

# **Early life determinants of Beta-cell function in the sheep**

**Siti Aishah Sulaiman, BSc (Biotechnology), BHSc (Hons)**

Discipline of Obstetrics and Gynaecology

School of Paediatrics and Reproductive Health

Faculty of Health Sciences

The University of Adelaide

South Australia

Australia

Thesis submitted for the fulfilment for the degree of

Doctor of Philosophy (PhD)

December 2014



THE UNIVERSITY  
*of* ADELAIDE

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

*Albert Einstein*

---

---

**TABLE OF CONTENTS**

**LIST OF TABLES AND FIGURES ..... XI**

**ACKNOWLEDGEMENTS ..... XV**

**STATEMENT OF ORIGINALITY AND AUTHENTICITY..... XVII**

**TABLE OF ABBREVIATIONS AND BIOCHEMICAL NAMES.....XVIII**

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

**ABSTRACT.....XXIV**

**1 CHAPTER 1 INTRODUCTION.....29**

1.1 OVERVIEW .....29

1.2 PREVALENCE OF TYPE 2 DIABETES (T2D) .....31

1.3 T2D AND INSULIN ACTION .....32

1.4 SIZE AT BIRTH AND T2D .....34

1.5 EARLY LIFE ORIGINS OF IMPAIRED INSULIN ACTION .....34

1.6 DETERMINANTS OF  $\beta$ -CELL FUNCTION, MASS AND PLASTICITY ..38

1.6.1 Regulation of  $\beta$ -cell function, mass and plasticity.....38

1.6.2 *PDX1*.....43

1.6.3 MicroRNA .....44

1.6.4 Epigenetic regulation of  $\beta$ -cell mass and function .....47

1.6.5 Adiponectin regulation of  $\beta$ -cell mass and function.....53

1.7 EFFECTS OF EXPERIMENTAL IUGR DUE TO RESTRICTED PLACENTAL FUNCTION ON INSULIN ACTION .....55

1.8	SEX SPECIFIC IUGR EFFECTS ON POSTNATAL INSULIN SECRETION AND ACTION.....	59
1.9	NEONATAL EXENDIN-4 AS AN INTERVENTION AFTER IUGR .....	60
1.10	SUMMARY .....	64
1.11	HYPOTHESIS AND AIMS .....	65
1.11.1	Hypothesis .....	65
1.11.2	General Aim.....	65
1.11.2.1	Aim 1.....	65
1.11.2.2	Aim 2.....	65
1.11.2.3	Aim 3.....	66
1.12	SIGNIFICANCE.....	66
<b>2</b>	<b>CHAPTER 2 MATERIALS AND METHODS .....</b>	<b>68</b>
2.1	THEORETICAL FRAMEWORK .....	68
2.2	ANIMALS AND TREATMENTS .....	69
2.2.1	Animals.....	69
2.2.2	Treatments .....	70
2.2.3	Measurement of size at birth and postnatal growth rates .....	71
2.2.4	Insertion of vascular catheters .....	71
2.2.5	Post-mortem and tissue collection .....	72
2.3	<i>IN VIVO</i> INSULIN ACTION .....	72
2.3.1	Immunohistochemical analysis of pancreas morphology.....	72
2.3.1.1	<i>Staining of insulin-positive cells.....</i>	<i>72</i>
2.3.1.2	<i>Morphometric analysis.....</i>	<i>73</i>

---

2.3.2	Assessment of the insulin axis .....	73
2.3.2.1	<i>In vivo insulin sensitivity</i> .....	73
2.3.2.2	<i>In vivo glucose tolerance, insulin secretion and action</i> .....	74
2.3.2.3	<i>Analysis of plasma hormones and metabolites</i> .....	75
2.3.3	<i>In vivo</i> assessment of pancreatic $\beta$ -cell function .....	76
2.4	<i>IN VITRO</i> ASSESSMENT OF PANCREATIC ISLET FUNCTION .....	76
2.4.1	Isolation and purification of pancreatic islets .....	76
2.4.2	<i>In vitro</i> insulin secretion .....	77
2.5	PANCREATIC ISLET GENE EXPRESSION.....	78
2.5.1	Pancreatic islet RNA extraction.....	78
2.5.2	RNA quantity and quality assessments.....	78
2.5.3	DNase treatment .....	78
2.5.4	Reverse transcription .....	79
2.5.5	Primer design .....	79
2.5.6	Quantitative real time polymerase chain reaction .....	81
2.5.6.1	<i>Preparation of cDNA</i> .....	81
2.5.6.2	<i>Cloning reaction and plasmid standard extraction</i> .....	81
2.5.6.3	<i>Sequencing of cDNA inserts</i> .....	82
2.5.6.4	<i>Real Time PCR</i> .....	83
2.5.6.5	<i>Optimisation of Real Time PCR reactions</i> .....	84
2.5.6.6	<i>Quantification of pancreatic mRNA levels</i> .....	84
2.6	PANCREATIC ISLET MICRORNA EXPRESSION .....	85
2.6.1	Pancreatic microRNA extraction, quantity and quality assessments .....	85
2.6.2	Exiqon microarray labelling .....	85
2.6.3	Exiqon microarray hybridisation .....	86

