pellet was allowed to air dry briefly at 65°C before being dissolved in 25 µL of 1X hybridisation buffer (Exiqon, Vedbaek, Denmark).

2.6.3 Exiqon microarray hybridisation

All hybridisation samples were performed on an Exiqon miRNA array version 11 specific for human samples. Each microarray consists of 1700 antisense microRNA probes (miRCURY microRNA Array probe set, Exiqon, Vedbaek, Denmark), printed in triplicate on microarray slides (Corning, Massachusetts, USA) with a VersArray ChipWriter Pro system (BioRad, California, USA) using tungsten pins (PointTech, La Aurora de Heredia, Costa Rica). The antisense probes were designed from the miRNA sequences annotated in Sanger sequence miRBase library v11. Each array contained 12 positive control probes (U6-snRNA, an antisense sequence of U6-small nuclear spliceosomal RNA 1 to 12), and 7 negative control probes (hsa_negative_control 1 to 7, synthetic sequences that share no known homology with any mammalian microRNAs). A dye swap design was implemented to remove the effect of dye bias (180), with RNA from each treatment group labelled as described previously (179). Hybridisation buffer used to reconstitute RNA contained labelled RNA that was mixed to the matching RNA

paired samples with the opposite fluorescent dye to the treatment group. Pancreatic islet microRNA was analysed between treatment groups of same sex for the effect of IUGR (CON *cf*. IUGR+Veh) and effect of exendin-4 treatment (IUGR+Veh *cf*. IUGR+Ex-4).

Before the hybridisation process, the position of the microarray was scored with a diamond cutter and the array was blocked in hot molecular grade water for 30 seconds, and allowed to spin-dry for 5 min at 500 rpm. The hybridisation buffer containing the labelled RNA was heated at 95ºC for 3 min and loaded (25 µL) to the array in Corning hybridization chambers. Seventeen µL of 4X salt buffer (Exiqon, Vedbaek, Denmark) was added to each chamber and microarrays were incubated overnight at 60°C in the dark. Microarray slides were then washed once with pre-warmed Buffer A (5 mL of 20X salt buffer, 44 mL of DEPC-treated molecular grade water) at 60°C, and 1 mL of 10% detergent solution (Exiqon, Vedbaek, Denmark) for 2 min, two washes with Buffer B (2.5 mL of 20X salt buffer and 47.5 mL of DEPC-treated molecular grade water) for 2 min at room temperature and 1 wash with Buffer C (0.5 mL of 20X salt buffer and 49.5 mL of DEPC-treated molecular grade water) for 2 min at room temperature. Microaray slides were then dried by centrifuging at 1000 rpm for 5 min and scanned at 10 µm resolution with a GenePix 4000B Scanner (Molecular Devices, California, USA) using photomultiplier tube settings to balance the channel histograms.

2.6.4 Exiqon miRNA data analysis

All Exiqon Microarray data were extracted with GenePix Pro 6.0 (Molecular Devices, California, USA) which was used to produce scanned images for each array. The foreground and background pixel intensities were extracted from the scanned images and processed with Spot v.3 plugin (CSIRO, SA, Australia) using R software

(The R Foundation for Statistical Computing, Vienna, Austria). Mean intensities were log^2 transformed to produce a single Cy5/Cy3 ratio for each probe on the microarray. The ratio values were normalised and the replicate arrays were normalised to each other to give similar ranges of miRNA expression values and raw P-values. Raw P-values were then adjusted using the Benjamin-Hochberg method (181) to control for false discovery rate. Adjusted P-values below 0.05 were considered statistically significant.

2.7 ANALYSIS OF ADIPONECTIN ABUNDANCE AND EXPRESSION

2.7.1 Plasma adiponectin abundance

Although there is a diurnal variation of adiponectin concentration in humans (182), plasma adiponectin concentrations do not vary diurnally in horses (183). Because of this and limitations on blood volume that could be taken from each lamb, we measured adiponectin in only a single sample from each sheep at a given age, and all samples were collected at similar times of day (0800h – 1000h). Routine blood samples were taken at birth (within 24 h after birth) and at 6, 11 and 15 d of ages in non-fasted lambs and were centrifuged at 4000 rpm for 10 min at 4ºC to collect plasma for subsequent measurements. Plasma adiponectin levels were measured using a commercially available sheep adiponectin (ADIPOQ) ELISA kit (MyBioSource, California, USA). The minimum detectable concentration was 3.13 $ng.mL^{-1}$ and the intra- and inter- assay coefficients of variation for the ELISA assay were 1.9% and 2.3% respectively. Due to loss of CON samples, data for plasma adiponectin concentrations was successfully obtained for 3 CON, 8 IUGR+Veh and 8 IUGR+Ex-4 lambs.

2.7.2 Adipose tissue RNA extraction, quantity and quality assessments

RNA was extracted from omental and subcutaneous adipose tissues (CON, n = 6, IUGR+Veh, n = 8 and IUGR+Ex-4, n = 8) using a Precellys®24 Lyse machine (Bertin Technologies, Montigny le Bretonneux, France) and a modified Trizol-based approach for adipose tissues (8, 184). For every 0.1 g of adipose tissue, 1 mL of Trizol (Invitrogen, Victoria, Australia) was added and samples were homogenized with 0.58 g of 1.4 mm ceramic beads (Bertin Technologies, Montigny le Bretonneux, France) with Precellys®24 at 3000 rpm (2 cycles x 10 seconds with 10 second interval) at 4ºC. Samples were then incubated for 5 min on ice to allow complete dissociation of nucleoprotein complexes. Chloroform (0.2 mL) was added; samples were vigorously shaken for 15 seconds and again incubated on ice for 3 min, before centrifuging for 30 min at 12000 *g* at 4 ºC. The aqueous phase was transferred to new collection tubes and mixed by inversion with 0.5 mL of isopropyl alcohol. Samples were allowed to precipitate overnight at -20ºC, and then centrifuged at 12000 *g* for 40 min at 4ºC to pellet the RNA. The supernatant was removed and the RNA pellet was washed twice with 1 mL of 75% ethanol and re-pelleted by centrifugation at 12000 *g* for 30 min at 4ºC. The supernatant was removed and the RNA pellet was allowed to air dry for 15 min and then was dissolved in 25 µL of molecular grade water and stored at -80ºC for later analysis. The quality and quantity assessments of omental and subcutaneous adipose tissue RNA samples were performed as described previously for pancreatic islet RNA (refer 2.5.2).

2.7.3 DNAse treatment and cDNA synthesis

Omental and subcutaneous adipose tissues RNA samples were DNAse-treated to remove contamination with DNA using Turbo DNA-free ambion kit (Life

Technologies, Victoria, Australia). Briefly, aliquots of 10 µL containing 10 µg of RNA was mixed with 2 µL of 10X Turbo DNAse I buffer and 1 µL of DNAse I and incubated at 37ºC for 30 min. DNAse inactivation reagent (2.5 µL) was added, mixed and incubated at room temperature for 2 min with occasional mixing. Samples were centrifuged at 10000 *g* for 2 min at room temperature and supernatants were transferred to new tubes (DNAse-treated RNA). Samples were then measured for quantity and quality (refer to 2.5.2). DNAse-treated RNA samples were reverse-transcribed to cDNA using an Applied Biosystems Conventional Thermocycler 9700 (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. For each reverse transcription reaction, two negative controls were used; one was the RNA negative control where no RNA was added and the other was the Mastermix negative control where no Mastermix was added. Samples were stored at -20ºC for later analysis.

2.7.4 Adiponectin gene expression

Adiponectin cDNA was amplified using previously published bovine primer sequences (185), listed in Table 2.2, and Platinum *Taq* DNA polymerase (Invitrogen, Victoria, Australia) and Applied Biosystems Conventional Thermocycler 9700 (Applied Biosystems, Warrington, UK) (refer 2.5.6.1). Adiponectin cDNA was cloned into StrataClone PCR Cloning Kit (Intergrated Sciences, NSW, Australia) to generate Real Time PCR standards as previously described (refer 2.5.6.2 to 2.5.6.3).

Table 2.2 Primer sequences used for Real Time PCR for adiponectin expression in omental and subcutaneous adipose tissues.

Gene	Species	Predicted amplicon size (bp)	Primer sequence 5' → 3'	GenBank accession No.
β-actin, *ACTB*	Ovine	157	Forward : atgtaccctggcatcgca Reverse : atccacatctgctggaaggtgg	U39357.1
Adiponectin *ADIPOQ*	Bovine	203	Forward : ctggagagaagggagagaaag Reverse : tgggtacattgggaacagtg	NM174742

2.7.5 Quantification of adiponectin mRNA

The expression of adiponectin mRNA in omental and subcutaneous adipose tissues was measured by Real Time PCR (refer 2.5.8) using 1 in 5 dilution of cDNA (refer 2.5.9). The relative expression of adiponectin gene was determined by normalising the absolute expression of adiponectin gene to the expression of the reference gene, *ACTB*. The expression of *ACTB* did not differ between treatment groups.

2.8 STATISTICAL ANALYSES

Data for non-repeated measures on each animal were analysed by the mixed models procedure in SPSS for effects of treatment (fixed effect) and including dams as a random (block) effect in the model to account for common maternal environment in twins. Where treatment effects or trends were apparent ($P < 0.1$), means were compared by the LSD method, based on a *priori* questions to determine: 1. effects of IUGR (CON *cf*. IUGR+Veh groups), 2. effects of exendin-4 in IUGR lambs (IUGR+Veh *cf*.

IUGR+Ex-4 groups), and 3. whether exendin-4 restored outcomes in IUGR lambs to control levels (CON *cf*. IUGR+Ex-4 groups). Neonatal growth patterns, glucose and insulin concentrations, and insulin: glucose ratios overall, and during the 1st (0-30 min) and 2nd phases (30-210 min) of insulin secretion during the IVGTT were analysed by repeated measures ANOVA for effects of treatment (between factor), time (within factor) and interactions, and including dams as a random (block) effect in the model to account for common maternal environment in twins. Glucose-stimulated *in vitro* insulin secretion was analysed by repeated measures ANOVA for effects of treatment (between factor), glucose concentration (within factor) and interactions. Stimulation and inhibition of *in vitro* insulin secretion were analysed using repeated measures ANOVA for effects of treatment (between factor), stimulation (within factor, 11.1 mM glucose *cf*. KCl) or inhibition (within factor, 11.1 mM glucose *cf*. epinephrine and control at 0°C) and interactions, and by mixed model analysis as described above for incubations with individual secretagogues. Plasma adiponectin measured at multiple ages was analysed by repeated measures ANOVA for effects of treatment (between factor), age (within factor) and interactions, and including dams as a random (block) effect in the model to account for common maternal environment in twins. Relationships between *in vivo* measures, *in vitro* measures, gene expression and other outcomes were analysed by Pearson's correlation. Due to limited numbers of females in each treatment group, it was not possible to analyse outcomes on a sex-specific basis or to include sex as a factor in models. In our previous studies of PR lambs at similar ages, postnatal β-cell mass and function, insulin secretion and action did not differ between sexes in the first month of life (32).

CHAPTER 3

STATEMENT OF AUTHORSHIP

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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Siti Aishah Sulaiman		
Contribution to the paper	Assisted with or performed in vivo and in vitro experiments including surgery, glucose tolerance tests and hyperinsulinaemic clamps and islet insulin secretion studies. Analysed plasma samples for insulin and performed all immunohistochemical staining and counting of pancreas sections. Conducted statistical analysis of data and contributed to data interpretation, wrote first draft of manuscript. Edited manuscript and approved final version for submission.		
Signature		Date	20/8/14

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Contribution to the paper	Obtained external grant funding for project, designed and managed study, performed catheterisation surgeries and assisted with in vivo experiments. Trained students in required techniques, including data analysis and contributed to data interpretation and first draft of manuscript. Wrote final manuscript version with input from all coauthors. Approved final version for submission.		
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Contribution to the paper	Contributed to experimental design and funding application. Assisted in data analysis and interpretation. Edited manuscript and approved final version for submission.		
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3 CHAPTER 3 *IN VIVO* AND *IN VITRO* OUTCOMES

3.1 ABSTRACT

Background: IUGR increases the risk of type 2 diabetes mellitus (T2DM) in later life, due to reduced insulin sensitivity and impaired adaptation of insulin secretion. In IUGR rats, development of T2DM can be prevented by neonatal administration of the GLP1 analogue exendin-4. We therefore investigated effects of neonatal exendin-4 administration on insulin action and β-cell mass and function in the IUGR neonate in the sheep, a species with a more developed pancreas at birth.

Methods: Twin IUGR lambs were injected s.c. daily with vehicle (IUGR+Veh, n=8) or exendin-4 (1 nmol.kg^{-1}, IUGR+Ex-4, n=8), and singleton control lambs were injected with vehicle (CON, n=7), from d 1 to 16 of age. Glucose-stimulated insulin secretion and insulin sensitivity were measured *in vivo* during treatment (d 12-14). Body compositions, β-cell mass and *in vitro* insulin secretion of isolated pancreatic islets were measured at d 16.

Principal Findings: IUGR+Veh did not alter *in vivo* insulin secretion or insulin sensitivity or β-cell mass, but, increased glucose-stimulated insulin secretion *in vitro*. Exendin-4 treatment of the IUGR lamb impaired glucose tolerance *in vivo*, reflecting reduced insulin sensitivity, and normalised glucose-stimulated insulin secretion *in vitro*. Exendin-4 also reduced neonatal growth and visceral fat accumulation in IUGR lambs, known risk factors for later T2DM.

Conclusions: Neonatal exendin-4 induces changes in IUGR lambs that might improve later insulin action. Whether these effects of exendin-4 lead to improved insulin action in adult life after IUGR in the sheep, as in the PR rat, requires further investigation.

3.2 INTRODUCTION

Small size at birth or intrauterine growth restriction (IUGR) consistently predicts increased risk of type 2 diabetes mellitus (T2DM) in human studies (1, 2), including independent of gestation length (42). This relationship is consistent and significant, with ~18% of the lifetime risk of T2DM accounted for by poor growth before birth (28). Impaired insulin sensitivity and inadequate insulin secretion are each implicated as contributing to this increased risk of T2DM in the IUGR human (1, 6, 7, 50).

Poor fetal growth commonly reflects restricted fetal supply of oxygen and nutrients due to impaired placental growth and/or function (31). In the sheep, surgically-induced restriction of placental growth (PR) from before mating, and small size at birth, increase insulin sensitivity in early neonatal life in association with catch-up growth and increased fat deposition (141, 176). PR nevertheless impairs glucose-stimulated insulin disposition before weaning at 1 month of age, and this progresses to impaired insulin sensitivity and blunted basal and glucose-stimulated insulin disposition in young adult males at 1 year of age (8, 32). Impaired β-cell function is the primary cause of this inadequate insulin secretion, which occurs despite increases in β-cell mass in 1-year-old males (8). Similarly, PR late in pregnancy in rats produces progeny with normal circulating glucose and insulin levels at 1 week of age, but mild fasting hyperglycaemia and hyperinsulinaemia at 7–10 weeks and frank diabetes by 26 weeks (3, 4). Impaired β-cell function with later reduction in β-cell mass is also implicated in decreased insulin secretion in the PR rat postnatally (3, 4). Excitingly, administration of the GLP1 analogue exendin-4 to neonatal PR rats normalised subsequent β-cell mass and insulin secretion and prevented later development of T2DM (9). Prevention of T2DM by neonatal exendin-4 treatment in PR rats is at least partially due to induction and normalisation of expression of the transcription factor *PDX1* (9,

33), which regulates β-cell function as well as adaptive increases in β-cell mass (66, 67), and is epigenetically down-regulated in PR rat progeny (21).

The timing of pancreatic development and maturation of β-cell function, and therefore developmental stages of exposure to IUGR and neonatal interventions, differs between species. In humans and sheep, most pancreatic development takes place before birth, with β-cells present by 0.25 gestation, islets present in mid-gestation and substantial remodelling to a mature endocrine pancreas by near term (10-13). In both species, β-cell function is present and matures from mid-gestation onwards (14-17). This functional maturation in humans and sheep may be driven in part by their pre-partum surge in cortisol. In contrast, rodents undergo later development of β-cells than sheep or humans, with β-cells first appearing in late gestation (0.6) and pancreatic remodelling at ~10-17 d postnatal age (18-20). Neonatal surges in corticosterone and β-cell maturation in rodents are marked by increased expression of key molecular determinants of glucose-induced insulin secretion coupling (186) and mitochondrial enzymes of the NADH shuttle, essential for stimulation of insulin secretion by oxidative metabolism (187). Exendin-4 may in part be effective in preventing PR programming of reduced β−cell mass and function in rodents, because it occurs before and during such maturation. In the present study, we have therefore treated neonatal IUGR sheep with exendin-4 and assessed whether it is able to induce changes in growth, insulin action and β-cell mass and function after IUGR in a species in which the pancreas undergoes most maturation before birth.

3.3 MATERIALS AND METHODS

3.3.1 Ethics statement

All procedures in this study were approved by the University of Adelaide Animal Experimentation and Ethics Committee (approval M-84-2007) and complied

with the Australian code of practice for the care and use of animals for scientific purposes (171).

3.3.2 Animal, treatments and surgery

Australian Merino ewes underwent a timed-mating program, and pregnancies were confirmed by ultrasound scanning at ~60 d gestational age (term ~ 150 d). Delivery occurred naturally at term and the lambs were housed in floor pens with their mothers throughout the study and allowed to suckle freely, with access to their mother's feed and water, except during experimental protocols as described below. Natural twinning was used to induce IUGR. Sibling twin lambs were injected with vehicle (0.5% methanol in 0.9% saline s.c., IUGR+Veh) or exendin-4 (1 nmol.kg^{-1} s.c., IUGR+Ex-4, n = 8), with the first twin pair randomly allocated to treatments and then the heavier and lighter birth weight twin alternately allocated in order to balance birth weights between the two treatments. Exendin-4 (Bachem, Buberndorf, Germany) was prepared as a 5 nM stock in 0.5% methanol and 0.9% saline, and stored at -20ºC in single use aliquots, which were thawed immediately prior to injection. Singleton lambs were injected daily with vehicle (CON, n = 7). All lambs (singletons and twins) were supplemented with whey protein (Resource Beneprotein instant protein powder, Nestle, Australia) given orally in two equal feeds (at 0900 – 1000 h and 1600 – 1700 h), commencing at 1.25 g.kg^{-1}.d^{-1} on d 4 and increasing to 5 g.kg^{-1}.d^{-1} on and after d 7. Feeding this supplement during this period of maximal catch-up growth in IUGR lambs (176) was intended to minimise the potential for limitation of neonatal growth by milk availability in twins (174) by providing ~25% of the protein expected to be available through milk, and allowing lambs to self-regulate their milk intake to appetite. On d 4, catheters were inserted into the lamb's femoral artery and vein under general anaesthesia, induced and maintained by fluothane inhalation anaesthetic, as described

previously (176). Basal blood samples were collected from arterial catheters every second morning before supplement feeding. Lambs were weighed at birth and then every 2 d throughout the study. Lamb size was measured at birth and then every 4 d, and absolute (AGR) and fractional (FGR) growth rates from birth to d 16 fitted by linear regression (176).

3.3.3 *In vivo* measures of insulin secretion, sensitivity, and action.

Glucose tolerance and glucose-stimulated insulin secretion were measured during an intravenous glucose tolerance test (IVGTT) at d 14, and indices of glucose tolerance and insulin secretion calculated as described previously (32, 176, 177). The whole body insulin sensitivity of glucose metabolism was measured by hyperinsulinaemic euglycaemic clamp at d 12 (177). Insulin $sensitivity_{glucose}$, the metabolic clearance rate (MCR) of insulin, basal and maximal post-hepatic insulin delivery rates, and basal and maximal insulin disposition indices (IDI) were calculated as described previously (177).

3.3.4 Analysis of plasma insulin and metabolites.

Plasma insulin concentrations were measured in duplicate by a double antibody, solid phase radioimmunoassay using a commercially available kit (Human insulin-specific RIA, HI-14K, Linco Research Inc., St Charles, MO, USA), which has 100% cross-reactivity with ovine insulin. The intra-assay coefficients of variation (CV) for the insulin assay were 7.2% and 5.3%, and inter-assay CV were 7.0% and 19.6% for QC samples containing 9.9 and 35.9 $mU.L^{-1}$ insulin respectively (n=10 assays). Plasma glucose concentrations were measured by colorimetric enzymatic analysis on a Hitachi 912 automated metabolic analyser using Roche/Hitachi Glucose/HK kits (Roche Diagnostics GmbH, Mannheim, Germany).

3.3.5 Post-mortem

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

3.3.6 Pancreas and islet isolation and morphometric analysis

Each pancreas was rapidly dissected and weighed. Representative mixed aliquots were fixed for 48 h in 4% paraformaldehyde before embedding in paraffin wax. One section per block was immune-stained to detect insulin-positive cells, and morphometric analysis of β-cells was performed as described previously, in 20 fields of view per sheep selected by random-systematic sampling (8). Measures of *in vivo* β-cell function were calculated by dividing total, 1st phase and 2nd phase glucose-stimulated insulin secretion and basal and maximal IDI by β-cell mass. Pancreatic islets were obtained by collagenase digestion of pancreas at 35ºC for 40 min, washing and handpicking of islets >100 μm in diameter, with purity confirmed by immunostaining of aliquots as previously described (178). Islet aliquots were cultured overnight at 37ºC in 95% O_2/5% CO_2 in RPMI 1640 media (Sigma Aldrich, Sydney, Australia).

3.3.7 *In vitro* β-cell secretion and responses

Static islet incubation and experiments were performed as previously described (178). Briefly, for each animal and incubation condition, triplicate preparations of 10 islets were handpicked into 1.5 mL tubes. Static incubations were performed at 37ºC for 1 h in KRB/BSA/Forskolin media containing 0, 1.1, 11.1 mM glucose, or 15 mM

KCl, or 11.1 glucose plus 5mM Lysine, 11.1 glucose plus 5mM Arginine, 1.1 glucose plus 10mM Leucine, 11.1 glucose plus 10μM Epinephrine, or at 0ºC for 1 h in KRB/BSA/Forskolin media containing 11.1 glucose. Islets were then centrifuged, supernatant collected for insulin analysis and DNA was ethanol-extracted from pellets and quantified by PicoGreen dsDNA Quantification kit (Invitrogen, Melbourne, Australia). *In vitro* insulin secretion for each replicate was calculated as insulin concentration divided by DNA concentration. *In vitro* data for an animal was included in analyses provided that insulin secretion in incubations with KCl (test of maximal release) was greater than those obtained from incubations with epinephrine or at 0ºC (inhibitory quality controls). Due to technical difficulties with some preparations, *in vitro* insulin secretion data was obtained successfully for 5 CON, 5 IUGR+Veh and 6 IUGR+Ex-4 lambs.

3.3.8 Statistical analysis

Data for non-repeated measures on each animal were analysed by the mixed models procedure of SPSS for effects of treatment (fixed effect) and including dam as a random (block) effect in the model to account for common maternal environment in twins. Where treatment effects or trends were apparent ($P < 0.1$), we then compared means by the LSD method, based on a priori questions to determine: 1. effects of IUGR (CON *cf*. IUGR+Veh groups), 2. effects of exendin-4 in IUGR lambs (IUGR+Veh *cf*. IUGR+Ex-4 groups), and 3. to assess whether exendin-4 restored values to those of controls (CON *cf*. IUGR+Ex-4 groups). We also confirmed these comparisons between IUGR+Veh and IUGR+Ex-4 groups using a paired t-test to compare twin siblings, and the significance of this test was consistent with that for LSD comparisons for all measures (data not shown). Neonatal growth patterns and glucose, insulin and insulin:glucose ratios overall and during 1st phase (0-30 min) and 2nd phase (30-210

min) of insulin secretion during the IVGTT were analysed by repeated measures for effects of treatment (between factor), time (within factor) and interactions, and including dam as a random (block) effect in the model to account for common maternal environment in twins. Glucose-stimulated in vitro insulin secretion was analysed by repeated measures for effects of treatment (between factor), glucose concentration (within factor) and interactions. Stimulation and inhibition of in vitro insulin secretion were analysed using repeated measures models for effects of treatment (between factor), stimulation (within factor, 11.1 mM glucose or KCl) or inhibition (within factor, 11.1 mM glucose or epinephrine) and interactions, and by mixed model as described above for incubations with individual secretagogues.

3.4 RESULTS

3.4.1 Size at birth, neonatal growth and body composition

Lamb weight, abdominal circumference and body mass index at birth were reduced in twin lambs (all IUGR groups) compared to singleton lambs (each $P<0.001$, Table 3.1). Absolute and fractional growth rates for weight and abdominal circumference, and absolute but not fractional growth rate for shoulder height, differed with treatment (Table 3.1). IUGR+Veh lambs had higher FGR for weight and abdominal circumference than CON lambs ($P = 0.022$ and $P = 0.001$ respectively), and by d 16, there was no difference in weight between these two groups (Figure 3.1A). In control and IUGR+Veh lambs, FGR for weight increased as birth weight decreased (combined: $R = -0.700$, $P = 0.002$, $n = 15$; Figure 3.1B), whereas in IUGR+Ex4 lambs, neonatal FGR was not related to birth weight ($P > 0.3$; Figure 3.1B). Neonatal exendin-4 treatment reduced neonatal growth rates (Table 1) including for weight (AGR_{weight}, -35%, $P < 0.001$), linear growth ($AGR_{shoulder\ height}$, -20%, $P = 0.031$), and organ growth ($AGR_{abdominal\ circumference}$, -30%, $P = 0.007$), and this group were lighter than CON and

IUGR+Veh lambs at d16 (Figure 3.1). Neonatal exendin-4 reduced body weight (-18%, P = 0.016) and relative visceral fat mass (-57%, P < 0.001) at post-mortem compared to IUGR+Veh lambs (Table 3.1). IUGR+Ex-4 lambs had lower absolute liver weights than CON (-28%, P = 0.001) or IUGR+Veh (-25%, P = 0.009) lambs, and lower relative liver weights (as a proportion of body weight) than IUGR+Veh lambs (-9%, P = 0.021). Absolute summed muscle mass was lower in IUGR+Veh lambs (-3.7%, P = 0.017) relative to CON, and was decreased by exendin-4 treatment relative to CON (-27%, P < 0.001) and IUGR+Veh (-25%, P = 0.004) groups. Relative summed muscle weight also tended to be lower in IUGR+Veh (-7.6%, P = 0.093) and was lower in IUGR+Ex-4 (-9.5%, P = 0.019) compared to CON lambs (Table 3.1).

Table 3.1 Effect of IUGR and neonatal exendin-4 treatment on size at birth, postnatal growth and body composition in young lambs.

Neonatal growth rates are from d 0 to 16. NS: P > 0.1, * different from CON (P < 0.05), ^ different from IUGR+Veh (P < 0.05).

	CON	IUGR+Veh	IUGR+Ex-4	Significance (treatment effect)
Number of animals	7	8	8	
Size at birth				
Birth weight (kg)	6.01 ± 0.21	4.82 ± 0.17*	4.84 ± 0.15*	< 0.001
Crown rump length (cm)	56.3 ± 1.4	54.6 ± 1.1	55.1 ± 1.1	NS
Shoulder height (cm)	44.0 ± 0.7	40.1 ± 0.8*	40.9 ± 0.7*	0.008
Abdominal circumference (cm)	40.1 ± 0.4	35.1 ± 0.9*	36.1 ± 0.6*	< 0.001
Body mass index ($kg.m^{-2}$)	19.2 ± 1.1	16.3 ± 0.8*	16.0 ± 0.7*	0.040
Neonatal Growth				
AGR_{weight} ($g.day^{-1}$)	309 ± 29	327 ± 14	211 ± 17*^	0.001
FGR_{weight} ($\%.day^{-1}$)	5.17 ± 0.48	6.86 ± 0.39	4.35 ± 0.32^	0.001
$AGR_{shoulder\ height}$ ($cm.day^{-1}$)	0.390 ± 0.027	0.507 ± 0.037*	0.403 ± 0.038^	0.030
$FGR_{shoulder\ height}$ ($\%.day^{-1}$)	0.89 ± 0.06	1.17 ± 0.19	1.00 ± 0.11	NS
$AGR_{abdominal\ circumference}$ ($cm.day^{-1}$)	0.473 ± 0.075	0.782 ± 0.042*	0.544 ± 0.048^	0.002
$FGR_{abdominal\ circumference}$ ($\%.day^{-1}$)	1.18 ± 0.19	2.25 ± 0.17*	1.52 ± 0.15^	0.001
Postmortem (d 16)				
Body weight (kg)	11.0 ± 0.5	10.1 ± 0.3	8.33 ± 0.25*^	< 0.001
Total liver weight (g)	296 ± 19	285 ± 17	214 ± 7*^	0.002
Total liver weight (% of body weight)	2.70 ± 0.10	2.82 ± 0.14	2.57 ± 0.07^	NS
Summed muscle mass (g)	265 ± 13	228 ± 8*	183 ± 9*^	< 0.001
Summed muscle mass (% of body weight)	2.42 ± 0.07	2.26 ± 0.04	2.19 ± 0.08*	0.055
Visceral fat (g)	132 ± 19	118 ± 11	41.7 ± 6.3*^	< 0.001
Visceral fat (% of body weight)	1.19 ± 0.17	1.16 ± 0.09	0.495 ± 0.062*^	< 0.001

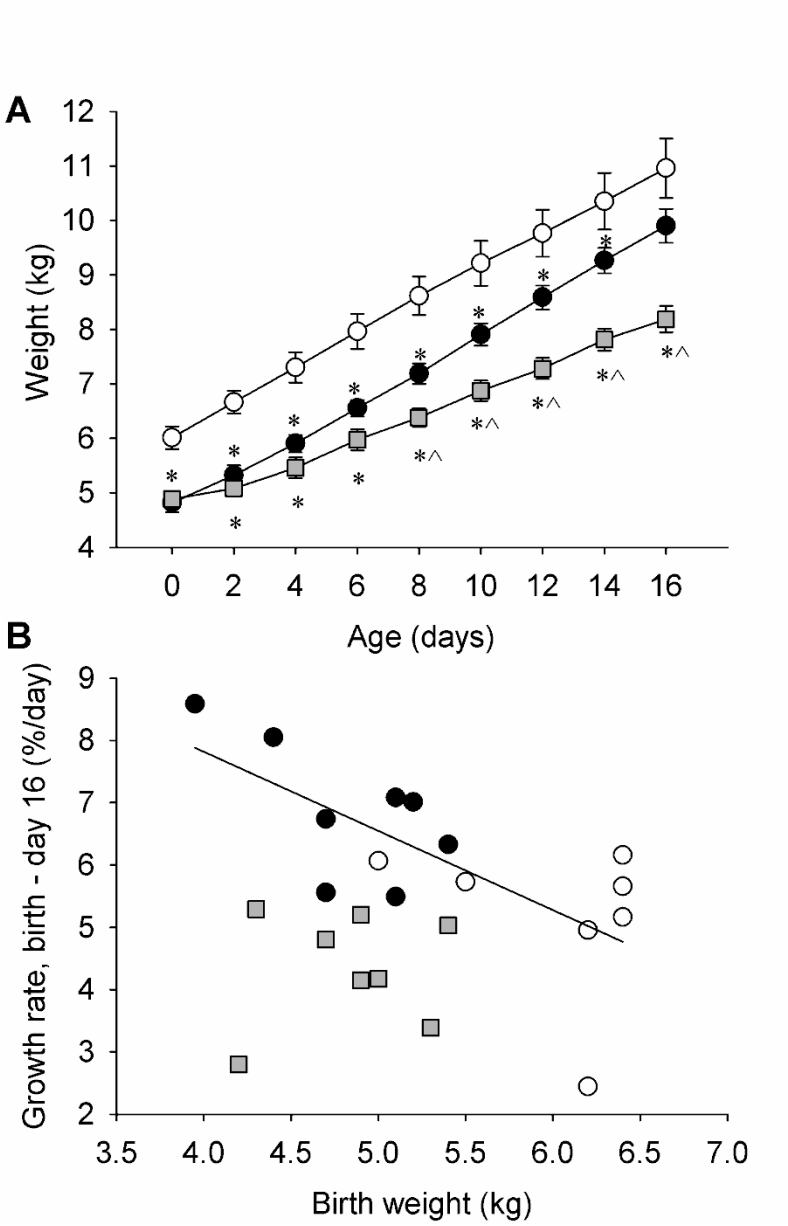

Figure 3.1 Effect of IUGR and neonatal exendin-4 treatment on (A) lamb growth and (B) relationships between birth weight and neonatal fractional growth rate.

CON (white circle) and IUGR+Veh (black circle) lambs were treated once daily with vehicle (0.5 % methanol in saline s.c.) and IUGR+Ex-4 (grey square) lambs were treated once daily with exendin-4 (1 nmol.kg^{-1} s.c.). Data in Figure 1A are means ± SEM, and data in Figure 1B are individual animal outcomes. * different from CON ($P < 0.05$), ^ different from IUGR+Veh ($P < 0.05$).

3.4.2 Insulin secretion, sensitivity and action

Fasting glucose and insulin levels, glucose tolerance and overall, 1st phase and 2nd phase insulin secretion *in vivo* were similar in IUGR+Veh and CON lambs (each P > 0.1, Table 3.2). Fasting plasma glucose (d 14) was reduced in IUGR+Ex-4 lambs compared to CON (-10%, P = 0.022) and IUGR+Veh lambs (-9%, P = 0.019, Table 3.2). Conversely, glucose tolerance was impaired (increased glucose AUC) in IUGR+Ex-4 lambs overall (+132%, +156% respectively), during first phase insulin secretion (+41%, +57%), and during second phase insulin secretion compared to CON and IUGR+Veh lambs (each P ≤ 0.02, Table 3.2). Across the whole of the IVGTT, and within the 1st phase of insulin secretion, plasma glucose (Figure 3.2) changed with time (each P < 0.001). Fasting plasma glucose in fasting samples was lower in IUGR+Ex4 than in IUGR+Veh lambs (P < 0.001), and tended to be lower in IUGR+Ex4 than in CON lambs (P = 0.091). Conversely, plasma glucose during the 1st phase of insulin secretion was higher in IUGR+Ex4 than in IUGR+Veh lambs (P < 0.001), and plasma glucose during the 2nd phase of insulin secretion did not differ between groups (P > 0.3). The pattern of change in plasma glucose with time differed between groups overall (P < 0.001) and during the 1st phase of insulin secretion (P = 0.003). Fasting plasma insulin in absolute terms and relative to glucose, and insulin secretion (assessed relative to the glucose stimulus as AUC insulin/AUC glucose) did not differ between the groups (Table 3.2 and Figure 3.2). Plasma insulin (Figure 3.2) changed with time throughout the IVGTT (P < 0.001), and within 1st (P < 0.001) and 2nd phase (P = 0.008) of insulin response. The ratio of plasma insulin to glucose (Figure 3.2), an index of insulin secretion, similarly changed with time throughout the IVGTT (P<0.001), and within 1st (P = 0.015) and 2nd phase (P = 0.005) of insulin response. Plasma insulin concentrations and the ratio of plasma insulin to glucose ratios during the IVGTT

(Figure 3.2) were higher in IUGR+Ex4 than in IUGR+Veh lambs overall (each $P < 0.001$) and during the 2nd phase of insulin secretion (each $P < 0.001$), and did not differ between other treatment groups. IUGR+Ex-4 lambs had lower insulin sensitivity compared to CON (-44%, $P = 0.004$) and IUGR+Veh lambs (-46%, $P = 0.002$, Table 3.2). Basal and maximal insulin disposition indices did not differ between groups (Table 3.2).

3.4.3 Pancreas morphology and β-cell function

Absolute and relative pancreas weights, and numbers of β-cells per islet, β-cell volume density and absolute β-cell mass did not differ with treatment (Table 3.3). β-cell mass relative to body weight was greater in IUGR+Ex-4 lambs than CON lambs (+36%, $P = 0.039$, Table 3.3). IUGR+Ex-4 lambs also tended to have higher relative β-cell mass than IUGR+Veh lambs (+28%, $P = 0.083$, Table 3.3). Measures of β-cell function did not differ between treatments (Table 3.3).

Table 3.2 Effect of IUGR and neonatal exendin-4 treatment on insulin secretion and action in young lambs.

Glucose and insulin AUC were measured during an IVGTT (0.25 g glucose.kg^{-1}) at d 14. 1st and 2nd phase values for insulin and glucose were measured from 0-30 and from 30-210 min after glucose administration, respectively. Insulin sensitivity was measured during a hyperinsulinaemic euglycaemic clamp (2 mU insulin.kg^{-1}.min^{-1}) at d 12. NS: P > 0.1, * different from CON (P < 0.05), ^ different from IUGR+Veh (P < 0.05).

	CON	IUGR+Veh	IUGR+Ex-4	Significance (treatment effect)
Number of animals	7	8	8	
Fasting				
Plasma glucose (mmol.L^{-1})	6.47 ± 0.26	6.40 ± 0.11	5.81 ± 0.12*^	0.008
Plasma insulin (mU.L^{-1})	20.4 ± 6.0	15.4 ± 2.2	16.4 ± 2.2	NS
Plasma insulin:glucose (mU.mmol^{-1})	3.30 ± 1.11	2.40 ± 0.35	2.83 ± 0.38	NS
AUC glucose (mmol.min.L^{-1})				
Total	62 ± 6	56 ± 3	143 ± 28*^	0.003
1st phase	60.9 ± 5.4	54.8 ± 2.9	86.2 ± 5.7*^	< 0.001
2nd phase	1 ± 1	1 ± 1	57 ± 24*^	0.017
AUC insulin (mU.min.L^{-1})				
Total	587 ± 184	590 ± 181	863 ± 178	NS
1st phase	499 ± 133	579 ± 180	650 ± 128	NS
2nd phase	88 ± 61	12 ± 6	213 ± 119	NS
AUC insulin:AUC glucose (mU.mmol^{-1})				
Total	10.8 ± 4.3	10.9 ± 3.6	7.6 ± 1.9	NS
1st phase	8.9 ± 2.8	10.7 ± 3.5	7.8 ± 1.6	NS
2nd phase	26.5 ± 25.9	0.3 ± 0.3	9.6 ± 2.8	NS
Insulin sensitivity (mg.L.mU^{-1}.kg^{-1}.min^{-1})	0.097 ± 0.010	0.100 ± 0.011	0.047 ± 0.009*^	0.003
Basal IDI (mg.mL.kg^{-2}.min^{-2})	69.7 ± 31.2	39.5 ± 5.5	28.4 ± 10.0	NS
Maximal IDI (mg.mL.kg^{-2}.min^{-2})	138 ± 28	119 ± 27	97 ± 37	NS

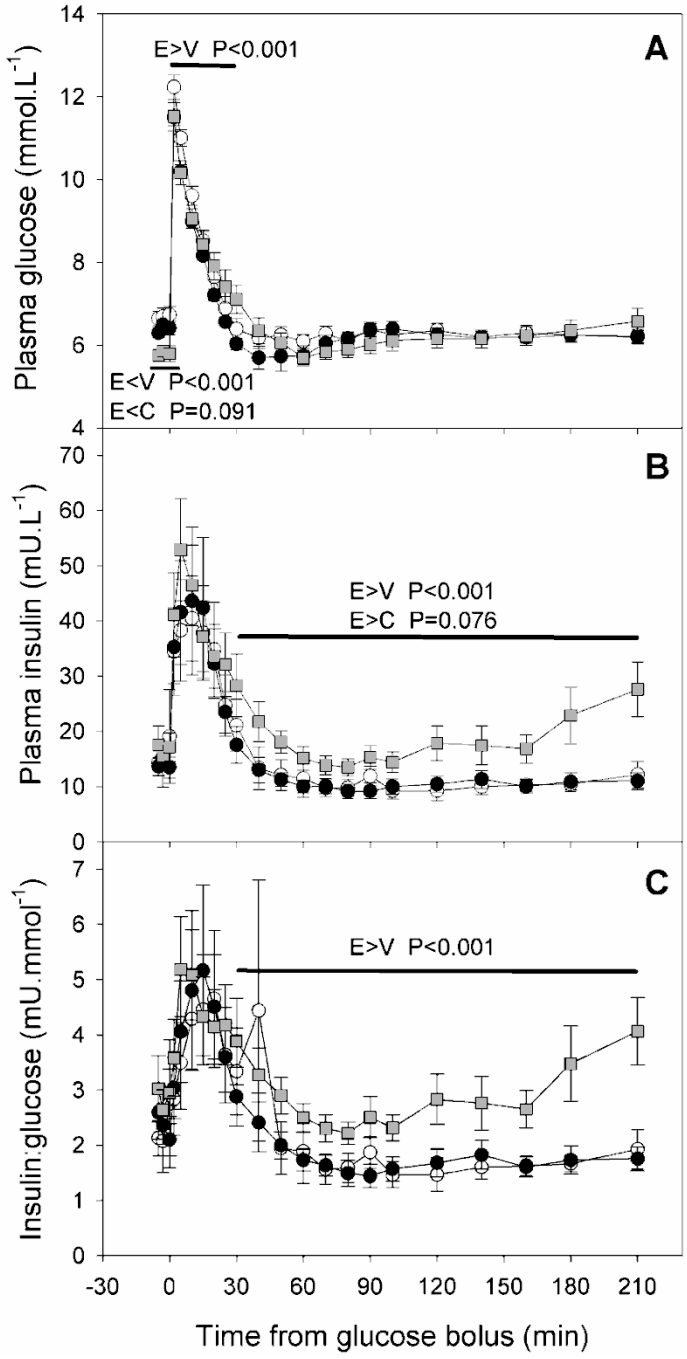

Figure 3.2 Effect of IUGR and neonatal exendin-4 treatment on glucose tolerance (A), glucose-stimulated insulin secretion (B) and relative glucose-stimulated insulin secretion (C) in young lambs.

CON (white circle, n = 7), IUGR+Veh (black circle, n = 8) and IUGR+Ex-4 (grey square, n = 8). Data are means ± SEM. * different from CON ($P < 0.05$).

Table 3.3 Effect of IUGR and neonatal exendin-4 treatment on pancreas morphology and β-cell function.

NS: P > 0.1, * different from CON (P < 0.05).

	CON (7)	IUGR+Veh (8)	IUGR+Ex-4 (8)	Significance (treatment effect)
Pancreas morphology				
Pancreas weight (g)	10.8 ± 1.5	8.53 ± 0.93	7.90 ± 0.55	NS
Pancreas (% of body weight)	0.103 ± 0.019	0.085 ± 0.009	0.096 ± 0.007	NS
β-cell volume density	0.033 ± 0.005	0.040 ± 0.003	0.049 ± 0.007	NS
β-cell mass (g)	0.326 ± 0.038	0.345 ± 0.054	0.387 ± 0.055	NS
β-cell mass (% of body weight)	0.0030 ± 0.0004	0.0034 ± 0.0005	0.0047 ± 0.0006*	0.070
Islet density ($no.mm^{-2}$)	66.3 ± 9.7	76.9 ± 10.3	91.6 ± 10.5	NS
β-cells/islets	10.9 ± 1.4	10.5 ± 1.3	12.9 ± 1.3	NS
% of islets with <5 β-cells	27.7 ± 6.3	23.8 ± 6.4	31.3 ± 6.7	NS
β-cell function				
Insulin secretion (AUC ins) per β-cell mass ($mU.min.L^{-1}.g^{-1}$)	1682 ± 413	1944 ± 588	2190 ± 286	NS
Basal IDI per β-cell mass ($mg.mL.kg^{-2}.min^{-2}.g^{-1}$)	187 ± 60	129 ± 25	85.4 ± 26.5	NS
Max IDI per β-cell mass ($mg.mL.kg^{-2}.min^{-2}.g^{-1}$)	441 ± 85	389 ± 89	269 ± 94	NS

3.4.4 *In vitro* β-cell secretory function

Islet insulin secretion (Figure 3.3) increased with increasing glucose concentration between 0 and 11.1 mM overall (P = 0.006). Glucose-stimulated insulin secretion tended to be higher overall in IUGR+Veh compared to CON lambs (+420%, P = 0.081), did not differ between IUGR+Ex4 lambs and CON lambs (P = 0.9) and tended to be higher in IUGR+Veh lambs than in IUGR+Ex4 lambs (+20%, P = 0.087). At the highest glucose concentration (11.1 mM), IUGR+Veh lambs had higher insulin secretion than CON lambs (+66%, P = 0.046) and tended to have higher insulin secretion than IUGR+Ex-4 lambs (+58%, P = 0.066 respectively, Figure 3.3). Within each group of lambs, *in vitro* insulin secretion at 11.1 mM glucose was between 1.6 and 2-fold higher than that at 0 mM glucose (Figure 3.3). *In vitro* insulin secretion was similar from islets incubated with 15mM KCl or 11.1 mM glucose (P > 0.5), and the response to KCl was similar between treatments (P > 0.8). *In vitro* insulin secretion was suppressed by epinephrine treatment compared to glucose-stimulated insulin secretion (-62%, P = 0.001). Suppression of glucose-stimulated insulin secretion by epinephrine was greater in IUGR+Veh than CON lambs in absolute terms (-173 *cf*. -47 μU insulin/μgDNA, P=0.044), but not as a proportion of insulin secretion in the absence of epinephrine (-28.8% *cf*. -7.8%, P=0.274). Epinephrine suppression of glucose-stimulated insulin secretion was similar in islets from IUGR-Ex4 to that in other groups (P>0.1 for each). Lysine-, arginine- and leucine-stimulated *in vitro* islet insulin secretion did not differ between treatment groups (each P > 0.3, data not shown).

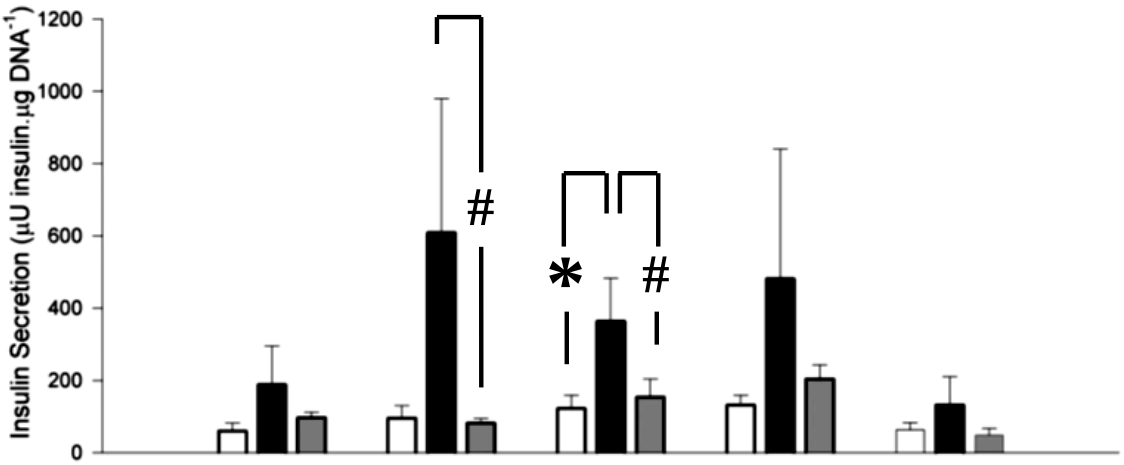

Figure 3.3 Effect of IUGR and neonatal exendin-4 treatment on *in vitro* insulin secretion from isolated islets in response to glucose and potassium chloride.

CON (white bar, n = 5), IUGR+Veh (black bar, n = 5) and IUGR+Ex-4 (grey bar, n = 6). Data are means ± SEM. Specific contrasts: * $P < 0.05$, # $P < 0.10$.

3.5 DISCUSSION

In the present study, twin IUGR lambs caught up in weight by 16 d of age, and had normal *in vivo* insulin action in their second week of life, with similar β-cell mass to singleton control lambs. Glucose-stimulated *in vitro* insulin secretion was increased in the IUGR twin lamb relative to controls, suggesting up-regulated β-cell function at this age. Daily exendin-4 treatment of twin IUGR lambs during neonatal life prevented catch-up growth and fat accumulation, and normalised *in vitro* insulin secretion from their islets, relative to untreated IUGR twins, which may retain adaptive capacity for later life. Glucose tolerance of IUGR lambs was impaired during exendin-4 treatment however, reflecting decreased insulin sensitivity and occurred despite greater *in vivo* insulin secretion. This may be due to central actions of exendin-4 to inhibit food intake and insulin sensitivity (24-26). Nevertheless, the reduction in fat accumulation and normalised β-cell action *in vitro* of IUGR lambs suggest that neonatal exendin-4 might have beneficial effects on insulin-regulated glucose homeostasis in later life. These outcomes also demonstrate the biological activity of exendin-4 for the first time in the sheep, at least in the context of individuals who had undergone growth-restriction before birth.

We found similar growth and metabolic responses to IUGR induced by twinning in this study to those seen previously after IUGR induced by restriction of placental growth and function (PR) in sheep. Like the PR lamb, the twin IUGR lambs in the present study experienced accelerated neonatal catch-up growth, achieving a normal body weight by 16 d of age in this study and by 30 d of age in our studies in PR lambs (141). Accelerated fat deposition occurs during accelerated neonatal growth, and in humans catch-up growth is a risk factor for later obesity (188). PR lambs have fat stores proportionate to their reduced body weight in late gestation (189), and similar to

our twin lambs at 16 days in the present study, fat mass relative to body weight is similar in PR and CON lambs at 21 days despite their catch up growth (151). By 43 days of age, however, the accelerated fat deposition results in greater visceral fat in PR lambs than their control counterparts (141). Small size at birth in humans consistently induces insulin resistance in adults and adolescents (1), but this is preceded by enhanced insulin sensitivity in neonates, which reverses to resistance in association with catch-up growth in the first few years of life (7). There is similar evidence of a reversal from insulin sensitivity to insulin resistance in the lamb following IUGR induced by restriction of placental growth and function (PR). The young PR lamb at 21 days of age has increased expression of insulin receptors and insulin signalling molecules in skeletal muscle (190), although *in vivo* insulin action was not measured. At 30 days, glucose tolerance of PR lambs is normal, despite decreased insulin action caused by falls in both *in vivo* insulin secretion and insulin sensitivity (141, 191). The latter reflects decreased expression of insulin-signalling pathways in skeletal muscle (191). Impaired glucose tolerance and elevated fasting glucose emerge by 1 year of age in IUGR sheep (140). The normal insulin sensitivity and glucose tolerance seen here in the twin IUGR lamb may therefore reflect the beginnings of the reversal from insulin sensitivity to insulin resistance occurring during the neonatal catch-up growth they are experiencing at this age.

Neither IUGR nor neonatal exendin-4 treatment in IUGR lambs altered relative β-cell mass at 16 days in the present study, consistent with the lack of effect of PR and neonatal exendin-4 treatment on β-cell mass in young postnatal rats at 2 weeks of age (9). In the rat, reduced β-cell mass after IUGR emerges by 3 months of age in young adults, and neonatal exendin-4 treatment normalises adult β-cell mass at this age in this model (9). We hypothesise that these beneficial effects of exendin-4 treatment

after IUGR might also emerge with ageing in the sheep. This lack of an immediate response may also reflect the collection of pancreas soon after completion of exendin-4 treatment here and in PR rats. Previous rodent studies have reported increased β-cell replication after similar exendin-4 treatment durations, but differences in β-cell mass are sometimes not apparent until several weeks later (reviewed by 153, 192). Many of the actions of exendin-4 and GLP1 on insulin secretion are mediated via stimulation of *PDX1* expression, a transcription factor important for regulation of β-cell mass as well as function, and which is required for plasticity of β-cell mass and function to increase insulin secretion in response to demand. In the PR rat, prevention of later diabetes following neonatal exendin-4 treatment reflects reversal of epigenetic changes induced by PR in the *PDX1* promoter by late gestation, that normally worsen with age and lead to decreased *PDX1* expression, loss of β-cell function and subsequent loss of β-cell mass postnatally (9, 21, 33). Intriguingly, although neonatal exendin-4 induces epigenetic changes such as increased acetylation and lysine 4 trimethylation at histone H3 in control as well as PR rat juveniles, it only increases *PDX1* expression and β-cell mass and improves glucose tolerance in the PR progeny (9, 21, 33). Indeed, the *PDX1* promotor becomes methylated and hence partially silenced by adulthood in untreated PR rat progeny, but not in control progeny regardless of exendin-4 treatment, which implies that the levels of histone 3 acetylation and lysine 4 trimethylation in untreated control progeny is already sufficient to prevent later promoter methylation (33). We do not yet know whether neonatal exendin-4 treatment will affect outcomes in control sheep progeny, as the aim of the present study was to evaluate its efficacy only in the context of IUGR. Whether neonatal exendin-4 acts similarly in the IUGR lamb as in the PR rat, by reversing epigenetic changes in the *PDX1* promotor and improves adult β-cell mass and function to delay or prevent the subsequent loss of insulin secretory

capacity observed after IUGR in young adult male sheep (32) remains to be determined, and will require separate animal cohorts with long-term follow-up of functional and molecular outcomes.

In this study, the decrease in fasting plasma glucose (~9%) and more sustained insulin secretion during exendin-4 treatment in the IUGR neonatal lamb compared to untreated IUGR siblings were generally consistent with responses to exendin-4 in rodents and humans. Medium- to long-term exendin-4 treatment in human T2DM patients (daily 5-10 μg injections for 30 and 82 weeks) (193-195), in the obese diabetic *db/db* mouse (1 $nmol.kg^{-1}.d^{-1}$ as daily injections for 14 days) (196), and in the obese *ob/ob* mouse (20 $\mu g.kg^{-1}.d^{-1}$, ~ 5 $nmol.kg^{-1}.d^{-1}$ as twice daily injections for 60 days) (197) reduces fasting blood glucose as well as HbA1c, a marker of chronic hyperglycaemia. Earlier studies in humans also demonstrated acute decreases in fasting and post-prandial glucose concentrations after a single exendin-4 dose and after 5 days of twice daily injections with 5 μg exendin-4 (198). Infusions with GLP1 and chronic exendin-4 treatment enhance post-prandial and glucose-stimulated insulin secretion in human patients with T2DM, including restoration of 1^{st} phase insulin secretion response to glucose, and sustained elevation of 2^{nd} phase insulin secretion in T2DM patients (198-200). In the diabetic rat, four weeks of twice-daily exendin-4 injections (105 $pmol.kg^{-1}$) increased 1^{st} and 2^{nd} phase insulin secretion during a hyperglycaemic clamp (201). Whilst we similarly observed increases in second phase insulin secretion in IUGR lambs during exendin-4 treatment, their first phase insulin secretion was unchanged. This apparent difference may be because first phase insulin secretion is normal in the IUGR lamb at this age, whereas previous reports of increased first phase insulin responses after exendin-4 or GLP1 treatment have all been in the context of

diabetes, when first phase secretion is impaired. The effects of exendin-4 on insulin secretion in IUGR sheep AFTER cessation of treatment remain to be investigated.

We performed *in vitro* testing to measure intrinsic β-cell function independent of systemic input from endocrine and nervous systems (178). In this study, IUGR neonatal lambs had enhanced *in vitro* glucose-stimulated insulin secretion, or β-cell hyper-secretion relative to control lambs, which occurs in obese individuals, as well as early in the pathogenesis of type 2 diabetes (202-204). Interestingly, exendin-4 treatment of IUGR lambs abolished this *in vitro* insulin hyper-secretion from isolated islets, suggesting some normalisation of intrinsic β-cell function and its determinants. Together with increased β-cell mass, this suggests that neonatal exendin-4 may improve insulin secretory capacity after IUGR.

In contrast with the improved insulin sensitivity seen after chronic GLP1 or exendin-4 treatment in human patients with extreme obesity (205) or T2DM (206), insulin sensitivity was profoundly decreased on the 11^{th} day of exendin-4 treatment in neonatal IUGR lambs, relative to their untreated IUGR twins. In studies of exendin-4 action in rodents, direct measures and calculated indices of insulin sensitivity have either been increased (197, 201, 207), or not altered (196), immediately following or during chronic (2 – 9 weeks) exendin-4 treatment. We propose that the differential effects of exendin-4 on insulin sensitivity may depend on whether the latter is assessed during treatment or after, whether the subjects are obese and on their developmental stage and growth rate. Exendin-4 reduced weight gain in the IUGR lambs in the present study, consistent with its actions including decreased food and caloric intake, reduced gastric emptying and induced weight loss or slowed weight gain in mice and rats (208, 209) and in adolescent and adult humans (193-195, 205, 210-212). It appears that restricted nutrition reduces insulin sensitivity in growing animals, possibly partly due to

reduced mass of insulin-responsive tissues, whereas in older or obese animals the net effect of restricted feeding and consequently reduced fatness is to increase insulin sensitivity. Thus, feed restriction *increases* insulin-stimulated glucose metabolism and insulin sensitivity in adult sheep (213), but decreases insulin-stimulated glucose uptake in muscle of young growing pigs (214). In mice, exendin-4 can cross the blood-brain barrier (215), and acts centrally to suppress femoral blood flow and whole body insulin sensitivity, via the GLP-1 receptor and activation of PKC-δ signalling pathways in the hypothalamus (24, 25), suggesting an additional mechanism for decreased peripheral insulin sensitivity during exendin-4 treatment. As a consequence of their reduced insulin sensitivity, and despite the increased 2nd phase insulin secretion that maintained insulin disposition, glucose tolerance was impaired in IUGR+Ex-4 lambs compared to IUGR+Veh and CON lambs. This contrasts with improved glucose tolerance observed 24 h after completion of medium- to long-term exendin-4 treatment in mature rats (197, 200, 201, 207), during continued long-term exendin-4 treatment in β-cell depleted rats (201), and acutely in T2DM human patients (200). In some of these studies, the improved glucose tolerance during or after exendin-4 treatment reflects marked improvement of deficient insulin secretion due to stimulation of β-cell regeneration (201) or up-regulation of β-cell function in T2DM patients (200). Long-term exendin-4 treatment increases insulin sensitivity in obese humans, genetically-obese rodents and diabetic humans and rodents, measured either during or 16-24 h after completion of treatment (197, 205, 207, 216). Improved whole-body insulin sensitivity is probably also due to improvements in hepatic insulin sensitivity, with lower post-prandial endogenous glucose production after or during exendin-4 treatment (217). To our knowledge, this is the first study of the effects of exendin-4 on insulin action treatment in young growing animals.

The profound reduction in visceral fat deposition after IUGR in response to exendin-4 is also of particular potential importance for later glucose homeostasis, given that obesity and particularly visceral fat deposition are strong risk factors for impaired glucose tolerance and T2DM (218, 219). In the PR rat, neonatal exendin-4 reduces later weight gain in conjunction with prevention of later diabetes, and this may particularly reduce the risk of T2DM in IUGR subjects (9), since catch-up growth after IUGR is a risk factor for T2DM and for adult obesity (188, 220). Intriguingly, neonatal exendin-4 treatment abolished the negative relationship between birth weight and fractional growth rate in IUGR lambs in the current study. In contrast to its metabolic effects, exendin-4 reduced adult size in both control and PR rat progeny (9). This suggests that exendin-4 may act in part, but not only, via the pathway/s responsible for catch-up after IUGR, which include neonatal hyperphagia, elevated insulin sensitivity and increased abundance of thyroid hormones in IUGR lambs (141, 176, 221). Longer-term evaluations of growth and composition after cessation of exendin-4 are needed to determine whether this decrease in central adiposity persists in the IUGR sheep.

3.6 CONCLUSION

In conclusion, neonatal exendin-4 treatment increased 2nd phase insulin secretion *in vivo*, normalised *in vitro* insulin secretion and decreased visceral fat at the end of treatment in the IUGR lamb. Neonatal exendin-4 treatment also improves insulin secretion and glucose tolerance in adolescent and adult rat progeny following IUGR, preventing development of diabetes in these animals (9), although the effects during treatment were not measured in the latter study. Investigation of the long-term effects of neonatal exendin-4 on glucose homeostasis and insulin action in the IUGR lamb into adulthood should be a priority for the future.

CHAPTER 4

4 CHAPTER 4 EFFECT OF IUGR DUE TO TWINNING AND NEONATAL EXENDIN-4 TREATMENT ON MOLECULAR DETERMINANTS OF β-CELL FUNCTION AND MASS IN YOUNG LAMBS

4.1 INTRODUCTION

Intrauterine growth restriction (IUGR) increases the risk of type 2 diabetes (T2D) in later life, due to impaired insulin sensitivity and inadequate insulin secretion or action (1, 2). In a study of 19-year-old Caucasian men, reduced insulin action was observed in IUGR men despite no evidence of insulin resistance (6), which indicates that impaired insulin secretion can be present before insulin resistance emerges following IUGR. In experimentally-induced IUGR, reduced insulin secretion was evident in PR rats from 2 weeks of age and followed by reduced β-cell mass, impaired glucose tolerance and insulin action at 3 months of age (4). Importantly, administration of the GLP1 analogue, exendin-4, to neonatal PR rats normalised subsequent β-cell mass and insulin secretion and prevented later development of T2D (9). Because fetal growth is substantially determined by substrate availability, IUGR and prenatal nutrient deprivation causes the fetus to adapt and survive by slowing growth and preserving critical organs at the expense of others (44). Restored nutrition after birth following IUGR may expose any defects in insulin action programmed by IUGR (49). Understanding how these processes and their underlying molecular determinants are influenced by nutrient deprivation and IUGR before birth is needed to develop effective interventions.