---

---

2.6.4	Exiqon miRNA data analysis.....	87
2.7	ANALYSIS OF ADIPONECTIN ABUNDANCE AND EXPRESSION .....	88
2.7.1	Plasma adiponectin abundance .....	88
2.7.2	Adipose tissue RNA extraction, quantity and quality assessments .....	89
2.7.3	DNase treatment and cDNA synthesis .....	89
2.7.4	Adiponectin gene expression.....	90
2.7.5	Quantification of adiponectin mRNA.....	91
2.8	STATISTICAL ANALYSES .....	91
<b>3</b>	<b>CHAPTER 3 <i>IN VIVO</i> AND <i>IN VITRO</i> OUTCOMES.....</b>	<b>97</b>
3.1	ABSTRACT.....	97
3.2	INTRODUCTION .....	98
3.3	MATERIALS AND METHODS.....	99
3.3.1	Ethics statement .....	99
3.3.2	Animal, treatments and surgery .....	100
3.3.3	<i>In vivo</i> measures of insulin secretion, sensitivity, and action. ....	101
3.3.4	Analysis of plasma insulin and metabolites.....	101
3.3.5	Post-mortem.....	102
3.3.6	Pancreas and islet isolation and morphometric analysis.....	102
3.3.7	<i>In vitro</i> $\beta$ -cell secretion and responses.....	102
3.3.8	Statistical analysis.....	103
3.4	RESULTS .....	104
3.4.1	Size at birth, neonatal growth and body composition.....	104
3.4.2	Insulin secretion, sensitivity and action .....	108
3.4.3	Pancreas morphology and $\beta$ -cell function .....	109

---

3.4.4	<i>In vitro</i> $\beta$ -cell secretory function .....	113
3.5	DISCUSSION .....	115
3.6	CONCLUSION .....	121
<b>4</b>	<b>CHAPTER 4 EFFECT OF IUGR DUE TO TWINNING AND NEONATAL EXENDIN-4 TREATMENT ON MOLECULAR DETERMINANTS OF B-CELL FUNCTION AND MASS IN YOUNG LAMBS.....</b>	<b>123</b>
4.1	INTRODUCTION .....	123
4.2	MATERIALS AND METHODS .....	126
4.2.1	Animals and treatments. ....	126
4.2.2	Post-mortem, pancreatic islet RNA and microRNA extraction and their quality and quantity assessments .....	126
4.2.3	Statistical analyses .....	127
4.3	RESULTS .....	128
4.3.1	Islet mRNA expression of molecular determinants of $\beta$ -cell mass .....	128
4.3.2	Islet mRNA expression of molecular determinants of $\beta$ -cell function...	130
4.3.3	Islet mRNA expression of epigenetic machinery .....	132
4.3.4	Islet microRNA expression.....	132
4.3.5	Relationships between $\beta$ -cell mass and function and islet mRNA expression of molecular regulators of $\beta$ -cell mass and function .....	134
4.3.5.1	<i>Relationships between pancreas morphology and <math>\beta</math>-cell mass and islet mRNA expression .....</i>	<i>134</i>
4.3.5.2	<i>Relationships between in vivo insulin secretion and disposition and islet mRNA expression .....</i>	<i>136</i>