Altered expression of molecular determinants of β-cell mass and function following IUGR contributes to risk of impaired insulin action and diabetes in later life. *PDX1* is a master regulator of β-cell growth and development as well as β-cell postnatal

adaptation (54, 222) and directly regulates expression of insulin (*INS*, 62), glucose transporter 2, (*SLC2A2*, 63) and glucokinase (*GCK*, 64), which are determinants of insulin secretion by β-cells. In PR-induced IUGR rats, impaired insulin secretion and loss of β-cell mass are partly due to reduced expression of *PDX1* (4), which is epigenetically down-regulated (21). Moreover, heterozygous *PDX1* knockout mice have attenuated *in vivo* glucose-stimulated insulin secretion (66) and this *PDX1* deficiency causes impaired glucose-stimulated insulin secretion and glucose tolerance in response to insulin resistance (66). This indicates that deficiency of *PDX1* expression leads to failure of compensatory increases in β-cell function, especially insulin secretion, in response to insulin demand or resistance. Other molecular regulators of β-cell function may also be programmed by IUGR. In experimentally-induced IUGR sheep, β-cell function and insulin secretion was impaired in adult male IUGR sheep, and gene expression of an L-type voltage-gated Ca^{+2} channel subunit (*CACNA1D*) was reduced in IUGR young lambs (8). Importantly, β-cell function in the young IUGR lambs correlated positively with *CACNA1D* mRNA expression (8), suggesting that *CACNA1D* may be critical in regulating β-cell function and insulin secretion. Another potential regulatory mechanism for altered gene regulation of β-cell mass and function is through microRNAs (miRNA), which are short single-stranded non-coding RNAs (18-22 nucleotides in length) that can regulate gene expression by binding to target messenger mRNA (69). Several miRNAs regulate β-cell development and function, including miRNA-124a (miR-124a) which targets *FOXA2*, a transcriptional activator of the *PDX1* gene (75), miRNA-375 (miR-375) which down-regulates mRNA expression of the Myotrophin (*MTPN*) gene required for exocytosis of insulin vesicles (76) and miRNA-7 (miR-7). Suppression of miR-7 expression by antisense miRNA in mice embryos leads to reduced insulin synthesis and β-cell numbers in newborn pups, and

these mice developed impaired glucose tolerance postnatally (78). Whether IUGR alters gene expression of miRNAs or other determinants of β-cell mass and function is unknown, and thus requires further investigation to determine whether this is a mechanism for impaired insulin secretion or action following IUGR.

IUGR due to twinning in sheep increased catch up growth in young lambs from birth to 16 d of age, whilst neonatal exendin-4 treatment prevented catch-up growth and halved visceral fat mass at 16 d of age (Chapter 3, 172). Exendin-4 treated IUGR lambs had proportionally less lean mass than control lambs, but did not differ in lean mass compared to vehicle-treated IUGR twin lambs (Chapter 3, 172). Although we did not observe any effect of IUGR due to twinning on *in vivo* β-cell function and mass, IUGR increased *in vitro* glucose-stimulated insulin secretion from islets isolated from 16 d-old lambs (Chapter 3, 172). This *in vitro* insulin hyper-secretion after IUGR was abolished by neonatal exendin-4 treatment (Chapter 3, 172). In the present study, we therefore measured expression of key molecular determinants of β-cell mass and function in the same cohort of animals; *PDX1*, *IGF1* and *IGF2* and their receptors, *INS* and its receptor, *GCK*, *SLC2A2*, *CACNA1D*, *KCNJ11* and insulin signalling molecules (*PIK3CB* and *PIK3R1*). We also investigated expression of key regulatory microRNAs (miR-7, miR-375, miR-124a) and expression of epigenetic machinery enzymes (*DNMT1*, *DNMT3A*, *DNMT3B*). We hypothesised that IUGR would up-regulate islet expression of key determinants of β-cell function but not determinants of β-cell mass at 16 d of age, consistent with observed effects of IUGR in these young lambs (Chapter 3, 172). We further hypothesised that neonatal exendin-4 treatment would normalise expression of these key determinants of β-cell function. We also hypothesised that expression of these molecular regulators following IUGR and exendin-4 treatment would correlate with *in vivo* and *in vitro* outcomes.

4.2 MATERIALS AND METHODS

4.2.1 Animals and treatments.

Animals and treatments are described more fully in Section 2.2.1 and 2.2.2 of Chapter 2 and 3 (172). In brief, ewes underwent a timed-mating program with ultrasound scanning performed at G60 to confirm singleton or twin pregnancies. Delivery occurred naturally at term and lambs were allocated to 3 treatment groups, singleton lambs with vehicle treatment, CON (n = 7), and twin lambs. Within each set of twins, sibling twin lambs were alternately allocated to vehicle treatment, IUGR+Veh (n = 8) or exendin-4 treatment, IUGR+Ex-4 (n = 8). Vehicle (0.5% methanol in 0.9% saline) and exendin-4 (1 $nmol.kg^{-1}$ in vehicle, Bachem, Buberndorf, Germany) were injected s.c. daily from d 1 to d 16 of age. All lambs (singletons and twins) were supplemented with whey protein (Resource Beneprotein instant protein powder, Nestle, Australia) given orally in two equal feeds (at 0900 – 1000 h and 1600 – 1700 h), commencing at 1.25 $g.kg^{-1}.d^{-1}$ on d 4 and increasing to 5 $g.kg^{-1}.d^{-1}$ on and after d 7. Feeding this supplement during this period of maximal catch-up growth in IUGR lambs (176) was intended to minimise the potential for limitation of neonatal growth by milk availability in twins (174) by providing ~25% of the protein expected to be available through milk, and allowing lambs to self-regulate their milk intake to appetite.

4.2.2 Post-mortem, pancreatic islet RNA and microRNA extraction and their quality and quantity assessments

Post-mortem, pancreatic islets RNA and microRNA extraction and their quantity and quality assessments are described in full in Sections 2.2.5, 2.5.1, 2.5.2, and 2.6.1 of Chapter 2. Due to technical difficulties with one preparation, RNA samples were obtained successfully for 6 CON, 8 IUGR+Veh and 8 IUGR+Ex-4 lambs.

Methods for Real Time PCR (RT PCR) of target gene mRNA are described fully in Sections 2.5.4 to 2.5.6 in Chapter 2. In this study, we measured mRNA expression of key molecular determinants of β-cell mass (*PDX1, IGF1, IGF2, IGF1R, IGF2R, INS, INSR*), β-cell function and insulin signalling (*PDX1*, *INS*, *INSR*, *GCK*, *SLC2A2*, *CACNA1D*, *KCNJ11*, *PIK3CB* and *PIK3R1*), and other regulators (epigenetic machinery enzymes: *DNMT1*, *DNMT3A*, *DNMT3B*). Analysis of islet microRNA expression (miR-7, miR-375 and miR-124a) was as described in full in Sections 2.6 in Chapter 2. Pairwise array comparisons were made between CON lambs *cf*. IUGR+Veh lambs to assess the effect of IUGR and between IUGR+Veh lambs *cf*. IUGR+Ex-4 lambs to assess the effect of neonatal exendin-4 treatment.

4.2.3 Statistical analyses

Statistical analyses used in this chapter are described fully in Section 2.8 of Chapter 2. Data were analysed by the mixed models procedure in SPSS for effects of treatment (fixed effect) and including the dam as a random (block) effect in the model to account for the common maternal environment in twins. Where treatment effects or trends ($P < 0.1$) were apparent, we then compared means by the LSD method, based on *a priori* questions to determine: 1. the effect of IUGR (CON *cf*. IUGR+Veh groups), 2. the effect of exendin-4 in IUGR lambs (IUGR+Veh *cf*. IUGR+Ex-4 groups), and 3. whether exendin-4 restored values to those of controls (CON *cf*. IUGR+Ex-4 groups). Relationships between *in vivo* measures, *in vitro* measures and gene expression were analysed by Pearson's correlation analysis within each treatment group.

4.3 RESULTS

4.3.1 Islet mRNA expression of molecular determinants of β-cell mass

IUGR due to twinning did not alter islet mRNA expression of molecular determinants of β-cell except that it tended to increase *INSR* expression (+89%, P = 0.098) when compared to CON lambs (Figure 4.1). Neonatal exendin-4 treatment of IUGR lambs also did not alter expression of molecular determinants of β-cell mass when compared to IUGR+Veh lambs. However, exendin-4 treatment of IUGR twin lambs increased islet mRNA expression of *IGF2R* (+63%, P = 0.005) and tended to increase islet mRNA expression of *IGF1* (+62%, P = 0.050) compared to CON lambs (Figure 4.1).

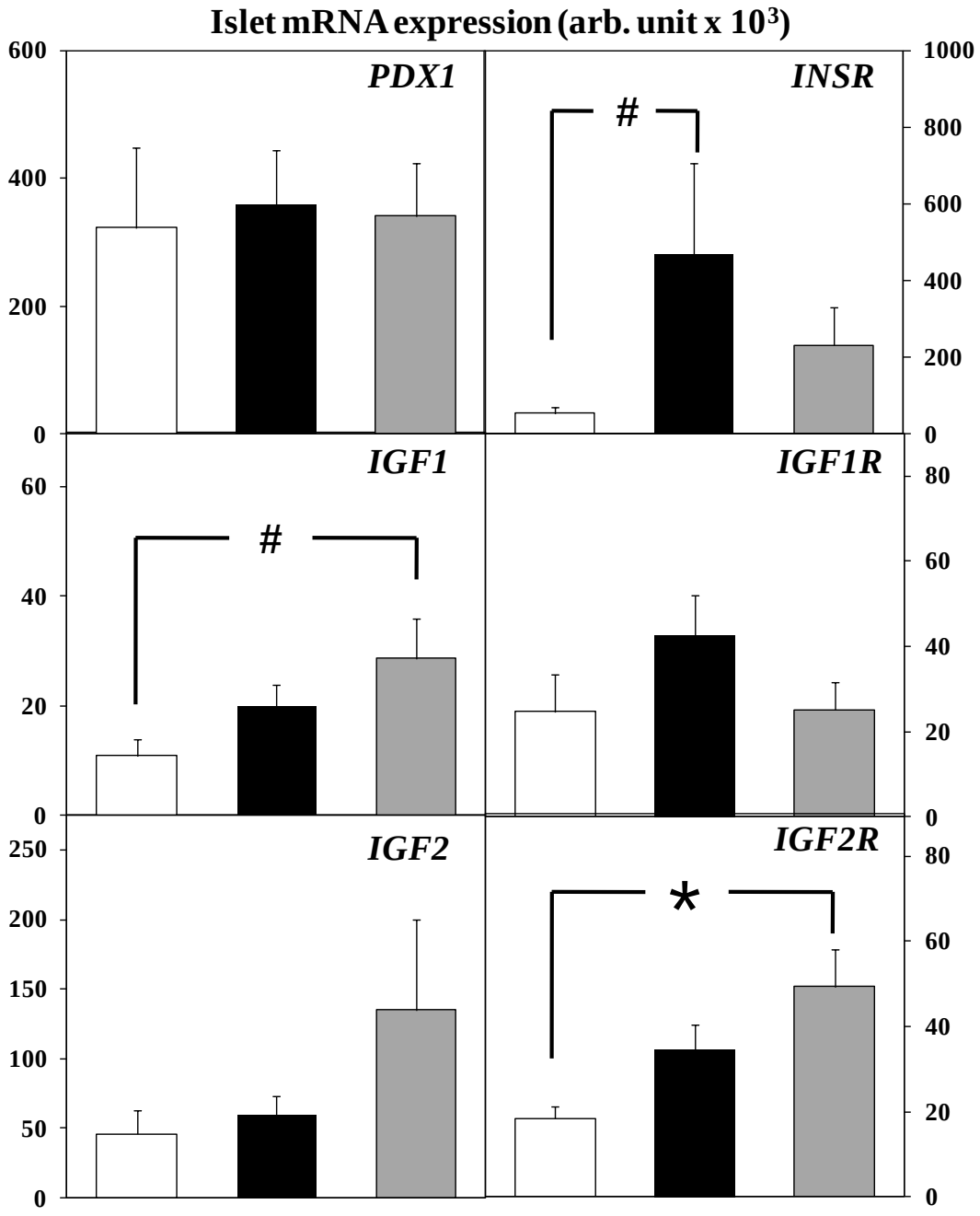

Figure 4.1 Effects of IUGR and neonatal exendin-4 treatment on islet mRNA expression of genes that regulate β-cell mass.

Control (white bar, n = 6), IUGR + Veh (black bar, n = 8) and IUGR + Ex-4 (grey bar, n = 8). Relative gene expression (normalised to *ACTB*) is expressed as mean ± SEM, and differences between groups are indicated by *, $P < 0.05$, # $P < 0.1$.

4.3.2 Islet mRNA expression of molecular determinants of β-cell function

IUGR due to twinning increased islet mRNA expression of *GCK* (+80%, P = 0.017) and *PIK3CB* (+85%, P = 0.019), and tended to increase islet mRNA expression of *SLC2A2* (+78%, P = 0.064) and *INSR* (+89%, P = 0.098), compared to CON lambs (Figure 4.2). Neonatal exendin-4 treatment reduced the islet mRNA expression of *GCK* (-62%, P = 0.047) and tended to reduce islet mRNA expression of *PIK3CB* (-73%, P = 0.073) in IUGR twin lambs (Figure 4.2). Neonatal exendin-4 treatment of IUGR+Ex-4 lambs also tended to increase islet mRNA expression of *PIK3R1* (Figure 4.2) when compared to IUGR+Veh lambs (+62%, P = 0.053) and increased this compared to CON lambs (+79%, P = 0.023). Gene expression of *CACNA1D, KCNJ11, PDX1* and *INS* did not differ between treatments.

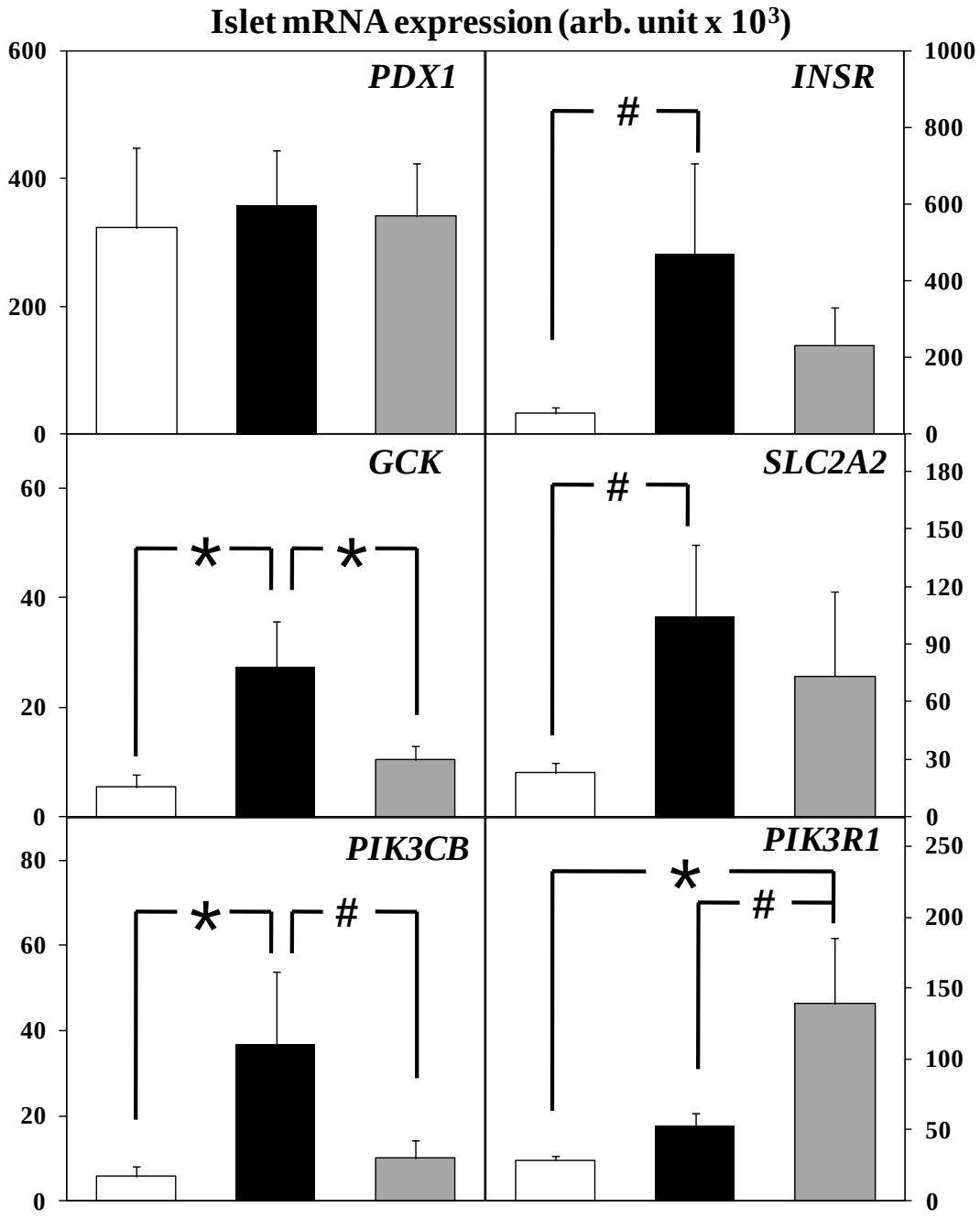

Figure 4.2 Effects of IUGR and neonatal exendin-4 treatment on islet mRNA expression of genes that regulate β-cell function.

Control (white bar, n = 6), IUGR + Veh (black bar, n = 8) and IUGR + Ex-4 (grey bar, n = 8). Relative gene expression (normalised to *ACTB*) are expressed as mean ± SEM, and differences between groups are indicated by *, $P < 0.05$, # $P < 0.1$.

4.3.3 Islet mRNA expression of epigenetic machinery

In IUGR+Veh lambs, islet expression of *DNMT3B* (+96%, P = 0.027) was increased and islet expression of *DNTM3A* (+75%, P = 0.085) tended to increase when compared to CON lambs (Figure 4.3). Conversely, islet expression of *DNMT1* was decreased in IUGR+Ex-4 lambs when compared to IUGR+Veh lambs (-81%, P = 0.027) with a similar trend compared to CON lambs (-50%, P = 0.050). Islet expression of *DNTM3A* and *DNMT3B* were not different in IUGR+Ex-4 lambs compared to other groups.

4.3.4 Islet microRNA expression

IUGR due to twinning did not alter islet microRNA expression measured by microarray compared to that in CON lambs (all P > 0.99, data not shown). Similarly, neonatal exendin-4 treatment of IUGR lambs did not alter islet miRNA expression compared to IUGR+Veh lambs (all P >0.7, data not shown).

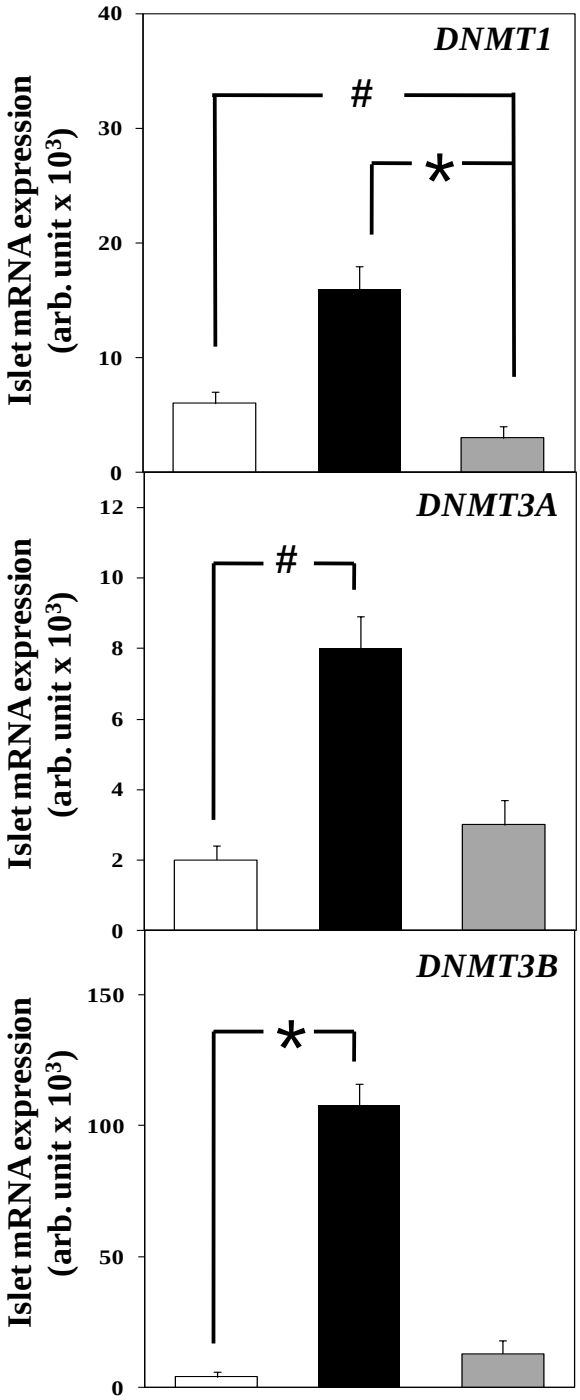

Figure 4.3 Effects of IUGR and neonatal exendin-4 treatment on islet mRNA expression of DNA methyl transferase enzymes.

Control (white bar, n = 6), IUGR + Veh (black bar, n = 8) and IUGR + Ex-4 (grey bar, n = 8). Relative gene expression (normalised to *ACTB*) are expressed as mean ± SEM, and differences between groups are indicated by *, $P < 0.05$, # $P < 0.1$.

4.3.5 Relationships between β-cell mass and function and islet mRNA expression of molecular regulators of β-cell mass and function

4.3.5.1 Relationships between pancreas morphology and β-cell mass and islet mRNA expression

In CON lambs, measures of pancreas morphology and β-cell mass did not correlate with islet mRNA expression of each of the analysed molecular determinants of β-cell mass, except that β-cell mass correlated positively with islet mRNA expression of *INS* ($r = 0.86$, $P = 0.028$, $n = 6$). In IUGR+Veh lambs, measures of pancreas morphology and β-cell mass did not correlate with islet mRNA expression of each of the analysed molecular determinants of β-cell mass. In IUGR+Ex-4 lambs (Figure 4.4), β-cell mass and β-cell volume density correlated or tended to correlate positively with islet mRNA expression of *IGF1* ($r = 0.71$, $P = 0.049$, $n = 8$ and $r = 0.71$, $P = 0.051$, $n = 8$ respectively). Also in IUGR+Ex-4 lambs, the percentage of all islets with < 5 β-cells, an indicator of neogenesis, correlated positively with islet mRNA expression of *PDX1* ($r = 0.83$, $P = 0.010$, $n = 8$).

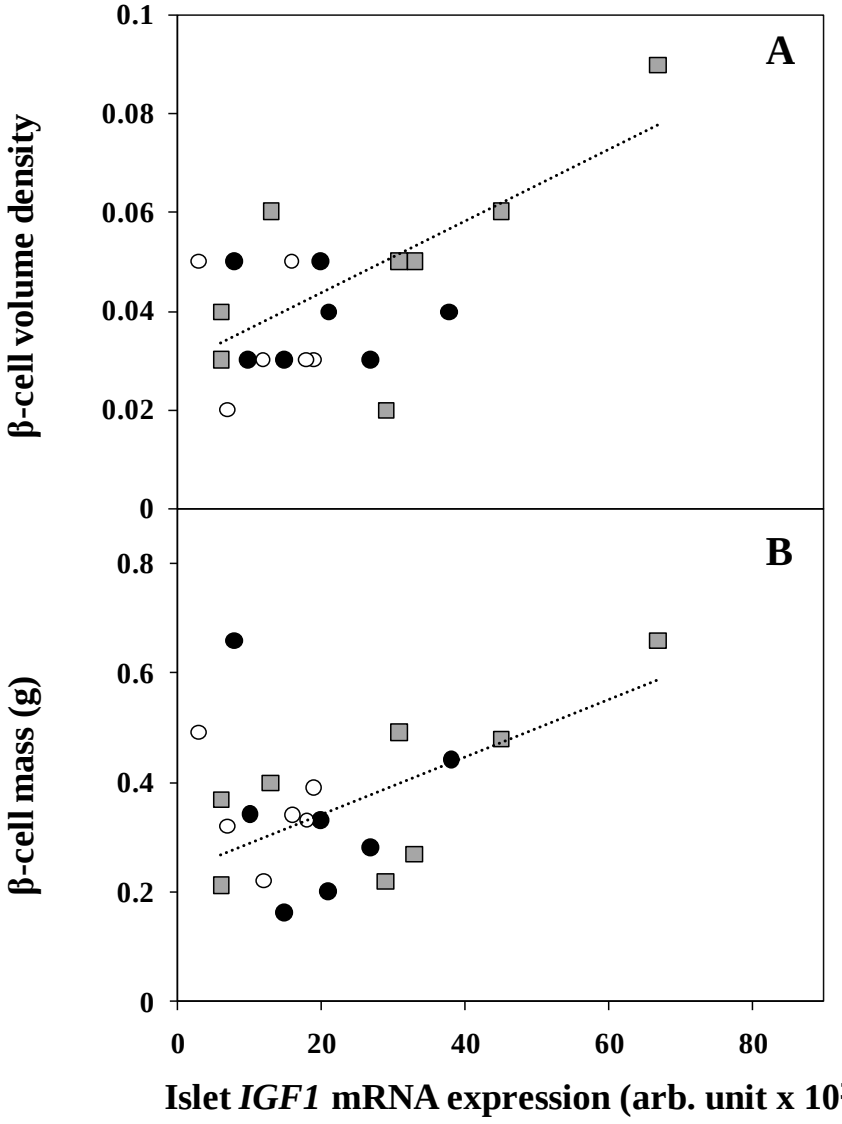

Figure 4.4 Relationships between (A) β-cell volume density and (B) β-cell mass and islet *IGF1* mRNA expression in the young lambs.

Control (white circle, n = 6), IUGR + Veh (black circle, n = 8), IUGR + Ex-4 (grey square, n = 8) and linear correlation in IUGR+Ex-4 lambs (dotted line). Points show individual animal outcomes.

4.3.5.2 Relationships between in vivo insulin secretion and disposition and islet mRNA expression

In CON and IUGR+Veh groups, *in vivo* insulin secretion did not correlate with islet mRNA expression of each of the analysed molecular determinants of β-cell function. In IUGR+Ex-4 lambs, *in vivo* insulin secretion correlated or tended to correlate negatively with islet mRNA expression of *PDX1* and *INSR* (*PDX1:* r = -0.72, P = 0.046, n = 8, and *INSR:* r = -0.64, P = 0.090, n = 8). In CON lambs, basal and maximal insulin disposition correlated negatively with islet mRNA expression of *GCK* and *INSR* (Figure 4.5, Basal insulin disposition: *GCK*: r = -0.93, P = 0.008, n = 6, *INSR*: r = -0.93, P = 0.007, n = 6 and Maximal insulin disposition: *GCK*: r = -0.85, P = 0.034, n = 6, *INSR*: r = -0.87, P = 0.025, n = 6). However, this was at least partially reversed in IUGR+Veh, where basal and maximal insulin disposition correlated positively with islet mRNA expression of *GCK* (Figure 4.5, Basal: r = 0.75, P = 0.033, n = 8 and Maximal: r = 0.78, P = 0.022, n = 8), and were not correlated with *INSR*. In IUGR+Ex-4 lambs, basal and maximal insulin disposition did not correlate with islet mRNA expression of molecular regulators of β-cell function.

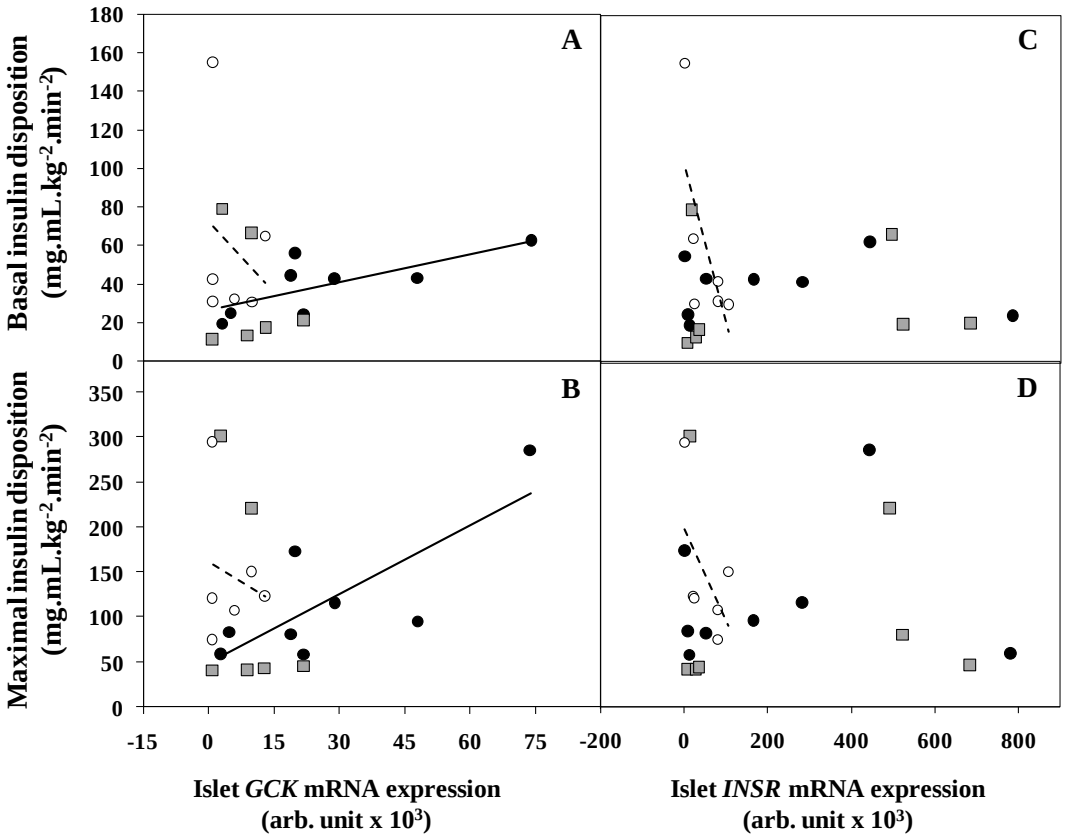

Figure 4.5 Relationships between (A) Basal and (B) Maximal insulin disposition and islet *GCK* mRNA expression, and between (C) Basal and (D) Maximal insulin disposition and islet *INSR* mRNA expression in the young lambs.

Control (white circle, n = 6), IUGR + Veh (black circle, n = 8), IUGR + Ex-4 (grey square, n = 8), linear correlation in CON lambs (dashed line) and linear correlation in IUGR+Veh lambs (solid line). Points show individual animal outcomes.

4.3.5.3 Relationships between in vitro insulin secretion and action and islet mRNA expression

In CON lambs, basal *in vitro* insulin secretion at 0 mM glucose correlated negatively with islet mRNA expression of *CACNA1D* (Figure 4.6, r = -0.96, P = 0.010, n = 5). In IUGR+Veh lambs, basal *in vitro* insulin secretion at 0 mM glucose correlated negatively with islet mRNA islet expression of *GCK* (Figure 4.6, r = -0.96, P = 0.010, n = 5). Basal *in vitro* insulin secretion at 0 mM glucose did not correlate with islet mRNA expression of each of the analysed molecular determinants of β-cell function in IUGR+Ex-4 group.

When challenged with glucose (1.1 mM), glucose-stimulated *in vitro* insulin secretion correlated negatively with islet mRNA expression of *CACNA1D* in the CON group only (Figure 4.6, r = -0.93, P = 0.021, n = 5). Glucose-stimulated *in vitro* insulin secretion at 1.1 mM glucose correlated negatively with islet mRNA expression of *GCK* in IUGR+Veh (r = -0.95, P = 0.014, n = 5) and IUGR+Ex-4 groups (r = -0.96, P = 0.010, n = 5) but not in the CON group (Figure 4.6). In contrast, at the higher concentration of glucose (11.1 mM), no correlations were observed between *in vitro* glucose-stimulated insulin secretion and expression of each of the analysed molecular determinants of β-cell function in any group.

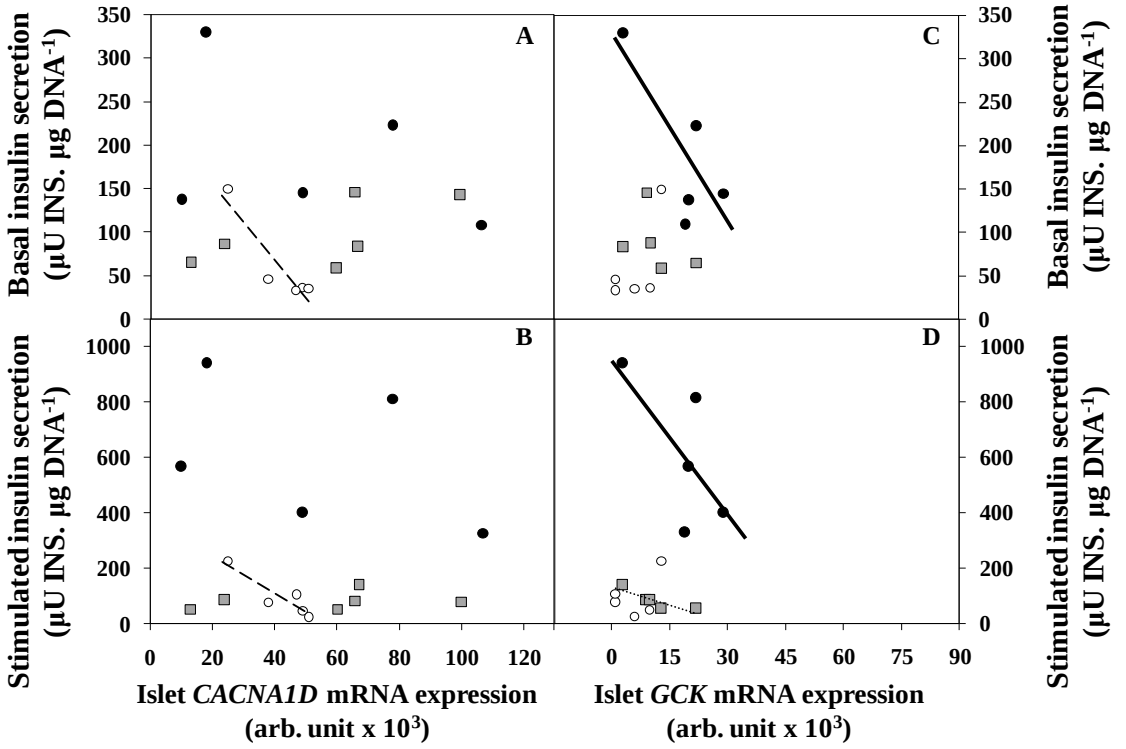

Figure 4.6 Relationships between *in vitro* insulin secretion at (A) basal 0 mM glucose and (B) stimulated 1.1 mM glucose and islet *CACNA1D* mRNA expression, and between *in vitro* insulin secretion at (C) basal 0 mM glucose and (D) stimulated 1.1 mM glucose and islet *GCK* mRNA expression.

Control (white circle, n = 6), IUGR + Veh (black circle, n = 8), IUGR + Ex-4 (grey square, n = 8), linear correlation in CON lambs (dashed line), linear correlation in IUGR+Veh lambs (solid line) and linear correlation in IUGR+Ex-4 lambs (dotted line). Points show individual animal outcomes.

4.3.6 Relationships between islet mRNA expression of *PDX1* and other regulators of β-cell mass and function

Islet mRNA expression of *IGF1R* correlated positively with *PDX1* mRNA expression in all groups (Figure 4.7); CON (r = 0.91, P = 0.032, n = 5), IUGR+Veh (r = 0.78, P = 0.022, n = 8) and IUGR+Ex-4 (r = 0.85, P = 0.007, n = 8). In IUGR+Ex-4, but not in other groups, islet mRNA expression of *IGF2* also correlated positively with *PDX1* mRNA expression (Figure 4.7, r = 0.74, P = 0.037, n = 8). Islet mRNA expression of *INS* did not correlate with expression of *PDX1* in any group (each P > 0.1, data not shown).

In CON lambs, islet mRNA expression of *SLC2A2*, *GCK*, *INSR* and *KCNJ11* did not correlate with islet mRNA expression of *PDX1*. In IUGR+Veh and IUGR+Ex-4 lambs (Figure 4.8), islet mRNA expression of *SLC2A2*, *GCK*, *INSR* and *KCNJ11* each correlated positively with islet mRNA expression of *PDX1 (SLC2A2*, IUGR+Veh: r = 0.85, P = 0.014, n = 8 and IUGR+Ex-4: r = 0.91, P = 0.005, n = 7, *GCK*, IUGR+Veh: r = 0.71, P = 0.050, n = 8 and IUGR+Ex-4: r = 0.77, P = 0.043, n = 7, *INSR*, IUGR+Veh: r = 0.77, P = 0.025, n = 8 and IUGR+Ex-4: r = 0.90, P = 0.003, n = 8, and *KCNJ11*, IUGR+Veh: r = 0.89, P = 0.003, n = 8 and IUGR+Ex-4: r = 0.94, P <0.001, n = 8).

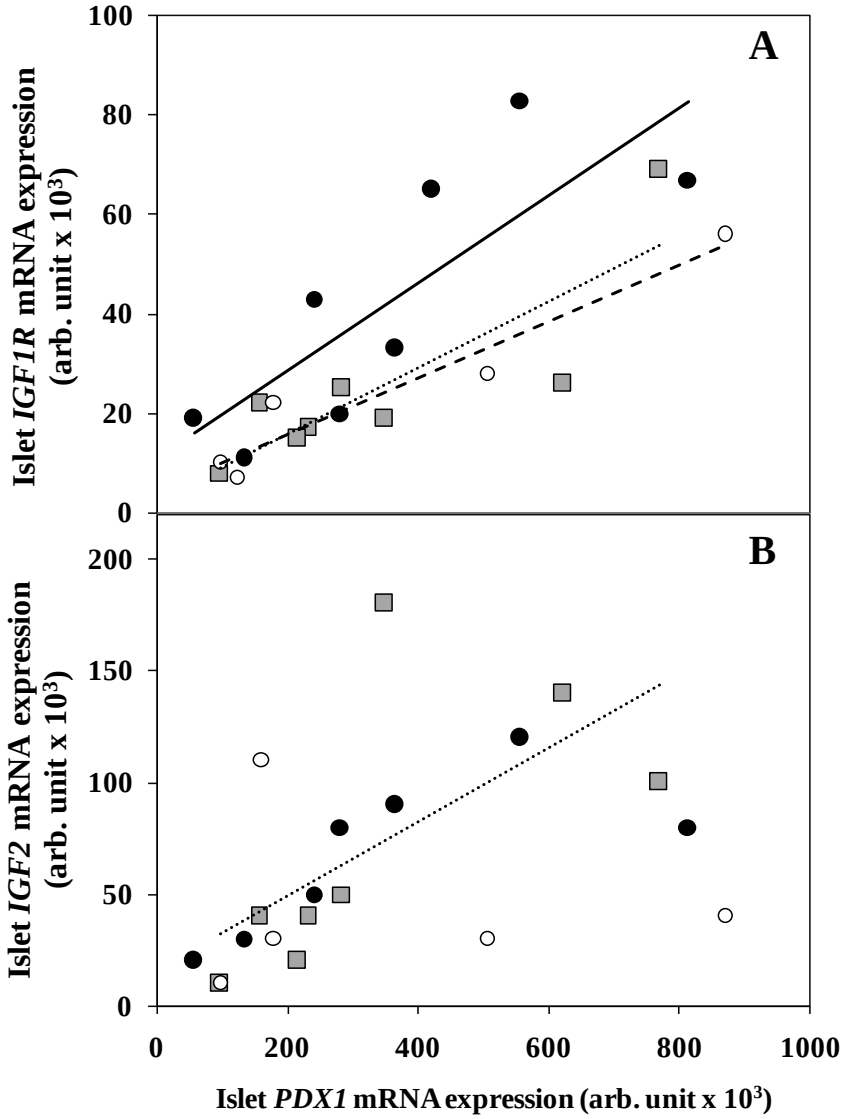

Figure 4.7 Relationships between islet mRNA expression of (A) *IGF1R* and (B) *IGF2* and islet *PDX1* mRNA expression in the young lambs.

Control (white circle, n = 6), IUGR + Veh (black circle, n = 8), IUGR + Ex-4 (grey square, n = 8), linear correlation in CON lambs (dashed line), linear correlation in IUGR+Veh lambs (solid line) and linear correlation in IUGR+Ex-4 lambs (dotted line). Points show individual animal outcomes.

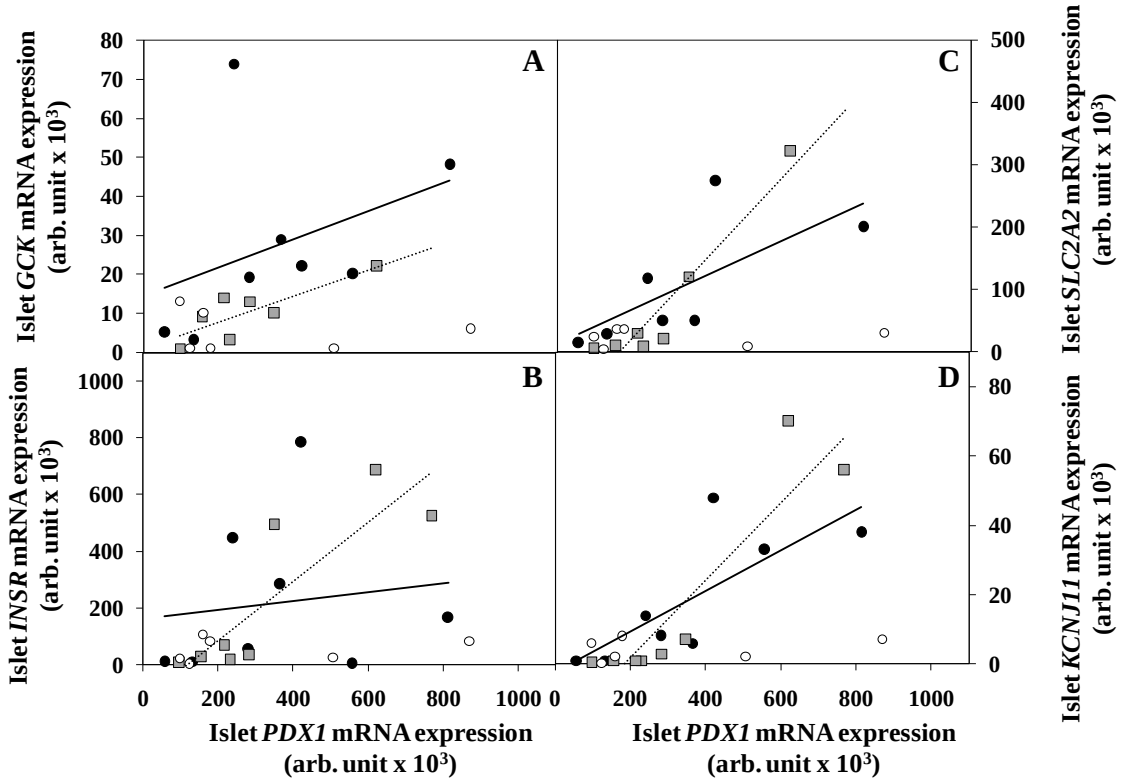

Figure 4.8 Relationships between islet *PDX1* mRNA expression and islet mRNA expression of (A) *GCK*, (B) *SLC2A2*, (C) *INSR* and (D) *KCNJ11* in young lambs.

Control (white circle, n = 6), IUGR + Veh (black circle, n = 8), IUGR + Ex-4 (grey square, n = 8), linear correlation in IUGR+Veh lambs (solid line) and linear correlation in IUGR+Ex-4 lambs (dotted line). Points show individual animal outcomes.

4.3.7 Relationships between islet mRNA expression of *PDX1* and other regulators of β-cell mass and secretory function and islet mRNA expression of epigenetic machinery

In CON lambs, islet mRNA expression of *PDX1* did not correlate with islet mRNA expression of *DNMT1, DNTM3A* or *DNMT3B*. Islet mRNA expression of *PDX1* correlated positively with that of *DNMT3A* in IUGR+Veh lambs (r = 0.83, P = 0.011, n = 8) and in IUGR+Ex-4 lambs (r = 0.81, P = 0.026, n = 7, Figure 4.9).

In the present study, islet mRNA expression of other genes that are epigenetically regulated correlated positively with mRNA expression of *DNMT3A*. Islet mRNA expression of *GCK* correlated positively with *DNMT3A* expression in all groups (CON: r = 0.85, P = 0.031, n = 6, IUGR+Veh: r = 0.77, P = 0.024, n = 8; IUGR+Ex-4: r = 0.85, P = 0.014, n = 7). Islet mRNA expression of *IGF1R* expression also correlated positively with *DNMT3A* expression in both IUGR groups (IUGR+Veh: r = 0.93, P = 0.001, n = 8; IUGR+Ex-4: r = 0.83, P = 0.021, n = 7), but not in CON (P > 0.1, Figure 4.9). Islet mRNA expression of *GCK* correlated positively with *DNMT3B* mRNA expression in IUGR+Veh lambs (r = 0.82, P = 0.024, n = 7), but not in the other two groups (each P > 0.1, Figure 4.10). Islet mRNA expression of *DNMT1* did not correlate with that of genes that regulate β-cell function in any group.

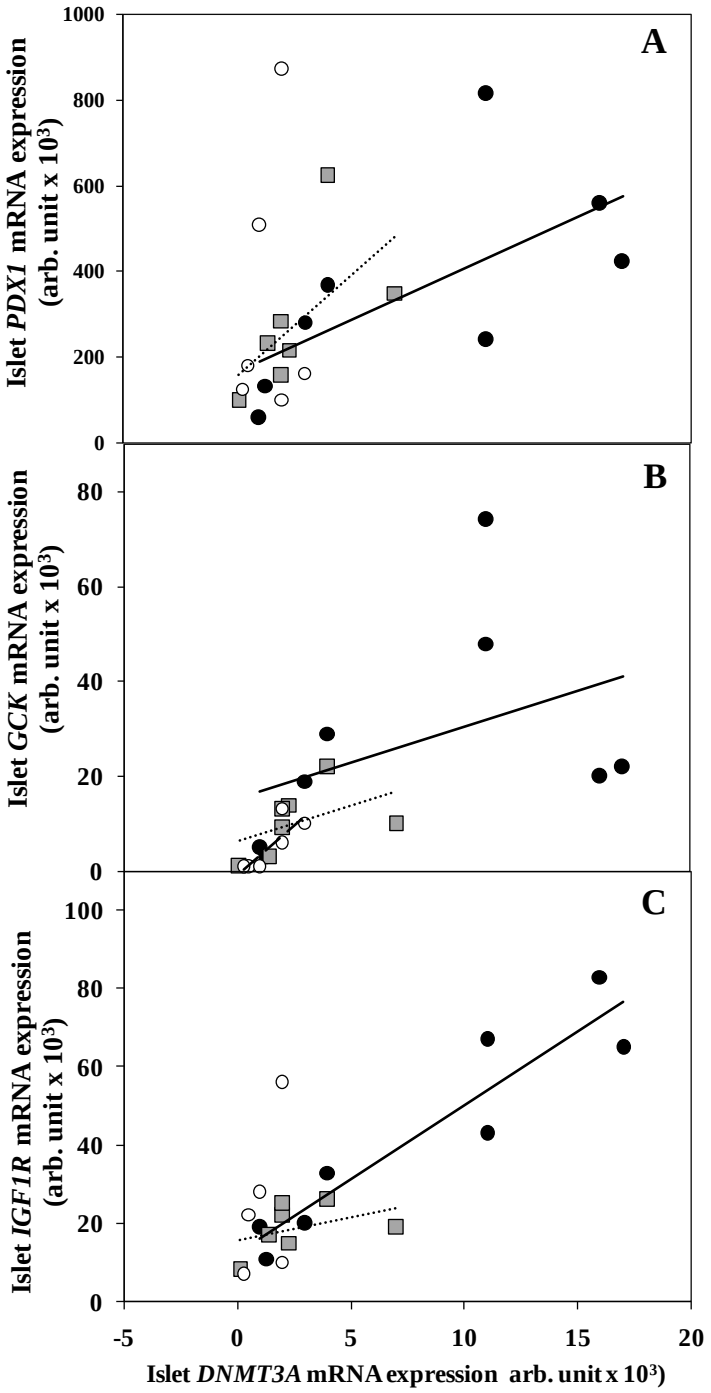

Figure 4.9 Relationships between islet mRNA expressions of (A) *PDX1*, (B) *GCK* and (C) *IGF1R* and islet *DNMT3A* mRNA expression in the young lambs.

Control (white circle, n = 6), IUGR + Veh (black circle, n = 8), IUGR + Ex-4 (grey square, n = 8), linear correlation in CON lambs (dashed line), linear correlation in IUGR+Veh lambs (solid line) and linear correlation in IUGR+Ex-4 lambs (dotted line). Points show individual animal outcomes.

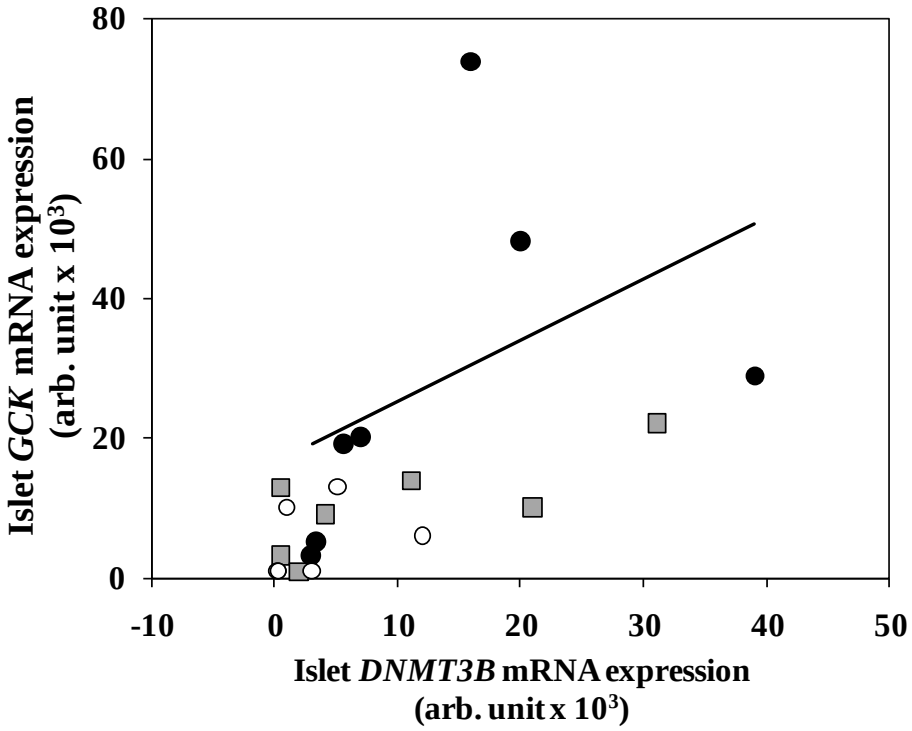

Figure 4.10 Relationship between islet *GCK* mRNA expression and islet *DNMT3B* mRNA expression in the young lambs.

Control (white circle, n = 6), IUGR + Veh (black circle, n = 8), IUGR + Ex-4 (grey square, n = 8) and linear correlation in IUGR+Veh lambs (solid line). Points show individual animal outcomes.

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4.4 DISCUSSION

This study provides the first evidence that *GCK* may be a critical modulator of altered insulin secretion after IUGR. Islet mRNA expression of *GCK*, a molecular regulator of β-cell function, was up-regulated concurrent with *in vitro* insulin hyper-secretion in young lambs after IUGR. Neonatal exendin-4 treatment of IUGR twin lambs normalised both islet mRNA expression of *GCK* and *in vitro* insulin secretion to that of control lambs, also consistent with a critical role of this gene. Expression of molecular regulators of β-cell mass was unchanged by IUGR, consistent with the lack of IUGR effect on β-cell mass at this age. Finally, vehicle-treated IUGR lambs had increased islet mRNA expression of *DNMT3B*, which is responsible for *de novo* DNA methylation, compared to control lambs, and islet mRNA expression of *GCK* was positively correlated with that of *DNMT3B* in this group of IUGR lambs, suggesting that altered islet *GCK* expression after IUGR may occur in part via epigenetic changes that can persist and maintain altered gene expression throughout life.

Up-regulation of islet *GCK* mRNA expression, a determinant of β-cell function (61), in young IUGR twin lambs was consistent with the enhanced *in vitro* β-cell function that was observed in these animals (Chapter 3, 172). GCK is a rate-limiting enzyme for glycolysis, and genetic mutations in the *GCK* gene in humans are associated with hyper-insulinaemia (223, 224), maturity-onset diabetes of the young (MODY, 225) and neonatal diabetes mellitus (226, 227). These mutations increase affinity of the GCK enzyme towards glucose and increase the rate of insulin release from β-cells (223). Thus, increased islet mRNA expression of *GCK* in the present study may explain insulin hyper-secretion of β-cells after IUGR, however the protein abundance and activity of GCK should be further investigated to confirm this potential mechanism. Heterozygous *GCK* knockout mice ($GCK^{+/-}$) develop severe hyper-

glycaemia and hypo-insulinaemia (228, 229), because their β-cells are unable to sense glucose and secrete insulin and failure of β-cell mass expansion postnatally (230). Similarly, reduced *GCK* expression in $GCK^{+/-}$ mice prevents compensatory expansion of β-cell mass in response to insulin resistance induced by high fat-feeding (231). The findings of these two studies suggest a role of *GCK* not only in regulation of β-cell function, but also in regulation of β-cell mass. Thus, whether altered islet *GCK* mRNA expression and/or early life enhanced insulin secretion *in vitro* in these young IUGR twin lambs at 16 d of age will persist with improved β-cell capacity with ageing or potentially exhaust the endocrine pancreas sooner due to increased β-cell function, leading to β-cell death, is unknown. In T2D patients, prolonged stimulation of insulin production in which the demands exceed the folding capacity of the endoplasmic reticulum of β-cells, results in accumulation of mis-folded proteins and stress, and eventually leads to β-cell dysfunction and death (232, 233).

In the present study, islet mRNA expression of *GCK* was positively correlated with that of *DNMT3B* in IUGR+Veh lambs, suggesting that altered islet *GCK* expression after IUGR may occur in part via epigenetic changes that may persist throughout life. The *GCK* gene in the rat contains CpG islands in its promoter region, and hyper-methylation of *GCK* in rat liver occurs in response to ageing (234) or consumption of a high fat diet (235) and reduces *GCK* expression. This shows that the methylation of this gene is labile and can respond to environmental factors, but also suggests that increased *DNMT3B* expression should decrease *GCK* gene expression, in contrast to the positive relationship that we observed. Perinatal exposure to the endocrine disruptor bisphenol A (BPA) in the rat, increased hepatic *DNMT3B* expression at 3-weeks of age, and induced hyper-methylation of the *GCK* promoter with subsequent reduction in its hepatic expression at 21 weeks of age (236). Our

results therefore suggest that changes in *DNMT3B* expression do not consistently predict *GCK* expression. These differential outcomes in association between islet expression of *GCK* and that of *DNMT3B* may be partly due to the fact that *DNMT3B de novo* methylation can be tissue-specific rather than global (237). For example, global over-expression of *DNMT3B* in mice did result in hyper-methylation of Wnt pathway inhibitor gene, *Sfrp2* and subsequently silenced its expression in colon but not in spleen (237). Effects of altered *DNMT3B* expression can also vary between genes, as in the same study (237), the tumor suppressor gene *Cdx2*, which contains methylation sites, was not methylated in the presence of over-expression of *DNMT3B* in colon tumors. Interestingly, in IUGR induced by feeding a maternal low protein diet in the rat, hepatic *GCK* expression in offspring was reduced during fasting and was increased on re-feeding, relative to that of control animals, without any changes in DNA methylation status (238). Together these findings may suggest that IUGR due to twinning could be affecting *GCK* expression by mechanisms other than *de novo* DNA methylation and therefore may also explain the varying relationships between islet *DNMT3B* and *GCK* expression between the present study and that in other studies in the rat. However, further analyses are needed to investigate this relationship between *DNMT3B* and *GCK* methylation and expression, the underlying mechanisms, and their consequences for β-cell function and insulin action following IUGR.

Consistent with the hypothesis that neonatal exendin-4 treatment normalised *in vitro* β-cell hyper-secretion (Figure 3.3, Chapter 3), islet expression of the β-cell function determinant, *GCK* was normalised in IUGR+Ex-4 lambs compared to their IUGR counterparts. Reduced *GCK* expression in exendin-4-treated IUGR lambs may be partly due to prevention of excess weight gain that was observed in the IUGR lambs treated with exendin-4 when compared to vehicle-treated IUGR lambs (Chapter 3,

172), presumably due to suppression of appetite. Exendin-4 treatment suppresses appetite causing reduced nutrient intake and slows gastric emptying in mice and rats (208, 209) and in adolescent and adult humans (193-195, 205, 210-212). Unfortunately, for logistical reasons, it was not possible to measure lamb milk intake and feeding behaviour during the present study to test this hypothesis. By reducing appetite, exendin-4 treatment would therefore be expected to reduce demand for insulin and the stimulus for increased β-cell function in these young IUGR lambs. Moreover, in comparison to vehicle-treated IUGR twin lambs, exendin-4-treated IUGR twin lambs had a tendency for increased β-cell mass (Chapter 3, 172), which would further reduce the need to increase their β-cell function. This prevention of up-regulated β-cell function following IUGR by neonatal exendin-4 treatment may preserve the capacity for up-regulation of β-cell function at later ages in response to developing insulin resistance.

Consistent with our previous finding that IUGR due to twinning did not alter β-cell mass at 16 d of age (Chapter 3, 172), islet gene expression of regulators of β-cell mass, including *PDX1* and *IGF2*, did not differ between groups in the present study. This is consistent with a lack of an effect of PR-induced IUGR on β-cell mass or pancreatic *PDX1* expression observed previously in lambs at 42 d of age (8). β-cell mass is increased in the adult PR sheep, though is inadequate to compensate for increasing insulin resistance, given their impaired insulin action by 1 year of age (8). In contrast to the lack of change in islet *PDX1* mRNA expression in young IUGR sheep, *PDX1* expression was reduced in the pancreas (9) and islets (33) of PR rats, although with normal β-cell mass, at 2 weeks of age. This reduced *PDX1* expression in PR rats was followed by reduced β-cell mass, impaired glucose tolerance and insulin action at 3 months of age (9). These differential effects of IUGR/PR on *PDX1* expression and its

consequences for subsequent β-cell mass may also be a consequence of the insult occurring at different stages of β-cell development, due to differences in the timing of β-cell development in these species. In humans and sheep, most pancreatic development takes place before birth, with β-cells present as early as at 0.25 gestation (10-13), and development of a mature (glucose-responsive) phenotype before birth (14-17). Rodents undergo later development of β-cells than sheep or humans, with β-cells first appearing in late gestation (0.6 gestation) and the majority of pancreatic remodelling occurring at ~10-17 d postnatal age (18-20). Thus for the human and sheep, IUGR may impose greater effects on the β-cell and its determinants because the exposure to restriction insults occur at earlier developmental stages, while for rats, the IUGR may impose different effects because some key pancreatic developmental events occur postnatally.

Neonatal exendin-4 treatment increased islet mRNA expression of *IGF1* and *IGF2R* in IUGR+Ex-4 lambs compared to CON lambs, but not compared to IUGR+Veh lambs. Even though IUGR induced by twinning did not alter islet mRNA expression of *IGF1* and *IGF2* in these young lambs at 16 d of age, a previous study of PR lambs reported increased pancreatic expression of *IGF2* and *IGF1R* in PR lambs at 43 d of age, and expression of *IGF2* was positively correlated with β-cell mass at that age (8). This suggests that activation of the *IGF* axis may be a mechanism underlying subsequent increases in β-cell mass seen in adult PR sheep (239). Further evidence for a role of *IGF2* in regulation of β-cell mass comes from studies in transgenic mice, where β-cell-specific over-expression of the *IGF2* gene resulted in bigger islets (240, 241). Consistent with positive regulation of β-cell mass by IGFs in previous studies (240, 241), in the IUGR+Ex-4 lambs in the present study, islet expression of *PDX1* correlated strongly and positively with that of the *IGF1R*, while *IGF1* expression

correlated strongly and positively with β-cell volume density, though β-cell mass does not yet differ between exendin-4-treated and vehicle-treated IUGR lambs at 16 d of age. Our results suggest that expression of the *IGF* axis is important for regulation of β-cell mass in young lambs, as shown in previous studies in rodents (242, 243), and in PR lambs (8). However, further studies are required to address whether and how *PDX1* and *IGF* signalling promote β-cell hyperplasia and proliferation and subsequently increase β-cell mass following IUGR.

In the current study, IUGR and exendin-4 treatment did not affect microRNA expression, which may suggest no involvement of microRNA in regulating β-cell mass and function in our IUGR model. Since previous studies have observed discrepancies in results of microRNA array analysis, in which expression measures for some microRNAs were inconsistent between different RNA preparation techniques (244, 245), confirmation of this microRNA array expression data by quantitative PCR is needed to further strengthen the analysis.

4.5 CONCLUSION

In conclusion, *in vitro* insulin hyper-secretion in young IUGR lambs at 16 d of age may be in part due to up-regulation of islet *GCK* expression. Consistent with our second hypothesis, neonatal exendin-4 treatment normalised islet *GCK* expression and *in vitro* insulin secretion. Neonatal exendin-4 treatment also increased islet expression of known regulators of β-cell mass without any changes in β-cell mass. Together, these effects of neonatal exendin-4 treatment may preserve the future capacity for β-cell plasticity in later life and enable adaptation to developing insulin resistance after IUGR. A long term investigation is required to assess how these molecular changes following

IUGR and neonatal exendin-4 treatment at 16 d of age affect β-cell function and mass and insulin action in the IUGR lamb into adulthood.