---

---

---

4.3.5.3	<i>Relationships between in vitro insulin secretion and action and islet mRNA expression.....</i>	<i>138</i>
4.3.6	Relationships between islet mRNA expression of <i>PDX1</i> and other regulators of $\beta$ -cell mass and function.....	140
4.3.7	Relationships between islet mRNA expression of <i>PDX1</i> and other regulators of $\beta$ -cell mass and secretory function and islet mRNA expression of epigenetic machinery.....	143
4.4	DISCUSSION .....	146
4.5	CONCLUSION.....	151
<b>5</b>	<b>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 .....</b>	<b>154</b>
5.1	INTRODUCTION .....	154
5.2	MATERIALS AND METHODS.....	156
5.2.1	Animals and treatments .....	156
5.2.2	Surgery and routine blood sampling .....	157
5.2.3	Plasma adiponectin analysis .....	158
5.2.4	Growth measurements and post-mortem.....	158
5.2.5	Omental and subcutaneous adipose tissues RNA extraction and adiponectin gene expression analysis .....	158
5.2.6	<i>In vivo, in vitro</i> insulin action and gene expression measures.....	159
5.2.7	Statistical analyses .....	160
5.3	RESULTS .....	161

---

5.3.1	Plasma adiponectin concentrations .....	161
5.3.2	Adiponectin mRNA expression in omental and subcutaneous fat .....	161
5.3.3	Relationships between circulating plasma adiponectin and adiponectin mRNA expression in omental and subcutaneous adipose tissues .....	164
5.3.4	Relationships between growth, metabolic outcomes and circulating plasma adiponectin and its expression in omental and subcutaneous fat .....	166
5.3.4.1	<i>Relationships between neonatal growth and plasma adiponectin concentration and its expression in omental and subcutaneous fat .....</i>	<i>166</i>
5.3.4.2	<i>Relationships between insulin sensitivity and plasma adiponectin and its expression in omental and subcutaneous fat .....</i>	<i>168</i>
5.3.4.3	<i>Relationships between in vivo insulin secretion and plasma adiponectin and its expression in omental and subcutaneous fat.....</i>	<i>168</i>
5.3.4.4	<i>Relationships between in vitro insulin secretion and plasma adiponectin and its expression in omental and subcutaneous fat.....</i>	<i>168</i>
5.3.4.5	<i>Relationships between <math>\beta</math>-cell mass and plasma adiponectin and its expression in omental and subcutaneous fat .....</i>	<i>169</i>
5.3.4.6	<i>Relationships between pancreatic islet mRNA expression of molecular regulators of <math>\beta</math>-cell mass and function and plasma adiponectin and its expression in omental and subcutaneous fat .....</i>	<i>172</i>
5.4	DISCUSSION .....	174
5.5	CONCLUSION.....	178
<b>6</b>	<b>GENERAL DISCUSSION .....</b>	<b>181</b>
6.1	<i>IN VIVO AND IN VITRO RESPONSES TO IUGR DUE TO TWINNING AND NEONATAL EXENDIN-4 TREATMENT.....</i>	<i>182</i>

6.2	MECHANISM OF IUGR PROGRAMMING – GENE EXPRESSION .....	187
6.3	MECHANISM OF IUGR PROGRAMMING – EPIGENETIC REGULATION .....	191
6.4	MECHANISM OF IUGR PROGRAMMING – ADIPONECTIN REGULATION .....	193
6.5	STUDY LIMITATIONS .....	195
6.6	IMPLICATIONS AND FUTURE DIRECTIONS .....	197
6.7	CONCLUSION .....	198
	<b>REFERENCES .....</b>	<b>200</b>
	<b>APPENDIX.....</b>	<b>240</b>

**LIST OF TABLES AND FIGURES**

Table 2.1 Primer details and sequences used for quantitative Real Time PCR of islet gene expression.....80

Table 2.2 Primer sequences used for Real Time PCR for adiponectin expression in omental and subcutaneous adipose tissues. ....91

Table 3.1 Effect of IUGR and neonatal exendin-4 treatment on size at birth, postnatal growth and body composition in young lambs. ....106

Table 3.2 Effect of IUGR and neonatal exendin-4 treatment on insulin secretion and action in young lambs. ....110

Table 3.3 Effect of IUGR and neonatal exendin-4 treatment on pancreas morphology and  $\beta$ -cell function.....112

Figure 1.1 Hyperbolic relationship between insulin sensitivity and insulin secretion, modified from (29). ....33

Figure 1.2 Regulation of  $\beta$ -cell mass and its molecular determinants. ....40

Figure 1.3 Regulation of glucose-stimulated insulin secretion in pancreatic  $\beta$ -cells, adapted from (61). ....42

Figure 1.4 Bio-synthesis of microRNAs, adapted from (70). ....46

Figure 1.5 Schematic diagram of gene expression regulation by epigenetic mechanisms, adapted from (85-87). ....49