CHAPTER 5

5 CHAPTER 5 EFFECT OF IUGR DUE TO TWINNING AND NEONATAL EXENDIN-4 TREATMENT ON CIRCULATING PLASMA ADIPONECTIN AND ADIPONECTIN GENE EXPRESSION IN ADIPOSE TISSUES IN YOUNG LAMB

5.1 INTRODUCTION

Intrauterine growth restriction (IUGR) consistently predicts increased risk of type 2 diabetes (T2D), impaired glucose tolerance, insulin resistance and inadequate insulin secretion in humans (1, 2) and in some animal studies (3, 4). Importantly, most IUGR humans who develop insulin resistance had accelerated or catch up growth (CUG) in early life (50, 246-248). CUG is also an independent risk factor for development of insulin resistance (249). Moreover, IUGR children that undergo CUG in terms of weight have the propensity to store visceral fat rather than subcutaneous fat when compared to AGA children (116), suggesting that CUG may affect the dynamic changes in adiposity and fat deposition following IUGR. Since CUG after IUGR is a risk factor for T2D and adult obesity (188, 220), these dynamic changes in adiposity and visceral fat deposition may contribute to later development of insulin resistance in life, and imply that altered fat deposition or composition contributes to risk of T2D after IUGR.

Adiponectin is an adipokine secreted by fat tissues that can promote insulin sensitivity (98, 99), and its circulating levels are reduced in obesity-related insulin resistance (250). Importantly, adiponectin can also regulate insulin secretion and action. Adiponectin stimulates insulin secretion from isolated mice pancreatic islets by increasing exocytosis of insulin granules as well as enhancing *INS* gene expression (119). In a similar study, incubation of mice islets with adiponectin for 24 h increased

PDX1 gene expression and insulin secretion (120), which may suggest that *PDX1* is the target molecule or pathway of induction by adiponectin in β-cells. Rats that were semi-starved for 2 weeks and re-fed for a week, showed a recovery of body fat mass, presence of CUG and increased circulating plasma adiponectin together with hyperinsulinaemia, compared to control animals (251). The results of this study (251) suggest that following nutrient deprivation, the recovery of fat mass during subsequent CUG may be due to glucose redistribution to adipose tissue, and these insulin-sensitive adipose tissues signal to the pancreas to increase insulin secretion by increasing adiponectin release. These findings therefore imply that adiponectin is not just involved in insulin resistance and adiposity, but may also play a role in regulating insulin secretion and that increased circulating adiponectin may contribute to changes in β-cell function following IUGR.

The impact of IUGR on adiponectin levels in early postnatal life is unclear. Some studies have found normal plasma adiponectin concentrations in SGA when compared to AGA neonates (100, 111), while other studies found reduced plasma adiponectin in SGA in comparison to AGA neonates (112-114). These discrepancies in the findings may be due to the facts that all the above studies did not consistently classify IUGR, with some defining SGA as having birth weight below the 3rd percentile (100, 113, 114) or 10th percentile (112), or having birth weight less than 2.5 kg (111). Subsequently, plasma adiponectin is reduced in children aged 6-8 years old following IUGR, together with the presence of visceral adiposity and insulin resistance when compared to control children (115, 116). Intriguingly, in a study of SGA prepubertal children, serum adiponectin was increased in lean SGA compared to lean control children, while overweight SGA children had reduced adiponectin serum and were more insulin resistant than lean SGA (118). These results are consistent with the

hypothesis that lower adiponectin contributes to risk of insulin resistance after IUGR. Therefore, adiponectin deficiency in early life and in childhood may be a predisposing factor for later development of insulin resistance, which in turn contributes to the risk of T2D following IUGR.

In Chapters 3 and 4, we showed that IUGR lambs catch-up in terms of body weight and have normal fat mass, insulin sensitivity and enhanced *in vitro* β-cell function (Chapter 3, 172), and up-regulation of gene expression for molecular determinants that regulate β-cell function in the third week of life (Chapter 4). This is consistent with changes that might reflect increased adiponectin responses. Moreover, we showed that these changes were normalised by neonatal exendin-4 treatment. Previous studies have shown that exendin-4 can directly induce expression of adiponectin in 3T3-L1 adipocyte cell lines (252), and so this normalisation of the metabolism induced by exendin-4 treatment in IUGR lambs may also be mediated by increased adiponectin expression. Therefore in this study, we investigated whether adiponectin was increased in young IUGR lambs and its association with growth, β-cell mass and function and insulin sensitivity, and effects of neonatal exendin-4 treatment on these outcomes.

5.2 MATERIALS AND METHODS

5.2.1 Animals and treatments

Animals and treatments are described more fully in Section 2.2.1 and 2.2.2 of Chapter 2 (172). In brief, ewes underwent a timed-mating program with ultrasound scanning performed at G60 to confirm singleton or twin pregnancies. Delivery occurred naturally at term and lambs were allocated to 3 treatment groups, singleton lambs with vehicle treatment, CON (n = 7), and twins lambs. Within each set of twins, sibling twin

lambs were alternately allocated to vehicle treatment, IUGR+Veh (n = 8) or exendin-4 treatment, IUGR+Ex-4 (n = 8). Vehicle (0.5% methanol in 0.9% saline) and exendin-4 (1 nmol.kg^{-1} in vehicle, Bachem, Buberndorf, Germany) were injected s.c. daily from d 1 to d 16 of age. All lambs (singletons and twins) were supplemented with whey protein (Resource Beneprotein instant protein powder, Nestle, Australia) given orally in two equal feeds (at 0900 – 1000 h and 1600 – 1700 h), commencing at 1.25 g.kg^{-1}.d^{-1} on d 4 and increasing to 5 g.kg^{-1}.d^{-1} on and after d 7. Feeding this supplement during this period of maximal catch-up growth in IUGR lambs (176) was intended to minimise the potential for limitation of neonatal growth by milk availability in twins (174) by providing ~25% of the protein expected to be available through milk, and allowing lambs to self-regulate their milk intake to appetite.

5.2.2 Surgery and routine blood sampling

Surgery procedures and routine blood sampling are described in full in sections 2.2.4 and 2.7.1 of Chapter 2 and 3 (172). Briefly, at 4 ± 1 d of age, catheters (1.52 mm OD x 0.86 mm ID, Biocorp, Victoria, Australia) were inserted into the lamb's right femoral artery and vein under general anaesthesia, induced and maintained by Fluothane inhalation anaesthetic (Independent Veterinary Supplies, SA, Australia) as previously described (176). Routine blood samples were taken via jugular venepuncture on the day of birth (day 0) and then via arterial catheter in non-fasted lambs at 6, 11 and 15 d of age, centrifuged at 4000 rpm for 10 min at 4ºC, and plasma stored at -20ºC for subsequent analyses.

5.2.3 Plasma adiponectin analysis

Analysis of plasma adiponectin is described in full in section 2.7.1 of Chapter 2. Briefly, plasma adiponectin levels were measured in samples collected between 0800 and 1000 h, within 24 h of birth and then on 6, 11 and 15 d postnatal age, using a commercially available sheep adiponectin (ADIPOQ) ELISA kit (MyBioSource, California, USA). The intra-assay and inter-assay coefficient of variation (CV) for the ELISA assay were 1.9% and 2.3% respectively. Plasma adiponectin concentration was measured in 3 CON, 8 IUGR+Veh and 8 IUGR+Ex-4 lambs, due to loss of CON samples.

5.2.4 Growth measurements and post-mortem

Growth measurements, post-mortem and collection of fat depots are described in full in sections 2.2.3 and 2.2.4 of Chapter 2 and 3 (172). For each growth parameter, measurements were performed in duplicate at each age and then averaged. The absolute growth rate (AGR) and the fractional growth rate (FGR) were calculated as previously described (176). At 16 ± 1 d of age, lambs (CON, n = 7, IUGR+Veh, n = 8 and IUGR+Ex-4, n = 8) were humanely killed by an overdose of sodium pentobarbitone (Pentobarbitone sodium 325 $mg.mL^{-1}$ Lethabarb, Lyppards, Victoria, Australia). Omental and subcutaneous fat depots were dissected and samples of each were stored at -80ºC for later analysis.

5.2.5 Omental and subcutaneous adipose tissues RNA extraction and adiponectin gene expression analysis

Extraction of RNA from omental and subcutaneous adipose tissues and adiponectin gene expression analysis are described in full in sections 2.7.2 to 2.7.5 of

Chapter 2. Briefly, RNA samples were extracted from omental and subcutaneous adipose tissues (CON, n=6, IUGR+Veh, n=8 and IUGR+Ex-4, n=8) using a modified Trizol-based approach for adipose tissue (8, 184). RNA samples were DNAse-treated, and 2 µg of RNA was reverse transcribed to generate cDNA. Quantitative Real Time PCR was performed on a Corbett RotorGene Real Time PCR 6000 (Corbett Life Sciences, San Francisco, USA) using 5 Prime MasterMix SyBR Rox (Quantum Scientific, Queensland, Australia) according to manufacturer's instructions. Real Time PCR reactions were held at 95ºC for 2 min, followed by 40 cycles of (95ºC for 15 seconds, melting at 72-95ºC for 1 second, 60ºC for 45 seconds). Oligonucleotide primers for adiponectin were designed according to previously published ovine sequences (Table 2.3 of Chapter 2, 185) and *Taq* polymerase (Invitrogen, Victoria, Australia) amplified PCR products were cloned using a StrataClone PCR Cloning Kit (Intergrated Sciences, NSW, Australia). Plasmid DNA was purified by NucleoSpin Plasmid (Macherey Nagel, Duren, Germany) and sequenced to confirm the identity of the amplicon. The relative expression of each gene of interest was determined by normalising the absolute expression of that gene to the expression of the reference gene, *ACTB*. *ACTB* expression did not differ between treatment groups.

5.2.6 *In vivo, in vitro* insulin action and gene expression measures

In vivo and *in vitro* measures of insulin action including insulin sensitivity, insulin secretion and β-cell mass as well as analysis of gene expression of their molecular determinants were described in full in sections 2.3 to 2.5 of Chapter 2. In brief, *in vivo* insulin sensitivity was measured using hyperinsulinaemic euglycaemic clamps (HEC) and *in vivo* insulin secretion was determined by intravenous glucose tolerance tests (IVGTT) as previously described (177). The metabolic clearance rate of

insulin, basal and maximal post-hepatic insulin delivery rates, and basal and maximal insulin disposition were calculated from HEC and IVGTT data as described previously (177). *In vitro* insulin secretion was measured by static incubation and experiment of isolated pancreatic islets with various inducers of insulin secretion and concentrations as previously described (172). β-cell mass was determined by staining of insulin positive cells and calculated as described previously (8, 172). Gene expression of molecular determinants of β-cell mass and function in pancreatic islets was performed by quantitative Real Time PCR (RT PCR) on a Corbett RotorGene RT PCR 6000 (Corbett Life Sciences, San Francisco, USA) using 5 Prime MasterMix SyBR Rox (Quantum Scientific, Queensland, Australia) according to manufacturer's recommendations. The relative expression of the gene of interest was determined by normalising the absolute expression of that gene to the expression of reference gene, *ACTB*. *ACTB* expression did not differ between treatment groups.

5.2.7 Statistical analyses

Statistical analyses for this chapter are described in full in Chapter 2.8. Data for non-repeated measures on each animal were analysed by the mixed models procedure in SPSS for effects of treatment (fixed effect) and including the dam as a random (block) effect in the model to account for common maternal environment in twins. Where treatment effects ($P < 0.05$) or trends ($P < 0.1$) were apparent, means were compared by least squared differences, based on *a priori* questions to determine: 1. the effect of IUGR (CON *cf*. IUGR+Veh groups), 2. the effect of exendin-4 in IUGR lambs (IUGR+Veh *cf*. IUGR+Ex-4 groups), and 3. to assess whether exendin-4 restored values to those of controls (CON *cf*. IUGR+Ex-4 groups). Concentrations of plasma adiponectin were analysed by repeated measures ANOVA for effects of treatment

(between factor), age (within factor) and interactions, including the dam as a random (block) effect in the model to account for common maternal environment in twins. Relationships between *in vivo* measures, *in vitro* measures, gene expression, and other outcomes were analysed by Pearson's correlation.

5.3 RESULTS

5.3.1 Plasma adiponectin concentrations

At birth, plasma adiponectin did not differ between treatment groups (Figure 5.1). Plasma adiponectin tended to increase with age (P = 0.058), peaking at 6 d of age, and this pattern of increased in plasma adiponection was greater in IUGR+Veh lambs than CON lambs (P = 0.012). IUGR+Veh lambs had higher plasma adiponectin at 11 d (+29%, P = 0.019) and tended to be increased at 15 d (+29%, P = 0.083), compared to CON lambs. Neonatal exendin-4 treatment of IUGR twin lambs did not alter plasma adiponectin concentrations when compared to IUGR+Veh lambs or CON lambs overall, however plasma adiponectin concentration at 11 d of age tended to be higher in IUGR+Ex-4 lambs (+22%, P = 0.099) than in CON lambs (Figure 5.1).

5.3.2 Adiponectin mRNA expression in omental and subcutaneous fat

IUGR due to twinning increased mRNA expression of adiponectin in omental fat (+72%, P = 0.008), but not in subcutaneous fat (P > 0.2), compared to CON lambs (Figure 5.2). Neonatal exendin-4 treatment of IUGR twin lambs did not alter mRNA expression of adiponectin in omental fat when compared to IUGR+Veh (P = 0.10) or CON lambs (P = 0.22), but did increase adiponectin expression in subcutaneous fat when compared to IUGR+Veh lambs (+91%, P = 0.007), with a similar trend (+89%, P = 0.089) relative to CON lambs (Figure 5.2).

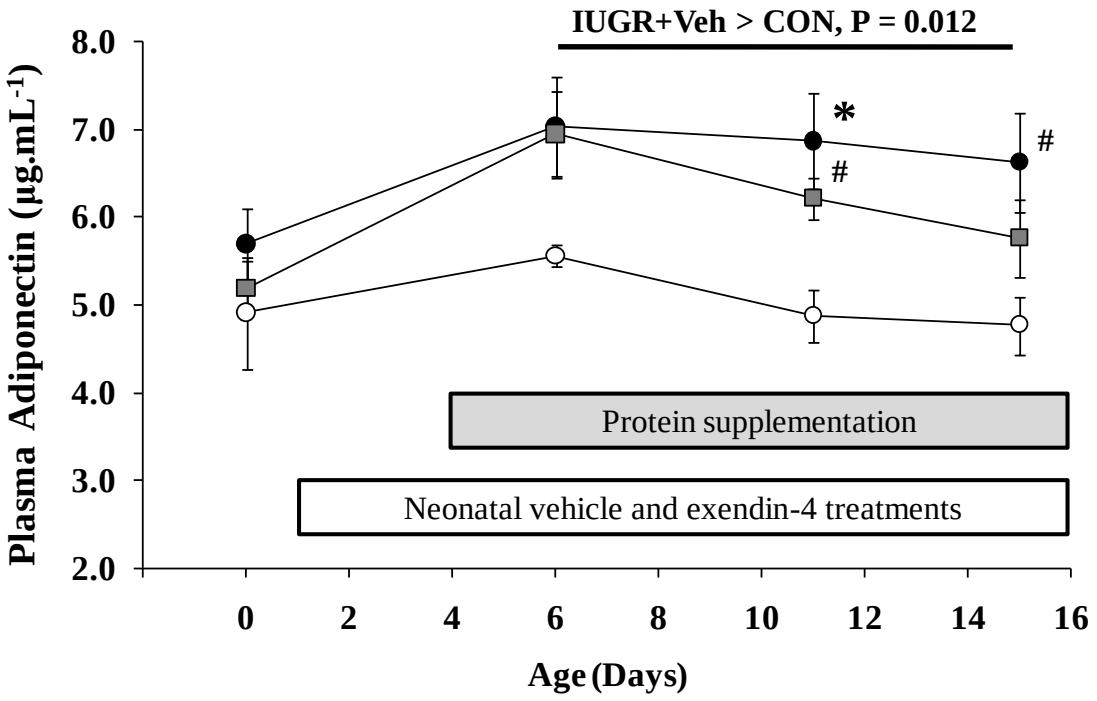

Figure 5.1 Effects of IUGR due to twinning and neonatal exendin-4 treatment on circulating plasma adiponectin from birth to 15 d of age in the young lambs. Control (white circle, n = 3), IUGR+Veh (black circle, n = 8), IUGR+Ex-4 (grey square, n = 8). All lambs were fed a protein supplement (Beneprotein) from day 4 to 16 of postnatal life to minimise the potential for restricted nutrient availability in twin lactation. Data are expressed as mean ± SEM. * $P < 0.05$, $^{\#}$ $P < 0.1$ when compared to CON.

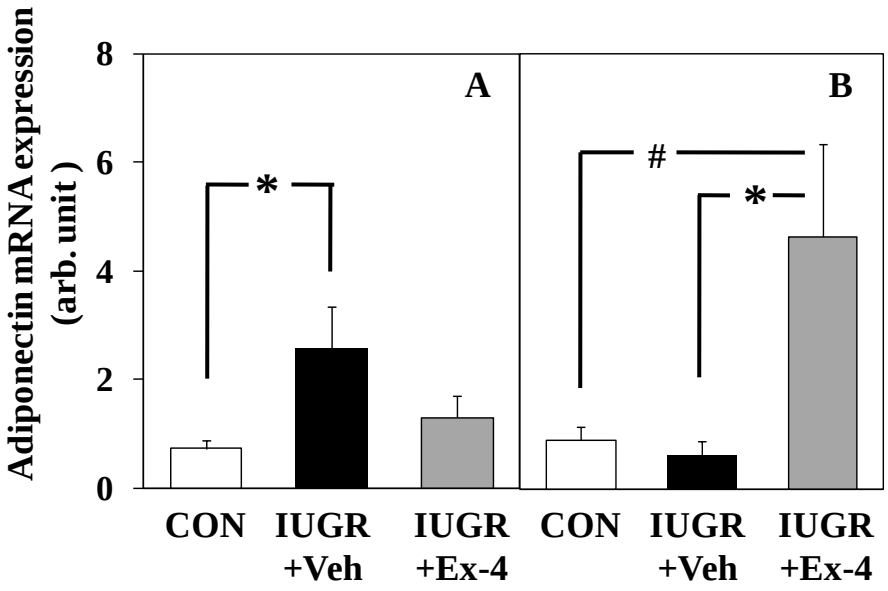

Figure 5.2 Effects of IUGR due to twinning and neonatal exendin-4 treatment on adiponectin mRNA expression in (A) omental and (B) subcutaneous fat in the young lambs at 16 d of age.

Control (white bar, n = 6), IUGR+Veh (black bar, n = 8) and IUGR+Ex-4 (grey bar, n = 8). Adiponectin mRNA expression (normalised to *ACTB*) is expressed as mean ± SEM, and differences between groups are indicated by * $P < 0.05$, $^{\#}$ $P < 0.1$.

5.3.3 Relationships between circulating plasma adiponectin and adiponectin mRNA expression in omental and subcutaneous adipose tissues

Because circulating adiponectin concentrations were only available in a small number of CON lambs (n = 3) due to loss of samples, all lambs that were not treated with exendin-4 as neonates, i.e. CON and IUGR+Veh lambs, were combined for subsequent correlation analyses (CON+IUGR). Plasma adiponectin concentration at 15 d of age did not correlate with omental fat adiponectin mRNA expression at 16 d of age in CON+IUGR or IUGR+Ex-4 groups. Plasma adiponectin concentration at 11 d of age correlated positively with omental fat adiponectin mRNA expression at 16 d of age in all lambs combined ($r = 0.69$, $P = 0.002$, $n = 18$), as well as in CON+IUGR lambs ($r = 0.63$, $P = 0.020$, $n = 11$, Figure 5.3). In CON+IUGR, plasma adiponectin concentration at 15 and 11 d of age correlated negatively with subcutaneous fat adiponectin mRNA expression at 16 d of age (15 d: $r = -0.62$, $P = 0.021$, $n = 11$ and 11 d: $r = -0.61$, $P = 0.023$, $n = 11$, Figure 5.3). No correlations were observed between plasma adiponectin at 15 and 11 d of ages and omental and subcutaneous fat adiponectin mRNA expression in IUGR+Ex-4 lambs.

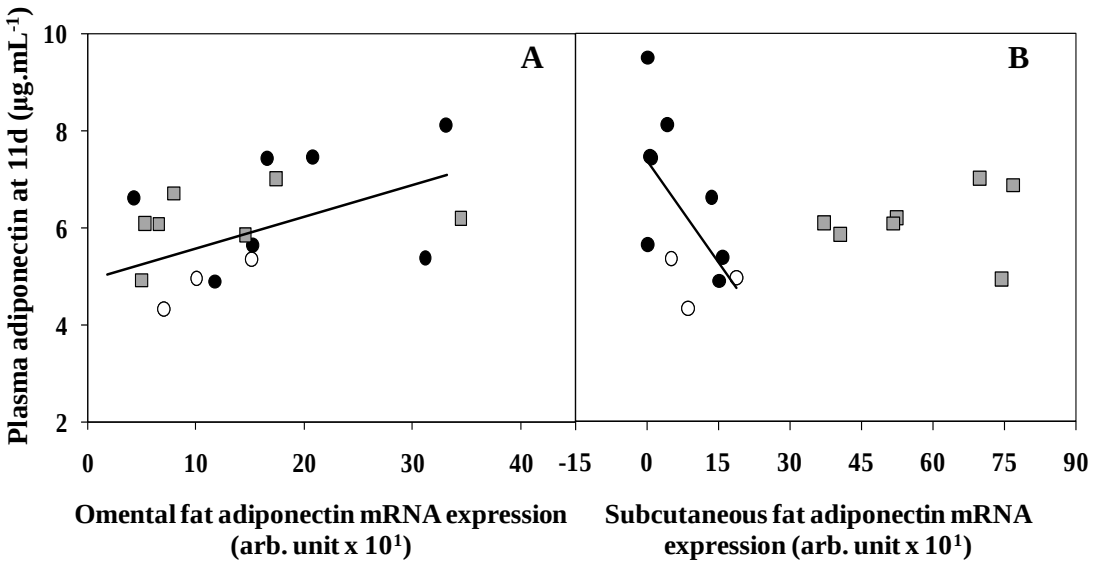

Figure 5.3 Relationships between plasma adiponectin concentration at 11 d of age and (A) omental and (B) subcutaneous fat adiponectin mRNA expression.

Control (white circle, n = 3), IUGR + Veh (black circle, n = 8), IUGR + Ex-4 (grey square, n = 8), linear correlation in CON+IUGR lambs (solid line). Points show individual animal outcomes.

5.3.4 Relationships between growth, metabolic outcomes and circulating plasma adiponectin and its expression in omental and subcutaneous fat

5.3.4.1 Relationships between neonatal growth and plasma adiponectin concentration and its expression in omental and subcutaneous fat

In CON+IUGR lambs, FGR_{weight} correlated positively with average plasma adiponectin concentration from birth to 15 d of age ($r = 0.54$, $P = 0.045$, $n = 11$) but not in IUGR+Ex-4 lambs (Figure 5.4). Absolute and fractional rates of weight gain from birth to 16 d of age did not correlate with adiponectin mRNA expression in omental fat and subcutaneous fat. However, FGR_{weight} during the protein supplementation period (from 4 to16 d of age) correlated positively with omental fat mRNA expression of adiponectin ($r = 0.56$, $P = 0.018$, $n = 14$) in CON+IUGR lambs only (Figure 5.4).

Similar relationships were also observed between average plasma adiponectin concentration from birth to 15 d of age and growth rates for shoulder height. In combined CON+IUGR lambs only, $AGR_{shoulder\ height}$ tended to correlate positively with average plasma adiponectin concentration from birth to 15 d of age ($r = 0.50$, $P = 0.060$, $n = 11$). $FGR_{shoulder\ height}$ during the nutrient supplementation period (from 4 to 16 d of age) also correlated positively with average plasma adiponectin from birth to 15 d of age ($r = 0.71$, $P = 0.011$, $n = 10$) in combined CON+IUGR lambs, and not in IUGR+Ex-4 lambs. No correlation was observed between absolute or fractional rates of shoulder height gain and adiponectin mRNA expression in either adipose depot in IUGR+Ex-4 lambs.

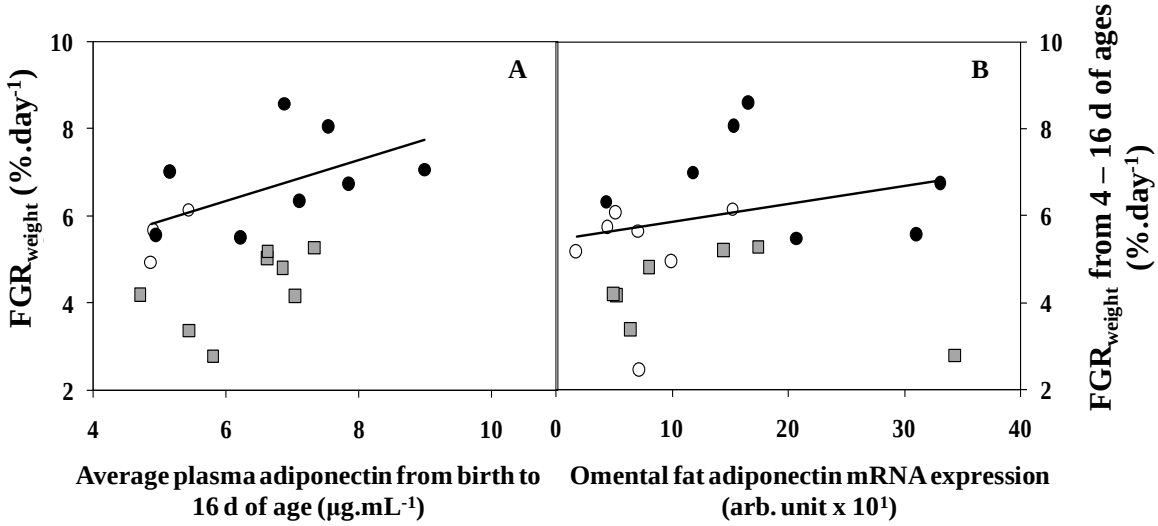

Figure 5.4 Relationships between neonatal fractional weight growth rate and (A) average plasma adiponectin from birth to 16 d of age and (B) omental fat adiponectin mRNA expression.

Control (white circle, n = 3), IUGR + Veh (black circle, n = 8), IUGR + Ex-4 (grey square, n = 8), linear correlation in CON+IUGR lambs (solid line). Points show individual animal outcomes.

5.3.4.2 Relationships between insulin sensitivity and plasma adiponectin and its expression in omental and subcutaneous fat

Insulin sensitivity at 14 d of age did not correlate with average plasma adiponectin concentration from birth to 15 d of age or with omental fat adiponectin mRNA expression either in CON+IUGR or IUGR+Ex-4 lambs. Insulin sensitivity at 14 d of age correlated negatively with subcutaneous fat adiponectin mRNA expression in IUGR+Ex-4 lambs ($r = -0.85$, $P = 0.016$, $n = 8$) and not in CON+IUGR lambs ($P > 0.3$).

5.3.4.3 Relationships between in vivo insulin secretion and plasma adiponectin and its expression in omental and subcutaneous fat

Maximal insulin disposition correlated negatively with average plasma adiponectin concentration from birth to 15 d of age in CON+IUGR lambs ($r = -0.66$, $P = 0.014$, $n = 11$) and not in IUGR+Ex-4 lambs ($P > 0.9$). Basal insulin disposition did not correlate with average plasma adiponectin concentration from birth to 15 d of age in either group. No correlation was observed between basal and maximal insulin disposition and omental and subcutaneous fat adiponectin mRNA expression in either group.

5.3.4.4 Relationships between in vitro insulin secretion and plasma adiponectin and its expression in omental and subcutaneous fat

In vitro insulin secretion at all glucose concentrations did not correlate with average plasma adiponectin concentration from birth to 15 d of age in either CON+IUGR or IUGR+Ex-4 lambs. However in CON+IUGR lambs, *in vitro* insulin secretion at all glucose concentrations correlated positively with plasma adiponectin at 6 d of age (Basal: $r = 0.74$, $P = 0.030$, $n = 6$, Stimulated 1.1mM glucose: $r = 0.78$, $P =$

0.019, n = 7 and Stimulated 11.1mM glucose: r = 0.72, P = 0.034, n = 7, Figure 5.5). In IUGR+Ex-4 lambs, stimulated *in vitro* insulin secretion at 11.1mM glucose correlated positively with subcutaneous fat adiponectin mRNA expression at d 16 (r = 0.97, P = 0.005, n = 8). No correlation was observed between *in vitro* insulin secretion and omental and subcutaneous fat adiponectin mRNA expression in CON+IUGR lambs.

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5.3.4.5 Relationships between β-cell mass and plasma adiponectin and its expression in omental and subcutaneous fat

Absolute β-cell mass and β-cell mass relative to bodyweight did not correlate with average plasma adiponectin from birth to 15 d of age in either CON+IUGR or IUGR+Ex-4 lambs. However, absolute and relative β-cell mass correlated positively with plasma adiponectin at d 11 of age in CON+IUGR lambs (absolute: r = 0.44, P = 0.088, n = 11 and relative: r = 0.55, P = 0.039, n = 11), with similar trends observed in IUGR+Ex-4 lambs (absolute: r = 0.66, P = 0.073, n = 8 and relative: r = 0.64, P = 0.086, n = 8, Figure 5.6). Absolute and relative β-cell mass also correlated positively with omental fat adiponectin mRNA expression in CON+IUGR lambs (absolute: r = 0.64, P = 0.005, n = 15 and relative: r = 0.62, P = 0.007, n = 15) and not in IUGR+Ex-4 lambs (P > 0.1, Figure 5.6). Absolute and relative β-cell mass did not correlate with subcutaneous fat adiponectin mRNA expression in either CON+IUGR or IUGR+Ex-4 lambs (data not shown).

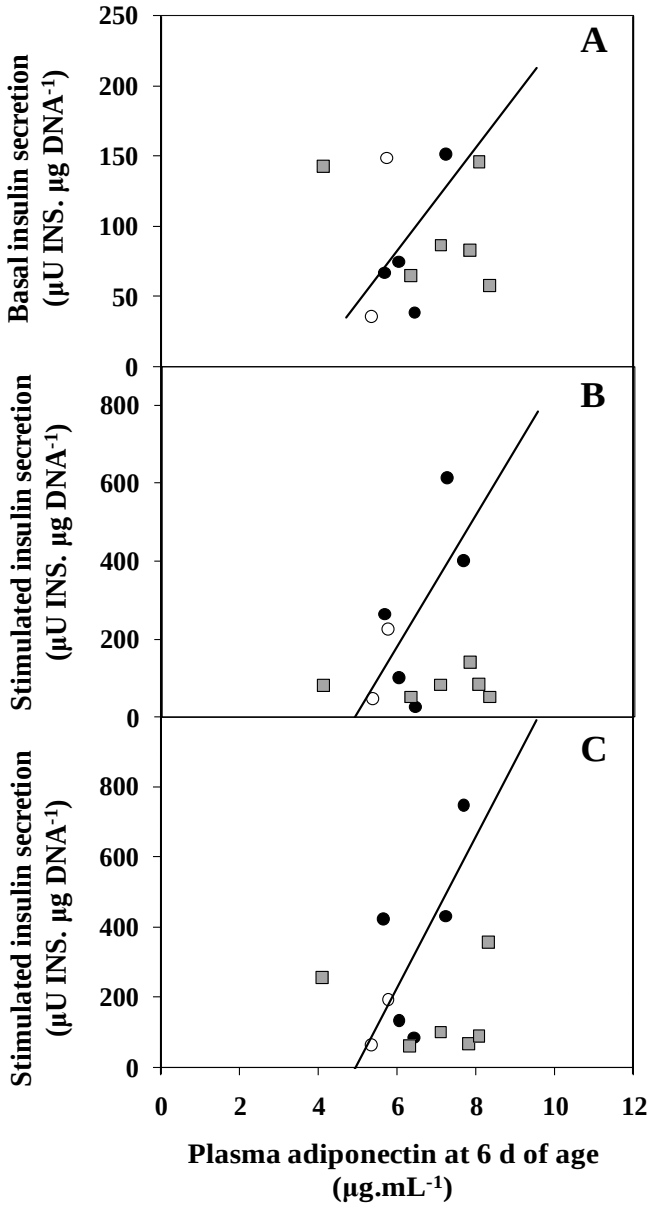

Figure 5.5 Relationships between *in vitro* insulin secretion at (A) basal 0mM glucose, (B) stimulated 1.1mM glucose and (C) stimulated 11.1mM glucose and plasma adiponectin at 6 d of age.

Control (white circle, n = 3), IUGR + Veh (black circle, n = 8), IUGR + Ex-4 (grey square, n = 8), linear correlation in CON+IUGR lambs (solid line). Points show individual animal outcomes.

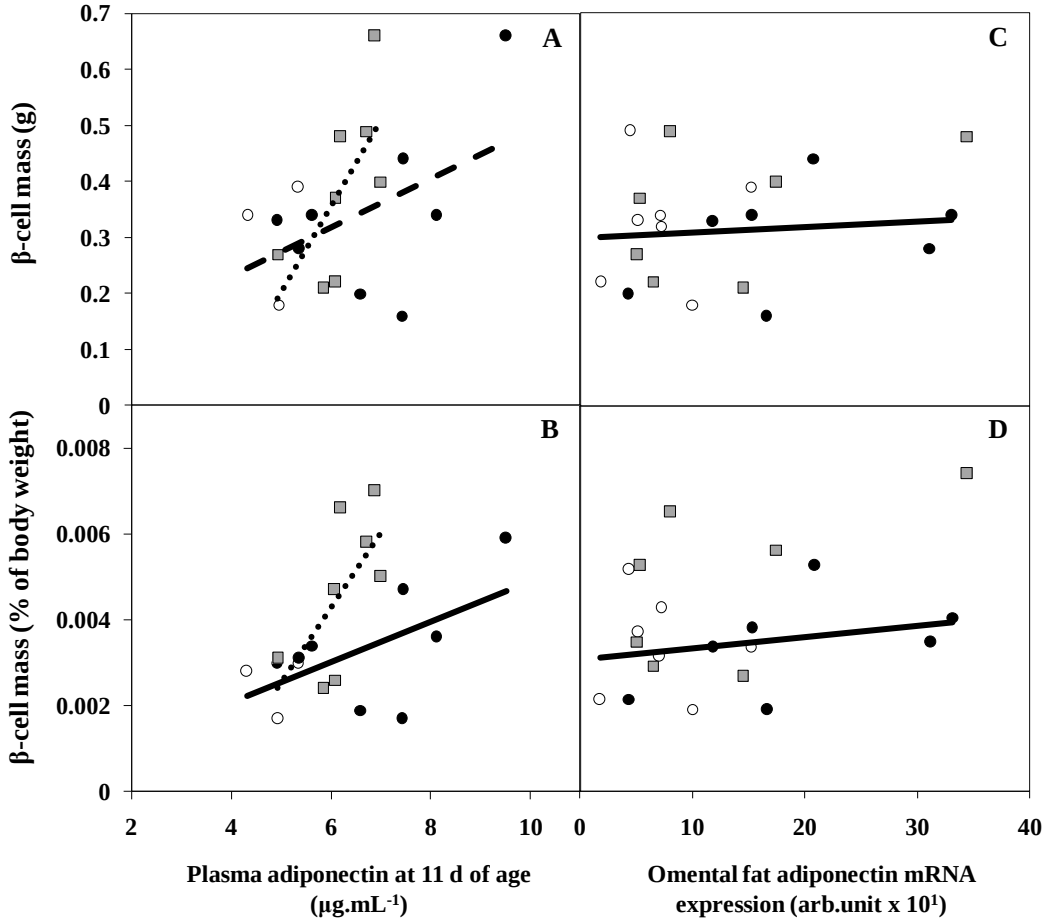

Figure 5.6 Relationships between (A) absolute β-cell mass and (B) relative β-cell mass and plasma adiponectin at 11 d of age, and between (C) absolute β-cell mass and (D) relative β-cell mass and omental fat adiponectin mRNA expression. Control (white circle, n = 3), IUGR + Veh (black circle, n = 8), IUGR + Ex-4 (grey square, n = 8), significant linear correlation in CON+IUGR lambs (solid line), trend linear correlation in CON+IUGR lambs (dashed line), and trend linear correlation in IUGR+Ex-4 lambs (dotted line). Points show individual animal outcomes.

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5.3.4.6 Relationships between pancreatic islet mRNA expression of molecular regulators of β-cell mass and function and plasma adiponectin and its expression in omental and subcutaneous fat

In CON+IUGR lambs, islet mRNA expression of genes that regulate β-cell mass and function did not correlate with average plasma adiponectin concentration from birth to 15 d of age. In IUGR+Ex-4 lambs, however, islet mRNA expression of *PDX1* (r = -0.79, P = 0.019, n = 8), *INSR* (r = -0.72, P = 0.043, n = 8), and *KCNJ11* (r = -0.72, P = 0.046, n = 8) correlated negatively with average plasma adiponectin concentration from birth to 15 d of age.

In CON+IUGR lambs, islet mRNA expression of *PIK3CB* and *GCK* correlated positively with omental fat adiponectin mRNA expression (*PIK3CB*: r = 0.58, P = 0.019, n = 13 and *GCK*: r = 0.38, P = 0.050, n = 14, Figure 5.7). Also in CON+IUGR lambs islet mRNA expression of *INS* correlated positively and islet mRNA expression of *CACNA1D* correlated negatively with subcutaneous fat adiponectin mRNA expression (*INS*: r = 0.46, P = 0.055, n = 13 and *CACNA1D:* r = -0.58, P = 0.015, n = 14). In IUGR+Ex-4 lambs, islet mRNA expression of *PIK3CB, INS* and *IGF2* correlated positively with adiponectin mRNA expression in omental fat (*PIK3CB*: r = 0.76, P = 0.059, n = 7, Figure 5.7, *INS*: r = 0.85, P = 0.033, n = 6 and *IGF2*: r = 0.72, P = 0.069, n = 7) and did not correlate with adiponectin expression in subcutaneous fat (all P > 0.4).

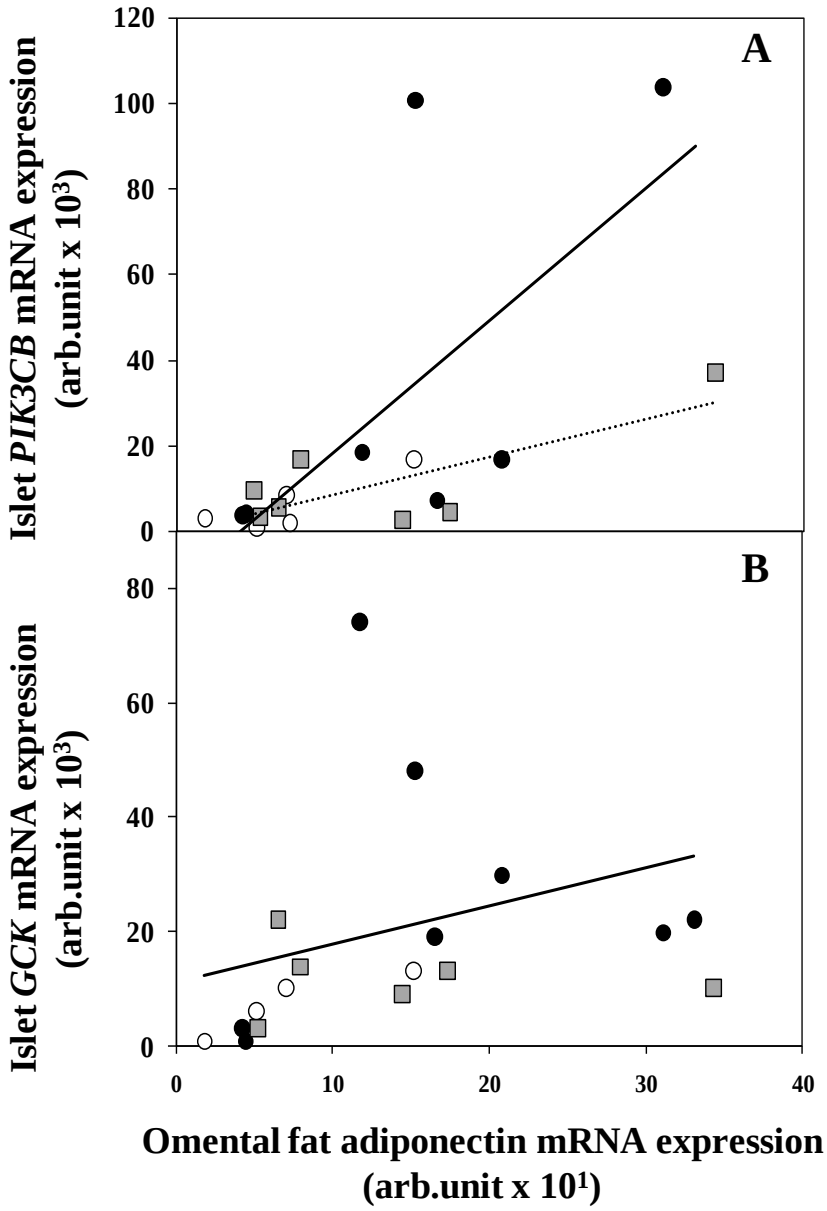

Figure 5.7 Relationships between (A) islet *PIK3CB* mRNA expression and (B) islet *GCK* mRNA expression and omental fat adiponectin mRNA expression.

Control (white circle, n = 6), IUGR + Veh (black circle, n = 8), IUGR + Ex-4 (grey square, n = 8), significant linear correlation in CON+IUGR lambs (solid line), and trend for a linear correlation in IUGR+Ex-4 lambs (dotted line). Points show individual animal outcomes.

5.4 DISCUSSION

In contrast with previous reports of reduced adiponectin in human IUGR neonates and infants, IUGR due to twinning in sheep up-regulated adiponectin expression in omental fat and increased circulating adiponectin levels during the neonatal period. Circulating adiponectin concentration and expression in omental fat correlated positively with glucose-stimulated insulin secretion and β-cell mass in combined control and IUGR+Veh lambs. Despite the profound reduction in visceral fat mass induced by neonatal exendin-4 treatment, circulating adiponectin concentrations were not reduced in exendin-4-treated IUGR lambs, possibly due to up-regulation of adiponectin expression in subcutaneous fat in these animals. These results suggest that elevated adiponectin after IUGR may contribute to up-regulation of insulin secretion.

IUGR induced by twinning increased plasma adiponectin concentrations between 11 and 15 d of age and up-regulated adiponectin expression in omental fat of IUGR+Veh lambs when compared to CON lambs. In a study of non-diabetic humans, serum adiponectin was negatively correlated with central body fat area rather than subcutaneous fat area (253), and in another study of obese non-diabetic humans, serum adiponectin was positively correlated with visceral fat adiponectin mRNA expression (254). This suggests that adiponectin secretion into circulation is predominantly from visceral fat rather than subcutaneous fat. Further evidence for a role of omental fat on regulation and production of circulating adiponectin comes from a study of isolated human omental and subcutaneous adipose tissues, in which a 12 h incubation with insulin *in vitro* increased adiponectin secretion in omental adipose tissues, but not in subcutaneous adipose, while basal adiponectin secretion did not differ between the two tissues (255). This further confirms that visceral fat is a major source of circulating adiponectin in the body. Therefore, the up-regulation of adiponectin expression in the

omental fat of the young IUGR+Veh lambs in the current study may be the source of their elevated circulating plasma adiponectin. The elevated plasma adiponectin we observed in IUGR+Veh lambs, compared to controls, differs from reports in human IUGR neonates and children, in whom plasma adiponectin was lower in IUGR group when compared to controls (112-116). In other studies however, no difference in circulating adiponectin was observed between IUGR and control groups (118, 256, 257). Increasing adiponectin between samples collected at birth and those at d 6 in the present study may reflect developmental changes and/or effects of the protein supplement. During weight loss in obese humans, adiponectin increased to a greater extent in those fed a high-protein diet than in those fed a high-carbohydrate diet (258). Additionally, adiponectin fluctuates diurnally in humans (182), and this may contribute to variable results between studies. Although adiponectin does not vary diurnally in horses (183), whether there are daily changes in sheep has not yet been established. As all lambs were sampled at similar times of day this is unlikely to explain the between-group differences in circulating adiponectin in the present study. Variable effects of IUGR in circulating adiponectin in human studies suggests that adiponectin production in IUGR neonates and children may also be affected by other factors such as prematurity status, postnatal growth rates, diet and adiposity, and these factors may also contribute to the differing results between the present study in young IUGR lambs and previous reports in human IUGR neonates and children.

In the present study, the *in vitro* insulin hyper-secretion (Chapter 3, 172) and increased islet mRNA expression of regulators of β-cell function (Chapter 4) observed in vehicle-treated young lambs correlated positively with plasma adiponectin. Importantly, young IUGR twin lambs had increased weight gain during CUG and at d 16 had normal percentage body fat as compared to controls (Chapter 3, 172), similar to

the pattern seen in re-fed rats after nutrient deprivation (251). These suggest that recovery of fat mass after nutrition depletion or IUGR may induce metabolic changes in adipose tissue and pancreatic islets, to increase glucose uptake in adipocytes to replenish energy stores; these changes include increasing insulin sensitivity of adipose tissue and increasing insulin secretion from β-cells. The positive relationships we observed suggest that these processes may be mediated in part via actions of adiponectin (251). This hypothesised role of adiponectin in the IUGR lamb is further supported by previous findings that adiponectin can up-regulate insulin secretion and action (119-121) and thus may contribute to the changes in β-cell function following IUGR. Conversely, in the present study, *in vivo* β-cell insulin secretion measures were negatively associated with plasma adiponectin in IUGR lambs, although β-cell mass was positively correlated with plasma adiponectin. We hypothesised that adiponectin may not positively stimulate *in vivo* insulin action in the absence of insulin resistance. This is partly supported by the study of isolated mice islets, in which adiponectin did not affect insulin secretion in isolated islets of control mice, but selectively induced insulin secretion at high glucose level (16.7 nM) and inhibited insulin secretion at low glucose level (2.8 nM) in islets isolated from insulin-resistant mice (121). Whether the elevated adiponectin in young IUGR lambs will stimulate and preserve the capacity of β-cell to secrete insulin in response to later development of insulin resistance (191) and hence increased demand is unknown, and therefore requires further investigation.

Neonatal exendin-4 treatment for 16 days did not alter circulating plasma adiponectin or its expression in omental fat despite profoundly reducing visceral fat mass at 16 d of age (Chapter 3, 172), probably due to up-regulation of adiponectin expression in subcutaneous fat in these animals. Although visceral fat is a major source of circulating adiponectin (253-255), adiponectin expression in isolated subcutaneous

fat was higher than in isolated visceral fat in lean women (259) and serum adiponectin was positively correlated with adiponectin mRNA expression in subcutaneous fat of women with polycystic ovary syndrome (260). Moreover, in obese human adults, a combination of diet and weight loss decreased visceral fat mass, but induced an increase in adiponectin expression in subcutaneous fat in parallel with increased circulating adiponectin (261). These findings suggest that subcutaneous tissue may also play a role in regulating circulating adiponectin, and this may account for maintenance of circulating adiponectin in exendin-4-treated lambs. Consistent with this hypothesis, *in vitro* incubation of 3T3-L1 adipocyte cell lines with exendin-4 (2.5 nM) for 8 h, increased adiponectin protein level and its gene expression when compared to non-treated 3T3-L1 cell lines, and this induction by exendin-4 occurred via glucagon-like-peptide 1 receptor (GLP1R) activation (252). In study of 4-week-old rats, high dose exendin-4 treatment (2 $\mu g.kg^{-1}$ twice daily) increased adiponectin expression in white adipose tissue and its circulating plasma levels in high-fat diet fed rats compared to both control diet and non-treated high-fat diet groups (208), confirming an *in vivo* stimulatory effect of exendin-4 on adiponectin expression. Together these results suggest that neonatal exendin-4 treatment may change the main source of circulating adiponectin, and that increased subcutaneous fat adiponectin mRNA expression explains the normal adiponectin abundance in these animals.

Consistent with presence of CUG, normalisation of insulin hyper-secretion (Chapter 3, 172) and of expression of molecular determinants of β-cell function (Chapter 4) in IUGR lambs by neonatal exendin-4 treatment, relationships between insulin secretion and islet expression of β-cell functional determinants and circulating adiponectin and adiponectin expression in these young IUGR lambs were abolished by neonatal exendin-4 treatment. Exendin-4 directly induces adiponectin gene and protein

expression in the 3T3-L1 adipocyte cell line (252), and increases plasma adiponectin in T2D patients (262) and diet-induced obese rats (263), showing that exendin-4 can directly up-regulate adiponectin production. Moreover, exendin-4 treatment also improves insulin sensitivity and insulin action in extremely obese (205) and T2D (206) humans and rodents (197, 201, 207). The abolition of relationships between insulin secretion and circulating concentrations and gene expression of adiponectin by neonatal exendin-4 treatment may reflect the positive effects of exendin-4 on insulin action, adiponectin or both in these young IUGR twin lambs. Whether this normalisation of increased adiponectin and its association to β-cell function will prevent later IUGR-induced failure of β-cell function (8) and insulin resistance (191) is unknown and hence will require long-term follow-up study.

5.5 CONCLUSION

In conclusion, circulating plasma adiponectin and its expression in omental fat positively correlated with *in vitro* insulin secretion and β-cell mass in control and PR lambs, suggesting a role of adiponectin in regulating neonatal insulin secretion. Neonatal exendin-4 treatment did not alter plasma adiponectin levels or its expression in omental fat, but increased adiponectin expression in subcutaneous fat, which may explain maintenance of normal plasma adiponectin concentrations in IUGR+Ex-4 lambs, despite a halving of their visceral fat mass. Whether IUGR and exendin-4 treatment will alter adiponectin abundance and action including its regulation of insulin secretion in adult life where adiposity and insulin resistance become evident is unknown. Therefore, a long term investigation is required to address how these molecular changes following IUGR and neonatal exendin-4 treatment at 16 d of age

will affect adiponectin and its regulation of β-cell function and mass and insulin action in IUGR lamb to adulthood.

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CHAPTER 6

6 GENERAL DISCUSSION

Low birth weight or intrauterine growth restriction (IUGR) consistently predicts increased risk of type 2 diabetes (T2D) through impairment of glucose tolerance, insulin resistance and inadequate insulin secretion in humans (1, 2), as well as in many experimental studies in other species (3, 4). However, how IUGR exactly 'programs' development of diabetes in later life and the mechanistic basis of those impairments of β-cell mass and function that follow IUGR has required more investigation; this is a research theme of the work described in this thesis. Interestingly, postnatal administration of exendin-4 to neonatal IUGR rats normalises subsequent β-cell mass, insulin secretion and prevents later development of T2D (9); this is at least partially due to normalisation of pancreatic expression of the β-cell master regulator, *PDX1* (21). Whether this treatment would have similar effects in IUGR in a species such as the sheep, where major events in pancreatic maturation occur before birth (14, 16, 18-20) is unknown and is the second research theme within this thesis.

This thesis has addressed the effects of IUGR due to twinning and neonatal exendin-4 treatment on neonatal growth, pancreatic β-cell *in vivo* and *in vitro* insulin secretory function, β-cell mass and their expression of key regulatory genes including those of microRNAs and epigenetic pathways, and the possible involvement of altered circulating abundance and adiponectin expression in adipose tissue in the young lamb. How these various *in vivo*, *in vitro* and molecular changes in key glucoregulatory tissues following IUGR due to twinning and neonatal exendin-4 treatment may affect insulin action into adulthood is discussed. Also reported for the first time is how IUGR affects β-cell mass and function soon after birth in the young lamb, and also for the first time, the efficacy and biological activity of exendin-4 in affecting these and other measures in the sheep, at least in the context of IUGR.

6.1 *IN VIVO* AND *IN VITRO* RESPONSES TO IUGR DUE TO TWINNING AND NEONATAL EXENDIN-4 TREATMENT

Excessive visceral fat deposition during catch up growth (CUG) after IUGR is a strong risk factor for later obesity (188) and insulin resistance (218-220). In the present study, IUGR due to twinning reduced size at birth and these young IUGR twin lambs experienced accelerated neonatal CUG, achieving normal body weight (Figure 3.1, Chapter 3) and fat mass (Table 3.1, Chapter 3), compared to control lambs at 16 d of age. CUG in neonatal life in these IUGR twin lambs is consistent with neonatal growth patterns in PR lambs (141, 176) and after IUGR in humans (264, 265). Although in the present study, IUGR twin lambs had normal fat mass relative to body weight compared to control lambs, a previous study of PR lambs at 43 d of age showed that accelerated CUG and fat deposition occurred following birth and resulted in greater visceral fat in PR lambs than in their control counterparts (141). This suggests that there is a risk of obesity with ageing in the IUGR twin lambs following accelerated CUG. In the present study, young IUGR twin lambs had normal insulin sensitivity, glucose tolerance and *in vivo* insulin action when compared to control lambs (Table 3.2 and Figure 3.2, Chapter 3). However, in older PR lambs (8, 140, 141, 191), there is a shift to insulin resistance following IUGR in association with CUG, as observed in human IUGR (1, 2, 7). Similarly, previous studies in various experimental models of IUGR/PR in lambs and the rat also show that accelerated neonatal CUG and fat deposition occur in association with increased insulin action in early life (4, 141, 176, 266) but that this reverses with age. Thus, reduced insulin action was observed in PR lambs at 30 d of age (141, 191) and this became worse by 1 year of age (140). Hence, the normal insulin sensitivity, glucose tolerance and *in vivo* insulin action observed in young IUGR twin lambs in the present study may reflect the beginnings of the reversal from increased insulin

sensitivity in fetal IUGR sheep (140, 267) to insulin resistance, and this reversal may occur during the neonatal CUG that the IUGR twin lambs were experiencing at this age. It is also possible that the metabolic phenotype of twinning is milder than that of PR, although the 20% reduction in birth weight we observed in twin lambs compared to control singletons is comparable with the birth weight reduction achieved by PR (32, 141, 176, 268).

We performed *in vitro* testing to measure intrinsic β-cell function independent of systemic input from endocrine and nervous systems (178). In the present study, IUGR due to twinning enhanced *in vitro* glucose-stimulated insulin secretion (Figure 3.3, Chapter 3) or β-cell insulin hyper-secretion, relative to control lambs at 16 d of age. β-cell insulin hyper-secretion is observed in obese individuals, as well as early in the pathogenesis of T2D and is considered to be an adaptation to worsening insulin resistance and increased insulin demand (202-204). Whether this early life enhanced *in vitro* insulin secretion in our IUGR twin lambs at 16 d of age will persist as improved β-cell capacity with ageing, or potentially exhaust the endocrine pancreas sooner due to a high level of β-cell activity is unknown, however there is evidence in humans that β-cell insulin hyper-secretion precedes β-cell failure (202-204, 232, 233). *In vitro* insulin secretion was highly variable in these vehicle-treated IUGR lambs, and additional numbers would be required to assess potential factors contributing to this variability. Although PR has different effects on adult metabolic outcomes in sheep (8, 141), *in vivo* insulin secretion and effects of IUGR due to PR on insulin secretion did not differ between sexes in previous studies of young lambs (32), and we consider that sex differences are therefore unlikely to underlie the variability in insulin secretion in this group.

Prevention of excess visceral fat deposition in the neonate and possibly of β-cell hyper-secretion after IUGR are therefore priorities, since development of obesity and visceral fat deposition are strong risk factors for insulin resistance and T2D (218-220). In response to daily neonatal exendin-4 treatment for 16 days, the IUGR-induced accelerated CUG and visceral fat deposition (Figure 3.1 and Table 3.1, Chapter 3) in the young IUGR twin lambs was prevented. Since accelerated CUG and visceral fat deposition are risk factors for later obesity and T2D (188, 220), this may reduce the risk of T2D following IUGR, similar to the prevention of T2D in PR rats following 6 days of neonatal exendin-4 treatment (9). It should be noted that in these neonatal PR rats, excess weight gain or CUG does not occur before weaning (4, 9, 266, 269), in contrast to neonatal CUG and visceral fat deposition which occurred both in the present study of young twin lambs and in older PR lambs to 43 d of age (141, 176) as well as in IUGR humans (264, 265). Prevention of CUG and excess weight gained in neonatal IUGR twin lambs following neonatal exendin-4 treatment may be due to the fact that exendin-4 can cross the blood-brain barrier (215), and acts centrally via the GLP1 receptor in the hypothalamus (24, 25). These exendin-4 actions suppress appetite, decrease food and caloric intake, reduce gastric emptying and induce weight loss or slow weight gain in mice and rats (208, 209) and in adolescent and adult humans (193-195, 205, 210-212). Hence in the present study, exendin-4 treatment of the young IUGR lamb limits growth and weight and fat gain in early postnatal life, probably by reducing appetite and nutrient intake; whether this may provide beneficial outcomes in prevention of later obesity remains to be determined.

Despite the prevention of accelerated CUG and visceral fat deposition, insulin sensitivity was decreased on the 11^{th} day of exendin-4 treatment in IUGR twin lambs (Table 3.2, Chapter 3), when compared to their vehicle-treated IUGR counterparts. In

contrast, improved insulin sensitivity and glucose tolerance was observed after and during acute or chronic GLP1 or exendin-4 treatment in humans with extreme obesity (205) or T2D (200, 206), and in obese or diabetic rodents (197, 201, 207). These differential effects of exendin-4 on insulin sensitivity may depend on whether the subjects are obese before treatment, and on their stage of development and growth and possibly species. In agreement with this hypothesis, insulin sensitivity was reduced after 4 weeks of nutrient restriction in young growing animals, which may be partly due to a reduced mass of insulin-responsive tissues (214). Also consistent with the hypothesis, in older or obese animals, the net effect of restricted feeding and consequently reduced fatness is to increase insulin sensitivity (213). Improved whole-body insulin sensitivity is also due to improvements in hepatic insulin sensitivity, with lower post-prandial endogenous glucose production after 2 weeks of daily exendin-4 treatment observed in T2D patients (217). In addition to the above, exendin-4 can act centrally via the GLP1 receptor and activation of PKC-δ signalling pathways in the hypothalamus, to suppress femoral blood flow and whole body insulin sensitivity in mice (24, 25), suggesting an additional mechanism for decreased peripheral insulin sensitivity during exendin-4 treatment. Although neonatal exendin-4 treatment reduced absolute weights of lean tissues, relative weight of lean tissue was reduced in exendin-4 treated twins only relative to the control group, and not relative to vehicle-treated twin IUGR lambs, implying that exendin-4 treatment affects lean tissue deposition to a lesser extent than adipose deposition. This is important, as muscle is the site of ~80% of insulin-stimulated glucose uptake (270) and mass of lean tissue correlates positively with insulin sensitivity in healthy (271) and obese humans (272), so that loss of lean mass would be expected to adversely affect insulin sensitivity.

As a consequence of their reduced insulin sensitivity, glucose tolerance was impaired in IUGR twin lambs treated with exendin-4, even though they exhibited increased 2nd phase insulin secretion that maintained their insulin disposition (Figure 3.2 and Table 3.2, Chapter 3). This contrasts with the improved glucose tolerance observed during and after exendin-4 treatment in mature rats (197, 200, 201, 207), in β-cell depleted rats (201), and in T2D human patients (200). In some of these studies, the improved glucose tolerance during or after exendin-4 treatment reflects marked improvement of deficient insulin secretion, with stimulation of β-cell regeneration observed in β-cell depleted rats (201) and up-regulation of β-cell function during exendin-4 treatment in T2D patients (200). Interestingly, exendin-4 treatment of IUGR twin lambs normalised *in vitro* insulin hyper-secretion (Figure 3.3, Chapter 3), suggesting some normalisation of intrinsic β-cell function and its molecular determinants and possibly prevention of increased insulin action in early age. To our knowledge, this is the first study of the effects of exendin-4 on insulin action in young growing animals during the treatment. An exendin-4-treated control group was not included in the present study because our primary aim was to evaluate exendin-4 as an intervention. We would predict that acute responses to exendin-4 including suppressed appetite and therefore insulin sensitivity would occur to at least some extent in un-restricted control lambs. Based on previous studies in rats, where neonatal exendin-4 treatment improved later metabolic outcomes in PR offspring and had no effect on outcomes in control offspring (9), we would expect that 'reprogramming' effects of neonatal exendin-4 treatment would be evident primarily in PR lambs. Further studies, including of feed and nutrient intake are needed to define the underlying mechanisms for the reduced insulin sensitivity of exendin-4-treated twin IUGR lambs during treatment. Thus, despite decreased insulin sensitivity during treatment, prevention of fat

accumulation and CUG together with normalisation of the β-cell hyper-secretion in the young IUGR twin lamb suggests that neonatal exendin-4 treatment has the potential to be beneficial to insulin-regulated glucose homeostasis in later life.