Figure 1.6 Potential pathways for action of GLP1 to stimulate or increase  $\beta$ -cell mass via *PDX1*, modified from (154). ....62

---

---

Figure 2.1 Schematic flow diagram and timeline of *in vivo* studies. ....68

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. ....107

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. ....111

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. ....114

Figure 4.1 Effects of IUGR and neonatal exendin-4 treatment on islet mRNA expression of genes that regulate  $\beta$ -cell mass. ....129

Figure 4.2 Effects of IUGR and neonatal exendin-4 treatment on islet mRNA expression of genes that regulate  $\beta$ -cell function. ....131

Figure 4.3 Effects of IUGR and neonatal exendin-4 treatment on islet mRNA expression of DNA methyl transferase enzymes. ....133

Figure 4.4 Relationships between (A)  $\beta$ -cell volume density and (B)  $\beta$ -cell mass and islet *IGF1* mRNA expression in the young lambs. ....135

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. ....137

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. ....139

---



---

Figure 4.7 Relationships between islet mRNA expression of (A) *IGF1R* and (B) *IGF2* and islet *PDX1* mRNA expression in the young lambs. ....141

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. ....142

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. ....144

Figure 4.10 Relationship between islet *GCK* mRNA expression and islet *DNMT3B* mRNA expression in the young lambs. ....145

  

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. ....162

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. ....163

Figure 5.3 Relationships between plasma adiponectin concentration at 11 d of age and (A) omental and (B) subcutaneous fat adiponectin mRNA expression. ....165

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. ....167

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. ....170

Figure 5.6 Relationships between (A) absolute  $\beta$ -cell mass and (B) relative  $\beta$ -cell mass and plasma adiponectin at 11 d of age, and between (C) absolute  $\beta$ -cell mass and (D) relative  $\beta$ -cell mass and omental fat adiponectin mRNA expression. ....171

Figure 5.7 Relationships between (A) islet *PIK3CB* mRNA expression and (B) islet *GCK* mRNA expression and omental fat adiponectin mRNA expression. ....173

## **ACKNOWLEDGEMENTS**

I am very grateful to have met and worked with numerous people, without whom I believe this thesis could not have been written.

First of all, I would like to say thank you to all my supervisors, Professor Julie Owens, Dr Kathy Gatford and Dr Miles De Blasio, for their excellent guidance, support and encouragement throughout my whole candidature. I am eternally grateful to Julie for bringing me into her lab, the memories will always be with me till the end. Thank you for your exceptional expertise and knowledge, and your share of jokes, which are always the best as there are no words to describe them. I am so honoured to learn and work with you, thank you very much. Kathy, I am so thankful for the very decision that you made in accepting my honours application, which allowed me to take my first step into this research world and the JAO lab. I am much honoured to have worked and learnt from you, there are so many things I learnt from you that this page of acknowledgement alone does not suffice in describing them all. Miles, like most of the PhD students in the JAO lab, I also considered you as a mentor as well as a big brother of the lab. I am very grateful to all of your help and knowledge.

I would also want to thank Ms Lyn Harland for all technical help especially in regards to pancreas RNA extraction and islet isolations, and your expertise in primer sequencing and cloning. Thanks also to Ms Patricia Grant, for all the paper works and applications, without you, I would probably still be stuck in the madness of forms and paperwork. And, most grateful thank you to my beloved sister in research, Ms Saidatul Mohammed, for assisting me in many aspects of the experiments and working together for our PhD. Thank you also to Mr Simon Morretta and Ms Tasma How, for helping with sheep *in vivo* experiments and post-mortem.

Thank you to the rest of JAO lab members, for keeping me positive and hopeful every day. Special thank you to Ms Ezani Mohammed Jamil and Mr Vincent Chu, for our special friendship and unforgettable memories. Thank you also to Mr Himawan Harryanto for constantly helping me when I had no one else to ask. And thank you to the rest of the lab members, Ms Wee-Ching Kong, Mrs Tulika Sundanathan, Mr Hong Liu, Mr. Gary Heinemann and Ms Amy Wooldridge, for making JAO lab more fun than ever. Lastly but not last, the rest of School of Paediatrics and Reproductive Health (SPRH), thank you for the help and exceptional encouragement.

Thank you to the University of Adelaide for giving me the scholarship and Professor Julie Owens and Dr Kathy Gatford, for additional scholarship, of which I believe, without these financial supports, I would not be able to complete my candidature.