6.2 MECHANISM OF IUGR PROGRAMMING – GENE EXPRESSION

Altered molecular determinants of β-cell mass and function following IUGR may contribute to the increased risk of impaired insulin action and diabetes in later life. At 16 d of age, IUGR due to twinning did not alter β-cell mass (Chapter 3, 172), islet gene expression of regulators of β-cell mass (Figure 4.1, Chapter 4), including *PDX1* and *IGF2*, in the present study. These results are consistent with a lack of PR-induced IUGR effects on β-cell mass or pancreatic *PDX1* expression in previous study of lambs at 42 d of age (8). In contrast to the lack of change in islet *PDX1* mRNA expression in young IUGR twin lambs in the present study, *PDX1* expression was reduced in the pancreas (9) and islets (33) of PR rats, albeit with normal β-cell mass, at 2 weeks of age. Therefore, it appears that in both rats and sheep, IUGR/PR does not affect β-cell mass in early postnatal life, however with ageing, differences in effects on β-cell mass emerge. Adult PR rats had reduced β-cell mass together with impaired glucose tolerance and insulin action by 3 months of age (9), in contrast to increased β-cell mass in adult PR lambs at 1 year of age, though is insufficient to compensate for increasing insulin resistance resulting in impaired insulin disposition (8). These different consequences of IUGR/PR on β-cell mass in adult animals between the species may reflect the differences in the effects of prenatal restriction on *PDX1* expression in early life, since reduced *PDX1* expression impairs the capacity to increase β-cell mass in response to demand (273). Programming of impaired β-cell mass or capacity later in life may also be influenced by the timing of IUGR insult occurring at different stages of

β-cell development, and these differ between the species. In humans and sheep, most pancreatic and β-cell development occurs before birth (10-13), whereas rodents undergo later development of β-cells than sheep or humans, with the majority of pancreatic remodelling occurred at postnatal ages (18-20). Thus for the humans and sheep, IUGR may inflict greater effects on the β-cell and its molecular determinants, because the exposure to restriction occurs at earlier developmental stages, whereas in rodents, the key pancreatic developmental events that occur postnatally will not occur in a restricted environment. There is limited evidence that severe IUGR reduces the β-cell mass at birth in humans (68) and also in IUGR sheep fetuses in late gestation, with those of low weight having the lowest β-cell mass (8).

Enhanced early life β-cell function and expression of its molecular determinants following IUGR may contribute to later β-cell dysfunction and exhaustion, in response to insulin resistance and ageing. In the present study, consistent with the fact that IUGR due to twinning induced *in vitro* insulin hyper-secretion (Figure 3.3, Chapter 3), up-regulation of islet *GCK* expression was observed in the young IUGR twin lamb (Figure 4.2, Chapter 4). GCK is a rate-limiting enzyme for glycolysis and mutations in the *GCK* gene are associated with hyperinsulinaemia (223, 224) when these mutations increase affinity of the GCK enzyme towards glucose and increase the rate of insulin release from β-cells (223). Up-regulation of islet mRNA expression of *GCK* in the present study thus may in part explain insulin hyper-secretion of β-cells, though *GCK* protein abundance and activity should be further investigated to confirm this potential mechanism. Altered *GCK* mRNA expression and early life β-cell insulin hyper-secretion may also affect β-cell function and insulin action later in life. Heterozygous *GCK* knockout mice ($GCK^{+/-}$) develop hyperglycaemia and hypoinsulinaemia (228, 229), because their β-cells are unable to sense glucose and secrete insulin postnatally

(230). In an *in vitro* study, chronic incubation of $GCK^{+/-}$ mice islets with high glucose concentration enhanced their glucose-stimulated insulin secretion, due to increased GCK protein and activity levels in comparison to those measured before the incubation (274). Importantly, in contrast to the responses in $GCK^{+/-}$ islets , similar chronic high glucose incubation in the control mice ($GCK^{+/+}$) islets increased basal insulin levels, but reduced glucose-stimulated insulin secretion and reduced GCK glucose sensing activity in comparison to before the incubation (274). These findings may suggest that prolonged exposure to high glucose in GCK-deficient islets induces compensatory mechanisms to improve β-cell function, whereas this same exposure can be detrimental to normal islets due to constant insulin production. This is consistent with previous studies in T2D patients, where constant stimulation of insulin production in response to increased insulin demand resulted in accumulation of β-cell endoplasmic reticulum stress, which eventually leads to β-cell dysfunction and death (232, 233). Whether altered islet *GCK* mRNA expression and/or early life enhanced insulin secretion *in vitro* following IUGR in these young IUGR lambs at 16 d of age, will persist with improved β-cell capacity later in life, or potentially exhaust the endocrine pancreas earlier due to a high performance of β-cell activity are unknown, thus require more investigation.

Up-regulation of pathways known to be important in β-cell survival and replication might contribute to later increases in β-cell mass after neonatal exendin-4 treatment. At 16 d of age, neonatal exendin-4 treatment did not alter β-cell mass (Table 3.3, Chapter 3) or islet *PDX1* expression (Figure 4.1, Chapter 4) in the young IUGR twin lamb in the present study. A previous study of PR rats showed that despite neonatal exendin-4 treatment not affecting β-cell mass shortly after treatment (9), it did restore their islet *PDX1* expression at 2 weeks of age (33). This rescue of islet *PDX1*

expression in early life of the PR rats had favourable effects with aging, as the adult PR rats that had received neonatal exendin-4 treatment had normal β-cell mass, while PR rats treated with saline as neonates had substantial reductions in β-cell mass and developed T2D (9). We hypothesise that similar beneficial effect of exendin-4 treatment after IUGR might also emerge with ageing in the sheep. This is supported by previous studies in rodents in which β-cell replication was increased after similar duration of exendin-4 treatment in young and adult animals, but increases in β-cell mass were sometimes not apparent until several weeks later (reviewed by 153, 192). Moreover, in the exendin-4-treated IUGR twin lamb, islet expression of *PDX1* correlated strongly and positively with that of *IGF1R*, while *IGF1* expression correlated strongly and positively with β-cell volume density. This is consistent with hyperplastic effects of *IGF* action on β-cell mass seen in other models (240, 241), although trends for differences in β-cell mass between exendin-4-treated and vehicle-treated IUGR lambs are not yet significant at 16 d of age. Our results thus suggest that expression of the *IGF* axis is important for regulation of β-cell mass in young lambs, consistent with earlier studies in rodents (242, 243), and in PR lambs (8).

Consistent with our observation that neonatal exendin-4 treatment normalised *in vitro* β-cell hyper-secretion (Figure 3.3, Chapter 3), islet mRNA expression of *GCK*, a determinant of β-cell function (Figure 4.2, Chapter 4) was normalised in IUGR twin lambs treated with exendin-4 compared to their IUGR counterparts. These normalisation effects may be partly due to suppression of appetite during exendin-4 treatment in these young IUGR lambs (Chapter 3, 172). Exendin-4 treatment suppresses appetite, and decreases caloric intake and gastric emptying in mice and rats (208, 209) and in adolescent and adult humans (193-195, 205, 210-212). Reduced appetite would therefore be expected to reduce demand for insulin and the need for

increased β-cell function in these young IUGR lambs during exendin-4 treatment. Moreover, in comparison to vehicle-treated IUGR lambs, exendin-4 treated IUGR lambs had a tendency for increased β-cell mass (Chapter 3, 172), which would further reduce the need to increase their β-cell function. Whether this prevention of up-regulated β-cell function and expression of islet determinants of β-cell function, including *GCK* following IUGR by neonatal exendin-4 treatment would preserve the capacity for up-regulation of β-cell function at later ages in response to developing insulin resistance is unknown.

6.3 MECHANISM OF IUGR PROGRAMMING – EPIGENETIC REGULATION

Programming of β-cell growth and development in early life by IUGR, leading to functional impairment later in life, may occur in part via epigenetic changes that persist and maintain altered gene expression throughout life. In the present study, the IUGR twin lamb had increased islet mRNA expression of *DNMT3B* (Figure 4.3, Chapter 4), which is responsible for *de novo* DNA methylation (22, 23), and islet mRNA expression of *GCK* was positively correlated with that of *DNMT3B* in this group (Figure 4.10, Chapter 4). Methylation of the *GCK* gene is labile and can be influenced by maternal low protein diets (238), ageing (234) or consumption of a high fat diet (235). Hyper-methylation of the *GCK* gene in rat liver reduces *GCK* expression (234, 235) and this hepatic *GCK* expression is negatively correlated with *DNMT3B* expression (236), which suggests that increased *DNMT3B* expression should decrease *GCK* gene expression, contradicting the positive relationship observed in the present study. Our results therefore suggest that changes in *DNMT3B* expression do not consistently predict *GCK* expression. These differential outcomes in relationship

between islet expression of *GCK* and that of *DNMT3B* may be partly because *DNMT3B* *de novo* methylation can be tissue-specific rather than global (237). For example, global over-expression of *DNMT3B* in mice increased hyper-methylation of the Wnt pathway inhibitor gene, *Sfrp2* and consequently silenced its expression in colon but not in spleen (237). Moreover, impacts of altered *DNMT3B* expression can also differ between target genes, as shown in the same study (237), where the tumour suppressor gene *Cdx2*, which also contains methylation sites, was not methylated in colon tumours in the presence of *DNMT3B* global over-expression. Interestingly, in IUGR induced by feeding a maternal low protein diet in the rat, hepatic *GCK* expression in offspring was reduced during fasting and was increased on re-feeding, relative to that of control animals, without any changes in DNA methylation status (238). Based on these findings, we suggest that IUGR due to twinning may be affecting islet *GCK* expression by mechanisms other than *de novo* DNA methylation which may also explain the varying relationships between islet *DNMT3B* and *GCK* expression, between the present study and that in other studies in the rat. However, further analyses are needed to investigate this relationship between islet *DNMT3B* and *GCK* methylation and expression, and the underlying mechanisms, and their effects on β-cell function following IUGR.

Consistent with normalisation of β-cell function measured as insulin hyper-secretion (Chapter 3, 172), and of islet expression of its determinant, *GCK* (Chapter 4), neonatal exendin-4 treatment abolished this positive relationship between islet expression of *DNMT3B* and *GCK*. Evidence for epigenetic mechanisms underlying long-term effects of neonatal exendin-4 was also evident from the study of how neonatal exendin-4 treatment of IUGR rats for 6 days rescued *PDX1* expression, acting by normalisation of histone acetylation activity on *PDX1* promoter region in the islets

(33). In this latter study (33), IUGR caused the loss of histone acetylation (21, 33) and triggered later DNA methylation through binding of DNMT1 to the promoter region, which progressively reduced *PDX1* expression in islets (21, 33). Exendin-4 restoration of histone acetylation on *PDX1* promoter region in one-week-old rats following IUGR persisted into adulthood and prevented DNA methylation by DNMT1 (33). Therefore, exendin-4 action in the islets may occur through histone modifications in early life before altering DNA methylation of *GCK*. Intriguingly, islet expression of *DNMT1* was reduced in IUGR twin lambs treated with exendin-4 compared to IUGR twin lambs (Figure 4.3, Chapter 4). To what extent IUGR could lead to permanent changes in β-cell genes and epigenetic status in non-rodent species, including humans, is still unknown, however this provides the possibility of these adverse molecular changes in islets following IUGR can be normalised in early life by neonatal exendin-4 treatment in young lambs.

6.4 MECHANISM OF IUGR PROGRAMMING – ADIPONECTIN REGULATION

Recovery of fat mass after nutrient depletion or IUGR induces metabolic changes in adipose tissue and pancreatic islets, to increase glucose uptake in adipocytes to replenish energy stores. These changes include increasing insulin sensitivity of adipose tissue and increasing insulin secretion from β-cells, mediated in part via actions of adiponectin (251). Consistent with this hypothesis, up-regulation of adiponectin expression in omental fat (Figure 5.2, Chapter 5) and increased circulating levels (Figure 5.1, Chapter 5) were evident in IUGR twin lambs at 16 d of age, and this may partly mediate the enhanced *in vitro* insulin hyper-secretion (Figure 3.3, Chapter 3) and up-regulation of its expression of some of its determinants in islets (Figure 4.2, Chapter

4). We also observed that *in vitro* insulin hyper-secretion correlated positively with plasma adiponectin in the IUGR twin lambs, which also had had increased weight gain during CUG, reaching similar body fat at 16 d of age compared to controls (Chapter 3, 172), similar to the growth pattern seen in re-fed rats after nutrient deprivation (251). This potential role of adiponectin in the IUGR lamb is further supported by previous findings that adiponectin can up-regulate insulin secretion and action (119-121) and thus may contribute to the changes in β-cell function following IUGR. However, in the present study, measures of *in vivo* insulin secretion were negatively associated with plasma adiponectin in IUGR lambs, though β-cell mass was positively correlated with plasma adiponectin. We hypothesised that adiponectin may not positively stimulate *in vivo* insulin action in the absence of insulin resistance. In agreement with this hypothesis, adiponectin selectively induced insulin secretion at a high glucose concentration (16.7 nM) but inhibited insulin secretion at a low glucose concentration (2.8 nM) in islets isolated from insulin-resistant mice (121). Thus, how this elevated adiponectin in IUGR twin lambs at 16 d of age will stimulate and preserve the capacity of β-cell function to secrete insulin in response to later development of insulin resistance (191) will require further investigation.

Consistent with normalisation of insulin hyper-secretion and CUG (Chapter 3, 172) and β-cell function molecular determinant expression (Chapter 4), relationships between insulin secretion and islet expression of β-cell functional determinants and circulating adiponectin and its expression in these young IUGR twin lambs were abolished by neonatal exendin-4 treatment. Exendin-4 treatment of IUGR twin lambs also did not alter circulating adiponectin concentrations and its expression in omental fat (Figure 5.1 and Figure 5.2, Chapter 5), possibly due to up-regulation of adiponectin expression in subcutaneous fat (Figure 5.2, Chapter 5) in these animals. Exendin-4

directly induces adiponectin expression and its protein production *in vitro* of 3T3-L1 adipocytes cell lines (252) and increased plasma adiponectin *in vivo* of T2D patients (262) and diet-induced obese rats (263), showing that exendin-4 can directly regulate adiponectin production. Moreover, exendin-4 treatment improves insulin sensitivity and insulin action in extremely obese (205) and T2D (206) humans and rodents (197, 201, 207). Normalisation of relationships between insulin secretion and adiponectin in IUGR twin lambs treated with exendin-4 may therefore reflect the positive effects of exendin-4 on insulin action, adiponectin or both. How neonatal exendin-4 treatment will affect adiponectin expression and circulating abundance into later postnatal life after IUGR is as yet unknown.

6.5 STUDY LIMITATIONS

Interpretation of findings of the present study needs to acknowledge several limitations. One limitation of the present study is the lack of feed intake or composition measures able to be collected on these lambs. Protein supplementation was given to the young lambs on a per body weight basis to prevent growth constraint due to potential limitation in maternal milk protein supply in twin litters (173-175). As the lambs were also able to suckle to appetite, provision of the supplement will have changed diet composition in supplemented lambs compared to un-supplemented lambs in our studies. Protein supplementation may have altered metabolic outcomes, independent of effects of IUGR or exendin-4. Protein supplementation independently promotes beneficial effects in terms of weight loss and blood glucose level in diabetic humans (275), improves fasting lipids and insulin sensitivity in obese or overweight humans (276), and inhibits appetite and increases satiety, with increased GLP1 response to feeding (277). Moreover, whey protein supplementation induced greater β-cell

response to feeding, as plasma insulin, pro-insulin and C-peptide concentrations were higher in whey supplemented group compared to casein supplemented group in diabetic humans (278). Our experimental design did not allow us to control for potential interactions between dietary composition and IUGR or exendin-4 treatment. Although the similar growth and metabolic responses to IUGR induced by twinning and IUGR induced by PR in previous studies (141, 176, 268) suggest that the protein supplement did not differentially affect metabolism in singleton control and twin IUGR lambs, additional control groups would have been required in this study to differentiate the independent effects of protein supplementation and exendin-4 treatment.

Another limitation of the present study is the unfortunate loss of control singleton plasma samples for adiponectin analysis, which limits interpretations of the circulating adiponectin concentrations and its correlations with other outcomes. Moreover, plasma adiponectin can exist in different forms such as a total/globular or high or medium or low molecular weight forms (105) and previous human study has shown that high molecular weight adiponectin is positively correlated with glucose tolerance (110). These may suggest that more extensive investigation is needed to accurately determine the abundance of adiponectin in plasma following IUGR, specifically those of different forms of adiponectin. Although a previous study of obese or overweight humans showed that whey protein or high protein diet did not alter total adiponectin concentrations despite substantial weight loss (279), another study of obese humans showed that the high and medium molecular weight form of adiponectin was increased after medium weight loss (280). Measures of the various forms of adiponectin may be warranted in future studies.

6.6 IMPLICATIONS AND FUTURE DIRECTIONS

Young IUGR twin lambs exhibited CUG in conjunction with early life up-regulation of β-cell function and islet expression of its determinant, *GCK* at 16 d of age. These molecular changes may be mediated in part by increased circulating adiponectin and its expression in omental fat, as part of the adipose tissue response to accelerated fat deposition occurring during CUG. β-cell hyper-insulin secretion occurs in obese individuals, as well as early in the pathogenesis of T2D (202-204), showing that excessive early β-cell function following IUGR can lead to subsequent β-cell dysfunction and exhaustion in response of insulin resistance and ageing later in life. Thus, the relationship between up-regulation of *GCK* expression in the islets and *in vitro* β-cell hyper-insulin secretion, between islet expression of *GCK* and *DNMT3B* in IUGR twin lambs as well as reduced *DNMT1* expression in IUGR lambs treated with exendin-4 should be further investigated. In particular, additional measures are needed to determine whether changes in islet gene expression following IUGR are reflected in changes in protein abundance. Moreover, whether these *in vivo*, *in vitro* and molecular changes in β-cell mass and function at 16 d of age of the young IUGR lambs will persist into adulthood, thus contributing to development of T2D is unknown and will require separate animal cohorts with long-term follow-up of functional and molecular outcomes.

Neonatal exendin-4 treatment prevented this IUGR-induced CUG and visceral fat deposition, increased 2nd phase insulin secretion *in vivo*, normalised *in vitro* insulin secretion and islet expression of its determinant, *GCK*, and abolished the associations between circulating adiponectin and adiponectin expression and *in vitro* β-cell hyper-insulin, at the end of treatment in the IUGR lamb. We do not yet know whether neonatal exendin-4 treatment will affect outcomes in control lambs, as the aim of the

present study was to evaluate its efficacy only in the context of IUGR. Whether these changes following neonatal exendin-4 treatment will affect outcomes in adulthood of sheep progeny, and whether it will improve adult β-cell mass and function to delay or prevent the subsequent loss of insulin secretory capacity observed after IUGR in young adult male sheep (8, 32) remains to be determined, and will require separate animal cohorts with long-term follow-up of functional and molecular outcomes following the end of exendin-4 treatment.

6.7 CONCLUSION

In conclusion, IUGR due to twinning induced CUG, early life up-regulation of *in vitro* β-cell insulin secretion and islet expression of its determinant, *GCK*, despite unaltered *in vivo* insulin action, glucose tolerance and β-cell mass in the young lambs at 16 d of age. These metabolic and molecular changes may be partly mediated by increased circulating adiponectin and its expression in omental fat, as part of adipose tissue response to drivers of CUG. Consistent with our hypothesis, neonatal exendin-4 treatment prevented this IUGR-induced CUG and decreased visceral fat deposition, increased 2^{nd} phase insulin secretion *in vivo*, normalised *in vitro* insulin secretion and islet expression of its determinants at the end of treatment in the IUGR twin lambs. Although exendin-4 treatment only tended to increase β-cell mass in young IUGR lamb, the up-regulation of islet expression of β-cell mass determinants after 16 days of exendin-4 treatment may suggest beneficial effects of exendin-4 to subsequently expand β-cell mass. This may protect the exendin-4-treated IUGR individual from a need to increase β-cell function, and preserve the capacity of β-cell for later plasticity of insulin secretion in response to development of insulin resistance with ageing. Whether these increases in β-cell function and islet expression of its determinants

following IUGR at 16 d of age and normalisation of these changes by neonatal exendin-4 treatment can affect β-cell function and mass in the IUGR lambs with ageing, especially in response to elevated insulin demand or insulin resistance is still unclear. Thus, a long term investigation is required to assess how these metabolic and molecular changes in β-cell function and mass initiated by IUGR and neonatal exendin-4 treatment during the first 16 of life will affect β-cell function and mass and insulin action in the IUGR sheep into adulthood.

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APPENDIX

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Neonatal Exendin-4 Reduces Growth, Fat Deposition and Glucose Tolerance during Treatment in the Intrauterine Growth-Restricted Lamb

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Abstract

Background: IUGR increases the risk of type 2 diabetes mellitus (T2DM) in later life, due to reduced insulin sensitivity and impaired adaptation of insulin secretion. In IUGR rats, development of T2DM can be prevented by neonatal administration of the GLP-1 analogue exendin-4. We therefore investigated effects of neonatal exendin-4 administration on insulin action and β-cell mass and function in the IUGR neonate in the sheep, a species with a more developed pancreas at birth.

Methods: Twin IUGR lambs were injected s.c. daily with vehicle (IUGR+Veh, n = 8) or exendin-4 (1 nmol.kg^{-1}, IUGR+Ex-4, n = 8), and singleton control lambs were injected with vehicle (CON, n = 7), from d 1 to 16 of age. Glucose-stimulated insulin secretion and insulin sensitivity were measured *in vivo* during treatment (d 12–14). Body composition, β-cell mass and *in vitro* insulin secretion of isolated pancreatic islets were measured at d 16.

Principal Findings: IUGR+Veh did not alter *in vivo* insulin secretion or insulin sensitivity or β-cell mass, but increased glucose-stimulated insulin secretion *in vitro*. Exendin-4 treatment of the IUGR lamb impaired glucose tolerance *in vivo*, reflecting reduced insulin sensitivity, and normalised glucose-stimulated insulin secretion *in vitro*. Exendin-4 also reduced neonatal growth and visceral fat accumulation in IUGR lambs, known risk factors for later T2DM.

Conclusions: Neonatal exendin-4 induces changes in IUGR lambs that might improve later insulin action. Whether these effects of exendin-4 lead to improved insulin action in adult life after IUGR in the sheep, as in the PR rat, requires further investigation.

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Introduction

Small size at birth or intrauterine growth restriction (IUGR) consistently predicts increased risk of type 2 diabetes mellitus (T2DM) in human studies [1,2], including independently of gestation length [3]. This relationship is consistent and significant, with ~18% of the lifetime risk of T2DM accounted for by poor growth before birth [4]. Impaired insulin sensitivity and inadequate insulin secretion are each implicated as contributing to this increased risk of T2DM in the IUGR human [1,5,6,7].

Poor fetal growth commonly reflects restricted fetal supply of oxygen and nutrients due to impaired placental growth and/or function [8]. In the sheep, surgically-induced restriction of placental growth (PR) from before mating, and small size at birth, increase insulin sensitivity in early neonatal life in association with catch-up growth and increased fat deposition [9,10]. PR nevertheless impairs glucose-stimulated insulin disposition before weaning at 1 month of age, and this progresses to impaired insulin sensitivity and blunted basal and glucose-stimulated insulin disposition in young adult males at 1 year of age [11,12]. Impaired β-cell function is the primary cause of this inadequate insulin secretion, which occurs despite increases in β-cell mass in 1-year-old males [12]. Similarly, PR late in pregnancy in rats produces progeny with normal circulating glucose and insulin levels at 1 week of age, but mild fasting hyperglycemia and hyperinsulinemia at 7–10 weeks and frank diabetes by 26 weeks [13,14]. Impaired β-cell function with later reduction in β-cell mass is also implicated in decreased insulin secretion in the PR rat postnatally [13,14]. Excitingly, administration of the GLP-1 analogue exendin-4 to neonatal PR rats normalised subsequent β-cell mass and insulin secretion and prevented later development of T2DM [15]. Prevention of T2DM by neonatal exendin-4

treatment in PR rats is at least partially due to induction and normalisation of expression of the transcription factor Pdx-1 [15,16], which regulates b-cell function as well as adaptive increases in b-cell mass [17,18], and is epigenetically down-regulated in PR rat progeny [19].

The timing of pancreatic development and maturation of b-cell function, and therefore developmental stages of exposure to IUGR and neonatal interventions, differs between species. In humans and sheep, most pancreatic development takes place before birth, with b-cells present by 0.25 gestation, islets present in mid-gestation and substantial remodelling to a mature endocrine pancreas by near term [20,21,22,23]. In both species, b-cell function is present and matures from mid-gestation onwards [24,25,26,27]. This functional maturation in humans and sheep may be driven in part by their pre-partum surge in cortisol. In contrast, rodents undergo later development of b-cells than sheep or humans, with b-cells first appearing in late gestation (0.6) and pancreatic remodelling at ~10-17 d postnatal age [28,29,30]. Neonatal surges in corticosterone and b-cell maturation in rodents are marked by increased expression of key molecular determinants of glucose-induced insulin secretion coupling [31] and mitochondrial enzymes of the NADH shuttle, essential for stimulation of insulin secretion by oxidative metabolism [32]. Exendin-4 may in part be effective in preventing PR programming of reduced b-cell mass and function in rodents, because it occurs before and during such maturation. In the present study, we have therefore treated neonatal IUGR sheep with exendin-4 and assessed whether it is able to induce changes in growth, insulin action and b-cell mass and function after IUGR in a species in which the pancreas undergoes most maturation before birth.

Materials and Methods

Ethics statement

All procedures in this study were approved by the University of Adelaide Animal Experimentation and Ethics Committee (approval M-84-2007) and complied with the Australian code of practice for the care and use of animals for scientific purposes [33].

Animal, treatments and surgery

Australian Merino ewes underwent a timed-mating program, and pregnancies were confirmed by ultrasound scanning at ~60 d gestational age (term ~150 d). Delivery occurred naturally at term and the lambs were housed in floor pens with their mothers throughout the study and allowed to suckle freely, with access to their mother's feed and water, except during experimental protocols as described below. Natural twinning was used to induce IUGR. Sibling twin lambs were injected with vehicle (0.5% methanol in 0.9% saline s.c., IUGR+Veh) or exendin-4 (1 $nmol.kg^{-1}$ s.c., IUGR+Ex-4, n = 8), with the first twin pair randomly allocated to treatments and then the heavier and lighter birth weight twin alternately allocated in order to balance birth weights between the two treatments. Exendin-4 (Bachem, Buberndorf, Germany) was prepared as a 5 nM stock in 0.5% methanol and 0.9% saline, and stored at −20°C in single use aliquots, which were thawed immediately prior to injection. Singleton lambs were injected daily with vehicle (CON, n = 7). All lambs (singletons and twins) were supplemented with whey protein (Resource Beneprotein instant protein powder, Nestle, Australia) given orally in two equal feeds (at 0900–1000 h and 1600–1700 h), commencing at 1.25 $g.kg^{-1}.d^{-1}$ on d 4 and increasing to 5 $g.kg^{-1}.d^{-1}$ on and after d 7. Feeding this supplement during this period of maximal catch-up growth in IUGR lambs [10] was intended to minimise the potential for limitation of neonatal growth by milk availability in twins [34] by providing ~25% of the protein expected to be available through milk, and allowing lambs to self-regulate their milk intake to appetite.

On d 4, catheters were inserted into the lamb's femoral artery and vein under general anaesthesia, induced and maintained by fluothane inhalation anaesthetic, as described previously [10]. Basal blood samples were collected from arterial catheters every second morning before supplement feeding. Lambs were weighed at birth and then every 2 d throughout the study. Lamb size was measured at birth and then every 4 d, and absolute (AGR) and fractional (FGR) growth rates from birth to d 16 fitted by linear regression [10].

In vivo measures of insulin secretion, sensitivity, and action

Glucose tolerance and glucose-stimulated insulin secretion were measured during an intravenous glucose tolerance test (IVGTT) at d 14, and indices of glucose tolerance and insulin secretion calculated as described previously [10,11,35]. The whole body insulin sensitivity of glucose metabolism was measured by hyperinsulinemic euglycemic clamp at d 12 [35]. Insulin sensitivity glucose, the metabolic clearance rate (MCR) of insulin, basal and maximal post-hepatic insulin delivery rates, and basal and maximal insulin disposition indices (IDI) were calculated as described previously [35].

Analysis of plasma insulin and metabolites

Plasma insulin concentrations were measured in duplicate by a double antibody, solid phase radioimmunoassay using a commercially available kit (Human insulin-specific RIA, HI-14K, Linco Research Inc., St Charles, MO, USA), which has 100% cross-reactivity with ovine insulin. The intra-assay coefficients of variation (CV) for the insulin assay were 7.2% and 5.3%, and inter-assay CV were 7.0% and 19.6% for QC samples containing 9.9 and 35.9 $mU.L^{-1}$ insulin respectively (n = 10 assays). Plasma glucose concentrations were measured by colorimetric enzymatic analysis on a Hitachi 912 automated metabolic analyser using Roche/Hitachi Glucose/HK kits (Roche Diagnostics GmbH, Mannheim, Germany).

Post-mortem

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

Pancreas and islet isolation and immunostaining and morphometric analysis

Each pancreas was rapidly dissected and weighed. Representative mixed aliquots were fixed for 48 h in 4% paraformaldehyde before embedding in paraffin wax. One section per block was immunostained to detect insulin-positive cells, and morphometric analysis of b-cells was performed as described previously, in 20 fields of view per sheep selected by random-systematic sampling [12]. Measures of in vivo b-cell function were calculated by dividing total, 1st phase and 2nd phase glucose-stimulated insulin secretion and basal and maximal IDI by b-cell mass. Pancreatic islets were obtained by collagenase digestion of pancreas at 37°C for 40 min, washing and handpicking of islets ~100 mm in diameter, with

purity confirmed by immunostaining of aliquots as previously described [36]. Islet aliquots were cultured overnight at 37°C in 95% O_2/5% CO_2 in RPMI 1640 media (Sigma Aldrich, Sydney, Australia).

In vitro b-cell secretion and responses

Static islet incubation and experiments were performed as previously described [36]. Briefly, for each animal and incubation condition, triplicate preparations of 10 islets were handpicked into 1.5 mL tubes. Static incubations were performed at 37°C for 1 hr in KRB/BSA/Forskolin media containing 0, 1.1, 11.1 mM glucose, or 15 mM KCl, or 11.1 glucose plus 5 mM Lysine, 11.1 glucose plus 5 mM Arginine, 1.1 glucose plus 10 mM Leucine, 11.1 glucose plus 10nM Epinephrine, or at 0°C for 1 hr in KRB/BSA/Forskolin media containing 11.1 glucose. Islets were then centrifuged, supernatant collected for insulin analysis and DNA was ethanol-extracted from pellets and quantified by PicoGreen dsDNA Quantification kit (Invitrogen, Melbourne, Australia). In vitro insulin secretion for each replicate was calculated as insulin concentration divided by DNA concentration. In vitro data for an animal was included in analyses provided that insulin secretion in incubations with KCl (test of maximal release) was greater than those obtained from incubations with epinephrine or at 0°C (inhibitory quality controls). Due to technical difficulties with some preparations, in vitro insulin secretion data was obtained successfully for 5 CON, 5 IUGR+Veh and 6 IUGR+Ex-4 lambs.