Special thanks to my lovely friends to whom I have known for the past four years in Adelaide. Kak Alin, Kak Kay, Kak Shifa', Kak Nik, Intan, Ummu, Nasruna, Baitul Abrar, Zahratul Afia, Zahratul Hamra' and the rest (long list here!); thank you for supporting me and feeding me, I love the foods from you all.

Lastly, to my beloved parents, Sulaiman Yusoff and Inonushiah Abu Bakar, I am so grateful for everything. There is no word to describe how much both of you are to me, and I hope I can return all of these cares, love and supports. To my younger siblings, Zaharah and Faris, thanks for keeping up with this weird sister of yours, I love both of you so much.

**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

Date: 18/12/2014

**TABLE OF ABBREVIATIONS AND BIOCHEMICAL NAMES**

ACTB	Beta cytoskeletal actin / $\beta$ -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

---

*Table of Abbreviations and Biochemical Names*

---

GCK	Glucokinase
GIR	Glucose infusion rate
GLP1	Glucagon-like-peptide 1
HAT	Histone acetyltransferase
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 <sup>+</sup> 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

---

---

*Table of Abbreviations and Biochemical Names*

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 <sub>d</sub>	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**

Kathryn L Gatford<sup>1,3</sup>, Siti A Sulaiman<sup>1,3</sup>, Saidatul N B Mohammad<sup>1</sup>, Miles J De Blasio<sup>1</sup>, M Lyn Harland<sup>1</sup>, Rebecca A Simmons<sup>2</sup>, Julie A Owens<sup>1</sup>

<sup>1</sup>Research Centre for Early Origins of Health and Disease, Robinson Institute, and School of Paediatrics and Reproductive Health, University of Adelaide, SA 5005, Australia.

<sup>2</sup>Department of Paediatrics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA.

<sup>3</sup>KLG and SAS are equal joint first authors.

PLOS ONE journal, 2013, volume 8, issue 2, page e56553. (Attached in Appendix)

**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)

September 2011: *ESA/APEG Annual Scientific Meeting*, Perth, Australia. (Oral presentation by Siti A Sulaiman)

November 2011: *7<sup>th</sup> 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.

February 2011: *Fetal Neonatal Workshop of Australia and New Zealand*, Hobart, Australia. (Oral presentation by Kathryn L Gatford)

**Neonatal Exendin-4 Treatment Increases  $\beta$ -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

September 2010: *ESA/SRB Scientific Meeting*, Sydney, Australia (Oral presentation by SA Sulaiman)

**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

June 2010: *ASMR SA Scientific Meeting*, Adelaide, Australia. (Poster presentation by Siti A Sulaiman)

**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

2010: *PSANZ Scientific Meeting*, Wellington, New Zealand. (Oral presentation by KL Gatford)

**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

2009: *PSANZ Scientific Meeting*, Darwin, Australia. (Oral presentation by KL Gatford)

**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  $\beta$ -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  $\beta$ -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  $\beta$ -cell development between species and therefore in the developmental stages during exposure to IUGR and neonatal interventions. In humans and sheep, most pancreatic and  $\beta$ -cell development occurs before birth (10-17). In contrast, rodents undergo later development of  $\beta$ -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  $\beta$ -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  $\beta$ -cell *in vivo* and *in vitro* insulin secretory function,  $\beta$ -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,  $\beta$ -cell mass or islet mRNA expression of  $\beta$ -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  $\beta$ -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  $\beta$ -cell function after IUGR may occur in part via epigenetic changes that may persist throughout life. In conjunction with enhanced  $\beta$ -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  $\beta$ -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  $\beta$ -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).  $\beta$ -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  $\beta$ -cell volume density, which may suggest hyperplastic effects of the *IGF* axis on  $\beta$ -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*  $\beta$ -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*  $\beta$ -cell insulin secretion and islet expression of its determinant, *GCK*, but did not alter *in vivo* insulin action, glucose tolerance or  $\beta$ -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<sup>nd</sup> 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  $\beta$ -cell mass in young IUGR lamb, the up-regulation of islet expression of  $\beta$ -cell mass determinants after 16 days of exendin-4 treatment may suggest beneficial effects of exendin-4 to subsequently expand  $\beta$ -cell mass. This may protect the exendin-4-treated IUGR individual from a need to increase  $\beta$ -cell function, and preserve the capacity of  $\beta$ -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  $\beta$ -cell function and mass and insulin action and their regulation in the IUGR sheep to adulthood.