Statistical analysis

Data for non-repeated measures on each animal were analysed by the mixed models procedure of SPSS for effects of treatment (fixed effect) and including dam as a random (block) effect in the model to account for common maternal environment in twins. Where treatment effects or trends were apparent ($P < 0.1$), we then compared means by the LSD method, based on a priori questions to determine: 1. effects of IUGR (CON cf. IUGR+Veh groups), 2. effects of exendin-4 in IUGR lambs (IUGR+Veh cf. IUGR+Ex-4 groups), and 3. to assess whether exendin-4 restored values to those of controls (CON cf. IUGR+Ex-4 groups). We also confirmed these comparisons between IUGR+Veh and IUGR+Ex-4 groups using a paired t-test to compare twin siblings, and the significance of this test was consistent with that for LSD comparisons for all measures (data not shown). Neonatal growth patterns and glucose, insulin and insulin:glucose ratios overall and during 1st phase (0–30 min) and 2nd phase (30–210 min) of insulin secretion during the IVGTT were analysed by repeated measures for effects of treatment (between factor), time (within factor) and interactions, and including dam as a random (block) effect in the model to account for common maternal environment in twins. Glucose-stimulated in vitro insulin secretion was analysed by repeated measures for effects of treatment (between factor), glucose concentration (within factor) and interactions. Stimulation and inhibition of in vitro insulin secretion were analysed using repeated measures models for effects of treatment (between factor), stimulation (within factor, 11.1 mM glucose or KCl) or inhibition (within factor, 11.1 mM glucose or epinephrine) and interactions,

Table 1. Effect of IUGR and neonatal exendin-4 on size at birth, postnatal growth and body composition in lambs.

	CON	IUGR+Veh	IUGR+Ex-4	Significance (treatment effect)
Number of animals	7	8	8	
Size at birth				
Birth weight (kg)	6.0±0.21	4.8±0.17*	4.8±0.15*	< 0.001
Crown rump length (cm)	56.3±1.4	54.6±1.1	55.1±1.1	NS
Shoulder height (cm)	44.8±0.7	40.1±0.8*	40.9±0.7*	0.008
Abdominal circumference (cm)	40.1±0.4	35.1±0.9*	36.1±0.6*	< 0.001
Body mass index ($kg.m^{-2}$)	19.2±1.1	16.3±0.8*	16.0±0.7*	0.040
Neonatal Growth				
AGR_{weight} ($g.day^{-1}$)	309±29	327±14	211±17#	0.001
FGR_{weight} ($\%.day^{-1}$)	5.1±0.4#	6.8±0.3$	4.3±0.3#	0.001
$AGR_{shoulder\ height}$ ($cm.day^{-1}$)	0.39±0.02#	0.50±0.03*$	0.40±0.03#	0.030
$FGR_{shoulder\ height}$ ($\%.day^{-1}$)	0.8±0.06	1.1±0.1$	1.0±0.1$	NS
$AGR_{abdominal\ circumference}$ ($cm.day^{-1}$)	0.47±0.07$	0.78±0.04*$	0.54±0.04#	0.002
$FGR_{abdominal\ circumference}$ ($\%.day^{-1}$)	1.1±0.1$	2.2±0.1*$	1.5±0.1#	0.001
Postmortem (d 16)				
Body weight (kg)	11.8±0.5	10.1±0.3	8.3±0.25#	< 0.001
Total liver weight (g)	296±19	285±17	214±7#	0.002
Total liver weight (% of body weight)	2.7±0.1$	2.8±0.1$	2.5±0.07	NS
Summed muscle mass (g)	265±13	228±8*	183±9#	< 0.001
Summed muscle mass (% of body weight)	2.4±0.07	2.2±0.04	2.1±0.08*	0.055
Visceral fat (g)	132±19	118±11	41.7±6.3#	< 0.001
Visceral fat (% of body weight)	1.1±0.17	1.1±0.09	0.49±0.062#	< 0.001

Neonatal growth rates are from d 0 to 16. NS: $P > 0.1$, * different from CON ($P < 0.05$), $ different from IUGR+Veh ($P < 0.05$).

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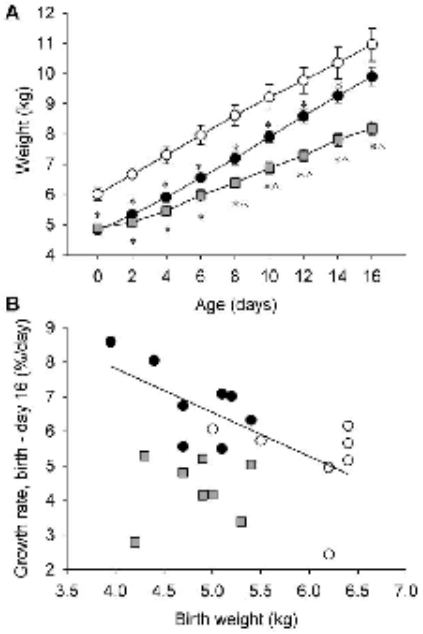

Figure 1. Effect of IUGR and neonatal exendin-4 treatment on neonatal growth. Neonatal exendin-4 treatment reduced weight of twin IUGR lambs from 8 days of age (A), and abolished the negative relationship between birth weight and neonatal fractional growth rate (B). CON (white circle) and IUGR+Veh (black circle) lambs were treated once daily with vehicle (0.5% methanol in saline s.c.) and IUGR+Ex4 (gray square) lambs were treated once daily with exendin-4 (1 nmol.kg^{-1} s.c.). Data in Figure 1A are means ± SEM, and data in Figure 1B are individual animal outcomes. * different from CON (P < 0.05), ^ different from IUGR+Veh (P < 0.05).
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and by mixed model as described above for incubations with individual secretagogues.

Results

Size at birth, neonatal growth and body composition

Lamb weight, abdominal circumference and body mass index at birth were reduced in twin lambs (all IUGR groups) compared to singleton lambs (each P < 0.001, Table 1). Absolute and fractional growth rates for weight and abdominal circumference, and absolute but not fractional growth rate for shoulder height, differed with treatment (Table 1). IUGR+Veh lambs had higher FGR for weight and abdominal circumference than CON lambs (P = 0.022 and P = 0.001 respectively), and by d 16, there was no difference in weight between these two groups (Figure 1A). In control and IUGR+Veh lambs, FGR for weight increased as birth weight decreased (combined: R = 20.700, P = 0.002, n = 15; Figure 1B), whereas in IUGR+Ex4 lambs, neonatal FGR was not related to birth weight (P > 0.3; Figure 1B). Neonatal exendin-4 treatment reduced neonatal growth rates (Table 1) including for weight (AGR_{weight}, 235%, P < 0.001), linear growth ($AGR_{shoulder\ height}$, 220%, P = 0.031), and organ growth ($AGR_{abdominal\ circumference}$, 230%, P = 0.007), and this group were lighter than CON and IUGR + Vehlambs at d16 (Figure 1). Neonatal exendin-4 reduced body weight (218%, P = 0.016) and relative visceral fat mass (257%, P < 0.001) at post-mortem compared to IUGR+Veh lambs (Table 1). IUGR+Ex-4 lambs had lower absolute liver weights than CON (228%, P = 0.001) or IUGR+Veh (225%, P = 0.009) lambs, and lower relative liver weights (as a proportion of body weight) than IUGR+Veh lambs (29%, P = 0.021). Absolute summed muscle mass was lower in IUGR+Veh lambs (23.7%, P = 0.017) relative to CON, and was decreased by exendin-4 treatment relative to CON (227%, P < 0.001) and IUGR+Veh (225%, P = 0.004) groups. Relative summed muscle weight also tended to be lower in IUGR+Veh (27.6%, P = 0.093) and was lower in IUGR+Ex-4 (29.5%, P = 0.019) compared to CON lambs (Table 1).

Insulin secretion, sensitivity and action

Fasting glucose and insulin levels, glucose tolerance and overall, 1st phase and 2nd phase insulin secretion in vivo were similar in IUGR+Veh and CON lambs (each P > 0.1, Table 2). Fasting plasma glucose (d 14) was reduced in IUGR+Ex-4 lambs compared to CON (210%, P = 0.022) and IUGR+Veh lambs (29%, P = 0.019, Table 2). Conversely, glucose tolerance was impaired (increased glucose AUC) in IUGR+Ex-4 lambs overall (+132%, +156% respectively), during first phase insulin secretion (+41%, +57%), and during second phase insulin secretion compared to CON and IUGR+Veh lambs (each P##0.02, Table 2). Across the whole of the IVGTT, and within the 1st phase of insulin secretion, plasma glucose (Figure 2) changed with time (each P < 0.001). Fasting plasma glucose in fasting samples was lower in IUGR+Ex4 than in IUGR+Veh lambs (P < 0.001), and tended to be lower in IUGR+Ex4 than in CON lambs (P = 0.091). Conversely, plasma glucose during the 1st phase of insulin secretion was higher in IUGR+Ex4 than in IUGR+Veh lambs (P < 0.001), and plasma glucose during the 2nd phase of insulin secretion did not differ between groups (P > 0.3). The pattern of change in plasma glucose with time differed between groups overall (P < 0.001) and during the 1st phase of insulin secretion (P = 0.003). Fasting plasma insulin in absolute terms and relative to glucose, and insulin secretion (assessed relative to the glucose stimulus as AUC insulin/AUC glucose) did not differ between the groups (Table 2 and Figure 2). Plasma insulin (Figure 2) changed with time throughout the IVGTT (P < 0.001), and within 1st (P < 0.001) and 2nd phase (P = 0.008) of insulin response. The ratio of plasma insulin to glucose (Figure 2), an index of insulin secretion, similarly changed with time throughout the IVGTT (P < 0.001), and within 1st (P = 0.015) and 2nd phase (P = 0.005) of insulin response. Plasma insulin concentrations and the ratio of plasma insulin to glucose ratios during the IVGTT (Figure 2) were higher in IUGR+Ex4 than in IUGR+Veh lambs overall (each P < 0.001) and during the 2nd phase of insulin secretion (each P < 0.001), and did not differ between other treatment groups. IUGR+Ex-4 lambs had lower insulin sensitivity compared to CON (244%, P = 0.004) and IUGR+Veh lambs (246%, P = 0.002, Table 2). Basal and maximal insulin disposition indices did not differ between groups (Table 2).

Pancreas morphology and b-cell function

Absolute and relative pancreas weights, and numbers of b-cells per islet, b-cell volume density and absolute b-cell mass did not differ with treatment (Table 3). b-cell mass relative to body weight was greater in IUGR+Ex-4 lambs than CON lambs (+36%,

Table 2. Effect of IUGR and neonatal exendin-4 on insulin action in lambs.

	CON	IUGR+Veh	IUGR+Ex-4	Significance (treatment effect)
Number of animals	7	8	8	
Fasting				
Plasma glucose (mmol.L^{-1})	6.4760.26	6.4060.11	5.8160.12$^{\$}$	0.008
Plasma insulin (mU.L^{-1})	20.466.0	15.462.2	16.462.2	NS
Plasma insulin:glucose (mU.mmol^{-1})	3.3861.11	2.4060.35	2.8360.38	NS
AUC glucose (mmol.min.L^{-1})				
Total	6266	5663	143628$^{\$}$	0.003
1st phase	60.965.4	54.862.9	86.265.7$^{\$}$	,0.001
2nd phase	161	161	57624$^{\$}$	0.017
AUC insulin (mU.min.L^{-1})				
Total	5876184	5906181	8636178	NS
1st phase	4996133	5796180	6506128	NS
2nd phase	88661	1266	2136119	NS
AUC insulin:AUC glucose (mU.mmol^{-1})				
Total	10.864.3	10.963.6	7.661.9	NS
1st phase	8.962.8	10.763.5	7.861.6	NS
2nd phase	26.5625.9	0.360.3	9.662.8	NS
Insulin sensitivity (mg.L.mU^{-1}.kg^{-1}.min^{-1})	0.09760.010	0.10060.011	0.04760.009$^{\$}$	0.003
Basal DI (mg.mL.kg^{-2}.min^{-2})	69.7631.2	39.565.5	28.4610.0	NS
Maximal DI (mg.mL.kg^{-2}.min^{-2})	138628	119627	97637	NS

Glucose and insulin AUC were measured during an IVGTT (0.25 g glucose.kg^{-1}) at d 14. 1st and 2nd phase values for insulin and glucose were measured from 0–30 and from 30–210 minutes after glucose administration, respectively. Insulin sensitivity was measured during a hyperinsulinemic euglycemic clamp (2 mU insulin.kg^{-1}.min^{-1}) at d 12. NS: P . 0.1, $ different from CON (P , 0.05), - different from IUGR+Veh (P , 0.05).

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P = 0.039, Table 3). IUGR+Ex-4 lambs also tended to have higher relative b-cell mass than IUGR+Veh lambs (+28%, P = 0.083, Table 3). Measures of b-cell function did not differ between treatments (Table 3).

In vitro b-cell secretory function

Islet insulin secretion (Figure 3) increased with increasing glucose concentration between 0 and 11.1 mM overall (P = 0.006). Glucose-stimulated insulin secretion tended to be higher overall in IUGR+Veh compared to CON lambs (+420%, P = 0.081), did not differ between IUGR+Ex4 lambs and CON lambs (P = 0.9) and tended to be higher in IUGR+Veh lambs than in IUGR+Ex4 lambs (+20%, P = 0.087). At the highest glucose concentration (11.1 mM), IUGR +Vehlambs had higher insulin secretion than CON lambs (+66%, P = 0.046) and tended to have higher insulin secretion than IUGR+Ex-4 lambs (+58%, P = 0.066 respectively, Figure 3). Within each group of lambs, in vitro insulin secretion at 11.1 mM glucose was between 1.6 and 2-fold higher than that at 0 mM glucose (Figure 3). In vitro insulin secretion was similar from islets incubated with 15 mM KCl or 11.1 mM glucose (P . 0.5), and the response to KCl was similar between treatments (P . 0.8). In vitro insulin secretion was suppressed by epinephrine treatment compared to glucose-stimulated insulin secretion (262%, P = 0.001). Suppression of glucose-stimulated insulin secretion by epinephrine was greater in IUGR+Veh than CON lambs in absolute terms (2173 cf. 247 mU insulin/mgDNA, P = 0.044), but not as a proportion of insulin secretion in the absence of epinephrine (228.8% cf. 27.8%, P = 0.274). Epinephrine suppression of glucose-stimulated insulin secretion was similar in islets from IUGR-Ex4 to that in other groups (P . 0.1 for each). Lysine-, arginine- and leucine-stimulated in vitro islet insulin secretion did not differ between treatment groups (each P . 0.3, data not shown).

Discussion

In the present study, twin IUGR lambs caught up in weight by 16 d of age, and had normal in vivo insulin action in their second week of life, with similar b-cell mass to singleton control lambs. Glucose-stimulated in vitro insulin secretion was increased in the IUGR twin lamb relative to controls, suggesting up-regulated b-cell function at this age. Daily exendin-4 treatment of twin IUGR lambs during neonatal life prevented catch-up growth and fat accumulation, and normalised in vitro insulin secretion from their islets, relative to untreated IUGR twins, which may retain adaptive capacity for later life. Glucose tolerance of IUGR lambs was impaired during exendin-4 treatment however, reflecting decreased insulin sensitivity and occurred despite greater in vivo insulin secretion. This may be due to central actions of exendin-4 to inhibit food intake and insulin sensitivity [37,38,39]. Nevertheless, the reduction in fat accumulation and normalised b-cell action in vitro of IUGR lambs suggest that neonatal exendin-4 might have beneficial effects on insulin-regulated glucose homeostasis in later life. These outcomes also demonstrate the biological activity of exendin-4 for the first time in the sheep, at least in the context of individuals who had undergone growth-restriction before birth.

We found similar growth and metabolic responses to IUGR induced by twinning in this study to those seen previously after IUGR induced by restriction of placental growth and function (PR) in sheep. Like the PR lamb, the twin IUGR lambs in the

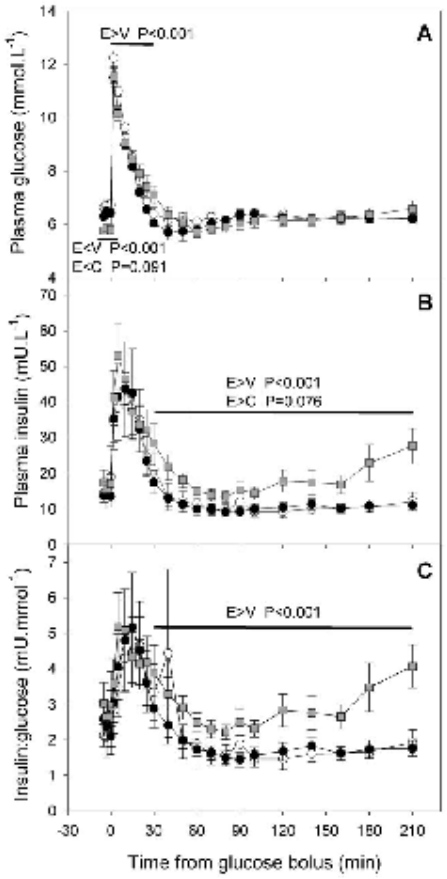

Figure 2. Effect of IUGR and neonatal exendin-4 treatment on in vivo metabolism in young lambs. Glucose tolerance (A), glucose-stimulated insulin secretion (B) and relative glucose-stimulated insulin secretion (C) were measured during an intravenous glucose tolerance test 14 days of age. CON (white circle, n = 7), IUGR+Veh (black circle, n = 8) and IUGR+Ex4 (gray square, n = 8). Data are means ± SEM. * different from CON (P < 0.05).
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present study experienced accelerated neonatal catch-up growth, achieving a normal body weight by 16 d of age in this study and by 30 d of age in our studies in PR lambs [40]. Accelerated fat deposition occurs during accelerated neonatal growth, and in humans catch-up growth is a risk factor for later obesity [41]. PR lambs have fat stores proportionate to their reduced body weight in late gestation [42], and similar to our twin lambs at 16 days in the present study, fat mass relative to body weight is similar in PR and CON lambs at 21 days despite their catch up growth [43]. By 43 days of age, however, the accelerated fat deposition results in greater visceral fat in PR lambs than their control counterparts [40]. Small size at birth in humans consistently induces insulin resistance in adults and adolescents [44], but this is preceded by enhanced insulin sensitivity in neonates, which reverses to resistance in association with catch-up growth in the first few years of life [45]. There is similar evidence of a reversal from insulin sensitivity to insulin resistance in the lamb following IUGR induced by restriction of placental growth and function (PR). The young PR lamb at 21 days of age has increased expression of insulin receptors and insulin signalling molecules in skeletal muscle [46], although in vivo insulin action was not measured. At 30 days, glucose tolerance of PR lambs is normal, despite decreased insulin action caused by falls in both in vivo insulin secretion and insulin sensitivity [40,47]. The latter reflects decreased expression of insulin-signalling pathways in skeletal muscle [47]. Impaired glucose tolerance and elevated fasting glucose emerge by 1 year of age in IUGR sheep [48]. The normal insulin sensitivity and glucose tolerance seen here in the twin IUGR lamb may therefore reflect the beginnings of the reversal from insulin sensitivity to insulin resistance occurring during the neonatal catch-up growth they are experiencing at this age.

Neither IUGR nor neonatal exendin-4 treatment in IUGR lambs altered relative b-cell mass at 16 days in the present study, consistent with the lack of effect of PR and neonatal exendin-4 treatment on b-cell mass in young postnatal rats at 2 weeks of age [49]. In the rat, reduced b-cell mass after IUGR emerges by 3 months of age in young adults, and neonatal exendin-4 treatment normalises adult b-cell mass at this age in this model [49]. We hypothesise that these beneficial effects of exendin-4 treatment after IUGR might also emerge with ageing in the sheep. This lack of an immediate response may also reflect the collection of pancreas soon after completion of exendin-4 treatment here and in PR rats. Previous rodent studies have reported increased b-cell replication after similar exendin-4 treatment durations, but differences in b-cell mass are sometimes not apparent until several weeks later [50,51]. Many of the actions of exendin-4 and GLP-1 on insulin secretion are mediated via stimulation of Pdx-1 expression, a transcription factor important for regulation of b-cell mass as well as function, and which is required for plasticity of b-cell mass and function to increase insulin secretion in response to demand. In the PR rat, prevention of later diabetes following neonatal exendin-4 treatment reflects reversal of epigenetic changes induced by PR in the Pdx-1 promoter by late gestation, that normally worsen with age and lead to decreased Pdx-1 expression, loss of b-cell function and subsequent loss of b-cell mass postnatally [15,16,19]. Intriguingly, although neonatal exendin-4 induces epigenetic changes such as increased acetylation and lysine 4 trimethylation at histone H3 in control as well as PR rat juveniles, it only increases Pdx-1 expression and b-cell mass and improves glucose tolerance in the PR progeny [15,16,19]. Indeed, the Pdx-1 promotor becomes methylated and hence partially silenced by adulthood in untreated PR rat progeny, but not in control progeny regardless of exendin-4 treatment, which implies that the levels of histone 3 acetylation and lysine 4 trimethylation in untreated control progeny is already sufficient to prevent later promoter methylation [16]. We do not yet know whether neonatal exendin-4 treatment will affect outcomes in control sheep progeny, as the aim of the present study was to evaluate its efficacy only in the context of IUGR. Whether neonatal exendin-4 acts similarly in the IUGR lamb as in the PR rat, by reversing epigenetic changes in the Pdx-1 promotor and improves adult b-cell mass and function to delay or prevent the subsequent loss of insulin secretory capacity observed after IUGR in young adult male sheep [11] remains to be determined, and will require separate animal

Table 3. Effect of IUGR and neonatal exendin-4 on pancreas morphology and b-cell function.

	CON (7)	IUGR+Veh (8)	IUGR+Ex-4 (8)	Significance (treatment effect)
Pancreas morphology				
Pancreas weight (g)	10.161.5	8.5360.93	7.9060.55	NS
Pancreas (% of body weight)	0.10360.019	0.08560.009	0.09660.007	NS
b-cell volume density	0.03360.005	0.04860.003	0.04960.007	NS
b-cell mass (g)	0.32660.038	0.34560.054	0.38760.055	NS
b-cell mass (% of body weight)	0.00360.0004	0.00346 0.0005	0.00476 0.0006*	0.070
Islet density (no.mm^{-2})	66.369.7	76.9610.3	91.6610.5	NS
b-cells/islets	10.961.4	10.561.3	12.961.3	NS
% of islets with $\geq$ 5b-cells	27.766.3	23.866.4	31.366.7	NS
b-cell function				
Insulin secretion (AUC ins) per b-cell mass (nU.min.L^{-1}.g^{-1})	16826413	19446588	21906286	NS
Basal DI per b-cell mass (mg.nL.kg^{-2}.min^{-2}.g^{-1})	187660	129625	85.4626.5	NS
Max DI per b-cell mass (mg.nL.kg^{-2}.min^{-2}.g^{-1})	441685	389689	269694	NS

NS: $P > 0.1$, * different from CON ($P < 0.05$).
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cohorts with long-term follow-up of functional and molecular outcomes

In this study, the decrease in fasting plasma glucose (~9%) and more sustained insulin secretion during exendin-4 treatment in the IUGR neonatal lamb compared to untreated IUGR siblings were generally consistent with responses to exendin-4 in rodents and humans. Medium- to long-term exendin-4 treatment in human T2DM patients (daily 5–10 mg injections for 30 and 82 weeks) [52,53,54], in the obese diabetic db/db mouse (1 nmol.kg^{-1}.d^{-1} as daily injections for 14 days) [55], and in the obese ob/ob mouse (20 mg.kg^{-1}.d^{-1}, ~ 5 nmol.kg^{-1}.d^{-1} as twice daily injections for 60 days) [56] reduces fasting blood glucose as well as HbA1c, a marker of chronic hyperglycemia. Earlier studies in humans also demonstrated acute decreases in fasting and post-prandial glucose concentrations after a single exendin-4 dose and after 5 days of twice daily injections with 5 mg exendin-4 [57]. Infusions with GLP-1 and chronic exendin-4 treatment enhance post-prandial and glucose-stimulated insulin secretion in human patients with T2DM, including restoration of 1st phase insulin secretion response to glucose, and sustained elevation of 2nd phase insulin secretion in T2DM patients [57,58,59]. In the diabetic rat, four weeks of twice-daily exendin-4 injections (105 pmol.kg^{-1}) increased 1st and 2nd phase insulin secretion during a hyperglycemic clamp [60]. Whilst we similarly observed increases in second phase insulin secretion in IUGR lambs during exendin-4 treatment, their first phase insulin secretion was unchanged. This apparent difference may be because first phase insulin secretion is normal in the IUGR lamb at this age, whereas previous reports of increased first phase insulin responses after exendin-4 or GLP-1 treatment have all been in the context of diabetes, when first phase

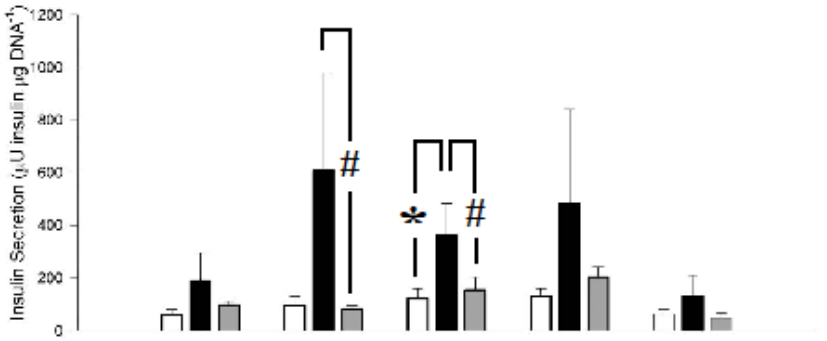

Figure 3. Effect of IUGR and neonatal exendin-4 treatment on in vitro insulin secretion from isolated islets in response to glucose and potassium chloride. CON (white bar, n = 5), IUGR+Veh (black bar, n = 5) and IUGR+Ex4 (gray bar, n = 6). Data are means ± SEM. Specific contrasts: * $P < 0.05$, # $P < 0.10$.
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secretion is impaired. The effects of exendin-4 on insulin secretion in IUGR sheep AFTER cessation of treatment remain to be investigated.

We performed in vitro testing to measure intrinsic b-cell function independent of systemic input from endocrine and nervous systems [36]. In this study, IUGR neonatal lambs had enhanced in vitro glucose-stimulated insulin secretion, or b-cell hypersecretion relative to control lambs, which occurs in obese individuals, as well as early in the pathogenesis of type 2 diabetes [61,62,63]. Interestingly, exendin-4 treatment of IUGR lambs abolished this in vitro insulin hypersecretion from isolated islets, suggesting some normalisation of intrinsic b-cell function and its determinants. Together with increased b-cell mass, this suggests that neonatal exendin-4 may improve insulin secretory capacity after IUGR.

In contrast with the improved insulin sensitivity seen after chronic GLP-1 or exendin-4 treatment in human patients with extreme obesity [64] or T2DM [65], insulin sensitivity was profoundly decreased on the 11th day of exendin-4 treatment in neonatal IUGR lambs, relative to their untreated IUGR littermates. In studies of exendin-4 action in rodents, direct measures and calculated indices of insulin sensitivity have either been increased [56,60,66], or not altered [55], immediately following or during chronic (2 – 9 weeks) exendin-4 treatment. We propose that the differential effects of exendin-4 on insulin sensitivity may depend on whether the latter is assessed during treatment or after, whether the subjects are obese and on their developmental stage and growth rate. Exendin-4 reduced weight gain in the IUGR lambs in the present study as well as in PR rat neonates [15], consistent with its actions including decreased food and caloric intake, reduced gastric emptying and induced weight loss or slowed weight gain in mice and rats [67,68] and in adolescent and adult humans [52,53,54,64,69,70,71]. It appears that restricted nutrition reduces insulin sensitivity in growing animals, possibly partly due to reduced mass of insulin-responsive tissues, whereas in older or obese animals the net effect of restricted feeding and consequently reduced fatness is to increase insulin sensitivity. Thus, feed restriction increases insulin-stimulated glucose metabolism and insulin sensitivity in adult sheep [72], but decreases insulin-stimulated glucose uptake in muscle of young growing pigs [73]. In mice, exendin-4 can cross the blood-brain barrier [74], and acts centrally to suppress femoral blood flow and whole body insulin sensitivity, via the GLP-1 receptor and activation of PKC-d signalling pathways in the hypothalamus [37,38], suggesting an additional mechanism for decreased peripheral insulin sensitivity during exendin-4 treatment. As a consequence of their reduced insulin sensitivity, and despite the increased 2nd phase insulin secretion that maintained insulin disposition, glucose tolerance was impaired in IUGR+Ex-4 lambs compared to IUGR+Veh and CON lambs. This contrasts with improved glucose tolerance observed 24 h after completion of medium- to long-term exendin-4 treatment in mature rats [56,59,60,66], during continued long-term exendin-4 treatment in b-cell depleted rats [75], and acutely in T2DM human patients [76]. In some of these studies, the improved glucose tolerance during or after exendin-4 treatment reflects marked improvement of deficient insulin secretion due to stimulation of b-cell regeneration [75] or up-regulation of b-cell function in T2DM patients [76]. Long-term exendin-4 treatment increases insulin sensitivity in obese humans, genetically-obese rodents and diabetic humans and rodents, measured either during or 16–24 h after completion of treatment [56,64,77,78]. Improved whole-body insulin sensitivity is probably also due to improvements in hepatic insulin sensitivity, with lower post-prandial endogenous glucose production after or during exendin-4 treatment [79]. To our knowledge, this is the first study of the effects of exendin-4 on insulin action treatment in young growing animals. Further studies are needed to define the underlying mechanisms for their reduced insulin sensitivity during treatment.

The profound reduction in visceral fat deposition after IUGR in response to exendin-4 is also of particular potential importance for later glucose homeostasis, given that obesity and particularly visceral fat deposition are strong risk factors for impaired glucose tolerance and T2DM [80,81]. In the PR rat, neonatal exendin-4 reduces weight gain in conjunction with prevention of later diabetes, and this may particularly reduce the risk of T2DM in IUGR subjects [15], since catch-up growth after IUGR is a risk factor for T2DM and for adult obesity [82,83]. Intriguingly, neonatal exendin-4 treatment abolished the negative relationship between birth weight and fractional growth rate in IUGR lambs in the current study. In contrast to its metabolic effects, exendin-4 reduced neonatal growth and adult size in both control and PR rat progeny [15]. This suggests that exendin-4 may act in part, but not only, via the pathway/s responsible for catch-up after IUGR, which include neonatal hyperphagia, elevated insulin sensitivity and increased abundance of thyroid hormones in IUGR lambs [40,84,85]. Longer-term evaluations of growth and composition after cessation of exendin-4 are needed to determine whether this decrease in central adiposity persists in the IUGR sheep.

In conclusion, neonatal exendin-4 treatment increased 2nd phase insulin secretion in vivo, normalised in vitro insulin secretion and decreased visceral fat at the end of treatment in the IUGR lamb. Neonatal exendin-4 treatment also improves insulin secretion and glucose tolerance in adolescent and adult rat progeny following IUGR, preventing development of diabetes in these animals [15], although the effects during treatment were not measured in the latter study. Investigation of the long-term effects of neonatal exendin-4 on glucose homeostasis and insulin action in the IUGR lamb into adulthood should be a priority for the future.

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Author Contributions

Conceived and designed the experiments: KLG JAO. Performed the experiments: KLG SAS SNBM MJD MLH. Critically revised manuscript for intellectual content: KLG MJD MLH RAS JAO. Analyzed the data: KLG SAS JAO. Wrote the paper: KLG SAS JAO.

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